The expression profile of a multi-stress inducible transient receptor potential vanilloid 4 (TRPV4) in Pacific oyster *Crassostrea gigas*

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\textbf{ABSTRACT}

Transient receptor potential vanilloid 4 (TRPV4) is one of the major non-selective cation channel proteins, which plays a crucial role in sensing biotic and abiotic stresses, such as pathogen infection, temperature, mechanical pressure and osmotic pressure changes by regulating Ca\textsuperscript{2+} homeostasis. In the present study, a TRPV4 homologue was identified in Pacific oyster *Crassostrea gigas*, designated as CgTRPV4. The open reading frame (ORF) of CgTRPV4 was of 2298 bp encoding a putative polypeptide of 765 amino acid residues with three typical ankyrin domains and six conserved transmembrane domains of TRPV4 subfamily proteins, as well as multiple N-glycosylation sites, cAMP- and cGMP-dependent protein kinase phosphorylation sites, protein kinase C phosphorylation sites, casein kinase II phosphorylation sites, and prokaryotic membrane lipoprotein lipid attachment site. The deduced amino acid sequence of CgTRPV4 shared 20.5%-26.2% similarity with TRPV4s from other species. During the early ontogenesis stages of oyster, the mRNA transcripts of CgTRPV4 were detectable in all the stages with the highest expression level in fertilized eggs and the lowest in D-hinged larvae. In adult oyster, the CgTRPV4 mRNA could be detected in all the examined tissues, including gill, hepatopancreas, adductor muscle, labial mantle and haemocyte, with the highest expression level in gill (45.08-fold of that in hepatopancreas, \textit{p} < 0.05). In immunocytochemical assay, the CgTRPV4 positive signals were distributed in both endoplasmic reticulum and cytoplasmic membrane of oyster haemocytes. The mRNA expression of CgTRPV4 in gill was significantly up-regulated after high temperature stress at 30°C (\textit{p} < 0.05) and after \textit{Vibrio splendidus} stimulation (\textit{p} < 0.05). These results indicated that CgTRPV4 was a classical member of TRPV4 family in oyster, which was induced by either biotic or abiotic stimulations and involved in mediating the stress response of oysters.

\textbf{1. Introduction}

Transient receptor potential (TRP) channel is a class of non-selective cation channel proteins containing six transmembrane domains, and forms ion channels in the form of homologous or heterotetramers to regulate intracellular calcium homeostasis \cite{1}. According to different structural characteristics, TRP superfamily can be divided into seven subfamilies, including TRPC (C stands for canonical or classical), TRPV (V stands for the vanilloid receptor), TRPM (M stands for melastatin), TRPA (A stands for the protein denoted ankyrin-like with transmembrane domains), TRPP (P stands for polycystin), TRPN (N stands for the no mechanoreceptor potential C) and TRPML (ML stands for mucolipin) subfamily \cite{1}. The TRPV subfamily is named for its ability to be activated by chemicals containing vanilloid groups. By far, a total of six subfamily members have been identified (named TRPV1–6), among which TRPV4 is mostly studied.

Studies in model animals including mouse, human, nematode and fruit fly have shown that TRPV4 plays vital roles in sensing biotic stresses (such as pathogenic bacteria) and abiotic stresses (such as temperature, mechanical pressure, chemical pressure, and osmotic pressure) \cite{2}. The classical TRPV4 is consisted of a conserved six transmembrane helical domain at the C-terminal and three to five...
ankyrin repeats (ANK) domains at the N-terminal [3], in which the latter ANK domain plays an important role in the assembly and transportation of TRPV4 [4], and determines the sensitivity of TRPV4 channel [5,6]. In mammalian, TRPV4 is broadly expressed in various tissues such as heart, arteries, lung, skin, bone, brain, urinary bladder, kidney, intestine, liver and pancreas, as well as in a wide range of cells such as neuron cells, epidermal cells, macrophages, and muscle cells [7–12]. The TRPV4 in different types of cells may play different functions in a variety of physiological and biochemical processes, such as signal transduction between neuron cells, endoplasmic reticulum (ER) stress, lipid peroxidation, inflammatory response, phagocytosis, and so on [13–16]. Compared to the vast knowledge on TRPV4 channel regulation, little is known about the induction mechanism of TRPV4 transcription [17].

Compared with the relatively in-depth research in model animals, the research on TRPVs in aquatic animals is very limited, mainly focusing on the identification of fish and shellfish TRPV subfamily members and analysis of their expression level under osmotic pressure [18–23,26], mechanical pressure [19,24], and temperature stress [25,26]. At present, TRPV4s have been identified in zebrafish Danielio rerio, half-smooth tongue sole Cynoglossus semilaevis, chum salmon Oncorhynchus keta, tilapia Oreochromis mossambicus, sea bass Dicentrarchus labrax, and Pacific oyster Crassostrea gigas [18–26]. Similar to their homologues in mammals, aquatic TRPVs all harbor typical six transmembrane domains and three ANK domains [18–26]. The DTrPV4 is expressed in various sensory cells of Danielio rerio such as retinal cells, ciliated sensory neurons, lateral line cells, and many tissues such as brain, kidney and heart, and its expression is affected by external osmotic pressure, drugs and other factors [19–22]. The OTRPV4 can be activated by agonist GSK1016790A, hypotonic solution, and temperature (cold and hot) stimulation [23]. DTRPV4 and GsTRPV4 is involved in sensing osmotic pressure and mechanical stimulation, respectively [22,24]. The expression level of CgTRPV4.7 mRNA in gill was higher than in other tissues, which can be induced by high temperature stress [25]. Interestingly, OkTRPV4 generates two transcript variants through alternative splicing, TRPV4 x1 and TRPV x2, and their expression levels significantly increased with the increase of salinity and temperature and the decrease of temperature, respectively [26]. However, the functional characters and regulation mechanism of TRPV4s in aquatic animals especially in lower aquatic invertebrates under various stressors are still not clear.

The Pacific oysters C. gigas, a typical representative of intertidal organisms, have to cope with a series of biotic threats such as pathogens, and has been regarded as an attractive experimental model for studying the mechanism of environmental adaptation [27,28]. Timely sensing biotic and abiotic stresses and regulating the mechanism of environmental adaptation [27,28]. The expression level of CgTRPV4 α in sensing biotic and abiotic stresses and regulating the mechanism of environmental adaptation 

2. Materials and methods

2.1. Oysters and sample collection

All experiments were performed in accordance with the approval and guidelines of the Ethics Review Committee of Dalian Ocean University. Two-year-old Pacific oysters C. gigas with an average shell length of 12–14 cm were collected from a commercial farm in Dalian, China. They were cultured in aerated seawater at 16°C for seven days and fed with diatom once a day before following experiments. Tissues including gill, muscle, labial palp, hepatopancreas and mantle were collected from six oysters as parallel samples, and haemolymph from these oysters was extracted and centrifuged at 800 × g. 4°C for 10 min to harvest the haemocyte. After addition of 1 mL Trizol reagent (Invitrogen), all the samples were stored at -80°C for further RNA extraction.

2.2. RNA extraction, gene cloning and sequence analysis of CgTPRV4

Total RNA isolation, cDNA synthesis and gene cloning were performed according to the previous reports [29]. The primers of CgTPRV4-F and CgTPRV4-R (Table 1) were designed according to the sequence information of CgTPRV4 (GenBank accession No. XM_034442984.1). The PCR product was gel-purified, cloned into pMD19-T simple vector (Takara), and confirmed by DNA sequencing.

The conserved domains of CgTPRV4 were predicted using the simple modular architecture research tool (SMART) (http://smart.embl-heidelberg.de/), and the conserved domain database (CDD) (https://www.ncbi.nlm.nih.gov/cdd). The amino acid sequence analyses were carried out using the BLAST algorithm (http://www.ncbi.nlm.nih.gov/BLAST). Multiple sequence alignments were conducted using the Cluster X 1.81 program. The molecular weight and hydrophobicity of CgTPRV4 were predicted using the ProtParam Tool (https://web.expasy.org/protparam/). The secondary structure was predicted using the SOPMA (https://npsa-prabi.ibcp.fr/NPSA/npsa_sopma.html). The N-glycosylation site, cAMP- and cGMP-dependent protein kinase phosphorylation site, Protein kinase C phosphorylation site, Casein kinase II phosphorylation site and Prokaryotic membrane lipoprotein attachment site were predicted using the SoftBerry-Psite (http://linux1.softberry.com/berry.phtml?topic=psite&group=&subgroup=proloc). The neighbor-joining (NJ) phylogenetic tree of CgTPRV4 was constructed using MEGA 6.0 software. Bootstrap trials were replicated 1000 times to derive the confidence value for phylogeny analysis [24].

2.3. Detection of CgTPRV4 mRNA transcripts in different developmental stages of oyster larvae using quantitative real-time PCR (qRT-PCR)

All embryos and larvae were sampled from the Yipintang farm in Dalian, Liaoning Province, China in May, 2022 as previously described [30]. Briefly, the embryo or larvae at different stages were identified microscopically and collected as fertilized eggs, 4-cell embryos, 8-cell embryos, morula (6 h post-fertilization, hpf), blastula (11 hpf), gastrula (18 hpf), trophophore (22 hpf), D-hinged larvae (2 day post-fertilization, dpf). For each developmental stage, six aliquot samples were collected, re-suspended in 1 mL Trizol reagent (Invitrogen), and stored in liquid nitrogen immediately. RNA isolation and cDNA synthesis were carried out as described above. The cDNA mix was diluted to 1:100 and stored at -80°C for subsequent SYBR Green qRT-PCR.

The mRNA transcripts of CgTPRV4 in different tissues were analyzed by SYBR Green qRT-PCR. All reactions were performed as previously described [31]. The information of the gene specific primers for CgTPRV4 (CgTPRV4-RTFand CgTPRV4-RTR) and CgEF-α (CgEF-α-RTF and CgEF-α-RTR) used in the qRT-PCR was shown in Table 1. The expression of CgTPRV4 was normalized to that of the elongation factor α

Table 1

| Primer name               | Primer Sequence (5'-3')     |
|--------------------------|----------------------------|
| Primers for CgTPRV4      | (Accession number XM_034442984.1) |
| CgTPRV4-ANK-F            | ATCGAAGCTTCTTCCATATACACCA |
| CgTPRV4-ANK-R            | ATTTGAACTTTATCATCAATGAGTA |
| CgTPRV4-ANK-EX-F         | CCAGATATGGAGATTATCATACGATAA |
| CgTPRV4-ANK-EX-R         | CCGGAGCTTATGGATATGGTGTG |
| CgTPRV4-ANK-R            | CCTTCTCAACCAAAGGACCAGG |
| CgTPRV4-ANK-RTR          | GCAGATTAACGGCAGGGTGC |
| CgEF-α-RT-F              | AGTCCACAGGGAGTCCAGAGAAAG |
| CgEF-α-RTR              | TCCGGTATTTCTTCTTTGATG |
Table 2

| Protein name | Organism       | Accession number |
|--------------|----------------|------------------|
| TRPV4        | Homo sapiens   | XP_011536936.2   |
| TRPV4        | Mus musculus   | XP_011246537.1   |
| TRPV4        | Gallus gallus  | NP_990023.2      |
| TRPV4        | Xenopus tropicalis | XP_002932129.1 |
| TRPV4        | Physoster canadensis | XP_028356654.1 |
| TRPV4        | Danio rerio     | XP_005165208.1   |
| TRPV4        | Larimichthys crocea | XP_019133001.6 |
| TRPV4        | Oncorhynchus mykiss | XP_021476579.1  |
| TRPV4        | Salmo salar     | XP_014016423.1   |
| TRPV4        | Monopterus albus | XP_020449957.1   |
| TRPV4        | Trichinella spiralis | KJ295620.1      |
| TRPV4        | Crassostrea gigas | XP_034298875.1   |

(CgEF-a) gene (GenBank accession No. NM_001305313.2) for each sample, and the relative expression levels of CgTPRV4 were analyzed using the comparative Ct method (ΔΔCt method) [31,32].

2.4. Detection of CgTPRV4 mRNA transcripts in different tissues of adult oysters using qRT-PCR

The mRNA transcripts of CgTPRV4 in different tissues were analyzed by SYBR Green qRT-PCR. The SYBR Green qRT-PCR was carried out, and the relative expression level of CgTPRV4 was analyzed as previously described [31,32].

2.5. Recombinant expression and polyclonal antibody preparation of CgTPRV4

Recombinant protein of CgTPRV4 fused with GST tag was expressed in Escherichia coli using the pCold™-GST DNA vector (Takara). The cDNA sequence encoding the ANK domains of CgTPRV4 was amplified using gene specific primers rCgTPRV4-ANK-F and rCgTPRV4-ANK-R (Table 1) with restriction enzyme sites Nde I and Hind III. The PCR products were digested, gel-purified, and ligated into the expression vector pCold-GST (Takara). The recombinant plasmids pCold™-GST-CgTPRV4-ANK were extracted and transformed into E. coli Transetta (DE3). The positive transformants were cultured in LB medium and induced using Isoprropyl β-D-1-Thiogalactopyranoside (IPTG), and the cell lysates were separated using SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The purification of the recombinant protein of CgTPRV4 (designated rCgTPRV4) and the purity and concentration analysis of rCgTPRV4 were performed as previously described [30]. To prepare polyclonal antibody, the purified rCgTPRV4-ANK was subcutaneously stimulated six-week-old female mice according to the previous description [30]. The anti-rCgTPRV4 serum was stored at -80°C for subsequent experiments.

2.6. Western blot analysis

Proteins were extracted from haemocytes of oysters as previously described [33]. The specificity of polyclonal antibody against endogenous CgTPRV4 in haemocytes was examined using Western blot analysis previously described [30]. The proteins extracted from haemocytes were separated using 15% SDS-PAGE. The antibodies against rCgTPRV4 (1:1000 dilution) and the HRP-conjugated goat anti-mouse IgG (Sangon, diluted 1:1500 in 3% BSA) were used as primary and secondary antibodies, respectively. The western blotting ECL substrate (Bio-Rad) was used to visualize the protein bands of CgTPRV4 as previously described [34]. The positive transformants were cultured in LB medium and induced using Isoprropyl β-D-1-Thiogalactopyranoside (IPTG), and the purity and concentration analysis of rCgTPRV4 were performed as previously described [31,32]. The purified rCgTPRV4-ANK was subcutaneously stimulated six-week-old female mice according to the previous description [30]. The anti-rCgTPRV4 serum was stored at -80°C for subsequent experiments.

2.7. Immunofluorescence assay

Immunofluorescence assay was carried out to investigate the subcellular localization of CgTPRV4 in haemocytes as previously described with some modification [34]. The haemocytes were collected from five oysters, re-suspended in L15 culture media, deposited on to dishes precoated with poly-Lysine (a drop on each) in the wet chamber, and fixed with 4% paraformaldehyde. After blocked with 3% bovine serum albumin (BSA) at room temperature for 30 min, the samples were incubated with anti-rCgTPRV4 (diluted 1:1000 (v/v) in 3% BSA) antibody at 37°C for 1 h. Alexa Fluor 488-conjugated goat anti-mouse IgG (Sangon, diluted 1:1500 in 3% BSA) was used instead of secondary antibodies. The secondary antibodies, respectively. The western blotting ECL substrate (Bio-Rad) was used to visualize the protein bands of CgTPRV4 as previously described [34].

2.8. Analysis of CgTPRV4 mRNA expression under different temperature using qRT-PCR

The different temperature treatment was performed by transferring 60 oysters from aerated seawater at 16°C to aerated seawater at 22°C, 26°C, 30°C and 34°C. Nine oysters were sampled from each group at 12 h post-treatment, and the gill collected from three individuals were pooled together as one sample, and there were three samples for each time point. Nine untreated oysters were used as blank samples. The SYBR Green qRT-PCR was carried out, and the relative expression level of CgTPRV4 was analyzed as previously described [30,31].

2.9. Analysis of CgTPRV4 mRNA expression after Vibrio splendidus stimulation using qRT-PCR

The V. splendidus stimulation experiment was performed as previously described [35]. Briefly, the oysters were sawed a narrow notch in the oyster shell adjacent to the adductor muscle, and then were acclimated for one week. Seventy oysters were equally separated into two groups. The oysters in two groups received a 100 µL injection of sterile seawater and V. splendidus (1 x 10⁵ CFU mL⁻¹) into the adductor muscle, respectively. Afterwards, nine oysters were sampled from each group at 0, 6 and 12 h post-injection, and the gill collected from three individuals were pooled together as one sample, and there were three samples for each time point. The SYBR Green qRT-PCR was carried out, and the relative expression level of CgTPRV4 was analyzed as previously described [30,31].

2.10. Statistical analysis

All data were analyzed using ANOVA followed by a multiple comparison. The results were given as mean ± SD (N ≥ 4), and statistically significant difference was designated at p < 0.05.

3. Results

3.1. Molecular characteristics and phylogenetic evolution of CgTPRV4

The open reading frame (ORF) of CgTPRV4 was of 2298 bp, encoding a polypeptide of 765 amino acid residues with an isoelectric point (pI) of 8.52 and molecular weight (MW) of 89.3 kDa. There were three typical ANK domains and six transmembrane domains in CgTPRV4 (Fig. 1). Furthermore, there were seven N-glycosylation sites (NTNR) (138-141), NKG5G250-253, NCTR237-239, NETT388-391, NRST667-670, NDT171-713, NKS651-744, and two cAMP- and cGMP-dependent protein kinase phosphorylation sites (KRKT724-727, KKS773-774), ten protein kinase C phosphorylation sites (SLR7-5, TLR5-37, TPK149-151, TSK239-241, SAK260-263, TKR129-321, SVE69-671, TDR62-674, TKR723-725, SSK733-735), nine casein kinase II phosphorylation sites (TESE102-105).
Multiple sequence alignment revealed that CgTRPV4 shared low similarity with TRPV4s from other organisms, including Trichinella spiralis (KRZ95620.1, 20.5%), Homo sapiens (XP_011536936.2, 21.3%), Mus musculus (XP_011246537.1, 22.8%), Physeter catodon (XP_028336047.1, 25.4%), Monopterus albus (XP_020449937.1, 25.7%), Larimichthys crocea (XP_019130016.1, 26.0%), Xenopus tropicalis (XP_002932129.1, 26.0%), Danio rerio (XP_005165208.1, 26.1%), Salmo salar (XP_014016243.1, 26.1%), Galleus gallus (NP_990023.2, 26.1%), and Oncorhynchus mykiss (XP_021476679.1, 26.2%). However, the ANK domains (27.1%-31.6%) and transmembrane domains (28.0%-36.8%) of CgTRPV4 shared relatively higher similarity with their homologues from other organisms (Fig. 2). The phylogenetic NJ trees were constructed to analyze the phylogenetic evolution of TRPV4s. Though CgTRPV4 shared similar domain composition with other selected TRPV4s from vertebrates (H. sapiens, M. musculus, P. catodon, G. gallus, L. crocea, M. albus, D. rerio, S. salar), the ANK domains (27.1%-31.6%) and transmembrane domains (28.0%-36.8%) of CgTRPV4 shared relatively higher similarity with their homologues from other organisms (Fig. 2).

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mykiss and X. tropicalis) and invertebrate (T. spiralis), it showed relatively far evolutionary relationships with them (Fig. 3).

### 3.2. The mRNA expression of CgTRPV4 in different developmental stages

In order to elucidate the possible role of CgTRPV4 in the ontogenesis, its mRNA expression level in different development stages was monitored by qRT-PCR. The mRNA transcript of CgTRPV4 was detected in the development stages from fertilized eggs to D-hinged larvae with the highest expression level in fertilized eggs (Fig. 4). The expression level of CgTRPV4 decreased gradually in the following development stages and reached the lowest in D-hinged larvae (0.01-fold of that in fertilized eggs, \( p < 0.05 \)) (Fig. 4).

### 3.3. The distribution of CgTRPV4 mRNA in different tissues of adult oyster

The CgTRPV4 transcripts were detected in all the examined tissues, including hepatopancreas, mantle, gill, muscle, labial palp and haemocyte. The highest mRNA expression level of CgTRPV4 was detected in gill, which was 45.08-fold \( (p < 0.05) \) of that in the hepatopancreas (Fig. 5). The expression level of CgTRPV4 mRNA in mantle (19.09-fold, \( p < 0.05 \)) was also significantly higher than that in the hepatopancreas (Fig. 5).

### 3.4. The subcellular distribution of CgTRPV4 in haemocytes

The cDNA sequence encoding the ANK domains of CgTRPV4 was amplified and ligated into the pCold™-GST DNA vector (Fig. 6A). The recombinant plasmid (pCold-GST-CgTRPV4-ANK) was transformed into E. coli Transetta (DE3). After IPTG induction for 4 h, the whole cell lysate...
was analyzed by SDS-PAGE, and a distinct band about 55.2 kDa was revealed, which was consistent with the predicted molecular weight of fusion rCgTRPV4 with GST-tag (Fig. 6B, lane2). The rCgTRPV4 was further purified for preparing the polyclonal antibody (Fig. 6B, lane3). The antibody specificity of the anti-rCgTRPV4 was examined using endogenous protein of oyster haemocytes by Western blot analysis. A distinct immune-precipitated band with a molecular weight of approximately 89.30 kDa was revealed with a similar molecular weight predicted by the target sequence (Fig. 6C, lane1).

The subcellular distribution of CgTRPV4 in oyster haemocytes was detected using immunofluorescence assay. The morphology of the haemocytes was observed in a bright field, and the nucleus stained by DAPI was shown in blue fluorescence. The positive signals of CgTRPV4 labeled by FITC were indicated in green fluorescence, and both ER and cytoplasmic membrane labeled by ER-Tracker Red and Dil were indicated with red fluorescence. The co-localization of signals for CgTRPV4 (green) and ER (red) was observed (Fig. 7). The co-localization of positive green signals for CgTRPV4 and red signals for cytoplasmic membrane was also observable (Fig. 7), indicating that CgTRPV4 were located in ER and cytoplasmic membrane of oyster haemocytes.

3.5. Temporal expression of CgTRPV4 mRNA in gill under different temperature

The expression profiles of CgTRPV4 in gill at 12 h after different temperature treatment (22°C, 26°C, 30°C and 34°C) were investigated using qRT-PCR. The mRNA transcripts of CgTRPV4 in gill were significantly up-regulated after high temperature stress at 30°C, which was 1.85-fold of that in the Blank group (p < 0.05). However, there was no significant difference between the blank group and the three experimental groups (22°C, 26°C and 34°C) (Fig. 8).
3.6. Temporal expression of CgTRPV4 mRNA in gill after V. splendidus stimulation

The expression profiles of CgTRPV4 in gill after V. splendidus stimulation were investigated using qRT-PCR. Compared to the control group, the mRNA transcripts of CgTRPV4 were significantly up-regulated at 6 h after V. splendidus stimulation (3.07-fold of that in the control group, \( p < 0.05 \)). There was no significant difference between the control group and the V. splendidus stimulation group at 0 h and 12 h post-stimulation (Fig. 9).

4. Discussion

TRPV4 usually plays an important role in sensing biological and abiotic stresses, such as pathogen infection, temperature, mechanical and osmotic pressure changes by regulating Ca\(^{2+}\) homeostasis \([1,2,17]\). In the present study, one TRPV4 was identified from Pacific oyster, which was consisted of three typical ANK domains and six transmembrane domains. The similar domain composition and conserved six transmembrane helical domains identified at the C-terminal of CgTRPV4 and mammalian TRPV4s indicated that they might perform similar...
might be some functional differentiation between them. It was reported the sensitivity of TRPV4 channel \[5, 6\], the different number of ANK assemble and transportation of TRPV4 \[3, 4\] as well as in determining stimulation. Relative expression levels of \textit{CgTRPV4} mRNA in gill were normalized to that of \textit{CgEF-\textalpha}, and the significant difference is indicated by different letters (a and b). Each value is shown as mean ± S.D. (N ≥ 4).

functions. Considering the important roles of ANK domain in the assemble and transportation of TRPV4 \[3, 4\] as well as in determining the sensitivity of TRPV4 channel \[5, 6\], the different number of ANK domains in \textit{CgTRPV4} and mammalian TRPV4s indicated that there might be some functional differentiation between them. It was reported that N-glycosylation and phosphorylation regulated TRP destination to the specialized structures \[36\], and the phosphorylation of (cGMP)-dependent kinase protein kinase phosphorylation sites would inactivate TRP \[37\], while the phosphorylation of Casein kinase II site would modulate the function of TRP \[38\]. In addition to the typical domains and motifs, there were seven N-glycosylation sites, two cAMP- and cGMP-dependent protein kinase phosphorylation sites, ten protein kinase C phosphorylation sites, nine casein kinase II phosphorylation sites, and one prokaryotic membrane lipoprotein lipid attachment site identified in \textit{CgTRPV4}, suggesting that the activity of \textit{CgTRPV4} is subjected to complex and precise regulation. In the phylogenetic NJ tree, the invertebrate TRPV4s (\textit{CgTRPV4} and \textit{TSTRPV4}) showed relatively far evolutionary relationships with TRPV4s from other vertebrates, and this was consistent with the results of multiple sequence alignment, which revealed that \textit{CgTRPV4} shared low similarity with TRPV4s from other organisms (20.5%-26.2%). The structural and evolutionary characteristics collectively suggested that \textit{CgTRPV4} was a typical member of the TRPV4 family in invertebrates, which might be involved in regulating Ca\(^{2+}\) homeostasis similar to their homologues in other species, and meanwhile, there might be a certain degree of variation in \textit{CgTRPV4}.

TRPV4 is broadly expressed in various tissues and a wide range of cells in mammalian, and can be significantly regulated by either biotic or abiotic stimulations followed by the regulation of Ca\(^{2+}\) influx \[7–16, 18–26, 39\]. It is indicated that the mRNA expression profile of \textit{CgTRPV4} is associated with their physiological function. In order to further understand the potential function of \textit{CgTRPV4}, its expression profile in different development stages, different tissues and subcellular component of oyster haemocytes was investigated. The mRNA transcripts of \textit{CgTRPV4} could be detected during the tested developmental stages with the highest level in fertilized eggs, and was significantly down-regulated from 4-cell embryos to D-hinged larvae. It was suspected that the higher level of \textit{CgTRPV4} transcripts in fertilized eggs might be endowed by parents for the capability of sensing stress. It has been demonstrated that the immune tissues and organs of bivalves usually begin to form in gastrula and mature in D-hinged larvae and veliger larvae \[40, 41\]. The level of \textit{CgTRPV4} transcripts decreased along with the developmental stages, which may be explained by the observation that the sensory organs are not fully developed till D-hinged larvae. In the adult oysters, the \textit{CgTRPV4} mRNA was constitutively expressed in all the examined tissues, and this wide distribution profile was consistent with that of mammalian TRPV4s \[7–12\]. Similar to \textit{CgTRPV4.4} with incomplete domain composition \[25\], the highest mRNA expression level of \textit{CgTRPV4} was also observed in gill, which function as the primarily sensitive tissue of molluscs responding to environmental stress \[42, 43\]. These results indicated that \textit{TRPV4} might play a key role in resistance to environmental stress in oysters. The co-localization of \textit{CgTRPV4} signals with ER and cytoplasmic membrane were observable, indicating that \textit{CgTRPV4} were located on both ER and cytoplasmic membrane of oyster haemocytes. This result was consistent with the subcellular localization of mouse TRPV4 \[44\], suggesting that \textit{CgTRPV4} might function as a Ca\(^{2+}\) channel in both cytoplasmic membrane and ER membrane of oysters. These results collectively suggested that \textit{CgTRPV4} might play a key role in resistance to environmental stress by regulating cytoplasmic Ca\(^{2+}\) homeostasis through channels on plasma membrane and ER membrane.

Accumulating evidences have shown that TRPV4 plays an important role in sensing both biotic and abiotic stresses \[2\]. In the present study, the expression profiles of \textit{CgTRPV4} mRNA under different temperature or after \textit{V. splendidus} stimulation was investigated to deduce its potential function. The mRNA transcripts of \textit{CgTRPV4} in gill were significantly up-regulated after high temperature stress at 30°C, which favored the previous conclusion that TRPV4 is a temperature sensitive channel protein \[2\]. Similarly, the heat-induced mRNA expression of TRPV4 was also observed in other TRPV4s in aquatic animals, such as \textit{O. tetrathalamus} \[23\], \textit{CgTRPV4.7} \[25\] and \textit{O. kroyeri} \[26\]. However, there was no significant change after high temperature stress at 34°C, probably due to the different temperature activation threshold of the thermoTRPV channels (TRPV1–4). The previous studies indicated that TRPV1–4 could be activated by temperatures ranging from innocuous warmth (<37°C) to noxious heat (>50°C) in heterologous systems \[2, 23, 25, 26, 45\]. It is suspected that four TRPV1s and eight TRPV4s identified in oyster genome may be responsible for sensing different temperature range \[25\]. In addition, the mRNA transcripts of \textit{CgTRPV4} were significantly up-regulated at 6 h after \textit{V. splendidus} stimulation. It was reported that the mammalian TRPV4 in different types of cells might play different functions in a variety of immune reaction, such as lipid peroxidation, inflammatory response, and phagocytosis \[13–16\]. The TRPV4 in airway epithelial cells and macrophages can be activated by LPS stimulation, which triggered downstream protective responses and anti-inflammatory pathways \[13, 14\]. Moreover, TRPV4 could protect lung from injury upon intratracheal \textit{Pseudomonas aeruginosa} in mice.

**Fig. 8.** The temporal expression of \textit{CgTRPV4} mRNA in gill under different temperature. Relative expression levels of \textit{CgTRPV4} mRNA in gill were normalized to that of \textit{CgEF-\alpha}, and the significant difference is indicated by different letters (a and b). Each value is shown as mean ± S.D. (N ≥ 4).

**Fig. 9.** The temporal expression of \textit{CgTRPV4} mRNA in gill after \textit{V. splendidus} stimulation. Relative expression levels of \textit{CgTRPV4} mRNA in gill were normalized to that of \textit{CgEF-\alpha}, and the significant difference is indicated by different letters (a, and b). Each value is shown as mean ± S.D. (N ≥ 4).
through a novel mechanism of molecular switching of LPS signaling [46], and there was a significant increase in TRPV4 in the hippocampus of a depression mouse model induced by LPS [47]. These results indicated that CgTRPV4 might share a similar role in sensing both biotic and abiotic stresses with their homologues in other species.

In conclusion, CgTRPV4, a typical member of the TRPV4 family, was identified in Pacific oyster. The CgTRPV4 transcripts were constitutively expressed in all the examined tissues with the highest expression level in gill, and CgTRPV4 located in both ER and cytoplasmic membrane of oyster haemoocytes. The expression level of CgTRPV4 mRNA in gill was significantly up-regulated after high temperature stress at 30°C or V. splendidus stimulation. All above results indicated that CgTRPV4 shared a similar role in sensing either biotic or abiotic stresses with their homologues in other species.

Declaration of Competing Interest

The authors have declared that no competing interests exist.

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