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Caspase-3C gene from red swamp crayfish, *Procambarus clarki*: Characterization and expression in response to pathogenic infection

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ABSTRACT

The caspase is an essential module in the *Drosophila* immune deficiency (IMD) pathway, which plays a crucial role in countering pathogen infection. In this study, a gene named *PcCaspase-3C* was found in *Procambarus clarkia* with a full-length of 4684 bp, including a 1572 bp opening reading frame, which encoded a putative protein of 523 amino acids. PcCaspase-3C contained a CASc domain constituted of 237 amino acids. The *PcCaspase-3C* gene was primarily expressed in heart, stomach, and intestine, while less in gonad, hepatopancreas, gills, and hemocytes, with the least expression in muscle. Infection with *Staphyloccocus aureus*, *Vibrio parahaemolyticus* or white spot syndrome virus (WSSV) induced an up-regulated expression of *PcCaspase-3C* in intestine or stomach to varying degrees. When *PcCaspase-3C* was silenced by double-stranded RNA, the expression of some antimicrobial peptides such as *ALF2*, *ALF5*, *ALF6*, *Cru3, Cru4,* and *Lys* was significantly inhibited. In addition, silencing of *PcCaspase-3C* accelerated infection with WSSV *in vivo*. According to these results, we suggest that *PcCaspase-3C* might play a crucial role in the immune response of *P. clarkia* against pathogenic bacterial and viral infections.

1. Introduction

The white spot syndrome virus (WSSV), which was first discovered as a pathogen in China, is now widespread in aquaculture worldwide [1]. Beyond that, some bacteria are also pathogenic agents in aquatic animals. Crustacean, a kind of invertebrates, have no adaptive immunity, but have a deficient innate immune system, by which the pathogens are mainly defended [2,3]. In recent years, with the rapid development of crayfish farming, the consumer market and the industrial chain continue to expand [4]. However, the cultivation of red swamp crayfish has been increasingly affected by various pathogens such as WSSV, *Staphylococcus aureus*, and *Vibrio parahaemolyticus*, causing huge economic losses to the crayfish aquaculture industries [5,6]. Hence, it is urgent to unravel the innate immune system of crayfish and to confirm its anti-pathogen activity and related molecular mechanism.

In most organisms, apoptosis is one of the vital biological processes to withstand pathogenic bacteria and maintain normal cellular homeostasis [7,8]. As programmed cell death [9], apoptosis, can maintain the health of organisms by removing the impaired, dangerous and useless cells [10]. Nevertheless, only some pathogens can elicit host apoptotic response during infection which may evolve some defense and response mechanisms [11]. It is reported that apoptosis could be detected on a large scale in shrimp after infection by pathogenic microorganisms, and this also leads to the shrimp mortality [12–15].

In recent years, many researchers have paid their attention to caspases, an aspartate-specific cysteine protease playing essential roles in the process of apoptosis [8,16–18]. Some studies have verified that the elimination of the caspases activity can reduce apoptosis [19]. In mammals, the identified caspases can be sorted into three types: upstream initiator apoptotic caspases (caspase-2, -8, -9, -10), downstream executioner or effector apoptotic caspases (caspase-3, -6, -7), and pro-inflammatory caspases (caspase-1, -4, -5, -11, −12) [20,21]. Commonly, the apoptosis is induced by two pathways: one is extrinsic apoptotic pathway mediated by caspase-8, the other is an intrinsic

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apoptotic pathway mediated by the activation of caspase-9 [21,22]. Both of them can activate caspases-3, making it the vital executioner member of apoptosis. In invertebrates, researchers have demonstrated that the activity of caspase-3 increases significantly after pathogenic infection or stress treatment. A high expression level of *caspase-3* gene can be detected when *Penaeus merguiensis* is infected with WSSV [23]. The expression of caspase from *Marsupenaeus japonicus* is upregulated in survivors of WSSV-challenged shrimp and WSSV-induced apoptosis is significantly suppressed when the gene is silenced [11]. Caspase-3C in the gill of *Macrobrachium nipponense* is regulated by hypoxia in a time-dependent manner, suggesting that caspase-3C participates in the apoptotic pathway [20].

In *Procambarus clarkia*, whether caspases-3 plays a role in innate immunity is still elusive. In this study, we isolated a homologue of caspase-3, named as *PcCaspase-3C* and detected the tissue distribution profile as well as the expression patterns of *PcCaspase-3C* in response to bacterial and viral infection. Besides, the RNA interference was implemented to determine the function of PcCaspase-3C in innate immunity of *P. clarkia*. Our study suggests that *PcCaspase-3C* played an important role in the immune response of *P. clarkia* against both bacterial and viral pathogen infections.

2. Materials and methods

2.1. Experimental animals and immune challenge

Healthy adult crayfish were purchased from an aquaculture farm in Zhenjiang, Jiangsu Province, China, and each body was about 15 g in weight. They were cultured in the laboratory with enough freshwater and aeration at 23–25 °C for 10 days before the experiments.

The crayfish were randomly divided into three groups: normal group, challenge group, and control group. The stomach and intestine were collected at different time points. For the normal group, crayfish were allowed to grow without any further processing. For bacteria challenge group, bacteria (*Staphylococcus aureus* or *Vibrio parahaemolyticus*) were shaking cultured for 16 h in Luria-Bertani (LB) broth at 37 °C, and then diluted with PBS to a concentration of approximately 3.0×10^6 Colony-Forming Units (CFU) per milliliter. Each crayfish was injected with 100 μL bacteria suspension using 1 mL sterile syringe. Tissues were collected at 2 h, 6 h, 12 h, and 24 h post-infection. For virus challenge group, WSSV (approximately 3×10^7 copies per milliliter) was diluted to 3.0×10^6 copies per milliliter with PBS,and each crayfish was injected with 100 μL virus suspension. Tissues were collected at 12 h, 24 h, 36 h, and 48 h post-infection. Bacteria and WSSV in this study were all obtained from our laboratory. For the control group, 100 μL PBS was injected individually, and the tissues were collected at 2 h, 6 h, 12 h, 24 h, 36 h, and 48 h. Three crayfish were randomly sampled to eliminate individual differences and the experiments were performed in triplicate. All samples were stored at −80 °C for further use.

2.2. Total RNA extraction and cDNA synthesis

Different tissues in healthy crayfish (hemocytes, heart, hepatopancreas, gills, stomach and intestine) and infected crayfish (stomach and intestine) were sampled from three crayfish to extract total RNA via an RNA pure high-purity total RNA rapid extraction kit (Spin-column, BioTeke, Beijing, China) according to the manufacturer's protocol. The PrimeScript® 1st Strand cDNA Synthesis Kit (Takara, Dalian, China) was used to synthesize the first-strand cDNA with an oligo(dT) primer for the quantitative real-time PCR (qRT-PCR) analysis in accordance with the manufacturer's protocol (Takara, Code No. 6210 A).

2.3. Cloning of the full-length of PcCaspase-3C

Two gene-specific primers, PcCaspase-3C–F for 3′ fragment and PcCaspase-3C-R for 5′ fragment of *PcCaspase-3C* (Table 1) were designed based on the unigenes from *P. clarkia* transcriptome data. The 5′ and 3' fragments were cloned by using a Clontech SMARTer™ RACE cDNA Amplification Kit (Takara). The reaction mixture was subjected to the following amplification program on a thermocycler (Bioteke): five cycles at 94 °C for 30 s and 72 °C for 3 min; 5 cycles at 94 °C for 30 s, 70 °C for 30 s, and 72 °C for 3 min; and 20 cycles at 94 °C for 30 s, 68 °C for 30 s, and 72 °C for 3 min. Full-length cDNA of *PcCaspase-3C* was obtained by overlapping 5′ and 3′ fragments.

Table 1
List of primers used in this study.

Primer name	Sequences (5′-3′)
PcCaspase-3C–F	TCACAGGCACAATGCTACAAATGGGAGA
PcCaspase-3C-R	GCAAGAAACAACGGCAAGTCGTGGGT
PcCaspase-3C-qRT-F	AGGCACTTTTGAACAAACCA
PcCaspase-3C-qRT-R	CGAGATGAGGCAGTCACTCT
Pc-Cru3-qRT-F	TACGTCTTGCCCTCGTCTTA
Pc-Cru3-qRT-R	CAGCGTCCTCCTCTTTGTAATC
Pc-Cru4-qRT-F	CTCTGACTGCCAGGTGTTT
Pc-Cru4-qRT-R	TGCGAGCTGTGATGGTTAG
Pc-ALF2-qRT-F	CGTGGGAGTGTTTGTGGTGGT
Pc-ALF2-qRT-R	TTGGACTGTAACTGTAGCGGC
Pc-ALF5-qRT-F	ATGGGGAGGTGAGGCTACT
Pc-ALF5-qRT-R	CCTTCCTGCTCGGTGATGA
Pc-ALF6-qRT-F	ACAAATGAACACAAGCCACCC
Pc-ALF6-qRT-R	TGATAAACCTGTCCTCCCAAC
Pc-Lys-qRT-F	GTCAACCCACCCTCAATAAC
Pc-Lys-qRT-R	CTTGTGAATCAGGGCGTA
Pc-18S rRNA-qRT-F	ACCGATTGAATGATTTAGTGAG
Pc-18S rRNA-qRT-R	TACGGAAACCTTGTTACGAC
PcCaspase-3C-iF	GCGTAATACGACTCACTATAGGGCAACAGGACACCTGCACC
PcCaspase-3C-iR	GCGTAATACGACTCACTATAGGGGACACTGCACATAGCCAC
GFP-iF	GCGTAATACGACTCACTATAGGTGGTCCCAATTCTCGTGGAAC
GFP-iR	GCGTAATACGACTCACTATAGGCTTGAAGTTGACCTTGATGCC
WSSV-F	TTGGTTTCAGCCCGAGATT
WSSV-R	CCTTGGTCAGCCCCTTGA
WSSV-TaqMan probe	FAM-TGCTGCCGTCTCCAA-TAMRA

2.4. Bioinformatics analysis

The online program of BLAST (http://www.ncbi.nlm.nih.gov/blast) was used to analyze the cDNA and deduced amino acid sequences of PcCaspase-3C. The predicted isoelectric point (pI) and calculated a molecular weight (MW) of PcCaspase-3C were obtained by ExPASy (http://web.expasy.org). Signal peptide and domain organization were predicted with the Simple Modular Architecture Research Tool (http://smart.embl-heidelberg.de/). Multiple sequence alignments were performed with ClustalW and visualized with Genedoc program. A phylogenetic tree was built using the neighbor-joining method in MEGA 6.06 software [24]. Bootstrap values (%) of 1000 replicates were calculated for each node of the consensus tree obtained.

2.5. Tissue distribution and expression patterns of PcCaspase-3C after challenge

The tissue distribution of *PcCaspase-3C* in hemocytes, heart, hepatopancreas, gills, stomach, intestine, gonad, and muscle was analyzed via qRT-PCR using the specific primers PcCaspase-3C-qRT-F and PcCaspase-3C-qRT-R. *Pc-18S rRNA* was used for internal standardization. The expression patterns of *PcCaspase-3C* in stomach and intestine

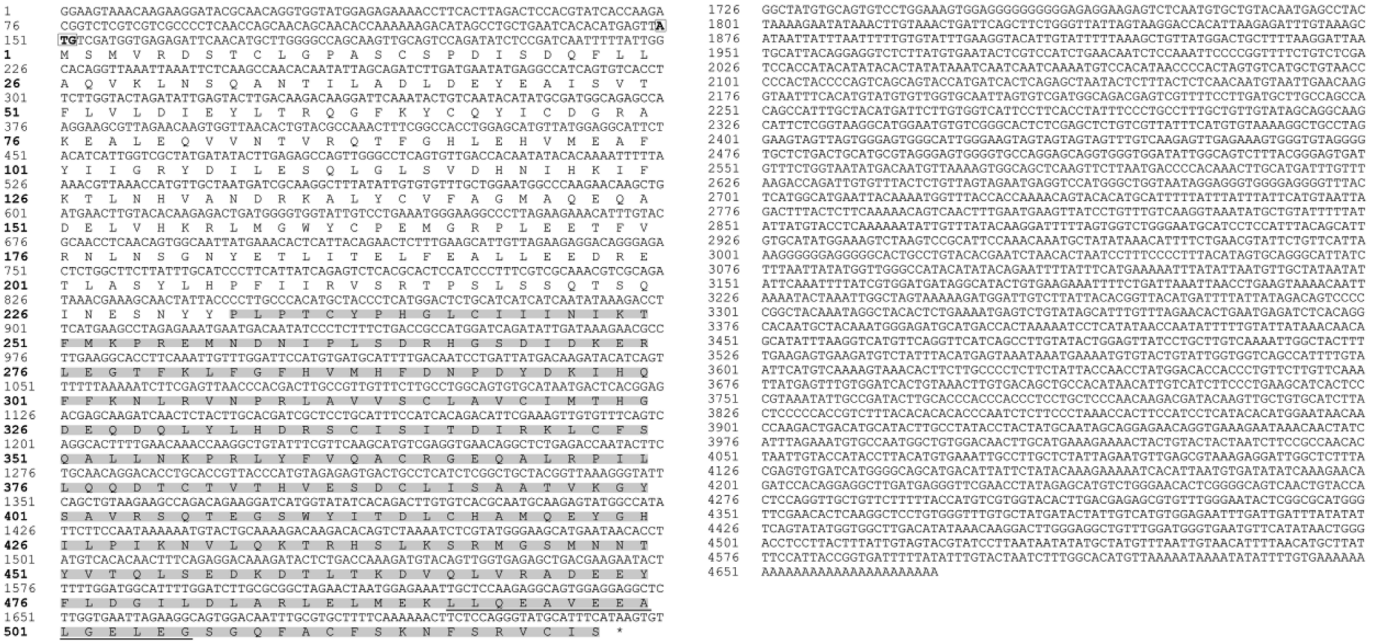

Fig. 1. Nucleotide and deduced amino acid sequence of PcCaspase-3C from *P. clarkia*. Deduced amino acid residues were numbered in bold on the left. A low complexity region sequence was underlined, and the CASc domain was shown in gray shade. The initiation codon (ATG) was shown in box.

after *S. aureus*, *V. parahaemolyticus*, WSSV or PBS challenge were also analyzed via qRT-PCR. According to the manufacturer's instructions, the TransStart Top Green qPCR SuperMix (2 ×) (TransGen Biotech, Beijing) was used in the qRT-PCR assay. The amplification procedure was as follows: 94 °C for 30 s, followed by 40 cycles of 94 °C for 5 s, and 60 °C for 30 s. The obtained data were calculated using the $2^{-\triangle\triangle CT}$ method [25]. All primers are listed in Table 1.

2.6. RNA interference of PcCaspase-3C

Tow specific primers (PcCaspase-3C-iF, PcCaspase-3C-iR) were synthesized for *PcCaspase-3C* RNA interference (RNAi) assay. The MEGAscript RNAi Kit (Thermo Fisher Scientific, USA) was used to synthesize the dsRNA *in vitro* following the instructions of the manufacturer. *GFP*-dsRNA was also synthesized for scrambled control.

Healthy adult crayfish were randomly divided into three groups (just like the immune challenge): normal group, challenge group, and control group. For challenge group, crayfish were injected twice with 30 μg of *PcCaspase-3C*-dsRNA (dissolved in RNase-free phosphate-buffered saline (PBS)) each time with an interval of 12 h. As a control group, *GFP*-dsRNA was injected into crayfish in the same manner. The intestine was collected at 48 h after the first injection, and the RNA extraction and cDNA synthesis were conducted as mentioned above. The qRT-PCR assay was conducted to determine the efficiency of RNAi. All primers used are listed in Table 1.

2.7. Expression patterns of AMPs after RNAi

Another RNAi plus bacterial or WSSV challenge experiment was conducted to evaluate its effect on AMP expressions. When crayfish interfered for 48 h, each group was divided into two sets. One was injected with 100 μL of *V. parahaemolyticus* (approximately 3×10^6 CFU/mL), whereas the other was injected with 100 μL of WSSV (approximately 3×10^6 copies/mL). Then the intestine in the first set was collected at 24 h post-infection for cDNA synthesis, while the other was collected at 48 h for genomic DNA extraction. The expression levels of AMP genes (*ALF2*, *ALF5*, *ALF6*, *Cru3*, *Cru4,* and *Lys*) were determined by using qRT-PCR.

2.8. Determination of WSSV copies and cumulative mortality of crayfish

To quantify the WSSV copies in *P. clarkia* after RNAi, the genomic DNA was extracted with Genomic DNA Extraction Kit (Takara, Dalian, China) following the manufacturer's instructions. WSSV-specific primers (WSSV–F, WSSV-R) and a TaqMan probe (WSSV-TaqMan probe) were used in qRT-PCR assay with the Premix Ex Taq Kit (Takara, Dalian, China). The program was run as follows: 95 °C for 1 min, followed by 50 cycles of 95 °C for 5 s, 52 °C of 20 s, and 72 °C for 20 s. All primers used above are listed in Table 1.

To analyze cumulative mortality of crayfish, four groups of 20 individuals were kept in tanks for 10 days and the populations were counted and recorded every day. Dead crayfish were removed immediately, and aquatic water was replaced with clean water.

2.9. Statistical analysis

All biological experiments were repeated thrice independently. Numerical data were analyzed using a one-way analysis of variance (ANOVA). The statistical significance between treatments was analyzed using Student's *t*-test. Significant difference accepted (*P*-value) was less than 0.05.

3. Results

3.1. Cloning and characterization of PcCaspase-3C

The *PcCaspase-3C* cDNA obtained from *P. clarkia* was 4684 bp in length, including an open reading frame (ORF) of 1572 bp which encode for a 523-amino (aa) protein, a 5′-untranslated region (UTR) of 161 bp, and a 3′-UTR of 2951 bp with a poly(A) tail (Fig. 1A). SMART analysis indicates that PcCaspase-3C protein contained a CASc domain constituted of 237 amino acids and a low complexity region (Fig. 1B). The theoretical pI and molecular weight of PcCaspase-3C were 5.42 and 59.79 kDa, respectively.

3.2. Phylogenetic analyses and multiple alignments of PcCaspase-3C

The neighbor-joining method with 1000 bootstrap replicates was

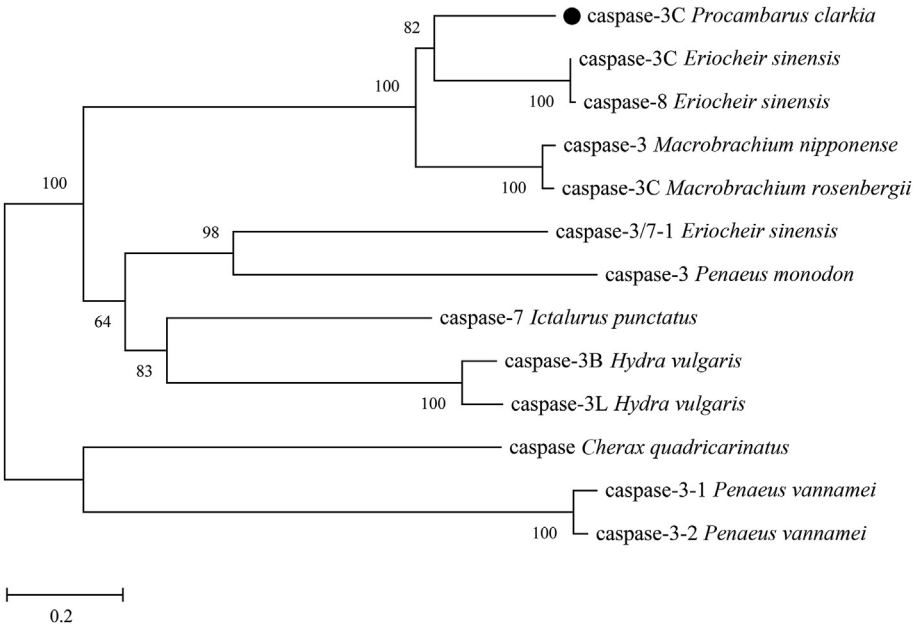

Fig. 2. Phylogenetic analysis of PcCaspase-3C with other proteins that contain CASc domain. The corresponding GenBank accession numbers were shown as below: caspase-3C from *Eriocheir sinensis* (AGT29868.1); caspase-8 from *Eriocheir sinensis* (AKS36884.1); caspase-3 from *Macrobrachium nipponense* (AMZ03555.1); caspase-3C from *Macrobrachium rosenbergii* (AET34920.1); caspase-3/7-1 from *Eriocheir sinensis* (AGT29867.1); caspase-3 from *Penaeus monodon* (ADV17345.1); caspase-7 from *Ictalurus punctatus* (XP_017338664.1); caspase-3B from *Hydra vulgaris* (AAF98012.1); caspase-3L from *Hydra vulgaris* (NP_001296683.1); caspase from *Cherax quadricarinatus* (AYU65464.1); caspase-3 from *Penaeus vannamei* (ROT80957.1, ROT80956.1).

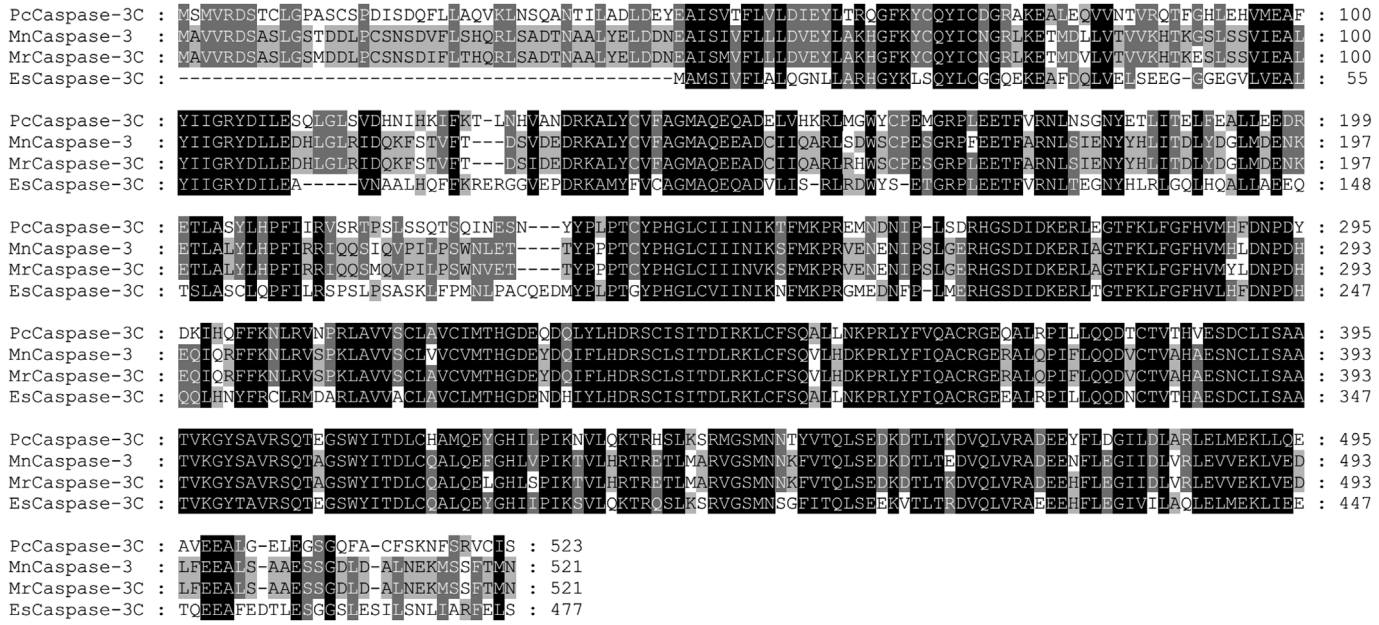

Fig. 3. Multiple alignment of PcCaspase-3C with counterparts from other species. The GenBank accession numbers of sequences used for alignment were as follows: caspase-3 from *Macrobrachium nipponense* (MnCaspase-3, AMZ03555.1); caspase-3C from *Macrobrachium rosenbergii* (MrCaspase-3C, AET34920.1); caspase-3C from *Eriocheir sinensis* (EsCaspase-3C, AGT29868.1).

used to build a phylogenetic tree for analyzing the relationship between PcCaspase-3C with other proteins containing CASc domain. Fig. 2 shows that PcCaspase-3C was clustered into the crustacean group. Multiple sequence alignment was set up on three caspases of crustacean. As shown in Fig. 3, caspase from different species was highly conserved. In particular, PcCaspase-3C shared 58.22%, 62.02% and 62.02% identity with Mncaspase-3, Mrcaspase-3C and Escaspase-3C, respectively.

3.3. Tissue distribution and expression patterns of PcCaspase-3C after pathogenic infection

qRT-PCR analysis was used to determine the tissue-specific expression profile of *PcCaspase-3C* in crayfish, and to investigate the transcript level of *PcCaspase-3C* in different tissues. Fig. 4 reveals that *PcCaspase-3C* was widely distributed in all selected tissues. Specifically, *PcCaspase-3C* was primarily expressed in heart, stomach, and intestine, and less in the gonad, hepatopancreas, gills, and hemocytes, with the least expression in muscle. When challenged by *S. aureus*, the expression of *PcCaspase-3C* in stomach increased rapidly, reached a peak at 2 h and then returned to normal, whereas that in intestine increased gradually until 24 h post-infection (Fig. 5A). When challenged by *V. parahaemolyticus*, the gene expression levels in stomach and intestine increased significantly at different time points post-infection (Fig. 5B). When challenged by WSSV, *PcCaspase-3C* expression in stomach kept growing until 36 h and dropped at 48 h, whereas that in the intestine was slightly upregulated at 24 h and decreased to the normal at 48 h (Fig. 5C). Furthermore, no significant change in *PcCaspase-3C* expression was detected in both tissues of the PBS group (Fig. 5D).

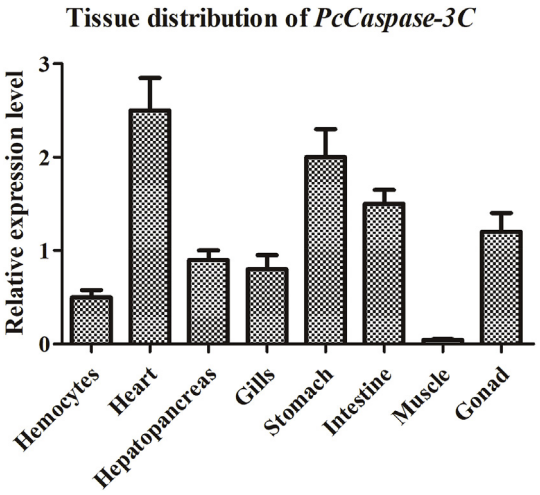

Fig. 4. Tissue distribution of *PcCaspase-3C* in hemocytes, heart, hepatopancreas, gills, stomach, intestine, gonad and muscle of *P. clarkia*.

3.4. Expression of AMP genes after RNAi of PcCaspase-3C

To further assess the roles of PcCaspase-3C in the immune response of crayfish, *PcCaspase*-3C-specific dsRNA was injected into *P. clarkia*. Fig. 6A shows that the expression of *PcCaspase-3C* was significantly reduced when compared with the control groups. *V. parahaemolyticus* infection induced an increment of *PcCaspase-3C* expression in the normal group. However, *PcCaspase-3C* kept at an extremely low level in *PcCaspase-3C*-dsRNA group even after *V. parahaemolyticus* infection (Fig. 6B). It is worth noting that *GFP*-dsRNA injection downregulated the expression level of *PcCaspase-3C* to some extent when compared with the group injected only with *Vibrio*, which might be attributed to the stress response of dsRNA injection in crayfish. In addition, silencing of *PcCaspase-3C* significantly decreased the expression of AMP genes (*ALF2*, *ALF5*, *ALF6*, *Lys*, *Cru3*, *Cru4*) when compared with the *GFP*-dsRNA group (Fig. 7).

3.5. Effects of PcCaspase-3C on WSSV infection

Viral infections can result in the initiation of apoptosis, and multiple caspases are involved in this process [26]. To determine the effects of PcCaspase-3C on viral infection, *PcCaspase-3C* expression was knocked down and WSSV copies were detected at 48 h post-infection. Fig. 8A shows that silencing of *PcCaspase-3C* led to an increment of WSSV copies when compared with the *GFP*-dsRNA-injection and WSSV only groups. To evaluate the effects of PcCaspase-3C on the mortality of WSSV-challenged crayfish, dsRNA-injected crayfish were observed for ten days. Interestingly, 100% mortality was recorded in the group injected only with WSSV on day 8, while *PcCaspase-3C* silencing group reached the mortality of 100% on day 6 (Fig. 8B). These results indicate that *PcCaspase-3C* played a positive role in the innate immunity of *P. clarkia* against viral infection.

4. Discussion

Crustacean have no adaptive immune but have a deficient innate immune system, by which the pathogens are mainly defensed [27]. Apoptosis is considered as a kind of programmed cell death, and it also participates in cell growth, differentiation and innate immunity [11,28]. In order to explore the anti-bacterial and anti-viral properties of caspase, we cloned and characterized a *PcCaspase-3C* in *P. clarkia* which was highly homologous to the caspase-3C in *Eriocheir sinensis*, caspase-3 in *M. nipponensis* and caspase-3C in *Macrobrachium rosenbergii* [29]. The high sequence similarity of these proteins suggests that the

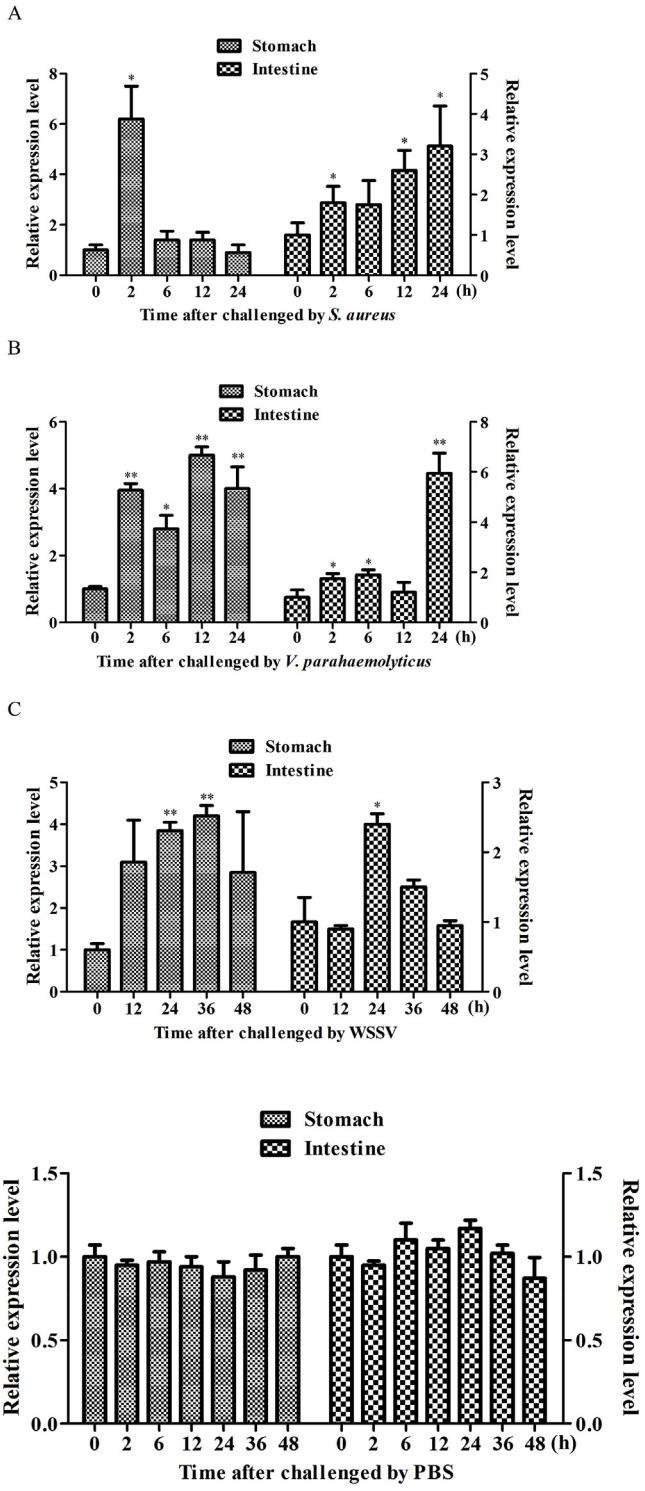

Fig. 5. Expression patterns of *PcCaspase-3C* in stomach and intestine of *P. clarkia* challenged by *S. aureus* (A), *V. parahaemolyticus* (B), WSSV (C) or PBS (D). Asterisks indicated significant differences (*$P < 0.05$, **$P < 0.01$) compared with values of control. Error bars represent ± S.D. of three independent PCR amplifications and quantifications.

caspase proteins might have a similar function in the innate immune system of crustacean. A previous study revealed that caspase 3C and 3 share a similar protein structure and the two genes are considered to be located at the same chromosome, but at different locus [30]. Therefore, it is difficult to identify and classify homologue genes into caspase 3C or 3 based on their protein structures. In the phylogenetic tree, PcCaspase-

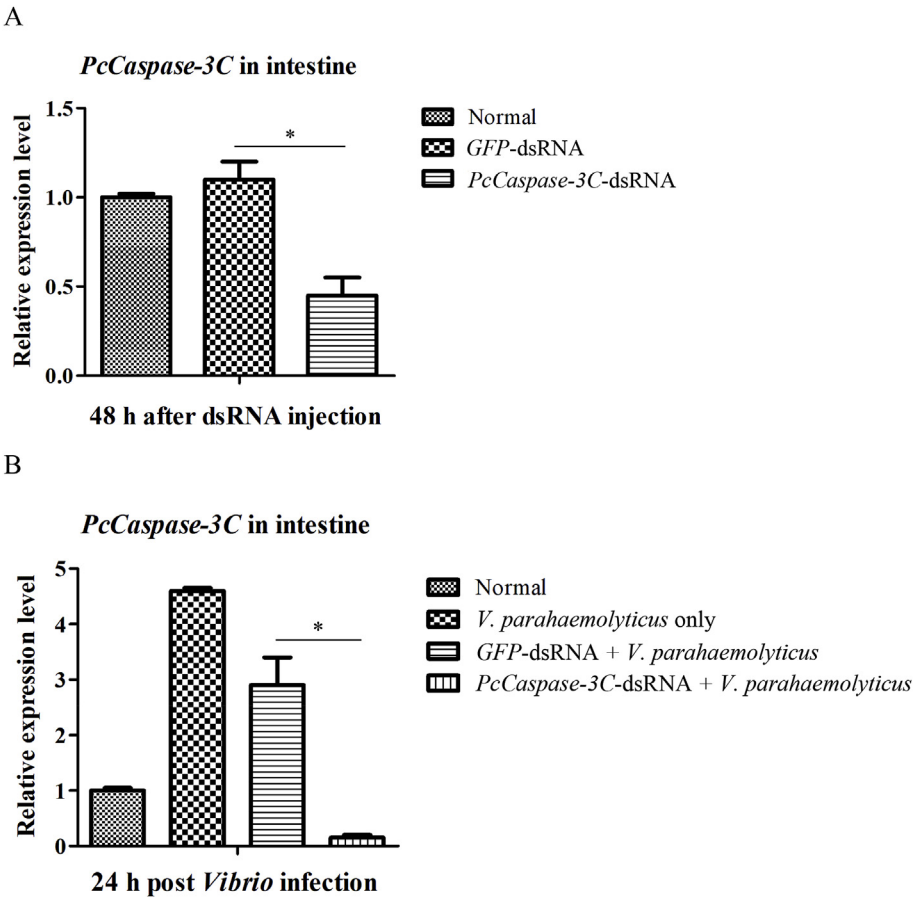

Fig. 6. Silencing of *PcCaspase-3C* by *PcCaspase-3C*-specific dsRNA. (A) Transcript level of *PcCaspase-3C* in intestine of crayfish at 48 h after dsRNA injection. (B) Expression of *PcCaspase-3C* in intestine of *V. parahaemolyticus*-injected crayfish after dsRNA injection. Asterisks indicate significant differences (*$P < 0.05$) compared with values of control. Error bars represent ± S.D. of three independent PCR amplifications and quantifications.

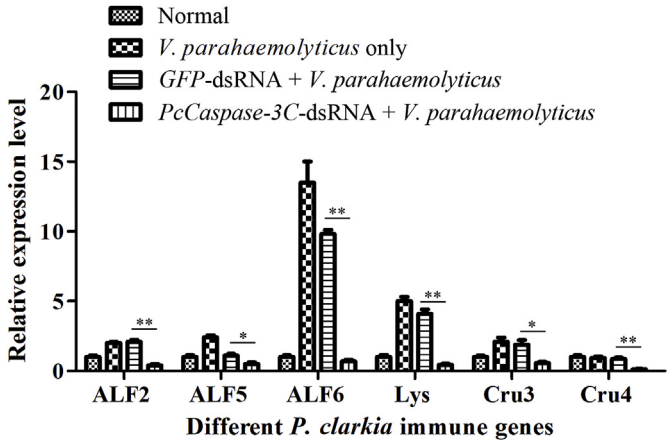

Fig. 7. AMPs expression in intestine of *V. parahaemolyticus*-injected crayfish after *PcCaspase-3C* silencing. Asterisks indicate significant differences (*$P < 0.05$, **$P < 0.01$) compared with values of control. Error bars represent ± S.D. of three independent PCR amplifications and quantifications.

3C was clustered and had high similarity with other caspases 3C from *E. sinensis* and *M. rosenbergii*. Hence, it suggests that PcCaspase-3C belonged to caspase 3C family.

Damaged or unnecessary cells are cleared by apoptosis in almost all organs of organisms [31]. In this study, *PcCaspase-3C* was found widely distributed in various tissues with the high expression in heart, stomach, and intestine, which was similar with the expression pattern of caspase-3 in *M. nipponense* [20], *Pseudosciaena crocea* [32], and *E. sinensis* [21]. In rat and mice, caspase-3 is engaged in signaling pathway activation during heart disease occurrence [33,34]. High expression of PcCaspase-3C in heart suggests that PcCaspase-3C played a vital role in physiological and pathological processes of heart. Stomach and intestine are organs for food digestion and nutrition absorption and serve as the first defense barrier against pathogen invasion [35]. When food from a complex environment is ingested by the crayfish, pathogens may infect the host *in situ* [30].

Challenge test indicates that the PcCaspase-3C in *P. clarkia* had an enormous effect on pathogen infection. As reported, crayfish are most likely to be infected by viruses and bacteria [36,37]. To explore the role of PcCaspase-3C in the innate immunity of *P. clarkia* against infection, the expression patterns of *PcCaspase-3C* in stomach and intestine were detected. The gene was significantly upregulated at different levels at certain time points after challenge. Specifically, PcCaspase-3C was engaged in the early stage of *S. aureus* infection in the stomach and the whole infection process in intestine. *Es-casp* in hemocytes of *E. sinensis* is upregulated after exposing to *Vibrio anguillarum* for 4 h, 8 h and 12 h [38], whereas *cap-3* in the hemolymph of *Penaeus merguiensis* increases at 24 h post-WSSV injection [23]. In general, caspase-3 might play a role in the innate immune response of *P. clarkia* against bacterial and viral infection.

Antimicrobial peptides (AMPs) are important components of the innate immune system of crustacean [39]. The known AMPs in the crustacean, such as anti-lipopolysaccharide factor (ALF), crustin and lysozyme [40], are involved in innate immunity in different ways [41]. A previous study reveals that IMD pathway regulated the expression of these AMP genes in crayfish [42]. In this study, in order to verify the relationship between AMPs and PcCaspase-3C (an essential module in the IMD pathway) in *P. clarkia*, *PcCaspase-3C* was knocked down by dsRNA. Silencing of *PcCaspase-3C* significantly inhibited the expression level of AMPs such as *ALF2*, *ALF5*, *ALF6*, *Lys*, *Cru3* and *Cru4*.

To date, a few studies have been reported on the role of caspase in the innate immunity of crustacean. In this study, we showed that the

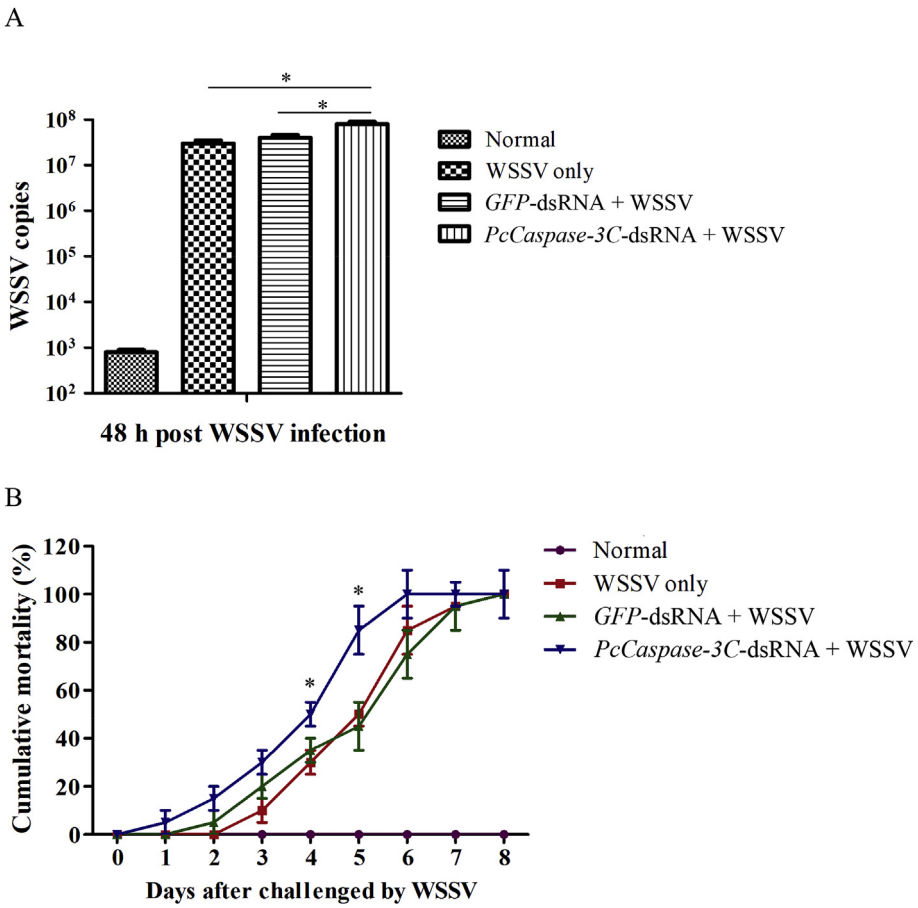

Fig. 8. Detection of WSSV copies in intestine (A) and cumulative mortality (B) of WSSV-infected crayfish after *PcCaspase-3C* silencing. Asterisks indicate significant differences (*$P < 0.05$) compared with values of control. Error bars represent ± S.D. of three independent experiments.

interference of *PcCaspase-3C* significantly increased the WSSV copies in the intestine and the cumulative mortality of crayfish, indicating that PcCaspase-3C was involved in the innate immunity of *P. clarkia* by inhibiting WSSV replication. Similarly, Yan-yao et al. [43] reported that delivery of recombinant caspase-3 of *Cherax quadricarinatus* into hematopoietic cells results in significantly decreased expression of WSSV proteins, suggesting that CqCaspase, possibly by the enhanced apoptotic activity, has a strong negative effect on WSSV infection. Hence, data in this study further confirm that caspases could play essential roles in innate immunity of crustacean mainly by regulating the apoptotic activity. As reported, Caspase-3/7-1 from *E. sinensis* acts as an LPS receptor and plays an important role in the regulation of immune homeostasis [44]. Further studies are recommended to explore the molecular mechanism of PcCaspase-3C in innate immunity of *P. clarkia* and its exact role in the process of apoptosis.

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