Identification, characterization and expression profiling of the *Tollip* gene in Yesso scallop (*Patinopecten yessoensis*)

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Toll-interacting protein (Tollip) is a critical regulator of Toll-like receptor (TLR)-mediated innate immune responses. However, the *Tollip* gene has not been systematically characterized in shellfish. In this study, we identified and characterized a *Tollip* gene, *PyTollip*, in Yesso scallop (*Patinopecten yessoensis*). Phylogenetic and protein structural analyses were conducted to determine its sequence identities and evolutionary relationships. Compared with *Tollip* genes from other invertebrate and vertebrate species, the *PyTollip* gene is highly conserved in its sequence and structural features, except that a unique asparagine residue was found at a conserved site in the C2 domain of *PyTollip*. Quantitative real-time PCR was used to investigate the expression profiles of *PyTollip* in different developmental stages, healthy adult tissues, and in hemolymph after *Micrococcus luteus* and *Vibrio anguillarum* bacterial infection. Real-time PCR analysis demonstrated differential expression of *PyTollip* at the acute phase (3 h) after infection with Gram-negative (*V. anguillarum*) and Gram-positive (*M. luteus*) bacteria. A second strong response of *PyTollip* expression was observed 24 h after challenge with *V. anguillarum*. Collectively, these results provide novel insights into the specific role and response of *Tollip* and TLR signaling pathways in host immune responses against different bacterial pathogens in bivalves.

Key words: Gram-positive and Gram-negative infection, Innate immunity, *Patinopecten yessoensis*, TLR signaling pathways, Tollip

INTRODUCTION

The innate immune system contributes the first line to the fundamental defense strategy of most organisms in response to various infectious agents, playing a crucial role in host survival (Parkin and Cohen, 2001). As the key steps in the initiation of immune defense mechanisms, Toll-like receptor (TLR) signaling pathways and the Interleukin-1 receptor (IL-1) signaling cascade, which facilitate the activation of downstream immune-related genes, have naturally become a hotspot in immunology research. Several intrinsic and extrinsic regulators have been found that can specifically modulate TLR signaling. These regulators include full-length and truncated forms of protein adaptors, transcriptional regulators, ubiquitin ligases, deubiquitinases and microRNAs (Kawai and Akira, 2010), which are essential to TLR-induced responses

for suppressing harmful excessive immune activation. Among these endogenous modulators, the Toll-interacting protein (Tollip) is a critical regulator of TLR-mediated innate immune responses.

Tollip is a ubiquitin-binding protein that interacts with several TLR signaling cascade components, and is highly conserved in evolution from invertebrates to vertebrates and involved in the inflammatory response. The *Tollip* gene was first described in the mouse (Burns et al., 2000) and has since been identified and reported in various invertebrates and vertebrates (Lo et al., 2009; Rebl et al., 2011; Lu et al., 2013; Wang et al., 2013). In mammals, Tollip usually consists of three characterized functional domains: an N-terminal Target of Myb1 (Tom1) binding domain (TBD), a central conserved 2 (C2) domain, with several conserved aspartic acid residues critical for Ca²⁺ and phospholipid binding, and a C-terminal coupling of ubiquitin to endoplasmic reticulum degradation (CUE) domain, which serves as a ubiquitin-binding site that mediates intramolecular monoubiquitylation (Burns et

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al., 2000). In invertebrates, C2 and CUE domains have both been found in the Tollips of various species (Lu et al., 2013; Wang et al., 2013), but a TBD has not yet been discerned due to high sequence diversity.

As a multitasking protein in innate immunity and protein trafficking, two major functions of Tollip have been reported in several higher vertebrates. The first is that Tollip plays an important role in blocking IL-1 receptor associated kinase (IRAK) phosphorylation (Burns et al., 2000), which triggers the suppression both of IL-1R-mediated signaling pathways and of TLR-mediated cellular responses (Zhang and Ghosh, 2002), and also in negatively regulating the IL-1 β and TNF- α signaling pathways (Brissoni et al., 2006). The second function concerns the role of Tollip in protein sorting through its interaction with Tom1, ubiquitin and clathrin (Yamakami et al., 2003; Brissoni et al., 2006), and the control of both nuclear and cytoplasmic protein trafficking (Huang et al., 2012). In invertebrates, several studies have observed the differential expression of *Tollip* after bacterial infection, for example in *Litopenaeus vannamei* and *Apostichopus japonicus*. In *L. vannamei*, the *Tollip* gene displayed diverse expression patterns in different tissues challenged with *Vibrio alginolyticus*, e.g., up-regulation was observed in gill and hepatopancreas, but down-regulation was observed in hemocytes and intestine (Wang et al., 2013). *Tollip* expression declined significantly in coelomocytes after *V. splendidus* infection in *A. japonicus* (Lu et al., 2013). Although the importance of *Tollip* in immunity is being reported in more and more species, systematic analysis of the *Tollip* gene has not been conducted in bivalves, notably in Yesso scallop (*Patinopecten yessoensis*), which is an ancient marine shellfish contributing tremendously to the aquaculture industry of northern China. In the study reported here, we therefore characterized the *Tollip* gene, *PyTollip*, of Yesso scallop, compared its deduced amino sequence with those of other species, and determined its expression profiles in different developmental stages, in healthy scallop tissues and in hemolymph after *Micrococcus luteus* and *V. anguillarum* infections, thereby providing insights into the innate immune mechanisms of scallops.

MATERIALS AND METHODS

Database mining, gene identification and sequence analysis To identify the *Tollip* gene, the transcriptome (Hou et al., 2011) and whole genome sequence databases of Yesso scallop (unpublished data) were searched using all available Tollip protein sequences of invertebrates including sea cucumber (*Apostichopus japonicus*), purple sea urchin (*Strongylocentrotus purpuratus*), mosquito (*Anopheles gambiae*), kuruma shrimp (*Marsupenaeus japonicus*), Pacific oyster (*Crassostrea gigas*), Mediterranean mussel (*Mytilus galloprovincialis*), nematode

(*Caenorhabditis elegans*), and vertebrates including zebrafish (*Danio rerio*), frog (*Xenopus laevis*), chicken (*Gallus gallus*), mouse (*Mus musculus*) and human (*Homo sapiens*) as queries from NCBI (<http://www.ncbi.nlm.nih.gov>), Ensembl (<http://useast.ensembl.org>), Echinobase (<http://www.echinobase.org/Echinobase/>) and OysterBase (<http://www.oysterdb.com/>). TBLASTN was used to obtain an initial pool of *Tollip* transcriptome sequences in Yesso scallop, and a BLASTN search was then performed to verify the cDNA sequence by comparing the transcriptome sequences with the whole genome sequences. ORF (open reading frame) finder (<http://www.ncbi.nlm.nih.gov/gorf/gorf.html>) and DNASTAR (version 7.1) were used to predict amino acid sequence. Furthermore, the predicted amino acid sequence was 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 and molecular weight were computed using the Compute pI/Mw tool (http://web.expasy.org/compute_pi/). The secondary structure of Yesso scallop Tollip protein was predicted using Geneious 7.0.6 (<http://www.geneious.com/>).

Phylogenetic analysis Tollip proteins from other invertebrates, including *A. japonicus*, *S. purpuratus*, *A. gambiae*, *M. japonicus*, *C. gigas*, *M. galloprovincialis* and *C. elegans*, and from human, mouse, chicken and zebrafish were chosen for phylogenetic analysis with the Yesso scallop *PyTollip* gene. The amino acid sequences of Tollips from these species were retrieved from the NCBI, Ensembl Genome Browser, OysterBase and Echinobase databases. Multiple Tollip protein sequences were aligned using the ClustalW2 program (Larkin et al., 2007). A phylogenetic tree was constructed using MEGA 6 (Tamura et al., 2011) with the neighbor-joining method (Saitou and Nei, 1987). Bootstrapping with 1000 replications was conducted to evaluate the phylogenetic tree.

Scallop collection and bacterial challenge All the procedures involved in handling and treatment of scallops during this study were approved by the Ocean University of China Institutional Animal Care and Use Committee prior to the initiation of the study. A total of 600 two-year-old *P. yessoensis* were obtained in January 2014 from the Dalian Zhangzidao Fishery Group Corporation (Liaoning Province, China). After collection, scallops were used for *M. luteus* and *V. anguillarum* challenge experiments as described by Cong et al. (2013). Briefly, *M. luteus* and *V. anguillarum* were cultured (Tryptone 5 g l⁻¹, yeast extract 1 g l⁻¹, C₆H₅Fe·5H₂O 0.1 g l⁻¹, pH 7.6) at 28 °C to OD₆₀₀ = 0.2 and centrifuged at 2,000 g for 5 min to harvest the bacteria. Scallops were randomly divided into three groups with 40 scallops per group. One group served as the control, and the other

two treatment groups underwent immersion infection with *M. luteus* and *V. anguillarum* at concentrations of 2×10^7 and 1×10^7 CFU ml⁻¹, respectively, in seawater. At each time point of 0 h (scallop were sampled immediately after the immersion infection), 3 h, 12 h and 24 h post infection, 10 individuals were collected from each group. Hemolymph samples were collected from all the individuals, flash-frozen in liquid nitrogen and stored at -80 °C prior to RNA extraction.

To obtain samples representing different developmental stages, spawning, fertilization and larval culture were conducted following previously described protocols (Wang and Wang, 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 12–13 °C until they developed into juvenile mollusks. Samples including eggs, zygotes, multiple cells, blastulae, gastrulae, trochophore larvae, D-shaped larvae, umbo-larvae, eyespot larvae and juvenile mollusks were collected, preserved in RNAlater (Sigma–Aldrich, MO, USA) and stored at -80 °C until use.

RNA extraction, cloning of full-length *PyTollip* and quantitative real-time PCR analysis

Total RNA was isolated following the method described by Hu et al. (2006), and then digested with DNase I (TaKaRa, Shiga, Japan). RNA concentration and purity were determined using a Nanovue Plus spectrophotometer (GE Healthcare, NJ, USA). RNA integrity was determined by agarose gel electrophoresis. First-strand cDNA was synthesized using moloney murine leukemia virus reverse transcriptase (Thermo, CA, USA) following the manufacturer's protocol. All of the cDNA products were then diluted to 250 ng/μl for gene cloning and real-time PCR. According to the sequences deduced from alignment between our sequencing results and homologous *Tollip* sequences in NCBI, the gene-specific primers 5'-CCACACTGGAACAAAGAGGTCAGT-3' and 5'-TGTATCTCCACCCAGCACTCCC-3' were used for 5' and 3' RACE, respectively, of *PyTollip*. 5'- and 3'-RACE cDNA libraries were generated using a SMARTer RACE 5'/3' Kit (Clontech, CA, USA), following the manufacturer's user manual. The products were ligated with pMD18-T vector and transferred into *Escherichia coli* DH5α, and then sent to Sangon Biotech (Shanghai, China) for sequencing. Real-time PCR was conducted using the SsoFast EvaGreen Supermix on a LightCycler 480 Real-time PCR System (Roche Diagnostics, Mannheim, Germany). The running program was: 50 °C for 2 min and 94 °C for 10 min, followed by 40 cycles of 94 °C for 15 s and 62 °C for 1 min. Primers of Cytochrome B, DEAD-box RNA helicase and β-actin were separately designed as internal reference genes for normalization of gene expression in embryos, healthy adults and test sub-

jects during real-time PCR experiments (Feng et al., 2013). The specificity of primers was assessed by alignment with the *P. yessoensis* draft genome assembly (unpublished data) using BLASTN with a threshold E-value of $1E^{-10}$. Melting curve analysis was also performed to verify that each primer set amplified a single product. All the primers used in real-time PCR are listed in Table 1. Real-time PCR data were analyzed using the Relative Expression Software Tool (REST) version 2009 (Pfaffl et al., 2002). In the analysis of gene expression, healthy tissue and the developmental stage having the lowest Ct value were generally selected as the control group in REST, and the relative expression levels of *PyTollip* were then calculated based on the control group. Statistical analysis of the data was performed with SPSS (version 16.0) software using one-way analysis of variance followed by Fisher's Least Significant Difference tests.

RESULTS

Gene identification and sequence characterization of *PyTollip*

A single *Tollip* gene was identified in *P. yessoensis* and was named *PyTollip*, following the nomenclature of mouse (Burns et al., 2000). The genomic DNA, cDNA and predicted amino acid sequences of *PyTollip* were submitted to GenBank with the accession number KP115208. As the schematic diagram (Fig. 1) shows, the *PyTollip* gene is composed of seven exons, separated by six introns. The full-length cDNA of *PyTollip* is 3,815 bp with a 73-bp 5'-UTR, a 2,875-bp 3'-UTR, and an 867-bp ORF encoding 288 amino acids. The secondary structure of *PyTollip* protein predicted by Geneious 7.0.6 is shown in Fig. 2, and indicates that this protein consists of 11 alpha helices, 20 beta strands, 30 coils and 27 turns. The predicted molecular weight of *PyTollip* is 32,007.30 Da, with an isoelectric point (pI) of 4.93.

According to previous research (Li et al., 2004; Azurmendi et al., 2010; Ankem et al., 2011) and the results given by SMART and Phyre², two unambiguous functional domains with multiple conserved sites and motifs (Fig. 3) were recognized in the amino acid sequence

Table 1. List of primers used in real-time PCR

Primer	Primer sequence (5'-3')
<i>PyTollip</i> -F	CCACACTGGAACAAAGAGGTCAGT
<i>PyTollip</i> -R	TGTATCTCCACCCAGCACTCCC
Cytochrome B-F	CCTCTCCACCCCTTCTAGTCCTTG
Cytochrome B-R	CTCCTGGTCTTCTCGTCTTTCTCC
DEAD-box RNA helicase-F	CCAGGAGCAGAGGGAGTTCCG
DEAD-box RNA helicase-R	GTCTTACCAGCCCGTCCAGTTC
β-actin-F	CCAAAGCCAACAGGGAAAAG
β-actin-R	TAGATGGGGACGGTGTGAGTG

Fig. 1. Structural features of *PyTollip* sequences. Top and middle: light gray boxes represent the 3' UTR and 5' UTR, and dark gray boxes represent exons. Bottom: black boxes represent amino acid sequences lacking predicted functional domains, and light gray boxes represent the successfully predicted C2 and CUE domains.

Fig. 2. Secondary structure of *PyTollip* predicted by Geneious 7.0.6. Pink cylinders represent alpha helices; yellow bars represent beta strands; wavy gray lines represent coils and blue curved arrows/bars represent turns.

of the *PyTollip* protein. From our analysis, the conserved C2 and CUE domains of *PyTollip* were located in the 65–162 and 244–286 residue regions, with many conserved amino acid residues. Moreover, from the multiple sequence alignment analysis, *PyTollip* showed 50% and 49% amino acid identity to human and mouse *Tollip*, respectively, and 64% identity to that of *M. galloprovincialis*.

Phylogenetic analysis of Tollip polypeptides To confirm the sequence identities of the *PyTollip* gene in *P. yessoensis*, a neighbor-joining phylogenetic tree was constructed using the full-length amino acid sequences of Tollip from various species. Tollips from bivalve species were grouped closest together, and the generated clades formed into progressively larger clusters. Such relationships were also consistent with the phylogeny of these invertebrates. For instance, *P. yessoensis* is phylogenetically closer to other mollusks than to echinoderms and arthropods; so is its *Tollip* gene (Fig. 4). Based on all the sequence information that has been acquired so far, *PyTollip* showed the highest identity to Tollips from other bivalves, i.e., *C. gigas* and *M. galloprovincialis*; and the Tollip of *A. japonicus* exhibited the closest relationship

with those of the bivalves compared with others.

Spatiotemporal expression analysis of *PyTollip*

The expression of *PyTollip* was first analyzed in the ten developmental stages of Yesso scallop by quantitative RT-PCR. As shown in Fig. 5A, the expression levels of *PyTollip* displayed a roughly 'W'-shaped trend, possessing two valleys and one conspicuous peak. In the first three stages, *PyTollip* demonstrated moderate expression in the incipient egg phase, and this gradually declined to the minimum level during the multiple cells phase. Expression then started to rise stage by stage from the embryo and reached the maximum level (10.3-fold higher than expression in the multiple cells phase) in the period of D-shaped larvae, corresponding to the conspicuous peak mentioned above. Thereafter, the expression level dramatically dropped in the next two stages, umbo-larvae and eyespots larvae; however, *PyTollip* expression rose again in the juvenile mollusk.

The expression profile of *PyTollip* was then studied in ten tissues of healthy adult scallops: mantle, gill, gonad, kidney, hepatopancreas, smooth muscle, striated muscle, foot, eye and hemolymph. As Fig. 5B shows, *PyTollip*

Fig. 3. Multiple sequence alignment of Tollips from *Patinopecten yessoensis*, *Homo sapiens* (ENST00000317204), *Mus musculus* (ENSMUST00000001950), *Gallus gallus* (ENSGALT00000010823), *Xenopus laevis* (Q3B8H2.1), *Danio rerio* (ENSART000000126917), *Apostichopus japonicus* (AHA83602.1), *Strongylocentrotus purpuratus* (SPU_021673.3a or SPU_026252.3a), *Anopheles gambiae* (AGAP003615-PA), *Marsupenaeus japonicus* (BAK19511.1), *Litopenaeus vannamei* (AET79206.1), *Crassostrea gigas* (EKC34473.1), *Mytilus galloprovincialis* (AH117285.1) and *Caenorhabditis elegans* (F25H2.1) downloaded from Ensembl and NCBI. Conserved amino acid residues are shaded in black. The gray-shaded regions represent similar amino acid residues. Gaps introduced to improve the alignment are represented by dashes. The three regions marked with vertical lines and arrows represent the deduced TBD, C2 and CUE domains in vertebrates. Sequence conservation and functional motifs of the two domains are also marked. Red arrows indicate the conserved basic residues responsible for PtdIns3P and PtdIns(4,5)P₂ recognition and binding in the C2 domain. The red boxes denote (i) the distinct asparagine residue at position 161 of PyTollip in the C2 domain, and (ii) the conserved ubiquitin binding motifs found in the CUE domain.

Fig. 4. Molecular phylogenetic tree of Tollips constructed by the neighbor-joining method. The black diamond indicates PyTollip from *P. yessoensis*. Bootstrapping values are indicated by numbers at the nodes and the scale represents the amino acid substitution rate.

Fig. 5. Relative expression levels of PyTollip in *P. yessoensis* among different developmental stages (A) and healthy adult tissues (B). The healthy tissue and developmental stage samples having the lowest Ct values were selected as the controls. Error bars represent the mean ± S.E. (N = 3). Significant differences (P < 0.05) exist between any two samples labeled with different single letters.

Fig. 6. Expression levels of PyTollip in hemolymph after *V. anguillarum* (Gram-negative) or *M. luteus* (Gram-positive) challenge. Vertical bars represent the mean ± S.E. (N = 10). * and **, significant difference from control groups (P < 0.05 and P < 0.01, respectively). Separate controls were used for each time point and the expression values in each group were compared to their own control.

was expressed in all tissues examined, most prominently in kidney, in which expression was 11.6-fold higher than that in gonad, followed by muscles (smooth and striated), gill, hemolymph, mantle, foot, eye and hepatopancreas. The lowest expression level was observed in gonad.

Temporal expression of PyTollip after bacterial challenge To further explore the expression pattern of PyTollip during the immune response, Yesso scallop was challenged with *M. luteus* and *V. anguillarum*. As shown in Fig. 6, significantly differential expression of PyTollip was observed in challenges with *M. luteus* and *V. anguillarum* in hemolymph, reflecting its important role during innate immune responses in scallop (Costa et al., 2009). After infection with *M. luteus*, the expression of PyTollip increased rapidly and reached its highest level of 9.4-fold that of the control at 3 h, and then declined gradually after this acute increase. After infection with *V. anguillarum*, PyTollip expression also peaked at 3 h (15.5-fold) and then sharply decreased at 12 h (1.8-fold); it displayed another notable rise at 24 h, to a level comparable to that at 3 h, which did not occur when the scallops were challenged with *M. luteus*. Compared to the Gram-positive bacterial infection, PyTollip demonstrated a higher expression and response level against invasion by the Gram-negative bacterium *V. anguillarum*.

DISCUSSION

As important components of innate immune systems, TLR signaling pathways and the IL-1 signaling cascade have naturally become hotspots in immunology. However, as research progresses, more and more evidence suggests that host defense responses through activation of the innate and adaptive immune systems must be

under tight control by their regulators (Wang et al., 2009) to avoid excessive immune activation that would otherwise lead to pathogenesis of infectious, chronic inflammatory and autoimmune diseases (Melmed et al., 2003; Cook et al., 2004; Shah et al., 2012). As one of the most crucial regulators and mediators in the TLR/IL-1R signal transduction pathways, Tollip has been confirmed to be able to control the extent of inflammatory cytokine production in response to IL-1 β and lipopolysaccharide, as well as to inhibit IL-1R- and TLR-mediated immune responses by directly binding to IRAK-1 and preventing its autophosphorylation without promoting its degradation (Burns et al., 2000; Zhang and Ghosh, 2002). Systematic studies of *Tollip* in bivalve species are essential to establish a full comprehension of immune regulation and to elaborate the evolution of such a multi-pronged, integrated system against pathogens. In the present study, we scanned the genome and transcriptome of *P. yessoensis* and successfully identified a single *PyTollip* gene, conducted phylogenetic analysis, and determined the expression of the *PyTollip* gene in different developmental stages, and in healthy adult tissues as well as in individuals encountering bacterial infections, to provide insight into the sequence identities, orthology, expression and involvement of *PyTollip* in early development and immune responses in scallop.

Tollip is a modular molecule which, in vertebrates, consists of three conserved and annotated domains with corresponding functions: a TBD at the N terminus, a C2 domain in the central region and a CUE domain at the C terminus. The TBD is responsible for mediating protein-protein interactions (Yamakami et al., 2003); the C2 and CUE domains are involved in positioning Tollip into the endosome (Martin, 1998) and binding with monoubiquitin, respectively (Katoh et al., 2004; Oguro et al., 2011). Based on previous research and sequence information, multiple sequence alignments were carried out to deduce the structure and functions of *PyTollip* protein (Fig. 3). In this study, the conserved C2 and CUE domains, including several functional residues, were successfully recognized in *PyTollip*. Sequence analysis showed that both C2 and CUE domains of *PyTollip* display highly conserved structural characteristics, being most similar to the C2 and CUE domains of oyster Tollip protein with 78% and 79% identity, respectively. Even the lowest similarities were still 46% and 45% with the respective domains of *C. elegans* Tollip. Amino acid residue 150 of vertebrate Tollip, which is lysine, glutamic acid or aspartic acid in most available species, was reported as a key factor that realized the inhibitory role of this gene in TLR-mediated innate immune responses of mammals (Li et al., 2004). However, from the alignment constructed in this study, asparagine was surprisingly found in this position of *PyTollip* (Fig. 3). This result suggests that a distinct role or function is conferred by

this residue in *PyTollip*, since lysine is positively charged and glutamic acid is negatively charged, whereas asparagine is neutral. Although a Tollip-mediated negative loop of TLR signaling pathways has been suggested to be conserved in non-mammalian organisms (Reynolds and Rolff, 2008; Rebl et al., 2011), further studies and analyses are needed to test this proposal in invertebrates. In addition to the C2 domain, two highly conserved motifs of the CUE domain (the Met-Phe-Pro and Leu-Leu motifs), which are considered to be required for ubiquitin binding (Kang et al., 2003; Prag et al., 2003; Shih et al., 2003), were both identified in *PyTollip* protein. It is noteworthy that a TBD was not predicted in *PyTollip*, which is consistent with *L. vannamei* and *A. japonicus* (Lu et al., 2013; Wang et al., 2013), perhaps due to the highly variable sequence composition of this region in invertebrates.

Phylogenetic analysis revealed that *PyTollip* from *P. yessoensis* was most closely related to its orthologs in bivalves and to a lesser extent with those in chordate and echinoderm species. This result postulated that the molecular evolution of Tollips was consistent with species taxonomy and confirmed the identification of *PyTollip*. Further research and more detailed information about Tollip structure and function will be required to know more precisely when and how the *Tollip* genes evolved. Our findings supplement the evolutionary analysis of Tollips (Luiz et al., 2014) with additional information.

Spatiotemporal expression patterns of *PyTollip* measured by real-time PCR demonstrated abundant expression of this gene in different developmental stages and adult tissues except for a few cases, which suggested that multitasking functions of this gene contribute to many biochemical pathways. During embryo and larval development, *PyTollip* mRNA appeared to be moderately abundant in the original egg, representing maternal expression. Subsequently, *PyTollip* expression decreased until the multiple cells phase, implying that the embryo relies on maternal *PyTollip* mRNA at the beginning of embryonic development and does not undertake new expression until the blastula stage (Zhang et al., 2013b; Garbarino et al., 2014; Škugor et al., 2014). The following stepwise increase of *PyTollip* expression is relevant to its involvement in protein trafficking and metabolic processes, which are physiologically required during the growth of scallops from blastula to the D-shaped larvae stage (Brissoni et al., 2006; Ciarrocchi et al., 2009; Capelluto, 2012; Zhu et al., 2012). In addition, the expression level of *PyTollip* decreased significantly after peaking in the D-shaped larvae stage, and then increased in the juvenile stage, which was also observed in sea urchin (Arenas-Mena et al., 1998), implying the depletion and recovery of Tollip during embryonic development. In healthy adult tissues, *PyTollip* was strongly expressed in kidney and gill, which was not unexpected because these two tissues are usually targeted as the entry points dur-

ing bacterial infection, coinciding with previous research (Abreu et al., 2001; Rautava et al., 2005). Relatively high expression of *PyTollip* was also displayed in smooth and striated muscle of Yesso scallop, concordant with results in *A. japonicus* and *L. vannamei* (Lu et al., 2013; Wang et al., 2013), implying the potential involvement of invertebrate Tollip in metabolic processes.

In a preliminary exploration of the role of *PyTollip* in scallop innate immune defense, Gram-positive (*M. luteus*) and Gram-negative (*V. anguillarum*) bacterial challenges were performed. Previous studies have shown that hemolymph is one of the major immune components and the location where the recognition and elimination of bacterial pathogens occurs in mollusks (Wootton et al., 2003; Bachère et al., 2004), so the hemolymph was chosen here as the test component.

The expression patterns of *PyTollip* confirmed its induction in the acute phase after infection, the same time point as the inhibition of excessive activation of TLR/IL-1R signaling pathways during the innate immune response in human, sheep, Pacific oyster and Mediterranean mussel (Li et al., 2004; Herman et al., 2013; Toubiana et al., 2013; Zhang et al., 2013a). This highly consistent pattern between *PyTollip* expression and TLR/IL-1R signaling pathways confirmed the important regulatory role of the *Tollip* gene in the innate immune response of scallop. Not coincidentally, our observation that the expression level of *PyTollip* after infection with *V. anguillarum* was higher than that with *M. luteus* is relevant to the different response level of shellfish TLR signaling pathways in combating Gram-positive and Gram-negative bacteria. For instance, in Pacific oyster, infection with Gram-negative bacteria such as *Listeria monocytogenes* and *V. parahaemolyticus* elicited significantly higher expression of multiple *CgTLRs* and *CgMyD88* than did infection with all tested Gram-positive bacteria (Zhang et al., 2013a). A similar pattern was also observed in Mediterranean mussel (Toubiana et al., 2013). Interestingly, a second strong response of *PyTollip* at 24 h post *V. anguillarum* challenge was observed, which has not been reported in previous *Tollip* studies. This specific expression pattern of *Tollip* in Yesso scallop may also imply novel features and functions of *PyTollip* during the innate immune response against the invasion of Gram-negative bacteria.

In conclusion, we identified a *Tollip* gene in *P. yessoensis*, named *PyTollip*, and successfully cloned its full-length cDNA. Two functional domains and several conserved sites of biological significance in the *PyTollip* protein sequence were detected in bioinformatics analyses. Moreover, expression profiles of this gene were analyzed in healthy tissues, developmental stages and hemolymph after bacterial infection, affirming the participation of *PyTollip* in TLR/IL-R immune signaling pathways. Although many questions remain, especially regarding

features that are unique to *PyTollip*, our work provides insights into the innate immune mechanisms of scallops, paving the way for subsequent in-depth exploration of the negative control system of *Tollip* in marine invertebrates.

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