Fish and Shellfish Immunology 84 (2019) 404–413

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

# Functional characterization of a *matrix metalloproteinase 2* gene in *Litopenaeus vannamei*

Yu-Ying Lian[a,c], Hong-Hui He[b,c], Chao-Zheng Zhang[d], Xin-cang Li[e,**], Yi-Hong Chen[f,*]

[a] Key Laboratory of Marine Resources and Coastal Engineering in Guangdong Province/South China Sea Bio-Resource Exploitation and Protection Collaborative Innovation Center (SCS-REPIC)/ School of Marine Sciences, SunYat-senUniversity, 135 Xingang Road West, Guangzhou, 510275, PR China
[b] School of Life Sciences, Sun Yat-sen University, 135 Xingang Road West, Guangzhou, 510275, PR China
[c] State Key Laboratory for Biocontro / Institute of Aquatic Economic Animals and Guangdong Province Key Laboratory for Aquatic Economic Animals, SunYat-senUniversity, 135 Xingang Road West, Guangzhou, 510275, PR China
[d] Guangdong Provincial Center for Disease Control and Prevention, 160 QunXian Road, Guangzhou, 511430, PR China
[e] East China Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences, Shanghai, 200090, PR China
[f] Guangdong Provincial Key Laboratory for Healthy and Safe Aquaculture, College of Life Science, South China Normal University, Guangzhou, 510631, PR China

ARTICLE INFO

*Keywords:*
Matrix metalloproteinase 2
Oxidative stress
Antimicrobial peptide gene
*Litopenaeus vannamei*

ABSTRACT

Matrix metalloproteinases (MMPs) contribute to both normal and pathological tissue remodeling. They act as regulatory molecules by working in enzyme cascades as well as processing matrix proteins, cytokines, growth factors and adhesion molecules to generate fragments with biological effects. So MMPs could play distrinct roles in the process of pathogen infection. In present study, we cloned a *MMP-2* (*LvMMP-2*) gene from *Litopenaeus vannamei*. *LvMMP-2*, highly expressed in epidermis, located to endoplasmic reticulum in S2 cells. Results of real-time RT-PCR assay showed that *LvMMP-2* was induced in shrimp hemocytes upon unfolded protein response or oxidative stress, but not via heat shock treatment. It is proved that the promoter activity of *LvMMP-2* was enhanced by NF-E2-related factor 2 and AP-1 factor c-Jun. Further research showed that down-regulated *LvMMP-2* contributing to oxidative stress injury, could reduce the cumulative mortality of shrimps under oxidative stress. Besides, our study also indicated that *LvMMP-2* was accelerated by lipopolysaccharides injection. LvMMP-2 in S2 could increase the promoter activity of several *antimicrobial peptide* genes, and knocked-down expression of *LvMMP-2* depressed the expression of *penaeidin2* and *β-Defensin*. Moreover, we showed that down-regulated *LvMMP-2* suppressed the cumulative mortality of shrimp infected with white spot syndrome virus (WSSV) or with *Vibrio alginolyticus*. Collecting results suggested that *LvMMP-2* involves in shrimp innate immune response, and also contributes to tissue injury caused by WSSV infection.

## 1. Introduction

Metalloproteinase (MP) is the protease that catalytic mechanism contains a metal. Most metalloproteases require zinc, and some need cobalt or calcium [1]. The metal ion is coordinated to the protein via three ligands, which the metal ion may be form covalent bond with histidine, glutamate, aspartate, lysine, or arginine [1]. And the metal ion also has the fourth coordination position that taken up by a labile water molecule. So far, MPs have been divided into more than 50 families [2]. About half of them contain an HExxH motif, which has been shown in crystallographic studies to form part of the metal-binding site. The HExxH motif is relatively conservative, and it is more stringently defined as 'abXHEbbHbc' for metalloproteases, where 'a' usually is valine or threonine, 'b' is an uncharged residue, and 'c' indicates a hydrophobic residue [3]. Most of MPs distribute in cytoplasm, and a few of them locate to specific organelle in cells, for example, MPs from family M48 are integral membrane proteins associated with the endoplasmic reticulum (ER) and Golgi [4].

Matrix metalloproteinases (MMPs) are calcium-dependent zinc-containing endopeptidases [5]. They were first described in vertebrate, including humans, and then found in invertebrate and plant [2]. All MMPs are synthesized in the latent form (Zymogen) [6]. They are secreted as proenzymes and require extracellular activation. They can be activated *in vitro* by various mechanisms including organomercurials, chaotropic agents and other proteases. MMPs have three common domains: the pro-peptide, the catalytic domain, and the haemopexin-like

* Corresponding author.
** Corresponding author.
*E-mail addresses:* lixin8687@163.com (X.-c. Li), chenyh18@m.scnu.edu.cn (Y.-H. Chen).

https://doi.org/10.1016/j.fsi.2018.10.021
Received 22 August 2018; Received in revised form 5 October 2018; Accepted 7 October 2018
Available online 11 October 2018

**Table 1**
Summary of primers used in this study.

| Primers | Sequence (5′-3′) |
|---|---|
| **For cDNA cloning** | |
| LvMMP-1 5′ RACE1 | TCACACGTGTCAGGCTTCTCCGTGTTCT |
| LvMMP-2 5′ RACE2 | TGGGCTTCTTGGGGGTCTCCTTGGTGGG |
| LvMMP-1 3′ RACE1 | AAACCGAAGTCACCTGCCCTTTCAGCAC |
| LvMMP-2 3′ RACE2 | CCCAACAACATCGAAAACCCGTCCTATG |
| **For Genome Walking-PCR** | |
| 5 GW-LvMMP-2-R2 | CGTCTCCACACGCCCTTGGCACCCTCCT |
| 5 GW-LvMMP-2-R1 | CGCATTATCGTCGATGATTTCCCTCCTT |
| **For genes expression** [a] | |
| pACB-LvMMP-2-EcoRI-F | CCG**GAATTC**TATGCTCTTGGCTGTAGTGATGT |
| pACB-LvMMP-2-Xba I-R | ATT**TCTAGA**CTCAGCCTAAACCTCCATGATGC |
| pACB-LvNRF2-EcoR I-F | CCG**GAATTC**TATGGAAGGCCCTGTAATTGAA |
| pACB-LvNRF2-Xba I -R | ATT**TCTAGA**CTCTGCTTGGGGTCATCCTTCC |
| pACB-Lvc-Jun-Kpn I-F | ATA**GGTACC**TATGGAGGCAACCATGTACGAG |
| pACB-Lvc-Jun-Xba I-R | ATT**TCTAGA**CTCTGGTGCGTTACGAAGGGGAT |
| **For report genes**[a, b] | |
| pGL3-LvMMP-2-full-Kpn I-F | ATA**GGTACC**TATATATTAATATATAAATATATAAACCAA |
| pGL3-LvMMP-2-1500-Kpn I-F | ATA**GGTACC**TTCTCATGGCTTCCCCATATC |
| pGL3-LvMMP-2-1300-Kpn I-F | ATA**GGTACC**ATGGTAACACAGAGGCAGCG |
| pGL3-LvMMP-2-1100-Kpn I-F | ATA**GGTACC**ATCAATGGGTTCACACACGGT |
| pGL3-LvMMP-2-900-Kpn I-F | ATA**GGTACC**CTCGATGAGATCGGTAACTATGC |
| pGL3-LvMMP-2-700-Kpn I-F | ATA**GGTACC**ACTGGCCTGACTGTTTTTGCTC |
| pGL3-LvMMP-2-500-Kpn I-F | ATA**GGTACC**ACTAATATTTTTGTTCAACTGCGAT |
| pGL3-LvMMP-2-300-Kpn I-F | ATA**GGTACC**CTCTTCCTCACCACCATCACC |
| pGL3-LvMMP-2-Bgl II –R | TAT**AGATCT**CTTGGCACCAGCAGGCACATCACss |
| pGL3-LvMMP-2mAP-1–F | gagcatggGCCGAGAGAGCCAATACACAAAAC |
| pGL3-LvMMP-2mAP-1–R | GCAGTTGCCAGACACTTCCTTTC |
| pGL3-LvMMP-2mARE–F | Gatcatgcaggttgc TGTACCCGCTGCCTCTGTGT |
| pGL3-LvMMP-2mARE–R | GTGCCTGCCAAGGTCGCCTCC |
| **For dsRNA templates amplification** | |
| DsRNA-LvMMP-2-595-T7-F1 | GGATCCTAATACGACTCACTATAGGGTAGTGATGTGCCTGCTGGTG |
| DsRNA-LvMMP-2-595-R1 | CTCCGTGGTATCCTCGTTTG |
| DsRNA-LvMMP-2-595-F2 | GTAGTGATGTGCCTGCTGGTG |
| DsRNA-LvMMP-2-595-T7-R2 | GGATCCTAATACGACTCACTATAGGCTCCGTGGTATCCTCGTTTG |
| **For RT-PCR** | |
| SQPCR-LvMMP-2-689-F | GCGTTCTCCCGTGTTCCTC |
| SQPCR-LvMMP-2-689-R | CACCCGGATAGAAGGCATG |
| SQ-LvEF1α-F | AAGGCCCTCAAGAAGAAGTAAAT |
| SQ-LvEF1α-R | TTGACAACCATACCTGGCTTC |
| **For real-time RT-PCR** | |
| QPCR-LvMMP-2-F | CCCTATTGGCTTGGCTGTCC |
| QPCR-LvMMP-2-R | CCACTACCAGAGGTGTCCGTTAT |
| QPCR-LvPEN2-F | CCACCACCCTATGGAAGACC |
| QPCR-LvPEN2-R | GACAACAACGTCCGAACCTG |
| QPCR-Lv*β*-Def-F | GCCATTGTGGTAGCTTTTGGA |
| QPCR-Lv*β*-Def-R | ATCCATTGCTGATTTTGCTGAC |
| QPCR-LvEF1α-F | GCTGATTGCGCCGTACTCAT |
| QPCR-LvEF1α-R | TCACGGGTCTGTCCGTTCTT |

[a] Nucleotides in bold indicate restriction sites introduced for cloning.
[b] Lowercase letters represent mutated nucleotides.

C-terminal domain, which is linked to the catalytic domain by a flexible hinge region [7]. MMPs are capable of degrading all kinds of extracellular matrix proteins [8]. They are also involved in the cleavage of cell surface receptors, the release of apoptotic ligands (such as the FAS ligand), and chemokine/cytokine inactivation [9]. Besides, MMPs are also reported to play a major role in cell behaviors, such as cell proliferation, migration (adhesion/dispersion), differentiation, angiogenesis, apoptosis as well as host defense [9].

The basement membrane is important for maintaining tissue organization, providing structural support for cells, influencing cell signaling and polarity. MMP-2 (along with MMP-9) is capable of degrading type IV collagen, the most abundant component of the basement membrane [10]. Because of this, MMP-2 has a close relationship with tumors. For examples, it plays an important role in the formation of new blood vessels within tumors, a process known as angiogenesis [11]. This process is essential for tumor progression, for the growth of tumors need increasing supplies of oxygen and nutrients; MMP-2 alters tumor viability and invasion by regulating lymphangiogenesis [12,13]. Furthermore, as reported, MMP-2 can initiate primary innate immune response by inducing mitochondrial-nuclear stress signaling with activation of the pro-inflammatory NF-κB pathway, NFAT pathway and IRF transcriptional pathway [14].

In this study, we have identified and characterized a cDNA that encodes *Litopenaeus vannamei matrix metalloproteinases 2*, which are designated as *LvMMP-2* (GenBank accession no. MH778698). We demonstrated that *LvMMP-2* was induced by UPR in *L. vannamei*, and its promoter activity is enhanced by [Nuclear factor (erythroid-derived 2)-like 2, NRF2] as well as c-Jun of *L. vannamei*. Also it was induced by lipopolysaccharide. Additionally, it is investigated that LvMMP-2 increased the activity of *antimicrobial peptide* (*AMP*) gene promoters, and knocked-down expression *LvMMP-2* not only decreased promoters activity of *penaeidin 2* (*PEN2*) and *β-Defensin* (*β-Def*), but also cut down the cumulative mortality of *Vibrio alginolyticus*-infected shrimp.

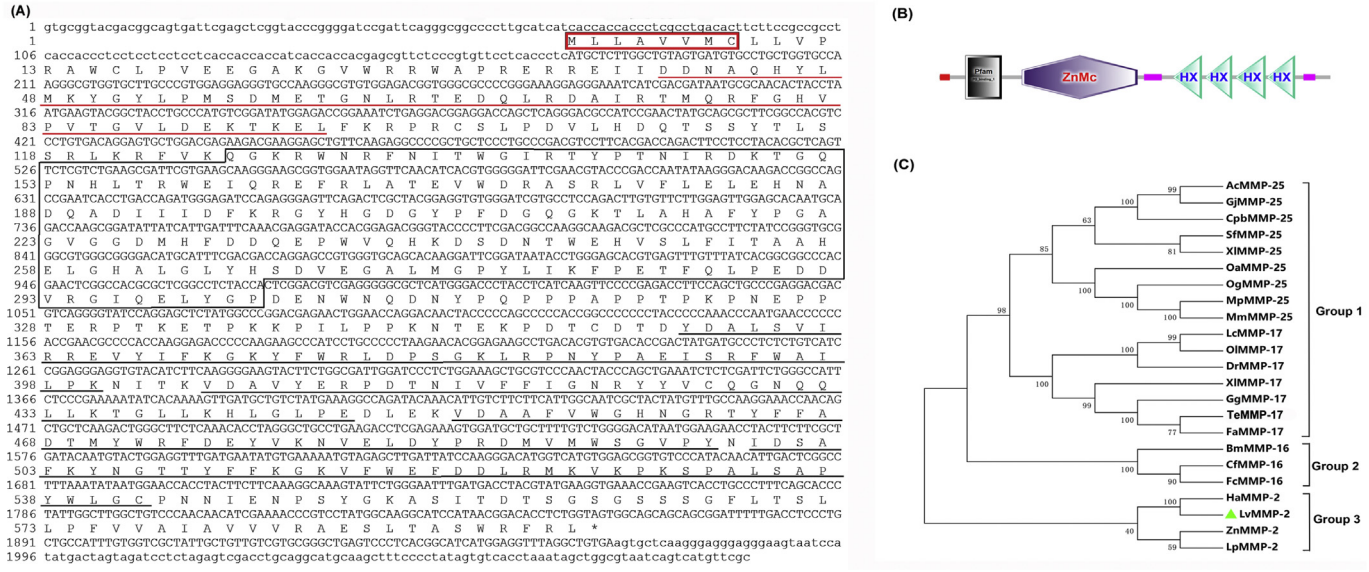


**Fig. 1. Characterization of *LvMMP-2*.** (A) The ORFs of the nucleotide sequences are presented in uppercase letters; the 5′- and 3′-UTRs are shown in lowercase letters. Nucleotides and amino acids are numbered on the left of the sequences. The signal peptide is showed in red. ZnMC domains are boxed. The PGBD is indicated with a red underline. And the four HX motifs are indicated with black underline. (B) Schematic representation of the structural motifs of LvMMP-2 proteins. (C) Phylogenetic tree of MMP proteins from invertebrates and vertebrates. The tree was constructed by an N-J algorithm using the Mega 6.0 program based on the multiple sequence alignment via ClusterW indicates the genetic distance. LvMMP-2 is indicated with a green triangle. The tree could be divided into three classes as indicated. AcMMP-25, *Anolis carolinensis* matrix metalloproteinase-25 (GenBank accession no. XP_008119204.1); GjMMP-25, *Gekko japonicus* matrix metalloproteinase-25 (GenBank accession no. XP_015261861.1); CpbMMP-25, *Chrysemys picta bellii* matrix metalloproteinase-25 (GenBank accession no. XP_008175955.1); SfMMP-25, *Scleropages formosus* matrix metalloproteinase-25 (GenBank accession no. XP_018592412.1); XlMMP-25, *Xenopus laevis* matrix metalloproteinase-25 (GenBank accession no. XP_018094874.1); OaMMP-25, *Ornithorhynchus anatinus* matrix metalloproteinase-25 (GenBank accession no. XP_001515193); OgMMP-25, *Ornithorhynchus anatinus* matrix metalloproteinase-25 (GenBank accession no. XP_012661153.1); MpMMP-25, *Mus pahari* matrix metalloproteinase-25 (GenBank accession no. XP_021077520.1); MmMMP-25, *Mus musculus* matrix metalloproteinase-25 (GenBank accession no. EDL22252); LcMMP-17, *Larimichthys crocea* matrix metalloproteinase-17 (GenBank accession no. XP_019115675.1); OlMMP-17, *Oryzias latipes* matrix metalloproteinase-17 (GenBank accession no. XP_011480221.2); DrMMP-17, *Danio rerio* matrix metalloproteinase-17 (GenBank accession no. XP_698601.6); XlMMP-17, *Xenopus laevis* matrix metalloproteinase-17 (GenBank accession no. XP_018117899.1); GgMMP-17, *Gallus gallus* matrix metalloproteinase-17 (GenBank accession no. XP_015131123); TeMMP-17, *Tauraco erythrolophus* matrix metalloproteinase-17 (GenBank accession no. KFV18061.1); FaMMP-17, *Ficedula albicollis* matrix metalloproteinase-17 (GenBank accession no. XP_016157548); BmMMP-16, *Ficedula albicollis* matrix metalloproteinase-16 (GenBank accession no. ELR61828); CfMMP-16, *Camelus ferus* matrix metalloproteinase-16 (GenBank accession no. XP_006194011); FcMMP-16, *Camelus ferus* matrix metalloproteinase-16 (GenBank accession no. XP_004000005); HaMMP-2, *Hyalella azteca* matrix metalloproteinase-2 (GenBank accession no. XP_004000005); HaMMP-2, *Hyalella azteca* matrix metalloproteinase-2 (GenBank accession no. XP_004000005); LvMMP-2, *L. vannamei* matrix metalloproteinase-2 (GenBank accession no. MH778698); ZnMMP-2, *Zootermopsis nevadensis* matrix metalloproteinase-2 (GenBank accession no. XP_021938955.1); LpMMP-2, *Limulus polyphemus* matrix metalloproteinase-2 (GenBank accession no. XP_013794718.1). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

## 2. Materials and methods

### 2.1. Experimental shrimp

Juvenile shrimps (~7 g) were raised in a shrimp farm in Zhuhai City, Guangdong Province, China. The shrimps were acclimated for at least one week in a recirculating water tank system that was filled with air-pumped seawater (2.5% salinity) at a temperature of about 27 °C prior to experimentation.

### 2.2. Cloning full-length LvMMP-2 cDNA of L. vannamei

Based on the EST sequence of the *LvMMP-2* in GenBank (accession No. JP42743), primers were designed to obtain the 5′ and 3′ ends of *LvMMP*-2 by rapid amplification of cDNA ends (RACE). The cDNA template for RACE-polymerase chain reaction (PCR) was prepared using the BD SMART RACE cDNA Amplification Kit (Clontech, Japan). The 5′ and 3′ untranslated region (UTRs) were amplified via primers *LvMMP-2* 5′ RACE1 and *LvMMP-2* 3′ RACE1, respectively. Nested PCR was conducted using the first round PCR products as templates and *LvMMP-2* 5′RACE2 or *LvMMP-2* 3′ RACE2 together with nested universal primer (NUP) A, respectively, and the PCR protocol is the same as that of the first round PCR. The primer sequences are listed in Table 1.

### 2.3. Bioinformatics analysis

The protein domains of LvMMP-2 were predicted by the SMART program (http://smart.embl-heidelberg.de/). The MMP proteins of other species in GenBank were searched and analyzed by online software BLAST (http://www.ncbi.nlm.nih.gov/BLAST/). MMPs multiple sequence alignment and neighbor-joining (NJ) phylogenic construction were carried out by MEGA 6.0. Bootstrap sampling was reiterated 5000 times.

### 2.4. Expression profile investigation and subcellular localization of LvMMP-2

For *LvMMP-2* expression profiling investigation, 15 shrimps were sacrificed and their muscles, hemocytes, gills, hearts, pyloric caecum, eyestalks, hepatopancreas, epidermis, nerves, stomachs, and intestines were collected. The shrimps were then divided into three parallel samples for tissue expression analysis.

Total RNA was extracted using RNeasy Mini Kit (Qiagen, Germany) and then reverse transcribed into cDNA by PrimeScript RT reagent kit (TaKaRa, Japan). Real-time RT-PCR assays were then performed on a LightCycler 480 System (Roche, Germany). Results were calculated using the $2^{-\Delta\Delta Ct}$ method after normalization to *L. vannamei elongation factor 1 α* (*LvEF1a*) (GenBank Accession No. GU136229). The primers

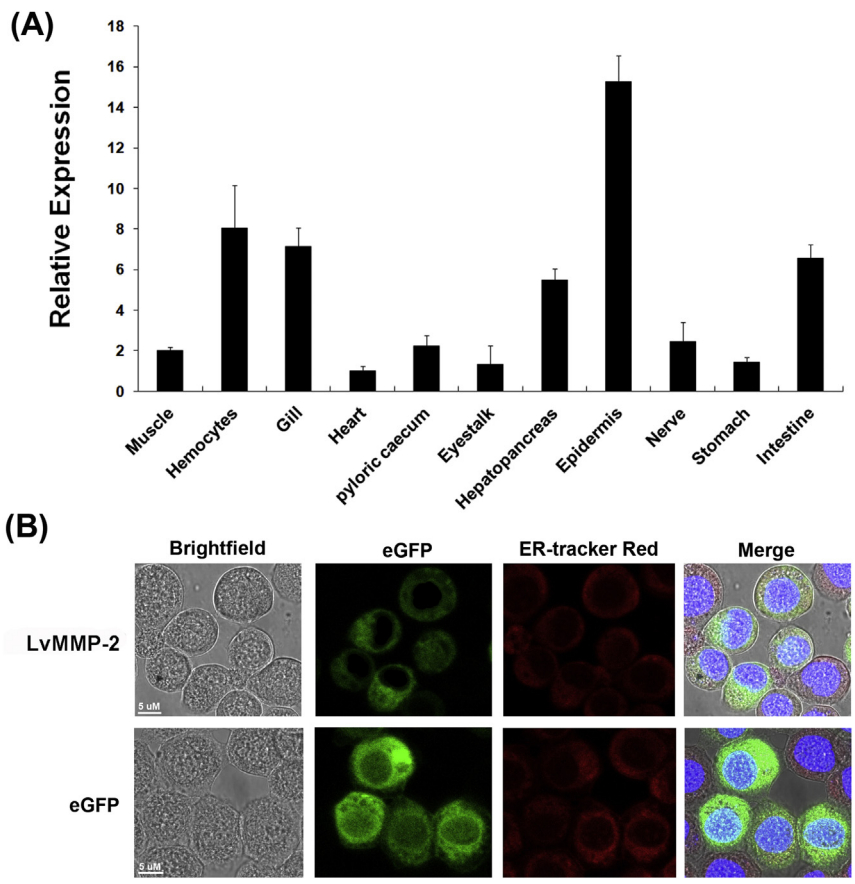


**Fig. 2. Tissue expression pattern and subcellular localization of *LvMMP-2*.** (A) The relative expression of *LvMMP-2* in various tissues were compared against that in pyloric caecum. The results were based on three independent experiments and expressed as mean values ± SD. (B) The location of the proteins were visualized under a Leica laser scanning confocal microscope.

QPCR-LvMMP-2-F/QPCR-LvMMP-2-R and QPCR-LvEF-1α-F/QPCR-LvEF-1α-R were used to detect *LvMMP-2* and *LvEF-1a*, respectively (Table 1).

The expression vector of pIZ-eGFP-LvMMP-2 was constructed by inserting the eGFP DNA fragment into the recombinant vectors of pIZ-LvMMP-2 (primers in Table 1). *Spodoptera frugiperda* 9 (Sf9) cells were maintained at 28 °C in Grace serum-free medium (Sigma Aldrich, USA) supplemented with 10% fetal bovine serum (FBS). Cells were seeded onto cover slips treated with poly-L-lysine in 24-well plates. After 24 h, the cells were transfected with the pIZ-eGFP-LvMMP-2. At 48 h post transfection, cells on the cover slips were washed twice with PBS and stained with Hoechst 33258 solution (Beyotime, China) and ER Tracker Red Kit (Beyotime). The treated cells were observed under a laser scanning confocal microscope (Leica TCS SP5).

### 2.5. Synthesis of double-stranded RNA

To investigate the function of *LvMMP-2*, RNA interference (RNAi) is used to repress the expression of *LvMMP-2* in *L. vannamei*. The DNA templates of *LvMMP-2* dsRNA (designated as dsLvMMP-2) were prepared via PCR using the primer pairs DsRNA-LvMMP-2-T7-F/DsRNA-LvMMP-2-R and DsRNA-LvMMP-2-F/DsRNA-LvMMP-2-T7-R (Table 1). DsLvBip DNA templates were prepared via PCR using the primer pairs, DsRNA-LvBip-T7-F/DsRNA-LvBip-R and DsRNA-LvBip-F/DsRNA-LvBip-T7-R. Products with a T7 promoter were confirmed via sequencing. Subsequently, the products were used as templates for the sense and antisense RNA strands, which were then subjected to *in vitro* transcription and purification by using RiboMAX™ Large Scale RNA Production System-T7 (Promega, USA), following the protocol of the manufacturer. The dsLvMMP-2 is 537 bp in length. DNA templates for *enhanced green fluorescence protein* (dseGFP) dsRNA synthesis were prepared as described elsewhere [15].

### 2.6. Immune challenges, real-time RT-PCR analysis and histolopathologic analysis

To investigate the expression profiles of *LvMMP-2* in infected shrimp, each healthy *L. vannamei* was injected intramuscularly in the second abdominal segment with 50 μL of *V. alginolyticus* ($7.0 \times 10^5$ CFU/g), 50 μL of peptidoglycan (PG, 0.2 mg/mL, 69554 from *Bacillus subtilis,* Sigma-Aldrich), or PBS as control. Total RNAs were isolated from hemocytes at 0, 3, 6, 9, 12, 18, 24, 30, 36, 48, 72, and 96 h post infection (hpi).

For UPR activation, shrimps ($n = 200$) injected with dsLvBip or dseGFP (control) were prepared. Each healthy L. *vannamei* was injected intramuscularly at the second abdominal segment with about 7 μg dsLvBip or dseGFP (50 μL in volume) [16]. Total RNAs from the hemocytes were extracted immediately and at 4, 8, 12, 24, 36, 48, 72 and 96 hpi. Each time point with three parallel samples. And each sample was converged from hemocytes of five shrimps. The total RNAs samples were obtained by RNeasy Mini Kit (Qiagen, Germany), and then reversely transcribed into cDNA via PrimeScript RT Reagent Kit (TaKaRa, Japan) for real-time RT-PCR assays. And for hemocytes protein collecting, total proteins from hemocytes of five shrimps were extracted at 12, 24, 48 and 96 hpi using EpiQuik Whole Cell Extraction Kit (Epigentek, USA). Real-time RT-PCR was performed on a LightCycler 480 System (Roche, Germany). The optimized thermal cycling parameters were as follows: 95 °C for 2 min to activate the polymerase, followed by 40 cycles of 95 °C for 15 s, 60 °C for 15 s, and 72 °C for 10 s. After the cycling protocol, melting curves were obtained by increasing the temperature from 72 °C to 95 °C (0.5 °C/s) to denature the double-stranded DNA.

For exploring the role of LvMMP-2 in oxidative damage in shrimp upon oxidative stress, we induced the oxidative stress by injecting shrimps ($n = 30$) with 5 μg/50 μL β-glucan, whereas the control shrimps were injected with PBS [17]. 24 h later, hepatopancreas of the

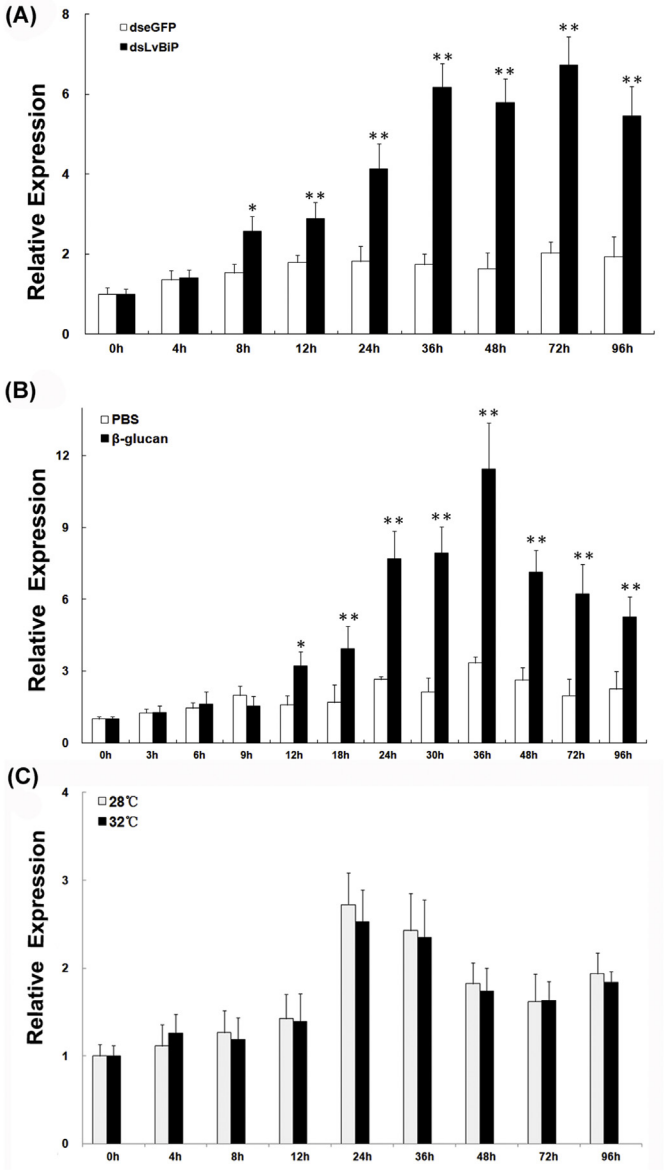


**Fig. 3. Relative expression of *LvMMP-2* under stimulations.** The expression of ***LvMMP-2*** in hemocytes at various time points post UPR activation (A), oxidative stress (B) and heat shock treatment (C) was determined by real time RT-PCR assays. The relative expression of the ten unigenes in hemocytes were compared against time zero. Relative expression levels were normalized to *LvEF-1a*. The results were based on three independent experiments and expressed as the mean values ± SD. The statistical significance was calculated by the Student's *t*-test (* indicates $p < 0.05$, ** indicates $p < 0.01$ compared with controls at each time point, respectively).

shrimps were fixed in Davidson's AFA solution (33% ethanol, 22% formalin, 11.5% acetic acid, 33.5% $H_2O$) for 24 h, then transferred to 70% ethanol and stored until histologic analysis. Paraffin sections of embedded tissue were stained with hematoxylin and eosin (H&E).

### 2.7. Dual-luciferase report gene assays

For investigating the influence of LvMMP-2 on *antimicrobial peptide* (*AMP*) gene expression, dual-luciferase report gene assays were carried out. The expression vectors for the full-length open reading frame (ORF) of *LvMMP-2*, *NF-E2-related factor 2* (*LvNRF2*) and *c-Jun* (*Lvc-Jun*) of *L. vannamei* were constructed by using pAc5.1/V5-His B (Invitrogen, USA), and PCR products were amplified with primers pAcB-LvMMP-2-Kpn I-F/pAcB-LvMMP-2-Xho I-R, pAcB-LvNRF2- EcoR I -F/pAcB-LvNRF2-Xba I-R, pAcB-Lvc-Jun-Kpn I-F/pAcB-Lvc-Jun-Xba I-R (Table 1). Promoters of *LvMMP-2* was obtained by genome-walking PCR, which is 1, 833 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 200–295 bp intervals). The ARE sites AGCAGGATCATGGTA at −1309 bp ~ -1294 bp was replaced by GATCATGCAGGTTGC. And AP-1 binding site at TGA GTCA at −800 bp ~ -793 bp was repalced by GAGCATGG using MutanBEST Kit (TAKARA, Japan). Other report gene vectors: pGL3-AttA, pGL3-Drs, and pGL3-Mtk were constructed by our laboratory and used in the luciferase assays previously [18]. Report gene assays were conducted according to the manufacturer's instructions.

### 2.8. Cumulative mortality test of LvMMP-2 knockdown shrimps upon V. alginolyticus- or white spot syndrome virus (WSSV) infection

The expression of *LvMMP-2* was down-regulated by RNAi using sequence-specific dsRNA, and RT-PCR was performed to investigate the RNAi efficiency at 72 h post dsRNA injection. *LvEF1a* was used as internal control. To determine the cumulative mortality of *LvMMP-2* knocked-down shrimps, healthy shrimps were injected at the second abdominal segment with 7 μg of dsLvMMP-2, dseGFP, or PBS ($n = 150$). Approximately 48 h later, the shrimps were injected again with 50 μL of *V. alginolyticus*, WSSV inoculum or PBS ($n = 50$). Cumulative mortality was recorded every 12 h.

### 2.9. Statistical analysis

Numerical datas were presented as mean ± standard deviation (SD). The means of two samples were compared by Student's *t*-test. The * indicates $p < 0.05$, ** indicates $p < 0.01$in all cases. All experiments were repeated at least three times. In addition, the differences in mortality levels between treatments were analyzed by the Kaplane-Meier plot (log-rank $X^2$ test).

## 3. Results and discussions

### 3.1. Cloning and sequence analysis of LvMMP-2

The full-length cDNA of *LvMMP-2* was 2072 bp, which included a 196 bp 5′-untranslated region (UTR), and a 85 bp 3′-UTR with a poly (A) tail (Fig. 1A). The ORF of *LvMMP-2* is 1791 bp in length and encodes a putative protein of 597 amino acids with a calculated molecular weight of 68.9 kDa. Conserved domain analysis illustrated that LvMMP-2 contained a signal peptide, peptidoglycan binding domain (PGBD), Zinc-dependent metalloprotease (ZeMc) domain and four Hemopexin-like repeats (Fig. 1B). PGBD has a core structure consisting of a closed, three-helical bundle with a left-handed twist. Usually, PGBD is found at the amino terminal-, or carboxyl terminal-terminus of a variety of enzymes involved in bacterial cell wall degradation. ZeMc domian endow MMP zinc ion binding activity and metallopeptidase activity. Hemopexin-like repeats occur in vitronectin and some MMPs family (matrixins). The Hemopexin-like repeats of some matrixins bind tissue inhibitor of metalloproteinases (TIMPs). Therefore, LvMMP-2 with conservative structure is a member of the MMP family, of while its exact function still need further investigation.

### 3.2. Phylogenetic analysis

To estimate the relationship between LvMMP-2 and its homologs, multiple sequence alignment was conducted (Fig. S1). LvMMP-2 showed 52% identity to MMP-2 of an amphipods *Hyalella azteca*, and 49% identity to MMP-2 of an ant *Cryptotermes secundus*. Furthermore, the phylogenetic tree showed that these MMPs could be divided into three groups (Fig. 1C). Group 1 contained McMMP-25, GjMMP-25,

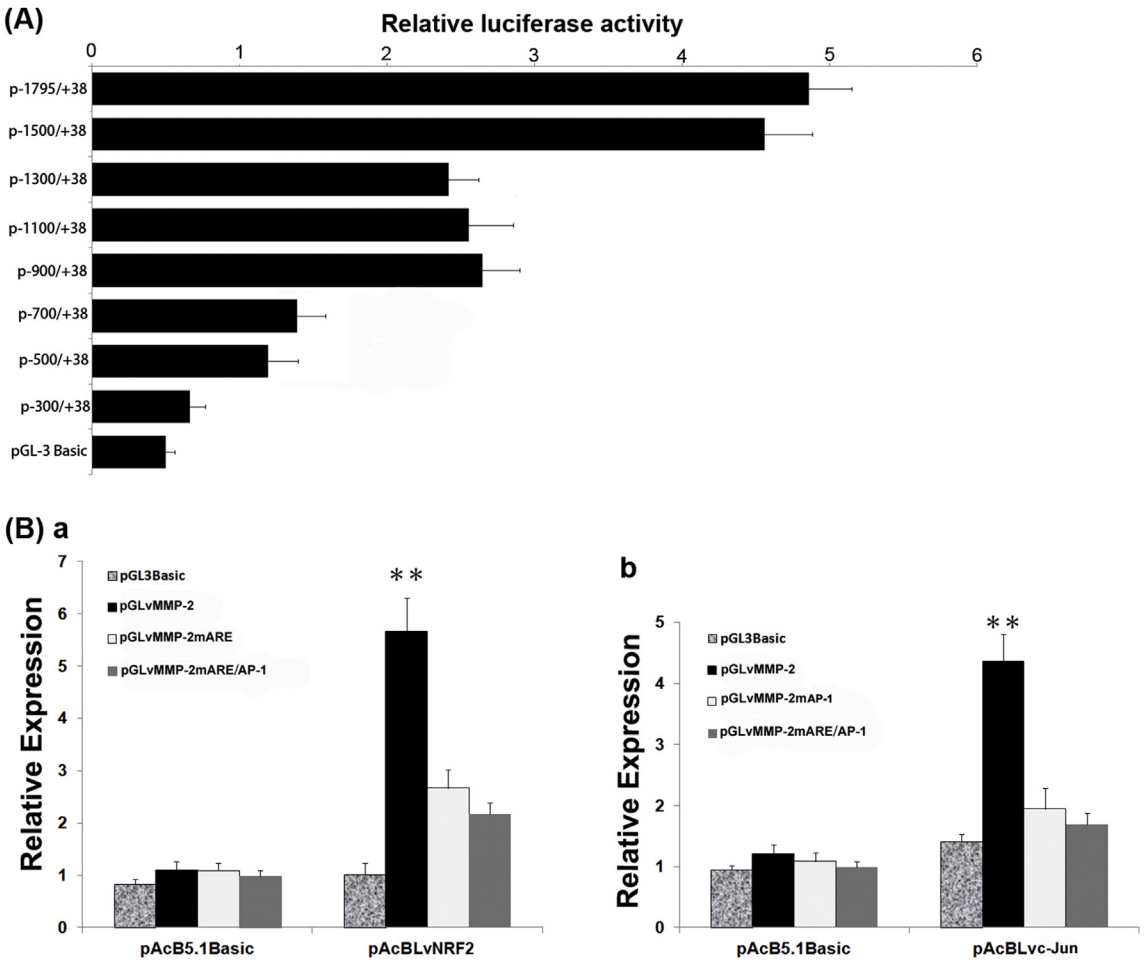


**Fig. 4. LvNRF2 or Lvc-Jun activated the promoter of *LvMMP-2* in S2 cells.** (A) The ***LvMMP-2*** promotersactivity of different fragments in S2 cells were detected by dual-luciferase reporter gene assay. The effect of LvNRF2 [(B) a] and Lvc-Jun [(B) b] on activity of ***LvCruU*** promoter were evaluated in S2 cells. The bars indicate mean values ± SD 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.

CpbMMP-25, SfMMP-25, XlMMP-25, OaMMP-25, OgMMP-25, MpMMP-25, LcMMP-17, OlMMP-17, XlMMP-17, GgMMP-17, MmMMP-17, FaMMP-17 and DrMMP-17. Group 2 comprised BmMMP-16, CfMMP-16 and FcMMP-16. Group 3 included LvMMP-2, ZnMMP-2, and LpMMP-2. These findings indicate that MMPs are highly conserved proteins in both invertebrates and vertebrates, even they are divided into different families. And LvMMP2 was gathered in the group of MMP-2, which is the most well studied subfamily of MMPs. As reported, MMP-2 plays an important role in tumor progression, such as angiogenesis, lymphangiogenesis, tumor cells invasion and so on [11,12]. And it also takes part in primary innate immune response. For example, MMP-2 affects the clearance of recruited immune cells, which is necessary to resolve inflammatory reactions, as part of an interleukin 13 (IL-13)-dependent regulatory loop, it dampens inflammation by promoting the egress of inflammatory cells into the airway lumen [19].

### *3.3. LvMMP-2 is constitutively transcribed in various tissues and located to the ER*

Real-time RT-PCR analysis confirmed that *LvMMP-2* was expressed in all tissues examined in this study. *LvMMP-2 was highly expressed in* epidermis, and lowly expressed in heart. The former expression was respectively 15.2-fold than the latter expression (Fig. 2A). *LvMMP-2* also had relatively high expression in hemocyte, gills and intestine. It is well-known that MMP-2 could degrade type IV collagen, the major structural component of basement membranes. Thus, the high expression of *LvMMP-2* in shrimp epidermis related to its function of collagen degradation. Analysis by PSORT II Prediction (https://psort.hgc.jp/form2.html), LvMMP-2 with an possible vacuolar targeting motif ILPK, was suggestedto be located to ER with 44.4% possiblity. In present study, a subcellular distribution assay suggested that LvMMP-2-eGFP is expressed in Sf9 cells and predominantly aggregated in the cytoplasm. And the ER Tracker Red staining confirmed that LvMMP-2-eGFP was located to the ER in Sf9 cells (Fig. 2B). In contrast, unfused eGFP was widely distributed in cytoplasm. Most MMPs are secreted or anchored to the cell surface. For LvMMP-2, we found that it has an possible vacuolar targeting motif ILPK. And in this study we determined its location to ER in Sf9 cells.

### *3.4. LvMMP-2 is successively induced by oxidative stress and UPR*

In our previous study, we had shown that $\beta$-glucan (5 μM/μL) causes oxidative stress in shrimp [20]. The expression profile of *LvMMP-2* in hemocytes was detected by real-time RT-PCR, and revealed that upon oxidative stress, expression of *LvMMP-2* increased from 12 hpi to 96 hpi, and peaked at 36 hpi, which was 3.0-fold of the control (Fig. 3B). And for activating UPR, shrimps were injected with dsLvBip. 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 hemocytes, *LvMMP-2* was induced 8 h post dsLvBip injection, and reached the peak values at 72 h post dsLvBip injection, which was about 3.3-fold of the control group (Fig. 3A). In this study, the expression of

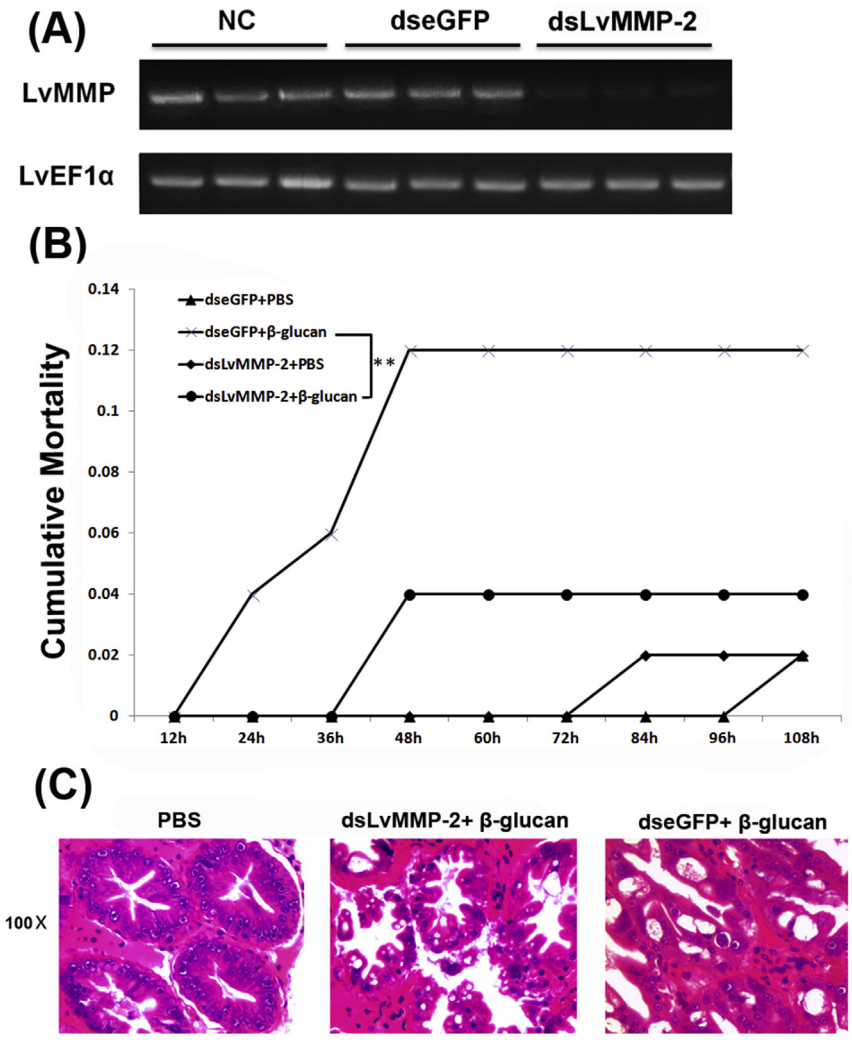


**Fig. 5. Histolopathologic analysis and cumulative mortality test upon oxidative stress in *L. vannamei*.** (A) The RNAi efficiency of dsRNA injection was detected by RT-PCR. (B) Shrimp ($n = 50$) were intramuscularly injected with dsLvMMP-2, dseGFP, or PBS (as a control). 72 h after the initial injection, shrimps were injected with $\beta$-glucan or PBS (control) again. Cumulative mortality was recorded every 12 h post-challenge. Data are derived from three independent experiments and shown as the mean ± SD. Differences in mortality levels between treatments were analyzed by *Kaplane-Meier* plot (log-rank $X^2$ test). (C) Fluorescence value indicated the protein aggregates in hemacytes. Statistical significance was determined by one-way ANOVA. Bars with different letters indicate statistical differences ($p < 0.05$). (C) At 48 h post $\beta$-glucan injection, structure of shirmp hepatopancrea cells was more seriously damaged in dseGFP than that in dsLvMMP-2 group.

*LvMMP-2* upon heat shock treatment also be investigated. And the results indicated that heat shock treatment did not significantly influenced the expression of *LvMMP-2* (Fig. 3C). Growing evidences showed that oxidative stress activated MMP-2. For examples, human MMP-2 could be activated by very low concentrations of $ONOO^-$ [21]; oxidative stress activated MMP-2 in cultured human coronary smooth muscle cells [22]. In L. *vannamei*, we found that *LvMMP-2* was enhanced by oxidative stress, though whether oxidative stress influence the activity of LvMMP-2 still needed further investigation.

We have showed that oxidative stress increased the expression of *LvMMP-2*, yet the regulatory mechanism still remained unclear. Results of report gene assay illustrated that the activity of *LvMMP-2* promoter was dramatically changed between −1500 bp and-1300 bp, and between −900 bp ~ -700 bp (Fig. 4A). In fact, there are two BRLZ transcription factor binding sites in the promoters of *LvMMP-2*, the ARE site (−1309 ~ -1294: AGCAGGATCATGGTA) and AP-1 bind site (−800 bp ~ -793 bp:TGAGTCA), corresponding to the regions significantly affected the promoters activity of *LvMMP-2* as showed in the report gene assay, respectively. It has been provided that *LvMMP-2* was induced by oxidative stress, and the NRF2-Keap1 pathway was the key pathway for oxidative stress response. We restored BRLZ transcription factor NRF2 binding to the ARE site to investigate whether or not the promoter activity of LvMMP-2 was enhanced by NRF2 in S2 cells. And results of report gene assay showed that LvNRF2 evidently increased the expression of *luciferase*, which is about 5.7-fold of the control (Fig. 4B). And once the ARE site in the promoter of *LvMMP-2* was mutated, LvNRF2 lost most its ability to enhance the expression of reprot genes. These results proved that promoter activity of *LvMMP-2* was enhanced by LvNRF2 in a ARE site dependent-manner. And by over-expression Lvc-Jun in S2 cells, we demonstrated that AP-1 was also involved in LvMMP-2 regulation (Fig. 4B). In fact, it has been found that MMP-9, another well-studied MMPs, was upregulated in mesangial cells by NF-κ B and AP-1 [23].

### 3.5. Knock-down expression of LvMMP-2 decreases shrimp cumulative mortality upon oxidative stress

Oxidative stress can cause oxidative injury. And MMP-2 was involved in these processes. For example, acute release of MMP-2 during reperfusion after ischemia contributes to cardiac mechanical dysfunction [24]. However, the influence of MMP-2 in oxidative stress injury to *L. vannamei* was still unknown. Here, *LvMMP-2* was knocked-down, and its effecting on shirmp cumulative mortality upon oxidative stress was investigated. RT-PCR assay showed that dsLvMMP-2 injection effectively suppressed the expression of *LvMMP-2* (Fig. 5A). And at 36 h post $\beta$-glucan injection, the shrimp cumulative mortality of dseGFP plus $\beta$-glucan group, dsLvMMP-2 plus $\beta$-glucan group, dsLvMMP-2 plus PBS group, and dseGFP plus PBS group were 6%, 0%, 0% and 0%, respectively; at 48 h post $\beta$-glucan injection were 12%, 4%, 0%, and 0%, respectively; and at 108 h post $\beta$-glucan injection were 12%, 4%, 2%, and 2%, respectively (Fig. 5B). Additionally, histopathological analysis also showed that oxidative stress resulted in hepatopancreas injury, and shrimps injected with dsLvMMP-2 exhibited more minor hepatopancreas damage than the control group (Fig. 5C). These results suggested

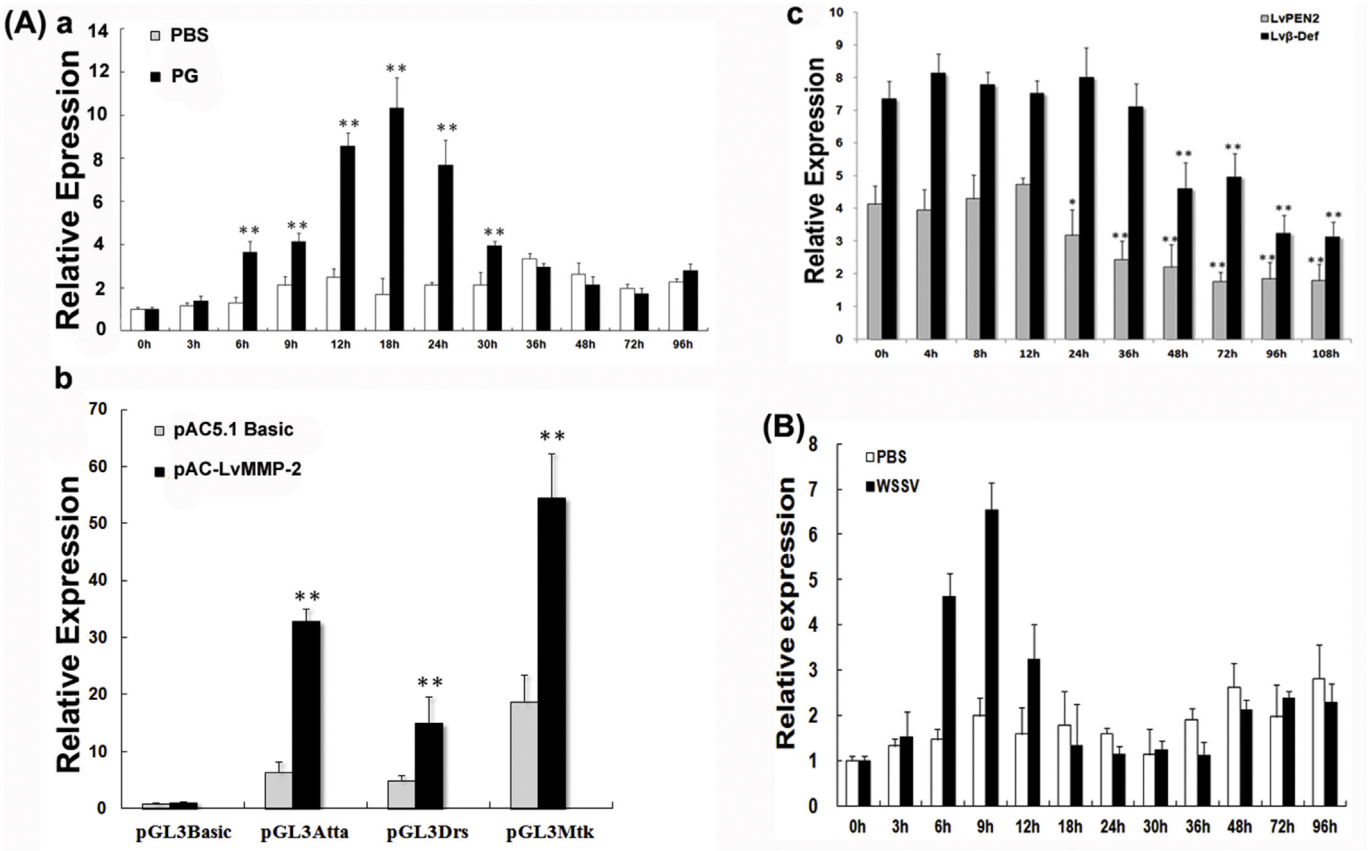


**Fig. 6. LvMMP-2 played a role in innate immunity resposne.** (A) Real time RT-PCR assays were conducted to measure the expression of *LvMMP-2* in hemocytes at various time points post PG injection. (B) Dual-luciferase reporter gene assays were carried out to validate luciferase activity of pGL3Mtk, pGL3Atta, and pGL3Drs in S2 cells. (C) Knocked-down expression of *LvMMP-2* depressed the expression of *pen2* and *β-Def*.

that LvMMP-2 contributes to oxidative injury upon oxidative stress.

### 3.6. Expression of LvMMP-2 is strengthened by PG, and LvMMP-2 can upregulate the expression of AMPs

MMPs contain a peptidoglycan binding-like domian with predicted peptidoglycan binding function, which is found at the amino terminal- or carboxyl terminal-terminus of a variety of enzymes involved in bacterial cell wall degradation, such as muramoyl-pentapeptide carboxypeptidase and autolytic lysozyme. It also has been reported that *Borrelia burgdorferi* induces MMPs from human chondrocytes [25]. So in this study, *LvMMP-2* expression in PG-injected shrimp hematocytes was investigated. The expression level of the gene at 0 h was used as the baseline. The relative expression of *LvMMP-2* in the PG-injected shrimp was increased significantly by 6.0-fold that of the control at 18 hpi, which is the peak value [Fig. 6 A (a)]. And report gene assay showed the LvMMP-2 could increase the expression of *AttA*, *Drs* and *Mtk* about 6.1-, 1.3- and 1.7-fold, respectively [Fig. 6A (b)]. We knocked-down expression of *LvMMP-2* in shrimps by dsLvMMP-2 injection and the expression of two *L. vannamei AMPs* were investigated. Results of real-time RT-PCR assay showed that decreased the expression of *LvMMP-2* obviouslyweakened the expression of *Pen2* and *β-Defensin* (*β-Def*) from 24 hpi and 48 hpi, respectively [Fig. 6A (c)]. Here, we not only showed that LvMMP-2 was heightened under PG challenging, but also proved that it was engaged in *AMPs* regulation in shrimp.

### 3.7. Knock-down expression of LvMMP-2 decreased the cumulative mortality of V. alginolyticus- or WSSV-infected shrimp

MMPs, were proved to take part in innate immune response, also aggravated the infection and illness in many cases [26,27]. And from the above experimental results, we discovered that LvMMP-2, not only had a role in oxidative stress injury, but also was involved in innate immune response in *L. vannamei*. So we aimed to study its influence in the process of pathogen infection. For WSSV infection, the cumulative mortality of shrimp injected with dsLvMMP-2 at 72, 84, 96, 108, 120, 132 and 144 hpi were 43.4%, 40.5%, 42.6%, 50.6%, 62.3%, 70.9% and 78.9%, respectively; and the cumulative mortality of shrimp injected with dseGFP at 72, 84, 96, 108, 120, 132 and 144 hpi were 43.6%, 65.5%, 85.5%, 100%, 100%, 100% and 100%, respectively (Fig. 7B). For *V. alginolyticus* infection, the cumulative mortality of shrimp injected with dsLvMMP-2 at 12, 24, 36, 48 and 60 hpi were 8.0%, 16.4%, 26.0%, 34.0% and 36.0%, respectively; and the cumulative mortality of shrimp injected with dseGFP at 12, 24, 36, 48 and 60 hpi were 12.2%, 30.0%, 40.0%, 46.0% and 50%, respectively (Fig. 7A). Consequently, both upon WSSV infection and *V. alginolyticus* infection, the depression of LvMMP-2 reduced the damage to *L. vannamei*, and therefore cut down the shrimp cumulative mortality.

## 4. Conclusions

In the present study, we cloned and functionally characterized a *MMP-2* gene in *L. vannamei*. *LvMMP-2* was highly expressed in epidermis, and located to ER in S2 cells. It was induced in hemocytes by UPR, oxidative stress and WSSV- or *V. alginolyticus* infection. Promoter activity of *LvMMP-2* was enhanced by LvNrf2 as well as the AP-1 factor Lvc-Jun. Besides, suppressing *LvMMP-2* reduced the oxidative stress injury and cumulative mortality of shrimp under oxidative stress. Moreover, *LvMMP-2* induced by LPS could strengthen the promoter activity of several *AMPs* in S2; knocked-down expression of *LvMMP-2*

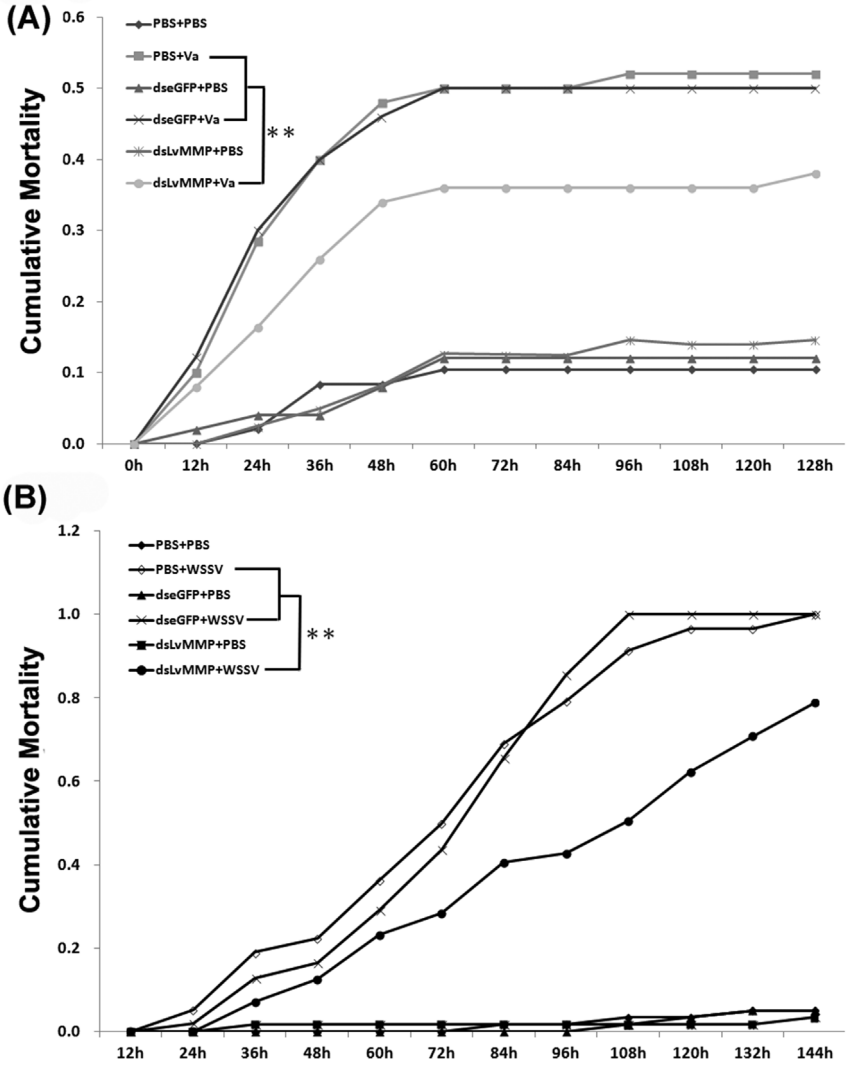


**Fig. 7. Cumulative mortality following dsLvMMP-2 injection plus immune challenges.** The cumulative mortality of shrimps with dsLvMMP-2 together with WSSV-infection (A) and *V. alginolyticus* infection (B) were investigated.

depressed the expression of *Pen2* as well as *β-Def*. Additionally, down-regulation *LvMMP-2* decreased the cumulative mortality of shrimps that infected with WSSV- or *V. alginolyticus*. These results suggested *LvMMP-2* might be involved in shrimp innate immune response, and contributed to tissue injury caused by pathogenic infection.

## Acknowledgements

This research was supported by Guangdong Natural Science Funds for Distinguished Young Scholar (2015A030306030); National Natural Science Foundation of China (31472301); National Natural Science Foundation of China (31772895); Tip-top Scientific and Technical Innovative Youth Talents of Guangdong special support program (2015TQ01N220); Pearl River Nova Program of Guangzhou (201610010061). The nucleotide sequences of *LvMMP-2* had been submitted to NCBI database and with GenBank accession No. MH778698.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2018.10.021.

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