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Functional characterization of an ER-stress responding *Crustin* gene in *Litopenaeus vannamei*

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ABSTRACT

Shrimp in culture ponds are challenged by various pathogens as well as harsh water environment. The innate immune system and environmental stress response system of shrimp paly an important role in shrimp survival and growth. For remission the endoplasmic reticulum (ER)-stress caused by environmental stress, unfolded protein response (UPR) may reduce the synthesis of most proteins, including great mass of immune factors, which could weaken the immune function of shrimp. Therefore, how cells keep appropriate amount of immune factor synthesis under such a situation is critical important for shrimp health and growth. In this study, we cloned a new *Crustin* gene (*LvCruU*) from *Litopenaeus vannamei*. We showed that LvCruU has antibacterial activity, and reducing its expression would increase the cumulative mortality of *L. vannamei* upon the *Vibrio parahemolyticus* infection. In addition, we found that promoter activity of *LvCruU* was enhanced not only by the deformed epidermal autoregulatory factor-1 (Deaf1), but also by activating transcription factor 3 (LvATF3) of shrimp UPR. Real-time RT-PCR showed that *LvCruU* and *LvATF3* both were induced upon UPR activation. And moreover, in Thapsigargin plus dsLvCruU injection test, we showed that down-regulation of *LvCruU* increased the cumulative mortality of *V. parahemolyticus*-infected shrimp under ER-stress. These results suggest that LvCruU work as a downstream effector of UPR, and contribute to antimicrobic immune response upon ER-stress in *L. vannamei.*

1. Introduction

Like other invertebrates, shrimp depends mainly on its innate immune system for resisting pathogenic infections. Nowadays, we know that shrimp innate immunity consists of two important system: the cell immune reaction and the humoral immune reaction [1,2]. For shrimp cell immune reaction, haemocytes play a central role in it. Once there is microbials invasion, non-self-recognition factors of shrimp innate immune system, such as β-1, 3-glucan-binding protein could activate immediate defence systems mediated by haemocytes [3]. Among them, the prophenoloxidase (proPO) cascade is the best characterized one in crustaceans. Some components associated with this immediate defense process, which result in the reaction of melanisation and haemolymph coagulation, both are rapid and powerful reaction in crustaceans. Shrimp cell immune reaction also includes the haemocytic process of encapsulation, phagocytosis and the microbicidal mechanism that based on the production of cytotoxic reactive oxygen intermediate [4,5]. Comparing to cell immune reaction, humoral immune reaction of shrimp got more in-depth researches. Currently, the main pathways of shrimp humoral immune system, such as Toll-like receptor (TLR) pathway, immune deficiency (IMD) pathway, JAK-STAT pathway, RNA interference (RNAi) pathway, p38 mitogen-activated protein kinase (MAPK) pathway and JNK pathway have been proved to play important roles in shrimp innate immune response [6]. As the well-studied immune signal pathways in invertebrate, TLRs pathway and IMD pathway are involved in Gram-positive bacteria and Gram-negative bacteria

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response, respectively, by inducing some immune-relative genes, such as *antimicrobial peptide genes* (*AMPs*), *lysozymes* as well as *anti-lipopolysaccharide factor* (*ALF*) [7–9].

So far, several AMP families in crustacean have been investigated, such as penaeid, ALF, Defensin, Crustin and so on [10]. Among them, penaeids are the best study crustacean AMP, and they were involved in shrimp antibacterial immune response. And in recently, more and more attention has been paid to the function of Crustin in crustacean. Crustins are antimicrobial polypeptides containing 1–3 whey acidic protein (WAP) domains, with molecular weight in the range of 6–22 kDa [11]. They mainly distributed in crustaceans, and also in some species of insect as report. Crustins are currently divided into three types, namely, type I—, type II—, and type III crustin. Members of type I Crustin are reported to share a consensus motif arrangement as "—C-X(3)—C-X(8–12)—C—C-X(16–17)—C-X(6)—C-X(9–10)-", type II Crustins with a long Gly-rich reagion at is N-terminal, and type III Crustins are a group of WAP domain-containing proteins, which lacks both of the Gly-rich domain of type II Crustin molecules and the Cys-rich region present in type I— and type II Crustin [11,12].

In addition to pathogens, shrimp has to face to the constant changing water environment. And the biochemical, physiological and pathological stimuli that can disrupt the equilibrium state of ER, lead to ER-stress [13]. For survival, living organisms have developed strategies to cope with ER-stress. And the unfolded protein response (UPR) is the central system performs such function in eukaryotic cells. UPR is a cascade of response caused by the accumulation of unfolded or misfolded proteins in the lumen of the ER. This mechanism has been observed in mammalian species, as well as in worms and yeasts [14]. UPR consists of three signal pathways, each of them contains a class of transmembrane ER-resident signaling components, named inositolrequiring enzyme 1 (IRE1), double-stranded RNA-activated protein kinase (PKR)-like ER kinase (PERK), and activating transcription factor 6 (ATF6), respectively [15]. UPR can relieve ER-stress through diverse strategies: increasing the production of molecular chaperones involved in protein folding, inhibits protein translation to restore normal cell functions, and activates the signal pathways that result in the targeting of misfolded proteins in the ER for ubiquitination and subsequent degradation by proteasomes [16]. While, if ER-stress is too seriously or last for a long time, UPR may lead to apoptosis [13].

Accumulating evidences suggested that there was a close relationship between UPR and innate immune response. In one hand, UPR is a stimulant for innate immunity pathways, for examples, UPR genes *pqn/abu* that regulated in a CED-1-dependent manner, are involved in the *C. elegans* immune response to bacteria [17]; UPR in cystic fibrosis airway cells contributes to p38 MAPK-mediated innate immune responses [18]. In another hand, UPR depresses innate immune responses, as an example, ER-stress depresses NF-κB activation in mesangial cells through preferential induction of C/EBPβ [19]. In fact, sometimes these two systems look likely forming network, and members of UPR involve in the immune signal transduction or even work as the pattern recognition protein (PRP). A typical example is that TLR-activated XBP1 was required for optimal and sustained production of proinflammatory cytokines in macrophages [20]; and IRE1α senses bacterial proteins invading the ER to activate RIG-I and innate immune signaling [21]. While there is a contradiction between the two systems: the activation of PERK-eIF2α signal pathway inhibits the translation of most proteins for ER-stress remission, including those are involved in innate immune response, and may depress the antibacterial function.

In previous studies, we had shown that UPR was involved in shrimp innate immune response [22,23]. Yet the mechanism to avoid innate immunity overinhibition in shrimp when UPR is activated still unknown. In this study, a novel *Crustin* gene (*LvCruU*) in *L. vannamei* was cloned. It was induced by *Vibrio parahemolyticus* or *Staphylococcus aureus* infection. And antimicrobial activity assays showed that the purified recombinant-LvCruU inhibited the growth of *V. parahemolyticus* or *S. aureus*. Down-regulated expression of *LvCruU* resulted in a higher cumulative mortality in *V. alginolyticus* infected *L. vannamei*. Moreover, we showed that *LvCruU* was induced by *L. vannamei* UPR, and its promoter was activated by Deaf1 (LvDeaf1) of NF-κB pathway as well as activating transcription factor 3 (LvATF3) of UPR in S2 cells. By treating with UPR activator thapsigargin (Tg) or dsLvCruU, we proved that *LvCruU* could provide antimicrobial protection under UPR activation. In conclusion, we reported a novel *Crustin* gene *LvCruU* as downstream effector of shrimp UPR, which contributes to antibacterial function maintenance under ER-stress.

2. Materials and methods

2.1. Cloning of the crustin gene (LvCruU) and bioinformatics analysis

Samples for transcriptome analysis were prepared and collected as describe in our previous publication [23]. In brief, shrimps injected with dsLvBip, which had been proved that could activate the UPR system. 72 h post dsLvBip injection, hemocytes were collected and total RNAs were islated for transcriptional analysis. The fragment of *LvCruU* form transcriptional data is 572 bp, and we obtained the full-length open reading frame (ORF), which is 591 bp in length by rapid amplification of cDNA ends (RACE). Protein domain is analyzes using the SMART program (http://smart.embl-heidelberg.de/). The Crustin protein sequences from other species in the database were obtained from Genbank (https://www.ncbi.nlm.nih.gov/genbank/) and then evaluated by Clustal Omega Multiple Sequence Alignment (http://www.ebi.ac.uk/Tools/msa/clustalo/). A neighbor-joining (NJ) phylogenic tree is constructed using the deduced amino acid sequences of Crustins by employing MEGA 6.0 software. Bootstrap sampling is reiterated 5000 times. The conservative motif in the Crustin proteins were analyzed with MEME 4.11.1 (http://meme-suite.org/tools/meme).

2.2. Culture of cells and western-blot assay

Drosophila Schneider 2 (S2) cells were maintained at 27 °C in *Drosophila* SDM (Serum-Free Medium; Invitrogen, USA) supplemented with 10% fetal bovine serum (Invitrogen). For DNA transfection, cells were seeded overnight, and then plasmids were transfected by using the FuGEN (Invitrogen) according to the manufacturer's instructions. For dual-luciferase report gene assays, S2 cells in 6-well plates (TPP, Switzerland) were transfected using 2 μg expression plasmids. At 48 h post-transfection, culture medium was collected, and the cells were harvested and lysed on ice in 0.6 mL of NP-40 lysis buffer (Beyotime, China). Then the supernatants and the collected medium, which has been concentrated were subjected to SDS-PAGE and Western-blot analyses with anti-V5 monoclonal antibody (CST, USA), and horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG antibody (Pierce, USA). Signal is detected with a SuperSignal West Pico Trial Kit (Pierce).

2.3. Recombination protein expression and purification

The *LvCruU* was amplified using primers pAcLvCruU-EcoR I—F with an EcoR I site, pAcLvCruU-Not I-R with a Not I site (Table 1). PCR product was inserted into pPICZα-A vector that was digested with EcoR I and Not I. Then the linearized recombinant plasmid obtained by digestion with Sac I was transformed into the X33 strain (Invitrogen) *Pichia pastoris* by electroporation (10 μg of each plasmid mixed with 80 μL of the cells per each transformation). Spread the cell on YPDS plates containing 100 μg/mL Zeocin, and incubate plates 5 days at 30 °C until colonies form. The genomic DNA isolated from the *P. pastoris* transformants was used as the template for PCR to confirm the integration of the recombinant plasmid. The positive transformants were cultured in BMGY medium for growth, and then transferred to BMMY medium for induction according to the producer's manual (Invitrogen). A final concentration of 1% (w/w) methanol was added to maintain

Table 1
Summary of primers used in this study.

Primers	Sequence (5′-3′)
For cDNA cloning	
LvCruU-5race-R1	TGCACCAGTAACGGCATGTGGAGGGAGG
LvCruU-5race-R2	ACAGGAGTGAATGCAGGATTTACTCCTC
LvCruU-3race-F1	ACCCAACACTTGGAGGAGGATTCGGATT
LvCruU-3race-F2	TCTTGCGGGCGGATTTGGAGTAAACCCT
For Genome Walking-PCR	
5 GW-LvCruU-R1	CGTCGTCAGCAACCACGAGGGCGAAAAT
5 GW-LvCruU-R2	GCGACCAGCAACAAAAGTCTCTTCATCT
For genes expression [a]	
pAcLvCruU-EcoR I—F	CCGGAATTCTATGAAGAGACTTTTGTTGCTGGTCGCG
pAcLvCruU-Not I-R	ATTGCGGCCGCTCGCCCAATGCCCAGAGGAGCCTTG
pACB-LvCruU-EcoR I-F	ATAGAATTCTATGAAGAGACTTTTGTTGCTGG
pACB-LvCruU-Xho I-R	TTACTCGAGCG TCGCCCAATGCCCAGAGGAG
pACB-LvDeaf1-EcoR I-F	ATAGAATTCTATGGACGCGAGTTTAGGGGA
pACB- LvDeaf1-Xba I-R	TTATCTAGACTGTCAGAAAGCATAAATATATATCCAG
pACB-LvATF3-EcoR I-F	ATAGAATTCTATGTCCGATTTCCCGACCT
pACB LvATF3-Xho I-R	TTATCTAGACTGCTGAGTGGCGCCGGGACGT
For report genes [a]	
pGL3- LvCruU-full-Kpn I—F	ATAGGTACC TTCAGTGACTATCACCTTTTCAGA
pGL3- LvCruU-1500-Kpn I—F	ATAGGTACCCAGTTAGTTTTATTAATGGTAAAGATTA
pGL3- LvCruU-1250-Kpn I—F	ATAGGTACCTACTATTGGCAGTAGTGCTGATT
pGL3- LvCruU-1000-Kpn I—F	ATAGGTACCAAATCCTTGTACGATGCCAG
pGL3- LvCruU-750-Kpn I—F	ATAGGTACCTCAAAGTGTATCACAGTGTTAGGG
pGL3- LvCruU-500-Kpn I—F	ATAGGTACCATTATCATAACGAGACTAATACCAATGT
pGL3- LvCruU-250-Kpn I—F	ATAGGTACCTTCACGACCTACATCATGTGG
pGL3- LvCruU-Bgl II —R	TATAGATCTCTGAAATTCAATATGAAGCATAGG
For dsRNA templates amplification	
DsRNA-LvCruU-428-T7-F1	GGATCCTAATACGACTCACTATAGGAGGGTCGTTCCAGAAGCAAA
DsRNA-LvCruU-428-R1	GCCGTCGTTAGAGCAAGTGA
DsRNA-LvCruU-428-F2	GTCGTTCCAGAAGCAAA
DsRNA-LvCruU-428-T7-R2	GGATCCTAATACGACTCACTATAGGAGGGCCGTCGTTAGAGCAAGTGA
For RT-PCR	
SQPCR-LvCruU-537-F	ACAATGACGCAACAGATGGC
SQPCR-LvCruU-537-R	TCTATCGCCCAATGCCCAG
SQ-LvEF1α-F	AAGGCCCTCAAGAAGAAGTAAAT
SQ-LvEF1α-R	TTGACAACCATACCTGGCTTC
For real-time RT-PCR	
QPCR-LvCruU-F	GACGACAATGACGCAACAGAT
QPCR- LvCruU-R	GACCTCCACCCAATCCAAATC
QPCR-LvEF1α-F	GCTGATTGCGCCGTACTCAT
QPCR-LvEF1α-R	TCACGGGTCTGTCCGTTCTT

[a] Nucleotides in bold indicate restriction sites introduced for cloning.

induction every 24 h. After 4 days induction, the recombinant protein was purified from the medium using Ni-Sepharose High Performance (Amersham Biosciences). Purified protein was dialyzed against 1 × PBS at 4 °C for three times. And the purified rLvCruU was detected by SDS-PAGE and Western-blot assay (Fig. S1).

2.4. Antimicrobial activity assays

Antimicrobial activity assays were preferred as describe by Ai-Qing Yu et al. [24]. In brief, for the growth curves assay, microorganisms were cultured in the Luriae-Bertani (LB) medium overnight until the OD600 reached 0.8–1.0. A sample of the overnight bacterial culture (20 μL) was diluted in LB medium (4 mL), and the purified rLvCruU proteins were added (final concentration 20 μg/mL). An equal volume of TBS was used as a control. Samples were incubated at optimum temperature (*S. aureus*, 30 °C; *V. parahemolyticus*, 36 °C) with rotation at 150 rpm, and the OD600 was measured every hour. And for the agar diffusion test, the microorganisms cultured overnight were plated on an agar petri dish (100 mm × 20 mm), containing four pores (10 mm diameter) generated using the perforex. The purified rLvCruU (0.2 μg/mL, 100 μL) or ampicillin (0.2 μg/mL, 100 μL) was added to the pores, with TBS and rTrx as negative controls. The plates were incubated for 16 h at the optimum temperature (*V. parahemolyticus*, 36 °C; *S. aureus*, 30 °C) for each microorganism. A transparent ring around the pores indicated antimicrobial activity.

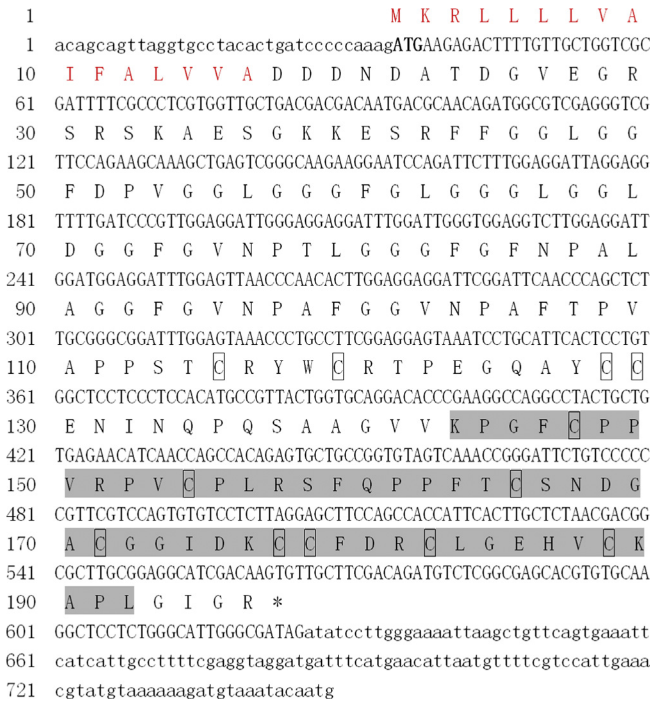
2.5. Synthesis of double-stranded RNAs

The DNA templates of *LvCruU* dsRNA (designated as dsLvCruU) were prepared via PCR using the primer pairs, DsRNA-LvCruU-T7-F/DsRNA-LvCruU-R and DsRNA-LvCruU-F/DsRNA-LvCruU-T7-R (Table 1). The PCR products were used as templates for the RNA strands and subjected to *in vitro* transcription and purification using Ribo-MAXTM Large Scale RNA Production System-T7 (Promega, USA) following the manufacturer's protocol. The dsLvCruU was 428 bp in length. DNA templates for eGFP dsRNA (dseGFP) or *L. vannamei* immunoglobulin heavy chain binding protein dsRNA (dsLvBip) synthesis were prepared as described in our previous report [15].

2.6. DsRNAs injection, immune challenge and preparation of templates for real-time RT-PCR

L. vannamei (~7 g) were raised in a shrimp farm in Zhuhai City, Guangdong Province, China. Shrimps were acclimated in a recirculating water tank system that was filled with air-pumped seawater (2.5% salinity) at about 28 °C. Shrimp were cultured for at least one week before the experiments.

For UPR activation, shrimps (n = 200) were injected with dsLvBip or dseGFP (control) were prepared. Each healthy L. *vannamei* was injected intramuscularly at the second abdominal segment with about 8 μg dsLvBip or dseGFP (50 μL in volume). Total RNA from the



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1                                      M  K  R  L  L  L  L  V  A
1   acagcagttaggtgcctacactgatcccccaaagATGAAGAGACTTTTGTTGCTGGTCGC
10   I  F  A  L  V  V  A  D  D  D  N  D  A  T  D  G  V  E  G  R
61  GATTTTCGCCCTCGTGGTTGCTGACGACGACAATGACGCAACAGATGGCGTCGAGGGTCG
30   S  R  S  K  A  E  S  G  K  K  E  S  R  F  F  G  G  L  G  G
121 TTCCAGAAGCAAAGCTGAGTCGGGCAAGAAGGAATCCAGATTCTTTGGAGGATTAGGAGG
50   F  D  P  V  G  G  L  G  G  G  F  G  L  G  G  G  L  G  G  L
181 TTTTGATCCCGTTGGAGGATTGGGAGGAGGATTTGGATTGGGTGGAGGTCTTGGAGGATT
70   D  G  G  F  G  V  N  P  T  L  G  G  G  F  G  F  N  P  A  L
241 GGATGGAGGATTTGGAGTTAACCCAACACTTGGAGGAGGATTCGGATTCAACCCAGCTCT
90   A  G  G  F  G  V  N  P  A  F  G  G  V  N  P  A  F  T  P  V
301 TGCGGGCGGATTTGGAGTAAACCCTGCCTTCGGAGGAGTAAATCCTGCATTCACTCCTGT
110  A  P  P  S  T  C  R  Y  W  C  R  T  P  E  G  Q  A  Y  C  C
361 GGCTCCTCCCTCCACATGCCGTTACTGGTGCAGGACACCCGAAGGCCAGGCCTACTGCTG
130  E  N  I  N  Q  P  Q  S  A  A  G  V  V  K  P  G  F  C  P  P
421 TGAGAACATCAACCAGCCACAGAGTGCTGCCGGTGTAGTCAAACCGGGATTCTGTCCCCC
150  V  R  P  V  C  P  L  R  S  F  Q  P  P  F  T  C  S  N  D  G
481 CGTTCGTCCAGTGTGTCCTCTTAGGAGCTTCCAGCCACCATTCACTTGCTCTAACGACGG
170  A  C  G  G  I  D  K  C  C  F  D  R  C  L  G  E  H  V  C  K
541 CGCTTGCGGAGGCATCGACAAGTGTTGCTTCGACAGATGTCTCGGCGAGCACGTGTGCAA
190  A  P  L  G  I  G  R  *
601 GGCTCCTCTGGGCATTGGGCGATAGatatccttgggaaaattaagctgttcagtgaaatt
661 catcattgccttttcgaggtaggatgatttcatgaacattaatgttttcgtccattgaaa
721 cgtatgtaaaaaagatgtaaatacaatg
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Fig. 1. Nucleotide and deduced amino acid sequences of *LvCruU*. The ORF of the nucleotide sequences are shown in upper-case letters, the 5'and 3′-UTRs are shown in lower-case letters. Nucleotides and amino acids are numbered on the left of the sequences. The signal peptide is shown in red; the conserved cysteines are boxed; the WAP domain is shaded. The initiation codons are in bold. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

hemocytes were isolated immediately and at 4, 8, 12, 24, 36, 48, 72 and 96 hpi (hours post infection). Each time point with three parallel samples. And each sample was converged from hemocytes of five shrimps. The total RNA was extracted using RNeasy Mini Kit (Qiagen, Germany), and then reverse transcribed into cDNA by using PrimeScript RT Reagent Kit (TaKaRa, Japan) for real-time RT-PCR assays. And for hemocytes protein collection, total proteins from hemocytes of five shrimps were extracted at 12, 24, 48 and 96 hpi unsing EpiQuik Whole Cell Extraction Kit (Epigentek, USA).

For immune challenge experiments, each healthy *L. vannamei* was intramuscularly injected at the third abdominal segment with 50 mL of *V. parahemolyticus* (7.0×10^5 CFU/g), 50 mL of *S. aureus* (4.5×10^5 CFU/g), and 50 mL of phosphate-buffered saline (PBS; control), respectively. The hemocytes and gills of the challenged *L. vannamei* were sampled at 0, 4, 8, 12, 24, 36, 48, and 72 h hpi, and three shrimps from each group were randomly selected.

For Tissue distribution investigation, healthy shrimps were killed and their eyestalks, gills, hearts, hepatopancreas, stomachs, intestines, nerves, muscles, pyloric caecum, hemocytes, and epidermis were collected for tissue expression analysis.

Real-time RT-PCR assays were performed with a Roche LightCycler 480 thermal cycler (Roche Applied Science, Germany). The fold changes in gene expression were calculated using the relative standard curve method [16]. Three replicate real-time RT-PCR were performed per sample. *LvEF-1a* (GenBank Accession No. GU136229) was used as internal control. The expression level at 0 h was used as the baseline, and the corresponding expressions in the PBS injection group were used as the control group. The primer sequences are provided in Table 1. The LvCurU in *L. vannamei* hemocytes was detected with anti-LvCruU monclone antibody prepared from Abmart (China). The primer sequences are listed in Table 1.

2.7. Dual-luciferase report gene assay

The promoter of *LvCruU* was obtained by genome-walking PCR, which is 1, 777 bp in length. Report gene vectors were constructed based on pGL3-Basic (Promega, USA), and PCR primers were designed to amplified promoters of different lengths (about 250 bp interval) are listed in Table 1. The *LvCruU* promoter with Deaf1 binding sites and/or ATF3 binding site mutants were synthesed by Guangzhou IGE Biotechnology LTD. The five Deaf1 binding sites, CACGAA at −1669～-1663 (the standing initial of initiation codon A was set as +1), CTCGGC at −1026～-1020, CTCGTG at −783～-777, TTCCGA at −724～-718, and AACGAG at −492～-486 were replaced by AGTTCC, AGTATA, ACTTCA, GGAATC and CGATCT, respectively. And ATF3 binding site at TGATGCAA at −824～-816 was replaced by GAGCATGG. The S2 cells were cultured at 28 °C with Schneider Insect Medium (Sigma) supplemented with FBS (10%) in 96-well plates for 24 h. Then, 100 ng of firefly luciferase report gene plasmid, 20 ng of pRL-TK renilla luciferase plasmid, and 200 ng or the indicated amount of expression plasmid (pAC-eGFP and pAC5.1-Basic plasmid as a negative control) were transfected into S2 cells in a single well using the FuGEN Transfection Reagent (Promega). The luciferase report assays were measured at 48 h post transfection, according to the manufacturer's instructions. All of the experiments were repeated three times.

2.8. Cumulative mortality test

For the cumulative mortality test, six groups of shrimps were used in this experiment (PBS plus PBS, PBS plus *V. parahemolyticus*, dseGFP plus PBS, dseGFP plus *V. parahemolyticus*, dsLvCruU plus PBS, and dsLvCruU plus *V. parahemolyticus*, $n = 50$). Shrimps were intramuscularly injected with dsLvCruU, dseGFP, or PBS. At 48 h later, the second injection followed, with either PBS or *V. parahemolyticus* (7.0×10^6 CFU/g). Then, cumulative mortality was recorded every 12 h. And for testing the function of LvCruU in shrimp antibacterial immunity, six groups of shrimps were used (PBS plus *V. parahemolyticus* group, the Tg plus dsLvCruU plus *V. parahemolyticus* group, Tg plus *V. parahemolyticus* group, Tg plus dseGFP plus *V. parahemolyticus* group, and the Tg plus PBS, $n = 50$). The shrimps were intramuscularly injected with PBS, Tg plus dsLvCruU, Tg or Tg plus dseGFP. At 48 h later, the second injection followed, with either PBS or *V. parahemolyticus*. Then, cumulative mortality was recorded every 12 h. To confirm the RNAi efficiency of this test, hemocytes of shrimps ($n = 18$) intramuscularly injected with dsLvCruU, dseGFP, or PBS were collected at 72 h post injection. Then the total RNA of five shrimps was extracted and reverse transcribed into cDNA. Finally, RT-PCR assays were performed as previously described [14]. *LvEF1a* was used as the internal reference.

3. Results

3.1. Characterization of LvCruU

The cDNA of *LvCruU* is 748 bp in length, and the open reading frame (ORF) of *LvCruU* is 591 bp, encods a putative protein of 197 amino acids with a calculated molecular weight about 13.7 kDa (Fig. 1). By blasting to GenBank with LvCruU, we confirmed that homologous proteins of LvCruU mainly distribute in crustacean and insect, such as *Camponotus floridanus*. LvCruU contains a signal peptide at its N-terminal (1–16 aa), and a WAP domain (143–192 aa), which is the four-disulfide core domain can be found in proteins with antimicrobial activities or anti-protease activities (Fig. 2B). MEME was performed to discover conserved motifs within the Crustin proteins. It can be observed that most of Crustin proteins be investigated with all the three conservative motifs; and their sequences are as follows, motif 1: CSHDGACGGLDKCCYDACLKEHVCK, motif 2: RYWCKTPEGQAYCCENANEPE, and motif 3: PVGVKPGDCPPVRPTCPRFR (Fig. S3).

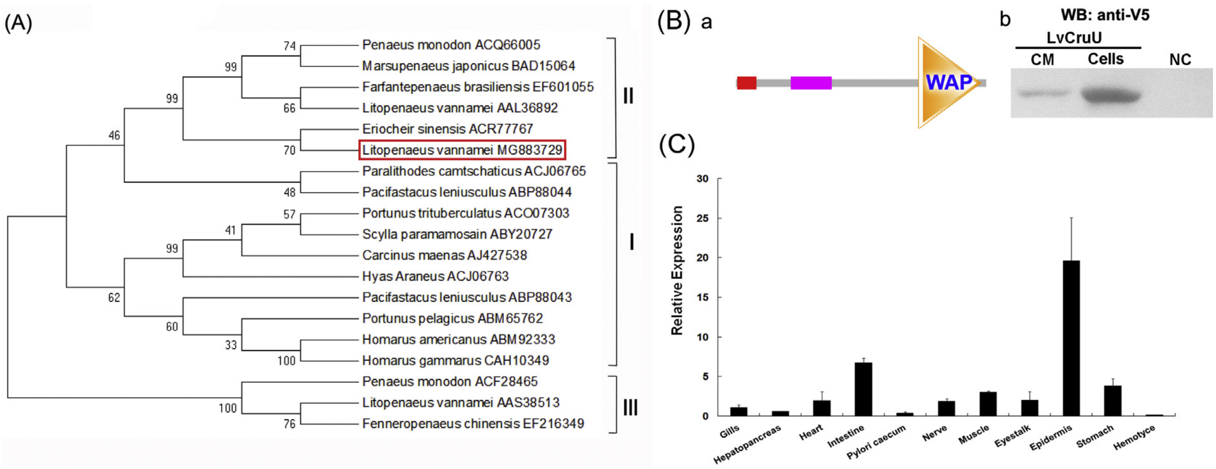

Fig. 2. Basic information of LvCruU. (A) Phylogenetic analysis of the Crustin proteins. Consensus neighbor-joining (N–J) tree based on the sequences of Crustins from different species. The numbers at the forks indicate the bootstrap. The bootstrap trials were replicated 5, 000 times. [(B) a] A motif block diagram showed motifs distribution secreted effector indicated by bars. [(B) b] S2 cells were transfected with pACB-LvCruU. And 48 h later, the LvCruU can be detected both in S2 cells and culture medium with anti-V5 antibody. [(B) c] Relative expression of *LvCruU* in different tissues. Total RNA extracted from different tissues was reverse-transcribed into cDNA to serve as templates. Ten shrimps were used for tissue expression analysis. The relative expression levels were normalized to *LvEF-1a*, and the relative expression of *LvCruU* in various tissues was compared against that in the pylori caecum. The results are based on three independent experiments and expressed as mean values ± S.D.

3.2. Phylogenetic analysis

To investigate the relationship between LvCruU and its homologues, multiple sequence alignment was performed (Fig. S2). And the results showed that only at the signal peptides and the WAP domians, Crustin proteins with high homology, but not at other positions. And the 19 Crustin proteins be investigated belong to the three types Crustin family, respectively (Fig. 2A). As the phylogenetic tree showed, Type I Crustin family with the largest number of member, it also includes *Paralithodes camtschaticus* ACJ06765, *Pacifastacus leniusculus* ABP88044, *Portunus trituberculatus* ACO07303, *Scylla paramamosain* ABY20727, *Carcinus maenas* AJ427538, *Hyas Araneus* ACJ06763, *P. leniusculus* ABP88043, *Portunus pelagicus* ABM65762, *Homarus americanus* ABM92333 and *H.gammarus* CAH10349. LvCruU (MG993729), *Eriocheir sinensis* ACR77767,*P. monodon* ACQ66005, *M. japonicus* BAD15064, *Farfantepenaeus brasiliensis* EF601055 and *L. vannamei* AAL36892 all belong to Type II Crustin family. Type III Crustin family contains the *P. monodon* ACF28465, *L. vannamei* AAS38513 and *Fenneropenaeus chinensis* EF216349 (Fig. 2A).

3.3. LvCruU was constitutively transcribed in various tissues of shrimp and could secretory expression in S2 cells

Results of real-time RT-PCR showed that *LvCruU* was expressed in all tissues examined [Fig. 2B (b)]. High expressions of *LvCruU* were detected in intestine and epidermis, with approximately 8.3-, and 22.2-fold higher than that in the pylori caecum, which has the lowest expression level in all tissues examined. Both intestine and epidermis are the important tissues for shrimp immunity, so the tissue distribution of *LvCruU* is related to its function. And there is a signal peptide at the N-terminal of LvCruU, which suggests that LvCruU may be a secretory protein. We over-expressed the *LvCruU* in S2 cells, then the culture medium and the cells were submitted to Western-blot assay. The result indicated that LvCruU could be detected in both culture medium sample and the cells sample [Fig. 2B (a)], which confirmed that it could be excreted into the extracellular space when express in S2 cells. In fact, most of the AMPs are secretory protein.

3.4. LvCruU was induced with V. parahemolyticus or S. aureus infection

In this study, expressions of *LvCruU* in intestine and epidermis upon *V. alginolyticus* or *S. aureus* infection were detected. The expression level of the gene at 0 h was used as the baseline. For *V. alginolyticus* infection, expression of *LvCruU* in shrimp both epidermis and intestine were up-regulated from 8 hpi, and reached the peak values at 36 hpi, which was 24.5-fold and 9.4-fold of the control, respectively; and then, in these two tissues, expression of *LvCruU* continued to decline (Fig. 3A). For *S. aureus* infection, expression of *LvCruU* in epidermis was increased from 12 hpi, and then kept on about 3-fold of the control during 24–96 hpi; the expression profile of *LvCruU* in intestine is similar to that in epidermis, while it was induced at 8 hpi (Fig. 3B).

3.5. Antimicrobial activity assays

To evaluate the antimicrobial activity of rLvCruU, its inhibitory effects on the growth of microbes (*V.parahemolyticus* and *S. aureus*) were examined (Fig. 4). The growth curves of *V. parahemolyticus* and *S. aureus* were generated as describe in Section 2.4. After incubation with 100 μg/mL rLvCruU for 12 h, the OD600 values of *V. parahemolyticus* and *S. aureus* increased to 1.62 and 1.39, respectively [Fig. 4A (a)]. In contrast, after incubation of *V. parahemolyticus* and *S. aureus* with 200 μg/mL rLvCruU for 12 h, the OD600 values reached 1.24 and 1.21, respectively [Fig. 4A (b)]. The results showed that rLvCruU at the final concentration of 200 μg/mL exhibited higher inhibitory effects on the *S. aureus* than *V. parahemolyticus*. The antibacterial activities of rLvCruU protein were also detected in agar diffusion assays (Fig. 4B). Comparing to the effects of the rTrx control protein and TBS, the transparent rings around the pores contained the rLvCruU protein were bigger in size (*V. parahemolyticus*: ~3.0 cm; *S. aureus*: ~3.1 cm), and smaller than those contained ampicillin (*V. parahemolyticus*: ~3.9 cm; *S. aureus*: ~4.0 cm), which indicates rLvCruU with antibacterial activity (Fig. 4B).

3.6. Knocked-down expression of LvCruU increased the cumulative mortality of V. parahemolyticus infected shrimp

As the results of RT-PCR showed, dsLvCruU but not dseGFP injection significantly reduced expression of dsLvCruU in *L. vannamei* hemotyces at 72 h post dsRNA injection (Fig. 5A). To study the antibacterial function of *LvCruU*, cumulative mortality of *L. vannamei* infected *by V. parahemolyticus* plus *LvCruU* knocked-down was investigated. In *V. parahemolyticus* infection shrimps, cumulative mortality reached 77% at 72 hpi for dsLvCruU plus *V. parahemolyticus*

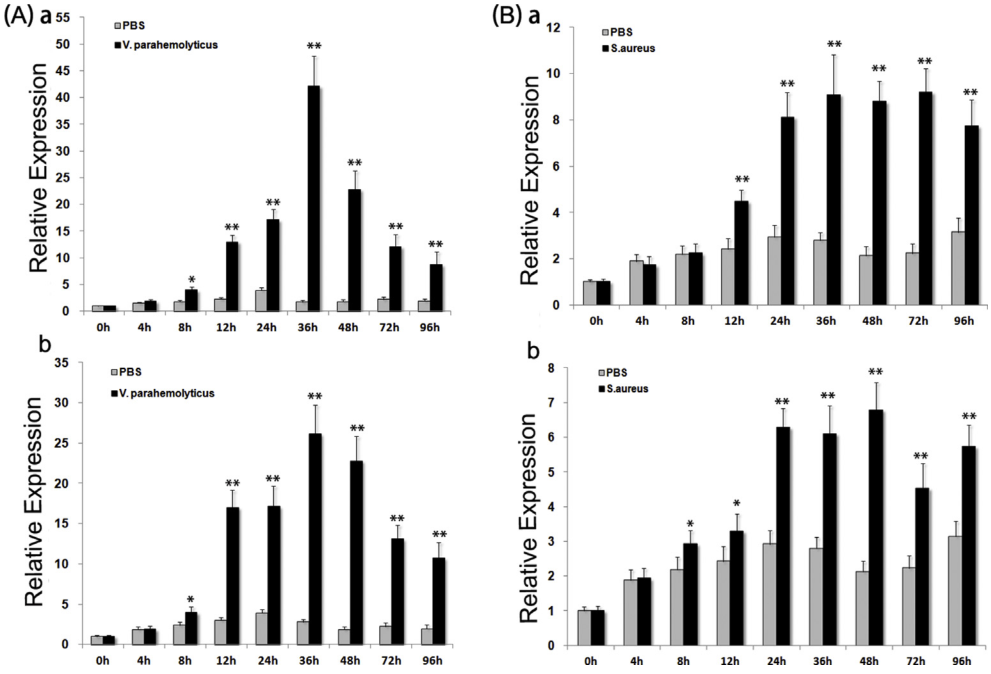

Fig. 3. Temporal expression of *LvCruU* in immune challenged *L. vannamei*. The relative expression of *LvCruU* in the groups treated with *V. parahaemolyticus* in epidermis [A(a)] and intestine [A(b)], and *S. aureus* in epidermis (B(a)) and intestine [B (b)] were compared with that in the control group. The relative expression level of the target genes was normalized to *LvEF1a*. The results are based on three independent experiments and expressed as mean values S.D. Statistical significance was calculated using Student's *t*-test (* indicates $p < 0.05$ and ** indicates $p < 0.01$ compared with the control).

group, 26% in dseGFP plus *V. parahemolyticus* group, and 29% in PBS plus *V. parahemolyticus* group (Fig. 5B). This result was similar to that knocked-down expression of *LvSpz4* in *V. alginolyticus* infected *L. vannamei* [25]. And for the control groups, the cumulative mortality of dsLvCruU group, dseGFP group and PBS group were 4%, 4% and 0% at 72 h post injection, respectively. These results confirmed that LvCruU worked at *L. vannamei* pathogenic bacteria resistance. *S. aureus* is not the pathogenic bacteria for *L. vannamei*, though it also induced the expression of LvCruU (Fig. 3B), it did not cause severe death in this experiment (Fig. 5B).

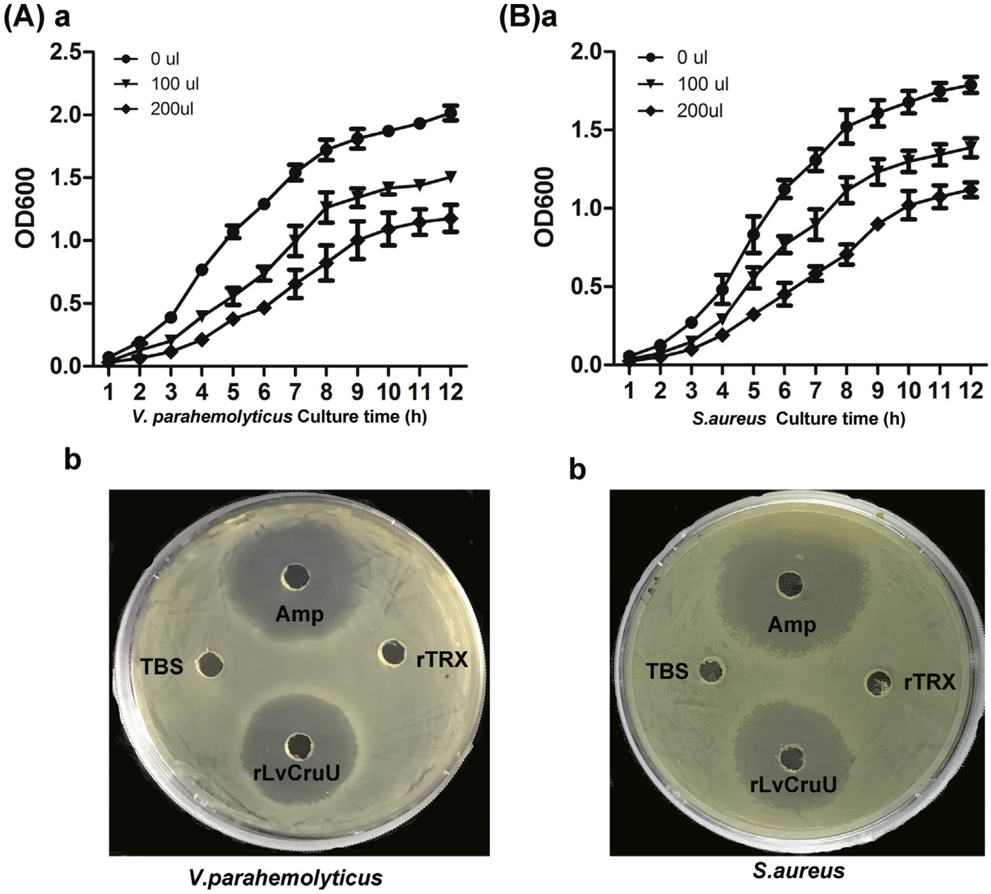

Fig. 4. Antimicrobial activity assays of rLvCruU. Growth inhibition assay of the antimicrobial activity of rLvCruU. Growth inhibition curves showing the antimicrobial activity of rLvCruU. Purified rLvCruU (final concentration, 100 μg/mL or 200 μg/mL) was added to cultures of *V. parahaemolyticus* [A (a)] and *S. aureus* [B (a)]. An equal volume of TBS was added to the control cultures. Growth curves were constructed from OD600 values measured at 1 h intervals. Agar diffusion assay of antimicrobial activity of rLvCruU. Microorganisms were added to the pores on the agar plates in the presence of 100 μL TBS solution with 20 μg rLvCruU protein or rTrx control. TBS solution (100 μL) or 20 μg ampicillin (Amp) was added as controls. The plates were the incubated at 36 °C for 16 h (*V. parahemolyticus*) or 30 °C for 16 h (*S. aureus*). The transparent ring around the pores signifies antibacterial activity.

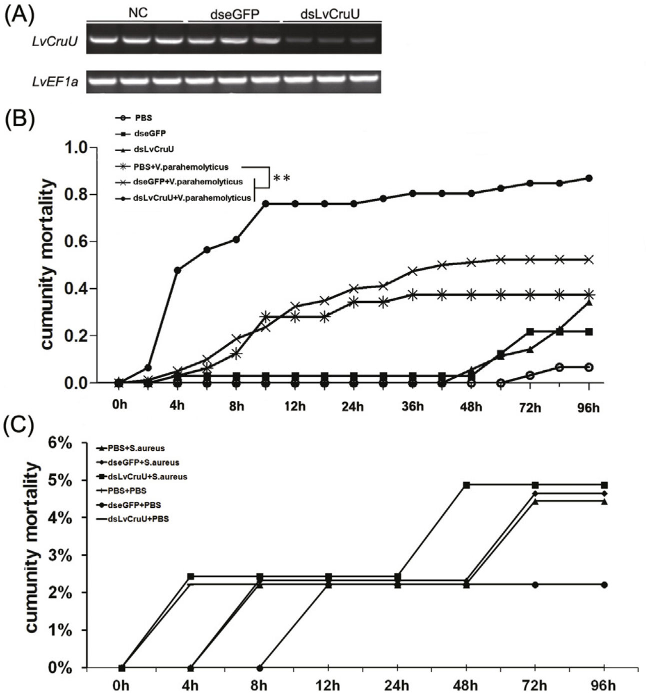

Fig. 5. Knocked-down expression of *LvCruU* by RNAi inecreased the cumulative mortality in *V. parahaemolyticus* infected shrimps. (A) RT-PCR analysis of *LvCruU* expression with *LvEF-1a* as the internal control. Shrimps ($n = 50$) were intramuscularly injected with dsLvCruU, dseGFP or PBS (as a control). 72 h after the initial injection, shrimps were infected with *V. parahaemolyticus* (B), *S. aureus* (C) or PBS (control) again. Cumulative mortality was recorded every 12 h post-challenge. Datas are derived from three independent experiments and showed as mean ± S.D. Differences in mortality levels between treatments were analyzed by *Kaplane-Meier* plot (log-rank X^2 test).

3.7. Upregulated expression of LvCruU by UPR in L. vannamei

The expression level of the gene at 0 h was used as the baseline, and the corresponding expression in the dseGFP group was used as the control. In the intestine, *LvCruU* was induced 12 h post dsLvBip injection, and reached the peak values at 72 h post dsLvBip injection, which was about 6.1-foled of the control group (Fig. 6A). And in epidermis, *LvCruU* also was increased upon UPR activation. And there two peak value at 8 h and 48 h post dsLvBip injection, which were about 1.9-fold and 17.3-fold of the control group, respectively (Fig. 6B). And in hemotyces, *LvCruU* with the most dramatic inducing, at 72 h post dsLvBip injection, it was about 65-fold of the control group (Fig. 6C). Moreover, the expression of LvCruU in the hemotyces also confirmed by Western-blot assay, which showed that expression of LvCruU gradually increased in the duration of 12 hpi ~ 96 hpi (Fig. 6D).

3.8. LvATF3 and LvDeaf1 activated the promoters of LvCruU in S2 cells

By genome-walking PCR assay, we obtained the promoter sequences of *LvCruU*, which was 1777 bp in length. And report genes assays were carried out to investigate the transcriptional regulation of *LvCruU*. As the results showed, activity of *LvCruU* promoter was significant change between −1000 bp ~ -750 bp and −750 bp ~ 500 bp (Fig. 7A). Analysis by JASPAR database (http://jaspar.genereg.net/), we found there are Deaf1 binding site and BRLZ transcription factor binding site at −1000 bp ~ -750 bp and −750 bp ~ 500 bp of the *LvCruU* promoter. By an BRLZ transcription factor scanning, LvATF3, which also induced upon UPR activation (Fig. S4), was found that it increased the activity of *LvCruU* promoter significantly (data not show). And report gene assay results showed that transcription factor of NF-κB pathway LvDeaf1 could also significantly increase the activity of *LvCruU* promoter. And the mutation of Deaf1 binding site or BRLZ transcription factor binding site would greatly reduce the influence of LvDeaf1 and LvATF3 on *LvCruU* promoter activity, respectively (Fig. 7B). And the results of co-expression assay showed that LvDeaf1 and LvATF3 with forward coordination effecte on *LvCruU* promoter activity (Fig. 7C).

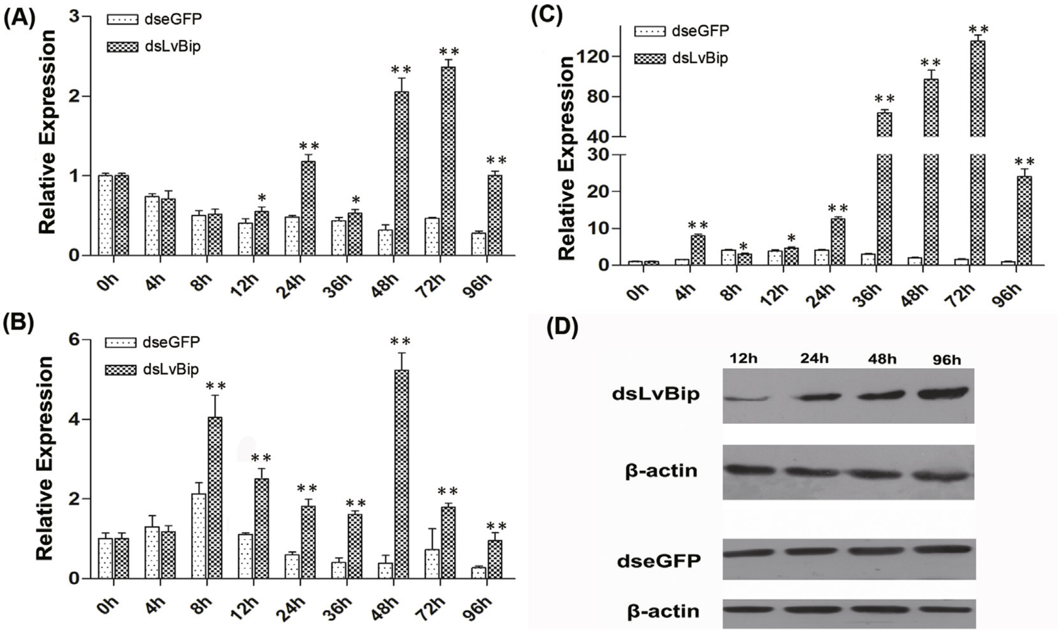

Fig. 6. Relative expression of *LvCruU* upon UPR activation. The mRNAs were collected at 0, 4, 8, 12, 24, 36, 48, 72 and 96 h after dsLvBip or dseGFP injection. Then expression levels of ***LvCruU*** in epidermis (A), intestine (B) and hemocytes (C) at various times were measured using real-time RT-PCR. The relative expressions of *LvCruU* in hemocytes were normalized to *LvEF-1a* and compared against time zero. The results were based on three independent experiments and expressed as mean values ± S.D. Statistical significance was determined by Student's *t*-test (* indicates $P < 0.05$, ** indicates $P < 0.01$ compared with controls at each time point). (D) The protein samples of hemocytes were collected at 12, 24, 48 and 96 h after dsLvBip or dseGFP injection, and the expression of LvCruU was detected by anti-V5 antibody.

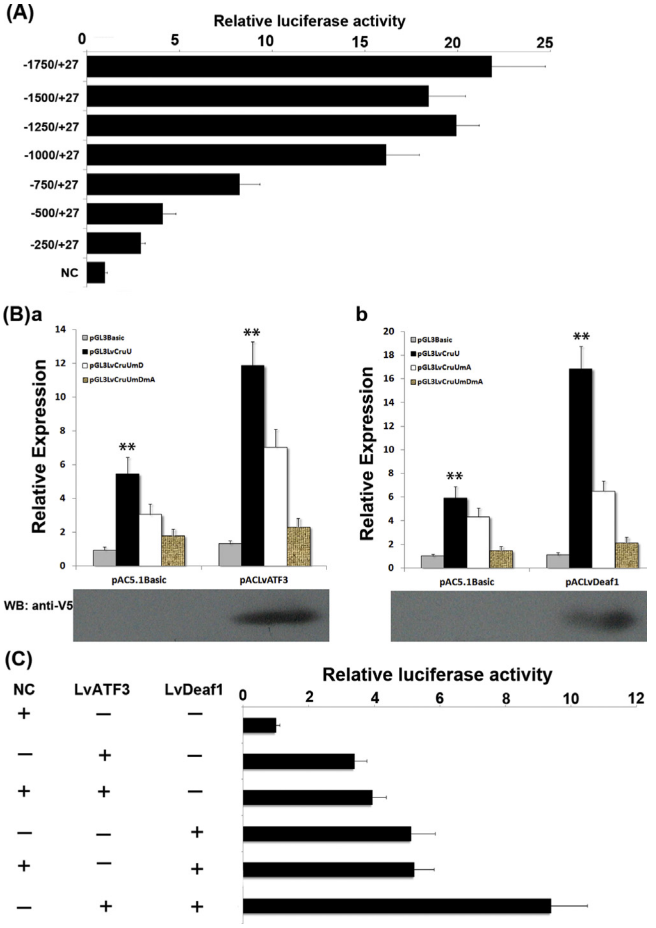

Fig. 7. Activation of the *LvCruU* promoter by LvDeaf1 and LvATF3. (A) Relative luciferase activity of ***LvCruU*** promoters of different lengths in S2 cells. Relative luciferase activity of ***LvCruU*** promoter induced by LvATF3 [(B) a] or LvDeaf1 [(B) b] in S2 cells. (D) And the influence of LvATF3 and LvDeaf1 co-expression on ***LvCruU*** promoter activity. The bars indicate mean values ± S.D. of the luciferase activity ($n = 3$). Statistical significance was determined by one-way ANOVA (**$p < 0.01$). (D) **Significant difference ($p < 0.01$) from the pGL3Basic group.

3.9. LvCruU is important for antibacterial immunity upon ER-stress in L. vannamei

In our study, we found that 0.2 μg/g shrimp Tg injection did not cause death in *L. vannamei.* The results of RT-PCR indicated that LvCruU was down-regulated 72 h post dsLvCruU injection (Fig. 8A). Tg injection significantly increased the expression of *LvCruU* in the duration of the test (Fig. 8B). And far more important is that results of cumulative mortality assay showed that 12 h post *V. parahemolyticus* infection, the cumulative mortality of the PBS plus *V. parahemolyticus* group, the Tg plus dsLvCruU plus *V. parahemolyticus* group, Tg plus *V. parahemolyticus* group, Tg plus dseGFP plus *V. parahemolyticus* group, and the Tg plus PBS were 72%, 44%, 42%, 40% and 2%, respectively. And at 24 h post *V. parahemolyticus* infection, the cumulative mortality of the PBS plus *V. parahemolyticus* group, the Tg plus dsLvCruU plus *V. parahemolyticus* group, Tg plus *V. parahemolyticus* group, Tg plus dseGFP plus *V. parahemolyticus* group, and the Tg plus PBS were 72%, 58%, 52%, 54% and 2%, respectively (Fig. 8C). These results suggested that *LvCruU* contributed to antibacterial immune response upon ER-stress in *L. vannamei.*

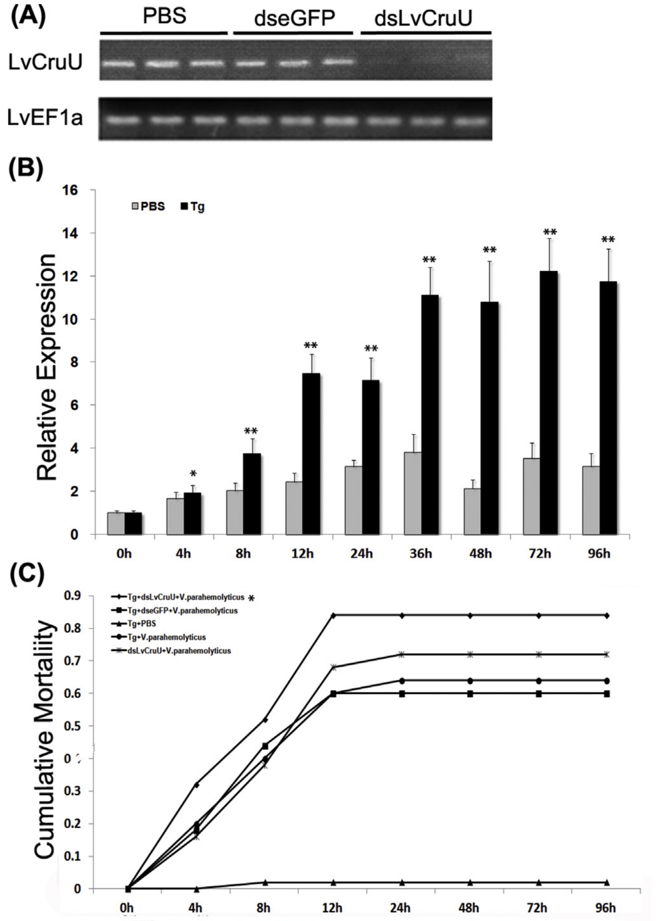

Fig. 8. DsLvCruU injection decreased the cumulative mortality of *V. parahaemolyticus*-infected shrimps upon ER-stress. (A) RT-PCR analysis of *LvCruU* expression with *LvEF-1a* as the internal control. (B) Shrimps ($n = 50$) were intramuscularly injected with Tg, dsLvCruU, dseGFP, PBS (as a control) or their combinations. 48 h after the initial injection, shrimps were infected with *V. parahaemolyticus* or PBS (control) again. Cumulative mortality was recorded every 12 h post-challenge. Data are derived from three independent experiments and showed as mean ± S.D. Differences in mortality levels between treatments were analyzed by *Kaplane-Meier* plot (log-rank X^2 test).

4. Discussions

UPR is the main system for ER-stress remission in eukaryotic cell, and contributes to cell's survival upon environmental stress. Yet UPR activation could reduce the protein translation of most proteins and therefore influence various physiological functions of cells, including immune response. In this study, we found that LvCruU in shrimp showed antibacterial activity was downstream gene of UPR. Down-regulated its expression would decrease the *L. vannamei* cumulative mortality under *V. parahemolyticus* infction. We found that the prompter activity of *LvCruU* not only enhanced by NF-κB pathway transcription factor Deaf1, but also by UPR transcription factor LvATF3. By Tg plus dsLvCruU injection, we proved that *LvCruU* played a vital role in *L. vannamei* antibacterial immune response. All these results uncovered the function of *LvCruU* in maintaining balance between immune response and ER-stress response in *L. vannamei.*

Crustins are mainly divided into three types base on their sequence patterns. For type I Crustins, they have motif characterized by a series of specific intervals of cysteine: "—C-X(3)—C-X(8–12)—C—C-X(16–17)—C-X(6)—C-X(9–10)-". Type II Crustin family is defined as Crustins that contains a glycine-rich hydrophobic domain at the N-terminal position ahead of the cysteine-rich domain. While type III Crustin is a group of

WAP domain-containing proteins, which lacks both of the Gly-rich domain in Crustin II molecules and the Cys-rich region present in type I− and type II Crustin [11]. LvCruU with motif "−C-X(3)−C-X(8)−C−C-X(17)−C-X(6)−C-X(10)-", and it also contains a glycine-rich region (27 glycines between 45aã101aa) ahead the cysteine-rich domain, which perfect matching the definition of type II Crustin. And on the phylogenetic three, it was gathered together with other known type II Crustin as a group, so it is obviously that LvCruU is a member of type II Crustin [26]. As report, Type I− and II crustins are abundant in crustaceans, and exhibit antimicrobial activity mainly against Gram-positive bacteria. Type III crustin has only been reported in shrimp, and it not only exhibits antibacterial activity against several Gram-positive, but also showed antiprotease activity that inhibited subtilisin A from bacterial [27]. In this study, we showed that LvCruU could against both Gram-positive bacteria and Gram-negative bacteria *in vitro*. While it does not mean the situation is the same *in vivo*.

As immune factor, The expressions of *AMPs*, such as *Crustins* are mainly regulated by the TLR/IMD−NF−κB signal pathway at the transcriptional level in invertebrate [28]. In *Drosophila*, TLR and IMD receive the upstream immune signal and activate the downstream effector, such as *AMPs*, via NF-κB factor drosal, dif or relish [29]. While for survival in fungal infection, flies required another transcription factors the Deaf1 which acts downstream of the NF-κB factors dorsal and dif, to regulate the expression of *Drosomycin* [30]. In our study, we found that promoter region of *LvCruU* contains sereral Deaf1 binding site, which suggested that Deaf1 may be involved in *LvCruU* expression regulation. Immune challenge, which had been proved that activated the NF-κB factors, also induced the expression of *LvCruU*. And results of report gene assay showed that LvDeaf1 enhanced the activity of *LvCruU* promoter. These results suggest that difference from *Drosophila*, *L. vannamei* Deaf1 is engaged in the regulation of antimicrobial factors *LvCruU*. Whether LvCruU has antifungal function remains to be studied. While it should be noted that several studies also suggested that *AMPs* could be regulated at the posttranslational modification level in shrimp [1]. Whether LvCruU also is regulated at the posttranslational modification is still unclear.

For resisting the invasion of pathogens, production of immune-related factors is indispensable. While in some cases, cells have to reduce the synthesis of new proteins for survival. For example, once cells suffer from severe ER-stress, PERK-eIF2α pathway is activated to reduce the production of most proteins, including the *AMPs*, for ER-stress remission in cells [13]. Unfortunately, in the harsh water environment, pathogens and environmental stimulus always come together. In such case, the coordination between immune response and UPR is essential for shrimp survival. Many studies have shown that immune response and UPR were inseparable from each other [17–19,21]. And in shrimp, in previous studies, we also proved that UPR could increase the expressions of several immune-related factors, such as *solute carrier family 15 member 4* and *Spatzle 4* [25,31]. While we cannot answer the question until then: how its innate immune function avoids being excessive to weaken upon UPR activation. In this study, we found that a BRLZ transcription factor LvATF3 was increased under ER-stress, and it is involved in the regulation of *LvCruU*, which has antibacterial function. Moreover, *LvCruU* also be induced by ER-stress, and plays a role in antibacterial immune response under ER-stress. These results make us to put forward a conjecture: in shrimp, UPR activation inhibits the expressions of most proteins, while it induced some immune factors, such as LvCruU, for maintaining basic immune response function upon ER-stress.

To sum up, in this study we cloned a type II Crustin *LvCruU* with antibacterial function. It was regulated by the NF-κB pathway transcription factor LvDeaf1, as well as the UPR transcription factor LvATF3. Both LvATF3 and *LvCruU* were induced with UPR. And these results suggest that under stress, *LvCruU* is induced and played an antimicrobial role.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2018.10.047.

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