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Characterization, subcellular localization and function analysis of myeloid differentiation factor 88 (*Pt*-MyD88) in swimming crab, *Portunus trituberculatus*

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ABSTRACT

Myeloid differentiation factor 88 (MyD88) is a universal and essential adaptor protein required for the Toll-like receptors (TLRs) pathway activation in invertebrates as well as in vertebrates. Herein, we characterized a MyD88 (*Pt*-MyD88) cDNA sequence in the swimming crab (*Portunus trituberculatus*). The *Pt*-MyD88 ORF is predicted to encode 469 peptides with an N-terminal death domain and a typical C-terminal TIR domain. Real-Time quantitative PCR analysis showed that the *Pt*-MyD88 transcriptions were constitutively expressed in hemocytes, gill, intestine, heart and muscle in normal crab. The expressions of *Pt*-MyD88 would be down-regulated by *V. alginolyticus* or LPS challenge, and be up-regulated by WSSV infection in hemocytes. Intracellular localization showed *Pt*-MyD88 was distributed mainly in the cytoplasm when it was over-expressed in human cell HEK293T or in *Drosophila* Schneider 2 (S2). Functionally, over-expression of *Pt*-MyD88 could either activate the NF-κB in HEK293T cells or activate the promoters of *Drosophila* antimicrobial peptide genes (AMPs) in S2 cell. In primary cultured hemocytes of swimming crab, after *Pt*-MyD88 was knocked-down by specific long double strand RNA, the expression of anti-lipopolysaccharide factor1 (ALF1), hyastatin3, crustin1 and crustin3 have been significantly inhibited, while the expression of other AMPs is normal compared to non-specific dsRNA treated cells.

1. Introduction

The innate immune system is an older evolutionary defense strategy and centrally involved in the recognition and defense of various infection in all animals [1]. The process depends on a set of pattern-recognition receptors (PRRs) that are activated by pathogen-associated molecular patterns (PAMPs) specific in microbes [2]. The Toll-like receptor (TLR) family is one of the most extensively studied PRRs, and it plays a fundamental role in pathogen recognition and the activation of innate immunity [3]. Myeloid differentiation factor 88 (MyD88) is a universal and essential adaptor protein required for the TLRs pathway activation in invertebrates as well as in vertebrates [4,5]. After the recognition of PAMPs by TLRs, MyD88 will be recruited to activate TLR signal pathway. MyD88 consists of a C-terminal Toll/interleukin-1 receptor (TIR) domain and N-terminal a death domain (DD), the TIR domain mediates the interaction with other TIR domain-containing proteins and the death domain associates with the IRAK family members through homotypic DD interactions [4–6]. In mammals, the PAMPs were directly recognized by TLRs, and then it will activate the transcription of the downstream target genes including pro-inflammatory cytokines or type I IFN [3,4]. Comparably, in *Drosophila*, the Toll recognizes its endogenous ligand and the downstream effector of TLR-MyD88 pathway is antimicrobial peptides (AMPs) [7,8].

Shrimp and crab farming is one of the major aquaculture industries worldwide, especially in Asian countries. During the past decade, various diseases caused by bacteria and viruses have hampered the further development of the industry [9,10]. However, vaccine cannot be used for disease control and prevention in crustacean species due to the absence of immunological memory. It has long been recognized that innate immunity is the main strategy used to defend against various pathogens in invertebrate animals [11]. Therefore, plenty of studies on molecular or signal pathway involved in innate immune response in shrimp were performed for immune control against various pathogens [12–15]. As a critical component in innate immune signaling, MyD88 and its orthologs have been described in many aquatic animal species including fish [16–18], crustacean [19,20] and molluscs [21,22]. Functionally, the fish MyD88 can activate the mammals NF-κB promoter [16–18]. In shrimp, it reported that the MyD88 induced the

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promoter activities of both *Drosophila* and shrimp AMPs in *Litopenaeus vannamei* [19]. In a freshwater crab, overexpression of the MyD88 in *Drosophila* Schneider 2 (S2) cells also led to the activation of antimicrobial peptide (AMPs) genes [23].

In present study, the MyD88 (*Pt*-MyD88) gene were characterized in an economic marine crab species *Portunus trituberculatus*, and its function in signal transduction was studied in both mammal and *Drosophila* cell line models. Moreover, RNA interference experiments were also conducted to investigate the effect of *Pt*-MyD88 on expression of several AMPs in *P. trituberculatus* primary cultured hemocytes. These data will greatly help us to understand the role of the Toll-MyD88 pathway in regulating the innate immunity of crab.

2. Materials and methods

2.1. Tissue collection, RNA extraction and cDNA synthesis

Swimming crabs (*P. trituberculatus*; mean body weight ± SD, 138 ± 22 g, n = 6) purchased from a local seafood market in Ningbo, Zhejiang province, China. Six individuals (three females and three males) were randomly taken out for tissue preparation. Animal handling and experimental procedures were approved by the Animal Ethics Committee of Ningbo University. The crab tissues including hemocyte, gill, muscle, heart and intestine were dissected out and preserved in −80 °C for RNA extraction. Total RNAs were extracted from the tissues of the crabs using total RNA kit (Omega) according to the manufacturer's instructions. Total RNAs were checked by spectrophotometric method as well as by agarose gel electrophoresis. Approximately 5 μg of total RNA extracted from hemocytes was reverse-transcribed with PrimeScript™ 1st strand cDNA Synthesis Kit (TaKaRa) according to the manufacturer's instructions.

2.2. Cloning and sequencing the cDNA and DNA of Pt-MyD88

The cDNA fragment of *Pt*-MyD88 was initially amplified by PCR with degenerate primers in Table 1. Based on the cDNA fragments, the full-length *Pt*-MyD88 cDNA was obtained via the 5′ and 3′ rapid amplification of cDNA ends (RACE) using the SMART RACE cDNA Amplification Kit (Clontech) according to the manufacturers' instructions. To analyze the genomic structure of *Pt*-MyD88, DNA encoding the MyD88 was amplified from genomic DNA. All primers used for cDNA and DNA cloningwere listed in Table 1. The PCR product was then subcloned into plasmid vector pMD19-T simple (TaKaRa) and transformed into competent *Escherichia coli* DH5α cells for sequencing.

2.3. Bioinformatics analyses

Nucleotide sequences were translated to amino acid sequences by the ExPASy Translate tool (http://www.expasy.ch/tools/dna.html). The predicted amino acid sequences were blasted against the NCBI database using BLASTP program (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to identify sequence identities and similarities. The identification and annotation of protein domains were performed using the web-based SMART program (http://smart.embl-heidelberg.de/). Phylogenetic analyses were conducted on amino acid using the MEGA 5 program.

2.4. Immune challenge

Several groups (mean body weight ± SD, 126 ± 18 g, n = 15) were set for challenge experiments. Experimental crabs reared in a recirculating water tank system filled with air-pumped seawater (24‰ salinity) at 24 ± 0.5 °C. The crabs were allowed to acclimatize for at least 5 days before the experiments were conducted. Challenge experiments were performed as previously described [24,25]. Briefly, each group was injected with 0.2 mL of *V. alginolyticus* (5.5×10^7 CFU/mL, 1.1×10^7 CFU/crab), LPS (0.5 μg/mL), WSSV (approximately 3.2×10^7 copies/mL, quantified by Real-time PCR) and PBS, respectively. The hemocytes of three crabs from each group were randomly collected at 0, 6, 12 and 24 h for RNA extraction. Animal handling and experimental procedures were approved by the Animal Ethics Committee of Ningbo University.

2.5. Real-time quantitative PCR

All samples were lysed to purify total RNA using the RNA extraction kit (Omega) according to the manufacturer's instruction. cDNAs for Real-time quantitative PCR (qRT-PCR) analysis were synthesized using PrimeScript RT reagent kit with gDNA Eraser (TaKaRa). Primers for *Pt*-MyD88 were designed using Beacon Designer 7.80 software in this study and primers of the internal gene were used as previously described [25]. Moreover, the amplicon of *Pt*-MyD88 was cloned to the T-vector to detect the amplification efficiency of the primer set used for qRT-PCR analysis. The efficiencies of the qRT-PCR primers for the, *Pt*-MyD88 genes were 1.941.

qRT-PCR was performed with Roche LightCycler 480 Real-time PCR System (Roche) using LightCycler 480 SYBR Green I Master (Roche). Each run included a no template control to test the assay reagents for contamination. The cycling protocol was pre-denaturation at 95 °C for 5 min followed by 40 cycles of 95 °C for 15 s, 60 °C for 15 s, and 72 °C for 10 s. Specificity of PCR products was detected by melting curve analysis and sequencing. Each sample was done in triplicate. The relative expression ratio of the cytokines gene were analyzed using the $2^{-\triangle\triangle CT}$ method in triplicates for three independent parallel samples [26].

2.6. Construction of the expression plasmids and reporter gene plasmids

The full-length *Pt*-MyD88 ORF was amplified by primers sets with (for luciferase reporter assay) or without the stop codon (for cellular location) and cloned to pMD18-T vector. To create the confusion plasmids, *Pt*-MyD88 with or without the stop codon were cloned into pEGFP-N2 vector or pAc5.1-EGFP vector by Gibson Assembly [27] using the Gibson Assembly® Master Mix kit (NEB, USA), respectively. To construct Luciferase reporter vectors, the promoter sequences of Attacin A (*AttA*) and Metchnikowin (*Mtk*) were amplified from DNA of S2 cells and then were cloned into pGL3 basic plasmids, respectively. All primer sets used in plasmids construction were list in Table 1.

2.7. Cell culture and transfection

HEK293T cells were cultured at 37 °C under a 5% (v/v) CO_2 atmosphere in DMEM (Gibco, USA) containing 10% (v/v) fetal bovine serum (FBS, Gibco, USA) and sub-cultured at intervals of 2 days. *Drosophila* Schneider 2 (S2) cells (Invitrogen, USA) were grown at 26 °C under normal atmosphere in Schneider's Drosophila Medium (Gibco, USA) containing 10% (v/v) FBS and sub-cultured at intervals of 2–3 days. For transfection, cells were seeded into 24- or 96-well plates and cultured for 24 h, which made 80–90% cells confluent at the time of transfection. Plasmids were transfected with Lipofectamine™ 3000 Reagent (Invitrogen, USA) following the manufacturer's protocol.

2.8. Laser confocal fluorescence microscope

For the cellular localization analysis, HEK293T or S2 cells were seeded onto poly-L-lysine-treated cover slips in a 24-well plate, and they were transfected with recombinant plasmids not contain the stop codon of *Pt*-MyD88 (pEGFP-N2-MyD88 for HEK293T, and pAC5.1-MyD88-EGFP for S2), respectively. After 24 h post-transfection, the cells on the coverslips were washed twice with PBS, fixed by Immunol Staining Fix Solution (Beyotime, China), and then stained with 40, 6-diamidino-2-phenylindole (DAPI) for 5 min. The coverslips were washed with PBS three times and transferred to glass slides, with the cells between the

Table 1
PCR primers used in this study.

Primer	Sequence (5′-3′)
Degenerate PCR	
Pt-MyD88 DF	ATGTCTATTCACCGDGGAGAAGTAG
Pt-MyD88 DR	TATAATCACTDGATTCCCCAGAAG
RACE PCR	
5′ *Pt*-MyD88-RACE1	ATGTCTATTCACCGGGGAGAAGTAG
5′ *Pt*-MyD88-RACE2	TCAATAACATCAAAGCGATCCAT
3′ *Pt*-MyD88-RACE1	TCAACATTGTAACTACACCGTCTCC
3′ *Pt*-MyD88-RACE2	AGATGTTCCTACAACTCCACCCTG
qRT-PCR analysis	
Pt-MyD88 F	TTGTACCAAGCCTCCTGTTAT
Pt-MyD88 R	CCGTATTCTTACTCCAGACTCA
18s RNA F	TCCGATAACGAACGAGACT
18s RNA R	TAAGAAGAAGCTGCGAACTG
GADPH F	TGAGGTGAAGGTAGAGGAT
GADPH R	CCAGTGAAGTGAGCAGAG
Pt-ALF1 F	ATGAGGCTCTGGTAACTTCC
Pt-ALF1 R	AGACGACACAATACTTACACCT
Pt-ALF3 F	GGTGGTGATGTGCCTGTA
Pt-ALF3 R	AAACTTGCCCTCGTGGTA
Pt-ALF4 F	CCTGGCTGGACCATCATTA
Pt-ALF4 R	CCGCTTCTTCCTTAGTGACT
Pt-ALF7 F	GCTCTGACGGCTGCTATT
Pt-ALF7 R	GACCCTGACCTGCTCTTG
Pt-LYZ F	GCCCAGTATGAGTCTACCTTC
Pt-LYZ R	TCGTTCTTGTGAGTGTTACCA
Pt-AST2 F	CCTGCTGATTGTCCTGCTAGTGT
Pt-AST2 R	TCTCACGAAGCGGCGACTAC
Pt-HYT1 F	TTGACCAGACCAGCAGTAGTG
Pt-HYT1 R	GCCTGATGGAGCCTGTGATG
Pt-HYT3 F	CGGCTGTGGGCAATGCTTAC
Pt-HYT3 R	GCTGGTCTGGTCAAGGATGGATT
Pt-CRUT1 F	GCAAGGACAACTACGATAAACACT
Pt-CRUT1 R	GGAAAGTCACAAGCACCATCAT
Pt-CRUT3 F	ACCTATCCACCTTATACCGAGCG
Pt-CRUT3 R	CACAGGCGTCATAACAGCACTT
Expression plasmids construction for location	
pEGFP-N2-MyD88 F	AAAAACCTAATGGTGAGCAAGGGCGAG
pEGFP-N2-MyD88 R	AATAGACATGGTGGCGACCGG
MyD88-pEGFP-N2 F	TCGCCACCATGTCTATTCACCGGGGAGAAGT
MyD88-pEGFP-N2 R	TGCTCACCATTAGGTTTTTTTTTTTGCCTTTAAAAATTTTCTCTAGAATTCGT
pAc5.1-EGFP-MyD88 F	AAAAAACCTAGGTAAGCCTATCCCTAACCCT
pAc5.1-EGFP-MyD88 R	GAATAGACATTTCGTTACTTGTACAGCTCGTCCATG
MyD88-pAc5.1-EGFP F	AAGTAACGAAATGTCTATTCACCGGGGAGAAGTAG
MyD88-pAc5.1-EGFP F	TAGGCTTACCTAGGTTTTTTTTTTTGCCTTTAAAAATTTTCTCTAGAATTCGT
Expression plasmids construction for function analysis	
pEGFP-N2-MyD88 F	AACCTATAGATGGTGAGCAAGGGCGAG
pEGFP-N2-MyD88 R	AATAGACATGGTGGCGACCGG
MyD88-pEGFP-N2 F	TCGCCACCATGTCTATTCACCGGGGAGAAGT
MyD88-pEGFP-N2 R	TGCTCACCATCTATAGGTTTTTTTTTTTGCCTTTAAAAATTTTCTCTAGAATTCG
pAc5.1-MyD88 F	AAACCTATAGGGTAAGCCTATCCCTAACCCTCT
pAc5.1-MyD88 R	GAATAGACATTTCGAACCGCGGGCCC
MyD88-pAc5.1 F	CGGTTCGAAATGTCTATTCACCGGGGAGAAGTAG
MyD88-pAc5.1 R	TAGGCTTACCCTATAGGTTTTTTTTTTTGCCTTTAAAAATTTTCTCTAGAATTCG
Promoter cloning	
AttAF	GGTACCGCCATCAGGCCACCACCCATTCTG
AttAR	AGATCTGTTGCTGAACTGGATTGCTGGAGCTGAC
Mtk F	TCGACTTTTCTGCCATTAGCCATCG
Mtk R	CTTAGCTCGGTGGCGGGAATTGATT
Luciferase reporter vector	
pGL-Atta F	CAACAGATCTATGGAAGACGCCAAAAACATAAAGAAAGG
pGL-Atta R	GCGGTACCGGTGGCTTTACCAACAGTACCG
Atta-pGL F	AGCCACCGGTACCGCCATCAGGCC
Atta-pGL R	CGTCTTCCATAGATCTGTTGCTGAACTGGATTGCT
pGL-Basic-Mtk F	ATTCCCGCCACCGAGCTAAGCCCGGGCTCGAGATCTGC
pGL-Basic-MtkR	GCTAATGGCAGAAAAGTCGACTAGCACGCGTAAGAGCTCG
Mtk- pGL-Basic F	CGAGCTCTTACGCGTGCTAGTCGACTTTTCTGCCATTAGCCATCG
Mtk- pGL-Basic R	TCGCAGATCTCGAGCCCGGGCTTAGCTCGGTGGCGGG
DsRNA synthesis	
dsR-MyD88 F	TAATACGACTCACTATAGGGCGGGGAGAAGTAGCTGAGGC
dsR-MyD88 R	TAATACGACTCACTATAGGGCATATGCAGCCACATCAGTCACT
dsR EGFP-F	TAATACGACTCACTATAGGGCAGTGCTTCAGCCGCTACCC
dsR EGFP-R	TAATACGACTCACTATAGGGAGTTCACCTTGATGCCGTTCTT

Note: D = A + T + G; F indicates forward; R, reverse.

slide and the coverslip. Cellular localization of the *Pt*-MyD88 protein was observed using a laser-scanning confocal microscope (Zeiss, LSM880, Germany).

2.9. Luciferase reporter assays

For luciferase reporter assays, HEK293T cells or S2 cells were seeded in a 96-well plate respectively. HEK293T cells were transfected with 200 ng plasmids containing 150 ng pEGFP-N2-MyD88 that contain the stop codon, 40 ng NF-κB reporter plasmids and 10 ng pRL-TK Renilla (*Renilla reniformis*) luciferase plasmids. pEGFP-N2 plasmids or a NF-κB positive plasmid pcDNA 3.1-EcMyD88 (kindly provided by Dr. Y.W. Li, South China Agricultural University, China) were also transfected respectively for control. S2 cells were transfected with 500 ng plasmids containing 350 ng pAc5.1-MyD88 or pAc5.1 empty plasmids, 150 ng reporter gene plasmids and 10 ng pRL-TK Renilla (*Renilla reniformis*) luciferase plasmids. Each sample was done in quintuplicate. After 24 h, the culture medium was discarded and cells were washed with PBS. The firefly (*Photinus pyralis*) and Renilla luciferase activities were determined with a Dual Luciferase Reporter Gene Assay Kit (Beyotime, China) according to the manufacturer's instructions.

2.10. Double strands RNA (dsRNA) preparation

Primer set targeted to *Pt*-MyD88 with T7 promoter sequence (shown in Table 1) were designed and used to amplify the specific DNA fragment (394 bp) of *Pt*-MyD88 based on the cDNA template. The polymerase chain reaction (PCR) was performed as follows: pre-

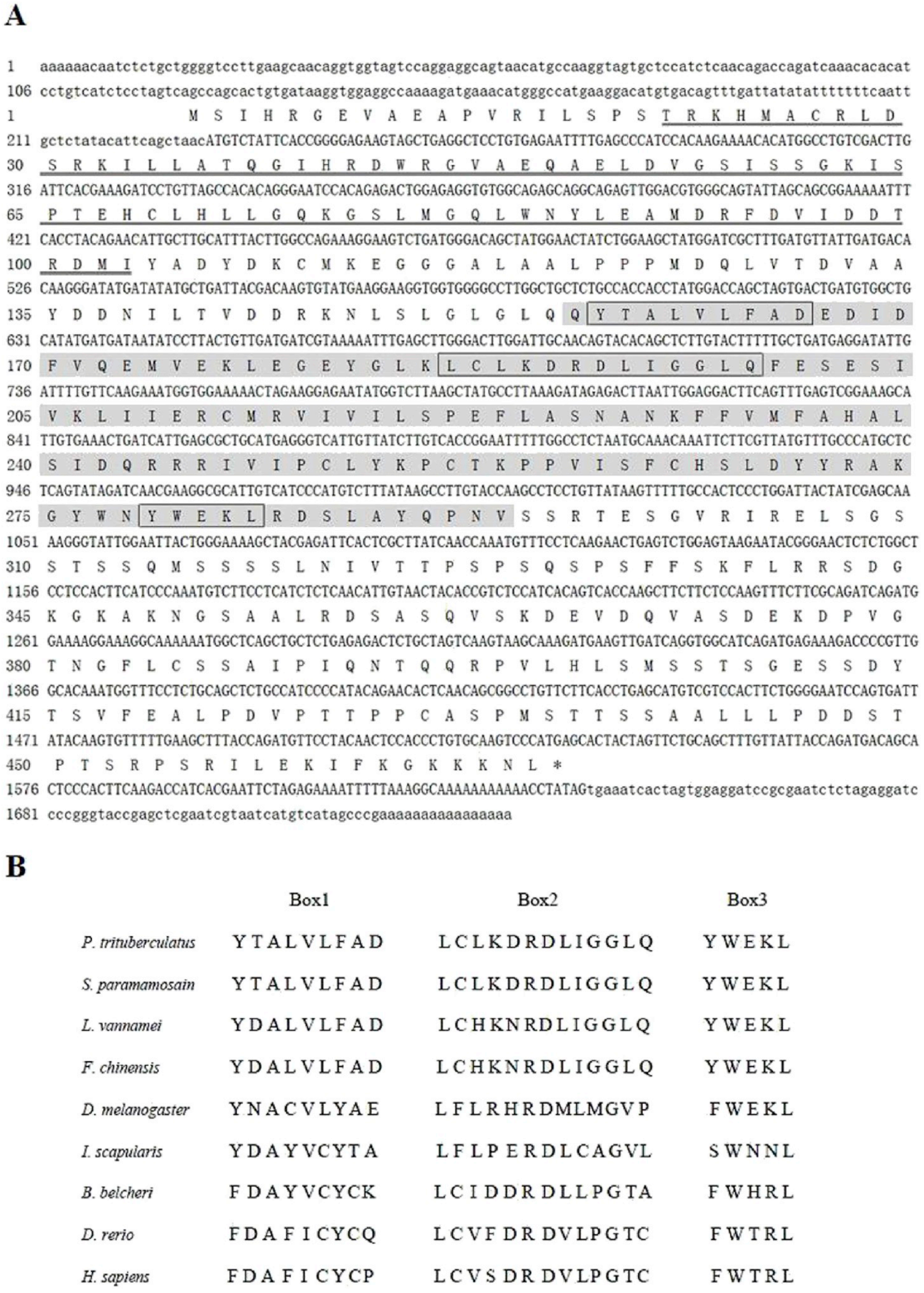

Fig. 1. Nucleotide and deduced amino acid sequences of the Pt-MyD88. (A) The conserved DD is underlined and the TIR domain is shown in shadow. Conserved Box1, Box2 and Box 3 in TIR domain are framed. (B) It showed the conserved Box1, Box2 and Box 3 in TIR domain from *P. trituberculatus* with other invertebrates and vertebrates species.

denaturation at 94 °C for 5 min followed by 35 cycles of 95 °C for 30 s, 60 °C for 20 s, and 72 °C for 20 s. The PCR products were purified by and used as the template for synthesis of dsRNA of *Pt*-MyD88 with TranscriptAid T7 High Yield Transcription kit (Thermo Fisher Scientific, USA). Non-specific dsRNA targeted enhanced green fluorescent protein (EGFP, 289 bp) was preparation for mock group as Wen et al. described previously [28]. The dsRNA was treated with ribonuclease (RNase) A (TaKaRa, China) to digest the single strand RNA. The dsRNAs were checked by spectrophotometric method as well as by agarose gel electrophoresis.

2.11. Primary culture hemocytes and in-vitro RNA interference by dsRNA

The hemocytes were drawn from the normal crab with some anticoagulant. After a short centrifuge, the cell pellets were resuspended in L15 medium (HyClone, USA) which supplemented with 17‰ NaCl. Then, cells were adjusted to 1×10^6 cells per mL and seeded into 12-well plates which maintained in L15 medium (HyClone, USA) supplemented with 17‰ NaCl and 10% FBS (Gibco, USA) at 26 °C in an incubator. After the hemocytes attached to the plates, cells were incubated with 2 μg dsRNA of *Pt*-MyD88 or EGFP for 48 h. Each sample was done in triplicate. Total RNA were extracted as the method described in 2.1. qPCR was used to test the relative expression level of *Pt*-MyD88 after specific or non-specific dsRNA interference, GADPH was used as reference gene. Moreover, the expression of several kinds of AMPs including anti-lipopolysaccharide factor, hyastatin, crustin, lysozyme and asatin were also analyzed to investigate the effect of *Pt*-MyD88 on expression of AMPs in swimming crab. qPCR method were conducted as described in 2.5.

2.12. Statistical analysis

All data were analyzed with SPSS software (version 16.0) and expressed as mean ± standard error and the significance. Student's *t*-test was used to determine statistical significance of differences, and $p < 0.05$ was considered statistically significant.

3. Results

3.1. Sequence analysis of Pt-MyD88

The full-length cDNAs of *Pt*-MyD88 (GeneBank accession number: KM521426) were obtained using homology cloning and SMART RACE. The *Pt*-MyD88 cDNA was 1736 bp in length consisted of an ORF of 1410 bp encoding 469 amino acid residues, 230 bp 5′-UTR and 96 bp 3′-UTR. Sequence homology analysis revealed that the predicted *Pt*-MyD88 protein sequences were 94% identical to the *S. paramamosain* ortholog, and 63–69% to MyD88 of shrimps. In *Pt*-MyD88, the N-terminal death domain is composed of 85 amino acids (19-103aa) and the C-terminal TIR domain contains 138 amino acids (156-293aa), with the presence of three highly conserved motifs: box 1 (F/YDA), box 2 (RDχΦ1Φ2G, where χ represents any amino acid and Φ represents a hydrophobic residue) and box 3 (a conserved W surrounded by basic residues) (Fig. 1). The Box 1 in *Pt*-Myd88 was YTA, which was different from FDA in vertebrate or YDA in shrimp (Fig. 1). Different with shrimp, *Drosophila* and other vertebrate animals [19], no intron was found in *Pt*-MyD88 genomic sequence.

3.2. Phylogenetic analyses

Phylogenetic analyses were performed on the full-length amino acid sequences of the predicted *Pt*-MyD88 molecules using the neighbor-joining (NJ) method. A phylogenetic tree constructed using 15 MyD88 protein sequences from invertebrates and vertebrates. The MyD88 proteins sequences in the tree was split into two groups (Fig. 2), and *Pt*-MyD88 grouped in a cluster containing MyD88 protein sequences of other invertebrates, with a close relationship to other crustaceans (Fig. 2).

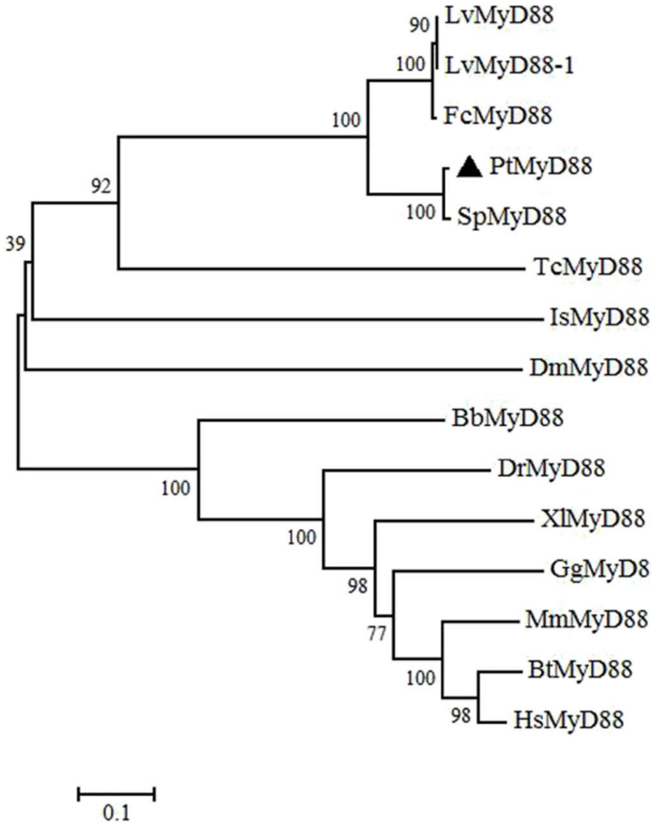

Fig. 2. Phylogenetic tree of MyD88s used full length amino acid sequences, and the Pt-MyD88 is marked by▲. The rooted trees were constructed via the neighbor-joining method and bootstrapped 1000 times using the MEGA 5.0 software (http://www.megasoftware.net/index.html). Sequences used for phylogenetic tree analysis of MyD88s included: LvMyD88 (AFP49300.1), LvMyD88-1 (AFP49301.1), FcMyD88 (AFU61120.1), PtMyD88 (KM521426), SpMyD88 (AGC65453.1), TcMyD88 (EFA01304.1), DmMyD88 (AAF58953.1), IsMyD88 (XP_002407372.1), BbMyD88 (ABQ32299.1), DrMyD88 (NP_997979.2), XlMyD88(NP_001081001.1), GgMyD88 (NP_001026133.1), MmMyD88 (AAC53013.1), BtMyD88 (DAA17130.1), HsMyD88 (AAC50954.1). Abbreviations are: Bb, *Branchiostoma belcheri*; Bt, *Bos taurus*; Dm, *Drosophila melanogaster*; Dr, *Danio rerio*; Es, *Eriocheir sinensis*; Fc, *Fenneropenaeus chinensis*; Gg, *Gallus gallus*; Hs, *Homo sapiens*; Is, *Ixodes scapularis*; Lv, *Litopenaeus vannamei*; Mm, *Mus musculus*; Pt, *Portunus trituberculatus*; Sp, *Scylla paramamosain*; Tc, *Tribolium castaneum*; Xl, *Xenopus laevis*.

3.3. Expression analysis in tissues and in response to immune challenge

The abundance of *Pt*-MyD88 transcripts was determined in different tissues of the crabs by real-time PCR that were normalized by 18S rRNA. The results showed that *Pt*-MyD88 gene was constitutively expressed in different tissues in the healthy male or female crab, and a higher expression of *Pt*-MyD88 gene were detected in hemocytes, gill, intestine and heart than that in muscle (Fig. 3). Upon challenge, the *Pt*-MyD88 expression was suppressed in hemocytes after injection of the *V. alginolyticus* or LPS. However, WSSV challenge could apparently upregulated the *Pt*-MyD88 expression at 12 h in hemocytes (Fig. 4).

3.4. Intracellular localization of Pt-MyD88 protein

To determine the cellular localization of *Pt*-MyD88, *Pt*-MyD88 was subcloned into the pEGFP-N2 or pAc5.1-EGFP vector and then transfected into HEK293T cells or S2 cells, respectively. As shown in Fig. 5,

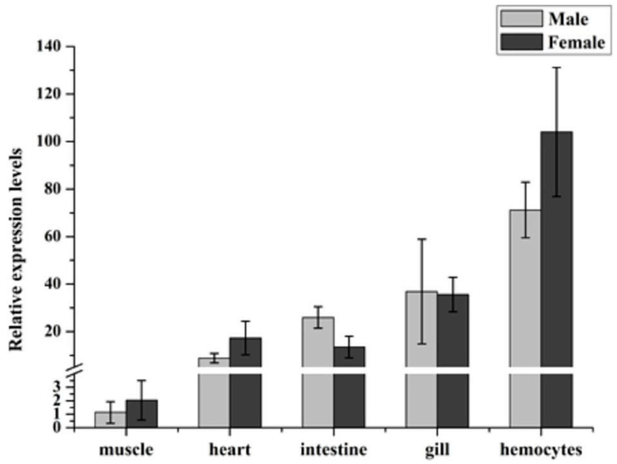

Fig. 3. Tissue distributions of *Pt*-MyD88 gene in the normal male and female *P. trituberculatus* by qRT-PCR analysis. The mRNA expression level was normalized to 18S rRNA using the relative standard curve method. Data was shown as mean values ± standard deviations.

the green fluorescence of the *Pt*-MyD88-GFP fusion protein was located in cytoplasm.

3.5. Pt-MyD88 activates mammals NF-κB and Drosophila AMP promoters

The results showed that over-expression of the full length of *Pt*-MyD88 induces a slightly activation of NF-κB in HEK293T cell models (1.99-fold), which compared to a NF-κB positive plasmid pcDNA3.1-EcTRAF6 (8.80-fold) (Fig. 6A). Moreover, three type promoters of antimicrobial peptide genes were subcloned to pGL3.0 basic as the luciferase reporter vectors. In S2 cells, *Pt*-MyD88 significantly induced *Drosophila* AMPs promoter activities of *AttA* (2.09-fold), and *Mtk* (2.71-fold) (Fig. 6B).

3.6. Knock-down efficiency of Pt-MyD88 dsRNA and its effect on expression of AMPs in crab

DsRNAs of *Pt*-MyD88 and EGFP with size of 394 bp and 289 bp were synthesized successfully (Fig. 7A). After the crab primary cultured hemocytes were incubated with dsRNAs for 48 h, the expression of *Pt*-MyD88 was significantly suppressed in specific *Pt*-MyD88 dsRNA treated cells compared to EGFP dsRNA treated cell and negative control (Fig. 7B). The expression of crab AMPs were also detected in present study. The results showed that the expression of ALF1, hyastatin3, crustin1 and crustin3 have been significantly inhibited, while the expression of other AMPs including ALF3, ALF4, ALF7, lysozyme, asatin2 and hyastatin1 is normal compared to non-specific dsRNA treated cells (Fig. 7C).

4. Discussion

From insects to mammals, the MyD88 protein acts as a conserved adaptor to mediate canonical Toll/TLR dependent NF-κB activation [4,5]. In this study, we first cloned the cDNA of *Pt*-MyD88 from the swimming crab, in which the complete ORFs encoded a deduced 469 amino acids protein. Prediction of the protein domains revealed that the *Pt*-MyD88 possessed an N-terminal death domain that is required for interaction with and recruitment of IRAK kinase, and a typical C-terminal TIR domain, which allows for interaction with TLRs. Previous studies have indicated that three short, sequence motifs, called box 1–3 motifs, which are F/YDA, RDχΦ1Φ2G, and a conserved W surrounded by basic residues, respectively, are conserved between TIR domains [29]. These highly conserved sequence motifs were also found in *Pt*-MyD88 (Fig. 3). The first conserved box (box1), which had been proposed to be essential for the interaction of MyD88 with TLRs and IL-1R [30], was YTA in two crab MyD88, which was different from YDA in most invertebrate and FDA in vertebrate. The conserved sequences and loci of these functional motifs in *Pt*-Myd88 implied a similar function in Toll signaling transduction.

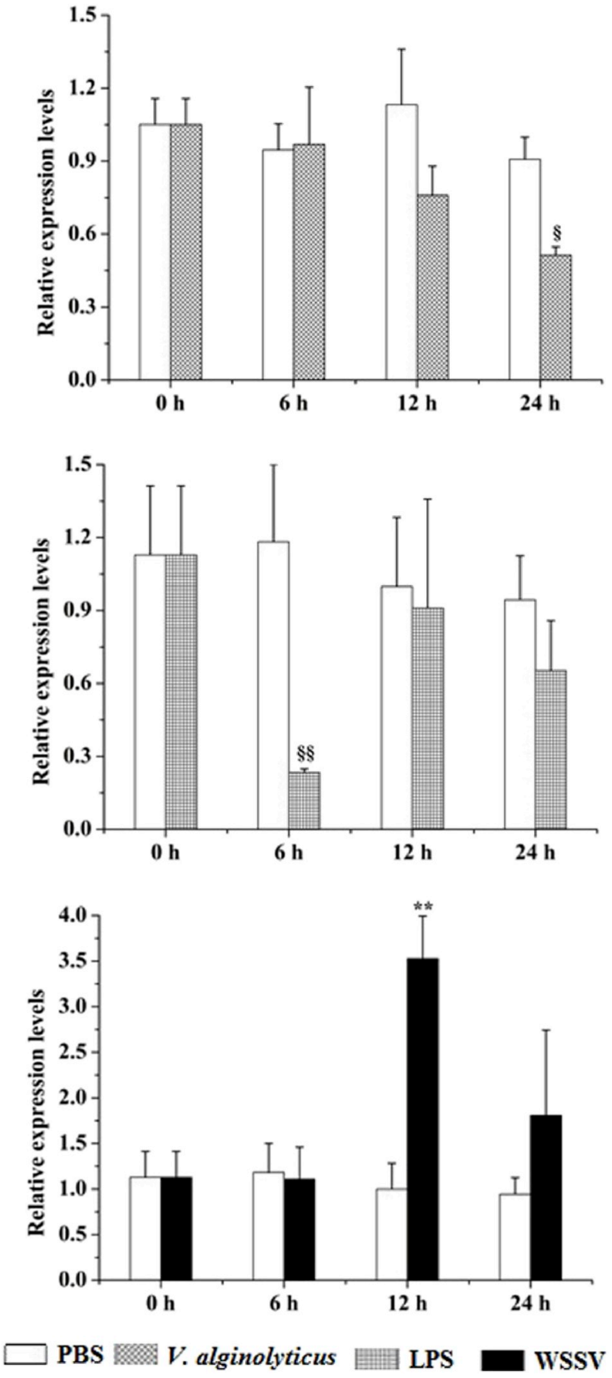

Fig. 4. qRT-PCR analysis of Pt-MyD88 mRNA expression in hemocytes in response to *V. alginolyticus*, LPS or WSSV challenges. The relative expression level of *Pt*-MyD88 was normalized to GADPH using the relative standard curve method. Data was shown as mean values ± standard deviations. * (up-regulation) or § (down-regulation) indicate a significant difference between PBS group and challenge group: * or §p < 0.05; ** or §§p < 0.01.

The innate immune system is one and only immunity strategy found in invertebrates due to the absence of adaptive response. In the past years, increasing evidence has confirmed that the insect Toll-MyD88 pathway displays anti-bacterial, anti-fungal and anti-viral functions by

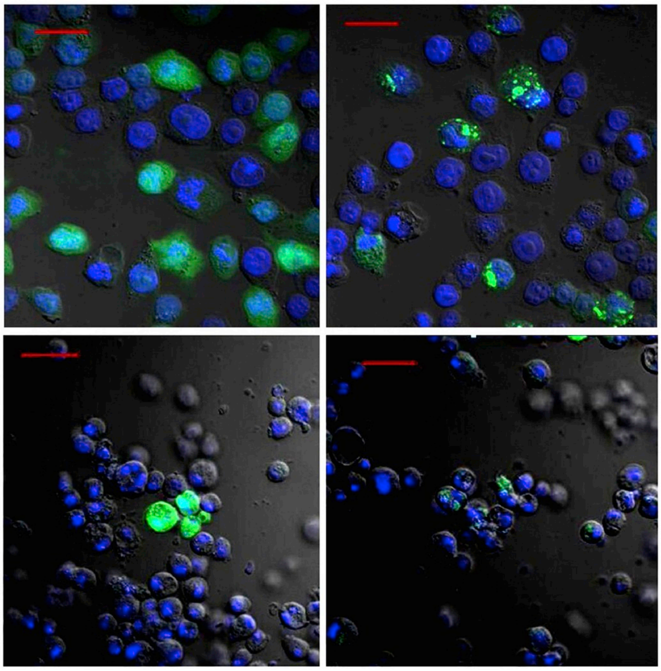

Fig. 5. Subcellular localization of *Pt*-MyD88 detected by over-expressed *Pt*-MyD88-EGFP fusion protein in HEK-293 T cells or S2 cells using a laser-scanning confocal microscope. HEK293T cells or S2 cells were transfected with (upper row) pEGFP-N2 (left) or pAc5.1-EGFP (right) or (lower row) pEGFP-N2-*Pt*-MyD88 (left) or pAc5.1- *Pt*-MyD88-EGFP (right), and nuclei were stained with DAPI, the bar = 25 μm.

regulating the expression of the immune-related genes such as AMPs [31–33]. In swimming crab, the *Pt*-MyD88 is constitutive expression in the male or female crab tissues and was shown relative high expression levels in hemocytes. The hemocytes have been shown to be the key immune cells in invertebrate which involved in protection of the host from infection, via phagocytosis and the release of antibacterial peptide or others [34]. The high expressions *Pt*-MyD88 in hemocytes suggested it may play an important role in the immune defense in the crab. Swimming crab shared the same aquatic ecosystems with many microbes, especially with many Gram negative bacteria such as vibrios. In the immune challenge experiments, the *Pt*-MyD88 expression were remarkably decreased in contact with LPS and slightly reduced after being challenged by *V. alginolyticus* in the crab hemocytes. This result is similar to scallop MyD88 expression after injection of LPS that was found in another study [21]. The WSSV has been recognized an important viral pathogen in both shrimp and crab [35,36]. To date, the immune responses of MyD88 to WSSV have studied in several shrimp species. In *L. vannamei*, it showed the expression of MyD88 was induced after challenge with WSSV [19,20]. Similarly, it has shown that the *Pt*-Myd88 transcriptions was significantly induced upon to the WSSV challenge at 12 h post-infection in the hemocytes of swimming crab, suggesting the Toll-MyD88 pathway involvement in response to virus infection.

MyD88 function as an adaptor molecule and was identified as a cytoplasmic protein [4–6]. Sequence analysis showed that the predicted *Pt*-MyD88 protein has neither signal peptides nor transmembrane domain which suggested *Pt*-MyD88 is soluble cytoplasmic protein. To determine the cellular localization and its functions in signal transduction, *Pt*-MyD88 was overexpressed in a mammal cell model and an arthropod cell model. Confocal microscopic analysis revealed that the *Pt*-MyD88-EGFP fusion protein was found aggregated mainly in cytoplasm of HEK293T or S2 cells. The cellular localization of *Pt*-MyD88 is consistent with its function as an adaptor protein. The Toll-MyD88 signaling pathways are remarkably conserved between mammalian and *Drosophila* [3,8]. When it was activated, the expression of main effectors of the humoral immune responses will be induced via NF-κB activation. To date, the function of MyD88 on signal transduction has been reported on plenty of aquatic animal species including reptile [37], fish [17] and mollusks [22], using a mammal NF-κB reporter plasmid in HEK-293 T cells. In present study, *Pt*-MyD88 could also activate mammal NF-κB compared to negative control group, but the level of NF-κB activation induced by *Pt*-MyD88 is markedly lower than a fish MyD88 protein. The results suggested that the crab MyD88 shared partial function on signal transduction with the vertebrate animals. More than one reference has reported the similarity of the Toll-MyD88 signal pathway between shrimp and *Drosophila*. In shrimp, overexpression of Toll or MyD88 protein in S2 cell led to activation of *Drosophila* AMP prompters, using a luciferase reporter vector [19,23,38]. As expected, *Pt*-MyD88 could significantly activate the prompters from two different *Drosophila* AMPs, compared to negative control vector. All of the results suggested that the function of *Pt*-MyD88 is quite conservative in signal transduction.

In *Drosophila*, the activation of the Toll signaling pathway was demonstrated to induce the expression of genes related to immunefunction, including AMPs [3,31,32]. Several studies have reported that the Toll-MyD88 signal pathway regulated the expression of AMPs in crustaceans. In *Litopenaeus vannamei*, overexpressed of LvMyD88 in S2 cells could activate the promoters of the penaeidin in shrimp suggested that the expression of penaeidin was regulated by Toll-MyD88 signal

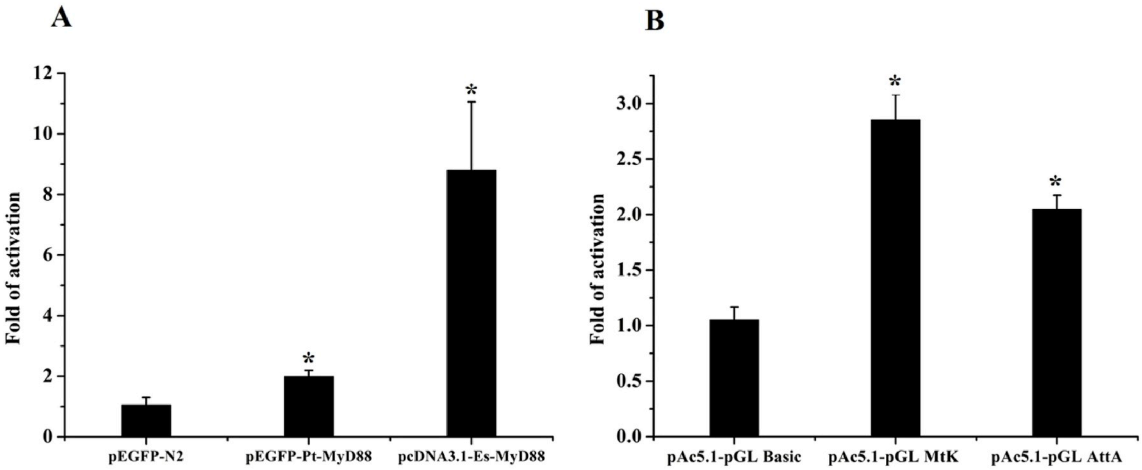

Fig. 6. Effects of *Pt*-MyD88 on the activity of NF-κB reporter gene in HEK293T cells (A) or on the promoter activities of *Drosophila* antimicrobial peptide genes in S2 cells (B). Luciferase activities were tested at 36 h post transfection. * indicate a significant difference (*$p < 0.05$, **$p < 0.01$).

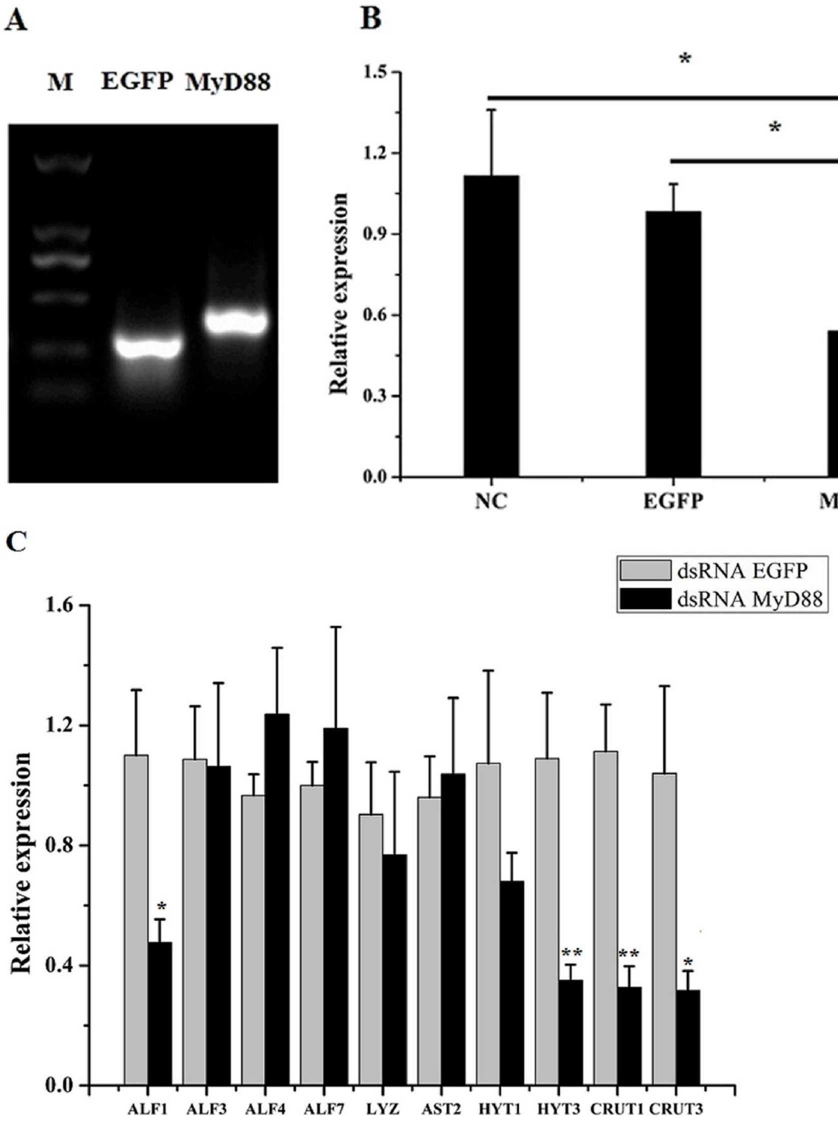

Fig. 7. Interference efficiency of *Pt*-MyD88 dsRNA and its effect on expression of AMPs in primary cultured hemocytes in swimming crab. A. Detection of purified double strand RNA of EGFP and *Pt*-MyD88 by gel electrophoresis. M represents DNA marker (DL 2000). B. The expression level of *Pt*-MyD88 in crab primary cultured hemocytes at 48 h after incubation with specific or non-specific ds RNA. C. The expression level of AMPs in crab primary cultured hemocytes at 48 h after incubation with specific or non-specific ds RNA * indicate a significant difference (*$p < 0.05$, **$p < 0.01$).

pathway in shrimp [19]. In Chinese mitten crab, when the MyD88 was knocked down and challenged by *V. parahaemolyticus*, the expression of a series of AMPs including ALF1 and ALF2, crustin1 and crustin2, and lysozyme were inhibited [23]. In present study, the expression of *Pt*-MyD88 was successfully knocked-down by specific dsRNA. Meanwhile, the expression of ALF1, hyastatin3, crustin1 and crustin3 also have been significantly inhibited, which suggested that *Pt*-MyD88 is involved in regulating the transcription of ALF1, hyastatin3, crustin1 and crustin3 in swimming crab.

In summary, we have cloned the whole *Pt*-MyD88 cDNA from *P. trituberculatus* and then characterized their expression profiles when challenged by different microbial pathogens. Moreover, the cell localization and its functions in signal transduction were studied in two different cell models. The results showed that the overexpressed *Pt*-MyD88 was distributed in cytoplasm, and it could either activate mammal NF-κB or promoters of *Drosophila* AMPs. Moreover, RNAi assay showed that *Pt*-MyD88 is involved in regulating the transcription of ALF1, hyastatin3, crustin1 and crustin3 in swimming crab. The findings in this study will help us to obtain a better understanding of the role of Toll-MyD88 signaling pathway in marine crab immunity.

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