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A new crustin is involved in the innate immune response of shrimp *Litopenaeus vannamei*

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ABSTRACT

Crustin is an antimicrobial peptide (AMP) that plays a key role in the innate immunity of crustaceans. This study cloned a new crustin from Pacific white shrimp *Litopenaeus vannamei*, which we designated as LvCrustinB, using rapid amplification of cDNA ends (RACE). The full-length cDNA of LvCrustinB is 751 bp with an open reading frame (ORF) of 591 bp encoding a peptide of 196 amino acids that includes a putative signal sequence. LvCrustinB is a type II crustin that has a glycine-rich region and a single whey acidic protein domain (WAP) domain. The mRNA transcript of LvCrustinB was detected in all examined tissues and was found to be most abundantly expressed in the epithelium and muscle. The expression of LvCrustinB in hemocytes was significantly upregulated after *L. vannamei* was challenged with LPS, *Vibrio parahaemolyticus*, and white spot syndrome virus (WSSV). When LvCrustinB was knocked down with RNAi, the mortality rate of *L. vannamei* significantly increased after *V. parahaemolyticus* or WSSV infection. Recombinant LvCrustinB was produced using *Pichia pastoris* GS115 and was shown to bind to 2 g-positive bacteria (*Staphylococcus aureus* and *Bacillus subtilis*) and 2 g-negative bacteria (*Escherichia coli* and *V. parahaemolyticus*) via polysaccharides, which included PGN, LTA, and LPS. *In vivo*, the recombinant LvCrustinB remarkably protected *L. vannamei* from *V. parahaemolyticus* infection. These results suggest that LvCrustinB plays an important role in innate immunity and may be potentially utilized as antibacterial agents in shrimp.

1. Introduction

Unlike vertebrates, invertebrates largely rely on innate immunity to defend themselves from pathogenic invasion [1], which primarily involves mechanisms such as phagocytosis, encapsulation, clotting, and a variety of soluble antimicrobial peptides (AMPs) [2]. AMP systemic production has been extensively studied in insect innate immune response, which is regarded as the most important characteristic of humoral immunity [3,4]. AMPs act as frontline effectors of host defense against a wide range of microbes, including bacteria, fungi, and viruses [5–8]. Multiple types of AMPs have been isolated from a wide variety of invertebrate phyla, including insects [9], ascidians [10,11], chelicerates [12], annelids [13], and mollusks [14–16]. Due to their small size, AMPs can be synthesized without elevated metabolic cost and can rapidly diffuse to infected sites. Moreover, because of their remarkable specificity for prokaryotes and their low toxicity for eukaryotic cells, many AMPs have been investigated and exploited as novel antibiotics [17].

Based on their important economic value, research on crustaceans and their innate immunity is highly valued. To date, several AMPs from crustaceans have been identified and characterized, including defensins, crustins, penaeidins, and anti-LPS factor [18–20]. Crustins are AMPs that are widespread among crustaceans. These humoral factors have diversity functions in the innate immunity in different species [1,4,21]. Crustins are cationic cysteine-rich AMPs that contain a glycine-rich, cysteine-rich, or proline-rich region at the N-terminus, and

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one or more whey acidic protein (WAP) domains at the C-terminus [21–23].

Most crustins are divided into three groups based on their structures (types I–III) [21]. Type I crustins are reported to contain a signal sequence in the N-terminal and a WAP domain in C-terminal, as well as a region harboring cysteine-rich residues in the middle. Type II crustins possess not only a cys-rich region but also a long gly-rich domain of approximately 40–80 aa adjacent to the signal region [21]. Type III crustins are a group of WAP domain-containing proteins that lack both the Gly-rich domain of type II crustin molecules and the Cys-rich region present in type I crustin and II crustin [2]. However, some new types of crustin-like proteins, including double WAP domain-containing proteins (type IV) [24–27] and insect crustin-like proteins with an extra aromatic amino acid-rich region between the cysteine-rich domain and WAP domain (type V) [28], have also been discovered in invertebrates. Approximately 50 crustin-like genes have been found in 20 different crustacean species, including some penaeid shrimp [21].

The Pacific white shrimp, *Litopenaeus vannamei*, an important commercial crustacean that is naturally distributed along the Pacific coasts of Central and South America, has become the primary species currently cultured in Pacific rim countries [29]. Due to the rapid expansion of the aquaculture industry, world shrimp production has increased in recent decades. However, industrial development has been severely affected by outbreaks of viral and bacterial diseases [30,31]. Under these circumstances, investigating the mechanisms of immune defense against diseases may be beneficial to shrimp culture [31,32].

In this study, a new crustin gene named LvCrustinB from shrimp *L. vannamei* was cloned and characterized. LvCrustinB, which is a type II crustin, possesses a long Gly-rich domain between the signal peptide and the WAP domain. Upregulation of LvCrustinB mRNA was observed when shrimp were challenged with lipopolysaccharide (LPS), *Vibrio parahaemolyticus*, and white spot syndrome virus (WSSV). Higher mortality caused by *V. parahaemolyticus* or WSSV was also observed when LvCrustinB was knocked down. Recombinant LvCrustinB (rLvCrustinB) was proven to be effective in reducing the mortality of shrimp challenged with *V. parahaemolyticus in vivo*. These observations indicate that the LvCrustinB plays an important role in the defense mechanism of *L. vannamei*.

2. Materials and methods

2.1. cDNA cloning

A sequence predicted to encode a crustin was obtained from the shrimp *L. vannamei* transcriptome [33], and specific primers were designed to clone the LvCrustinB gene (Table 1). Total RNA was extracted from *L. vannamei* tissues (hemocytes, hepatopancreas, gills, stomach, intestine, and epithelia) using TRIzol (Invitrogen, USA). The full-length sequence of LvCrustinB was amplified using the SMARTer RACE cDNA Amplification kit (Clontech, Japan) according to the manufacturer's protocol by a two-step nested PCR. To amplify the 5′ and 3′ ends of the LvCrustinB, the mRNA was reverse-transcribed to cDNA. First, buffer Mix 1 (4.0 μL 5× buffer, 0.5 μL DTT, 1 μL dNTPs) was prepared. Second, buffer Mix 2 (1.0–10.0 μL RNA, 1.0 μL 5′-CDS Primer A or 3′-CDS Primer A, 0.0–10.0 μL sterile H_2O) was prepared and incubated in tubes at 72 °C for 3 min, then cooled to 42 °C for 2 min. Third, buffer Mix 3 (5.5 μL buffer Mix 1, 0.5 μL RNA inhibitor, 2.0 μL reverse transcriptase) was prepared. Fourth, 8.0 μL of buffer Mix 3 were mixed with 12 μL of buffer Mix 2 (1 μL of SMARTer II A Oligonucleotide was added for 5′ RACE cDNA synthesis), the tubes were incubated at 42 °C for 90 min, and then the first-strand cDNA synthesis reaction product was diluted with 10.0–90.0 μL of Tricine-EDTA buffer. 5′-Rapid amplification of cDNA ends (RACE)-PCR amplification was performed with Universal Primer A Mix (UPM) and gene-specific reverse primer LvCrustinB-5RACE1. Nested PCR was subsequently performed with Nested Universal Primer A (NUP) and LvCrustinB-5RACE2 using the first-round PCR product as template. 3′-RACE-PCR was performed using UPM together with a gene specific forward primer LvCrustinB-3RACE1, and the nested PCR was subsequently performed with NUP and LvCrustinB-3RACE2. The second PCR products were cloned into the pEASY-T1 vector (TransGen Biotech, China), and 12 positive clones were selected and sequenced (BGI Tech, China).

2.2. Bioinformatics analysis

Multiple sequence alignments with amino acid sequences of LvCrustinB and other known crustins were performed using ClustalW (https://www.genome.jp/tools-bin/clustalw) [34]. Phylogenetic reconstruction of selected crustins was performed with MEGA 5.0 (http://www.megasoftware.net) using the neighbor-joining (NJ) method. Protein domains were predicted using the SMART program (http://smart.embl-heidel-berg.de/).

2.3. Tissue expression analysis by quantitative RT-PCR

The experimental shrimp (4–6 *g* each, average: 5 *g*) were obtained from the National SPF Shrimp Farm in Fangchenggang, Guangxi Province, China, and were cultured in laboratory aquaria with aerated seawater at 27 °C. The shrimp were fed daily with commercial feed equal to 5% of their body weight. For the tissue expression analysis experiment, tissues such as eyestalks, epithelia, gills, hepatopancreases, hemocytes, intestines, muscles, nerves, posterior capsule, tentacles, hearts, and stomachs were collected from 15 shrimps, and 5 shrimps pooled together.

2.4. Immune challenge analysis by quantitative RT-PCR

In the immune stimulation experiment, the treated shrimp were injected intramuscularly with 50 μL of WSSV (1×10^5 copies), 50 μL of *V. parahaemolyticus* suspension (1×10^6 colony-forming units, CFUs), and 50 μL of LPS (2 μg/μL) per shrimp, and the control group was injected with 50 μL of PBS solution. Hemocytes of the challenged shrimp were collected at 0, 4, 8, 12, 24, 36, 48, and 72 h post-injection (hpi), and 15 shrimp at each time point were collected, and 5 shrimps pooled together. Total RNA extraction and quantitative RT-PCR were performed as previously described [35]. Expression levels of LvCrustinB were calculated using the Livak ($2^{-\Delta\Delta CT}$) method after normalization to EF-1α (GU136229). The primer sequences used in this study are listed in Table 1.

2.5. RNA interference and pathogenic challenge assay

dsRNA synthesis was performed as previously described [36]. The experimental group was injected with LvCrustinB dsRNA (2 μg/*g* shrimp) at the second abdominal segment, while the control groups were injected with GFP dsRNA (2 μg/*g* shrimp) or with 50 μL phosphate-buffered saline (PBS). The hemocytes were collected from nine shrimp of each challenge group at 48 hpi, and three shrimp were pooled together. Total RNA was extracted and analyzed by RT-PCR to assess the effect of RNAi. The primer sequences used in the synthesis of dsRNAs or checking RNAi are listed in Table 1.

Experimental shrimp (4–6 *g* each, $n = 40$ in each group) were injected with 10 μg of dsRNA (LvCrustinB dsRNA or *GFP* dsRNA) or 50 μL of PBS. After 48 h, the shrimp were injected again with 50 μL of *V. parahaemolyticus* (1×10^6 CFU), 50 μL of WSSV (1×10^6 copies), or 50 μL of PBS as a control. The shrimp were cultured using standard procedures, and their cumulative mortalities were recorded every 8 h. The assays were performed in triplicate.

2.6. Expression and purification of rLvCrustinB

To generate a recombinant LvCrustinB (rLvCrustinB), a DNA

Table 1
Summary of primers used in this study.

Primers	Sequences (5′–3′)
RACE	
LvCrustinB-3RACE1	GACGA CGACAATGACGCAACAGA
LvCrustinB-3RACE2	GAGGGTCGTTCCAGAAGCAAA GCT
LvCrustinB-5RACE1	ACCTCCACCCAATCCAAATCCT
LvCrustinB-5RACE2	CCGAAGGCAGGGTTTACTCCAA
Quantitative RT-PCR	
LvEF-1α-F	TATGCTCCTTTTGGACGTTTTGC
LvEF-1α-R	CCTTTTCTGCGGCCTTGGTAG
LvCrustinB-F	GGTCGTTCCAGAAGCAAA
LvCrustinB-R	GGGTTGAATCCGAATCCT
dsRNA synthesis	
LvCrustinB-T7-F	GGATCCTAATACGACTCACTATAGGACGACAATGACGCAACAGATGGC
LvCrustinB-T7-R	GGATCCTAATACGACTCACTATAGGAGCAAGTGAATGGTGGCTGGAAG
LvCrustinB-F	ACGACAATGACGCAACAGATGGC
LvCrustinB-R	AGCAAGTGAATGGTGGCTGGAAG
dsRNA-GFP-T7-F	GGATCCTAATACGACTCACTATAGGCGACGTAAACGGCCACAAGTT
dsRNA-GFP-T7-R	GGATCCTAATACGACTCACTATAGGATGGGGGTGTTCTGCTGGTAG
dsRNA-GFP-R	ATGGGGGTGTTCTGCTGGTAG
dsRNA-GFP-F	CGACGTAAACGGCCACAAGTT
Protein expression	
rLvCrustinB-F	GGGAATTCCATCATCACCATCACCACGA
rLvCrustinB-R	GGTCTAGATTATCTACCGATACCCAATGGA

The *EcoR*I and *Xho*I restriction sites are underlined.

fragment, including encoded protein sequence of LvCrustinB and double enzyme cutting site (*EcoR*I and *Xba*I) was prepared using the chemical synthesis method. After digestion with endonucleases *EcoR*I and *Xba*I, the cDNA fragment of LvCrustinB was cloned into yeast plasmid pYE-GAPα. The recombinant plasmid pYE-GAPα-LvCrustinB were transformed into an *Escherichia coli* Top10 strain, and then extracted and verified by restriction analysis and sequencing. The pYE-GAPα-LvCrustinB plasmid was linearized by enzyme AVrII, and transformed into the *Pichia pastoris* GS115 strain by electrotransformation at 1.5 kV, 25 μF, and 200 Ω for 4.8 ms. Transformants were selected on MD plates, and then His^+ transformants were selected through YPD plates (2% peptone, 1% yeast extract, 2% glucose, 1.5% AGAR powder) with 100 μg/mL zeocin. The positive resistant strains were subsequently verified by PCR. The positive colonies were inoculated in 100 mL of PYD (2% peptone, 1% yeast extract, 2% glucose, 0.1 mol/L sorbitol) at 30 °C with constant shaking at 220 rpm. The culture fluid was sampled at 0 h, 24 h, 48 h, 72 h, and 96 h and detected by sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The rLvCrustinB was purified using the Ni-IDA method and verified by Western blotting using an anti-His antibody (Proteintech, USA, 1:3000 dilution).

2.7. Binding of rLvCrustinB to microorganisms

Gram-negative bacteria (*E. coli* and *V. parahaemolyticus*) and Gram-positive bacteria (*Staphylococcus aureus* and *Bacillus subtilis*) were used to detect rLvCrustinB binding activity to microorganisms. The bacteria were prepared as previously described [8] and were resuspended with TBS to OD_{600} of 1.0. The purified rLvCrustinB (100 μg) or His-tag fused protein of Trx (as a control) [37] was incubated with bacteria (500 μL) for 20 min at 25 °C with rotation, collected by centrifugation, and washed four times with triethanolamine-buffered saline (TBS). Finally, the binding proteins were eluted with 7% SDS for 1 min and separated by 12.5% SDS-PAGE. The proteins in the gel were transferred onto a nitrocellulose membrane and detected by Western blotting using an anti-his antibody (Proteintech, USA, 1:3000 dilution).

To detect the binding activity of rLvCrustinB to bacterial cell wall components, enzyme-linked immunosorbent assay (ELISA) was performed as previously described [38]. Lipopolysaccharides (LPS; *E. coli* 055: B5, Sigma), lipoteichoic acid (LTA; *B. subtilis* 56411-57-5, Sigma), and peptidoglycan (PGN; *B. subtilis* 69554, Sigma) were selected for the assay. Polysaccharides (4 μg) were added to each well of the microplate and incubated at 37 °C overnight. After incubation at 60 °C for 30 min, the microplate was blocked with BSA (1 mg/mL, 200 μL) at 37 °C for 2 h and was washed thrice with TBS (200 μL). The purified rLvCrustinB (final concentration 0–25 μg/mL in TBS) was added to the corresponding wells. The microplate was incubated at 37 °C for 3 h and was then washed four times with TBS. Subsequently, HRP-conjugated mouse anti-his antibody (1:3000 dilution in binding buffer) was added (100 μL per well) and incubated at 37 °C for 1 h. The plate was washed four times with TBS, and color reactions were developed with TMB Solution (100 μL per well). After 30 min, 1 M HCl (100 μL per well) was added, and the OD value was subsequently read at a wavelength of 450 nm. Each binding assay was performed in triplicate.

2.8. The anti-V. parahaemolyticus activity of rLvCrustinB in vivo

To test the anti-pathogenic microorganism activity of rLvCrustinB *in vivo*, *V. parahaemolyticus* was prepared as earlier described [39]. The shrimp were divided into four groups (three tanks for each group), and 50 shrimp were cultured in each 500-L tank. Two different fermented feeds were prepared as follows: The first feed was prepared by adding the liquid culture of an rLvCrustinB expressing yeast strain to the shrimp feed at a concentration of 2×10^6 CFU/kg, and then it was kept in a 30 °C airtight culture for two weeks. The second feed was prepared by adding a His tag protein-expressing yeast strain to shrimp feed following the method described above. The shrimp in the experimental groups were fed daily with rLvCrustinB or His tag protein (as a control) fermented feed equal to 5% of their body weights. The negative and positive control groups were fed with unfermented feed normally. After feeding the shrimp for a week, *V. parahaemolyticus* was added to the experimental groups and to the positive control groups at a final concentration of 4×10^4 CFU/L, whereas in the negative control group, no *V. parahaemolyticus* was added. The cumulative mortality was recorded each day. Differences in mortality between the experimental and control groups were tested for statistical significance using the Kaplan-Meier plot (log-rank χ^2 test).

3. Results

3.1. Characteristics of LvCrustinB

The full-length cDNA of LvCrustinB was 751 bp, containing a 5′

Fig. 1. Full-length cDNA sequence of LvCrustinB. The signal peptide is underlined (residues 1–17), and the whey acidic protein (WAP) domain (residues 143–191) is highlighted in gray. The 32 glycine residues are underlined in red, and the eight conserved cysteine residues are encircled. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

terminal untranslated region (UTR) of 34 bp, a 3′ UTR of 126 bp within a poly (A) tail, and an open reading frame (ORF) of 591 bp (GenBank No. MK593455) (Fig. 1). The ORF of LvCrustinB encoded a protein of 196 amino acids, and its calculated molecular mass is approximately 19.9 kDa. The LvCrustinB protein contained a putative signal peptide (residues 1–17), suggesting it could be a secreted protein. The LvCrustinB peptide sequence contained a long WAP domain between 143 and 191 along with a WAP-type "four-disulfide core" structure. These four disulfide bonds are formed by eight characteristically spaced cysteine residues (Cys^{147}, Cys^{154}, Cys^{165}, Cys^{171}, Cys^{177}, Cys^{178}, Cys^{182}, and Cys^{188}). Thirty-two glycine residues were distributed in LvCrustinB between the signal peptide and the WAP, which suggests that LvCrustinB belongs to the type II crustins.

3.2. Homology and phylogenetic analysis of crustins

Comparison of the LvCrustinB with other crustins showed that LvCrustinB has a single WAP domain that is highly homologous to other selected crustins from other species (Fig. 2A). Eight conserved cysteine residues were found in all the analyzed crustins. Phylogenetic tree constructed by the NJ method revealed that LvCrustinB was closely related to these crustin homologs from *Fenneropenaeus subtilis* (ABO93323.1), *Fenneropenaeus brasiliensis* (ABQ96197.1), *Marsupenaeus japonicus* (BAD15066.1), and from *Macrobrachium rosenbergii* (AGF92153.1) (Fig. 2B).

3.3. Tissue distribution of LvCrustinB

Quantitative RT-PCR showed that LvCrustinB was ubiquitously distributed in all examined tissues, and it was expressed at highest abundance in the epithelium, followed by the muscles, eyestalk, stomach, intestines, hemocytes, hepatopancreases, tentacles, nerves, gills, hearts, and posterior capsule (Fig. 3A). The high expression levels of LvCrustinB in epithelium, muscles, eyestalk, stomach, and intestines, suggest that these tissues could be important for LvCrustinB mediated local immune response. As for systemic immunity, the induced AMPs are regarded as the most important characteristic. Considering that the hemocytes are important immune-related tissues for most AMPs expression, and hemocytes are easy to collect and treat, the hemocytes was selected as target tissues for the subsequent experiments.

3.4. Expression of LvCrustinB in hemocytes of pathogen- or stimulant-challenged shrimp

Quantitative RT-PCR was used to detect the expression of LvCrustinB in hemocytes from shrimp challenged with LPS, *V. parahemolyticus*, WSSV, and PBS (as control). After injecting LPS, the expression of LvCrustinB in the hemocytes gradually increased and reached a peak of 126-fold at 48 hpi (Fig. 3C). In response to *V. parahemolyticus*, LvCrustinB was upregulated and reached a peak of 18.6-fold at 24 hpi and then reverted to levels of 1.8-fold at 72 hpi (Fig. 3D). During WSSV challenge, the expression of LvCrustinB continuously increased, with a peak of 282-fold at 48 hpi, and then returned to 132-fold at 72 hpi (Fig. 3E). The control group injected with PBS showed no obvious change of LvCrustin*B* expression (Fig. 3B).

3.5. Crucial protective function of LvCrustinB against WSSV and V. parahaemolyticus infections

To test the RNA interference efficiency of LvCrustinB-dsRNA, the experimental shrimp were injected with LvCrustinB-dsRNA and GFP-dsRNA (as control). The results showed that LvCrustinB-dsRNA significantly downregulated the mRNA level of LvCrustinB at 48–72 h after dsRNA injection compared to the GFP-dsRNA group, which showed no detectable interference effect on LvCrustinB expression (Fig. 4A). To evaluate the effects of LvCrustinB on mortality, pathogen-challenged shrimp were injected with LvCrustinB dsRNA and then challenged with WSSV or *V. parahaemolyticus*. The results showed that WSSV-infected shrimp treated with LvCrustinB-dsRNA had significantly higher mortality at 24–120 h post-infection compared to shrimp challenged with WSSV alone or with WSSV + GFP-dsRNA ($P = 0.0012 < 0.01$; Fig. 4B). Similar results were observed in the *V. parahaemolyticus*-challenged groups. Shrimp treated with LvCrustinB-dsRNA also showed significantly higher mortality at 8–120 hpi compared to shrimp challenged with *V. parahaemolyticus* alone or with *V. parahaemolyticus* + GFP-dsRNA ($P < 0.001$; Fig. 4C). All the negative controls showed a cumulative mortality of zero (Fig. 4B and C), indicating that LvCrustinB-dsRNA itself was non-toxic to shrimp. Overall, these results indicated that LvCrustinB was vital to survival after *V. parahaemolyticus* or WSSV infection.

3.6. rLvCrustinB binds to bacteria and exhibits antibacterial activity in vivo

The recombinant protein rLvCrustinB was successfully expressed in *P. pastoris* GS115. The purified rLvCrustinB was further validated by SDS-PAGE and Western blotting assays (Fig. 5A and B).

The binding activities of LvCrustinB were analyzed using rLvCrustinB. The results showed that rLvCrustinB could bind to 2 g-positive bacteria (*S. aureus* and *B. subtilis*) and two Gram-negative bacteria (*E. coli* and *V. parahaemolyticus*) (Fig. 5C). An ELISA was also

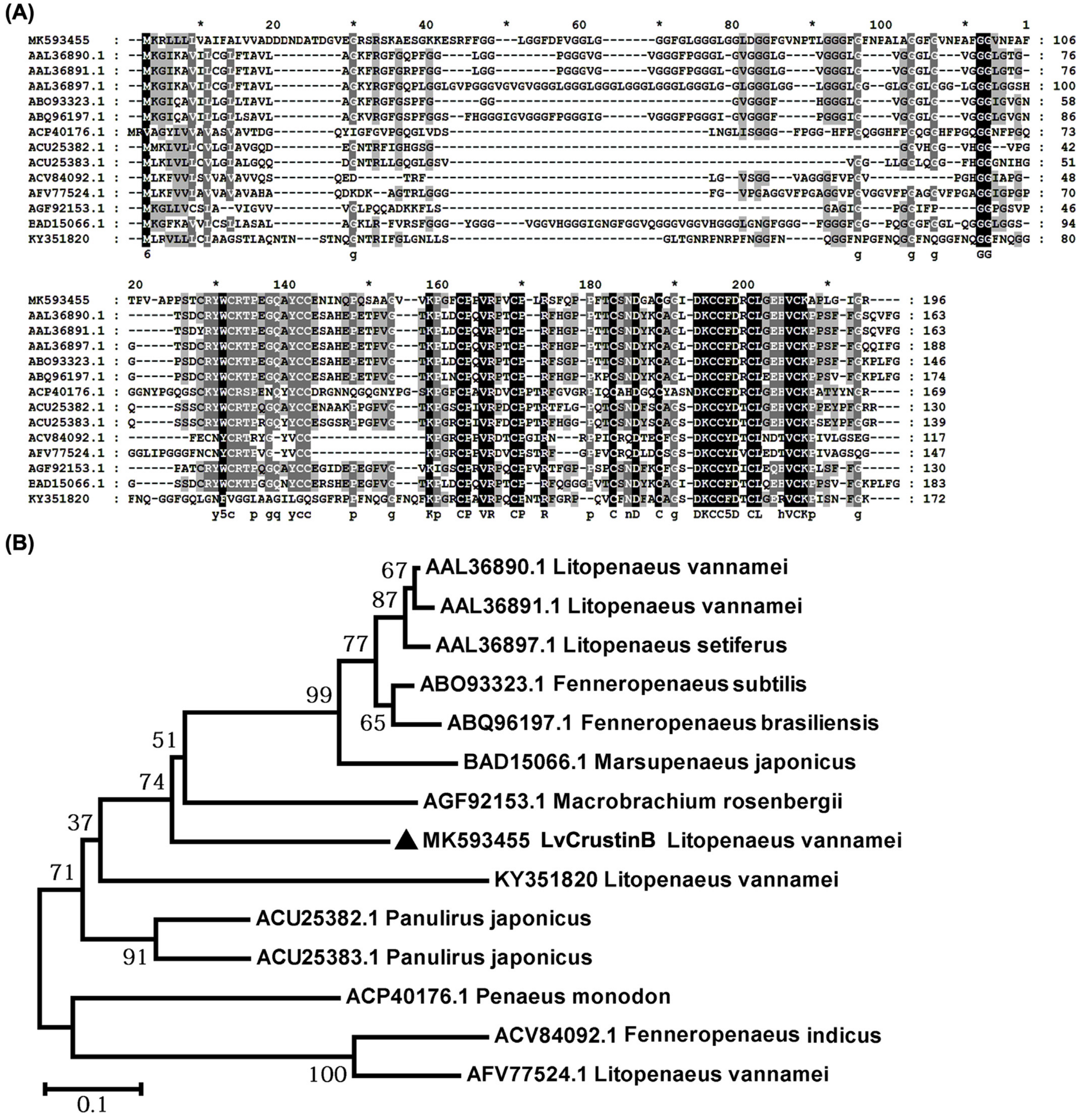

Fig. 2. Multiple sequence alignment and phylogenetic tree construction of crustins and crustin-like proteins. (A) Multiple sequence alignment of LvCrustinB with other crustins or crustin-like proteins. The identical residues are shown in black, while similar residues are shown in gray. (B) Phylogenetic relationships of the deduced amino acid sequences of the LvCrustinB with other crustins or crustin-like proteins. All amino acid sequences were collected from NCBI, and the tree was constructed with MEGA 5.0 (bootstrap = 1000). LvCrustinB is marked with a black triangle.

performed to detect the direct binding activity of rLvCrustinB to polysaccharides, including PGN, LTA, and LPS. The results showed that rLvCrustinB could bind to PGN, LTA, and LPS (Fig. 5D). These results suggested that rLvCrustinB bound to different bacteria, perhaps by binding to the bacterial cell wall components.

To determine whether rLvCrustinB also has anti-pathogenic microorganism activity *in vivo*, healthy shrimp *L. vannamei* were fed with three kinds of feed, including rLvCrustinB, His tag protein-fermented, or normal feed, and then infected with *V. parahaemolyticus*. The mortality of the shrimp was monitored daily for 15 days. Initial mortality was assessed after the second day post-injection in all groups of infection shrimp. On the fifth day post-infection, the mortality of the rLvCrustinB group (78%) was significantly lower ($P = 0.023 < 0.05$) than that of the His-tag-protein group (92%) and of the positive group (96%), and the mortality rate of the negative control shrimp was 0 (Fig. 5E). These results suggest that rLvCrustinB can protect shrimp from *V. parahaemolyticus* infection *in vivo*.

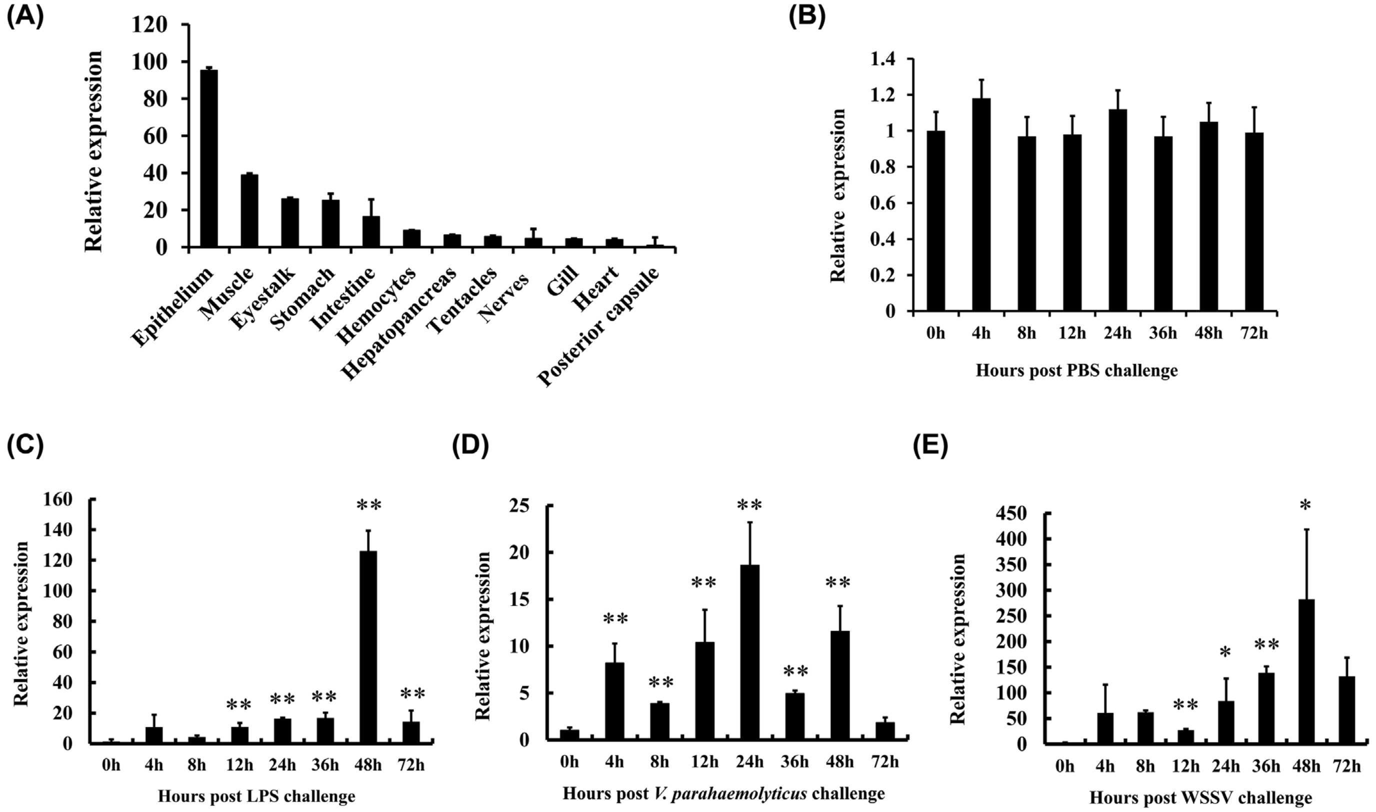

Fig. 3. The expression patterns of LvCrustinB in response to immune stimuli. (A) Tissue distribution of LvCrustinB in healthy shrimp. Quantitative RT-PCR was used to determine the transcriptional levels of LvCrustinB in different tissues. The data were provided as the means ± SD of triplicate assays ($n = 5$ shrimp each assay). The expression level in hepatopancreas was set to 1.0. (B–E) Expression profiles of LvCrustinB in PBS (B), LPS (C), *V. parahemolyticus* (D), and WSSV (E) challenged shrimp. Quantitative RT-PCR was performed in triplicate for each sample. Expression values were normalized to those of EF-1α using the Livak ($2^{-\triangle\triangle CT}$) method. The results are expressed as the mean ± SD of triplicate samples, and the statistical significance was calculated using Student's *t*-test ($^{*}p < 0.05$, $^{**}p < 0.01$).

4. Discussion

Disease prevention remains the key to shrimp culture. To prevent shrimp disease, it is essential to study their immune mechanisms. In this study, a new AMP gene from *L. vannamei*, named LvCrustinB, was cloned and identified. LvCrustinB contains a characteristic WAP structure and a glycine-rich region (32 glycine residues). Based on the presence or absence of structural domains lying at the N-terminal region, Smith et al. [21] classified crustins into three sub-groups, designated as types I – III. Type II crustins are mainly present in penaeid shrimp, and

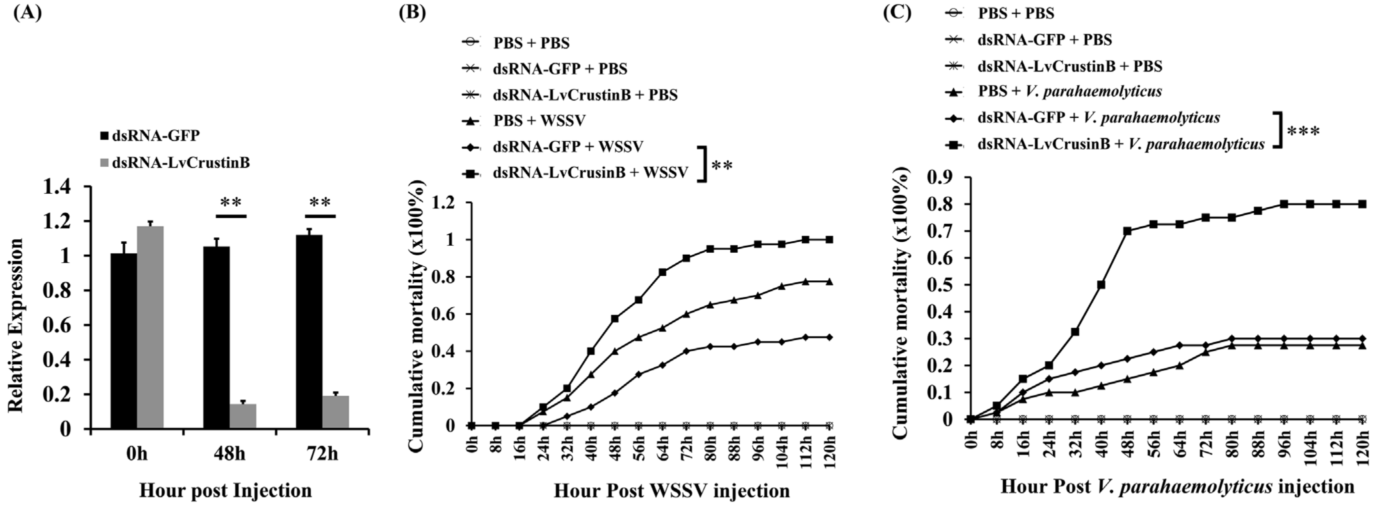

Fig. 4. Cumulative mortality of challenged shrimp after RNA interference. (A) Quantitative RT-PCR analysis of the knock down efficiency of LvCrustinB; the internal control was EF1α. Samples were collected at 48 and 72 h after injection with indicated dsRNAs; (B–C) Shrimp were injected intramuscularly with dsRNA-LvCrustinB or dsRNA-GFP. At 48 h after the initial injection, the shrimp were infected with WSSV (B) or *V. parahemolyticus* (C), and PBS was injected into a group of shrimp as a negative control. Statistical significances between dsRNA-LvCrustinB and dsRNA-GFP (as a positive control) groups were calculated using the log-rank χ^2 test ($^{**}p < 0.01$, $^{***}p < 0.001$). The results are representative of three independent experiments.

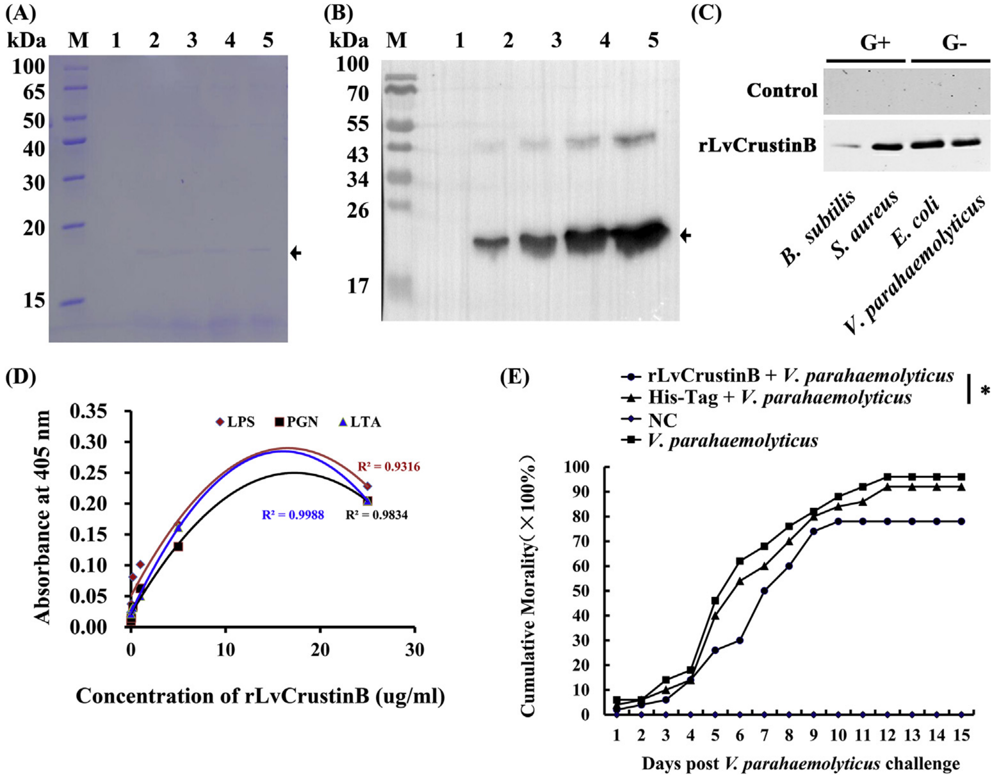

Fig. 5. Recombinant LvCrustinB binds to bacteria and shows antibacterial activity *in vivo*. (A–B) Purified rLvCrustinB from *P. pastoris* GS115 at 0, 12, 24, 48, and 72 h (Lines 1–5, respectively) was shown in Coomassie blue staining (A) and Western blotting (B). (C) Western blotting assay was used to analyze the binding activity of rLvCrustinB or His-tagged TRX (as a control) to four bacteria. (D) The ELISA method was performed to detect the binding activity of rLvCrustinB to different carbohydrates (PGN, LPS, and LTA). (E) *In vivo* antibacterial activity of rLvCrustinB. Statistical significances between the rLvCrustinB and the control groups were calculated using the Kaplan–Meier plot (log-rank χ^2 test, $^*p < 0.05$). Experiments were performed thrice and generated similar results. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

harbor a glycine-rich region and a cysteine-rich domain between the signal peptide and the C-terminal single WAP domain. According to the above classification criteria, LvCrustinB is a type II crustin. Most crustins contain a single WAP domain, which was considered to play a key role in its antibacterial effect [21]. However, it has been observed that type II crustins with an incomplete WAP domain have impaired antimicrobial activities [40]. The presence of a highly conserved WAP domain suggests that LvCrustinB may play a role in the defense against microbial infection.

In crustaceans, most crustins are mainly expressed in hemocytes, but they are able to be detected in other tissues due to the infiltration or adherence of hemocytes in these tissues [8,21,41]. Besides, emerging studies have shown that different crustins have distinct tissue distributions. For example, two crustin isoforms, MjCRS8 and MjCRS9 from kuruma shrimp *M. japonicus*, are expressed only in gill tissue [42]. CrustinPm5 from *Penaeus monodon* is mainly expressed in eyestalks but not in hemocytes [43]. In *L. vannamei*, the *crustin Lv* gene (type IIa) was found to have the highest expression levels in circulating hemocytes, while the *crustin-like Lv* gene (type IIb) has been shown to be predominantly expressed in the gills [44]. In addition, we have previously reported that another crustin named LvCrustinA is mainly expressed in the gills [36]. In this study, LvCrustinB is found to be highly expressed in several immune-responsive tissues, including epithelium, stomach, intestine, and hemocytes. The differences in the tissue distribution of the crustins transcripts probably indicate the diverse functions of crustins as immune effectors or mediators in response to various physiological stresses [45].

The response of crustin expression to pathogenic challenges may reflect its involvement in anti-pathogenic activity [46–49]. In this study, the expression of LvCrustinB was significantly upregulated after challenged with LPS, *V. parahemolyticus*, or WSSV. This finding may suggest that LvCrustinB is involved in defense against gram-negative bacteria and DNA virus. The induced expression of crustins is also frequently observed in previous reports. For instance, diverse crustins, including the crustin-like mRNA of *M. japonicas* [50], the MrCrs of *M. rosenbergii* [51], and the LvCrustinA of *L. vannamei* [36], were upregulated post-challenge with viruses (IHHNV and/or WSSV) and pathogenic bacteria strains (*Vibrio alginolyticus*, *V. parahemolyticus*, *Aeromonas hydrophila*, and/or *Enterococcus faecium*). Nevertheless, there is one exception: crustins MjCRS8 and MjCRS9 showed no significant upregulation in response to WSSV and *V. parahaemolyticus* infection [42]. The observed variations in the responses of crustins stimulated by pathogens may be implicated with their functional differences. Indeed, it has been reported that most crustins possess protease inhibition and antibacterial activity [1,41,52], but some of them have other important biological functions in physiological stress, wound repair, and tissue regeneration or ecdysis [53].

To further investigate the function of LvCrustinB, RNAi, which has been extensively used in investigating protein function in shrimp immunity [54], was performed. Knocking down LvCrustinB led to shrimp with higher susceptibility to WSSV infection, which demonstrates that LvCrustinB plays a protective role against WSSV. However, the antiviral mechanism of LvCrustinB remains uncovered. A previous study has shown that knocking down crustin-like mRNA leads to a significant downregulation of the innate immune factors p53, Rab7, IMD, L-lectin, Rho, proPO, and MAPK, whereas myosin, NOS, and TNF-α were significantly upregulated, thereby suggesting that a crustin-like peptide influences the processes of apoptosis and phagocytosis [50]. The exact antiviral action of LvCrustinB is worthy of further study.

Next, we explore the function of LvCrustinB during bacterial infection. In our study, rLvCrustinB is able to bind to gram-positive bacteria (*S. aureus* and *B. subtilis*) and gram-negative bacteria (*E. coli* and *V. parahaemolyticus*). ELISA also reveals that rLvCrustinB directly binds to polysaccharides, including PGN, LTA, and LPS. These findings indicate that LvCrustinB may be involved in the defense against Gram-positive and -negative bacteria. This is supported by the following RNAi experiment that LvCrustinB is crucial for shrimp against *V. parahaemolyticus*. Furthermore, shrimp fed with rLvCrustinB fermented feed had a higher survival rate than those fed with His-tag protein-fermented fermented feed or unfermented feed after these were infected with *V. parahaemolyticus*. These results strongly demonstrate that LvCrustinB can protect shrimp from *V. parahaemolyticus* infections, perhaps because that LvCrustinB has the ability to directly bind to some polysaccharides from bacteria. Many crustins have been reported to have antimicrobial functions, but their antimicrobial actions may vary. All fairly active AMPs interact with membranes by folding into amphipathic three-dimensional structures, which insert into membranes at the membrane interface [6]. Scanning electron microscopy (SEM) has shown that crustins can directly destroy the bacterial cell wall structure [8,55]. Whether LvCrustinB plays a similar role of fighting bacteria remains unclear.

In summary, a new crustin homolog from *L. vannamei* (LvCrustinB) was cloned and identified. LvCrustinB is highly expressed in several immune-related tissues such as epithelium, stomach, intestine, and hemocytes. LvCrustinB expression in hemocytes is able to respond to *V. parahaemolyticus* and WSSV infections. In addition, we provide evidence to indicate that LvCrustinB is involved in the host defense against WSSV infection. Furthermore, LvCrustinB can directly bind to several polysaccharides, thereby exhibiting a strong antibacterial activity against bacteria such as *V. parahaemolyticus*.

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