ภาคผนวก

Fish & Shellfish Immunology 32 (2012) 26–34

Contents lists available at SciVerse ScienceDirect

Fish & Shellfish Immunology

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

Gene silencing reveals a crucial role for anti-lipopolysaccharide factors from *Penaeus monodon* in the protection against microbial infections

Sirikwan Ponprateep [a], Sirinit Tharntada [b], Kunlaya Somboonwiwat [a], Anchalee Tassanakajon [a,*]

[a] Center of Excellence for Molecular Biology and Genomics of Shrimp, Department of Biochemistry, Faculty of Science, Chulalongkorn University, 254 Phayathai Road, Bangkok 10330, Thailand

[b] Faculty of Veterinary Technology, Kasetsart University, Bangkok 10900, Thailand

ARTICLE INFO

Article history:
Received 11 August 2011
Received in revised form
6 October 2011
Accepted 7 October 2011
Available online 20 October 2011

Keywords:
Anti-lipopolysaccharide factor
Vibrio harveyi
White spot syndrome virus *Penaeus monodon*
Gene silencing

ABSTRACT

Anti-lipopolysaccharide factors (ALFs) are antimicrobial peptides previously identified in various crustaceans. Out of five isoforms identified in *Penaeus monodon*, ALF*Pm*3 is the best characterized, exhibits antibacterial and antifungal activities and can protect the shrimp from viral infections. Herein, the most recent identified ALF*Pm*, called ALF*Pm*6, is characterized for its potential role in the shrimp's immunity. RNA interference-mediated gene silencing was used to study the function of ALF*Pm*6 in comparison to ALF*Pm*3. Knockdown of ALF*Pm*3 gene led to rapid death with a cumulative shrimp mortality of 86% within 7 days, accompanied by a 12- and 50-fold higher bacterial count after 2 days in the haemolymph and hepatopancreas, respectively, compared to the control shrimp injected with GFP dsRNA. In contrast, gene silencing of ALF*Pm*6 alone had no effect on the shrimp mortality, but led to a significant increase in the cumulative mortality and a faster mortality rate following *Vibrio harveyi* and white spot syndrome virus (WSSV) infections, respectively. These results support the roles of ALF*Pm*6 and ALF*Pm*3 in the protection of shrimp against microbial infections.

1. Introduction

Invertebrates lack an adaptive immune system but have various protective mechanisms from their innate immune system to fight against invading microorganisms. Antimicrobial peptides (AMPs) represent the major defense system of invertebrate innate immune system and display diverse and complex antimicrobial activities [1,2]. AMPs are typically small cationic amphipathic molecules (15–100 amino acid residues) that differ in their primary sequences and tertiary structures. Some of these AMPs directly kill the pathogens, but some appear to function indirectly by modulating the immunity so as to induce protection from infection via other mechanisms [2,3].

Numerous AMPs have been identified in invertebrates, including shrimp and other crustaceans, and they have been shown to play a key role in the host defense responses against invading pathogens. The major shrimp AMPs are represented by the three cationic peptide families: penaeidins, crustins and anti-lipopolysaccharide factors (ALFs). These shrimp AMPs display considerable amino acid sequence diversity and constitute multiple isoforms or subgroups [4]. Penaeidins exhibit antimicrobial activity against fungi and Gram-positive bacteria [5,6], whilst crustins generally display anti-Gram positive bacterial activity [7].

In the black tiger shrimp, *Penaeus monodon*, five different isoforms of ALF (ALF*Pm*1-5) have previously been reported [8], of which the major isoform is ALF*Pm*3. Recombinant ALF*Pm*3 (rALF*Pm*3) exhibits a broad antimicrobial activity spectrum with antifungal properties against filamentous fungi and antibacterial activities against both Gram-positive and Gram-negative bacteria, including a high potency against the natural shrimp pathogen, *Vibrio harveyi* [9]. The antiviral property of ALF against the major shrimp viral pathogen, WSSV, was first reported in the freshwater crayfish *Pacifastacus leniusculus* [10]. Subsequently, rALF*Pm*3 has been shown to inhibit WSSV propagation in crayfish hematopoietic cell culture and in shrimp [11]. These results then potentially implicate the role of ALF*Pm*3 protein in the defense mechanism against WSSV infection and vibriosis in *P. monodon* shrimp. Recently, a novel ALF*Pm*, namely ALF*Pm*6, has been identified by suppression subtractive hybridization as an up-regulated gene in *P. monodon* hemocyte in response to the yellow head virus infection [12] implying its potential role in the antiviral immunity of shrimp.

In this study, ALF*Pm*6 was chosen for further study of its potential role in the antiviral response of *P. monodon* shrimp in comparison with ALF*Pm*3. The deduced amino acid sequence and

* Corresponding author. Tel.: +66 2 218 5439.
E-mail address: anchalee.k@chula.ac.th (A. Tassanakajon).

1050-4648/$ – see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.fsi.2011.10.010

the gene organization of ALF*Pm*6 were analyzed and compared with those of the other ALF*Pm* isoforms, whilst the RNA interference (RNAi)-mediated gene silencing of ALF*Pm*3 and ALF*Pm*6 was performed by injection of the respective specific double-stranded RNA (dsRNA). The efficiency and specificity of gene knockdown were determined at the transcript level by semi-quantitative RT-PCR, and the ALF*Pm*6- and ALF*Pm*3-silenced shrimp were then assayed for susceptibility to *V. harveyi* and WSSV infections.

2. Materials and methods

2.1. Animals

Subadult *P. monodon* (about 2 g wet body weight for Sections 2.7–2.9 and 15–20 g for Sections 2.4 and 2.5) were obtained from a local shrimp farm in Thailand and acclimatized in the laboratory aquaria at a temperature of 28 ± 1 °C and a salinity of 15 ppt for at least 3 days before use.

2.2. Sequence and phylogenetic analysis

Nucleotide sequences of several ALFs from shrimp and other crustaceans were retrieved from the GenBank database. The accession numbers are listed in Fig. 1b. The putative cleavage site of the signal peptide was predicted using the SignalP 3.0 Server (http://www.cbs.dtu.dk/services/SignalP/). Multiple sequence alignments of the predicted amino acid sequences were performed using the ClustalX program [13].

Phylogenetic analysis of the mature peptide amino acid sequences was performed by the neighbor-joining (NJ) distance algorithm in the MEGA 4 program [14]. Aligned sequences were bootstrapped 1000 times to obtain the confidence value for the analysis.

2.3. Amplification of the genomic ALFPm6 gene

Genomic DNA was prepared from the pleopods of *P. monodon* using a standard phenol-chloroform extraction method. The PCR primers for the amplification of ALF*Pm*6 from genomic DNA, ALF*Pm*6GF/R (Table 1), were designed from the corresponding cDNA sequences. PCR amplification was performed in a 50 μl reaction containing 1 U of RBC *Taq* DNA Polymerase (RBC Bioscience), 1 × reaction buffer (10 mM KCl, 2 mM $MgSO_4.7H_2O$, 20 mM Tris-HCl pH 8.8, 0.1% Triton X-100, 10 mM $(NH_4)_2SO_4$ and 0.1 mg/ml BSA), 200 μM of each dNTP and 0.2 μM of each of the ALF*Pm*6GF/R primers. After an initial 94 °C for 10 min, 35 cycles of 94 °C for 40 s, 65 °C for 40 s and 72 °C for 90 s were performed and then a final 72 °C for 10 min. The PCR products were cloned into the TA cloning vector (RBC Bioscience) and subjected to nucleotide sequencing by the commercial service (Macrogen Inc.).

2.4. Tissue distribution of ALFPm3 and ALFPm6 transcripts

The expression levels of ALF*Pm*3 and ALF*Pm*6 transcripts were measured in 10 different tissues: antennal gland, hemocytes, stomach, gill, intestine, lymphoid organ, heart, eye stalk, epipodite and hepatopancreas, from 3 individuals of unchallenged *P. monodon* by RT-PCR. The total RNA was extracted from those shrimp tissues using the TRI Reagent® (Molecular Research Center, Inc.), and then treated with RQ1 RNase-free DNase (Promega). The first stand cDNA was synthesized from 0.5 μg of DNase-treated total RNA by RevertAid™ First Strand cDNA Synthesis Kit (Fermentas) according to the manufacturer's instructions. The gene-specific ALF*Pm*3F/R and ALF*Pm*6F/R primers (Table 1) were used to amplify each gene fragment, and as an internal control, a fragment of the β-actin gene was amplified using the β-ACTINF/R gene-specific primers designed from *P. monodon* β-actin gene (accession no. JN808449) (Table 1). The PCR reaction contained 75 mM Tris–HCl pH 8.8, 50 mM $(NH_4)_2SO_4$, 0.1% (v/v) Triton X-100, 2.5 mM $MgCl_2$, 200 μM of each dNTP, 0.2 μM of each specific primer, 1 U *Taq* DNA polymerase (Fermentas) and 3 μl of a 1:10 dilution of the cDNA template in a total reaction volume of 25 μl. PCR conditions started with an initial 94 °C for 5 min, followed by 30 (ALF*Pm*3), 35 (ALF*Pm*6) or 26 (β-actin) cycles of 94 °C for 30 s, 58 °C for 30 s and 72 °C for 30 s, and a final extension at 72 °C for 10 min. From each reaction, 5 μl of the PCR products were analyzed following electrophoresis through a TBE-2% (w/v) agarose gel, stained with ethidium bromide and visualized by UV transillumination.

2.5. ALFPm3 and ALFPm6 gene expression analysis by quantitative real-time PCR

To examine the temporal expression of ALF*Pm*3 and ALF*Pm*6 genes in response to pathogens, shrimp were injected with 100 μl of sterile saline solution (SSS; 0.85% (w/v) NaCl) containing 10^6 colony forming units (CFU) of live *V. harveyi* 639, a lethal dose (86.6% mortality within seven days), whilst the control shrimp were injected with 100 μl of SSS only. Haemolymph was collected from the ventral sinus, using 10% (w/v) sodium citrate as an anti-coagulant solution, at 0, 6, 24 and 48 h post-injection (hpi). Total RNA was then extracted from the hemocyte pellet as described in Section 2.4. At each time point, equal amounts of total RNA from three individual shrimp were pooled and subsequently used as the template for the first strand cDNA synthesis, performed as described in Section 2.4.

For WSSV challenge, the shrimp were injected with WSSV in 100 μl lobster haemolymph medium (LHM: 486 mM NaCl, 15 mM $CaCl_2$, 10 mM KCl, 5 mM $MgCl_2$, 0.5 mM Na_2HPO_4, 8.1 mM $MgSO_4$, 36 mM $NaHCO_3$, 0.05% (w/v) dextrose in Minimum Essential Medium (Invitrogen)), and the control group was injected with 100 μl of LHM only. The WSSV titer used (a 1/8000-fold dilution in LHM) was empirically determined to be sufficient to kill all the shrimp in about 4 days (data not shown). Haemolymph was collected at 0, 12, 24 and 48 hpi. Total RNA extracted from the hemocyte was used as the template for the first strand cDNA synthesis as above.

The temporal expression levels of ALF*Pm*3 and ALF*Pm*6 transcripts in the *V. harveyi*- and WSSV- infected shrimp were evaluated by quantitative real time RT-PCR (qRT-PCR). The transcript expression levels of the two ALF*Pm*s genes were each normalized to that of the β-actin gene. The specific primers used for qRT-PCR analysis, ALF*Pm*3QF/R, ALF*Pm*6F/R and β-ACTINQF/R, are shown in Table 1. The amplification was done in a 20 μl reaction volume containing 10 μl of 2 × iQ™ SYBR® Green Supermix (Bio-Rad), 0.1 μM each of the forward and reverse primer, and 5 μl of a 1:10 dilution of the cDNA template. All runs employed a negative control without the addition of the cDNA template. The SYBR green quantitative RT-PCR amplification was performed in triplicate in an iCycler Thermal Cycler (Bio-Rad), as follows: 95 °C for 8 min followed by 40 cycles of 95 °C for 15 s, 60 °C for 20 s and 72 °C for 10 s. The threshold cycle (C_t) of each sample was analyzed by the mathematical model described by Pfaffl [15]. Statistical analysis was done using the independent samples *t*-test, with differences between means being considered as significant at $P < 0.05$.

2.6. Synthesis of dsRNA

The dsRNA targeted to the ALF*Pm*3, ALF*Pm*6 and green fluorescent protein (GFP, as a control) genes were synthesized by

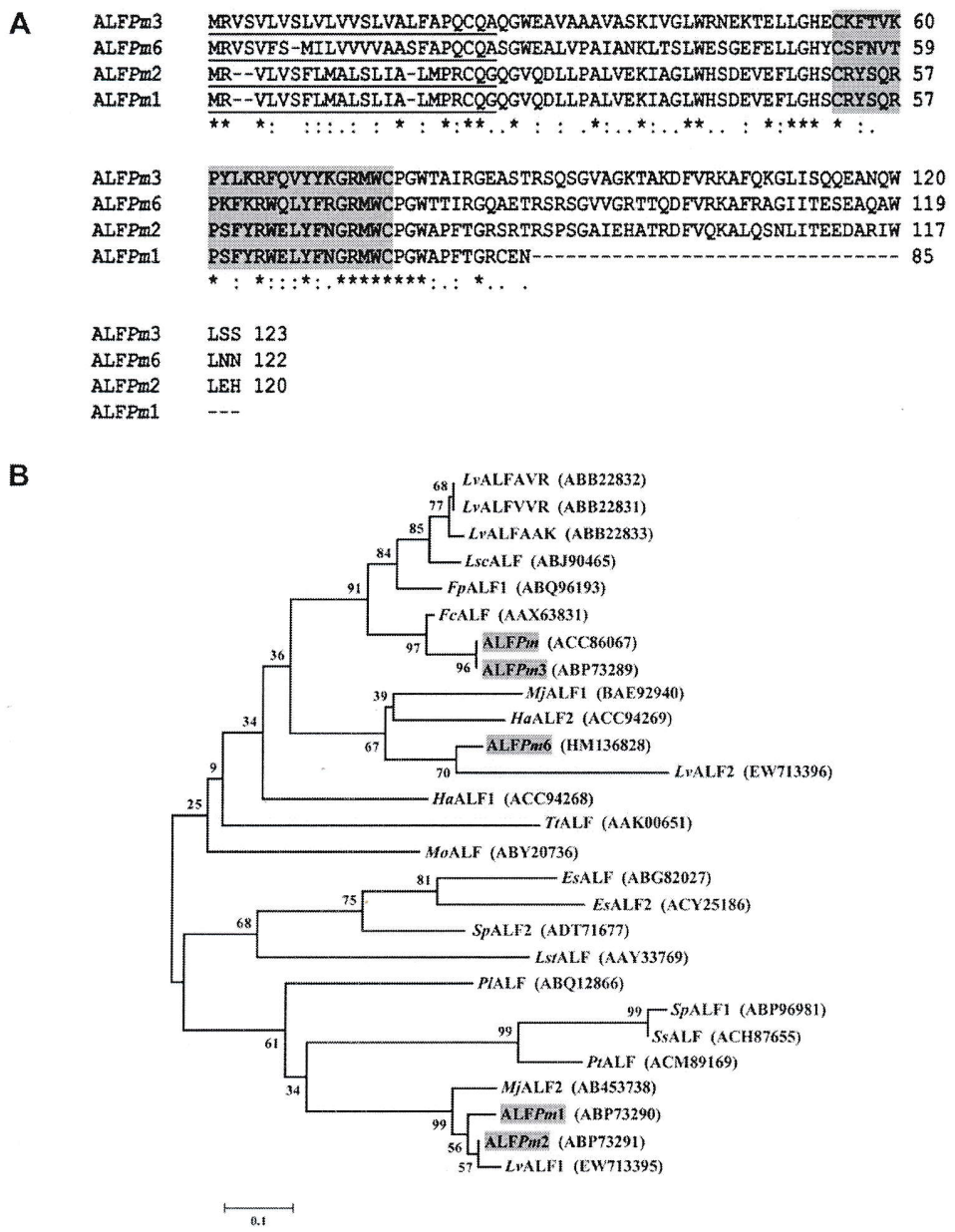

Fig. 1. Amino acid sequence analysis of ALFs from *P. monodon* (ALF*Pm*s). (A) Multiple amino acid alignment of ALF*Pm*1-3 and 6. The putative signal sequences are underlined, whilst the putative LPS-binding domains, harboring two conserved cysteine residues responsible for a disulfide bond, are shaded in grey. (B) A NJ distance based phylogenetic tree of the mature ALF*Pm*6, the other ALF*Pm*s (highlighted in grey) and various other selected ALFs including *Eriocheir sinensis* (*Es*ALF, *Es*ALF2), *Farfantepenaeus paulensis* (*Fp*ALF1), *Fenneropenaeus chinensis* (*Fc*ALF), *Homarus americanus* (*Ha*ALF1, *Ha*ALF2), *Litopenaeus schmitti* (*Lsc*ALF), *L. stylirostris* (*Lst*ALF), *L. vannamei* (*Lv*ALF1, *Lv*ALF2, *Lv*ALFAVR, *Lv*ALFAAK, *Lv*ALFVVR:), *Macrobrachium olfersii* (*Mo*ALF), *Marsupenaeus japonicas* (*Mj*ALF1, *Mj*ALF2), *P. monodon* (ALF*Pm*, ALF*Pm*1, ALF*Pm*2, ALF*Pm*3, ALF*Pm*6), *Portunus trituberculatus* (*Pt*ALF), *Scylla paramamosain* (*Sp*ALF1, *Sp*ALF2), *S. serrata* (*Ss*ALF:), *Pacifastacus leniusculus* (*Pl*ALF) and *Tachypleus tridentatus* (*Tt*ALF). GenBank accession numbers are shown in the parenthesis. Bootstrap values (per 1000 trees) are shown above the node.

in vitro transcription. DNA templates for ALF*Pm*3, ALF*Pm*6 and GFP dsRNA preparation were performed using the gene-specific primers for ALF*Pm*3F/R, ALF*Pm*6F/R and GFPF/R, respectively (Table 1). One μg of each DNA template was used for *in vitro* transcription using the T7 RiboMAX™ Express Large Scale RNA Production System (Promega), according to the manufacturer's instructions. The quality and amount of dsRNAs was verified by agarose gel electrophoresis and UV spectrophotometer, respectively.

2.7. *In vivo* gene silencing

Healthy juvenile *P. monodon* shrimp were used for this experiment. Each shrimp was intramuscularly injected into the third abdominal segment with dsRNA. The appropriate amount of dsRNA used for knockdown both ALF*Pm*3 and ALF*Pm*6 genes was preliminary tested (data not shown). 7.5 μg of ALF*Pm*3 or 5 μg of ALF*Pm*6 and the same amount of GFP dsRNA for the control (per *g* wet body weight) in 30 μl of SSS were used. Haemolymph was collected from

Table 1
Nucleotide sequences of the PCR primers.

Gene	Primer sequences(5′–3′)	Purpose
ALF*Pm*3F/R	TTTCCTAGTTTAGAAGATGC/CTGACAATTCTTCATAGAGC	RNAi, RT-PCR
ALF*Pm*3QF/R	CCCACAGTGCCAGGCTCAA/TGCTGGCTTCTCCTCTGATG	Real time PCR
ALF*Pm*6F/R	AGTCAGCGTTTAGAGAGGTT/GCTCGAACTCTCCACTCTC	RNAi, RT-PCR, Realtime PCR [12]
ALF*Pm*6GF/R	GTCCATCATGCCGAGTGTCAGTCTTCAG/TCGTCGCCTTAATTATTCAGCCAAG	Gene organization
GFPF/R	ATGGTGAGCAAGGGCGAGGA/AGAAGGAAGGGCGCTGAC	RNAi
β-ACTINF/R	GCTTGCTGATCCACATCTGCT/ATCACCATCGGCAACGAGA	RT-PCR
β-ACTINQF/R	GAACCTCTCGTTGCCGATGGTG/GAAGCTGTGCTACGTGGCTCTG	Real time PCR

Primers for the production of ssRNA, designated as above but with T7 (e.g. ALF*Pm*3R is ALF*Pm*3T7R). The T7 sequence (5′TAATACGACTCACTATAGG3′) was inserted at the 5, end of the shown sequence.

individual shrimp at 24 h after injection and immediately centrifuged at 800 × *g* for 10 min at 4 °C to separate the hemocytes from the plasma. Total RNA was then extracted from the hemocytes as described in Section 2.4. The level of ALF*Pm*3 and ALF*Pm*6 transcripts was determined by semi-quantitative RT-PCR and standardized to that of β-actin.

2.8. Cumulative mortality of ALFPm3-silenced shrimp and the total viable bacterial count in their haemolymph and hepatopancreas

Shrimp were divided into two groups of 10 shrimp each and injected with 7.5 μg of ALF*Pm*3 (gene silenced group) or GFP (control group) dsRNA per *g* wet body weight. The shrimp mortality was then observed daily for seven days. The experiment was performed in triplicate.

To determine the bacterial count in the shrimp, the haemolymph and hepatopancreas were collected from the individual shrimp at 48 h after dsRNA injection. The number of viable bacterial cells in the haemolymph was determined as a CFU by a modified total plate count method, as previously reported [16]. The hepatopancreas was homogenized in 200 μl of SSS and serial diluted in SSS. Each 10 μl aliquot from each of the diluted samples was dropped onto an LB/2% (w/v) NaCl-agar plate and incubated at 30 °C overnight, and then the number of bacterial colonies was counted and converted to CFU per tissue.

2.9. Cumulative mortality of ALFPm6-silenced shrimp after pathogen challenge

The pathogenic *V. harveyi* 639 isolate, a kind gift from Assoc. Prof. Dr. Sirirat Rengpipat, Department of Microbiology, Chulalongkorn University, was grown in tryptic soy broth (TSB) supplemented with 2% (w/v) NaCl at 250 rpm and 30 °C to an OD_{600} of 0.6 at which point the cell density was determined to be 10^8 CFU/ml by total plate count method.

The WSSV stock solution was purified from infected shrimp gills by a modification to the method of Xie et al. [17]. Gills were homogenized in TNE buffer (50 mM Tris-HCl, 400 mM NaCl and 5 mM EDTA, pH 8.5) containing 1 mM PMSF. The tissue debris was removed by pelleting and the supernatant was collected. The supernatant was then centrifuged at 30,000 × *g* for 30 min at 4 °C. The pellet was rinsed with TM buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, pH 7.5) and centrifuged at 3,500 × *g* for 5 min. The pellet was then suspended in TM buffer. The supernatant was centrifuged at 30,000 × *g* for 30 min at 4 °C and the pellet was resuspended in TM buffer and stored at −80 °C until use.

Each group of 10 shrimp was used in these experiments. For the *V. harveyi* challenged group, two groups were injected with 5 μg of ALF*Pm*6 or GFP dsRNA per *g* wet body weight, respectively, in 30 μl of SSS. Twenty-four hours later, the shrimp were injected with 2×10^5 CFU of *V. harveyi* 639 in 100 μl of SSS. The control was shrimp injected with SSS on both occasions.

For the WSSV-challenged group, three groups of shrimp were initially injected with 30 μl of SSS (challenged control), 5 μg of ALF*Pm*6 or GFP dsRNA per *g* wet body weight diluted in SSS, and 24 h later with 30 μl of a 10^{-6} dilution of the purified WSSV stock in TN buffer (20 mM Tris-HCl, 400 mM NaCl, pH 7.4). In the unchallenged control group, shrimp were first injected with 30 μl of 5 μg/g shrimp ALF*Pm*6 dsRNA in SSS and then 24 h later with 30 μl TN buffer. The WSSV titer used was empirically determined to be sufficient to kill all the shrimp in about 3–5 days (data not shown).

The cumulative mortality was recorded daily for seven and ten days post-*V. harveyi* and -WSSV challenges, respectively. The experiment was performed in triplicate.

3. Results

3.1. Analysis of the ALFPm6 sequence

Previously, five different isoforms of *P. monodon* ALF (ALF*Pm*1-5) have been identified from the hemocyte EST libraries [8]. However, searching against the *P. monodon* EST database (http://pmonodon.biotec.or.th), we identified a full-length gene of the latest ALF*Pm*, ALF*Pm*6, which had been recently reported by Prapavorarat et al. [12], from the gill cDNA library. The homology searching against the NCBI GenBank database revealed that the ALF*Pm*6 gene shared 75% nucleotide sequence identity to an ALF from the Kuruma shrimp, *Marsupenaeus japonicus*, *Mj*ALF1. The likely open reading frame (ORF) of ALF*Pm*6 obtained from the EST clone contains 369 bp that are predicted to encode for 122 amino acid residues (Fig. 1a), with a calculated molecular mass and pI of 11.3 kDa and 9.77, respectively. The 24 residues at the N-terminus were predicted to be a putative signal peptide. Previously, the LPS binding domain of ALF*Pm*3 was identified based on the alignment with the *Limulus* ALF [9]. Thereafter, the putative LPS binding site of several ALFs have been identified in the same way. In this study, amino acid sequence alignment with the other ALF*Pm*s (ALF*Pm*1-5) revealed a putative novel LPS binding domain, having the sequence of CSFNVTPKFKRWQLYFRGRMWC, located at amino acid positions 54-75. The amino acid composition of which was different from those in the putative LPS-binding domain of ALF*Pm*1 and ALF*Pm*2 or ALF*Pm*3-5. There was a clear difference in the total number of positively charged amino acid residues (Arg and Lys) among the ALFs from *P. monodon*; 18.2%, 27.3% and 22.7% for ALF*Pm*1/ALF*Pm*2, ALF*Pm*3-5 and ALF*Pm*6, respectively. Nevertheless, the putative LPS binding sequences of ALF*Pm*3-5 and ALF*Pm*6 contained no negatively charged residues whereas those of ALF*Pm*1 and ALF*Pm*2 have one Glu residue.

The amino acid sequence based phylogenetic analysis (NJ distance method) of 27 isoforms of mature ALFs (i.e. after removal of the predicted signal peptide sequence) from shrimp and other organisms was performed (Fig. 1b). ALF*Pm*6 was distantly different from the other ALF*Pm*s but closely related to *Lv*ALF2, *Mj*ALF1 and *Ha*ALF2; it was placed in the same group as ALF*Pm*3 but in different

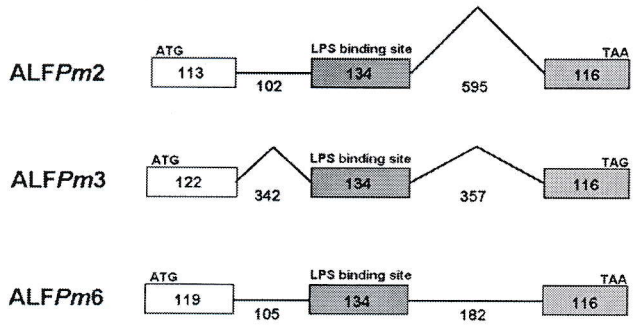

Fig. 2. Genomic structure of ALF*Pm*6 in comparison to that of other ALF*Pm*s. The genomic sequences of ALF*Pm*6 are shown in the supplementary material. The numbers in the boxes (exons) and under the lines (introns) indicate the nucleotide length of each.

subgroups. The closely related ALF*Pm*1 and ALF*Pm*2 were in different group.

3.2. Gene organization of ALFPm6

The genomic organization of ALF*Pm*6 gene was determined by PCR amplification of the genomic DNA and sequencing of the resultant amplicons. The results (GenBank accession no. JN562340) showed that the ALF*Pm*6 gene contained three exons (119, 134 and 116 bp) interrupted by two introns (105 and 182 bp) (Supplement data) (Fig. 2). All splice junctions were composed of classic GT/AG donor/acceptor splice sites. The signal peptide was encoded at the 5′ end of exon 1 whereas the mature peptide was encoded by the 3′ end of exon 1 to exon 3. The gene organization of ALF*Pm*6 is comparable to those of ALF*Pm*2 and ALF*Pm*3 [27]. The putative LPS binding sequences of all three ALF*Pm*s were located within their exon 2 regions (Fig. 2).

3.3. Tissue distribution of ALFPm3 and ALFPm6 transcripts

The distribution of ALF*Pm*3 and ALF*Pm*6 transcripts in different shrimp tissues including antennal gland, hemocytes, stomach, gill, intestine, lymphoid organ, heart, eyestalk, epipodite and hepatopancreas, was determined by RT-PCR (Fig. 3). The ALF*Pm*3 transcripts were found to be highly expressed in hemocytes only, and not significantly detected in the other examined tissues of the unchallenged shrimp (Fig. 3a), which is consistent with its reported discovery in the hemocyte EST libraries. In contrast, the ALF*Pm*6 transcript was expressed in all tissues examined (Fig. 3b). It expressed most in heart followed by hepatopancreas and epipodite. Less expression of ALF*Pm*6 mRNA was observed in the hemocytes, stomach, intestine, lymphoid organ and eyestalk and barely detectable in the antennal gland and gill.

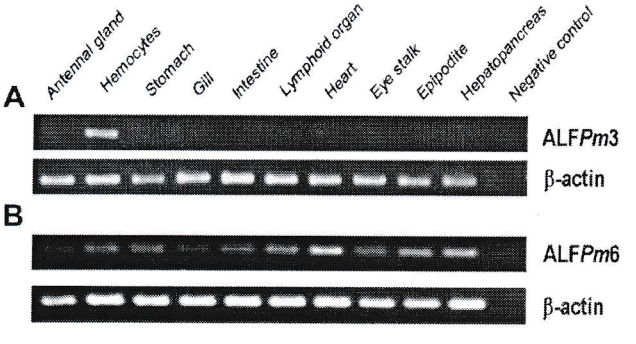

Fig. 3. Expression analysis of ALF*Pm*3 and ALF*Pm*6 transcript levels. The tissue distribution of (A) ALF*Pm*3 and (B) ALF*Pm*6 transcripts was measured by RT-PCR. Total RNAs of the antennal gland, hemocytes, stomach, gill, intestine, lymphoid organ, heart, eyestalk, epipodite and hepatopancreas were extracted from normal shrimp (15–20 g wet body weight). The β-actin gene was amplified from the same templates and used as the internal reference control.

3.4. Expression analysis of ALFPm genes by quantitative real-time RT-PCR

The temporal expression of the ALF*Pm*6 transcript in *V. harveyi* challenged shrimp was analyzed by qRT-PCR. ALF*Pm*3 transcript expression levels have been reported to be highly (seven-fold) induced at 6 hpi in *V. harveyi*-challenged shrimp [18]. Here, the ALF*Pm*6 transcript levels were also found to be significantly (four-fold) increased at 6 hpi and then decreased towards the normal level at 24 and 48 hpi (Fig. 4a).

Upon challenge with WSSV, the ALF*Pm*3 transcript levels remained essentially the same at 12 hpi but significantly (~5-fold) increased at 24 hpi and then gradually decreased to about 4-fold elevated level at 48 hpi (Fig. 4b). In contrast, ALF*Pm*6 transcript expression levels showed different magnitudes of elevation, rising quicker and attaining a higher level than that of ALF*Pm*3 transcript. The ALF*Pm*6 transcript expression attained about 11-fold higher expression level at 12 hpi, and then decreasing rapidly to about 3-fold higher at 24 hpi before being highly elevated (~22-fold) again at 48 hpi (Fig. 4c).

3.5. Gene silencing of ALFPm3 and ALFPm6 in P. monodon shrimp

It was previously shown that the recombinant ALF*Pm*3 protein could inhibit the replication of WSSV both *in vivo* and *in vitro* [11]. Here, we further investigated the function of ALF*Pm*6 in comparison to ALF*Pm*3 against WSSV by gene silencing using RNA interference. The systemic injection of 7.5 μg and 5 μg of ALF*Pm*3 or ALF*Pm*6 dsRNA (per *g* wet body weight of shrimp), respectively, could knock down the corresponding gene expression by about 85% and 73% at 24 hpi, respectively, whilst the control injection of GFP dsRNA had no effect on the ALF*Pm* gene expression (Fig. 5). The specificity of dsRNA-mediated gene knockdown was verified by RT-PCR using gene-specific primers for ALF*Pm*2, ALF*Pm*3 and ALF*Pm*6. The RT-PCR results indicated that, for the genes evaluated at least, the ALF*Pm*3 and ALF*Pm*6 genes were specifically silenced by their corresponding dsRNA.

3.6. Cumulative mortality and the total viable bacterial count in the haemolymph and hepatopancreas of ALFPm3-silenced P. monodon shrimp

Unexpectedly, injection of the ALF*Pm*3 dsRNA into shrimp caused rapid mortality, with the cumulative mortality in the ALF*Pm*3-silenced shrimp reaching 86% within 4 days post-dsRNA injection, compared to less than 20% in the GFP dsRNA-injected control shrimp (Fig. 6a). To assess the potential cause of the high mortality in ALF*Pm*3-silenced shrimp, the number of total viable bacteria (CFU) in the haemolymph and hepatopancreas of apparently healthy shrimp at two days post-dsRNA injection was derived from total plate counts. The number of viable bacteria (as CFU) in the haemolymph and hepatopancreas were 12- and 50-fold higher than the control shrimp injected with GFP dsRNA, respectively (Fig. 6b). Since the shrimp had not been systemically injected or deliberately exposed externally to bacterial sources, just kept in clean but not aseptic culture conditions, we assume that there was invasion of bacteria from external sources, perhaps through the injection site or other wounds, but penetration through the gills or gut could not be excluded.

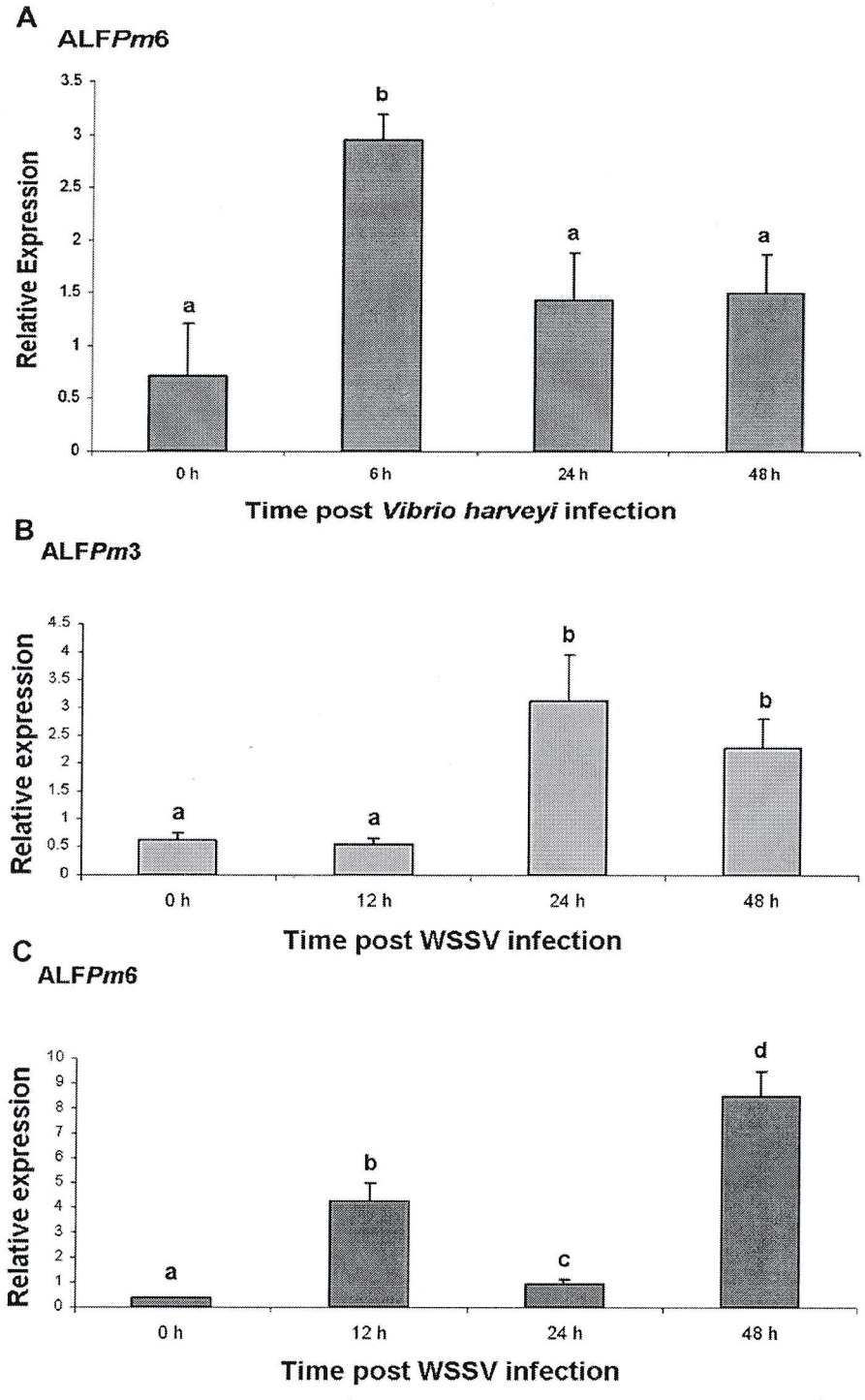

Fig. 4. Time course analysis of transcript expression levels in the hemocytes of *P. monodon* shrimp (15–20g wet body weight) for (A) ALF*Pm*6 after *V. harveyi* challenge and (B) ALF*Pm*3 and (C) ALF*Pm*6 after WSSV challenge. The mRNA levels were evaluated by qRT-PCR and were normalized to that of the β-actin gene. Each histogram represents the mean expression fold change relative to the control, ± 1 S.D. and is derived from three independent repeats. Significant differences ($P < 0.05$) are indicated by a different letter (a–d).

3.7. Cumulative mortality of ALFPm6-silenced P. monodon shrimp after WSSV challenge

Unlike that found in the ALF*Pm*3-silenced shrimp, injection of ALF*Pm*6 dsRNA had no significant effect on the shrimp mortality (data not shown). Thus, the potential role of ALF*Pm*6 in the shrimp defense against WSSV infection was evaluated. The ALF*Pm*6-silenced shrimp were systemically challenged with WSSV at 24 h after the ALF*Pm*6 dsRNA injection, and the mortality rate was recorded for a period of ten days after challenge. The two control

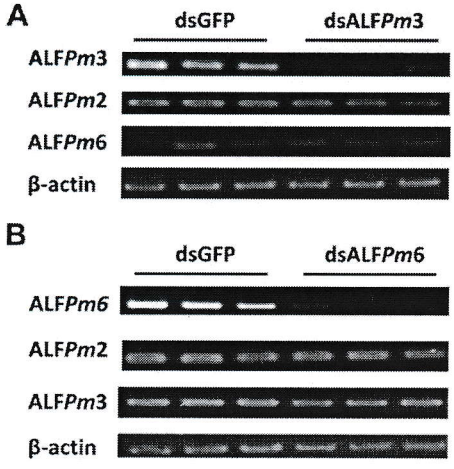

Fig. 5. Gene-specific dsRNA-mediated knockdown of ALF*Pm*3 and ALF*Pm*6 transcript expression levels in 1–3 g wet body weight *P. monodon* hemocytes. Shrimp were injected with (per *g* wet body mass) (A) 7.5 μg of ALF*Pm*3 or GFP dsRNA, (B) 5 μg of ALF*Pm*6 or GFP dsRNA. After twenty-four hpi, the hemocytes were collected from each shrimp, total RNA was extracted and the expression levels of the indicated gene transcripts were detected by RT-PCR.

shrimp groups were injected with either GFP dsRNA or with TN buffer. At 3–5 days post-WSSV challenge, the cumulative mortality was significantly higher in the ALF*Pm*6-silenced shrimp compared to the two control groups, and reached 100% by day 8 (Fig. 7). Although the increased cumulative mortality of the TN-injected (the challenged control group) of shrimp was delayed relative to that of the ALF*Pm*6 down-regulated shrimp, it rose quickly from 4- to 6- days post-WSSV challenge, reaching ~84%, 90% and 100% mortality after 6, 8 and 10 days, respectively. However, a lower mortality was observed in the GFP dsRNA-injected shrimp with, for example, only ~60% and ~80% cumulative mortality being observed by 8 and 10 days post-WSSV challenge, respectively. The results indicate the importance of ALF*Pm*6 in the shrimp antiviral response and the likely protection effect of the non-specific dsRNA injection (in this case GFP) in priming the immune response against WSSV (a dsDNA virus) infection.

3.8. Cumulative mortality of ALFPm6-silenced P. monodon shrimp after V. harveyi 639 challenge

To further examine the potential role of ALF*Pm*6 as an essential effector molecule in the immunity of *P. monodon* shrimp, its potential role in the defense against the pathogenic bacterium *V. harveyi* was examined. To this end the cumulative mortality of the ALF*Pm*6-knockdown shrimp was examined post-*V. harveyi* infection. The two control shrimp groups were injected with either GFP dsRNA in SSS or SSS alone. Twenty-four hours after the dsRNA or SSS injection, the shrimp were challenged by intramuscular injection of *V. harveyi* and the cumulative mortality was then monitored for seven days.

A significant increase in the cumulative mortality level two days after the bacterial challenge, from 20% in the control shrimp to 90% in ALF*Pm*6-knockdown shrimp, was observed (Fig. 8). The cumulative mortality thereafter remained at these respective levels from day 2 over the remaining seven day assay period. Thus, the mortality was principally induced within the first two days and was significantly higher in the ALF*Pm*6-knockdown shrimp, suggesting the important role of ALF*Pm*6 in the defense against *V. harveyi* infection.

4. Discussion

ALFs were originally isolated from the hemocytes of the horseshoe crabs, *Limulus polyphemus* (LALF) and *Tachypleus*

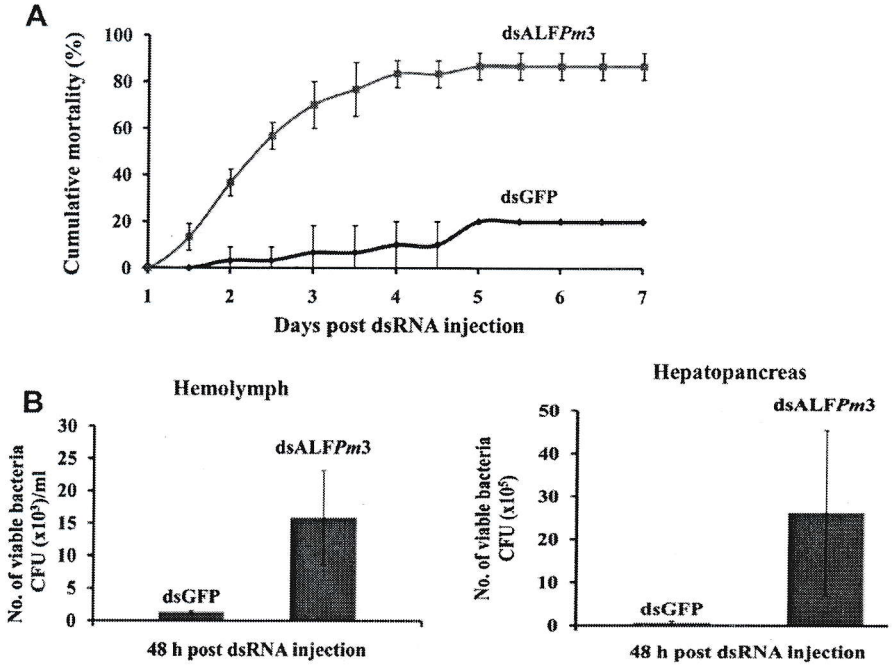

Fig. 6. (A) The effect of ALF*Pm*3silencing in 1–3 g wet body weight *P. monodon* shrimp on their cumulative mortality. Shrimp were injected with 7.5 μg of ALF*Pm*3 or GFP dsRNA per *g* wet body weight and the cumulative mortality was observed over seven days. Data are shown as the mean ± 1 S.D. and are derived from three replicate experiments. (B) The number of viable bacterial colonies (as CFU) from the haemolymph and hepatopancreas of *P. monodon* shrimp 48 hpi with ALF*Pm*3 or GFP dsRNA (5 μg/g shrimp). Data represent the mean ± 1 S.D. and are derived from three independent repeats with three shrimp per condition.

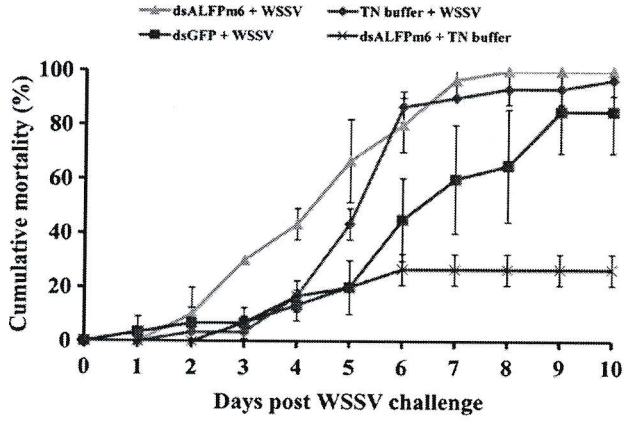

Fig. 7. The effect of ALF*Pm*6 gene silencing in *P. monodon* shrimp (1–3 g wet body weight) on the subsequent WSSV challenge. Shrimp were injected with 30 μl of TN buffer or 5 μg of ALF*Pm*6 or GFP dsRNA per *g* wet body weight and 24 h later injected with a lethal dose of WSSV. The cumulative mortality was then recorded daily for seven days. Data are shown as the mean ± 1 S.D. and are derived from three independent repeats.

tridentatus (TALF) [19,20], and have been reported to inhibit the LPS-mediated activation of the *Limulus* coagulation system [19,21], and to also exhibit an antibacterial effect on the growth of the Gram-negative R-type bacteria. Subsequently, the ALF cDNAs have been reported in various crustaceans, including shrimp [4].

So far, six isoforms of ALF has been discovered from *P. monodon* [12,22]. Of those, the ALF*Pm*6 is the most recent isoform; however, there is no report on ALF*Pm*6 characterization. This isoform was the second most abundant isoform identified from the *P. monodon* EST database (http://pmonodon.biotec.or.th) [8]. Phylogenetic analysis indicated that ALF*Pm*6 was different from the other *P. monodon* ALFs (ALF*Pm*1-5) as it formed a distinctly separate group along with *Lv*ALF2, *Mj*ALF1 and *Ha*ALF2. However, the pattern of gene organization was conserved among the ALF*Pm*s in that they contained three exons interrupted by two introns of variable lengths. Like ALF*Pm*s, the ALFs from crabs such as *Sp*ALF1 and *Sp*ALF2 from the mud crab, *Scylla paramamosain* [23,24], *Es*ALF1 from Chinese mitten crab, *Eriocheir sinensis* [25], have the same pattern of genomic organization. From the ALF genome structure either of shrimp or crabs, the LPS-binding domain located only on the exon 2 [23–25,27]. In addition, these ALF*Pm* genes are likely to be encoded by different genomic loci and do not arise from alternative splicing.

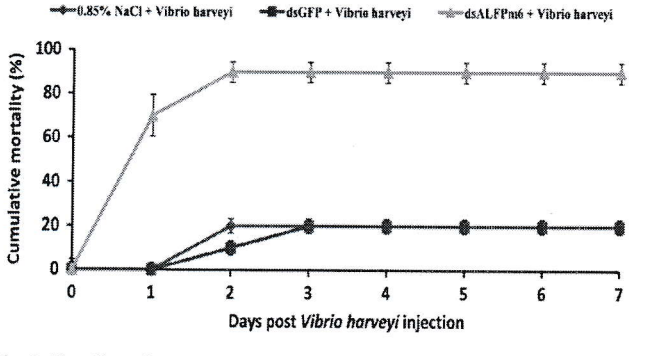

Fig. 8. The effect of ALF*Pm*6 gene silencing in *P. monodon* shrimp (1–3 g wet body weight) on the subsequent *V. harveyi* 639 challenge. Shrimp were injected with 30 μl of SSS or 5 μg of ALF*Pm*6 or GFP dsRNA per *g* wet body weight in SSS, and 24 h later injected with a lethal dose (2×10^5 CFU) of *V. harveyi* 639. The cumulative mortality was then recorded daily for seven days. Data are shown as the mean ± 1 S.D. and are derived from three independent repeats.

Like other ALF*Pm*s, ALF*Pm*6 contained a signal peptide at the N-terminus and a predicted LPS binding site, in this case with the novel sequence CSFNVTPKFKRWQLYFRGRMWC. Previously, the five different *P. monodon* ALF sequences (ALF*Pm*1–5) were compared and were divided into two groups based upon their LPS binding site sequence. ALF*Pm*1 and ALF*Pm*2 formed one group (group A) with the LPS binding site sequence CRYSQRPSFYRWELYFNGRMWC, whilst ALF*Pm*3-5 formed another group (group B) with the LPS binding site sequence CKFTVKPYLKRFQVYYKGRMWC [27]. Since the putative LPS binding site of ALF*Pm*6 was different from those of groups A and B, ALF*Pm*6 should belong to a new ALF*Pm* group, designated here as group C. The differences in the LPS binding site sequences might indicate the ability of these different ALF*Pm* isoforms to bind to different microbial cell wall components.

Unlike the ALF*Pm*3 or *Mj*ALF1 [26], which were mainly expressed in the hemocytes, ALF*Pm*6 appeared to be expressed in several tissues including antennal gland, hemocytes, stomach, gill, intestine, lymphoid organ, heart, eyestalk, epipodite and hepatopancreas. The differences in expression levels of ALF*Pm*6 in different tissues are similar to that observed for *Mj*ALF2 [29]. Previously, it was reported that the expression of ALF is induced in bacterial-challenged shrimp [18,22,26] and in WSSV-challenged *P. leniusculus* [10]. In this study, we found that the expression of ALF*Pm*6 was also induced in both *V. harveyi*- and WSSV-challenged *P. monodon* shrimp. Moreover, it has been shown recently that ALF*Pm*6 gene is up-regulated upon YHV infection [12]. These suggest a potential role of ALF*Pm*6 in the shrimp immunity.

ALF*Pm*3 transcripts are abundantly expressed in shrimp and their gene-silencing resulted in the rapid death of the knockdown shrimp and in the presence of a high bacterial count in the hepatopancreas and haemolymph. This result suggested that this protein was essential for controlling bacterial infection, as reported previously [30], and provided systemic protection against microorganisms surrounding the animal. In contrast, ALF*Pm*6 gene silencing did not cause shrimp mortality. However, after challenge with either *V. harveyi* or WSSV, a significantly higher mortality was observed in the ALF*Pm*6-silenced shrimp compared to the control shrimp. Their mortality rate reached over 90% after two days of infection whereas only 20% mortality was observed in the control shrimp. This result strongly supported a role for ALF*Pm*6 in the shrimp immunity against bacterial infection.

ALF*Pm*6-silenced shrimp had a higher mortality rate at the early stage (days 3–5) of WSSV infection compared with the control shrimp, although the cumulative mortality of this control group also reached 100% by 7 days after WSSV infection. It was found also that GFP dsRNA injection actually increased the protection to WSSV infection, presumably by activation of the antiviral immune response in shrimp in a sequence-independent manner. Certainly a dsDNA sequence-independent protection mechanism against viral infection of shrimp has been reported before [28]. ALF*Pm*3 has been reported to mediate anti-WSSV activity by inhibiting the replication of WSSV [11]. According to our results, it is likely that ALF*Pm*6 might also be involved in the anti-WSSV infection response in shrimp. In contrast to the role of ALF*Pm*6 in bacterial immunity seen here, it has been reported that dsRNA-mediated *Lv*ALF1 gene silencing in *L. vannamei* shrimp resulted in a significant increase in their mortality when subsequently challenged with the bacterium *Vibrio penaeicida* and the fungus *Fusarium oxysporum* but not with WSSV [31]. Thus, *Lv*ALF1 has a likely role in protecting shrimp from bacterial and fungal infections, but not from WSSV infection.

In conclusion, ALF*Pm*3, the most abundant *P. monodon* ALF, plays a major role in the protection of shrimp from microbial and viral infections and is essential for their survival, clearing or controlling the number of microorganisms and providing systemic protection against pathogens. Additionally, another ALF isoform, ALF*Pm*6,

though less crucial, provides an additional protection against bacterial infection.

Acknowledgements

This work was supported by research grants from the Thailand National Center for Genetic Engineering and Biotechnology (BIOTEC) and the Higher Education Research Promotion and National Research University Project of Thailand, Office of the Higher Education Commission (FW643A). We thank Chulalongkorn University for the support to the Center of Excellence for Molecular Biology and Genomics of Shrimp under the Ratchadaphisek Somphot Endowment and the Faculty of Science, Chulalongkorn University (Grant No.RESA1B1-11) to Kunlaya Somboonwiwat. We acknowledge the support from the Thailand Research Fund (TRF), the Office of the Higher Education Commission and Kasetsart University (Grant No. MRG5380020) to Sirinit Tharntada. Equipments used in this project are partially supported by Thai Government Stimulus Package 2 (TKK2555) under the Project for Establishment of Comprehensive Center for Innovative Food, Health Products and Agriculture. We would like to thank Dr. Robert Douglas John Butcher at the Publication Counseling Unit (PCU), Faculty of Science, Chulalongkorn University, for English language corrections of this manuscript.

References

[1] Jenssen H, Hamill P, Hancock REW. Peptide antimicrobial agents. Clin Microbiol Rev 2006;19:491–511.
[2] Hancock REW, Brown KL, Mookherjee N. Host defence peptides from invertebrates - emerging antimicrobial strategies. Immunobiol 2006;211:315–22.
[3] Hancock REW, Diamond G. The role of cationic antimicrobial peptides in innate host defences. Trends Microbiol 2000;8:402–10.
[4] Tassanakajon A, Amparyup P, Somboonwiwat K, Supungul P. Cationic antimicrobial peptides in penaeid shrimp. Mar Biotechnol 2010;12:487–505.
[5] Destoumieux D, Bulet P, Loew D, Van Dorsselaer A, Rodriguez J, Bachère E. Penaeidins, a new family of antimicrobial peptides isolated from the shrimp *Penaeus vannamei* (Decapoda). J Biol Chem 1997;272:28398–406.
[6] Destoumieux D, Bulet P, Strub JM, Van Dorsselaer A, Bachère E. Recombinant expression and range of activity of penaeidins, antimicrobial peptides from penaeid shrimp. Eur J Biochem 1999;266:335–46.
[7] Smith VJ, Fernandes JMO, Kemp GD, Hauton C. Crustins: enigmatic WAP domain-containing antibacterial proteins from crustaceans. Dev Comp Immunol 2008;32:758–72.
[8] Tassanakajon A, Klinbunga S, Paunglarp N, Rimphanitchayakit V, Udomkit A, Jitrapakdee S, et al. *Penaeus monodon* gene discovery project: the generation of an EST collection and establishment of a database. Gene 2006;384:104–12.
[9] Somboonwiwat K, Marcos M, Tassanakajon A, Klinbunga S, Aumelas A, Romestand B, et al. Recombinant expression and anti-microbial activity of anti-lipopolysaccharide factor (ALF) from the black tiger shrimp *Penaeus monodon*. Dev Com Immunol 2005;29:841–51.
[10] Liu H, Jiravanichpaisal P, Söderhäll I, Cerenius L, Söderhäll K. Anti-lipopolysaccharide factor interferes with white spot syndrome virus replication in vitro and in vivo in the crayfish *Pacifastacus leniusculus*. J Virol 2006;80:10365–71.
[11] Tharntada S, Ponprateep S, Somboonwiwat K, Liu H, Söderhäll I, Söderhäll K, et al. Role of anti-lipopolysaccharide factor from the black tiger shrimp, *Penaeus monodon*, in protection from white spot syndrome virus infection. J Gen Virol 2009;90:1491–8.
[12] Prapavorarat A, Pongsomboon S, Tassanakajon A. Identification of genes expressed in response to yellow head virus infection in the black tiger shrimp, Penaeus monodon, by suppression subtractive hybridization. Dev Comp Immunol 2010;34:611–7.
[13] Chenna R, Sugawara H, Koike T, Lopez R, Gibson TJ, Higgins DG, et al. Multiple sequence alignment with the Clustal series of programs. Nucleic Acids Res 2003;31:3497–500.
[14] Tamura K, Dudley J, Nei M, Kumar S. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol Biol Evol 2007;24:1596–9.
[15] Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 2001;29:e45.
[16] Liu H, Jiravanichpaisal P, Cerenius L, Bok LL, Söderhäll I, Söderhäll K. Phenoloxidase is an important component of the defense against *Aeromonas hydrophila* infection in a crustacean, *Pacifastacus leniusculus*. J Biol Chem 2007;282:33593–8.
[17] Xie X, Li H, Xu L, Yang F. A simple and efficient method for purification of intact white spot syndrome virus (WSSV) viral particles. Virus Res 2005;108:63–7.
[18] Somboonwiwat K, Bachère E, Rimphanitchayakit V, Tassanakajon A. Localization of anti-lipopolysaccharide factor (ALF*Pm*3) in tissues of the black tiger shrimp, *Penaeus monodon*, and characterization of its binding properties. Dev Comp Immunol 2008;32:1170–6.
[19] Tanaka S, Nakamura T, Morita T, Iwanaga S. Limulus anti-LPS factor: an anticoagulant which inhibits the endotoxin mediated activation of Limulus coagulation system. Biochem Biophys Res Commun 1982;105:717–23.
[20] Aketagawa J, Miyata T, Ohtsubo S, Nakamura T, Morita T, Hayashida H, et al. Primary structure of limulus anticoagulant anti-lipopolysaccharide factor. J Biol Chem 1986;261:7357–65.
[21] Muta T, Nakamura T, Furunaka H, Tokunaga F, Miyata T, Niwa M, et al. Primary structures and functions of anti-lipopolysaccharide factor and tachyplesin peptide found in horseshoe crab hemocytes. Adv Exp Med Biol 1990;256:273–85.
[22] Supungul P, Klinbunga S, Pichyangkura R, Hirono I, Aoki T, Tassanakajon A. Antimicrobial peptides discovered in the Black Tiger Shrimp *Penaeus monodon* using the EST approach. Dis Aquat Org 2004;61:123–35.
[23] Imjongjirak C, Amparyup P, Tassanakajon A, Sittipraneed S. Anti-lipopolysaccharide factor (ALF) of mud crab *Scylla paramamosain*: molecular cloning, genomic organization and the antimicrobial activity of its synthetic LPS binding domain. Mol Immunol 2007;44:3195–203.
[24] Imjongjirak C, Amparyup P, Tassanakajon A. Molecular cloning, genomic organization and antibacterial activity of a second isoform of anti-lipopolysaccharide factor (ALF) from the mud crab, *Scylla paramamosain*. Fish Shellfish Immunol 2010;30:58–66.
[25] Li C, Zhao J, Song L, Mu C, Zhang H, Gai Y, et al. Molecular cloning, genomic organization and functional analysis of an anti-lipopolysaccharide factor from Chinese mitten crab *Eriocheir sinensis*. Dev Comp Immunol 2008;32:784–94.
[26] Nagoshi H, Inagawa H, Morii K, Harada H, Kohchi C, Nishizawa T, et al. Cloning and characterization of a LPS-regulatory gene having an LPS binding domain in kuruma prawn *Marsupenaeus japonicus*. Mol Immunol 2006;43:2061–9.
[27] Tharntada S, Somboonwiwat K, Rimphanitchayakit V, Tassanakajon A. Anti-lipopolysaccharide factors from the black tiger shrimp, *Penaeus monodon*, are encoded by two genomic loci. Fish Shellfish Immunol 2008;24:46–54.
[28] Robalino J, Browdy CL, Prior S, Metz A, Parnell P, Gross P, et al. Induction of antiviral immunity by double-stranded RNA in a marine invertebrate. J Virol 2004;78:10442–8.
[29] Mekata T, Sudhakaran R, Okugawa S, Kono T, Sakai M, Itami T. Molecular cloning and transcriptional analysis of a newly identified anti-lipopolysaccharide factor gene in kuruma shrimp, *Marsupenaeus japonicus*. Lett Appl Microbiol 2010;50:112–9.
[30] Ponprateep S, Somboonwiwat K, Tassanakajon A. Recombinant anti-lipopolysaccharide factor isoform 3 and the prevention of vibriosis in the black tiger shrimp, *Penaeus monodon*. Aquaculture 2009;289:219–24.
[31] de la Vega E, O'Leary NA, Shockey JE, Robalino J, Payne C, Browdy CL, et al. Anti-lipopolysaccharide factor in *Litopenaeus vannamei* (*Lv*ALF): a broad spectrum antimicrobial peptide essential for shrimp immunity against bacterial and fungal infection. Mol Immunol 2008;45:1916–25.