ORIGINAL PAPER

Identification of a novel inducible cytosolic Hsp70 gene in Chinese shrimp *Fenneropenaeus chinensis* and comparison of its expression with the cognate Hsc70 under different stresses

Wei Luan · Fuhua Li · Jiquan Zhang · Rong Wen · Yutao Li · Jianhai Xiang

Abstract The heat shock protein 70 (Hsp70) family is widely expressed in eukaryotic cells as the major chaperone protein. In this study, the full-length complementary DNA (cDNA) of a novel inducible cytosolic Hsp70 family member (FcHsp70) was cloned from *Fenneropenaeus chinensis*. *FcHsp70* full-length cDNA consists of 2,511 bp with a 1,890-bp open reading frame encoding 629 amino acids. Three Hsp70 protein family signatures, IDLGTTYS, IIDLGGGTFDVSIL, and IVLVGGSTRIPKVQK, were found in the predicted FcHsp70 amino acid sequence. Phylogenetic analysis showed that FcHsp70 was categorized together with the inducible HSP70s reported in other crustaceans. Compared to the previously identified cognate Hsp70 (FcHsc70) in *F. chinensis*, the expression of FcHsp70 showed quite different expression profiles when the shrimp were subjected to different stresses including heat shock and heavy metal treatments. Under heat shock treatment, the expression of FcHsp70 showed much higher up-regulation than FcHsc70. Copper treatment also induced higher up-regulation of FcHsp70 than FcHsc70. Cadmium treatment did not induce the expression of FcHsp70, but caused down-regulation of FcHsc70. The different expression profiles of FcHsp70 and FcHsc70 in shrimp may indicate their different reactions to different stresses. Therefore, Hsp70 or Hsc70 could be developed as a biomarker to indicate different stresses in shrimp.

Keywords Heat shock proteins · Hsp70 · Heat shock · Heavy metal · Stress · *Fenneropenaeus chinensis*

Wei Luan and Fuhua Li contributed equally to this work.

W. Luan · F. Li (✉) · J. Zhang · R. Wen · J. Xiang (✉)
Institute of Oceanology, Chinese Academy of Sciences,
Qingdao 266071, People's Republic of China
e-mail: fhli@ms.qdio.ac.cn
e-mail: jhxiang@ms.qdio.ac.cn

W. Luan · R. Wen
Graduate University, Chinese Academy of Sciences,
Beijing 100039, People's Republic of China

Y. Li
CSIRO Livestock Industries, Queensland Bioscience Precinct,
306 Carmody Road,
St Lucia, Queensland 4067, Australia

Introduction

Heat shock proteins (Hsps) are ubiquitous chaperone proteins that are conserved evolutionarily in most organisms. Heat shock proteins in eukaryotes are normally classified into Hsp90, Hsp70, Hsp60, and small heat shock protein families according to their molecular weights. As one of the most abundant and widely investigated families in high eukaryotes, the Hsp70 family is made up of cytosolic Hsp70 including the inducible Hsp70 and the cognate Hsc70, glucose-regulated protein 78 (Grp78), and mitochondrion Hsp70. As potential biomarkers, Hsp70s can be induced generally by various environmental stressors such as hyperthermia, hypoxia, and heavy metals to protect the organisms from damages caused by an overload of unfolded proteins in cells (Ellis 1987; Gething and Sambrook 1992; Tsan and Gao 2004).

The functionally conserved structure of Hsp70 protein contains two major domains: the N-terminal ATPase domain (Flaherty et al. 1990) and the C-terminal peptide-binding domain (Wang et al. 1993). Depending on the ATP cycle, Hsp70 binds to the substrate peptides and refolds the

unfolded or nascent peptides either in resting status or under stressed situations (Szabo et al. 1994; Bukau et al. 1998). Under heat shock, cells initiate the heat shock response by up-regulating the expression of Hsps including Hsp70, in order to survive the stresses (Lindquist 1986). However, the cognate form of Hsp70 (Hsc70) is constitutively expressed regardless of stress (Feder and Hofmann 1999).

An aquatic environment is a very complex system in which temperature, salinity, pollutant content, and oxygen will vary greatly depending on season, weather, or human activity. Therefore, both invertebrate and vertebrate aquatic animals have to cope with widely varied environments to survive. In recent years, more studies have focused on the Hsps of aquaculture animals due to their importance in coping with stress-induced denaturation of client proteins, as well as their essential roles including folding, assembly, degradation of other proteins, and the regulation of gene expression (Piano et al. 2005; Terasawa et al. 2005). In aquatic animals, the Hsp70 family is the most widely studied family in Hsps. In mollusks, the complementary DNA (cDNA) of Hsp70 genes has been cloned and its expression has been studied in the European flat oyster, *Ostrea edulis* (Piano et al. 2005), *Mytilus galloprovincialis* (Cellura et al. 2006), the bay scallop *Argopecten irradians* (Song et al. 2006), and the abalone *Haliotis tuberculata* (Farcy et al. 2007). In contrast, fewer reports exist on crustacean species. To date the cDNAs of Hsp70 and Hsc70 have been cloned from the giant freshwater prawn *Macrobachium rosenbergii* (Liu et al. 2004). In the black tiger shrimp *Penaeus monodon*, the full-length cDNA of Hsc70 has been reported by Lo et al. (2004), and its promoter structure and expression have been studied by Chuang et al. (2007). In the Chinese shrimp *Fenneropenaeus chinensis*, the cDNA cloning and expression of Hsc70 and glucose-regulated protein 78 (GRP78, endoplasmic reticulum-resided protein) have been reported previously (Jiao et al. 2004; Luan et al. 2007). To our knowledge, there is no report about inducible Hsp70 in the Penaeid shrimp. In the present study, an inducible HSP70 gene was cloned and its expression was analyzed by real-time polymerase chain reaction (PCR) in comparison with Hsc70 when the shrimp were subjected to different stresses.

Materials and methods

RNA extraction and cDNA synthesis for gene cloning

Juvenile shrimp with body length of 4–5 cm were reared in the aquarium of our institute. The shrimp were kept at normal seawater temperature at approximately 25°C and then were transferred to water at high temperature (35°C). After 2 h, the cephalothoraxes of shrimp which underwent heat shock treatment were dissected and preserved in liquid nitrogen for further RNA extraction. Total RNA was extracted from cephalothorax with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) as described in the manufacturer's protocol. RNA quality was assessed by electrophoresis on 1% agarose gel.

For reverse transcription, the cDNA was synthesized in a 25-μl reaction volume containing 2 μg of DNase I-treated total RNA, 1× Moloney murine leukemia virus (MMLV) buffer, 0.5 mM deoxyribonucleotide triphosphate (dNTP), 0.4 mM oligo-dT, 20 U of RNase inhibitor (Promega, Madison, WI, USA), 200 U of MMLV reverse transcriptase (Promega), two adaptor primers, oligo-d(T) [5′GGCCAC GCGTCGACTAGTAC(T)16(A/C/G)] and BD-Oligo (5′ AAGCAGTGGTATCAACGCAGAGTACGCGGG) with 10 mM. The cDNA was then diluted five times, and 1 μl of the dilution was used for each reverse transcriptase (RT)-PCR reaction.

FcHsp70 cDNA cloning

A pair of degenerate primers FcHsp70Df [5′CAGAACGA CATGAAA(/G)CAT(/C)TG] and FcHsp70Dr1 [5′GTGG GT(/C)TCGTTGATGATG(/A)C] were designed based on the alignment of inducible Hsp70 cDNA identified previously: *M. rosenbergii* AY466497, *Procambarus clarkia* DQ301506, *Rimicaris exoculata* DQ534065, *Drosophila melanogaster* NM_169469, and *Homo sapiens* NM_002155. PCR reaction was performed as follows: an initial denaturation (94°C, 5 min), followed by 35 cycles of (94°C, 40 s; 60°C, 40 s; 72°C, 40 s) and a final extension at 72°C for 10 min. After electrophoresis on 1% agarose gel, PCR products were subsequently purified with MinElute gel extraction kit (Qiagen, Hilden, Germany). Purified DNA of PCR products were ligated into pMD-19T vector (Takara, Dalian, China) and then transformed into Top10 competent cell (Invitrogen). Positive clones were screened out for DNA sequencing (Sangon, Shanghai, China). The sequences of PCR products were analyzed by Blast X (http://www.ncbi.nlm.nih.gov) to confirm whether it is a partial sequence of inducible type Hsp70 gene.

The whole cloning strategy for FcHsp70 was shown in Fig. 1. A pair of specific primers FcHsp70f1 (5′CCGG GTGGTGAGCGAGGAC) and FcHsp70r1 (5′GATGAT GCGCACCACGTTC) were designed based on the fragment sequence above, and a 281-bp fragment was amplified. Another degenerate primer FcHsp70Dr2 (5′TTTGA TGAGGGCIGTCATIAC) was designed based on the alignment of HSP70s from species described above. The degenerate primer FcHsp70Dr2 combined with the specific primer FcHsp70f1 was used to amplify the fragment as follows: an initial denaturation (94°C, 5 min), with 35 cycles of (94°C, 50 s; 59°C, 50 s; 72°C, 90 s) with a final extension (72°C, 10 min). Based on the sequence of previously amplified fragment, another specific primer

Fig. 1 The schematic diagram of FcHsp70 cloning. Primers were illustrated with locations in PCR amplification. *Dotted arrows* indicate the degenerate primers. *Straight arrows* indicate the specific primers
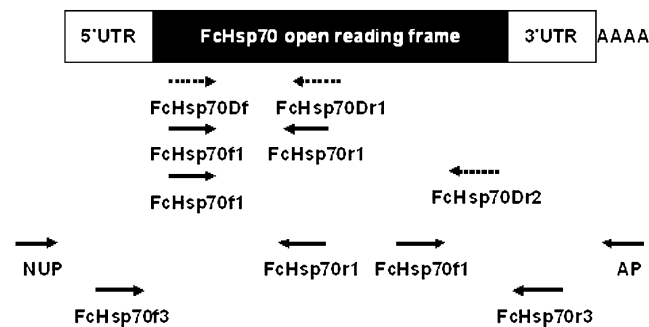
FcHsp70f2 (5′TTCACTGGGAATTGAAACAGCT) was designed for 3′ rapid amplification of cDNA ends (RACE). The complete 3′ terminal of FcHsp70 cDNA was obtained through the amplification of FcHsp70f2 and the anchor primer AP (5′GGCCACGCGTCGACTAGTAC). The amplification follows an initial denaturation (94°C, 5 min), 35 cycles of (94°C, 50 s; 60°C, 50 s; 72°C, 2 min) with a final extension (72°C, 10 min). The specific primer FcHsp70r1 and the anchor primer NUP (5′AAGCAGTGG-TATCAACGCAGAGT) were used to amplify for 5′ RACE with an initial denaturation (94°C, 5 min), 35 cycles of amplification (94°C, 50 s; 62°C, 50 s; 72°C, 90 s) with a final extension (72°C, 10 min). A pair of FcHsp70 specific primers FcHsp70f3 (5′AAACCGGCAAAGTGTTCTTG) and FcHsp70r3 (5′CAAGTCACATTGTGCTCCAA) were designed to confirm the full-length of FcHsp70 cDNA sequence. The PCR amplification condition is as follows: an initial denaturation of 94°C for 5 min, 35 cycles of 94°C, 1 min; 60°C, 1 min and 72°C, 150 s, followed by a final extension of 72°C for 10 min.

Sequence analysis

The *FcHsp70* cDNA sequence was analyzed and the deduced amino acid sequence was predicted by BioEdit (version 7.0.1) software. The deduced amino acid sequence was analyzed by the Simple Modular Architecture Research Tool (http://smart.embl-heidelberg.de). The Compute pI/Mw tool (http://ca.expasy.org/tools/pi_tool.html) was used to calculate the theoretical isoelectric point (p*I*) and molecular weight (Mw). The amino acid sequences of different species were obtained from the GenBank database (www.ncbi.nih.gov) and a multiple sequence alignment with ClustalX (version 1.83) and subsequent neighbor-joining phylogeny tree were constructed by Bioedit (Protdist/neighbor phylogenetic tree).

Shrimp for heat shock treatment

The procedures and sampling methods for culturing the juvenile shrimp during heat shock treatment are the same as those described previously (Li et al. 2009). Briefly, six shrimp were randomly collected at 1, 2, 3, 4, and 6 h, respectively, during heat shock treatment at 35±1°C. After heat shock treatment, the shrimp were transferred to 25±1°C to be cultured for recovery for 48 h. During the recovery time, six shrimp were sampled at 6, 24, and 48 h, respectively. The cephalothoraxes of the shrimp at each sampling time were separated, frozen immediately in liquid nitrogen, and stored at −80°C until RNA extraction for later gene expression analysis.

Shrimp for copper and cadmium treatment

The shrimp of the same age and size as above were used for copper or cadmium treatment. Prior to the treatment, six individuals were collected as the control group and preserved in liquid nitrogen for RNA extraction.

For copper treatment, 500 mM initial stock solution of CuSO₄ was prepared and then mixed with seawater to make the 50 μM working solution. For cadmium treatment, 50 mM stock solution of CdCl₂ was prepared and then mixed with seawater to make the final solution of 2.5 μM. Two groups of 30 juvenile shrimp with body length of 4–5 cm were transferred into a 600-l tank with working solutions for copper or cadmium treatment. Six shrimp from each group were collected randomly at 6, 12, 24, and 72 h post-exposure to Cu^{2+} or Cd^{2+}. The cephalothoraxes of shrimp at each sampling point were collected, frozen immediately in liquid nitrogen, and stored at −80ºC for later RNA extraction. No mortality was observed during the collection.

RNA extraction and cDNA synthesis for real time RT-PCR

Total RNA was extracted from cephalothorax with TRIzol Reagent (Invitrogen) following the manufacturer's protocol. RNA quality was assessed by electrophoresis detection on 1% agarose gel, combined with measuring the $OD_{260/280}$ value by spectrophotometer (GeneQuant *pro*, Amersham Biosciences, Freiburg, Germany). RNA with good quality was used for the following reverse transcription.

For reverse transcription, 2 μg total RNA was treated with 2 U RQ1 RNase-free DNase (Promega) at 37°C for 30 min following the manufacturer's protocol for the first

step. The cDNA templates were synthesized using MMLV reverse transcriptase (Promega) by hexa primer (Sangon) according to the manufacture's protocol and diluted before use in real-time RT-PCR.

FcHsp70 and FcHsc70 gene expression quantification by real-time RT-PCR

In order to study the gene expression levels of FcHsp70 and FcHsc70, two sets of specific primers were designed. They were FcHsp70RTf (5′ATCTCGTCCGTGGTGCTG) and FcHsp70RTr (5′GCCTTTCCTACGCTCTGC) for inducible FcHsp70 (with the predicted amplification fragment size of 203 bp), and FcHsc70RTf (5′GGACCTGTTGCTGTTGGA) and FcHsc70RTr (5′CTGGCTGGTTGTCAGAGTAG) for the heat shock protein cognate 70 (Hsc70) (FcHSC70, accession number: AY748350, the predicted size being 143 bp). For expression analysis, 18s rRNA gene of *F. chinensis* was used as the reference gene. The specific primers 18Sf (5′AGTAGCCGCCCTGGTTGTAGAC) and 18Sr (5′TTCTCCATGTCGTCCCAGT) were designed to amplify a 147-bp-long fragment of 18s rRNA. Nuclease-free water (Promega) was used as PCR negative control instead of cDNA templates.

Real-time RT-PCR reactions were carried out using the PCR machine (Mastercycler *ep realplex* 4, Eppendorf, Hamburg, Germany), in 25 µl reaction systems containing 1 U Takara Ex taq hot start, 1× Ex taq buffer (plus Mg^{2+}), 0.2 mM dNTP mixture, 1× SYBR Green Master Mix (Applied Biosystems, Framingham, MA, USA), 0.2 mM forward primer, 0.2 mM reverse primer, and 1 µl cDNA template. The thermal profile was 95°C for 2 min, followed by 40 cycles of 95°C 15 s, 60°C (for *FcHsp70*) or 59°C (for *FcHsc70*) 15 s and 68°C 15 s. One additional melting cycle (68°C to 100°C, total 20 min) followed each thermal profile and produced the melting curves to confirm that only one specific PCR product was amplified. Triplicates were performed at each time point for each gene. The fluorescent real-time PCR data were analyzed by *RealPlex* Software (Eppendorf).

Calculations and statistics

Expression levels of target genes (*FcHsp70* and *FcHsc70*) were calculated based on the comparative C_T (cycle threshold) value to the reference gene 18S. Considering each ratio equal to 1 at the start point of the control group, normalization of the ratios was calculated. The χ-fold curves were produced as follows: if the ratio (χ) of one particular time point was less than 1, χ-fold of this time point was calculated as $(-1/\chi)$; if the ratio (χ) was greater than 1, χ-fold would be considered as χ. Data were presented as arithmetical mean of triplicates ±SE. SPSS

11.0 software was used to determine whether there is a significant variation in the real-time RT-PCR data. The Turkey HSD test was performed with one-way analysis of variance by the SPSS software. Significance was declared if $P<0.05$.

Results

cDNA cloning and sequence analysis of FcHsp70

A fragment of 281 bp was generated by FcHsp70Df and FcHsp70Dr1. Through Blast online analysis, the fragment was confirmed to be a part of Hsp70 gene sequence. The primer pair of FcHsp70Df and FcHsp70Dr2 produced a 976-bp fragment which had an overlap of 281 bp to the initial fragment. The 2,511-bp full-length cDNA sequence of FcHsp70 was obtained by 5′ and 3′ RACE, containing an 1,890-bp open reading frame (ORF) encoding 629 amino acids, a 274-bp 5′ untranslated region (UTR) and a 347-bp 3′ UTR. The predicted molecular weight of FcHsp70 is 69.1 kDa and the theoretical p*I* is 5.40. Three Hsp70 protein family signatures, IDLGTTYS (8–15), IIDLGGGTFDVSIL (198–211), and IVLVGGSTRIPKVQK (335–349), were identified in the predicted FcHsp70 amino acid sequence (Fig. 2). The deduced amino acid sequence of FcHsp70 protein was functionally divided into the ATPase domain (1–386) near the N-terminal and the peptide-binding domain (387–629). The EEVD motif appeared at the end of the C-terminal (shown in Fig. 2).

Homology analysis of FcHsp70

Through alignment with the cognate form of the cytosolic Hsp70 protein (FcHsc70) and glucose-regulated protein 78 (FcGrp78) which resides in the endoplasmic reticulum (ER) in *F. chinensis*, FcHsp70 was found to have 86% homology with FcHsc70 and 74% with FcGrp78 (Fig. 3). FcHsp70 also shared 90% and 82% homology, respectively, with two decapod species, *P. clarkia* and *M. rosenbergii*, in addition to 86% and 81% with *H. sapiens* and *D. melanogaster*, respectively (data not shown). The comparison of deduced amino acid sequences among Hsp70 family members, FcHsp70, FcHsc70, and FcGRp78, was performed within the ATPase domain (Fig. 3a) and the peptide-binding domain (Fig. 3b), respectively. A conserved nuclear location signal (QRKYKKDLTSNKRALRR, 247–263) was found, and a conserved phosphorylation residue (T199) together with some residues (Y145, N147, D148, N170, and T173) interacting with Hsp40 which is a Hsp70 co-chaperone was also identified in the ATPase domain of Hsp70 (Fig. 3a). The linker region (DMLLLD, 391–396) was located in the peptide-binding domain. Several key

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1    GAATCACGGTCGGAACGATACACGAAGGAAAGAAAAGAGGCCAAGAGGAAAGTGCAAGAATATTACATACATCAAGGGATTTCTCAACGA 90
91   GCAAACCGGCAAAGTGTTCTTGAAACAATAACCGGTTACTACATATACCCGAGGAGACCCAAAGATTTTCATAAGGTGCAGACGAGAAAAA 180
181  CCTTATTGGATTGATTCGATTATTTCTACGTAAGAGAAGAGACCACCCTGAAATAAATTCGACTTTAACTGGAGTGCAATTAGAAGGCCG 270
271  GACGATGGCGGGTCCCGTGATTGGCATCGACCTGGGCACCACGTACTCGTGCGTGGGCGTGTTCCAGCAGGGCAAGGTGGAGATCCTGGC 360
1        M  A  G  P  V  I  G  I  D  L  G  T  T  Y  S  C  V  G  V  F  Q  Q  G  K  V  E  I  L  A 29
361  CAACGACCAGGGCAACAGGACCACCCCCTCGTACGTGGCCTTCGGCGACGCTGAGCGCCTGGTGGGTGACGCCGCCAAGAACCAGCGGC 450
30       N  D  Q  G  N  R  T  T  P  S  Y  V  A  F  G  D  A  E  R  L  V  G  D  A  A  K  N  Q  A  A 59
451  CATGAACCCGTGCAACACCGTCTTCGACGCCAAGAGGCTGATCGGACGCAAGTTCGAGGACGCCGCCGTCCAGAGCGACATGAAGCACTG 540
60       M  N  P  C  N  T  V  F  D  A  K  R  L  I  G  R  K  F  E  D  A  A  V  Q  S  D  M  K  H  W 89
541  GCCGTTCCGGGTGGTGAGCGAGGACGGCAAGGCCAAGATCAGCGTGCGCTTCAAGGGCGAGGACAAGAGCTTCAACCCGGAGGAGATCTC 630
90       P  F  R  V  V  S  E  D  G  K  A  K  I  S  V  R  F  K  G  E  D  K  S  F  N  P  E  E  I  S 119
631  GTCCGTGGTGCTGGCCAAGATGAAGGAGACAGCCGAAGCCTACTTGGGGAACAGTGTGAAGAATGCCGTGATCACGGTGCCCGCCTACTT 720
120      S  V  V  L  A  K  M  K  E  T  A  E  A  Y  L  G  N  S  V  K  N  A  V  I  T  V  P  A  Y  F 149
721  TAATGACTCTCAGCGACAAGCCACCAAGGACGCAGGAACCATCGCGGGCATCAACGTTCTTAGGATCATCAATGAACCCACAGCCGCCGC 810
150      N  D  S  Q  R  Q  A  T  K  D  A  G  T  I  A  G  I  N  V  L  R  I  I  N  E  P  T  A  A  A 179
811  CATTGCTTATGGTCTGGACAAGCAGAGCGTAGGAAAGGCTGAGCGGAACGTGTTGATCATCGACCTGGGAGGCGGTACCTTCGACGTGTC 900
180      I  A  Y  G  L  D  K  Q  S  V  G  K  A  E  R  N  V  L  I  I  D  L  G  G  G  T  F  D  V  S 209
901  CATCCTCAGCATCGACGACGGAGTGTTCGAGGTGAAGTCGACCGCAGGAGACACTCACTTGGGCGGCGAAGACTTCGATAACAGGATGGT 990
210      I  L  S  I  D  D  G  V  F  E  V  K  S  T  A  G  D  T  H  L  G  G  E  D  F  D  N  R  M  V 239
991  TACTCATTTCGTTCAAGAGTTTCAGAGGAAATACAAGAAGGATCTGACGAGCAACAAACGCGCACTTCGACGTCTCCGAACTGCCTGTGA 1080
240      T  H  F  V  Q  E  F  Q  R  K  Y  K  K  D  L  T  S  N  K  R  A  L  R  R  L  R  T  A  C  E 269
1081 ACGAGCCAAGCGAACGCTGTCTTCGTCCACACAAGCTAGTCTTGAAATCGACTCTCTCTTCGAGGGAATTGACTATTATACATCCATCAC 1170
270      R  A  K  R  T  L  S  S  S  T  Q  A  S  L  E  I  D  S  L  F  E  G  I  D  Y  Y  T  S  I  T 299
1171 TCGTGCAAGATTCGAAGAACTTTGTTCCGACCTTTTCCGAGGAACTCTAGAACCAGTGGAGAAAGCTCTTCGAGACGCCAAGTTAGACAA 1260
300      R  A  R  F  E  E  L  C  S  D  L  F  R  G  T  L  E  P  V  E  K  A  L  R  D  A  K  L  D  K 329
1261 GAGTAGTATCCATGATATCGTCCTGGTTGGAGGCTCAACACGCATCCCTAAGGTGCAAAAACTGCTTCAAGGTTTCTTCAACGGCAAGGA 1350
330      S  S  I  H  D  I  V  L  V  G  G  S  T  R  I  P  K  V  Q  K  L  L  Q  G  F  F  N  G  K  E 359
1351 ACTGAACAAGTCCATCAACCCAGACGAAGCTGTAGCTTACGGAGCTGCAGTTCAGGCAGCCATATTGCGAGGAGACCAGTCTGAAGCAGT 1440
360      L  N  K  S  I  N  P  D  E  A  V  A  Y  G  A  A  V  Q  A  A  I  L  R  G  D  Q  S  E  A  V 389
1441 GAAAGACATGCTACTTCTTGATGTGGCTCCCCTTTCACTGGGAATTGAAACAGCTGGCGGGGTCATGACGGCCCTCATCAAACGCAACAC 1530
390      K  D  M  L  L  L  D  V  A  P  L  S  L  G  I  E  T  A  G  G  V  M  T  A  L  I  K  R  N  T 419
1531 CACAATTCCCACCAAACATTCTCAGATCTTTACTACATATTCGGATAATCAGCCTGGAGTATCGATTCAAGTGTACGAGGGCGAGCGAAC 1620
420      T  I  P  T  K  H  S  Q  I  F  T  T  Y  S  D  N  Q  P  G  V  S  I  Q  V  Y  E  G  E  R  T 449
1621 CATGACCAGAGATAACAACTTGCTTGGTAAGTTCGAGCTGAGTGGTATCGCCCCAGCTCCACGCGGAGTTCCACAGATCGAAGTCACCTT 1710
450      M  T  R  D  N  N  L  L  G  K  F  E  L  S  G  I  A  P  A  P  R  G  V  P  Q  I  E  V  T  F 479
1711 CGACATCGACGCCAACGGCATCCTGAACGTAAGCGCGGTGGACAAGTCCACTGGGAAAGAGAACAGGATCTCCATCAACAACGACAAGGG 1800
480      D  I  D  A  N  G  I  L  N  V  S  A  V  D  K  S  T  G  K  E  N  R  I  S  I  N  N  D  K  G 509
1801 CCGTCTCAGCAAAGAGGAAATCGAGAGGATGGTCAATGAGGCTGAAAACTATCGAGAGGAAGACGCCAAACAGCGAGAACGCATCGAGGC 1890
510      R  L  S  K  E  E  I  E  R  M  V  N  E  A  E  N  Y  R  E  E  D  A  K  Q  R  E  R  I  E  A 539
1891 CAAAAACCGCCTTGAAGCACTTTGCTATAGCATCAAATCTTCTCTTTCTGACACTGCCGTGGCTGACAAACTGCCATCAGATGAAAAACG 1980
540      K  N  R  L  E  A  L  C  Y  S  I  K  S  S  L  S  D  T  A  V  A  D  K  L  P  S  D  E  K  R 569
1981 CCTCGTGGAAGAGAAAGCTGAAGAAACGCTGAAATGGTTAGATAATAACCAGCTGGCAGAAAAGGAGGAGTACGAGTACAAGGTGAAGGA 2070
570      L  V  E  E  K  A  E  E  T  L  K  W  L  D  N  N  Q  L  A  E  K  E  E  Y  E  Y  K  V  K  E 599
2071 GCTGGAGCAGGTGTGGCGACCCCTTGCTAGCAAATTGCATGGATCGGGGACAGGCTCCTCAGGCACGGGTCCAACAGTAGAGGAAGTTGA 2160
600      L  E  Q  V  W  R  P  L  A  S  K  L  H  G  S  G  T  G  S  S  G  T  G  P  T  V  E  E  V  D 629
2161 TTAAATTTCATCATGTGATGAGTACAATCTATTGGAGCACAATGTGACTTGTGTGATAGATCGACTTCGTACATGTATTTCAATACTGATC 2250
                 *
2251 TCTTTCTGTGTACCTGCATAGCCTATACTGTTTTACCCTATTACTGTACATTGTGTGTTAAAATATTTAGAAAGCATACTGTGATATTAA 2340
2341 TATAAATTTGATATTTATCATTGTAAGCTATGATTCTAAATGTACATTTGATTGTATTATCACGCTTATAAGACAAAATGTACATTTCATC 2430
2431 ATAAAGTATTCCCTCTGAAAATGTACTATTCAATGTAATAAGTGAATAGACATACCTGTTATCTGAAAAAAAAAAAAAAAAA 2511
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Fig 2 The full-length cDNA sequence of FcHsp70 with its deduced amino acid sequence. The start codon is *boxed* and the stop codon is indicated with an *asterisk*. Three Hsp70 protein family signatures are *shaded*. The conserved EEVD motif at the C-terminal is *underlined*

residues (I404, T406, V410, T412, F429, T430, Q436, G438, and I441, illustrated with a dot in Fig. 3b), which may be involved in the interaction with client proteins of Hsp70, were found in the peptide-binding domain of FcHsp70. Three conserved residues A407, Y432, and V439 (indicated with arrows in Fig. 3b) are located at the key positions in forming hydrophobic arch and pocket for determining that the Hsp70 substrate-binding activity also exists in the peptide-binding domain. Unlike the ER retention signal KDEL at the C-terminal of FcGrp78,
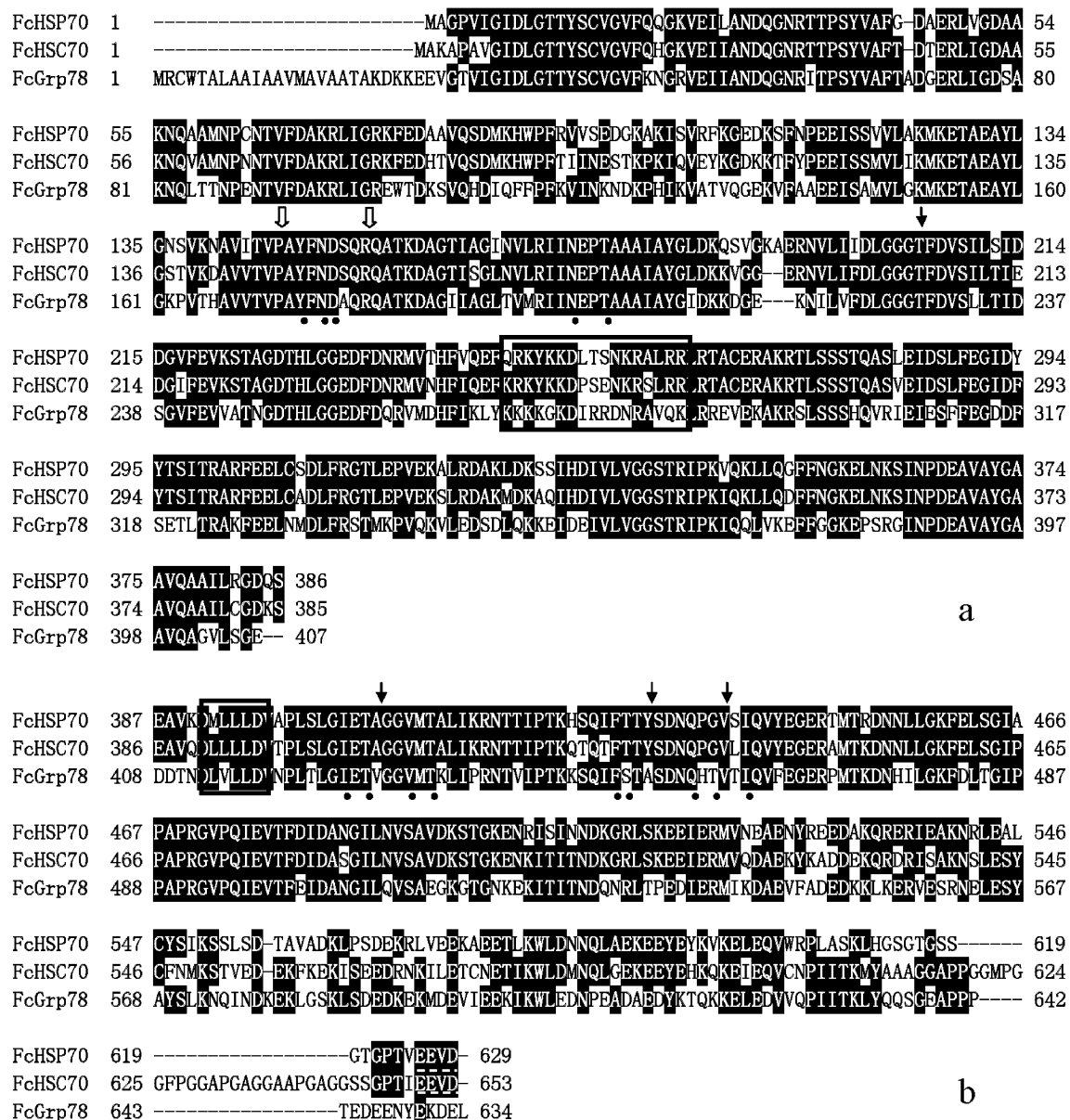
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FcHSP70   1   ------------------------MAGPVIGIDLGTTYSCVGVFQQGKVEILANDQGNRTTPSYVAFG-DAERLVGDAA   54
FcHSC70   1   ----------------------MAKAPAVGIDLGTTYSCVGVFQHGKVEIIANDQGNRTTPSYVAFT-DTERLIGDAA   55
FcGrp78   1   MRCWTALAAIAAVMAVAATAKDKKEEVGTVIGIDLGTTYSCVGVFKNGRVEIIANDQGNRITPSYVAFTADGERLIGDSA  80

FcHSP70  55   KNQAAMNPCNTVFDAKRLIGRKFEDAAVQSDMKHWPFRVVSEDGKAKISVRFKGEDKSFNPEEISSVVLAKMKETAEAYL  134
FcHSC70  56   KNQVAMNPNNTVFDAKRLIGRKFEDHTVQSDMKHWPFTIINESTKPKIQVEYKGDKKTFYPEEISSMVLIKMKETAEAYL  135
FcGrp78  81   KNQLTTNPENTVFDAKRLIGREWTDKSVQHDIQFFPFKVINKNDKPHIKVATVQGEKVFAAEEISAMVLGKMKETAEAYL  160

FcHSP70 135   GNSVKNAVITVPAYFNDSQRQATKDAGTIAGINVLRIINEPTAAAIAYGLDKQSVGKAERNVLIFDLGGGTFDVSILSID  214
FcHSC70 136   GSTVKDAVVTVPAYFNDSQRQATKDAGTISGLNVLRIINEPTAAAIAYGLDKKVGG--ERNVLIFDLGGGTFDVSILTIE  213
FcGrp78 161   GKPVTHAVVTVPAYFNDAQRQATKDAGIIAGLTVMRIINEPTAAAIAYGIDKKDGE---KNILVFDLGGGTFDVSLLTID  237

FcHSP70 215   DGVFEVKSTAGDTHLGGEDFDNRMVTHFVQEFQRKYKKDLTSNKRALRRLRTACERAKRTLSSSTQASLEIDSLFEGIDY  294
FcHSC70 214   DGIIFEVKSTAGDTHLGGEDFDNRMVNHFIQEFKRKYKKDPSENKRSLRRLRTACERAKRTLSSSTQASVEIDSLFEGIDF  293
FcGrp78 238   SGVFEVVATNGDTHLGGEDFDQRVMDHFIKLYKKKKGKDIRRDNRAVQKLRREVEKAKRSLSSSHQVRIEIESFFEGDDF  317

FcHSP70 295   YTSITRARFEELCSDLFRGTLEPVEKALRDAKLDKSSIHDIVLVGGSTRIPKVQKLLQGFFNGKELNKSINPDEAVAYGA  374
FcHSC70 294   YTSITRARFEELCADLFRGTLEPVEKSLRDAKMDKAQIHDIVLVGGSTRIPKIQKLLQDFFNGKELNKSINPDEAVAYGA  373
FcGrp78 318   SETLTRAKFEELNMDLFRSTMKPVQKVLEDSDLQKKEIDEIVLVGGSTRIPKIQQLVKEFFGGKEPSRGINPDEAVAYGA  397

FcHSP70 375   AVQAAILRGDQS  386
FcHSC70 374   AVQAAILCGDKS  385
FcGrp78 398   AVQAGVLSGE--  407
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a

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FcHSP70 387   EAVKDMLLLDVAPLSLGIETAGGVMTALIKRNTTIPTKHSQIFTTYSDNQPGVSIQVYEGERTMTRDNNLLGKFELSGIA  466
FcHSC70 386   EAVQDLLLLDVTPLSLGIETAGGVMTALIKRNTTIPTKQTQIFTTYSDNQPGVLIQVYEGERAMTKDNNLLGKFELSGIP  465
FcGrp78 408   DDTNDLVLLDVNPLTLGIETVGGVMTKLIPRNTVIPTKKSQIFSTASDNQHTVTIQVFEGERPMTKDNHILGKFDLTGIP  487

FcHSP70 467   PAPRGVPQIEVTFDIDANGILNVSAVDKSTGKENRISINNDKGRLSKEEIERMVNEAENYREEDAKQRERIEAKNRLEAL  546
FcHSC70 466   PAPRGVPQIEVTFDIDASGILNVSAVDKSTGKENKITITNDKGRLSKEEIERMVQDABKYKADDEKQRDRISAKNSLESY  545
FcGrp78 488   PAPRGVPQIEVTFEIDANGILQVSAEGKGTGNKEKITITNDQNRLTPEDIERMIKDAEVFADEDKKLKERVESRNELESY  567

FcHSP70 547   CYSIKSSLSD-TAVADKLPSDEKRLVEEKABETLKWLDNNQLAEKEEYEYKVKELEQVWRPLASKLHGSGTGSS------  619
FcHSC70 546   CFNMKSTVED-EKFKEKISEEDRNKILETCNETIKWLDMNQLEKEEYEHKQKEIEQVCNPIITKMYAAAGGAPPGGMPG  624
FcGrp78 568   AYSLKNQINDKEKLGSKLSDEDKEKMDEVIEEKIKWLEDNPEADAEDYKTQKKELEDVVQPIITKLYQQSGEAPPP----  642

FcHSP70 619   ------------------GTGPTVEEVD-  629
FcHSC70 625   GFPGGAPGAGGAAPGAGGSSGPTIEEVD-  653
FcGrp78 643   ------------------TEDEENYEKDEL  634
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b

Fig. 3 The homology analysis of Hsp70 family members identified in *F. chinensis*. **a** The ATPase domain. The nuclear localization signal is *boxed*. The *black arrow* shows T199 phosphorylation site. The P143 and R151 indicated with *blank arrows* are important for the interdomain function (Vogel et al. 2006). Residues *underlined with dots* are responsible for interaction of Hsp70 with hsp40 (Suh et al. 1998). **b** The peptide-binding domain. The linker interface where hsp70 and hsp40 interaction occurs is *boxed* (Jiang et al. 2005). *Residues with dot* are in contact with the substrate proteins (Zhu et al. 1996). *Arrows* show the constitutive hydrophobic arch and hydrophobic pocket (Mayer et al. 2000). EEVD (*broken lines above*) and KDEL (*underlined*) are the cytosolic and ER retention signals, respectively (Pelham 1989; Johnson et al. 1998). The accession number of FcGrp78 is DQ205426, for FcHsc70 it is AY748350

FcHsp70 and FcHsc70 have the EEVD motif at their C-terminal (Fig. 3b).

When considering the phylogeny tree, three subclasses were formed based on the multiple alignments. They were inducible, cognate, and the ER groups of Hsp70 proteins. FcHsp70 was clustered into the inducible Hsp70 group and was much closer to its homologs in *P. clarkia* and *M. rosenbergii* (Fig. 4). From the phylogenetic tree, it was demonstrated that cytosolic type Hsp70 proteins were sorted into one subgroup against the ER resident forms.

Expression profiles of FcHsp70 and FcHsc70 under heat shock treatment

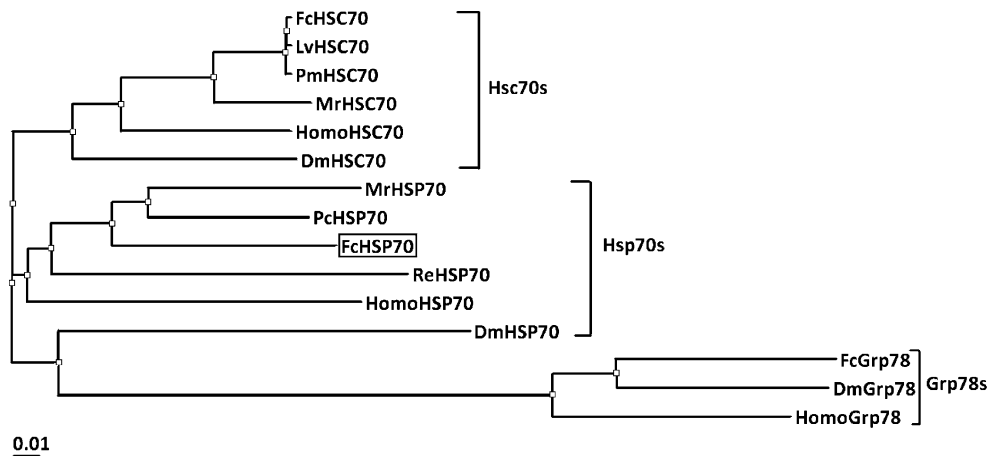The expression profiles of FcHsp70 and FcHsc70 after shrimp experienced heat shock treatment are shown in

Fig. 4 The neighbor-joining phylogeny tree (bootstrap=1,000) of FcHsp70 with other Hsp70 family members. FcHsp70 is *boxed*. Three subclasses were classified as inducible, cognate, and ER groups. Abbreviations and accession numbers are FcHSC70, *F. chinensis* Hsc70, AY748350; LvHSC70, *Litopenaeus vannamei* Hsc70, ABP01681; PmHsc70, *Penaeus monodon* Hsc70, AAQ05768; HomoHSC70, *Homo sapiens* Hsc70, NP_006588; MrHSP70, *Macro-* *brachium rosenbergii* Hsp70, AY466497; PcHSP70 *Procambarus clarkia* Hsp70, DQ301506; ReHSP70, *Rimicaris exoculata* Hsp70, DQ534065; DmHSP70, *Drosophila melanogaster* Hsp70, NM_169469; HomoHSP70, *Homo sapiens* Hsp70, NM_002155; FcGrp78, *F. chinensis* Grp78, DQ205426; DmGrp78, *Drosophila melanogaster* Grp78, NP_511132; HomoGrp78, *Homo sapiens* Grp78, NP_005338

Fig. 5. It is clear that the expression profile of FcHsp70 was quite different from that of FcHsc70. The relative expression level of FcHsp70 increased rapidly by more than 60-fold at 1 h post-heat shock treatment in comparison to that of the control shrimp without any treatment. It continued rising to a level nearly 100 times higher at 2 h post-heat shock treatment than that of the control. From 3 h post-shock, the expression of FcHsp70 decreased dramatically and reached a level lower than that in the initial couple of hours, but was still significantly higher than that of the control group. However, when the shrimp were placed in the seawater at 25°C to recover after 6 h heat shock treatment, significant declines of FcHsp70 expression were observed.

Unlike the inducible FcHsp70, the cognate form FcHsc70 did not show such a huge variation in expression. Instead, the expression of FcHsc70 increased gradually and reached the highest level (about eight times of that of the control group) at 4 h heat shock treatment. During the recovery period, the expression level of FcHsc70 was still higher than that of the control shrimp.

Expression profiles of FcHsp70 and FcHsc70 after heavy metal treatments

The expression profiles of FcHsp70 and FcHsc70 when shrimp were exposed to Cu^{2+} treatment are shown in Fig. 6. The expression of FcHsp70 in juvenile shrimp was significantly up-regulated at 6, 12, and 24 h after exposure to $CuSO_4$, and the highest expression was detected at 12 h, which is 17 times higher than that of the control group. A down-regulation of FcHsp70 expression was detected at 72 h post-exposure. However, a quite different expression profile was observed for FcHsc70 (Fig. 6). The $CuSO_4$ exposure did not cause any significant difference in the expression of FcHsc70 at 12 h post-treatment. An up-regulation of FcHsc70 expression was detected at 24 h, and its expression then returned to the same level as that of the control at 72 h post-treatment.

The expression profile of FcHsc 70 was shown in Fig. 7 when shrimp were exposed to Cd^{2+}. The expression of FcHsc70 was greatly inhibited by Cd^{2+} exposure, but exposure to $CdCl_2$ did not induce any over-expression of FcHsp70.
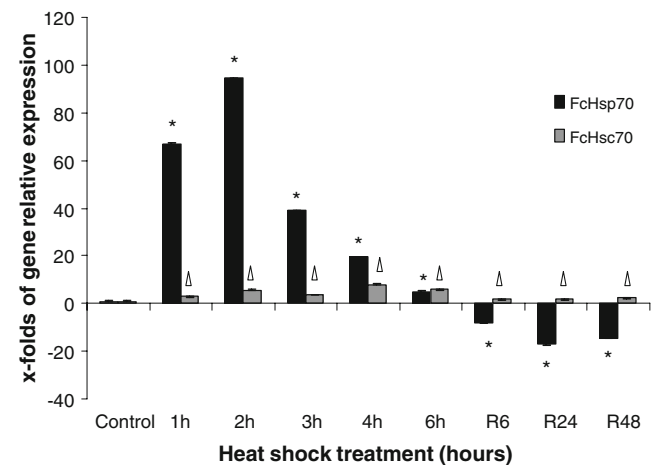

Fig. 5 Real-time RT-PCR analyses of FcHsp70 and FcHsc70 expression in *F. chinensis* juveniles under heat shock treatment ($n=6$). The ratio of the control group was considered as 1. Data were presented as arithmetical mean ± SE (*error bar*). *Asterisks* indicate significant differences in FcHsp70 expression compared to the control ($P<0.05$). *Triangles* indicate significant differences in FcHsc70 expression compared to the control ($P<0.05$)
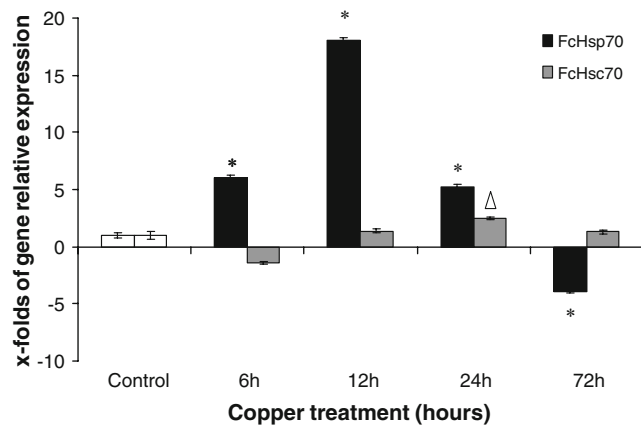
Fig. 6 Real-time RT-PCR analyses of FcHsp70 and FcHsc70 expression in *F. chinensis* juveniles under CuSO$_4$ treatment (*n*=6). The ratio of the control group was considered as 1. Data were presented as arithmetical mean ± SE (*error bar*). Asterisks indicate significant differences in FcHsp70 expression compared to the control (*P*<0.05). *Triangle* indicates significant differences in FcHsc70 expression compared to the control (*P*<0.05)

Discussion

As described in the other inducible forms of Hsp70 genes, no intron region was found in FcHsp70 (Wu et al. 1985; Boutet et al. 2003). Instead, FcHsc70 and other Hsc70 previously identified contain an intron region close to the 5′ terminal of the ORFs (Jiao et al. 2004; Chuang et al. 2007; Daugaard et al. 2007). Without the hydrophobic signal peptide at the 5′ terminal of FcGrp78 precursor (Luan et al. 2007), FcHsp70 possesses functionally conserved ATPase and peptide-binding domains in the structure. In FcHsp70, no G-G-X-P motif was found near the 3 terminal. In contrast, FcHsc70 contains several repeats of G-G-X-P. The tetrapeptide motif G-G-X-P repeats are thought to be involved in co-chaperone-binding activities of Hsc70 (Demand et al. 1998). The EEVD motif at the C-terminal of FcHsp70 suggests that FcHsp70 is a cytosolic Hsp70. The EEVD motif can facilitate the binding ability of FcHsp70/FcHsc70 with co-chaperones such as Hop, through the interaction of Hsp70 with Hsp90 (Johnson et al. 1998). The EEVD possibly plays important roles in the peptide-binding activity (Ramya et al. 2006). The existence of nuclear localization signal in the ATPase domain might make the FcHsp70 protein transfer from the cytoplasm into the nucleus (Robbins et al. 1991). Actually, recent evidence indicates that Hsp70 could be detected in the extracellular space and might participate in the host immune response (Asea et al. 2002; Thériault et al. 2005). Some studies have shown that the linker region as the interaction point of Hsp70 with Hsp40 (the co-chaperone protein of Hsp70), plays a key role in the function of Hsp70 (Jiang et al. 2005). The conserved linker region ensures the function of

Hsp70 in its 3D structure (Shonhai et al. 2005). Autophosphorylation of T199 residue plays a key role in regulating the Hsp70 chaperone activity (McCarty and Walker 1991). In fact, the existence of many threonine and serine residues is crucial for the multiple functions of Hsp70 by either autophosphorylation or phosphorylation with the kinases (Kappes et al. 1993; Sherman and Goldberg 1993). The P143 and R151 were suggested to be important for the inter-domain function (Vogel et al. 2006).

The phylogenetic analysis of Hsp70, Hsc70, and Grp78 demonstrates not only the high similarity among these Hsp70 family proteins, but also the close relationship of these homologs among species within subclasses in the phylogeny tree. In both Hsp70 and Hsc70 subclasses, FcHsp70 and FcHsc70 of shrimp were closer to their homologs in crustaceans than to those from human beings. In addition, Hsp70s, Hsc70s, and Grp78s were clustered into three separate subclasses, which might show how Hsp70 family proteins evolve in the subcellular locations of eukaryotic cells.

Although both FcHsp70 and FcHsc70 showed apparent up-regulations at the messenger RNA (mRNA) transcription level of shrimp, different expression profiles were obtained. The expression of FcHsp70 is very sensitive to heat shock treatment. At 1–2 h post-heat shock, the expression of FcHsp70 increased approximately 60–100-fold compared with that in shrimp without heat shock. After the shrimp were returned to 25°C, the expression of FcHsp70 dropped very quickly and became down-regulated in comparison to that of the control. However, the expression of FcHsc70 gradually increased and kept up-regulating even though the shrimp had been returned to water at 25°C. The data showed that the transcription level of FcHsp70 was very sensitive to
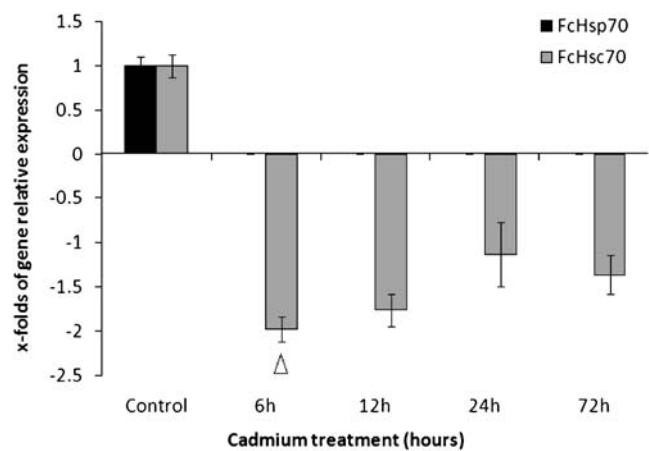

Fig. 7 Real-time RT-PCR analyses for FcHsc70 expressions in juveniles of *F. chinensis* under CdCl$_2$ treatment (*n*=6). The ratio of the control group was considered as 1. Data were presented as arithmetical mean ± SE (*error bar*). *Triangle* indicates significant differences in FcHsc70 expression compared to the control (*P*<0.05)

temperature variations. Elevation of temperature induced the over-expression of FcHsp70 in a short period of time, and a reduction in temperature inhibited the over-expression of FcHsp70 very quickly. In contrast to FcHsp70, the variation of FcHsc70 transcription was much slower and at lower levels when shrimp experienced temperature variation. Based on the present data, we inferred that FcHsp70 played more crucial roles in protecting the cells from damage than FcHsc70 during heat shock treatment, while FcHsc70 may be more important in protecting the cells when shrimp were transferred from high- to low-temperature water. In our previous study, the data also showed that the expression of HSP90 of Chinese shrimp (FcHsp90) was dramatically up-regulated and kept at high levels during the heat shock treatment and became down-regulated after the shrimp were returned to water at 25°C from 36°C (Li et al. 2009). When considering another HSP70 family member Grp78 of *F. chinensis*, we found that its expression level increased gradually during heat shock treatment, then returned to the same level as that of controls after the shrimp were returned back to water at 25°C (Luan et al. 2007). The expression profile of FcHsc70 in this study is similar to that of FcGrp78. In *M. rosenbergii*, the expression of Hsp70 was induced under heat shock, but the Hsc70 mRNA level remained unchanged (Liu et al. 2004). A similar case was also observed in flesh fly *Sarcophaga crassipalpis*, where Hsp70 was significantly up-regulated under heat shock in nondiapausing flies, while the cognate Hsc70 was not affected (Rinehart et al. 2000). However, there are also reports to show that the transcription of Hsc70 in the rat cortex and cerebellum was up-regulated after heat shock, but the extent of variation is much lower than that of Hsp70 (Miller et al. 1991). The rapid reaction of FcHsp70 might protect shrimp from acute stresses, during which a large number of denatured proteins are necessary to be chaperoned with Hsps in the cells, and then to reduce the overload of unfolded proteins in the cells (Franzellitti and Fabbri 2005).

Heavy metal pollution becomes a serious problem in aquatic ecosystems for the aquaculture animals. Considerable data presented so far illustrate the effects of heavy metals on aquaculture animals such as shrimp (Sadiq and Zaidi 1982; Rainbow and White 1989; Moksnes et al. 1995; Guhathakurta and Kaviraj 2000). Heavy metal contaminants can affect the growth or reproduction of different organisms (Calabrese et al. 1977; Spurgeon et al. 1994). Copper is an abundant element in nature and vital for diverse metabolic processes in a wide range of organisms. The elevated copper concentration may become toxic (Flemming and Trevors 1989). Moreover, exposure to the elevated levels of cadmium can lead to oxidative damage in cells (Stohs and Bagchi 1995). FcHsp70 can be induced by copper exposure at transcription level in Chinese shrimp *F. chinensis*. It has been reported that Hsp70 could be

responsive to a wide spectrum of heavy metal pollutants such as Pb, Hg, Ag, Co, or Ni at either the protein or the mRNA levels (Murata et al. 1999; Nadeau et al. 2001). Studies in aquatic invertebrates have shown that heat shock proteins contribute significantly to both organism adaptation to the environments and to survival under acute environmental stresses (Sanders 1993).

In the present study, FcHsp70 exhibited a dramatic up-regulation in shrimp after 6–24 h of exposure to copper treatment, while the expression of FcHsc70 was less sensitive to copper treatment. The different expression profiles of FcHsp70 and FcHsc70 suggest that Hsp70 has a potential to become a biomarker for the indication of copper exposure. Previous studies have shown that the copper treatment with 0–500 µg l^{-1} can lead to a two- to threefold increase of Hsp70 gene transcription level at 24 h relative to the control level in zebra mussels *Dreissena polymorpha* (Clayton et al. 2000). In *Chironomus tentans*, sublethal concentrations of copper as high as 2 mg l^{-1} can also result in the up-regulation of Hsp70 protein at 24 h (Karouna-Renier and Zehr 2003). In rainbow trout, no significant changes of Hsc70 were detected in the primary cultured hepatocytes under 12.8 mg l^{-1} exposure for 24 h (Boone and Vijayan 2002). But in the pacific oyster *Crassostrea gigas*, both inducible and cognate Hsp70s were down-regulated in gills and digestive glands after exposure to Cu^{2+} (0.025 and 0.25 mg·l^{-1}) for 15 days. The difference might be caused by different exposure times to copper.

Unlike the up-regulation induced by copper, cadmium led to a significant decrease of FcHsc70 at mRNA level. No expression of FcHsp70 was detected when shrimp were exposed to cadmium. The failure to detect the expression of FcHsp70 was probably caused by the depression from cadmium treatment, since FcHsp70 in shrimp was expressed at a very low transcription level at 25°C. It has been reported in oysters that both Hsp70 and Hsc70 can be down-regulated at the transcription level after cadmium treatment (Boutet et al. 2003). It was demonstrated that the depression of Hsp70 by cadmium exposure might be regulated through a cAMP-responsive regulatory pathway (Vilaboa 1995), which is possibly similar to that reported in yeast (Boorstein and Craig 1990). The high sensitivities exhibited by inducible FcHsp70 identified in this study under either heat shock or heavy metal exposure indicate its potential to be developed as an ideal biomarker for *F. chinensis* in aquaculture applications.

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