The expression profile of calnexin in *Patinopecten yessoensis* after acute high temperature stress

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ABSTRACT

Calnexin (CNX) is one of the major calcium-binding proteins in endoplasmic reticulum (ER) and plays a crucial role in regulating Ca\textsuperscript{2+} homeostasis and unfolded protein response (UPR). In the present study, *PyCNX* was identified in Yesso Scallop *Patinopecten yessoensis*. The open reading frame (ORF) of *PyCNX* was of 1794 bp encoding a putative polypeptide of 598 amino acid residues with an N-terminal signal peptide, a typical calreticulin (CRT) domain and a C-terminal transmembrane domain. The deduced amino acid sequence of *PyCNX* shared 47.91%-70.16% identities with CNXs from other species. The mRNA transcripts of *PyCNX* were constitutively expressed in all the examined tissues, including gills, gonad, hepatopancreas, mantle and haemocytes, with the highest expression level in gills. The mRNA transcripts of *PyCNX* in gills were significantly up-regulated at 1 h (*p* < 0.01), down-regulated to similar level of Blank group at 3 h (*p* > 0.05) and 6 h (*p* > 0.05), and decreased significantly from 12 to 48 h (*p* < 0.05) after acute high temperature stress (25 °C). *PyCNX* was mainly located in the ER of haemocytes. The expression profiles of *PyATF6*, *PyIRE1* and *PyGRP78* in the gills of scallops after acute high temperature stress were investigated using quantitative real-time PCR. The mRNA transcripts of *PyATF6* increased significantly at 1 h, 3 h and 6 h after acute high temperature stress (*p* < 0.01), then down-regulated to similar level of Blank group at 12 h (*p* > 0.05) and 24 h (*p* > 0.05), and finally significantly up-regulated again at 48 h (*p* < 0.05). Similar to *PyATF6*, the mRNA transcripts of *PyIRE1* were significantly up-regulated at 1 h (*p* < 0.05), 3 h (*p* > 0.01), 6 h (*p* < 0.05) and 48 h (*p* < 0.05) after acute high temperature stress. The mRNA transcripts of *PyGRP78* were significantly up-regulated at 3 h (*p* < 0.05), reached the highest level at 6 h (*p* < 0.01) after acute high temperature stress, and kept significant higher level at 12-48 h (*p* < 0.05). These results indicated that *PyCNX* was involved in the response upon the acute high temperature stress of scallops probably by regulating UPR.

1. Introduction

Calci um ion (Ca\textsuperscript{2+}) is an intracellular messenger that couples a wide-range of extracellular signals to specific responses [1]. Most of the intracellular calcium ions are stored in the endoplasmic reticulum (ER) under normal physiological conditions. However, the ER luminal Ca\textsuperscript{2+} concentration is easily affected by environmental factors, pathogens and various other factors, which subsequently results in the accumulation of unfolded and misfolded protein in the ER lumen and the occurrence of ER stress (ERS), and glucose regulated protein 78 kD (GRP78) is a major marker of ERS response [2-4]. The accumulation of misfolded and unfolded protein in ER will trigger unfolded protein response (UPR) [3]. There are three well-characterized UPR signaling pathways mediated by three ER adaptor proteins named inositol-requiring enzyme 1 (IRE1), activated transcription factor 6 (ATF6) and protein kinase R-like ER kinase (PERK), respectively [5]. For alleviating the UPR reaction caused by the disruption of Ca\textsuperscript{2+} homeostasis, there are many luminal and integral membrane proteins [2] contributing for the maintenance of the
ER luminal Ca\(^{2+}\) homeostasis, such as calnexin (CNX), one of the major calcium-binding proteins in ER of mammalian cells [5, 6]. CNX is an integral ER membrane chaperone conserved in almost all eukaryotes, which is implicated to play vital roles in many cellular functions. The mammalian CNXs are composed of three distinct structural and functional domains, a globular N-domain, an extended P-domain, and an acidic C-domain [7, 8]. The N-domain is a Ca\(^{2+}\)-binding globular \(\beta\)-sandwich, and endows CNX with monovalent glycan binding capabilities [9]. The P-domain is proline-rich, and usually contains four pairs of repeat A (IXDPXA/DXKPEDWDX) [5] and C-domain (GXWXPPXXIXNPXYX) [5]. Moreover, the characteristic KPEDWDE motifs among repeat A represent the high-affinity calcium binding domain of CNX [10]. The ER positioning signal in mammal is usually Arg-Lys-Pro-Arg-Arg-Glu (Abbreviated as RKPRRE) [11]. CNXs are involved in many cellular processes, such as protein folding and unfolded protein response (UPR), depending on their multiple functions. They usually function as key ER chaperones to promote protein folding and prevent protein aggregation by binding to the nascent glycoproteins [12, 13]. Moreover, they are involved in regulating UPR to relieve ER stress [10, 14]. It was reported that the expression of chaperone protein including CNX is mainly regulated at the transcriptional level [15, 16]. The mRNA transcripts of CNX1 from Schizosaccharomyces pombe were up-regulated upon heat shock stress [10], and CNX in Aspergillus nidulans was involved in the ER stress response by regulating Ca\(^{2+}\) homeostasis [14]. There were motifs associated with UPR controlled genes identified in the promoter of CNX genes from S. cerevisiae and Aspergillus niger [10, 17]. These evidences indicated that the CNXs could be up-regulated under ER stress and participated in the regulation of UPR.

CNXs have been identified in species ranging from yeast to humans [18]. The researches on CNXs in aquatic invertebrates mainly focused on the gene identification, expression profile analysis and potential function investigation. By far, a large number of CNXs have been identified from various aquatic invertebrates, such as sea hare Aplysia californica [19], Pacific oyster Crassostrea gigas [20], Chinese mitten crab Eriocheir sinensis [22], and so on. It was reported that CgCNX and EsCNX are located in the ER of haemocytes [20, 21]. The mRNA transcripts of CgCNX in the adductor muscle, mantle and gills of C. gigas were induced under air exposure stress [20], and those of MjCNX were up-regulated in M. japonicus challenged with Vibrio anguillarum [21]. Both mRNA and protein expression levels of EsCNx were significantly altered in E. sinensis challenge with lipopolysaccharides (LPS), peptidoglycans (PGN), Staphylococcus aureus, and V. parahaemolyticus [22]. Moreover, the recombinant EsCnx (rEsCnx) was able to bind various bacteria (Gram-positive and Gram-negative) and different polysaccharides (LPS and PGN), and assisted in the clearance of V. parahaemolyticus in vivo [22]. However, the functional characters and regulation mechanism of molluscan CNXs under high temperature stress are still not clear.

Yesso scallop Patinopecten yessoensis is a cold-water bivalve species, which has become one of the major mariculture mussels in the North Yellow Sea of China [23]. Recently, the global warming makes scallops often suffering serious summer mortalities, and the high temperature is suspected to be one of the main environmental inducers [24]. Considering the important role of CNXs in the regulation of ER calcium homeostasis which is considered as one key mechanism of the vertebrates response to high temperature stress, a CNX was identified from scallop P. yessoensis (PyCNX) in the present study with the objectives to investigate its expression profile in the gills of scallops under acute high temperature stress and to explore its involvement in regulating UPR in the response upon stress.

2. Materials and methods

2.1. Scallops, acute high temperature stress, and sample collection

Two-year-old Yesso scallops P. yessoensis with an average shell length of 4-6 cm were collected from a commercial farm in Dalian, China. They were cultured in aerated seawater at 10 °C for seven days and fed with diatom once a day before following experiments. Tissues including gill, gonad, hepatopancreas and mantle were collected from six scallops as parallel samples, and haemolymph from these scallops were extracted and centrifuged at 800 × g, 4 °C for 10 minutes to harvest the haemocytes. After addition of 1 mL Trizol reagent (Invitrogen), all the samples were stored at -80 °C for RNA extraction.

The acute high temperature stress experiment was performed by transferring 110 scallops from aerated seawater at 10 °C to aerated seawater at 25 °C according to the previous study [25, 26]. Gills were collected from six randomly selected scallops at 1, 3, 6, 12, 24 and 48 h after acute high temperature stress. Six untreated scallops were used as blank samples.

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

Total RNA isolation, cDNA synthesis and gene cloning were performed according to the previous reports [27]. The primers of PyCNX-F and PyCNX-R (Table 1) were designed according to the sequence information of PyCNX (GenBank accession No. XM_021506467.1). The PCR product was gel-purified, cloned into pMD19-T simple vector (Takara), and confirmed by DNA sequencing.

The conserved domains of PyCNX 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 phosphorylation sites were predicted using the NetPhos 1.0 Server (http://www.cbs.dtu.dk/services/NetPhos/). The N-glycosylation sites were predicted using the NetNGlyc 1.0 Server (http://www.cbs.dtu.dk/services/NetNGlyc/). The palmitoylation sites were predicted using the CSS-palm Server (http://csspalm.biocuckoo.gov/BLAST).

The neighbor-joining (NJ) phylogenetic tree of PyCNX was constructed using MEGA 6.0 software. Bootstrap trials were replicated 1000 times to derive the confidence value for phylogeny analysis [28].

2.3. Analysis of mRNA expression using quantitative real-time PCR (qRT-PCR)

The SYBR Green qRT-PCR was carried out on an ABI 7500 Real-time Thermal Cycler platform according to the manufacturer’s protocol (TaKaRa). All primers used in this assay were listed in Table 1. The

| Primer name | Primer sequences (5’-3’)
|-------------|-------------------|
| PyCNX-F | GAGGTGTGTTCTGCTGATTGG |
| PyCNX-R | TAGGGTCCCCTTCTGATTGG |
| For protein expression | |
| rPyCNX-F | CGCGGATCCGAGAAGTTTACCAACGAGGCAA |
| rPyCNX-R | CGGCTGAGTATGATGATGATGAGGTCAGTAC |
| For qRT-PCR | |
| PyEF-α-RF | GCCGCGTGTATGTAAGGAGAGA |
| PyEF-α-RTF | CTGGCATTGCTGATGGCAGT |
| PyCNX-RF | GTGGGGAATGAGGGTAATGTTGCTG |
| PyCNX-RTR | TGCCGCTGATTGAGGTCAGTAC |
| PyATF6-RF | CACAGAGCTAACCAGTATGAGG |
| PyATF6-RTF | GATGGTGCGTCTCCTTGTGAG |
| PyGRP78-RF | TGAACCTGTCGGCGTATGATG |
| PyGRP78-RTF | CCTGGGTAATGCTGATGCCAG |
| PyIRE1-RTF | AATAGATTTCAAACCTAAGG |
| PyIRE1-RTR | CTAGAATATGGGGTAATTG |
| For sequencing | |
| M13-47 | TATATTGACCTACATATAAGG |
| M13-Rv | TGATCGTATTTGCTGATGCCAGG |
elongation factor α (PyEF-α) gene (GenBank accession No. NW_018406511.1) was employed as internal control, and the relative expression levels of PyCNX, inositol-requiring enzyme-1α (PyIRE1) (GenBank accession No. XM_021515702.1), glucose-regulated protein 78 (PyGRP78) (GenBank accession No. MF318508) and activating transcription factor 6 (PyATF6) (GenBank accession No. MF499125.1) were analyzed using the comparative Ct method (2^ΔΔCt method) [28, 29]. Data were statistically analyzed through one-way ANOVA followed by multiple comparisons. Significant differences were accepted at p < 0.05.

2.4. Recombinant expression and polyclonal antibody preparation of PyCNX

Recombinant protein of PyCNX was expressed in Escherichia coli as previously described [28]. The cDNA sequence encoding the ER luminal terminal of PyCNX was amplified using gene specific primers rPyCNX-F and rPyCNX-R (Table 1) with restriction enzyme sites BamHI and XhoI. The PCR products were digested, gel-purified, and ligated into the expression vector pET-30a (Novagen). The recombinant plasmids were transformed and transformed into E. coli Transetta (DE3). The positive transformants were cultured in LB medium and induced using Isopropyl 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 PyCNX (designated rPyCNX) and the purity and concentration analysis of rPyCNX were performed as previously described [28].

To prepare polyclonal antibody, the purified rPyCNX was subcutaneously stimulate six-week-old female mice according to the previous description [28]. The anti-rPyCNX serum was stored at -80 °C for subsequent experiments.

2.5. Western blot analysis

Proteins were extracted from gills of scallops as following described. The gills (50 mg) were ground and lysed in 1 mL cell extraction buffer with 1 mM Phenylnethanesulfon fluoride (PMSF) and a protease inhibitor cocktail on ice for 30 min. The homogenates were centrifuged at 12,000 × g, 4 °C for 10 min, and the supernatant fractions were collected for following Western blot analysis.

The specificity of polyclonal antibody against endogenous PyCNX in gills was examined using Western blot analysis as previously described [28]. The proteins extracted from gills were separated using 15% SDS-PAGE. The antibodies against rPyCNX (1:1500 dilution) and the HRP-conjugated goat anti-mouse (1:2000 dilution, Sangon Biotech) were used as primary and secondary antibodies, respectively. The western lighting ECL substrate system (Thermo Scientific) was used to detect the immune-blotted protein bands of PyCNX. The results were visualized using a chemiluminescent imaging system (Amersham Imager 600).

2.6. Immunofluorescence assay

Immunofluorescence assay was carried out to investigate the subcellular localization of PyCNX in haemocytes as previously described with some modification [30]. The haemocytes were collected from five scallops, re-suspended in L15 cell culture media, deposited on 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-PyCNX (diluted 1:1000 (v/v) in 3% BSA) and commercial anti-GRP78 (ER marker molecule) (Beyotime, diluted 1:1000 in 3% BSA) antibodies at 37 °C for 1 h. The commercial anti-GRP78 antibody is produced by immobilizing rabbits with a synthetic peptide corresponding to human GRP78 which shares high identity of 81% with that of PyGRP78. Alexa Fluor 488-conjugated goat anti-mouse IgG (Sangon, diluted 1:1500 in 3% BSA) and Alexa Fluor 555-conjugated goat anti-rabbit IgG (Sangon, diluted 1:1500 in 3% BSA) were used separately to incubate with the samples at 37 °C for 1 h. After incubated with 4, 6-diamidino-2-phenylindole hydrochloride (DAPI, diluted at 1:500 with 3% BSA) for 10 min to stain the nuclei, the fluorescent images of the scallop haemocytes were obtained using a Laser Scan Confocal Microscope (ZEISS).

2.7. Statistical analysis

All data were analyzed using ANOVA followed by multiple comparisons. 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 PyCNX

The open reading frame (ORF) of PyCNX was of 1794 bp, encoding a polypeptide of 598 amino acid residues with an isoelectric point (pI) of 4.55 and molecular weight (MW) of 67.7 kDa. There was an N-terminal signal peptide, a typical CRT domain and a C-terminal transmembrane domain in PyCNX (Fig. 1A and B). Furthermore, PyCNX could be divided into N-domain, P-domain, and C-domain using CDD analysis (Fig. 1B). There were three repeat A (IXDPXA/DXKPE/SDWDE/D) and four repeat B (GXWXXP/KXI/VXNPXY/CX) identified in the P-domain, and one characteristic KPEDWDE motif was identified in repeat A (Fig. 1A and Fig. 2). The ER positioning signal RKPRKD was located in the C-domain (Fig. 1A). There were three potential phosphorylation sites (Ser98, Thr203 and Thr513), two potential N-glycosylated sites (Asn396 and Asn544) and three potential palmitoylation sites (Cys251, Cys358 and Cys497) identified in PyCNX (Fig. 1A).

Multiple sequence alignment revealed that PyCNX shared high similarity with CNXs from other organisms, including Crassostrea gigas (XP_023041211.1, 70.16%), Xenopus tropicalis (NP_001005668.1, 59.83%), Takifugu rubripes (XP_029702775.1, 59.24%), Danio rerio (XP_009306088.1, 56.72%), Homo sapiens (NP_001350923.1, 53.72%) and Mus musculus (NP_011245658.1, 51.70%). The similarity of PyCNX with CNXs from Caenorhabditis elegans (NP_499176.1, 47.91%) were relatively low (Fig. 2).

The phylogenetic NJ trees were constructed to analyze the phylogenetic evolution of CNXs. PyCNX was firstly clustered with CNXs from other mollusces (Pecten maximus, C. gigas, and Octopus vulgaris), then gathered with the CNX from Brachiospoda (Lingula anatina), and finally gathered with CNXs from the vertebrates (Mus musculus, Homo sapiens, Gallus gallus, Danio rerio, Salom salar and Takifugu rubripes). PyCNX shared relatively far evolutionary relationships with CNXs from other invertebrates (Eriocheir sinensis and Acanthaster planci) (Fig. 3).

Table 2

| Protein name | Organism     | Accession number       |
|--------------|--------------|------------------------|
| calnexin     | Homo sapiens | NP_001350923.1         |
| calnexin     | Mus musculus | NP_031623.1            |
| calnexin     | Bos taurus   | NP_001099802.1         |
| calnexin     | Gallus gallus| XP_025010618.1         |
| calnexin     | Xenopus tropicalis | NP_001005668.1 |
| calnexin     | Danio rerio  | XP_009306088.1         |
| calnexin     | Crassostrea gigas | XP_034031211.1       |
| calnexin     | Taijia rugos | XP_029702775.1         |
| calnexin     | bioden salar | XP_014067454.1         |
| calnexin     | Caenorhabditis elegans | XP_499176.1 |
| calnexin     | Patinoptecen yessoensis | XP_021362412.1 |
| calnexin     | Eriocheir sinensis | ANN4688.1 |
| calnexin     | Acanthaster planci | XP_022084498.1 |
| calnexin     | Octopus vulgaris | XP_029643676.1 |
| calnexin     | Pecten maximus | NP_033742114.1 |
| calnexin     | Lingula anatina | XP_013414188.1 |
3.2. The distribution of PyCNX mRNA in different tissues

The PyCNX transcripts were detected in all the examined tissues, including hepatopancreas, gonad, mantle, gills, and haemocytes. The highest mRNA expression level of PyCNX was detected in gills, which was 2.64-fold \( (p < 0.01) \) of that in the mantle (Fig. 4). The expression level of PyCNX mRNA in hepatopancreas (1.79-fold, \( p < 0.05 \)) and gonad (1.55-fold, \( p < 0.05 \)) was also significantly higher than that in the mantle (Fig. 4).

3.3. The subcellular distribution of PyCNX in haemocytes

The recombinant plasmid (pET-30a-PyCNX) 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 67.7 kDa was revealed, which was consistent with the predicted molecular weight of fusion rPyCNX with His-tag (Fig. 5A, lane 3). The rPyCNX was further purified for preparing the polyclonal antibody. The antibody specificity of the anti-rPyCNX was examined using endogenous protein of scallop gills by Western blot analysis. A distinct immune-precipitated band with a molecular weight of approximately 90 kDa was revealed with a similar molecular weight predicted by the target sequence (Fig. 5B, lane 1).
The subcellular distribution of PyCNX in scallop 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 PyCNX labeled by FITC were indicated in green fluorescence, and those of GRP78 labeled by propidium iodide (PI) were indicated in red fluorescence. PyCNX were mainly located in cytoplasm of haemocytes, and the co-localization of signals for PyCNX (green) and GRP78 (red) were observable, indicating that PyCNX protein located in the ER of scallop haemocytes (Fig. 6).

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3.4. Temporal expression of PyCNX mRNA in gills after acute high temperature stress

The expression profiles of PyCNX in gills after acute high temperature stress at 25°C were investigated using qRT-PCR. The mRNA transcripts of PyCNX were significantly up-regulated at 1 h after acute high temperature stress, which was 2.17-fold of that in the Blank group \((p < 0.01)\) (Fig. 7). Then, they were down-regulated to the similar level of Blank group at 3 h \((1.72\text{-fold}, p > 0.05)\) and 6 h \((1.17\text{-fold}, p > 0.05)\), and were significantly down-regulated from 12 to 48 h \(0.19\text{-}0.54\text{-fold}\) of that in the Blank group, \(p < 0.05\) after acute high temperature stress (Fig. 7).

3.5. Temporal mRNA expression of UPR key genes in gills after acute high temperature stress

The expression profiles of three UPR genes including PyATF6, PyIRE1 and PyGRP78 in gills after acute high temperature stress at 25°C were investigated using qRT-PCR. The mRNA transcripts of PyATF6 increased significantly at 1 h, 3 h and 6 h after acute high temperature stress, which were 5.09-fold \((p < 0.01)\), 6.39-fold \((p < 0.01)\) and 7.97-fold \((p < 0.01)\) of that in the Blank group (Fig. 8A). Then, they were down-regulated to the similar level of Blank group at 12 h \((p > 0.05)\) and 24 h \((p > 0.05)\), and finally up-regulated again at 48 h \(2.69\text{-fold}, p < 0.05\). Similar to PyATF6, the mRNA transcripts of PyIRE1 were significantly up-regulated at 1 h \(2.53\text{-fold}, p < 0.05\), 3 h \(5.89\text{-fold}, p < 0.01\), 6 h \(4.41\text{-fold}, p < 0.05\) and 48 h \(1.83\text{-fold}, p < 0.05\) after acute high temperature stress (Fig. 8B). The mRNA transcripts of PyGRP78 were significantly up-regulated at 3 h \(3.20\text{-fold}, p < 0.05\), reached the
highest level at 6 h (28.67-fold, \( p < 0.01 \)) after acute high temperature stress, and kept significant higher level at 12-48 h (3.17~5.47-fold of that in the Blank group, \( p < 0.05 \)) (Fig. 8C).

4. Discussion

CNX, a conserved ER membrane chaperone, usually plays an important role in promoting protein folding, preventing protein aggregation and regulating UPR to relieve ER stress in a \( \text{Ca}^{2+} \) dependent manner \cite{5,10,12,13,17}. In the present study, one \( Py\text{CNX} \) was identified from Yesso scallop, which was consisted of three conserved domains including an N-domain, a P-domain, and a C-domain. There were three repeat A (IXDPXA/DXPE/SDWDE/D) and four repeat B (GXWXXP/KXI/VXNPXY/CX) in the P-domain, which were different from mammalian to some extent \cite{5,8}, indicating that there might be some functional differentiation between \( Py\text{CNX} \) and mammalian CNX. Moreover, one characteristic KPEDWDE motif was identified in repeat A of \( Py\text{CNX} \), which was suggested to be responsible for the high-affinity calcium binding activities of CNX \cite{10}. Different from the ER positioning signal of mammalian CNXs “RKPRRE” \cite{11}, ER positioning signal “RKPRKD” was located in the C-domain of \( Py\text{CNX} \), which was consistent with that of \textit{Xenopus tropicalis} (No. NP_001005668.1). It was reported that CNX phosphorylation linked cytoplasmic signaling to ER luminal functions \cite{31}, and N-glycosylation help protein to be properly folded \cite{32}, while palmitoylation was the switch that assigned CNX either to ER \text{Ca}^{2+} \) signaling or to protein chaperoning \cite{33}. In addition to the typical domains and motifs, there were three potential phosphorylation sites, two potential N-glycosylated sites and three potential palmitoylation sites identified in \( Py\text{CNX} \), suggesting that \( Py\text{CNX} \) might play important roles in protein folding, \text{ER Ca}^{2+} \) signaling, and protein chaperoning. In the phylogenetic NJ tree, \( Py\text{CNX} \) was firstly clustered with CNXs from other molluscs, then gathered with the CNX from \textit{brachiopoda} and vertebrates, and finally gathered with CNXs from arthropod and echinoderm, indicating that molluscan CNXs shared closer evolutionary relationships with their vertebrate homologues.

![Fig. 5. SDS-PAGE and western blotting analysis of rPyCNX.](image)

(A) Lane M: protein molecular standard; Lane 1: negative control for rPyCNX (without induction); Lane 2: induced rPyCNX; Lane 3: purified rPyCNX; (B) Lane M, protein molecular standard; Lane 1: Western blot analysis of the polyclonal antibody against endogenous of PyCNX in gills.

![Fig. 6. Subcellular localization of PyCNX in haemocytes of P. yessoensis.](image)

The nucleus of haemocytes stained by DAPI is shown in blue fluorescence. The positive signals of PyCNX and PyGRP78 labeled by FITC and PI are indicated in green and red fluorescence. Bar = 10 μm.

![Fig. 7. The temporal expression of PyCNX mRNA in gills after acute high temperature stress.](image)

Relative mRNA expression levels of PyCNX in gills were normalized to that of PyEF-α, and the significant difference is indicated by * (\( p < 0.05 \)) and ** (\( p < 0.01 \)). Data are shown as mean ± S.D. (\( N \geq 4 \)).
expression levels of investigated. The mRNA transcripts of gills were all significantly up-regulated after acute high temperature stress, the mRNA expressions of three UPR key genes were [41, 42], which was consistent with the previous study [43]. It is found that the up-regulation of both PyATF6 and PyIRE1 would further regulate the expression of more ER chaperones, such as CNX [8, 44]. Consistent with the down-regulation of PyCNX to normal or lower level from 3 h to 48 h, the mRNA expression of the UPR key genes recovered to normal or lower level from 6 h to 48 h after acute high temperature stress, indicating that there was a mechanism limiting UPR signaling and maintaining it within a physiologically appropriate range [45].

In conclusion, PyCNX, a highly conserved CNX homologue, was identified in Yesso scallop. The mRNA transcripts of PyCNX were constitutively expressed in all the examined tissues with the highest expression level in gills, and PyCNX was mainly located in the ER of haemocytes. PyCNX, PyATF6, PyIRE1 and PyGRP78 showed similar expression profiles in gills after acute high temperature stress. All above results indicated that PyCNX was involved in the acute high temperature stress response of scallops probably by regulating UPR.

Declaration of Interests
The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Fig. 8. The relative mRNA expression levels of PyATF6 (A), PyIRE1 (B) and PyGRP78 (C) in gills of scallops after acute high temperature stress. Relative mRNA expression levels of PyATF6, PyIRE1 and PyGRP78 in gills were normalized to that of PyEF-a, and the significant difference is indicated by * (p < 0.05) and ** (p < 0.01). Data are shown as mean ± S.D. (N ≥ 4).

C. Yang et al.
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