Identification of a novel cognate cytosolic Hsp70 gene (MnHsc70-2) from oriental river prawn *Macrobrachium nipponense* and comparison of its expressions with the first cognate Hsc70 (MnHsc70-1) under different stresses

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Abstract The 70-kDa family of heat-shock proteins (Hsp70) plays an important role in the host immunity, which is widely expressed in eukaryotic cells as a major chaperone protein. In the present study, the full-length complementary DNA (cDNA) of a second cognate cytosolic Hsp70 family member (MnHsc70-2) was cloned and characterized from *Macrobrachium nipponense*, which is an economically and nutritionally important crustacean. The cDNA was 2,717 bp, containing an open reading frame (ORF) of 1,950 bp, which encodes a protein of 649 amino acids with a theoretical molecular weight of 71.1 kDa and an isoelectric point of 5.27. Sequence alignment showed that the MnHsc70-2 shared 75–97 % identity with other heat-shock

Highlights 1. This is first report of the second Hsc70 cDNA from crustacean organism (MnHsc70-2).
2. MnHsc70-2 was cloned, and the characteristics of two MnHsc70s were analyzed.
3. Heat-shock treatment was done to study expression profiles of two MnHsc70s.
4. Expression changes of two MnHsc70s were studied when challenged with *A. hydrophila*.
5. The results above implied that two Hsc70 genes have distinct biological tasks.

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proteins. Compared to the previously identified cognate Hsp70 (MnHsc70-1) in *M. nipponense*, MnHsc70-2 showed quite different expression profiles under unstressed conditions in all tested tissues, including the hemocytes, heart, hepatopancreas, gill, intestine, nerve, and muscle. The phylogenetic analysis demonstrated that MnHsc70-2 showed the closest relationship with MnHsc70-1. Heat-inducibility assays showed that two isolated messenger RNAs (mRNAs) displayed different expression profiles in both the hepatopancreas and gill tissues. MnHsc70-1 mRNA expression level decreased at first and then increased to the normal level, whereas MnHsc70-2 mRNA level increased at first and then decreased. The expressions of two MnHsc70s showed substantial obvious heat-inducible regulation in both the hepatopancreas and gill. Under bacterial challenge by *Aeromonas hydrophila*, both MnHsc70-1 and MnHsc70-2 mRNA level was up-regulated moderately. The results suggested that two cognate Hsc70s may play essential functions in mediating responses to heat-shock and bacterial challenge.

Keywords *Macrobrachium nipponense* · Heat-shock protein · Heat-shock treatment · *Aeromonas hydrophila* · Immune response

Abbreviations

Hsp70	Heat-shock protein 70
Hsc70	Cognate cytosolic Hsp70
HSPs	Heat-shock proteins
TLR	Toll-like receptors
Grp78	Glucose-regulated protein 78
<i>M. nipponense</i>	<i>Macrobrachium nipponense</i>
<i>A. hydrophila</i>	<i>Aeromonas hydrophila</i>
RT-PCR	Reverse-transcriptase polymerase chain reaction

RACE	Rapid amplification of cDNA ends
AAP	Abridged anchor primer
AUAP	Abridged universal amplification primer
NJ	Neighbor-joining
<i>F. chinensis</i>	<i>Fenneropenaeus chinensis</i>
<i>P. monodon</i>	<i>Penaeus monodon</i>
<i>C. gigas</i>	<i>Crassostrea gigas</i>

Introduction

Heat-shock proteins (Hsps), first described in *Drosophila busckii* in 1962 by Ritossa (Ritossa 1962), have been shown to be the most phylogenetically conserved proteins present in all prokaryotes and eukaryotes (Tsan and Gao 2004). Hsps perform essential biological functions under both normal and stress conditions (Kiang and Tsokos 1998; Sørensen et al. 2003). The primary function of Hsps appears to serve as molecular chaperones in which they recognize and bind to nascent polypeptide chains and partially folded intermediates of proteins, preventing their aggregation and misfolding, or as chaperonins that directly mediate protein folding (Hartl and Hayer-Hartl 2002). In addition to serving as molecular chaperones, Hsps have been implicated in autoimmune diseases (Pockley 2003), antigen presentation, and tumor immunity (Yue et al. 2011). Moreover, several studies have indicated that Hsps, acting as ligands for the toll-like receptors (TLR), are critical to the induction of both innate and adaptive immune responses (Asea et al. 2002; Vabulas et al. 2002).

Hsps have been classified into three major families according to their molecular weights: Hsp90 (85–90 kDa), Hsp70 (68–73 kDa), and low-molecular-weight Hsps (16–47 kDa) (Ming et al. 2010). As one of the most abundant and widely investigated families in higher eukaryotes, the Hsp70 family is made up of cytosolic Hsp70 including the inducible Hsp70 and the cognate Hsc70, glucose-regulated protein 78 (Grp78) located in the endoplasmic reticulum, and mitochondrion Hsp75 (Lindquist and Craig 1988). The inducible Hsp70s express at extremely low levels under normal conditions and increase significantly in response to stresses (Hartl 1996). However, the cognate Hsc70s are constitutively expressed under normal conditions and change relatively little on exposure to stressors (Kregel 2002; Park et al. 2001).

In recent years, many 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). Up to now, several Hsc70s have been cloned from many kinds of crustaceans, including the pacific white shrimp *Litopenaeus vannamei* (Wu et al.

2008), tiger shrimp *Penaeus monodon* (Lo et al. 2004), Chinese shrimp *Fenneropenaeus chinensis* (Jiao et al. 2004), and giant freshwater prawn *Macrobrachium rosenbergii* (Liu et al. 2004). However, there are only a little data on Hsc70 of the oriental river prawn, *Macrobrachium nipponense*.

It has been reported that some lower vertebrates and mammals appear to contain more than one isoform for both Hsp70 and Hsc70 (Ali et al. 1996b, 2003; Giebel et al. 1988; Walsh et al. 1994, 1997), but there is no report from a crustacean. In the present study, for the first time, we identified and characterized the second cytosolic member of the Hsp70 family (MnHsc70-2) from *M. nipponense*. The deduced amino acid sequences were compared with other known Hsp70s, and the distribution of MnHsc70-1 and MnHsc70-2 in various tissues was also investigated. By means of analysis of the expression changes in response to heat-shock and *Aeromonas hydrophila* challenge, we hope to learn if two cognate Hsc70s play different potential immune-related functions in *M. nipponense*.

Materials and methods

Animal and RNA extraction

The oriental river prawns, *M. nipponense* (2–3 g in weight, 4–5 cm in length) purchased from a market in Nanjing, China, were cultivated in 100-L aquaculture tanks at 15 °C with freshwater and an aeration system. The experimental prawns were fed with minced prawn once daily. The prawns were acclimated for 10 days before processing. The hemocytes and hepatopancreas from randomly selected specimens were checked using *A. hydrophila* diagnostic PCR (Pollard et al. 1990) and were all found to be PCR negative. Prawn health status was assessed daily during acclimation by monitoring both general activity and food intake.

RNA from different tissues was extracted using TRIzol Reagent (Invitrogen, USA) and then treated with RNase-free DNase I (Invitrogen, USA) following the manufacturer's protocol. RNA quality was assessed by electrophoresis on 1.2 % agarose gel, and the total RNA concentration was determined by measuring the absorbance at 260 nm on a spectrophotometer.

cDNA synthesis and gene cloning

The complementary DNA (cDNA) was synthesized using about 5 µg total RNA using the Takara PrimerScript™ First Strand cDNA Synthesis kit (TaKaRa, China) according to the manufacturer's instructions. Degenerate primers, MnHsc70-2F1 and MnHsc70-2R1, designed based on the highly conserved nucleotide region of other known Hsp70s (Luan et al. 2010) were used in the reverse-transcriptase polymerase chain reaction (RT-PCR). Amplification primers for MnHsc70-1 and MnHsc70-2 were shown in Tables 1 and 2.

Table 1 Primers used in the RACE

Name	Sequence (5'–3')
MnHsc70-2F1	CAGAACGACATGAAA(G)CAT(C)TG
MnHsc70-2R1	GTGGGT(C)TCGTTGATGATG(A)C
MnHsc70-2R2	TGACGACAGCATCCTT
MnHsc70-2R3	CTTAATAAGCACCATTGAGGAG
MnHsc70-2R4	GGTTTTGGTCTCTCCTTTGTAA
MnHsc70-2F2	TGGGTGGTACTGTAAAGGATGCTGTCGT
MnHsc70-2F3	GACTCCCAGCGTCAGGCCACCAAAGAT
AAP	GGCCACGCGTCGACTAGTACGG GIIGGGIIGGGIIG
AUAP	GGCCACGCGTCGACTAGTAC
Oligo-d(T) ₁₈ ACP	CTGTGAATGCTGCGACTACGA(T) ₁₈
3'-RACE primer	CTGTGAATGCTGCGACTACGA

Rapid amplification of cDNA ends (RACE) of MnHsc70-2

About 5 µg of total RNA was reverse-transcribed with MnHsc70-2R2 primer to generate 5'-RACE template. MnHsc70-2R2 primer was designed based on the sequence amplified by the degenerate primers. For the 5'-RACE, part of the MnHsc70-2 gene was obtained using a 5'-RACE System (Invitrogen, USA) according to the manufacturer's instructions. The primer set consisted of MnHsc70-2R3 with abridged anchor primer (AAP) for the first-run PCR and MnHsc70-2R4 with the abridged universal amplification primer (AUAP) for the second-run PCR. For the 3'-RACE, the RT-PCR was performed using the oligo-d(T)₁₈ ACP primer with MnHsc70-2F2, and the nested PCR was performed using MnHsc70-2F3 with 3'-RACE primer. The amplified products were cloned into pMD19-T vector and sequenced by Springen (Nanjing) Biotechnology Company.

Sequence analysis

The cDNA sequences of MnHsc70-1 and MnHsc70-2 were analyzed using the basic local alignment search tool (BLAST) algorithm (<http://www.ncbi.nlm.nih.gov/blast>), and the deduced amino acid sequence was analyzed with the

ExPASy tools (<http://us.expasy.org/>). The ClustalW multiple alignment program (<http://www.ebi.ac.uk/clustalw/>) was used for the multiple sequence alignment. A phylogenetic tree was constructed based on the amino sequences alignment by the neighbor-joining (NJ) algorithm embedded in the MEGA 5 program (Tamura et al. 2011). The reliability of the branching was tested by bootstrap resampling (1,000 pseudoreplicates).

Tissue expression of MnHsc70-1 and MnHsc70-2

Tissue distribution of MnHsc70-1 and MnHsc70-2 messenger RNAs (mRNA) in the hemocytes, heart, hepatopancreas, gill, intestine, nerve, and muscle was demonstrated by quantitative real-time PCR analysis. Total RNA was extracted as described above; 5 µg of total RNA was used to synthesize the first strand cDNA. For quantification of MnHsc70-1 and MnHsc70-2 expression, two pairs of gene-specific primers (MnHsc70-1QF, MnHsc70-1QR and MnHsc70-2QF, MnHsc70-2QR) were used, respectively. The primers β -actin F and β -actin R (Zhao et al. 2011) were used to amplify β -actin as an internal control (Table 2). The different gene expression was calculated by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001). Statistical analysis was performed using SPSS software (Ver11.0). Data are presented as the mean $\pm$ standard error ($n=3$). Statistical significance was determined by two-way ANOVA and post hoc Duncan multiple range tests.

Heat-shock treatment of *M. nipponense*

In order to study the effect of temperature on MnHsc70-1 and MnHsc70-2 gene expression, individuals were exposed to 25 °C for 12 h and then returned to the control temperature for a period of 48 h. Time course was performed at 0, 2, 6, 12, 24, or 48 h during the recovery period at 15 °C. The hepatopancreas and gill of five prawns at each sampling time were collected, respectively, frozen immediately in TRIzol Reagent, and stored at –80 °C until RNA extraction. Gene expression of MnHsc70-1 and MnHsc70-2 was determined by quantitative real-time RT-PCR.

Table 2 Primers used in the qRT-PCR

Name	Sequence (5'–3')	Annealing temperature (°C)	Size of product (bp)
MnHsc70-1QF	GTTATGACAGCACTTATTAA	56	232
MnHsc70-1QR	AGATACCATTTGGCATCAATATC		
MnHsc70-2QF	ATATTGATGCTAATGGTATTC	57	207
MnHsc70-2QR	TTGAAACAGTAAGACTCCAGGT		
β -actin F	AATGTGTGACGACGAAGTAG	56	176
β -actin R	GCCTCATCACCGACATAA		

Bacterial challenge

For bacterial challenge, the *M. nipponense* were cultivated at 25 °C for 10 days before processing. In the experimental group, the prawns were injected individually with 50 µl of bacterial suspension (10^4 cells/ml) which had been dissolved in 0.85 % NaCl solution. The bacterium *A. hydrophila* was purchased from China General Microbiological Culture Collection Center and was grown at 28 °C in a TSB medium. At the same time, the prawns injected with 50 µl saline (0.85 % NaCl) (pH=7.0) were used as the control group. *M. nipponense* were sampled at 0, 1, 12, 24, 36, and 48 h post-injection. For each treatment and each exposure time, the hepatopancreas of five prawns were sampled, and total RNA was extracted as described above. Quantitative real-time RT-PCR was employed to determine expression profile of MnHsc70-1 and MnHsc70-2. In order to confirm the infection of the prawns, the hepatopancreas and hemolymph were chosen for bacterium culture. At the same time, PCR assay was also used to confirm the infection.

Results

Characterizations of MnHsc70-1 and MnHsc70-2 cDNA

MnHsc70-1 cDNA and amino acid sequences were found in GenBank accession number ABG45886. The full-length MnHsc70-1 cDNA was 2,553-bp long, including a 1,950-bp coding sequence and a 98- and 505-bp flanking region at the 5' and 3' ends, respectively. Different from MnHsc70-2 cDNA, the consensus polyadenylation site (AATAAA) was not found upstream of the polyA tail in MnHsc70-1. The deduced amino acid sequence encodes a 649 aa protein with a calculated molecular mass of 71.06 kDa and a *pI* of 5.27 (Fig. 1).

A fragment of 242 bp was generated by MnHsc70-2F1 and MnHsc70-2R1. The full-length MnHsc70-2 cDNA (GenBank accession number KC460343) was 2,717 bp in length including 252 bp in the 5'-untranslated region (UTR), an open reading frame (ORF) of 1,950 bp encoding a protein of 649 amino acids, and 515 bp in the 3'-untranslated region. A canonical polyadenylation signal (AATAAA) and a polyA tail were located within the 3'-UTR. The predicted molecular weight is 71.1 kDa with an estimated *pI* of 5.27 (Fig. 2).

Homology analysis of MnHsc70-1 and MnHsc70-2

The BLAST analysis showed that two MnHsc70s amino acid sequences shared closest homology with the deduced proteins

of other known constitutive Hsc70s. The alignment analysis between the MnHsc70-1 and MnHsc70-2 amino acid sequences showed 99 % identity. The MnHsc70-2 amino acid exhibited 94–97 % sequence identity with constitutive Hsc70s from other crustacean species, including *M. rosenbergii* Hsc70 (97 %), *F. chinensis* Hsc70 (94 %), *P. monodon* Hsc70 (94 %), and *L. vannamei* Hsc70 (94 %). Meanwhile, MnHsc70-2 was found to have 82 % homology with *Procambarus clarkii* Hsp70, 80 % with *F. chinensis* Hsp70, and 75 % with *M. rosenbergii* Hsp70.

Deduced amino acid sequence alignment indicates that three highly conserved signatures including IDLGTTYS, IFDLGGGTFDVSIL, and IVLVGGSTRIPKIQK (Zdobnov and Apweiler 2001) were all identified in most organisms (Fig. 3). In addition, the potential non-organelle eukaryotic consensus motif RARFEEL and cytoplasmic characteristic motif EEVD were also identified.

Phylogenetic analysis

A phylogenetic tree was constructed by analyzing the amino acid sequences of two MnHsc70s and HSPs from a variety of species (Fig. 4). The phylogenetic analysis, which was consistent with the alignment results, showed that MnHsc70-1 clustered with MnHsc70-2 on a unique branch. This branch showed the closest relationship with *M. rosenbergii* Hsc70 and other crustacean Hsc70s. In addition, Hsp70 and Hsc70s tended to cluster into two separate subclasses except for one especial sequence, which might show how Hsp70 family proteins evolved in the eukaryotic cells.

Tissue distribution of MnHsc70-1 and MnHsc70-2

In order to compare the distribution of MnHsc70-1 and MnHsc70-2 mRNA in different tissues, total RNA was extracted from the hemocytes, heart, hepatopancreas, gill, intestine, nerve, and muscle. The relative expression level of gene was calculated by $2^{-\Delta\Delta CT}$ method, with the expression level of MnHsc70-1 mRNA in the hemocytes used as a calibrator and set at 1.0. The relative expression levels of other MnHsc70s mRNA at different tissues were indicated as *n*-fold differences relative to the calibrator. As determined by quantitative real-time PCR, the mRNA transcripts of MnHsc70-1 and MnHsc70-2 were widely detected in all examined tissues (Fig. 5). A predominant MnHsc70-1 transcript was found in the intestine, with slightly less in the hepatopancreas, and even less in other tissues. Different from MnHsc70-1, MnHsc70-2 transcript was most abundant in the hepatopancreas, with a moderate expression in the gill, intestine, and muscle, and a lower expression in the hemocytes, heart, and nerve. Significant differences between the two MnHsc70s were obvious, and a higher expression level of MnHsc70-2 was found

[illegible]

MnHsc70-2 within the same tissue, significant differences were found in all tested tissues except for the hemocytes.

1 atggagagtctagcgtgttttccatcgccgcgaacatgcaagttttacgagcggtacgtgcttagcgggagcatttccttcacata
 91 ttttaatcgaccataggagtgccattttcttcttcttcccccgagtcgatctttctccgtctcgttaccgggttaaatacgagcgtag
 181 accacgagaccggcctgagccccacttaattcgttttccgactaagttacttcgaaattgctgacttttggaac
 253 atggctaaaaagtgcagctgtcgggattgacctgggtaccacctactcatgtgtagggtgtgttccagcatggaaaggtagaatcattgcc
 1 N A K S A 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
 343 aatgatcaggcgcaaccgaaccactccatcatatgttgcctttaccgacacagagcgtcttattggtagctgccaagaaccaagtgtct
 31 N D Q G N R T T P S Y V A F T D T E R L I G D A A K N Q V A
 433 atgaatcccaacaacactgtatttgatgccagaggctcattggctgtaagtttgaagatcatgtagtgcagtcgacatgaaacattgg
 61 M N P N N T V F D A K R L I G R K F E D H V V Q S D M K H W
 523 cccctttacagtcatttaagacagcacgaagcccaaatcaagttgattacaaggagagacaaaaccttttccagagaagatctcc
 91 P F T V I N D S T K P K I Q V D Y K G E T K T F F P E E I S
 613 tcaatgggtgcttatttaagatgaaagaaactgcagaagccttttgggtggtaactgttaaaggatgctgtctgacagtccttgcctatttc
 121 S M V L I K M K E T A E A F L G G T V K D A V V T V P A Y F
 703 aatgactcccagcgtcaggccaccaaaagatgctgggtacaatctctgggtcctaatgtgtctccgtatcatcaatgagcccaactgctgctgcc
 151 N D S Q R Q A T K D A G T I S G L N V L R I I N E P T A A A
 793 atcgcatatgggtcggacaagaaagtgggtggagaacgtaagtgtctgatttttgatttaggtgggtggcacttttgatgtatctatttta
 181 I A Y G L D K K V G G E R N V L I F D L G G G T F D V S I L
 883 actattgaagatggcatttttgaagtgaagtctactgctgggtgacacacatttaggtgggtgaagattttgataacagaatgggttaacct
 211 T I E D G I F E V K S T A G D T H L G G E D F D N R M V N H
 973 ttcatccaagaattcaagagaaaaatacaagaaagatccttccgaaacaagcgtgctctacgtcgtttacgtactgctgtgtgagcgcgcc
 241 F I Q E F K R K Y K K D P S E N K R A L R R L R T A C E R A
 1063 aagcgtactcttccagcctctgctcaagctagtggtgagattgattctctgtatgaaggtattgatttttaccactctattaccctgtgcc
 271 K R T L S A S A Q A S V E I D S L Y E G I D F Y T S I T R A
 1153 aggttttgaggaaactttgtgggtgacttgttccgtggccacctgggagccagttgagaagctctgagagatgcaaaaatggacaagcccaa
 301 P F E E L C G D L F R G T L E P V E K S L R D A K M D K A Q
 1243 atccatgacattgtccttgttggagggttccaccagaattccaagattcagaagcgtgttacaggatttcttcaatggaaaggagctcaac

 331 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 L N
 1333 aaatccatcaatcctgatgaagctgtagcttattgggtcagctgtacaggcagccatattgtgtgggtgataagctgaagcagttcaagat
 361 K S I N P D E A V A Y G A A V Q A A I L C G D K S E A V Q D
 1423 ttgttgccttttggatgtaacaccactctctcttgggtattgaaactgctgggtggtgctcatgactgcccattataaagaacacacacatt
 391 L L L L D V T P L S L G I E T A G G V M T A L I K R N T T I
 1513 ccaaccaaacagaccagacctttaccacctattctgacaaccagcctggagtgctcattcaagtttacgaaggagaactgccatgaca
 421 P T K Q T Q T F T T Y S D N Q P G V L I Q V Y E G E R A M T
 1603 aaagacaataacattttgggtgaagtttgagctgactgggtatccccccagcccaagggtgccacaatcgaagtaacattttgatatt
 451 K D N N I L G K F E L T G I P P A P R G V P Q I E V T F D I
 1693 gatgctaattgggtatttcaatgtatctgctgctgacaagtcacaggcaagagaacaagatcaccatcactaatgataaggccgcttg
 481 D A N G I L N V S A A D K S T G K E N K I T I T N D K G R L
 1783 agcaaggaagagattgagcgaatgggtgcaagagccgagaagtlacaagctgacgatgagaacacggggagcgtatctctgccaagaac
 511 S K E E I E R M V Q E A E K Y K A D D E K Q R E R I S A K N
 1873 aacctggagctcttactgtttcaatatgaagtcactgttgaagatgacaaattcaaggataaggttccctgaggatgatcgcaacaagatc
 541 N L E S Y C F N M K S T V E D D K F K D K V P E D D R N K I
 1963 atggaagcctgtaattgatccatcaagtggctcgacaccaatcagcttggagagaaggaagaatatgaacacaaactgaaggaaattgaa
 571 M E A C N D A I K W L D T N Q L G E K E E Y E H K L K E I E
 2053 cagatatgtaatcctatcatcaccaaaatgtaccaagcagctgggtgggtcctcctcagggtgggtatgccaggaggttttccagggtgccca
 601 Q I C N P I I T K M Y Q A A G G A P P G G M P G G F P G A P
 2143 ggggctgggtgctgctcctggcgggtgttcatcaggccccacaattgaagggtcgat
 631 G A G A A P G G G S S G P T I E E V D *
 2203 atgtgctcgactcattgaaacttactgtttccagctcttggaaataaccgaatgtaggaaatttttaagtaaaatttagtttgcaaacgtg
 2293 aacactggatgactacatcacatcatttagtgcaatgcaccactcacttagatcaaaaatgttgatgccacatttaattcccaaatgttgca
 2383 ctttgatattaaggttactcacattttacatagaatgagtatgaaatctctttaaagggtgtgggaatgtgccttattttgaagtgggttt
 2473 taagtggactcatgtaccaagaaatttcatccagtttaaccttgttcttttttgggaattatgttaattgagctcattttattttttgta
 2563 tttttctattattaaatttccatccattcctgtgagaacctgagtaataagaaaaccattaaaagtatttttgagaaaagtgtttaatgat
 2653 ttgaatttttagtgcattccaacaaaatacattatatacatatataaaaaaaaaaaaaaaaaaaaaa

◀ **Fig. 2** Nucleotide sequence and its deduced amino acid sequence of MnHsc70-2. Three characteristic Hsp70 family signatures are shown in **bold letters**; the putative ATP-GTP binding site is underlined and in *italics*; the putative bipartite nuclear localization signal (KK and RRLRT) is shown in a *box*; the potential non-organelle eukaryotic consensus motif RARFEEL is indicated in *italics*; three tetrapeptides of GGXP at the carboxyl terminal region are in *gray*; the conserved EEVD motif at the C-terminal is underlined; and the consensus polyA signal is indicated by a double-underline

Expression analysis of MnHsc70-1 and MnHsc70-2 mRNA under heat shock

The expression profiles of MnHsc70-1 and MnHsc70-2 in the hepatopancreas and gill after heat-shock treatment are shown in Fig. 6. It was clear that the expression profile of MnHsc70-1 was quite different from that of MnHsc70-2 in both the hepatopancreas and gill tissues. In the hepatopancreas, MnHsc70-1 mRNA level decreased at first and then increased to the normal level, whereas MnHsc70-2 mRNA expression level increased at first and then decreased. MnHsc70-1 mRNA level slightly decreased at 0 h to 84.93 % of the control level. At 12 h, the mRNA expression level of MnHsc70-1 significantly decreased to 2.83 % of the control level. After that, MnHsc70-1 mRNA level increased gradually and reached the normal level at 48 h (Fig. 6a). MnHsc70-2 mRNA level increased moderately after heat shock and reached the highest level at 2 h which was 1.67-fold higher compared with that in the control group (Fig. 6b).

In the gill, MnHsc70-1 and MnHsc70-2 mRNA showed substantial obvious heat-inducible regulation. For MnHsc70-1 mRNA, the lowest level at 2 h was 11.69 % of the control group, and thereafter, it gradually increased. The MnHsc70-1 mRNA significantly increased at 12 h which was 2.20-fold compared with that in the control level (Fig. 6c). At 12 h, the mRNA expression level of MnHsc70-2 significantly increased to 11.67-fold and reached a peak (Fig. 6d).

Expression analysis of MnHsc70-1 and MnHsc70-2 mRNA after *A. hydrophila* challenge

The bacterium culture and PCR assay showed that the prawns had been successfully infected by *A. hydrophila*. After *A. hydrophila* challenge, the *M. nipponense* tail turned red, and subsequently, the swim foot and entire abdomen were affected. Fifty percent mortality was shown during this experiment. The temporal expression pattern of MnHsc70-1 and MnHsc70-2 in the hepatopancreas after being challenged by *A. hydrophila* was shown in Fig. 7. The results showed that after infection of *A. hydrophila*, two MnHsc70s mRNA levels increased slightly at first and

then decreased. MnHsc70-1 mRNA levels moderately increased and reached a peak at 1 h, when the mRNA levels increased to 2.26-fold higher than that in the control. The expression of MnHsc70-2 was slightly up-regulated at 1 h and reached the highest level at 12 h post-injection, which was 2.72-fold higher than that in the control groups. Then, the expression level dropped as time progressed; the mRNA levels of MnHsc70-1 and MnHsc70-2 decreased to 21.04 and 75.9 % of the control at 24 h, respectively. Another up-regulation was detected at 36 h, and the expression returned to the normal level at 48 h. Although the MnHsc70-1 and MnHsc70-2 expression in the control group fluctuated slightly at different time points, no significant differences were found among them.

Discussion

In this article, the gene encoding MnHsc70-2 was identified from *M. nipponense*, and the characteristics of two MnHsc70s were analyzed. Amino acid sequence analysis showed that both of the isolated cDNAs conserved the main typical structural features of eukaryotic cytoplasmic HSP70 family members. In addition, MnHsc70-1 and MnHsc70-2 sequences included two additional specific motifs indicative of the HSP70 cytosolic localization: a nonorganelle stress protein motif (RARFEEL) and the extreme C-terminal domain EEVD, which is involved in binding with different co-chaperones (Demand et al. 1998; Johnson et al. 1998). It has been suggested that the functional differences between inducible and constitutive HSP70 members include the α -helical subdomain, the tetrapeptide GGXP repeats, and the EEVD motif (Demand et al. 1998; Fuertes et al. 2004). The tetrapeptide motif GGXP repeats are thought to be involved in co-chaperone-binding activities of Hsc70 (Demand et al. 1998); and likewise, in our results, three tetrapeptides of GGXP at the carboxyl terminal were found in both MnHsc70-1 and MnHsc70-2.

There are a number of examples where more than one Hsc70 have been described from the same organism (Ali et al. 1996a, b, 2003; Graser et al. 1996; Santacruz et al. 1997). However, to our knowledge, there have been no reports of two or more Hsc70s in a crustacean organism, and this is the first time that two closely related Hsc70s have been identified in the oriental river prawn, *M. nipponense*. Furthermore, comparison between our peptide sequences and those of known Hsc70s in other crustaceans showed stronger homology than that of the heat-inducible form. A phylogenetic tree was constructed using the amino acid sequences of the MnHsc70s along with Hsc70 and Hsp70 sequences from a number of other species. As shown in

MnHsc70-1	MAKSAAVG DLGTTYS CVGVFQHGKVEI I ANDQGNRTT PSYVAFDTERLI GDAAKNQVA	60
MnHsc70-2	MAKSAAVG DLGTTYS CVGVFQHGKVEI I ANDQGNRTT PSYVAFDTERLI GDAAKNQVA	60
MrHsc70	MAKSAAVG DLGTTYS CVGVFQHGKVEI I ANDQGNRTT PSYVAFDTERLI GDAAKNQVA	60
PmHsc70	MAKAPAVG DLGTTYS CVGVFQHGKVEI I ANDQGNRTT PSYVAFDTERLI GDAAKNQVA	60
FcHsc70	MAKAPAVG DLGTTYS CVGVFQHGKVEI I ANDQGNRTT PSYVAFDTERLI GDAAKNQVA	60
LvHsc70	MAKAPAVG DLGTTYS CVGVFQHGKVEI I ANDQGNRTT PSYVAFDTERLI GDAAKNQVA	60
PcHsp70	. MADPVI G DLGTTYS CVGVFQHGKVEI I ANDQGNRTT PSYVALDTERLI GDAAKNQTA	59
FcHsp70	. MAGPVI G DLGTTYS CVGVFQHGKVEI LANDQGNRTT PSYVAFGDAERLVGDAAKNQAA	59
MrHsp70		0
Consensus		
MnHsc70-1	MNPNTVFDAKRLI GRKFEDHVCSDMKHWPFTVI NDSTKPKI QVDYKGETKTFFPEEIS	120
MnHsc70-2	MNPNTVFDAKRLI GRKFEDHVCSDMKHWPFTVI NDSTKPKI QVDYKGETKTFFPEEIS	120
MrHsc70	MNPNTVFDAKRLI GRKFDDGVVCSDMKHWPFTVI NDNTKPKI QVDYKGETKTFFPEEIS	120
PmHsc70	MNPNTVFDAKRLI GRKFEDHTVCSDMKHWPFTI I NESTKPKI QVEYKGDKKTFYPEEIS	120
FcHsc70	MNPNTVFDAKRLI GRKFEDHTVCSDMKHWPFTI I NESTKPKI QVEYKGDKKTFYPEEIS	120
LvHsc70	MNPNTVFDAKRLI GRKFEDHTVCSDMKHWPFTI I NESTKPKI QVEYKGDKKTFYPEEIS	120
PcHsp70	LNPSNTVFDAKRLI GRKFNDPTVCNDKKHWPFDVVDSDNGKPKI QVEYKGERKTFFNPEEIS	119
FcHsp70	MNPNTVFDAKRLI GRKFEDAAVCSDMKHWPFRVVSDEGKAKI SVRFKGEDKSFNPEEIS	119
MrHsp70	MNPNTVFNAKRLI GRKFDDPTVCSDMKHWPFTVVSDEGKPKI QVEYKGERKTFFNPEEIS	60
Consensus	np nt f akrli grkf d vq d khwpf k k v kg k f peei s	
MnHsc70-1	SMVLI KMKETA EAF LGSTVKDAVVTVPAYFNDSQRQATKDAGTI SGLNVLRI I NEPTAAA	180
MnHsc70-2	SMVLI KMKETA EAF LGSTVKDAVVTVPAYFNDSQRQATKDAGTI SGLNVLRI I NEPTAAA	180
MrHsc70	SMVLI KMKETA EAF LGSTVKDAVVTVPAYFNDSQRQATKDAGTI SGLNVLRI I NEPTAAA	180
PmHsc70	SMVLI KMKETA EAYL GSTVKDAVVTVPAYFNDSQRQATKDAGTI SGLNVLRI I NEPTAAG	180
FcHsc70	SMVLI KMKETA EAYL GSTVKDAVVTVPAYFNDSQRQATKDAGTI SGLNVLRI I NEPTAAA	180
LvHsc70	SMVLI KMKETA EAYL GSTVKDAVVTVPAYFNDSQRQATKDAGTI SGLNVLRI I NEPTAAA	180
PcHsp70	SMVLT KMKETA EAYL GQKVKDAVVTVPAYFNDSQRQATKDAGTI SGLNVLRI I NEPTAAA	179
FcHsp70	SMVLT KMKETA EAYL GNSVKNVAVTVPAYFNDSQRQATKDAGTI SGLNVLRI I NEPTAAA	179
MrHsp70	SMVLT KMKETA EAYL GKTVKDAVVTVPAYFNDSQRQATKDAGTI SGLNVLRI I NEPTAAA	120
Consensus	s vl krnketae a lg stvk d av vt vp ay f nds qr qat kd ag ti s gl n vl ri i nept aa a	
MnHsc70-1	IAYGLDKKVG. . GERNVLI FDLGGGTFDVSI LTI EDGI FEVKSTAGDTHLGGEDFDNRMV	238
MnHsc70-2	IAYGLDKKVG. . GERNVLI FDLGGGTFDVSI LTI EDGI FEVKSTAGDTHLGGEDFDNRMV	238
MrHsc70	IAYGLDKKVG. . GERNVLI FDLGGGTFDVSI LTI EDGI FEVKSTAGDTHLGGEDFDNRMV	238
PmHsc70	IAYGLDKKVG. . GERNVLI FDLGGGTFDVSI LTI EDGI FEVKSTAGDTHLGGEDFDNRMV	238
FcHsc70	IAYGLDKKVG. . GERNVLI FDLGGGTFDVSI LTI EDGI FEVKSTAGDTHLGGEDFDNRMV	238
LvHsc70	IAYGLDKKVG. . GERNVLI FDLGGGTFDVSI LTI EDGI FEVKSTAGDTHLGGEDFDNRMV	238
PcHsp70	IAYGLDKKVGGRGERNVLI FDLGGGTFDVSI LTI EDGI FEVKSTAGDTHLGGEDFDNRMV	239
FcHsp70	IAYGLDKQSVGKAERNVLI FDLGGGTFDVSI LTI EDGI FEVKSTAGDTHLGGEDFDNRMV	239
MrHsp70	IAYGLDKKVGRLRGERNVLI FDLGGGTFDVSI LTI EDGI FEVKSTAGDTHLGGEDFDNRMV	180
Consensus	i aygl dk e nvl f dl g ggt f d s l i g f evk t agdt hl gged f d nrmv	
MnHsc70-1	NHFI QEFKRKYKKDPSENKRAL RRLRTACERAKRTLSSAQASVEI DSLFEGDFYTSIT	298
MnHsc70-2	NHFI QEFKRKYKKDPSENKRAL RRLRTACERAKRTLSSAQASVEI DSLFEGDFYTSIT	298
MrHsc70	NHFI QEFKRKYKKDPSENKRAL RRLRTACERAKRTLSSAQASVEI DSLFEGDFYTSIT	298
PmHsc70	NHFI QEFKRKYKKDPSENKRS LRLRTACERAKRTLSSAQASVEI DSLFEGDFYTSIT	298
FcHsc70	NHFI QEFKRKYKKDPSENKRS LRLRTACERAKRTLSSAQASVEI DSLFEGDFYTSIT	298
LvHsc70	NHFI QEFKRKYKKDPSENKRS LRLRTACERAKRTLSSAQASVEI DSLFEGDFYTSIT	298
PcHsp70	NHFI QEFKRKYKKDLGKNRAVRLRTACERAKRTLSSAQASVEI DSLFEGDYTSIT	299
FcHsp70	THFVQEFORKYKKDLTSNKRAL RRLRTACERAKRTLSSAQASLEI DSLFEGDYTSIT	299
MrHsp70	HHFVQEFORKYKKDLTTNKRAL RRLRTACERAKRTLSSAQASLEI DSLFEGDYTSIT	240
Consensus	hf qef rkykkd nkr rrlrtacerakrtls s qas eidsl eg d yts t	
MnHsc70-1	RARFEELCGDLFRGTLPEVEKSLRDAKMDKAQHDI VLVGGSTRI PKIQKLLQDF FNGKE	358
MnHsc70-2	RARFEELCGDLFRGTLPEVEKSLRDAKMDKAQHDI VLVGGSTRI PKIQKLLQDF FNGKE	358
MrHsc70	RARFEELCGDLFRGTLPEVEKSLRDAKMDKAQHDI VLVGGSTRI PKIQKLLQDF FNGKE	358
PmHsc70	RARFEELCADLFRGTLPEVEKSLRDAKMDKAQHDI VLVGGSTRI PKIQKLLQDF FNGKE	358
FcHsc70	RARFEELCADLFRGTLPEVEKSLRDAKMDKAQHDI VLVGGSTRI PKIQKLLQDF FNGKE	358
LvHsc70	RARFEELCADLFRGTLPEVEKSLRDAKMDKAQHDI VLVGGSTRI PKIQKLLQDF FNGKE	358
PcHsp70	RARFEELCSDLFRGTLPEVEKALRDAKMDKAQHDI VLVGGSTRI PKIQKLLQDF FNGKE	359
FcHsp70	RARFEELCSDLFRGTLPEVEKALRDAKMDKSSIHDI VLVGGSTRI PKIQKLLQDF FNGKE	359
MrHsp70	RARFDELCSDLFRGTLPEVEKALRDAKMDKSSIAEI VLVGGSTRI PKIQKLLQDF FNGKE	300
Consensus	rarf el c dl frgt lepvek l rdak dk a q h d i vlv ggstri pk i q k ll q df f ngke	

Fig. 4, the MnHsc70s clusters closely with other crustacean Hsc70s on a unique branch of the tree, and they in turn form a

larger node with Hsc70 sequences from *Xenopus*, human, *Rattus*, fish, and *Crassostrea*. Finally, taken together,

MnHsc70-1	L N K S I N P D E A V A Y G A A V Q A A I L C G D K S E A V Q D L L L L D V T P L S L G I E T A G G V M T A L I K R N T	418
MnHsc70-2	L N K S I N P D E A V A Y G A A V Q A A I L C G D K S E A V Q D L L L L D V T P L S L G I E T A G G V M T A L I K R N T	418
MrHsc70	L N K S I N P D E A V A Y G A A V Q A A I L C G D K S E A V Q D L L L L D V T P L S L G I E T A G G V M T A L I K R N T	418
PmHsc70	L N K S I N P D E A V A Y G A A V Q A A I L C G D K S E A V Q D L L L L D V T P L S L G I E T A G G V M T A L I K R N T	418
FcHsc70	L N K S I N P D E A V A Y G A A V Q A A I L C G D K S E A V Q D L L L L D V T P L S L G I E T A G G V M T A L I K R N T	418
LvHsc70	L N K S I N P D E A V A Y G A A V Q A A I L C G D K S E A V Q D L L L L D V T P L S L G I E T A G G V M T A L I K R N T	418
PcHsp70	L N K S I N P D E A V A Y G A A V Q A A I L H G D Q S E G V K D V 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
FcHsp70	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 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
MrHsp70	L N K S I N P D E A V A Y G A A V Q G A I L R G D Q S E G V K D V 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	360
Consensus	l n k s i n p d e a v a g a a v q a i l g d s e v d l l l d v p l s l g i e t a g v n t a l i k r n t	
MnHsc70-1	T I P T K Q T Q T F T T Y S D N Q P G V L I Q V Y E G E R A M T K D N N L L G K F E L S G I P P A P R G V P Q I E V T F	478
MnHsc70-2	T I P T K Q T Q T F T T Y S D N Q P G V 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	478
MrHsc70	T I P T K Q T Q T F T T Y S D N Q P G V L I Q V Y E G E R A M T K D N N L L G K F E L S G I P P A P R G V P Q I E V T F	478
PmHsc70	T I P T K Q T Q T F T T Y S D N Q P G V L I Q V Y E G E R A M T K D N N L L G K F E L S G I P P A P R G V P Q I E V T F	478
FcHsc70	T I P T K Q T Q T F T T Y S D N Q P G V L I Q V Y E G E R A M T K D N N L L G K F E L S G I P P A P R G V P Q I E V T F	478
LvHsc70	T I P T K Q T Q T F T T Y S D N Q P G V L I Q V Y E G E R A M T K D N N L L G K F E L S G I P P A P R G V P Q I E V T F	478
PcHsp70	T I P T K Q Q V F T T Y S D N Q P G V L I Q V Y E G E R A M T K D N N L L G K F E L S G I P P A P R G V P Q I E V T F	479
FcHsp70	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 M T R D N N L L G K F E L S G I P P A P R G V P Q I E V T F	479
MrHsp70	T I P T K Q Q I F T T Y A D N Q P G V L I Q V Y E G E R P M T K D N N L L G K F E L S G I P P A P R G V P Q I E V T F	420
Consensus	t i p t k q f t t y d n q p g v i q v y e g e r n t d n n l g k f e l g i p a p r g v p q i e v t f	
MnHsc70-1	D I D A N G I L N V S A A 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 V Q E A E K Y K A D D E K O R D R I A A	538
MnHsc70-2	D I D A N G I L N V S A A 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 V Q E A E K Y K A D D E K O R E R I S A	538
MrHsc70	D I D A N G I L N V S A A 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 V Q E A E K Y K A D D E K O R D R I A A	538
PmHsc70	D I D A N G I L N V S A 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 V Q D A E K Y K A D D E K O R D R I S A	538
FcHsc70	D I D A S G I L N V S A 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 V Q D A E K Y K A D D E K O R D R I S A	538
LvHsc70	D I D A N G I L N V S A 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 V Q D A E K Y K A D D E K O R D R I S A	538
PcHsp70	D I D A N G I L N V S A A 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 V N E A E K Y H E D D A K O R E R V E A	539
FcHsp70	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 T N D K G R L S K E E I E R M V N E A E N Y R E E D A K O R E R I E A	539
MrHsp70	D I D A N G I L N V S A 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 K M V N D V E R Y K E E D D K O R E R V E A	480
Consensus	d i d a g i l n v s a d k s t g k e n i t i n d k g r l s k e e i e m v e y d k q r r a	
MnHsc70-1	K N S L E S Y C F N M K S T V E D D K F K D K V P E E D R N K I M E A C N D A I K V L D T N Q L G E K E E Y E H K L K E	598
MnHsc70-2	K N N L E S Y C F N M K S T V E D D K F K D K V P E E D R N K I M E A C N D A I K V L D T N Q L G E K E E Y E H K L K E	598
MrHsc70	K N S L E S Y C F N M K S T V E D D K F K D K V P E E D R N K I M E A C N D A I K V L D S N Q L G E K E E Y E H K L K E	598
PmHsc70	K N S L E S Y C F N M K S T V E D K F K E K I S E E D R N K I L E T C N E T I K V L D M N Q L G E K E E Y E H K Q K E	598
FcHsc70	K N S L E S Y C F N M K S T V E D K F K E K I S E E D R N K I L E T C N E T I K V L D M N Q L G E K E E Y E H K Q K E	598
LvHsc70	K N S L E S Y C F N M K S T V E D K F K E K I S E E D R N K I L E T C N E T I K V L D M N Q L G E K E E Y E H K Q K E	598
PcHsp70	K N R L E A L C F S I K S A V S E P S V G D K L S A E E K R S V E E K A Q E T L N V L D A N Q L A E K E E Y E H Q V K E	599
FcHsp70	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 L V E E K A E T L K V L D N N Q L A E K E E Y E Y K V K E	599
MrHsp70	K N R L E S V C Y S I K S A V C D P S V A D K L S A D E K R C V E Q A E E T L R V L E A N Q L A E K E E F E Y Q V N E	540
Consensus	k n l e c k s k k w l n q l e k e e e e	
MnHsc70-1	I E Q I C N P I I T K I V Y Q A A G G A P P G G M P G G F P G . A P G A G . A A P G . G G S S G P T I E E V D	649
MnHsc70-2	I E Q I C N P I I T K I V Y Q A A G G A P P G G M P G G F P G . A P G A G . A A P G . G G S S G P T I E E V D	649
MrHsc70	I E Q I C N P I I T K I V Y Q A A G G A P P G G M P G G F P G . A P G G G . A A P G . G G S S G P T I E E V D	649
PmHsc70	I E Q V C N P I I T K I V Y A A A G G A P P G G M P G G F P G G A P G A G G A A P G A G G S S G P T I E E V D	652
FcHsc70	I E Q V C N P I I T K I V Y A A A G G A P P G G M P G G F P G G A P G A G G A A P G A G G S S G P T I E E V D	652
LvHsc70	I E Q V C N P I I T K I V Y A A A G G A P P G G M P G G F P G G A P G A G G A A P G A G G S S G P T I E E V D	652
PcHsp70	L E R V V R P L A S K V H G Q G A G G G Q G G A S A S S G . P T V E E V D	635
FcHsp70	L E Q V V 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
MrHsp70	L E K V V R P L A S K V H G Q G G Y A G N A G Y L G S T G G P T V E E V D	577
Consensus	e p k p t e e v d	

Fig. 3 (continued)

Hsp70s and Hsc70s were clustered into two separate sub-classes except for one especial sequence, which might show

◀ **Fig. 3** Alignment result of the deduced amino acid sequences: MnHsc70-1, ABG45886; MnHsc70-2, KC460343; MrHsc70, AY466445; PmHsc70, AAQ05768; FcHsc70, AY748350; LvHsc70, ABP01681; PcHsp70, ABC01063; FcHsp70, FJ167398; and MrHsp70, AY466497. Three signatures are shown in red boxes. The potential non-organelle eukaryotic consensus motif RARFEEL and cytoplasmic characteristic motif EEVD are shown in the green and blue box, respectively

how Hsp70 family proteins evolved in the subcellular locations of eukaryotic cells.

Whether a heat-shock gene is heat-inducible (Hsp gene) or a cognate gene (Hsc gene) depends on the regulation of this gene in normal somatic cells: a heat-inducible gene is efficiently expressed only after heat induction, whereas a cognate gene has a high basal level of expression and is not or is weakly heat-inducible (David 1993). In our study, the expression of the MnHsc70 genes under unstressed conditions was

Fig. 4 Phylogenetic tree showing the relationship of MnHsc70-1 and MnHsc70-2 amino acid sequences to other Hsc70 family members from a variety of species. Sequences used in the tree were followed by their GenBank accession number. The number at each node indicated the percentage of bootstrapping after 1,000 replications

performed by a quantitative real-time PCR method. Results revealed that two MnHsc70s mRNA transcripts were present in all tested tissues. The different expression profiles between MnHsc70-1 and MnHsc70-2 were obvious, and the level of MnHsc70 expression in different tissues of *M. nipponense* was also varied. Differential expression of Hsc70 also has been reported in the tissues of rats, *Xenopus*, carp, and shrimp (Ali et al. 1996a; Lo et al. 2004; O'Malley et al. 1985). The

expression pattern of the two MnHsc70s led us to speculate that under normal conditions, the expression of MnHsc70-2 was dominant, while the expression of MnHsc70-1 was just supplementary.

In the present study, MnHsc70-1 exhibited a dramatic down-regulation in the hepatopancreas and gill after heat shock. By contrast, the expression of MnHsc70-2 was slightly up-regulated in the hepatopancreas, whereas the MnHsc70-2

Fig. 5 Expression profiles of MnHsc70-1 and MnHsc70-2 mRNA in different tissues. Quantitative real-time RT-PCR was carried out with RNA samples from the hemocytes, heart, hepatopancreas, gill, intestine, nerve, and muscle of the *M. nipponense*. Different lowercase letters above the bars indicate significant differences ($P < 0.05$) at different tissues of the same group; asterisks above the bars show significant differences ($P < 0.05$) between two groups of the same tissue

Fig. 6 Real-time RT-PCR analyses of MnHsc70-1 and MnHsc70-2 expressions in *M. nipponense* under heat-shock treatment ($n=3$). Graphs **a** and **b** represent expression profiles of MnHsc70-1 and MnHsc70-2 in the hepatopancreas, respectively. Graphs **c** and **d** represent expression profiles of MnHsc70-1 and MnHsc70-2 in the gill, respectively. Significant differences are indicated, * $P<0.05$ and ** $P<0.01$. Expression of MnHsc70-1 and MnHsc70-2 mRNA is relative to β -actin

of the gill was relatively sensitive to the stressor producing an 11.67-fold induction. The depression of Hsc70 has been demonstrated in Pacific oyster *Crassostrea gigas* (Boutet et al. 2003) and Chinese shrimp *F. chinensis* by cadmium exposure (Luan et al. 2010). It was demonstrated that the depression of Hsp70 by cadmium exposure might be regulated through a cAMP-responsive regulatory pathway (Vilaboa et al. 1995) which implied that the down-regulation of MnHsc70-1 may also be regulated by cAMP. In previous studies, Hsc70 expression in most species was not increased by heat-shock treatment (Kiang and Tsokos 1998). However, an increasing number of studies have demonstrated that heat-shock treatment does increase mRNA levels several fold (<20-fold) in a variety of cell types, including hemocytes of *P. monodon* (Lo et al. 2004), Pacific oyster *C. gigas* hemocytes (Gourdon et al. 2000), human peripheral blood monocytes (Jacquier-Sarlin et al. 1995), human lymphocytes (Hansen et al. 1991), and human HeLa cells (O'Malley et al. 1985). Several cognate Hsc70s increase several fold in stressed animals, indicating that they are moderately inducible (Tavaria et al. 1996).

In recent years, due to infection by various pathogenic microorganisms especially *A. hydrophila* (Shen et al. 2000), mass mortality of *M. nipponense* occurred, which caused heavy economic losses. It is necessary to better understand the mechanism of stress tolerance or pathogen resistance of *M. nipponense*. Previous studies showed that the expression levels of Hsp70 were significantly induced by pathogen stimulation (Cui et al. 2010; Rungrasamee et al. 2010). Likewise, Hsp70 showed specific immune responses against infectious agents; both MnHsc70-1 and MnHsc70-2 exhibited a moderate up-regulation when challenged with *A. hydrophila*. Similar expression profiles were found in previous studies: the expression of FcHsc70 was less sensitive to copper treatment (Luan et al. 2010), and no significant changes of Hsc70 were detected in the primary-cultured hepatocytes in rainbow trout under heavy metal exposure (Boone and Vijayan 2002).

In conclusion, two MnHsc70 members from *M. nipponense* have been identified and characterized for the first time. The expression levels of MnHsc70s under normal conditions are consistent with their cognate nature: the basal

Fig. 7 Relative expressions of MnHsc70-1 and MnHsc70-2 in the hepatopancreas at different time points under *A. hydrophila* challenge analyzed by quantitative RT-PCR. Graphs **a** and **b** represented expression profiles of MnHsc70-1 and MnHsc70-2, respectively. Asterisks indicate there was a significant difference between control prawns and bacteria-challenged prawns. Expression of MnHsc70-1 and MnHsc70-2 mRNA is relative to β -actin

expression levels are substantial. The different expression profiles between MnHsc70-1 and MnHsc70-2 support the hypothesis that two Hsc70 genes have distinct biological tasks. The results presented here provide useful insights for investigating stress-related response in *M. nipponense*.

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