Fish and Shellfish Immunology 94 (2019) 924–933

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

Differential expressions of HSP70 gene between golden and brown noble scallops *Chlamys nobilis* under heat stress and bacterial challenge

Dewei Cheng[a,b,c], Hongxing Liu[a,b,c], Hongkuan Zhang[a,b,c], Tan Kar Soon[a,b,c], Ting Ye[a,b,c], Shengkang Li[a,b,c], Hongyu Ma[a,b,c], Huaiping Zheng[a,b,c,*]

[a] *Key Laboratory of Marine Biotechnology of Guangdong Province, Shantou University, Shantou, 515063, China*
[b] *Mariculture Research Center for Subtropical Shellfish & Algae of Guangdong Province, Shantou, 515063, China*
[c] *STU-UMT Joint Shellfish Research Laboratory, Shantou University, Shantou, 515063, China*

ARTICLE INFO

Keywords:
Scallop
Chlamys nobilis
Carotenoids
CnHSP70
Heat stress
Vibrio parahaemolyticus

ABSTRACT

Heat shock proteins (HSPs) are a family of conserved proteins that enhance stress resistance and protect cells from external damage. In the present study, the full-length HSP70 cDNA from the noble scallop *Chlamys nobilis* (designated *CnHSP70*) was first cloned and characterized. Then, the expression of *CnHSP70* in golden and brown scallops with different carotenoid content was evaluated under heat stress and *Vibrio parahaemolyticus* challenge. The complete *CnHSP70* cDNA is 2621 bp, including a 1971 bp open reading frame (ORF) encoding a polypeptide of 656 amino acids with an estimated molecular weight of 71.55 kDa and an isoelectric point of 5.32. Based on amino acid sequence and phylogenetic analysis, the *CnHSP70* gene was identified as a member of the cytoplasmic HSP70 family. The *CnHSP70* was ubiquitously expressed in all examined tissues, including intestines, hemocytes, mantle, adductor and gills, with the highest expression in gills. After heat stress and *V. parahaemolyticus* injection, the expression levels of *CnHSP70* in gills and hemocytes of golden and brown scallops were both significantly increased, indicating that the gene was involved in resistance or immune response. Moreover, under both conditions, similar expression profiles of *CnHSP70* were observed between gills and hemocytes from the same color scallop, but different expression levels were detected in the same tissue from the different color scallop, which may be related to difference in their carotenoids content.

1. Introduction

Aquatic animals live in the complexity and variability of the marine environment and often experience a variety of environmental stresses, including water temperature fluctuations, pathogen infections and water quality deterioration, which could lead to reduced productions and significant economic losses in marine aquaculture. As important members of aquatic animals, studies have shown that bivalve mollusks are particularly vulnerable to environmental changes due to the lack of specific immune responses and immune memory [1]. In the process of adaptation, these bivalves have developed a fundamental self-protection innate immunity system that prevents potential damage from the harmful environments [2]. Therefore, exploring their innate immunity will contribute to better understand the bivalve immune mechanism and promote the development of aquaculture.

Heat shock proteins (HSPs) are a group of highly conserved proteins that help organisms regulate stress responses and protect organisms from environmentally induced cellular damage [3,4]. According to the sequence similarity and molecular size, the HSPs have been classified into five major families, including HSP100, HSP90, HSP70, HSP60, and small HSPs [5]. Among them, the HSP70 family is ubiquitous, abundant and well-studied proteins and is involved various cellular processes including protein folding/unfolding, translocation, targeting, degradation, and protein complex remodeling [6,7]. In bivalves, studies have shown that HSP70 involved not only in protein folding and cytoprotection after heat shock [8], but also in specific immune responses against bacterial infection [9,10]. For instance, up-regulation of the HSP70 genes has been observed in Asiatic hard clam *Meretrix mertrix* [11], Pacific oyster *Crassostrea gigas* [12], and mussel *Mytilus coruscus* [13] after *Vibrio* bacterial infection etc.

The noble scallop *Chlamys nobilis* Reeve is famous for their brilliant shell colors including orange/golden, yellow, purple, and brown, orange-purple, etc, which has become an importantly cultured bivalve in China since 1980s. Unfortunately, massive mortality often occurred in the cultured scallops in recent years during summer, especially from July to September when the seawater temperature reached 26–30 °C.

* Corresponding author. STU-UMT Joint Shellfish Research Laboratory, Shantou University, Shantou, 515063, China.
E-mail address: hpzheng@stu.edu.cn (H. Zheng).

https://doi.org/10.1016/j.fsi.2019.10.018
Received 6 August 2019; Received in revised form 1 October 2019; Accepted 7 October 2019
Available online 08 October 2019
1050-4648/

Table 1
Primers used in the study of *CnHSP70*.

Primer name	Sequence (5′-3′)	Tm (°C)
cDNA synthesis		
Oligo (dT)	GGCCACGCGTCGACTAGTACT $_{17}$	
Gene cloning		
UPM (long)	CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT	
UPM (short)	CTAATACGACTCACTATAGGGC	
NUP	AAGCAGTGGTATCAACGCAGAGT	
3′ RACE primers		
HSP-GSP-F1	CCTGACGAGGCTGTTGCTTATGGC	66
HSP-GSP-F2	AAGCAGACGCAGACCTTCACCACC	65
5′ RACE primers		
HSP-GSP-R1	AGCCAGGCGATTACTTCTGAACACTT	62
HSP-GSP-R2	AATGGTCTTCTTGTCCTCTTCGCTGA	60
RT-PCR primers		
qHSP70-F	ACCCAAACATTCACCACATA	53
qHSP70-R	CCTCCTTAGACAAACGACCT	53
β-actin-F	CAAACAGCAGCCTCCTCGTCAT	60
β-actin-R	CTGGGCACCTGAACCTTTCGTT	60
Vector primers		
M13-47	CGCCAGGGTTTTCCCAGTCACGAC	66
RV-M	GAGCGGATAACAATTTCACACAGG	57

Since 2008, a genetic improvement program for the noble scallop has been carried out in China, and a new variety named "Nan'ao Golden Scallop" was successfully bred by selection breeding and authorized by the National Aquatic Protospecies and Improved Variety Approval Committee of China in 2015 [14]. These golden scallops contain significantly higher total carotenoids content than others. It is well known that carotenoids not only can be used as potent antioxidants to scavenge free radicals caused by environmental stress [15,16], but also can improve the efficiency of immune responses by stimulating both innate and adaptive components of animals' immunity [17,18]. Our previous studies found a positive correlation between total carotenoid content and total antioxidant capacity in the scallop [19], and have reported that golden scallops have higher cold tolerance than others, which may be related to carotenoids [20].

To unravel the potential contributions of HSP70 and carotenoids to against environmental stress and pathogen invasion in noble scallop. The heat and *Vibrio parahaemolyticus* stress experiments were separately conducted by using two shell-colored scallops from same stock with different carotenoids content in their muscle. The molecular characteristics of HSP70 (designated *CnHSP70*) were identified and their expression profiles in different tissues were examined. In addition, the expression pattern of CnHSP70 after heat and bacterial challenges were also analyzed. The findings of present study provide valuable information on improving stress and disease resistance in bivalves, in which will beneficial to the development of bivalve aquaculture.

2. Materials and methods

2.1. Experimental animals and tissues collection

In the present study, 300 golden and 300 brown noble scallops (12 months old) originated from the same stock, which had been cultured under the same environment at sea-field of the Nan'ao Marine Biology Experimental Station of Shantou University (23.4786°E, 117.1132°N) were collected. Prior to the experiment, all scallops were cleaned and equally acclimated in three 500 L tanks (each tank contains 100 golden and 100 brown individuals) filled with aerated filtered seawater at room temperature of 24.0 ± 0.5 °C for one week. Seawater was changed 50% daily, and scallops were fed with diatoms (*Nitzschia closterium f. minutissima*) and tetraselmis (*Platymonas subcordiformis*) during acclimation period. Tissues including intestine, hemocytes, mantle, adductor and gills were sampled from two individuals of each color from each tank (2 individuals × 3 tanks = 6 samples) for gene tissue distribution. After adding 1 mL of Trizol reagent (Invitrogen), all samples were stored at −80 °C for subsequent RNA extraction.

2.2. Experiment design and treatments

2.2.1. Heat stress

Prior to the heat stress experiment, gills and hemocytes of two individuals of each color from each tank were sampled and served as control (24.0 °C ± 0.5 °C). Subsequently, 20 golden scallops and 20 brown scallops were randomly collected and transferred into a heat preservation tank with the temperature of 30.0 °C ± 0.5 °C (the highest temperature of seawater in summer) as experiment group. Scallops were not fed during the experiments. The gills and hemocytes were sampled at 3, 6, 12, 24, 36 h post-exposure from two individuals of each color of each tank. All samples were preserved in liquid nitrogen until RNA extraction. Three replicates were employed.

2.2.2. Vibro parahaemolyticus challenge

In this experiment, 40 golden and 40 brown scallops were equally divided and maintained in two 500 L tanks filled with filter seawater (24 ± 0.1 °C) (each tank containing 20 golden and 20 brown scallops). The scallops in one of the tanks (experimental tank) were injected with 100 μL of *V. parahemolyticus* suspension (5×10^7 cfu mL^{-1} in PBS), while the scallops in another tank (control tank) were injected with 100 μL of PBS. Two golden and brown scallops were sampled from the experimental and control tanks at 3, 6, 12, 24, 36 h after injection, respectively. The gills and hemocytes of each sample were extracted for further analysis. Experiments were conducted in triplicate.

2.3. Cloning of the full-length cDNA of CnHSP70

Total RNA was extracted from the tissues using the Trizol Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The RNA quantity, purity and integrity were verified by native RNA electrophoresis on a 1% agarose gel in 50 × TAE buffer (Acetic acid 1 mol L^{-1}, tris 2 mol L^{-1}, EDTA 0.1 mol L^{-1}, pH 8.0) and the UV absorbance ratio at 260 nm and 280 nm (Ultrospec 2100 pro, Amersham, USA). Then, 1 μg of total RNA was used as template for cDNA synthesis using SuperScript III reverse transcriptase (Invitrogen, USA) and Oligo-dT as primers to obtain first-strand following the protocol of the manufacturer.

A cDNA fragment of *CnHSP70* was obtained from high throughput transcriptome sequencing [21]. Based on this known sequence, Gene-specific primers (Table 1) were designed for 3′ RACE and 5′ RACE of *CnHSP70* using the SMART™ RACE cDNA amplification kit (Clontech, USA) and LA Taq polymerase (TaKaRa). The preparation methods of the cDNA were conducted according to the instructions of manufacturers. A touchdown PCR program was performed to obtain the 3′end and 5′ end of *CnHSP70* gene, the profile was 95 °C for 2 min followed by 35 cycles of 94 °C for 30 s, 70-60 °C for 30 s in the initial 10 cycles decrementing 1 °C/cycle, 60 °C for 30 s for the remaining 25 cycles, 72 °C for 60 s, and a final extension step at 72 °C for 10 min. Purified DNA fragment was sub-cloned into vector pMD18-T(TaKaRa, Guangzhou, China), then transformed into *E.coli*. And fragment from positive clones was sequenced (Sangon Biotech, Shanghai, China). Then the 3′- and 5′-RACE PCR fragment sequences were aligned to assemble the full nucleotide sequence of the scallop putative *CnHSP70* cDNA. The resulting sequences were verified by the amplification of the whole full length and further subjected to cluster analysis.

2.4. Bioinformatics analysis

The acquired *CnHSP70* nucleotide sequence and the deduced amino acid sequence was analyzed using BLAST program(http://blast.ncbi.nlm.nih.gov/Blast.cgi). The theoretical pI/Mw were determined by Expasy-ProtParam online tool Compute PI/Mw program (http://web.expasy.org/compute_pi/). The deduced amino acid sequence of *CnHSP70* was aligned with other species using the Clustal X multiple alignment program (version1.83) and software of Genedoc. In addition, the protein motif features were analyzed by SMART program [22]. A phylogenetic tree was generated by Boostrapped Neighbor-Joining (NJ) method from a distance matrix with the software of MEGA 5.0 [23]. All the analyzed sequences in this tree were retrieved from GenBank database, whose accession numbers listed in Table 2.

2.5. Relative quantitative real-time PCR analysis

Transcripts level of *CnHSP70* were performed in the LightCycler 480 (Roche) using the SYBR®Premix Ex Taq™ II Kit (Perfect Real Time) (Takara, Japan). Primers for real-time PCR were listed in Table 1. According to the instruction, qRT-PCR amplificatons were carried out in a total volume of 20 μL containing 10 μL SYBR®Premix Ex Taq™ II, 2 μL of the 1:10 diluted cDNA, 0.8 μL each of qHSP70-F and qHSP70-R primer to amplify *CnHSP70* gene, 0.8 μL *β*-actin-F and *β*-actin-R to amplify the *β*-actin gene, and 6.4 μL PCR-grade water. The qRT-PCR program was 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s, 60 °C for 30 s. Melting curve analysis of amplification products was performed at the end of each PCR reaction to confirm that only one PCR product was amplified and detected, and the program was set as: 95 °C for 15 s, 60 °C for 30 s, 95 °C for 15 s. Each sample was run in triplicate. Relative mRNA expression value of *CnHSP70* was determined using the $2^{-\Delta\Delta CT}$ algorithm with *β*-actin from *Chlamys nobilis* as the internal control [24]. The amplification efficiency of CnHSP70 and *β*-actin was 95.36% and 98.01%, respectively.

2.6. Statistical analysis

Data were presented as relative expression levels (mean ± S.D, n = 6). Differences in relative expression levels of *CnHSP70* among tissues and different time points were analyzed by one-way Analysis of Variance (ANOVA).

A General Linear Model was used to evaluate the effects of genetic group (G, golden scallop VS Brown scallop), stress time (T) and their interaction on mRNA transcripts in tissues according to Zheng et al. [25]. The model was:

$$Y_{ijm} = \mu + G_i + T_j + (G \times T) + e_{ijm}$$

Where, Y_{ijm} = the *CnHSP70* transcript level of the m replicate in the *i* genetic group from the *j* treatment time; μ = overall constant; G_i = the fixed effect of genetic group (*i* = 1, 2); T_j = treatment time (*j* = 1, 2, 3, 4, 5, 6); $(G \times T)_{ij}$ = interaction effect between genetic group and treatment time; e_{ijm} = random observation error.

All statistical analyses were performed by SPSS vision 20 (SPSS Inc. Chicago, USA) and the significance of all analyses was set to $P < 0.05$ unless noted otherwise.

3. Results

3.1. Sequence analysis of CnHSP70 cDNA

A 2621 bp cDNA, containing a 1971 bp open reading frame (ORF), a 110 bp 5′-terminal untranslated region (UTR) and a 540 bp 3′-UTR, was determined by 5′ and 3′ RACEs (Fig. 1). A canonical poly-adenylation signal (AATAAA) and a poly(A) tail were located within the 3′-UTR. The ORF encoded a polypeptide of 656 amino acid residues with a theoretical molecular mass of 71.55 kDa and an isolectric point (pI) of 5.32. The predicted amino acids sequence of *CnHSP70* contains three conserved signature sequences of HSP70 family (IDLGTTYS, IFDLGGGTFDVSIL, IVLVGGSTRIPKIQK) and the cytoplasmic characteristic motif EEVD. A putative ATP-GTP binding site motif and a putative bipartite nuclear localization signal (NLS) were also observed in the sequence. At the carboxyl terminal region, *CnHSP7*0 has five terapeptides of GGXP (where X is any aliphatic residue), four of them are GGMP, another one is GGAP (Fig. 1). The cDNA sequence was submitted to GenBank under the accession number MK992915.

3.2. Phylogenetic tree analysis and multiple sequence alignments

The phylogenetic tree based on an amino acid sequences alignment

Table 2
Information of the sequences used in the phylogenetic analyze.

Species	Style	Accession number	Species	Style	Accession number
Crassostrea gigas	HSC70	CAC83683.1	*Crassostrea hongkongensis*	HSP70	ACH95805.1
Crassostrea gigas	HSC71	BAD15287.1	*Crassostrea gigas*	HSP70	BAD15286.1
Ostrea edulis	HSC70	CAC83684.1	*Crassostrea virginica*	HSP70	CAB89802.1
Septifer virgatus	HSC70	BAS29643.1	*Cristaria plicata*	HSP70	ADM64336.1
Mytilus galloprovincialis	HSC71	CAH04109.1	*Mytilus galloprovincialis*	HSP70-1	CAE51348.1
Solen grandis	HSC70	AFU72268.1	*Mytilus galloprovincialis*	HSP70-2	CAH04107.1
Rattus norvegicus	HSC71	NP_077327.1	*Mytilus galloprovincialis*	HSP70-3	CAH04108.1
Bos taurus	HSC71	NP_776770.2	*Bos taurus*	HSP70	NP_001161367.1
Danio rerio	HSC71	NP_001103873.1	*Homo sapiens*	HSP70	NP_005518.3
Ctenopharyngodon idella	HSC70	AEM75093.1	*Rattus norvegicus*	HSP70	AAA17441.1
Megalobrama amblycephala	HSC70	ACC93993.2	*Danio rerio*	HSP70	BAB72170.1
Mizuhopecten yessoensis	HSP70	AAS17724.1	*Megalobrama amblycephala*	HSP70	ACG63706.2
Azumapecten farreri	HSP70	AAO38780.1	*Ctenopharyngodon idella*	HSP70	ADX32514.1
Ruditapes philippinarum	HSP70	AKE47619.1			

```
        GGGCATAACGCCGGGAACGAAGAACAAACCGGATCGAGGGGTTTTAGGCTGTGACAAGAACTTAAATTTCTGGCTCTACTATTCAAGTTAACACACCAAAACACAGCAAC 110
ATGAGTACCAAAGTAGCAACAGCAGTCGGAATTGACTTAGGAACCACTTATTCCTGTGTTGGAGTGTTCCAGCATGGAAAAGTAGAAATCATTGCAAATGACCAGGGAAACAGAACAACC 230
 M  S  T  K  V  A  T  A  V  G  I  D  L  G  T  T  Y  S  C  V  G  V  F  Q  H  G  K  V  E  I  I  A  N  D  Q  G  N  R  T  T  40
CCAAGTTATGTAGCATTCACAGACACTGAACGTCTTGTAGGAGATGCCGCAAAGAACCAAGTGGCTATGAACCCAACAAACACCATCTTTGATGCCAAGCGTCTCATAGGAAGAAAATAC 350
 P  S  Y  V  A  F  T  D  T  E  R  L  V  G  D  A  A  K  N  Q  V  A  M  N  P  T  N  T  I  F  D  A  K  R  L  I  G  R  K  Y  80
AATGAACCCTGTGTCGCCTCCGATAAGAAACATTGGCCATTTGAAGTGGTCTGTGATGACGGTAAACCAAAACTGCAGGTCAGCTACAAGACTGAAATGAAGACTTTCTTCCCAGAAGAA 470
 N  E  P  C  V  A  S  D  K  K  H  W  P  F  E  V  V  C  D  D  G  K  P  K  L  Q  V  S  Y  K  T  E  M  K  T  F  F  P  E  E  120
ATCTCCTCTATGGTTCTCAACAAGATGAAGGAGACAGCAGAAGCCTATCTTGGCAAGACGGTATCAAACGCAGTGGTAACTGTCCCAGCTTACTTCAATGACTCTCAGAGACAGGCAACA 590
 I  S  S  M  V  L  N  K  M  K  E  T  A  E  A  Y  L  G  K  T  V  S  N  A  V  V  T  V  P  A  Y  F  N  D  S  Q  R  Q  A  T  160
AAAGATGCCGGAACAATTTCTGGATTGAATGTTCTACGTATCATCAACGAACCAACAGCAGCTGCTATCGCATATGGTCTTGACAAGAAGGTTGGAACCGAAAGGAATGTACTCATCTTT 710
 K  D  A  G  T  I  S  G  L  N  V  L  R  I  I  N  E  P  T  A  A  A  I  A  Y  G  L  D  K  K  V  G  T  E  R  N  V  L  I  F  200
GATTTAGGTGGTGGTACCTTTGATGTATCCATCCTGACGATAGAGGATGGAATCTTTGAAGTGAAATCCACATCTGGGGACACCCACTTGGGTGGTGAGGACTTTGATAACCGTATGGTG 830
 D  L  G  G  G  T  F  D  V  S  I  L  T  I  E  D  G  I  F  E  V  K  S  T  S  G  D  T  H  L  G  G  E  D  F  D  N  R  M  V  240
AACCACTTTGTACAGGAATTCAAACGCAAACACAAGAAAGACATCACAGATAACAAAAGGGCTGTCCGTCGTCTGAGAACAGCCTGTGAACGAGCAAAGAGAACCCTATCATCAAGTGCT 950
 N  H  F  V  Q  E  F  K  R  K  H  K  K  D  I  T  D  N  K  R  A  V  R  R  L  R  T  A  C  E  R  A  K  R  T  L  S  S  S  A  280
CAGGCCAGCGTTGAGATCGACTCTCTCTATGAAGGTATTGATTTCTACACCAGTATAACTCGTGCTCGTTTTGAGGAGTTGAATGCTGACCTTTTCAGAGGAACCCTGGAACCTGTAGAA 1070
 Q  A  S  V  E  I  D  S  L  Y  E  G  I  D  F  Y  T  S  I  T  R  A  R  F  E  E  L  N  A  D  L  F  R  G  T  L  E  P  V  E  320
AAGTCCCTGCGTGATGCCAAGATGGACAAATCCAAGATCCACGACATCGTACTGGTTGGAGGGTCCACACGTATTCCAAAGATCCAGAAACTTCTTCAGGATTTCTTCAACGGCAAAGAA 1190
 K  S  L  R  D  A  K  M  D  K  S  K  I  H  D  I  V  L  V  G  G  S  T  R  I  P  K  I  Q  K  L  L  Q  D  F  F  N  G  K  E  360
CTGAACAAGTCTATCAATCCTGATGAGGCTGTTGCCTATGGTGCAGCTGTGCAGGCCGCCATCCTTTCTGGAGATAAGTCTGAGGAGGTCCAGGATCTGCTGTTGTTGGACGTCGCTCCA 1310
 L  N  K  S  I  N  P  D  E  A  V  A  Y  G  A  A  V  Q  A  A  I  L  S  G  D  K  S  E  E  V  Q  D  L  L  L  L  D  V  A  P  400
TTGTCCCTGGGTATCGAGACCGCTGGAGGCGTGATGACATCACTTATCAAACGTAACACAACTGTTCCCACCAAACAGACCCAAACATTCACCACATATTCTGACAATCAACCTGGTGTG 1430
 L  S  L  G  I  E  T  A  G  G  V  M  T  S  L  I  K  R  N  T  T  V  P  T  K  Q  T  Q  T  F  T  T  Y  S  D  N  Q  P  G  V  440
TTGATCCAGGTGTACGAGGGAGAACGAGCAATGACCAAAGACAACAATTTGCTCGGAAAGTTTGAACTGACCGGAATTCCACCCGCACCAAGAGGTGTACCCCAGATTGAGGTTACATTT 1550
 L  I  Q  V  Y  E  G  E  R  A  M  T  K  D  N  N  L  L  G  K  F  E  L  T  G  I  P  P  A  P  R  G  V  P  Q  I  E  V  T  F  480
GATGTTGACGCCAACGGAATTCTGAACGTTTCTGTTGTTGACAAGAGTACTGGCAAAGAAAACAAAATCACCATCACCAATGACAAAGGTCGTTTGTCTAAGGAGGAGATCGAGAGGATG 1670
 D  V  D  A  N  G  I  L  N  V  S  V  V  D  K  S  T  G  K  E  N  K  I  T  I  T  N  D  K  G  R  L  S  K  E  E  I  E  R  M  520
GTGAACGATGCCGAGAAATACAAGGCAGAAGATGATGTACAACGTAACCGTGTCTCCGCAAAGAATTCTCTTGAGAGCTATGCTTTCCAAATGAAATCCACCTCTGAGGATGACAATCTG 1790
 V  N  D  A  E  K  Y  K  A  E  D  D  V  Q  R  N  R  V  S  A  K  N  S  L  E  S  Y  A  F  Q  M  K  S  T  S  E  D  D  N  L  560
AAGGGCAAGATCAGCGAAGAGGACAAGAAGACCATTGTCGATAAGTGTTCAGAAGTAATCGCCTGGCTAGATGCTAACCAGTTGGCTGAGAAGGAAGAGTTTGAACACAAACAGAAGGAG 1910
 K  G  K  I  S  E  E  D  K  K  T  I  V  D  K  C  S  E  V  I  A  W  L  D  A  N  Q  L  A  E  K  E  E  F  E  H  K  Q  K  E  600
TTGGAGGGAGTCTGTAATCCTATTATCACCAAACTGTACCAGGGCGCTGGTGGAGCTCCAGGAGGAATGCCCGGTGGCATGCCCGGAGGTATGCCTGGTGGCATGCCAGGTGGTGCTGAT 2030
 L  E  G  V  C  N  P  I  I  T  K  L  Y  Q  G  A  G  G  A  P  G  G  M  P  G  G  M  P  G  G  M  P  G  G  M  P  G  G  A  D  640
GGTGCTTCAACTGGTGGCAGCCAAGGCCCAACCATCGAGGAGGTTGATTAGATAGGCTTCTTATACAAAATACATTCAGGACTTTGTAAGATCATGTCGGTAACATTCAAGACTTGTTAT 2150
 G  A  S  T  G  G  S  Q  G  P  T  I  E  E  V  D  *                                                                      656
AACTTCATTTCTACGAACTTGTTCAAATGTTAATGATTTGGAAGAAAACAAATCCTATAAAATCAACCAAATCAATGTTTATAAATCTAGTTGCCACCAAACTGTTATTTAGAGAAATCA 2270
ACAGTGAATGCTTGATTGCTTGTAATTGTGTTGGCGAAAAGCTCATTTGTTATTCATTAGTTCTTTATTCACATTTAAAGTGAGGAAAATAATTATTTTAAATGACAAACGTATCTGATG 2390
GGTTTTTCATATTTTACATCTTATTGACTTTTTTTCATCATTCCACTAGCAACAAAATGAAAACTAACAATATATTGTCAAGCTTGCGAAAAACCCTCTGTGAAATGCAAATGTGACAAA 2510
TCATTAAGATGCTATTTCAAACTCTGAAAATGTGTCTTTAAAAAAATGGAAAAAATTAACTTTAAAAAAATAGAATAAAAGAGCCAGAACAAAAAAAAAAAAAAAAAAAA           2621
```

Fig. 1. Nucleotide and deduced amino acid sequences of *CnHSP70* cDNA. The polyadenylation signal (AATAAA) was shown in gray and boxes. The characteristic motifs of the HSP70 family were shown as followed. Three signatures were underlined; a putative ATP-GTP binding site was bold in gray; a putative bipartite nuclear localization signal was bold in italics and highlighted with wave; four terapeptides of GGXP at carboxyl terminal region were underlined and in gray; and the cytoplasmic HSP70 carboxyl terminal EEVD was indicated in gray.

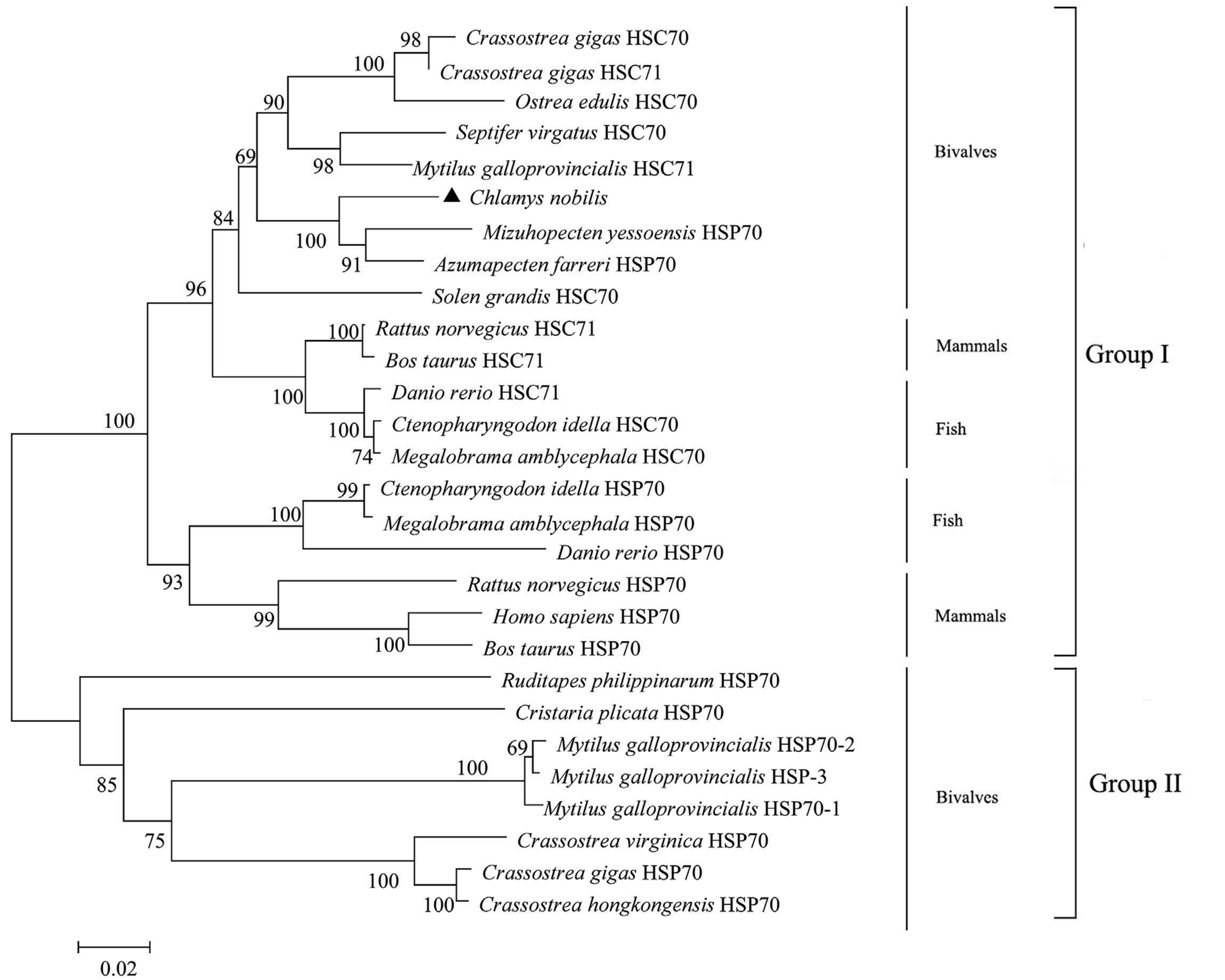

Fig. 2. A phylogenetic tree constructed using the neighbor-joining method based on amino acids sequence deduced from HSP70 gene of *Chlamys nobilis* and other species. *Chlamys nobilis* was labeled with "▲". The scale bar under the tree represents the amino acid substitution rate, and the bootstrap values (%) of 1000 replicates are listed at the nodes. The GenBank accession numbers of all the HSP70/HSC70 were shown in Table 2.

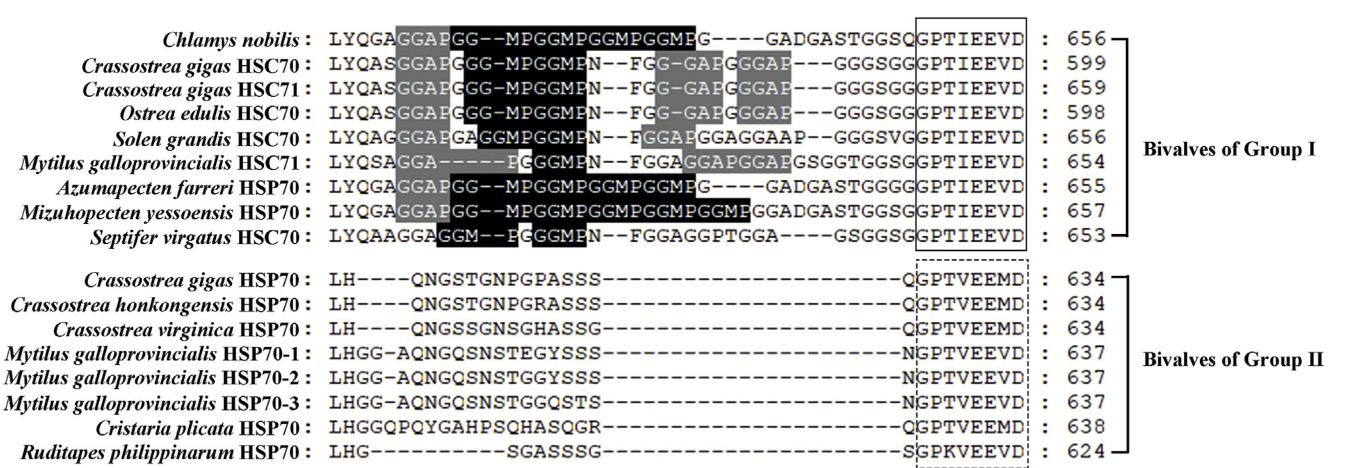

Fig. 3. Alignment of the C-terminal region of amino acid sequences from *CnHSP70* and other bivalve HSP70s presenting in Table 2. "GGMP" were highlighted with dark gray; "GGAP" were highlighted with light gray. The "GPTIEEVE" motifs in bivalves of group I were in solid line box and "GPT(K)VEEM(V)D" motifs in bivalves of group II were in dash line box.

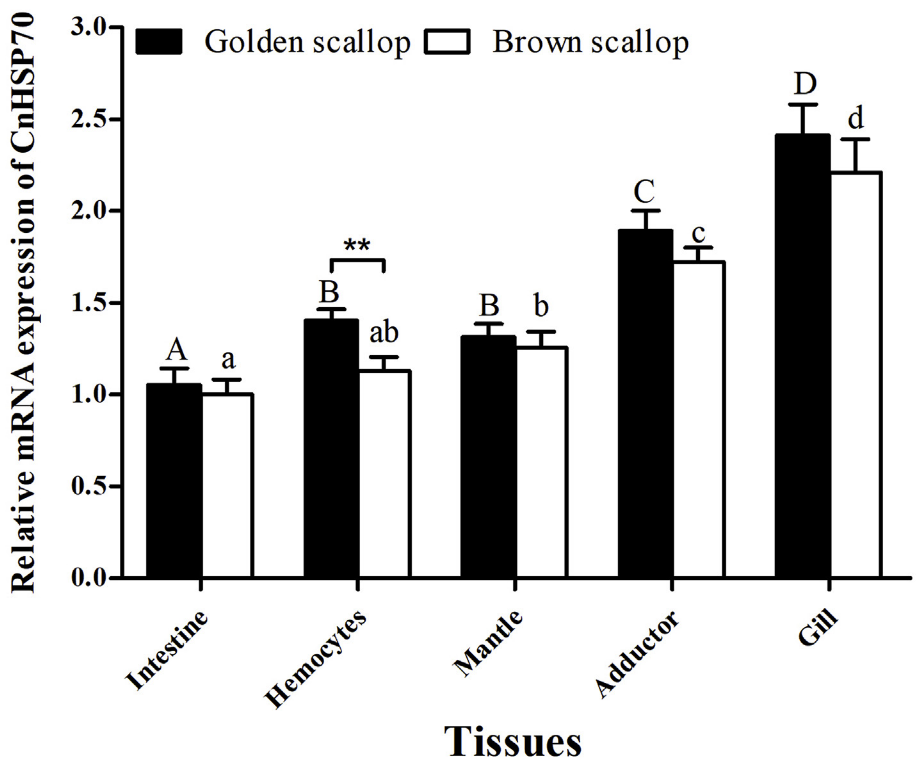

Fig. 4. Tissue distribution of *CnHSP70* mRNA expression. The mRNA expression of *CnHSP70* was determined by real-time PCR with β-actin as internal control gene. The relative expression of *CnHSP70* in each tissue (intestine, hemocytes, mantle, adductor and gill) was determined compared to the expression in the intestine. Vertical bars represent means ± SD (N = 6). Significant differences are indicated by ** ($P < 0.01$). Different upper letters on bars indicate significant difference ($P < 0.05$) among different tissues in the same golden scallops, while different lower letters on bars indicate significant difference ($P < 0.05$) among different tissues in the same brown scallops.

between *CnHSP70* and other bivalves HSP70/HSC70 showed that all sequences were divided into two major groups, group I and group II (Fig. 2). *CnHSP70* was placed in group I and had the closest relationship to *Mizuhopecten yessoensis* HSP70 and *Azumapecten farreri* HSP70. Interestingly, the inducible (HSP70) and cognate (HSC70) genes were found in group I, whereas only the inducible genes (HSP) gene was found in group II. In addition, all bivalve sequences in group I contained more than one GGXP motif and GPTIEEVD motif in the C-terminal of the sequence, and no two motifs were found in the sequences of bivalves in group II (Fig. 3).

3.3. Expression profile of CnHSP70 in different tissues

Real-time quantitative PCR was used to investigate the tissue distribution and expression level of *CnHSP70* under normal conditions. Expression levels of *CnHSP70* were detected in all tested tissues, including intestine, hemocytes, mantle, adductor and gills (Fig. 4). The highest expression level of *CnHSP70* was observed in gills, which significantly higher than that in other tissues, and the basal *CnHSP70* expression level in hemocytes of golden scallops was significantly higher than that in brown scallops ($P < 0.05$).

3.4. Comparison of CnHSP70 transcript profiles between the golden and brown scallops in response to heat stress

In gills, the expression levels of *CnHSP70* in golden and brown scallops were both significantly upregulated after acute heat stress ($P < 0.05$), and respectively reached the highest at 12 h and 6 h (Fig. 5A). Furthermore, the value at 3 and 6 h in golden scallops was lower than that in brown ones, but after 12 h, which was significantly higher in former than that in latter ($P < 0.05$). Analysis of variance demonstrated (Table 3) that genetic group and stress time made significant impact on the gene expression level ($P < 0.001$), and their interaction was also significant ($P < 0.001$).

In hemocytes, the expression levels of *CnHSP70* in golden and brown scallops were also both significantly upregulated after acute heat stress ($P < 0.05$) (Fig. 5B). The expression level in golden scallops increased with stress time lengthening, whereas the value in brown scallops was firstly increased significantly ($P < 0.05$), peaked at 12 h, and then declined. Moreover, the gene expression level from 3 to 12 h in golden scallops was lower than that in brown ones, but at 24 and 36 h, which was significantly higher in former than that in latter ($P < 0.05$). Analysis of variance demonstrated (Table 3) that both genetic group and stress time made significant impact on the gene expression level ($P < 0.001$), and their interaction was significant ($P < 0.001$).

3.5. Comparison of CnHSP70 transcript profiles between golden and brown scallops in response to pathogen challenge

In gill, the expression level of *CnHSP70* in golden and brown scallops at 3–24 h post-infection was significantly higher than ($P < 0.05$) control and reached a maximum level at 12 h (Fig. 6A). After 12 h, the expression level dropped significantly and returned to control level at 36 h after injection. There was no significant difference in the gene expression level between golden and brown scallops ($P > 0.05$). Analysis of variance in Table 4 demonstrated that only stress time had a significantly effect on the gene expression level ($P < 0.001$).

In hemocytes, the expression level of *CnHSP70* in hemocytes of both color scallops was significantly upregulated at 3 h post-injection and reached the highest level at 12 h post-injection (Fig. 6B). The expression level of *CnHSP70* in golden scallops was lower than that in brown scallops at 3–12 h post-injection. After 24 h post-infection, the expression level of *CnHSP70* in golden scallops was significantly higher than that in brown scallops ($P < 0.05$). Analysis of variance demonstrated (Table 4) that both genetic group and stress time made significant impact on the gene expression level ($P < 0.001$), and their interaction was also significant ($P < 0.001$).

4. Discussion

In this study, *CnHSP70* was cloned and characterized for the first time in the noble scallop *Chlamys nobilis*, and the nucleotide sequence was submitted to the GenBank under accession number of MK992915.

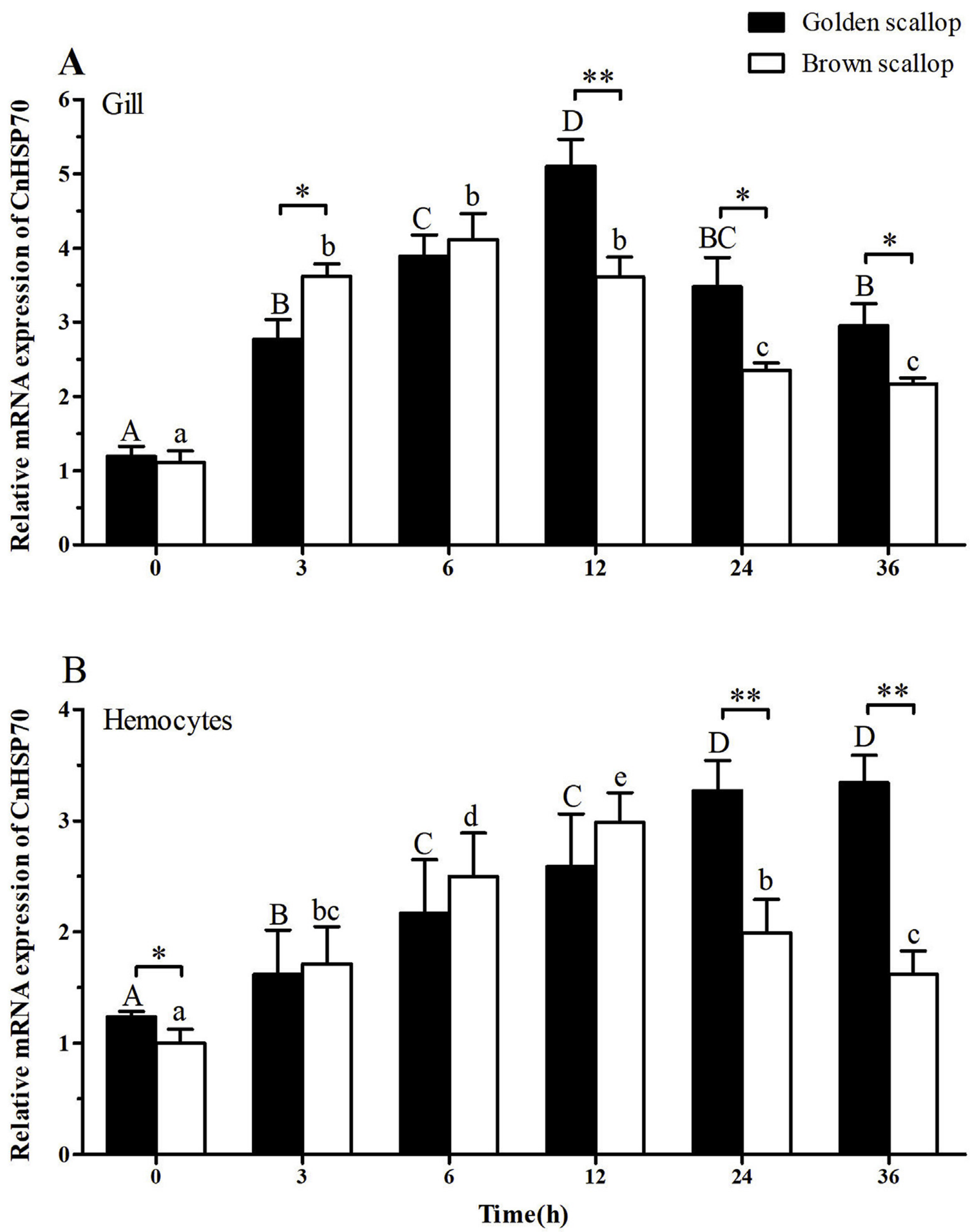

Fig. 5. The expression levels of *CnHSP7*0 mRNA in gill and hemocytes from golden and brown scallop after acute heat stress. Vertical bars represent means ± SD (N = 6). Significant differences between golden and brown scallops at same time point are indicated by * ($P < 0.05$), ** ($P < 0.01$). Different upper letters on bars indicate significant difference ($P < 0.05$) among different stress times in the same golden scallop, while different lower letters on bars indicate significant difference ($P < 0.05$) among different stress times in the same brown scallop.

Table 3
Analyses of variance for *CnHSP70* expression level in response to acute heat stress in gill and hemocytes of *C. nobilis*.

Source	*df*	Gill			Hemocytes		
		MS	F	*P*	MS	F	*P*
Genetic group (G)	1	2.195	16.382	< 0.001	2.896	28.071	< 0.001
Time (T)	5	13.017	97.167	< 0.001	4.989	48.360	< 0.001
G × T	5	2.884	21.530	< 0.001	2.376	23.035	< 0.001
Error	60	0.134			0.103		

The amino acid sequence of *CnHSP7*0 has three different signature sequences (IDLGTTYS, IFDLGGGTFDVSIL, and IVLVGGSTRIPKIQK), which are well conserved in the eukaryotic HSP70 family [26], indicating that the sequence is a member of the HSP70 family. An ATP-GTP binding motif (AEAYLG) and a putative bipartite nuclear localization signal (KRKHKKDITDNKRAVRR) were observed in *CnHSP7*0 that were required for the selective translocation of HSPs into the nucleus [27,28]. In addition, a conserved cytoplasmic specific motif, EEVD was present at the gene C-terminal, indicating *CnHSP70* could be classified as a member of the cytoplasmic HSP70 family [29].

Phylogenetic analysis between *CnHSP70* and other species HSP70/HSC70 showed that all sequences were divided into group I and group II. Alignment of the C-terminal of the sequences showed that all the sequences of bivalves in group I contained terapeptides of GGXP, none of the sequences of bivalves in group II contained GGXP. A previous report indicated that GGXP is possibly present in the C-terminal region of the vertebrate HSC70 [30], and the absence of GAP/GGMP repeats is the main characteristic in sequence discrepancy between HSP70s and

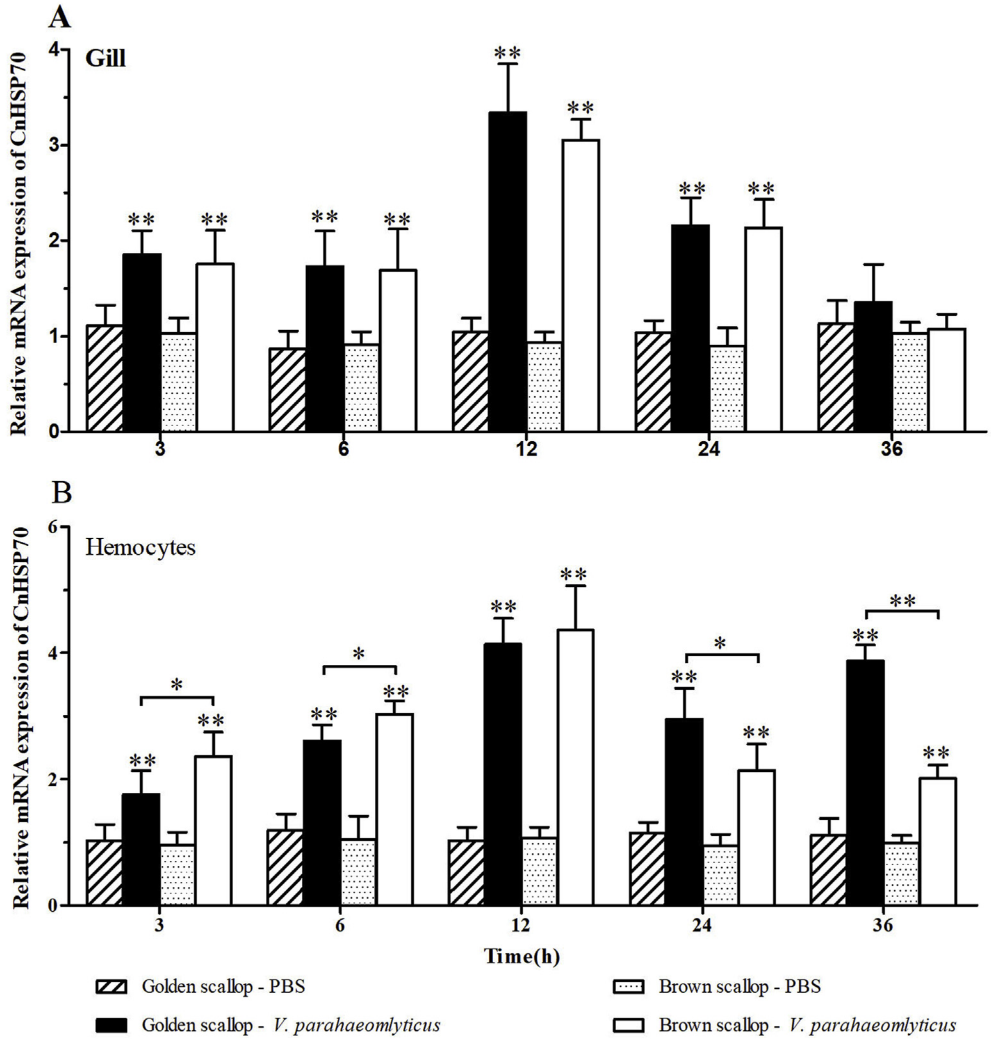

Fig. 6. The expression levels of *CnHSP7*0 mRNA in gill and hemocytes from golden and brown scallops after *V. parahaemolyticus* challenge. Vertical bars represent the mean ± SD (N = 6). Significant differences are indicated by * ($P < 0.05$), ** ($P < 0.01$).

Table 4
Analyses of variance for *CnHSP70* expression level in response to bacterial infection in gill and hemocytes of *C. nobilis*.

Source	*df*	Gill			Hemocytes		
		MS	F	*P*	MS	F	*P*
Genetic group (G)	1	0.318	2.708	> 0.05	1.199	7.546	< 0.001
Time (T)	4	6.567	55.879	< 0.001	7.979	50.235	< 0.001
G × T	4	0.049	0.421	> 0.05	3.232	20.349	< 0.001
Error	50	0.118			0.159		

HSC70s [31]. In our present study, *CnHSP70* contains one GGAP tetrapeptides and three GGMP tetrapeptide in the C-terminal region. In addition, a GPTIEEVD octapeptide was also found in the *CnHSP70* C-terminal region. The GPTIEEVD octapeptide was specific for HSC70s [11,32]. And it was believed to be responsible for interacting with tetratricopeptide repeat (TPR) domains of the HSP-organizing protein (HOP), which is an adapter protein that mediates the association of HSP70 and HSP90 into a multi-chaperone complex [30]. Taken together, we can conclude that the characteristics of *CnHSP70* were more closely related to those of HSC70s.

The distribution of *CnHSP70* gene expression can provide useful information for analyzing its biological function in mollusks. Real time RT-PCR analysis showed that *CnHSP70* was ubiquitously expressed in all tested tissues of *C. nobili*, and the highest expression level was found in gill. These results are consistent with previous findings in Manila clam *Ruditapes philippinarum* [8]. It is well known that gill consists of only a single layer of fragile cells and is covered with a thin protective mucus layer with extensive environmental exposure. The high expression level of *CnHSP70* in the gills of golden and brown scallops indicates that *CnHSP70* significantly promotes the regulation of environmental stress response. Additionally, the present study showed the basal mRNA level of *CnHSP70* in hemocytes of golden scallops was significantly higher than that of brown scallops ($P < 0.05$), suggesting that the regulation mechanism of *CnHSP70* in two color scallops is different.

Since sedentary filter feeders are broadly inhabit in coastal and estuarine areas, scallops are susceptible to temperature fluctuations. When organisms are continuously exposed to high temperature, excessive amounts of reactive oxygen species (ROS) are generated in the body [33], which severely impairs the normal cells function and may indirectly act as a signal molecule for DNA damage [34]. HSP70 is a general molecular chaperone involved in repairing or degrading damaged proteins [35], thereby protecting cells from environmental stress. In the present study, the expression levels of *CnHSP70* in gills and hemocytes of both scallops were significantly upregulated after heat shock compared with the control group ($P < 0.05$). Similar results were reported in the mussels *Mytilus coruscus* [13] and Manila clam *Ruditapes philippinarum* [8]. The present findings suggest that *CnHSP70* is involved in the response to heat stress, and up-regulation of *CnHSP70* presumably reflect the need for more *CnHSP70* proteins to repair damaged or misfolded proteins.

Vibrio parahaemolyticus is a common bacterium in marine aquaculture system and is associated with mass mortality of marine mollusks, including scallop *Pecten maximus* [36], abalone *Haliotis diversicolor supertexta* [37] and clam *Meretrix meretrix* [38]. In recent years, HSP70s have received the most attention in aquatic animals because they regulate cellular immune responses and protect organisms from pathogenic stress [39]. For instance, in bay scallop *Argopecten irradians*, the expression level of HSP70 in the hemocytes was up-regulated and reached a maximum level at 8 h after *Vibrio anguillarum* injection [40]. In pacific abalone *Haliotis discus hannai*, the expression of HSP70 mRNA in muscle and gill tissues was significantly increased after challenged with *Vibrio anguillarum* [41]. In pearl oyster *Pinctada fucata*, the HSP70 gene was inducible and reached a maximum level at 4 h after *Vibrio alginolyticus* injection [42]. In the present study, the expression levels of *CnHSP70* in gills and hemocytes of both color scallops were up-regulated after challenge with *V. parahaemolyticus* compared with that control group. The findings suggested that *CnHSP70* was involved in the response of scallops to pathogen stress. Up-regulation of *CnHSP7*0 mRNA expression following infection indicated that the HSP70 gene was inducible and may play important role in resistance to pathogen attack.

In addition, some interesting results were observed by comparing the expression levels of *CnHSP70* between golden and brown scallops. Under heat stress, the expression levels of *CnHSP70* in gills and hemocytes of golden scallops were lower than those of brown scallops at 3–6 h and 3–12 h, respectively. Our previous study had shown that golden scallops contain significantly higher TCC than brown scallops [25]. Since carotenoids are powerful antioxidants, which efficiently scavenges oxygen free radicals to protects cell organelles from oxidative damage [43–45]. Therefore, this could partly explain the less sensitive of golden scallops than brown scallops. However, over time, the expression levels of *CnHSP70* in gill and hemocytes of golden scallops were significantly higher than those of brown scallops at 12–36 h and 24–36 h, respectively. Similar results were also observed in hemocytes after infected with *V. parahaemolyticus*. It is well known that carotenoids can regulate the immune gene expression [46], including, *CuZnSOD* [20], *CnTRX* [47], *Cnlec-1* [48] and *Ferritin* gene [49]. Therefore, we speculate that the TCC could also regulate the expression of *CnHSP70* in the golden scallop.

In conclusion, *CnHSP70* was cloned and identified in the noble scallop *Chlamys nobilis* for the first time. The *CnHSP70* was involved in the responses to heat and pathogen stress. Under both conditions, similar expression profiles of *CnHSP70* were observed between gills and hemocytes from the same color scallop, but different expression levels were detected in the same tissue from the different color scallop. This work provides valuable information on improving stress and disease resistance in bivalves.

Acknowledgements

This work was financially supported by the National Natural Science Foundation of China (31872563), China Modern Agro-industry Technology Research System (CARS-49), Guangdong Provincial Yangfan Plan (14600706) and Department for Education (2017KCXTD014), China.

References

[1] L. Song, L. Wang, L. Qiu, H. Zhang, Bivalve immunity, Adv. Exp. Med. Biol. 708 (2010) 44–65.
[2] L. Cerenius, K. Söderhäll, The prophenoloxidase-activating system in invertebrates, Immunol. Rev. 77 (1) (2004) 21–26.
[3] O. Bensaude, Protein denaturation during heat shock and related stress, in: O. Bensaude (Ed.), Stress Proteins, Springer, Berlin Heidelberg, 1990, pp. 89–99.
[4] S. Lewis, R.D. Handy, B. Cordi, Z. Billinghurst, M.H. Depledge, Stress proteins (HSP's): methods of detection and their use as an environmental biomarker, Ecotoxicology 8 (5) (1999) 351–368.
[5] J.D. Trent, A review of acquired thermotolerance, heat-shock proteins, and molecular chaperones in archaea, FEMS Microbiol. Rev. 18 (1996) 249–258.
[6] M. Daugaard, M. Rohde, Marja Jäättelä, The heat shock protein 70 family: highly homologous proteins with overlapping and distinct functions, FEBS Lett. 581 (19) (2007) 3702–3710.
[7] S. Thanaphum, D.S. Haymer, A member of the hsp70 gene family from the Mediterranean fruit fly, *Ceratitis capitata*, Insect Mol. Biol. 7 (1) (2010) 63–72.
[8] H. Nie, L. Liu, Z. Huo, P. Chen, J. Ding, F. Yang, X. Yan, The HSP70 gene expression responses to thermal and salinity stress in wild and cultivated Manila clam *Ruditapes philippinarum*, Aquaculture 470 (2017) 149–156.
[9] L. Wang, C. Yang, L. Song, The molluscan HSP70s and their expression in hemocytes, Invertebr. Surviv. J. 10 (1) (2013) 77–83.
[10] L. Song, L. Wang, H. Zhang, M. Wang, The immune system and its modulation mechanism in scallop, Fish Shellfish Immunol. 46 (1) (2015) 65–78.
[11] X. Yue, B. Liu, L. Sun, B. Tang, Cloning and characterization of a hsp70 gene from Asiatic hard clam *Meretrix meretrix* which is involved in the immune response against bacterial infection, Fish Shellfish Immunol. 30 (3) (2011) 791–799.
[12] B. Genard, P. Miner, J.L. Nicolas, D. Moraga, P. Boudry, F. Pernet, R. Tremblay, Integrative study of physiological changes associated with bacterial infection in Pacific oyster larvae, PLoS One 8 (5) (2013) e64534.
[13] H. Liu, J. He, C. Chi, J. Shao, Differential HSP70 expression in *Mytilus coruscus* under various stressors, Gene 543 (2014) 166–173.
[14] H.P. Zheng, H.L. Liu, X.Q. Chen, B. Zhang, Q. Zhang, Y.J. Wang, Z.C. Guo, H.K. Zhang, Q. Wang, Y.Q. Lu, J.Q. Yang, A new variety "nan'ao golden scallop" of the noble scallop *Chlamys nobilis* (bivalve: pectinidae) (ed by the national aquaculture technique extension central station of China, 123-132), Guide for Extending Aquatic Varieties, Chinese Agricultural Press, 2015.
[15] W. Stahl, H. Sies, Antioxidant activity of carotenoids, Mol. Asp. Med. 24 (6) (2003) 345–351.
[16] A.V. Rao, L.G. Rao, Carotenoids and human health, Pharmacol. Res. 55 (3) (2007) 207–216.
[17] J.D. Blount, N.B. Metcalfe, T.R. Birkhead, P.F. Surai, Carotenoid modulation of immune function and sexual attractiveness in zebra finches, Science 300 (5616) (2003) 125–127.
[18] K.J. McGraw, D.R. Ardia, Carotenoids, immunocompetence, and the information content of sexual colors: an experimental test, Am. Nat. 162 (6) (2003) 704–712.
[19] H. Zheng, Q. Zhang, H. Liu, W. Liu, Z. Sun, S. Li, T. Zhang, Cloning and expression of vitellogenin (Vg) gene and its correlations with total carotenoids content and total antioxidant capacity in noble scallop *Chlamys nobilis* (Bivalve: pectinidae), Aquaculture 366–367 (2) (2012) 46–53.
[20] J. Han, Y. Lu, H. Zheng, H. Liu, H. Deng, B. Zhang, Differential expression of CuZnSOD gene under low temperature stress in noble scallop *Chlamys nobilis* with different carotenoid content, Fish Shellfish Immunol. 54 (2016) 30–39.
[21] H. Liu, H. Zheng, H. Zhang, L. Deng, W. Liu, S. Wang, F. Meng, Y. Wang, Z. Guo, S. Li, G. Zhang, A de novo transcriptome of the noble scallop, *Chlamys nobilis*, focusing on mining transcripts for carotenoid-based coloration, BMC Genomics 16 (1) (2015) 44.
[22] I. Letunic, R. Copley, B. Pils, S. Pinkert, J. Schultz, P. Bork, Smart 5: domains in the context of genomes and networks, Nucleic Acids Res. 34 (2006) 257–260.
[23] K. Tamura, D. Peterson, N. Peterson, G. Stecher, M. Nei, S. Kumar, MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods, Mol. Biol. Evol. 28 (10) (2011) 2731–2739.
[24] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method, Methods 25 (4) (2001) 402–408.
[25] H. Zheng, H. Liu, T. Zhang, S. Wang, Z. Sun, W. Liu, Y. Li, Total carotenoid differences in scallop tissues of *Chlamys nobilis* (Bivalve: pectinidae) with regard to gender and shell colour, Food Chem. 122 (4) (2010) 1164–1167.
[26] R.S. Gupta, B. Singh, Phylogenetic analysis of 70 kD heat shock protein sequences suggests a chimeric origin for the eukaryotic cell nucleus, Curr. Biol. 4 (12) (1995) 1104–1114.
[27] M. Kodiha, A. Chu, O. Lazrak, U. Stochaj, Stress inhibits nucleocytoplasmic shuttling of heat shock protein hsc70, Am. J. Physiol. Cell Physiol. 289 (4) (2005) C1034–C1041.
[28] M. Köhler, H. Haller, E. Hartmann, Nuclear protein transport pathways, Exp. Nephrol. 7 (4) (1999) 290–294.
[29] I. Boutet, A. Tanguy, S. Rousseau, M. Auffret, D. Moraga, Molecular identification and expression of heat shock cognate 70 (hsc70) and heat shock protein 70 (hsp70) genes in the Pacific oyster *Crassostrea gigas*, Cell Stress Chaperones 8 (1) (2003) 76–85.
[30] S.J. Wu, F.H. Liu, S.M. Hu, C. Wang, Different combinations of the heat-shock cognate protein 70 (hsc70) C-terminal functional groups are utilized to interact with distinct tetratricopeptide repeat-containing proteins, Biochem. J. 359 (2) (2001) 419–426.
[31] M. Vayssier, G. Le, J.F. Fabien, H. Philippe, C. Vallet, P. Ortega, C. Soule, C. Perret, M. Liu, L. Vegal, P. Boireau, Cloning and analysis of a *Trichinella britovi* gene encoding a cytoplasmic heat shock protein of 72 kDa, Parasitology 119 (1) (1999) 81–93.
[32] E.E. Deane, N.Y.S. Woo, Cloning and characterization of the hsp70 multigene family from silver sea bream: modulated gene expression between warm and cold temperature acclimation, Biochem. Biophys. Res. Commun. 330 (3) (2005) 776–783.
[33] J. Wang, B. Dong, Z. Yu, C. Yao, The impact of acute thermal stress on green mussel *Perna viridis*: oxidative damage and responses, Comp. Biochem. Physiol. A 222

(2018) 7–15.
[34] S. Kwiecien, K. Jasnos, M. Magierowski, Z. Sliwowski, R. Pajdo, T. Mach, D. Wojcik, T. Brzozowski, Lipid peroxidation, reactive oxygen species and antioxidative factors in the pathogenesis of gastric mucosal lesions and mechanism of protection against oxidative stress - induced gastric injury, J. Physiol. Pharmacol. 65 (5) (2014) 613–622.
[35] M.E. Feder, G.E. Hofmann, Heat-shock proteins, molecular chaperones, and the stress response: evolutionary and ecological physiology, Annu. Rev. Physiol. 61 (1999) 243–282.
[36] L. Torkildsen, C. Lamebert, A. Nylund, T. Magensen, Bergh Ø, Bacteria associated with early life stages of the great scallop, Pecten maximus: impact on larval survival, Aquacult. Int. 13 (2005) 575–592.
[37] J.P. Cai, Y. Han, Z. Wang, Isolation of *Vibrio parahaemolyticus* from abalone(*Haliotis diversicolor supertexta* L.) postlarvae associated with mass mortalities, Aquaculture 257 (2006) 161–166.
[38] X. Yue, B. Liu, J. Xiang, J. Jia, Identification and characterization of the pathogenic effect of a *Vibrio parahaemolyticus*-related bacterium isolated from clam *Meretrix meretrix* with mass mortality, J. Invertebr. Pathol. 103 (2010) 109–115.
[39] V.G. Encomio, F.L. Chu, Characterization of heat shock protein expression and induced thermotolerance in *P. marinus* parasitized eastern oysters: lab and field studies, J. Shellfish Res. 23 (2004) 289–290.
[40] L.S. Song, L.T. Wu, D.J. Ni, Y.Q. Chang, W. Xu, K.Z. Xing, The cDNA cloning and mRNA expression of heat shock protein 70 gene in the haemocytes of bay scallop (*Argopecten irradians*, Lamarck 1819) responding to bacteria challenge and naphthalin stress, Fish Shellfish Immunol. 21 (2006) 335–345.
[41] P. Cheng, X. Liu, G. Zhang, J. He, Cloning and expression analysis of a HSP70 gene from pacific abalone (*Haliotis discus hannai*), Fish Shellfish Immunol. 22 (2007) 77–87.
[42] Z. Wang, Z. Wu, J. Jian, Y. Lu, Cloning and expression of heat shock protein 70 gene in the haemocytes of pearl oyster (*Pinctada fucata*, Gould 1850) responding to bacterial challenge, Fish Shellfish Immunol. 26 (2009) 639–645.
[43] N.I. Krinsky, Antioxidant functions of carotenoids, Free Radic. Biol. Med. 7 (6) (1989) 617–635.
[44] N.I. Krinsky, Carotenoids as antioxidants, Nutrition 17 (10) (2001) 815–817.
[45] K. Miyashita, T. Yoshikawa, Function of marine carotenoids, Forum Nutr. 61 (2009) 136.
[46] J.S. Bertram, Carotenoids and gene regulation, Nutr. Rev. 57 (6) (1999) 182–191.
[47] H.K. Zhang, D.W. Cheng, H.X. Liu, H.P. Zheng, Differential responses of a thioredoxin-like protein gene to, *Vibrio parahaemolyticus*, challenge in the noble scallop, *Chlamys nobilis*, with different total carotenoids content, Fish Shellfish Immunol. 72 (2018) 377–382.
[48] Y.Q. Lu, H.K. Zhang, D.W. Cheng, H.X. Liu, S.K. Li, H.Y. Ma, H.P. Zheng, A multi-CRD C-type lectin gene Cnlec-1 enhance the immunity response in noble scallop *Chlamys nobilis* with higher carotenoids contents through up-regulating under different immunostimulants, Fish Shellfish Immunol. 83 (2018) 37–44.
[49] H.K. Zhang, D.W. Cheng, K.S. Tan, H.X. Liu, T. Ye, S.K. Li, H.Y. Ma, H.P. Zheng, Identification of two ferritin genes and their expression profiles in response to bacterial challenge in noble scallop *Chlamys nobilis* with different carotenoids content, Fish Shellfish Immunol. 88 (2019) 9–16.