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Antimicrobial peptides discovered in the black tiger shrimp *Penaeus monodon* using the EST approach

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ABSTRACT: Two cDNA libraries were prepared from hemocytes of normal and *Vibrio harveyi*-challenged black tiger shrimp *Penaeus monodon*. A total of 1062 expressed sequence tag (EST) clones were sequenced unidirectionally. ESTs representing the antimicrobial peptide (AMP) homologues, antilipopolysaccharide factors (ALF), penaeidins and crustins were discovered. They predominated among immune-related genes, representing 29.2% and 64.0% of the normal and challenged libraries, respectively. Several types of each AMP homologue were found. Sequence alignments of ALF in *P. monodon* (ALF*Pm* 1 to 5) implied possible alternative splicing of different exons at both NH_2 and COOH-termini. Only one major type of penaeidin (pen*Pm*3) was found in *P. monodon*. In addition, crustin homologues (crus*Pm*s1 to 4) and a newly identified glycine-rich antibacterial peptide (GAMP*Pm*1) were also isolated and characterized. Using RT-PCR analysis, expression of ALF, penaeidin and crustin transcripts was detected in various tissues but the main expression site was in hemocytes. Expression of these antimicrobial peptides in *P. monodon* subjected to *V. harveyi* challenge revealed a significant increase in expression of ALF*Pm*s ($p < 0.05$) but a decrease in expression of crustins and penaeidins.

KEY WORDS: Antimicrobial peptides · *Penaeus monodon* · Hemocytes · RT-PCR · EST

INTRODUCTION

Farming of the black tiger shrimp *Penaeus monodon* has been the most important aquacultural activity in Thailand for the last 2 decades. The country has contributed at least 20% of the total global aquacultural production of *P. monodon* annually (Rosenberry 2003). Nevertheless, the production of farmed shrimp is regularly affected by outbreaks of infectious disease caused by bacteria and viruses (Lightner & Redman 1998, Bachere et al. 2000). Genetic selection and breeding programs to improve brood stock performance and seed production of *P. monodon* in Thailand have been conducted for a period of time (Jarayabhand et al. 1998, Withyachumnarnkul et al. 1998). Although growth rate is the desired trait most frequently targeted in penaeid shrimp (Hetzel et al. 2000, Argue et al. 2002, Goyard et al. 2002), selection for disease resistance has also been proposed to overcome low production efficiency associated with disease outbreaks (Bachere et al. 1995, Bachere et al. 2000).

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Efficient strategies for quantitative evaluation and monitoring of immune related genes in *P. monodon* would be useful in future selection of resistance lines.

Host defense in crustaceans and other invertebrates relies on innate, non-adaptive mechanisms (Lemaitre et al. 1997). Antimicrobial peptides (AMPs) are generally small cationic molecules that play an important role in the innate immune defense against bacterial and fungal pathogens (Boman 1995, Hancock & Scott 2000) but some also exhibit antiviral or antiparasitic activity (Murakami et al. 1991, Hancock & Diamond 2000, Pan et al. 2000) and even antitumor activity (Cruciani et al. 1991). AMPs have been isolated from diverse organisms ranging from plants and insects to animals (Bartlett et al. 2002). Their primary structures are highly variable but they share some common features including an amphipathic secondary structure, cationic properties at physiological pH and a broad spectrum of antimicrobial activity (Bulet et al. 1999, Lehrer & Ganz 1999). Most antimicrobial peptides are membrane active agents and are either inducible or constitutive. They kill microbial pathogens by permeating and disrupting the outer cell membrane (Shai 1999, Yeaman & Yount 2003).

Previously, a small basic protein, anti-lipopolysaccharide factor (ALF) exhibiting strong antibacterial activity against Gram-negative bacteria, was isolated and characterized from hemocytes of the horseshoe crab (Morita et al. 1985, Wainwright et al. 1990). More recently, crustacean AMPs including penaeidins, cysteine- and proline-rich antibacterial proteins and astacidin 1 have been isolated and characterized from the white shrimp *Litopenaeus vannamei* (Destoumieux et al. 1997, Bachere et al. 2000), the crab *Carcinus maenas* (Schnapp et al. 1996, Relf et al. 1999) and the crayfish *Pacifastacus leniusculus* (Lee et al. 2002), respectively. Moreover, homologues of a cysteine-rich 11.5 kDa antibacterial peptide crustin from the shore crab were identified in *L. vannamei* and *L. setiferus* while homologues of penaeidin 2 and bacternectin 11 were identified in the kuruma shrimp *Marsupenaeus japonicus* using the expressed sequence tag (EST) approach (Bartlett et al. 2002).

We established cDNA libraries from hemocytes of normal and of *Vibrio harveyi*-challenged *Penaeus monodon*. Several immune-related genes were identified, including AMP homologues of ALF, penaeidins and crustins. The EST sequences were analyzed and compared to those reported in other species. Tissue distribution and expression of these AMP transcripts were examined in response to *V. harveyi* challenge with *P. monodon*.

MATERIALS AND METHODS

Experimental shrimp, hemocyte collection and total RNA preparation. *Penaeus monodon* juveniles (16 to 20 g body weight) were purchased from local farms in Chachoengsao, eastern Thailand and divided to 2 groups: normal and challenged shrimp. *Vibrio harveyi* 1526 (provided by Shrimp Culture Research Center, Charoenpokaphand Group of Companies, Thailand) was cultured in tryptic soy broth with 1% (w/v) NaCl at 30°C for 8 to 10 h and diluted 1:100 with sterile normal saline solution [0.8% (w/v) NaCl]. The number of bacterial cells ml^{-1} present in the inoculum was determined by plate count method in tryptic soy agar with 1% (w/v) NaCl (modified from Austin 1988). A known quantity (100 µl) of the inoculum (10^6 cfu ml^{-1}) were injected intramuscularly into the 4th ventral abdominal segment of each challenged shrimp.

For construction of cDNA libraries, approximately 10 ml hemolymph from 10 normal and 10 challenged shrimp (48 h post-infection) was collected individually in an anticoagulant solution of 10% (w/v) of trisodium citrate dihydrate adjusted to pH 7.0 by addition of citric acid. Hemocytes were separated from the plasma of each shrimp by centrifugation at 800 × *g* for 10 min at room temperature. Hemocytes from each group were pooled and total RNA was immediately extracted using TRIZOL reagent (GIBCO BRL). Extracted RNA was kept in 75% ethanol at –80°C until needed.

To determine tissue-specific expression of AMP transcripts, total RNA was isolated from hemocytes, hearts, gills, lymphoid organs, intestines and hepatopancreata of freshly killed unchallenged juvenile *Penaeus monodon*. For a time-course study of mRNA expression 25 juvenile *P. monodon* were injected with *Vibrio harveyi* (10^5 cfu). Approximately 5 ml hemolymph was collected from 5 individuals and pooled at 3, 6, 12, 24 and 48 h post-injection. Unchallenged shrimp (5) were used as the control. This time-course experiment was repeated 3 times.

Construction of cDNA libraries and EST analysis. Two cDNA libraries were constructed, 1 from hemocytes of normal shrimp (Supungul et al. 2002) and 1 from *Vibrio harveyi*-challenged shrimp (Supungul 2002). Insert sizes of recombinant clones were verified by colony PCR or by digestion of extracted plasmids (Supungul 2002). EST clones containing insert sizes > 500 bp were randomly selected and partially sequenced unidirectionally using a Thermo Sequenase Fluorescent Labeled Primer Sequencing Kit (Amersham Pharmacia Biotech) with M13 forward (5′-CAC GAC GTT GTA AAA CGA C-3′) or reverse (5′-GGA CAA CAA TTT CAC ACA GG-3′) primers on an automated DNA sequencer LC4000 (LICOR).

Semiquantitative RT-PCR and tissue-specific expression analysis. The first-stranded cDNA was synthesized from total RNA of hemocytes using an AMV Reverse Transcriptase First-Strand cDNA Synthesis Kit (Life Sciences). Semi-quantitative RT-PCR was carried out in a 25 µl reaction volume containing 10 mM Tris-HCl, pH 8.8, 50 mM KCl, 0.1% Triton X-100, an appropriate $MgCl_2$ concentration (1.5, 2.5 and 4 mM for crustins, penaeidins and ALF, respectively), 0.2 mM of each dNTP, 50 ng of the first-stranded cDNA template, 1 U of Dynazyme™ II DNA polymerase and 0.5 M of each primer. Primers for amplification of ALF was designed based on the sequences of ALF*Pm*3 whereas those of penaeidin can amplify all penaeidin variants. Primers for amplification of crustin was designed based on the sequences of a complete ORF of Crus*Pm*1. Primers were 5′-CGC CAG CAA GAT CGT AGG GTT G-3′ and 5′-AGG CCT ATG AGC TGA GCC ACT G-3′ for ALF, 5′-AGG ATA TCA TCC AGT TCC TG-3′ and 5′-ACC TAC ATC CTT TCC ACA AG-3′ for penaeidins and 5′-TCC CTG GAG GTC AAT TGA GTG-3′ and 5′-AGT CGA ACA TGC AGG CCT ATC C-3′ for crustins, while primers 5′-GCT TGC TGA TCC ACA TCT GCT-3′ and 5′-ACT ACC ATC GGC AAC GAG A-3 served for the β-actin internal control. Semiquantitative RT-PCR was standardized according to Marone et al. 2001. PCR was initially performed by pre-denaturation at 94°C for 1 min followed by 24 cycles (ALF and crustins) or 27 cycles (penaeidins) of 94°C denaturation for 30 s, 53°C annealing for 30 s and 72°C extension for 1 min. A final extension was carried out at 72°C for 5 min. Amplicons were electrophoretically analyzed through 1.4% agarose gels.

For tissue-specific expression, RT-PCR of each transcript was carried out for 40 cycles to reach a plateau level of amplification using comparable conditions as those described above. An 18S rRNA gene amplicon was included as a positive control (primers 5′-GAG ACG GCT ACC ACA TCT AAG-3′ and 5′-ATA CGC TAG TGG AGC TGG A-3′). Five µl of each PCR sample were size-fractionated through a 1.5% gel and visualized with a UV transilluminator after ethidium bromide staining.

Data analysis. EST sequences were compared with data at GenBank using BLASTN and BLASTX (Altschul et al. 1997, available at www.ncbi.nlm.nih.gov). Significant probabilities and numbers of matched nucleotides/ proteins were considered when E-values were $<10^{-4}$ and the match included >100 nucleotides for BLASTN or >10 amino acid residues for BLASTX. Deduced amino acid sequences of ALF, crustin and penaeidin homologues were translated using GENETYX and multiple alignments were carried out using CLUSTALX (Thompson et al. 1997). Aligned sequences were bootstrapped 1000 times using SEQBOOT. Sequence divergence between pairs of deduced protein sequences was calculated using PRODIST. A bootstrapped neighbor-joining tree (Saitou & Nei 1987) was constructed to illustrate the relationship among different sequences of each AMP using NEIGHBOR and CONSENSE. All phylogenetic programs described were routine in PHYLIP (Felsenstein 1993).

The AMP expression level in response to bacterial challenge was determined by a semi-quantitative RT-PCR. The AMP transcript and the internal control (β-actin) were amplified in the same reaction tube and the PCR product was semi-quantitatively analyzed at the exponential phase of amplification (Marone et al. 2001). The ratio between the intensity of electrophoresed target and β-actin gene products was recorded using a gel documentation system (GeneCam FLEX1, SynGene) and further quantified by Genetool Analysis Software. The time-course of expression for each transcript was normalized with that of β-actin (i.e. expression of β-actin at each interval was 100) and tested using 1-way analysis of variance (ANOVA) (Steel & Torrie 1980) followed by Duncan's new multiple range test (Duncan 1955) using SPSS software.

RESULTS AND DISCUSSION

Identification and characterization of genes encoding antimicrobial peptides in *Penaeus monodon*

A total of 1062 clones from cDNA libraries established from hemocytes of normal (615 clones) and *Vibrio harveyi*-challenged *Penaeus monodon* (447 clones) were analyzed (Supungul 2002, Supungul et al. 2002). One hundred and fifteen clones (10.8%) corresponded to 30 different genes identified as putative immune-related genes (Table 1). ESTs homologues of AMP (ALF, crustins, penaeidins and lysozymes) were the most abundant, representing 29.2% and 64.0% of the putative immune genes found in the normal and *V. harveyi*-challenged libraries, respectively (Table 1). Penaeidins predominated among AMP in the normal library (36.84%) followed by crustins and ALF (26.3% each). In contrast, ALF predominated (50.0%) in the challenged library while crustins (25.0%) and penaeidins (18.8%) were found at relatively lower levels. The abundance of these AMP in hemocytes of infected *P. monodon* particularly, suggested that they played a major role in host-defense against bacterial infection. In ESTs of cDNA libraries from hemocytes of normal *Litopenaeus vannamei* and *L. setiferus*, Gross et al. (2001) found that 14.3% and 26.6%, respectively, encoded immune-related genes amongst which AMP were the most abundant (86.1% in *L. vannamei* and 80.7% in *L. setiferus*). Penaeidins predominated (75.0% in *L. vannamei* and 75.2% in *L. setiferus*) and

Table 1. *Penaeus monodon*. Immune related genes isolated from haemocytes of normal and *Vibrio harveyi*-challenged shrimp

Putative identification	Closest species	Probability	Matched (%)	Sequence length (bp)	Frequency (normal shrimp)	Frequency (challenged shrimp)	Total clones
1. Antimicrobial molecules[a]							
antilipopolysaccharide factor	*Limulus polyphemus*	7.00E-19	64	512	5	16	21
Crustin	*Litopenaeus setiferus*	1.00E-42	65	515	5	8	13
penaeidin3a	*Litopenaeus vannamei*	1.00E-18	66	412	7	6	13
lysozyme	*Mus musculus*	2.00E-26	67	541	2	2	4
2. ProPO systems and oxidative enzyme[b]							
clottable protein	*Penaeus monodon*	E-130	98	458	3	3	6
cytosolic manganese superoxide dismutase precursor	*Callinectes sapidus*	E-129	86	930	–	1	1
glutathione peroxidase	*Homo sapiens*	3.00E-18	76	331	1	–	1
transglutaminase	*Tachypleus tridentatus*	2.00E-48	76	746	4	–	4
Peroxidase	*Aedes aegypti*	1.00E-21	52	747	1	–	1
prophenoloxidase	*Penaeus monodon*	1.00E-51	98	465	1	–	1
prophenoloxidase activating factor	*Holotrichia diomphalia*	7.00E-18	70	550	1	2	3
Catalase	*Campylobacter jejuni*	3.00E-09	93	575	1	–	1
3. Proteinases and inhibitor[c]							
Kazal-type serine proteinase inhibitor	*Pacifastacus leniusculus*	5.00E-31	60	503	8	4	12
hemocyte protease-1	*Manduca Sexta*	1.00E-12	57	700	1	–	1
cathepsin B-like cysteine proteinase	*Sarcophaga perregrina*	6.00E-18	88	659	1	–	1
serine protease	*Pacifastacus leniusculus*	4.00E-79	79		1	1	2
Whey acidic protein	*Trichosurus vulpecula*	5.00E-06	48	712	1	1	2
4. Heat shock protein[d]							
heat shock protein 10	*Gallus gallus*	2.00E-25	73	665	1	–	1
heat shock cognate 70	*Oncorhynchus mykiss*	9.00E-95	76	772	1	1	2
heat shock protein 70	*Hydra magripapillata*	2.00E-56	90	806	1	1	2
heat shock protein 90	*Gallus gallus*	9.00E-27	70	618	2	–	2
5. Other immune molecules[e]							
Fc fragment of IgE	*Homo sapiens*	5.00E-07	67	533	–	1	1
protein c	*Mus musculus*	3.00E-10	63	708	1	–	1
protein-kinase c inhibitor	*Bos taurus*	2.00E-38	78	756	1	–	1
thymosin beta-9 and beta 8	*Bos taurus*	2.00E04	77	706	2	–	2
thymosin beta-11	*Oncorhynchus mykiss*	4.00E-27	64	872	3	–	3
perlucin	*Haliotis cuniculus*	7.00E-16	54	575	2	–	2
cyclophilin 18	*Oryctolagus cuniculus*	8.00e-58	72	756	1	–	1
chaperonin containing t-complex polypeptide 1	*Homo sapiens*	9.00E-67	77	637	3	–	3
peptide-prolyl cis-trans isomer 5	*Drosophila melanogaster*	3.00E-68	81	816	4	3	7

[a]GenBank sequences BI784448, BI784449, BI018071, BI784451, CF415871, CD766060, BI784444, BI784445, CF415873, BI784446, BI784459, BI784441, BI018079, BI018081
[b]GenBank sequences CF415874, BI784454, BI018091, BI018082, BI018083, BI018084, BI018093, BI018092, BI018090, BI018087, BI784455, BI018085
[c]GenBank sequences BI784456, BI018098, BI018075, BI018078, CF415875, CF415876, CF415872, BI784457
[d]GenBank sequences BI018100, BI018094, BI784458, BI018097, BI784452, BI018095, BI018096
[e]GenBank sequences BI784442, BI018099, BI018088, BI018086, CF415877, CF415878, CF415879, CF415880, CF415881

mainly penaeidin 3. Crustins were the second most common AMP but comprised only 9.7% in *L. vannamei* and 4.1% in *L. setiferus*. Two ALF homologues were found in *L. setiferus* but none in *L. vannamei*. In contrast, EST of penaeidin 2 precursor, lysozyme type c and bactinectin were found in normal and WSSV-challenged cDNA libraries of *Marsupenaeus japonicus* hemocytes (Rojtinnakorn et al. 2002).

Multiple genes encoding ALF in *Penaeus monodon* and possible alternative splicing

ALF, has been reported from hemocytes of horseshoe crabs *Limulus polyphemus* and *Tachypteus tridentatus* (Morita et al. 1985, Aketagawa et al. 1986). They bind and neutralize lipopolysaccharides and have strong antibacterial effects on Gram negative R-type bacteria (Wainwright et al. 1990).

Twenty one EST representing ALF were identified in *Penaeus monodon*. Of these, 17 contained complete open reading frames (ORF) of 252, 360, 369, 396 and 465 bp encoding 84, 120, 123, 132 and 155 deduced amino acids, respectively. These EST exhibited 57% to 65% amino acid homology with those of the horseshoe crab *Tachypleus tridentatus* and *Limulus polyphemus*. Sequence alignment revealed that at least 5 different types — ALF*Pm*1 to ALF*Pm*5, with accession numbers BI784448, BI784449, BI018071, BI784451 and CF415871, respectively — exist in *P. monodon* hemocytes (Fig. 1). These clones were re-sequenced 2 to 3 times to ensure accuracy of their nucleotide sequences.

ALF*Pm*3 predominated (13 clones) and was found in both cDNA libraries. Other ALF (ALF*Pm*s1, 2, 4 and 5) were found only in the hemocyte library of challenged *Penaeus monodon*. A putative signal peptide of ALF*Pm* 1, 2, 3 and 5 was located at the NH_2-terminus between Ala/Gly (A/G) and Gln (Q). However, this putative cleavage site was not found in ALF*Pm*4. All ALF*Pm* contain 2 cysteine residues bracketing somewhat conserved amino acids within the loop that would result from their disulfide linkage (**C**RYSQRPSFYRWELYFNGRMW**C** for ALF*Pm*s1 and 2 and **C**KFTVKPYLKRFQVYYKGRMW**C** for ALF*Pm*s3 to 5) (Fig. 1.). These differed from ALF in *Limulus polyphemus* and *Tachypteus tridentatus* [**C**HYR(V/I)(N/K)PT(V/F)(K/R)RLKWKY-KG-KFW**C**] (Fig. 1). This segment of the protein forms the basic face in the 3-dimensional structure of ALF*Tt* that was proposed to play an important role in Lipid A binding (Hoess et al. 1993). Although ALF*Pm* lacked some of the positively charged residues present in ALF*Tt*, 5 residues in ALF*Pm*1 to 2 and 3 residues in ALF*Pm*3 to 5, the arginine residue at the center of the segment and other positively charged residues were conserved. Moreover, we found that most of the bulky hydrophobic residues were conserved, and this implied a crucial structural role in this domain.

The N-terminus of ALF*Pm*1 to 2, ALF*Pm*3 to 5, and ALF of the horseshoe crabs were found to be divergent in their amino acid sequences. However, they all contained conserved hydrophobic amino acid residues at common positions along the primary structure. These residues formed a conserved hydrophobic face on the amphipathic β-helix 1 of ALF (Hoess et al. 1993), that may be involved in the membrane insertion ability of ALF. Nevertheless, heterogeneous ALF*Pm* were found in *Penaeus monodon* hemocytes.

Pairwise alignment of ALF*Pm* indicated 100% amino acid sequence homology between ALF*Pm*1 and ALF*Pm*2 (residues 1 to 82). As a result, ALF*Pm*1 should be an allelic

Fig. 1. Multiple alignments of ALF homologues in *Penaeus monodon* (ALF*Pm*1 to 5) and those of *Limulus polyphemus* (ALF*Lp*1 and 2) and *Tachypleus tridentatus* (ALF*Tt*) (Aketagawa et al. 1986, Muta et al. 1987). * indicates amino acid identity and · indicates amino acid similarity. The putative signal peptides and a cleavage site in ALF-*Pm*s1, 2, 3 and 5 are underlined and indicated by an arrowhead, respectively. The boxes indicate cysteine residues

variant of ALF*Pm*2 (Fig. 2A). In addition, perfect homology was observed for the NH_2 terminal sequences (residues 1 to 79) of ALF*Pm*3 to 5 (Fig. 2B–D) although polymorphism was found at the COOH terminus. On the other hand, ALF*Pm*3 and ALF*Pm*4 perfectly shared the COOH-terminal portion (residues 51 to 88). ALF*Pm*3 to 5 shared a core sequence of LWRNEKTELLGHECK-FTVKPYLKRFQVYYKGRMWCPGW but this sequence stretch was not found in ALF*Pm*1 and 2, indicating that ALF*Pm* may have been encoded from more than 1 locus. Polymorphism found among ALF*Pm*3 to 5 ESTs may have resulted from alternative splicing of different exons in the NH_2 and COOH-terminal regions of ALF in *Penaeus monodon*.

Sequence divergences between different ALF*Pm* were 0.0330 (ALF*Pm*1 and ALF*Pm*2) to 1.6257 (ALF*Pm*2 and ALF*Pm*5). Phylogenetic analysis indicated large genetic differences between ALF*Pm* and their outgroups from *Limulus polyphemus* and *Tachypleus tridentatus* (Fig. 2E). At the intraspecific level, ALF*Pm*s1 to 5 can be phylogenetically allocated to 2 different groups: A (ALF*Pm*1 and ALF*Pm*2) and B (ALF*Pm*s3 to 5), where the molecular evolution from AFL*Pm*4 to ALF*Pm*5 (or vice versa) required contribution of ALF*Pm*3 (Fig. 2E). This supports the hypothesis that ALF*Pm* does not arise from a single copy nuclear gene but may be encoded by 2 different loci. Nevertheless, antibacterial peptides are typically encoded by single-copy genes (Hancock & Diamond 2000). As a result, organization of ALF*Pm* genes should be further characterized at the genomic level.

One major penaeidin EST found in normal and challenged *Penaeus monodon*

Penaeidins are a unique family of AMP previously reported in *Litopenaeus vannamei*. They exhibit anti-Gram-positive and antifungal activities (Destoumieux et al. 1999). Ten of 13 penaeidin ESTs isolated from *Penaeus monodon* hemocyte cDNA (Table 1) contain complete ORF. The deduced amino acid sequences suggested the existence of 3 penaeidin variants in *P. monodon* (pen*Pm*). These variants contained an identical ORF of 225 bp coding for a 74 amino acid protein with 2 single amino acid variations at residues 34 and 64 (Fig. 3A). They were regarded as homologues (pen*Pm*3a, 3b and 3c, GenBank accession numbers BI784459, BI784441 and BI018079, respectively) of penaeidin3 of *L. vannamei* (pen*Lv*3a) with 48 to 50% sequence similarity. However, they did not contain a conserved chitin binding domain (C-X5-C-C-S-X2-G-X-CG-X5-C) as found in their homologue (Destoumieux et al. 2000) and common to some plant chitin-binding proteins (Raikhel et al. 1993). This suggested that pen*Pm*3a, 3b and 3c may not posses antifungal activity.

A putative signal peptide was predicted at the NH_2-terminus comprising 19 amino acid residues that were almost identical to those of *Litopenaeus vannamei* (Fig. 3A). A proline-rich region was also found, containing a conserved PRP motif and 6 cysteine residues at the COOH-terminal region as typically observed in *L. vannamei* (Destoumieux et al. 1997) and insects (Hetru et al. 1998). Pairwise sequence divergence between pen*Pm*3a, 3b and 3c was 0.0123 to 0.0276. The bootstrapped neighbor-joining tree supports the classification of pen*Pm*3a, 3b and 3c as allelic variants rather than different isoforms (Fig. 3B).

Crustins and a newly isolated Gly-rich antibacterial peptide

Crustin was initially isolated and characterized in the shore crab *Carcinus maenas* as an 11.5 kDa antibacterial peptide potentially acting against Gram positive bacteria (Relf et al. 1999). Recently, 6 and 3 complete ORF of crustins were identified from hemocyte cDNA libraries of *Litopenaeus vannamei* and *L. setiferus*, respectively (Bartlett et al. 2002).

Thirteen *Penaeus monodon* hemocyte clones (5 clones from normal shrimp and 8 clones from challenged shrimp, GenBank accession numbers CD766060, BI784444, BI784445, CF415873 and BI784446) significantly matched crustins of other crustaceans (Table 1). Two contained an identical, complete ORF of 435 bp encoding a putative protein of 145 amino acid residues (crus*Pm*1, accession no. CD766060) that showed 65% homology to a crustin of *Litopenaeus sertiferus* (crus*Ls*, Fig. 4A). In addition, 3 different sequences (crus*Pm*2 to 4) showed Gly-rich and Pro/Cys-rich sequences at the NH_2 and COOH-termini. Alignment of these ESTs with those from *L. vannamei* (crus*Lv*) and *L. setiferus* (crus*Ls*1 and crus*Ls*2) revealed 12 conserved Cys residues and a consensus pattern of C1-X3-C2-X8-C3-C4-X16-C5-X5/6-C6-X9/11/12/13-C7-X5-C8-X5-C9-C10-X3-C11-X5-C12 where X is any amino acid residue and Xn is a stretch of n residues.

An additional EST (called GAMP*Pm*1, GenBank accession no. BI018079) significantly matched with crustins of *Litopenaeus vannamei* (E value = 6×10^{-9}) and exhibited 9 conserved Cys residues (C3 to C12) following the consensus sequences of crustin homologues and a whey acidic protein (WAP) domain (conserved C5 to C12 forming 4 disulfide core, 4DSC). However, C1, C2 and C3 were replaced with Val, Gly and Glu, respectively. Therefore, it was not regarded as a member of the crustin group. Generally, proteins containing the WAP

A

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ALFPm2          MRVLVSFLMALSLIALMPRCQGQGVQDLLPALVEKIAGLWHSDEVEFLGHSCRYSQRPSF
ALFPm1          MRVLVSFLMALSLIALMPRCQGQGVQDLLPALVEKIAGLWHSDEVEFLGHSCRYSQRPSF
                ************************************************************

ALFPm2          YRWELYFNGRMWCPGWAPFTGRSRTRSPSGAIEHATRDFVQKALQSNLITEEDARIWLEH
ALFPm1          YRWELYFNGRMWCPGWAPFTGRCE-----------------------------------
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B

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ALFPm4          MYLSSYLISLTVTVLVKYHSSFSPSLFLCHFFLIPRLHFSNLFVRSPPTRLWRNEKTELL
ALFPm3          ---------MRVSVLVSLVLVVSLVALFAPQCQAQGWEAVAAAVASKIVGLWRNEKTELL
                         : *:***.    .*   ::.        .     * *  . **********

ALFPm4          GHECKFTVKPYLKRFQVYYKGRMWCPGWTAIRGEASTRSQSGVAGKTAKDFVRKAFQKGL
ALFPm3          GHECKFTVKPYLKRFQVYYKGRMWCPGWTAIRGEASTRSQSGVAGKTAKDFVRKAFQKGL
                ************************************************************

ALFPm4          ISQQEANQWLSS
ALFPm3          ISQQEANQWLSS
                ************
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C

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ALFPm5          MRVSVLVSLVLVVSLVAVFAPQCQAQGWEAVAAAVASKIVGLWRNEKTELLGHECKFTVK
ALFPm3          MRVSVLVSLVLVVSLVALFAPQCQAQGWEAVAAAVASKIVGLWRNEKTELLGHECKFTVK
                ****************:*******************************************

ALFPm5          PYLKRFQVYYKGRMWCPGWDGHQRRSQHTQSVRGSWKDSQRLRSESFPERSHLSTGGQPV
ALFPm3          PYLKRFQVYYKGRMWCPGWTAIRGEASTRSQSGVAGKTAKDFVRKAFQKGLISQQEANQW
                ****************** . : .:.  ..   : * :: :  ::* :    .  .:

ALFPm5          AQLIGLLLYEELSVFSCSWQWKLYHFDFLCFSFQY
ALFPm3          LSS--------------------------------
                 .
```

D

```
ALFPm5          ---------MRVSVLVSLVLVVSLVAVFAPQCQAQGWEAVAAAVASKIVGLWRNEKTELL
ALFPm4          MYLSSYLISLTVTVLVKYHSSFSPSLFLCHFFLIPRLHFSNLFVRSPPTRLWRNEKTELL
                         : *:***.    .*   .:.        .     * *  . **********

ALFPm5          GHECKFTVKPYLKRFQVYYKGRMWCPGWDGHQRRSQHTQSVRGSWKDSQRLRSESFPERS
ALFPm4          GHECKFTVKPYLKRFQVYYKGRMWCPGWTAIR--------------------GEASTRS
                ************************** . :                     . . **

ALFPm5          HLSTGGQPVAQLIGLLLYEELSVFSCSWQWKLYHFDFLCFSFQY
ALFPm4          QSGVAGKTAKDFVRKAFQKGLISQQEANQWLSS-----------
                : ...*:.. :::   : : *   . : **
```

E

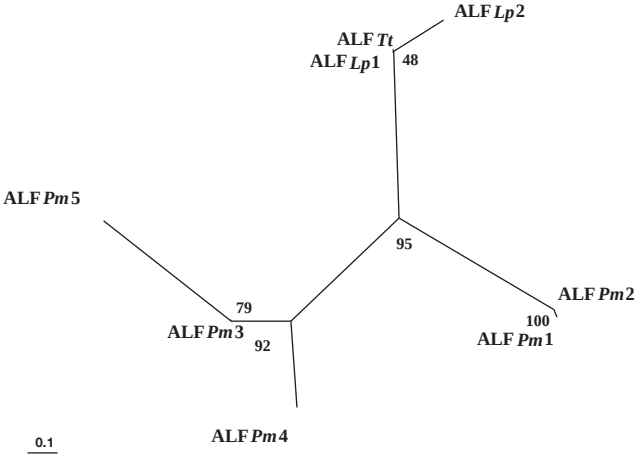

Fig. 2. Pairwise alignments of deduced amino acid sequences of ALF*Pm*1 to 2 (A), ALF*Pm*3 to 4 (B), ALF*Pm*3 to 5 (C) and ALF*Pm*4 to 5 (D). * indicates amino acid identity and · indicates amino acid similarity. A conserved core region (bold-italics) was found in ALF*Pm*3, 4 and 5 but not in ALF*Pm*1 and 2 (Aketagawa et al. 1986, Muta et al. 1987). A bootstrapped neighbor-joining tree summarizing relationships of ALF*Pm*, ALF*Lp*1, ALF*Lp*2 and ALF*Tt* is illustrated (E). Values at the node represent the percentage of times that the particular node occurred in 1000 trees generated from bootstrapping of the original amino acid sequences (*Lp = Limulus polyphemus*, *Tt = Tachypleus tridentratus Pm = Penaeus monodon*)

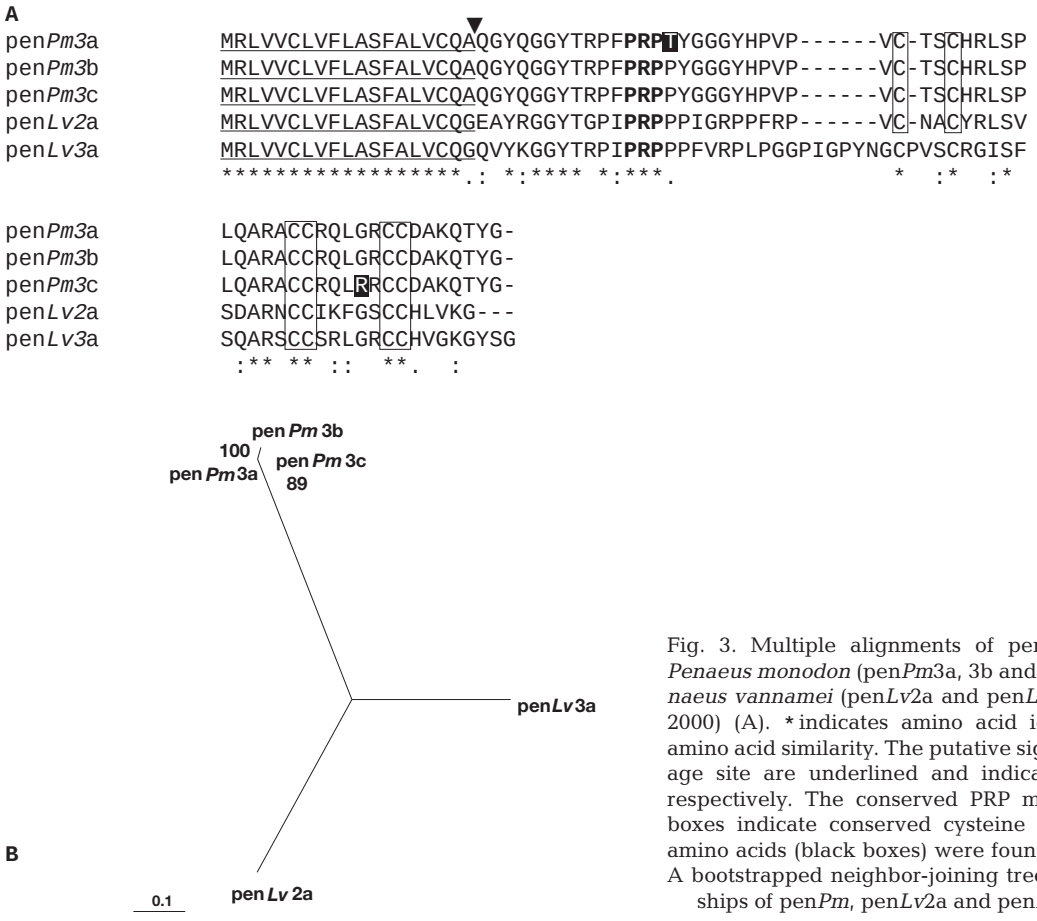

Fig. 3. Multiple alignments of penaeidin homologues in *Penaeus monodon* (pen*Pm*3a, 3b and 3c) and those in *Litopenaeus vannamei* (pen*Lv*2a and pen*Lv*3a, Destoumieux et al. 2000) (A). * indicates amino acid identity and · indicates amino acid similarity. The putative signal peptides and cleavage site are underlined and indicated by an arrowhead, respectively. The conserved PRP motifs are bold and the boxes indicate conserved cysteine residues. The different amino acids (black boxes) were found at residues 34 and 64. A bootstrapped neighbor-joining tree summarizing relationships of pen*Pm*, pen*Lv*2a and pen*Lv*3a is illustrated (B)

signature domain are primarily secreted proteinase inhibitors (Larsen et al. 1998). Proteinase inhibitors in the hemolymph of shrimp regulate the proteinase initiated host defense cascades (e.g. coagulation and prophenol oxidase or proPO systems) against bacterial and fungal pathogens (Bartlett et al. 2002).

Phylogenetic analysis clearly indicated monophyletic cladding of crustin families born after gene duplication and showed that crustin homologues in *Penaeus monodon* fell into 2 different groups called A (crus*Pm*1 and 2 that were closely related to crus*Ls*1) and B (crus*Pm*3 and 4 that were closely related to crus*Lv* and crus*Ls*2). The gene tree also confirmed a distant relationship between crus*Pm*1 to 4 and GAMP*Pm*1 (Fig. 4B).

Tissue distribution and expression of AMP transcripts in normal and challenged *Penaeus monodon*

Tissue expression patterns for *Penaeus monodon* ALF, penaeidin, and crustin were similar (Fig. 5). They were constitutively expressed at high levels in hemocytes. Lower levels of mRNA expression were found in hearts, gills, intestines and lymphoid organs and no transcript was detected in the hepatopancreas. Results were concordant to those found in other arthropods where hemocytes are the major sites of AMP synthesis (Iwanaga & Kawabata 1998). Secondary expression sites such as intestines or gonads have been found for antibacterial peptides in in-sects (Hoffmann et al. 1997, Manetti et al. 1998). In *Litopenaeus vannamei*, penaeidin mRNAs were also detected mainly in hemocytes (Destoumieux et al. 2000, Munoz et al. 2002).

Comparison of expression levels of *Penaeus monodon* AMP (Table 2) revealed that crustin expression was highest in unchallenged *P. monodon* followed by penaeidins and ALF. Upon challenge with *Vibrio harveyi*, the expression level of ALF*Pm* significantly increased within 3 h post-injection ($p < 0.05$) and returned to normal at 48 h post-injection ($p < 0.05$, Table 2 and Fig. 6A). The highest transcriptional level of ALF*Pm* was observed at 6 h after *V. harveyi* injection (1.6 fold above that of normal shrimp).

In contrast, injection of *Vibrio harveyi* caused significant decreases of crustin and penaeidin expression in

A

```
crusPm2         ----------------------------------------------------RFRHEASQ
crusPm1         ---------------------------------------------------------------
crusPm3         GGGAYGGGLGGGLGGGGVNGGGLGGGLGGGVNGGGLGGGLGGGVNGGGLGGGLGGGVHGG
crusPm4         ----------------------------------------------------------W
crusLs1         ---------------------------------------------------------------
crusLs2         -------------------------------------GKYRGFGQPLGGLGVPGGGVGVG
crusLv          -------------------------------------GKFRGFGRPFGGLGGPGGGVGVG

crusPm2         FTGFRSHSFRKHLAVVSAHGGRPGARPGGFPAGVPGGFPGGVPGEFPAPHLGGFLSVTAP
crusPm1         ------------------QSWHGGRPGGFPGGGRP--GGFPGGGRPGGRPGGFPSVTAP
crusPm3         GLGGGLGGGVNGGGLGGGVHGGGLGGGLGGGLSGGLGGGLGRPGGGLGRPGGGLRPGSRG
crusPm4         RFRGGVNGGGLGGGLGGGVHGGGLGGGLGGGLSGGLGGGLGRPGGGLGRPGGGLRPGSRG
crusLs1         ------------------------GPGGFSGGVPGGFPGGRPGGFPGGVPGGFPSATAP
crusLs2         VGGGLGGGLGGGLGGGLGGGLGGGLGGLGGGLGGLGGGLGGGLGGGLGGGLGGGLGGSHG
crusLv          GG-----------------------------------FPGGGLGVGGGLGVGGGLGTG
                                                       *   .   *

crusPm2         PATCRRWCRTPEDAVYCCESKYEPEAPVGTKPLDCPRVRDTCPPVRFGGLAP-VTCSSDL
crusPm1         PASCRRWCETPENAFYCCESRYEPEAPVGTKILDCPKVRDTCPPVRFLAVEQPVPCSSDY
crusPm3         PSTCRYWCTTPEGKQYCCEDKNEPEIPVGTKPLDCPQVR-TCPRFQGPP----VTCSHDF
crusPm4         PSTCRYWCTTPEGKQYCCEDKNEPEIPVGTKPLDCPQVRPTCPRFQGPP----VTCSHDF
crusLs1         PATCRRWCKTPENQAYCCETIFEPEAPVGTKPLDCPQVRPTCPPTRFG--GRPVTCSSDY
crusLs2         TSDCRYWCKTPEGQAYCCESAHEPETPVGTKPLDCPQVRPTCPRFHGPP----TTCSNDY
crusLv          TSDCRYWCKTPEGQAYCCESAHEPETPVGTKPLDCPQVRPTCPRFHGPP----TTCSNDY
                .: ** ** ***.  ****   *** ***** ****:** ***  :       ..** *

crusPm2         KCGGLDKCCFDRCLKEHVCKPPSFYSHFA--
crusPm1         KCGGLDKCCFDRCLGQHVCKPPSFYEFFA--
crusPm3         KCAGLDKCCFDRCLGEHVCKPPSFYGRNVKG
crusPm4         KCAGLDKCCFDRCLGEHVCKPPSFYGRNVKG
crusLs1         KCGGLDKCCFDRCLGEHVCKPPSFYSQFR--
crusLs2         KCAGLDKCCFDRCLGEHVCKPPSFFGQQIFG
crusLv          KCAGLDKCCFDRCLGEHVCKPPSFFGSQVFG
                **.*********** :********:
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B

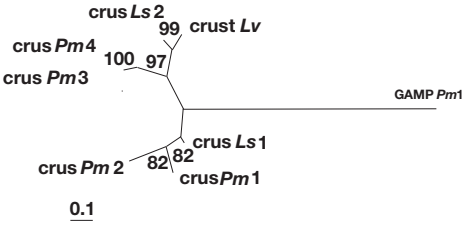

Fig. 4. Multiple alignments of crustin homologues in *Penaeus monodon* (crus*Pm*s1 to 4) and those in *Litopenaeus vannamei* (crus*Lv*) and *L. setiferus* (crus*Ls*1 to 2, Bartlett et al. 2002). * indicates amino acid identity and · indicates amino acid similarity. Twelve conserved Cys residues (boxes) were found in the carboxy-terminal region of each putative crustin EST. A bootstrapped neighbor-joining tree summarizing relationships between crus*Pm*, crus*Ls*1, crus*Ls*2, crus*Lv* and a Glyrich antimicrobial peptide GAMP*Pm*1 is illustrated (B)

challenged *Penaeus monodon* within 3 h after injection of *V. harveyi* ($p < 0.05$, Table 2, Fig. 6B,C). The lowest expression level of crus*Pm* and pen*Pm* was observed at 6 h (0.16-fold below the normal level) and 12 h (0.35-fold below the normal level), respectively. Following this, crus*Pm* and pen*Pm* levels significantly increased after 24 h post-injection ($p < 0.05$) and were still significantly different from that of unchallenged *P. monodon* at 48 h ($p < 0.05$). Time-course transcriptional levels of proPO and heat shock protein (HSP) 70 were also examined using the same experimental procedures but the transcriptional levels were not significantly different between normal and challenged *P. monodon* throughout the test period ($p > 0.05$, data not shown).

Due to limited facilities, we did not include a control of shrimp injected with normal saline at different time intervals in the first experiment. Therefore, we cannot yet exclude the possibility that the genes expressed after *Vibrio harveyi* injection could possibly be induced by the injection of other materials and even neutral substances such as saline solution. Nonetheless, we performed an additional experiment to compare the expression at different time intervals

(0, 3, 6, 12, 24 and 48 h post injection) of juvenile *Penaeus monodon* injected with 100 µl of normal saline solution (N = 3 for each group) with that of normal shrimp (N = 3) and *V. harveyi*-injected shrimp (0 h post injection, N = 9). No significant differences in expression of AMP transcripts between these groups were observed ($p > 0.05$). Quantitative assay for measuring AMP transcripts by real-time PCR and determination of the number of hemocytes expressing AMP by *in situ* hybridization would be useful for further investigation of changes in AMP expression in response to microbial challenge.

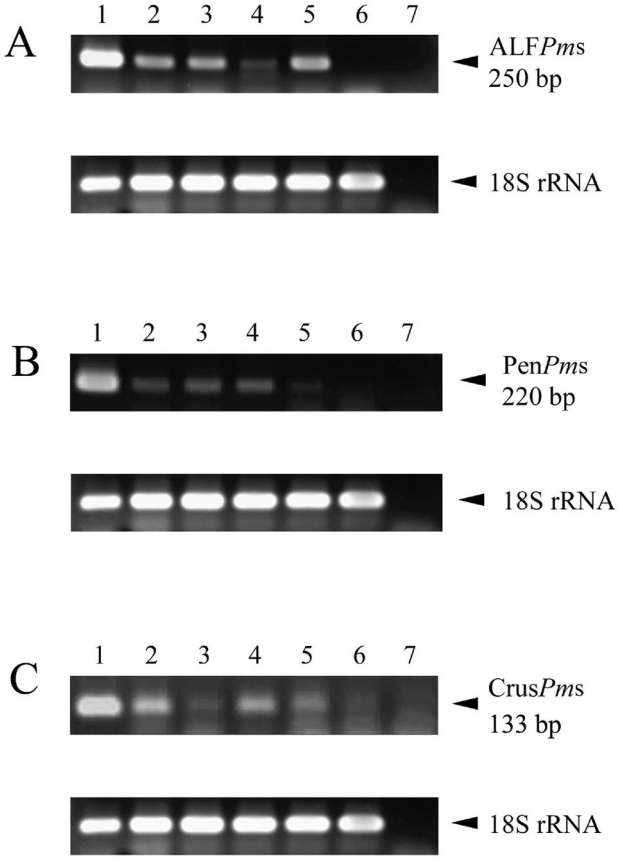

Fig. 5. *Penaeus monodon*. RT-PCR expression analysis of ALF*Pm* (A), pen*Pm* (B) and crus*Pm* (C) in hemocytes, the midgut, heart, gills, lymphoid organ, and hepatopancreas (lanes 1 to 6, respectively). Lane 7 was a negative control with no RNA template. The 18S rRNA gene product was included as control

There have been no reports on transcriptional regulation of ALF and crustins in other penaeid shrimp. However, Destoumieux et al. (2000) studied *in vivo* microbial experimental challenge using heat-killed bacteria (*Aerococcus viridans* and *Vibrio alginolyticus*) and the fungus (*Fusarium oxysporum*) and reported that transcriptional levels of penaeidins in *Litopenaeus vannamei* were not significantly increased by these microorganisms.

Our results suggest that expression of ALF*Pm* was up-regulated whereas the expression of crus*Pm* and pen*Pm* were down-regulated *in vivo* upon injection with the pathogenic Gram-negative bacterium, *Vibrio harveyi*. This may indicate some level of specificity in the innate immune system in *Penaeus monodon* since ALF is active against Gram negative bacteria like *V. harveyi*. Challenge of juvenile *Litopenaeus vannamei* with heat-killed microorganisms resulted in a 4 to 5 fold decrease of penaeidin mRNA levels at 3 h post-injection. This may have resulted from a partial decrease in penaeidin-producing hemocytes from the bloodstream or alternatively, from degranulation and release of penaeidins into the circulatory system (Destoumieux et al. 2000).

EST analysis is a potential approach for isolation and characterization of new AMP genes in *Penaeus monodon* where limited information at the genomic level is presently available. The presence of several isoforms of ALF, crustin and penaeidin ESTs in *P. monodon* reflects the diversity and possible broad spectrum of potential response against various types of microorganisms. Genome organization of these AMP genes should be further studied to clarify whether they are

Table 2. *Penaeus monodon*. A time-course analysis of ALF*Pm*, crus*Pm* and pen*Pm* expression levels using semiquantitative RT-PCR. The same superscripts between different time interval data are not significantly different ($p > 0.05$)

Gene	Relative expression level*					
	0 h	3 h	6 h	12 h	24 h	48 h
ALF*Pm*s	72.67 ± 7.09^{a}	104.00 ± 7.81^{b}	116.33 ± 23.18^{b}	106.67 ± 7.64^{b}	101.33 ± 8.145^{b}	90.67 ± 6.11^{ab}
Crus*Pm*s	252.36 ± 10.54^{a}	78.85 ± 37.55^{bc}	39.61 ± 12.42^{c}	43.62 ± 12.44^{c}	90.57 ± 11.31^{b}	154.92 ± 38.14^{d}
Pen*Pm*s	137.33 ± 17.62^{a}	76.00 ± 12.12^{b}	57.00 ± 11.36^{bc}	48.33 ± 8.74^{c}	79.33 ± 9.29^{b}	107.67 ± 23.01^{d}

*The expression of β-actin was normalized to 100. The expression of ALFs, crustins and penaeidins was determined as the percentage of signal ratio between the target gene and β-actin

encoded by single or multiple loci and to provide an understanding of their regulation in the shrimp immune response.

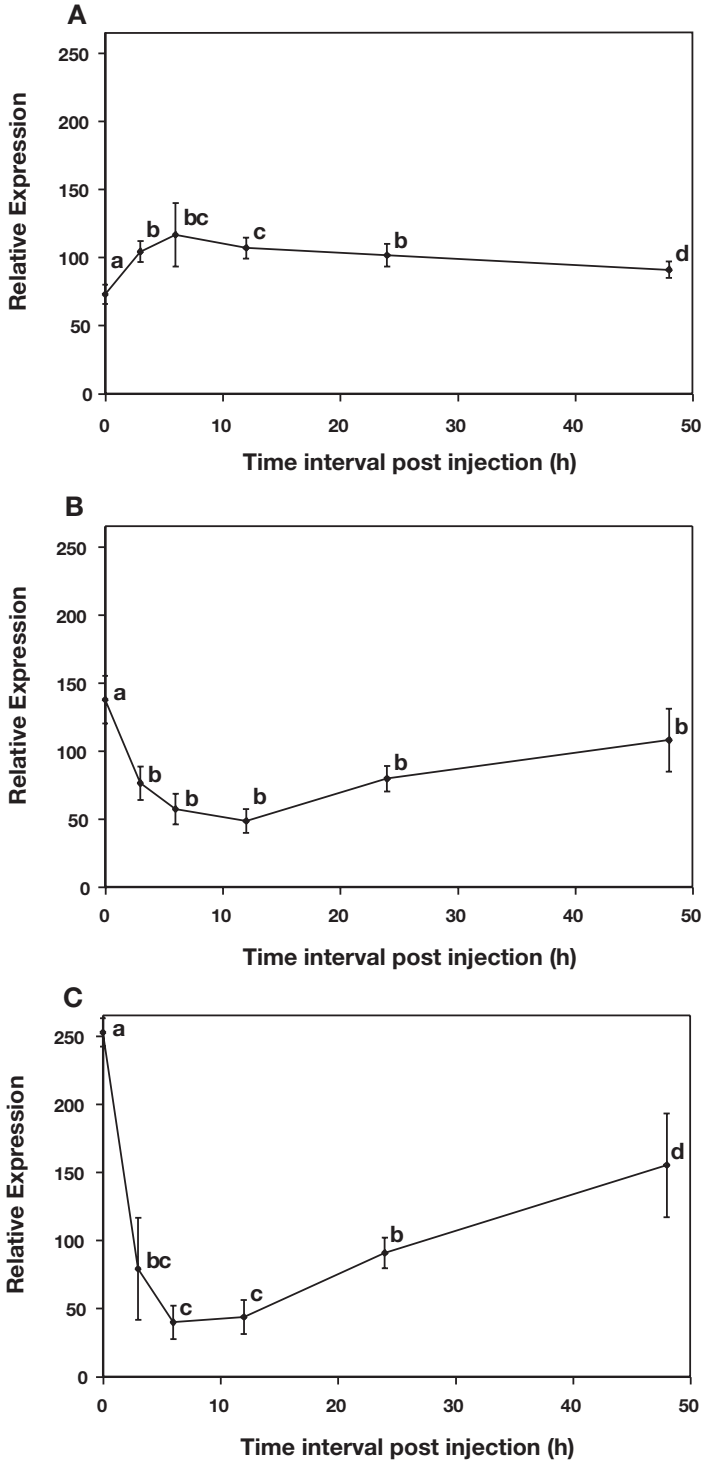

Fig. 6. Relative expression levels of (A) *Penaeus monodon* ALF, (B) penaeidins and (C) crustins at different time intervals after *Vibrio harveyi* challenge. Different letters between time interval data indicate significant differences ($p < 0.05$)

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