FUNCTIONAL CHARACTERIZATION OF INHIBITOR OF KAPPA B KINASE IN BLACK TIGER SHRIMP *Penaeus monodon* ANTIVIRAL SIGNALING PATHWAY

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ลักษณะสมบัติเชิงหน้าที่ของตัวยับยั้งแคปปาบีไคเนสในวิถีส่งสัญญาณต้านไวรัสของกุ้งกุลาดำ *Penaeus monodon*

นายสิทธิพงษ์ ณ นคร

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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Thesis Title	FUNCTIONAL CHARACTERIZATION OF INHIBITOR OF KAPPA B KINASE IN BLACK TIGER SHRIMP *Penaeus monodon* ANTIVIRAL SIGNALING PATHWAY
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สิทธิพงษ์ ณ นคร : ลักษณะสมบัติเชิงหน้าที่ของตัวยับยั้งแคปปาบีไคเนสในวิถีส่งสัญญาณต้านไวรัสของกุ้งกุลาดำ *Penaeus monodon*. (FUNCTIONAL CHARACTERIZATION OF INHIBITOR OF KAPPA B KINASE IN BLACK TIGER SHRIMP *Penaeus monodon* ANTIVIRAL SIGNALING PATHWAY) อ.ที่ปรึกษาหลัก : ศ. ดร.อัญชลี ทัศนาขจร, อ.ที่ปรึกษาร่วม : ดร.ปิติ อ่ำพายัพ

วิถีการส่งสัญญาณ IKK-NF-κB เป็นหนึ่งในกลไกการตอบสนองที่สำคัญในระบบภูมิคุ้มกันโดยมีโปรตีนตัวยับยั้งแคปปาบีไคเนส (IKK) ทำหน้าที่เป็นตัวกลางในการกระตุ้นการส่งสัญญาณ ในงานวิจัยนี้ได้ทำการบ่งชี้บริเวณถอดรหัสของยีน IKK ในกุ้งกุลาดำ *Penaeus monodon* (*Pm*IKK) จำนวน 3 ชนิดคือ *Pm*IKKβ, *Pm*IKKε1 และ *Pm*IKKε2 และพบว่ายีนทั้ง 3 ชนิดมีการแสดงออกในทุกเนื้อเยื่อที่นำมาทดสอบ นอกจากนี้ยังได้ศึกษาบทบาทของ *Pm*IKK ทั้งสามในการตอบสนองเมื่อกุ้งติดเชื้อไวรัสและแบคทีเรีย ซึ่งพบว่ามีเพียง *Pm*IKKε1 และ *Pm*IKKε2 เท่านั้น ที่แสดงออกเพิ่มขึ้นอย่างมีนัยสำคัญในสภาวะที่กุ้งติดเชื้อไวรัสตัวแดงดวงขาว (WSSV), ไวรัสหัวเหลือง (YHV) รวมถึงเชื้อแบคทีเรีย *Vibrio harveyi* แต่ไม่พบความเปลี่ยนแปลงของยีน *Pm*IKKβ ต่อการติดเชื้อไวรัสหรือแบคทีเรียที่ทดสอบ เมื่อทำการยับยั้งการแสดงออกของยีน *Pm*IKKβ และ *Pm*IKKε โดยเทคนิค dsRNA-mediated RNA interference (RNAi) พบว่าส่งผลให้กุ้งที่ติดเชื้อมีอัตราการรอดลดลงเมื่อเทียบกับกุ้งกลุ่มควบคุม และยังส่งผลลดระดับการแสดงออกของยีน *Pm*Vago4 ซึ่งมีบทบาทคล้าย interferon (IFN-like) ในสัตว์มีกระดูกสันหลัง ขณะเดียวกันพบว่ายีนในระบบภูมิคุ้มกันบางชนิดมีการแสดงออกเพิ่มขึ้นเช่น เปปไทด์ต้านจุลชีพ ALF*Pm*3 และ Crustin*Pm*5 รวมถึง transcription factor *Pm*Dorsal และบางชนิดที่ไม่เปลี่ยนแปลงเช่น ALF*Pm6*, Crustin*Pm1*, Crustin*Pm7*, *Pm*Vago1, *Pm*Relish และ *Pm*Cactus เป็นต้น เมื่อยับยั้งการแสดงออกของยีน *Pm*MyD88 และ *Pm*IMD ซึ่งเป็นยีนที่สำคัญในวิถีการส่งสัญญาณ Toll และ IMD พบว่ายีน*Pm*IKKβ และ *Pm*IKKε ไม่ได้รับผลกระทบและไม่เกี่ยวข้องกับวิถีการส่งสัญญาณทั้งสอง นอกจากนี้การแสดงออกของยีน *Pm*IKKβ และ *Pm*IKKε ในเซลล์ HEK293T ยังส่งผลกระตุ้นให้ promoter ของยีน NF-κB และ IFNβ ทำงานมากขึ้นตามลำดับอีกด้วย ผลการทดลองดังกล่าวบ่งชี้ว่า *Pm*IKKβ และ *Pm*IKKε อาจมีบทบาทสำคัญโดยเป็นหนึ่งในหลายตัวกลางเพื่อส่งผ่านสัญญาณจากหลายวิถีที่เกิดขึ้น ดังนั้น *Pm*IKKβ และ *Pm*IKKε อาจมีบทบาทเกี่ยวข้องกับระบบ cytokine โดยกระตุ้น *Pm*Vago4 ในระบบภูมิคุ้มกันโดยกำเนิด (innate immune system) ของกุ้งกุลาดำในการตอบสนองต่อเชื้อโรค

สาขาวิชา	ชีวเคมีและชีววิทยาโมเลกุล	ลายมือชื่อนิสิต .....
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Zittipong Nhnhkorn : FUNCTIONAL CHARACTERIZATION OF INHIBITOR OF KAPPA B KINASE IN BLACK TIGER SHRIMP *Penaeus monodon* ANTIVIRAL SIGNALING PATHWAY. Advisor: Prof. Anchalee Tassanakajon, Ph.D. Co-advisor: Dr. Piti Amparyup

The IKK-NF-κB signaling cascade is one of the crucial responsive mechanisms in inflammatory and immune responses. The key kinase proteins called inhibitor of kappa B kinases (IKKs) serve as the core elements involved in cascade activation. Here, the complete open reading frames of IKK homologs including *Pm*IKKβ, *Pm*IKKε1 and *Pm*IKKε2, from the black tiger shrimp *Penaeus monodon* were identified and characterized for their functions in shrimp antiviral responses. The *Pm*IKK transcripts were widely expressed in various examined tissues and the *Pm*IKKε protein was detected in all three types of shrimp hemocytes. Only the *Pm*IKKε1 and *Pm*IKKε2 were responsive to white spot syndrome virus (WSSV), yellow head virus (YHV) and a bacterium *Vibrio harveyi* infection, while the *Pm*IKKβ exhibited no significant response to pathogen infection. On the contrary, suppression of *Pm*IKKβ and *Pm*IKKε by dsRNA-mediated RNA interference (RNAi) resulted in a rapid death of WSSV-infected shrimp and the significant reduction of an IFN-like *Pm*Vago4 transcript. Whereas the mRNA levels of the antimicrobial peptides, *ALFPm3* and *CrustinPm5*, and a transcription factor, *PmDorsal* were significantly increased, those of *ALFPm6*, *CrustinPm1*, *CrustinPm7*, *PmVago1*, *PmRelish* and *PmCactus* were unaffected. Suppression of *Pm*MyD88 and *Pm*IMD which disrupt Toll and IMD signaling pathways showed no consequent effect on *Pm*IKKβ and *Pm*IKKε transcript levels. Overexpression of *Pm*IKKβ and *Pm*IKKε in HEK293T cells differentially activated the NF-κB and IFNβ promoter activities, respectively. These results suggest that the *Pm*IKKβ and *Pm*IKKε may act as the common factors regulating the expression of immune-related genes from various signaling pathways. Interestingly, the *Pm*IKKs may also contribute a possible role in shrimp cytokine-like system and cross-talking between signaling transductions in innate immune responses.

Field of Study:	Biochemistry and Molecular Biology	Student's Signature .....
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Zittipong Nhnhkorn

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℃	degree Celsius
bp	base pairs
BSA	bovine serum albumin
cDNA	complementary deoxyribonucleic acid
CFU	colony forming unit
dsRNA	double stranded ribonucleic acid
EF1-α	elongation factor 1 alpha
EMS	early mortality syndrome
GFP	green fluorescence protein
h	hour
hpi	hours post infecdtion
HEK293T	human embryonic kidney 293 T
IFNβ	interferon beta
IPTG	isopropyl-beta-D-thiogalactopyranoside
kb	kilobase
kDa	kiloDalton
LB	Luria-Bertani
Lv	*Litopenaeus vannamei*

μM	micromolar
μg	microgram
μl	microlitre
M	molar
mg	milligram
min	minute
ml	mililitre
O.D.	optical density
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PEI	polyethyleneimine
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
Pm	*Penaeus monodon*
r	recombinant
RACE	rapid amplification of cDNA ends
RNAi	ribonucleic acid interference
RT-PCR	reverse transcription/polymerase chain reaction
SD	standard deviation

SDS	sodium dodecyl sulfate
s	second
WSSV	white spot syndrome virus
YHV	yellow head virus

CHAPTER I

INTRODUCTION

1.1 Shrimp aquaculture

Shrimp aquaculture has been expanded rapidly in decades along with the increasing economic activities in several world countries. Many factors responsible for rapid expansion are mainly involved with the high profits and high demand in international markets for the global consumption. Shrimp farming production is mainly taken place in Asia like China, Thailand, Malaysia, Vietnam and Indonesia leading world production up to 3,118,971 tons in 2012 (Chowdhury, 2013). Among several fishery products, farmed penaeid shrimp such as *Penaeus monodon* or *Litopenaeus vannamei* have been the most products traded internationally and about 25% or 700,000 metric tons came from aquaculture in 1996 (Briggs *et al.*, 2004). In Southeast Asia, Thailand was one of the largest exporters for farmed shrimp and prawns. The contribution of farmed shrimp is more than 50% to the world total shrimp supply and is growing up in the major international markets including the USA, Japan and the European Union (EU) (Dierberg and Kiattisimkul, 1996). As local economies grow and consumers demand more seafood, Asian fishery markets such as in China, Korea, Thailand or Malaysia will expand. Shrimp production grew steadily in east Asia, averaging 6 percent annual growth from 2008 through 2011 (FAO, 2012). In 2010, the world top ten shrimp producers were China (26.7%) followed by Thailand (18.2%), Vietnam (15.3%), Indonesia (12.2%), Ecuador (7.2%), India (3.4%), Mexico (3.4%), Malaysia (2.8%), Brazil (1.2%) and Philippines (1.8%) (FAO, 2012) (**Figure 1.1**).

(2001-2010), production=x1000tons

Countries	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010
1 China	267	337	432	468	546	640	710	725	796	833
2 Thailand	280	265	331	360	401	494	523	507	575	567
3 Viet Nam	150	181	232	276	327	349	377	381	411	479
4 Indonesia	149	160	191	239	280	340	330	408	337	379
5 Ecuador	45	64	77	90	119	149	150	150	179	223
6 India	103	115	113	118	131	132	99	80	97	106
7 Mexico	48	46	46	62	90	112	112	130	126	105
8 Malaysia	27	26	26	31	33	35	35	51	69	87
9 Brazil	40	60	90	76	63	65	65	70	65	69
10 Philippines	42	37	37	38	40	41	43	50	54	56
Total	1152	1290	1575	1757	2030	2357	2444	2554	2710	2904
The World	1311	1466	1791	1998	2304	2654	2726	2847	2934	3119

Figure 1.1 Shrimp aquaculture production trends from world top ten producers in the last decade. (Sources: FAO 2012)

Since the rapid expanding of shrimp aquaculture in the last few decades, the sustainable for shrimp farming has been concerned. Because shrimp farming is threatened by several disease problems, shrimp production has declined and questions have been raised for quality of shrimp in the market. Following disease outbreaks, the global shrimp production was decreased and as many affected countries, Thailand moved from the second to the fifth place of the world exporters (**Figure 1.2**). The outbreaks of early mortality syndrome (EMS) or officially named acute hepatopancreatic necrosis disease (AHPND) caused huge economic losses throughout China and expanded to Vietnam, Malaysia and reached Thailand in 2012 (Tran *et al.*, 2013). In Thailand, disease outbreaks have resulted in huge national income losses up to billions of dollars annually and at least USD 20 billion globally (Barbier and Sathirathai, 2004). Nowadays, several infectious diseases cause shrimp aquaculture unsustainable and become a big concern for most of shrimp producing countries.

Figure 1.2 Shrimp aquaculture production in major Asian farming nations.
Sources: FAO (2011-2014) and GOAL Surveys (2012-2018)

1.2 Black tiger shrimp *Penaeus monodon* and taxonomy

Black tiger shrimp *Penaeus monodon* is an economically important species in tropical countries including Thailand. It was found originally in the Indian Ocean and western Pacific (Indo-West Pacific) and was distributed from Africa to Australia, Japan, and Pakistan (Dore and Frimodt, 1987). *P. monodon* is found at depths from 0 to 110 meter in bottom mud and sand. In its natural habitat, *P. monodon* live in water with salinities of 5-45 ppt and temperatures of 18-34.5°C (Branford, 1981) and also cultured commercially at salinities of 1-5 ppt (Musig *et al.*, 1998). Adult *P. monodon* live in offshore waters on sandy bottom at depths of 20-40 meter. The larvae move towards the coast, entering estuaries and mangrove swamps as nursery grounds. They migrate to the deeper sea level when becoming adolescent to mature and mate. Marine shrimp are omnivorous scavengers, as they grow, their feed are including polychaetes, nematodes, algae, animal tissues and vegetable matters (Briggs *et al.*, 2004). In the most environmentally responsible operations, their life cycle is raised initially from eggs hatch and grown to adult shrimp. *P. monodon* has six nonfeeding naupliar stages, three protozoeal stages and three mysis stages (Abubakr and Jones, 1992) (**Figure 1.3**).

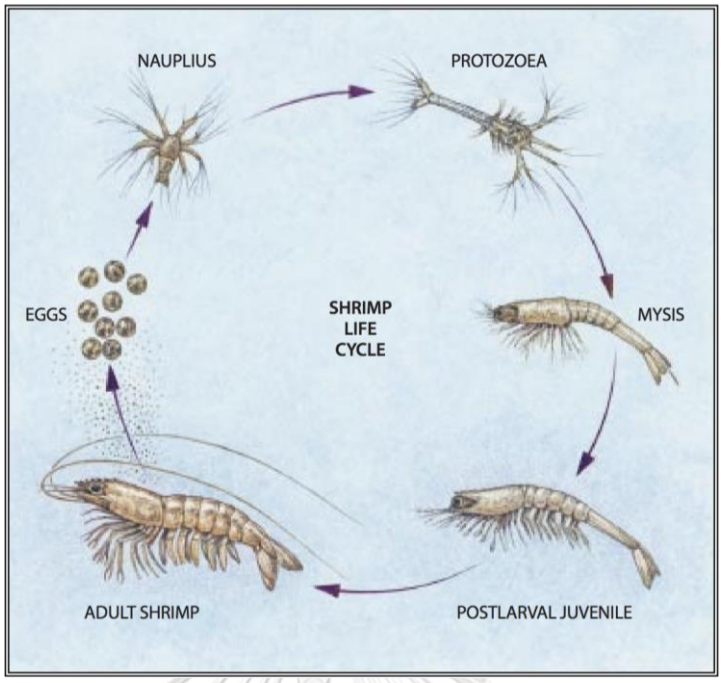

Figure 1.3 Shrimp life cycle. After shrimp eggs are fertilized and hatched, the resulting nauplius larvae are released into the water. They grow to the second major stage, protozoea larvae where several shrimp-like features are developed. At mysis and postlarval stages, they are carried shoreward by wind-driven currents and settle to feed until adulthood (Source: FAO 1998).

The appeared anatomy of a black tiger shrimp is covered with a protective exoskeleton shell made of cuticle with jointed appendages. Their external morphology is generally dark colored with transversely black and white bands on the carapace and abdomen like tiger traits. The rest body color is variable ranging from light brown to blue or red, some smaller specimens show a dull red dorsal strip from rostrum to abdominal segment regions (Grey *et al.*, 1983). The head part is called thorax linked to six segments of abdomen holding a pair of fins on the ventral sides for forward swimming called pleopods. The last abdominal segment is telson which allows shrimp to jump backwards in an escape reflex. The spine at the thorax is called rostrum holding a pair of eyes, two pairs of antennae, five pairs of walking legs and three pairs of maxillipeds for feeding (Motoh, 1985; Braak, 2002; Freitas *et al.*, 2007) (**Figure 1.4**).

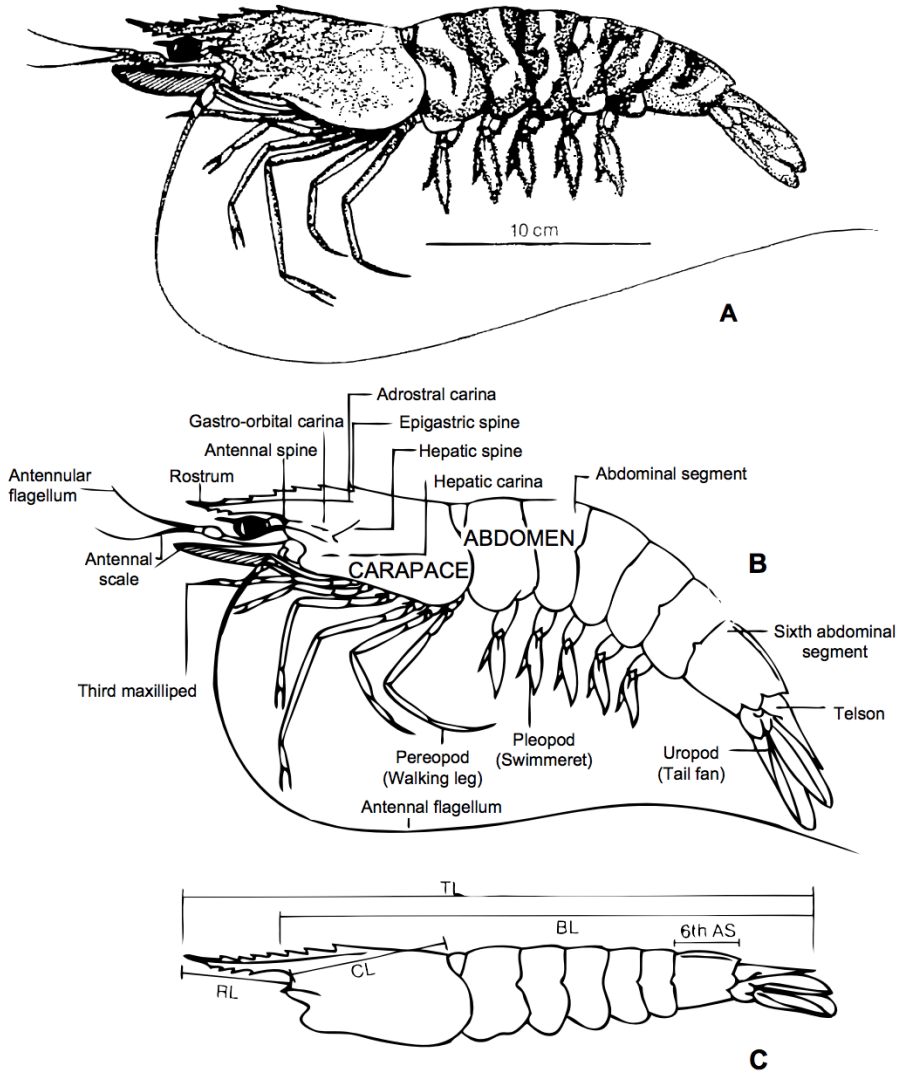

Figure 1.4 Schematic drawing of the external anatomy of a black tiger shrimp *Penaeus monodon*. (**A**) adult *P. monodon* (**B**) External anatomical features of *P. monodon* (**C**) Approximated rational lengths of *P. monodon* body segments (TL: total length, RL: rostrum length, CL: carapace length, BL: body length, 6th AS: length of 6th abdominal segment) (Motoh, 1985).

The taxonomic features of *P. monodon* are classified as below (Solis, 1988)

Kingdom: Animalia
Phylum: Arthropoda
Subphylum: Crustacea
Class: Malacostraca
Subclass: Eumalacostraca
Order: Decapoda
Suborder: Natantia
Superfamily: Penaeoidea
Family: Penaeidae Rafinesque, 1815
Genus: *Panaeus* Fabricius, 1798
Species: *monodon*

Scientific name: *Penaeus* (*Penaeus monodon*) *monodon* Fabricius, 1798.
Common names: 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) (Solis, 1988).

1.3 Shrimp pathogens and diseases

Infectious diseases from several pathogens including bacteria, viruses and fungi are the big concerns in shrimp aquaculture for all farming areas. These diseases have caused serious difficulties in China, Taiwan, Thailand and other shrimp farming countries (Flegel, 2012). The pathogens travel through countries even in hemisphere, shipments of infected hatchery-produced shrimp or processed frozen shrimp (Lightner, 1999). The spreading of causative pathogens is one of the main factors affecting shrimp production to decrease in many countries (**Figure 1.5**).

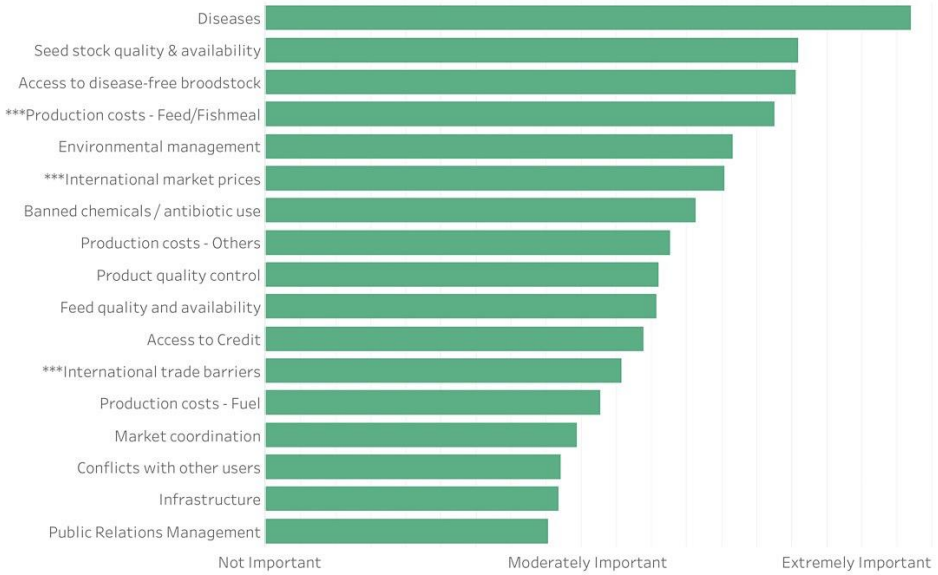

Figure 1.5 Factors affecting shrimp aquaculture in all countries. The survey results of different factors affecting shrimp industry in 2017 that showed the problem with diseases has become the top-challenge among various challenges (Source: GOAL 2017).

1.3.1 Viral diseases

1.3.1.1 White spot syndrome

White spot syndrome remains one of the most serious viral infections in a wide range of crustacean hosts caused by white spot syndrome virus. The disease was first reported in the 1990s and has been a major impact in economic and production losses for shrimp aquaculture sector (Verbruggen *et al.*, 2016). White spot syndrome is highly lethal as the viral outbreaks have wiped out many shrimp farms entirely within a few days (Karunasagar and Ababouch, 2012). The clinical signs of WSSV-infected shrimp are reduced food consumption, loosening of the cuticle and discoloration of hepatopancreas (Pradeep *et al.*, 2012). Moreover, white calcified spots appearing on shrimp carapace exoskeleton are diagnostic WSSV infection **(Figure 1.6)** but not in all host species (Chou *et al.*, 1995; Rajan *et al.*, 2000). The

disease causes a rapid mortality up to 100% after 3-10 days of the infection (Zhan *et al.*, 1998; Wang *et al.*, 1999).

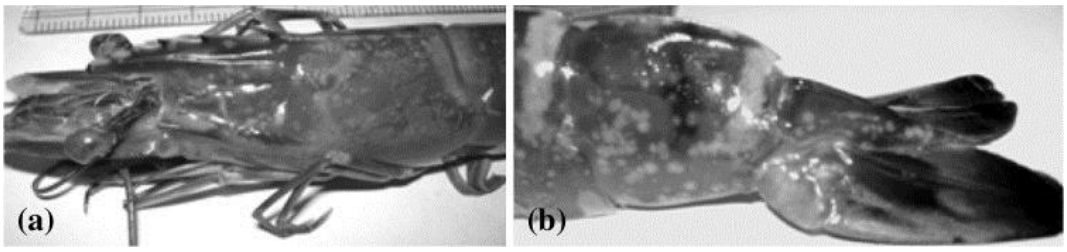

Figure 1.6 White calcified spots as a clinical sign of WSSV infection. White spot syndrome virus infected *Penaeus monodon* showing white spot symptoms on (**a**) carapace and (**b**) last abdominal segment (Pradeep *et al.*, 2012).

White spot syndrome virus (WSSV) is a double-stranded DNA (dsDNA) virus of approximately 300 kilobase pairs (Nadala and Loh, 1998; Pradeep *et al.*, 2012). The virus belongs to the member of the *Nimaviridae* family (Haq *et al.*, 2012). Morphologically, the virion consists of an enveloped rod-shaped nucleocapsid with a single tail-like filamentous (Wang *et al.*, 1995; Durand *et al.*, 1997). The average size of the WSSV particle from transmission electron micrograph is 80×350 nm with nucleocapsid double-layered envelope (Huang *et al.*, 2001). The virion nucleocapsid core is assembled with two parallel striations consisting of 14 globular capsomeres. (Huang *et al.*, 2001; Lightner, 2003) (**Figure 1.7**).

WSSV was initially reported as a non-occluded baculovirus but DNA sequence analysis shown that it is not related to the baculoviruses (Hulten *et al.*, 2001; Yang *et al.*, 2001). The viral genome has been differently reported from different isolates: 305,107 bp (GenBank Accession No. AF332093) from China, 292,967 bp (GenBank Accession No. AF369029) from Thailand and 307,287 bp (GenBank Accession No. AF440570) from Taiwan (Lightner, 1999; Lightner, 2003).

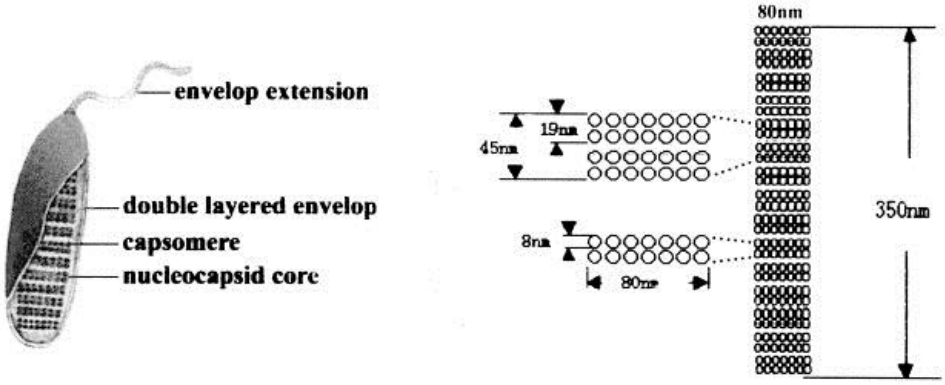

Figure 1.7 Nucleocapsid structure of WSSV virion. As shown in transmission electron micrograph, the average size of WSSV nucleocapsid within the double-layered envelope is 80×350 nm. The nucleocapsid core is a ring-like structure assembled with parallel striations from globular capsomeres (Huang *et al.*, 2001; Lightner, 2003).

WSSV has a broad host range such as shrimp, crayfish, lobsters and crab (Chakraborty *et al.*, 2002). It is highly lethal to most of the commercially important species of penaeid shrimp including *P. monodon* and *P. vannamei* (Håstein and Blancou, 1997). Virus infection occurs in all tissues of ectodermal and mesodermal embryonic origin including gill, lymphoid organ, stomach, cuticular epithelium and subcuticular connective tissues (Lightner, 1996; Wang *et al.*, 1999). The infection occurs in stomach, gills, cuticular epidermis and hepatopancreas in the early stage. In the late stage, lymphoid organ, antennal gland, muscle, hematopoietic tissue, heart and intestine get infected (Chang *et al.*, 1996). At the very late stage of infection, the nervous system is infected and shrimp become necrotic (Lo *et al.*, 1997). However, there is no effective treatment available to limit the occurrence and spread of the disease.

1.3.1.2 Yellow head disease

Yellow head disease or YHD was first identified in 1991 as an outbreaks have been reported from shrimp farming countries in Asia (Limsuwan, 1991; Ahmad, 2016). The disease is caused by a single-stranded RNA (ssRNA) virus called yellow head virus (YHV) which is a member of the genus *Okavirus*, family *Roniviridae* in

the order *Nidovirales* (Senapin *et al.*, 2010; Chen *et al.*, 2018). The virions are enveloped and rod-shaped with 40-60 nm × 150-200 nm in dimensions (Chantanachookin *et al.*, 1993). The envelope surrounds a viral particle by spike-like projections with 7-9 nm and spacing between the projections is 4-7 nm (**Figure 1.8**).

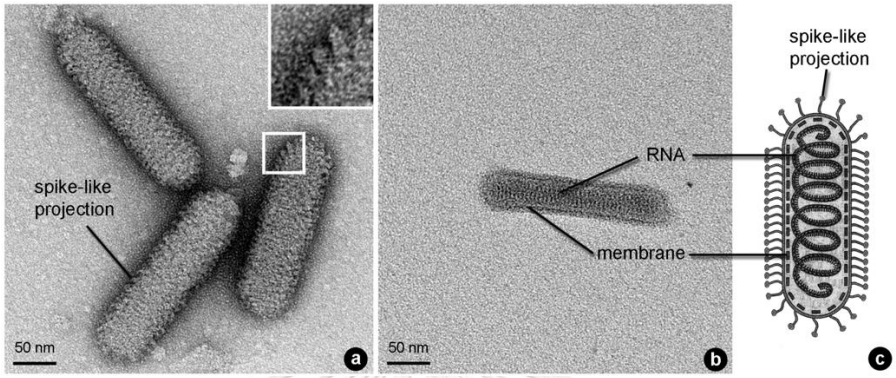

Figure 1.8 Micrographs from TEM and a schematic diagram of intact and nucleocapsid of yellow head virus. (**a**) High magnification micrograph of YHV showing enveloped particles with spike-like projections (**b**) YHV nucleocapsid with rod-shaped helical structure (**c**) illustrated diagram of whole viral particle (Duangsuwan *et al.*, 2011).

Yellow head disease is potentially lethal to most of the commercially cultivated penaeid shrimp species (Ahmad, 2016). The disease outbreaks caused significant economic loss for shrimp industry in Thailand (Chainarong *et al.*, 1997). Shrimp with YHV infection is characterized by a high mortality rate accompanied by yellowing gross signs on carapace and body color bleaching (Lightner, 2003) (**Figure 1.9**). In Thailand, shrimp from 20 farms were observed with faded body color and 60-70% mortality from the end of 2007 through early 2008 (Senapin *et al.*, 2010). The targeted tissues are from ectoderm and mesoderm origins (Kasornchandra *et al.*, 1995). The virus mainly infects lymphoid organ and forms lymphoid spheroids in the chronic infection stage (Soowannayan *et al.*, 2003). The lymphoid organ with chronic infection shows an extensive abnormalities and tubule cell degradation leading shrimp lumen become occluded (Khanobdee *et al.*, 2002; Duangsuwan *et al.*, 2011).

Figure 1.9 Yellow head disease in black tiger shrimp (*Penaeus monodon*). Shrimp with YHV infection exhibit yellowing gross on carapace and body color bleaching (left) compared with Healthy Shrimp (right). (Source: Department of Agriculture, Fisheries and Forestry, Australian Government, Photo: DV Lightner).

1.3.2 Bacterial diseases

1.3.2.1 Vibriosis

Vibriosis has become a serious concern for shrimp bacterial infection because of the association with low survival rates in hatcheries or grow-out ponds in the infected areas. The intensification of penaeid shrimp aquaculture industry has likely been developed simultaneously with the infectious diseases caused by bacterial infection. Several outbreaks caused by bacterial pathogens belonging to *Vibrio* species including *Vibrio alginolyticus, Vibrio parahaemolyticus, Vibrio vulnificus, Vibrio campbellii and Vibrio harveyi* were reported (Wang *et al.*, 2015). Larval mortalities of *P. monodon* and *P. vannamei* are commonly reported with the presence of *V. harveyi* as a causative agent in many countries including Indonesia, Thailand and Taiwan (Saulnier *et al.*, 2000; Wang *et al.*, 2015). *Vibrio harveyi* is a major bacterial pathogen among *Vibrio* species for other aquatic animals including finfish and lobster larvae *Jasus verreauxi* (Diggles *et al.*, 2000; Shivu *et al.*, 2007; Haldar *et al.*, 2010). The bacterium *V. harveyi* is a Gram-negative and rod-shaped (Thompson *et al.*, 2004) with luminous colony by morphological test on agar plate (**Figure 1.10**)

and caused luminous vibriosis in shrimp (Liu *et al.*, 1996; Oakey *et al.*, 2002; Shivu *et al.*, 2007).

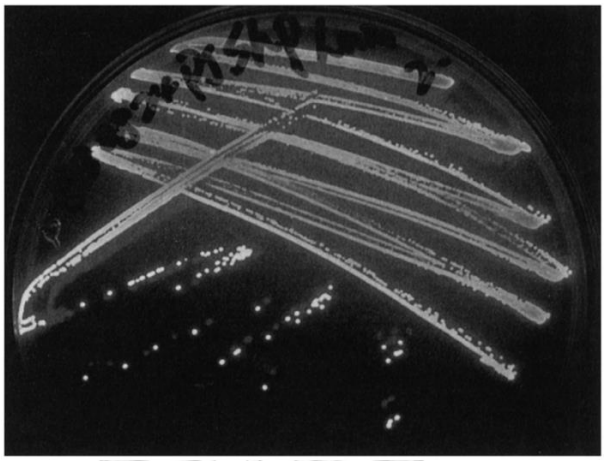

Figure 1.10 Isolated *Vibrio harveyi* showing luminous colonies on tryptic soy agar (Liu *et al.*, 1996).

The pathology is characterized by the degeneration of hepatopancreas tissues forming balls that eventually move to the upper gut (Soto-Rodriguez *et al.*, 2003). Gross examination of shrimp containing vibriosis reveal dark color of hepatopancreas in cephalothorax region with whitish muscle or smoky body coloration (Longyant *et al.*, 2008) (**Figure 1.11**). Scanning electron microscopic shows bacterial colonization specifically on feeding apparatus and oral cavity of shrimp larvae, suggesting an infection initially via an oral route (Aguirre-Guzmán *et al.*, 2010). Furthermore, the pathogenicity occurred in *P. monodon* was observed from infection with both live bacteria and extracellular products including cysteine protease, phospholipase and haemolysin (Soto-Rodriguez *et al.*, 2003; Austin and Zhang, 2006).

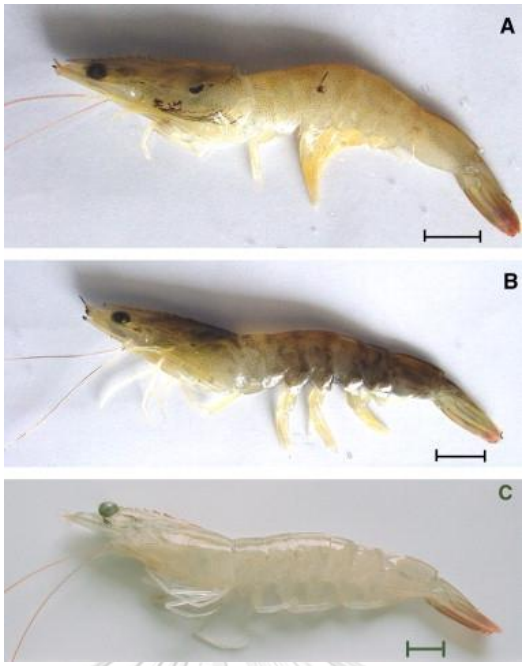

Figure 1.11 Vibriosis in *Penaeus vannamei*. The gross signs of disease demonstrated (**A**) black stripes on lateral cephalothorax regions and with whitish muscle or (**B**) dark color of hepatopancreas with smoky body coloration. (**C**) Uninfected shrimp. (Longyant *et al.*, 2008) (bar=1 cm.)

1.3.2.2 Acute hepatopancreatic necrosis disease (AHPND) or early mortality syndrome (EMS)

A new disease known as acute hepatopancreatic necrosis disease (AHPND) or early mortality syndrome (EMS) appeared in shrimp farms located in China and by early 2011 (Lightner *et al.*, 2012) the disease was reported subsequently in Vietnam, Malaysia and Thailand (Zorriehzahra and Banaederakhshan, 2015). Thai Department of Fisheries (DOF) reported the effect of disease outbreaks on the reduction of total shrimp production from 94,400 tons in 2012 to 63,500 tons in 2013, indicating a production decline of approximately 30,900 tons (FAO, 2011). Typically, postlarvae stages of both *P. monodon*, and *L. vannamei* are affected by EMS/AHPND. The disease causes up to 100% mortality within 20-30 days after stocking (De Schryver *et al.*, 2014).

A bacterium *Vibrio parahaemolyticus* from *Vibrio* species is specifically reported as a causative agent for EMS/AHPND (Zorriehzahra and Banaederakhshan, 2015). The obvious signs of pathology are observed in hepatopancreases with a reduction of oil/fat storage and the decreased activity of secretory cells (Lightner *et al.*, 2012). The hardening of hepatopancreas, reduction in dark spots and size often appear together with whitish due to pigment loss in the connective tissues (Soto-Rodriguez *et al.*, 2015) (**Figure 1.12**). The pathological virulence is the effects of bacterial toxins PirA and PirB which were first described as the cause of acute hepatopancreatic necrosis disease in Chinese cultivated shrimp (Wangman *et al.*, 2018).

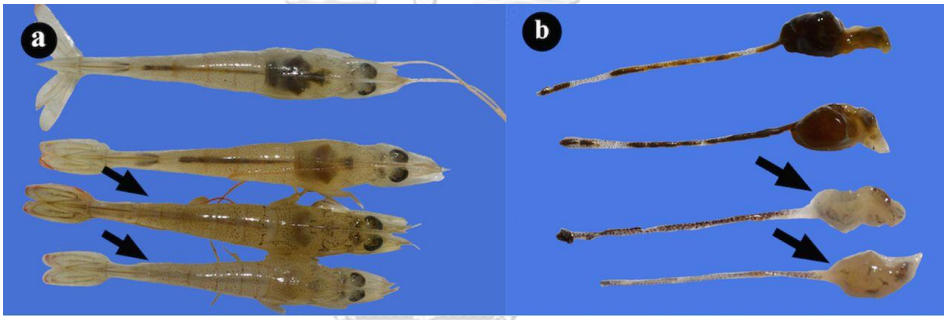

Figure 1.12 Pathogenicity of *Vibrio parahaemolyticus* AHPND infected shrimp. (**a**,**b**) Clinical signs of hepatopancreases in healthy shrimp and shrimp naturally infected with *Vibrio parahaemolyticus* AHPND (arrows). The whitish hepatopancreas are obviously observed in *V. parahaemolyticus* infected shrimp compared to healthy shrimp (Soto-Rodriguez *et al.*, 2015)**.**

1.4 Shrimp innate immune system

Shrimp are classified as crustaceans which lacking an adaptive immune system (Bachère, 2000). Their immune system relies on innate or natural immune responses involving diverse humoral and cellular activities (Li and Xiang, 2013b). Cellular immune reactions include phagocytosis, nodulation and encapsulation, while several immune molecules and their syntheses are involved in humoral responses (Tassanakajon *et al.*, 2013; Wang and Wang, 2013). Innate immune system is the first line of defense responding against large range of pathogens including bacteria, viruses

and fungi (Bachère *et al.*, 2004). The system helps to limit infection by recognizing molecular patterns present on invading microbes (Lee and Söderhäll, 2002). Immune reactions of crustaceans including shrimp, mainly occur in hemolymph, where three different types of hemocytes take place including hyaline, granular and semigranular hemocytes (Martin and Graves, 1985) (**Figure 1.13**).

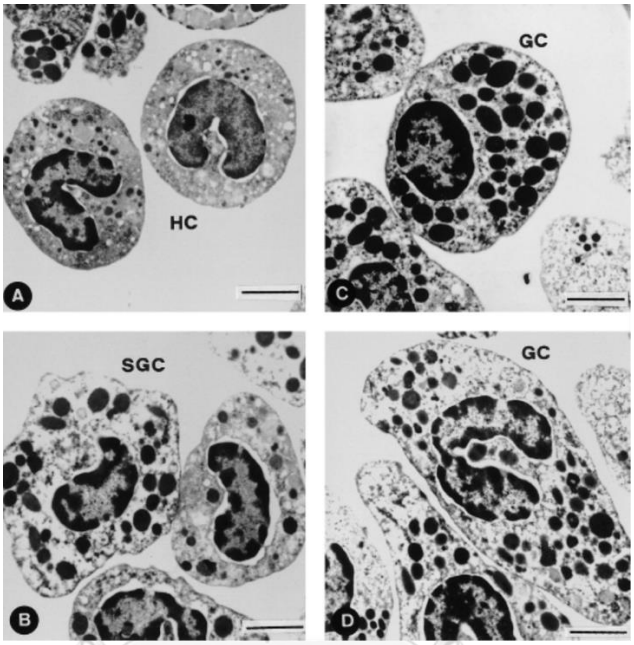

Figure 1.13 Transmission electron micrographs of three different hemocytes from *Penaeus monodon*. (**A**) Hyaline cells (HC) are small round or ovoid hemocytes (4.4–6.9 µm) with a large nucleus surrounded by a thin cytoplasmic layer. (**B**) Semigranular cells (SGC) are irregular in shape (6.2–9.2 µm) and contain spherical or ovoid granules (0.31–0.9 µm). (**C**,**D**) Granular cells (GC), are round (8.3–12 µm) or ovoid (5.8 × 13.4 µm) in shape with relatively small nuclei and contain several big granules (Sung and Sun, 2002). (bar=2.2 µm).

Typically, hyaline cells are involved in phagocytosis and blood clotting (Söderhäll and Smith, 1983; Lin and Söderhäll, 2011), while granular cells are responsible for melanization (Lavine *et al.*, 2002; Amparyup *et al.*, 2013), nodulation, and apoptosis (Kobayashi *et al.*, 1990; Strand, 2008). Moreover, semigranular cells are found to hold the actions of encapsulation, early non self-recognition and coagulation. The proportion of different hemocyte types varies among crustacean

species and is influenced by environmental conditions (Söderhäll *et al.*, 2003). The number of immune molecules are accumulated in secretory granules of hemocytes before being released upon microbial invasion. (Iwanaga, 2002; Tassanakajon *et al.*, 2013). Recently, several immune responses such as prophenoloxidase (proPO) system-mediated melanization, clotting cascade and the activities of immune molecules are reported in shrimp innate immune system (Amparyup *et al.*, 2013; Tassanakajon *et al.*, 2013) (**Figure 1.14**)

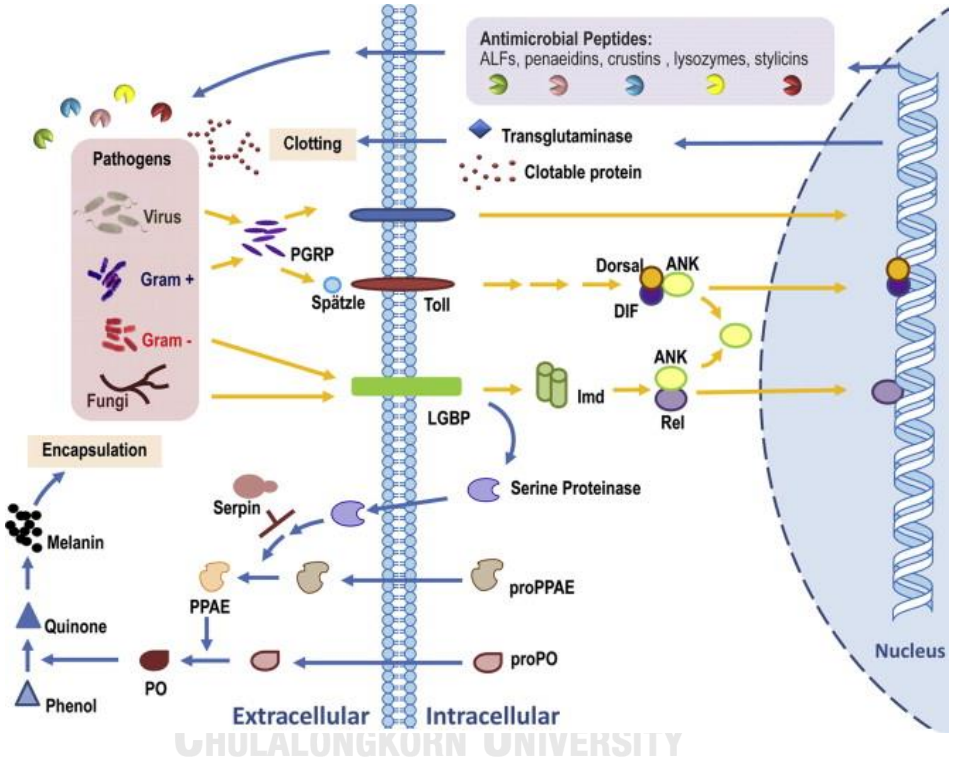

Figure 1.14 A schematic model of shrimp innate immune system. Shrimp immune responses are triggered upon invading pathogens including bacteria, viruses and fungi. The defense mechanisms are initiated after molecular patterns from invading pathogens are recognized by pattern recognition receptors (PRRs) and sending the signal through innate immune signaling pathways such as Toll and IMD pathways to activate the synthesis or release of several immune molecules (Tassanakajon *et al.*, 2013).

1.4.1 Antimicrobial peptides (AMPs)

Shrimp innate immune response also relies on the production of antimicrobial peptides that are released to destroy a broad range of invading microorganisms (Bachère, 2000). Antimicrobial peptides are found widely in bacteria, plants, vertebrates and invertebrates (Rathinakumar *et al.*, 2009). They are generally small in size, less than 150-200 amino acid residues, consisting of an amphipathic structure with cationic or anionic properties (Hancock and Diamond, 2000). AMPs are produced and stored in shrimp hemocytes before being released targeting several pathogens involving viruses, Gram-negative and/or Gram-positive bacteria, fungi, parasites and even cancer cells (Bachère, 2000; Krepstakies *et al.*, 2012).

Several AMPs were identified and characterized in shrimp such as penaeidins, crustins and ALFs (Somboonwiwat *et al.*, 2005; Amparyup *et al.*, 2008; Tassanakajon *et al.*, 2011). Penaeidins are a unique AMP family identified in penaeid shrimp (Tassanakajon, 2013). They are normally characterized as the 5.5-6.6 kDa peptides containing an N-terminal proline-rich domain and six cysteine residues at C-terminus. Moreover, Panaeidins exhibited antimicrobial activity against Gram-positive bacteria and fungi (Amparyup *et al.*, 2008). In *P. monodon*, Penaeidin class 5 (*Penmon*PEN5) was significantly up-regulated at 24 hours post WSSV infection and showed increased susceptibility to viral infection after it was suppressed (Woramongkolchai *et al.*, 2011). Crustins are characterized as a cysteine-rich with approximately 11.5 kDa molecular weight and exhibit antimicrobial activity against Gram-positive bacteria (Relf *et al.*, 1999). They were reported in several crustaceans including *L. vannamei*, *P. monodon*, *M. japonicus*, *F. chinensis* and *P. leniusculus* (Amparyup *et al.*, 2008; Supungul *et al.*, 2008). Crustin*Pm*1 is the most abundant isoform in *P. monodon* that exhibited bactericidal effect in inhibition mechanism of antimicrobial activity (Supungul *et al.*, 2008). Regulatory pathways of crustin*Pm*1 and crustin*Pm*7 were investigated and revealed that crustin*Pm*1 was mediated through Toll pathway, while crustin*Pm*7 was regulated via Toll and IMD pathways (Arayamethakorn *et al.*, 2017). Furthermore, antilipopolysaccharide factors or ALFs have been first described in horseshoe crab *Limulus polyphemus* and later characterized in shrimp (Tassanakajon

et al., 2015). They were identified as the cationic polypeptides of approximately 100 residues with a hydrophobic N-terminal region in shrimp. Investigation of regulatory pathway demonstrated that ALF*Pm*3 in *V. harveyi*-infected *P. monodon* was regulated by Toll and IMD pathways, while the ALF*Pm*6 was regulated by Toll pathway (Kamsaeng *et al.*, 2017). These peptides are produced with a broad-spectrum activities including antibacterial, antifungal, antiviral and antiprotozoal properties to modulate the inflammatory process for innate defenses (Hancock and Diamond, 2000).

1.4.2 Pattern recognition receptors (PRRs) and signal transduction

Signal transduction following microbial infection involves the binding of extracellular microbial components to the receptors that generate intracellular events (Li and Xiang, 2013b). In the process, the recognition of pathogen-associated molecular patterns (PAMPs) by host pattern recognition receptors (PRRs) is the first step of innate immunity. PRPs are capable of binding to a variety of microbial cell wall components including lipopolysaccharide (LPS), lipoteichoic acid, and peptidoglycan (PGN) from Gram-positive bacteria Gram-negative and and β-1,3-glucan from fungi (Charles A. Janeway and Medzhitov, 2002; Iwanaga and Lee, 2005). As the consequence, several cascades are generated in order to defend against the invading pathogens (Wang and Wang, 2013). Recently, several types of PRRs have been identified in black tiger shrimp such as lipopolysaccharide- and β-1,3-glucan-binding protein (*Pm*LGBP). The *Pm*LGBP recognizes LPS and β-1,3-glucan and enhanced prophenoloxidase (proPO) activating system (Amparyup *et al.*, 2012).

Toll and immunodeficiency (IMD) are reported as the main signaling pathways regulating immune response of invertebrates (Anderson, 2000; Li and Xiang, 2013b). Toll pathway generally responses to Lys-type peptidoglycans (PNGs) on Gram-positive bacteria and β-1,3-glucan from fungi (Yang *et al.*, 2007; Amparyup *et al.*, 2012) or lipopolysaccharide (LPS) of Gram-negative bacteria (Anderson, 2000). Activation of Toll receptor by molecular recognition recruits a group of cytoplasmic proteins including MyD88, Tube, TRAF6 and Pelle (Hoffmann and Reichhart, 2002; Li and Xiang, 2013b). The activation of downstream proteins results

in phosphorylation of inhibitor Cactus leading to nuclear translocation NF-κB transcription factor Dorsal. The migration of Dorsal finally activates immune-related gene expression (Wen *et al.*, 2013; Tassanakajon *et al.*, 2018).

On the contrary, IMD pathway is preferentially activated by Gram-negative bacteria, some Gram-positive *Bacilli* and some RNA viruses by membrane-bound PRPs (Wang and Wang, 2013; Udompetcharaporn *et al.*, 2014). In *Drosophila*, a transcription factor Relish is identified and play the important roles in regulation of immune response (Li and Xiang, 2013b). Recently, a homolog IMD and Relish were identified in shrimp, indicating the presence of IMD pathway in shrimp immune responses (Li and Xiang, 2013a). However, Relish and Dorsal are involved in the response upon bacterial and viral infection suggesting the possible association between two pathways in shrimp innate immune system (Li and Xiang, 2013b; Tassanakajon *et al.*, 2018). The overview of shrimp Toll and IMD pathway is shown in **figure 1.15**.

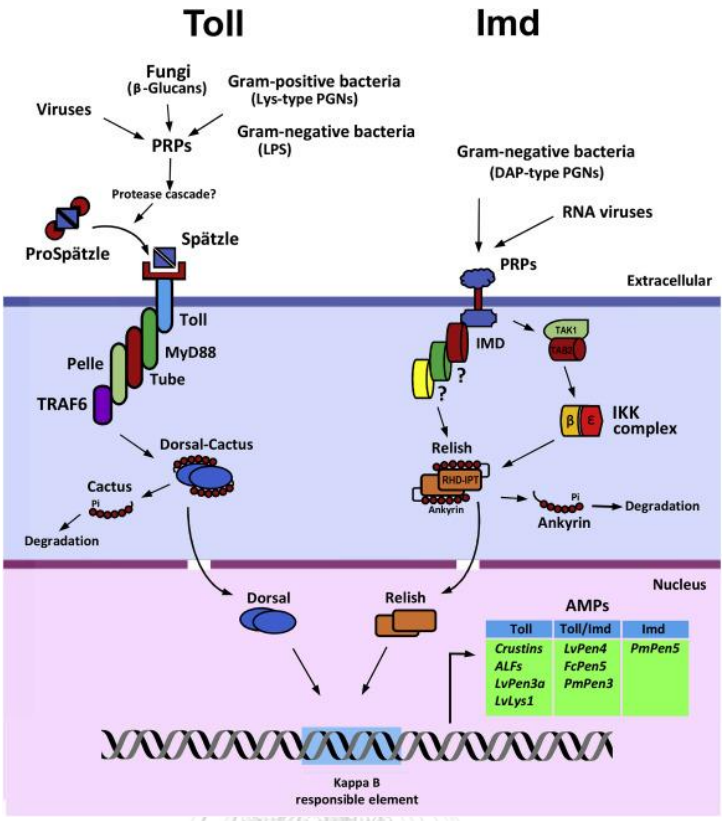

Figure 1.15 Overview of Toll and IMD signaling pathways from Penaeid shrimp
Toll pathway is stimulated by fungi and Gram-positive bacteria while IMD pathway is activated upon Gram-negative bacteria. Signal transduction is generated following the recognition of microbial components by PRRs. Several cytoplasmic proteins including essential transcription factors are activated due to phosphorylation cascade. The activated transcription factors migrate into the nucleus and regulate the expression of immune-related genes such as AMPs to defense against invading microbes. The question marked icons are the extrapolation data from *Drosophila* (Tassanakajon *et al.*, 2018).

1.5 Cytokine-like system in crustacean

As the result of evolution, the multicellular organisms are sustained by complex systems of intercellular communications (Lin *et al.*, 2010). Cytokines are generally considered to function as classical soluble molecules in these cell-to-cell contacts (Lin and Söderhäll, 2011). They are key mediators of inflammation

prominently involved in autoimmune and inflammatory diseases which regulate several cell functions through the members of cytokine receptor superfamily (Ihle, 1995). In mammals, cytokines constitute a network and are conserved for the fundamental processes they control which can be considered as the real controller of the effects on immune or developmental functions. Information of cytokines has been growing impressively in the past five years including for invertebrates or crustaceans (Malagoli, 2010). Several reports on invertebrate immunity have used the terms "putative cytokine" or "cytokine-like" to represent their existence of possible counterparts of vertebrate cytokines in comparative immunology (Malagoli and Ottaviani, 2007).

Recently, Astakine-1, a homologue to vertebrate prokineticins, was first identified in *Pacifastacus leniusculus*, and was found to be necessary for new hemocyte synthesis and release (Söderhäll *et al.*, 2005). The presence of a variety of cytokine-like molecules such as tumor necrosis factor alpha, TNF-α found in the hemocytes with phagocytic activity of two molluscs including *Planorbarius corneus* and *Viviparus ater* were reported (Ottaviani *et al.*, 1993). In addition, an AMP, penaeidin from *Penaeus monodon* possesses functional cytokine to promote integrin-β-mediated cytokine feature to shrimp granulocyte and semi-granulocyte adhesion (Li *et al.*, 2010). The Vago gene from Pacific white shrimp, *Litopenaeus vannamei* which encodes a viral-activated secreted peptide, was induced in a similar manner to that of vertebrate IFNs to restrict virus infection through activating the JAK-STAT pathway (Li *et al.*, 2015). This suggested that shrimp might possess an IFN system-like antiviral mechanism. Taken together, these data with recent reports on the presence of cytokine-like molecules in other invertebrates suggest that cytokines are important, ancestral and functionally conserved molecules through invertebrates to vertebrates.

1.6 IKK-NF-κB signaling pathway

The IKK-NF-κB pathway has been studied and reported to regulate pro-inflammatory cytokine production, leukocyte recruitment or cell survival in vertebrates (Lawrence, 2009). The signaling cascade is responsible for stresses and critical diseases from viral and bacterial infections. Upon the invasion, the key

mediator proteins called inhibitor of kappa B kinases (IKKs) act as the mediators to drive the signaling cascade (Häcker and Karin, 2006; Sun, 2011; Hinz and Scheidereit, 2014). In vertebrates, the pathogen signature molecules called PAMPs are recognized by pattern recognition receptors (PRRs) and trigger the IKK-NF-κB pathway. The pathway commonly comprises an essential role in coordinating the expression of type I interferons (IFNs), pro-inflammatory cytokines and chemokines. Following the activation, the elimination of invading viruses and bacteria commences by initiating innate and adaptive immune systems (Akira *et al.*, 2006; Lawrence, 2009; Takeuchi and Akira, 2010). Primarily, IKK proteins function to phosphorylate their substrate NF-κB inhibitor designated IκBα which is subsequently undergone the degradative ubiquitination (Israël, 2010). The NF-κB transcription factor is then recruited and translocated into the nucleus where it regulates transcription of target immune genes which are generally involved in the pro-inflammatory responses (Dale *et al.*, 2006; Liu *et al.*, 2017). It is also clear that the NF-κB is an important contributor to the immune responses and feedback control of inflammation via various mechanisms (Baldwin, 1996) (**Figure 1.16**).

Previous studies revealed a group of immune-related genes in the Toll and IMD pathways of *Drosophila melanogaster* were stimulated following the activation of *Dm*IKKs in the NF-κB signaling (Ertürk-Hasdemir *et al.*, 2009; Myllymäki *et al.*, 2014). Moreover, several kinds of antimicrobial peptides and an IFN-like molecule called *Dm*Vago are up-regulated by NF-κB transcription factor in responses to pathogen invasion (Li *et al.*, 2015) In arthropods including *Culex* mosquito and honey bees *Apis mellifera*, the viral infection was responded via NF-κB ortholog activation that induced by signaling cascade from PRRs (Paradkar *et al.*, 2012; Ryabov *et al.*, 2014). The study in Pacific white shrimp *Litopenaeus vannamei* also demonstrated the response of *Lv*IKK against WSSV infection (Wang *et al.*, 2013). In addition, NF-κB, IRF3/7 and IFNβ are stimulated simultaneously following the recognition of extrinsic dsRNA by Toll-like receptors 3 (TLR3), TLR4 and RIG-I (Yamamoto *et al.*, 2002; Bakshi *et al.*, 2017). These results demonstrated that essential peptides or interferons in immune system are characterized and regulated via NF-κB transcription factor in responses to different stimuli (Lawrence, 2009).

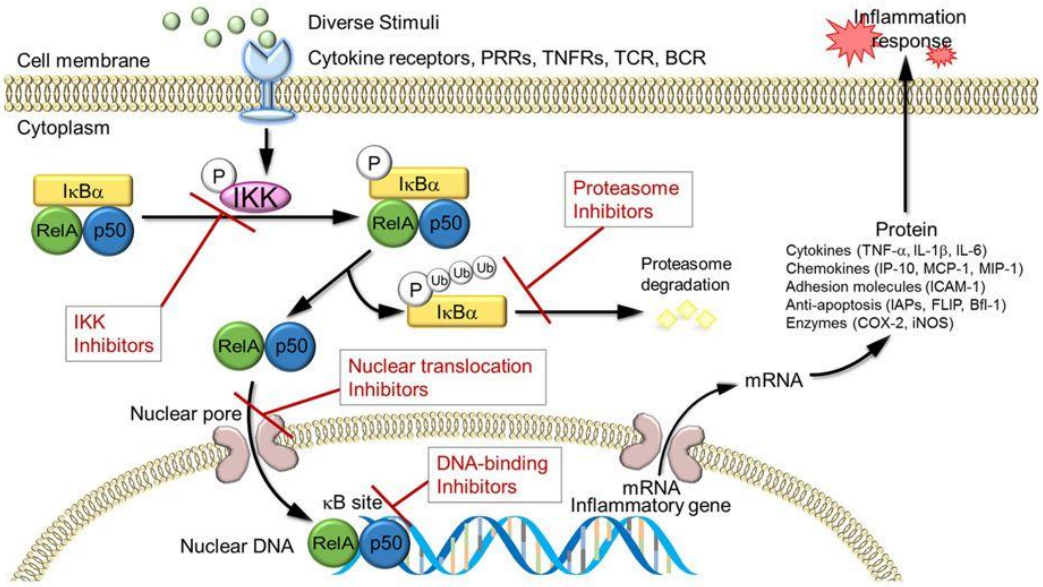

Figure 1.16 IKK-NF-κB signaling pathway in inflammatory responses. The NF-κB signaling plays a crucial role in various inflammatory diseases. A common signaling event is the activation of the canonical NF-κB pathway by diverse stimuli, which is responsible for transcriptional induction of pro-inflammatory cytokines, chemokines and additional inflammatory mediators in different types of immune cells. The pathway has been implicated in the pathogenesis of a number of inflammatory diseases. Several steps considered as the feedback control of inflammation aimed at blocking NF-κB activity via various mechanisms (Liu *et al.*, 2017).

In mammals, two inhibitors of kappa B kinases including IKKα and IKKβ are required for mediating the phosphorylation of IκBα and function to connect among the cascades of most signal transduction pathways (Silverman *et al.*, 2003; Hinz and Scheidereit, 2014). The p100-like NF-κB precursor protein or Relish is phosphorylated by IKKβ and acts as a central component in the IMD pathway to promote the expression of immune-related genes (Ertürk-Hasdemir *et al.*, 2009; Lawrence, 2009). IKKα functions independently to the NF-κB pathway and seems to show much more diverse functions. Mice deficient with IKKα subunit die a few days after birth and show defects affecting multiple morphogenetic events which are not relied on the NF-κB pathway (Israël, 2010). In the immune response, the IKKβ

compensated for the lack of IKKα to modulate the NF-κB cascade for pro-inflammatory stimulation (Häcker and Karin, 2006; Hinz and Scheidereit, 2014). These results proposed that the IKK-NF-κB signaling pathway provided a cross-talking within proteins in the IKK family and they might not be modulated solely in one signaling pathway.

1.7 Research hypothesis and purposes

Although several studies of IKK-NF-κB signaling pathway have been investigated in vertebrates and fruit fly *D. melanogaster*, less attention has been paid for the mechanism in crustaceans. Previous studies have demonstrated that the IKK-NF-κB signaling pathway is essential to the modulation of immune system. The pathway involves the stimulation of immune molecules in both vertebrates and invertebrates (Lawrence, 2009; Takeuchi and Akira, 2010; Wang *et al.*, 2013; Hinz and Scheidereit, 2014). In the past decades, the information of shrimp immune system has been extensively investigated but the overall perspective of shrimp defense mechanisms remains obscure. Thus, to take a new insight in shrimp immunity, the IKK-NF-κB signaling pathway from the black tiger shrimp *Penaeus monodon* will be revealed using biochemical approaches. The key mediator *Pm*IKKs will be functionally characterized for their roles in the immune pathway. The *Pm*IKK expression profiles are examined analyze the responses against the invading pathogens including WSSV, YHV viruses and a bacterium *Vibrio harveyi*. The involvement of *Pm*IKKs in the signal transduction of Toll and IMD pathways will be elucidated. The results from this research on the IKK-NF-κB signaling pathway will provide a better understanding in shrimp immunity and regulation upon pathogen infection.

CHAPTER II

MATERIALS AND METHODS

2.1 Materials

2.1.1 Equipment

-20°C Refrigerator Freezer (SHARP)

-80°C Freezer (Thermo Forma)

ABI PRISM Genetic Analyzer (Applied Biosystems)

Axygen® 0.2 mL Thin Wall PCR Tubes with Flat Cap

Axygen® 0.6 mL MaxyClear Snaplock Microcentrifuge Tube

Axygen® 1.5 mL MaxyClear Snaplock Microcentrifuge Tube

Balance (METTLER TOLEDO)

Bio-Rad low-profile 0.2 ml 8-tube strips

Bio-Rad flat PCR tube 0.2 ml 8-cap strips

Corning® 15 ml centrifuge tubes

Corning® 50 ml centrifuge tubes

Corning® 96 Well TC-Treated Microplates

Gel documentation (SYNGENE)

Gilson™ PIPETMAN Classic™ Pipets

Hettich® Universal 320R centrifuge

IKA® C-MAG HS 7 Magnetic stirrer with heating ceramic plate (IKA® WERKE)

IKA® mini G minicentrifuge (IKA® WERKE)

Incubator (Memmert)

Innova 4080 incubator shaker (New Brunswick Scientific)

Insulin syringes U100 (Becton Dickinson and Company)

Labo autoclave (Sanyo)

Millex syringe-driven filter unit 0.22 μM (Millipore)

Millex syringe-driven filter unit 0.45 μM (Millipore)

New Brunswick™ Scientific C24KC Refrigerated Incubator Shaker

NuAire LabGard® ES NU-540 Class II, Type A2 Biosafety Cabinet

NuAire NU-S813-300 Laboratory Fume Hood (NuAire)

Orbital shaker SO3 (Stuart Scientific, Great Britain)

Parafilm PM996 Wrap, 4" Wide × 125 Ft/Roll

PCR Mastercycler (BIO-RAD)

pH meter Model # SA720 (Orion)

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

Power supply PAC 3000 (BIO-RAD)

Semi-dry Trans-Blot® (BIO-RAD)

SpectraMax® M5 Microplate Reader (Molecular Devices)

Spectrophotometer Spectronic 2000 (Bausch & Lomb)

Thermo Scientific™ Forma™ 8600 Series -86°C Ultra-Low Temperature Chest Freezers

Thermo Scientific™ Sorvall™ Legend™ Micro 17 Microcentrifuge

Thermo Scientific™ Sorvall™ Legend™ Micro 21R Microcentrifuge

Touch mixer Model # 232 (Fisher Scientific)

TriStar^2 LB 942 Modular Multimode Microplate Reader (Berthold)

Vertical electrophoresis system (Hoefer™ miniVE)

Water bath (Memmert)

2.1.2 Chemicals and reagents

β-Mercaptoethanol (AppliChem)

Absolute ethanol, CH_3CH_2OH (HAYMAN)

Acrylamide page (GE Healthcare)

Agar powder (HIMEDIA)

Agarose (Research organics)

Ampicillin sodium salt (BIO BASIC INC.)

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

BigDye® Terminator v3.1 (Thermo Scientific)

Boric acid, BH_3O_3 (MERCK)

Bromophenolblue sodium salt (USB)

Bovine serum albumin (SIGMA-ALDRICH)

Calcium chloride, $CaCl_2$ (MERCK)

Coomassie brilliant blue R250 (BIO BASIC INC.)

Chloroform, $CHCl_3$ (RCI Labscan)

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

Diethyl pyrocarbonate (DEPC), $C_6H_{10}O_5$ (SIGMA)

Dipotassium hydrogen orthophosphate (AJAX Finechem)

Dithiothereitol (DTT), $C_4H_{10}O_2S_2$ (BIO BASIC INC.)

Ethidium bromide (SIGMA)

Ethylene diaminetetraacetic acid disodium salt dehydrate, EDTA (Ajax Finechem)

GENEzol™ Reagent (Geneaid Biotech)

Glacial acetic acid, CH_3COOH (MERCK)

Glycerol, $C_3H_8O_3$ (Scharlau)

Hydrochloric acid, HCl (MERCK)

Isopropanol, C_3H_7OH (MERCK)

Isopropyl-β-D-thiogalactoside (IPTG), $C_9H_{18}O_5S$ (Thermo Scientific)

Magnesium chloride, $MgCl_2$ (MERCK)

Methanol, CH_3OH (Burdick&Jackson)

N-N dimethyl formamide (Carlo Erba)

Opti-MEM (Life Technologies)

Paraformaldehyde (SIGMA)

Potassium chloride, KCl (Ajax Finechem)

Potassium dihydrogen orthophosphate (Ajax Finechem)

Sodium acetate, CH_3COONa (Carlo Erba)

Sodium citrate (Ajax Finechem)

Sodium chloride, NaCl (Ajax Finechem)

Sodium dihydrogen orthophosphate, $NaH_2PO_4.H_2O$ (Carlo Erba)

Sodium hydroxide, NaOH (MERCK)

Thermo Scientific™ dNTP Set 100 mM Solutions

Thermo Scientific™ GeneRuler 100 bp Plus DNA Ladder

Thermo Scientific™ GeneRuler 1 kb DNA Ladder

Tris-(hydroxyl methyl)-aminomethane, $NH_2C(CH_2OH)_3$ (Vivantis)

Triton®X-100 (MERCK)

Tryptone type I (HIMEDIA)

X-gal (5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside) (Fermemtas)

Yeast extract powder (HIMEDIA)

2.1.3 Enzymes and kits

Advantage® 2 Polymerase Mix (Clontech)

DNase I (RNase-free) (NEB)

Dual-Glo® Luciferase Assay System (Promega)

FavorPrep™ GEL/PCR Purification Kit

FavorPrep™ Plasmid DNA Extraction Mini Kit

Luna® Universal qPCR Master Mix (NEB)

*Nco*I-HF® (NEB)

*Not*I-HF® (NEB)

SMARTer™ RACE cDNA Amplification Kit (Takara Bio)

RBC T&A Cloning Kit (RBC Bioscience)

RBC *Taq* DNA polymerase (RBC Bioscience)

T4 DNA ligase (NEB)

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

Thermo Scientific™ RevertAid First Strand cDNA Synthesis Kit

*Xho*I (NEB)

2.1.4 Experimental shrimp, microorganisms, cells and viruses

Black tiger shrimp *Penaeus monodon*

Escherichia coli strain BL21-CodonPlus (DE3)-RIPL

Escherichia coli strain JM109

Vibrio harveyi 639

Human embryonic kidney 293T cells (HEK293T)

White spot syndrome virus

Yellow head virus

2.1.5 Software

BLAST® (https://blast.ncbi.nlm.nih.gov/Blast.cgi)

CFX Manager™ Software (Bio-Rad)

Clustal Omega (https://www.ebi.ac.uk/Tools/mas/clustalo/)

ExPASy-Translate tool (https://web.expasy.org/translate/)

GENETYX® 7.0.3 (GENETYX Corporation)

GraphPad Prism 6 (GraphPad Software)

ImageJ IJ 1.46r (NIH Image)

MEGA 6.0 (MEGA software)

SMART 8.0 (http://smart.embl-heidelberg.de/)

IBM® SPSS® Statistics 17.0 (IBM Corporation)

2.2 Methods

2.2.1 Shrimp aquaculture and animal ethics

Healthy black tiger shrimp, *Penaeus monodon* were purchased from a local shrimp farm with the average body mass of 10-15 g for gene expression analysis and that of 3-5 g for dsRNA-mediated RNA interference experiments. Shrimp were cultivated in recirculating aquaria filled with air-pumped seawater with a salinity of 20 ppt at an ambient temperature of about 29 ± 1°C. They were fed with a commercial diet twice a day for at least 7 days for acclimation without clinical signs of diseases before experiments. This study was conducted under the ethical principles and guidelines according to the animal use protocol approved by Chulalongkorn University Animal Care And Use Committee (CU-ACUC).

2.2.2 RNA extraction and cDNA synthesis

Sampled tissues from healthy or pathogen infected shrimp were collected and homogenized using GENEzol™ reagent (Geneaid Biotech). The total RNA was isolated according to manufacturer's protocol. In brief, 50-100 mg of sampled tissue was homogenized in 1 mL GENEzol™ reagent and incubated at room temperature for 5 minutes. Following the incubation, 200 µl of chloroform per 1 mL GENEzol™ reagent was added to the homogenized sample. The sample was mixed vigorously and centrifuged at 12,000×*g*, 4°C for 15 minutes. The upper aqueous phase was transferred to a new tube and mixed with 1 volume of isopropanol for RNA

precipitation. The mixture was centrifuged at 12,000×*g*, 4°C for 10 minutes to collect the RNA pellet. The pellet was washed with 70% (v/v) ethanol and centrifuged at 12,000×*g*, 4°C for 5 minutes before air-dried for 5-10 minutes. Total RNA was resuspended with diethylpyrocarbonate-treated water (DEPC water) and treated with DNase I (RNase-free) (NEB) to remove contaminating DNA. The DNase was removed by phenol/chloroform extraction and RNA concentration was measured using NanoDrop™ 2000c Spectrophotometer (Thermo Scientific). The equal amounts of RNA from three individual shrimp were pooled for first strand cDNA synthesis.

One microgram RNA was reverse transcribed to first strand cDNA using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific). As described in the manufacturer's instructions, the extracted RNA was reverse transcribed in 20 µl reaction containing 1 µg RNA, 1 µl of 100 µM Oligo(dT)$_{18}$ primer, 4 µl of 5X Reaction Buffer, 1 µl RiboLock RNase Inhibitor (20 U/µl), 2 µl of 10 mM dNTP mix, 1 µl RevertAid M-MuLV RT (200 U/µl) and nuclease-free water. The reaction was mixed gently, spun down and incubated at 42°C for 60 minutes followed by termination at 70°C for 5 minutes. RNA extract and first strand cDNA were stored at -80°C until use.

2.2.3 Sequence retrieving and cloning of *Pm*IKKβ and *Pm*IKKε

The open reading frames (ORFs) of *Pm*IKKβ, *Pm*IKKε1 and *Pm*IKKε2 were obtained from hemocyte cDNA by PCR amplification and specific primers (**Table 2.1**). The total hemolymph was drawn from shrimp ventral sinus with a syringe containing 10% (w/v) tri-sodium citrate solution as an anticoagulant. Total hemocytes was separated from fluid plasma by centrifugation at 800×*g*, 4°C for 10 minutes and resuspended in GENEzol™ Reagent (Geneaid Biotech) for RNA extraction and first strand cDNA synthesis. Based on a partial sequence of EST *Pm*IKKβ (accession no. PM53485) from *Penaeus monodon* EST database (http://pmonodon.biotec.or.th) (ref) and ORF of *Lv*IKKβ (accession no. JN180642) from *Litopenaeus vannamei*, specific primers were designed and the complete ORF of *Pm*IKKβ was successfully cloned. PCR reaction was performed in 50 µl total volume containing 2 µl cDNA template, 1X Advantage 2 PCR buffer, 1X dNTP mix, 0.2 µM primer mix and 1X Advantage 2

Polymerase Mix (Takara Bio). The reaction was carried out with the initial denaturation at 95°C for 1 min followed by 35 cycles of 95°C for 30 sec and 68°C for 1 min and final extension at 68°C for 1 min.

In order to obtain the ORF of *Pm*IKKε, specific primers were designed based a partial sequence of EST *Pm*IKKε (accession no. PM42457) and RACE-PCR approach was performed using SMARTer™ RACE cDNA Amplification Kit (Takara Bio). The PCR reaction was carried out using 2.5 µl of RACE-Ready cDNA template in 50 µl reaction containing 1X Advantage 2 PCR buffer, 1X dNTP mix, 1X UPM primer, 0.2 µM GSP primer and 1X Advantage 2 polymerase Mix (Takara Bio). The reaction was incubated under the following conditions: 5 cycles of 94°C for 30 sec and 72°C for 3 min followed by 5 cycles of 94°C for 30 sec, 70°C for 30 sec and 72°C for 3 min and 25 cycles of 94°C for 30 sec, 68°C for 30 sec and 72°C for 3 min. To further confirm the amplified region, nested RACE PCR was performed using Nested Universal Primer A and nested GSP primer. The amplicons of *Pm*IKKβ, *Pm*IKKε1 and *Pm*IKKε2 ORFs were purified from 1% (w/v) agarose-TBE gel electrophoresis using FavorPrep™ GEL/PCR Purification Kit. Each purified ORF was ligate to RBC T&A cloning vector (RBC Bioscience) with a ratio of 1:3 plasmid:insert and transformed into *E. coli* JM109 competent cells. The recombinant plasmids were extracted from 16-18 hours grown transformant bacteria using FavorPrep™ Plasmid DNA Extraction Mini Kit and sequenced by Macrogen, Korea using M13 universal and M13 reverse primers.

2.2.4 Bioinformatics analysis

Similarities of nucleotide and protein sequences of IκB kinase (IKK) genes from *Penaeus monodon* and IKK family genes from other typical species from GenBank® genetic sequence database (NIH) were analyzed with GENETYX® 7.0.3 (GENETYX Corporation) and BLAST® algorithm at the National Center for Biotechnology Information (NCBI) (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Multiple sequence alignments and conserved domain analysis were performed using Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo). Amino acid sequences of *Pm*IKKβ, *Pm*IKKε1 and *Pm*IKKε2 were deduced from nucleotide sequences by

ExPASy-Translate tool (https://web.expasy.org/translate) and analyzed for protein motif features using Simple Modular Architecture Research Tool, SMART 8.0 (http://smart.embl-heidelberg.de). The neighbor joining phylogenic tree was constructed in MEGA 6.0 software (http://www.megasoftware.net/index.html) based on the amino acid sequences of IKK and IKK-family proteins in typical species. Bootstrap sampling was reiterated for 1000 times.

Table 2.1 Primer sequences for cloning of *Pm*IKKβ and *Pm*IKKε

Primer	Sequence (5′ to 3′)	purpose
ORF *Pm*IKKβ-F1	GGTGTGAGGTGCAACATGGCA	ORF cloning
ORF *Pm*IKKβ-R1	GCCTGCTCATCATAGTAGTCGAG	
ORF *Pm*IKKβ-F2	CTGAGGGCATGACGCGACCAC	ORF cloning
ORF *Pm*IKKβ-R2	TCAGCAGAAGACTACAAGGAAGTT	
ORF *Pm*IKKε-R2	ACCGTCTCGAGAAAAGGGTCCTA	ORF cloning
ORF *Pm*IKKε-R2	TCCGTCTGGACTCGCTGGACT	
*Pm*IKKεRACE-F	GAAACCCTCCTGGCCTCCGTCACAG	RACE PCR
*Pm*IKKεRACE-nested-F	CAGGACACCTTAGGCAATACAGAG	Nested-RACE PCR

2.2.5 Production of recombinant *Pm*IKKβ and *Pm*IKKε1 proteins and antibody cross-reactivity examination

2.2.5.1 Expression of recombinant *Pm*IKKβ and *Pm*IKKε1 proteins

To detect the localization of *Pm*IKKβ and *Pm*IKKε proteins in shrimp hemocytes, the recombinant *Pm*IKKβ and *Pm*IKKε proteins were expressed for antibody raising. In order to obtain the particular antibodies, the recombinant *Pm*IKKβ (r*Pm*IKKβ) and *Pm*IKKε1 (r*Pm*IKKε1) proteins were expressed in *E. coli*

system. The open reading frames (ORF) of *Pm*IKKβ and *Pm*IKKε1 were amplified from cDNA templates using specific primers attached with restriction recognition sites (**Table 2.2**). The amplified amplicons attached with *Nco*I and *Xho*I for *Pm*IKKβ and with *Nco*I and *Not*I for *Pm*IKKε1 were separated and purified from 1% (w/v) agarose-TBE gel electrophoresis using FavorPrep™ GEL/PCR Purification Kit. They were further introduced to restriction enzymes supplied from New England Biolabs, United states corresponding to the attached sites and ligated into pET-28b(+) with hexa-histidine tag sequence (Novagen®) (**Figure 2.1**). The ligation was carried out at 4°C overnight in 10 µl reaction containing 1X T4 DNA Ligase Buffer, T4 DNA Ligase (NEB), pET-28b(+) and an insert of *Pm*IKKβ or *Pm*IKKε1 with a molar ratio of 1:3 plasmid to insert. The recombinant plasmids were prior transformed into *E. coli* strain JM109. The resulting pET-28b(+)-*Pm*IKKβ-6xHis and pET-28b(+)-*Pm*IKKε1-6xHis recombinant plasmids were extracted by FavorPrep™ Plasmid DNA Extraction Mini Kit for sequencing at Macrogen, Korea using T7 promoter and T7 terminator primers.

Table 2.2 Primer sequences for *Pm*IKKβ and *Pm*IKKε1 recombinant protein expression

Primer	Sequence (5′ to 3′)	purpose
*Pm*IKKβ*Nco*IF	AGTTCCATGGCAGCAGCAGAAGACCGCC	protein expression
*Pm*IKKβ*Xho*IR	AGTCCTCGAGCAAGGAAGTTTCAACTGCC TTCT	
*Pm*IKKε*Nco*IF	CTGAGGGCATGACGCGACCAC	protein expression
*Pm*IKKε*Not*IR	TCAGCAGAAGACTACAAGGAAGTT	

The verified recombinant plasmids were transformed to *E. coli* strain BL21-CodonPlus (DE3)-RIPL as an expression host. A bacterium *E. coli* strain BL21-CodonPlus (DE3)-RIPL harboring pET-28b(+)-*Pm*IKKβ-6xHis or pET-28b(+)-*Pm*IKKε1-6xHis expression plasmids was grown in Luria-Bertani (LB) medium containing 50 µg/ml kanamycin at 37°C with agitation at 250 rpm until the OD_{600} reached approximately 0.6. Protein expression was induced with 1 mM IPTG and

cells were harvested by centrifugation at 10,000×*g*, 4°C for 10 minutes at 0, 2, 4, 6 hours after induction. The collected cells were resuspended in 1X PBS, pH7.4 and disrupted by sonication at 25% amplitude. Cell lysate was centrifuged at 12,000×*g*, 4°C for 10 minutes to separate inclusion and soluble fractions. The supernatant containing soluble proteins was stored on ice while the inclusion bodies were completely dissolved in 8 M urea at 70°C.

2.2.5.2 Immunostaining of r*Pm*IKKβ and r*Pm*IKKε1 proteins and cross-reactivity examination using human anti-IKK antibodies

To analyze the expression of recombinant proteins r*Pm*IKKβ and r*Pm*IKKε1, Western blot analysis was performed. In addition, cross reactivity from commercial human anti-IKK antibodies was further verified prior to immunofluorescence staining. The inclusion and soluble fractions prepared from crude lysate containing r*Pm*IKKβ and r*Pm*IKKε1 were measured for total protein concentration by Bradford protein assay (Bradford, 1976). For polyacrylamide gel electrophoresis, 10 μg total proteins was separated in 10% SDS-PAGE using Mini-PROTEAN® Tetra Vertical Electrophoresis Cell (Bio-Rad) and stained with Coomassie brilliant blue R-250 reagent. Moreover, Western blot analysis was performed by electrophoretic transfer of polyacrylamide gel-separated proteins to nitrocellulose membrane using transfer buffer (25 mM Tris, 192 mM glycine pH8.3 and 20% (v/v) methanol). Gels and membranes were prewet and equilibrated in transfer buffer before the gel/membrane sandwich is placed in Trans-Blot® SD Semi-Dry Transfer Cell (Bio-Rad) with the directed orientation at a constant voltage of 110V for 1:30 hours (**Figure 2.2**). The transferred membrane was soaked for 1 hour with 5% (w/v) skimmed milk in 1X PBS pH7.4 containing 0.05% (v/v) Tween® 20 (PBST). After blocking, the membrane was washed 3 times with PBST and incubated with rabbit anti-human IKKβ (Thermo Scientific) or mouse anti-IKKε (Abcam) primary antibodies with a 1:5000 ratio in PBST containing 1% (w/v) skimmed milk. The unbound component was removed by washing with PBST before probed with goat anti-rabbit or goat anti-mouse secondary antibodies (Millipore) conjugated with alkaline phosphatase with a ratio of 1:10,000 in PBST. For the detection using color development, the membrane was washed 3 times followed by incubation with NBT and BCIP substrates.

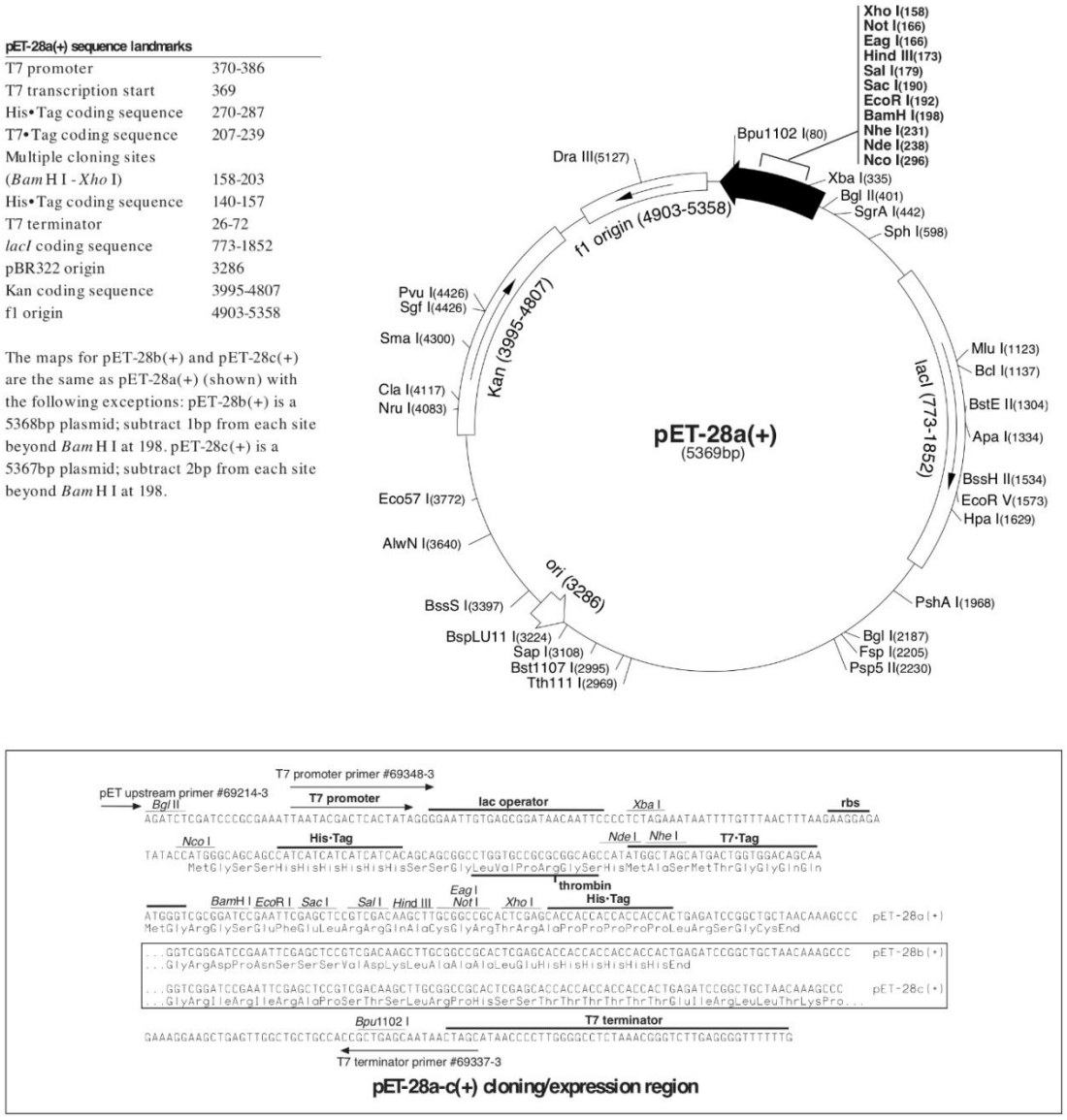

Figure 2.1 Sequence map of pET-28a-c(+) expression vectors (Novagen®).

(Source; http://www.m erckmillipore.com/TH/en/product/pET-28b+-DNA-Novagen, EMD_BIO-69865#anchor_VMAP)

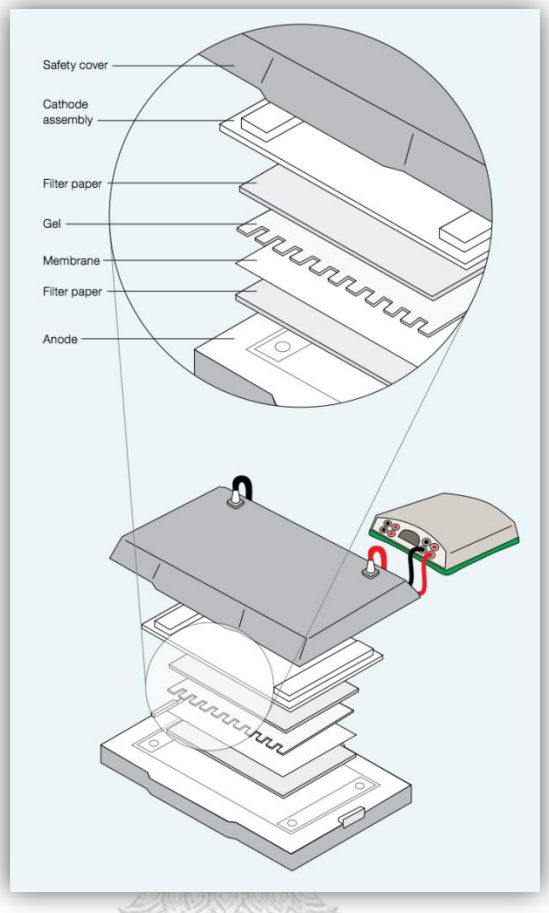

Figure 2.2 Direction of gel/membrane sandwich for electrophoretic transfer in semi-dry blotting. Gels and membranes were prewet and equilibrated in transfer buffer before placed in Trans-Blot® SD Semi-Dry Transfer Cell (Bio-Rad) with the directed orientation with buffer-soaked filter papers (Source: Bio-Rad).

2.2.6 Tissue-specific expression and protein detection of *Pm*IKKβ and *Pm*IKKε

2.2.6.1 Tissue-specific expression of *Pm*IKKβ and *Pm*IKKε in *Penaeus monodon*

To determine the expression of *Pm*IKKβ and *Pm*IKKε transcripts in *Penaeus monodon*, various tissues from healthy shrimp including hemocyte, lymphoid organ, gill, hepatopancreas, heart, intestine, muscle, eyestalk and stomach were collected. Total hemocytes was prepared by collecting hemolymph in 10% (w/v) tri-sodium

citrate solution and centrifugation at 800×*g*, 4°C for 10 minutes. The selected tissues were collected individually from three healthy shrimp for total RNA extraction and pooled for cDNA synthesis. The expression of *Pm*IKKβ and two isoforms of *Pm*IKKε including *Pm*IKKε1 and *Pm*IKKε2 was examined. Semi-quantitative RT-PCR reaction was performed using specific primers (**Table 2.3**) in 25 µl reaction volume containing 1 µl of first stand cDNA template, 1X reaction buffer (50 mM KCl, 1.5mM $MgCl_2$, 10 mM Tris-HCl, 0.1 mg/ml BSA, 10 mM $(NH_4)_2SO_4$, 0.1 µM dNTP mix, 0.2 µM forward and reverse primers and 1.25 units RBC *Tag* DNA polymerase (RBC Bioscience). The reaction was carried out by initial denaturation at 94°C for 2 min followed by 30 cycles of 94°C for 30 sec, 55°C for 30 sec and 72°C for 30 sec and final extension at 72°C for 7 min. The elongation factor-1α gene (*EF-1α*) was used as an internal control. The amplified PCR products were analyzed by 2% (w/v) agarose-TBE gel electrophoresis and visualized by UV-transillumination.

2.2.6.2 Detection of *Pm*IKKβ and *Pm*IKKε protein expression in three types of shrimp hemocytes

Protein expression of *Pm*IKKβ and *Pm*IKKε in shrimp hemocytes was detected using monoclonal mouse antibody specific to human IKKε (Abcam) and monoclonal rabbit antibody specific to human IKKβ (Invitrogen). Total hemolymph was drawn from healthy *P. monodon* (8-10 g) and fixed with 4% (w/v) paraformaldehyde in 1X PBS pH 7.4 (137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, and 2 mM KH_2PO_4) at a 1:1 ratio. Hemocytes were separated by centrifugation at 800×*g*, 4°C for 10 min before washed and resuspended in 1X PBS before counting with hemocytometer. The prepared hemocyte cells (1×10^6) were mounted on the poly-L-lysine-coated coverslips in 24-well plate and washed three times with 1X PBS containing 0.02% Triton(R) X-100 followed by permeabilization with 1X PBS containing 100 mM glycine, 0.02% Triton(R) X-100 and 1% BSA for 30 min, room temperature. Cells were then washed three times and blocked with 1X PBS containing 0.02% Triton(R) X-100, 10% FBS and 1% BSA at 4°C, overnight. Cells were washed again and probed with 1:500 anti-IKKβ or anti-IKKε primary antibody in 1X PBS containing 0.02% Triton(R) X-100, 10% FBS and 1% BSA at 4°C, overnight followed by washing and incubation with 1:5000 goat anti-mouse or anti-rabbit

secondary antibody conjugated with Alexa Fluor® 488 at room temperature for 2 hours. Cell were washed before nuclear staining with 1:5000 Hoechst 33342 (Thermo Scientific) in 1X PBS containing 0.02% Triton(R) X-100, 10% FBS and 1% BSA for 15-30 min. The coverslips were washed and mounted with Fluoro-KEEPER Antifade Reagent (Nacalai Tesque) and sealed on glass slides. Fluorescence images were detected by LSM 700 laser scanning confocal microscope (Carl Zeiss).

Table 2.3 Primer sequences for tissue-specific expression of *Pm*IKKβ, *Pm*IKKε1 and *Pm*IKKε2

primer	Sequence (5′ to 3′)	purpose
EST *Pm*IKKβ-F	CTGAGGGCATGACGCGACCAC	RT-PCR
EST *Pm*IKKβ-R	GCCTGCTCATCATAGTAGTCGAG	
EST *Pm*IKKε1-F	ACCGTCTCGAGAAAAGGGTCCTA	RT-PCR
EST *Pm*IKKε1-R	TCAGCAGAAGACTACAAGGAAGTT	
EST *Pm*IKKε2-F	ACCGTCTCGAGAAAAGGGTCCTA	RT-PCR
EST *Pm*IKKε2-R	TCCGTCTGGACTCGCTGGACT	
EF-1α-F	GGTGCTGGACAAGCTGAAGGC	RT-PCR
EF-1α-R	CGTTCCGGTGATCATGTTCTTGA	

2.2.1 Gene expression analysis of *Pm*IKKβ, *Pm*IKKε1 and *Pm*IKKε2 upon viral and bacterial immune challenges

2.2.1.1 Expression analysis of *Pm*IKKβ, *Pm*IKKε1 and *Pm*IKKε2 in responses to viral infection from WSSV and YHV

In immune challenge with WSSV, the virus stock was prepared according to the purification method modified from Xie *et al*., 2005. Briefly, gills from moribund

WSSV- or YHV-infected shrimp were collected and homogenized in TNE buffer (50 mM Tris-HCl pH 8.5, 400 mM NaCl and 5 mM EDTA) containing 1 mM phenylmethylsulfonyl fluoride (PMSF). The debris was removed by centrifugation at 3,500×*g*, 4°C for 15 min and pass the supernatant through 0.45 µm Millex®-HP Syringe Filter Unit. The filtrate was centrifuged at 30,000×*g*, 4°C for 30 min to form the virion pellet and washed twice with TM buffer (50 mM Tris-HCl pH 7.5 and 10 mM $MgCl_2$). The pellet was resuspended with TM buffer and stored at -80°C in aliquots until use.

To determine the transcript levels of *Pm*IKKβ, *Pm*IKKε1 and *Pm*IKKε2 in response to WSSV infection, healthy shrimp were injected intramuscularly in the third abdominal segment with 30 µl of phosphate-buffered saline (1X PBS, 137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, and 2 mM KH_2PO_4, pH 7.4) as a control, 1×10^5 copies of purified WSSV or YHV. Three shrimp from each group were randomly collected at 0, 6, 12, 24, 48 hours post injection (hpi) and total hemocyte was collected individually for RNA extraction and cDNA synthesis. The expression profiles of *Pm*IKKβ, *Pm*IKKε1 and *Pm*IKKε2 were examined by qRT-PCR analysis using specific primers and prepared cDNA templates (**Table 2.3**). The reactions were performed in 20 µl volume containing 1 µl of cDNA template, 10 µl of 2X Luna® Universal qPCR Master Mix (NEB) and 0.25 µM primer mix using CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad). Quantitative RT-PCR was carried out with the following condition: 1 cycle of 95°C for 1 min followed by 40 cycles of 95°C for 15 sec and 60°C for 30 sec. The expression of elongation factor-1α gene (*EF-1α*) was used as an internal control. Melt curve analysis was performed at the end of PCR thermal cycle for determining the specificity of amplification. The reactions were carried out in triplicates and the relative expression of *Pm*IKKβ, *Pm*IKKε1 and *Pm*IKKε2 was calculated using a comparative method described by Pfaffl (2001) as shown below. Data were shown as means±standard deviations (SD). Statistical analysis was performed using one-way ANOVA followed by Duncan's new multiple range test. The data was considered for statistical differences with the significance at $P<0.05$.

To calculate the relative expression according to a comparative method described by Pfaffl (2001), the following equation was used:

$$\text{Expression ratio} = \frac{(E_{target})^{\Delta C_T(control\text{-}sample)}}{(E_{ref})^{\Delta C_T(control\text{-}sample)}}$$

Where, E is amplification efficiency

C_T is threshold cycle

2.2.1.2 Expression analysis of *Pm*IKKβ, *Pm*IKKε1 and *Pm*IKKε2 upon *Vibrio harveyi* infection

In bacterial infection experiment, a bacterium *Vibrio harveyi* strain 639 was prepared and introduced to black tiger shrimp. *V. harveyi* 639 was grown by inoculating a single colony in tryptic soy broth (TSB) supplemented with 2% (w/v) NaCl at 30°C and 250 rpm orbital shaking for overnight. The culture was diluted in TSB supplemented with 2% (w/v) NaCl with a ratio of 1:200 and grown until the optical density at 600 nm (OD_{600}) reached 0.6, where cell density was approximately 10^8 CFU/ml. The culture was diluted to make a cell suspension of 2×10^5 CFU/ml for bacterial injection. Healthy black tiger shrimp were injected intramuscularly with 30 µl of phosphate-buffered saline as a control or *V. harveyi* 639 inoculum in the third abdominal segment. Hemocytes were collected from three individual shrimp at 0, 6, 12, 24, 48 hours post injection (hpi) for RNA extraction and cDNA synthesis. The expression of *Pm*IKKβ, *Pm*IKKε1 and *Pm*IKKε2 transcripts was determined by qRT-PCR analysis using specific primers in **table 2.3**. The reactions were performed in triplicates using 2X Luna® Universal qPCR Master Mix (NEB) and 0.25 µM primer mix in CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad). Elongation factor-1α gene (*EF-1α*) was used as an internal control and melt curve analysis was performed at the end of thermal cycle to determine the amplification specificity. The data were shown as relative expression ratio according to a comparative method described by Pfaffl (2001) and standard deviations (SD). Statistical analysis was performed using one-way ANOVA followed by Duncan's new multiple range test and considered for statistical significant difference at $P<0.05$.

2.2.2 *In vivo* gene silencing

2.2.2.1 Preparation of double-stranded RNAs (dsRNAs)

To analyze the consequent effects of *Pm*IKKβ and *Pm*IKKε silencing on shrimp innate immune system, double-stranded RNA (dsRNA)-mediated RNA interference was investigated. Double-stranded RNAs (dsRNAs) correspond to *Pm*IKKβ, *Pm*IKKε and GFP sequences (ds*Pm*IKKβ, ds*Pm*IKKε and dsGFP respectively) were synthesized by *in vitro* transcription using T7 RiboMAX™ Express Large Scale RNA Production System (Promega) according to manufacturer's protocol. DNA templates for *in vitro* transcription of sense and antisense RNA strands were amplified separately by PCR reactions containing gene specific primers attached with T7 RNA polymerase binding site (**Table 2.4**).

The reactions were carried out in 100 µl reaction volume with 100 ng plasmid bearing either *Pm*IKKβ or *Pm*IKKε gene using RBC *Tag* DNA polymerase (RBC Bioscience). Each reaction was carried out in thermal cycles with initial denaturation at 94°C for 2 min followed by 35 cycles of 94°C for 30 sec, 60°C for 30 sec and 72°C for 30 sec and final extension at 72°C for 7 min. The dsGFP for an internal control was amplified from pEGFP-1 vector (Clonetech) which harbors a gene fragment of green fluorescent protein (GFP). The amplicons were purified and further transcribed to single-stranded RNAs by *in vitro* transcription. Briefly, 1 µg of purified DNA template was transcribed in 20 µl reaction containing RiboMAX™ Express T7 2X Buffer, Enzyme Mix (T7 Express) and nuclease-free water. The reaction was incubated at 37°C for 30 min and treated with RQ1 RNase-Free DNase at 37°C for 15 min to remove DNA templates. The equal volumes of complementary single-stranded RNAs were incubated at 70°C for 10 minutes and annealed by slowly cool at room temperature for 20 min to generate the consequent double-stranded RNAs. One volume isopropanol was added to the reactions in the presence of 0.1 volume of 3M sodium acetate (pH 5.2) to precipitate the dsRNA. The pellets were collected by centrifugation at 13,000×*g*, 4°C for 10 min and washed with cold 70% (v/v) ethanol. The purified dsRNAs were resuspended in nuclease-free water before determine the concentration and qualified by agarose gel electrophoresis. The dsRNAs were stored

at -20°C until use. The specific regions on mRNAs of *Pm*IKKβ (bp-706 to bp-1,059) and *Pm*IKKε (bp-630 to bp-1,058) for dsRNA binding are drawn in **figure 2.3**.

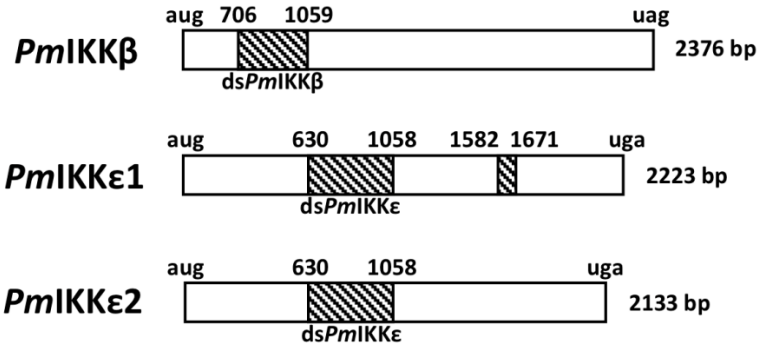

Figure 2.3 Double-stranded RNA binding sites on *Pm*IKKβ and *Pm*IKKε mRNAs. Schematic diagram of specific regions on mRNAs at bp-706 to bp-1,059 of *Pm*IKKβ and bp-630 to bp-1,058 of *Pm*IKKε. The dsRNA for *Pm*IKKε binds nonspecifically to both *Pm*IKKε1 and *Pm*IKKε2 due to their highly identical mRNAs sequences. The additional 90-nucleotide region on *Pm*IKKε1 at bp-1,582 to bp-1,671 are drawn.

2.2.2.2 *In vivo* gene silencing of *Pm*IKKβ and *Pm*IKKε by dsRNA-mediated RNA interference (RNAi)

To verify the silencing efficiency of dsRNAs *in vivo*, shrimp were double-injected with 30 µl total volume containing ds*Pm*IKKβ or ds*Pm*IKKε (10 µg/g shrimp) dissolved in 150 mM NaCl by intramuscular injection, whereas the control group was injected with dsGFP. The interval before a second dsRNA injection was 24 hours. At 24 hours after final dsRNA injection, shrimp total hemolymph was drawn individually and hemocytes were separated by centrifugation at 800×*g*, 4°C for 10 min for RNA extraction and first strand cDNA synthesis. To verify gene silencing efficiency, transcript levels of *Pm*IKKβ and *Pm*IKKε were determined by quantitative RT-PCR (qRT-PCR). The reactions were carried out in triplicates using 2X Luna® Universal qPCR Master Mix (NEB), 1 ul cDNA template and 0.25 µM specific primers (**Table 2.4**). Elongation factor-1α gene (*EF-1α*) was amplified as an internal control for normalization. Thermal cycles were performed with 1 cycle of 95°C for 1

min followed by 40 cycles of 95°C for 15 sec and 60°C for 30 sec in CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad).

Table 2.4 Primer sequences for double-stranded RNA production

primer	Sequence (5′ to 3′)	purpose
ds*Pm*IKKβ-F	GAATGGATGAAGCGTGTACGCAC	RNAi
ds*Pm*IKKβ-R	ACTGTCACGTGCAACCCACTGCT	
ds*Pm*IKKβ-T7F	TAATACGACTCACTATAGGGAATGGATGAAGCGTGTACGCAC	RNAi
ds*Pm*IKKβ-T7R	TAATACGACTCACTATAGGACTGTCACGTGCAACCCACTGCT	
ds*Pm*IKKε-F	AATAGGTGTGACACTTTACCACGT	RNAi
ds*Pm*IKKε-R	TGGTTGACTGGATTCATGTCTGTC	
ds*Pm*IKKε-T7F	TAATACGACTCACTATAGGAATAGGTGTGACACTTTACCACGT	RNAi
ds*Pm*IKKε-T7R	TAATACGACTCACTATAGGTGGTTGACTGGATTCATGTCTGTC	
dsGFP-F	AGTGCTTCAGCCGCTACCC	RNAi
dsGFP-R	GCGCTTCTCGTTGGGGTC	
dsGFP-T7F	TAATACGACTCACTATAGGAGTGCTTCAGCCGCTACCC	RNAi
dsGFP-T7R	TAATACGACTCACTATAGGGCGCTTCTCGTTGGGGTC	

2.2.3 Silencing effects of *Pm*IKKβ and *Pm*IKKε on shrimp innate immune system and WSSV infection

2.2.3.1 Silencing effects of *Pm*IKKβ and *Pm*IKKε on immune-related genes after WSSV infection

For examining the silencing effects of *Pm*IKKβ and *Pm*IKKε on shrimp immune-related genes, dsRNA-mediated gene silencing was performed followed by WSSV infection. Shrimp were double-injected with either 10 μg/g shrimp of dsGFP for control group, ds*Pm*IKKβ or ds*Pm*IKKε dissolved in 150 mM NaCl by intramuscular injection. Following gene silencing, shrimp were injected with 1×10^5 copies of purified WSSV at 24 hours post dsRNA injection. Three shrimp per a treatment group were randomly collected at 24 hours post WSSV infection. Total RNA was extracted from collected shrimp hemocytes for cDNA synthesis. The expression profiles of immune-related genes including *Pm*Vago, *Pm*Cactus, *Pm*Dorsal, *Pm*Relish, ALF*Pm* and *Pm*Crustin were determined by qRT-PCR using specific primers (**Table 2.5**). The reactions were performed in 20 μl reaction volume with 2X Luna® Universal qPCR Master Mix (NEB), 1 μl cDNA template and 0.25 μM primer mix using CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad). Quantitative RT-PCR was carried out in triplicates with following conditions: 1 cycle of 95°C for 1 min followed by 40 cycles of 95°C for 15 sec and 60°C for 30 sec. The expression of elongation factor-1α gene (*EF-1α*) was used as an internal control. Melt curve analysis was performed at the end of PCR thermal cycle for determining the specificity of amplification. The relative expression of *Pm*IKKs was calculated using a comparative method described by Pfaffl (2001). The data were shown as means±standard deviations (SD). Statistical analysis was performed using one-way ANOVA followed by Duncan's new multiple range test. The data was considered for statistical differences with the significance at $P<0.05$.

Table 2.5 Primer sequences for gene expression analysis of after *Pm*IKKβ and *Pm*IKKε silencing

primer	Sequence (5′ to 3′)	purpose
EST *Pm*IKKβ-F	CTGAGGGCATGACGCGACCAC	qRT-PCR
EST *Pm*IKKβ-R	GCCTGCTCATCATAGTAGTCGAG	
EST *Pm*IKKε-F	ACCGTCTCGAGAAAAGGGTCCTA	qRT-PCR
EST *Pm*IKKε-R	CGGATCGTCCAGAATGTTGAAGAG	
EF-1α-F	GGTGCTGGACAAGCTGAAGGC	qRT-PCR
EF-1α-R	CGTTCCGGTGATCATGTTCTTGA	
*Pm*Relish-F	TCTCCAGGTGAGCACTCAGTTGGC	qRT-PCR
*Pm*Relish-R	GCTGTAGCTGTTGCTGTTGTTGAG	
ALF*Pm*3-F	CCCACAGTGCCAGGCTCAA	qRT-PCR
ALF*Pm*3-R	TGCTGGCTTCTCCTCTGATG	
ALF*Pm*6-F	ATGCTACGGAATTCCCTCCT	qRT-PCR
ALF*Pm*6-R	ATCCTTGCAACGCATAGACC	
Crustin*Pm*1-F	CTGCTGCGAGTCAAGGTATG	qRT-PCR
Crustin*Pm*1-R	AGGTACTGGCTGCTCTACTG	
Crustin*Pm*5-F	ATCAGCAGGGGAACAAGAGA	qRT-PCR
Crustin*Pm*5-F	CGGACTCGCAGCAATAGACT	
Crustin*Pm*7-F	GGCATGGTGGCGTTGTTCCT	qRT-PCR
Crustin*Pm*7-F	TGTCGGAGCCGAAGCAGTCA	

Table 2.5 (continued) Primer sequences for gene expression analysis of after *Pm*IKKβ and *Pm*IKKε silencing

primer	Sequence (5′ to 3′)	purpose
*Pm*Vago1-F	GCATCAAGTTCGGAAGCTGT	qRT-PCR
*Pm*Vago1-R	ACCCACATCCTTTCCACAAG	
*Pm*Vago4-F	CTCTGGCTTGTGGAATGGAT	qRT-PCR
*Pm*Vago4-R	GCATGGATTCACTTCCTCGT	
*Pm*Dorsal-F	TCACTGTTGACCCACCTTAC	qRT-PCR
*Pm*Dorsal-R	GGAAAGGGTCCACTCTAATC	

2.2.3.2 Shrimp survival after *Pm*IKKβ and *Pm*IKKε silencing and WSSV infection

To further investigate the roles of *Pm*IKKβ and *Pm*IKKε upon WSSV infection, shrimp (3-5 g) were double-injected with 10 μg/g shrimp of *in vitro*-transcribed ds*Pm*IKKβ, ds*Pm*IKKε, dsGFP or 30 μl of 150 mM NaCl with an interval of 24 hours. At 6 hours following a second dsRNA injection, shrimp were injected intramuscularly with 1×10^5 copies of purified WSSV or 1X PBS, pH7.4 using insulin syringe for immune challenge. The experiment was carried out in triplicates consisting of 10 shrimp per group. After WSSV infection, cumulative mortalities were recorded daily for a period of 10 days. Statistical analysis was performed using one-way ANOVA with the significance at $P<0.05$.

2.2.3.3 Quantification of WSSV copy number in *Pm*IKKβ- and *Pm*IKKε-silenced shrimp

To study the effect of *Pm*IKKβ and *Pm*IKKε suppression on WSSV replication, the copy number of WSSV in *Pm*IKKβ- and *Pm*IKKε-silenced shrimp was determined. Shrimp were double-injected with either dsGFP, ds*Pm*IKKβ or

ds*Pm*IKKε dissolved in 150 mM NaCl with an interval of 24 hours followed by 1×10^5 copies of purified WSSV at next 24 hours. Shrimp were collected at 120 hpi and total genomic DNA was extracted from gill using FavorPrep™ Tissue Genomic DNA Extraction Mini Kit (Favorgen). Total genomic DNA was quantified by NanoDrop™ 2000c Spectrophotometer (Thermo Scientific) and prepared for 15 ng/μl genomic DNA to use in viral copy number analysis. Quantitative RT-PCR was performed in triplicates using Luna® Universal qPCR Master Mix (NEB) with 1 μl genomic DNA (15 ng/μl) and VP28 primers (**Table 2.6**). Thermal conditions were performed with initial denaturation at 95°C for 1 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 30 sec. Melt curve analysis was performed at the end to determine the specificity of amplification. The experiment was investigated in triplicates and the number of *VP28* gene was calculated regarding to absolute quantification. Recombinant plasmid containing using a conserved region of WSSV *VP28* was used to generate a standard curve for data analysis. Data were shown as means±standard deviations (SD). Statistical analysis was performed using one-way ANOVA followed by Duncan's new multiple range test. The data was considered for statistical differences with the significance at $P<0.05$.

Table 2.6 Primer sequences of *VP28* gene for WSSV copy quantification

primer	Sequence (5′ to 3′)	purpose
VP28-F	GGGAACATTCAAGGTGTGGA	qRT-PCR
VP28-R	GGTGAAGGAGGAGGTGTTGG	

2.2.4 Activation of NF-κB signaling cascade and cytokine-like system by *Pm*IKKβ, *Pm*IKKε1 and *Pm*IKKε2

2.2.4.1 Cells, reagents and plasmids

HEK293T cells were purchased from CH3 BioSystems and cultured in Dulbecco's modified Eagle's medium (Nacalai Tesque) supplemented with 10% fetal bovine serum (Invitrogen) at 37°C in a humidified 5% CO_2/95% air atmosphere. To

construct protein expression plasmids, cDNA fragments coding *Pm*IKKβ, *Pm*IKKε1 and *Pm*IKKε2 were amplified by PCR reactions using KOD FX (TOYOBO). The reactions were carried out in 100 µl volume containing 2 µl cDNA template, 1X PCR buffer for KOD FX, 0.4 mM dNTPs, 0.3 µM forward and reverse primers and 2 µl KOD FX (1U/µl). ORF fragments of *Pm*IKKβ, *Pm*IKKε1 and *Pm*IKKε2 were amplified under following thermal conditions started by initial denaturation at 94°C for 2 min, followed by 40 cycles of 98°C for 10 sec, 60°C for 30 sec and 68°C for 3 min. PCR products were purified by 1% (w/v) agarose-TAE gel electrophoresis using FavorPrep™ GEL/PCR Purification Kit. The purified amplicons were cloned into pcDNA3-Myc with 2X TOYOBO ligation reagent to generate pcDNA-*Pm*IKKβ-Myc, pcDNA-*Pm*IKKε1-Myc and pcDNA-*Pm*IKKε2-Myc protein expression plasmids, respectively. Luciferase reporter plasmid of IFN-β was constructed by cloning a fragment of murine IFN-β promoter region (-125 to +55) as described previously (Sato *et al.*, 2000). The endothelial cell-leukocyte adhesion molecule (ELAM)-1 promoter-derived luciferase reporter plasmid (NF-κB luciferase reporter) has been prepared (Yamamoto *et al.*, 2002). The insert cDNAs of all constructs were confirmed using BigDye® Terminator v3.1 (Thermo Scientific) in an ABI PRISM Genetic Analyzer (Applied Biosystems).

2.2.4.2 Overexpression of *Pm*IKKβ, *Pm*IKKε1 and *Pm*IKKε2 in HEK293T cells and luciferase reporter assay

For reporter assays, HEK293T cells (5×10^4 cells/well) seeded on 24-well plates for 24 hours were transiently co-transfected with 50 ng of pGL3-IFNβ or pGL3-NF-κB luciferase reporter plasmids and 1 µg of each protein expression plasmids or empty control plasmid using polyethylenimine (PEI) at a ratio of 1:3 (µg:µl) in Opti-MEM (Life Technologies). As an internal control, 10 ng of pRL-TK *Renilla* luciferase reporter plasmid was transfected simultaneously. Twenty-four hours after transfection, cells were harvested and lysed for the assessment of protein expression and luciferase reporter assay using Dual-Glo® Luciferase Assay System (Promega) according to the manufacturer's instructions. Luciferase activities were measured using a TriStar^2 LB 942 Modular Multimode Microplate Reader (Berthold).

CHAPTER III

RESULTS

3.1 Cloning and sequence characterization of *Pm*IKKβ and *Pm*IKKε

Two partial nucleotide sequences related to IκB kinases (IKKs) including *PmIKKβ* (753 bp) and *PmIKKε* (380 bp) were retrieved from the *Penaeus monodon* EST database (http://pmonodon.biotec.or.th) (Tassanakajon *et al.*, 2006). The complete open reading frames (ORFs) of *PmIKKβ* and *PmIKKε* were obtained by PCR amplification using specific primers (**Table 2.1**) and cDNA template prepared from healthy shrimp hemocytes. Analysis of nucleotide sequences in ExPaSy bioinformatics resource demonstrated that a 2,376 bp of *PmIKKβ* ORF encoded a 791-amino acid protein (**Figure 3.1A**) with predicted molecular mass of 89.373 kDa and isoelectric point (pI) of 7.56. The ORF of *PmIKKβ* was deposited in the GeneBank with the accession number MK331816. Sequence analysis using BLAST® from NCBI database showed 95% and 30% identity with Pacific white shrimp *Litopenaeus vanamei* IKKβ and *Drosophila* IKKβ, respectively. In addition, two isoforms of *PmIKKε* including *PmIKKε1* and *PmIKKε2* were identified using RACE approach and deposited in GeneBank with the accession numbers MK331817 and MK331818, respectively. The amplification resulted in two isoforms of *Pm*IKKε namely, *Pm*IKKε1 and *Pm*IKKε2. The ORF of *Pm*IKKε1 contains 2223 bp which encoded a 740-amino acid protein with predicted molecular mass of 83.562 kDa and pI of 5.85. *Pm*IKKε1 and *Pm*IKKε2 were 99.58% identical. *Pm*IKKε2 lacks a 30-amino acid sequence at positions 528 to 557 found in *Pm*IKKε1 (boxed), which made the predicted molecular mass and pI of 80.268 kDa and 6.03, respectively (**Figure 3.1B**). *Pm*IKKε1 and *Pm*IKKε2 share 93% and 94% sequence identity with *Lv*IKKε1 and *Lv*IKKε2, respectively. Moreover, the deduced amino acid sequences of *Pm*IKKε1 and *Pm*IKKε2 exhibit 27% identity with *Pm*IKKβ. Protein domain characterization using SMART database revealed N-terminal kinase domains (KDs) from amino acid residues 13 to 286 in *Pm*IKKβ and 13 to 266 in *Pm*IKKε1 and *Pm*IKKε2 (**Figure 3.2**).

A ***Pm*IKKβ**

```
 M  A  A  A  E  D  R  P  P  T  Y  P  W  L  K  D  K  V  L  G  T  G  G  F  G  T  V  T  L  W     30
atggcagcagcagaagaccgcccaccgacataccccctggcttaaagacaaggttttggggactggtggatttggtacagtaacattatgg     90

 R  H  N  D  T  G  E  T  I  A  L  K  K  C  R  W  G  T  P  G  T  G  T  E  N  I  L  T  P  K     60
cgtcacaacgatactggagaaactattgccctgaaaaaatgccgctggggaacaccaggaactggcacagaaaatatcctaaccccaaag     180

 H  V  E  R  W  E  K  E  V  E  I  M  N  R  L  N  H  Q  A  V  V  R  C  F  P  V  P  D  E  L     90
catgttgagcgttgggaaaaggaagtagagatcatgaatcgcttaaatcatcaagcagttgtgagatgtttcccagttcccgatgagcta     270

 T  G  P  Q  G  D  L  P  M  L  C  M  E  Y  C  S  G  G  D  L  R  K  V  L  N  K  P  E  N  C    120
actgggcctcagggagatcttcccatgctttgtatggaatactgtagtggtggtgaccttcgcaaggttttaaataagccagagaattgc     360

 C  G  L  R  E  A  A  V  R  S  C  I  R  D  M  T  E  A  V  A  Y  L  H  S  M  R  I  I  H  R    150
tgtggtttacgggaagctgctgtccgttcctgcataagagacatgactgaagctgtggcctaccttcactctatgcgtatcattcatcga     450

 D  L  K  P  E  N  I  V  L  Q  D  V  D  G  K  T  V  Y  K  L  I  D  L  G  Y  A  K  E  L  E    180
gatctgaagcctgagaacattgttctacaggatgttgatgggaaaacagtctacaaacttattgaccttggatatgcgaaagaattagaa     540

 Q  S  S  V  C  T  S  F  V  G  T  L  Q  Y  L  A  P  E  L  F  L  S  K  R  Y  T  C  T  V  D    210
caaagcagtgtctgcacctcctttgttggcacgctccagtacttggctccagaactgttcctcagcaaacgatacacttgcactgttgac     630

 Y  W  S  L  G  L  V  T  H  E  I  I  T  G  I  R  P  F  L  P  N  M  T  P  V  E  W  M  K  R    240
tactggagtctaggtcttgttacacatgaaattattacaggaattcgccccttttcttccaaatatgactccagttgaatggatgaagcgt     720

 V  R  T  K  Q  S  H  H  V  C  V  Y  E  G  R  N  G  E  I  Q  F  S  S  H  M  F  P  E  C  H    270
gtacgcacaaagcagtcccatcatgtatgtgtatatgaaggtcgcaatggagaaattcagttcagttcccacatgtttccagaatgtcat     810

 I  S  Q  P  L  R  T  R  I  E  E  W  L  R  I  M  L  E  W  D  P  V  L  R  G  Q  V  L  D  E    300
atctcacagcctctgagaacaagaatagaggagtggttgcgcatcatgctggagtgggaccctgttttgcgaggccaggtgctggacgag     900

 G  G  A  K  Q  F  V  A  F  N  M  I  N  D  I  L  N  K  K  M  I  K  V  F  V  V  D  L  C  R    330
ggcggggccaagcagtttgtggctttcaacatgatcaacgacattttaaacaagaagatgattaaagtgttcgtagtggacttgtgtcgc     990

 L  L  E  Y  E  V  T  E  S  T  S  L  S  E  V  Q  Q  W  V  A  R  D  S  G  V  L  V  D  D  Q    360
ttgctggaatatgaagtgacagagtccacatccctgtctgaggtgcagcagtgggttgcacgtgacagtggggtgttggtggatgaccaa    1080

 R  P  L  L  P  R  G  Q  P  P  D  P  T  R  P  A  I  Q  C  W  A  P  P  D  E  D  E  W  L  L    390
aggcccctactcccacgtgggcagccccccgaccccactaggcctgccatccagtgctgggcccctccggatgaagatgaatggttgttg    1170

 Y  I  F  A  E  G  M  T  R  P  Q  V  P  P  H  F  P  P  L  V  E  A  M  L  R  E  P  R  T  A    420
tatatatttgctgagggcatgacgcgaccacaggtgccaccacactttccacctttagtagaggcaatgttaagagaaccccgaacggct    1260

 V  E  Y  Q  T  Q  R  R  M  W  A  H  A  V  F  F  L  H  R  E  A  R  L  L  T  L  L  T  Q  A    450
gtcgaataccagacgcagcgtagaatgtgggcacatgctgtgttcttccttcatcgggaagcccgtctccttaccctgttaacgcaagca    1350

 Q  K  V  S  M  L  H  L  M  S  G  H  A  Q  L  T  K  T  G  Q  R  M  L  S  D  I  A  K  L  Q    480
cagaaagtttcaatgcttcacctaatgtctggccatgctcagttaaccaaaaccggccagagaatgcttagtgacattgcaaaacttcag    1440

 A  R  H  H  L  F  M  E  A  L  N  T  D  L  D  Y  Y  D  E  Q  A  S  S  G  R  L  T  S  E  K    510
gcccgtcaccatctcttcatggaggcgctcaacacggacctcgactactatgatgagcaggcttcttcaggacgccttacctcagagaag    1530

 L  Y  S  G  W  R  E  M  G  E  V  T  L  R  Q  V  H  A  V  V  E  R  V  Q  Q  L  E  G  S  L    540
ctatacagtggctggagagaaatgggagaggtaaccctaagacaagtacatgctgtggtcgaacgtgtccagcaactagaaggctcactc    1620

 T  A  L  N  T  R  I  L  E  L  Q  C  S  P  F  A  R  A  R  A  I  D  S  L  D  S  V  L  T  A    570
actgccttaaacactcgcatcctggaactacagtgttcaccatttgcccgagcaagagctattgattctctggattctgtattaacagct    1710

 G  E  D  H  Y  C  N  L  R  R  R  N  K  E  Q  R  A  T  P  H  D  N  T  D  M  C  K  L  L  L    600
ggagaagaccattattgtaatctaagaagaaggaataaagagcagcgtgctacaccacatgataatacagacatgtgcaagttgctgcta    1800

 Q  A  L  R  K  R  D  R  L  Q  Q  D  L  Y  K  H  V  E  K  Q  S  E  C  C  S  E  V  A  A  L    630
caagctttgagaaaaagggacagattgcaacaagatctttataaacacgtagagaaacagagtgaatgttgcagtgaagtagctgctctc    1890

 S  S  P  L  E  A  V  L  Q  D  A  A  R  T  A  Q  H  I  S  S  L  Q  K  Q  R  Q  K  D  I  W    660
tcttcaccattagaagcagttttgcaagatgcagcaagaaccgcacaacacatatcttcacttcagaagcaacgccagaaggacatttgg    1980

 K  I  M  E  I  A  I  N  H  S  R  T  A  G  T  A  A  G  M  A  Q  A  P  Q  T  I  P  N  A  S    690
aagatcatggaaattgcaattaaccacagccgtactgcggggacagcagctggaatggcccaggcgccccagaccattccaaatgcttcg    2070

 Q  L  P  K  K  P  P  S  P  A  V  L  S  S  L  N  N  L  L  E  K  S  K  K  E  S  D  A  I  I    720
cagttgccaaagaaaccccccatcaccagcagtcttgagctcactcaataatttgcttgaaaagtcaaagaaggaatcagatgccattata    2160

 A  E  N  R  A  L  R  C  Q  M  V  E  M  L  S  G  N  V  N  S  N  L  V  I  G  A  A  R  S  P    750
gctgaaaatcgtgctcttcgttgccagatggttgagatgctctcaggaaatgtaaactccaaccttgtaataggcgcagccaggtctcca    2250

 T  G  L  S  P  P  V  T  A  D  G  L  M  L  P  P  A  L  P  E  K  R  T  P  S  P  T  S  Q  A    780
actggcctgagccctccagtcacagcagatggcctcatgctgccaccagccttgcctgagaagaggactccttccccaaccagtcaagca    2340

 A  L  N  K  K  A  V  E  T  S  L  *                                                          791
gcactgaataagaaggcagttgaaacttccttgtag                                                        2376
```

B ***Pm*IKKε**

```
 M  G  S  F  L  R  G  S  A  N  Y  V  W  C  T  T  S  V  L  G  K  G  A  T  G  A  V  F  Q  G     30
atggttcatttctgcgaggatcagccaactatgtctggtgtacgacttctgtcttggggaaggggccacgggagctgtctttcagggc     90

 V  N  R  H  T  G  E  P  V  A  V  K  T  F  N  Q  L  S  H  M  R  P  H  E  V  Q  M  R  E  F     60
gtcaacaggcatacgggagaaccagttgctgtcaagacgtttaatcagctctcacacatgcggccacacgaagtacaaatgcgtgagttt    180

 E  V  L  K  K  V  N  H  E  N  I  V  K  L  L  A  I  E  E  E  Q  E  G  R  G  K  V  I  V  M     90
gaagtacttaagaaggtcaaccatgagaatattgtaaaactcctggctatagaagaagagcaagaaggccgggggaaggtgattgtgatg    270

 E  L  C  T  G  G  S  L  F  N  I  L  D  D  P  E  N  S  H  G  L  E  E  D  E  F  I  L  V  L    120
gagctttgtaccggaggctcactcttcaacattctggacgatccagaaaatagtcatggcctggaggaggatgagtttatcttggttctg    360

 S  H  L  A  A  G  M  K  H  L  R  D  N  S  L  V  H  R  D  L  K  P  G  N  I  M  K  F  T  D    150
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 V  D  G  S  T  I  Y  K  L  T  D  F  G  A  A  R  E  L  Q  D  D  Q  Q  F  M  S  L  Y  G  T    180
gtcgacggatctactatatataagttaacagattttggtgctgctcgagaattgcaagatgaccagcagttcatgtctctatatggaaca    540

 E  E  Y  L  H  P  D  M  Y  E  R  A  V  L  R  K  P  V  G  K  T  F  G  A  R  V  D  L  W  S    210
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 I  G  V  T  L  Y  H  V  A  T  G  Q  L  P  F  R  P  Y  G  G  R  R  N  K  E  T  M  Y  H  I    240
ataggtgtgacactttaccacgtggccacaggtcagcttcctttccggccgtatggaggtcggcgaaacaaagagaccatgtaccatata    720

 T  T  E  K  A  P  G  V  I  S  G  V  Q  T  S  E  N  G  P  I  D  W  C  T  E  L  P  E  T  C    270
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 R  L  S  L  G  L  R  K  L  V  T  P  L  L  A  G  L  L  E  V  D  P  Q  R  M  W  N  F  E  R    300
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 F  F  Q  E  V  T  M  I  L  S  K  K  V  V  H  I  F  F  V  N  K  V  Q  P  I  T  V  Y  M  D    330
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 H  L  S  D  I  V  A  P  D  Q  P  S  S  S  Y  P  S  T  T  P  R  T  P  L  V  L  F  S  K  Q    390
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 D  D  D  I  T  L  T  L  P  E  T  P  A  V  K  F  G  S  F  P  T  L  V  S  V  E  H  D  A  A    420
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 V  G  K  S  M  C  S  V  G  H  A  I  K  R  K  I  D  Y  F  S  K  C  V  H  L  M  D  Y  S  V    450
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 L  M  F  I  E  V  I  V  T  Q  L  T  T  L  Q  D  R  V  G  H  V  Q  S  L  T  S  A  V  S  D    480
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 Q  L  Y  E  R  V  V  R  G  G  Q  L  R  R  Q  W  Q  Q  A  G  N  N  A  V  A  V  E  R  A  P    600
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 H  T  T  D  N  L  A  D  W  C  K  V  A  K  V  Q  R  V  Q  T  E  I  E  E  A  D  V  E  K  H    690
cacactacagataacttggccgactggtgcaaagtcgccaaagtccagcgagtccagacggagattgaagaggcggacgttgagaagcac   2070

 E  G  L  L  S  S  F  Q  D  T  L  G  N  T  E  D  Q  Y  H  Q  T  L  S  G  L  L  A  A  I  K    720
gaggggctgttgtcttctttccaggacaccttaggcaatacagaggaccagtaccaccaaaccctctctggactcctggcagccattaag   2160

 D  K  K  L  Q  D  D  P  R  L  Q  T  E  N  P  A  A  A  L  E  *                               740
gacaagaagttgcaggacgacccgagattgcaaactgagaatccagcggccgcactcgagtga                               2223
```

Figure 3.1 Nucleotide and deduced amino acid sequences of *PmIKKβ*, *PmIKKε1* and *PmIKKε2* genes from *Penaeus monodon*. The ORFs of (**A**) *PmIKKβ* and (**B**) *PmIKKε1* and *PmIKKε2* were cloned and sequenced successfully. The start codons

(ATG) are bold and stop codons (TAG, TGA) are indicated with an asterisks (*). The 90-bp nucleotide sequence which is absent in *PmIKKε2* is boxed. The important kinase domains (KDs) were predicted using SMART program. The N-terminal KDs are underlined from amino acid residues 13 to 286 in *Pm*IKKβ and 13 to 266 in *Pm*IKKε1 and *Pm*IKKε2.

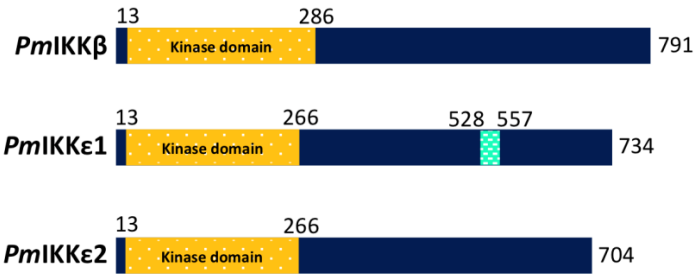
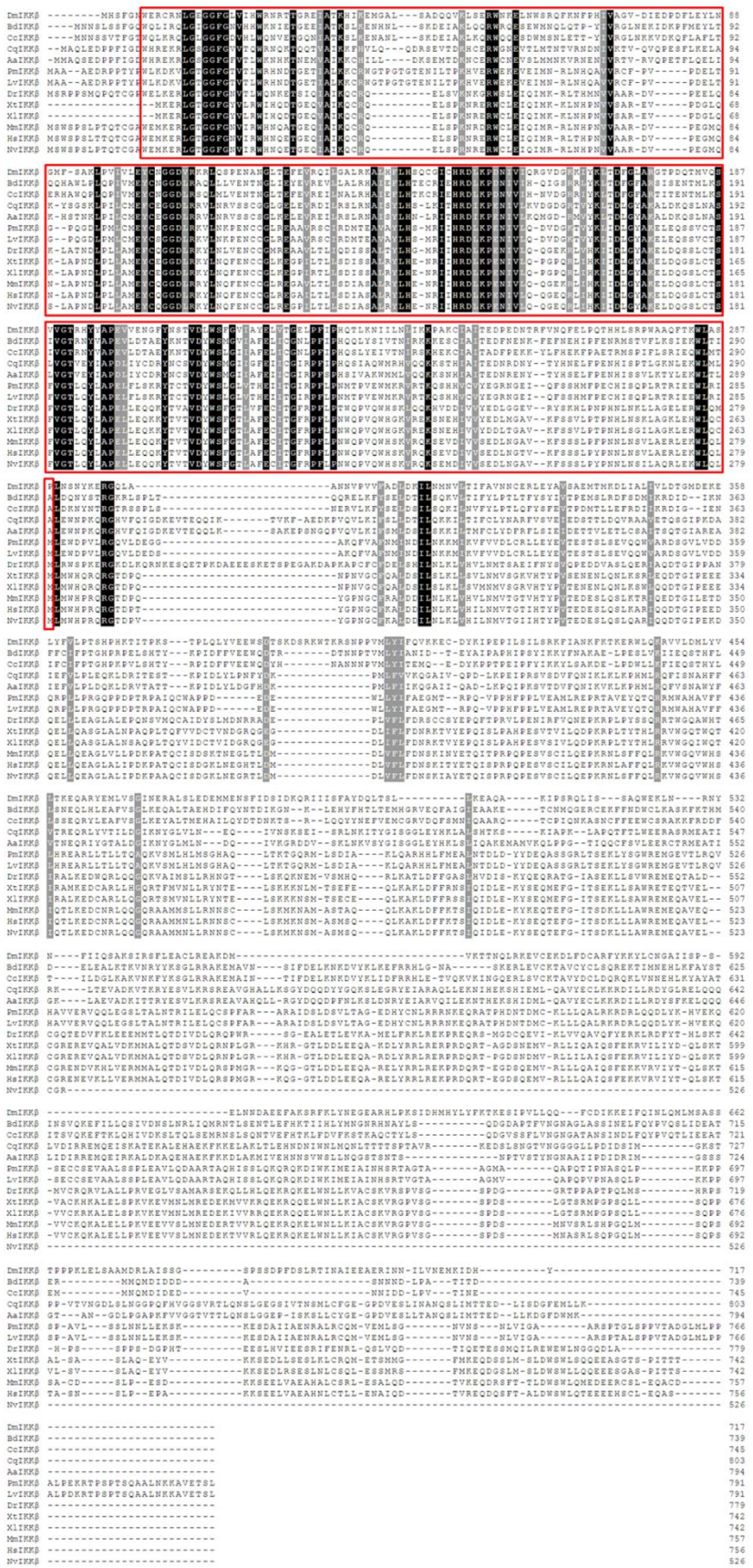


Figure 3.2 Schematic diagram of structural domain topology of *Pm*IKKβ, *Pm*IKKε1 and *Pm*IKKε2 analyzed by SMART 8.0 program. N-terminal KDs are marked as yellow from amino acid residues 13 to 286 of *Pm*IKKβ and amino acid residues 13 to 266 of *Pm*IKKε1 and *Pm*IKKε2. *Pm*IKKε1 contains unidentified domain from amino acid residues 528 to 557 at the C-terminal region.

3.2 Multiple sequence alignment and phylogenetic analysis

To examine the evolutionary relationship of IκB kinases among various organisms, the deduced amino acid sequences of *Pm*IKKβ, *Pm*IKKε1 and *Pm*IKKε2 were aligned with IKK and IKK-family proteins from various species. Multiple sequence alignment performed using Clustal Omega revealed the important N-terminal kinase domains which is conserved among IKK and IKK family proteins from the examined species (**Figure 3.3A and 3.3B**). The phylogenetic analysis was performed in MEGA 7.0 software to construct an unrooted neighbor-joining phylogenetic tree based on the deduced amino acid sequences. The bootstrap sampling was reiterated for 1000 times and demonstrated the divided clusters comprising species of mammalian, arthropod and mollusk. As the results, *Pm*IKKβ and *Pm*IKKε from *Penaeus monodon* were grouped with the closely related *Lv*IKKβ and *Lv*IKKε from *Litopenaeus vannamei*, respectively (**Figure 3.4**).

A



B

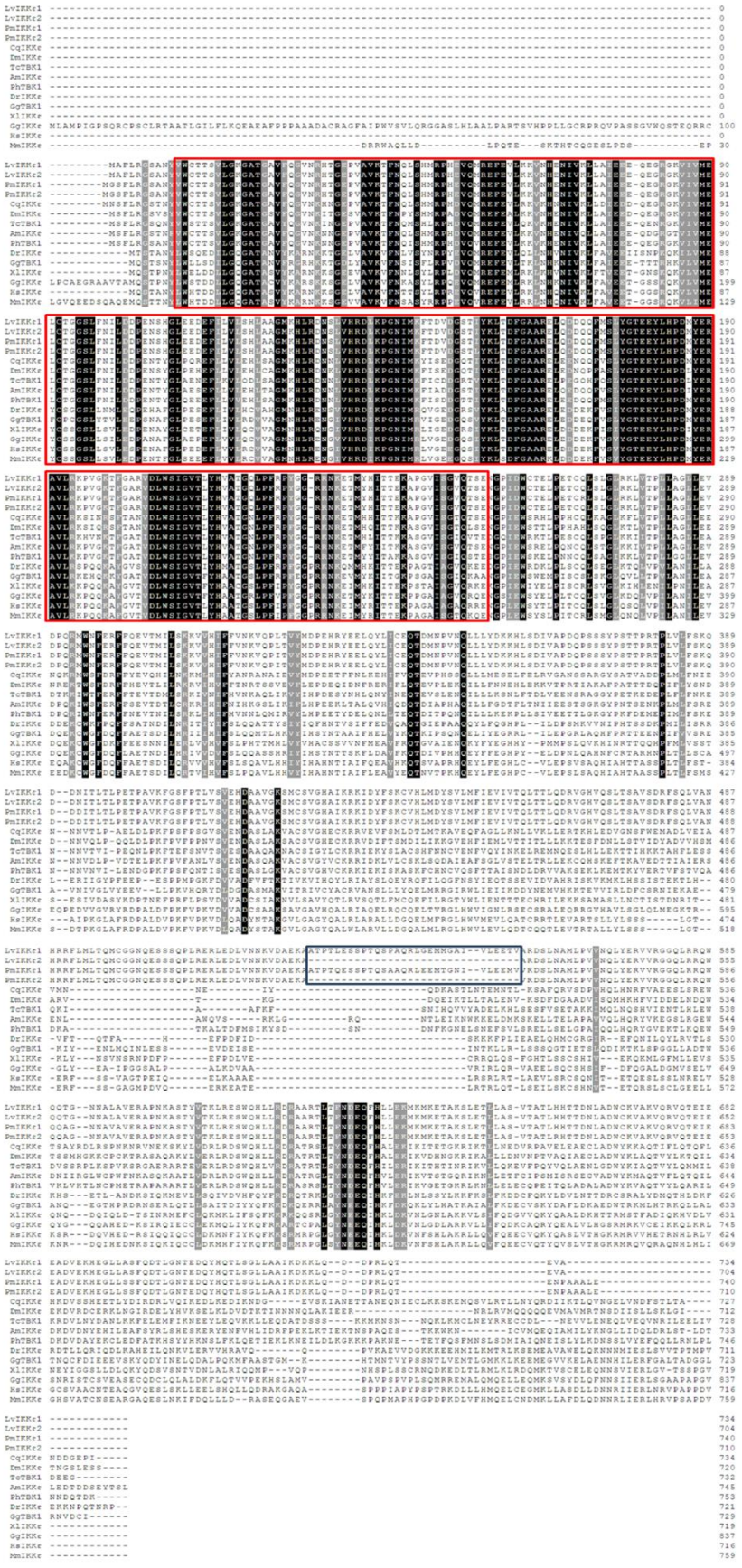


Figure 3.3Sequence analysis of *Pm*IKK and IKK family proteins from various species. Multiple sequence alignments of (**A**) *Pm*IKKβ and (**B**) *Pm*IKKε with IKK-family proteins were performed using Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo). The amino acid sequences of *Pm*IKKβ, *Pm*IKKε1 and *Pm*IKKε2 were deduced by ExPASy-Translate tool (https://web.expasy.org/translate). Important protein motif features were predicted using Simple Modular Architecture Research Tool, SMART 8.0 (http://smart.embl-heidelberg.de). The conserved residues are shaded in black and grey. The important kinase domains at the N-termini are in the red boxes and the 30-amino acid regions of *Pm*IKKε1 and *Lv*IKKε1 are in the blue box below. The amino acid sequences include; *Penaeus monodon* IKKβ (*Pm*IKKβ, MK331816); *Litopenaeus vannamei* IKKβ (*Lv*IKKβ, AEK86518); *Mus muculus* IKKβ (*Mm*IKKβ, NP_001153246); *Homo sapiens* IKKβ (*Hs*IKKβ, NP_001547); *Xenopus tropicalis* IKKβ (*Xt*IKKβ, NP_001005651); *Xenopus laevis* IKKβ (*Xl*IKKβ, NP_001085125); *Danio rerio* IKKβ (*Dr*IKKβ, NP_001116737); *Drosophila melanogaster* IKKβ (*Dm*IKKβ, AAG02485); *Culex quinquefasciatus* IKKβ (*Cq*IKKβ, XP_001865661); *Mustela putorius* IKKβ (*Mp*IKKβ, XP_004775760); *Bactrocera dorsalis* IKKβ (*Bd*IKKβ, XP_011211311); *Ceratitis capitatal* IKKβ (*Cc*IKKβ, XP_004537145); *Aedea aegypti* IKKβ (*Aa*IKKβ, XP_001656614); *Penaeus monodon* IKKε1 (*Pm*IKKε1, MK331817); *Penaeus monodon Pm*IKKε2 (*Pm*IKKε2, MK331818); *Litopenaeus vannamei* IKKε1 (*Lv*IKKε1, AEK86519); *Litopenaeus vannamei* IKKε2 (*Lv*IKKε2, AEK86520); *Culex quinquefasciatus* IKKε (*Cq*IKKε, XP_001848400); *Drosophila melanogaster* IKKε (*Dm*IKKε, NP_724278); *Apis mellifera* IKKε (*Am*IKKε, XP_396937); *Xenopus laevis* IKKε (*Xl*IKKε, NP_001089830); *Danio rerio* IKKε (*Dr*IKKε, NP_001002751); *Gallus gallus* IKKε (*Gg*IKKε, XP_428036); *Homo sapiens* IKKε (*Hs*IKKε, NP_054721); *Mus musculus* IKKε (*Mm*IKKε, EDL39711); *Gallus gallus* TBK1 (*Gg*TBK1, NP_001186487), *Tribolium castaneum*TBK1 (*Tc*TBK1, XP_969718) and *Pediculus humanus* TBK1 (*Ph*TBK1, XP_002428501).

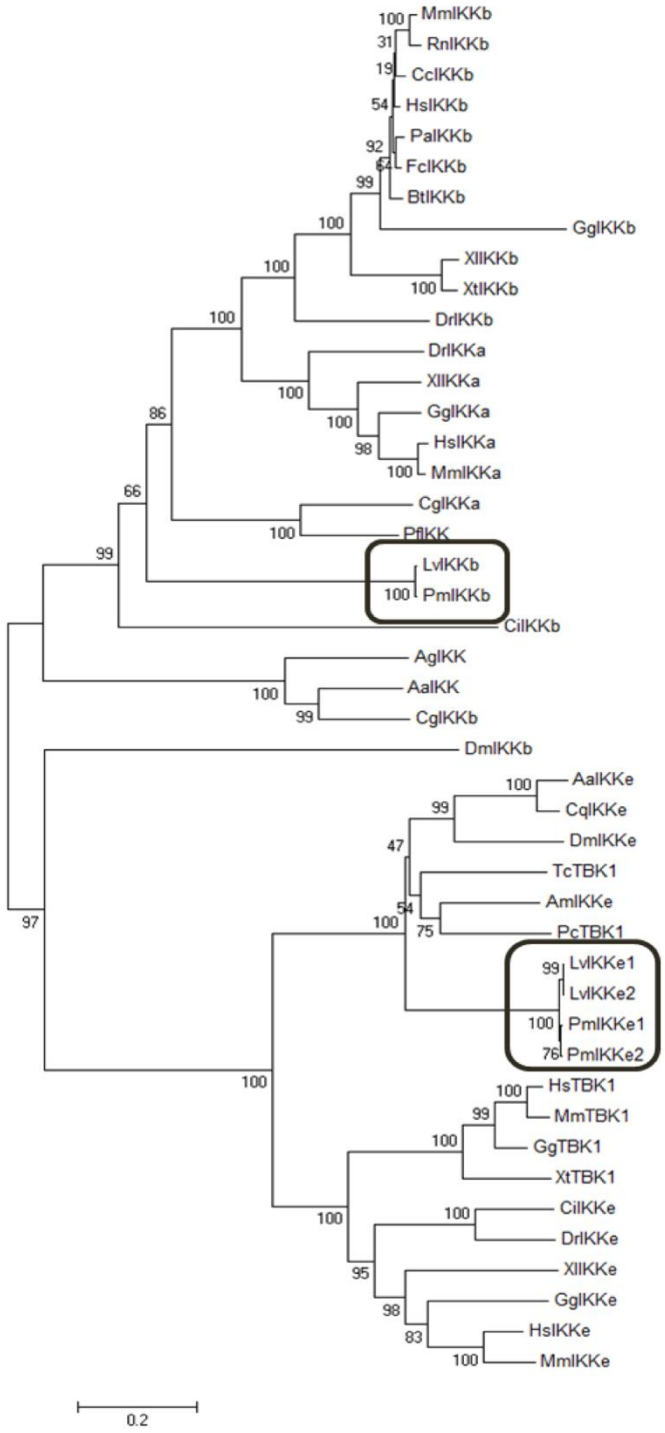


Figure 3.4 Phylogenetic analysis of IκB kinases (IKKs) and IKK-family proteins from *Penaeus monodon* and various species. The neighbor joining phylogenic tree was constructed in MEGA 7.0 software based on the amino acid sequences of IKKβ, IKKε and IKK-family proteins from vertebrates and invertebrates. Bootstrap sampling

was reiterated for 1000 times. The deduced amino acid sequences retrieved from various species include; *Penaeus monodon* IKKβ (*Pm*IKKβ, MK331816); *Litopenaeus vannamei* IKKβ (*Lv*IKKβ, AEK86518); *Mus muculus* IKKβ (*Mm*IKKβ, NP_001153246); *Rattus norvegicus* IKKβ (*Rn*IKKβ, AAF21978); *Castor canadensis* IKKβ (*Cc*IKKβ, P_020011901); *Homo sapiens* IKKβ (*Hs*IKKβ, NP_001547); *Pongo abelii* IKKβ (*Pa*IKKβ, XP_024106853); *Felis catus* IKKβ (*Fc*IKKβ, XP_003984800); *Bos tausus* IKKβ (*Bt*IKKβ, NM_174353); *Danio rerio* IKKβ (*Dr*IKKβ, NP_001116737); *Xenopus tropicalis* IKKβ (*Xt*IKKβ, NP_001005651); *Xenopus laevis* IKKβ (*Xl*IKKβ, NP_001085125); *Cricetulus griseus* IKKβ (*Cg*IKKβ, XP_027293025); *Gallus gallus* IKKβ (*Gg*IKKβ, NP_001026568); *Drosophila melanogaster* IKKβ (*Dm*IKKβ, AAG02485); *Aedes aegypti* IKK (*Aa*IKK, EAT45468); *Anopheles gambiae* IKK (*Ag*IKK, XP_553095); *Ciona intestinalis* IKK (*Ci*IKK, XP_002125567); *Pinctada fucata* IKK (*Pf*IKK, AAX56336); *Mus musculus* IKKα (*Mm*IKKα, AAC52589); *Homo sapiens* IKKα (*Hs*IKKα, NP_001269); *Crassostrea gigas* IKKα (*Cg*IKKα, NP_001295815); *Xenopus laevis* IKKα (*Xl*IKKα, NP_001086127); *Danio rerio* IKKα (*Dr*IKKα, AAW68010); *Gallus gallus* IKKα (*Gg*IKKα, NP_001012922); *Penaeus monodon* IKKε1 (*Pm*IKKε1, MK331817); *Penaeus monodon Pm*IKKε2 (*Pm*IKKε2, MK331818); *Litopenaeus vannamei* IKKε1 (*Lv*IKKε1, AEK86519); *Litopenaeus vannamei* IKKε2 (*Lv*IKKε2, AEK86520); *Culex quinquefasciatus* IKKε (*Cq*IKKε, XP_001848400); *Drosophila melanogaster* IKKε (*Dm*IKKε, NP_724278); *Apis mellifera* IKKε (*Am*IKKε, XP_396937); *Aedes aegypti* IKKε (*Aa*IKKε, XP_001650774); *Homo sapiens* IKKε (*Hs*IKKε, NP_054721); *Mus musculus* IKKε (*Mm*IKKε, EDL39711); *Xenopus laevis* IKKε (*Xl*IKKε, NP_001089830); *Danio rerio* IKKε (*Dr*IKKε, NP_001002751); *Ciona intestinalis* IKKε (*Ci*IKKε NP_001072034); *Gallus gallus* IKKε (*Gg*IKKε, XP_428036); *Xenopus tropicalis* TBK1 (*Xt*TBK1, NP_001135652); *Homo sapiens* TBK1 (*Hs*TBK1, NP_037386); *Macaca mulatta* TBK1(*Mm*TBK1, NP_001248122); *Phalacrocorax carbo* (*Pc*TBK1, XP_009506274.1); *Gallus gallus* TBK1 (*Gg*TBK1, NP_001186487) and *Tribolium castaneum*TBK1 (*Tc*TBK1, XP_969718).

3.3 Tissue-specific expression of *PmIKKβ* and *PmIKKε*

Several tissues were collected from healthy *P. monodon* to investigate the mRNA expression of *PmIKKβ*, *PmIKKε1* and *PmIKKε2*. Total RNAs were extracted from selected tissues and reverse transcribed to cDNA. The expression was observed by semi-quantitative RT-PCR using elongation factor-1α gene (*EF1-α*) as an internal control. The amplicon of 322 bp was detected from the gene-specific cDNA fragment of *PmIKKβ*. Moreover, using specifically designed primers for amplification, the amplicons of 249 bp and 230 bp from *PmIKKε1* and *PmIKKε2* were amplified distinguishably. The *PmIKKβ*, *PmIKKε1* and *PmIKKε2* transcripts were detected in all examined tissues with high mRNA expression of *PmIKKβ* and *PmIKKε1* in hemocytes (Hc) which is an immune-related tissue. However, the *PmIKKε2* transcript was moderately expressed in hemocytes (**Figure 3.5**).

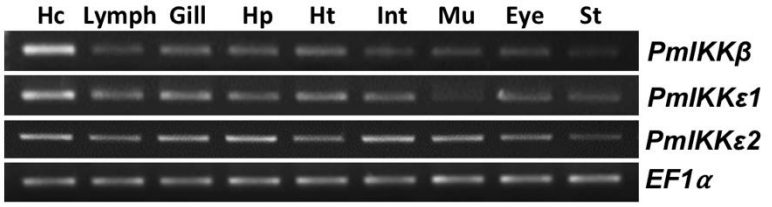


Figure 3.5Tissue-specific gene expression of *PmIKKβ*, *PmIKKε1* and *PmIKKε2* by semi-quantitative RT-PCR. Various tissues were collected from healthy *P. monodon* including; hemocyte (Hc), lymphoid organ (Lymp), gill (G), hepatopancreas (Hp), heart (Ht), intestine (Int), muscle (Mu), eyestalk (Eye) and stomach (St). Total RNA was extracted for tissue distribution analysis using semi-quantitative RT-PCR. The elongation factor1-α gene (*EF-1α*) was used as an internal control.

3.4 Recombinant protein expression and localization of *Pm*IKKβ and *Pm*IKKε in shrimp hemocytes

3.4.1 Plasmid construction and expression of *Pm*IKKβ and *Pm*IKKε recombinant proteins for validating antibody specificity

To examine the expression of *Pm*IKKβ and *Pm*IKKε proteins in different types of shrimp hemocytes, immunofluorescence staining and confocal microscopy were performed using anti-IKKβ and anti-IKKε antibodies specific to human IKKβ and IKKε, respectively. In order to obtain the particular antibodies, the recombinant *Pm*IKKβ (r*Pm*IKKβ) and *Pm*IKKε1 (r*Pm*IKKε1) proteins were expressed in *E. coli* system. The cDNA fragments with attached restriction recognition sites of *Nco*I and *Xho*I for *Pm*IKKβ (2400 bp) and of *Nco*I and *Not*I for *Pm*IKKε1 (2241 bp) were amplified and cloned into pET-28b(+) (**Figure 2.1**) as an expression vector with hexa-histidine tag sequence to construct pET-28b(+)-*Pm*IKKβ-6xHis and pET-28b(+)-*Pm*IKKε1-6xHis. The recombinant plasmids were prior screened using restriction digestions with *Nco*I and *Xho*I for pET-28b(+)-*Pm*IKKβ-6xHis and that with *Nco*I and *Not*I for pET-28b(+)-*Pm*IKKε1-6xHis (**Figure 3.6**). They were further verified by nucleotide sequencing before introduced to *E. coli* strain BL21-CodonPlus (DE3)-RIPL as an expression host.

To express the recombinant proteins, a bacterium *E. coli* strain BL21-CodonPlus (DE3)-RIPL harboring pET-28b(+)-*Pm*IKKβ-6xHis or pET-28b(+)-*Pm*IKKε1-6xHis expression plasmids was grown in LB medium containing kanamycin until the OD_{600} reached approximately 0.6. Cells were harvested and disrupted at 0, 2, 4, 6 hours after induction with 1 mM IPTG. The r*Pm*IKKβ and r*Pm*IKKε1 were used to test the cross reactivity with commercial antibodies in western blot analysis prior to immunofluorescence staining. The inclusion and soluble fractions from crude lysate containing r*Pm*IKKβ and r*Pm*IKKε1 were measured for total protein concentration by Bradford protein assay (Bradford, 1976). The total protein of 10 μg was detected in 10% SDS-PAGE with Coomassie brilliant blue R-250 staining reagent and immunostaining. The commercial human anti-IKKβ and anti-IKKε antibodies were used as the primary antibodies for the detection. The

r*Pm*IKKβ and r*Pm*IKKε1 which exhibit molecular masses of 90.44 and 84.35 kDa, respectively were expressed at 37°C with 250 rpm shaking mainly as the inclusion bodies at 2, 4 and 6 hours post induction (**Figure 3.7**). Since the commercial antibodies exhibited high specificity to shrimp IKK recombinant proteins, the large-scale protein production for antibody raising was not further carried out.

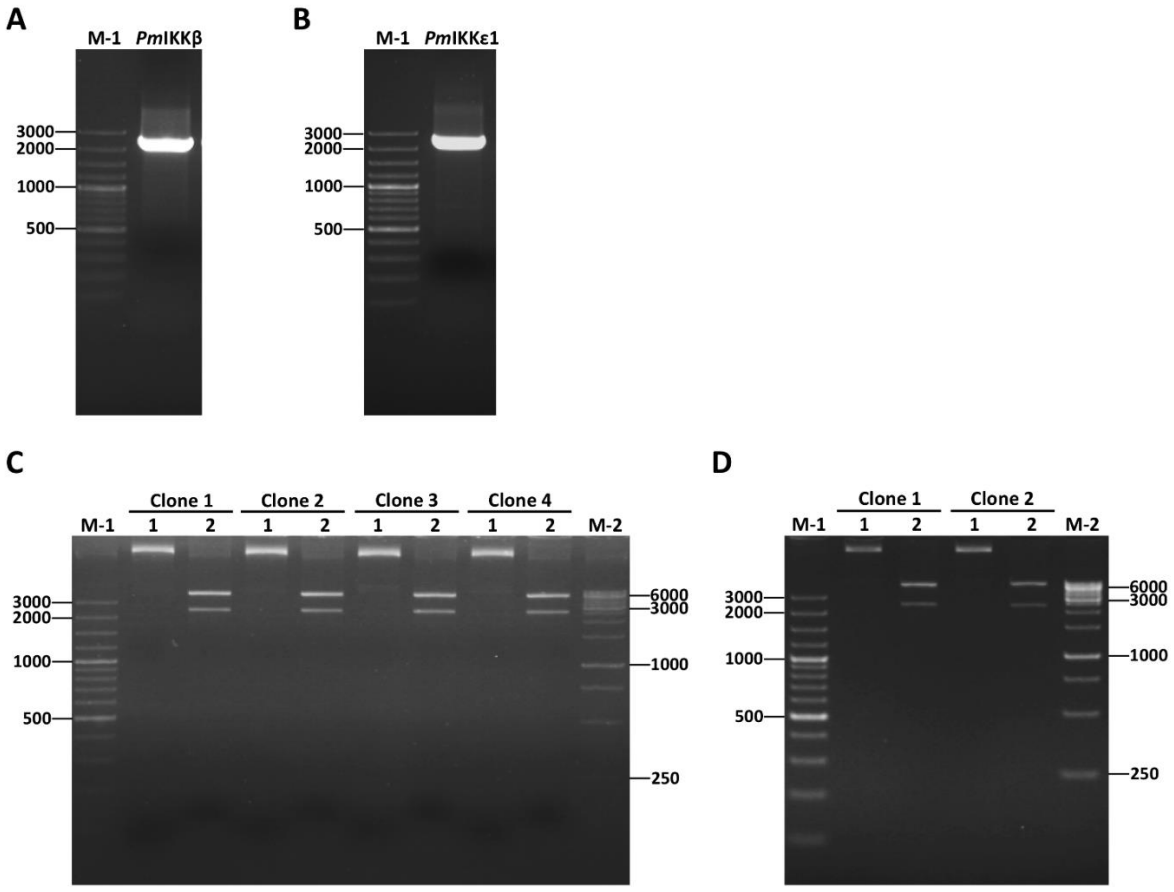


Figure 3.6 PCR amplification of *PmIKKβ* and *PmIKKε1* and plasmid DNA screening for protein expression. The ORFs of (A) *PmIKKβ* (2400 bp) and (B) *PmIKKε1* (2241 bp) were amplified in the PCR reactions with attached hexa-histidine tag sequence and cloned into pET-28b(+) to construct protein expression vectors. The recombinant plasmids pET-28b(+) harboring (C) *Pm*IKKβ were digested with *Nco*I and *Xho*I while that harboring (D) *Pm*IKKε1 were screened by *Nco*I and *Not*I. They were visualized under UV transilluminator following 1% (w/v) agarose-TBE gel electrophoresis. M-1 is Thermo Scientific™ GeneRuler 100 bp Plus DNA Ladder. M-

2 is Thermo Scientific™ GeneRuler 1 kb DNA Ladder. Lanes1 and 2 represent uncut and digested plasmid DNAs, respectively.

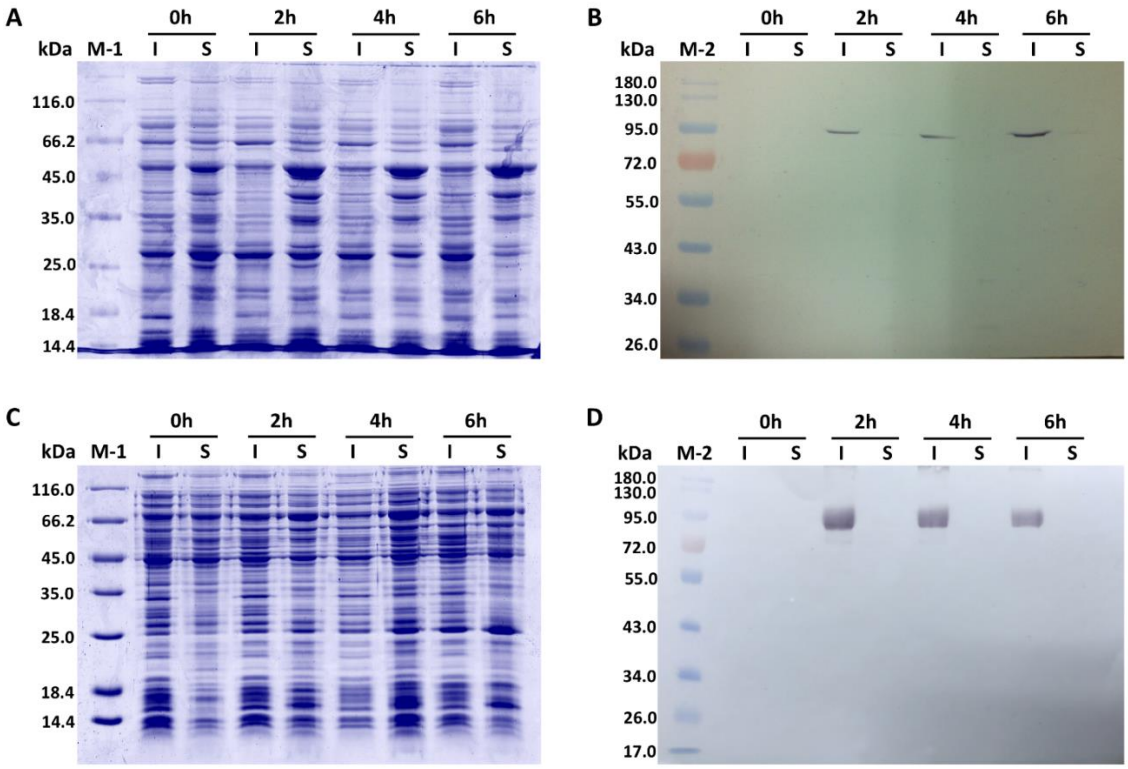


Figure 3.7 Specificities of anti-IKKβ and anti-IKKε antibodies in detection of recombinant *Pm*IKKβ and *Pm*IKKε1 proteins. The overexpressed recombinant (A) *Pm*IKKβ (90.44 kDa) and (C) *Pm*IKKε1 (84.35 kDa) in 10 μg total protein from *E. coli* strain BL21-CodonPlus (DE3)-RIPL were analyzed by 10% SDS-PAGE. Cells were harvested at the indicated time points and detected for protein expression using Coomassie brilliant blue staining. Inclusion and soluble fractions containing recombinant (B) *Pm*IKKβ and (D) *Pm*IKKε were transferred to nitrocellulose membrane. The specificities of anti-IKKβ and anti-IKKε antibodies were examined as the primary antibodies in immunoblotting. M-1 is Thermo Scientific™ Pierce™ Unstained Protein MW Marker. M-2 is Thermo Scientific™ PageRuler™ Prestained Protein Ladder. Lanes I and S represent inclusion and soluble fractions, respectively. 0h, 2h, 4h, 6h indicate hours post induction with 10 mM IPTG.

3.4.2 Detection of endogenous *Pm*IKKβ and *Pm*IKKε proteins in shrimp hemocytes using immunofluorescence microscopy

In order to perform immunofluorescence and confocal microscopy, shrimp hemocytes were collected, fixed and processed for the detection of endogenous *Pm*IKKβ and *Pm*IKKε proteins. Nuclei were stained blue with Hoechst 33342 while the *Pm*IKKβ and *Pm*IKKε were visualized in green with Alexa Fluor® 488. The fluorescent microscopic images revealed that *Pm*IKKε protein was expressed mainly in cytoplasm and slightly in nucleus of all three types of hemocytes including hyaline cells (HC), granular cells (GC) and semi-granular cells (SGC) (**Figure 3.9**). Unfortunately, the human anti-IKKβ antibody was not suitable for examining protein expression with fluorescent immunostaining in shrimp hemocytes as a result of weak fluorescent signal even with higher concentration used (data not shown).

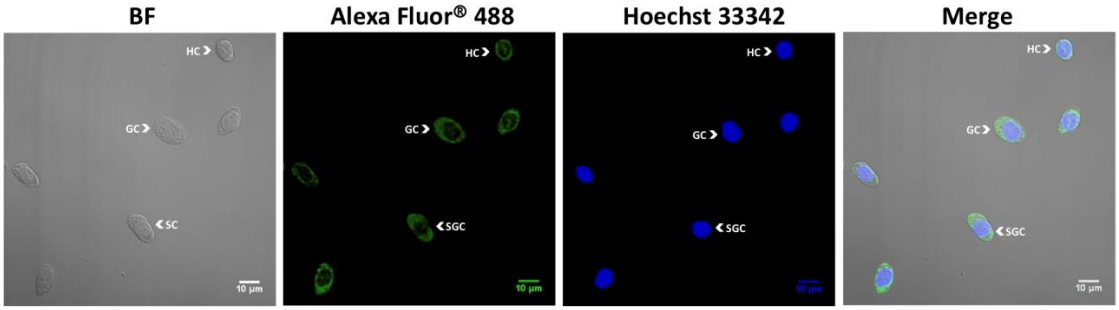


Figure 3. 8 Protein localization of *Pm*IKKε in three different types of *P. monodon* hemocytes. Total hemocytes including hyaline cells (HC), granular cells (GC) and semi-granular cells (SGC) were drawn from three healthy shrimp and fixed immediately in 1X PBS containing 4% paraformaldehyde. Cells were washed, counted with hemocytometer and mounted on coverslips for immunofluorescence staining. *Pm*IKKε was detected using a monoclonal antibody specific to human IKKε. The nuclei were stained blue with Hoechst 33342 and *Pm*IKKε was stained green with Alexa Fluor® 488. The detection was performed using an LSM700 laser scanning confocal microscope (Carl Zeiss).

3.5 Temporal expression of *PmIKKβ* and *PmIKKε* mRNAs after pathogen challenges

The transcript levels of *PmIKKβ*, *PmIKKε1* and *PmIKKε2* were determined to investigate the effects of infection by viruses including WSSV and YHV and a bacterium *Vibrio harveyi*. Following the infection with 1×10^5 copies of purified WSSV or YHV or 2×10^5 CFU/ml of *Vibrio harveyi* strain 639, total shrimp hemocytes was collected for RNA isolation at 0, 6, 12, 24, 48 hpi. Quantitative RT-PCR showed that after WSSV infection, *PmIKKε1* was up-regulated by 2.23-fold and 1.7-fold at 6 and 24 hours post infection, respectively, while *PmIKKε2* was up-regulated at 24 hours by 2.1-fold. YHV infection also induced the expression of *PmIKKε1* by 58-fold and 12-fold at 6 and 24 hours, respectively, while *PmIKKε2* was up-regulated at 6 hours by 3.27-fold compared to the PBS-injected group (**Figure 3.9A and 3.9B**). Moreover, *PmIKKε1* was up-regulated at 24 hpi upon *V. harveyi*, whereas, down-regulation was detected at 6 and 48 hpi. *PmIKKε2* was also slightly down-regulated by 0.5-fold at 6 hpi ($P<0.05$) (**Figure 3.9C**). However, there was no significant difference in the expression level of *PmIKKβ* after infection with WSSV, YHV or *V. harveyi* compared to the control PBS-injected shrimp ($P<0.05$).

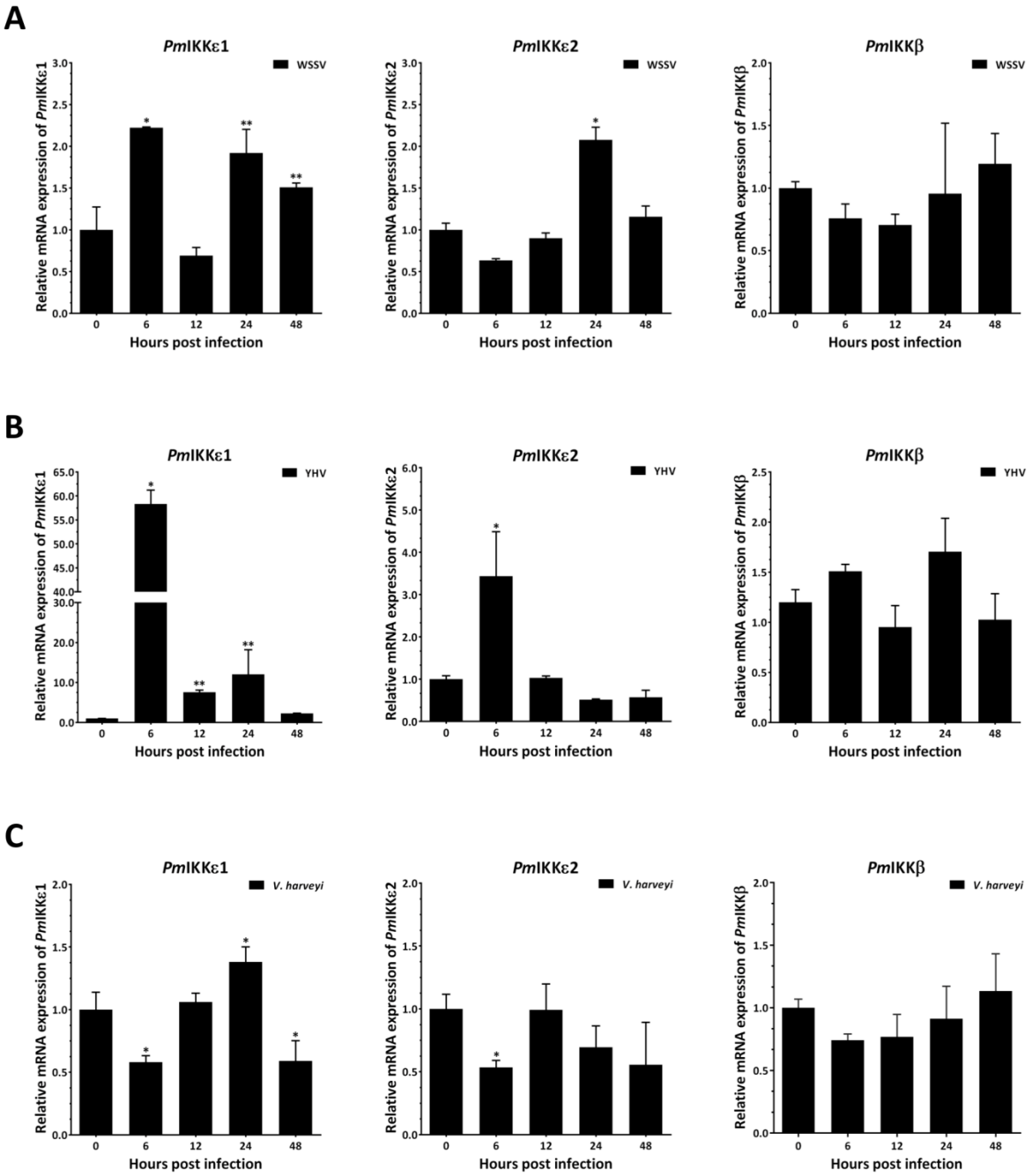


Figure 3.9 Temporal expression of *PmIKKβ*, *PmIKKε1* and *PmIKKε2* in shrimp hemocyte upon immune challenge with WSSV, YHV and *Vibrio harveyi*. Healthy *P. monodon* (10-15 g) were injected intramuscularly at the third abdominal segment with 30 µl of PBS as a control, 1×10^5 copies of purified WSSV, 1×10^5 copies of purified YHV or 30 µl of 1×10^6 CFU/ml of *V. harveyi* 639. Three shrimp were randomly collected at 0, 6, 12, 24 and 48 hpi from each group and total hemocyte was

obtained for qRT-PCR analysis. Expression levels of *PmIKKβ*, *PmIKKε1* and *PmIKKε2* in hemocytes of shrimp challenged with (**A**) WSSV, (**B**) YHV, and (**C**) *Vibrio harveyi* was normalized with those of control PBS group and set to 1.0 at 0 hpi. Calculation of relative mRNA expression was performed according to Pfaffl method (2001) using *EF1-α* as a reference gene. Data are derived from three independently triplicate experiments and shown as the means ± SDs. Asterisks indicate significant differences of mean values ($P<0.05$).

3.6 *In vivo* gene knockdown of *PmIKKβ* and *PmIKKε* by dsRNA-mediated RNA interference

3.6.1 Preparation of double-stranded RNA (dsRNA)

The dsRNA-mediated RNA interference was performed to characterize the roles of *Pm*IKKβ and *Pm*IKKε in shrimp innate immune system. Double-stranded RNAs (dsRNAs) corresponding to *Pm*IKKβ, *Pm*IKKε and GFP sequences (ds*Pm*IKKβ, ds*Pm*IKKε and dsGFP respectively) were synthesized by *in vitro* transcription using T7 RiboMAX™ Express Large Scale RNA Production System (Promega). DNA templates for *in vitro* transcription of sense and antisense RNA strands were amplified separately by PCR reactions containing gene specific primers attached with T7 RNA polymerase binding site (Table 1) and analyzed in 1% (w/v) agarose-TBE gel electrophoresis (**Figure 3.10**).

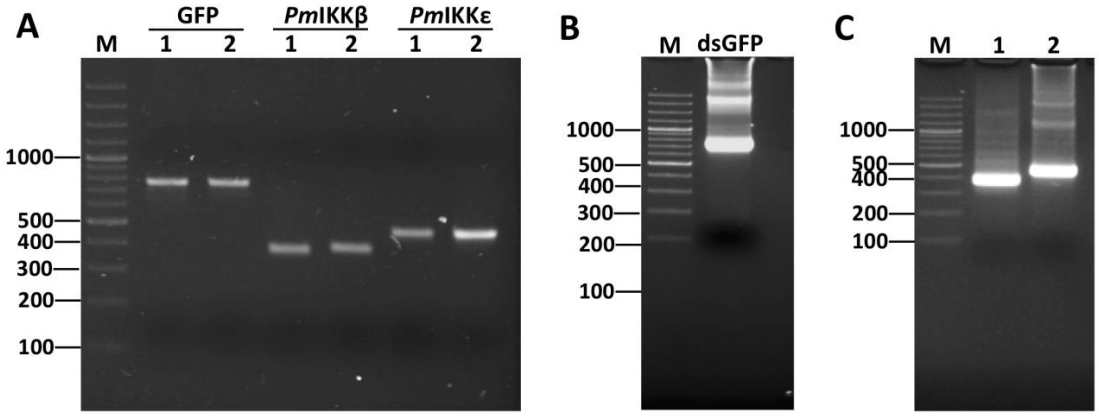


Figure 3.10 Preparation and purification of *Pm*IKKβ, *Pm*IKKε and GFP double-stranded RNAs. DNA templates for *in vitro* transcription of sense and anti-sense RNA strands were amplified by PCR reactions and purified separately. (A) Purified DNA templates for dsGFP, ds*Pm*IKKβ and ds*Pm*IKKε, sense; lane 1 and anti-sense; lane 2. Double-stranded RNAs (dsRNAs) were synthesized using T7 RiboMAX™ Express Large Scale RNA Production System (Promega) according to manufacturer's protocol. (B) Purified double-stranded RNA specific to *GFP* gene. (C) Purified ds*Pm*IKKβ; lane 1 and ds*Pm*IKKε; lane 2. M is Thermo Scientific™ GeneRuler 100 bp Plus DNA Ladder.

3.6.2 Optimization of gene knockdown using dsRNA

The dsRNA injection was optimized *in vivo* to obtain the optimal concentration for gene silencing. A single injection was performed to juvenile shrimp (3-5 g) at the third abdominal segment with different dsRNA concentrations of including 5 μg/ g shrimp and 10 μg/ g shrimp. Following the dsRNA injection, shrimp were randomly selected for hemocyte RNA extraction. Transcription levels of *PmIKKβ* and *PmIKKε* were determined using semi-quantitative RT-PCR and showed no significant suppression after single injection (**Figure 3.11**). However, the double injection was investigated and showed the significant suppression of *PmIKKβ* and *PmIKKε* transcripts after injection with 10 μg/g shrimp dsRNA. Quantitative RT-PCR was performed and showed the significant decrease of *PmIKKβ* transcript by 0.4-fold in ds*PmIKKβ*-injected shrimp compared to that observed in the control ds*GFP*-injected

group. Moreover, the expression *PmIKKε* was significantly decreased to 0.1-fold in ds*PmIKKε*-injected shrimp compared to the level observed in the control ds*GFP*-injected group (**Figure 3.12**).

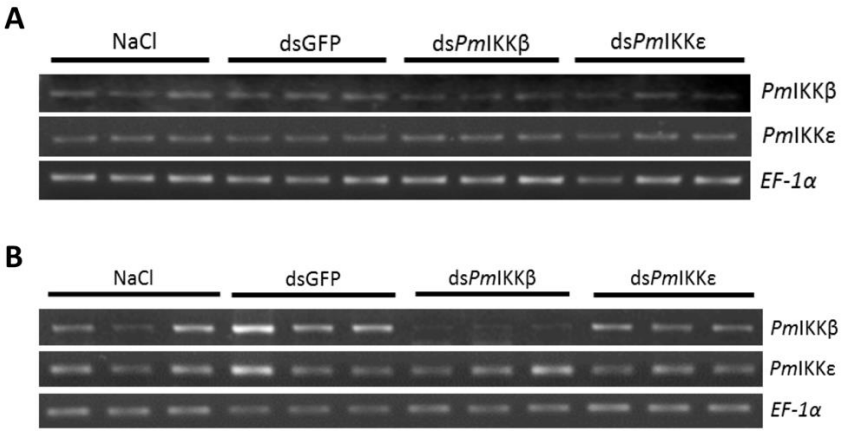


Figure 3.11 Optimization of dsRNA concentration for *in vivo* gene silencing. A single injection was performed to juvenile shrimp (3-5 g) with different dsRNA concentrations of (A) 5 μg/ g shrimp and (B) 10 μg/ g shrimp. The dsRNAs were dissolved in 150 mM NaCl solution for injecting intramuscularly at the third abdominal segment. At 24 hours post injection, total RNA was extracted and transcription levels of *PmIKKβ* and *PmIKKε* were determined using semi-quantitative RT-PCR. The elongation factor-1α gene (*EF-1α*) was used as an internal control. The amplified PCR products were analyzed in 2% (w/v) agarose-TBE gel electrophoresis and visualized by UV-transillumination.

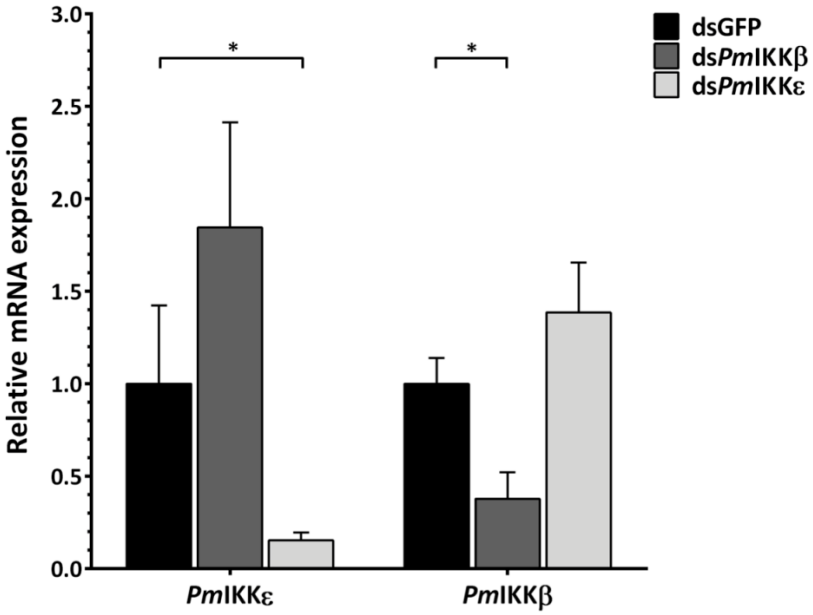


Figure 3.12 Expression profiles of *PmIKKβ* and *PmIKKε* after gene silencing by dsRNA-mediated RNAi. Juvenile shrimp (3-5 g) p were doubly injected with 10 μg/ g shrimp ds*GFP* (control), ds*PmIKKβ* or ds*PmIKKε* with an interval of 24 hours. Total hemocytes were collected at 24 hours after final dsRNA injection for total RNA isolation and cDNA synthesis. The mRNA expression levels of *PmIKKβ* and *PmIKKε* were determined by qRT-PCR independently in three triplicates. The expression was normalized with *EF1-α* as a reference gene. Data are shown as the mean fold change (means ± SDs, *n*=3) relative to a control ds*GFP*-injected group. Asterisks indicate significant differences of mean values ($P<0.05$).

3.7 Survival rate of WSSV-infected shrimp and viral copy number after *Pm*IKKβ and *Pm*IKKε silencing

To examine the effect of *PmIKKβ* and *PmIKKε* silencing on shrimp mortality upon WSSV infection, shrimp were injected with dsRNAs as described in 2.2.7. Twenty-four hours following the dsRNA injection, shrimp were infected with 1×10^5 copies of purified WSSV and the cumulative mortalities were recorded daily over a period of 10 days. The results revealed that, after viral infection, *PmIKKβ*- and

PmIKKε-silenced shrimp were susceptible to 100% death within 7 days, while the control ds*GFP*-injected group exhibited 100% death at 10 days after infection (**Figure 3.13**). In addition, both the *PmIKKβ*- and *PmIKKε*-silenced shrimp exhibited 50% cumulative mortalities at 3.5 and 4 days, respectively.

Compared to the ds*GFP*-injected group, the higher mortality rates suggest the essential roles of *Pm*IKKβ and *Pm*IKKε in shrimp immune system against WSSV infection. Moreover, the consequence of *PmIKKβ* and *PmIKKε* suppression on WSSV infection was investigated. The viral copy number of WSSV in *PmIKKβ*- and *PmIKKε*-silenced shrimp was quantified by the detection of a conserved *VP28* gene using qRT-PCR. The WSSV copy number was quantified at 120 hpi in the correlation with shrimp cumulative mortalities. It was found that the viral copy number was significantly higher in the *PmIKKβ*- and *PmIKKε*-silenced shrimp, compared to the ds*GFP*-injected group (**Figure 3.14**).

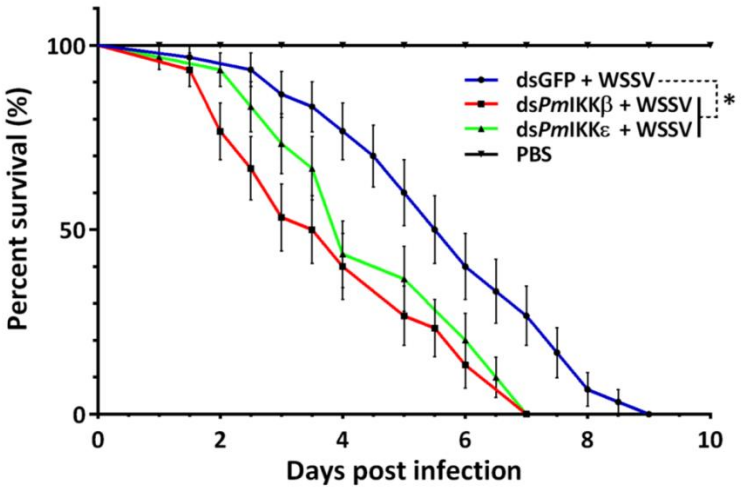


Figure 3.13 Effects of *PmIKKβ* and *PmIKKε* silencing on shrimp survival after challenged with WSSV. Shrimp were doubly injected with PBS, 10 μg/ g shrimp ds*GFP* (control), ds*PmIKKβ* or ds*PmIKKε* with an interval of 24 hours followed by 1 × 10^5 copies of purified WSSV or PBS as a control. The cumulative mortalities were recorded daily over a period of 10 days after the infection.

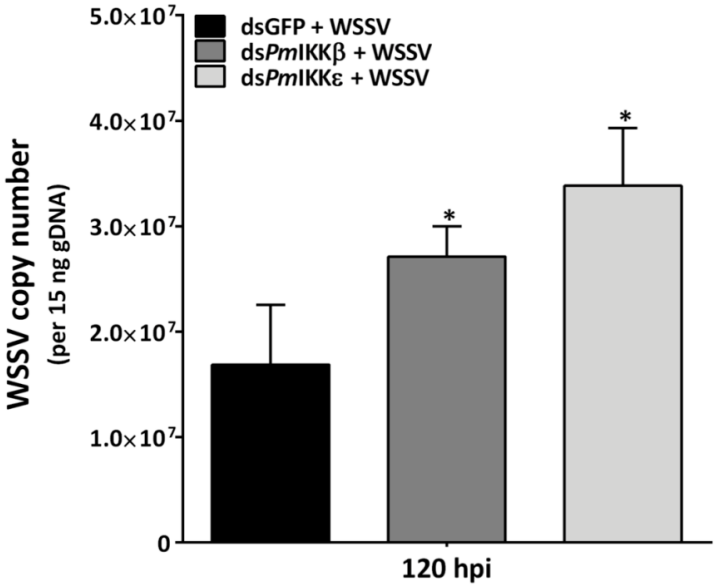


Figure 3.14 Effects of *PmIKKβ* and *PmIKKε* gene silencing on the WSSV copy number. Experimental shrimp (3-5g) were doubly injected with 10 μg/g shrimp of ds*GFP*, ds*PmIKKβ*, ds*PmIKKε* or PBS with an interval of 24 hours prior to 1×10^5 copies WSSV or PBS injection. For WSSV copy number, the genomic DNA from gill was extracted at 120 hpi for qRT-PCR analysis. The experiment was performed in three independent triplicates of 10 shrimp per group. Data are shown as the means ± SDs with *n*=3.

3.8 Effect of *in vivo Pm*IKKβ and *Pm*IKKε silencing on immune-related genes upon WSSV infection

To further investigate the functions of *Pm*IKKβ and *Pm*IKKε in shrimp immune response, juvenile shrimp (3-5g) were doubly injected with 10 μg/g shrimp of ds*GFP* (control), ds*PmIKKβ* or ds*PmIKKε* with an interval of 24 hours. Following the dsRNA injection, shrimp were subsequently injected with 1×10^5 copies of purified WSSV and the mRNA levels of immune-related genes were determined by qRT-PCR. The expression of genes in signal transduction pathways *PmDorsal*, *PmRelish* and *PmCactus*), antimicrobial peptides (*ALFPm3, ALFPm6, CrustinPm1*, *CrustinPm5* and *CrustinPm7*) and IFN-like genes (*PmVago1* and *PmVago4*) were analyzed. When compared with the ds*GFP* control group, the expression of *PmVago4* was

significantly decreased by 0.3-fold in both *PmIKKβ*- and *PmIKKε*-silenced shrimp, while that of *PmVago1* was not affected. The expression of *PmDorsal* was increased by 1.3-fold after *PmIKKβ* suppression, whereas those of *PmCactus* and *PmRelish* remained unaffected. Moreover, the *CrustinPm5* was up-regulated by 2.45-fold in *PmIKKβ*-silenced shrimp, while the *ALFPm3* was up-regulated by 3.05- and 4.45-fold in *PmIKKβ*- and *PmIKKε*-silenced shrimp, respectively. The expression of *ALFPm6*, *CrustinPm1* and *CrustinPm7* were not affected in both *PmIKKβ* and *PmIKKε*-silenced shrimp when compared with the ds*GFP* control group (**Figure 3.15**).

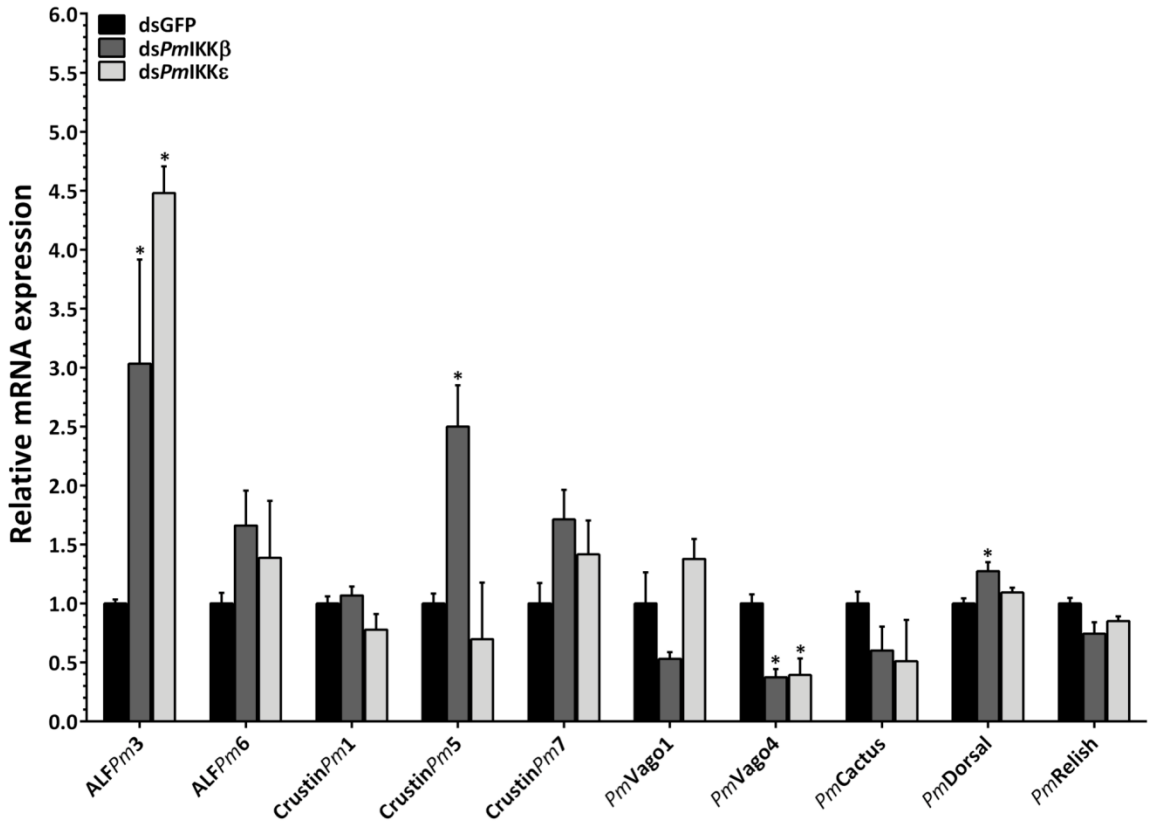


Figure 3.15 Expression of immune-related genes upon WSSV infection following *PmIKKβ* and *PmIKKε* silencing. The transcript levels of antimicrobial peptides, cytokines and transcription factors were examined upon *PmIKKβ* and *PmIKKε* silencing. Juvenile shrimp (3-5g) were doubly injected with 10 μg/g shrimp of ds*GFP* (control), ds*PmIKKβ* or ds*PmIKKε* with an interval of 24 hours. Following suppression, shrimp were injected with 1×10^5 copies of purified WSSV inoculum. Total RNA was isolated from the hemocytes for qRT-PCR analysis. The expression

levels of genes involved in signal transduction pathway (*PmRelish*, *PmCactus*, and *PmDorsal*), antimicrobial peptides (*ALFPm3*, *ALFPm6*, *CrustinPm1*, *CrustinPm5* and *CrustinPm7*) and IFN-like molecules (*PmVago1* and *PmVago4*) were determined using *EF1-α* as an internal control. Data are shown as the means ± SDs from three triplicate experiments relative to the control ds*GFP* group. Asterisks indicate significant differences of mean values ($P<0.05$).

3.9 Involvement of *Pm*IKKβ and *Pm*IKKε in Toll and IMD regulatory pathways

3.9.1 Preparation of *Pm*MyD88 and *Pm*IMD double-stranded RNAs by *in vitro* transcription

To investigate the involvement of *Pm*IKKβ and *Pm*IKKε in regulatory pathways of shrimp immune system, two immune-related pathways were disrupted. The important target genes including *Pm*MyD88 and *Pm*IMD in Toll and IMD pathways, respectively, were suppressed by dsRNA-mediated RNA interference technique. Double-stranded RNAs (dsRNAs) specific to *Pm*IMD and *Pm*MyD88 sequences (ds*Pm*IMD and ds*Pm*MyD88) were synthesized by *in vitro* transcription using T7 RiboMAX™ Express Large Scale RNA Production System (Promega). DNA templates for *in vitro* transcription of sense and antisense RNA strands were amplified by PCR reactions containing specific primers attached with T7 RNA polymerase binding site (Table 1). The amplified DNA templates were analyzed in 1% (w/v) agarose-TBE gel electrophoresis (**Figure 3.16**).

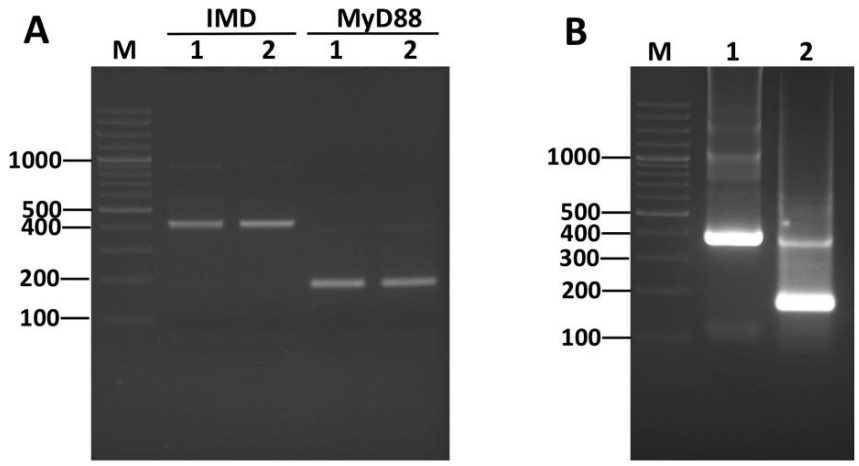


Figure 3.16 Preparation and purification of *Pm*IMD and *Pm*MyD88 double-stranded RNAs. Double-stranded RNAs were synthesized by *in vitro* transcription using amplified DNA templates. (A) Purified sense; lane 1 and anti-sense; lane 2. Double-stranded RNAs (dsRNAs) were synthesized using T7 RiboMAX™ Express Large Scale RNA Production System (Promega) according to manufacturer's protocol. (B) Purified ds*Pm*IMD; lane 1 and ds*Pm*MyD88; lane 2. M is Thermo Scientific™ GeneRuler 100 bp Plus DNA Ladder.

3.9.2 *In vivo Pm*MyD88 and *Pm*IMD suppression by RNAi and effect on *Pm*IKKβ and *Pm*IKKε expression

The dsRNA injection was performed *in vivo* for gene silencing. A single injection was performed to juvenile shrimp (3-5 g) at the third abdominal segment with dsRNA concentrations of including 7.5 μg/ g shrimp for *Pm*IMD and 10 μg/ g shrimp for *Pm*MyD88, respectively. Following the dsRNA injection, shrimp were randomly selected for hemocyte RNA extraction. Significant suppression of *Pm*IMD and *Pm*MyD88 transcript levels were observed using semi-quantitative RT-PCR after dsRNA injection. Moreover, the subsequent effect on *Pm*IKKβ and *Pm*IKKε expression was performed to demonstrate the involvement in Toll and IMD signaling pathways. Following ds*Pm*IMD and ds*Pm*MyD88 injection, semi-quantitative RT-PCR showed no effect on *Pm*IKKβ and *Pm*IKKε expression suggesting that *Pm*IKKβ

and *Pm*IKKε are not involved in both Toll and IMD signaling pathway of black tiger shrimp (**Figure 3.17**).

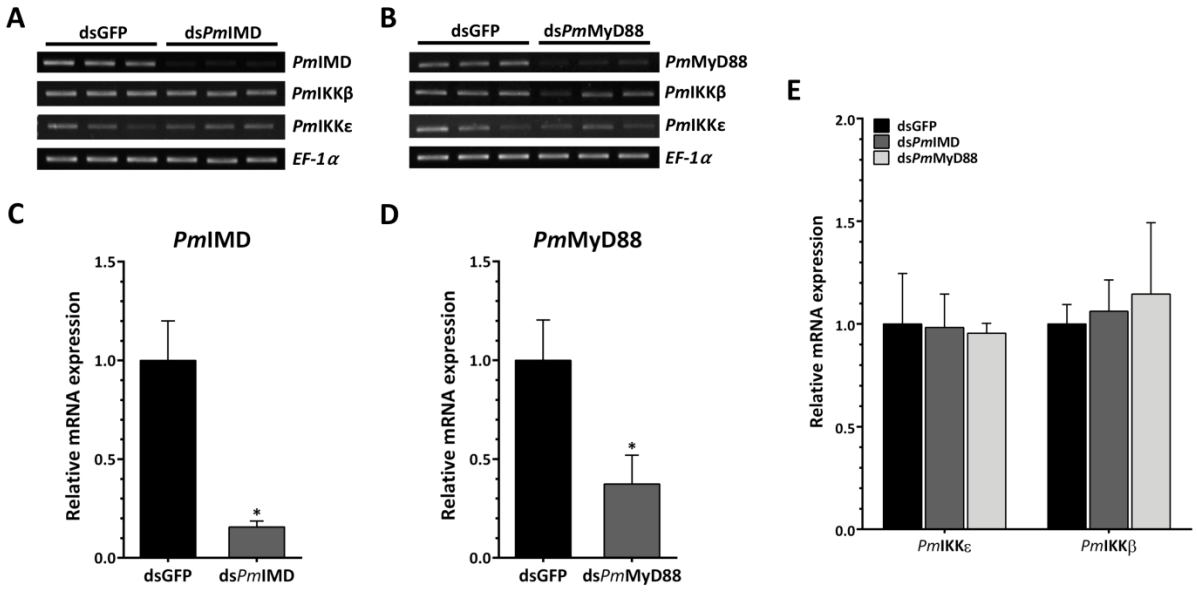


Figure 3.17 Expression profile of *Pm*IKKβ and *Pm*IKKε following suppression of *Pm*IMD and *Pm*MyD88 by dsRNA-mediated RNAi. A single injection of dsRNAs specific to *Pm*IMD and *Pm*MyD88 genes was performed to juvenile shrimp (3-5 g). Different dsRNA concentrations including 7.5 μg/g shrimp for *Pm*IMD and 10 μg/g shrimp for *Pm*MyD88 were introduced for IMD and Toll pathway signaling disruption, respectively. (**A**,**B**) At 24 hours post dsRNA injection, total RNA was extracted to confirm the suppression of *Pm*IMD and *Pm*MyD88 together with the expression of *Pm*IKKβ and *Pm*IKKε by semi-quantitative RT-PCR. (**C**,**D**) Realtime PCR analysis exhibited successful suppression of *Pm*IMD and *Pm*MyD88 genes with no significant effect on (**E**) *Pm*IKKβ and *Pm*IKKε. Elongation factor-1α gene (*EF-1α*) was used as an internal control. The amplified PCR products were analyzed in 2% (w/v) agarose-TBE gel electrophoresis and visualized by UV-transillumination. Data from quantitative RT-PCR are shown as the means ± SDs from three triplicate experiments relative to the control ds*GFP* group. Asterisks indicate significant differences of mean values ($P<0.05$).

3.10 Overexpression of *P. monodon Pm*IKKs in HEK293T and promotor activity assay

To further evaluate the roles of *Pm*IKKβ and *Pm*IKKε in the regulation of shrimp cytokine-like system and NF-κB signaling, the HEK293T were transiently transfected with NF-κB or IFNβ reporter plasmids simultaneously with each construct of protein expression plasmids for *Pm*IKKβ, *Pm*IKKε1 or *Pm*IKKε2 **(Figure 3.18A**) and luciferase activities were measured. Compared with the pcDNA3-Myc control group, the overexpression of *Pm*IKKβ, *Pm*IKKε1 and *Pm*IKKε2 induced the promoter activities of NF-κB approximately 204.45-, 22.13- and 4.91-fold, respectively (**Figure 3.18B**). Moreover, *Pm*IKKε1 and *Pm*IKKε2 but not *Pm*IKKβ overexpression significantly induced the IFNβ promoter activities approximately 152.90- and 17.92-fold, respectively (**Figure 3.18C**), suggesting the possible roles of *Pm*IKKε1 and *Pm*IKKε2 as the immune-stimulatory factors for an IFN-like system in shrimp.

On the contrary, *Pm*IKKβ showed no significant induction on IFNβ promoter activity in HEK293T cells. The result suggested that *Pm*IKKβ might serve mainly as a positive regulator of NF-κB signaling pathway in shrimp innate immune responses. In addition, the greater activation from *Pm*IKKε1 in HEK293T cells was possibly a result from the additional 30 amino acid segment whose function remained to be elucidated.

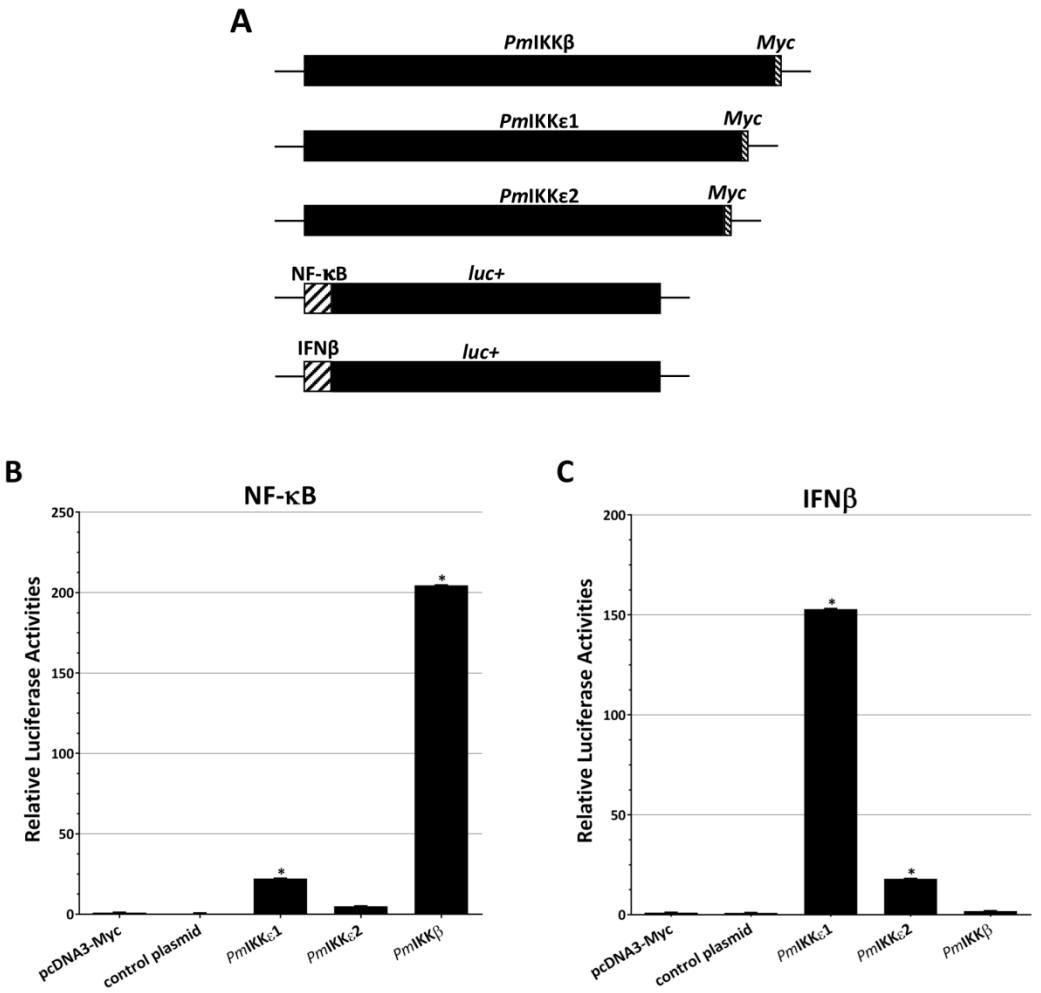


Figure 3.18 Overexpression of *Pm*IKKβ, *Pm*IKKε1 or *Pm*IKKε2 in HEK293T cells and luciferase activity assay. The HEK293T cells were transiently co-transfected with luciferase reporter plasmids and control plasmid, no plasmid or a construct of protein expression plasmids as indicated (**A**). Luciferase activities from (**B**) NF-κB and (**C**) IFNβ reporter plasmids were determined and normalized on the basis of *Renilla* expression with Dual-Glo® Luciferase Assay System (Promega). Data are shown as the means ± SDs of luciferase activities from triplicate experiments with n=3. Asterisks indicate significant differences of mean values (P<0.01).

CHAPTER IV

DISCUSSION

Innate immune response is generally the first line of defense responding to the invading pathogens (Janeway and Medzhitov, 2002; Tassanakajon, 2013). During host infection, the endogenous ligands from pathogens trigger pattern recognition receptors. leading to activation of signal transduction pathways that resulted in the production of several antimicrobial peptides (AMPs) (Hornung and Latz, 2010). The IKK-NF-κB pathway has been studied and reported to regulate pro-inflammatory cytokine production, leukocyte recruitment or cell survival in vertebrates (Lawrence, 2009). It is clear that the transcription factor NF-κB is an important contributor to the immune responses and feedback control via various mechanisms. NF-κB in vertebrates is induced by different types of stimuli and participates in the regulation of different target genes (Baldwin, 1996; Häcker and Karin, 2006). Since a large variety of pathogens activate NF-κB, it has often been termed a 'central mediator for immune responses' (Pahl, 1999). In addition, the key IκB kinase proteins, IKKs, serve as the core elements to drive the pathway and integrate the NF-κB activation signals (Hinz and Scheidereit, 2014). Primarily, IKK proteins function to phosphorylate the IκBα inhibitor to release the NF-κB transcription factor upon the stimulation (Sun, 2011; Hinz and Scheidereit, 2014). Although several studies in IKK-NF-κB signaling pathway have been investigated in vertebrates and fruit fly *D. melanogaster*, less attention has been paid for the mechanism in crustaceans. In this study, the IKKs from black tiger shrimp *Penaeus monodon* (*Pm*IKKβ, *Pm*IKKε1 and *Pm*IKKε2) were identified and characterized in the innate immune system of the black tiger shrimp *Penaeus monodon*.

In mammalian, the inhibitor of kappa B kinases (IKKs) exhibit the crucial roles to trigger a wide variety of NF-κB-independent signaling events that regulate various physiological functions and impact disease states of cells (Hinz and Scheidereit, 2014). In addition to its specific function, IKKα is a major cofactor in a signaling pathway that is required for cell cycle exit and induction of terminal

differentiation (Descargues *et al.*, 2008). Despite IKK proteins in vertebrates showed the extensive sequence similarity, they comprise distinct functions with different substrate specificities and modes of regulation. IKKβ and IKKγ are essential for rapid NF-κB activation by proinflammatory signaling cascades, such as those triggered by tumor necrosis factor alpha (TNFα) or lipopolysaccharide (LPS). In contrast, IKKα activates a specific form of NF-κB in response to a subset of TNF family members and serve to attenuate IKKβ-driven NF-κB activation (Häcker and Karin, 2006; Israël, 2010). In terms of vertebrates, IKKε and IKK-related kinase TANK-binding kinase 1 are the components which phosphorylate IRF-3 and IRF-7 triggering host antiviral responses (Sato *et al.*, 2000; Bakshi *et al.*, 2017).. In black tiger shrimp, the sequences of *Pm*IKKs shared high similarities approximately 95-97% at the protein level with those of the white shrimp *Lv*IKKs, which were reported as an invertebrate homologs of mammalian IKKα/IKKβ and strongly induced the NF-κB activity in human HEK293T cells (Wang *et al.*, 2013). In this study, the complete open reading frames of *PmIKKβ*, *PmIKKε1* and *PmIKKε2* from the *P. monodon* were successfully cloned and sequenced. Phylogenetic analysis demonstrated the clusters of *Pm*IKKs from *P. monodon* with *Lv*IKKs from the Pacific white shrimp *Litopenaeus vannamei* indicating an evolutionary relationship between the two species. Moreover, the pairwise alignments showed that two isoforms of *Pm*IKKε namely, *Pm*IKKε1 and *Pm*IKKε2, were highly similar to those of *Lv*IKKε from *L. vannamei*. *Pm*IKKε1 carried an extra 30 amino acid region which was absent in the *Pm*IKKε2 and the function is still unclear. However, Luciferase reporter assays revealed the higher IFNβ promoter activation from *Pm*IKKε1 for which the additional 30 amino acid region was believed to be an important responsive element as for the higher NF-κB promoter activity (Wang *et al.*, 2013). *Pm*IKKβ and *Pm*IKKε differentially stimulated the distinct target immune genes including NF-κB and IFNβ, respectively, suggesting that there are diverse functions in the pathway contributed by these two proteins.

According to the protein feature analysis from the SMART database, *Pm*IKKβ, *Pm*IKKε1 and *Pm*IKKε2 from shrimp only contained the important kinase domains located in their N-termini. In addition to kinase domains, the IKK and IKK-family proteins from various species also contain a NEMO-binding domain (NBD),

ubiquitin-like domain (ULD) or leuzine zipper domain (LZ) which are essential for IKK complex assembly and their catalytic activities (Sun, 2011; Hinz and Scheidereit, 2014). The ULD was previously predicted as a critical element of IKKβ required to be involved in the exact positioning of the kinase substrate IκBα. Most likely, NEMO, which ensures specific IκBα substrate recognition is recruited through intramolecular interaction with IKKβ (Xu *et al.*, 2011; Schröfelbauer *et al.*, 2012). However, the information of the interdependence for the domains within IKK proteins from crustacean is of a great issue to be uncovered. Therefore, the domain compositions of *Pm*IKKβ, *Pm*IKKε1 and *Pm*IKKε2 were needed to be investigated for further understanding their functions in signaling cascade.

Tissue-specific expression analysis revealed that the transcripts of *Pm*IKKβ, *Pm*IKKε1 and *Pm*IKKε2 were expressed in various shrimp tissues. The particular high mRNA expression levels of *Pm*IKKβ, *Pm*IKKε1 were found in hemocytes (Hc) which is the important tissue where several immune reactions took place (Tassanakajon, 2013; Tassanakajon *et al.*, 2013). Crustacean hemocytes play crucial roles in several host immune responses including phagocytosis, recognition, melanization, cytotoxicity and cell–cell communication (Johansson *et al.*, 2000). In recent years, several immune-related molecules that participate in shrimp innate immune system were identified including antimicrobial peptides, serine proteinases and inhibitors, phenoloxidases, pattern recognition proteins and other humoral factors (Tassanakajon *et al.*, 2013). Upon immune challenges with shrimp pathogens including viruses and a bacterium, the *Pm*IKKε1 and *Pm*IKKε2 transcripts were up-regulated whereas the *Pm*IKKβ expression was unaffected. Previous report showed that both IKKε and IKK-family protein TBK1 were considered to be upstream of IKKβ in the NF-κB activation pathway (Harris *et al.*, 2006). In immune mechanisms, the IKK-NF-κB signaling cascade is targeted and interrupted by various pathogens to favor the diseases (Sarkar *et al.*, 2008; Lawrence, 2009; Wang *et al.*, 2013). The immune challenges suggested that the *Pm*IKKs may contribute to the immune responses against dsDNA virus (WSSV), dsRNA virus (YHV) and also a bacterium *V. harveyi* in shrimp innate immune system.

Among several pathogens, the WSSV is considered as one of the most pathogenic and destructive viruses to the shrimp aquaculture industry. The viral outbreaks caused up to 100% accumulative mortality within 3-10 days to shrimp farming (Lo *et al.*, 1996; Flegel and Alday-Sanz, 1998; Walker and Mohan, 2009). In this study, the black tiger shrimp with suppression of *Pm*IKKβ and *Pm*IKKε were more susceptible to WSSV infection as a more rapid death and higher viral copy number were detected, suggesting the essential roles in shrimp antiviral response against WSSV. This further reflected the essential roles of the *Pm*IKKβ and *Pm*IKKε in pathogen defense as the fact that the NF-κB signaling cascade is probably the most frequently targeted intracellular pathway by a wide spectrum of pathogens (Rahman and McFadden, 2011; Liu *et al.*, 2017). In addition, following the *Pm*IKKβ and *Pm*IKKε silencing, the mRNA level of *PmVago4* which is an IFN-like molecule was reduced significantly. The *Lv*Vago4 from *L. vannamei* was induced in a similar manner as type I IFNs in vertebrates during virus infection and initiate an antiviral state in mammalian cells, suggesting that shrimp Vago might function as an IFN-like molecule in invertebrates (Li *et al.*, 2015). In *Drosophila melanogaster*, the dsRNA-mediated *Dm*IKK silencing inhibited the immune response leading to reduction of both IFN-β mRNA level and protein production (Ertürk-Hasdemir *et al.*, 2009). The *Dm*Vago was reported as an antiviral molecule targeting the virion or a cytokine which subsequently triggered an infected-state in the neighboring cells (Ertürk-Hasdemir *et al.*, 2009; Sabin *et al.*, 2010). In other arthropods, the *Cx*Vago from *Culex* was induced and secreted as a peptide that restricts the West Nile virus (WNV) infection. The *Cx*Vago acted as a homolog of interferon activating JAK-STAT pathway and limiting virus replication in neighboring cells. Thus, the Vago was demonstrated to function as an IFN-like in mosquito antiviral cytokine system (Paradkar *et al.*, 2014).

In the Pacific white shrimp *L. vannamei*, the *Lv*IKKβ and *Lv*IKKε are the central regulators of the IKK-NF-κB signaling pathway and represent the points of convergence for the most signal transduction leading to NF-κB activation (Wang *et al.*, 2013). Toll and IMD pathways are two important signaling cascades in which their components activate AMP luciferase reporters and bind to NF-κB-binding sites

in the AMP promoter regions (Sabin *et al.*, 2010). Endogenous ligands from pathogens may trigger pattern recognition receptors during host infection. This activation may act to promote signal transduction mechanisms that include the production of several anti-microbial peptides (AMPs) through IKK complex (Ertürk-Hasdemir *et al.*, 2009).

The shrimp IMD pathway is involved for sensing of RNA viruses and Gram-negative bacteria to activate a transcription factor Relish (Tassanakajon *et al.*, 2013). The signal-induced transcription factor Relish translocates into the nucleus and regulates the expression of shrimp penaeidins, crustins, and antilipopolysaccharide factors (ALF) (Tassanakajon *et al.*, 2013; Tassanakajon *et al.*, 2018). Moreover, knockdown of the *Pm*Relish suppresses the *PmPEN5* transcript level, whereas the *PmPEN3* is slightly up-regulated. These results demonstrate that the expression of *PmPEN5* and *PmPEN3* are regulated by *Pm*Relish through shrimp IMD pathway (Visetnan *et al.*, 2015). In this study, neither silencing of *PmIKKβ* nor *PmIKKε* have affected the expression of *PmRelish*. The Crustin*Pm*1 and crustin*Pm*7 are the two cationic AMPs that are identified from the hemocytes of *Penaeus monodon*. Suppression of *PmRelish* and *PmMyD88* in regulatory pathways reveals that the expression of *CrustinPm*1 is regulated by the Toll pathway, while that of *CrustinPm*7 is regulated by both the Toll and IMD signaling pathways (Arayamethakorn *et al.*, 2017). Moreover, the *IKK*-silenced *P. monodon* showed no significant difference in the expression of *CrustinPm1*, *CrustinPm7* and *ALFPm6* suggesting that the *Pm*IKKs might act as the points in several alternative regulatory factors for immune-related gene expression. In the immune response, the IKKβ compensated for the lack of IKKα to modulate the NF-κB cascade for pro-inflammatory stimulation (Häcker and Karin, 2006; Hinz and Scheidereit, 2014). These results proposed that the IKK-NF-κB pathway provided a cross-talking within proteins in the IKK family and they might not be modulated solely in one signaling pathway.

Overexpression of *Pm*IKKβ and *Pm*IKKε1 highly activated the NF-κB reporter in luciferase assay. Likewise, the IFNβ reporter was significantly induced by the *Pm*IKKε1 and *Pm*IKKε2 in HEK293T cells. The majority of target proteins

encoded by NF-κB transcription factor participate in the host immune responses including different cytokines and chemokines, as well as receptors required for immune recognition such as MHC molecules (Pahl, 1999). Similar to NF-κB, the IFNβ is identified as a pro-inflammatory cytokine activated in the presence of infectious diseases. The activated IFNβ stimulates the inflammatory responses in neighboring cells leading to the expression of immune-related genes involved in innate and adaptive immune responses (Sato *et al.*, 2000; Yamamoto *et al.*, 2002). The results from the reporter assays indicated the potential roles of *Pm*IKKβ and *Pm*IKKε for driving immune responses through the NF-κB signaling and cytokine-like system in shrimp defense mechanism.

More recently, *Pm*DDX41, a cytosolic DNA sensor, has been firstly identified in shrimp (Soponpong *et al.*, 2018). Interestingly, suppression of *PmDDX41* resulted not only in a significant increase of cumulative mortality against WSSV infection but also significantly affected the expression of several immune-related genes especially *PmIKKβ* and *PmIKKε*. The DDX41 is identified as a dsDNA-sensing receptor in mouse dendritic cells and involved in type I interferon regulation via interferon regulatory factors (IRF3 and IRF7) (Deddouche *et al.*, 2008). Following bacterial or viral infection, the DDX41 acted as a sensor for recognizing the invaders DNA or cyclic-di-GMP using DEAD-box and induced type I interferons (IFN) production (Jiang *et al.*, 2017). In recent years, DExD/H-box helicases have been reported to contribute to the antiviral immunity either by acting as sensors for viral nucleic acids or by facilitating downstream signaling events (Schmidt *et al.*, 2012). The silencing of *PmDDX41* affected several downstream immune genes including transcription factors and AMPs (Soponpong *et al.*, 2018). These results demonstrate the signaling cascade generated from the *Pm*DDX41 as a sensing molecule through *Pm*IKKβ and *Pm*IKKε for shrimp immune response.

In summary, the *Pm*IKKβ, *Pm*IKKε1 and *Pm*IKKε2 were identified and characterized in shrimp antiviral responses. The *PmIKKε1* and *PmIKKε2* but not *PmIKKβ* were up-regulated in responses to viruses and a bacterium suggesting the essential roles against pathogen infection. Suppression of *PmIKKβ* and *PmIKKε*

resulted in subsequent reduction of an IFN-like *PmVago4*. Moreover, the *PmIKKβ*- and *PmIKKε*-silenced shrimp were more susceptible to WSSV infection and showed the higher viral copy number indicating that the *Pm*IKKβ and *Pm*IKKε participated in the regulation of viral infection. In addition, the overexpression of *Pm*IKKβ and *Pm*IKKε in HEK293T cells enhanced the NF-κB and IFNβ promoter activities, respectively. Therefore, this study demonstrated the potential roles of *Pm*IKKs in an IFN-like system through *PmVago4* and cross-talking between signaling transductions for regulating antiviral responses in shrimp.

CHAPTER V
CONCLUSIONS

1. Inhibitor of kappa B kinases (IκB kinases) including *PmIKKβ*, *PmIKKε1* and *PmIKKε2* were identified in the black tiger shrimp and their open reading frames were obtained successfully. The *PmIKKβ*, *PmIKKε1* and *PmIKKε2* transcripts were widely expressed in various tissues with high mRNA expression of *PmIKKβ* and *PmIKKε1* in hemocytes (Hc) which is an immune-related tissue.

2. *PmIKKε1* and *PmIKKε2* but not *PmIKKβ* responded to pathogen infection including white spot syndrome virus, yellow head virus and a bacterium *Vibrio harveyi*. Moreover, the *PmIKKβ*- and *PmIKKε*-silenced shrimp were more susceptible to WSSV infection as a rapid death and higher WSSV copy number were detected, suggesting the essential roles in shrimp antiviral response against WSSV infection.

3. Silencing of *PmIKKβ* and *PmIKKε* resulted in the significant reduction of *PmVago4* mRNA level which is an IFN-like molecule. Moreover, *CrustinPm5* and *ALFPm3* were up-regulated, while the expression of *ALFPm6*, *CrustinPm1* and *CrustinPm7* were not affected suggesting that the *PmIKKβ* and *PmIKKε* might act as the points of several alternative regulatory factors for immune-related gene expression.

4. Investigation for *PmIKKβ* and *PmIKKε* regulatory pathways was performed using suppression of *Pm*IMD and *Pm*MyD88 by dsRNA-mediated RNAi. Following the suppression, the transcripts of *Pm*IMD and *Pm*MyD88 which are the two important genes in IMD and Toll pathways were significantly decreased. However, these signaling disruptions have no effect on the

expression of *PmIKKβ* and *PmIKKε*, suggesting that they may not directly regulated by Toll and IMD signaling pathways of the black tiger shrimp.

5. Luciferase reporter assays showed that overexpression of *Pm*IKKβ and *Pm*IKKε1 highly activated the NF-κB reporter in HEK293 cells. Likewise, the IFNβ reporter was significantly induced by the *Pm*IKKε1 and *Pm*IKKε2. These results indicated the involvement of *Pm*IKKβ, *Pm*IKKε1 and *Pm*IKKε2 in activation of immune responses. Moreover, *Pm*IKKβ and *Pm*IKKε differentially stimulated the distinct target immune genes including NF-κB and IFNβ, respectively, suggesting that they might contribute to the diverse functions independently in the immune pathways of shrimp.

6. Taken together, these results demonstrated that *Pm*IKKβ and *Pm*IKKε may act as the potent factors stimulating an IFN-like system through *PmVago4.* Meanwhile, they also provide the integration and cross-talking between distinct signaling transductions for regulating antiviral responses in shrimp innate immune system.

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