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Alternative splicing, spatiotemporal expression of TEP family genes in Yesso scallop (*Patinopecten yessoensis*) and their disparity in responses to ocean acidification

Qiang Xing[a,b], Jing Wang[a,c], Qiang Zhao[a,d], Huan Liao[a], Xiaogang Xun[a,b], Zujing Yang[a], Xiaoting Huang[a,b,*], Zhenmin Bao[a,b]

[a] MOE Key Laboratory of Marine Genetics and Breeding, College of Marine Life Sciences, Ocean University of China, 5 Yushan Road, Qingdao, 266003, China
[b] Laboratory for Marine Fisheries Science and Food Production Processes, Qingdao National Laboratory for Marine Science and Technology, Qingdao, 266237, China
[c] Laboratory for Marine Biology and Biotechnology, Qingdao National Laboratory for Marine Science and Technology, Qingdao, 266237, China
[d] Yantai Marine Economic Research Institute, Yantai, 264000, China

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ABSTRACT

The complement system constitutes a highly sophisticated and powerful body defense machinery acting in the innate immunity of both vertebrates and invertebrates. As central components of the complement system, significant effects of thioester-containing protein (TEP) family members on immunity have been reported in most vertebrates and in some invertebrates, but the spatiotemporal expression and regulatory patterns of TEP family genes under environmental stress have been less widely investigated in scallops. In this study, expression profiling of TEP family members in the Yesso scallop *Patinopecten yessoensis* (designated *PyTEPs*) was performed at all developmental stages, in different healthy adult tissues, and in mantles during exposure to different levels of acidification (pH = 6.5 and 7.5) for different time points (3, 6, 12 and 24 h); this profiling was accomplished through *in silico* analysis of transcriptome and genome databases. Spatiotemporal expression patterns revealed that *PyTEPs* had specific functional differentiation in all stages of growth and development of the scallop. Expression analysis confirmed the inducible expression patterns of *PyTEPs* during exposure to acidification. Gene duplication and alternative splicing events simultaneously occurred in *PyTEP1*. Seven different cDNA variants of *PyTEP1* (designated PyTEP1-A–PyTEP1-G) were identified in the scallop mantle transcriptome during acidic stress. These variants were produced by the alternative splicing of seven differentially transcribed exons (exons 18–24), which encode the highly variable central region. The responses to immune stress may have arisen through the gene duplication and alternative splicing of *PyTEP1*. The sequence diversity of *PyTEP1* isoforms and their different expression profiles in response to ocean acidification (OA) suggested a mechanism used by scallops to differentiate and regulate *PyTEP1* gene expression. Collectively, these results demonstrate the gene duplication and alternative splicing of TEP family genes and provide valuable resources for elucidating their versatile roles in bivalve innate immune responses to OA challenge.

1. Introduction

The complement system is a major defense effector component of the immune response in both vertebrates [1] and invertebrates [2] and is composed of more than 30 soluble plasma and cell surface proteins [3] that function as part of a highly sophisticated and powerful body defense mechanism [4]. As important constituents of innate immunity, the thioester-containing protein family members involved in the complement system were thought to be involved in defense as pattern recognition receptors (PRRs), performing key regulatory functions in immune recognition [5,6] and elimination of non-self particles [3]. This evolutionarily conserved family comprises (1) α2-macroglobulins (A2M), (2) C3/C4/C5 components of the complement factors, and (3) thioester-containing proteins (TEPs) [7]. Previous studies demonstrated that complement system members shared a common ancestor, but they separated from each other at a rather early lineage, playing important and widespread roles in the immune response [2,8]. Among these family members, the structural thioester first appeared in the broad-

* Corresponding author. MOE Key Laboratory of Marine Genetics and Breeding, College of Marine Life Sciences, Ocean University of China, 5 Yushan Road, Qingdao, 266003, China.
E-mail address: xthuang@ouc.edu.cn (X. Huang).

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spectrum endopeptidase inhibitors, A2M, which have to date been characterized in metazoans (vertebrates and invertebrates) and Gram-negative bacteria and are involved in the immobilization and entrapment of proteases [9–11]. A2M proteins, together with their receptors, have an opsonic function in the clearance of proteases and cytokines and in the delivery of antigens to antigen presenting cells [12]. Complement components C3, C4 and C5 are synthesized as single chains with an approximate molecular mass of 180 kDa, showing overall phylogenetic sequence similarity to A2M [13]. C3 arose from a gene duplication of A2M during evolution and was considered central to both the activation and function of complement. A second component, C4, is involved in the activation of C3 via the antibody and mannan binding lectin (MBL) pathways [14]. Another component, C5, sequentially activates complexes of C6–C9, which form the membrane attack complex (MAC) that inserts into lipid bilayers and lyses susceptible targets [9]. In addition, thioester-containing proteins (TEPs) appeared early in animal evolution; members of this subfamily have been identified in diverse organisms such as nematodes, mollusks, insects, fish, birds and mammals [15]. As relatively large proteins (1400–1800 amino acids), TEPs are characterized by the unique intrachain β-cysteinyl-γ-glutamyl thioester bond and a propensity for multiple conformationally sensitive binding interactions [7,12].

Considering the vital importance of the complement system in innate immunity, numerous investigations of complement members in immune recognition and signal transduction have been broadly conducted, from vertebrates to invertebrates [2,4,6,12,16–18]. The original member of complement system, C3a, was identified as a human serum protease inhibitor and acted as the central component of the complement system [19]. Since then, many genes in Eumetazoa have been shown to contain the thioester-encoding region and are defined as thioester-containing protein (TEP) family members. For example, in vertebrates, seven members of this family are encoded in the human genome: C3, C4, C5, A2M, pregnancy zone protein (PZP), cell surface antigen (CD109) and PZP-like A2M domain-containing 8 (CPAMD8) [20]. In addition, similar TEP family members have been identified and evolutionarily analyzed in species of *Mus musculus*, *Gallus gallus*, *Xenopus laevi*, etc. [21]. Moreover, the complement system members were investigated with their phylogeny, expression patterns and evolutionary analysis in the host defense of some invertebrate model organisms. For instance, in *Drosophila*, the TEP family is composed of six genes named *Tep1-Tep6*, which are clustered in three distinct branches and function in the innate response to bacterial and fungal infections [22]. Additionally, the widespread presence of TEP family members was identified in coral (*Swiftia exserta*) [23], clam (*Ruditapes decussates*) [24], squid (*Euprymna scolopes*) [25], crab (*Carcinoscorpius rotundicauda*) [18], sea squirt (*Ciona intestinalis*) [26] and nematode (*Caenorhabditis elegans*) [27], indicating that the origin of the complement system is extremely ancient. Molecular characterization and phylogeny analyses have only been conducted in a minority of mollusk species, including *Chlamys farreri* [28–30] and *R. decussates* [23], for *CfTEP*, *CfA2M* and *Rd-C3*. Additionally, eight thioester-containing genes, including a *C3/4/5-like* gene (designated *CgC3*), two *A2M-like* genes, two *CD109-like* genes and three *TEPs*, were identified in the Pacific oyster (*Crassostrea gigas*) genome [31].

Interest in bivalve immunity has continuously increased in recent years due to serious diseases and mortality problems threatening the healthy development of bivalve aquaculture [2]. Moreover, rising CO_2 concentrations observed in this century have made seawater more acidic and reduced calcium carbonate availability, which is critical for many marine calcifiers and likely to have transformative effects on many coastal marine organisms, such as scallops [32,33]. Research has demonstrated that ocean acidification (OA) influences the growth, reproduction and immune response of bivalves [33,34]. In particular, OA weakens the immune responses of bivalves and may constitute a potential threat by increasing the susceptibility of bivalves to diseases [35–37]. As a consequence, exposure to acidic environments could be a major stress to which scallops' innate immune responds in the ocean of tomorrow.

The Yesso scallop (*Patinopecten yessoensis* Jay, 1859) is a major economic shellfish cultured in Asian countries and consumed worldwide [38]. The scallop is cultured in an open seawater environment and has been proven to be affected by acidification stress, resulting in the decrease of thermal tolerance and even the death of scallops, potentially threatening the scallop aquaculture industry [34,38,39]. As ancient genes involved in the innate immune system, *PyTEPs* were particularly investigated in the Yesso scallop, including our previous study that focused on the identification, phylogeny, protein structure and expression profiles of the five *PyTEPs* in response to bacterial challenges [40]. However, the spatiotemporal expression patterns of TEP family members in Yesso scallop still remain unclear. Furthermore, seven different cDNA variants of *CfTEP* were cloned from the gonad of the Zhikong scallop *C. farreri* by RT-PCR technique, and the expression profiles of these genes were different, displaying sex and bacterial dependencies [28]. We wondered whether alternative splicing of *PyTEPs* occurred upon immune-elicited challenge in the Yesso scallop, which has a close phylogenetic relationship with the Zhikong scallop. As a consequence, *PyTEPs* provide a novel model for investigating the diversity of TEP family members in scallop and determining whether/how transcript isoforms function in the immune response [38]. In the present study, *PyTEPs* and different splicing forms of *PyTEP1* in the Yesso scallop were specifically analyzed with RNA-seq datasets; these studies examined the expression patterns of *PyTEPs* at different development stages and in healthy adult tissues and the expression profiles of *PyTEPs* and *PyTEP1* in mantles after being challenged with different levels of acidification (pH = 6.5 and 7.5). In brief, further analyses of TEP family genes aimed (1) to identify different transcripts of *PyTEP1*, (2) to characterize the spatiotemporal expression patterns of *PyTEPs* at different developmental stages and in healthy adult tissues, and (3) to investigate the regulation of *PyTEPs* and each transcript of *PyTEP1* in response to acidification exposure. This work may provide evidence to elucidate the roles of TEP family members in the scallop immune response, the regulation of TEP family members of scallops during ocean acidification, and the evolutionary origin of this important, widespread and functionally diversified family of proteins.

2. Materials and methods

2.1. Sample collection and acidification exposure experiment

Two-year-old healthy Yesso scallops (n > 500) were collected from natural populations at Dalian Zhangzidao Fishery Group Co. (Dalian, Liaoning Province, China). Approximately six hours were required for the collected specimens to reach the laboratory (Qingdao, Shandong Province, China). Prior to the experiments, the active animals were acclimated in the laboratory for one week in filtered and aerated seawater maintained at approximately 8 °C, which is within the optimum temperature range for their growth [38].

For spatiotemporal expression analysis of *PyTEPs*, oocytes, zygotes, 2–8 cells, blastulae, gastrulae, trochophores, D-shaped larvae, umbo larvae, eyespot larvae and juvenile scallops were preserved in RNAlater (Sigma-Aldrich, St. Louis, MO, USA) and stored at −80 °C for subsequent analysis. Additionally, the mantle, gill, male gonad, female gonad, kidney, hepatopancreas, smooth muscle, striated muscle, foot, hemocytes, eye and ganglia of healthy Yesso scallops were dissected, immediately frozen in liquid nitrogen, and subsequently stored at −80 °C for further RNA-seq analysis.

For ocean acidification exposure stimulation, a specially designed gas proportionator system (Cole Parmer® Flowmeter system, multitube frame) was used, and a pH regulation protocol was followed according to Talmage and Gobler [32]. A total of 200 scallops were randomly divided into three groups (groups C, P and N). Group P (pH = 6.5) and group N (pH = 7.5) were used to evaluate the immune response of the

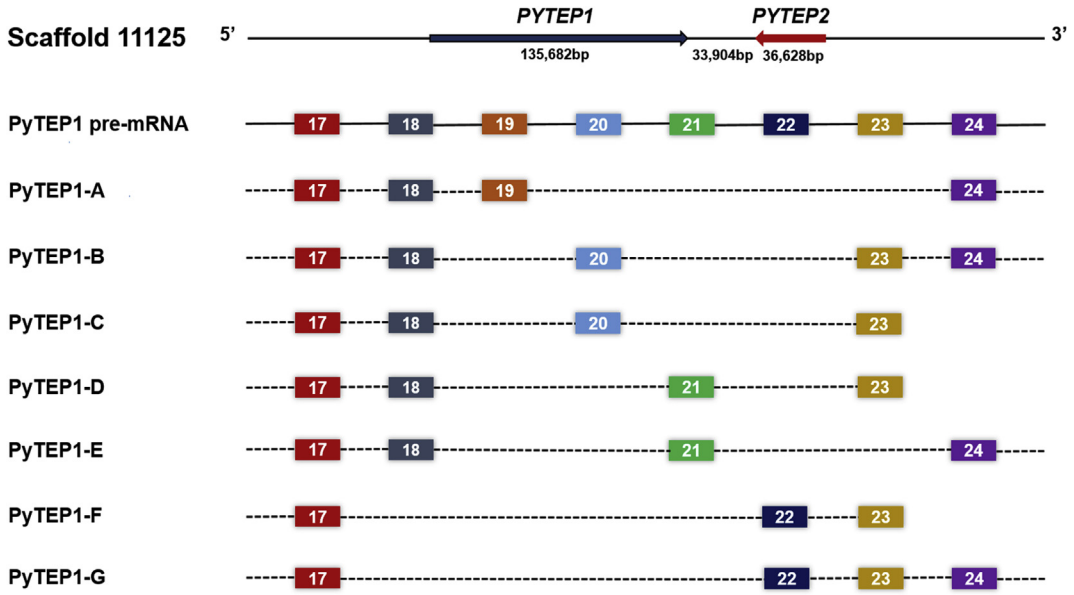

Fig. 1. *PyTEP1* duplication member and duplication patterns in *P. yessoensis* scaffold 11125 located in chromosome 7. Schematic of the *PyTEP1* pre-mRNA (exons 17–24), which has a set of mutually exclusive exons, and the seven distinct mRNA transcripts (PyTEP1-A–PyTEP1-G) generated by alternative splicing.

scallops after acidification exposure stimulation, which imitated changes in seawater pH over the twenty-first century [41]. The individuals were immersed in seawater, which was adjusted to pH = 6.5 and pH = 7.5, respectively, and three scallops from each group were sampled at 0, 3, 6, 12 and 24 h, respectively. Mantle tissue from each sampled individual was dissected and stored in liquid nitrogen for further RNA-seq analysis. Group C was designated as the control group and maintained in filtered and aerated seawater with normal pH (8.1) at 8 °C.

2.2. RNA isolation and RNA-seq analysis

Total RNA was isolated following the method described by Hu et al. [42]. RNA-seq data, generated from larvae at different developmental stages (oocytes, zygotes, 2–8 cells, blastulae, gastrulae, trochophores, D-shaped larvae, umbo larvae, eyespot larvae and juvenile scallops, n > 500) from three tubes and the sampled adult scallop tissues (mantle, gill, male gonad, female gonad, kidney, hepatopancreas, smooth muscle, striated muscle, foot, hemocytes, eye and ganglia), were used to spatiotemporally examine the expression profiles of *PyTEPs*. Specially, acidification transcriptomes (pH = 6.5 and 7.5) were assembled with RNA-seq data generated from the dissected mantles following acidification exposure [43].

2.3. Expression pattern analysis of PyTEPs

RNA-seq data generated from the normal and immune-elicited samples were used to examine the expression profiles of *PyTEPs* at different developmental stages and in healthy adult scallop tissues, as well as in response to ocean acidification. Barcodes were used to discriminate the sequencing reads from different sampled individuals. The estimated expression of *PyTEPs* determined via RNA-seq was normalized and represented in the form of RPKM (Reads Per Kilobase of exon model per Million mapped reads), and fold changes were calculated as $(RPKM_{test}\text{-}RPKM_{control})/RPKM_{control}$.

In embryos and larvae, healthy tissues, the relative expression levels were calibrated against the expression levels in the D-shaped larvae and ganglia of each group, respectively. To determine significance ($P < 0.05$, N = 3), one-way ANOVA with a post-hoc test was performed among different developmental stages and healthy adult scallop tissues. In the immune treatment groups, the control samples (Group C) were used for normalization. Significant differences between the test and control groups were determined with SPSS (version 21.0) using independent-sample T-tests ($P < 0.05$, N = 3).

2.4. Identification and expression analysis of PyTEP1 scripts

Sequence identification of TEP family genes was performed according to Liao et al. [40]. Five TEP family members, *PyC3*, *PyA2M*, *PyTEP1*, *PyTEP2* and *PyCD109*, were further confirmed to be present in the Yesso scallop genome sequences. For further identification and expression analysis of *PyTEP1* scripts, Stringtie (v1.3.1) [43] was utilized to assemble different transcripts of *PyTEP1* in the acidification transcriptome of the Yesso scallop with expression levels. In particular, diverse transcripts of *PyTEP1* were searched, and only the complete and unique transcripts were preserved for downstream analyses. ORF (open reading frame) 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 GT-AG rule was used to distinguish intron-exon boundaries [44,45]. The putative isoelectric (PI) point and molecular weight of protein encoded by transcripts/splices were computed using the Compute pl/Mw tool (http://web.expasy.org/compute_pi/). The ClustalW2 Multiple Alignment program [46] was used to create a multiple sequence alignment of seven diverse splices of *PyTEP1*. Geneious 7.1.7 (http://www.geneious.com/) was used to predict the secondary structure of alternative splices.

For expression analysis of *PyTEP1* scripts at 0, 3, 6, 12 and 24 h following acidification exposure, the absolute RPKM value was used. Statistical analysis of the data was performed with SPSS (version 21.0) software using an independent T-test (N = 3). Differences were considered significant at $P < 0.05$.

3. Results

3.1. Sequence identification and analysis of PyTEP1 splices

Five TEP family members (*PyC3*, *PyA2M*, *PyTEP1*, *PyTEP2* and *PyCD109*) were identified in the Yesso scallop genome and transcriptome database in our previous study [40]. Specially, at the genome level, a reverse tandem gene duplication of *PyTEP1* occurred, with

Table 1
The sequence features of seven splices of *PyTEP1* from Yesso scallop *Patinopecten yessoensis*.

PyTEP1_splices	PyTEP1-A	PyTEP1-B	PyTEP1-C	PyTEP1-D	PyTEP1-E	PyTEP1-F	PyTEP1-G
Total length (bp)	135,682	135,682	135,682	135,682	135,682	135,682	135,682
5′ UTR length (bp)	322	322	322	322	322	322	322
3′ UTR length (bp)	192	192	192	192	192	192	192
ORF length (bp)	4620	4365	2364	2409	4554	2334	4485
Amino acids length	1539	1454	787	802	1517	777	1494
Weight (kDa)	168.21	159.52	86.67	87.90	165.69	85.83	163.77
Theoretical pI	6.10	6.06	6.54	6.52	6.05	6.38	5.98
Number of exons	35	36	19	19	35	18	35
Number of introns	34	35	18	18	34	17	34
Number of alpha helixes	66	61	31	31	65	30	64
Number of beta strands	111	109	64	60	109	59	108
Number of coils	136	124	71	72	130	69	128
Number of turns	113	103	54	56	110	56	111

PyTEP1 and *PyTEP2* both located in the same scaffold (11125) of chromosome 7 [47] (Fig. 1). Stringtie (v1.3.1) assembly results showed that, of the five *PyTEPs* members, only seven transcripts of *PyTEP1* were identified. As a result, gene duplication and alternative splicing events simultaneously occurred in *PyTEP1*. Moreover, exons 18–24 of *PyTEP1* encoded the highly variable central region and formed seven different splice isoforms (designated PyTEP1-A to PyTEP1-G). The basic information (total length, 5′ UTR length, 3' UTR length, ORF length, amino acids length, protein weight, theoretical pI, exon number, intron number, alpha helix number, beta strand number, coils number and turns number) of the seven *PyTEP1* splices is summarized in Table 1.

Analysis of genome sequences showed that all of the exon-intron boundaries in the seven *PyTEP1* splices were consistent with the GT/AG rule for distinguishing intron-exon boundaries [44,45]. The number of exons/introns in the seven *PyTEP1* splices varied from 19/18 to 37/36 (Supplementary Table 1). The highly diverse region located mainly in the N-terminus was caused by alternative splicing, while the deduced C-terminal 17 exons of the seven isoforms were similar. The first exon was composed of at least 316 bp of the 5′-terminal untranslated region (UTR). The second exon was composed of a 6-bp 5′UTR and 53-bp codon, which encoded the signal peptide. Exons 18–24 encoded the highly variable central region of PyTEP1-A–PyTEP1-G, respectively, confirming that the different transcripts of *PyTEP1* resulted from alternative splicing. Interestingly, the PyTEP-C/D/F transcripts were short of the highly variable central coding region, the absence of which introduced a premature termination codon. Although the gene structures of the seven splice isoforms of *PyTEP1* varied, several evolutionarily conserved domains and motifs were found by the ClustalW program [48] (Fig. 2). Generally, aligning the results revealed the identification of seven different transcripts, with the exception of a continuous sequence of 66–2286 nucleotides that exactly encoded the highly variable central region of 22–762 amino acids.

3.2. Spatiotemporal expression of PyTEPs

RNA-seq data generated from larvae at different developmental stages and from sampled adult scallop tissues were used to examine the spatiotemporal expression profiles of *PyTEPs*. As shown in Fig. 3 A, *PyA2M* and *PyCD109* displayed similar distribution patterns during all developmental stages. The expression of both genes was highest in the oocytes (27.53 and 4.57 folds compared to D-shaped larvae, respectively), then gradually decreased as larval development stages proceeded and reached the lowest levels in the D-shaped larvae (*PyA2M*, 1.00 fold) and juvenile mollusk (*PyCD109*, 0.35 fold) stages, respectively. In contrast, *PyC3* and *PyTEP1/2* displayed disparate expression patterns, with trace expression in the first seven developmental stages (oocytes, zygotes, 2–8 cells, blastulae, gastrulae, trochophores and D-shaped larvae). Subsequently, an approximately inverted "V" trend of the three TEP family members in the last three development stages (umbo larvae, eyespots larvae and juvenile mollusks) could be observed. A dramatic increase in expression was observed in the umbo larvae of the three TEP family members (14.79, 14.92 and 25.30 folds compared to D-shaped larvae), reaching a maximum in eyespots larvae (19.08, 43.73 and 48.24 folds compared to D-shaped larvae), and then showing a significant reversal with a reduced expression pattern in the juvenile mollusks (16.58, 4.45 and 18.51 folds compared to D-shaped larvae).

The expression profiles of *PyTEPs* were determined in twelve tissues, including the mantle, gill, male gonad, female gonad, kidney, hepatopancreas, smooth muscle, striated muscle, foot, hemocytes, eye and ganglia from healthy adult scallops. As shown in Fig. 3 B, the expression of *PyTEPs* was ubiquitous in all tissues examined but was predominantly detected in the mantle, gill, female gonad, kidney, hepatopancreas, foot, hemocytes and eye. *PyC3* was expressed at the highest level in the female gonad (1.60 folds compared to the corresponding expression in the ganglia), followed by the striated muscle (0.78 fold), male gonad (0.50 fold), kidney (0.47 fold), foot (0.46 fold), mantle (0.38 fold) and gill (0.30 fold). *PyA2M* was most highly expressed in the hemocytes, and its expression was 2.70 folds higher than that in the ganglia. In addition to the hemocytes, *PyA2M* was mainly expressed in the smooth (1.54 folds) and striated muscles (1.14 folds). *PyTEP1* showed significantly higher expression in the tissues of the female gonad (134.92 folds) and hepatopancreas (406.79 folds) but low or barely detectable expression in other tissues. Similar to *PyTEP1*, the expression of *PyTEP2* was mainly detected in hepatopancreas (422.03 folds), and this expression was markedly higher than that in other tissues. Unlike the other TEP family members, *PyCD109* expression was generally high in most tissues, and the highest expression was detected in the hepatopancreas (7.80 folds increase relative to the ganglia), followed by the kidney (7.29 folds), gill (5.49 folds), eye (5.33 folds), mantle (3.82 folds), foot (3.73 folds) and female gonad (2.23 folds).

3.3. Expression regulation of PyTEPs after acidification exposure

To assess the challenge responses to acidification exposure, Yesso scallops (30 individuals, 3 individuals for each time point) were sampled before and after acidification exposure (pH = 6.5 and 7.5) and analyzed to determine the expression pattern of *PyTEPs*. Barcodes were used to distinguish the data from different individuals, and the mantle was used for gene expression investigation because it is considered to be the first barrier against pathogens [49]. Generally, all five *PyTEPs* displayed notable expression patterns in response to acidification exposure at pH = 6.5 and 7.5, including up- and down-regulated responses at different time points. Moreover, the down-regulated expression of *PyTEPs* was more dramatic than the up-regulated expression, except for that at 3 h at pH = 6.5 (Fig. 4). Upon pH = 6.5 challenge, *PyC3* showed a slightly up-regulated expression pattern, with relatively mild and stable levels (1.02–2.32 folds relative to the

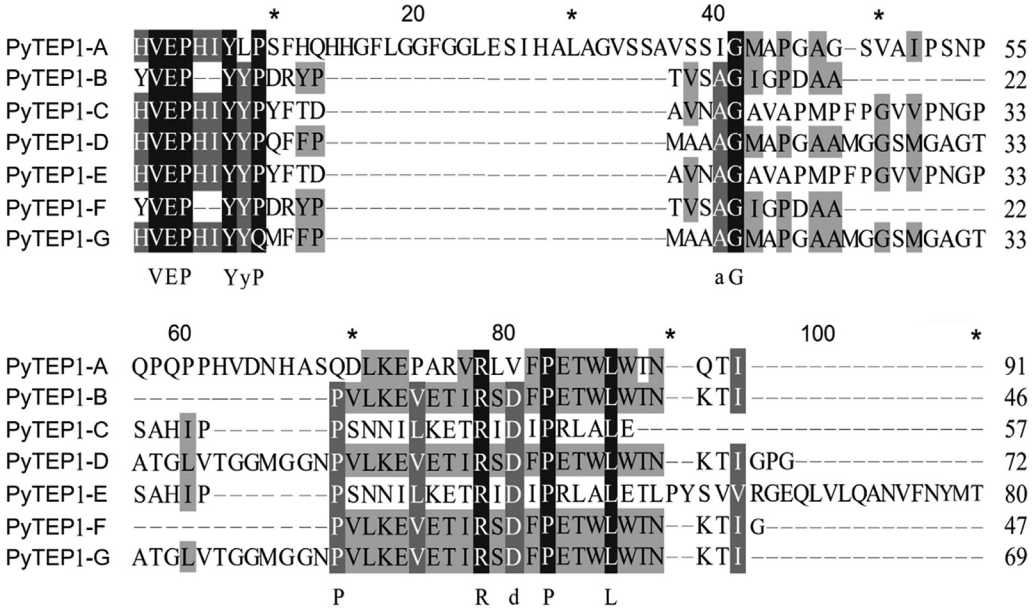

Fig. 2. Multiple alignment of the amino acid sequences of highly variable central region of PyTEP1 in seven isoforms. The identical and similar residues are shaded. Amino acid residues that are conserved in at least 50% sequences are shaded in dark, and similar amino acids are shaded in grey.

control). In contrast, *PyA2M* and *PyCD109* were notably ($P < 0.05$) down-regulated and reached their lowest levels (−3.03 and −2.42 folds) within 3 h. After reaching this minimum, the response levels of *PyA2M* and *PyCD109* gradually increased and became up-regulated as stimulation continued until 24 h post-challenge, when it reached the maximum (1.29 and 1.69 folds, down- and up-regulated chronologically). A completely reversed expression pattern (up- and down-regulated chronologically) was observed in *PyTEP1* and *PyTEP2*, both of which were promptly up-regulated at 3 h (12.83 and 4.59 folds). Subsequently, notably down-regulated expression was displayed at 6, 12 and 24 h, with extremely significant ($P < 0.05$ or $P < 0.01$) decreased levels of *PyTEP1* or *PyTEP2*. It is worth noting that, among all *PyTEPs*, *PyTEP1* and its tandem duplication *PyTEP2* showed the highest expression levels, varying from −32.95 to 12.83 folds and −57.37 to 4.59 folds, respectively, with significant ($P < 0.05$ or $P < 0.01$) up- or down-regulated expression levels during the whole period of duress. In addition, *PyTEP1* and *PyTEP2* displayed distinct and continuous down-regulated expression patterns after challenge for 3 h, with significant suppression levels relative to the control. Similar *PyTEPs* expression patterns, including expression levels and trends, could be observed at pH = 7.5, except for the down-regulated expression of *PyTEP1* (−2.18 folds) and *PyTEP2* (−57.74 folds) at 3 h, which was completely reversed compared to the up-regulated patterns at pH = 6.5 (12.83 and 4.59 folds, respectively). Overall, all *PyTEPs* were differentially expressed at one or several time points post-infection.

3.4. Expression regulation of PyTEP1 splices after acidification exposure

While fold change in the expression levels of *PyTEP1* were determined, the absolute amount of each *PyTEP1* variant splice in response to acidification exposure remained unclear. Therefore, the seven splices containing the highly variable central region of *PyTEP1* were investigated in the mantle of Yesso scallops at different pH levels (6.5 and 7.5) (Supplementary Table 2, Fig. 5). In normal pH seawater (0 h, pH = 8.1), the seven *PyTEP1* splices displayed slightly elevated expression levels with RPKM values varying from 0 to 0.97, among which PyTEP1-A and PyTEP1-E showed no expression. Under pH = 6.5 duress, most *PyTEP1* splices, except PyTEP1-B, were promptly elevated and reached the highest levels of expression (19.01, 0.11, 2.23, 0.03, 0.74 and 133.01 folds, respectively) within 3 h. The expression level of PyTEP1-A was notably up-regulated, reaching the most significant point (19.01 folds) at 3 h. Then, a tremendous decrease in the levels, even reaching zero, was observed at 6, 12 and 24 h. A similar expression pattern was also exhibited by PyTEP1-D and PyTEP1-G, with the highest levels at 3 h (2.23 and 133.01 folds, respectively). In addition, PyTEP1-B displayed continuous trace expression levels at all challenge time points, varying from 0.03 to 0.29 fold. Furthermore, PyTEP1-C, PyTEP1-E and PyTEP1-F were expressed at relatively lower levels and even showed no expression at most time points, showing some degree of fluctuation in the expression. Under pH = 7.5 acidification exposure, *PyTEP1* splice expression was not as intense as that at pH = 6.5, with no expression of PyTEP1-A, PyTEP1-C and PyTEP1-E at all challenge time points. In addition to PyTEP1-G, which showed significantly higher levels at 3, 6 and 12 h, the other splices of *PyTEP1* maintained relatively minor and stable expression levels at one or several challenge points. Overall, the disparate expression patterns of *PyTEP1* splices at different levels of acidification exposure suggested different responses of gene regulation in innate immunity.

4. Discussion

Innate immunity, which provides nonspecific defense, is believed to be the first line of host defense against pathogenic organisms and foreign materials [50,51]. In invertebrates, the absence of adaptive immunity makes innate immunity even more important because it frequently means the difference between life and death [52]. The innate immune responses in bivalves have been investigated in a complex network of evolutionarily conserved signaling pathways [2] and multiple critical members are involved in immune defense upon invasion or exposure to stimuli that threaten the healthy development of bivalve aquaculture [53–59]. In particular, the complement system consists of a family of proteins, mainly including complement factors, α_2-macroglobulins and TEPs, that participate in innate inflammatory and immune responses in three distinct but overlapping pathways: the classical, lectin and alternative pathways [7,19]. Our work represents the first genome-wide identification of *TEP* duplication accompanied by alternative splicing in mollusk, providing evidence of molecular evolution and elucidating how the expression profiles of these genes/splices change in response to ocean acidification.

Gene duplication is crucial for supplying raw genetic material for

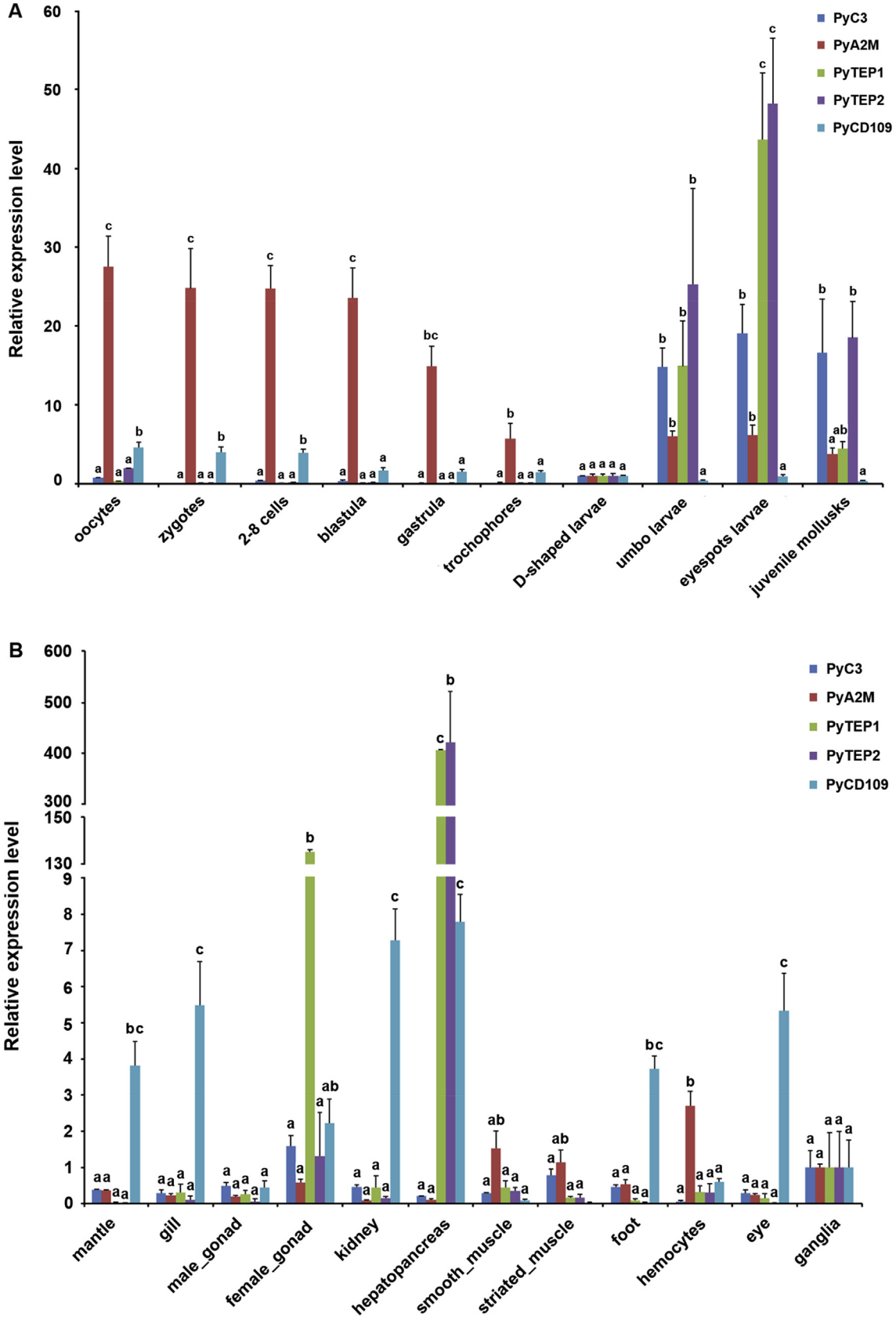

Fig. 3. The expression profiles at different developmental stages (A) and in healthy adult tissues (B). A. Relative expression levels of *PyTEPs* at different embryonic and larval stages. The relative expressions are shown as fold difference relative to the expression in D-shaped larvae. Cytochrome B was used as the internal control. B. Relative expression levels of *PyTEPs* in healthy adult tissues. The relative expressions are shown as fold difference relative to the expression in the ganglia. DEAD-box RNA helicase was used as the internal control. Vertical bars represent the mean ± S.E. (N = 3). Bars with different superscripts indicate significant differences ($P < 0.05$).

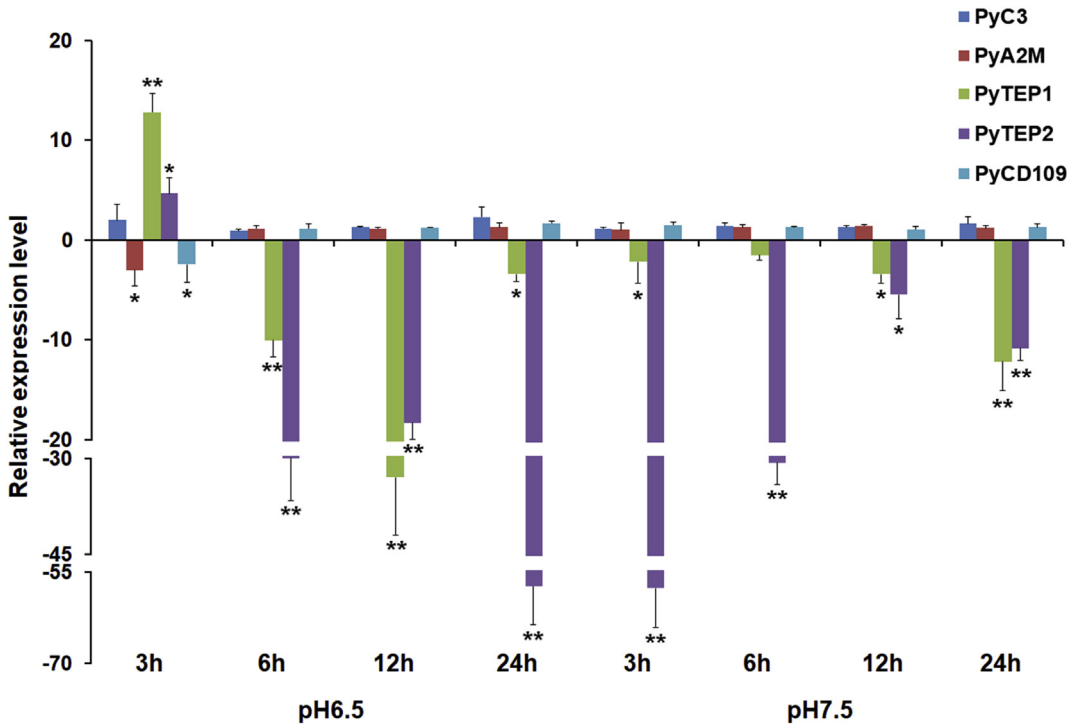

Fig. 4. Expression of *PyTEPs* in Yesso scallop mantles after acidifying exposure (pH = 6.5 and pH = 7.5) at different time points (3, 6, 12 and 24 h) in fold change. The vertical bars represent the mean ± S.E. (N = 3). '*' and '**' indicate differences that are statistically significant ($P < 0.05$ and $P < 0.01$, respectively). β-actin was used as an internal control.

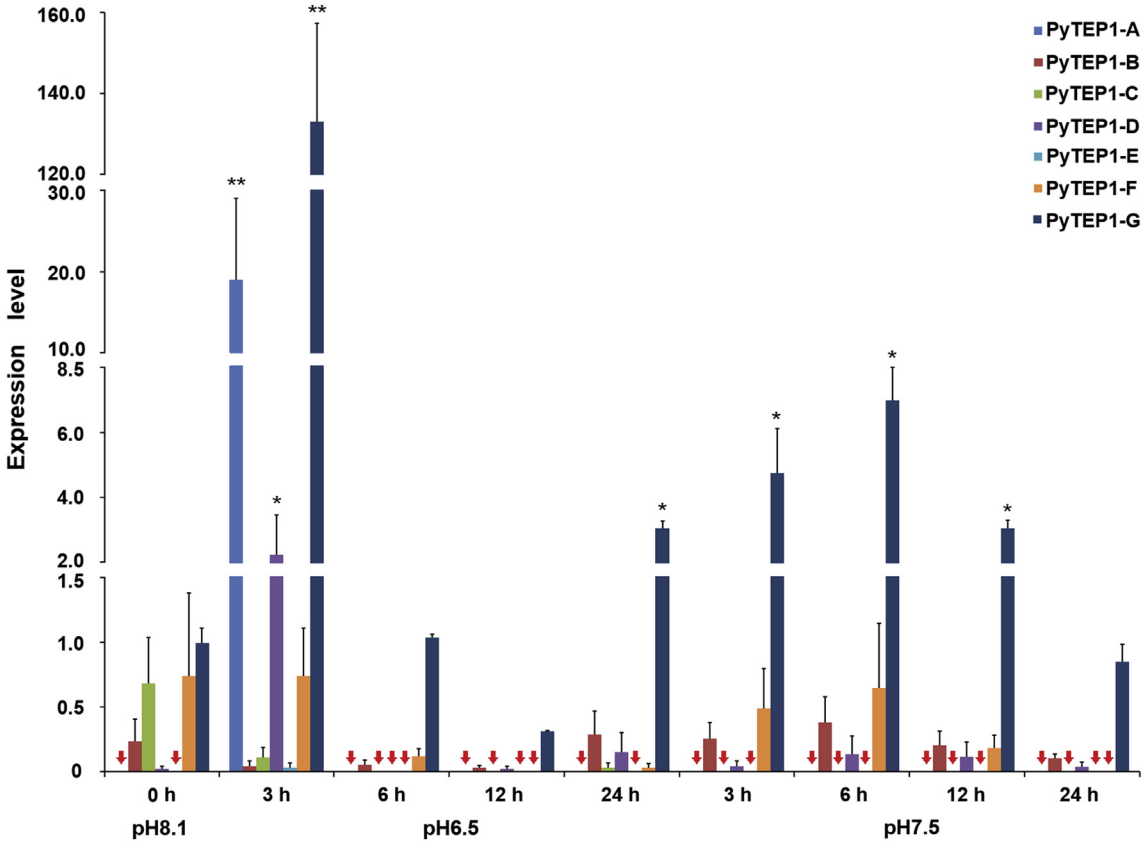

Fig. 5. Expression of seven alternative splices of *PyTEP1* gene in Yesso scallop mantles after acidifying exposure (pH = 6.5 and 7.5) at different time points (0, 3, 6, 12 and 24 h). The vertical bars represent the mean ± S.E. (N = 3). '*' and '**' indicate significant difference from the control group ($P < 0.05$ and $P < 0.01$, respectively). β-actin was used as an internal control. Red arrows indicate no expression of the corresponding *PyTEP1* splice(s). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

biological evolution [60], and numerous duplicated genes have been reported in vertebrates [61,62] and invertebrates [63,64]. Specifically, it is well known that duplication of immune-related genes provides opportunities for the host to recognize the variability of pathogens [65–67]. In the Yesso scallop genome, *PyTEP1* and *PyTEP2* (conversely located in the same scaffold of chromosome 7) are closely related, a phenomenon caused by gene duplication [59,68]. Similarly, in the Yesso scallop innate immune system, tandem or proximal (in a nearby but not adjacent chromosomal region) duplications have been widely reported in immune-related genes involved in immune recognition and immune signaling pathways [43,51,52,61], inferring functional expansion in response to various stressful environmental stimuli. In addition, seven different transcripts produced by alternative splicing were observed in the duplicated gene *PyTEP1*, suggesting that seven adjacent exons encoding the highly variable central region were alternatively spliced, and only two or three of these exons were included in the final product, eventually allowing seven different transcripts to be produced. Similarly, seven different cDNA variants of *CfTEP* were cloned from the Zhikong scallop (*C. farreri*), while CfTEP-A–CfTEP-G were produced by alternative splicing of six mutually exclusive exons (exons 19–24), with only one/none of these exons being included to encode the highly variable central region [28]. The differences in the highly variable central region of TEPs exactly corresponded to the bait-like regions in A2M, which were involved in ligand recognition as reported in *CfTEP* [28] and *dTEP2* [22]. The alternative splicing at these regions might introduce structural changes to *PyTEP1*, producing different affinities to various ligands. Gene duplication and alternative splicing have been widely considered to be ubiquitous and crucial mechanisms for generating protein diversity and regulating protein expression, both of which were discovered in *PyTEP1* for the first time, providing a molecular model for investigating molecular evolutionary mechanisms in the ancient and/or slow-evolving bilaterian lineage, Yesso scallop.

PyTEPs were expressed in all developmental stages and in all adult tissues of healthy Yesso scallops, which indicated that *PyTEPs* had specific functions in all stages of development and growth of the scallop. The five *PyTEPs* had various expression patterns in the different developmental stages, indicating that *PyTEPs* are involved in multi-task functions throughout scallop development. The five *PyTEPs* can be categorized into two groups based on their expression patterns. In early developmental stages, *PyA2M* and *PyCD109* were detected with the highest expression levels in oocytes and zygotes, suggesting that the transcripts might be maternally derived and involved in the early development of scallop embryos. Both of these genes exhibited an approximate trend of reduced expression, with a decrease during the D-shaped larvae and juvenile mollusk stages, indicating that maternally derived transcripts decreased during the period of increased immune function in the early developmental stage. Conversely, the other three *PyTEPs* showed minor expression levels from oocytes to D-shaped larvae and displayed another reversed 'V'-shaped pattern in the final developmental stages (umbo larvae, eyespots larvae and juvenile mollusks), with the highest expression levels during the eyespots larval period. This result may suggest that *PyA2M* and *PyCD109* are maternally derived, whereas *PyTEP1/2* and *PyC3* are synthesized *de novo* from this stage. Such diverse expression patterns suggested that *PyTEPs* show functional differentiation throughout all developmental stages of scallops.

The tissue expression patterns of TEP family genes have been widely characterized in various species, and high expression of these genes has been detected in multiple immune-related tissues [24,28,29], reflecting their crucial roles in the host immune response. In this study, the ubiquitous expression of all *PyTEPs* was observed in most healthy tissues, with particularly high abundance in the mantle, gill, female gonad, kidney, hepatopancreas, foot, hemocytes and eye, which are tissues that are all relevant to the innate immune system. The scallop mantle, gill and foot are generally considered to be the first barrier against pathogens [49]. Additionally, the kidney is the one of the major tissues involved in immune-related events [69]. Furthermore, functional genes and related pathways in the hepatopancreas play critical roles in metabolism and the immune system, as reported in the marine bivalves *C. farreri* [70], *Pinctada fucata* [71] and *Mytilus galloprovincialis* [72]. As a result, relatively high levels of *PyTEPs* expression ensure the realization of their immune-related functions in scallops [49]. Moreover, relatively high expression was observed in the hemocytes, which have been widely reported to be one of the most important immune tissues in mollusks and the location where the recognition and elimination of bacterial pathogens occurs [73]. Interestingly, a relatively higher expression level of *PyCD109* was observed in the eyes, suggesting that scallops may have innate immune function in their visual organ, which is controlled by a hierarchical organization of multilayered mirrors [74]. In vertebrates, numerous types of antibodies activated by immunologic stimuli have been reported in eyes [75]; similarly, the first report of immune protein expression in mantle eyes of bivalves was conducted with *PyTNFR* (-like) [53]. As a result, *PyCD109* is a major immunologic protein of the complement system in bivalve mantle eyes, playing an important role in the realization of accustomed foraging and defense behavior [47,74,76]. Furthermore, remarkable up-regulated expression of *PyTEP1* was observed in the female gonad relative to the male gonad and other detected tissues (except hepatopancreas), implying *PyTEP1* was constitutively expressed in tissues but highly expressed in female gonad and perhaps played a critical role in related functions and/or pathways involved in female gametogenesis. A similar study was conducted for *CfTEP* [29], which is highly related to *PyTEP1* according to phylogenetic analysis [40]. The ubiquitous expression patterns of *PyTEPs* in multiple immune-related tissues revealed the widespread TEP family members involved in the fundamental immune response of scallops.

To provide insight into the function of *PyTEPs* in the innate immune response of scallops, expression analyses were performed after exposure to acidification with pH = 6.5 and 7.5 seawater, respectively. As a major immune tissue in mollusks and the first barrier against pathogens, the mantle was selected for immune-elicited expression pattern investigation [49]. This study demonstrates that different elevated levels of CO_2 negatively impact adult Yesso scallops. The expression patterns of *PyTEPs* in response to acidification exposure confirmed their induction in the acute phase after infection. During the acidification exposure process, all inducible *PyTEPs* were either up- or down-regulated after acidification exposure at pH = 6.5 and 7.5 relative to the normal pH = 8.1. A similar phenomenon was also observed in *PyTLRs* [59], *Pyhsp70* [68], *PyMyD88* [58], etc., which are genes involved in the innate immune system of the Yesso scallop. Interestingly, the highly consistent expression patterns of *PyTEP1* and *PyTEP2*, especially their tremendously down-regulated levels at most time points, implied a weakened immune response under pH stress, which means this organism may be more susceptible to attacks by viruses and bacteria. In contrast, the other three *PyTEPs* mainly showed a reversed and slightly up-regulated trend at each challenge point. Although the molecular mechanisms underlying different patterns of immune challenge in mammals are almost completely understood, the diverse gene regulation expression patterns of *PyTEPs* are unclear, and this interesting phenomenon is worthy of further research.

Alternative splicing, as an important transcription mechanism, can generate a large number of mRNA and protein isoforms with relatively lower numbers of genes [77], resulting in a high degree of diversity and flexibility of functional expression in response to changing environmental conditions [78]. As a result, splices of *PyTEP1* were further investigated during ocean acidification challenge at pH = 6.5 and 7.5. The results showed that most *PyTEP1* splices were significantly induced during the acute phase (3 h) after acidification exposure at pH = 6.5, which was the same phenomenon observed at the time point when the excessive activation of other distinct groups of PRRs in bivalves, such as peptidoglycan recognition proteins (PGRPs), Gram-negative binding proteins (GNBPs), C-type lectins, galectins, scavenger receptors (SRs)

and Toll-like receptors (TLRs), where inhibited [2]. Moreover, except for the acute immune response (3 h) at pH = 6.5, PyTEP1-A–PyTEP1-F displayed trace/zero expression levels at other challenge time points, while PyTEP1-G was the main isoform of *PyTEP1* transcripts in the scallops challenged by acidification exposure. Similarly, CfTEP-G was the only and most highly expressed transcript of *CfTEP* splices in the gonad of male scallops upon *Listonella anguillarum* challenge, with no clones of the other six splices (CfTEP-A–CfTEP-F) detected. In the gonads of female scallops, four and five of the seven alternative splices were detected in *CfTEP* cDNA from *L. anguillarum*- and *M. luteus*-challenged groups, respectively [29]. The sequence diversity of the scallop *PyTEP1* isoforms and their different expression profiles in response to OA suggested a mechanism by which scallops differentiate and regulate the *PyTEP1* gene expression. In addition, the post-transcriptional regulation of *PyTEP1* mediated by the alternative splicing mechanism in response to time-dependent immune challenge suggests its essential role as a PRR in innate immunity. One possibility is that TEP family members perform their functions through expression allocation to maintain physiology and response to immune challenge. Alternatively, these proteins may be required in host defense against specific, but currently unidentified, natural pathogens of bivalves. Furthermore, diverse alternative splices of *PyTEP1* act in a significantly dominant manner in post-transcriptional regulation, guaranteeing the realization of specific immune functions, and their absence can be compensated by other components of the immune response. TEP family members in bivalves may thus provide a subtle selective advantage/strategy during evolution. In summary, we hypothesized that PyTEPs were constitutive and inducible acute-phase proteins and pH stress responses may have arisen through tandem duplication and alternative splicing of *PyTEP1* due to exposure to environmental stress. *PyTEP1* gene duplication and regulated expression may play crucial roles in the maintenance of homeostasis of the Yesso scallop under stress conditions.

In conclusion, this study represents a comprehensive analysis of TEP family genes in the Yesso scallop. This is the first report of gene duplications accompanied by seven different splices that simultaneously occurred in *PyTEP1* in bivalves. The alternative splicing and highly variable central region of PyTEP1 protein sequences were detected and specifically identified via bioinformatics analysis. The spatiotemporal expression profiles of the five *PyTEPs* were examined at various developmental stages and in healthy tissues of Yesso scallops. Importantly, the regulation of *PyTEPs* and each transcript of *PyTEP1* were further investigated in the mantles after exposure to different levels of acidification (pH = 6.5 and 7.5). Our expression analysis not only confirms the involvement of *PyTEPs* in the scallop immune response with distinct response patterns upon OA challenge but also provides potential clues that the selection pressure derived from the OA and/or other environmental stimuli is a potentially major factor leading to the functional allocation of TEP family members in the scallop. Future exploration of the activities of mollusk TEP family gene duplication and alternative splice generation, especially molecular mechanisms, will contribute to a better understanding of mollusk defensive mechanisms against OA stress.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.10.026.

References

[1] S.K. Law, A.W. Dodds, The internal thioester and the covalent binding properties of the complement proteins C3 and C4, Protein Sci. 6 (2) (1997) 263–274.
[2] L. Song, L. Wang, L. Qiu, H. Zhang, Bivalve immunity, Adv. Exp. Med. Biol. 708 (708) (2010) 44–65.
[3] M. Nonaka, F. Yoshizaki, Primitive complement system of invertebrates, Immunol. Rev. 198 (1) (2010) 203–215.
[4] J.E. Volanakis, M.M. Frank, The Human Complement System in Health and Disease, Crc Press, 1998.
[5] V. Buresova, O. Hajdusek, Z. Franta, G. Loosova, L. Grunclova, E.A. Levashina, P. Kopáček, Functional genomics of tick thioester-containing proteins reveal the ancient origin of the complement system, J. Innate Immun. 3 (6) (2011) 623–630.
[6] G.K. Christophides, E. Zdobnov, C. Barillasmury, E. Birney, S. Blandin, C. Blass, P.T. Brey, F.H. Collins, A. Danielli, G. Dimopoulos, Immunity-related genes and gene families in *Anopheles gambiae*, Science 298 (5591) (2002) 159–165.
[7] S. Blandin, E.A. Levashina, Thioester-containing proteins and insect immunity, Mol. Immunol. 40 (12) (2004) 903–908.
[8] L.J. Dishaw, S.L. Smith, C.H. Bigger, Characterization of a C3-like cDNA in a coral: phylogenetic implications, Immunogenetics 57 (7) (2005) 535–548.
[9] A.W. Dodds, S.K. Law, The phylogeny and evolution of the thioester bond-containing proteins C3, C4 and alpha 2-macroglobulin, Immunol. Rev. 166 (1) (1998) 15–26.
[10] G. Salvesen, J.J. Enghild, α-macroglobulins: detection and characterization, Methods Enzymol. 223 (5) (1993) 121–141.
[11] I. Garcia-Ferrer, A. Marrero, F.X. Gomis-Rüth, T. Goulas, α2-macroglobulins: structure and function, Subcell. Biochem. 83 (2017) 149–183.
[12] C.T. Chu, S.V. Pizzo, Alpha 2-macroglobulin, complement, and biologic defense: antigens, growth factors, microbial proteases, and receptor ligation, Lab. Investig.; J. Tech. Methods Pathol. 71 (6) (1995) 792–812.
[13] L. Sottrupjensen, T.M. Stepanik, T. Kristensen, P.B. Lønblad, C.M. Jones, D.M. Wierzbicki, S. Magnusson, H. Domdey, R.A. Wetsel, A. Lundwall, Common evolutionary origin of alpha 2-macroglobulin and complement components C3 and C4, Proc. Natl. Acad. Sci. U.S.A. 82 (1) (1985) 9–13.
[14] S.K.A. Law, A.W. Dodds, The internal thioester and the covalent binding properties of the complement proteins C3 and C4, Protein Sci. 6 (2) (2010) 263–274.
[15] M. Nonaka, Origin and evolution of the complement system, Curr. Top. Microbiol. Immunol. 248 (12) (2000) 37–50.
[16] B.J. Janssen, P. Gros, Structural insights into the central complement component C3, Mol. Immunol. 44 (1–3) (2005) 3–10.
[17] M. Nonaka, A. Kimura, Genomic view of the evolution of the complement system, Immunogenetics 58 (9) (2006) 701–713.
[18] Y. Zhu, S. Thangamani, B. Ho, J.L. Ding, The ancient origin of the complement system, EMBO J. 24 (2) (2005) 382–394.
[19] M.W. Dodds, L.S.K. Alex, The phylogeny and evolution of the thioester bond-containing proteins C3, C4 and α2-macroglobulin, Immunol. Rev. 166 (1) (1998) 15–26.
[20] L. Sottrup-Jensen, J. Folkersen, T. Kristensen, B.F. Tack, Partial primary structure of human pregnancy zone protein: extensive sequence homology with human alpha 2-macroglobulin, Proc. Natl. Acad. Sci. 81 (23) (1984) 7353–7357.
[21] E.A. Levashina, S. Blandin, L.F. Moita, M. Lagueux, F.C. Kafatos, Thioester-containing Proteins of Protostomes, Humana Press, 2003.
[22] A.R. Bou, C. Hetru, L. Troxler, D. Doucet, D. Ferrandon, N. Matt, Analysis of thioester-containing proteins during the innate immune response of *Drosophila melanogaster*, J. Innate Immun. 3 (1) (2011) 52–64.
[23] L.J. Dishaw, S.L. Smith, C.H. Bigger, Characterization of a C3-like cDNA in a coral: phylogenetic implications, Immunogenetics 57 (7) (2005) 535–548.
[24] M. Pradoalvarez, J. Rotllant, C. Gestal, B. Novoa, A. Figueras, Characterization of a C3 and a factor B-like in the carpet-shell clam, *Ruditapes decussatus*, Fish Shellfish Immunol. 26 (2) (2009) 305–315.
[25] M.G. Castillo, M.S. Goodson, M. Mcfallngai, Identification and molecular characterization of a complement C3 molecule in a lophotrochozoan, the Hawaiian bobtail squid *Euprymna scolopes*, Dev. Comp. Immunol. 33 (1) (2009) 69–76.
[26] P. Lemaire, R.K. Campbell, M. Celpke, A.D. Cregorio, W. Huang, K. Hotta, D.M. Coodstein, B. Davidson, T. Kawashima, B. Degnan, The draft genome of *Ciona intestinalis*: insights into chordate and vertebrate origins, Science 298 (5601) (2002) 2157–2167.
[27] C.E.S. Consortium, Genome sequence of the nematode *C. elegans*: a platform for investigating biology, Science 282 (5396) (1998) 2012–2018.
[28] H. Zhang, L. Wang, L. Song, J. Zhao, L. Qiu, Y. Gao, X. Song, L. Li, Y. Zhang, L. Zhang, The genomic structure, alternative splicing and immune response of *Chlamys farreri* thioester-containing protein, Dev. Comp. Immunol. 33 (10) (2009) 1070–1076.
[29] C. Li, Molecular cloning and characterization of a thioester-containing protein from Zhikong scallop *Chlamys farreri*, Mol. Immunol. 44 (14) (2007) 3492–3500.
[30] H. Ma, K. Mai, W. Xu, Z. Li, Molecular cloning of alpha 2-macroglobulin in sea scallop *Chlamys farreri* (Bivalvia, Mollusca), Fish Shellfish Immunol. 18 (4) (2005) 345–349.
[31] L. Wang, H. Zhang, L. Wang, D. Zhang, Z. Lv, Z. Liu, W. Wang, Z. Zhou, L. Qiu, H. Wang, The RNA-seq analysis suggests a potential multi-component complement system in oyster *Crassostrea gigas*, Dev. Comp. Immunol. 76 (2017) 209–219.
[32] S.C. Talmage, C.J. Gobler, Effects of elevated temperature and carbon dioxide on the growth and survival of larvae and juveniles of three species of northwest

atlantic bivalves, PLoS One 6 (10) (2011) 47–56.
[33] L. Alma, K.E. Kram, G.W. Holtgrieve, A. Barbarino, C.J. Fiamengo, J.L. Padillagamino, Ocean Acidification and Warming Effects on the Physiology, Skeletal Properties, and Microbiome of the Purple-Hinge Rock Scallop, Comparative Biochemistry and Physiology, (2019), https://doi.org/10.1016/j.cbpa.2019.110579 Part A published online.
[34] B. Schalkhausser, C. Bock, K. Stemmer, T. Brey, Hans-O. Pörtner, G. Lannig, Impact of ocean acidification on escape performance of the king scallop, *Pecten maximus*, from Norway, Mar. Biol. 160 (8) (2013) 1995–2006.
[35] Q. Wang, R. Cao, X. Ning, L. You, C. Mu, C. Wang, L. Wei, M. Cong, H. Wu, J. Zhao, Effects of ocean acidification on immune responses of the Pacific oyster *Crassostrea gigas*, Fish Shellfish Immunol. 49 (2016) 24–33.
[36] W. Su, S. Zha, Y. Wang, W. Shi, G. Xiao, X. Chai, H. Wu, G. Liu, Benzo[a]pyrene exposure under future ocean acidification scenarios weakens the immune responses of blood clam, *Tegillarca granosa*, Fish Shellfish Immunol. 63 (2017) 465–470.
[37] N. Castillo, L.M. Saavedra, C.A. Vargas, C. Gallardo-Escárate, C. Détrée, Ocean acidification and pathogen exposure modulate the immune response of the edible mussel *Mytilus chilensis*, Fish Shellfish Immunol. 70 (2017) 149–155.
[38] S.E. Shumway, G.J. Parsons, Scallops: Biology, Ecology and Aquaculture, 2, Developments in Aquaculture & Fisheriesence 164 Elsevier, 1991, pp. 280–281.
[39] R.G. Richards, A.T. Davidson, Jan-Olaf Meynecke, K. Beattie, V. Hernaman, Effects and mitigations of ocean acidification on wild and aquaculture scallop and prawn fisheries in Queensland, Australia, Fish. Res. 161 (2015) 42–56.
[40] S. Wang, J. Zhang, W. Jiao, J. Li, X. Xun, Y. Sun, X. Guo, P. Huan, B. Dong, L. Zhang, et al., Scallop genome provides insights into evolution of bilaterian karyotype and development, Nat. Ecol. Evol. 1 (2017) 0120.
[41] H. Liao, X. Xun, L. Zhao, Z. Yang, X. Zhu, Q. Xing, X. Huang, Z. Bao, Identification and characterization of TEP family genes in Yesso scallop (*Patinopecten yessoensis*) and their diverse expression patterns in response to bacterial infection, Fish Shellfish Immunol. 79 (2018) 327–339.
[42] J.C. Orr, V.J. Fabry, O. Aumont, L. Bopp, S.C. Doney, R.A. Feely, A. Gnanadesikan, N. Gruber, A. Ishida, F. Joos, Anthropogenic ocean acidification over the twenty-first century and its impact on calcifying organisms, Nature 437 (7059) (2005) 681–686.
[43] X. Hu, Z. Bao, J. Hu, M. Shao, L. Zhang, K. Bi, A. Zhan, X. Huang, Cloning and characterization of tryptophan 2,3-dioxygenase gene of Zhikong scallop *Chlamys farreri* (Jones and Preston 1904), Aquacult. Res. 37 (12) (2006) 1187–1194.
[44] M. Pertea, G.M. Pertea, C.M. Antonescu, T.C. Chang, J.T. Mendell, S.L. Salzberg, StringTie enables improved reconstruction of a transcriptome from RNA-seq reads, Nat. Biotechnol. 33 (3) (2015) 290–295.
[45] M.W. Pfaffl, G.W. Horgan, L. Dempfle, Relative expression software tool (REST©) for group-wise comparison and statistical analysis of relative expression results in real-time PCR, Nucleic Acids Res. 30 (30) (2002) e36.
[46] P.A. Sharp, Speculations on RNA splicing, Cell 23 (3) (1981) 643–646.
[47] M.A. Larkin, G. Blackshields, N. Brown, R. Chenna, P.A. McGettigan, H. McWilliam, F. Valentin, I.M. Wallace, A. Wilm, R. Lopez, Clustal W and clustal X version 2.0, Bioinformatics 23 (21) (2007) 2947–2948.
[48] A.M. Alzohairy, ClustalW©: widespread Multiple sequences alignments program, J. Cell Mol. Biol. 7 (1) (2008) 81–82.
[49] J. Su, L. Qiu, L. Li, L. Liu, L. Wang, V.S. Siva, D. Yu, L. Song, cDNA cloning and characterization of a new member of the tumor necrosis factor receptor family gene from scallop, *Chlamys farreri*, Mol. Biol. Rep. 38 (7) (2011) 4483–4490.
[50] S. Akira, S. Uematsu, O. Takeuchi, Pathogen recognition and innate immunity, Cell 124 (4) (2006) 783–801.
[51] J.A. Hoffmann, F.C. Kafatos, C.A. Janeway, R.A.B. Ezekowitz, Phylogenetic perspectives in innate immunity, Science 284 (5418) (1999) 1313–1318.
[52] L. Qiu, L. Song, Y. Yu, W. Xu, D. Ni, Q. Zhang, Identification and characterization of a myeloid differentiation factor 88 (MyD88) cDNA from Zhikong scallop *Chlamys farreri*, Fish Shellfish Immunol. 23 (3) (2007) 614–623.
[53] Q. Xing, Q. Yu, H. Dou, J. Wang, R. Li, X. Ning, R. Wang, S. Wang, L. Zhang, X. Hu, Genome-wide identification, characterization and expression analyses of two *TNFRs* in Yesso scallop (*Patinopecten yessoensis*) provide insight into the disparity of responses to bacterial infections and heat stress in bivalves, Fish Shellfish Immunol. 52 (2016) 44–56.
[54] J. Zou, R. Wang, R. Li, Y. Kong, J. Wang, X. Ning, L. Zhang, S. Wang, X. Hu, Z. Bao, The genome-wide identification of mitogen-activated protein kinase kinase (MKK) genes in Yesso scallop *Patinopecten yessoensis* and their expression responses to bacteria challenges, Fish Shellfish Immunol. 45 (2) (2015) 901–911.
[55] J. Wang, R. Wang, S. Wang, M. Zhang, X. Ma, P. Liu, M. Zhang, X. Hu, L. Zhang, S. Wang, Z. Bao, 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 (1) (2015) 545–555.
[56] R. Zhang, R. Li, J. Wang, S. Wang, M. Zhang, X. Hu, L. Zhang, S. Wang, R. Wang, Z. Bao, Identification, characterization and expression profiling of the *Tollip* gene in Yesso scallop (*Patinopecten yessoensis*), Genes Genet. Syst. 90 (2015) 99–108.
[57] R. Li, R. Zhang, L. Zhang, J. Zou, Q. Xing, H. Dou, X. Hu, L. Zhang, R. Wang, Z. Bao, Characterizations and expression analyses of *NF-κB* and *Rel* genes in the Yesso scallop (*Patinopecten yessoensis*) suggest specific response patterns against Gram-negative infection in bivalves, Fish Shellfish Immunol. 44 (2) (2015) 611–621.
[58] X. Ning, R. Wang, X. Li, S. Wang, M. Zhang, Q. Xing, Y. Sun, S. Wang, L. Zhang, X. Hu, Genome-wide identification and characterization of five *MyD88* duplication genes in Yesso scallop (*Patinopecten yessoensis*) and expression changes in response to bacterial challenge, Fish Shellfish Immunol. 46 (2) (2015) 181–191.
[59] Q. Xing, H. Liao, X. Xun, J. Wang, Z. Zhang, Z. Yang, X. Huang, Z. Bao, Genome-wide identification, characterization and expression analyses of *TLRs* in Yesso scallop (*Patinopecten yessoensis*) provide insight into the disparity of responses to acidifying exposure in bivalves, Fish Shellfish Immunol. 68 (9) (2017) 280–288.
[60] J. Zhang, Evolution by gene duplication: an update, Trends Ecol. Evol. 18 (6) (2003) 292–298.
[61] P. Mombaerts, The human repertoire of odorant receptor genes and pseudogenes, Genom. Hum. Genet. 2 (2) (2001) 493–510.
[62] X. Zhang, The olfactory receptor gene superfamily of the mouse, Nat. Neurosci. 5 (2) (2002) 124–133.
[63] S. Ohno, Evolution by gene duplication, Am. J. Hum. Genet. 23 (5) (1971) 541–701.
[64] Z. Gu, A. Cavalcanti, F. Chen, P. Bouman, W.H. Li, Extent of gene duplication in the genomes of *Drosophila*, Nematode, and Yeast, Mol. Biol. Evol. 19 (3) (2002) 256–262.
[65] H. Zhou, J. Gu, S.J. Lamont, X. Gu, Evolutionary analysis for functional divergence of the toll-like receptor gene family and altered functional constraints, J. Mol. Evol. 65 (2) (2007) 119–123.
[66] E. Peatman, Z. Liu, Evolution of CC chemokines in teleost fish: a case study in gene duplication and implications for immune diversity, Immunogenetics 59 (8) (2007) 613–623.
[67] B. Korithoski, O. Kolaczkowski, K. Mukherjee, R. Kola, C. Earl, B. Kolaczkowski, Evolution of a novel antiviral immune-signaling interaction by partial-gene duplication, PLoS One 10 (9) (2015) e0137276.
[68] J. Cheng, X. Xun, Y. Kong, S. Wang, Z. Yang, Y. Li, D. Kong, S. Wang, L. Zhang, X. Hu, *Hsp70* gene expansions in the scallop (*Patinopecten yessoensis*) genome and their expression regulation after exposure to the toxic dinoflagellate *Alexandrium catenella*, Fish Shellfish Immunol. 58 (2016) 266–273.
[69] W. Liu, C. He, W. Li, Z. Zhou, X. Gao, L. Fu, Discovery of host defence genes in the Japanese scallop *Mizuhopecten yessoensis* Jay by expressed sequence tag analysis of kidney tissue, Aquacult. Res. 41 (11) (2010) 1602–1613.
[70] H. Zhang, Y. Zhai, L. Yao, Y. Jiang, F. Li, Comparative transcriptomics reveals genes involved in metabolic and immune pathways in the digestive gland of scallop *Chlamys farreri* following cadmium exposure, Chin. J. Oceanol. Limnol. 5 (35) (2017) 603–612.
[71] G. Jing, Y. Li, L. Xie, R. Zhang, Metal accumulation and enzyme activities in gills and digestive gland of pearl oyster (*Pinctada fucata*) exposed to copper, Comp. Biochem. Physiol. C Toxicol. Pharmacol. 144 (2) (2006) 184–190.
[72] C. Barmo, C. Ciacci, B. Canonico, R. Fabbri, K. Cortese, T. Balbi, A. Marcomini, G. Pojana, G. Gallo, L. Canesi, *In vivo* effects of n-TiO_2 on digestive gland and immune function of the marine bivalve *Mytilus galloprovincialis*, Aquat. Toxicol. 132–133 (2013) 9–18.
[73] E. Bachère, Y. Gueguen, M. Gonzalez, L.J. De, J. Garnier, B. Romestand, Insights into the anti-microbial defense of marine invertebrates: the penaeid shrimps and the oyster *Crassostrea gigas*, Immunol. Rev. 198 (1) (2004) 149–168.
[74] B.A. Palmer, G.J. Taylor, V. Brumfeld, D. Gur, M. Shemesh, N. Elad, A. Osherov, D. Oron, S. Weiner, L. Addadi, The image-forming mirror in the eye of the scallop, Science 358 (6367) (2017) 1172–1175.
[75] C.E.O. Fraser, D.E. Mccomb, E.S. Murray, A.B. Macdonald, Immunity to chlamydial infections of the eye, Arch. Ophthalmol. 93 (7) (1975) 518–521.
[76] Y. Li, X. Sun, X. Hu, X. Xun, J. Zhang, X. Guo, W. Jiao, L. Zhang, W. Liu, J. Wang, et al., Scallop genome reveals molecular adaptations to semi-sessile life and neurotoxins, Nat. Commun. 8 (1) (2017) 1721.
[77] O. Kelemen, P. Convertini, Z. Zhang, Y. Wen, M. Shen, M. Falaleeva, S. Stamm, Function of alternative splicing, Gene 514 (1) (2013) 1–30.
[78] K.W. Lynch, Consequences of regulated pre-mRNA splicing in the immune system, Nat. Rev. Immunol. 4 (12) (2004) 931–940.