ลักษณะสมบัติของโปรตีนชนิดใหม่ที่มีโดเมน TBC1 ในกุ้งกุลาดำ *Penaeus monodon* และบทบาทในการตอบสนองต่อการติดเชื้อไวรัสตัวแดงดวงขาว

นางสาววันฉัตร ยิ่งวิลาศประเสริฐ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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ปีการศึกษา 2554

CHARACTERIZATION OF A NOVEL PROTEIN CONTAINING TBC1 DOMAIN IN THE BLACK TIGER SHRIMP *Penaeus monodon* AND ITS ROLE IN RESPONSE TO WHITE SPOT SYNDROME VIRUS INFECTION

Miss Wanchart Yingvilasprasert

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Biochemistry
Department of Biochemistry
Faculty of Science
Chulalongkorn University
Academic Year 2011

Thesis Title	**CHARACTERIZATION OF A NOVEL PROTEIN CONTAINING TBC1 DOMAIN IN THE BLACK TIGER SHRIMP *Penaeus monodon* AND ITS ROLE IN RESPONSE TO WHITE SPOT SYNDROME VIRUS INFECTION**
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วันฉัตร ยิ่งวิลาศประเสริฐ : ลักษณะสมบัติของโปรตีนชนิดใหม่ที่มีโดเมน TBC1 ในกุ้งกุลาดำ *Penaeus monodon* และบทบาทในการตอบสนองต่อการติดเชื้อไวรัสตัวแดงดวงขาว Characterization of a novel protein containing TBC1 domain in the black tiger shrimp *Penaeus monodon* and its role in reponse to white spot syndrome virus infection อ.ที่ปรึกษาวิทยานิพนธ์หลัก : ศ.ดร.อัญชลี ทัศนาขจร, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม : ดร.เปรมฤทัย สุพรรณกูล, 115 หน้า

โปรตีนโดเมน TBC (TRE2/BUB2/CDC16) เป็นโปรตีนขนาดประมาณ 200 กรดอะมิโนซึ่งพบในกลุ่มโปรตีนที่กระตุ้นการทำงานของ Rab GTPase ในการขนส่งสารถุงหุ้มภายในเซลล์ จากฐานข้อมูล EST และผลการวิเคราะห์ทาง microarray ของกุ้งกุลาดำ พบยีนชนิดใหม่ คือ *Pm*TBC1D20 ซึ่งมีความคล้ายคลึงกับโปรตีน TBC1D20 ในสิ่งมีชีวิตหลายชนิด และยีนดังกล่าวมีการตอบสนองต่อการติดเชื้อไวรัสตัวแดงดวงขาว ในการศึกษาครั้งนี้ ได้ทำการหาลำดับนิวคลีโอไทด์ที่ครบสมบูรณ์ของยีน โดยมีขนาดทั้งหมด 2004 คู่เบส สามารถแปลเป็นโปรตีนที่มีลำดับกรดอะมิโน 480 ตัว เมื่อศึกษาการแสดงออกของยีนนี้ในอวัยวะต่างๆของกุ้งกุลาดำด้วยเทคนิค semi-quantitative RT-PCR พบว่า ยีนนี้มีการแสดงออกในทุกอวัยวะรวมทั้งเม็ดเลือด และพบการแสดงออกของยีนนี้เพิ่มขึ้นในเม็ดเลือดกุ้งประมาณ 2.3 และ 2.0 เท่าหลังจากกุ้งติดเชื้อไวรัสตัวแดงดวงขาวที่ 24 และ 48 ชั่วโมงตามลำดับหลังการติดเชื้อ โดยใช้เทคนิค quantitative real-time RT-PCR สำหรับการศึกษาผลของการยับยั้งยีน *Pm*TBC1D20 ด้วยเทคนิค RNA interference ต่อการติดเชื้อไวรัสตัวแดงดวงขาว พบว่า ในเซลล์เม็ดเลือดปฐมภูมิของกุ้งกุลาดำที่ยับยั้งยีนนี้แล้วทำให้ติดเชื้อไวรัสดังกล่าวปริมาณยีน VP28 ซึ่งเป็นโปรตีนเปลือกหุ้มของไวรัสตัวแดงดวงขาวลดลงประมาณ 50% และพบกุ้งที่ถูกยับยั้งการแสดงออกของยีนนี้แล้วทำให้ติดเชื้อไวรัสตัวแดงดวงขาวมีการแสดงออกของยีน VP28, ie1 และ WSV477 ลดลงมากกว่า 90% เมื่อพิจารณาเซลล์เม็ดเลือดกุ้งภายใต้กล้องจุลทรรศน์ด้วยเทคนิค immunofluorescense hybridization โดยใช้แอนติบอดี้ที่จำเพาะกับโปรตีน *Pm*TBC1D20 พบโปรตีนนี้แสดงออกในเม็ดเลือดทุกชนิด โดยพบอยู่ในไซโตพลาสซึมและเยื่อหุ้มนิวเคลียส และมีปริมาณโปรตีนนี้เพิ่มมากขึ้นเมื่อกุ้งติดเชื้อไวรัสตัวแดงดวงขาว นอกจากนี้เมื่อใช้เทคนิคนี้ศึกษาผลของการยับยั้งการแสดงออกของยีน *Pm*TBC1D20 ในกุ้งแล้วทำให้ติดเชื้อไวรัสตัวแดงดวงขาว พบปริมาณของโปรตีน VP28 ในเซลล์เม็ดเลือดกุ้งน้อยกว่าในกุ้งกลุ่มควบคุม จากผลการทดลองทั้งหมดแสดงให้เห็นว่า *Pm*TBC1D20 น่าจะเกี่ยวข้องกับการติดเชื้อไวรัสตัวแดงดวงขาวในกุ้งกุลาดำ

ภาควิชา ชีวเคมี ลายมือชื่อนิสิต .....

สาขาวิชา ชีวเคมี ลายมือชื่อ อ. ที่ปรึกษาวิทยานิพนธ์หลัก .....

ปีการศึกษา 2554 ลายมือชื่อ อ. ที่ปรึกษาวิทยานิพนธ์ร่วม .....

5172577123 : MAJOR BIOCHEMISTRY

KEYWORDS : *Penaeus monodon* / TBC DOMAIN / TBC1D20 / WHITE SPOT VIRUS

WANCHART YINGVILASPRASERT : CHARACTERIZATION OF A NOVEL PROTEIN CONTAINING TBC1 DOMAIN IN THE BLACK TIGER SHRIMP *Penaeus monodon* AND ITS ROLE IN RESPONSE TO WHITE SPOT SYNDROME VIRUS INFECTION

ADVISOR: PROF. ANCHALEE TASSANAKAJON, Ph.D., CO-ADVISOR : PREMRUETHAI SUPUNGUL, Ph.D., 115 pp.

TBC domain (**T**RE2/**B**UB2/**C**DC16) is a ~200-amino-acid protein containing a Rab-GTPase-activating protein (GAP) homology domain required for regulating the activity of Rab proteins which are center of endocytic membrane trafficking in cells. From cDNA microarray analysis, a novel *Pm*TBC1D20 gene with deduced amino acid sequences that showed significant similarity with TBC1D20 protein in many organisms was up-regulated in response to White Spot Syndrome Virus (WSSV) in the black tiger shrimp (*Penaeus monodon*). In this study, a full-length sequence of *Pm*TBC1D20 gene was determined by Rapid Amplification of cDNA Ends technique. The full-length sequence contained 2,004 bp with an open reading frame encoding a 480 amino acid protein. The *Pm*TBC1D20 mRNA was expressed in many tissues including hemocytes and up-regulated about 2.3 and 2.0 fold at 24 and 48 h after WSSV infection, respectively. The effect of *Pm*TBC1D20 suppression upon WSSV infection in primary hemocyte cell culture using RNA interference technique was investigated. The result revealed that the transcription level of VP28 gene, an envelope protein of WSSV, was decreased by about 50%. Moreover, the suppression of *Pm*TBC1D20 transcript in WSSV-infected shrimp reduced the expression of three WSSV genes; VP28, ie1, and WSV477 by 90% compared to the control shrimp. When observed under the fluorescence microscope using coimmunofluorescense technique, the VP28 transcription level in *Pm*TBC1D20 suppressed WSSV-infected hemocyte cells detected with anti-VP28 antibody was also decreased. Localization of *Pm*TBC1D20 protein using anti-TBC antibody in shrimp hemocyte revealed that *Pm*TBC1D20 protein localized both in cytoplasm and nuclear membrane in all types of hemocyte cells and the *Pm*TBC1D20 protein signal in hemocytes of 48 h-WSSV-infected shrimp was higher than in the hemocytes of uninfected shrimp.These results suggested that *Pm*TBC1D20 is involved in WSSV infection.

Department : Biochemistry Student's Signature .....

Field of Study: Biochemistry Advisor's Signature .....

Academic Year : 2011 Co-advisor's Signature .....

ACKNOWLEDGEMENTS

I would like to express my deepest gratitude to my advisor Professor Dr. Anchalee Tassanakajon, and my co-advisors Dr. Premruethai Supungul for their advice, vision, encouragement and every supports throughout my study.

Many appreciates are given to Professor Dr. Piamsook Pongsawasdi, Assistant Professor Rath Pichyangkura and Assistant Professor Dr. Sarawut Jitrapakdee for serving as thesis committee, for their comments and also useful suggestions.

Moreover, I would like to thank to Associate Professor Dr. Vichien Rimpanitchayakit, and Miss Sirikwan Pornprateep for very useful comments and suggestions. Many thanks to all my friends of the Department of Biochemistry, Chulalongkorn University especially my colleagues in center of excellence of molecular and gentic of shrimp (CEMS) for their help in the laboratory and friendships throughout my study.

Finally, I would like to express my deepest gratitude to my family for their love, care, understanding and encouragement extended throughout my study.

I wish to acknowledge to the Thailand Graduated Institute of Science and Technology (TGIST) programme of National Science and Technology Development Agency (NSTDA) for grant support (grant no. TGIST 01-51-018).

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μg	microgram
μl	microliter
ALF	anti-lipopolysaccharide factor
AP-1	activator protein 1
Bp	base pair
C-terminal	carboxyl terminal
dATP	deoxyadenosine triphosphate
dCTP	deoxycytosine triphosphate
DEPC	diethylpyrocarbonate
dGTP	deoxyguanosine triphosphate
DNA	deoxyribonucleic acid
dTTP	deoxythymidine triphosphate
EF-1α	elongation factor-1 alpha
EST	expressed sequence tag
EtBr	ethidium bromide
H	hour
Hpi	hour-post injection
Hpt	hematopoietic tissue
Kb	kilobase
LPS	lipopolysaccharide
M	molar
Mg	milligram
Min	minute
Ml	milliliter
mM	millimolar
Ng	nanogram
Nm	nanometer
N-terminal	amino terminal
^{o}C	degree Celcius

OD	optical density
ORF	open reading frame
PCR	polymerase chain reaction
ppt	part per thousand
RNA	ribonucleic acid
RNAi	ribonucleic acid interference
RNase	ribonuclease
RT	reverse transcription
sec	second
UTR	untranslated region
β-actin	beta actin

CHAPTER I
INTRODUCTION

1.1 General introduction

Black Tiger shrimp (*Penaeus monodon*) is one of the most important aquaculture in Thailand which exportation can earn a billion of income each year. Shrimp aquaculture has started in Thailand in the 1970s and grown rapidly in the mid of 1980s (Tookwinas, 1991) as an increase in the number of hatcheries and farms. Shrimp industry has increased in many countries worldwide especially in the tropical countries in South East Asia such as, Central and south America. (Rosenberry, 1997) Thailand's shrimp trading partners spreads out in every continent such as Asia, Europe, North America and also Australia and New Zealands. By the early of 1990s, Thailand emerged as the world's leading farmed shrimp producer and exporter. The black tiger shrimp was traditionally farmed in Thailand till the out breaks of shrimp diseases caused by various microbes especially virus in the 1990s (Flegel, 2006). In the first period of outbreaks, the yellow-head virus was the major cause of disease which the production was decreased about 40 million US dollars in lost export revenue. Later in 1997, the second virus with more seriously harmful, white spot syndrome virus (WSSV) was spreaded out. That outbreak damaged many shrimp aquacultures in all area by causing rapid morality. This led into high economic loss in shrimp farming industry (Chou et.al, 1995). The production of black tiger shrimp had reduced since 1997 as a result from the smaller size and high mortality of shrimp. In early 2000, while the production of the black tiger shrimp was still decreasing, the pacific white shrimp (*Litopenaeus vannamei*) was more popular, and then become the main cultured shrimp species instead of the black tiger shrimp.

During 2001-2010, shrimp aquaculture exportation from Thailand has reached an average exportation at 309,256 tons per year valued to 83,200 million baht per year. The shrimp aquaculture exportation increased every year and the exportation rate was 6% (Fig. 1.1). Shrimp exportation shared 41% of all fisheries products exportation in Thailand.

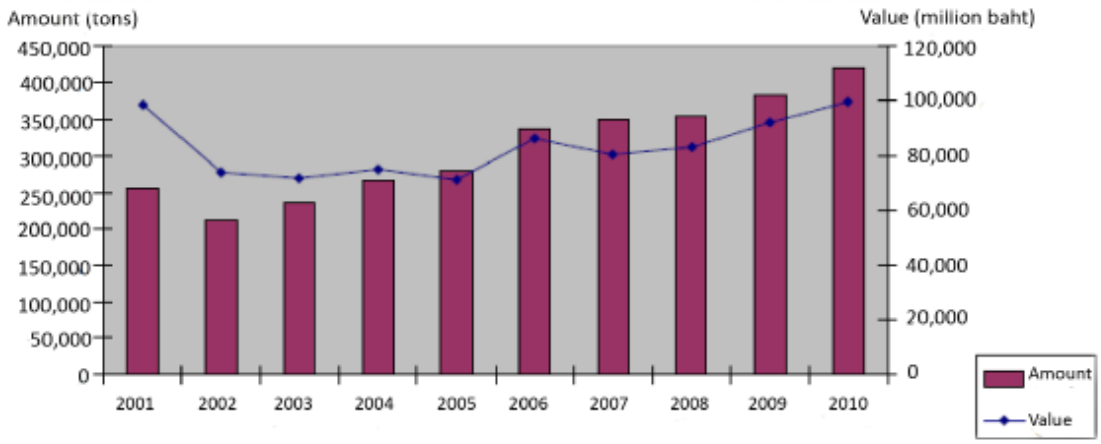


Fig. 1.1 Thailand's shrimp exportation during 2001-2010 (Source: Fisheries Foreign Affairs Division, Department of Fisheries, Thailand)

Shrimp products were exported to many countries all over the world. The United States of America was the top traders which shared 53% of the shrimp market share. Japan and the European union were the second and third rank of traders which had the market share of 21% and 7% respectively (Fig. 1.2).

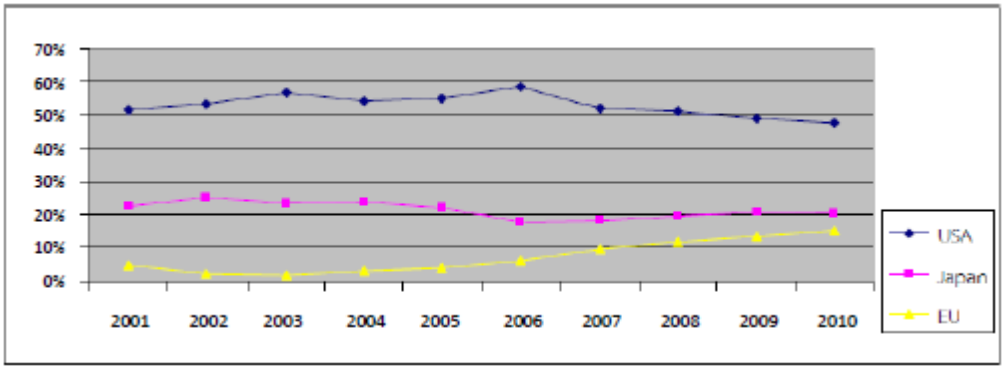


Fig. 1.2 Shrimp aquaculture exported market share in USA, Japan and European Union during 2001-2010 (Source: Fisheries Foreign Affairs Division, Department of Fisheries, Thailand)

For the black tiger shrimp, the exportation trend had decreased continuously. In 2010, the black tiger shrimp exportation was dropped to 17,799 tons valued to

3,849 million baht from 180,616 tons valued to 63,823 million baht in 2003, assuming that the black tiger shrimp exportation rate decreased 21% in total amounts and 26% of exportation value each year (Fig. 1.3). In the other hand, the pacific white shrimp exportation rate had increased 9% per year and the exportation value had risen to 11% per year since 2007 (Fig. 1.4)

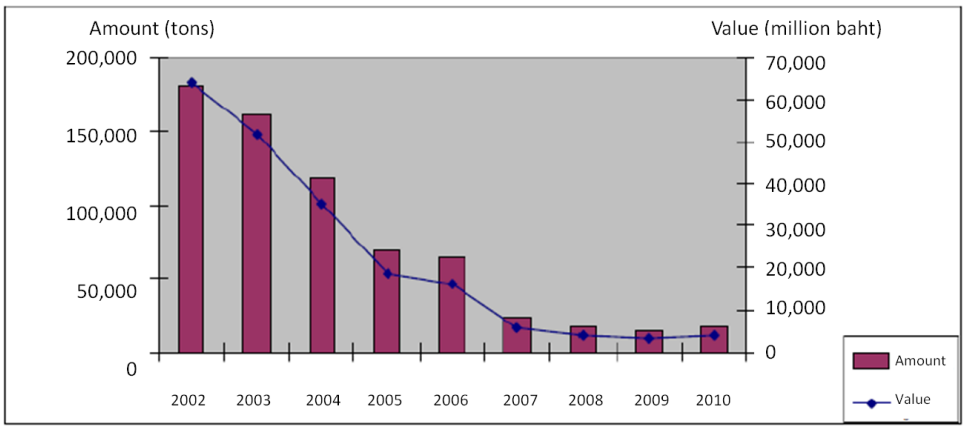


Fig. 1.3 Thailand's black tiger shrimp (*Penaeus monodon*) exportation during 2001-2010 (Source: Fisheries Foreign Affairs Division, Department of Fisheries, Thailand)

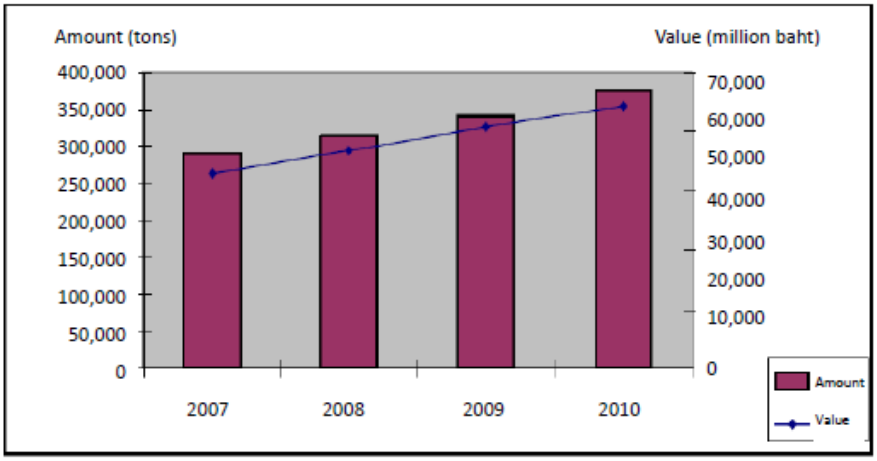


Fig. 1.4 Pacific white shrimp *(Litopenaeus vannamei)* exportation during 2007-2010 (Source: Fisheries Foreign Affairs Division, Department of Fisheries, Thailand)

1.2 Taxonomy of *Penaeus monodon*

Penaeus monodon, the giant tiger shrimp, is a penaeid shrimp species that are classified into the largest phylum in the animal kingdom, the Arthropoda. This group of animal is characterized by the presence of pair appendages and a protective cuticle or exoskeleton that covers the whole animal. The taxonomic definition of the black tiger shrimp, *P. monodon* is as follows (Bailey-Brook and Moss, 1992):

Phylum Arthropoda
Subphylum Crustacea Brünnich, 1772
Class Malacostraca Latreille, 1802
Subclass Eumalacostraca Grobben, 1892
Order Decapoda Latreille, 1802
Suborder Dendrobranchiata Bate, 1888
Superfamily Penaeoidea Rafinesque, 1815
Family Penaeidae Rafinesque, 1985
Genus Penaeus Fabricius, 1798
Species Penaeus monodon Fabricius, 1798

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

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

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

1.3 Shrimp diseases

Diseases are one of the main problems in shrimp aquaculture industry. Shrimp diseases can be divided into two main groups, noninfectious and infectious (Lightner and Redman, 1998). The examples for noninfectious diseases are environmental extremes, nutritional imbalances, toxicants and genetic factors (Lightner, 1988; Johnson, 1995). Infectious diseases is the most serious problem which caused by various microorganisms such as viruses, bacteria, fungi and parasites. Viral and bacterial outbreaks are the most crucial microbes which can cause serious diseases affect to the shrimp farming industries worldwide.

Thailand is facing problems in shrimp farming industry and exportation due to shrimp disease caused by various microbes (Flegel, 2006) especially the viruses. In Thailand, seven viral pathogens are discovered and studied: white spot syndrome virus (WSSV), yellow-head virus (YHV), hepatopancreatic parvovirus (HPV), monodon baculovirus (MBV), Taura syndrome virus (TSV), Laem Singh virus (LSNV) and infectious hypodermal and hematopoeitic virus (IHHNV).White spot syndrome virus (WSSV) and yellow-head virus (YHV) are two of the major viral pathogens of the shrimp which cause white spot syndrome and yellow head disease, respectively.

Yellow head virus which cause yellow head disease is a 22 kbp single-stranded RNA virus. Normally, the YHV-infected shrimp often exhibits light yellow coloration of the dorsal cephalothorax area and the shrimp body turns to a generally pale or bleached (Limsuwan, 1991). Yellow head disease occurs in the juvenile to sub-adult stages of shrimp, especially at 50 - 70 days of grow-out (Lightner, 1996). The critical infection proceeds to 100% mortality within 3 - 9 days post-infection.

(Lu, 1996). The target organ of YHV are lymphoid organs, gills, nerve cord, heart, midgut, hepatopancreas, head soft tissues, abdominal muscle, eyestalks and hematopoietic tissue (Boonyaratpalin et al., 1993; Chantanachookin et al., 1993; Lu et al., 1995). YHV may occur as late and asymptomatic infections in broodstock shrimp and can possibly transfer from the parental shrimp to their offspring (Chantanachookin et al., 1993). For WSSV, more information will be mentioned in the next topic. Moreover, not only virus can cause high loss of shrimp aquaculture, but bacteria can also cause serious loss of shrimp aquaculture. This bacteria is in *Vibrionacea* family such as *Vibrio harveyi*, *V. parahaemolyticus*, *V. alginolyticus* and *V. anguillaram*. Almost infections from these bacteria often result from the environmental stresses or viral diseases (Nash, 1990). The disease caused from *V. harveyi* is called luminous vibiosis which made the shrimp head and body glow after infection and easily noticed in the dark that the luminous moribunded shrimp will swim at the top of the water. Luminous vibriosis is a major disease that affects commercially-farmed penaeid prawns (Austin and Zhang, 2006; Harris and Owens, 1999; Ruangpan et al., 1998). The gram negative rod shape bacteria *V. harveyi* can be found free-swimming in tropical marine waters, commensally in the gut microflora of marine animals, and as both a primary and opportunistic pathogen of marine animals (Austin and Zhang, 2006).

These microbes cause rapid mortality and high economic loss in shrimp farming industry (Chou et al, 1995), whilst the effective approach for preventing the viral infection has not yet been discovered. Shrimp diseases have affected the shrimp aquaculture in loss of production in several agricultural countries including Thailand, then affect to loss of shrimp exportation income which leads to economical problem. Hence, the disease prevention and control is of prime important to the shrimp industry. Improvement of farm management and study of the shrimp immunity can be applied in cultivation of shrimp.

1.4 White spot syndrome

White spot syndrome is a viral disease resulting from WSSV infection, affecting most of the commercially globally cultivated marine shrimp species. The origin of white spot syndrome outbreak began in Taiwan shrimp farms in 1992 and rapidly spread throughout Asia, subsequently, the disease crossed over the Pacific Ocean and spread in North, Central and South America, creating by far the greatest economic damage (Chou et al., 1995; Lightner, 1996; Flegel, 1997; Lotz, 1997; Span and Lester, 1997). WSSV is a major shrimp pathogen causing the onset of a rapid and mass mortality within 2 - 7 days post-infection (Chou et al., 1995, Flegel, 2006). A clinical sign of white spot syndrome typically is the development of many white spots on the carapace of the infected shrimp which results from white calcium accumulated in shell (Chou et al., 1995). However, the disease can occur without the presence of white spots. Other signs of the WSSV infection in shrimp also include the body surface and appendages turning to red or pink, loosing shell, lower food consumption and show lethargic behavior (Liu et al, 2009).

Initially, this virus was called white spot syndrome baculovirus because its morphological characteristic was similar to the insect baculovirus (Lightner, 1996). However, phylogenetic analysis of ribonucleotide reductase and protein kinase gene revealed that WSSV did not share a common ancestor with baculovirus (Van Hulten et al., 2000b, 2001). WSSV is belongs to the genus *Whispovirus* in the viral family *Nimaviridae*, containing a circular double-stranded DNA of about 305 kb, and is an enveloped rod-shaped particle with a single filamentous appendage-like tail at one end of the nucleocapsid (Vlak et al., 2004, Yang et al., 2001). The average size of the virus is about 298 ± 21 nm long and 107 ± 8 nm in diameter (Wang et al., 1997). They contain one nucleocapsid with a typical striated appearance and five majors and at least other 39 structural proteins (Chang et al., 1996; Lu et al., 1997; Park et al., 1998; Rajendran et al., 1999; van Hulten et al., 2000a; van Hulten et al., 2000b; van Hulten et al., 2002) and one non-structural protein VP9 (Liu et al., 2006) (Fig. 1.5). The viral envelope is the external cover of the mature virus that protects the virion from degradation. It is 6-7 nm thick and is a lipidic bilayer membrane structure with two electrontransparent layers divided by an electronopaque layer (Wongteerasupaya et al., 1995; Durand et al., 1997). The nucleocapsid is situated inside the envelope and is

stacked ring structure composed of globular protein subunits (Durand et al., 1997; Nadala and Loh 1998). Inside the nucleocapsid, there is a highly electrondense core containing the DNA binding protein VP15 and the viral DNA (Durand et al., 1997; Wang et al., 1999; van Hulten et al., 2001a).

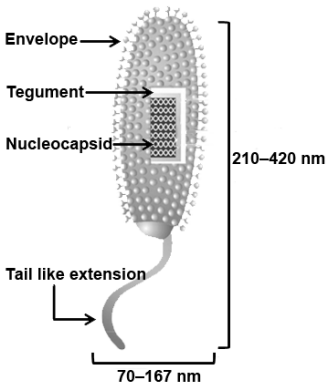


Fig. 1.5 Schematic diagram of WSSV (modified from Sánchez-Paz, 2010)

Some of the most studied genes and encode proteins of the WSSV genome are classified into four groups according to their assumed function (Table 1.1). The first group is composed of the structural genes encoding the envelope and the nucleocapsid or tegument. The second group contains functional or physiological genes that involves in virus proliferation or life cycle functions, such as replication and phosphorylation of host proteins, and nuclease activity. The third group consists of latency and sequesters genes, whose expression can be detected even though the structural genes might not be active. Moreover these genes involve in the persistence of the virus within a host cell. The temporary regulatory genes belong to the fourth group, which participate at specific times during infection. Indeed, they do not require viral protein for their transcriptional processes and are expressed using the host molecular machinery in the first hours after infection (Flint et al., 2000; Sánchez-Martínez et al., 2007). Transcription of viral genes during infection can be broadly divided into three kinetic phases of gene expression according to an ordered cascade of events. Three phase are immediate-early (IE), early (E) and late (L). The IE genes are expressed relying primarily on host proteins and factors for their expression, which occur in the absence of viral DNA replication. Thus, the proper expression of these genes, during the early stages of infection, controls the accurate cascade of viral

replication. The E gene expression mainly encodes enzymes about the synthesis of viral DNA and the proteins which can regulate the expression of L genes. Synthesis of viral DNA is initiated, L gene expressions are encoded enzymes and structural proteins necessary for virion assembly (reviewed in Sánchez-Paz, 2010).

WSSV has a broad host range such as shrimp, crab, crayfish and lobsters (Lo et al., 1996), and can infect various tissues including antennal grand, cuticular epidermis, gill, muscle, lymphoid organ, nervous tissue, hematopoietic tissue, connective tissues of some organs (Chang et al., 1996, Wang et al., 1997). However, the major target tissues for replication are gills, stomach and body cuticular epithelium, hematopoietic tissues, lymphoid organ and antennal glands (Tan et al., 2001; Durand and Lightner, 2002; Escobedo-Bonilla et al., 2007).

The WSSV infection can be detected by several diagnostic methods such as PCR, *in situ* hybridization, mini-array, observation of tissues subjected to staining, and immunological methods using specific antibodies to WSSV proteins (Okumura et al., 2004)

Table 1.1 Functions of WSSV genes (Sánchez-Martínez et al., 2007)

Type	Function/protein encoded	gene/ORF/PROTEIN	Report
Structural	Envelope	VP19, VP466, VP281	Rout *et al.* (2004), Huang *et al.* (2002), Van Hulten *et al.* (2002)
	Envelope/actin interaction	VP26/VP22	Xie and Yang (2005)
	Envelope/attachment and cell-penetration	VP28/VP27.5	Yi *et al.* (2004)
	Envelope	VP110/*wssv092*	Li *et al.* (2006)
	Nucleocapsid	VP15, VP24	Witteveldt *et al.* (2005), Zhang *et al.* (2004), Van Hulten *et al.* (2001a)
	Nucleocapsid/assembly	VP664/*wssv419*	Leu *et al.* (2005)
	Nucleocapsid	VP35/*wssv019*/ORF687, ORF5	Tsai *et al.* (2004), Chen *et al.* (2002), Lo *et al.* (1999)
		VP136B/*wssv524*	Tsai *et al.* (2004)
	Envelope, cytokine receptor	VP76/ORF112 or 220	Huang *et al.* (2005)
	Actin interaction	VP51C/*wssv364*	Tsai *et al.* (2004)
Functional	Met Prim/ATPase S/ER	VP95/*wssv502*	Tsai *et al.* (2004)
		VP75/*wssv388*	Tsai *et al.* (2004)
		VP73/*wssv275*	Tsai *et al.* (2004)
	Vitellogenin-like	VP60A/*wssv381*	Tsai *et al.* (2004)
		VP60B/*wssv474*	Tsai *et al.* (2004)
		VP55/*wssv051*	Tsai *et al.* (2004)
	Hemocyanin	VP53A/*wssv067*	Tsai *et al.* (2004)
		VP53B/*wssv171*	Tsai *et al.* (2004)
		VP53C/*wssv324*	Tsai *et al.* (2004)
		VP51A/*wssv294*	Tsai *et al.* (2004)
		VP51B/*wssv311*	Tsai *et al.* (2004)
		VP41A/*wssv293*	Tsai *et al.* (2004)
		VP41B/*wssv298*	Tsai *et al.* (2004)
		VP39A/*wssv362*	Tsai *et al.* (2004)
		VP39B/*wssv395*	Tsai *et al.* (2004)
		VP38A/*wssv314*	Tsai *et al.* (2004)
		VP38B/*wssv449*	Tsai *et al.* (2004)
		VP36A/*wssv134*	Tsai *et al.* (2004)
	Photosystem reaction	VP36B/*wssv309*	Tsai *et al.* (2004)
		VP32/*wssv253*	Tsai *et al.* (2004)
	RING-H2 motif/sequester ligase	*wssv249*	Wang *et al.* (2005)
		VP24/*wssv480*	Tsai *et al.* (2004)
		VP13A/*wssv339*	Tsai *et al.* (2004)
		VP13B/*wssv377*	Tsai *et al.* (2004)
		VP12B/*wssv445*	Tsai *et al.* (2004)
		VP12A/*wssv065*	Tsai *et al.* (2004)
		VP11/*wssv394*	Tsai *et al.* (2004)
	dUTPase, Nucleotide metabolism	*wsv112*/*wdut*	Liu and Yang (2005), Li *et al.* (2005a)
	Non-specific nuclease	*wsv191*	Li *et al.* (2005a)
	Nucleotide metabolism	*wsv067, 172, 188, 395*	Yang *et al.* (2001)
	Anti-apoptotic	ORF390	Wang *et al.* (2004)
Temporal	Putative transcription factor	ORF126/*ie1*	Liu *et al.* (2005)
	–	ORF242/*ie2*	Liu *et al.* (2005)
	–	ORF418/*ie3*	Liu *et al.* (2005)
	GTP-binding activity	*wsv447*	Han *et al.* (2007)
Latency	Shrimp phosphatase interact	ORF427	Lu and Kwang (2004)
	Auto-repressor	ORF89/ORF151	Hossain *et al.* (2004)
	Protein kinase	*Pk wssv*	Liu *et al.* (2001)

Nomenclature: Genes appear call in italic letters. Name of proteins appear in capital letters and open reading frames (ORFs) appear according to original reports.

1.5 Rab GTPase

Rab (Ras analog in brain) GTPase is the largest subgroup of Ras superfamily of small GTPase, usually about 25 kDa in size, which involved in a various signal transduction pathways that regulate cell growth, intracellular trafficking, cell migration and also apoptosis. Rab GTPases are key regulators in intracellular trafficking including endocytosis and exocytosis transport processes, control membrane traffic within the cells, identification of cellular compartments, and also involved in membrane fushion (Zerial and McBride, 2001) (Fig. 1.6). More than 60 members of Rab protein was identified in human cells. Rab GTPase works as molecular switches and interact with a wide variety of effectors. When Rab GTPase works together with their effectors, they would result in regulatory function in coordinating the consecutive stages of membrane transport including the formation of transport carriers and their tethering-docking to target membrane (Chavrier and Goud, 1999; Zerial and McBride, 2001). Moreover, several roles in cellular transport processes are sorting function in vesicle formation, motor and motor adaptors in intracellular transport processes, vesicle tethering, and membrane fusion.

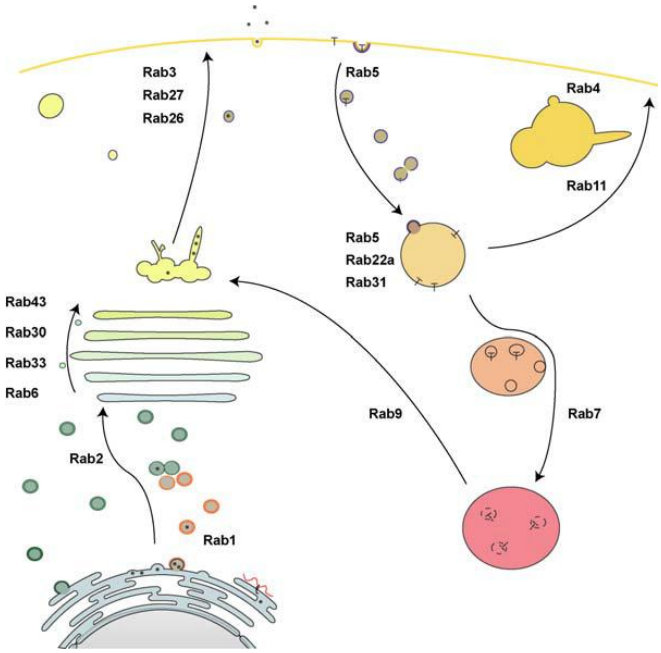


Fig 1.6 Rab proteins provide membrane identity. A selection of human Rab proteins linked to processes and organelles they regulate is depicted (Hass, 2008)

Rab protein works by cycling between an GTP-bound from called “active stage” and GDP-bound called “inactive stage”. Only when Rab proteins are activated, they can interact with downstream effectors. Both stages are regulated by three different classes of regulatory proteins named guanine-nucleotide exchange factors (GEFs), GTPase activating proteins (GAPs), and GDP dissociation inhibitors (GDIs). GEFs promote GDP-GTP exchange of Rab GTPase activity. The GAP proteins acts as negative regulators to Rab GTPase by increasing intrinsic GTPase activity of Rab proteins and therefore inducing the conversion of the active GTP-bound state to the inactive GDP-stage (Clabecq et al., 2000; Haas et al., 2005; Strom et al., 1993; Will and Gallwitz, 2001). The GDI proteins also act as negative regulators by blocking GDP dissociation via interactions with the isoprenylated carboxyterminus of Rab proteins (DerMardirossian and Bokoch, 2005; Pfeffer and Aivazian, 2004). The schematic diagram described brief Rab cycle as in Fig. 1.7. In this study we focus on GAPs of which their details are described as follow.

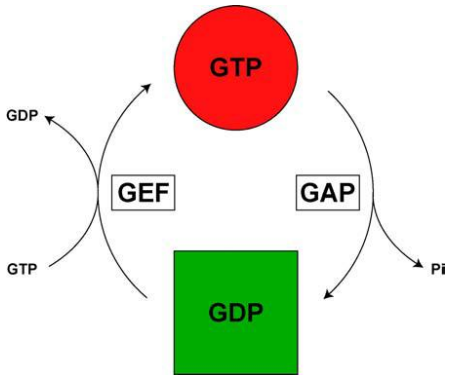


Fig. 1.7 GTPases cycle between an active conformation bound to GTP (red) and an inactive conformation (green) bound to GDP. Hydrolysis of GTP to GDP is stimulated by GAPs. The exchange of GDP to GTP is catalysed by GEFs (Haas, 2008).

1.6 GTPase Activating Proteins (GAPs)

GTPase activators (GAPs) are key regulator of Rab protein. (Haas, 2008) They have an important role in promoting GTP hydrolysis of specific GTPase, then shut the GTPase down. In active state, GTPase which are bound with the membrane promote the assembly of effector complexes which can be cytosolic or membrane protein at the membrane surface. These effectors display various biological functions important for signalling such as vesicle formation and fusion (Barr, 2009). Rab-GAP first indentified in *S.cerevisiae* (Storm, 1993). GYP6 was the first GAP for Ypt6 yeast protein. Many Ypt GAPs were indentified later which can be noticed that these proteins shared a common protein domain. From Tre2 gene of human Oncogene, Bub2 gene of *S.cerevisiae*, and Cdc16 gene of *S.pombe*, this domain was called as TBC domain. (Neuwald, 1997; Richardson et al, 1995) The important amino acid in TBC domain is an Arginine which is required in catalytic of GTP hydrolysis which is similar to RasGAP (Fig. 1.8) (Albert et.al, 1999).

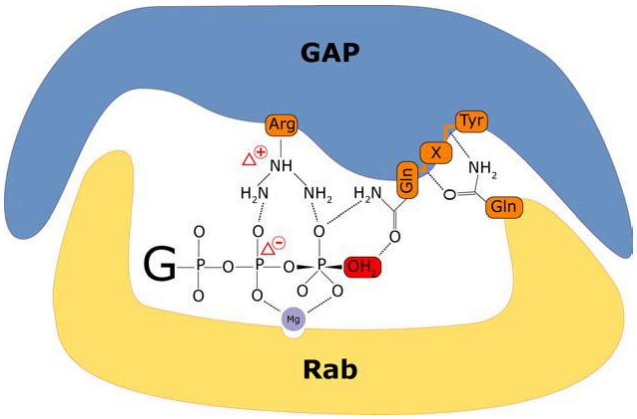


Fig. 1.8 Rab-GAP mediated GTP hydrolysis. This schematic depicts the probable transition state in TBC domain activated GTP hydrolysis. The amino acids involved directly or indirectly in catalysis are shown in orange. They are labelled with the common three letter abbreviations. The lavender sphere indicates the magnesium ion required for nucleotide coordination by the Rab. The letter "G" indicates the guanine nucleotide attacking water molecule in red. Solid lines indicate bonds dashed lines indicate hydrogen bonds or ionic interactions (Haas, 2007).

TBC domain proteins contain an about 200-amino-acid motif initially identified in the TRE2/BUB2/CDC16 genes. (Richardson et al., 1995) TBC domain has 3 signature motifs : Rxxxw in motif A, IxxDxxR in motif B (The Arginine required for GTPase hydrolysis is found in this motif), and YxQ in motif C. These 3 signature motifs are located at the N-terminus of the domain. TBC domain has been proposed the mechanism in catalyzing GTP hydrolysis by dual-finger mechanism (Pan et al., 2006) (Fig. 1.9). Describe briefly, there are 2 amino acid residues involved in this mechanism. A conserve glutamine (Q) in motif C positioning the nucleophilic water molecule required for GTP hydrolysis. A conserve Arginine (R) in Motif B promotes hydrolysis by polarizing the develop partial negatively charge of gamma phosphate of GTP. (Bos et. al, 2007)

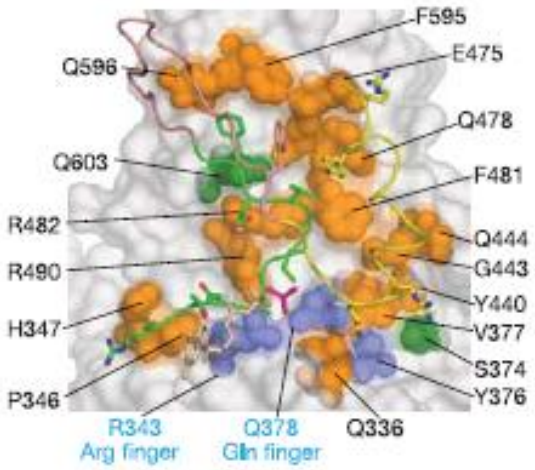


Fig. 1.9 Interaction epitope of TBC domain of Gyp1p protein from yeast with Rab33. Blue area showed the conserved region of dual-finger catalysite site as arginine finger and glutamine finger respectively (Pan et al., 2006).

Like all TBC family members, TBC1D20 contains a Rab-GTPase-activating protein (GAP) homology domain required for regulating the activity of Rab proteins (Barr, 2009; Bernards and Settleman, 2004) as a molecular switch off that hydrolyze the Rab-GTP bound form (active form) back to Rab-GDP bound form (inactive form). Rab GTPases are central elements in endocytic membrane trafficking, organizing

effector proteins into specific membrane subdomains, as well as in excocytic membrane trafficking in eukaryotic cell (Armstrong, 2000; Rink et al., 2005; Takai et al., 2001). In *C.elegans*, TBC domain played roles in cytokine systems and innate immunity (Alper et al., 2008). In human, TBC1D20 was found as the protein that mediated Hepatitis C virus replication by binding to viral NS5A protein involed in replicating the viral genome. Depleteion of TBC1D20 transcript severly impaired HCV replication and prevent new infection, with minimal effects on cell viability (Sklan et al., 2007a). Later that, TBC1D20 was found as a Rab-GTPase-activating protein for Rab1 GTPase and located in the Endoplasmic Reticulum (ER) (Sklan et al., 2007b). Rab1 GTPase regulates anterograde transport of cargo between the ER and the Golgi apparatus (Barnekov et al., 2009). Thus, TBC1D20 is the GAP that blocking the delivery of secretory cargo from the ER to the cell surface (Haas et.al, 2007).

However, there is no report of TBC1D20 in crustaceans. There are only few studies of GTPases in shrimp such as in kuruma shrimp, *Marsupeneaus japonicas*. *M. japonicas* Rab GTPase (*Pj*Rab) which similar to Rab6 GTPase was up-regulated when infected by the virus, suggesting that the *Pj*Rab protein might play an important role in shrimp immune response against virus infection (Wu and Zhang, 2007). Rab GTPase from Chinese white shrimp, *Fenneropenaeus chinensis* also showed response to WSSV infection. Moreover, recombinant protein of Rab GTPase from Chinese white shrimp could interact with VP28 which was an envelope protein of WSSV. This sugguested the probable roles of Rab GTPase in shrimp innate immunity (Ren et al., 2012)

1.7 Aim of study

The EST clone homologue of TBC1D20 was up-regulated at 6 h after WSSV infection by microarray analysis. Therefore, the aim of this study is to characterize and evaluate its role in WSSV infection. The full-length sequence of this gene is analyzed by Rapid Amplification of cDNA Ends (RACE) technique. The WSSV response gene is investigated by semi-quantitative RT-PCR and real-time PCR. The effect of *Pm*TBC1D20 depletion upon WSSV challenge is explored. In addition, localization of *Pm*TBC1D20 protein is also demonstrated in this study.

1.8 Techniques used in this study

1.8.1 Reverse transcription-polymerase chain reaction (RT-PCR)

RT-PCR is a rapid and quantitative method in analyzing the level of expression of gene. It is a highly sensitive and specific method useful for the detection of rare transcripts or using for analysis of samples available for limited amounts (Cardings et al., 1992 and Marone et al., 2001). The RNA cannot be used directly as a template for PCR, so complementary DNA (cDNA) is synthesized from RNA which can be performed PCR. The combination of both techniques is colloquially referred to RT-PCR (Fig. 1.10). The necessity to reverse transcribes mRNA into a cDNA prior to subjecting the RNA template to PCR is given by the fact that the polymerase used in PCR is a DNA-dependent polymerase. Reverse transcription of mRNA requires choosing a reverse transcriptase, a means of priming the mRNA to initiate polymerization and supplying optimal condition for the enzymatic reaction. Reverse transcriptase are RNA-dependent DNA polymerases which have been used predominantly to catalyze first strand synthesis (synthesis of a complementary DNA-cDNA), but are also capable of synthesizing a DNA strand complementary to a primed single stranded DNA. The RT-PCR can be used for semi-quantitative also known as relative or quantitative analysis.

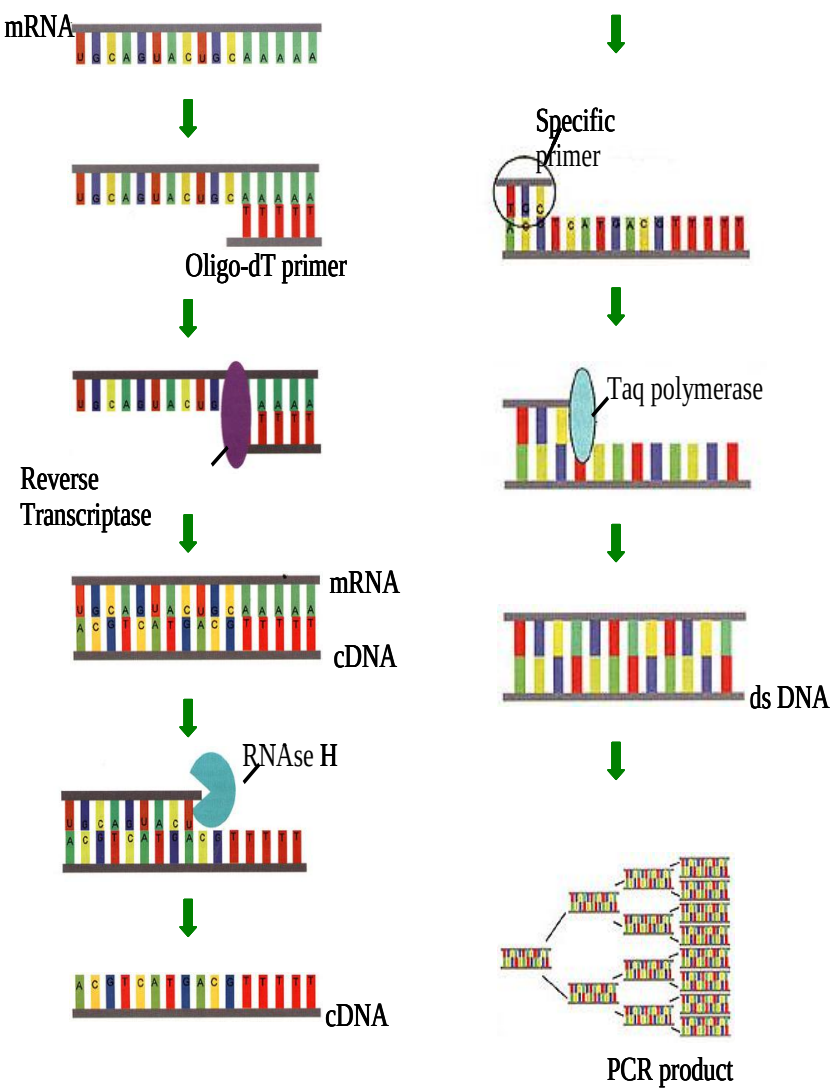


Fig. 1.10 Overview of the RT-PCR technique

(Source:http://ccm.ucdavis.edu/cpl/Tech%20updates/RTPCR%20folder/RTPCRweb.jpg)

The semi-quantitative RT-PCR method is based on the used of an internal control which is included in the polymerase chain reaction with the gene specific primers. In the majority of cases, the internal control is a housekeeping gene expressed at a very high level, which is assumed to be expressed at a constant level throughout all samples analyzed. Also, it is assumed that the expression levels of the control RNA are not changed by the experimental conditions, thus acting as an experimental control. Common internal controls are β-actin, elongation factor 1 alpha (EF-1α), and 18s rRNA. The PCR products, including the internal control, are then separated by agarose gel electrophoresis, stained with Ethidium Bromide and analyzed to observe relative expression of the target transcript. However, semi-quantitative RT-PCR is only able to compare either higher or lower expressed transcripts of interested transcripts among samples. The semi-quantitative RT-PCR method is one of generally methods that use for confirmation of differential expression from gene analysis. Since, this method is highly sensitive and specific method for the detection of rare transcript or for analysis of samples available for limited amounts.

1.8.2 Quantitative real-time polymerase chain reaction (qrt-RT-PCR)

Quantitative real-time polymerase chain reaction, also called kinetic polymerase chain reaction (KPCR), is a technique based on the conventional polymerase chain reaction (PCR), which is used to amplify and simultaneously quantify a targeted DNA molecule. For one or more specific sequences in a DNA sample, qrt-RT-PCR enables both detection and quantification. The quantity can be either an absolute number of copies or a relative quantitation when normalized to reference DNA or additional normalizing genes. Frequently, real-time PCR is combined with reverse transcription to quantify messenger RNA which is reverse-transcripted to cDNA. The success of this technique comes from the development of PCR products determination in the solution which is using of fluorescence reporters and the development of the real time thermocycler machines to detect the fluorescence by adding the light source to stimulate the emission of PCR products and the florescence detector to detect the PCR product in the reaction.

The procedure follows the general principle of polymerase chain reaction that the amplified DNA is detected as the reaction progresses in real time. This is more accuracy and easier compare to conventional PCR which the product of the reaction is only detected at its end.

Two common methods for detection of products in real-time PCR are: (1) non-specific fluorescent dyes that intercalate with any double-stranded DNA, and (2) sequence-specific DNA probes consisting of oligonucleotides that are labeled with a fluorescent reporter which permits detection only after hybridization of the probe with its complementary DNA target.

The most common method for calculating the relative quantitation is the $2^{-\Delta\Delta CTCT}$ method (Livak and Schmittgen, 2001). This method relies on two assumptions. The first is that the reaction is occurring with 100% efficiency.. This assumption is also one of the reasons for using a low cycle number when the reaction is still in the exponential phase. In the initial exponential phase of PCR, substrates are not limiting and there is no degradation of products. In practice, this requires setting the crossing threshold (CT) at the earliest cycle possible. The CT is the number of cycles that it takes each reaction to reach an arbitrary amount of fluorescence. The second assumption of the $2^{-\Delta\Delta CT}$ method is that there is a gene (or genes) that is expressed at a constant level between the samples. This endogenous control will be used to correct for any difference in sample loading. The equation is in Fig. 1.11

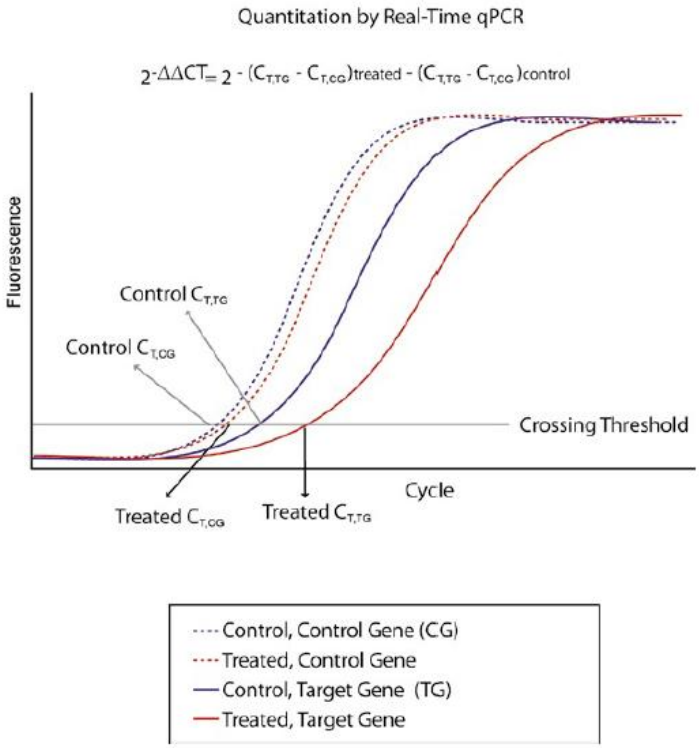


Fig. 1.11 Mathematical basis of the 2-ΔΔCT method. The 2-ΔΔCT method enables relative quantitation (treated sample is X fold of control sample) through measurements of crossing thresholds (CT). As described in the text, the comparative differences between the gene of interest and an endogenous controlfor each sample enable a relative quantitative comparison between the samples. Note that the endogenous control genes (dotted lines) do not vary significantly in CT with treatment. While this is an assumption of the method, it can be empirically tested by the experimenter (VanGuilder et al., 2008).

1.8.3 RNA interference technique (RNAi)

RNA interference (RNAi) or RNA-based gene silencing is a process within living cells that moderates the activity of their gene. This pathway was first discovered in Tobacco (Matzke et al., 1989), but it was known by other names, including co-suppression, post transcriptional gene silencing (PTGS), and quelling. Only after these apparently unrelated processes were fully understood make it become clear that they all described the RNAi phenomenon. The RNAi interference pathway and its effect of dsRNA involed in gene silencing was clearly desecribed in the nematode worm, *Caenorhabditis elegans* (Fire et al., 1998), which later got the Nobel Prize in Physiology or Medicine in 2006. It soon turns out that RNAi and post-translational gene silencing mechanism can be found in various eukaryote organisms such as fungi, insects, and vertebrates (Mello and Conte, 2004).

Two types of small RNA molecules, microRNA (miRNA) and small interfering RNA (siRNA), are central to RNA interference. RNAs are the direct products of genes, and these small RNAs can bind to other specific messenger RNA (mRNA) molecules and either increase or decrease their activity, for example by preventing an mRNA from producing a protein. RNAi is mediated by small interfering RNAs (siRNA) that are generated from long dsRNAs of exogeneous or endogenous orgin. Long dsRNAs are clevaged by ribonuclease III (RNase III) type protein dicer. Dicer homologues can be found in *C.elegans*, Drosophila, plants, and mammals, suggesting that small RNA-mediated regulation is evolutionarily ancient and may have critical biological roles (Elbashir et al., 2001; Agrawal et al., 2003; Bernstein et al., 2003). The siRNA generated by Dicer is an about 22-nucleotides short RNA with 2 bases overhang at each 3´ end. Each strand contains a 5´ phosphate group and a 3´ hydroxyl group. The siRNA is incorporated into a nuclease complex called RISC (RNA Induced Silencing Complex) that targets and cleaves mRNA that is complementary to the siRNA. The initial RISC containing a siRNA duplex is still inactive until it is transformed into an active form (RISC) which involves loss of one strand of the duplex by an RNA helicase activity (Fig. 1.12). The identity of the RNA helicase is currently unknown. Dicer has a conserved helicase domain but it remains to be seen whether dicer actually catalyzed this reaction.

RNA interference has an important role in defending cells against parasitic genes, viruses, and transposons but also in directing development as well as gene expression in general. Because of this exquisite specificity and efficiency, RNAi has drown much more attention as a powerful gene knockdown technique.

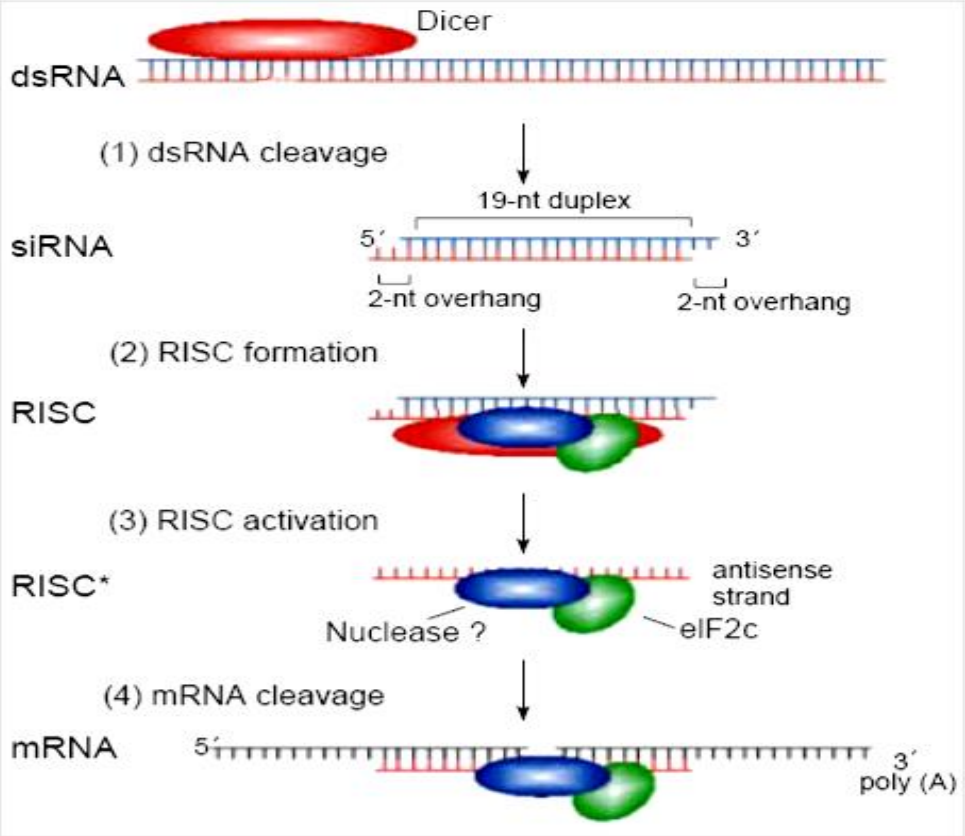


Fig. 1.12 Current models for RNA interference**.** RNAi process can be divided into four stages: (1) dsRNA cleavage by Dicer and generation of siRNA duplex, (2) recruitment of RNAi factors and formation of RISC (RNA-induced silencing complex), (3) siRNA unwinding and RISC activation and (4) mRNA targeting and degradation (Kim, 2003)

1.8.4 Rapid Amplification of cDNA Ends (RACE)

Rapid Amplification of cDNA ends (RACE) is a procedure for amplification of nucleic acid sequences from an mRNA template between a defined internal site and unknown sequence at either the 5′ or the 3′ ends to obtain the full length sequence of an mRNA transcript. RACE results in the production of a cDNA copy of the RNA sequence of interest, produced through reverse transcription, followed by PCR amplification of the cDNA copies. The amplified cDNA copies are then sequenced and, if long enough, should map to a unique mRNA already described, the full sequence of which is known. RACE procedures have been used for amplification and cloning of rare mRNAs that may escape or prove challenging for conventional cDNA cloning methodologies. Additionally, RACE may be applied to existing cDNA library. Random hexamer-primer cDNA has also been adapted to 5′ RACE for amplification and cloning multiple genes form a single first strand synthesis reaction. Products generated by the 5′ and 3′ RACE procedures may be utilized in conjution with exon trapping methods to enable amplification and subsequent characterization of unknown coding sequences.

In RACE-PCR, gene specific primer (GSP) designed from the known mRNA sequence was used together with a general primer complementary either to the mRNA poly A tail for 3′-RACE or to a homopolymer added to the 3′-end ofthe cDNA for 5′-RACE. Because homopolymers do not make good PCR primers, and to facilitate cloning of RACE products, the general primers contain a sequence with a restriction endonuclease site at their 5′-end. The cDNA template for the PCR may be produced either using an oligo-dT primer (for 3′- or 5′-RACE) or using a primer complementary to the known sequence within the mRNA (for 5′-RACE only). Where RACE gives a mixture of products, an aliquot of this mixture may be used as the template for another PCR using a second gene specific primer, GSP2 (nested within the first) and the original general primer (nested RACE).

CHAPTER II
MATERIALS AND METHODS

2.1 Materials

2.1.1 Equipments

-20 °C Freezer (Whirlpool)

-80 °C Freezer (Thermo Electron Corporation)

96 well cell culture cluster, flat bottom with lid (Costar)

Amicon Ultra-4 concentrators (Millipore)

Autoclave model # MLS-3750 (SANYO E&E Europe (UK Branch) UK Co.)

Automatic micropipette P10, P20, P100, P200, and P1000 (Gilson Medical Electrical)

Balance PB303-s (Mettler Teledo)

Biological safty cabinets (Nuaire)

Biophotometer (Eppendrof)

Centrifuge 5804R (Eppendrof)

Centrifuge Avanti™ J-301 (Beckman Coulter)

Gel document (Syngene)

GelMate2000 (Toyobo)

Hitrap SP HP (Amersham Biosciences)

Hoefer™ miniVE (Amersham Biosciences)

Incubator 37 °C (Memmert)

Incubator 30 °C (Memmert)

Microcentrifuge tubes 0.6 ml and 1.5 ml (Bio-RAD Laboratories, USA)

Microscope eclipse TS100 (Nikon)

Minicentrifuge (Costar, USA)

Nipro disposable syringes (Nissho)

PCR thin wall microcentrifuge tubes 0.2 ml (Perkin Elmer)

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

pH meter model # SA720 (Orion)

Pipette tips (10, 20, 200, and 1000 µl (Axygen)

Power supply: Power PAC 300 (Bio-RAD Laboratories)

iCycler iQ™ Real-Time Detection system (Bio-Rad, USA)

CX31 Biological Microscope (Olympus)

Inverted microscope (Nikon)

Refigerated incubator shaker (New Brunswick Scientific)

Sterring hot plate (Fisher Scientific)

Thermal cycler mastercycler gradient (Eppendrof)

Touch mixer model # 232 (Fisher Scientific)

Vacuum pump (Bio-RAD Laboratories, USA)

2.1.2 Chemicals and reagents

0.45 µm millipore membrane filter (Millipore)

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

2-(*N*-morpholino)ethanesulfonic (Sigma)

2-Mercaptoethanol, C_2H_6OS (Fluka)

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

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

Absolute ethanol, C_2H_5OH (BDH)

Acetic acid glacial, CH_3COOH (BDH)

Acrylamide, C_3H_5NO (Merck)

Agarose (Sekem)

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

Amplicilin (Biobasic)

Bacto agar (Difco)

Bacto tryptone (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 R-250, $C_{45}H_{44}N_3O_7S_2Na$ (Sigma)

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

Dipotassium hydrogen phosphate, K_2HPO_4 (Ajax)

Ethidium bromide (Sigma)

Ethylene diamine tetraacetic acid (EDTA)

Formaldehyde, CH_2O (BDH)

GeneRuler™ 100bp DNA ladder (Fermentas)

Glucose (Merck)

Glycerol, $C_3H_8O_3$ (BDH)

Hydrochloric acid, HCl (Merck)

IPTG (Fermentus)

Isoamylalcohol, $C_5H_{12}O$ (Merck)

Isopropanol, C_3H_7OH (Merck)

Magnesium chloride, $MgCl_2$ (Ajax)

Magnesium sulfate, $MgSO_4$ (Carlo Erba)

Methanol, CH_3OH (Merck)

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

Phenol crystals, C_6H_5OH (Carlo Erba)

Phenol:chloroform:isoamyl alcohol (Sigma)

Potassium chloride, KCl (Ajax)

Potassium dihydrogen phosphate, KH_2PO_4 (Ajax)

Prestained protein molecular weight marker (Fermentus)

Sodium acetate, CH_3COONa (Merck)

Sodium chloride, NaCl (BDH)

Sodium citrate, $Na_3C_6H_5O_7$ (Carlo Erba)

Sodium dodecyl sulfate (Sigma Chemical Co., USA)

Sodium hydroxide, NaOH (Eka Nobel)

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

TRI Reagent® (Molecular Research Center)

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

Tryptic soy broth (Difco)

Unstained protein molecular weight marker (Fermentus)

Whatman 3 MM™ filter paper (Whatman)

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

2.1.3 Enzymes

*Bam*HI (Biolabs, UK)

DNaseI (Promega)

*Hind*III (Biolabs, UK)

*Not*I (Biolabs, UK)

Phusion hot start Pfu polymerase (Finzyme)

RBC *Taq* DNA polymerase (RBC Bioscience)

RNase A (Sigma)

RQ1 RNase-free DNase (Promega)

T4 DNA ligase (New England Biolabs)

2.1.4 Microorganisms

E. coli strain XL-1 blue

E. coli strain Rosetta (DE3)

White spot syndrome virus

2.1.5 Kits

High-Speed Plasmid Mini Kit (Geneaid)

NucleoSpin® Extract II kit (Macherey-Nagel)

RevertAid™ First Strand cDNA Synthesis Kits (Fermentas)

SMART™ RACE Amplification kit (BD)

SYBR® Green (Bio-Rad, USA)

T & A Cloning vector kit (RBC)

T7 RiboMAX(TM) Express RNAi System (Promega)

2.1.6 Vectors

pET19b(+) (Novagen)

T&A cloning vector (RBC)

2.2 Software

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

ClustalX (Thompson et al., 1997)

Genesnap (SYNGENE)

Genetools (SYNGENE)

GENETYX version 7.0.3 program (Software Development Inc.)

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

SECentral (Scientific & Education software)

SignalP (http://www.cbs.dtu.dk/services/SignalP/)

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

TMHTMM (http://www.cbs.dtu.dk/services/TMHMM/)

2.3 Samples

2.3.1 Shrimp samples

Shrimps were obtained from a local shrimp farm in Thailand and acclimatized in laboratory aquaria, at a temperature of 28 ± 4 °C and a salinity of 20 ppt, for at least 7 days before use in experiment. Healthy sub-adult *P. monodon* shrimps of about 16 g body weight were used in every experiments except in the RNAi experiments, *P. monodon* weight of 3 - 5 g were purchased from local farms in Thailand, and were maintained as above.

2.3.2 Preparation of white spot syndrome virus

The hemolymph was collected from WSSV-infected *P. monodon* and mixed with TNE buffer (50mM Tris–HCl, 400mM NaCl, and 5mM EDTA, pH 8.5) in the ratio 1:1. The mixed solution was centrifuged at 3,500 x *g* for 5 min at 4 ºC to gain the supernatant. After centrifugation of the supernatant at 30,000 x *g* for 30 min at 4 ºC, the pellet was washed with TM buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, pH 7.5) and continued centrifugation at 3,500 x *g* for 5 min at 4 ºC. The pellet was collected and suspened in TM buffer. Then, the supernatant was harvested using centrifugation at 30,000 x *g* for 30 min at 4 ºC and dissolved in TM buffer to split the aliquots and stored at -80 °C until used

2.4 Identification and characterization of the full-length cDNA of *Pm*TBC1D20 gene by Rapid Amplification of cDNA Ends (RACE)

Rapid Amplification of cDNA Ends (RACE) technique is used widely for the preparation of full-length cDNA. *Pm*TBC1D20 gene from EST library is lack of start and stop codon. So, both 5´ RACE and 3´ were carried out to obtain full-length sequence of *Pm*TBC1D20 gene

2.4.1 First-stranded RACE-cDNA library synthesis

The first-stranded RACE-cDNA was synthesized according to the manufacturer's instructions (Fig.) Describe briefly, two µg of mRNA obtained from shrimp hemocyte were mixed with SMART II™ A oligonucleotide and 5´-RACE CDS primer for 5´ RACE cDNA library and mixed with 3´-RACE CDS primer which is a special oligo(dT) for 3´ RACE cDNA library. The mixtures were preheated for 10 min at 80 ˚C and immediately chilled on ice for 10 min. The first strand cDNA was

synthesized using Powerscript reverse transcriptase at 42°C for 2 h in a hot-lid thermal cycler. The reaction contains 1x First strand buffer (250 mM Tris-HCl, pH8.3, 375 mM KCl and 30 mM $MgCl_2$), 20 mM DTT, 10 mM dNTP MIX and 1 µl of BD powerscript™ Reverse transcriptase. Finally, the RACE cDNA library were diluted with 250 µl of Tricine-EDTA buffer (10 mM Tricine-KOH pH 8.5, and 1 mM EDTA) and heated at 72 ◦C for 10 min. The 5´ and 3´ cDNA libraries were kept at -20 ◦C.

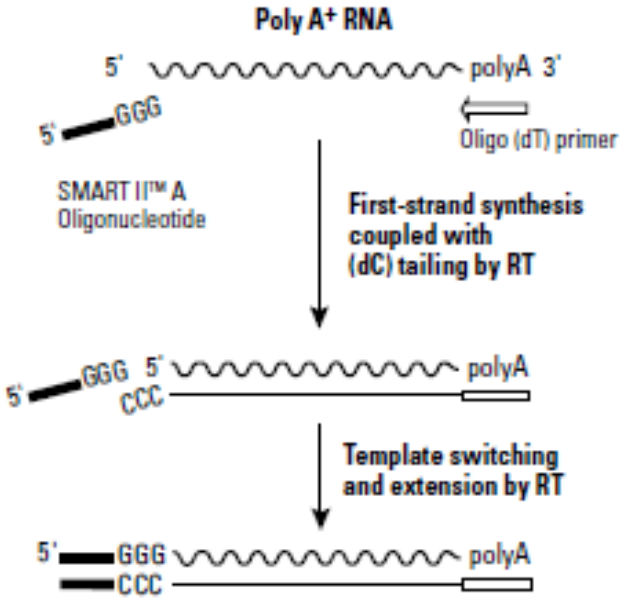


Fig. 2.1 First stand RACE-cDNA library synthesis procedure according to the SMART™ RACE Amplification kit (Clontech)

2.4.2 *Pm*TBC1D20 gene specific primer design

For *Pm*TBC1D20 gene, the 5´ and 3´ gene specific primers were designed from EST library sequence of *Pm*TBC1D20 singleton (HC-N-N01-2807) from hemocyte cDNA library of *P. monodon*. (http://pmonodon.biotec.or.th). SECentral program (Sciectific & Educational Software) was used to design *Pm*TBC1D20 gene specific primers (GSP) as listed in the table 2.1. The gene specific primers were designed to produce overlapping RACE products as shown in Fig. 2.2.

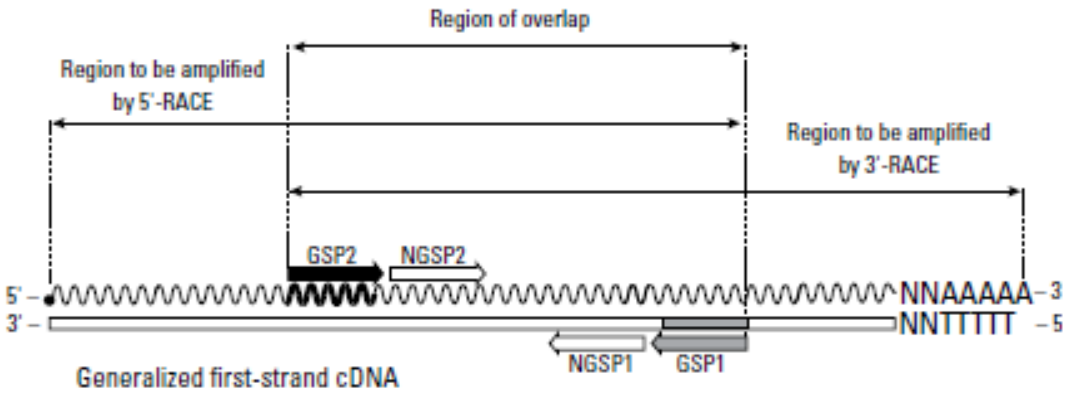


Fig. 2.2 Schematic diagram of specific primer design according to the SMART™ RACE Amplification kit (Clontech)

Table 2.1 Lists of Primers

Primer name	Sequence (5´→3´)
5R6817	AAGACCGCCATCGCCAATTGCAAGTT
3R6817	TGCGGCCAACAATGGAGAAGACCACA
Sg8917real_F	AGTCAGCGTTTAGAGAGGTT
Sg6817real_R	GCTCGAACTCTCCACTCTC
EF-1F	GGTGCTGGACAAGCTGAAGGC
EF-1R	CGTTCCGGTGATCATGTTCTTGATG
TBC_F	CCATGGGCCATCATCATCATCATCAC-GACGAGTTACGCCGCAA
TBC_R	GGATCCTAG-ATCTTCTTTCTTGTGTAAAACAA
Sg6817_F	CAATTGGCGATGGCGGTCTT
Sg6817_R	TGTTGGCCGCATGAACTCAC
Sg6817_FT7	GGATCCTAATACGACTCACTATAGGCAATTGGCGATGGCGGTCTT
Sg6817_RT7	GGATCCTAATACGACTCACTATAGGTGTTGGCCGCATGAACTCAC
GFP_F	ATGGTGAGCAAGGGGGAGGA
GFP_R	TTACTTGTACAGCTCGTCCA
GFP_FT7	GGATCCTAATACGACTCACTATAGGATGGTGAGCAAGGGGGAGGA
GFP_RT7	GGATCCTAATACGACTCACTATAGG TTACTTGTACAGCTCGTCCA
VP28F	TCACTCTTTCGGTCGTGTCG
VP28R	CCACACACAAAGGTGCCAAC
VP28Q_F	GGGAACATTCAAGGTGTGGA
VP28Q_R	GGTGAAGGAGGAGGTGTTGG

2.4.3 Rapid amplification of cDNA ends-polymerase chain reaction (RACE-PCR)

The 5´ and 3´ RACE libraries were used as template for PCR to determine the full-length sequence of *Pm*TBC1D20 gene using gene specific primer sg6817F and 3R_6817. The 5´ RACE- PCR reaction was performed using Advantage® 2 polymerase Mix (Clontech). The 50 µl of RACE-PCR was consisted of 1x PCR buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl, 0.001% gelatin), 1.5mM $MgCl_2$, 200 µM of dNTP mix , 0.4 µM of RACE gene specific primers, 5 µl of 10x UPM and 2.5 µl of 5´ or 3´ RACE cDNA library template. PCR condition were as follow: For 5´ RACE, 5 cycles of 94 ˚C for 30 sec and 72 ˚C for 3 min, 5 cycles of 94 ˚C for 30 sec, 70 ˚C for 30 sec, and 72 ˚C for 3 min and 25 cycles of 94 ˚C for 30 sec, 68 ˚C for 30 sec and 72 ˚C for 3 min.

The nested PCR strategy was performed to increase specificity using NUP and nested specific primer (5R6817 for 5´ RACE and N_3R6817 for 3´ RACE) (Table 2.1). The RACE product was diluted 1:50 dilution before used as template for the nested PCR. The PCR condition was as followed: 25 cycles of 94 ˚C for 30 sec, 68 ˚C for 30 sec, and 72 ˚C for 3 min

The PCR product was analyzed by 1.5% agarose gel electrophoresis and the specific bands of interest were then purified from agarose gel using NucleoSpin® Extract II kit (Clontech) according to the manufacturer's instructions. The purified fragment is cloned into the RBC T & A Cloning Vector (RBC Bioscience) and then transformed into *E.coli* XL1-blue by heat shock, followed by the standard blue/white screening method. The recombinant plasmid was isolated from the positive clone using High-Speed Plasmid Mini Kit (Geneaid) according to the manufacturer's instructions and sequenced using T7 promoter and M13 reverse primer at Macrogen INC., South Korea.

2.4.4 Competent cell preparation

A single colony of *E. coli* XL-1 blue was cultured in 10 ml of LB broth (1% (w/v) bacto tryptone, 0.5% (w/v) bacto yeast extract, and 1% (w/v) NaCl) to prepare the starter inoculum. The culture was incubated at 37 °C with shaking at 250 rpm overnight. One percent of starter culture was inoculated into 1 L of LB broth and incubated at 37 °C with vigorous shaking for 2-3 h until OD_{600} of the cells reached

0.4-0.6. Cells were then incubated on ice for 10 min and harvested by centrifugation at 5,000 rpm for 5 min at 4 °C. The supernatant was eliminated as much as possible. Cell pellet was washed with cold 10 mM $CaCl_2$ in a total volume of 500 ml, and then centrifuge at 5,000 rpm for 5 min at 4 °C. The pellet was resuspended in a total volume of 50 ml of cold 10 mM $CaCl_2$, chilled on ice for 30 min. This cell suspension was divided into 100 µl aliquots for immediately used or mixed with 60% (v/v) glycerol to a final concentration of 15 % glycerol before divided into 100 µl aliquots and stored at -80 °C until used.

2.4.5 Cloning of the amplified genomic DNA fragments

The DNA fragment was ligated into the T&A cloning vector (Fig. 2.3). The reaction component contained 1 µl of each 10x Rapid A and B ligation buffers, 50 ng of T&A cloning vector, proper amount of PCR product, 1 µl of T4 DNA ligase (3 units/ µl) and distilled water to a final volume of 10 µl. The reaction was mixed, quickly spun and incubated at 4 °C overnight. The amount of inserted DNA fragment was calculated by using the equation as follow :

$$\text{ng of insert} = \left[\frac{\text{ng of vector} \times \text{kb size of insert}}{\text{kb size of vector}}\right] \times \text{insert:vector molar ratio}$$

2.4.6 Calcium chloride transformation

The ligation reaction was transformed into an *E. coli* XL-1 blue using $CaCl_2$ method. The aliquot competent cells were gently thawed on ice, mixed with 5-10 µl of ligation mixture and then chilled on ice for 30 min. The mixture was incubated at 42 °C for 1 min and added 0.9 ml of LB broth. The reaction was incubated at 37 °C with shaking at 250 rpm for 1 h. The mixture reaction was spread onto a LB agar plate containing 100 µg/ml of amplicillin, 20 µg/ml of X-gal and 30 µg/ml of IPTG and then incubated at 37 °C for overnight. After incubation, the recombinant clone was identified by colony PCR using universal T7 and M13R primers.

2.4.7 Screening of transformant by colony PCR

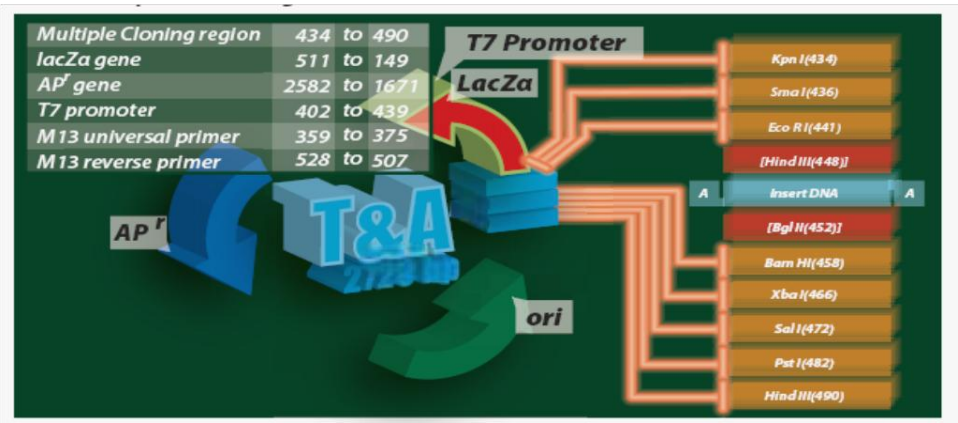
T7 and M13R primers were used to amplify and analyze the DNA insert cloned size into the multiple cloning regions. Colony PCR was carried out in a 25 µl reaction volume including 1× reaction buffer (10 mM KCl, 2 mM $MgSO_4.7H_2O$, 20 mM Tris-HCl pH 8.8, 10 mM $(NH_4)_2SO_4$, 0.1 mg/ml BSA, 0.1% (v/v) Triton X-100 and 2 mM $MgCl_2$), 0.2 mM of each dNTP, 0.2 mM of each primer, 0.7 unit of RBC Taq DNA Polymerase (RBC Bioscience). For the DNA template, white colonies were picked and resuspended in the reaction mixture. The PCR profile was performed at 94ºC for 3 min, 30 cycles at 94 °C for 30 sec, 55 °C for 30 sec, 72 °C for 1 min and a final extension at 72 °C for 7 min. The PCR products were analyzed by 1.0% agarose gel electrophoresis. The clones that contain an expected size of insert were selected to isolate the recombinant plasmid.

2.4.8 Recombinant plasmid preparation

A positive colony was inoculated into 2 ml of LB broth and incubated at 37 °C with shaking at 4 °C overnight. The culture was spun at 8,000 rpm for 3 min and collected the pellet to isolate and purify the plasmid using High-Speed Plasmid Mini Kit (Geneaid) described the handbook. This kit is based on modified alkaline lysis method and RNase treatment to obtain clear lysate of bacterial cutured cells. The overnight cultured bacteria cells was resuspended in 200 µl of PD1buffer containing RNase A, then lysis buffer, PD2, was added and mixed gently by inverting the tube 4-6 times and stand at room temperature for 2 minutes or until the lysate is homologus. Then, 300 µl of PD3 buffer was added and mixed immediately and thoroughly by inverting the tube 10 times. After centrifugation at 13,000 rpm for 10 min, the supernatant was transfer to column. plasmid DNA in the supernatant binded to the galss fiber matrix of the spin column. The column was centrifuged for 1 min, and then the flow-through was discarded. The spin column was pre-washed by adding 400 µl of W1 buffer and centrifuged for 1 min, then the flow-through was discarded. Later that, the column was washed with 600 µl of Wash buffer and centrifuged twice to discard the flow through. After that, the column was placed in a new 1.5 ml microcentrifuge tube. The plasmid DNA was eluted by adding 50 µl of Elution buffer buffer to the center of each column. After incubation at room temperature for 2 min, the DNA eluted fraction was collected by centrifugation for 2 min.

The recombinant plasmid containing interested gene was examined with restriction enzyme digestion using *Eco*RI and *Bam*HI. The digested plasmid was analyzed by agarose gel electrophoresis. The size of DNA fragment was compared with standard DNA ladder (100 bp ladder marker). The recombinant plasmid was sequenced by the commercial service (Macrogen Inc., Korea).

(A)



(B)

Fig. 2.3 The circular map of the T&A Cloning vector (A) and the linear map that represent the multiple cloning site sequences (B). (Source: T&A Cloning vector kit User Manaul: RBC)

2.4.9 Sequence analysis

Partial cDNA sequence of *Pm*TBC1D20 gene was identified in *P. monodon* EST database (http://pmonodon.biotec.or.th) and translated using the GENETYX 7.0.3 program. The cDNA sequence and deduced amino acid sequence of this gene were analyzed using the NCBI BLAST programs in GenBank. The signal peptide and domain identification were analyzed by SignalP 3.0 program (http://www.cbs.dtu.dk/services/SignalP/), and SMART database (http://smart.embl-heidelberg.de/), respectively. Related deduced amino acid sequences of the TBC domain protein in various organisms were aligned using ClustalX program.

2.5 Gene expression analysis of *Pm*TBC1D20

After obtaining the full-length sequence of *Pm*TBC1D20gene, gene expression analysis was performed to investigate the expression phenomena in various tissues of *P. monodon* shrimp and transcriptional expression levels changed in hemocyte when infected the WSSV virus compared to uninfected state.

2.5.1 Tissue distribution of *Pm*TBC1D20 gene

2.5.1.1 Shrimp tissue collection and total RNA preparation

Hemocyte was collect using 10% Sodium citrate as anti co-agulant. Other tissues including eye stalks, lymphoid organ, hepatopancreas, antennal gland, gill, heart, epipodite, and intestine were collected. The hemocyte was immediately centrifuged at 800 ×g for 10 min at 4 ^{o}C to separate the hemocytes from the hemolymph. The harvested hemocyte pellet was then immediately resuspended in 1 ml of TRI Reagent® (Molecular Research Center) and briefly homogenized. Other tissues were frozen by liquid nitrogen before homogenized in 1 ml of TRI Reagent. The homogenate was incubated at room temperature for 5 min to completely separate of nucleoprotein complexes. Then 0.2 ml of chloroform was added into the sample and vigorously shaken for 15 sec. The mixture was incubated at room temperature for 2-5 min and centrifuged at 12,000 × *g* for 15 min at 4 °C resulting two phases, a lower red of phenol chloroform phase and colorless aqueous phase. The RNA remains in the aqueous phase that was transferred into a fresh 1.5 ml centrifuge tube and the same volume of isopropanol was added to precipitate total RNA. The sample was stored at room temperature for 10 min and centrifuged at 12,000 ×*g* for 15 min at 4 °C for

removing the supernatant. The RNA pellet was washed with 1 ml of ice-cold 75% ethanol and centrifuged at 12000 ×g for 15 minutes at 4 °C then discard the supernatant. The RNA pellet was shortly air-dried for 10 min, and dissolved with an appropriate amount of diethyl pyrocarbonate (DEPC)-treated water and kept at -80 °C until used.

The total RNA was further treated with 1 µl of RNase-free DNase (Promega, 1 unit/µl) at 37 °C for 30 min to eliminate the contaminated DNA and extracted again with TRI Reagent as described above. The RNA pellet was air-dried and resuspended in DEPC-treated water.

The concentration of total RNA was determined by measuring the OD_{260} and estimated in ng/µl using the following equation:

$$[\text{RNA}] = OD_{260} \times \text{dilution factor} \times 40$$

Whereas, one OD_{260} corresponds to approximately 40 ng/µl of RNA (Sambrook et al., 1989). The relative purity of RNA samples was investigated by measuring the ratio of $A_{260/280}$. The maximum absorption of protein is at 280 nm. The good quality of RNA sample should have an $OD_{260/280}$ ratio above 1.7.

The quality of the extracted RNA was analyzed using 1.2% (w/v) formamide agarose gel electrophoresis that prepared in 1× MOPS buffer (20 mM of 3-(N-morpholino) propanesulfonic acid (MOPS), 5 mM sodium acetate, 1mM EDTA, and adjust to pH 7.0 with NaOH). The agarose slurry was melted by a microwave oven until completely dissolving and placed to cool down at room temperature to 60 °C. Then adding the formalmyde and formaldehyde and poured the solution into a casting tray with a well-forming comb rinse with 3% H_2O_2, absolute Ethanol and DEPC-treated water respectively. The gel was immersed in a chamber that holds enough amount of 1× MOPS buffer for covering the gel.

Approximate 200 ng of the total RNA was mixed with one-sixth volume of 6x loading dye (0.25% bromophenol blue, EDTA, formamide, formaldehyde, 2.5% ethidium bromide (EtBr), 30% glycerol in DEPC-treated water and 1X MOPS) before loading into the well. A RNA marker was used as the standard markers. Electrophoresis was carried out in 1× MOPs buffer at 100 volts about 40 min that the

bromophenol blue front was migrated to approximately ¾ of the length. The gel was destained in a deionized water for 1 min.The total RNA was visualized as fluorescent bands under a UV transilluminator.

2.5.1.2 First strand cDNA synthesis

One microgram of the DNA-free total RNA sample was used and incubated with 0.5 µg of oligo$(dT)_{18}$ primer for first-strand cDNA synthesis using a RevertAid™ First Strand cDNA Synthesis Kit (Fermentas). The reaction was adjusted to 12 µl by nuclease free water and shortly incubated at 65 °C for 5 min. After on ice for 5 min, the reverse transcription reaction mix (4 µl of 5× reaction buffer, 2 µl of 10 mM each of the dNTP, 1 µl of RevertAid™ M-MuLV reverse transcriptase (200 U/µl) and 1 µl of RiboLock™ RNase inhibitor (20 U/µl),) was added and gently mixed. The reaction was incubated at 42 °C for 60 min and then terminated reverse transcriptase activity at 70 °C for 10 min. All cDNA samples were stored at –20 ºC until use.

2.5.1.3 Semi-quantitative RT-PCR of *Pm*TBC1D20 gene distribution in shrimp tissues

The expression level of *Pm*TBC1D20 transcripts in various shrimp tissues was determined by semi-quantitative RT- PCR. Semi-quantitative PCR was performed to determine the level of *Pm*TBC1D20mRNA using the gene specific primers, sg6817_F and sg6817_R (Table 2.1), which were designed using the SECentral computer program (Scientific & Educational Software, Durham, NC, USA). EF-1α was used as the internal reference control for cDNA levels and was detected with the gene specific primers, EF-1F and EF-1R. One microliter of the ten-fold diluted cDNA was subjected to PCR in a 25 µl reaction volume containing 10mM KCl, 2mM $MgSO_4.7H_2O$, 20mM Tris-HCl (pH 8.8), 0.1% Triton X-100, 10mM $(NH_4)_2SO_4$, 0.1mg/ml BSA, 0.2 mM of each dNTP, 0.2 µM of each specific primers and 1.5 unit of Taq DNA polymerase (RBC Bioscience). The reaction was predenatured at 94 °C for 2 min following by 30 cycles of denaturing at 94 °C for 30 sec, annealing at 60 °C for 30 sec and extension at 72 °C for 30 sec. amplification product was analyzed by resolution through a 1.5% (w/v) agarose gel and visualized by UV transillumination after ethidium bromide visualization.

2.5.2 Expression profile of *Pm*TBC1D20 transcript upon WSSV infection

2.5.2.1 WSSV-infected hemocyte preparation and total RNA extraction

Healthy sub-adult shrimp (about 16g body weight) were divided into 2 groups, the control and the WSSV-infected group. For WSSV-infected group, shrimp were separated into 3 groups and were injected with 100µl of a 1:8000 dilution of the WSSV stock in lobster hemolymph medium (LHM) (15mM $CaCl_2$, 10mM KCl, 5mM $MgCl_2$, 8.1mM $MgSO_4$, 486mM NaCl, 0.5mM Na_2HPO_4, 36mM $NaHCO_3$ and 0.05% (w/v) dextrose in Minimum Essential Medium (Invitrogen)). The control shrimp were injected with 100 µl of LHM. The hemocyte was collected from three individual shrimp/time/group from the ventral sinus using a sterile 1 ml syringe containing 10% (w/v) sodium citrate as the anti-coagulant at 0.25, 12, 24, and 48 h post infection (hpi). The hemolymph was centrifuged immediately at 800 × g for 15 min at 4 °C to separate the hemocyte from plasma. Total RNAs were extracted from the hemocytes using TRI Reagent® (Molecular Research Center) followed by DNase I (Promega) treatment as describe above in 2.5.1.1 and then used to further synthesize single-stranded cDNAs with the First-stranded cDNA synthesis kit (Fermentas) as describe in 2.5.1.2.

2.5.2.2 Semi-quantitative RT-PCR of *Pm*TBC1D20 transcript expression profile in WSSV-infected shrimp

The expression levels of *Pm*TBC1D20 gene of normal and WSSV-infected hemocyte of shrimp were determined using semi-quantitative RT-PCR using cDNA from WSSV-infected hemocyte at 0.25, 12, 24, and 48 hpi. The PCR condition and PCR cycle was described above. The amplification product was then analyzed by resolution through a 1.5% (w/v) agarose gel and visualized by UV transillumination after ethidium bromide visualization. The relative expression levels of *Pm*TBC1D20 in WSSV-infected shrimp were normalized with the internal control (EF-1α) and were normalized against the expression of this gene in the control shrimp and tested the statistics using one-way analysis of variance (ANOVA) (Steel and Torrie, 1980) followed by Duncan's new multiple range test (Duncan, 1955) using SPSS software.

2.5.2.3 Confirmation of *Pm*TBC1D20 transcript expression profilie in response to WSSV infection by quantitative real-time RT-PCR (qrt-RT-PCR)

According to the expression profile of *Pm*TBC1D20 gene in WSSV-infected shrimp determined by RT-PCR, *Pm*TBC1D20 gene showed up-regulation when infected with WSSV, therefore this result was confirmed by using qrt-RT-PCR and the expression of EF-1α was used as an internal control.

The qrt-RT-PCR was performed using an equal amount of cDNAs in an iCycler iQ^{TM} Real-Time Detection system using iQ^{TM} SYBR Green Supermix (Bio-Rad) with the following conditions: 95 °C for 9 min, 40 cycles of 95 °C for 30 sec, 58 °C for 30 sec and 72 °C for 45 sec. The specific sg6817REAL_F and sg6817REAL_R primers (Table 2.1) were used in the experiment. For each sample, the ΔC_t was calculated from the threshold PCR cycle (C_t) of *Pm*TBC1D20 gene normalized relatively by the C_t of the reference EF-1α gene in the same sample. The $\Delta\Delta C_t$ value was then calculated from ΔC_t of WSSV-infected group - ΔC_t of control group (LHM injected). Each real-time PCR reaction was done in triplicate. The results were presented as the relative expression ratios which come from $2^{-\Delta\Delta Ct}$. The data were subjected to One-way ANOVA. Significance was accepted at $P < 0.05$.

2.6 *Pm*TBC1D20 gene silencing using RNA interference technique

To investigate the inhibition of *Pm*TBC1D20 transcripts against white spot syndrome virus (WSSV) propagation, the primary cell culture of *P. monodon* hemocyte and 3-5 g shrimp were used. The WSSV infection was detected using RT-PCR and qrt-RT-PCR of a major envelope protein VP28 expression in the host cells. The presence of the VP28 transcript indicated viral propagation.

2.6.1 Primer design and DNA template preparation

Double stranded RNA (dsRNA) that correlated with nucleotide sequence of *Pm*TBC1D20 gene was synthesized by *in vitro* transcription and performed by T7 RiboMAX$^{(TM)}$ Express RNAi System (Promega). Oligonucleotide primers of this gene were incorporated with T7 promoter sequences (5′GGATCCTAATACGACTCACTATAGG 3′) at the 5′ ends. The template DNA for generating dsRNA was amplified in two separate PCR reactions (Fig. 2.4). The specific primers were designed from the nucleotide sequence of *Pm*TBC1D20 gene.

The sense strand template was synthesized from sg6817_F and sg6817_RT7 primers (Table 2.1), whereas the anti- sense strand template was synthesized from the sg6817_FT7 and sg6817_R primers (Table 2.1). Both PCR reactions were performed with 0.02 units/µl of *Taq* DNA Polymerase (RBC) with PCR cycle parameters of an initial denaturation at 94 °C for 2 min, followed by 30 cycles of 94 °C for 30 sec, 60 °C for 30 sec and 72 °C for 30 sec, and with a final extension at 72 °C for 5 min. For internal control, GFP gene was used to synthesized double stranded RNA. Like the experimental group of *Pm*TBC1D20 gene, the specific primers of GFP gene incorperated with T7 promotor sequence were used to synthesized the template; GFP_F and GFP_RT7, GFP_FT7 and GFP_R (Table 2.1). The PCR reactions were performed with 0.02 units/µl of *Taq* DNA Polymerase (RBC) with PCR cycle parameters of an initial denaturation at 94 °C for 2 min, followed by 30 cycles of 94 °C for 30 sec, 58 °C for 30 sec and 72 °C for 30 sec, and with a final extension at 72 °C for 5 min.

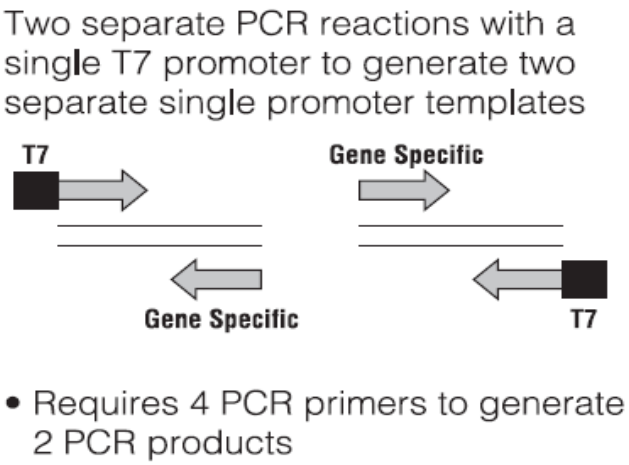


Figure 2.4 Strategy for adding T7 promoters to DNA templates by PCR. (Source: T7 RiboMAX$^{(TM)}$ Express RNAi System (Promega))

The PCR product was separated by 1.5% (w/v) agarose-TBE gel electrophoresis and the desired band was eluted and purified using NucleoSpin® Extract II Kits. The obtained DNA template with T7 promoter sequences were futher used for synthesizing dsRNA

One point five microgram of each template was used in an *in vitro* transcription of T7 RiboMAX^{TM} Express Large Scale RNA Production Systems (Promega, USA), according to the manufacturer's protocol. The sense and anti-sense single stranded RNA were then mixed at equimolar amounts and annealed to construct the dsRNA. The DNA template was then eliminated by treatment with RNase-free DNaseI.

2.6.2. Synthesizing the double stranded RNA

The dsRNA was synthesized as revealed in Fig. 2.5. The DNA template of 1.5 µg each was used as template for synthesis of large quantities of ssRNA. The reaction contained 10 µl at the components of RiboMAX express T7 2x buffer, 1 to 8 µl of linear DNA template , 2 µl of enzyme mix-T7 Express and nuclease-free water was added to the final volume of 20 µl. The reaction was stood at 37◦C for 0.5-2 h. Then the single stranded RNA was synthesized.

To anneal the RNA strands, equal volumes of complementary RNA reactions were mixed together and incubated at 70 °C for 10 minutes, and then the reaction was slowly cooled to room temperature (~20 min). This allows annealing of the double-stranded RNA. The supplied RQ1 RNase-Free DNase was added to the mixture in order to remove the remainng DNA template, leaving only dsRNA by adding 1 µl per 1 µg of DNA template. The reaction was mixed, and incubated for 30 min at 37 °C. The dsRNA solution was added with 0.1 volume of 3M Sodium Acetate (pH 5.2) and 1 volume of isopropanol or 2.5 volumes of 95 % ethanol to precipitate the dsRNA. The reaction was mixed and placed on ice for 5 min. Spun at top speed in a microcentrifuge for 10 min. A white pellet was washed with 0.5 ml of cold 70 % ethanol, air-dried and resuspended in nuclease-free water. The dsRNA was stored at -80°C.

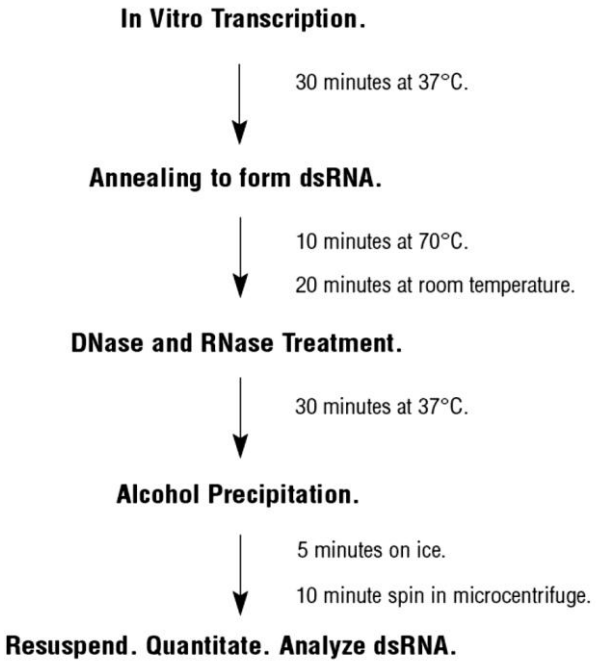


Figure 2.5 Outline of procedure for the production and purification of dsRNA using the T7 RioMAX express RNAi System. (Source: T7 Ribomax$^{(TM)}$ Express RNAi System (Promega)

2.6.3 Determination of quality and quantity of double-stranded RNA

The quality of dsRNA was examined by 1.5 % (w/v) agarose gel electrophoresis. Half of one microliter of dsRNA and each stand template were loaded and run on agarose gel electrophoresis in 1× TBE buffer. The gel was stained with ethidium bromide and visualized under the UV light to estimate the size of product.

The concentration of dsRNA was determined by measuring the OD_{260} and estimated in ng/µl using the following equation:

$$[\text{dsRNA}] = OD_{260} \times \text{dilution factor} \times 40$$

One OD_{260} corresponds to approximately 40 ng/µl of DNA. The relative purity of RNA samples was investigated by measuring the ratio of $A_{260/280}$. The maximum absorption of protein is at 280 nm. The good quality of RNA sample should have an $A_{260/280}$ ratio above 1.7.

2.6.4 Preparation of primary culture of hemocyte of *P. monodon* cells

Haemolymph of *P. monodon* was collected from 3 healthy sub-adult shrimp (16g body weight) at ventral sinus using 24-inch needle fitted onto 1.0 ml syringes pre-loaded with 0.5 ml of an anticoagulant solution (0.82% (w/v) Sodium Chloride, 0.55% (w/v) citric acid, 1.98% (w/v) glucose, 0.88% (w/v) sodium citrate and adjust the pH to 5.6 by 10N sodium hydroxide) each and then the haemocyte cells were harversted by centrifugation at 800 x g for 15 minutes at 4°C. The haemocyte cells were resuspended in 0.7 ml of L-15 culture medium (1.6x Leibovitz L-15 medium (Gibco) supplemented with 20% (v/v) fetal bovine serum (FBS), 1% (w/v) glucose, 0.4% (w/v) sodium chloride, 100 IU/ml penicillin and 100 µg streptomycin; pH 7.6; adjusting the osmotic pressure to 750 ± 15 mOsm/kg with sodium chloride solution). The concentration of a cell suspension was determined using Hemocytometer Slide under a light microscope. Ten micro liter of cell suspension was added to an assembled slide (The coverslip was pressed down onto slide), viewed on microscope and the cell lysate was counted within this 1-mm^2 area that stand on top-left, top-right, bottom-left, bottom-right, and middle of the area. The cell suspension was calculated the average of the the five counts and derived the concentration using the following formula

$$c = n / v$$

Whereas c is the cell concentration (cells/ml), n is the number of cells counted, and v is the volume counted (ml). For this slide, the depth of the chamber is 0.1 mm, and assuming that only the central 1 mm^2 is used, v is the 1 x 10^4 ml. The formula then become

$$C = n \times 5 \times 10^4$$

The cell suspension was subsequently seeded at 5 x 10^5 cells per 150 µl in a 96-well plate and incubated at room temperature for at least 2 h. Then the hemocytes were ready to used.

2.6.5 Specific gene silencing of *Pm*TBC1D20 gene transcript in primary culture of hemocyte

From above, the pre-treatment hemocyte of *P.monodon* was fixed on a 96-well plate and then incubated at room temperature at least 2 h. Then the pre-treatment hemocytes were ready to use for gene silencing using RNA interference technique. The dsRNA solution containing 5 µg of *Pm*TBC1D20 double-stranded RNA and H2A Histone protein 0.2 µg as RNA carrier in 10 µl of L-15 medium was added in the 150 µl of pre-treatment hemocyte and re-incubated at 28 °C for a further 24 h. Total RNA was extracted from the hemocytes using the TRI Reagent® (Molecular Research Center) followed by DNaseI (Promega) treatment, and then used to synthesize single-stranded cDNA (Fermentas). RT-PCR was performed using the TBC, and EF-1α primers (Table 2.1) with the conditions of 10mM KCl, 2mM $MgSO_4.7H_2O$, 20mM Tris-HCl (pH 8.8), 0.1% Triton X-100, 10mM $(NH_4)_2SO_4$, 0.1mg/ml BSA, 0.2 mM of each dNTP, 0.2 µM of each specific primers and 1.5 unit of *Taq* DNA polymerase (RBC Bioscience). The reaction was predenatured at 94 °C for 2 min following by 30 cycles of denaturing at 94 °C for 30 sec, annealing at 58 °C for 30 sec and extension at 72 °C for 30 sec. The PCR product was analyzed by 1.5% (w/v) agarose gel electrophoresis, and the differential expression level was reported as relative to that of EF-1α.

2.6.6 The effect of suppression of the *Pm*TBC1D20 transcript on WSSV challenge in primary culture of hemocyte

To determine the effect of suppression of the *Pm*TBC1D20 transcript on WSSV infection, the primary cell culture was transfected with the double-stranded RNA solution containing 5 µg of *Pm*TBC1D20 double-stranded RNA and H2A Histone protein 0.2 µg as RNA carrier in 10 µl of L-15 medium was added in the 150 µl of pre-treatment hemocyte and re-incubated at 28 °C for a further 24 h. After that, 50 µl of L-15 culture medium was removed from each well, and replaced with new 50 µl of L-15 culture medium with 1:250 of purified WSSV and re-incubated at 28 °C for a further 24 h. Total RNA was extract and then reverse transcribed to first strand cDNA, as described above

The effect of suppression of the *Pm*TBC1D20 transcript on WSSV infection was investigated by semi-quantitative RT-PCR analysis using the VP28 primers

(Table 2.1) with the conditions of 10mM KCl, 2mM $MgSO_4.7H_2O$, 20mM Tris-HCl (pH 8.8), 0.1% Triton X-100, 10mM $(NH_4)_2SO_4$, 0.1mg/ml BSA, 0.2 mM of each dNTP, 0.2 µM of each specific primers and 1.5 unit of *Taq* DNA polymerase (RBC Bioscience). The reaction was predenatured at 94 °C for 2 min following by 30 cycles of denaturing at 94 °C for 30 sec, annealing at 60 °C for 30 sec and extension at 72 °C for 30 sec. The PCR product was analyzed by 1.5% (w/v) agarose gel electrophoresis, and the differential expression level was reported as relative to that of EF-1α. Moreover, this result was confirmed by using qrt-RT-PCR and the expression of EF-1α was used as an internal control.

The qrt-RT- PCR was performed using an equal amount of cDNAs in an iCycler iQ^{TM} Real-Time Detection system using iQ^{TM} SYBR Green Supermix (Bio-Rad) with the following conditions: 95 °C for 9 min, 40 cycles of 95 °C for 30 sec, 60 °C for 30 sec and 72 °C for 45 sec. The specific VP28QF and VP28QR primers (Table 2.1) were used in the experiment. For each sample, the ΔC_t was calculated from the threshold PCR cycle (C_t) of *Pm*TBC1D20 gene normalized relatively by the C_t of the reference EF-1α gene in the same sample. The $\Delta\Delta C_t$ value was then calculated from ΔC_t of ds*Pm*TBC1D20 group - ΔC_t of control group (dsGFP). Each real-time PCR reaction was done in triplicate. The results were presented as the relative expression ratios which come from $2^{-\Delta\Delta Ct}$. The data were subjected to One-way ANOVA. Significance was accepted at $P < 0.05$.

2.6.7 The effect of *Pm*TBC1D20 transcript silencing in WSSV-infected shrimp

To investigate the specific knockdown effect of ds*Pm*TBC1D20 in the WSSV-infected shrimp *P.monodon*, the concentration of ds*Pm*TBC1D20 and the control and GFP dsRNA were optimized. After optimization, double injections of 10 µg of dsRNA per 1 g shrimp were used. The shrimp *P. monodon* weight of 4 g were used and each group of sample and control consisted of 3 triplicates of 2 pooled shrimp. The 40 µg of ds*Pm*TBC1D20 or dsGFP were dissolved in 0.85% (w/v) NaCl to a final volume of 30 µl and were injected into each shrimp in the lateral area of the fourth abdominal segment using a 0.5 ml insulin syringe with a 29-gauge needle. An additional control group of shrimps were injected with 0.85% (w/v) NaCl only. At 24 h after the first injection, the injection of dsRNA (10 µg per 1 g shrimp) or NaCl

combine with 50 µl of 1:10000 WSSV dilution in 0.85% (w/v) NaCl were repeated. The hemolymph of three individual shrimps from each group were collected and used for total RNA extraction and first strand cDNA synthesis, as described above.

The specificity of dsRNA-mediated gene knockdown of *Pm*TBC1D20 was analyzed by semi-quantitative RT-PCR using the sg6817F/R (Table 2.1) with the same condition as semi-quantitative RT-PCR of *Pm*TBC1D20 gene expression profile in WSSV-infected shrimp in section 2.5.2.2.

In order to investigate the effect of WSSV gene transcription level on *Pm*TBC1D20 transcript suppression. VP28 gene, ie1 gene, and WSV477 gene of WSSV were analyzed using qrt-RT- PCR. The quantitative real-time PCR was performed using an equal amount of cDNAs in an iCycler iQ^{TM} Real-Time Detection system using iQ^{TM} SYBR Green Supermix (Bio-Rad). The condition for VP28 gene (VP28QF/VP28QR) (Table 2.1) was the same as in section 2.6.6, and for ie1(ie1-qrtF/ie1-qrtR) (Table 2.1) and WSV477(477-qrtF/477-qrtR) (Table 2.1) gene were the same as in section 2.5.2.3. The calculation of relative expression was the same as section 2.6.6. All samples were normalized relatively by the C_t of the reference EF-1α gene in the same sample.

2.7 Immunolocalization of *Pm*TBC1D20 protein in shrimp hemocyte

In order to localization of *Pm*TBC1D20 protein in shrimp hemocyte, the specific anti-TBC antibody was prepared by overexpression of TBC protein in E.coli Rosetta (DE3) system.

2.7.1 Preparing anti-TBC antibody

2.7.1.1 Construction of the TBC expression plasmid

The TBC domain sequence of *Pm*TBC1D20 gene was amplified by using specific primers, TBC_F (Table 1) which introduced a *Nco*I site and a 6_His-Tag at the amino-terminus, and TBC_R (Table 2.1) which introduced a *BamH*I site at the carboxyl-terminus. The PCR amplification was performed in a final volume of 50 ml containing 1x pfusion buffer containing 1.5mM MgCl2, 200 mM dNTPs (each), 2 uM each primer, 5 U Phusion *Taq* DNA polymerase (Finzyme) and 250 ng of cDNA . The PCR reaction was conducted with the initial denaturation step at 94 ˚C for 10 sec,

followed by 30 cycles at 94 °C for 30 sec, 55 °C for 30 sec and 72 °C for 15 sec, and the final extension step at 72 °C for 5 min. The purified PCR product and the pET19b(+) expression vector (Novagen) were digested with *Nco*I and *BamH*I at 37 °C for 3 h, followed by being purified using NucleoSpin® Extract II kit. The TBC domain fragment was ligated into the pET19b(+) between *Nco*I and *BamH*I cloning sites by using T4 DNA ligase (New England Biolabs) in the reaction volume of 10 µl comprising 300 ng of the DNA insert and 50 ng of the cut-vector, and the ligation reaction occurred at 16 °C overnight. The recombinant plasmid was transformed into *E. coli* XL-1 blue competent cells, followed by screening on Luria-Bertani (LB) agar medium containing 100 µg/ml ampicillin at 37 °C overnight. Then TBC-recombinant pET19b(+) was isolated from the positive clones using Hi-Speed plasmid mini kit (Geneaid) according to the manufacturer's instructions and sequenced using T7 promoter at Macrogen INC., South Korea to examine the correctness of the inserted TBC DNA sequence. The recombinant plasmid was transformed into an *E. coli* Rosetta (DE3) for the production of the recombinant TBC.

2.7.1.2 Expression of recombinant TBC protein

The *E. coli* Rosetta was transformed with the expression plasmid containing the TBC1 domain sequence and the transformants were selected on the LB agar plates containing ampicillin (100 mg/ml). The cells containing the recombinant expression plasmid were cultured under vigorous shaking at 37°C. Expression of TBC protein was induced by adding IPTG to the final concentration of 1mM when the optical density at 600 nm of the cell culture reached 0.4–0.6, Before the induction, the split culture was served as an uninduced control. Protein expression was induced by adding IPTG to final concentration of 1 mM. The cells were harvested at 1, 2, 4 and 6 h post-induction by centrifugation at 10,000 x g for 10 min at 4 °C, then the cells were resuspended in SDS-PAGE sample buffer followed by heating at 100 °C for 10 min. The overexpression of recombinant TBC (rTBC) was analyzed by SDS-PAGE (18% (w/v) acrylamide) followed by coomassie brilliant blue staining.

2.7.1.3 Solubility analysis of the recombinant TBC protein in *E. coli*

The TBC-transformed *E. coli* was grown in LB medium containing 100 µg/ml ampicillin and 34 µg/ml chloramphenicol at 37 °C overnight with agitation at 250 rpm. The culture was diluted 1:100 in fresh LB medium supplemented with 100 µg/ml ampicillin and 34 µg/ml chloramphenicol and grown until an OD_{600} of the cultures reached 0.6. The cells were harvested at 4 h post-induction by centrifugation at 10,000 x g for 10 min at 4 °C, then the cells were resuspended in in phosphate-buffered saline (PBS) pH 7.4 (PBS; 137mM NaCl, 2.7mM KCl,10mM Na_2HPO_4, 2mM KH_2PO_4) by vortex. The cell suspension was disrupted by three rounds of freeze/thaw followed by sonication. After centrifugation at 10,000 x g for 10 min, supernatant and inclusion bodies were resuspended in SDS-PAGE sample buffer, followed by heating at 100 °C for 10 min. The solubility of rTBC was analyzed by SDS-PAGE (18% (w/v) acrylamide) followed by coomassie brilliant blue staining.

2.7.1.4 Purification of recombinant TBC protein

After 4 h of IPTG induction, the Rosetta cells containing the recombinant TBC domain protein were harvested, resuspended in 1x PBS and sonicated for 2–4 min. The cell lysate was centrifuged at 5000 rpm for 10 min at 4 °C to collect the inclusion bodies. The inclusion bodies were solubilized in a denaturing solution containing 0.1% SDS, 50mM phosphate buffer, pH 7.4, 8M urea and 20mM imidazol. The recombinant TBC domain protein was purified using a Ni-Sepharose column (GE) and eluted stepwise with the denaturing solution containing 300mM imidazole. Consequently, the purified protein was dialyzed against 50mM carbonate buffer, pH 10, and analysed using SDS-PAGE. The concentration of the eluted protein was determined by the Bradford method. Ater confirmation with western blot analysis, TBC domain protein was further used to generate rabbit polyclonal antibodies by a commercial service at Biomedical Technology Research Unit, Chiang Mai University, Thailand.

2.7.1.5 Western blot analysis

Standard 15% SDS-PAGE was used to analyze and follow the expressed TBC domain protein upon expression and purification. The protein bands were visualised by staining with Coomassie Brilliant Blue. Western blot analysis was used to confirm the identity of the expressed recombinant TBC protein. The proteins separated by the

SDS-PAGE were electro-transferred on to a nitrocellulose membrane (GE) in a semi-dry electrophoretic transfer cell (Trans-blot SD, Bio-Rad) at 90 mA for 90 min. The membrane was then washed twice with phosphate-buffered saline (PBS), pH 7.4 at room temperature for 10 min, and incubated in a blocking buffer (3% BSA in a PBS buffer) at room temperature for 1 h. The membrane was subsequently washed twice with PBS containing 0.05% (v/v) Tween20 at room temperature for 10 min and incubated with anti-His antibodies (GE) or anti-TBC antibody at room temperature for 1 h. After washing with PBS, the secondary antibodies conjugated with alkaline phosphatse were then added and incubate for 1 h. The recombinant protein was visualized as a purple band with the NBT/BCIP solution in detection buffer (100mM Tris-HCl pH 9.5, 100mM NaCl, 100mM $MgCl_2$).

2.7.2 Immunolocalization of *Pm*TBC1D20 protein in shrimp hemocyte upon WSSV infection

2.7.2.1 Rabbit anti-TBC antibody purification

In order to probe the *Pm*TBC1D20 protein in shrimp hemocyte with the anti-TBC antibody, the antibody had to be purified before probing. Protein A sepharose CL-4B (GE Healthcare) was used to purified the antibody. One gram of the protein A sepharose CL-4B powder was swirled in 4-5 ml of deionized water before packed in the column. The column was washed with 10 volume of PBS as the binding buffer. After loading the crude rabbit anti-TBC antibody, the column was eluted with 100 mM glycine buffer pH 2.5. The purified rabbit anti-TBC was analysed in 12% SDS PAGE.

2.7.2.2 Localization of *Pm*TBC1D20 protein in WSSV-infected hemocyte

Six healthy sub-adult shrimps were injected with the diluted WSSV solution (100 µl, 1:10000 dilution) whilst unchallenged shrimp were injected with 100 µl of virus-free LHM. After 24 hpi, hemolymph was drawn from each shrimp using a sterile 1 ml syringe with 500 µl of an anticoagulant solution. The hemocyte was fixed in 4% (w/v) paraformaldehyde. The hemocyte was fixed in the paraformaldehyde for 10 min at room temperature and centrifuged at 800 x g for 10 min at 4°C. After that,

the hemocyte was resuspended in PBS pH 7.4 and attached onto the poly-L-lysine coated slide. Then the slide was washed with PBS pH 7.4 for 5 min 3 times. The fixed hemocyte was permeabilized by adding 0.1% triton X 100 in PBS pH 7.4, leave it for 5 min at room temperature and washed 3 times with PBS pH 7.4.

The fixed hemocytes were incubated with a 1:500 dilution of rabbit anti-TBC and 1: 50 dilution of mouse anti-VP28 polyclonal antibody in PBS, pH 7.4, containing 1% (v/v) FBS) at 37 °C for 1 h whilst the negative control (non-infected) hemocytes were incubated with PBS, pH 7.4 containing 1% (v/v) FBS. Blots were then washed three times with PBS, pH 7.4, and then incubated with a 1:1000 dilution of Alexa Fluor 488 and 568 goat anti-rabbit IgG antibodies (Invitrogen) in PBS, pH 7.4 at room temperature for 1 h in the dark, and washed three times with PBS, pH 7.4. Subsequently, the nucleus was stained with a 1:500 dilution of TO-PRO-3 iodide (Invitrogen) in PBS, pH 7.4, at room temperature for 1 h in the dark, and washed three times with PBS, pH 7.4. The cover slips contained the stained and fixed hemocyte samples were then coated with Prolong Gold Antifade Reagent (Invitrogen) before detecting the fluorescent signal by confocal microscopy.

2.7.2.3 Effect of *Pm*TBC1D20 depletion on WSSV challenge

To determine the effect of suppression of the *Pm*TBC1D20 mRNA on WSSV infection, 6 shrimps were injected with ds*Pm*TBC1D20 RNA (10 µg/g shrimp) for the test group and 12 shrimps were injected with dsGFP RNA (10 µg/g shrimp) or 0.85% (w/v) NaCl for the control groups at the lateral area of the fourth abdominal segment. After 24 h of the first injection, shrimps were second injection together with WSSV. After a further 24 h, the hemolymph was collected from individual shrimp of each group and then fixed in 4% (w/v) paraformaldehyde for 10 min. The resulting hemocytes were then analyzed effect of *Pm*TBC1D20 depletion in vivo on WSSV challenge by co-immunolocalization under a confocal laser scanning microscope (Nikon) as mention in section 2.7.2.2.

CHAPTER III
RESULTS

3.1 Tissues and total RNA preparation

Gill, epipodite, antennal gland, heart, hemocytes, hepatopancreas, intestine, lymphoid organ, eye stalk, and stomach were collected for total RNA isolation. The A_{260}/A_{280} ratio of the total RNA samples were between 1.5 and 1.8 which indicated acceptable quality of total RNA. The average quantity of total RNA obtained from different tissues ranged from 24 to 90 µg per individual shrimp and depended on each tissues. The quality of total RNA was also monitored by running on a denaturing formaldehyde/agarose/Ethidium Bromide gel. Each tissue revealed a predominant band of 18S rRNA (1.9 kb) as shown in Fig. 3.1.

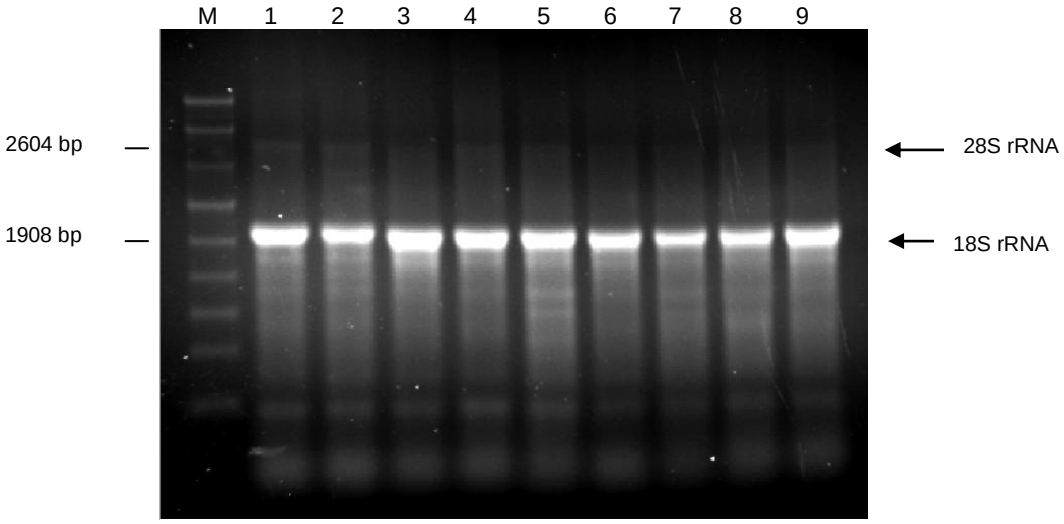


Fig. 3.1 Total RNA from *Penaeus monodon* tissues were analysed in 1.2% denaturing formaldehyde/agarose/Ethidium Bromide gel electrophoresis. A major band revealed to 18S rRNA at 1908 bp. Tissues were described as follows,

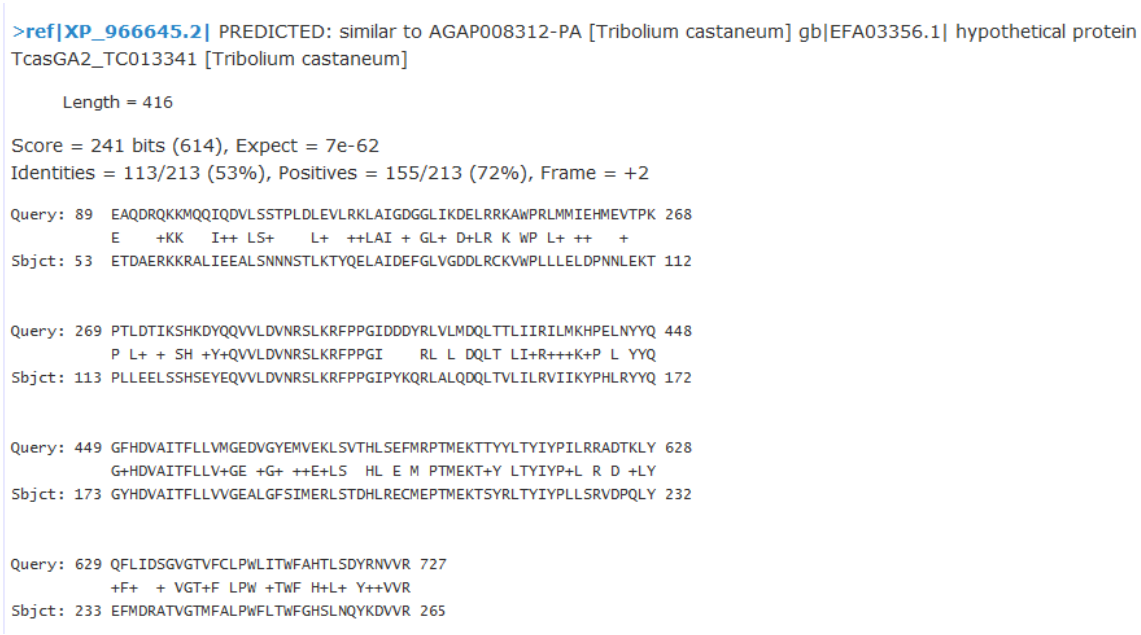
Lane M : RNA marker
Lane 1 : Lymphoid organ
Lane 2 : Eye stalk
Lane 3 : Gill
Lane 4 : Hemocyte
Lane 5 : Hepatopancreas
Lane 6 : Heart
Lane 7 : Intestine
Lane 8 : Epipodite
Lane 9 : Antennal gland

3.2 Determination of a full-length cDNA of the *Pm*TBC1D20 gene

A novel *Pm*TBC1D20 gene was first discovered in the *Penaeus monodon* EST database (http://pmonodon.biotec.or.th). From sequence analysis, the sequence of the *Pm*TBC1D20 gene from EST library (HC-N-N01-2807-LF) was a partial cDNA sequence containing 728 bp which lack the nucleotides coding for both start and stop codons. The partial nucleotide and deduced amino acid sequences of *Pm*TBC1D20 were shown in Fig. 3.2. The deduced amino acid of *Pm*TBC1D20 had 53% identity to AGAP008312-PA protein which contained TBC domain of red flour beetle (*Tribolium castaneum*) at E-value of 7e-62 as shown in Fig 3.3. In order to obtain a full-length cDNA of *Pm*TBC1D20, A Rapid Amplification of cDNA ends (RACE) technique of both 5´ and 3´ sites were conducted using specific primers.

```
AACAGGATCATCATCGGAAAATGATGCTTCAGAAACGGCTGAGAAAACCATGAAGAGAAG
 T  G  S  S  S  E  N  D  A  S  E  T  A  E  K  T  M  K  R  R
AAAGAAAAGGCCCAAGAATGTATCGCAAGAGGCTCAGGACCGTCAAAAGAAAATGCAGCA
 K  K  R  P  K  N  V  S  Q  E  A  Q  D  R  Q  K  K  M  Q  Q
AATTCAAGATGTACTGTCTTCCACTCCACTAGATTTAGAAGTCCTTCGAAAACTTGCAAT
 I  Q  D  V  L  S  S  T  P  L  D  L  E  V  L  R  K  L  A  I
TGGCGATGGCGGTCTTATAAAAGACGAGTTACGCCGCAAGGCTTGGCCGCGCCTCATGAT
 G  D  G  G  L  I  K  D  E  L  R  R  K  A  W  P  R  L  M  M
GATTGAGCATATGGAAGTGACCCCCAAACCCACCCTGGACACGATCAAAAGCCACAAGGA
 I  E  H  M  E  V  T  P  K  P  T  L  D  T  I  K  S  H  K  D
TTACCAGCAAGTGGTGCTGGATGTCAACCGGTCCCTCAAACGCTTCCCTCCTGGAATAGA
 Y  Q  Q  V  V  L  D  V  N  R  S  L  K  R  F  P  P  G  I  D
TGATGATTACAGGCTGGTTCTGATGGACCAGCTCACCACTCTCATTATTAGGATTCTCAT
 D  D  Y  R  L  V  L  M  D  Q  L  T  T  L  I  I  R  I  L  M
GAAGCATCCCGAACTGAATTATTACCAGGGGTTTCATGATGTTGCTATAACATTCCTTCT
 K  H  P  E  L  N  Y  Y  Q  G  F  H  D  V  A  I  T  F  L  L
GGTCATGGGTGAAGATGTTGGTTATGAGATGGTGGAGAAGCTCTCCGTCACACACCTGAG
 V  M  G  E  D  V  G  Y  E  M  V  E  K  L  S  V  T  H  L  S
TGAGTTCATGCGGCCAACAATGGAGAAGACCACATATTACCTCACCTACATATATCCCAT
 E  F  M  R  P  T  M  E  K  T  T  Y  Y  L  T  Y  I  Y  P  I
TCTTCGAAGGGCAGACACCAAGCTGTACCAGTTTTTGATAGATTCAGGTGTAGGAACAGT
 L  R  R  A  D  T  K  L  Y  Q  F  L  I  D  S  G  V  G  T  V
GTTTTGTTTGCCCTGGCTTATTACATGGTTCGCTCACACACTTTCTGACTACCGCAATGT
 F  C  L  P  W  L  I  T  W  F  A  H  T  L  S  D  Y  R  N  V
GGTGAGGT
 V  R
```

Fig. 3.2 Partial nucleotide (above) and deduced amino acid (below) sequences of *Pm*TBC1D20 obtained from the *Penaeus monodon* EST database.



```
>ref|XP_966645.2| PREDICTED: similar to AGAP008312-PA [Tribolium castaneum] gb|EFA03356.1| hypothetical protein
TcasGA2_TC013341 [Tribolium castaneum]

     Length = 416

Score = 241 bits (614), Expect = 7e-62
Identities = 113/213 (53%), Positives = 155/213 (72%), Frame = +2

Query: 89  EAQDRQKKMQQIQDVLSSTPLDLEVLRKLAIGDGGLIKDELRRKAWPRLMMIEHMEVTPK 268
           E    +KK   I++ LS+    L+  ++LAI + GL+ D+LR K WP L+ ++   +
Sbjct: 53  ETDAERKKRALIEEALSNNNSTLKTYQELAIDEFGLVGDDLRCKVWPLLLELDPNNLEKT 112


Query: 269 PTLDTIKSHKDYQQVVLDVNRSLKRFPPGIDDDYRLVLMDQLTTLIIRILMKHPELNYYQ 448
           P L+ + SH +Y+QVVLDVNRSLKRFPPGI    RL L DQLT LI+R+++K+P L YYQ
Sbjct: 113 PLLEELSSHSEYEQVVLDVNRSLKRFPPGIPYKQRLALQDQLTVLILRVIIKYPHLRYYQ 172


Query: 449 GFHDVAITFLLVMGEDVGYEMVEKLSVTHLSEFMRPTMEKTTYYLTYIYPILRRADTKLY 628
           G+HDVAITFLLV+GE +G+ ++E+LS  HL E M PTMEKT+Y LTYIYP+L R D +LY
Sbjct: 173 GYHDVAITFLLVVGEALGFSIMERLSTDHLRECMEPTMEKTSYRLTYIYPLLSRVDPQLY 232


Query: 629 QFLIDSGVGTVFCLPWLITWFAHTLSDYRNVVR 727
           +F+  + VGT+F LPW +TWF H+L+ Y++VVR
Sbjct: 233 EFMDRATVGTMFALPWFLTWFGHSLNQYKDVVR 265
```

Fig. 3.3 Sequence analysis of the partial *Pm*TBC1D20 gene. The BLASTX result showed the highest sequence identity (53%) to the AGAP008312-PA protein which contained TBC domain of red flour beetle (*Tribolium castaneum*) (acc. No. XP_966645).

3.2.1 5´ Rapid Amplification of cDNA Ends (5´ RACE)

To obtain the full-length cDNA of *Pm*TBC1D20, 5´ RACE cDNA library were synthesized from total RNA from *P. monodon* hemocyte. The specific primer sg6817_R was used to perform the first round PCR with the universal primer (provided by RACE kit). The product was then used as the template for the second round PCR (nested PCR) with nested specific primer 5R6817 and nested universal primer. The PCR products were analyzed in 1.5% agarose gel electrophoresis as shown in Fig 3.4. The first round PCR product could not be observed while the second (nested) round PCR product was a DNA band at about 700 bp. The expected band was purified and ligated to T&A cloning vector kit (RBC). The purified plasmid containing this nested PCR product was further sequencing to obtain the sequence in the 5´ site of *Pm*TBC1D20 gene. The additional sequence obtained from 5´ RACE was 453 bp including that coding for the start codon (Fig.3.5).

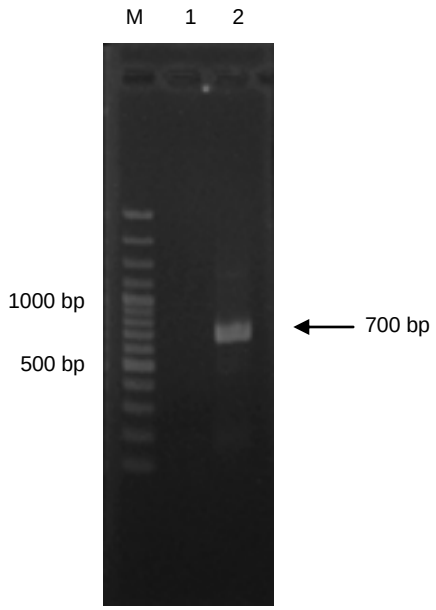


Fig. 3.4 5´ RACE PCR of *Pm*TBC1D20 gene using specific primers designed from partial sequence of *Pm*TBC1D20 gene of *Penaeus monodon*. The first round PCR product and second round (nested) PCR products of 5´ RACE PCR was analysed by 1.5% agarose gel electrophoeresis. The first round PCR product was in lane 1 and the second round (nested) PCR product was in lane 2 as a band of about 700 bp was observed. Lane M ; 100 bp marker.

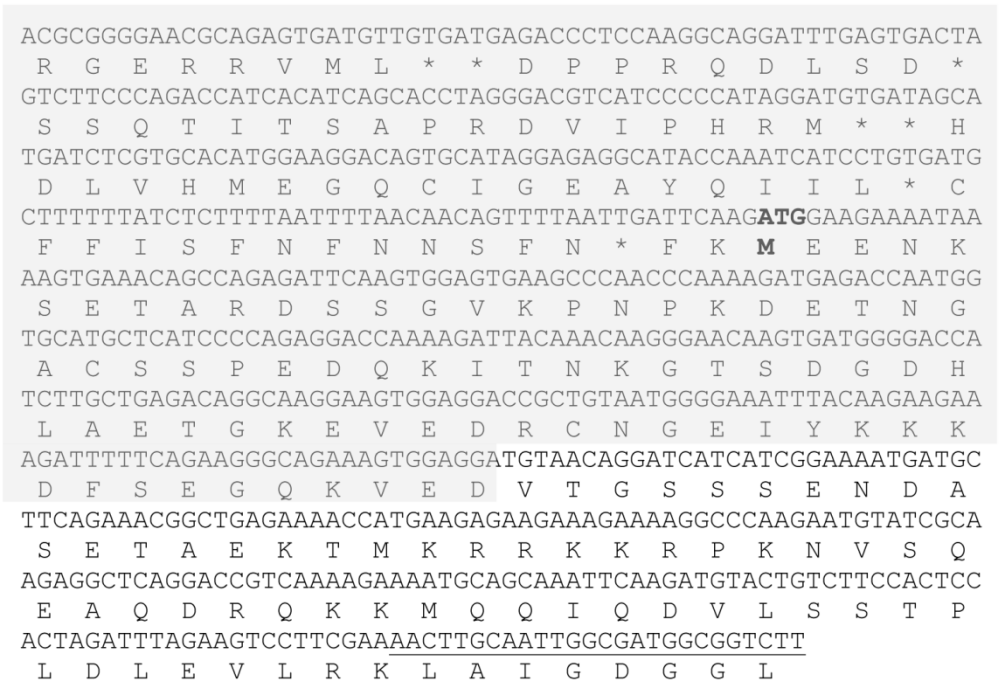


Fig. 3.5 Sequence of *Pm*TBC1D20 gene obtained from 5´ RACE PCR. The additional sequence of 453 bp in the 5´ site of *Pm*TBC1D20 gene was obtained as highlighted. The start codon sequence was bolded and the primer sequence of 5R6817 was underlined.

3.2.2 3´ Rapid Amplification of cDNA Ends (3´ RACE)

The 3´ RACE was performed to obtain the remaining complete cDNA including those coding for the stop codon. First, 3´ RACE cDNA library were synthesized from total RNA from *P. monodon* hemocyte. The specific primer 3R6817 was used to perform the first round PCR with the universal primer (provided by RACE kit). The product from the first round PCR was used as the template for the second round PCR (nested PCR) with the nested specific primer N_3R6817 and the nested universal primer. The PCR products were analyzed by 1.5% agarose gel electrophoresis. The first round PCR product showed smear pattern while the second (nested) round PCR product contained two DNA bands at about 600 and 1000 bp. Those observed bands were purified and ligated to T&A cloning vector kit (RBC). The purified plasmid containing these nested PCR products (Fig. 3.7) was further sequenced to obtain the 3´ site of *Pm*TBC1D20 gene. The additional sequence

obtained only from the clone of 1000 bp including those coding for the stop codon, polyadenylate signal, and poly A tail (Fig. 3.8).

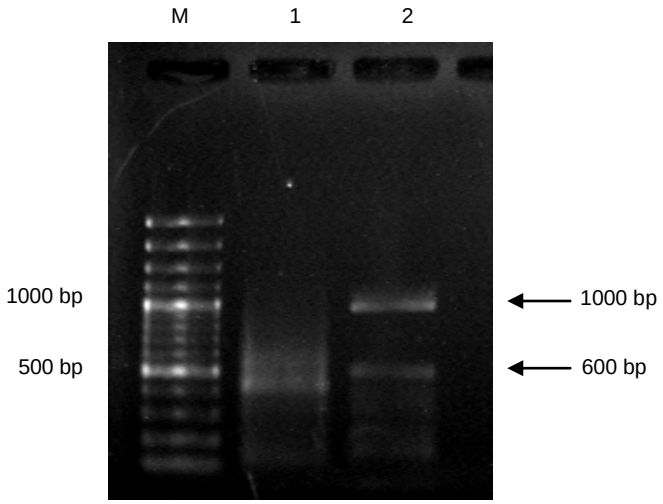


Fig. 3.6 3´ RACE PCR of *Pm*TBC1D20 gene using specific primers designed from the partial sequence of the *Pm*TBC1D20 gene of *Penaeus monodon*. The first round PCR product and the second round (nested) PCR products of the 3´ RACE PCR were analysed by 1.5% agarose gel electrophoeresis. The first round PCR product was in lane 1 and the second round (nested) PCR product was in lane 2.

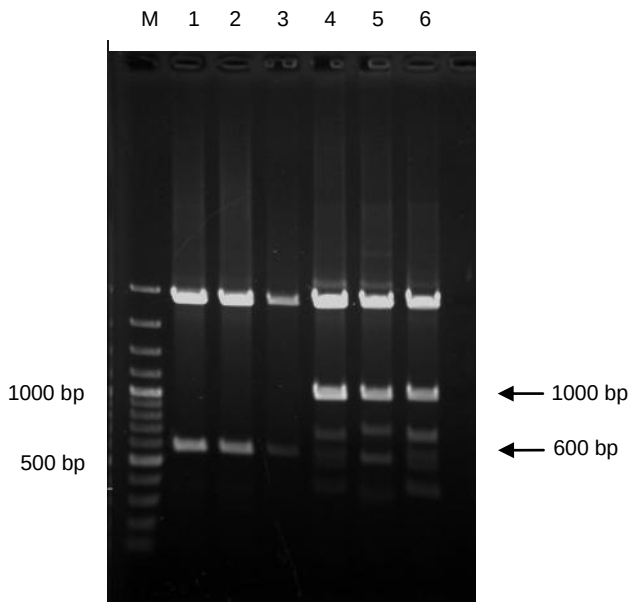


Fig. 3.7 Positive clones of 3´RACE products in T&A cloning vector cut with *Hind*III and analyzed in 1.2% agarose gel electrophoresis. The major band revealed the inserted size of 600 bp in lanes 1-3 and 1000 bp in lanes 4-6 of 3´RACE products respectively.

ATGCGGCCAACAATGGAGAAGACCACATATTACCTCACCTACATATATCCCATTCTTCGA
M R P T M E K T T Y Y L T Y I Y P I L R
AGGGCAGACACCAAGCTGTACCAGTTTTTGATAGATTCAGGTGTAGGAACAGTGTTTTGT
R A D T K L Y Q F L I D S G V G T V F C
TTGCCCTGGCTTATTACATGGTTCGCTCACACACTTTCTGACTACCGCAATGTGGTGAGG
L P W L I T W F A H T L S D Y R N V V R
CTATACGACTGCTTCTTGGCTTCCCCTTATCTCATGCCCATGTACATAGCTGCAGCAATT
L Y D C F L A S P Y L M P M Y I A A A I
GTTTTACACAAGAAAGAAGATGTTTTAGCTGGAGAATGTGATATGGCAATGCAACATTAT
V L H K K E D V L A G E C D M A M Q H Y
ATTCTTTCACAGGTTCCTGATACATTACCTCTGGAGCGCATCCTAGTAGATGCAGGGGAA
I L S Q V P D T L P L E R I L V D A G E
TTATATAACCTTTATCCACCTGACACTTTACAAGAGGAAGCCAACGAGTTTCTGCAGAAA
L Y N L Y P P D T L Q E E A N E F L Q K
AAGGCTGAAGAAGAGAAGCTGGAGAAGCAGCAAGTCCTCGAGAGACGTAAGAAGTTTGAA
K A E E E K L E K Q Q V L E R R K K F E
GCAGCAAGGAGAGGTCAGAATTACCGGGGGATCCTTGCAACGGTGTTCAGATACCTTCCA
A A R R G Q N Y R G I L A T V F R Y L P
CTGGTGCCAGCTCAGAGGAACATGCTTGTGAAGGTGGCTGCCTTTGCAGTAACAGCATAC
L V P A Q R N M L V K V A A F A V T A Y
ATTGCCACTAGCCTTTATAGCTACTATTCCAATTCAGCTCTTGTGTTTCCTTTTGTAGCA
I A T S L Y S Y Y S N S A L V F P F V A
AAACGA**TGA**TAATATATGAATTACAGTGCATAGCTCTTAGTTCCTAGTCAGTTTCATCAC
K R *****
CTAGTCTTTCACTCTGCTCGATTATTTTAAAAAAATTGAATGATGGGTAGTAAACTTTAT
TATCTTTTTGCTCTGTAAACTAATAAAATAATGGAAGAAGTATGTTAAATGTATATAACA
TAACTATATTGTACAGAGTTTTAGAGTTTTTTGTTTTTTTGTATTTTCAGAATATATGAT
ATTAGCATGTCAGTTTGTTATATTCAGGGCTCATAACTTAGAGTTTTTGTAAAGTTTACT
ATGG***AATAAA***GTGGTTGTTTGAAAAAAAAAAAAAAAAAAAAAGT

Fig. 3.8 Sequence of *Pm*TBC1D20 gene obtained from 3´ RACE PCR. The additional sequence of 824 bp at the 3´ site of *Pm*TBC1D20 gene was obtained as highlighted. The stop codon sequence was bolded. The primer sequence (N_3R6817) was underlined. The polyadenylated signal was in bold and italic and the poly A tail was italicized.

3.2.3 Sequence analysis of the full-length cDNA of *Pm*TBC1D20 gene

From 5´ RACE and 3´ RACE of the *Pm*TBC1D20 gene, the full-length of *Pm*TBC1D20 gene was obtained as shown in Fig. 3.9, consisted of 5´ untranslated region (5´ UTR), open reading frame (ORF), and 3´ untranslated region (3´UTR). The full-length sequence of *Pm*TBC1D20 was 2004 bp with the complete open reading frame encoding 480 amino acid protein (Fig. 3.9). From protein structural analysis using SMART (Simple Modular Architecture Research Tool, available on-line), this protein has a transmembrane region at the C-terminus and a TBC domain located at the middle of the protein. Besides, there was no signal peptide in this protein

(determined using SignalP). Schematic diagram of the TBC protein was as in Fig. 3.10.

The BLAST homology search indicated that the putative predicted protein sequence encoded by *Pm*TBC1D20 gene had a significant amino acid sequence identity to the TBC domain family, member 20-like from the acorn worm, *Saccoglossus kowalevskii*as 45% identity, hypothetical protein AaeL_AAEL001505 from the yellow fever mosquito, *Aedes aegypti* (39%), hypothetical protein BRAFLDRAFT_121942 of the Florida lancelet, *Branchiostoma floridae* (50%), TBC1D20 protein from the purple sea urchin, *Branchiostoma floridae* (42%) (Table 3.1). When only TBC domain sequence of *Pm*TBC1D20 protein was used to perform the BLAST search, it was found that the TBC domain of *Pm*TBC1D20 shared 55% identity to hypothetical protein TcasGA2_TC013341 from the red flour beetle, *Tribolium castaneum*, which also contain TBC domain within the sequence.

Amino acid sequence alignment of the TBC domain of the *Pm*TBC1D20 protein and other TBC domain from the TBC1D20 protein from both invertebrates (*Aedes aegypti, Drosophila melanogaster, Tribolium castaneum, Strongylocentrotus purpuratus, Branchiostoma floridae, Saccoglossus kowalevskii, Penaeus monodon,*) and invertebrates (*Homo sapiens, Mus musculus, Danio rerio*) showed 6 similar motifs including 3 significant motifs (RxxxW, I/VxxDxxR, and YxQ) which were contained within this domain as in Fig. 3.11. This result indicated that the TBC domain was highly conserved in various organisms.

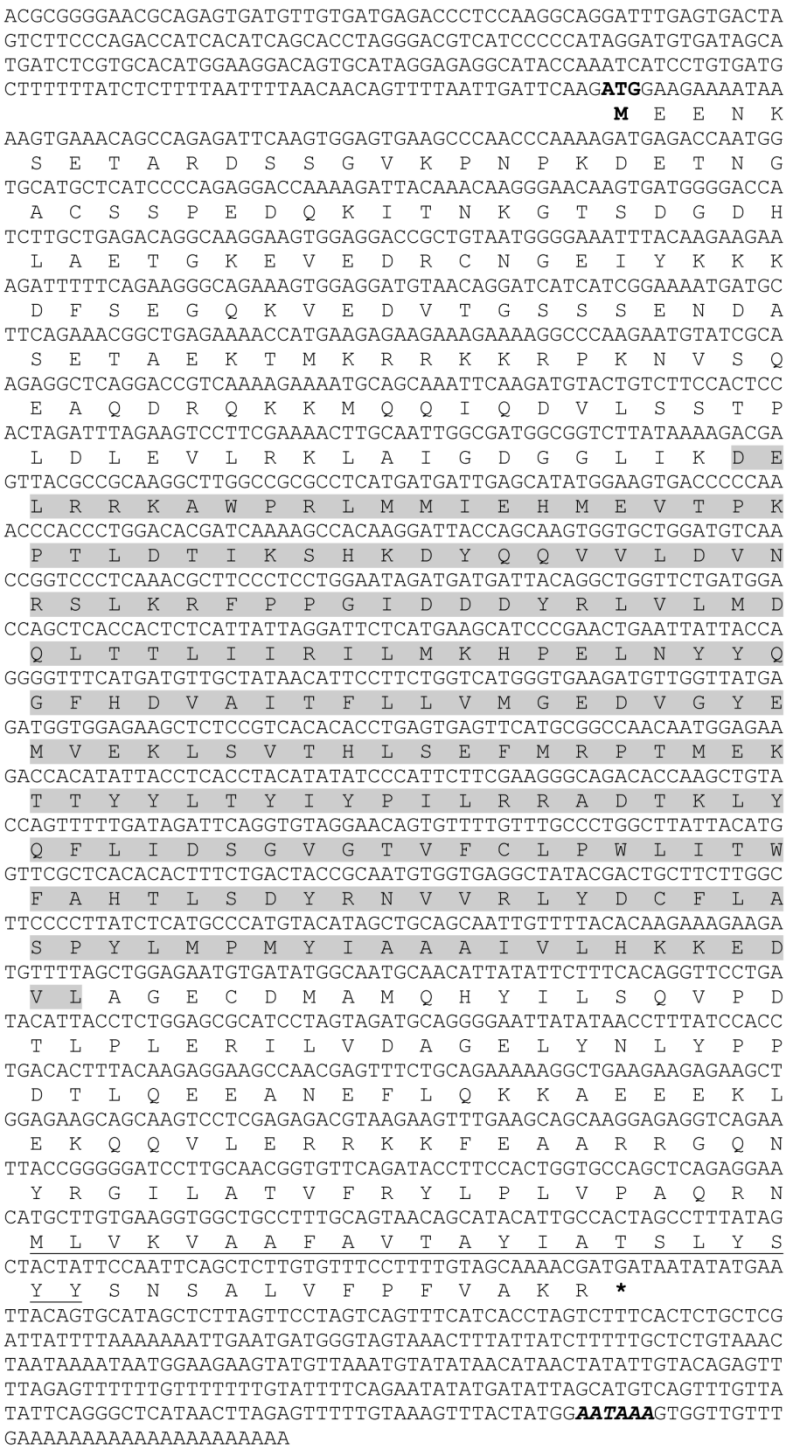


Fig. 3.9 Full-length sequence of the *Pm*TBC1D20 gene with 2004 bp translated to a 480 amino acid protein. The predicted TBC domain is indicated in grey hilight and the transmembrane region is underline at the C-terminus. The bold amino acid indicates the start codon. An asterisk is the stop codon and a polyadenylation signal is indicated by bold and italic prints.

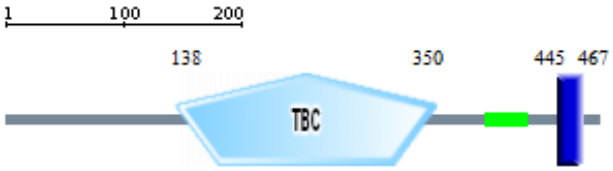


Fig. 3.10 SMART diagram of the *Pm*TBC1D20 protein showing the TBC domain in pentagon. The transmembrane segment is predicted at the C-terminus in blue rectangle.

Table 3.1 BLASTX results and percentages of identity of the *Pm*TBC1D20 deduced amino acid to the homologue sequences in the Genbank database

Accession no.	**Description**	Total score	E value	Max identity
XP_001653390.1	>gb|EAT47398.1| conserved hypothetical protein [*Aedes aegypti*]	296	5e-78	39%
XP_002736159.1	PREDICTED: TBC1 domain family, member 20-like [*Saccoglossus kowalevskii*] hypothetical protein AaeL_AAEL001505 [*Aedes aegypti*]	310	4e-82	45%
XP_002612373.1	hypothetical protein BRAFLDRAFT_121942 [*Branchiostoma floridae*] >gb|EEN68382.1| hypothetical protein BRAFLDRAFT_121942 [*Branchiostoma floridae*]	294	3e-77	50%
XP_782379.2	PREDICTED: similar to TBC1D20 protein [*Strongylocentrotus purpuratus*] >ref|XP_001181455.1| PREDICTED: similar to TBC1D20 protein [*Strongylocentrotus purpuratus*]	294	3e-77	42%
XP_966645.2	PREDICTED: similar to AGAP008312-PA [*Tribolium castaneum*] >gb|EFA03356.1| hypothetical protein TcasGA2_TC013341 [*Tribolium castaneum*]	286	7e-75	47%

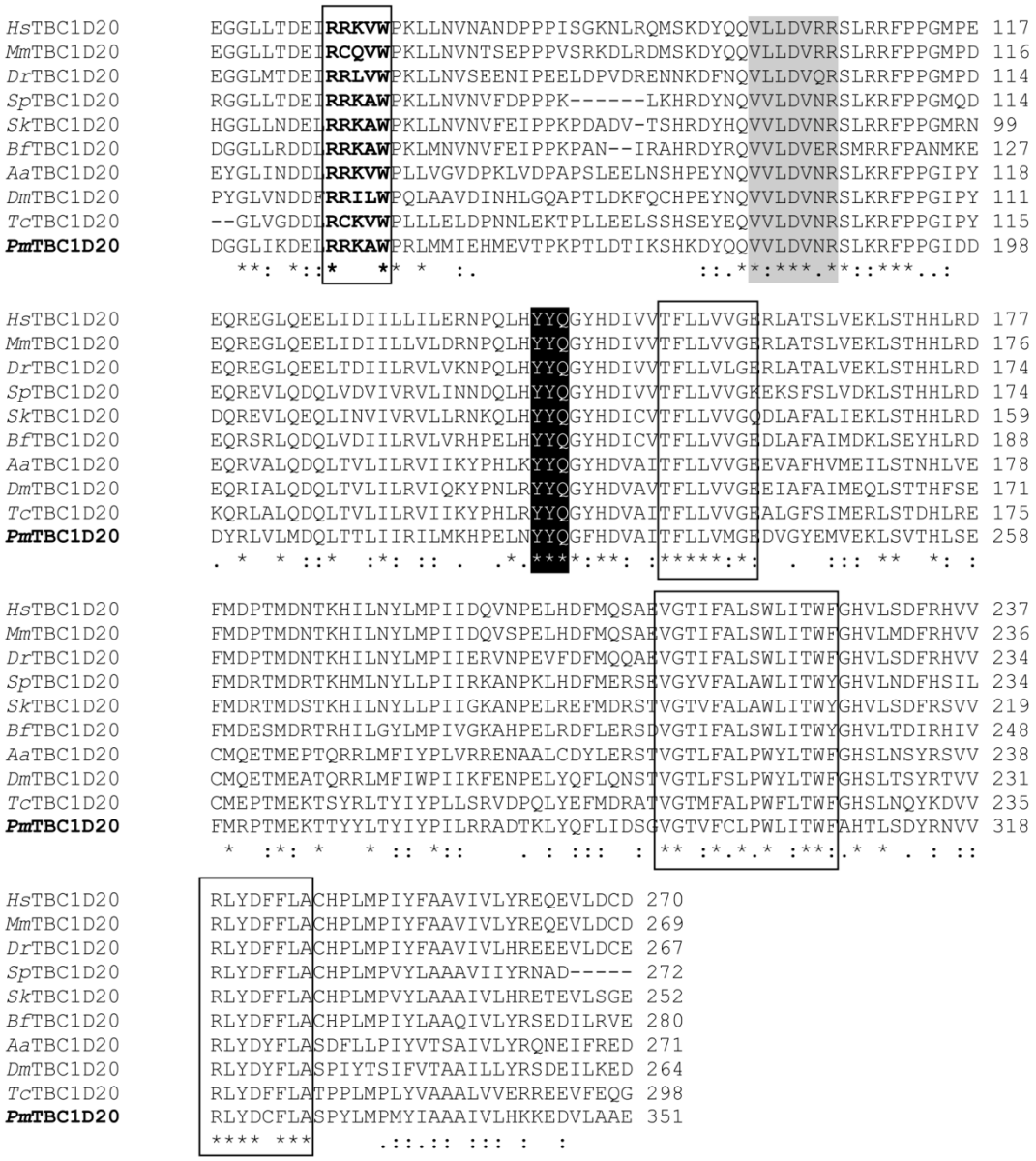


Fig. 3.11 Sequence alignment of the TBC domain of *Penaeus monodon* TBC1D20 with those of various organisms. 3 Signature motifs are shown. Motif A (Rxxxw) are shown in the box with bold characters. Motif B with catalytic arginine residue (VxxDxxR) are shown in the grey highlighted. Motif C (YxQ) are shown in the black highlighted.Other motifs (D-F) are in the boxes, respectively. Amino acid conservation across alignments is shown as (*), (:) and (.) for identical, conserved and semi-conserved, respectively. The Abbreviations represent Scientific names ; *Hs-Homo sapiens* (acc. No. NM_144628.2), *Mm-Mus musculus* (acc. No. BC040089.1),

Dr-Danio rerio (acc. No. NM_001025172.1), *Sp- Strongylocentrotus purpuratus* (acc. No. XM_001178307.1), Sk- *Saccoglossus kowalevskii* (acc. No. XM_002736113.1), Bf- *Branchiostoma floridae* (acc. No. XM_002612327.1), *Aa -Aedes aegypti* (acc. No. XM_001653340.1), *Dm -Drosophila melanogaster* (acc. No, NM_001042984.1), Tc- *Tribolium castaneum* (acc. No. XM_961552.2) *Pm- Penaeus monodon* (acc. No, XXXXXX).

3.3 Tissue distribution of the *Pm*TBC1D20 transcript

Semi-quantitative RT-PCR was performed to determine the *Pm*TBC1D20 mRNA expression level in shrimp tissues. EF-1α was used as the internal reference control. Total RNA from shrimp tissues; lymphoid organ, eye stalk, gill, hemocyte, hepatopancreas, heart, intestine, epipodite, antennal gland, and stomach were extracted and then synthesized to cDNA by using First Strand cDNA synthesis kit (Fermentas). After RT-PCR analysis, the *Pm*TBC1D20 transcripts were found to be expressed in many tissues including hemocyte, gill, hepatopancreas, heart, lymphoid organ, stomach, epipodite, eyestalk, antennal gland, and also expressed highly in intestine (Fig. 3.12).

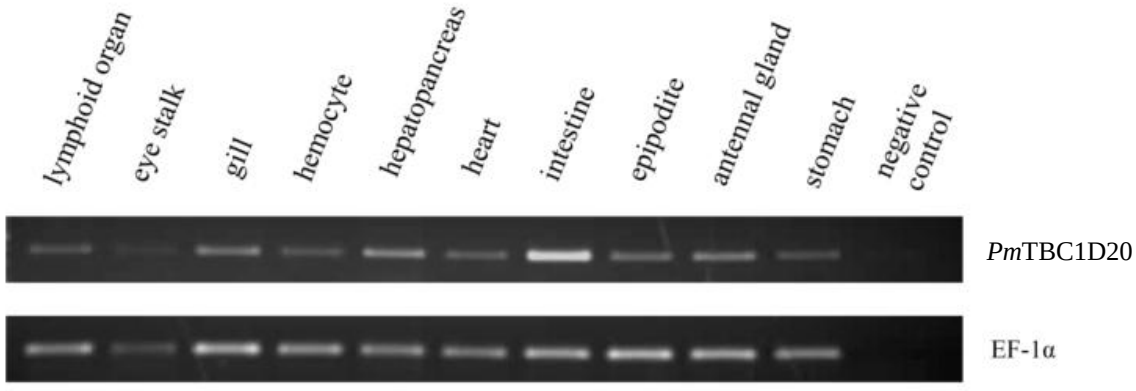


Fig. 3.12 Expression of the *Pm*TBC1D20 transcripts in various shrimp tissues. EF-1α was used as an internal control.

3.4 *Pm*TBC1D20 gene expression profile in WSSV-infected *P. monodon*

3.4.1 Expression profile of *Pm*TBC1D20 transcripts after WSSV-infection by semi-quantitative RT-PCR

Expression level of the *Pm*TBC1D20 transcript in hemocytes of WSSV-infected *P. monodon* was determined by using semi-quantitative RT-PCR. The relative expression ratio of the *Pm*TBC1D20 transcript was determined at 0.25,12, 24, and 48 hpi compared with the control shrimp injected with LHM, normalized to EF-1α. The results showed that the expression of *Pm*TBC1D20 was not significantly increased until 24 hpi. Then, the *Pm*TBC1D20 transcript was significantly up-regulated at 48 hpi by about 2.38 fold (Fig. 3.13).

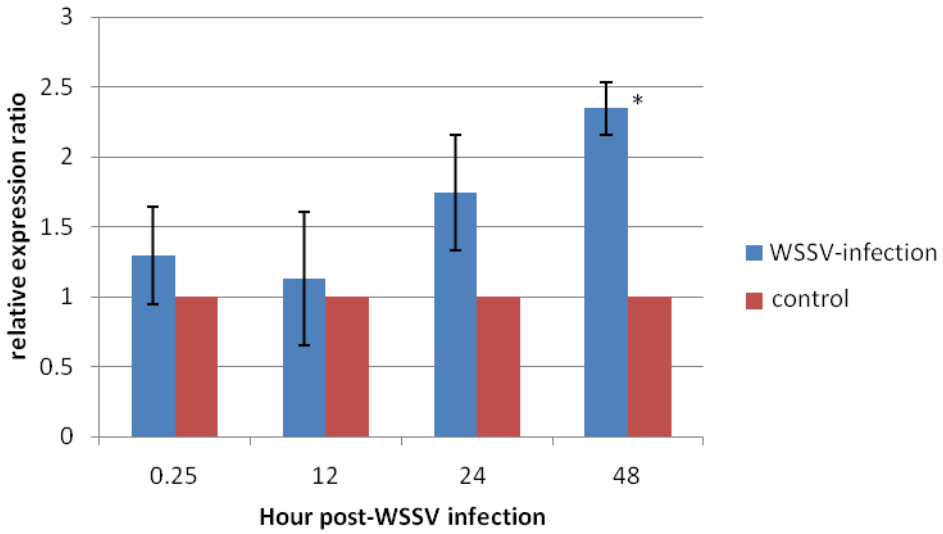


Fig. 3.13 Relative expression ratio of the WSSV-infected *P. monodon* analysed by semi-quantitative RT-PCR. The relative expression ratio at different time point after infection were normalized with EF-1α and then normalized against the control group (LHM-injected). The data shown as the mean (± 1 SD) are derived from three groups. One-Way ANOVA was used as the statistic and significant differences ($p < 0.05$) are marked with an asterisk.

3.4.2 Confirmation of *Pm*TBC1D20 gene expression profile upon WSSV infectoin by quantitative real time RT-PCR

To substantiate the expression level of the *Pm*TBC1D20 transcript in hemocytes of WSSV-infected *P. monodon*, quantitative real time RT-PCR was examined at 0.25,12, 24, and 48 h post-infection compared with the control shrimp

injected with LHM, and normalized to EF-1α. The results revealed that expression of the *Pm*TBC1D20 transcript was up-regulated at 24 and 48 hpi by about 2.3 and 2.0 fold respectively (Fig. 3.14).

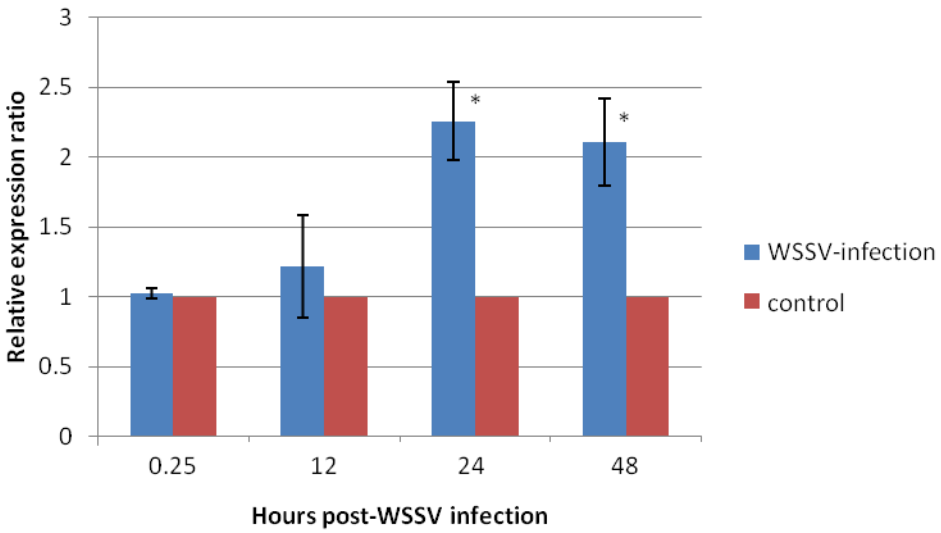


Fig. 3.14 *Pm*TBC1D20 mRNA expression in the hemocytes of WSSV-infected *P.monodon* at various time point (0.25, 12, 24 and 48 hpi) by qrt-RT-PCR. Data were shown as the mean (±1 SD) of three replicates and represent the fold change of *Pm*TBC1D20 after normalization relative to the EF1-α transcript levels and then compared relatively to the control group (set as = 1). Significant differences ($p < 0.05$) are marked with an asterisk.

3.5 The effect of *Pm*TBC1D20 transcript silencing in WSSV-infected primary hemocyte cell culture.

From the expression profile of the *Pm*TBC1D20 transcript in response to WSSV infection, it was revealed that the *Pm*TBC1D20 gene was up-regulated after WSSV infection. In order to investigate its fuction involved in WSSV infection, RNA interference (RNAi) technique was conducted.

3.5.1 dsRNA synthesis

DNA template with T7 promotor sequence of the *Pm*TBC1D20 gene and GFP gene were amplified. Each DNA templates (1.5 µg) was used for RNA synthesis by *in vitro* transcription and performed by T7 RiboMAX$^{(TM)}$ Express RNAi System

(Promega). After purification of dsRNAs, they were analysed by 1.2% agarose gel electrophoresis. The size of ds*Pm*TBC1D20 and dsGFP were about 400 and 800 bp respectively (Fig. 3.15).

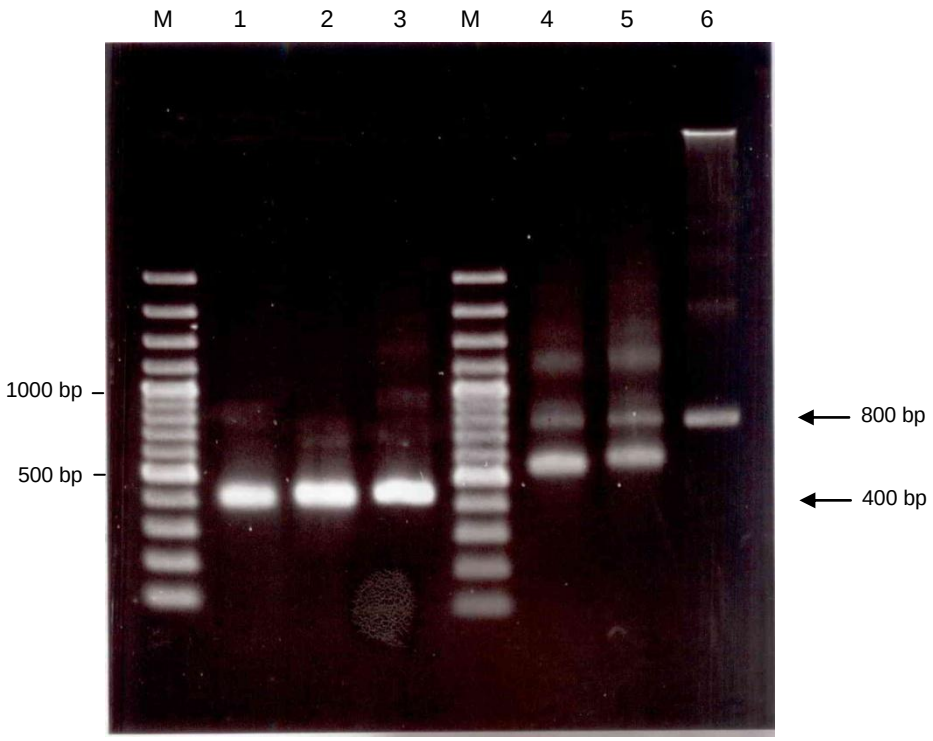


Fig. 3.15 Double stranded RNA of *Pm*TBC1D20 and GFP were analysed by 1.2% agarose gel electrophoresis.

Lane M : 100 bp DNA marker
Lane 1,2 : single stranded RNA of *Pm*TBC1D20
Lane 3 : double stranded RNA of *Pm*TBC1D20
Lane 4,5 : single stranded RNA of GFP
Lane 6 : double stranded RNA of GFP

3.5.2 Gene specific silencing of *Pm*TBC1D20 transcript levels in *P. monodon* primary hemocytes.

After optimization, $5x10^5$ pre-incubated primary hemocytes cells were transfected with 5µg of ds*Pm*TBC1D20 using histone-2A as a carrier and incubated for the next 24 hours. Total RNA was extracted and used as template to synthesize cDNA. GFP dsRNA and 150mM NaCl were used as internal controls. This result

indicated that ds*Pm*TBC1D20 specifically suppressed the *Pm*TBC1D20 transcription levels in primary hemocytes at 24 hour post transfection. Transfection of dsGFP and 150 mM NaCl as controls did not affect the mRNA expression level of *Pm*TBC1D20 (Fig. 3.16)

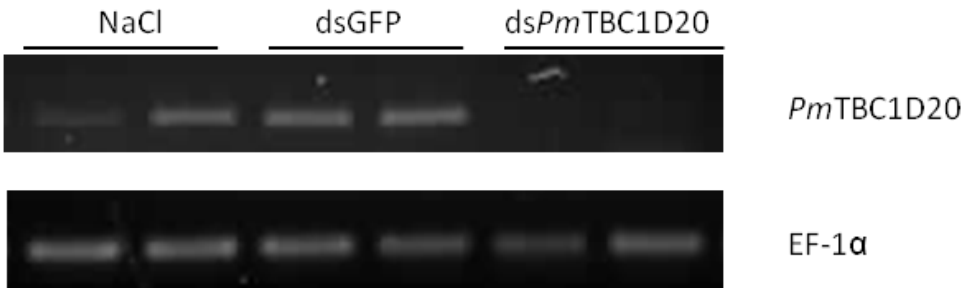


Fig. 3.16 Gene specific silencing of *Pm*TBC1D20 transcript levels in primary hemocyte cell culture. Pre-incubated cells were transfected with 5 µg of ds*Pm*TBC1D20, dsGFP per $5x10^5$ cells/ml or NaCl using Histone-2A protein as a carrier. Total RNA was extracted after 24 h after incubation and assayed for the transcript expression levels of *Pm*TBC1D20 gene by RT-PCR. EF-1α was used as an internal control to normalized the amount of cDNA template in each reaction.

3.5.3 Relative expression levels of VP28 mRNA after silencing of *Pm*TBC1D20 in WSSV infected primary hemocytes of *P.monodon*

The function of *Pm*TBC1D20 transcript against WSSV infection in shrimp hemocyte was determined by using RNA interference technique (RNAi) to mediate *Pm*TBC1D20 gene silecncing. Hemocytes of *P. monodon* from 2 sets of shrimp (3 shrimp per set) in concentration of 5×10^5 cells/ml were fixed on 96-well plate for at least 2 hours prior to addition of the double-stranded RNA solution which contained 2.5 µg and 5 µg of ds*Pm*TBC1D20 as experimental group or dsGFP as control group and incubated for 24 h. the cell culture was then infected with WSSV as described in material and methods section. Semi-quantitative RT-PCR was performed to examine the effect of gene suppressing using the TBC, VP28, and EF-1α primers (Table 2.1). It was found that suppression of *Pm*TBC1D20 resulted in a decrease of WSSV

infection as seen by the decrease of VP28 transcripts compared with the controls (Fig. 3.17).

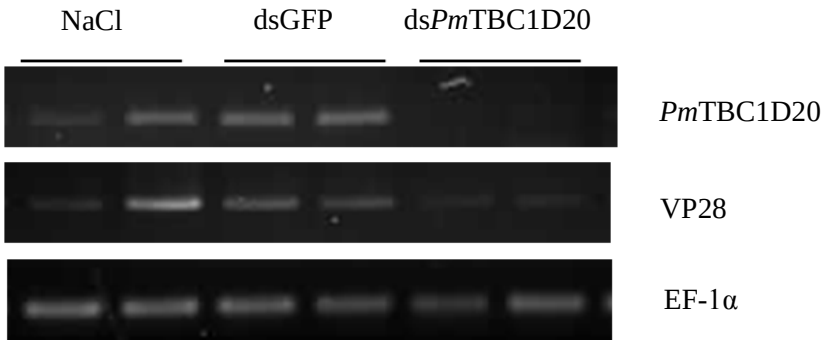


Fig. 3.17 Suppression of *Pm*TBC1D20 gene by specific dsRNA resulted in a decrease of VP28 transcript levels in primary hemocytes of *P. monodon*. Pre-incubated primary hemocytes cells were transfected with ds*Pm*TBC1D20, dsGFP and 150mM NaCl respectively. After 24 hour of incubation, primary hemocytes were infeceted with 1:250 dillution of WSSV in L-15 medium and continued incubating for the next 24 hours. Total RNA of primary hemocyte was collected. The relative expression of VP28 was revealed using semi-quantitative RT-PCR method and normalized against EF-1α gene. The experiment was examined using two sets of shrimp (3 shrimp per set).

In order to verify the relative expression levels of VP28 in primary hemocyte of *P. monodon*, qrt-RT-PCR was used. The cDNA sample of the experiment group transfected with ds*Pm*TBC1D20 and cDNA sample of the control group transfected with dsGFP were used to perform qrt-RT-PCR. The relative expression of VP28 gene was normalized against EF-1α. The significant differences of any data between groups was analysed statistically by using One-Way Anova method. Quantitative real-time PCR showed a decrease of VP28 transcripts of WSSV in the two experimental groups by 45% and 53%, respectively as compared with the control transfected with dsGFP (Fig. 3.18), suggesting that *Pm*TBC1D20 gene may be essential for virus infection.

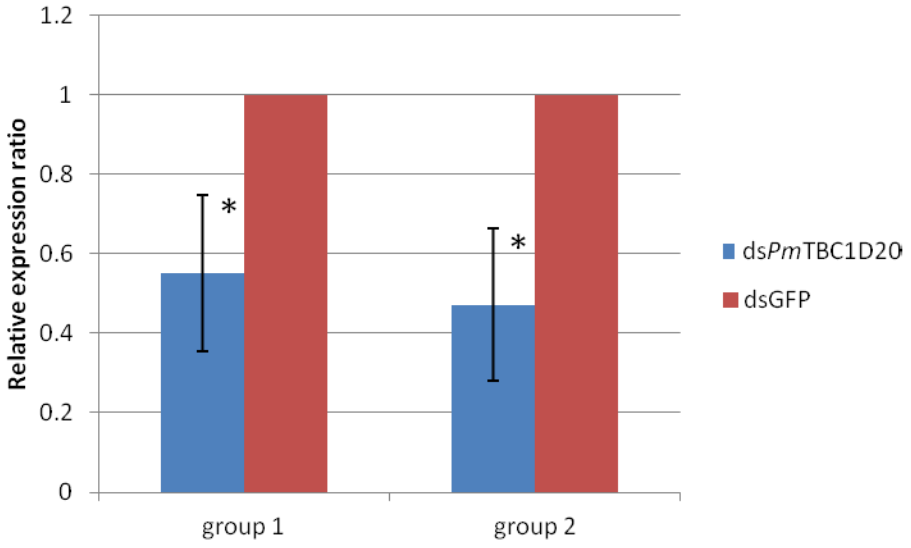


Fig. 3.18 Suppressing of the *Pm*TBC1D20 transcript mediated by RNAi resulted in an decrease of VP28 transcripts in WSSV infected primary hemocyte of *P. monodon*. Two sets of pre-incubated primary hemocyte of shrimp were transfected with ds*Pm*TBC1D20 and then added the 1:250 dilution of purified WSSV. The transcript expression levels of VP28 was determined by quantitative realtime RT-PCR. Data were shown as the mean (±1 SD) of three replicates and represent as the fold change of VP28 after normalization relative to the EF-1α transcript levels and then compared relatively to the control group transfected with dsGFP (set as = 1). Significant differences ($p < 0.05$) were marked with an asterisk.

3.6 The effect of *Pm*TBC1D20 gene silencing in WSSV infection shrimp

The effect of *Pm*TBC1D20 gene silencing on WSSV infection was also examined in shrimp (in *vivo*). Shrimp (about 4 g body weight) were separated into 3 groups and then injected with 10µg/ 1g shrimp ds*Pm*TBC1D20 while the control groups were injected with dsGFP or NaCl. After 24 h of the first injection, the same amount of dsRNA combined with 1:10000 dillution of WSSV was secondly injected. The hemolymph was collected after 24 h of the second injection. After cDNA synthesis, semi-quantitative RT-PCR was performed to investigate the *Pm*TBC1D20 gene. *Pm*TBC1D20 transcript suppression was observed in shrimp injected with

ds*Pm*TBC1D20 whereas suppression was not observed in the control shrimp (Fig. 3.19). Then, the amount of viral genes was determined by qrt-RT-PCR. The results showed a decrease in transcripts of WSSV genes; ie1, WSV477, and VP28 in the knockdown shrimp (Fig. 3.20).

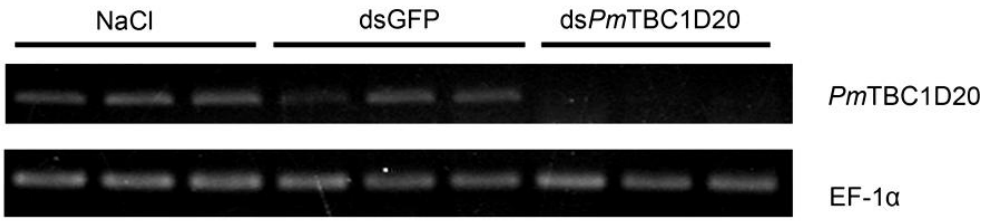


Fig. 3.19 *Pm*TBC1D20 gene silencing in *P.monodon*. ds*Pm*TBC1D20 or dsGFP or NaCl were double injected to *P.monodon* shrimp. The specific knockdown of *Pm*TBC1D20 was determined by RT-PCR using EF-1α as the control. Three set (2 shrimp per set) of shrimp were used.

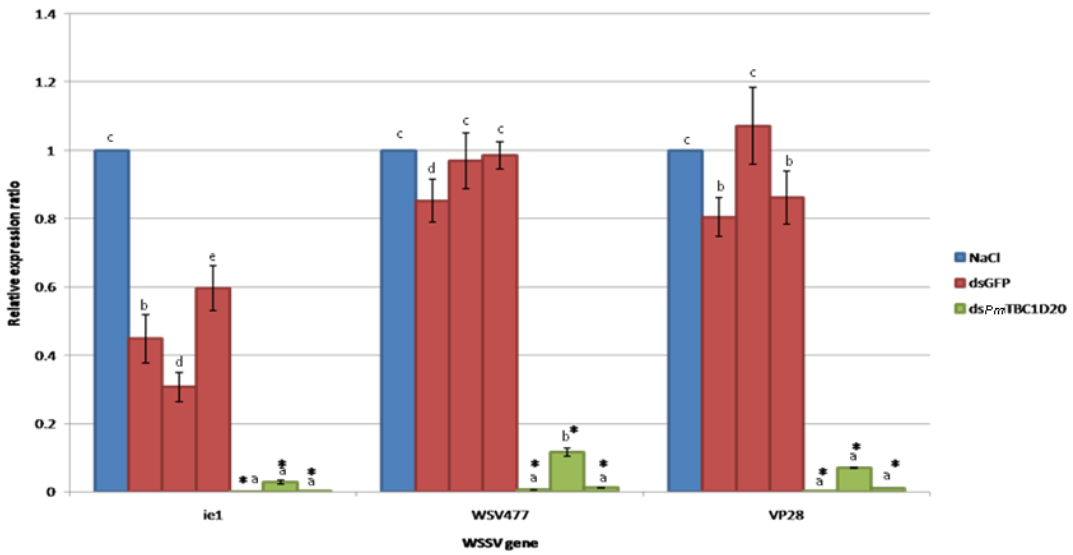


Fig. 3.20 *Pm*TBC1D20 gene silencing resulted in a decrease of WSSV transcripts in WSSV-infected *P.monodon*. After double injection of ds*Pm*TBC1D20 in WSSV-infected shrimp, the transcript expression levels of VP28, ie1, and WSV477 genes of WSSV were analyzed using qrt-RT-PCR. Data were shown as the mean (±1 SD) of three replicates and represent as the fold change of VP28, ie1, and WSV477 after normalization relative to the EF-1α transcript levels and then compared

relatively to the NaCl as the control groups (set as = 1). Significant differences within group ($p < 0.05$) were marked with alphabets and asterisk. This experiment was done in triplicate using three sets of shrimp (2 shrimp per set).

3.7 Immunolocalization of *Pm*TBC1D20 protein

3.7.1 Preparation of anti-TBC antibody

3.7.1.1 Preparation of TBC domain of *Pm*TBC1D20 gene

The sequence of TBC domain of *Pm*TBC1D20 at location 430-1035 bp was amplified from *P. monodon* hemocyte using specific primers. The specific primers designed from the cDNA sequence of *Pm*TBC1D20 included the restriction sites of *Nco*I and 6xHistidine Tag in the forward primer and stop codon and restriction site of *BamH*I in the reverse primer. The PCR product was analysed and separated by 1.2% agarose gel electrophoeresis, the size of this PCR product was observed at 650 bp (Fig 3.21). This PCR product could translate to a 208 amino acid protein with predicted molecular weight of 24.9 kDa. The PCR product of TBC domain sequence was later purified and ligated to T&A cloning vector to increase the amount of PCR product before ligated with the expression vector (Fig. 3.22).

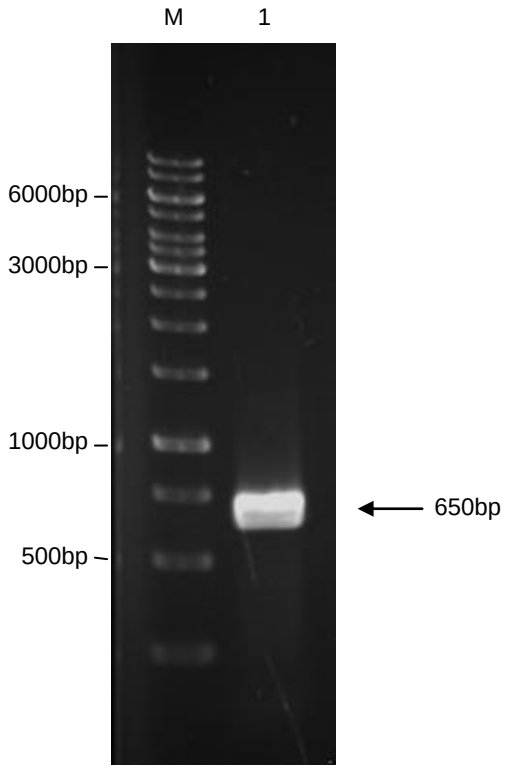


Fig. 3.21 Agarose gel electrophoresis of TBC domain sequence of *Pm*TBC1D20 amplified by Phusion hot start *Tag* DNA polymerase (Finzyme).

Lane M : 1 kb marker

Lane 1 : amplified TBC sequence product

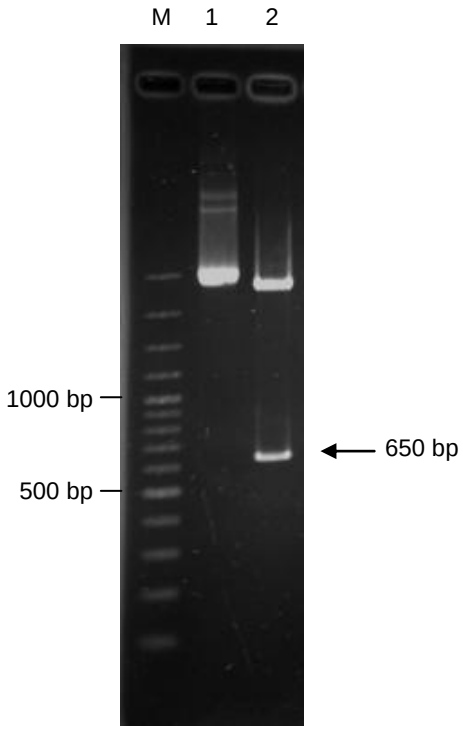


Fig. 3.22 Ligation of TBC sequence to T&A cloning vector. Amplified TBC sequence of *Pm*TBC1D20 was ligated to T&A cloning vector (RBC bioscience) and transformed to *E.coli* XL-1 blue to increase the amount of inserted DNA. After gaining the ligated vector, TBC sequences was digested by *Hind*III restriction enzyme to obtain only inserted DNA fragment. The product was analysed by 1.2% agarose gel electrophoresis. The TBC sequence was eluted from the agarose gel for further use.

Lane M : 100bp marker

Lane 1 : uncut T&A cloning vector containing TBC sequence

Lane 2 : T&A cloning vector digested with *Hind*II

3.7.1.2 Preparation of pET 19b expression vector

pET19b expression vector (novagen) was used to produce the recombinant TBC protein of *Pm*TBC1D20. As the expression vector, pET19b which carried TBC domain sequence could be transformed to *E.coli* rosetta and then expressed the TBC protein within the cells. pET19b had T7 promotor for induction at the high rate protein expression. pET19b was cleavaged at *Nco*I and *BamH*I site in the multiple cloning site (MCS) and separated on 0.8% agarose gel as shown in Fig. 3.23 the product size of digested pET19b was about 5640 bp (Fig. 3.23).

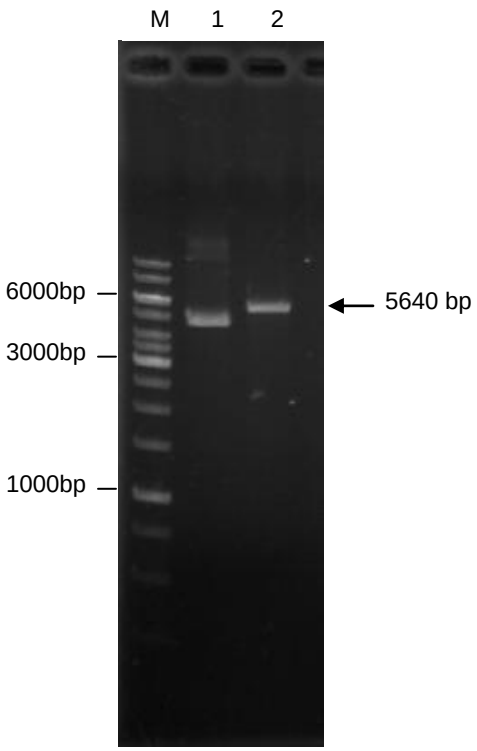


Fig. 3.23 Expression vector, peT19b, was double digested with *Nco*I and *BamH*I restriction enzymes and analysed on 0.8% agarose gel. The digested pET19b was eluted from the agarose gel for further usage.

Lane M : 1kb marker

Lane 1 : pET19b uncut

Lane 2 : pET19b digested with *Nco*I and *Bam*HI

3.7.1.3 Construction of the recombinant plasmid pET19b-TBC

The TBC sequence in 3.8.1 was digested with restriction enzymes *Nco*I and *BamH*I before ligated with the digested pet19b in 3.8.2 by T4 DNA ligase. The ligated product was transformed to *E.coli* XL-1 blue cells to gain the complete ligation of TBC sequence and pET19b. The white *E.coli* cells which contained pET19b-TBC plasmid were used and the completed pET19b-TBC plasmid was extracted for further use. The pET19b-TBC was checked for the inserted DNA by digestion with restriction enzyme *Nco*I and *BamH*I and analysed on 1.2% agarose gel. The result showed that expression vector pET19b certainly contained TBC sequence

as the inserted DNA (Fig. 3.24). The plasmid pET19b-TBC was further sequencing to check the accuracy of the inserted TBC sequence.

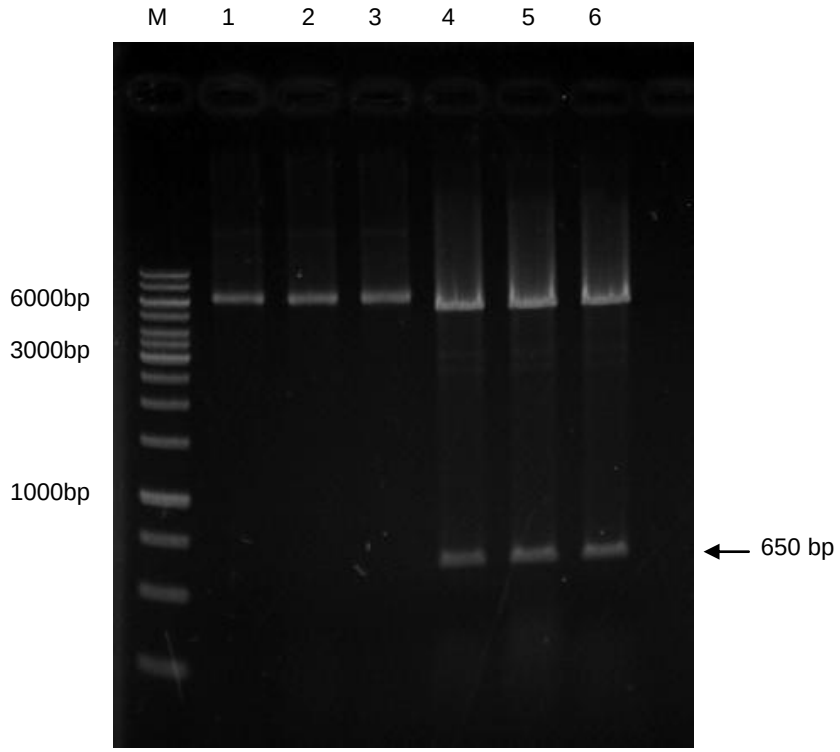


Fig. 3.24 TBC sequence was ligated to pET19b with T4 DNA ligase and transformed to *E.coli* XL-1blue. The extracted plasmid pET19b-TBC from 3 colonies were digest with *Nco*I and *BamH*I to check the inserted DNA size. The products were analysed on 1.2% agarose gel.

Lane M : 1kb marker

Lane 1-3 : uncut pET19b-TBC

Lane 4-6 : pET19b-TBC digested with *Nco*I and *BamH*I

3.7.1.4 Transformation of pET19b-TBC and expression of the recombinant TBC protein (rTBC)

After gaining the expression vector pET19b-TBC, the vector was then transformed to *E.coli* Rosetta (DE3) competent cells by heat shock method. The colony of transformed *E.coli* was selected on LB-agar plate containing chloramphenicol and ampicillin antibiotic. The selected colony was further used for

the recombinant protein expression. The recombinant TBC protein (rTBC) with histidine tag (His_6) at N-terminus was produced in the *E. coli* induced by adding IPTG to final concentration of 1 mM and then incubated for 0, 1, 2, 3,4 and 6 h. The overexpression of rTBC was analyzed by 15% SDS-PAGE and western blot analysis using anti-His antibody. The result showed that the rTBC was expressed at the highest level at 4 h post-IPTG induction, while its expression detected in trace in *E. coli* without IPTG-induction (Fig. 3.25). Moreover, the rTBC was found to be principally located in inclusion bodies, not in soluble form (Fig. 3.26).

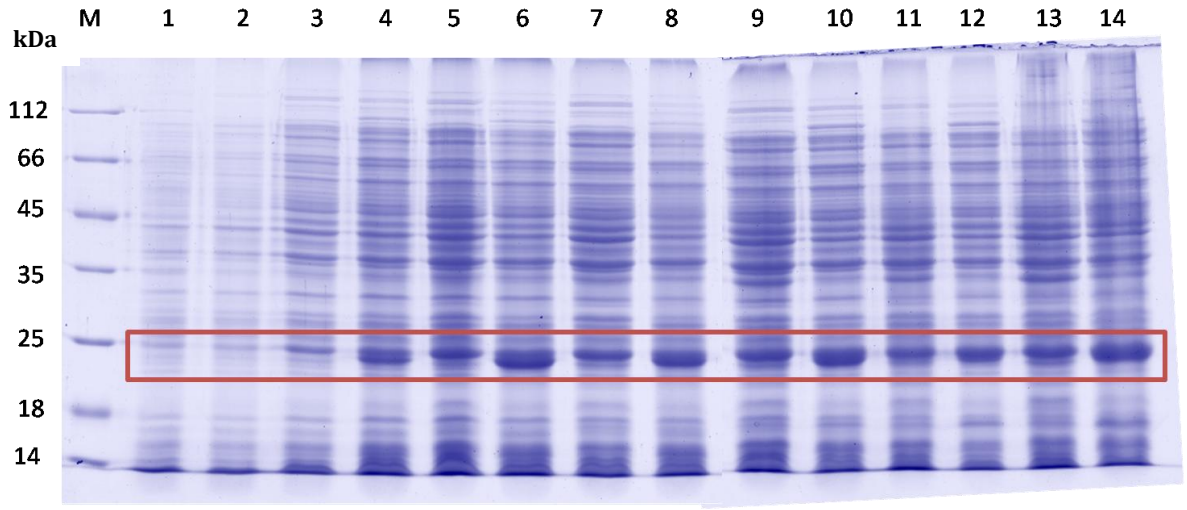


Fig. 3.25 15% SDS PAGE analysis of the recombinant TBC protein expression in *E.coli* Rosetta (DE3) with respective induction time. *E.coli* colony containing pET19b-TBC was inoculated in LB medium with antibiotic of chloramphenicol and ampicillin. The overexpression of the recombinant TBC protein was induced with 1mM IPTG. The *E.coli* culture was collected at the time of 0, 1, 2, 3, 4, 6 hour and overnight after induction.

Lanes 1, 3, 5, 7, 11, and 13 are uninduced cells at 0, 1, 2, 3, 4, 6, and overnight, respectively.

Lanes 2, 4, 6, 8, 10, and 12 are induced cells at 0, 1, 2, 3, 4, 6, and overnight, respectively

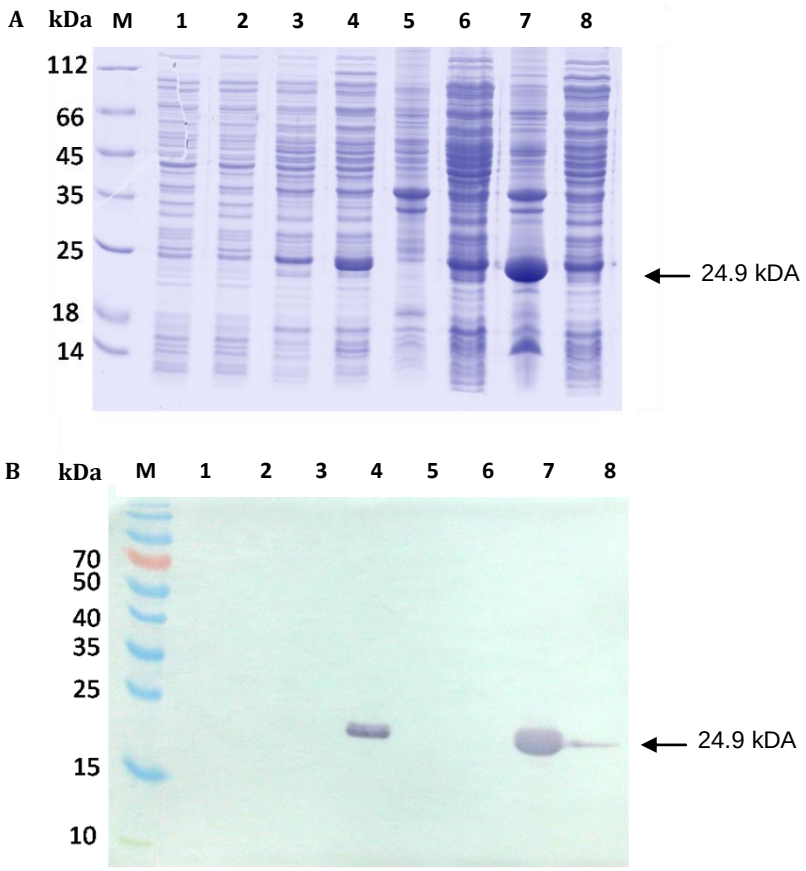


Fig. 3.26 Protein expression profile of the TBC protein. A: SDS-PAGE analysis of the TBC protein. B: Western blot analysis using anti-His antibody revealed major band of the TBC protein at molecular weight of 24.9 kDa.

Lane 1: un-induced cells at 0 h,

Lane 2: 1mM IPTG induced cells at 0 hr

Lane, 3: un-induced cells at 4 h,

Lane 4: 1mM IPTG induced cells at 4 hr,

Lane 5: inclusion body of un-induced cells at 4 h,

Lane 6: supernatant of un-induced cells at 4 h,

Lane 7: inclusion body of induced cells at 4 h,

Lane 8: supernatant of induced cells at 4 h,

Lane M: PageRuler™ unstained and Prestained Protein Ladder (fermentas) respectively.

3.7.1.5 Purification of the recombinant TBC protein

After overexpression of rTBc at 4 hrs, the *E.colli* cells were harvested by centrifugation and resuspended in 1X PBS buffer before sonication. The pellet obtained from the sonicated cells were washed and then resuspended in denaturing buffer containing 8M urea to solubilize the protein pellet before purified by Ni-Sepharose 6 fast-flow (GE healthcare) column. The rTBC was eluted with the stepwise gradient of different concentration of imidazole in the eluted buffer of 100, 300, and 500 mM. The eluted fraction of purified protein was analysed on 15% SDS PAGE. The result showed that the rTBC protein was eluted at 300 mM imidazole (Fig. 3.27). After purified with Ni-sepharose and eluted at 300 mM imidazol, the purified rTBC protein was further dialyzed against 50mM carbonate buffer, pH 10, the protein was also diazlyzed against deionized water for further synthesized anti-TBC antibody. Western blot analysis using anti-His antibody revealed the major band which belongs to TBC protein. After that, the recombinant TBC protein was used for synthesis of specific antibody. Western blot analysis using anti-TBC antibody revealed the major band which belongs to the TBC protein. Anti-TBC antibody was used for further localization of *Pm*TBC1D20 protein (Fig. 3.28).

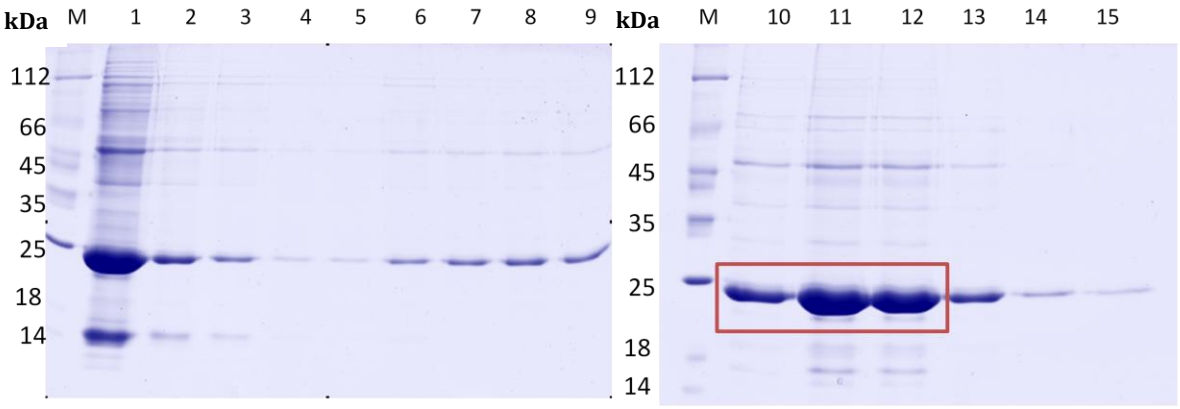


Fig. 3.27 Stepwise gradient elution fractions of the purified recombinant TBC protein by Ni-Sepharose column. Recombinant TBC protein was eluted from the column with different concentration of imidazole in elution buffer at 50, 100, 300, and 500 mM imidazole. The purified recombinant protein was eluted at the concentration of 300 mM imidazole.

Lane M : unstained protein marker

Lane 1 : Flow through

Lane 2 : denaturing buffer (loading buffer)

Lane 3-5 : elution of 50 mM imidazole

Lane 6-9 : elution of 100 mM imidazole

Lane 10-12 : elution of 300 mM imidazole

Lane 13-15 : elution of 500mM imidazole

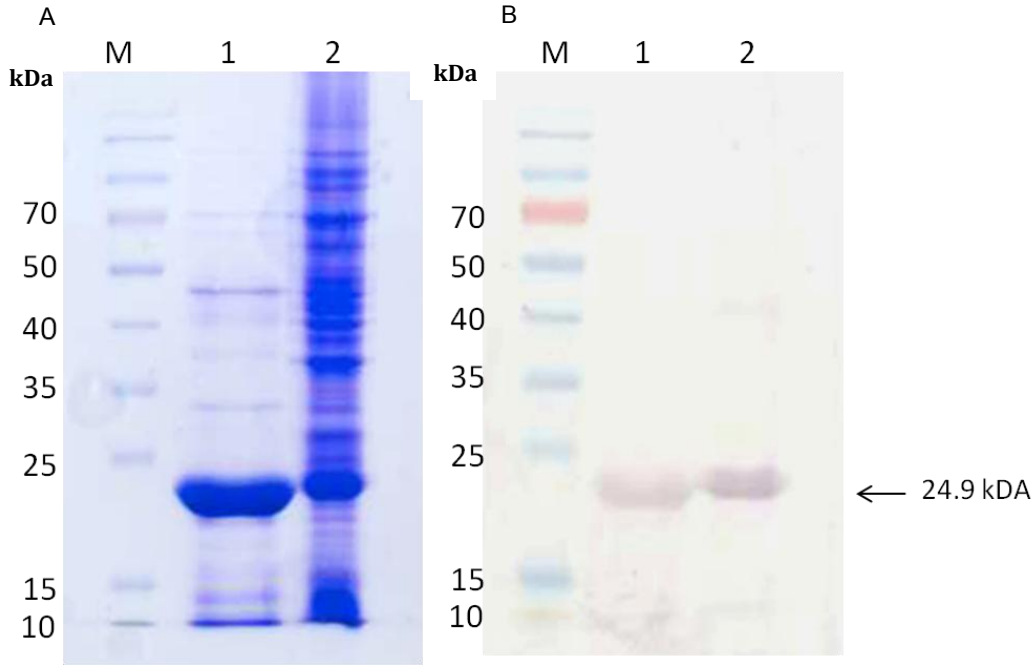


Fig. 3.28 (A) SDS-PAGE analysis of TBC protein. (B) Western blot analysis using anti-TBC antibody revealed major band refered to TBC protein.

Lane M : PageRuler™ Prestained Protein Ladder (fermentas)

Lane 1 : purified TBC protein

Lane 2: crude cells with 1 mM IPTG induction at 4 h.

3.7.2. Localization of *Pm*TBC1D20 protein

In order to identify the type of hemocytes that produced the *Pm*TBC1D20 protein, a confocal laser scanning microscopy and immunofluorescence technique were performed. Hemolymph from three healthy shrimp was collected and fixed on the poly-L-lysine coated slide. The fixed hemocyte slide was incubated with the purified anti-TBC antibody for the test sample and incubated with BSA as a negative control. After incubated with secondary antibody conjugated with fluorescence dye, Alexa 488, the nucleus of the hemocyte was stained with the To-Pro-III-iodide. The *Pm*TBC1D20 protein was observed as green while the hemocyte nucleus was adjusted to the blue color. In the normal hemocytes, the bright field and immunofluorescence image were observed positive signal of TBC1D20 in cytoplasm and a nuclear membrane in all types of hemocyte cells; hyaline, semi-granular and granular cells as demonstrated in Fig. 3.29A.

3.7.3 Co-localization of *Pm*TBC1D20 and VP28 protein after WSSV challenge

The effect WSSV infection on *Pm*TBC1D20 protein in shrimp was further investigated. Six Shrimp were injected with WSSV whereas the control group was uninfected. The hemocytes were collected from each group at 24 h later for colocalization of the *Pm*TBC1D20 and the VP28 protein within hemocyte cells. The confocal immunofluorescence microscopy illustrated colocalization of the two partner proteins within cell (Fig. 3.29B). The *Pm*TBC1D20 signal in the 48 h-WSSV-infected hemocytes was higher than that in the uninfected hemocytes. In addition, the VP28 protein was detected at 48 h in the nucleus of infected hemocytes which detected a *Pm*TBC1D20 signal in those cells (Fig. 3.29B). Moreover, the WSSV-infected cells detecting with VP28 signal was in the apoptosis state, so they lost their cytoplasm.

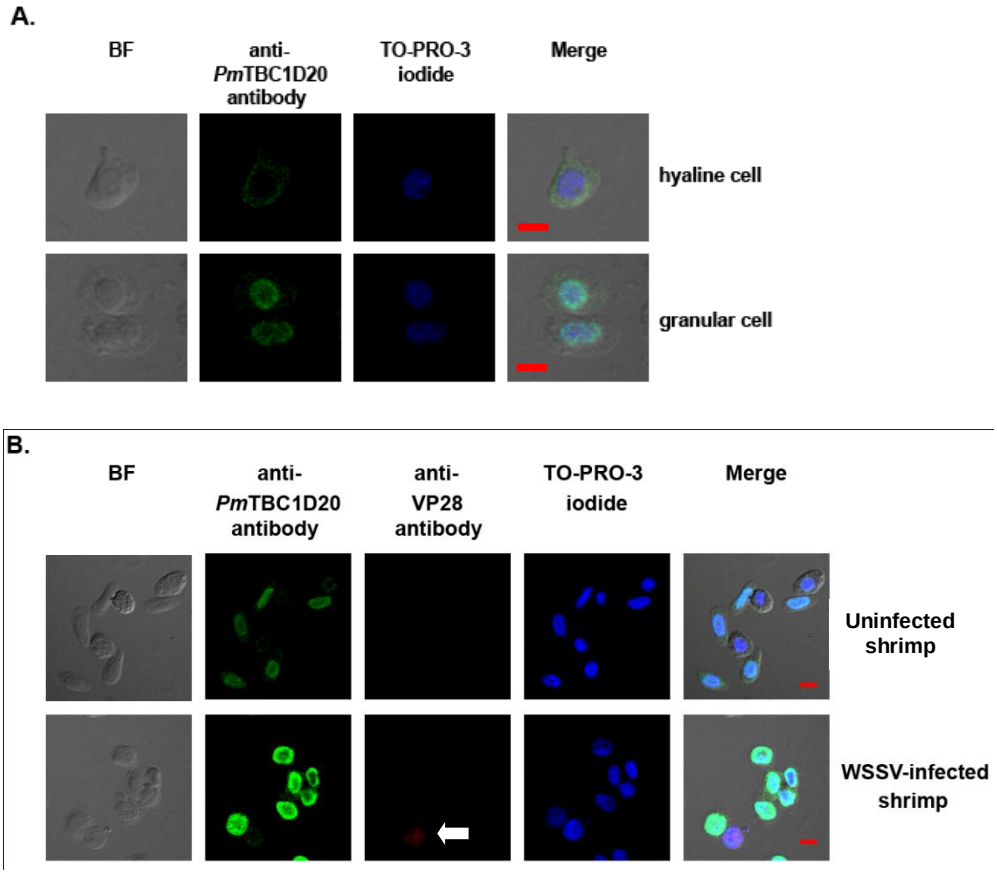


Fig. 3.29 Localization of *Pm*TBC1D20 and VP28 in uninfected and WSSV-infected *P. monodon* hemocytes by immunofluorescence confocal laser scanning microscopy. (A) *Pm*TBC1D20 was localized in uninfected shrimp hemocyte (hyaline and granular cell). (B) Uninfected, WSSV- infected at 48 h were visualized the TBC1D20 and VP28 using purified polyclonal anti- *Pm*TBC1D20 and monoclonal anti-VP28, respectively. The fluorescence was developed using secondary antibodies conjugated with Alexa Flour 488 (green) for TBC1D20 and Alexa Flour 568 (red) for VP28. Nuclei (adjusted to blue color) were stained with TO-PRO-3 iodide. BFs are bright field images. Bars = 5µm.

3.7.4 Subcellular localization of *Pm*TBC1D20 protein on *Pm*TBC1D20 depletion in WSSV-infected shrimp

Since *Pm*TBC1D20 suppression led to decrease the transcript of ie1, WSV 477, and VP28 gene, the expression of *Pm*TBC1D20 protein in hemocyte of *Pm*TBC1D20 depletion was explored. Six Shrimp per group were injected with ds*Pm*TBC1D20 or dsGFP or NaCl. After 24 h, all shrimp were secondly injected with WSSV. The hemocytes were collected from each group at 24 h later for colocalization of the *Pm*TBC1D20 and the VP28 protein within hemocyte cells (Fig. 3.30). The results demonstrated that the *Pm*TBC1D20 signal was reduced in the hemocytes of ds*Pm*TBC1D20-infected shrimp compared to the signal in the hemocytes of dsGFP- or NaCl-treated shrimp after WSSV infected shrimp. The hemocyte cells of ds*Pm*TBC1D20 or dsGFP treated cells remained healthy during the 48 h after WSSV injection compared to the control, WSSV- and NaCl-treated cells. The number of WSSV-infected hemocyte cells was observed the highest in the NaCl-treated shrimp followed by the dsGFP- and dsTBC1D20-treated shrimp, respectively.

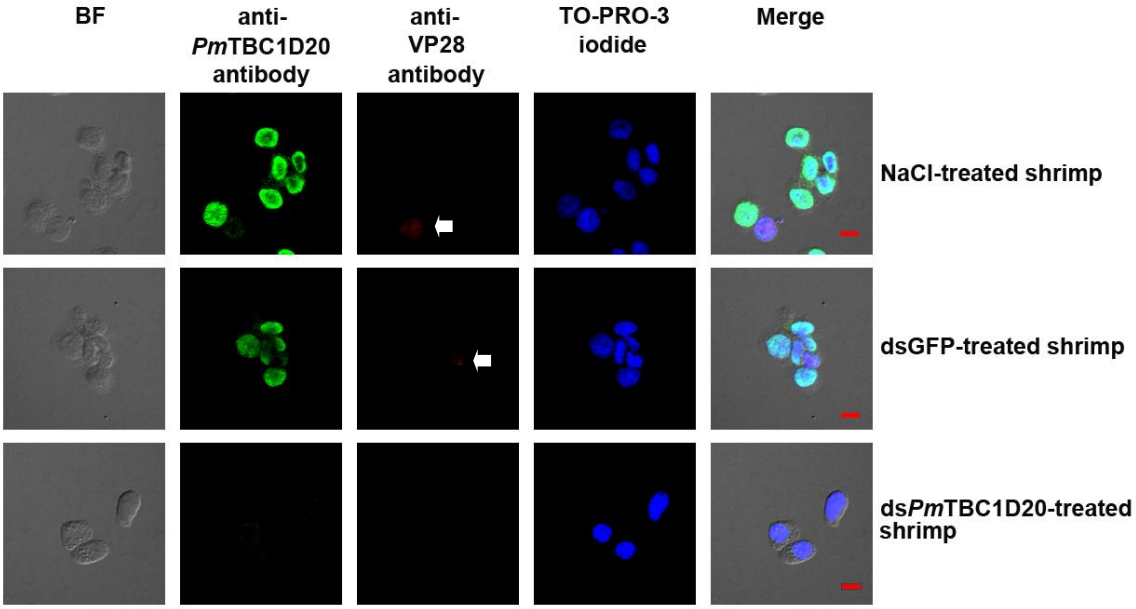


Fig. 3.30 Suppression of *Pm*TBC1D20 reduced WSSV-infection in shrimp. Shrimp were injected with dsTBC1D20, dsGFP or NaCl and then WSSV was also injected. The hemocytes were collected and the TBC1D20 and VP28 were visualized using purified polyclonal anti- *Pm*TBC1D20 and monoclonal anti-VP28, respectively. The fluorescence was developed using secondary antibodies conjugated with Alexa Flour 488 (green) for TBC1D20 and Alexa Flour 568 (red) for VP28. Nuclei (adjusted to blue color) were stained with TO-PRO-3 iodide. BFs are bright field images. Bars = 5μm.

CHAPTER IV
DISCUSSION

Penaeus monodon farming industry in Thailand is facing the problem of infectious disease outbreaks which leads to massive loss of shrimp and affect to income earning from shrimp exportation. These outbreaks are mainly caused by virus infection. One of the most harmful viruses which cause a lot of mortality loss is White spot syndrome virus (WSSV). The syndrome caused by WSSV has no effective prevention or appropriate solution enough to control the outbreak. Shrimp farming management such as Biosecurity is the most effective way now to prevent the virus infection from the environment whereas chemical substance or antibiotic drug is used to protect or cure from the syndrome. Understanding the mechanism of virus-host infection and immune system of shrimp will finally lead to a discovery of the effective solution of the virus outbreak in shrimp farming.

When shrimp was infected with WSSV, many genes were altered their expression level to response to the infection. So, these genes were called viral responsive genes. Viral responsive genes which were upregulated in different times after WSSV infection showed different function response to the virus. The immune-related genes almost up-regualted at the early or middle phase of infection then down-regulated at the late phase such as in many antimicrobial peptides (AMPs). Crustin-1, crustin-2 and penaeidin-5 could be observed during early phase of WSSV infection and a noticeable down-regulation during late phase of infection (Swapna et al., 2011; Woramongkolchai et al., 2011). While, antilipopolysachharide factors (ALFs) which had antiviral potential against WSSV in penaeid shrimp by interfering the virus replication was up-regulated from the early to late phase of WSSV infection. (Swapna, 2011; Aoki et al., 2011). However, the viral responsive genes which both up and down regulated at the late phase of infection could refer that virus used these genes to work for its maintenance and production for more virus particle in the host cells. Viral responsive gene related to signaling and communication such as genes involved in G protein signal cascade, were up-regulated in the late phase of infection.

These G protein cascade was important in signal transduction for molecules within the cells (Pongsomboon et al., 2011).

The G-protein family, such as Rab GTPase family, is made up of a diverse range of molecules that control a complex array of biological processes but structurally homologues to GTP-binding domain. They act as molecular switches that cycle between the active, GTP-bound form and the inactive, GDP-bound form (Bourne et al., 1990; Bourne et al., 1991; Boguski et al., 1993). In the active form, they are competent to interact with a broad range of effector molecules. Whereas the lifetime of this active state is determined by the combination of slow intrinsic GTPase activity and the action of GTPase-activating proteins (GAPs), which can accelerate GTP hydrolysis. GTP hydrolysis causes conformational changes in the G protein that are localized to two distinct regions of the molecule. The cycling between GTP and GDP can lead to many processes in cells such as membrane trafficking pathway which used to transport any membrane-bound protein or cargo within the cells from one organelle to another for processing or modifying the protein or sending the protein or cargo out of the cells (Barr, 2006). In shrimps, some of Rab GTPases were found to have more function involved in WSSV infection, such as MjRab protein in *Marsupenaeus japonicus* was up-regulated against WSSV infection. This protein was bound to envelop ptotein VP466 of the virus and formed complex with β-actin and tropomyosin, and was the intracellular virus recognition protein to increase the phagocytic activity by regulating the hemocytic phagocytosis via this complex (Wu and Zhang, 2007; Wu et al, 2008), Rab GTPase from Chinese white shrimp *Fenneropenaeus chinensis* had been reported to up-regulate against both *Vibrio harveyi* and WSSV. Pull-down assay indicated that Rab GTPase interact with VP28 which is the envelope protein of WSSV (Ren et al., 2011), and knocking down of Rab7 GTPase, a receptor of VP28 envelope protein of WSSV, in *P. monodon* has antiviral effect to WSSV and Leam-Singh virus (Ongvarrasopone et al, 2006; Ongvarrasopone et al, 2010).

GTPase activating proteins (GAPs) were thought to limit Rab GTPase activity (Barr and Lambright, 2010). TBC domains, as the outstanding domain in the GAPs, play roles in regulating many Rab GTPases. More than 40 kinds of TBC protein had been reported and studied mostly in human. TBC proteins have many different

functions such as acting as oncoprotein, formation of primary cilia (Yoshimura et. al., 2007), macropinocytosis (Frittoli et al,2007; Lanzetti et al., 2004), immunological synapse formation (Patino-Lopez et al., 2008), cytokinesis (Yoshimura et al., 2007; Faitar et al., 2005; Faitar et al., 2006) and autophagy (Behrends et al., 2010). Moreover, TBC protein involved in Shiga toxin uptake from *Shigella dysenteriae*, a bacterial which its toxin inhibit protein synthesis (Fuchs et al, 2007). However, there were few researches of TBC protein that involved in virus infection. TBC1D20 which specific to Rab1 GTPase has more function not only to inactivate Rab1 GTPase but also involved in virus replication as reported in human Hepatitis C virus (HCV) (Sklan et al., 2007a). In addition, in Dorsophila, CG17883 gene was predicted to have TBC domain and transmembrane region at the C-terminus which is similar to TBC1D20 in human. This gene was found to localize to the ER similar to the report in human cells. The result confirmed that CG17883 was likely to be a genuine component of the secretory pathway in dorsophila cells (Wendler et al., 2010). However, there is less understanding of viral infection especially in shrimp.

Here, a novel gene named *Pm*TBC1D20 obtained from EST library and found to be reported to WSSV as analysed by microarray (unpublished data). This gene was up-regulated when the shrimp was infected with the WSSV and show high similarity to TBC1D20 protein. So these led us to study further for its function which is the first study of TBC domain in shrimp.

Tissue distribution of *Pm*TBC1D20 transcript analyzed using semi-quantitative RT-PCR was found to be expressed in all tissues examined including hemocyte. Quantitative real time RT-PCR showed that *Pm*TBC1D20 gene was up-regulated by 2.2 and 2.0 fold after WSSV-challenged shrimp at 24 and 48 hpi, respectively. In addition, co-immunofluorescence technique demonstrated that *Pm*TBC1D20 protein was up-regulated after WSSV-infection.

From the full-length sequence obtained from Rapid Amplification of cDNA ends, the open reading frame contained 480 amino acid including a TBC domain of about 213 amino acid located at middle of the protein. From structure analysis, this protein also contained transmembrane region at the C-terminus and had no signal peptide which is similar to TBC1D20 from human which contain transmembrane region at the C-terminus (Sklan et al., 2007b). From data of human TBC1D20,

transmembrane region is important in anchoring TBC1D20 protein with the ER. This also confirmed that TBC1D20 is a GAP for a Rab1 GTPase, because Rab1 transport protein from ER to Golgi. When blast the whole protein sequence with the Genbank database, it was found that *Pm*TBC1D20 protein show highest similarity of 45% to TBC1D20 protein from *S. kowaleskii*, but for only the TBC domain showed highest similarity to hypothetical protein TcasGA2_TC013341 which contain TBC domain from the red flour beetle from *T. casenum* at 58% and followed by 55% from TBC1D20 protein from *S. kowaleskii*. The sequence alignment of TBC domain of *Pm*TBC1D20 protein among TBC domains from other TBC protein from many organisms showed 6 motifs, including the important three signature motifs (A-C) (Fig. 3.11) suggesting that this protein was highly conserved in various organisms in both invertebrates and vertebrates. When determining the signature motifs of TBC domain, *Pm*TBC1D20 protein contains the catalytic arginine in motif B called arginine finger which is key amino acid in enhancing the hydrolysis rate of Rab protein as described in the research from Pan et al. (2007). While glutamine in the motif C helped glutamine in the Rab GTPase to mediate polar interaction equivalent in the complexes between GAP and Rab GTPase (Rittinger et. al., 2006). The amino acid of TBC domain in motif B is similar to human TBC1D20 that the first amino acid in motif B is valine (V) instead of other TBC protein which is isoleucine (I).

In functional analysis in order to further understand the effect of *Pm*TBC1D20 depletion upon WSSV infection, RNA interference technique and primary cell culture technique were used. dsRNA of *Pm*TBC1D20 gene was transfected using transfection protein to the hemocyte of *P.monodon* and incubate for 24 hour prior to knock down the *Pm*TBC1D20 gene before infected WSSV to the primary cells. The dsGFP was used as the internal control of this experiment. The result by RT-PCR showed that after 24 hour of infection, the mRNA level of *Pm*TBC1D20 was decreased and the mRNA level of VP28 gene of WSSV was also decreased. The quantitative real time PCR showed that mRNA expression level of VP28 gene of WSSV was decreased to 50% from the control. Moreover, the silencing of *Pm*TBC1D20 transcript in WSSV-infected shrimp resulted in decreasing WSSV gene at all states including intermediate early gene (ie1), early gene (WSV477), and late gene (VP28). This result also indicated that *Pm*TBC1D20 gene involved in virus infection that knocking down this

gene could prevent new infection as little intermediate early gene expression level was detected in the WSSV-infected shrimp. In addition, the depletion of *Pm*TBC1D20 in shrimp was also illustrated the lower WSSV-infected hemocytes by coimmunofluorescense technique. However, the ie1 transcriptional level in the dsGFP control group was also decreased. The reason might be that the non-sequence specific dsRNA, such as dsGFP, might have antiviral activity against WSSV infection as mention by Hirono et al. in 2011.This experiment was observed at 24 hpi which was in the late phase of infection while ie1 gene and WSV477 gene responsed at early phase. So, more observation at early phase of infection at 3 and 6 hpi would be better for more significant results. Moreover, the strong signal of the *Pm*TBC1D20 protein in the WSSV-infected hemocyte under the confocal microscope by coimmunofluorescense technique could be assumed that this protein was expressed much more than the uninfected hemocyte. These results suggested that *Pm*TBC1D20 was not only upregulated in the transcriptional stage but also in the translational stage when infected to the WSSV. In human, depletion of TBC1D20 led to dramatically decrease of the human hepatitis C (HCV) mRNA level to 50% and inhibition of HCV infection (Sklan et al., 2007a). The interaction between the N terminal of HCV nonstructural protein NS5A and *Hs*TBC1D20 may subvert host cell machinery for mediating the endocytosis, trafficking and also sorting of the viral proteins (Sklan et al., 2007b). For the herpes simplex virus which is RNA virus, TBC1D20 and RN-tre which are partners of two Rab GTPase, Rab 1a/b and Rab43, respectively are important for the trafficking of the virus envelope proteins from the ER to Golgi (Zenner et al., 2011). These suggested that *Pm*TBC1D20 protein which contain TBC domain might help virus to infect and reproduce using host system. However, the mechanism of this protein in WSSV infection and replication remains unknown. One hypothesis that can possibly be is that virus used host membrane trafficking system to work for its maintenance and reproduction (Sklan et al., 2007a; Sklan et al., 2007b).

From the data mention above, *Pm*TBC1D20 containing TBC domain was the first GAP discovered in *P.monodon* and played role in WSSV infection. Previous study in shrimp, most of them reported only the Rab GTPase, but not their effectors. This discovery of this Rab-GAP involed in WSSV infection might lead us to better

understanding to the molecular events involved in shrimp and virus infection and replication mechanism.

CHAPTER V
CONCLUSIONS

*Pm*TBC1D20 was first found as a novel gene responded to WSSV infection in shrimp and its partial sequence showed high degree of similarity to TBC1D20 protein which is GTPase activating protein. The full-length cDNA of *PmTBC1D20* was 2004 bp which translated to a 480 amino acid protein. This protein contains a TBC domain of 213 amino acid located at the middle and also contained transmembrane region at C-terminus and had no signal peptide. The deduced amino acid of *Pm*TBC1D20 revealed 45% similarity to the TBC1D20 protein from *Saccglossus kowaleskii*. However, only TBC domain of this protein demonstrated 58% homology to TcasGA2_TC013341 which contain TBC domain from the red flour beetle from *Tribolium casenum.*

☐ Tissue distribution showed that *Pm*TBC1D20 gene was expressed in all tissues, including hemocyte. Transcription level of *Pm*TBC1D20 transcript was up-regulated by 2.2 and 2.0 fold at 24 and 48 h after WSSV infetction, respectively. Moreover, immunofluorescence hybridization approach illustrated that the signal of the *Pm*TBC1D20 protein in hemocytes of the WSSV-infected shrimp was significant increased comparing with that in hemocytes of the uninfected shrimp. This result proved that *Pm*TBC1D20 was a viral responsive gene.

☐ Suppression of *Pm*TBC1D20 gene by RNAi resulted in a decrease in the VP28 transcript by about 50% in the WSSV-infected primary hemocyte cells and a decrese in the VP28, ie1, and WSV477 transcript at approximately 90% in the WSSV infected shrimp. Moreover, under the fluorescence microscope, the VP28 protein was detected less in the hemocytes of *Pm*TBC1D20 suppressed shrimp. This result indicated that *Pm*TBC1D20 was involved in virus infection.

Localization of *Pm*TBC1D20 protein in shrimp hemocyte with anti-TBC antibody revealed that *Pm*TBC1D20 localized in both cytoplasm and plasma membrane in every cell types of hemocytes; hyaline cell, semi-granular cell, and granular cell.

Taken together, it can be concluded that *Pm*TBC1D20 was a viral responsive protein and involved in WSSV infection probably by promoting the replication of the virus.

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APPENDICES

Appendix A

1. Preparation for SDS-PAGE electrophoresis

Stock reagents

30 % Acrylamide, 0.8% bis-acrylamide, 100 ml

Acrylamide 29.2 g

N,N′-methylene-bis-acrylamide 0.8 g

Adjust volume to 100 ml with distilled water.

1.5 M Tris-HCl pH 8.8

Tris (hydroxymethyl)-aminomethane 18.17 g

Adjust pH to 8.8 with 1 M HCl and adjust volume to 100 ml with distilled water.

2.0 M Tris-HCl pH 8.8

Tris (hydroxymethyl)-aminomethane 24.2 g

Adjust pH to 8.8 with 1 M HCl and adjust volume to 100 ml with distilled water.

0.5 M Tris-HCl pH 6.8

Tris (hydroxymethyl)-aminomethane 6.06 g

Adjust pH to 6.8 with 1 M HCl and adjust volume to 100 ml with distilled water.

1.0 M Tris-HCl pH 6.8

Tris (hydroxymethyl)-aminomethane 12.1 g

Adjust pH to 6.8 with 1 M HCl and adjust volume to 100 ml with distilled water.

Solution B (SDS PAGE)

2.0 M Tris-HCl pH 8.8 75 ml

10% SDS 4 ml

Distilled water 21 ml

Solution C (SDS PAGE)

1.0 M Tris-HCl pH 8.8 50 ml

10% SDS 4 ml

Distilled water 46 ml

SDS-PAGE

15 % Seperating gel

30 % Acrylamideml solution 5.0 ml

Solution B 2.5 ml

Distilled water 2.5 ml

10% (NH4)2S2O8 50 µl

TEMED 10 µl

5.0 % Stacking gel

30 % Acrylamideml solution 0.67 ml

Solution C 1.0 ml

Distilled water 2.3 ml

10 % (NH4)2S2O8 30 µl

TEMED 5.0 µl

5X Sample buffer

1 M Tris-HCl pH 6.8 0.6 ml

50% Glycerol 5.0 ml

10% SDS 2.0 ml

2-Mercaptoethanol 0.5 ml

1 % Bromophenol blue 1.0 ml

Distilled water 0.9 ml

One part of sample buffer was added to four parts of sample. The mixture was heated 5 min. in boiling water before loading to the gel.

Electrophoresis buffer, 1 litre

(25 mM Tris, 192 mM glycine)

Tris (hydroxymethyl)-aminomethane 3.03 g

Glycine 14.40 g

SDS 1.0 g

Dissolve in distilled water to 1 litre. Do not adjust pH with acid or base (final pH should be 8.3).

Appendix B

_Pm_TBC1D20 mRNA expression in the hemocytes of WSSV infected shrimp at various time point

ANOVA

VAR00002					
	Sum of Squares	Df	Mean Square	F	Sig.
Between Groups	3.482	3	1.161	14.813	.001
Within Groups	.627	8	.078		
Total	4.109	11			

Duncan

VAR00001	N	Subset for alpha = 0.05	
		1	2
0.25h	3	1.0239161E0	
12h	3	1.2136977E0	
48h	3		2.1094516E0
24h	3		2.2559724E0
Sig.		.430	.539

Means for groups in homogeneous subsets are displayed.

Suppression of the *Pm*TBC1D20 transcript in WSSV infected primary hemocytes of *P.monodon*

ANOVA

VAR00002

	Sum of Squares	Df	Mean Square	F	Sig.
Between Groups	.487	2	.244	9.673	.013
Within Groups	.151	6	.025		
Total	.638	8			

Duncan

VAR00001	N	Subset for alpha = 0.05	
		1	2
group2	3	.4715038	
group1	3	.5508520	
control	3		1.0000000
Sig.		.563	1.000

Means for groups in homogeneous subsets are displayed.

VP28 transcription level in ds*Pm*TBC1D20 suppression in WSSV infected shrimp *P. monodon*

ANOVA

VAR00002

	Sum of Squares	df	Mean Square
Between Groups	4.368	6	.728
Within Groups	.044	14	.003
Total	4.412	20	

VAR00002

Duncan

VAR00001	N	Subset for alpha = 0.05		
		1	2	3
PmTBC!D20-1	3	.0026		
PmTBC1D20-3	3	.0103		
PmTBC1D20-2	3	.0705		
GFP1	3		.8043	
GFP3	3		.8610	
control	3			1.0000
GFP2	3			1.0710
Sig.		.180	.236	.143

Means for groups in homogeneous subsets are displayed.

Ie1 transcription level in ds*Pm*TBC1D20 suppression in WSSV infected shrimp *P. monodon*

ANOVA

VAR00002					
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2.521	6	.420	263.126	.000
Within Groups	.022	14	.002		
Total	2.543	20			

VAR00002

Duncan

		Subset for alpha = 0.05				
VAR00001	N	1	2	3	4	5
PmTBC!D20-1	3	.0006				
PmTBC1D20-3	3	.0024				
PmTBC1D20-2	3	.0282				
GFP2	3		.3063			
GFP1	3			.4482		
GFP3	3				.5958	
control	3					1.0000
Sig.		.437	1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

WSV477 transcription level in ds*Pm*TBC1D20 suppression in WSSV infected shrimp *P. monodon*

ANOVA

VAR00002

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	4.294	6	.716	406.606	.000
Within Groups	.025	14	.002		
Total	4.319	20			

VAR00002

Duncan

		Subset for alpha = 0.05			
VAR00001	N	1	2	3	4
PmTBC!D20-1	3	.0058			
PmTBC1D20-3	3	.0112			
PmTBC1D20-2	3		.1159		
GFP1	3			.8522	
GFP2	3				.9683
GFP3	3				.9845
control	3				1.0000
Sig.		.876	1.000	1.000	.395

Means for groups in homogeneous subsets are displayed.

Biography

Miss Wanchart Yingvilasprasert was born on May 5, 1986 in Bangkok. She graduated with the degree of Bachelor of Science in 2008. She has studied for the degree of Master of Science at the Department of Biochemistry, Chulalongkorn University since 2008. In 2012, she had an oral presentation on her work in the topic of "Characterization of a novel protein containing TBC domain in the black tiger shrimp *Penaeus monodon* and its role in reponse to white spot syndrome virus infection" at the 1st ASEAN Plus three Graduate Reserch Congress (ARGC 2012) which organized by Consortium of the Graduate Studies Administrators of Public and Autonomous Universtites (CGAU) Thailand at the Empress hotel, Chiang Mai on March 1-2, and publish in the conference proceedings.