Differentially Expressed Genes in Hemocytes of *Vibrio harveyi*-challenged Shrimp *Penaeus monodon*

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Differential Display PCR technique (DD-PCR) was used for the analysis of altered gene expression in hemocytes of *Vibrio harveyi*-infected *Penaeus monodon*. Forty-four combinations of arbitrary and oligo(dT) primers were used to screen for differentially expressed genes. A total of 79 differentially expressed bands could be identified from 33 primer combinations. These included 48 bands (61%) whose expression level increased and 31 bands (39%) decreased after *V. harveyi* challenge. Subsequently, forty-eight differential display fragments were successfully reamplified and cloned. A total of 267 clones were randomly selected and sequenced. The sequence analysis showed that 85 (31%) out of 267 clones were matched with sequences in the GenBank database which represented 24 different genes with known functions. Among the known genes, glucose transporter 1, interferon-related developmental regulator 1, lysozyme, profilin, SERPINB3, were selected for further confirmation of their differentially expression patterns by real-time PCR. The results showed increasing in expression level of the selected genes in shrimp hemocytes after microbial challenge suggesting the involvement of such genes in bacterial response in shrimp. The anti-lipopolysaccharide factor type 3 (ALFPm3) gene, previously reported in *P. monodon* (Supungul *et al.*, 2002) was found among the up-regulated genes but diversity due to amino acid changes was observed. Increase in ALFPm3 transcripts upon *V. harveyi* injection is in accordance with that found in the previous study.

Introduction

For aquaculture of crustaceans, one of the most commercially significant species is the black tiger shrimp, *Penaeus monodon*. It is unfortunate that the production of aquacultured *P. monodon* has been seriously affected due to the outbreak of infectious diseases, mainly from viruses, white-spot syndrome virus (WSSV) and yellow-head virus (YHV), and luminescent bacteria, *Vibrio* species (Saulnier *et al.*, 2000; Soowannayan *et al.*, 2003; Wongteerasupaya *et al.*, 2003). The most prevalent bacterial disease is vibriosis which causes a mass mortality both in larval cultures and shrimp production (Saulnier *et al.*, 2000). The major virulent strains of vibrios in shrimp are *Vibrio alginolyticus*, *V. anguillarum*, *V. harveyi* and *V. parahaemolyticus*. For the sustainability of shrimp farming, scientific approaches to prevent and control the disease are of paramount importance. The understanding of the molecular responses and defense mechanisms in shrimp against invading microorganisms is thus needed.

In arthropods, predominantly insects, crustaceans and chelicerates, innate immune system provides the major defense mechanisms against pathogenic agents. In crustaceans, the immune responses occur mainly in hemolymph. The host defense mechanisms, cellular and humoral reactions, include phagocytosis, encapsulation, nodule formulation, clotting, agglutination, prophenoloxidase (proPO) cascade and antimicrobial activity (Sritunyalucksana *et al.*, 2000; Iwanaga, 2002; Smith *et al.*, 2003).

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For immediate immune response to pathogens, several proteins and peptides are synthesized, stored in the hemocytes and released into the hemolymph upon infections. Subsequently, more biosynthesis of the proteins is required for continuing the defense or restoring the hemocyte defense proteins, leading to the changes in gene expression as the response progresses. Such cellular responses to pathogen invasion are of interest. To better understanding of the innate immune system, the cellular responses should be unveiled and the clues are believed to lie in the changes in gene expression. In order to identify and characterize of the differentially expressed genes, several techniques are available, for example suppression subtractive hybridization (SSH), differential hybridization (HD) and differential display PCR (DD-PCR). The SSH, a sensitive PCR-based subtraction, and HD approaches have been adopted to isolate genes, up-regulated in hemocytes of virus-infected shrimp *Penaeus japonicus* (Diatchenko *et al.*, 1996). Recently, Luo *et al.* (2003) adopted the DD-PCR technique to compare the mRNAs from hepatopancreas of virus-sensitive and virus-resistant shrimps, *P. monodon*, and the first shrimp antiviral gene, *PmAV*, was identified.

In this study, we determine changes in gene expression patterns in hemocytes of *P. monodon* upon *V. harveyi* infection by using the advantage of DD-PCR approach. We have identified several up- and down-regulated genes, and postulated that they may be involved in defense mechanisms against bacteria invasion.

Materials and Methods

Animals. Subadults *P. monodon* (approximately 3 months old, 20 g of body weight) were purchased from local farms and separated into 3 groups. The first group was the unchallenged or normal shrimps. The second group was *P. monodon* injected with 0.85% (w/v) NaCl. The last group was *P. monodon* infected with *V. harveyi* 1526. All groups were acclimatized in aquaria at ambient temperature ($28 \pm 4°C$) and salinity of 15 ppt for at least 1 day before used in the experiments.

Shrimps were intramuscularly injected into the forth abdominal segment with 10^6 CFU *V. harveyi* 1526 (kindly provided by Shrimp Culture Research Center, Charoenpokaphand Group of Companies). For differential display PCR, hemolymph was collected at 40 hour post-injection (hpi) and the shrimps were tested whether the infection was successful by culturing the suspensions of hepatopancreas on TSA plates supplemented with 2% NaCl, incubating at 30°C overnight and examining the strong luminescence from the colonies of *V. harveyi* 1526 in the dark. For a time-course study of mRNA expression by real-time PCR, hemolymph was collected from individual *V. harveyi*- and saline-injected *P. monodon* at times 0, 6, 24 and 48 hpi. Saline-injected shrimps were used as the control.

Total RNA preparation. Hemolymph was collected from the ventral sinus of shrimp using a 27 G/1/2 inch needle fitted onto a 1.0 ml syringe pre-loaded with 200 μl of anti-coagulant (10% sodium citrate). Hemocytes of 5 individuals for differential display PCR and 10 individuals for real-time PCR experiments were pooled after isolation by centrifugation at $800 \times g$ for 10 minutes. Total RNA was isolated from the hemocytes using TriReagent® (Molecular Research Center, Inc.) according to the manufacturer's instructions. Chromosomal DNA contamination in total RNA samples was removed by treating 25 μg of total RNA with 5 units of RQ1 RNase-free DNase (Promega) at 37°C for 1 hour and purified by phenol/chloroform extraction and ethanol precipitation.

Differential display PCR. The first-stranded cDNAs were generated from 2 μg of DNA-free total RNA and 0.1 mM of oligo(dT) primers according to Delta® Differential Display Kit (Clontech) protocol. The cDNA was then diluted with sterile H_2O to 1 : 10 and 1 : 30 dilutions and used as template for differential display PCR (DD-PCR). Forty-four combinations of oligo(dT) T primers and arbitrary P primers provided in the kit, were used for DD-PCR reactions. For each differential display experiment, 1 μl of each diluted cDNA sample, 1 μl of 20 μM P primer and 1 μl of 20 μM T primer were mixed with a 17 μl of master mix containing 2 μl of 10× KlenTaq PCR reaction buffer, 14.2 μl of sterile H_2O, 0.2 μl of dNTP mix (5 mM each; final concentration of 50 μM), 0.2 μl of [α-^{33}P]dATP (1,000-3,000 Ci/mmole; 3.3 μM; final concentration of 50 nM) (Amersham Biosciences), 0.4 μl 50× Advantage KlenTaq Polymerase Mix. One drop of mineral oil was added on top of the PCR mixture and the tubes were tightly capped. The cycling parameters were one cycle at 94°C for 5 min, 40°C for 5 min, 68°C for 5 min, two cycles at 94°C for 2 min, 40°C for 5 min, 68°C for 5 min, 25 cycles at 94°C for 1 min, 58°C for 1 min, 68°C for 2 min and a final extension at 68°C for 7 min. A molecular weight marker, λDNA/*Eco*RI + *Hin*dIII marker (Promega), was labeled with [α-^{33}P]dATP by end-repairing reaction with a Klenow polymerase (New England Biolabs) (Sambrook *et al.*, 1989). The PCR products and the labeled molecular weight marker were electrophoresed through a 6% denaturing polyacrylamide gel. The gel was dried *in vacuo* and subjected to autoradiography. DD-PCR patterns with visually different signal intensities between normal and infected shrimps were compared for each primer combination. The level of expression was evaluated by measuring the intensity of bands with the gel documentation system (GeneCam FEXI, Syngene).

To isolate differentially expressed cDNA fragments, regions of dried gels corresponding to the cDNAs were excised. Gel slices were rehydrated in 40 μl sterile water and incubated at 100°C for 10 min. The eluted cDNAs were reamplified with the same pairs of primers and condition used in DD-PCR reaction. Amplified cDNAs were purified using QIAGEN gel extraction kit (QIAGEN) and directly cloned into the pGEM-T Easy vector (Promega). Recombinant clones were screened by colony PCR using T7 and SP6 primers and 4 to 8 clones for each cDNA were randomly selected for further nucleotide sequencing.

DNA sequencing and data analysis. DNA sequencing was carried out using a ThermoSequenase Fluorescent Labeled Primer Sequencing kit (Amersham Biosciences) with M13 forward and reverse primers on an automated DNA sequencer LC4000 (Li-cor Biosciences). The nucleotide sequences were compared to those in the GenBank database using the BLASTN and BLASTX programs (Altschul *et al.*, 1990; Gish and States, 1993). The annotation of the differential display clones is considered when significant probability (E) is $< 10^{-4}$ with a match more than 100 nucleotides for BLASTN and a

match more than 10 amino acid residues for BLASTX. Multiple sequence alignments of nucleotides and translated amino acids were performed using Clustal W (Thompson *et al.*, 1994).

Quantitative real-time RT-PCR. The first-strand cDNAs were generated from 1 μg of DNA-free total RNA sample and 0.5 μg of oligo(dT)$_{18}$ primers and ImProm-II™ Reverse Transcriptase System kit (Promega) according to the manufacturer's protocol. The SYBR Green I real-time RT-PCR assay was carried out using the iCycler iQ™ Real-Time Detection system (Bio-Rad). The amplifications were performed in a 96-well plate using a 20 μl reaction volume containing 10 μl of 2× SYBR Green supermix (Bio-Rad), the appropriate amount of forward and reverse primers, 1 μl of 1 : 10 diluted cDNA from each reverse transcription reaction as template and sterile water to adjust the reaction volume. The required final concentration of primers is shown in Table 1. The thermal profile was 95°C for 3 min followed by 40 cycles of denaturation, annealing and extension as indicated in Table 1. Fluorescent data were collected at the end of extension step. Each sample was conducted in triplicate. Sterile water was added in place of template as a negative control.

The specificity of PCR amplification of each primer pair was determined by constructing a melting curve immediately after the PCR amplification. The amplification reactions were incubated at 95°C for 1 min and subsequently at 50°C for 1 min, followed by 80 repeats of heating for 10 seconds starting at 50°C with 0.5°C increments.

Fluorescence signal was analyzed by the data analysis software of iCycler iQ™ Real-Time Detection system (Bio-Rad) using PCR base line subtracted curve fit method. For each *V. harveyi*-injected sample, the threshold cycle or Ct value was normalized with the saline-injected control. The elongation factor 1-alpha gene (EF-1α) in the same sample as *V. harveyi*-infected shrimp hemocytes was used as an internal reference. The PCR efficiency of each gene was determined by constructing a standard curve. Serial dilutions of the pooled cDNA of normal animal were appropriately made. For each run, the amplification was performed in triplicate including a negative control, which used water in place of template. The data were automatically analyzed, and the amplification plots and a calibration curve of Ct values against the input quantities on log scale were constructed for both reference (EF-1α) and the target gene. In the calibration curve, a linear graph with a correlation coefficient more than 0.990 was obtained. The slope of the curve was used to calculate the PCR efficiency, as it is equal to $10^{-1/\text{slope}}$.

The relative expression ratio (R), the real-time PCR efficiency of target gene transcript (E_{target}) relatively to that of reference gene transcript (E_{ref}), was calculated using the equation by Pfaffl (2001):

$$R = (E_{\text{target}})^{\Delta Ct \text{ target}^{(\text{control-sample})}}/(E_{\text{ref}})^{\Delta Ct \text{ ref}^{(\text{control-sample})}}$$

where R is the relative expression ratio, $\Delta Ct_{\text{target}}$ is the Ct deviation of control (saline-injected) minus sample (*V. harveyi*-injected) of the target gene transcript and ΔCt_{ref} is the Ct deviation of control (saline-injected) minus sample (*V. harveyi*-injected) of the reference gene transcript.

Results and Discussions

Identification of *Vibrio harveyi*-responsive genes in *Penaeus monodon* hemocytes. Identification of genes involved in the immune response is an important step towards the understanding of the immune system. It is expected that microbial infection affects the gene expression in the cells, particularly those immune-related and target cells. Techniques have been developed to isolate the genes involved in such infection by taking the advantage of the differences in gene expression (Astrofsky *et al.*, 2002; He *et al.*, 2005). The effect of microbial invasion on gene expression of shrimp has been elucidated in the hemocytes which are the main site of

Table 1. Primer pairs and conditions for real-time PCR

Gene name	Primers	Final primer concentration (μM)	Temperature profile		
			Denaturation (°C/sec)	Annealing (°C/sec)	Elongation (°C/sec)
GLUT1	Forward: GCGAAACGACGATGATTGTG Reverse: TGAAGAAGCGAGCGATGATG	200	95/10	56/20	72/10
IFRD1	Forward: GGAAGGTCTGTCGGAGATGG Reverse: ACGGGTGAGAGGCTTACAAC	100	95/10	58/20	72/10
Lysozyme	Forward: GCTGCTGGTTGGGCTTCTG Reverse: TGCGGTTGCGGTTGATGG	100	95/10	58/15	72/10
Profilin	Forward: GCTGGTGGAATCTGGAAACG Reverse: GCGGTGTTAGTCTTGGTGATG	100	95/10	58/20	72/10
SERPINB3	Forward: CCCTCAAACGCCTCAAAGTC Reverse: GTCGGTCCACGGTGAAGG	100	95/10	62/15	72/10
ALF*Pm*3	Forward: CCCACAGTGCCAGGCTCAA Reverse: TGCTGGCTTCTCCTCTGATG	500	95/10	58/20	72/10
EF-1α	Forward: GGTGCTGGACAAGCTGAAGGC Reverse: CGTTCCGGTGATCATGTTCTTGATG	500	95/10	58/20	72/10

Table 2. Changes in mRNA level of differential display bands identified in hemocytes of *Vibrio harveyi*-challenged shrimps

Clone	Primers used in differential display*	Size (bp)	Fold change	Clone	Primers used in differential display*	Size (bp)	Fold change
DD1	P2/T2	1200	+2.6	DD40	P6/T3	1500	-1.41
DD2	P2/T2	750	+1.55	**DD41**	P6/T3	800	-2.56
DD3	P2/T2	600	-4	DD42	P8/T2	700	-2.56
DD4	P2/T2	450	+2.33	**DD43**	P8/T2	640	+3.48
DD5	P2/T2	2300	+1.5	**DD44**	P8/T2	590	-2.38
DD6	P2/T2	600	-2.44	**DD45**	P8/T2	500	+2.11
DD7	P2/T2	750	+1.94	**DD46**	P10/T2	3000	-2.0
DD8	P3/T3	600	-1.96	**DD47**	P10/T2	1400	-2.04
DD9	P3/T3	500	+0.52	DD48	P10/T1	600	+1.95
DD10	P8/T8	550	+1.28	**DD49**	P10/T1	480	+2.84
DD11	P8/T8	2900	-3.70	**DD50**	P4/T3	750	+2.14
DD12	P8/T8	500	-3.13	**DD51**	P4/T3	740	+2.13
DD13	P8/T8	500	-4.55	DD52	P6/T4	2000	+1.31
DD14	P8/T1	1400	-1.92	DD53	P5/T4	550	+1.67
DD15	P8/T1	650	+2.79	**DD54**	P9/T4	580	-2.38
DD16	P3/T1	450	-2.94	**DD55**	P9/T4	530	+1.46
DD17	P3/T1	680	+3.36	DD56	P8/T4	1200	+2.12
DD18	P3/T1	550	-2.17	**DD57**	P8/T4	500	+1.26
DD19	P6/T6	1600	+1.91	DD58	P2/T4	1200	+1.6
DD20	P6/T6	800	-1.35	**DD59**	P2/T4	1100	+1.64
DD21	P10/T1	2000	-1.82	**DD60**	P2/T4	650	+2.08
DD22	P10/T1	700	+2.26	DD61	P1/T4	2300	+1.77
DD23	P1/T2	2000	-1.82	**DD62**	P1/T4	1500	+1.6
DD24	P1/T2	980	+4.53	DD63	P1/T4	880	+1.76
DD25	P1/T2	930	+2.03	**DD64**	P3/T4	550	-2.33
DD26	P1/T2	790	+1.35	DD65	P1/T3	1200	+2.8
DD27	P1/T2	750	+2.23	DD66	P9/T2	700	+2.86
DD28	P3/T2	750	+2.75	**DD67**	P5/T3	700	+2.49
DD29	P4/T2	1400	-1.82	**DD68**	P10/T3	520	-2
DD30	P5/T2	2400	+2.37	**DD69**	P9/T3	1400	+2.07
DD31	P5/T2	1100	-2.22	**DD70**	P9/T3	1200	+1.87
DD32	P5/T2	800	-2.13	DD71	P9/T3	1180	-1.82
DD33	P5/T2	400	-2.5	**DD72**	P9/T3	1000	-1.61
DD34	P2/T3	1250	+2.75	DD73	P9/T3	750	+1.62
DD35	P2/T3	780	+2.6	**DD74**	P9/T3	550	+1.96
DD36	P2/T3	630	-1.30	DD75	P7/T4	850	-2.22
DD37	P2/T3	500	-1.8	DD76	P7/T2	480	+12.08
DD38	P2/T3	480	+2.3	DD77	P8/T2	1502	-2.63
DD39	P5/T3	630	+2.54	**DD78**	P7/T1	850	+2.43
				DD79	P7/T1	650	-2.70

Remark: The clone names in bold indicate those that were successfully reamplified and cloned. *The sequences of primers can be found in the Delta® Differential Display Kit user manual.

immune response (Astrofsky *et al.*, 2002; Supungul *et al.*, 2004; de Lorgeril *et al.*, 2005). The DD-PCR is a technique of choice used to identify genes potentially responded to *V. harveyi* infection in *P. monodon* hemocytes.

The differential display PCR was carried out using 44 combinations of primers (10 arbitrary primers in random combination with 9 oligo(dT) primers). In theory, about 34% of the expressed genes in hemocytes of *V. harveyi*-infected *P. monodon* should be observed [$1-(0.96)^n$ where n is the number of arbitrary primers] (Astrofsky *et al.*, 2002). The DD-PCR patterns of normal and *V. harveyi* infected *P. monodon* were compared for each primer combination. A total of 79

Table 3. *Vibrio harveyi* responsive gene homologues identified in hemocytes of *Penaeus monodon* by DD-PCR

Sequence homology	Closest species	Accession no. of closest species	Expected value	Positive	Up or down*
Gene expression and regulation					
balbiani ring protein3 precursor	*Chironomus tentans*	Q03376	1.00E-05	50/111 (45%)	+
argonaute protein	*Drosophila melanogaster*	NP_523734	8.00E-43	98/129 (75%)	+
ADP-ribosylation factor1	*Anopheles gambiae*	XP_320516	E-101	183/183 (100%)	-
glycyl-tRNA synthetase	*Homo sapiens*	P41250	4.00E-20	60/85 (70%)	+
Potential immune function					
anti-lipopolysaccharide factor	*Limulus polyphemus*	A23931	4.00E-16	56/95 (58%)	+
caspase 3B	*Hydra vulgaris*	AAF98012	4.00E-22	107/217 (49%)	+
lysozyme	*Penaeus monodon*	AAN16375	9.00E-83	143/143 (100%)	+
serine protease inhibitor B3	*Rattus norvegicus*	NP_001008887	1.00E-23	110/184 (59%)	-
transglutaminase	*Penaeus monodon*	AAL78166	9.00E-29	64/64 (100%)	+
Metabolism					
carbonic anhydase1	*Drosophila melanogaster*	NP_523561	4.00E-52	123/199 (61%)	+
kynurenine aminotransferase	*Drosophila melanogaster*	AAN71426	1.00E-35	94/125 (75%)	+
ligase family member	*Anopheles gambiae*	XP_309685	6.00E-13	47/64 (73%)	+
phosphogluconate dehydrogenase	*Danio rerio*	AAQ91261	3.00E-70	143/165 (86%)	-
Structure and motility					
alpha-spectrin	*Drosophila melanogaster*	AAL39886	5.00E-67	137/154 (88%)	+
myosin like protein	*Anopheles gambiae*	XP 314222	3.00E-63	141/172 (81%)	+
profilin	*Branchiostoma belcheri*	AAL75810	2.00E-20	72/111 (64%)	+
Rc12 protein	*Drosophila melanogaster*	EAL27028	1.00E-22	77/124 (62%)	-
Signaling and communication					
asialoglyco protein receptor2	*Rattus norvegicus*	NP_058885	3.00E-05	50/199 (42%)	+
calmodulin	*Metridium senile*	P02596	3.00E-17	44/44 (100%)	-
Transport					
autotransporter adhesin	*Magnetococcus sp. MC-1*	ZP_00288950	2.00E-13	70/134 (52%)	-
Glucose Transporter1	*Drosophila melanogaster*	NP_728559	6.00E-28	90/152 (59%)	+
Miscellaneous function					
interferon-related developmental regulator 1	*Homo sapiens*	AAH01272	3.00E-21	95/177 (55%)	+
putative type IV aminophospho-lipid transporting ATPase	*Mus musculus*	NP_700438	2.00E-53	128/192 (66%)	+
Huntingtin interacting protein E	*Drosophila melanogaster*	NP_609026	4.00E-79	181/235 (77%)	+
Unidentified-similar to other cDNA/DNA					
ENSANGP00000016412	*Anopheles gambiae*	XP_306944	4.00E-53	133/201 (66%)	+
ENSANGP00000021966	*Anopheles gambiae*	XP_309498	2.00E-87	158/189 (83%)	+
ENSANGP00000022625	*Anopheles gambiae*	XP_308486	4.00E-29	92/156 (58%)	+
hypothetical protein MG03029.4	*Magnaporthe grisea*	EAA47786	6.00E-29	78/119 (65%)	-

* (+) and (-) indicate up or down regulation of putative genes observed by DD-PCR technique.

differentially expressed bands from 33 combinations of primers were identified. The average fold change in the expression level was calculated from the band intensity (Table 2). The differentially expressed bands could be divided into two groups: 48 bands showing increased expression and 31 bands showing reduced expression. The approximate sizes of differentially expressed cDNA bands ranged from 400 to 3,000 bp.

The cDNAs from 48 (61%) out of 79 differentially expressed bands were successfully extracted from the acrylamide gels,

reamplified and cloned as indicated in Table 2. Among these, 30 bands were up regulated, while the other 18 bands were down regulated. Four to eight representative clones from each band were randomly selected and a total of 267 clones were sequenced. The resulting DNA sequences from the clones of each band were aligned to determine their sequence discrepancy. The sequence analysis of these clones showed that 21 (44%) out of 48 bands contained one unique DNA sequence, while the remaining bands contained a mixture of two to four DNA sequences. Mixture of similar size products obtained from one
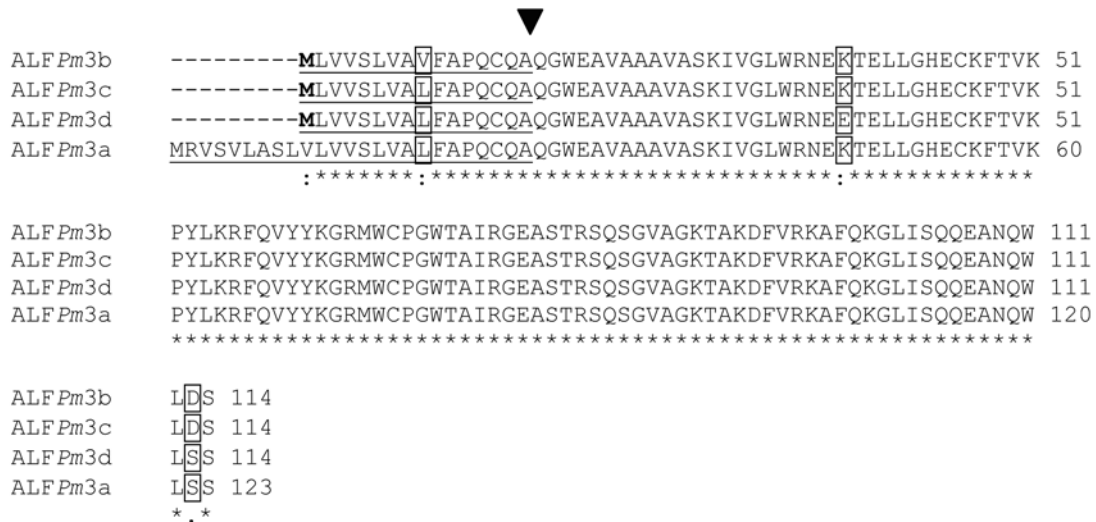
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                        ▼
ALFPm3b    ---------MLVVSLVAVFAPQCQAQGWEAVAAAVASKIVGLWRNEKTELLGHECKFTVK 51
ALFPm3c    ---------MLVVSLVALFAPQCQAQGWEAVAAAVASKIVGLWRNEKTELLGHECKFTVK 51
ALFPm3d    ---------MLVVSLVALFAPQCQAQGWEAVAAAVASKIVGLWRNEETELLGHECKFTVK 51
ALFPm3a    MRVSVLASLVLVVSLVALFAPQCQAQGWEAVAAAVASKIVGLWRNEKTELLGHECKFTVK 60
                     :*******:****************************:************

ALFPm3b    PYLKRFQVYYKGRMWCPGWTAIRGEASTRSQSGVAGKTAKDFVRKAFQKGLISQQEANQW 111
ALFPm3c    PYLKRFQVYYKGRMWCPGWTAIRGEASTRSQSGVAGKTAKDFVRKAFQKGLISQQEANQW 111
ALFPm3d    PYLKRFQVYYKGRMWCPGWTAIRGEASTRSQSGVAGKTAKDFVRKAFQKGLISQQEANQW 111
ALFPm3a    PYLKRFQVYYKGRMWCPGWTAIRGEASTRSQSGVAGKTAKDFVRKAFQKGLISQQEANQW 120
           ************************************************************

ALFPm3b    LDS 114
ALFPm3c    LDS 114
ALFPm3d    LSS 114
ALFPm3a    LSS 123
           *.*
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Fig. 1. Multiple alignment of the deduced amino acid sequences of ALF*Pm*3 homologues obtained from DD-PCR and the ALF*Pm*3 previously identified by Supungul *et al.*, 2004. (*) indicates amino acid identity and (.) and (:) indicate amino acid similarity. The putative signal peptides are underlined and the cleavage sites are indicated by an arrowhead. Open boxes indicate the variation at residues 18, 47 and 122.

cDNA band is possible because of the co-migration of other non-homologous cDNAs (Mathieu-Daude *et al.*, 1996). Mixture of sequences was also detected in differentially expressed genes from white spot virus infected *Litopenaeus stylirostris* (Astrofsky *et al.*, 2002).

Homology search against the GenBank database using the BLAST search program showed that the 85 clones (31%) derived from 24 different bands significantly matched with 24 different known genes with known function. These DNA sequences could be divided into 7 categories according to the major functions of their encoded proteins including gene expression and regulation, potential immune function, metabolism, structure and motility, signaling and communication, transport and miscellaneous function (Table 3). Among the known genes, 8 genes including balbiani ring protein3 precursor, argonaute protein, caspase 3B, serine protease inhibitor B3 (SERPINB3), Rc12 protein, glucose transporter 1 (GLUT 1), interferon-related developmental regulator 1 (IFRD 1) and huntingtin interacting protein E, were found for the first time in the penaeid shrimp. Due to a large number of clones identified in this study, only the interesting differentially expressed genes, including GLUT 1, IFRD 1, lysozyme, profilin and SERPINB3, were selected for the real-time RT-PCR in order to study their response at various times after bacterial injection.

Among the known genes analyzed, an immune effector, namely anti-lipopolysaccharide factor (ALF*Pm*), the same gene previously identified and characterized (Supungul *et al.*, 2002; Somboonwiwat *et al.*, 2005), was found as the up-regulated gene. This finding agreed well with the previous study by RT-PCR (Supungul *et al.*, 2004) showing that the injection of *V. harveyi* caused significant increase of ALF expression in the challenge shrimp. In the EST library

prepared from hemocytes of *V. harveyi*-challenged shrimp, the ALF*Pm* transcripts were found to predominate and increased suggesting their importance in shrimp immune system (Supungul *et al.*, 2002). In this study, they were identified from the 8 clones derived from 2 differentially expressed bands (DD17-2, DD17-4, DD17-5, DD64-2, DD64-3, DD64-4, DD64-5 and DD64-6). The deduced amino acid sequences of these clones exhibited 57% to 66% homology with the ALF of the horseshoe crab *Limulus polyphemus*. Comparing with the ALF*Pm*1-5 previously reported by Supungul *et al.*, (2002), all of these clones encoded for proteins almost identical to ALF*Pm*3 (accession number BI018071), the major isoform identified in *P. monodon*, with 97 to 99% identity (Fig. 1). The amino acid sequences are however nine amino acids shorter than the ALF*Pm*3. This is due to the primer used in the DD-PCR. The amino acid sequence comparison suggested to us that the complete ORF of these clones should be 123 amino acid residues like that of the ALF*Pm*3. In addition, the comparison revealed the ALF*Pm*3 variants, ALF*Pm*3a-ALF*Pm*3d. Of these, the designated ALF*Pm*3a (DD64-6) variant was the first isolate ALF*Pm*3 (Supungul *et al.*, 2002). ALF*Pm*3b, 3c and 3d possessed variations at amino acid residues 18, 47 and 122 (Fig. 1).

The GLUT 1 homologue was identified from clones DD51-1, DD51-2, DD51-3, DD51-5, DD51-6 and DD51-8 with 59% homology to that of *Drosophila melanogaster*. GLUT proteins belong to a Major Facilitator Superfamily (MFS) of structurally related glycoproteins (Hruz and Mueckler, 2001). The amino acid sequences of homologue clones covered only the MFS domain (data not shown). GLUT family is composed of GLUT 1-12 gene members with variable tissue-specific expression, subcellular localization, and substrate specificity (Joost *et al.*, 2002). There is a report showing that GLUT 1
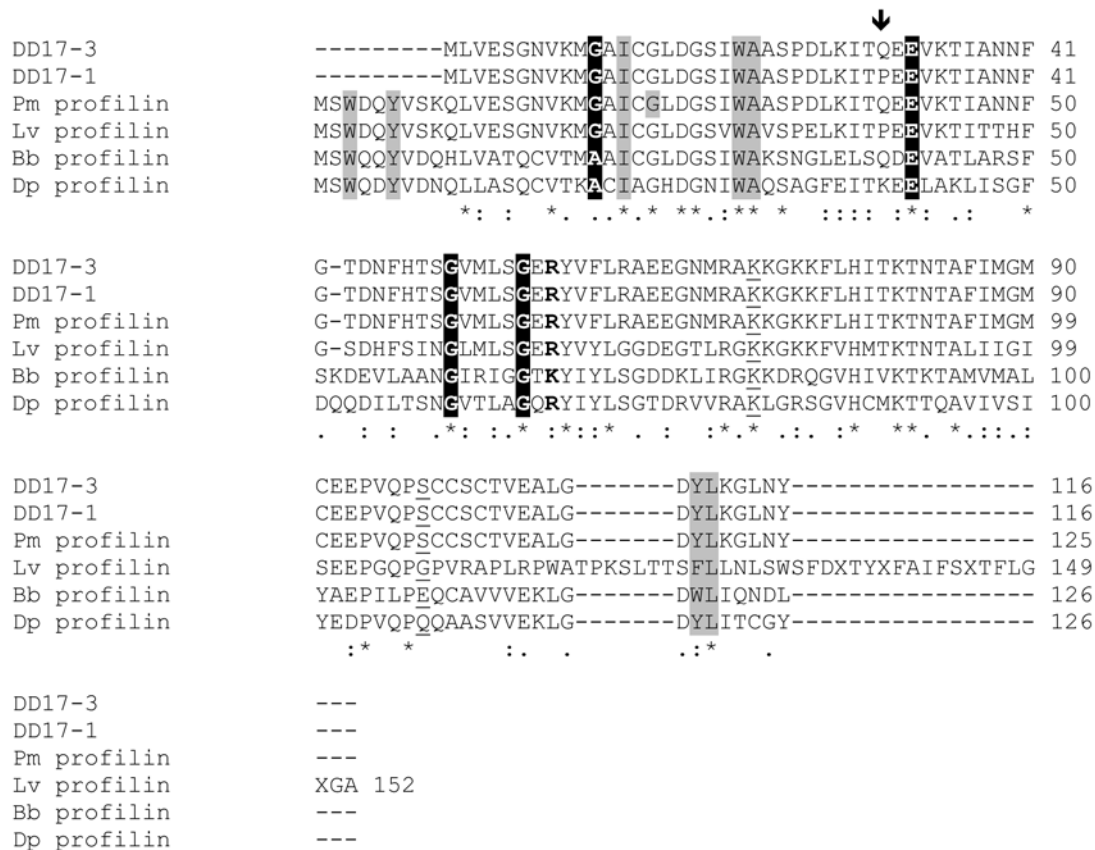
Fig. 2. Alignment of profilin deduced amino acid sequences. The putative profilin (DD17-1 and DD17-3) obtained from the DD-PCR was compared to a full-length clone from the *Penaeus monodon* hemocyte EST library (Pm profilin) and to those of *Litopenaeus vannamei* (Lv profilin, accession BE188423), *Drosophila pseudoobscura* (Dp profilin, accession EAL34274) and *Branchiostoma belcheri* (Bb profilin, accession AAL75808). (*) indicates amino acid identity and (.) and (:) indicates amino acid similarity. The arrow indicates variation of *P. monodon* profilin gene. The gray and black boxes indicate amino acid residues involved in PLP binding and fold conservation, respectively. The bold letters represent the conserved (+) patch. The underlined residues function as the actin binding residues.

mRNA in rat is regulated by stress and is proposed as a stress protein like glucose-regulated protein 78 (GRP 78) (Wertherimer *et al.*, 1991).

IFRD1 is the human homologue of the rat nerve-growth factor-inducible PC4 protein and murine homologue TIS7 (Buanne *et al.*, 1998; Micheli *et al.*, 2005). The amino acid sequences of human IFRD1, rat PC4 and mouse TIS7 are highly conserved suggesting they may have a common function. The exact function of IFRD1 is unknown but it has been shown that PC4 is necessary for muscle differentiation and that it may have a role in signal transduction. Furthermore, the carboxyl terminal region of human IFRD1 protein shows significant similarity to interferon-γ, a molecule known for its role in cellular differentiation (Buanne *et al.*, 1998). The IFRD 1 homologue was identified from clones DD78-2, DD78-3 and DD78-5 which showed 55% homology to that of *Homo sapiens*. The homologues contained 216 deduced amino acid residues constituting the first conserved domain of the proteins in this family (Buanne *et al.*, 1998).

The lysozyme, an antimicrobial protein in invertebrate immunity (Hikima *et al.*, 2003), was identified from clones DD19-2, DD19-3, DD19-4, DD19-5, DD19-6, DD20-3 and DD20-4. Lysozyme genes have been identified in many penaeid species, such as *Litopenaeus vannamei*, *P. monodon*, *Marsupenaeus japonicus*, *P. semisulcatus* and *L. stylirostris* (Rojtinnakorn *et al.*, 2002; Supungul *et al.*, 2002; Sotelo-Mundo *et al.*, 2003; de Lorgeril *et al.*, 2005). The kuruma shrimp lysozyme shows lytic activity on infectious pathogens in various *Vibrio* strains (Hikima *et al.*, 2003). However, the activity against *V. harveyi* has not been determined. Analysis of the amino acid sequence of *P. monodon* lysozyme indicates that it belongs to the C-type family as described in *L. vannamei* and *M. japonicus*.

Profilin, a small actin-binding protein, which has a major role in actin polymerization, was encoded by the two clones, DD17-1 and DD17-3. Profilin also interacts with several ligands other than actin including poly-L-proline (PLP), phosphatidylinositol 4, 5-bisphosphate (PIP₂), an actin-related protein complex, vasodilator-stimulated phosphoprotein (VASP)-like proteins, and formin-related proteins. Profilin also functions
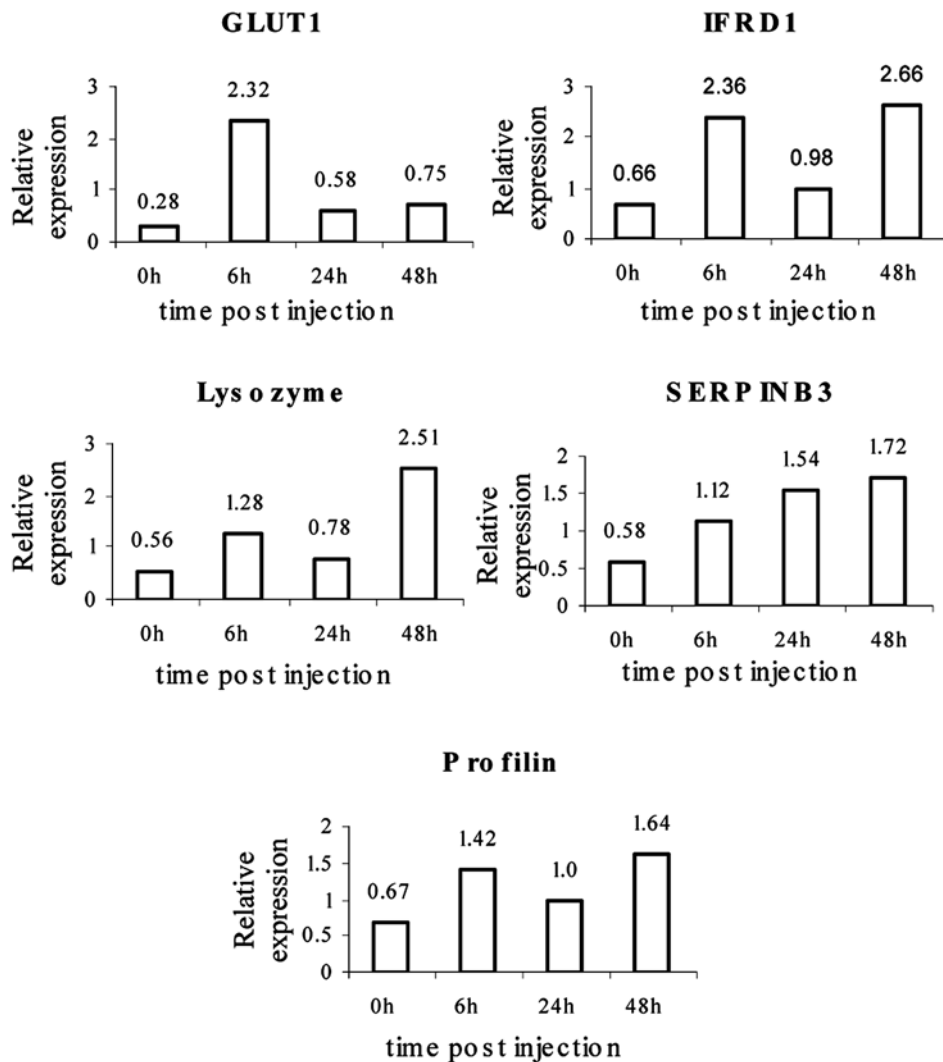
Fig. 3. Relative expression of selected genes, glucose transporter 1 (GLUT1), interferon-related developmental regulator 1 (IFRD 1), lysozyme, serine proteinase inhibitor B3 (SERPINB3), and profilin, at 0, 6, 24, and 48 hours after *Vibrio harveyi* injection determined by using real-time PCR.

as hubs that control complex network of molecular interactions such as membrane trafficking, small GTPase signalling and nuclear activities. The profilin family is highly conserved in terms of the protein folds, whereas the homology of the primary sequences of different species is low (Witke, 2004). Alignment of the differential display profilin clones with *P. monodon* and those of other species profilins revealed several conserved residues involved in poly-L-proline binding, actin binding, fold conservation and conserved (+) patch (Wilkes and Otto, 2000) (Fig. 2). Among the *P. monodon* profilins, variation of amino acid sequence at position 40 was observed in the clone DD17-1.

The serine proteinase inhibitor B3 (SERPINB3) homologues derived from clones DD46-1, DD46-3 and DD46-5 showed 51% homology to that of *Rattus norvegicus*. They did not have the complete ORF but the C-terminus 186 amino acid residues. The proteinase inhibitory effect of serpins normally require the reactive site loop (RSL) near their carboxyl terminus which can be divided functionally into two segments: a proximal (P15-P9) hinge region and a distal (P9-P5') variable region. The latter region is critical for inhibition and defining target specificities. Cleavage of a specific peptide bond between residues designated P1 and P1', results in a large conformational rearrangement in the serpin and formation of a stable serpin-proteinase complex. The P1 residue, for most serpin-serine proteinase interactions, and the P2 residues, for SERPINB3, dictate the inhibitory activity of the molecule (Schick *et al.*, 1998). The putative RSL (P15-P5') of the SERPINB3 homologues identified in this study was designated according to Schechter-Berger numbering scheme. A conserved Gly was P15, a consensus cleavage site was between P1Ile and P1'Gly, and Arg was designated as P2 (data not shown). However, the actual functional P1, P2 residues for this SERPINB3 homologue should be determined experimentally with different target proteinases (Schick *et al.*, 1998).

Analysis of gene expression by real-time RT-PCR. Four up-regulated genes homologue to glucose transporter 1 (GLUT 1), interferon-related developmental regulator 1 (IFRD 1), lysozyme and profilin, and one down-regulated gene homologue to serine protease inhibitor B3 (SERPINB3), were further analyzed by real-time PCR in order to verify the changes in expression level of the selected genes upon *V. harveyi* challenge observed by mRNA differential display technique and to determine their expression level in hemocytes at various time post-injection. The cDNAs derived from the pooled total RNAs of 10 individuals at each time point (0, 6, 24 and 48 hpi) from both saline- and *V. harveyi*-injected hemocytes were used for real-time RT-PCR reactions. For accurate assessment of gene expression by real-time RT-PCR, the PCR efficiency and the PCR specificity of each gene should be taken into consideration. The real-time RT-PCR efficiencies revealed the unequal efficiencies among the genes, all of which had imperfect amplification (E < 2.00) except for the IFRD 1 gene (data not shown). Therefore, the differences in PCR efficiency were corrected in the calculation of relative expression ratios. The specificity of real-time PCR judging by the dissociation curve analysis indicated that the target gene was amplified and there was no non-specific amplification or primer-dimers (data not shown). The expression ratios of the genes at each time point were calculated relative to the housekeeping gene, elongation factor 1 alpha (EF-1α) (Fig. 3).

For the selected genes, we found that all of them were up-regulated at all time points after *Vibrio* injection although different patterns of responses were obtained. Fig. 3 shows the expression levels of the genes at various times after *Vibrio* challenge. The results indicated that the GLUT1 gene responded strongly to the invasion at the early phase of infection, while the SERPINB3 transcript was increased gradually from 6 to 48 hpi. Interestingly, lysozyme, profilin and IFRD1 mRNA expression rose higher at 6 hpi and fell down at 24 hpi before climbing up again at 48 hpi. The real-time PCR results, therefore, showed that the selected genes were differentially expressed in hemocytes of *P. monodon* upon microbial infection.

Table 4 compares the real-time PCR results of the selected genes with those of DD-PCR. Since the results of gene expression in DD-PCR experiment were from the shrimp hemocytes at 40 hpi, the real-time PCR results at 48 hpi which also represented the changes in gene expression at the late phase of infection were used in the comparison. The more reliable real-time PCR results showed that all selected genes were up-regulated upon *V. harveyi* challenge, similar to the DD-PCR results except the SERPINB3 gene, which was down-regulated in DD-PCR. The discrepancy was probably due to the lower accuracy of DD-PCR technique as observed by other research groups as false positives. It has been postulated that the high proportion of false positives generated by DD-PCR could result in part from several factors such as degraded RNA or contaminating DNA, the use of short

Table 4. Validation of the DD-PCR results by real-time PCR

Gene name	Fold change		Expression of gene upon microbial challenged[c]
	DD-PCR[a]	Real time PCR[b]	
GLUT1	+2.13	+2.68	up
IFRD1	+2.43	+4.03	up
Lysozyme	+1.91/-1.35*	+4.48	up
Profilin	+3.36	+2.45	up
SERPINB3	-2.0	+2.97	up

[a]Fold change of differentially expressed band corresponding to each gene at 40 hour post-injection (hpi).
[b]Fold chage of relative expression ratios of *Vibrio harveyi*-injected sample at 48 hpi to that of 0 hpi by real-time PCR.
[c]The expression of gene upon microbial challenge according to the more reliable real-time PCR result.
*A putative lysozyme cDNA obtained from 2 differentially expressed bands, DD19 and DD20, which had different fold changes.

primers and low annealing temperatures for PCR amplification. However, the major problem is the fact that a single cDNA band on a gel may be resulted from co-migration of similar size cDNAs (Mathieu-Daude *et al.*, 1996; Vögeli-Lang *et al*, 1996). Some of these cDNAs may be derived from differentially expressed genes, others from constitutive expressed genes, which may contribute to the misinterpretation.

The putative GLUT 1 gene, a glucose transporter gene, in hemocytes of *P. monodon* was identified as the up-regulated transcript after *V. harveyi* injection. Real-time RT-PCR analysis revealed that the expression of GLUT 1 reached the highest level early after infection and thereafter decreased significantly to the level which was still higher than normal. The high level of GLUT 1 after infection indicated that the hemocytes required more energy for cellular immune processes than in the normal state. GLUT isoforms are differentially expressed and regulated in human leukocytes. Previous study indicated that GLUT 1 protein in monocytes was increased 1.9 fold after lipopolysaccharide activation and high GLUT 1 and GLUT 3 expression could provide cellular fuel for the immune response (Fu *et al.*, 2004).

The expression profile of IFRD1 upon microbial infection is very similar to those of lysozyme and profilin (Fig. 3). The gene expression is relatively higher in the early phase, lower in the intermediate phase and much higher in the late phase of *V. harveyi* infection. In vertebrate, interferon-γ is a pleiotrophic cytokine that regulates expression of hundreds of diverse genes, some of which are involved in innate immunity (Shtrichman and Samuel, 2001), and may have a role in cellular differentiation (Buanne *et al.*, 1998). IFRD1 may be involved in the regulation of innate immune response in shrimp.

Using *Vibrio* challenged *Litopenaeus stylirostris*, de Lorgeril *et al.* (2005) found that early after infection, the lysozyme mRNA was significantly lower than that of unchallenged

animals and elevated in surviving shrimps thereafter. On the other hand, low level of lysozyme transcript is increased readily in *V. harveyi* infected *P. monodon*. Though slightly decreased as the infection progressed, the lysozyme transcript is finally increased dramatically afterwards. The different expression profile may be due to the difference in methods used to infect the animals. *L. stylirostris* is infected with *V. penaeicida* by immersion which may result in slower increase of lysozyme transcripts (de Lorgeril *et al.*, 2005), while direct injection of *P. monodon* with *V. harveyi* results in more rapid increase in lysozyme expression as observed in this study.

As an actin-binding protein, profilin is believed to involve in infiltrating hemocytes through the shrimp tissues, especially muscle, by regulating the actin polymerization during the hemocyte migration. The higher expression of profilin at the early phase of infection suggests that hemocytes are in the process of migrating to the site of injection to perform their immune functions. The detectable transcripts are then lower after the initial phase, which may be due to the reduction of profilin-synthesizing hemocytes in the circulation. The expression is however increased to higher level late after infection. The increase in profilin mRNA in this late phase of shrimp immune response probably corresponds to the proliferation of the hemocytes and their response to further systemic infection (Bachere *et al.*, 2004).

SERPINs, proteinase inhibitors, are involved in a number of biological processes, such as blood coagulation cascades, complement activation, fibrinolysis, angiogenesis, inflammation, tumor suppression and hormone transport. Serpins have been identified as hemolymph protein in several arthopod species, including horseshoe crabs, crayfish, insects, and recently chinese shrimp (Kanost, 1999; Shen *et al.*, 2004). In horseshoe crabs, three serpins are synthesized in hemocytes and released in response to infection, and regulate proteinases in hemolymph coagulation pathway (Iwanaga *et al.*, 1998). In lepidopteran insect, *Manduca sexta*, three serpins have been identified and one of them, serpin-3, is a regulator of the pro-PO activation reaction in response to microbial infection by inhibiting prophenoloxidase-activating proteinases (Zhu *et al.*, 2003). In *P. monodon*, only the low molecular weight Kazal type-serine proteinase inhibitors were identified and characterized (Baily-Brook and Moss, 1992; Jarasrassamee *et al.*, 2005). By using DD-PCR technique, the SERPINB3 was identified for the first time in this species. Expression analysis by real-time PCR of SERPINB3 revealed that upon microbial challenge, mRNA level is gradually increased as the infection progresses.

Although the functions of the above up-regulated proteins can be speculated, the exact natures and their relation to the immune response remains to be further investigated. The full-length genes are also needed as the DD-PCR does not give complete genes because the technique relies on the RT-PCR amplification of cDNAs complementary to the 3'-terminal mRNA sequences (Kornmann *et al.*, 2001). To access the complete nucleotide sequences of such genes, other molecular biology techniques such as Rapid Amplification of cDNA Ends (RACE) PCR or screening of the cDNA library should be performed based on the sequences of DD-PCR clones.

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