CONSTRUCTION OF cDNA LIBRARY OF LYMPHOID ORGAN OF YHV INFECTED SHRIMP (*PENAEUS MONODON*)

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CONSTRUCTION OF cDNA LIBRARY OF LYMPHOID ORGAN OF YHV INFECTED SHRIMP (*PENAEUS MONODON*).

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ABSTRACT

The yellow head virus (YHV) is a causative agent of yellow head disease in shrimp farming. This virus was first observed in Thailand and causes mass mortality in shrimp farming. This study was undertaken to investigate the gene expression profile of YHV infected shrimp. The cDNA library was constructed from the samples of lymphoid organs of YHV-infected shrimp inorder to identify the genes that are associated with shrimp immunity. From this library, 887 clones were randomly selected for EST (expressed sequence tags) sequencing, then they were searched for homologies in the GenBank database with the BlastX program. 452 clones (51.0%) showed significant homology with known genes in the database, whereas 435 EST clones (49.0%) did not show any significant homology to the known genes. The putative defense and homeostasis proteins accounted for 3.3% (15 clones). These genes might play a role during viral infection due to chelonianin (basic protease inhibitor, serine protease analogue), cathepsin B-like cysteine protease precursor, and lipoprotein localization factor, translational controlled tumor protein (TCTP), cyclophilin, peritrophin and thrombospondin. Whether these gene products are involved in response to viral infection, functional studies of these genes remain to be proven.

ASF1 (anti-silencing factor1) is the histone chaperone H3/H4 binding protein, it plays an important role in assembly and disassembly of the chromatin. ASF1 is required for transcriptional activation in yeast (*Saccharomyces cerevisiae*). Shrimp ASF1 cDNA consists of 957 nucleotides encoding 200 amino acid residues. The amino acid sequence of shrimp ASF1 was similar to those of fruit flies, and other invertebrate and vertebrate ASF1 sequences. RT-PCR analysis has show that ASF1 expression was down-regulated in moribund shrimp, especially in the muscle, gill and hepatopancreas. A previous study indicated that these organs were a primary site of YHV infection and showed different degrees of cellular apoptosis. This study indicated that the down regulation of ASF1 expression was induced by viral infection and this may affect transcriptional regulation of the host cells.

KEY WORDS: *PENAEUS MONODON* / YHV / LYMPHOID ORGAN

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การสร้างห้องสมุด cDNA จากอวัยวะน้ำเหลืองของกุ้งกุลาดำที่ติดเชื้อไวรัสหัวเหลือง
(CONSTRUCTION OF cDNA LIBRARY OF LYMPHOID ORGAN OF YHV INFECTED SHRIMP (*PENAEUS MONODON*).)

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บทคัดย่อ

การเพาะเลี้ยงกุ้งกุลาดำเป็นอุตสาหกรรมที่มีความสำคัญทางเศรษฐกิจต่อประเทศไทย โดยไทยสามารถส่งออกกุ้งกุลาดำได้เป็นอันดันหนึ่งของโลก ต่อมาในระยะหลังปริมาณการส่งออกกุ้งของไทยลดลงเนื่องจากการเกิดโรคระบาดซึ่งมีสาเหตุมาจากเชื้อไวรัส โดยเฉพาะเชื้อไวรัสหัวเหลือง (YHV) ทำให้เกิดการตายของกุ้งเป็นจำนวนมาก เพื่อศึกษาการแสดงออกของยีนในกุ้งกุลาดำที่ถูกกระต้นด้วยเชื้อไวรัสหัวเหลือง จึงสร้างห้องสมุด cDNA จากอวัยวะน้ำเหลืองของกุ้งที่ติดเชื้อไวรัสหัวเหลือง เมื่อสุ่มเลือกโคลนจำนวน 887 โคลน มาหาลำดับนิวคลีโอไทด์ และเปรียบเทียบกับยีนที่มีรายงานแล้วใน GenBankโดยใช้โปรแกรม BlastX พบว่า 452 โคลน (51%) มีลำดับนิวคลีโอไทด์เหมือนกับยีนที่รายงานแล้วในขณะที่ 435 โคลน (49%) ไม่เหมือนกับยีนที่มีรายงานใน GenBank พบยีนที่เกี่ยวข้องกับระบบภูมิคุ้มกันจำนวน 15 โคลน (3.3%) และยีนที่น่าจะเกี่ยวข้องกับการตอบสนองต่อไวรัส เช่น chelonianin, cathepsin B-like cysteine protease precursor, translational controlled tumor protein (TCTP), cyclophilin, peritrophin และ thrombospondin เป็นต้น อย่างไรก็ตามหน้าที่ในการตอบสนองต่อเชื้อไวรัสของยีนเหล่านี้จำเป็นต้องมีการศึกษาต่อไป

นอกจากนี้ยังพบยีน ASF1 (anti-silencing factor1) ซึ่งพบครั้งแรกในยีสต์ (*Saccharomyces cerevisiae*) ทำหน้าเกี่ยวข้องกับการเปลี่ยนแปลงโครงสร้างของโครมาตินซึ่งมีผลต่อการกระตุ้นการแสดงออกของยีนในยีสต์ จากการแยกและศึกษาลำดับนิวคลีโอไทด์พบว่า ASF1 ที่พบในกุ้ง ประกอบด้วย 957 นิวคลีโอไทด์และแปลรหัสให้โปรตีนที่มีกรดอะมิโนจำนวน 200 ตัว และจากการศึกษาระดับการแสดงออกพบว่า ASF1 มีการแสดงออกกระจายอยู่ในหลายอวัยวะ โดยพบระดับการแสดงออกสูงที่สุดในกล้ามเนื้อของกุ้งที่ปกติและในเม็ดเลือดของกุ้งที่ติดเชื้อ YHV ระดับการแสดงออกที่ลดลงของ ASF1 สามารถพบได้ในเกือบทุกอวัยวะของกุ้งที่ติดเชื้อ ยกเว้นในเม็ดเลือดซึ่งมีการแสดงออกเพิ่มขึ้นเล็กน้อย การแสดงออกที่ลดลงอย่างมากพบในกล้ามเนื้อเหงือก และตับ จากการศึกษาก่อนหน้านี้แสดงให้เห็น ทั้งสามอวัยวะนี้เป็นอวัยวะแรกๆที่สามารถพบการติดเชื้อไวรัสหัวเหลือง และมีการตายของเซลล์เกิดขึ้น จากการแสดงให้เห็นว่า การแสดงออกที่ลดลงของ ASF1 เนื่องมาจากการติดเชื้อไวรัส และอาจจะมีผลกระทบต่อการควบคุมการแสดงออกของยีนที่ทำหน้าที่ต่างๆในเซลล์

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CONTENTS

CONTENTS (CONTINUED)

CONTENTS (CONTINUED)

Page

CONTENTS (CONTINUED)

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES (CONTINUED)

LIST OF ABBREVIATIONS

ASF1	=	Anti-silencing function 1
ATP	=	adenosine triphosphate
bp	=	base pair
BGBP	=	β-1,3-glucan binding protein
BSA	=	bovine serum albumin
^{o}C	=	degree celcius
CAF-1	=	chromatin assembly factor 1
cDNA	=	complementary deoxyribonucleic acid
cm^2	=	square centimeter
CP	=	clotting protein
DEPC	=	diethyl pyrocarbonate
DIG	=	digoxigenin
DMSO	=	dimethylsulfoxide
DNA	=	deoxyribonucleic acid
dNTP	=	deoxynucleotide triphosphate
DTT	=	dithiothreitol
EDTA	=	ethylenediamine tetra-acetic acid
ESTs	=	expressed sequence tags
g	=	gram
GAV	=	gill associated virus
h	=	hour
H&E	=	hematoxylin and eosin
Hel	=	helicase
HP	=	hepatopancreas
HPV	=	hepatopancreatic parvovirus
HST	=	head soft tissue
kb	=	kilo-base pair
kDa	=	kilodalton

LIST OF ABBREVIATIONS (CONTINUED)

LO	=	lymphoid organ
LOVV	=	lymphoid organ vacuolization virus
LOS	=	lymphoid organ spheroid
LPS	=	lipopolysaccharide
LPV	=	lymphoidal parvo-like virus
M	=	molar
mA	=	milliampaire
MDH	=	malate dehydrogenase
mg	=	milligram
$MgCl_2$	=	magnesium chloride
$MgSO_4$	=	magnesium sulfate
MIB	=	metal ion-binding
MIC	=	minimum inhibitory concentration
min	=	minute
ml	=	millilitre
mm	=	millimetre
mM	=	millimolar
MMLV	=	Moloney murine leukemia virus reverse transcriptase
MOI	=	multiplicity of infection
MOPs	=	3-[*N*-morpholino]propane-sulfonic acid
mRNA	=	messenger ribonucleic acid
MW	=	molecular weight
NADH	=	nicotinamide adenine dinucleotide
ng	=	nanogram
nM	=	nanomolar
nm	=	nanometre
OD	=	optical density
ORF	=	open reading frame
PBS	=	phosphate buffer saline
PCR	=	polymerase chain reaction

LIST OF ABBREVIATIONS (CONTINUED)

pfu	=	plaque-forming units
PGRP	=	pattern recognition molecules
PMSF	=	phenylmethylsulfonyl fluoride
RNA	=	ribonucleic acid
RLM-RACE	=	RNA ligase mediated rapid amplification of cDNA ends
ROIs	=	reactive oxygen intermediates
rpm	=	revolutions per minute
RPS	=	Rhabdovirus of penaeid shrimp
RT-PCR	=	reverse transcriptase polymerase chain reaction
sec	=	second
ssRNA	=	single stranded RNA
SDS	=	sodium dodecyl sulfate
STI	=	soybean trypsin inhibitor
TEM	=	transmission electron microscopy
TGase	=	transglutaminase
Tris	=	Tris-(hydroxymethyl)methylamine
TSV	=	taura syndrome virus
U	=	unit
UTR	=	untranslated region
v/v	=	volume by volume
w/v	=	weight by volume
YHD	=	yellow head disease
YHV	=	yellow head virus
WSSV	=	white spot syndrome virus
µg	=	microgram
µl	=	microliter

CHAPTER I
INTRODUCTION

1. Shrimp farming

The penaeid shrimp culture is a worldwide economic activity especially important for intertropical developed and developing countries, with the consumer demand for shrimp continue to grow at a rate of 7-9% annually (1). This industry has generated employment both on farms and indirectly through servicing industries. It also provides augmentation of the world's food supply. There are two major areas in the world, where shrimps aquaculture has been well developed including Asia especially the Southeastern Asia, and the Latin America especially the Central America and northern part of South America. Almost 80% of cultured shrimp come from Asia with Thailand, China, Indonesia, and India as the top of the producers (Figure 1). In the western hemisphere, Ecuador is the major shrimp producing country. In the past few years, Thai shrimp aquaculture production has grown steadily (Figure 2). Thailand entered the culture shrimp business in the mid-1980s and become the leading country for cultured production in Eastern Hemisphere in 1991 (Table 1) such as over 80,000 hectares in Thai shrimp farm produced over 200,000 metricton. During a period from 1985 - 1995, the number of brackish shrimp farms was rapidly increased along the gulf of Thailand provinces close to Bangkok; Samut Sakon, Samut Songkram and Samut Prakarn. Moreover, in 1995 shrimp farming then developed to Sounthern Thai Gulf particularly in Nakorn Si Thammarat province and Eastern Thai Gulf nearby to Cambodian border Chuntaburi and Trat (Figure 3). Although Thailand has many shrimp species suitable for culture, the popular cultured shrimp are P.vannamei and the black tiger shrimp (*Penaeus monodon*) it is relatively resistant to environmental changes, has a high growth rate and a high economic value on exported markets. This species is the main product accounting for 56% (Figure 4). Concomitant with the growth of shrimp culture industry has been the recognition of the increasing importance of disease, particularly those caused by infectious agent. The most

important diseases of cultured penaeid shrimp have had viral or bacterial in which some diseases cause serious and economic losses. Due to viral etiology, twenty kinds of shrimp viruses both DNA and RNA (Table 2). Two major viral diseases of cultured penaeid shrimp that emerged during early 1990s have resulted in a serious devastation in shrimp farms of Asia and Indo-Pacific. Those diseases caused by the either White spot syndrome virus (WSSV) and the Yellow head virus (YHV) which are potentially serious pathogens in many species of penaeid shrimp and have been associated with severe epizootic of Western Hemisphere penaeid shrimps also (1).

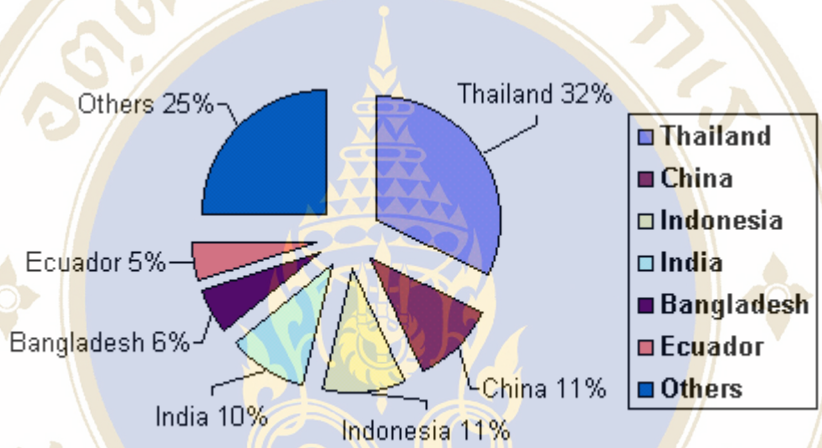

Figure 1. Major producers of aquaculture shrimp in the world (Year 2001) (2).

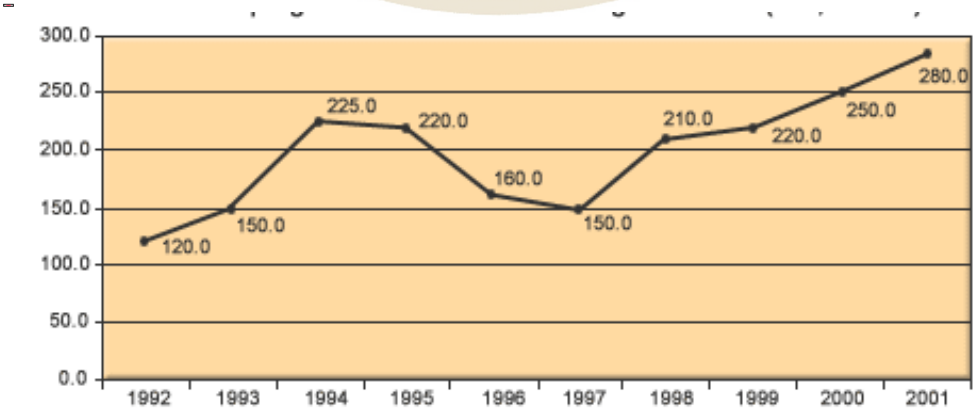

Figure 2. Thai shrimp aquaculture production during 1992-2001 (in 1,000 MT) (3).

Table 1. World shrimp production statistic in 1999 (1).

	Pond area (ha)	Production (t)	Relative prod. (%)	Productivity (kg/ha)
Thailand	80 000	200 000	24,6%	2 500
China	180 000	110 000	13,5%	610
Indonesia	350 000	100 000	12,3%	290
India	130 000	70 000	8,6%	540
Philippines	60 000	40 000	4,9%	670
Vietnam	200 000	40 000	4,9%	200
Taiwan	5 000	20 000	2,5%	4 000
Malaysia	4 000	6 000	0,7%	1 500
Iran	4 000	2 500	0,3%	630
Australia	600	2 400	0,3%	4 000
Other (Eastern hemisphere)	100 450	51 850	6,4%	520
Ecuador	100 000	85 000	10,4%	850
Mexico	11 700	35 000	4,3%	3 000
Brazil	6 000	15 000	1,8%	2 500
Nicaragua	6 000	4 000	0,5%	670
Venezuela	2 000	4 000	0,5%	2000
Panama	3 000	2 000	0,2%	670
United States	400	1 500	0,2%	3 750
Other (Western hemisphere)	11 300	27 000	3,3%	2 340
TOTAL	1 251 450	814 250		651

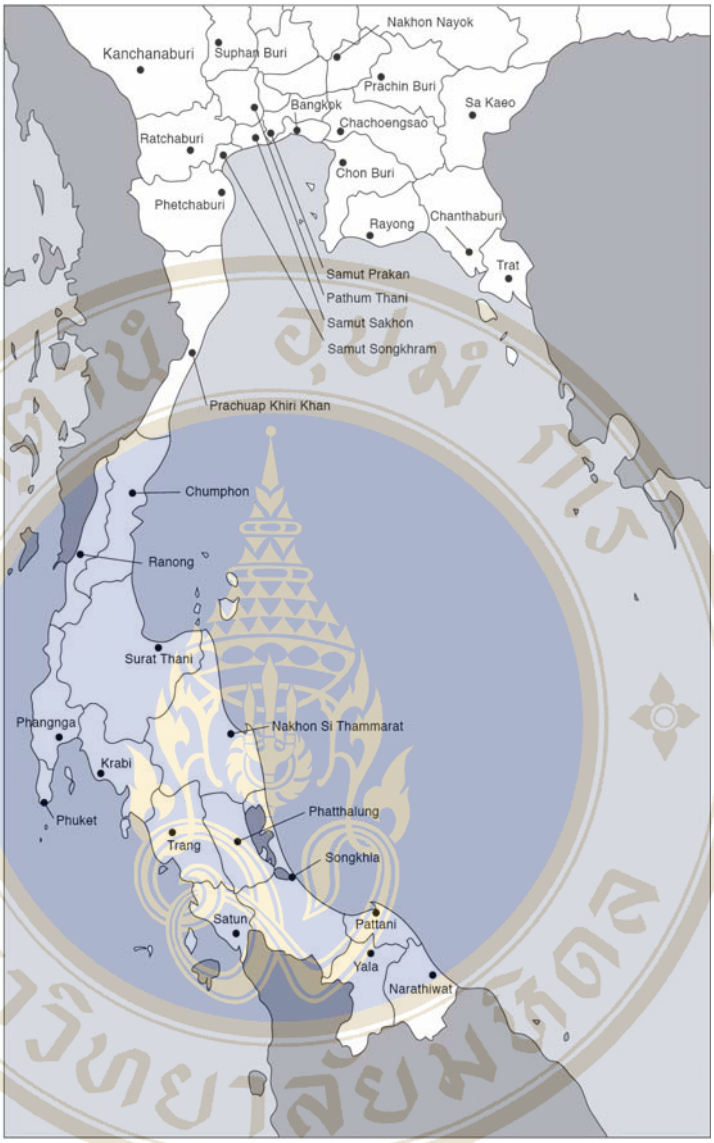

Figure 3. Geographic distribution of shrimp farm in Thailand: 1995 (4)**.**

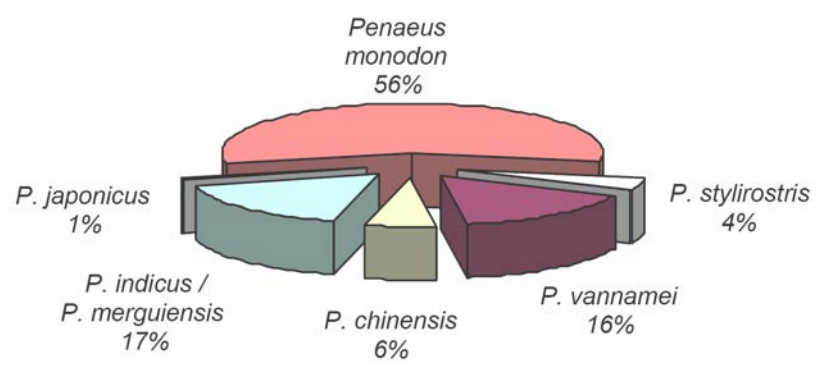

Figure 4. Relative importance of penaeid shrimp species to global aquaculture production in 1999. Source: Rosenberry (1999) (1).

Table 2. The DNA and RNA viruses of Penaeid shrimp (5).

The DNA viruses of penaeid shrimp (as of May 1996): grouped by major types and with complete names	
Abbreviation/full name	Key Refs.
DNA viruses	
Parvoviruses	
IHHNV = infectious hypodermal and hematopoietic necrosis virus	Lightner et al., 1983a,b; Bonami et al., 1990
HPV = hepatopancreatic parvovirus	Lightner and Redman, 1985
LPV = lymphoidal parvo-like virus	Owens et al., 1991
Penaeid baculo-like viruses	
BP-type = *Baculovirus penaei* type viruses (PvSNPV type sp.):	
BP from the Gulf of Mexico	Couch, 1974a,b
BP from Hawaii	Brock et al., 1986
BP from the Eastern Pacific	Lightner et al., 1985
MBV-type = *Penaeus monodon*-type baculoviruses (PmSNPV type sp.):	
MBV from S.E. Asia	Lightner et al., 1983c
MBV from Italy	Bovo, 1984 in Lightner et al., 1985
PBV = *Penaeus plebejus* baculovirus	Lester et al., 1987
BMN-type = baculoviral midgut gland necrosis type viruses:	
BMN = from *P. japonicus* in Japan	Sano et al., 1981
TCBV = type C baculovirus of *P. monodon*	Brock and Lightner, 1990a
WSSV-type = white spot syndrome baculoviruses (PmNOBII-type):	
SEMBV = systemic ectodermal and mesodermal baculovirus	Wongteerasupaya et al., 1995
RV-PJ = rod-shaped virus of *P. japonicus*	Takahashi et al., 1994
HHNBV = hypodermal and hematopoietic necrosis baculovirus	Huang et al., 1995
WSBV = white spot baculovirus	
Large baculo-like viruses	
PHRV = hemocyte-infecting nonoccluded baculovirus	Owens, 1993
Iridovirus	
IRDO = shrimp iridovirus	Lightner and Redman, 1993

The RNA viruses of penaeid shrimp (as of May 1996): grouped by major types and with complete names	
RNA viruses	
Picornavirus	
TSV = Taura syndrome virus	Lightner et al., 1995; Brock et al., 1995; Hasson et al., 1995
Reoviruses	
REO-III = type III reo-like virus	Tsing and Bonami, 1987
REO-IV = type IV reo-like virus	Adams and Bonami, 1991
Toga-like virus	
LOVV = lymphoid organ vacuolization virus	Bonami et al., 1992
Rhabdoviruses and ssRNA rod-shaped virus	
YHV/'YBV' = yellowhead virus of *P. monodon*	Boonyaratpalin et al., 1993; Flegel et al., 1995
RPS = rhabdovirus of penaeid shrimp	Nadala et al., 1992

2. Yellow Head Virus

Yellow head disease (YHD) is caused by yellow head virus (YHV), was first described as distinct disease from epizootic of cultured *P.monodon* in the central part of Thailand in 1991(6, 7). It had a wide distribution in cultures stock of *P.monodon* that reported mostly from cultivated shrimp in the Southeast Asia and Indo-Pacific countries especially Malaysia, Sri Lanka, Indonesia, Philippines, China and Taiwan. Furthermore, YHV has also been reported in farmed shrimp from the Americas in 1995. These virus was introduced from Asia where the first report in 1992 via the transportation of infected live stocks, by birds acting as vectors or by the importation and reprocessing of frozen food products (8, 9). YHV is capable of infecting several penaeid shrimp species. Natural infection occur in *P.monodon* while experimental infections have shown that YHV can cause high mortality in *P.japonicus, P.vannamei, P.setiferus, P.aztecus, P.duorarum* and *P.styirotris. P.merguiensis* it appears to be resistant to disease. In the other crustacean, Palemon has been shown to be carrier of viable virus including *Euphausia spp*. (krill), Acetes spp. and other and other small shrimp species could also carry the disease (10, 11).

The virus was named from the gross signs of disease which included a pale overall body coloration and a yellowish-cephalothoraxes. The virus is also potential of infection in several tissues which are the branchial portion of the gill, nerve cord, lymphoid organ (Oka), heart, midgut, hepatopancreas, head soft tissue (HST), abdominal muscle and eye stalk. It is remarkable that oka, gill and HST are the most susceptible targets and they should be the primary tissue used for YHD diagnosis.

2.1 Sign of Yellow head disease

YHD can infect cultured shrimp from post larval stages onwards, but mass mortality is usually encountered from early to late juvenile stage. The infected shrimp consume feed at an abnormality high rate for several days. Then they cease feeding entirely and within a day, a few moribund shrimp aggregate at pond edge. It appears swimming slowly. Affected shrimp exhibited a bleached overall appearance and a yellow discoloration of cepharothorax caused by the underling yellow HP, which was exceptionally soft when compared with the brow HP of the normal shrimp. In many cases, total crop loss has experienced within a few day of the appearance of shrimp showing gross-sign of YHD (6, 12, 13).

Figure 5. The sign of YHV infected *P. monodon* (A) shows pale to yellowish coloration of cephalothorax area along its body compared with normal shrimp (B) (6, 12, 13).

Table 3. Summary of Yellow Head Virus (YHV) (6, 7, 11, 13-17)

Order (Proposed name)	*Nidovirales* Okavirus
Closely related viral species	- Gill associated virus (GAV) - Lymphoid organ virus (LOV)
Clinical signs	Pale to yellowish coloration of the cephalothorax and Gills
Basophilic inclusions	Yes
Virion morphology	A pleomorphic enveloped - spike virus with bacilliform morphology enclosed the helical nucleocapsid
Size of the virion Size of nucleocapsid in virion Size of free nucleocapsid	173±13 nm x 44±6 nm 135±25 nm x 22±7 nm 14-18 nm
Nucleic acid	Positive polarity single stranded RNA with 22 kb in size
Structural protein composition by SDS-PAGE	- 170, 135, 67 and 22 kDa (Nadala, 1997) in which 170 and 135 kDa were glycosylated - 110, 63 and 20 kDa (Chang and Wang, 2000)
Site of replication	Cytoplasm
Potential carrier species	Penaeid shrimps *P. monodon, P. setiferus, P. aztecus, P. merquiensis, P. Stylirostris, P. vannamei* and *P. duorarum* Other shrimps *Metapenaeus ensis, palaeman styliferus and euphasia supertia*
Target tissues	Main target tissues - Lymphoid organ (Oka), head soft tissue, gills Other infected tissues - Midgut, abdominal muscle, heart, nerve cord, hepatopancreas, eyestalk

2.2 YHV Morphology

YHV is a positive-sense, single stranded RNA (ssRNA) virus, originally through to be rhabdoviruses but thereafter know to be related to coronaviruses and arteriviruses. Nowadays YHV has now been formally classified in the new genus Okavirus and new family Roniviridae (10, 11, 15, 18). within the viral order Nidovirales. Transmission electron microscopy (TEM) of purified YHV virions demonstrates the enveloped particles with bacilliforms or rod shape (Figure6). They range from approximately 150 nm to 200 nm in length and from 40 nm to 50 nm in diameter consist of two structural units, nucleocapsid and envelope with a trilaminar unit membrane. Moreover negatively stained YHV virions reveal regular arrays of short spike on the viral envelope. Agarose gel electrophoresis indicates a genome size of approximately 22 kb and more information about YHV show in Table 3. (10, 11, 15-17, 19).

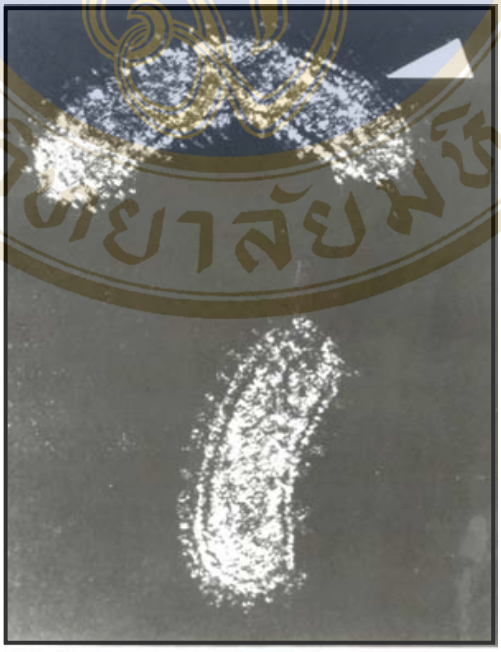

Figure 6. Transmission electron micrograph of YHV virion shows the bacilliform shape. Arrow indicates the spike-like projection.

2.3 YHV genome organization

The genome of YHV is single-stranded RNA with positive polarity. Its genome is large (26 kb). The GAV RNA genome has been extensively characterized. The genome comprises of two long overlapping open reading frames (ORFs), ORF1a and ORF1b, and the other three ORFs encoding the structural proteins. The overall nucleotide sequence identity of GAV ORF1b and YHV ORF1b is 80.5% in which the YHV ORF1b sequence contains 9 nucleotides (3 codons) insertion, 3 nucleotides (1 codon) deletion and 3 nucleotides (1 codon) extension immediately preceding the UAA stop codon. The deduced amino acid sequences of YHV and GAV ORF1b share 88.9% identity. They encode conserved polymerase, metal ion-binding (MIB) and helicase domain and 2 sequences that have undefined function (motif 1 and 3) (Figure 7) (101).

Downstream of ORF1a/b of GAV was reported to contain three genes which encoded at least three structural proteins. The corresponding region of the GAV genome contains three ORFs between ORF1a-1b and 3'-poly(A) tail (named ORF2, ORF3 and ORF4). The ORF2 and ORF3 encode the three main structural components of viral particle. ORF2 possesses a high proportion of proline residues, is highly hydrophilic and, in size and structure, shows some resemblance to the nucleocapsid protein (N) of toroviruses. Recent study comfirm that YHV nucleocapsid protein (p20) is also encoded in ORF2 and this gene comprises 441 nucleotides and encodes a polypeptide 146 amini acids (103). The function of theYHV ORF2 gene is evident from the deduced amino acid sequence, which shares a high level identity (84%) with the GAV nucleoprotein (103). ORF3 appears functionally equivalent to the S glycoprotein of corona- and toroviruses. The YHV ORF3 gene (5 kb) encodes a 1666 aa (185.7 kDa) protein that contains six highly hydrophobic regions that are likely to be transmembrane domains and has the predicted membrane topology shown in Figure 8 (100). The N-terminal sequence analyses have shown that the YHV virion gp116 and gp64 proteins are encoded in the ORF3 gene and generated by post-translational processing from a precursor polyprotein (100). the 0.6 kb region between ORF3 and 3'-poly(A) tail, ORF4, contains only one ORF of 83 amino acids of which the function of ORF4 protein is still unidentified (97).

The International Committee on Taxonomy of Viruses (ICTV) has recently ratified the classification of YHV, together with GAV as a type species in new genus Okavirus of the new family Roniviridae within the Nidovirales (18, 98). The name Okavirus is derived from the obserbvation that the viruses are commonly detected in the shrimp lymphoid or "Oka"organ. Roniviridae (single rod shap nidovirus) recognize their distinctive rod-shaped virion morphology (18, 97, 99). Classification within the Nidovirales was supported by identified phylogenetic relationships between GAV and nidoviruses in the viral replicase genes in the 5'-terminal 20 kb of the GAV (+) ssRNA genome (98). The discovery that GAV synthesizes 3'coterminal subgenomic (sg) mRNAs (98) consistent with the gene transcription strategy used by coronaviruses, toroviruses and arteriviruses also supports taxonomic classification in the Nidovirales.

Figure 7. Schematic illustration of the organization of GAV and YHV genomes. Boxes represent the open reading frames (ORFs) of the subgenomic RNA. The discontinuous boxes between ORF1a and ORF1b represent the overlapped (-1) ribosomal frameshift site. N, nucleocapsid; S proteins, structural proteins; Pol, polymerase domain; MIB, metal ion-binding domain; Hel, helicase domain; m1, conserved Motif 1 and m3, conserved Motif 3. The location of nucleotide insertions (+9, +3, +263) and deletion (-3) in the YHV genome compared to GAV are shown. (modified from Sittidilokratna, 2002)

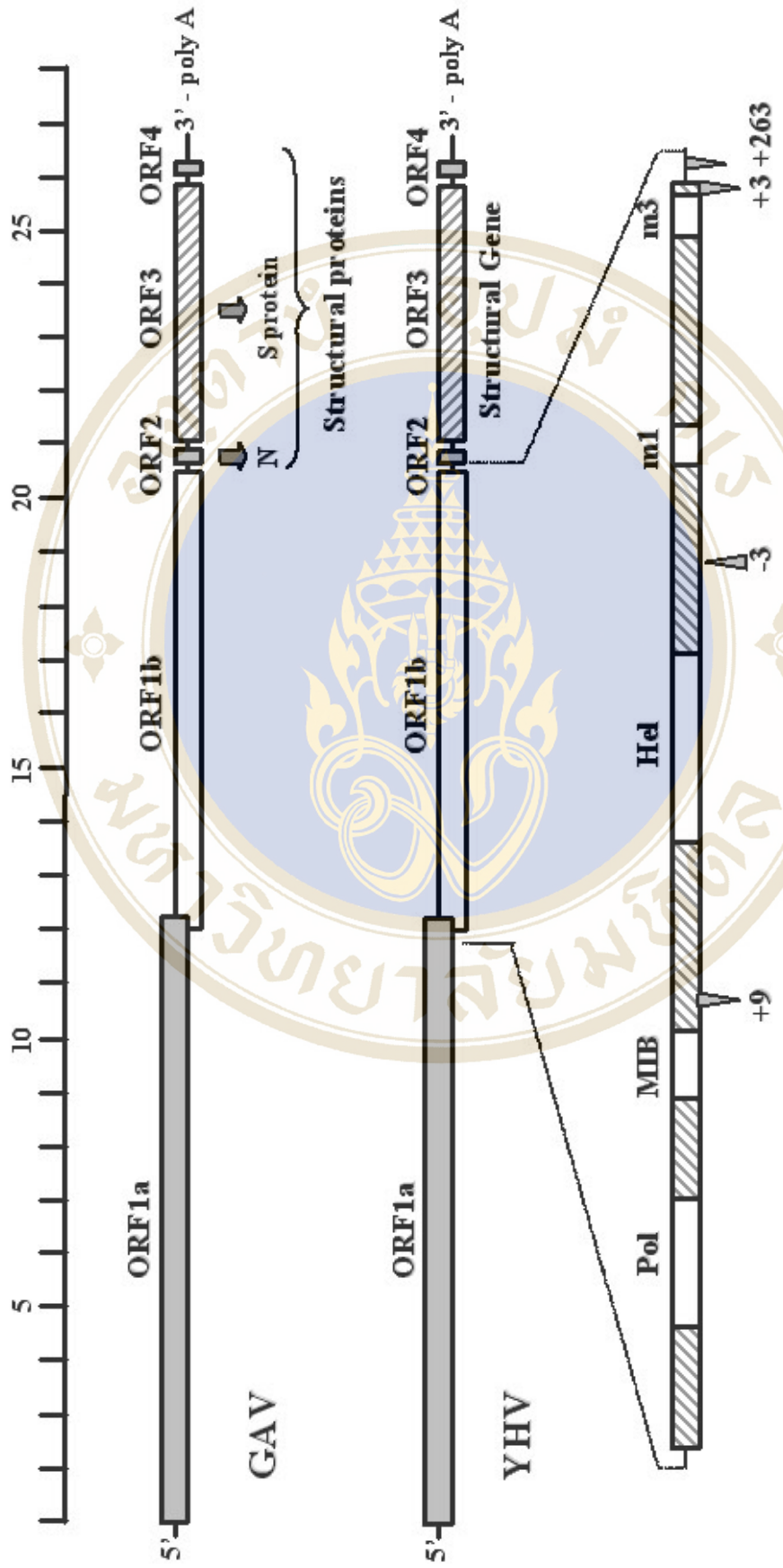

Figure 7.

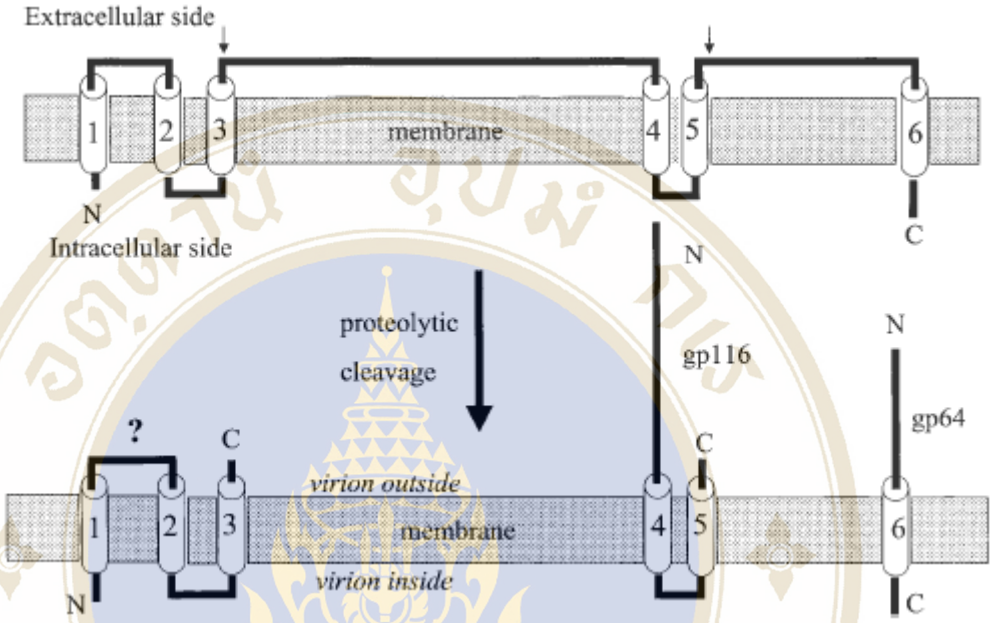

Figure 8. Predicted topology of YHV gp116 and gp64. Topology predictions were determined using TMHMM (Krogh et al.,2001). Cylinders with the number represent the predicted transmembrane domains while the solid lines represent predicted ectodomain or intra-virion domains. Arrows represent the proteolytic cleavage sites (100).

3. Immune system of shrimp

Immune systems have developed to protect multicellular organisms from foreign substances, during evolution, two types of immune systems have been developed to detect foreign substances namely innate (natural) immunity and adaptive (acquired) immunity (20, 21)

The innate immune system is phylogenetically a more ancient defense mechanism and can be found in all multicellular organisms. This system is the first line defense that helps to limit infection at the early stage and relies on germ line encoded receptors that recognize conserved molecular patterns present on microorganism.The adaptive immune system evolved about 400 million years ago and is found only in vertebrates. The immune response of vertebrates has developed more sophisticated and complicated mechanism including an immunological memory with generation of large repertoire of antigen recognition receptors and innate immune systems such as phagocytosis, natural killer cells and complement system for both recognizing and eliminating foreign invaders. While adaptive immunity occurs only in vertebrate, invertebrate have a raapid and efficient innate system to recognize and destroy non-self material including pathogens. Although they cannot produce antibodies and therefore have no immune memory. In addition to their rigid and wax-covered cuticle, which serves as a physical barrier, they can also rapid produce effective innate immune responses during infection (20-22).

Similar to other invertebrates, shrimp lack a true adaptive immune response system and rely stead on various innate immune responses against invading pathogen. The shrimp immune system is futhure subdivided into humoral and cellular defense response. Both cellular and humoral production immunity plays important roles in defense against microorganism in invertebrates. The cellular immune response refer to hemocyte mediated response like phagocytosis, encapsulation and nodule formation whereas the humoral immune response include the clotting cascade, the synthesis of a wide array of antimicrobial peptides and the phenoloxidase activating system (proPO system) due to melanin production (22-24).

3.1 Hemocyte mediated immune response

Crustaceans have open circulatory systems that are used both for blood cell circulation and nutrient and hormone distribution throughout the body (Figure 9).

Shrimp immune system is mainly occurring in hemolymph. The circulating hemocytes, which play a central role in host, defense. Firstly, they remove foreign materials in the hemocoel by phagocytosis, encapsulation and nodular aggregation. Secondly, hemocytes that part in wound healing by cellular clumping and initiation of coagulation process through the release of factors required for plasma gelation, and carriage and release of prophenoloxidase (proPO) system. They are also involved in the synthesis and dischage in hemolymp of important molecules such as agglutinins and antimicrobial peptide (20, 23-26). Three main hemocyte populations are usually identified based on the presence of cytoplasmic granules, i.e. the hyaline or agranular, the semigranular (with small granules) and the granular (with large granules) hemocytes (30). By combining morphological, cytochemical, and functional studies, two groups or lineages were shown in shrimps: the hyaline hemocytes involved in coagulation processes and the granular hemocytes active in phagocytosis, encapsulation and storage of the prophenoloxidase (proPO) system (27-30). The morphological different of hemocyte were shown in Figure 10.

In penaeid shrimp most studies concerning phagocytosis have been performed through observation of clearance processes of injected bacterial or particulate material. During this process, particle or microorganisms are engulfing in to the cell which later forms a digestive vacuole called the phagosome. The elimination of phagocyted particles involves the release of degradative enzymes into the phagosome and the generation of reactive oxygen intermediates (ROIs). On encunter with foreign particle, formation of capsule or nodule requires that circulating hemocytes change from non adhesive cells that are able to bind to target and one another. The encapsulated pathogen was eliminated by several factors including production of toxic intermediate via proPO system, ROIs and antimicrobial peptides (20, 29, 31-35).

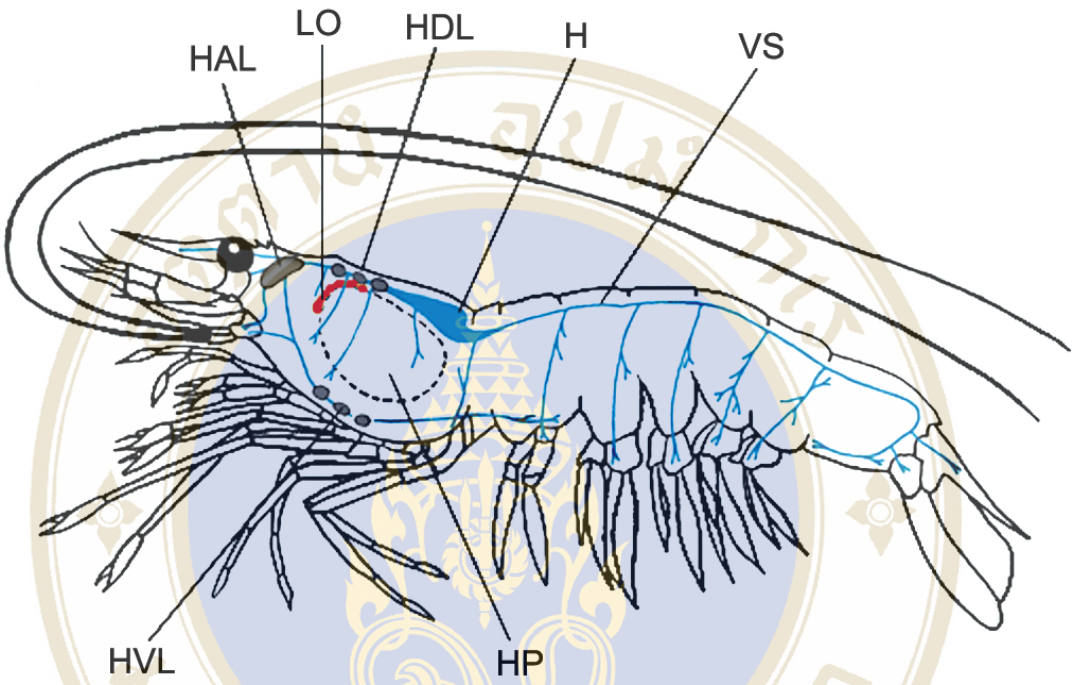

Figure 9. Open circulatory systems of shrimp (34).

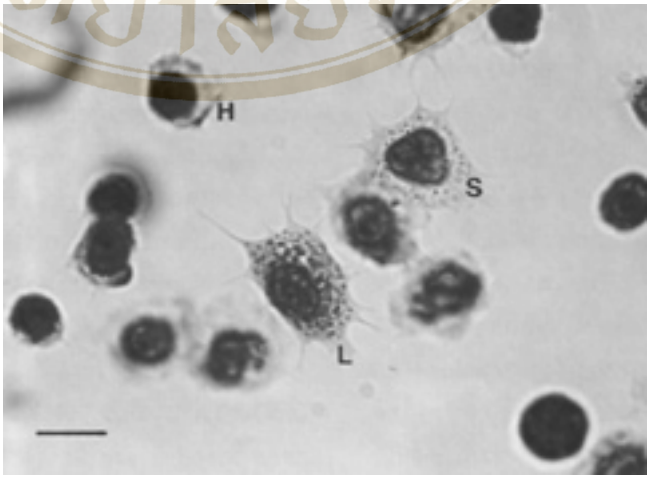

Figure 10. The morphologically different of shrimp hemocyte, which are hyalin cell (H), semi granular and granular cells by light microscopy (104).

3.2 Activation of shrimp immune system

Invertebrates do not contain antigen-specific lymphocytes and do not produce immunoglobulins. However, they contain a number of soluble molecules that bind to and lyse microorganisms. Typically such molecules are lectin-like proteins, which bind to carbohydrates present on microbial cell walls and hence initiate several immune responses as well as agglutinate the invading microorganisms. The innate immune system is activated by pathogens or environmental antigens and is mediated by interaction between receptors or pattern recognition molecules and pathogens. The recognition molecules for foreign material have been named pattern-recognition proteins (PRPs) by Janeway because the host primitive effector cells would recognize molecular patterns rather than particular structures of the invading micro-organisms. Examples of pathogen-associated molecules, which are not found in other multicellular organisms, are LPS or peptidoglycans of bacterial cell walls, β-1,3-glucan of fungal cell walls, and double-stranded RNA of viruses. Most current research has emphasized the possible role of non-self recognition molecules in the vertebrate and the invertebrate immune system (20, 21, 27, 28, 36-39).

Recognition of carbohydrates may have evolved because they are common constituents of microbial cell walls and have structures that are distinct from those of carbohydrates of eucaryotic cells. Therefore, LPS or/and β-1,3-glucan binding proteins (LBP, β-GBP, or LGBP), peptidoglycan recognition protein (PGRP), several kinds of lectins, and haemolin have been found in a variety of invertebrates and different biological functions have been proposed for these molecules following their binding to their targets (20, 22). Some of the characterized pattern recognition molecules in invertebrates are summarized in Table 3.

Table 3. Pattern recognition proteins of invertebrates (20).

Proteins	Properties/References
LPS-binding proteins	
Cockroach *Periplaneta americana* LBP	Opsonin [86, 92, 117]
Fall webworm *Hyphantria cunea* GNBP	Binding to LPS [118, 119]
Horseshoe crab *T. tridentatus* Factor C	Initiation of the coagulation system [120–123]
Horseshoe crab LPS-binding protein	Negative regulator of coagulation [124]
Mosquito *Anopheles gambiae* GNBP	LPS binding activity [58]
Silkworm *bombyx mori* BmLBP	Hemocyte nodule formation; bacterial clearance [88, 125]
Silkworm *bombyx mori* GNBP	Binding to G(−) bacteria [126]
Ig-like protein	
Moth *Hyalophora cecropia* Hemolin	IgG-like protein; binding to LPD and G(−) bacteria; prevents haemocyte aggregation; enhances phagocytosis [106]
Snail *Lymnaea stagnails* MDM	IgG-like protein [111]
Tobacco hornworm *M. sexta* Hemolin	IgG-like protein; prevents haemocyte aggregation; enhances phagocytosis [110]
β-1,3-glucan binding proteins	
Black tiger shrimp *P. monodon* βGBP	Binding to β-1,3-glucan [127]
Brown shrimp *P. californiensis* βGBP	Involvement in proPO system [128]
Cockroach *Blaberus craniifer* βGBP	Involvement in proPO system [129]
Crayfish *P. leniusculus* βGBP	Involvement in proPO system; plasma high-density lipoprotein; opsonin; degranulation factor [87, 102, 104, 105, 130, 131]
Horseshoe crab *T. tridentatus* Factor G	Initiation of the coagulation system [132–134]
Silkworm *Bombyx mori* βGRP	Involvement in proPO system [99]
Tobacco hornworm *M. sexta* βGBP	Involvement in proPO system, agglutinin [100]
LPS & β-1,3-glucan binding proteins	
Crayfish *P. leniusculus* LGBP	Involvement in proPO system [97]
Crayfish *P. leniusculus* mas-like protein	Opsonin; cell adhesion [54, 55]
Earthworm *Eisenia foetida* CCF-1	Involvement in proPO system; cytolytic factor; opsonin; TNF-*a* function [101, 136]
Fruit fly *Drosophila melanogaster* GNBP	Induction of antimicrobial peptide production [98]
Peptidoglycan (PG) binding proteins (T3-lysozyme-like protein)	
Fruit fly *D. melanogaste* PGRP-SA	Binding to PG
PGRP-SC1B	Binding to PG [137]
Moth *Trichoplusia ni* PGRP	Binding to PG and G(+) bacteria [138]
Silkworm *Bombyx mori* PGRP	Involvement in proPO system [135, 139]
Lectins	
Cockroach *Blaberus discoidalis* BDL 1	Binding to mannose
BDL 2	Opsonin; binding to D-GlcNAc/D-GalNAc
BDL 3	Opsonin; binding to D-GalNAc
GSL	Binding to β-1,3-glucan [78]
Fruit fly *Drosophila melanogaster* Lectin	Binding to galactose [140]

Table 3. Pattern recognition proteins of invertebrates (continued).

Proteins	Properties/References
Flesh fly *S. peregrina* Granulocytin	Agglutinin; binding to mucin [141]
Horseshoe crab *T. tridentatus* Lectin	
Tachylectin 1 (L6)	Antibacterial activity against G(−); binding to 2-keto-3-deoxyoctanate disaccharide, agarose, and dextran [142]
Tachylectin 2 (L10)	Haemagglutinating activity; recognition of G(−) bacteria; binding to D-GlcNAc/D-GalNAc, staphylococcal lipoteichoic acid and LPS [143]
Tachylectin 3 and Tachylectin 4	Haemagglutinating activity; binding to S-type LPS through O-specific polysaccharide [144, 145]
Tachylectin 5	Enhancing the antibacterial activity of big defensin; binding to N-Acetyl group [13, 146]
Tachylectin-P (TL-P)	Haemagglutinating activity; presence in perivitelline fluid [147]
Plasma lectin 1 (TPL-1)	Sepharose CL-4B binding activity [148]
Plasma lectin 2 (TPL-2)	LPS and protein A binding activity [148]
Tobacco hornworm *M. sexta* immulectin 1	Agglutinin; Involvement in proPO system [94]
immulectin 2	Agglutinin; Involvement in proPO system [95]

3.2.1 Recognition molecules

In shrimp, two kinds of proteins are involved in the recognition of microbial products, and their activation of cellular functions has been. The first group is constitutes multivalent sugar-binding agglutinins, also named hemagglutinins or lectins. The second group constitutes molecules that are apparently monovalent and do not induce agglutination, even though they are able to bind sugar residues. Agglutinating activities have been detected in plasma of *Penaeus monodon* , *Penaeus stylirostris* , *Penaeus californiensis* , *Penaeus japonicus* and *Penaeus indicus* (Maheswari et al. 1997). Lectins/agglutinin are glycoproteins usually without catalytic activity that have the ability to bind to specific carbohydrates and they are present in almost all living organisms. They can bind cells and an agglutination reaction occurs. Interaction between lectins and carbohydrates is involved in various biological activities, for instance the cellular and tissue transport of carbohydrates and glycoproteins, cell adhesion, opsonization and nodule formation. The biological

function of these LPS binding proteins was shown to be a bacterial clearance activity and an opsonic effect, respectively .

The second recognizing protein detected in shrimp plasma has the capability to react with beta glucan, and therefore it is named beta glucan binding protein or BGBP. The first crustacean BGBP was reported in crayfish (*Pacifastacus leniusculus*)
BGBP has been purified from *P. californiensis*, *P.vannamei* and *P. stylirostris* plasma as a 100-kDa monomeric protein. The amino acid swquence comparison among BGBP can only be clone with the N-terminal of the protein, the sequence appearred to be highly conserved between shrimp and crayfish (Figure 11).

Shrimp BGBP appears to be a constitutive plasma protein that after binding to β-glucans reacts with hemocyte surface and stimulates the release of hemocytic granules. The contents of the granules become activated in presence of plasma Ca^{2+}, leading to PO activity. Obviously, the activating reaction does not occur solely by the presence of BGBP, although its interaction with hemocyte membrane could exist.
In shrimp, both LPS and β -glucans binding proteins are present as possible recognition proteins. Besides differences in specificity, microbial products are recognized by proteins showing common characteristics. Both kinds of proteins are normally present in serum and its cellular receptors have been described and cloned. In addition, they appear to have two biologically active sites: one for the microbiological component, and other for the circulating cells. The second active site seems to be only available after reaction with LPS or β-glucans, respectively. The case of LPS-binding agglutinin is clear, because this protein is, at least, divalent which is a basic requirement for the induction of agglutination. Normally, the agglutinin does not induce hemocyte agglutination, but when the agglutinin reacts with an LPS-containing particle it is capable of reacting with the hemocyte surface and increasing the phagocytic activity. In the same manner, BGBP itself is unable to induce release and activation of the proPO system, but the protein–glucan complex is able to react with the circulating cells and increase the effect of glucans on the proPO system. Thus, these recognition proteins are capable of activating cellular activities only after reaction with the microbial carbohydrate LPS peptidoglycans or glucans (24, 33, 39).

3.2.2 proPhenoloxidase (proPO) system The proPO activating system consists of several proteins involved in the immune defence in invertebrates leading to

melanin production, cell adhesion, encapsulation, and phagocytosis. It is an efficient immune system for non-self recognition and is initiated by recognition of lipopolysaccharides or peptidoglycans from bacteria and β-1,3-glucans from fungi (20). This system contains a proteinase cascade composed of pattern recognition protein and prophenoloxidase (Figure 12.) The biochemical and molecular signals involved in these events are gradually being elucidated and a number of key proteins have been characterized as summarized in Table 4.

In shrimp, as in all crustaceans, a dark pigmented spot appears after an animal is injured. This is due to the action of phenoloxidase PO, which promotes hydroxylation of phenols and oxidation of *o*-phenols to quinones, necessary for the melanization process observed in response to foreign intruder in the hemocoele and during wound healing. Quinones are subsequently transformed, by a non-enzymatic reaction, to melanin and often deposited around encapsulated objects, in hemocyte nodules and at sites of fungal infections in the cuticle. Although a direct antimicrobial activity has been described for melanin and its precursors, the production of reactive oxygen species such as superoxide anions and hydroxyl radicals during the generation of quinoids also has an important antimicrobial role. In addition, biological reactions such as phagocytosis, encapsulation and nodulation are also activated, (20, 23, 32, 37, 40).

Crustacean PO is located inside hemocytic granules as an inactive pro-enzyme called prophenoloxidase (proPO) and its transformation from proPO to PO involves several reactions known as the proPO activating system. As in other crustaceans, the shrimp proPO system is specifically activated by β-1,3-glucans and LPS. Therefore, the crustacean proPO system has been considered as a recognition system. In addition, the proPO system has been proposed as the invertebrate counterpart of the vertebrate complement system since it can be activated by β-1,3-glucans, has a cascade reaction, and involves proteinases. The proposed proPO activation model for crustaceansinvolves a proteolytic cleavage mediated by a serine-proteinase namely proPO activating enzyme (PPAE). Although in shrimp, PPAE has been not purified its presence and participation on proPO activation has been detected. Shrimp PPAE is contained as a zymogen inside the hemocyte granules, together with proPO and is also released during microbial stimulus, centrifugation or cellular lysis. The activation of

PPAE is Ca^{2+}-dependent and the active enzyme can be inhibited by either melittin or soybean trypsin inhibitor (STI). In shrimp, the activation of proPO involves in two steps. The first one is the degranulation that occurs when hemocytes are stimulated by bacteria, LPS or β-glucans, and inactive forms of both proPO and PPAE are released. The second one requires the participation of Ca^{2+} for the conversion of inactive PPAE to an active proteinase that, in turn, transforms proPO to active PO. Thus, under *in vivo* conditions, PPAE is activated by plasmatic Ca^{2+}after hemocyte degranulation, which is induced by external, like LPS and β-glucans (20, 22, 23, 25, 29, 31, 40).

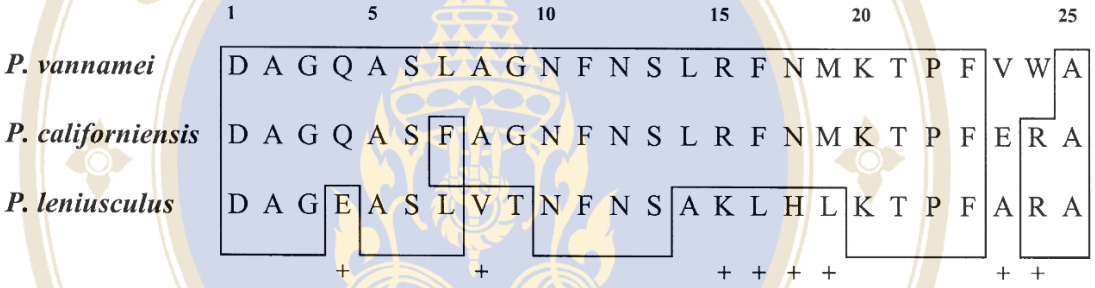

Figure 11. Alignment of *P. vannamei, P. californiensis Pac. leniusculus* BGBP *N*-terminal sequences. Identical sequences are boxed. Plus signs indicate conservative replacement between white shrimp and crayfish (39).

Table 4. The characterized effector involve in crustaceans immunity (22).

Key proteins of the crustacean immune system that have been purified, cloned and sequenced

Protein	Confirmed immune role?	Published sequence information	Species*	Reference
Prophenoloxidase(proPO)	Yes	mRNA	*Pacifastacus leniusculus*	[74]
		mRNA	*Penaeus monodon*	[75]
		mRNA	*Marsupenaeus japonicus*	Adachi et al., 2001† (unpublished) Rojtinnakorn et al., 2001† (unpublished)
Peroxinectin precursor	Yes	mRNA	*Pacifastacus leniusculus*	[76]
Peroxinectin	Yes	mRNA	*Pacifastacus leniusculus*	[67]
			Penaeus monodon	[77]
Cell surface superoxide dismutase peroxinectin binding protein	Yes	mRNA	*Pacifastacus leniusculus*	[76]
β-1,3-glucan binding protein (β-GBP)	Yes	mRNA	*Pacifastacus leniusculus*	[80]
Lipolysaccharide and β-1,3-glucan binding protein (L-GBP)	Yes	mRNA	*Pacifastacus leniusculus*	[81]
Prophenoloxidase activating enzyme (serine proteinase ppA)	Yes	mRNA	*Pacifastacus leniusculus*	[82]
11.5 kDa antibacterial peptide	Yes	mRNA, partial	*Carcinus maenas*	[69]
6.5 kDa antibacterial peptide	Yes	amino acid fragment	*Carcinus maenas*	[70]
Penaeidin 2, antibacterial peptide	Yes	mRNA	*Penaeus vannamei*	[83]
Penaeidin 3a, antibacterial peptide	Yes	mRNA	*Penaeus vannamei*	[83]
Penaeidin 3 family, antibacterial peptides	Yes	mRNA	*Litopenaeus vannamei*	Cuthbertson et al., 2001† (unpublished)
Penaeidin 4 family, antibacterial peptides	Yes	mRNA	*Litopenaeus vannamei*	Cuthbertson et al., 2001† (unpublished)
α-2-macroglobulin‡	Yes/No?	terminal and thiolester amino acid sequence only	*Pacifastacus leniusculus*	[84]
		mRNA	*Carcinus maenas, Homarus gammarus*	Hammond et al., in prep
α-2-macroglobulin homologue	Yes/No?	N terminal and thiolester amino acid sequence only	*Homarus americanus*	[85]
Crustacean masquerade like protein	Yes	mRNA	*Pacifastacus leniusculus*	[86]
Clotting protein precursor	Yes	mRNA	*Pacifastacus leniusculus*	[87]

* Species are referred to according to the names used in the sequence databases.
† Sequence information published on Genbank only.
‡ Complete mRNA sequences for *Ciona intestinalis* (Hammond et al., 2002† and *Limulus* spp. [88]α-2-macroglobulin have been published.

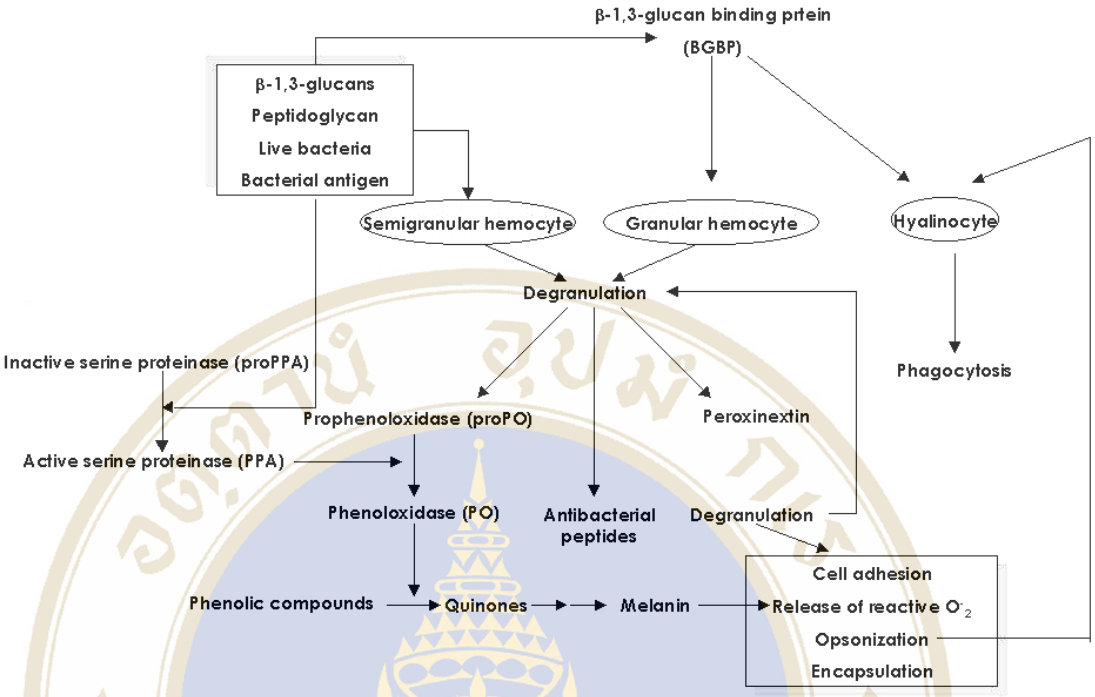

Figure 12. Simplified flow diagram of the crustacean host defense system (22)**.**

3.2.3 Coagulation system

Another system activated by microbial products is coagulation.This is an essential defence response for crustaceans because it prevents both loss of hemolymph through breaks in the exoskeleton and the dissemination of bacteria throughout the body. Only two different coagulation mechanisms have been characterized which are the hemocytes derived clotting cascade in hoseshoe crab (*Tachypleus tridentatus)* and the transglutaminase (Tgase)- dependent clotting reaction in crayfish (*P. leniusculus*). The key plasma protein, which constitutes the clot has been named clotting protein or CP. It appears to be present in relatively high concentrations in hemolymph (23, 33, 37). This clotting reaction occurs when TGase is released from hemocytes, upon wounding that becomes activated by Ca^{2+} in hemolymph and starts to cross-link the CP molecules into large aggregates (Figure13). In crustaceans, the clotable protein were found in several species that are *P. monodon*, *P. vannamei*, lobster (*Panulirus interruptus*), and the sand crayfish (*Ibacus ciliatus*). In all case, CP was reported as lipoglycoprotein with a molecular mass about 420 kDa and with identical subunits linked by disulfide bridges. Moreover, these proteins were shown similar amino acid composition and N-terminal sequences among crustaceans (Figure 14), (23, 32).

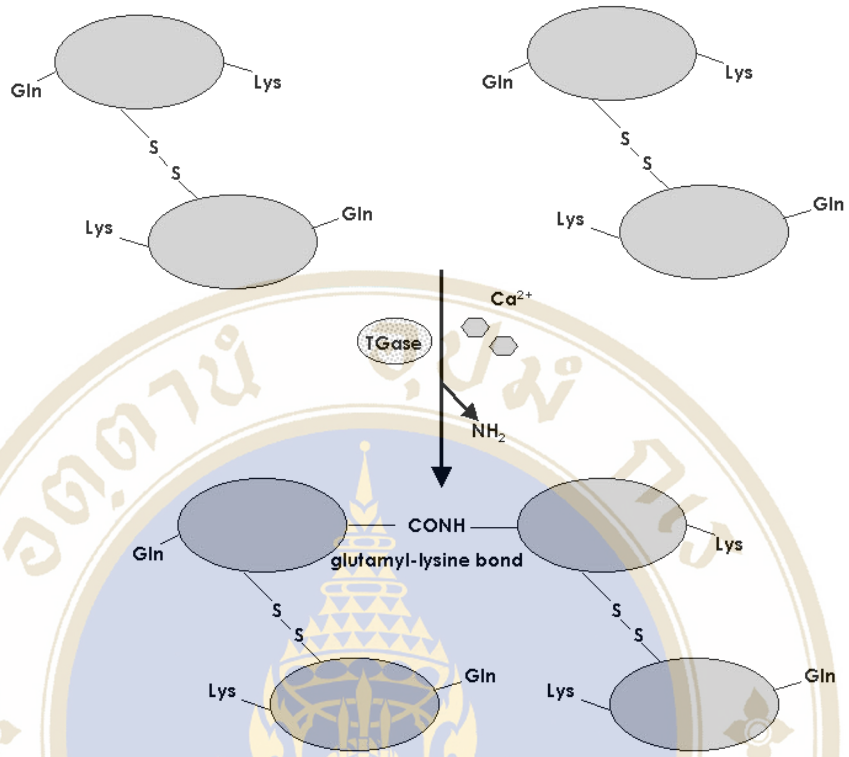

Figure 13. The TGase-mediated clotting reaction in crayfish. CP, a dimeric protein consisting of two identical 210-kDa subunits held together by disulfide bond -s-s- are cross-linking by hemocyte TGase in the presence of Ca^{2+} filled hexagon (23).

P. monodon CP	**LQPGLEYQYR**
P. leniusculus CP	**LHSNLEYQYR**
I. ciliatus VHDL	**LQPGLEYQYR**
P. interruptus fibrinogen	**LQPKLEYQYK**

Figure 14. Alignment of N-terminal sequence of *P. monodon* CP compared with that of *P. leniusculus* CP , *I. ciliatus* VHDL and *P. interruptus* fibrinoge. The corresponding residues in the shrimp CP are in boldface (23).

3.3.3 Antimicrobial peptides (AMPs)

It is widely known that AMPs are major players in innate immunity, conserved in evolution and present in all phyla of the living kingdom. These effectors present a great diversity in terms of structural features, biological properties and functions, and also in their tissue distribution and expression. AMPs are conventionally described as cationic and amphipathic molecules encoded by genes, but there are now striking evidence that other AMPs or polypeptides may originate from various other sources, such as from the hydrolysis of large inactive proteins involved in various functions. More than 700 AMPs have been discovered in plants, vertebrates, and invertebrates, where most originate from the insects (28, 34, 41).

In penaeid shrimp the AMPs were initially characterized from *Litopenaeus vannamei* by biochemical approaches and molecular cloning, they were named penaeidin. The three peptides (Litvan-Pen2-2, Litvan-Pen-2-1, and Litvan-Pen-3-1; initially named Pen-1, Pen-2, and Pen-3) were isolated in their active and mature form (5.48–6.62 kDa) from the hemocytes of animals collected from intensive shrimp farms. They are highly cationic molecules with calculated isoelectric points ranging from 9.34 to 9.84. The penaeidins are composed of an N-terminal proline-rich region (domain), followed by a COOH-terminal region containing six cysteine residues organized in two doublets. This overall structure is quite unique among the AMP families, and this originality has led to the identification of the new family of penaeidins (42, 43). Indeed, recent studies based on a genomic approach have revealed the presence of penaeidins in different penaeid shrimp species (30, 34, 44, 45). To date, penaeidin sequences have been described in *L. vannamei* (pacific white shrimp), *L. setiferus* (atlantic white shrimp), *L. stylirostris* (blue shrimp), Penaeus semisulcatus (green tiger prawn), *P. japonicus* (kuruma shrimp), *P. monodon* (black tiger shrimp) and *Fenneropenaeus chinensis* (fleshy prawn). All these peptides share high sequence similarities that have allowed the establishment of a penaeidin signature (46). The family appears to be characterized by (i) a highly conserved signal peptide (ii) an N-terminal proline-rich domain with the following signature (Y,F)T(R,G)P(X)2(R,K)P, and (iii) a C-terminal cysteine-rich domain with the following signature C(X)2_3C(X)7RXCC(X)5CC (46) (Figure 15). Penaeidins are constitutively expressed in their mature and active form in granular hemocytes of naive shrimps i.e.

that have not been experimentally stimulated by microbial challenge. About 30–40% of the circulating hemocytes express penaeidins(34, 42). The peptides are stored within cytoplasmic granules of granular hemocyte populations, namely hemocytes with large granules and to a lesser extend also in hemocytes with small granules. The population of hyaline cells is devoid of penaeidins. Such a location of constitutively expressed AMPs in granular cells is similar to that observed in hemocytes of many invertebrates, such as horseshoe crabs, mussels, tunicates or spiders and also in mammalian phagocytes and intestinal cells (34, 47-52). In shrimp, hemocytes are the main source of penaeidin production. The distribution of penaeidin transcripts and proteins is restricted to hemocytes that are present strikingly in almost all the shrimp tissues, both circulating in blood vessels irrigating the tissues or infiltrating tissues (34, 53).

Only the properties of penaeidins isolated from *L. vannamei* have been studied in detail. Litvan-Pen2-1 and Litvan-Pen3-1 have a broad spectrum of anti-fungal activities against filamentous fungi with minimum inhibitory concentration (MIC) below 10 mM (Table 3). The fungicidal activity against different fungus strains have been associated with an inhibition of spore germination, whereas at lower concentration (<5 mM), the peptides cause a growth inhibition, resulting in abnormal morphology (34, 53). Penaeidin anti-bacterial activity is predominantly directed against Gram-positive bacteria via a strain-specific inhibition mechanism and through multiple modes of action. Litvan-Pen3-1 displays potent growth inhibition properties against various bacteria (MIC=0.3–0.6 mM) (Table 6). In some cases, this bacteriostatic effect is associated with a slow killing of the bacteria (34). Additionally, the peptides can have a rapid bactericidal effect, as shown against *Bacillus megaterium*, which is killed after only few minutes incubation with Litvan-Pen3-1 (at 10-fold higher than the MIC which is 2.5–5 mM). Similar spectra of activities have been shown for Litvan-Pen2-1 and Litvan-Pen-3-1, the latter appearing to be more efficient. Penaeidins display weak anti-bacterial activity in vitro against Gram-negative strains (up to 20 mM) including Vibrionaceae species, a family of bacteria commonly associated with shrimp mortalities or, conversely, known as commensals for penaeid shrimps (Table 6).

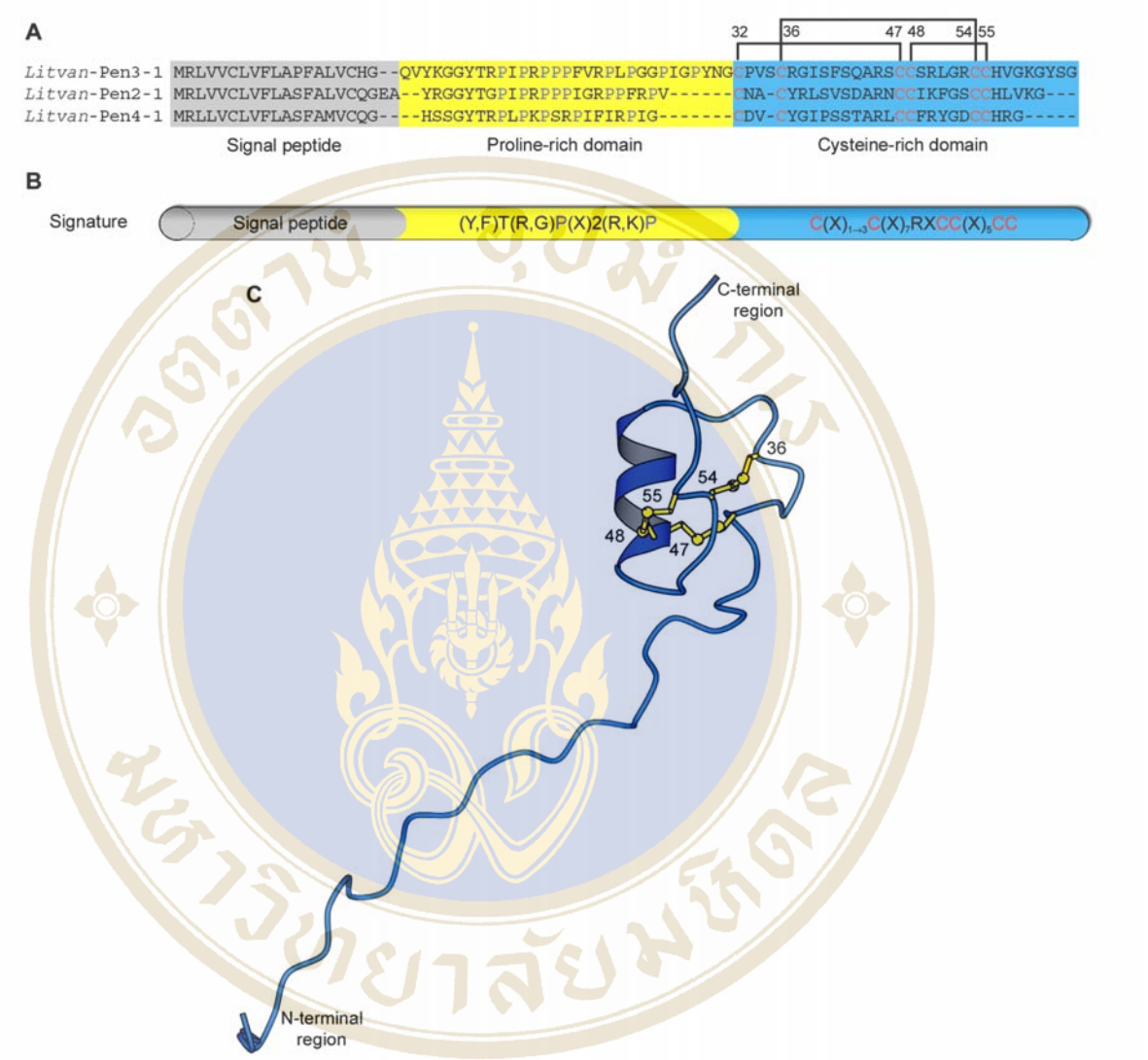

Figure 15. Structure and properties of penaeidins isolated from Penaeid shrimp. (A) Sequence comparison of three penaeidins from Litopeneaus vannamei [Litvan-Pen2-1 (Y14925), Litvan-Pen3-1 (Y14926), and Litvan-Pen4-1 (BE188446)], representing the three subgroups of the penaeidin family. The disulfide bridge pattern determined for Litvan-Pen3-1 is shown over the sequence. (B) A signature of the penaeidin family has been established using all the available peptide sequences (PENBASE, a penaeidin database). (C) 3D structure of Litvan-Pen3-1 (34, 54).

Table 6. Anti-microbial activity of Livan-Pen3-1 (34, 53).

Microorganisms	Minimum inhibitory concentration (MIC) (μM)
Gram(+) bacteria	
Aerococcus viridans	0.3–0.6
Micrococcus luteus	1.25–2.5
Bacillus megaterium	2.5–5
Gram(–) bacteria	
Escherichia coli 363	5–10
Vibrio harveyi	>40
Vibrio penaeicida	>40
Salmonella thyphimurium	>40
Enterobacter cloacae	>40
Filamentus fungi	
Neurospora crassa	1.25–2.5
Fusarium oxysporum	5–10
Botrytis cinerea	5–10

MIC is expressed as the interval *a*–*b*, where *a* is the highest concentration tested at which the growth of the microorganism is not inhibited and *b* is the lowest concentration that causes 100 growth inhibition. Adapted from (43).

4. Lymphoid organ with the potential of immune function

4.1 Structure and function of lymphoid organ

The Lymphoid organ (LO) is considered to be an integral part of the penaeid shrimp circulatory system. The organ, absent in most of the crustaceans, is located at the anterior part of the hepatopancreas. The histology of LO is a bi-lobe network of arterioles usually referred to as tubules. It recieves hemolymph from the heart via paired afferent vessels branching from the subgastric artery. Hemocytes passage through tubule walls into intertubular hemal sinuses has been documented (55). Histopathology examined the LO structure show in Figure 16.

Because of the hemolymph filtration and elimination function, LO has been shown to participate in particle uptake and clearancefor instance. LO processing 2 primary components, 'arteriolar tubules' or 'shealth arterioles' (LO tubules) and 'nodular structures with many vacuolated cells' lymphoid organ spheroid (LOS) (Figure 17) that having a characteristic of exocytosed, granular hemocytes and apoptotic cells, have been considered as a major mechanism of penaeid antiviral defense or as an unexplored and significant branch of cell-mediated immune response of penaied shrimp.

There are three distinct LOS morphotypes, type A, B and C. The research on Tuara syndrome virus infection in *P. vannamei* revealed the progression of LOS morphotypes through the stage of viral infection and elimination (55).

The earliest detectable, type A, appeared to evolve from activated LO tubule phagocytes that has sequestered virus. Cells in this stage can be characterized by a lightly basophilic, homogeneous cell mass containing few or no necrotic cells or cell debris. The cytoplasm to nuclear volumetric ratio ranged from ~2:1 to 3:1. Figure 18 A&B revealed the homogeneous appearance of each cell mass at this stage and gene probed negative were determined.

The succeeding, type B spheroid appeared to evolve from type A, but differed by displaying increased numbers of necrotic cell/ debris and few to moderate numbers of cytoplasmic vacuoles. The necrotic cells were consistently with the virus replication taken place in an acute phase of infection as the accumulation of viruses can be determined as the strong TSV-probe-positive signal (black precipitated) (Figure 18 C&D).

In the chronic of infection; 9 wk p.i., the terminal type C LOS displayed an overall increase in basophilliia, containing highly basophillic niclei ~33 to 50% smaller than those observed in the type A and B forms, a reduced cytoplasm to nuclear volumetric ratio (ranging from ~0.5:1 to 1:1) and few to numerous cytoplasmic vacuole. Cells in this stage were not found any viruses when determined by *in situ* hybridization (Figure 18 E&F). But the undetectable virus in the LOS C type may result on the virus elimination at this organ, thus, the virus may continue to replicate within LOS cells unchecked, or it may be eliminated by LOS cells or viral replication and elimination may occur concurrently, resulting in persistent infection. (55)

Bonami *et al.* (1992) found that LOS were common placed and had been observed within at least 7 different penaied shrimp species; *P. vannamei, P. monodon, P. penicillatus, P. esculentus, P. stylirostris, P. chinensis and P. merguiensis*. The development of LO has also been associated with at least 6 different penaied shrimp viral infection including Lymphoid organ vacuolization virus (LOVV), Lymphoid parvo-like virus (LPV), lymphoid organ virus (LOV), Rhabdovirus of Penaied shrimp (RPS), Yellow head virus (YHV) and tusra syndrome virus (TSV). Indeed, the morphology of LOS induced by all 6 viruses mentioned aboved is virtually identical, suggesting that LOS development is a general of penaeid shrimp in response to viral agents (55)

Taken together, even the LO involved in the clearance of foreign components, the lymphoid organ of moribund shrimp suffering from virus infection usually has extensive abnormalities, including many degenerated cells in the stromal matrix of the tubules. These have condensed pyknotic and karyorhectic nuclei, and cytoplasmic basophilic inclusions. The dysfunction of LO during YHV infection which caused mortality to host is necessary to study what factor(s) which responsed to viral infection in the way to leading apoptosis.

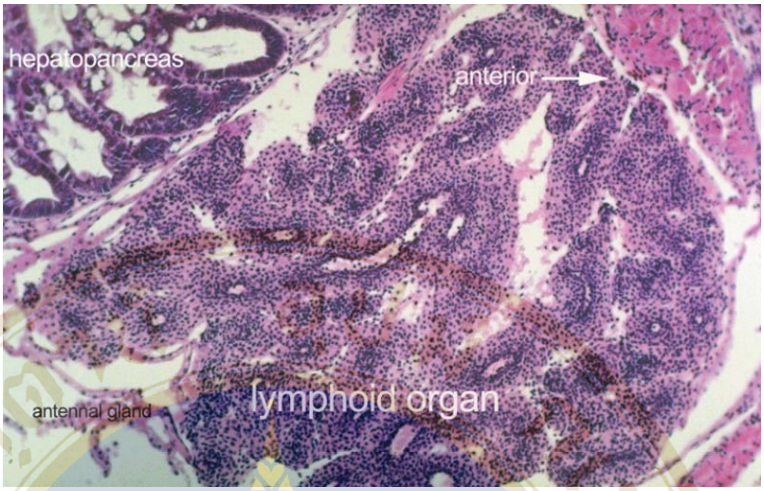

Figure 16. Light micrograph of the longitudinal section of *P. monodon* LO. Arrow head points the LO tubule (59).

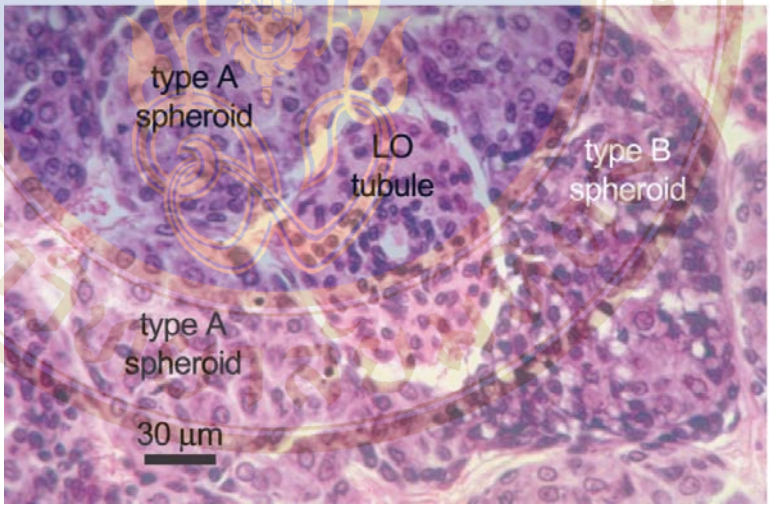

Figure 17. Light micrograph show the different LOS morphotypes (Type A and B) were identified by light microscopy (59).

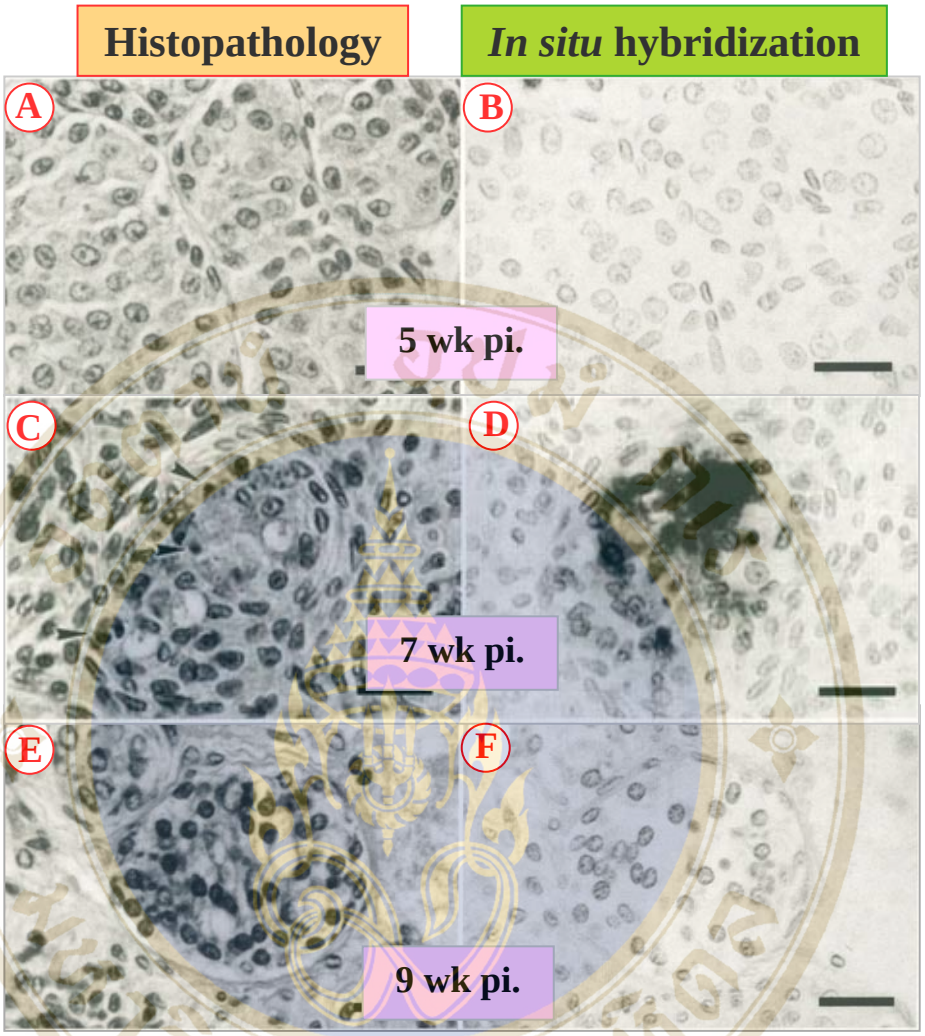

Figure 18. Photomicrographs of consecutive H&E-stained (Left column) and Taura syndrome virus gene probed (Right column) lymphoid organ (LO) tissue sections illlustrating the lymphoid spheroid (LOS) monotype (Type A to C). (A, B) Type A LOS in a specimen collected 5 wk p.i. (C&D) Early Type B LOS from a specimen 7 wk p.i. (E&F) Type C LOS from a specimen 9 wk p.i.. Scale bars = 20 µm (Hasson *et. al*, 1999).

CHAPTER II
OBJECTIVES

1. To study the gene expression profile from lymphoid organ (LO) of YHV-infected shrimp (*Penaeus monodon*) through the cDNA library construction.
2. To analyze the inferred amino acid of cDNA sequences using bioinformatics tools.
3. To study expression pattern of some genes selected from shrimp upon YHV infection.

CHAPTER III
MATERIALS AND METHODS

I. Materials

1. Virus, Bacteria strains and Plasmid vector

Table7. Virus, Bacteria strains and Plasmid used in this experiment.

Bacteria strains and vectors	Properties
Yellow head virus Isolated from *P. monodon*	The causative agent of Yellow head disease in Penaeid shrimp
Escherichia coli: -DH5α: *supE*44 Δ*lac*U169 (ϕ80 *lac*ZΔM15) *hsd*R17 *recA*1 *endA*1 *gyrA*96 *thi*-1 *relA*1]	A recombinant-deficient amber suppressing strain used for recombinant DNA manipulation
- XL1-Blue MRF' Δ(*mcrA*)183 Δ(*mcrCB-hsdSMR-mrr*)173 *endA1 supE44 thi-1 recA*1 *gyrA*96 *relA*1 *lac* [F' *proAB lacl*^{q}Z ΔM15 Tn10 (Tet^{r})]	A strain used for cDNA library construction that susceptible for lambda phage library infection.
- SOLR® e14^{-}(McrA^{-}) Δ(*mcrCB-hsdSMR-mrr*)171 *sbcC recB recJ uvrC umuC*::Tn5 (Kan^{r}) *lac recA*1 *gyrA*96 *relA*1 *thi-1 endA*1 λ^{R} [F' *proAB lacl*^{q}Z ΔM15] Su^{-} (nonsupressing)	A strain used for efficient excision of the pBluescript phagemid from the Uni-ZAP XR vector.
Vectors; PGEM®-T easy (Promega)	The vector for cloning PCR products; ampicillin as and selectable marker
-Uni-ZAP® XR vector (Stratagene)	The vector used for cloning cDNA; it can be converted to pBluescript®

2. Chemicals

Bacterial culture media; tryptone or peptone, yeast extract, bacto agar were purchased from Difco. Amplicillin, Tetracycline and Kanamycin were purchased from Sigma. The following chemicals were purchased from Fermentas: IPTG, X-GAL and dNTPs mix. Other chemicals and solvents were purchased from Merk:Germany, Fluka:Switzerland, Sigma:USA.

3. Enzymes and markers

Restriction endonuclease, *Eco*RI was purchased from New England Biolabs. Heat-activated thermostable DNA polymerase was obtained from Bioline. Reverse transcriptase (MMLV) was purchased from Invitrogen. The Ribonuclease A was purchased from Sigma. 1 kb ladder and 100 bp ladder were purchased from Fermentas.

4. Oligonucleotide primers

Two universal primers for amplifying cDNA fragments in pBluescript phagmid are T7 and T3 they were synthesized by the Bioservice Unit (BSU), National Science and Technology Development Agency (NSTDA). The specific oligonucleotide primers used for generating the 5' end and partial fragment of ASF1 were synthesized by Proligo Singapore Pty Ltd. These primers were designed based on 5'ESTs sequencing of clone number LP-S01-0712-LF that obtained from cDNA library of YHV infected shrimp lymphoid organ.

5. Kits

- QIAquick Gel Extraction Kit and QIAprep Miniprep Kit were purchased from QIAGEN
- ZAP-cDNA® Synthesis Kit and ZAP-cDNA® Gigapack® III Gold Cloning Kit were purchased Stratagene.
- BD Advantage™ 2 PCR Kit was purchased from BD Bioscience
- GeneRacer™ Kit was obtained from Invitrogen.

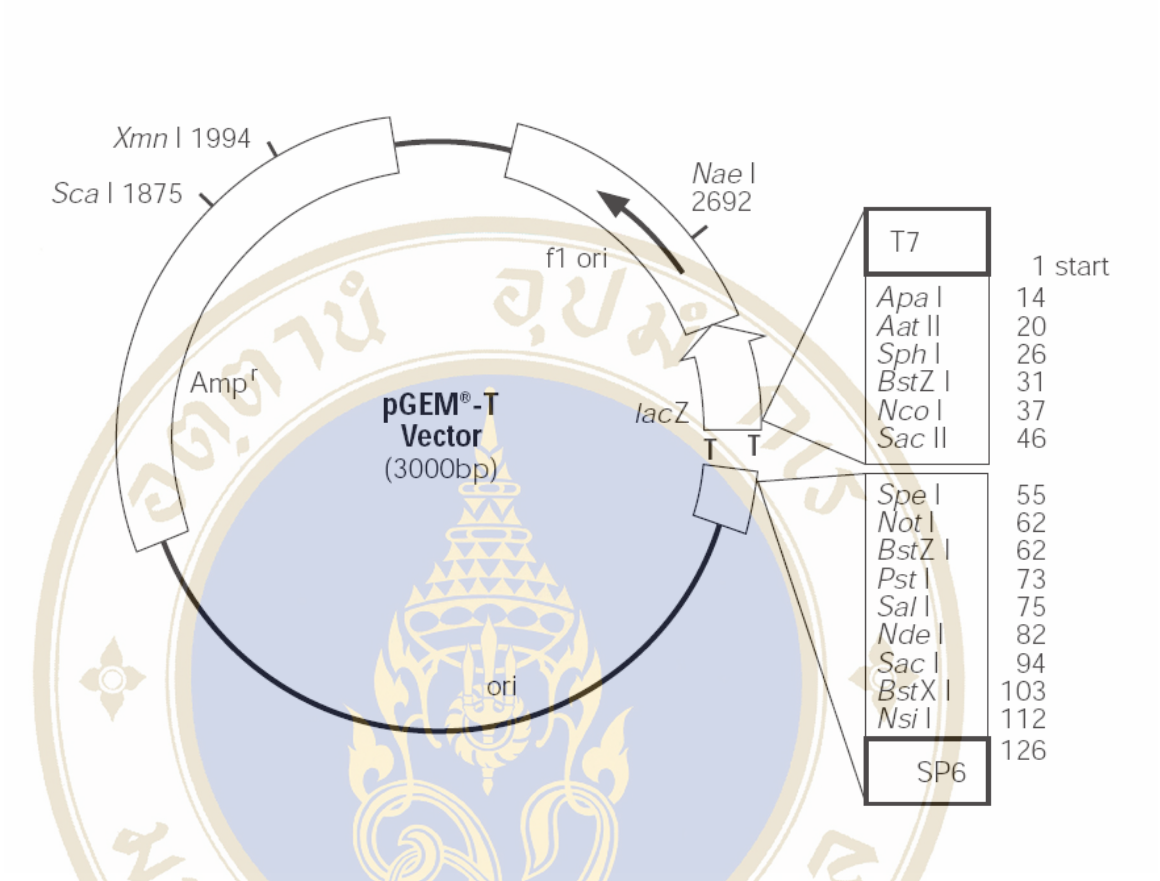

Figure 19. The physical map of pGEM®-T easy (Promega, USA)

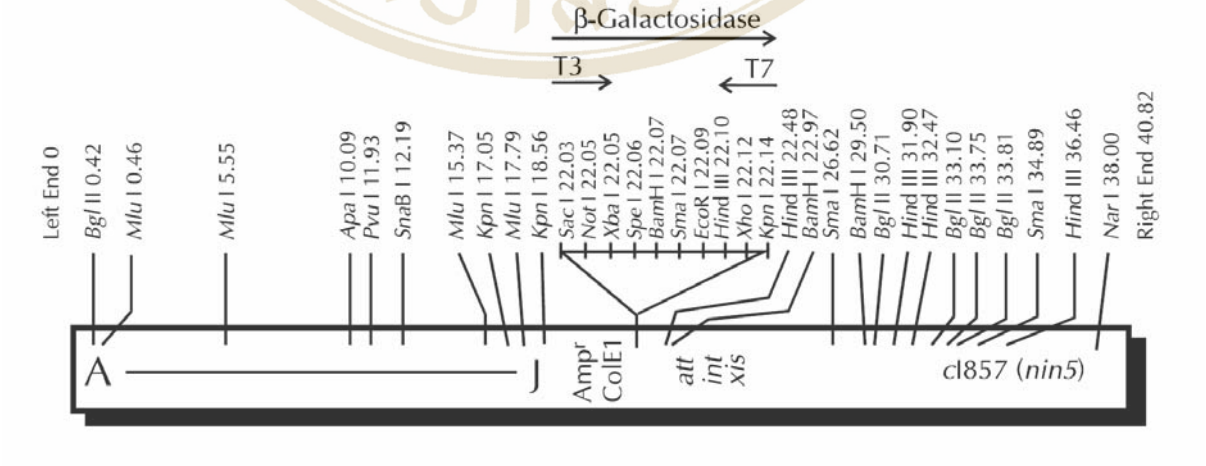

Figure 20. Map of the Uni-ZAP XR insertion vector**.**

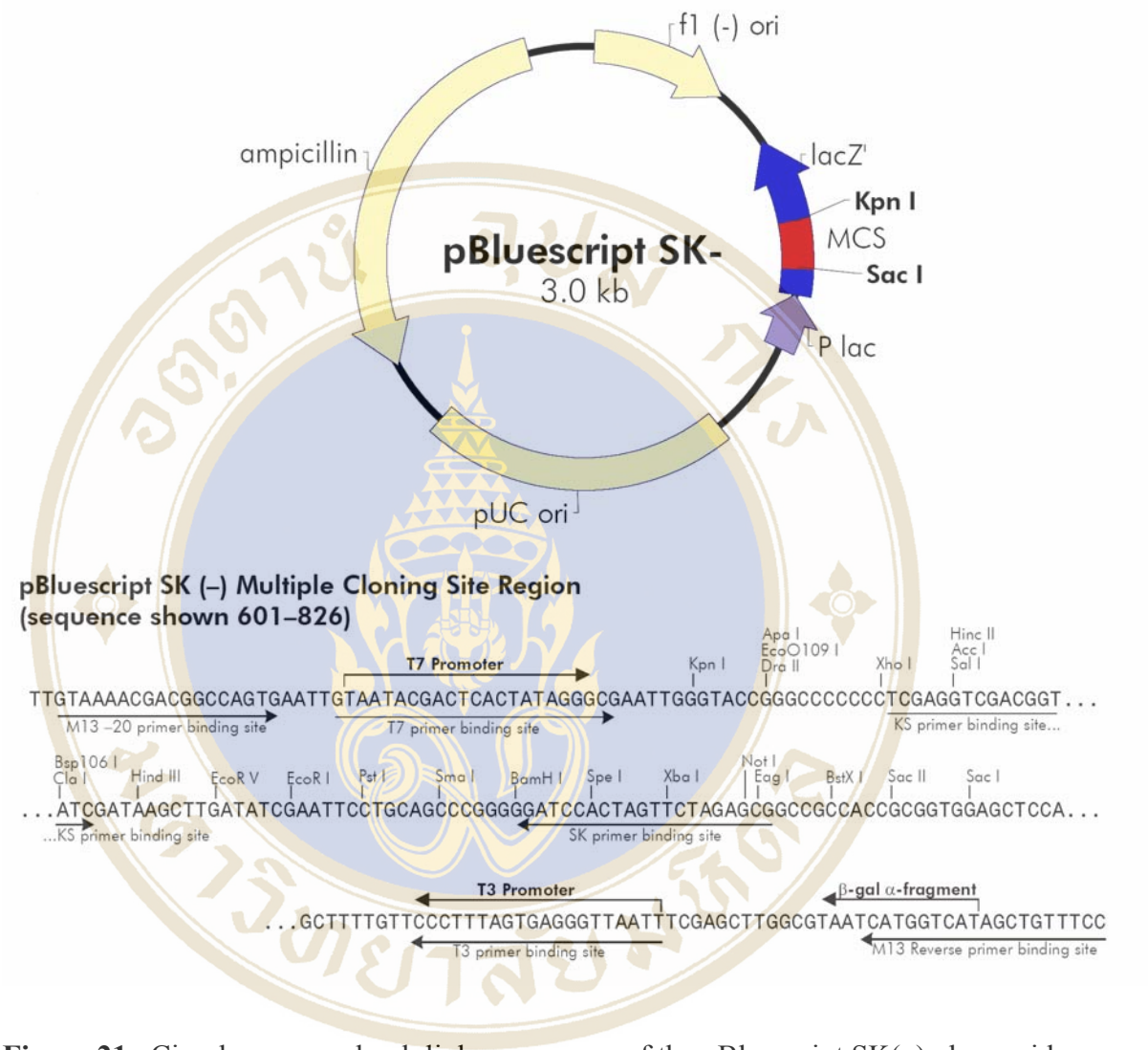

Figure 21. Circular map and polylinker sequence of the pBluescript SK(–) phagemid.

Methods

Part I: Construction of the lymphoid organ (LO) cDNA library from YHV-infected shrimp (*P. monodon*) and sequence analysis

1. Sample Collection

The black tiger shrimp (*Penaeus monodon*) approximately 20-25 g obtained from local farm were placed into 10 ppt brackish aquarium to acclimatize for at least one day. After testing for the absence of WSSV and YHV by PCR and RT-PCR diagnosis (described in the following step), each shrimp was intramuscularly (IM) inoculated with 100 µl of yellow head virus suspension (approximately 5 x 10^4 particles: measured by PCR method) which were supplied by SCRC.

Pooled lymphoid organs were collected at 12 and 24 hr post infection, washed in sterile PBS, added 500 µl TRI Reagent, and snap frozen in liquid nitrogen or deep freeze (-80°C).

1.1 Yellow Head Virus RT-PCR Diagnosis

Total RNA was extracted from TRI Reagent according to the manufacturer's instruction. The YHV diagnostic was performed by one-step RT-PCR (Invitrogen) with specific primers based on the gp64-YHV structural protein. Twenty five microlitres of RT-PCR reaction was consisted of 1 µl of SUPERSCRIPT™ II RT/Taq DNA polymerase mix, 1x reaction mix (0.2 mM of each dNTP, 1.2 mM $MgSO_4$), 200 nmole of each forward and reverse primers (YHVD12 and YHVD15), 1-5 µl of RNA template, 0.5 U of RNase inhibitor. The reaction conditions were shown in Table 7. PCR products (10 µl) were analyzed by 1.5% (w/v) agarose gel electrophoresis.

1.2 White Spot Syndrome Virus Diagnosis

The DNA from pleopod preserved in absolute ethanol was extracted by adding an equal volume of 50 mM NaOH and 0.025% (w/v) SDS, definitely ground. Samples were boiled for 5 min and centrifuged at 10,000 rpm for 5 min to separate the cellular debris. Supernatant was used as the template for PCR in a 50 µl final reaction volume containing 1x reaction buffer (10 mM Tris-Cl, pH 8.3, 50 mM KCl and 0.01% gelatin), 1 mM dNTP, 0.5 U *Taq* polymerase, 1.5 mM $MgCl_2$, 0.5 mM of each forward and

reverse primer and 5 µl of extracted DNA. PCR products (10 µl) were analyzed by 1.5% (w/v) agarose gel electrophoresis.

2. Isolation of RNA (TRIzol® Reagent)

Total RNAs were isolated from approximately 50 mg of shrimp organs; lymphoid organs, gill and hemocyte. The tissues were homogenized in 500 µl of Tri reagent. The homogenate was incubated at room temperature for 5 min to permit the complete dissociation of nucleoprotein complex. One hundred microlitres of chloroform were added and shacked vigorously for 15 sec and incubated at room temperature for 2-3 min. After centrifugation at 10,000 rpm at 4°C for 15 min, the mixture was separated into a red, phenol-chloroform phase, an interphase, and a colorless upper aqueous phase containing RNA. An aqueous phase was then transferred to a new microcentrifuge tube and RNA precipitated by mixing with 250µl 100% isopropyl alcohol. The mixture was incubated at -80°C for 1 hr or -20 °C overnight and centrifuged at 10,000 rpm for 10 min at 4°C. The supernatant was discarded and the RNA pellet was washed with 500 µl of cold 75% ethanol. The sample was mixed and centrifuged at 10,000 rpm for 5 min at 4°C. The pellet containing RNA was briefly dried and subsequently dissolved in 20 µl of DEPC-treated water and stored at -80°C.

3. Agarose Gel Electrophoresis Analysis

The cDNA, the DNA fragments and the plasmids were separated by electrophoresis. The different concentrations of gel [0.7 – 2 % (w/v)] allow the optimal resolution of fragments in different size ranges. Generally, 0.8 % gel in TBE (Tris Borate buffer; 89 mM Tris – HCl, pH 8.0, 89 mM Boric acid and 2.5 mM EDTA) was used. Other concentrations of agarose gel were used in specific purposed as described in each method. The DNA samples were mixed with 1/3 volume of DNA loading dye [25 mg/ml bromphenol blue, 25 mg/ml xylene cyanol, 30 %(v/v) glycerol] before loading into the slots of gel, which were submerged in the TBE in electrophoretic chamber. The power supply was switched at constant voltage (10 v/cm) and the DNAs were allowed to migrate through the gel in separate lanes. The duration of running depends on the size of DNA.

After electrophoresis, DNAs in the gel were stained with 2.5 µg/ml ethidium bromide solution for 5 min and subsequently destained with water. The DNA bands were visualized as an orange band under the UV transilluminator and photographed with gel documentation (BioRad).

4. Formaldehyde gel electrophoresis

The quality of RNA was analyzed by formaldehyde gel elctrophoresis. After RNAs were measured for their concentrations by spectrophotometer at wavelength 260 and 280. Approximately 2-10 µg of RNA samples in sample mixture (1x MOPS, 3.5 µl formaldehyde and 10 µl formamide) were denatured by heating at 65°C for 15 min and immediately chilled on ice. Prior loading, the samples were added with 0.5 µl of 10 mg/ml EtBr and 2 µl of RNA loading dye [25 mg/ml bromphenol blue, 25 mg/ml xylene cyanol, 30 %(v/v) glycerol].

The formaldehyde gel was prepared by using 1g of agarose in 20 ml of 5X MOPS buffer and 63 ml RNase-free water, boiled agarose and then added 17.8 ml of formaldehyde. Gel solution was poured to the gel tray, stand for 30 min. After gel was rigid, the gel was moved to chamber containing 1X MOPS. Rinse each well better prior loaded RNA samples and run gel at 100V.

5. Digestion of contaminated DNA with DNaseI

Total RNA 25 µg were added into 1.5 ml microcentrifuge tube. The reaction mixture (10 mM Tris-Cl pH7.5, 10 mM $MgCl_2$, 0.02-1U of DnaseI) was added to RNA sample with 50 µl final volume and then incubated at 37 °C for 1 h. The reaction was terminated by adding 2.5 µl of 0.2M EDTA and 2 µl of 3 M NaoAc. The RNA sample was purified by adding 54.5 µl of phenol/ chloroform/ isoamyl (25:24:1), vortexed and centrifuged at 13,000 rpm for 10 min. The aqueous phase was carefully separated to new tube and added equal volume of phenol: isoamyl alcohol (24:1), vortexed and centrifuged at 13,000 rpm for 10 min. The aqeous phase was transferred to new tube and subsequently added 0.1V of 3M NaoAc and 2.5 V of absolute ethanol, mixed and centrifuged at 13,000 for 20 min. The supernatant was discarded and the pellet was washed with 200 µl of 80% ethanol, centrifuged at 13,000 rpm for min, air -

dried. Twenty microlitre of DEPC-H_2O were added to sample and collected sample at -80°C.

6. Purification of mRNA (Oligotex; QIAGEN)

The total RNA after treated with DNaseI was determined for its concentration using spectrophotometer at absorbance 260 and 280 nm. The mRNA purification was performed according to manufacturer instruction. Briefly, 250 µg total RNA were added with DEPC-H_2O to make 250 µl final volume. Equal volume of Buffer OBB were added to the sample and subsequently added 15 µl oligotex suspension. After mixing, the mixture was incubated at 70°C for 3 min, leaved at room temperature for 10 min and the supernatant was removed by centrifugation at 13,000 rpm for 2 min. The pellet was added with 400 µl of buffer OW2, mixed, pipetted the mixture to the spin column and spinned at high-speed for 1 min. 400 µl of buffer OW2 were added and then centrifuged at 13,000 rpm for 10 min. One hundred microlitre of hot (70°C) OEB buffer were applied to the column and centrifuged at 13,000 rpm for 1 min. The supernatant from this step was applied to the column and repeated as previous step. The eluent, mRNA, was then kept at -80 °C.

7. cDNA library construction

cDNA was prepared from purified mRNA and subjected to a directional cloning into the UniZAP® XR Vector (Stratagene) using the ZAP-cDNA synthesis kit (Stratagene) was used according to the manufacturers intructions. The cDNA ligated into UniZAP® XR Vector was packaged using the Gigapack® III Gold Cloning Kit (Stratagene) packaging system.

7.1 cDNA synthesis

First-strand cDNA synthesis reaction was performed in a 50 µl mixture containing 5 µl of 10X first-strand buffer, 3 µl of first methyl nucleotide mixture, 2µl of linker-primer (1.4 µg/µl), DEPC-treated water, 1 µl of RNase Block Ribonuclease Inhibitor (40 U/µl) and 5 µg of mRNA. The reaction was incubated at room temperature for 10 min then added 1.5 µl of StrataScript RT (50U/µl). After that the reaction was incubated at 42°C for 1 h using automated thermal cycler GeneAmp PCR

system model 2400 (Perkin Elmer Cetus, USA). After 1 hr, the first-strand synthesis reaction was removed from 42°C and placed on ice.

Second-strand synthesis reaction was performed by adding the following components in order, 200 µl of 10X second-strand buffer, 6 µl second-strand dNTP mixture, 114 µl of sterile distilled water, 2 µl of RNaseH (1.5U/µl) and 11 µl of DNA polymerase I (9.0 U/µl). The contents of the tube were gently mixed, centrifuged and incubated at 16°C for 2.5 hr, before immediately placing the reaction on ice. The uneven termini of the double stranded cDNA were filled in by adding 2 µl of cloned *Pfu* DNA polymerase (2.5U/µl) and 23 µl of blunting dNTP mix into the second-strand synthesis reaction, quickly mixed and briefly centrifuged. The reaction tube was incubated at 72°C for 30 min. The blunted cDNA were precipitated by adding 20 µl of 3 M sodium acetate and 400 µl of 100% (v/v) ethanol, incubated overnight at -20°C. The pellet was collected by centrifugation at 13,000 rpm for 60 min at 4°C. The pellet was washed by adding 500 µl of 70% (v/v) ethanol and air dried the pellet. The pellet of blunted cDNA was resuspended with 9 µl of *Eco*RI adapter then the adapter ligation was carried out by adding 1 µl of 10x ligase buffer, 1 µl of rATP and 1 µl of T4 DNA ligase. After adapter ligation was completed and the T4 ligase has been heat inactivated at 70°C for 30 min, spin down and placed the reaction at room temperature for 5 min. The phosphorylation of ligted adapters were performed as the same tube of ligated *Eco*RI adapter reaction by adding 1 µl of 10x ligase buffer, 2 µl of 10 mM rATP, 5 µl of sterile water and 2 µl of T4 polynucleotide kinase (5 U/µl) then incubated at 37°C for 30 min. After that kinase was heat-inactivated at 70°C for 30 min, the reaction centrifuged and placed at room temperature for 5 min. The *Xho*I digestion was performed subsequently releasing of the *Eco*RI adapter and residual linker-primer from the 3' end of the cDNA. These two fragments were separated on a drip column containing Sepharose® CL-2B gel filtration medium (Figure 23). The size fractionated cDNA was precipitated and ligated to the Uni-ZAP® XR vector.

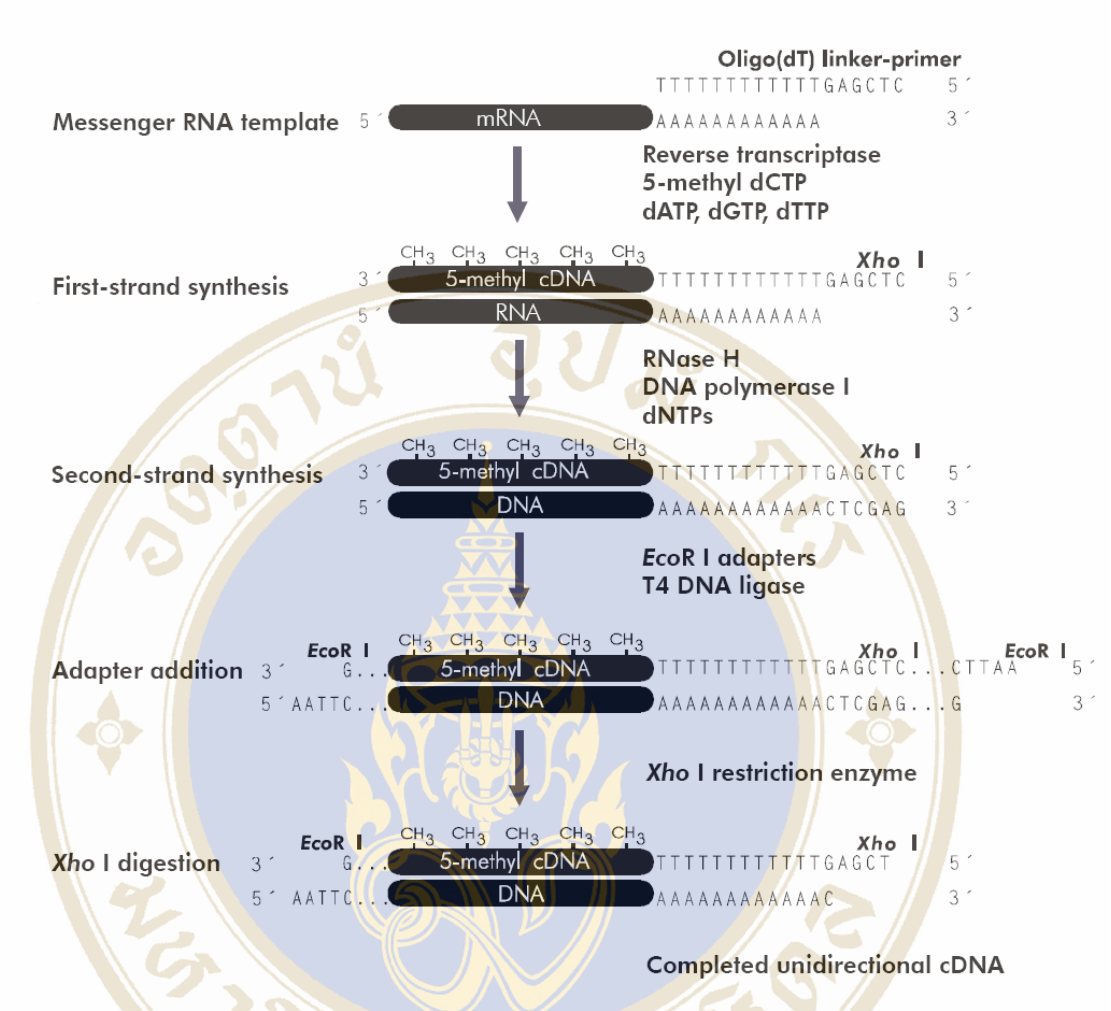

Figure 22. The cDNA synthesis flow chart.

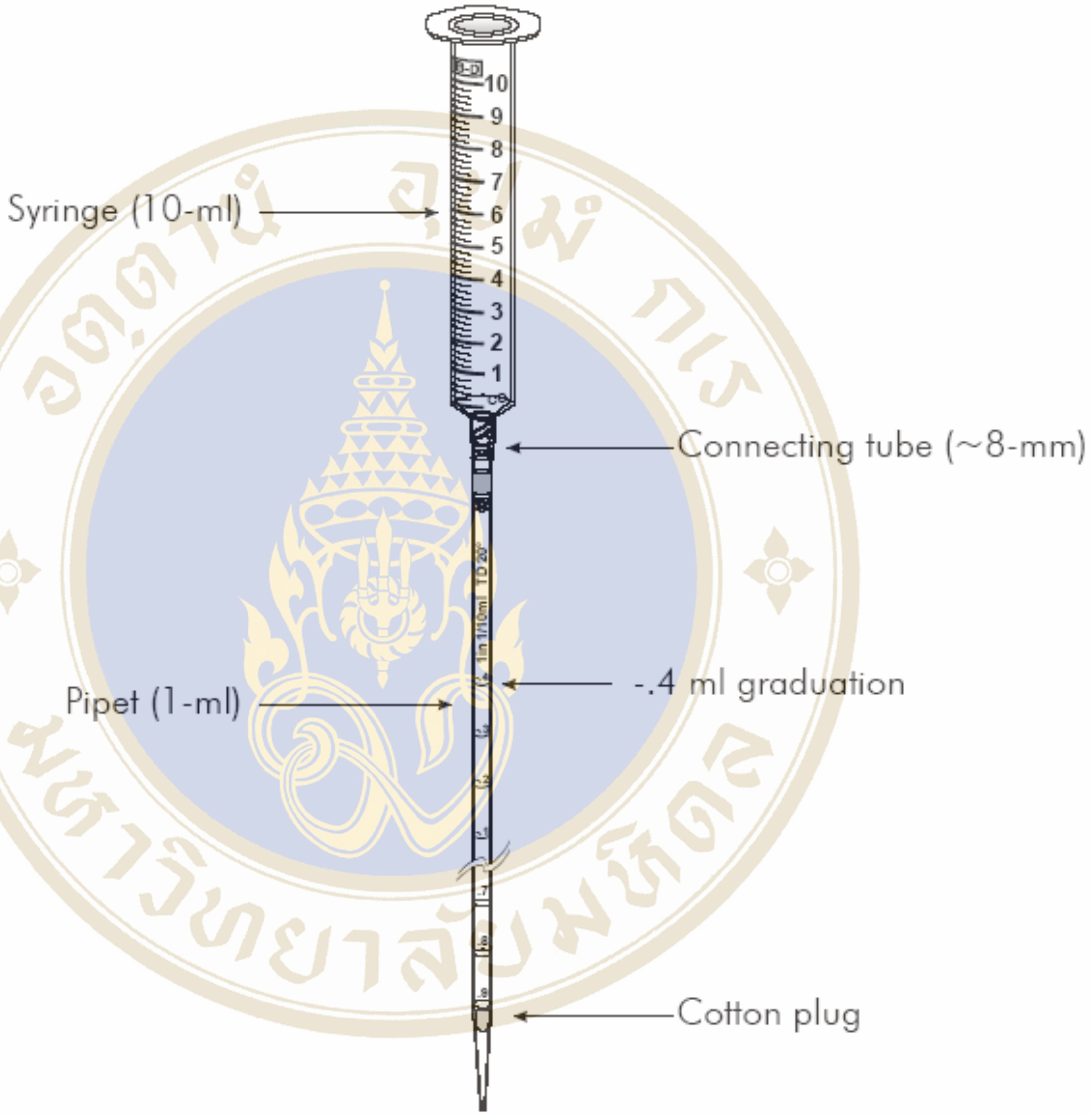

Figure 23. Assembly of the drip column used for size fractionated cDNA.

7.2 Packaging reaction

The cDNA ligated to the Uni-ZAP® XR vector was packaged using the Gigapack® III Gold packaging extract. The packaging extract was removed from -80°C freezer and quickly thawed then immediately 1-4 µl containing 0.1-1 µg of ligated cDNA were mixed with packaging reaction. The reaction was incubated at room temperature for 2 before adding 500 µl of SM buffer (0.1 M NaCl, 0.1 M MgSO4, 0.05 M TrisHCl, pH 7.5, 0.01% Gelatin) and 20 µl of Chloroform, gently mixed then briefly centrifuged. The supernatant which contained lambda phage library was transferred to the new tube.

7.3 Tittering the lambda library

The titer of the packaged product should be determined. This process started with mixing 1 µl of final packaged reaction with 200 µl of XL1-Blue MRF' cells (grow in 50 ml of LB-broth with 0.2% maltose and 10 mM $MgSO_4$ at 30°C overnight, shaking at 200 rpm, the culture was diluted to an OD_{600} of 0.5 with sterile 10 mM $MgSO_4$). Then the mixture was incubated at 37°C for 15 min. After that 3 ml of NZY top agar (0.7% top agarose/NZY medium) that melted and cooled to ~48°C, were added immediately onto prewarmed NZY agar plates. These plates were allowed to set at room temperature for 10 min, they were then inverted and incubated at 37°C for 6-8 hr. Subsequently, plaque formation could be observed as a clear zone of bacterial cells on NZY plates. So that, the titer of primary library was determined by counting the number of plaques and using this equation for calculated the titer.

$$\left[\frac{\text{Number of plaques (Pfu) x dilution factor}}{\text{Volume plated (µl)}}\right] \text{x 1000 µl/ml}$$

The phage library was amplified by combine aliquots of library suspension containing ~5×10^4 pfu of bacteriophage with 600 µl of XL1-Blue MRF' cells (the bacteria cells were prepared as same as tittering library) at OD_{600} of 0.5 in 15 ml tubes, incubated at 37°C for 15 min. Thereafter the reaction tubes were plated on NZY plates by mixing with 6.5 µl of NZY top agar that melted and cooled to ~48°C, then were spreaded onto a 150-mm NZY agar plate. These plates were allowed to set for 10 min at room temperature, inverted the plate and incubated at 37°C for 6-8 hr. After

incubation, the plaques had been observed, they were recovered by adding 8-10 ml SM buffer on NZY plates then they were incubated at 4° C overnight. The phage particles that diffuse in SM buffer were collected and pooled together in sterile 50 ml tube.

7.4 In *vivo* excision (mass excision)

The in vivo excision using ExAssist® Helper Phage was performed on a small aliquot of phage library. These two strains of *E.coli* are XL1-Blue MRF' and SOLR were grown separately into 50 ml LB broth with supplement (0.2% maltose and 10 mM $MgSO_4$) at 30°C, 200 rpm overnight. The cell pellet was collected by centrifuged at 1000×g for 10 min. Each of the cell pellet was resuspended with 25 ml 10mM $MgSO_4$ then adjusted the concentration of cells to OD_{600} of 1.0 (8×10^8 cell/ml). The reaction was performed in a 50 ml conical tube containing the amplified library (1-100 fold above the primary library size), XL1-Blue MRF' cell (1:10 lambda phage to cell ratio), ExAssist® helper phage (10:1 helper phage to cell ratio). The reaction tube was incubated at 37°C for 15 min added 20 ml LB broth with supplement and incubated at 37°C for 2.5-3 h shaking. The tube was then heated at 65-70°C for 20 min, centrifuged at 1000×g for 10 min to remove the cell debris and decanted the supernatant into 50 ml sterile conical tube. 1 µl of this supernatant was combined with 200 µl of SOLR cells in 1.5 microcentrifuge tube and incubated at 37°C for 15 min. The reaction was plated onto LB ampicilin agar plates (100 µg/µl) incubated at 37°C overnight. At this step, the lambda library was converted to pBluescript SK phagmid.

8. Selection of cDNA clone for EST sequencing

8.1 PCR screening

The cDNA clones were randomly selected for EST sequencing by PCR method. The inserted cDNA was amplified using T7 and T3 primers. The picked colony was used as template for PCR in a 25 µl final reaction volume containing 1x ImmoBuffer (16 mM $(NH_4,)_2SO_4$, 100 mM Tris-HCl pH 8.3, 50 mM KCl and 0.01% Tween-20), 1 mM dNTP, 0.5 U *Taq* polymerase (IMMOLASE™ DNA polymerase), 1.5 mM $MgCl_2$, 0.5 mM of each forward and reverse primer and the picked colony. PCR products (5 µl) were analyzed by 1.5% (w/v) agarose gel electrophoresis.

8.2 Plasmid preparation (QIAGEN® Miniprep)

E.coli DH5α containing plasmid vector or recombinant plasmid was grown in 3 ml LB-broth containing 100 µg/µl ampicillin in the 37˚C with shaking incubator overnight. The cell supernatant was transferred to 1.5 ml microcentrifuge tube then centrifuged at 13,000 rpm for 2 min. The cell pellet was resuspended with 250 buffer P1 followed by adding 250 µl buffer P2 and gentle inverted the tube 4-6 times. The bjffer N3 (350 µl) was added and the tube was immediately inverted 4-6 times. Thereafter the flow-through was discarded, the spin column was washed with 0.75 ml of buffer PE and centrifuged at 10,000 rpm for 30-60s. The flow-through was discarded and centrifuged for an addition 1 min to remove residue wash buffer. The QIAprep columns was placed to the new 1.5 ml microcentrifuge tube and were then added 20µl of sterile water to the center of the column. This column was standed for 1 min before centrifuged for 1 min, 13,000 rpm.

9. Sequencing and sequence analysis

Plasmid samples were partially sequenced from the 5' end using dideoxy termination method using Big Dye (ABI). The sequencing was performed at Shrimp Molecular Biology Center, Chulalongkon University. All sequences were examined for possible sequencing errors, vector sequences were removed, and the EST sequence was converted into FASTA format. The EST sequences were compared with sequences in GenBank (National Center of Biotecnology and Information, NCBI) using BLASTX and BLASTN. Sequences were considered to be significantly matched when the possibility value was less than 10^{-4}.

Part II: Isolation of the full-length transcript of shrimp anti-silencing factor1 (ASF1)

To obtain the full-length transcript of *P.monodon* ASF1 cDNA, the 5'RACE (Rapid Amplification of cDNA ends) PCR was performed by using the GeneRacer™ Kit (Invitrogen).

1. Primer designation for 5'RACE PCR

Two gene specific primer (GSP) were designed based on the 600 bp cDNA sequence of *P.monodon* ASF1 clone (LP-Y-S01-712). The primers should be between 23-28 nucleotides in length, and the GC content of primers should be 50-70% that gives a high annealing temperature (Tm) it should be greater than 72°C. In addition, the Tm of GSP2 should be similar to Tm for the GeneRacer™ 5' Nested Primer and far enough from the GSP1 so that the product of original and nested GSP can be distinguished by size distance. The PrimerPremier 5 program was used for designing and calculating the Tm of difference two primers.

2. Construction of 5'RACE ready cDNA

This experiment was carried out using the GeneRacer™ Kit. This technique is based on RNA ligase-mediated (RLM-RACE) and oligo-capping rapid amplification of cDNA ends (RACE) methods, and results in selective ligation of an RNA oligonucleotide to the 5' ends of decapped mRNA using T4 RNA ligase.

2.1 Dephosphorylating RNA

Total RNA or mRNA was treated with calf intestinal phosphatase (CIP) to remove the 5' phosphates. This eliminates truncated mRNA and non-mRNA from subsequent ligation with GeneRacer™ RNA Oligo.

Dephosphorylation reaction was carried out in a 10 µl reaction volume containing 5 µg of DNase-trated total RNA, 1X CIP buffer, 4 U of RNaseOut™ and 1 U of CIP. The reaction was mixed by pipe ting, briefly centrifuged and then incubated at 50°C for 1 hour. After that the reaction was removed, briefly centrifuge and placed on ice.

2.1.1 Precipitating RNA

To precipitate the dephosphorylated RNA, 90 µl of DEPC water and 100 µl of phenol: chloroform were added to the dephosphorylation reaction tube. The mixture was mixed by vortexing for 30 sec then centrifuged at 13,000 rpm for 5 min at room temperature. The aqeous phase was transferred to a new 1.5 ml microcentrifuge tube and then 2 µl of 10 µg/ µl glycogen and 10 µl of 3 M sodium acetate (pH 5.2) were added and mixed. After that 220 µl of 95% ethanol were added to the mixture, vortex briefly. The RNA pellet were obtained by incubating the reaction tube at -20°C overnight and the pellet was collected by centrifugation at 13,000 rpm for 20 min at 4°C. The supernatant was carefully removed by pipeting then 500 µl of 70 % ethanol were added and centrifugation at 13,000 rpm for 2 min at 4°C. The pellet was washed used 70% ethanol before being dried and resuspended with 7 µl of DEPC water. This RNA solution was subjected to decapping reaction.

2.2 Removing the mRNA Cap structure

The dephosphorylated RNA was treated with tobacco acid pyrophosphatase (TAP) in order to remove the 5' cap structure from intact full-length mRNA. This treatment leaves 5' phosphate required for ligation GeneRacer™ RNA Oligo. Decapping reaction was performed in 10 µl of reaction volume containing 7 µl of dephosphorylated RNA, 1X TAP buffer, 4 U of RNaseOut™ and 5 U of TAP. The reaction was mixed by pipeting and then incubated at 37°C for 1 hour that allowed the enzyme to become activated. After that the reaction tube was removed and briefly centrifuged then placed on ice. The decapped RNA reaction was subjected to RNA precipitation by performing in the step 2.1.1 that shown in dehosphorylation reaction.

2.3 Ligating the RNA Oligo to decapped mRNA

In this step, the GeneRacer™ RNA Oligo was ligated to the 5' end of decapped mRNA. This oligo contains the priming site for GeneRacer™ 5' Primer and GeneRacer™ 5'Nested Primer that allowed the amplification of the 5' end of cDNAs with GSP1 and GSP2.The ligation reaction was performed in a 10 µl reaction volume containing 7 µl of dephosphorylated and decapped RNA was added to the tube containing 0.25 ng of GeneRacer™ RNA Oligo, pipetted up and down to mix and resuspend RNA Oligo was then briefly centrifuged to collect the fluid. The reaction was incubated at 65°C for 5 min to destroy the RNA secondary structure and then

placed on ice for 2 min and centrifuged. 1 µl of these reagents which are 10X ligase buffer, 10mM ATP, RNaseOut™ (40U/ µl) and T4 RNA ligase (5 U/ µl) were added to the reaction tube, mixed by pipetting and then the reaction tube was incubated at 37°C for 1 hour. After that the reaction was removed and the solution was collected by brief centrifugation before placing on ice. The ligated RNA was precipitated using the same methods that shown in the previous step, acception only the volume of DEPC water was used for resuspend the RNA pellet. For ligated RNA pellet, this pellet was resuspended with 10 µl of DEPC water.

2.4 Reverse transcription of mRNA

In this experiment, to obtain 5'RACE ready cDNA that subjected to amplification of 5' end cDNA. The ligated mRNA was reverse transcribed with SuperScript™ III RT (Invitrogen) with random primers. The reverse transcription (RT) was carried out in a 20 µl reaction volume containing 10 µl of ligated RNA, 1 µl of Random primers (100 ng), 1 µl of dNTP Mix (10 mM each, in 1mM Tris-HCl, pH 7.5) and 8 µl sterile distilled. The reaction tube was incubated at 65°C for 5 min that allow removing the secondary structure of RNA and was then placed on ice at least 1 min. To collect the solution, the reaction tube was briefly centrifuged. The 13 µl of ligated and primer mixture were mixed with 4 µl of 5X First strand buffer [250mM Tris-HCl (pH 8.3), 375 mM KCl and 15 mM $MgCl_2$], 1 µl of 0.1 mM DTT, 1 µl of RNaseOut™ (40 U/ µl) and 1 µl of 200 U/ µl of SuperScript™ III RT. The RT reaction was performed at 25°C for 5 min to allow efficient binding of the random primers to the template followed by incubation at 50°C for 30-60 min. The reaction was terminated by heating at 70°C for 15 min. After that the RT reaction was placed on ice for 2 min and briefly centrifuged to collect the reaction. 1 µl of RNaseH (2 U) was added to RT reaction and further incubated at 37°C for 20 min. The RT reaction containing the first strand cDNA that was used as the template for amplify the 5' end of shrimp ASF1 cDNA.

3. Amplification of the 5' end of the shrimp ASF1 cDNA

The GeneRacer™ kit contains two primers designed to amplified 5' end of full-length cDNA. Two primers are homologous to sequence in the GeneRacer™ RNA Oligo. The 5' end of shrimp ASF1 cDNA was obtained using the reverse GSP

and the GeneRacer™ 5’ Primer. Only mRNA that has the GeneRacer™ RNA Oligo ligated to the 5’ end and is completely reverse transcribed will be amplified using PCR. The nested GSP and GeneRacer™ 5’ Nested Primer were used for performing nested PCR in order to identify real PCR products if the first PCR product could not be observed or observed with multiple bands. The amplified 5’ end cDNA reaction was carried out in a 50 µl reaction volume containing 1 µl of 1:10 dilution RT reaction, 1X BD Advantage2™ PCR buffer[40 mM Tricine-KOH (pH 8.7), 15 mM KOAc, 3.5 mM $Mg(OAc)_2$, 3.75 µg/µl BSA, 0.05% Tween 20 and 0.05% Nonidet-P40], 200 nM of ASF-189NR (reverse GSP), 600 nM of GeneRacer™ 5’ Primer and 1X BD Advantage™ 2 polymerase Mix. The amplification was performed in a GeneAmp 2400 thermal cycler using touch down PCR which is show in the Table8. This specificity of PCR condition should be increase to reduce background amplification. The 5’RACE PCR product were analyzed on 1.2 % agarose electrophoresis. All of primer sequences that used for generate the 5’ end ASF1 are shown in the Table9.

Table 8. List the primer sequence of ASF1 GSP and adapter primer for 5' RACE PCR

Primer name	Sequence 5'→ 3'	Bases	Tm °C
ASF-NR189 (GSP1)	TTTTCAAACTGTGGGACATCAGGTGGGG	28	73.6
ASF-NR 87 (GSP2)	CCGCAAGTTAACAGCACAACTGTCACACC	29	72.6
GeneRacer™ 5' Primer	CGACTGGAGCACGAGGACACTGA	23	74
GeneRacer™ 5' Nested Primer	GGACACTGACATGGACTGAAGGAGTA	26	78

Table 9. The thermal cycle profile of touch down PCR used to amplify 5'end ASF1 cDNA.

Temperature	Time	Cycles
94°C	2 min	1
94°C	30 sec	5
72°C	3 min	
94°C	30 sec	5
70°C	3 min	
94°C	30 sec	25
68°C	30 sec	
72°C	3 min	
72°C	10 min	1

4. Cloning the 5'end partial cDNA of ASF1

4.1 Purification of cDNA from agarose gel

QIAGEN Gel extraction Kit (QIAGEN) was used in this experiment. The DNA fragment in the agarose gel was excised with clean, sharp blade with minimal exposure to the short wave length UV light. A slice of gel was placed in pre-weight colorless tube. The gel slice was weighted and dissolved in 3 volumes of QG Buffer to 1 volume of gel (100 mg~100 µl). The tube was then incubated at 50°C for 10 min or until the gel slice was completely dissolved. The solution was mixed by vortexing every 2-3 min during incubation to dissolve gel. One volume of 100% Isopropanol was added to the dissolved gel, mixed and applied the mixture to the QIAquick column that placed in a 2 ml collection tube. The PE Buffer (750 µl) was added to QIAquick column, centrifuged for 1 min, discarded flowthrough, and centrifuged for 1 min. The spun column was placed into a new 1.5 ml microfuge tube before 20 µl of sterile water were added to the center of QIAquick column. The tube was incubated for 1 min and centrifuged at 13,000 rpm for 2 min.

4.2 TA-cloning

In this study, The PCR product was cloned into vector using pGEM®-T Easy vector (Promega). Prior to cloning, the thermostable DNA polymerase, oligonucletides, primer-dimer and excess dNTPs were removed from PCR products using Gel extraction kit (QIAGEN) as step 4.1. The ligation reaction of 5' end ASF1 cDNA to the pGEM®-T Easy vector was performed in 10 µl reaction mixture containing a 1X rapid ligation buffer (30mM Tris-HCl, pH 7.8, 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP and 5% polyethylene glycol), 2 µl of gel purifed PCR product, 50 ng of pGEM®-T Easy vector and 0.3 U of T4 DNA ligase. The ligation was performed at 16°C for 14-16 hours.

4.3 Preparation and Transformation of *E. coli* (DH5α) Competent cell.

4.3.1 Competent cell preparation using Rubidium Chloride

A single colony of *E. coli* (DH5α) from LB agar plate was grow in 2 ml LB broth at 37°C in shaker incubator for 14-16 hours. Five hundred microlitres of overnight culture were added to 100 ml fresh LB broth and incubated at 37°C shaker incubator, until the optical density at 600 nm (OD_{600}) reaches 0.5-0.6. The culture was placed on ice for 5 min and the cells collected by centrifugation at 3,000 xg for 15 min.

The cells were then resuspended in 20 ml TFB1buffer (50 mM manganese chloride, 30 mM potassium acetate, 97 mM rubidium chloride and 38.125 mM calcium chloride), mixed well placed on ice for 10 min. The cells were centrifuged at 3,000 xg for 15 min. Four microlitres of TFB2 (10 mM MOPs, 10 mM rubidium chloride and 285.6 mM calcium chloride) buffer were added, mixed, left on ice for 5 min and 110 µl aliquot of cells were kept at -80°C.

4.3.2 Transformation into *E. coli* (DH5α) competent cell

One hundred microlitres of *E. coli* competent cells were mixed with 10 µl of ligated DNA incubated on ice for 30 min. The mixture was heat at 42°C in water bath for 2 min, then quickly cooled on ice for 2 min before adding 800 µl of LB broth. The transformed cells were incubated at 37°C with shaking for 1 hour before plating on LB agar containing 100 µg/µl ampicillin, which had been spread with 40 µl 20 mg/ml X-gal and 4 µl 200 mg/ml IPTG then incubated at 37°C overnight. The recombinant *E. coli* were visualized as white colonies and selected for further analysis.

5. Plasmid DNA extraction

5.1 Alkaline lysis plasmid extraction

The *E. coli* (DH5α) containing plasmid vectors or recombinant plasmids were grown in 3 ml LB broth containing 100 µg/ µl ampicillin in the 37°C shaking incubator overnight. The cell suspension was harvested by centrifugation at 13,000 rpm for 2 min in the 1.5 ml microcentrifuge tubes. Cell pellets were resuspended in the 100 µl of suspension solution (50 mM glucose, 25 mM Tris-HCL, pH 8.0) on ice for 30 min. Then 200 µl of freshly prep rated 0.2 N NaOH and 1% SDS solution were added, gently mixed by inverting for 4-6 times and incubated on ice for 5 min. 150 µl of 3 M potassium acetate pH 4.8 were then added, gently mixed and the tube was centrifuged at 13,000 rpm for 15 min. The supernatant was collected in a new 1.5 ml microcentrifuge tube containing 4 µl of 10 ng/ml RNase A and further incubated at 37°C for 30 min. The plasmid DNA was then precipitated by adding 2 volumes of absolute Ethanol (800 µl) then stored at -20°C for 1 hour. The plasmid DNA was collected by centrifugation at 13,000 rpm and washed once with cold 75% Ethanol and

air dried or speed vac. This pellet was dissolved in 20 µl sterile water and store at -20°C.

5.2 QIAGEN® Miniprep (as the same as 8.2)

6. Restriction Enzyme Digestion for determining recombinant clones

Generally, the restriction endonuclease (*Eco*RI digestion is useful for check the inserted fragment in the T-vector recombinant clones). A typical reactions contain 1-2 µg of plasmid DNA, 10 U of *Eco*RI, 1x reaction buffer [10 mM NaCl, 20 mM Tris-HCl, 2 mM $MgCl_2$, 0.005% TritonX-100 (pH7.5)], 0.2 mg/ml BSA and sterile distilled water to the final volume of 20 µl. The reactions were incubated in 37°C for 2 hours. After the digestions have been completed, the digested products were analyzed by agarose gel electrophoresis.

7. Quantitative of gene expression by RT-PCR

7.1 One-step PT-PCR

One effective method used to study gene expression is reverse transcriptase polymerase chain reaction (RT-PCR). Since, RT-PCR method is a semi-quantitative measurement therefore it only reveals that one is relatively more or less than another. In this experiment, the SuperScript™ III one-step RT-PCR system with Platinum® *Taq* DNA Polymerase were used to amplify shrimp ASF1 gene. This system allows performing both cDNA synthesis and PCR amplification in a single tube. Two sets of primer were used to amplify 377 bp fragments from β-actin gene and 473 bp from ASF1 gene. All of primer sequences used in RT-PCR is shown in Table9 include their position and Tm.

One-step RT reaction was performed in a 25 µl reaction volume containing 100 ng of each total RNA whereas 5 ng of the same RNA sample were used as template for β-actin gene fragment, 12.5 µl of 2X Reaction buffer (0.4 mM of each dNTP, 3.2 mM $MgSO_4$), 2 µl of 5 mM $MgSO_4$ and 0.5 µl of SuperScript™ III RT/Platinum® *Taq* Mix. The reaction was performed using a GeneAmp® 2400 thermal cycler (Perkin Elmer). The thermal cycler profile was continued of 1 cycle of 50°C for 30 min followed by denaturation at 94°C for 2 min and 26 cycles of 94°C for 30 s, 55°C for 30 s and 68°C for 30 s followed by the final extension at 70°C for 5 min.

7.2 Varying number of PCR cycle

Kinetic analysis of PCR at various cycles was performed to obtain linear amplification between target and control gene expression. One hundred nanograms and 5 µg of total RNA from shrimp lymphoid organ were used to amplify ASF1 and β-actin genes respectively. By using annealing temperature at 55°C for 30 sec and varying numbers of PCR from 20 to 26 for both ASF2 and its internal control β-actin were amplified and monitored for their products. At the same time, both ASF1 and β-actin RT-PCR reaction were colleted at the following cycle 20, 24, 28 and 32. Three microlitres of each PCR product were analyzed on 1.2% agarose gel electrophoresis. Bands were visualized by UV transilluminator, using ethidium bromide staining prior to photography. Densitometer model Gel-doc 2000 (Bio-rad, USA) was used to measure the intensity of each PCR product upon staining with ethidiumbromide. The intensity of each PCR band was plotted on against number of cycle on the x-axis. Cycle in the mid-exponential curve of PCR product were thereafter, selected for RT-PCR study.

7.3 RT-PCR analysis of shrimp ASF1 from different tissues

Several black tiger shrimp tissues were examined for expression of the shrimp ASF1 gene by RT-PCR analysis. Each tissue was duplicated using the two sets of tissue sample were prepared by pooling each of various tissues that obtained from five shrimps. Total RNA was extracted by Trizol reagent from gill, heart, hemocyte, hapatopancreas, intestine, pleopod, lymphoid organ and muscle. Single-stranded cDNAs and PCR amplification were performed using SuperScript™ III one-step RT-PCR system with Platinum® *Taq* DNA Polymerase kit. PT-PCR analysis was conducted using specific primers for the shrimp ASF1, ASF1-48F and ASF1-480R. The black tiger shrimp β-actin primer, Actin-F and Actin-R, were used as an internal control. One-step RT reaction was performed in a 25 µl reaction volume containing 100 ng of each total RNA whereas 5 ng of the same RNA sample were used as template for β-actin gene fragment, 12.5 µl of 2X Reaction buffer (0.4 mM of each dNTP, 3.2 mM $MgSO_4$), 2 µl of 5 mM $MgSO_4$ and 0.5 µl of SuperScript™ III RT/Platinum® *Taq* Mix. The cDNA synthesis was done at 1 cycle of 50°C for 30 min followed by denaturation at 94°C for 2 min, thereafter amplification was achieved within 26 cycles consisting of 30 s at 94°C, 30 s at 55 °C and 5 min at 70°C. The

expected sizes for the amplified products are 433 bp for shrimp ASF1 and 377 bp for β-actin.

Table 10. List the primer sequence of ASF1 and β-actin genes.

Primer name	Sequence 5'→ 3'	Bases	Tm °C
ASF1-480R	TTC CGA ATCCTT TGCATCATCCC	20	62
ASF1-48F	GCCGTTCTTCAACCCGTTCCA GT	23	61
Actin-F	TGACGGCCAGGTGATCACCA	20	55
Actin-R	GAAGCACTTCCTGTGAACGA	20	55

CHAPTER IV
RESULTS

PART I: The cDNA library construction and sequences analysis

1. Determination of RNA quality

Total RNA was extracted from LO of YHV infected shrimp by TRIZOL reagent then treated with DNaseI to remove the contaminated DNA. The RNA stability was determined by 1% formaldehyde gel electrophoresis. The band of 28s rRNA was clearly observed on formaldehyde gel (Figure 24). This total RNA will be used for mRNA purification.

2. Analysis of cDNA library

From cDNA library construction, 5 μg of mRNA were performed using Oligo(dT) cellulose spin columns and cDNA was synthesized and directionally cloned into the UniZAP vector using the ZAP cDNA synthesis kit. The resulting primary library contained 2.5×10^5-indepent phage and was amplified to 9×10^{10} pfu/ml.

To assess the sequence content, phagemid inserts were excised in *vivo* from small aliquot of the library and a number of randomly selected clones were sequenced by single pass ESTs sequencing from the 5' region of the clones. GenBank database was searched for homologies with shrimp ESTs using the BLASTX program generated conceptual translation that is shown in Figure 25. P values less than 10^{-4} were considered significant. From this cDNA library, a total of 887 clones were sequenced. Of these total ESTs, 435 clones (49.0%) did not match to the sequences in GenBank whereas the remaining 452 ESTs (51.0%) showed significant similarity with deposited sequences. The matched ESTs represented different protein types that can be categorized according to the major function of their deduced protein in eleven clusters. These were putative genes of (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, there is a group of other genes which did not fit appropriately into the listed categories and (11) unidentified (hypothetical) similar to other cDNA/DNA which are ESTs with significant homology to genes of unknown function. The percentages of functional categories of ESTs matched in the database ranged from 0.9%-32.3%. The large number of matched sequence falling into the unidentified (hypothetical) similar to other cDNA/DNA (32.2%) whereas the small portion of these sequences encoded for protein in the internal/external structure and motility group (0.9%). The sequences that have function in defense and homeostasis account for 3.3% and the dominant genes found in this group were for peptidyl-propyl cis-trans isomerase (2 clones), cathepsin B (2 clones) and oxidoreductase (2 clones). The proportions of the clones in each category are showed in the Figure 26. The group of gene that represented in this cDNA library was shown in the Table10. The putative genes that were mostly expressed in the *P.monodon* LO infected library were the mitochondria proteins (10.2%) and the highest redundant putative genes were the cytochrome C oxidase subunit II (10 clones). Moreover two dominant genes found in signaling and communication categories (4.4%) are retinol binding protein (7 clones) and thrombospondin (6 clones). The redundancies of the each identified putative gene are shown in Table11.

In addition, all of these EST and their corresponding functional classification are available on as well as a complete list of BlastX matches and contig assemblies. (http://pmonodon.biotec.or.th)

Figure 24. 1 %formaldehyde gel electrophoresis revealed the quality of the two sets of total RNA sample. Two micrograms of RNA were loaded on the gel. The 28S rRNA was observed in these two samples that showed in lane 1 and 2.

(A)

gi|45549139|ref|NP_523366.2| CG9916-PA [Drosophila melanogaster]
gi|47117835|sp|P25007|CYPH_DROME (Cyclosporin A-binding protein)

```
Length=227
Score =  244 bits (624),  Expect = 1e-63
Identities = 122/157 (77%), Positives = 140/157 (89%), Gaps = 0/157 (0%)
Frame = +2

Query 2   FDITADNQPVGRIIMELRADVVPKTAENFRSLCTGEKGFGYKGSCFHRVIPNFMCQGGDF 181
          FD+TADN+P+GRI+MELR+DVVPKTAENFR+LCTGEKGFGYKGS FHRVIPNFMCQGGDF
Sbjct 71  FDMTADNEPLGRIVMELRSDVVPKTAENFRALCTGEKGFGYKGSIFHRVIPNFMCQGGDF 130

Query 182 TXXNGTGGKSIYGNKFEDENFALKHTGPGTLSMANAGPNTNGSQFFICTVKTPWLDNKHV 361
          T  NGTGGKSIYGNKF DENF LKHTG G LSMANAG NTNGSQFFICTVKT WLDNKHV
Sbjct 131 TNHNGTGGKSIYGNKFPDENFELKHTGSGILSMANAGANTNGSQFFICTVKTAWLDNKHV 190

Query 362 VFGSVVEGMDIVRQVEGFGTPNGSCKRKVMIANCGQL  472
          VFG VVEG+D+V+++E +G+ +G   +K+++AN G L
Sbjct 191 VFGEVVEGLDVVKKIESYGSQSGKTSKKIIVANSGSL  227
```

(B)

gi|56199605|gb|AAV84282.1| translationally controlled tumor protein [Fenneropenaeus merguiensis]Length=168

```
Score =  208 bits (530),  Expect = 4e-53
Identities = 103/103 (100%), Positives = 103/103 (100%), Gaps = 0/103 (0%)
Frame = +2

Query 2   VDVVIYMRLQETGFQVKKDYLAYMKEYLKNVKAKLEGTPEASKLTSIQKPLTDLLKKFKD 181
          VDVVIYMRLQETGFQVKKDYLAYMKEYLKNVKAKLEGTPEASKLTSIQKPLTDLLKKFKD
Sbjct 66  VDVVIYMRLQETGFQVKKDYLAYMKEYLKNVKAKLEGTPEASKLTSIQKPLTDLLKKFKD 125

Query 182 LQFFTGESMDPDGMVVLMDYKDIDGEERPVLYFPKYGLTEEKL 310
          LQFFTGESMDPDGMVVLMDYKDIDGEERPVLYFPKYGLTEEKL
Sbjct 126 LQFFTGESMDPDGMVVLMDYKDIDGEERPVLYFPKYGLTEEKL 168
```

Figure 25. BLASTX analysis of the putative peptides of (A) LP-Y-S01-0189-LF clone to *Drosophila melanogaster* Cyclophilin A, (B) LP-Y-S01-0906-LF clone to *Fenneropenaeus merguiensis* translationally controlled tumor protein (TCTP).

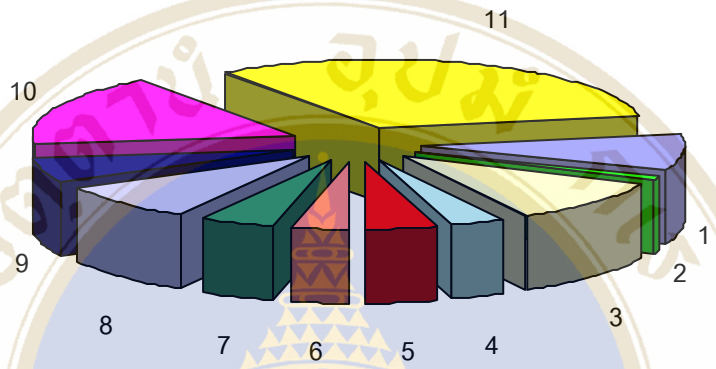

Figure 26. Distribution of ESTs by functional classes. Functional classes included: (1) gene expression, regulation and protein synthesis, (2) internal/external structure and motility, (3) metabolism, (4) defense and homeostasis, (5) signaling and communication, (6) cell division/DNA synthesis, repair and replication, (7) ribosomal protein and rRNA, (8) mitochondrial protein, (9) transport, (10) miscellaneous function, there is a group of other genes which did not fit appropriately into the listed categories and (11) unidentified (hypothetical) similar to other cDNA/DNA

Table 11. The putative gene of YHV-infected LO ESTs of *P.monodon*.

Black tiger shrimp ESTs			Matching sequences			
Clone no.	Putative genes	Sequence size (bp)	Species	Accession number	E value	Score
1. Gene expression, regulation and protein synthesis						
LP-Y-S01-0090-LF	RNA polymerase II	600	*Homo sapiens*	I38186	4e-21	102
LP-Y-S01-0091-LF	elongation factor 1-beta	600	*Homo sapiens*	NP_001950	4e-50	199
LP-Y-S01-0105-LF	U1small ribonucleoprotein	600	*Homo sapiens*	AAF19255	6e-26	119
LP-Y-S01-0147-LF	VspA	207	*Mycoplasma bovis*	AAK94952	6e-08	57
LP-Y-S01-0152-LF	regulator for leucine	542	*Shigella flexneri 2a*	NP706774	1e-19	97
LP-Y-S01-0159-LF	translation initiation factor 4C	558	*Anopheles gambiae*	AAD47075	6e-60	231
LP-Y-S01-0189-LF	cyclophilin 1	600	*Drosophila melanogaster*	NP_523366	3e-71	269
LP-Y-S01-0274-LF	repressor protein	600	*Bacteriophage VT2-Sa*	BBA89781	5e-37	115
LP-Y-S01-0300-LF	Eukaryotic translation initiation factor 4A	467	*Homo sapiens*	AAH15842	8e-76	283
LP-Y-S01-0308-LF	yfjP protein	600	*Escherichia coli K12*	B65042	1e-100	358
LP-Y-S01-0316-LF	similar to QM protein	600	*Apis mellifera*	XP_393092	1e-90	312
LP-Y-S01-0427-LF	Trp1p	511	*Stx1 converting bacteriophage*	NP_859200	1e-25	117
LP-Y-S01-0449-LF	elongation factor-1 alpha	568	*Apis mellifera*	XP_393092	1e-68	260
LP-Y-S01-0482-LF	Trp1p	800	*Saccharomyces cerevisiae*	CCA88068	1e-08	62
LP-Y-S01-0562-LF	elongation factor 1-alpha	276	*Libinia emarginata*	AAC03149	6e-45	180
LP-Y-S01-0593-LF	elongation factor 1-beta	597	*Bombyx mori*	P29522	1e-53	187
LP-Y-S01-0594-LF	yejO	696	*Escherichia coli*	AAA16385	8e-47	188
LP-Y-S01-0603-LF	elongation factor 2b	600	*Drosophila melanogaster*	NP_724357	1e-91	337
LP-Y-S01-0612-LF	elongation factor 1-alpha	504	*Schizophyllum commune*	O42820	4e-52	201
LP-Y-S01-0623-LF	similar to QM protein	565	*Apis mellifera*	XP_393092	9e-82	200
LP-Y-S01-0631-LF	CI protein	600	*Stx1 converting bacteriophage*	NP_859200	5e-37	155
LP-Y-S01-0707-LF	transcriptional regulatory protein	495	*Escherichia coli K12*	NP_415648	2e-58	227
LP-Y-S01-0712-LF	anti-silencing factor 1	600	*Drosophila melanogaster*	NP_524163	9e-34	144
LP-Y-S01-0716-LF	Hnr protein	598	*Escherichia coli CFT073*	NP_753604	6e-35	142
LP-Y-S01-0725-LF	similar to QM protein	623	*Apis mellifera*	XP_393092	5e-86	318
LP-Y-S01-0726-LF	serine tRNA synthetase	600	*Escherichia coli O157:H7 EDL933*	NP_286770	3e-73	276
LP-Y-S01-0730-LF	putative NAGC-like transcriptional regulator	547	*Escherichia coli K12*	NP_415637	2e-41	170
LP-Y-S01-0732-LF	elongation factor 1 beta	577	*Plutella xylostella*	BAD26687	6e-53	208
LP-Y-S01-0744-LF	Gal4, aa 1-147	609	*synthetic construct*	CAA59966	4e-37	155
LP-Y-S01-0757-LF	repressor protein	554	*Bacteriophage VT2-Sa*	BAA89781	3e-57	222
LP-Y-S01-0819-LF	longation factor 1-beta	655	*Bombyx mori*	P29522	6e-59	228
LP-Y-S01-0845-LF	translation elongation factor 1-alpha	600	*Pilobolus umbonatus*	AAG29027	8e-40	101
LP-Y-S01-0861-LF	similar to U1 small nuclear ribonucleoprotein	529	*Rattus norvegicus*	XP_341858	7e-05	48
LP-Y-S01-0884-LF	Similar to eukaryotic translation elongation factor 1 beta 2	600	*Danio rerio*	AAH46042	4e-37	155
LP-Y-S01-0905-LF	Xanthosine operon Regulatory protein	600	*Escherichia coli CFT073*	NP_754819	6e-28	125
LP-Y-S01-0909-LF	HLA class II region expressed gene KE2; KE2 protein	545	*Homo sapiens*	NP_055075	4e-27	122

Table11. Identified putative gene of YHV-infected LO ESTs of *P. monodon.* (continued)

Black tiger shrimp ESTs			Matching sequences			
Clone no.	Putative genes	Sequence size (bp)	Species	Accession number	E value	Score
1. Gene expression, regulation and protein synthesis (continued)						
LP-Y-S01-0922-LF	putative transcriptional regulator LYSR-type similar to QM protein serine tRNA synthetase	600	*Escherichia coli K12*	NP_416043	1e-54	214
LP-Y-S01-0949-LF	elongation factor 1-alpha	512	*Schizophyllum commune*	O42820	3e-56	219
2. Internal/ external structure and motility						
LP-Y-S01-0132-LF	tubulin-alpha 1	600	*Patella vulgata*	P41383	2e-29	115
LP-Y-S01-0164-LF	cuticle protein	511	*Locusta migratoria*	P81225	3e-12	72
LP-Y-S01-0817-LF	periplasmic glucans	600	*Escherichia coli K12*	NP_415566	6e-89	328
LP-Y-S01-0892-LF	flagellin	600	*Pseudomonas stutzeri*	AAC63950	1e-61	237
3. Metabolism						
LP-Y-S01-0010-LF	CTP:CMP-3-deoxy-D-manno-octulosonate transferase	504	*Escherichia coli K12*	NP_415438	2e-54	213
LP-Y-S01-0014-LF	methylmalonyl-CoA mutase	800	*Escherichia coli K12*	NP_417392	3e-55	216
LP-Y-S01-0018-LF	citrate lyase synthetase	600	*Escherichia coli K12*	NP_415151	1e-28	127
LP-Y-S01-0026-LF	citrate lyase synthetase	600	*Escherichia coli K12*	NP_415151	2e-08	60
LP-Y-S01-0033-LF	enterobactin synthetase	600	*Escherichia coli*	AAB40785	1e-113	410
LP-Y-S01-0062-LF	3-deoxy-D-arabinoheptulosonate-7-phosphate synthase	800	*Shigella flexneri 2a*	NP_706487	5e-40	86
LP-Y-S01-0081-LF	enterobactin synthetase	217	*Escherichia coli*	AAB40785	5e-34	144
LP-Y-S01-0085-LF	citrate lyase synthetase	600	*Escherichia coli K12*	NP_415151	9e-29	128
LP-Y-S01-0093-LF	formate acetyltransferase	450	*Shigella flexneri 2a*	NP_709751	3e-39	103
LP-Y-S01-0120-LF	CTP:CMP-3-deoxy-D-manno-octulosonate transferase	322	*Escherichia coli K12*	NP_415438	2e-54	213
LP-Y-S01-0240-LF	Lactoylglutathione lyase	498	*Lycopersicon esculentum*	Q42891	8e-27	80
LP-Y-S01-0289-LF	diadenosine tetraphosphatase	600	*Shigella flexneri 2a str. 301*	NP_709064	5e-58	175
LP-Y-S01-0294-LF	metallothionein 2	600	*Callinectes sapidus*	Q9U620	5e-24	113
LP-Y-S01-0296-LF	Chain A, Crystal Structure Of Beta-Lactamase Inhibitor Protein-Ii In Complex With Tem-1 Beta-Lactama	600	*Escherichia coli*	1JTD	5e-17	89
LP-Y-S01-0317-LF	Epoxide hydrolase 1 (Microsomal epoxide hydrolase)	592	*Oryctolagus cuniculus*	P04068	5e-37	115
LP-Y-S01-0349-LF	N-(5'-phosphoribosyl)anthranilate isomerase	593	*Saccharomyces cerevisiae*	P00912	5e-38	159
LP-Y-S01-0385-LF	quinolinate synthetase A protein	556	*Escherichia coli K12*	NP_415271	3e-79	295
LP-Y-S01-0391-LF	ubiquitin conjugating enzyme E2, J2 isoform 3; ubiquitin conjugating enzyme 6; yeast UBC6 homolog	600	*Homo sapiens*	NP_919439	3e-43	176
LP-Y-S01-0394-LF	cystathionine gamma-lyase	600	*Bdellovibrio bacteriovorus HD100*	NP_970501	3e-09	63
LP-Y-S01-0400-LF	putative hexulose-6-phosphate isomerase	600	*Escherichia coli K12*	NP_418618	4e-93	342
LP-Y-S01-0411-LF	metallothionein 2	551	*Callinectes sapidus*	Q9U620	3e-24	113
LP-Y-S01-0421-LF	COG0626: Cystathionine beta-lyases/cystathionine gamma-synthases	600	*Rhodopseudomonas palustris*	ZP_00011905	1e-08	60

Table 11. Identified putative gene of YHV-infected LO ESTs of *P. monodon*. (continued)

Black tiger shrimp ESTs			Matching sequences			
Clone no.	Putative genes	Sequence size (bp)	Species	Accession number	E value	Score
3. Metabolism (continued)						
LP-Y-S01-0425-LF	D-erythrose 4-phosphate dehydrogenase	600	*Shigella flexneri 2a str. 301*	NP_708687	2e-75	283
LP-Y-S01-0426-LF	Glyoxylase I Activity Enhancing protein	600	*Saccharomyces cerevisiae*	P33440	1e-29	131
LP-Y-S01-0466-LF	D-erythrose 4-phosphate dehydrogenase	596	*Shigella flexneri 2a str. 301*	NP_708687	1e-70	236
LP-Y-S01-0487-LF	farnesoic acid O-methyltransferase	600	*Metapenaeus ensis*	AAP79984	2e-37	152
LP-Y-S01-0560-LF	ubiquitin specific protease 2	600	*Rattus norvegicus*	NP_446226	7e-16	85
LP-Y-S01-0575-LF	phosphoglycerate kinase	598	*Shigella flexneri 2a str. 301*	NP_708686	3e-93	194
LP-Y-S01-0588-LF	Enoyl-CoA hydratase/carnithine racemase	600	*Pseudomonas fluorescens PfO-1*	ZP_00084395		
LP-Y-S01-0595-LF	diadenosine tetraphosphatase	600	*Shigella flexneri 2a str. 301*	NP_709864	5e-58	175
LP-Y-S01-0641-LF	metallothionein 2	550	*Callinectes sapidus*	Q9U620	3e-24	113
LP-Y-S01-0658-LF	heptosyl transferase	600	*Escherichia coli K12*	NP_418078	1e-58	227
LP-Y-S01-0686-LF	Chain A, Structure Of The Mutant F174a T Form Of The Glucosamine-6- Phosphate Deaminase	752	*Escherichia coli*	1JT9	2e-54	214
LP-Y-S01-0702-LF	farnesoic acid O-methyltransferase	818	*Metapenaeus ensis*	AAP79984	4e-37	156
LP-Y-S01-0718-LF	putative hexulose-6-phosphate isomerase	600	*Escherichia coli K12*	NP_418618	2e-61	236
LP-Y-S01-0787-LF	glutamate dehydrogenase	551	*NAD(P)*	S42919	5e-51	201
LP-Y-S01-0789-LF	similar to deoxyribose-phosphate aldolase	547	*Listeria monocytogenes EGD-e*	NP_465519	2e-22	107
LP-Y-S01-0813-LF	diadenosine tetraphosphatase	600	*Shigella flexneri 2a str. 301*	NP_709864	6e-62	238
LP-Y-S01-0824-LF	the sequence of TRP1 from strain S228C, which is the sequence stored in SGD	800	*Saccharomyces cerevisiae*	NP_010290	1e-95	351
LP-Y-S01-0849-LF	COG0155: Sulfite reductase, beta subunit (hemoprotein)	410	*Pseudomonas fluorescens PfO-1*	ZP_00265988	1e-36	152
LP-Y-S01-0868-LF	putative fructose 1,6-bisphosphate aldolase	600	*Homalodisca coagulata*	AAT01078	6e-26	119
LP-Y-S01-0899-LF	farnesoic acid O-methyltransferase	402	*Metapenaeus ensis*	AAP79984	8e-38	156
LP-Y-S01-0918-LF	enterobactin synthetase	600	*Escherichia coli*	AAB40785	8e-78	291
LP-Y-S01-0934-LF	D-erythrose 4-phosphate dehydrogenase	600	*Shigella flexneri 2a str. 301*	NP_708687	1e-74	280
LP-Y-S01-0989-LF	putative dehydrogenase	512	*Escherichia coli O157:H7 EDL933*	NP_286603	1e-62	152
LP-Y-S01-1029-LF	farnesoic acid O-methyltransferase	600	*Metapenaeus ensis*	AAP79984	2e-37	156
4. Defense and homeostasis						
LP-Y-S01-0012-LF	chaperonin	323	*Plasmodium yoelii*	EAA21693	2e-06	52
LP-Y-S01-0092-LF	peptidyl-prolyl cis-trans isomerase	546	*Zea mays*	P21596	2e-11	70
LP-Y-S01-0122-LF	**lysozyme**	600	*Penaeus monodon*	AAN16375	1e-79	265
LP-Y-S01-0137-LF	peptidyl-prolyl cis-trans isomerase	504	*Zea mays*	P21569	1e-11	70
LP-Y-S01-0225-LF	**serine protease homologue**	384	*Pseudomonas fluorescens*	BAA36466	1e-64	205
LP-Y-S01-0288-LF	**cathepsin B**	600	*Glossina morsitans*	AAK07477	5e-12	72
LP-Y-S01-0369-LF	putative oxidoreductase	598	*Shigella flexneri 2a str. 301*	NP_707630	7e-24	112
LP-Y-S01-0465-LF	P109 protein	564	*Bombyx mori*	T00207	2e-20	100

Table 11. Identified putative gene of YHV-infected LO ESTs of *P. monodon*. (continued)

Black tiger shrimp ESTs			Matching sequences			
Clone no.	Putative genes	Sequence size (bp)	Species	Accession number	E value	Score
4. Defense and homeostasis (continued)						
LP-Y-S01-0453-LF	stress-associated endoplasmic reticulum protein 1	600	*Homo sapiens*	AAH29067	5e-18	89
LP-Y-S01-0672-LF	Heat-responsive regulatory protein	600	*Escherichia coli K12*	BAA35397	1e-110	397
LP-Y-S01-0709-LF	COG0542: ATPases with chaperone activity, ATP-binding subunit	446	*Cytophaga hutchinsonii*	ZP_00308874	5e-63	240
LP-Y-S01-0775-LF	**putative oxidoreductase**	537	*Shigella flexneri 2a str. 301*	NP_707630	5e-24	112
LP-Y-S01-0791-LF	putative oxidoreductase, major subunit	127	*Escherichia coli O157:H7 EDL933*	NP_288022	2e-10	66
LP-Y-S01-0906-LF	**translationally controlled tumor protein**	462	*Penaeus monodon*	AAO61938	4e-53	207
LP-Y-S01-0977-LF	**cathepsin B**	403	*Glossina morsitans morsitans*	AAK07477	1e-12	72
5. Signaling and communication						
LP-Y-S01-0001-LF	Rhodopsin	212	*Procambarus clarkii*	P35356	6e-11	67
LP-Y-S01-0023-LF	RhsB protein	600	*Escherichia coli*	P16917	5e-71	154
LP-Y-S01-0059-LF	Extracellular-regulated kinase A	600	*Drosophila melanogaster*	NP_524763	4e-65	248
LP-Y-S01-0117-LF	retinol binding protein	600	*Metapenaeus ensis*	AAL68638	9e-35	148
LP-Y-S01-0314-LF	sensor protein PhoQ	526	*Shigella flexneri 2a str. 301*	NP_707058	9e-79	262
LP-Y-S01-0423-LF	retinol binding protein	600	*Metapenaeus ensis*	AAL68638	2e-37	96
LP-Y-S01-0508-LF	retinol binding protein	597	*Metapenaeus ensis*	AAL68638	3e-31	136
LP-Y-S01-0541-LF	Thrombospondin	483	*Penaeus monodon*	AAN17670	5e-42	171
LP-Y-S01-0579-LF	retinol binding protein	600	*Metapenaeus ensis*	AAL68638	3e-31	136
LP-Y-S01-0636-LF	retinol binding protein	592	*Metapenaeus ensis*	AAL68638	1e-18	94
LP-Y-S01-0677-LF	Histidine kinase	623	*Dictyostelium discoideum*	AAS38743	1e-12	75
LP-Y-S01-0689-LF	Thrombospondin	427	*Penaeus monodon*	AAN17670	6e-54	210
LP-Y-S01-0790-LF	Prosaposin	584	*Bos taurus*	NP_776586	1e-28	87
LP-Y-S01-0798-LF	Thrombospondin	492	*Penaeus monodon*	AAN17670	1e-64	246
LP-Y-S01-0801-LF	Thrombospondin	490	*Penaeus monodon*	AAN17670	1e-64	246
LP-Y-S01-0926-LF	retinol binding protein	600	*Metapenaeus ensis*	AAL68638	2e-22	107
LP-Y-S01-0944-LF	sensor histidine protein kinase UhpB	432	*Escherichia coli*	E91204	5e-15	58
LP-Y-S01-0965-LF	Thrombospondin	492	*Penaeus monodon*	AAN17670	1e-64	246
LP-Y-S01-1022-LF	Thrombospondin	492	*Penaeus monodon*	AAN17670	1e-64	246
LP-Y-S01-1025-LF	retinol binding protein	359	*Metapenaeus ensis*	AAL68638	5e-19	94
6. Cell division/DNA synthesis, repair and replication						
LP-Y-S01-0057-LF	endonuclease IV		*Shigella flexneri 2a*	NP_708056	4e-46	185
LP-Y-S01-0067-LF	ORF1b polyprotein	600	*yellow head virus*	AAL14793	1e-106	385
LP-Y-S01-0133-LF	RNA polymerase	552	*Vairimorpha necatrix*	T31435	3e-37	155
LP-Y-S01-0216-LF	DNA-directed RNA polymerase, alpha subunit/40 kD subunit	380	*Ralstonia metallidurans*	ZP_00024747	1e-82	306
LP-Y-S01-0219-LF	Cell division protein ftsK	471	*Escherichia coli CFT073*	NP_752956	7e-22	220
LP-Y-S01-0223-LF	cell division protein	470	*Shigella flexneri 2a str. 301*	NP_706775	1e-84	313
LP-Y-S01-0481-LF	Replicative DNA helicase	600	*Cytophaga hutchinsonii*	ZP_00117368		
LP-Y-S01-0537-LF	Chain B, Mechanism Of Processivity Clamp Opening of DNApolymerase III	600	*Escherichia coli*	1JQL	1e-40	167

Table 11. Identified putative gene of YHV-infected LO ESTs of *P. monodon*. (continued)

Black tiger shrimp ESTs			Matching sequences			
Clone no.	Putative genes	Sequence size (bp)	Species	Accession number	E value	Score
6. Cell division/DNA synthesis, repair and replication (continued)						
LP-Y-S01-0533-LF	MukB	600	*Escherichia coli K12*	NP_415444	7e-60	214
LP-Y-S01-0627-LF	MukB	600	*Escherichia coli*	BBA06510	4e-73	275
LP-Y-S01-0829-LF	MukB	600	*Escherichia coli*	BBA06510	2e-70	266
LP-Y-S01-0837-LF	Similar to XPMC2H protein	600	*Apis mellifera*	XP_396631	2e-25	117
LP-Y-S01-0864-LF	ExoP-related protein	572	*Deinococcus radiodurans*	NP_285357	5e-41	120
LP-Y-S01-0874-LF	UvrB protein	800	*Escherichia coli K12*	BBA35437	1e-58	228
LP-Y-S01-0907-LF	UvrB protein.	600	*Escherichia coli K12*	BBA35437	1e-106	386
LP-Y-S01-0924-LF	DNA repair; excision nuclease subunit B	600	*Escherichia coli O157:H7 EDL933*	NP_286542	2e-99	361
LP-Y-S01-0930-LF	Proable DNA-directed RNA Polymerase (alpha cahian) protein	600	*Ralstonia solanacearum*	NP_521114	3e-48	192
7. Ribosomal protein and rRNA						
LP-Y-S01-0041-LF	ribosomal protein L17	595	*Rattus norvegicus*	P24049	1e-62	150
LP-Y-S01-0097-LF	ribosomal protein S26	362	*Rattus norvegicus*	NP_037356	4e-46	184
LP-Y-S01-0102-LF	ribosomal protein L30	440	*Argopecten irradians*	AAN05584	5e-48	190
LP-Y-S01-0128-LF	ribosomal protein S3	600	*Spodoptera frugiperda*	AAL26579	2e-76	155
LP-Y-S01-0129-LF	ribosomal protein L5	600	*Gallus gallus*	P22451	8e-84	274
LP-Y-S01-0131-LF	ribosomal protein S23	515	*Spodoptera frugiperda*	AAK92191	1e-75	283
LP-Y-S01-0174-LF	ribosomal protein S23	507	*Carabus granulatus*	CAH04342	9e-76	283
LP-Y-S01-0214-LF	ribosomal protein S13	583	*Escherichia coli O157:H7 EDL933*	NP_289856	1e-55	217
LP-Y-S01-0236-LF	ribosomal protein S26	416	*Rattus norvegicus*	NP_037356	3e-46	184
LP-Y-S01-0293-LF	ribosomal protein L10	600	*Bombyx mandarina*	O96647	1e-77	290
LP-Y-S01-0567-LF	ribosomal protein L5	426	*Branchiostoma lanceolatum*	AAN73355	6e-56	216
LP-Y-S01-0609-LF	ribosomal protein S12	452	*Dermacentor variabilis*	AAP04352	8e-47	186
LP-Y-S01-0643-LF	ribosomal protein S4	437	*Spodoptera frugiperda*	AAL26580	8e-58	223
LP-Y-S01-0799-LF	ribosomal protein S3a	483	*Oryzias latipes*	O73813	3e-47	112
LP-Y-S01-0839-LF	ribosomal protein L5	600	*Branchiostoma lanceolatum*	AAN13355	1e-78	293
LP-Y-S01-0847-LF	ribosomal protein L32	600	*Plutella xylostella*	BAD26685	3e-55	216
LP-Y-S01-0877-LF	ribosomal protein L26	452	*Spodoptera frugiperda*	AAK92162	2e-56	218
LP-Y-S01-0902-LF	ribosomal protein S18	408	*Cherax destructor*	AAG47944	4e-62	237
LP-Y-S01-0927-LF	ribosomal protein S1	800	*Escherichia coli O157:H7 EDL933*	NP_286786	7e-94	298
LP-Y-S01-994-LF	ribosomal protein S1	770	*Escherichia coli O157:H7 EDL933*	NP_286786	1e-106	385
8. Mitochondria protein						
LP-Y-S01-0005-LF	carnitine o-palmitoyltransferase I	569	*Rattus norvegicus*	Q63704	4e-08	59
LP-Y-S01-0068-LF	cytochrome b	600	*Penaeus monodon*	NP_038299	5e-90	331
LP-Y-S01-0101-LF	ATP-binding component	600	*Escherichia coli K12*	NP_45406	1e-14	65
LP-Y-S01-0111-LF	cytochrome c oxidase subunit I	294	*Fenneropenaeus chinensis*	AAK82961	2e-40	165
LP-Y-S01-0115-LF	ATP synthase	600	*Penaeus monodon*	NP_038292	6e-71	268
LP-Y-S01-0144-LF	Ubiquinone	494	*Mus musculus*	NP_079628	4e-14	79
LP-Y-S01-0163-LF	cytochrome c oxidase subunit II	448	*Penaeus monodon*	NP_038290	7e-65	168
LP-Y-S01-0279-LF	cytochrome c oxidase subunit Va	635	*Rhyzopertha dominica*	AAL17604	5e-52	205

Table 11. Identified putative gene of YHV-infected LO ESTs of *P. monodon*. (continued)

Black tiger shrimp ESTs			Matching sequences			
Clone no.	Putative genes	Sequence size (bp)	Species	Accession number	E value	Score
8. Mitochondria protein (continued)						
LP-Y-S01-0419-LF	cytochrome c oxidase subunit I	600	*Fenneropenaeus chinensis*	AAK82961	1e-50	148
LP-Y-S01-0424-LF	Vacuolar ATP synthase subunit G (V-ATPase G subunit)	600	*Manduca sexta*	Q25532	3e-27	123
LP-Y-S01-0440-LF	Acyl-CoA dehydrogenases	408	*Thermobifida fusca*	ZP_00058724		
LP-Y-S01-0486-LF	cytochrome c oxidase subunit III	600	*Penaeus monodon*	NP_038293	6e-3	308
LP-Y-S01-0489-LF	ATPsyn-gamma	530	*Drosophila yakuba*	AAR09946	4e-30	132
LP-Y-S01-0492-LF	Vacuolar ATP synthase subunit G (V-ATPase G subunit)	600	*Manduca sexta*	Q25532	3e-27	123
LP-Y-S01-0564-LF	putative dehydrogenase	486	*Shigella flexneri* 2a str. 301	NP_706713	5e-83	307
LP-Y-S01-0569-LF	NADH dehydrogenase subunit 6	502	*Penaeus monodon*	NP_038298	4e-72	271
LP-Y-S01-0583-LF	NADH dehydrogenase subunit 6	351	*Penaeus monodon*	NP_038298	2e-47	188
LP-Y-S01-0590-LF	cytochrome c oxidase subunit I	449	*Fenneropenaeus chinensis*	AAK82961	4e-62	175
LP-Y-S01-0616-LF	cytochrome c oxidase subunit II	600	*Penaeus monodon*	NP_038290	1e-94	326
LP-Y-S01-0619-LF	cytochrome c oxidase subunit III	552	*Penaeus monodon*	NP_038293	4e-89	328
LP-Y-S01-0630-LF	cytochrome c oxidase subunit I	600	*Fenneropenaeus merguiensis*	AAK82976	2e-97	356
LP-Y-S01-0668-LF	cytochrome c oxidase subunit II	600	*Penaeus monodon*	NP_038290	6e-95	342
LP-Y-S01-0676-LF	Vacuolar ATP synthase subunit G (V-ATPase G subunit)	600	*Manduca sexta*	Q25532	3e-27	123
LP-Y-S01-0733-LF	cytochrome c oxidase subunit III	600	*Penaeus monodon*	NP_038293	1e-92	340
LP-Y-S01-0806-LF	cytochrome c oxidase subunit II	600	*Penaeus monodon*	NP_038290	2e-82	294
LP-Y-S01-0853-LF	ATP-binding component of cytochrome-related transport, Zn sensitive	600	*Escherichia coli* O157:H7 EDL933	NP_286764	1e-77	158
LP-Y-S01-0865-LF	cytochrome b	600	*Penaeus monodon*	NP_03899	7e-85	294
LP-Y-S01-0866-LF	cytochrome c oxidase subunit II	572	*Coelopa pilipes*	AAP23094	5e-66	203
LP-Y-S01-0896-LF	cytochrome c oxidase subunit I	411	*Fenneropenaeus chinensis*	AAK82961	2e-60	231
LP-Y-S01-0925-LF	cytochrome c oxidase subunit II	600	*Penaeus monodon*	NP_038290	3e-89	314
LP-Y-S01-0946-LF	cytochrome b	462	*Penaeus monodon*	AAF36100	6e-69	260
LP-Y-S01-0966LF	cytochrome b	600	*Penaeus monodon*	NP_038299	1e-100	365
LP-Y-S01-0983-LF	cytochrome c oxidase subunit III	409	*Penaeus monodon*	NP_038293	8e-61	233
LP-Y-S01-1001-LF	cytochrome c oxidase subunit II	600	*Penaeus monodon*	NP_038290	3e-99	362
LP-Y-S01-1013-LF	cytochrome c oxidase subunit II	550	*Penaeus monodon*	NP_038290	1e-90	333
LP-Y-S01-1019-LF	Vacuolar ATP synthase subunit G (V-ATPase G subunit)	442	*Manduca sexta*	Q25532	9e-28	123
LP-Y-S01-1020-LF	cytochrome c oxidase subunit II	681	*Penaeus monodon*	NP_038290	1e-109	397
LP-Y-S01-1032-LF	cytochrome c oxidase subunit II	600	*Penaeus monodon*	NP_038290	1e-88	177
9. Transport						
LP-Y-S01-0121-LF	SEC61, gamma subunit	473	*Mus musculus*	NP_035473	8e-27	120
LP-Y-S01-0204-LF	putative transport protein	600	*Shigella flexneri 2a str. 301*	NP_706523	8e-84	279
LP-Y-S01-0224-LF	putative high-affinity potassium transporter	543	*Oryza sativa (japonica cultivar-group)*	BAC84607		
LP-Y-S01-0276-LF	putative transport protein	600	*Shigella flexneri 2a str. 301*	NP_706726	1e-107	390
LP-Y-S01-0306-LF	putative transport	731	*Escherichia coli K12*	NP_415419	3e-70	266
LP-Y-S01-0545-LF	ABC-type multidrug transport system, permease component	680	*Actinobacillus pleuropneumoniae serovar*	ZP_00135663	2e-53	210

Table 11. Identified putative gene of YHV-infected LO ESTs of *P. monodon*. (continued)

Black tiger shrimp ESTs			Matching sequences			
Clone no.	Putative genes	Sequence size (bp)	Species	Accession number	E value	Score
9. Transport (continued)						
LP-Y-S01-0548-LF	Chain A, Crystal Structure Of The Lipoprotein Localization Factor	566	*Lola pdb|1UA8|A Chain A, Crystal*	1IWL	1e-50	200
LP-Y-S01-0586-LF	putative transport protein	580	*Shigella flexneri 2a str. 301*	NP_706523	5e-74	233
LP-Y-S01-0608-LF	nascent polypeptide-associated complex alpha polypeptide	450	*Danio rerio*	AMM21714	3e-18	91
LP-Y-S01-0662-LF	Permease	600	*Propionibacterium acnes KPA171202*	YP_054796	3e-87	322
LP-Y-S01-0667-LF	transport periplasmic protein	584	*Escherichia coli K12*	NP_415826	1e-106	385
LP-Y-S01-0678-LF	outer membrane protein	800	*Escherichia coli K12*	NP_415460	1e-129	462
LP-Y-S01-0693-LF	SapD	600	*Shigella dysenteriae*	AAF28110	6e-36	152
LP-Y-S01-0706-LF	transport of dicarboxylates	600	*Shigella flexneri 2a str. 301*	NP_706588	5e-89	308
LP-Y-S01-0720-LF	putative high-affinity potassium transporter	534	*Oryza sativa (japonica cultivar-group)*	BAD31835	3e-12	73
LP-Y-S01-0727-LF	Multidrug translocase mdfA	566	*Escherichia coli CFT073*	NP_752857	2e-94	295
LP-Y-S01-0739-LF	peptide ABC transporter, periplasmic peptide-binding protein	600	*Chlamydophila caviae GPIC*	NP_829016	4e-07	40
LP-Y-S01-0753-LF	putative outer membrane receptor for iron transport	600	*Escherichia coli K12*	NP_415968	8e-86	317
LP-Y-S01-0764-LF	permease	545	*Propionibacterium acnes KPA171202*	YP_054796	2e-75	271
LP-Y-S01-0814-LF	Arginine transport ATP-binding protein artP	600	*Escherichia coli CFT073*	NP_752927	5e-95	349
LP-Y-S01-0850-LF	transport periplasmic protein	600	*Escherichia coli K12*	NP_45826	1e-59	127
LP-Y-S01-0862-LF	nascent polypeptide-associated complex alpha polypeptide	447	*Danio rerio*	AMM21714	3e-18	91
LP-Y-S01-0939-LF	probable membrane protein ybiO	596	*Escherichia coli K12*	H64817		
LP-Y-S01-0950-LF	putative transport protein	600	*Shigella flexneri 2a str. 301*	NP_706726	3e-81	302
LP-Y-S01-0976-LF	oligopeptide ABC transporter	600	*Chlamydophila pneumoniae TW-183*	NP_876895	8e-07	55
LP-Y-S01-0980-LF	Dolichyl diphosphooligosaccharide-protein glycosyltransferase 67 kDa subunit precursor	717	*Apis mellifera*	NP_395304	2e-16	87
10. Miscellaneous function						
LP-Y-S01-0009-LF	tail fiber protein		*Escherichia coli O157:H7 EDL933*	NP_287397	3e-16	86
LP-Y-S01-0036-LF	Oligomycin	600	*Drosophila melanogaster*	XP_081326	8e-51	119
LP-Y-S01-0060LF	Chelonianin	600	*Caretta caretta*	P00993	3e-06	52
LP-Y-S01-0065-LF	reticulon 1	442	*Homo sapiens*	NP_066959	2e-15	74
LP-Y-S01-0075-LF	outer membrane protein	600	*Escherichia coli O157*	NP_309297	2e-48	150
LP-Y-S01-0078-LF	tail component	573	*Bacteriophage lambda*	NP_040590	2e-56	218
LP-Y-S01-0110-LF	tail component	338	*Bacteriophage lambda*	NP_040597	7e-96	352
LP-Y-S01-0141-LF	peroxisomal membrane protein	800	*Homo sapiens*	NP_000309	4e-09	62
LP-Y-S01-0153-LF	Unknown	507	*Cloning vector pPW78*	CAB76939	7e-07	40
LP-Y-S01-0185-LF	Selenoprotein T	600	*Homo sapiens*	Q9NZJ3	4e-34	145
LP-Y-S01-0186-LF	putative ORF4	600	*gill-associated virus*	AAK84671	3e-08	48
LP-Y-S01-0200-LF	structural glycoprotein	500	*Yellow head virus*	AAO83987	5e-97	355
LP-Y-S01-0220-LF	Chitinase	600	*Aspergillus nidulans*	BAA36223	1e-07	38

Table 11. Identified putative gene of YHV-infected LO ESTs of *P. monodon*. (continued)

Black tiger shrimp ESTs			Matching sequences			
Clone no.	Putative genes	Sequence size (bp)	Species	Accession number	E value	Score
10. Miscellaneous function (continued)						
LP-Y-S01-0226-LF	Transposase	517	*Klebsiella sp. KCL-2*	NP_620616	1e-42	173
LP-Y-S01-0231-LF	proactivator polypeptide	500	*Gallus gallus*	O13035	1e-34	147
LP-Y-S01-0238-LF	Zfp294 protein	591	*Mus musculus*	AAH27795	9e-31	134
LP-Y-S01-0244-LF	ribophorin I	574	*Xenopus laevis*	AAH44106	8e-12	71
LP-Y-S01-0313-LF	Psmb1-prov protein	532	*Xenopus laevis*	AAH43739	5e-05	47
LP-Y-S01-0315-LF	outer membrane protein A precursor	445	*Shigella boydii*	AAT02227	1e-109	346
LP-Y-S01-0320-LF	putative structural protein	600	*Escherichia coli O157:H7 EDL933*	NP_285859	3e-55	216
LP-Y-S01-0346-LF	structural glycoprotein	661	*Yellow head virus*	AAO83987	3e-38	159
LP-Y-S01-0357-LF	zinc finger	600	*Arabidopsis thaliana*	NP_187692	5e-10	65
LP-Y-S01-0373-LF	Bor protein precursor	515	*Bacteriophage lambda*	NP_597780	4e-43	176
LP-Y-S01-0389-LF	aspartic protease family member (5T521)	600	*Caenorhabditis elegans*	NP_741676	4e-06	51
LP-Y-S01-0393-LF	tail component	456	*Bacteriophage lambda*	NP_040590	4e-39	162
LP-Y-S01-0401-LF	endomembrane protein 70, putative	600	*Arabidopsis thaliana*	NP_187991	7e-44	105
LP-Y-S01-0446-LF	DHSB protein	598	*Dendronephthya klunzingeri*	CAC80855	5e-68	238
LP-Y-S01-0460-LF	peritrophin 2	593	*Penaeus monodon*	AMM44050	3e-72	26
LP-Y-S01-0463-LF	outer membrane protein 3a	600	*Escherichia coli O157:H7 EDL933*	NP_286832	1e-115	416
LP-Y-S01-0473-LF	pva1	600	*Plasmodium vivax*	CAA63219	3e-14	77
LP-Y-S01-0477-LF	ybiX protein	695	*Escherichia coli K12*	D64817	9e-44	178
LP-Y-S01-0479-LF	antifreeze glycopeptide AFGP polyprotein precursor	600	*Boreogadus saida*	T44768	1e-07	58
LP-Y-S01-0495-LF	peritrophin 1	600	*Penaeus monodon*	AMM44049	1e-111	370
LP-Y-S01-0520LF	Integrase	600	*Bacteriophage 434*	P27078	5e-40	165
LP-Y-S01-0528-LF	Dnaja1-prov protein	600	*Xenopus laevis*	AAH42291	1e-78	294
LP-Y-S01-0561-LF	putative senescence	563	*Pisum sativum*	BAB33410	4e-97	355
LP-Y-S01-0582-LF	putative protease	549	*Escherichia coli K12*	NP_415788	7e-54	211
LP-Y-S01-0597-LF	outer membrane protein A precursor	600	*Shigella boydii*	AAT02227	1e-111	318
LP-Y-S01-0598-LF	peritrophin 1	356	*Penaeus monodon*	AMM44049	7e-21	100
LP-Y-S01-0600-LF	Granulins precursor (Acrogranin) (Proepithelin) (PEPI)	600	*Homo sapiens*	P28799	6e-09	62
LP-Y-S01-0649-LF	rhsB protein in rhs element	591	*Escherichia coli K12*	YP_026224	1e-59	231
LP-Y-S01-0657-LF	outer membrane protein A precursor	600	*Shigella boydii*	AAT02227	1e-115	416
LP-Y-S01-0664-LF	outer membrane protein A precursor	600	*Shigella boydii*	AAT02227	1e-100	246
LP-Y-S01-0669-LF	GVG	573	*Binary vector pINDEX1*	AAG35029	2e-30	133
LP-Y-S01-0671-LF	heat-sensitive lambda citS857 repressor protein/VP16 fusion protein	547	*synthetic construct*	AAN86071	8e-20	98

Table 11. Identified putative gene of YHV-infected LO ESTs of *P. monodon*. (continued)

Black tiger shrimp ESTs			Matching sequences			
Clone no.	Putative genes	Sequence size (bp)	Species	Accession number	E value	Score
10. Miscellaneous function (continued)						
LP-Y-S01-0680-LF	Excisionase	404	*Bacteriophage lambda*	NP_040610	2e-16	70
LP-Y-S01-0697-LF	outer membrane protein A precursor	600	*Shigella boydii*	AAT02227	1e-106	385
LP-Y-S01-0717-LF	Excisionase	528	*Bacteriophage lambda*	NP_040610	4e-36	152
LP-Y-S01-0722-LF	outer membrane protein A precursor	548	*Shigella boydii*	AAT02227	1e-106	384
LP-Y-S01-0731-LF	peritrophin 1	600	*Penaeus monodon*	AMM44049	4e-65	249
LP-Y-S01-0740-LF	peritrophin 1	600	*Penaeus monodon*	AMM44049	2e-88	326
LP-Y-S01-0743-LF	putative EC 1.2 enzyme	600	*Escherichia coli O157:H7 EDL933*	NP_288790	2e-48	189
LP-Y-S01-0751-LF	integrase	600	*Bacteriophage 434*	P27078	1e-37	157
LP-Y-S01-0765-LF	Leucine-rich repeat (LRR) protein	491	*Pseudomonas fluorescens PfO-1*	ZP_00264431	4e-13	75
LP-Y-S01-0779-LF	IS30 transposase encoded within prophage CP-933T	586	*Escherichia coli O157:H7 EDL933*	NP_288371	6e-91	334
LP-Y-S01-0781-LF	BCS-1	458	*Balanus amphitrite*	BAA99543	3e-20	98
LP-Y-S01-0794-LF	cyclophilin-RNA interacting protein	467	*Paramecium tetraurelia*	CAC35733	1e-18	94
LP-Y-S01-0807-LF	putative ORF4	474	*gill-associated virus*	AAK84671	2e-08	48
LP-Y-S01-0830-LF	putative pectinase	600	*Escherichia coli K12*	NP_415215	3e-88	243
LP-Y-S01-0842-LF	outer membrane protein A precursor	600	*Shigella boydii*	SST02227	1e-115	416
LP-Y-S01-0844-LF	LtrG*	604	*Shuttle vector pCOMT-Kan*	AAP60028	1e-20	96
LP-Y-S01-0872-LF	proactivator polypeptide	571	*Gallus gallus*	O13035	2e-31	137
LP-Y-S01-0883-LF	Periplasmic glucans biosynthesis protein mdoG precursor	600	*Escherichia coli CFT073*	NP_753226	4e-75	282
LP-Y-S01-0885-LF	minor tail protein	534	*phage lambda*	TLBPTL	2e-78	268
LP-Y-S01-0897-LF	peritrophin 2	600	*Penaeus monodon*	AMM44050	1e-117	401
LP-Y-S01-0908-LF	outer membrane protein A precursor	579	*Shigella boydii*	AAT02227	1e-109	397
LP-Y-S01-0914-LF	UDP-N-acetylmuramoylalanine--D-glutamate ligase	600	*Photorhabdus luminescens subsp. laumondii TTO1*	NP_980864	5e-63	169
LP-Y-S01-0948-LF	Alu subfamily J sequence contamination warning entry	537	*Homo sapiens*	P39188	2e-13	77
LP-Y-S01-0951-LF	UDP-N-acetylmuramoylalanine-D-glutamate ligase	580	*Escherichia coli O157:H7 EDL933*	NP_285784	1e-103	375
LP-Y-S01-0967LF	aspartic protease family member (5T521)	458	*Caenorhabditis elegans*	NP_741676	4e-06	51
LP-Y-S01-0971-LF	Ren protein	600	*Bacteriophage 933W*	NP_049490	2e-35	150
LP-Y-S01-0979-LF	Peptidoglycan hydrolase flgJ	800	*Escherichia coli CFT073*	NP_753261	1e-105	381
LP-Y-S01-0984-LF	Excisionase	600	*Bacteriophage lambda*	NP_040610	6e-36	152
LP-Y-S01-0992-LF	nitrate reductase 1 alpha subunit	712	*Escherichia coli K12*	NP_415742	1e-111	404
LP-Y-S01-1002-LF	Outer membrane receptor for ferric coprogen and ferric-rhodotorulic acid	600	*Pseudomonas aeruginosa UCBPP-PA14*	ZP_00140121	5e-78	291
LP-Y-S01-1030-LF	antifreeze glycopeptide AFGP polyprotein precursor	600	*Boreogadus saida*	T44768	4e-08	59

Table 11. Identified putative gene of YHV-infected LO ESTs of *P. monodon*. (continued)

Black tiger shrimp ESTs			Matching sequences			
Clone no.	Putative genes	Sequence size (bp)	Species	Accession number	E value	Score
11. Unidentified (hypothetical) similar to other cDNA/DNA						
LP-Y-S01-0003-LF	Hypothetical 12.1 kDa protein	600	*Manduca sexta*	Q9U516	2e-40	167
LP-Y-S01-0004-LF	hypothetical protein	204	*Plasmodium yoelii yoelii*	EAA21093	0.0001	47
LP-Y-S01-0006-LF	hypothetical protein XP_177119	512	*Mus musculus*	XP_177119	2e-28	125
LP-Y-S01-0027-LF	hypothetical protein	600	*Plasmodium falciparum 3D7*	NP_701571	9e-05	48
LP-Y-S01-0032-LF	Enzyme	600	*Shigella flexneri 2a*	NP_706693	1e-103	376
LP-Y-S01-0040-LF	hypothetical protein	600	*Biomphalaria glabrata*	PC1123	2e-60	233
LP-Y-S01-0044-LF	hypothetical protein	600	*Carassius auratus*	JC1348	5e-05	42
LP-Y-S01-0046-LF	agCP14148	449	*Anopheles gambiae*	EAA09099	3e-06	51
LP-Y-S01-0052-LF	agCP4709	320	*Anopheles gambiae*	EAA15143	3e-21	100
LP-Y-S01-0054-LF	ENSANGP00000000338	190	*Anopheles gambiae*	EAA02026	1e-13	75
LP-Y-S01-0080-LF	hypothetical protein	361	*Plasmodium falciparum*	NP_702308	3e-06	52
LP-Y-S01-0082-LF	CG8860-PA	224	*Drosophila melanogaster*	NP_610738	1e-06	53
LP-Y-S01-0083-LF	CG7291-PA	600	*Drosophila melanogaster*	NP_608637	2e-20	100
LP-Y-S01-0089-LF	hypothetical protein	600	*Escherichia coli*	NP_308668	5e-57	222
LP-Y-S01-0096-LF	hypothetical protein	600	*Caenorhabditis elegan*	T20630	2e-48	158
LP-Y-S01-0098-LF	hypothetical protein	600	*Bacteriophage I*	NP_612984	2e-53	209
LP-Y-S01-0099-LF	hypothetical protein	204	*Plasmodium yoelii yoelii*	EAA21093	0.0001	47
LP-Y-S01-0107-LF	ENSANGP00000000338	600	*Anopheles gambiae*	EAA02026	9e-16	84
LP-Y-S01-0108-LF	hypothetical protein	600	*Plasmodium falciparum 3D7*	NP_701307	6e-07	55
LP-Y-S01-0118-LF	hypothetical protein	202	*Escherichia coli O157:H7 EDL933*	NP_286555	1e-25	116
LP-Y-S01-0126-LF	hypothetical protein	600	*Carassius auratus*	JC1348	5e-05	42
LP-Y-S01-0145-LF	hypothetical protein	600	*Bacteriophage I*	NP_612984	2e-53	209
LP-Y-S01-0154-LF	unnamed protein product	600	*Tetraodon nigroviridis*	CAG13317	8e-07	55
LP-Y-S01-0168-LF	unknown protein	591	*Escherichia coli*	NP_287365	1e-26	115
LP-Y-S01-0171LF	Unknown	520	*Rattus norvegicus*	AAH64030	1e-66	253
LP-Y-S01-0176-LF	CG2839-PA	600	*Drosophila melanogaster*	NP_608540	3e-13	76
LP-Y-S01-0178-LF	unnamed protein product	385	*Tetraodon nigroviridis*	CAF93717	7e-13	73
LP-Y-S01-0179-LF	Unknown	523	*Rattus norvegicus*	AAH64030	8e-67	254
LP-Y-S01-0182-LF	unnamed protein product	532	*Tetraodon nigroviridis*	CAG13317	6e-57	55
LP-Y-S01-0205-LF	orf, hypothetical protein	670	*Escherichia coli O157:H7 EDL933*	NP_286556	2e-523	210
LP-Y-S01-0215-LF	ORF X; putative	551	*Escherichia coli*	AAA83842	6e-72	271
LP-Y-S01-0237-LF	CG3283-PA	600	*Drosophila melanogaster*	NP_477101	1e-87	321
LP-Y-S01-0271-LF	hypothetical protein XP_216384	469	*Rattus norvegicus*	XP_216384	2e-18	92
LP-Y-S01-0273-LF	orf, conserved hypothetical protein	725	*Shigella flexneri 2a str. 301*	NP_416039	2e-97	338
LP-Y-S01-0275-LF	hypothetical protein Tfus02001166	489	*Thermobifida fusca*	BAB22644	2e-05	50
LP-Y-S01-0277-LF	hypothetical protein b1522	600	*Escherichia coli K12*	NP_755591	7e-32	116
LP-Y-S01-0280-LF	unnamed protein product	535	*Mus musculus*	NP_286033	3e-09	43
LP-Y-S01-0282-LF	Hypothetical protein	577	*Escherichia coli CFT073*	AAL39561	4e-35	130
LP-Y-S01-0283-LF	orf, hypothetical protein	600	*Escherichia coli O157:H7 EDL933*	NP_706671	1e-111	403
LP-Y-S01-0284-LF	LD11211p	600	*Drosophila melanogaster*	XP_306301	1e-29	130

Table 11. Identified putative gene of YHV-infected LO ESTs of *P. monodon*. (continued)

Black tiger shrimp ESTs			Matching sequences			
Clone no.	Putative genes	Sequence size (bp)	Species	Accession number	E value	Score
11. Unidentified (hypothetical) similar to other cDNA/DNA (continued)						
LP-Y-S01-0290-LF	orf, conserved hypothetical protein	712	*Shigella flexneri 2a str. 301*	NP_706671	4e-73	276
LP-Y-S01-0291-LF	ENSANGP00000022728	600	*Anopheles gambiae*	XP_306301	5e-13	75
LP-Y-S01-0295-LF	unnamed protein product	474	*Mus musculus*	BAC31047	6e-06	51
LP-Y-S01-0299-LF	similar to Ac1873	600	*Rattus norvegicus*	XP_342268	1e-08	61
LP-Y-S01-0305-LF	hypothetical protein b3376	485	*Escherichia coli K12*	NP_417835	6e-55	214
LP-Y-S01-0311-LF	CG7415-PA	600	*Drosophila melanogaster*	NP_649749	1e-24	70
LP-Y-S01-0321-LF	Leucine-rich repeat (LRR) protein	595	*Pseudomonas fluorescens*	ZP_00085882	1e-10	67
LP-Y-S01-0332-LF	containing human KIAA0714 protein	567	*Homo sapiens*	CAB90429	9e-31	134
LP-Y-S01-0338-LF	orf, hypothetical protein	600	*Escherichia coli K12*	NP_416064	2e-77	289
LP-Y-S01-0339-LF	CG3283-PA	600	*Drosophila melanogaster*	NP_477101	6e-95	348
LP-Y-S01-0341-LF	gp173	541	*Mycobacteriophage Omega*	NP_818473	2e-11	70
LP-Y-S01-0344-LF	unnamed protein product	442	*Tetraodon nigroviridis*	CAF88611	4e-15	47
LP-Y-S01-0345-LF	Unknown	450	*Silurana tropicalis*	AAH63365	5e-18	90
LP-Y-S01-0350-LF	Unknown (protein for IMAGE:7028390)	492	*Xenopus tropicalis*	AAH77022	3e-13	75
LP-Y-S01-0356-LF	hypothetical protein ECs0699	501	*Escherichia coli O157:H7*	NP_308726	2e-74	279
LP-Y-S01-0361-LF	gene 11-1 protein precursor	600	*Plasmodium falciparum 3D7*	NP_700847	1e-15	84
LP-Y-S01-0379-LF	similar to Plasmodium falciparum (isolate 3D7). Hypothetical protein	600	*Dictyostelium discoideum*	AAO51829	3e-12	73
LP-Y-S01-0397-LF	ENSANGP00000005174	565	*Anopheles gambiae*	XP_314867	3e-10	66
LP-Y-S01-0413-LF	ENSANGP00000024243	600	*Anopheles gambiae*	XP_310017	2e-35	150
LP-Y-S01-0420-LF	hypothetical protein	600	*Escherichia coli*	NP_308668	1e-111	362
LP-Y-S01-0422-LF	hypothetical RNA methyltransferase	756	*Salmonella enterica subsp. enterica serovar Typhi*	NP_455409	7e-65	184
LP-Y-S01-0429-LF	hypothetical protein	600	*Pseudomonas syringae pv. tomato str. DC3000*	NP_749660	0.0001	48
LP-Y-S01-0445-LF	hypothetical protein	461	*Dictyostelium discoideum*	AAO50882	1e-05	51
LP-Y-S01-0459-LF	hypothetical protein	600	*Streptomyces avermitilis MA-4680*	NP_823107	1e-45	184
LP-Y-S01-0462LF	conserved hypothetical protein	600	*Corynebacterium efficiens YS-314*	NP_738153	2e-09	63
LP-Y-S01-0467-LF	CG30476-PA	600	*Drosophila melanogaster*	NP_725413	3e-23	110
LP-Y-S01-0471-LF	insertion element	588	*Escherichia coli*	P51026	1e-23	96
LP-Y-S01-0497-LF	KIAA0714 protein	576	*Homo sapiens*	BBA34434	9e-31	134
LP-Y-S01-0499-LF	unnamed protein product	470	*Mus musculus*	BAC31047	6e-06	51
LP-Y-S01-0500-LF	ENSANGP00000010006	600	*Anopheles gambiae*	XP_313976	8e-12	72
LP-Y-S01-0505-LF	hypothetical protein	600	*Pseudomonas syringae pv. syringae B728a*	ZP_00127686	2e-58	226
LP-Y-S01-0511-LF	hypothetical protein	600	*Pseudomonas syringae pv. syringae B728a*	ZP_00127686	e-53	207
LP-Y-S01-0519-LF	hypothetical protein MG08761.4	600	*Magnaporthe grisea 70-15*	EAA51239	1e-05	51
LP-Y-S01-0521-LF	ENSANGP00000025348	600	*Anopheles gambiae*	XP_321574	4e-24	112
LP-Y-S01-0525-LF	orf, conserved hypothetical	147	*Shigella flexneri 2a str. 301*	NP_706671	4e-19	95
LP-Y-S01-0526-LF	Hypothetical protein ybjS	600	*Escherichia coli CFT073*	NP_752931	7e-55	214
LP-Y-S01-0529-LF	hypothetical protein	382	*Homo sapiens*	BAA74876	0.0001	46
LP-Y-S01-0546-LF	ORF X; putative	600	*Escherichia coli*	AAA83842	3e-73	276

Table 11. Identified putative gene of YHV-infected LO ESTs of *P. monodon*. (continued)

Black tiger shrimp ESTs			Matching sequences			
Clone no.	Putative genes	Sequence size (bp)	Species	Accession number	E value	Score
11. Unidentified (hypothetical) similar to other cDNA/DNA (continued)						
LP-Y-S01-0549-LF	ORF X; putative	449	*Escherichia coli*	AAA83842	2e-62	239
LP-Y-S01-0553-LF	hypothetical protein b1177	600	*Escherichia coli K12*	NP_415695	2e-24	113
LP-Y-S01-0555-LF	YbiC	600	*Escherichia coli*	AAA53657	1e-112	405
LP-Y-S01-0559-LF	hypothetical protein b0707	800	*Escherichia coli K12*	NP_415235	5e-78	292
LP-Y-S01-0570-LF	gene 11-1 protein precursor	386	*Plasmodium falciparum 3D7*	NP_700847	1e-11	70
LP-Y-S01-0572-LF	expressed protein	555	*Arabidopsis thaliana*	NP_200300	1e-30	134
LP-Y-S01-0585-LF	hypothetical protein b0644	715	*Escherichia coli K12*	NP_415177	2e-99	363
LP-Y-S01-0591-LF	conserved hypothetical protein	241	*Xanthomonas axonopodis pv. citri str. 306*	NP_642162	1e-10	67
LP-Y-S01-0592-LF	hypothetical protein Efae03002652	600	*Enterococcus faecium*	ZP_00285449	0.0001	48
LP-Y-S01-0601-LF	orf156	430	*Paramecium aurelia*	NP_059418	0.0002	45
LP-Y-S01-0602-LF	PREDICTED: similar to hypothetical protein FLJ20457	474	*Gallus gallus*	XP_419070	8e-19	94
LP-Y-S01-0607-LF	hypothetical protein b0663	600	*Escherichia coli K12*	E64801	3e-51	202
LP-Y-S01-0610-LF	Hypothetical protein ydhH	664	*Escherichia coli CFT073*	NP_753927	2e-55	217
LP-Y-S01-0621-LF	hypothetical protein b3160	560	*Escherichia coli K12*	NP_417629	3e-49	196
LP-Y-S01-0622-LF	conserved hypothetical protein	545	*Pseudomonas aeruginosa PA01*	NP_252474	3e-18	57
LP-Y-S01-0634-LF	reading frame S1	643	*Escherichia coli*	CAA23630	3e-95	349
LP-Y-S01-0646-LF	hypothetical protein ECs0699	600	*Escherichia coli O157:H7*	NP_308726	2e-74	279
LP-Y-S01-0648-LF	Unknown (protein for MGC:55504)	600	*Danio rerio*	AHH48043	4e-07	56
LP-Y-S01-0652-LF	similar to Plasmodium falciparum. Hypothetical protein	591	*Dictyostelium discoideum*	AAS38793	1e-11	71
LP-Y-S01-0653-LF	ENSANGP00000022463	810	*Anopheles gambiae*	XP_321040	1e-68	261
LP-Y-S01-0660-LF	orf, hypothetical protein	600	*Escherichia coli O157:H7 EDL933*	NP_286001	2e-61	171
LP-Y-S01-0665-LF	ENSANGP00000023986	600	*Anopheles gambiae*	XP_316923	5e-09	62
LP-Y-S01-0666-LF	hypothetical protein	790	*Pseudomonas syringae pv. syringae B728a*	ZP_00127686	1e-56	221
LP-Y-S01-0673-LF	hypothetical protein b1967	718	*Escherichia coli K12*	NP_416476	1e-104	337
LP-Y-S01-0690LF	similar to hypothetical proteins	600	*Bacillus subtilis*	NP_391934	2e-23	110
LP-Y-S01-0700-LF	hypothetical protein b3160	696	*Escherichia coli K12*	NP_417629	5e-49	196
LP-Y-S01-0724-LF	RIKEN cDNA 9330155M09 gene	600	*Mus musculus*	NP_796228	3e-07	57
LP-Y-S01-0737-LF	Unknown (protein for MGC:82154)	566	*Xenopus laevis*	AAH75189	3e-25	116
LP-Y-S01-0745-LF	hypothetical protein MGC75969	597	*Xenopus tropicalis*	NP_989225	2e-17	90
LP-Y-S01-0750-LF	orf, conserved hypothetical protein	800	*Shigella flexneri 2a str. 301*	NP_706525	1e-37	158
LP-Y-S01-0761-LF	reading frame S1	800	*Escherichia coli*	CAA23630	3e-94	346
LP-Y-S01-0772-LF	similar to ENSANGP00000021507	573	*Apis mellifera*	XP_392881	2e-29	130
LP-Y-S01-0773-LF	ENSANGP00000024398	584	*Anopheles gambiae*	XP_308512	2e-83	291
LP-Y-S01-0776-LF	hypothetical protein b0703	456	*Escherichia coli K12*	NP_415231	7e-59	226
LP-Y-S01-0777-LF	hypothetical protein	657	*Escherichia coli*	AAB40755	4e-29	129
LP-Y-S01-0783-LF	hypothetical protein ECs0699	447	*Escherichia coli O157:H7*	NP_308726	2e-68	258
LP-Y-S01-0784-LF	COG0177: Predicted EndoIII-related endonuclease	492	*Pseudomonas syringae pv. syringae B728a*	ZP_00126535	9e-56	217
LP-Y-S01-0795-LF	unnamed protein product	600	*Tetraodon nigroviridis*	CAG03719	8e-06	52

Table 11. Identified putative gene of YHV-infected LO ESTs of *P. monodon*. (continued)

Black tiger shrimp ESTs			Matching sequences			
Clone no.	Putative genes	Sequence size (bp)	Species	Accession number	E value	Score
11. Unidentified (hypothetical) similar to other cDNA/DNA (continued)						
LP-Y-S01-0812-LF	hypothetical protein b0703	454	*Escherichia coli K12*	NP_415231	1e-63	242
LP-Y-S01-0828-LF	unnamed protein product	439	*Kluyveromyces lactis*	XP_453836	2e-16	52
LP-Y-S01-0833-LF	hypothetical protein MG08761.4	600	*Magnaporthe grisea 70-15*	EAA51239	1e-05	51
LP-Y-S01-0851-LF	COG2847: Uncharacterized protein conserved in bacteria	600	*Azotobacter vinelandii*	ZP_0009063	4e-18	55
LP-Y-S01-0854-LF	hypothetical protein b0358	800	*Escherichia coli K12*	NP_414892	6e-69	168
LP-Y-S01-0858-LF	unnamed protein product	600	*Tetraodon nigroviridis*	CAG11019	5e-53	208
LP-Y-S01-0859-LF	CG3662-PA	504	*Drosophila melanogaster*	NP_608549	7e-24	111
LP-Y-S01-0870-LF	CG7415-PB	600	*Drosophila melanogaster*	NP_731238	4e-29	129
LP-Y-S01-0881-LF	hypothetical protein	473	*Pseudomonas syringae pv. tomato str. DC3000*	NP_794660	0.0001	47
LP-Y-S01-0891-LF	hypothetical protein	398	*Streptomyces avermitilis MA-4680*	NP_827173	2e-09	62
LP-Y-S01-0898-LF	hypothetical protein MGC75969	600	*Xenopus tropicalis*	NP_989225	7e-32	138
LP-Y-S01-0913-LF	hypothetical protein b0703	452	*Escherichia coli K12*	NP_415231	7e-53	198
LP-Y-S01-0919-LF	Hypothetical protein b1001	720	*Escherichia coli K12*	NP_415521	6e-45	120
LP-Y-S01-0921-LF	YbiC	600	*Escherichia coli*	AAA53657	1e-112	405
LP-Y-S01-0928-LF	Unknown	488	*Rattus norvegicus*	AAH64030	2e-61	236
LP-Y-S01-0938-LF	Hypothetical protein in bioA 5	600	*Escherichia coli K12*	BAA35436	5e-36	152
LP-Y-S01-0963-LF	hypothetical protein	632	*Yarrowia lipolytica*	XP_56130	8e-08	58
LP-Y-S01-0968-LF	expressed protein	600	*Arabidopsis thaliana*	NP_200300	1e-30	134
LP-Y-S01-0969-LF	ORF	600	*Mus musculus*	BAA00447	3e-10	66
LP-Y-S01-0986-LF	ENSANGP00000024398	594	*Anopheles gambiae*	XP_308512	4e-11	69
LP-Y-S01-0988-LF	zgc:86791	271	*Danio rerio*	NP_001002683	2e-17	57
LP-Y-S01-0990-LF	expressed protein	583	*Arabidopsis thaliana*	NP_200300	2e-30	134
LP-Y-S01-0996-LF	orf, conserved hypothetical protein	600	*Shigella flexneri 2a str. 301*	NP_07467	4e-38	159
LP-Y-S01-0998LF	similar to ENSANGP00000021507	600	*Apis mellifera*	XP_392881	6e-34	145
LP-Y-S01-1004-LF	hypothetical protein	343	*Carassius auratus*	JC1348	9e-05	42
LP-Y-S01-1014-LF	hypothetical C4-type zinc finger protein TraR-family	699	*Escherichia coli O157:H7*	NP_308832	4e-35	149
LP-Y-S01-1026-LF	Hypothetical protein	600	*Escherichia coli CFT073*	NP_752852	2e-56	219
LP-Y-S01-1027-LF	hypothetical protein ECs0699	600	*Escherichia coli O157:H7*	NP_308726	5e-59	228
LP-Y-S01-1031-LF	hypothetical protein	600	*Thermobifida fusca*	ZP_00056820	5e-06	52
LP-Y-S01-1036-LF	ENSANGP00000010006	600	*Anopheles gambiae*	XP_31397670	2e-11	70

Table 12. The Redundancy of identified putative genes**.**

Putative genes	Redundancy
1. Gene expression, regulation and protein synthesis	
RNA polymerase II	1
elongation factor 1-beta	4
U1small ribonucleoprotein	1
VspA	1
regulator for leucine	1
translation initiation factor 4C	1
cyclophilin 1	1
repressor protein	1
Eukaryotic translation initiation factor 4A	1
yfjP protein	1
similar to QM protein	4
Trp1p	2
elongation factor-1 alpha	4
yejO	1
elongation factor 2b	1
CI protein	1
transcriptional regulatory protein	1
anti-silencing factor 1	1
Hnr protein	1
serine tRNA synthetase	2
putative NAGC-like transcriptional regulator	1
repressor protein	1
translation elongation factor 1-alpha	1
Similar to eukaryotic translation elongation factor 1 beta 2	1
Xanthosine operon Regulatory protein	1
HLA class II region expressed gene KE2; KE2 protein	1
putative transcriptional regulator LYSR-type	1
2. Internal/ external structure and motility	
tubulin-alpha 1	1
cuticle protein	1
periplasmic glucans	1
flagellin	1
3. Metabolism	
CTP:CMP-3-deoxy-D-manno-octulosonate transferase	2
methylmalonyl-CoA mutase	1
citrate lyase synthetase	3
enterobactin synthetase	3
3-deoxy-D-arabinoheptulosonate-7-phosphate synthase	1
formate acetyltransferase	1
Lactoylglutathione lyase	1

Table 12. The Redundancy of identified putative genes. (continued)

Putative genes	Redundancy
3. Metabolism (continued)	
diadenosine tetraphosphatase	3
metallothionein 2	3
Chain A, Crystal Structure Of Beta-Lactamase Inhibitor Protein-Ii In Complex With Tem-1 Beta-Lactamase	1
Epoxide hydrolase 1 (Microsomal epoxide hydrolase)	
N-(5'-phosphoribosyl)anthranilate isomerase	1
quinolinate synthetase A protein	1
ubiquitin conjugating enzyme E2, J2 isoform 3; ubiquitin conjugating enzyme 6	1
cystathionine gamma-lyase	1
putative hexulose-6-phosphate isomerase	1
COG0626: Cystathionine beta-lyases/cystathionine gamma-synthases	1
D-erythrose 4-phosphate dehydrogenase	3
Glyoxylase I Activity Enhancing protein	1
farnesoic acid O-methyltransferase	4
ubiquitin specific protease 2	1
phosphoglycerate kinase	1
Enoyl-CoA hydratase/carnithine racemase	1
heptosyl transferase	1
Chain A, Structure Of The Mutant F174a T Form Of The Glucosamine-6- Phosphate - Deaminase	1
putative hexulose-6-phosphate isomerase	1
glutamate dehydrogenase	1
similar to deoxyribose-phosphate aldolase	1
the sequence of TRP1 from strain S228C, which is the sequence stored in SGD	1
COG0155: Sulfite reductase, beta subunit (hemoprotein)	1
putative fructose 1,6-bisphosphate aldolase	1
putative dehydrogenase	1
4. Defense and homeostasis	
chaperonin	1
peptidyl-prolyl cis-trans isomerase	2
lysozyme	1
serine protease homologue	1
cathepsin B	2
putative oxidoreductase	2
P109 protein	1
stress-associated endoplasmic reticulum protein 1	1
Heat-responsive regulatory protein	1
COG0542: ATPases with chaperone activity, ATP-binding subunit	1
putative oxidoreductase, major subunit	1
translationally controlled tumor protein	1

Table 12. The Redundancy of identified putative genes. (continued)

Putative genes	Redundancy
5. Signaling and communication	
Rhodopsin	1
RhsB protein	1
Extracellular-regulated kinase A	1
retinol binding protein	7
sensor protein PhoQ	1
Thrombospondin	6
Histidine kinase	1
Prosaposin	1
sensor histidine protein kinase UhpB	1
6. Cell division/DNA synthesis, repair and replication	
endonuclease IV	1
ORF1b polyprotein	1
RNA polymerase	1
DNA-directed RNA polymerase, alpha subunit/40 kD subunit	1
Cell division protein ftsK	1
Cell division protein	1
Replicative DNA helicase	1
Chain B, Mechanism Of Processivity Clamp Opening of DNApolymerase III	1
MukB	3
Similar to XPMC2H protein	1
ExoP-related protein	1
UvrB protein	2
DNA repair; excision nuclease subunit B	1
Proable DNA-directed RNA Polymerase (alpha cahian) protein	1
7. Ribosomal protein and rRNA	
ribosomal protein L17	1
ribosomal protein S26	2
ribosomal protein L30	1
ribosomal protein S3	1
ribosomal protein L5	3
ribosomal protein S23	2
ribosomal subunit S13	1
ribosomal protein L10	1
ribosomal protein S12	1
ribosomal protein S4	1
ribosomal protein S3a	1
ribosomal protein L32	1
ribosomal protein L26	1
ribosomal protein S18	1
ribosomal protein S1	2

Table 12. The Redundancy of identified putative genes. (continued)

Putative genes	Redundancy
8. Mitochondria protein	
carnitine o-palmitoyltransferase I	1
cytochrome b	4
ATP-binding component	1
cytochrome c oxidase subunit I	5
ATP synthase	1
Ubiquinone	1
cytochrome c oxidase subunit II	10
cytochrome c oxidase subunit Va	1
Vacuolar ATP synthase subunit G (V-ATPase G subunit)	3
Acyl-CoA dehydrogenases	1
cytochrome c oxidase subunit III	4
ATPsyn-gamma	1
Vacuolar ATP synthase subunit G (V-ATPase G subunit)	1
putative dehydrogenase	1
NADH dehydrogenase subunit 6	2
ATP-binding component of cytochrome-related transport, Zn sensitive	1
9. Transport	
SEC61, gamma subunit	1
putative transport protein	4
putative high-affinity potassium transporter	2
putative transport	1
ABC-type multidrug transport system, permease component	1
Chain A, Crystal Structure Of The Lipoprotein Localization Factor	1
nascent polypeptide-associated complex alpha polypeptide	2
Permease	2
transport periplasmic protein	2
outer membrane protein	1
SapD	1
transport of dicarboxylates	1
Multidrug translocase mdfA	1
peptide ABC transporter, periplasmic peptide-binding protein	1
putative outer membrane receptor for iron transport	1
Arginine transport ATP-binding protein artP	1
probable membrane protein ybiO	1
oligopeptide ABC transporter	1
Dolichyl-diphosphooligosaccharide--protein glycosyltransferase 67 kDa subunit precursor	1
10. Miscellaneous function	
tail fiber protein	1
Oligomycin	1
Chelonianin	1

Table 12. The Redundancy of identified putative genes. (continued)

Putative genes	Redundancy
10. Miscellaneous function (continued)	
reticulon 1	1
outer membrane protein	1
tail component	2
peroxisomal membrane protein	1
Unknown	1
Selenoprotein T	1
putative ORF4	2
structural glycoprotein	2
Chitinase	1
Transposase	1
proactivator polypeptide	2
Zfp294 protein	1
ribophorin I	1
Psmb1-prov protein	1
outer membrane protein A precursor	8
putative structural protein	1
structural glycoprotein	2
zinc finger	1
Bor protein precursor	1
aspartic protease family member (5T521)	2
endomembrane protein 70, putative	1
DHSB protein	1
peritrophin 2	2
outer membrane protein 3a	1
pva1	1
ybiX protein	1
antifreeze glycopeptide AFGP polyprotein precursor	2
peritrophin 1	4
Integrase	2
Dnaja1-prov protein	1
putative senescence	1
putative protease	1
Granulins precursor (Acrogranin) (Proepithelin) (PEPI)	1
rhsB protein in rhs element	1
GVG	1
heat-sensitive lambda citS857 repressor protein/VP16 fusion protein	1

PART II: Isolation and characterization of the full-length transcript of shrimp ASF1

1. Cloning the 5'end cDNA of shrimp ASF1 by the 5'RACE technique

From the sequence analysis cDNA clones using BLASTX program showed that a 600 bp ESTs clone has significant similarity to the 3' end of the open reading frame (ORF) of the *D. melanogaster* ASF1. This indicates that the shrimp ASF1 partial clone (LP-Y-S01-0712-LF) was missing approximately 210 bp (71 amino acids) from the 5' end of the coding region in the cDNA compared with *D. melanogaster* ASF1 (Figure 27).

The 5' end of shrimp ASF1 cDNA was generated by an RNA ligase-mediated RACE (RLM-RACE) method. The internal primers which are GSP1 (ASF-189R) and GSP2 (ASF-87R) were designed based on the 3'end of ESTs sequence (LP-Y-S01-0712-LF). These two primers were used in conjunction with the RLM 5' RACE adapter primer to amplify the 5' end of shrimp ASF1 cDNA. This fragment was obtained by nested PCR reaction and determined their size on 1.2% agarose gel electrophoresis. From this gel showed that two bands of 5' RACE product were observed as 500 bp and 900 bp (Figure 28). A 500 bp fragment was extracted and cloned to the pGEM-T Easy vector. The recombinant clone was named pASF-500. When pASF-500 was digested by E*coR*I, the insert with size of 500 bp was determined (Figure 29 , lanes 3 and 4) and this clone was then sequenced. The sequence that obtain from 5' RACE ASF1 (pASF-500) clone was subjected to homology search against the known sequence in the GenBank database using BLASTX program.

(A)

gi|6525170|gb|AAF15354.1| ASF1 [Drosophila melanogaster]

```
Length=218 Score =  144 bits (364),  Expect = 1e-33
Identities = 77/146 (52%), Positives = 97/146 (66%), Gaps = 19/146 (13%)
Frame = +2

Query 2   FVFQADPPDHTKIPVGDAVGVTVVLLTCGYKGQEFVRVGYYVNNSYTDPELQETPPDVPQ 181
          FVFQADPPD +KIP  DAVGVT+VLLTC Y+GQEFVRVGYYVNN Y DPE++E PP  P
Sbjct 72  FVFQADPPDVSKIPEPDAVGVTIVLLTCSYRGQEFVRVGYYVNNDYADPEMRENPPTKPL 131

Query 182 FEKLQRNILGTQPRVTKFKIDWDDAKDSENIPPSENA-----ASDGASTSQNSTDL---- 334
          FEKL RNIL ++PRVT+FKI+WD    + N    EN      A+DG STS+ ++ +
Sbjct 132 FEKLTRNILASKPRVTRFKINWDYGHINGNGNGVENGHQDEMATDGPSTSEAASAVIHPE 191

Query 335 ----------KGITAMESSNSACAME  382
                     GI A+  ++++ AME
Sbjct 192 DDNSLAMPMENGIKALNENSNSLAME  217
```

(B)

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DMEL            MAKVHITNVVVLDNPSSFFNPFQFELTFECIEELKEDLEWKMIYVGSAESEEHDQVLDTI
60
pASF1-600       ------------------------------------------------------------


DMEL            YVGPVPEGRHIFVFQADPPDVSKIPEPDAVGVTIVLLTCSYRGQEFVRVGYYVNNDYADP
120
pASF1-600       -----------FVFQADPPDHTKIPVGDAVGVTVVLLTCGYKGQEFVRVGYYVNNSYTDP
49
                ********* :***  ******:*****.*:*************.*:**

DMEL            EMRENPPTKPLFEKLTRNILASKPRVTRFKINWDYGHINGNGNGVENGHQDEMATDGPST
180
pASF1-600       ELQETPPDVPQFEKLQRNILGTQPRVTKFKIDWDDAKDSENIPPSEN-----AASDGAST
104
                *::*.**  * **** ****.::****.***:** .: . *    **      *:**.**

DMEL            SEAASAVIHPEDDNSLAMPMENGIKALNENSNSLAMEC 218
pASF1-600       SQNSTDLK-------------GITAMESSNSACAME- 127
                *: :: :              **.*::....: ***
```

Figure 27. BLASTX analysis of the putative peptides of LP-Y-1-0712-LF clone to *Drosophila melanogaster* ASF1 (A). Pair wise alignment of partial translated sequence shrimp ASF1 (pASF-600) with the complete translated sequence *D. melanogaster* ASF1 (DMEL) indicate the aspect size of 5' end shrimp ASF1 as 210 bp it will be obtained by 5'RACE technique(B).

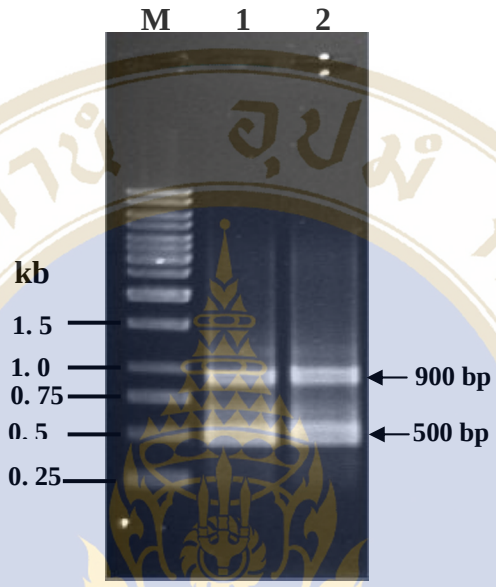


Figure 28. 5'RACE nested PCR amplification of 5'end ASF1 fragment on 1.2% agarose gel, stained with ethidium bromide. Lanes1 and 2 show the same result of 5' RACE ASF1 product that used the different of template (1st PCR product) dilution, 1:100 and 1:250 respectively. Two fragments of 5'RACE ASF1 were determined as 500 and 900 bp in length.

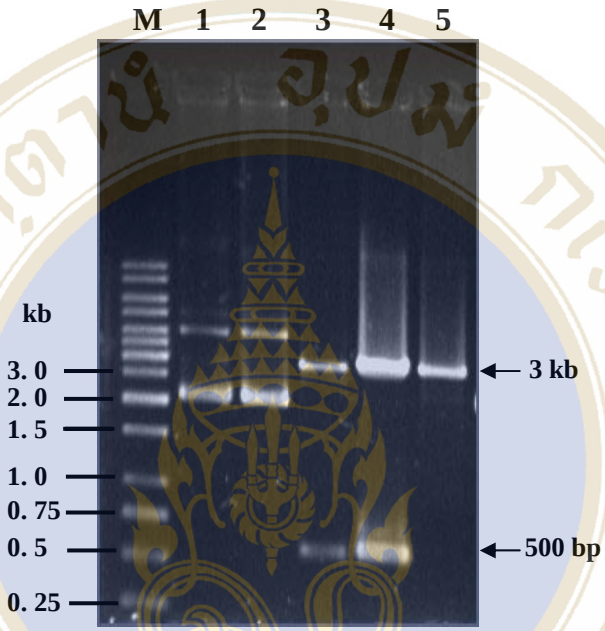


Figure 29. 1.2% agarose gel electrophoresis of restriction enzyme digestion analysis of T-vector cloned the amplified fragment. The 500 bp fragment of 5'RACE ASF1 fragment was ligated with pGEM-T easy vector. Four recombinant clones (pASF-500) were selected for *EcoR*I digestion. The uncut pASF-500 was showed in Lane1. The insert PCR product was determined as 500 bp in lanes 3 and 4.

2. Determination of the cDNA and sequences analysis of shrimp ASF1

ASF1 belongs to the H3-H4 family of histone chaperones. It was firstly identified through genetic studies in budding yeast as a factor that antagonized chromatin-mediated gene silencing when over expressed (86, 87). Studies in a various organisms have defined Asf1's role as a histone chaperone during DNA replication through specific interactions with histones H3/H4 and the histone deposition factor CAF-1. Several biochemical studies have directly linked Asf1 to chromatin assembly after DNA replication. For example, *Drosophila* Asf1 binds newly synthesizwd histone H3/H4 tetramers to form a complex that stimulates the histone deposition activity of chromatin assembly factor-I (CAF-I) (80, 87). Moreover, ASF1 is required for chromatin disassembly during activation of the *PHO5* gene and that this nucleosome disassembly is essential for transcriptional activation. Chromatin disassembly is essential for transcriptional activation because *PHO5* cannot be activated in yeast lacking ASF1 (85, 88). ASF1 is not formally a transcription factor, so it does not posses sequence specific DNA binding properties, but rather it is likely to influence chromatin structure of the promoter and open reading frame of its transcriptional targets, as was shown recently at the *PHO5* and *PHO8* genes (82, 88). Therefore, direct role for ASF1 in gene expression via a role in assembly and disassembly chromatin in a dynamic and gene specific manner during transcriptional regulation (83).

By the RLM 5'RACE technique, a 500 bp of ASF1 cDNA was amplified. Sequencing and searching against database with BLASTX were performed to analyze the 5' RACE clone. From the homology search demonstrate a 500 bp of this clone also shown the similarity to the 5' end ORF of *D. melanogaster* ASF1 (Figure27) that corresponds to the 600 bp ESTs

Shrimp ASF1 cDNA composed of 957 nucleotides. The probable initiation codon is in the sequence AGAGATGG. It exhibits limited homology with the consensus sequence by Kozak (89) that acts as a signal for efficient translation initiation in eukaryotes. However, the critical purine in the position-3 is conserved. The deduce protein is comprised of 200 amino acid residues, has a calculated molecular mass of 22.16 kDa and isoelectric point of 4.02. It dose not contain a typical signal sequence. In addition, the full length of shrimp ASF cDNA contained a 5'

untranslated region (UTR) of 145 bases followed by an ORF of 600 bases beginning with a methionine codon at position 146 and ending with a TAA termination codon at nucleotide position 745 and a 3' UTR of 210 bases (Figure 30).

The amino acid sequence comparison of shrimp ASF1with other ASF1 homologue protein of human, mouse, frog, insect, yeast and plant using ClustalW and BOXSHADE programs. It is revealed that shrimp ASF1 had the highest identity/similarity (62%/76%) (Figure 31) to *Drosophila* ASF1. Moreover shrimp ASF1 exhibits significant identity/similarity with other species. Among these organisms ASF1 show highly conserved especially at the N-terminal sequence while the C-terminal region is poorly conserved.

```
  1 - GAAACAGTCTACGCTCACCCGCCGCCCGGTCCGCGTGTGCGTGTCTTCTCTCCTCCCCCC - 60
 61 - TCCCCCCGTGGCGCGCGCGTGGAACCGACCCTGAAAAGCGGGGAAAAGATAAAAACTAAA - 120
121 - TACAAAAATAAAGTGAGAGAGAGAGATGGCCAAGGTGCACATAGTGAACGTGGTGGTGCT - 180
                            M  A  K  V  H  I  V  N  V  V  V  L  - 12
181 - AGACAACCCCTCGCCGTTCTTCAACCCGTTCCAGTTCGAAATTACCTTCGAATGCGTCGA - 240
    -  D  N  P  S  P  F  F  N  P  F  Q  F  E  I  T  F  E  C  V  E  - 32
241 - GGACCTGCAGGAAGATCTGGAGTGGAAAATAATATATGTAGGTAGTGCAGAAAGTGAAGA - 300
    -  D  L  Q  E  D  L  E  W  K  I  I  Y  V  G  S  A  E  S  E  D  - 52
301 - CTATGATCAGACCCTTGATACAGTGTATGTTGGACCAGTTCCAGAAGGGAAACACATGTT - 360
    -  Y  D  Q  T  L  D  T  V  Y  V  G  P  V  P  E  G  K  H  M  F  - 72
361 - TGTATTTCAGGCTGACCCACCAGATCATACAAAAATACCAGTTGGTGATGCAGTAGGTGT - 420
    -  V  F  Q  A  D  P  P  D  H  T  K  I  P  V  G  D  A  V  G  V  - 92
421 - GACAGTTGTGCTGTTAACTTGCGGATACAAAGGACAGGAGTTTGTGAGGGTGGGTTATTA - 480
    -  T  V  V  L  L  T  C  G  Y  K  G  Q  E  F  V  R  V  G  Y  Y  - 112
481 - TGTGAATAATTCTTATACAGACCCTGAACTTCAAGAAACCCCACCTGATGTCCCACAGTT - 540
    -  V  N  N  S  Y  T  D  P  E  L  Q  E  T  P  P  D  V  P  Q  F  - 132
541 - TGAAAAGCTTCAGAGAAATATCTTGGGAACACAGCCACGTGTCACAAAGTTCAAAATTGA - 600
    -  E  K  L  Q  R  N  I  L  G  T  Q  P  R  V  T  K  F  K  I  D  - 152
601 - CTGGGATGATGCAAAGGATTCGGAAAATATCCCACCCTCGGAGAATGCAGCAAGCGATGG - 660
    -  W  D  D  A  K  D  S  E  N  I  P  P  S  E  N  A  A  S  D  G  - 172
661 - TGCCTCCACATCACAGAATTCTACGGATCTCAAAGGCATAACTGCGATGGAGAGCAGTAA - 720
    -  A  S  T  S  Q  N  S  T  D  L  K  G  I  T  A  M  E  S  S  N  - 192
721 - TAGTGCTTGTGCTATGGAGGTGGCATAAATTTTTGATTTTTAGGTCAATTTTTCTATACT - 780
    -  S  A  C  A  M  E  V  A  *                                  - 200
781 - GCTTAAACCCTTTCATATTGTAATTGTGAATGTTTTGTATTCAGTGCAACTAGATAAGCT - 840
841 - GGTATTGTACAGTCTTTTTGCCTCCCATTCTGACATGATTTTGTGATAAAATTTTGCATA - 900
901 – GGGAATAGTTTTTCCAGTTAATTACTACATTTTATATGAAGCAATATTAAGATTCGT    - 957
```

Figure 30. Nucleotide and deduce amino acid sequences of *P. monodon* ASF1 cDNA. The nucleotide sequence is numbered from the 5' end and the single-letter amino acid code is presented below the corresponding codon. The asterisk indicates the stop codon.

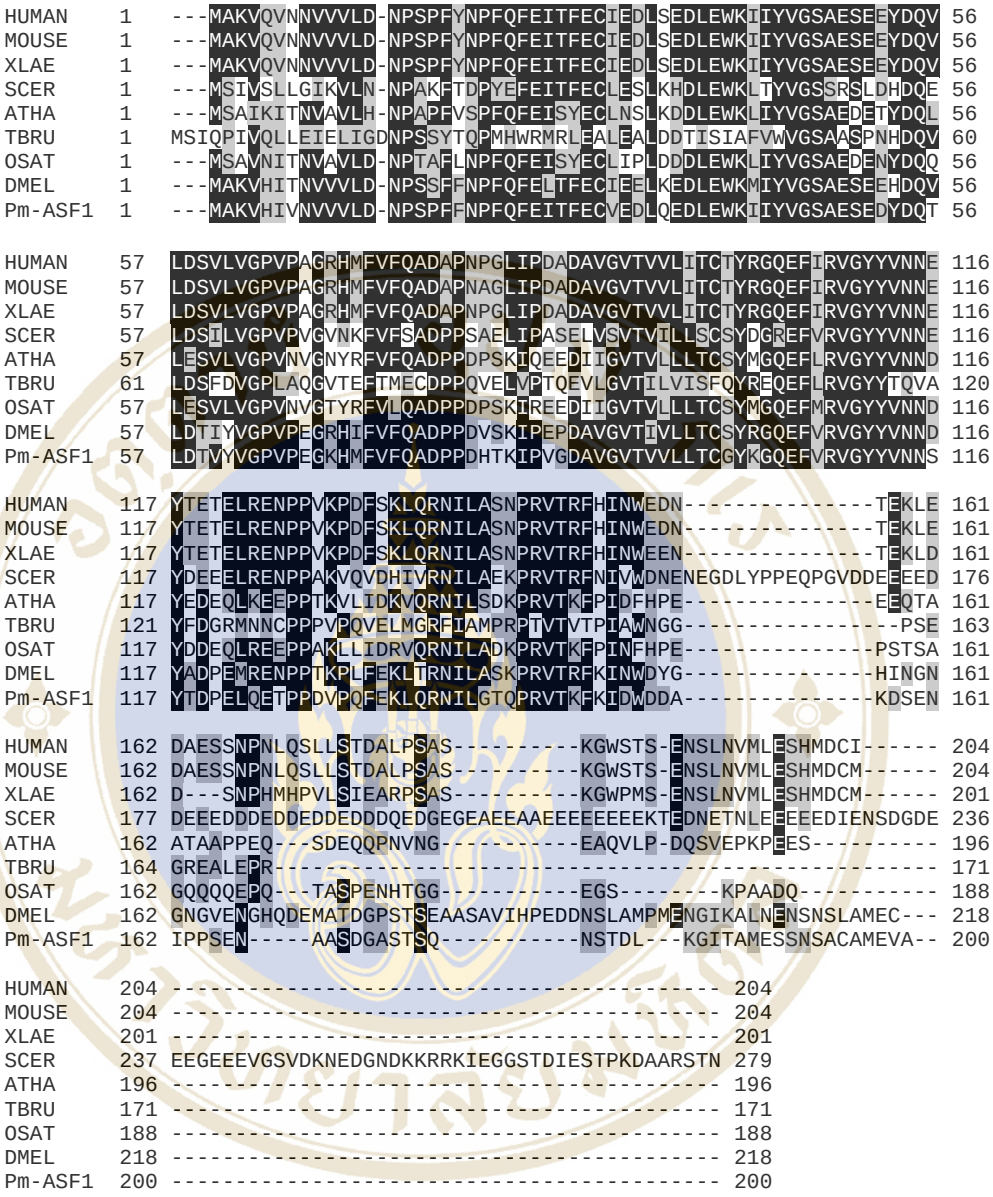


Figure 31. Multiple alignment of amino acid sequences of ASF1 from *P.monodon* (Pm-ASF) with that of various ASF1; *Homo sapiens* (HUMAN,CAG33628.1), *Mus musculus* (MOUSE, NP_079817.1), *Xenopus laevis* (XLAE, AAR08148.1), *Saccharomyces cerevisiae* (SCER, P32447), *Arabidopsis thaliana* (ATHA, BAC54103.1), *Trypanosoma brucei* (TBRU, CAB95356.1), *Oryzae sativa* (OSAT, XP_475872.1), *Drosophila melanogaster* (DMEL, AAO45225.1). The dash represents a gap at the indicated proteins. The numbers of the left indicate the amino acid position of ASF1 in corresponding to species. The black shading indicates identical amino acids, and the gray shading indicates conservative replacements.

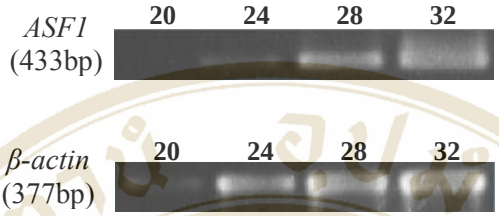
3. Expression of ASF1 transcript in infected shrimp

3.1 Optimization of PCR cycles

Kinetic analysis of PCR reaction at various cycles must be performed in order to investigate appropriate points in the exponential phase for comparison between internal control target and gene expression. By limiting PCR amplification to the linear range (mid-exponential phase), usually 25 cycles or less, it is possible to establish a direct correlation between the internal control and target mRNA. However, the absolute number of target mRNA molecules in the sample greatly affects the number of cycles needed to detect the product within the linear range.

In order to obtain the suitable condition for RT-PCR, the cycle number should be optimized. PCR cycles for ASF1 and β-actin genes were varied from 20, 24, 28 and 32. This experiment was performed in uninfected (normal) and YHV infected (moribund) shrimp. PCR product of 433 bp of ASF1 and 377 bp of β-actin from these two samples were shown in Figure 31. A 433 bp PCR product of ASF1 was detected after 24 cycles on ward of amplification in both of normal and moribund shrimp. The intensity value of all PCR products were quantified by a densitometer as show in the Table12. The plots of these intensities on the Y-axis versus number of cycle on the X-axis were also show in Figure32. From these results, the optimal cycles where PCR products at the mid-exponential phase of amplification for ASF1 was determined and selected. The suitable cycle number is 26 cycles that will be used for determine the expression level of ASF1 by RT-PCR.

(A) Normal shrimp



(B) Moribund shrimp

Figure 32.1.2% agarose gel showing the PCR products from different numbers of PCR cycles of ASF1 and its internal control β-actin gene within lymphoid organ (LO) of normal (A) and moribund (B) shrimp.

Table 12. The intensity of RT-PCR products amplified from ASF1 and β-actin genes of LO from normal (A) and moribund (B) shrimps when the numbers of PCR cycle were varied from 20-32 cycles.

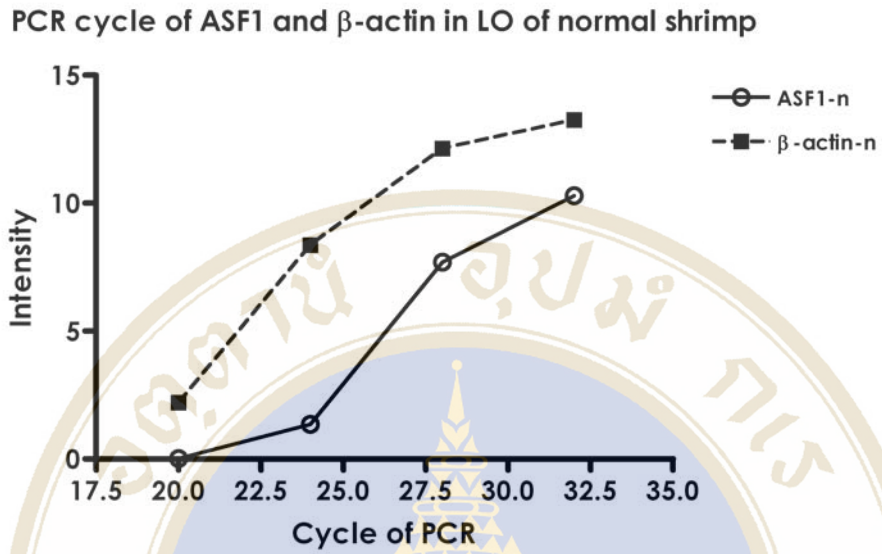
(A)

PCR cycles	Intensity of ASF1	Intensity of β-actin
20	0.02	2.21
24	1.36	8.37
28	7.70	12.15
32	10.29	13.25

(B)

PCR cycles	Intensity of ASF1	Intensity of β-actin
20	0.06	0.66
24	1.03	6.63
28	5.51	11.81
32	8.06	11.37

(A)



(B)

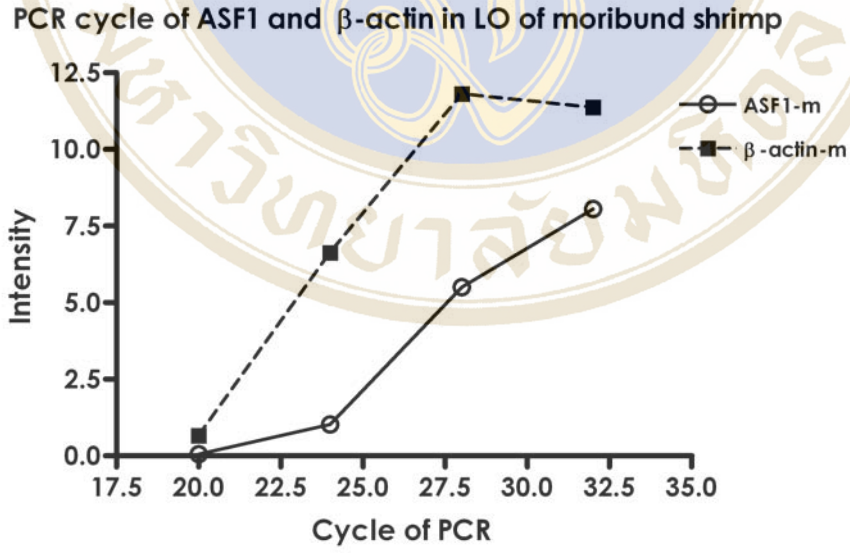


Figure 33. Graphs showing kinetic curves of amplification of ASF1 and β-actin genes in LO of normal (A) and moribund (B) shrimps by PT-PCR analysis at various numbers of cycles.

3.2 Tissue distribution of ASF1 in various organs of shrimp

To investigate whether the gene encoding ASF1 was indeed induced by viral infection, we performed RT-PCR analyses and semi-quantitative assay on total RNA of LO extracts from normal and YHV infected shrimp using specific primers designed from the full-length transcript of shrimp ASF1. RT-PCR of β-actin gene was used as internal control in the semi-quantitative analysis and to ensure that the RT-PCR for each sample contained the same amount of total RNA. Following the optimization of PCR condition of ASF1 and β-actin genes, the suitable cycle number is 26 cycles that used for RT-PCR.

In order to determine the expression of shrimp ASF1 gene in various tissues including gill, heart, hemocyte, hapatopancrease, intestine, pleopod, lymphoid organ and muscle. The ASF1 gene expression was observed in normal and moribund shrimp, these experiment were performed from two independent experiments using two sets of tissue samples from normal and moribund shrimps. The RT-PCR was performed to analyze the ASF1 expression, the PCR product of ASF1 and its internal control, β-actin were then analyzed by 1.2 % agarose gel electrophoresis. The expected size for ASF1 and β-actin amplification fragment was 433 and 377 bp, respectively (Figure 34). The ASF1 expression was found in various tissues of normal and moribund shrimps. Moreover the relative expression of ASF1 gene was compared between normal and moribund samples among various tissues that is shown as the bar graph and calculated for the mean value of two independent experiments (Figure 35). The RT-PCR analysis clearly showned that the ASF1 expression of moribund seem mostly decreased in all tissues but except in hemocyte that ASF1 expression was seem to be slightly increased. Interestingly, the greatly decreased of ASF1 occurred after YHV infection were found in gill, HP and muscle, of these tissue, muscle shown the highest decreasing of ASF1 expression. In addition, the relative expression of shrimp ASF1 has been found to the highest expressed in the muscle and hemocyte of normal and moribund shrimp respectively. The relative intensity of shrimp ASF1 normalized with their internal control β-actin were show in Table13.

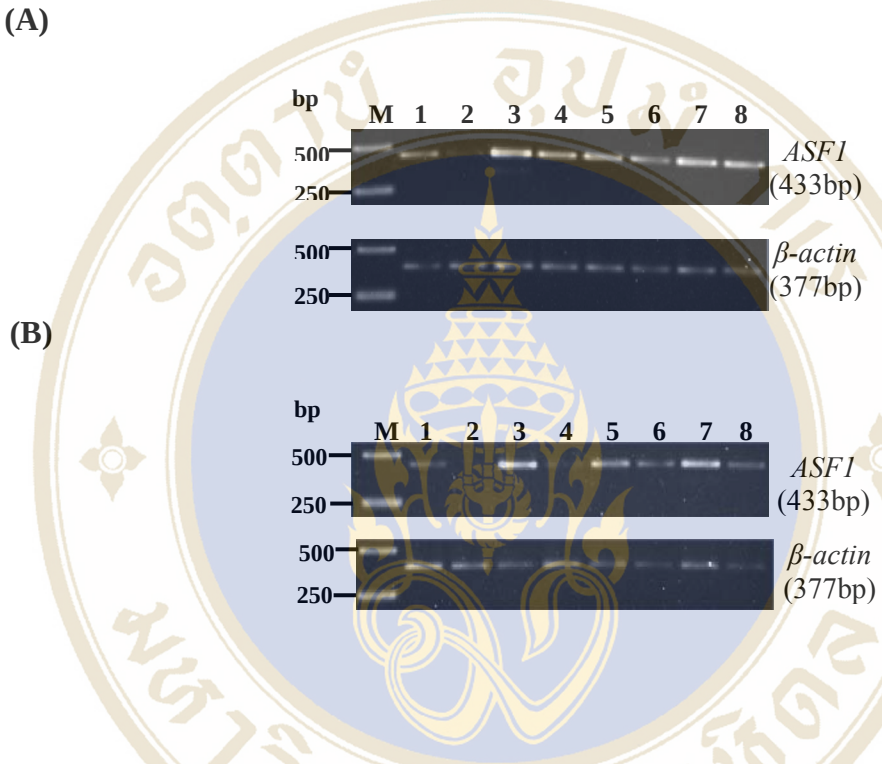


Figure 34. Ttissue distribution of shrimp ASF1 was revealed by 1.2% agarose gel electrophoresis, the RT-PCR analysis of β-actin and ASF1 expression in normal (A) and moribund shrimps (B). The expected size for the actin and ASF1 amplification fragment was 377 and 433 bp, respectively. M;1 kb ladder marker; lane1; gill, lane2; heart (HA), lane3; hemocyte (HC), lane4; hepatopancres, lane5;intestine (Int), lane6; pleopod (Ple), lane7; lymphoid organ (LO), lane8; muscle (Mus).

Table 13. Relative intensity values of RT-PCR products of ASF1 in various organs of normal and infected (moribund) shrimps.

Type of Tissue	The relative intensity of shrimp ASF1	
	Normal	Moribund
Gill	1.98 ± 0.08	0.40 ± 0.04
Heart	0.32 ± 0.11	0.02 ± 0.01
Hemocyte	2.22 ± 0.05	3.45 ± 0.25
Hepatopancreas	2.24 ± 0.17	0.36 ± 0.27
Intestine	2.81 ± 0.93	1.28 ± 0.36
Pleopod	2.43 ± 0.63	1.73 ± 1.14
Lymphoid oragn	3.35 ± 0.47	2.62 ± 0.22
Muscle	3.70 ± 0.22	1.15 ± 0.07

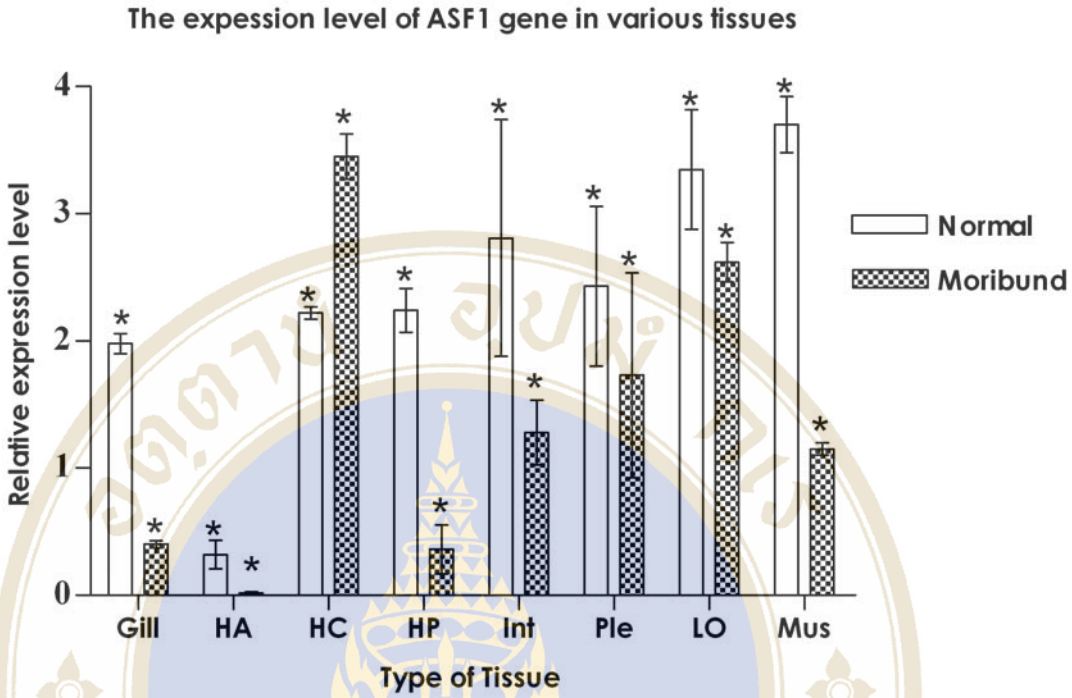


Figure 35. Histograms showing the shrimp ASF1 expression in the other tissues in addition to lymphoid organ. The investigated tissues are gill, heart (HA), hemocyte (HC), hepatopancreas (HP), intestine (Int), pleopod (Ple), lymphoid organ (LO) and muscle (Mus). The relative expression levels of shrimp ASF1 are plotted on the y-axis and various types of tissues are located on the x-axis. These values represent the average of two independent experiments.

CHAPTER V
DISCUSSION

Penaeid shrimp culture is an important economic industry worldwide. However, this industry has suffered many diseases especially from viral origin. Among the shrimp viruses, YHV is currently one of the most important pathogens. It caused massive losses in Thailand from 1991 to 1993. YHV was first characterized in 1993 by Boonyaratpalin *et al*. and Chantanachookin *et al*., then it was first through to be a baculovirus but was subsequently found to be an RNA virus by Wongyeerasupaya *et al*. (1995). Up to date it is classified in the new family Roniviridae (10, 18). The virus was named from the gross signs of disease, which included a yellowish cephalothorax, and very pale overall coloration of moribund infected shrimp. Shrimp, like other invertebrates, lack a true adaptive immune system and rely instead on various innate immune responses against invading pathogens. While the intricate of the shrimp immune system has been revealed with respect to a number of bacteria and fungal pathogens, little is known about the response of shrimp to the viral infection. Additionally, information about genes involved in the antiviral ability of shrimp has been identified such as TCTP (60, 61), interferon-like protein (IntlP), hepatic lectin (61) and PmAV (62).The previous study shown that tissue extracts of blue crab (*Callinectes sapidus*), shrimp (*Penaeus setiferus*), and crayfish (*Procambarus clarkii*) contained antiviral activities that inhibit a variety of DNA and RNA (63). Recently, Robalino *et al.* (64) have demonstrated that injection of dsRNA induced resistance to white spot syndrome virus and Taura syndrome virus in pacific white shrimp, P. vannamei, by nonspecific dsRNA. In blacktiger shrimp, dsRNA administered to the primary lymphoid cell culture (Oka cell) gave protection against YHV infection resembled to RNA interference (RNAi) mechanism in many invertebrate species (65).

The shrimp immune systems mainly occur in hemolymph. Hemocytes are involved in the immediate defensive reactions such as nodulation, encapsulation and phagocytosis (57-59). The circulating cells are also implicated different immune

response such as melanization and coagulation, which is mediated by the release of hemocytic effectors, including the proPO activating system, trans-glutaminase or antimicrobial peptides. In addition, other immune related proteins have been described in shrimp such as a clotting protein, lysozyme, LPS-agglutinin and β-glucan binding protein(20, 26, 34).

The lymphoid organ (LO) of penaeid shrimps is believed to function within the immune system, but its actual role is still debatable. The production of spheroids within the LO has been associated with disease states but again is unknown. Recently, the evidence present that the spheroids are made of accumulations of large granular haemocytes in the haemal sinuses of LO. LO spheroid (LOS) formation has been determined at the onset of experimentally induced chronic Taura syndrome virus (TSV) in *P. vannamei*. These spheroids developed from phagocytes in the tubule walls that ha sequestered the virus (55, 56, 57). The spheriod cell in the LO of *P. monodon* are most likely accumulations of degranulated haemocytes especially large granular cells, contain phagocytosed material and show phenoloxidase and peroxidase activity. So that the spheroids are associated with so many systemic viral infections in penaeid shrimp, it has been suggested that they represent a major mechanism for sequestering virus (55, 56). However, as shown in the present study other, bacterial antigens are also located in the spheroids. Thus the very active spheroid cells might indirectly develop from the circulating haemocytes and contribute to the degranulation of both viral and bacteria material.

To identify the molecules involved in LO response to viral infection, the cDNA library was constructed in order to obtain the gene expression profile in the LO of YHV infected shrimp. From the cDNA library analysis, a total of 1030 randomly selected clones in phagemid form, were single pass sequenced from the 5' end yielding 887 ESTs that were longer than 300 bp after elimination of vector sequence. The average size of the insert was estimated to be 850 bp by PCR amplification of insert from total ESTs sequence. BLASTX analysis was performed characterize the ESTs clones. Comparison of 887 ESTs againsts GenBank database revealed that 452 clones (51.0%) were significantly similar (E-value $<10^{-4}$) to known gene. Of these, 48 clones showed the highest similarities to previously identified shrimp gene (*P. monon, M.ensis and F. chinensis*). The EST sequence were classified into two main groups

first group yielded no hits or had poor similarity (E-value $>10^{-4}$) to genes in the database and in the second group sequences with significant homologue to known gene. In second group, these known sequences were clustered into ten categories including [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, there is a group of other genes which did not fit appropriately into the listed categories and [11] unidentified (hypothetical) similar to other cDNA/DNA. The known sequence mostly found to be hypothetical protein accounting for 32.2%.

Comparisons of cDNA libraries prepared from normal (from black tiger shrimp genome database) and YHV infected *P. monodon* showed the different proportion of the clones in each category (Figure 36). All categories of ESTs sequence were significantly different between the normal and YHV infected shrimp except in the putative genes involved in metabolism seem to be slightly different. The number of ESTs found to code for categories of [1] gene expression, regulation and protein synthesis, [2] internal/external structure and motility, [4] defense and homeostasis, [7] ribosomal protein and rRNA, [8] mitochondrial protein were greatly decreased in the YHV infected library especially there are involved in the defense and homeostasis. The cDNAs encoding lysozyme, serine protease homologue, putative oxidoreductase, chelonianin (serine protease inhibitor) and QM protein were only found in YHV infected library but not the normal library. In the defense and homeostasis category of normal library, those with the greatest number of sequences belonged to anti-lipopolysaccharide (18 clones) were not found in YHV infected library whereas cathepsin B (14 clones) found in normal library was found more often in the YHV infected library. In this group the putative defense gene mostly seem to be down regulation after viral infection such as cathepsin B, TCTP, P109, chaperonin, cyclophilin and selenoprotein. It is remarkable that various types of defense and homeostasis proteins and most of the identified proteins were different between the two libraries.

Gene involved in apoptosis found in our library is QM protein (4 clones) a putative tumor suppressor, has also been shown to associate with apoptosis (30, 67,

68). Therefore, in response to the virus infection, some apoptotic protein may be up-regulated in the virus infected shrimp, leading to an increase of apoptosis. As a result, those initially infected cells may be cleared away by apoptosis (30).

A number of ESTs corresponding to the translational controlled tumor protein (TCTP) was found only one clone in this library. TCTP was initially identified in mouse ascetic tumor and mouse erythro leukemic cell (69). Actually TCTP was not expressed only in tumor cell, it has been found subsequently in a various normal human cells and TCTP homologue have been found in a number of other species, including nematodes, amphibians, plant and yeast (60, 69). A series of recent reports demonstrated the importance of TCTP for cell cycle progression, malignant transformation and anti-apoptotic activity. Moreover the expression level of TCTP were seem to slightly up regulated in early WSSV-infected shrimp whereas in moribund shrimp correlates with the significant down regulation of TCTP (60). From this evidence, TCTP may be the key factor involve with shrimp response to viral infection (60).

Protease and protease inhibitor are important in many extracellular processes in addition to their roles in nutrient digestion. It has become clear that protease and their inhibitors are important in invertebrate anti-infectious response (30, 70). They may be expressed during humoral immune response to inactivate proteases that are produced by invading pathogens. Among arthropod, serine protease and protease inhibitor act as potent activators of diverse immune cascade. In crustaceans, the activity of a series of serine protease activators has been implicated in the activation of the proPO system, leading to melanization of invaders (30, 31, 71). Protease inhibitors are necessary in the regulation of immune cascades, as well as potential inhibiting invader protease. In this library, only one of serine protease was identified and another protease is Cathepsin B (cysteine protease) (30, 72). Its main function is the degradation of proteins that have entered the lysosomal system from outside the cell (via endocytosis or phagocytosis). It has been implicated as a mediator of apoptosis (30). The serine protease belonging to the Kunitz family of inhibitor one with homology to chelonianin from the sea turtle (30, 74).

The lysozyme also found in this library, this molecule is widely distributed among eukaryotes and prokaryotes. It catalyzes the hydrolysis of bacterial cell walls and act

as a non-specific innate immunity molecule against the invasion of pathogens. Lysozyme is commonly included in the family of the anti-bacterial peptides based on molecular weight and it bacteriolytic effect. This molecule has been found in the other shrimp species including *L. vannamei, L. setiferus and M. japonicas* (30, 44, 73, 75).

The metal binding proteins were found in this library, as metallothioneines (2 clones). These molecules are scavengers of reactive oxygen intermediates and are up-regulated during immune response in vertebrates as well as in invertebrates.

A shrimp cyclophilin was also identified. Cyclophilins have diverse regulatory functions in mammalian cells, but it is interesting to note that they can be involved in viral attachment to the cells and in the stress response to oxygen depletion (30, 77, 78). Moreover, cyclophilin A (CypA) has been found to interact with apoptosis inducing factor (AIF) these complex capable chromatinolysis or DNA degradation that involved in apoptosis (79).

The other putative gene that might be participates in immune response there are selenoproteins (80). Many of the selenoproteins have been identified and characterized in vertebrates, they have function as anti-oxidant or cellular redox functions. These molecules include the glutathione peroxidase. Possible anti-oxidant roles have been suggested for selenoprotein P and W that are likely to be found for newly identified selenoprotein (80). The selonoprotein T has been found in this library, about its function was not identified.

For the gene that responsible for stress, a homologue for the stress related heat shock protein, chaperonin (HSP60) was identified. It serves as a cellular protective role that function to facilities the mature structural conformation of proteins, preventing denaturation of protein under stress conditions and renature denatured proteins. Level of HSP60 can increase 3-5 folds in response to cell stress (30, 81, 82). It is a constitutive protein found within the mitochondrial matrix, although 15-20% of the protein has also been detected in the cytosol. HSP60 participates in the folding of mitochondrial proteins and facilities the proteolytic degradation of misfolded or denatured in an ATP dependent manner (81, 82). The chaperone function of HSP60 is regulated by HSP10, which binds to HSP60 and regulates substrate binding and ATPase activity. A pro-apoptotic role for HSP60 and HSP10 has been demonstrated that associate with procaspase-3 and stimulate pro-enzyme maturation. In contrast, the

cytosolic HSP60 was described an anti-apoptotic function it was suggested that HSP60 could bind the Bcl-2-related protein Bax and Bak, thereby inhibiting their mitochondria translocation (81-83). In addition to their protective roles, HSP60 are also know to support viral protein folding during viral replication and have hypothesized role in mediating virulence in some viruses (30).

The ASF1 gene was originally identified in yeast by its ability, when overexpressed to repress silencing at the *HMR* and *HML* mating-type loci and at telomere (84, 85).

The full-length cDNA of shrimp ASF1 was identified by comparing to the previously characterized. Shrimp ASF1 was shown the highest similarity with the *Drososphila* ASF1. In addition, the first 155 amino acid are highly conserved among eukaryotic species (65-90% identities) and have predicted secondary structure elements (85, 93). The C-terminal region (<15% identity) is poorly conserved and is predicted to consist largely of disordered coils (85). They suggested that a minimal core region comprising the conserved N-terminal 155 amino acids might be biologically functional (85). The function of ASF1 has come largely from studied in yeast. Showing that *Drosophila* ASF1 and the two human ASF1 proteins can function in yeast demonstrates that the function of ASF1 is highly similar in higher eukaryotes and yeast (85,86). Interestingly, it has also been shown that loss of function mutations in yeast ASF1 gene derepress transcription from silenced loci, when combined with mutations in the largest subunit of yeast CAF-1 complex. Because of this, the role of ASF1 in silencing is thought to be in the assembly of silenced chromatin (89). Chromatin assembly has been studied almost exclusively using biochemical approaches (87, 88). In this manner, ASF1 was found biochemically as a protein that facilitates CAF-1 mediated assembly of newly replicated DNA into chromatin in *vitro* (86, 94). ASF1 and CAF-1 are highly conserved among eukaryotes, and their inactivation leads to broad range of cellular defects (87, 89, 93). The molecular basis of which is unclear (88). ASF1 also has functions that are independent of CAF-1. In the absence of CAF-1 has no preference for assembling newly replicated DNA into chromatin, being fully able to assemble non replicating DNA (86, 88). In yeast, ASF1 has been additional CAF-1 independent role in the cell during the disassembly of chromatin during transcriptional activation of the *PHO5* and *PHO8* gene (92, 95). As

such, detect in chromatin assembly or chromatin disassembly should influence gene expression (88). ASF1 is required for chromatin disassembly during activation of the *PHO5* gene and that this nucleosome disassembly is essential for transcriptional activation. Chromatin disassembly is essential for transcriptional activation because *PHO5* cannot be activated in yeast lacking ASF1 (95). ASF1 is not formally a transcription factor, so it does not posses sequence specific DNA binding properties, but rather it is likely to influence chromatin structure of the promoter and open reading frame of its transcriptional targets, as was shown recently at the *PHO5* and *PHO8* genes (89). The possible function of ASF1 in gene expression via a role in assembly and disassembly chromatin in a dynamic and gene specific manner during transcriptional regulation (89, 90).

In this study, RT-PCR analysis of ASF1 mRNA in normal and moribund shrimp using ASF1 specific primers showed that expression is widespread except in the heart of moribund shrimp. From this result suggest that ASF1 gene was not restricted to the LO. The ASF1 expression was found to be decreased when shrimp showed the mortality characteristic in all investigated tissue examined where it was seems to be slightly up-regulated in hemocyte while it seem to be strongly down-regulated in muscle, hepatopancreas and gill. These three tissues showed the lower ASF1 expression than LO. From the previous study these three organs had been found as the primary site of YHV infection in addition to LO and hemocyte (17). Histopathological examinations showed different degrees of cellular apoptosis in many organs and tissues including gill, midgut, connective tissues, hemocytes, hematopoietic tissues, heart, hepatopancreas and LO (17). This study revealed the presence of the infectious YHV in a wide variety of tissues and organs examined. The present study shown the results of ASF1 expression was downregulated in various tissues suggest that shrimp it might be affected on transcriptional regulation of host cell resulting in change the gene expression profile during viral infection. It is possible that the defect of shrimp ASF1 gene exhibits transcriptional inactivation of several genes that essential to maintain the biological activity of the cell. It is widely accepted that changes in chromatin structure can be regulate gene expression (89, 95). As such, defects in chromatin assembly or chromatin disassembly should influence gene expression. This dynamic disassembly

and reassembly of chromatin may be widely employed to enable the rapid and tight control of eukaryotic gene expression (95).

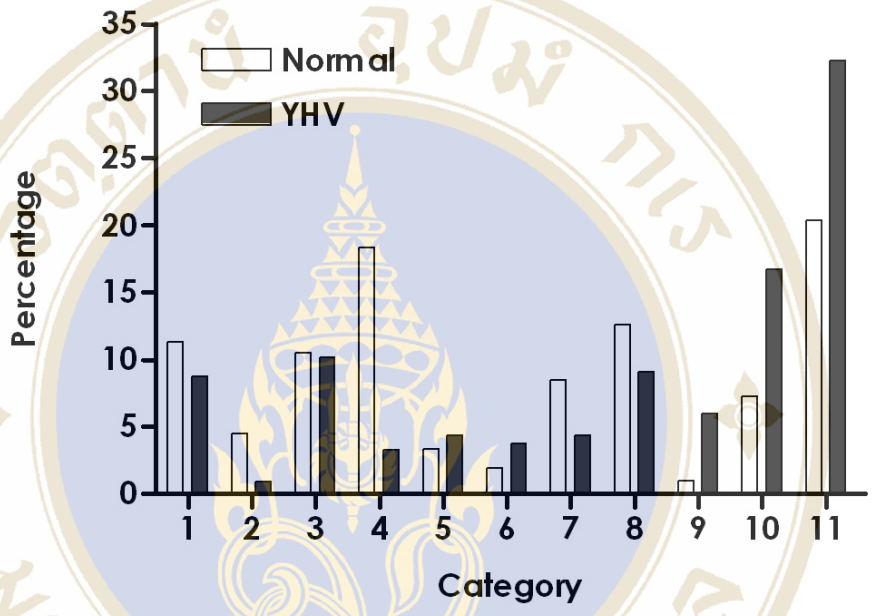


Figure 36. Comparison the distribution of putative gene in each functional category between normal YHV infected shrimp lymphoid organ libraries. [1] gene expression, regulation and protein synthesis, [2] internal/external structure and motility, [3] metabolism, [4] defense and homeostasis, [5] signaling and communication, [6] cell division/DNA synthesis, repair and replication, [7] ribosomal protein and rRNA, [8] mitochondrial protein, [9] transport, [10] miscellaneous function, there is a group of other genes which did not fit appropriately into the listed categories and [11] unidentified (hypothetical) similar to other cDNA/DNA.

CHAPTER VI
CONCLUSION

1. Homology search of the 887 cDNA sequences against GenBank database using BLASTX program revealed 452 clones (51.0%) were significant matched with the sequences in database while the 435 (49.0%) clones were not identical with the deposited sequences.
2. The known sequences (452 clones) were classified according to their functions. These sequences were mostly found as hypothetical protein (32.2%). The putative defense and homeostasis proteins accounted for 3.3%. The serine protease homologue was identified in this group, it participates in proPO system.
3. The other immune effectors of shrimp such as lipopolysaccharide binding protein, β-1,3-glucan binding protein and anti-microbial peptides had not identified in this library. However, the putative protein that play role in apoptosis pathway is cyclophilin which is possibility involved in viral infection.
4. Identification and characterization of the full-length cDNA of shrimp ASF1 using 5' RACE technique and bioinformatics revealed it showed the highest sequence portion of the protein identity and similarity to fruit fly ASF1. It also exhibited highly conserved especially at the N-terminal region through various organisms.
5. Shrimp ASF1 was expressed in all tissue examined but being the highest in muscle and hemocyte from normal and moribund samples respectively. Down regulation of shrimp ASF1 has been found in several tissues and organs upon YHV infection except in the hemocyte when the level of ASF1 was increased.
6. ASF1 expression in moribund shrimp was significantly decreased in muscle, gill and hepatopancreas. Of these, muscle showed the highest reduction of ASF1 followed by gill and hepatopancreas.

7. Previous study demonstrated that the different degree of cellular apoptosis occur various tissue of infected shrimp together these result suggest that loss of ASF1 may be affected transcriptional regulation of several genes in the host cell.

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