ลักษณะสมบัติเชิงหน้าที่ของโปรตีนรีลิชจากกุ้งกุลาดำ *Penaeus monodon*

นางสาวสุวัฒนา วิเศษนันท์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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FUNCTIONAL CHARACTERIZATION OF RELISH PROTEIN FROM BLACK TIGER SHRIMP *Penaeus monodon*

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สุวัฒนา วิเศษนันท์ : ลักษณะสมบัติเชิงหน้าที่ของโปรตีนรีลิชจากกุ้งกุลาดำ *Penaeus monodon* (FUNCTIONAL CHARACTERIZATION OF RELISH PROTEIN FROM BLACK TIGER SHRIMP *Penaeus monodon*) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ดร. วิเชียร ริมพณิชยกิจ, 149 หน้า.

การติดเชื้อจากแบคทีเรียแกรมลบและไวรัสบางชนิดของสัตว์จะไปกระตุ้นการส่งสัญญาณของวิถี Imd แทรนส์คริปชันแฟกเตอร์ NF-**K**B ชนิด Relish เป็นโปรตีนที่มีความสำคัญในการควบคุมการสังเคราะห์โปรตีนต้านจุลชีพ ยีน *PmRelish* ซึ่งแปลรหัสให้โปรตีนที่มีกรดอะมิโนจำนวน 1,195 ตัว ถูกโคลนจากซีดีเอ็นเอของกุ้งที่ติดเชื้อ YHV ที่เวลา 48 ชั่วโมง การศึกษาระดับการแสดงออกของยีน *PmRelish* ด้วยวิธี RT-PCR พบว่ายีน *PmRelish* มีการแสดงออกในเนื้อเยื่อทุกชนิดและมีการแสดงออกที่มากขึ้นเมื่อติดเชื้อ YHV จากการทำ real-time RT-PCR พบว่า ยีน *PmRelish* มีการแสดงออกเพิ่มขึ้นอย่างมีนัยสำคัญหลังจากการกระตุ้นด้วยเชื้อแบคทีเรียและไวรัส เมื่อมีการยับยั้งการแสดงออกของยีน *PmRelish* พบว่ากุ้งมีความไวต่อการติดเชื้อ YHV อย่างมาก โดยกุ้งมีอัตราการรอดตายลดลง 50% จาก 76 ชั่วโมง เป็น 42 ชั่วโมง จากรูปแบบการแสดงออกของยีน *ALFPm3*, *crustinPm1*, *penaeidin3* และ *penaeidin5* โดยใช้เชื้อ YHV และ *V. harveyi 639* เป็นตัวกระตุ้นวิถี Imd พบว่าการแสดงออกของยีน *penaeidin3* และ *penaeidin5* อยู่ภายใต้การควบคุมของโปรตีน *Pm*Relish สามารถตรวจสอบโปรตีน *Pm*Relish ได้ในไซโตพลาสซึมของเซลล์เม็ดเลือดกุ้งทุกชนิดทั้งที่ติดเชื้อและไม่ติดเชื้อ YHV ด้วยเทคนิค immunolocalization จากเทคนิค Western blot สามารถตรวจพบโปรตีน *Pm*Relish ที่ถูกกระตุ้นแล้วในเซลล์เม็ดเลือดกุ้งที่ติดเชื้อ YHV เทคนิค RNA interference (RNAi) และ suppression subtractive hybridization (SSH) เป็นเทคนิคที่ถูกนำมาใช้ร่วมกันในการระบุหายีนที่เกี่ยวข้องกับภูมิคุ้มกันที่มีการแสดงออกเพิ่มขึ้นและตอบสนองต่อการติดเชื้อ YHV และอยู่ภายใต้การควบคุมของโปรตีน *Pm*Relish โดย cDNA โคลน จำนวน 252 และ 99 โคลนจากห้องสมุด forward และ reverse ถูกนำไปหาลำดับเบส และทำการค้นหาลำดับที่คล้ายคลึงกันในฐานข้อมูล GenBank ด้วยโปรแกรม BLAST พบยีนที่เกี่ยวข้องกับการป้องกันและธำรงสมดุลอย่างมากในห้องสมุด forward และ reverse โดยคิดเป็น 31% และ 23% ตามลำดับ โดยโปรตีนในกลุ่มดังกล่าวประกอบด้วย โปรตีนต้านจุลชีพ โปรตีเอสและตัวยับยั้งโปรตีเอส โปรตีน chaperones โปรตีนที่ช่วยในการแข็งตัวของเลือด และโปรตีนที่ทำหน้าที่ในการป้องกันและธำรงสมดุล อื่น ๆ

ภาควิชา ชีวเคมี ลายมือชื่อนิสิต .....

สาขาวิชา ชีวเคมีและชีววิทยาโมเลกุล ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

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In animals, infection by Gram-negative bacteria and certain viruses activates the Imd signaling pathway wherein the a NF-**K**B transcription factor, Relish, is a key regulatory protein for the synthesis of antimicrobial proteins. A *PmRelish* gene which encoded a protein of 1,195 amino acids was cloned from the cDNA of 48-h YHV-infected shrimp. RT-PCR study of the *PmRelish* gene revealed that it was constitutively expressed in all tissues tested and mostly up-regulated upon YHV infection. Real-time RT-PCR of *PmRelish* gene after bacterial and viral challenges showed that the expression of *PmRelish* gene was significantly increased. Under *PmRelish* silencing, the shrimp were more susceptible to infection by YHV with the 50% survival rate reduced from about 72 h to 42 h. The expression profiles of *ALFPm3, crustinPm1*, *penaeidin3* and *penaeidin5* determined using YHV and *V. harveyi* 639, as an inducer of the Imd pathway indicate that the expression of *penaeidin*3 and *penaeidin*5 is under the *Pm*Relish regulation. Immunolocalization of *Pm*Relish protein in shrimp hemocytes showed that the *Pm*Relish was detected in the cytoplasm of all the hemocytes from both uninfected and YHV-infected shrimp. The activated *Pm*Relish was detected in the YHV-infected hemocytes by Western blot analysis. The RNA interference (RNAi) and suppression subtractive hybridization (SSH) was employ to identify the immune genes up-regulated in response to YHV infection and under the *Pm*Relish regulation. Sequencing of 252 and 99 cDNA clones from the respective forward and reverse libraries were done and annotated through blast search against the GenBank sequences. Genes involved in defense and homeostasis were abundant in both libraries, 31% and 23% in the forward and reverse libraries, respectively. They were predominantly antimicrobial proteins, proteinases and proteinase inhibitors, chaperones, clotting protein, and other defense and homeostasis proteins.

Department:	Biochemistry	Student's Signature	.....
Field of Study:	Biochemistry and Molecular Biology	Advisor's Signature	.....
Academic Year:	2014		

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bp	base pair
dATP	deoxyadenosine triphosphate
dCTP	deoxycytosine triphosphate
DEPC	diethylpyrocarbonate
dGTP	deoxyguanosine triphosphate
DNA	deoxyribonucleic acid
dTTP	deoxythymidine triphosphate
EtBr	ethidium bromide
h	hour
kb	kilobase
M	molar
mg	milligram
ml	millilitre
mM	millimolar
ng	nanogram
nm	nanometre
O.D.	optical density
^{o}C	degree Celcius
ORF	open reading frame
PCR	polymerase chain reaction
RNA	ribonucleic acid
RT	reverse transcription

sec	second
µg	microgram
µl	microlitre
µM	micromolar
UPS	untranslated region

CHAPTER I

INTRODUCTION

1.1 General introduction

Shrimp have been one of the most economically important aquatic species in aquaculture up till now. The shrimp farming region of Southeast Asia and China supports the largest and most productive shrimp farming industry in the world. In Thailand, the shrimp species produced is the Pacific white shrimp, *Penaeus vannamei*, though it was once the black tiger shrimp, *Penaeus monodon*. Since then, Thailand has been the world leader for exporting shrimp produces in the forms of frozen and value-added products to several countries, e.g. Japan, USA and the European Union. The industry has been worth approximately 300,000-420,000 metric tons annually providing an income of nearly 80,000 million baht yearly for the country (Figure 1.1) (Source: Department of fisheries, Thailand).

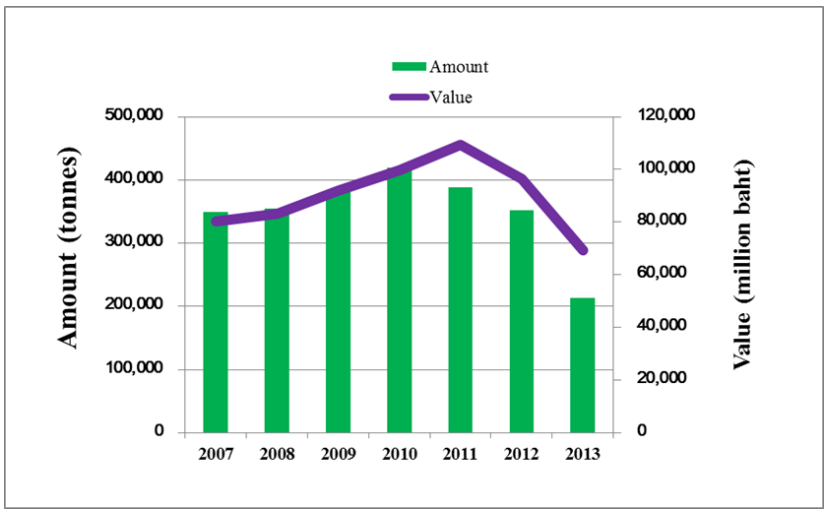


Figure1. 1 Cultured shrimp export from Thailand from 2007 to 2013**.** (**Source**: Department of fisheries, Thailand)

From 2007 till 2013, the once successful shrimp farming industry has been seriously affected by many dramatic factors, for example, the outbreaks of bacterial and viral diseases, the water quality problem and the very rare high-quality bloodstocks. Since then, the shrimp industry has been affected in terms of shrimp production and collapse of the black tiger shrimp farming. Recently, a new emerging disease called 'early mortality syndrome' or 'EMS' (more descriptively called acute hepatopancreatic necrosis syndrome or AHPNS; (Tran et al. 2013; Liu et al. 2007) is a cause of significant production losses in southern China in 2009. By 2010, the outbreak of EMS disease has expanded from China to Vietnam, Malaysia and reached Thailand in 2012 (Tran et al. 2013). Consequently, to solve the disease outbreak problems and maintain the production of *P. monodon*, the efficient domestication, great farm management, genetic improvement and the invention of the effective

disease control measures are required. Although the overall biological systems of *P. monodon* are progressively studied at the molecular level, the immune system should be intensively studied considering its importance in fighting the shrimp diseases. The knowledge is not only applied for the protection of shrimp from the diseases but also for the selective breeding for healthy shrimp for the industry in Thailand.

1.2 Shrimp diseases

Disease problems and health management in shrimp farms are related to three fundamental major problems: management of culture ponds, the epidemic and the use of drugs or chemicals. These problems will affect the cost of shrimp production. Nevertheless, the diseases are the major problem and causes by viruses, bacteria, fungi and protozoa. In order to solve the problems on the spot and on time, it is needed to understand the underlying cause of the diseases (http://www.arda.or.th/kasetinfo/south/shrimp/controller/01-04.php).

The major disease outbreaks from both viruses (WSSV and YHV) and *Vibrio* spp. have been detrimental to the industry from time to time for many years. Viral disease outbreaks are generally more severe. Most bacterial infections are usually resulted from extreme stress. The most common bacterial infection is *Vibrio spp*. *Vibrio* infection often occurs following the environmental stresses or viral diseases, and is not the primary disease problem (Nash 1990).

Four microbial pathogens including white spot syndrome virus (WSSV), yellow head virus (YHV), *Vibrio harveyi* and *Vibrio parahaemolyticus* are the main hazards to the cultivated *P. monodon* in Thailand.

1.2.1 Viral diseases

1.2.1.1 White spot syndrome virus (WSSV)

White spot disease (WSD) is well known and caused by a white spot syndrome virus (WSSV). The WSSV is an important viral pathogen and it is one of the most serious diseases and responsible for huge economic losses in the shrimp culture industry worldwide (Tsai et al. 2000; Balakrishnan et al. 2011). The WSSV is an enveloped, double-stranded DNA virus, which shows little genetic variation among WSSV isolates from around the world (Tsai et al. 2000)

The first outbreak of WSSV was reported in the shrimp farms in Taiwan in 1990-1992, and has spread rapidly to the shrimp-farming areas of South East Asia, Middle East, North, Central and South America (Tsai et al. 2000; Balakrishnan et al. 2011). The WSSV infection has been observed in several commercial penaeid species, including *P. monodon*, *M. japonicus*, *P. chinensis*, *P. indicus*, *P. merguiensis*, *P. vannemei*, *P. stylirostris*, *P. penicillatus* and *P. setiferus* (Chou et al. 1995). This virus can cause 100% accumulative mortality within 2-10 days after the onset of symptoms. At present, no treatment is available to cure the disease and mortality (Lightner 1996; Tsai et al. 2000). In addition, the mechanism of WSSV infection and

the spreading of the virus in the crustacean body is not fully understood. It is believed that the envelop protein of virus may play important roles in virus infection (Zhang et al. 2004; Tsai et al. 2000).

Detection of virus is necessary to reduce damage from the WSSV infection. Several diagnostic methods have been described. The WSSV can be detected in early larvae stages of *P. monodon* although the significant mortality was observed in post-larvae and juveniles shrimp (Yoganandhan et al. 2003). The neutralization experiments of antibodies against six envelope proteins (VP22/VP26, VP28, VP68, VP281, VP292 and VP466), as well as WSSV virions, indicates that four envelope proteins (VP28, VP68, VP281 and VP466) might play key roles in the initial steps of WSSV infection in shrimp (Tsai et al. 2000). The uses of hyperbranched rolling circle amplification (HRCA) assay and a strip-based test suggest that these methods will provide a rapid detection of WSSV and also a basis for field-based detection of animal diseases (Zhao et al. 2014). Using the secondary cell cultures from the lymphoid organ, it is shown that the progenies of WSSV are released gradually from 12 hpi and peaked at 18 hpi (Li et al. 2015). Indirect immunofluorescence assay and Far-Western blotting indicates that a protein LvRPL7 from *L. vannamei* mainly located on the surface and cytoplasm of hemocytes is able to bind with VP51 (Liu et al. 2015; Li et al. 2015).

To protect the shrimp or other crustaceans against WSSV, the WSSV subunit vaccines, the WSSV envelop proteins VP19 and VP28, have been studied. The VP19

and VP28 fused to the maltose binding protein (MBP) provide significant better survival rate for the treated shrimp than the control shrimp (Witteveldt et al. 2005).

To explore the innate immune molecules of shrimp with suitable experimental evidences together with the evolution of “specific immune priming” of invertebrates, the baculovirus displayed rVP28 (Bac-VP28) was used to study its vaccine efficacy by oral route. The orally vaccinated shrimp with Bac-VP28 transduced successfully in the shrimp cells as well as provided highest survival rate (Syed Musthaq and Kwang 2014).

1.2.1.2 Yellow head virus (YHV)

First report of the yellow head disease in *P. monodon* suggests that the virus should be classified as a granulosis-type baculovirus, because it is a viral core consisted of DNA (Chantanachookin et al. 1993). Based on the morphology of negatively stained virions by TEM, RNA content and biochemical data strongly suggest that YHV is an RNA virus rather than a DNA virus but it cannot be classified in any family. Which of the 3 known families of rod-shaped viruses is appropriate for YHV depends first upon whether it contains plus-strand RNA (Coronaviridae) or minus-strand RNA (Rhabdoviridae and Paramyxoviridae) (Ishihama and Barbier 1994; Lightner 1999). However, from these families, rhabdovirus and paramyxovirus have been reported in crustaceans, but coronaviruses have not (Wongteerasupaya et al. 1995). It has been shown that the genome of highly purified YHV is an unsegmented single-stranded RNA with negative polarity and approximately 22 kb in size. The YHV has

many of the properties of rhabdoviruses such as size, shape, general ultrastructural morphology and buoyant density in sucrose gradients but it does not exhibit the characteristic of bullet shaped morphology typical of rhabdoviruses infecting vertebrate hosts. It is formally classified as rhabdoviruses (Nadala Jr et al. 1997) but is reclassified recently as Okavirus (Flegel and Sritunyalucksana 2011). The yellow head virus (YHV) has caused significant losses of cultured shrimp *P. monodon* throughout Asia (Chantanachookin et al. 1993; Flegel 1997). This syndrome occurs in the juvenile to sub-adult stages of shrimp 5 to 15 grams in size, especially at 50-70 days of grow out (Lightner 1996). Clinical signs of YHV occur in *P. monodon* within 7-10 days after exposure. The affected shrimp exhibit light yellow coloration in the cephalothorax area and a generally pale or bleached appearance. The shrimp die within a few hours afterward (Chantanachookin et al. 1993; Lightner 1996).

Several diagnostic methods are invented to detect YHV infection in shrimp, for example monoclonal antibody, enzyme-linked immunosorbent assay (ELISA), dot blotting (de la Rosa-Velez et al. 2006; Chaivisuthangkura et al. 2014), Western blotting, immunohistochemistry, immunochromatographic strip tests (Lu et al. 1996; Nadala and Loh 2000; Sithigorngul et al. 2000; Sithigorngul et al. 2002; Soowannayan et al. 2003), *in situ* hybridization (Tang and Lightner 1999; Tang et al. 2002; Spann et al. 2003), conventional RT-PCR (Wongteerasupaya et al. 1997; Tang and Lightner 1999; Cowley et al. 1999; Cowley et al. 2000), real-time RT-PCR (Dhar et al. 2002) and dsRNA injection (Sanitt et al. 2014).

1.2.2 Bacterial disease

Vibrio spp. is bacterial flora which commonly found in aquatic environments and formerly considered to be mostly opportunistic pathogens (Lightner and Redman 1998). Especially the luminescence bacteria, *V. harveyi*, has been implicated as the main bacterial pathogens of shrimps (Baticados 1990) and cause of shrimp luminous disease resulting in the extreme losses of cultured *P. monodon* in hatcheries and shrimp farms. The other species of *Vibrio* is *V. parahaemolyticus* which contains the 69 kb plasmid pVPA3-1 and can cause acute hepatopancreatic necrosis disease (AHPND). This disease is responsible for mass mortalities in farmed penaeid shrimp and is referred to as early mortality syndrome (EMS) (Photorhabdus insect-related (Pir) toxin-like genes in a plasmid of Vibrio parahaemolyticus, the causative agent of acute hepatopancreatic necrosis disease (AHPND) of shrimp (Han et al. 2015).

1.2.2.1 *V. harveyi*

The *V. harveyi* is Gram-negative bacterium. The *V. harveyi* is a rod shape, motile (via polar flagella), with 0.5-0.8 μm in width and 1.4-2.6 μm in length. This bacterium is able to emit a blue-green color light. It is a pathogen of fish and many invertebrates, including sharks, seabass, seahorses, lobster, and shrimp. *V. harveyi* was found in the gut of shrimp and presumptive diagnosis can be made by culture of the suspensions of hepatopancreas on TCBS (Thiosulfate Citrate Bile salt Sucrose) agar plate supplemented with 2% (w/v) NaCl. The luminescence of *V. harveyi* was detected in the dim light. Diseases caused by *V. harveyi* include eye-lesions, gastro-

enteritis, vasculitis, and luminous vibriosis. Luminous vibriosis is a leading cause of death among commercially farmed shrimp and other aquaculture. Contamination can spread all the way to egg and larval tanks, thus causing an even bigger problem for shrimp farmers. Mortality occurs when penaeid shrimp is exposed to a concentration of *V. harveyi* at 10^2 cells/milliliter (Lightner 1993). Antibiotics have been used in several attempts to control the bacteria but it might have led to the problems of drug resistance.

The use and selection of probiotics bacterial groups: *Vibrio*, *Pseudomonas*, *Bacillus*, and several *Lactobacilli*, for aquaculture of shrimp, crab, oyster and fish, is still needed. This process is in an early stage of development and much work. It can alleviate the infection for it can produce some compounds which have inhibitory property against shrimp pathogen (Gomez-Gil et al. 2000).

1.2.2.2 *V. parahaemolyticus*

V. parahaemolyticus is a curved, rod-shaped, Gram-negative bacterium found in brackish saltwater which, when ingested, causes gastrointestinal illness in humans. *V. parahaemolyticus* is motile, with a single polar flagellum.

A new emerging disease in shrimp, first reported in China in 2009, was initially named “early mortality syndrome (EMS)” which was monitored and reported the situation of the disease by Lightner’s team from the University of Arizona, United State. In 2011, a more descriptive name for the acute phase of the disease was proposed as acute hepatopancreatic necrosis syndrome (AHPNS). The disease is,

then, emerging in Southeast Asia and then affecting both *L. vannamei* and *P. monodon* (Tran et al. 2013) (www.gaalliance.org; www.aquathai.org).

Lightner's team identified the EMS/AHPNS pathogen as a unique strain of a relatively common bacterium, *V. parahaemolyticus*. The bacterium is infected by a phage viruscausing it to release a potent toxin. The bacterium can colonize in the shrimp gastrointestinal tract and produces a toxin that causes tissue destruction and dysfunction of the shrimp digestive organ the hepatopancreas. It does not affect humans.

Since EMS was first reported in China, it has spread to Vietnam, Malaysia and Thailand, and now causes annual losses more than US $1 billion. The EMS outbreaks typically occur within the first 30 days after stocking a newly prepared shrimp pond, and mortality can exceed 70%.

1.3 Immunity

Immunity is the balanced state of having adequate biological defenses to fight with a variety of infectious microorganism, disease, or other unwanted invasion, while having adequate tolerance to avoid inflammation, allergy and autoimmune diseases (https://en.wikipedia.org/wiki/Immunity_(medical)). Traditionally, the immune system can be divided into two parts, innate and adaptive immunity (Kim 2006).

1.3.1 Adaptive immunity

The adaptive immunity is a highly specific to the particular pathogen that induced them and can also provide long-lasting protection. The function of adaptive

immune system in vertebrates is essential to promote protective immunity and to avoid immunopathology. Adaptive immune responses are carried out by white blood cells called lymphocytes. There are two types of adaptive immune responses: humoral immunity, mediated by antibodies produced by B lymphocytes, and cell-mediated immunity, mediated by T lymphocytes (Alberts et al. 2002).

1.3.2 Innate immunity

The innate immune system is the first line of defense against bacterial, fungal, and viral pathogens (Hoebe et al. 2004) that helps to limit infection at an early stage. This defense system is essential for the survival and perpetuation of all multicellular organisms (Hoffmann et al. 1999; Salzet 2001). Innate immune responses include phagocytosis, complement, antimicrobial peptides and proteinase cascades, which lead to melanization and coagulation (Kim 2006). The vertebrates possess both adaptive and innate immune system whereas the invertebrates have only the innate immunity. The innate immunity detects and responds to microbial surface antigens such as lipopolysaccharides (LPS), lipoteichoic acids, lipoproteins, peptidoglycan (PNG) and (1→3) β-D-glucans (Iwanaga and Bok 2005).

1.4 The crustacean immune system

The major defense system of invertebrates including crustaceans is grouped into humoral and cellular responses. The humoral responses include prophenoloxidase (proPO) system, the clotting cascade and the generation of circulating antimicrobial peptides whereas the cellular responses include apoptosis,

phagocytosis, nodule formation and encapsulation of the intruders (Flegel and Sritunyalucksana 2011). Major defense systems are carried out in the hemolymph containing cells called “hemocytes”. Hemocytes are the effectors of the cellular immune response and they are also involved in the synthesis of the majority of humoral effectors. Crustacean hemocytes are generally classified into three types; hyaline (agranular), semigranular (small granular) and granular (large granular) hemocytes (Johansson et al. 2000; Tsing et al. 1989).

1.4.1 Pattern recognition proteins

When foreign entities attack animals, the first immune process is the recognition of a broad spectrum of factors that present on the surface of invading microorganisms. There is little knowledge about the molecular mechanisms that mediate the recognition; however, in crustaceans, several types of modulator proteins recognizing cell surface components of pathogens have been identified. The common structures shared among pathogens are recognized to be non-selves in the immunity (Ochiai and Ashida 1999). This type of recognition is termed pattern recognition and is recognized by “pattern recognition proteins (PRPs) or peptidoglycan recognition proteins (PGRPs)” (Janeway 1989).

The PRPs and/or PGRPs specifically binds to peptidoglycan and plays an important role as a pattern recognition receptor in the innate immunity of insects. There is an essential component for peptidoglycan to trigger the prophenoloxidase cascade that is now recognized to be an important insect defense mechanism.

These patterns include the lipopolysaccharide (LPS) of Gram-negative bacteria, the lipoteichoic acids of Gram-positive bacteria, the glycolipids of mycobacteria, the mannans of yeasts, the ß-1,3-glucan of fungi and the double-stranded RNA of viruses (Ochiai and Ashida 1999; Yang et al. 2015).

Most current researches have emphasized on the possible roles of non-self-recognition molecules in the vertebrate and the invertebrate immune system. The LPS or/and ß-1,3-glucan-binding proteins (LBP, ßGBP, or LGBP), peptidoglycan recognition protein (PGRP), several kinds of lectins, and hemolin have been identified in a variety of invertebrates and show different biological functions after binding to their targets (Young Lee and Söderhäll 2002). In shrimp, the LPS-binding protein has been reported as a multivalent carbohydrate-binding agglutinin, besides its bacterial agglutination ability and phagocytic induction (Francisco 1995). The other protein involved in the recognition of microbial products and the activation of cellular functions is the ß-glucan binding protein. It is apparently monovalent and does not induce agglutination, but activates degranulation as well as the proPO system. Thus, these recognition proteins are capable of activating cellular activities after reacting with the microbial carbohydrates (LPS, peptidoglycan or glucan) (Vargas-Albores and Yepiz-Plascencia 2000).

Tachylectins are found to have hemagglutinating and antibacterial activities which are important in the immune system (Kawabata and Iwanaga 1999). So far, the C-type lectins from shrimp have been reported to be involved in antibacterial,

antifungal and antiviral processes. For instance, FC-hsl and LvLec displayed immune activities against some bacteria and fungi (Sun et al. 2008; Zhang et al. 2009), whilst Fclectin exhibits up-regulated expression levels after challenge with bacteria, lipopolysaccharide or WSSV (Liu et al. 2007). Likewise, LvCTL1 and PmAV exhibit antiviral activity against infection with WSSV and grouper iridovirus, respectively (Luo et al. 2003; Zhao et al. 2009). The PmLT plays a role in the pattern recognition receptor for initial recognition of WSSV, and can also activate the cellular defense mechanism of shrimp hemocytes (Ma et al. 2008).

1.4.2 Cell-mediated defense reactions

Cellular defense reactions include various biological processes such as phagocytosis, encapsulation and nodule formation (Holmblad and Söderhäll 1999) Phagocytosis is a natural phenomenon in all organisms, includes the foreign body attachment, ingestion and destruction. Encapsulation defined as a process wherein layers of cells surround the foreign material occurs when a parasite is too large to be ingested by phagocytosis. Nodule formation is a similar process to capsule formation, which occurs when the bacterial penetrate into the host cells. These structures, capsules and nodules are always melanized in arthropods.

1.4.3 The prophenoloxidase (proPO) system

The proPO activating pathway is a phenoloxidation cascade comprising of pattern recognition proteins, several serine proteases, and their inhibitors and terminates with the zymogens, proPO (Söderhäll and Cerenius 1998; Sritunyalucksana

and Söderhäll 2000; Li and Xiang 2013). The phenoloxidase (PO) exists as an inactive precursor, prophenoloxidase (proPO), which is activated by a stepwise process involving serine proteases activated by microbial cell wall components, such as low quantities of lipopolysaccharides or peptidoglycans from bacteria and ß-1,3-glucans from fungi, through pattern-recognition proteins (PRPs) (Ariki et al. 2004).

Activation of proPO is mediated by a proteinase cascade. This system is similar to the vertebrate complement system, although the components involved are not homologous to the complement factors (Cerenius and Söderhäll 2004). An enzyme that is able to activate the proPO *in vivo* is termed prophenoloxidase activating enzyme or factor (ppA, PPAE, PPAF). In crayfish, ppA is a trypsin-like proteinase present as an inactive form in the hemocyte granules. The active ppA converts proPO to an active form, phenoloxidase (PO) (Aspan 1991, 1995).

PO is a copper-containing protein and a key enzyme in melanin synthesis (Söderhäll and Cerenius 1998; Shiao et al. 2001). PO catalyzes the early steps in the pathway to melanin formation (Söderhäll and Cerenius 1998). The both catalyses O-hydroxylation of monophenols to diphenols and oxidises diphenols to quinones, which can polymerize non-enzymatically to melanin. The PO is a sticky protein and can adhere to the surface of parasites, which will lead to melanisation of the pathogen. In addition to their role in innate immunity, it is important for pigmentation and sclerotization of many tissues (Söderhäll and Cerenius 1998; Li and Xiang 2013).

Melanisation is an important immune mechanism in arthropods and possibly among many other invertebrate (Cerenius et al. 2008). It is usually observed by blackening of the parasite in the hemolymph or black spots on the cuticle. This reaction provides toxic quinone substances and other short-lived reaction intermediates. These substances are involved in the formation of long-lived products such as the melanin that physically encapsulates pathogens (Cerenius et al. 2008). The melanin and intermediates in the melanin formation can inhibit growth of microbial parasites, such as the crayfish plague fungus, *Aphanomyces astaci* (Söderhäll and Ajaxon 1982). Moreover, reaction intermediates in the melanin pathway participate in the wound healing process by the formation of covalent links in damaged tissues resulting in sclerotisation (Theopold et al. 2004; Cerenius et al. 2008). To prevent excessive activation of the proPO cascade, proteinase inhibitors are needed for its regulation.

In the penaeid shrimp, enzymes of the proPO system are localized in the semigranular and granular cells (Perazzolo and Barracco 1997). This is in agreement with a recent study showing that *P.monodon* proPO mRNA is expressed only in the hemocytes (Sritunyalucksana and Söderhäll 2000).

1.4.4 Proteinase inhibitors

In multicellular organisms, serine proteinase inhibitors (SPIs) are essential factors involving in controlling the various proteinase mediated biological processes, such as the complement system, blood coagulation, melanization, apoptosis, etc.

(Iwanaga and Bok 2005; Jiravanichpaisal et al. 2006). Not only do they control the extent of deleterious protease digestion in such processes, they potentially fight as part of the humoral defense of the innate immune system against the invading pathogens (Christeller 2005). Injury and microbial infection in vertebrates lead to activation of the blood coagulation and proPO systems. Both of these systems employ cascades of serine proteinases to amplify an initial signal (wounded tissue or the presence of microbial polysaccharides) resulting in rapid and efficient responses to the threats to health (O'Brien 1993; Whaley 1993). Blood clotting and phenoloxidase activation can also be harmful to the host if they are not limited as local and transient reactions. For this reason, the proteinases in these systems are tightly regulated by proteinase inhibitors.

The SPIs are also involved in direct defense against proteinases from invading pathogens. For example, a subtilisin inhibitor, BmSPI, from *Bombyx mori* might function as an inhibitor to the microbial proteases and protected the silkworm pupae from infection by pathogens (Zheng et al. 2007). Some microbial pathogens and parasites use the SPIs to counter defense the host protective proteinases.

The obligate intracellular parasite of human *Toxoplasma gondii* produces a serine protease inhibitor to protect itself from the digestive enzymes during its residency in small intestine (Morris et al. 2002). For haematophagous insects such as *Dipetalogaster maximus* and *Triatoma infestans*, they secrete potent thrombin

inhibitors, dipetalogastin and infestin, to prevent blood clotting during blood meal (Campos et al. 2002; Mende et al. 2004).

Based on the primary and three-dimensional structures, topological functional similarities (Laskowski M et al. 1988; Bode 1992) and inhibition mechanisms, the proteinase inhibitors are classified into at least 18 families according to Laskowski and Qasim (Laskowski Jr and Qasim 2000). Among them, the following six families: Kazal, BPTI-Kunitz, α-macroglobulin, serpin, pacifastin and bombyx (Pham et al. 1996; Kanost 1999; Simonet et al. 2002) have been described in invertebrate hemolymph or in saliva. One of the well-known SPIs is the Kazal-type SPIs which are usually multi-domain proteins containing more than one Kazal domain (Van de Locht et al. 1995; Rawlings et al. 2004; Rawlings et al. 2008). Structural studies reveal that there are several contact positions responsible for the interactions between Kazal domains and the proteinases (Lu et al. 1997; Bode and Huber 2000). However, the inhibitory specificity is determined mainly by the P1 amino acid residue resided at the second amino acid residue after the second cysteine residue of the domain.

1.4.5 The coagulation system/the clotting system

Hemolymph coagulation is part of the crustacean innate immune response that prevents both leakage of hemolymph from sites of injury and dissemination of bacteria throughout the body (Martin et al. 1991; Li and Xiang 2013). It is a proteolytic cascade and is activated by microbial cell wall components. The coagulation system involves a plasma-clotting protein (CP) and a hemocyte-derived

transglutaminase (TG) (Kopacek et al. 1993; Yeh et al. 1998). It has been shown that polymerization of clottable proteins (CPs) by the hemocyte transglutaminases (TG) triggers hemolymph clotting in crustaceans (Sritunyalucksana and Söderhäll 2000). The crayfish CP is a dimeric protein, which have both free lysine and glutamine residues for covalently linking to each other by TGs. The CP is synthesized in the hepatopancreas and released into the hemolymph.

Transglutaminase (TG) are Ca^{2+}-dependent enzymes capable of forming covalent bonds between the side chains of free lysine and glutamine residues on clotting protein molecules in the presence of calcium ion to form a soft gel at the wound sites (Wang et al. 2001; Shibata et al. 2013). In shrimp, TG is important for blood coagulation and post-translation remodeling of proteins (Huang et al. 2004). The coagulation system is initiated by the activation and lysis of hyaline cells (deposit cells), and then release hemocyte components to react with plasma factors (Omori et al. 1989; Chen et al. 2005).

Recently, a shrimp second TG (STG II) was found from the tiger shrimp hemocyte cDNA. The STG II was characterized as a hemocyte TG that is involved in coagulation (Chen et al. 2005). A novel transglutaminase (*Fc*TG) gene has been cloned from the hemocytes of Chinese shrimp *F. chinensis*. In situ hybridization confirmed that the FcTG is constitutively expressed in the circulatory system and lymphoid organ (Liu et al. 2007). A TG from *P. monodon* was isolated from the hemocyte cDNA library. The TG signals are stronger in mitotic cells, indicating that

cell proliferation and TG synthesis are associated. Preliminary data showed that the recombinant TG has the enzyme activity but lacks coagulation activity (Huang et al. 2004).

1.4.6 Antimicrobial peptides (AMPs)

Antimicrobial peptides (AMPs) are a group of immune active proteins in humoral response capable of inhibiting and eradicating the pathogens (Visetnan et al. 2015). The functions of AMPs have been important for the innate immunity of all animals (Amparyup et al. 2008). Most of the AMPs are small in size, generally less than 150-200 amino acid residues with amphipathic structure and cationic property. However, the anionic peptides also exist.

The AMPs rapidly diffuse to the point of infection. There is evidence that one of the targets for the peptide is the lipid bilayer of the membrane (Yeaman and Yount 2003). In addition, most of the antimicrobial peptides are cationic and their interaction with anionic phospholipids would provide a ready explanation for their specificity for bacterial membranes. With regard to the mechanism by which the peptide breaks down the membrane permeability barrier, it is possible that the peptide induces complete lysis of the organism by rupturing the membrane or perturbing the membrane lipid bilayer that allows the leakage of certain cellular components as well as dissipating the electrical potential of the membrane (Yeaman and Yount 2003).

AMPs are active against a large spectrum of microorganisms; bacteria and filamentous fungi. In addition, some AMPs have antiviral or antiparasitic activities (Hancock and Diamond 2000; Murakami et al. 1991) and may also exhibit an anti-tumor property (Cruciani et al. 1991). Their production was regulated by Toll and IMD pathway in *Drosophila* (Tzou et al. 2002). There are a few reports on antimicrobial peptides in shrimp. Penaeidins which act against Gram-positive bacteria and fungi were reported in *L. vannamei* (Destoumieux et al. 1997). The cDNA clones of penaeidin isoform are also isolated from the hemocytes of *L. vannamei*, *P. setferus* (Gross et al. 2001) and *P. monodon* (Supungul et al. 2004). Crustins are identified from *L. vannamei* and *L. setiferus*. Crustins from shrimp show no homology with other antibacterial peptides, but possess sequence identity with a family of proteinase inhibitory proteins, the whey acidic protein (WAP). Peptide derived from the hemocyanin of *L.vannamei, P. stylirostris* and *P. monodon* possessed antiviral activity has been identified (Destoumieux-Garzon et al. 2001; Patat et al. 2004; Zhang et al. 2004). Recently, the histones and histone-derived peptides from *L. vannamei* have been reported as an innate immune effectors because they can inhibit growth of Gram-positive bacteria (Patat et al. 2004). Till present, different types of antimicrobial peptides or proteins have been reported in shrimp.

1.4.6.1 Penaeidins

Penaeidins are a family of shrimp AMPs that contain the unique characteristic in that they are composed of two domains, the N-terminal proline-rich domain (PRD)

and the C-terminal cysteine-rich domain (CRD) (O'Leary and Gross 2006; Woramongkolchai et al. 2011). Penaeidins are initially characterized from the Pacific white shrimp *L. vannamei* and appear to be expressed in all penaeid shrimps (Li and Xiang 2013). Based on their primary amino acid sequence diversity, four classes of penaeidins such as PEN2, PEN3, PEN4 and PEN5 were identified from the black tiger shrimp *P. monodon*, while three classes of penaeidins such as PEN2, PEN3 and PEN4 are identified in the Pacific white shrimp *L. vannamei* (Ho and Song 2009; Woramongkolchai et al. 2011). Penaeidins possess Gram-positive antibacterial and antifungal activities (Ho and Song 2009), but it appears that different classes exhibit variations in their potency and target specificity against various strains of microorganisms. The recombinant Ch-penaeidin was expressed in *Pichia pastoris*. It exhibits activities against some Gram-negative and Gram-positive bacteria, but has a relatively low activity against fungi (Li et al. 2005). Recombinant Fenchipenaeidin 5–1 in *P. pastoris* exhibits activities against both Gram-positive and negative bacteria and fungi (Kang et al. 2007).

Recently, penaeidin from *P. monodon* is reported to possess an integrin-ß-mediated cytokine feature that promotes shrimp granulocyte and semi-granulocyte adhesion (Li et al. 2010a). However, no activity of penaeidin against shrimp viral pathogens has been reported to date (Woramongkolchai et al. 2011).

1.4.6.2 Crustins

Crustins are cysteine-rich antimicrobial proteins containing a whey acidic protein (WAP) domain. The first crustin was isolated from the granular hemocytes of the shore crab *C. maenas*, and found to be active against only Gram-positive bacteria (Vatanavicharn et al. 2009; Li and Xiang 2013). They are homologues of carcinin that was characterized as a cysteine-rich 11.5 kDa AMP with antimicrobial activity against Gram-positive bacteria (Amparyup et al. 2008).

There are three types of crustins, namely type I, II and III, found among crustaceans, which different in the amino acid sequences between the signal peptides and the WAP domains. They contain Cys-rich, Gly-rich and Cys-rich, and Pro-Arg regions, respectively (Suthianthong et al. 2011). The type I crustin is a carcinin-type crustin first found in the shore crab *Carcinus maenas*. The type II crustin is mostly found in shrimp. The type III crustin has a short N-terminal Pro/Arg-rich sequence followed by the WAP domain (Smith et al. 2008). Most crustins are antimicrobial towards Gram-positive bacteria whereas the type III crustin also has protease inhibitory activity (Vatanavicharn et al. 2009; Donpudsa et al. 2014).

About 50 crustin-like genes have been found in 20 different crustacean species (Smith et al. 2008). Different types of crustins are reported in several types of crustacean. One crustin-like gene (CruFc) cloned from the hemocytes of *F. chinensis* contains a whey-acidic protein (WAP) domain at the C-terminal. The recombinant CruFc can inhibit the growth of Gram-positive bacteria in vitro (Zhang et al. 2007).

Then a new member of crustin family was reported. It is constitutively expressed in hemocytes and dramatically up-regulated after bacteria injection (Liu et al. 2008). The above crustins reported in *F. chinensis* have a common characteristic that one WAP domain exists in them.

In *M. japonicus*, a gene with two WAP domains (Mj-DWD) is isolated. The expression level of Mj-DWD is rapidly upregulated by WSSV infection. The recombinant Mj-DWD shows specific protease inhibitory activity on *Bacillus subtilis* (Chen et al. 2008; Li and Xiang 2013).

In black tiger shrimp *P. monodon*, several isoforms of crustin have been identified from EST database (Tassanakajon et al. 2013). The four isoforms, crustin*Pm*1, crustin*Pm*4, crustin*Pm*5 and crustinlike*Pm* (crustin*Pm*7) have been studied (Amparyup et al. 2008; Vatanavicharn et al. 2009; Krusong et al. 2012; Donpudsa et al. 2014). The crustin*Pm*1 and 5 have the antimicrobial activity against only Gram-positive bacteria whilst the crustin*Pm*4 and crustin*Pm*7 have the antimicrobial activity against both Gram-positive and Gram-negative bacteria. The crustin*Pm*4 can reduce the expression of WSSV early gene *ie1* and able to prolong the lives of WSSV-infected shrimp by more than one day (Donpudsa et al. 2014).

1.4.6.3 Antilipopolysaccharide

Antilipopolysaccharide factor (ALF), a small basic protein, was initially isolated and characterized in the hemocytes of horseshoe crab *Limulus polyphenus* (LALF)

and *Tachypleus tridentatus* (TALF) (Tharntada et al. 2008; Li and Xiang 2013). The ALFs have broad spectra of antimicrobial activity, an ability to kill or neutralize Gram-negative and Gram-positive bacteria, fungi, parasites and viruses. It binds to lipopolysaccharide (LPS) or endotoxin mediated coagulation system and, thus, exhibits strong anti-bacterial activity against the Gram-negative bacteria in particular (Alpert et al. 1992; Tharntada et al. 2008).

An antimicrobial protein gene of the anti-lipopolysaccharide factor family (tentatively named as ALFFc) is cloned from hemocytes of *F. chinensis*. Significant enhancement of ALF transcription appears in shrimp after *Vibrio* infection (Liu et al. 2005).

1.5 Signaling pathway

The synthesis of AMPs in innate immunity in response to infection is signaled by two signaling pathways, the Toll and immune deficiency (Imd) pathways. The two signaling pathways have been studied extensively in insects, particularly the *Drosophila* (Naitza and Ligoxygakis 2004; Ganesan et al. 2011).

The pathways begin with the sensing of infection in the circulating system by some pattern recognition proteins (PRPs) that recognize the common constituents of the pathogens such as peptidoglycans of both Gram-positive and Gram-negative bacteria, lipopolysaccharide of Gram-negative bacteria and ß-glucans of fungi, bind and are activated. The signals of infection as the activated recognition proteins are carried to the AMP-synthesizing cells by binding to the cell surface receptors which,

in turn, activate in series the intracellular proteins involved in the pathways. The signals are relayed as the proteins in the pathways are activated. In the end, the NF-κB transcription factors, Dorsal/DIF of Toll pathway and Relish of Imd pathway are activated and are able to move into the nucleus to commence the synthesis of AMPs (Ganesan et al. 2011). The Toll pathway is responsible for sensing the invasion of Gram-positive bacteria with the Lys-type peptidoglycan and fungi. The Imd pathway is for the Gram-negative bacteria with the diaminopimelic acid (DAP)-type peptidoglycan as well as some Gram-positive bacteria that contain the DAP-type peptidoglycan (Ganesan et al. 2011).

1.5.1 Toll pathway in *Drosophila*

Toll is a transmembrane receptor which relays the stimulation signals into the cells to activate the two Rel factors, Dif and Dorsal (Hultmark 2003). It was first reported as an essential component in dorsal-ventral embryonic development in *Drosophila* (Wu and Anderson 1997; Tanji and Ip 2005). Most of the information on Toll signaling pathway derives from the initial studies of *Drosophila melanogaster* embryo (Ferrandon et al. 2007). The Toll pathway is also stimulated by Gram-positive bacteria through a circulating peptidoglycan recognition protein (PRGP)-SA (Michel et al. 2001). This pathway is related to the Il-1 and Toll-like receptor (TLR) pathways (Hultmark 2003).

Interaction of Spätzle with Toll leads to dimerization of Toll and its TIR domains. *In vivo* experiment showed that the function of Spätzle is to induce the

dimerization of Toll (Tanji and Ip 2005). Toll acts through three death domain-containing (DD) proteins, the kinase Pelle and the adapters Tube and MyD88 (Myeloid differentiation factor 88). The complex of MyD88 and Tube form is localized to the embryonic plasma membrane and promotes Pelle activation, leading to degradation of the IκB-like inhibitor, Cactus (Sun et al. 2004). The Bomanins (Boms) are specifically induced by Toll. Genome engineering indicates that inactivation of these genes decreases survival upon microbial infection. Assay of bacterial load post-infection in wild-type and mutant flies, shows that the Boms are required for resistance to, rather than tolerance of, infection (Clemmons et al. 2015). Using *Drosophila* X virus (DXV) system to screen *Drosophila*'s antiviral activity shows inactivation of this pathway instigates a rapid onset of anoxia induced death in infected flies and increases in viral titers (Zambon et al. 2005). Suppression of the degalactosylation by a novel UDP-galactose transporter called "Senju" results in reduced induction of Toll-dependent expression of an antimicrobial peptide, Drosomycin, and increased susceptibility to infection with Gram-positive bacteria (Yamamoto-Hino et al. 2015).

The Dorsal is translocated from the cytoplasm into the nucleus of the fat body and a Toll pathway target gene reporter is upregulated in response to *Drosophila* C Virus (DCV) infection. This pathway also mediates resistance to several other RNA viruses (Cricket paralysis virus, Flock House virus, and Nora virus). The viral titres are highly increased in mutant flies (Ferreira et al. 2014).

1.5.2 Imd pathway in *Drosophila*

The Imd pathway shares striking similarities with the tumor necrosis factor receptor 1 (TNFR1) signaling cascade (Leulier et al. 2006). The Imd pathway regulates the response to Gram-negative bacterial peptidoglycan (diaminopimelic peptidoglycan-DAP-PGN) (Tanji et al. 2007; Hetru and Hoffmann 2009). This peptidoglycan is recognized by upstream receptors called "peptidoglycan recognition proteins LC (PGRP-LC) and LE" (Gottar et al. 2002; Choe et al. 2002; Chang et al. 2005). The recognition leads to activation of the Rel factor "Relish". Within a short time after infection, Relish is cleaved into two parts. The active N-terminal domain, which contains the DNA-binding motif, translocates into the nucleus to modulate the expression of antimicrobial peptide genes such as *Diptericin* and *AttacinA* (Hultmark 2003; Tanji et al. 2007). RNA interference (RNAi) assays and promoter analyses demonstrate that cooperation of different NF-κB-related transcription factors mediates the synergy (Tanji et al. 2007). Using transgenic fly strains, it is shown that the overexpression of REL-68 is sufficient to activate strong constitutive transcription of the *Diptericin* gene, but little constitutive or inducible transcription of *Attacin* and *Cecropin* genes (Wiklund et al. 2009). Intra-abdominal injection of Cricket Paralysis virus (CrPV) into wild type flies indicates that antimicrobial peptides (AMPs) are not induced and hemocytes are depleted in the course of infection. Loss-of-function mutations in several Imd pathway genes display increased sensitivity to CrPV infection and higher CrPV loads (Costa et al. 2009). Mutation of a novel

suppressor, *caspar*, of the *Drosophila* immune system shows increased antibacterial immune responses including *diptericin* after infection. They response by blocking translocation of an NF-κB transcription factor, Relish, into nucleas. It is highly specific for the Imd pathway and does not affect the Toll pathway (Kim et al. 2006). Rudra is a new regulator of the IMD pathway, which is identified from PGRP-LC by two-hybrid screening method. The expression of rudra is rapidly increased after stimulation with the insect pathogen *Erwinia carotovora carotovora*. It binds with both PGRP-LC and PGRP-LE (Aggarwal et al. 2008). Using deletion mutants, they show that Relish is specifically required for the induction of the humoral immune response, including both antibacterial and antifungal peptides. Relish mutants are very sensitive to *E. cloacae* infection and susceptibility to fungal infection. Relish plays a key role in the humoral response in Drosophila immunity (Hedengren et al. 1999).

1.5.3 Toll pathway in crustaceans

In crustaceans, the two signaling pathways have only recently been recognized. Protein components of the pathways have been identified. For the Toll pathway, the Spätzle proteins from *F. chinensis* Fc-Spz and *L. vannamei* LvSpz1–3 are identified and they are up-regulated against *Vibrio* species and WSSV infection. They also affect the expression of certain antimicrobial proteins (AMPs) (Shi et al. 2009; Wang et al. 2012). The Toll receptors from *P. monodon* PmToll, *L. vannamei* LvToll1-3 and *M. japonicus* MjToll are found to express in all tissues (Yang et al.

2007; Arts et al. 2007; Mekata et al. 2008; Wang et al. 2012). The MyD88 from *S. paramamosain* SpMyD88 is able to bind SpToll while that from *F. chinensis* FcMyD88 is up-regulated transcriptionally against bacterial infection (Li et al. 2013; Wen et al. 2013). The LvPelle was cloned for functional study. It is up-regulated in gills against WSSV infection and able to activate *P. monodon* penaeidin promoter (Wang et al. 2011). The Cactus from *F. chinensis* can regulate the expression of some AMPs and antiviral factor (Wang et al. 2013a). The Dorsal from *L. vannamei* LvDorsal can regulate the transcription of shrimp *penaeidin-4* gene (Huang et al. 2010). Dorsal and/or DIF (Dorsal-related immunity factor), as a Rel/NF-κB transcription factor, can be activated in the successive signaling cascade of Toll pathway for antifungal and antibacterial responses (Stet and Arts 2005; Li and Xiang 2013). Depending on the developmental stage, Toll can activate two closely related NF-κB proteins, DIF and Dorsal and/or DIF in adults and in larvae, respectively (Hoffmann 2003). The end effect of Toll signaling is the dissociation of NF-κB protein from the ankyrin-repeat inhibitory protein Cactus, a homologue of mammalian IκBs (Belvin and Anderson 1996; Towb et al. 2001; Hoffmann 2003). This process releases the NF-κB transcription factors into the nucleus and activate transcription of a variety of genes such as antimicrobial proteins and other immune related genes. It is unclear how activation of the Toll receptor–adaptor complex leads to these various processes.

A Dorsal homolog (FcDorsal) has been cloned from *F. chinensis*. The FcDorsal contains a Rel homolog domain (RHD) and an IPT/TIG (Ig-like, plexins and

transcriptions factors) domain. The expression profiles in the hemocytes and lymphoid organ are apparently modulated when shrimp are stimulated by bacteria or WSSV. The transcription of Penaeidin5 was suppressed in the absence of FcDorsal (Li et al. 2010b).

1.5.4 Imd pathway in crustaceans

For the Imd pathway, the IMDs from *L. vannamei* is able to induce the expression of *penaeidin-4* (Wang et al. 2009). Two IMDs from *F. chinensis* FcIMD and *P. clarkia* PcIMD are involved in regulating the expression of crustins, anti-lipopolysaccharide factors and lysozymes in shrimp and crayfish, respectively (Lan et al. 2013). The Relish from horseshoe crab *C. rotundicauda* CrRelish is up-regulated by *P. aeruginosa* infection (Fan et al. 2008). Two isoforms of LvRelish, short and full length, can be found in *L. vannamei*. The LvRelish can regulate the transcription of *penaeidin-4* (Huang et al. 2009). A Relish from *F. chinensis* FcRelish is essential for the expression of *penaeidin-5* (Li et al. 2009). The expression of Relish from *E. sinensis* EsRelish is induced by fungi and bacteria (Li et al. 2010c).

Similar to Dorsal/DIF, the NF-κB/Rel transcription factor Relish is a key signal mediator and a regulatory factor for the synthesis of cognate AMPs. The Relish is able to move into the nucleus and activate the synthesis of AMPs providing that it is activated. Normally, the full length Relish consists of a Rel homology domain (RHD), a nucleus localization signal, an IκB-like domain containing six ankyrin repeats and a death domain. The Relish is activated by cleaving away its C-terminal half, ankyrin

repeats and a death domain, with a caspase enzyme (Dushay et al. 1996; Kim et al. 2014).

In *Drosophila*, it has been shown that the Toll and Imd pathways are involved in antiviral responses. In particular, the Imd pathway responds to the RNA virus. (Zambon et al. 2005). Studies in shrimp show that the Toll pathway and Imd pathway are also responses to viral infections. The expression of LvDorsal and LvRelish is significantly up-regulated by WSSV (Qiu et al. 2014).

1.6 Suppression subtractive hybridization (SSH)

A PCR-based cDNA subtraction method, also called suppression subtractive hybridization (SSH), is a powerful technique that offers the advantage of being able to compare two mRNA populations and obtain clones of genes that are expressed in one population but not in the other (Diatchenko et al. 1996; Li and Xiang 2013). There are several different methods, the basic theory behind subtraction is simple. First, both mRNA populations are converted into cDNA: we refer to the cDNA that contains specific (differentially expressed) transcripts as tester, and the reference cDNA as driver. Tester and driver cDNAs are hybridized, and the hybrid sequences are then removed. Consequently, the remaining unhybridized cDNAs represent genes that are expressed in the tester yet absent from the driver mRNA. Although traditional subtractive hybridization methods have been successful in some cases, they require several round of hybridization and are not well suited for the

identification of rare messages (Sargent and Dawid 1983; Davis et al. 1984; Hedrick et al. 1984; Duguid and Dinauer 1990; Hara et al. 1991).

The information from SSH approach provides a new insight for further study in the shrimp innate immunity (Li and Xiang 2013).

1.7 Objectives of the thesis

- To identify the *PmRelish* from the black tiger shrimp *Penaeus monodon*.
- To determine the expression of *PmRelish* gene in tissues and in response to bacterial and viral infection.
- To determine the importance and function of *Pm*Relish in regulating antimicrobial peptide synthesis.
- To investigate the cellular localization and the activation of *Pm*Relish in YHV-infected shrimp hemocytes.
- To verify the molecular mechanism of *Pm*Relish, the interaction between *Pm*Relish and DNA was examined.
- To explore the consequence of YHV infection in shrimp and to fish out genes that are under the influence of the Imd pathway using RNA interference (RNAi) and suppression subtractive hybridization (SSH) techniques.

CHAPTER II

MATERIALS AND METHODS

2.1 Materials

2.1.1 Equipment

- 20 ºC Freezer (Whirlpool), - 80 ºC Freezer (ThermoForma)

96-well plate (Bio-Rad)

Amicon Ultra-4 concentrators (Millipore)

Autoclave model # LS-2D (Rexall industries Co. Ltd., Taiwan)

Automatic micropipettes P10, P100, P200 and P1000 (Gilson Medical Electrical S.A., France)

Balance Satorius 1702 (Scientific Promotion Co.)

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

CX31 Biological Microscope (Olympus)

Gel documentation (SYNGENE)

Hyperfilm MP (Amersham International, England)

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

Incubator 30 ºC (Memmert), Incubator 37 ºC (Memmert)

Innova 4080 incubator shaker (New Brunswick Scientific)

LABO Autoclave (SANYO)

Laminar Airflow Biological Safety Cabinets Class II Model NU-440-400E (NuAire, Inc., USA)

Microcentrifuge tubes 0.6 ml and 1.5 ml (BIO-RAD)

Microtiter plate reader (BMG Labtech)

Minicentrifuge (Costar, USA)

NanoDrop™ 2000c Spectrophotometers (Thermo Scientific)

Nipro disposable syringes (Nissho)

Orbital shaker SO3 (Stuart Scientific, Great Britain)

PCR Mastercycler (Eppendorf AG, Germany)

PCR thermal cycler: Gene Amp PCR System 2400 (Perkin Elmer)

PCR thermal cycler: DNA Engine (MI Research, USA)

PCR thermal cycler: S1000™ (Bio-Rad)

PCR thin wall microcentrifuge tubes 0.2 ml (Perkin Elmer)

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

PD-10 column (GE Healthcare)

pH meter model # SA720 (Orion)

Pipette tips 10, 20, 200, and 1000 µl (Bio-Rad)

Power supply Power PAC 3000 (Bio-Rad)

Refrigerated microcentrifuge Kubota 1300 (Kubota, Japan)

Refrigerated microcentrifuge MIKRO 22R (Hettich Zentrifugen, Germany)

Spectrophotometer: Spectronic 2000 (Bausch & Lomb)

Spectrophotometer DU 650 (Beckman, USA)

Stirring hot plate (Fisher Scientific)

Transilluminator 2011 Macrovue (LKB)

Trans-Blot® SD (Bio-Rad)

Vacuum blotter Model # 785 (Bio-Rad)

Vacuum pump (Bio-Rad)

Vertical electrophoresis system (Hoefer™ miniVE)

Whatman® 3 MM Chromatography paper (Whatman International Ltd., England)

White/UV transilluminator: UVP ImageStore 7500 (Mitsubishi Electric Corporation, Japan)

2.1.2 Chemicals and reagents

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

2-Mercaptoethanol, C_2H_6OS (Fluka)

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

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

Absolute ethanol, C_2H_5OH (BDH)

Acetic acid glacial, CH_3COOH (BDH)

Acrylamide (Plus one)

Adenosine-5'-triphosphate potassium salt (ATP) (Sigma)

Agarose (Sekem)

Alexa Fluor 568 goat anti-mouse IgG antibody (Invitrogen)

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

Ammonium persulfate, $(NH_4)_2S_20_8$ (USB)

Amplicillin (BioBasic)

Anti-His antiserum (GE Healthcare)

Bacto agar (Difco)

Bacto tryptone (Scharlau)

Bacto yeast extract (Scharlau)

Boric acid, BH_3O_3 (Merck)

Bovine serum albumin (Fluka)

Bromophenol blue (Merck, Germany)

Calcium chloride, ($CaCl_2$) (Merck)

Chloramphenicol (Sigma)

Chloroform, $CHCl_3$ (Merck)

Coomassie brilliant blue G-250 (Fluka)

Coomassie brilliant blue R-250 (Sigma)

Dextran sulfate (BioBasic Inc.)

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

Dimethyl sulfoxide (DMSO), C_6H_6SO (Amresco)

di-Sodium hydrogen orthophosphate anhydrous, Na_2HPO_4 (Carlo Erba)

Dithiothreitol (Pharmacia)

Ethidium bromide (Sigma)

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

Formaldehyde (BDH)

GeneRuler™ 100bp DNA ladder (Fermentas)

Glucose (Ajax chemicals)

Glycerol, $C_3H_8O_3$ (BDH)

Glycine (Scharlau)

Hydrochloric acid (MERCK)

Imidazole (Fluka)

Isoamylalcohol (Merck)

Isopropanol (Merck)

Isopropyl-ß-D-thiogalactoside (IPTG)

Kanamycin (BIO BASIC Inc.)

Leibovitz's L-15 Medium (GIBCO)

Magnesium chloride (MERCK)

Methanol (MERCK)

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

N, N'-methylene-bisacrylamide, $C_7H_{10}N_2O_2$ (USB)

Ni Sepharose 6 Fast Flow (GE Healthcare)

Nitroblue tetrazolium (NBT) (Fermentas)

Nytrans® super charge nylon membrane (Schleicher & Schuell)

Paraformaldehyde (Sigma)

Phenol crystals, C_6H_5OH (Carlo Erba)

Potassium chloride, KCl (Ajax)

Prestained protein molecular weight marker (Fermentas)

Prolong Gold Antifade Reagent (Invitrogen)

RNase A (Sigma)

RNA markers (Promega)

Skim milk powder (Mission)

Sodium acetate, CH_3COONa (Merck)

Sodium chloride, NaCl (BDH)

Sodium citrate, $Na_3C_6H_5O_7$ (Carlo Erba)

Sodium dihydrogen orthophosphate (Carlo Erba)

Sodium dodecyl sulfate (Sigma Chemical Co., USA)

Sodium hydroxide, NaOH (Eka Nobel)

TEMED $(CH_3)_2NCH_2CH_2N(CH_3)_2$. (Amresco)

TO-PRO-3 iodide (Invitrogen)

Tris-(hydroxy methyl)-aminomethane, $NH_2C(CH_2OH)_3$ (USB)

Tryptic soy broth (Difco)

Triton® X-100 (MERCK)

Trizol reagent (Gibco BRL)

Tween™-20 (Flula)

Urea (Fluka)

Xylene cyanol FF, $C_{25}H_{27}N_2O_6S_2Na$ (Sigma)

X-Gal (5-Bromo-4-Chloro-3-Idolyl-ß-D-Galactopyranose)

2.1.3 Kits

FavorPrep™ Blood/Cultured Cell Total RNA Purification Mini Kit (Favorgen)

FavorPrep Plasmid DNA Extraction Mini Kit (Flavogen)

iQ™ SYBR® Green Supermix (BIO-RAD)

NucleoSpin® Extract II kit (Macherey-Nagel)

PCR-Select™ cDNA Subtraction Kit (Clontech)

pGEM-T easy vector (Promega)

RevertAid™ First Strand cDNA Synthesis Kits (Fermentas)

Sso Fast™ Evagreen® Supermix (Bio-Rad)

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

2.1.4 Enzymes

Advantage® 2 PCR Enzyme (Clonetech)

Ex Taq® (TaKaRa)

RBC *Taq* DNA polymerase (RBCBioscience)

*Bam*HI (Biolabs)

*Cla*I (Biolabs)

*Eco*RI (Biolabs)

*Hin*dIII (Biolabs)

*Kpn*I (Biolabs)

*Nco*I (Biolabs)

*Nde*I (Biolabs)

*Not*I (Biolabs)

*Pst*I (Biolabs)

*Xho*I (Biolabs)

RNase A (Sigma)

RQ1 RNase-free DNase (Promega)

T4 DNA ligase (Biolabs)

2.1.5 Antibiotics

Ampicillin

Chloramphenicol

Kanamycin

Tetracycline

Penicillin and Streptomycin

2.1.6 Bacterial strains

Escherichia coli XL-1 Blue MRF'

Escherichia coli competent cell (StrataClone™SoloPack® competent cell, Algilent)

Escherichia coli Rosetta (DE3) pLysS

Escherichia coli BL21 codon plus

White spot syndrome virus

Yellow head virus

Vibrio harveyi 639

2.1.7 Softwares

BlastX (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi)

Clustal X (Thompson, 1997)

ExPASy ProtParam (http://au.expasy.org/tools/protparam.html)

GENETYX version 7.0 program (Software Development Inc.)

SECentral (Scientific & Educational Software)

SMART (http://smart.emblheidelberg.de/smart/set_mode.cgi.GENOMIC=1)

Penaeus monodon EST database (http://pmonodon.biotec.or.th/home.jsp)

SPSS statistics 17.0 (Chicago, USA)

2.1.8 Vectors

pET-28b(+)

pVR500, a pET-32a(+) derivative

2.2 Shrimp, bacterium and viruses

Healthy black tiger shrimp with body weight of 5-10 g for gene silencing experiment and 10-15 g for other experiments were purchased from local farms Chachoengsao Province and Nakhon Si Thammarat Province, Thailand. The animals were reared in laboratory tanks at ambient temperature (29 ± 4 °C) and maintained in aerated water with a salinity of 15 ppt for at least 7 days before use.

The *V. harveyi* 639 solution was prepared by culturing the bacterium in tryptic soy broth (TSB) supplemented with 2% (w/v) NaCl at 30 °C with shaking at 250 rpm until the OD_{600} reached 0.6 where the cell density was 10^8 CFU/ml. The number of colonies was determined by agar plating method. Shrimp were divided into 3 groups of 10 individuals. The first group, as a control group, was intramuscularly injected with 50 µl of sterile 0.85% (w/v) NaCl (normal saline). The second and third groups were intramuscularly injected with 50 µl of 10^6 and 10^7 CFU/ml *V. harveyi* 639 in normal saline, respectively. The mortality rates were observed every 12 hours. The *V. harveyi* 639 solution dose, which point the cell density was determined to be 10^6 CFU/ml induce a cumulative mortality of ~ 50% within three days post-injection was used in this study.

The yellow head virus (YHV) solution was the hemolymph drawn with anticoagulant from the moribund YHV-infected *P. monodon* according to Pongsomboon et al. (2008) (Pongsomboon et al. 2008).

In this study, the YHV solution was diluted 1:1000 in PBS or normal saline. The diluted YHV solution (15 µl) was injected into each shrimp in the abdominal muscle (20 shrimp). The YHV solution used induced a cumulative mortality of 50% within 3 days post-injection. In all cases, YHV infection or its absence in the shrimp was determined by RT-PCR.

The WSSV was prepared from gills of WSSV-infected *L. vannamei* according to Xie et al. (2005) (Xie et al. 2005), and the number of virions was determined by real time PCR.

2.3 Total RNA extraction by TRI reagent, and DNase treatment

Cells or tissues harvested from shrimp were homogenized in 1 ml of TRI Reagent® (Molecular Research Center). Subsequently, two hundred µl of chloroform was added and the mixture was vigorously shaken for 15 sec. After incubation at room temperature for 5 min, the mixtures were centrifuged at 12,000 × g for 15 min at 4 °C, and, then, the RNA-containing aqueous (upper) phase was transferred to a fresh tube. Total RNA was precipitated by adding a volume of absolute isopropanol, followed by incubating at - 20 °C for 15 min. The supernatant was removed by centrifugation at 12,000 × g for 15 min at 4 °C. The RNA pellet was washed with 1 ml of 70% (v/v) ethanol in diethyl pyrocarbonate (DEPC)-treated water. The total RNA was stored in 70% (v/v) ethanol at -80 °C until used. The ethanol supernatant was completely removed by centrifugation at 12,000 × g for 15

min at 4 ºC. The RNA pellet was air-dried at room temperature for 10 - 15 min, and dissolved in an appropriate volume of DEPC-treated water. The obtained total RNA was further treated with RQ1 RNase-free DNase I (Promega) (1 unit/5 µg of total RNA) at 37 ºC for 30 min to remove the contaminating chromosomal DNA. Then, the RNA pellet was re-purified using TRI Reagent as described above.

2.4 Total RNA extraction by FavorPrep™ Blood/Cultured Cell Total RNA Purification Mini Kit (Favorgen)

Hemocytes were collected from hemolymph by centrifugation at 800 × g for 10 min and the plasma was all removed. Three hundred and fifty microliters of FARB Buffer with ß-mercaptoethanol added were added to the cell pellet and vortexed vigorously. After incubation at room temperature for 5 min, the mixtures were transferred to filter column set centrifuged at 10,000 × g for 4 min at 4 ºC and then transferred the clarified supernatant to a new micro-centrifuge tube. A volume of 70% (v/v) ethanol in diethyl pyrocarbonate (DEPC)-treated water was added to the clear lysate and mixed well by pipetting. The ethanol added sample (including any precipitate) was transferred to FARB Mini Column set (FARB Column), followed by centrifugation at 10,000 × g for 4 min at 4 ºC, and the flow-through was discarded. Two hundred and fifty µl of Wash Buffer were added and centrifuged at 10,000 × g for 4 min at 4 ºC. To eliminate genomic DNA contamination, 30 µl of RNase-free DNase 1 solution (1 unit/µl) was added to the membrane center of FARB Column,

incubated at room temperature for 5 min. Two hundred and fifty µl of Wash Buffer were added and centrifuged at 10,000 × g for 4 min at 4 ºC. The flow-through was discarded. The FARB Column was washed twice with 700 µl of Wash Buffer 2, followed by centrifuged at 10,000 × g for 4 min. The FARB Column was centrifuged at 10,000 × g for 4 min to dry the FARB Column. The FARB Column was transferred to a fresh tube and then 50 µl of RNase-free Water was added to the membrane center of FARB Column. After standing the FARB Column at room temperature for 2 min, the FARB Column was centrifuged at 10,000 × g for 5 min to elute the total RNA which was stored at -80 ºC until used.

2.5 First strand cDNA synthesis using RevertAid RT Reverse Transcription Kit (Thermo Scientific)

The DNase-treated RNA (1 µg) was combined with 5 µM of Oligo$(dT)_{15}$ primer in nuclease-free water for a final volume of 12 µl per a reverse transcription reaction. The sample was added with 8 µl of the reverse transcription reaction mixture, containing 5×Reaction buffer (250 mM Tris-HCl pH 8.3, 250 mM KCl, 20 mM $MgCl_2$, 50 mM DTT), 1 mM dNTP mix, 1 unit of RiboLock RNase Inhibitor, 10 units of RevertAid Reverse Transcriptase, mixed gently and immediately centrifuged briefly. The mixture was incubated at temperature in a series of 42 ºC for 60 min, 70 ºC for 5 min and 10 ºC for 10 min. The reverse transcription reaction product can be directly used in PCR applications or stored at -20 ºC for less than one week.

2.6 Plasmid DNA extraction using FavorPrep Plasmid DNA Extraction Mini Kit (Flavogen)

The plasmid was isolated from the bacterial cell culture using a FavorPrep Plasmid DNA Extraction Mini Kit (Flavogen). The recombinant plasmid was transformed into *Escherichia coli* XL-1 Blue using a $CaCl_2$ method. The transformants were selected using blue-white screening; the agar plates contained 50 µg/ml ampicillin, 100 µl 100 mM IPTG and 20 µl of 50 mg/ml X-gal. The white colonies were inoculated into 3 ml of Luria-Bertani (LB) medium containing an appropriate antibiotic, usually 50 µg/ml of ampicillin and cultured overnight at 37 ºC. The culture was centrifuged at 800*g* at 4 ºC for 2 min and the supernatant was removed.

Bacterial cells were re-suspended in 200 µl FAPD1 Buffer containing RNase A. Then, 200 µl of FAPD2 Buffer was added and mixed thoroughly by inverting the tube 10 times to lyse the cells and incubate at room temperature for 2 min. The cell lysate was neUPSalized by adding 300 µl of FAPD3 Buffer and, then, inverted the tube 10 times immediately. The mixture was centrifuged at 10,000 × g for 10 min at 4 ºC. After centrifugation, the supernatant containing the plasmid was transferred to a FAPD Column by pipetting. The FAPD Column was centrifuged for 60 sec and the flow-through was discarded. The FAPD Column was washed twice with 400 µl of W1 Buffer and 600 µl of Wash Buffer, respectively, and then centrifuged to remove residual ethanol from the Wash Buffer. Finally, the plasmid DNA was eluted by adding 50 µl of heated-Elution Buffer to the membrane center of FAPD Column,

incubated at room temperature for 2 min and centrifuged for 4 min. The flow-through containing the plasmid was then stored at -20 ºC until use.

The concentration of plasmid DNA was determined by measuring the A_{260} and estimated in µg/ml using an equation: [DNA] (µg/ml) = A_{260} × dilution factor × 50 for an A_{260} corresponds to 50 µg/ml of DNA (Sambrook 1989).

2.7 Primer design

PCR primer pairs were designed based on nucleotide sequences of the template DNAs using the SECentral program (Scientific & Educational Software). The primers in the pair should have about the same T_m values. They were checked for minimal self-priming and primer dimer formation. A housekeeping gene, EF-1α was generally used as an internal control (Table 2.1).

Table 2. 1 Primers used to this study

Primer	Sequence (5′-3′)	Product size (bp)	Usage
PmRelish_F1	CCGGTATTGTCAGCTTACG	1,730	Cloning of *PmRelish*
PmRelish_R1	GAAGCAGCTAGGCAGGTC		
PmRelish_F2	CCATACATCCAGGCTCTG	2,300	
PmRelish_R2	CTGCACTTATGATATGCAC		
PmRelish_RT_F	TCTCCAGGTGAGCACTCAGTTGGC	223	RT-PCR and qRT-PCR of *PmRelish*
PmRelish_RT_R	GCTGTAGCTGTTGCTGTTGTTGAG		
EF-1α_F	GGTGCTGGACAAGCTGAAGGC	150	Internal control
EF-1α_R	CGTTCCGGTGATCATGTTCTTGA		
KD_PmRelish_T7F	GGATCCTAATACGACTCACTATAGGCTCGTGGTCA GGAAGACTCAAT	434	dsRNA knockdown
KD_PmRelish_R	GACTGGAGATGGAGACTGAATG		
KD_PmRelish_F	CTCGTGGTCAGGAAGACTCAAT		
KD_PmRelish_T7R	GGATCCTAATACGACTCACTATAGGGACTGGAGAT GGAGACTGAATG		
KD_GFP_T7F	GGATCCTAATACGACTCACTATAGGATGGTGAGCA AGGGGGAGGA	720	
KD_GFP_R	TTACTTGTACAGCTCGTCCA		
KD_GFP_F	ATGGTGAGCAAGGGGGAGGA		
KD_GFP_T7R	GGATCCTAATACGACTCACTATAGGTTACTTGTACA GCTCGTCCA		
ALFPm3_F	CCCACAGTGCCAGGCTCAA	202	qRT-PCR of antimicrobial peptide genes
ALFPm3_R	TGCTGGCTTCTCCTCTGATG		
CrustinPm1_F	CTGCTGCGAGTCAAGGTATG	118	
CrustinPm1_R	AGGTACTGGCTGCTCTACTG		
Penaeidin3_F	GGCTTAGCCCCTTACA	353	
Penaeidin3_R	GACCCATACCTACAAATAAC		
Penaeidin5_F	ATCCCGACCTATTAGTACTC	133	
Penaeidin5_R	TTATCCTTTCAATGCAGAACAA		

Table 2.1 (Continued) Primers used to this study

Primer	Sequence (5′-3′)	Product size (bp)	Usage
PmRelish_F	GGCCCCATGGGACATCATCATCATCATCATGTGAG AGGTGACAGAGGTGG	3,582	Production of recombinant *Pm*Relish protein
Pmrelish_R	GGAAGCGGCCGCTTATGCCTGGTCCAGCACAG		
PmN-terminal_F	CCGGGAATTCATGCATCATCATCATCATCATGTGAG AGGTGACAGAGGTGG	1709	Production of recombinant *Pm*N-terminal protein
PmN-terminal_R	CCGGCTCGAGTTAGTCAGCAACTTCTAACATAT		
PmC-terminal_F	AATTCCATGGGACCTGACACCTTGAAACGCAG	2502	Production of recombinant *Pm*C-terminal protein
PmC-terminal_R	GATTCTCGAGTGCCAGGTCCAGCACAGCAA		
PmRel_F	GGCCCCATGGGACATCATCATCATCATCATGTGAG AGGTGACAGAGGTGG	1,080	Production of recombinant *Pm*Rel RHD protein
PmRel_R	GGAAGCGGCCGCTTACATGAATGTGAAGTCCA		
CPm7UPS_F	TCCAGTCTAAAACGCGACAGGTGATCTC	737	Upstream sequence of *crustinPm*7
CPm7UPS_R	CAGCCACAGCGACAACGGATAATACTAC		
Pen3(411)UPS_F	CTGACCATGGGACATTTACATGAAATTGAAAAGAAC TG	835	Upstream sequence of *penaeidin*3(411)
Pen3(411)UPS_R	TCCTTCCTGTGTCCGCCATGCTCGAGAATG		
Pen5UPS_F	TTGGTCTTCATTACGTGTGGTATATG	459	Upstream sequence of *penaeidin*5
Pen5UPS_R	CGAGACGCATGGCGGACTCAGGAAG		
Kunitze_F	GCCATGGGCCTACTCCGCCCA	212	Confirmation of SSH
Kunitze_R	GCTCGAGTCGGCCGCAGAC		
Transglutaminase_F	ACGACGACTGGGACATAAGG	237	
Transglutaminase _R	CATACTCCTGGCGCATTTTT		
SWDPm2_F	CGGCATCATCACCACGTGCGAG	212	
SWDPm2_R	TCAGTAACCTTTCCAGGGAGAC		
HHAP_F	GCAACAGGAGAACCTGTGGATA	243	
HHAP_R	GGTTCCAGAATCGCCTCCTATA		
SPI*Pm*2_F	ATGCAACCACGTCTGTACTG	192	
SPI*Pm*2_R	CTGCAAGGTTCCACATCT		

2.8 Cloning and nucleotide sequencing of *PmRelish*

The *PmRelish* was PCR amplified from the cDNA of 48-h YHV-infected shrimp using specifically designed primers (Table 2.1). The cDNA was prepared from the shrimp hemocytes. The hemolymph was drawn from shrimp ventral sinus and centrifuged 250 × g for 10 min at 4 °C to collect the hemocytes for total RNA extraction. The total RNA was extracted using Trizol® Reagent (Molecular Research Center) and treated with RNase-free DNase I (Promega). First-strand cDNA was synthesized from 1 μg of total RNA by a cDNA First-strand Synthesis Kit (Fermentas) according to the manufacturer's instruction.

For the ease of cloning the gene fragments and DNA sequencing, the *PmRelish* was amplified as two overlapping fragments. Two primer pairs of *PmRelish*, PmRelish_F1/PmRelish_R1 and PmRelish_F2/PmRelish_R2, were designed based on the Relish sequences from two shrimp species, *LvRelish* and *FcRelish* (Huang et al. 2010; Li et al. 2009). The PmRelish_F1 and PmRelish_R1 (Table 2.1) annealed a few bases upstream of start codon and downstream of stop codon, respectively. The PmRelish_F2 and PmRelish_R2 (Table 2.1) annealed at around the middle of the complete Relish gene.

The PCR amplification was performed using 1 μl cDNA template in 50 μl reaction volume containing 1× buffer, 0.2 mM dNTP, 0.2 μM each primer and 1× Advantage 2 Polymerase Mix (Clontech). The mixtures were subjected to denaturation at 94 °C for 2 min, 5 cycles of 94 °C for 25 s, 62 °C for 45 s and 72 °C

for 3 min, 30 cycles of 94 ºC for 25 s, 60 ºC for 45 s and 67 ºC for 3 min, and the final extension step at 67 ºC for 10 min. The uses of Relish_F1/Relish_R2 and Relish_F2/Relish_R1 in PCR gave rise to two gene fragments with overlapping sequences. The two gene fragments were cloned into pGEM-T easy vector (Promega) and sequenced using T7 promoter and M13 reverse primer by Macrogen Inc., South Korea. Then, the two sequences were connected using a restriction site *Kpn*I in the overlapping sequence. The connection of the *PmRelish* fragments also removed the primer sequences in the overlapping sequence.

Nucleotide and protein sequence similarities were analyzed with BLAST algorithm at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/blast). The protein motifs features were predicted using Simple Molecular Architecture Research Tool SMART (http://smart.embl.heidelberg.de/).

2.9 Tissue distribution of *PmRelish* in normal and YHV-infected shrimp

The expression of *PmRelish* in six different shrimp tissues of normal and YHV-infected shrimp was determined by RT-PCR using the primers PmRelish_RT_F and PmRelish_RT_R (Table 2.1). Experiments were performed in triplicate for statistical analysis. Shrimp were challenged with YHV by injecting 100 µl of YHV suspension in 1× PBS into each shrimp through the abdominal muscle. The control shrimp were injected with 100 µl 1× PBS.

At 48 hours post infection (hpi) when the shrimp were in fully YHV-infected stage, the various tissues: hearts, hepatopancreata, hemocytes, gills, lymphoid organs and stomachs, were dissected out, homogenized in TRI® Reagent (Molecular Research Center) and total RNA isolated. The total RNAs obtained were subjected to cDNA synthesis as described above.

The RT-PCR analysis was performed in 25 µl reaction volume containing appropriate amount of first-stand cDNA, 500 mM KCl, 15 mM $MgCl_2$, 100 mM Tris-HCl, 1 mg/ml BSA, 100 mM $(NH_4)_2$ SO_4, 0.2 mM each dNTP, 0.4 µM of each primer and 1.5 unit *Tag* DNA polymerase (RBC Bioscience). The reaction was denatured at 94 ºC for 2 min followed by 36 cycles of 94 ºC for 30 s, 60 ºC for 30 s and 72 ºC for 30 s, and the final extension step at 72 ºC for 10 min. The PCR products were analyzed with 2.5% agarose gel electrophoresis. The shrimp *EF-1α* gene was used as an internal control.

The band intensities of the PCR products were analyzed using the Genetools analysis software. The relative expression was calculated as the expression of *PmRelish* normalized against that of *EF-1α*. The data were shown as means ± standard deviations. The statistical significance of the data was evaluated using one-way ANOVA followed by Duncan's new multiple range test. Values were considered statistically significant at $P < 0.05$.

2.10 Expression analysis of *PmRelish* in response to viral and bacterial challenges using shrimp hemocyte primary cell culture

The expression of *PmRelish* transcript in shrimp hemocytes upon pathogen challenges was investigated using shrimp hemocyte primary cell culture. The shrimp hemocyte primary cell culture was prepared according to Ponprateep et al. (2011) (Ponprateep et al. 2011). Briefly, hemolymph from 40 normal shrimp was collected using a modified Alsever's solution as an anticoagulant, pooled and centrifuged to collect the hemocytes. The hemocytes were resuspended in 2× L15 medium supplemented with 20% fetal bovine serum (GIBCO) and counted under a light microscope.

The hemocyte suspension was aliquot into each well of a 24-well plate with 1×10^6 cells and the volume adjusted to 400 µl. The plate was incubated at 28 ºC for 3 h for the cells to attach to the plate. The hemocyte cell cultures were divided into four groups and each group was incubated with 1× PBS, 10^6 CFU/ml *V. harveyi* 639 (bacterial cell:hemocyte ratio of 1:1), half the amount of YHV preparation used for the shrimp and 1×10^5 copies of purified WSSV (multiplicity of infection of 1:10). The cultures were collected at 1, 2, 3, 6 and 12 hpi for *V. harveyi* challenge; 1, 2, 6, 12, 24 and 48 hpi for YHV challenge; and 1, 3, 6, 12, 24 and 48 hpi for WSSV challenge. The hemocytes were extracted for total RNA using a FavorPrep™

Blood/Cultured Cell Total RNA Purification Mini Kit (Favorgen) and the total RNAs were subjected to cDNA preparation as described above.

The expression of *PmRelish* was examined by the real-time RT-PCR using the CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad). The primers were Relish_RT_F and Relish_RT_R (Table 2.1). The elongation factor-1α gene (*EF-1α*) was used as an internal control. The real-time RT-PCR reactions were carried out in 20 µl reaction system with appropriate amount of cDNA template, 1× iQ™ SYBR® Green Supermix (BIO-RAD), 0.12 µM each primers, Relish_RT_F and Relish_RT_R. The real-time RT-PCR was carried out with 1 cycle of 94 ºC for 2 min, 40 cycles of 94 ºC for 30 s, 65 ºC for *PmRelish* or 52 ºC for *EF-1α* for 30 s and 72 ºC for 40 s. Each sample was analyzed in triplicate. The relative expression of *PmRelish* was calculated using a comparative method described by Pfaffl (Pfaffl 2001). The data were shown as means ± standard deviations. Statistical analysis was done using the one-way ANOVA followed by Duncan's new multiple range test. Data differences were considered statistically significant at $P < 0.05$. The standard curves for *PmRelish* and *EF-1α* were generated by running triplicate real-time RT-PCR reactions of 5 different 2-fold serial diluted concentrations of cDNAs. The efficiencies for *PmRelish* and *EF-1α* were 100.5 and 99.2%, respectively.

2.11 *PmRelish* gene knockdown

To investigate the importance of *PmRelish* on YHV infection, double-stranded RNA (dsRNA)-mediated gene knock-down of *PmRelish* was employed. The *PmRelish* dsRNA and *GFP* dsRNA were generated by *in vitro* transcription. Primers KD_PmRelish_T7F/KD_PmRelish_R and KD_PmRelish_F/KD_PmRelish_T7R for the synthesis of upper and lower RNA strands, respectively, were designed from the specific *PmRelish* gene region covering the nucleotide sequence 1259-1692 (Figure 3.1) for the synthesis of dsRNA counterpart. The *GFP* dsRNA as a control for dsRNA treatment was produced using primers KD_GFP_T7F/KD_GFP_R and KD_GFP_F/KD_GFP_T7R.

The specific gene regions of *PmRelish* and *GFP* were amplified form respective plasmid DNA templates as PCR products using *Taq* DNA polymerase (RBC Bioscience) as described above. Then, 1 µg of PCR products were used as templates for in vitro transcription using T7 RiboMAX™ Express Large Scale RNA Production Systems (Promega) following the manufacturer's protocol for the synthesis of respective dsRNAs. The quality and amount of dsRNAs were verified by 1.5% agarose gel electrophoresis.

The dsRNAs were tested for their efficacy in gene silencing. Three groups of eight shrimp were given injection of 20 µl of 1× NACl, *PmRelish* dsRNA (5 µg/g

shrimp) and *GFP* dsRNA (5 µg/g shrimp). After 24 h, the shrimp in each group were injected again with the same solutions plus YHV.

At 3, 6, 24 and 48 h post-second injection, the hemolymph of two individual shrimp from each group were collected at each time point for total RNA extraction by a FavorPrep™ Blood/Cultured Cell Total RNA Purification Mini Kit (Favorgen). One microgram of total RNA was synthesized cDNA according to the manufacturer's instructions.

RT-PCR was performed to evaluate the level of gene transcript silencing using the specific primers PmRelish_RT_F and PmRelish_RT_R (Table 2.1) with the conditions as a mention above. The PCR product was analyzed by 2.5% agarose gel electrophoresis, and the expression level of *PmRelish* was reported as a relative to *EF-1α*.

2.12 Mortality assay of *PmRelish*-silenced shrimp upon YHV infection

To test the importance of *PmRelish* in the shrimp immune defense against YHV, three groups of 12 shrimp were intramuscularly injected with 20 µl of 1× PBS, 5 µg/g shrimp *PmRelish* dsRNA in 1× PBS and 5 µg/g *GFP* dsRNA in 1× PBS. After 24 h, they were given the same second injection along with 20 µl YHV solution. The shrimp in control group were injected with 1× PBS followed by 1× PBS instead of YHV. The mortality was, then, recorded every 6 hpi up to 90 hpi. This experiment was performed in triplicate. Data were analyzed using GraphPad Sigma Plot, and

presented as Kaplan-Meier survival plots with the *P* values calculated by log-rank test.

2.13 Effect of *PmRelish* gene knockdown in conjunction with YHV infection on the expression of antimicrobial peptides

The expression of four AMPs: *ALFPm3*, *crustinPm1*, *penaeidin3* and *penaeidin5*, were investigated in the *PmRelish*-knockdown shrimp in conjunction with YHV infection. Two groups of six Juvenile shrimp of 5 g were given double injection with 20 µl of *PmRelish* dsRNA (5 µg/g shrimp) and *GFP* dsRNA (5 µg/g shrimp) followed 24 h thereafter by the second injection of the same solutions plus the YHV.

The hemolymph was collected from 3 shrimp each at 3 and 6 h after the second injection. The transcript levels of *ALFPm3*, *crustinPm1*, *penaeidin3*, and *penaeidin5* were analyzed by real-time PCR as described above using specific primer pairs (Table 2.1). The *EF-1α* was served as an internal control. The amplification of each gene was done in triplicate. Relative expression was calculated using a mathematic model of Pfaffl (2001) (Pfaffl 2001). The standard curves for *ALFPm3*, *crustinPm1*, *penaeidin3*, *penaeidin5* and *EF-1α* were evaluated as described above. The efficiencies for *ALFPm3*, *crustinPm1*, *penaeidin3*, and *penaeidin5* and *EF-1α* were 99.4, 99.4, 99.6, 99.6 and 99.8%, respectively. Data were analyzed for statistically significance using one-way ANOVA followed by Duncan's new multiple ranges test and presented as means ± standard deviations.

2.14 Effect of *PmRelish* gene knockdown on the transcription of antimicrobial peptides after *V. harveyi* 639 infection

The expression level of four antimicrobial peptides: *ALFPm*3, *crustinPm*1, *penaeidin*3 and *penaeidin*5, were examined in the *PmRelish* silencing shrimp after *V. harveyi* 639 infections. Shrimp were divided into two groups, a *PmRelish* knockdown group and *GFP* control group of six shrimp. Each group was double injected with 20 µl of *PmRelish* dsRNA (5 mg/g shrimp) and *GFP* dsRNA (5 mg/g shrimp), respectively. After 24 h post-primary injection, two groups of six shrimp were given a second injection of the same solutions plus *V. harveyi* 639.

The hemocytes were collected at 3 and 6 h post-second injection. The transcript levels of four antimicrobial peptides were analyzed by real-time PCR as described above using specific primer pairs (Table 2.1). The *EF-1α* was served as an internal control. The experiments were performed in triplicate for statistical analysis.

The standard curves and the efficiencies for *ALFPm*3, *crustinPm*1, *penaeidin*3, *penaeidin*5 and *EF*-1*α* were generated as described above. The calculation of the relative expression level was also performed as described above. All samples were normalized relative to the Ct of the reference *EF-1α* gene in the same sample.

2.15 Cloning and over-expression of *Pm*Relish

2.15.1 Construction of the protein expression clones

To express the recombinant *Pm*Relish, four primer pairs, PmRelish_F/PmRelish_R, PmN-terminal_F/PmN-terminal_R, PmC-terminal_F/PmC-terminal_R and PmRel_F/PmRel_R were designed from the specific *PmRelish* gene sequence (Table 2.1) for PCR amplification of the *Pm*Relish, *Pm*N-terminal, *Pm*C-terminal and *Pm*Rel, respectively. They were produced with a 6×His tag at the N-terminus but except for *Pm*C-terminal which was produced with 6×His tag at the C-terminus. Two primer pairs, PmRelish_F/PmRelish_R and PmC-terminal_F/PmC-terminal_R were conjugated with NcoI and XhoI sites while the primer pair (PmRel_F/PmRel_R) for PmRel was fused with NcoI and NotI sites at the 5′ and 3′ ends of the gene fragment. The specific primer pair (PmN-terminal_F/PmN-terminal_R) was added *EcoR*I and *Xho*I sites at the 5′ and 3′ ends of the gene fragment.

The *Pm*Relish-recombinant plasmid was used as DNA template. The *Pm*Relish, *Pm*N-terminal, *Pm*C-terminal and *Pm*Rel fragments were PCR amplified in a final reaction volume of 100 µl containing 300 ng of plasmid template, 1× buffer, 0.4 µM of each primer, 0.4 mM of dNTPs and 1× Advantage 2 Polymerase Mix (Clontech). The PCR amplification was carried out of 94 °C for 2 min, followed by 5 cycles of 94 °C for 25 s, 62 °C for 45 s and 72 °C for 3 min, and then followed by

30 cycles of 94 ºC for 25 s, 60 ºC for 45 s and 67 ºC for 3 min, and final extension at 67 ºC for 10 min. The amplified products were analyzed using 1.5% agarose gel electrophoresis, excised and purified using NucleoSpin® Extract II Kits (Macherey-Nagel) according to the manufacturer's instruction. The purified DNA fragments were cloned into the *Nco*I–*Xho*I digested pET-28b(+) expression vector (Novagen) for *Pm*Relish fragment, the *Nco*I–*Xho*I digested pET-32a(+) expression vector (Novagen) for *Pm*C-terminal fragment, the *EcoR*I–*Xho*I digested pET-28b(+) for *Pm*N-terminal, the *Nco*I–*Not*I digested pET-28b(+) for *Pm*Rel fragment and by using T4 DNA ligase (New England Biolabs) in the reaction volume of 10 µl comprising 150 ng of the DNA insert and 50 ng of the plasmid. The ligation reactions were incubated at room temperature for 1 hour. The recombinant plasmid was transformed into *Escherichia coli* XL-1 Blue MRF', followed by screening on Luria-Bertani (LB) agar medium containing 100 µg/ml ampicillin at 37 ºC overnight. The expression clones were named r*Pm*Relish, r*Pm*N-terminal, r*Pm*C-terminal and r*Pm*Rel, respectively. They were isolated from the positive clones using FavorPrep Plasmid DNA Extraction Mini Kit (Flavogen) according to the manufacturer's instructions and sequenced using T7 promoter and T7 terminater at Macrogen Inc., South Korea to examine the correctness of the inserted r*Pm*Relish, r*Pm*N-terminal, r*Pm*C-terminal and r*Pm*Rel DNA sequences.

For some reasons, the r*Pm*N-terminal was not expressed well; it was then subcloned into a pET-32a(+) derivative, pVR500, and expressed as fusion protein to the thioredoxin.

2.15.2 Recombinant protein expression

The recombinant plasmids, r*Pm*Relish, r*Pm*N-terminal, r*Pm*C-terminal and r*Pm*Rel, were transformed into the expression host, *E. coli* BL21 codon plus for r*Pm*Relish, r*Pm*N-terminal and r*Pm*Rel, an *Escherichia coli* Rosetta (DE3) pLysS for r*Pm*N-terminal and r*Pm*C-terminal by $CaCl_2$ method, followed by screening on LB agar medium containing 70 µg/ml kanamycin and 34 µg/ml chloramphenicol for r*Pm*Relish, r*Pm*C-terminal, and r*Pm*Rel, 100 µg/ml ampicillin and 34 µg/ml chloramphenicol for r*Pm*N-terminal at 37 °C overnight. The starter cultures were prepared by inoculating a single colony from each a freshly plate into a 2 ml LB medium containing 70 µg/ml of kanamycin for r*Pm*Relish, r*Pm*C-terminal, and r*Pm*Rel or 100 µg/ml of ampicillin for r*Pm*N-terminal and 34 µg/ml of chloramphenicol and incubating at 37 ºC overnight with shaking at 250 rpm.

The culture was diluted 1:100 in fresh LB medium supplemented with antibiotics and grown at 37 ºC with shaking at 250 rpm until an OD_{600} of the cultures reached 0.6. Before the induction, the split culture was served as an uninduced control. Protein expression was induced by the addition of 1 M isopropyl-ß-D-thiogalactopyranoside to a final concentration 1 mM, and incubated at 37 ºC with

shaking. The cells were harvested at 0, 2, 4, 6 and 8 h for r*Pm*Relish, at 0, 2, 3, 4 and 24 h for r*Pm*N-terminal, at 0, 1, 2, 3, 4 and 6 h for r*Pm*C-terminal and at 0, 1, 2, 3 and 4 h for r*Pm*Rel post-induction by centrifugation at 5,000 × g for 4 min at 4 °C, then the cells were resuspended in SDS-PAGE sample buffer (or stored at -80 °C for further analysis) followed by heating at 100 °C for 10 min. The overexpression of r*Pm*Relish, r*Pm*N-terminal, r*Pm*C-terminal and r*Pm*Rel were analyzed by SDS-PAGE (15% (w/v) acrylamide) followed by coomassie brilliant blue staining, and western blot analysis.

2.16 Protein analysis and western blot detection

Protein samples from the overexpression of r*Pm*Relish, r*Pm*N-terminal, r*Pm*C-terminal and r*Pm*Rel were prepared by resuspending the protein samples in 1× sample buffer (12 mM Tris-HCl, pH 6.8, 5% glycerol, 0.4% SDS, 2.88 mM 0.02% bromophenol blue, 2-mercaptoethanol). The samples were then boiled for 10 min and either held at room temperature or kept at 0 °C until loaded into the gel.

The samples were separated by SDS-PAGE (15% (w/v) acrylamide), and transferred onto PROTRAN™ nitrocellulose transfer membrane (Whatman) by a semi-dry blotter. After transferring the proteins from the gel to the membrane, the orientation of the gel was marked on the membrane. The membrane was immersed in blocking buffer (1× phosphate buffered saline (PBS), pH 7.4 containing 5% (w/v) skim milk and 0.05% (v/v) Tween 20) at room temperature for an overnight with

gentle shaking, and then washed 3 times for 10 min each in washing buffer (1× phosphate buffered saline (PBS), pH 7.4 and 0.05% (v/v) Tween 20). The membrane was incubated with a 1:3,000 dilution of an anti-His antibody solution in PBS, pH 7.4 containing 1% (w/v) skim milk and 0.05% (v/v) Tween 20 at 37 ºC with gentle mixing for 1 h. Then, the membrane was washed 3 times with washing buffer (1× phosphate buffered saline (PBS), pH 7.4 and 0.05% (v/v) Tween 20), and incubated with a 1:2,500 dilution of alkaline phosphatase-conjugated goat anti-mouse IgG antibody in washing buffer containing 1% (w/v) skim milk at room temperature for 1 h. The membrane was washed 3 times with washing buffer, and the color was developed with NBT/BCIP solution (Fermentas) as substrate dissolving in 100 mM Tris-HCl, 100 mM NaCl and 50 mM $MgCl_2$, pH 9.5.

2.17 Purification of recombinant protein and the antibody production

After inducing the overexpression of r*Pm*Relish, r*Pm*N-terminal, r*Pm*C-terminal and r*Pm*Rel with 1 mM IPTG for 4 h, the r*Pm*Relish, r*Pm*N-terminal and r*Pm*C-terminal can't be expressed but only the r*Pm*Rel can be expressed in an *E. coli* expression system.

The r*Pm*Rel was produced and found located in the inclusion bodies, so it was solubilized with 25 mM sodium hydroxide buffer (NaOH), pH 14, at room temperature for 1-2 h. The solubilized protein was dialysis against 20 mM sodium carbonate buffer (Na_2CO_3), pH 10, at 4 ºC overnight. The dialyzed protein was

collected and then centrifuged at 10,000g at 4 ºC for 30 minutes to separate the soluble fraction. The soluble fraction was purified under non-denaturing condition using nickel affinity chromatography (GE Healthcare). The Ni-NTA agarose was packed into the PD-10 column and washed 3 times with distilled water, respectively. The Ni-NTA was equilibrated using binding buffer (20 mM carbonate buffer, pH 10, containing 20 mM imidazole). The soluble fraction was incubated with equilibrated Ni-NTA agarose at room temperature for 30 min by shaking at 50 rpm. The mixer solution was loaded into the PD-10 column at room temperature. The flow through was collected by a gravity flow. The column was washed with binding buffer followed by the wash buffer (20 mM carbonate buffer pH 10 containing 50 mM imidazole) to remove unbound proteins. After washing, the protein was eluted with an elution buffer (20 mM carbonate buffer, pH 10, containing 300 mM imidazole). The presence and purity of the purified protein was evaluated by 15% SDS-PAGE. The imidazole was removed by dialysis for at least 10 h at 4 ºC against 10 mM carbonate buffer, pH 10 twice. The purify protein was directly used or kept at 4 ºC until used. The r*Pm*Rel was confirmed by Western blot analysis using anti-His tag antibody (Merck). The protein content was determined by the Bradford assay. The purified r*Pm*Rel was used to generate mouse polyclonal antibody by a service at Biomedical Technology Research Unit, Chiang Mai University, Thailand.

2.18 Immunolocalization of *Pm*Relish protein in shrimp hemocytes

The cellular location of *Pm*Relish in the hemocytes of normal and YHV-infected shrimp was investigated using a confocal laser scanning microscopy. The samples were prepared from three groups of 36 shrimp. The first group was untreated. Shrimp in the second and third groups were injected with 20 µl 1× PBS and 20 µl YHV suspension, respectively. At time points 12, 24, and 36 hpi, the hemolymph from 4 shrimp per group per time point was withdrawn and fixed in 4% (w/v) paraformaldehyde. The hemocytes were separated by centrifugation at 250 × g at 4 ºC for 10 min. The hemocytes were washed three times with 1× PBS, counted and coated 10^6 cells/slide onto the poly-L-lysine slides (Thermo Scientific) by centrifuged at 1,000 × g for 10 min.

The slides were washed three times with 1× PBS for 5 min. The hemocytes were permeabilized by adding 250 µl 0.1% Triton X-100 in 1× PBS for 5 min at room temperature and washed three times with 250 µl of 1× PBS for 5 min. The slides were blocked with 10% fetal bovine serum (FBS) in 1× PBS at room temperature for 1 h and probed with the purified mouse IgG polyclonal antibody specific to *Pm*Relish protein, diluted 1:50 in 1× PBS containing 1% FBS at room temperature for 2 h, and then washed three times with 1× PBS for 5 min. The slides were incubated with 1:500 dilution of Alexa 568 conjugated goat-anti-mouse antibody (Invitrogen) at room temperature for 1 h. The slides were then washed three times with 1× PBS for 5 min

to remove non-specific binding, and subsequently incubated with TOPRO-3 iodide (Invitrogen) diluted 1:1,500 in 1× PBS at room temperature for 10 min to stain the nuclear DNA and washed once with 1× PBS. ProLong® Gold Antifade mounting medium (Invitrogen) was applied, and the slides were examined under a Nikon confocal laser scanning microscope to collect the images.

2.19 Detection of activated *Pm*Relish from YHV-infected shrimp

To detect the activated *Pm*Relish, the membrane was incubated in blocking buffer (1× PBS pH 7.4, 0.05% (v/v) Tween® 20 and 5% (w/v) non-fat dry milk) at 4 °C for an overnight. The membrane was washed three times for 10 min each in washing buffer (1× PBS pH 7.4 and 0.05% (v/v) Tween® 20) and incubated in an anti-*Pm*Rel antibody solution (1:50 dilution of anti-*Pm*Rel in washing buffer with 1% (w/v) non-fat dry milk(at room temperature with gentle mixing for 3 h. The membrane was washed three times for 10 min each in washing buffer and further incubated in a secondary antibody solution (1:500 dilution of alkaline phosphatase-conjugated goat anti-mouse IgG (Jackson ImmunoResearch) in washing buffer with 1% (w/v) non-fat dry milk) with agitation for 1 h. The membrane was, finally, washed three times for 10 min each in washing buffer. The bound antibody was detected by incubating the membrane with NBT/BCIP (Fermentas) solution (NBT/BCIP in 100 mM Tris-HCl pH 9.5, 100 mM NaCl and 50 mM $MgCl_2$) for color development.

2.20 Detection of Protein-DNA interaction by DNA binding assay

To investigate the interaction between *Pm*Relish and upstream sequence of three antimicrobial peptides (Crustin*Pm*7, Penaeidin3(411), Penaeidin5), so the 5′ upstream sequence of Crustin*Pm*7, Penaeidin3(411) and Penaeidin5 were constructed.

Three primer pairs, CPm7UPS_F/CPm7UPS_R, Pen3(411)UPS_F/Pen3(411) UPS_R and Pen5UPS_F/Pen5UPS_R were designed based on the upstream sequence of three antimicrobial peptides (crustin*Pm*7, *penaeidin3* (or 411 in Ho and Song 2009 or called "penaeidin3(411)") and *penaeidin*5) from two shrimp species, *L. vannamei* and *P. monodon* (Amparyup et al. 2008; Ho and Song 2009; Woramongkolchai et al. 2011) for PCR amplification of the CPm7UPS, Pen3(411)UPS and Pen5UPS, respectively (Table 2.1). The cDNA of 12-h YHV-infected shrimp hemocyte was used as template for PCR amplification in a final volume of 100 µl containing an appropriate amount of cDNA 1× buffer, 0.4 µM of each primer, 0.4 mM of dNTPs and 1× Advantage 2 Polymerase Mix (Clontech). The PCR amplification was carried out of 94 ºC for 2 min, followed by 5 cycles of 94 ºC for 25 s, 62 ºC for 45 s and 72 ºC for 3 min, and then followed by 30 cycles of 94 ºC for 25 s, 60 ºC for CPm7UPS and Pen5UPS, 55 ºC for Pen3(411), for 45 s and 67 ºC for 3 min, and final extension at 67 ºC for 10 min. The PCR amplified products were analyzed using 1.5% agarose gel electrophoresis, excised and purified using NucleoSpin® Extract II Kits (Macherey-

Nagel) according to the manufacturer's instruction. The purified DNA fragments were cloned into a pGEM-T easy vector (Promega) by using T4 DNA ligase (New England Biolabs), transformed into *Escherichia coli* XL-1 Blue MRF', followed by screening on Luria-Bertani (LB) agar medium containing 100 µg/ml ampicillin, 100 µl of 100 mM IPTG and 20 µl of 50 mg/ml X-gal and incubated at 37 °C overnight. The recombinant plasmid DNAs were purified by FavorPrep Plasmid DNA Extraction Mini Kit (Flavogen) according to the manufacturer's instructions. The recombinant plasmid DNAs were named rCPm7UPS, rPen3(411)UPS and rPen5UPS, respectively. They were sequenced using T7 promoter and M13 reverse by Macrogen Inc., South Korea to evaluate the correctness of the three inserted (rCPm7UPS, rPen3(411)UPS and rPen5UPS) DNA sequence.

Nucleotide similarities were analyzed with BLAST algorithm at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/blast) and CrustalX program.

Gel-retardation experiments were performed by mixing 100 ng of each recombinant plasmid DNA (rCPm7UPS, rPen3(411)UPS and rPen5UPS) with increasing the amount of r*Pm*Relish protein in a final volumn 20 µl of binding buffer [5% glycerol, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 1mM DTT, 20 mM KCl and 50 mg/ml BSA]. The reaction mixtures were incubated at room temperature for 1.5 h. Subsequently, 4 µl of native loading buffer was added [10% Ficoll 400, 10 mM Tris-HCl (pH 7.5), 50 mM EDTA, 0.25% bromophenol blue and 0.25% xylene cyanol]

and applied all to a 1% (w/v) agarose gel electrophoresis in 0.5× Tris borate-EDTA buffer for 90 min (Park et al. 1998). The Bovine serum albumin (BSA) was used as internal control.

2.21 Suppression subtractive hybridization (SSH) technique

2.21.1 Viral challenge and cDNA preparation

Shrimp were separated into two groups (*GFP*+YHV and *PmRelish* knockdown+YHV groups) with three shrimp per group. The shrimp are injected with 5 µg/g shrimp of *GFP* dsRNA in 30 µl normal saline solution for *GFP* group and 5 µg/g shrimp of *PmRelish* dsRNA in 30 µl normal saline solutions for *PmRelish* knockdown group. After 24 h post-injection, each shrimp from *GFP* and *PmRelish* knockdown groups were injected with 30 µl of the same solutions plus the YHV. Haemocytes are collected at 3 and 6 h post-second injection. Total RNA is extracted from the haemocytes using Favor Prep™ blood/cultured cell total RNA Purification Mini Kit (Favorgen) and then the RNA quality was assessed by 1.5% agarose gel electrophoresis.

Total RNA was used to synthesize cDNA by SMARTer™ PCR cDNA Synthesis Kit (Clontech, America) according to the manufacturer's instructions for cDNA subtraction. Briefly, 1 µg of total RNA from each sample (tester and/or driver group) was combined with 1.2 µM of SMART CDS Primer II A (5′–AAGCAGTGGTATCAACGCAGAGTAC$T_{(30)}N_{-1}$N–3′) in nuclease-free water for a final

volume of 4.5 µl per a first-strand reaction, mix and spin the tubes briefly. The mixture was incubated at 72 ºC for 3 min, and then reduced the temperature to 42 ºC for 2 min. The sample was added with 5.5 µl of the reverse transcription reaction mixture, containing 5× reaction buffer (250 mM Tris-HCl pH 8.3, 375 mM KCl, 30 mM $MgCl_2$, 2.5 mM DTT, 1 mM dNTP mix, 1.2 µM of SMARTer II A Oligonucleotide primer (5′–AAGCAGTGGTATCAACGCAGAGTACXXXXX–3′), 1 unit of RNase Inhibitor, 10 units of SMARTScribe Reverse Transcriptase, mixed gently and immediately centrifuged briefly. The mixture was incubated at 42 ºC for 60 min followed by 70 ºC for 5 min to terminate the reaction. The first-strand reaction product (ss cDNA), Tester_cDNA for tester group, Driver_cDNA for driver group, could be directly used in cDNA amplification. If necessary, cDNA samples could be stored at -20 ºC (for up to three months) until it was ready to proceed with cDNA amplification. The *Rsa*I site (underlined) was added into the 3′ ends of the SMART CDS Primer II A and the SMARTer II A primers.

To prepare the cDNA sample for cDNA amplification, the first-strand reaction products (Tester_cDNA and Driver_cDNA) were added with 40 µl of TE buffer (10 mM Tris-HCl pH 8.0, 0.1 mM EDTA) and used as a cDNA template for PCR amplification. The cDNA amplification was done using 10 µl (200 ng of RNA) of the diluted cDNA in a final reaction volume of 100 µl containing 1× buffer of Ex Taq (TaKaRa), 0.24 µM of 5′ PCR Primer II A, 0.2 mM of dNTP mix and 10 units of Ex Taq (TaKaRa). The PCR

mixtures were subjected to denaturation at 95 ºC for 1 min, 15 cycles of 95 ºC for 15 s, 65 ºC for 30 s and 68 ºC for 3 min, and the final extension step at 68 ºC for 10 min. The PCR products were analyzed by 1.2% (w/v) agarose gel electrophoresis and then added 2 µl of 0.5 M EDTA to each tube to terminate the reaction. The PCR products were purified by column chromatography provided in SMARTer™ PCR cDNA Synthesis Kit (Clontech) according to the manufacturer's instruction. The purified PCR products were analyzed by 1.2% (w/v) agarose gel electrophoresis again to confirm the presence of purified PCR product in the purified ds cDNA fraction.

The purified ds cDNAs were digested with *Rsa*I restriction enzyme to generate shorter, blunt-ended ds cDNA fragments, which are necessary for both adaptor ligation and subtraction. After that, *Rsa*I-digested tester and driver cDNAs were purified by phenol:chloroform extraction method.

2.21.2 Construction of cDNA libraries using SSH

To identify the immune genes up-regulated in response to YHV infection and under the *PmRelish* regulation, the SSH was performed to generate two libraries in forward and reverse directions using the Clontech PCR-select™ cDNA Subtraction Kit (Takara) according to the manufacturer's instructions. The cDNAs were from the shrimp injected with dsRNA of either *GFP* or *PmRelish* and YHV for 3 h and 6 h. For the forward subtraction, cDNAs of *PmRelish* dsRNA-YHV group were used as driver

(Driver_cDNA) to subtract from those of tester, *GFP* dsRNA-YHV group (Tester_cDNA). For the reverse subtraction, cDNAs of *GFP* dsRNA-YHV group were used as driver (Driver_cDNA) to subtract from those of tester *PmRelish* dsRNA-YHV group (Tester_cDNA).

The *Rsa*I-digested tester cDNA was subdivided into two portions, and each was ligated with a different adaptor (adaptor 1 and adaptor 2R) at the 5′ ends of the cDNA using 400 units of T4 DNA ligase while the driver cDNA had no adaptor. The mixtures were incubated at 16 ºC overnight. The samples were heated at 72 ºC for 5 min to inactivate the ligase.

The adaptor 1-ligated and adaptor 2R-ligated tester cDNAs were then separately hybridized with an excess amount of the driver cDNA at 68 ºC for 8 h after denaturation at 98 ºC for 90 sec in a S1000™ Thermal cycler (Bio-Rad). Then, the two hybridization samples were mixed together without denaturation and hybridized at 68 ºC overnight with an excess amount of fresh denatured driver cDNA. The resulting mixture was diluted in 50 µl of dilution buffer (Clontech) composed of 20 mM HEPES, 20 mM NaCl and 0.2 mM EDTA followed by nested PCR amplification.

In the primary PCR, the diluted cDNAs were amplified with a primer against adaptor 1 and adaptor 2R, which were used to selectively amplify differentially expressed cDNA. The primary PCR reaction consisted of 1 µl of the diluted cDNA, 0.2 mM dNTP mix, 0.4 µM of the PCR Primer 1(5′-CTAATACGACTCACTATAGGGC-3′), 2.5 units of Ex Tag polymerase (5 U/µl) (TaKaRa), 1× PCR buffer of Ex Taq (TaKaRa).

The reaction mixture was denatured at 94 ºC for 2 min followed by 27 cycles of 94 ºC for 30 s, 55 ºC for 30 s and 72 ºC for 90 s, and the final extension step at 72 ºC for 10 min after incubate the reaction mixture at 75 ºC for 7 min in a thermal cycler to extend the adaptors.

Then, the product of first PCR was used as template in the second PCR with nested primers, Nested PCR primer 1 (5′-TCGAGCGGCCGCCCGGGCAGGT-3′) and Nested PCR primer 2R (5′-AGCGTGGTCGCGGCCGAGGT-3′), to generate subtracted cDNA (Figure 2.1). The secondary PCR reaction consisted of 1 µl of the ten-fold diluted product from the first PCR, 0.2 mM dNTP mix, 0.4 µM of each primer, 2.5 units of Ex Tag polymerase (5 U/µl) (TaKaRa), 1× PCR buffer. The reaction mixture was denatured at 94 ºC for 2 min followed by 12 cycles of 94 ºC for 30 s, 68 ºC for 30 s and 72 ºC for 90 s, and the final extension step at 72 ºC for 10 min. The products of primary and secondary PCRs were analyzed by 2% agarose gel electrophoresis. The PCR product is now enriched for differentially expressed cDNAs.

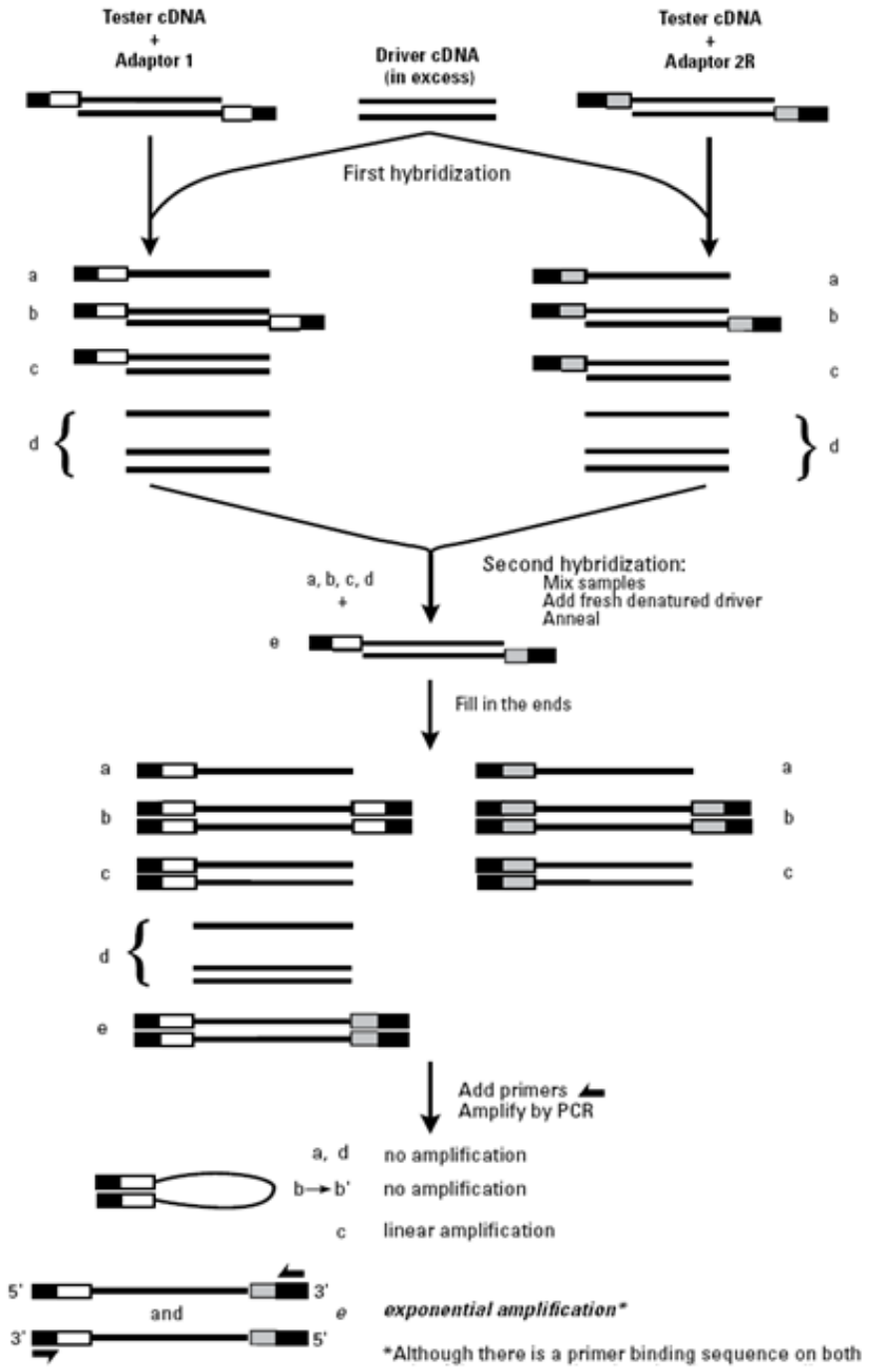


Figure 2. 1 PCR-Select™ cDNA Subtraction procedure (Clontech).

2.21.3 Evaluation of subtraction efficiency

To estimate the efficiency of subtraction, a housekeeping gene, *EF-1α*, was used to comparing the transcript abundant cDNA before and after subtraction by PCR.

After finish the SSH process, the secondary PCR product of subtracted and unsubtracted were diluted by added an RNase-free Water to make a 10-fold diluted samples. The PCR analysis was performed in 25 µl reaction volume containing 1 µl of 10-fold diluted sample, 1× buffer, 0.2 mM dNTP, 0.2 µM each primer and 1× Advantage 2 Polymerase Mix (Clontech). The mixtures were subjected to denaturation at 94 ºC for 2 min and 25 cycles of 94 ºC for 25 s, 52 ºC for 30 s and 72 ºC for 39 s, and the final extension step at 72 ºC for 10 min. Subsequently, the abundance of PCR products between unsubtracted and subtracted samples was compared using 2.5% agarose gel electrophoresis followed by ethidium bromide staining and UV-transillumination.

2.21.4 cDNA cloning

The resulting SSH mixture of the first PCR was diluted in 50 µl of dilution buffer (Clontech) composed of 20 mM HEPES, 20 mM NaCl and 0.2 mM EDTA followed by nested PCR amplification with nested PCR primer pair. The resulting PCR products were purified using phenol-chloroform method, cloned into a pGEM-T easy vector (Promega), transformed into an *Escherichia coli* StrataClone™ SoloPack® competent cell (Algilent) and followed by plating onto an agar medium containing

amplicillin, X-gal and IPTG. The positive clones were randomly selected for plasmid preparation by FavorPrep Plasmid DNA Extraction Mini Kit (Flavogen).

2.21.5 Sequencing and analysis of the subtracted cDNA

To analyze the subtracted cDNA libraries, the positive clones were sequenced using M13 forward primer by Macrogen Inc., South Korea. The sequences obtained were analyzed against the NCBI Genbank database for homology with the BLASTN and BLASTX programs (http://www.ncbi.nlm.nih.gor/ BLAST/). The significant matches present the *E*-values lower than 1×10^{-7}.

2.22 Verification of gene up-regulation in response to YHV infection

To verify the differential expression of genes regulated by *PmRelish* and in response to YHV infection, the seven up-regulated genes from SSH libraries, including Kunitz-type serine proteinase inhibitor "*Kunitz*", *penaeidin*3, *penaeidin*5, *transglutaminase,* single whey acidic protein domain-containing protein isoform 2 "*SWDPm2*", from the forward library, hemocyte homeostasis-associated protein "*HHAP*" and Kazal-type serine proteinase inhibitor isoform 2 "SPI*Pm2*" from the reverse library (Table 2.1) were selected and verified by real-time PCR. The shrimp elongation factor-1α gene (*EF-1*α) gene was used as an internal control.

The *GFP* control and *PmRelish* knockdown groups were prepared as described above. Hemocytes of shrimp from two groups were collected at 3 and 6 h post-second injection. Total RNAs preparation and cDNA synthesis were performed as

mentioned above. The real-time PCR was performed using an appropriate amount of cDNAs in an iCycler iQ™ Real-Time Detection system using iQ™ SYBR Green Supermix (Bio-Rad) with the following conditions: 95 ºC for 9 min, 40 cycles of 95 ºC for 30 s, 58 ºC for 30 s and 72 ºC for 45 s with the oligonucleotide primers specific to seven up-regulate genes from subtracted libraries and *EF-1*α (Table 2.1).

Each real-time PCR reaction was done in triplicate. The standard curves for seven up-regulate genes and *EF-1*α was generated by running triplicate reactions of 5 different cDNA concentrations prepared by 2-fold serial dilution (Visetnan et al. 2015). The efficiencies for *Kunitz*, *penaeidin*3, *penaeidin*5, *transglutaminase*, *SWDPm2*, *HHAP*, SPI*Pm2* and *EF*-1α were 101.2, 99.6, 99.6, 99.2, 98.7, 101.5, 101.6 and 99.8%, respectively. Relative expression was calculated using a mathematic model of Pfaffl (2001) (Pfaffl 2001). Data were analyzed for statistically significance using one-way ANOVA followed by Duncan's new multiple ranges test and presented as means ± standard deviations. Significant differences were accepted at $P < 0.05$.

2.23 Expression analysis of seven up-regulate genes from subtracted libraries in response to YHV infection

To explore the expression profiles in response to YHV infection of the seven immune genes identified from SSH libraries, the seven up-regulate genes were analyzed by real-time PCR. *EF-1*α gene was used as an internal control. The control and YHV-infected shrimp groups were prepared as described above. Briefly,

six Juvenile shrimp of 10 g from the both groups (control and YHV-infected shrimp) were given double injection with 20 µl of normal saline followed 24 h thereafter by the second injection with 20 µl of normal saline for control group and YHV in 20 µl of normal saline for YHV-infected shrimp group.

The hemolymph was collected from 3 shrimp each at 3 and 6 h post-second injection. Total RNAs preparation and cDNAs synthesis were performed as mentioned above. Each real-time PCR reaction was done in triplicate. Relative expression was calculated using a mathematic model of Pfaffl (2001) (Pfaffl 2001). Data were analyzed for statistically significance using one-way ANOVA followed by Duncan's new multiple ranges test and presented as means ± standard deviations. Significance was accepted at $P < 0.05$.

CHAPTER III

RESULTS

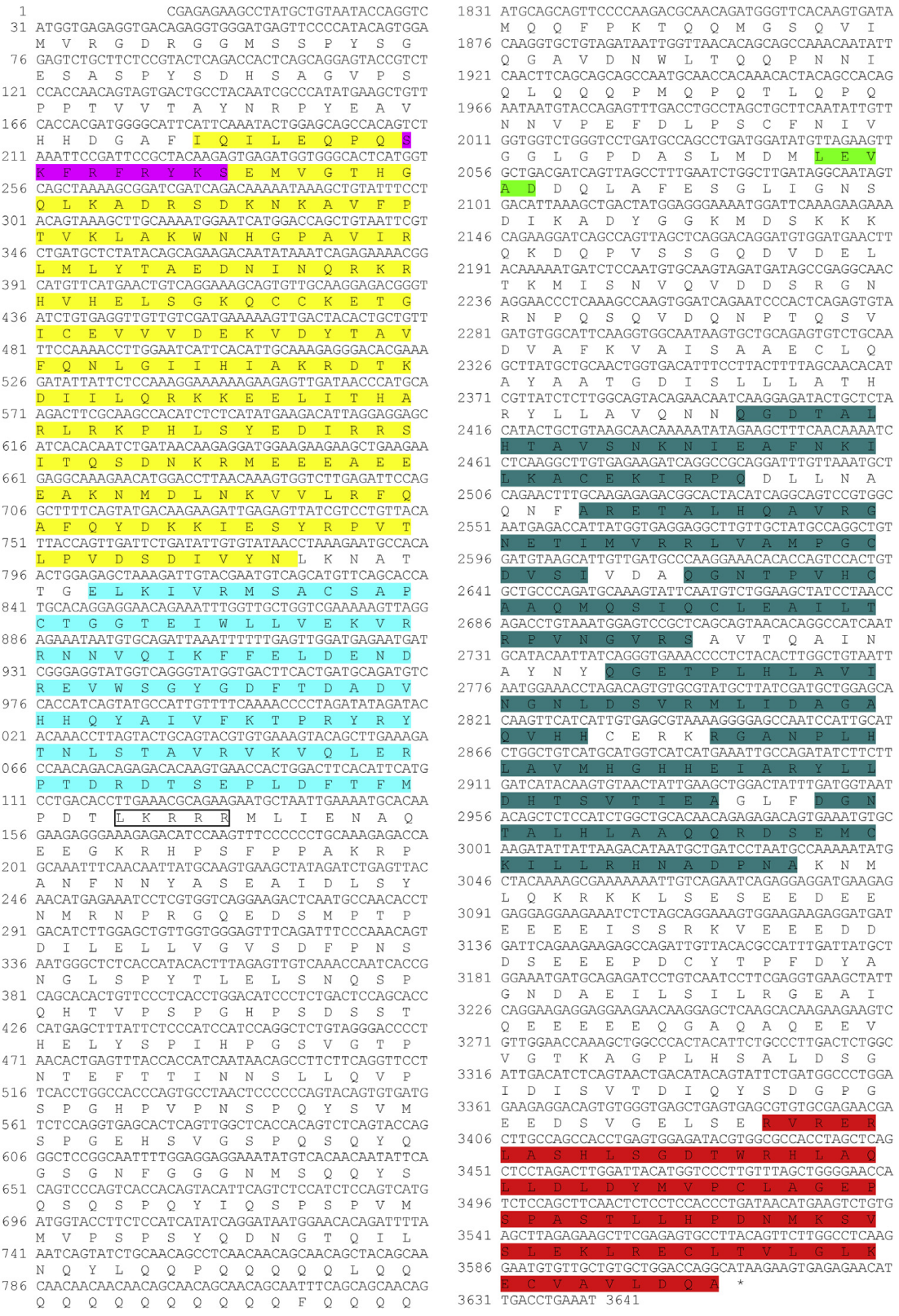
3.1 Cloning and sequence analysis of *Pm*Relish

To pursue a study of Imd pathway in *P. monodon*, the *Penaeus monodon* database was searched for a *PmRelish* clone as a first gene to begin with. An RNA sequence from RNA-Seq, namely NODE_276629, was identified as Relish. The RNA-Seq sequence carried the N-terminal half of the complete *PmRelish* gene. Sequence comparison using the GenBank BLAST search revealed 97-98% match both the nucleotide and amino acid sequences with those Relish sequences of *L. vannamei* and *F. chinensis* Relish sequences (data not shown). Owing to this high homology, primers were designed from the *LvRelish* and *FcRelish* nucleotide sequences (Huang et al. 2009; Li et al. 2009). The *PmRelish* gene were amplified from the hemocyte cDNA preparing from the 48-h YHV-infected shrimp into two fragments of about 2,300 bp and 1,730 bp with overlapping sequence in the middle (data not shown).

The gene fragments were cloned and sequenced. Then, the two parts of *PmRelish* segments were connected by DNA cloning through a restriction site *Kpn*I in the overlapping sequence. The nucleotide sequences from the two segments were also combined and analyzed. The *PmRelish* cDNA sequence obtained was 3,641 bp containing an open reading frame of 3,585 bp that encoded a protein of 1,195 amino

acids. The nucleotide sequence of *PmRelish* and the deduced amino acid sequence were shown in Figure 3.1. The predicted molecular weight of the deduced amino acids of *Pm*Relish is 133.2 kDa, and its theoretical p*I* is 5.42. The sequence has been deposited into the GenBank with accession number KM204120.

With the complete *PmRelish* sequence blasting against the GenBank database, the identities against those Relishes of shrimp, *L. vannamei* (GenBank accession EF432734), *F. chinensis* (GenBank accession EU815055) and another *P. monodon* (GenBank accession JQ728539, deposited after our *Pm*Relish cloning) were 97-99%. That against the Chinese mitten crab *E. sinensis* (GenBank accession GQ871279) was down to 68%. The amino acid sequence of *Pm*Relish contains a Rel homology domain (RHD) with a putative DNA binding motif (SKFRFRYKS), IPT domain, nucleus localization signal (NLS), a putative cleavage site, an IκB-like domain with six ankyrin repeats, and a death domain.


1 CGAGAGAAGCCTATGCTGTAATACCAGGTC
31 ATGGTGAGAGGTGACAGAGGTGGGATGAGTTCCCCATACAGTGGA
M V R G D R G G M S S P Y S G
76 GAGTCTGCTTCTCCGTACTCAGACCACTCAGCAGGAGTACCGTCT
E S A S P Y S D H S A G V P S
121 CCACCAACAGTAGTGACTGCCTACAATCGCCCATATGAAGCTGTT
P P T V V T A Y N R P Y E A V
166 CACCACGATGGGGCATTCATTCAAATACTGGAGCAGCCACAGTCT
H H D G A F I Q I L E Q P Q S
211 AAATTCCGATTCCGCTACAAGAGTGAGATGGTGGGCACTCATGGT
K F R F R Y K S E M V G T H G
256 CAGCTAAAAGCGGATCGATCAGACAAAAATAAAGCTGTATTTCCT
Q L K A D R S D K N K A V F P
301 ACAGTAAAGCTTGCAAAATGGAATCATGGACCAGCTGTAATTCGT
T V K L A K W N H G P A V I R
346 CTGATGCTCTATACAGCAGAAGACAATATAAATCAGAGAAAACGG
L M L Y T A E D N I N Q R K R
391 CATGTTCATGAACTGTCAGGAAAGCAGTGTTGCAAGGAGACGGGT
H V H E L S G K Q C C K E T G
436 ATCTGTGAGGTTGTTGTCGATGAAAAAGTTGACTACACTGCTGTT
I C E V V V D E K V D Y T A V
481 TTCCAAAACCTTGGAATCATTCACATTGCAAAGAGGGACACGAAA
F Q N L G I I H I A K R D T K
526 GATATTATTCTCCAAAGGAAAAAAGAAGAGTTGATAACCCATGCA
D I I L Q R K K E E L I T H A
571 AGACTTCGCAAGCCACATCTCTCATATGAAGACATTAGGAGGAGC
R L R K P H L S Y E D I R R S
616 ATCACACAATCTGATAACAAGAGGATGGAAGAAGAAGCTGAAGAA
I T Q S D N K R M E E E A E E
661 GAGGCAAAGAACATGGACCTTAACAAAGTGGTCTTGAGATTCCAG
E A K N M D L N K V V L R F Q
706 GCTTTTCAGTATGACAAGAAGATTGAGAGTTATCGTCCTGTTACA
A F Q Y D K K I E S Y R P V T
751 TTACCAGTTGATTCTGATATTGTGTATAACCTAAAGAATGCCACA
L P V D S D I V Y N L K N A T
796 ACTGGAGAGCTAAAGATTGTACGAATGTCAGCATGTTCAGCACCA
T G E L K I V R M S A C S A P
841 TGCACAGGAGGAACAGAAATTTGGTTGCTGGTCGAAAAAGTTAGG
C T G G T E I W L L V E K V R
886 AGAAATAATGTGCAGATTAAATTTTTTGAGTTGGATGAGAATGAT
R N N V Q I K F F E L D E N D
931 CGGGAGGTATGGTCAGGGTATGGTGACTTCACTGATGCAGATGTC
R E V W S G Y G D F T D A D V
976 CACCATCAGTATGCCATTGTTTTCAAAACCCCTAGATATAGATAC
H H Q Y A I V F K T P R Y R Y
1021 ACAAACCTTAGTACTGCAGTACGTGTGAAAGTACAGCTTGAAAGA
T N L S T A V R V K V Q L E R
1066 CCAACAGACAGAGACACAAGTGAACCACTGGACTTCACATTCATG
P T D R D T S E P L D F T F M
1111 CCTGACACCTTGAAACGCAGAAGAATGCTAATTGAAAATGCACAA
P D T L K R R R M L I E N A Q
1156 GAAGAGGGAAAGAGACATCCAAGTTTCCCCCCTGCAAAGAGACCA
E E G K R H P S F P P A K R P
1201 GCAAATTTCAACAATTATGCAAGTGAAGCTATAGATCTGAGTTAC
A N F N N Y A S E A I D L S Y
1246 AACATGAGAAATCCTCGTGGTCAGGAAGACTCAATGCCAACACCT
N M R N P R G Q E D S M P T P
1291 GACATCTTGGAGCTGTTGGTGGGAGTTTCAGATTTCCCAAACAGT
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1471 AACACTGAGTTTACCACCATCAATAACAGCCTTCTTCAGGTTCCT
N T E F T T I N N S L L Q V P
1516 TCACCTGGCCACCCAGTGCCTAACTCCCCCCAGTACAGTGTGATG
S P G H P V P N S P Q Y S V M
1561 TCTCCAGGTGAGCACTCAGTTGGCTCACCACAGTCTCAGTACCAG
S P G E H S V G S P Q S Q Y Q
1606 GGCTCCGGCAATTTTGGAGGAGGAAATATGTCACAACAATATTCA
G S G N F G G G N M S Q Q Y S
1651 CAGTCCCAGTCACCACAGTACATTCAGTCTCCATCTCCAGTCATG
Q S Q S P Q Y I Q S P S P V M
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1741 AATCAGTATCTGCAACAGCCTCAACAACAGCAACAGCTACAGCAA
N Q Y L Q Q P Q Q Q Q Q L Q Q
1786 CAACAACAACAACAGCAACAGCAACAGCAATTTCAGCAGCAACAG
Q Q Q Q Q Q Q Q Q Q F Q Q Q Q
1831 ATGCAGCAGTTCCCCAAGACGCAACAGATGGGTTCACAAGTGATA
M Q Q F P K T Q Q M G S Q V I
1876 CAAGGTGCTGTAGATAATTGGTTAACACAGCAGCCAAACAATATT
Q G A V D N W L T Q Q P N N I
1921 CAACTTCAGCAGCAGCCAATGCAACCACAAACACTACAGCCACAG
Q L Q Q Q P M Q P Q T L Q P Q
1966 AATAATGTACCAGAGTTTGACCTGCCTAGCTGCTTCAATATTGTT
N N V P E F D L P S C F N I V
2011 GGTGGTCTGGGTCCTGATGCCAGCCTGATGGATATGTTAGAAGTT
G G L G P D A S L M D M L E V
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2236 AGGAACCCTCAAAGCCAAGTGGATCAGAATCCCACTCAGAGTGTA
R N P Q S Q V D Q N P T Q S V
2281 GATGTGGCATTCAAGGTGGCAATAAGTGCTGCAGAGTGTCTGCAA
D V A F K V A I S A A E C L Q
2326 GCTTATGCTGCAACTGGTGACATTTCCTTACTTTTAGCAACACAT
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2371 CGTTATCTCTTGGCAGTACAGAACAATCAAGGAGATACTGCTCTA
R Y L L A V Q N N Q G D T A L
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2461 CTCAAGGCTTGTGAGAAGATCAGGCCGCAGGATTTGTTAAATGCT
L K A C E K I R P Q D L L N A
2506 CAGAACTTTGCAAGAGAGACGGCACTACATCAGGCAGTCCGTGGC
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D V S I V D A Q G N T P V H C
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A Y N Y Q G E T P L H L A V I
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2821 CAAGTTCATCATTGTGAGCGTAAAAGGGGAGCCAATCCATTGCAT
Q V H H C E R K R G A N P L H
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D H T S V T I E A G L F D G N
2956 ACAGCTCTCCATCTGGCTGCACAACAGAGAGACAGTGAAATGTGC
T A L H L A A Q Q R D S E M C
3001 AAGATATTATTAAGACATAATGCTGATCCTAATGCCAAAAATATG
K I L L R H N A D P N A K N M
3046 CTACAAAAGCGAAAAAAATTGTCAGAATCAGAGGAGGATGAAGAG
L Q K R K K L S E S E E D E E
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3271 GTTGGAACCAAAGCTGGCCCACTACATTCTGCCCTTGACTCTGGC
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I D I S V T D I Q Y S D G P G
3361 GAAGAGGACAGTGTGGGTGAGCTGAGTGAGCGTGTGCGAGAACGA
E E D S V G E L S E R V R E R
3406 CTTGCCAGCCACCTGAGTGGAGATACGTGGCGCCACCTAGCTCAG
L A S H L S G D T W R H L A Q
3451 CTCCTAGACTTGGATTACATGGTCCCTTGTTTAGCTGGGGAACCA
L L D L D Y M V P C L A G E P
3496 TCTCCAGCTTCAACTCTCCTCCACCCTGATAACATGAAGTCTGTG
S P A S T L L H P D N M K S V
3541 AGCTTAGAGAAGCTTCGAGAGTGCCTTACAGTTCTTGGCCTCAAG
S L E K L R E C L T V L G L K
3586 GAATGTGTTGCTGTGCTGGACCAGGCATAAGAAGTGAGAGAACAT
E C V A V L D Q A *
3631 TGACCTGAAAT 3641

Figure 3. 1 The DNA sequence of *PmRelish* and its deduced amino acid sequence. The important sequences at the N-terminal half are the Rel homology domain (RHD) which contains a DNA binding subdomain shaded in yellow with a DNA binding motif in pink and an immunoglobulin-like/plexins/transcription factor (IPT) dimerization subdomain shaded in blue followed by the nuclear localization signal (NLS) in a box. The C-terminal half contains six ankyrin repeats (IκB-like domain) shaded in bluish green and a death domain (DD) in red. The putative caspase cleavage site (L-E-V-A-D) is shaded in green. The primer sequences at both ends were not included. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.2 Tissue distribution of *PmRelish* mRNA

Tissue distribution of *PmRelish* was analyzed by RT-PCR to see if the YHV infection could up-regulated the expression of *PmRelish* in various tissues. It was found that the *PmRelish* was constitutively expressed in all tissues tested. In PBS-injected shrimp, the expression of *PmRelish* was higher in lymphoid organ and hepatopancreas compared to hemocytes, gill and heart. With YHV infection, the expression of *PmRelish* was increased in all tissues except gill. When the expression in YHV-infected shrimp was compared to that of PBS-injected shrimp, the expression ratios were higher in hemocytes and heart (Figure 3.2).

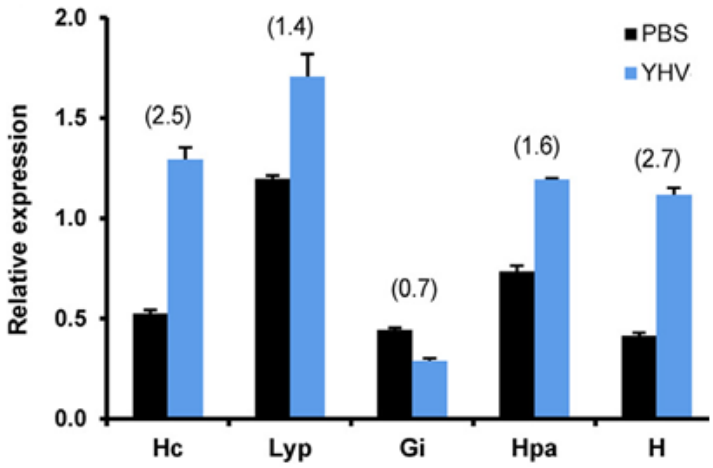

Figure 3. 2 The relative transcription levels of *PmRelish* in shrimp tissues of YHV-infected shrimp. The tissues: hemocytes (Hc), lymphoid organ (Lyp), gill (Gi), hepatopancreas (Hpa) and heart (H), were collected from PBS- and YHV-infected shrimp for RT-PCR analysis of *PmRelish*. The numerals in parentheses are the expression ratios of *PmRelish* in YHV-infected shrimp against that of PBS-injected shrimp. The PBS injection was done as a control. The *PmRelish* expression in infected shrimp was calculated relative to that of the control shrimp. Data are presented as means ± standard deviations. Different letters indicate significant differences accepted at $P < 0.05$.

3.3 Expression of *PmRelish* in response to viral and bacterial challenges

In crustaceans, it has been shown that Relish is involved in immune responses against bacteria, fungus and WSSV (Li et al. 2009; Li et al. 2010c; Qiu et al. 2014). In this study, the expression of *PmRelish* was elucidated whether it was responded to infection by *V. harveyi*, WSSV and YHV. The experiment was done using the shrimp primary hemocyte cell culture. The hemocyte cell culture was infected

with appropriate number of *V. harveyi* 639, YHV or WSSV. At various time points, the cell culture was collected for qRT-PCR analysis of *PmRelish* gene expression.

It was observed that the expression of *PmRelish* gene was stimulated by *V. harveyi* 639 for about 3-fold at 1-2 hpi and 8-fold at 6 hpi. Interestingly, the expression was diminished below normal level at 3 and 12 hpi (Figure 3.3A). For both YHV and WSSV infection, the transcription of *PmRelish* was down-regulated in early hours of infection, up-regulated through 12 hpi and decreased to about normal at 24 hpi onwards (Figure 3.3B and 3.3C). Therefore, the expression of *PmRelish* was modulated in response to *V. harveyi*, YHV and WSSV.

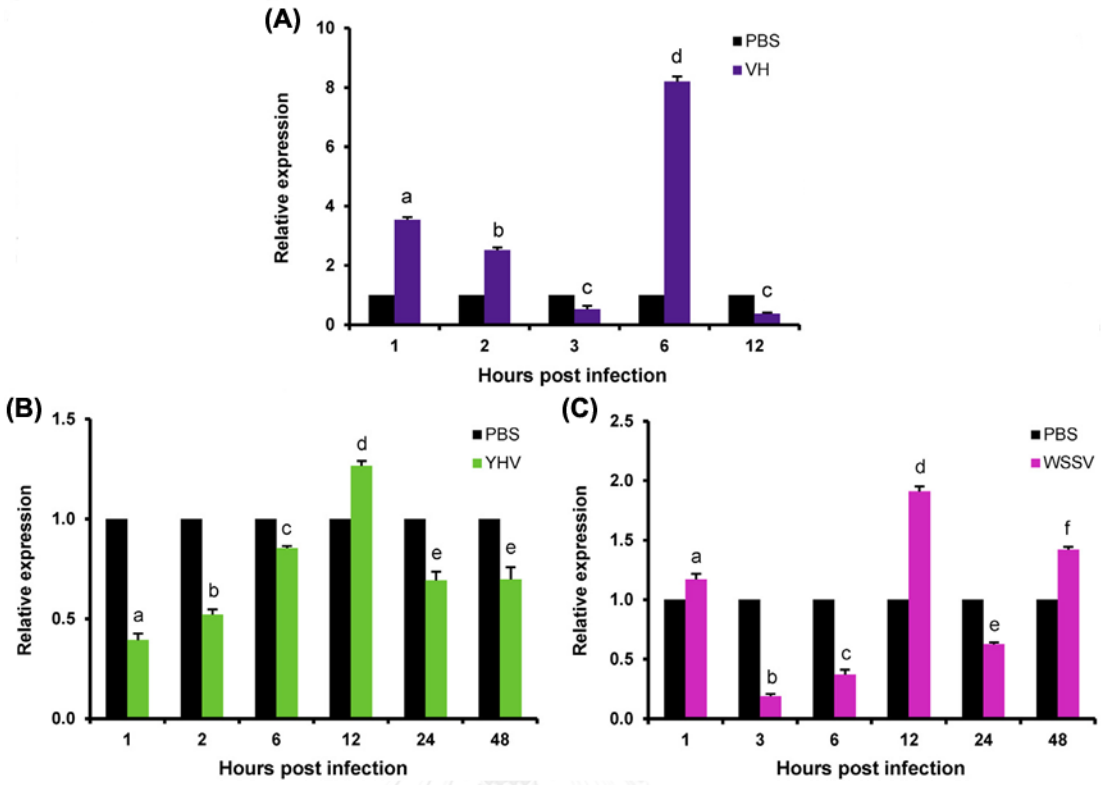

Figure 3. 3 The relative transcription levels of *PmRelish* in hemocytes of *V. harveyi*-, YHV- and WSSV-infected shrimp. The numerals in parentheses are the expression ratios of *PmRelish* in YHV-infected shrimp against that of PBS-injected shrimp. (A-C) After various hours of *V. harveyi* (A), YHV (B) and WSSV (C) infections, the shrimp hemocytes were collected for qRT-PCR analysis of *PmRelish* expression. The PBS injection was done as a control. The *PmRelish* expression in infected shrimp was calculated relative to that of the control shrimp. The expression of *PmRelish* of control shrimp at each time point was presented for comparison. Data are presented as means ± standard deviations. Different letters indicate significant differences accepted at $P < 0.05$.

3.4 Detection on the silencing efficiency of *PmRelish* dsRNA

RNAi-mediated-gene knock-down was used to study the effect of suppression of the *PmRelish* transcript level on the YHV infection in shrimp hemocytes. Initially,

the efficiency of the *PmRelish* knock-down was ascertained. Shrimps were double-injected with either *PmRelish* dsRNA (5 μg/g shrimp) or, as controls, with *GFP* dsRNA (5 μg/g shrimp) or normal saline alone, and then 24 h later all shrimp were injected with same solutions plus YHV. The hemolymph of two individual shrimp from each group were collected at 3, 6, 24 and 48 h post-second injection for detected the expression level of *PmRelish* transcripts by RT-PCR to check the effectiveness of gene knock-down by RT-PCR. The results indicated that suppression of the *PmRelish* transcript was observed in the *PmRelish*-injected shrimps but not in either control groups (*GFP* dsRNA or normal saline-injected shrimps). The expression of *PmRelish* was almost nil at 3, 6 and 24 h, and regained slightly at 48 h after gene silencing (Figure 3.4).

The cDNAs were prepared from *GFP*-injected and *PmRelish* knockdown shrimp infected with *V. harveyi* 639 or called "VH" for 3 h and 6 h and checked for the silencing of *PmRelish* by RT-PCR. As compared to the cDNAs isolated from NaCl control and *GFP* dsRNA-VH shrimp, it was found that the *PmRelish* gene expression was suppressed in *PmRelish* dsRNA-YHV shrimp at both time points (Data not show).

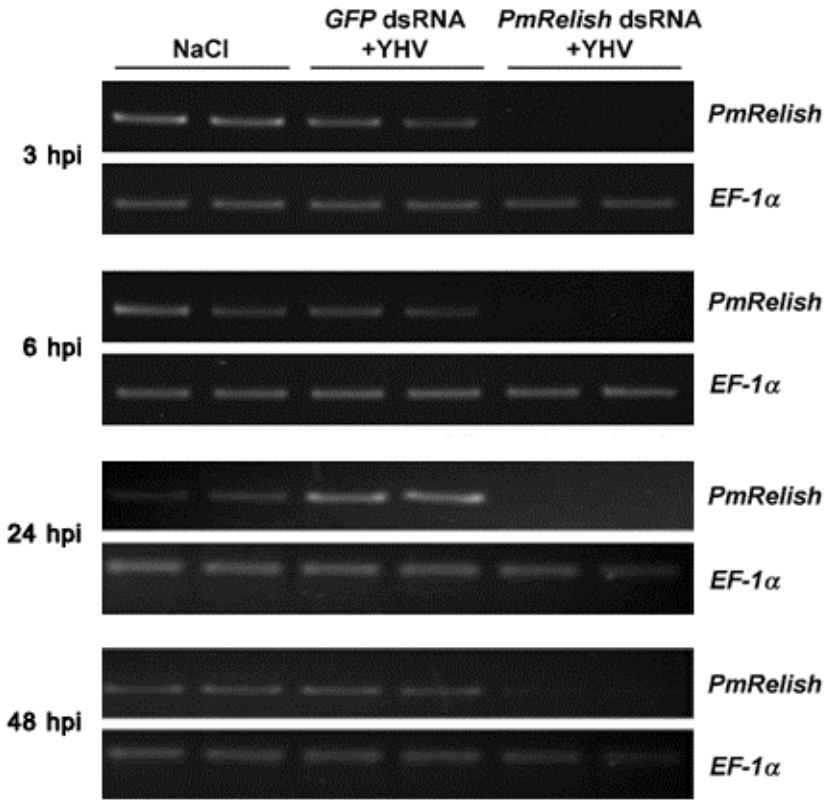

Figure 3. 4 Silencing of *PmRelish* by dsRNA interference. Shrimp were doubly injected with normal saline (NaCl), *GFP* dsRNA and *PmRelish* dsRNA. YHV was included in the second injection of *GFP* dsRNA and *PmRelish* dsRNA. For 3, 6, 24 and 48 h after the second injection (hpi), hemolymph was collected and processed for RT-PCR analysis of *PmRelish* expression. The expression of *EF-1α* was used as an internal control.

3.5 The *PmRelish* gene knockdown rendered the shrimp more susceptible to YHV infection

Our results indicated that the *PmRelish* expression was modulated by YHV infection and the *PmRelish* expression might be important for immune responses against YHV. If this was the case, the shrimp might be more susceptible to YHV

infection when the *PmRelish* expression was knocked down. The shrimp survival experiment against the YHV was, then, carried out to observe how the shrimp treated with *Pm*Relish dsRNA survived the YHV infection. In this experiment, shrimp injected with 1× PBS and GFP dsRNA were the control groups. It was observed that with 1× PBS injection, no death was observed. With the addition of YHV, shrimp injected with 1× PBS and GFP dsRNA survived 50% at about 72 h. Injected with *Pm*Relish dsRNA along with YHV reduced the 50% survival rate to about 42 h (Figure 3.5). These results showed that the *Pm*Relish and, hence, the Imd pathway responded against YHV in the shrimp.

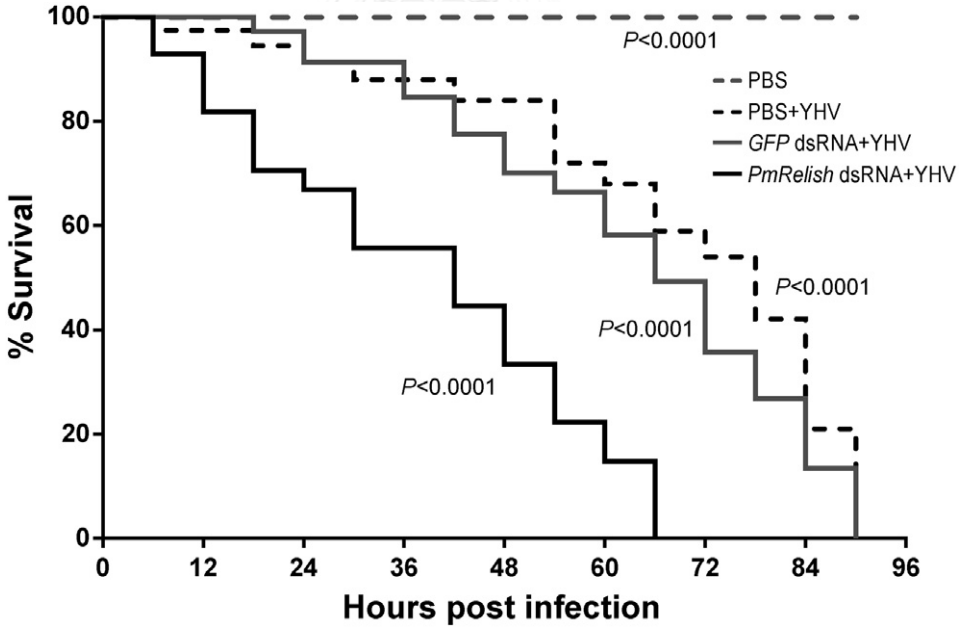

Figure 3. 5 The KaplaneMeier survival plots of *PmRelish* knockdown shrimp infected with YHV. The shrimp injected with 1 × PBS, 1 × PBS + YHV and GFP dsRNA + YHV were the different controls. The P values indicated were calculated by log-rank test in the GraphPad Sigma Plot.

3.6 The expression of *penaeidin5* upon YHV infection was regulated by the Imd pathway

Since the Imd pathway regulates the synthesis of antimicrobial proteins in response to pathogenic infection, the synthesis of antilipopolysaccharide ALF*Pm*3, crustin*Pm*1, penaeidin3 and penaeidin5 were elucidated in *PmRelish* silencing shrimp to determine the AMP under the regulation of Imd pathway. The YHV was used to stimulate the pathway. The dsRNA-mediated gene knock-down of *PmRelish* was done by injections of 5 μg/g shrimp *Pm*Relish dsRNA two times, 24 h apart. The YHV was injected along at the second time. At 3 h and 6 h after the second injection, the hemocytes were collected to examine the expression level of AMP by qRT-PCR.

Assay of AMP expression at 3 h and 6 h after the second YHV and *Pm*Relish dsRNA injection by qRT-PCR showed that *ALFPm3*, *crustinPm1* and *penaeidin3* were still expressed albeit there was very low or no *PmRelish* expression (Figure 3.6). However, *penaeidin5* was not expressed at both time points in the absence of *PmRelish* expression indicating that the biosynthesis of *penaeidin5* was under the positive control of *Pm*Relish. It should be noted here that the expression of *crustinPm1* and *penaeidin3* were down-regulated at 3 h upon YHV infection and regained their expression at 6 h after YHV and *Pm*Relish dsRNA injection.

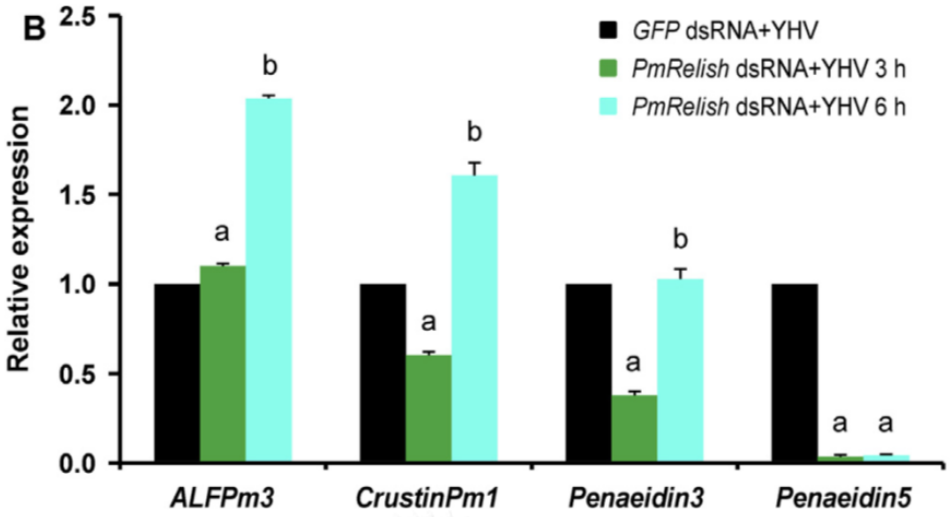

Figure 3. 6 The silencing of *PmRelish* affects the expression of *penaeidin5* but not *ALFPm3*, *crustinPm1* and *penaeidin3*. After *PmRelish* silencing and YHV infection for 3 h and 6 h, the hemolymph was collected for qRT-PCR analysis of antimicrobial protein expression. The antimicrobial protein genes analyzed were *ALFPm3*, *crustinPm1*, *penaeidin3* and *penaeidin5*. The expression was calculated relative to that of the control shrimp injected with GFP dsRNA and YHV. The experiment was done in triplicate. The expression of *EF-1α* was used as an internal control. The expression of antimicrobial protein genes in control shrimp was presented for comparison. Data are presented as means ± standard deviations. Different letters indicate significant differences between data at 3 h and 6 h accepted at $P < 0.05$.

3.7 Antimicrobial proteins under the regulation of *Pm*Relish

A few genes of antimicrobial proteins were identified in the SSH libraries: *crustinPm1*, *penaeidin3*, *penaeidin5* in the forward library and antilipopolysaccharide *ALFPm3* in the reverse library. To check whether they were under or not under the regulation of *PmRelish*, we undertook another *PmRelish* silencing experiment. A Gram-negative bacterium, *V. harveyi*, was used instead of YHV for it was well

known that the Gram-negative bacteria stimulated the Imd pathway. If the expression of an antimicrobial protein was under the regulation of the Imd pathway, it should be up-regulated in response to both *V. harveyi* and YHV infection, and was not responsive under *PmRelish* silencing. The expression of the four genes has already been tested with YHV infection (Visetnan et al. 2015). In this report, they were tested with *V. harveyi*.

The *PmRelish* knockdown shrimp along with their controls, GFP dsRNA injected shrimp were injected with *V. harveyi* 639. The shrimp hemocytes were collected at 3 h and 6 h after *V. harveyi* infection. The cDNAs were prepared and the expression of four antimicrobial proteins were analyzed by qRT-PCR. It was found that at 3 h after *V. harveyi* infection, the expression of all four antimicrobial proteins was severely decreased (Figure 3.7). The expression resumed at 6 h after *V. harveyi* infection. The expression of *ALFPm3* and *crustinPm1* was up-regulated whereas that of *penaeidin3* and *penaeidin5* was still at the subnormal level. Considering these results along with those reported previously with YHV infection (Visetnan et al. 2015), it indicated that the *penaeidin3* and *penaeidin5* were under the regulation of *Pm*Relish. In contrast, the expression of *ALFPm3* and *crustinPm1* was not regulated by the Imd pathway although the *crustinPm1* was detected in forward library.

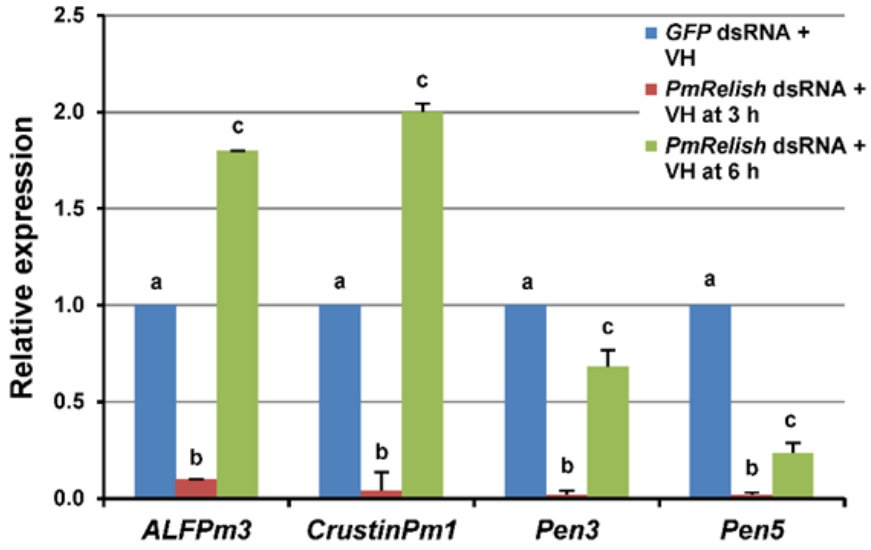

Figure 3. 7 The effect *PmRelish* silencing on the expression of *ALFPm3*, *crustinPm1*, *penaeidin3* and *penaeidin5*. After *PmRelish* silencing and *V. harveyi* (VH) infection for 3 h and 6 h, the hemocytes were collected and processed for the analysis of antimicrobial protein expression by qRT-PCR. The antimicrobial protein genes analyzed were *ALFPm3*, *crustinPm1*, *penaeidin3* (*Pen3*) and *penaeidin5* (*Pen5*). The expression was calculated relative to that of the control shrimp injected with GFP dsRNA and *V. harveyi*. The experiment was done in triplicate. The expression of antimicrobial protein genes in the control shrimp was presented for comparison. Data are presented as means ± standard deviations. Different letters indicate significant differences between data at 3 h and 6 h accepted at $P < 0.05$.

3.8 Recombinant expression of *Pm*Relish

3.8.1 Construction of the recombinant plasmid r*Pm*Relish, r*Pm*N-terminal, r*Pm*C-terminal and r*Pm*Rel

The *Pm*Relish was interesting because its molecular function is to activate the production of antimicrobial peptides and/or other immune proteins related to microbial infection. The *Pm*Relish and its domains (*Pm*N-terminal, *Pm*C-terminal and

*Pm*Rel domains) contained the open reading frames of 3585, 1709, 2505 and 1080 bp that encoded polypeptides of 1195, 677, 835 and 360 amino acid residues with the predicted molecular weights of 133.2, 76.59, 91.78 and 41.42 kDa, respectively. The theoretical p*I* of four forms of *Pm*Relish was 5.42, 6.05, 4.76 and 8.41, respectively.

To explore the activities of four *Pm*Relish forms (*Pm*Relish, *Pm*N-terminal, *Pm*C-terminal and *Pm*Rel), the four *Pm*Relish open reading frames were cloned into the *Nco*I–*Xho*I digested pET-28b(+) expression vector (Novagen) for *Pm*Relish and *Pm*C-terminal fragments, *EcoR*I–*Xho*I digested pET-28b(+) for *Pm*N-terminal and the *Nco*I–*Not*I digested pET-28a(+) for *Pm*Rel fragment. The expression clones were named r*Pm*Relish (A), r*Pm*N-terminal (B), r*Pm*C-terminal (C) and r*Pm*Rel (D), respectively (Figure 3.8). The four expression clones were transformed into *E. coli* BL21 CodonPlus for r*Pm*Relish, r*Pm*N-terminal and r*Pm*Rel, and *E. coli* Rosetta(DE3)pLysS for r*Pm*N-terminal and r*Pm*C-terminal, respectively.

For some reasons, the r*Pm*N-terminal was not expressed well. It was then subcloned into a pET-32a(+) derivative, pVR500, then transformed into *E. coli* Rosetta(DE3)pLysS and expressed as fusion protein to the thioredoxin.

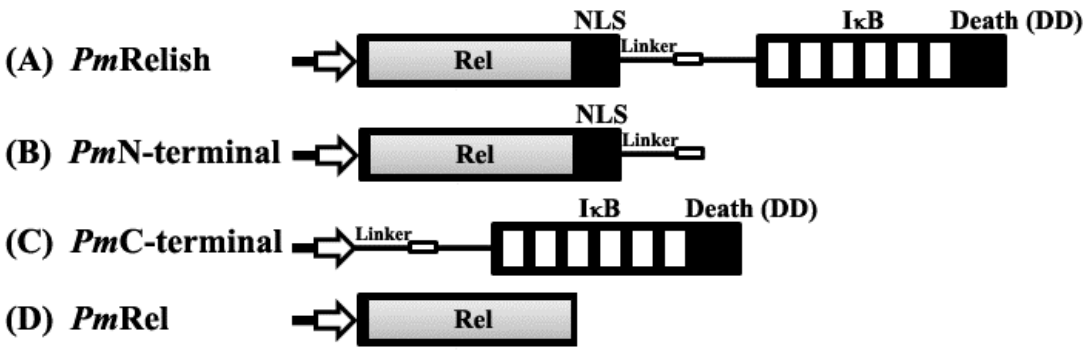

Figure 3. 8 Schematic representation of the expression vectors used in recombinant expression of *Pm*Relish experiments: full-length *Pm*Relish (**A**), *Pm*N-terminal (Rel with the NLS and the linker between the NLS and the putative caspase cleavage site) (**B**), *Pm*C-terminal (the linker with the IκB) (**C**), and *Pm*Rel (Rel only) (**D**). The putative caspase cleavage site is shown as a white small box located at the central of the linker of *PmRelish* gene (▭). (Modified from Fan et al., Infect. Immun, 2008) (Fan et al. 2008)

Three expression clones, r*Pm*Relish, r*Pm*N-terminal and r*Pm*Rel, with histidine tag (His_6) at the N-termini, one expression clone, the r*Pm*C-terminal, with histidine tag (His_6) at its C-terminus, were produced by adding IPTG to final concentration of 1 mM to induce the expression. The cells were harvested at 0, 2, 4, 6 and 8 h for r*Pm*Relish, at 0, 2, 3, 4 and 24 h for r*Pm*N-terminal, at 0, 1, 2, 3, 4 and 6 h for r*Pm*C-terminal and at 0, 1, 2, 3 and 4 h for r*Pm*Rel and then analyzed by 15% SDS-PAGE followed by Coomassie brilliant blue staining, and western blot analysis.

The results showed that the r*Pm*Relish cannot be expressed in an *E. coli* expression system after induction with IPTG. The expression of r*Pm*Relish was not detected by Coomassie brilliant blue staining and western blot analysis (Figure 3.8.1).

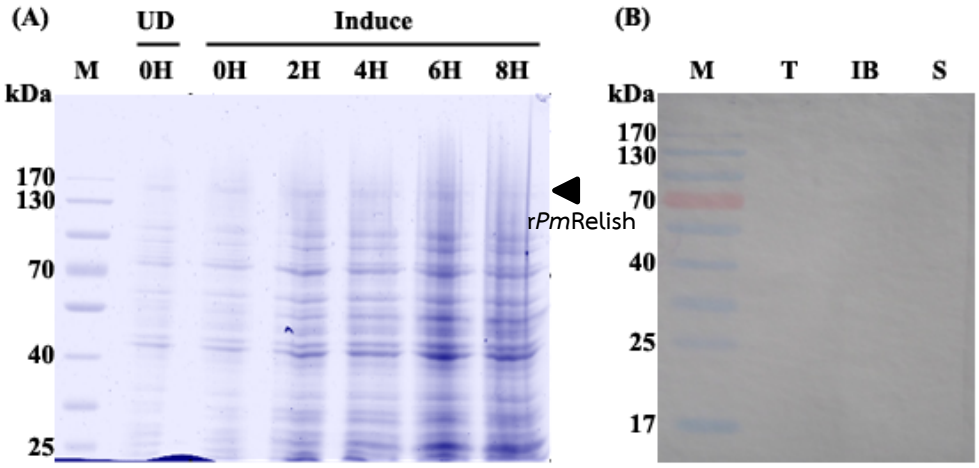

Figure 3. 8. 1 SDS-PAGE analysis of r*Pm*Relish. The r*Pm*Relish was expressed in *E. coli* BL21-CodonPlus at various times of induction. For r*Pm*Relish (A), lanes 0 (UD), 0 (I), 2, 4, 6 and 8 are the lysate of cells carrying r*Pm*Relish from uninduced cells at 0 h, induced cells at 0 h, induced cells at 2, 4, 6 and 8 h, respectively. For r*Pm*Relish (B), lanes T, I and S are the lysate of cells carrying r*Pm*Relish from induced cells at 4 for test total protein, 4h for test inclusion body and 4 h for test soluble protein, respectively. Lanes M is prestained protein marker (Fermentas).

The results revealed also that the r*Pm*N-terminal cannot be expressed in both *E. coli* BL21 CodonPlus and *E. coli* Rosetta(DE3)pLysS expression hosts (Data not shown). Then, the r*Pm*N-terminal was subcloned into *EcoR*I–*Xho*I digested pET-32a(+) derivative, pVR500, and transformed into *E. coli* Rosetta(DE3)pLysS. After IPTG induction, the cell pellets were collected at 0 (un-induced or UD), 2, 3, 4 and 24 h (overnight), respectively. The results indicated that the r*Pm*N-terminal was continuously increased after 2 h of IPTG induction but it was cleaved into two

fragments, while its expression was not detected in *E. coli* without IPTG induction (Figure 3.8.2).

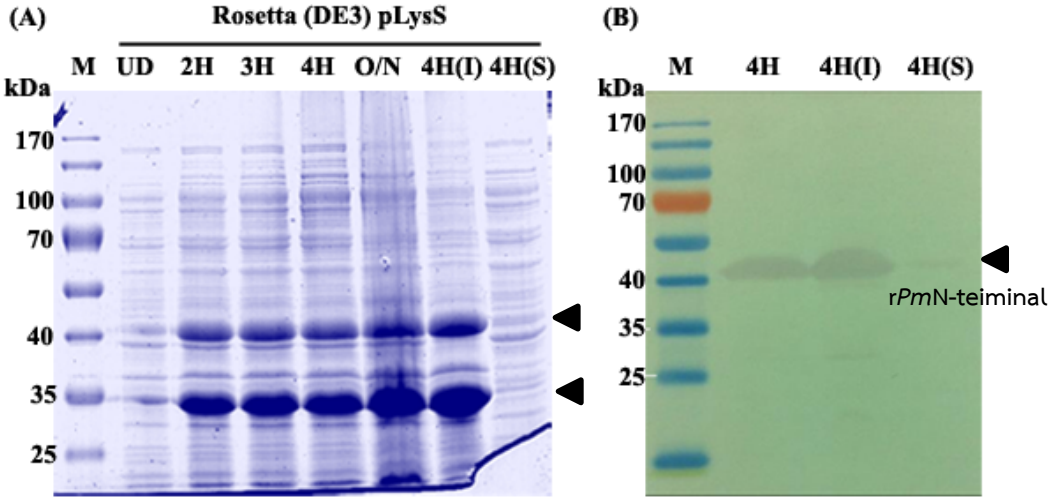

Figure 3. 8. 2 SDS-PAGE analysis of r*Pm*N-terminal. The r*Pm*N-terminal was expressed in *E. coli* Rosetta(DE3)pLysS at various times of induction. For r*Pm*N-terminal (A), lanes UD, 2H, 3H, 4H, 0/N, 4H(I) and 4H(S) are the cells carrying r*Pm*N-terminal from uninduced cells, induced cells at 2, 3, 4, overnight (24 h), 4 h inclusion body and 4 h soluble protein, respectively. For r*Pm*N-terminal (B), lanes 4H, 4H(I) and 4H(S) are total proteins, inclusion bodies and soluble proteins from the 4-h induced cells, respectively. Lanes M is prestained protein marker (Fermentas).

For the r*Pm*C-terminal, the protein expression was continuously increased after 2 h of IPTG induction but it was degraded into two major bands (Figure 3.8.3). This result was the same as those of the r*Pm*N-terminal.

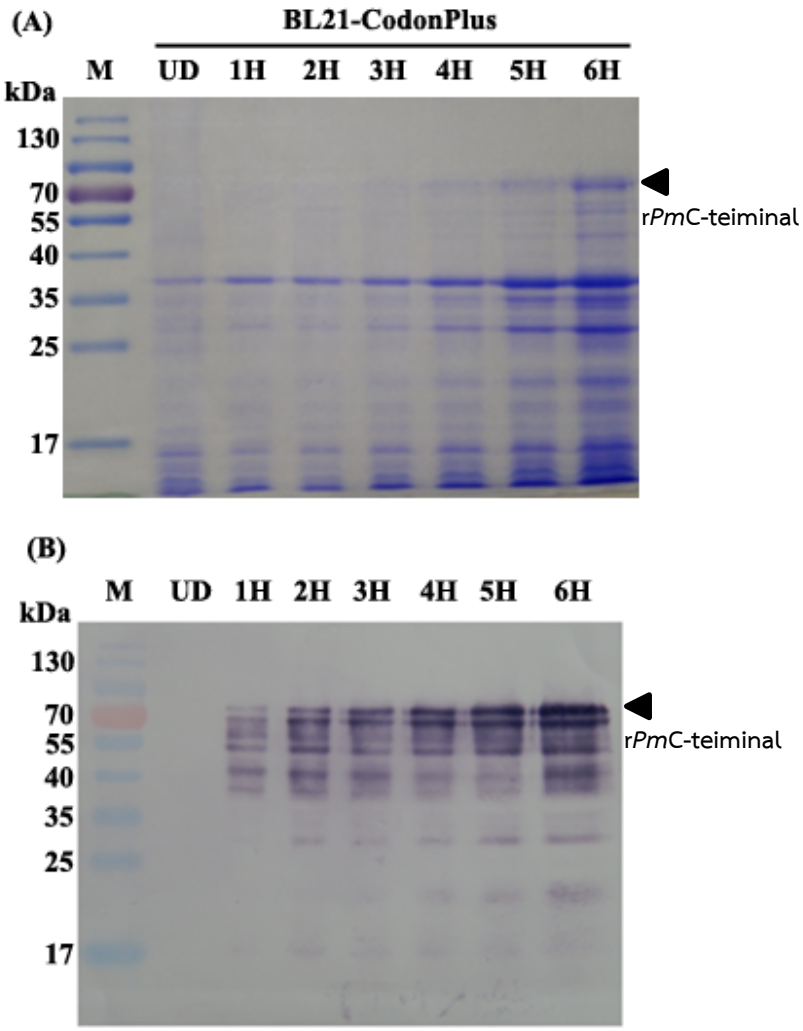

Figure 3. 8. 3 SDS-PAGE analysis of r*Pm*C-terminal. The r*Pm*C-terminal was expressed in *E. coli* Rosetta(DE3)pLysS at various times of induction. For r*Pm*C-terminal (A) and (B), lanes UD, 1H, 2H, 3H, 4H and 6H are the cells carrying r*Pm*C-terminal from uninduced cells, induced cells at 1, 2, 3, 4 and 6 h, respectively. Lanes M is prestained protein marker (Fermentas).

For the r*Pm*Rel, the recombinant protein r*Pm*Rel was produced in *E. coli* system at 4 h post IPTG induction but it was unstable and degraded rapidly. Moreover, the r*Pm*Rel was found to be principally located in inclusion bodies,

but not in soluble form (Figure 3.8.4). Therefore, this condition was employed for a large-scale expression of the recombinant r*Pm*Rel.

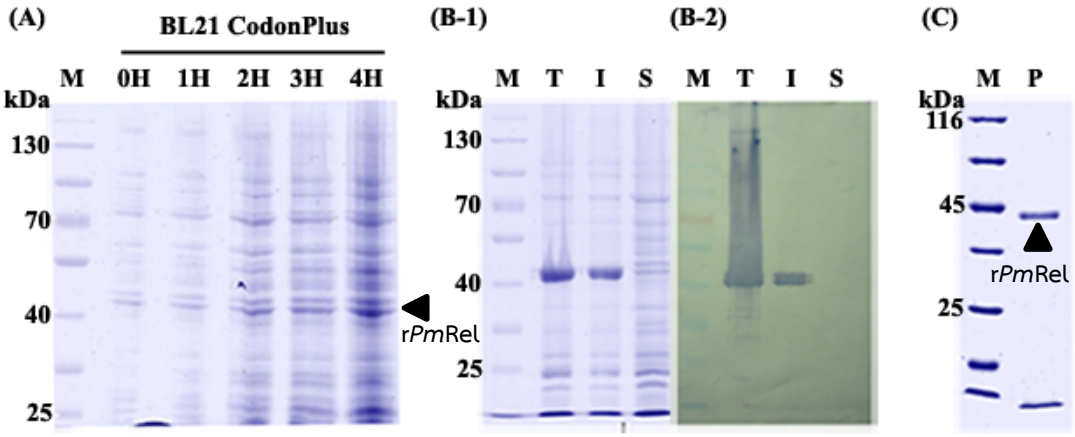

Figure 3. 8. 4 SDS-PAGE analysis of r*Pm*Rel. The r*Pm*Rel was expressed in *E. coli E. coli* CodonPlus at various times of induction. For r*Pm*Rel (A), lanes 0H, 1H, 2H, 3H and 4H are the cells carrying r*Pm*Rel from uninduced cells, induced cells at 1, 2, 3 and 4 h, respectively. For r*Pm*Rel (B-1) and r*Pm*Rel (B-2), the expression was analyzed by Coomassie brilliant blue staining and western blot analysis, respectively; lanes T, I and S from are the total proteins, inclusion bodies and soluble proteins of 4-h induced cells carrying r*Pm*Rel. The r*Pm*Rel was purified through nickel-NTA column (C). Lane P is the purified r*Pm*Rel. Lanes M is protein size marker.

3.9 Purification of recombinant protein

The recombinant protein (r*Pm*Rel) was efficiently purified by a single purification step using a nickel-NTA affinity column under non-denaturing condition. The r*Pm*Rel was expressed as inclusion bodies of about 41.42 kDa and its was solubilized with 25 mM sodium hydroxide at room temperature for 1-2 h. Then, the solubilized protein was dialysed against 20 mM sodium carbonate buffer (Na_2CO_3)

pH 10, at 4 ℃ overnight and purified under non-denaturing condition using nickel affinity chromatography (GE Healthcare). The purify protein was confirmed and analyzed for its identity and purity by Western blot analysis using anti-His tag antibody (Merck) and directly used or kept at 4 ℃ until used (Figure 3.8.4). The purified r*Pm*Rel was used to generate mouse polyclonal antibody in immunized mice.

3.10 Immunolocalization of *Pm*Relish protein in shrimp hemocytes

In *Drosophila*, the Relish is activated by proteolytic cleavage into two fragments (Stöven et al. 2000; Stöven et al. 2003; Kim et al. 2014). While the C-terminal fragment remains in the cytoplasm awaiting for degradation, the N-terminal fragment containing the RHD translocates into the nucleus to regulate the synthesis of cognate AMPs (Stöven et al. 2000; Kim et al. 2006). To investigate whether the *Pm*Relish can be activated by YHV and translocates into the nucleus, the immunocytochemistry of *Pm*Relish was carried out. The recombinant *Pm*Rel protein containing only the RHD was over-produced in an *E. coli* expression system for polyclonal antibody production.

At various time points after 1× PBS and YHV injection, the hemocyte samples were prepared from the shrimp. The hemocyte sample from untreated shrimp was also prepared. The hemocytes were coated onto the microscope slides and processing for visualization under the confocal laser scanning microscope. In Fig. 5,

the hemocyte images from 1× PBS-injected shrimp and 12-h YHV-injected shrimp are shown as representatives. The hemocyte images from the untreated shrimp and the 1× PBS-injected shrimp at various time points are not different (data not shown). The hemocyte images from 12-h YHV-injected shrimp reveals a portion of larger hemocytes with swollen nuclei characteristic of apoptotic cells (Khanobdee et al. 2002) (Figure 3.10). At 24 hpi and 36 hpi, the numbers of larger hemocytes are reduced to almost none (data not shown). The *Pm*Relish seen as red color in the images is detected in the cytoplasm of all hemocytes. Upon YHV infection, however, we do not or are unable to observe any accumulation of activated *Pm*Relish into the nuclei (Figure 3.9).

Ones would ask also that which hemocytes in the images were infected by the YHV. Unfortunately, the detection of YHV-infected cells could not be done because the antibody against the YHV gp64 envelope protein we had was from mouse like the anti-*Pm*Rel. Thus, the detection of infected cells and *Pm*Relish could not be done simultaneously for the secondary antibody to mouse IgG would detect both *Pm*Relish and YHV. With reference to the work of Jatuyosporn et al. (2014), it was observed that all the hemocytes were infected by YHV under the condition similar to the one we used (personal communication) (Jatuyosporn et al. 2014).

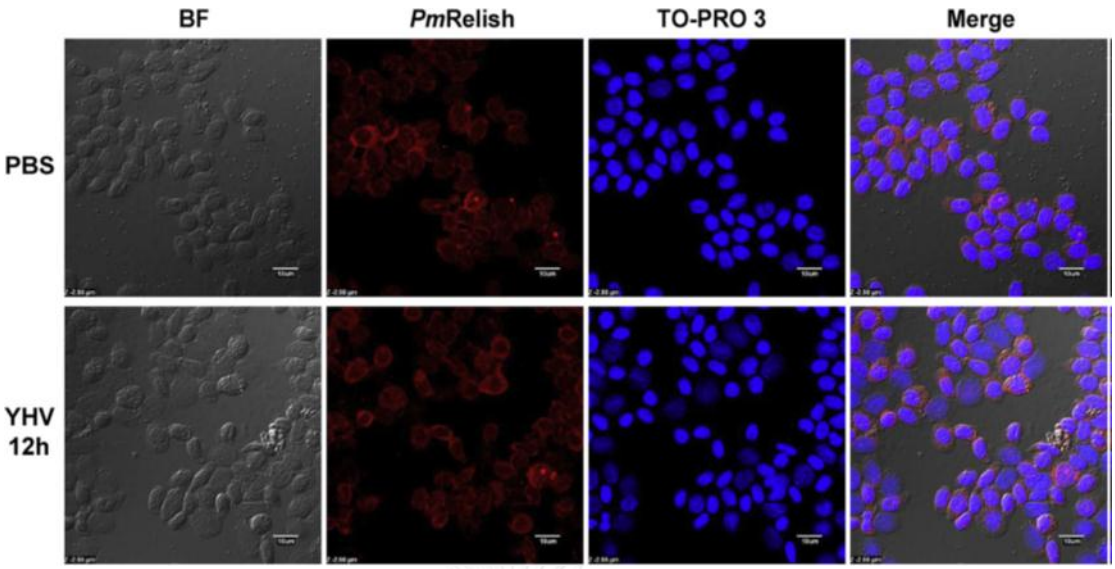

Figure 3. 9 Immunocytochemical detection of *Pm*Relish in the shrimp hemocytes. Hemocytes were collected from shrimp injected with 1 × PBS and YHV at various time points andmprocessed for viewing under a confocal laser scanning microscope. The images from 1 × PBS and 12-h YHV injected samples are shown as representatives. The bright field (BF), *Pm*Relish (red) and TO-PRO 3 (blue) images were collected and merged (Merge). The white bars represent 10 mm.

3.11 The *Pm*Relish is activated by YHV infection

The results in Figure 3.10 reveals that the activated *Pm*Relish with molecular weight (MW) of 70 kDa is detected in the hemocyte lysates from YHV-injected and 1× PBS-injected shrimp (lanes 2 and 3, respectively) but not that from the untreated shrimp (lane 1). The size of activated *Pm*Relish observed (I in Figure 3.10) agrees well with the calculated size of about 76 kDa. Lower amount of activated *Pm*Relish is observed in shrimp injected with 1× PBS suggests the possibility of *Pm*Relish activation by injury. For unknown reason, the full length *Pm*Relish with the calculated MW of 133.2 kDa is not observed. Two positive protein bands in which

sizes are about 45 and 30 kDa (II and III in Figure 3.10, respectively) are perhaps the degraded products of activated *Pm*Relish.

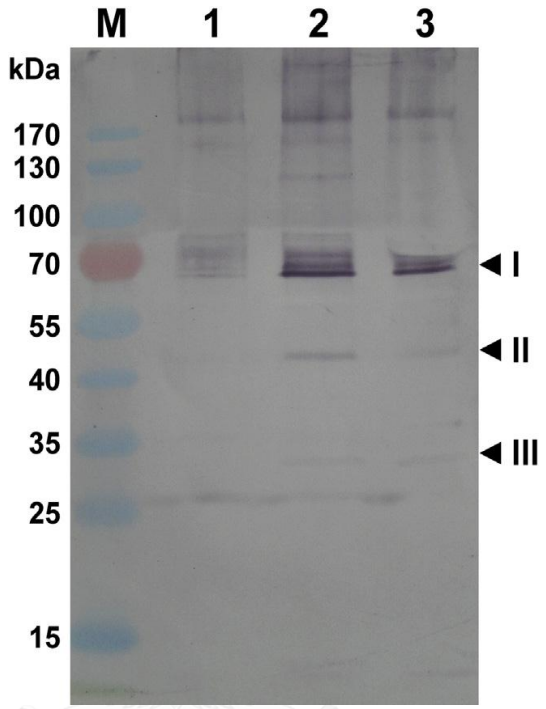

Figure 3. 10 Western blot detection of activated *Pm*Relish from the YHV-infected shrimp. Hemocyte lysates were prepared from untreated shrimp (lane 1), YHV-injected shrimp (lane 2) and 1 × PBS-injected shrimp (lane 3) and subjected to Western blot analysis using anti-*Pm*Rel to detect the *Pm*Relish and its activated product. I, II and III indicate the activated *Pm*Relish and its degraded products. Lane M is a protein size marker.

3.12 Detection of Protein-DNA interaction by DNA binding assay

In an attempt to verify the molecular mechanism of *Pm*Relish, the interaction between *Pm*Relish and DNA was examined. The DNA binding ability of r*Pm*Relish was examined by analyzing the electrophoretic mobility of DNA band after incubating the

DNA at various weight ratios of r*Pm*Relish to DNA using 1% agarose gel electrophoresis (w/v) (Park et al. 1998). The result showed that the Protein-DNA interaction cannot be detected by Gel-retardation because the shifted band can't be observed after increased a various weight ratios of r*Pm*Relish to DNA (Data not show).

3.13 Evaluation of subtraction efficiency

Total RNA of *GFP*+YHV and *PmRelish* knockdown+YHV groups (at 3 and 6 h post-YHV infection) were collected for cDNA preparation and subjected to SSH to generate two SSH libraries, forward and reverse SSH libraries, to enrich the immune genes up-regulated in response to YHV infection and under the *PmRelish* regulation.

The success of SHH was estimated by comparing the transcript abundance of a non-differentially expression gene, *EF-1*α, before and after subtraction. The result showed that the level of *EF-1*α was depleted in the subtracted cDNA samples from both forward and reverse libraries, whilst the presence of *EF-1*α was discovered in the un-subtracted cDNA samples (Figure 3.11). The efficient subtraction indicated that the SSH was successful.

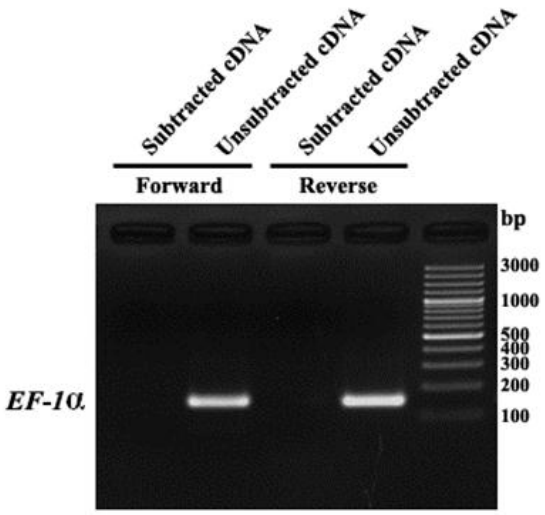

Figure 3. 11 Subtraction efficiency assay of forward and reverse libraries by comparing the transcript abundant of non-differentially expression gene, *EF-1α*, in subtracted and unsubtracted cDNA samples examined by PCR.

3.14 Sequencing and analysis of the subtracted cDNA

To analyze the subtracted cDNA libraries, the positive clones were sequenced using M13 forward primer by Macrogen Inc., South Korea. The sequences obtained were analyzed against the NCBI Genbank database for homology with the BLASTN and BLASTX programs (http://www.ncbi.nlm.nih.gor/ BLAST/). The significant matches present *E*-values lower than 1×10^{-7}.

3.15 Suppression subtractive hybridization

Previously, we used the dsRNA-mediated gene knockdown to study the effect on antimicrobial protein synthesis of *PmRelish* suppression in YHV-infected shrimp (Visetnan et al. 2015). To be able to pick up more genes involved in YHV infection

that were under the influence of *Pm*Relish activity, the suppression subtractive hybridization technique was used. Since the *PmRelish* expression is increased by YHV infection and peaked at 12 h after infection, the appropriate time to search for YHV responsive genes would be within this 12-h period (Visetnan et al. 2015). The 3-h and 6-h time points after YHV infection were chosen for the SSH experiment.

There are two possible subtractions we can make the forward subtraction and reverse subtraction. The forward direction subtracts the cDNAs of the *PmRelish* dsRNA-YHV shrimp from those of the *GFP* dsRNA-YHV shrimp. The remaining cloneable cDNAs are the YHV responsive genes under the influence of *Pm*Relish. The reverse subtraction does the opposite. Logically, the reverse subtraction should not give any cloneable genes. However, without the activity of *Pm*Relish in the knockdown shrimp, it is deemed possible for the shrimp to adapt alternative conditions by expressing other alternative defense genes for their survival upon YHV infection.

Two SSH cDNA libralies, forward and reverse libraries were constructed to identify the immune genes up-regulated in response to YHV infection and under the *PmRelish* regulation, respectively.

The suppression subtractive hybridization was performed the combined cDNAs from the two time points and the remaining cloneable cDNAs after subtraction were cloned into a plasmid vector. Since we were interested in the forward subtraction than the reverse subtraction, we selected randomly about 350 clones

from forward library but only 126 clones from reverse library. The plasmid clones were subjected to nucleotide sequencing. Only 252 and 99 good quality sequences were obtained from the forward and reverse libraries, respectively. The sequences were blast searched for sequence homology against the NCBI GenBank database with BLASTN and BLASTX programs. Significant matches (*E*-values < 1×10^{-7}) were obtained with 125 (49%) and 34 (34%) cDNA sequences for annotatable known genes, the remaining 127 (51%) and 65 (66%) cDNA sequences were hypothetical and unknown genes, and the annotatable cDNA sequences represented 60 and 19 different genes in the forward and reverse libraries, respectively (Tables 3.1, 3.2 and Figure 3.12).

The genes were categorized according to their putative functions into groups which were defense and homeostasis, transport, regulation and gene expression, cell cycle, energy and metabolism, signaling and communication, and miscellaneous functions (Tables 3.1 and 3.2). The numbers of genes involved in defense and homeostasis were abundant in both libraries, 78 (31%) and 23 (23%) copies in the respective forward and reverse libraries (Figure 3.12). The genes in this group can be antimicrobial proteins, proteinases and their inhibitors, chaperones, clotting protein, and other defense and homeostasis proteins. A few of these genes that were abundant or interesting antimicrobial proteins were selected for further studied to validate the SSH technique.

Table 3. 1 Up-regulated genes in forward libraries of YHV-infected *P. monodon* hemocytes identified from suppression subtractive hybridization.

Highest homology	Accession no.	*E* value	Closet species	No. of clones
Defense & homeostasis				
Antimicrobial proteins				
Crustin*Pm*1	ABW82154	8.00E-42	*Penaeus monodon*	3
Crustin*Pm*4	ACQ66005	3.00E-03	*Penaeus monodon*	3
Cysteine-rich protein 1-like	XP_967808	8.00E-27	*Tribolium castaneum*	1
Lysozyme	AAN16375	5.93E-91	*Penaeus monodon*	3
Penaeidin3	ACH70378	3.45E-16	*Penaeus monodon*	3
Penaeidin5	ACQ66008	5.00E-16	*Penaeus monodon*	5
Single whey acidic protein domain-containing protein 2 (SWD*Pm*2)	ACF28465	6.49E-38	*Penaeus monodon*	11
Stylicine 2	ABW24769	1.62E-27	*Litopenaeus stylirostris*	1
Pattern recognition protein				
QM protein	ACV72062	2.00E-78	*Penaeus monodon*	2
Proteinases & their inhibitors				
Clip domain serine protease	ACP19733	1.00E-35	*Eriocheir sinensis*	2
Kazal-type proteinase inhibitor (SPI*Pm5*)	ACB47427	4.00E-43	*Penaeus monodon*	2
Kunitz-type serine protease inhibitor	AGU16239	5.14E-42	*Fenneropenaeus chinensis*	15
Mas-like protein	AAT42131	9.64E-105	*Penaeus monodon*	2
Masquerade-like serine proteinase-like protein 2	ABO33174	2.64E-59	*Penaeus monodon*	1
Serine protease snake-like	XP_008547524	3.00E-14	*Microplitis demolitor*	1
Serine proteinase inhibitor (Serpin)	CAA57964	7.00E-23	*Pacifastacus leniusculus*	2
Chaperones				
Glutathione-dependent prostaglandin D synthase	AFJ11393	1.00E-23	*Penaeus monodon*	1
Heat shock protein 70	AAW71958	2.45E-66	*Fenneropenaeus chinensis*	3
Mitochondrial import inner membrane translocase subunit Tim9	XP_008192294	1.00E-28	*Tribolium castaneum*	3
Clotting protein				
Transglutaminase	AAL78166	5.45E-82	*Penaeus monodon*	10
Other defense and homeostasis proteins				
CD82 antigen-like isoform X2	XP_012263853	5.00E-27	*Athalia rosae*	1
DEAD(Asp-Glu-Ala-Asp) box polypeptide 5	ADB44900	1.00E-20	*Macrobrachium nipponense*	1
Prophenoloxidase 2-like protein	AIE45536	1.00E-136	*Penaeus monodon*	2
Cell cycle/DNA synthesis, repair & replication				
Apoptosis				
Caspase 3	AEW91437	3.00E-54	*Penaeus monodon*	1
Caspase 3c	AET34920	5.00E-30	*Macrobrachium rosenbergii*	1
Cell cycle				
E3 ubiquitin-protein ligase Nedd-4 isoform X3	XP_012260927	6.00E-10	*Tribolium castaneum*	1
Oncoprotein nm23	AFL02665	2.00E-53	*Penaeus monodon*	1
Ubiquitin-conjugating enzyme E2 c	AFU93453	2.00E-100	*Litopenaeus vannamei*	1

Table 3.1 (Continued)

Highest homology	Accession no.	*E* value	Closet species	No. of clones
DNA replication				
Proliferating cell nuclear antigen	AEJ89927	2.00E-41	*Litopenaeus vannamei*	1
Gene expression, regulation & protein synthesis				
Transcription				
H/ACA ribonucleoprotein complex subunit 2-like protein	XP_012345208	2.00E-90	*Apis florea*	1
Protein spinster-like	XP_005987471	1.00E-07	*Latimeria chalumnae*	1
U5 small nuclear ribonucleoprotein 200 kDa helicase-like protein	XP_008933667	6.00E-94	*Merops nubicus*	1
Protein synthesis				
39S ribosomal protein L2	KFM83087	6.00E-27	*Stegodyphus mimosarum*	1
60S ribosomal protein L27	ACY66537	8.00E-29	*Scylla paramamosain*	1
Aspartyl-tRNA synthetase	XP002423869	1.00E-74	*Pediculus humanus corporis*	1
Elongation factor 1-alpha	BAH28836	1.00E-129	*Marsupenaeus japonicus*	1
Elongation factor 2	ABR01223	7.00E-25	*Penaeus monodon*	1
Eukaryotic translation initiation factor 3 subunit D-like	XP_011432044	9.00E-69	*Crassostrea gigas*	1
Eukaryotic translation initiation factor 4 gamma 2-like	XP_003396125	2.00E-90	*Bombus terrestris*	1
Poly A binding protein, cytoplasmic 1 a	ACO51197	2.90E-30	*Hypophthalmichthys nobilis*	1
Ribonuclease P/MRP 30 subunit	XP_004349060	6.00E-14	*Capsaspora owczarzaki*	1
Ribosomal protein L4	ADD21621	1.00E-51	*Mya truncata*	2
Ribosomal protein L7	ACQ91224	6.00E-12	*Penaeus monodon*	1
Ribosomal protein P1	ACR54112	1.00E-28	*Palaemon varians*	2
Transport				
Beta actin	ACZ60616	4.00E-101	*Panulirus argus*	6
Tubulin-alpha	AGV09111	4.42E-28	*Torbia viridissima*	1
Energy & metabolism				
ADP/ATP translocase II	AEK78307	1.83E-71	*Litopenaeus vannamei*	1
Cytochrome c oxidase subunit iii	NP038293	3.00E-61	*Penaeus monodon*	4
Glutamate dehydrogenase	ACC95446	3.61E-42	*Litopenaeus vannamei*	1
Mitochondrial ATP synthase gamma subunit precursor	AEB40302	1.00E-62	*Litopenaeus vannamei*	1
NADH dehydrogenase subunit 1	NP038300	4.37E-48	*Penaeus monodon*	1
Propionyl-CoA carboxylase beta chain	KDQ71473	1.00E-38	*Zootermopsis nevadensis*	1
Saposin- isoform a	ADK94870	2.00E-67	*Penaeus monodon*	1
Signaling & communication				
Extracellular signal-regulated kinase 1/2	ADT80930	4.13E-97	*Penaeus monodon*	1
Miscellaneous functions				
Adaptin, alpha/gamma/epsilon	XP_001649235	9.00E-22	*Aedes aegypti*	1
O-sialoglycoprotein endopeptidase	XP_002434405	8.00E-43	*Ixodes scapularis*	1
SocE	EDX54372	7.00E-20	*Bacillus cereus*	1
Hypothetical proteins				78
Unknown proteins				52
Total				252

Table 3. 2 Up-regulated genes in reverse libraries of YHV-infected *P. monodon* hemocytes identified from suppression subtractive hybridization.

Highest homology	Accession no.	*E* value	Closet species	No. of clones
Defense & homeostasis				
Antimicrobial proteins				
Anti-lipopolysaccharide factor (ALF*Pm*3)	ACC86067	2.34E-62	*Penaeus monodon*	1
Single whey acidic protein domain-containing protein 1 (SWD*Pm*1)	ACF28464	3.00E-28	*Penaeus monodon*	1
Homeostasis				
Hemocyte homeostasis-associated protein (*Pm*HHAP)	ADN43412	4.00E-60	*Penaeus monodon*	2
Proteinases & their inhibitors				
Kazal-type proteinase inhibitor (SPI*Pm*2)	AAP92779	1.00E-140	*Penaeus monodon*	7
Viral responsive protein				
*Pm*VRP15	AHN10846	2.00E-22	*Penaeus monodon*	5
Chaperones				
Heat shock protein 70	ABF83607	2.00E-135	*Marsupenaeus japonicus*	2
Sacsin-like	XP_006822247	1.00E-19	*Saccoglossus kowalevskii*	2
Other defense and homeostasis proteins				
Cyclophilin a	ABV90639	3.00E-104	*Penaeus monodon*	3
Gene expression, regulation & protein synthesis				
Peptidyl-prolyl cis-trans isomerase 5	XP_004535714	1.00E-63	*Ceratitis capitata*	1
Protein disulfide isomerase	ACN89260	2.00E-104	*Litopenaeus vannamei*	1
Ribosomal protein S23e	ACY66586	9.00E-90	*Scylla paramamosain*	1
Zinc finger protein 84	KFM83369	2.00E-17	*Stegodyphus mimosarum*	1
Transport				
Beta-actin	AAF82308	2.00E-56	*Metridium senile*	1
Tubulin beta-1 chain	ABL75455	2.00E-37	*Homarus americanus*	1
Energy & metabolism				
Cytochrome c oxidase subunit VIIA putative	ACY66414	1.00E-12	*Scylla paramamosain*	1
Signaling & communication				
Ubiquitin-activating enzyme E1	AFK65746	5.00E-54	*Eriocheir sinensis*	1
Miscellaneous functions				
26S proteasome non-ATPase regulatory subunit 2	XP_967560	3.00E-30	*Tribolium castaneum*	2
Granulins-like isoform X2	XP_009862363	4.00E-46	*Ciona intestinalis*	1
Protein bcn92	P82116	8.00E-21	*Drosophila subobscura*	1
Protein Dom3Z	KFM80607	5.00E-11	*Stegodyphus mimosarum*	1
Hypothetical proteins	CAM36311	4.00E-11	*Thermobia domestica*	25
Unknown proteins				38
Total				99

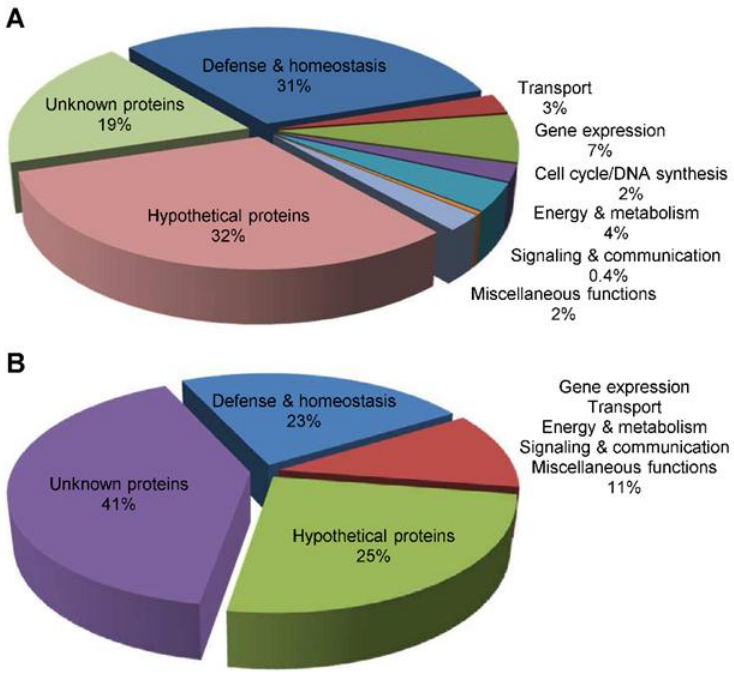

Figure 3. 12 Percentage proportion of gene groups identified from SSH libraries. A total of 252 and 100 clones from forward (A) and reverse (B) SSH libraries, respectively, were sequenced, annotated and categorized into nine groups of genes. Owing to a low number of sequencing clones, not all gene groups were identified in reverse library.

3.16 Differential expression of genes from SSH libraries by real-time RT-PCR

Since it was the subtraction of cDNAs of *PmRelish* dsRNA-YHV shrimp from those cDNA of *GFP* dsRNA-YHV shrimp, the genes in the forward library were genes that were up-regulated by YHV infection and also under the influence of *Pm*Relish (Table 3.1). Without the response from the Imd pathway in the *PmRelish* dsRNA-YHV shrimp, the shrimp might adopt alternative means to defense and survive the YHV infection. This resulted in up-regulated genes identified in the reverse libraries (Table 3.2). To validate that the genes were up-regulated by YHV infection and might be

under or not under the modulation of *Pm*Relish, seven responsive genes from the forward SSH library: *Kunitz* (a Kunitz-type serine proteinase inhibitor gene), *penaeidin3*, *penaeidin5*, *transglutaminase, SWDPm2* (single whey acidic protein domain-containing protein isoform 2), and two alternative responsive genes from the reverse library: *HHAP* (hemocyte homeostasis-associated protein), *SPIPm2* (Kazal-type serine proteinase inhibitor isoform 2) were selected and tested by real-time RT-PCR. The shrimp elongation factor-1α gene (*EF-1α*) was used as an internal control.

It was expected that the three genes, *Kunitz*, *penaeidin3* and *penaeidin5* would be up-regulated by YHV infection but would not be responsive to the infection under *PmRelish* silencing. The results revealed that the three genes were up-regulated similarly in YHV-injected shrimp and *GFP* dsRNA-YHV shrimp. As expected, they did not respond to the YHV infection if the *PmRelish* was silenced (Figure 3.13).

Although the *transglutaminase* was detected in the forward library, the expression profile in response to YHV infection was different from those of *Kunitz*, *penaeidin*3 and *penaeidin*5 in that the expression of *transglutaminase* was up-regulated at 3 h and, then, decreased at 6 h. Strangely enough, the expression of transglutaminase, as opposed to that of *Kunitz*, *penaeidin3* and *penaeidin5*, was responsive to YHV infection in *PmRelish* silenced shrimp; it was down-regulated at 3 h but increased dramatically at 6 h. The transglutaminase gene was also the major transcript detected in the group of defense and homeostasis (Figure 3.13).

The *SWDPm2* was detected in both libraries, and as a major transcript in forward library. The expression was up-regulated slightly in YHV-infected and *GFP* dsRNA-YHV shrimp. It was only up-regulated substantially at 6 h after YHV infection in *PmRelish* dsRNA-YHV shrimp (Figure 3.13). Logically, from the YHV up-regulation in *PmRelish* silenced shrimp, this gene transcript should be detected more in the reverse library than the forward library.

The *SWDPm2* was also detected as the major transcript in the forward library. The expression was up-regulated slightly in YHV-infected and *GFP* dsRNA-YHV shrimp. In *PmRelish* dsRNA-YHV shrimp, it was down-regulated at 3 h and, then, up-regulated substantially at 6 h after YHV infection (Figure 3.13). Logically, considering from the up-regulation of this gene in *PmRelish* dsRNA-YHV shrimp, it should be detected more in the reverse library than the forward library.

The *HHAP* and *SPIPm2* were alternative gene up-regulated in response to YHV infection without the *Pm*Relish influence or the Imd pathway response. The expression of *HHAP* was extremely high in response to YHV infection in YHV-infected and *GFP* dsRNA-YHV shrimp. The expression was decreased substantially below basal level at 6 h in *PmRelish* dsRNA-YHV shrimp (Figure 3.13). The expression profile of *HHAP* was similar to that of *Kunitz*, *penaeidin3* and *penaeidin5* in the forward library but the *HHAP* transcript was not detected at all in the forward library.

In contrast, the expression of *SPIPm2* was not up-regulated at 3 h and 6 h after YHV infection in YHV-injected and *GFP* dsRNA-YHV shrimp. When the *PmRelish*

was silenced, the expression was increased in response to YHV infection. Thus, the result suggested that the shrimp increased the synthesis of SPI*Pm2* as an alternative protein in the defense against the YHV disease if the Imd pathway was blocked. (Figure 3.13).

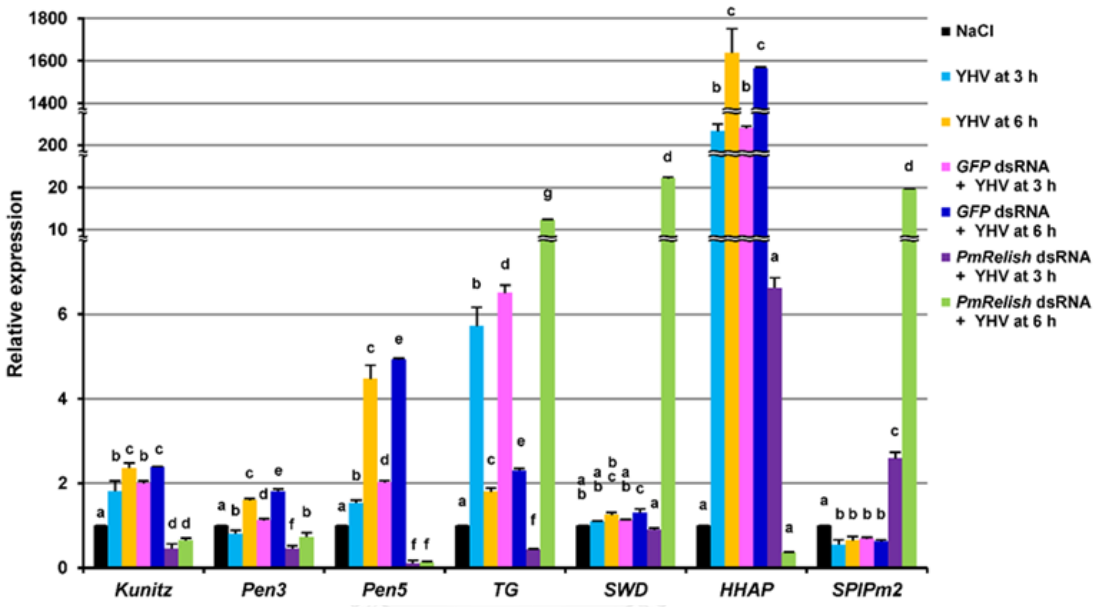

Figure 3. 13 Expression of selected genes from SSH libraries in YHV-infected, GFP dsRNA-YHV and PmRelish dsRNA-YHV shrimp. The *Kunitz*, *penaeidin3* (*Pen3*), *penaeidin5* (*Pen5*), *transglutaminase* (*TG*) and *SWDPm2* (*SWD*) were from the forward library whereas the *HHAP* and *SPIPm2* were from the reverse libraries. The cDNAs from the shrimp were analyzed for the transcription level of each gene. The expression was calculated relative to that of the control shrimp injected with normal saline (NaCl) either at 3 h or 6 h. The experiment was done in triplicate. Data are presented as means ± standard deviations. Different letters indicate significant differences between data at 3 h and 6 h accepted at $P < 0.05$.

CHAPTER IV
DISCUSSION

In the defense against pathogenic infections, shrimp and crustacean-alike respond solely by innate immunity which includes the cellular and humoral responses. The Toll and Imd pathways are two major humoral innate immune response pathways that regulate the synthesis of antimicrobial proteins against bacteria and viruses. Infection signals are sensed by the immune cells and relayed through the pathways. The Dorsal/Dif in Toll pathway and Relish in Imd pathway are the cytosolic NF-κB transcription factors that are activated upon infection signal relay and translocate into the nuclei to regulate the synthesis of AMPs (Ganesan et al. 2011).

In shrimp, the Relishes from *F. chinensis*, FcRelish, and *L. vannamei*, LvRelish, had been identified. Their expression was shown to be responsive to vibriosis and WSSV infection (Li et al. 2009; Qiu et al. 2014). In this study, we were interested in the Relish from *P. monodon*, *Pm*Relish, and its expression in response to YHV infection. Although the *PmRelish* was expressed in all tissues tested, the hemocytes was used in our study for the *PmRelish* was relatively highly expressed in the hemocytes. The expression of *PmRelish* was highly up-regulated to about 8-fold against *V. harveyi* infection. This was in contrast to the expression of *PmRelish* against the viruses. Upon YHV and WSSV infection, the expression was decreased in the early

hours and up-regulated as the infection progressed. Qui et al. (2014) found instead that the expressions of LvDorsal and LvRelish in gills of *L. vannamei* were significantly up-regulated by WSSV challenge (Qiu et al. 2014). The discrepancy may be due to the different tissues analyzed. Their results indicated that the activation of LvDorsal and LvRelish seemed to promote the WSSV replication.

In *Drosophila*, it has been shown that the Imd pathway mediates an antiviral response to RNA viruses (Costa et al. 2009; Avadhanula et al. 2009). In shrimp, the YHV is a pathogenic RNA virus and it is not known whether the Imd pathway responds to the YHV infection. To answer the question, we used the dsRNA silencing method to suppress the expression of *PmRelish*. Without *PmRelish* expression, the shrimp were more susceptible to the pathogenicity of the virus and perished faster. This result strongly indicated the antiviral function of the Imd pathway against the YHV.

The expression of four AMPs, *ALFPm3*, *crustinPm1*, *penaeidin3* and *penaeidin5*, was investigated to see which AMP was under the regulation of *Pm*Relish upon YHV infection. It was found that under the *Pm*Relish silencing condition, the expression of *penaeidin5* but not other three AMPs was considerably suppressed. The similar phenomenon was observed in *F. chinensis* for the expression of *penaeidin5* was also suppressed when the *Fc*Relish-silencing shrimp were infected with Gram-negative bacterium *V. anguillarium* (Li et al. 2009). Whether the reduced

amount of *penaeidin5* had any connection to the faster dying rate after YHV infection was not known.

The Toll and Imd pathways are known to regulate AMP synthesis in response to infection (Tassanakajon et al. 2013). In this work, a few AMP genes supposedly under and not under the Imd regulation are identified from the SSH libraries; relatively abundant expression are *crustinPm1*, *crustinPm4*, *penaeidin3*, *penaeidin5*, *SWDPm2* from the forward library and *ALFPm3*, *SWDPm1* from the reverse library. The expression profiles of *ALFPm3*, *crustinPm1*, *penaeidin3* and *penaeidin5* determined using Gram-negative bacterium *V. harveyi* as an inducer of the IMD pathway indicate that the expression of *penaeidin5* is under the *Pm*Relish regulation in agreement with previous report using YHV as an inducer of Imd pathway (Visetnan et al. 2015). From its low expression under *PmRelish* silencing, *penaeidin3* is very likely under the *Pm*Relish regulation as well. The expression of *ALFPm3* and *crustinPm1* is not under the regulation of *Pm*Relish.

The *PmRelish* gene encoding a 133.2 kDa protein of 1,195 amino acids was also cloned. The amino acid sequence of *Pm*Relish is fairly similar to that of LvRelish with an identity of 97%. We had attempted to over-produce the full-length *Pm*Relish protein, the N-terminal fragment corresponding to the activated *Pm*Relish, the RHD fragment (*Pm*Rel) and the C-terminal fragment using an *E. coli* expression system. The r*Pm*Relish was not over-produced. The r*Pm*N-terminal and r*Pm*C-terminal fragments could be over-produced but the proteins were cleaved readily in *E. coli*

into 2 fragments (Figure 3.8.2 and Figure 3.8.3). Only the r*Pm*Rel was successfully produced and was used for the production of anti-*Pm*Rel. The anti-*Pm*Rel could be used to detect the full-length *Pm*Relish and activated *Pm*Relish in the cells.

To see if the YHV infection would induce the activation and translocation of *Pm*Relish into the nuclei of hemocytes from infected shrimp, hemocytes were collected from uninfected and YHV-infected shrimp and stained using immunocytochemical technique and visualized under a confocal laser scanning microscopy. The *Pm*Relish was observed in all hemocytes from both uninfected and infected shrimp. Unlike those observed in *Drosophila* that the activated Relish translocates into the nuclei of fat cells (Stöven et al. 2000; Kim et al. 2014), we hardly observed the accumulation of activated *Pm*Relish, if any, in the nuclei of shrimp hemocytes. It is possible that the activated *Pm*Relish is only a small proportion of the *Pm*Relish compared to the inactivated *Pm*Relish in the cytoplasm and this makes it difficult to detect the activated *Pm*Relish clearly against the cytoplasmic *Pm*Relish in the images.

Nevertheless, we were able to detect the activation of *Pm*Relish by Western blot analysis. Although the full-length *Pm*Relish was not detected, the activation of *Pm*Relish was clearly observed. The cleavage of the full-length *Pm*Relish gave one major protein band along with two minor protein bands. This is similar to the activation of Relish from *Drosophila* for its activation also gives a few protein bands

indicating that the activation protease Dredd caspase may be able to cleave a peptide bond more than one position at the cleavage site (Stöven et al. 2000).

Therefore, it is concluded here that an RNA virus YHV can activate the *Pm*Relish of the shrimp Imd pathway. The activated *Pm*Relish regulates in the synthesis of penaeidin5 and probably other cognate AMPs. Without *Pm*Relish from the Imd pathway, the shrimp are more susceptible to YHV infection. Importantly, the Imd pathway responds against the YHV infection in *P. monodon*.

Several Rel/NF-kB transcription factors such as DIF, Dorsal and Relish are important regulators of innate immunity in both vertebrate and invertebrate (Stöven et al. 2000; Huang et al. 2009). The Rel/NF-κB transcription factor Relish is crucial for the humoral immune response in Drosophila by induce the production of antimicrobial peptides (AMPs) to defense against bacterial and fungi infection (Stöven et al. 2000). Recent studies suggest that Relish binds tightly to DNA. It binds to the κB site in the promoter of the antimicrobial peptide genes and probably also those of other genes that depend on the Relish for their induction (Stöven et al. 2000). However, in gel retardation experiment, the Protein-DNA interaction was not detected because the shifted band can not be observed even after using high mole ratios of r*Pm*Relish to DNA.

In order to reveal the whole scheme of gene expression under certain condition such as infection by pathogens, the SSH technique has been employed in several studied. Prapavorarat et al. (2010) (Prapavorarat et al. 2010b) used SSH to

identify genes expressed in response to 24 h-72 h yellow head virus infection in the black tiger shrimp (Prapavorarat et al. 2010a). Wang et al. (2013) used the same technique to screen for the genes regulated by *Fc*Relish in 1-h *Vibrio anguillarium*-infected shrimp (Wang et al. 2013b). Herein, the same technique is used to look for a group of genes that are involved in YHV infection under the regulation of Imd signaling pathway.

When the shrimp are infected by YHV, it is thought that several genes are modulated either up-regulated or down regulated to prepare the shrimp for their defense against the virus, the stress from the disease, tissue damages from the infection, the multiplication of virus, etc. The expression of regular housekeeping genes may not be changed. One pathway that is responsive to YHV infection is the Imd pathway (Visetnan et al. 2015). The Relish is a key NF-κB transcription factor of the Imd pathway that regulates the synthesis of defense genes, particularly the antimicrobial proteins. If Relish is knockdown by RNAi technique, the Imd response would be forfeited. To fish out the genes that are up-regulated against YHV infection under the Imd regulation in the forward library, the expressed cDNAs from YHV infected shrimp are subtracted with those from YHV-infected and Relish-silenced shrimp. In the reverse situation, if there is no Imd pathway response, there should be an alternative gene expression to respond to the infection in addition to the Imd pathway-independent responses already exist. The reverse subtraction may provide us with alternative genes that are expressed for the shrimp to withstand the infection

for sometimes. Nevertheless, it should be noted that the shrimp are more susceptible to the YHV if the *PmRelish* expression is silenced (Visetnan et al. 2015).

Another important factor is the duration of infection. For the shrimp to defense themselves from infection, the Imd response should be immediate. After infection, the expression of *PmRelish* is increased gradually from the subnormal level and peaked at 12 h (Visetnan et al. 2015). It suggests that the constitutive level of *Pm*Relish protein has been used in the early hours and requires replenishment through gene expression. Therefore, the Imd pathway actually responds quite early after the infection. We, thus, chose to collect the shrimp blood samples at 3 h and 6 h after infection in this experiment.

After the SSH and sequencing were done, we obtained as many as 252 clones from the forward library and 99 clones of alternate responsive genes from the reverse library. The forward library is far more interesting for it contains the YHV responsive genes expressed under the influence of *Pm*Relish regulation. The results from the mining of sequencing data and classification of annotatable genes seem to confirm our expectation that the Imd pathway responds early in YHV infection. At 3-6 h after infection, the genes in defense and homeostasis are abundantly expressed as major expressed genes, and are accounted for 31% and 23% of all cDNAs sequenced in forward and reverse libraries, respectively. Majority of the genes in defense and homeostasis belong to the subgroup antimicrobial proteins and subgroup proteinases and their inhibitors. Ones should bear in mind that not all the

genes in the forward library are actually under direct regulation of *Pm*Relish; they may be the accessories to the Imd response.

A few genes were tested by qRT-PCR to validate the SSH technique. The *Kunitz*, *penaeidin3*, *penaeidin5*, *transglutaminase* and *SWDPm2* from the forward library and the *HHAP* and *SPIPm2* from the reverse library are chosen for they are particularly high abundant and/or interesting genes. The *Kunitz*, *penaeidin3*, *penaeidin5*, *transglutaminase* and *SWDPm2* exhibit expression profiles more or less as expected; in particular, the *Kunitz*, *penaeidin3* and *penaeidin5*. Their expression was up-regulated by YHV infection. Once the *PmRelish* gene is knocked down, the expression is decreased. On the contrary, the *SPIPm2* from the reverse library is somewhat down-regulated by the YHV infection but up-regulated substantially after the silencing of *PmRelish* gene.

When the expression profiles of *transglutaminase* and *SWDPm2* and *HHAP* are considered in details, it can say that they may be partly under the influence of *Pm*Relish regulation at early hours of infection, 3-6 h post infection in our experiment. The expression of *transglutaminase* gene, for instance, is highly up-regulated at 3 h and subsided afterwards. However, without *Pm*Relish in silencing shrimp, the expression is down-regulated at 3 h and, then, highly increased at 6 h. At the early hours of infection, the expression of *SWDPm2* is barely increased from the basal level expression in control shrimp but is highly increased at 6 h in *PmRelish* silencing shrimp. As in the fly, the expression of *transglutaminase* may be increased

under the activation of Imd signaling pathway for it is needed to regulate the said pathway via its crosslinking activity on specific transcription factors, *Pm*Relish in this case (Shibata et al. 2013).

It has been shown that the *HHAP* gene is highly up-regulated upon white spot syndrome virus infection and is important for maintaining the level of circulating hemocytes in shrimp. Silencing of *HHAP* results in a significant decrease in the number of circulating hemocytes, deformation of the hemocytes and, finally, 100% shrimp mortality (Prapavorarat et al. 2010b). In this study, the YHV infection also results in high up-regulation of *HHAP* but the expression is reduced to subnormal level in *PmRelish* silenced shrimp. The expression profile is similar to those of *Kunitz*, *penaeidin3* and *penaeidin5* but it is not known why the *HHAP* gene is only detected in the reverse library. Moreover, Prapavorarat et al. (2010) did not find *HHAP* gene in their forward SSH libraries either (Prapavorarat et al. 2010a). What we know is that there is an increase in the number of apoptotic hemocytes at 12 h after YHV infection which means a reduction in hemocytes (Khanobdee et al. 2002; Visetnan et al. 2015). At 3-6 hours after YHV infection, it is possible that the *HHAP* gene is expressed at high rate for the shrimp to protect the hemocyte cellular integrity.

CHAPTER V

CONCLUSIONS

1. A *PmRelish* gene is successfully cloned. The *PmRelish* gene is 3,677 bp containing an open reading frame of 3,585 bp encoding a 1,195 amino acid residue. The predicted molecular weight of the deduced amino acids of *Pm*Relish is 133.2 kDa, and its theoretical p*I* is 5.42.
2. The *PmRelish* is constitutively expressed in all tissues tested and mostly up-regulated upon YHV infection.
3. The expression of *PmRelish* gene is significantly increased after *V. harveyi* 639, YHV and WSSV challenges.
4. Under *PmRelish* silencing, the shrimp are more susceptible to infection by YHV with the 50% survival rate reduced from about 72 h to 42 h.
5. The expression profiles of, *penaeidin3* and *penaeidin5* determined using YHV and *V. harveyi* 639, as an inducer of the Imd pathway indicate that the expression of *penaeidin3* and *penaeidin5* but not *ALFPm3* and *crustinPm1* is under the *Pm*Relish regulation.
6. The four fragments of *Pm*Relish: *Pm*Relish, *Pm*N-terminal, *Pm*C-terminal and *Pm*Rel, are over-expressed. Only the r*Pm*Rel is produced successfully in *E. coli* system and used to produce anti-*Pm*Relish antibody. By using gel retardation assay, the r*Pm*Rel has no DNA-binding activity.

7. The *Pm*Relish is detected in the cytoplasm of all the hemocytes from both uninfected and YHV-infected shrimp. The accumulation of activated *Pm*Relish in the nuclei is not clearly observed but can be detected in the YHV-infected hemocytes by Western blot analysis.
8. By using suppression subtractive hybridization, the immune genes up-regulated in response to YHV infection and under the *Pm*Relish regulation were identified. Sequences of 252 and 99 cDNA clones from the forward and reverse libraries, respectively, were obtained and annotated through blast search against the GenBank sequences. Genes involved in defense and homeostasis are abundant in both libraries, 31% and 23% in the forward and reverse libraries, respectively. They were predominantly antimicrobial proteins, proteinases and proteinase inhibitors, chaperones, clotting protein, and other defense and homeostasis proteins.

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APPENDIX

Publications

1. **Visetnan S**, Supungul P, Hirono I, Tassanakajon A, Rimphanitchayakit V (2015) Activation of PmRelish from Penaeus monodon by yellow head virus. Fish and Shellfish Immunology 42(2):335-344.
2. **Visetnan S**, Donpudsa S, Supungul P, Tassanakajon A, Rimphanitchayakit V (2014) Domain 2 of a Kazal serine proteinase inhibitor SPI*Pm*2 from *Penaeus monodon* possesses antiviral activity against WSSV. Fish and Shellfish Immunology 41 (2):526-530.
3. Donpudsa S, **Visetnan S**, Supungul P, Tang S, Tassanakajon A, Rimphanitchayakit V (2014) Type I and type II crustins from *Penaeus monodon*, genetic variation and antimicrobial activity of the most abundant crustin*Pm*4. Developmental and Comparative Immunology 47 (1):95-103.
4. Donpudsa S, Ponprateep S, Prapavorarat A, **Visetnan S**, Tassanakajon A, Rimphanitchayakit V (2010) A Kazal-type serine proteinase inhibitor SPI*Pm*2 from the black tiger shrimp *Penaeus monodon* is involved in antiviral responses. Developmental and Comparative Immunology 34 (10):1101-1108.
5. **Visetnan S**, Donpudsa S, Supungul P, Tassanakajon A, Rimphanitchayakit V (2010) Kazal-type serine proteinase inhibitors from the black tiger shrimp *Penaeus monodon* and the inhibitory activities of SPI*Pm4* and 5. Fish and Shellfish Immunology 27 (2):266-274.

VITA

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She had published her works in the research journals on the topics of

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She had submitted her works in the research journals on the topics of

"YHV-responsive gene expression under the influence of PmRelish regulation".