CHAPTER III
RESULTS

3.1 Lymphoid organ and total RNA preparation

Lymphoid organs were dissected from the internal thorax of normal and *Vibrio harveyi* 1526-injected shrimps, *P. monodon*. The lymphoid organs were immediately immersed and blended in Trizol reagent for total RNA preparation as described in chapter II. The A_{260}/A_{280} ratios of the total RNA samples were 1.5-1.8, the expected ratio for the acceptable quality of the total RNA. The average amount of the total RNA was approximately 240 μg per individual for normal shrimps and 23 μg per individual for *V. harveyi*-infected shrimps.

The total RNA samples from normal and *V. harveyi*-injected shrimps were examined by electrophoresis on a denaturing formaldehyde/agarose/EtBr gel to check their integrity. Similar to that of normal shrimps, the total RNA from the lymphoid organs of *V. harveyi*-infected shrimps at various times post-injection (6-48 hours) revealed a predominant band of 18S rRNA (1.9 kb) (Figure 3.1).

3.2 Construction of lymphoid organ cDNA libraries

3.2.1 The normal library

The lymphoid organ cDNA library of normal shrimps was constructed by a directional cloning approach using a Uni-ZAP XR cloning system. The cDNAs were cloned between the *Eco*RI and *Xho*I sites of the λ Uni-ZAP XR. The titer of this library was approximately 3.2×10^5 pfu. The library was amplified to increase the titer to approximately 1×10^{10} pfu/ml. The average insert length of the EST clones in this library was 1100 bp as determined by colony PCR (Figure 3.2).

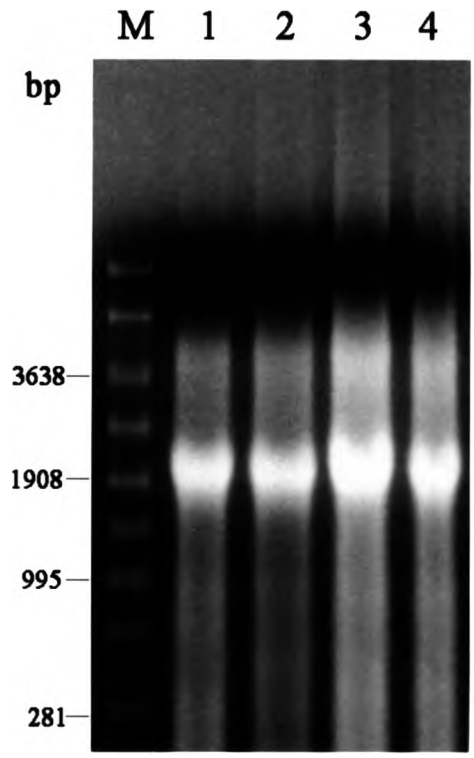


Figure 3.1 Total RNA from lymphoid organs of *V. harveyi* infected shrimps at various times post-injection (6-48 hours) electrophoresed on a 1% formaldehyde agarose gel

Lane M : RNA marker
Lane 1 : 6 hours post-injection
Lane 2 : 12 hours post-injection
Lane 3 : 24 hours post-injection
Lane 4 : 48 hours post-injection

3.2.2 The infected library

The lymphoid organ cDNA library of *V. harveyi*-infected shrimps was constructed by a directional cloning procedure using the same cloning system and strategy as described above. The titer of the infected library was 3.1×10^6 pfu, and became 2×10^{10} pfu/ml after amplification. The average insert length of the EST clones in this library was 1000 bp as determined by colony PCR (Figure 3.3).

3.3 EST analysis

3.3.1 Homology search

The 446 EST clones from the normal library were selected randomly and partially sequenced from the 5' side of the cDNA clones using MegaBACE DNA Analysis Systems (Amersham Biosciences). An average nucleotide sequence of cDNA insert is 1100 bp, corresponding to a total insert sequence length of 490 kb. After sequence editing, the nucleotide sequence of each clone was analyzed by homology searching against the protein sequence database in the GenBank of the translated protein sequences of the clone (BLASTX) (Altschul *et al.*, 1997). A putative function of each EST was assigned according to the highest significant similarity of a particular EST with that of the matched genes in the GenBank.

Homology search showed that 180 EST clones (40.41%) represented gene homologues whereas the remaining 266 clones (59.6%) did not match to any sequence data in the GenBank.

For the infected library, 642 EST clones were randomly selected and partially sequenced from the 5' side of the cDNA clones. An average nucleotide sequence of the cDNA inserts is 1000 bp, corresponding to a total insert of 642 kb. The nucleotide sequence of each clone from the infected library was analyzed as described above for the normal library.

The 286 EST clones (44.5%) from the infected library showed significant similarity with the deposited sequences whereas 356 EST clones (55.5%) did not match with any genes in the GenBank. The homology search results of the two libraries were summarized in Table 3.1.

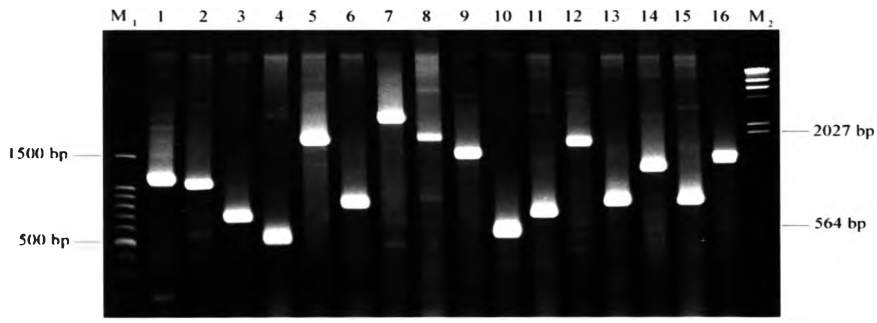


Figure 3.2 Determination of the insert size of the recombinant clones from normal library, using colony PCR. The PCR reactions from the clones were electrophoresed on 1.2% agarose gel (lanes 1-16). The 100 bp DNA ladder (M_1) and λ/*Hin*dIII (M_2) were used as DNA markers.

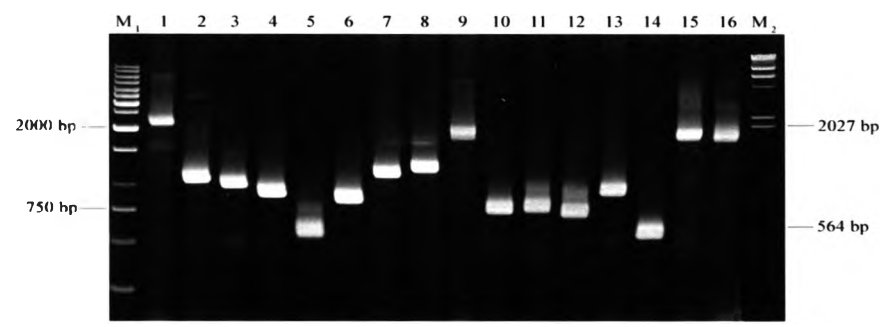


Figure 3.3 Determination of the insert size of the recombinant clones from *V. harveyi*-injected library, using colony PCR. The PCR reactions from the clones were electrophoresed on 1.2% agarose gel (lanes 1-16). The 1 Kb DNA ladder (M_1) and λ/*Hin*dIII (M_2) were used as DNA markers.

The EST clones in both libraries significantly matched the genes previously identified in many organisms. Those that matched the genes in *Penaeid specises* were 13 clones in the normal library, and 54 clones in the infected library; *P. monodon* (7 clones in the normal library and 44 clones in the infected library), *Fenneropenaeus chinensis* (2 clones in the normal library), *F. indicus* (1 clone in the normal library), *Litopenaeus stylirostris* (1 clone in the normal library and 1 clone in the infected library), *Metapenaeus ensis* (2 clones in the normal library and 1 clone in the infected library), *Marsupenaeus japonicus* (2 clones in the infected library), and *P. semisulcutus* (6 clones in the infected library). The ESTs that were homologues of genes in other shrimp were 1 clone (0.6%) and 6 clones (2.1%) from normal and infected libraries, respectively. The number of clones homologue to gene in other arthropods were 39 clones (21.7%) of normal library and 88 clones (30.8%) of infected library. Among these, the homologues of genes in *Drosophila melanogaster*genes were the largest group in the infected library (25 clones). In normal library, the major homologues were those of *Anopheles gambiae*, *Caenorhabditis elegans* and *Sarcophaga peregrine* (9 clones each).

Homologous genes in other animals were 95 clones (52.8%) and 110 clones (38.5%) from normal and infected library, respectively. In this group, homologous genes in *Homo sapiens* were the largest group in both libraries (30 clones in normal library and 28 clones in infected library). Homologues of genes in non-animal were 32 clones (17.8%) and 28 clones (9.8%) from normal and infected libraries, respectively.

To determine whether the sequencing of the EST clones should be continued or stopped because of high redundancy, a plot between the number of sequenced clones versus the number of newly identified unique sequences was carried out. The results indicated that the newly unique genes were progressively identified as the curves did not reach a plateau of identification. The ability to isolate new sequences of the normal library was 30%, 40%, 23% and 15% when 100, 200, 300 and 400 recombinant clones were sequenced. Likewise, 40%, 25%, 35%, 27%, 18% and 20% newly unique sequences of the *Vibrio*-infected library could be identified when 100, 200, 300, 400, 500 and 600 recombinant clones were sequenced (Figure 3.4).

Table 3.1 Summary of the homology search results of the two cDNA libraries from the shrimp lymphoid organs.

	Normal library	*V. harveyi*-infected library
Library titer (pfu/ml)	3.20E+05	3.10E+06
No. of sequenced clones	446	642
Matched clones	180 (40.4 %)	286 (44.5 %)
Penaeus shrimps	13 (7.2%)	54 (18.9%)
Other shrimps	1 (0.6%)	6 (2.1%)
Other arthopods	39 (21.7%)	88 (30.8%)
Other animals	95 (52.8%)	110 (38.5%)
Non-animal	32 (17.8%)	28 (9.8%)
Unmatched clones	266 [59.6 %]	356 [55.5 %]
Total nucleotide sequence (kb)	490	642
Average sequenced length (bp)	1100	1000

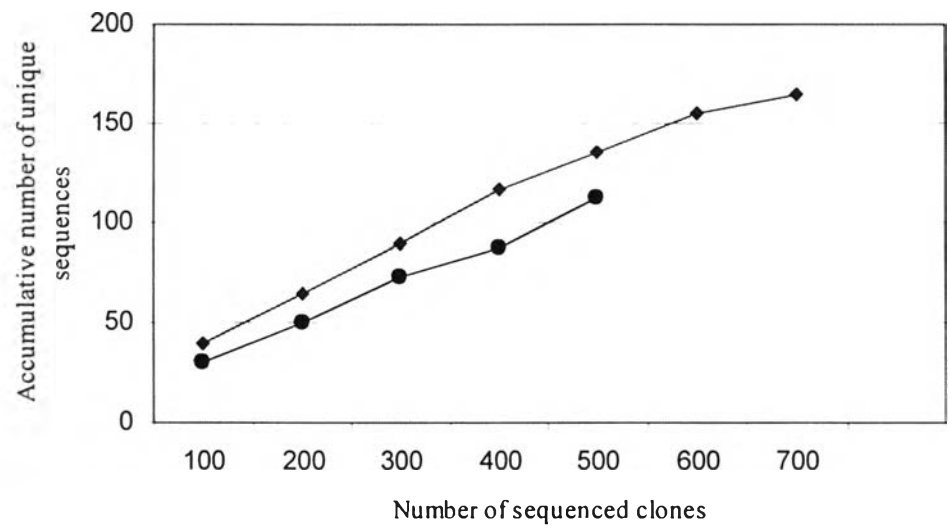


Figure 3.4 The possibility to isolate newly unique sequences of the normal cDNA library (•) and *V. harveyi*-infected library (♦) as determined by the relationship between the numbers of sequenced clones and the accumulative numbers of unique sequences.

3.3.2 Classification of the putative identified clones

The matched EST clones of the two cDNA libraries from the shrimp lymphoid organ were classified into 12 functional categories based on the significant sequence homology according to the criteria proposed by the *Penaeus monodon* EST project. These were genes involved in (1) gene expression, regulation and protein synthesis; (2) internal/external structure and motility; (3) metabolism; (4) defense and homeostasis; (5) signaling and communication; (6) cell division/DNA synthesis, repair and replication; (7) ribosomal protein and rRNA; (8) mitochondrial protein; (9) transport; (10) miscellaneous function; (11) unidentified (hypothetical)-similar to other cDNA/DNA; and (12) unknown function. The matched EST clones were summarized in Table 3.2.

In the normal library, the percentage of EST clones in defense and homeostasis (8.7%) was the largest category whereas the internal/external structure and motility (0.9%) was the smallest group. For the infected library, ribosomal proteins and rRNAs (8.4%) was the major category, whereas cell division/DNA synthesis, repair and replication category (0.8%) was the smallest group. ESTs in five categories: gene expression, regulation and protein synthesis; defense and homeostasis; cell division/DNA synthesis, repair and replication; transport; and unidentified (hypothetical)-similar to other cDNA/DNA, of the normal library were more abundant than those in the infected library. In contrast, for the remaining categories, the percentage of EST clones in infected library was more abundant than that in normal library.

The 466 matched ESTs, represented 283 different putative proteins, and the frequency of each identified protein in the normal and *V. harveyi*-infected libraries were shown in Tables 3.3-3.13.

Table 3.2 Matched ESTs in each functional category

Functional category	Normal library		*V.harveyi*-infected library	
	No. of clone	% of ESTs analysed	No. of clone	% of ESTs analysed
1. Gene expression, regulation and protein synthesis	21	4.7	26	4
2. Internal/external structure and motility	4	0.9	11	1.7
3. Metabolism	13	2.9	19	3
4. Defense and homeostasis	39	8.7	30	4.7
5. Signaling and communication	15	3.4	37	5.8
6. Cell division/DNA synthesis, repair and replication	4	0.9	3	0.5
7. Ribosomal protein and rRNA	16	3.6	54	8.4
8. Mitochondrial protein	18	4	29	4.5
9. Transport	5	1.1	6	0.9
10. Miscellaneous function	11	2.5	36	5.6
11. Unidentified (hypothetical)-similar to other cDNA/DNA	34	7.6	35	5.5
12. Unknown	266	59.6	356	55.5
Total ESTs	446	100	642	100

3.3.2.1 Gene expression, regulation and protein synthesis

This category includes the putative proteins that are involved in gene expression, regulation and protein synthesis. The 47 clones, classified into this category, represented 35 different genes (Table 3.3). Elongation factor was a major putative protein, and was found 8 clones in the normal library and 7 clones in the infected library. Other genes were activating transcription factor, ADP-ribosylation factor, ATP-dependent RNA-helicase, exonuclease, etc. Like all other EST works, redundancy was expected. Among the EST clones in this category, elongation factor 1-alpha had the highest redundancy as 7 clones in the normal library and 6 clones in the infected library were observed.

Table 3.3 The putative gene transcripts in the gene expression, regulation and protein synthesis category, isolated from the normal and *V. harveyi*-infected *P. monodon* lymphoid organs.

Putative gene	Closest species	Score	E Value	Length (bp)	Frequency		
					Normal	Infected	Total
activating transcription factor	*Bombyx mori*	69	3.00E-11	600	1	-	1
ADP-ribosylation factor	*Caenorhabditis elegans*	171	2.00E-83	800	2	-	2
ADP-ribosylation Factor related ARF-1	*Caenorhabditis elegans*	282	2.00E-77	0	1	-	1
ATP-dependent RNA-helicase	*Mus musculus*	73	2.00E-12	600	-	1	1
cleavage and polyadenylation specific factor 5	*Homo sapiens*	322	3.00E-87	600	-	1	1
DEAD box polypeptide 17 isoform p82	*Homo sapiens*	332	2.00E-90	600	-	1	1
elongation factor 1-alpha	*Armadillidium vulgare*	411	0	785	7	6	13
elongation factor 1-beta	*Bombyx mori*	176	6.00E-44	366	1	-	1
elongation factor 1-gamma	*Artemia sp.*	273	6.00E-73	525	-	1	1
eukaryotic initiation factor 2B-delta protein	*Drosophila melanogaster*	135	3.00E-31	600	-	1	1
eukaryotic initiation factor eIF-4A	*Marsupenaeus japonicus*	356	3.00E-97	600	-	1	1
eukaryotic translation elongation factor 1-beta	*Homo sapiens*	144	1.00E-33	766	1	-	1
eukaryotic translation initiation factor 3	*Mus musculus*	206	8.00E-53	495	-	1	1
exonuclease	*Phytophthora sojae*	86	5.00E-22	600	-	1	1
lysyl-tRNA ligase	*Cricetulus longicaudatus*	239	1.00E-62	600	-	1	1
prefoldin 5	*Mus musculus*	154	6.00E-37	600	-	1	1
protease regulatatory 26S subunit 4	*Drosophila melanogaster*	369	0	584	-	1	1
protease regulatory 26S subunit 8	*Manduca sexta*	68	2.00E-11	367	1	-	1
proteasome 26S	*Anopheles stephensi*	318	4.00E-86	800	1	-	1
proteasome N3	*Oncorhynchus mykiss*	214	5.00E-55	600	1	-	1
proteinase regulatory 26S complex	*Drosophila melanogaster*	128	7.00E-29	682	-	1	1
reverse transcriptase	*Caenorhabditis elegans*	43	4.00E-10	800	1	-	1
RNA polymerase beta subunit		179	4.00E-44	800	1	-	1
RNA polymerase II	*Homo sapiens*	101	5.00E-21	560	-	1	1
RNA recognition motif	*Caenorhabditis elegans*	175	3.00E-43	600	-	1	1
small nuclear ribonucleoprotein auxiliary factor	*Takifugu rubripes*	104	7.00E-22	600	-	1	1
splicing factor	*Homo sapiens*	47	7.00E-05	511	-	1	1
suppressor of Ty 4	*Homo sapiens*	48	6.00E-05	600	1	-	1
transcription initiation factor IIA gamma chain	*Oncorhynchus mykiss*	58	2.00E-16	600	-	1	1
transcriptional regulatory protein	*Escherichia coli K12*	390	0	684	1	-	1
translation elongation factor 1-gamma	*Locusta migratoria*	279	2.00E-74	600	-	1	1
translation initiation factor 4C	*Anopheles gambiae*	148	5.00E-35	600	1	-	1
triosephosphate isomerase	*Archaeopotamobius sibiriensis*	234	4.00E-61	600	-	1	1
zinc finger protein	*Homo sapiens*	90	1.00E-17	600	-	1	1

3.3.2.2 Internal/external structure and motility

The 15 matched EST clones, representing 12 different genes, were homologues of genes in the internal/external structure and motility (Table 3.4). The major putative proteins found in this category were actin-related proteins (4 clones), followed by calponin (3 clones) and kinesin (2 clones). Other genes were caldesmon protein, cofilin, fibropellin I precursor, glutenin, innexin-2 and radixin.

Table 3.4 The putative gene transcripts in the internal/external structure and motility category, isolated from the normal and *V. harveyi*-infected *P. monodon* lymphoid organs.

Putative gene	Closest species	Score	E Value	Length (bp)	Frequency		
					Normal	Infected	Total
actin-beta	*Artemia sp.*	143	4.00E-70	521	1	2	3
actin-related protein 2	*Homo sapiens*	252	1.00E-70	600	-	1	1
caldesmon protein	*Oryctolagus cuniculus*	58	4.00E-08	600	1	-	1
calponin	*Caenorhabditis briggsae*	161	4.00E-39	565	-	2	2
calponin 3	*Rattus norvegicus*	154	8.00E-40	600	-	1	1
cofilin	*Schizosaccharomyces pombe*	66	2.00E-10	600	1	-	1
fibropellin I precursor	*Strongylocentrotus purpuratus*	42	5.00E-08	600	-	1	1
glutenin	*Triticum aestivum*	50	2.00E-05	600	-	1	1
innexin-2	*Penaeus monodon*	155	2.00E-37	584	-	1	1
kinesin	*Loligo pealei*	115	5.00E-25	600	-	1	1
kinesin heavy chain	*Loligo pealei*	293	8.00E-79	600	1	-	1
radixin	*Mus musculus*	159	3.00E-38	600	-	1	1

3.3.2.3 Metabolism

The 32 matched EST clones, representing 27 different putative genes, were involved in the metabolism (Table 3.5). Major putative protein in this category was ubiquitin (3 clones). Other genes were adenylate kinase, amidase, cytidylate kinase, formate acetylransferase, etc. Redundancy was not observed in this category.

Table 3.5 The putative gene transcripts in the metabolism category, isolated from the normal and *V. harveyi*-infected *P. monodon* lymphoid organs

Putative gene	Closest species	Score	E Value	Length (bp)	Frequency		
					Normal	Infecte	Total
3-hydroxyacyl-CoA dehydrogenase	*Caenorhabditis elegans*	66	2.00E-10	356	-	1	1
adenylate kinase	*Caenorhabditis elegans*	219	2.00E-56	561	-	2	2
amidase	*Oceanobacillus iheyensis*	176	2.00E-47	600	-	1	1
ATP lipid-binding protein	*Marsupenaeus japonicus*	182	2.00E-45	600	-	1	1
beta-phosphoglucomutase	*Shigella flexneri*	132	5.00E-30	800	1	-	1
CDP-diacylglycerol phosphotidylhydrolase	*Shigella flexneri*	389	0	800	1	-	1
cytidylate kinase	*Escherichia coli*	142	5.00E-33	220	1	-	1
formate acetyltransferase	*Escherichia coli K12*	410	0	600	1	-	1
kinase	*Homo sapiens*	195	4.00E-49	600	1	-	1
lactate dehydrogenase	*Columba livia*	217	6.00E-56	560	-	1	1
mannose-6- phosphate isomerase	*Homo sapiens*	71	8.00E-12	600	1	-	1
mannose-P-dolichol utilization defect 1	*Mus musculus*	134	1.00E-30	600	1	-	1
metallothionein	*Homarus americanus*	131	5.00E-30	502	-	2	2
metallothionein 2	*Callinectes sapidus*	127	1.00E-28	549	1	2	3
methionine adenosyltransferase Complexed		168	5.00E-41	600	-	1	1
palmitoyl-protein hydrolase		272	4.00E-72	800	1	-	1
phospholipid hydroperoxide glutathione peroxidase	*Gallus gallus*	207	1.00E-52	600	-	1	1
phosphopyruvate hydratase	*Penaeus monodon*	361	0	600	-	1	1
polyubiquitin	*Tribolium castaneum*	194	6.00E-49	600	-	1	1
protein phosphatase-1	*Herdmania curvata*	281	3.00E-75	600	-	1	1
ribophorin I	*Danio rerio*	58	4.00E-08	600	1	-	1
succinate dehydrogenase complex, subunit B	*Homo sapiens*	271	5.00E-72	600	-	1	1
ubiquitin C	*Mus musculus*	384	0	600	-	1	1
ubiquitin conjugating enzyme	*Caenorhabditis elegans*	291	5.00E-78	582	1	-	1
ubiquitin-conjugating enzyme E2A	*Mus musculus*	150	1.00E-45	419	1	-	1
UMP-CMP kinase	*Mus musculus*	92	5.00E-33	800	-	1	1
vitellogenic carboxypeptidase precursor	*Aedes aegypti*	90	2.00E-17	600	1	1	2

3.3.2.4 Defense and homeostasis

A total of 69 clones from both cDNA libraries, representing 28 different putative immune related genes, were obtained. For the normal library, the

majority of the transcripts (36 clones) were in this category. The defense and homeostasis were categorized into 5 different subgroups as shown in Table 3.6.

The first subgroup was antimicrobial protein/peptides, which consisted of anti-lipopolysacchride factor (ALF) and lysozyme. The second subgroup was prophenoloxidase systems and oxidative enzyme that consisted of haem peroxidase, prophenoloxidase activating factor-III and transglutaminase. The third subgroup was proteinases and inhibitors. It was a group of cathepsins (A, B, C, D and L) and serine proteases. The fourth subgroup was heat shock proteins (HSP) that consisted of heat shock cognate 71 and HSP70. The last subgroup was other immune-related molecules, such as chaperonin, cyclophilin, peptidyl-prolyl cis-trans isomerase, profilin, etc.

Of these immune related genes, cathepsin family were the predominant genes in both normal and infected libraries (71.8% and 33.3%, respectively). Interestingly, the EST homologues of the ALF and lysozyme, and the proPO systems and oxidative subgroups were only found in infected library.

3.3.2.5 Signaling and communication

The 15 match EST clones, representing 28 different genes, were classified as putative genes of signaling and communication (Table 3.7). Redundant clones, found in the infected library, were thrombospondin 4 (11 clones). Other putative genes in this category were calcium binding protein, cartilage oligomeric matrix protein, integrin beta subunit, LDL-related protein, etc.

3.3.2.6 Cell division/DNA synthesis, repair and replication

This category was the smallest group, composed of 7 clones, representing 6 different genes (Table 3.8). They were ATP-dependent RNA helicase, cell division cycle2, cyclin A2, histone H1, histone H3 and retrovirus-related POL polyprotein.

Table 3.6 The putative gene transcripts in the defense and homeostasis category, isolated from the normal and *V. harveyi*-infected *P. monodon* lymphoid organs.

Putative gene	Closest species	Score	E Value	Length (bp)	Frequency		
					Normal	Infected	Total
1. Anitimicrobial molecules							
anti-lipopolysaccharide factor (ALF)	*Limulus polyphemus*	90	3.00E-17	800	-	3	3
lysozyme	*Penaeus monodon*	332	2.00E-90	600	-	3	3
2. ProPO systems and oxidative							
haem peroxidase	*Caenorhabditis elegans*	144	8.00E-34	600	-	1	1
prophenoloxidase activating factor-III	*Holotrichia diomphalia*	128	4.00E-29	600	-	1	1
transglutaminase	*Pacifastacus leniusculus*	98	5.00E-20	598	-	1	1
3. Proteinases and inhibitor							
cathepsin A	*Homo sapiens*	216	1.00E-55	600	-	1	1
cathepsin B	*Sarcophaga peregrina*	317	1.00E-85	800	14	7	21
cathepsin C	*Homo sapiens*	238	4.00E-62	600	1	-	1
cathepsin D	*Apriona germari*	235	3.00E-61	550	-	2	2
cathepsin L	*Maize weevil*	318	4.00E-86	600	13	1	14
serine protease 14D	*Anopheles gambiae*	131	7.00E-30	600	2	-	2
4. Heat shock protein							
heat shock cognate 71	*Bos taurus*	157	3.00E-38	418	1	-	1
heat shock protein 70	*Ambystoma mexicanum*	271	2.00E-76	800	1	-	1
5. Other immune molecules							
chaperonin	*Plasmodium yoelii*	55	1.00E-06	600	1	-	1
chaperonin containing T-complex	*Ochlerotatus triseriatus*	149	5.00E-72	600	-	1	1
chaperonin containing TCP1 beta	*Homo sapiens*	241	5.00E-63	600	1	-	1
copper homeostasis protein	*Caenorhabditis elegans*	135	6.00E-31	600	1	-	1
cyclophilin 18	*Oryctolagus cuniculus*	267	6.00E-71	600	1	-	1
cyclophilin 5	*Caenorhabditis elegans*	244	9.00E-64	600	1	-	1
cysteine protease caspase-2	*Gallus gallus*	68	4.00E-11	600	-	1	1
P109 protein	*Bombyx mori*	117	6.00E-26	539	-	2	2
peptidyl-prolyl cis-trans isomerase 5	*Caenorhabditis elegans*	243	1.00E-63	600	-	1	1
profilin	*Branchiostoma belcheri*	127	7.00E-29	600	-	1	1
survivin	*Sus scrofa*	92	3.00E-18	720	-	1	1
techylectin-5B	*Tachypleus tridentatus*	91	7.00E-18	566	-	1	1
thymosin beta-4	*Caenorhabditis elegans*	74	7.00E-13	600	-	1	1
TNF-induced protein GG2-1	*Homo sapiens*	105	9.00E-22	800	1	-	1
translationally controlled tumor protein	*Danio rerio*	133	2.00E-30	600	1	1	2

Table 3.7 The putative gene transcripts in the signaling and communication category, isolated from the normal and *V. harveyi*-infected *P. monodon* lymphoid organs.

Putative gene	Closest species	Score	E Value	Length (bp)	Frequency		
					Normal	Infected	Total
calcium binding protein	*Halocynthia roretzi*	94	1.00E-18	600	-	1	1
cartilage oligomeric matrix protein	*Equus caballus*	248	4.00E-65	600	-	1	1
ecdysone dependent glycoprotein	*Drosophila melanogaster*	116	2.00E-25	600	1	-	1
integrin beta subunit	*Anopheles gambiae*	181	6.00E-45	716	1	2	3
LDL-related protein	*Drosophila melanogaster*	49	7.00E-06	420	-	1	1
lysosomal cofactor/neurotrophic factor prosaposin	*Danio rerio*	112	3.00E-24	600	1	-	1
MAP kinase-interacting serine/threonine kinase 1	*Mus musculus*	124	1.00E-27	763	1	-	1
methylosome protein 50	*Homo sapiens*	78	5.00E-14	600	-	1	1
molybdenum cofactor	*Mus musculus*	172	8.00E-44	800	1	-	1
molybdenum cofactor biosynthesis protein A	*Homo sapiens*	211	7.00E-58	600	-	1	1
molybdopterin biosynthesis	*Escherichia coli*	390	0	800	1	-	1
mucin-like glycoprotein 900	*Cryptosporidium parvum*	53	1.00E-06	600	-	2	2
pallidin	*Homo sapiens*	86	2.00E-16	600	-	1	1
PDGF/VEGF-related factor 1	*Drosophila melanogaster*	50	1.00E-05	600	-	1	1
peroxisomal targeting signal type 2 receptor	*Arabidopsis thaliana*	48	4.00E-05	600	1	-	1
polyglutamine tract binding protein-1	*Mus musculus*	72	4.00E-12	692	-	1	1
prosaposin	*Gallus gallus*	131	7.00E-30	600	2	3	5
receptor for activated protein kinase C	*Danio rerio*	178	3.00E-44	405	-	1	1
retinol binding protein	*Metapenaeus ensis*	246	1.00E-64	600	2	1	3
rhsC protein	*Escherichia coli*	283	1.00E-90	800	1	-	1
seven transmembrane helix receptor	*Homo sapiens*	75	3.00E-13	600	1	-	1
tetratricopeptide	*Homo sapiens*	63	2.00E-09	587	-	1	1
tetratricopeptide repeat domain 2	*Homo sapiens*	145	3.00E-34	600	-	1	1
thrombospondin 1	*Bos taurus*	193	8.00E-54	600	-	5	5
thrombospondin 3	*Homo sapiens*	160	1.00E-38	600	-	2	2
thrombospondin 4	*Drosophila melanogaster*	229	2.00E-59	600	-	11	11
transient receptor protein 2	*Mus musculus*	213	5.00E-72	800	1	-	1
zinc finger protein/hormone receptor	*Caenorhabditis elegans*	80	1.00E-14	600	1	-	1

Table 3.8 The putative gene transcripts in cell division/DNA synthesis, repair and replication category, isolated from the normal and *V. harveyi*-infected *P. monodon* lymphoid organs.

Putative gene	Closest species	Score	E-Value	Length (bp)	Frequency		
					Normal	Infected	Total
ATP-dependent RNA helicase	*Dictyostelium discoideum*	81	1.00E-14	800	1	-	1
cell division cycle 2	*Homo sapiens*	225	4.00E-69	571	-	1	1
cyclin A2	*Bos taurus*	117	1.00E-25	600	-	1	1
histone H1	*Strongylocentrotus purpuratus*	165	4.00E-40	600	-	1	1
histone H3	*Homo sapiens*	265	4.00E-70	600	2	-	2
retrovirus-related POL polyprotein	*Mus musculus*	60	1.00E-08	395	1	-	1

3.3.2.7 Ribosomal protein and rRNA

Putative genes in this category were the highest abundant EST clones found in the infected library (54 clones, 8.4% of the EST homologues). For the normal library, 16 clones (3.6% of the EST homologues) were found. A total of 70 clones represented 43 different putative ribosomal proteins. Redundancy was not observed in this category.

3.3.2.8 Mitochondrial protein

The 47 clones, representing 19 different putative mitochondrial proteins were identified (Table 3.10). The major transcripts were cytochrome c oxidase subunits. Among these, the highest redundant clones were the cytochrome c oxidase subunit I in normal library (5 clones) and cytochrome c oxidase subunit II in infected library (6 clones). Other mitochondrial proteins were ATP synthase, ATP-binding protein, cytochrome b, NADH dehydrogenase and oligomycin sensitivity-conferring protein.

3.3.2.9 Transport

This group was small with only 11 clones, representing 9 putative transport proteins (Table 3.11). These were ADP-ATP translocator, balbiani RING protein 3, calreticulin, chloride conductance regulatory protein, karyopherin alpha2, etc.

Table 3.9 The putative gene transcripts in the ribosomal protein and rRNA category, isolated from the normal and *V. harveyi*-infected *P. monodon* lymphoid organs.

Putative gene	Closest species	Score	E Value	Length (bp)	Frequency		
					Normal	Infected	Total
40s ribosomal protein s12	*Schizosaccharomyces pombe*	49	4.00E-11	600	-	1	1
60s ribosomal protein L5A		234	4.00E-61	591	-	1	1
acidic ribosomal protein P1	*Artemia salina*	154	2.00E-37	421	-	2	2
ribosomal protein L10	*Bombyx mandarina*	297	4.00E-80	543	-	3	3
ribosomal protein L10a	*Spodoptera frugiperda*	315	3.00E-85	600	-	1	1
ribosomal protein L11	*Drosophila melanogaster*	149	9.00E-59	558	-	1	1
ribosomal protein L17	*Rattus norvegicus*	256	2.00E-67	688	1		1
ribosomal protein L18	*Plasmodium yoelii*	49	9.00E-06	311	-	1	1
ribosomal protein L18a	*Homo sapiens*	249	1.00E-65	597	1	1	2
ribosomal protein L21	*Ictalurus punctatus*	211	3.00E-54	538	-	3	3
ribosomal protein L23	*Homo sapiens*	257	3.00E-68	456	-	1	1
ribosomal protein L24	*Spodoptera frugiperda*	194	3.00E-49	501	-	1	1
ribosomal protein L27a	*Oncorhynchus mykiss*	249	1.00E-65	511	-	1	1
ribosomal protein L28	*Spodoptera frugiperda*	139	1.00E-32	514	-	1	1
ribosomal protein L3	*Spodoptera frugiperda*	138	6.00E-32	345	-	1	1
ribosomal protein L34	*Spodoptera frugiperda*	143	1.00E-33	426	1	1	2
ribosomal protein L37	*Spodoptera frugiperda*	155	1.00E-37	334	1	-	1
ribosomal protein L39	*Spodoptera frugiperda*	94	3.00E-19	257	-	2	2
ribosomal protein L44	*Aedes triseriatus*	182	6.00E-46	383	-	2	2
ribosomal protein L5	*Gallus gallus*	226	2.00E-75	600	2	1	3
ribosomal protein L7	*Spodoptera frugiperda*	229	2.00E-59	600	-	2	2
ribosomal protein L8	*Anopheles gambiae*	199	5.00E-51	385	-	1	1
ribosomal protein L9	*Drosophila melanogaster*	248	3.00E-65	590	-	1	1
ribosomal protein S10	*Mus musculus*	210	8.00E-54	516	3	1	4
ribosomal protein S12	*Branchiostoma belcheri*	211	8.00E-54	521	-	1	1
ribosomal protein S15	*Homo sapiens*	249	8.00E-66	475	1	1	2
ribosomal protein S17	*Ictalurus punctatus*	219	7.00E-57	429	-	3	3
ribosomal protein S19	*Mya arenaria*	196	1.00E-49	519	-	1	1
ribosomal protein S2	*Urechis caupo*	373	0	600	-	2	2
ribosomal protein S20	*Chlamys farreri*	192	1.00E-48	470	-	2	2
ribosomal protein S23	*Spodoptera frugiperda*	283	7.00E-76	500	1	1	2
ribosomal protein S25	*Drosophila melanogaster*	141	2.00E-33	302	-	1	1
ribosomal protein S26	*Rattus norvegicus*	170	8.00E-42	396	1	-	1
ribosomal protein S27	*Homarus americanus*	177	2.00E-44	376	-	4	4
ribosomal protein S27a	*Branchiostoma belcheri tsingtaunese*	117	4.00E-26	264	-	1	1
ribosomal protein S28	*Ictalurus punctatus*	95	9.00E-21	337	2	-	2
ribosomal protein S3	*Spodoptera frugiperda*	269	2.00E-71	600	1	2	3
ribosomal protein S30	*Spodoptera frugiperda*	96	3.00E-22	338	-	1	1
ribosomal protein S4	*Argopecten irradians*	149	6.00E-66	600	-	1	1
ribosomal protein S7	*Mus musculus*	162	3.00E-39	600	-	1	1
ribosomal protein S9	*Ictalurus punctatus*	310	1.00E-83	654	1	-	1
ribosomal protein XL1a	*Xenopus laevis (African clawed frog)*	281	3.00E-75	600	-	1	1
ribosomal prtein L17	*Mus musculus*	203	8.00E-52	523	-	1	1

Table 3.10 The putative gene transcripts in the group of mitochondrial protein, isolated from the normal and *V. harveyi*-infected *P. monodon* lymphoid organs.

Putative gene	Closest species	Score	E Value	Length (bp)	Frequency		
					Normal	Infected	Total
ATP synthase	*Penaeus monodon*	338	2.00E-92	600	-	3	3
ATP synthase F0 subunit 6	*Anopheles gambiae*	120	3.00E-42	600	-	1	1
ATPase 6	*Anastrepha suspensa*	108	8.00E-44	600	-	1	1
ATPase of high-affinity potassium transport system	*Escherichia coli K12*	414	0	800	1	-	1
ATP-binding protein	*Caenorhabditis elegans*	184	9.00E-46	600	-	1	1
cytochrome b	*Penaeus monodon*	146	5.00E-35	283	1	-	1
cytochrome c oxidase	*Mus musculus*	69	9.00E-12	409	1	1	2
cytochrome c oxidase subunit I	*Penaeus monodon*	370	0	600	5	5	10
cytochrome c oxidase subunit II	*Penaeus monodon*	364	0	580	3	6	9
cytochrome c oxidase subunit III	*Penaeus monodon*	358	3.00E-98	600	1	3	4
cytochrome c oxidase subunit IV	*Urechis caupo*	114	1.00E-24	600	-	1	1
cytochrome c oxidase subunit Va	*Rhyzopertha dominica*	209	2.00E-53	736	1	1	2
cytochrome c oxidase subunit VII	*Mus musculus*	65	2.00E-10	461	1	-	1
cytochrome c reductase complex	*Homo sapiens*	72	4.00E-12	600	1	-	1
cytochrome oxidase subunit I	*Pagurus longicarpus*	68	2.00E-21	286	-	1	1
cytochrome P450-3	*Musa acuminata*	63	2.00E-09	600	-	1	1
NADH dehydrogenase subunit 1	*Penaeus monodon*	333	9.00E-91	721	1	4	5
NADH dehydrogenase subunit 5	*Penaeus monodon*	350	1.00E-95	660	1	-	1
oligomycin sensitivity-conferring protein	*Drosophila melanogaster*	57	4.00E-08	418	1	-	1

Table 3.11 The putative gene transcripts in the transport category, isolated from the normal and *V. harveyi*-infected *P. monodon* lymphoid organs.

Putative gene	Closest species	Score	E Value	Length (bp)	Frequency		
					Normal	Infected	Total
ADP-ATP translocator	*Ethmostigmus rubripes*	345	3.00E-94	690	-	1	1
balbiani RING protein 3	*Chironomus tentans*	53	2.00E-06	600	-	2	2
calreticulin	*Anopheles gambiae*	255	0	600	-	1	1
chloride conductance regulatory protein	*Xenopus laevis*	107	1.00E-22	600	-	1	1
karyopherin alpha 2	*Homo sapiens*	249	2.00E-65	600	2	-	2
low density lipoprotein	*Mus musculus*	75	6.00E-13	600	1	-	1
N-ethylmaleimide	*Drosophila melanogaster*	119	2.00E-26	600	-	1	1
S-methylmethionine permeas		331	4.00E-90	655	1	-	1
zinc transporter	*Homo sapiens*	85	9.00E-16	762	1	-	1

3.3.2.10 Miscellaneous function

The 47 clones, representing 19 different genes, were identified as having miscellaneous function (Table 3.12). The most redundant transcript in the infected library was peritrophin 1 (14 clones). Likewise, peritrophin 2 was redundant in the infected EST library. Other genes were breast carcinoma, epididymal secretory protein, gag-pol polyprotein, glutaredoxin 1 redox coenzyme, etc.

3.3.2.11 Unidentified (hypothetical)-similar to other cDNA/DNA

Another 69 clones were homologous to the other genes with unidentified function (Table 3.13). The clones in this category showed that the most number of closest species as *Anopheles gambiae* was found.

3.3.2.12 Unknown genes

As many as 266 unmatched ESTs were identified from the normal library (59.6%) and 356 from the infected library (55.5%) as shown in Table 3.2.

Table 3.12 The gene transcripts in the miscellaneous function category, isolated from the normal and *V. harveyi*-infected *P. monodon* lymphoid organs.

Putative gene	Closest species	Score	E Value	Length (bp)	Frequency		
					Normal	Infected	Total
breast carcinoma	*Homo sapiens*	192	3.00E-48	600	-	1	1
epididymal secretory protein	*Danio rerio*	86	2.00E-16	600	2	1	3
gag-pol polyprotein	*Oryza sativa*	60	3.00E-13	585	-	1	1
glutaredoxin1 redox coenzyme	*Escherichia coli*	131	5.00E-30	518	1	-	1
hepatitis B virus x-interacting protein	*Homo sapiens*	73	2.00E-12	597	-	1	1
I factor protein 1	*Drosophila teissieri*	58	7.00E-08	600	1	-	1
lysosomal protective protein	*Mus musculus*	119	2.00E-32	600	-	1	1
membrane protein	*Caenorhabditis elegans*	87	9.00E-17	600	1	-	1
nucleoporin p54	*Rattus norvegicus*	97	1.00E-19	800	1	-	1
ocular development-associated gene	*Homo sapiens*	186	2.00E-46	514	1	-	1
oligomycin	*Drosophila melanogaster*	211	4.00E-54	600	2	-	2
peritrophin 1	*Penaeus monodon*	429	0	600	-	14	14
peritrophin 2	*Penaeus monodon*	346	1.00E-94	600	-	11	11
pol protein	*Aedes aegypti*	68	4.00E-11	600	1	-	1
polyprotein precursor	*gill-associated virus*	415	1.00E-115	600	-	3	3
semaphorin 2A precursor	*Schistocerca gregaria*	216	1.00E-55	600	1	-	1
testis specific protein	*Rattus norvegicus*	129	2.00E-29	524	-	1	1
transmembrane protein	*Drosophila melanogaster*	67	9.00E-11	600	-	1	1
Vitellogenin		54	7.00E-07	502	-	1	1

Table 3.13 The gene transcripts in the unidentified (hypothetical)-similar to other cDNA/DNA category, isolated from the normal and *V. harveyi*-infected *P. monodon* lymphoid organs.

Putative gene	Species	Score	E Value	Length (bp)	Frequency		
					Normal	Infected	Total
agCP14035	*Anopheles gambiae str. PEST*	62	5.00E-09	600	-	1	1
agCP14148	*Anopheles gambiae*	52	1.00E-06	442	-	1	1
agCP4709	*Anopheles gambiae*	114	1.00E-24	659	-	1	1
agCP6418	*Anopheles gambiae str. PEST*	180	1.00E-44	600	-	1	1
agCP8467	*Anopheles gambiae str. PEST*	252	3.00E-66	600	-	1	1
CDNA FLJ12480 FIS	*Dictyostelium discoideum*	71	2.00E-12	600	-	1	1
CG2839-PA	*Drosophila melanogaster*	102	4.00E-21	573	-	1	1
CG32464-PK	*Drosophila melanogaster*	135	4.00E-31	600	-	1	1
ebiP4168	*Anopheles gambiae str. PEST*	60	5.00E-09	435	1	-	1
expressed sequence AI649009	*Mus musculus*	89	3.00E-17	600	1	-	1
homeobox domain	*Caenorhabditis elegans*	60	2.00E-08	600	-	1	1
HSPC177	*Homo sapiens*	193	2.00E-59	600	-	1	1
hypothetical protein	*Anopheles gambiae*	86	2.00E-16	575	-	1	1
hypothetical protein	*Anopheles gambiae str. PEST*	85	6.00E-16	600	1	-	1

Table 3.13 (continue).

Putative gene	Species	Score	E Value	Length (bp)	Frequency		
					Normal	Infected	Total
hypothetical protein	*Anopheles gambiae str. PEST*	68	2.00E-11	454	1	-	1
hypothetical protein	*Anopheles gambiae str. PEST*	99	3.00E-20	624	1	-	1
hypothetical protein	*Anopheles gambiae str. PEST*	53	1.00E-06	600	-	1	1
hypothetical protein	*Anopheles gambiae str. PEST*	90	1.00E-17	600	-	1	1
hypothetical protein	*Anopheles gambiae str. PEST*	54	8.00E-07	600	-	1	1
hypothetical protein	*Anopheles gambiae str. PEST*	99	1.00E-20	482	-	1	1
hypothetical protein	*Anopheles gambiae str. PEST*	139	3.00E-32	539	-	1	1
hypothetical protein	*Anopheles gambiae str. PEST*	90	1.00E-17	600	-	1	1
hypothetical protein	*Arabidopsis thaliana*	56	2.00E-07	600	2	-	2
hypothetical protein	*Bacteriophage I*	302	3.00E-81	800	1	-	1
hypothetical protein	*Carassius auratus*	42	1.00E-05	600	5	6	11
hypothetical protein	*Dictyostelium discoideum*	97	1.00E-19	600	-	1	1
hypothetical protein	*Dictyostelium discoideum*	74	6.00E-13	488	-	1	1
hypothetical protein	*Drosophila melanogaster*	69	3.00E-11	600	1	-	1
hypothetical protein	*Drosophila melanogaster*	127	3.00E-30	492	1	-	1
hypothetical protein	*Drosophila melanogaster*	87	3.00E-21	394	1	-	1
hypothetical protein	*Homo sapiens*	108	5.00E-23	600	1	-	1
hypothetical protein	*Homo sapiens*	73	2.00E-12	600	1	-	1
hypothetical protein	*Mus musculus*	55	8.00E-07	600	1	-	1
hypothetical protein	*Neurospora crassa*	69	5.00E-11	800	1	-	1
hypothetical protein	*Plasmodium falciparum 3D7*	50	1.00E-05	679	1	-	1
hypothetical protein CaO19.64	*Candida albicans SC5314*	100	5.00E-44	683	-	1	1
hypothetical protein	*Homo sapiens*	97	8.00E-20	600	1	-	1
hypothetical protein FLJ20174	*Homo sapiens*	73	2.00E-12	600	1	-	1
hypothetical protein XP_058990	*Homo sapiens*	84	9.00E-16	600	1	-	1
hypoxia induced gene HIG1	*Rattus norvegicus*	78	5.00E-14	600	-	1	1
insertion element	*Escherichia coli*	270	2.00E-71	800	1	-	1
hypothetical protein	*Caenorhabditis elegans*	50	2.00E-05	451	1	-	1
ORF167	*shrimp white spot syndrome virus*	96	1.00E-19	513	1	-	1
orf265_2	*Paramecium aurelia*	52	2.00E-06	380	-	1	1
hypothetical protein	*Homo sapiens*	63	1.00E-09	600	1	-	1
RIKEN cDNA	*Mus musculus*	65	1.00E-09	600	-	3	3
RIKEN cDNA 0610012D14	*Mus musculus*	179	4.00E-44	800	1	-	1
RIKEN cDNA 1110061A19	*Mus musculus*	98	4.00E-20	483	1	-	1
RIKEN cDNA 1810034M08	*Mus musculus*	134	1.00E-30	607	-	1	1
RIKEN cDNA 2010003O14	*Mus musculus*	162	4.00E-39	600	1	-	1
RIKEN cDNA 2610510L01	*Mus musculus*	105	5.00E-22	800	1	-	1
RIKEN cDNA 2610510L01	*Mus musculus*	85	4.00E-16	597	-	1	1
SD04906p	*Drosophila melanogaster*	51	7.00E-06	600	1	-	1
hypothetical protein	*Homo sapiens*	192	2.00E-48	600	-	1	1
TB2		211	5.00E-54	600	-	1	1
unnamed protein product	*Homo sapiens*	47	5.00E-05	213	1	-	1

3.4 Microarray analysis

3.4.1 Shrimp cDNA microarray preparation

The shrimp cDNA microarray slide, containing 1026 cDNA clones, was successfully prepared from the cDNA clones of two penaeid shrimps, *P. monodon* (718 clones) and *Marsopenaeus japonicus* (308 clones). The complete data set has been submitted to and accepted by the ArrayExpress, the microarray database of the European Bioinformatics Institute (http://www.ebi.ac.uk/ arrayexpress/). The accession number is A-MEXP-306.

3.4.2 Fluorescent labeled cDNA probe preparation

Haemolymph, approximately 1 ml per individual, was collected from the control (0.85% NaCl and LHM) and pathogens (WSSV or *V. harveyi*)-injected shrimps. The haemolymph was centrifuged to separate the haemocytes from the plasma. The haemocytes were used to prepare a total RNA using Trizol reagent. The A_{260}/A_{280} ratio of the total RNA preparation was 1.6-1.8 indicating the acceptable quality of the total RNA used in this study. The average total RNA obtained from the 0.85% NaCl-injected and LHM-injected shrimps were approximately 22 μg and 18 μg per individual, respectively, whereas those obtained from *V. harveyi*-challenged and WSSV-challenged shrimps were 17 μg and 15 μg per individual, respectively. The total RNAs from 10 individual shrimps at each time point of each experiment were pooled, and used in a cDNA probe preparation.

3.4.3 Microarray hybridization

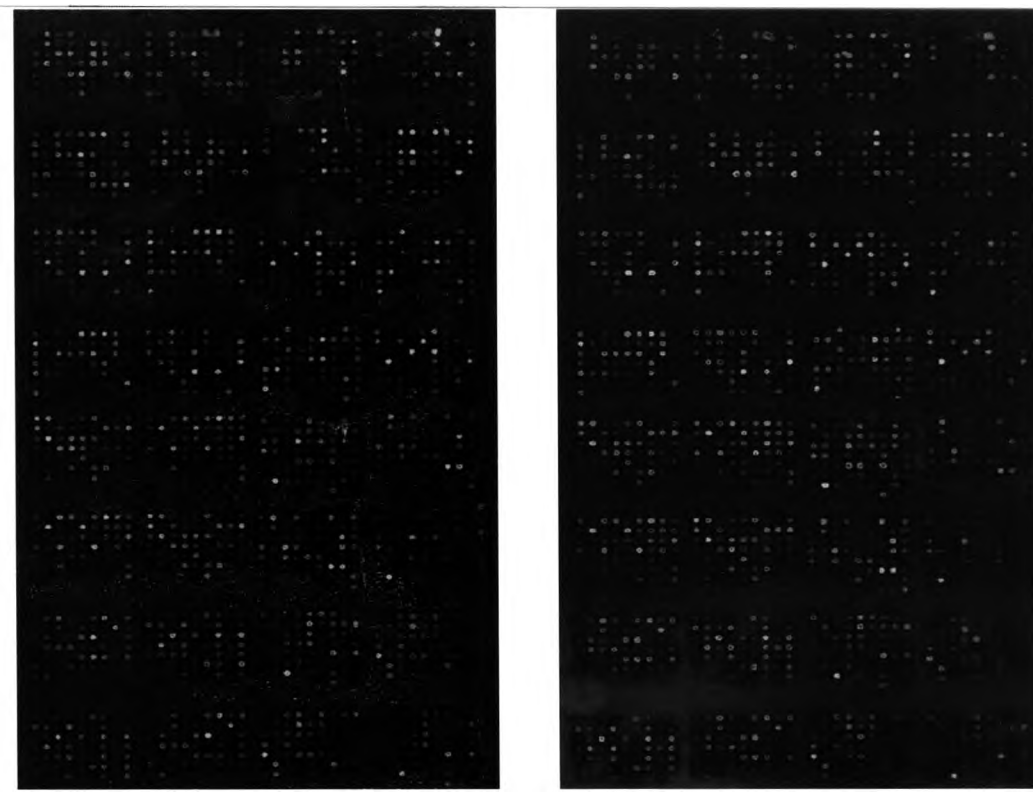
The fluorescent cDNA probes were successfully generated using the commercial kit as described above. The Cy3 probes from the control and Cy5 probes from the pathogen-challenged experiments were used to hybridize onto the cDNA microarray slides. The hybridized slides were washed, dried and immediately scanned.

The resulting images were analyzed by measuring the fluorescence of all features (spots) on the slides using the GenePix Pro 3.0 software (Axon

Instruments). The fluorescence of each pixel within each feature was determined, and the median fluorescence of these pixel measurements was taken as the measure of fluorescence for the whole feature. Image artifacts as a result of contamination, impurities in the surface, or very bright spots noted in features during visual inspection of arrays were flagged as 'bad features', and rejected from the analysis. After subtracting the background fluorescence, differences in the Cy5 and Cy3 incorporation efficiencies were corrected by global normalization. The results were reported as the gene expression ratio that was the ratio of the intensities of Cy5/Cy3. The Cy5/Cy3 signal intensity was calculated from the intensity means of duplet spots. Genes with feature ratios over 2.0 and under 0.5 were considered as up-regulated genes and down-regulated genes, respectively. The data management and expression analysis were performed according to the MIAME (Minimum Information About a Microarray Experiment) checklist (Brazma *et al.*, 2001). Each feature image represented each cDNA clone as shown in Fig 3.5. The red and the green colors represented the Cy5 and Cy3 fluorescent dyes, respectively. An equal combination of the two dyes resulted in a yellow feature.

A total of 1,026 distinct genes: 718 genes from *P. monodon* and 308 genes from *M. japonicus*, in the shrimp cDNA microarrays were used in this study. Among these, the functions of 489 genes were unknown. After examination, the numbers of up- and down-regulated genes in the haemocytes of pathogen-challenged shrimps were recorded as shown in Table 3.14.

In WSSV-challenged shrimps, the total numbers of up- and down-regulated genes were 30 (2.92%), 135 (13.16%), 123 (11.99%) and 129 (12.57%) at 6, 24, 48 and 72 hours post injection (hpi), respectively. The highest number of up-regulated gene was observed at 72 hpi (82 genes) whereas a large number of down-regulated gene was found at 24 hpi (72 genes). The fold changes of the gene expression in shrimp haemocytes due to WSSV challenge are shown in Table 3.15.



(a) (b)

Figure 3.5 The analysis of gene expression profile of black tiger shrimp at 6 hours post WSSV (a) and *Vibrio harveyi* (b) injection.

Table 3.14 The numbers of up- and down-regulated genes in haemocytes of the black tiger shrimp after injection with WSSV and *V. harveyi*

		• WSSV				• VH		
		6h	**24h**	**48h**	**72h**	**6h**	**24h**	**48h**
Up	Known	19	28	32	38	18	0	9
	Unknown	9	35	33	44	47	12	26
	Total	28	63	65	82	65	12	35
Down	Known	1	13	18	10	34	2	18
	Unknown	1	59	40	37	57	3	17
	Total	2	72	58	47	91	5	35
• **Total**		**30** (2.92%)	**135** (13.16%)	**123** (11.99%)	**129** (12.57%)	**156** (15.20%)	**17** (1.66%)	**70** (6.82%)

For *V. harveyi*-challenged shrimps, only 3 time points (6, 24 and 48 hours) of the haemocytes sampling were examined. The numbers of up- and down-regulated genes were 156 (15.20%), 17 (1.66%) and 70 (6.82%) at 6, 24 and 48 hpi, respectively. The highest numbers of up- and down-regulated genes were observed at 6 hpi. The fold changes of the gene expression in shrimp haemocytes due to *V. harveyi* challenge are shown in Table 3.16.

Since the cDNA microarray chips was prepared from the cDNA clones of the 2 penaeid shrimps, *P. monodon* and *Marsupenaeus japonicus*, and the cDNA probes was from the *P. monodon*, it was quite surprising that only a small number of *M. japonicus* gene spots hybridized with the cDNA probes.

3.4.4 cDNA microarray data mining

The microarray data were further analyzed by Gene Cluster 3 analysis to explore the relationship among the expressed genes in the haemocytes of the WSSV and *V. harveyi*-challenged shrimps. The data was filtered and further examined by hierarchical clustering analysis. A resulting clustering tree represents the relationships among genes, whose branch lengths reflect the degree of similarity among the genes. The hierarchical clustering and dendrogram of genes from the haemocytes of WSSV-injected shrimp at four different time points were shown in Fig 3.6. The 96 genes were filtered and grouped into two clusters according to the following expression patterns. Group A, consisting of 74 genes, was characterized by elevated expression at a late time (72 hpi). This group, with increased expression at 72 hpi, mainly encoded ribosomal proteins and several unknown genes. In addition, nucleoside diphosphate kinase, actin, and MAC25 protein were also found.

Cluster of 22 genes in group B were characterized by their increase in expression at 24 to 48 hpi. Some ribosomal proteins were transiently expressed at 24 and 48 hpi. Asialoglycoprotein receptor (ASGPR), an endocytic glycoprotein receptor, and cytochrome C oxidase subunit VIIc were also found in this group.

The hierarchical clustering of microarray data from the *V. harveyi*-challenge experiments failed to identify gene clusters due to an inconsistent hybridization results across the three different time points (data not shown).

Table 3.15 List of genes responded to white spot syndrome virus challenge at different time points.

WSSV challenge at 6 hpi

Description	Fold change	Description	Fold change
40s ribosomal protein S24	10.35	60s acidic ribosomal protein P2	2.59
asialoglycoprotein recepter	7.12	RpS25 gene product	2.55
cellular nucleic acid binding protein	5.05	40S ribosomal protein S19	2.30
tubulin beta-1 chain	4.70	60S ribosomal protein L6	2.22
60S ribosomal protein L27	4.62	kupffer cell receptor	0.43
60S ribosomal protein L7A	4.30	uk (7-c12)	4.18
elongation factor 2	4.24	uk (6-B11)	4.07
von Hippel-Lindau binding protein 1	3.67	uk (7-H3)	3.86
40S ribosomal protein S9	3.43	uk (2-A12)	3.04
cytosolic manganese superoxide dismutase percursor	3.41	uk (2-B12)	2.40
b-actin	3.17	uk (4-A4)	2.21
40s ribosomal protein S25	3.06	uk (7-F4)	2.12
60S ribosomal protein L38	2.98	uk (1-H11)	2.03
60Sacidic ribosomal protein P2	2.78	uk (2-F4)	2.01
40s ribosomal protein S10	2.61	uk (8-B8)	0.09

WSSV challenge at 24 hpi

Description	Fold change	Description	Fold change
60S ribosomal protein L34	24.10	uk (2-H3)	2.46
ribosomal protein L30	21.66	uk (4-H11)	2.37
14-3-3-like protein	19.33	uk (7-B4)	2.36
Rat insulinoma gene-Rig	8.92	uk (2-G4)	2.33
asialoglycoprotein recepter	8.72	uk (2-G10)	2.31
ribosomal protin eL12	7.51	uk (2-F2)	2.26
60s ribosomal protein L13	6.16	uk (3-B12)	2.08
60S ribosomal protein L7A	5.80	uk (3-A12)	2.05
calmodulin	5.63	uk (4-H4)	2.03
60a ribosomal protein L27A	5.37	uk (5-G3)	2.02
proteasome subunit beta type 2	4.49	uk (3-G8)	2.02
QM protein	3.84	uk (2-G5)	0.49
chain H, cytochrome Bc1 complex	3.81	uk (2-H9)	0.49
calcium-sensitive chloride channel 2	3.27	uk (9-H1)	0.48
ATP synthase alpha chain, mitochondrial precursor	3.24	uk (3-E1)	0.48
asialoglycoprotein recepter	3.16	uk (3-D7)	0.46
60Sacidic ribosomal protein P2	3.02	uk (6-F5)	0.46
60S ribosomal protein L7A	2.90	uk (7-H9)	0.46
S3 ribosomal protein	2.68	uk (5-E9)	0.46
40S ribosomal protein S27	2.65	uk (6-F6)	0.45
ribosomal protein S18	2.52	uk (4-B1)	0.45
integral membrance protein 2A	2.39	uk (5-H8)	0.45
40s ribosomal protein S10	2.37	uk (7-G1)	0.44
cytochrome c oxidase subunit VIIc	2.24	uk (3-D10)	0.44
		uk (5-F3)	0.44

WSSV challenge at 24 hpi (continue)

Description	Fold change	Description	Fold change
ribosomal protein S5	2.19	uk (3-C5)	0.44
60S ribosomal protein L19	2.16	uk (5-F12)	0.43
60S ribosomal protein L6	2.10	uk (5-D7)	0.43
hypothetical protein	0.49	uk (7-C3)	0.43
beta tub 56D gene product (alt 1)	0.47	uk (7-E12)	0.42
gene MAC25 protein - human	0.42	uk (1-B10)	0.41
CG6848 gene product	0.38	uk (7-G7)	0.41
actin related protein	0.34	uk (8-B10)	0.40
hemocyte protein-glutamine gamma-glutamyltransferase	0.34	uk (4-G12)	0.39
tubulin alpha-1 chain	0.26	uk (7-F10)	0.39
gene MAC25 protein	0.21	uk (7-G11)	0.39
probable reverse transcriptase	0.13	uk (3-H5)	0.38
Penaeidin-2 precusor	0.07	uk (3-E7)	0.37
agrin precursor	0.07	uk (6-G6)	0.37
penaeidin-3k	0.06	uk (6-C2)	0.37
11.5 kDa antibacterial protein	0.06	uk (3-A2)	0.35
uk (5-H10)	22.66	uk (1-F3)	0.35
uk (3-D8)	20.31	uk (6-A6)	0.35
uk (8-C7)	14.66	uk (1-G10)	0.34
uk (1-H12)	11.55	uk (8-B3)	0.33
uk (3-H8)	9.81	uk (6-F11)	0.32
uk (2-C6)	8.74	uk (6-H5)	0.32
uk (4-E10)	8.37	uk (3-A1)	0.32
uk (1-H11)	8.31	uk (6-F7)	0.32
uk (2-D10)	6.45	uk (1-C1)	0.31
uk (1-H7)	6.38	uk (6-G1)	0.30
uk (1-G3)	4.66	uk (2-G1)	0.28
uk (4-G8)	4.55	uk (8-D8)	0.24
uk (2-H11)	4.36	uk (5-F9)	0.24
uk (2-H7)	4.04	uk (3-H9)	0.18
uk (2-F10)	3.91	uk (2-A7)	0.14
uk (7-H4)	3.75	uk (7-F4)	0.13
uk (1-E7)	3.23	uk (3-E3)	0.11
uk (5-G4)	3.18	uk (6-B7)	0.09
uk (2-F7)	3.17	uk (5-C10)	0.08
uk (7-C8)	3.14	uk (4-G10)	0.07
uk (2-D3)	2.97	uk (7-H5)	0.07
uk (1-E9)	2.93	uk (7-F5)	0.06
uk (1-C11)	2.69	uk (2-D9)	0.05
uk (7-E3)	2.66	uk (5-H2)	0.03
uk (5-A3)	2.63	uk (5-C9)	0.02
uk (2-F4)	2.61	uk (8-F8)	0.02
uk (5-G11)	2.51	uk (5-F8)	0.02
uk (1-H6)	2.50	uk (8-B8)	0.02
		uk (6-E1)	0.02

WSSV challenge at 48 hpi

Description	Fold change	Description	Fold change
translocon-associated protein	24.33	uk (2-E12)	5.77
calmodulin	22.90	uk (6-G7)	5.20
tubulin beta-1 chain	15.54	uk (6-G4)	4.84
60S ribosomal protein L7A	7.85	uk (1-D3)	4.39
ribosomal protin eL12	6.70	uk (5-A3)	4.29
arginine kinase	6.41	uk (2-C6)	3.83
60S ribosomal protein L34	6.15	uk (3-D1)	3.57
60S ribosomal protein L10A	5.85	uk (7-H4)	3.27
60a ribosomal protein L27A	4.90	uk (3-C7)	3.09
myosin regulatory light chain	4.87	uk (7-F9)	3.08
ribosomal protein S18	4.81	uk (1-G3)	2.90
40S ribosomal protein	4.56	uk (8-B5)	2.81
elongation factor 2	4.45	uk (7-E3)	2.78
alpha-2-tubulin	4.24	uk (3-A12)	2.71
ribosomal protein S5	4.23	uk (8-C11)	2.40
ubiquitin-like protein / ribosomal protein S30	3.97	uk (5-G10)	2.36
actin 1	3.86	uk (2-B12)	2.28
H3	3.66	uk (2-F7)	2.28
60Sacidic ribosomal protein P2	3.65	uk (8-D4)	2.19
60s ribosomal protein L13	3.33	uk (2-F4)	2.17
ribosomal protein L24	3.22	uk (6-B1)	2.16
integral membrance protein 2A	3.04	uk (5-C3)	2.14
40S ribosomal protein S24	2.83	uk (12-A7)	2.12
ribosomal protein S28	2.73	uk (6-D3)	0.49
alpha-tubulin	2.47	uk (7-E6)	0.48
actin, cytoplasmic A3	2.40	uk (7-G6)	0.48
60S ribosomal protein L6	2.33	uk (1-C9)	0.48
hemocyte protein-glutamine gamma-glutamyltransferase	2.32	uk (6-F6)	0.47
acyl coenzyme A dehydrogenase, long chain	2.29	uk (6-E2)	0.46
40s ribosomal protein s7	2.21	uk (7-A11)	0.46
14-3-3-like protein	2.17	uk (5-E6)	0.46
QM protein	2.11	uk (1-G2)	0.46
hypothetical 36.9 kDa protein	2.00	uk (9-H1)	0.46
mitochondrial 1rRNA gene, partial 3' end	0.48	uk (6-C6)	0.45
mitochondrial rRNA gene	0.48	uk (2-G5)	0.45
16S rRNA gene	0.47	uk (7-H9)	0.42
gene MAC25 protein	0.46	uk (3-E9)	0.41
partial mitochondrial 16S rRNA gene	0.46	uk (3-C5)	0.40
mitochondrial, complete genome	0.46	uk (2-D7)	0.39
probable reverse transcriptase	0.46	uk (5-F9)	0.38
16S rRNA gene	0.45	uk (6-E11)	0.37
profilin	0.44	uk (8-B3)	0.35
16S rRNA gene	0.43	uk (2-H2)	0.31
40S ribosomal protein S	0.42	uk (5-A2)	0.31
16S ribosomal RNA gene	0.41	uk (7-D7)	0.27
actin related protein	0.31	uk (6-F7)	0.24
agrin precursor	0.23	uk (5-H2)	0.13

WSSV challenge at 48 hpi (continue)

Description	Fold change	Description	Fold change
11.5 kDa antibacterial protein	0.12	uk (2-A7)	0.12
cytochrome c oxidase subunit 2	0.06	uk (8-D2)	0.11
Penaeidin-2 precusor	0.02	uk (3-E3)	0.08
penaeidin-3k	0.01	uk (5-C9)	0.08
uk (4-A7)	31.02	uk (9-F8)	0.06
uk (7-F6)	21.11	uk (5-C10)	0.04
uk (3-B3)	17.21	uk (8-F8)	0.04
uk (2-F11)	16.40	uk (6-B7)	0.03
uk (1-H7)	14.34	uk (6-E1)	0.02
uk (3-H7)	12.69	uk (7-H5)	0.02
uk (7-F2)	12.36	uk (7-F5)	0.01
uk (7-A6)	10.72	uk (4-G10)	0.01
uk (4-D11)	9.56	uk (5-F8)	0.01
uk (2-F10)	5.90	uk (8-F11)	0.01
		uk (7-A12)	0.01

WSSV challenge at 72 hpi

Description	Fold change	Description	Fold change
gene MAC25 protein	22.51	uk (2-E11)	5.49
calmodulin	20.78	uk (7-E12)	5.41
60S ribosomal protein L14	18.57	uk (7-B4)	4.79
60S ribosomal protein L34	17.51	uk (1-B10)	4.68
putative large subunit ribosomal protein rpL 44	17.35	uk (2-F10)	4.66
60S ribosomal protein L7A	15.35	uk (7-G1)	4.07
heat shock cognate 70 kDa protein	13.78	uk (3-A1)	3.97
ribosomal protein L31	12.42	uk (6-G4)	3.84
cytoplasmic beta chain	12.37	uk (2-F8)	3.78
ribosomal protein L28	12.08	uk (7-E10)	3.71
40S ribosomal protein	11.01	uk (8-B4)	3.64
gene MAC25 protein	8.00	uk (7-C12)	3.41
beta tub 56D gene product (alt 1)	6.50	uk (4-B1)	3.17
hypothetical protein F08F1.8	6.44	uk (1-D1)	3.14
60S ribosomal protein L37A	5.34	uk (3-A2)	3.03
16S rRNA gene	5.08	uk (7-D12)	2.96
60S ribosomal protein L10A	3.96	uk (3-D10)	2.92
ATP synthase FO subunit 6	3.74	uk (5-G11)	2.85
ubiquitin-like protein / ribosomal protein S30	3.52	uk (38323)	2.85
60Sacidic ribosomal protein P2	3.27	uk (1-C4)	2.82
heterogeneous ribonucleoprotein A, A2,B1	3.20	uk (2-G6)	2.72
beta - actin	3.10	uk (1-F1)	2.68
5S ribosomal protein	3.00	uk (1-C1)	2.54
40S ribosomal protein S3a	2.99	uk (2-H2)	2.35
nucleoside diphosphate kinase	2.96	uk (3-D7)	2.32
40s ribosomal protein S10	2.86	uk (2-D3)	2.24
60S ribosomal protein -like	2.72	uk (3-E7)	2.05
H3	2.66	uk (2-G1)	2.04
60Sacidic ribosomal protein P2	2.60	uk (1-E7)	2.03

WSSV challenge at 72 hpi (continue)

Description	Fold change	Description	Fold change
double stranded RNA binding nuclear protein, ILF3	2.59	uk (6-E9)	0.49
60a ribosomal protein L27A	2.44	uk (8-A5)	0.49
H3 histone	2.43	uk (8-A12)	0.48
40S ribosomal protein S27	2.42	uk (7-D11)	0.46
ribosomal protin eL12	2.27	uk (2-H9)	0.46
actin 3	2.24	uk (2-D7)	0.45
60S ribosomal protein L19	2.22	uk (6-F1)	0.44
40S ribosomal protien S4	2.22	uk (4-A6)	0.43
ribosomal protein S5	2.21	uk (4-G12)	0.43
40S ribosomal protein S32	2.04	uk (1-G1)	0.42
histone 1	0.49	uk (1-G1)	0.42
16S rRNA gene	0.49	uk (7-H9)	0.41
eukaryotic translation initiation factor 3, subunit 2	0.47	uk (8-F6)	0.40
mitochondrial 1rRNA gene, partial 3' end	0.40	uk (7-C8)	0.38
NADH ubiquinone oxidoreductase chain 6	0.33	uk (5-G4)	0.36
11.5 kDa antibacterial protein	0.27	uk (6-F6)	0.35
CG6848 gene product	0.14	uk (3-C5)	0.33
cytochrome c oxidase subunit VIIc	0.13	uk (6-F7)	0.24
Penaeidin-2 precusor	0.04	uk (8-B3)	0.23
penaeidin-3k	0.02	uk (7-D7)	0.22
uk (1-D3)	47.71	uk (5-C9)	0.21
uk (8-G9)	31.78	uk (5-D10)	0.20
uk (2-F11)	22.44	uk (5-F9)	0.18
uk (8-D4)	21.97	uk (5-A2)	0.10
uk (2-H7)	13.83	uk (8-F8)	0.10
uk (2-H11)	10.99	uk (3-E3)	0.09
uk (6-D2)	10.89	uk (8-D2)	0.07
uk (1-B6)	10.03	uk (9-F8)	0.05
uk (3-D1)	9.56	uk (4-B8)	0.05
uk (3-H5)	9.16	uk (2-H5)	0.05
uk (4-D11)	7.71	uk (7-F5)	0.04
uk (3-B3)	7.52	uk (6-B7)	0.03
uk (6-B8)	6.83	uk (4-G10)	0.03
uk (6-D9)	6.74	uk (6-E1)	0.03
uk (8-D8)	6.41	uk (7-H5)	0.03
		uk (7-A12)	0.02
		uk (5-F8)	0.01

Table 3.16 List of genes responsed to *V. harveyi* challenge at different time points.

***V. harveyi* challenge at 6 hpi**

Description	Fold change	Description	Fold change
arginine kinase	21.85	uk (3-H8)	3.03
selenoprotein w muscle 1	16.87	uk (8-A10)	3.00
actin 1	13.00	uk (1-C9)	2.99
cytoplasmic beta chain	11.44	uk (1-C1)	2.95
60s ribosomal protein L13	10.80	uk (1-H6)	2.90
beta - actin	10.11	uk (6-D2)	2.88
tubulin beta-1 chain	7.34	uk (2-F5)	2.85
60s ribosomal protein L39	5.31	uk (8-D8)	2.76
60S ribosomal protein L6	4.80	uk (2-H11)	2.73
calmodulin	4.67	uk (2-E1)	2.72
actin, cytoplasmic A3	3.74	uk (1-E7)	2.58
60S ribosomal protein L7A	3.57	uk (4-D11)	2.54
16S ribosomal RNA gene	3.20	uk (3-A1)	2.53
Na/K-ATPase beta subunit isoform 3	3.12	uk (2-F10)	2.36
gamma-actin	2.59	uk (3-A2)	2.31
60S ribosomal protein L19	2.27	uk (6-G7)	2.31
tubulin beta-1 chain	2.09	uk (7-G6)	2.22
actin	2.01	uk (3-D10)	2.20
actin related protein	0.42	uk (2-F7)	2.18
alpha-tubulin	0.41	uk (3-H5)	2.11
tubulin alpha-1 chain	0.40	uk (10-F6)	2.09
40S ribosomal protein S27	0.39	uk (2-C2)	2.01
dynein light chain, cytoplasmic	0.39	uk (2-D3)	0.49
nucleoside diphosphate kinase	0.36	uk (6-F11)	0.49
mitochondrial 1rRNA gene, partial 3' end	0.34	uk (10-B12)	0.47
16S rRNA gene	0.34	uk (2-H2)	0.45
40S ribosomal protien S4	0.29	uk (4-G8)	0.44
16S rRNA gene	0.28	uk (4-H11)	0.43
16S rRNA gene	0.27	uk (7-E1)	0.42
cytochrome c oxidase subunit VIIc	0.23	uk (8-A12)	0.39
NADH dehydrogenase subunit 4	0.23	uk (3-H7)	0.38
Ef2b gene product	0.22	uk (8-A11)	0.37
ribosomal protein S28	0.21	uk (8-B3)	0.36
mitochondrial genome, partial 3' end	0.21	uk (7-D7)	0.35
16S rRNA gene	0.18	uk (3-G8)	0.34
ribosomal protein L37	0.16	uk (6-F7)	0.33
5S ribosomal protein	0.16	uk (2-D7)	0.33
60S ribosomal protein L14	0.15	uk (5-H2)	0.32
40S ribosomal protein S20	0.14	uk (6-C11)	0.31
putative large subunit ribosomal protein rpL 44	0.14	uk (4-G12)	0.30
heterogeneous ribonucleoprotein A, A2,B1	0.13	uk (7-D11)	0.30
60S ribosomal protein L17	0.10	uk (6-E11)	0.29
NADH ubiquinone oxidoreductase chain 6	0.09	uk (3-C7)	0.26
gamma-aminobutyric acid (GABA-A) receptor	0.08	uk (8-F11)	0.23

V. harveyi challenge at 6 hpi (continue)

Description	Fold change	Description	Fold change
cytochrome c oxidase subunit 2	0.06	uk (5-F9)	0.21
KIAA0924 protein	0.03	uk (5-D3)	0.21
Mo23 gene product	0.03	uk (3-B3)	0.18
chromatin-specific transcription elongation factor	0.02	uk (2-G6)	0.17
brain protein I3	0.01	uk (8-B5)	0.16
sex-lethal protein homolog	0.01	uk (4-A4)	0.15
clottable protein	0.01	uk (9-F7)	0.14
hypothetical protein F 26E4.9	0.01	uk (2-H5)	0.12
uk (3-B7)	17.31	uk (5-H10)	0.11
uk (7-E10)	16.53	uk (5-D10)	0.11
uk (8-D7)	16.28	uk (7-A11)	0.10
uk (6-C9)	16.04	uk (8-F6)	0.09
uk (8-C2)	15.56	uk (6-D3)	0.09
uk (2-F11)	12.34	uk (6-E1)	0.08
uk (3-D1)	8.25	uk (6-D7)	0.07
uk (7-F4)	6.61	uk (3-E12)	0.07
uk (8-D4)	6.37	uk (7-C3)	0.06
uk (4-B1)	5.67	uk (3-D8)	0.05
uk (7-B4)	5.19	uk (5-D11)	0.05
uk (7-H4)	4.85	uk (9-F8)	0.04
uk (5-E6)	4.62	uk (3-D4)	0.03
uk (8-B4)	4.52	uk (3-F2)	0.02
uk (2-H8)	4.36	uk (10-B7)	0.02
uk (1-F1)	4.31	uk (5-A2)	0.02
uk (2-H3)	4.16	uk (7-A6)	0.02
uk (7-C12)	4.16	uk (4-F11)	0.02
uk (1-G10)	4.11	uk (8-F8)	0.02
uk (2-C9)	3.54	uk (8-H7)	0.01
uk (6-G4)	3.36	uk (1-B2)	0.01
uk (2-H7)	3.28	uk (38294)	0.01
uk (4-D5)	3.25	uk (10-D2)	0.01
uk (5-C3)	3.24	uk (3-F10)	0.01
uk (1-H7)	3.08	uk (5-F8)	0.01
		uk (7-A12)	0.01

V. harveyi challenge at 24 hpi

Description	Fold change	Description	Fold change
histone 1	0.42	uk (6-B7)	4.39
hypothetical protein F 26E4.9	0.01	uk (8-D5)	3.86
uk (3-D10)	14.99	uk (3-A1)	2.84
uk (2-G5)	14.56	uk (8-B10)	2.38
uk (1-B11)	10.26	uk (4-G10)	2.36
uk (1-D6)	7.65	uk (6-E9)	2.22
uk (3-D7)	7.19	uk (8-A5)	0.43
uk (1-G1)	4.98	uk (5-H10)	0.35
		uk (5-F8)	0.01

V. harveyi **challenge at 48 hpi**

Description	Fold change	Description	Fold change
60Sacidic ribosomal protein P2	15.09	uk (7-F5)	5.51
Penaeidin-2 precusor	14.66	uk (6-B7)	3.94
partial mitochondrial 16S rRNA gene	7.39	uk (1-B11)	3.80
RpL46 gene product	3.64	uk (3-H5)	3.60
60S ribosomal protein	3.21	uk (8-D5)	3.31
ribosomal protein S15	2.56	uk (1-C9)	2.88
gamma-actin	2.31	uk (6-E8)	2.69
penaeidin-3k	2.20	uk (1-10)	2.67
60S ribosomal protein L19	2.17	uk (3-A2)	2.63
mitochondrial genome, partial 3' end	0.39	uk (5-H3)	2.49
ribosomal protein L26	0.30	uk (4-H1)	2.42
hypothetical protein	0.28	uk (8-B10)	2.39
ribosomal protein S8	0.27	uk (2-E1)	2.31
mitochondrial 1rRNA gene, partial 3' end	0.21	uk (3-E9)	2.27
SPARC-related protein	0.19	uk (3-D9)	2.22
asialoglycoprotein receptor	0.12	uk (7-E10)	2.13
60S ribosomal protein L18A	0.12	uk (2-E10)	2.08
Ef2b gene product	0.06	uk (2-G5)	2.02
NADH ubiquinone oxidoreductase chain 6	0.06	uk (7-B12)	2.01
60S ribosomal protein L17	0.05	uk (6-B1)	0.49
ATP synthase subunit C	0.05	uk (8-A12)	0.48
RpS 9 gene product	0.04	uk (7-C8)	0.47
profilin	0.04	uk (7-H9)	0.47
tubulin alpha-1 chain	0.04	uk (5-A2)	0.40
cytochrome c oxidase subunit VIIc	0.01	uk (6-D9)	0.37
Mo23 gene product	0.01	uk (7-E12)	0.32
clottable protein	0.01	uk (5-F3)	0.19
uk (2-C2)	34.94	uk (5-H2)	0.19
uk (2-A7)	19.59	uk (6-F11)	0.16
uk (7-H5)	15.84	uk (5-F12)	0.16
uk (1-D6)	12.37	uk (6-F7)	0.11
uk (6-F5)	11.12	uk (7-A11)	0.10
uk (5-C9)	8.99	uk (2-D7)	0.10
uk (7-D11)	7.71	uk (5-C10)	0.06
uk (6-H3)	6.35	uk (6-E11)	0.03
uk (3-D10)	5.63	uk (8-A11)	0.02

Upon pathogen challenge, the expression of several genes in shrimp haemocytes has been altered including the immune related genes. After WSSV challenge, the up-regulated transcripts were GeneMAC25 protein and heat shock protein70 at the late phase (72 hpi), whereas the transcripts for the antimicrobial molecules, penaeidin and 11.5 kDa protein (crustin) were down-regulated after 24 and 48 hpi. For other immune-related genes, prophenoloxidase activating factor (PPAF) and anti-lipopolysaccharide factor (ALF) did not respond to WSSV infection. For *V. harveyi*-challenged shrimps, penaeidin transcripts were up-regulated at 48 hpi (Table 3.15 and 3.16). Besides the changes in the expression of these immune genes in shrimp haemocytes, several genes with unknown functions were up-regulated throughout the pathogen infections (data not shown).

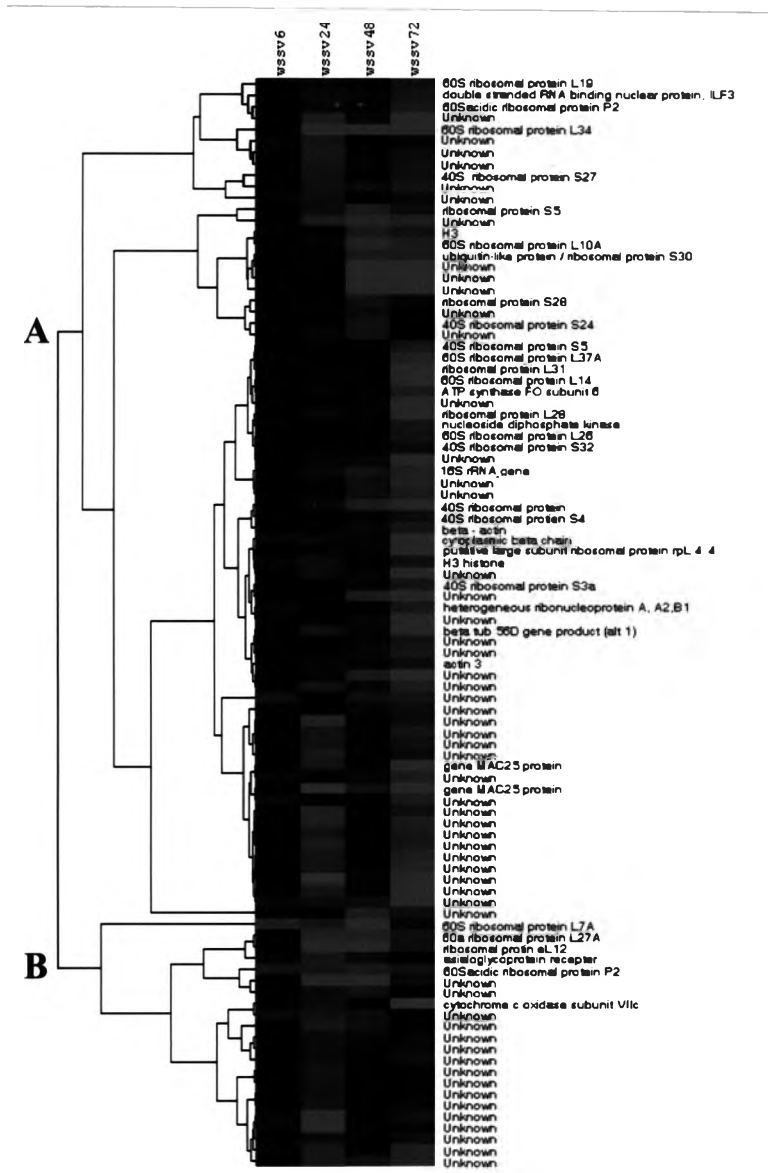


Figure 3.6. Hierarchical clustering analysis of the 96 genes in haemocytes of WSSV-challenged shrimps at 4 time points (6, 24, 48 and 72 hpi). Each row represents a single gene and each column an experimental sample. Genes were linked by the dendrogram shown on the left to illustrate similarity in their expression pattern. The brackets on the left represent the identified clusters, namely, group A and group B. Up-regulated genes are red, and down-regulated genes are green.

Among the responded genes, the transcripts of calmodulin (CaM), asialoglycoprotein receptor (ASGPR) and tubulin were highly up - regulate upon *Vibrio harveyi* and WSSV injections. Especially, the expression of CaM increased more than 20 fold at 48 and 72 hours after WSSV injection. For *V. harveyi*-challenged shrimps, the up-regulation of CaM was found at 6 hpi (4.6 fold). The above 3 gene homologues of CaM, ASGPR and β-tubulin were further analyzed by real-time PCR in order to confirm their expression in response to pathogen challenge.

3.4.4.1 Calmodulin (CaM)

The putative gene of calmodulin (CaM), CALcium MODULated proteIN, was up regulated upon pathogen challenge. This clone exhibited 98% deduced amino acid homology with CaM of human, bovine, *Bos taurus* and mouse *Mus musculus* (Fig 3.7). The clone contained a complete open reading frame (ORF) of 447 bp or 149 deduced amino acids. CaM is a sensor of intracellular calcium fluxes, and functions by binding to and regulating CaM binding proteins (CaMBPs). In addition to its other intracellular functions, CaM controls metabolism through the regulation of glycolysis and mitochondrial oxidative metabolism (Myre & O'Day, 2004). Moreover, CaM is implicated in plant pathogen interactions (Yamakawa *et al.*, 2001). CaM, a highly conserved protein through evolution, has four EF-hand motifs that change conformation upon binding Ca^{2+}. The calmodulin-Ca^{2+} complex then binds the target proteins, initiating various signaling cascades. The three dimensional structure of CaM was shown in Fig 3.8.

3.4.4.2 Asialoglycoprotein receptor (ASGPR)

The asialoglycoprotein receptor belongs to the family of calcium-dependent (C-type) animal lectins. The mouse ASGPR is involved in removing cells undergoing apoptosis in liver cells (Dalton *et al.*, 2003), and mediates endocytosis and degradation of several plasma proteins (Baricevic *et al.*, 2002). The microarray analysis of ASGPR transcript in haemocytes of pathogen-challenged shrimps showed an increase in its expression (8.7 fold in WSSV challenged shrimp)

and Figure 3.9 shows the deduced amino acid sequence alignment of the ASGPRs from selected species. The complete ORF of the shrimp's gene was not obtained.

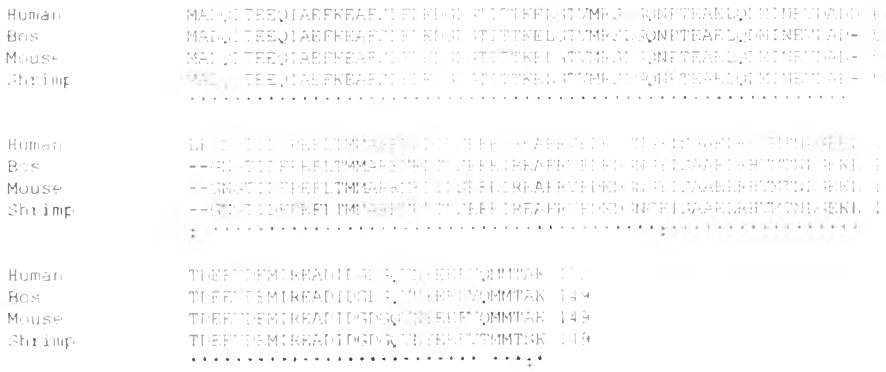


Figure 3.7 Multiple alignments of the deduced amino acid sequence of shrimp calmodulin (CaM) homologue with human, bovine and mouse CaMs. (*) indicates amino acid identity and (·) as well as (:) indicate amino acid similarity.

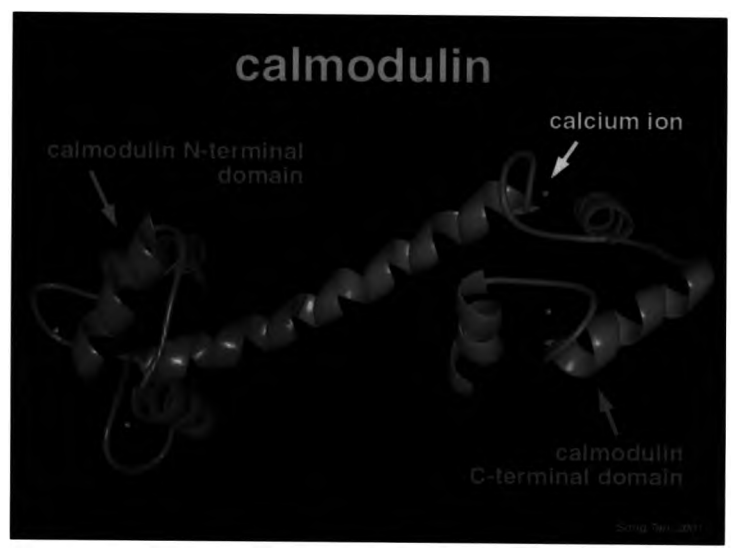


Figure 3.8 The three-dimensional structure of calmodulin showing an EF-hand motif that binds a calcium ion (source: http://www.bmb.psu.edu/faculty/tan/lab/gallery).

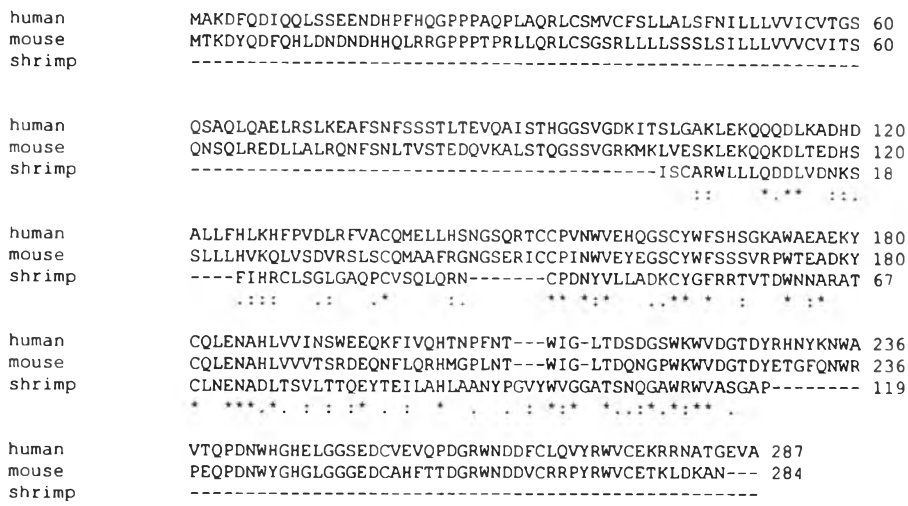


Figure 3.9 Multiple sequence alignment of the deduced amino acid sequence of shrimp asialoglycoprotein receptor (ASGPR) homologues with the human and mouse ASGPRs. (*) indicates amino acid identity and (·) as well as (:) indicate amino acid similarity.

Figure 3.10 Multiple alignment of the deduced amino acid sequence of shrimp β–tubulin homologues with urchin and silk moth β–tubulin. (*) indicates amino acid identity and (·) as well as (:) indicate amino acid similarity.

3.4.4.3 β-Tubulin

Tubulin is composed of a heterodimer of two closely related 55 kDa proteins called α- and β-tubulin. These two proteins are encoded by two different genes in a small gene family, whose sequences are highly conserved throughout the eukaryotic kingdom. Tubulin polymerizes into long chains or filaments that form microtubules. Microtubules have the ability to shift through various formations, which enables a cell to undergo mitosis or to regulate intracellular transport. The β-tubulin homologue was up-regulated in haemocytes of the pathogen-challenged shrimps. The shrimp β-tubulin in this study encoded a protein of 329 amino acid residues. The deduced amino acid sequence showed approximately 68% and 80% identity to the β-tubulin sequences from bombyx and urchin, respectively (Fig 3.10).

3.5 Verification of microarray analysis by real-time RT-PCR

Verification of the changes in expression level of the 3 up-regulated genes upon *V. harveyi* and WSSV challenges observed by cDNA microarray was carried out. These up-regulated genes, calmodulin, asialoglycoprotein receptor and tubulin, were further evaluated by real-time RT-PCR using SYBR Green chemistry. In this study, the housekeeping gene, elongation factor 1-alpha, was used as a reference gene. In addition to the 3 up-regulated genes, the expression of CaM – binding and candidate binding proteins, calcineurin, CDC like kinase 2, and protein phosphatase 1 were also examined in the same cDNAs from the pooled total RNA of 10 individuals at each time point from both saline- and *V. harveyi*-injected shrimp haemocytes (0, 6, 24, 48 hpi) and from LHM- and WSSV-injected shrimp haemocytes (0, 6, 24, 48 and 72 hpi) were used as templates for a real-time RT-PCR reaction.

For accurate assessment of gene expression by real time RT-PCR, the PCR efficiency of each gene should be taken into consideration. Determination of the PCR efficiency of selected genes was performed. The serial dilutions of the pooled cDNA of normal animal were made (5 to 5^5 dilutions). Real time PCR efficiency was calculated from the slope that was obtained from the curve plotted between the five dilutions of the cDNA (log scale) with the threshold cycle (Ct) (Figure 3.11, 3.12), using an equation, $E = 10^{[-1/slope]}$. The correlation coefficients, melting temperature and real time PCR efficiencies were shown in Table 3.17. Unequal of the PCR efficiencies between them was found to vary between 1.86 and 2.00 (the 2.00 of PCR efficiency represent 100% amplification at each cycle). Therefore, the relative expression ratios of certain genes were calculated using an equation described by Pfaffl to correct differences in efficiency.

The specificity of the real-time PCR reactions was determined by the dissociation curve analysis. The corresponding dissociation curves of the targeted genes; CaM, tubulin, ASGPR, calcineurin, CDC like-kinase 2 and protein phosphatase 1 and the reference gene, EF-1α, were shown in Figure 3.13. Single peak at expected melting temperature of dissociation curve of each gene was found indicating that the intended gene was amplified, and there is no contamination, non-specific amplification or primer-dimers. Negative controls that have no any template did not provide any amplification for each reaction.

The mRNA expression level of each gene was determined by normalizing the Ct values of the pooled sample of WSSV-injected shrimp haemocytes with that of LHM-injected shrimp haemocytes and *V. harveyi*-injected shrimp haemocytes with that of saline-injected shrimp haemocytes. The expression ratios of certain gene at each time point after pathogen challenge were calculated relative to EF-1α. The results were shown in Figures 3.14 and 3.15.

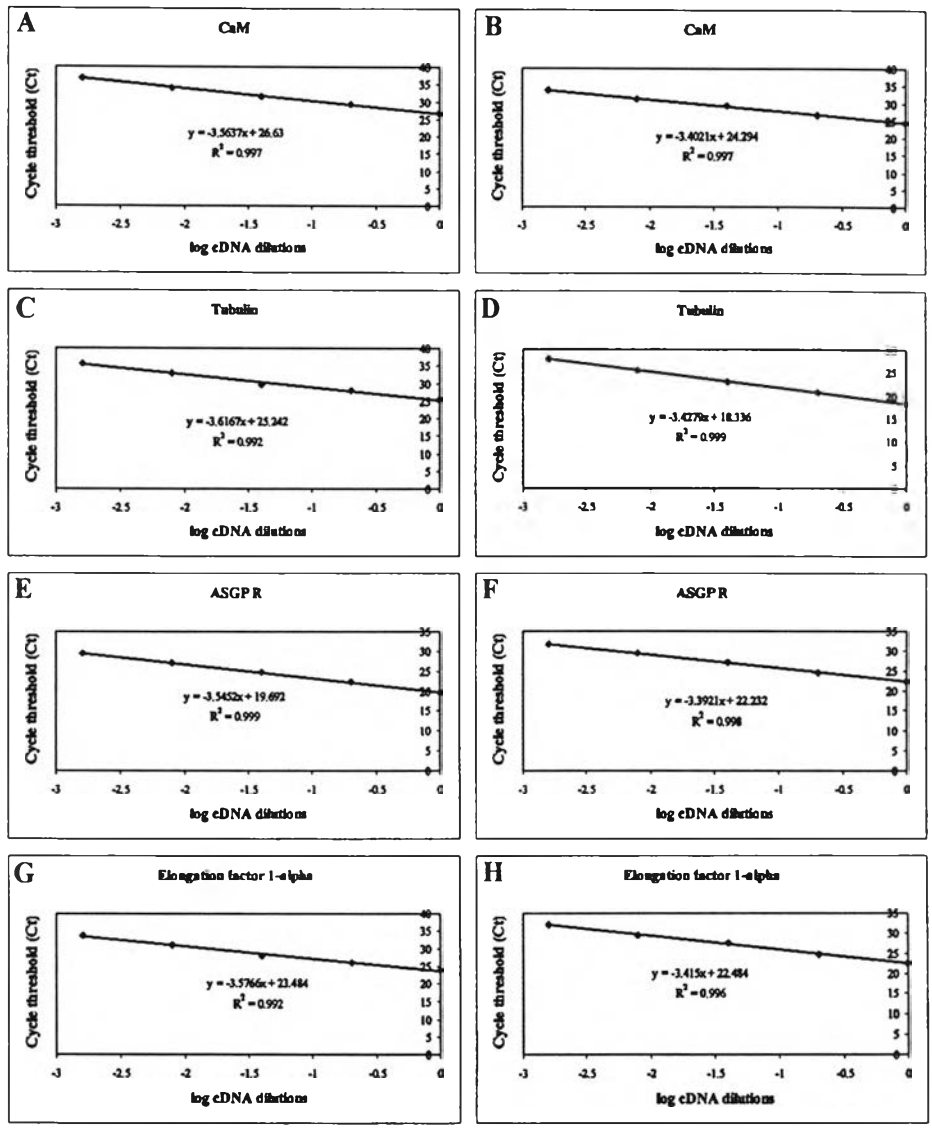


Figure 3.11 Amplification efficiency curves of the target genes. Calmodulin (CaM, A-B), tubulin (C-D), asialoglycoprotein receptor (ASGPR, E-F), and reference gene elongation factor 1-alpha (EF-1α, G-H) for WSSV (A, C, E and G) and *V. harveyi* (B, D, F and H) challenge experiments.

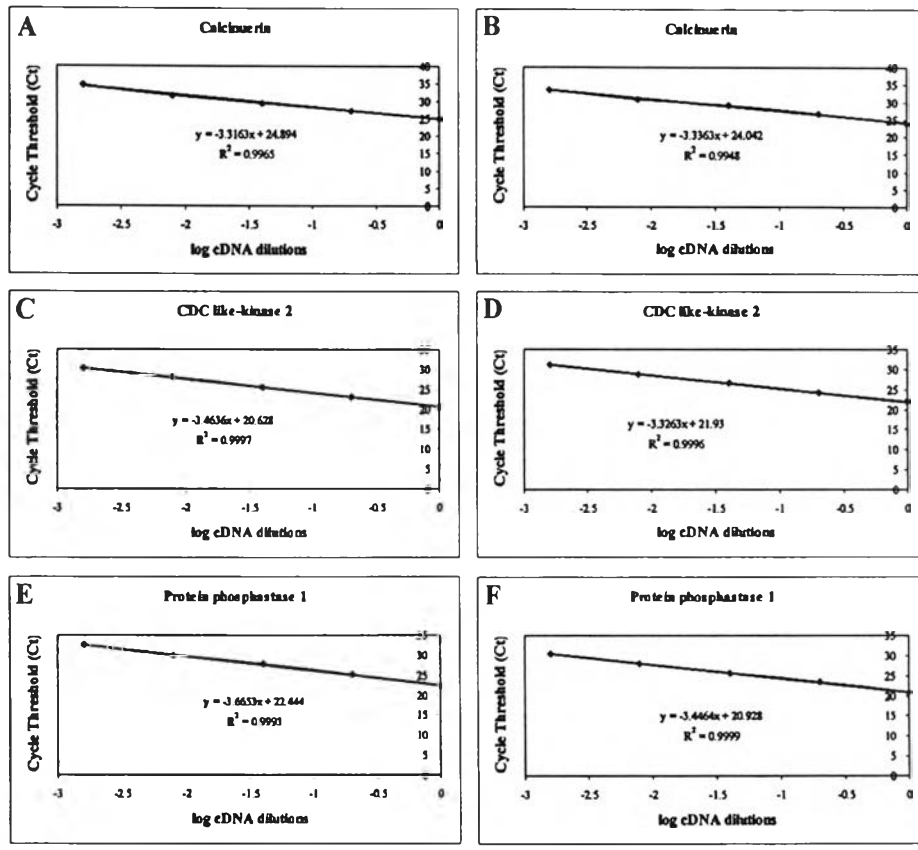


Figure 3.12 Amplification efficiency curves of calcineurin (A-B), CDC like-kinase 2 (C-D), and protein phosphatase 1 (E-F) for WSSV (A, C and E) and *V. harveyi* (B, D and F) challenge experiments.

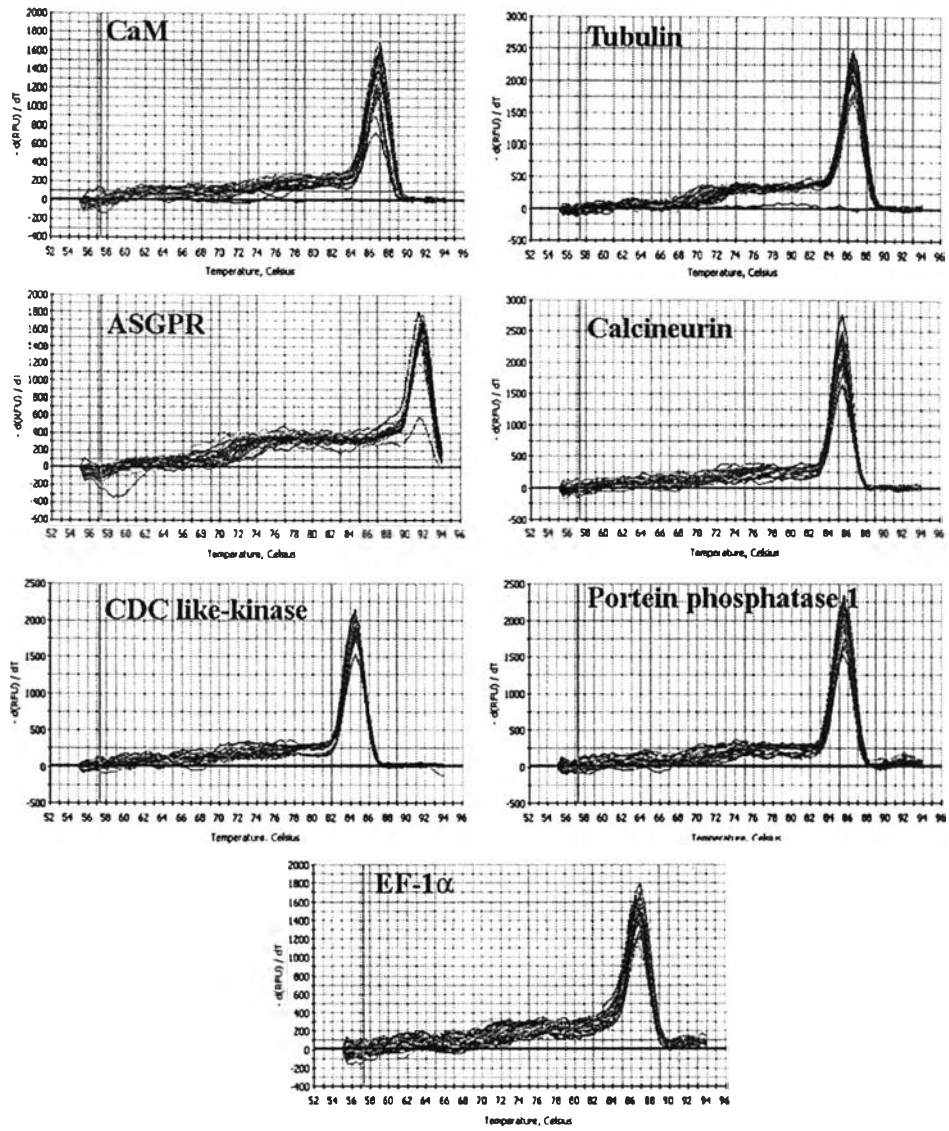


Figure 3.13 The dissociation curves of the target genes; CaM, tubulin, ASGPR, calcineurin, CDC like-kinase 2, and protein phosphatase 1 and the reference gene EF-1α.

Table 3.17 The PCR efficiency for each amplified gene, and the melting temperature (T_m) of its product.

Gene name	T_m (°C)	Correlation coefficient		Slope		PCR efficiency	
		WSSV injection	*V. harveyi* injection	WSSV injection	*V. harveyi* injection	WSSV injection	*V. harveyi* injection
Calmodulin	86.5	0.997	0.997	-3.5637	-3.4021	1.87	1.99
Asialoglycoprotein receptor	91.5	0.999	0.998	-3.5452	-3.3921	1.91	2.00
Tubulin	86.5	0.992	0.999	-3.6167	-3.4279	1.88	1.96
Calcineurin	85.5	0.996	0.995	-3.3163	-3.3363	1.99	1.99
CDC like kinase 2	84.5	1.000	1.000	-3.4636	-3.3263	1.95	2.00
protein phosphatase 1	85.5	0.999	1.000	-3.6653	-3.4464	1.86	1.95
EF-1alpha	87	0.997	0.999	-3.5766	-3.4150	1.86	1.86

For the WSSV challenged shrimp, the expression of CaM mRNA was increased after 24 hpi and peaked at 72 hpi (Figure 3.14A). For the *V. harveyi*-challenged shrimp, the expression of the CaM gene was increased at 6 hpi and different in expression were found at 24 and 48 hpi (Figure 3.14B). The expression of tubulin mRNA was not increased in haemocytes of WSSV challenged shrimp until 48 hpi, and the peaked expression was observed at 72 hpi (Figure 3.14C). For the *V. harveyi*-challenged shrimp, the up-regulation of the tubulin mRNA was observed at the early phase of injection (Figure 3.14D). The expression of ASGPR transcripts was also increased after viral and bacterial injection (Figure 3.14E and F). The temporal expression of the calcineurin transcript in WSSV challenged shrimp haemocytes was shown in Figure 3.15A. The expression of calcineurin was decreased after viral challenge but it was then up regulated at 48 hpi. For *V. harveyi* challenge, the expression of calcineurin transcripts was increased at 6 hpi, and showed slightly decreased in expression at 48 hpi (Figure 3.15B).

For the CDC like-kinase 2 and protein phosphatase 1 after WSSV challenge, their expressions were decreased 0.61 and 0.96 fold, respectively, at 24 hpi. Then, they were up regulated 1.33 and 1.98 folds for CDC like-kinase 2, and 1.92 and 1.71 folds for protein phosphatase 1 at 48 and 72 hpi, respectively (Figure 3.15C,E). For *V.*

harveyi challenge, the two genes showed the same pattern of regulation. They were up regulated 1.17 and 1.31 folds at 6 hpi with for CDC like-kinase 2 and protein phosphatase 1, respectively, and then decreased in expression at 24 and 48 hpi (Figure 3.15D,F). The results of real-time PCR indicated that the selected genes were differentially expressed in the haemocytes of *P. monodon* upon pathogen challenge.

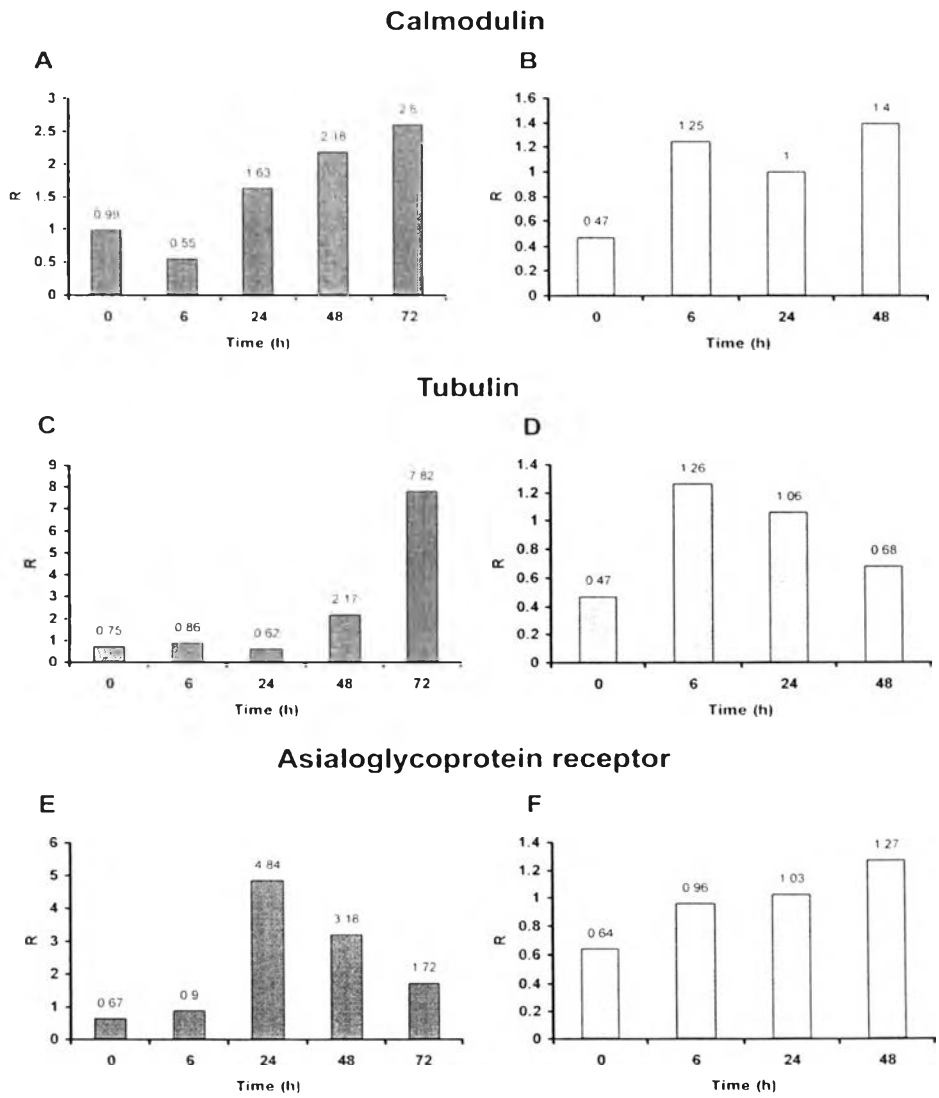


Figure 3.14 Verification of the highly expressed genes in WSSV- (A, C, E) and *V. harveyi*- (B, D, F) challenged shrimp haemocytes using real-time PCR.

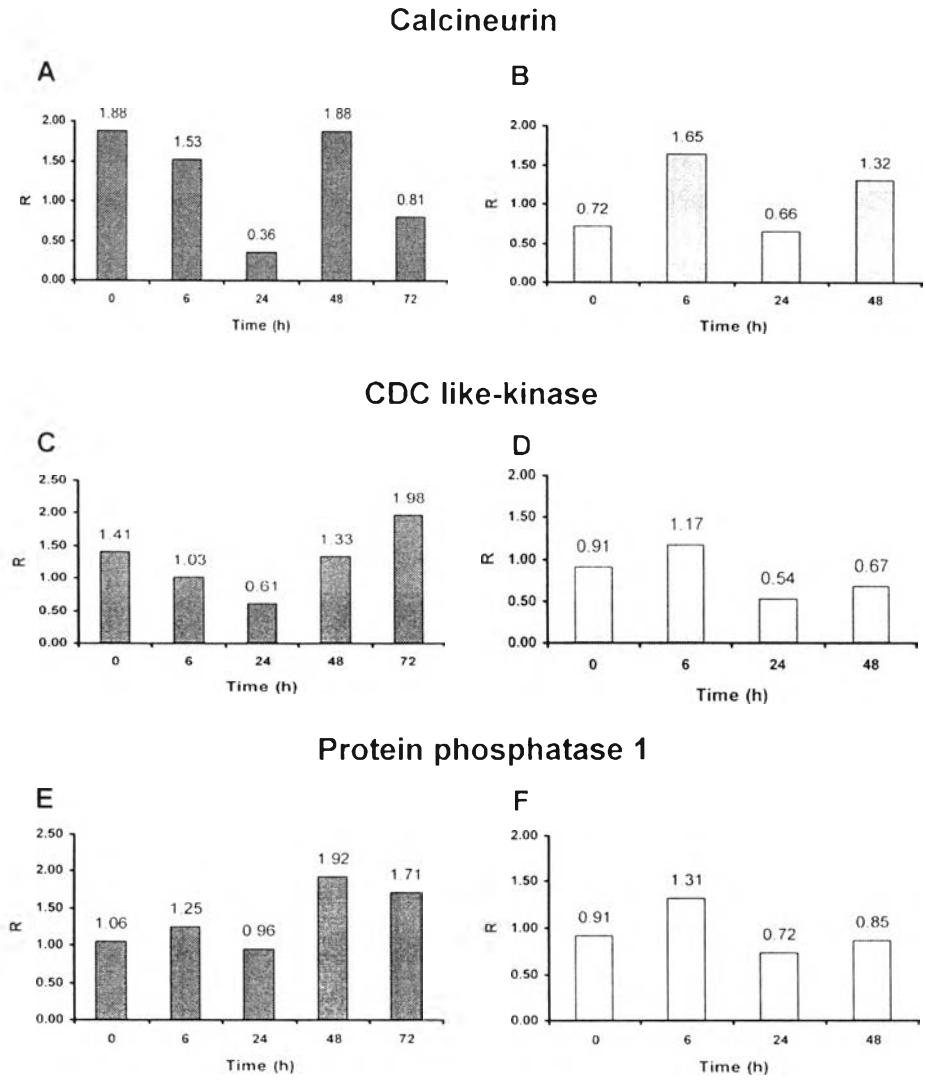


Figure 3.15 Time course analyses of calcineurin, CDC like-kinase 2 and protein phosphatase 1 transcripts in WSSV- (A, C, E) and *V. harveyi*- (B, D, F) challenged shrimp haemocytes using real-time PCR.

3.6 Localization of calmodulin (CaM) transcript and protein

3.6.1 Localization of CaM transcripts in shrimp haemocytes by *in situ* hybridization

The change in the population of circulating haemocytes expressing CaM was assessed in order to verify the involvement of CaM in pathogen response. The time-course analysis of the percentages of haemocytes expressing CaM was carried out by *in situ* hybridization. Circulating haemocytes of each individual shrimp were collected at various times post injection (0, 6, 24, 48 and 72 hr). They were fixed, counted and cytocentrifuged onto the poly-L-lysine-coated slides as described in section 2.13.1 for the determination of CaM expressed cells. The haemocytes were probed with DIG-labeled CaM-antisense and -sense riboprobes, and detected with alkaline phosphatase-conjugated anti-DIG antibodies. The positive haemocytes with dark purple color were identified by alkaline phosphatase using the NBT/BCIP solution as substrate. The sense probe was used as a control for signal specificity. In addition, RNAse A pretreatment of the haemocytes was performed prior to the probe hybridization to assure that the signal was indeed from the hybridization probe.

Hybridization signals of CaM transcripts could be detected in the shrimp haemocytes. An example of positive cells of *V. harveyi*-challenge shrimp haemocytes for CaM-antisense riboprobes was shown in Figure 3.16A. Hybridization using sense probe gave no positive signal in the cytocentrifuged cells (Figure 3.16B). The positive haemocytes at each time point were counted for the calculation of the percentage of positive haemocytes. For WSSV-challenged shrimps, the percentage of positive haemocytes was highest at 6 hpi (27.2%), and gradually fell off thereafter at 24, 48 and 72 hpi (24.6%, 18.9% and 12.8%, respectively) (Figure 3.17A). For *V. harveyi*-challenged shrimp, the highest and lowest percentages of positive haemocytes was 63.4% at 24 hpi, and 8.5% at 72 hpi, respectively (Figure 3.17B). The control shrimps injected with LHM and NaCl showed lower percentages of positive haemocytes than those of WSSV- and *V. harveyi*-challenged shrimps (Figure 3.17A,B).

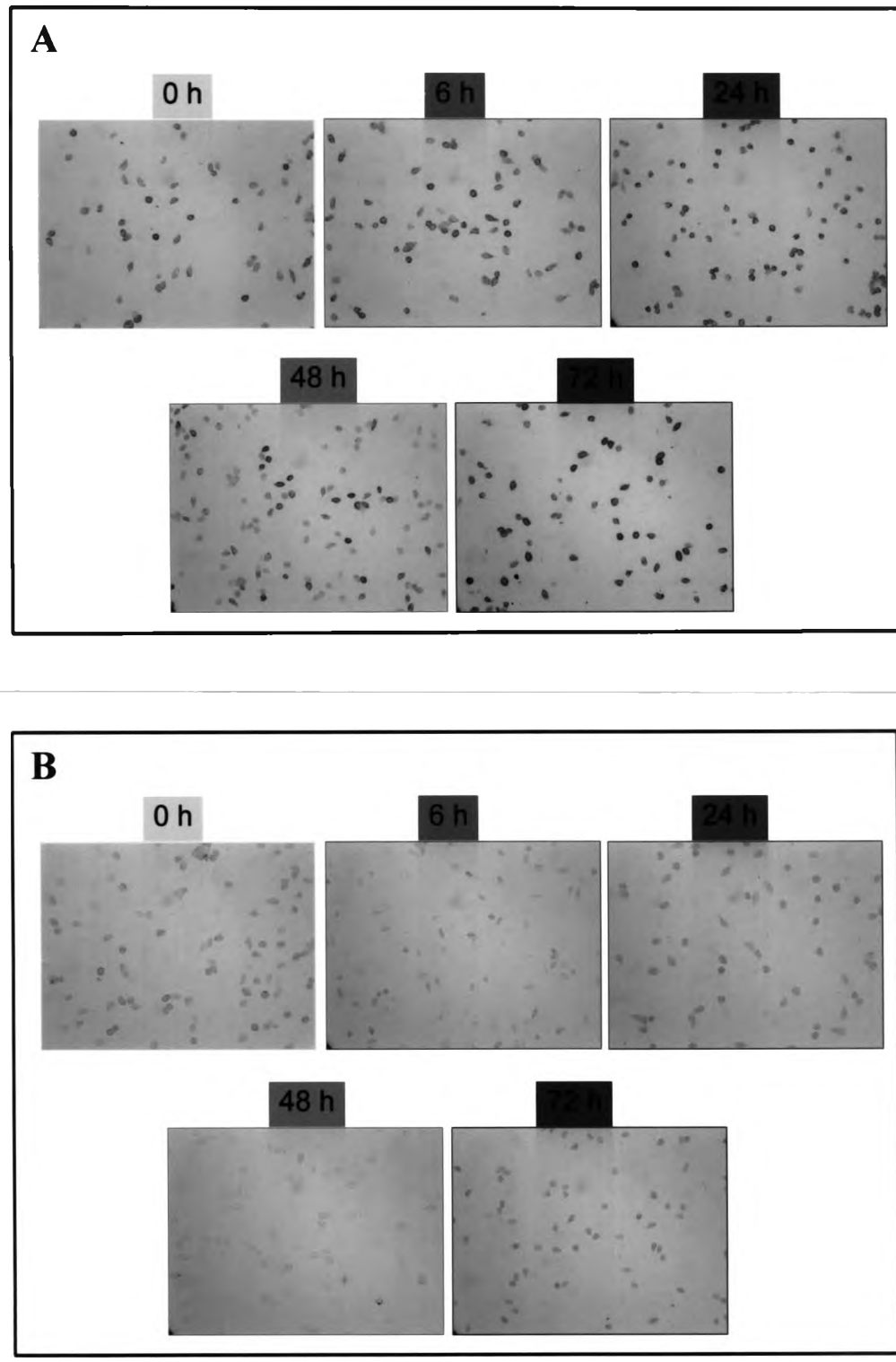


Figure 3.16 *In situ* hybridization of haemocytes at different time points of *V. harveyi*-challenged shrimps, probed with DIG-labeled CaM-antisense riboprobe (A) and –sense riboprobe (B).

3.6.2 Localization of CaM protein

To localize the CaM protein in shrimp tissues, immunohistochemistry was carried out using a commercial mouse monoclonal CaM antibody. The alkaline phosphatase conjugated secondary antibody was used to facilitate the colorimetric immunodetection using NBT/BCIP solution. Histological evaluation of the stained tissues indicated that CaM protein could be detected in the lymphoid organ (Figure 3.18-L1-4, l 1-4). Figure 3.18-l 2 revealed that the strongest CaM immunoreactivity was detected in the tubule epithelial cell of lymphoid organ at 6 hours post *V. harveyi* injection. Thereafter a fewer positive cells were observed in this tissue. For gills, after bacterial injection, there were many CaM-positive haemocytes in this tissue, especially at 48 hpi (Figure 3.18-g1-5). The tissue sections gradually showed more and more infiltrated positive haemocytes up to 48 hpi, which were then subsided at 72 hpi. The anti-CaM also reacted positively with cuticular epithelium of the gills. In addition, immunodetection showed that mature haemocytes were detected in the hematopoeitic tissue (Figure 3.18-Hae1-4, hae1-5). At 48 hpi, the number of mature haemocytes was highest (Figure 3.18-hae4). Similar results were observed in the hepatopancreas sections. The highest number of positive haemocytes appeared at 48 hpi in the tissue section (Figure 3.18 hep4). For the hepatocytes, the weak positive signal was observed at 0 hpi. The increasingly stronger signal was detected at 6, 24 and 48 hpi (Figure 3.18-hep1-4).

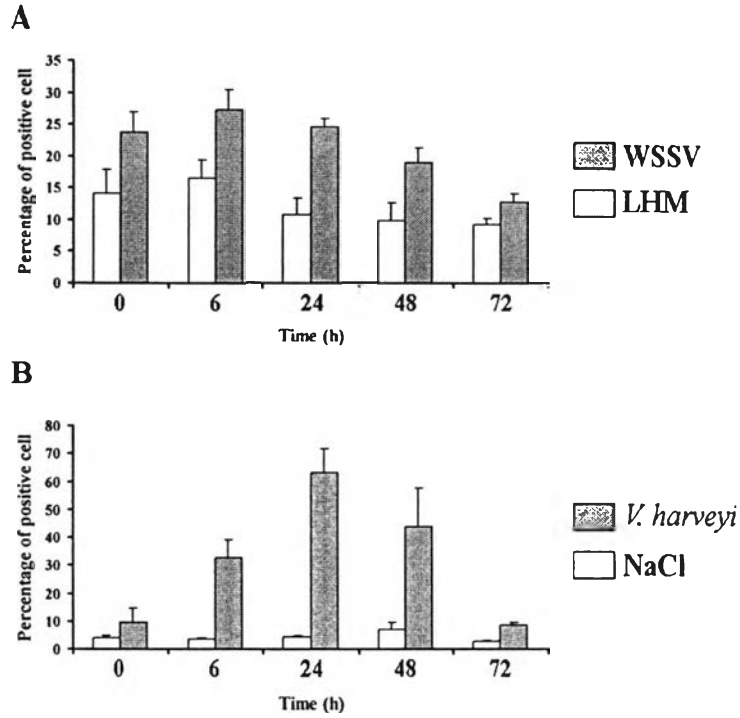


Figure 3.17 Time-course analyses of the CaM expressing haemocytes after WSSV (A) and *V. harveyi* (B) challenge.

Table 3.18 Percent of positive cell of the CaM expressing haemocytes at 0, 6, 24, 48 and 72 hour post injection.

Hour post injection	NaCl	*V. harveyi*	LHM	WSSV
0	3.89±0.70	9.29±5.22	14.08±3.19	23.73±3.78
6	3.34±0.67	32.53±6.82	16.49±3.22	27.25±2.93
24	4.43±0.50	63.38±8.31	10.68±1.42	24.59±2.66
48	6.73±2.81	43.76±13.81	9.77±2.27	18.91±2.86
72	2.59±0.48	8.54±0.79	9.13±1.31	12.75±1.04

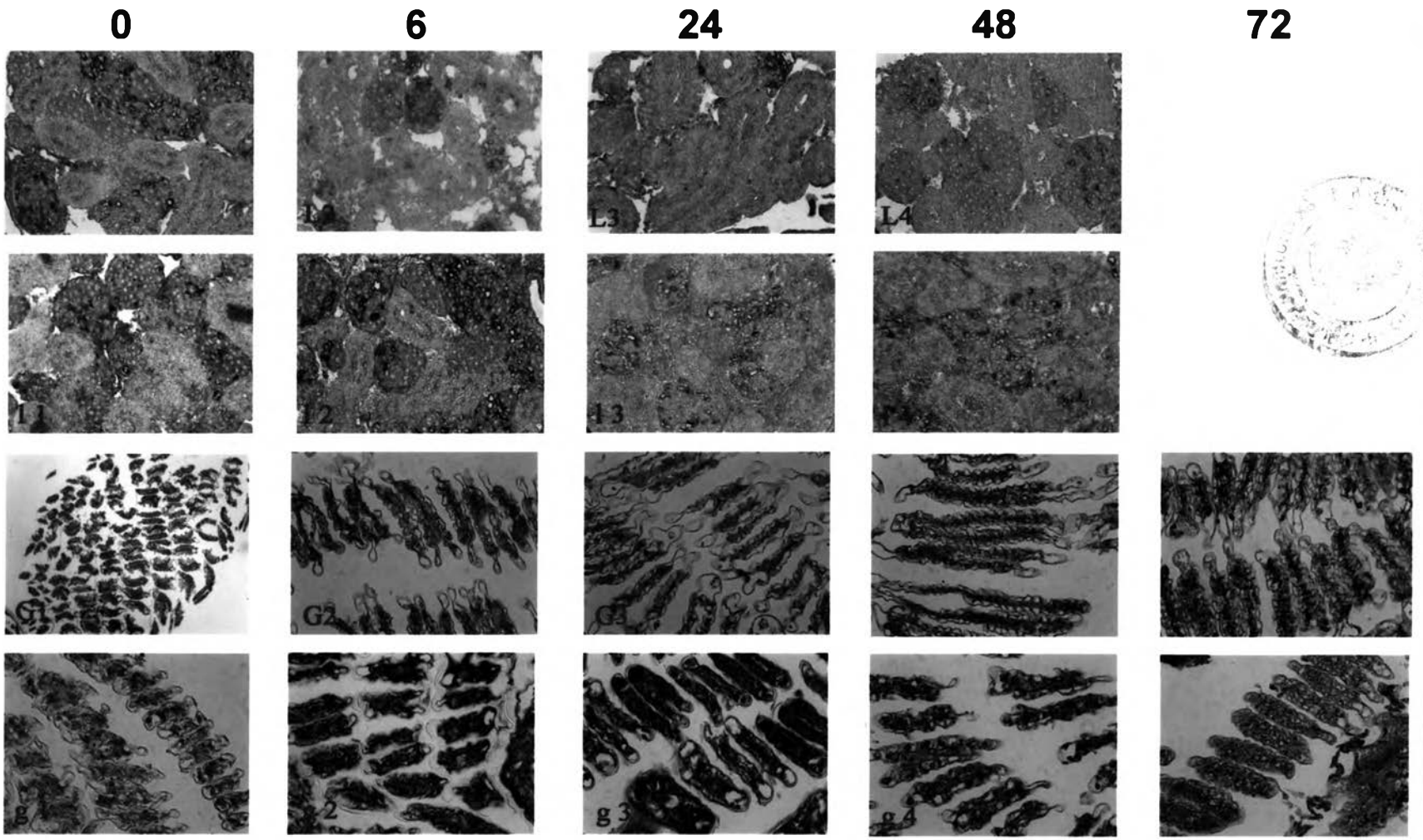


Figure 3.18 Immunohistochemical detection of CaM protein in the cepharothorax of *V. harveyi* and NaCl injected shrimps at 0, 6, 24, 48 and 72 hour post injection. The tissue sections of the cepharothorax were incubated with anti-CaM IgG followed by alkaline phosphatase-conjugated anti-mouse IgG. The observed organs included lymphoid organs (L1-4 and l1-4), gills (G1-5 and g1-5), hematopoietic tissue (Hae1-4 and hae1-5) and hepatopancreas (Hep1-5 and hep1-4).

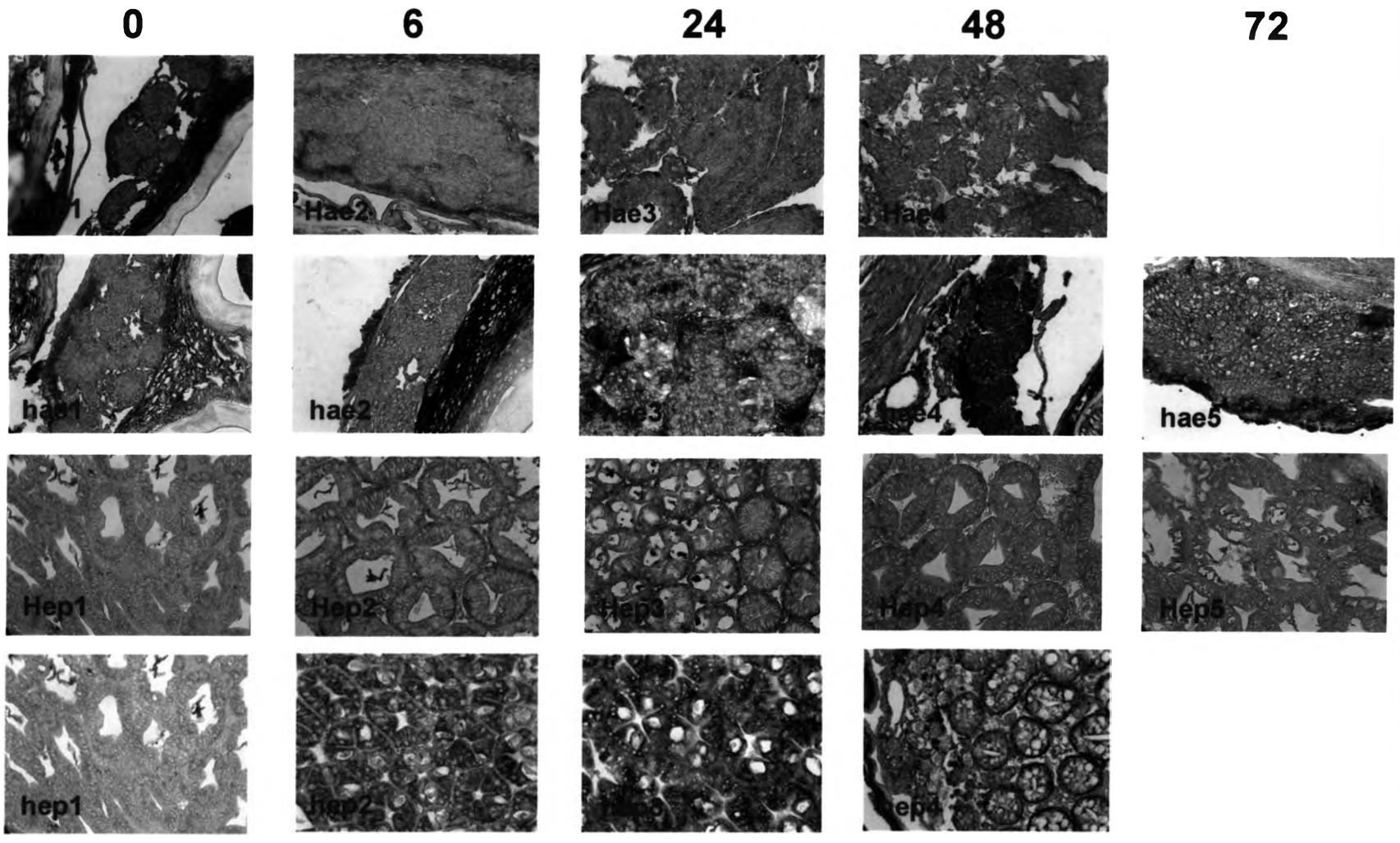


Figure 3.18 (continue) Immunohistochemical detection of CaM protein in the cepharothorax of *V. harveyi*- and NaCl-injected shrimps at 0, 6, 24, 48 and 72 hpi.