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Molecular characterization and functional analysis of *Akirin* from black tiger shrimp (*Penaeus monodon*)

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ABSTRACT

Akirin, which are members of the NF-κB signaling pathway, play critical roles in regulating the expression of antimicrobial peptides. In the present study, the *Akirin* gene from *Penaeus monodon* was identified from a transcriptome database and designated as *PmAkirin*. The complete sequence of the *PmAkirin* cDNA was 1508 bp, encoding a protein of 213 amino acids, and it showed 99% amino acid identity to the *Litopenaeus vannamei* Akirin. Two predicted nuclear localization signals (NLSs) were found, and the amino acid sequence alignments showed that PmAkirin was highly conserved at the N-terminus and C-terminus. *PmAkirin* expression was found to be the highest in the hemolymph, followed by the heart, gill, stomach, hepatopancreas, intestine, and muscle. When challenged with *Vibrio parahaemolyticus* infection, the *PmAkirin* mRNA and three antimicrobial peptides (AMPs: *PmALF2, PmALF3,* and *PmCrus4*) were upregulated. However, another five AMPs (*PmALF6, PmCrus1, PmPEN3a, PmPEN3b,* and *PmPEN5*) were downregulated by *V. parahaemolyticus* infection. Silencing *PmAkirin* by dsRNA significantly decreased the expression of the eight AMPs, which lead to an increase in the blood concentration of *V. parahaemolyticus* and higher mortality in the shrimp. In contrast, the overexpression of *PmAkirin* significantly increased the expression of the eight AMPs, which led to a reduction in the blood concentration of *V. parahaemolyticus* and promoted the survival of the shrimp. Taken together, we concluded that *PmAkirin* plays an important role in regulating the expression of AMPs in black tiger shrimp to defend against *V. parahaemolyticus* infection.

1. Introduction

Akirin, first discovered and identified in *Drosophila* by Goto, is a nuclear factor in the innate immune system that plays key roles in diverse biological processes, including immunity, myogenesis, development, and the cellular stress response [1,2]. In many vertebrates, including mammals and amphibians, the Akirin family is composed of two homologs (Akirin1 and Akirin2), whereas two to eight Akirins exist in teleost species, and only a single *Akirin* gene is retained in insects, birds, and invertebrates [3]. Akirin is a highly conserved nuclear protein in different species. Usually, the Akirin proteins consist of 180–204 amino acid residues with highly conserved regions at the N- and C-termini and a less conserved sequence in the middle region [4]. As a nuclear factor, the Akirin protein is strictly localized in the nucleus and has a highly conserved N-terminal nuclear localization signal (NLS) [5]. The Akirin family members can interact with other cofactors to regulate the mRNA transcription of downstream genes despite inconspicuous DNA-binding motifs [6].

Since it was first identified as a key regulator of innate immunity in *Drosophila*, Akirin has been shown to play a crucial role in the innate immune systems of both vertebrates and invertebrates. In mammals, Akirin2 is required for the secretion of NF-κB-dependent cytokines to regulate Toll-like receptor (TLR), tumor necrosis factor (TNF) receptor, and interleukin (IL)-1β receptor pathways [2]. For example, Akirin2 in macrophages and neutrophils is essential for the clearance of infecting bacteria and the proliferation of B cells in mice [7,8]. In bony fishes, Akirins have stronger and wider responsiveness to bacterial and viral immunological challenges [9–12]. With regard to invertebrates, Akirin2

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Table 1
Primers used in this study.

Primers	Sequence(5'→3')	Usage
Akirin-F	ATGGCATGTGTAACTCTAAAAAGGT	ORF cloning
Akirin-R	TTATGAAAGATAGCTCGGTAGGG	ORF cloning
UPM-long	CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT	3′RACE
UPM-short	CTAATACGACTCACTATAGGGC	3′RACE
NUP	AAGCAGTGGT AACAACGCAGAGT	3′RACE
Akirin-3′GSP1	GAGGTGAAGATCCGTGAGGAGTATGAC	3′RACE
Akirin-3′GSP2	GAGTGGCAGCTTGGGTGACAAG	3′RACE
qAkirin-F	CACCCTCATCATCCACATCATTC	qRT-PCR
qAkirin-R	ATACCTTGTCATACTCCTCACGGA	qRT-PCR
qEF-1α-F	AAGCCAGGTATGGTTGTCAACTTT	qRT-PCR
qEF-1α-R	GCTTCGTGGTGCATCTCCACAGAC	qRT-PCR
r-Akirin-F1	GTGCGGCCGCAAGCTTATGGCATGTGTAACTCTAAAAAGGT	Recombinant expression
r-Akirin-R1	AATGGGTCGCGGATCCTTATGAAAGATAGCTCGGTAGGG	Recombinant expression
r-Akirin-F2	CGAGCTCATGGCATGTGTAACTCTAAAAAGGT	Recombinant expression
r-Akirin-R2	ACGCGTCGACTTATGAAAGATAGCTCGGTAGGG	Recombinant expression
dsAkirin-F	CGAGCTCCTTCTCCTCCATCTCCAGC	RNA interference
dsAkirin-R	ACGCGTCGACAAGCCAATACCTTGTCATACTC	RNA interference
dsGFP-F	CGAGCTCTGGAGTGGTCCCAGTTCTTGTTGA	RNA interference
dsGFP-R	ACGCGTCGACGCCATTCTTTGGTTTGTCTCCCAT	RNA interference
pD7-F	CAGTGAGCGAGGAAGCGGAAG	Recombinant expression
pD7-R	GGATGTGCTGCAAGGCGATTAAGT	Recombinant expression
M13-F	TGTAAAACGACGGCCAGT	PCR verification
M13-R	CAGGAAACAGCTATGACC	PCR verification
PmPEN3a-F	CATGCGTCTCGTGGTCTG	qRT-PCR
PmPEN3a-R	CATGAAGTGCAAACAGGAACTG	qRT-PCR
PmPEN3b-F	CATGCGTCTCGTGGTCTG	qRT-PCR
PmPEN3b-R	GTGGCATGAAGTACAAACAGG	qRT-PCR
PmPEN5-F	GCGGTTACACAGGTTCATACTCC	qRT-PCR
PmPEN5-R	GGTTTTCATTGCCTTCTCCATC	qRT-PCR
PmCrus1-F	TGCGAAACTCCAGAGAATGC	qRT-PCR
PmCrus1-R	GCAGGTGTCACGGACTTTTG	qRT-PCR
PmCrus4-F	TGCTGCGAGGATAAGAATGAAC	qRT-PCR
PmCrus4-R	TTGAAGTCGTGGGAACAGGTTA	qRT-PCR
PmALF3-F	GTTCACCGTCAAGCCTTATTTG	qRT-PCR
PmALF3-R	AGTCTTTGGCTGTCTTTCCAG	qRT-PCR
PmALF2-F	TTCAATGGAAGGATGTGGTGTC	qRT-PCR
PmALF2-R	CAATGCTTTCCTTGTGCTCTGA	qRT-PCR
PmALF6-F	GTTCGAGCTGTTAGGACACTAC	qRT-PCR
PmALF6-R	CATGCGACCCCTGAAGTATAG	qRT-PCR
qEF-1α-F	AAGCCAGGTATGGTTGTCAACTTT	qRT-PCR
qEF-1α-R	GCTTCGTGGTGCATCTCCACAGAC	qRT-PCR

in the Hong Kong oyster (*Crassostrea hongkongensis*) can activate the transcriptional activities of the NF-κB reporter gene [13]. *Akirin* in *Litopenaeus vannamei* activated the expression of antimicrobial peptides (AMPs), and the silencing of *Akirin* significantly increased the cumulative mortality of the shrimp [14]. In both vertebrates and invertebrates, the selective activation of NF-κB target genes by Akirin requires chromatin remodeling factors. Studies have revealed that the selective activation of NF-κB depends on the interaction of Akirin with the 60 kDa Brahma-associated protein (Bap60), which is a component of the chromatin remodeling switch/sucrose nonfermentable (SWI/SNF) complex and NF-κB [7,15].

The black tiger shrimp (*Penaeus monodon*) is one of the three most important cultured species worldwide and is a popular farmed shrimp in China, particularly in southern China [16]. However, frequent outbreaks of disease have caused enormous economic losses in shrimp aquaculture in recent years. *Vibrio parahaemolyticus*, a Gram-negative bacterium, is the causative agent of acute hepatopancreatic necrosis disease (AHPND), which has caused large-scale losses in farmed shrimp production [17–20]. There are still no effective measures to control the spread of AHPND. Since Akirin is a highly conserved nuclear protein required for NF-κB-dependent genes which respond to Gram-negative bacterial infections [2]. Meanwhile, shrimp lack an adaptive immune system and their defense mechanisms mainly rely on the innate immune system. Hence, understanding the function, immunoprotective effect, and mechanism of Akirin with regard to resisting *V. parahaemolyticus* infection is quite important for *P. monodon* aquaculture. Here, we successfully cloned and characterized a full-length cDNA of the *Akirin* gene (*PmAkirin*) from *P. monodon* and determined the expression of *PmAkirin* transcripts in various tissues. RNAi and mRNA overexpression approaches were used to study the function of PmAkirin in regulating the transcription of AMPs post *V. parahaemolyticus* challenge. The blood concentration of *V. parahaemolyticus* and cumulative mortality were also investigated in *PmAkirin*-silenced and *PmAkirin*-overexpressed shrimp post *V. parahaemolyticus* challenge.

This study may help us to better understand the immune function of PmAkirin as it relates to defense against *V. parahaemolyticus* infection.

2. Materials and methods

2.1. Ethics statement

P. monodon is not an endangered or protected species, and there is no requirement for permission to undertake experiments involving this species in China.

2.2. Experimental animals, bacteria, and sample preparation

The *P. monodon* used in the experiments (mass: 16 ± 1 g) were collected from the Zhuhai experimental base of the South China Sea Fisheries Research Institute, China Academy of Fisheries Sciences (Guangdong, China). A total of 500 shrimp were cultured in aerated sea water (salinity: 3.3%) at 24–26 °C for 3 days, and two-thirds of the

water was replaced daily. Twenty-four hours prior to the experiment, the shrimp were fed with pelleted feed to suit the environment. Healthy tissues from three randomly selected *P. monodon* were examined to determine the distribution of *PmAkirin* in the heart, hepatopancreas, intestine, muscle, gill, stomach, and hemolymph. The samples were snap frozen in liquid nitrogen and kept at −80 °C until use.

V. parahaemolyticus was inoculated into high-salt LB medium (1% tryptone, 0.5% yeast extract, 3.5% NaCl) with constant shaking at 220 rpm at 37 °C overnight and then transferred to a logarithmic phase at a ratio of 1:100. After centrifugation to remove the supernatant, the sediment was diluted with sterile phosphate-buffered saline (PBS, pH 7.4), washed twice, and then resuspended to adjust the concentration to 1×10^7 cfu/mL.

2.3. Total RNA extraction and cDNA synthesis

Total RNA was extracted from approximately 50 mg of tissue obtained from the shrimp using TRIZOL reagent (Invitrogen, USA) according to the manufacturer's instructions. The quality of the total RNA was checked by electrophoresis with a 1.2% agarose gel, and the total RNA concentration was estimated at 260 nm using a NanoDrop-2000 (Thermo Fisher, USA). The PrimeScript reverse transcriptase kit (TaKaRa, Japan) was used for reverse transcription with 1 μg of total RNA according to the manufacturer's instructions. The cDNA template for rapid amplification of the cDNA ends (RACE) was synthesized according to the instructions of the SMART RACE 5′/3′ kit (Clontech, Japan). All OD_{260}/OD_{280} ratios were between 1.8 and 2.0. The original solution was stored at −80 °C. The working solution of the cDNA sample was diluted to about 30 ng/μL and stored at −20 °C until use.

2.4. Amplification of the full-length cDNA of PmAkirin

The partial cDNA sequence of *PmAkirin* was screened from the transcriptome database. The open reading frame (ORF) sequence was verified using the PmAkirin-F/R primers (Table 1). Next, 3′ RACE was performed using the SMARTER RACE 5′/3′ kit (TaKaRa) and the SMART RACE method. This step reaction was performed by the polymerase chain reaction (PCR) using the *PmAkirin*-3GSP1/3 GSP2 primers and UPM/NUP (Table 1). The resulting PCR product was further verified by electrophoresis using a 2.0% agarose gel and purified by PCR (Sangon Biotech, China). The product was ligated into the pMD19-T vector (TaKaRa) and transformed into DH5α cells, and the positive clones were picked for sequencing (Ruibiotech, China) (see Table 1).

2.5. Bioinformatics analysis of PmAkirin

The *PmAkirin* sequence was analyzed using the BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi) from the NCBI server. Protein domain analysis of PmAkirin was performed using the SMART 4.0 tool (http://smart.embl-heidelberg.de/smart/set_mode). The molecular size and theoretical isoelectric point of PmAkirin were analyzed online using the ExPASy software (http://www.expasy.org/). Multiplex sequence alignment of PmAkirin was performed using the ClustalX2.1 and TEXshade software, and a phylogenetic tree was constructed using the neighbor joining (NJ) method in MEGA 7.0 (http://www.megasoftware.net/) with the BioEdit software package. All *PmAkirin* alignment sequences were from the GenBank database.

2.6. Preparation of the dsRNA and overexpression vector for PmAkirin

In order to explore the effect of *PmAkirin* on the expression of antimicrobial peptides and its important role in the innate immunity of *P. monodon*, we performed RNA interference and overexpression experiments. For the RNAi assay, the primers were designed using Snap Dragon tools (http://www.flyrnai.org/cgi-bin/RNAi_find_primers.pl) and are shown in Table 1 dsRNA-Akirin and dsRNA-GFP were synthesized in vitro using the Transcription T7 Kit (TaKaRa) following the manufacturer's protocol. Recombinant plasmids (pD7-Akirin and pD7-GFP) were established. The sense and anti-sense DNA templates used in the in vitro transcription were generated via PCR using the pD7-*Akirin* and pD7-GFP recombinant plasmids as templates. The quality of the dsRNA was evaluated using 1.2% agarose gel electrophoresis.

For the overexpression assay, the ORF of *PmAkirin* was amplified using the primers rAkirin-F2 and rAkirin-R2 (Table 1) and then ligated into the pD7 vector containing a T7 promoter. The double-stranded DNA containing the *PmAkirin* sequence was used as a template to transcribe the capped *PmAkirin* mRNA with the mMESSAGE mMACHINE™ T7 kit (Ambion Europe LTD, Cambridgeshire, U.K.) according to the manufacturer's instructions.

2.7. Design of the injection experiment

The experiment included five groups with 90 shrimp in each group: the mRNA-Akirin, dsRNA-Akirin, dsRNA-GFP, PBS, and blank groups. The blank group did not receive any treatment. The remaining four groups were injected with 30 μL of mRNA-Akirin (3–5 μg of mRNA-Akirin per gram of shrimp), dsRNA-Akirin (3–5 μg of dsRNA-Akirin per gram of shrimp), dsRNA-GFP (3–5 μg of dsRNA-GFP per gram of shrimp), and PBS, respectively. Twenty-four hours after injection, the hepatopancreas tissues from five randomly selected *P. monodon* were sampled for RNA extraction and in situ hybridization (ISH) experiments to test the RNA interference and mRNA-Akirin overexpression effect.

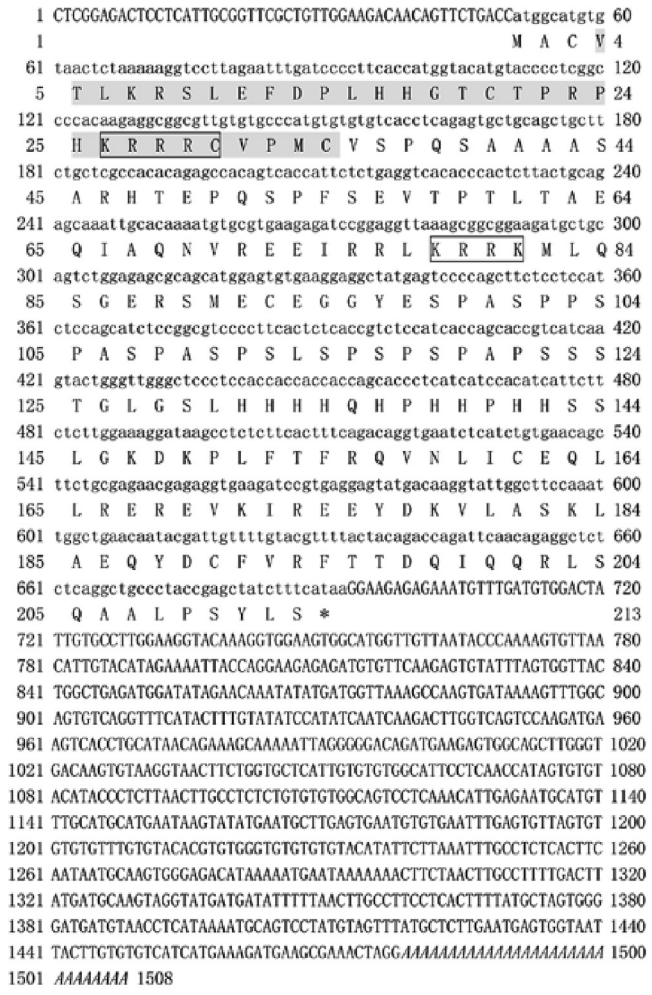
After 24 h of interference, the shrimp in the mRNA-Akirin, dsRNA-Akirin, and dsRNA-GFP groups were injected with 20 μL of *V. parahaemolyticus* (1×10^7 cfu/mL). The PBS group was divided into two groups: one group was injected with 20 μL of PBS, and the other group was injected with 20 μL of *V. parahaemolyticus* (1×10^7 cfu/mL). After that, the above groups were divided into two parts. One part was used for sampling the hepatopancreas tissues of five shrimp from each group 0, 6, 12, 24, and 48 h post injection (hpi) and storing them in liquid nitrogen, and 30 μL of hemolymph was also sampled and stored 1 hpi to detect the blood concentration of *V. parahaemolyticus*. The other part was used to calculate the cumulative mortality in each group.

2.8. Spatial and temporal expression analysis of PmAkirin and AMPs

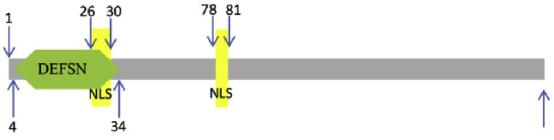
In this experiment, the template concentration was diluted to 30 ng/μL. Reverse transcription quantitative PCR (RT-qPCR) was used to detect the mRNA expression profiles of the *PmAkirin* gene using the qPmAkirin-F/R primers (Table 1). The *EF-1α*-F/R gene primers (GenBank No. DQ021452.1) in Table 1 were used to amplify the reference gene. RT-qPCR was performed in triplicate for each concentration using

Table 2
Number of *V. parahaemolyticus* colonies on the hemolymph plate.

Group	Numbers of *V. parahaemolyticus*				Mean
dsRNA-Akirin + V. parahaemolyticus	53	54	58	56	55.25
mRNA-Akirin + V. parahaemolyticus	9	11	12	16	12
dsRNA-GFP + V. parahaemolyticus	27	33	39	25	31
PBS + V. parahaemolyticus	24	26	32	40	30.5
PBS	0	0	0	0	0



A



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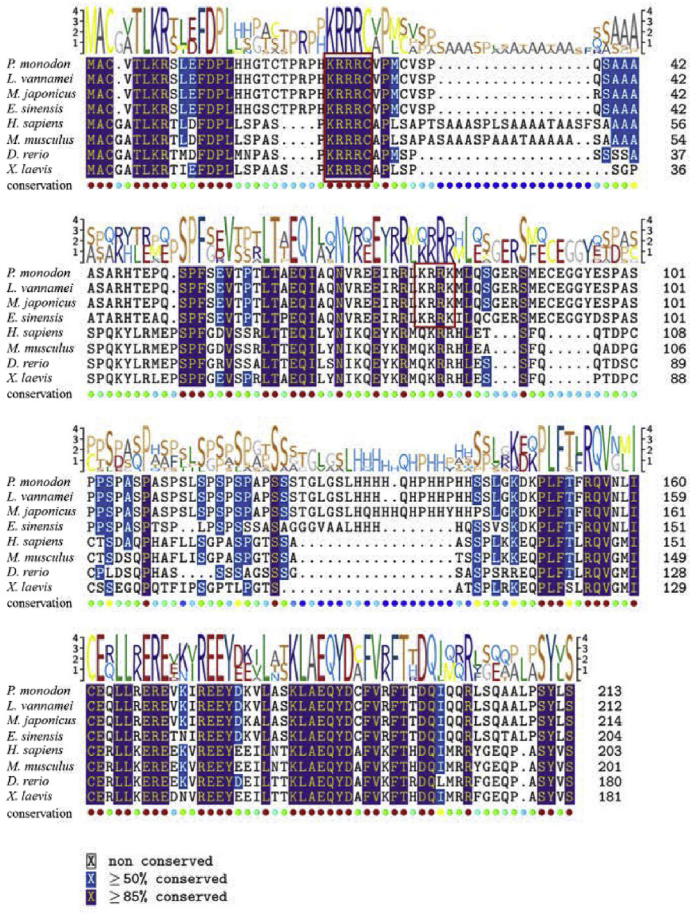
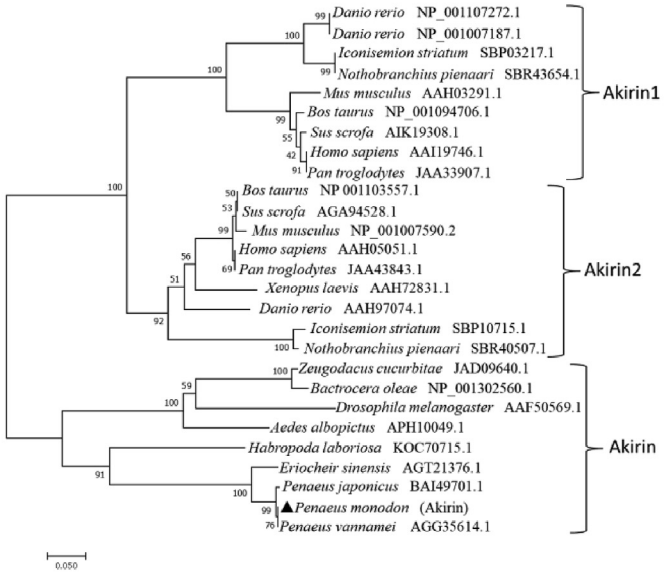


C



D

Fig. 1. Molecular characterization of the *PmAkirin* cDNA. (A) Nucleotide and amino acid sequences of PmAkirin. The black box represents the nuclear localization signal peptide sequence and the shaded sequence represents the DEFSN domain. (B) The predicted domain of the PmAkirin protein. (C) Multiple alignment of Akirin amino acid sequences between *P. monodon* and other species. (D) Phylogenetic analysis of *Akirin* family members. The black triangle marks the Akirin of *P. monodon*. The phylogenetic tree has a total of 27 sequences, which include vertebrate *Akirin1* and *Akirin2* and invertebrate *Akirin*. The relevant GenBank registration numbers are shown in the figure.

384-well plates with a total volume of 12.5 μL, including 6.25 μL of 2X SYBR Preix Ex Taq II (TaKaRa), 0.5 μL of each specific primer, 1 μL of the cDNA template, and 4.25 μL of DEPC water. The reaction conditions were 95 °C for 30 s, followed by 42 cycles of 95 °C for 5 s, 62 °C for 30 s, 95 °C for 1 s, and 65 °C for 5 s. The relative expression level of *PmAkirin* was analyzed by the $2^{-\Delta\Delta Ct}$ method by normalizing to *EF-1α*.

In order to know whether *PmAkirin* participates in the expression of AMPs after *V. parahaemolyticus* infection, the transcription levels of anti-lipopolysaccharide factors (*PmALF2*, GenBank No. BI784449; *PmALF3*, GenBank No. EF523559; *PmALF6*, GenBank No. AER45468.1), crustins (*PmCrus1*, GenBank No. GQ334395; *PmCrus4*, GenBank No. FJ686015), and penaeidins (*PmPen3a*, GenBank No. FJ686016.1; *PmPen3b*, GenBank No. FJ686017.1; *PmPen5*, GenBank No. FJ686018.1) were detected in the dsRNA-Akirin group with the *V. parahaemolyticus* injection (dsRNA-Akirin + *V. parahaemolyticus*), in the mRNA-Akirin group with the *V. parahaemolyticus* injection (mRNA-Akirin + *V. parahaemolyticus*), in the dsRNA-GFP group with the *V. parahaemolyticus* injection (dsRNA-GFP + *V. parahaemolyticus*), in the PBS group with the *V. parahaemolyticus* injection (PBS + *V. parahaemolyticus*), and in the PBS group. Specific primers were designed for the three AMPs and *EF-1α* (Table 1). The real-time quantitative PCR and data analysis methods are described above.

2.9. ISH analysis

The hepatopancreases from healthy *P. monodon* and from *P. monodon* 24 h after injection with PBS, mRNA-Akirin, dsRNA-Akirin, and dsRNA-GFP were dissected and fixed in 4% paraformaldehyde solution overnight. Subsequently, the fixed tissues were embedded in paraffin and sectioned into 5 μm pieces prior to mounting onto 3-aminopropyltriethoxysilane-coated glass slides. After drying, the paraffin sections were treated with xylene (2 × 10 min) to remove the wax and washed with gradient ethanol (100%, 90%, 80%, 75%, 50%, and 30% for 5 min each) for dehydration. The sections were treated with diluted protease K (0.1 mol/L, pH 7.4) for 20 min at room temperature and washed with 2X SSC twice for 10 min. Every section was prehybridized with 20 μL of prehybridization solution (5X SSC, 5X Denhardt's solution, 50% formamide, 10 μg/mL SDS, and 1 μg/μL salmon sperm DNA) at 42C for 1 h in a wet box. After samples probes were denatured. These samples were then co-incubated with 20 μL of the PmAkirin probe, TCAGCCAATTTGGAAGCCAATACCTTGTCATACTCCTCACGGATCTTCACCTCT, premixed with prehybridization solution at 42 °C overnight. The glass slide was washed thrice with 5X SSC and then dyed with DAPI counterstaining. Finally, the glass slide was sealed with anti-fluorescence attenuation sealer and mounted and photographed by fluorescence microscopy.

2.10. Statistical analysis

All data are depicted as means ± SD. One-way analysis of variance (ANOVA) tests were performed to calculate the P values using SPSS22.0 software. P values of < 0.05 indicated significant differences. The results of the analyses were plotted using the SigmaPlot 12.5 software.

3. Results

3.1. Molecular characteristics and phylogenetic analysis of the full-length PmAkirin gene

The cDNA sequence of the *PmAkirin* gene (GenBank No. MN104804) from *P. monodon* was 1508 bp in length, including a 50-bp 5′ untranslated region (UTR), an 816-bp 3′ UTR, and an open reading frame of 213 amino acids, which produced a protein with a theoretical molecular weight of 23.82 kDa and a theoretical isoelectric point of 9.05. The amino acid sequence of PmAkirin contained two nuclear localization signal (NLS), which were located at positions of 26–30 (KRRRC) and 78–81 (KRRK), respectively (Fig. 1A and B).

Analysis by SMART and SignalP4.1 showed that the PmAkirin polypeptide had no signal peptide and transmembrane domain, but it contained a 31-amino acid defensin/corticostatin family (DEFSN) domain (Fig. 1B).

The alignment of the deduced amino acid sequences of PmAkirin with known Akirins from other species is shown in Fig. 1C. The predicted protein sequence shared the highest homology with *Litopenaeus vannamei* (99%), followed by *Marsupenaeus japonicas* (98%) and *Eriocheir sinensis* (85%). However, the amino acid sequences of PmAkirin shared a relatively low similarity with those of invertebrates such as *Xenopus laevis* (41%), *Homo sapiens* (39%), *Danio rerio* (39%), and *Mus musculus* (38%). The result of the multiple sequence alignments revealed that PmAkirin was highly conserved at its N-terminus and C-terminus (Fig. 1C).

To assess the evolutionary relationship of *PmAkirin*, a phylogenetic tree was constructed using the NJ method. Phylogenetic analysis showed that the Akirins grouped into two major categories: vertebrates and invertebrates. The vertebrate Akirins contained two subtypes, which were Akirin1 and Akirin2. The *P. monodon* PmAkirin protein grouped with the invertebrates and had the closest evolutionary relationship with *P. vannamei* (Fig. 1D).

3.2. Tissue distribution of PmAkirin

The spatial expression pattern of the *PmAkirin* mRNA was determined by qRT-PCR using *EF-1α* as an internal control. The results showed that *PmAkirin* was ubiquitously expressed in all tested tissues, but the expression level varied among these different tissues, which means that PmAkirin may play a multifunctional role in the different tissues of *P. monodon*. The highest relative expression was observed in the hemolymph, followed by the heart, gill, stomach, hepatopancreas, intestine, and muscle (Fig. 2).

3.3. Expression of PmAkirin and AMPs in response to V. parahaemolyticus

To investigate the correlation with *V. parahaemolyticus* infection, the expression of *PmAkirin* was investigated in vivo during *V. parahaemolyticus* infection. In the hepatopancreas, the expression level of

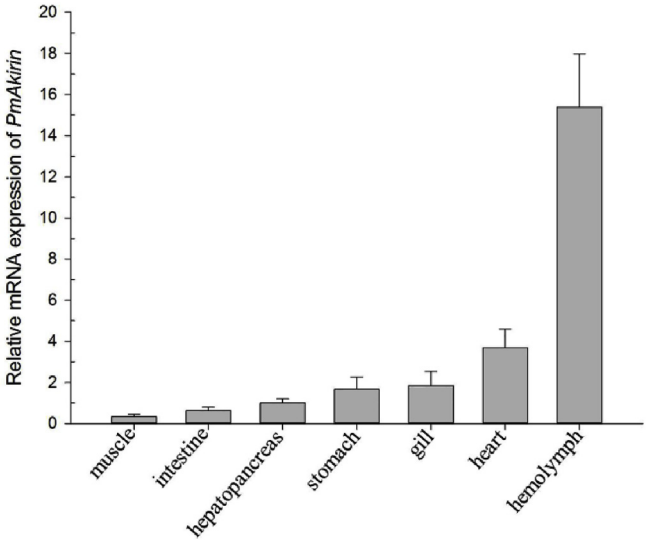


Fig. 2. Relative expression of the *PmAkirin* mRNA in different tissues. The relative expression levels of *PmAkirin* in the muscle, intestine, hepatopancreas, stomach, gill, heart, and hemolymph tissues were analyzed by real-time quantitative PCR. The relative expression levels in the different tissues were calculated by the $2^{-\Delta\Delta Ct}$ method and expressed as the mean ± standard deviation (n ≥ 3).

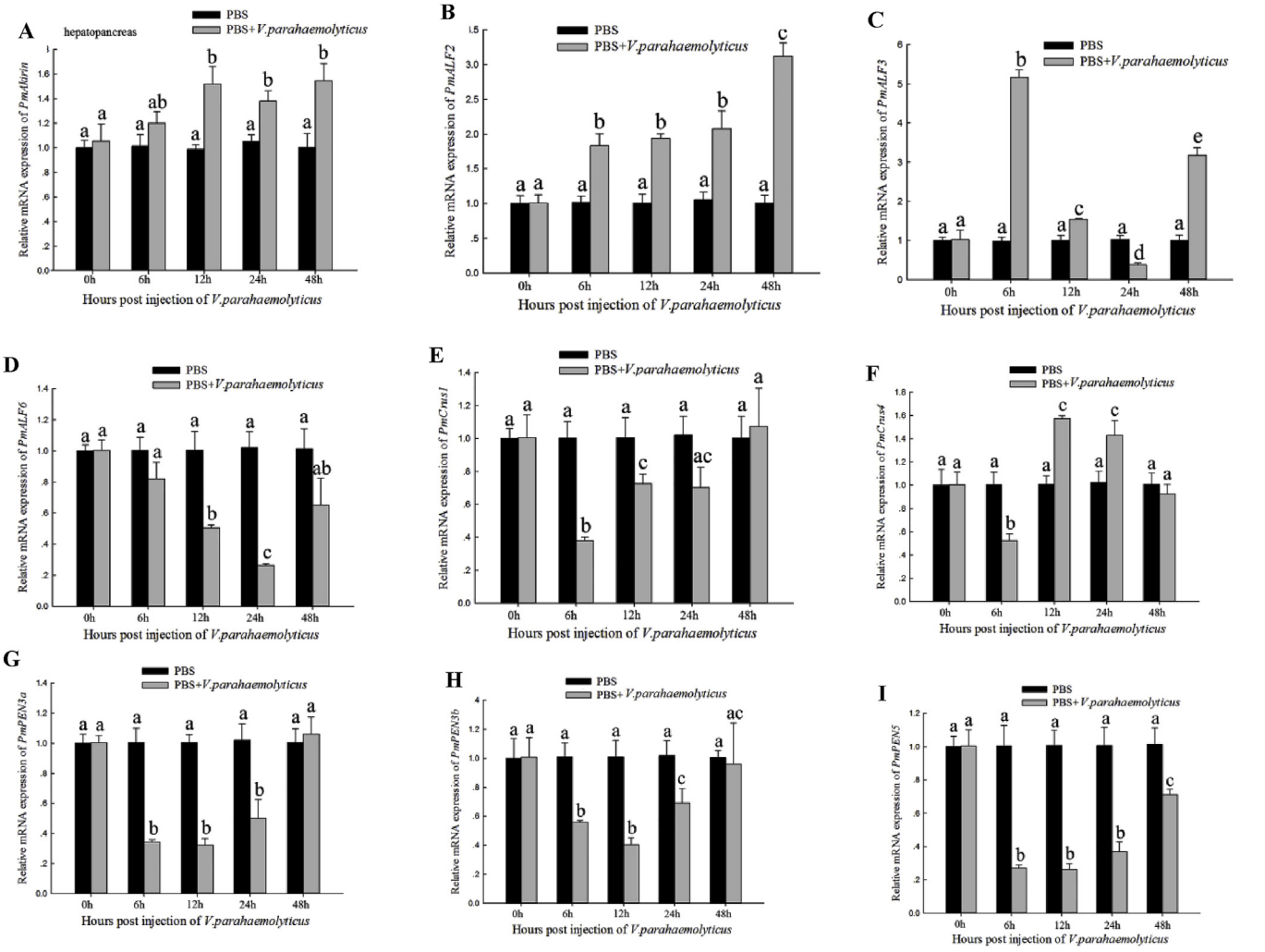


Fig. 3. Expression levels of *PmAkirin* and the AMPs in response to *V. parahaemolyticus*. (A) The relative expression of *PmAkirin* after *V. parahaemolyticus* infection. (B–I) The relative expression levels of the AMPs after *V. parahaemolyticus* infection. The values in the histogram are the mean ± mark difference (n = 3), and the different lowercase letters indicate significant differences ($P < 0.05$).

PmAkirin mRNA increased significantly after infection with *V. parahaemolyticus*. The relative expression level of *PmAkirin* at 6 h, 12 h, 24 h, and 48 h were 1.79, 1.93, 2.07, and 3.11 times those in the PBS group, respectively (Fig. 3A).

Because invertebrates mainly rely on the innate immune system to produce AMPs to resist bacterial invasion, we investigated whether some known shrimp AMPs can respond to *V. parahaemolyticus* by measuring the expression of AMPs after *V. parahaemolyticus* infection. We found that the relative expression levels of *PmALF2, PmALF3,* and *PmCrus4* were upregulated by *V. parahaemolyticus* infection (Fig. 3B,C, and F). However, the relative expression levels of *PmALF6, PmCrus1, PmPEN3a, PmPEN3b,* and *PmPEN5* were downregulated by *V. parahaemolyticus* infection (Fig. 3D, E, and G–I).

3.4. Effect of RNA interference and overexpression detected by ISH and RT-PCR

To examine the efficiency of the dsRNA-Akirin interference and mRNA-Akirin overexpression, the expression of *PmAkirin* was investigated by ISH and RT-PCR after *V. parahaemolyticus* infection.

As shown in Fig. 4F, the RT-PCR revealed that the expression level of *PmAkirin* in the hepatopancreas significantly increased after the overexpression of mRNA-Akirin at 24 hpi, which was 2.36 times that in the blank control group. In contrast, the expression level of *PmAkirin* significantly decreased after the interference of dsRNA-Akirin at 24 hpi, which was 0.53 times that in the blank control group. Meanwhile, the *PmAkirin* mRNA expression levels in the dsRNA-GFP group and PBS control group were not significantly different from that in the blank control group.

Similar results were also found in the ISH experiments, in which the positive signal in the mRNA-Akirin group (Fig. 4E) was stronger than that in the PBS and blank control groups (Fig. 4A and C). In addition, the positive signal in the dsRNA-Akirin group (Fig. 4D) was weaker than that in the blank control, dsRNA-GFP, and PBS groups (Fig. 4A–C). These results indicated that the expression level of *PmAkirin* in *P. monodon* increased after mRNA-Akirin overexpression and was knocked down after dsRNA-Akirin interference.

3.5. Expression of PmAkirin and AMPs in Akirin-silenced and -overexpressed shrimp after infection by V. parahaemolyticus

Twenty-four hours post dsRNA-Akirin and mRNA-Akirin injection, we infected the treated shrimp with *V. parahaemolyticus* and probed the expression levels of the eight selected AMPs at 0, 6, 12, 24, and 48 hpi. The results showed that the expression level of the *PmAkirin* mRNA in the dsRNA-Akirin + *V. parahaemolyticus* group was significantly lower than that in the PBS + *V. parahaemolyticus* group at 0–48 hpi (Fig. 5A). However, the expression level in the mRNA-Akirin + *V.*

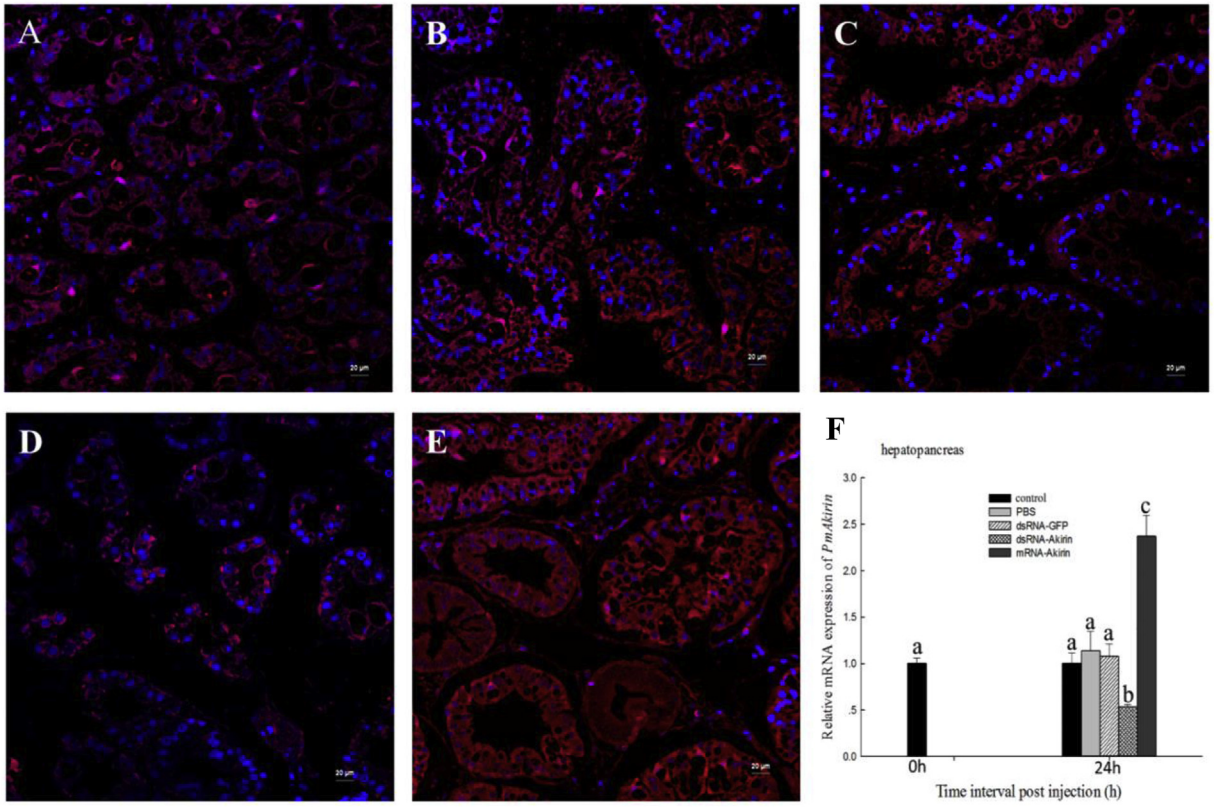


Fig. 4. Efficiency of the RNA interference and overexpression detected by ISH and RT-PCR. (A–E) Detection of the *PmAkirin* expression level by ISH; A, the blank control group of the ISH assay; B and C, the dsRNA-GFP and PBS groups of the ISH assay; D and E, the dsRNA-Akirin and mRNA-Akirin groups of the ISH assay. (F) RT-PCR detection of the relative expression of *PmAkirin* in the hepatopancreas after RNA interference or overexpression.

parahaemolyticus group was significantly higher than that in the PBS + *V. parahaemolyticus* group at 0–24 hpi (Fig. 5A). However, there was no significant difference between the mRNA-Akirin + *V. parahaemolyticus* group and the PBS + *V. parahaemolyticus* group at 48 hpi (Fig. 5A).

Since Akirins are involved in the IMD pathway, which leads to the synthesis of AMPs against the invasion of Gram-negative bacteria, we detected the expression levels of the eight AMPs (*PmALF6*, *PmCrus1*, *PmPEN3a*, *PmPEN3b*, *PmPEN5*, *PmALF2*, *PmALF3*, and *PmCrus4*) in Akirin-silenced and -overexpressed shrimp after *V. parahaemolyticus* infection. Similar to the expression patterns of *PmAkirin*, the expression levels of all AMPs in the dsRNA-Akirin + *V. parahaemolyticus* group were significantly lower than those in the PBS + *V. parahaemolyticus* group at 0–48 hpi (Fig. 5B–I). In addition, the expression levels of the AMPs in the mRNA-Akirin + *V. parahaemolyticus* group were significantly higher than those in the PBS + *V. parahaemolyticus* group at 0–24 hpi (Fig. 5B–I). Moreover, there was no significant difference between the mRNA-Akirin + *V. parahaemolyticus* group and the PBS + *V. parahaemolyticus* group at 48 hpi (Fig. 5B–I). These results suggest that *V. parahaemolyticus* infection in *P. monodon* activates *PmAkirin* expression and *PmAkirin* induces the production of the eight selected AMP genes.

3.6. Detection of *V. parahaemolyticus* clearance in Akirin-silenced and -overexpressed shrimp by the spread plate method

Since *PmAkirin* induces the expression of AMPs in *P. monodon*, we explored whether PmAkirin helps the shrimp clear the *V. parahaemolyticus* infection from the blood. We detected the blood clearance of *V. parahaemolyticus* in *Akirin*-silenced and -overexpressed shrimp after *V. parahaemolyticus* infection. As shown in Fig. 6, there were no *V. parahaemolyticus* colonies in the PBS-group hemolymph. The concentration of *V. parahaemolyticus* in the mRNA-Akirin + *V. parahaemolyticus* group was significantly lower than that in the PBS + *V. parahaemolyticus* group, which was 0.41 times that in the PBS + *V. parahaemolyticus* group (Fig. 6, Table 2). In contrast, the concentration of *V. parahaemolyticus* in the dsRNA-Akirin + *V. parahaemolyticus* group was significantly lower than that in the PBS + *V. parahaemolyticus* group, which was 1.87 times that in the PBS + *V. parahaemolyticus* group (Fig. 6, Table 2). These results indicated that PmAkirin helped the shrimp resist *V. parahaemolyticus* infection.

3.7. Survival rate in Akirin-silenced and -overexpressed shrimp post *V. parahaemolyticus* infection

To further investigate whether PmAkirin could protect the shrimp against *V. parahaemolyticus* infection and improve survival rate, the survival rates of the shrimp in the mRNA-Akirin + *V. parahaemolyticus* and dsRNA-Akirin + *V. parahaemolyticus* groups were recorded during *V. parahaemolyticus* infection. We found that *V. parahaemolyticus* is highly lethal to *P. monodon*, and the peak of death is between 6 and 12hpi (Fig. 7, Table 3). The survival rate in the dsRNA-Akirin + *V. parahaemolyticus* group (6%) was lower than that in the PBS + *V. parahaemolyticus* (26.7%) and dsRNA-Akirin + *V. parahaemolyticus* groups (23.3%) (Fig. 7, Table 3). However, the survival rate in the mRNA-Akirin group was 43.3%, which was higher than that in the PBS + *V. parahaemolyticus* group (26.7%). Hence, we concluded that PmAkirin protects shrimp from bacterial invasion by inducing the production of AMPs.

4. Discussion

In the present study, *PmAkirin* cDNA was cloned and characterized from *P. monodon*. The amino acid sequence of PmAkirin contained two

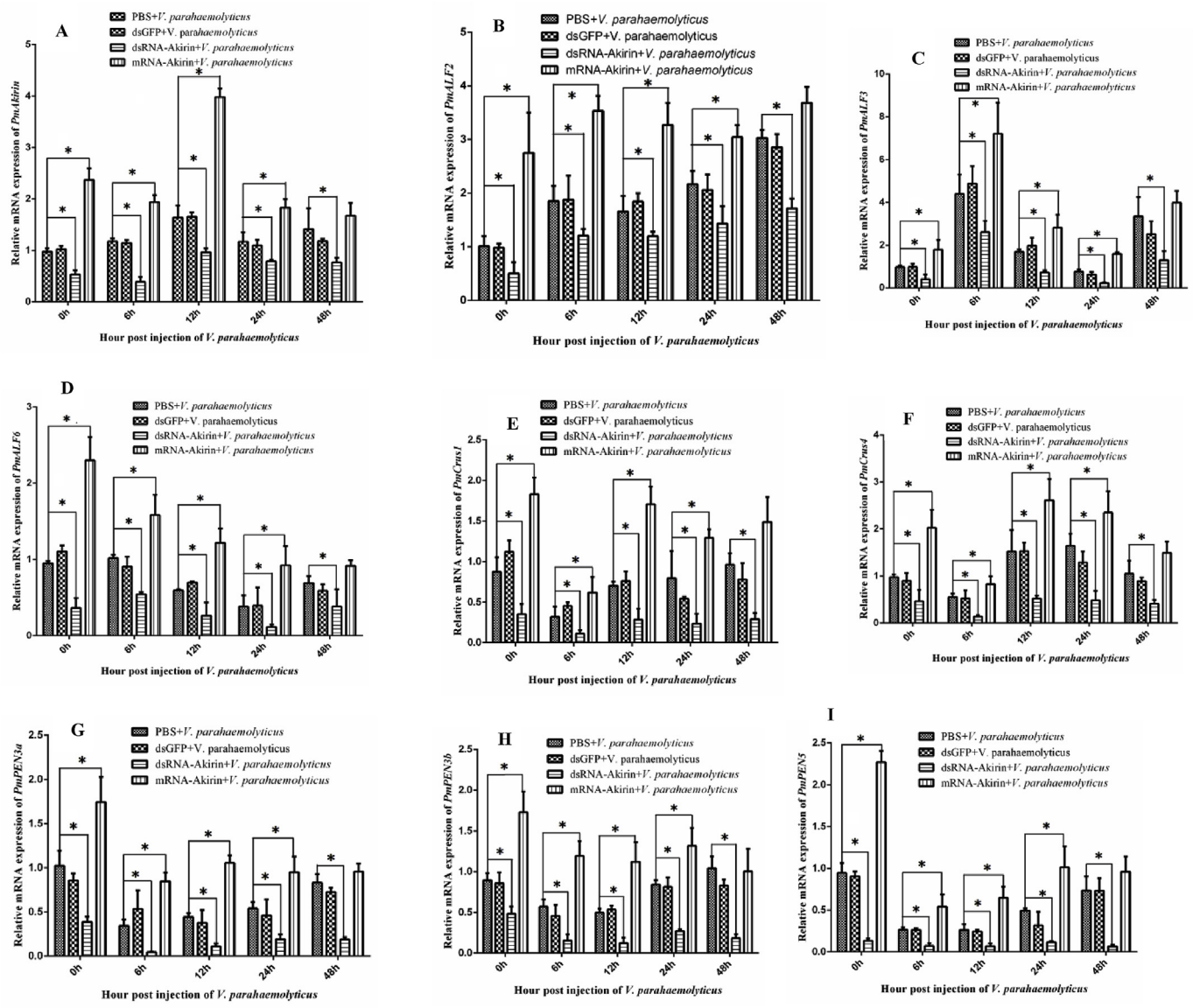


Fig. 5. Expression levels of *PmAkirin* and the AMPs in *Akirin*-silenced and -overexpressed shrimp after infection by *V. parahaemolyticus*. (A) The relative expression of *PmAkirin;* (B–I) The relative expression levels of the AMPs. The values in the histogram are mean ± SD (n = 3), and the * indicates significant differences ($P < 0.05$).

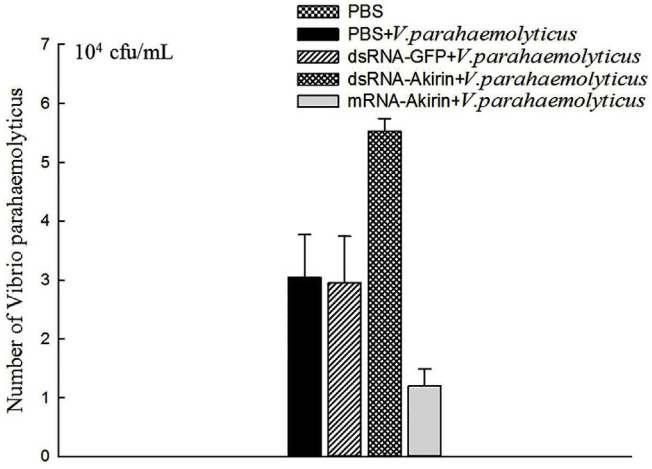


Fig. 6. Blood clearance of *V. parahaemolyticus* in *Akirin*-silenced and -overexpressed shrimp after *V. parahaemolyticus* infection.

NLSs. One of the NLSs located at amino acid position 26–30 has been demonstrated to play an important role in nuclear localization [2]. The function of another NLS at position 78–87 is unclear [4]. Moreover, PmAkirin showed an antibacterial domain DEFSN at position 3–34. The DEFSN domain is a cysteine-rich defensin domain, which can dissociate fungi, enveloped viruses, and Gram-negative and Gram-positive bacteria by forming multimeric transmembrane channels [21], indicating that PmAkirin may have an antibacterial function associated with innate immunity. The multiple sequence alignment result showed that PmAkirin was highly conserved in its N-terminus and C-terminus and was relatively not conserved in the middle region. This may be due to the faster evolution of the middle region in Akirins [5]. The phylogenetic tree analysis indicated that *PmAkirin* clustered with the invertebrate group, and the vertebrate Akirin1 and Akirin2 clustered into another two subtypes. The function of Akirin has been evolutionarily conserved, and Goto et al. found that the absence of Akirin in *Drosophila* caused a phenotypic change, while human Akirin could prevent this change [2]. These data indicated that the *Akirin* gene is highly conserved from invertebrates to vertebrates.

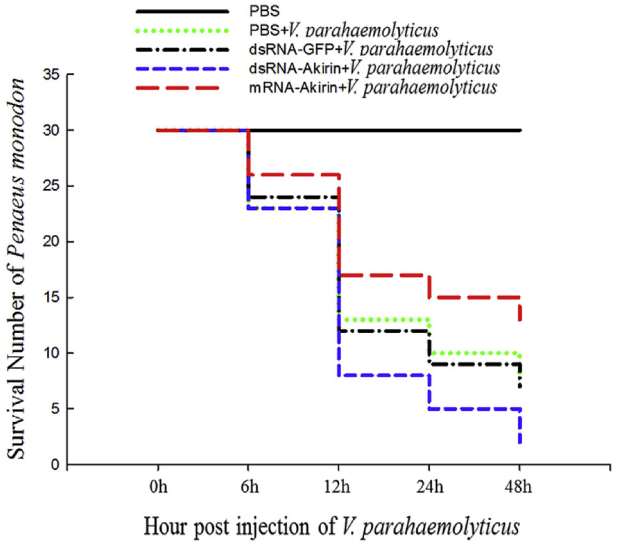


Fig. 7. Survival rate of *Akirin*-silenced and -overexpressed shrimp post *V. parahaemolyticus* infection.

Earlier studies suggested that *Akirin* mRNA is expressed in a variety of tissues in vertebrates [22–26]. In this study, the highest relative expression was observed in the hemolymph, followed by the heart, gill, stomach, hepatopancreas, intestine, and muscle (Fig. 2). The widespread expression of *PmAkirin* in different tissues suggested that *PmAkirin* may paly multiple functions in *P. monodon*. In invertebrates, hemolymph is considered to be the primary immune tissue, and it plays a key role in the recognition and elimination of pathogens via phagocytosis, bursts of reactive oxygen species (ROS), and the production of antimicrobial peptides [27,28]. The high expression level in the hemolymph indicated that *PmAkirin* may have an immune-related function.

Accumulating evidence indicates that pathogen infection could induce the production of *Akirin*. For example, in *Scophthalmus maximus* [9], *Oplegnathus fasciatus* [10], *Paramisgurnus dabryanus* [11], *Crassostrea hongkongensis* [13], *Hippocampus abdominalis* [29], and *Litopenaeus vannamei* [14], the expression of *Akirin* was upregulated by bacterial or viral immunological infections. In the present study, the expression level of the *PmAkirin* mRNA was also significantly upregulated by *V. parahaemolyticus* infection, which means that *PmAkirin* may be involved in the immune response against *V. parahaemolyticus*. Shrimp AMPs play an irreplaceable role in defense against bacterial infection. Our results showed that three out of eight AMPs were upregulated after *V. parahaemolyticus* infection, and the remaining five AMPs were downregulated after *V. parahaemolyticus* infection. This indicated that AMP expression was regulated by *V. parahaemolyticus* infection.

To further study the function of PmAkirin, we used RNA interference and overexpression technology. First, ISH and RT-PCR detection showed that the interference and overexpression were effective (Fig. 4). Twenty-four hours post RNAi interference, the expression levels of *PmAkirin* and the eight selected AMPs significantly decreased after *V. parahaemolyticus* infection. In contrast, the expression levels of *PmAkirin* and the eight selected AMPs significantly decreased after *V. parahaemolyticus* infection in the mRNA overexpression group (Fig. 5). Similar results have also been found in other species. For example, in fish and mollusks, the overexpression of *Akirin* in cells can activate the transcriptional activities of NF-κB and lead to the upregulation of proinflammatory cytokines, including TNF-a, IL-1b, and IL-4 [11,13]. And overexpression of *Akirin* in cells can activate the transcriptional activities of the NF-κB and lead to the up-regulation of pro-inflammatory cytokines [30]. Furthermore, knock down of *Akirin* has been shown to suppress the expression of NF-κB [31] and AMPs [14,30]. Since the *Drosophila* Akirin acts at the level of NF-κB in the immune deficiency (IMD) pathway and is critical for the production of AMPs [2], some researchers predicted that the decreased expression of AMPs is partly because the silenced Akirin reduces the possibility of Dorsal/Relish binding to the transcriptional region of the AMPs. That is, Akirin, as a co-factor, might be needed to bind Dorsal/Relish to the gene transcriptional region [14,32]. Hence, we concluded that *PmAkirin* also regulated the expression of AMPs via the IMD signal pathway.

NF-κB-dependent signaling pathways, which include Toll and IMD pathways in *Drosophila,* as well as TLR and TNF pathways in mammals, are of critical importance to defend against infectious microorganisms. A previous study showed that the RNAi-mediated knock down of Akirin led to impaired IMD pathway signaling and enhanced sensitivity of flies to Gram-negative bacterial infection [2]. When *PmAkirin* was silenced in *P. monodon,* both the blood concentration of *V. parahaemolyticus* and mortality increased significantly. In contrast, when *PmAkirin* was overexpressed, the blood concentration of *V. parahaemolyticus* and mortality decreased significantly. Hence, we conclude that PmAkirin can inhibit the replication of *V. parahaemolyticus* in shrimp and increase the survival rate of *P. monodon* after *V. parahaemolyticus* infection. However, studies on *L. vannamei* have shown different results, in which silencing *Akirin* did not affect the survival of adult shrimp [14].

In the present study, we successfully cloned and characterized the full-length cDNA of *PmAkirin* from *Penaeus monodon*. PmAkirin is evolutionarily conserved, and its expression is responsive to *V. parahaemolyticus* infection. RNAi-mediated knock down of *PmAkirin* significantly reduced the expression of AMPs and increased the blood concentration of *V. parahaemolyticus* and mortality post *V. parahaemolyticus* infection. Overexpression of *PmAkirin* mRNA significantly increased the expression of AMPs and decreased the blood concentration of *V. parahaemolyticus* and mortality post *V. parahaemolyticus* infection. Hence, we suggest that PmAkirin functions as a positive activator in the induction of AMP expression in shrimp innate immunity.

Conflicts of interest

The *authors* declare that they have no competing interests.

Acknowledgements

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Table 3
Results of the artificial infection experiments.

Group	Numbers of tested shrimps	Numbers of death					Number of survivors
		0h	6h	12h	24h	48h	
dsRNA-Akirin + V. parahaemolyticus	30	0	7	15	3	3	2
mRNA-Akirin + V. parahaemolyticus	30	0	4	9	2	2	13
dsRNA-GFP + V. parahaemolyticus	30	0	6	12	3	2	7
PBS + V. parahaemolyticus	30	0	7	10	3	2	8
PBS	30	0	0	0	0	0	30

Scientific Institution Basal Research Fund, CAFS (2018HY-ZD0204), Guangdong Provincial Science and Technology Foundation for Marine and Fishery Development (A201701A04), Central Public interest Scientific Institution Basal Research Fund, South China Sea Fisheries Research Institute, CAFS (No. 2019TS11), Key Research and Development Program of Hainan Province (ZDYF2018163),Natural Science Foundation of Hainan Province (319QN335), National Key R& D Program of China (2018YFD0900103), Innovative Research Team Project of Hainan Natural Science Foundation (2017CXTD021),Modern Agricultural Industrial Technology System Construction Special Shrimp and Crab System (CARS-48), and the High Health Spot Shrimp Seedling Cultivation Technology Integration and Demonstration Promotion Project of Guangdong Provincial Science and Technology Department (2017A040405001).

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