Molecular characteristics of a novel HSP60 gene and its differential expression in Manila clams (*Ruditapes philippinarum*) under thermal and hypotonic stress

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Abstract The Manila clam *Ruditapes philippinarum* inhabits the intertidal zone and must therefore tolerate broad fluctuations in water temperature and salinity. Heat shock protein 60 (HSP60) is an evolutionarily conserved, multi-functional protein that plays a significant role in protecting organisms from harmful stress conditions. We cloned the *R. philippinarum* HSP60 (RpHSP60) gene and analyzed its transcriptional responses to thermal and low-salinity stresses. The complete sequence of RpHSP60 cDNA was 1777 nucleotides, containing a 1728-bp open reading frame encoding a polypeptide of 576-amino acids, with a calculated molecular mass of 61.25 kDa and predicted isoelectric point of 5.08. Comparisons of amino acid sequences and three-dimensional structures of HSP60 revealed that RpHSP60 was highly conserved in the signature HSP60-family domains. RpHSP60 mRNA was detected in all the tested tissues of *R. philippinarum*, with the highest expression levels in hemocytes. We measured RpHSP60 mRNA levels in the gills under thermal and low-salinity stresses using quantitative real-time reverse transcription-polymerase chain reaction. Following the thermal challenge, RpHSP60 mRNA was significantly up-regulated at 6 h, and then progressively downregulated under

high-temperature stress (30 °C), while only slight fluctuations were observed under low-temperature stress (−1 °C). Under low-salinity (17 ppt) stress, RpHSP60 mRNA levels were significantly increased at 3, 72, and 96 h ($P < 0.05$). These results suggest that HSP60 of *R. philippinarum* may play important roles in responding to high-temperature and low-salinity stresses.

Keywords *Ruditapes philippinarum* · HSP60 · Temperature stress · Low-salinity stress · Expression profile

Introduction

Heat shock proteins (HSPs) are evolutionarily highly conserved proteins with a range of important physiological functions. These proteins exist in all cells, ranging from bacteria to humans, and are involved in numerous essential cellular functions and immune responses (Ranford et al. 2000; Ranford and Henderson 2002). They can also be induced by denatured proteins produced as a result of environmental-stress conditions, such as heat or cold, osmotic imbalance, toxins, heavy metals, and microbial damage (Ranford et al. 2000; Chen et al. 2007; Zhou et al. 2010; Rhee et al. 2009). These proteins serve as chaperones to repair nuclear proteins, and thus protect cells from the damaging effects of environmental stresses (Bensaude 1990; Lewis et al. 1999). HSPs is classified into families based on their molecular weights, including HSP100, HSP90, HSP70, HSP60, HSP40, and several smaller HSPs (Kregel 2002).

HSP60 proteins are chaperones that are mainly localized in the mitochondria of eukaryotic cells. They consist of two back-to-back rings with a total of 14–18 subunits,

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forming a protected environment for protein folding (Zhang et al. 2010; Horwich et al. 2006). Three domains of the subunit architecture, the equatorial, intermediate, and apical domains, are preserved in all chaperones (Ditzel et al. 1998). HSP60 has been suggested to aid in folding and maintaining the conformation of 15–30% of all cellular proteins (Ranford et al. 2000). As a typical HSP, HSP60 has been shown to play important roles in cellular processes, such as development, reproduction, thermoprotection, toxic-stress response, and immune defense (Kozlova et al. 1997; Timakov and Zhang 2001). *HSP60* genes have been studied in some aquatic invertebrates, including sea anemones (*Anemonia viridis*) (Choresht et al. 2001; Choresht et al. 2004), white shrimp (*Litopenaeus vannamei*) (Zhou et al. 2010), crabs (*Portunus trituberculatus*) (Xu and Qin 2012), and the mud crab (*Scylla paramamosain*) (Yang et al. 2013). However, the stress response in mollusks has been relatively poorly studied, except the zebra mussel (*Dreissena polymorpha*) (Clayton et al. 2000), golden apple snail (*Pomacea canaliculata*) (Xu et al. 2014), Japanese scallop (*Mizuhopecten yessoensis*) (Fandong et al. 2015), and Pacific oyster (*Crassostrea gigas*) (Zhu et al. 2016), and information about the role of HSP60 in the stress response in mollusks is therefore limited.

The Manila clam, *Ruditapes philippinarum*, is a commercial mollusk species native from southern Siberia to China. They are cultured along the shore, where they burrow partially or completely in the sediment. As bivalves inhabiting the intertidal zone, clams are inevitably exposed to temperature fluctuations associated with diurnal/tidal cycles and seasonal changes (Ivanina et al. 2009), as well as to dramatic changes in water salinity as a result of the ebb and flood of the tide, combined with freshwater inputs from rivers or during periods of heavy rain. These fluctuations can cause mass mortality of cultured bivalves (Carregosa et al. 2014; Matozzo et al. 2007; Kim et al. 2001b). A better understanding of the capacities of clams to adapt to temperature and salinity stresses may thus provide new insights for improving health management and disease control in aquaculture clams. Many members of the HSP family have been identified in *R. philippinarum*, including *HSP22*, *HSP40*, *HSP70*, and *HSP90*, and have been shown to play important roles in responding to pathogen challenge and toxic stress (Liu et al. 2015; Li et al. 2011; Li et al. 2010). However, reports of HSPs in relation to environmental adaptation in clams are lacking.

The present study aimed to clone and characterize a novel *HSP60* complementary DNA (cDNA) from Manila clams (*RpHSP60*) and to examine its temporal and tissue-specific expression following thermal and low-salinity challenges. These results will further our understanding of the response mechanism of *R. philippinarum* to temperature stress and hypotonic stimulation.

Material and methods

Animals

About 200 individuals of *R. philippinarum* (25.5 ± 0.46 mm) were collected from a commercial farm in Dalian and transported to the laboratory. The clams were reared in 250-L flat-bottomed tanks in aerated seawater (salinity 32 ppt, temperature 22 ± 1 °C, no sand was added into tanks to avoid affecting the quality of stress), at a density of 60 animals per tank, for 7 days prior to the experiments.

Molecular cloning of *RpHSP60* cDNA

We identified a 513-bp sequence in our transcriptome database of *R. philippinarum* (unpublished) with high similarity to the known HSP60 protein sequence. Contiguity of the clone was confirmed by a single PCR using primers (Table 1) on 5' and 3' ends of cDNA and sequence analysis. Gene-specific primers (Table 1) were designed based on the sequence, and nested polymerase chain reaction (PCR) was performed using a SMART™ RACE cDNA amplification kit (Clontech, CA, USA) to obtain the 3' and 5' ends of *RpHSP60*, respectively. The 5' and 3' RACE PCR products were gel-purified, cloned into the pMD18-T simple vector (TaKaRa, Dalian, China), and sequenced. The full-length gene was assembled using DNAMAN 5.2 software (Lynnon Biosoft, Quebec, Canada).

Experimental design

To investigate the effect of temperature stress on *RpHSP60* gene expression, 120 individuals of *R. philippinarum* were randomly divided into two groups and placed in separate tanks at -1 °C (low temperature) and 30 °C (high temperature), respectively. Gill samples from five individuals were

Table 1 Primer names and sequences used in this study

Primer name	Sequences (5' → 3')
Contiguity-Hsp60F	GCATCGAATTTACCACTTGTACG
Contiguity-Hsp60R	CTGGAGAGTCATGTAAGGCCGGTA
5'RACE-HSP60-1	AGTATTCTTACGATTGCCACC
5'RACE-HSP60-2	TAATGACAAGTGGTTTACGGG
3'RACE-HSP60-1	CCAAATGTGAGTTTCAGGATG
3'RACE-HSP60-2	AACCACTTGTCATTATTGCTG
RT-HSP60F	CCAAATGTGAGTTTCAGGATG
RT-HSP60R	TAATGACAAGTGGTTTACGGG
β-actin-F	CTCCCTTGAGAAGAGCTACGA
β-actin-R	GATACCAGCAGATTCCATACCC

designated as control (0 h) samples, and samples from five other randomly selected individuals from each group were collected after 3, 6, 12, 24, 48, 72, and 96 h, respectively.

To study the expression profile of RpHSP60 under low-salinity stress, 60 individuals of *R. philippinarum* were put into a tank containing seawater at 17 ppt salinity at 21 ± 1 °C. Five untreated clams were used as the control samples (0 h), and another five clams were randomly collected at 3, 6, 12, 24, 48, 72, and 96 h, respectively, and gill tissues were dissected and analyzed.

RNA extraction and cDNA synthesis

To determine the expression levels of RpHsp60 in different tissues in *R. philippinarum*, hemolymph was collected from the posterior adductor muscle sinus using a syringe fitted with a 22-G needle, and hemocytes were harvested immediately by centrifugation (3000×g for 10 min at 4 °C). The animals were then placed on ice and samples of gill, digestive tract, mantle, foot, and muscle tissue were dissected, snap-frozen in liquid nitrogen, and stored at -80 °C for further analysis.

Total RNA from hemocytes and tissues was extracted using an RNA Extraction kit (Tiangen, Beijing, China) and treated with DNase-I to remove contaminating DNA. The quality of the total RNA was detected using a nucleic acid spectrometer (Bio-Rad, Hercules, CA, USA). A sample of 1-μg purified RNA was used to synthesize cDNA using a PrimeScript™ RT reagent kit (TaKaRa, Dalian, China), according to the manufacturer's instructions.

Bioinformatic analysis

The amino acid sequence of each HSP gene was deduced using ORF Finder in NCBI (<http://www.ncbi.nlm.nih.gov/gorf/gorf.html>) and analyzed using the ExPASy stands for Expert Protein Analysis System (<http://www.expasy.org>). The deduced amino acid sequence of RpHSP60 was aligned with that of other homologous proteins using the Clustal Omega Multiple Alignment program (<http://www.ebi.ac.uk/Tools/msa/clustalo/>). A phylogenetic tree was constructed using MEGA 7.0 (Sudhir Kumar, Arizona State University, Tempe, AZ, USA) using the neighbor-joining method with 5000 cycles of bootstrapping. The three-dimensional (3D) protein structures of HSP60 from *C. gigas*, *R. philippinarum*, and *Escherichia coli* were generated using the I-TASSER online server (<http://zhanglab.cmb.med.umich.edu/I-TASSER/>) and further analyzed with PyMOL version 1.3 molecular graphics software (DeLano 2002).

Expression profile analysis of RpHSP60 by quantitative PCR

All gene-specific primers were designed using Primer premier 5.0 software (Premier Biosoft International, Palo Alto, CA)

(Table 1). Real-time reverse transcription (RT)-PCR amplifications were carried out in triplicate on a 96-well rotor in a total volume of 20 μL containing 10 μL $2 \times$ SYBR PremixEx Taq (TaKaRa), 2 μL cDNA template, 0.8 μL of 10 μmol/L of each forward and reverse primer, and 6.4 μL Milli-Q water. The ultra-purified water was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA). The real-time RT-PCR program was 95 °C for 30 s followed by 40 cycles of 95 °C for 5 s, and 60 °C for 30 s (Light Cycler480 II, Roche Diagnostics, Penzberg, Germany).

Taking β-actin as the internal control, the relative expression levels of RpHSP60 were calculated using the $2^{-\Delta\Delta CT}$ method. The statistical significance of differences between groups was analyzed by one-way analysis of variance, and paired comparisons were made using Tukey-Kramer test for multiple paired comparisons, using SPSS 11.5 software (SPSS Inc., Chicago, IL, USA). Differences were considered significant at $P < 0.05$.

Results

Characterization of RpHSP60 cDNA

The complete sequence of RpHSP60 cDNA was 1777 bp long (GenBank accession no. KT987978), containing a 1728-bp open reading frame encoding a polypeptide of 575 amino acids, a 21-bp 5' untranslated region (UTR), and a 49-bp 3' UTR with a poly(A) tail (Fig. 1). The calculated molecular mass of RpHSP60 was 61.25 kDa and the predicted isoelectric point was 5.08. Searching the NCBI database and using the patmatmotif program in EMBOSS identified a 26-amino acid presequence required for import into the mitochondria at the N terminus, typical of mitochondrial HSP60s, the ATP-binding motif, and a conserved GGM repeat at the C-terminal end (Supplementary Fig. 1).

Sequence comparisons and phylogenetic analysis of RpHSP60

The deduced amino acid sequence of RpHSP60 showed a high degree of similarity to other reported HSP60s (Supplementary Fig. 2). RpHSP60 exhibited significant sequence identity of 78.15–81.55% with HSP60 proteins from other mollusks, and 76.06 and 76.58% identity with *Rattus norvegicus* (CAA38564.1) and *Gallus gallus* (NP_001012934.1), respectively. Multiple sequence alignment analysis revealed that the functional structure of the deduced HSP60 protein, including an ATP/Mg²⁺ binding site, hinge regions, and ring oligomerization interface, was also conserved in RpHSP60.

The 3D model of RpHSP60 was modeled employing I-TASSER online platform, using a number of sequences which

Fig. 1 Comparison of monomeric structures of HSP60 proteins from *Escherichia coli* (a), *Crassostrea gigas* (b), and *Ruditapes philippinarum* (c). The oligomeric and monomeric structure of HSP60 from *E. coli* (PDB ID codes 4aaq and 1kp8A, respectively) are shown in

a, and the three domains of the HSP60 monomers from *C. gigas* and *R. philippinarum* are boxed with green (equatorial domain), yellow (intermediate domain) and blue (apical domain)

share 48.5–62.6% sequence identity with the query sequence as templates, including the HSP60 sequences of *C. gigas* and *E. coli*. The C-score (confidence score) was used for estimating the quality of the predicted models by I-TASSER (Zhang 2008). The 3D model of RpHSP60 was predicted adopting the same approach. Molecular graphic software (PyMOL) was applied for decorating the retrieved models of HSP60 from *C. gigas*, *R. philippinarum*, and *E. coli* (Fig. 1). The results indicated the three crucial domains including the apical, equatorial, and intermediate of HSP60 were nearly in the same positions, suggesting that they may form similar crystal structure and perform comparable functions (Nisemblat et al. 2015).

Based on the amino acid sequences of HSPs from other organisms, we generated a phylogenetic tree using the

neighbor-joining method and the maximum-likelihood method, and both phylogeny methods produced the same results, but only one representative tree is shown in Fig. 2. HSP60 family members were divided into two sub-groups, one including vertebrates, mollusks and arthropods, and the other comprising bacteria. As expected, RpHSP60 was most closely related to HSP60 sequences from other mollusks.

Tissue-specific expression of RpHSP60 mRNA

The results of real-time RT-PCR revealed that RpHSP60 was expressed in all the tested tissues, including the mantle, gill, digestive gland, adductor muscle, foot, and hemocyte. The

Fig. 2 Neighbor-joining phylogenetic tree of RpHSP60 protein sequences and other selected species. The RpHSP60 is marked by the black triangle. Aligned sequences were bootstrapped 5000 times. The

GenBank accession numbers for amino acid sequences of all the HSP60 are shown next to each species

highest expression levels were detected in hemocyte ($P < 0.05$) (Fig. 3).

Expression analysis of RpHSP60 mRNA under temperature stress

Under high-temperature stress, RpHSP60 mRNA expression dropped slightly at 3 h, increased to reach a peak at 6 h ($P < 0.05$), and was then downregulated gradually to normal levels (Fig. 4a). In contrast, only slight fluctuations in RpHSP60 mRNA levels were observed under low-temperature conditions (Fig. 4b).

Expression analysis of RpHSP60 mRNA under low-salinity stress

Under conditions of low salinity, RpHSP60 mRNA expression levels increased to peaks at 3, 72, and 96 h of 2.18-, 3.23-, and 2.90-fold that in the pretreatment group, respectively, and fluctuated within a narrow range at other time points (Fig. 5) ($P < 0.05$).

Discussion

In this study, we cloned the full-length cDNA of HSP60 from *R. philippinarum*. Multiple-sequence-alignment analysis

Fig. 3 The tissue distribution of RpHSP60 mRNA in mantle, gill, digestive gland, adductor muscle, foot, and hemocyte by qRT-PCR using β -actin as a reference gene. The values are shown as mean $\pm$ standard deviation ($n = 4$; *significantly different from mantle, $p < 0.05$)

Fig. 4 Temporal expression profile of *RpHSP60* in gills under high-temperature stress (a) and low-temperature conditions (b). Relative fold change in expression was compared to that of 0 h at different time points. Vertical bars represent the mean $\pm$ S.D. ($n = 4$) and significant differences are indicated with lowercase letters at $P < 0.05$

showed that the conserved sequences and characteristic motifs, including HSP60 family signatures, ATP-binding sites,

Fig. 5 Temporal expression profile of *RpHSP60* in gills under low-salinity stress. Relative fold change in expression was compared to that of 0 h at different time points. Vertical bars represent the mean $\pm$ S.D. ($n = 4$) and significant differences are indicated with lowercase letters at $P < 0.05$

and GGM repeats at the C-terminal end, were highly conserved in *RpHSP60* (Brocchieri and Karlin 2000; Tsugeki et al. 1992). The typical structural and functional domains of Hsp60 proteins, including the apical, equatorial, and intermediate domains, were also present in *RpHSP60*, as demonstrated by 3D-structure comparisons (Pereira et al. 2012). Construction of a phylogenetic tree showed that *RpHSP60* clustered with sequences from other mollusks. Molecular-feature analysis, multiple sequence alignment, 3D-structure comparisons, and phylogenetic analysis confirmed *RpHSP60* as a new member of the HSP60 family. The high degree of similarity in the crucial domains suggests that *RpHSP60* might share similar regulatory functions with other known HSP60s.

The distribution *RpHSP60* gene expression could provide useful information for analyzing its biological function in mollusks. We detected constitutive *RpHSP60* mRNA expression in all the tested tissues. Constitutive expression of *HSP60* has also been reported in several taxa of marine invertebrates, including the mud crab *S. paramamosain* and *P. trituberculatus* (Yang et al. 2013; Xu and Qin 2012), white shrimp *L. vannamei* (Zhou et al. 2010), sea anemones *A. viridis* (Choresch et al. 2001), Mediterranean sponges *Tetilla* sp (Choresch et al. 2004), and scallops *M. yessoensis* (Fandong et al. 2015). Particularly high expression levels of *RpHSP60* were found in hemocytes, which play an important role in the response to bacterial challenge and environmental stress. This was in accord with the results in *S. paramamosain* (Yang et al. 2013), indicating that *RpHSP60* was required to maintain cell homeostasis in *R. philippinarum*. In addition, some studies show that hemocyte of mollusca may be one of the important tissue induces new synthesis of the heat shock protein to cope with stress (Cellura et al. 2006; Gao et al. 2007; Farcy et al. 2007; Wang et al. 2009; Gao et al. 2008).

HSPs are known to be involved in preventing protein denaturation and in processing proteins denatured as a result of oxidative damage in response to thermal stress (Kregel 2002). Reactive oxygen species (ROS) in the mitochondria of the Antarctic bivalve *Laternula elliptica* were significantly increased under heat stress (Heise et al. 2003), while ROS production in hemocytes was increased following acute temperature elevation in eastern oysters, *Crassostrea virginica*, and in *Chlamys farreri* (Hégaret et al. 2003; Chen et al. 2007). In the intertidal mud clam *Mya arenaria*, mitochondrial function was reported to be temperature dependent and to correlate significantly with the production of ROS (Abele et al. 2002). High-temperature stress and the concomitant increase in ROS production may lead to an accumulation of denatured proteins in the cells, thus triggering HSP expression. HSP60 is thought to play a key role in the resistance to oxidative stress (Lee et al. 2008). A *Hsp60* gene was induced by higher temperatures in the mud crab *S. paramamosain* (Yang et al. 2013). In our study, *RpHSP60* expression in the gill of *R. philippinarum* were increased after 6 hours of high temperature stress.

Similarly, elevated *HSP60* expression in the gills to increase their resistance to heat stress have been reported in rainbow trout *O. mykiss* and mussel *Mytilus edulis* (Shi et al. 2015; Sanders et al. 1992). This suggests that RpHSP60 may be involved in the adaptive response to heat stress by maintaining correct protein folding (Oksala et al. 2014). In contrast, cold treatment had little effect on RpHSP60 expression in the current study, in accordance with a previous study of *HSP60* expression levels in the gills of *P. canaliculata* (Xu et al. 2014). This may be because low temperature reduced ROS-production rates in *R. philippinarum*, in line with the general perception of the effects of body temperature on biochemical and enzymatically catalyzed cellular reactions (Abele and Puntarulo 2004). However, low temperature resulted in decreased expression of one *HSP60* family member in *C. gigas* and increased expression of another, with maximal effects at 7 days (Zhu et al. 2016). The apparent differences in the responses of *HSP60* to low-temperature stress may be associated with their different locations in cells or differences between specific organs (Lee et al. 2008; Eder et al. 2009).

Salinity stress is considered to affect the folding and transformation of proteins, and HSPs are thought to play important roles in the refolding of stress-denatured proteins in organisms subjected to osmotic stress (Smurov et al. 2007; Xu and Qin 2012). In bivalves, the gills are in direct contact with the external environment and are thus assumed to be sensitive to environmental changes (Freas and Grollman 1980; Powell et al. 1982; Raftopoulou and Dimitriadis 2011; Gliński and Jarosz 1996). In our study, RpHSP60 expression levels in *R. philippinarum* gills peaked at 3, 72, and 96 h during low-salinity stress (17 ppt), compared to the *HSP60* gene in *P. trituberculatus*, which demonstrated a similar expression pattern after low-salinity challenge (Xu and Qin 2012). Notably, the increases in *HSP60* expression in both *R. philippinarum* and *P. trituberculatus* were not continuous, and the changes may reflect changes in osmolarity of the body fluid in mollusks. Bivalves are often subject to frequent and rapid changes in the osmolarity of their external medium, and utilize their shell-closing mechanism to help them withstand sudden osmotic stress, and free intracellular amino acids to regulate the osmolarity of the intracellular medium (Powell et al. 1982; Hoyaux et al. 1976; Carregosa et al. 2014), resulting in discontinuous osmotic-pressure changes in the body fluid. In the gastropod *Littorina littorea*, the osmolarities of both the perivisceral fluid and the blood decreased rapidly from 0 to 5 h and 136–150 h after direct transfer from pure sea water to 40% sea water (Hoyaux et al. 1976). These results suggest that HSP60 may be involved in adaptive responses to low salinity and indicate a possible positive relationship between RpHSP60 induction and rapid changes in salinity. Further studies are needed to confirm this speculation.

Conclusion

In this study, we identified a *HSP60* gene in the Manila clam *R. philippinarum*, with constitutive expression in various tissues. The dramatic upregulation of RpHSP60 expression following exposure to high temperature and low salinity suggests that it participates in essential survival responses to environmental stresses in mollusks.

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