ลักษณะสมบัติเชิงหน้าที่ของโปรตีนที่เกี่ยวข้องกับภาวะธำรงดุลของเซลล์เม็ดเลือด เอชเอชเอพี ใน
สองสปีชีส์ของครัสเตเชียน

นางสาวกัณฑมาศ อภิธันยาศัย

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

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FUNCTIONAL CHARACTERIZATION OF HEMOCYTE HOMEOSTASIS ASSOCIATED PROTEIN , HHAP IN TWO SPECIES OF CRUSTACEANS

Miss Kantamas Apitanyasai

A Dissertation Submitted in Partial Fulfillment of the Requirements
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Department of Biochemistry
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กัณฑมาศ อภิธันยาศัย : ลักษณะสมบัติเชิงหน้าที่ของโปรตีนที่เกี่ยวข้องกับภาวะธำรงดุลของเซลล์เม็ดเลือด เอช เอช เอ พี ในสองสปีชีส์ของครัสเตเชียน (FUNCTIONAL CHARACTERIZATION OF HEMOCYTE HOMEOSTASIS ASSOCIATED PROTEIN, HHAP IN TWO SPECIES OF CRUSTACEANS) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ศ. ดร.อัญชลี ทัศนาขจร, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ดร.ปิติ อ่ำพายัพ, ศ. ดร.เคนเน็ท โซเดอร์ฮอลล์, 147 หน้า.

Hemocyte homeostasis-associated protein (*Pm*HHAP) เป็นโปรตีนที่ตอบสนองต่อเชื้อไวรัสและมีบทบาทสำคัญในการควบคุมภาวะธำรงดุลของเม็ดเลือดในกุ้งกุลาดำ *Penaeus monodon* ในงานวิจัยนี้ได้ทำการศึกษากลไกการทำงานของ *Pm*HHAP ในการควบคุมการเกิดอะพอพโทซิสในกุ้ง เมื่อทำการยับยั้งการแสดงออกของยีน *Pm*HHAP ส่งผลให้มีการเกิดอะพอพโทซิสสูงขึ้นในเม็ดเลือดกุ้ง และมีผลต่อระดับการแสดงออกของยีนที่เกี่ยวข้องกับอะพอพโทซิสโดยเฉพาะ *Pm*Casp และ *Pm*Caspase นอกจากนี้ยังพบว่าโปรตีน *Pm*HHAP สามารถจับกับโปรตีน *Pm*Casp และรีคอมบิแนนท์โปรตีน *Pm*HHAP สามารถลดการเกิดอะพอพโทซิสในเม็ดเลือดที่ถูกกระตุ้นด้วยรีคอมบิแนนท์โปรตีน *Pm*Casp จากผลการทดลองแสดงให้เห็นว่า *Pm*HHAP เป็นโปรตีนที่ยับยั้งการเกิดอะพอพโทซิสโดยการควบคุมธำรงดุลของเม็ดเลือดป้องกันไม่ให้เม็ดเลือดเกิดอะพอพโทซิสในกุ้ง จากการใช้เทคนิค yeast two-hybrid และ co-immunoprecipitation พบว่า *Pm*HHAP สามารถจับกับโปรตีน WSSV134 ซึ่งมีหน้าที่ยับยั้งการเกิดอะพอพโทซิสของไวรัสตัวแดงดวงขาวได้ WSSV134 ถูกพบว่ามีการแสดงออกของยีนในระยะท้ายของการติดเชื้อไวรัส โดยสามารถตรวจพบได้ตั้งแต่ 24 ชั่วโมงหลังจากติดเชื้อ และน่าจะมีความเกี่ยวข้องกับการแพร่กระจายของไวรัส หลังจากฉีดไวรัสและทำการยับยั้งการแสดงออกของยีน *Pm*HHAP ควบคู่กับยีน WSSV134 พบว่าการเกิดอะพอพโทซิสในเม็ดเลือดสูงกว่ากุ้งเมื่อเทียบกับที่ถูกยับยั้งการแสดงออกเพียงแค่ยีนใดยีนหนึ่ง แสดงให้เห็นว่า *Pm*HHAP และ WSSV134 น่าจะทำงานร่วมกันในการควบคุมอะพอพโทซิสในระหว่างการติดเชื้อไวรัสตัวแดงดวงขาว นอกจากนี้ยังได้ทำการสร้างห้องสมุด suppression subtractive hybridization (SSH) จากเม็ดเลือดของกุ้งที่ถูกยับยั้งการแสดงออกของยีน *Pm*HHAP และระบุยีนที่มีการแสดงออกเปลี่ยนแปลงไป โดยพบยีนจำนวนมากมีหน้าที่เกี่ยวข้องกับตอบสนองต่อไวรัสและการรักษาภาวะธำรงดุลของเม็ดเลือด ได้แก่ transcription factor ATF-β, cathepsin L, lactate dehydrogenase, ferritin and inhibitor of apoptosis (IAP) ซึ่งน่าจะต้องคัดเลือกยีนเหล่านี้ เพื่อศึกษาหน้าที่ต่อไป นอกจากนี้ยังได้ทำการศึกษาหน้าที่ของ homolog ของ HHAP ในกุ้งน้ำจืดเครย์ฟิช *Pacifastacus leniusculus* โดยโปรตีนทั้งสองตัวมีความเหมือนกัน 47% โดยพบว่า *Pl*HHAP มีการแสดงออกในหลายเนื้อเยื่อของกุ้งเครย์ฟิช แต่มีการแสดงออกสูงสุดที่ลำไส้และเหงือก *Pl*HHAP มีบทบาทสำคัญในการต่อต้านเชื้อแบคทีเรียแต่ไม่มีบทบาทในการต่อต้านไวรัสซึ่งตรงข้ามกับ *Pm*HHAP โดยพบว่าภายหลังจากติดเชื้อไวรัสตัวแดงดวงขาวไม่มีผลให้การแสดงออกของยีน *Pl*HHAP เปลี่ยนแปลงไป แต่พบว่าการแสดงออกของยีน *Pl*HHAP เพิ่มขึ้นเป็นอย่างมากเมื่อติดเชื้อแบคทีเรีย *Aeromonas hydrophila* B1 เมื่อทำการศึกษาต่อพบว่าการยับยั้งการแสดงออกของยีน *Pl*HHAP มีผลให้ปริมาณแบคทีเรียในลำไส้ของกุ้งเครย์ฟิชเพิ่มขึ้น แต่ไม่มีผลให้เม็ดเลือดเกิดอะพอพโทซิสและไม่ส่งผลต่อระดับการแสดงออกของยีน CHF, astakine1, astakine2, และ IGFBP7 ที่เกี่ยวข้องกับการสร้างและรักษาภาวะธำรงดุลของเลือด ดังนั้นจึงสรุปได้ว่า HHAP ในสิ่งมีชีวิตต่างสปีชีส์กันมีบทบาทการทำงานที่แตกต่างกันในการป้องกันการรุกรานของเชื้อโรค

ภาควิชา	ชีวเคมี	ลายมือชื่อนิสิต	.....
สาขาวิชา	ชีวเคมีและชีววิทยาโมเลกุล	ลายมือชื่อ อ.ที่ปรึกษาหลัก	.....
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KEYWORDS: HEMOCYTE HOMEOSTASIS / APOPTOSIS / WSSV / BACTERIAL / PENAEUS MONODON / PACIFASTACUS LENIUSCULUS

KANTAMAS APITANYASAI: FUNCTIONAL CHARACTERIZATION OF HEMOCYTE HOMEOSTASIS ASSOCIATED PROTEIN, HHAP IN TWO SPECIES OF CRUSTACEANS. ADVISOR: PROF. ANCHALEE TASSANAKAJON, Ph.D., CO-ADVISOR: PITI AMPARYUP, Ph.D., PROF. KENNETH SöDERHäLL, Ph.D., 147 pp.

Hemocyte homeostasis-associated protein (*Pm*HHAP) has been reported as a viral responsive protein that plays an important role in controlling hemocyte homeostasis in shrimp *Penaeus monodon*. In this study, the role of *Pm*HHAP in regulating apoptosis in shrimp was investigated. *In vivo* gene silencing of *Pm*HHAP could induce the high level of apoptosis in shrimp hemocytes and altered the expression of apoptosis-related genes particularly *Pm*Casp and *Pm*Caspase. Moreover, *Pm*HHAP was able to bind to *Pm*Casp and the r*Pm*HHAP protein could decrease apoptosis in the r*Pm*Casp-treated hemocytes cells. These results indicated that *Pm*HHAP is an anti-apoptosis protein that regulates hemocyte homeostasis by inhibiting apoptosis in *P.monodon*. Yeast two-hybrid and co-immunoprecipitation revealed that *Pm*HHAP binds to an anti-apoptosis protein of white spot syndrome virus, WSSV134. The viral protein WSSV134 is a late protein of WSSV which is initially expressed after 24-hour post infection (hpi) and is likely involved in viral propagation. Co-silencing of *Pm*HHAP and WSSV134 prior to WSSV infection showed significant increase of caspase activity, which was higher than silencing only *Pm*HHAP or WSSV134, suggesting that these two proteins might work concordantly to control apoptosis during WSSV infection. Suppression subtractive hybridization (SSH) of *Pm*HHAP silenced shrimp hemocytes identified several viral responsive genes as well as hemocyte homeostasis-related genes such as the transcription factor ATF-β, cathepsin L, lactate dehydrogenase, ferritin and inhibitor of apoptosis (IAP), some of which should be selected for further investigation. In addition, a homolog of HHAP in crayfish *Pacifastacus leniusculus* with 47% similarity to *Pm*HHAP was functional characterized. *Pl*HHAP was detected in all examined tissues but highly expressed in intestine and gill. Surprisingly, *Pl*HHAP was found to play important role in bacterial but not viral defense in contrast to *Pm*HHAP. *Pl*HHAP transcript remained unchanged upon WSSV infection but dramatically increased upon bacterial *Aeromonas hydrophila* B1 challenge. Furthermore, suppression of *Pl*HHAP resulted in increasing the number of bacteria in intestine but did not affected hemocyte apoptosis or the expression of genes (CHF, astakine1, astakine2, and IGFBP7) involved in crayfish hematopoiesis and hemocyte homeostasis suggested that HHAP from different crustacean species have different functions in defense against invading pathogens.

Department:	Biochemistry	Student's Signature	.....
Field of Study:	Biochemistry and Molecular Biology	Advisor's Signature	.....
Academic Year:	2016	Co-Advisor's Signature	.....
		Co-Advisor's Signature	.....

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LIST OF ABBREVATIONS

°C	Degree celcius
AHPND	Acute Hepatopancreatic Necrosis Disease
BSA	Bovine serum albumin
cDNA	Complementary deoxyribonucleic acid
CFU	Colony forming unit
CO-IP	Co-immunoprecipitation
dsRNA	Double stranded ribonucleic acid
EF1-α	Elongation factor 1 alpha
EMS	Early mortality syndrome
GFP	Green fluorescence protein
h	Hour
HHAP	Hemocyte homeostasis-associated protein
HLS	Hemocyte lysate
IPTG	isopropyl-beta-D-thiogalactopyranoside
Kb	Kilobase
LB	Luria-Bertani
μM	Micromolar
μg	Microgram
μl	Microlitre
M	Molar
mg	Milligram
min	Minute
ml	Mililitre
O.D.	Optical density
ORF	Open reading frame

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PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
Pm	*Penaeus monodon*
r	recombinant
RNAi	Ribonucleic acid interference
RT-PCR	Reverse transcription/polymerase chain reaction
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SSH	Suppression subtractive hybridization
s	Second
THC	Total hemocyte number
TSB	Tryptic soy broth
WSSV	White spot syndrome virus
YHV	Yellow head virus

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CHAPTER 1
INTRODUCTION

1.1 Shrimp Aquaculture and Production

Over the last few decades, aqua farming has been greatly expanding to reach higher demands of the world consumption rate. Among those aquaculture, shrimp farming has become an important economic activity in several countries in Asia and South America. Thailand was the leader for exporting shrimp especially black tiger shrimp, *Penaeus monodon*, of the world market in shrimp and prawn (Wyban, 2007). The shrimp farms distributed along the coastal areas especially in Suratthani and Nakhornsrithammarat provinces are the majority of shrimp harvested. The shrimp farms are also located in the central and the eastern provinces such as Samutsongkharm, Samutsakhorn, Chachoengsoa and Chantaburi. Unfortunately, the production of the black tiger shrimp has rapidly declined due to the outbreaks of severe pathogens which resulted in the change of farmed species to the Pacific white shrimp *Litopenaeus vannamei*. The genetic selection of white shrimp was successfully performed to tolerate diseases and gain high survival rate which caused white shrimp become the main aquaculture species instead of black tiger shrimp (Figure 1.1). However, in recent years, diseases also have a great impact on the farming of the white shrimp especially the Early mortality syndrome (EMS) (more descriptively called acute hepatopancreatic necrosis disease or AHPND (Tran et al., 2013)). This outbreak caused huge economic losses in Southern China then expanded to Vietnam, Malaysia and reached Thailand in 2012 (Tran et al., 2013). Accordingly, to solve the diseases problems and maintain the production of black tiger shrimp, the great farm management, efficient domestication, genetic improvement and invention of effective disease control are

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urgently required. The knowledge is not only applied for protection of diseases but also for selective breeding of healthy shrimp for Thai industry.

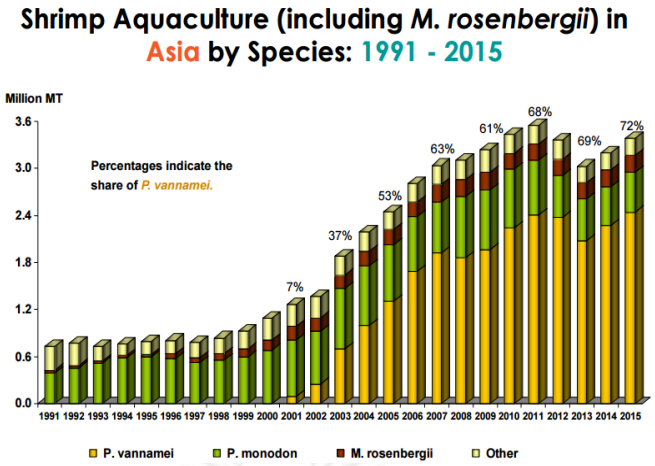

Figure 1.1 Shrimp aquaculture in Asia by shrimp species during 1991-2015. (Sources: FAO (2013) for 1991-2011; Goal (2013) for 2012-2015).

1.2 Taxonomy of black tiger shrimp, *Penaeus monodon*

The black tiger shrimp *Penaeus monodon* is an economically important aquaculture animal in Thailand. Naturally, *P. monodon* is widely distributed to the South East Asia, South Africa, Australia and the Pacific and Indian Oceans. The apparent aspect of the *P. monodon* are distinct black and white or yellow stripes on their cuticles appearing like tiger traits, tails and abdomens. The general body pattern is a head tail, five pairs of pleopods (swimming legs), five pairs of pereopods (walking legs) and presence of pair appendages and a protective exoskeleton that covers the whole body. The taxonomy is identified as below (Baily-Brock, 1992).

Kingdom: Animalia

Phylum: Arthropoda

Subphylum: Crustacea

Class: Malacostraca

Subclass: Eumalacostraca

Order: Decapoda

Suborder: Natantia

Superfamily: Penaeoidea

Family: Penaeidae Rafinesque, 1985

Genus: *Panaeus* Fabricius, 1798

Species: *Penaeus monodon*

Scientific name: *Penaeus (Penaeus) monodon* Fabricius, 1798.

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).

The FAO names are Crevette giante tigre (French), Caramon tigre gigante (Spanish) and Giant tiger prawn (English) (Noel B. Solis, 1988).

Figure 1.2 Black tiger shrimp (*Penaeus monodon*)

(http://www.sea-ex.com/fishphotos/prawn,3.htm)

1.3 Major diseases in shrimp

The production of shrimp in many countries continues to decrease due to the main problems caused by the outbreaks of pathogens including bacteria, fungi and viruses (Walker and Winton, 2010) (Figure 1.3).

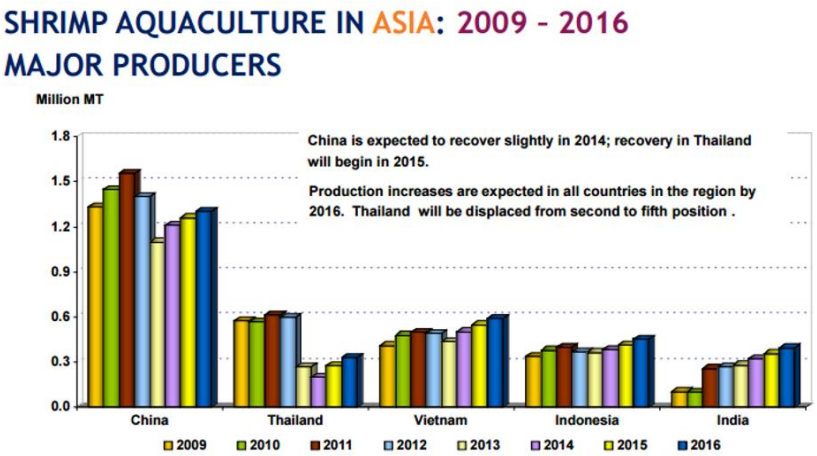

Figure 1.3 Shrimp aquaculture by major producing regions during 2009-2016. (Sources: FAO (2014) for 2009-2012; Goal (2014) for 2013-2016). Shrimp production in some countries still declined due to the diseases outbreak.

1.3.1 Bacterial diseases

1.3.1.1 Vibriosis

Vibriosis is one of the major diseases in shrimp aquaculture caused by bacterium *Vibrio harveyi*. The disease contributes to morbidity and mass mortality in shrimp (Austin and Zhang, 2006; Lightner, 1975). It results in almost 100% cumulative mortality which usually occurred in post-larvae and young juvenile shrimp (Flegel, 2006; Lightner et al., 1983). There have been occasional reports that Vibriosis also caused by other *Vibrio* species (Lightner, 1996). The apparent characteristics of *V. haeveyi* is a Gram-negative, motile, rod shape and luminescent (Lavilla-Pitogo, 1990). Disease transmission can occur via water or as a result of ingestion of infective material. The evident symptoms caused by *V. haeveyi* in the infected shrimp are called luminous vibriosis (disorder development of basal tissues in the digestive system) which pathogen will releases exotoxins to destroy the wall of gastrointestinal tract and the host's immune cells (Peddie, 2005). High mortalities were observed particularly in juvenile shrimp, moribund shrimp appear hypoxic and often come to the pond surface

or edge, reddish discoloration and show black spots of melanization on the cephalothorax.

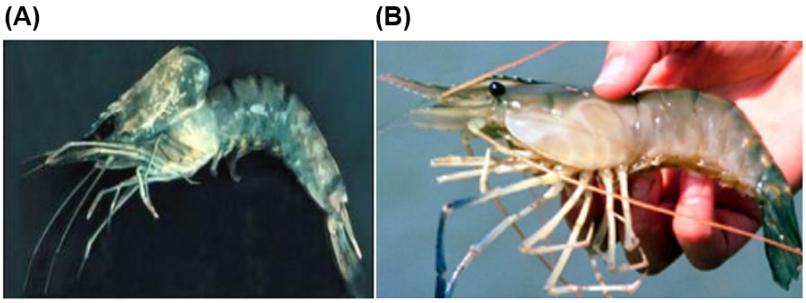

Figure 1.4 Vibriosis in shrimp farming. (A) Black spots of melanization was observed on the cephalothorax. (B) Shrimp display a pale reddish discoloration on the cuticle in infected shrimp. (http://www.viralinfections.info/article/664934838/disease-prevention-and-treatment)

1.3.1.2 Early mortality syndrome or Acute Hepatopancreatic Necrosis Disease (EMS/AHPND)

Recently, an emerging disease known as early mortality syndrome or Acute Hepatopancreatic Necrosis Disease (EMS/AHPND) was first reported in China and subsequently in Vietnam, Thailand and Malaysia. Mortalities can reach up to 100% in shrimp post-larvae during 20-30 days of culture (De Schryver et al., 2014). The causative agent of EMS/AHPND has been reported to be a *Vibrio parahaemolyticus.* Recently, the unique plasmid was identified from virulent strain of *V. parahaemolyticus*. This plasmid encodes two genes, PirA and PirB which are homologous to the *Photorhabdus* insect-related (Pir) toxin genes that are lethal to insect (Lee, 2015; Lightner, 2014; Yang, 2014). However, the non-virulent strains carrying the plasmid without the PirA and PirB genes (Flegel, 2014; Sirikharin, 2014). The PCR detection methods were developed in order to detect this disease, AP1, AP2, AP3 and AP4 PCR primer pairs were designed based on conserved sequences of the plasmid to developed the PCR methods in order to detect AHPND-related *V. parahaemolyticus* (Flegel, 2014; Sirikharin et al., 2015;

Sritunyalucksana, 2015). This bacterium can transmit orally, colonize in the shrimp gastrointestinal tract and produces a toxin causes tissue destruction and dysfunction of the shrimp digestive organ known as the hepatopancreas (Tran et al., 2013). Clinical signs of this disease are slow growth, corkscrew swimming, soft texture of the exoskeleton, being reduced in size of hepatopancreas and discolored hepatopancreas.

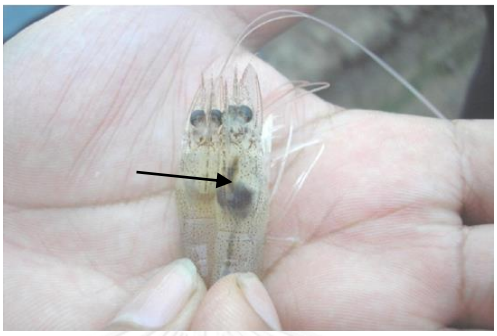

Figure 1.5 The infected shrimp caused by early mortality syndrome or Acute Hepatopancreatic Necrosis Disease (EMS/AHPND). The black arrow indicated the pale hepatopancreas of infected shrimp (right) compared to the brown hepatopancreas of normal shrimp (left). (Tran et al., 2013).

1.3.2 Viral diseases

1.3.2.1 Taura syndrome

Taura Syndrome (TS) was first reported in Ecuador in 1992 (Jimenez, 1992), and then quickly spread to the USA (Lightner, 1996), and later to Southeast Asia, where it is responsible for acute mortalities of penaeid shrimp in Taiwan (Yu, 2000). The pacific white shrimp *Litopenaeus vannamei* is highly sensitive to TS (Lightner, 1996). Taura syndrome is considered to be infected by Taura Syndrome Virus (TSV). This virus is a small single RNA virus, which the particles are non-enveloped icosahedral measuring 32 nm diameter and replicate within the cytoplasm of host cells. The genome of TSV consists of a linear, positive sense single-stranded RNA and it contains two large open reading frames (Lightner, 2011). TSV can be transmitted by contaminated water or by

horizontal transmission. Shrimp have a general pale reddish coloration with the tail fan and pleopods appearing hyperpigmented. The cuticle is soft and the gut empty and also exhibited irregularly shapes melanised cuticular lessions. Moribund shrimp accumulate at the pond surfaces and edges and typically result in cumulative mortalities between 40-100% in shrimp population. The selective breeding program was developed in order to select the resistance species to TSV in *L. vannamei*. This breeding program resulted in an increase of pond final survival equal to the levels to those prior to the initial TS outbreak (Cock, 2009), which has proven to be the most successful in preventing and controlling of TS disease. As a consequence, TSV is no longer considered as a major risk.

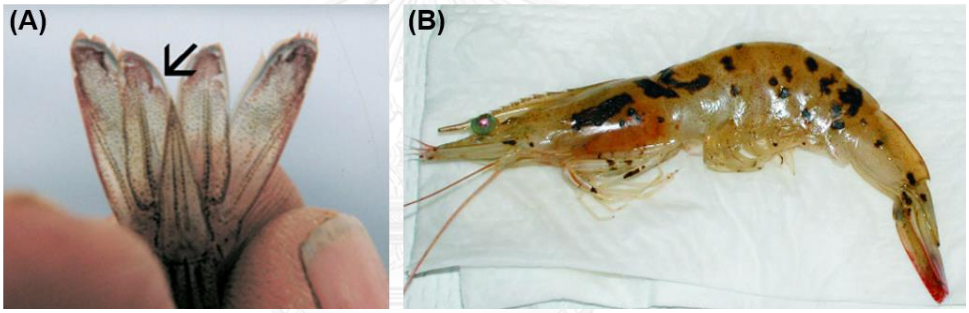

Figure 1.6 Taura syndrome disease in shrimp. (A) The characteristic of red tail fan was observed in infected shrimp. (B) The epidermal necrosis will cause black spots on the body of infected shrimp. (European Community Reference Laboratory for Crustacean Diseases Leaflet, 2008)

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1.3.2.2 Yellow head disease

The yellow head disease was first described as an epizootic from black tiger shrimp farms in Thailand which caused by the virus called yellow head virus (YHV) (Boonyaratpalin, 1993; Chantanachookin, 1993; Limsuwan, 1991). YHV is an invertebrate nidovirus containing single-stranded RNA of about 22 kb, and is an enveloped rod-shaped particle (Sittidilokratna, 2002), and is replicates within the cytoplasm of infected

host's cells (Cowley, 1999). Three YHV structural proteins have been discovered including two envelope glycoproteins gp116 and gp64 and a nucleocapsid protein p20 (Jitrapakdee et al., 2003; Sittidilokratna et al., 2006). The major organ being infected by YHV is the lymphoid organ (Chantanachookin, 1993; Khanobdee et al., 2002). The signs of yellow head disease are the development of yellowish cephalothorax and brown gills in the infected shrimp, increase in feeding and then suddenly decline and the mortality can reach to 100% within 3-5 days of the first appearance (Boonyaratpalin, 1993; Chantanachookin, 1993; Lightner, 1996). The YHV-infected shrimp also exhibits light yellow coloration of the dorsal cephalothorax area and has a generally pale or bleached appearance (Limsuwan, 1991). Lymphoid organ spheroids are commonly observed in infected shrimp (Pantoja and Lightner, 2001). Various diagnostic methods were developed for the detection of YHV such as RT-PCR (Cowley, 1999), *in situ* hybridization (Tang, 2002), loop mediated isothermal amplification (RT-LAMP) (Mekata et al., 2006) and real-time RT-LAMP (Mekata et al., 2009). A comprehensive assessment of host susceptibility to YHV has recently been completed by EFSA (EFSA 2008).

Figure 1.7 Yellow head disease in shrimp. The black arrow indicated the YHV-infected shrimp which shows a yellowish discoloration of the cephalothorax compare to the black color of normal shrimp (left). (Source: AGDAFF-NACA (Photo D. V. Lightner))

1.3.2.3 White spot syndrome

White spot syndrome has been one of the most serious diseases in shrimp aquaculture worldwide (Flegel, 1997; Lightner, 1996). This disease caused by the White Spot Syndrome Virus (WSSV) which belongs to the member of the Nimaviridae family (Mayo, 2002). The principle clinical sign of this disease is the development of white spots on the carapace of the infected shrimp which results from accumulated of calcium in cuticle but not all host species (Chou, 1995). The other symptoms including lower food consumption, the body surface and appendages turning to red or pink, loosing shell and lethargy. This disease caused 80-100% mortalities within 2-10 days after infection (Flegel, 1997; Lo, 1996).

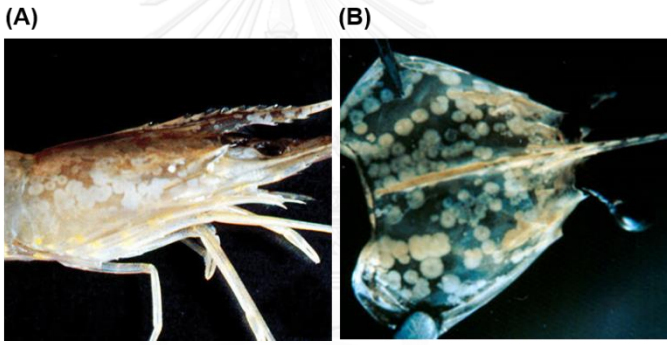

Figure 1.8 White spot syndrome disease. (A) and (B) The white spots occur on the cuticle of infected shrimp at the late phase of infection (Lightner, 1996).

White spot syndrome virus (WSSV) is a double-stranded DNA (dsDNA) virus. WSSV has a broad host range such as shrimp, crayfish, lobsters and crab (Lo, 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, 1996; Wang, 1997). The virions are large enveloped rod-shaped particle in the range of 80-120 × 250-380 nm (Durand, 1997; Nadala et al., 1998). The viral envelop has the structure of an apparently lipidic bilayer membrane. The nucleocapsid is tightly packed within the virion and surrounded by a trilaminar envelop

with a tail-like appendage (Figure 1.8) (Durand, 1997). The virion is formed by a complex of macromolecules specifically folded and assembled for the protection and delivery of viral genomes. Several proteins of WSSV have been characterized (Leu, 2009) as shown in Table 1.1. For example, VP28 is most likely located on the surface of the virus particle and plays a crucial role in WSSV infection (Van Hulten, 2001). The tegument protein VP26 was found to be useful for WSSV to move toward the nucleus by interacting with actin (Xie and Yang, 2005). The VP15 has been reported as one of major structural proteins located in the nucleocapsid and involved in packaging of viral genome (Van Hulten et al., 2002). VP664 is a major structural protein that forms the stacked rings of the WSSV nucleocapsid (Chai, 2013; van Hulten et al., 2001). Up to date, several proteins from both WSSV and shrimp were characterized for their interaction upon WSSV infection (Table 1.2).

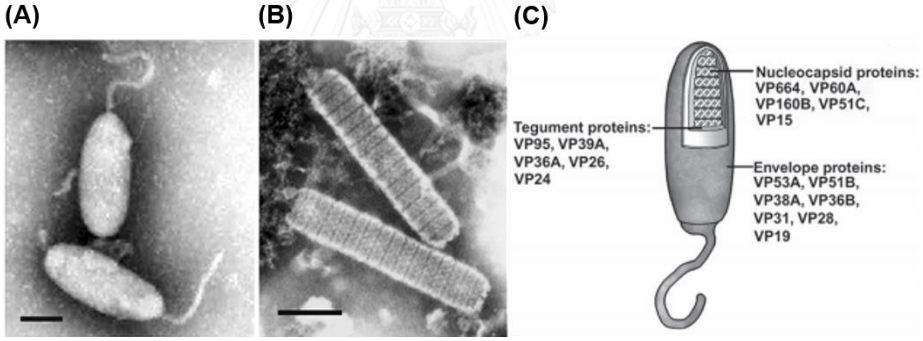

Figure 1.9 Purified virions of WSSV. (A) The completed virion with a characteristic tail-like. (B) The nucleocapsid prior to being enveloped (Leu, 2005). (C) Schematic diagram of WSSV that shows the layered structures of a virion including envelope, tegument and nucleocapsid (Leu et al., 2009).

The WSSV genome size is about 300 kbp. It is one of the largest sequenced animal viral genomes available. The complete genomes of WSSV were isolated from different geographical locations which includes (i) 292.9 kb isolated from Thailand (WSSV-TH) (AF369029), (ii) 307.2 kb isolated from Taiwan (WSSV-TW) (AF440570) and

(iii) 305.1 kb isolated from China (WSSV-CN) (AF332093) (Chen, 2002; Van Hulten et al., 2001; Yang, 2001). The WSSV-TH genome shows 184 ORFs could be assigned a putative function and the structural proteins of WSSV were identified and summarized as shown in Table 1.1.

Table 1.1 WSSV structural protein genes (Leu et al., 2009).

ORF name	Size (a.a.)	Protein name	Function/characteristics	References
wsv009	95	VP95	Structure protein	Huang et al. 2002
wsv026	507	VP507	Structure protein	Zhang et al. 2004
wsv115	968	VP53B	Structure protein	Tsai et al. 2004
wsv129	357	VP357	Structure protein	Huang et al. 2002
wsv137	337	VP337	Structure protein	Zhang et al. 2004
wsv198	278	VP32	Structure protein	Tsai et al. 2004; Xie et al. 2006
wsv199	856	VP320	Structure protein	Zhang et al. 2004
wsv249	216	VP216	Structure protein	Zhang et al. 2004
wsv260	387	VP387	Structure protein	Zhang et al. 2004
wsv269	489	VP53C	Structure protein	Tsai et al. 2004
wsv284	100	VP13A	Structure protein	Tsai et al. 2004
wsv293	60	VP14	Structure protein	Xie et al. 2006
wsv303	184	VP184	Structure protein	Huang et al. 2002
wsv332	786	VP75	Structure protein	Tsai et al. 2004
wsv338	433	VP11	Structure protein	Tsai et al. 2004
wsv390	321	ORF390	Structure protein	Tsai et al. 2004
wsv465	1243	VP136B	Structure protein	Tsai et al. 2004
wsv502	362	VP362	Structure protein	Zhang et al. 2004
wsv526	448	VP448	Structure protein	Huang et al. 2002
wsv001	1684	VP1684, Collagen-like	Structure protein, envelope	Huang et al. 2002; Li et al. 2004
wsv011	1301	VP53A, VP150	Structure protein, envelope	Tsai et al. 2006; Xie et al. 2006
wsv035	972	VP110	Structure protein, envelope	Tsai et al. 2004; Li et al. 2006a; Xie et al. 2006
wsv209	1606	VP187	Structure protein, envelope	Li et al. 2006b; Xie et al. 2006
wsv216	1194	VP124	Structure protein, envelope	Xie et al. 2006
wsv237	292	VP41A	Structure protein, envelope	Huang et al. 2002; Tsai et al. 2004; Xie et al. 2006
wsv238	486	VP51A,VP52A	Structure protein, envelope	Tsai et al. 2004; Xie et al. 2006
wsv242	300	VP300, VP41B	Structure protein, envelope	Huang et al. 2002; Tsai et al. 2004; Xie et al. 2006
wsv254	281	VP281,VP36B,VP33	Structure protein, envelope	Huang et al. 2002; Tsai et al. 2004; Xie et al. 2006
wsv256	384	VP384, VP51B, VP52B	Structure protein, envelope	Huang et al. 2002; Tsai et al. 2004; Xie et al. 2006
wsv259	309	VP38A,VP38	Structure protein, envelope	Tsai et al. 2004; Xie et al. 2006
wsv321	117	VP13B,VP16	Structure protein, envelope	Tsai et al. 2004; Xie et al. 2006
wsv325	465	VP60A, VP56	Structure protein, envelope	Tsai et al. 2004; Xie et al. 2006
wsv327	856	VP90	Structure protein, envelope	Xie et al. 2006
wsv339	283	VP39B,VP39	Structure protein, envelope	Tsai et al. 2004; Xie et al. 2006
wsv340	261	VP31	Structure protein, envelope	Tsai et al. 2004; Xie et al. 2006
wsv386	68	VP68, VP12B	Structure protein, envelope	Huang et al. 2002; Tsai et al. 2004; Zhang et al. 2004
wsv414	121	VP19	Structure protein, envelope	Huang et al. 2002; van Hulten et al. 2002
wsv421	204	VP28	Structure protein, envelope	van Hulten et al. 2000b
wsv002	208	VP24	Structure protein, tegument	van Hulten et al. 2000a; Tsai et al. 2006
wsv077	297	VP36A	Structure protein, tegument	Tsai et al. 2004; 2006
wsv306	419	VP39A	Structure protein, tegument	Tsai et al. 2004; 2006
wsv311	204	VP26	Structure protein, tegument	van Hulten et al. 2000; Tsai et al. 2006; Xie et al. 2006
wsv442	800	VP95	Structure protein, tegument	Huang et al. 2002; Tsai et al. 2004, 2006; Xie et al. 2006
wsv037	1280	VP160B	Structure protein, capsid	Tsai et al. 2006
wsv214	80	VP15	Structure protein, capsid, DNA-binding	Zhang et al. 2001; Witteveldt et al. 2005
wsv220	674	VP76,VP73	Structure protein, capsid	Huang et al. 2002; Tsai et al. 2006; Xie et al. 2006
wsv271	1218	VP136,VP136A	Structure protein, capsid	Tsai et al. 2006; Xie et al. 2006
wsv289	1565	VP160A, VP190	Structure protein, capsid	Tsai et al. 2006; Xie et al. 2006
wsv308	466	VP466, VP51C, VP51	Structure protein, capsid	Huang et al. 2002; Tsai et al. 2006; Xie et al. 2006
wsv360	6077	VP664	Structure protein, capsid	Leu et al. 2005; Tsai et al. 2004, 2006

The life cycle of WSSV has been reported and divided into three phases: entry into the host cell (directly or through host mechanisms), uncoating of the genome followed by replication and particle assembly and release (Escobedo-Bonilla et al., 2008). There are several molecules involved in this process and these molecules interaction occur between WSSV and its host (Figure 1.10) (Table 1.2).

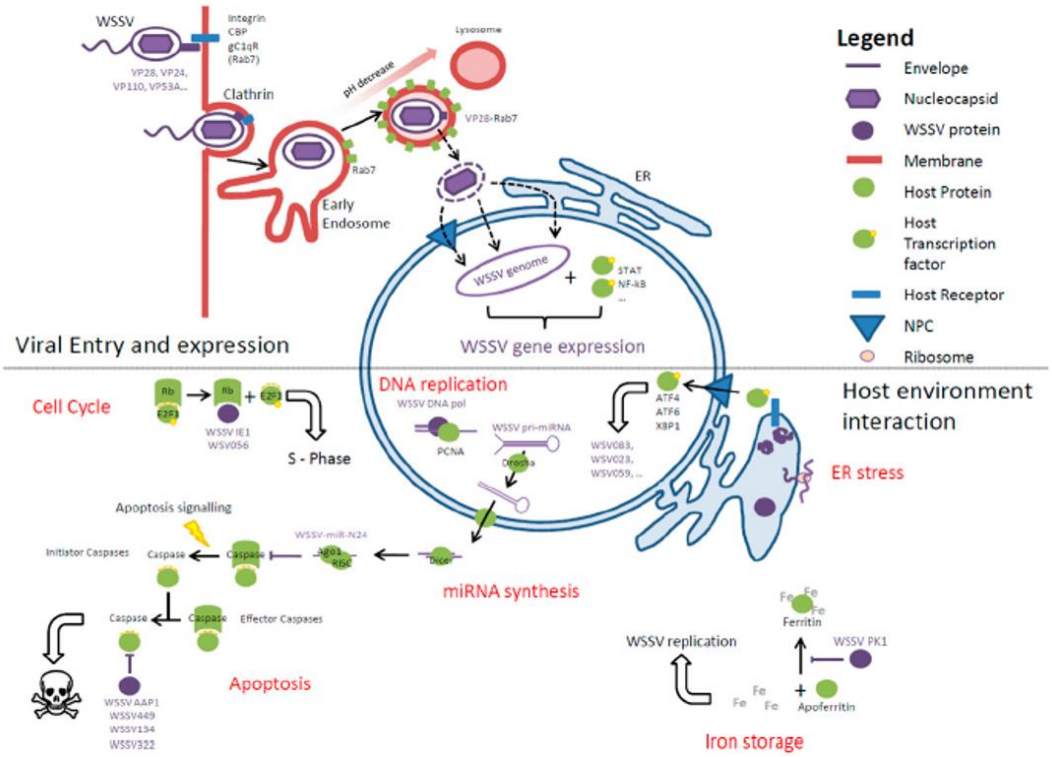

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Figure 1.10 Overview of WSSV entry and environment interactions. Top panel: Viral entry into the host cell. WSSV proteins interact with host receptors, which leads to induction of clathrin-mediated endocytosis. WSSV then travels through endosomes. During maturation, the pH decreases, a cue for viruses to exit the endosomes. This stage probably involves an interaction between VP28 and Rab7. Once in the nucleus, host transcription factors bind the WSSV genome and initiate expression of viral genes. Bottom panel: Intracellular interactions between WSSV and the host cell. WSSV DNA replication requires host machinery and to make these available WSSV can act to halt the cell cycle in the S-phase through E2F1. A high level of viral protein production can lead to ER stress, e.g., activation of unfolded protein response (UPR) pathways.

Transcription factors of the UPR can activate expression of viral genes, which in turn may inhibit translation through eIF2. WSSV replication requires essential nutrients including iron. To prevent the host from withholding iron, WSSV can inhibit the binding of iron to ferritin. WSSV can influence apoptosis signaling either through miRNA-mediated inhibition of initiator caspases or through viral proteins that inhibit effector caspase activity (Verbruggen, 2016).

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Table 1.2 White spot syndrome virus (WSSV) protein/gene interaction with host (Shekhar and Ponniah, 2015).

WSSV genes/proteins	Molecular interaction with host genes/proteins	Species	Reference
Virus-binding proteins			
VP53A (envelope protein)	Chitin-binding protein (*Pm*CBP)	*Penaeus monodon*	Chen *et al.* (2007)
VP24, VP53A, VP110, VP53B, VP337, VP32, VP124, VP41A, VP51B, VP60A and VP39B (envelope proteins)	Chitin-binding protein (*Pm*CBP)	*P. monodon*	Chen *et al.* (2009)
VP15, VP26, VP28 (nucleocapsid, envelope protein)	*Plg*C1qR	*Pacifastacus leniusculus*	Watthanasurorot *et al.* (2010)
VP95, VP28, VP26, VP24, VP19, VP14 (envelope proteins)	C-type lectin (*Lv*CTL1)	*Litopenaeus vannamei*	Zhao *et al.* (2009)
VP28 (envelope protein)	C-type lectin (*Fc*Lec3)	*Fenneropenaeus chinensis*	Wang *et al.* (2009a)
VP28, VP26 (envelope proteins)	C-type lectins (*Mj*LecA, *Mj*LecB, *Mj*LecC)	*Marsupenaeus japonicus*	Song *et al.* (2010)
VP28 (envelope protein)	C-type lectins (LdlrLec1, LdlrLec2)	*M. japonicus*	Xu *et al.* (2014)
Receptors			
VP187 (envelope protein)	Integrin	*M. japonicus*	Li *et al.* (2007)
VP26, VP31, VP37, VP90, VP136 (envelope nucleocapsid, protein)	Integrin	*L. vannmei*	Zhang *et al.* (2014)
VP28 (envelope protein)	Rab7	*P. monodon*	Sritunyalucksana *et al.* (2006)
Signalling pathways			
WSSV ie1 (immediate-early gene)	NF-κB homologue, *Lv*Relish	*L. vannamei*	Huang *et al.*(2010)
WSSV449 (anti-apoptosis protein)	TLR-mediated NF-kB pathway activation	*L. vannamei*	Wang *et al.* (2011a)
WSSV069 (ie1) promoter (immediate-early gene)	TLR-mediated NF-kB pathway activation	*L. vannamei*	Wang *et al.*(2011a)
WSSV303 promoter	TLR-mediated NF-kB pathway activation	*L. vannamei*	Wang *et al.*(2011a)
WSSV371 promoter	TLR-mediated NF-kB pathway activation	*L. vannamei*	Wang *et al.*(2011a)
WSV083 (immediate-early gene)	Focal adhesion kinase (integrin-mediated signal transduction)	*M. japonicus*	Lu *et al.* (2011)
Apoptosis			
WSSV449 (anti-apoptosis protein)	Caspase	*P. monodon*	Leu *et al.* (2010)
Inhibition of WSSV-DNA polymerase gene expression	Fortilin	*P. monodon*	Nupan *et al.* (2011)
Inhibition of VP15 (nucleocapsid protein) gene expression	Fortilin	*P. monodon*	Nupan *et al.* (2011)
Inhibition of VP28 (envelope protein) gene expression	Fortilin	*P. monodon*	Nupan *et al.* (2011)
VP38 (envelope protein)	Transcriptional repression of caspase gene	*M. japonicus*	Zuo *et al.* (2011)
VP41B (envelope protein)	Transcriptional activation of caspase gene	*M. japonicus*	Zuo *et al.* (2011)
Ubiquitination			
WSSV249 (E3 ligase, RING-H2 protein)	Interaction with ubiquitin-conjugating enzyme, PvUbc via the ubiquitination pathway	*L. vannamei*	Wang *et al.* (2005)
WSSV222 (anti-apoptosis protein)	Ubiquitin-mediated proteolysis of shrimp tumour suppressor-like protein	*L. vannamei*	He *et al.* (2006)
Other interactions			
VP53A (envelope protein)	Glucose transporter 1 (Glut1)	*P. monodon* and *L. vannamei*	Huang *et al.* (2012)
ICP11 (DNA mimic)	Histone-binding	*P. monodon*	Wang *et al.* (2008b)
VP9 (non-structural protein)	Receptor for activated protein kinase C1 (*Pm*-RACK1)	*P. monodon*	Tonganunt *et al.* (2009)
ORF427 (latency-related)	Protein phosphatase	*P. monodon*	Lu & Kwang (2004)
VP15 (nucleocapsid protein)	Immunophilin like protein (*Pm*FKBP46)	*P. monodon*	Sangsuriya *et al.* (2011)
VP26 (envelope protein)	Actin	*Procambarus clarkii*	Xie & Yang (2005)
WSSV ie1 (immediate-early gene)	TATA box-binding protein	*Penaeus monodon*	Liu *et al.* (2011)

1.4 The immune responses in shrimp

Crustaceans including shrimp lack of an adaptive immune system, therefore the defense mechanism relies mainly on the innate immune system (Bachère, 2004). The innate immune system is the first line of inducible host defense against invalid microorganisms including bacteria, virus and fungi (Salzet, 2001). Hemocyte is considered as an immune-responsive cell that plays the most important role in immobilizing or destroying invasive microorganisms (Amparyup et al., 2013; Jiravanichpaisal et al., 2006; Johansson, 2000; Lavine and Strand, 2002; Tassanakajon et al., 2013). Hemocytes are generally classified into three types base on the presence and size of cytoplasmic granules: hyaline (agranular), semi-granular (small granular) and granular (large granula) hemocytes (Bauchau, 1980; Martin, 1985; Tsing, 1989). The functions of different hemocytes in the immune system are previously reported. The hyaline cells are involved in phagocytosis and blood clotting (Smith and Soderhall, 1983), while granular cells are generally function in apoptosis, melanization, encapsulation and nodulation (Kobayashi, 1990; Pech and Strand, 2000; Sung, 1998).

The innate immune system comprises of two major responses including cellular and humoral immune responses (Jiravanichpaisal et al., 2006). The cellular defense performed directly by hemocytes which involved in phagocytosis, encapsulation, nodule formation and apoptosis. Otherwise, the humoral responses involve the prophenoloxidase (proPO)-activating system, the clotting cascade and the activity of immune-related proteins (Holmblad, 1999; Jiravanichpaisal et al., 2006). The overview of shrimp immune system is shown in Figure 1.11.

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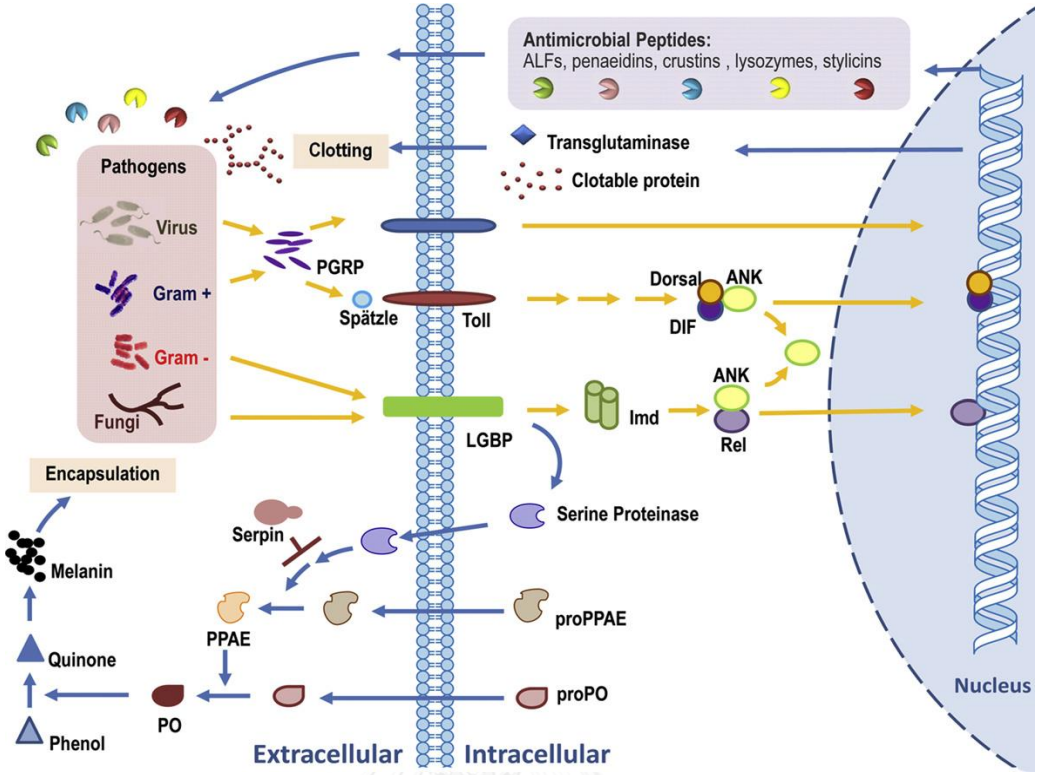

Figure 1.11 A schematic model of the shrimp immune system. (Tassanakajon et al., 2013). The innate immune system is triggered upon binding of pattern recognition receptors (PRRs) to the invading pathogens and subsequently activates host signaling pathways which leads to the activation of cellular and humoral immune responses.

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1.5 Cell-mediated defense mechanism

The cell-mediated immune defense involves various biological processes including phagocytosis, encapsulation and nodule formation (Millar, 1994). Hemocytes in the circulating system play an important role in these mechanisms with different reactions depend on the hemocytes type. Phagocytosis, a common process shared by several organisms, includes foreign body attachment, internalization and destruction. In case of the big invaders, hemocytes form multiple layers surrounded the foreign particle to diminish its severity, and this process called encapsulation (Gillespie, 1997). Another mechanism is called nodule formation. Nodule formation appears to be similar to capsule formation which occurs when the number of invading

microorganisms is high. The pathogens are destroyed by the prophenoloxidase-activating system (Jiravanichpaisal et al., 2006).

1.6 Pattern recognition proteins

Invading of microorganisms such as bacteria, virus and fungi, stimulates the host cell immune responses. The surface components of the foreign substances, called pathogen associated molecular patterns (PAMPs), were recognized by the pattern recognition proteins (PRPs) of the host cells (Hoffmann et al., 1999). These PAMPs include the lipopolysaccharide (LPS) of Gram-negative bacteria, the lipoteichoic acids (LTA) of Gram-positive bacteria, peptidoglycans (PGN) of microbial cell wall, the glycolipids of mycobacteria, the β-1,3-glucan of fungi, the mannans of yeasts and double-stranded RNA of viruses (Hoffmann et al., 1999). During recognition, these receptors activate distinct signaling cascade that regulates specific immune-related proteins in order to eliminating pathogenic microorganisms (Lee and Soderhall, 2001). In crustaceans, several pattern recognition proteins have been identified such as LPS binding proteins (LBPs), β-1,3-glucan binding proteins (βGBPs), peptidoglycan recognition protein, lectins and hemolin (Lee and Soderhall, 2002). Moreover, LPS and β-glucan binding proteins (βGBP and LGBP) have been characterized in black tiger shrimp *P. monodon* (Amparyup et al., 2012).

1.7 The prophenoloxidase (proPO) system

The proPO activating system is one of the important innate immune response in crustaceans. This system composes of several proteins participating in the immune cascade to finally generate the cytotoxic products, oxygen species and melanin as a final product, so-called melanization. It has been reported that the proPO activating system involved in phagocytosis, cell adhesion and encapsulation (Söderhäll and

Cerenius, 1998). Initially, the pathogen-associated molecular patterns (PAMPs) of invading pathogen was stimulated via pattern recognition proteins. The activation leads to a cascade of stepwise activation of proteinases (the prophenoloxidase-activating factors (ppA, PPAEs, PPAFs, PPAPs) in the proPO system, and produces prophenoloxidase (PO) (Ariki et al., 2004). This process controlled by serine proteases and their inhibitors. Then, the PO catalyzes the reaction through its cascade to produce the DOPA-quinone, precursor of the melanin. Melanization 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 restrain the pathogen growth (Söderhäll, 1982). In addition, production of melanin is important for the process of wound healing, sclerosis and encapsulation (Theopold et al., 2004). In the black tiger shrimp, the members of the proPO activating system, *Pm*proPO1, *Pm*proPO2, *Pm*PPAE1 and *Pm*PPAE2 have been identified and characterized. Silencing of these genes result in significant reduction of total PO activity and also enhanced the mortality rate after bacterial infection (Amparyup et al., 2009; Charoensapsri et al., 2009, 2011).

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1.8 The coagulation system

The clotting system is an important reaction to prevent hemolymph loss from sites of injury and obstructing pathogen dissemination throughout the body (Martin, 1991). The cross-linking reaction of coagulation-associated proteins in plasma is catalyzed by hemocyte-derived factors (Cerenius and Söderhäll, 2011). In invertebrate, after stimulation of pathogen-associated molecular patterns (PAMPs), the enzymes involved in clotting are released via exocytosis and subsequently converted to proteolytically active forms. These enzymes are responsible for the activation of proclotting enzyme to clotting enzyme which activated coagulin to form a large insoluble gel to immobilize the invalid microorganisms (Cerenius and Söderhäll, 2011).

It has been reported that polymerization of clottable proteins (CPs) by transglutaminase (TG) triggers hemolymph clotting in crayfish (Sritunyalucksana, 2000), whereas the process in horseshoe crab is regulated by a proteolytic cascade activated by bacterial elicitors through specific recognition proteins. In shrimp, coagulation is believed to rely on the formation of CP polymer that is catalyzed by the Ca^{2+} dependent covalent linkage of the large dimeric CP by TG (Tassanakajon et al., 2013). There are two types of TG found in black tiger shrimp, *Pm*STG I (Huang et al., 2004) and two members of *Pm*STG II (Chen, 2005; Yeh, 2006). The *Pm*STG II is reported to be involved in the coagulation, while *Pm*STG I lacks of coagulation activity. In addition, *Pm*STG II can interacted with receptor domain of *Pm*A2M, knockdown of *Pm*A2M significantly reduced the bacterial seizing of the clotting system, suggested the role of *Pm*A2M in protecting blood clots against fibrinolysis (Chaikeeratisak et al., 2012). Moreover, TG from *F. chinensis* was up-regulated after bacterial infection, implied that *Fc*TG might have a roles in shrimp immunity (Liu, 2007). In *M. Japonicus*, silencing of TG caused down-regulation of AMP, suggesting that the release of AMPs may depend on the coagulation system or mainly on the TG activity (Fagutao, 2012).

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1.9 Antimicrobial peptides (AMPs)

Antimicrobial peptides (AMPs) are products of immune response which play an important role in killing or cleaning the infected pathogens directly (Bachère, 2004). AMPs are found in a variety of organisms such as vertebrate, invertebrate and microorganisms. AMPs are originated from many immune related tissues and various cell types. These peptides have a broad spectrum killing activity such as Gram-negative and Gram-positive bacteria, fungi, enveloped viruses and parasites (Hancock, 2000; Pan, 2000). These molecules typically have a small size approximately less than 20 amino acid residues, with cationic net charge and amphipathic structure. Their small sizes

make them easily to be synthesized and rapidly translocate to the wound sites and also attach and insert themselves into membrane bilayers forming pores and resulting in the leakage of cellular components. To date, there are three distinct models of pores forming mechanism; barrel-stave, carpet and toroidal-pore models (Brogden, 2005).

Several AMPs have been identified and studied their biological functions in shrimp such as penaeidins, crustins, lysozymes, anti-lipopolysaccharide factors (ALFs), hemocyanin and stylicin (Tassanakajon et al., 2010). Among these proteins, the anti-lipopolysaccharide factor isoform 3 (ALF3) from *P. monodon* has a broad spectrum antimicrobial activity (Jaree et al., 2012; Somboonwiwat et al., 2005). It has been reported that ALF*Pm*3 can kills bacteria through bacterial membrane permeabilization (Jaree et al., 2012). In addition, ALF*Pm*3 also has the antiviral activity against WSSV by binding to the envelope protein, WSSV189 of virus (Suraprasit et al., 2014; Tharntada et al., 2009).

1.10 Proteinases and their inhibitors

Proteinases can be classified by their active-site catalytic residue into main proteinase families as follows: metalloproteinase, serine proteinase, cysteine proteinase, threonine proteinase, aspartic proteinase and unidentified proteinase families. Proteinases in some pathogens can be function to help them penetrate the cuticle of their host. In black tiger shrimp, several proteinases have been identified. Recently, *Pm*ClipSP2 was shown to act as a pattern-recognition protein and activate PO activity in *P. monodon* (Amparyup et al., 2013). The serine proteinase homologue, *Pm*MasSPHs is important in activation of shrimp proPO system, particularly *Pm*MasSPH1 which can specifically interact with *Pm*PPAE2 (Jearaphunt et al., 2015). In addition,

*Pm*Snake protein was shown to activate the proPO system via serine proteinase cascade in shrimp (Monwan et al., 2016).

In order to control the biological processes mediated by proteinase, proteinase inhibitors are required (Iwanaga and Lee, 2005; Jiravanichpaisal et al., 2006). Proteinase inhibitors are essential to protect host from microbial proteinases and regulate the proteinase cascade for example the proPO and coagulation system. Injury and microbial infection lead to activation of the blood coagulation and proPO systems. These systems employ cascades of proteinases to amplify an initial signal, result in rapid and efficient responses to the threats to health (O'Brien, 1993; Whaley, 1993). Previous study showed that pacifastin and **α**-macroglobulin inhibited crayfish proPO activation (Aspen et al., 1990). Recently, *Pm*Pacifastin-like was identified in *P. monodon* and have important role in shrimp proPO. It was shown that depletion of *Pm*Pacifastin-like strongly increased PO activity (Sangsuriya et al., 2016). In insect, Kunitz family inhibitors can interfere the proPO activation (Aso et al., 1994; Saul and Sugumaran, 1986; Sugumaran et al., 1985). The reports from *M. sexta* showed that, Serpin-1J and Serpin-6 isolated from hemolymph can inhibits the proPO activation (Jiang and Kanost, 1997; Wang, 2004). In black tiger shrimp, *Pm*SERPIN8 has been shown to be able to inhibit the Gram-positive bacterium and inhibit the proPO activation in shrimp (Somnuk et al., 2012). Moreover, *Pm*SERPIN3 was reported to function as a regulator of the proPO activating system (Wetsaphan et al., 2013).

1.11 Apoptosis

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., 1972; Opferman and Korsmeyer, 2003; Thompson, 1995). Apoptosis can be detected by characteristic

morphological features, such as chromatin condensation, cell shrinkage, blebbing of the plasma membrane and fragmentation of the cell body (Kerr et al., 1972; Wyllie et al., 1980). Caspases play a key role at various stages of apoptotic process (Thornberry, 1998). Caspase-8 is required for mediated apoptotic, whereas caspase-3 has been recognized as the crucial executioner caspase (Jin and El-Deiry, 2005). In shrimps, several genes involved in apoptosis are discovered. For example, it has been reported that caspase from *M. japonicas* is important for apoptosis during WSSV infection (Wang et al., 2008). Moreover, overexpression of recombinant caspase (*Pm*Caspase) in *P. monodon* can induces apoptosis in Sf-9 cells (Wongprasert et al., 2007). In addition, other caspases have been identified, include cap-3 and *Lv*caspase2-5 from *L. vannamei* (Rijiravanich et al., 2008; Wang, 2013). Cap-3 from *P. merguiensis* (Phongdara, 2006) and *Pm*casp from *P. monodon* (Leu, 2008).

However, over apoptosis can lead to shrimp death, so apoptotic inhibitor is necessary. In shrimps, several inhibitors of apoptotic proteins have been characterized, including the inhibitor of apoptosis protein (IAP), the defender against apoptotic death 1 (DAD1), translationally controlled tumor protein (TCIP/fortilin) and survivin (Bangrak et al., 2004; Graidist, 2006; Molthathong et al., 2008b). IAP can block apoptosis by directly inhibit the caspases activity (Ceveraux and Reed, 1999). In *P. monodon*, *Pm*IAP can block the apoptosis induced by the *Drosophila* Reaper protein (Leu et al., 2008). In addition, *Lv*IAP1 from *L. vannamei* has been reported to be a central regulation of hemocytes apoptosis (Leu et al., 2012).

1.11.1 Apoptosis during WSSV infection

Apoptosis plays a key role in the animal defense mechanism against viral infection by limiting virus production and decrease or eliminate spread of progeny virus in the host (Aubert and Jerome, 2003; Leu et al., 2013; Liu et al., 2009). Many viruses

have the mechanisms to inhibit host cell apoptosis during infection, so that they can prolong the host cell viability until the progeny viruses are successfully produced (Koyama et al., 2000). Although, some viruses induce apoptosis in order to facilitate the spread of progeny virus to neighboring cells at the late stage of infection (Best, 2008). It has been reported that *L. vannamei* shrimp infected with white spot syndrome virus (WSSV) showed increased numbers of apoptotic cells (Henderson, 1999). *P. monodon* infected with WSSV also showed signs of apoptosis and the extent of apoptosis was proportionally related to the severity of the WSSV infection (Sahtout et al., 2001). In addition, the apoptotic characteristics were observed only in cells that were virion-free and not in cells that contained the virions in Kuruma shrimp (Wu, 2004). Several researches showed that the extent of apoptosis is related to the dose of WSSV infection, varies among different tissues and depends on the species of the infected host (Hameed, 2006; Wongprasert et al., 2003).

WSSV can produces the anti-apoptosis proteins to regulate apoptosis in host cells upon infection. AAP-1 (ORF390 or WSSV449) can bind to and cleaved by *Pm*Caspase to inhibit *Pm*Caspase activity (Leu et al., 2008). WSSV222 was reported to function as an anti-apoptosis protein in WSSV-infected shrimp via ubiquitin-mediated degradation of the tumor suppressor-like protein (TLS) (He et al., 2009). Recently, two new anti-apoptotic proteins of WSSV were identified, WSSV134 and WSSV322 have been shown to bind with P20 domain of *Pm*Casp and can suppressed the apoptosis induced by *Pm*Casp in Sf-9 cells (Lertwimol et al., 2014). In addition, WSSV-miR-N24 can regulate apoptosis by inhibiting apoptosis through down-regulation of caspase-8 expression (Huang, 2014). Not only anti-apoptosis proteins but WSSV can also produce the inducer of apoptosis. For example, ICP11 has been shown to have the apoptosis-inducing activity by destabilization of nucleosome (Wang, 2008).

Leu et al. (2013) has proposed the model of the apoptotic interaction between shrimp and WSSV. When host was infected by WSSV, the cellular sensors detect the

invading virus and trigger the signaling pathways to induce the expression of pro-apoptotic proteins, including *Pm*Casp, *Mj*Caspase and VDAC. Whereas, WSSV also induced mitochondrial membrane permeabilization and increases oxidative stress. This possibly induces the production of WSSV ICP11 as well as other unknown mechanism to initiate apoptosis process (Figure 1.12A). Meanwhile, the WSSV anti-apoptosis proteins are expressed. AAP-1 is directly inhibit caspase, whereas WSV222 blocks apoptosis through ubiquitin-mediated degradation of shrimp TLS protein. WSSV also induces the expression of shrimp anti-apoptosis protein, *Pm*-fortilin, to inhibit mitochondrial-triggered apoptosis. Thus, WSSV is able to complete its replication cycle when these three proteins function together to block apoptosis (Figure 1.12B) (Leu et al., 2013).

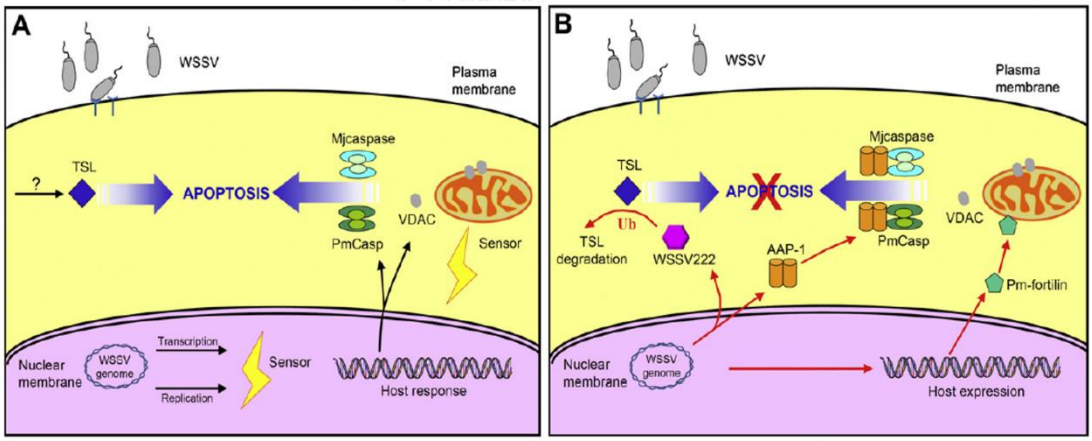

Figure 1.12 A model of the apoptotic interactions between a shrimp host cell and WSSV. (A) The apoptotic-related proteins from shrimp are produced upon WSSV infection. (B) WSSV proteins are expressed to inhibit apoptosis in order to complete replication cycle (Leu et al., 2013).

1.12 Hematopoiesis and hemocyte homeostasis

Hematopoiesis is a process where hemocytes synthesis takes place in the hematopoietic tissue (HPT), and the maturing hemocytes become functional and then

released into the circulatory system (Chaga, 1995; Söderhäll et al., 2003). Hemocytes number vary after pathogen infection, injury or stress (Jiravanichpaisal et al., 2001; Johansson, 2000; Persson, 1987; Smith and Söderhäll, 1983), and this variability is essential to maintaining the number of hemocytes in circulation. Homeostasis, achieved by the continuous proliferation and apoptosis, is thus required for the maintenance of hemocytes balance in the circulation. Hemocyte homeostasis is a complex process by which the hemocyte numbers are maintained at an appropriate level.

Crustaceans have an open circulatory system and main three types of hemocytes are identified including hyaline cells, semigranular cells (SGCs) and granular cells (GCs) (Lin et al., 2008; Söderhäll et al., 2003; Sricharoen et al., 2005; Wu et al., 2008). Hyaline cells are small, no granules or very few, and proposed as a pro-stage for the two hemocyte lineages (Roulston and Smith, 2011; Van de Braak et al., 2002; Wu et al., 2008). The SGC cells contains a variable number of small granules, while GC cells contain a densely packed with eosinophilic granules (Cerenius, 2010) (Figure 1.12).

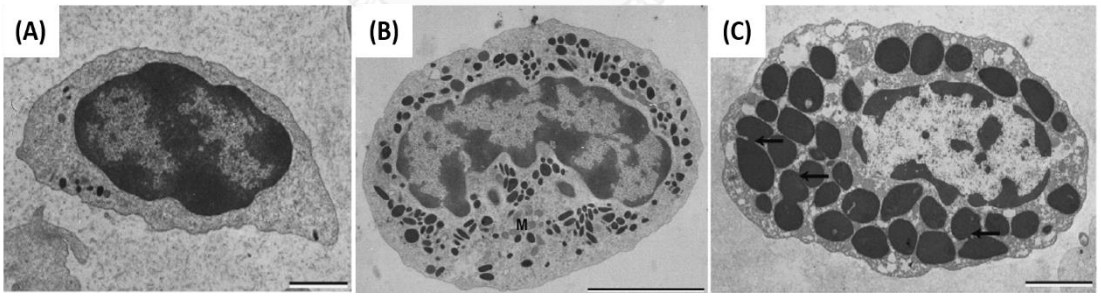

Figure 1.13 Classification of crustacean hemocytes. Transmission electron microscopy of hemocytes from crayfish, *Astacus leptodotylus*. (A) Hyaline cells. (B) Semi-granular cells. (C) Granular cells (Modified pictures from (Giulianini et al., 2007)).

In recent years, several genes involved in hematopoiesis and hemocyte homeostasis have been characterized in crustaceans (Lin and Söderhäll, 2011; Lin et al., 2011; Prapavorarat et al., 2010; Saelee et al., 2013; Söderhäll et al., 2003; Söderhäll

et al., 2005). Previously, the cytokine astakine was first identified in *Pacifastacus leniusculus* (Söderhäll et al., 2005) and was subsequently identified in the shrimp *Penaeus monodon* (Hsiao and Song, 2010). These astakines are found to be required for cell proliferation and differentiation in HPT of two crustacean species (Hsiao and Song, 2010; Lin X et al., 2010; Söderhäll et al., 2005). Recently, an astakine-like factor was reported to be involved in hemocytes proliferation in the pacific oyster, *Crassostrea gigas* (Li et al., 2016). Interestingly, transglutaminase in crayfish is reported to participate in preventing hematopoietic stem cells differentiation (Lin et al., 2008). A crustacean hematopoietic factor (CHF) was first identified in *P. leniusculus* and was shown to be vital for survival of the hemocytes and HPT cells by preventing apoptosis (Lin et al., 2011). The CHF-like protein was found in *L. vannamei* and was shown to bind to a laminin receptor (Lamr) and proposed to be involved in hemocyte homeostasis in shrimp (Charoensapsri et al., 2015). Recently, two β-thymosin proteins (β-thymosin 1 and β-thymosin 2) have been shown to regulate hemocyte homeostasis in *P. leniusculus* (Saelee et al., 2013). The Runt homologue and CBFβ were reported to play a role in production of hemocytes in the scallop, *Chlamys farreri* (Yue, 2014). In addition, ROS has been reported to be involved in hematopoiesis by help to maintain the progenitor cells in HPT and reduce hemocytes release in crayfish *P. leniusculus* (Junkunlo et al., 2016).

In shrimp, a hemocyte homeostasis-associated protein (HHAP) has been identified as a viral-responsive protein and plays a role in hemocyte homeostasis in the black tiger shrimp *Penaeus monodon* (Prapavorarat et al., 2010). Silencing of the *Pm*HHAP transcripts resulted in a significant decrease in the total hemocytes number together with hemocytes deformation and lysis (Prapavorarat et al., 2010). Moreover, suppression of *Pm*HHAP also led to 100% shrimp mortality within 30 h, indicating the importance of *Pm*HHAP in shrimp viability (Prapavorarat et al., 2010). Furthermore, it

has been shown that HHAP in *L. vannamei* is participates in hemocyte homeostasis in shrimp (Charoensapsri et al., 2015).

The model of hemocyte production in crustaceans has been studied in crayfish *Pacifastacus leniusculus* (Chaga, 1995; Noonin et al., 2012; Söderhäll, 2013, 2016). Five different cell types are identified in the HPT. Type I is a cell with large nuclei surrounded by a thin layer of cytoplasm (stem cells). Type 2 is a proliferation cell and a SGC and GC precursor. Type 3 and 4 are GC precursor, while type 5 is a SGC precursor (Chaga, 1995; Söderhäll, 2013). Two astakines are identified to play important role in controlling hematopoiesis, astakine 1 and astakine 2. Astakine 1 stimulates the proliferation of HPT cells, induces differentiation of SCG and stimulates the release of hemocytes into the circulating system (Söderhäll et al., 2005). Astakine 1 is also able to regulate the transcription of PCNA, KPI and CHF I HPT cells (Lin et al., 2010, 2011). On the other hand, astakine 2 is responsible for GC differentiation (Lin et al., 2010). There are also other proteins which expressed in different cell types in this process as shown in Figure 1.14. 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.

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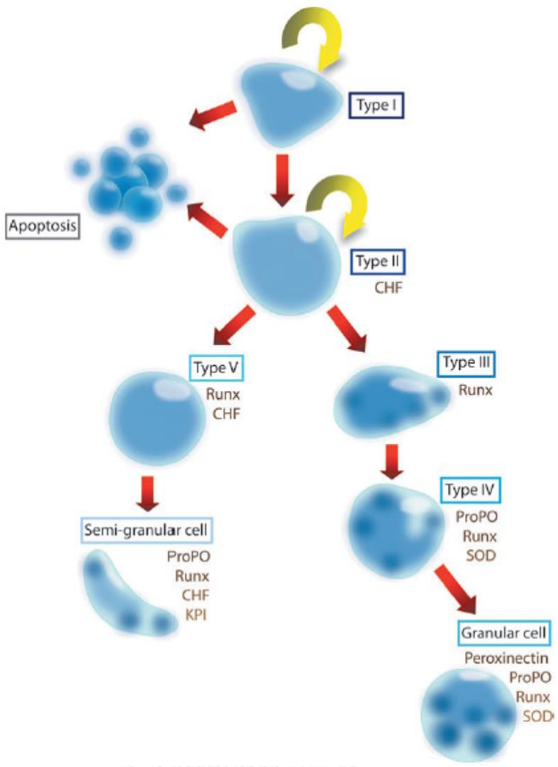

Figure 1.14 Schematic overview of the hematopoietic lineages in crayfish. Hemocytes are produced in hemotopoetic tissue (HPT) which five different cell types are identified. The cell type I is a stem cell and type II is a SGC/GC precursor. Cells in the HPT follow 2 main cell lineages: one is from type I-II (the stem cells) via type V cells to SGCs, and the other is from type III to types III and IV cells to GCs (Noonin et al., 2012).

1.13 Purpose of this thesis research

In crustaceans including shrimp, controlling of hemocyte homeostasis is very important for animals to survive as well as to protect themselves from invading pathogens. Our previous study in shrimp demonstrated that *Pm*HHAP highly responds upon WSSV infection and plays an important role in hemocyte homeostasis (Prapavorarat et al., 2010). However, the mechanism underlying the function of *Pm*HHAP in mediating hemocyte homeostasis in shrimp remains unclear. In the present study, the potential role of *Pm*HHAP in the regulation of hemocyte homeostasis in *P.*

monodon shrimps was mainly focused. In order to gain more insight into the function of *Pm*HHAP upon WSSV infection, we aimed to identify the partner proteins of *Pm*HHAP and characterized their function. In addition, HHAP has also been identified in fresh water crayfish, *Pacifastacus leniusculus* but the function is still in its fancy (Prapavorarat et al., 2010). In an attempt to address this issue, this present study also investigates the role of *Pl*HHAP in crayfish. The acquired results of this research will provide a better understanding of shrimp immunity and reveal the regulation of shrimp hemocyte homeostasis and its function upon viral infection to bring about the prevention and treatment of diseases in shrimp.

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CHAPTER 2

MATERIALS AND METHODS

2.1 Equipment and Chemicals

2.1.1 Equipment

-20°C Refrigerator Freezer (SHARP), -80°C Freezer (Thermo Forma)

24-well plate Costar® (Corning Incorporation)

96-well plate Costar® (Corning Incorporation)

96-well Black plate Costar® (Corning Incorporation)

Amicon Ultra-4 concentrators (Vivaspin)

Autoclave LABO (SANYO)

Automatic micropipette (Gilson Medical Electrical S.A.)

Balance (METTLER TOLEDO)

Centrifuge (Thermo Scientific)

Centrifuge 5804R (Eppendorf)

Centrifuge tube CentriStar™ 15 ml, 50 ml (Corning Incorporation)

Gel documentation (SYNGENE)

Gene Pulser (BIO-RAD)

Hybridization oven (Hybrid)

Incubator (Memmert)

Innova 4080 incubator shaker (New Brunswick Scientific)

Insulin syringes U 100 (Becton, Dickinson and Company)

Laminar Airflow Biological Safety Cabinets (NuAire, Inc.)

Microcentrifuge tube (Axygen Scientific)

Millex syringe-driven filter unit 0.22, 0.45 μM (Millipore, MERCK)

Orbital shaker SO3 (Stuart Scientific, Great Britain)

PCR Mastercycler (BIO-RAD, Eppendorf AG, Germany)

PCR Thin wall microcentrifuge tubes 0.2 ml (Axygen Scientific, USA)

PCR workstation Model # P-036 (Scientific Co., USA)

PCR strip tube white (BIO-RAD)

PCR cover strip (BIO-RAD)

PD-10 Column (GE Healthcare)

pH meter Model # SA720 (Orion)

Pipetman Classic™ (Gilson Incorporation)

Pipette tips 0.2-10, 20-200, 1000 µl (Axygen Scientific)

Power supply, Power PAC 3000 (BIO-RAD)

Spectrophotometer Spectronic 2000 (Bausch & Lomb)

Spectrophotometer DU 650 (Beckman, USA)

Sterring hot plate (Fisher Scientific)

Semi-dry Trans-Blot® (BIO-RAD)

Touch mixer Model # 232 (Fisher Scientific)

Ultra Sonicator (SONICS Vibracell)

Vertical electrophoresis system (Hoefer™ miniVE)

Water bath (Memmert)

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2.1.2 Chemicals, Reagents and Biological substance

100 mM dATP, dCTP, dGTP and dTTP (Thermo Scientific)

100 bp PlusGeneRuler™ (Thermo Scientific)

1 kp GeneRuler™ (Thermo Scientific)

2-Mercaptoethanol (Fluka)

5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) (Fermentas)

5-bromo-4-chloro-3-indolyl-a-D-galactopyranoside (X-alpha-gal) (Clontech)

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

Acrylamide/Bis Solution (BIO-RAD)

Agar powder (HIMEDIA)

Agarose (Research organics)

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

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

Ammonium persulfate (USB)

Amplicillin (BioBasic)

Anti-actin mouse Clone C4 (Millipore, MERCK)

Anti-His antiserum (GE Helthcare)

Boric acid (MERCK)

Bovine serum albumin (SIGMA-ALDRICH)

Bromophenol blue sodium salt (USB)

Calcium chloride (MERCK)

Chloramphenicol (Sigma)

Chloroform (RCL Labscan)

Collagenase Type I (SIGMA-ALDRICH®)

Collagenase Type IV (SIGMA-ALDRICH®)

Coomassie brilliant blue G-250, R-250 (BIO BASIC INC.)

Diethyl pyrocarbonate (DEPC) (Sigma)

Di-Sodium hydrogen orthophosphate anhydrous (Carlo Erba)

Dithiothereitol (DTT) (BIO BASIC INC.)

Ethanol (MERCK)

Ethidium bromide (Sigma)

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Ethylene diamine tetraacetic acid disodium salt dehydrate (EDTA) (Ajax Finechem)

Formaldehyde (BDH)

Genezol reagent (Geneaid)

Glacial acetic acid (MERCK)

Glucose (Ajax chemicals)

Glutaraldehyde (SIGMA®)

Glycerol (Scharlau)

Glycine (Scharlau)

Hydrochloric acid (MERCK)

Imidazole (Fluka)

Isopropanol (MERCK)

Isopropyl-β-D-thiogalactoside (IPTG) (Thermo Scientific)

Kanamycin (BIO BASIC Inc.)

L-15 Medium (Leibovitz) (SIGMA-ALDRICH®)

Magnesium chloride (MERCK)

Methanol (MERCK)

N, N-dimethyl formamide (Carlo Erba)

N, N-methylenebisacrylamide (Fluka)

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

Ni Sepharose 6 Fast Flow (GE Healthcare)

Nitrobluetetrazolium (NBT) (Fermentas)

Paraformaldehyde (SIGMA)

pEGFP vector (Clontech)

pET15b(+) vector (Novagen)

pET22b(+) vector (Novagen)

pGBKT7 vector (Clontech)

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pIZ/V5-His Vector (Invitrogen)

Phenol, saturated (MERCK)

Potassium chloride, KCl (Ajax Finechem)

Potassium dihydrogen orthophosphate (Ajax Finechem)

Prestained protein molecular weight marker (Fermentas)

Prolong Gold Antifade Reagent (Invitrogen)

Skim milk powder (HIMEDIA)

Sodium acetate (Carlo Erba)

Sodium citrate (Carlo Erba)

Sodium chloride (Ajax Finechem)

Sodium dihydrogen orthophosphate (Carlo Erba)

Sodium dodecyl sulfate (Vivantis)

Sodium hydroxide (MERCK)

Tris-(hydroxy methyl)-aminomethane (USB)

Triton® X-100 (MERCK)

Tryptone type I (HIMEDIA)

Tween™ 20 (Ajax Finechem)

Urea (SIGMA-ALDRICH)

Yeast extract powder (HIMEDIA)

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2.1.3 Enzymes and Kits

Advantage® 2 Polymerase Mix (Clontech)

Annexin-V-FLOUS Staining Kit (Roche)

Apoptotic DNA Ladder Detection Kit (Abcam)

BamHI (Biolabs® Inc.)

Caspase-Glo® 3/7 Assay (Promega)

DNase I (RNase-free) (Biolabs® Inc.)

MEGAscript® Kit (Ambion®)

NcoI (Biolabs® Inc.)

NdeI (Biolabs® Inc.)

NotI (Biolabs® Inc.)

NucleoSpin® Extract II Kits (MACHEREY-NAGEL)

PCR-Select™ cDNA Subtraction Kit (Clontech)

Plasmid mini prep (Geneaid)

QuantiTect SYBR® Green PCR Kit (QIAGEN)

RBC T&A Cloning Vector (RBC Bioscience)

Revert Aid First Strand cDNA Synthesis kit (Thermo Scientific)

RNase A (SIGMA)

SsoFast™ Evagreen® Supermix (BIO-RAD)

T4 DNA ligase (Biolabs® Inc.)

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

Taq DNA polymerase (RBC Bioscience)

ThermoScript (Invitrogen)

XhoI (Biolabs® Inc.)

Yeastmaker™ Yeast Trnasformation System (Clontech)

Zeocin™ (Invitrogen)

2.1.4 Microorganisms

Aeromonas Hydrophila B1

Escherichia coli strain BL21

Escherichia coli strain Rosetta (DE3)

Escherichia coli strain JM109

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Vibrio harveyi 639

White spot syndrome virus

2.1.5 Software

Blast programs (http://www.ncbi.nlm.nih.gov/BLAST/)

ClustalW multiple sequence alignment program (http://www.ebi.ac.uk/Tools/clustalw2/)

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

GENETYX 7.0.3 program (GENETYX Corporation)

GraphPad Prism 6 (GraphPad Software, Inc.)

FV10-ASW 3.0 viewer

SECentral (Scientific & Educational Software)

SMART version 4.0 (http://www.smart.emblheidelberg.de/)

SPSS statistics 17.0 (Chicago, USA)

2.2 Shrimp samples

Healthy black tiger shrimp, *Penaeus monodon*, of about 15-20 g bodyweight were purchased from shrimp farms in Surat Thani Province, Thailand. Moreover, shrimp also obtained from the Shrimp Genetic Improvement Center, BIOTEC, Thailand. Shrimp were maintained in tanks with aerated water with a salinity of 20 ppt for at least 7 days before use. For RNAi experiments, shrimp of about 3-5 g bodyweight were purchased from local farms and maintained as above.

2.3 *In vivo* gene silencing

2.3.1 Preparation of double-stranded RNAs (dsRNAs)

To investigate the role of *Pm*HHAP in hemocyte homeostasis in shrimp, the double-stranded RNA (dsRNA)-mediated gene knockdown was performed. Double-stranded RNAs (dsRNAs) were generated as described previously (Prapavorrarat et al., 2010). The plasmid containing *Pm*HHAP gene was amplified using sense and anti-sense *Pm*HHAP gene specific primers (Table 2.1) flanked by the T7 promoter site, while a GFP fragment from pEGFP-1 plasmid was produced as a negative control gene. After PCR amplification, the two types of PCR product were used as templates for *in vitro* transcription using T7 Ribomax™ Express Large Scale RNA Production System (Promega) according to the manufacturer's instruction. Briefly, a plasmid containing either *Pm*HHAP gene or GFP gene was used as a template for PCR amplification. Then, equal amounts of sense and anti-sense single stranded RNA were mixed together and incubated at 70°C for 10 min, and slowly cooled down at room temperature to form dsRNA. The solution was treated with 1 U of RQ1 RNase-free DNase (Promega) at 37°C for 30 min then purified by standard phenol-chloroform extraction. The quality and amount of dsRNAs were verified by 1.5% agarose gel electrophoresis with UV visualization following ethidium bromide staining, and UV spectrophotometry, respectively.

Table 2.1 Nucleotide sequences of the primers used for gene silencing.

Gene name	Primer sequences (5′-3′)	Accession no.
*Pm*HHAPi-F	GCAACAGGAGAACCTGTGGATA	HQ130431
*Pm*HHAPi-R	GGCCTTGTGACGTTCTTCCATT	HQ130431
T7*Pm*HHAPi-F	TAATACGACTCACTATAGGGCAACAGGAGAACCTGTGGATA	HQ130431
T7*Pm*HHAPi-R	TAATACGACTCACTATAGGGGCCTTGTGACGTTCTTCCATT	HQ130431
WSSV134i-F	GGGAATGTTTCTGCTGCCCTAC	AAL89002
WSSV134i-R	ATAGCCTTGAGCCAGTCCTTTG	AAL89002
T7WSSV134i-F	TAATACGACTCACTATAGGGGGAATGTTTCTGCTGCCCTAC	AAL89002
T7WSSV134i-R	TAATACGACTCACTATAGGATAGCCTTGAGCCAGTCCTTTG	AAL89002
GFPi-F	ATGGTGAGCAAGGGCGAGGA	U55761
GFPi-R	TTACTTGTACAGCTCGTCCA	U55761
T7GFPi-F	TAATACGACTCACTATAGGATGGTGAGCAAGGGCGAGGA	U55761
T7GFPi-R	TAATACGACTCACTATAGGTTACTTGTACAGCTCGTCCA	U55761
*Pl*HHAPi-F	TAATACGACTCACTATAGGGCCATACCAGGGCAAGAGGAG	HQ130432
*Pl*HHAPi-R	TAATACGACTCACTATAGGGCCACAGAACTGCCCTATTTG	HQ130432

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2.3.2 *In vivo* gene silencing of *Pm*HHAP mediated by RNA interference

For dsRNA-mediated gene knockdown, 2 μg of *Pm*HHAP dsRNA was dissolved in 25 μl of 150 mM NaCl and an equal volume of saline solution containing 2 μg of GFP dsRNA served as a dsRNA control. The dsRNA was intramuscularly injected into the third abdominal segment. Hemocytes were collected from individual shrimp at 12 h post-dsRNA injection (three shrimp per group) and immediately centrifuged at 800 × g for 10 min at 4°C to separate the hemocyte from the plasma. Then, hemocytes were subsequently collected to extract total RNA and synthesis cDNA to test the gene silencing efficiency.

2.3.3 Total RNA extraction and first strand cDNA synthesis

Total RNA was extracted from hemocytes using GeneZol Reagent (Geneaid) according to the manufacturer's protocol. Briefly, hemocytes were homogenized in GeneZol reagent, then incubated on ice 30 min after added 200 μl of chloroform. After centrifugation at 12,000 × g for 15 min at 4°C, the RNA-containing aqueous (upper) phase was transferred to a new tube and precipitated with 1 volume of isopropanol. Next, the sample was centrifuged at 12,000 × g for 15 min at 4°C, pellet was washed with 1 ml of 75% (v/v) ethanol in diethyl pyrocarbonate (DEPC)-treated water and centrifuged again. The RNA pellet was air-dried and dissolved in an appropriate volume of DECP-treated water followed by DNase I (Biolabs) treatment to remove any DNA contamination. Then RNA pellet was re-purified using GeneZol reagent as described above. The equal amount of RNA from each sample was used as a template for cDNA synthesis using Revert Aid First Strand cDNA Synthesis kit (Thermo Scientific). The mixture was incubated at temperature in a series of 25°C for 5 min, 42°C for 60 min and 70°C for 10 min, respectively, then the samples were stored at -20°C for further investigation.

2.3.4 Semi-quantitative RT-PCR analysis of gene expression in silenced shrimp

The efficiency of *Pm*HHAP gene transcript silencing was verified by semi-quantitative RT-PCR using gene specific primers for *Pm*HHAP gene (Table 2.2). A fragment of elongation factor1-α (EF1-α) gene was also amplified and used as an internal control. The cDNA from each RNA sample was used as template for the PCR amplification. The total amount of PCR reaction was 25 µl reaction consisting of 1 µl of 10-fold diluted cDNA as a template, 2.5 mM of dNTP, 5 µM of each gene specific primers and 1 unit of RBC Taq DNA polymerase (RBC Bioscience). The PCR conditions were 94°C for 1 min, followed by 30 cycles of 94°C for 30 s, 58°C for 30 s, 72°C for 30 s, and subsequently a final 72°C for 5 min, except only 22 cycles were performed for the EF1-α gene. The PCR products were analyzed by 1.5% (w/v) agarose gel electrophoresis and visualized by UV-transillumination.

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Table 2.2 Nucleotide sequences of the primers used for RT-PCR and qRT-PCR.

Gene name	Primer sequences (5′-3′)	Accession no.
*Pm*HHAP-F	GCAACAGGAGAACCTGTGGATA	HQ130431
*Pm*HHAP-R	GGTTCCAGAATCGCCTCCTATA	HQ130431
*Pm*Casp-F	TAAACTTCACGGCTGAACGG	DQ846887
*Pm*Casp-R	TCAGCATGGATGGGAATCAC	DQ846887
*Pm*Caspase-F	CGTGGTTCATTCAGTCGCTG	EF114674
*Pm*Caspase-R	AACCTTTCGCATCAGGGTTG	EF114674
Cat B-F	ATGAGTGATCGGCAGTGTATCC	EF213113
Cat B-R	GATGCAGATCACTCTCGTAGTC	EF213113
Cat D-F	GACCAAGTCCTCCACATACAAG	EF213114
Cat D-R	GTGACTCCATCAACAGCAATCC	EF213114
DAD1-F	ACTCCTTCCTGTCTGGGTTCA	EF581986
DAD1-R	AAATCAGCAAAGCCTCGTTC	EF581986
Survivin-F	CCTGACCTCGTCCGTTGCTTTG	GU903910
Survivin-R	GGTTGTCTCACTCTCTGCCTTG	GU903910
WSSV134-F	CGAAGCGGAACATACAGTTGAAGG	AAL89002
WSSV134-R	GCTCCTTCGATTTCCGGTAAGTTG	AAL89002
VP28-F	GGGAACATTCAAGGTGTGGA	DQ979320
VP28-R	GGTGAAGGAGGAGGTGTTGG	DQ979320
ie1-F	AGCAAGTGGAGGTGCTATGT	AF332093
ie1-R	CCATGTCGATCAGTCTCTTC	AF332093
wsv477-F	GGCCAAGTCATGGAGATCTA	DQ121373
wsv477-R	CCATCCACTTGGTTGCAGTA	DQ121373
WSSV362-F	GGCACCGAAGAGGACAATAAAG	AAL89229
WSSV362-R	CGCGTAGATACATCATGTTGCC	AAL89229

Table 2.2 Nucleotide sequences of the primers used for RT-PCR and qRT-PCR. (Continued)

Gene name	Primer sequences (5′-3′)	Accession no.
WSSV395-F	GGACTCCATGGGCTCGTCTAACGGAGATGAG	AAL89263
WSSV395-R	GGTCTAGGATCCCTAATGATGATGATGATGATGAAA	AAL89263
ATF-F	GCCCAGGTCCAGGTCTGTCTGAAGG	AHY82567
ATF-R	ACATCATAGGCCTCACACACGACGA	AHY82567
Cat L-F	CTCGCAAGTTTGGCAACAATGG	ABQ10739
Cat L-R	CAGCCTTCTTCAGTGCATGTTC	ABQ10739
LDH-F	AGCAGCATGTCTTCCTTCTC	AEC12821
LDH-F	ATTGGTCAGCTCCAACAGTC	AEC12821
Ferritin-F	CGGGTCACCAGTGTGTGGACGAGCA	ABP68819
Ferritin-R	AGAACTTGGCAAAACCAGGCAGAGC	ABP68819
IAP-F	ACAGATGGGACACTGAGTAAGAGCG	ABQ38431
IAP-R	AAGCCAAGGAGATGACGAGGCAATG	ABQ38431
EF1-α-F	CGTGCTGGACAAGCTGAAGGC	DQ021452
EF1-α-R	CGTTCCGGTGATCATGTTCTTGATG	DQ021452
*Pl*HHAP-F	AGTGCAGTTTAGGAACACAT	HQ130432
*Pl*HHAP-R	ACACATACATTCTTGCAGTC	HQ130432
CHF-F	ATGCGGAGCAGCTACCGCTTGAGGTG	GQ497446
CHF-R	GGCCGACATGTGAATGAG	GQ497446
AST1-F	GGCTATGGCTGGCTCTTGTAA	AY787656
AST1-R	AGGTGGCTGAAGGTCGGGTA	AY787656
AST2-F	ATGGTGTTAGTGTTGATGTGTGGC	EF568370
AST2-R	GGGACTCAGCGGAGATTTTGC	EF568370
IGFBP7-F	GCAGAAGAGGTCTCTGGTAAGG	

Table 2.2 Nucleotide sequences of the primers used for RT-PCR and qRT-PCR. (Continued)

Gene name	Primer sequences (5′-3′)	Accession no.
IGFBP7-R	CCCGCATAATACATGATCTGCTCC	
40S-F	GACGAATGGCATACACCTGAGAGG	CF542417
40S-R	CAGGACTCTGCAGTTCAAGCTGATG	CF542417

2.4 Apoptosis analysis in hemocyte of the *Pm*HHAP-silenced shrimp

2.4.1 Analysis of cell death by fluorescent staining

To investigate whether *Pm*HHAP is involved in hemocyte apoptosis in shrimp, percentage of apoptotic cells was measured using the Annexin-V-FLOUS Staining Kit (Roche). The dsRNA-mediated RNAi knockdown of *Pm*HHAP gene was performed as described above. At 12 h post-dsRNAs injection, hemolymph was withdrawn from the shrimp and hemocytes were subsequently collected by centrifugation at 800 × g for 10 min at 4°C. Cells were washed twice with the phosphate buffer saline (1XPBS, pH 7.4) and centrifuged at 800 × g for 10 min at 4°C. The number of apoptotic hemocytes in the *Pm*HHAP knockdown shrimp and control were then analyzed using the Annexin-V-FLUOS Staining Kit according to the manufacturer's protocol. Briefly, the hemocyte number was counted under a light microscope using the hematocytometer. The hemocyte cells were adjusted the concentration to 10^4 cells per well and applied into each well of a 96-well plate. Then, the hemocyte cells were incubated with 100 µl of Annexin-V-FLOUS labeling reagent in presence of propidium iodide for 10-15 min in dark at room temperature. Afterwards, cells were analyzed by fluorescence microscopy (Olympus). The positive green fluorescent signal of Annexin-conjugated FITC from the apoptotic cells were scored as apoptotic positive cells and the

percentage of positive cells was determined. The apoptotic hemocyte rate was expressed as the mean ± S.D. of three replicates.

2.4.2 Apoptotic DNA ladder detection in shrimp hemocyte

In vivo RNAi knockdown of *Pm*HHAP gene transcripts was carried out as described above. After 12 h post-dsRNA injection, the hemocytes from three shrimp were collected, pooled and subjected to isolate the apoptotic DNA fragments using the Apoptotic DNA Ladder Detection Kit (Abcam) according to the manufacturer's protocol. Briefly, hemocyte cells were washed with 1XPBS and pellet cells by centrifugation at 800 × g for 10 min at 4°C. Cells were lysed with 35 µl of TE lysis buffer by gently pipetting and then incubated with 5 µl of enzyme A at 37°C for 10 min. Afterwards, 5 µl of enzyme B was added into each sample and incubated at 50 °C for 30 min. The DNA was precipitated by adding ammonium acetate and isopropanol, mixed, and kept at -20°C for 10 min. The DNA pellets were washed with 70% (v/v) ethanol after centrifugation. DNA samples were dissolved in DNA suspension buffer and subjected to analyze on 1.2% (w/v) agarose gel and visualized by under UV light after ethidium bromide staining.

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2.4.3 Caspase activity assay

To test the effect of *Pm*HHAP to the level of key member of the apoptotic pathway (caspase-3 and -7), the caspase activity was determined in hemocytes of *Pm*HHAP knockdown shrimp. Hemocytes were collected from each shrimp at 12 h post-dsRNAs injection by centrifugation, the cell pellets were washed with 1XPBS and the cell suspension was subjected to determine the total protein using Bradford Reagent (Bio-Rad). An equal concentration of shrimp hemocytes (as protein) from each of three shrimp per treatment was pooled and used for caspase-3 activity assay using

the Caspase-Glo 3/7 Assay Kit (Promega) according to the manufacturer's instruction. Briefly, 100 µl of the pooled hemocyte cells suspension was seeded in the 96-well white plate, and then 100 µl of Caspase-Glo reagent was added to each well, gently mixed and incubated for 15 min. The luminescence of each sample was measured in a plate-reading luminometer (Molecular devices). After subtracting with blank (buffer only), the fold increase in caspase-3 activity was determined by comparing the caspase activity level of *Pm*HHAP dsRNA-injected shrimp with that of the GFP dsRNA-injected shrimp. The data were reported as the mean ± S.D. of three replicates.

2.5 Effect of *Pm*HHAP gene silencing on expression of apoptotic related-genes

The effect of the dsRNA-mediated *Pm*HHAP gene silenced on the transcript levels of the apoptotic related-genes were evaluated using quantitative real-time RT-PCR (qRT-PCR). Shrimp was injected with *Pm*HHAP dsRNA or GFP dsRNA (control) to suppress gene expression of *Pm*HHAP as described above. Shrimp hemocytes (three individuals each) were collected at 12 h post-dsRNAs injection. Total RNA was extracted from the hemocytes, and used to synthesize cDNA according to the manufacturer's instructions. Quantitative real-time RT-PCR was performed using CFX96 Touch™ Real-Time PCR Detection system (Bio-Rad) using SsoFast™ EvaGreen® Supermix (Bio-Rad). The thermal profile was 95°C for 8 min, 40 cycles of 95°C for 10 s, 58°C for 15 s and 72°C for 30 s. The gene specific primers used for amplification of *Pm*Casp (DQ846887), *Pm*Caspase (EF114674), cathepsin B (EF213113), cathepsin D (EF213114), defender against apoptotic death (DAD1) (EF581986) and survivin (GU903910) are shown in Table 2.2. Amplification of the EF1-α served as the internal control for cDNA template for normalization. Each real-time PCR reaction was done in triplicate. The specificity of PCR was verified by measuring the melting curve of the PCR product at the end of the reaction. The data was calculated using a comparative method described by Pfaffl

(Pfaffl, 2001). The results were presented as relative expression ratios of the target genes expressed in hemocytes of *Pm*HHAP-silenced shrimp versus the GFP control shrimp, normalizing to the expression level of a reference gene (EF1-**α**) in the same cDNA sample.

Real expression ratio (R) = $(E_{target})^{\Delta ct_{target}^{(control-sample)}}/(E_{ref})^{\Delta ct_{ref}^{(control-sample)}}$

2.6 Protein-Protein interaction of *Pm*HHAP with shrimp protein

2.6.1 Yeast two-hybrid assay

The yeast two-hybrid assay for detecting interaction between *Pm*HHAP and the p20 domain of *Pm*Casp, p10 domain of *Pm*Casp or *Pm*Casp was carried out basically according to the strategies from the Matchmaker GAL4 Two-Hybrid System (Clontech). The coding sequences of p20-*Pm*Casp, p10-*Pm*Casp and *Pm*Casp were amplified from total RNA extracted from shrimp hemocytes using SuperScript One-Step RT/Platinum Taq Mix (Invitrogen) with the specific primer pairs F-p20-NdeI/R-p20-BamHI, *Pm*Casp-p10-F-NdeI/R-*Pm*Casp-BamHI and F*Pm*Casp-NdeI/R-*Pm*Casp-BamHI, respectively (Table 2.3). The amplification reaction and PCR temperature profiles were carried out as described previously (Lertwimol et al., 2014). Except for *Pm*HHAP, which was digested with NdeI and XhoI, all amplified products were digested with NdeI and BamHI restriction nucleases. The bait plasmid was generated by cloning the purified *Pm*HHAP fragment into the NdeI/SalI site downstream of GAL4 DNA-binding domain (BD) of pGBKT7 (Clontech), while prey plasmids were constructed by cloned the p20-*Pm*Casp, p10-*Pm*Casp and *Pm*Casp fragments into the NdeI/BamHI sites downstream of GAL4 DNA-activation domain vector (AD) of pGADT7 (Clontech). The constructs were subsequently sequenced to confirm gene sequences and correct reading frames. Yeast two-hybrid assays were then performed by co-transforming the bait and prey plasmids simultaneously into the yeast cells *Saccharomyces cerevisiae* strain AH109 using the

lithium acetate/dimethyl sulfoxide method (Gietz et al., 1995). Yeast cells were grown on low stringency media lacking leucine and tryptophan (-L/-W) and the binding was tested in the high stringency medium lacking adenine, histidine, leucine and tryptophan (-A/-H/-L/-W) supplemented with X-α-gal (Apollo Scientific). The positive clones were picked-up and culture to rescue the recombinant plasmid and then subjected to sequencing, annotation and analysis using bioinformatics tools.

Table 2.3 Nucleotide sequences of the primers used for Yeast two-hybrid screening.

Gene name	Primer sequences (5′-3′)	Accession no.
*Pm*HHAP-F-NdeI	CATATGAGCGCCGAAATGAAAACC	HQ130431
*Pm*HHAP-R-XhoI	CTCGAGGCGTTTATAGGAGGCGATTCT	HQ130431
F-*Pm*Casp-p20-NdeI	GGAATTCCATATGCCGCGGGGCGCTGCTCT	DQ846887
R-*Pm*Casp-p20-BamHI	CGCGGATCCGTCGGGGCCCCTGCATGCCTG	DQ846887
F-*Pm*Casp-p10-NdeI	GGAATTCCATATGTACCCAGGATTCCCGGC	DQ846887
R-*Pm*Casp-p10-BamHI	CGCGGACGTCGGGGCCCCTGCATGC	DQ846887
F-*Pm*Casp-NdeI	GGAATTCCATATGAGCGACGCCGACGACTC	DQ846887
R-*Pm*Casp-BamHI	CGCGGATCCTCAGAAGTAAATTTCACGAAG	DQ846887

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2.6.2 Production and purification of recombinant *Pm*HHAP and *Pm*Casp protein

To investigate the biological role of the *Pm*HHAP and *Pm*Casp proteins, recombinant (r) *Pm*HHAP and p20 domain of *Pm*Casp were expressed. The gene fragments of *Pm*Casp and p20 domain of *Pm*Casp were amplified with specific primers (Table 2.3), the NdeI/BamHI digested *Pm*Casp and p20-*Pm*Casp fragment were inserted inframe with the NdeI/BamHI sites of pET-15(b) expression vector (Novagen). The recombinant plasmids were transformed into *E. coli* JM109 competent cells, and

positive clones were analyzed by nucleotide sequencing (Macrogen, Korea). Afterwards, the selected recombinant plasmid (pET15b-rP20-*Pm*Casp) was transformed into *E. coli* XL1-Blue, while the r*Pm*HHAP protein was production and purification as described previously (Prapavorarat et al., 2010). The recombinant clones were grown in Luria-Bertani (LB) medium containing 100 μg/ml ampicillin at 37°C overnight with agitation at 250 rpm. The culture was diluted 1:100 in fresh LB medium and cultured until the OD_{600} reached to 0.6, then the protein expression was induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG) to final concentration of 1 mM. The cells were harvested at 4 h post induction by centrifugation at 10,000 × g for 10 min at 4°C, and then resuspended in 20 mM Tris-HCl (pH 8.0). Sonication was used to break cell and separated soluble fractions and inclusion bodies by centrifugation. The proteins were found to be located in the inclusion bodies, so the proteins were solubilized with 8 M Urea for completely dissolved. The solubilized proteins were purified using the affinity column containing Ni Sepharose™ 6 Fast Flow (GE Healthcare) according to the manufacture's manual. Then, recombinant proteins were dialyzed with 20 mM Tris-HCl (pH 8.0) and concentrated with VivaSpin® (GE Heathcare) molecular weight cut off 10 kDa column. The purified proteins were analyzed by SDS-PAGE (18% (w/v) acrylamide) and stained with coomassie brilliant blue.

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2.6.3 Co-immunoprecipitation

To confirm the protein-protein interaction of *Pm*HHAP and the p20 domain of *Pm*Casp, the co-immunoprecipitation (Co-IP) was carried out. The r*Pm*HHAP protein and p20 domain (rP20) of *Pm*Casp were produced and purified as described above. The polyclonal antibody specific to *Pm*HHAP was immobilized and cross-linked with protein A-sepharose CL4B (GE Healthcare) using dimethyl pimelimidate as described by Meyer (Meyer et al., 1984). Fifty microlitres of cross-linked bead was equilibrated

with binding buffer (1×PBS, pH 7.4 and 1 M NaCl) and then 40 µg of r*Pm*HHAP and/or 60 µg of rP20 of *Pm*Casp were added and incubated at 4°C overnight. The column was washed with binding buffer for 10 times to remove non-specific binding. The proteins were eluted with 100 mM glycine (pH 2.5). The immunoprecipitated complexes were then separated by SDS-PAGE (18% (w/v) acrylamide) and transferred onto nitrocellulose membrane (GE-Healthcare) by semi-dry blotter (Bio-Rad) at a constant 100 mA for 2 hours. The membrane was blocked in PBST buffer (1XPBS, pH 7.4 and 0.05% (v/v) tween 20) containing 5% of skim milk at room temperature with shaking for 1 hour. The membrane was washed with PBST 3 times for each 10 min, then probed with mouse anti-His tag monoclonal antibody (1:5000; Millipore) and incubated at room temperature for 2 h. Then, the membrane was later incubated with goat anti-mouse IgG-AP conjugate (1: 10,000; Millipore), a secondary antibody for additional 2 h at room temperature. The positive bands were visualized by color development using NBT/BCIP solution as a substrate.

2.7 Analysis of *Pm*HHAP-suppressed caspase activation in shrimp hemocyte

In order to investigate whether *Pm*HHAP can suppress caspase activity in shrimp hemocytes, the caspase activity was detected using Caspase-Glo 3/7 assay (Promega). Hemocytes were collected from five individual shrimp, homogenized in 1XPBS then washed with 10 mM sodium cacodylate buffer (CAC buffer) twice. The solution was separated by centrifugation at 13,000 rpm for 10 min at 4°C. The total protein concentration of each resultant shrimp hemocyte lysate supernatant (HLS) was measured by the Bradford method. Each shrimp HLS was separated into four groups (150 µg of HLS per group made up to a final volume of 100 µl with PBS), the first group was incubated with 5 mM of r*Pm*HHAP, the second group with 5 mM of control protein (bovine serum albumin, BSA) and other groups contain only HLS. After incubation of

proteins with actinomycin D (ActD; 50 mg/ml), 100 µl of Caspase-Glo reagents was added and incubated for 15 min at room temperature. Then, the luminescent signals were measured using microplate reader (Molecular devices). Moreover, to investigate whether *Pm*HHAP can inhibit the caspase activation directly by *Pm*Casp, the caspase activity detected using Caspase-Glo 3/7 assay (Promega) was performed. After HLS preparation, shrimp HLS was separated into 4 groups (150 µg of HLS per group made up to a final volume of 100 µl with PBS), the first group was incubated with 10 mM of r*Pm*HHAP, the second group with 7 mM of r*Pm*Casp, the third group with 10 mM of r*Pm*HHAP and 7 mM of r*Pm*Casp and the remaining groups contain HLS with and without BSA. After proteins incubation for 30 min, 100 µl of Caspase-Glo reagents was added to each well, gently mixed and incubated for 15 min. The luminescence of each sample was measured in a plate-reading luminometer (Molecular devices). The relative caspase activity was expressed as the mean ± S.D. of three replicates.

2.8 Identification of *Pm*HHAP-interacting protein with WSSV proteins

2.8.1 Yeast two-hybrid screening

The yeast two-hybrid assay was carried out based on the Matchmaker GAL4 Two-Hybrid System (Clontech) to identify which WSSV proteins possibly interact with *Pm*HHAP. The recombinant plasmid of *Pm*HHAP/pGBKT7 was prepared as described above. Meanwhile, the interacting proteins (prey) from a WSSV DNA library was fused with GAL4 DNA-activation domain vector (AD) of pGADT7 (Clontech). Yeast two-hybrid assay was then performed by co-transformed into the yeast cells *Saccharomyces cerevisiae* strain AH109 using the lithium acetate/dimethyl sulfoxide method. Yeast cells were grown on low stringency media lacking leucine and tryptophan (-L/-W) and the binding was tested in the high stringency medium lacking adenine, histidine, leucine and tryptophan (-A/-H/-L/-W) supplemented with X-α-gal (Apollo Scientific). Library

plasmids from positive colonies were rescued in *E. coli* XL1-blue and then subsequence to DNA sequencing. The nucleotide sequences were compared with the deduced amino acid sequences against the GenBank™ database using the BLASTX program.

2.8.2 Production and purification of recombinant WSSV proteins

The *E. coli* Rosetta (DE3) strain containing recombinant plasmid pET22b-*Pm*HHAP was cultured, then the recombinant protein was produced and purified as described previously. The gene encoding WSSV395 was amplified using Advantage® Polymerase Mix (Clontech) with the gene specific primers (Table 2.3). The purified PCR product was digested with NcoI and XhoI, and then ligated into the pET28b vector (Novagen), respectively. Afterwards, ligation mixture was transformed into competent *E. coli* JM109, and positive clones were analyzed by nucleotide sequencing. Meanwhile, the recombinant plasmid pET15b-WSSV134 was kindly obtained from Dr. Sangchan Senapin. The recombinant pET15b-WSSV134 and pET28b-WSSV395 were transformed into an *E. coli* BL21 (DE3). The single colony was grown in LB medium, and induced with 1 mM IPTG for 4 h. Cells were collected and resuspended in 20 mM Tris-HCl pH 8 then sonicated and purified through Ni-NTA column (GE Healthcare). The protein was analyzed by SDS-PAGE (15% (w/v) acrylamide) followed by coomassie brilliant blue staining.

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2.8.3 Co-immunoprecipitation

In order to confirm the interaction between *Pm*HHAP protein with its targets, WSSV proteins, the co-immunoprecipitation (Co-IP) was performed. The r*Pm*HHAP protein, rWSSV134 and rWSSV395 were produced in *E. coli* system as described above. The polyclonal *Pm*HHAP antibody was immobilized and cross-linked with protein A-

sepharose CL4B (GE Healthcare) using dimethyl pimelimidate. The cross-linked bead was equilibrated with binding buffer (1× PBS, pH 7.4 and 1 M NaCl) followed by added 40 µg of r*Pm*HHAP and/or 60 µg of WSSV proteins then incubated at 4°C overnight. The protein complex was eluted with 100 mM glycine (pH 2.5) and then the immunoprecipitated complex was separated by SDS-PAGE (18% (w/v) acrylamide) and analyzed the protein band using western blot analysis. The r*Pm*HHAP and WSSV134 or WSSV395 were detected using anti-His tag antibody (1:5000; Millipore) as primary antibody and goat anti-mouse IgG-AP conjugate (1:10,000; Millipore) as secondary antibody then the positive bands were detected using NBT/BCIP solution.

2.9 Virus preparation

The WSSV stock was prepared according to the method of Xie (Xie et al., 2005). The gills of WSSV-infected shrimp were homogenized in TNE buffer (50 mM Tris-HCl, 0.1 M NaCl, 1 mm EDTA, pH 7.5) containing 1 mM phenylmethysulfonyl fluoride (PMSF) and centrifuged at 3,500 × g for 5 min at 4°C. The supernatant was filtered through a 0.45 µm pore size filter and then centrifuged at 30,000 × g for 30 min at 4°C. The pellet was suspended in TM buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, pH 7.5) containing 0.1% NaN_3 and kept at -80°C until used. The titer of the WSSV stock was determined by *in vivo* infection. The WSSV suspension was diluted in PBS and then, 50 µl of WSSV solution with optimal dilution level was injected into the fourth abdominal segment using an insulin syringe. The mortality was recorded daily for 7 days, the dose of WSSV that caused 50% mortality within 3 days after injection was used for all viral infection experiments.

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2.10 Expression profile of viral genes in WSSV challenged shrimp hemocytes

The transcription of viral genes including WSSV134, WSSV362 and WSSV395 in WSSV-infected shrimp at various time points were investigated using semi-quantitative RT-PCR. Shrimp were injected with 50 µl of diluted WSSV solution. Hemocytes were collected from 3 individual shrimp at 0, 3, 6, 12, 24 and 48 hpi then subjected to extract the total RNA. The first strand cDNA was synthesized after treated with RNase-free DNase (Biolabs) using the RevertAid First Strand cDNA Synthesis kit (Fermentas). The expression level of viral genes, WSSV134, WSSV362, WSSV395, ie-1 (immediate early gene), WSSV477 (early gene) and VP28 (late gene) were examined using gene specific primers (Table 2.2). EF1-α gene was used as an internal control gene. The reaction was pre-denatured at 94°C for 2 min and followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s or 58°C for EF1-α and extension at 72°C for 30 s, and final extension at 72°C for 10 min. The PCR products were analyzed by 1.5% (w/v) agarose gel electrophoresis and visualized by under UV light after ethidium bromide staining.

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2.11 Functional analysis of WSSV134 by RNAi-mediated double stranded RNA

2.11.1 Preparation of double-stranded RNA (dsRNA)

Double-stranded RNAs (dsRNA) of WSSV134 was generated *in vitro* using T7 RiboMAX™ Express Large Scale RNA Production System (Promega). Briefly, WSSV134 dsRNA was prepared using recombinant pET15b-WSSV134 plasmid as a template for producing sense and anti-sense strands DNA templates separately. Then 1 µg of each gel purified PCR products was used as a template for *in vitro* transcription according to the instructions provided by the T7 RiboMAX™ Express Large Scale RNA Production Systems (Promega) as described above. The quality of dsRNAs was verified by agarose

gel electrophoresis with UV visualization followed by the ethidium bromide staining and quantified by using UV spectrophotometer.

2.11.2 *In vivo* gene knockdown of WSSV134

Shrimp were divided into two groups of three individuals each. Shrimp were intramuscularly injected with 25 µl of 150 mM NaCl solution containing with 7 µg of WSSV134 dsRNA while the control group was injected with 7 µg of GFP dsRNA. After 3 h post-dsRNAs injection, diluted WSSV solution was then injected into the respective group of shrimp. Afterwards, hemocytes were collected at 48 h post infection and subjected to extracted total RNA as described above in order to determine the efficiency of WSSV134 gene transcript silencing. The RT-PCR was performed to check the transcription of gene using the specific primers for WSSV134 gene (Table 2.2). One microlitre of diluted cDNA was used as template for the PCR amplification, a fragment of EF1-α gene was amplified and used as an internal control, while VP28 was also amplified and used to quantify the viral replication in shrimp. The PCR conditions were 94°C for 2 min, followed by 35 cycles of 94°C for 30 s, 60°C (for WSSV134 and VP28) or 58°C (EF1-α) for 30 s, and 72°C for 30 s, and then a final extension at 72°C for 5 min. The PCR products were analyzed by 1.5% (w/v) agarose gel electrophoresis and visualized by UV-transillumination.

2.11.3 Viral propagation after silencing of WSSV134 transcription in shrimp hemocytes

To investigate the expression level of representative genes of WSSV after silencing of WSSV134, the RNAi experiment was performed and analyzed the expression level of tested genes by qRT-PCR. Two groups of shrimp, experimental and control group, were injected with 25 µl of 150 mM NaCl containing either WSSV134

dsRNA (7 μg/g shrimp) and GFP dsRNA (7 μg/g shrimp). The diluted WSSV solution was injected after 3 h post-dsRNAs injection into each shrimp and then harvested hemocytes at 48 h to extract the total RNA as described above. After cDNA synthesized, qRT-PCR was performed using SsoFast™ EvaGreen® Supermix (Bio-Rad) on CFX96 touch real-time PCR detection system (Bio-Rad), each sample was analyzed in triplicate. The specific oligonucleotide primers of viral genes which are ie-1, WSSV477 and VP28 was used to verify the gene expression level and EF1-α gene was served as an internal control and reference standard to verify the quantitative real time PCR reaction. The PCR conditions consisted of 95 °C for 8 min, followed by 40 cycles (22 cycles for EF1-α) of 95 °C for 10 s, 60 °C for 15 s and 72 °C for 30 s. The expression level of viral genes after knockdown of WSSV134 was calculated by normalized to the control injected with PBS. The results were presented as relative expression ratios of the target genes expressed in hemocytes of ie-1, WSSV477 and VP28 as means ± S.D. of three replicates.

2.12 The effect of *Pm*HHAP and WSSV134 gene knockdown in hemocyte apoptosis in shrimp

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2.12.1 *In vivo* gene silencing of shrimp *Pm*HHAP gene and viral gene WSSV134

Shrimp were divided into 4 groups three individuals each, the first group was injected with WSSV134 dsRNA (dose 7 μg/g shrimp), the second group was injected with *Pm*HHAP dsRNA (dose 5 μg/g shrimp), the third group was injected with both WSSV134 and *Pm*HHAP dsRNA, and the last group was injected with GFP dsRNA (dose 7 μg/g shrimp). At 3 h post-dsRNAs injection, diluted WSSV solution was injected to all shrimp groups and reared for further 48 h. In addition, for shrimp which was subjected to injection with *Pm*HHAP dsRNA, the *Pm*HHAP dsRNA was injected at 24 h post WSSV

injection. Afterwards, hemocytes were collected at 48 h post WSSV infection to extract the total RNA and subjected to synthesize the first strand cDNA as described above. The efficiency of genes knockdown was verified by semi-quantitative RT-PCR using the specific primers for *Pm*HHAP gene and WSSV134 gene (Table 2.2). The EF1-**α** was amplified and used as an internal control gene, while VP28 was also amplified and used to quantify the viral replication in shrimp. The thermal cycling conditions were of 94°C for 2 min, followed by 35 cycles (30 cycles for *Pm*HHAP and 22 cycles EF1-**α**) of 94°C for 30 s, 58°C (*Pm*HHAP and EF1-**α**) or 60°C (WSSV134 and VP28) for 30 s, 72°C for 30 s and 72°C for 5 min. The PCR product was analyzed by 1.5% (w/v) agarose gel electrophoresis and visualized by UV-transillumination.

2.12.2 Caspase activity assay

Shrimp were divided into 5 groups nine individuals each, the first group was injected with WSSV134 dsRNA (dose 7 μg/g shrimp), the second group was injected with *Pm*HHAP dsRNA (dose 5 μg/g shrimp), the third group was injected with both WSSV134 and *Pm*HHAP dsRNA, and the last two groups were injected with GFP dsRNA (dose 7 μg/g shrimp) and PBS only (control for apoptosis in normal condition), respectively. The gene knockdown was performed as described above. Hemolymph was withdrawn from each shrimp at 48 h post WSSV challenge, hemolymph from each of three shrimp per treatment was pooled and subjected to prepare HLS as described above. Then, the cell suspension was determined the total protein using Bradford Reagent (Bio-Rad). An equal concentration of protein in shrimp hemocytes was used for caspase-3 activity assay using the Caspase-Glo 3/7 Assay Kit (Promega) according to the manufacturer's instruction. Briefly, 100 μl of the pooled hemocyte cells suspension was seeded in the 96-well white plate, and then 100 μl of Caspase-Glo reagent was added to each well, gently mixed and incubated for 15 min. The luminescence of each

sample was measured in a plate-reading luminometer (Molecular devices). After subtracting with blank (buffer only), the changed in caspase-3 activity was determined by comparing the caspase activity level of treated shrimp with that of the PBS control shrimp. The data were reported as the mean ± S.D. of three replicates.

2.13 Suppression subtractive hybridization (SSH)

The SSH cDNA library was constructed from hemocytes of *Pm*HHAP-silenced shrimp and control GFP-silenced shrimp using the PCRSelect™ cDNA Subtraction Kit (Clontech), according to the manufacturer's instructions. Shrimp were divided into two groups, *Pm*HHAP dsRNA injected shrimp and control GFP dsRNA injected shrimp. After 12 h post-dsRNAs injection, hemocytes were collected and used for total RNA extraction. Then, double-stranded cDNAs were synthesized form poly(A)+RNAs of the control GFP-silenced shrimp hemocytes (driver) and *Pm*HHAP-silenced shrimp hemocytes (tester), then they were digested with Rsa I to generate short, blunt-ended double-stranded cDNA fragments. The Rsa I-digested tester cDNA was ligated with a different adaptor (adaptor 1 and adaptor 2R) at the 5'-ends of each strand of tester 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 samples from first hybridization were mixed together without denaturation and hybridized at 68°C overnight with an excess of fresh denatured driver cDNA. The final hybridization was diluted in 50 µl of dilution buffer (20 mM HEPES, 20 mM NaCl and 0.2 mM EDTA), heated at 68°C for 7 min, the expression of the differentially expressed cDNAs was enhanced by performing two steps of 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. The products from primary and secondary PCRs were analyzed by 1.8% agarose gel electrophoresis. The PCR product is now enriched for differentially expressed cDNAs.

To evaluate the subtraction efficiency, a house keeping gene, EF1-α gene was amplified compared the transcript abundant cDNA on subtracted and unsubtracted cDNA with the following conditions: 94℃ for 3 min, 18 cycles of 94 ℃ for 30 s, 55 ℃ for 30 s, 72 ℃ for 30 s, and then a final extension at 72 ℃ for 5 min. Remove 5 µl and amplified more 5 additional cycles. The abundance of PCR products between unsubtracted and subtracted samples from each reaction after 18, 23, 28 and 33 cycles was compared using a 1.5% (w/v) agarose gel followed by ethidium bromide staining and UV-transillumination.

The subtracted mixture was cloned into pGEM-T easy vector (Promega) and then transformed into *E.coli* JM109 competent cells. The positive clones were selected by blue/white screening method, then plasmids were extracted using QIAprep® spin Miniprep kit (QIAGEN) and sequenced using M13 reverse primer by Macrogen Inc., South Korea.

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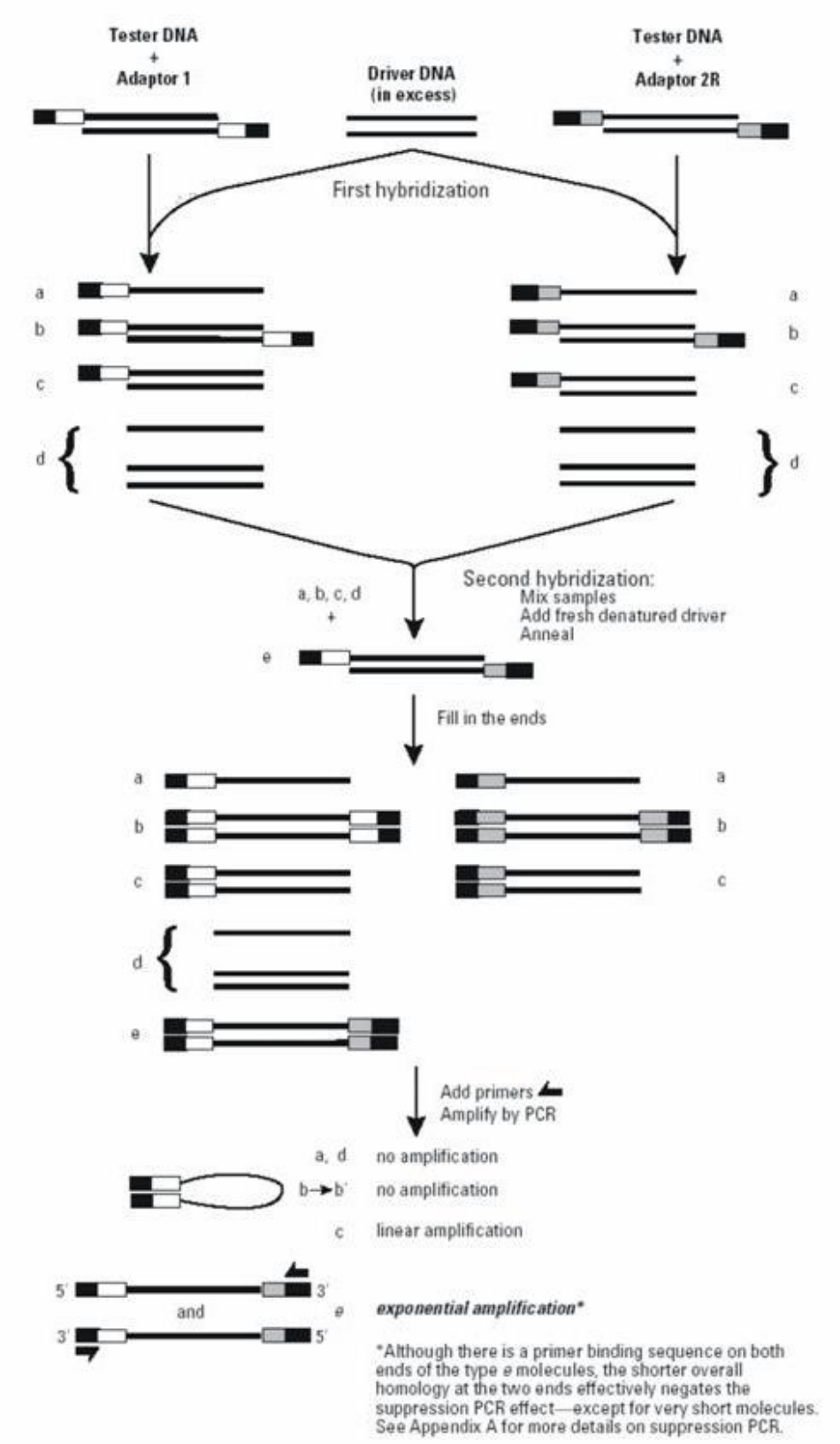

Figure 2.1 PCR-Selected^{TM} cDNA Subtraction procedure (Clontech).

2.14 Bioinformatics analysis of genes obtained from the SSH library

The consensus sequences obtained from the SSH library were processed by removing the unwanted vector and primer sequences and, then, searched against the NCBI GenBank database for homology with the BLASTN and BLASTX programs (http://www.ncbi.nlm.nih.gov/BLAST/) for clone annotation. The significant matches present *E*-values lower than 1×10^{-4}. Genes were categorized by its function and recorded the numbers after analyzed.

2.15 Validation of genes expression in response after *Pm*HHAP-silenced condition from the SSH library by real time RT-PCR

To verify the differential expression of genes identified from the SSH library, qRT-PCR was performed. The samples from control GFP and *Pm*HHAP-silenced shrimp were prepared as described above. Hemocytes of shrimp (three individuals each) were collected at 12 h post-dsRNAs injection. Total RNAs were extracted using GeneZol Reagent (Geneaid) followed by DNase I (Biolabs) treatment. First-stranded cDNA was synthesized with the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific) as mentioned above. The qRT-PCR was performed using SsoFast^{TM} EvaGreen® Supermix (Bio-Rad) on CFX96 touch real-time PCR detection system (Bio-Rad), each sample was analyzed in triplicate. The transcription level of target genes was identified by qRT-PCR with gene-specific primers. The specific oligonucleotide primers to selected genes including transcription factor ATF-β (Accession no. AHY82567); cathepsin L (Accession no. ABQ10739); lactate dehydrogenase (Accession no. AEC12821); ferritin (Accession no. ABP68819) and inhibitor of apoptosis (IAP) (Accession no. ABQ38431). The EF1-α gene was served as an internal control and reference standard to verify the quantitative real time PCR reaction. The PCR conditions consisted of 95 ℃ for 8 min, followed by 40 cycles of 95 ℃ for 10 s, 58 ℃ for 15 s and 72 ℃ for 30 s. The results were presented

as the relative expression ratios of the target genes expressed in the hemocytes of *Pm*HHAP-silenced shrimp versus the GFP control shrimp, with normalization to a reference gene (EF1-α) transcript levels in the same cDNA sample.

2.16 Tissue expression analysis of *Pl*HHAP in crayfish

Freshwater crayfish (*Pacifastacus leniusculus*) were obtained from lake Erken, Sweden. Crayfish were maintained in tanks with aerated water at 10°C. Only healthy animals were used in the experiments.

In order to investigate the tissue-specific expression of *Pl*HHAP, semi-quantitative RT-PCR was conducted using *Pl*HHAP gene specific primer (Table 2.2). Total RNA was extracted from muscle, anterior proliferation center (APC) in HPT, brain, hematopoietic tissue, hemocyte, eyestalk, hepatopancreas, gill, intestine, heart, nerve and abdominal nerve of crayfish using the TRI Reagent (Genezol) according to the manufacturer's protocol. The equal amounts of total RNA was used for cDNA synthesis with ThermoScript (Invitrogen) according to the manufacturer's instructions. Then, *Pl*HHAP transcript levels in each tissue were analyzed and the 40S ribosomal protein was used as an internal control. The PCR thermal cycling conditions consisted of 94°C for 1 min, followed by 30 cycles for *Pl*HHAP or 25 cycles for 40S of 94°C for 30 s, 58°C for 30 s and 72°C for 30 s, plus a final extension step at 72°C for 5 min. The PCR products were analyzed on 1.5% agarose gel stained with GelRed and visualized by UV-transillumination.

2.17 Preparation of hematopoietic tissue (HPT) cell culture

The HPT cells were isolated from freshwater crayfish as described previously with minor modifications (Lin et al., 2009). Briefly, the hematopoietic tissue was dissected from the dorsal side of stomach, washed with crayfish phosphate buffered

saline (CPBS) (10 mM Na_2HPO_4, 10 mM KH_2PO_4, 150 mM NaCl, 10 mM $CaCl_2$ and 10 mM $MnCl_2$, pH 6.8), and then incubated in 700 µl of collagenase Type I and Type IV (Sigma) in CPBS at room temperature (RT) for 30 min. The tissue was centrifuged at 800×g for 5 min at RT to remove the collagenase solution. The cells were washed twice with CPBS, and the undigested tissue was removed. The isolated HPT cells were resuspended in L-15 medium (Sigma-Aldrich) supplemented with 5 µM 2-ME, 1 µM phenylthiourea, 60 µg/ml penicillin, 50 µg/ml streptomycin, 50 µg/ml gentamicin and 2 mM L-glutamine, and subsequently seeded in 96 well plates at a density of 4×10^4 cells/100 µl. The cells were supplemented with 3 µl of cell-free crayfish plasma and incubated at 16°C. One-third of medium was changed every second day.

2.18 Transcription of *Pl*HHAP in response to WSSV infection *in vitro* and *in vivo*

A WSSV stock suspension was prepared from gills of viral infected crayfish (Xie et al., 2005). In the *in vitro* experiment, HPT cell cultures were prepared and incubated at 16°C for 16 h. The medium was replaced with 50 µl of L-15 medium containing 5 µl of the WSSV stock suspension, the control group was supplement with 5 µl of CBPS, followed by incubation for 0, 12, 24, 48 and 72 h at 16°C. Total RNA was extracted from the cells harvested at each time point. For *In vivo* treatment, WSSV was diluted ten times in CPBS and then 100 µl of this WSSV suspension or CPBS as a control was injected via the base of a walking leg. The hemolymph of crayfish from each group (three individuals each) were bled at 0, 12, 24, 48 and 72 h post infection, and separated hemocytes to extracted total RNA. After RNase-Free DNase I (Biolabs) treatment, cDNA was then synthesized by ThermoScript (Invitrogen) and the transcription level of *Pl*HHAP was determined by qRT-PCR. The mRNA encoding 40S ribosomal protein gene was used as an internal control. The qRT-PCR reactions contained with diluted cDNA template, 1× QuantiTect® SYBR Green PCR master mix

(QIAGEN) and 5 μM forward and reverse primers. The following amplification profile was used: 95 ℃ for 15 min, followed by 40 cycles of 95 ℃ for 15 s, 58 ℃ for 30 s and 72 ℃ for 30 s. All qRT-PCR reactions were performed in duplicate and all experiments were repeat twice. The results are presented as the relative expression ratios, which were normalized to 40S gene transcript levels in the same sample.

2.19 Analysis of *Pl*HHAP transcription levels after bacterial challenge

The bacteria *Aeromonas hydrophila* B1 was grown in LB medium overnight, and the OD_{600} of the bacterial cultures was adjusted to approximately 0.5-0.6. Then, the bacteria were harvested and resuspended in sterile 0.85% NaCl. Crayfish were divided into two group with four individual in each group, crayfish was injected with 100 μl of bacterial suspension containing 2×10^6 CFUs/ml (for high dose) or 5×10^5 CFUs/ml (for low dose) or 100 μl of 0.85% NaCl for the control groups. The hemocytes were collected at different time points post-bacterial challenge for total RNA extraction and single-stranded cDNA synthesis as described above. Quantitative real-time RT-PCR was performed using a QuantiTect® SYBR® Green PCR Kit (Qiagen) with the following conditions: 95℃ for 15 min, 40 cycles of 95℃ for 15 s, 64℃ (for *Pl*HHAP) or 58℃ (for 40S ribosomal protein) for 30 s, and 72℃ for 30 s. The 40S ribosomal protein was used as an internal control gene. Each real-time reaction was performed in duplicate. The results are presented as the relative expression ratios, which were normalized to 40S gene transcript levels in the same sample.

2.20 Gene expression analysis of *Pm*HHAP in response to *Vibrio harveyi* challenge

The *V. harveyi* strain 639 was grown on a trypic soy agar (TSA) supplemented with 2% (w/v) NaCl and incubated at 30℃ overnight. A single colony was inoculated in a trypic soy broth (TSB) supplemented with 2% (w/v) NaCl and cultured at 30℃

overnight with shaking at 250 rpm. The culture was diluted 1:200 in TSB and grown until an OD_{600} of the cultures reached 0.6. The *V. harveyi* cultured was diluted in 0.85% (w/v) NaCl to make a cell suspension of 2×10^5 colony forming unit (CFU). Then, the diluted culture was intramuscular injection into shrimp, control group was injected with 0.85% (w/v) NaCl solution. Hemocytes were collected at 0, 6, 12, 24 and 48 h after bacterial challenged to extract the total RNA. The equivalent amount of total RNA was used as a template to synthesize the first-strand cDNAs as described above. The qRT-PCR was performed using the specific primers of *Pm*HHAP and EF1-α (Table 1). The PCR conditions consisted of 95 °C for 8 min, followed by 40 cycles of 95 °C for 10 s, 58 °C for 15 s and 72 °C for 30 s. The results were presented as relative expression ratio of *Pm*HHAP genes expressed in hemocytes of bacterial infection versus the control shrimp using EF1-α as an internal control gene.

2.21 Effect of *Pl*HHAP-mediated gene knockdown in hemocyte apoptosis and hematopoiesis in crayfish

2.21.1 Preparation of double-stranded RNAs (dsRNAs) for *Pl*HHAP

The dsRNA specific to the *Pl*HHAP gene fragment and GFP fragment from the pd2EGFP-1 vector (Clontech) were incorporated with T7 promoter at the 5' end and used for amplify PCR products with gene specific primers (Table 2.1). The PCR was performed at 94°C for 1 min, followed by 30 cycles of 94°C for 30 s, 64°C for 30 s and 72°C for 30 s, and then a final extension at 72°C for 5 min. The PCR products were then purified, followed by *in vitro* transcription using Megascript kit (Ambion) according to the manufacturer's instruction. Briefly, the template DNA were mixed with enzyme and the ribonucleotides, then incubated at 37°C overnight. The reactions were precipitated by LiCl and chilled for 1 h at -20°C. The pellets were washed with 70% (v/v) ethanol after centrifugation at maximum speed for 15 min at 4°C. The RNA pellets

were resuspended with DECP-treated water and subjected to quantify the amount of dsRNAs by UV light spectrophotometry, followed by checked the quality with 1.5% agarose gel electrophoresis and UV visualization, respectively.

2.21.2 Effects of *Pl*HHAP gene silencing on the mRNA levels of genes involved in hematopoiesis and hemocyte homeostasis

For the *in vitro* RNAi experiment, dsRNA was transfected into HPT cells as described previously, with minor modifications (Liu and Söderhäll, 2007). Briefly, 1 μg of dsRNA was mixed with 3 μl calf histone H2A (1 mg/ml of histone type II-A from calf thymus in modified L-15 medium) (Sigma-Aldrich) and incubated for 10 min at RT. Then, a mixture of dsRNA and H2A was combined with 20 μl of cell culture medium and added to one well of HPT cell culture (96-well plates) before incubation at 16°C. One-third of the total volume of the medium was changed every other day during incubation. To determine the efficiency of the *Pl*HHAP gene transcript silencing, total RNA was extracted from the HPT cells at 24 and 48 h and subjected to semi-quantitative RT-PCR analysis. In addition, the cell morphology of the HPT cells was observed every day for 5 days after transfection.

For the *in vivo* RNAi treatments, small intermolt crayfish (18-25 g fresh weight) were divided into two groups with three crayfish in each group. Briefly, 100 μl of crayfish saline solution containing 100 μg of *Pl*HHAP or GFP dsRNA was injected into the base of a walking leg of each crayfish. Hemocytes of individual crayfish were collected at 24 h post-dsRNAs injection followed by total RNA extraction and synthesis of cDNA samples. Semi-quantitative RT-PCR analysis was performed to determine the efficiency and specificity of gene knockdown. A fragment of the 40S ribosomal protein was used as an internal control. The cycling conditions consisted of 94°C for 1 min, followed by 30 cycles for *Pl*HHAP or 25 cycles for 40S of 94°C for 30 s, 58°C for 30 s and 72°C for

30 s, plus an additional final extension at 72°C for 5 min. The PCR products were analyzed on 1.5% agarose gel stained with GelRed and visualized by UV-transillumination.

Afterwards, the genes involved in hematopoiesis and hemocyte homeostasis were determined in both *Pl*HHAP-silenced animals and GFP-silenced animals by semi-quantitative RT-PCR analysis using gene-specific primers for CHF (GQ497446), astakine1 (AY787656), astakine2 (EF568370) and IGFBP7 (Table 2.2). Amplification of the 40S ribosomal protein served as the internal control. The PCR conditions consisted of 94°C for 1 min, followed by 30 cycles for tested genes or 25 cycles for 40S of 94°C for 30 s, 58°C for 30 s and 72°C for 30 s, plus an additional final extension at 72°C for 5 min. The PCR products were analyzed on 1.5% agarose gel stained with GelRed and visualized by UV-transillumination.

2.21.3 Total hemocyte count (THC) after silencing of *Pl*HHAP

Crayfish were separated into two groups with 5 crayfish per group. Hemolymph from each crayfish were collected and fixed with 10% formalin in crayfish saline before the injection of dsRNA. Subsequently, the crayfish were injected with *Pl*HHAP dsRNA or GFP dsRNA at the base of a walking leg. After 24 h post-dsRNAs injection, hemolymph samples were taken from each crayfish and fixed with 10% formalin, and the total hemocyte number was counted in a hemocytometer. The hemocyte number after injection of each crayfish was then divided by the hemocyte number before injection, and the value is reported as the hemocyte index of mean ± S.D. of five replicates.

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2.21.4 Analysis of hemocyte apoptosis in *Pl*HHAP-silenced crayfish

To determine the effects of *Pl*HHAP on hemocyte apoptosis, gene knockdown of *Pl*HHAP was performed as described above and analyzed the caspase 3/7 activity using the Caspase-Glo 3/7 Assay Kit (Promega) according to the manufacturer's instructions. Briefly, hemolymph was harvested from each crayfish and subjected to centrifuge at 800 × g for 10 min at 4°C. The hemocyte pellet was suspended in 500 µl of CPBS and counted the number under the microscopy. The same amount of cell suspensions from each crayfish was seeded in a white 96-well plate in the total volume of 100 µl, and 100 µl of Caspase-Glo reagent was then added to each well, gently mixed with the cells and incubated for 1 h at RT. The luminescence of each sample was measured in a plate-reading luminometer. After subtracting with blank (buffer only), the fold increase in caspase 3/7 activity was determined by comparing the caspase activity level of *Pl*HHAP dsRNA-injected shrimp with that of the GFP dsRNA-injected shrimp. The data were reported as the mean ± S.D. of five replicates.

2.22 Determination of bacterial number in the intestine of *Pl*HHAP-silenced crayfish

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To elucidate the effect of *Pl*HHAP knockdown on bacterial number in crayfish intestines, RNAi experiment was performed followed by an assay of the bacteria count. Crayfish was injected with 100 µl of crayfish saline solution containing 100 µg of *Pl*HHAP or GFP dsRNA. At 24 h post-dsRNAs injection, crayfish were injected with dsRNAs as before, and then the intestine was collected from each crayfish after 24 h of second-dsRNAs injection. The intestine was homogenized in 0.15 M NaCl, the intestinal lysate was then serially diluted. Ten microliter aliquots were taken and dropped on LB agar plate and incubated overnight at 37°C. The surviving colonies were counted to

calculate the CFU per ml and compared between two groups of crayfish. Five crayfish were used in each group in this experiment.

In order to confirm and monitor the effect of *Pl*HHAP-silenced crayfish in the number of bacteria in intestine, scanning electron microscopy (SEM) was performed. The intestines of crayfish injected with *Pl*HHAP dsRNA or GFP dsRNA were collected individually 24 h after the second dsRNAs injection. The tissues were immediately fixed in 0.1 M phosphate buffer, pH 7.4, containing 3% (V/V) glutaraldehyde at 4°C in the darkness overnight. The samples were washed twice with 0.1 M phosphate buffer for 15 min followed by distilled water once for 15 min. Then, each intestine was dehydrated for 15 min in a series of 30%, 50%, 70%, 95% (V/V) ethanol followed by 15 min in absolute ethanol and this was repeated twice. Next, the samples were dried using a critical point dryer and then mounted on stubs. During mounting, the samples were split longitudinally to expose the inner layer of intestine and coated with gold before being observed under a scanning electron microscope. The images from the SEM were analyzed by blind tests. The images without labeling were given to 4 blinded independent analysts, who assessed each image.

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2.23 Determination of bacterial number in the intestine of *Pm*HHAP-silenced shrimp

To elucidate the effect of *Pm*HHAP knockdown on bacterial number in shrimp intestines, RNAi experiment was performed followed by an assay of the bacteria count. Shrimp was injected with 25 µl of shrimp saline solution containing 2 µg of *Pm*HHAP or GFP dsRNA. At 12 h post-dsRNAs injection, the intestine was collected from each shrimp and subjected to homogenize in 0.15 M NaCl, the intestinal lysate was then serially diluted. Ten microliter aliquots were taken and dropped on LB agar plate and incubated overnight at 37°C. The surviving colonies were counted to calculate the CFU

per ml and compared between two groups of shrimp. Five shrimp were used in each group in this experiment.

2.24 Statistical analysis

Statistical analysis was performed by one-way ANOVA (analysis of variance) followed by Duncan's test, whereas two treatments were compared by a Student's paired t-test. The cutoff for significance was $P < 0.05$, and the results are presented as the mean ± 1 standard deviation (SD).

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CHAPTER 3

RESULTS

3.1. Effect of RNAi-mediated gene silencing of *Pm*HHAP on the level of *Pm*HHAP transcript and protein in *P. monodon* hemocyte

It has been reported that suppression of *Pm*HHAP gene caused a severe damage of hemocyte cells and led to a significant decrease in circulating hemocytes of the knockdown shrimp (Prapavorarat et al., 2010). So, we hypothesized that *Pm*HHAP might be involved in hemocyte apoptosis. To understand the functional role of *Pm*HHAP in shrimp hemocyte apoptosis, the gene silencing was performed to suppress the *Pm*HHAP transcript and protein levels in shrimp. Initially, the efficiency of the *Pm*HHAP knockdown was determined. At 12 h after dsRNAs injection, the hemocytes of knockdown and control shrimp were collected (three individuals each). Total RNA and first-strand cDNA was prepared and subjected to analysis by semi-quantitative RT-PCR. The result indicated that gene silencing of *Pm*HHAP in shrimp was achieved by injection of *Pm*HHAP dsRNA (2 μg dsRNA/g shrimp) but not in control group injected with GFP dsRNA (Figure. 3.1A, Figure. 3.1B). According to the *Pm*HHAP/EF1-α ratio of DNA band intensity in the knockdown shrimp compared to the control shrimp, approximately 88% reduction of *Pm*HHAP transcript level was observed (Figure. 3.1B).

Moreover, the efficiency of gene knockdown at the protein level was observed by the Western blot analysis using anti-*Pm*HHAP antibody. After 12 h post dsRNAs injection, hemocytes were collected and then hemocyte lysates was prepared from each group of the knockdown and control shrimp. Then, the expression of *Pm*HHAP protein was detected by immunoblotting. A marked decrease in the *Pm*HHAP protein level was found in the knockdown shrimp when compared with GFP control. The β-actin was served as an internal control protein (Figure. 3.2).

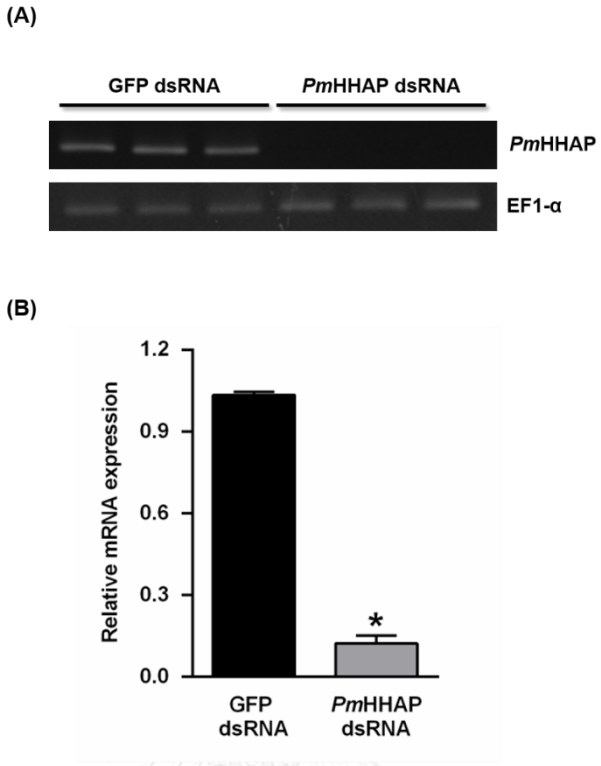

Figure 3.1 Gene silencing of *Pm*HHAP transcript level in *P. monodon* hemocytes. (A) Semi-quantitative RT-PCR analysis of the gene silencing efficiency of *Pm*HHAP in shrimp hemocytes. Lanes 1-3 shrimp were injected with GFP dsRNA and Lanes 4-6 shrimp were injected with *Pm*HHAP dsRNA. EF1-α was amplified and used as an internal control. (B) The relative expression ratio was analyzed. The average relative expressions are representative of three replicates ±1 S.D. (error bars). Significant difference compared with control indicated by an asterisk *(*P < 0.05)*.

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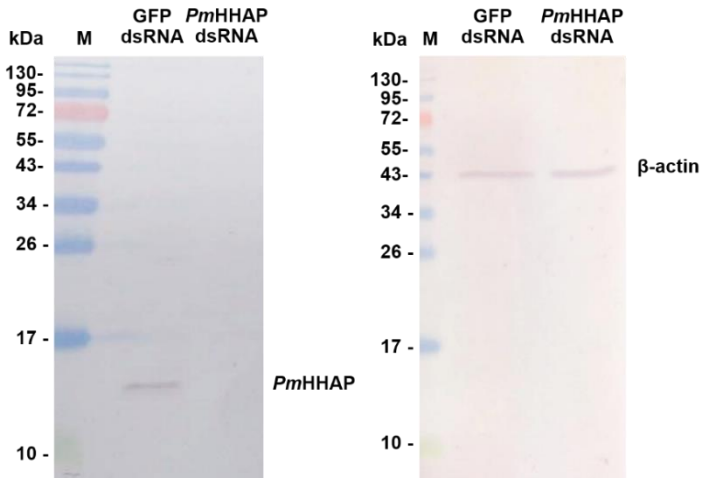

Figure 3.2 Western blot analysis of the *Pm*HHAP silencing experiment at the protein level. *Pm*HHAP protein was detected in hemocyte lysates (HLS) from *Pm*HHAP-silenced shrimp and GFP-silenced shrimp with rabbit anti-*Pm*HHAP antibody. The β-actin was used as an internal control. Each lane represents the protein from the pooled sample of three individual shrimp. Lane M is the protein marker.

3.2. Effect of *Pm*HHAP gene silencing on hemocyte apoptosis in shrimp *P. monodon*

3.2.1 Induction of apoptosis in hemocytes of *Pm*HHAP-knockdown

The level of apoptosis in hemocytes of the *Pm*HHAP-knockdown and control (GFP dsRNA-treated) shrimp was determined by the annexin-V-FLUOS staining assay. Hemocytes were collected from the *Pm*HHAP knockdown and control GFP dsRNA injected shrimp at 12 h after dsRNAs injection and stained with annexin-V-FLUOS. The annexin-V positive cells were counted under the microscope and the apoptosis rate of *Pm*HHAP-silenced shrimp and that of the GFP control was reported. Hemocyte cells of the *Pm*HHAP silencing shrimp clearly displayed an enhanced FITC-conjugated annexin-V signal compared to the control samples (Figure. 3.3A). The apoptotic rate, as the proportion of annexin-V positive cells, was increased 2.4-fold in the *Pm*HHAP knockdown shrimp compared to that in the control GFP (Figure. 3.3B).

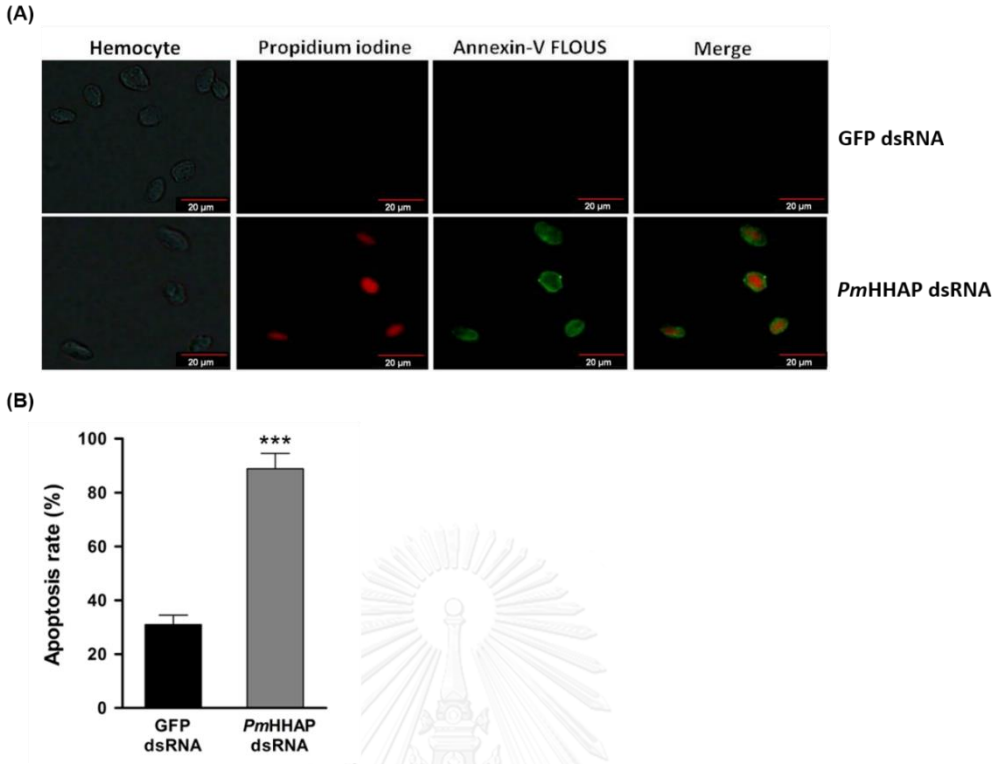

Figure 3.3 Detection of apoptosis in hemocyte of *Pm*HHAP-silenced *P. monodon* by annexin-V staining. (A) The propidium iodide (PI) was used to counter stain the hemocyte nucleus. The annexin-V positive cells were counted under the microscope and comparing between *Pm*HHAP-silenced shrimp and GFP control. (B) The proportion of annexin-V positive cells, counted under a fluorescent microscope, in *Pm*HHAP-silenced and GFP control shrimp, shown as the mean ± 1 S.D. (error bar), and derived from triplicate experiments. Asterisk indicates a statistical significant difference between means (****P < 0.001*).

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3.2.2. Apoptotic DNA fragmentation in the *Pm*HHAP knockdown shrimp hemocytes

DNA fragmentation is a key characteristic of late apoptosis. To confirm that silencing of *Pm*HHAP induces apoptosis in shrimp hemocytes, the gene silencing experiment was performed and then the DNA fragmentation was observed comparing between *Pm*HHAP-silenced shrimp and GFP-silenced shrimp. The characteristic of DNA ladder pattern of apoptosis was detected in the hemocytes of the *Pm*HHAP dsRNA-injected shrimp (Figure. 3.4), whereas no DNA fragmentation was found in GFP dsRNA-injected shrimp control. This result suggests the role of *Pm*HHAP in controlling apoptosis in hemocytes.

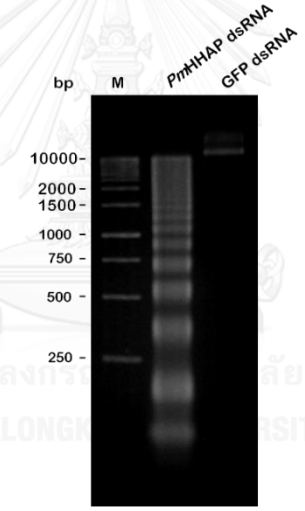

Figure 3.4 Analysis of apoptotic DNA ladders of the *Pm*HHAP-silenced shrimp. DNA was isolated from hemocytes of the *Pm*HHAP-silenced shrimp and the GFP-injected shrimp (control) after 12 h post dsRNAs injection. The apoptotic DNA ladders from hemocytes were then separated on agarose gel. The figure shown is representative of the triplicate experiments. Lane M is a 1 kb molecular weight DNA marker.

3.2.3. Activation of caspase activity in hemocytes of *Pm*HHAP knockdown *P. monodon*

Based on the above results, we hypothesized that *Pm*HHAP might be associated with the activation of caspase activity levels. To determine whether or not *Pm*HHAP is involved in the caspase-dependent apoptosis, the activation of caspase 3/7 in hemocyte cells of the knockdown shrimp was analyzed. Hemocytes were collected from each shrimp at 12 h post-dsRNAs injection, the caspase 3/7 activity was measured after incubation of hemocyte cells with Caspase-Glo reagent. It was found that the activity of caspase 3/7 in hemocyte of the *Pm*HHAP-silenced shrimp was increased 3.9-fold compared with the control shrimp (Figure. 3.5). This supports that hemocyte apoptosis was significantly induced by *Pm*HHAP dsRNA knockdown, and is congruent with the results of the annexin V-positive apoptotic cells and DNA fragmentation.

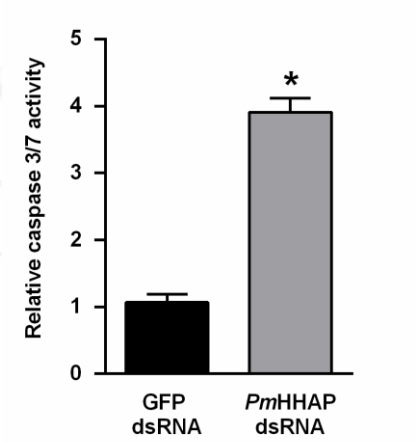

Figure 3.5 Caspase 3/7 activity in hemocytes of *Pm*HHAP-silenced *P. monodon*. Hemocytes were collected from *Pm*HHAP and GFP dsRNA-injected shrimp, and the caspase 3/7 activity was measured. The column represents the mean ± 1 S.D. (error bar) derived from three separated shrimp. Asterisk indicates a statistical significant difference between means (*$P < 0.05$).

3.3. Effect of *Pm*HHAP suppression on expression of effector caspases

To investigate whether *Pm*HHAP transcript effect to the mRNA expression of apoptotic-related genes in shrimp, dsRNA-mediated gene knockdown of *Pm*HHAP was performed as described above. The different apoptotic related-genes including two effector caspases *Pm*Casp and *Pm*Caspase, cathepsin B, cathepsin D, defender against apoptotic death (*Pm*DAD1) and survivin were selected for qRT-PCR based evaluation of their relative expression levels. After silencing of *Pm*HHAP for 12 h, the expression of *Pm*casp was slightly decreased while the *Pm*caspase was significantly down-regulated (3.4-fold) (Figure. 3.6A). Moreover, the expression of cathepsin B and cathepsin D showed significantly decreased about 5-fold and 7.5-fold, respectively (Figure. 3.6A). Transcript levels of anti-apoptotic genes, *Pm*DAD1 and survivin, were significantly lower by about 3.6-fold and 2.9-fold, respectively (Figure. 3.6A).

Furthermore, the effect of suppressing *Pm*HHAP on the expression level of the two isoforms of effector caspases in shrimp *P. monodon*, *Pm*Casp (Wongprasert et al., 2007) and *Pm*Caspase (Leu et al., 2008) was evaluated at various time points post dsRNAs injection using the qRT-PCR analysis. It was found that the gene silencing of *Pm*HHAP observed at 12, 18, and 24 h post dsRNAs injection (Figure. 3.6B) was accompanied with a reduction of *Pm*Caspase transcript levels at the same time points (Figure. 3.6B). Interestingly, the mRNA level of *Pm*Casp was significantly increased at 18 h in hemocyte of the *Pm*HHAP-silenced shrimp (Figure. 3.6B). These results suggested that suppression of *Pm*HHAP can alter the transcription of effector caspases in the apoptosis pathway.

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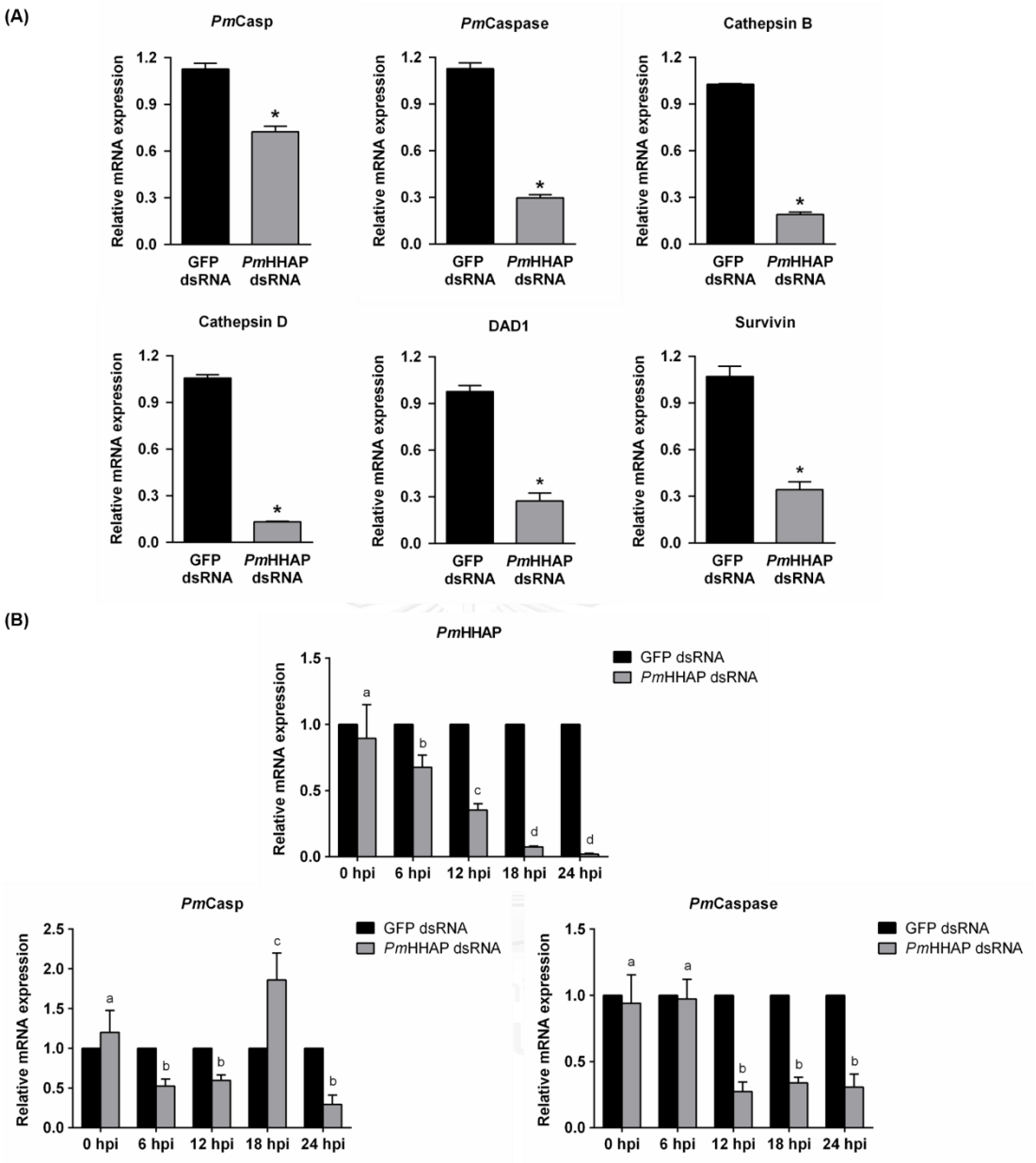

Figure 3.6 Effect of *Pm*HHAP silencing on expression of apoptotic-related genes in shrimp. (A) The effect of *Pm*HHAP knockdown at 12 h post dsRNAs injection on the expression level of genes involved in apoptosis (*Pm*Casp, *Pm*Caspase, cathepsin B, cathepsin D, DAD1 and survivin) were evaluated by qRT-PCR. (B) The effect of *Pm*HHAP gene knockdown on the transcript expression level of *Pm*HHAP, *Pm*Casp and *Pm*Caspase at each time point after dsRNAs injection was evaluated by qRT-PCR. EF1-α was served as the internal reference gene to normalize the amount of cDNA template. The data represent the mean ± 1 S.D. (error bar) derived from three

independent experiments. Asterisk indicates a statistical significant difference between means (*$P < 0.05$), while different letters indicate significant difference accepted at $P < 0.05$.

3.4 Protein-protein Interaction of *Pm*HHAP and *Pm*Casp

Yeast two-hybrid system was performed to detect the interaction of *Pm*HHAP with the effector caspase, *Pm*Casp, or with p20 domain or p10 domain of *Pm*Casp. The results showed that *Pm*HHAP was able to interact with the p20 domain of *Pm*Casp (Figure. 3.7A), but not with a mature protein or a p10 domain of *Pm*Casp, indicating that *Pm*HHAP is capable of interacting with *Pm*Casp via its p20 subunit. Furthermore, the co-immunoprecipitation assay was applied to verify the existence of the physical association between *Pm*HHAP and p20 subunit of *Pm*Casp. The result confirmed the interaction of *Pm*HHAP with the p20 subunit of *Pm*Casp (Figure. 3.7B). These results implied that *Pm*HHAP might function with *Pm*Casp to control apoptosis in shrimp.

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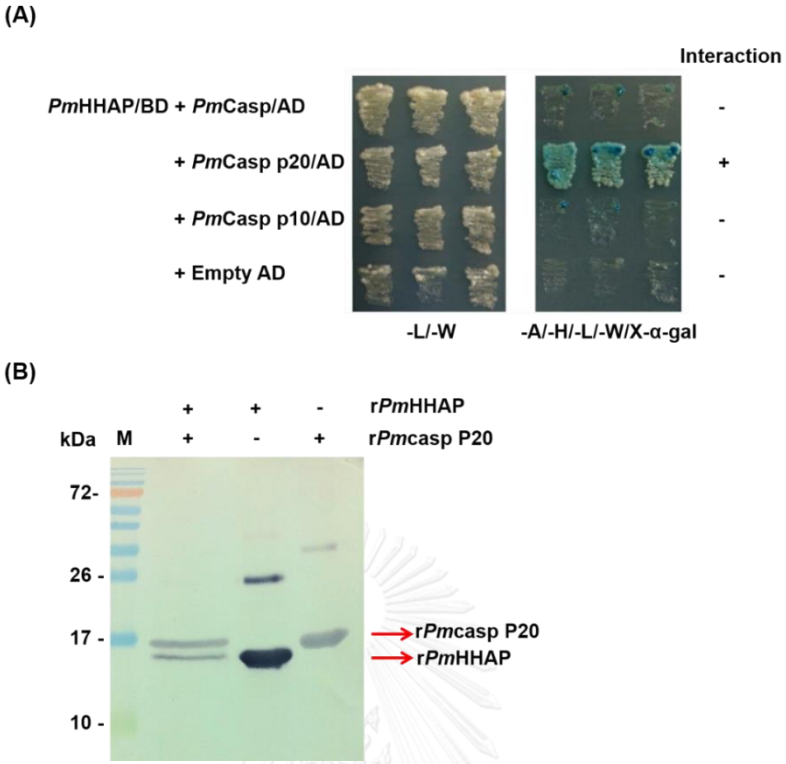

Figure 3.7 Interaction of *Pm*HHAP with *Pm*Casp protein. (A) The protein-protein interaction between *Pm*HHAP and the mature protein, p20 and p10 domains of *Pm*Casp, as determined by yeast two-hybrid assay. Interaction (+) is indicated by blue yeast colonies and no interaction (-) by no growth. BD and AD constructs co-transformed in yeast are indicated. (B) The protein-protein interaction between *Pm*HHAP and the p20 domain of *Pm*Casp was confirmed by Co-immunoprecipitation assay. The immunoprecipitated complex between r*Pm*HHAP and p20 domain (rP20) of *Pm*Casp was detected using the polyclonal anti-*Pm*HHAP antibody. The proteins were separated by SDS-PAGE and analyzed by Western blotting with anti-His antibody.

3.5. Inhibition of caspase activation in shrimp hemocyte by r*Pm*HHAP

Initially, the ability of *Pm*HHAP to inhibit the apoptosis induced by actinomycin D was evaluated in shrimp hemocyte lysates (HLS). The caspase activation analysis was performed by incubating the r*Pm*HHAP and shrimp HLS with actinomycin D, followed by measuring the caspase activity. The results revealed that the caspase 3/7 activity

were significantly decreased (58%), compared with the control group (BSA) (Figure. 3.8A). Moreover, no significant differences in caspase 3/7 activity were noticed among the groups of actinomycin D-treated HLS in the presence or absence of BSA. However, the level of caspase activity in actinomycin D-treated HLS was significantly increased (87%), compared to non-treated HLS.

In addition, the inhibition of apoptosis by r*Pm*HHAP through caspase activation was performed by incubating the r*Pm*HHAP and r*Pm*Casp with shrimp HLS, then measuring the caspase activity. It was found that the caspase 3/7 activity in r*Pm*HHAP and r*Pm*Casp treated-HLS were significantly decreased (67%), compared with the r*Pm*Casp-treated HLS (Figure. 3.8B). No significant differences in caspase 3/7 activity was observed among the groups of HLS treated with and without r*Pm*HHAP and with BSA. This result suggested that *Pm*HHAP likely inhibits shrimp hemocyte apoptosis via inhibition of *Pm*Casp activity.

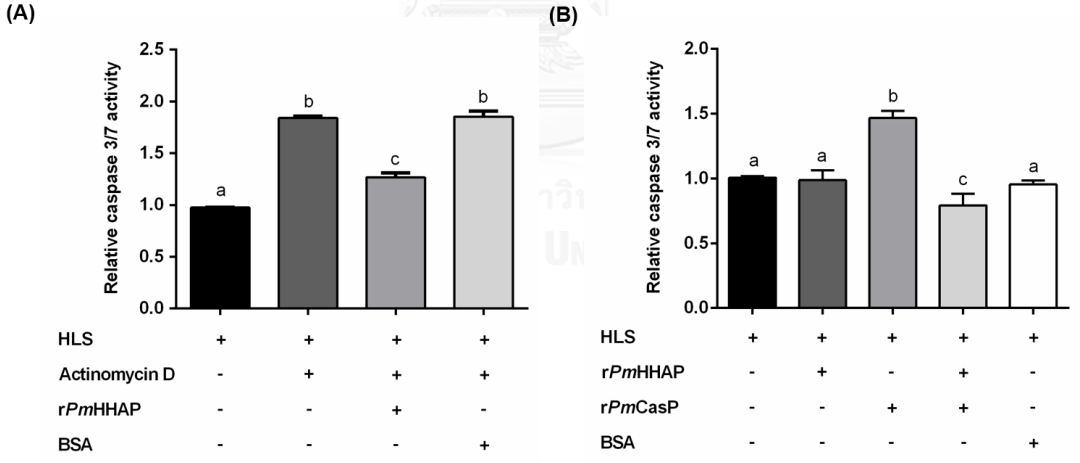

Figure 3.8 *In vitro* inhibition of caspase activation by r*Pm*HHAP. (A) Hemocyte lysate supernatant (HLS) was incubated with r*Pm*HHAP in the presence or absence of actinomycin D. Control groups were HLS only and HLS incubated with BSA. (B) Hemocyte lysate supernatant (HLS) was incubated with r*Pm*HHAP, r*Pm*Casp and co-incubated with both r*Pm*HHAP and r*Pm*Casp, control group was HLS with and without BSA protein control. Total caspase activity was detected using the Caspase-Glo 3/7

assay kit. The results are expressed as the mean ± S.D. (error bar) of three replicates. Different letters indicate significant difference accepted at $P < 0.05$.

3.6 Interaction of *Pm*HHAP with WSSV proteins

3.6.1 Yeast two-hybrid screening for binding target of *Pm*HHAP

From our previous result, *Pm*HHAP was extremely up-regulated upon WSSV infection (Prapavorarat et al., 2010) and in this study also found that *Pm*HHAP plays a role in preventing hemocyte apoptosis. Thus, it is interesting to identify the *Pm*HHAP interacting protein from WSSV using yeast two-hybrid screening. The *Pm*HHAP was used as a bait protein for screening of library constructed from the open reading frame of WSSV genome. Three positive clones were identified and then subjected to DNA sequencing. The obtained sequences were analyzed by searching against the GenBank database using BLASTX. The three positive WSSV ORF clones showed a sequence matching to WSSV134 (Accession No. AAL89002), WSSV362 (Accession no. AAL89229) and WSSV395 (Accession No. AAL89263) (Figure 3.9A).

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3.6.2 Interaction of r*Pm*HHAP and WSSV proteins by co-immunoprecipitation (Co-IP)

To confirm the interaction between *Pm*HHAP and the identified interacting partner, co-immunoprecipitation (Co-IP) was performed. The recombinant proteins of *Pm*HHAP, WSSV134 and WSSV395 were produced in *E. coli* system. While, the production of recombinant of WSSV362 was unsuccessful. The immunoprecipitated complex was detected using anti-His antibody according to the Western blotting analysis. The r*Pm*HHAP and rWSSV134 and/or rWSSV395 could be detected in the elution step indicating that *Pm*HHAP can bind with both WSSV134 and WSSV395 (Figures 3.9B and 3.9C). Among these proteins, WSSV134 was selected for further study

because it was reported to function as an anti-apoptotic protein in WSSV (Lertwimol et al., 2014).

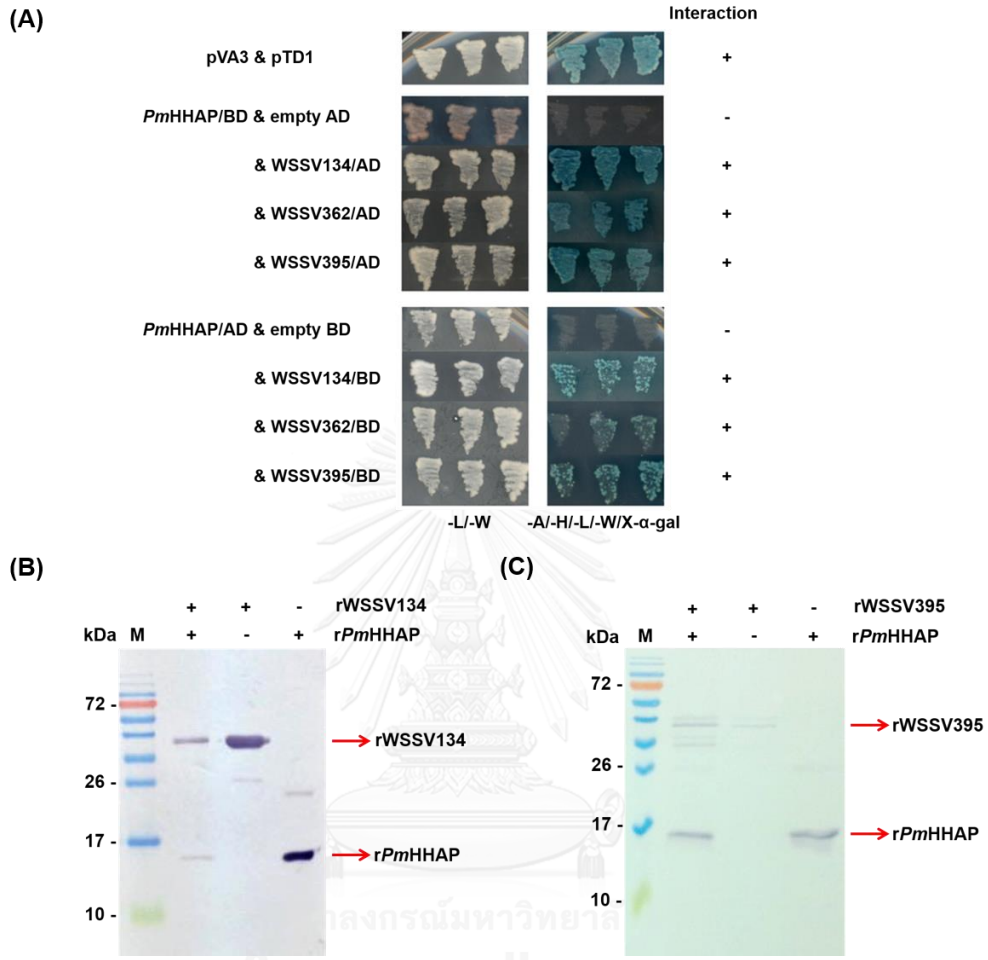

Figure 3.9 Interaction between *Pm*HHAP and WSSV proteins. (A) The protein-protein interaction between *Pm*HHAP and WSSV proteins (WSSV134, WSSV362 and WSSV395), as determined by yeast two-hybrid assay. Interaction (+) is indicated by blue yeast colonies and no interaction (-) by no growth. (B) and (C) The protein-protein interaction between r*Pm*HHAP and rWSSV134 or rWSSV395, respectively by co-immunoprecipitation assay. The proteins complexes were separated by SDS-PAGE and analyzed by Western blotting with anti-His antibody.

3.7 Expression profile of viral genes in WSSV challenged shrimp hemocytes

The expression levels of viral genes obtained from yeast two-hybrid screening at various time points after WSSV infection were determined by semi-quantitative RT-PCR analysis. In this study, the viral genes at different stages of viral replication, which are ie-1 (immediate early gene), wsv477 (early gene) and VP28 (late gene), were used as marker genes. The hemocytes were collected at 0, 3, 6, 12, 24 and 48 hpi and the gene expression was analyzed by semi-quantitative RT-PCR. EF1-α was used as an internal gene control. The results revealed that WSSV134, WSSV362 and WSSV395 transcripts were detected at 24 and 48 hpi, and the expression profile of these three genes was similar to that of VP28. Therefore, this indicating that WSSV134, WSSV362 and WSSV395 were late genes which expressed at late stage of WSSV infection (Figure 3.10).

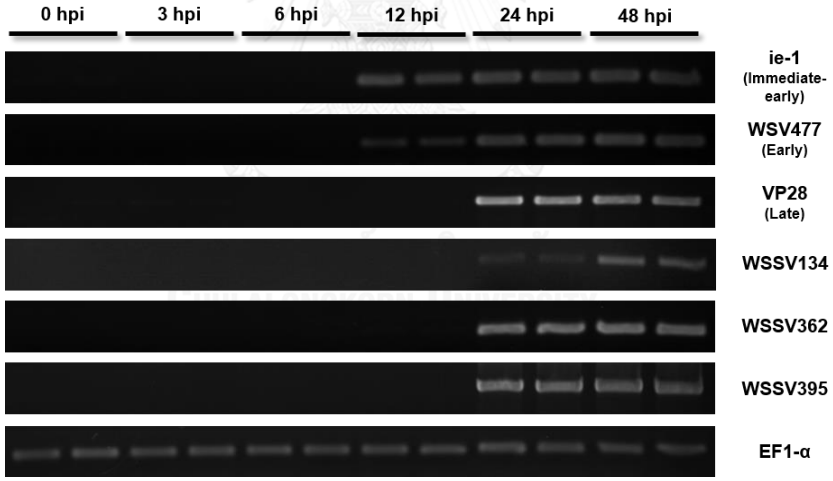

Figure 3.10 Expression profile of WSSV134, WSSV362 and WSSV395 genes in hemocytes of WSSV-infected shrimp. Hemocytes from both WSSV-infected shrimp and control PBS were collected at 0, 3, 6, 12, 24 and 48 hpi and subjected to analyze the transcript levels by semi-quantitative RT-PCR. Ie-1 (immediate-early gene), wsv477 (early gene) and VP28 (late gene) were amplified as a positive control, while EF1-α was used as an internal control. Three individuals shrimp were analyzed for each group. The representative data of two individuals per group is shown.

3.8 Effect of WSSV134 gene silencing in WSSV-infected shrimp

3.8.1 Functional analysis of WSSV134 by RNA-interference (RNAi)

Protein-protein interaction analysis revealed that *Pm*HHAP can interact with WSSV134, an anti-apoptosis of WSSV (Lertwimol et al., 2014). Therefore, the role of WSSV134 and *Pm*HHAP in hemocyte apoptosis was then further investigated. Initially, the efficiency of dsRNA-mediated knockdown of WSSSV134 was determined by semi-quantitative RT-PCR analysis. After injection of dsRNAs and followed by WSSV injection for 48 h, samples were collected and the transcript levels of WSSV134 was evaluated. The result showed that the WSSV134 transcript levels were significantly decreased in WSSV134-silenced shrimp, whereas injection of GFP dsRNA had no discernable effect on the WSSV134 transcript levels (Figure 3.11). Interestingly, knockdown of WSSV134 affected the expression level of VP28 gene compared to that of control injected with GFP dsRNA (Figure 3.11). Thus, the result suggested that WSSV134 might be involved in viral propagation.

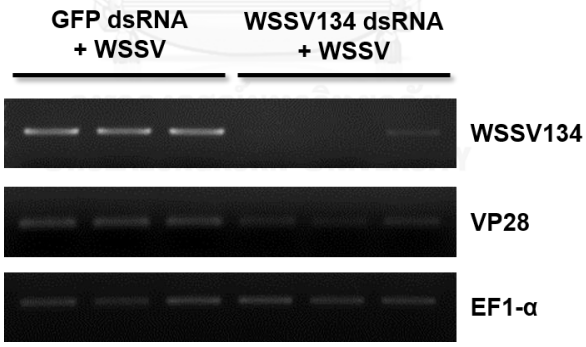

Figure 3.11 *In vivo* gene silencing of WSSV134 by RNA-interference (RNAi). Expression level of WSSV134 was examined by semi-quantitative RT-PCR to check the efficiency of gene knockdown. The VP28 and EF1-α were served as a marker gene and internal control gene, respectively. Each lane represents the result from individual shrimp with three individuals per group.

3.8.2 Effect of WSSV134 gene knockdown on viral propagation in shrimp hemocytes

Previous result showed that silencing of WSSV134 affected the transcript levels of VP28, suggesting that WSSV134 might be important in viral propagation. To investigate the important of WSSV134 in WSSV infection, the expression of representative WSSV genes for three stages of WSSV infection including ie-1 (immediate-early gene), wsv477 (early gene) and VP28 (late gene) were determined after WSSV134 knockdown in WSSV-infected shrimp using qRT-PCR. Silencing of WSSV134 caused dramatically decrease in the expression of ie-1, wsv477 and VP28 (87.3%, 86.0% and 82.3%, respectively) compared to the control shrimp (Figure 3.12). The decrease in WSSV transcript levels indicated that WSSV134 is important in WSSV propagation process.

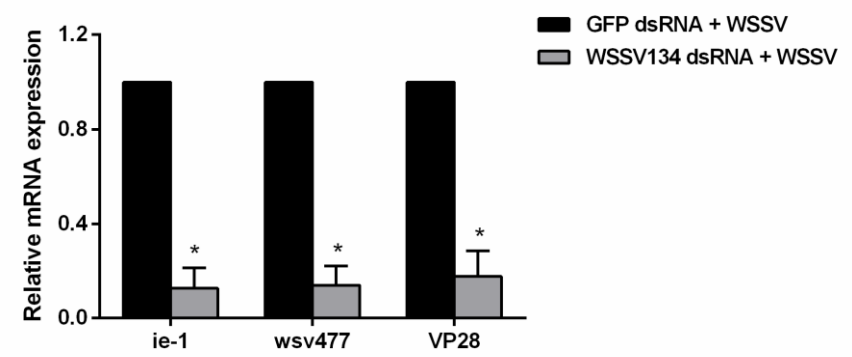

Figure 3.12 The effect of WSSV134 gene silencing on WSSV propagation in shrimp hemocytes. Expression level of representative WSSV genes for three stages of WSSV infection including ie-1 (immediate-early gene), wsv477 (early gene) and VP28 (late gene) was determined after WSSV134 knockdown in WSSV-infected shrimp using qRT-PCR. The data was shown as the mean ± 1 S.D. (error) derived from three independent shrimp. EF1-α was used as an internal control gene to normalization with same cDNA sample. Asterisk indicates a statistical significant difference between means (*$P < 0.05$).

3.9 Characterization of biological function of *Pm*HHAP and WSSV134 in hemocyte apoptosis upon WSSV infection

3.9.1 Co-silencing of *Pm*HHAP and WSSV134 by RNA interference (RNAi)

To investigate how *Pm*HHAP and WSSV134 function together upon viral infection, co-silencing of *Pm*HHAP and WSSV134 transcription were performed by RNAi. The efficiency of the gene knockdown was examined by semi-quantitative RT-PCR analysis. Shrimp were injected with either *Pm*HHAP dsRNA (5 μg/g shrimp) or WSSV134 dsRNA (7 μg/g shrimp) and *Pm*HHAP dsRNA (5 μg/g shrimp) together with WSSV134 dsRNA (7 μg/g shrimp) and GFP dsRNA (7 μg/g shrimp) (control). WSSV was injected after 3 h post dsRNAs injection and hemocytes were collected at 48 h post WSSV challenged. The expression level of *Pm*HHAP transcript was significantly decreased in the *Pm*HHAP-silenced shrimp, while WSSV134 transcript was significantly decreased in the WSSV134-silenced shrimp. Moreover, both of the *Pm*HHAP and WSSV134 transcript were decreased in co-silencing of *Pm*HHAP and WSSV134 compared to that of the control group (Figure 3.13A). In addition, the relative mRNA expression of VP28 derived from VP28/EF1-α ratio of DNA band intensity from each group was calculated. The result showed that knockdown of both *Pm*HHAP and WSSV134 caused a substantially decreased in VP28 transcripts as compared to that of GFP control. However, knockdown *Pm*HHAP gene only did not affect the VP28 transcript level when compared to WSSV134-silenced shrimp (Figure 3.13B).

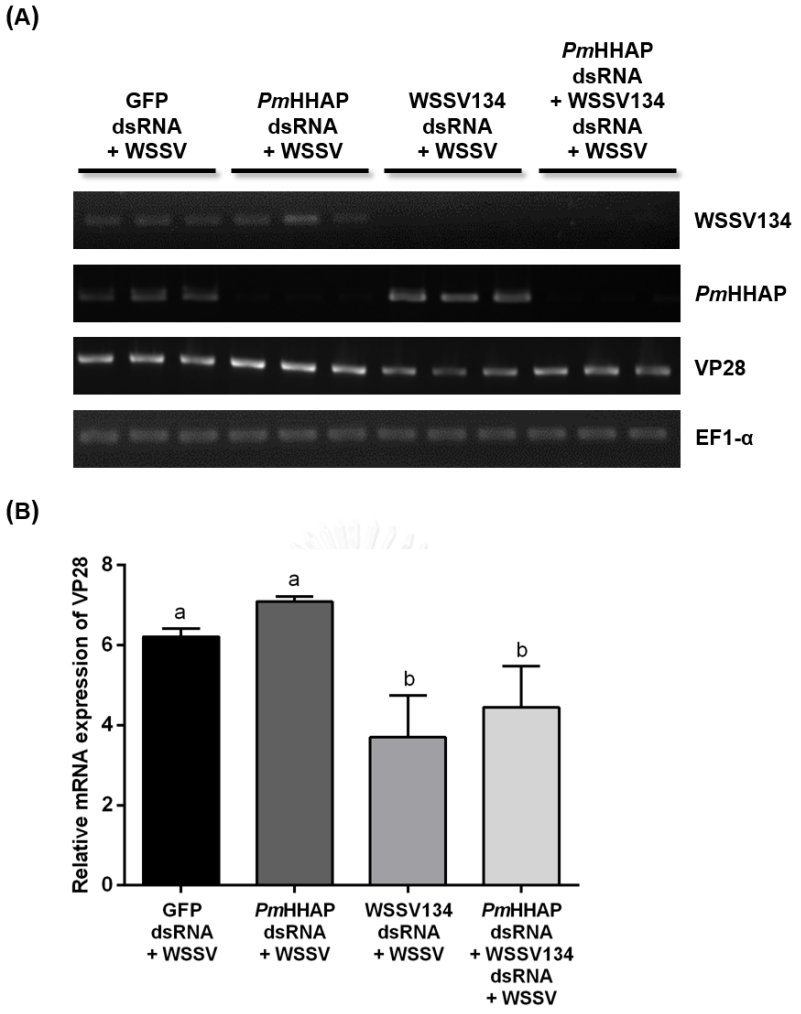

Figure 3.13 Co-silencing of *Pm*HHAP and WSSV134 by RNAi. (A) The efficiency of dsRNA-mediated gene knockdown was examined by semi-quantitative RT-PCR. The transcript levels of *Pm*HHAP and WSSV134 were shown in the gel, for each condition represents the result from three individuals shrimp. VP28 and EF1-α were used as a marker gene and internal control gene, respectively. (B) Relative expression ratio of VP28 transcripts was evaluated by semi-quantitative RT-PCR. Band intensities of VP28 and EF1-α from agarose gel electrophoresis were determined. Then, the VP28 band intensity was normalized with EF1-α band intensity and reported as relative mRNA expression. The data shown are the mean ± S.D. (error bar), and are derived from three individuals shrimp. Different letters indicate significant difference accepted at $P < 0.05$.

3.9.2 Apoptosis analysis in hemocytes of co-silencing of *Pm*HHAP and WSSV134

To better understand the function of *Pm*HHAP and WSSV134 in hemocyte apoptosis upon viral infection, the level of enzyme caspase 3/7 activity was measured in shrimp co-silencing with *Pm*HHAP and WSSV134 compared to that of the controls. The results revealed that the caspase 3/7 activity was significantly increased after WSSV infection compared to the control PBS-injected shrimp (Figure 3.14). A significant increase was observed in the *Pm*HHAP knockdown group by 47% and 112% compared with the controls GFP knockdown and PBS group, respectively (Figure 3.14). The caspase 3/7 activity also increased in WSSV134 knockdown group by 54.33% and 120% compared with the controls GFP knockdown and PBS group, respectively (Figure 3.14). In addition, the highest caspase 3/7 activity was found in the co-silencing of *Pm*HHAP and WSSV134 compared to the *Pm*HHAP-silenced shrimp (129%), WSSV134-silenced shrimp (121%) and other control groups, suggesting that these two proteins were important for hemocyte apoptosis during WSSV infection with unknown mechanism.

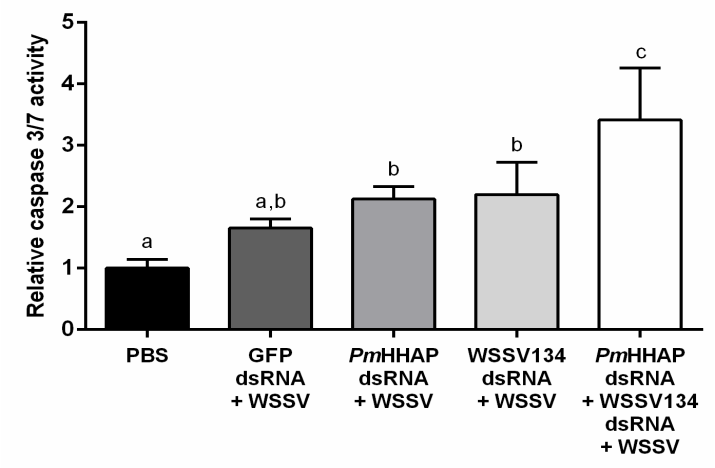

Figure 3.14 Caspase activity assay in hemocyte of shrimp co-silenced with *Pm*HHAP and WSSV134. The hemocytes were collected at 48 h post WSSV challenged and subjected to prepare the HLS followed by measuring the caspase 3/7 activity. The

column represents the mean ± 1 S.D. (error bar) derived from three individuals shrimp. Different letters indicate significant difference accepted at $P < 0.05$.

3.10. Construction of suppression subtractive cDNA library and evaluation of subtraction efficiency

To identify genes which are regulated by *Pm*HHAP, suppression subtractive hybridization between *Pm*HHAP-silenced shrimp and GFP-silenced shrimp control was performed. Initially, shrimp were injected with *Pm*HHAP dsRNA and GFP dsRNA (control) to suppress the expression of *Pm*HHAP. At 12 h post dsRNAs injection, hemocytes of *Pm*HHAP-silenced shrimp and GFP-silenced shrimp were collected and subjected to synthesize cDNAs for generate SSH library. After SSH library was constructed and subtracted, the efficiency of the SSH was evaluated by PCR to compare the transcript abundance of a house-keeping gene, EF1-α, before and after subtraction. The transcription level of EF1-α was reduced in subtracted cDNA sample, while the high transcript level of EF1-α was observed in unsubtracted cDNA samples (Figure. 3.15). It suggested that the SSH was successful.

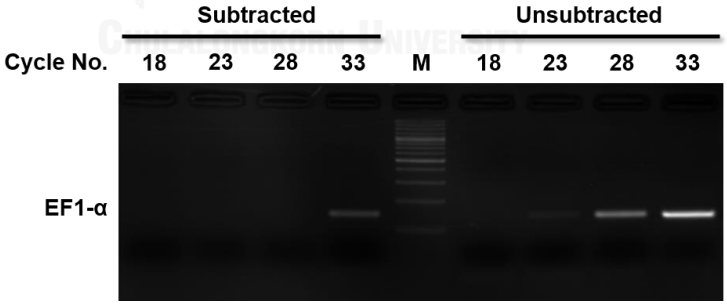

Figure 3.15 Subtraction efficiency analysis of the subtracted cDNAs. Transcription abundant of non-differentially expression gene, EF1-α, was amplified by PCR comparing between subtracted and unsubtracted cDNAs samples.

3.11 Identification of *Pm*HHAP-responsive genes by SSH

SSH was employed to identify genes that are up-regulated after suppression of *Pm*HHAP transcript in shrimp *P. monodon*. From the subtracted library, one hundred clones were randomly selected and subjected to nucleotide sequencing. The sequences were searched for sequence homology against NCBI GenBank database with BLASTN and BLASTX program. Significant matches (E-values < 1×10^{-4}) were obtained with 85% cDNA sequences for annotatable known genes, the remaining 4% and 11% were hypothetical proteins and unknown genes, respectively. The genes were categorized according to their putative functions including apoptosis, cell defense and homeostasis, DNA synthesis, repair and replication, transcription and RNA processing, protein synthesis and processing, transporters and channels, signaling and communication, energy and metabolism, cell growth and development, viral infection and miscellaneous protein (Table 3.1). A few of these genes that were abundant or interesting were selected for further analysis.

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Table 3.1 Up-regulated genes in the hemocyte of *Pm*HHAP-silenced *P. monodon* identified from suppression subtractive hybridization.

Genes and putative functions	The closest species	Accession no.	E-Value	No. of clones
Apoptosis				
Cathepsin L	*Peneaus monodon*	ABQ10739	2.00E-11	6
Oncoprotein nm23	*Litopenaeus vannamei*	ABI93176	3.00E-54	1
Inhibitor of apoptosis	*Peneaus monodon*	ABQ38431	2.00E-79	1
Cell defense and homeostasis				
Ferritin	*Penaeus monodon*	ABP68819	1.00E-56	14
Ubiquitin	*Culicoides soonorensis*	AAV84266	5.00E-103	2
Sacroplasmic calcium-binding protein	*Litopenaeus vannamei*	ACM89179	3.00E-26	1
AN1-type zinc finger protein 6	*Acromyrmex echinatior*	EGI68194	2.00E-21	1
Selenoprotein M	*Peneaus monodon*	AFS60116.1	6.00E-81	1
Sacsin	*Crassostrea gigas*	EKC39753	5.00E-37	2
DNA synthesis, repair and replication				
Structural maintenance of chromosomes protein 1A	*Acromyrmex echinatior*	EGI69340	6.00E-25	1
S-adenosylmethionine synthase	*Daphnia magna*	XP_001942800	6.00E-50	2
Chromatin modification-related protein MEAF6	*Zootermopsis nevadensis*	KDR23621	7.00E-16	1
Transcription and RNA processing				
Glutaminyl-tRNA synthetase	*Clupea harengus*	XP_018026094	4.00E-59	4
DNA-binding protein RFX2-like	*Acyrthosiphon pisum*	XP_016659561	6.00E-05	2
Putative nucleolar GTP-binding protein 1	*Zootermopsis nevadensis*	KDR12537	4.00E-103	1
Nascent polypeptide-associated complex alpha	*Penaeus monodon*	ACJ47904	6.00E-85	1
Prohibitin 2	*Penaeus monodon*	ACD13589	1.00E-64	1
Splicing factor 3A subunit 3	*Zootermopsis nevadensis*	KDR18912	4.00E-82	1
Extra sex combs	*Macrobrachium nipponense*	AGI50961	7.00E-115	1
Ornithine decarboxylase antizyme	*Cerapachys biroi*	EZA47396	9.00E-29	1
Protein synthesis and processing				
Eukaryotic translation initiation factor 2 subunit α	*Litopenaeus vannamei*	AGI197278	7.00E-81	1
Eukaryotic translation initiation factor 3	*Scylla paramamosain*	AEI88048	1.00E-92	1
Ribosomal protein L29	*Chrysomela tremula*	ACY71314	2.00E-20	1
Putative translation elongation factor ef-1 alpha	*Amblyomma aureolatum*	JAT95691	7.00E-43	1
40S ribosomal protein S4	*Salmo salar*	ACI69831	3.00E-31	1
Transporters and channels				
Sodium potassium-transporting ATPase subunit β	*Litopenaeus vannamei*	AEE25938	3.00E-58	1
Na+/K+ ATPase alpha subunit	*Penaeus monodon*	AGV55413	1.00E-69	1
Vacuole membrane protein 1-like isoform X2	*Hyalella azteca*	XP_018017043	3.00E-04	1
Innexin inx2-like	*Hyalella azteca*	XP_018017761	1.00E-13	1
Transmembrane emp24 domain-containing protein 2-like	*Hyalella azteca*	XP_018012499	1.00E-91	1
Beta tubilin	*Penaeus monodon*	ABU49604	1.00E-114	1

Table 3.1 (Continued)

Genes and putative functions	The closest species	Accession no.	E-Value	No. of clones
Signaling and communication				
Integrin	*Litopenaeus vannamei*	ACY82398	3.00E-79	2
signal recognition particle receptor subunit alpha homolog	*Ceratosolen solmsi marchali*	XP_611496400	8.00E-142	1
Energy and metabolism				
Lactate dehydrogenase	*Litopenaeus vannamei*	AEC12821	1.00E-65	11
Succinate gehydrogenase iron sulfur subunit	*Orchesella cincta*	ODN01472	7.00E-107	1
Beta-1,4-mannosyltransferase egh-like	*Hyalella azteca*	XP_01802741	2.00E-21	1
Cytochrome c	*Litopenaeus vannamei*	AGX27197	1.00E-07	1
Cell growth and development				
Protein phosphatase 2A regulatory subunit B	*Scylla paramamosain*	AFK24473	2.00E-40	1
Myophilin isoform X1	*Cephus ciactus*	XP_015599745	1.00E-31	1
Viral infection				
Transcription factor ATF-β	*Litopenaeus vannamei*	AHY82567	8.00E-71	8
Miscellanoeus functions				
Strongly chitin-binding protein-1	*Procambarus clarkii*	BAM99303	3.00E-47	1
Protein lifeguard 1-like	*Hyalella azteca*	XP_018022505	1.00E-51	1
Hypothetical protein				4
Unknown sequences				11
			Total clones	100

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3.12 Confirmation of the differential expressed genes by real time RT-PCR

To confirm the SSH result, qRT-PCR was used to analyze the expression of differentially expressed genes in hemocytes of *Pm*HHAP-silenced shrimp compared to the control injected with GFP dsRNA. Hemocytes were collected after 12 h post dsRNAs injection and subsequently to perform qRT-PCR. Five different genes, transcription factor ATF-β (Accession no. AHY82567); cathepsin L (Accession no. ABQ10739); lactate dehydrogenase (Accession no. AEC12821); ferritin (Accession no. ABP68819) and inhibitor of apoptosis (IAP) (Accession no. ABQ38431), were selected for mRNA expression analysis after *Pm*HHAP knockdown compared to the GFP control. The result revealed that transcription factor ATF-β and cathepsin L were extremely up-regulated, while lactate dehydrogenase and ferritin were significant increased. Whereas the transcript levels of IAP was not significant up-regulated after suppression of *Pm*HHAP (Figure 3.16). These results confirmed the efficiency of the SSH and some of these genes should be selected for further study in order to understand their immune function which might be related to *Pm*HHAP.

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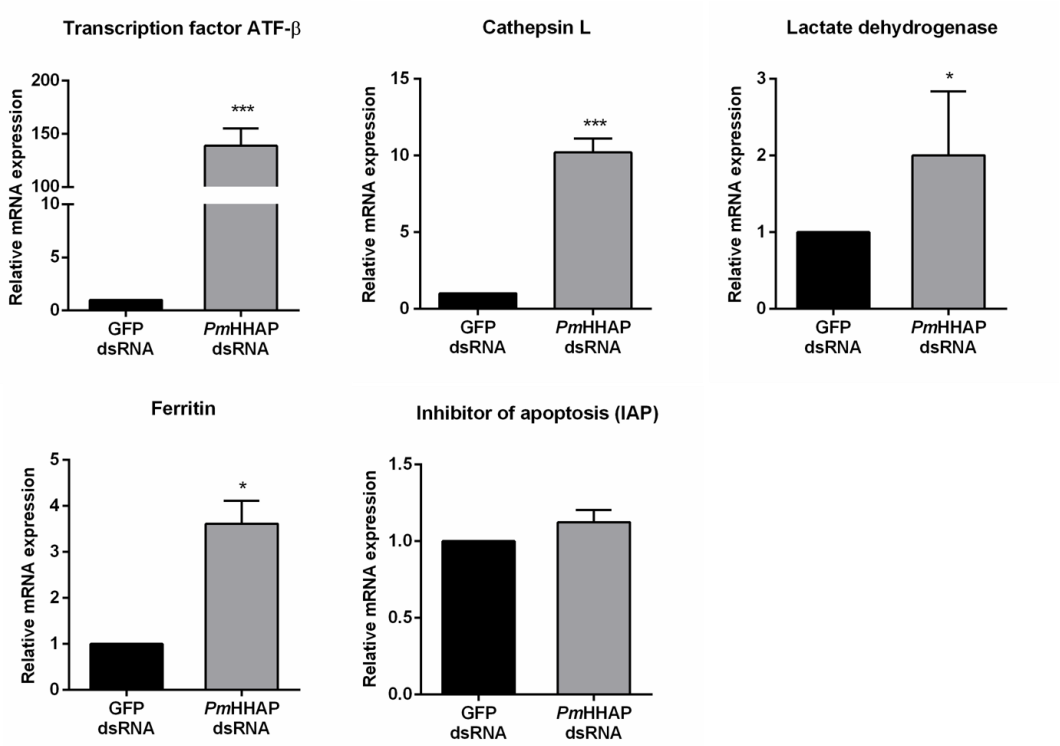

Figure 3.16 Analysis of transcription level of selected differentially expressed genes by qRT-PCR. EF1-α was used as an internal reference gene to normalize the amount of cDNA template. The data represent the mean ± 1 S.D. (error bar) derived from three independent experiments. Asterisk indicates a statistical significant difference between means (***P < 0.001* and **P < 0.05*).

3.13 Functional characterization of crayfish *Pl*HHAP

HHAP has also been identified in the freshwater crayfish *Pacifastacus leniusculus* (Prapavorarat et al., 2010). The *Pm*HHAP and *Pl*HHAP proteins share 47% identity and have eight conserved cysteine residues. However, the function of *Pl*HHAP is still unknown, thus it is interesting to investigate the role of *Pl*HHAP in freshwater crayfish.

3.13.1 Expression profile of *Pl*HHAP in crayfish tissues

To observe the transcription of *Pl*HHAP in different tissues of crayfish, cDNAs were prepared from various tissues and examined by semi-quantitative RT-PCR using gene specific primers. The crayfish *Pl*HHAP was transcribed in all tested tissues but was highly expressed in intestine and gill (Fig. 3.17). The expression of *Pl*HHAP in hemocytes was at relative low levels similar to *Pm*HHAP in shrimp but the expression in the hematopoietic tissue of crayfish was very low compared to *Pm*HHAP in shrimp.

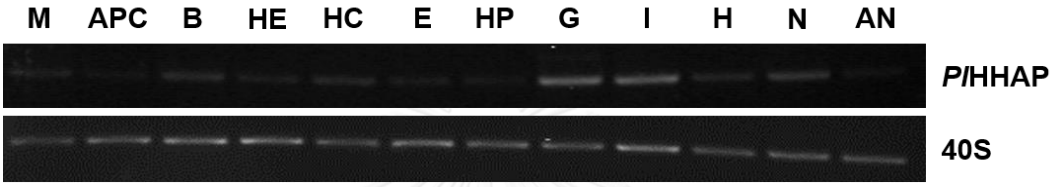

Figure 3.17 Distribution of *Pl*HHAP transcript in various tissues of *P. leniusculus* by semi-quantitative RT-PCR. The tissues examined were muscle (M), anterior proliferation center (APC), brain (B), hematopoietic tissue (HE), hemocyte (HC), eyestalk (E), hepatopancreas (HP), gill (G), intestine (I), heart (H), nerve (N) and abdominal nerve (AN). 40S ribosomal protein was used as an internal control gene. The cDNAs from three individual crayfish were pool and used for the experiment.

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3.13.2 Transcription of *Pl*HHAP in response to WSSV infection *in vitro* and *in vivo*

In shrimp, *Pm*HHAP was reported to respond to WSSV and YHV infections (Prapavorarat et al., 2010; Visetnan et al., 2015). After WSSV infection, the *Pm*HHAP transcript was highly up-regulated in shrimp hemocytes at both the mRNA and protein levels (Prapavorarat et al., 2010). To investigate the response of WSSV infection in both HPT cell cultures and crayfish hemocytes, *Pl*HHAP expression was detected at various time points after WSSV challenged by qRT-PCR. In the HPT cell culture, the addition of WSSV showed significant increase in the transcription levels of *Pl*HHAP at 48 hpi

then decreased afterward (Figure 3.18A). Whereas, the *Pl*HHAP transcript levels was not significant difference in hemocytes of crayfish *in vivo* (Figure 3.18B). This is in sharp contrast to that occurring in shrimp.

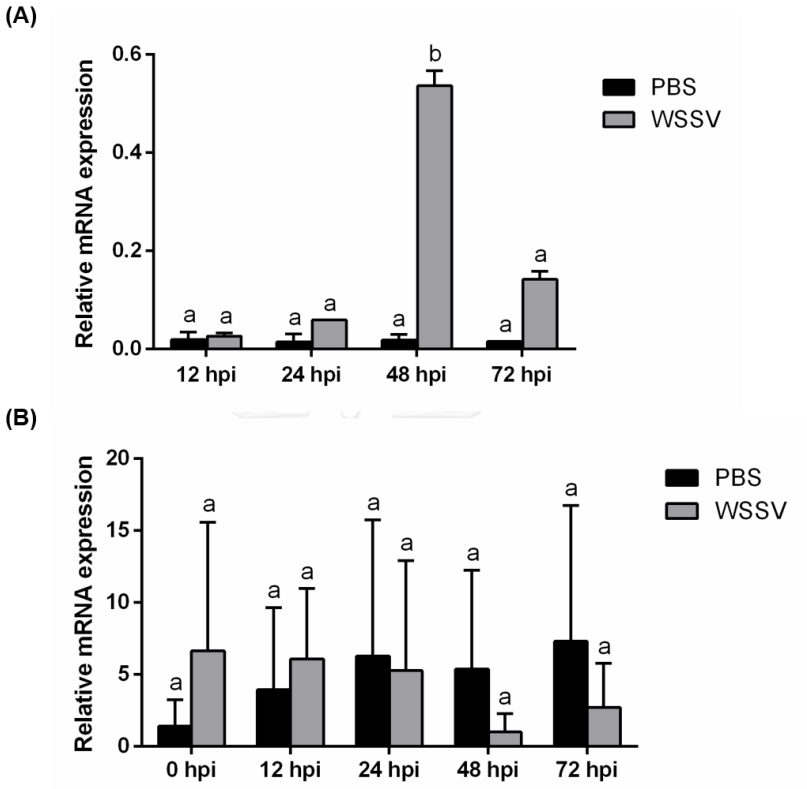

Figure 3.18 *Pl*HHAP transcripts in response to WSSV infection in crayfish by real-time RT-PCR. (A) Transcription level of *Pl*HHAP in HPT (in vitro) upon WSSV infection at 0, 12, 24, 48 and 72 hpi, respectively. (B) Expression of *Pl*HHAP from hemocytes cDNA (*in vivo*) of 3 individual WSSV-challenged crayfish at 0, 12, 24, 48 and 72 hpi, respectively. The data was shown as the mean ± 1 S.D. (error) derived from three independent experiments (*in vitro*) and three individual crayfish (*in vivo*). 40S ribosomal protein was used as an internal control gene to normalization with same cDNA sample. Different letters indicate significant difference accepted at $P < 0.05$.

3.13.3 Increased of *Pl*HHAP transcript levels in response to pathogenic bacteria infection

To determine whether the expression of *Pl*HHAP gene was influenced by a bacterial infection, the hemocytes were collected after *A. hydrophila* B1 infection and qRT-PCR was carried out. The transcript levels of *Pl*HHAP was significantly increased at 2 hpi and then decreased at 4 and 6 hpi but still significantly higher compared to the control (Figure 3.19A). Because with this dose of bacteria (high dose, 2×10^6 CFUs/ml), after 6 hours post bacteria challenged, crayfish started to moribund and reached 100% mortality within 8 hours post injection. In contrast, the control group had no crayfish deaths over the time course studied. To obtain better evidence that *Pl*HHAP is increased in expression in response to bacterial infection, we decreased the dose of bacteria used (low dose, 5×10^5 CFUs/ml) for injection and with this dose crayfish could survive until approximately 32 h post injection. With low dose, the transcription of *Pl*HHAP was significantly up-regulated at 6 hpi and reached the highest level at 12 hpi, then started to decreased at 18 hpi and attained the basal level at 24 hpi (Figure 3.19B). Taken together, these results show that *Pl*HHAP did respond to a pathogenic bacteria infection.

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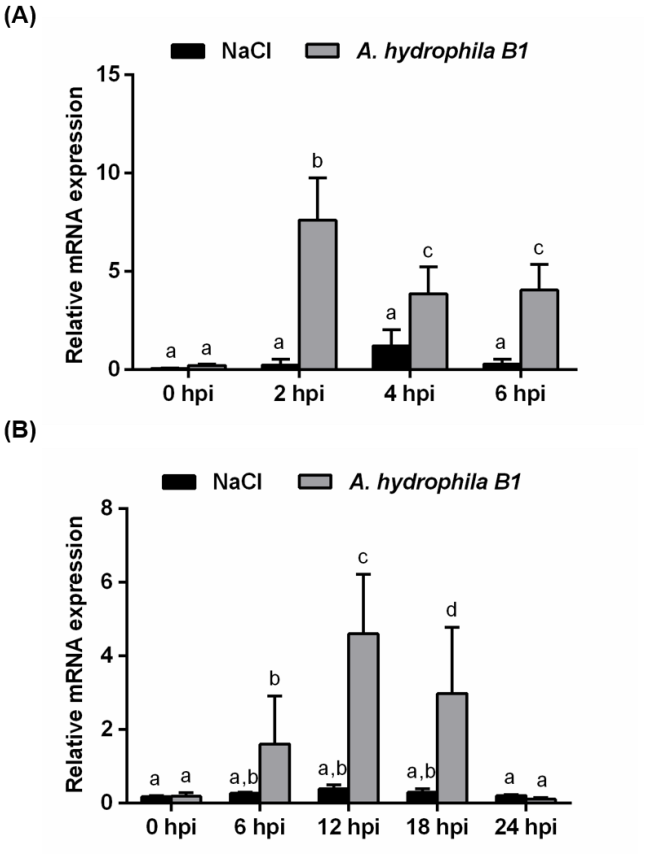

Figure 3.19 Up-regulation of *Pl*HHAP transcript in response to *A. hydrophila* B1 challenge. Relative expression of *Pl*HHAP in hemocytes of bacteria injected crayfish was determined by qRT-PCR and compared to those of the control. (A) *Pl*HHAP transcript after injection with high dose (2×10^6 CFUs/ml) of *A. hydrophila* B1. (B) Expression levels of *Pl*HHAP in response to low dose (5×10^5 CFUs/ml) of *A. hydrophila* B1 infection. The expression level of *Pl*HHAP was standardized against expression of 40S ribosomal protein and presented as relative expression. The data represent the mean ± S.D. (error bar) derived from four individual crayfish. Different letters indicate significant difference accepted at $P < 0.05$.

3.13.4 Characterization of *Pl*HHAP in hemocyte apoptosis and hematopoiesis in crayfish

3.13.4.1 Effects of *Pl*HHAP gene silencing on the mRNA levels of genes involved in hematopoiesis and hemocyte homeostasis

Due to *Pm*HHAP was reported to play an important role in hemocyte homeostasis in shrimp, it is possible that *Pl*HHAP might be implicated in hemocyte homeostasis in crayfish. To investigate whether *Pl*HHAP is involved in this process in crayfish, *in vitro* RNAi experiments of *Pl*HHAP was conducted. HPT cells were treated with either *Pl*HHAP dsRNA or GFP dsRNA control. Then, total RNA was extracted from the HPT cells at 24 and 48 h and subjected to semi-quantitative RT-PCR analysis. The semi-quantitative RT-PCR analysis showed that the *Pl*HHAP transcription level was specifically and significantly decreased in *Pl*HHAP-treated HPT cells, while in GFP-treated HPT cells no effect on gene silencing was observed (Figure 3.20A). Moreover, the efficiency of *Pl*HHAP gene silencing was evaluated *in vivo,* hemocytes of individual crayfish were collected at 24 h post-dsRNAs injection followed by total RNA extraction and synthesis of cDNA samples. The result showed that the transcript levels of *Pl*HHAP were significantly decreased in *Pl*HHAP knockdown group, when compared to that in the control GFP knockdown (Figure 3.20B). Interestingly, suppression of *Pl*HHAP did not cause any mortality of any crayfish even ten days post dsRNAs infection which is opposite to shrimp which die within 30 h post dsRNAs injection.

The transcription of genes involved in hemocyte homeostasis and hematopoiesis was further analyzed in *Pl*HHAP-silenced crayfish compared to GFP-silenced crayfish. Suppression of *Pl*HHAP gene had no significant effect on the transcription of the tested genes including CHF, astakine1, astakine2 and IGFBP7 in both *in vitro* and *in vivo* experiment (Figure 3.20A and Figure 3.20B).

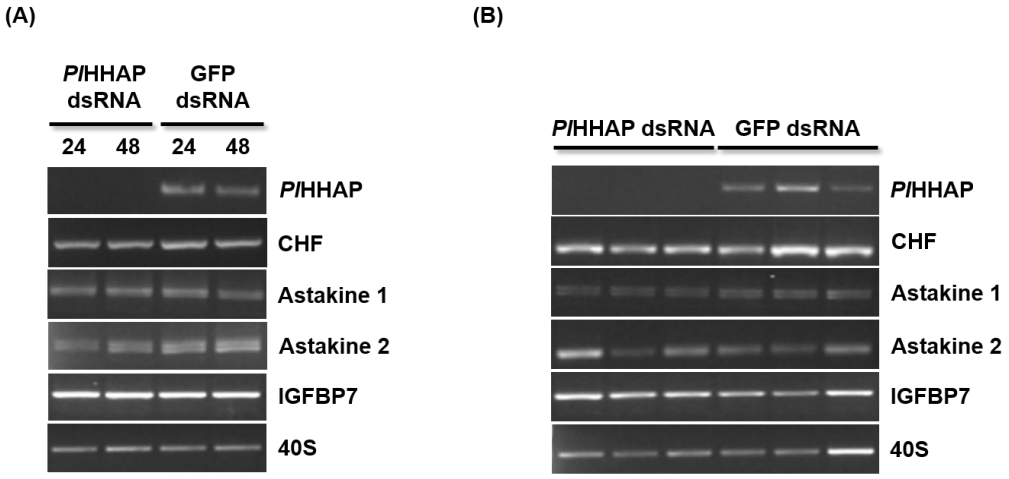

Figure 3.20 Effect of RNAi-mediated suppression of *Pl*HHAP. (A) The expression level of *Pl*HHAP after knockdown in HPT cells culture in both *Pl*HHAP dsRNA and GFP dsRNA control. Moreover, the transcription of genes involved in hematopoiesis and hemocyte homeostasis of crayfish including CHF, astakine 1, astakine 2 and IGFBP7 were evaluated by semi-quantitative RT-PCR. 40S ribosomal protein was used as a reference gene. (B) The efficiency of dsRNA-mediated gene knockdown of *Pl*HHAP gene after 24 hour post-dsRNAs injection. The effect of *Pl*HHAP dsRNA on genes involved in hematopoiesis and hemocyte homeostasis of crayfish (CHF, astakine1, astakine2 and IGFBP7) transcript was also elucidated by semi-quantitative RT-PCR. 40S ribosomal protein served as the internal control gene.

3.13.4.2 Total hemocyte count (THC) after silencing of *Pl*HHAP

The total hemocyte numbers in the circulatory system of *Pl*HHAP-silenced crayfish was verified. The hemocytes were collected after 24 h post-dsRNAs injection and subsequently to count the total hemocytes numbers. It was shown that silencing of *Pl*HHAP had no impact to the number of circulating hemocytes when compared to the total hemocyte number of GFP-injected crayfish (Figure 3.21). This again is in contrast to shrimp where THC decreases dramatically in *Pm*HHAP-silenced shrimp (Prapavorarat et al., 2010).

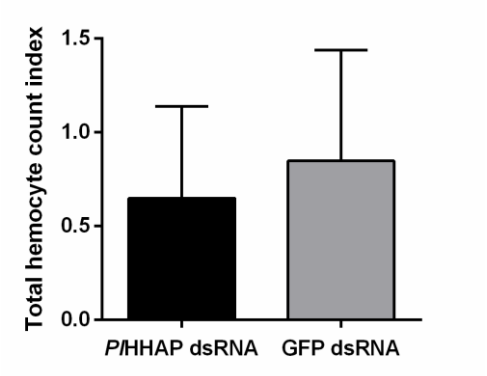

Figure 3.21 Total hemocyte number in *Pl*HHAP-silenced crayfish. After 24 h post injection of *Pl*HHAP dsRNA or GFP dsRNA, the total hemocyte count index was determined as the total number of hemocytes after injection divided by the total number of hemocytes before injection. Five animals were used in each experimental group. The data represent the mean ± 1 S.D. (error bar) derived from five independent experiments.

3.13.4.3 Analysis of hemocyte apoptosis in *Pl*HHAP-silenced crayfish

In addition, we also measured the caspase 3/7 activity comparing between *Pl*HHAP-silenced crayfish and GFP-silenced crayfish. After knockdown *Pl*HHAP *in vivo*, the caspase 3/7 activity of *Pl*HHAP dsRNA-injected crayfish remain unchanged when compared to GFP dsRNA-injected crayfish (Figure 3.22). When compared the effect of *Pm*HHAP depletion in shrimp, it has been shown that suppression of *Pm*HHAP caused hemocyte undergo apoptosis which is contrast from that found in crayfish. Taken together, these results indicated that *Pl*HHAP is not involved in hemocyte homeostasis of crayfish as *Pm*HHAP is in shrimp.

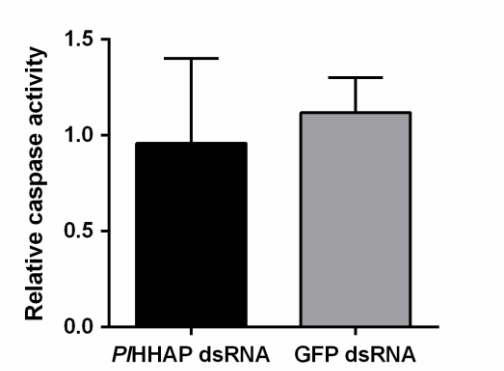

Figure 3.22 Caspase 3/7 activity in hemocytes of *Pl*HHAP-silenced crayfish. Hemocytes were collected from *Pl*HHAP and GFP dsRNA-injected crayfish, and the caspase 3/7 activity was measured. The column represents the mean ± 1 S.D. (error bar) derived from five individual crayfish.

3.13.5 Higher number of bacteria in the intestine of *Pl*HHAP-silenced animals

Tissue distribution result showed that *Pl*HHAP was highly expressed in crayfish intestine and gill. Therefore, we tried to find a possible function of this protein in intestine. Initially, we knockdown *Pl*HHAP and checked the expression of *Pl*HHAP at 24 h post dsRNAs injection in intestine. The result showed that *Pl*HHAP was significantly decreased at 24 h post-second dsRNAs injection (Figure 3.23A). Afterwards, the bacterial count was performed after silencing of *Pl*HHAP. The bacterial colonies were observed after 24 h post-second dsRNAs injection and the result showed that viable bacterial CFUs in intestine from the *Pl*HHAP-silenced crayfish was significantly higher compared to that for the control GFP-silenced crayfish (Figure 3.23B). We also visualized the normal flora inside of the intestine of knockdown *Pl*HHAP and control GFP using scanning electron microscopy (SEM). The small pieces of intestine that was processed for SEM was cut to check the efficiency of gene specific knockdown and it was displayed that expression of *Pl*HHAP mRNA was lower than control (Figure 3.24A). The intestine from *Pl*HHAP-silenced crayfish showed a large number of bacterial cells in

the inner surface and, some of them were gathered together like a cluster but some were spread in few cells over the intestine (Figure 3.24B). Whereas, a few bacterial cells were found in some areas of the luminal surface of intestine from the GFP control (Figure 3.24B). Taken together, these results indicated that depletion of *Pl*HHAP affected the number of bacteria in crayfish intestine with an unknown mechanism.

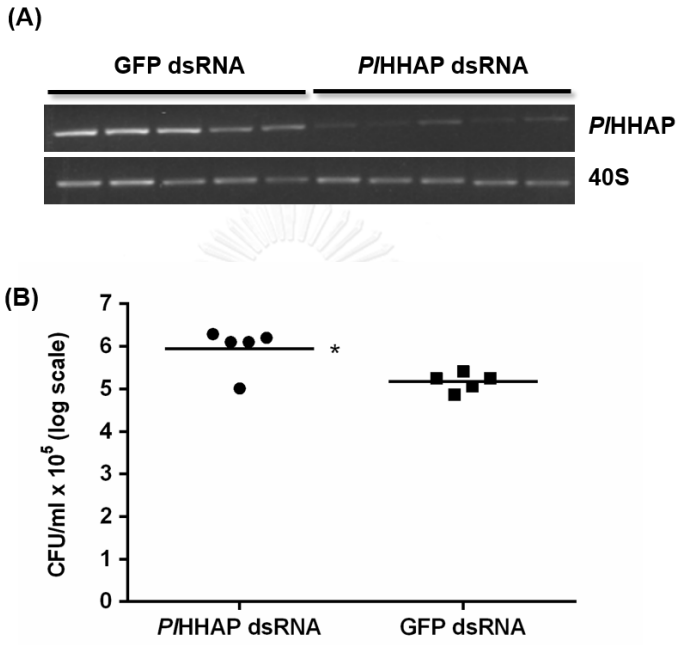

Figure 3.23 Depletion of *Pl*HHAP caused a higher number of bacteria in crayfish intestines. (A) Transcription levels of *Pl*HHAP after 24 h post-second dsRNAs injection in the crayfish intestine was determined by semi-quantitative RT-PCR. The 40S ribosomal protein was used as an internal control. (B) The bacterial colony forming units (CFUs) in the intestine of knockdown crayfish were determined at 24 h post-second dsRNAs injection and by a total plate count method. The value was derived from five crayfish. ● represents CFU of intestine from *Pl*HHAP-silenced crayfish each individual and ■ represents CFU of intestine from GFP-silenced crayfish. ─ represents mean CFU of each group. ** $P < 0.05$*, significant difference when compared to mean.

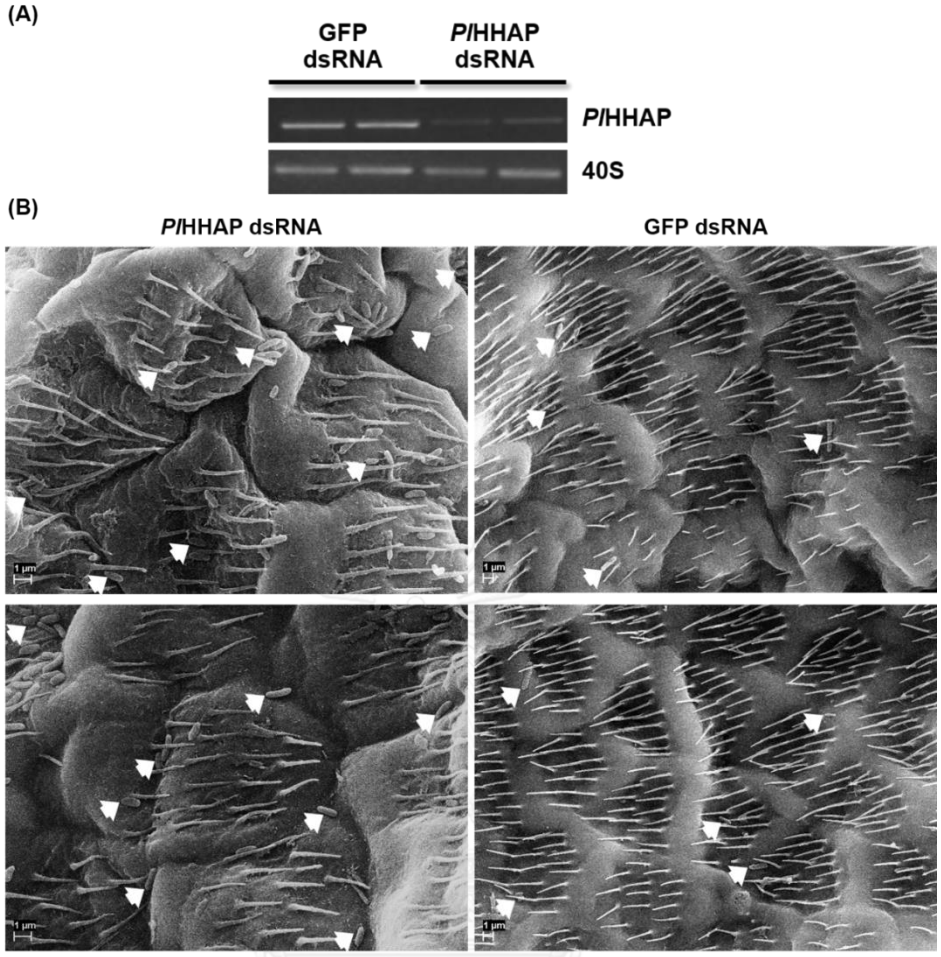

Figure 3.24 Morphology analysis of crayfish intestines in *Pl*HHAP-silenced crayfish and GFP-silenced crayfish using scanning electron microscope (SEM). (A) Transcription levels of *Pl*HHAP after 24 h post-second dsRNAs injection in the crayfish intestine was determined by semi-quantitative RT-PCR. The 40S ribosomal protein was used as an internal control. (B) The bacteria from intestine of *Pl*HHAP-silenced crayfish and the GFP-silenced crayfish control was visualized under scanning electron microscope (SEM). Bacteria can be observed within intestines of each group (white arrow). Micrographs shown are representatives of two fields of view per sample and four independent intestines each from an individual crayfish.

From the functional study of the crayfish *Pl*HHAP, it suggested that this protein plays a role in bacterial defense. Thus, we investigate further whether the shrimp *Pm*HHAP exhibits similar function. Since, *Pl*HHAP was up-regulated upon pathogenic bacterial infection but the response of *Pm*HHAP after bacterial infection has not been determined. To elucidate whether *Pm*HHAP is responded to bacterial challenge, the mRNA expression of *Pm*HHAP was determined in hemocytes of *V. harveyi*-infected shrimp using qRT-PCR. After systemic *V. harveyi* infection, the pooled hemocytes of three individual shrimp (nine shrimp at each time point) in both challenged shrimp and control shrimp were collected at various time points of 0, 6, 12, 24 and 48 h. The first-strand cDNAs were prepared and *Pm*HHAP expression was analyzed by qRT-PCR. EF1-α was used as a reference gene. The transcript of *Pm*HHAP was decreased at the very beginning of infection (0 hpi) about 2.15-fold compared with the control group and then continuously decreased. The lowest expression was observed at 12 hpi about 12.82-fold and remained down-regulated even at 48 hpi compared with the control group. (Figure 3.25). Therefore, the expression of *Pm*HHAP was also modulated in response to *V. harveyi*. However, it is in contrast to crayfish where *Pl*HHAP increases dramatically in *V. harveyi*-infected crayfish.

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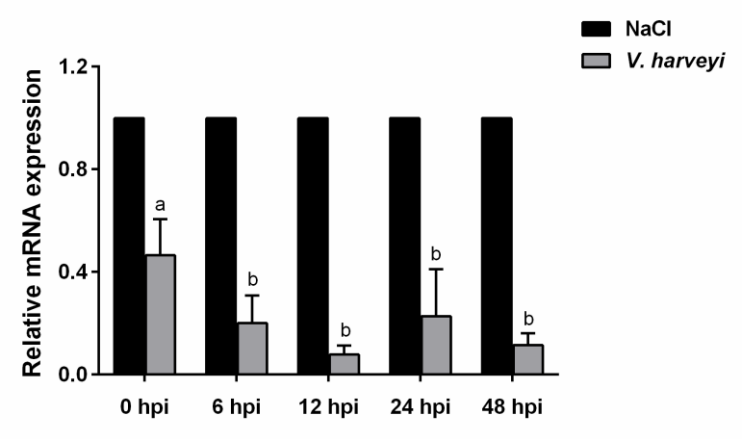

Figure 3.25 Temporal expression of *Pm*HHAP transcript in response to *V. harveyi* infection. The relative expression of *Pm*HHAP transcript in the hemocytes of *V. harveyi*-infected shrimp was determined by qRT-PCR at the indicated times post bacterial injection. The relative expression levels were calculated and EF1-α was served as the internal reference gene to normalize the amount of cDNA template. The data represent the mean ± 1 S.D. (error bar) derived from three independent experiments. Different letters indicate significant difference accepted at $P < 0.05$.

Furthermore, we asked whether knockdown of *Pm*HHAP affects the number of bacteria in shrimp intestine as *Pl*HHAP does in crayfish. To answer this question, the *Pm*HHAP transcript was first knockdown, and the number of bacteria in intestine of *Pm*HHAP-silenced shrimp and GFP-silenced shrimp were counted and compared. After 12 h post dsRNAs injection, the intestine was collected from *Pm*HHAP dsRNA-injected group and GFP dsRNA-injected group (five individuals each). The bacterial colonies were counted and the result showed that viable bacterial CFUs in intestine of the *Pm*HHAP-silenced shrimp was not significantly different compared to that of the control GFP-silenced shrimp (Figure 3.26). This again is in contrast from that found in crayfish. These results suggest that HHAP from different crustacean species might have different functions in defense against invalid pathogens

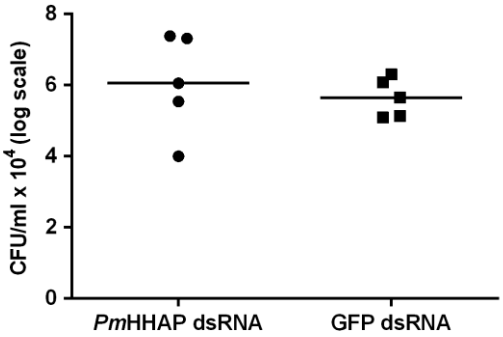

Figure 3.26 Bacterial number in intestine of *Pm*HHAP knockdown shrimp. The bacterial colony forming units (CFUs) in the intestine of knockdown shrimp were determined at 12 h post dsRNAs injection by a total plate count method. The value was derived from five individuals shrimp. ● represents CFU of intestine from each individual of *Pm*HHAP-silenced shrimp and ■ represents CFU of intestine from GFP-silenced shrimp. ─ represents mean CFU of each group.

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CHAPTER 4

DISCUSSION

Hemocyte is an immune-responsive cell that plays an important role in the immunity of crustacean (Amparyup et al., 2013; Jiravanichpaisal et al., 2006; Johansson, 2000; Lavine and Strand, 2002; Tassanakajon et al., 2013). The production of hemocytes and releases of the mature hemocytes into the circulatory system takes place in the hematopoietic tissue (HPT) (Chaga et al., 1995; Söderhäll et al., 2003). The number of hemocytes can decrease dramatically during infection (Jiravanichpaisal et al., 2001; Persson, 1987; Smith and Söderhäll, 1983). Homeostasis, achieved by the continuous proliferation and apoptosis, is thus required for the maintenance of hemocytes balance in the circulation.

In recent years, several genes involved in hematopoiesis and hemocyte homeostasis have been characterized in crustaceans (Söderhäll et.al 2003; Söderhäll et al. 2005; Prapavorarat et al., 2010; Lin et al., 2011; Lin et al., 2011; Saelee et al., 2013). Astakine, a homologue to vertebrate prokineticins, was first identified in fresh water crayfish, *P. leniusculus* (Söderhäll et al., 2005). Astakine 1 can stimulate proliferation of HPT, induce differentiation of cells in HPT into semi-granular cells and also necessary for release of new hemocytes in the circulatory system (Söderhäll et al., 2005). Moreover, the HPT cells cultured in the absence of astakine 1 are subsequently death by apoptosis, suggested that astakine 1 is a regulator of cell proliferation, differentiation and apoptosis (Lin and Söderhäll, 2011). Astakine 2, a homologue of astakine 1, has been reported to play an important role in granular cell differentiation (Lin et al., 2010). Later, astakine has also been found in shrimp and functions as a cytokine to promote cell proliferation in HPT of *P. monodon* (Hsiao and Song, 2010). Recently, an astakine-like factor has been reported to be involved in

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hemocytes proliferation in the pacific oyster, *Crassostrea gigas* (Li et al., 2016). The transcription factor, Runt, is important for hematopoiesis by inducing maturation and release of new hemocytes (Söderhäll et al., 2003, 2005). In addition, a Runt homologue and CBFβ have been reported to play a role in production of hemocytes in the scallop, *Chlamys farreri* (Yue et al., 2014). Transglutaminase (TGase) has been reported to be involved in hematopoiesis by keeping the HPT cells at undifferentiated stage inside the hematopoietic tissue and thereby affecting recruitment of new hemocytes to the circulation (Lin et al., 2008). Afterwards, a crustacean hematopoietic factor (CHF) has been identified in *P. leniusculus* and is vital for the survival of hemocytes and hematopoietic cells by preventing the apoptotic process (Lin et al., 2011). The CHF-like protein in *L. vannamei* binds to a laminin receptor (Lamr) and involves in hemocyte homeostasis in shrimp (Charoensapsri et al., 2015). Previously, two β-thymosin proteins (β-thymosin 1 and β-thymosin 2) have been shown to regulate hemocyte homeostasis in *P. leniusculus* (Saelee et al., 2013). In addition, ROS has been reported to be involved in hematopoiesis by helping to maintain the progenitor cells in HPT and reduces hemocytes release in crayfish *P. leniusculus* (Junkunlo et al., 2016).

Interestingly, a novel protein that is responsible for hemocyte homeostasis in shrimp has been discovered. A hemocyte homeostasis-associated protein (*Pm*HHAP) was initially identified as a viral responsive protein in black tiger shrimp *P. monodon* (Prapavorarat et al., 2010). Gene silencing of *Pm*HHAP resulted in a significant decrease in the number of circulating hemocytes and 100% shrimp mortality within 30 h. Moreover, severe damage of hemocytes was also observed in *Pm*HHAP- knockdown shrimp, indicating that *Pm*HHAP is essential for shrimp survival and plays an important role in hemocyte homeostasis (Prapavorarat et al., 2010). However, the mechanism of *Pm*HHAP in mediating hemocyte homeostasis in shrimp has not been clarified yet.

First, we hypothesized that *Pm*HHAP possibly controls hemocyte homeostasis via hemocytes apoptosis because depletion of *Pm*HHAP caused a decreasing and

damaging of hemocytes. It was found that dsRNA-mediated RNAi knockdown of the *Pm*HHAP transcript resulted in an increase of the apoptotic cells and also caspase 3/7 activity in the hemocytes of *Pm*HHAP knockdown, compared with that of GFP control shrimp. Additionally, DNA fragmentation which is a key characteristic of apoptosis was observed in hemocytes of the *Pm*HHAP knockdown shrimp, but this apoptotic sign was not found in hemocytes of control shrimp. These results supported our hypothesis that *Pm*HHAP plays role in controlling apoptosis in shrimp hemocytes.

Apoptosis participates in various biological processes including development, maintaining cells homeostasis and host defense to pathogenic infection (Bortner et al., 1995; Fuchs and Steller, 2011; Henson and Hume, 2006; Kerr et al., 1972). The features of apoptosis are characterized by series of hallmarks including nuclear condensation, DNA fragmentation, mitochondrial depolarization, phosphatidyl serine exposure on the plasma membrane, membrane blebbing and cell packaging into apoptotic bodies (Denault and Salvesen, 2002). The key enzyme of the execution of apoptosis are caspases, a family of cysteine proteases (Fischer et al., 2003). Caspases are synthesized as inactive zymogens in the cytosol which composed of a large subunit that contains the active-site cysteine (p20) and a carboxy-terminal small subunit (p10) linked by a flexible region highly susceptible to proteolysis (Denault and Salvesen, 2002; Riedl and Shi, 2004). Caspases are categorized as initiator or effector caspases (Salvesen and Dixit, 1999). After receiving apoptotic signals, the initiator caspases are auto-activated and then activate the effector caspases to function by cleaving many specific substrates and degrade cellular proteins, which results in the demise of cells (Adams and Cory, 2002; Riedl and Shi, 2004). In shrimp, several caspases have been identified including caspase-3 from *P. merguiensis* (Phongdara et al., 2006), *Pj*Caspase and caspase 8 from *P. japonicus* (Wang et al., 2008; Huang et al., 2014), caspase-3 and *Lv*caspase2-5 from *L. vannamei* (Rijiravanich et al., 2008; Wang et al., 2013), caspase-2 from *M. rosenbergii*

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(Youngcharoen et al., 2015), and two caspases, *Pm*Caspase and *Pm*Casp from *P. monodon* (Wongprasert et al., 2007; Leu et al., 2008).

In addition to caspases, the inhibitor of apoptosis protein or IAP also plays an important role in regulating apoptosis (Deveraux et al., 1997; Hisahara et al., 1998). IAP proteins contain three of the Baculovirus Inhibitor of apoptosis protein Repeat (BIR) domain which directly bind and inhibit caspases (Liston et al., 2003; O'Riordan et al., 2008). In shrimp, IAP was first characterized in *P. monodon* and this *Pm*IAP can block apoptosis induced by IAP antagonist Drosophila *Rpr* (Leu et al., 2008) and is able to bind with *Pm*HtrA2 and inhibits *Pm*Caspase activity (Saleeart et al., 2016). So far, three IAPs have been identified in *L. vannamei*: *Lv*IAP1, *Lv*IAP2 and *Lv*survivin (Leu et al., 2012). Silencing of *Lv*IAP1 affected shrimp mortality and also resulted in dramatically decrease in the number of circulating hemocytes caused by apoptosis (Leu et al., 2012). Interestingly, the effect of knockdown *Lv*IAP1 was similar to that of knockdown *Pm*HHAP, suggested that *Pm*HHAP might act similarly as an anti-apoptosis protein in shrimp.

Several apoptotic-associated proteins have been discovered in shrimp. Cathepsin, a lysosomal proteolytic enzyme including cathepsin B (Chwieralski et al., 2006; Liu et al., 2006) and cathepsin D, have been reported to be involved in apoptosis. Particularly, cathepsin D can function together with caspase and p53 to promote apoptosis (Chwieralski et al., 2006). However, the role of cathepsins in crustacean apoptosis is still unknown. The defender against apoptotic death (DAD1) is a negative regulator of apoptosis that has been reported to respond to YHV infection in *P. monodon* (Molthathong et al., 2008b). Survivin is an inhibitor of apoptosis that not only prevents apoptosis but also plays a role in mitotic regulation (Chen et al., 2012; Leu et al., 2008). Other genes such as fortilin (TCTP) (Bangrak et al., 2004), *Pm*-Alix (ALG-2-interacting protein X/ALG-2-interacting protein 1) (Sangsuriya et al., 2007), Ribophorin 1 (Molthathong et al., 2008a), the mitochondrial related voltage-dependent anion

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channel (VDAC) (Wang et al., 2010), *Lv*Daxx (Yan et al., 2015), and *Lv*HtrA2 (Peepim et al., 2016) were identified in shrimp. Although, even several genes/proteins in this pathway were characterized but there is still controversy remaining for the role of apoptosis in shrimp which needs to be clarified.

*Pm*HHAP appears to be involved in hemocyte apoptosis, therefore, silencing of *Pm*HHAP transcripts might alter the expression of apoptotic-related genes especially caspases. Study of caspases in shrimp mainly focus on shrimp infected by virus especially WSSV. Previous studies showed that most of the caspases in shrimp are up-regulated by viral infection. For example, *Pj*Caspase from *M. japonicus* and *Lv*caspase2-5 from *L. vannamei* transcript were significantly up-regulated after WSSV infection (Wang et al., 2008; Wang, 2013). In *P. merguiensis*, expression of caspase-3 between uninfected and infected shrimp was not significant different but caspase-3 transcripts was up-regulated in moribund shrimp infected with WSSV (Phongdara et al., 2006). Surprisingly, *Pm*Casp is up-regulated at 24 and 48 h upon WSSV infection in hemocytes (Wongprasert et al., 2007), whereas this gene is down-regulated during WSSV infection in gill (Shekhar et al., 2015). On the other hand, mRNA expression of *Pm*Caspase from *P. monodon* remains unchanged after WSSV infection (Leu et al., 2008). This present study demonstrated that expression levels *Pm*Casp was up-regulated after silencing of *Pm*HHAP at 18 h, which correlate to the increased of apoptosis after silencing of *Pm*HHAP transcripts. Whereas, silencing of *Pm*HHAP showed a decreasing in *Pm*caspase transcript. This result suggested that suppression of *Pm*HHAP could altered the mRNA expression of specific effector caspase and lead to an increase of apoptosis in hemocytes. Using yeast two-hybrid screening and co-immunoprecipitation, we found that *Pm*HHAP can interact with *Pm*Casp via p20 domain. Moreover, we showed that r*Pm*HHAP could reduce caspase activity in r*Pm*Casp-treated HLS which confirmed our hypothesis that *Pm*HHAP is an anti-apoptosis protein that plays an important role in controlling hemocyte homeostasis by inhibiting hemocytes apoptosis through caspase

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inhibition. Previously, the anti-apoptotic proteins which are responsible for inhibition of caspases activity have been reported. The caspase inhibitor, p35, could inhibit caspases specifically in nematodes, insects and humans by blocking caspase active sites (Bertin et al., 1996; Bump et al., 1995). The p35 can inhibits caspase-1 activity in *Spodoptera frugiperda* (Ahmad et al., 1997). The *Pm*caspase was shown to inhibit the activity by baculovirus anti-apoptosis protein, p35, *in vitro* (Leu et al., 2008). In human, survivin binds with caspase-3 and caspase-7 and inhibits their activity leading to suppression of apoptosis (Shin, 2001).

WSSV is one of the most virulent pathogens that caused devastating losses to shrimp industry worldwide (Shekhar and Ponniah, 2015). However, the information of interacting proteins between WSSV and host is still not well established. To entry host cells, viruses have developed several methods to pass through host's cell membrane via lipid fusion, perforation and endocytosis (Mercer et al., 2010). WSSV enters the host cell through activating the endocytotic processes. In *L. vannamei*, WSSV has been shown to enter hemocyte cells through caveolar endocytosis (Watthanasurorot et al., 2014), while the clathrin-mediated endocytosis pathway is used for WSSV to entry HPT cells in crayfish (Huang et al., 2015). Previously, several proteins involved in viral entry have been reported. For example, chitin-binding protein in *P. monodon* (*Pm*CBP) has been found to interact with various WSSV proteins (VP53A, VP24, VP110, VP53B, VP337, VP32, VP14, VP41A, VP51B, VP60A and VP39B) which suggested the role of *Pm*CBP as a cellular receptor of WSSV (Chen et al., 2009; Chen et al., 2007). In addition, *Pm*Rab7 has been demonstrated to be a receptor for VP28 which supported the recognition and entry of WSSV into shrimp cells (Sritunyalucksana et al., 2006). Recently, it has been reported that *Pm*Lamr can bind to VP31 and is responsible for host cell recognition and binding in WSSV infection (Liu et al., 2016). After entry through host cells, replication of viral genes was the next step. Since WSSV does not have its own transcription machinery, thus WSSV needs the host machinery to promote viral

replication. Host has also evolved the mechanisms to reduce the viral replication. For example, the cell might undergo apoptosis to prevent further viral replication. To counteract host cellular responses, viruses have to interact with host metabolism, stress response systems and apoptosis signaling to retain an appropriate environment for replication. After replication and production of structural proteins, these components come together to be assembled into new virions. For dsRNA viruses, DNA packaging is performed via the interactions of DNA and nucleocapsid proteins (Liu et al., 2010). However, the information of how WSSV virions are subsequently released from the host still undiscovered.

In shrimp, one of the powerful antiviral mechanisms is apoptosis, which implied to be the cause of mortality as a terminal result of viral infection (Li and Xiang, 2013). Viruses can perform both pro- and anti- apoptotic functions to facilitate different stages of infections (Galluzzi et al., 2008). In general, virus has the ability to avoid apoptosis of infected cells, so that they can prolong the host cells viability until the progeny viruses are successfully produced (Koyama et al., 2003; Koyama et al., 2000)., Some viruses induce apoptosis in order to facilitate the spread of progeny virus to neighboring cells at the late stage of infection (Best, 2008). To date, several proteins that involved in apoptosis either in host and WSSV are characterized. Suppression of *Pj*Caspase prior to WSSV infection led to an inhibition of apoptosis and increase in the WSSV copy number in *M. japonicus* (Wang et al., 2008). Moreover, VP41B and VP38 of WSSV bind to *Pj*Caspase promoter and activate or repress the *Pj*Caspase promoter activity, respectively (Zuo et al., 2011). Suppression of caspases from *L. vannamei* showed increasing of WSSV replication (Wang et al., 2013). The overexpression of recombinant *Pm*Caspase of *P. monodon* in Sf-9 cells caused cells undergo apoptosis (Leu et al., 2008). Viruses can produce their own proteins to regulate apoptosis in host cells upon infection. In WSSV, several proteins involved in apoptosis have been identified. AAP-1 (ORF390 or WSSV449), an anti-apoptosis protein of WSSV, can bind to and cleaved by

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*Pm*Caspase to inhibit *Pm*Caspase activity (Leu, 2008; Leu et al., 2010; Wang et al., 2008). In contrast to *Pm*Caspase, another effector caspase in *P. monodon*, *Pm*Casp, was reported to interact with other WSSV anti-apoptosis protein, WSSV134 and WSSV322 (Lertwimol et al., 2014). Another anti-apoptosis protein, WSSV222 functions as an anti-apoptosis protein in WSSV-infected shrimp via ubiquitin-mediated degradation of the tumor suppressor-like protein (TLS) (He et al., 2009). By preventing apoptosis, WSSV222 thus facilitates the propagation of WSSV. Most recently, two new anti-apoptotic proteins of WSSV, WSSV134 and WSSV322, have been identified (Lertwimol et al., 2014). WSSV-encoded ICP11 showed apoptosis-inducing activity by induced apoptosis when expressed in HeLa cells, and WSSV presumably benefits from preventing apoptosis induced by ICP11 (Wang et al., 2008; Leu et al., 2013). In addition, WSSV-miR-N24 can regulate apoptosis by inhibit apoptosis through down regulation of caspase-8 expression and promote viral replication (Huang, 2014).

Previously, it has shown that *Pm*HHAP was dramatically up-regulated upon WSSV infection and might be involved in shrimp immune response against viral infection (Prapavorarat et al., 2010). To gain more insight to the role of *Pm*HHAP responses after WSSV challenge, yeast two-hybrid and co-immunoprecipitation analysis were chosen to identify the interacting proteins between *Pm*HHAP and WSSV proteins. The results reveal that *Pm*HHAP can interact to WSSV134, WSSV362 and WSSV395. From the previous reports, WSSV134 (VP36A), WSSV362 (VP39A) and WSSV395 (VP39B) are structural proteins which WSSV134 and WSSV362 were identified as tegument proteins, while WSSV395 as an envelope protein (Li et al., 2007; Sangsuriya et al., 2014; Tsai et al., 2004; Tsai et al., 2006). Previously, WSSV134 was also identified as a novel anti-apoptosis protein in WSSV. This protein can bind to p20 domain of *Pm*Casp protein and reduces apoptosis induced by *Pm*Casp in Sf-9 cells (Lertwimol et al., 2014). Furthermore, yeast two-hybrid screening showed that, WSSV134 and WSSV395 can interact with WSSV517 but this interaction and their functions are not extensively

investigated (Sangsuriya et al., 2014). Recently, the recombinant WSSV134 has been shown to be able to inhibit *Pm*Casp in a dose-dependent manner. Site-directed mutagenesis of three potential caspase binding sites of WSSV134 revealed that residue D104A is important for inhibit *Pm*Casp activity (Bowornsakulwong, 2017). Unfortunately, the information of WSSV362 is still undiscovered.

According to the microarray analysis, transcripts of WSSV134, WSSV362 and WSSV395 are expressed in the late phase of infection (Tsai et al., 2004), which was similar to what we found in this present study. Semi-quantitative RT-PCR showed that transcription level of WSSV134, WSSV362 and WSSV395 were detected at late stage of infection, suggested that these three genes are late genes of WSSV. *Pm*HHAP transcript was also found to be highly up-regulated in the late phase of WSSV infection (48- 72 hpi) (Prapavorarat et al., 2010). It has been shown that after WSSV infection in *P. monodon*, the total hemocyte was dramatically decreased whereas the high level of apoptosis in hemocytes was dramatically increased as time passes (Wongprasert et al., 2003). Therefore, it might be possible that *Pm*HHAP responses during infection to maintain the hemocytes level in the circulation by preventing too rapid hemocytes apoptosis and help shrimp survive, and is also a way to prevent virus trigger apoptosis to release the viral progeny. In the case of WSSV134, this gene was also up-regulated in the late stage of WSSV infection and we found that WSSV134 can interact with *Pm*HHAP. Thus, it might be possible that WSSV134 binds to *Pm*HHAP to regulate host cells apoptosis so that virus can release to infect other cells.

Suppression of WSSV134 affected the transcription level of genes involved in viral replication including VP28, ie-1 and wsv477, indicating the importance of WSSV134 in viral propagation mechanism. Moreover, co-silencing of *Pm*HHAP and WSSV134 genes prior WSSV infection also showed a decreasing of VP28 transcripts, whilst knockdown only *Pm*HHAP gene did not affect to the mRNA expression of VP28. This results indicated that *Pm*HHAP is not directly involved in viral propagation. The finding

that *Pm*HHAP can interact with both WSSV134 and *Pm*Casp in the late phase of infection, implied that *Pm*HHAP might regulate apoptosis which induced by *Pm*casp to maintain hemocyte homeostasis of WSSV-infected shrimp in the presence of WSSV134. While WSSV134 was induced in late phase of infection to suppress this action in order to help virus release the viral progeny.

To better understand the function of *Pm*HHAP in innate immunity of shrimp, we identified which molecules are affected when *Pm*HHAP was knockdown. Suppression subtractive hybridization, a powerful approach for identifying differentially expressed genes, revealed several *Pm*HHAP-responsive genes including., the transcription factor ATF-β, cathepsin L, lactate dehydrogenase, ferritin and inhibitor of apoptosis (IAP).The transcription factor ATF-β is a member of activating transcription factor/cyclic adenosine 3', 5'-monophosphate response element binding protein (ATF/CREB) family which involved in viral infection (Gilchrist et al., 1996; Rosenberger et al., 2008). In *L. vannamei*, *Lv*ATFβ was reported to be highly up-regulated after WSSV infection and is able to promote expression of wsv059 and wsv166 genes of WSSV, suggested that *Lv*ATFβ is involved in WSSV replication by regulating the expression of wsv059 and wsv166 (Li et al., 2014). Cathepsin L is a member of cysteine proteases (Knop et al., 1993). In human, cathepsin L promotes apoptosis and participates in protein turnover (Levicar et al., 2003). In crustacean, cathepsin L was first characterized in *L. vannamei* (Boulay et al., 1998) then subsequently found in *P. monodon* (Lehnert, 2002). In *M. rosenbergii*, *Mr*CathL was up-regulated upon WSSV and *V. harveyi* challenge (Arockiaraj et al., 2013). However, the function of crustacean cathepsin L remains unclear. Lactate dehydrogenase (LDH) is a key enzyme of the anaerobic metabolism described in most organisms which produces lactate from pyruvate in the last step of glycolysis (Racotta, 2002). The invertebrate LDH is not yet well characterized. Two subunits of LDH have been identified in *L. vannamei*, LDHvan-1 and LDHvan-2 but the function of shrimp LDH is still infancy (Organis et al., 2012).

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Ferritin is an important storage protein, plays a key role in iron metabolism, detoxication and also involved in immunity processes (Connolly and Guerinot, 2002; Rocha et al., 1992). In shrimp, ferritin plays a role in immune system. *Fc*Fer was shown to respond to WSSV or metal ions challenge in *F. chinensis* (Zhang et al., 2006). In *L. vannamei*, ferritin administration caused shrimp susceptibility to WSSV (Hsieh et al., 2006; Ruan et al., 2010). Moreover, knockdown of ferritin increases susceptibility to WSSV, suggested that ferritin protected shrimp from WSSV by inhibits viral replication in *L. vannamei* (Ye et al., 2015). Ferritin was also discovered in *M. rosenbergii* (Qiu et al., 2008) and *M. japonicas* (Feng et al., 2014). The recombinant ferritin from *P. monodon* can reduce the mortality in shrimp infected with *V. harveyi* (Maiti et al., 2010). Lastly, the inhibitor of apoptosis protein or IAP which reported to be an essential molecule in apoptosis pathway. IAPs also plays a role in immune responses against microbial infection in mammals and *Drosophila* (Srinivasula and Ashwell, 2008). In *Drosophila*, silencing of *diap2* resulted in unsuccessful induction of the antimicrobial peptide production and are highly susceptible to infection by Gram-negative bacteria (Huh et al., 2007; Leulier et al., 2006). In *P. monodon*, transcription levels of *Pm*IAP remain unchanged upon WSSV infection (Leu et al., 2008). Suppression of *Pm*HHAP stimulates the transcription of the immune-related genes as well as homeostasis-related genes, some of which previously described to be involved in WSSV infection implying the crucial function of *Pm*HHAP during the viral infection. Some of these genes should be selected for further study in order to understand their immune function which might be related to *Pm*HHAP.

HHAP has also been identified in the freshwater crayfish *P. leniusculus* (Prapavorarat et al., 2010). The *Pm*HHAP and *Pl*HHAP proteins share 47% identity and have eight conserved cysteine residues. However, the function of *Pl*HHAP is contradictory from *Pm*HHAP in shrimp. While the *Pm*HHAP transcript is highly up-regulated, the *Pl*HHAP transcript levels did not significantly change in response to WSSV

infection. On the contrary, the significant increase of *Pl*HHAP mRNA expression in hemocytes upon bacterial *A. hydrophila* B1 was detected. The high up-regulation level of hemocytic *Pl*HHAP may suggest that *Pl*HHAP is involved in crayfish immune response against bacterial infection. On the other hand, *Pm*HHAP was down-regulated after *V. harveyi* infection. The role of *Pl*HHAP in bacterial response was further confirmed by determination of bacterial number in the intestine of *Pl*HHAP knockdown shrimp which showed a higher amount of bacterial than the control GFP injected shrimp. On the other hand, suppression of *Pm*HHAP did not affect to the number of bacterial in shrimp intestine which in contrast to that found in crayfish. These results indicated that HHAP in shrimp and crayfish have different functions in defense against different pathogens.

Moreover, *Pl*HHAP did not exhibit a role in hemocyte homeostasis. Suppression of *Pl*HHAP gene had no significant effect on the transcription of the hematopoiesis-related genes including CHF, astakine1, astakine2 and IGFBP7 in both *in vitro* and *in vivo* experiment, suggesting that *Pl*HHAP might not be involved in crayfish hematopoiesis. Accordingly, the total hemocyte numbers and caspase activity in hemocytes of *Pl*HHAP-silenced shrimp showed no significant difference to the control GFP-injected shrimp which is in contrast to *Pm*HHAP that affect dramatically to the loss of circulating hemocytes. Moreover, knockdown of *Pl*HHAP was not alter the caspase activity in crayfish hemocyte, while knockdown of *Pm*HHAP caused hemocyte undergo apoptosis. Taken together, these results confirmed that *Pl*HHAP does not display similar function in hemocyte homeostasis and is not involved in apoptosis in crayfish.

In summary, the function of *Pm*HHAP in regulation of hemocyte homeostasis and hemocytes apoptosis in shrimp was demonstrated. The possible interaction of *Pm*HHAP with WSSV134 is likely affect apoptosis in shrimp hemocytes via caspase inhibition (Figure 4.1). On the other hand, HHAP in crayfish possibly function in defense against bacterial infection. Our study provides novel insights to anti-apoptosis in shrimp

during WSSV infection which occurred via the host and virus interaction and also established a novel role of HHAP-like protein in crayfish.

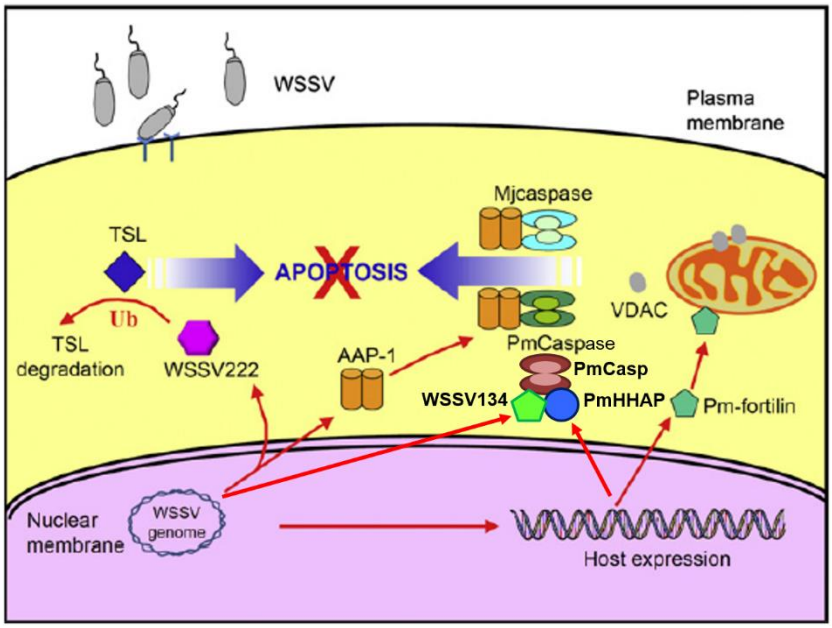

Figure 4.1 A schematic model of the apoptotic interactions between shrimp and WSSV. (Modified from Leu et al., 2013). During WSSV infection, both shrimp and virus produce the protein involved in apoptosis for its own benefit to either host survival or viral replication. Our study demonstrated that *Pm*HHAP is induced after viral infection and subsequently inhibits *Pm*Casp to prevent cell over apoptosis in order to protect cell death in shrimp. On the other hand, virus also produces WSSV134 to interact with this complex and regulate cell apoptosis.

CHAPTER 5

CONCLUSIONS

1. Silencing of *Pm*HHAP resulted in an increase of the number of annexin-V positive apoptotic cells and the combined caspase 3/7 activity and induced the characteristic of apoptotic DNA ladder in shrimp hemocytes. Moreover, suppression of *Pm*HHAP could altered the mRNA expression levels of apoptotic-related genes in hemocytes.

2. Yeast two-hybrid screening and Co-IP identified *Pm*Casp as the interacting protein of *Pm*HHAP. The recombinant *Pm*HHAP protein was able to reduce the caspase activity in the actinomycin D-treated hemocytes and r*Pm*Casp-treated hemocyte cells.

3. Yeast two-hybrid screening and Co-IP found the viral protein, WSSV134 (VP36A), interacted with *Pm*HHAP. WSSV134 was initially detected at 24 hpi in WSSV-infected hemocytes. *In vivo* gene silencing of WSSV134 affected the transcripts of genes involved in viral replication. In addition, co-silencing of *Pm*HHAP and WSSV134 prior to WSSV infection resulted in an increase of caspase activity in hemocytes more than when shrimp were knockdown with only *Pm*HHAP or WSSV134.

4. Suppression subtractive hybridization were successful to identify genes up-regulated after suppression of *Pm*HHAP in shrimp hemocytes, and the indeed up-regulations of selected genes were validated by qRT-PCR.

5. *Pl*HHAP was transcribed in various tissues, but principally expressed in intestine and gill. *Pl*HHAP responded to bacterial infection but not viral infection. Moreover, *Pl*HHAP

is not involved in hemocyte homeostasis but exhibited the function in control the number of bacteria in crayfish intestine.

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APPENDIX

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VITA

Ms. Kantamas Apitanyasai was born in January 12, 1989 at Bangkok, Thailand. She graduated with the degree of Bachelor of Science (B.Sc.) in the major of Biochemistry (Second class Honor) from the department of Biochemistry, Faculty of Science, Chulalongkorn University in 2009. Then, she started a Doctor of Philosophy degree (Ph.D.) of Science in the major of Biochemistry and Molecular Biology at Chulalongkorn University, Thailand. She got the scholarship from Royal Golden Jubilee Ph.D. Program Scholarship from Thailand research fund, and also the 90th Anniversary of Chulalongkorn University Fund. She had one publication in the Development & Comparative Immunology and one publication in the Fish & Shellfish Immunology.

Publications

1. Apitanyasai, K., Amparyup, P., Charoensapsri, W., Senapin, S., Tassanakajon, A., 2015. Role of Penaeus monodon hemocyte homeostasis associated protein (PmHHAP) in regulation of caspase-mediated apoptosis. Development and comparative immunology 53, 234-243.

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