Molecular characterization and transcriptional analysis of a crustacean heat shock protein 10 gene in shrimp *Litopenaeus vannamei*

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Abstract

Heat shock proteins (HSPs) are the most abundant and ubiquitous soluble intracellular proteins which conserved phylogenetically in all living organisms from archaeabacteria to humans. Recent research achievements indicated that HSP10s might not only be involved in the responses to environmental stresses, but also play a pivotal role in the host defenses mechanism. In the present study, a cDNA of 715 bp for the Pacific white shrimp *Litopenaeus vannamei* HSP10 (designated as *LvHSP10*) was cloned via rapid amplification of cDNA ends (RACE) technique. The complete cDNA sequence of *LvHSP10* contained an open reading frame (ORF) of 309 bp, which encoded a protein of 102 amino acids. The protein sequence of *LvHSP10* shared over 80% similarity with previously identified HSP10s. There were a CPN10 domain and a chaperonins HSP10/CPN10 signature in the protein sequence of *LvHSP10*. The mRNA transcripts of *LvHSP10* were constitutively expressed in all the tested tissues, including eyestalk, gill, gonad, heart, hemocytes, hepatopancreas, intestine, muscle, nerve and stomach, with the highest expression level in hepatopancreas. The mRNA expression profiles of *LvHSP10* in hepatopancreas could be significantly induced by the stimulation of *Vibrio parahaemolyticus*, white spot syndrome virus (WSSV), and low and high pH challenge. These results provided useful information of the potential roles of *LvHSP10* in the defense mechanism of shrimp against various biological stimulations and multiple environmental stresses.

Key Words: heat shock protein 10; hepatopancreas; *Litopenaeus vannamei*

Introduction

Heat shock proteins (HSPs) are the most abundant and ubiquitous soluble intracellular proteins which phylogenetically conserved in all living organisms from prokaryotes to eukaryotes (Schlesinger, 1990). In recent years, HSPs have attracted considerable interest among immunologists in the context of transcriptional regulation, evolution and innate immunity (Feder and Hofmann, 1999). According to the molecular mass, HSPs could be classified into several families, such as HSP100s, HSP90s, HSP70s, HSP60s, HSP40s, low molecular mass HSPs (small HSPs) and so on (Sørensen et al., 2003). To date, a large number of HSP members have been identified. Among them, HSP10 was first discovered and identified in the serum of pregnant women and displayed immunosuppressive properties (Morton et al., 1974). HSP10 is a highly conserved 10 kDa protein (Hartman et al., 1992), which co-chaperones with another heat shock protein HSP60 for protein folding as well as the assembly and disassembly of protein complexes to be involved in many biological processes, such as cell apoptosis (Lin et al., 2001), cellular differentiation (Cappello et al., 2005), cell proliferation (Sasu et al., 2001) and innate immunity (Chen et al., 1999). For example, mammalian HSP10 exhibited anti-inflammatory activity by inhibiting lipopolysaccharide (LPS) induced Toll-like receptor (TLR) signaling pathway via interactions with HSP60 (Dobbin et al., 2005). Moreover, HSP10 alone could be widely involved in protecting cells from stresses caused by infection, inflammation and so on (Jia et al., 2011).

Up to the present, most studies of HSP10 are
focused on typical model organisms (Sun and MacRae, 2005). And relatively little of gene information regarding HSP10 has been obtained from marine animals, such as Apostichopus japonicas (Xu et al., 2014), Lutjanus sanguineus (Zhang et al., 2011), Oryzias latipes (Hirayama et al., 2006), Penaeus monodon (Shi et al., 2016), Salmo salar (Andreasen et al., 2009), Scylla paramamosain (Ding et al., 2013) and Xenopus tropicalis (Klein et al., 2002). A clearly time-dependent mRNA expression pattern of HSP10 identified in Lutjanus sanguineus indicated that HSP10, might co-chaperoned with HSP60, played a pivotal role in the host defenses mechanism of marine animals (Zhang et al., 2011). However, information about HSP10s from marine animals is still few and fragmentary, and more research evidences are still needed to illustrate their roles in the innate immune system.

The Pacific white shrimp Litopenaeus vannamei has been widely cultured in the world as an important commercial species (Li and Xiang, 2013a). However, in the past two decades, outbreaks of infectious disease associated with bacteria, such as Vibrio parahaemolyticus, and viruses, such as white spot syndrome virus (WSSV), have become a major constraint, resulting in mass shrimp mortality, reductions in farmed shrimp production and considerable economic losses (Li and Xiang, 2013b; Zhang et al., 2016). Moreover, as an aquatic livestock, white shrimp are also suffered from multiple environmental stress during culture, and some of them, such as ammonia/nitrite accumulation, hypoxia stress and pH challenge, could be harmful to the survival of shrimps (Liang et al., 2016; Han et al., 2017a, b). A better understanding of mechanisms of stress tolerance is necessary for the health management of shrimp farming. In the present study, a novel HSP10 genes have been cloned and investigated in white shrimp (designated as LvHSP10), and the main objectives of the present study were (1) to characterize the molecular feature of LvHSP10 genes, (2) to detect the tissue distribution of its mRNA transcripts and (3) to investigate its temporal mRNA expression pattern after invading microbes and environmental factors stimulation and compare it with the previously identified white shrimp HSP60 (designated as LvHSP60).

Materials and Methods

Shrimp and tissues sample collection

The white shrimps used in the present study were obtained from Ruizi Seafood Development Co. Ltd., Qingdao, China, and all the experiments were conducted in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (NIH). The study protocol and all experimental design were conducted with approval from Experimental Animal Ethics Committee of Institute of Oceanology, Chinese Academy of Sciences. White shrimps, with body weight 8 - 12 g, were cultured placed in 640 L cylindrical tanks with 500 L air-pumped circulating seawater at 20 ± 1 °C for two weeks before processing. Hemolymph was extracted from the ventral sinus of at least three untreated shrimps using a sterile syringe preloaded with equal volume of anticoagulant buffer (NaCl 510 mmol L⁻¹, glucose 180 mmol L⁻¹, citric acid 200 mmol L⁻¹, tri-sodium citrate 30 mmol L⁻¹ and EDTA-2Na 10 mmol L⁻¹, pH 7.3). Then the hemocytes were collected by centrifugation at 800g for 10 min at 4 °C. Tissues including eyestalk, gill, gonad, heart, hepatopancreas, intestine (mid gut), muscle, nerve and stomach were collected from at least three untreated shrimps, kept in RNAlater (AM7020, Thermo Fisher Scientific, USA) and stored at -80 °C until RNA isolation.

Table 1 Oligonucleotide primers used in the present study

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’-3’)</th>
<th>Tm (°C)</th>
<th>Brief information</th>
</tr>
</thead>
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<tr>
<td>adaptor primer</td>
<td>GGCACGCCTCGACTAGTAC</td>
<td>60</td>
<td>Anchor primer for 3' RACE</td>
</tr>
<tr>
<td>adaptor primer-o (dT)</td>
<td>GGCACGGCTCGACTAGTAC</td>
<td>60</td>
<td>Oligo (dT) for cDNA synthetize</td>
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<td>LvEF-1α-qRT-F</td>
<td>GTATTGGAACAGTGCCGT</td>
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<td>Internal control for real-time PCR</td>
</tr>
<tr>
<td>LvEF-1α-qRT-R</td>
<td>CATCTCCACAGCTTACCTCAG</td>
<td>60</td>
<td>Internal control for real-time PCR</td>
</tr>
<tr>
<td>LvHSP10-CDS-F</td>
<td>ATGGCTGGTCTCTGAGAGTGGT</td>
<td>64</td>
<td>Gene specific primer for CDS</td>
</tr>
<tr>
<td>LvHSP10-CDS-R</td>
<td>TACTCGGCTCTCATCTTGCCAAAAG</td>
<td>64</td>
<td>Gene specific primer for CDS</td>
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<td>LvHSP10-qRT-F</td>
<td>GTTGTGGTGCAGAGGAGGA</td>
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<td>LvHSP10-qRT-R</td>
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<td>Gene specific primer for real-time PCR</td>
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<td>M13-47</td>
<td>CGCCAGGTTTTCAGTCACGAC</td>
<td>56</td>
<td>Vector primer for sequencing</td>
</tr>
<tr>
<td>RV-M</td>
<td>GAGCGGAATACATAATTCACACAGG</td>
<td>56</td>
<td>Vector primer for sequencing</td>
</tr>
</tbody>
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Immune stimulation, pH challenge assay and sample collection

Approximately 200 shrimps were employed for microbe stimulation assay. The V. parahaemolyticus suspension and WSSV stock were prepared according to previous reports (Yi et al., 2014; Xia et al., 2015; Sha et al., 2016). The shrimps were randomly divided into three groups and each group contained about 60 - 70 individuals. The shrimps were received an injection at the abdominal segment with 100 μL phosphate buffered saline (PBS, pH 7.4, Thermo Fisher Scientific, USA), V. parahaemolyticus suspension (1×10^5 CFUs μL^−1, in PBS) and WSSV stock (1×10^5 copies μL^−1, in PBS), respectively. The injected shrimps were returned to seawater tanks immediately and the hepatopancreas of at least three individuals were randomly sampled from each group at 3, 6, 12, 24 and 48 h post injection, kept in RNA later and stored at -80 °C until RNA isolation.

Approximately 200 shrimps were employed for pH challenge assay. The shrimps were randomly divided into three groups and each group contained about 60 - 70 individuals. Two kind of seawater at pH 6.7 and pH 9.7 were prepared using 4 mol L^−1 HCl or 4 mol L^−1 NaOH. Two groups of shrimps were amongst the seawater at pH 6.7 and pH 9.7 after acclimation in normal seawater (pH 8.2), respectively. The pH values were measured daily using a pH meter (PHB-4, Yidian Scientific, China). The hepatopancreas of at least three individuals in the control group and experiment groups were collected at 1, 3, 7, 14 and 28 d after the pH challenge, kept in RNA later and stored at -80 °C until RNA isolation.

RNA isolation and cDNA synthesis

Total RNA was isolated from various tissues using TRIzol reagent (15596026, Thermo Fisher Scientific, USA). The synthesis of first strand was carried out with Promega M-MLV using the DNase I (RQ1, M6101, Promega, USA) treated total RNA as template and adaptor primer - oligo (dT) as primer (Table 1). The reaction mixture was incubated at 42 °C for 1 h, terminated by heating to 95 °C for 5 min, and then stored at -80 °C.

Cloning the full-length cDNA of LvHSP10

The partial length sequence of LvHSP10 cDNA was obtained from the transcriptome database of white shrimp (Zhao et al., 2017). Two gene-specific primers, LvHSP10-RACE-F1/2, were designed using Primer Premier 5.0.0 based on this partial length sequence to clone the 3’ end of LvHSP10 cDNA by rapid-amplification of cDNA ends (RACE) technique. And the coding sequence (CDS) of LvHSP10 was amplified and confirmed using another two gene-specific primers, LvHSP10-CDS-F/R, which was also designed using Primer Premier 5.0.0. All PCR amplification was performed in a MJ Mini Personal Thermal Cycler (Bio-Rad, USA), and the PCR products were purified using Monarch DNA Gel Extraction Kit (T1020S, NEB, USA) and cloned into the pMD18-T simple vector (D103A, Takara, Japan). After being transformed into the competent cells Escherichia coli strain DH5α (CB101-03, Tiangen, China), the positive recombinants were identified via anti-ampicillin selection and verified by PCR screening using M13-47 and RV-M primers (Table 1). Three of the positive clones were sequenced using a

Fig. 1 Nucleotide and deduced amino acid sequences of LvHSP10. The nucleotides and amino acids were numbered along the left margin. The function domain was in shade. The asterisks indicated the stop codon.
PRISM 3730XL automated sequencer (Thermo Fisher Scientific, USA).

Bioinformatical analysis of LvHSP10 cDNA and protein sequences

The search for protein sequence similarity was conducted with blastp 2.6.0. The deduced protein sequences of LvHSP10 were analyzed by the EditSeq module in Lasergene program suite 14.0.0.88. The function domains of LvHSP10 were predicted with Simple Modular Architecture Research Tool (SMART) 7.0. Multiple sequence alignments were performed with Clustal Omega 1.2.4 and visualized using multiple alignment show module in Sequence Manipulation Suite 2.0. A Neighbor-Joining (NJ) phylogenetic tree was constructed with MEGA 7.0.21. To derive confidence value for the phylogeny analysis, bootstrap trials were replicated 1,000 times.

Expression pattern analysis via real-time quantitative RT-PCR

The mRNA transcripts of LvHSP10 and the previous identified LvHSP60 (Zhou et al., 2010) in different tissues or their temporal expression pattern in hepatopancreas of shrimps stimulated with various microbes or pH challenge were investigated by quantitative real-time PCR (qRT-PCR) technique. All qRT-PCR reactions were performed with the SYBR premix ExTaq (Tli RNaseH plus) (RR420, Takara, Japan) using 100 ng cDNA template in a LineGene K FQD-48A (A4) Fluorescence Quantitative PCR Detection System (Bioer, China). All the primers for qRT-PCR were designed using PerlPrimer 1.1.21 and listed in Table 1. The mRNA expression levels of LvHSP10 and LvHSP60 were normalized to those of elongation factor 1 α (EF-1α) for each sample. The relative mRNA expression levels of LvHSP10 and LvHSP60 were generated using comparative Ct method (2ΔΔCt method) (Schmittgen and Livak, 2008). The data were subjected to one-way analysis of variance (ANOVA) followed by a multiple comparison using IBM SPSS Statistics 23.0.0.0, and the p values less than 0.05 were considered statistically significant.

Results

Sequence features of LvHSP10

The full-length cDNA sequence of LvHSP10 was obtained by 3' RACE technique, and deposited in GenBank under the accession number MF062460. It comprised 715 bp, containing a 5' untranslated (UTR) of 80 bp, a 3' UTR of 326 bp with a poly A tail and an open reading frame (ORF) of 309 bp. The ORF encoded a polypeptide of 102 amino acid residues with a calculated molecular mass of approximately 11.04 kDa and a theoretical isoelectric point (pI) of 8.12. The deduced amino acid sequence of LvHSP10 contained a CPN10 domain (from R7 to M99, Fig. 1). The deduced protein sequence of LvHSP10 exhibited high similarity with other previously identified HSP10s, such as 93 % identity with that of P. monodon (ALS05376) (Shi et al., 2016) and 80 % with S. paramamosain (AGI74966) (Ding et al., 2013). An alignment of the protein sequence of LvHSP10 with those of previously identified HSP10s was shown in Figure 2, and a chaperonins HSP10/CPN10 signature (from F8 to I32) was revealed. The NJ phylogenetic tree based on protein sequences from multiple HSP10s was positioned separately into two main branches, and LvHSP10 was clustered with its homologue from the black tiger shrimp P. monodon (Fig. 3).

![Fig. 2](image-url)

Multiple alignments of LvHSP10 with previous known HSP10s. The black shadow region indicated positions where all sequences share the same amino acid residue. Similar amino acids were shaded in grey. Gaps were indicated by dashes to improve the alignment. The chaperonins HSP10/CPN10 signatures were boxed. The sequences and their accession numbers are as follows: *Daphnia magna*, KZS03047; *Hyalella azteca*, XP_018022648; *Nicrophorus vespilloides*, XP_017783781; *Penaeus monodon*, ALS05376 and *Scylla paramamosain*, AGI74966.
**Fig. 3** Consensus neighbor-joining phylogenetic based on the protein sequences of HSP10s from different organisms. The evolutionary history was inferred using the Neighbor-Joining method. The bootstrap consensus tree inferred from 1,000 replicates was taken to represent the evolutionary history of the taxa analyzed. All positions containing gaps and missing data were eliminated. The numbers at the forks indicated the bootstrap value. The sequences and their accession numbers are as follows: Apostichopus japonicas, AIF71190; Galeruca daurica, ARR95803; Gallus gallus, AAB86581; Homo sapiens, CAA53455; Ixodes pacificus, AAT92186; Lutjanus sanguineus, ADM63094; Monopterus albus, AAV37068; Musca domestica, AQY54358; Oryzias latipes, CAB40895; Paracyclopina nana, ADV59557; Paralichthys olivaceus, ABB76383; Penaeus monodon, ALS05376; Salmo salar, NP_001133144; Scylla paramamosain, AGI74966; Tigriopus japonicas, ACA03519 and Wuchereria bancrofti, EJW73405.

**Tissue distribution of LvHSP10**

The qRT-PCR was employed to detect the tissue distribution of LvHSP10 mRNA transcripts with EF-1α as internal control. The LvHSP10 mRNA transcripts could be detected in all the tested tissues, including eyestalk, gill, gonad, heart, hemocytes, hepatopancreas, intestine, muscle, nerve and stomach. The highest mRNA expression level was found in hepatopancreas, which was 9.12-fold ($p < 0.05$) of that in muscle, followed by hemocytes, gill and intestine, which were 5.19-fold, 4.51-fold and 4.27-fold of that in muscle ($p < 0.05$), respectively (Fig. 4).

**Expression profiles of LvHSP10 and LvHSP60 post microbe stimulation**

The mRNA expression levels of LvHSP10 and LvHSP60 were all up-regulated post the two kinds of microbe stimulation. The mRNA expression level of LvHSP10 was significantly up-regulated at 6 h post V. parahaemolyticus stimulation (3.37-fold compared with the origin level, $p < 0.05$), and the highest level was observed at 12 h (5.99-fold, $p < 0.05$, Fig. 5A). While after stimulated with V. parahaemolyticus, the mRNA scripts of LvHSP60 significantly increased at 3 h post stimulation (2.34-fold, $p < 0.05$) and reached the peak at 12 h (7.10-fold, $p < 0.05$), kept at a high level at 24 h (2.27-fold, $p < 0.05$) and then decreased to the origin level at 48 h (Fig. 5B). In the WSSV stimulation group, the mRNA transcripts of LvHSP10 significantly increased at 3 h post stimulation (1.97-fold, $p < 0.05$) and reached the maximum level at 12 h (8.15-fold, $p < 0.05$), kept at a high level at 24 h (4.19-fold, $p < 0.05$) and then decreased but still higher than the origin level at 48 h (2.17-fold, $p < 0.05$, Fig. 5C). While the mRNA expression level of LvHSP60 was significantly up-regulated at 6 h post stimulation (6.21-fold, $p < 0.05$) and reached the peak at 12 h (9.12-fold, $p < 0.05$), kept at a high level at 24 h (5.93-fold, $p < 0.05$) and then down-regulated but still higher than the origin level at 48 h (2.21-fold, $p < 0.05$, Fig. 5D).

**Expression profiles of LvHSP10 and LvHSP60 post high or low pH change**

In the high pH challenge experiments, the mRNA expression level of LvHSP10 was significantly up-regulated at 1 d post high pH challenge (3.97-fold, $p < 0.05$) and reached the peak at 12 h (7.10-fold, $p < 0.05$), kept at a high level at 24 h (2.21-fold, $p < 0.05$) and then down-regulated to a
Fig. 4 Tissue distribution of *Lv*HSP10 mRNA transcripts detected by qRT-PCR technique. The mRNA transcripts levels in eyestalk, gill, gonad, heart, hemocytes, hepatopancreas, intestine, muscle, nerve and stomach of three untreated shrimps were normalized to that of muscle. The EF-1α gene was used as an internal control to calibrate the cDNA template for each sample. Vertical bars represent mean ± SD (n = 3), and bars with different characters were significantly different (*p* < 0.05), while bars with same characters were not significantly different.

lower level at 28 d (0.43-fold, *p* < 0.05, Fig. 6A). During the high pH challenge experiments, the mRNA transcripts of *Lv*HSP60 significantly increased at 1 d post high pH challenge (3.39-fold, *p* < 0.05) and reached the peak at 7 d (9.10-fold, *p* < 0.05) and then decreased to a lower level at 28 d (0.39-fold, *p* < 0.05, Fig. 6B). In the low pH challenge group, the mRNA scripts of *Lv*HSP10 significantly increased at 3 d post low pH challenge (3.87-fold, *p* < 0.05) and reached the peak at 7 d (5.75-fold, *p* < 0.05), kept at a high level at 14 d (3.62-fold, *p* < 0.05) and then decreased to the origin level at 28 d (Fig. 6C). While the mRNA expression level of *Lv*HSP60 was significantly up-regulated at 1 d post low pH challenge (2.47-fold, *p* < 0.05) and reached the peak at 7 d (6.95-fold, *p* < 0.05), kept at a high level at 14 d (2.58-fold, *p* < 0.05) and was then down-regulated to the origin level at 28 d (Fig. 6D).

**Discussion**

HSPs are the most abundant and ubiquitous soluble intracellular proteins which conserved phylogenetically in all living organisms, including archaeabacterial, bacteria and eukaryotes (Schlesinger, 1990). Recent research achievements indicated that HSP10s might not only be involved in the responses to environmental stresses, but also play essential roles in the innate immune defenses mechanism (Jia et al., 2011). However, information about HSP10s from marine animals is still few and fragmentary. In the present study, the full-length cDNA of HSP10 was cloned from white shrimp *L. vannamei*. The deduced polypeptide of *Lv*HSP10 consisted of 102 amino acids, and its calculated molecular weight was 11.04 kDa, which was close to those from vertebrate and invertebrate. The protein sequence of *Lv*HSP10 shared over 80% similarities with other identified HSP10s. Moreover, a CPN10 domain and a chaperonins HSP10/CPN10 signature were revealed from the amino acid sequence of *Lv*HSP10. Additionally, in the NJ phylogenetic tree, *Lv*HSP10 were clustered with its homologue from the black tiger shrimp *P. monodon*. The conserved function domain and signature sequence of *Lv*HSP10, high similarity with other identified HSP10s and the phylogenetic relationship collectively suggested that *Lv*HSP10 was a novel member of invertebrate HSP10 family, and it could have similar functions to those from vertebrates and other invertebrates.

To investigate the potential function of *Lv*HSP10 in shrimp, the distribution of its mRNA transcripts in different tissues was detected by qRT-PCR technique. The mRNA transcripts of *Lv*HSP10 were observed to be constitutively expressed in all the detected tissues, including eyestalk, gill, gonad, heart,
Fig. 5 Temporal mRNA expression profiles of LvHSP10 and LvHSP60 detected via qRT-PCR technique in white shrimp hepatopancreas at 0, 3, 6, 12, 24 and 48 h post microbe stimulation. A: Temporal mRNA expression profiles of LvHSP10 post V. parahaemolyticus stimulation; B: Temporal mRNA expression profiles of LvHSP60 post V. parahaemolyticus stimulation; C: Temporal mRNA expression profiles of LvHSP10 post WSSV stimulation; D: Temporal mRNA expression profiles of LvHSP60 post WSSV stimulation. The EF-1α gene was used as an internal control to calibrate the cDNA template for each sample. Vertical bars represent mean ± SD (n = 3), and bars with different characters were significantly different (p < 0.05), while bars with same characters were not significantly different.
study, LvHSP10 mRNA transcripts could be significantly induced by the stimulation of V. parahaemolyticus and WSSV, and also significantly increased in the high or low pH challenge experiment, suggesting that LvHSP10 play a pivotal role in the host defenses mechanism not only against the cute phase microbe infection but also the long term environmental stresses. Moreover, it has been further observed that the change tendencies of LvHSP10 and LvHSP60 mRNA transcripts were similar to each other in most cases, which is consistent with the observation in previous reports (Cappello et al., 2005; Lin et al., 2009; Xu et al., 2014) and further confirmed the hypothesis that HSP10 might co-chaperones with HSP60.

In conclusion, the full-length cDNAs of LvHSP10 were obtained from white shrimp, and its mRNA expression profiles were detected when the shrimps were exposed to microbe stimulation and high/low pH challenge. LvHSP10 was found to be involved in responses to the cute phase microbe infection, as well as the long term environmental stresses. The results obtained from this study would provide useful information of the potential roles of LvHSP10 in the defense mechanism of shrimp against various biological stimulations and multiple environmental stresses.

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Reference


Xu DX, Sun LN, Liu SL, Zhang LB, Ru XS, Zhao Y, et al. Molecular cloning of heat shock protein 10 (Hsp10) and 60 (Hsp60) cDNAs and their


