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Identification and characterization of TEP family genes in Yesso scallop (*Patinopecten yessoensis*) and their diverse expression patterns in response to bacterial infection

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ABSTRACT

Thioester-containing protein (TEP) family members are characterized by their unique intrachain β-cysteinyl-γ-glutamyl thioesters, and they play important roles in innate immune responses. Although significant effects of TEP members on immunity have been reported in most vertebrates, as well as certain invertebrates, the complete TEP family has not been systematically characterized in scallops. In this study, five TEP family genes (*PyC3*, *PyA2M*, *PyTEP1*, *PyTEP2* and *PyCD109*) were identified from Yesso scallop (*Patinopecten yessoensis*) through whole-genome scanning, including one pair of tandem duplications located on the same scaffold. Phylogenetic and protein structural analyses were performed to determine the identities and evolutionary relationships of the five genes (*PyTEPs*). The vast distribution of *PyTEPs* in TEP subfamilies confirmed that the Yesso scallop contains relatively comprehensive types of TEP members in evolution. The expression profiles of *PyTEPs* were determined in hemocytes after bacterial infection with gram-positive (*Micrococcus luteus*) and gram-negative (*Vibrio anguillarum*) using quantitative real-time PCR (qRT-PCR). Expression analysis revealed that the *PyTEP* genes exhibited disparate expression patterns in response to the infection by gram bacteria. A majority of *PyTEP* genes were overexpressed after bacterial stimulation at most time points, especially the notable elevation displayed by duplicated genes after *V. anguillarum* challenge. Interestingly, at different infection times, *PyTEP1* and *PyTEP2* shared analogous expression patterns, as did *PyC3* and *PyCD109*. Taken together, these results help to characterize gene duplication and the evolutionary origin of *PyTEPs* and supplied valuable resources for elucidating their versatile roles in bivalve innate immune responses to bacterial pathogen challenges.

1. Introduction

The complement system is one of the major defense effector arms in immune responses in both vertebrates [1] and invertebrates [2], and it has therefore evolved into a highly complex system, comprising approximately 30 soluble components, along with cell surface inhibitors that limit damage to self and receptors on a range of cell types with which the system interacts [1]. As an essential bridge between innate and adaptive immunity, the complement system can be activated through four separate pathways, referred to as the classical, alternative, lectin [3] and coagulation [4] pathways. Among the above pathways, the central component of the complement system is the C3 protein, which is activated in higher vertebrates by C3 convertase [5]. C3 and its homologous complement proteins C4 and C5, along with the universal protease inhibitor α2-macroglobulin (A2M), the thioester-containing proteins (TEPs), and the cell surface antigen CD109s, belong to the so-called thioester-containing protein family [6–10]. All of these proteins are relatively large (1400–1800 amino acids) and have several marked functional features, such as a reactive thioester moiety enabling covalent attachment to target particles, a highly variable central segment likely involved in recognition, and the propensity to undergo conformational changes yielding distinct protein-binding interactions [11–14]. In the innate immune system, the invertebrate C3(-like) molecules and insect TEPs are thought to be involved in defense as pattern recognition receptors (PRRs), facilitating the recognition of pathogens and the subsequent activation of various immune modules, notably

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phagocytosis or the Toll pathway [15,16].

TEPs appeared early in animal evolution, and the family member C3 was first isolated in 1969 in human serum and named C3a, demonstrating smooth muscle contracting activity [17]. Since that study, a large number of members of this family have been identified in such diverse organisms as nematodes, insects, mollusks, fish, birds and mammals [18]. In vertebrates, the thioester-containing complement proteins, C3 and C4, act as opsonins of microorganisms or immune complexes for clearance by phagocytes bearing complement-receptors (CRs) [19,20]. For example, seven members of this family are encoded in the human genome: C3, C4, C5, A2M, pregnancy zone protein (PZP), CD109 and PZP-like A2M domain-containing 8 (CPAMD8) [21].

However, in invertebrates, the complement system is far from clear due to limited evidence. To date, C3-like molecules were found in the sea squirt coral *Swiftia exserta* [22], the nematode *Caenorhabditis elegans* [23], the clam *Ruditapes decussates* [24], the squid *Euprymna scolopes* [25], the crab *Carcinoscorpius rotundicauda* [26], and *Ciona intestinalis* [27], indicating that the origin of the complement system was extremely ancient. In addition, the number of TEP family genes in most invertebrates is varied, with six and nineteen TEP homologs being identified in *Drosophila melanogaster* and *Anopheles gamiae* [6], respectively, three in *C. intestinalis* [27], and only one in the *C. elegans* [23] genome. This dramatic difference in gene copy number and universality of species makes TEP family genes a potential ideal model for exploring innate immune divergence in invertebrates. Moreover, to our knowledge, the information about thioester-containing proteins in mollusks, one of the most important triploblastic protostomes in the lower lineage, remains deficient. Only three TEP gene members in mollusks have been identified from *Chlamys farreri* [10,28,29] and *R. decussates* [24] via molecular clone, namely *CfTEP*, *CfA2M* and *Rd-C3*, respectively. Recently, RNA-seq analysis identified eight thioester-containing proteins (CgTEPs) functioning in the *Crassostrea gigas* complement system [9]. These proteins all contained the canonical thioester motif GCGEQ, proteolytic cleavage sites and catalytic histidine residues.

The complement system is a highly sophisticated and powerful body defense mechanism acting in innate immunity [3]. Considering their importance in innate immunity, a study of the number, assortment and the roles of TEP members in immune recognition and signal transduction have been broadly conducted from vertebrates to invertebrates [2,3,5,6,12,15,26]. Nevertheless, to date, systematic analysis of the TEP family genes has not been performed in scallops, especially in Yesso scallop (*Patinopecten yessoensis*), which is a major economic aquaculture species cultured in Asian countries and consumed worldwide [30]. Yesso scallops are cultured in an open seawater environment that is threatened by numerous types of potential pathogens. It has been reported that bacterial disease pandemics can lead to a dire mortality rate in scallops [31], resulting in the scallop aquaculture industry experiencing great economic losses [32]. To facilitate the healthy development of the scallop industry, many potentially important scallop disease resistance genes have been identified and functionally characterized [33–36], but little is known concerning TEP family members in scallops. In the present study, TEP family genes of Yesso scallop were scanned and systematically characterized, and after being challenged by *Micrococcus luteus* and *Vibrio anguillarum* infection, their expression patterns in hemocytes were analyzed using quantitative real-time PCR. Simultaneously, molecular evolution of the PyTEPs were also analyzed. This work may help to elucidate the mechanisms of the complex scallop innate immune regulation system and help in developing strategies for long-term healthy sustainable Yesso scallop culture.

2. Materials and methods

2.1. Database mining, gene identification and sequence analysis

The transcriptome [37] and whole-genome sequence databases of Yesso scallop [38] were searched to identify TEP family genes using all available TEP protein sequences of invertebrates and vertebrates, including *Homo sapiens*, *Mus musculus*, *Danio rerio*, *Xenopus laevi*, *D. melanogaster*, *C. gigas*, *C. farreri*, *Lottia gigantean*, and *C. elegans* from NCBI (http://www.ncbi.nlm.nih.gov), Ensembl (http://useast.ensembl.org) and OysterBase (http://www.oysterdb.com/) databases. TBLASTN was used to obtain the initial pool of TEP family genes transcriptome sequences in Yesso scallop, and BLASTN was later used to verify the cDNA sequences by comparing the transcriptome sequences to the whole-genome sequences. Whole genome references and annotation information were utilized to eliminate the interference of the isoforms. The isoforms identified from Yesso scallop transcriptome were mapped to the whole genome and then the redundant isoforms that mapped to the same position with the same BLAST results and confirmed certain genome annotation were removed, and only the complete and unique transcripts were kept for the downstream analyses. Open reading frame (ORF) finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) and DNAstar (version 4.05) were used to predict amino acid sequences (Data S1). To confirm the predicted amino acid sequences, BLASTP was conducted 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 (PI) points and molecular weights were computed using the Compute pl/Mw tool (http://web.expasy.org/compute_pi/). Geneious 7.1.7 (http://www.geneious.com/) was used to predict secondary structure.

2.2. Phylogenetic analysis

TEP protein sequences were aligned using the ClustalW2 program [39] based on the amino acid sequences of the Yesso scallop TEP proteins and orthologs from other vertebrates and invertebrates, including *H. sapiens*, *M. musculus*, *Gallus gallus*, *X. laevis*, *D. rerio*, *D. melanogaster*, *Aedes aegypti*, *Strongylocentrotu spurpuratus*, *C. intestinalis*, *C. elegans*, *C. gigas*, *C. farreri* and *Branchiostoma glabrata* (Table S1). The deduced amino acid sequences of the TEP family genes from these species were retrieved from the NCBI, Ensembl Genome Browser and UniProt databases. Phylogenetic trees were then constructed using MEGA 6.06 with the neighbor-joining method [40]. Bootstrapping with 5000 replications was performed to evaluate the trees.

2.3. Sample collection and bacterial challenge experiment

Healthy Yesso scallops at the age of two years were collected from natural populations at Dalian Zhangzidao Fishery Group Co. (Liaoning Province, China) in January 2014. The animals were acclimated in the laboratory for one week prior to the experiments. Filtered and aerated seawater was maintained at approximately 8 °C, which is within the optimum temperature range for their growth.

For the bacterial challenge experiment, gram-positive (*M. luteus*) and gram-negative (*V. anguillarum*) bacteria were used to challenge scallops in our study [41,42]. *M. luteus* and *V. anguillarum* were cultured in liquid 2216 E broth (tryptone 5 g/L, yeast extract 1 g/L, $C_6H_5Fe{\cdot}5H_2O$ 0.1 g/L, pH = 7.6) at 28 °C to an OD_{600} of 0.2 and were harvested by centrifugation at 2000 × g for 5 min, as described by Cong et al. [43]. Next, the cell pellets were suspended in filtered seawater and adjusted to 2×10^7 and 1×10^7 CFU/mL, respectively. A total of 200 scallops were randomly divided into three groups (groups C, P and N). Group P and group N were used to evaluate the immune response of the scallops after bacterial stimulation. The individuals were immersed in *M. luteus* (G^+) and *V. anguillarum* (G^-) containing seawater, which was adjusted to 2×10^7 and 1×10^7 CFU/mL, respectively, and 3 scallops from each group were sampled at 3 h, 6 h, 12 h, and 24 h post-challenge [44–46]. Group C was used as the control group and kept in filtered and aerated seawater at 8 °C.