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

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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–κB (NF–κB) 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–κB 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
(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 TRAF3s in response to bacterial challenge 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/orffinder.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 pl/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), zebrasfish (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 12–13 °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, eyespots larvae and juvenile mollusks of P. yessoensis were collected 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, C₂H₄Fe·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. The pellet was suspended in filtered seawater and adjusted to 2 × 10⁷ CFU ml⁻¹ (M. luteus) or 1 × 10⁷ 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 × 10⁷ and 1 × 10⁷ 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 and DNA contamination was removed by DNase I (Takara, Dalian, China). After extraction, the RNA was treated with RNALater (Sigma-Aldrich, St. Louis, MO, USA) and stored at −80 °C.

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-GCTAACCCCTTTGTTAAAATC-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 DH5α, 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 μl containing 1 × Real-time PCR master mix with SYBR Green dye (Toyobo, Osaka, Japan), 0.2 μM each primer and 2 μl 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 (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 ± 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, 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).

**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    | ATAGTAGTGAACGAGATGGGTAGTAA       | 86.16%                        |
| PyTRAF3IP1-R    | GAATGATACGATGATGGCTGGAT          | 83.60%                        |
| Cytochrome B-F  | CCTCTCCACCCCTTCTAGTCTTG          | 83.60%                        |
| Cytochrome B-R  | CTCTGTTCTCCGTCTTTCTCC            | 83.60%                        |
| DEAD-box RNA helicase-F | CCAGGAGCAGAGGGAGTTCG         | 86.40%                        |
| DEAD-box RNA helicase-R   | GCTTTACAGCCCCGTCCAGTTC         | 86.40%                        |
| β-actin-F       | CCAAACCCACGAGGAAAAG             | 83.99%                        |
| β-actin-R       | TAGATGGGGACGATGTGAGT           | 83.99%                        |
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 TRAF3IP1s.

**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-κB, C/EBP (CCAAT/enhancer binding protein) and AP1 (activator protein 1) are important TFs in diverse cel-
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.

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.
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, significantly 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.
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 TRAFs 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 TRAFs 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 overexpression inhibited CD40-mediated NF-kB 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-kB 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 TRAFs or TRAF3-interacting proteins by pre-occupying the same binding domains in TRAFs 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

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<td>0 -- -- --</td>
<td>C. gigas</td>
<td>Huang et al., 2014</td>
</tr>
<tr>
<td>TRAF2</td>
<td>++ 0 0 --</td>
<td>++ ++ 0 ++</td>
<td>P. yessoensis</td>
<td>Wang et al., 2015</td>
</tr>
<tr>
<td>TRAF4</td>
<td>0 ++ 0 0 0</td>
<td>0 ++ ++ ++</td>
<td>P. yessoensis</td>
<td>Wang et al., 2015</td>
</tr>
<tr>
<td>TRAF6</td>
<td>0 ++ ++ 0</td>
<td>++ ++ ++</td>
<td>M. galloprovincialis</td>
<td>Toubiana et al., 2014</td>
</tr>
<tr>
<td>TRAF6</td>
<td>0 ++ ++ 0</td>
<td>++ ++ ++</td>
<td>P. yessoensis</td>
<td>Wang et al., 2015</td>
</tr>
<tr>
<td>TRAF7</td>
<td>++ 0 0 0</td>
<td>++ ++ 0 ++</td>
<td>P. yessoensis</td>
<td>Wang et al., 2015</td>
</tr>
</tbody>
</table>

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

In summary, in this study we scanned the genome and transcriptome of *Py. 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 confirmed 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 immune systems.

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REFERENCES


