การระบุและลักษณะสมบัติของยีนชนิดใหม่ที่ตอบสนองต่อไวรัสในกุ้งกุลาดำ

Penaeus monodon

นายอดิศักดิ์ ประภาวรรัตน์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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IDENTIFICATION AND CHARACTERIZATION OF NOVEL VIRUS-RESPONSIVE GENES IN THE BLACK TIGER SHRIMP *Penaeus monodon*

Mr. Adisak Prapavorarat

A Dissertation Submitted in Partial Fulfillment of the Requirements
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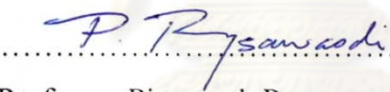
Thesis Title	IDENTIFICATION AND CHARACTERIZATION OF NOVEL VIRUS-RESPONSIVE GENES IN THE BLACK TIGER SHRIMP *Penaeus monodon*
By	Mr. Adisak Prapavorarat
Field of study	Biochemistey
Thesis Advisor	Professor Anchalee Tassanakajon, Ph.D.
Thesis Co-advisor	Professor Kenneth Söderhäll, Ph.D.

Accepted by the Faculty of Science, Chulalongkorn University in Partial Fulfillment of the Requirements for the Doctoral Degree

..... Dean of the Faculty of Science
(Professor Supot Hannongbua, Dr.rer.nat.)

THESIS COMMITTEE



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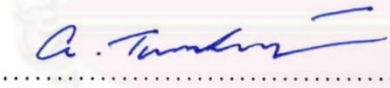

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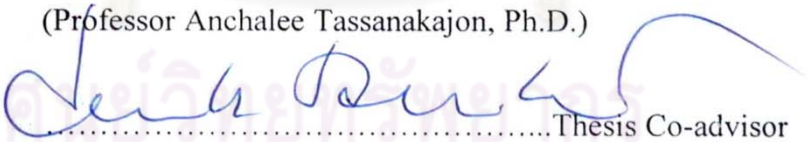

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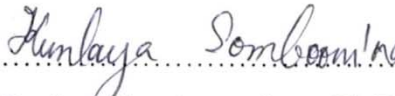

.....Examiner
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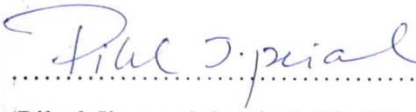

.....External Examiner
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อดิศักดิ์ ประภาวรรัตน์: การบ่งชี้และลักษณะสมบัติของยีนชนิดใหม่ที่ตอบสนองต่อไวรัสในกุ้งกุลาดำ *Penaeus monodon* (IDENTIFICATION AND CHARACTERIZATION OF NOVEL VIRUS-RESPONSIVE GENES IN THE BLACK TIGER SHRIMP *Penaeus monodon*) อ. ที่ปรึกษาวิทยานิพนธ์หลัก: ศ.ดร. อัญชลี ทัศนาขจร, อ. ที่ปรึกษาวิทยานิพนธ์ร่วม: Prof. Kenneth Söderhäll, Ph.D., 100 หน้า.

ไวรัสโรคตัวแดงดวงขาว และไวรัสโรคหัวเหลืองนับว่าเป็นเชื้อโรคหลักที่ก่อให้เกิดความเสียหายต่ออุตสาหกรรมการเพาะเลี้ยงกุ้งเป็นอย่างมาก ในการศึกษานี้ได้ใช้เทคนิค suppression subtractive hybridization (SSH) ในการระบุหายีนที่มีการแสดงออกเพิ่มขึ้นในเซลล์เม็ดเลือดของกุ้งกุลาดำ *Penaeus monodon* ที่ติดเชื้อไวรัสโรคตัวแดงดวงขาว และไวรัสโรคหัวเหลืองในระยะต้น (24 ชม.)และปลาย (48 และ 72 ชม.) ของการติดเชื้อ ยีนจำนวนมากถูกค้นพบจากห้องสมุด SSH ซึ่งส่วนหนึ่งมีหน้าที่เกี่ยวข้องกับกลไกการต้านไวรัสรวมถึงกลไกการติดเชื้อไวรัส ในจำนวนนั้นเราได้ค้นพบยีน/โปรตีนชนิดใหม่ 2 ชนิดเป็นครั้งแรก ชื่อว่า hemocyte homeostasis-associated protein (*Pm*HHAP) และ viral responsive protein 15 kDa (VRP15) ซึ่ง *Pm*HHAP และ VRP15 ได้ถูกนำมาศึกษาต่อ เพื่อหาหน้าที่ในการตอบสนองต่อเชื้อไวรัสในกุ้งกุลาดำ เมื่อทำการเปรียบเทียบลำดับนิวคลีโอไทด์ของ *Pm*HHAP ในฐานข้อมูล GenBank พบว่ามีความเหมือนกับ hypothetical proteins ในสิ่งมีชีวิตหลายๆชนิด โดยมีความเหมือนสูงสุด (54%) กับ hypothetical protein TcasGA2_TC006773 จากมอดแป้ง *Tribolium castaneum* ในงานนี้พบว่า *Pm*HHAP มีการแสดงออกในหลากหลายเนื้อเยื่อของกุ้ง โดยแสดงออกสูงสุดในเนื้อเยื่อสร้างเซลล์เม็ดเลือด และมีการแสดงออกที่เพิ่มขึ้นทั้งในระดับ mRNA และโปรตีนในเซลล์เม็ดเลือดกุ้งกุลาดำที่ติดเชื้อไวรัสตัวแดงดวงขาว การยับยั้งการแสดงออกของยีน *Pm*HHAP โดยเทคนิค RNA interference (RNAi) ส่งผลให้ปริมาณเซลล์เม็ดเลือดในกุ้งลดลงอย่างมาก และทำให้กุ้งตาย 100 % ภายในเวลาเพียง 30 ชั่วโมงหลังฉีด *Pm*HHAP dsRNA โดยผลดังกล่าวไม่เกิดในกุ้งกลุ่มควบคุม จึงกล่าวได้ว่า *Pm*HHAP มีความสำคัญอย่างยิ่งสำหรับการดำรงชีวิตของกุ้ง นอกจากนั้นยังพบว่าการยับยั้งการแสดงออกของยีน *Pm*HHAP ทำให้เซลล์เม็ดเลือดเกิดความเสียหายอย่างมากในระบบหมุนเวียนเลือดของกุ้ง (*in vivo*) รวมถึงใน primary hemocyte cell culture (*in vitro*) ผลการทดลองนี้บ่งชี้ว่า *Pm*HHAP มีความสำคัญต่อการรักษาสภาพ และสมดุลของเซลล์เม็ดเลือดกุ้ง ดั้งนั้นจึงอาจกล่าวได้ว่าการที่ *Pm*HHAP มีการแสดงออกเพิ่มขึ้นเพื่อที่จะควบคุมระดับสมดุลของเซลล์เม็ดเลือดขณะที่กุ้งติดเชื้อไวรัส สำหรับการศึกษา VRP15 พบว่าเมื่อทำการเปรียบเทียบลำดับนิวคลีโอไทด์กับข้อมูลในฐานข้อมูล GenBank ไม่พบยีนใดในฐานข้อมูลที่มีความเหมือนอย่างมีนัยสำคัญ (E-values > $1x10^{-4}$) จากการทำนายส่วนประกอบโครงสร้างของโปรตีนนี้ พบว่า VRP15 ประกอบด้วย transmembrane helix 1 ช่วงโดย VRP15 มีการแสดงออกมากที่สุดในเซลล์เม็ดเลือด และจะมีการแสดงออกเพิ่มขึ้นอย่างมากในเซลล์เม็ดเลือดกุ้งที่ติดเชื้อไวรัสตัวแดงดวงขาว นอกจากนั้นการยับยั้งการแสดงออกของยีน VRP15 ทำให้อัตราการเพิ่มจำนวนของไวรัสโรคตัวแดงดวงขาวในกุ้งลดลงอย่างมาก ส่งผลให้การติดเชื้อไวรัสในกุ้งกุลาดำลดต่ำลง

สาขาวิชา .....ชีวเคมี.....	ลายมือชื่อนิสิต .....
สาขาวิชา .....ชีวเคมี.....	ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์หลัก .....
ปีการศึกษา .....2553.....	ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์ร่วม .....

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KEYWORDS: VIRAL RESPONSIVE GENE/ INNATE IMMUNITY/ VIRAL INFECTION MECHANISM/ *Penaeus monodon*

ADISAK PRAPAVORARAT: IDENTIFICATION AND CHARACTERIZATION OF NOVEL VIRUS-RESPONSIVE GENES IN THE BLACK TIGER SHRIMP *Penaeus monodon.* THESIS ADVISOR: PROF. ANCHALEE TASSANAKAJON, Ph.D., THESIS CO-ADVISOR: PROF. KENNETH SÖDERHÄLL, Ph.D., 100 pp.

White spot syndrome virus (WSSV) and yellow head virus (YHV) are major viral pathogens for various crustaceans which cause severe losses to the shrimp aquaculture. Here, suppression subtractive hybridization (SSH) was employed to identify genes up-regulated in the hemocytes of WSSV- and YHV-infected *Penaeus monodon* at the early (24 h) and late phases (48 and 72 h) of the viral infections. A number of novel viral responsive genes regarding antiviral immunity and/or viral infection mechanism were uncovered. Among the viral responsive genes obtained from the SSH libraries, two novel genes/proteins first identified and named here, hemocyte homeostasis-associated protein (*Pm*HHAP) and viral responsive protein 15 kDa (VRP15), showed to be greatly abundant in the SSH libraries. Hence, *Pm*HHAP and VRP15 were further characterized for their roles in the shrimp's response to viral infection. The full-length cDNA of *Pm*HHAP showed high sequence identity to hypothetical proteins from various organisms, with the highest identity (54%) to the hypothetical protein TcasGA2_TC006773 from the red flour beetle, *Tribolium castaneum*. Transcripts of *Pm*HHAP were expressed in various shrimp tissues with the highest expression in hematopoietic tissue. Upon WSSV infection, a high up-regulation level of shrimp hemocytic HHAP mRNA and protein was observed. Gene silencing of *Pm*HHAP by RNA interference (RNAi) resulted in a significant decrease in the number of circulating hemocytes and 100% shrimp mortality within 30 h of the double-stranded *Pm*HHAP RNA injection (but not in control shrimp), indicating that *Pm*HHAP is essential for shrimp survival. Interestingly, severe damage of hemocytes was observed *in vivo* in the *Pm*HHAP knockdown shrimp and *in vitro* in shrimp primary hemocyte cell culture, suggesting that *Pm*HHAP plays an important role in hemocyte homeostasis. Thus, it is speculated that the upregulation of *Pm*HHAP is an important mechanism to control circulating hemocyte levels in crustaceans during viral infection. The full sequence of VRP15 was obtained and showed no significant similarity (*E*-values > $1x10^{-4}$) to any known gene in the GenBank database. A protein domain prediction indicated that its protein structure consisted of a transmembrane helix. VRP15 transcript was mainly expressed in the hemocytes of shrimp, and found to be greatly up-regulated post WSSV-infection. Importantly, the significant reduction of WSSV replication was observed in the VRP15-RNAi knockdown shrimp, indicating that the novel VRP15 is important for viral propagation as its knock-down leads to decrease in viral infection in *P. monodon*.

Department: .....Biochemistry..... Student's signature .....
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LIST OF ABBREVIATIONS

bp	Base pair
Da	Dalton
DNA	Deoxyribonucleic acid
dsRNA	Double-stranded RNA
EST	Expressed sequence tag
f	Femto
FBS	Fetal bovine serum
g	Gram
GFP	Green fluorescence protein
h	Hour, Hours
HHAP	Hemocyte homeostasis-associated protein
K	Kilo
l	Liter
LB	Luria-Bertani
LHM	Lobster hemolymph medium
m	Milli
M	Molar
min	Minute, Minutes
PAGE	polyacrylamide electrophoresis
PCR	Polymerase chain reaction
PBS	1x phosphate buffered saline
Pl	*Pacifastacus leniusculus*
Pm	*Penaeus monodon*
RACE	Rapid amplification of cDNA end
rHHAP	Recombinant HHAP
RNA	Ribonucleic acid
RNAi	RNA interference
RT	Reverse transcription
SD	Standard deviation
sec	Seconds

SPF	Specific pathogen free
SPI	Serine proteinase inhibitor
SSH	Suppression subtractive hybridization
ssRNA	Single-stranded RNA
THC	Total hemocyte number
µ	Micro
UV	Ultraviolet
VRP15	Viral responsive protein 15 kDa
WSSV	White spot syndrome virus.
YHV	Yellow head virus

CHAPTER I
INTRODUCTION

1.1 History of the shrimp culture industry

Shrimp farming is a multi-billion dollar industry contributing a major income to several countries in Asia and South America. Thailand has been the world's leading exporter of cultured shrimp since 1992, and used to be the largest producer of the black tiger shrimp supplying 20 percent of the world trade in shrimp and prawn (Wyban, 2007). The rapid growth of shrimp farming led to an economic boom and also enhanced employment distribution in the country. In the past, the most important cultured shrimp in Thailand is the black tiger shrimp, *Penaeus monodon.* The black tiger shrimp farms and hatcheries are dispersed along the coastal areas. Southern provinces such as Nakon Sri Thammarat and Surat Thani yield the majority of harvests, whereas eastern and central provinces such as Samut Sakhon and Samut Songkhram yield the minority in terms of number. Unfortunately, during the decade, the production of the black tiger shrimp has rapidly been decreased due to the main problems including the outbreaks of bacterial and viral diseases as well as the lack of high-quality shrimp broodstock (Mohan, 1998). Accordingly, *P. monodon* culture has latterly been replaced with the white shrimp, *Litopenaeus vannamei* since *L. vannamei* has several advantages over the *P. monodon*, including the rapid growth rate, availability of specific pathogen free stocks, disease resistance and high survival rate during larval rearing. However, there are disadvantages of *L. vannamei* is that its ability can act as a carrier of various viral pathogens such as *baculovirus penaei* and Taura syndrome virus, and these viruses can be transmitted to the native penaeid shrimp, *P. monodon*. Moreover, *L. vannamei* is a non-native species, and its broodstock has to be imported mainly from aboard that possibly acts as a carrier of various new pathogens to the culture areas. Consequently, to solve the disease outbreak problems and maintain the production of *P. monodon*, the efficient domestication, great farm management, genetic improvement and the invention of the effective disease control measures are urgently required.

1.2 Taxonomy of the black tiger shrimp, *Penaeus monodon*

Penaeid shrimp are classified into the largest phylum in the animal kingdom, the Arthropoda. This group of animal is characterized by the presence of pair appendages and a protective cuticle or exoskeleton that covers the whole animal. The subphylum Crustacea is made up of 42,000, predominantly aquatic species, which belong to the 10 classes. Within the class Malacostraca; shrimp, crayfish, lobster and crab belong to the order Decapoda. The taxonomic definition of the black tiger shrimp, *P. monodon* is as follows (Bailey-Brock and Moss, 1992):

P**hylum** Arthropoda
Subphylum Crustacea
Class Malacostraca
Subclass Eumalacostraca
Order Decapoda
Suborder Natantia
Infraorder Penaeidea
Superfamily Penaeoidea
Family Penaeidae Rafinesque, 1985
Genus Penaeus Fabricius, 1798
Subgenus Penaeus
Species monodon

Scientific name: *Penaeus monodon*

Common name: Jumbo tiger prawn, Giant tiger prawn, Blue tiger prawn, Leader prawn, Panda prawn (Australia), Jar-Pazun (Burma), Bangkear (Cambodia), Ghost prawn (Hong Kong), Jinga (India, Bombay region), Udang windu(Indonesia), Ushi-ebi (Japan), Kamba ndogo (Kenya), Kalri (Pakistan), Sugpo (Phillipines), Grass shrimp (Taiwan), Kung kula-dum (Thailand), Tim sa (Vietnam).

F.A.O. Names: Giant tiger prawn, Crevette giante tigre, Camaron tigre gigante.

1.3 Major viral diseases in shrimp

The infectious diseases, especially viral diseases, become serious problems in shrimp industry worldwide. The shrimp farming industry in Thailand encountered a severe problem from uncontrollable viral diseases for over a decade. White spot syndrome virus (WSSV) and yellow head virus (YHV) are two of the major viral pathogens of the shrimp which cause white spot syndrome and yellow head disease, respectively, leading to rapid mass mortality of the infected shrimp within few weeks, whilst up to now the effective approach for preventing the viral infection has not yet been established. Thus, the outbreaks of these viral diseases still cause great economic risks as well as severe losses of the shrimp production in several agricultural countries including Thailand. The details of the viral diseases are mentions as follows.

1.3.1 White spot syndrome

White spot syndrome is a viral disease resulting from WSSV infection, affecting most of the commercially globally cultivated marine shrimp species. The origin of white spot syndrome outbreak began in Taiwan shrimp farms in 1992 and rapidly spread throughout Asia, subsequently, the disease crossed over the Pacific Ocean and spread in North, Central and South America, creating by far the greatest economic damage (Chou et al., 1995; Lightner, 1996; Flegel, 1997; Lotz, 1997; Span and Lester, 1997). WSSV is a major shrimp pathogen causing the onset of a rapid and mass mortality within 2 - 7 days post-infection (Chou et al., 1995, Flegel, 2006). A clinical sign of white spot syndrome typically is the development of many white spots on the carapace of the infected shrimp which results from white calcium accumulated in shell (Chou et al., 1995). However, the disease can occur without the presence of white spots. Other signs of the WSSV infection in shrimp also include the body surface and appendages turning to red or pink, loosing shell, lower food consumption and show lethargic behavior.

Initially, this virus was called white spot syndrome baculovirus because its morphological characteristic was similar to the insect baculovirus (Lightner, 1996). However, phylogenetic analysis of ribonucleotide reductase and protein kinase gene revealed that WSSV did not share a common ancestor with baculovirus (Van Hulten et al., 2000, 2001). WSSV is the type specie of the genus *Whispovirus* in the viral

family *Nimaviridae*, containing a circular double-stranded DNA of about 305 kb, and is an enveloped rod-shaped particle with a single filamentous appendage-like tail at one end of the nucleocapsid (Vlak et al. 2004, Yang et al., 2001). The average size of the virus is about 298 ± 21 nm long and 107 ± 8 nm in diameter (Wang et al., 1997). WSSV has a broad host range such as shrimp, crab, crayfish and lobsters (Lo et al., 1996), and can infect various tissues including antennal grand, cuticular epidermis, gill, muscle, lymphoid organ, nervous tissue, hematopoietic tissue, connective tissues of some organs (Chang et al., 1996, Wang et al., 1997).

The WSSV infection can be detected by several diagnostic methods such as PCR, *in situ* hybridization, mini-array, observation of tissues subjected to staining, and immunological methods using specific antibodies to WSSV proteins (Okumura et al., 2005)

1.3.2 Yellow head disease

Yellow head disease resulting from YHV infection, one of the major shrimp pathogens, seriously affects the shrimp industry, causing mass mortality in shrimp farming in Asia. In Thailand, the disease was first reported in 1990. Yellow head disease occurs in the juvenile to sub-adult stages of shrimp, especially at 50 - 70 days of grow-out (Lightner, 1996). The initial clinical signs of yellow head disease typically are the development of a yellowish cephalothorax and brown gills in the infected shrimp, but critically the infection proceeds to 100% fatality within 3 - 9 days post-infection (Boonyaratpalin et al., 1993; Chantanachookin et al., 1993; Lu et al., 1995), leading to dramatic shrimp stock losses. The YHV-infected shrimp often exhibits light yellow coloration of the dorsal cephalothorax area and has a generally pale or bleached appearance (Limsuwan, 1991). At the onset of yellow head disease, food consumption of shrimp is at an abnormally high rate for several days, and then abruptly ceases feeding. The moribund shrimp appear to swim slowly near the surface at the pond edge.

YHV is an invertebrate nidovirus containing single-stranded RNA of about 22 kb, and is an enveloped rod-shaped particle of approximately 195 ± 5 nm long and 55 ± 5 nm in diameter (Sittidilokratna et al., 2002), and is primarily localized in the cytoplasm of the infected cells (Cowley et al., 1999). The target tissues of YHV are

diverse such as the lymphoid organs, gills, nerve cord, heart, midgut, hepatopancreas, head soft tissues, abdominal muscle, eyestalks and hematopoietic tissue (Boonyaratpalin et al., 1993; Chantanachookin et al., 1993; Lu et al., 1995). YHV may occur as latent and asymptomatic infections in broodstock shrimp and possibly transfer from the parental shrimp to their offspring (Chantanachookin et al., 1993).

The YHV infection diagnosis could be performed by using several methods such as RT-PCR, *in situ* hybridization, and immunological methods using specific antibodies to a surface glycoprotein and the nucleocapsid proteins of the virus. (Sanchez-Barajas et al., 2009; Wongteerasupaya et al., 1995; Tang and Lightner, 1999)

1.4 The immune responses in crustacean

Invertebrates, including crustaceans, rely on an effective innate immune system to fight against invading pathogens, which comprises cellular and humoral immune responses. The cellular responses, the actions with direct participation of blood cells, include phagocytosis, nodule formation and encapsulation, whilst the humoral responses involve the prophenoloxidase (proPO)-activating system, the clotting cascade and activity of immune-related proteins. The humoral factors act in the defense without direct involvement of the cells even though many of the factors are originally synthesized and stored in the blood cells. These factors include enzyme and proteins involved in proPO system, clotting proteins, agglutinins, hydrolytic enzymes, proteases, proteinase inhibitors, and antimicrobial peptides (Söderhäll, 1999; Hoffmann et al., 1999; Iwanaga and Lee, 2005). Moreover, the hard cuticle covering all external surfaces of crustaceans is the first line of defense between them and the environment.

Major defense systems are carried out in the hemolymph containing cells called hemocytes. The hemocyte is a major immune-responsive cell in crustaceans since it produces many immune effectors, and also participates in a number of immune activities. Crustacean hemocytes are generally classified into three types; hyaline (agranular), semigranular (small granular) and granular (large granular) hemocytes (Tsing et al., 1989; Bauchau, 1980). It is believed that the hyaline hemocyte is associated with phagocytosis (Smith and Söderhäll, 1983, 1983), whilst

the granular cells principally function in apoptosis, melanization, encapsulation and nodulation (Sung et al., 1998; Pech and Strand, 2000; Kobayashi et al., 1990).

So far, there are several research reports on the viral defense mechanisms in crustaceans (Liu et al., 2009). Some potential proteins have been identified as likely to be involved in the antiviral mechanism in shrimp, including caspase (Wang et al., 2008), hemocyanin (Zhang et al., 2004), Pm-fortilin (Tonganunt et al., 2008), Fclectin (Liu, et al., 2007), LvCTL1 (Zhao et al., 1999), PmAV (Lou et al., 2003) and PmLT (Ma et al., 2008). Nevertheless, the information concerning viral infection and antiviral mechanisms in crustaceans is still mostly unknown.

1.5 Pattern recognition proteins

When foreign substances attack animals, the first immune process is recognition of a broad spectrum of factors that present on the surface of invading microorganisms. This process is mediated by hemocytes and plasmatic proteins. There is little knowledge about the molecular mechanisms that mediate the recognition; however, in crustaceans, several types of modulator proteins recognizing cell surface components of pathogens have been identified. The target recognition of innate immunity, so-called “pattern recognition molecules (PRMs)”, is shared among groups of pathogens. Host organisms have developed the response to these PRMs by a set of receptors referred to as “pattern recognition proteins or receptors (PRPs or PRRs)”. These patterns include the lipopolysaccharides (LPS) of gram-negative bacteria, the glycolipids of mycobacteria, the lipoteichoic acids of gram-positive bacteria, the mannans of yeasts, the β-1,3-glucan of fungi, and double-stranded RNA of viruses (Hoffmann et al., 1999). Most current researches have emphasized the possible roles of non-self recognition molecules in the vertebrate and the invertebrate immune system.

Carbohydrate recognition is important for the immunity because carbohydrates are common constituents of microbial cell wall, and microbial carbohydrates have distinct structures from the carbohydrates of eukaryotic cells. Therefore, LPS or/and β-1,3-glucan-binding proteins (LBP, βGBP, or LGBP), peptidoglycan recognition protein (PGRP), several kinds of lectins, and hemolin have been identified in a variety of invertebrates and show different biological functions after binding to their targets

(Lee and Söderhäll, 2002). In shrimp, the LPS-binding protein has been reported as a multivalent carbohydrate-binding agglutinin, besides its bacterial agglutination ability and phagocytic induction (Vargas-Albores, 1995). A second protein involved in the recognition of microbial products and the activation of cellular functions is the β-glucan binding protein. It is apparently monovalent and does not induce agglutination, but activates degranulation as well as the proPO system. Thus, these recognition proteins are capable of activating cellular activities after reacting with the microbial carbohydrates (LPS, peptidoglycan or glucan) (Vargas-Albores and Yepiz-Plascencia, 2000).

Lectins are sugar-binding proteins that agglutinate cells and/or precipitate glycoconjugate molecules with a carbohydrate portion such as polysaccharide, glycoproteins, glycolipids, and the others. There are many different lectins, including tachylectins from hemolymph plasma of the horseshoe crab, *Tachypleus tridentatus* (Gokudan et al., 1999). They are involved in a variety of processes, including the innate immune response, and are critical for the detection and elimination of infectious microorganisms (Weis et al., 1998; Kilpatrick, 2000). Generally, they recognize sugar or carbohydrate structures on the surfaces of pathogens that do not present on host cell. It has been reported that lectins have an LPS-binding property (Koizumi et al., 1999; Jomori and Natori., 1992). These LPS-binding proteins have biological functions of a bacterial clearance activity and an opsonic effect. Lectins are responsible for promoting phagocytosis and stimulating the proPO system (Yu et al., 1999; Yu and Kanost, 2000). Tachylectins were found to have hemagglutinating and antibacterial activities which are important in the immune system (Kawabata and Iwanaga, 1999). So far, C-type lectins from shrimp have been reported to be involved in antibacterial, antifungal and antiviral processes. For instance, FC-hsl and LvLec displayed immune activities against some bacteria and fungi (Sun et al., 2008; Zhang et al., 2009), whilst Fclectin showed up-regulated expression levels after challenge with bacteria, lipopolysaccharide or WSSV (Liu et al., 2007). Likewise, LvCTL1 and PmAV exhibited antiviral activity against infection with WSSV and grouper iridovirus, respectively (Zhao et al., 2009; Luo et al., 2003) whilst PmLT played a role in the pattern recognition receptor for initial recognition of WSSV, and could also activate the cellular defense mechanism of shrimp hemocytes (Ma et al., 2008).

1.6 Cell-mediated defense reactions

Cellular defense reactions include various biological processes such as phagocytosis, encapsulation, and nodule formation (Millar and Ratcliffe, 1994). Phagocytosis, a common phenomenon in all organisms, includes foreign body attachment, ingestion and destruction. Encapsulation, a process wherein layers of cells surround the foreign material, occurs when a parasite is too large to be ingested by phagocytosis. Nodule formation, which appears to be similar to capsule formation, occurs when the number of invading bacteria is high. These structures, capsules and nodules are always melanized in arthropods.

1.7 The prophenoloxidase (proPO) system

The proPO activating system is composed of several proteins involved in melanin production, cell adhesion, encapsulation, and phagocytosis (Söderhäll and Cerenius, 1998). *In vitro* studies have shown that phenoloxidase (PO) exists as an inactive precursor, prophenoloxidase (proPO), which is activated by a stepwise process involving serine proteases activated by microbial cell wall components such as lipopolysaccharides or peptidoglycans from bacteria, and β-1,3-glucans from fungi through pattern-recognition proteins (PRPs) (Ariki et al., 2004). An enzyme, capable of activating the proPO *in vivo*, is called prophenoloxidase-activating enzyme (factor) (ppA, PPAE, PPAF). In crayfish, ppA is a trypsin-like proteinase presenting as an inactive form in the hemocyte granules. After degranulation, the enzyme is released together with proPO and becomes an active form in the presence of microbial elicitors. The active ppA can convert proPO to an active form, PO (Aspán and Söderhäll, 1991; Aspán et al., 1995). PO is a copper-containing protein and a key enzyme in melanin synthesis (Söderhäll and Cerenius. 1998; Shiao et al., 2001). It catalyses o-hydroxylation of monophenols to diphenols and oxidises diphenols to quinones, which can non-enzymatically polymerise to melanin. PO is a sticky protein and can adhere to the surface of parasites leading to melanisation of the pathogens. Melanisation is usually observed by blackening of the parasite in the hemolymph or black spots on the cuticle. The melanin and intermediates in the melanin formation can inhibit growth of microbial parasites such as crayfish plague fungus, *Aphanomyces astaci* (Söderhäll and Ajaxon, 1982). Thus, the generation of insoluble

melanin is important for the process of sclerotisation, wound healing, and encapsulation of foreign materials (Theopold et al., 2004).

In the crayfish, *P. leniusculus*, RNA interference-mediated depletion of crayfish proPO led to lower PO activity, increased bacterial growth, lower phagocytosis, lower nodule formation, and higher mortality when infected with a highly pathogenic bacterium, *Aeromonas hydrophila* (Liu et al., 2007). In contrast, RNA interference of pacifastin, an inhibitor of the crayfish proPO activation cascade, resulted in higher phenoloxidase activity, lower bacterial growth, increased phagocytosis, increased nodule formation, and delayed mortality of the infected crayfish (Liu et al., 2007). Moreover, in penaeid shrimp, enzymes in the proPO system are localized in the semigranular and granular cells (Perazzolo and Barracco, 1997). This is in accordance with the former studies showing that *P. monodon* proPO and PPAE mRNAs as well as a *L. vannamei* proPO mRNA are expressed only in hemocytes (Sritunyalucksana et al., 2000; Ai et al., 2009; Amparyup et al., 2009; Charoensapsri et al., 2009, 2011). Previous reports revealed that RNAi-mediated silencing of two *P.monodon* proPO genes (*Pm*proPO1 and *Pm*proPO2) and two *P.monodon* PPAE genes (*Pm*PPAE1 and *Pm*PPAE2) significantly decreased the total PO activity, leading to an increase in the bacterial number in *Vibrio harveyi*-infected shrimp and also enhanced their the mortality rate after the infection (Amparyup et al., 2009; Charoensapsri et al., 2009, 2011). These results indicate that proPO and PPAE are important in proPO system as well as the innate immune response in crustaceans.

1.8 The coagulation system/ the clotting system

Hemolymph coagulation is a defensive response of crustaceans preventing both loss of hemolymph through the breaks in the exoskeleton and the dissemination of bacteria throughout the body (Martin et al., 1991). It is a proteolytic cascade activated by microbial cell wall components. The coagulation system involves a plasma-clotting protein (CP) and a hemocyte-derived transglutaminase (Kopäcek et al., 1993a; Yeh et al., 1998).

Clotting has been most studied in two non-insect arthropod species with significantly different clotting reactions: freshwater crayfish and horseshoe crab. The clotting system in crayfish depends on the direct tranglutaminase (TGase)-mediated

cross linking of a specific plasma protein, whereas the process in horseshoe crab is regulated by a proteolytic cascade activated by bacterial elicitors through specific recognition proteins. In crayfish, clotting occurs through polymerization of a clotting protein in plasma. The crayfish CP is a dimeric protein and each subunit has both free lysine and glutamine. They are recognized and become covalently linked to each other by a calcium ion-dependent TGases (Hall et al., 1999; Wang et al., 2001b; Yeh et al., 1998). CPs are synthesized in the hepatopancreas and released to hemolymph. In crustaceans, CPs were found in several species: the freshwater crayfish (Kopacek et al., 1993b), the black tiger shrimp (Yeh et al., 1998), and the lobster, *Panulirus interruptus* (Doolittle and Fuller, 1972).

1.9 Antimicrobial peptides (AMPs)

The peptide antibiotics are defined as antimicrobial peptides. They are produced in animal including human and play important function in innate immunity. Most of the AMPs are small in size, generally less than 200 amino acid residues, with amphipathic structure and cationic property. However, the anionic peptides also exist. Their small sizes make them easily to be synthesized without dedicated cells or tissues, and therefore rapidly diffuse to the point of infection. For many of these peptides, there is evidence that one of the targets for the peptides is the lipid bilayer of the membrane because these peptides can often increase the rate of leakage of the internal aqueous contents of liposomes. In addition, most of the antimicrobial peptides are cationic and their interaction with anionic phospholipids would provide a ready explanation for their specificity for bacterial membranes. With regard to the mechanism by which the peptide breaks down the membrane permeability barrier, it is possible that the peptide induces complete lysis of the microorganisms by rupturing the membrane or perturbing the membrane lipid bilayer, allowing for leakage of certain cellular components as well as dissipating the electrical potential of the membrane.

AMPs are active against a large spectrum of microorganisms: bacteria and filamentous fungi. In addition, some AMPs have antiviral or antiparasitic activities (Hancock and Diamond, 2000; Murakami et al., 1991; Pan et al., 2000) and may also exhibit an anti-tumor property (Cruciani et al., 1991). There are some reports on

AMPs and their immune functions in shrimp (see a review by Tassanakajon et al., 2010). AMPs are primarily expressed in circulating hemocytes, which is the main site of the immune response, and hemocytes expressing AMPs probably migrate to infection sites to fight against pathogen invasion. Penaeidins, a family of antimicrobial peptides acting against gram- positive bacteria and fungi, had been reported in the white shrimp, *L. vannamei* (Destoumieux-Garzon et al., 1997). cDNA clones of penaeidins were also isolated from the hemocytes of *L. vannamei*, *P. setferus* and *P. monodon* (Gross et al., 2001, Supungul et al., 2004). Recently, a new function of penaeidin5 from the *P. monodon* has been reported to play a possible role in protection against viral infection (Woramongkolchai et al., 2011). Crustins, the crustacean antimicrobial peptides, were identified from two species of *Penaeid* shrimp, *L. vannamei* and *L. setiferus*. Several isoforms of crustins were observed in both shrimp species. The 11.5 kDa antibacterial protein from *Carcinus maenas*a and crustins from shrimp showed no homology with other known antibacterial peptides, but possessed sequence identity with a family of proteinase inhibitory proteins, the whey acidic protein (WAP). ALFs have broad antimicrobial activities towards gram-positive and gram-negative bacteria, filamentous fungi, and viruses (Liu et al., 2006; Li et al., 2008; Somboonwiwat et al., 2005). Formerly, it had been shown that recombinant ALF*Pm*3 could protect shrimp from vibriosis (Ponprateep et al., 2009) and WSSV infection (Tharntada et al., 2009). Besides, the ALF transcript level in the crayfish, *P. leniusculus,* was enhanced after WSSV injection, and its knockdown activated WSSV propagation in cell cultures (Liu et al., 2006). In addition, peptides derived from the hemocyanin of *L. vannamei, P. stylirostris,* and *P. monodon* have also been identified to be able to possess antiviral activity (Destoumieux-Garzon et al., 2001; Patat et al., 2004; Zhang et al., 2004). Previously, histones and histone-derived peptides of *L. vannamei* have been reported as innate immune effectors because they could inhibit growth of gram-positive bacteria (Patat et al., 2004).

1.10 Proteinase inhibitors

Proteinase inhibitors, produced by the hemocytes, are necessary to protect host from microbial proteinases and regulate the proteinase cascades (the proPO and coagulation system). Function of proteinases in many pathogenic fungi helps them

penetrate the cuticle of their arthropod hosts. Proteinases also contribute to the virulence of bacterial pathogens. Some of the proteinase inhibitors in hemolymph can defend the host against such microbial proteinases. For instance, the silk worm (*Bombyx mori*) serine proteinase inhibitor is active against proteinases from fungal pathogens (Eguchi et al., 1993). Several of *Manduca sexta* serpin gene-1 variants inhibit bacterial and fungal serine proteinases (Jiang et al., 1998). Proteinase inhibitors in the cuticle or at the surface of the integument might function in protection against fungal infection. An external secretion from grasshoppers has been shown to contain proteinase inhibitors with a wide range of specificity (Polanowski et al., 1997).

In vertebrates, injury and microbial infection lead to activation of the blood coagulation and proPO systems. Both of these systems employ cascades of serine proteinases to amplify an initial signal (wounded tissue or the presence of microbial polysaccharides), resulted in rapid and efficient responses to the threats to health (Whaley et al., 1993; O'Brien and McVey, 1993). Blood clotting and phenoloxidase activation can also be harmful to the host if they are not limited as local and transient reactions. For this reason, the proteinases in these systems are tightly regulated by proteinase inhibitors.

Like blood clotting, phenoloxidase activation is normally regulated *in vivo* as a local reaction with brief duration. Also comparable to blood clotting, the regulation may be partly due to serine proteinase inhibitors in plasma (Kanost et. al., 1996). Previous report revealed that pacifastin and α-macroglobulin inhibited crayfish proPO activation (Aspan et al., 1990). Among the low molecular weight inhibitors from insect hemolymph, Kunitz family inhibitors from *M. sexta, Sarcophaga bullata*, and *B. mori* (Sugumaran et al., 1985; Saul and Sugumaran, 1986 and Aso et al., 1994) and the 4 kDa locust inhibitors (Boigegrain et al., 1992) can also interfere in proPO activation. Serpin-1J from hemolymph of *M. sexta* inhibits the activity of a serine proteinase linked to prophenoloxidase activation (Jiang and Kanost, 1997). The *M. sexta* serpin-6 was isolated from hemolymph of the bacteria-challenged larvae, which selectively inhibited proPO-activating proteinase-3 (PAP-3) (Wang and Jiang, 2004). In addition, its structure and function were further characterized by cloning and expression in *E. coli* expression system (Zou and Jiang, 2005). The results indicated

that serpin-6 played important roles in the regulation of immune proteinases in the hemolymph. It is likely that each proteinase in the proPO cascade is regulated by one or more specific inhibitors present in plasma or in hemocyte granules.

1.11 Apoptotic and tumor proteins

Apoptosis or programmed cell death plays a major role in differentiation, development, tissue homeostasis and cell-mediated immunity as well as defense against environmental insults including pathogen attack (Kerr et al., 1977; Thompson, 1995). Previous studies showed that many pathogens exerted a control on the processes regulating apoptosis in the host. The induction of apoptosis upon infection results from a complex interaction of parasite proteins with cellular host proteins. In the infected cells, induction of apoptosis significantly imparts protection to the host from the pathogen (Hasnain et al., 2003). However, if over apoptosis, it will be implicated in shrimp death, so apoptosis inhibitor is necessary. For example, both survivin and P109 protein are involved in apoptosis inhibition (Liston et al., 1996; Uren et al., 1996; Tambunan et al., 1998). Besides, survivin is involved in regulation of cell division during HIV-1 infection (Zhu et al., 2003).

Apoptosis, which occurs after viral infections, plays an important role in the antiviral mechanism of crustaceans (Liu et al., 2009). However, this also leads to a significant reduction in the number of circulating hemocytes, probably resulting in a decline of antiviral immunity as well as mortality of crustaceans (Van de Braak et al., 2002; Wongprasert et al., 2003; Wang et al., 2008; Granja et al., 2003; Wang and Zhang, 2008). Therefore, maintenance of the hemocyte level in the blood-circulating system, including the rapid production of new hemocytes from hematopoietic tissue, is essential for the survival of the animals as is the capacity to protect against pathogenic invaders.

1.12 Other immune molecules

Cyclophilins are highly conserved proteins first identified as main binding proteins for cyclosporin A (CyPA) (Fischer et al., 1989). They were later identified as peptidyl-prolyl cis/trans isomerases and have been proposed to be involved in protein folding (Galat, 1993). Cyclophilins are involved in cellular processes and have many

clinical applications such as cell signaling (Mattila et al., 1990), apoptosis (Montague et al., 1997), oxidative stress (Jaschke et al., 1998), heat shock, and hypoxia (Andreeva et al., 1997). For example, CyPA is predominantly in cytosol but can be secreted by macrophages in response to stimulation with bacterial endotoxin (Sherry et al., 1992) or by vascular smooth muscle cells in response to oxidative stress (Jin et al., 2000). Cyclosporin B (CyPB) was found in the endoplasmic reticulum and secreted into milk and plasma (Spik et al., 1991; Allain et al., 1995; Arber et al., 1992). CyPB has been shown to enhance platelet adhesion to collagen (Allain et al., 1999), whereas CyPA shown to help protect cells from oxidative stress (Jaschke et al., 1998). A shrimp cyclophilin was also identified from *L. vannamei and L. setiferus* (Gross et al., 2001). Cyclophilins have diverse regulatory functions in mammalian cells, thus it is noteworthy how they can be responsible for viral attachment to cells (Saphire et al., 1999) and in the stress response to oxygen depletion (Santos et al., 2000).

Prostaglandins (PGs) are able to regulate the expressions of genes in various mechanisms such as cell protection, energy metabolism, lipid metabolism, signal transduction and proPO-activating cascade in insect cells (Downer et al., 1997; Stanley et al., 2008), as well as the expression of the antibacterial peptides (cecropine and lysozyme) in the silkworm (Morishima et al., 1997). Additionally, *Anopheles albimanus* prostaglandin E_2 (PGE2) modulated the expression of three antimicrobial peptides: *Aa*-Attacin, *Aa*-Cecropin and *Aa*-Gambicin (García Gil de Muñoz et al., 2008). Although, until now, there is no information on the functions of PGs in crustaceans, the results from previous studies strongly suggested that PGs acted as mediators in the immune response pathways of various invertebrates.

Rab GTPases belong to the *Ras* superfamily and are known to play key roles in phagocytotic development, and are probably involved in virus evasion. Certainly, Rab7 from *P. monodon* has previously been implicated in WSSV and YHV infections (Ongvarrasopone et al., 2008). It binds directly to the VP28 envelope protein of WSSV and is probably involved in the transport of viruses into the cells (Sritunyalucksana et al., 2006). In addition, the Rab6 protein homologue of the kuruma shrimp, *Penaeus (Marsupenaeus) japonicus*, was documented to be essential

in the regulation of hemocytic phagocytosis against WSSV and the bacterium *Vibrio parahemolyticus* (Wu et al., 2008; Zong et al., 2008).

1.13 Hematopoiesis in crustaceans

Hematopoiesis is a process where hemocyte synthesis takes place in the hematopoietic tissue (Hpt). In crustacean, hemocytes are produced and partially differentiated into young hemocytes in Hpt, and then become functional or mature hemocytes as they are released into circulation (Söderhäll et al., 2003). Previously, astakine, an invertebrate protein homologue to vertebrate prokineticin, was first identified in the freshwater crayfish, *P. leniusculus,* and found to be required for cell proliferation and differentiation in Hpt of two crustacean species (Söderhäll et al., 2005; Hsiao and Song, 2010). So far, two isoforms of astakines have been discovered in *P. leniusculus,* namely astakine 1 and astakine 2. Astakine 1 is able to encourage proliferation and differentiation of Hpt cell to semigranular cell (SGC), whilst astakine 2 is responsible for granular cell (GC) differentiation (Söderhäll et al., 2005; Lin et al., 2010). Specific marker genes for crayfish hemocytes have been determined and a proliferating cell nuclear antigen (PCNA), a specific Kazal proteinase inhibitor (KPI) and a superoxide dismutase (SOD) were established and found to be principally expressed in proliferating Hpt cell, semigranular cell (SGC) and granular cell (GC), respectively (Wu et al., 2008). Recently, a crustacean hematopoietic factor (CHF) was first identified in *P. leniusculus* and was shown to be vital for the survival of hemocytes and Hpt cells by preventing the apoptotic process (Lin et al., 2011). Astakine 1 was also able to regulate the transcription of PCNA, KPI and CHF in Hpt cells (Lin et al., 2010, 2011). Moreover, a former report revealed that ATP synthase in the plasma membrane of Hpt was functioning as a receptor for astakine 1 (Lin et al., 2009). Interestingly, a new function of transglutaminase in crayfish was disclosed, in that it participated in preventing differentiation of hematopoietic stem cells (Lin et al., 2008). Although there are some recent attempts to investigate crustacean hematopoiesis, the mechanisms regarding hemocyte synthesis and differentiation, the release of hemocytes into the circulation, and the maintenance of circulating hemocytes in crustaceans are still largely unknown.

1.14 Purposes of the thesis

In order to gain more insight into viral infection and/or antiviral mechanisms in crustaceans, we applied suppression subtractive hybridization (SSH) to identify viral responsive genes in the hemocytes of virus-challenged *Penaeus monodon* at the early and late phases of the infection. Among the genes obtained from SSH libraries, two of the highly up-regulated genes post-WSSV infection were selected for further investigation in this study. First, a novel gene encoding a protein with significant similarity to the hypothetical protein TcasGA2_TC006773 from the red flour beetle, *Tribolium castaneum* was characterized for the first time, and appeared to be involved in hemocyte homeostasis in *P.monodon*. Therefore, it was named as "hemocyte homeostasis-associated protein (HHAP)". Another novel viral responsive gene with no similarity to any genes in the database, named here "viral responsive protein 15 kDa (VRP15)" was first characterized in this study, and it was found to be involved in viral infection mechanism in *P.monodon*.

CHAPTER II
MATERIALS AND METHODS

2.1 Materials

2.1.1 Equipment

- 20 °C Freezer (Whirlpool), - 80 °C Freezer (ThermoForma)

Amicon Ultra-4 concentrators (Millipore).

Automatic micropipette (Gilson Medical Electrical S.A.)

Centrifuge 5804R (Eppendorf), Centrifuge Avanti^{TM} J-301 (Beckman Coulter)

CX31 Biological Microscope (Olympus)

Gel documentation (SYNGENE)

Gene Pulser (Bio-RAD)

Hybridization oven (Hybrid)

iCycler iQ™ Real-Time Detection System (Bio-Rad)

Incubator (Memmert)

Innova 4080 incubator shaker (New Brunswick Scientific)

LABO Autoclave (SANYO)

Laminar Airflow Biological Safety Cabinets (NuAire, Inc.)

Microcentrifuge tubes 0.5 ml and 1.5 ml (Bio-RAD Laboratories)

PCR Mastercycler (Eppendorf AG)

pH meter Model # SA720 (Orion)

Spectrophotometer (eppendorf)

Sterring hot plate (Fisher Scientific)

Trans-Blot® SD (Bio-RAD Laboratories)

Ultra Sonicator (SONICS Vibracell)

Vertical electrophoresis system (Hoefer™ miniVE)

Water bath (Memmert)

2.1.2 Chemicals, Reagents and Biological substance

100 mM dATP, dCTP, dGTP, and dTTP (Fermentas)

2-Mercaptoethanol (Fluka)

3-(N-morpholino) propanesulfonic acid (MOPS) (USB)

5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside (X-Gal) (Fermentas)

5-bromo-4-chloro-indolyl phosphate (BCIP) (Fermentas)

Acetic anhydride (Sigma)

Acrylamide (Plus one)

Agarose (Sekem)

Alexa Fluor 488 goat anti-rabbit IgG antibody (Invitrogen)

Alkaline phosphatase-conjugated rabbit anti-mouse IgG (Jackson ImmunoResearch Laboratories, Inc.)

Ammonium persulfate (USB)

Amplicillin (BioBasic)

Bacto agar (Difco)

Bacto tryptone (Scharlau)

Bacto yeast extract (Scharlau)

Bovine serum albumin (Fluka)

Bromophenol blue (MERCK)

Calcium chloride (MERCK)

Chloramphenicol (Sigma)

Chloroform (MERCK)

Coomassie brilliant blue G-250, R-250 (Fluka)

Diethyl pyrocarbonate (DEPC) (Sigma)

di-Sodium hydrogen orthophosphate anhydrous (Carlo Erba)

Ethanol (MERCK)

Ethidium bromide (Sigma)

Ethylene diamine tetraacetic acid disodium salt dihydrate (EDTA)(Fluka)

Formaldehyde (BDH)

Formamide deionized (Sigma)

DNA ladder (Fermentas)

Glacial acetic acid (J.T. Baker)

Glucose (Ajax chemicals)

Glycerol (Scharlau)

Glycine (Scharlau)

Hydrochloric acid (MERCK)

Imidazole (Fluka)

Isopropanol (MERCK)

Isopropyl-β-D-thiogalactoside (IPTG) (USBiological)

Kanamycin (BIO BASIC Inc.)

Magnesium chloride (MERCK)

Methanol (MERCK)

Minimum Essential Medium (Invitrogen)

N, N, N', N'-Tetramethylethylenediamine (TEMED) (BDH)

N, N', methylenebisacrylamide (Fluka)

Ni Sepharose 6 Fast Flow (GE Healthcare)

Nitroblue tetrazolium (NBT) (Fermentas)

Paraformaldehyde (Sigma)

Peptidoglycan from Staphylococcus aureus (Sigma)

pET22b(+) vector (Novagen)

Phenol, saturated (MERCK)

Potassium chloride, KCl (Ajax)

Prestained protein molecular weight marker (Fermentas)

Prolong Gold Antifade Reagent (Invitrogen)

RNA markers (Promega)

Skim milk powder (Mission)

Sodium acetate (Carlo Erba)

Sodium chloride (BDH)

Sodium dihydrogen orthophosphate (Carlo Erba)

Sodium dodecyl sulfate (Sigma)

Sodium hydroxide (Eka Nobel)

TO-PRO-3 iodide (Invitrogen)

Tris-(hydroxy methyl)-aminomethane (USB)

TRI Reagent® (Molecular Research Center)

Triton® X-100 (MERCK)

Tween™-20 (Flula)

Urea (Fluka)

2.1.3 Enzymes and Kits

ImProm-II™ Reverse Transcription system kit (Promega)

NucleoSpin® Extract II Kits (MACHEREY-NAGEL)

PCR-Select™ cDNA Subtraction Kit (Clontech)

QIAprep® Miniprep kits (QIAGEN)

QuickPrep™ Micro mRNA Purification Kit (Amersham Biosciences)

RBC T&A Cloning Vector (RBC Bioscience)

SMART™ RACE cDNA Amplification Kit (Clontech)

T7 RiboMAX™ Express Large Scale RNA Production System (Promega)

IQ™ SYBR Green Supermix (Bio-Rad)

*Nde*I (Biolabs)

RQ1 RNase-free DNase (Promega)

T4 DNA ligase (Promega)

Taq DNA polymerase (Fermentas)

*Xho*I (Biolabs)

2.1.4 Microorganisms

Escherichia coli strain Rosetta (DE3)

Escherichia coli DH5α competent cells (RBC Bioscience)

White spot syndrome virus

Yellow head virus

2.1.5 Software

BlastN and BlastX (http://www.ncbi.nlm.nih.gov/BLAST/)

DBS-Pred (http://www.netasa.org/dbs-pred/)

DiANNA 1.1 web server (http://clavius.bc.edu/~clotelab/DiANNA/)

EMBOSS Pairwise Alignment (http://www.ebi.ac.uk/Tools/emboss/align/)

GENETYX (Software Development Inc.)

SECentral (Scientific & Educational Software)

SMART (http://smart.embl-heidelberg.de/)

SPSS statistics 17.0 (Chicago, USA)

TMHMM Server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM/)

2.2 Animal cultivation

Specific pathogen free (SPF) black tiger shrimp, *Penaeus monodon*, of about 20 g bodyweight were obtained from a farm in Nakhon Si Thammarat Province, Thailand. The animals were reared in laboratory tanks at ambient temperature (28 ± 4 °C), and maintained in aerated water with a salinity of 20 ppt for at least 7 days before use. For RNAi experiments, *P. monodon* of about 3 - 5 g in weight were purchased from local farms in Thailand, and were maintained as above.

2.3 Total RNA extraction by TRI reagent, and DNase treatment

Cells or tissues harvested from shrimp were homogenized in 1 ml of TRI Reagent® (Molecular Research Center). Subsequently, two hundred µl of chloroform was added and vigorous shaken for 15 sec. After incubation at room temperature for 5 min, the mixtures were centrifuged at 12,000 x g for 15 min at 4 °C, and then transfer of the RNA-containing aqueous (upper) phase to a fresh tube. Total RNA was precipitated by adding a volume of absolute isopropanol, followed by incubating at -20 °C for 15 min. The supernatant was removed by centrifugation at 12,000 x g for 15 min at 4 °C. The RNA pellet was washed with 1 ml of 70% (v/v) ethanol in diethyl pyrocarbonate (DEPC)-treated water. The total RNA was stored in 70% (v/v) ethanol at - 80 °C until used. The ethanol supernatant was completely removed by centrifugation at 12,000 x g for 15 min at 4 °C. The RNA pellet was air-dried at room temperature for 10 - 15 min, and dissolved in an appropriate amount of DEPC-treated water. The obtained total RNA was further treated with RQ1 RNase-free DNase I (Promega) (1 unit/5 µg of total RNA) at 37 °C for 30 min to remove the contaminating chromosomal DNA. Then, the RNA pellet was purified using TRI Reagent as described above.

2.4 Single-stranded cDNA synthesis using ImPromp-II™ reverse transcription system (Promega)

The DNase-treated RNA (up to 1 µg) was combined with 0.5 µg of Oligo$(dT)_{15}$ primer in nuclease-free water for a final volume of 10 µl per a reverse transcription reaction. The sample was heated at 70 °C for 5 min, and immediately

chilled in ice-water for at least 5 min. Ten µl of the reverse transcription reaction mix, containing 2X ImProm-II™ reaction buffer, 6.5 mM $MgCl_2$, 1mM dNTP mix, 40 units of RNasin® ribonuclease inhibitor, 2X ImProm-II™ Reverse Transcriptase and nuclease-free water, was added into the total RNA sample. The mixture was incubated at temperature in a series of 25 °C for 5 min, 42 °C for 60 min and 70 °C for 15 min, respectively, whereupon the cDNA was ready to be used in the experiments.

2.5 WSSV challenge and diagnosis of WSSV infection

The WSSV solution was previously prepared from hemolymph of moribund WSSV-infected *P. monodon* as described in Pongsomboon et al., 2011, and was diluted 1:2000 in Lobster Hemolymph Medium (LHM) (1X Minimum Essential Medium (Invitrogen) supplemented with 0.2 mM NaCl, 6.1 mM $CaCl_2.2H_2O$, 5.4 mM KCl, 64.1 µM $NaH_2PO_4.2H_2O$ and 10 mM N-(2-Hydroxyethyl)piperazine-N'-(2-ethane sulfonic acid), adjusted to pH 7.5 with 7.5% (w/v) $NaHCO_3$ solution). The diluted WSSV solution (100 µl) was injected into each shrimp at the abdominal muscle (40 shrimp), the dose having been previously experimentally determined as that which would induce a cumulative mortality of ~ 50% within three days post-injection. As a control group, twenty shrimp were injected with 100 µl of virus-free LHM. In all cases WSSV infection, or its absence, in the shrimp was determined by RT-PCR.

Gills of control and WSSV-infected shrimp were collected and homogenized in 200 µl of lysis buffer containing 50 mM NaOH and 0.025% (w/v) SDS. The samples were heated at 100 °C for 5 min followed by chilling in ice for 2 min, and then centrifuged at 10,000 x g for 5 min at 4 °C. The supernatants were collected and diluted 1: 100 in distilled water for PCR analysis using VP466 primer (forward: 5′-CCCGAATAGTGTTTCCTCAGC-3′ and reverse: 5′-AACACAGCTAACCTTTATGAG-3′). The PCR condition comprised 94 °C for 3 min, 30 cycles of 94 °C for 30 sec, 60 °C for 30 sec and 72 °C for 30 sec, and then a final extension at 72 °C for 5 min. The positive and negative controls were amplified from the PCR samples with and without WSSV, respectively. The PCR products were analyzed by 1.5% (w/v) agarose gel electrophoresis.

2.6 YHV challenge and diagnosis of YHV infection

The YHV solution was previously prepared from hemolymph of moribund YHV-infected *P. monodon* as described in Pongsomboon et al., 2011, and was diluted 1:4000 in LHM. The diluted YHV solution (100 µl) was injected into each shrimp in the abdominal muscle (40 shrimp), the dose having been previously experimentally determined as that which would induce a cumulative mortality of ~ 50% within three days post-injection. As a control group, twenty shrimp were injected with 100 µl of virus-free LHM. In all cases YHV infection, or its absence, in the shrimp was determined by RT-PCR.

Gills of control and YHV-infected shrimp were collected, and then total RNAs were extracted from the tissues using TRI Reagent® (Molecular Research Center), followed by DNase I (Promega) treatment. The total RNAs (1 µg) were converted to single-stranded cDNA with the ImPromp-II™ reverse transcription system (Promega) according to the manufacturer's instruction. The cDNAs were subjected to RT-PCR to analyze a degree of YHV infection using GP64 primer (forward: 5′-TCACTATTACTCCAGTTATCA-3′ and reverse: 5′-CTAGGATCGTTTGGCTTT CGTTC-3′). The PCR condition comprised 94 °C for 3 min, 35 cycles of 94 °C for 30 sec, 50 °C for 30 sec and 72 °C for 30 sec, and then a final extension at 72 °C for 5 min. The positive and negative controls were amplified from the PCR samples with and without YHV, respectively. The PCR products were analyzed by 1.5% (w/v) agarose gel electrophoresis.

2.7 Hemocyte collection and preparation of poly(A)+RNA

Hemolymphs of control and virus-infected (surviving) shrimp were drawn at 24, 48 and 72 h post-injection from the ventral sinus using a sterile 1 ml syringe with 150 µl anticoagulant (10% (w/v) sodium citrate). The hemolymphs were centrifuged immediately at 5000 × g for 5 min at 4 °C to separate the hemocytes from plasma. Total RNAs were isolated from the hemocytes using TRI Reagent® (Molecular Research Center). Subsequently, poly(A)+RNAs were purified using a QuickPrep™ Micro mRNA Purification Kit (Amersham Biosciences) according to the manufacturer's protocol. Briefly, one ml of oligo(dT)-cellulose was homogeneously swirled and immediately pipetted into a 1.5 ml microcentrifuge tube. After

centrifugation for 1 min, the buffer was removed. The total RNA was applied onto the oligo (dT)-cellulose and gently mixed by inverting for 3 min. The mixture was centrifuged at the max speed for 1 min. The supernatant was removed and resin was washed five times with the high-salt buffer (10 mM Tris-HCl, pH 7.5 containing 1 mM EDTA and 0.5 M NaCl), followed by three times with the low-salt buffer (10 mM Tris-HCl; pH 7.5, 1 mM EDTA, 0.1 M NaCl). Next, the resin was resuspended with 300 μl of the low-salt buffer, and transferred to a microspin column placed on a microcentrifuge tube. After spin down, the column was washed three times with 500 μl of the low-salt buffer and centrifuged at the full speed for 1 min. The flow through in collection tube was discarded. After that, the column was placed in a fresh 1.5 ml microcentrifuge tube. Two hundred microliters of pre-warmed (65 °C) elution buffer (10 mM Tris-HCl, pH 7.5 containing 1 mM EDTA) was added to the top of the resin bed. The eluted mRNA was collected by centrifugation at the full speed for 1 min. The eluted mRNA was kept at -80 °C until used.

2.8 Suppression subtractive hybridization (SSH)

The SSH was performed to generate two SSH libraries (24I and 48/72I SSH libraries) of cDNA fragments enriched for virus-induced genes using the PCR-Select™ cDNA Subtraction Kit (Clontech), according to the manufacturer's instructions. Briefly, double-stranded cDNAs were systhesized form poly(A)+RNAs of the control shrimp hemocytes (driver) and virus-infected shrimp hemocytes (tester), then they were digested with *Rsa*I to generate short, blunt-ended double-stranded cDNA. After that *Rsa*I-digested tester cDNA was subdivided into two portions, and each was ligated with a different adaptor (adaptor 1 and adaptor 2R) at the 5' ends of the cDNA while the driver cDNA had no adaptor ligation. The adaptor 1-ligated and adaptor 2R-ligated tester cDNAs were then separately hybridized with an excess of the driver cDNA at 68 °C for 8 h after denaturation at 98 °C for 90 sec. The two hybridization samples were mixed together without denaturation and hybridized at 68^{o}C overnight with an excess of fresh denatured driver cDNA. The resulting mixture was diluted in 200 ml of dilution buffer (Clontech) composed of 20 mM HEPES, 20 mM NaCl and 0.2 mM EDTA followed by nested PCR

amplification. In the primary PCR, primers against adaptor 1 and adaptor 2R were used to selectively amplify differentially expressed cDNA, and then the product of the first PCR was used as template in the second PCR with nested primers to generate subtracted cDNA (Figure 2.1).

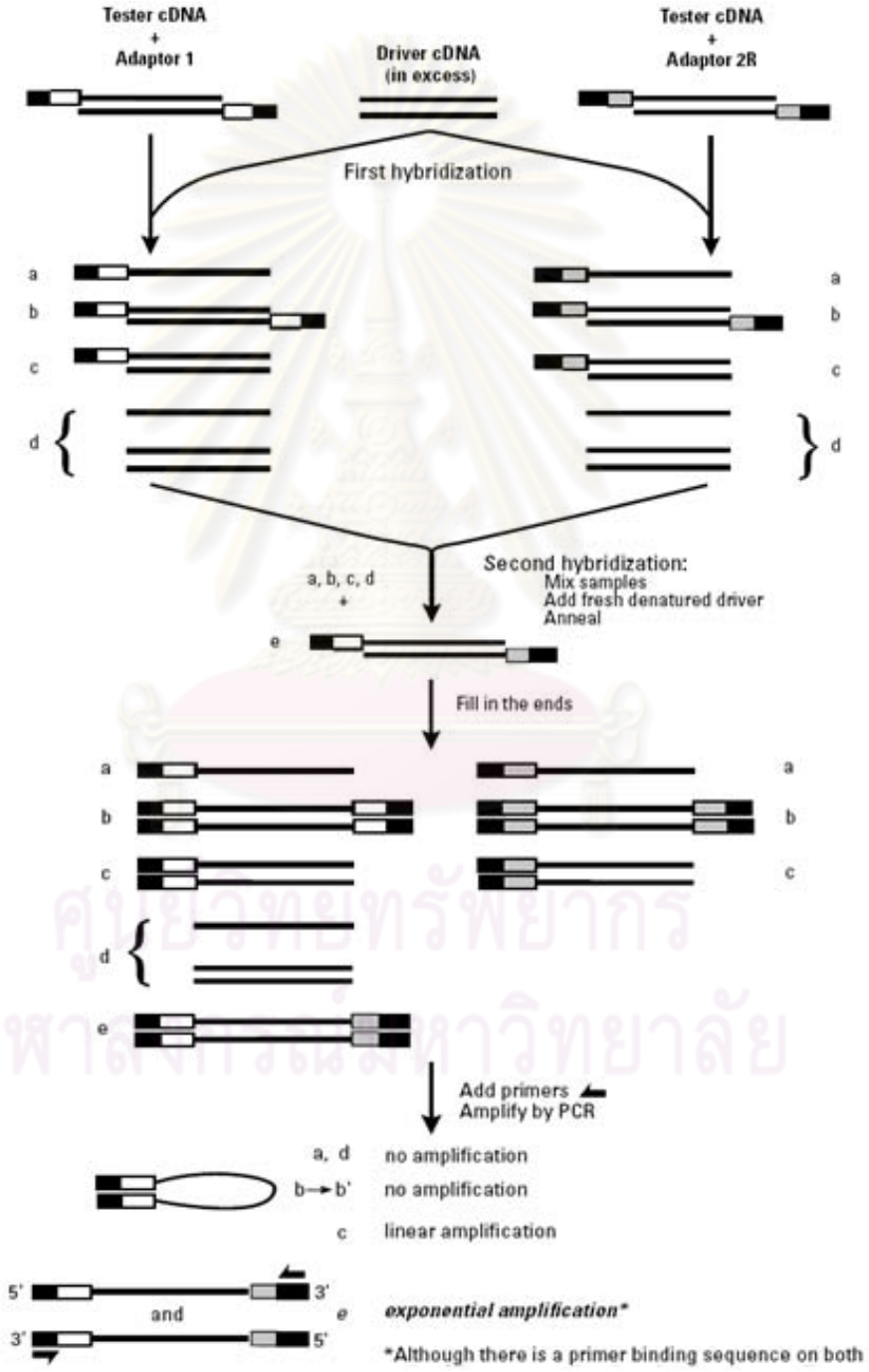

Figure 2.1 PCR-Select™ cDNA Subtraction procedure (Clontech).

2.9 Evaluation of subtraction efficiency

The SSH efficiency was evaluated by PCR with β-actin primer pair (forward : 5′-GCTTGCTGATCCACATCTGCT-3′ and reverse : 5′-ATCACCATCGGCAACGAGA-3′) performed on unsubtracted and subtracted cDNAs with the following conditions: 94°C for 3 min, 25 cycles of 94 °C for 30 sec, 55 °C for 30 sec, 72 °C for 30 sec, and then a final extension at 72 °C for 5 min. Subsequently, the abundance of PCR products between unsubtracted and subtracted samples was compared using a TBE-1.5% (w/v) agarose gel followed by ethidium bromide staining and UV-transillumination.

2.10 cDNA cloning, sequencing and homology analysis

The subtracted cDNA products were cloned into the RBC T&A Cloning Vector (RBC Bioscience) and then transformed into *E. coli* DH5α competent cells (RBC Bioscience), followed by the standard blue/white screening method. The recombinant plasmids were isolated from the positive clones using QIAprep® spin Miniprep Kit (QIAGEN) according to the manufacturer's instructions and sequenced using T7 promoter and/or M13 reverse primer at Macrogen INC., South Korea. The consensus sequences obtained were searched in the NCBI GenBank database for homology with the BLASTN and BLASTX programs (http://www.ncbi.nlm.nih.gov/BLAST/) (Altschul et al., 1990). The significant matches present *E*-values lower than 1×10^{-4}.

2.11 Confirmation of gene up-regulation in response to WSSV infection by RT-PCR

The control and WSSV-infected shrimp were prepared as described above. Hemocytes of shrimp (three individuals each) were collected at 24, 48 and 72 h post-injections. Total RNAs were extracted from the hemocytes using TRI Reagent® (Molecular Research Center) followed by DNase I (Promega) treatment, and then used to synthesize single-stranded cDNAs with the ImPromp-II™ reverse transcription system (Promega). The transcription level of target genes was identified by RT-PCR using an equal amount of cDNA template with gene-specific primers. The β-actin was used as an internal control. The specific oligonucleotide primers to

selected genes including VRP15 (forward: 5′-CGATCACCACTCTCGTTCTT-3′, reverse: 5′-GTACTAACAGCGAACCCATC-3′), *Pm*HHAP-1 (forward: 5′-GCAACA GGAGAACCTGTGGATA-3′ and reverse: 5′-GGTTCCAGAATCGCCTCCTATA-3′), histone H1 (forward: 5′-CCAGGCCATCCTCAAGTACA-3′ and reverse: 5′-TGGCTTCTTGGCAGGCTTAG-3′), non-muscle myosin heavy chain (forward: 5′-AGTGCGATCCAAGACCAAGG-3′ and reverse: 5′-GCGCTTCATTCCGAGGA AGT-3′), X-box-binding protein 1 (forward: 5′-TCAGCTTGAAGCGCAGATTG-3′ and reverse: 5′-TTGACTGTTGCTGGCAGGTA-3′) and β-actin (forward : 5′-GAACCTCTCGTTGCCGATGGTG-3′ and reverse : 5′-GAAGCTGTGCTACGTG GCTCTG-3′). The PCR conditions consisted of 94 °C for 3 min, followed by 33 (for VRP15), 30 (for *Pm*HHAP), 25 (for histone H1and X-box-binding protein 1) and 28 (for myosin-9 and β-actin) cycles of 95 °C for 30 sec, 58 °C for 30 sec and 72 °C for 30 sec, and then a final extension at 72 °C for 5 min. The PCR products were analyzed by 1.5% (w/v) agarose gel electrophoresis.

2.12 Confirmation of gene up-regulation in response to YHV infection by real-time RT-PCR

The control and YHV-infected shrimp were prepared as described above. Hemocytes of shrimp (three individuals each) were collected at 6, 24, 48 and 72 h post-injections. Total RNAs were extracted from the hemocytes using TRI Reagent® (Molecular Research Center) followed by DNase I (Promega) treatment, and then used to synthesize single-stranded cDNAs with the ImPromp-II™ reverse transcription system (Promega). The real-time PCR was performed using an equal amount of cDNAs in an iCycler iQ™ Real-Time Detection system using iQ™ SYBR Green Supermix (Bio-Rad) with the following conditions: 95 °C for 9 min, 40 cycles of 95 °C for 30 sec, 55 - 60 °C for 30 sec and 72 °C for 45 sec. The specific oligonucleotide primers to five selected genes including anti-lipopolysaccharide factor isoform 6 “ALF*Pm*6” (forward : 5′-AGTCAGCGTTTAGAGAGGTT-3′ and reverse : 5′-GCTCGAACTCTCCACTCTC-3′), crustin isoform 1 “crustin*P*m1” (forward : 5′-CTGCTGCGAGTCAAGGTATG-3′ and reverse : 5′-AGGTACTGGCTGCTCTA CTG-3′), transglutaminase (forward : 5′-ACGACGACTGGGACATAAGG-3′ and reverse : 5′-CATACTCCTGGCGCATTTTT-3′), Kazal-type serine proteinase

inhibitor isoform 2 “SPI*Pm*2” (forward : 5′-ATGCAACCACGTCTGTACTG-3′ and reverse : 5′-CTGCAAGGTTCCACATCT-3′) and β-actin (forward : 5′-GAACCTCTCGTTGCCGATGGTG-3′ and reverse : 5′-GAAGCTGTGCTACGT GGCTCTG-3′) were used in the experiment. Each real-time PCR reaction was done in triplicate. The results were presented as the relative expression ratios of the target genes expressed in the hemocytes of the sample (YHV-infected) shrimp versus the control shrimp, with normalization to a reference gene (β-actin). The relative expression ratios of target genes were calculated as described by Pfaffl, 2001. The data were analyzed using SPSS statistics 17.0 (Chicago, USA). Statistical significance was calculated by paired-samples *t*-test, one-way ANOVA and Scheffe *post-hoc* test. Significance was accepted at $P < 0.05$.

2.13 Identification of the full length cDNAs of *Pm*HHAP and VRP15 using 5′ Rapid Amplification of cDNA End

To Identification of the full length cDNAs of *Pm*HHAP and VRP15, the SMART^{TM} RACE cDNA Amplification Kit (Clontech) was used. Firstly, the first-strand RACE cDNA was synthesized according to the manufacturer’s instructions. Briefly, one µg of total RNA obtained from shrimp hemocyte were mixed with SMART II™ A oilgonucleotide and 5′-RACE CDS primer (Clontech), and then pre-heated at 70 °C for 2 min, and immediately cooled on iced for 2 min. The mixture was applied to synthesize the first-strand cDNA using MMLV reverse transcriptase (Clontech) in the reaction containing 1X first-strand buffer, 2 mM dithiothreitol, 1 mM dNTP Mix, and incubated at 42 °C for 1.5 h in a hot-lid thermal cycler. The RACE cDNA was diluted 1: 100 fold with Tricine-EDTA buffer (10 mM Tricine-KOH, pH 8.5 and 1 mM EDTA), and heated at 72°C for 7 min. The obtained 5′ RACE cDNA was kept at -20 °C until used.

The PCR is performed using the gene specific primers, including 5′-CCACAGGTTCTCCTGTTGCCCTGGTATG-3′ for *Pm*HHAP amplification and 5′-CGCCGCTCGCAGCTTCTTCTCTTGACAC-3′ for VRP15 amplification, and Universal primer A mix (Clontech) which is complementary to adaptor under the following condition: 5 cycles of 94 °C for 30 sec and 72 °C for 3 min; 5 cycles of 94 °C for 30 sec, 70 °C for 30 sec and 72 °C for 3 min; and 25 cycles of 94 °C for 30 sec,

68 °C for 30 sec and 72 °C for 3 min. The PCR reaction was composed of 1X advantage 2 PCR buffer, 0.2 mM dNTP, 1X Universal Primer A Mix (Clontech), 0.2 mM the gene specific primer, 1X Advantage 2 polymerase mix and 2.5 µl of 5′ RACE cDNA. The RACE PCR products were analyzed by 1.5% (w/v) agarose gel electrophoresis, and the specific bands of interest were then purified from agarose gel using NucleoSpin® Extract II kit (Clontech) according to the manufacturer's instructions. The purified fragment is cloned into the RBC T & A Cloning Vector (RBC Bioscience) and then transformed into *E. coli* DH5α competent cells (RBC Bioscience), followed by the standard blue/white screening method. The recombinant plasmid was isolated from the positive clone using QIAprep® spin Miniprep Kit (QIAGEN) according to the manufacturer's instructions and sequenced using T7 promoter and M13 reverse primer at Macrogen INC., South Korea.

2.14 Northern blot analysis of *Pm*HHAP and VRP15 mRNA

2.14.1 Northern blotting

Northern blot analysis was done to confirm the size of full-length cDNA obtained from RACE technique. Ten shrimp were infected with WSSV for 48 h as method described in section 2.5. Total RNA was extracted from hemocytes of WSSV-infected shrimps using TRI Reagent® (Molecular Research Center). Ten µg of the total RNA was electrophoresed in the 1.2% (w/v) formamide/formaldehyde-agarose gel containing 12.5 ng/ml ethidium bromide at 100 V for 60 min. After electrophoresis, total RNA was photographed by using UV-transillumination. The gel was soaked twice in 250 ml of 10 mM sodium phosphate buffer, pH 7.0 for 20 min each, followed by being immersed in distilled water overnight. The Hybond™-N+ nylon membrane (Amersham Biosciences) was soaked in distilled water. Four sheets of thick filter paper in the bottom of a plastic box and saturated with 20X saline-sodium phosphate-EDTA, pH 7.4 (SSPE) consisted of 0.2 M NaH_2PO_4, 20 mM Na_2EDTA and 3 M NaCl. The gel was placed on a stack of the wet filter papers, and then the wet nylon membrane was immediately overlaid onto the gel. One sheet of the thin filter paper wetted in 20X SSPE was overlaid onto the nylon membrane, followed by a dry thin filter paper and a stack of thick filter papers, respectively. The entire set-up was covered with plastic wrap to prevent evaporation, and the top was placed with

an object with weight of around 500 g. The set-up was left for 5 days to allow the complete transfer, and the stack of the thick filters on the top was changed every day. The membrane was placed, RNA-side up, on a piece of filter paper damped with 20X SSPE, and the RNA was fixed to the membrane by UV cross-linker. The blot can be used immediately or stored at room temperature until used. Subsequently, the RNA-linked membrane was hybridized with specific DNA probe labeled with digoxigenin-dUTP after that immunological detection method was used to visualize the specific RNA band using DIG High Prime DNA Labeling and Detection Starter Kit I (Roche) according to the manufacturer's instructions.

2.14.2 Digoxigenin (DIG)-DNA probe labeling

*Pm*HHAP and VRP15 DNA templates were generated by PCR using *Pm*HHAP-1 and VRP15 primers, respectively with the conditions as described in section 2.11, and then the PCR products were purified using NucleoSpin® Extract II kit (Clontech) according to the manufacturer's instructions. One µg of the DNA templates were diluted with distilled water to the final volume of 16 µl, and denatured by heating in a boiling water bath for 10 min and quickly chilling in an ice/water bath. Four µl of the DIG-High Prime (clontech) containing random primers, nucleotides, DIG-dUTP and Klenow enzyme was added into the diluted DNA template, and then the mixture was incubated at 37 °C overnight. The reaction was stopped by adding 2 µl of 0.2 M EDTA, pH 8.0 and heating at 65°C for 10 min.

2.14.3 Hybridization

DIG Easy Hybridization buffer (10 ml/100 cm^2 membrane) was pre-heated at 42 °C. The membrane was prehybridized in DIG Easy Hybridization buffer at 42 °C for 30 min with gentle agitation. The DIG-labeled DNA probe was denatured by boiling for 5 min and rapidly cooling in ice/water, and then it was added into pre-heated DIG Easy Hybridization buffer (3.5 ml/100 cm^2 membrane). The prehybridization solution was then removed and the probe/hybridization mixture was added to the membrane, and it was incubated at 42 °C overnight. The membrane was washed twice in washing solution 1 containing 2X SSC (Clontech) and 0.1% SDS at room temperature for 5 min each, followed by twice with washing solution 2

(prewarmed to 68 °C) containing 0.5X SSC (Clontech) and 0.1% SDS at 68 °C for 15 min each under constant agitation.

2.14.4 Immunological detection

After hybridization and stringency washes, the membrane was rinsed briefly with washing buffer, pH 7.5 (Clontech) containing 0.1 M maleic acid, 0.15 M NaCl, 0.3% (v/v) Tween 20) for 5 min and incubated in blocking solution (Clontech) at room temperature for 30 min, followed by incubating in antibody solution (Clontech) (1:5000 dilution of alkaline phosphatase-conjugated anti-DIG antibody in the blocking solution) at room temperature for 30 min. Subsequently, the membrane was washed twice with washing buffer, pH 7.5 for 15 min each, and equilibrated in detection buffer, pH 9.5 (Clontech) containing 0.1 M Tris-HCl and 0.1 M NaCl. Color development was carried out by adding color substrate solution (Clontech) to the membrane and incubating at room temperature in the dark until the desired band was detected, then the reaction was stopped by washing the membrane with distilled water for 5 min.

2.15 Analysis of *Pm*HHAP and VRP15 gene expression in shrimp tissues

Tissues of shrimp such as the antennal gland, epipodite, eye stalk, gill, heart, hemocytes, hematopoietic tissue, hepatopancreas, intestine, nerve and lymphoid tissues, were collected from normal shrimp, and then total RNA was extracted from each tissue using the TRI Reagent® (Molecular Research Center). After DNase I (Fermentas) treatment, the total RNA (1 µg) was converted to single-stranded cDNA with the ImPromp-II™ reverse transcription system (Promega) according to the manufacturer's instruction. The HHAP gene expression level in each tissue was identified by RT-PCR using 1 µl of cDNA template with *Pm*HHAP primers. The β-actin was amplified using the β-actin primer as internal control. The PCR conditions consisted of 94 °C for 3 min, followed by 31 (for *Pm*HHAP), 35 (for VRP15) and 25 (for β-actin) cycles of 95 °C for 30 sec, 58 °C for 30 sec and 72 °C for 30 sec, and then a final extension at 72 °C for 5 min. The PCR product was analyzed by 1.5% (w/v) agarose gel electrophoresis.

2.16 Production of recombinant *Pm*HHAP and anti-*Pm*HHAP polyclonal antibody

2.16.1 Preparation of *Pm*HHAP-recombinant pET22b(+)

The open reading frame of *Pm*HHAP conjugated with a 5′ *Nde*I and 3′ *Xho*I restriction site was amplified from the *Pm*HHAP-recombinant plasmid by PCR with the *Pm*HHAP expression primers (forward: 5′-CATATGAGCGCCGAAATGAAAACC-3′ and reverse: 5′-CTCGAGGCGTTTATAGGAGGCGATTCT-3′) for the following: 94 °C for 3 min, 30 cycles of 94 °C for 30 sec, 57 °C for 30 sec and 72 °C for 30 sec, and then a final extension at 72 °C for 5 min, and then was purified using NucleoSpin® Extract II kit (Clontech) according to the manufacturer's instruction. The purified PCR product and the pET22b(+) expression vector (Novagen) were digested with *Nde*I and *Xho*I at 37 °C for 3 h, followed by being purified using NucleoSpin® Extract II kit. The *Pm*HHAP fragment was ligated into the pET22b(+) between *Nde*I and *Xho*I cloning sites by using T4 DNA ligase (New England Biolabs) in the reaction volume of 10 µl comprising 90 *f*mole of the DNA insert and 30 *f*mole of the plasmid, and the ligation reaction occurred at 16 °C overnight. The recombinant plasmid was transformed into *E. coli* DH5α competent cells (RBC Bioscience), followed by screening on Luria-Bertani (LB) agar medium containing 100 µg/ml ampicillin at 37 °C overnight. Then *Pm*HHAP-recombinant pET22b(+) was isolated from the positive clones using QIAprep spin Miniprep Kit (QIAGEN) according to the manufacturer's instructions and sequenced using T7 promoter at Macrogen INC., South Korea to examine the correctness of the inserted *Pm*HHAP DNA sequence.

2.16.2 Optimization of the recombinant *Pm*HHAP expression condition

The *Pm*HHAP-recombinant pET22b(+) was transformed into *E. coli* Rosetta (DE3) competent cells by electroporation, followed by screening on LB agar medium containing 100 µg/ml ampicillin and 34 µg/ml chloramphenicol at 37 °C overnight. A colony of the transformed *E. coli* was grown in LB medium containing 100 µg/ml ampicillin and 34 µg/ml chloramphenicol at 37 °C overnight with agitation at 250 rpm. The culture was diluted 1:100 in fresh LB medium supplemented with 100 µg/ml ampicillin and 34 µg/ml chloramphenicol and grown until an OD_{600} of the

cultures reached 0.6. Before the induction, the split culture was served as an uninduced control. Protein expression was induced by adding IPTG to final concentration of 1 mM. The cells were harvested at 1, 2, 4 and 6 h post-induction by centrifugation at 10,000 x g for 10 min at 4 °C, then the cells were resuspended in SDS-PAGE sample buffer followed by heating at 100 °C for 10 min. The overexpression of recombinant *Pm*HHAP (r*Pm*HHAP) was analyzed by SDS-PAGE (18% (w/v) acrylamide) followed by coomassie brilliant blue staining.

2.16.3 Solubility analysis of the r*Pm*HHAP in *E. coli*

The *Pm*HHAP-transformed *E. coli* was grown in LB medium containing 100 µg/ml ampicillin and 34 µg/ml chloramphenicol at 37 °C overnight with agitation at 250 rpm. The culture was diluted 1:100 in fresh LB medium supplemented with 100 µg/ml ampicillin and 34 µg/ml chloramphenicol and grown until an OD_{600} of the cultures reached 0.6. The cells were harvested at 4 h post-induction by centrifugation at 10,000 x g for 10 min at 4 °C, then the cells were resuspended in 50 mM sodium phosphate buffer, pH 7.4 containing 50 mM NaCl by vortex. The cell suspension was disrupted by three rounds of freeze/thaw followed by sonication. After centrifugation at 10,000 x g for 10 min, supernatant and inclusion bodies were resuspended in SDS-PAGE sample buffer, followed by heating at 100 °C for 10 min. The solubility of r*Pm*HHAP was analyzed by SDS-PAGE (18% (w/v) acrylamide) followed by coomassie brilliant blue staining.

2.16.4 Purification of the recombinant *Pm*HHAP and the antibody production

After inducing the overexpression of r*Pm*HHAP with 1 mM IPTG for 4 h, the protein was found to be principally located in the inclusion bodies, so it was solubilized with 20 mM sodium phosphate buffer, pH 7.4, containing 8 M urea, 500 mM sodium chloride and 20 mM imidazole at room temperature overnight. The solubilized protein was purified using the affinity column containing Ni Sepharose™ 6 Fast Flow (GE Healthcare) according to the manufacturer's suggestion and, subsequently, the protein was refolded by dialyzing with 20 mM Tris-HCl, pH 8.0 containing 4, 2 and 0 M urea, respectively, for 3 h in each step. The purity of the

recombinant protein was analyzed by SDS-PAGE (18% (w/v) acrylamide) as above. The purified r*Pm*HHAP was used to generate rabbit polyclonal antibodies by a commercial service at Biomedical Technology Research Unit, Chiang Mai University, Thailand.

2.17 Western blot analysis of *Pm*HHAP

Total protein was extracted from the hematopoietic tissue of each shrimp using the TRI Reagent® (Molecular Research Center) according to the manufacturer's instructions. The purified r*Pm*HHAP, and the total protein from hematopoietic tissue, were separated by SDS-PAGE (18% (w/v) acrylamide), and transferred onto PROTRAN™ nitrocellulose transfer membrane (Whatman) by a semi-dry blotter. The membrane was immersed in 1X phosphate buffered saline (PBS), pH 7.4 containing 5% (w/v) skim milk and 0.05% (v/v) Tween 20 at room temperature overnight, and then incubated with a 1:1,000 dilution of rabbit anti-r*Pm*HHAP antibody in PBS, pH 7.4 containing 1% (w/v) skim milk and 0.05% (v/v) Tween 20 at 37 °C for 2 h. The membrane was washed three times with washing buffer (PBS, pH 7.4, containing 0.05% (v/v) Tween 20), and incubated with a 1:10,000 dilution of alkaline phosphatase-conjugated goat anti-rabbit IgG antibody in blocking buffer at room temperature for 1 h. Then the membrane was washed three times with washing buffer, and the color was developed with NBT/BCIP solution (Fermentas) according to the manufacturer's protocol.

2.18 *Pm*HHAP, VRP15 and SPI*Pm*2 mRNA expression in unchallenged- and WSSV-challenged shrimp hemocytes

WSSV was prepared from the gills of WSSV-challenged *P. monodon* according to the method described by Du et al., 2007, and then diluted in LHM. One hundred µl of the diluted WSSV solution (~80 viral copies/µl) was injected into each shrimp (~20 g body weight), a viral dose that had been previously determined as that which would induce a cumulative mortality of ~50% within 3 days post-injection. Control shrimp were injected with 100 µl of virus-free LHM. Hemocytes of shrimp (three individuals each) were collected at 24, 48 and 72 h post-injection, and total RNA was extracted from the hemocytes using the TRI Reagent® (Molecular Research

Center) followed by DNase (Fermentas) treatment, and used to synthesize single-stranded cDNA with the ImPromp-II™ reverse transcription system (Promega). Real-time RT-PCR was performed using an equal amount of cDNAs in iCycler iQ™ Real-Time Detection system using an IQ™ SYBR Green Supermix (Bio-Rad) with the following conditions: 95 °C for 9 min, 40 cycles of 95 °C for 30 sec, 58 °C (for *Pm*HHAP) or 60 °C (for VRP15, SPI*Pm*2 and β-actin) for 30 sec and 72 °C for 45 sec. The primers used in the experiment are *Pm*HHAP real-time PCR primer (forward: 5′-AAGAAGCCAAGCTGTCTG-3′ and reverse: 5′-CAGGAGAACCTGTGGATA-3′), VRP15 real-time PCR primer (forward: 5′-CGTCCTTCAGTGCGCTTCCATA-3′ and reverse: 5′-ACAGCGACTCCAAGGTCTACGA-3′), SPI*Pm*2 real-time PCR primer (forward: 5′-ATGCAACCACGTCTGTACTG-3′ and reverse: 5′-CTGCAAGGTTCCACATCT-3′) and β-actin real-time PCR primer (forward: 5′- GAACCTCTCGTTGCCGATGGTG-3′ and reverse: 5′- GAAGCTGTGCTACGTGGCTCTG-3′). The results are presented as the average relative expression ratio of *Pm*HHAP expressed in the hemocytes of the sample (WSSV-challenged) shrimp versus the control (unchallenged) shrimp, with normalization to the reference gene (β-actin). The relative expression ratios of *Pm*HHAP gene were calculated as described by Pfaffl, 2001. The data were analyzed using SPSS statistics 17.0 (Chicago, USA). Statistical significance was calculated by paired-samples *t*-test. Significance was accepted at $P < 0.05$.

2.19 Isolation and tissue culture pre-treatment of primary hemocyte cultures

Hemolymph was drawn from each shrimp (~20 g body weight) using a sterile 1 ml syringe with 500 µl of anticoagulant, pH 5.6 (0.82% (w/v) sodium chloride, 0.55% (w/v) citric acid, 1.98% (w/v) glucose and 0.88% (w/v) sodium citrate; adjusted to pH 5.6 with sodium hydroxide solution). The hemolymph-anticoagulant mixture was then centrifuged at 200 x g for 10 min at room temperature to separate the hemocytes from the plasma. The hemocyte pellet was resuspended in 1 ml of L-15 culture medium (1.6x Leibovitz L-15 medium (Gibco) supplemented with 20% (v/v) fetal bovine serum (FBS), 1% (w/v) glucose, 0.4% (w/v) sodium chloride, 100 IU/ml penicillin and 100 µg/ml streptomycin; pH 7.6; adjusting the osmotic pressure to 750 ± 15 mOsm/kg with sodium chloride solution). The hemocyte number was counted

under a light microscope, and then distributed into the wells of a 96-well plate at 10^5 cells per well. The total volume in each well was adjusted to 150 µl with L-15 culture medium, and the culture plate was incubated at 28 °C for 24 h, whereupon the pre-treated hemocytes were ready to be used in experiments.

2.20 *Pm*HHAP mRNA expression in unchallenged- and WSSV-challenged shrimp primary hemocyte cultures

After the pre-treatment of primary hemocyte cultures as described above, 50 µl of L-15 culture medium was removed from each well, and replaced with 50 µl of L-15 culture medium with (WSSV-challenged hemocytes) or without (SPF control hemocytes) ~10^6 WSSV viral copies, and re-incubated at 28 °C for a further 24 h. Total RNA was extracted from the hemocytes using the TRI Reagent® (Molecular Research Center) followed by DNase (Fermentas) treatment, and then used to synthesize single-stranded cDNA. RT-PCR was performed using the *Pm*HHAP-1, and β-actin primers with the conditions as described in section 2.11. The PCR product was analyzed by 1.5% (w/v) agarose gel electrophoresis, and the differential expression level was reported as relative to that of β-actin.

2.21 Protein expression and subcellular localization analysis of *Pm*HHAP in shrimp hemocytes

The diluted WSSV solution (100 µl, ~8 x 10^3 WSSV copies) was injected into shrimp (~20 g body weight) whilst unchallenged shrimp were injected with 100 µl of virus-free LHM. Sixty h post-injection, hemolymph was drawn from each shrimp using a sterile 1 ml syringe with 500 µl of the anticoagulant, pH 5.6. The anticoagulant-hemolymph (50 µl) mixture was added into 24-well plate containing a cover slip and 200 µl of L-15 culture medium in each well, and incubated at room temperature for 2 h. Subsequently, the medium was removed, and then 300 µl of 4% (w/v) paraformaldehyde in PBS, pH 7.4 was added instead. The plate was incubated at room temperature for 20 min, washed three times with PBS, pH 7.4, and then immersed in PBS, pH 7.4, and kept at 4 °C until the next treatment.

The fixed hemocytes were incubated with a 1:500 dilution of rabbit anti-r*Pm*HHAP polyclonal antibody in PBS, pH 7.4, containing 1% (v/v) FBS) at 37 °C

for 1 h whilst the negative control (non-infected) hemocytes were incubated with PBS, pH 7.4 containing 1% (v/v) FBS. Blots were then washed three times with PBS, pH 7.4, and then incubated with a 1:500 dilution of Alexa Fluor 488 goat anti-rabbit IgG antibody (Invitrogen) in PBS, pH 7.4 at room temperature for 1 h in the dark, and washed three times with PBS, pH 7.4. Subsequently, the nucleus was stained with a 1:500 dilution of TO-PRO-3 iodide (Invitrogen) in PBS, pH 7.4, at room temperature for 1 h in the dark, and washed three times with PBS, pH 7.4. The cover slips contained the stained and fixed hemocyte samples were then coated with Prolong Gold Antifade Reagent (Invitrogen) before detecting the fluorescent signal by confocal microscopy.

2.22 Production of *Pm*HHAP, VRP15 and GFP dsRNA

Two types of *Pm*HHAP DNA templates with a single T7 promoter at the 5' ends were PCR amplified from the *Pm*HHAP-recombinant plasmid by two separate PCR reactions using two primer sets including *Pm*HHAP-1 RNAi primers (forward: 5′-TAATACGACTCACTATAGGGCAACAGGAGAACCTGTGGATA-3′, reverse: 5′-GGCCTTGTGACGTTCTTCCATT-3′) and *Pm*HHAP-2 RNAi primers (forward: 5′-GCAACAGGAGAACCTGTGGATA-3′, reverse: 5′-TAATACGACTCACTATA GGGGCCTTGTGACGTTCTTCCATT-3′) with the following conditions: 94 °C for 3 min, 30 cycles of 94 °C for 30 sec, 57 °C for 30 sec and 72 °C for 30 sec, and then a final extension at 72 °C for 5 min. The two types of PCR product templates were *in vitro* transcribed using T7 RiboMAX^{TM} Express Large Scale RNA Production System (Promega) to produce two complementary ssRNAs according to the manufacturer's instruction. Then, equal amounts of each of the complementary ssRNAs were mixed together and incubated at 70 °C for 10 min, and slowly cooled down at room temperature to allow annealing to form dsRNA. The *Pm*HHAP dsRNA solution was treated with 2 units of RQ1 RNase-free DNase (Promega) at 37 °C for 15 min, and then purified by standard phenol-chloroform extraction.

VRP15 DNA templates with a single T7 promoter at the 5' ends were PCR amplified from the VRP15-recombinant plasmid by two separate PCR reactions using two primer sets including VRP15-1 RNAi primers (forward: 5′-GGATCC TAATACGACTCACTATAGGCGCGACCGAGCCAAGAGAACAT-3′, reverse: 5′-

TGAGCTGACGGAAGGCCACAGA-3′) and VRP15-2 RNAi primers (forward: 5′-CGCGACCGAGCCAAGAGAACAT-3′, reverse: 5′-GGATCCTAATACGACTCACTATAGGTGAGCTGACGGAAGGCCACAGA-3′) with the following conditions: 94 °C for 3 min, 30 cycles of 94 °C for 30 sec, 57 °C for 30 sec and 72 °C for 30 sec, and then a final extension at 72 °C for 5 min. Subsequently, VRP15 dsRNA was produced as above.

Green fluorescent protein (GFP) DNA templates with a single T7 promoter at the 5' ends were PCR amplified from the pd2EGFP-1 vector (Clontech) using the GFP1 RNAi primers (forward: 5′-TAATACGACTCACTATAGGATGGTGAGCAAGGGCGAGGA-3′, reverse: 5′-TTACTTGTACAGCTCGTCCA-3′) and GFP2 RNAi primers (forward: 5′-ATGGTGAGCAAGGGCGAGGA-3′, reverse: 5′-TAATACGACTCACTATAGGTTACTTGTACAGCTCGTCCA-3′) with the following conditions: 94 °C for 3 min, 30 cycles of 94 °C for 30 sec, 57 °C for 30 sec and 72 °C for 1 min, and then a final extension at 72 °C for 5 min. Subsequently, GFP dsRNA was produced as above.

2.23 Analysis of *Pm*HHAP gene silencing mediated by RNA interference

Shrimp were separated into four groups with three shrimp per group. Twenty-five µl of 150 mM sodium chloride solution alone (group 1) or containing 10 µg of GFP dsRNA (group 2) or 5 and 10 µg of *Pm*HHAP dsRNA (groups 3 and 4, respectively) was injected into each shrimp (~5 g body weight) of each respective group. Twenty-four h post-injection, hemocytes were collected and total RNA was extracted from them using the TRI Reagent® (Molecular Research Center) followed by DNase (Fermentas) treatment, and then single stranded cDNA was synthesized with the ImPromp-II™ reverse transcription system (Promega). RT-PCR was performed to evaluate the degree of gene transcript silencing using the *Pm*HHAP-1 and β-actin primers with the conditions as mentioned in section 2.11. The PCR product was analyzed by 1.5% (w/v) agarose gel electrophoresis, and *Pm*HHAP gene expression level was reported as relative to that of β-actin.

2.24 Analysis of VRP15 gene silencing mediated by RNA interference

Shrimp were separated into three groups with three shrimp per group. Twenty-five µl of 150 mM sodium chloride solution alone (group 1) or containing 9 µg of GFP dsRNA (group 2) or 9 µg of VRP15 dsRNA (group 3) was injected into each shrimp (~3 g body weight) of each respective group. Twenty-four h post-injection, hemocytes were collected and total RNA was extracted from them using the TRI Reagent® (Molecular Research Center) followed by DNase (Fermentas) treatment, and then single stranded cDNA was synthesized with the ImPromp-II™ reverse transcription system (Promega). RT-PCR was performed to evaluate the degree of gene transcript silencing using the VRP15 and β-actin primers with the conditions as mentioned in section 2.11. The PCR product was analyzed by 1.5% (w/v) agarose gel electrophoresis, and VRP15 gene expression level was reported as relative to that of β-actin.

2.25 Investigation of shrimp mortality after *Pm*HHAP gene knockdown

Shrimp were separated into four groups with 10 shrimp per group. Twenty-five µl of 150 mM sodium chloride solution alone (group 1) or containing 8 µg of GFP dsRNA (group 2) or 4 and 8 µg of *Pm*HHAP dsRNA (groups 3 and 4, respectively) was injected into each shrimp (~ 4 g body weight) in each respective group. Shrimp mortality was observed and recorded every 6 h post-injection for 36 h, and the results are reported as average cumulative percent mortality $\pm$ 1 SD from three experiments.

2.26 Identification of shrimp circulating hemocyte numbers after *Pm*HHAP gene knockdown

Shrimp were separated into three groups with 15 shrimp per group. Twenty-five µl of 150 mM sodium chloride solution (group 1) and 150 mM sodium chloride solution containing 4 µg of GFP dsRNA (group 2) or 4 µg of *Pm*HHAP dsRNA (group 3) was injected into each shrimp (~4 g body weight) in each group. At 12 and 24 h post-injection, 50 µl of hemolymph was drawn from 3 - 5 shrimp using a sterile 0.5 ml syringe with 50 µl of anticoagulant, pH 5.6. The total hemocyte number, (THC), in the hemolymph was counted with a hemocytometer under a light

microscope, and the average THC of each group at each time point was calculated and recorded. The experiment was done in triplicate, and the results are shown as the average THC $\pm$ 1 SD from triplicate samples.

2.27 Morphological analysis of *Pm*HHAP-depleted hemocytes by scanning electron microscopy

Twenty-five µl of 150 mM sodium chloride solution containing 5 µg of *Pm*HHAP dsRNA or GFP dsRNA was injected into each shrimp (~5 g body weight), and after either 12 or 24 h, the hemolymph was drawn from each group of shrimp using a sterile 1 ml syringe without anticoagulant, and immediately dropped onto a cover slip. Proteins in the hemolymph were washed out with 0.1 M sodium phosphate buffer, pH 7.2, and the cover slip was then immersed in 0.1 M sodium phosphate buffer, pH 7.2, containing 2.5% (w/v) glutaraldehyde at room temperature for 1 h to fix the cells. The cover slip was then washed with 0.1 M sodium phosphate buffer, pH 7.2, followed by distilled water and then dehydrated through immersion for 3 min each in a series of 30%, 50%, 70%, 90%, 100%, 100% and 100% (v/v) ethanol. Finally, the samples were dried using a critical point dryer, and coated with gold using ion sputter. The hemocyte morphology was then observed by scanning electron microscopy.

2.28 RNAi-mediated *Pm*HHAP transcript knockdown *in vitro* in primary hemocyte cultures

After pre-treatment of primary hemocyte cultures, as described above, the L-15 culture medium was completely removed from the wells and replaced with 100 µl of serum-free L-15 transfection reagent (1.6x Leibovitz L-15 medium (Gibco) supplemented with 1% (w/v) glucose, 0.4% (w/v) sodium chloride, 0.25% (v/v) Lipofectamine^{TM} 2000 (Invitrogen); pH 7.6; and the osmotic pressure adjusted to 750 ± 15 mOsm/kg with sodium chloride solution) containing 1 µg of either *Pm*HHAP or GFP dsRNA, respectively. The culture plate was incubated at 28 °C for 48 h, and then the hemocyte morphology was observed by light microscopy. To evaluate the degree of *Pm*HHAP gene knockdown, *Pm*HHAP transcript levels were evaluated. Total RNAs were extracted from the hemocytes using the TRI Reagent® (Molecular

Research Center), followed by DNase (Fermentas) treatment, and then used to synthesize single-stranded cDNA with the ImPromp-II™ reverse transcription system (Promega). The gene knockdown was analyzed by RT-PCR using the *Pm*HHAP-2 (forward: 5′-ATGAGCGCCGAAATGAAAACC-3′ and reverse: 5′-GGTTCCAG AATCGCCTCCTATA-3′) and β-actin primers as mentioned above. PCR condition for the *Pm*HHAP-2 primers consisted of 94 °C for 3 min, 33 cycles of 94 °C for 30 sec, 58 °C for 30 sec and 72 °C for 30 sec, and then a final extension at 72 °C for 5 min. The PCR product was analyzed by 1.5% (w/v) agarose gel electrophoresis, and *Pm*HHAP gene expression level was reported as relative to that of β-actin.

2.29 Investigation of VP28 transcript level after knocking down VRP15 expression in WSSV-infected hemocytes

Shrimp with different body weight were separated into four groups with two shrimp per group. Twenty-five µl of 150 mM sodium chloride solution containing 9 µg of VRP15 dsRNA (group 1) or 9 µg of GFP dsRNA (group 2) was injected into each shrimp (~3 g body weight) in each group, whilst 25 µl of 150 mM sodium chloride solution containing 15 µg of VRP15 dsRNA (group 3) or 15 µg of GFP dsRNA (group 4) was injected into each shrimp (~5 g body weight) in each group. After 24 h post-injection, 25 µl of the diluted WSSV solution (~2 x 10^3 WSSV copies) was injected into the shrimp. Hemocytes of the shrimp were collected at 24 h post-infection. Total RNAs were extracted from the hemocytes using TRI Reagent® (Molecular Research Center) followed by DNase I (Promega) treatment, and then used to synthesize single-stranded cDNAs with the ImPromp-II™ reverse transcription system (Promega). The transcription level of VRP15 and VP28 was examined by RT-PCR using the VRP15, VP28 (forward: 5′-TCACTCTTT CGGTCGTGTCG-3′ and reverse: 5′-CCACACACAAAGGTGCCAAC-3′) and β-actin primers with the conditions as mentioned in section 2.11. PCR condition for the VP28 primers consisted of 94 °C for 3 min, 30 cycles of 94 °C for 30 sec, 60 °C for 30 sec and 72 °C for 30 sec, and then a final extension at 72 °C for 5 min. The PCR product was analyzed by 1.5% (w/v) agarose gel electrophoresis, and VRP15 and VP28 gene expression level was reported as relative to that of β-actin.

CHAPTER III
RESULTS

3.1 Diagnosis of the viral infection in WSSV- and YHV-challenged shrimp

To determine the status of viral infection or its absence in the experimental shrimp, PCR and RT-PCR was adopted to detect a part of WSSV DNA genome (VP466) or YHV RNA genome (GP64), respectively in unchallenged and WSSV- or YHV-challenged shrimp. The results showed that VP466 DNA and GP64 RNA were identified in gills of WSSV- and YHV-challenged shrimp, respectively, and continuously increased following the infection time (24, 48 and 72 h post-infection), while those were not detected in unchallenged shrimp (Figure 3.1). Hence, these meant that the systemic WSSV and YHV challenges were successful.

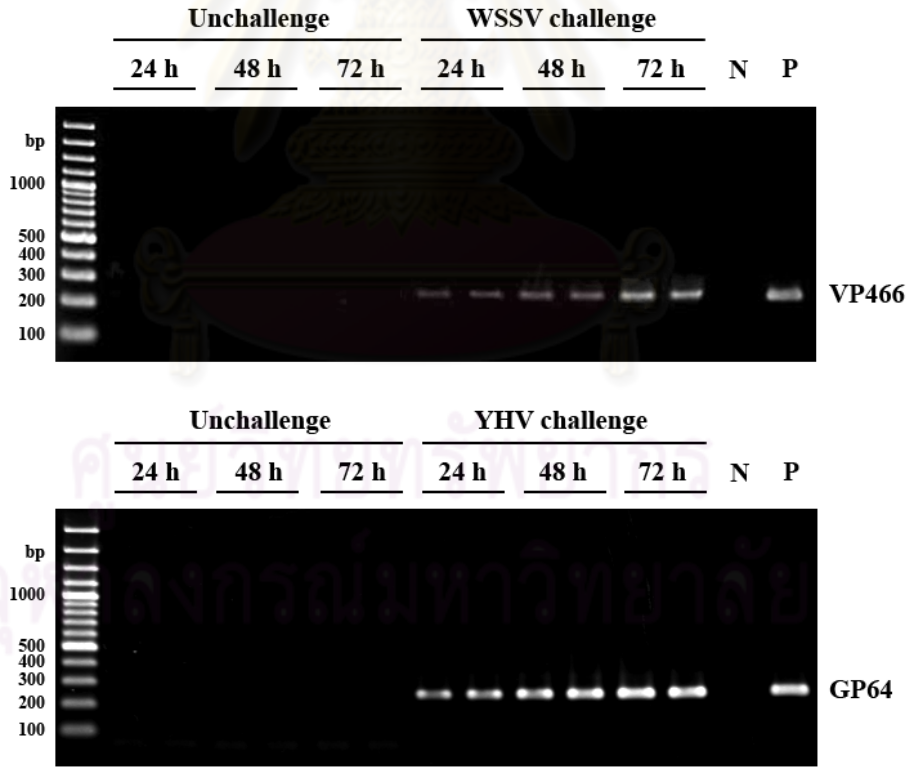

Figure 3.1 Analysis of WSSV and YHV infections in the experimental *P. monodon*. VP466 WSSV DNA and GP64 YHV RNA were examined by PCR and RT-PCR, respectively in unchallenged and virus (WSSV and YHV)-challenged shrimp at 24, 48 and 72 h post-infection. Lanes N and P are negative and positive controls, respectively.

3.2 Evaluation of SSH efficiency

Initially, poly(A)+RNAs of uninfected *P. monodon* hemocytes and virus (WSSV and YHV)-infected hemocytes (at 24, 48 and 72 h post-infection) were collected and subjected to SSH performing to generate two SSH libraries, 24I and 48/72I SSH libraries, for enriching early and late virus-induced genes, respectively. After finishing the SSH process, the success of SSHs was preliminarily evaluated by comparing the transcript abundance of a house-keeping gene, β-actin, in the cDNA samples before and after subtraction. It was found that β-actin transcript was hardly detectable in the subtracted cDNA samples of both WSSV and YHV challenge-SSH libraries, whilst the high transcript level of β-actin was still discovered in the unsubtracted cDNA samples, implying that the SSHs were successful (Figure 3.2).

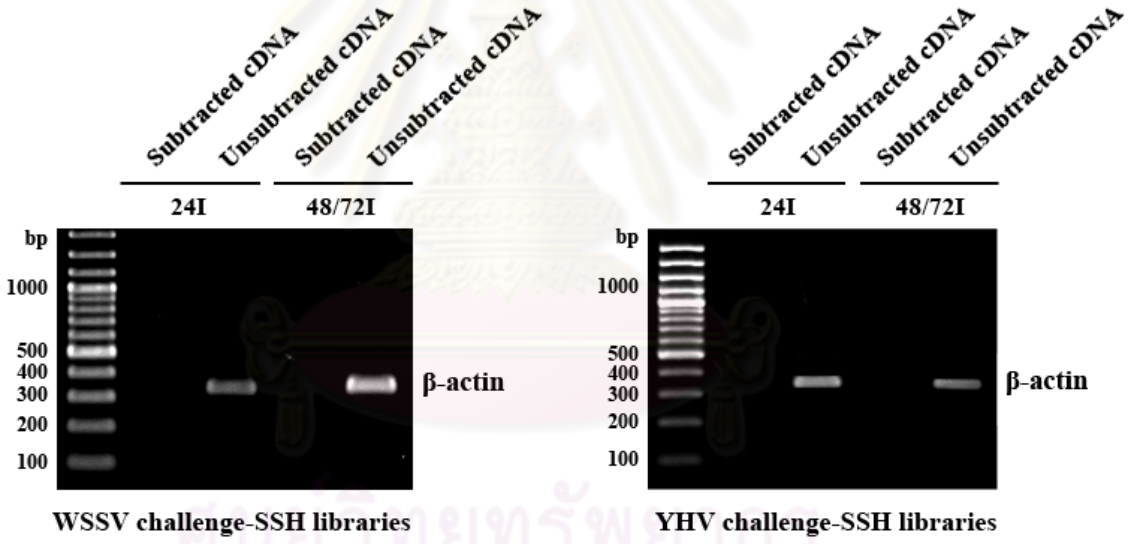

Figure 3.2 Analysis of SSH efficiency of (24I and 48/72I) WSSV challenge- and YHV challenge-SSH libraries by comparing the transcript abundance of β-actin in the unsubtracted and subtracted cDNA samples, examined by PCR.

3.3 Identification of WSSV-responsive genes by SSH and confirmation of their differential expression by RT-PCR

SSH was employed to identify viral responsive genes from the hemocytes of the black tiger shrimp, *P. monodon.* Two SSH cDNA libraries, constructed to identify viral responsive genes in the early (24I) and late (48/72I) phases of WSSV infection. Three-hundred clones from each library were randomly selected and sequenced. After searching for sequence homology in NCBI GenBank database with BLASTN and BLASTX programs, 110 and 228 non-redundant transcripts from the early and late libraries, respectively, were obtained which represent 11 and 12 known genes with significant matches (*E*-values < $1x10^{-4}$), and subsequently categorized according to their putative functions including apoptosis, cell defense and homeostasis, cytoskeletal component, energy metabolism, protein synthesis and processing, signal transduction, transcription and RNA processing, viral infection mechanism, and WSSV protein-coding genes (Table 3.1). Among the WSSV-responsive genes obtained from the SSH libraries, the viral responsive protein 15 kDa (VRP15) and *P. monodon* hemocyte homeostasis-associated protein (*Pm*HHAP) found to be greatly abundant in both early and late WSSV infection libraries, as well as moderate abundant genes, non-muscle myosin heavy chain, histone H1, Kazal-type proteinase inhibitor isoform 2, and X-box-binding protein 1, became the interesting candidates for being studied on their functions in response to viral infection. Moreover, high redundancy of WSSV-protein coding genes were observed, particularly in the 48/72I SSH library.

To confirm the differential expression of genes identified from the 24I and 48/72I SSH libraries, five different genes from both libraries, VRP15, *Pm*HHAP, histone H1, non-muscle myosin heavy chain and X-box-binding protein 1, were selected for RT-PCR. The results showed that all examined genes were up-regulated in response to WSSV infection. VRP15 and *Pm*HHAP were extremely up-regulated since 24 up to 72 h post-infection, while the transcript level of histone H1, non-muscle myosin heavy chain and X-box-binding protein 1 were significantly increased at 24 h post-infection, and then their expression were continuously decreased at 48 and 72 h post-infection (Figure 3.3). The differential expression and up-regulation of these WSSV-responsive genes verify the success of the SSHs. Among the WSSV-responsive genes, VRP15 and *Pm*HHAP have been found to show such a high up-regulation post-

WSSV infection. Thus, in this study, VRP15 and *Pm*HHAP were therefore selected to be further investigated on their functions implicated in viral infection and/or antiviral mechanisms in shrimp.

Table 3.1 Up-regulated genes in the hemocytes of WSSV-infected *P. monodon* identified from suppression subtractive hybridization.

Genes and putative functions	The closest species	GenBank accession no.	No. of clone in libraries 24I	48/72I	*E*-Value (%Identity)
Apoptosis					
Translationally controlled tumor protein	*Penaeus monodon*	EU492535	1	-	8e-55 (100%)
Cell defense and homeostasis					
Alpha-2-macroglobulin	*Penaeus monodon*	AAX24130	-	3	1e-12 (100%)
Heat shock cognate 70	*Litopenaeus vannamei*	ABP01681	1	-	4e-04 (100%)
Hemocyte homeostasis-associated protein (*Pm*HHAP)	*Penaeus monodon*	HQ130431	13	32	5e-100 (100%)
Histone H1	*Venerupis philippinarum*	ABV25905	-	18	4e-15 (65%)
Kazal-type proteinase inhibitor, isoform 2	*Penaeus monodon*	AY267200	17	-	1e-57 (100%)
Transglutaminase	*Penaeus monodon*	AAV49005	-	2	5e-29 (100%)
Sodium/potassium-transporting ATPase subunit beta	*Penaeus monodon*	EF672698	2	-	1e-64 (98%)
Cytoskeletal component					
Non-muscle myosin heavy chain	*Harpegnathos saltator*	EFN89532	33	-	1e-25 (60%)
Energy metabolism					
ATP synthase beta subunit	*Tethya actinia*	ACV88000	1	-	1e-13 (100%)
Cytochrome c oxidase subunit I	*Penaeus monodon*	EU139434	-	1	3e-112 (100%)
Protein synthesis and processing					
16S ribosomal RNA gene	*Penaeus monodon*	AF217843	-	6	6e-31 (100%)
Eukaryotic translation initiation factor 5A	*Penaeus monodon*	ABI30653	4	-	6e-10 (100%)
Signal transduction					
G protein, alpha subunit	Aplysia californica	ABD62078	-	2	8e-33 (40%)
Transcription and RNA processing					
X-box-binding protein 1	*Lycosa singoriensis*	ABX75460	8	-	8e-10 (85%)
Viral infection mechanisn					
Viral responsive protein 15 kDa (VRP15)	*Penaeus monodon*	EE661626	20	104	5e-58 (100%)
WSSV protein-coding genes					
ICP11	White spot syndrome virus	HM778020	-	11	1e-46 (100%)
Thymidine kinase	White spot syndrome virus	AF132668	-	2	3e-34 (100%)
VP15	White spot syndrome virus	DQ681072	4	37	3e-81 (100%)
VP68	White spot syndrome virus	EF534252	-	1	1e-67 (100%)
Unknown sequences			6	9	
		Total clones	**110**	**228**	

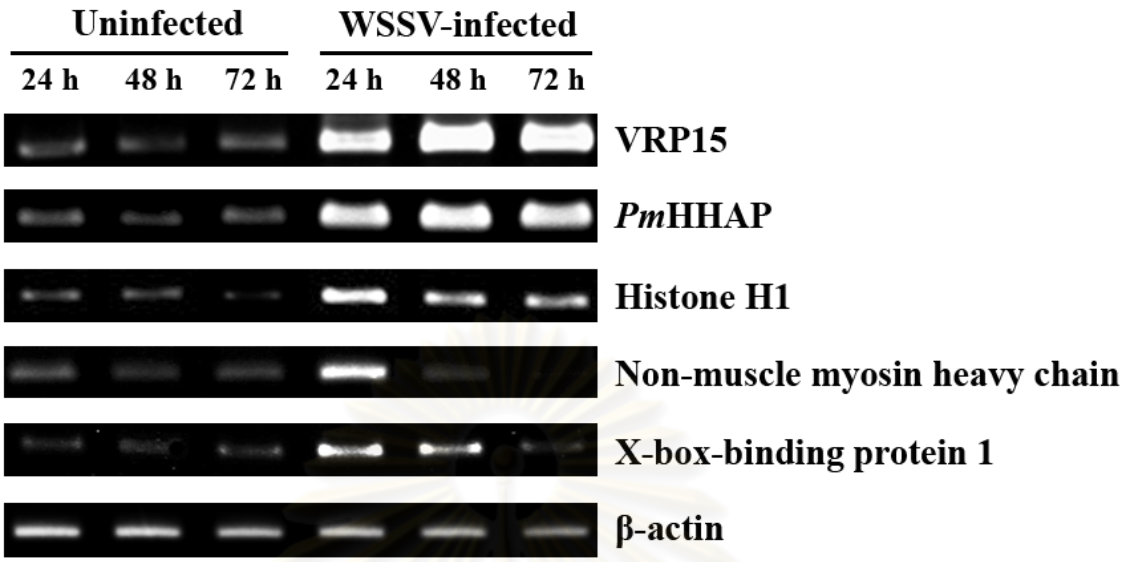

Figure 3.3 Differential expressions of five target genes, viral responsive protein 15 kDa (VRP15), *Penaeus monodon* hemocyte homeostasis-associated protein (*Pm*HHAP), histone H1, non-muscle myosin heavy chain and X-box-binding protein 1, in response to WSSV at 24, 48 and 72 h post-infection in *P. monodon* hemocytes compared to the gene expression in uninfected hemocytes, were analyzed by RT-PCR. β-actin was used as an internal reference.

3.4 Up-regulation of SPI*Pm*2 mRNA in response to WSSV infection

Serine proteinase inhibitor in the Kazal family, namely SPI*Pm*2, was among the known genes that were abundantly found in the early WSSV-infected SSH library (Table 3.1). To confirm the differential expression of SPI*Pm*2, a quantitative real-time RT-PCR was performed. The shrimp were injected with WSSV. At different time points, hemocytes were collected for total RNA preparation. Subsequently, real-time RT-PCR was adopted to determine relative expression ratio of SPI*Pm*2 expressed in the hemocytes of WSSV-infected *P. monodon* at 24, 48 and 72 h post-infection compared with the control shrimp injected with LHM, normalized to β-actin. The results indicated that SPI*Pm*2 expression was significantly up-regulated by about 1.9-fold at 24 h post-WSSV infection, and then its expression was continuously decreased at 48 and 72 h post-infection (Figure 3.4).

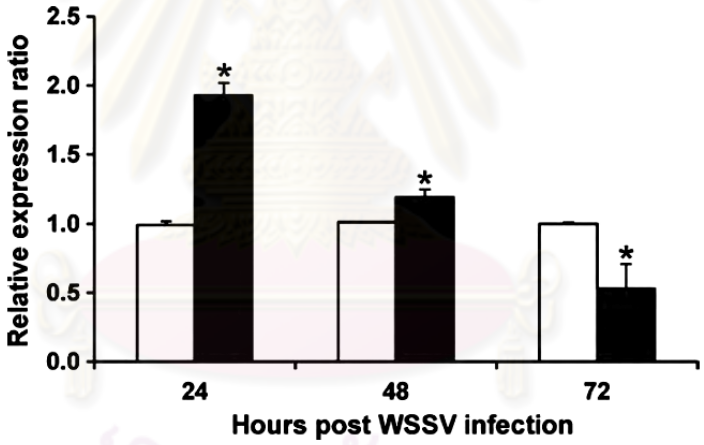

Figure 3.4 Up-rgulation of SPI*Pm*2 mRNA in response to WSSV infection. Relative expression ratios, as determined by real time RT-PCR, of SPI*Pm*2 transcript levels in the hemocytes of WSSV-infected *P. monodon* compared to that of the control (non-infected) shrimp and standardized against β-actin as the internal reference, at 24, 48 and 72 h post-WSSV infection. The data represent the mean (± 1 SD) relative expression of SPI*Pm*2 post-infection (solid bar) and the control (open bar), derived from three independent experiments. Means with an asterisk are significantly different ($P < 0.05$, analyzed by paired samples *t*-test). Relative expression ratio < 1, 1, and > 1 mean that the target gene expression is down-regulated, the same or up-regulated, respectively, in the hemocytes of WSSV-infected shrimp compared with the control.

3.5 Identification of YHV-responsive genes by SSH and confirmation of their differential expression by real-time RT-PCR

Two SSH cDNA libraries were constructed to identify YHV-induced genes in the early (24I) and late (48/72I) phases of YHV infection. From 240 randomly selected clones from each library, 154 and 223 obtained sequences with significant matches (*E*-values < $1x10^{-4}$) in the 24I and 48/72I SSH libraries, respectively, represented 72 and 16 known genes from shrimp and 3 and 7 YHV protein-coding genes, respectively (Table 3.2). High redundancy of YHV-coding genes were observed, particularly in the 48/72I SSH library. Known genes were categorized according to their putative functions (Table 3.2). The genes involved in cell defense and homeostasis were more abundant in the 24I SSH library than in the 48/72I SSH library, implying that most of immune-related genes were expressed to fight against YHV in the early stage of infection whereas their expressions were repressed in the late stage of infection. Additionally, it should be noted that a high redundancy of the gene coding for mitochondrial 16S rRNA was observed in both 24I (27 clones) and 48/72I (71 clones) SSH libraries.

To confirm the differential expression of genes identified from the 24I and 48/72I SSH libraries, 4 different immune-related genes from both libraries, anti-lipopolysaccharide factor isoform 6 (ALF*Pm*6), crustin isoform 1 (crustin*Pm*1), transglutaminase and hemocyte Kazal-type proteinase inhibitor isoform 2 (SPI*Pm*2), were selected for real-time RT-PCR based evaluation of their relative expression levels. The expression of ALF*Pm*6 was up-regulated from after 6 h up to 72 h post-infection (Figure 3.5). Crustin*Pm*1 showed the highest expression level at 6 h post-infection, and then the expression level dramatically decreased, but was still significantly up-regulated, by about 1.3-fold compared to the control shrimp, at 24 h post-infection. However, by 48 h post-infection, the expression was significantly down-regulated (Figure 3.5). The expression of transglutaminase appeared to be significantly up-regulated at only 24 h post-infection (up-regulated 1.3-fold) but was down-regulated prior to that at 6 h post-infection and after 24 h again decreased in expression levels to be significantly down-regulated at 48 and 72 h post-infection (Figure 3.5). Finally, the expression of SPI*Pm*2 was suppressed in the initial early stage (6 h) of infection, but

was increased thereafter until 48 h post-infection when it was 1.5-fold up-regulated relative to that of control shrimp, and then decreased drastically at 72 h (Figure 3.5).

Table 3.2 Up-regulated genes in the hemocytes of YHV-infected *P. monodon* identified from suppression subtractive hybridization.

Genes and putative functions	The closest species	GenBank accession no.	No. of clone in libraries		*E*-Value (%Identity)
			24I	48/72I	
Apoptosis					
Caspase-3	*Penaeus monodon*	GT067185	1	-	2e-79 (97%)
Cathepsin L	*Penaeus monodon*	EF213115	1	-	3e-50 (100%)
Histidine triad nucleotide-binding protein 2	*Esox lucius*	ACO14175	1	-	2e-18 (65%)
Programmed cell death-involved protein	*Culex quinquefasciatus*	EEC06780	1	-	1e-23 (59%)
Translationally controlled tumor protein	*Penaeus monodon*	EU492535	1	-	0.0 (100%)
Cell defense and homeostasis					
Anti-lipopolysaccharide factor, isoform 6	*Penaeus monodon*	EL595654	1	-	0.0 (99%)
C-type lectin	*Penaeus monodon*	GT067184	2	-	2e-19 (90%)
C-type lectin-like domain-containing protein PtLP	*Portunus trituberculatus*	ACC86854	1	-	2e-05 (32%)
Crustin , isoform 1	*Penaeus monodon*	FJ686014	2	-	1e-156 (99%)
Crustin, isoform 4	*Penaeus monodon*	FJ686015	1	-	2e-109 (100%)
Death domain protein	*Litopenaeus vannamei*	ACL37048	1	-	1e-11 (91%)
Epoxide hydrolase	*Caenorhabditis elegans*	ABV45407	1	-	2e-16 (38%)
Heat shock cognate 70	*Litopenaeus vannamei*	ABP01681	1	-	1e-55 (100%)
Hemolectin	*Drosophila melanogaster*	AAF49795	1	-	2e-13 (34%)
Kazal-type proteinase inhibitor, isoform 2	*Penaeus monodon*	AY267200	-	1	0.0 (98%)
Kazal-type proteinase inhibitor, isoform 5	*Penaeus monodon*	DW677898	1	-	0.0 (95%)
Phospholipid-hydroperoxide glutathione peroxidase	*Lepeophtheirus salmonis*	ABU41074	2	-	1e-27 (66%)
Prostaglandin E synthase	*Pediculus humanus*	EEB18185	-	1	6e-34 (56%)
Protein-disulfide isomerase	*Scylla paramamosain*	ACD44938	1	-	6e-87 (75%)
Sarco/endoplasmic reticulum Ca2+-ATPase	*Litopenaeus vannamei*	GR973897	2	-	8e-82 (100%)
Transglutaminase	*Penaeus monodon*	AY074924	5	-	0.0 (98%)
Tetraspanin D107	*Litopenaeus vannamei*	FE084500	1	-	0.0 (94%)
Cell growth and migration					
Membrane-associated protein gex-3	*Culex quinquefasciatus*	FE099108	1	-	8e-77 (84%)
Cytoskeletal component					
Adducin-like protein R1 isoform	*Acyrthosiphon pisum*	AAG01377	1	-	6e-11 (47%)
Arp2/3 complex 21 kD subunit	*Pediculus humanus*	EEB18691	1	-	1e-46 (69%)
Myosin IA	*Ixodes scapularis*	EEC09037	1	-	3e-05 (53%)
Nonmuscle myosin-II heavy chain	*Drosophila melanogaster*	AAB09048	4	-	5e-37 (61%)
Vinculin	*Synthetic construct*	AAX36196	1	-	2e-34 (72%)
DNA replication and repair					
Proliferating cell nuclear antigen	*Fenneropenaeus chinensis*	ABM66815	1	-	3e-23 (100%)
Energy metabolism					
ATP synthase F0 subunit 6	*Penaeus monodon*	AAF43372	-	1	6e-24 (83%)
Cytochrome b5	*Pediculus humanus*	GE615749	1	-	2e-48 (100%)
Cytochrome c oxidase subunit I	*Fenneropenaeus chinensis*	ABF83970	2	1	1e-75 (91%)
Cytochrome c oxidase subunit II	*Penaeus monodon*	AAF43375	1	-	9e-35 (91%)
Cytochrome c oxidase subunit III	*Penaeus monodon*	AAF43376	1	1	2e-65 (93%)
Dihydrolipoamide acetyltransferase	*Culex quinquefasciatus*	EDS43829	1	2	9e-51 (75%)
Dihydrolipoamide dehydrogenase precursor	*Cricetulus griseus*	AAN15202	1	-	4e-49 (82%)
Fatty acid elongation protein 3	*Litopenaeus vannamei*	FE146429	1	-	6e-72 (93%)
NADH dehydrogenase	*Aedes aegypti*	EAT41291	1	-	2e-20 (57%)
NADH dehydrogenase subunit 1	*Penaeus monodon*	AAF43378	1	-	4e-63 (88%)
NADH dehydrogenase subunit 6	*Penaeus monodon*	AAF43384	1	-	2e-22 (84%)
NADH ubiquinone dehydrogenase	*Penaeus monodon*	GO077965	1	1	1e-177 (99%)
Succinate-CoA ligase, alpha subunit	*Maconellicoccus hirsutus*	ABN12047	1	1	2e-16 (80%)
V-ATPase subunit A	*Aedes albopictus*	GO082065	1	-	1e-165 (99%)

Table 3.2 (Continued)

Genes and putative functions	The closest species	GenBank accession no.	No. of clone in libraries 24I	48/72I	*E*-Value (%Identity)
Protein synthesis and processing					
Eukaryotic translation initiation factor 3, subunit B	*Mus musculus*	AAH23767	1	-	5e-17 (60%)
Elongation factor 2	*Penaeus monodon*	EF426560	1	-	0.0 (99%)
40S ribosomal protein S23	*Penaeus monodon*	GT067183	-	1	2e-79 (99%)
Ribosomal protein S24	*Sipunculus nudus*	ABW90383	1	-	6e-13 (76%)
Ribosomal protein L3	*Penaeus monodon*	FJ997634	1	-	0.0 (99%)
Ribosomal protein L8	*Litopenaeus vannamei*	DQ316258	1	-	0.0 (91%)
Ribosomal protein L11	*Maconellicoccus hirsutus*	ABM55572	2	-	4e-45 (83%)
Ribosomal protein L18	*Homo sapiens*	EAW47614	1	-	2e-29 (46%)
Ribosomal protein L30	*Argopecten irradians*	AAN05584	1	1	4e-36 (84%)
Ribosome biogenesis protein NSA2 homolog	*Salmo salar*	ACI66914	1	-	1e-83 (73%)
Translation elongation factor EF-1 alpha/Tu	*Ixodes scapularis*	EEC19875	1	-	2e-25 (58%)
Mitochondrial 16S rRNA	*Penaeus monodon*	EF589684	27	71	5e-153 (99%)
Signal transduction					
cGMP-dependent protein kinase	*Aedes aegypti*	EAT40455	1	-	4e-43 (76%)
Cyclophilin 5 precursor	*Pediculus humanus*	EEB15506	-	1	9e-49 (73%)
EF hand-containing protein 1, isoform CRA_a	*Rattus norvegicus*	EDL75629	1	-	5e-04 (36%)
Integrin beta 1	*Plutella xylostella*	GQ178290	2	2	2e-08 (61%)
Phospholipase C gamma	*Ixodes scapularis*	EEC01651	1	-	7e-32 (59%)
Protein phosphatase 1 catalytic subunit gamma	*Taeniopygia guttata*	ACH46336	1	-	3e-105 (97%)
Rab11	*Litopenaeus vannamei*	FE130921	1	-	4e-87 (97%)
Rac GTPase-activating protein 1, isoform CRA_a	*Mus musculus*	EDL04119	1	-	9e-04 (43%)
Transcription and RNA processing					
ATP-dependent chromatin assembly factor	*Penaeus monodon*	GE615311	-	1	0.0 (98%)
General transcription factor IIH subunit 3	*Caligus clemensi*	EAW98427	1	-	1e-23 (50%)
Heterogeneous nuclear ribonucleoprotein L	*Pediculus humanus*	GE615970	1	-	4e-48 (100%)
High mobility group 20A, isoform CRA_b	*Litopenaeus vannamei*	FE179993	1	-	2e-34 (83%)
Muskelin 1	*Rattus norvegicus*	EDM15281	1	-	3e-04 (36%)
RNA polymerase II ctd phosphatase	*Pediculus humanus*	EEB15640	1	-	2e-39 (41%)
U2 small nuclear ribonucleoprotein auxiliary	*Carcinus maenas*	DY308616	1	-	5e-13 (82%)
Zinc finger protein 84 (HPF2) isoform 1	*Homo sapiens*	EAW54800	1	-	7e-25 (48%)
Hypothetical protein					
Similar to GG13705	*Penaeus monodon*	DW677926	1	-	7e-145 (98%)
Similar to AGAP006684-PA	*Litopenaeus vannamei*	FE089592	1	-	4e-51 (84%)
Conserved hypothetical protein	*Pediculus humanus*	EEB10667	1	-	3e-49 (39%)
HEAT repeat-containing protein 5B	*Ixodes scapularis*	EEC11700	1	-	3e-34 (45%)
Similar to transmembrane protein Tmp21	*Litopenaeus vannamei*	FE062382	1	-	4e-59 (92%)
Hypothetical protein BRAFLDRAFT_82353	*Branchiostoma floridae*	EEN62723	1	-	1e-59 (67%)
GI11059	*Drosophila mojavensis*	EDW05500	1	-	1e-11 (51%)
YHV protein-coding genes					
3C-like protease	Yellow head virus	ACH99403	-	5	6e-72 (100%)
Glycoprotein 64	Yellow head virus	ACH99409	27	44	4e-80 (100%)
Glycoprotein 116	Yellow head virus	ABL96315	-	4	2e-44 (100%)
Helicase	Yellow head virus	ACH99406	-	11	3e-153 (100%)
Nucleocapsid protein	Yellow head virus	ACA21303	2	8	1e-74 (100%)
Replicase polyprotein 1ab	Yellow head virus	ACA21302	13	62	6e-85 (100%)
RNA polymerase	Yellow head virus	ACH99405	-	3	1e-152 (100%)
Unknown sequences			80	7	
		Total clones	**234**	**230**	

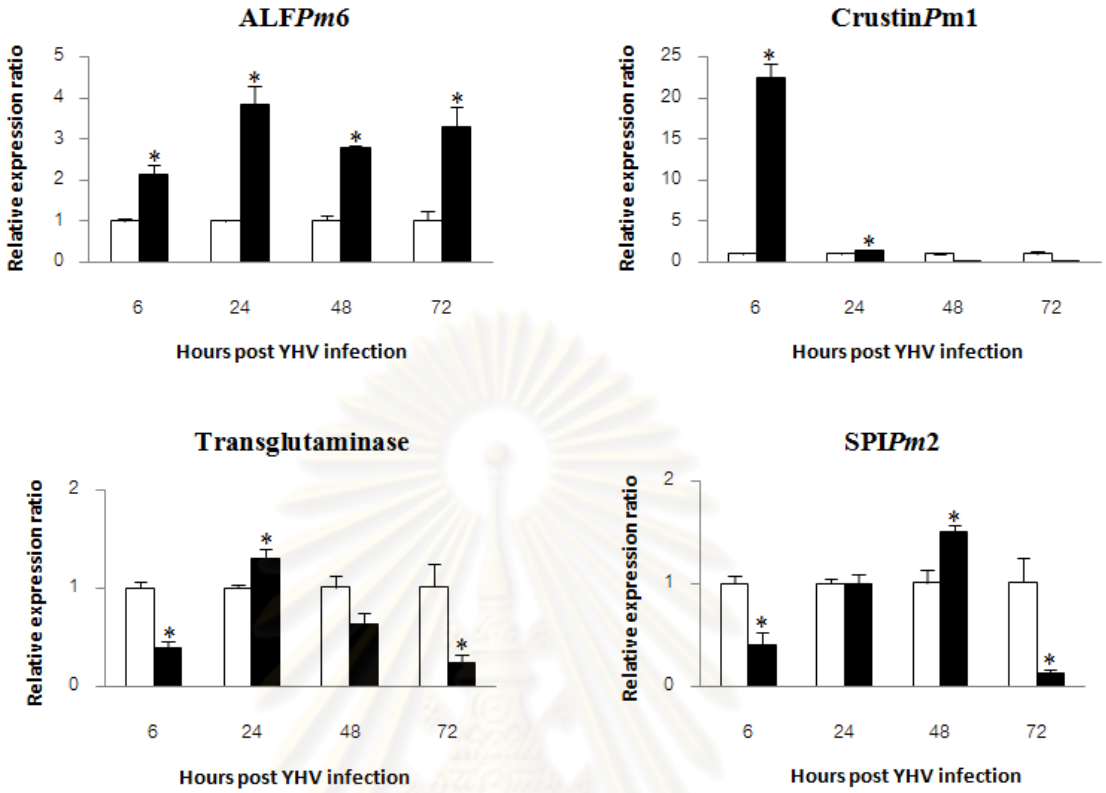

Figure 3.5 Relative expression ratios of four different target genes, anti-lipopolysaccharide factor isoform 6 (ALF*Pm*6), crustin antimicrobial peptide (crustin*Pm*1), transglutaminase and hemocyte Kazal-type proteinase inhibitor isoform 2 (SPI*Pm*2), expressed in the hemocytes of YHV-infected *P. momodon* compared to uninfected shrimp and standardized against β-actin as the internal reference, were analyzed by real-time RT-PCR at 6, 24, 48 and 72 h post-YHV infection. The data represent the mean (± 1 standard deviation) relative expression of the target genes post-infection (solid bar) and the controls (open bar), derived from three independent experiments. Means with an asterisk are significantly different ($P < 0.05$, analyzed by paired samples *t*-test). Relative expression ratio < 1, 1 and > 1 mean that the target gene expression is down-regulated, the same or up-regulated, respectively, in the hemocytes of YHV-infected *P. momodon* compared with the control.

From the results, these four target genes, selected from the two SHH libraries as up-regulated genes following YHV infection, were indeed significantly up-regulated in the hemocytes of YHV-infected shrimp in either the early, late or both stages of YHV infection. The RT-PCR expression profiles of these four gene transcripts mostly matched their corresponding presence or absence status in the two SSH libraries, the exception being that no up-regulation of ALF*Pm*6 was detected in the late SSH library unlike the clear up-regulation seen with the RT-PCR analysis. Thus, overall these RT-PCR expression profiles confirm the success of the SSHs.

3.6 The full-length cDNA of *Pm*HHAP and sequence analysis

The partial sequence of the *P. monodon* shrimp HHAP (*Pm*HHAP) cDNA was previously obtained from the SSH library of WSSV-challenged *P. monodon* hemocytes. The full-length cDNA of *Pm*HHAP identified using 5' RACE contains 643 base pairs with a complete open reading frame encoding 93 amino acids (Figure 3.6). The full *Pl*HHAP sequence has also been determined from the freshwater crayfish, *Pacifastacus leniusculus* (Prapavorarat et al., 2010). The pairwise alignment of the shrimp *Pm*HHAP and the crayfish *Pl*HHAP amino acid sequences exhibited 47% identity, and they share the eight conserved cysteine residues (Figure 3.7). The size of the *Pm*HHAP mRNA obtained was also confirmed by Northern blot analysis showing the corresponding size of about 643 base pairs (Figure 3.8). The BLAST homology search indicated that the putative predicted protein sequence encoded by *Pm*HHAP had a significant amino acid sequence similarity (54%) to the hypothetical protein TcasGA2_TC006773 from the red flour beetle, *Tribolium castaneum*. From a protein-structural analysis, the protein is expected to be a DNA-binding protein (99.2% possibility; DBS-Pred, available on-line) (Ahmad et al., 2004), and potentially can form four disulfide bridges (DiANNA 1.1 web server) (Ferrè and Clote, 2005), but it has no predicted signaling domain (Simple Modular Architecture Research Tool (SMART), available on-line) (Schultz et al., 1998).

Figure 3.6 Nucleotide and deduced amino acid sequences of *Pm*HHAP. An asterisk indicates the stop codon. The polyadenylation site is underlined.

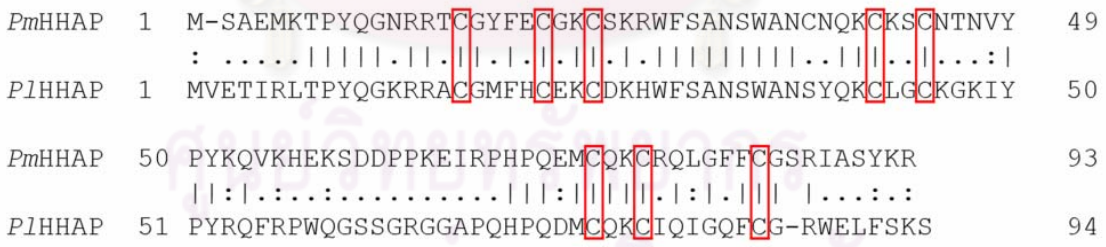

Figure 3.7 Pairwise alignments of deduced amino acid sequences of *Pm*HHAP and *Pl*HHAP were performed using EMBOSS Pairwise Alignment (available on-line). The conserved eight cysteine residues are boxed. The symbols (|, :, and .) indicate amino acid identity, similarity, and unrelated, respectively.

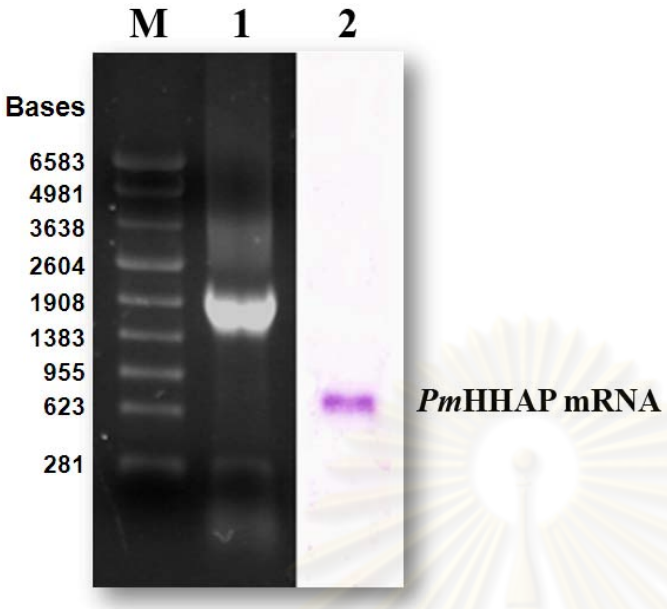

Figure 3.8 Northern blot analysis of *Pm*HHAP. Lane 1, total RNA extracted from hemocytes of WSSV-infected shrimps was electrophoresed in the 1.2% (w/v) formamide/formaldehyde-agarose gel. Lane 2, *Pm*HHAP mRNA was detected by Northern blot analysis probing with DIG-labeled *Pm*HHAP-specific DNA probe. Lane M is the RNA marker.

3.7 *Pm*HHAP expression in shrimp tissues

The tissue distribution of *Pm*HHAP transcript in the normal shrimp, *P. monodon* was examined by RT-PCR using the gene specific primers. The shrimp *Pm*HHAP was transcribed in all tested tissues but was highly expressed in the heart, hematopoietic and intestine tissues, whilst the transcript level in hemocytes was at relative low levels (Figure 3.9). In addition, the crayfish *Pl*HHAP transcript profiles had also been examined and found to be different from those of the shrimp (Prapavorarat et al., 2010). The *Pl*HHAP transcripts were identified in hematopoietic and nerve tissues of the crayfish, but at very low levels compared to that found in the shrimp tissues, and not at detectable level in the other tissues including hemocytes (Prapavorarat et al., 2010).

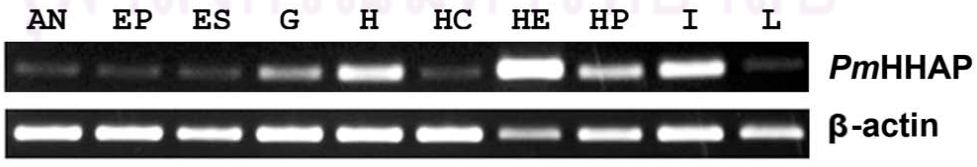

Figure 3.9 mRNA expression analysis of *Pm*HHAP in various shrimp tissues by RT-PCR. The figure shows antennal gland (AN), epipodite (EP), eye stalk (ES), gill (G), heart (H), hemocyte (HC), hematopoietic tissue (HE), hepatopancreas (HP), intestine (I), lymphoid (L). β-actin was used as an internal reference.

3.8 Production of recombinant *Pm*HHAP in *E. coli* expression system

Firstly, the *Pm*HHAP open reading frame was cloned into the pET22b(+) expression vector between *Nde*I and *Xho*I cloning sites, and the recombinant plasmid was transformed into *E. coli* Rosetta (DE3). r*Pm*HHAP with histidine tag (-Leu-Glu-His_6) at C-terminus was produced in the *E. coli* induced by adding IPTG to final concentration of 1 mM and then incubated for 0, 1, 2, 4 and 6 h. The overexpression of r*Pm*HHAP was analyzed by SDS-PAGE. The result showed that the r*Pm*HHAP was expressed in the highest level at 4 h post-IPTG induction, while its expression was not detected in *E. coli* without IPTG-induction (Figure 3.10A). Moreover, the r*Pm*HHAP was found to be principally located in inclusion bodies, but not in soluble form (Figure 3.10B).

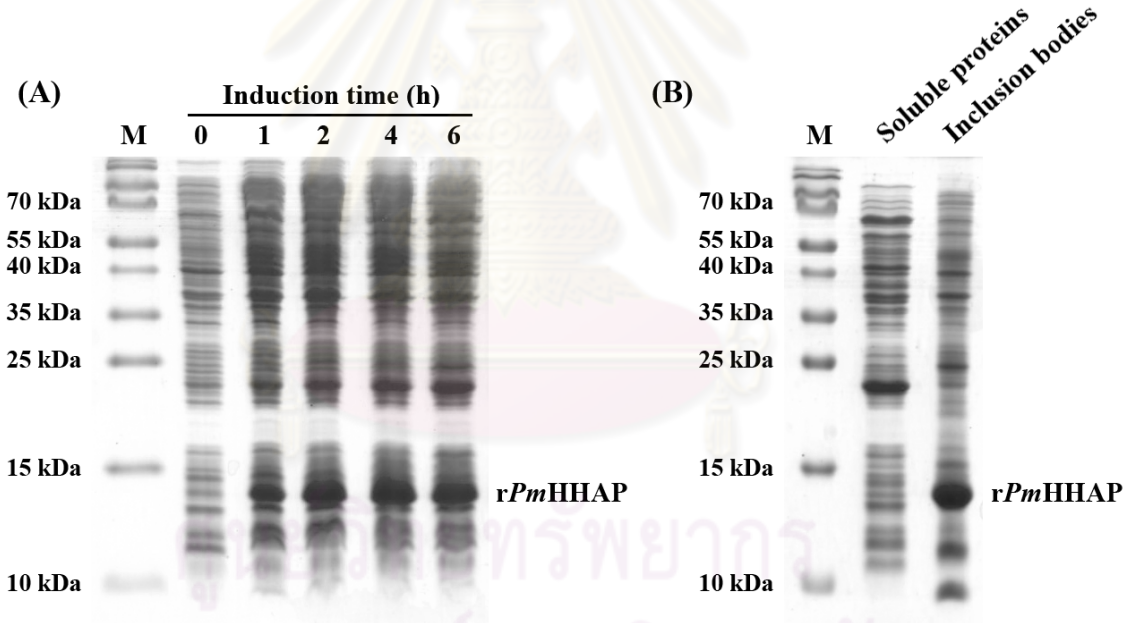

Figure 3.10 r*Pm*HHAP production in *E. coli* expression system. A, r*Pm*HHAP was expressed in *E. coli* Rosetta (DE3) induced with 1 mM IPTG for 0, 1, 2, 4 and 6 h. The overexpression of r*Pm*HHAP was analyzed by SDS-PAGE (18% (w/v) acrylamide) followed by coomassie brilliant blue staining. B, Solubility of r*Pm*HHAP expressed in the *E. coli* was examined in the fractions of soluble proteins and inclusion bodies, analyzed by SDS-PAGE (18% (w/v) acrylamide) followed by coomassie brilliant blue staining. Lane M is the protein marker.

3.9 *Pm*HHAP was found among hematopoietic proteins

Initially, the r*Pm*HHAP with histidine tag was expressed in a bacterial expression system by IPTG induction for 4 h (Figure 3.11, lane 1). Since the expressed r*Pm*HHAP was located in inclusion bodies, it was then purified by using Ni Sepharose-containing column under denaturing condition. After affinity chromatography purification, a purified r*Pm*HHAP was obtained (Figure 3.11, lane 2). The purified r*Pm*HHAP was subsequently used to generate an anti-r*Pm*HHAP polyclonal antibody in immunized rabbits. Western blot analysis indicated that the antibody reacted specifically with the r*Pm*HHAP (Figure 3.11, lane 4). The crude protein extracted from the shrimp hematopoietic tissue is shown in Figure 3.11, lane 3. Western blot analysis with anti-r*Pm*HHAP antibody identified the endogenous *Pm*HHAP as one of the proteins in the hematopoietic tissue of *P. monodon* shrimp (Figure 3.11, lane 5) with an approximately 1 kDa lower molecular mass than the recombinant protein.

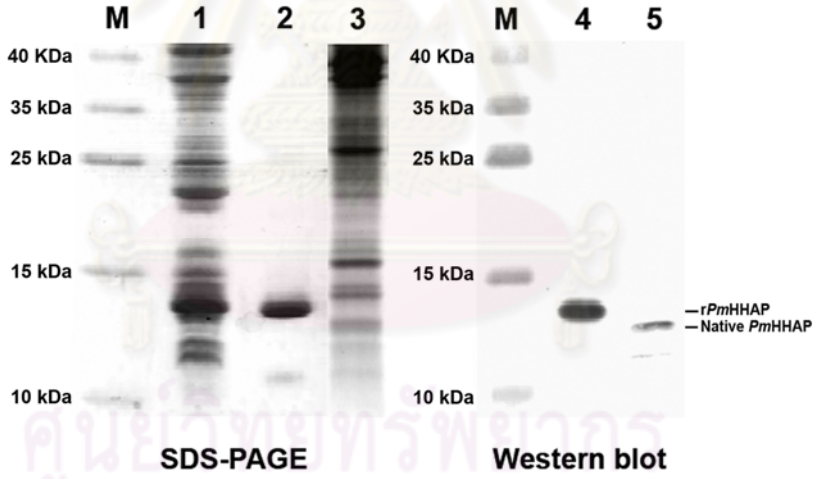

Figure 3.11 SDS-PAGE and Western blot analysis of r*Pm*HHAP and native *Pm*HHAP from *P. monodon* shrimp hematopoietic tissue. The crude protein extracted from *Pm*HHAP-transformed *E. coli* after IPTG induction for 4 h (lane 1), the purified r*Pm*HHAP (lane 2) and the crude protein extracted from shrimp hematopoietic tissue (lane 3) were separated by SDS-PAGE followed by coomassie brilliant blue staining. The purified r*Pm*HHAP and the crude protein from *P. monodon* hematopoietic tissue were transferred onto PROTRAN^{TM} nitrocellulose transfer membrane (Whatman) for Western blot analysis probing with rabbit anti-r*Pm*HHAP antibody: lane 4, r*Pm*HHAP; lane 5, native *Pm*HHAP. Lane M is the protein marker.

3.10 Up-regulation of *Pm*HHAP mRNA in response to WSSV infection

*Pm*HHAP transcript levels were evaluated by real-time RT-PCR. The results clearly demonstrated that *Pm*HHAP mRNA expression was highly up-regulated in shrimp hemocytes after WSSV challenge, increasing by about 3.0, 8.3 and 33.3 fold at 24, 48 and 72 h post-infection, respectively, as compared to the gene expression in non-infected SPF hemocytes (Figure 3.12A). Likewise, in the shrimp primary hemocyte cell culture, up-regulation of *Pm*HHAP transcript levels in WSSV-infected *in vitro* hemocytes was also detected when compared to non-infected SPF hemocyte cells (Figure 3.12B). These results strongly suggest that *Pm*HHAP transcript is significantly up-regulated in the mature *P. monodon* shrimp hemocytes in response to WSSV infection.

3.11 Differential expression and subcellular localization of *Pm*HHAP in shrimp hemocytes

The expression of *Pm*HHAP protein in hemocytes was evaluated with immunofluorescence and confocal microscopy. The hemocyte nucleus was stained with TO-PRO-3 iodide (blue fluorescent dye) whilst *Pm*HHAP were recognized with anti-r*Pm*HHAP antibody and visualized as a green (Alexa Fluor 488) fluorescent dye (Figure 3.13). The *Pm*HHAP signal in the WSSV-infected hemocytes was much stronger than that in the non-infected hemocytes, whereas the control where primary antibodies were not added had no signal (Figure 3.13). Taken together, the results reveal that the hemocytic *Pm*HHAP is not only up-regulated at the mRNA level but also at the protein level following WSSV infection. Moreover, it was also found that *Pm*HHAP was mainly distributed in the cytoplasm, and only partly located in a nuclear compartment of the hemocytes (Figure 3.13).

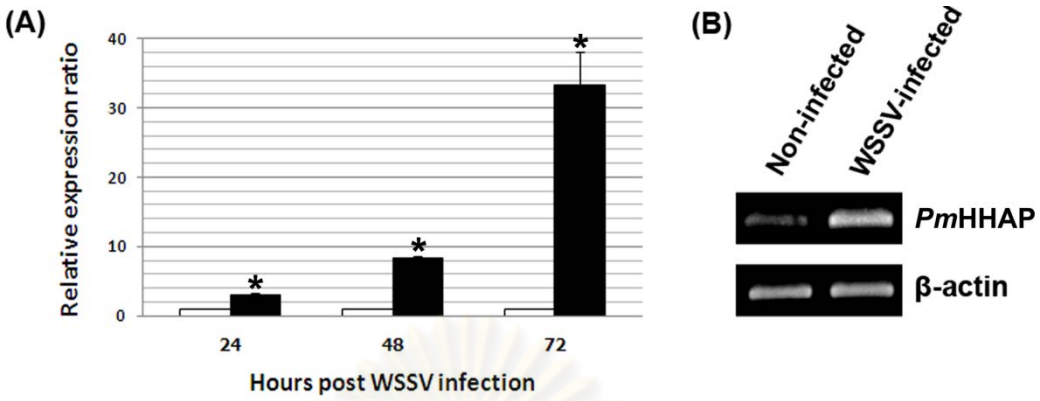

Figure 3.12 Up-regulation of *Pm*HHAP mRNA in response to WSSV infection. A, relative expression ratios, as determined by real time RT-PCR, of *Pm*HHAP transcript levels in the hemocytes of WSSV-infected *P. monodon* compared to that of the control (non-infected) shrimp and standardized against β-actin as the internal reference, at 24, 48 and 72 h post-WSSV infection. The data represent the mean (± 1 SD) relative expression of *Pm*HHAP post-infection (solid bar) and the control (open bar), derived from three independent experiments. Means with an asterisk are significantly different ($P < 0.05$, analyzed by paired samples *t*-test). Relative expression ratio < 1, 1, and > 1 mean that the target gene expression is down-regulated, the same or up-regulated, respectively, in the hemocytes of WSSV-infected shrimp compared with the control. B, differential expression of *Pm*HHAP mRNA in shrimp primary hemocyte cultures after WSSV infection compared with non-infected cells when examined by RT-PCR at 24 h post-WSSV infection. β-actin was used as internal reference.

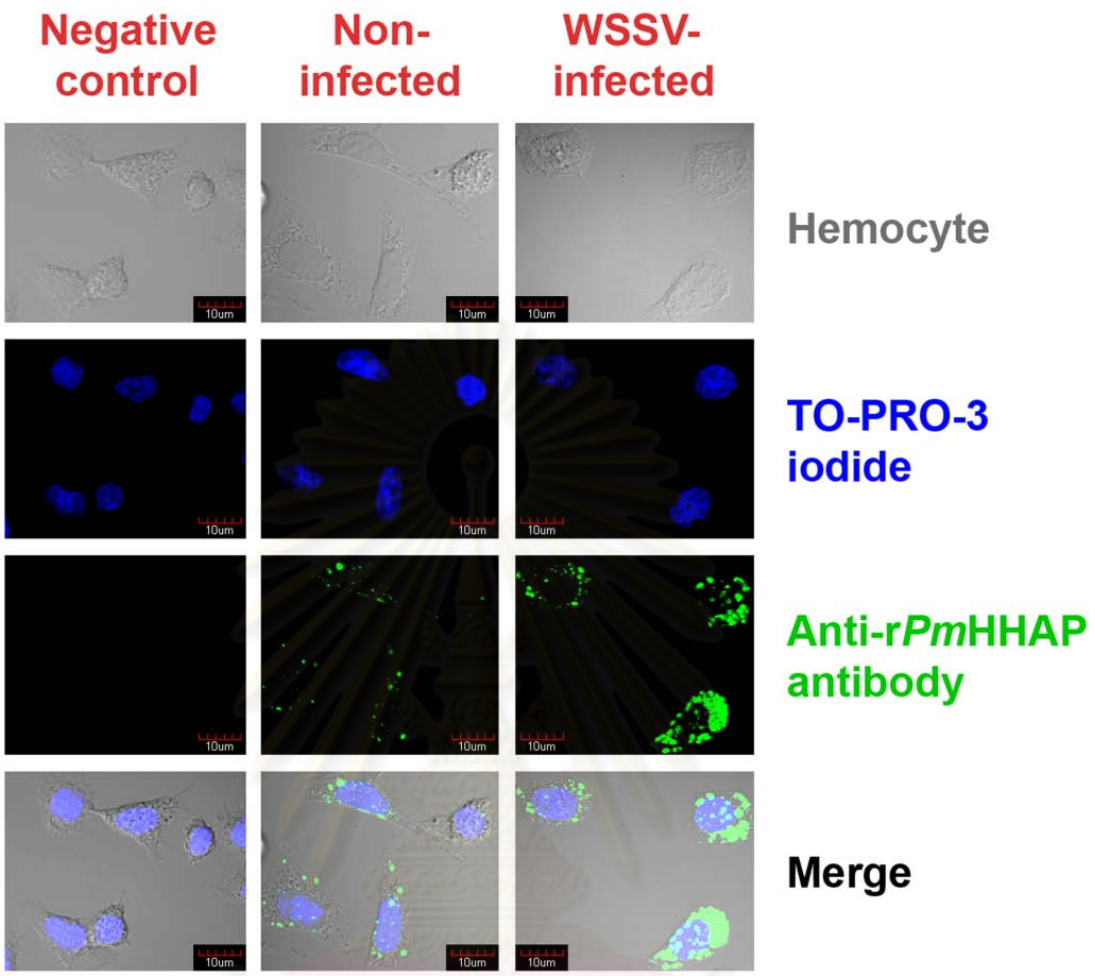

Figure 3.13 *Pm*HHAP protein expression analysis in non-infected and WSSV-infected *P. monodon* hemocytes by immunofluorescence microscopy. The hemocytes from non-infected and WSSV-infected shrimp were stained with anti-r*Pm*HHAP (green) and nuclei (blue) as detailed under the method section. The fluorescent signal was detected by confocal microscopy. Images shown are representative of at least three such fields of view per sample and three independent samples.

3.12 Double strand RNAi-mediated knockdown of *Pm*HHAP gene expression resulted in shrimp mortality

RNA interference (RNAi) has become a powerful tool to identify gene function by silencing the expression of specific genes with dsRNA, and then analyzing their loss-of-function phenotype. Here, the systemic injection of 5 µg (1 µg dsRNA/g of shrimp) and 10 µg (2 µg dsRNA/g of shrimp) of *Pm*HHAP dsRNA into shrimp of ~5 g body weight could knock down *Pm*HHAP gene expression by about 46% and 80%, respectively, while an injection of sodium chloride solution or GFP dsRNA had no effect on the gene silencing (Figure 3.14A). Surprisingly, the knocked down shrimp displayed a very rapid mortality since 12 h post-*Pm*HHAP dsRNA injection (Figure 3.14B). The shrimp challenged with 1 µg and 2 µg *Pm*HHAP dsRNA per gram of shrimp displayed a cumulative mortality of around 20% and 70% at 18 h post-the dsRNA injection, respectively, and reached 100% mortality within 36 and 30 h post-injection, respectively. In contrast, the control groups had no shrimp deaths over the 36 h time course studied (Figure 3.14B). Accordingly, the results indicate that *Pm*HHAP is indispensable for the survival of shrimp.

3.13 Significant decrease in circulating mature hemocyte numbers after *Pm*HHAP gene knockdown

After *Pm*HHAP dsRNA injection, peripheral (circulating) hemocytes in the shrimp hemolymph were reduced following dsRNA injection, whereas an injection of GFP dsRNA did not lead to any decrease in the hemocyte number (Figure 3.15A), and this affect was more marked at 24 h after injection than after 12 h suggesting a possible continuous decline in levels until death. Evaluation of this data in terms of the concentration of circulating hemocytes in the knocked down shrimp reveals the same pattern, namely a two- and four- fold lower level of circulating hemocytes, compared to the control shrimp, at 12 and 24 h post-*Pm*HHAP dsRNA injection, respectively (Figure 3.15B).

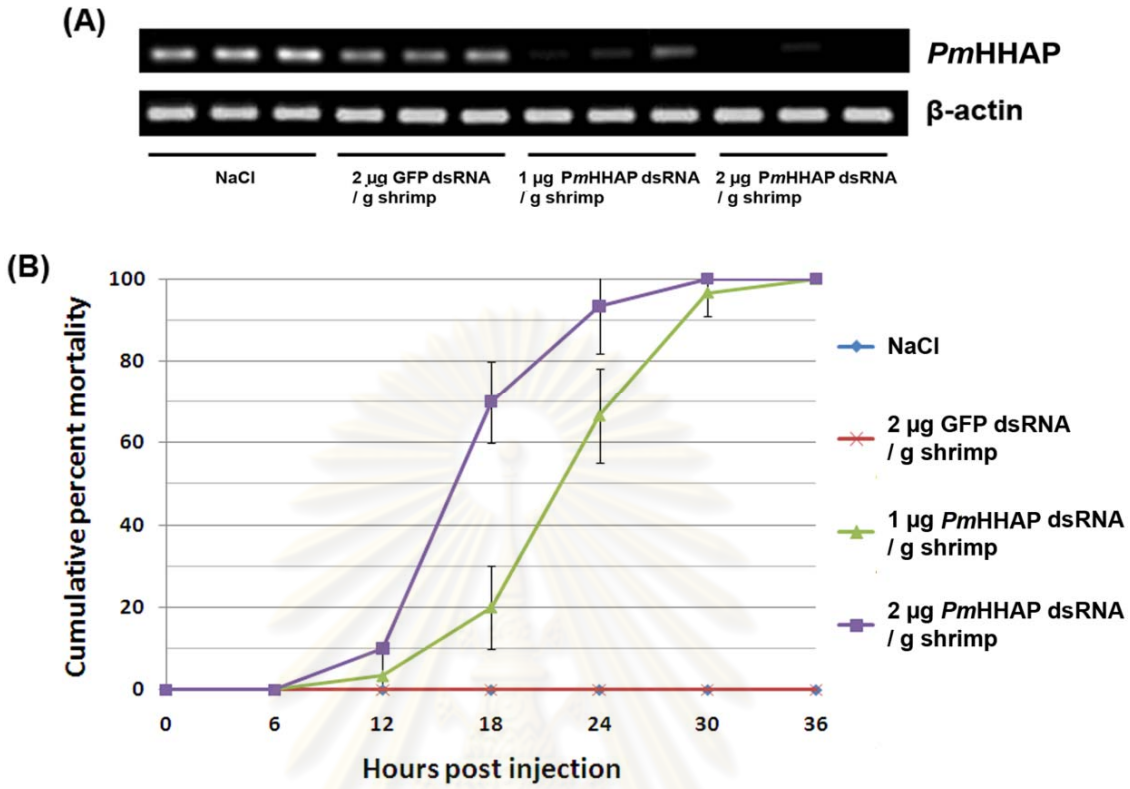

Figure 3.14 Knockdown of *Pm*HHAP expression resulted in shrimp mortality. A, gene-specific knockdown of *Pm*HHAP transcript levels in WSSV-uninfected *P. monodon* hemocytes. The hemocytes from shrimp injected with 150 mM sodium chloride solution alone (NaCl) or containing GFP dsRNA (2 μg / g shrimp) or *Pm*HHAP dsRNA (1 and 2 μg / g shrimp) for 24 h, were collected and used to analyze *Pm*HHAP gene expression levels by RT-PCR with β-actin as the internal reference. B, cumulative mortality analysis of *Pm*HHAP-depleted shrimp. Shrimp were injected with NaCl alone or containing GFP dsRNA (2 μg / g shrimp) or *Pm*HHAP dsRNA (1 and 2 μg / g shrimp), and their mortality was recorded every 6 h thereafter for 36 h. Data are reported as the average ± 1 SD and are derived from three independent trials.

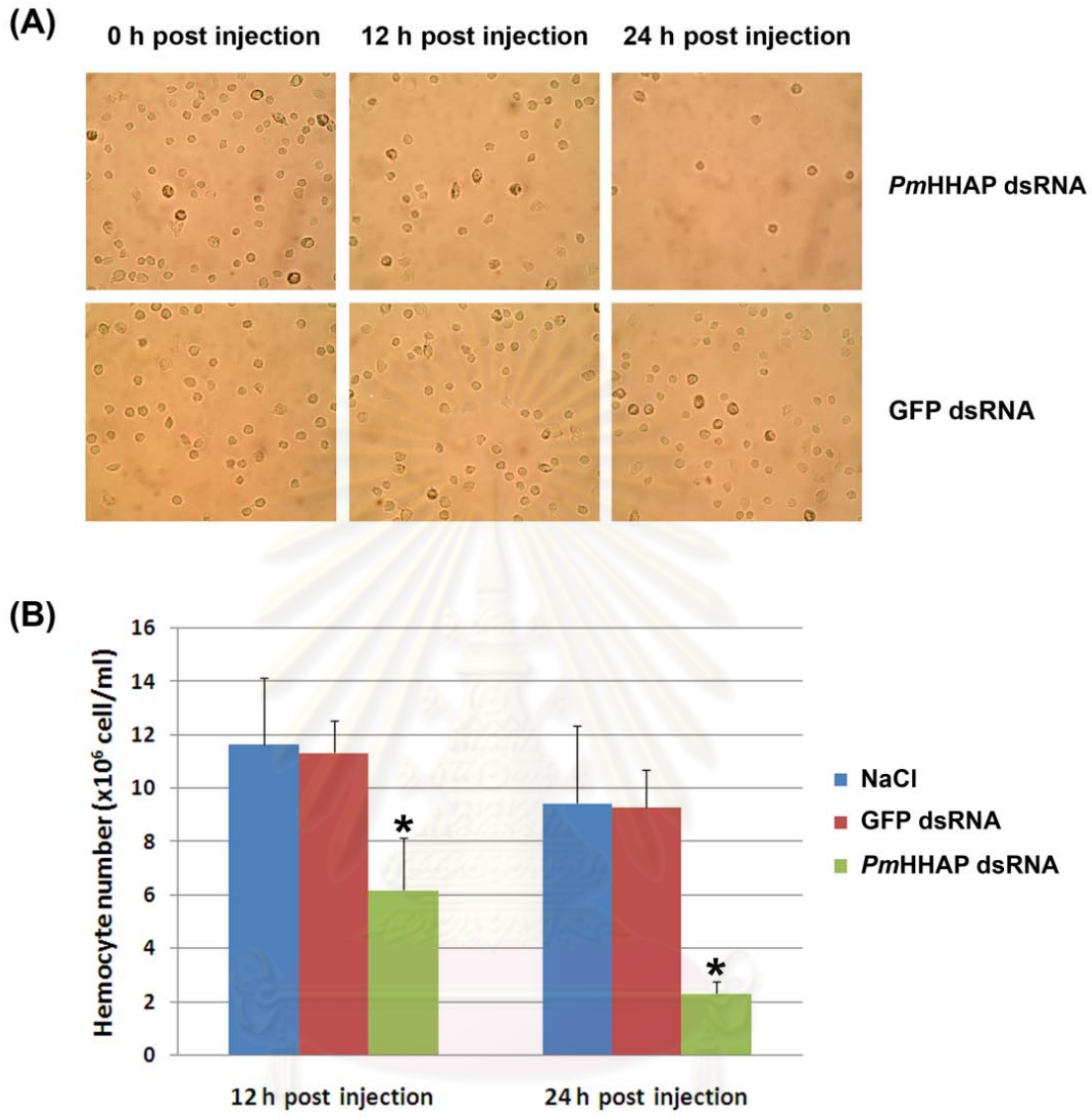

Figure 3.15 Reduction of *P. monodon* hemocyte numbers after *Pm*HHAP knockdown. The hemocyte numbers of shrimp injected with 150 mM sodium chloride solution (NaCl), *Pm*HHAP dsRNA or GFP dsRNA were examined at 0, 12 and 24 h post-injection. A, the hemocyte density in "as extracted and seen" hemolymph. Photographs shown are representative of at least two such fields of view per sample and three different samples. B, the hemocyte density in the shrimp hemolymph. Data are reported as the average ± 1 SD, derived from three independent trials. Means with an asterisk are significantly different ($P < 0.05$, analyzed by one-way ANOVA and Scheffe *post hoc* test).

3.14 Morphology changes in *Pm*HHAP-depleted hemocytes

To analyze the morphology of *Pm*HHAP-knocked down hemocytes, the scanning electron microscope was used in the experiment. All the observed hemocytes from the control group of shrimp, that is those that were injected with NaCl alone or containing GFP dsRNA for 12 and 24 h, were completely normal, and no morphological changes were observed. In contrast, clear changes in the morphology of the hemocytes were detected in the hemolymph of shrimp after *Pm*HHAP dsRNA-mediated gene knockdown (Figure 3.16). At 12 h post-*Pm*HHAP dsRNA injection, some hemocytes appeared abnormal and deformation was initiated, and by 24 h after the dsRNA injection, cell lysis and fragmentation into apoptotic-like bodies were observed (Figure 3.16). Moreover, *in vitro Pm*HHAP knockdown in the shrimp primary hemocyte cell culture corroborated the above *in vivo* results, with severe cell damage occurring in the *Pm*HHAP-depleted hemocytes while most of the control hemocytes still looked healthy (Figure 3.17). Altogether, our results reveal an essential function of *Pm*HHAP in hemocyte homeostasis.

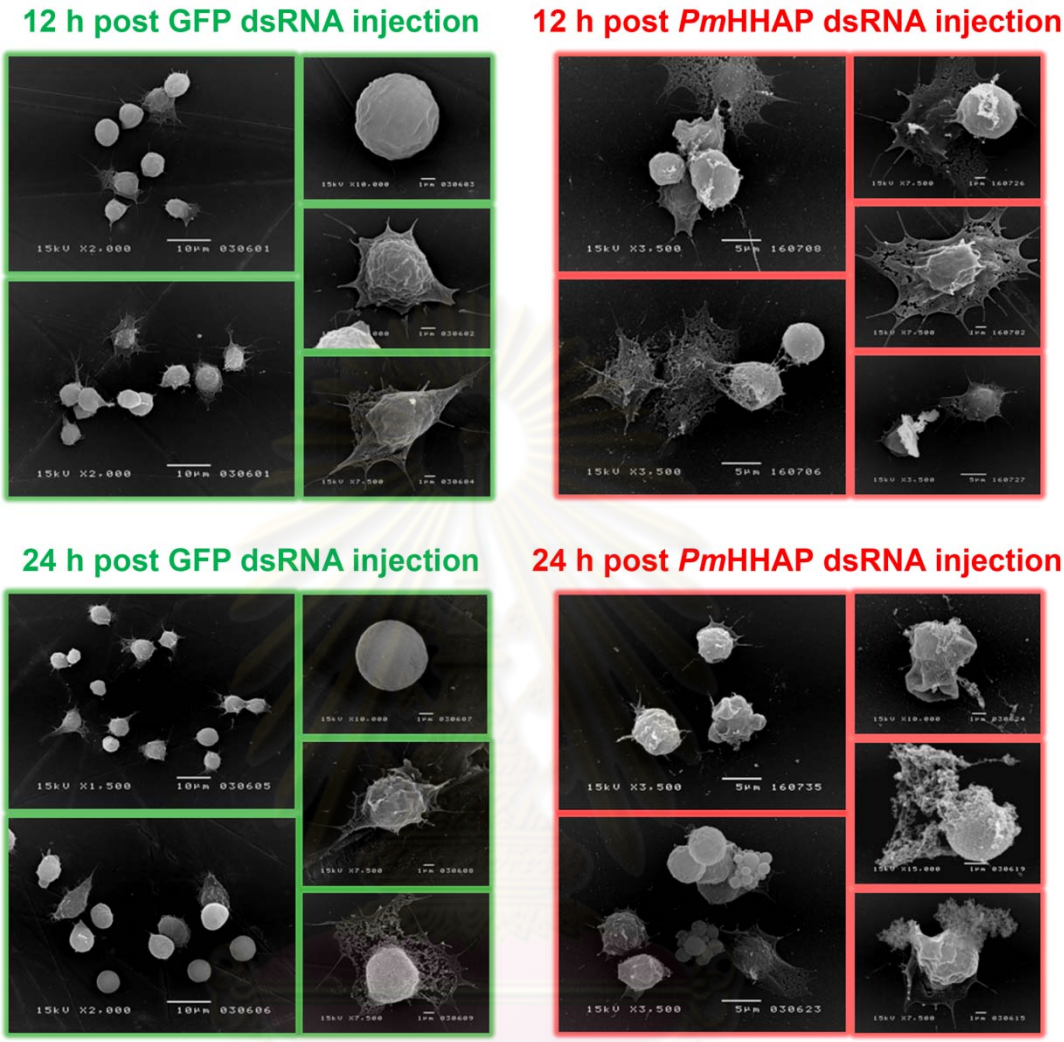

Figure 3.16 Morphological analysis of *P. monodon* hemocytes in *Pm*HHAP-knocked down and control (GFP dsRNA-injected) shrimp. The hemocytes were collected from shrimp 12 and 24 h after injection with GFP dsRNA or *Pm*HHAP dsRNA at 1 µg dsRNA / g shrimp, fixed in 2.5% (w/v) glutaraldehyde solution, and subsequentially prepared for and analyzed by scanning electron microscopy. Micrographs shown are representative of at least five fields of view per sample and three independent samples.

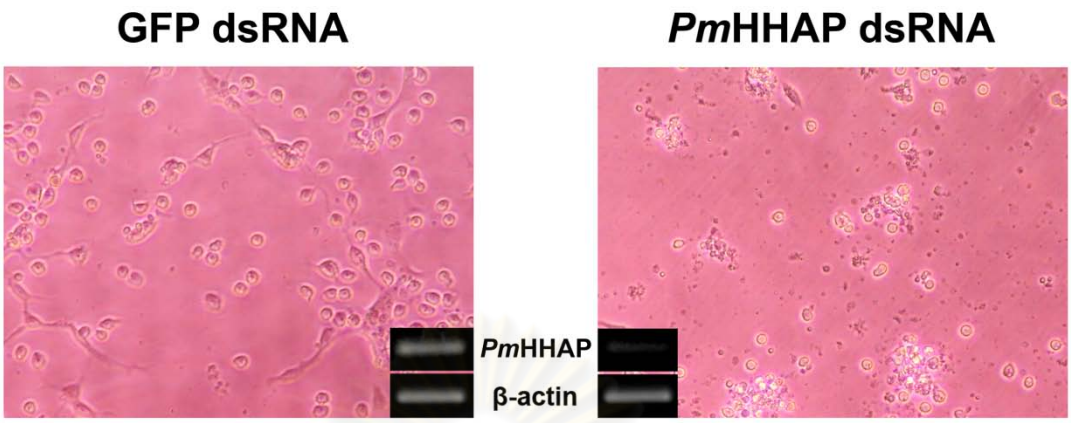

Figure 3.17 Severe cell damage occurred in the *Pm*HHAP-depleted primary hemocyte cell culture. Hemocytes from WSSV-uninfected SPF *P. monodon* were collected and precultured in L-15 culture medium before *in vitro* transfection with 1 µg of *Pm*HHAP or GFP dsRNA for 48 h. The hemocyte morphology was then observed by light microscopy. Total RNA was extracted from the transfected hemocytes, and subjected to RT-PCR for analysis of *Pm*HHAP gene knockdown, with β-actin as internal reference (Inset). Photographs shown are representative of at least two such fields of view per sample and three different samples

3.15 The full-length cDNA of VRP15 and sequence analysis

The partial sequence of the VRP15 cDNA was formerly obtained from the SSH library of WSSV-challenged *P. monodon* hemocytes as mentioned above. The full-length cDNA of VRP15 was then identified using 5′ RACE, and it contained 722 base pairs with a complete open reading frame encoding 137 amino acids (Figure 13.18). The size of the *Pm*HHAP mRNA obtained was confirmed by Northern blot analysis showing the corresponding size of about 722 base pairs (Figure 13.19). The BLAST homology search indicated that the putative predicted protein sequence encoded by VRP15 had no significant amino acid sequence similarity to any proteins in the database (*E*-value > 10^{-4}). From a protein-structural analysis, the protein is consisted of a transmembrane helix of 23 amino acids (TMHMM Server v. 2.0, available on-line) (Krogh et al., 2001) and it has no predicted signaling domain (Simple Modular Architecture Research Tool (SMART), available on-line) (Schultz et al., 1998).

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   1 AGCGGCCGCGACCGAGCCAAGAGAACGTTCACTCGATCACCACTCTCGTTCTTTGATCTA   60
  61 CGCAATGTTAACAGAGGACTTAGTAAACCTGGTGTACGAGGTGTGTCAAGAGAAGAAGCT  120
         M  L  T  E  D  L  V  N  L  V  Y  E  V  C  Q  E  K  K  L
 121 GCGAGCGGCGGTGAAATGCATCCTTCAGTGCGCTTCCATACCATTCGTCTCAACAATAGC  180
      R  A  A  V  K  C  I  L  Q  C  A  S  I  P  F  V  S  T  I  A
 181 CGTAGCTCTGTATATGGGCCCCTTGGGCGTCTTGCTGGGTGGCGCTGTAGGTACTGGGAT  240
      V  A  L  Y  M  G  P  L  G  V  L  L  G  G  A  V  G  T  G  I
 241 CTCCTACGTCTATGCTAGGGGGAAGTTCAAAAGCGTCGTTAGCATTATCAGGGACGACTT  300
      S  Y  V  Y  A  R  G  K  F  K  S  V  V  S  I  I  R  D  D  L
 301 GACTCCACAGGAAAGGGAGAGGCTCATGATGAGGGTGCGGGCCGCTCTCGTAGACCTTGG  360
      T  P  Q  E  R  E  R  L  M  M  R  V  R  A  A  L  V  D  L  G
 361 AGTCGCTGTCGGGGCCTCTGTGGCCTTCCGTCAGCTCACCGAGCCCATGAAGTCGGAGAT  420
      V  A  V  G  A  S  V  A  F  R  Q  L  T  E  P  M  K  S  E  I
 421 CGCTGCTACTGTCAAGAAGTACTTGGAGTATGACCACAACATGTCAGTAGAGCATTAAAT  480
      A  A  T  V  K  K  Y  L  E  Y  D  H  N  M  S  V  E  H  *
 481 GCCTAAAAGACTGTTCAGGTGAATGGCGAGAACGACGGTTTCTTTTCTGTTTGCATTTGT  540
 541 TAGCGAAGATGGGTTCGCTGTTAGTACTACTTTTGGAAATTGGATTTGTTTTATGTTCGA  600
 601 GGCAAAAATGTGAAAGAGACAGTTCCAAAATAAACAAATAAAACTATCAAAAAAAAAAAA  660
 661 AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA  720
 721 AA  722
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Figure 3.18 Nucleotide and deduced amino acid sequences of VRP15. An asterisk indicates the stop codon. A transmembrane domain is boxed. The polyadenylation site is underlined.

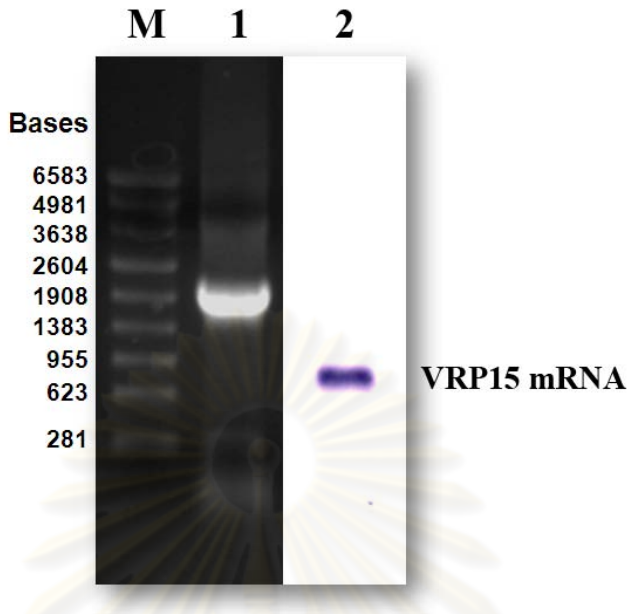

Figure 3.19 Northern blot analysis of VRP15. Lane 1, total RNA extracted from hemocytes of WSSV-infected shrimps was electrophoresed in the 1.2% (w/v) formamide/formaldehyde-agarose gel. Lane 2, VRP15 mRNA was detected by Northern blot analysis probing with DIG-labeled VRP15-specific DNA probe. Lane M is the RNA marker.

3.16 VRP15 expression in shrimp tissues

The tissue distribution of VRP15 transcript in normal shrimp was examined by RT-PCR using the gene specific primers. The shrimp VRP15 transcript was found in all tested tissues but was highly expressed in hemocytes, whilst the transcript level in antennal gland, epipodite, eye stalk was at relative low when compared to that of other tissues. (Figure 3.20).

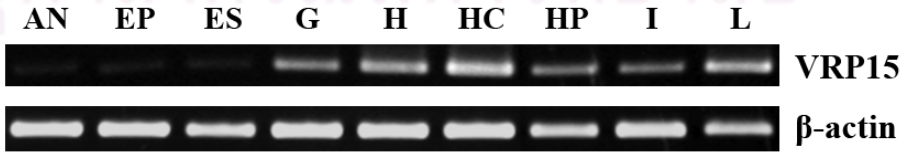

Figure 3.20 mRNA expression analysis of VRP15 in various shrimp tissues by RT-PCR. The figure shows antennal gland (AN), epipodite (EP), eye stalk (ES), gill (G), heart (H), hemocyte (HC), hepatopancreas (HP), intestine (I), lymphoid (L). β-actin was used as an internal reference.

3.17 Up-regulation of VRP15 mRNA in response to WSSV infection

VRP15 transcript levels were evaluated by real-time RT-PCR. The results clearly demonstrated that VRP15 mRNA expression was greatly up-regulated in shrimp hemocytes after WSSV challenge, increasing by about 3.6, 9410.1 and 1351.2 fold at 24, 48 and 72 h post-infection, respectively, as compared to the gene expression in non-infected SPF hemocytes (Figure 3.21). This result clearly suggests that VRP15 transcript is significantly up-regulated in the *P. monodon* shrimp hemocytes in response to WSSV infection, corresponding to the gene up-regulation status analyzed by RT-PCR as described previously (Figure 3.3).

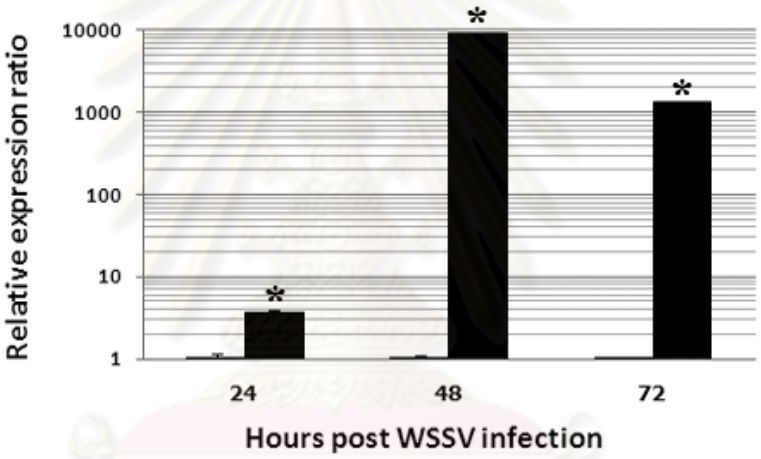

Figure 3.21 Up-regulation of VRP15 mRNA in response to WSSV infection. A, relative expression ratios, as determined by real time RT-PCR, of VRP15 transcript levels in the hemocytes of WSSV-infected *P. monodon* compared to that of the control (non-infected) shrimp and standardized against β-actin as the internal reference, at 24, 48 and 72 h post-WSSV infection. The data represent the mean (± 1 SD) relative expression of VRP15 post-infection (solid bar, right) and the control (open bar, left), derived from three independent experiments. Means with an asterisk are significantly different ($P < 0.05$, analyzed by paired samples *t*-test). Relative expression ratio < 1, 1, and > 1 mean that the target gene expression is down-regulated, the same or up-regulated, respectively, in the hemocytes of WSSV-infected shrimp compared with the control.

3.18 Double strand RNAi-mediated knockdown of VRP15 gene expression resulted in reducing viral replication in WSSV-infected shrimp

The systemic injection of 9 µg (3 µg dsRNA/g of shrimp) of VRP15 dsRNA into shrimp of ~3 g body weight could knock down VRP15 gene expression by about 70%, while an injection of sodium chloride solution or GFP dsRNA (3 µg dsRNA/g of shrimp) had no effect on the gene silencing (Figure 3.22). Interestingly, the VRP15 gene knockdown in WSSV-infected shrimp could reduce WSSV replication in shrimp. It was found that VP28 transcript was significantly decreased in two shrimp groups (~3 and ~5 g body weight) injected with VRP15 dsRNA (3 µg dsRNA/g of shrimp) prior WSSV injection (Figure 3.23). The results suggest that the novel VRP15 gene is important for viral propagation as its knock-down leads to decrease in viral infection.

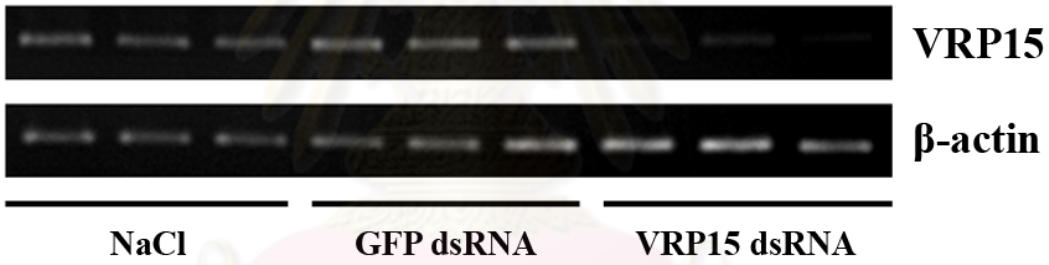

Figure 3.22 Gene-specific knockdown of VRP15 transcript levels in WSSV-uninfected *P. monodon* hemocytes. The hemocytes from shrimp injected with 150 mM sodium chloride solution alone (NaCl) or containing GFP dsRNA (3 µg / g shrimp) or VRP15 dsRNA (3 µg / g shrimp) for 24 h, were collected and used to analyze VRP15 gene expression levels by RT-PCR with β-actin as the internal reference.

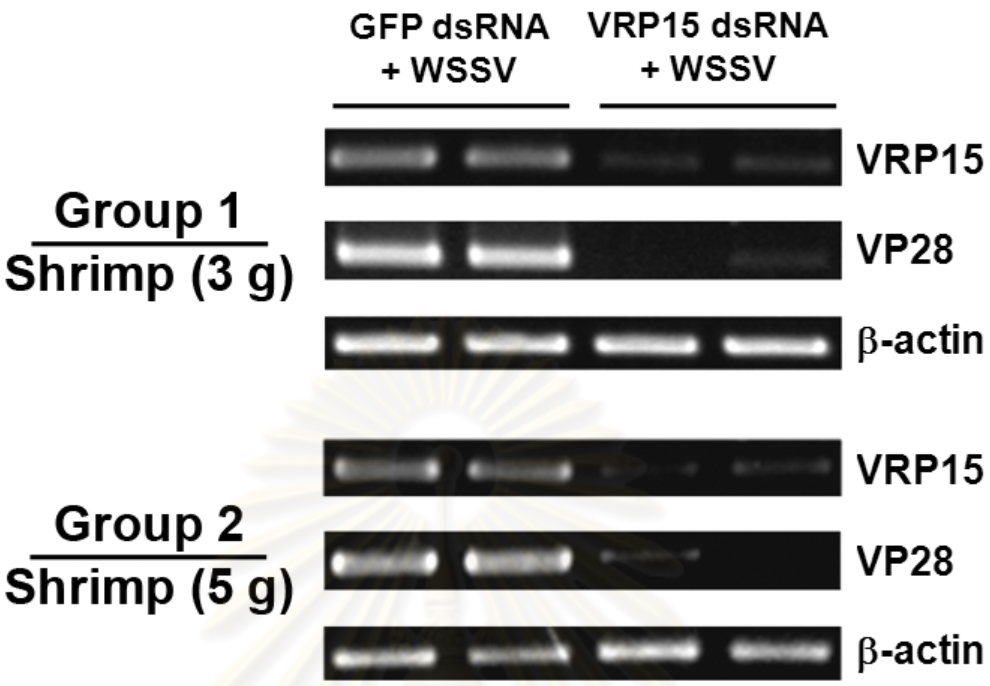

Figure 3.23 Knockdown of VRP15 gene expression leads to a reduction of WSSV replication in *P. monodon*. The hemocytes from two groups of shrimp (~3 and ~5 g body weight) injected with 150 mM sodium chloride solution containing GFP dsRNA (3 µg / g shrimp) or VRP15 dsRNA (3 µg / g shrimp) for 24 h followed by being injected with WSSV for 24 h, were collected and used to analyze VRP15 and VP28 gene expression levels by RT-PCR with β-actin as the internal reference.

CHAPTER IV
DISCUSSIONS

The outbreak of viral diseases resulting from WSSV and YHV caused severe losses to the shrimp culture industry worldwide, but up to now there is still neither the effective treatment for the infected shrimp nor the protection against the viral diseases. To develop a better understanding of shrimp antiviral defenses as well as viral infection mechanism, and possibly develop new control measures for WSSV and YHV infection, suppression subtractive hybridization, a powerful approach for identifying differentially expressed genes, was herein adopted to determine WSSV and YHV-responsive genes in shrimp. In this study, four SSH libraries were constructed, including 24I and 48/72I WSSV challenge-SSH libraries to identify genes up-regulated in early and late phases of WSSV infection, respectively, and 24I and 48/72I YHV challenge-SSH libraries to identify genes up-regulated in early and late phases of YHV infection, respectively. From the WSSV challenge-SSH libraries, a number of genes proposed to be involved in immune responses against WSSV infection were uncovered such as translationally controlled tumor protein, alpha-2-macroglobulin, heat shock cognate 70, *Penaeus monodon* hemocyte homeostasis-associated protein (*Pm*HHAP), Kazal-type proteinase inhibitor isoform 2 (SPI*Pm*2) and transglutaminase, and we also found some genes that facilitated WSSV infection such as viral responsive protein 15 kDa (VRP15) and ICP11. The actions of these genes regarding viral infection and/or anti-viral mechanisms had been discussed below. Among the WSSV-responsive genes obtained from the SSH libraries, two novel viral responsive genes/proteins found for the first time in crustacean, namely *Pm*HHAP, and the first time here, referred to as VRP15, showed to be greatly abundant in both early and late WSSV infection libraries, and RT-PCR confirmed their indeed high up-regulation post-WSSV infection. Hence, *Pm*HHAP and VRP15 have principally been focused in this research to reveal their functions associated with viral infection and/or antiviral mechanisms in *P. monodon* shrimp.

Furthermore, the YHV challenge-SSH libraries had unveiled genes implicated in various cellular functions, and they were found in the 24I SSH library more than in

the 48/72I SSH library where genes related to energy production and protein synthesis dominated. A high up-regulation of mitochondrial 16S rRNA found in early and especially in late phases of viral infection reflected requirements for high energy utilization which may serve viral propagation as indicated by high expression of viral (WSSV and YHV) proteins in the late infection period. The genes involved in the innate immune system were identified at high proportion in the early phase of YHV infection (24I SSH library) and lower in late phase of YHV infection (48/72I SSH library), probably implying that the protective molecules were highly expressed to participate in viral defense response in early phase of viral infection. A previous microarray research supporting this hypothesis disclosed that most genes related to defense and homeostasis were down-regulated in the mid and especially in the late stages of viral infection that may well reflect intensive viral propagation at the late stage of infection (Pongsomboon et al., 2008). However, few numbers of the genes found in WSSV challenge-SSH library and the small sample size of non redundant annotated genes presenting in YHV challenge-SSH libraries prevent any definitive comparative analysis.

Formerly, the studies using comparative EST analyses and cDNA microarray approaches reported several up-regulated genes in virus-infected shrimp (Pongsomboon et al., 2008; Leu et al., 2007; Wang et al., 2006). Most of these studies have concentrated on identification of genes involved in the host response to WSSV. In this study, we identified a number of novel YHV-responsive genes from the SSH libraries, several of which were previously reported to participate in defense against WSSV such as caspase, heat shock cognate 70, C-type lectin, hemocyte kazal-type proteinase inhibitor, anti-lipopolysaccharide factor, translationally controlled tumor protein, proliferating cell nuclear antigen, integrin and transglutaminase (Tonganunt et al., 2008; Zhao et al., 2009; Ma et al., 2008; Wang et al., 2008; Rijiravanich et al., 2008; Wanget al., 2006; Kong et al., 2009; Liu et al., 2006; Tharntada et al., 2009; Xie et al., 2008; Li et al., 2007; Zhang et al., 2009; Maningas et al., 2008). This could indicate that YHV infection in shrimp induced similar host immune responses as observed during WSSV infection. The potential involvements in host antiviral immune responses and/or viral infection of some interesting viral responsive genes isolated from the SSH libraries are discussed as follow.

Serine proteinase inhibitors (SPIs) play important roles as regulators of numerous biological processes. Here, the results from the SSH libraries and real-time RT-PCR indicated that SPI*Pm*2 was up-regulated in response to both WSSV and YHV infection. Moreover, a significantly higher expression level of Kazal-type SPI transcripts was also observed in WSSV-infected Chinese shrimp, *Penaeus (Fenneropenaeus) chinensis* (Kong et al., 2009). These information suggest a potential role of the Kazal-type SPI in viral defense mechanism in crustaceans. SPI*Pm*2, a *P. monodon* SPI with five kazal domains, was previously reported to be expressed only in the hemocytes of *P. monodon* (Jarasrassamee et al., 2005), and showed inhibitory activity against various proteinases such as subtilisin, elastase and trypsin (Somprasong et al., 2006), and possessed bacteriostatic activity against *Bacillus subtilis* by inhibiting subtilisin release (Donpudsa et al., 2009). Due to the resultant up-regulation of SPI*Pm*2 after viral infection obtained from this study, subsequently, the attempt to investigate the involvement of SPI*Pm*2 in antiviral process was first established in *P. monodon*. It was found that the injection of the recombinant SPI*Pm*2 prior to WSSV injection resulted in a significant inhibition of WSSV replication, and also prolonged the mortality rate of WSSV-infected *P. mododon* (Donpudsa et al., 2010). Therefore, the SPI*Pm*2 was involved in the innate immunity against WSSV infection in shrimp. Although the mechanism of Kazal-type SPIs in the defense system of shrimp is still largely unknown, one might speculate from their proteinase-inhibitory activities that Kazal-type SPIs might protect shrimp from microorganisms as well as viruses via obstructing the activity of proteinases participating in propagation.

The data from the SSH libraries showed that three antimicrobial peptides (AMPs): Anti-lipopolysaccharide factor isoform 6 (ALF*Pm*6), crustin*Pm*1 and crustin*Pm*4 were up-regulated in response to viral infection in *P. monodon*. ALFs have broad antimicrobial activities towards gram-positive and gram-negative bacteria, filamentous fungi, and viruses (Liu et al., 2006; Li et al., 2008; Somboonwiwat et al., 2005). Recently, it has been shown that recombinant ALF*Pm*3 protein can protect shrimp from vibriosis (Ponprateep et al., 2009) and WSSV infection (Tharntada et al., 2009). Besides, the ALF transcript level in crayfish, *Pacifastacus leniusculus,* was enhanced after WSSV injection, and its knockdown activated WSSV propagation in

cell cultures (Liu et al., 2006). Crustin*Pm*1, a 14.7 kDa peptide with a single WAP domain, exhibits antimicrobial activity against *Staphylococcus aureus* and *Streptococcus iniae* (Supungul et al., 2008) whereas crustin*Pm*4 has been reported as one of the major crustin isoforms in *P. monodon* (Tassanakajon et al., 2008) but its antibacterial activity has not yet been determined. Recently, crustin*Pm*5 has recently been characterized as a unique isoform that is highly expressed in the gill and epipodite tissues of *P. monodon* and exhibits strong antibacterial activity against *S. aureus* (Vatanavicharn et al., 2009). Despite the fact that crustins are wildly known to be involved in the immune system, the crustin mechanisms of actions and functions are still largely unknown, particularly with regard to viral defense responses. However, it has been reported that transcript levels of the shrimp single WAP domain-containing proteins (SWD*Pm*1 and SWD*Pm*2) were significantly up-regulated after WSSV injection (Amparyup et al., 2008). Our data suggest a potential role for crustins in viral responses, and raise the interesting question for further study of whether crustins, including other WAP domain-containing proteins might be also implicated in the antiviral response of crustaceans.

Caspases and translationally controlled tumor protein are widely known to be essential genes involved in apoptosis and anti-apoptosis, respectively. Previously, they were found to be up-regulated in a variety of penaeid shrimps after WSSV infection (Tonganunt et al., 2008; Wang et al., 2008; Wongprasert et al., 2007), but so far there is no report on the involvement of these genes in YHV infection. Here, the SSH libraries demonstrated that the apoptosis-related genes were not only up-regulated after WSSV infection but also YHV infection, implying the importance of apoptotic and anti-apoptotic process in antiviral immune response in crustacean. Caspase gene silencing caused an increase in the number of WSSV copies in shrimp (Wang et al., 2008) and the knockdown of caspase-3 reduces mortality in Pacific white shrimp *Penaeus (Litopenaeus) vannamei* challenged with a low dose of white-spot syndrome virus (Wongprasert et al., 2007). Interestingly, a preceding report proposed a mechanism controlling over viral-triggered apoptosis by the action of the translationally controlled tumor protein that was necessary for protecting the infected shrimp from death (Bangrak et al., 2004). Up till now, the role of apoptosis in the antiviral mechanism of shrimp is still controversial. The potential role of the apoptotic

and anti-apoptotic genes isolated from the SSH libraries in response against viruses was valuable to be further investigated.

Integrins have emerged as receptors or coreceptors for a large number of viruses (Bergelson et al., 1992; Summerford et al., 1999; Akula et al., 2002; Chu et al., 2004). In 2007, the first report revealed that shrimp β-integrin can bind to the WSSV envelope protein VP187, which may facilitate the entry of virus and the delivery of genome to nucleus, and also participate in the defense mechanism via the enhancement of the immune-cell adhesion (Li et al., 2007; Zhang et al., 2009). Additionally, the interaction of tetraspanin and integrin on hemocyte surface of *Manduca sexta* could initiate cellular innate immune responses in insect (Zhuang et al., 2007). In this research, we found that β-integrin and tetraspanin D107 were up-regulated following YHV infection; as a result, it was suggested that β-integrin might be implicated in YHV infection and/or innate immune responses in crustaceans, thus it is attractive to examine a function of integrin for being YHV receptor and tetraspanin-integrin interaction in antiviral mechanism.

Transglutaminases (TGases) play a critical role in crustacean hemolymph coagulation. The expression of TGase was reported to be up-regulated in the Chinese shrimp, *P.chinensis* infected with bacteria (Liu et al., 2007). Silencing of TGase in *P. japonicus* significantly enhanced mortality compared to control shrimp after challenge with *Vibrio penaecida* and WSSV (Maningas et al., 2008). Together, these data indicate that TGases play an essential role in the shrimp immune response to bacteria and viruses. The results from the SSH libraries and real-time RT-PCR suggested that the shrimp TGase was up-regulated in response to WSSV and YHV infection, thus data from this study supported a possible role of TGase in antiviral responses in shrimp.

Lectins are important pattern recognition receptors in innate immunity. They bind specifically to carbohydrate components on the surface of microbes and activate immune responses to eliminate invading pathogens. So far, C-type lectins from shrimp have been reported to be involved in antibacterial, antifungal and antiviral processes. For instance, FC-hsl and LvLec displayed immune activities against some bacteria and fungi (Sun et al., 2008; Zhang et al., 2009), whilst Fclectin showed up-regulated expression levels after challenge with bacteria, lipopolysaccharide or WSSV

(Liu et al., 2007). Likewise, LvCTL1 and PmAV exhibited antiviral activity against infection with WSSV and grouper iridovirus, respectively (Zhao et al., 2009; Luo et al., 2003) whilst PmLT played a role in the pattern recognition receptor for initial recognition of WSSV, and could also activate the cellular defense mechanism of shrimp hemocytes (Ma et al., 2008). Here, we found that three different lectin transcripts (two C-type lectins and a hemolectin) were up-regulated at the initial stage of YHV infection, possibly as a recognition response to YHV envelope proteins that would trigger the innate immune system.

One of WSSV gene, ICP11, transcript was found to be abundant in the SSH libraries. ICP11 is the most highly expressed WSSV nonstructural gene/protein revealed by both transcriptomic (cDNA microarray) and proteomic (2D electrophoresis) approaches (Wang et al., 2007, 2007), which implies its importance in WSSV infection. ICP11 acts as a DNA mimic preventing DNA from binding to histone proteins in hemocytes from WSSV-infected shrimp. ICP11 can disrupt nucleosome assembly and prevent histone from fulfilling its critical function of repairing DNA double strand breaks. Therefore, an abundance of ICP11 in the host cell deprives the cell nucleus of histone proteins, makes the host DNA in cell vulnerable to damage, and finally leads to a disruption of the genetic machinery in the nucleus (Wang et al., 2008).

Thus the SSH libraries provides novel information on shrimp genes that are potentially involved in WSSV and YHV infection and/or innate immune response against viral infection. The information may also be relevant to other viruses and other crustaceans. This baseline study can serve as a starting point for further work on viral defense mechanisms in crustaceans and may lead to new methods of viral disease control.

A number of WSSV-responsive genes were identified from the SSH libraries of *P. monodon* hemocytes, several of which were of unknown function. Among these genes, a novel viral responsive protein, named here hemocyte homeostasis-associated protein (HHAP), was highly up-regulated. A BLASTX search of the NCBI GenBank database revealed that the deduced amino acid sequence of *Pm*HHAP significantly matched a hypothetical protein homolog from various invertebrates and vertebrates, with the highest similarity to the hypothetical protein TcasGA2_TC006773 from the

red flour beetle, *Tribolium castaneum*, with 54% amino acid sequence identity. The full HHAP sequence has been determined from two crustacean species; the black tiger shrimp, *P. monodon*, and the freshwater crayfish, *P. leniusculus* (Prapavorarat et al., 2010). These two sequences exhibited 47% amino acid sequence identity to each other and shared eight conserved cysteine residues. Moreover, protein-structure analysis suggested that HHAP is likely to be a DNA-binding protein (99.2% possibility). The high predicted amino acid sequence identity of the HHAP sequences found in various organisms, and thus the potentially conserved function, and the possible DNA-binding properties, imply the potential importance of the HHAP protein.

The tissue distribution of HHAP transcripts in SPF *P. monodon* and *P. leniusculus* shrimp were different. Within *P. monodon*, *Pm*HHAP mRNA expression was detectable in a wide variety of tissues, but mainly in the hematopoietic tissue and a low expression level in hemocytes. However, in *P. leniusculus* crayfish *Pl*HHAP transcripts were only detected in nerve and hemopoietic tissues at low levels, and not at all in other tissues including hemocytes (Prapavorarat et al., 2010). Moreover, based on Western blot analysis, the endogenous *Pm*HHAP was also detected as one of the major proteins in the shrimp hematopoietic tissue (but not in circulating (mature) hemocytes themselves). In crustaceans, the hematopoietic tissue is reported to be an organ in which hemocyte proliferation and maturation takes place (Van de Braak et al., 2002; Chaga et al., 1995; Söderhäll et al., 2003). Hemocytes are produced and partially differentiated into young hemocytes in the hematopoietic tissue, and then became functional or mature hemocytes as they are released into circulation (Chaga et al., 1995; Söderhäll et al., 2003). The high expression levels of HHAP transcripts in hematopoietic tissue may suggest a possible role in hemocyte production or maturation (hematopoiesis). Formerly, a new function of TGase in crayfish was disclosed, in that it participated in preventing differentiation of hematopoietic stem cells (Lin et al., 2008). In addition, an endogenous cytokine-like factor, astakine, first described in crayfish (Söderhäll et al., 2005), was required for cell proliferation and differentiation in the hematopoietic tissue of two crustaceans (Söderhäll et al., 2005; Hsiao et al., 2010), and ATP synthase in the plasma membrane of hematopoietic cells functions as a receptor for astakine (Lin et al., 2009). Recently, a crustacean

hematopoietic factor was identified in *P. leniusculus* and was shown to be vital for the survival of hemocytes and hematopoietic tissue cells by preventing the apoptotic process (Lin et al., 2011). Although there are some recent attempts to unveil crustacean hematopoiesis, the mechanisms regarding hemocyte synthesis and differentiation, the release of hemocytes into the circulation, and the maintenance of circulating hemocytes in crustaceans are still very indistinct.

To uncover the functions of HHAP, RNAi knockdown of the *Pm*HHAP transcript expression in *P. monodon* shrimp was performed. The resultant partial average down regulation (46 and 80% for the two doses tested) of *Pm*HHAP transcript levels in circulating hemocyte cells correlated with a significant (two to four-fold) depletion of circulating hemocytes and a rapid mortality in the knocked down shrimp. The damage to hemocytes was demonstrated by scanning electron microscope, and also observed *in vitro* in the shrimp primary hemocyte cell cultures. These results suggest that *Pm*HHAP plays an important role in hemocyte homeostasis, at least in *P. monodon,* as its knockdown initiates the deformation and lysis of hemocytes and a greatly decreased number of circulating hemocytes, as well as subsequent increased shrimp mortality. Notably in shrimp, the reduced hemocyte number is unlikely to be a direct cause of the significant increase in shrimp mortality since depletion of up to 95% circulating hemocytes usually does not affect shrimp survival as new hemocytes are rapidly produced and released into the circulation. Therefore, we speculated that death in the HHAP knockdown shrimp might result from an inability of shrimp to recover circulating hemocytes due to loss of a critical function of HHAP that remains unclear and need further investigation. Nevertheless, HHAP is clearly important in both shrimp viability and hemocyte homeostasis.

Hemocytes are wildly admitted as a major cell type which plays several essential roles in the innate immunity (Söderhäll, 1999). The number of circulating hemocytes in various crustaceans has been observed to decrease after viral infection (Van de Braak et al., 2002; Wongprasert et al., 2003), or after challenge with bacterial components (Söderhäll et al., 2003; Lorenzon et al., 1999), but this leads to a rapid recovery in the circulating hemocyte level by the induction of hemocytic maturation in hematopoietic tissue and the release of new hemocytes into the circulation (Van de Braak et al., 2002; Söderhäll et al., 2003). From the results in *P. monodon* shrimp

primary cell culture, the up-regulation of *Pm*HHAP in WSSV-infected cultured hemocytes clearly indicate that the increase of HHAP expression in the hemocytes of WSSV-infected shrimp is a result of gene up-regulation in the mature hemocytes, not from gene expression of HHAP in new hemocytes released from the hematopoietic tissue.

The high up-regulation level of hemocytic *Pm*HHAP, both at the mRNA and at the protein level, in response to WSSV infection may suggest that *Pm*HHAP is involved in the shrimp's immune response against viral infections. In the past, a few reports have proposed possible mechanisms for maintaining circulating hemocyte levels and protecting the infected shrimp from death, such as the rapid production and release of new hemocytes from hematopoietic tissue after infection (Van de Braak et al., 2002; Söderhäll et al., 2003) and the control of over viral-triggered apoptosis by the action of the translationally controlled tumor protein (Bangrak et al., 2004). Our data clearly indicate that *Pm*HHAP has a direct role in hemocyte persistence so it is reasonable to speculate that the up-regulation of *Pm*HHAP is a mechanism for maintaining the hemocyte level in the circulation by preventing too rapid hemocyte degradation, and is also a way to control immune homeostasis in crustaceans during a viral infection.

Another interesting WSSV-responsive gene with unknown function discovered by the SSH libraries is named here as viral responsive protein 15 kDa (VRP15). It was found to be the most abundant transcript in the WSSV-challenge SSH libraries. A BLASTX search of the NCBI GenBank database revealed that VRP15 had no significant amino acid sequence similarity to any proteins in the database. The full sequence of the novel gene, VRP15, has been identified for the first time in *P. monodon*. Moreover, protein-structure analysis suggests that VRP15 is a transmembrane protein implying its possible function as a cellular receptor or transporter. Moreover, the VRP15 transcript was found in all tested shrimp tissues but principally expressed in hemocytes. The VRP15 transcript level in antennal gland, epipodite, eye stalk was detected at very low level when compared to that of other shrimp tissues, probably, the observed transcript might result from the PCR amplification of VRP15 transcript in contaminated hemocytes located in the tissues.

VRP15 expression was extremely up-regulated in *P. monodon* hemocytes in response to WSSV infection implying the critical role of VRP15 in the shrimp after WSSV challenge. So far, most studies have concentrated on investigation of genes involved in the host immune response against viruses, while the research studying on the host genes associated with viral infection mechanism is very rare. To determine the function of VRP15, RNAi knockdown of the VRP15 transcript expression in *P. monodon* shrimp was applied. The success of VRP15 gene expression knockdown in normal and WSSV-infected shrimp hemocytes was carried out by injecting 3 μg of VRP15 dsRNA per gram of shrimp into each experimental shrimp. Interestingly, VRP15 knockdown obviously decreased WSSV-structural gene, VP28, transcript level in various populations of WSSV-infected shrimp. These finding clearly indicate that the novel VRP15 gene is essential for viral infection mechanism to contribute viral propagation in *P. monodon* whilst its knock-down leads to decrease in viral infection. Notably, up to now VRP15 existence has only been discovered in *P. monodon* that is a host for WSSV (it may present in other crustaceans with WSSV as virulent pathogen but their genome sequences are not well established), but is not found in species which are not hosts of WSSV such as the insects, the invertebrates as well as the vertebrates whose genome sequences are well established. Due to the host specificity of VRP15, it might imply an importance of VRP15 to the host with WSSV as pathogen. However the mechanism of how VRP15 facilitates viral replication is still unknown, and needs further investigation.

CHAPTER V
CONCLUSIONS

1. Suppression subtractive hybridization were successful to identify genes up-regulated in early and late phases of WSSV and YHV infection in *P. monodon*, and the indeed up-regulations of the viral responsive genes were revealed by RT-PCR and/or real-time RT-PCR.
2. The full-length cDNAs of *Pm*HHAP and VRP15 were obtained, and their sizes were confirmed by Northern blot analysis.
3. *Pm*HHAP and VRP15 were transcribed in various tissues, but principally expressed in hematopoietic tissue and hemocytes, respectively.
4. Hemocytic *Pm*HHAP was up-regulated post-WSSV infection at both mRNA and protein levels, likewise, VRP15 was also greatly up-regulated in shrimp hemocytes after WSSV challenge.
5. *Pm*HHAP is essential for shrimp survival as well as hemocyte homeostasis in blood circulation system of shrimp.
6. VRP15 is important for WSSV propagation mechanism as its knock-down leads to decrease in the viral infection in *P. monodon*.

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BIOGRAPHY

Mr. Adisak Prapavorarat was born on January 10, 1985 in Bangkok. He graduated with the degree of Bachelor of Science from the Department of Biochemistry, Chulalongkorn University in 2005. Subsequently, He has received a fellowship from the Royal Golden Jubilee PhD program, Thailand Research Funds and Chulalongkorn University to study for the Doctor of philosophy (Ph.D.) degree in the Department of Biochemistry, Chulalongkorn University.

During his Ph.D. study, he has published three of his works in the international journals: one scientific paper in the Journal of Biological Chemistry, and two scientific papers in Developmental and Comparative Immunology.

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