Characterization of the *TRAF3IP1* gene in Yesso scallop (*Patinopecten yessoensis*) and its expression in response to bacterial challenge

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(Received 14 April 2016, accepted 28 July 2016; J-STAGE Advance published date: 19 December 2016)

Tumor necrosis factor receptor-associated factor 3 (TRAF3) is an important adaptor that transmits upstream activation signals to induce innate immune responses. TRAF3 interacting protein 1 (TRAF3IP1) interacts specifically with TRAF3, but its function in innate immunity remains unclear, especially in marine invertebrates. In this study, to better understand the functions of TRAFs in innate immune responses, we identified and characterized the first bivalve *TRAF3IP1* gene, *PyTRAF3IP1*, from Yesso scallop (*Patinopecten yessoensis*), one of the most important mollusk species for aquaculture. The *PyTRAF3IP1* cDNA is 2,367 bp, with an open reading frame of 1,629 bp encoding 542 amino acids. Phylogenetic and protein structural analysis confirmed the gene's identity and revealed that *PyTRAF3IP1* was more similar to vertebrate TRAF3IP1s than to those of invertebrates. *PyTRAF3IP1* was expressed in all the adult tissues and developmental stages sampled, implying that it plays versatile roles in many biological processes. Furthermore, *PyTRAF3IP1* expression was dramatically induced in the acute phase (3–6 h) after infection with both Gram-positive (*Micrococcus luteus*) and Gram-negative (*Vibrio anguillarum*) bacteria, even stronger induction being observed after *V. anguillarum* challenge. This is the first report of the characterization and immune response involvement of *TRAF3IP1* in marine invertebrates, and suggests that *TRAF3IP1* contributes to innate immunity in bivalves.

Key words: Gram-positive and Gram-negative infection, innate immunity, *TRAF3IP1*, Yesso scallop (*Patinopecten yessoensis*)

INTRODUCTION

Tumor necrosis factor receptor-associated factors (TRAFs) are essential intracellular adapter proteins that function in innate immune responses. They transmit upstream activation signals and mediate downstream signaling pathways, and finally activate a variety of transcription factors including nuclear factor- κ B (NF- κ B) and interferon regulatory factors to regulate cell survival, proliferation and stress responses in the immune and inflammatory systems (Bradley and Pober, 2001; He et al., 2007; Hacker et al., 2011). Among the seven known TRAFs, TRAF3 is a highly versatile regulator. Recent

work has revealed that TRAF3 functions as a negative regulator of alternative NF- κ B signaling (Hacker et al., 2011), and there is also increasing evidence for a critical role of TRAF3 as a positive regulator of the production of type I interferon (IFN), an important effector in innate immune responses which combats microbial infections at the front line (Oganesyan et al., 2006; Hacker et al., 2011).

As an important regulator of immune responses, TRAF3 needs to interact with binding partners to control its recruitment to various signaling complexes (He et al., 2007; Hacker et al., 2011). TRAF3IP1 (TRAF3 interacting protein 1), also termed MIP-T3 (microtubule-interacting protein associated with TRAF3), is one of only a few proteins that specifically interact with TRAF3 but not with other TRAF proteins (Ling and Goeddel, 2000). TRAF3IP1 is conserved from worms to human and interacts constitutively with TRAF3 through a C-terminal coiled-coil region

Edited by Hie Lim Kim

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DOI: <http://doi.org/10.1266/ggs.16-00019>

(Ling and Goeddel, 2000; Guo et al., 2010). A previous study has shown that TRAF3IP1 is a cellular inhibitor of the innate IFN response that impedes TRAF3 complex formation with critical downstream transducers and effectors (Ng et al., 2011), which supports the notion that TRAF3IP1 is biologically important in the regulation of immune responses. Moreover, TRAF3IP1 was also shown to bind microtubules and play an important role in the regulation of cytoskeleton dynamics in cells (Kunitomo and Iino, 2007; Guo et al., 2010).

Marine bivalves are constantly exposed to complex microbiota from the surrounding seawater and are thus subjected to attack by potential pathogens. The innate immune system is especially important to the survival of these organisms because they lack adaptive immunity (Bassim et al., 2015). With innate immunity, they have evolved elaborate mechanisms to protect themselves against pathogenic invasion. As an important gene that functions in innate immunity, *TRAF3* has been identified and characterized in several marine bivalves, such as *Pinctada fucata* (Huang et al., 2012), *Crassostrea gigas* (Huang et al., 2014), *Patinopecten yessoensis* (Wang et al., 2015) and *Mytilus galloprovincialis* (Toubiana et al., 2014). The regulated expression of bivalve *TRAF3*s in response to bacterial challenge that has been revealed in these studies implies the involvement of *TRAF3* and its related molecules in innate immunity of shellfish. However, as a protein that interacts specifically with TRAF3, TRAF3IP1 has mainly been identified in mammals (Ling and Goeddel, 2000; Bizet et al., 2015) and also in model organisms such as *Caenorhabditis elegans* (Kunitomo and Iino, 2007), but its characterization, expression pattern or possible function in marine invertebrates has not been reported.

Yesso scallop (*P. yessoensis*, Jay, 1857) is an important aquaculture species in Northern China. In recent years, like other mollusks, the scallop aquaculture industry has experienced bacterial diseases and suffered mass mortalities (Teng et al., 2012). Identifying the immune-related genes in scallops and revealing their expression in response to bacterial challenge could benefit our understanding of the innate immune system of marine bivalves, and promote genetic improvement of the species for disease resistance. In this study, we identified the first *TRAF3IP1* gene (*PyTRAF3IP1*) in mollusks, from *P. yessoensis*, investigated its expression patterns at different developmental stages and in adult tissues, and then characterized its temporal responses to bacterial challenge after scallops were exposed to *Micrococcus luteus* (Gram-positive) and *Vibrio anguillarum* (Gram-negative). Our results confirm the involvement of *PyTRAF3IP1* in scallop innate immunity and provide useful information for the further functional study of TRAF3 signaling in marine bivalves.

MATERIALS AND METHODS

Database mining, gene identification and sequence analysis To identify the *TRAF3IP1* gene, the *P. yessoensis* transcriptome (Hou et al., 2011) and genome sequence (unpublished; NCBI accession no.: PRJNA259405) were BLAST-searched using available *TRAF3IP1* sequences of other organisms from NCBI (<http://www.ncbi.nlm.nih.gov>), Ensembl (<http://www.ensembl.org>) and Echinobase (<http://www.echinobase.org/Echinobase/>). BLAST hits from TBLASTN against the *P. yessoensis* transcriptome with an e-value of $1e^{-5}$ were selected and a BLASTN search was then performed to confirm the existence of these cDNA sequences by mapping them to the whole-genome assembly. Identification of gene structure was performed by BLAST-searching the obtained cDNA sequences against the *P. yessoensis* genome sequence. ORF Finder (<http://www.ncbi.nlm.nih.gov/gorf/gorf.html>) and DNASTAR (version 7.1) were used to predict amino acid sequence, which was then confirmed by BLASTP against the NCBI non-redundant protein sequence database. The simple modular architecture research tool (SMART) (<http://smart.embl.de/>) was used to identify conserved domains. The putative isoelectric point (pI) and molecular weight were determined using the Compute pI/Mw tool (http://web.expasy.org/compute_pi/). PyTRAF3IP1 secondary structure was predicted using Geneious 9.1.2 (<http://www.geneious.com/>). The putative transcription initiation site was inferred using the BDGP tool (http://www.fruitfly.org/seq_tools/promoter.html). Potential transcription factor binding sites (TFBSs) in the promoter region were predicted using TFSEARCH and Alibaba tools (<http://www.gene-regulation.com/pub/programs.html#alibaba2>).

Multiple alignment and phylogenetic analysis TRAF3IP1 protein sequences from selected organisms, including human (*Homo sapiens*), mouse (*Mus musculus*), chicken (*Gallus gallus*), lizard (*Anolis carolinensis*), tropical clawed frog (*Xenopus tropicalis*), zebrafish (*Danio rerio*), stickleback (*Gasterosteus aculeatus*), sea urchin (*Strongylocentrotus purpuratus*), tunicate (*Ciona intestinalis*) (Deuterostome group), fruitfly (*Drosophila melanogaster*), mosquito (*Anopheles gambiae*), worm (*Caenorhabditis elegans*), daphnia (*Daphnia pulex*) (Ecdysozoan group), Zhikong scallop (*Chlamys farreri*), Pacific oyster (*Crassostrea gigas*) and owl limpet (*Lottia gigantea*) (Lophotrochozoan group), were retrieved from the NCBI, Ensembl Genome Browser and Echinobase databases and used for phylogenetic analysis with PyTRAF3IP1. The sequences of TRAF3IP1 from different species were aligned using the ClustalW2 program (Larkin et al., 2007). An amino acid percent identity matrix of each TRAF3IP1 protein from *P. yessoensis* and other organisms was generated by DNASTAR MegAlign software (<http://www.dnastar.com/t-dnastar-lasergene.aspx>). The JTT + G + I + F

model (Jones-Taylor-Thornton model with frequency and gamma distribution) was selected as the best fit model by MEGA 7.0 (Kumar et al., 2016). A phylogenetic tree was constructed using MEGA 7.0 with the maximum likelihood (ML) method based on the JTT + G + I + F model. Bootstrapping with 100 replications was conducted to evaluate the phylogenetic tree. To find conserved motifs in the TRAF3IP1 proteins among different species, Multiple EM for Motif Elicitation (MEME) version 3.5.4 (<http://meme.sdsc.edu/meme/website/intro.html>) (Bailey et al., 2006) was used with the following parameters: number of repetitions – any; maximum number of motifs – 8; optimum width of motif – 6-200.

Scallop collection All of the scallop handling and treatment during this study was conducted in accordance with the guidelines and regulations established by the Ocean University of China Institutional Animal Care and Use Committee and the local government. A total of 200 two-year-old *P. yessoensis* specimens were collected in January 2014 from the Dalian Zhangzidao Fishery Group Corporation (Liaoning Province, China). After collection, the scallops were acclimated in filtered and aerated seawater for one week before the start of the experiment. Samples from the mantle, gill, gonad, kidney, hepatopancreas, smooth muscle, striated muscle, foot, eye and hemocytes were collected from randomly selected individuals, flash-frozen in liquid nitrogen and stored at -80°C until RNA extraction.

To obtain samples representing different developmental stages, spawning, fertilization and larval culture were conducted following previously detailed protocols (Wang et al., 2008). Briefly, to induce spawning, sexually mature scallops were exposed to the air in darkness for 1 h, and then thermally stimulated by raising the seawater temperature from 9°C to 12°C . After fertilization, the embryos were incubated at $12\text{--}13^{\circ}\text{C}$ until they developed into juvenile mollusks. Samples from different developmental stages including the oocytes, fertilized eggs, morulae, blastulae, gastrulae, trochophores, D-shaped larvae, umbo larvae, eyespot larvae and juvenile mollusks of *P. yessoensis* were collected and preserved in RNALater (Sigma-Aldrich, St. Louis, MO, USA) and stored at -80°C for further use.

Bacterial challenge Bacterial challenge experiments were as described by Wang et al. (2015). Briefly, *M. luteus* (Gram-positive) and *V. anguillarum* (Gram-negative) were cultured in liquid 2216E broth (Tryptone 5 g l^{-1} , yeast extract 1 g l^{-1} , $\text{C}_6\text{H}_5\text{Fe}\cdot 5\text{H}_2\text{O}$ 0.1 g l^{-1} , pH 7.6) at 28°C to $\text{OD}_{600} = 0.2$ and centrifuged at $2,000\text{ g}$ for 5 min to harvest the bacteria. The pellet was suspended in filtered seawater and adjusted to $2 \times 10^7\text{ CFU ml}^{-1}$ (*M. luteus*) or $1 \times 10^7\text{ CFU ml}^{-1}$ (*V. anguillarum*). The scallops were randomly divided into three groups with 60 scallops per group. One group served as the control, and the other

two treatment groups received immersion infection with *M. luteus* and *V. anguillarum* at a concentration of 2×10^7 and $1 \times 10^7\text{ CFU ml}^{-1}$ in seawater, respectively. At each time point of 0, 3, 6, 12 and 24 h post infection, 10 individuals were collected from each of the three groups. The hemolymph of the sampled scallops was collected and the hemocytes were then used for gene expression analysis.

RNA extraction and cDNA synthesis Total RNA was extracted using the method described by Hu et al. (2006), and DNA contamination was removed by DNase I (Takara, Shiga, Japan) treatment. RNA concentration and purity were determined using a NanoVue Plus spectrophotometer (GE Healthcare, Chicago, IL, USA), and RNA integrity was verified by agarose gel electrophoresis. First-strand cDNA was synthesized from $2\text{ }\mu\text{g}$ RNA using oligo(dT)₁₈ and moloney murine leukemia virus reverse transcriptase (Thermo, Waltham, MA, USA) following the manufacturer's protocol. All of the cDNA products were then diluted to $250\text{ ng }\mu\text{l}^{-1}$ for gene cloning and real-time PCR.

Cloning full-length cDNA of *PyTRAF3IP1* To obtain full-length *PyTRAF3IP1* cDNA, 3' and 5' rapid amplification of cDNA ends (RACE) were performed using the SMART RACE cDNA Amplification Kit (Clontech, Mountain View, CA, USA) according to the manufacturer's instructions. The gene-specific primers 5'-CGTAGT-GCTAACCTCTTGGTAAATC-3' and 5'-TTCAGGAG-GAGGTGGGGCAGAT-3' were used for 3' and 5' RACE, respectively. 5' RACE and 3' RACE cDNA libraries were generated using a SMARTer RACE 5'/3' Kit (Clontech) according to the manufacturer's user manual. The products were ligated into pMD18-T vector, transferred into *Escherichia coli* DH5 α , and sent to Sangon Biotech for sequencing.

Expression analysis of *PyTRAF3IP1* Real-time quantitative reverse transcription PCR (qRT-PCR) was conducted using the SsoFast EvaGreen Supermix on a Light Cycler 480 Real-time PCR System (Roche Diagnostics, Mannheim, Germany) in a total volume of $20\text{ }\mu\text{l}$ containing $1 \times$ Real-time PCR master mix with SYBR Green dye (Toyobo, Osaka, Japan), $0.2\text{ }\mu\text{M}$ each primer and $2\text{ }\mu\text{l}$ of cDNA template. The running program was as follows: 50°C for 2 min, 94°C for 10 min, followed by 40 cycles of 94°C for 15 s and 62°C for 1 min. Cytochrome B (CB) and DEAD-box RNA helicase (HELI) were used as internal reference genes for normalization of gene expression in the embryos/larvae and tissues (Ct values: CB, 16.1 ± 0.7 ; HELI, 25.2 ± 0.9), respectively, because they were found to be the most stably expressed reference genes in *P. yessoensis* embryos/larvae and tissues (Feng et al., 2013). β -actin was chosen as the internal reference gene for normalization of gene expression in the bacterial challenge experiments (Ct value: 18.9 ± 2.8), because it was

found to be stably expressed in *P. yessoensis* hemocytes with bacterial infection (Bao et al., 2011) and has also been widely used as a reference control for gene expression analysis in bivalves with bacterial challenge (Shan et al., 2011; He et al., 2012; Wang et al., 2015; Wu et al., 2015). Primer specificity was assessed by alignment with the *P. yessoensis* transcriptome and draft genome assembly (unpublished data) using BLASTN with an e-value of $1e^{-10}$. Melting curve analysis was also performed to verify that each primer set amplified a single product. All primers used in qRT-PCR were designed by Primer Premier 5.0 and are listed in Table 1. Each sample was run in duplicate along with the internal control gene. The qRT-PCR amplification efficiency of each gene was calculated using the online software Real-time PCR Miner (<http://miner.ewindup.info/>) (Zhao and Fernald, 2005). Data from the qRT-PCR were analyzed using the Relative Expression Software Tool version 2009 (Pfaffl et al., 2002). The CT method ($2^{-\Delta\Delta Ct}$) was used to calculate relative changes in gene expression (Arocho et al., 2006). In the analysis of bacterial challenge experiments, separate controls were used for each time point and the expression fold changes in each group were compared to their own control. Data obtained from qRT-PCR are presented as the mean $\pm$ standard error ($n = 3$ for tissues; $n = 10$ for bacterial challenge). No standard error was obtained for embryos/larvae because only one mixed sample was used for each stage ($n = 1$). Statistical analysis of the data was performed with SPSS (version 16.0) software by two-way analysis of variance with the Bonferroni post test. Differences were considered significant if $P < 0.05$.

RESULTS AND DISCUSSION

Identification and characterization of *PyTRAF3IP1*

After extensive data mining using the transcriptome and genome assembly of *P. yessoensis*, one *TRAF3IP1* gene was identified and was named *PyTRAF3IP1*. The full-length cDNA of *PyTRAF3IP1* was 2,367 bp with a 226-bp 5'-UTR,

a 512-bp 3'-UTR and a 1,629-bp open reading frame encoding 542 amino acids. The predicted molecular weight of *PyTRAF3IP1* was 62.42 kDa, with an isoelectric point (pI) of 8.66. As the schematic diagram shows in Fig. 1A, the *PyTRAF3IP1* gene was composed of 14 exons separated by 13 introns. The genomic sequence of this gene spanned 20,248 bp (GenBank accession number KX609989). The cDNA and predicted amino acid sequences of *PyTRAF3IP1* were submitted to GenBank with the accession number KU984105. Secondary structure analysis using Geneious 9.1.2 indicated that *PyTRAF3IP1* consists of 25 α -helices, 16 β -strands, 36 coils and 35 turns (Fig. 1B). Multiple sequence alignment showed that the deduced amino acid sequence of *PyTRAF3IP1* had high identity with the sequences of other known TRAF3IP1s (Fig. 2), including those of *C. gigas* (63.4%), *L. gigantea* (62.4%), *M. musculus* (41.2%) and *H. sapiens* (39.6%) (Supplementary Table S1). According to previous research (Ling and Goeddel, 2000) and the results given by SMART and Phyre², the *PyTRAF3IP1* protein contains a MIP-T3 domain (amino acids 49-539; Pfam: PF10243; Supplementary Fig. S1), which is the characteristic structure of the identified TRAF3IP1s. Sequence alignment also showed that 38 amino acids near the C terminus of *PyTRAF3IP1* corresponded to those in the mammalian TRAF3IP1s which form a coiled-coil domain (amino acids 454-491) (Fig. 2), the region interacting specifically with the coiled-coil TRAF-N domain of TRAF3 (Ling and Goeddel, 2000).

Comparative structural and phylogenetic analysis

Phylogenetic analysis was conducted for the *PyTRAF3IP1* protein and those from other selected species. As shown in Fig. 3, *PyTRAF3IP1* was first clustered with orthologs from bivalves and gastropods (Lophotrochozoan group), and then fell into bigger clusters with echinoderms and vertebrates (Deuterostome group) and finally with the Ecdysozoan group. MEME analysis identified eight conserved motifs shared among the related TRAF3IP1 proteins (Fig. 3). The type, order, and number of motifs

Table 1. Primers used in qRT-PCR and their amplification efficiency for each gene

Primer	Sequence (5'-3')	Efficiency
PyTRAF3IP1-F	ATAGTAGTGAACCGAAGGATGGGTAGTAA	86.16%
PyTRAF3IP1-R	GAATGATAACGGTATGATGGCTGGAT	
Cytochrome B-F	CCTCTCCACCCCTTTCTAGTCCTTG	83.60%
Cytochrome B-R	CTCCTGGTTCTTCGTCTTTCTCC	
DEAD-box RNA helicase-F	CCAGGAGCAGAGGGAGTTTCG	86.40%
DEAD-box RNA helicase-R	GTCTTACCAGCCCGTCCAGTTC	
β -actin-F	CCAAAGCCAACAGGGAAAAG	83.99%
β -actin-R	TAGATGGGGACGGTGTGAGTG

Fig. 1. Sequence analysis of *PyTRAF3IP1*. (A) Gene structure of *PyTRAF3IP1*. Light blue boxes represent the 5' (left) and 3' (right) UTRs. Dark blue boxes represent the exons, and horizontal lines the introns. The gene is shown according to the lengths (bp) of the gDNA and mRNA sequences, which were obtained from the genome and transcriptome data of *P. yessoensis*. (B) The secondary structure of *PyTRAF3IP1* predicted by Geneious 9.1.2. Pink cylinders represent α -helices; yellow bars represent β -strands; wavy gray lines represent coils; and blue curved arrows/bars represent turns.

Fig. 2. Multiple sequence alignment of partial TRAF3IP1 proteins from *P. yessoensis* and other organisms at the coiled-coil region. Amino acid sequences were downloaded from Ensembl and NCBI, including human (AAH59174.1), mouse (AAI17869.1), chicken (XP_004942568.1), lizard (XP_008112167.1), tropical clawed frog (ENSXETG00000004877), zebrafish (NP_956894.1), stickleback (WBGene00001127), sea urchin (SPU_019927), tunicate (PTHR31363), fruitfly (FBgn0038221), mosquito (AGAP001429), worm (WBGene00001127), daphnia (DAPPUDRAFT_334039), Zhikong scallop (unpublished), Pacific oyster (CGI_10012204) and owl limpet (LotgiG159832).

were similar in proteins with closer phylogenetic relationships. For example, motif 7 was present in all the vertebrates but absent from invertebrates, which may indicate its specific role in the vertebrate lineages. All the TRAF3IP1s except that from *A. gambiae* contained motif 2 where the coiled-coil domain is located to interact with the coiled-coil TRAF-N domain of TRAF3. This suggests a conserved role for TRAF3IP1 interacting with TRAF3. Interestingly, compared to the Ecdysozoan group comprising worms and insects, the mollusk TRAF3IP1 proteins were more similar to those of vertebrates, which can be seen in both alignment and motif analysis (Figs. 2 and 3). This agrees with the phylogenetic relationship of TRAF3IP1s and implies that the mollusk *TRAF3IP1*

genes are derived from that of the common ancestor with vertebrates and may thus have similar functions to vertebrate *TRAF3IP1*s.

Putative promoter region of *PyTRAF3IP1* Identifying TFBSs in the promoter region is a first step toward understanding the transcriptional regulation of a gene. The 2-kb region upstream of a putative transcription initiation site of *PyTRAF3IP1* was subjected to prediction analysis. Potential TFBSs were identified, and many of them were consensus sequences for binding TFs (Fig. 4). Among these elements, Sp1 (stimulating protein 1), NF- κ B, C/EBP (CCAAT/enhancer binding protein) and AP1 (activator protein 1) are important TFs in diverse cel-

Fig. 3. Phylogenetic relationship and conserved motifs of TRAF3IP1s. (Left) Phylogenetic tree constructed by the ML method. The black circle indicates PyTRAF3IP1 from *P. yessoensis*. Bootstrapping values are indicated by numbers at the nodes. (Right) Conserved motifs in TRAF3IP1 proteins; each colored block represents a motif in the protein. The length of each protein and motif can be estimated using the scale at the bottom. The height of a block gives a qualitative indication of the significance of the site; taller blocks are more significant.

lular processes including the regulation of immune-related genes in response to pathological stresses (He et al., 2013; Li et al., 2015; Umasuthan et al., 2015). These TFBSs may be important *cis*-regulatory elements that can bind to their TFs and regulate the expression of *PyTRAF3IP1*, which implies that *PyTRAF3IP1* transcription may be tightly modulated following different immune stresses.

Spatiotemporal expression of *PyTRAF3IP1* The spatiotemporal expression profile of *PyTRAF3IP1* was measured by qRT-PCR in the developmental stages and adult tissues of healthy *P. yessoensis*. As shown in Fig. 5A, during the first two developmental stages sampled, the amount of *PyTRAF3IP1* mRNA was reduced in the fertilized eggs compared with the incipient oocytes, suggesting that the embryos took advantage of maternal *PyTRAF3IP1* at the beginning of embryo development. *PyTRAF3IP1* expression then increased stage by stage from the morulae phase and reached the maximum level (20.5-fold compared with the lowest expression level, which appeared in the umbo larvae stage) in the D-shaped larvae stage. This implies that *PyTRAF3IP1* is involved in the early development of scallop. After D-shaped larvae, the expression level dramatically dropped in the following stage of umbo larvae, and then slightly increased again in the eyespots larvae and juvenile stages (Fig. 5A). This suggests the consumption and replenishment of *PyTRAF3IP1* during the embryonic development of *P. yessoensis*.

PyTRAF3IP1 expression was ubiquitously detected in

all 10 tissues of healthy scallops (Fig. 5B): mantle, gill, gonad, kidney, hepatopancreas, smooth muscle, striated muscle, foot, eye and hemocytes. The highest expression was observed in gill (6.3-fold compared with the lowest expression level, which appeared in the hepatopancreas), which is constantly in contact with the external environment via water filtering and is targeted as the entry site during bacterial infection. *PyTRAF3IP1* was also highly expressed in gonad, and moderately expressed in hemocytes and mantle. As filter feeders, scallops are in permanent contact with a broad diversity of microorganisms whose ecology is largely unknown. The interplay between the scallop immune system and the associated microbiota is suggested to occur both at epithelial surfaces such as mantle and gills, which form the first line of defense against infection, and within the hemolymph (blood), where pathogens are recognized and eliminated by the immune cells (Bachere et al., 2004; Schmitt et al., 2012). A relatively high expression level of *PyTRAF3IP1* was also displayed in smooth and striated muscle of *P. yessoensis*, and the lowest expression was in the hepatopancreas. Other than binding to TRAF3, TRAF3IP1 can also interact with microtubular structures which may be regulated in coordination with the dynamic function of the microtubule cytoskeleton during processes such as cell growth and differentiation (Ling and Goeddel, 2000; Kunitomo and Iino, 2007). This may explain the ubiquitous expression of *PyTRAF3IP1* in many tissues and indicate its involvement in different immune responses and/or metabolic processes.

-1974 ATAATTTAATCAGCACCAGCATATGTGTATGACCTCTTTTGCAGAAACCTACATGTCTTCTC
c-Jun C/EBP α

-1914 TATTATCGGTTACATTTCTTCCGAAAGAAATAGAGAAACATCATTCTGTCCTTTTGGT

-1854 GATTTCATATAAGAGTTCTAGACTTCTAGGAAGTTATAAATAATCCCTGACCTAGTGTTC
RSRFC4 RAR- α

-1794 TGTAACATGTTCTGTAATAGACTTTAAGGAAGTTATAAATAAATCCCTCTTCTACACTCT
C/EBP α NF- κ B

-1734 GAAACATTGTCTGTAATAGACTTCTAGGAAGTTATAAATAATCTCTGTCCTATGTTCTGT
RSRFC4

-1674 AACATTGTCTGTAATAGACTTCTAGGAAGTTATAAATAATCTCTGTCCTATGTTCTGTAA
RSRFC4

-1614 CATGTTCTGTAATAGACTTTAAGGAAGTTATAAATAAATCCCTCTTCTACACTCTGAAAC
C/EBP α NF- κ B

-1554 ATTGTCTGTAATAGACTTCTAGGAAGTTATAAACACATTCCTGCTGACGATCTGAAATA
TEC1

-1494 TGTCTTTTGTACTAACGTATCAGCTGTAACATCAATCAGGTCACACTATCATCCCACT
ER

-1434 CACAGCAGCAGAGAAAGTATAATGAGTGAAGAGTAACAGATGGAAATACTCTTGAC
HNF-3B C/EBP α

-1374 GACGTCAAATGGTACATTAGAAATTTGACCACTGCTAATGTTTCCCGCATGTTTCTCT
USF

-1314 TTTATTCTTGAATTGCTGTTCCGGTGGTCCAGTCAACATCTTCTGATGTTCTTTAATAC

-1254 GTATCCCATATGTCAGCTTGTTCCTTGATATATGAAGTTGCGGTTGTACATAAAA

-1194 TTTGGTAAACCTTCCATCAGCGTTTCTGCTTTCGGATAAACTCATGGTTGCTTGTATGGC

-1134 GTTGATGGTCGAAGAGCGCTGTTGATGTGATGATGCTAACTCTTGAAGTCTCTCA
Sp1 AP-1 C/EBP

-1074 GACACTCCTTGTATGTATGGTATAGTGACCATGCCGTTAGTATGTTCTTGAGACATT

-1014 GTTACTTCGAGTGAATTGAGTCGACACCGATCATTTACCTCTTTCGCTTGACAAAGAAA

-954 GTTCGAAAGTTCAATAATGAGTGTCAATTACTGTTTGTGTTTAAACAACTTAGCAC
MEB-1

-894 CAGTATTTATCCCAACTGACGGTATCATCTTGAATGATACGATTTTGTGTTAATTG
C/EBP β

-834 TGGTGAACCGGTCGCTAGCTGTGCGGGGATAACAATGTAACGTTTAAACGATTCTTA

-774 TTATGTTACTGCATTCTTTCATTTAGTTTGAAGTAATTGATACCTTGTAAAGTATTA
HNF-1C

-714 ATTAAGCTCATAATATGGGAGAGACCGGGGCAAGATCGGCTTCGATGTAAGTTCCGGGCAC

-654 CCCTGATCTTAATTTGGTCTTGGAGCAATTTCAACATTTGAGGATGTCGCACTTCC
C/EBP α

-594 TTTGACATGATGGAACATTCAGTGAGAGAGAAAAGAGTTTGAACAACAAGGGA
IRF-1

-534 AAGTTGTGGCTTTTTTAGGATGCTAGAAAGTAGATTATCACCTCACTTCTCTACTTTTG
CGAATCTTAGTACAACAAATTGCTACTAATGATGTATGTCATAGGTGATCACACTGACGGC
ER

-414 TTCACATGTCACATGTCAAAGTCAATTGGTGTGTTGTTAGTTTGTGTTGATGTTTTC

-354 CTAATAAGTTACATGAAACCAAGAGTCACTGTAGACACTCTATAGCTTTTGAAACG
SRF

-294 GTGGCCCTGTCAAGAAATGTTAACTTAGATCCCGGTACAAATATACAACCGACTGTTGT

-234 GTATATGTACATATATATATATATATTGTTGTTGTAATAGGTAATTTGTGATATAA
TBP Pit-1a Pit-1a

-174 ATGTATTAATCTGTACGGGAGTTATTTCCCTTTGACCTAGATCTATGCATGATGACATG
REV-Erba Oct-1

-114 TTGAATATTTAAATCATGTCACTTTTCTTAAGATTTACAGTAAGGAGATGAACAAAGC

-54 AAAGCAGATATAAGAGACAGATAAATCCCGAAACACATATAATTCGGCTTAAGGTA
+1

+7 GGGGATATTTCAACGTGTTGCCGCCAGGCACCTGCTCCCTAGCAACTGTAAACATTAA

Fig. 4. Predicted TFBSs in the putative *PyTRAF3IP1* promoter region. The sequence numbers are relative to the predicted transcription initiation site (+1), which is boxed and in bold type. Possible TFBSs are differentially marked and identified beneath each site.

Temporal expression of *PyTRAF3IP1* in response to bacterial infection To provide insights into the function of *PyTRAF3IP1* in the innate immune response of scallop, expression was analyzed after infection with two major bacterial pathogens, *M. luteus* (Gram-positive) and *V. anguillarum* (Gram-negative). The induction level of *PyTRAF3IP1* was analyzed in the hemocytes, which have been reported to play critical roles in innate immune responses in bivalves (Costa et al., 2009). As shown in Fig. 6, *PyTRAF3IP1* expression increased substantially in the acute phase (3–6 h) in challenges by both *M. luteus* and *V. anguillarum*. After infection by *M. luteus*, *PyTRAF3IP1* expression dramatically increased and reached its highest level of a 10-fold increase at 6 h ($P = 0.045$), and then declined rapidly after the acute increase to a level of expression even lower than the normal value at the end of experiment (24 h). After *V. anguillarum* infection, signif-

Fig. 5. Relative expression levels of *PyTRAF3IP1* in *P. yessoensis* among (A) different developmental stages ($n = 1$) and (B) healthy adult tissues ($n = 3$). Vertical bars represent the mean $\pm$ S.E. ($n = 3$).

Fig. 6. Expression levels of *PyTRAF3IP1* in hemocytes after *Vibrio anguillarum* (G $-$) or *Micrococcus luteus* (G $+$) challenge. Vertical bars represent the mean $\pm$ S.E. ($n = 10$). Expression in experimental groups marked with asterisks differed significantly from that in the contemporaneous control ($P < 0.05$).

icantly more *PyTRAF3IP1* transcripts were detected, with a peak at 3 h (14-fold, $P = 0.019$) and a slight reduction at 6 h (12.5-fold, $P = 0.033$). Following a sharp drop at 12 h, *PyTRAF3IP1* expression displayed another notable increase at 24 h (5.5-fold, $P > 0.05$). Compared to the Gram-positive *M. luteus* infection, *PyTRAF3IP1* showed a stronger expression and response level against invasion by the Gram-negative *V. anguillarum*. This may correlate with different response levels of the innate immunity

Table 2. Expression regulation of *TRAF*s of several bivalve species in response to bacterial challenge, as found in bibliography

Gene	G+ challenge				G– challenge				Species	Reference
	3 h	6 h	9/12 h	24 h	3 h	6 h	9/12 h	24 h		
<i>TRAF3IP1</i>	+	++	0	0	++	++	0	+	<i>P. yessoensis</i>	This study
<i>TRAF3</i>	0	0	0	0	0	0	0	0	<i>M. galloprovincialis</i>	Toubiana et al., 2014
<i>TRAF3</i>	0	0	0	0	0	0	0	0	<i>P. yessoensis</i>	Wang et al., 2015
<i>TRAF3</i>	n.d.	n.d.	n.d.	n.d.	0	--	–	--	<i>P. putida</i>	Huang et al., 2012
<i>TRAF3L</i>	n.d.	n.d.	n.d.	n.d.	0	++	++	++	<i>C. gigas</i>	Huang et al., 2014
<i>TRAF3S</i>	n.d.	n.d.	n.d.	n.d.	0	--	--	--	<i>C. gigas</i>	Huang et al., 2014
<i>TRAF2</i>	++	0	0	--	++	++	0	++	<i>P. yessoensis</i>	Wang et al., 2015
<i>TRAF4</i>	0	++	0	0	0	++	++	++	<i>P. yessoensis</i>	Wang et al., 2015
<i>TRAF6</i>	0	++	++	0	+	++	++	+	<i>M. galloprovincialis</i>	Toubiana et al., 2014
<i>TRAF6</i>	0	++	++	0	++	++	++	++	<i>P. yessoensis</i>	Wang et al., 2015
<i>TRAF7</i>	++	0	0	0	++	+	0	++	<i>P. yessoensis</i>	Wang et al., 2015

0: no effect; +: up-regulated; ++: significantly up-regulated; –: down-regulated; --: significantly down-regulated; n.d.: not determined.

of shellfish in combating infections by Gram-positive and Gram-negative bacteria. A similar pattern has also been reported in several bivalve species. For instance, *PyRel* and *PyTollip* in *P. yessoensis* (Li et al., 2015; Zhang et al., 2015), *CgTLRs* and *CgMyD88* in *C. gigas* (Zhang et al., 2013) and *MgIKK-2* in *M. galloprovincialis* (Toubiana et al., 2014) all displayed higher expression levels after infection by Gram-negative bacteria than by Gram-positive bacteria. Interestingly, the second strong response of *PyTRAF3IP1* at 24 h post *V. anguillarum* challenge has also been reported for *CfRel* (Zhou et al., 2013) and *PyTollip* (Zhang et al., 2015). This expression pattern indicates that the up-regulation of *PyTRAF3IP1* expression represents a longer-lasting response against the invasion of Gram-negative bacteria.

Activation of *TRAF3IP1* and other *TRAF*s from bivalves To ensure a rapid but transient response, the production of type I IFNs must be tightly regulated to avoid excessive immune activation leading to pathogenesis of infectious, chronic inflammatory and autoimmune diseases (Melmed et al., 2003; Cook et al., 2004). A previous study has shown that *TRAF3IP1* interacts specifically with *TRAF3* and prevents it from forming protein complexes with downstream transducer and effector proteins (Ng et al., 2011). Here, transcription levels of several *TRAF*s measured in different bivalve species with different bacterial challenges, and resulting from bibliographic analysis, are summarized in Table 2. Unlike the up-regulation of *PyTRAF3IP1* in response to bacterial challenge, the expression of *P. yessoensis TRAF3* (*PyTRAF3*) was not significantly up- or even down-regulated during 0–24 h of bacterial infection (Wang et al., 2015). A similar expression pattern of *TRAF3* under bacterial

invasion was also reported in Mediterranean mussel (*M. galloprovincialis*) (Toubiana et al., 2014) and pearl oyster (*P. fucata*) (Huang et al., 2012). Interestingly, there are two *TRAF3* isoforms in *C. gigas*, which display contrary expression responses to Gram-negative bacterial challenge: *CgTRAF3-L* is significantly up-regulated, whereas *CgTRAF3-S* is significantly down-regulated (Huang et al., 2014). No such multiple isoforms of *TRAF3* have yet been isolated in other bivalve species. It would be of interest to investigate whether *TRAF3IP1* in *C. gigas* interacts in the same way with both isoforms. Except for *TRAF3*, most of the other *TRAF* members showed strong up-regulation after bacterial challenge, especially with Gram-negative bacteria (Table 2). *TRAF3* was first described as a CD40-interacting molecule whose over-expression inhibited CD40-mediated NF- κ B activation (Hacker et al., 2011; Yi et al., 2014), whereas both *TRAF2* and *TRAF6* could compete with *TRAF3* for CD40 binding, which limited the capacity of CD40 engagement to induce NF- κ B activation (Hacker et al., 2011; Wang et al., 2015). On the other hand, *TRAF3-TRAF3IP1* complex association can also be regulated by activation of CD40, as the complex can be dissociated by CD40 ligand stimulation and *TRAF3* is recruited to the CD40 receptor (Ling and Goeddel, 2000). Such interaction may be regulated in coordination with other *TRAF* members. It will be of great interest to understand whether *TRAF3IP1* competes with other *TRAF*s or *TRAF3*-interacting proteins by pre-occupying the same binding domains in *TRAF*s with higher affinity, speed or stability. Nevertheless, regarding whether *PyTRAF3* appears as a negative regulator of the innate defense against bacterial infection and whether *PyTRAF3IP1* modulates type I IFN production through its direct interaction with *PyTRAF3*, more experiments and

evidence are required.

CONCLUSION

In summary, in this study we scanned the genome and transcriptome of *P. yessoensis* and successfully identified a gene encoding a TRAF3-interacting protein, named *PyTRAF3IP1*, for the first time in marine invertebrates. Several potential regulatory motifs were found in the promoter region, and these may function in regulating *PyTRAF3IP1* gene expression. Expression profiles of this gene were analyzed in healthy tissues, at different developmental stages and in hemocytes after bacterial infection, which affirmed the participation of *PyTRAF3IP1* in scallop immune responses. Our results represent the first report of *TRAF3IP1* identification and its regulation in innate immune responses of scallops, and should provide valuable information for the understanding of their innate immunity systems.

This project was supported by the National Natural Science Foundation of China (31472276); Qingdao National Laboratory for Marine Science and Technology (2015ASKJ02); the Earmarked Fund for Modern Agro-industry Technology Research System (CARS-48); and the National Infrastructure of Fishery Germplasm Resources. The authors wish to thank Dalian Zhangzidao Fishery Group Corporation (Liaoning Province, China) for providing scallop materials. We also thank Prof. Xiaohua Zhang for providing bacterial strains and technical assistance in the bacterial challenge experiments.

REFERENCES

- Arocho, A., Chen, B., Ladanyi, M., and Pan, Q. (2006) Validation of the 2- $\Delta\Delta C_t$ calculation as an alternate method of data analysis for quantitative PCR of BCR-ABL P210 transcripts. *Diagn. Mol. Pathol.* **15**, 56–61.
- Bachere, E., Gueguen, Y., Gonzalez, M., De Lorget, J., Garnier, J., and Romestand, B. (2004) Insights into the anti-microbial defense of marine invertebrates: the penaeid shrimps and the oyster *Crassostrea gigas*. *Immunol. Rev.* **198**, 149–168.
- Bailey, T. L., Williams, N., Misleh, C., and Li, W. (2006) MEME: discovering and analyzing DNA and protein sequence motifs. *Nucleic Acids Res.* **34**, W369–W373.
- Bao, X., Liu, W., Jiang, B., Su, H., Li, Y., Shan, Z., Yu, H., and He, C. (2011) Expression stability of reference genes for quantitative PCR in Japanese scallop *Mizuhopecten yessoensis*. *Fish. Sci.* **30**, 603–608.
- Bassim, S., Genard, B., Gauthier-Clerc, S., Moraga, D., and Tremblay, R. (2015) Ontogeny of bivalve immunity: assessing the potential of next-generation sequencing techniques. *Reviews in Aquaculture* **7**, 197–217.
- Bizet, A., Becker-Heck, A., Ryan, R., Weber, K., Filhol, E., Krug, P., et al. (2015) Mutations in *TRAF3IP1/IFT54* reveal a new role for IFT proteins in microtubule stabilization. *Nat. Commun.* **6**, 8666.
- Bradley, J. R., and Pober, J. S. (2001) Tumor necrosis factor receptor-associated factors (TRAFs). *Oncogene* **20**, 6482–6491.
- Cook, D. N., Pisetsky, D. S., and Schwartz, D. A. (2004) Toll-like receptors in the pathogenesis of human disease. *Nat. Immunol.* **5**, 975–979.
- Costa, M., Prado-Alvarez, M., Gestal, C., Li, H., Roch, P., Novoa, B., and Figueras, A. (2009) Functional and molecular immune response of Mediterranean mussel (*Mytilus galloprovincialis*) haemocytes against pathogen-associated molecular patterns and bacteria. *Fish Shellfish Immunol.* **26**, 515–523.
- Feng, L., Yu, Q., Li, X., Ning, X., Wang, J., Zou, J., Zhang, L., Wang, S., Hu, J., Hu, X., and Bao, Z. (2013) Identification of reference genes for qRT-PCR analysis in Yesso Scallop *Patinopecten yessoensis*. *PLoS One* **8**, e75609.
- Guo, C., Xiong, S., Liu, G., Wang, Y., He, Q., Zhang, X., Zhang, Z., Ge, F., and Kitazato, K. (2010) Proteomic analysis reveals novel binding partners of MIP-T3 in human cells. *Proteomics* **10**, 2337–2347.
- Hacker, H., Tseng, P., and Karin, M. (2011) Expanding TRAF function: TRAF3 as a tri-faced immune regulator. *Nat. Rev. Immunol.* **11**, 457–468.
- He, C., Yu, H., Liu, W., Su, H., Shan, Z., Bao, X., Li, Y., Fu, L., and Gao, X. (2012) A goose-type lysozyme gene in Japanese scallop (*Mizuhopecten yessoensis*): cDNA cloning, mRNA expression and promoter sequence analysis. *Comp. Biochem. Physiol. B* **162**, 34–43.
- He, C., Wang, Y., Liu, W., Gao, X., Chen, P., Li, Y., and Bao, X. (2013) Cloning, promoter analysis and expression of the tumor necrosis factor receptor-associated factor 6 (TRAF6) in Japanese scallop (*Mizuhopecten yessoensis*). *Mol. Biol. Rep.* **40**, 4769–4779.
- He, J. Q., Oganessian, G., Saha, S. K., Zarnegar, B., and Cheng, G. (2007) TRAF3 and its biological function. *Adv. Exp. Med. Biol.* **597**, 48–59.
- Hou, R., Bao, Z., Wang, S., Su, H., Li, Y., Du, H., et al. (2011) Transcriptome sequencing and *de novo* analysis for Yesso scallop (*Patinopecten yessoensis*) using 454 GS FLX. *PLoS One* **6**, e21560.
- Hu, X., Bao, Z., Hu, J., Shao, M., Zhang, L., Bi, K., Zhan, A., and Huang, X. (2006) Cloning and characterization of tryptophan 2, 3-dioxygenase gene of Zhikong scallop *Chlamys farreri* (Jones and Preston 1904). *Aquac. Res.* **37**, 1187–1194.
- Huang, B., Zhang, L., Du, Y., Li, L., Qu, T., Meng, J., and Zhang, G. (2014) Alternative splicing and immune response of *Crassostrea gigas* tumor necrosis factor receptor-associated factor 3. *Mol. Biol. Rep.* **41**, 6481–6491.
- Huang, X., Liu, W., Guan, Y., Shi, Y., Wang, Q., Zhao, M., Wu, Z., and He, M. (2012) Molecular cloning, characterization and expression analysis of tumor necrosis factor receptor-associated factor 3 (TRAF3) from pearl oyster *Pinctada fucata*. *Fish Shellfish Immunol.* **33**, 652–658.
- Kumar, S., Stecher, G., and Tamura, K. (2016) MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. *Mol. Biol. Evol.* **33**, 1870–1874.
- Kunitomo, H., and Iino, Y. (2007) *Caenorhabditis elegans* DYF-11, an orthologue of mammalian Traf3ip1/MIP-T3, is required for sensory cilia formation. *Genes Cells* **13**, 13–25.
- Larkin, M. A., Blackshields, G., Brown, N., Chenna, R., McGettigan, P. A., McWilliam, H., Valentin, F., Wallace, I. M., Wilm, A., Lopez, R., et al. (2007) Clustal W and Clustal X version 2.0. *Bioinformatics* **23**, 2947–2948.
- Li, R., Zhang, R., Zhang, L., Zou, J., Xing, Q., Dou, H., Hu, X., Zhang, L., Wang, R., and Bao, Z. (2015) Characterizations and expression analyses of *NF- κ B* and *Rel* genes in the Yesso scallop (*Patinopecten yessoensis*) suggest specific response patterns against Gram-negative infection in bivalves. *Fish Shellfish Immunol.* **44**, 611–621.
- Ling, L., and Goeddel, D. V. (2000) MIP-T3, a novel protein linking tumor necrosis factor receptor-associated factor 3 to the microtubule network. *J. Biol. Chem.* **275**, 23852–23860.

- Melmed, G., Thomas, L. S., Lee, N., Tesfay, S. Y., Lukasek, K., Michelsen, K. S., Zhou, Y., Hu, B., Arditi, M., and Abreu, M. T. (2003) Human intestinal epithelial cells are broadly unresponsive to Toll-like receptor 2-dependent bacterial ligands: implications for host-microbial interactions in the gut. *J. Immunol.* **170**, 1406–1415.
- Ng, M. H. J., Ho, T. H., Kok, K. H., Siu, K. L., Li, J., and Jin, D. Y. (2011) MIP-T3 is a negative regulator of innate type I IFN response. *J. Immunol.* **187**, 6473–6482.
- Oganesyan, G., Saha, S. K., Guo, B., He, J. Q., Shahangian, A., Zarnegar, B., Perry, A., and Cheng, G. (2006) Critical role of TRAF3 in the Toll-like receptor dependent and -independent antiviral response. *Nature* **439**, 208–211.
- Pfaffl, M. W., Horgan, G. W., and Dempfle, L. (2002) Relative expression software tool (REST©) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. *Nucleic Acids Res.* **30**, e36.
- Schmitt, P., Rosa, R., Duperthuy, M., Lorgier, J., Bachere, E., and Destoumieux-Garzon, D. (2012) The antimicrobial defense of the Pacific oyster, *Crassostrea gigas*. How diversity may compensate for scarcity in the regulation of resident/pathogenic microflora. *Front. Microbiol.* **3**, 160.
- Shan, Z., Li, H., Bao, X., He, C., Yu, H., Liu, W., Hou, L., Wang, J., Zhu, D., Sui, L., Zhu, B., and Li, Y. (2011) A selenium-dependent glutathione peroxidase in the Japanese scallop, *Mizuhopecten yessoensis*: cDNA cloning, promoter sequence analysis and mRNA expression. *Comp. Biochem. Physiol. B* **159**, 1–9.
- Teng, W., Li, W., Zhang, M., Yu, Z., Li, S., Liu, X., et al. (2012) Isolation, identification and pathogenicity of *Vibrio chagasii* from *Patinopecten yessoensis*. *J. Fish China* **6**, 020.
- Toubiana, M., Rosani, U., Giambelluca, S., Cammarata, M., Gerdol, M., Pallavicini, A., Venier, P., and Roch, P. (2014) Toll signal transduction pathway in bivalves: Complete cds of intermediate elements and related gene transcription levels in hemocytes of immune stimulated *Mytilus galloprovincialis*. *Dev. Comp. Immunol.* **45**, 300–312.
- Umasuthan, N., Bathige, S. D. N. K., Revathy, K. S., Nam, B. H., Choi, C. Y., and Lee, J. (2015) Molecular genomic- and transcriptional-aspects of a teleost *TRAF6* homolog: possible involvement in immune response of *Oplegnathus fasciatus* against pathogens. *Fish Shellfish Immunol.* **42**, 66–78.
- Wang, R., Wang, Z., and Zhang, J. (2008) Artificial seed rearing and culture of scallops in: *Science of Marine Shellfish Culture*, (eds.: Li, J. and Deng, Z.), pp. 277–283. Ocean University of China Press, Qingdao, China.
- Wang, J., Wang, R., Wang, S., Zhang, M., Ma, X., Liu, P., Zhang, M., Hu, X., Zhang, L., Wang, S., and Bao, Z. (2015) Genome-wide identification and characterization of *TRAF* genes in the Yesso Scallop (*Patinopecten yessoensis*) and their distinct expression patterns in response to bacterial challenge. *Fish Shellfish Immunol.* **47**, 545–555.
- Wu, B., Liu, Z., Zhou, L., Ji, G., and Yang, A. (2015) Molecular cloning, expression, purification and characterization of vitellogenin in scallop *Patinopecten yessoensis* with special emphasis on its antibacterial activity. *Dev. Comp. Immunol.* **49**, 249–258.
- Yi, Z., Lin, W., Stunz, L., and Bishop, G. (2014) Roles for TNF-receptor associated factor 3 (TRAF3) in lymphocyte functions. *Cytokine Growth Factor Rev.* **25**, 147–156.
- Zhang, Y., He, X., Yu, F., Xiang, Z., Li, J., Thorpe, K. L., and Yu, Z. (2013) Characteristic and functional analysis of Toll-like receptors (TLRs) in the lophotrochozoan, *Crassostrea gigas*, reveals ancient origin of TLR-mediated innate immunity. *PLoS One* **8**, e76464.
- Zhang, R., Li, R., Wang, J., Wang, S., Zhang, M., Hu, X., Zhang, L., Wang, S., Wang, R., and Bao, Z. (2015) Identification, characterization and expression profiling of the *Tollip* gene in Yesso scallop (*Patinopecten yessoensis*). *Genes Genet. Syst.* **90**, 99–108.
- Zhao, S., and Fernald, R. D. (2005) Comprehensive algorithm for quantitative real-time polymerase chain reaction. *J. Comput. Biol.* **12**, 1047–1064.
- Zhou, Z., Wang, M., Zhao, J., Wang, L., Gao, Y., Zhang, H., et al. (2013) The increased transcriptional response and translocation of a Rel/NF- κ B homologue in scallop *Chlamys farreri* during the immune stimulation. *Fish Shellfish Immunol.* **34**, 1209–1215.