A THESIS

FOR THE DEGREE OF DOCTOR OF PHYLOSOPHY

TRANSCRIPTOME-WIDE DISCOVERY OF INNATE IMMUNE-RELATED GENES AND MOLECULAR CHARACTERIZATION OF THE INFLAMMATORY/APOPTOTIC PATHWAY IN MANILA CLAM (*Ruditapes philippinarum*)

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GRADUATE SCHOOL

JEJU NATIONAL UNIVERSITY

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요약문

바지락은 전세계적으로 매우 중요한 양식 이매패류 종으로 한국을 비롯한 동아시아 지역과, 유럽 및 북미에서 넓게 서식하고 있다. 최근 바지락의 대량 폐사에 대한 연구가 보고되고 있다. 이러한 바지락의 대량 패사는 다양한 환경 요인, 환경오염, 그리고 감염 등이 그 원인으로 보고되었으며, 그중에서 특히 brown ring disease (BRD)와 perkinsus 감염증이 중요 원인으로 알려져 있다. BRD 와 perkinsus 감염증과 같은 병원체에 의한 대량패사는 바지락의 생산량에 커다란 영향을 미치고 있어 경제적 손실 또한 커지고 있기 때문에 이러한 문제의 해결을 위하여 바지락의 면역체계에 대한 연구에 대한 관심이 증가하고 있다. 병원체에 의한 바지락내의 면역반응을 이해하기 위해서는 분자 수준에서의 접근이 필요한데, 이는 해당 종에 대한 유전정보가 필수적이다. 하지만 NCBI GenBanK database 에 등록되어 있는 바지락의 유전정보는 다른 양식 이매패류종인 굴이나 가리비 등에 비하여 매우 적은 7%에 불과한 실정이다.

바지락을 포함한 연체동물은 후천면역이 결핍되어 있다고 알려져 있다. 그렇기 때문에 바지락과 같은 연체동물에서의 선천면역은 매우 중요한 역할을 하지만 해양연체동물의 선천면역반응에 대한 메커니즘 연구는 매우 미비한 실정이다. 반면 포유류에서는 광범위하게 연구가 이루어지고 있으며, 이러한 자료는 연체동물의 면역 시스템을 연구하기 위한 방향을 제시하고 있다. 바지락과 같은 무척추동물에서는 선천면역이 외부 병원체감염으로부터 방어하기 위한 주요 시스템으로 작용한다. 이런 이유로, 분자수준에서 다양한 병원체의 감염에 대한 면역반응에 대한 이해는 매우 중요하다. 이 연구에서는 바지락의 면역체계에 대한 이해를 위하여 부족한 유전정보를 확보하고자 454 Genome Sequencer Flex titanium 을 이용하여 바지락의 transcriptome 을 분석하였고, 이를 통하여 117,347 개의 유전자에 대한 정보를 확보하였고, 이중에서 NCBI 에 등록된 단백질들과의 비교를 통하여 24,327 개의 unigene 을 확보하였다. 확보한 24,327 개의 unigene 에 대한 data 로부터 중요한 면역 관련 유전자로 알려진 MyD88, IκB, TNFα 와 세포사멸에 중요한 조절자로 작용하는 것으로 알려진 두 개의 Bcl-2 family 단백질의 전체서열을 확보하고 분자적 특성 및 면역자극을 통한 발현변화를 관찰하였다.

바지락의 RpMyD88 은 1416 bp 의 ORF 로 구성되어 있으며, 471 개의 아미노산을 암호화하고 있으며, RpMyD88 의 아미노산 서열에서 MyD88 단백질의 전형적인 특징인 death doman 과 TIR 도메인을 확인 할 수 있었다. RpMyD88 의 아미노산 서열은 가리비 MyD88 과 27%로 가장 높은 identity 를 나타내었다. RpMyD88 은 바지락의 모든 조직에서 발현이 관찰되었으며, 특히 주요 면역 유전자인 hemocyte 와 gill 그리고 mantle 에서 높은

발현량을 나타내었다. 반면 siphon, foot, adductor muscle 에서는 매우 적은 양의 발현을 나타내었다. RpMyD88 은 *Vibrio tapetis* 와 LPS 를 주사한 바지락의 hemocyte 와 gill 모두에서 유의적인 발현 증가를 나타내었으며, 이러한 서열특성 및 유전자 발현에 관련된 결과들은 바지락에서도 포유류와 같은 MyD88 dependent signaling pathway 가 존재하며 RpMyD88 역시 박테리아에 대한 선천 면역반응에서 중요한 역할을 수행하고 있을 것으로 예상할 수 있었다.

NF-kB signaling pathway 는 선천면역 시스템에서 가장 중요한 구성요소 중의 하나로, NF-kB 의 활성은 inhibitor of NF-κB (IκB)와의 물리적 상호작용에 의하여 조절되게 된다. IκB 가 NF-κB 에 결합하게 되면 NF-κB 의 nuclear localization signal 을 막음으로써 NF-κB 를 불활화 상태로 Cytoplasm 에 가둬두게 된다. 3 장에서는 NF-κB 의 활성을 조절하는 IκB 의 분자적 특성과 발현 정보를 나타내었다. 바지락 IκB (Rp-IκB)의 cDNA 는 1032 bp 의 ORF 로 구성되어있고, 343 개의 아미노산을 암호화 하였다. Rp-IκB 단백질은 IκB 단백질의 전형적인 특징인 IκB degradation motif, PEST sequence 그리고 6 개의 ankyrin repeats 로 구성되어 있었다. 또한 계통분류학적 분석을 통하여 무척추동물의 IκB 단백질들과 그룹을 이루는 것을 확인 하였다. Rp-IκB 는 모든 조직에서 지속적으로 발현을 나타내었으며, 주요 면역 세포인 hemocyte 와 gill 에서 *V. tapetis* 와 LPS 자극에 의하여 유의적인 발현 증가를 나타내었다. 이러한 결과는 포유류에서 NF-κB 의 key 조절자인 IκB 와 같이 Rp-IκB 가 박테리아의 침임에 대하여 중요한 역할을 수행할 것으로 예상할 수 있었고, 이는 Rp-IκB 는 포유류의 IκB orthologs 들과 비슷한 기능을 가지고 있음을 뒷받침하고 있다.

포유류의 TNFα 의 homology 를 바지락으로부터 확인하였고, RpTNFα 로 명명하였다. RpTNFα 는 다른 선행 연구에서 보고된 TNFα 의 전형 적인 특징인 Transmembrane 과 TNF-signature 를 가지고 있었다. qRT-PCR 을 통하여 조직 특이적 발현을 분석한 결과 모든 조직에서 발현이 관찰되었으며, V. tapetis, LPS 그리고 poly I:C 를 주사하였을 때 이에 반응하여 hemocyte 와 gill 에서 유의적인 발현 증가를 나타내었다.

Bcl-2 는 Mitochondrial membrane permiabilzation (MMP)의 억제를 통하여 세포 사멸을 억제하는 Anti-apoptotic Bcl-2 sub-family 다. Bax 는 pro-apoptotic Bcl-2 subfamily 다. GS-flx transcriptome 서열에 기초하여, Bcl-2 와 Bax like 단백질의 orthologue 로 예상되는 cDNA 전체 서열을 바지락에서 확인하였고, 이 서열들은 Bcl-2 homology domain 과 transmembrane (only RpBcl-2)을 포함하고 있었다. Real time PCR 을 통하여 분석한 조직특이적 발현에서는 모든 조직에서 발현이 관찰되었으며, gill 에서 가장 높은 발현을

나타내었다. *V. tapetis* 를 주사한 후 gill 과 hemocyte 에서 유의적인 발현 변화를 나타내었다. 이러한 결과는 RpBcl2 와 RpBax 가 바지락의 면역반에서 포유류의 apoptotic regulator 들과 비슷한 기능 수행과 함께 면역에 매우 깊이 관련 있는 것을 나타내고 있다.

Summary

Manila clam, *Ruditapes philippinarum*, is an important aquaculture species worldwide. Recent mass mortalities of manila clam landings, believed to be due to microbial infection, have significantly impacted production and sparked interest in immune-related research of this species. Genetic information is important to better understand the molecular basis for the host immune response against pathogen. However, genomic data of clam form just 7% in bivalve genomic database. The manila clam is an economically important species, but to date only a few fully-annotated genomic data are available in genomic databases.

The manila clam is known to lack an adaptive immune system, but its mechanisms of innate immunity have yet to be fully elucidated. In contrast, the innate immune system in mammals has been extensively studied and this data may be used to provide significant insights into the immune system used by molluscs. The innate immune system is the main host defense in manila clam like invertebrates. Hence, it is important to understand the immune responses and their mechanisms against different microbial challenge at molecular level. In this study, in order to fill the gap in transcriptome sequence data abailable for the manila clam, transcriptome analysis was performed by Next-Generation Sequenicng (NGS) technology, furthermore molecular analysis of immune genes in manila clam, *Ruditapes phillipinarum* was investigated using transcription profiling.

Myeloid differentiation factor 88 (MyD88) is a universal adaptor protein which is required for signal transduction of TLR/IL-1R family. In this study, a novel molluscan MyD88 family member protein (named as RpMyD88) was identified from

manila clam, *Ruditapes philippinarum*. It was identified using BLAST algorithm from GS-FLX^{TM} sequencing data. The cDNA of RpMyD88 consists of 1416 bp open reading frame (ORF) encoding 471 amino acid residues. The RpMyD88 contains death domain and Toll/interleukin-1 receptor (TIR) domain which are typical features of MyD88 family proteins. The predicted amino acid sequence of RpMyD88 shares 27% identity with scallop MyD88. The expression level of RpMyD88 mRNA was investigated in healthy and challenged clams by quantitative real-time RT-PCR. The RpMyD88 gene expression is ubiquitous in all selected tissues. The RpMyD88 mRNA was strongly expressed in hemocyte, gill and mantle. In contrast, it was weakly expressed in siphon, foot and adductor muscle. RpMyD88 was up-regulated in gill and hemocyte after immune challenge with both *Vibrio tapetis* and LPS challenge. All results considered, sequence characterization, comparison and gene expression data suggesting that MyD88 dependent signaling pathway is presence in manila clam and RpMyD88 plays an important role in innate immune response against bacteria.

The nuclear factor-kappaB (NF-κB) signaling pathway is one of the most important components of the innate immune system and its activity is regulated by physical interaction with the inhibitor of NF-κB (IκB). Upon binding, IκB protein masks the nuclear localization signal (NLS) of NF-κB, thereby sequestering inactive NF-kB in the cytoplasm. Thus, elucidating the expression profile of such NF-kB inhibitors in the agriculturally-important manila clam will advance our understanding of the species' immune response to pathogenic threats for preventing and controlling molluscan diseases. Our investigations led to the identification a novel IκB (Rp-IκB)

from the manila clam, *Ruditapes philippinarum*. The Rp-IκB cDNA is comprised of a 1032 bp ORF, which encodes 343 amino acids residues with a predicted molecular mass of 38 kDa. Rp-IκB protein exhibited typical features of IκB protein family members, including the IκB degradation motif, PEST sequence and six ankyrin repeats. Phylogenetic analysis showed that manila clam and other known molluscan IκB proteins grouped together in the invertebrate cluster. Tissue specific expression analysis revealed that Rp-IκB was ubiquitiously expressed in all tested tissues. Significant up-regulation of Rp-IκB expression was observed in gill and hemocytes following bacterial immune challenge with *Vibrio tapetis* and purified lipopolysaccharide endotoxin. These results indicated that as a key regulator of NF-κB in mammals, Rp-IκB might play an important role in manila clam defense against bacterial infection. Thus, Rp-IκB appears to function similarly to mammalian IκB family orthologs.

The RpTNF amino acid sequences showed their characteristic TNF family signature and N-terminal transmembrane domains. Phylogenic analysis results showed that AbTNF-α was closely related with their invertebrate counterparts. qRT-PCR results showed that RpTNF-α transcripts were constitutively expressed in Clam hemocytes, gills, mantle, adductor muscle, Siphon and foot in a tissue-specific manner. Transcription level of RpTNF-α was significantly ($p<0.05$) up-regulated in gills and hemocytes by *Vibrio tapetis* and LPS challenge.

Bcl-2 is conserved anti-apoptotic protein that belongs to the anti-apoptotic Bcl-2 sub-family, which inhibits cell death by preventing mitochondrial membrane permeabilization (MMP). Bax is belongs to the pro-apoptotic Bcl-2 sub-family

protein. Given the anti-apoptotic functions of these proteins in vertebrate and the involvement of apoptotic regulation in immune responses, we studied the sequences of this gene and its transcript expression in manila clam during innate immune responses to *V. tapetis* stimuli. Based on GS-FLX transcriptome sequence data, we identified full-length cDNA sequences of putative orthologues of manila clam Bcl-2 and Bax-like protein. The analysis of manila clam cDNA sequences, and comparisons of the clam deduced amino acid sequences to putative orthologues in other species, revealed the presence of highly conserved Bcl-2 homology (BH) and trnasmembrane (TM) domain (only RpBcl-2) in manila clam sequences. Quantitative RT-PCR was used to study tissue specific expression in six tissues of un-challenged clam. We observed that constitutively expression of these genes in all examined tissues and both genes highly expressed in gill. In challenge of *V. tapetis* , RpBcl-2 and RpBax mRNA expression were significantly up-regulated in gill and hemocytes.

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CHAPTER 1

Characterization of manila clam transcriptome using 454-pyrosequencing approaches

1.1. Introduction

Manila clam *Ruditapes philippinarum* is an infaunal bivalve mollusk constitutes natural population in intertidal zones of Yellow Sea bordering countries such as Korea, China and Japan and has worldwide distribution including the countries of Canada, America, Italy, France, the UK and Ireland [Goullertquer, 1997; Coughlan et al., 2009]. Manila clam is commercially important shellfish in Korea as well as in worldwide aquaculture. One of the most serious constraints to the clam aquaculture industry in Korea and worldwide is the occurrence of high mortality and poor growth due to several known infectious disease (brown ring disease, perkinsus infection), oxidative stress, physical factors and other unknown reasons resulting massive economic loss [Paillard et al., 2004; Park et al., 2001; Park et al., 2006].

In GenBank, more than 206,000 and 14,600 expressed Sequence Tags (ESTs) are available for the Pacific oyster *Crassostrea gigas* and the eastern oyster *Crassostrea virginica*, respectively, and more than 42,500 and 19,500 ESTs for the California mussel *Mytillus californiacus* and the Mediterranean mussel *Mytilus galloprovincialis*, respectively. Recently, functional analysis of large-scale EST sequencing efforts from *C. gigas* and *M. galloprovincialis* led to the publication of many ESTs that were compiled in publish-available databases such as the Gigas database and the MytliBase [Fleury et al., 2009; Venier et al., 2009]. In addition to these large-scale sequencing efforts, a project has been initiated to sequence the entire genome of the Pacific oyster using next-generation sequencing technique [Zhang et al., 2011a].

In contrast to the considerable genomic data available for some shellfish

species, there is still a lack of data available. The manila clam *R. philippinarum*, is also an economically important shellfish, but to date only a few fully-annotated genomic data are available in genomic databases.

In the last few years, global genomic approaches have been applied successfully to the immune cells of bivalve species to better understand the molecular basis for the host immune response to challenges by *Vibrio* species [Gueguen et al., 2003; Roberts et al., 2009; Araya et al., 2010; De Lorgeril et al., 2011]. The presence of previously-undescribed genes in these organisms has provided new insights into the molecular basis of host-pathogen interactions.

Invertebrate mollusk including clam defense mechanisms rely on an innate immune system, which employs circulating cells (hemocytes) and a large variety of molecular effectors. Bivalve immune defense against pathogens mainly occurs by major innate immune systems or components and pathways which include; pattern recognition receptors (PRRs), hemolymph coagulation, antimicrobial peptides (AMP), and respiratory burst activity. The mechanisms underlying these host defense components depend on the presence of functional proteins in appropriate quantities, within a crucial time window. These proteins are encoded by genes whose transcription is tightly coordinated by complex programs of gene expression. However, immune and stress response mechanism of manila clam is not understood well even though several immune genes have been cloned and functionally characterized.

In present study, we performed de novo transcriptome sequencing of manila

clam using Roche 454 GS-FLX platform. Annotation and gene ontology analysis were then performed on assembled contigs, providing the valuable resource for future genetic and genomic research on manila clam and closely related species.

1.2. Materials and methods

1.2.1. Biological materials

Manila clams were collected from the Eastern coastal region of Jeju Island (Republic of Korea) and there after maintained in 80 L tank with aerated seawater until tissues collection.

Hemolymph (1–2 mL/clam) was collected from the pericardial cavity of healthy, unchallenged clams using a 22G syringe and the samples were immediately centrifuged (3000*g* at 4 °C for 10 min) to harvest the hemocytes. In order to determine the tissue specific expression, adductor muscle, mantle, siphon, gill and foot tissues were collected from three healthy, unchallenged animals and snap frozen in liquid nitrogen before storage at −80 °C.

1.2.2. RNA isolation, cDNA synthesis and normalization

The total RNA was isolated from tissues using the TRi Reagent (Sigma–Aldrich, USA). The mRNA was purified by using an mRNA isolation kit (FastTrack® 2.0; Invitrogen, USA) according to the manufacturer's instructions. Concentration was determined at 260 nm in a UV-spectrophotometer (BioRad, USA).

Purified RNA samples, were diluted to 1 μg/μL and pooled to perform multi-tissue first strand cDNA synthesis using Creator SMART cDNA library construction kit (Clontech, USA). Then, amplification was carried out with the Advantage 2 PCR kit (Clontech, USA) and then resultant products were normalized using the Trimmer-Direct cDNA normalization kit (Evrogen, Russia) according to the manufacturer's instructions.

1.2.3. Next generation cDNA sequencing and assembling of 454 sequencing data

Sequencing of normalized manila clam cDNA was carried out on a GS-FLX titanium instrument (DNA linker, Korea). Sequencing yielded 1,240,820 raw reads. Low quality 454-sequencing reads were removed by the Roche signal processing software. The remaining 797,858 (64.30%) reads were then assembled into contigs. The output reads were processed and assembled by using the Arachne whole-genome shotgun assembler program. The basic local alignment search tool (BLAST) [Altschul et al., 1990] was used to find sequence similarity to entries in public databases, including the non-redundant (NR) protein database from NCBI (http://www.ncbi.nlm.nih.gov).

1.3. Results and discussion

Samples sequenced on three full picotitre plates using the Roche 454 GS FLX platform. Analysis summary of NGS sequencing was shown in table 1. After filtering the two three data sets (L1, L2 and L3), 428,668 reads of 123,616,280

nucleotides were obtained in Lane 1 (L1), 422,234 reads of 130,486,649 nucleotides were obtained in Lane 2 (L2) and 389,914 reads of 111,309,109 nucleotides were obtained in Lane 3 (L3). A total of 1,240,820 raw sequencing reads with average length of 340 bp were generated. After removal of partially assembled sequences (300,429 reads), singletons (314,408), repeat sequences (9,554 reads), too short sequences (62,993 reads) and outlier sequences (55,489 reads), a total of 496,947 reads ranging from 100 bp to 600 bp were assembled average length of 351.

After assemble, 117,347 contigs were obtained, after blastx 24,327 contigs hit with NCBI database sequence. The number of bases was 19,113,449 nucleotides was matched with protein in NCBI database, which showed 785 nucleotides of average contig size and the largest contig size was 7,063 nucleotide length.

Functional genomics technologies have been widely adopted in the biological research of both model and non-model species. An efficient functional annotation of DNA or protein sequences is a major requirement for the successful application of these approaches as functional information on gene products is often the key to the interpretation of experimental results. Therefore, there is an increasing need for bioinformatics resources which are able to cope with large amount of sequence data, produce valuable annotation results and are easily accessible to laboratories where functional genomics projects are being undertaken [Gotz et al., 2008].

Functional annotation of transcripts of the combination assembly was carried out by BLASTX search against the NCBI non-redundant protein database.

Blast result accessions were used to retrieve associated gene names and Gene ontology (GO) terms. Gene Ontology is widely used to standardize representation of genes across species and provides a controlled vocabulary of terms for describing gene product [Salem et al., 2010]. Molecular function constituted the largest class of GO assignment of the transcripts (17,290), followed biological process (14,959) and cellular components (13,611). Level 2 GO assignments within the three categories are summarized in Fig 2. From the molecular function categories, 46% of the genes are associated with binding and 34 % are related to catalytic activity. Within the biological process category, 17% are related to cellular process followed by 17 % metabolic process. Of the cellular components, 30% of the genes are related to the cell and 27% were related to cell parts.

The identified major immune related genes by the contigs are presented in table 3. Table 3 lists the predicted gene name of manila clam, followed by the putative identification sequence, and E-value. We found pattern-recognition receptors (PGRPs, TLRs), inflammatory response genes (TNF, MyD88 and IκB), apoptosis-related gene (Bcl-2, Bax like gene and caspases), antimicrobial peptides (defensins and theromacin) and anti-oxidant genes (GSTs, Mn-SOD).

Table 1. Reads summary of GS-FLX sequencing data

		Lane 1	Lane2	Lane 3	sum
run data	numReads	428,668	422,238	389,914	**1,240,820**
	numBases	123,616,280	130,486,649	111,309,109	
Align result	numAlignedReads	268366, 62.60%	278377, 65.93%	251115, 64.40%	
	numAlignedBases	74893828, 60.59%	83357386, 63.88%	71183747, 63.95%	
	inferredReadError	3.85%, 2880781	3.51%, 2924252	3.50%, 2492176	
consensus result	numAlignedReads	797858, 64.30%			
	numAlignedBases	229434961, 62.79%			
	inferredReadError	3.62%, 8297209			%
	numberAssembled	**496,947**			**40.05**
	numberPartial	300,429			**24.21**
	numberSingleton	315,408			**25.42**
	numberRepeat	9,554			**0.77**
	numberTooShort	62,993			**5.08**
	numberOutlier	55,489			**4.47**
contig	Number Of Contigs	**117,347**			
	Number Of Bases	47,568,607			%
	Number Of Hit Contigs	**24,327**			21
	Number Of Hit Bases	19,113,449			
	Avg Hit Contig Size	785			
	Largest Contig Size	7,063			

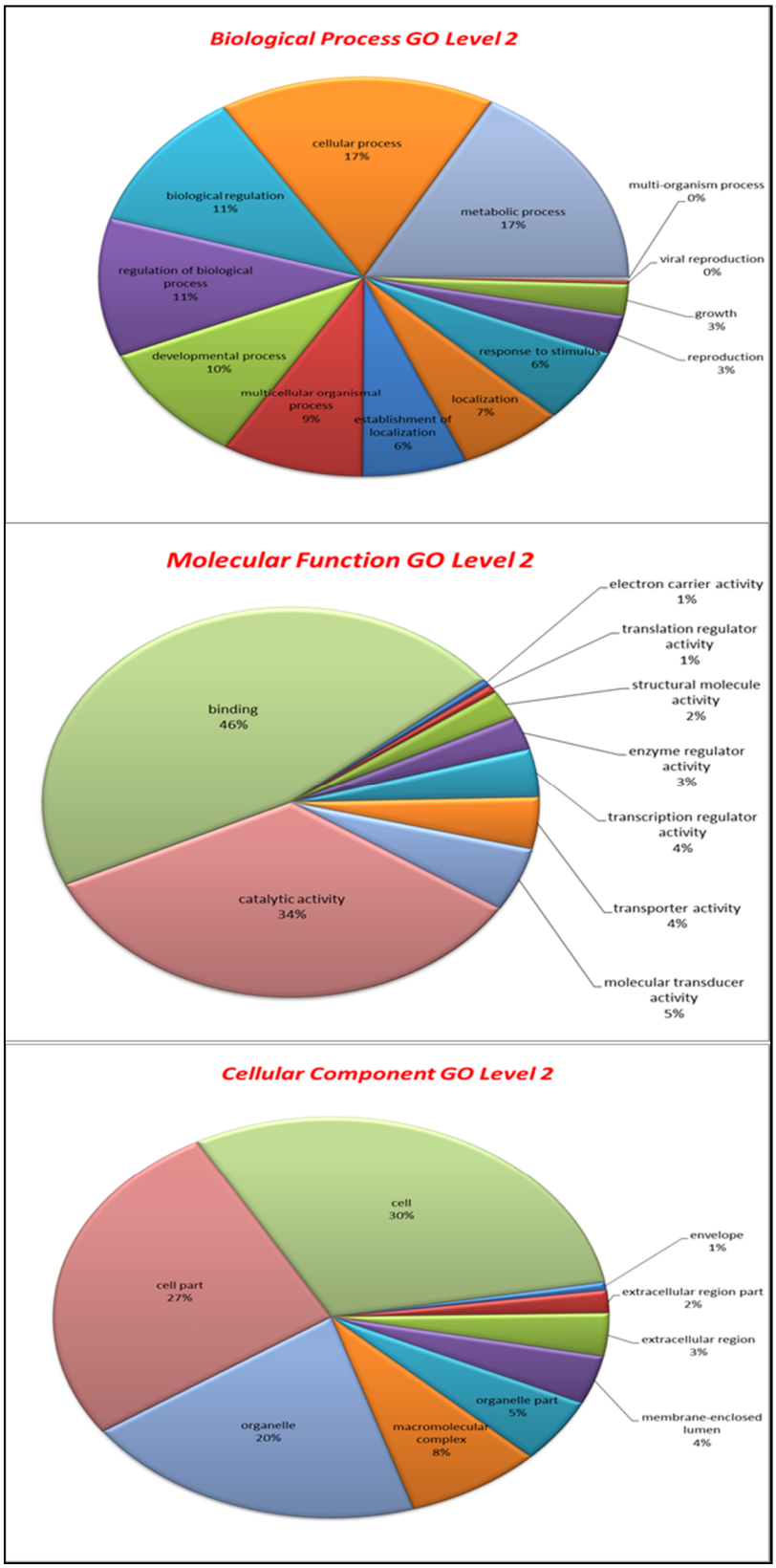

Figure 2. Gene Ontology (GO) analysis

Table 2. The innate immune-related genes from GS-FLX sequencing data

Gene description	Hit description	E-value
MyD88	myeloid/lymphoid or mixed-lineage leukemia 5 [Homo sapiens]	6.96E-27
Tumor necrosis factor superfamily memeber5-induced protein	PREDICTED: tumor necrosis factor (ligand) superfamily, member 15 [Taeniopygia guttata]	6.90E-07
IkB	nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (p105) [synthetic construct]	1.12E-11
Hydramacin (Theromacin)	PREDICTED: similar to antimicrobial peptide hydramacin [Hydra magnipapillata]	7.09E-16
Defensin A	defensin A [Ornithodoros moubata]	4.80E-08
Big defensin	BDEF_TACTR RecName: Full=Big defensin; Flags: Precursor	2.51E-09
Mn-SOD		
CystatinB	PREDICTED: similar to cystatin B [Equus caballus]	1.25E-14
Caspase3	PREDICTED: similar to caspase 3C [Hydra magnipapillata]	2.61E-23
Caspase-9	caspase 9, apoptosis-related cysteine protease [Homo sapiens]	9.51E-08
Caspase 6	Caspase 6 [Mus musculus]	8.04E-07
CathepsinC	cathepsin C precursor [Ixodes ricinus]	3.67E-77
Bcl-xL	Bcl-xL [Cricetulus griseus]	2.75E-13
Bax	BCL2-associated X protein [Xenopus (Silurana) tropicalis]	2.97E-07
defensin	defensin [Dreissena polymorpha]	1.60E-07
Glutathion S-transferase omega2	glutathione S-transferase omega 2 [Rattus norvegicus]	5.13E-13
Glutathion S-transferase	glutathione S-transferase [Branchiostoma belcheri tsingtauense]	1.29E-22
Relish	relish [Apis andreniformis]	2.20E-24
Toll-like receptor 2	toll-like receptor 2 [Sus scrofa]	3.86E-07
Toll-like receptor 3	toll-like receptor 3 [Sus scrofa]	4.87E-13
Toll-like receptor 6	toll-like receptor 6 [Gallus gallus]	2.11E-08
Toll-like receptor 7	toll-like receptor 7 [Xenopus (Silurana) tropicalis]	1.22E-07
Toll-like receptor 9	toll-like receptor 9 [Cynoglossus semilaevis]	1.15E-07
peptidoglycan-recognition protein S2	peptidoglycan-recognition protein S2 [Apis koschevnikovi]	4.40E-40
Mitogen-activated protein kinase	mitogen-activated protein kinase [Ajellomyces dermatitidis ER-3]	3.97E-64

CHAPTER II

Characterization of a novel molluscan MyD88 family protein from manila clam, *Ruditapes philippinarum*.

2.1. Introduction

Innate immunity is evolutionarily ancient, and predates adaptive immunity. As such, it is an organism's first line of defense against invading pathogens [Akira et al., 2006]. Innate immune responses are triggered when pathogen-associated molecular patterns (PAMPs) come into contact with host-expressed pattern recognition receptors (PRRs) [Medzhitov and Janeway, 2000; Qiu et al., 2007], which can detect and distinguish PAMPs derived from various microbial pathogens [Kawai and Akira, 2007]. These microbial components include bacterial membrane-expressed lipopolysaccharide (LPS), viral RNA, CpG DNA, flagellin, and lipoprotein.

The myeloid differentiation factor 88 (MyD88) is an evolutionarily conserved host expressed adaptor protein that is essential for proper TLR/Interluekin -1 receptor (IL-1R) immune-response signaling [Janssen and Beyaert 2002]. MyD88 consists of a Toll/IL-1 receptor (TIR) domain, located in the N-terminal region, and a death domain, at its C-terminus. TLRs are known to interact with the TIR domain and be activated through the death domain. MyD88 itself can also interact with the death domains of interleukin-1 receptor associated kinase (IRAK) family members, including IRAK1, IRAK2, IRAK4 and IRAK-M [Kawai and Akira, 2007, Akira and Takeda, 2004; West et al., 2006] to trigger downstream signaling cascades that lead to the activation of the transcription factor nuclear factor (NF)-κB.

MyD88 was first identified in 1990 as a protein that was induced during the terminal differentiation of $M1D^{+}$ myeloid precursors in response to IL-6 [Lord et al.,

1990]. Subsequently, orthologues of this protein have been found in several species, including human [Bonnert et al., 1997], mouse [Strausberg et al., 2002], frog [Prothmann et al., 2000], zebra fish [Wheaton et al., 2007], rock bream [Whang et al., 2011], and fruit fly [Tauszig-Delamasure et al., 2001]. However, to our knowledge, molluscan MyD88 has been only reported in scallop [Qiu et al., 2007].

Manila clam, *Ruditapes philippinarum*, is an infaunal bivalve mollusc that is an important constituent of the intertidal zones of the Yellow Sea bordering countries such as Korea and China and Japan [Goullet, 1997; Coughlan et al., 2009]. Its worldwide distribution as a human food commodity has made it an economically-important species of the Korean aquaculture industry. Recent mass mortalities of manila clam landings, believed to be due to microbial infection, have significantly impacted production and sparked interest in immune-related research of this species [Park and Choi, 2004; Lee et al., 2001]. The manila clam is known to lack an adaptive immune system, but its mechanisms of innate immunity have yet to be fully elucidated. In contrast, the innate immune system in mammals has been extensively studied and this data may be used to provide significant insights into the immune system used by molluscs. In mammals, the MyD88-dependent TLR signaling pathway relies completely on the presence and proper activity of MyD88 adaptor protein. Therefore, we presumed that MyD88 play a similar role in the immune system of manila clam.

This study was designed to first determine the presence of a MyD88 orthologue in manila clam and subsequently characterize the function of such in response to immune challenge. We describe here our successful cloning of the

complete ORF sequence of manila clam MyD88 and the results of comparative analysis with other known MyD88 genes to establish the phylogenetic and evolutionally relationship. In addition, we defined the tissue-specific expression and temporal expression profile in response to immune stimulation (*Vibrio tapetis* and LPS).

2.2. Materials and methods

2.2.1. Next-generation sequencing and identification of manila clam MyD88

We have previously established a manila clam cDNA sequence database based upon pyrosequencing data obtained from the 454 Genome Sequencer FLX platform (GS-FLX^{TM}; Roche, USA). Briefly, total RNA was isolated from whole body of healthy manila clams using the Trizol reagent (Sigma, USA). Poly(A) mRNA was then isolated using the FastTrack® 2.0 kit (Invitrogen, USA). First-strand cDNA was generated from 1.5 μg of poly(A^+) RNA using a Creator^{TM} SMART^{TM} cDNA library construction kit (Clontech, USA), which was then amplified with the 50X Advantage 2 polymerase mix (Clontech). The resultant cDNA library was normalized using the Trimmer-Direct cDNA normalization kit (Evrogen, Russia). Sequencing of normalized manila clam cDNA was carried out on a GS-FLX Titanium instrument (DNA linker, Korea), and the output reads were processed and assembled by using the Arachne whole-genome shotgun assembler program [Batzoglou et al, 2002; Jaffe et al, 2003; Gnerre et al., 2009]. MyD88 gene from

manila clam (designated as RpMyD88) was identified by using the Basic Local Alignment Search Tool (BLAST) algorithm [Altchul et al, 1990].

2.2.2. Sequence analysis of RpMyD88

The RpMyD88 gene ORF sequence was identified using DNAssist software (Version 2.2; http://www.dnassist.com). To determine the conserved domains of the RpMyD88 predicted protein, motif scan Pfam hidden Markov models (Local models) were used (http://hits.isb-sib.ch/cgi-bin/PFSCAN). Multiple alignment of protein sequences from different species was performed by the ClustalW program (http://www.ebi.ac.uk/Tools/clustalw2/index.html), and the identity and similarity percentage was calculated using EMBOSS pairwise alignment algorithms (http://www.ebi.ac.uk/Tools/emboss/align/). The phylogenetic tree was constructed by the Neighbor-Joining method using molecular evolutionary genetic analysis software (MEGA, v5.03; http://www.megasoftware.net).

2.2.3. Experimental animals and immune challenge

Manila clams, with average shell length of 3.5-4.0 cm, were collected from the Eastern coastal region of Jeju Island (Republic of Korea). In the laboratory, the clams were maintained at 21±1°C in a flat bottom tank with recirculating seawater. All animals were allowed to acclimatize for one week before any experimentation.

To determine the normal tissue-specific expression of the RpMyD88 gene the following tissues and hemocytes were collected from three un-challenged manila clams: adductor muscle, mantle, siphon, gill and foot. The clam hemolymph was

collected from the posterior adductor muscle sinus using 1 ml syringes with 26-gauge needles through the shell hinge. The hemolymph was immediately centrifuged (3500 rpm for 10 min at 4 °C) and the hemocytes were obtained after removal of supernatant.

To evaluate the immune-responsive expression of RpMyD88 gene, whole bacteria (*Vibrio tapetis*, a common manila clam gram-negative pathogen) and purified endotoxin (LPS; *E. coli* 0127:B8; Sigma, USA) were selected for controlled exposure. The *V. tapetis* [KCTC no. 12728] was obtained from the Korean Collection for Type Culture (KCTC). *V. tapetis* and LPS were diluted in phosphate buffer saline (PBS) to make stock. Clams were intramuscularly injected with 100 μL (3.2 X 10^7 cfu individual^{-1}) of *V. tapetis* and LPS (50 μg individual^{-1}) stock into live clam adductor muscle. A negative control group was established as un-injected, while positive control group was injected with an equal volume (100 μL) of PBS. Gill and hemocyte samples were taken from three animals at 3, 6, 12, 24 and 48 h post-challenge. PBS control samples also were isolated at each time point. All samples were immediately snap-frozen in liquid nitrogen and stored at -70℃ until use.

2.2.4. RNA extraction and cDNA synthesis

Total RNA was extracted from isolated tissues (50 mg each) using the Trizol reagent (Sigma). First-strand cDNA synthesis was carried out using 1 μg of total RNA as template with the PrimeScript^{TM} first strand synthesis kit (TaKaRa, Japan) by following the manufacturer's instruction. The cDNA product was diluted 10-fold and stored at -20℃ until further use. Subsequent, quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR) was performed to determine

tissue-specific expression and expression changes in response to immune stimulation.

2.2.5. qPCR analysis

Two PCR primers were designed based on the clam MyD88 coding sequence (forward primer, 5'-TGA GCT GGA AGT TAA ACG AGG GCT-3'; reverse primer, 5'-TCA CCA CTC TAC GGC ATC TTG CTT-3'). Two internal control primers were synthesized to amplify β-actin (forward primer, 5'-CTC CCT TGA GAA GAG CTA CGA-3'; reverse primer, 5'-GAT ACC AGC AGA TTC CAT ACC C-3') [Kim et al., 2008, Zhang et al., 2011]. qPCR was performed in the Thermal Cycler Dice real-time system (TP800; TaKaRa, Japan) under the following conditions: one denaturation cycle of 95℃ for 3 min, followed by 45 amplification cycles of 95℃ for 20 s, 58℃ for 20 s, 72 ℃ for 30 s. The baseline was set automatically by the accompanying system software (version 2.0). The relative expression of each gene was determined by the Livak ($2^{-\Delta\Delta CT}$) method [Livak and Schmittgen, 2001]. For tissue-specific expression analysis, relative expression of RpMyD88 mRNA was normalized to the expression detected in healthy adductor muscle samples. To determine expression fold-change after immune challenge, the relative expression was further compared to tissue-matched un-injected controls. All data are presented as relative mRNA expressed as means ± standard deviation (SD). Statistical difference between the control and treatment groups were determined by t-test using the SPSS 11.5 program, and differences were considered significant at $P < 0.05$.

2.3. Results

2.3.1. RpMyD88 sequence characterization

The nucleotide and predicted amino acid sequences of RpMyD88 (GenBank accession No: JF773572) are shown in Fig. 3. The RpMyD88 cDNA contains a 1,416 base pair (bp) open reading frame (ORF), which encodes a putative protein of 471 amino acid (aa) residues. The calculated molecular mass of RpMyD88 is 54 kDa and the predicted isoelectric point is 6.5.

Motif scan analysis was used to identify characteristic domains of MyD88 family members. RpMyD88 was found to contain the highly conserved death domain (^{56}DSD- RPI^{154}) and TIR domain (^{180}AFI-ALA^{263}). Multiple alignment analysis of RpMyD88 to other known MyD88 family members from different species revealed a distinct nature of RpMyD88 (Fig. 4). RpMyD88 possesses the longest sequence of all MyD88s examined but also has significant sequence gaps at several positions.

The calculated amino acid sequence identity and similarity demonstrated that RpMyD88 had the highest identity (27.0%) and similarity (42.7%) with *Chlamys farreri* MyD88. Identities with mandarin fish (25.9%), mouse (23.6%), and human (23.1%) were also significant (Table. 3.)

To determine the evolutionary position of RpMyD88 among the other MyD88 family members, a phylogenetic tree was constructed using Sponge MyD88 as the out-group (Fig. 5). Several other known MyD88 family members were selected from mammals, fish, and insects, along with the scallop. The results showed that MyD88 family proteins comprised two clades, those being populated by vertebrate and invertebrate MyD88s. The vertebrate clade consisted of two sub-

clades, which held the mammalian and teleost MyD88 proteins, respectively. The invertebrate clade also consisted of two sub-clades, which held mollusc and insect MyD88 proteins, respectively. The mullusc MyD88s grouped together, indicating that RpMyD88 was phylogenetically closest to scallop MyD88.

2.3.2. Tissue-specific expression of RpMyD88

To investigate the basal transcription level of RpMyD88 in several tissues of healthy manila clams, the mRNA level was determined by means of qRT-PCR. RpMyD88 was found to be constitutively expressed in all tissues examined (hemocytes, gill, mantle, siphon, foot and adductor muscle), although the expression level was variable among each (Fig 6). The most robust level of RpMyD88 expression was detected in gill tissue. Hemocytes and mantle also showed high levels of expression, and the lowest levels were in siphon, foot and adductor muscle.

2.3.3. Expression profile of RpMyD88 after V. tapetis and LPS challenge

Changes in the expression profile of RpMyD88 were determined in hemocyte and gill tissues following *V. tapetis* and LPS challenge of live, intact animals. Following *V. tapetis* and LPS challenge, transcriptional up-regulation of RpMyD88 was detected by qRT-PCR for both hemocytes and gill. The expression levels of RpMyD88 in hemocyte after *V. tapetis* and LPS challenge, they have similar but slightly different expression patterns. RpMyD88 mRNA level was significantly up-regulated after both *V. tapetis* and LPS stimulation (Fig. 7). The significant difference of expression was observed at 12h ($p<0.05$) and 6 h ($p < 0.05$) after *V.*

tapetis and LPS challenge, respectively. At the other time points after challenge, no significant difference in RpMyD88 level was found between the challenge and positive control group ($p > 0.05$), except that at 48 h after LPS challenge.

In gill tissue, mRNA of RpMyD88 was significantly up-regulated at 12 h after *V. tapetis* and LPS challenge, although it was down-regulated at 3 h after *V. tapetis* challenge (Fig. 8). Peak expression was 6.7 ($p<0.05$) and 2.0 ($p<0.05$) -fold increased compared to the control at 12 h after challenge. At the other time points after both *V. tapetis* and LPS challenge, we found no significant difference of RpMyD88 mRNA expression level.

2.4. Discussion

Molluscs have not evolved the adaptive immune system. Therefore, gaining a detailed understanding of the innate immune system is vital to developing methods to control species-related diseases. The TLR signaling pathway is one of the most important components of the mammalian and insect innate immune system; however, the molluscan Toll-like receptor signaling pathways remain under-researched and relatively unknown. When activated, all TLRs (except TLR3) recruit the adaptor protein MyD88 in order to propagate the signal. MyD88 activates IRAKs, which subsequently regulate downstream gene expression and modulate the immune response. In molluscs, only one MyD88 orthologue has been identified to date, that being from the scallop *Chlamys farreri* [Qiu et al., 2007].

In this study, we identified and characterized MyD88 cDNA (RpMyD88) from manila clam. Expression of this gene appears to play an important role in TLR-

mediated signal transduction. The novel RpMyD88 gene is comprised of a 1,416 bp sequence that encodes 471 aa residues. When compared to other known MyD88 aa sequences, RpMyD88 demonstrated relatively low identity and similarity. Detailed homology analysis also indicated the presence of many gaps, indicating significant genomic deviation throughout evolution. The RpMyD88 peptide is longer than other vertebrate MyD88s. Human (AAC50954), scallop (ABB76627) and chicken (NP_001026133) MyD88 proteins are comprised of 296, 367 and 376 amino acids, respectively. Nevertheless, RpMyD88 harbors two highly conserved MyD88 characteristic regions: the death domain and TIR domain.

MyD88 is known as a universal adaptor protein having an N-terminal death domain and a C-terminal TIR domain. These two conserved domains facilitate MyD88 function as an adaptor between members of the TLR/Il-1R superfamily and downstream signaling proteins, such as IRAK [Wesche et al., 1997; Medzhitove et al., 1998; Bums et al., 1998]. The TIR domain is found almost exclusively in eukaryotic host organisms and governs dimerization between TLRs and the association between TLRs and TIR domain–containing adaptors [Barton and Medzhitov, 2003]. The death domain-containing proteins mediate protein-protein interaction, and are critically involved in apoptosis and inflammatory response-related processes [Bums et al., 1998]. Death domains are typically located in the C-terminal region [Feinstein et al., 1995], however the death domain in MyD88 and IRAKs of TLR signaling pathway are located in the N-terminal regions [Medzhitove et al., 1998]. In this study, the RpMyD88 death domain was found to be located at positions 56-154 of the N-terminal sequence. Presence of both the death domain and TIR domain in RpMyD88

suggest that this newly-identified protein may function in the TLR signaling pathway of the clam immune defense system.

The tissue-specific expression analysis revealed that RpMyD88 gene expression was ubiquitous in healthy clams, although its level of expression was variable among different tissues. Several reports by others have indicated that MyD88 from various species is also ubiquitously expressed. Chicken MyD88 was demonstrated to be expressed in all tested tissues, including spleen, bursa of fabricius, liver, kidney, lung, and brain [Qiu et al., 2008]. In the lower order vertebrate rock bream, MyD88 transcripts were detected in blood, gill, liver, spleen, head kidney, skin muscle, brain and intestine [Whang et al., 2011]. Another *Chlamys farreri*, was found to express MyD88 in normal hemocytes, muscle, mantle, heart, gonad and gill [Qiu et al., 2007].

In order to better understand the functional role of RpMyD88, especially in regards to bacterial infection and endotoxin exposure, we investigated the expression profile of RpMyD88 mRNA after immune stimulation. *V. tapetis* is a known pathogen of manila clam and causative agent of brown ring disease (BRD). BRD has been implicated in previous mass mortalities in cultured clam beds [Fulye-Sainte-Marie et al., 2008]. The gram-negative endotoxin LPS has also been reported as a powerful stimulator of innate immunity in various eukaryotic organisms [Qiu et al., 2007; Iwanaga, 2002]. Rock bream MyD88 has been evidenced to become up-regulated in blood, spleen and head kidney in response to experimental challenge with LPS and *Edwardsiella tarda* [Whang et al., 2011]. In Japanese flounder peripheral blood leukocytes, MyD88 was also found to be strongly up-regulated in

response to experimental exposure to LPS, PGN, and poly I:C [Takano et al., 2006]. In scallop, MyD88 was up-regulated in primary cultured hemocytes after LPS and PGN treatment [Qiu et al., 2007]. In the present study, we also found that RpMyD88 was strongly up-regulated in both hemocytes and gill after *V. tapetis* and LPS challenge. These findings collectively indicate that MyD88 plays an important role in vertebrate and invertebrate species in transducing the immune-response signal during pathogen invasion.

In conclusion, we identified the cDNA of MyD88 from manila clam, *Ruditapes philippinarum*. The RpMyD88 contains death and TIR domains, which are typical features of MyD88 family proteins. RpMyD88 was ubiquitously expressed in healthy manila clam tissues, and was significantly up-regulated in immune-related tissues (hemocytes and gill) in response to whole bacteria (*V. tapetis*) and gram-negative endotoxin (LPS) challenge. This suggested that the manila clam MyD88 may be involved in immune defense against *V. tapetis* infection. Sequence characterization and phylogenetic analysis further suggested that RpMyD88 may have a similar function to known vertebrate MyD88s. However, MyD88 has only been identified in one other mollusc species to date and its detailed molecular role in marine invertebrates is not yet completely understood. In order to attain a better understanding of the immunological function of RpMyD88, studies should be carried out to identify its functional interactions with other molecules of the molluscan innate immune system.

Table 3. Pairwise ClustalW analysis and comparison of the RpMyD88 deduced amino acid sequence

	Identity (%)	Similarity (%)	Gaps (%)	Accession No.
Scallop	27.0	42.7	28.6	ABB76627
Mandarin fish	25.9	34.9	42.5	ADM25313
Mouse	23.6	33.1	39.5	AAC53013
Human	23.1	32.7	44.4	AAC50954
Fruit fly	19.0	33.1	36.4	NP_610479

```
                                                    gaaggcggaagttatgtagcattgagttaatctt   -76
taatcatttgaaatagttatagtggtcagaaacagttgtattttatgaacatttgatcaagctatatagaaaaac   -1
ATGGCTACGAGAATGGATGATGATGATACACTTCAACCAGATCTTGTCCTGCCAGAAATATATACTTTTACGCCT   75
 M  A  T  R  M  D  D  D  D  T  L  Q  P  D  L  V  L  P  E  I  Y  T  F  T  P    25
TTAAGGGCTCTAAGAGTAGCTTCACGACGGAAGTTGTCGGGGTTTCTGGATTTGGAAGGAGCTCTTGTTGTAGTT   150
 L  R  A  L  R  V  A  S  R  R  K  L  S  G  F  L  D  L  E  G  A  L  V  V  V    50
AACCATGAGGGTGGAGACTCCGACGTTGTCAATGACTATAGTGGTTTGGCAGAACTTGCTGGATTTGATTACAAC   225
                Death domain
 N  H  E  G  G  D  S  D  V  V  N  D  Y  S  G  L  A  E  L  A  G  F  D  Y  N    75
AGTATCATGAACATGAAAAGACAGAAAAGTCCAACTTTTGAATTACTTGAGAAGTGGACAGGACGGAATGGAACC   300
 S  I  M  N  M  K  R  Q  K  S  P  T  F  E  L  L  E  K  W  T  G  R  N  G  T   100
GTGGGAAATCTATGGACGTATCTATTTTTGATGGAAAGATTTGATGTTCTTGTTGACTGCCGGCGACCAATAATA   375
 V  G  N  L  W  T  Y  L  F  L  M  E  R  F  D  V  L  V  D  C  R  R  P  I  I   125
AGAGACTGTGAGAAATATGAAGAAAATCTGCAGCTAGCAGAAGATGCCGAAAGATACAGAATGGAACAAGATCCT   450
 R  D  C  E  K  Y  E  E  N  L  Q  L  A  E  D  A  E  R  Y  R  M  E  Q  D  P   150
ACAGTTACAACATCTGAGATGAGAGTTGATGAGACGAAATTTGCAGCAAAAGGAGATGTTATGACCGGGAAGAAA   525
 T  V  T  T  S  E  M  R  V  D  E  T  K  F  A  A  K  G  D  V  M  T  G  K  K   175
ACATTTTATGACGCCTTCATCTGTTGGTCATCTGAGGACGAGAAAGACCGCCAATTTCTGTATGAAATGATAAAT   600
                TIR domain
 T  F  Y  D  A  F  I  C  W  S  S  E  D  E  K  D  R  Q  F  L  Y  E  M  I  N   200
GAGCTGGAAGTTAAACGAGGGCTAAAATTGTTTGTACCTGGAAGAGACGACCTGCCTGGTTCAGCTGAACACACA   675
 E  L  E  V  K  R  G  L  K  L  F  V  P  G  R  D  D  L  P  G  S  A  E  H  T   225
ATCACAGCTTATCTCATAGAAGCAAGATGCCGTAGAGTGGTGATACTGATGTCCAGAACGTTTTTGCAGAGTTCT   750
 I  T  A  Y  L  I  E  A  R  C  R  R  V  V  I  L  M  S  R  T  F  L  Q  S  S   250
GCGTGTGACTTTCAGGTGAAATTCGCTCATGCTCTAGCACCAGGTGCAAGAAGCAAAAAACTTATACCAGTTATC   825
 A  C  D  F  Q  V  K  F  A  H  A  L  A  P  G  A  R  S  K  K  L  I  P  V  I   275
CGTGAAAAAAATACACCAATACCCAGAATCCTTCGCTTTCTGGCATTATGTGACTTCGGCAAGTCCGACATGGTT   900
 R  E  K  N  T  P  I  P  R  I  L  R  F  L  A  L  C  D  F  G  K  S  D  M  V   300
GAATGGGTTTGGGACAGACTTTCTACAGCCATAATTGCGCCAATAGGACCAAACACATATTTTGAACCGGAAGAT   975
 E  W  V  W  D  R  L  S  T  A  I  I  A  P  I  G  P  N  T  Y  F  E  P  E  D   325
GAGCACAATCCATTTGAACAGAGTGATGAAAGTTTAAAAGCTATAAGATTTCCAACACCCAGGCGTCTAGATGAG   1050
 E  H  N  P  F  E  Q  S  D  E  S  L  K  A  I  R  F  P  T  P  R  R  L  D  E   350
CATGATGAGAGATCGGTTAATTCCGGAGCCGAACGCATACCGACAAATGTAAGCAATAGGTCCCAATTGAGAGCG   1125
 H  D  E  R  S  V  N  S  G  A  E  R  I  P  T  N  V  S  N  R  S  Q  L  R  A   375
CCAACAGAACACGGTATGTCTTCAGAGGTACGGGGCCAACCAACATCCAATTATCAATCTCTTTCTTCATCTCCC   1200
 P  T  E  H  G  M  S  S  E  V  R  G  Q  P  T  S  N  Y  Q  S  L  S  S  S  P   400
ACAACTTTTACTGATCACACCAAGAAGGGGGCAACTAATAAATCAAAGACCGCTACATCACAAAAGCCAAGCAAA   1275
 T  T  F  T  D  H  T  K  K  G  A  T  N  K  S  K  T  A  T  S  Q  K  P  S  K   425
AAGGATAAGAAACGTGAGTCGGGCGGCATGTTCAATAAATTTAAAGAAAATTTGTGTGATCTCATTTGTACATTA   1350
 K  D  K  K  R  E  S  G  G  M  F  N  K  F  K  E  N  L  C  D  L  I  C  T  L   450
AAATCAGTATTGAAAAAACCTTTTAAAGAAGTAATTTTGCTTATTTATTTTTCCATTTCTCATTGACGCTATGTA   1425
 K  S  V  L  K  K  P  F  K  E  V  I  L  L  I  Y  F  S  I  S  H  *             471
Gtcaagctcaatcaatctgattaatgatttaaacacttttgtgaatcattgcagagcattttgtatgagcgttc   1500
tagatgacgatacaaatgacgtcagatacacaccaagaacaaaacaaacaacattattttatgtgttcgttgttc   1575
tagccagactcattaact                                                           1593
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Figure 3. Nucleotide and deduced amino acid sequence of RpMyD88. The ORF is shown in uppercase letters, and the 5'- and 3'-untranslated regions are in lowercase. Translated amino acid sequence is shown under the nucleotide sequence. The start codon (ATG) is underlined.

The stop codon at the end of the ORF is indicated by asterisk. Numbers to the right of each row refer to nucleotide or amino acid position, accordingly. The death domain is shaded and the TIR domain is boxed.

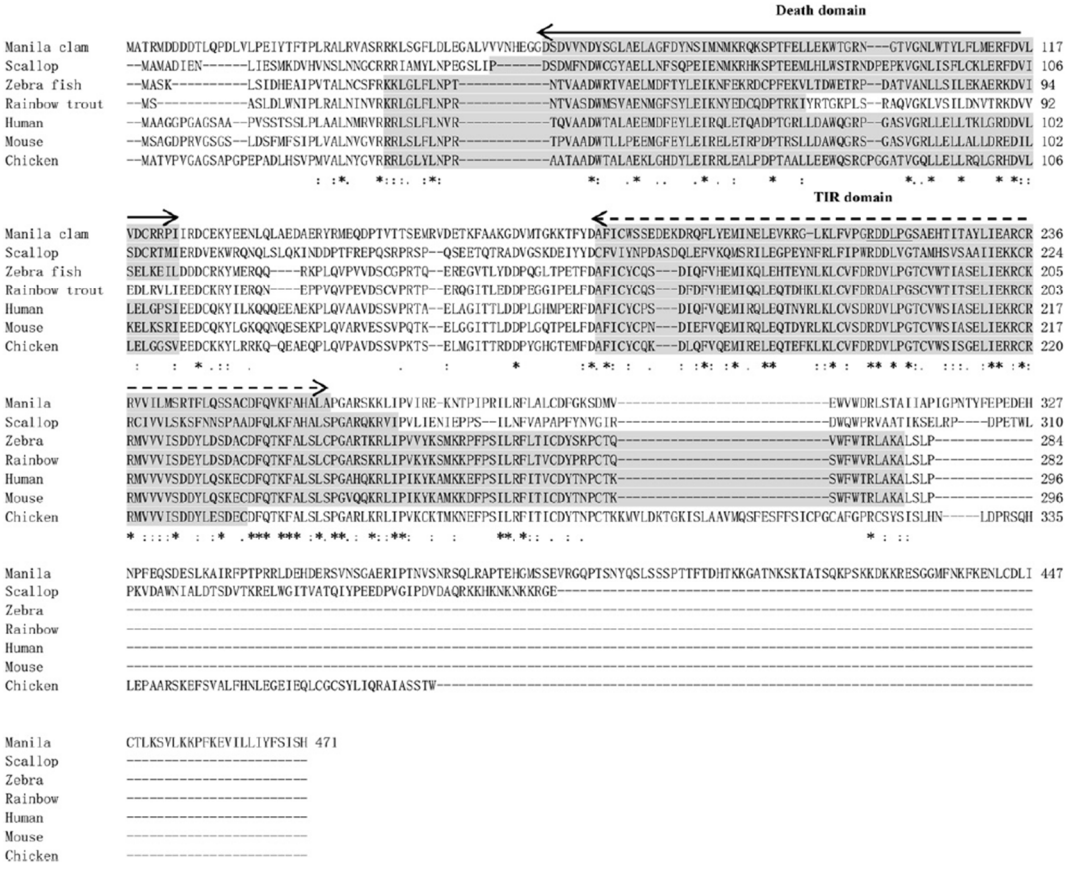

Figure 4. Multiple alignment of RpMyD88 with other known MyD88 amino acid sequences. The death and TIR domainsare indicated with continuous and discontinuous arrows, respectively. Scallop (ABB76627), mouse (AAC53013), human (AAC50954), chicken (NP_001026133), zebrafish (NP_997979), and fruit fly (NP_610479) are shown.

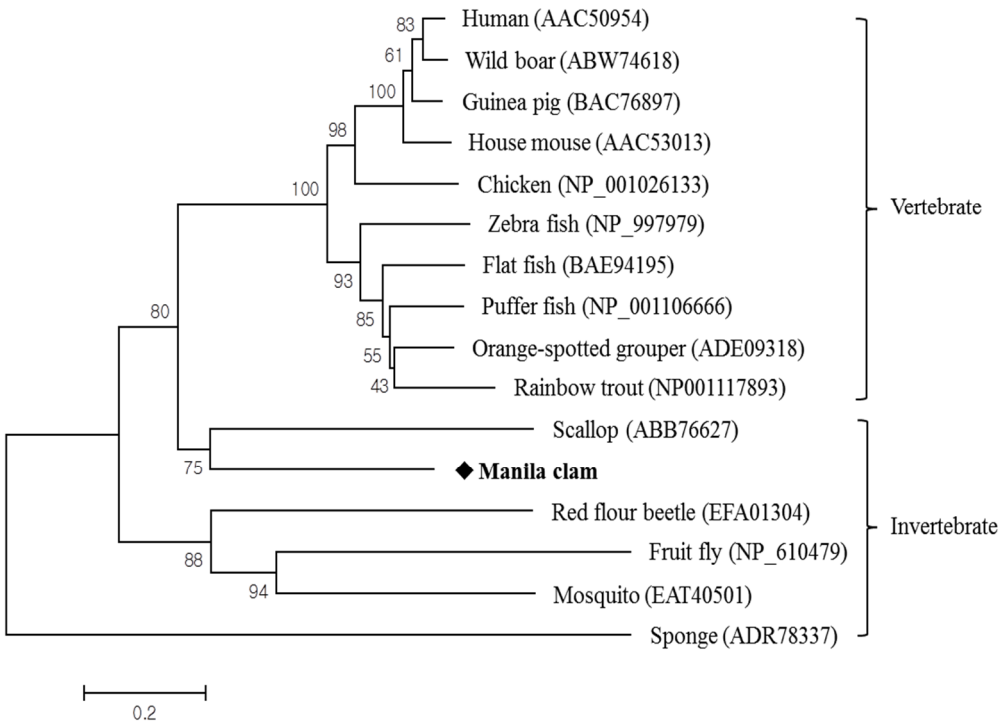

Figure 5. Phylogenetic analysis of RpMyD88. The number at each node indicates the percentage of bootstrapping after 1000 replications. The GenBank accession numbers of selected sequences are indicated within brackets.

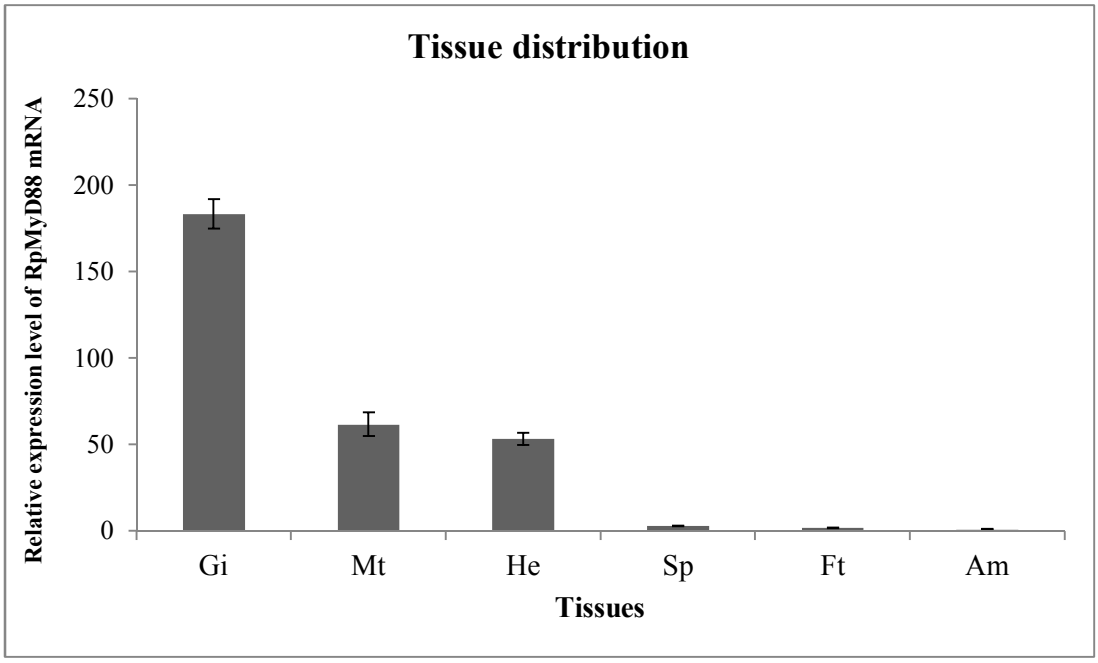

Figure 6. Tissue-specific expression analysis of RpMyD88 mRNA. The relative mRNA expression of each tissue was normalized with that in adductor muscle. Data are presented as the mean relative expression ± standard error (SE) for three individual real-time reactions using pooled tisssues from three individual clams. Gi, gill; Mt, mantle; He, hemocyte; Sp, Siphon; Ft, foot; Am, adductor muscle.

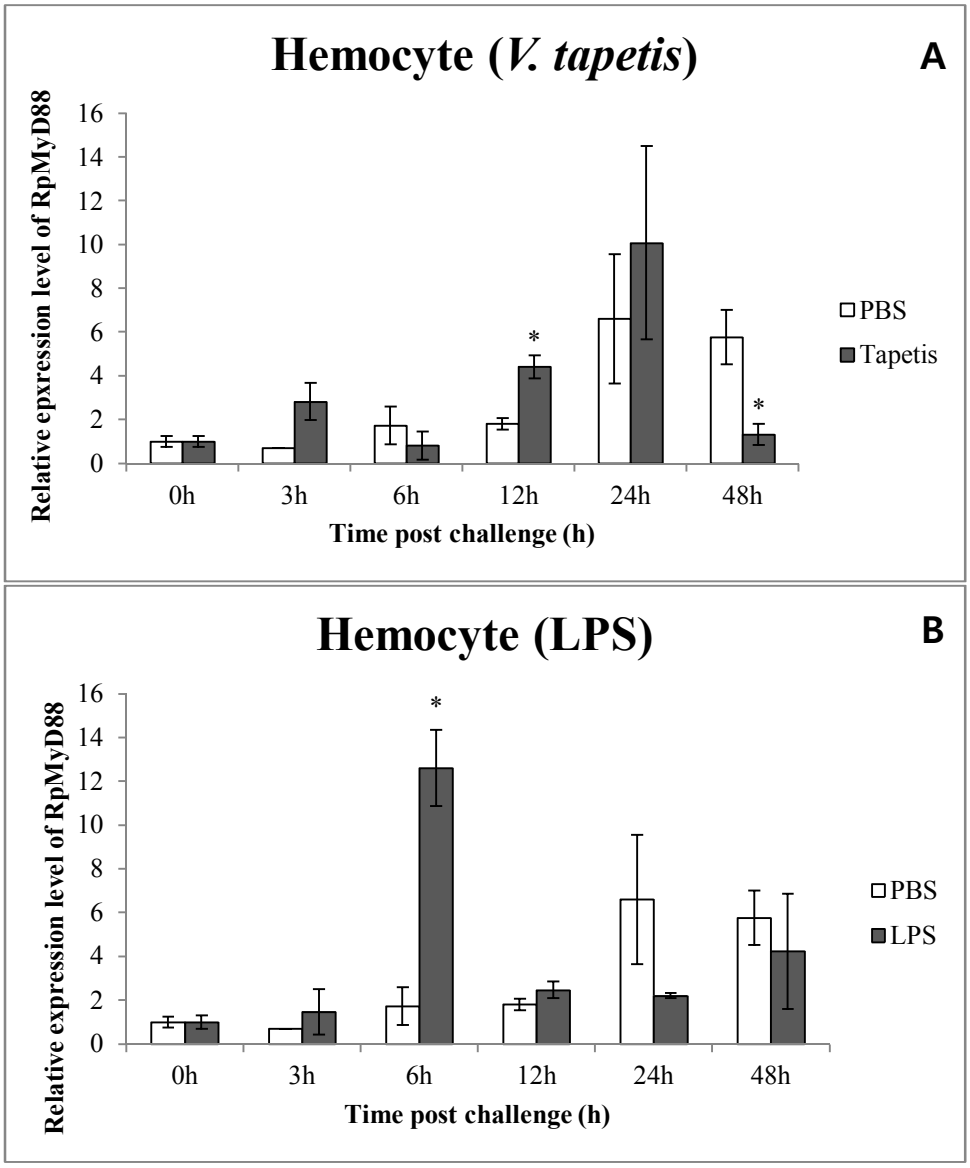

Figure 7. Expression profiles of RpMyD88 mRNA in manila clam hemocytes after *V. tapetis* (A) and LPS (B) challenge. The relative level of expression for each time point was compared to that of un-injected controls. Data are presented as the mean relative expression± SE. Significant difference ($p < 0.05$) across control were indicated with an asterisk.

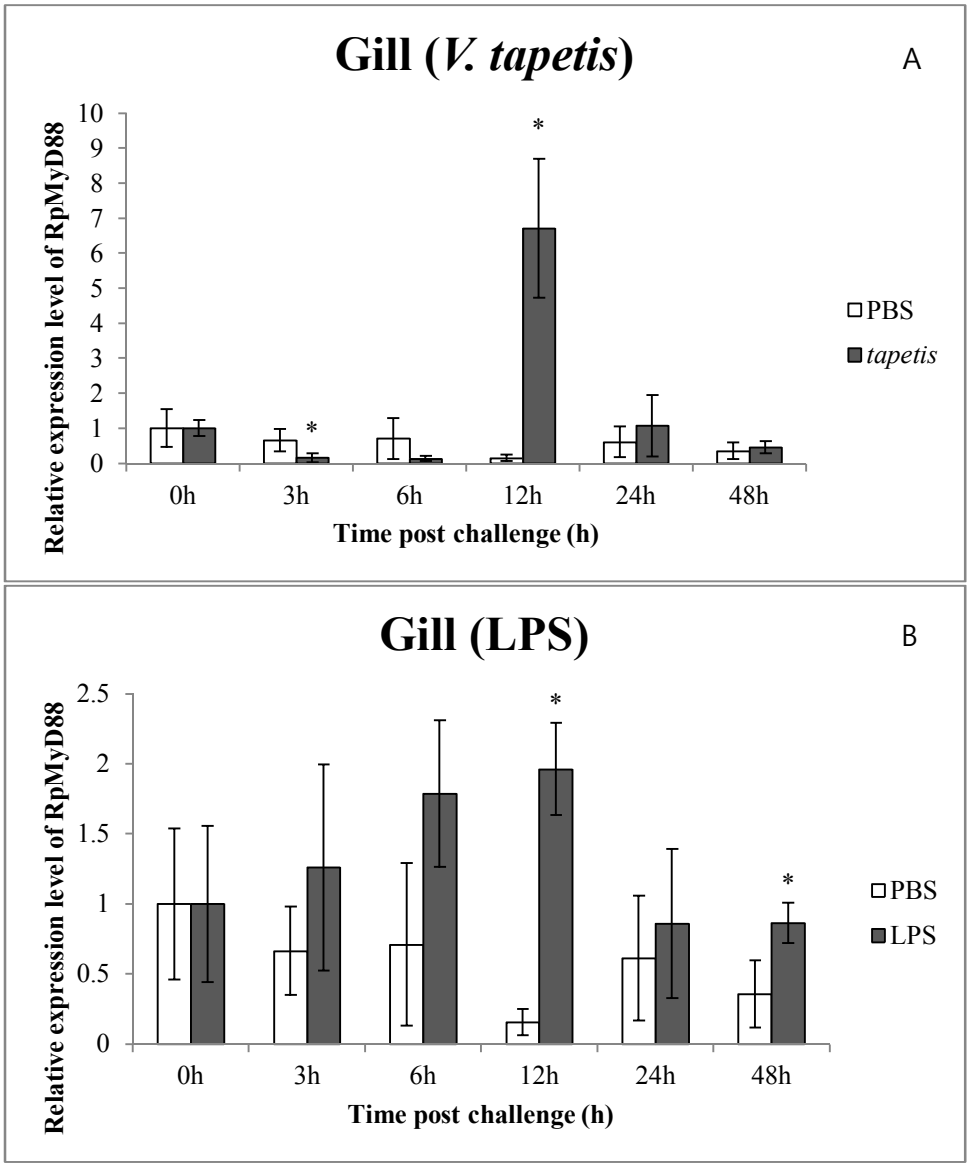

Figure 8. Expression profiles of RpMyD88 mRNA in manila clam gill tissue after *V. tapetis* (A) and LPS (B) challenge. The relative level of expression for each time point was compared to that of un-injected controls. Data are presented as the mean relative expression± SE. Significant difference ($p < 0.05$) across control were indicated with an asterisk.

Chapter III

Immune response-related gene expression of a novel molluscan IκB protein member from manila clam, *Ruditapes philippinaum*

3.1. Introduction

The nuclear factor-kappa B (NF-κB) proteins are dimeric transcription activators that contribute to regulation of many biological processes, such as immune response, inflammation, apoptosis, cell growth, differentiation, proliferation, tumorigenesis and embryonic morphogenesis [Pahl, 1999; Whang et al., 2009; Barkett and Gilmore, 1999]. In resting cells, NF-κB exists in its inactive form, sequestered in the cytoplasm by physical interaction with a member of the inhibitor of NF-κB (IκB) protein family. Upon IκB degradation by the IκB kinase (IKK) [Brown et al., 1993; Cordle et al., 1993; Traenckner et al., 1995], the unbound NF-κB rapidly translocates into the nucleus, where it binds to gene promoters in a sequence-specific manner and activates gene transcription [Kawagoe et al., 2009; Verma et al., 1995; Wang et al., 2001; Huxford et al., 1998].

IκB family members are characterized by the presence of three domain features: ankyrin repeats that mediate binding to NF-κB; an N-terminal regulatory region that contains two conserved serine residues and the IκB degradation motif; and, a region of sequence rich in proline (P), glutamine (E), serine (S), and threonine (T) residues (known as PEST) [Traenckner et al., 1995; Huxford et al., 1998; Rechsteiner et al., 1996]. In mammals, seven IκB members have been identified, including IκBα, IκBβ, IκBε, IκBγ, IκBδ, IκBζ, and Bcl-3 [Kawai and Akira 2007; Karin and Ben-Neriah, 2000]. IκB genes have also been found in several non-mammalian species, including the molluscs *Crassostrea gigas* [Montagnani et al., 2008, Zhang et al., 2011], *Pinctada fucata* [Zhang et al., 2009] and *Euprymna scolopes* [Goodson et al., 2005], but the biological functions of these homologues

remain to be fully characterizaed.

The manila clam *Ruditapes philippinarum* represents one of the most economically-important shellfish species of the aquaculture industry worldwide, and especially in Korea [Park et al., 2006]. Over the last decade, Korean clam landings have been decreasing dramatically, presumably due to the associated upsurge in prevalence of the marine protist *Perkinsus olseni* [Park et al., 1999; Park et al., 2001]. However, incidence of Gram-negative bacteria *Vibrio tapetis* infection has risen remarkably along the west coast of Korea over this same time period [Park et al., 2006]. *V. tapetis* is the causative agent of brown ring disease (BRD), named as such for the obvious brown conchiolin deposits that accumulate on the infected clam's inner shell surface [Borrego et al., 1996]. The underlying mechanism of this phenotype has been defined as *V. tapetis*-mediated disruption of normal periostracal lamina production, which leads to anomalous deposition of peristracum. In addition to this morphological symptom, *V. tapetis* infected clams experience significant weight loss, altered biochemical composition [Plana et al., 1996], depressed defense-associated activities [Allam et al., 2000; Paillard et al., 2004], and ultimately death [Allam et al., 2002].

In order to control such pathogen-associated diseases in the aquacultural mollosc population, and to enhance the yields of manila clam landings, it is necessary to research the innate immune defense mechanisms that protect clams. Since the NF-κB/IκB pathway is a very important mediator of innate immunity in molluscs, we investigated the factors involved in activating or suppressing this particular immune response. Using a genome-based sequencing approach, we

isolated a novel full-length IκB cDNA from manila clam, designated as Rp-IκB. The Rp-IκB mRNA expression profiles in response to bacterial challenge were determined in the immune-related tissues of gill and hemocytes.

3.2. Materials and methods

3.2.1. Next-generation sequencing

Total RNA was extracted from various tissues of manila clams using the Trizol reagent (Sigma-Aldrich, USA) and following the manufacturer's protocol. Poly(A) mRNA was isolated using the FastTrack® 2.0 kit (Invitrogen, USA), according to the manufacturer's instructions. Purified mRNA from various tissues was diluted to 1 μg/μL and then pooled in equal amounts for use as template to synthesize multi-tissue cDNA. The full-length, enriched first-strand cDNA was generated using the Creator™ SMART™ cDNA library construction kit (Clontech, USA) and following the manufacturer's instructions. Resultant cDNA was amplified in a reaction mix containing 50X Advantage 2 polymerase (Clontech) and normalized using the Trimmer-Direct cDNA normalization kit (Evrogen, Russia).

The normalized cDNA was used for parallel pyrosequencing in a GS-FLX titanium automated sequencing instrument (454 Life Sciences, USA). The creation of the cDNA GS-FLX shotgun library and subsequent pyrosequencing on the next-generation sequencing platform were performed by DNA Linker (Korea). Sequencing reads were processed and assembled by the Arachne assembler algorithm [Batzoglou et al., 2002; Jaffe et al., 2003; Gnerre et al., 2009]. Unique cDNAs that

showed homology to known IκB family members were identified by the Basic Local Alignment Tool (BLAST) algorithm [Altchul et al., 1990].

3.2.2. Sequence characterization of Rp-IκB

The nucleotide sequence was translated to protein sequence by DNAssist 2.2 software (http://www.dnassist.soft32.com). The conserved serine domain and PEST sequence were identified by the SMART sequence analysis program (http://smart.embl-heidelberg.de) and ePESTfind (http://emboss.bioinformatics.nl/cgi-bin/emboss/epestfind), respectively. Multiple alignment of protein sequences was carried out by the ClustalW program (http://www.ebi.ac.uk/Tools/clustalw2/index.html), and the identity and similarity percentage of Rp-IκB were calculated using EMBOSS pairwise alignment algorithms (http://www.ebi.ac.uk/Tools/emboss/align/). The phylogenetic tree was constructed by means of the Neighbor-Joining method using molecular evolutionary genetic analysis (MEGA) software (http://www.megasoftware.net).

3.2.3. Manila clam care and tissue collection

Manila clams (shell lengths of 3.5-4 cm) were collected from the Eastern coastal region of Jeju Island (Republic of Korea). Clams were maintained in the laboratory in 80 L flat-bottom tank filled with aerated and sand-filtered seawater having salinity of 34‰ and kept at 21±1°C. All animals were acclimatized to laboratory conditions for at least one week prior to initiation of any experimental

procedures.

To investigate the tissue-specific expression of Rp-IκB mRNA, hemocytes, adductor muscle, siphon, mantle, gill, and foot were dissected. The clam hemolymph was harvested from the posterior adductor muscle sinus by using 1 mL syringes with 26-gauge needles placed through the shell hinge. The hemolymph was immediately centrifuged (3000 x g for 10 min at 4°C) and the pelleted hemocytes were collected after removal of supernatant. All of the tissue samples and hemocytes were immediately snap-frozen in liquid nitrogen and stored at -70°C until RNA isolation.

3.2.3. Immune challenge

In order to determine the immune response of Rp-IκB, intact *V. tapetis* (no. 12728; Korean Collection for Type Culture) and purified lipopolysaccharide (LPS from *Escherichia coli* 0127:B8; Sigma-Aldrich) were injected directly into adductor muscle of manila clams. The *V. tapetis* was initially grown by culturing on solid marine agar (Difco, USA) at 25°C for 48 h, after which a single colony was selected and used to inoculate 15 mL of marine broth. The liquid culture was then grown at 25°C with shaking at 180 rpm; twenty-two hours later, the entire sample volume was centrifuged at 3500 rpm for 10 min. The resulting supernatant was removed and discarded, and the bacterial pellet was resuspended in 0.9% saline. Clams were intramuscularly injected with 100 μL (1.3 X 10^8 cfu individual^{-1}) of *V. tapetis* and LPS (100 μg individual^{-1}) stock into live clam adductor muscle. A negative control group established as un-injected, while positive control group was injected with an

equal volume (100 μL) of 0.9% saline. Gill and hemocyte samples were taken from five animals at 3, 6, 12, 24 and 48 h post-challenge. 0.9% saline control samples also were isolated at each time point. All samples were immediately snap-frozen in liquid nitrogen and stored at -70°C until use.

3.2.4. Total RNA isolation and cDNA synthesis

Total RNA was extracted from isolated tissues (40 mg each). First-strand cDNA was synthesized from 1 μg of total RNA by using the PrimeScript^{TM} first-strand synthesis kit (TaKaRa, Japan) and following the manufacturer's protocol. The cDNA product was diluted 10-fold and stored at -20°C until use as template for quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR) to analyze tissue-specific expression and expression changes in response to immune challenges.

2.5. qPCR analysis

Primers used in this study are listed in Table 1. qPCR was carried out using the Thermal Cycler Dice Real-Time System (TP800; TaKaRa) programmed with the following amplification conditions: one denaturation cycle of 95°C for 3 min, followed by 45 amplification cycles of 95°C for 20 s, 58°C for 20 s, 72°C for 30 s. The baseline was set automatically by the accompanying system software (version 2.0). The Livak ($2^{-\Delta\Delta CT}$ method) was used to analyze the expression levels of Rp-IκB

[30]. To determine expression fold-change after immune challenge, the relative expression was further compared to that detected in matched tissues from un-injected controls. All data are presented as relative mRNA expressed as means ± standard error. Statistical differences between the control and treatment groups were determined by the *t*-test using the SPSS 11.5 program. A *p*-value less than 0.05 was considered to indicate significant difference.

3.3. Results

3.3.1. Sequence characterization and phylogenetic analysis of the Rp-IκB

Manila clam whole genome pyrosequencing data was analyzed by BLAST, and an NF-κB inhibitor cDNA was identified. The Rp-IκB sequence was deposited in GenBank under Accession No. JF683414. Rp-IκB was found to be comprised of a 1032 base pair (bp) open reading frame (ORF) that encodes for a 343 amino acid (aa) protein having a predicted molecular mass of 38 kDa and theoretical isoelectric point of 4.6 (Fig. 9). SMART analysis revealed that the Rp-IκB protein contained six ankyrin repeats in its C-terminal region, and two signal recognition-related serine residues (Ser_{52} and Ser_{59}) encompassing the IκB degradation motif ($DS_{52}GVYS_{59}$) in the N-terminal region. In addition, the IκB characteristic PEST sequence was identified in the N-terminal region of Rp-IκB. When the deduced aa sequence of Rp-IκB was aligned against other known IκB orthologs, the IκB degradation motif and ARR functional domains were found to be conserved (Fig. 10).

The deduced aa sequence of Rp-IκB was analyzed to determine the extent of

similarity and identity with other known IκB protein family members. Coding full-length amino acid sequence of Rp-IκB showed the highest identities with *Pinctada fucata* (33.9%) followed by *Argopecten irradians* (32.6%), *Eupryma scolopes* (30.2%), *Crassostrea gigas* (30.1%), Metrix metrix (28.4%) and *Biomphalaria glabrata* (27.1%). The identity to human and mandarin fish IκB was 27.1% and 22.6%, respectively.

The deduced amino acid sequence of Rp-IκB was compared with that of the protein form other vertebrate and invertebrate IκBs. A phylogenetic tree was constructed based on the analysis of protein sequences by the ClustalW program. The Rp-IκB and invertebrate IκB proteins grouped together (Fig 11). The vertebrate IκBs that were most proximal to the invertebrate group, including Rp-IκB, were of the mammalian IκBα proteins. All other vertebrate IκB isoforms, including IκBβ and IκBε clustered together in another branch of the phylogenetic tree.

3.3.2. Basal expression level analysis of Rp-IκB mRNA

qRT-PCR was carried out to determine the normal expression profile of Rp-IκB in various clam tissues (Fig. 12.). Rp-IκB mRNA was ubiquitously expressed in all examined tissues (adductor muscle, mantle, siphon, gill, and foot) and hemocytes. The highest expression level of Rp-IκB mRNA was detected in hemocytes, with the next highest levels in gill and mantle. When compared to levels detected in the adductor muscle tissue, where expression was lowest, the Rp-IκB expressions in hemocytes, gill and mantle were found to be 184-, 63-, 33-fold higher, respectively.

3.3.3. Rp-IκB expression is altered in response to immune challenge

Temporal expression of Rp-IκB was examined in hemocyte and gill tissues following *V. tapetis* and LPS challenge (Fig.13). Transcriptional up-regulation of Rp-IκB occurred in both gill and hemocyte after LPS challenge. In hemocyte, Rp-IκB mRNA was gradually increased to peak at 6 h (25.7-fold). Then, expression level was decreased until 24 h post challenge, and again increased at 48 h after challenge (Fig. 13A). In gill tissue, Rp-IκB mRNA was immediately up-regulated (4.7-fold) at 3 h after challenge (Fig. 13B). Compared to saline injected control, significant difference of Rp-IκB mRNA expression level was observed at all the time point (except 6 h post challenge).

After the *V. tapetis* challenge Rp-IκB mRNA expression profiling showed different patterns to those observed with the LPS challenge. No significant difference of Rp-IκB was observed in both hemocyte and gill at 3 h after *V. tapetis* challenge, unlike LPS challenged clams. In hemocyte, significant up-regulation was observed at 6 (3.7-fold), 24 (2.6-fold) and 48 h (1.7-fold) posh challenge (Fig. 13A). In gill tissues, significant up-regulation was observed at only 12 h (2.5-fold) after challenge (Fig. 13B).

3.4. Discussion

In this study, we described the identification and characterization of a novel

IκB gene from manila clam. The Rp-IκB aa sequence contains domains characteristic of IκB protein family members, including the degradation motif, ARR and PEST sequence.

Previous studies on the mammalian IκB degradation motif have demonstrated that phosphorylation of the conserved serine residues can be mediated by a variety of extracellular stimuli [Luque et al., 2000]. In particular, kinase activity of the IκB kinase subunit beta (IKKβ) targeting the degradation domain leads to ubiquitination and proteasomal degradation of IκB [Schmid and Birbach, 2008]. The Rp-IκB also contains the conserved IκB degradation motif ($DS_{52}GVYS_{56}$) in its N-terminal region, which suggests that Rp-IκB may be similarly regulated by IKKβ and NF-κB activation.

Ankyrin repeats are present in many biologically-important proteins and typically function as regulators of specific protein-protein interactions [Ghosh et al., 1998; Mu et al., 2010]. The ankyrin repeat motif has been shown to be essential for physical interactions with the NF-κB protein in mammals; specifically, ARRs mediate binding with the Rel homology domain (RHD) of NF-κB, which masks the nuclear localization signal (NLS) and blocks the DNA-binding site [Yamamoto and Gaynor, 2004]. Five ankyrin repeats are present in IκBα from humans, cattle, and chickens. However, six ankyrin repeats were found in Rp-IκB, which is identical to the number found in other marine species, such as molluscan IκBs (pacific oyster [Montagnani et al., 2008; Zhang et al., 2011], scallop [Mu et al., 2010], and pearl oyster [Zhang et al., 2009]) and fish IκBα (rainbow trout [Yazawa et al., 2007]). The causes of different ankyrin repeats numbers are still unknown, and require further

study.

The PEST sequence was found in the N-terminal region of Rp-IκB. Human IκBα also has the PEST sequence, but it is located in the C-terminal. The only other molluscan IκB described to date that has the PEST sequence is the bobtail squid (AAY27980). All other molluscan IκBs contain only casein kinase II phosphorylation sites. Some IκBs from other species also contain the PEST sequence in the N-terminal region, including those from the fruit fly cactus (GenBank accession No. NP_476942) and mouse IκBε (AAH30923). In humans, degradation of free IκBα is not controlled by IKK or ubiquitination [Mathes et al., 2008], but is intrinsically mediated by the PEST sequence region. When human IκB is bound to NF-κB the PEST sequence is protected from proteasomal degradation [Huxford et al., 1998; Phelps et al., 2000]. The finding of PEST in Rp-IκB fits with the theory that Rp-IκB is regulated by NF-κB in resting cells.

Ubiquitously expressed IκBs have been previously reported in vertebrates and invertebrates [Wang et al., 2009; Montagnani et al.,2008; Zhang et al., 2009; Mu et al., 2010, Yazawa et al., 2007; Rupec et al., 1999; Sangrador-Vegas et al., 2005]. In the pacific oyster [Montagnani et al., 2008] and pearl oyster [Zhang et al., 2009] molluscs, IκB expression was reported to be highest in hemocytes. Similarly, we found that Rp-IκB mRNA was expressed in all tested tissues (adductor muscle, siphon, mantle, gill and foot), and the highest level was in hemocytes. Hemocytes represent the main cellular component of the molluscan immune system, wherein they mediate cellular internal defense through phagocytosis and encapsulation of invading microorganisms [Humphries and Yoshino et al., 2003].

To gain a better understanding of the biological function of Rp-IκB, we investigated the effects of immune challenge on Rp-IκB expression. Rp-IκB mRNA was found to be significantly up-regulated in gill and hemocytes in response to *V. tapetis* and LPS challenge. These results are consistent with previously reported data for both vertebrate and invertebrate IκB family members. In particular, the IκBα of the vertebrate Japanese flounder was demonstrated to be up-regulated in kidney in response to LPS stimulation [Yazawa et al., 2007], while the CgIκB1 and CgIκB2 of the invertebrate pacific oyster were both up-regulated rapidly in hemocytes in response to *V. alginolyticus* infection [Zhang et al., 2011]. Interestingly, expression of Rp-IκB mRNA was up-regulated by immune challenge, even though its structure suggests it is an inhibitor of NF-κB. In mammals, once NF-κB is released from the IκB complex it translocates to the nucleus, wherein it can activate gene transcription of the NF-κB gene itself. The IκBα gene is also a target of NF-κB; thus, increased transcription of IκBα can result from NF-κB activation. As a result, the newly-synthesized IκBα inhibitor would rebind NF-κB, thereby inactivating the transcriptional activity by removing NF-κB from the DNA and mediating its export from the nucleus [Hayden and Ghosh, 2004; Sue and Dyson, 2009]. Collectively, these findings suggest that the NF-κB/IκB pathway may be involved in the *V. tapetis*- and LPS- stimulated immune response. Future studies to identify the NF-κB pathway-related factors will provide further insight into the Rp-IκB function and its potential use as a therapeutic target.

In conclusion, we have described the cloning and characterization of the first IκB identified to date from the manila clam *Ruditapes philippinarum*. Sequence

characterization and phylogenetic analysis revealed that the Rp-IκB gene and protein have characteristic features similar to members of the vertebrate IκBα. We were able to confirm that the IκB homologue from manila clam belongs to a subset of the IκB family, based upon the presence of three characteristic protein domain features: IκB degradation motif, PEST sequence region, and six ankyrin repeats. Rp-IκB was ubiquitously expressed in all the examined tissues of un-injected manila clam. Furthermore, bacterial challenge caused Rp-IκB mRNA expression to be up-regulated in gill and hemocytes. Taken together, these results suggest that Rp-IκB represents a new member of the inhibitor of NF-κB protein family that is involved in the NF-κB signaling pathway and participates in innate immune responses against bacterial and LPS infection.

Table. 4. Primers used in this study

Primer	Sequence	Gene target
IkBF	5’-ATG TCC GAG TCT GCA CTT GCT CTT-3’	Rp-IκB
IkBR	5’-TTT CAG TTT GCC GTG TGA GCA CTG-3’	Rp-IκB
ActinF	5’-CTC CCT TGA GAA GAG CTA CGA-3’	β-actin
ActinR	5’-GAT ACC AGC AGA TTC CAT ACC C-3’	β-actin

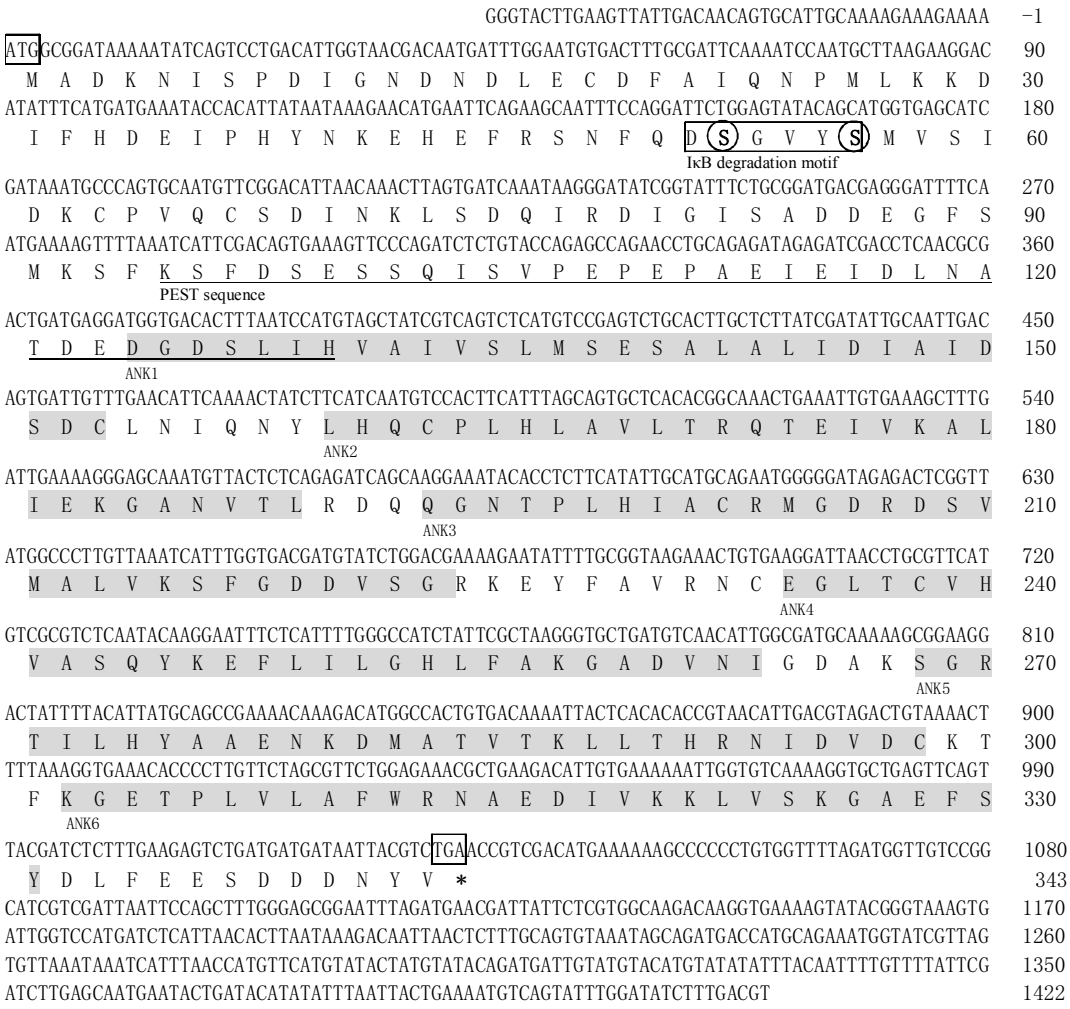

Figure 9. The complete nucleotide and deduced amino acid sequences of the manila clam IκB. The start codon (ATG) and stop codons (TGA) are indicated by boxes. The six putative ankyrin repeats are highlighted in gray. The PEST sequence is underlined. The IκB degradation motif is boxed and the two conserved serine residues are circled.

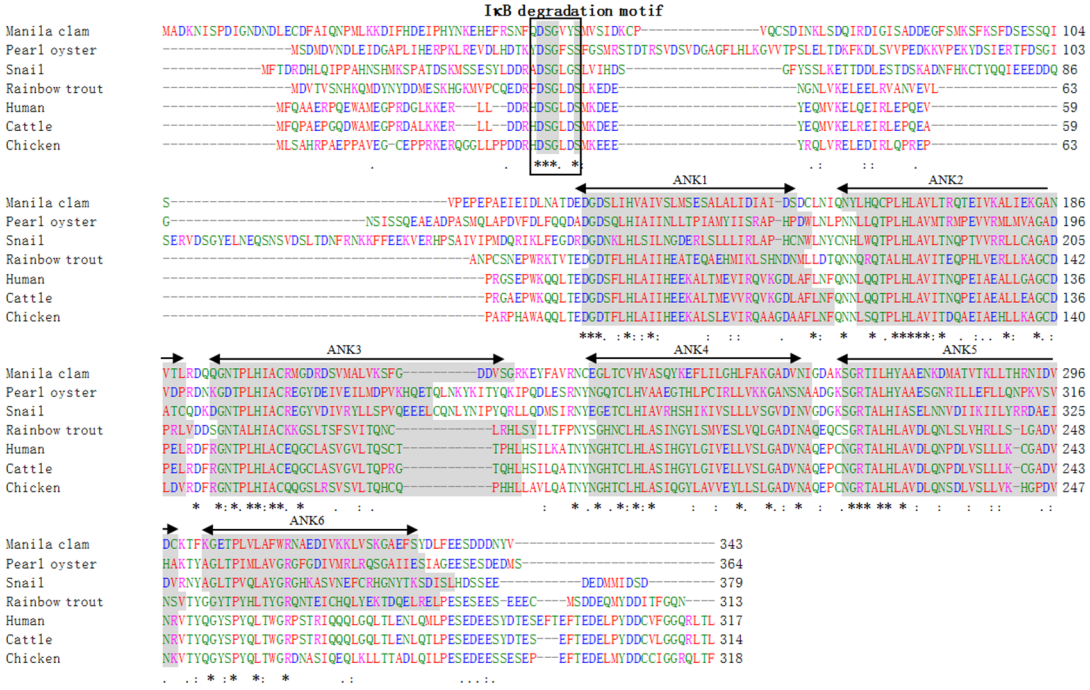

Figure 10. Multiple alignment of Rp-IκB with other selected species IκB amino acid sequences. The IκB degradation motif is boxed. The ankyrin (ANK) repeats are indicated by arrows. Human IκBα (AAH02601), cattle IκBα (AAI05485), chicken IκBα (Q91974), rainbow trout IκBα (NP_001117840), snail IκB (ABL74452), pearl oyster IκB (ACF93446) are shown.

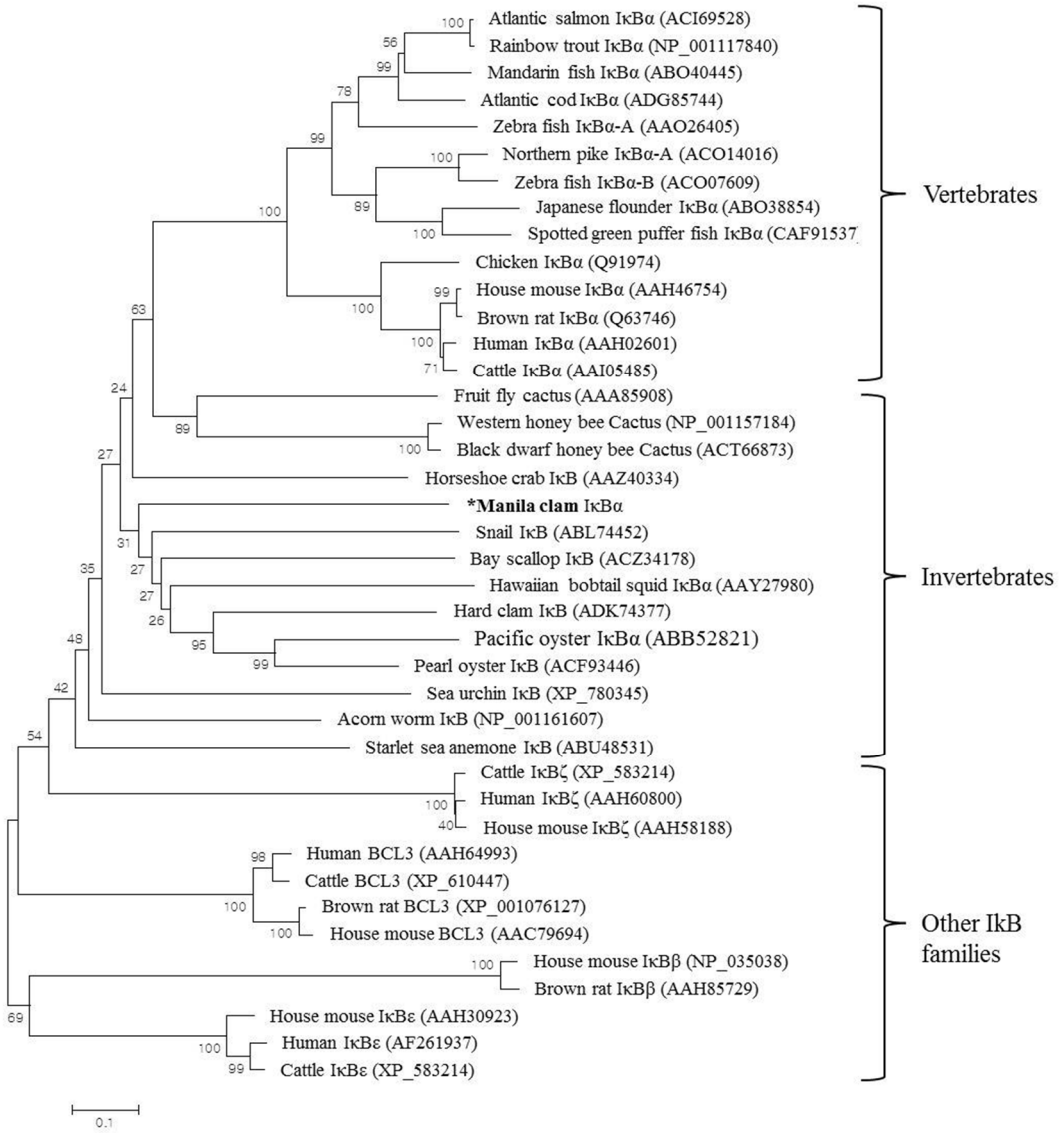

Figure 11. The phylogenetic relationship of Rp-IκB with other selected species IκB family members. The number at each node indicates the percentage of bootstrapping after 1000 replicates. The GenBank accession numbers of selected sequences are indicated within brackets.

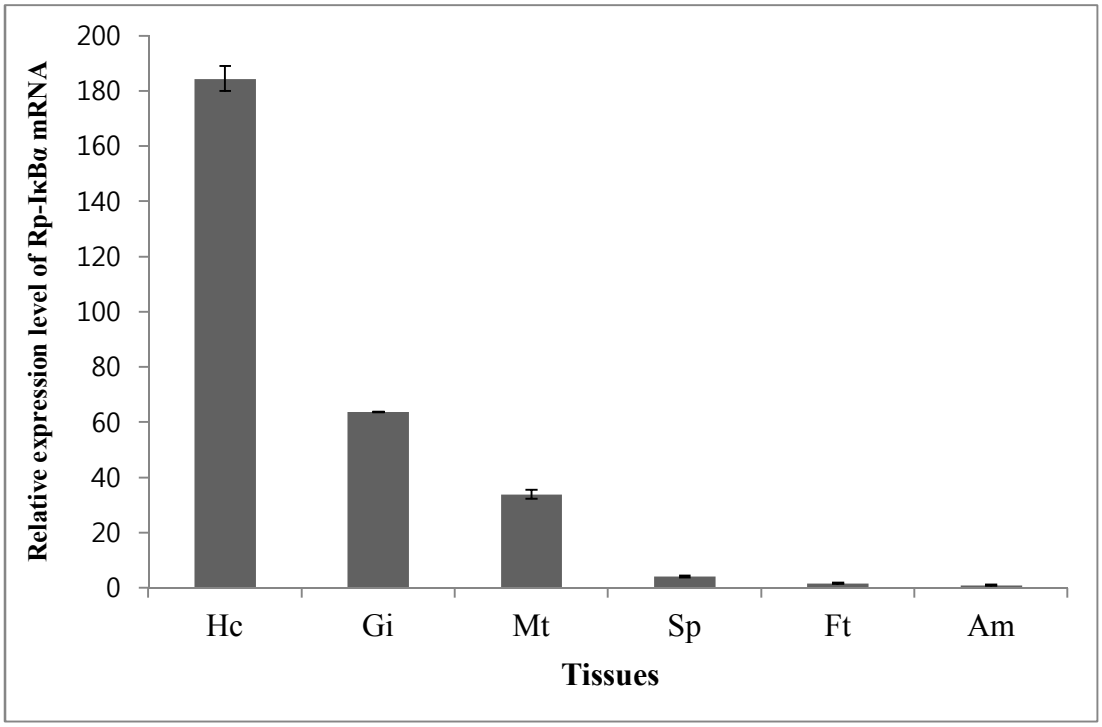

Figure 12. Tissue-specific expression of Rp-IκB mRNA. The relative mRNA expression detected by quantitative real-time RT-PCR for each tissue was normalized to adductor muscle mRNA expression. Data are presented as the mean relative expression ± standard error for three replicate real-time reactions from pooled tissues of five individual clams for each tissue. Hc, hemocytes; Gi, gill; Mt, mantle; Sp, siphon; Ft, foot; Am, adductor muscle.

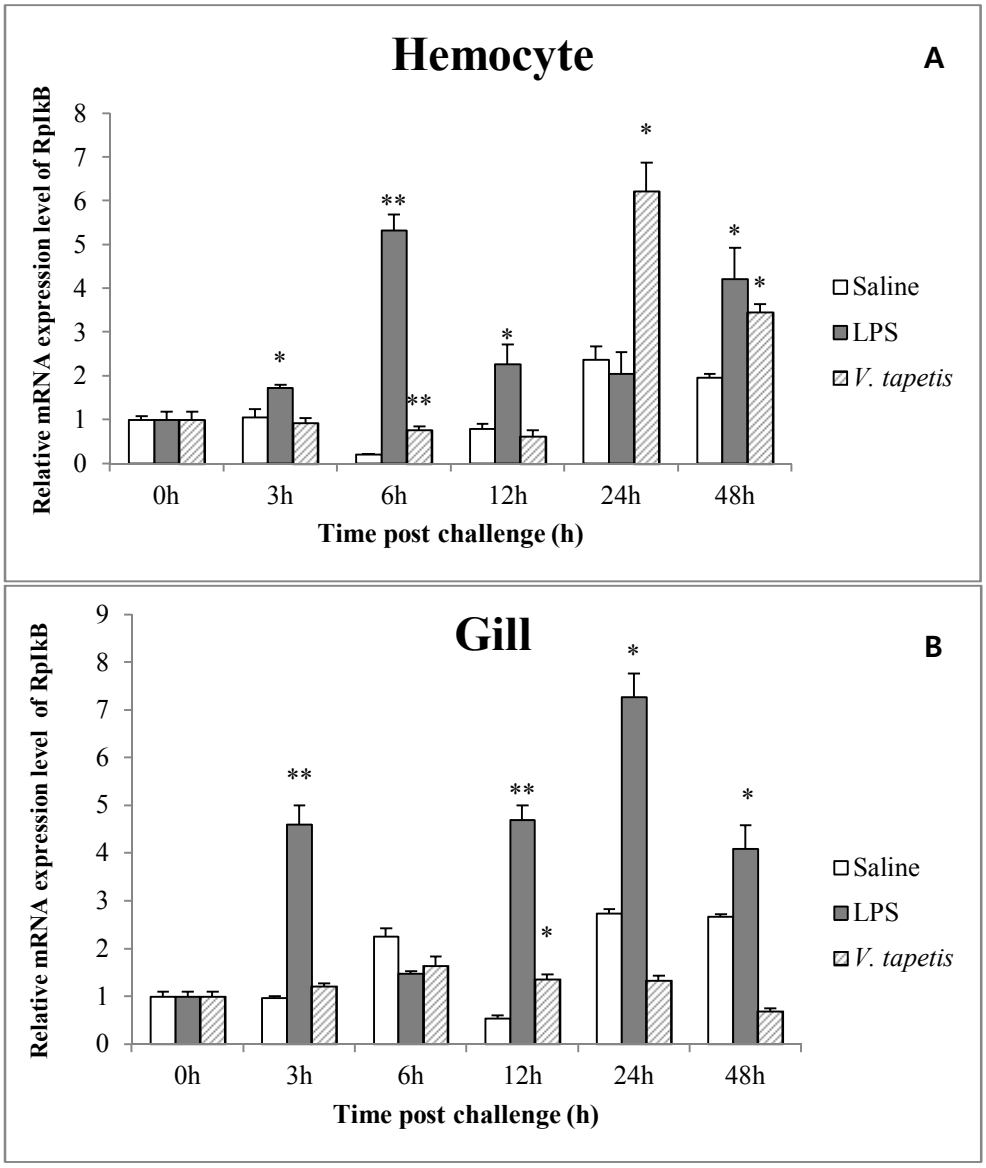

Figure 13. Rp-IκB mRNA expression in clam hemocyte (A) and gill (B) after LPS and *V. tapetis* challenge. The expression fold was calculated by the $2^{-\Delta\Delta CT}$ method using β-actin as the reference gene. The relative level of expression for each time point was compared to that of un-injected control. Data are presented as the mean relative expression ± SE for three replicate real-time reactions from pooled tissue of five individual clams at each time point.

Chapter IV

Molecular characterization and expression analysis of molluscan TNFα homologue from manila clam *Ruditapes philippinarum.*

4.1. Introduction

Tumor necrosis factor (TNF) is a pro-inflammatory cytokine which plays a key role in a wide variety of physiological processes, including inflammation, proliferation and programmed cell death and belongs to a large family of structurally related proteins called the "TNF ligand superfamily" [Goetz et al., 2004]. The actions of these factors are diverse and profound involving inflammation, apoptosis, cell proliferation and the stimulation of various aspects within the immune system [Goetz et al, 2004]. These pleiotropic biological effects of TNF result from its ability to initiate different intracellular signaling pathways [Wullaert et al., 2006]. TNF-α also can interact directly with certain parasites as sell as bacteria through a region contains the lectin-like activity for N, N'-diacetylchitobiose [Beschin et al., 2001; Lucas et al., 1994].

Innate immunity is a wide spread non-specific immune response which is associated with phagocytes, effector molecules, and cytokines. Cytokines are a group of small and soluble molecules, e.g. interleukins, interferons, and tumor necrosis factors which have a key role in the proliferation of immunocytes, apoptosis and immune modulation. In invertebrates, Eiger and Wengen genes have been identified as the first members of the TNFSF and TNFRSF proteins in *Drosophila*, respectively [Igaki et al., 2002, 2011; Kanda et al., 2002; Kauppila et al., 2003; Li et al., 2009; Babery and Schneider, 2010; Mekata et al., 2010; Moreno et al., 2002].

Innate immunity is a regulatory system conserved in all species of multicellular organisms through lower animals to higher animals. There is a considerable potential to use innate immune system for effective pathogenic control

without being use of antibiotics. Furthermore we have control the fish disease in cost effective manner in mariculture industry. Therefore, development of good method is a mandatory to prevent infection. Innate Immunity is initiated following the recognition of foreign bodies (such as bacterial components) and a response by immune cells, for example macrophages [Du Pasquier, 2001].

In this study, we identified manila clam TNF gene. We characterized the nucleotide sequence, putative amino acid sequences, and their expression pattern in various organs, and expression profiling in gill and hemocyte after immune challenge. The information presented in this study will facilitate the understanding of the immune system of manila clam.

4.2. Materials and methods

4.2.1. Manila clam cDNA library and identification of RpTNFα

We have established a normalized cDNA library using RNA isolation from multiple tissues of healthy clams. The basic procedure of cDNA library construction, normalization and initial GS-FLX sequencing strategies has been described in previous report [Revathy et al., 2011]. BLAST analysis of the clam cDNA shotgun sequence database identified a putative cDNA with high homology to known TNFα, designated as manila clam *RpTNFα* and further characterized.

4.2.3. Sequence characterization of RpTNFα

The nucleotide sequence was translated to protein sequence by DNAssist 2.2 software (http://www.dnassist.soft32.com). The conserved serine domain was identified by the SMART sequence analysis program (http://smart.embl-heidelberg.de). Multiple alignment of protein sequences was carried out by the ClustalW program (http://www.ebi.ac.uk/Tools/clustalw2/index.html), and the identity and similarity percentages of RpTNFα were calculated using EMBOSS pairwise alignment algorithms (http://www.ebi.ac.uk/Tools/emboss/align/). The phylogenetic tree was constructed by means of the Neighbor-Joining method using molecular evolutionary genetic analysis (MEGA) software (http://www.megasoftware.net).

4.2.4. Experimental animals

Manila clams (shell length 3.5-4.0 cm) were collected from the Eastern coastal region of Jeju Island (Republic of Korea). Clams were maintained in the laboratory in an 80 L flat bottom tank with aerated and send-filtered seawater having salinity of 34 ‰ at 21±1°C. All animals were acclimatized to laboratory conditions for at least a week prior to initiation of any experimental procedures. Animals were fed with a recommended commercial diet (shellfish diet 1800, Reed Mariculture INC, USA) during the acclimation period.

4.2.5. Collection clam tissues and hemocytes for tissue specific expression of RpTNFα mRNA

Gill, mantle, siphon, adductor muscle and foot tissues of clam were dissected from three animals to determine the RpTNFα mRNA expression by quantitative real-time RT-PCR. The clam hemolymph (approximately 1 mL per animal) was collected from the posterior adductor muscle sinus using 1 ml syringes with 26-gauge needles through the shell hinge. The hemolymph was immediately centrifuged (3500 x g for 10 min at 4 °C). The supernatant was removed and the hemocytes were immediately snap-frozen in liquid nitrogen and stored at -70°C.

4.2.6. Immune stimulation by V. tapetis, LPS and poly I:C

To determine the immune responses of RpTNFα, *V. tapetis,* LPS (bacterial lipopolysaccharide, Sigma) and poly I:C (double strand RNA mimic, Sigma) were injected directly into adductor muscle of manila clam. The *V. tapetis* [KCTC no. 12728] was obtained from the Korean Collection for Type Culture (KCTC). *V. tapetis* was initially cultured on solid marine agar (Difco, USA) at 25°C for 48 h, after which a single colony was selected and used to inoculate 15 mL of marine broth. The liquid culture was then grown at 25°C with shaking at 180 rpm; twenty-two hours later the entire sample volume was centrifuged at 3500 rpm for 10 min. The resulting supernatant was removed and discarded, and the bacterial pellet was resuspended in 0.9% saline. Bacterial CFU was quantified by spectrophotmetric measurement (OD_{650}) and stock solution was prepared (cell count of 3.2 x 10^9 cell/mL saline). LPS (1 mg/mL) and poly I:C (100 mg/mL) also dissolved in 0.9 % saline. Immune challenge was carried out by intramuscular injection of 100 μL LPS, poly I:C and

bacterial stock solution per clam. The control group was injected with an equal volume (100 μL) of 0.9% saline. Gill and hemocytes samples were collected from five animals in each group at 3, 6, 12, 24 and 48 h post-challenge in triplicates

4.2.7. Total RNA isolation and cDNA synthesis

Total RNA was extracted from collected tissues (50 mg per each tissue) using Qiazol reagent (Qiazen, Japan) according to the manufacturer's protocol. The RNA concentration was measured at 260 nm in a UV-spectrophotometer (Bio-Rad, USA). First-strand cDNA synthesis was carried out using 1 μg of total RNA as template with PrimeScript^{TM} first strand synthesis kit (TaKaRa, Japan) by following the manufacturer's instruction. The cDNA product was diluted 10- fold and stored at -20°C until further use.

4.2.8. mRNA expression analysis of RpTNFα by real-time RT-PCR

The RpTNFα mRNA expression was determined by quantitative real-time RT-PCR using gene specific primers RpTNF-F (5'- AGA ATG GTG CCA TGG TCG AGA ACT -3) and RpTNF-R (5'- CGC ATA TTT GCA CGG CTG GTA GAA- 3'). The gene coding the clam β-actin protein was selected as a reference gene and it amplified using RpActin-F (5'- CTC CCT TGA GAA GAG CTA CGA -3') and RpActin-R (5'- GAT ACC AGC AGA TTC CAT ACC C -3') gene specific primers [Zhang et al., 2011]. Tissue specific mRNA expression was analyzed in clam gill,

mantle, adductor muscle, foot and siphon tissues and hemocytes. Real-time RT-PCR was performed using the Thermal Cycler Dice Real-Time System (TP800; TaKaRa) under the following conditions: one denaturation cycle of 95°C for 3 min, and 45 amplification cycles of 95°C for 20 s, 58°C for 20 s, 72 °C for 30 s. The baseline was set automatically by the accompanying system software (version 2.0). The Livak ($2^{-\Delta\Delta CT}$ method) was used to analyze the expression levels of RpTNFα [Livak and Schmittgen, 2001]. To determine expression fold-change after immune challenge, the relative expression was further compared with un-injected controls. All data are represented as relative mRNA expressed as means ± standard error (SE). Statistical difference between the control and treatment groups were determined by t-test using the SPSS 11.5 program, and differences were considered significant at $P < 0.05$.

4.3. Results

4.3.1. Identification and characterization of RpTNFα cDNA sequence

The TNFα cDNA was identified from GS-FLX sequencing database of manila clam transcriptome by NCBI BLAST program. It was named as RpTNFα. The nucleotide and predicted amino acid sequences of RpTNFα cDNA are shown Fig.14. The RpTNFα cDNA consist 1429 bp with 792 ORF sequence encoding 265 amino acids. It contains 86 bp 5′-untranslate region (UTR) and 548 bp 3′-UTR. The RpTNFα has a putative molecular mass of 31 kDa with an isoelectric point (pI) of 8.9. The signal peptide was identified at the N-terminal sequence with the cleavage site between 25 and 26 amino acid positions. Cleavage at this site generates a mature

polypeptide of 240 amino acids. The predicted transmembrane domain and TNF family domain was observed at the 7-29, and 103-264 amino acids position, respectively. In addition, a sequence $_{154}$LVVPVSGTYYYIYSF$_{167}$ containing the TNF family signature ([LV]-x-[LIVM]-x_3-G-[LIVMF]-Y-[LIVMFY]$_2$-x_2-[QEKHL]) was located in the TNF family domain. (Fig.15.).

The deduced amino acid sequence of RpTNFα showed the highest identity (28 %) with that of bivalve pacific oyster, based on the EMBOSS pairwise alignment algorithm (Table. 6). Also, it was shown that RpTNFα that has 25.3 and 24.1 % identity with disk abalone and small abalone, respectively.

4.3.2. Phylogenetic analysis

A phylogenetic tree was generated by the MEGA 5.05 neighbor-joining method using different members of TNF superfamily ligands selected from vertebrates and invertebrates (Fig 16). The *Drosophila* "Eiger" protein, which is a member of TNF ligand superfamily, was used as the out-group. The result of phylogenetic tree indicated that there is a clear separation between vertebrate and invertebrate. One cluster includes vertebrate TNF α and β, the other one includes all invertebrates TNFα. The vertebrate cluster was divided in to three sub-clusters as mammalian TNF-α, mammalian LT-α and teleost TNF-α. RpTNFα was included in invertebrate TNFα cluster, very close to pacific oyster.

4.3.3. Tissue specific expression of RpTNFα

To determine the TNF-α expression profile in clam tissues, quantitative real-time RT-PCR was carried out using gene specific primers designed from the RpTNF-α coding sequence. The relative expression level of each transcript was normalized to that of adductor muscle to carry out the comparison. Constitutive expression of RpTNFα was detected in all examined tissues from un-challenged clams including hemocytes, gill, adductor muscle, mantle, siphon and foot (Fig 17). The highest expression level was found in hemocytes followed in a hierarchical fashion by gill, mantle and siphon.

4.3.4. Temporal expression of RpTNFα

Change of RpTNFα transcription was investigated in gill and hemocytes after challenge with *V. tapetis,* LPS and poly I:C using qPCR. In hemocytes, compare to saline control, significant up-regulation was detected at 48 h after all challenged (Fig 18a). After LPS challenge, the expression level was significantly up-regulated at 6 and 12 h. Except 48 h after challenge, no significant difference was detected at all the time point after *V. tapetis* and poly I:C.

In gill, expression of RpTNFα mRNA was significantly up-regulated at 3h and 12 h after *V. tapetis* challenge (Fig 18b). The expression level was significantly up-regulated 3 h after LPS challenge then, it was down-regulated at 6h. The expression of RpTNFα was up-regulated again from 12 to 24 h after challenge, and it showed peak level at 24 h after challenge. The expression pattern of RpTNFα after

poly I:C challenge, which is similar with expression pattern of LPS challenged clam. The RpTNF mRNA was induced by *V. tapetis*, LPS and Poly I:C.

4.4. Discussion

In this study, we identified the manila clam TNFα gene and analyzed its characteristics from nucleotide and amino acid sequences. Clam TNFα (named as RpTNFα) showed significant homology with TNFα of other vertebrates and invertebrates.

The average size of the mammalian TNFα is 234 amino acids with a range between 233 and 235, while the average size of TNFα in fish a range between 242, with a range between 225 and 256 amino acids [Goetz et al., 2004]. The TNFα of molluscan species disk abaloneconsist of 239 amino acids, which is similar to mammalian and fish TNFα. RpTNF consist of 264 amino acids, which is not similar to the range of both mammalian and fish TNFα. However, RpTNFα size is comparable with TNFα of pacific oyster which consist of 260 amino acids.

The fish and mammalian TNF share several conserved features. Among them the TNF family signature is highly conserved but varies with alternative amino acids [Goetz et al., 2004; Ordas et al., 2007]. Within this signature motif, three consistent amino acid differences have been observed previously: (i) at position 1, isoleucine and leucine; (ii) at position 3, isoleucine and valine; (iii) at position 10, phenylalanine and leucine. Fish do no completely obay to the typical signature. Although RpTNFα has conserved TNF signature motif the [LIVMF] at position 8

and [QEKHL] at position 13 are replaced by threonine and serine in that location. In disk abalone, De Zoysa et al. have reported the leucine at position 1 and valine at position 3 are replaced by isoleucine in both location [De Zoysa et al., 2009] .

Mammalian TNFα is a type II transmembrane glycoprotein having a single transmembrane domain and cleavage site for releasing of the mature soluble protein after proteolysis by TNFα converting enzyme (TACE) [Ordas et al., 2007; Kruys et al., 1990]. Also transmembrane domain comprises a cluster of hydrophobic amino acid residues from the N-terminus [Uenobe et al., 2007]. A similar transmembrane domain with relatively high hydrophobic amino acid residues is present in RpTNFα from 7-29 amino acid region. Therefore, it could be suggested that RpTNFα is a membrane bound precursor protein, and is released as a mature protein after propeptide proteolysis by TACE or a similar protein in the clam.

TNFα mRNA expression in distinct tissues has been previously reported in several fish species and disk abalone. For instance, constitutive TNFα expression was detected in the head kidney and gill tissues of the rainbow trout [Laing et al., 2001], the head kidney and liver of the catfish [Zou et al., 2003], and rainbow trout [Bridle et al., 2006], the head kidney, liver, and macrophages of the gilthead seabream [Garcia-Castillo et al., 2002]. Within the mollusc species, disk abalone, TNFα constitutive expressed in gill, mantle, muscle, digestive tract, hepatopancreas and hemocytes [De Zoysa et al, 2009] and also showed high expression level in hemocytes, gill and mantle, compare to muscle, digestive tract and hepatopancrease. In the present study, we observed constitutive RpTNFα expression in hemocytes, adductor muscle, mantle, siphon, gill and foot in a tissue specific manner. The

highest expression was observed in hemocytes and following gill and mantle. Tissue specific expression of manila clam showed similar results with disk abalone tissue specific expression. Our results indicate that TNF-α is expressed mainly in the immune cells (hemocytes) as well as non-immune tissues such as gill, mantle, siphon, adductor muscle and foot in clam. In disk abalone, the expression level of TNF was lower in hemocytes than in gill tissue. However, RpTNFα showed higher expression level in hemocytes compare to gill.

The TNFα mRNA expression in fish showed differential expression profile during viral [Tafalla et al., 2005], bacterial [Ordas et al., 2007], parasitic infections [Sigh et al., 2004] and LPS stimulation [Hirono et al., 2000]. Therefore, the study of TNFα and its specific transcriptional regulation in terms of cellular activation with different types of pathogens would greatly facilitate the understanding of the mechanism of pathogenesis in clam disease. Thus, we selected a *V. tapetis*, LPS and poly I:C to study TNFα responses in the clam hemocytes and gill. In previous studies, significant up-regulation of TNFα was observed in orange spot grouper thymus, heart, kidney, trunk kidney, brain muscle, liver, pancreas, spleen, stomach, and intestine after LPS challenge; in turbot liver and kidney after *Vibrio pelagius* strain Hq222 and VHSV. In mollusc disk abalone, TNFα was found to be significantly induced in gill by bacterial mixture (*Vibrio alginolyticus, Vibrio parahemolyticus*, and *Lysteria monocytogenes*), VHSV (viral haemorrhagic septicaemia virus) and LPS (lipopolysacchareide) challenge [De Zoysa et al, 2009]. It was reported that different pathogens induce the expression of pro-inflammatory cytokines in different ways [Thanawongnuwech et al., 2004]. Similarly, we also observed that RpTNFαwas

significantly up-regulated in the gill and hemocyte after bacteria, LPS and poly I:C stimulation. However, the induction levels and highest induction time after challenge varied with the type of induction in this study.

Table. 5. Primers used in this study for real-time RT-PCR

Primer	Sequence	Description
RpTNF-F	5'-AGA ATG GTG CCA TGG TCG AGA ACT-3'	TNFα real-time PCR forward primer
RpTNF-R	5'-CGC ATA TTT GCA CGG CTG GTA GAA-3'	TNFα real-time PCR reverse primer
Rpactin-F	5'-CTC CCT TGA GAA GAG CTA CGA-3'	β-actin real-time PCR forward primer
Rpactin-R	5'-GAT ACC AGC AGA TTC CAT ACC C-3'	β-actin real-time PCR reverse primer

Table. 6. Pair wise ClustalW analysis and comparison of the RpTNFα deduced amino acid sequence

Species	Identity (%)	Similarity (%)	Gap (%)	GenBank accession Number
Pacific oyster	28.3	44.8	16.8	ADW09010
Disk abalone	25.3	40.4	23.5	ACF75368
Small abalone	24.1	40.3	25.8	ADP24261
Japanese flounder	20.5	35.8	30.2	BAA94969
Human	18.9	32.9	34.9	AAA61198
Sea squirt	14.3	27.8	40.6	ABW76095

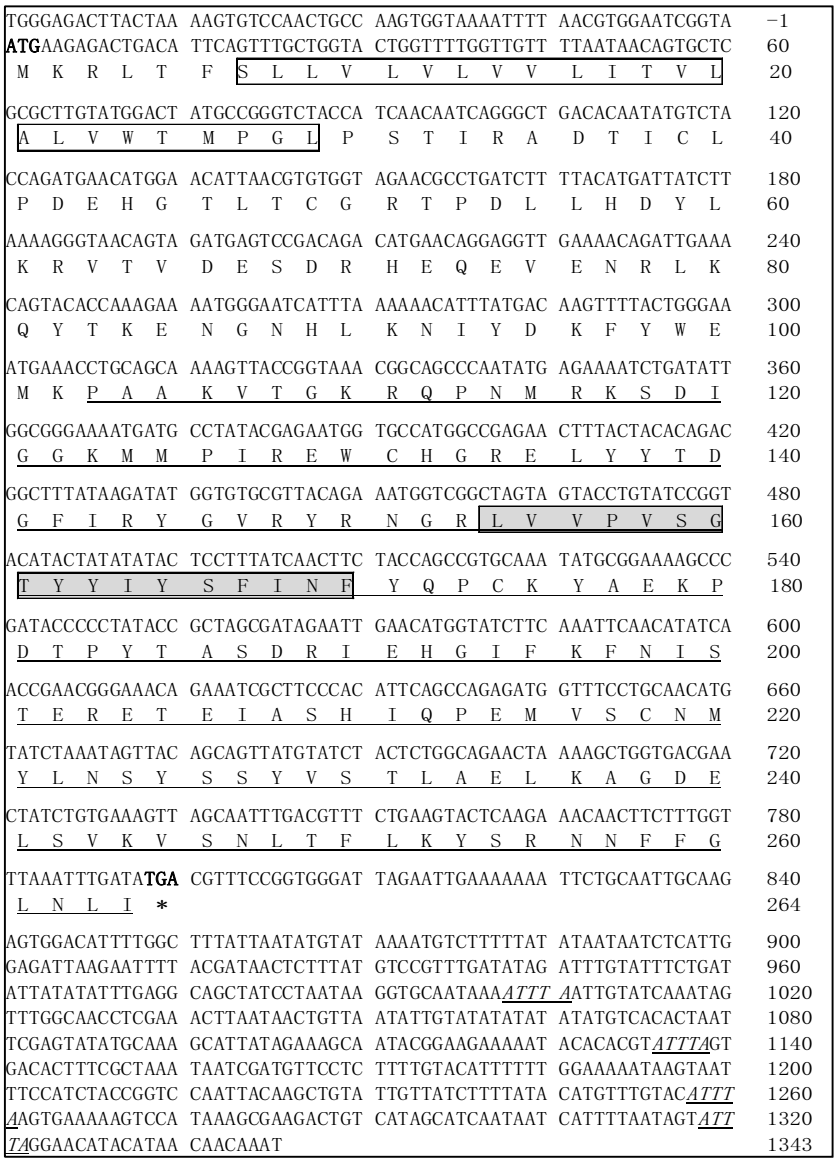

Figure 14. The complete nucleotide and deduced amino acid sequences of the RpTNFα. The start codon (ATG) and stop codons (TGA) are indicated by boxes. The transmembrane domain was boxed. The TNF homology domain is underlined. The TNF signature is grey boxed

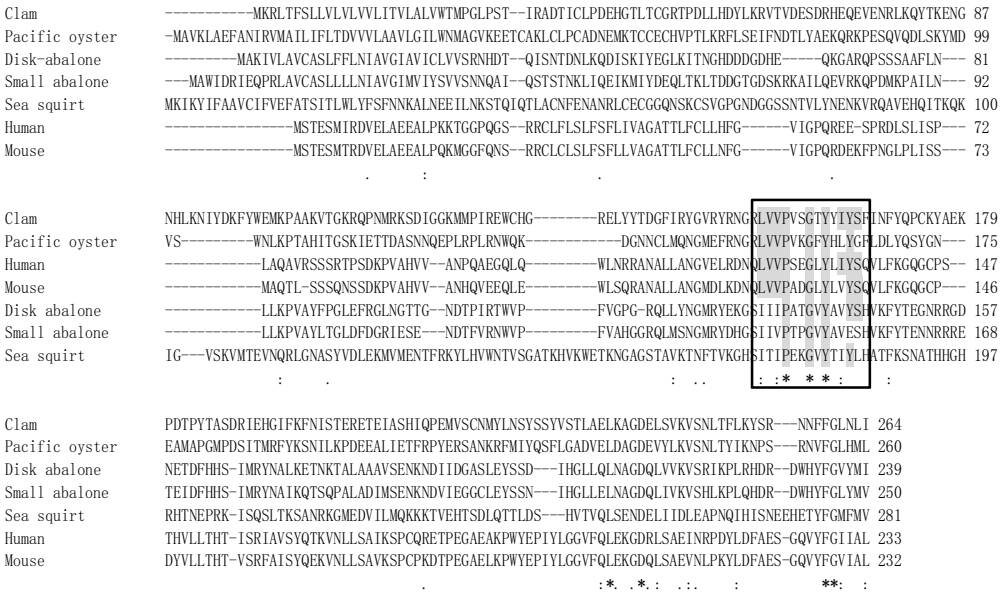

Figure 15. Multiple alignment of RpTNFα with other selected species TNF family amino acid sequences. Pacific oyster (ADW09010), disk abalone (ACF75368), small abalone (ADP24261), sea squirt (ABW76095), Human (AAA61198) and mouse (AAB65593)

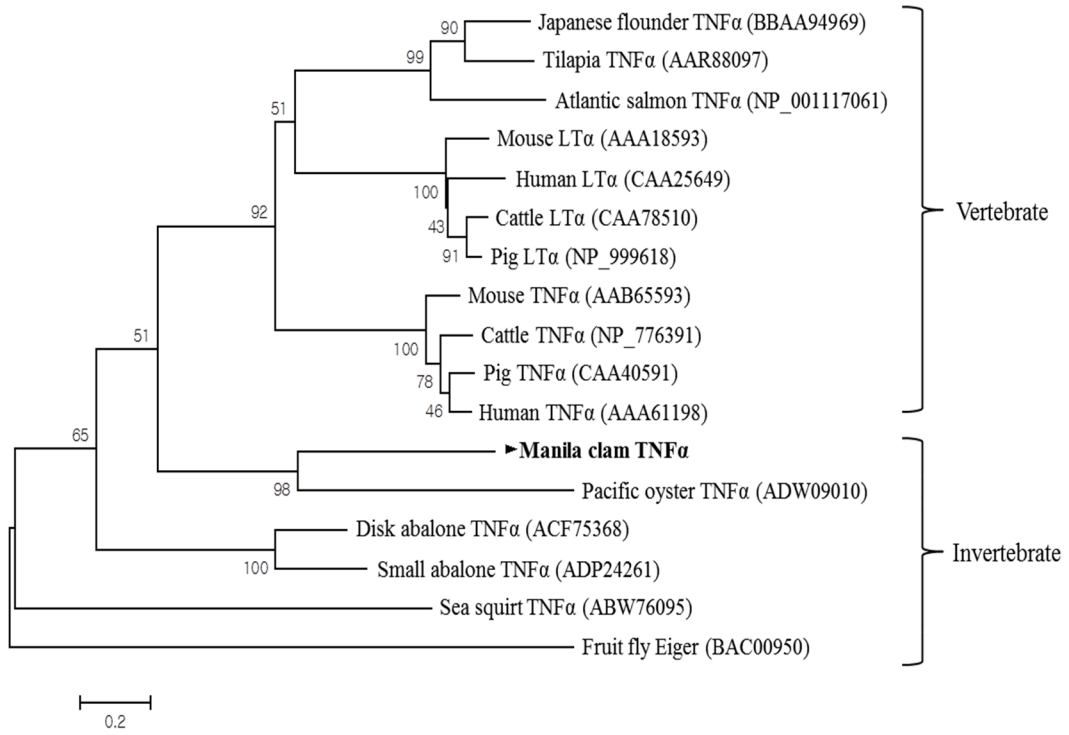

Figure 16. Phylogentic analysis of RpTNFα. The number at each node indicates the percentage of bootstrapping after 1000 replications. The GenBank accession numbers of selected sequences are indicated within brackets.

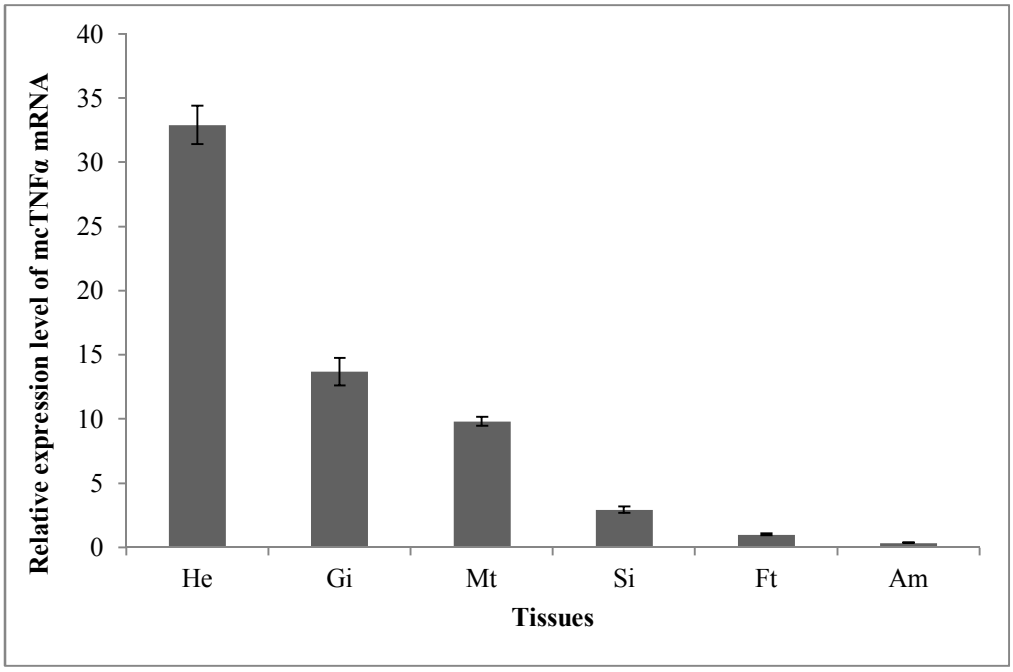

Figure 17. Tissue-specific expression analysis of RpTNFα. The relative mRNA expression of each tissue was normalized with that in adductor muscle. Data are presented as the mean relative expression ± standard error for three individual real-time reactions using pooled tisssues from three individual clams. Gi, gill; Mt, mantle; He, hemocyte; Sp, Siphon; Ft, foot; Am, adductor muscle.

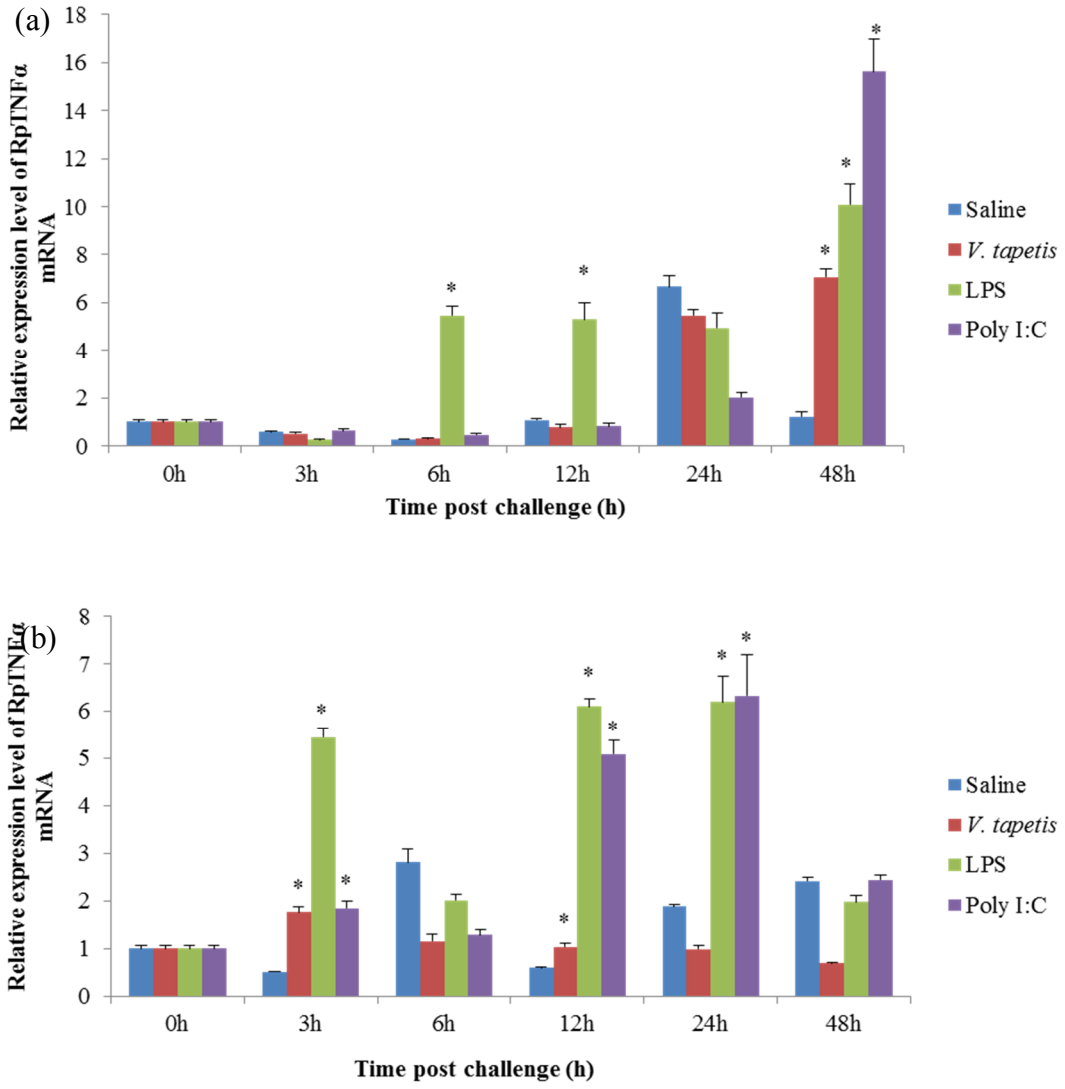

Fig. 18. RpTNFα mRNA expression in clam hemocyte (a) and gill (b) after *V. tapetis*, LPS and poly I:C challenge. The expression fold was calculated by the $2^{-\Delta\Delta CT}$ method using β-actin as the reference gene. The relative level of expression for each time point was compared to that of un-injected control. Data are presented as the mean relative expression ± SE for three replicate real-time reactions from pooled tissue of five individual clams at each time point

Chapter V

The first report of molluscan Bcl-2 family members from manila clam, *Ruditapes philippinarum*: gene expression analysis after *Vibrio tapetis* challenge

5.1. Introduction

Apoptosis is an evolutionarily conserved mechanism of programmed cell death, which is critically important for many biological processes such as development and homeostasis. In addition, a variety of pathogens have evolved abilities to either promote or inhibit apoptosis as part of their pathogenic mechanisms [Hay and kannourakis, 2002; Weinrauch and Zychlinsky, 1999].

The molecular mechanisms of programmed cell death are highly conserved throughout evolution. The B-cell lymphoma 2 (Bcl-2) family proteins are key regulator of this process. Bcl-2 family members possess characteristic Bcl-2 homology (BH) domains, which account for their ability to dimerize and function as apoptotic regulator [Borner et al., 1994]. Each member of Bcl-2 family possesses at least one of four Bcl-2 homology (BH) domains. Structurally, The Bcl-2 family is comprised of two subgroups, multi-domain (three or four BH domains) and BH3-only Bcl-2 family proteins. The group of multi-domain Bcl-2 family proteins consists of both pro- and anti-apoptotic members, whereas BH3-only proteins are pro-apoptotic. In mammalian cells, pro-and anti-apoptotic members of the Bcl-2 family act as molecular determinants for cell death decisions.

Bcl-2 gene was first described as being present in one of the most frequent hematologic malignancies, follicular lymphoma, which is associated with a reciprocal translocation in human [Tsujimoto et al., 1985]. This translocation results in deregulated expression of a gene in chromosome 18, which interferes with normal apoptosis of B-lymphocytes. Bcl-2 has been demonstrated to be an integral

mitochondrial membrane protein of around 25 kDa [Hockenbery et al., 1990], with two predominant splice variants in humans. In teleosts, Bcl-2 has been described in zebrafish [Langenau et al., 2005] and Atlantic cod [Feng and Rise, 2010], common carp [Vidal et al., 2008] and salmon [Matejusova et al., 2006]. However, in mollusc species, Bcl-2 family proteins have not characterized yet.

Here, we report the first identification and initial analysis of two critical genes involved in apoptosis, Bcl-2 and Bcl-2-associated X protein (Bax)-like in manila clam.

5.2. Materials and methods

5.2.1. Manila clam cDNA library and identification of RpBcl-2 and RpBax

We have established a normalized clam cDNA library using RNA isolated from multiple tissues of healthy clams. The basic procedure of cDNA library construction, normalization and initial GS-FLX sequencing strategies has been described in previous report [Revathy et al., 2011]. BLAST analysis of the clam cDNA shotgun sequence database identified a putative cDNAs with high homology to known Bcl-2 family members.

5.2.2. Characterization of RpBcl-2 and RpBax

The full-length nucleotides and amino acid sequences of RpBcl-2 and RpBax were analyzed using the NCBI BLAST program and Bcl-2 family homologs were

retrieved. DNAssist (2.2) was used to determine the open reading frames (ORF) and encoded amino acid sequences of RpBcl-2 and RpBax. The BH homology domains were determined by using NCBI conserved domain database. The transmembrane domain was identified using SMART sequence analysis program (http://smart.embl-heidelberg.de). Multiple alignment of protein sequences was carried out by the ClustalW program (http://www.ebi.ac.uk/Tools/clustalw2/index.html), and the identity and similarity percentage of RpBcl-2 and RpBax were calculated using EMBOSS pairwise alignment algorithms (http://www.ebi.ac.uk/Tools/emboss/align/). The phylogenetic tree was constructed by means of the Neighbor-Joining method using molecular evolutionary genetic analysis (MEGA) software (http://www.megasoftware.net).

5.2.3. Clams, immune challenges and tissue isolation

Clams with an average size of 35±5 mm were collected from the eastern coastal area of Jeju Island (Republic of Korea) and maintained in 80 L tanks of aerated sand-filtered seawater with 34±1‰ salinity and at 21±1°C. Clams were acclimatized to laboratory conditions for seven days prior experiment.

In order to evaluate the tissue specific distributions of the RpBcl-2 and RpBax mRNA, tissues from adductor muscle, mantle, siphon, gill and foot were isolated from five healthy, unchallenged individuals. Hemolymph (1 mL/clam) was also collected from each animal using a sterile syringe; hemocytes were immediately harvested from the biological fluid by centrifugation at 3000 x *g* for 10 min at 4°C.

In order to determine the immune responses of RpBcl-2 and RpBax, in vivo challenge was carried out using Gran-negative bacteria *V. tapetis*. *V. tapetis* was suspended in 0.9% saline. Clams were randomly chosen for intramuscular injection of 100 μL of *V. tapetis* (1.9 x 10^8 cells/clam). Un-injected clams were used as control and 100 μL of 0.9% saline injected clams were used as positive control.

5.2.4. Total RNA isolation and cDNA synthesis

The total RNA was extracted from 50 mg of tissue sample and hemocyte samples using the Qiazol (Qiagen). Purified RNA concentration was determined by measuring the absorbance at 260 nm in a UV-spectrophotometer (Bio-Rad). The total RNA was stored at -80°C until further use.

RNA samples were thawed and diluted to 1 μg/μL concentration and used as template in cDNA synthesis using PrimeScript™ first-strand cDNA synthesis kit (Takara). The cDNA synthesis reaction was carried out according to the manufacturer's instructions. The synthesized cDNA product was diluted appropriately and used in quantitative real-time RT-PCR (qRT-PCR) analysis.

5.2.5. RpBcl-2 and RpBax transcriptional profiling by qRT-PCR

The RpBcl-2 and RpBax mRNA expression profiles were determined by qRT-PCR. RpBcl-2 gene specific primers (forward primer 5'-TTG AGA AAC GGT ACG AGT CGC AGT-3' and reverse primer 5'- AGT ACA CTG ACA TAG CAC CCG CAA-3')

and RpBax gene specific primers (forward 5'-GTT GTT TAC GCG TTT GCT GCC AGA-3' and reverse 5'-CTG CAA ATG CAT CCC ATC CTC CAT-3') were used. The gene coding for clam β-actin was amplified by using specific primers (Forward 5'-CTC CCT TGA GAA GAG CTA CGA-3' and reverse 5'-GAT ACC AGC AGA TTC CAT ACC C-3') and used as an invariant control [Zhang et al., 2011]. Tissue-specific distribution of RpBcl-2 and RpBax were determined by measuring mRNA expression levels in adductor muscle, mantle, siphon, gill, foot and hemocytes. Pathogen inducibility was demonstrated in hemocytes and gill by using a time course experiment. qRT-PCR assays were performed in 20 μL reaction system containing 4 μL of diluted cDNA from different tissues, 10 μL of 2× Takara SYBR premix Ex *Taq*™, 0.8 μL of each primer (10 pmol/μL), and 5 μL dH_2O. A Thermal Cycler Dice™ Real-Time System (Takara) was used with the following cycling profile: a single denaturation cycle of 95 °C for 10 s; 45 amplification cycles of 95 °C for 5 s, 58 °C for 10 s, and 72 °C for 20 s; and a final single cycle of 95 °C for 15 s, 60 °C for 30 s, and 95 °C for 15 s. The relative RpBcl2 and RpBax mRNA expressions were determined by the Livak $2^{-\Delta\Delta CT}$ method, using the clam β-actin as a reference gene. To quantify the tissue-specific expression, the expression levels of RpBcl-2 and RpBax in hemocytes were used as the calibrator, to which the expression levels in all other tissues were compared. To determine the fold-change in expression induced by challenges with bacteria, the expressional level of tissues from injected animals were compared with those from 0.9% saline injected controls.

5.3. Results

5.3.1. Identification of manila clam Bcl-2 and Bax-like

In this study, we identified two clam Bcl-2-like genes that were named RpBcl-2 and RpBax. The nucleotide and amino acid sequence were shown in Fig. 19 and 20. The RpBcl-2 cDNA contains a 564 base pair open reading frame (ORF), which encodes a putative protein of 188 amino acid residues (Fig. 19). The calculated molecular mass of RpBcl-2 is 21 kDa and the predicted isoelectric point is 5.3. RpBcl-2 was found to contain the conserved Bcl2-homology domain and transmembrane domain (^{163}TNG-LII^{185}). The conserved Bcl2-honology domain consists of four BH regions including BH-4 (^{18}EYI-DYQ^{32}), BH-3 (^{58}VKK-EFE^{56}), BH-1 (^{86}QLI-SFA^{104}), and BH-2 (^{137}-NWI-IDF^{151}). The BH-3 homology region binding sites were found at ^{51}Leu-Cys^{52}, ^{62}Gln-Phe^{63}, ^{78}Ala-Asn^{79}, ^{81}Val-Ala^{82}, ^{86}Gln-leu^{87}, His^{93}, ^{96}Gly-Arg^{97}, ^{98}Val-Ala^{99}, Phe^{103} and Met^{107}.

The nucleotide and amino acid sequences were shown in Fig. 20. The RpBax consisted of 1013 bp with an ORF of 693 bp encoding a putative protein of 231 amino acid residues. The calculated molecular mass of RpBax is 26 kDa and the predicted isoelectric point is 5.8. RpBax also was found to contain the conserved Bcl2-homology domain, but transmembrane domain was not detected. Bcl2 homology domain of RpBax contains BH-3 (^{94}MRK-EVS^{102}), BH-1 (^{93}EIF-AFA^{142}) and BH-2 (^{184}RWI-DAF^{195}), but BH-4 region was lacked. In amino acid sequence of RpBax, the BH-3 homology region binding sites also were found at ^{97}Thr-Val^{98},

^{100}Glu-Val^{101}, H^{105}, ^{108}Ala-Leu^{109}, ^{125}Ala-Thr^{126}, ^{129}Asn-Val^{130}, ^{133}Glu-Ile^{134}, Asn^{141}, ^{143}Gly-Arg^{144}, ^{146}Ile-Val^{147} and Phe^{151}.

Multiple alignment analysis of RpBcl-2 with other known Bcl2-family members from different species revealed that BH1-4 regions are highly conserved (Fig. 21). Except BH1-4 region, the amino acid sequences are shown very low identity and similarity. RpBax also showed highly conserved at only BH-1, BH-2 and BH-3 regions (Fig. 22).

A phylogenetic tree of RpBcl-2 and RpBax were constructed with different members of the Bcl-2 family selected from both vertebrates and invertebrates (Fig. 23). We determined that different functional groups of Bcl-2 homologies from various species clustered within their respective Bcl-2 sub-family. However, RpBax wasn't group with other Bcl-2 family members.

5.3.2. Tissue specific expression analysis

Constitutive expressions of RpBcl-2 and RpBax transcripts were evaluated by quantitative real-time RT-PCR using tissues isolated from five individuals. The real-time RT-PCR analysis showed that the beta-actin was transcribed at a similar level in the following 6 tissues: hemocytes, adductor muscle, siphon, mantle, gill and foot. This finding supports the selection of beta-actin as the normalizer gene for quantifying the relative expression of target genes in this study. The tissue specific analysis results showed that two genes were constitutively expressed at detectable levels in all six tissues examined.

The highest tissue specific expression level of RpBcl-2 was detected in gill, followed by siphon and mantle, and it showed that the tissue specific expression of RpBcl-2 was significantly higher in the gill, siphon and mantle than in the hemocytes, adductor muscle and foot (Fig 24). The tissue specific expression level of RpBax showed similar expression pattern with RpBcl-2, although the expression level was different. Tissue specific expression level of RpBax also showed the highest in gill followed by siphon and mantle, and it was significantly higher in gill, siphon and mantle than hemocyte, adductor muscle and foot.

5.3.3. Temporal expression analysis

Quantitative real-time RT-PCR was used to examine the temporal expression of RpBcl-2 and RpBax. We evaluated their expression profiles in hemocyte and gill at different time points after *V. tapetis* challenge. Rp-Bcl-2 mRNA expression in hemocytes was significantly up regulated at 3, 6 and 12 h. The expression level was increased till 6h and then decreased at 12h, but significantly higher than saline challenged control (Fig 25A). We couldn't find significant difference of expression level of RpBcl2 at 24 and 48 h post challenge. In gill, expression level of RpBcl-2 showed no significant difference compared to saline injected control till 6 h post challenge. However it was significantly down-regulated from 12 h to 24 h post challenge (Fig 26A).

The expression level of RpBax was significantly up-regulated at 3h post challenge in hemocytes (Fig 25B). Afterwards, the expression level of RpBax was

down-regulated till 12 h, then again up-regulated till 48 h post challenge. In gill, expression level of RpBax was up-regulated at 3h post challenge however, no significant difference was detected at all the other time points (Fig 26B).

5.4. Discussion

Among mollusk, Bcl-2 family member has been identified only in pacific oyster, and no functional studies have been published to date. In this study, two Bcl-2 sub-family genes, RpBcl-2 and Rp-Bax, were identified in manila clam by GS-FLX pyrosequencing.

To study the expression of manila clam two Bcl-2 sub-family genes, we examined tissue specific expression in six tissues and the gene expression in immune tissues following the stimulations with pathogenic bacteria. The anti-apoptotic of pro-apoptotic functions, gene organizations, expression patterns, along with the presence of potential motif were discussed separately for each gene, and then integrated to examine the potential roles of these genes in manila clam immune responses.

Our analysis of GS-FLX sequencing database led to the identification of two manila clam transcripts representing members of the Bcl-2 sub-family. This allowed us to obtain the full-length cDNA sequences of RpBcl-2 and RpBax. These cDNA sequences are similar with putative orthologous sequences from other species, especially within the Bcl-2 homology (BH) domains that are critical for their anti-apoptotic functions [Antonsson and Martinou 2000]. In addition, RpBcl-2 cDNA

analysis observed the conserved transmembrane (TM) domains at their carboxyl termini, which are required for localization to intracellular membranes such as mitochondrial outer membrane, smooth endoplasmic reticulum, and nuclear envelope [Antonsson and Martinou, 200]. However, transmembrane domain was not detected at C-terminus of the Rp-Bax amino acid sequence. Igaki et al. reported Drob-1 in *Drosophila* EST database, which is showed the highest homology with mammalian pro-apoptotic Bcl-2 sub-family such as Bax, Bak and Bok/Mtd [Igaki and Miura, 2004; Zhang et al., 2000]. Drob-1 has BH1, BH2 and BH3 domains, and a weak BH4 domain, as well as a C-terminal transmembrane domain [Zhang et al., 2000]. In this study, the Rp-Bax also showed weak transmembrane domain at C-terminus.

Almost all anti-apoptotic Bcl-2 subfamily members contain four BH domains, however RpBax consist of only three BH domains including BH-1, BH-2 and BH-3. One of the unique features of Bcl-2 family proteins is heterodimerization between anti-apoptotic and pro-apoptotic proteins, which is considered to inhibit the biological activity of their partners [Tsujimoto and Shimizu, 2000]. This heterodimerization is mediated by the insertion of a BH3 region of a pro-apoptotic protein into a hydrophobic cleft composed of BH1, BH2 and BH3 from an anti-apoptotic protein [Tsujimoto and Shimizu, 2000]. In addition to the BH1 and BH2, the BH4 domain is required for anti-apoptotic activity [Belka and Budach, 2002]. In contrast, BH3 is essential and, itself, sufficient for pro-apoptotic activity [Kelekar and Thompson, 1998]. RpBcl-2 showed that typical features of anti-apoptotic Bcl-2 sub-family members.

BH-4 plays a crucial role in the prevention of apoptosis [Moissac et al., 1999]. In previous studies, deletion or mutation of BH-4 region was shown to render Bcl-2 defective for suppression of apoptosis [Tsujimoto and Shimizu, 2000], independent of its ability to dimerize with the pro-apoptotic factors Bax, Bak, or Bad [Kelekar and Thompson, 1998]. The RpBax lacked BH4 domain, which is similar with pro-apoptotic Bcl-2 sub-family members such as Bax, Bak and Bad.

Pairwise alignment was performed to confirm the extensive similarity between the manila clam and orthologs of different species. RpBcl-2 showed 49.2, 47.2 and 43.9 % similarities and 28.8, 25.7 and 27.9 identities with Pacific oyster, Acorn worm and Zebra fish, respectively. Bcl-2 showed the highest identity with Pacific oyster. The results from the pairwise alignment were supported by the Neighbor-Joining tree data, which grouped all the Bcl-2 ortholog sequences together. In phylogenetic tree, RpBax was grouped alone. RpBax sequence similar with Bcl-2 orthologs, but it has typical feature of Bax. It considered that no Bax sequences of mollusc are available in NCBI GenBank database.

In Atlantic cod, anti-apoptotic Bcl-2 was constitutively expressed in all tested tissues [Feng and Rise, 2010]. It was reported that anti-apoptotic Bcl2 sub-family, NR-13, Bcl-1, Bcl-X1 and Bcl-X2 from Atlantic cod were highly expressed in gill tissue. Interestingly, in spleen, the basal expression level was not significantly higher than other tissues. In this study, RpBcl-2 also showed the highest expression level in gill tissue, as well as expression level of hemocytes was the lowest. Predicted pro-apoptotic Rp-Bax also showed the highest expression level in the gill, as well as hemocytes also showed the lowest expression level.

Quantitative real-time RT-PCR was used to study RpBcl-2 and RpBax gene expression in manila clam immune tissues (gill and hemocytes) following challenge with *V. tapetis*. NR-13, Mcl-1, Bcl-x1 and Bcl-x2 in immune tissue of Atlantic cod was significantly up-regulated by ASAL (formalin-killed, atypical *Aeromonas salmonicida*) injection. The apoptotic effect of LPS on mammalian cells has been previously demonstrated. [Sano et al., 2005]. The expression of Bcl-2 family members, Bcl-xL, Bcl-2, Mcl-1 and Bax was investigated in delayed apoptosis of canine neutrophils induced by LPS. In this study, the significant up-regulation of RpBcl-2 and RpBax mRNA expressions were detected in hemocyte and gill after challenged with bacteria. The induction of predicted anti-apoptotic RpBcl-2 expression level was higher and longer than predicted pro-apoptotic RpBax. In, this study high expression of Bcl-2 provided a life signal against apoptotic signals, when the life signal is induced by stimulation of LPS.

In conclusion, RpBcl-2 and RpBax are belonging to the anti-apoptotic and pro-apoptotic Bcl-2 sub-family, respectively. RpBcl-2 and RpBax showed similarity in gene organization and predicted amino acid sequence to putative orthologous sequences in other species including vertebrates and invertebrates. Furthermore, the presence of similar BH domains in these anti- or pro-apoptotic Bcl-2 sub-family genes suggest that these genes may have arisen from a common ancestral gene. Although we did not fully characterize the RpBax gene, it showed little difference compare to other Bcl-2 and Bax like proteins. Furthermore, we demonstrated the up-regulation of RpBcl-2 and RpBax transcripts in manila clam immune tissues by *V. tapetis* challenge. Consistent with the expression pattern of these genes observed in

our study, further emphasize their potential roles in innate immune response in manila clam.

```
      GGGATCTGTCGATTATCGCTTGAAGGCAAACACGGTTAAACGAGTGTCGCGGCTA   -61
GTATCTCGCTTATTTGTTTAGTTTTTAGTTTAAGTTTGGTTTTTAAATTAACAGAAAGAT   -1
ATGAGGATGGACGAGGATGACTTTTCGATCGGTACTAGTGGAATAGTGGCGGAATATATT   60
 M  R  M  D  E  D  D  F  S  I  G  T  S  G  I  V  A  E  Y  I    20
                                                   BH-4
ATGAGGATGGACGAGGATGACTTTTCGATCGGTACTAGTGGAATAGTGGCGGAATATATT   120
 T  Y  K  V  Q  Q  A  G  F  D  Y  Q  V  S  P  T  R  N  C  P    40
ACTTATAAAGTACAACAAGCTGGATTTGATTACCAAGTCTCGCCAACAAGGAACTGCCCT   180
 N  N  K  V  I  D  T  V  K  K  L  C  S  E  F  E  K  R  Y  E    60
                         BH-3
AATAACAAAGTAATAGACACTGTGAAGAAACTGTGCAGCGAGTTTGAGAAACGGTACGAG   240
 S  Q  F  T  E  M  C  E  T  C  S  Q  V  E  L  N  S  A  N  Y    80
TCGCAGTTTACCGAAATGTGCGAAACTTGCTCGCAAGTTGAATTAAATTCTGCAAATTAT   300
 V  A  V  L  D  Q  L  I  V  N  G  L  H  W  G  R  V  V  A  I   100
                   BH-1
GTAGCTGTTTTGGACCAATTAATTGTCAACGGACTTCATTGGGGTAGGGTTGTTGCAATA   360
 F  S  F  A  G  A  M  S  V  Y  C  M  K  K  N  Q  K  E  R  V   120
TTTTCATTTGCGGGTGCTATGTCAGTGTACTGCATGAAAAAGAACCAGAAAGAGAGGGTT   420
 D  W  I  R  E  W  T  C  Q  F  M  D  S  K  V  E  N  W  I  N   140
                                                BH-2
GACTGGATCCGAGAGTGGACTTGCCAGTTCATGGACAGTAAAGTAGAAAACTGGATAAAT   480
 E  N  N  G  W  R  G  L  I  D  F  Y  T  N  G  I  K  K  P  E   160
GAAAATAATGGATGGAGAGGACTTATAGATTTTTACACGAATGGCATCAAGAAACCAGAA   540
 E  K  T  N  G  W  G  S  V  L  I  G  G  A  L  G  A  F  A  L   180
GAAAAAACAAATGGATGGGGATCTGTTCTCATCGGTGGTGCCCTCGGAGCTTTTGCGCTC   600
 G  A  L  I  I  N  K  A  *                                    188
GGTGCGCTCATAATCAATAAGGCATGAATGCTAGATTAGCCTACACTTGCTGTATTTCAA   660
ATTTAGATCTATTTTAAAATTCGTAGTATATTGTGAATAGGTCAGTCAATACGGGTAGGA   720
CAGGATTCTACTGTTCGAAAGTGCACTTCTTTCGCGTGATCGTATATGTGCTCGGATTGG   780
AGACAGACATTTGGGACGATTAGAAGTGATTTTCGAACGGACAACAAGAAAGGAGGGTTG   840
TAAAGAATACAAGGAGGTGATTCTGCTTCAAAGAACAGAGAGATACCCCCACAATTAACG   900
TGCTATAAATGACACGTGAGAATCTATGGCCAGTGCAATACAAACTCATAAAGTGCTTAA   960
TAGAAGAGCTTAAACATTAATACTGGTTACGGAAGTTCCAGTTAGCTGAAGAAAACATTG   1020
TAGAAAAAAAAAAAAAAAAAA                                         1023
```

Figure 19. The complete nucleotide and deduced amino acid sequences of the RpBcl-2. Bcl-2 homology domain is boxed and BH1-4 domains are grey boxed. Transmembrane is underlined.

```
                                        GGGGGCCATTTTGAACAACAAGAGAAGAAACTGGGA   -61
GGAATTTTTTGGCCGTTTATGGTCAGTTTTTTGCTTTTAGAAGGGCCCATATAAACTAAA   -1
ATGACGACAACAAGTCCAACATACGGTGGAATTAAATCATTAGGAGGGAATGGAGGTTTT   60
 M  T  T  T  S  P  T  Y  G  G  I  K  S  L  G  G  N  G  G  F    20
CATAACAATTCAAATCTGTTCGCTGAAATTCATAAACATTGGGGACTTTTACCAGGAAAC   120
 H  N  N  S  N  L  F  A  E  I  H  K  H  W  G  L  L  P  G  N    40
CAAGGGCTCAATAACATCAACCAGGAAACACAGACAACAGGTTCTATGTTATCACCATTA   180
 Q  G  L  N  N  I  N  Q  E  T  Q  T  T  G  S  M  L  S  P  L    60
GAAAATGTAAGAAAACAGGCAGAGATAATAGCAACGGCTGTTGTTGTTAATTTTGGTCTT   240
 E  N  V  R  K  Q  A  E  I  I  A  T  A  V  V  V  N  F  G  L    80
GACTGTGAAAAGAAATGTCCGGACAGATTCTGTAAAACAATGAGAAAAACAGTGCAAGAA   300
 D  C  E  K  K  C  P  D  R  F  C  K  T  M  R  K  T  V  Q  E   100
                                        BH-3
GTTTCAGATAGACATGACATAGCTTTGAAATCAATGGTCAACAAGCTGAATCTTGATGAT   360
 V  S  D  R  H  D  I  A  L  K  S  M  V  N  K  L  N  L  D  D   120
TCTAATATGTTTGCTACTTTTGTAAATGTTGCTGATGAAATCTTCGAAGACGGTCAAATA   420
 S  N  M  F  A  T  F  V  N  V  A  D  E  I  F  E  D  G  Q  I   140
                                        BH-1
AATTGGGGACGAGTCATTGTTGTTTACGCGTTTGCTGCCAGATTAAATGCCTACTTTAGA   480
 N  W  G  R  V  I  V  V  Y  A  F  A  A  R  L  N  A  Y  F  R   160
CAAGGAAATCCAACTTACGAAGAGAAGATATCTCTTTATGTTGGAAAGTATGTCGGTAAT   540
 Q  G  N  P  T  Y  E  E  K  I  S  L  Y  V  G  K  Y  V  G  N   180
AAACTTGGACGATGGATTTTAGATAATGGAGGATGGGATGCATTTGCAGACTTTTTCTCA   600
 K  L  G  R  W  I  L  D  N  G  G  W  D  A  F  A  D  F  F  S   200
          BH-2
GATGAAAATGCAGTAGAGGATAAAATTTGGAAAGGACTTTTATTTACAGCAACTCTTGGG   660
 D  E  N  A  V  E  D  K  I  W  K  G  L  L  F  T  A  T  L  G   220
GGATTGGGTGCATTAGCAGCTGTTATGTCCCGATGATCACCATACCTAACAAGTGCAATC   720
 G  L  G  A  L  A  A  V  M  S  R  *                           231
TTCATATGTTTTCACAGAGGAAAAGTGGAATAAACTATTATTGTAAATTTATATATGATT   780
TCTTTCTAAGTAGATATATTTAAATGAAAATATAAAAAAATTGTCATTTAACATACAGAC   840
GGTTTGACATTTAACTCAATTGTAAATTTATAATTTTAAAGAATTTGATTTGATGATACA   900
GTTATGGTAATAGTTTATTTTACTTTTTTTCTTACTTCATTGATGAGTATAT          952
```

Figure 20. The complete nucleotide and deduced amino acid sequences of the RpBax. The complete nucleotide and deduced amino acid sequences of the RpBcl-2. Bcl-2 homology domain is boxed and BH1-3 domains are grey boxed

Figure 21. Multiple alignment of RpBcl-2 with other selected species Bcl-2 family amino acid sequences

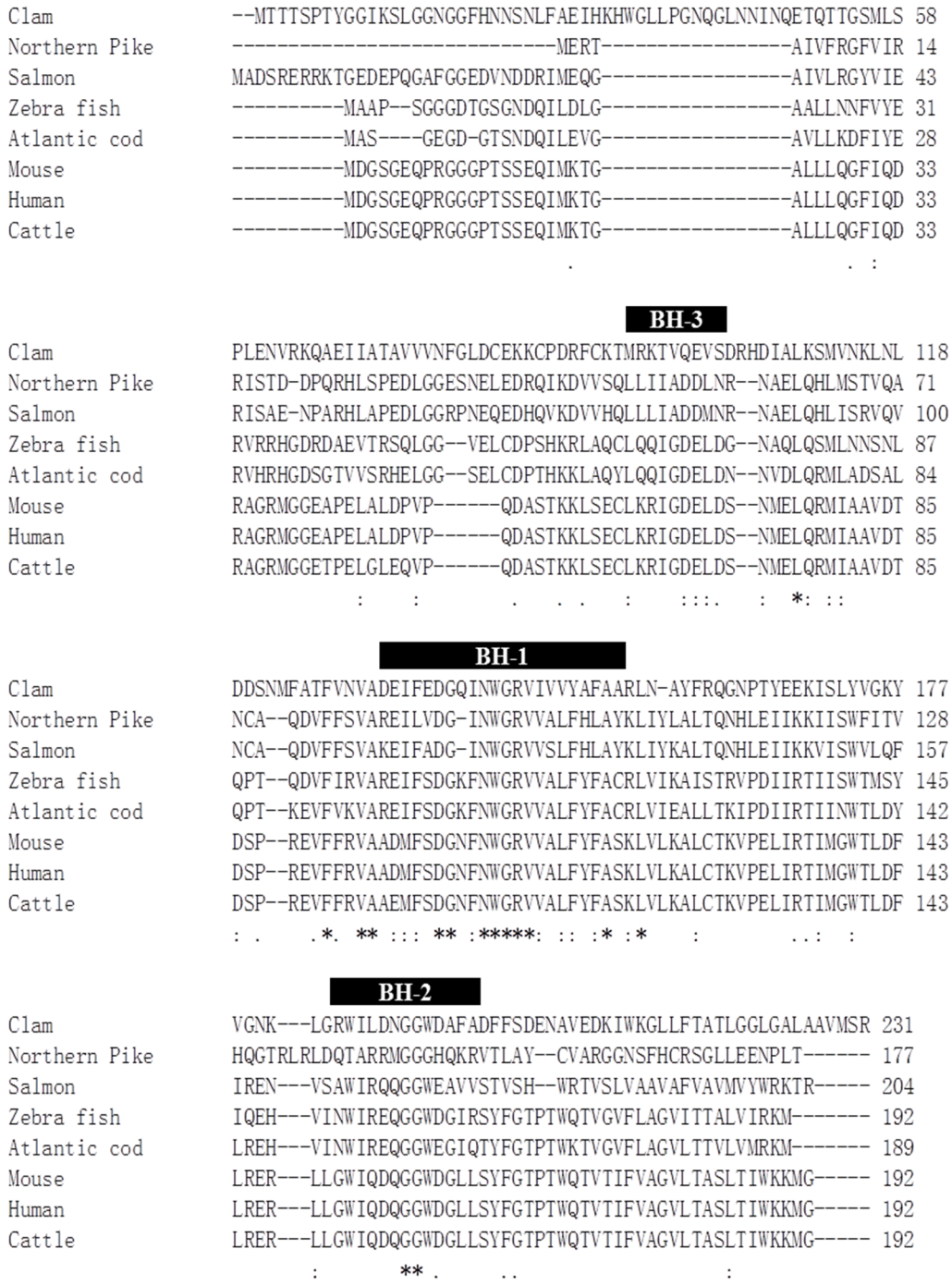

Figure 22. Multiple alignment of RpBax with other selected species Bax family amino acid sequences

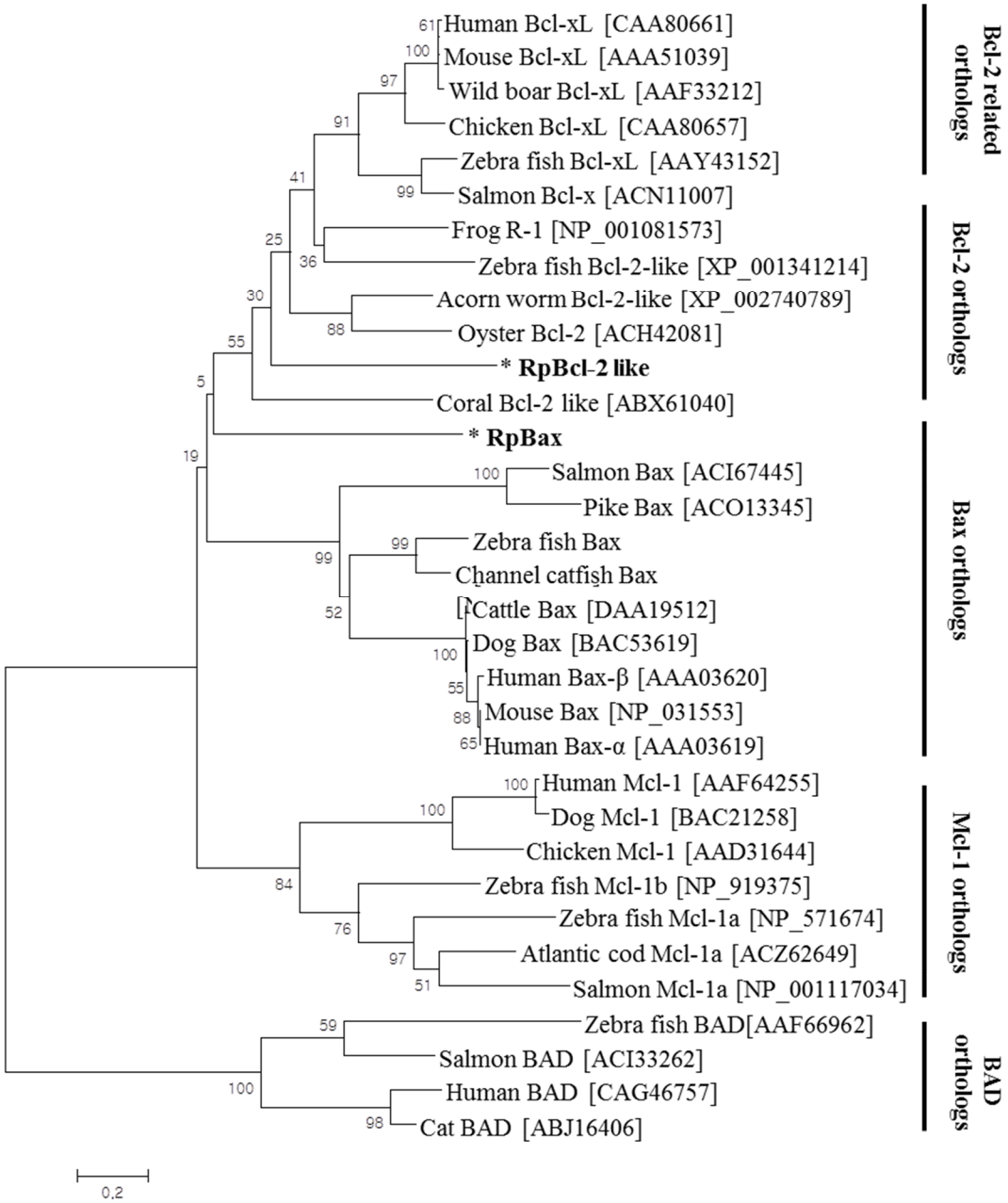

Figure 23. Phylogenetic analysis of RpBcl-2 and RpBax

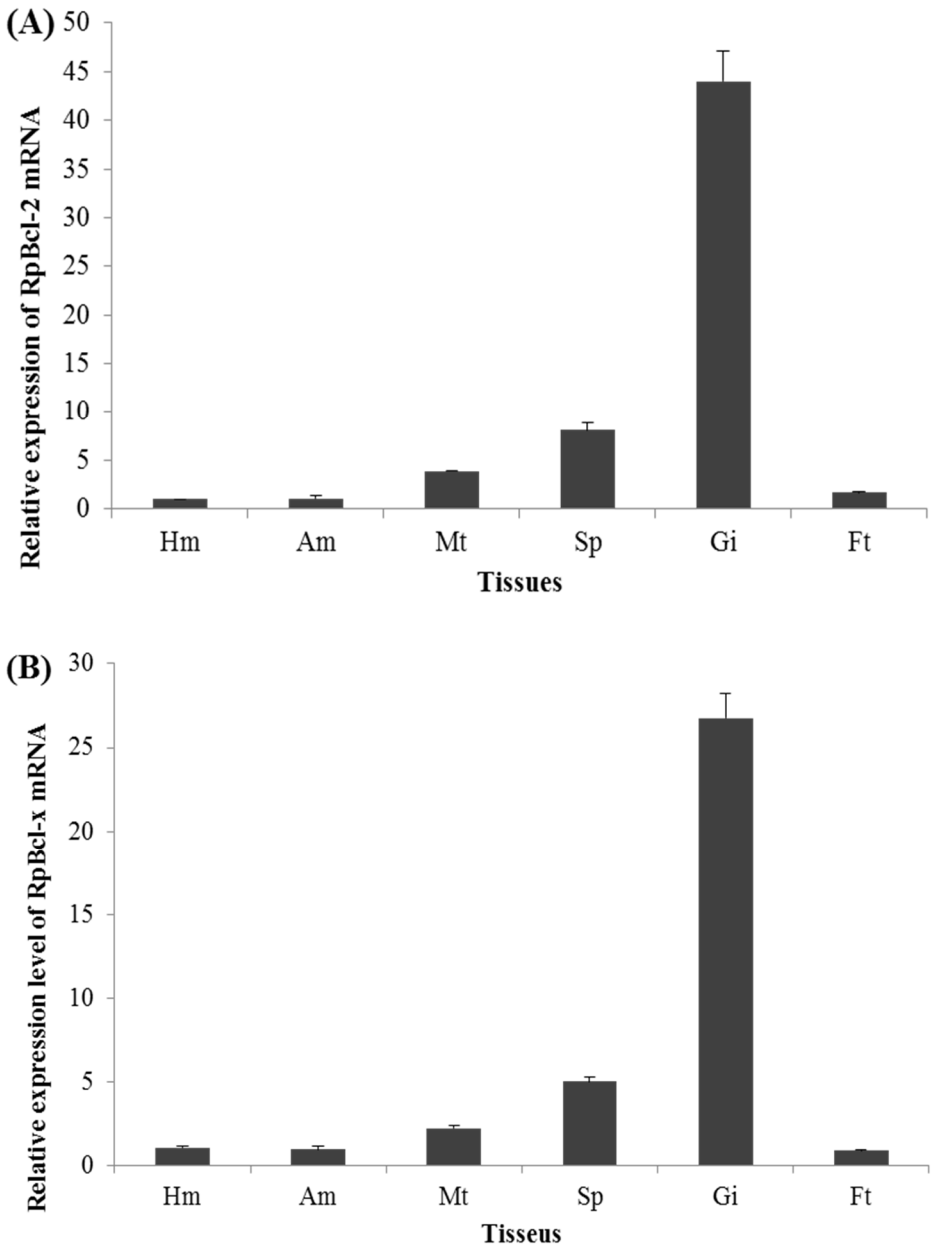

Figure 24. Tissue-specific expression analysis of RpBcl-2 (A) and RpBax (B) mRNA. The relative mRNA expression of each tissue was normalized with that in hemocyte. Data are presented as the mean relative expression ± standard error (SE) for three individual real-time reactions using pooled tisssues from five individual clams. Gi, gill; Mt, mantle; He, hemocyte; Sp, Siphon; Ft, foot; Am, adductor muscle.

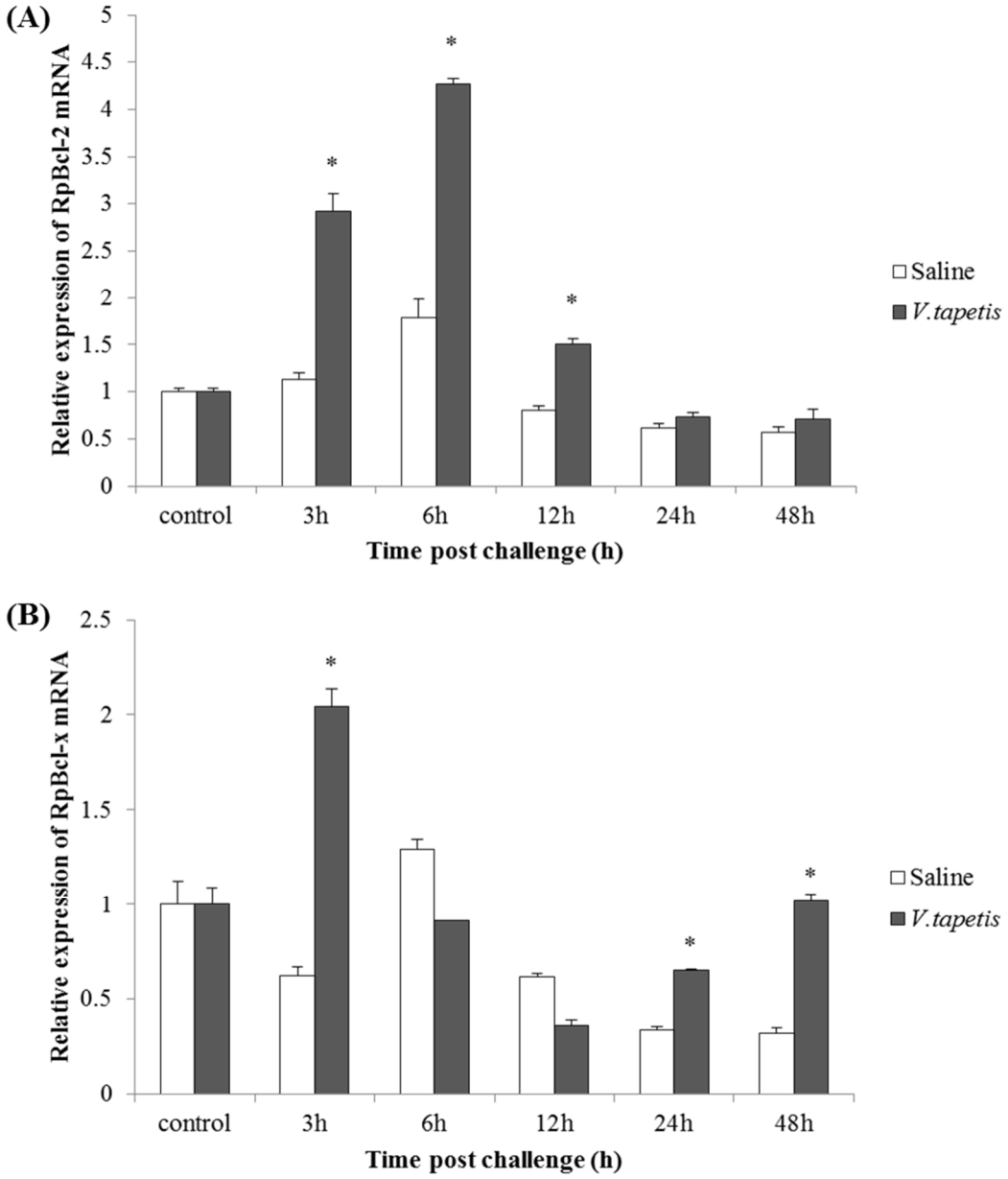

Fig 25. Expression profiles of RpBcl-2 (A) and RpBax (B) mRNAs in manila clam hemocytes after *V. tapetis* challenge. *V. tapetis* challenge, as determined by SYBR green RT-PCR. The relative expression was calculated by the $2^{-\Delta\Delta CT}$ method using Manila clam *β-actin* as reference gene and compared to 0.9% saline control. The error bars represent SE of $n = 3$. Data with different symbols are significantly different at $P < 0.05$, among the different time points.

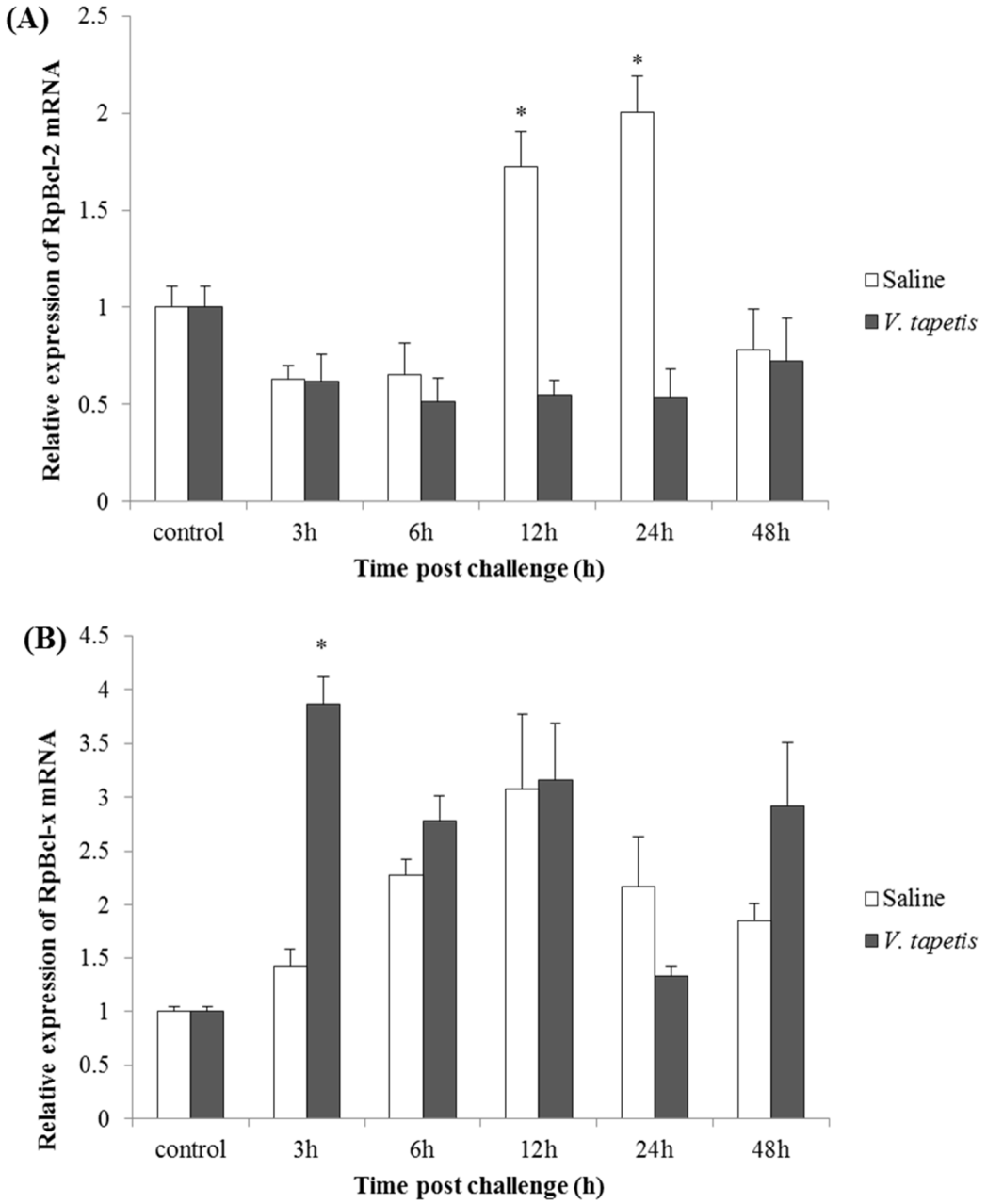

Figure 26. Expression profiles of RpBcl-2 (A) and RpBax (B mRNAs in manila clam gill after *V. tapetis* challenge. *V. tapetis* challenge, as determined by SYBR green RT-PCR. The relative expression was calculated by the $2^{-\Delta\Delta CT}$ method using Manila clam *β-actin* as reference gene and compared to 0.9% saline control. The error bars represent SE of $n = 3$. Data with different symbols are significantly different at $P < 0.05$, among the different time points.

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감사의 글

어느덧 3년 반이라는 시간이 훌 쩍 지나 비록 미숙하나마 작은 결실을 맺게 되었습니다. 박사학위 과정 동안에 저의 부족함을 채우기에는 너무 짧은 시간들이었고 아쉬움도 많이 남습니다. 그 동안 많은 즐거움과 기쁨도 있었고, 실패와 아픔도 있었지만 그 모든 것이 저에게는 소중한 경험이 되고 앞으로 살아가면 소중한 가르침이 되어줄 것이라고 생각합니다. 이 모든 시간들을 함께해 주시고 많은 조언과 격려를 아끼지 않은 모든 분들께 감사인사 드립니다. 아울러 부족한 논문이지만 논문이 완성될 수 있도록 오랜 기간 동안 저에게 많은 도움을 주신 분들에게 이렇게 짧게나마 글로써 진신어린 감사의 말씀을 드리고 싶습니다.

먼저 참으로 부족한 저를 잘 할 수 있다며 토닥거려 주시고 격려해주시고 항상 희망을 심어주셨던 존경하는 이제희 지도 교수님의 은혜에 고개 숙여 감사 드립니다. 그리고 바쁘신 가운데서도 부족한 저의 논문을 심사해 주신 김기영 교수님, 허문수 교수님, 정준범 교수님께 감사 드립니다. 제 논문이 완성될 수 있도록 항상 조언 해주신 심사위원 충남대 De Zoysa 교수님께도 감사 드립니다. 그리고 대학원 과정 동안 관심과 힘을 주신 해양생명과학과 최광식 교수님, 송춘복 교수님, 전유진 교수님, 여인규 교수님께도 감사의 마음을 전하고 싶습니다.

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