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Biology

Molecular characterization and expression analysis of heat shock proteins 40, 70 and 90 from kuruma shrimp *Marsupenaeus japonicus*

Threechada Danwattananusorn · Fernand F. Fagutao · Aiko Shitara · Hidehiro Kondo · Takashi Aoki · Reiko Nozaki · Ikuo Hirono

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Abstract Heat shock proteins (HSPs) are proteins that are expressed more strongly when the cells are exposed to physiological and stressful conditions. In this study, the full-length cDNAs of heat shock proteins 40 (*Mj*HSP40), 70 (*Mj*HSP70) and 90 (*Mj*HSP90) were cloned from kuruma shrimp *Marsupenaeus japonicus*. The open reading frames (ORFs) of the cDNA clones have lengths of 1,191, 1,959 and 2,172 bp and encode 396, 652 and 723 amino acid residues, respectively. The predicted *Mj*HSP40 amino acid sequence contains a J domain, a glycine/phenylalanine-rich region, and a central domain containing four repeats of a CxxCxGxG motif, indicating that it is a type I HSP40 homolog. The signature sequences of the HSP70 and HSP90 gene families are conserved in the *Mj*HSP70 and *Mj*HSP90 amino acid sequences. The deduced amino acid sequences of *Mj*HSP70 and *Mj*HSP90 share high identity with previously reported shrimp HSP70s and HSP90s, respectively. The expression of *Mj*HSP90 mRNA increased at 32°C. Additionally, the expressions of *Mj*HSP40, *Mj*HSP70 and *Mj*HSP90 mRNAs increased in defense-related tissues (i.e., hemocytes and lymphoid organ) when the shrimp were challenged with white spot syndrome virus.

Keywords HSP40 · HSP70 · HSP90 · *Marsupenaeus japonicus* · Heat shock proteins · Heat shock · WSSV challenge

T. Danwattananusorn · F. F. Fagutao · A. Shitara · H. Kondo · T. Aoki · R. Nozaki · I. Hirono (✉)
Laboratory of Genome Science, Tokyo University of Marine Science and Technology, Konan 4-5-7, Minato-ku, Tokyo 108-8477, Japan
e-mail: hirono@kaiyodai.ac.jp

Introduction

Cultured shrimp, like most commercially cultured aquatic species, are constantly exposed to stresses, such as elevated temperatures, high pressure, or the presence of toxic compounds, in their rearing environment. These stresses ultimately affect the various biological processes in shrimp, and how they respond to them is crucial to shrimp health. Heat shock proteins (HSPs) can help shrimp and other aquatic animals cope with various stresses in their environment.

Heat shock proteins are found in both eukaryotes and prokaryotes, where they function as molecular chaperones that are known to participate in protein folding, transport and assembly. They are synthesized in response to a variety of stresses, including extremes of temperature, cellular energy depletion, and extreme concentrations of ions, other osmolytes, gases and various toxic substances [1]. HSPs have been classified according to their sequence homology and molecular weight into several families, such as HSP110, HSP100, HSP90, HSP70, HSP60, HSP40 and HSP20 [2].

HSP40s (also called DnaJs) have been conserved throughout evolution and are important for protein homeostasis, where they stimulate the ATPase activity of the HSP70 proteins that are involved in protein translation, folding, unfolding, translocation, and degradation [3]. The HSP40 family is large. For example, the genomes of *Saccharomyces cerevisiae* and *Homo sapiens* encode 20 and 44 members, respectively [4–6]. All HSP40s contain a 70-amino acid-long J domain that is responsible for interactions with HSP70 [7, 8]. HSP40s can be classified into three subtypes [5]. Type I HSP40s have a glycine and phenylalanine (G/F)-rich region and a cysteine-rich, zinc finger-like region (ZFLR). Type II HSP40s have a G/F-rich region, while type III HSP40s have neither a G/F-rich region nor a ZFLR. In yeast, the J domain alone is sufficient

to enable HSP70 to perform its essential cellular function [9]. Recent studies suggest a mechanism where the substrate appears to be released from HSP40 and transferred to HSP70 [10–12]. Furthermore, HSP40 proteins can regulate the activities of other chaperones, such as HSP90 [13].

HSP70s play a role in protein synthesis under normal cellular conditions, fixing denatured proteins and preventing the misfolding or aggregation of proteins [14–16]. The HSP70 family is composed of several members, including heat-inducible HSP70, constitutively expressed heat shock cognate 70 (HSC70), glucose-regulated protein (GRP78), and others [17]. HSC70s, unlike HSP70s, have no introns. However, they share common structural features, including a 44-kDa N-terminal ATPase domain, an 18-kDa peptide-binding domain, and a 10-kDa C-terminal substrate-binding domain [17, 18].

HSP90 participates in the folding, maintenance of structural integrity, and the proper regulation of a subset of cytosolic proteins [19], and accounts for 1% of the soluble protein in most tissues, even in the absence of stress [20]. HSP90 also functions as a specialized chaperone for a set of signaling proteins, including several protein kinases and transcription factors [21]. HSP90 has roles in cell growth and differentiation, apoptosis, signal transduction and cell–cell communication. Eukaryotic HSP90 proteins consist of three domains: a 25-kDa N-terminal ATP-binding domain, a 40-kDa middle domain, and a 12-kDa C-terminal dimerization domain. The N-terminal ATP-binding domain is connected to the middle domain by a "linker" of variable length, and the C-terminal dimerization domain provides the binding site for a set of co-chaperone molecules that function with HSP90 as part of a multi-chaperone complex [21].

In recent years, there has been increasing interest in shrimp HSPs (mostly HSP70 and HSP90), because of their roles in shrimp immune response. HSP70 genes and their expressions have been studied in Chinese shrimp *Fenneropenaeus chinensis* [22], Pacific white shrimp *Litopenaeus vannamei* [23, 24], and black tiger shrimp *Penaeus monodon* [25, 26], while the expressions of HSP90 genes have been studied in *F. chinensis* [27], *P. monodon* [28], and greasyback shrimp *Metapenaeus ensis* [29]. In contrast, we were unable to find reports on HSP40 in shrimp. In the present study, HSP40, HSP70 and HSP90 were cloned from kuruma shrimp (*Marsupenaeus japonicus*), and their expressions were examined after heat shock and being challenged with white spot syndrome virus (WSSV).

Materials and methods

Shrimp

The kuruma shrimp used in this study were purchased from a commercial shrimp farm in Miyazaki, Japan. The shrimp were analyzed for signs of infectious diseases, kept in artificial seawater maintained at 25°C and 30–32 ppt, and fed daily with commercial shrimp feed prior to all experimental procedures.

Cloning of *Mj*HSP40, *Mj*HSP70 and *Mj*HSP90

The *Mj*HSP40, *Mj*HSP70 and *Mj*HSP90 cDNAs were amplified from a normal kuruma shrimp hepatopancreas cDNA library prepared in our laboratory. The pairs of specific primers designed based on the partial cDNA sequences of *Mj*HSP40, *Mj*HSP70 and *Mj*HSP90 previously identified in our laboratory were used for PCR amplification (Table 1). Moreover, the pair of primers used to amplify *Mj*HSP70 were also designed based on the cDNA sequence of kuruma shrimp HSP70 (GenBank accession no. ABK76338). The PCR reaction was performed as follows: an initial denaturation at 95°C for 5 min, followed by 35 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 1 min, and a final extension at 72°C for 5 min. After electrophoresis on 1% agarose gel, the PCR products were subsequently purified. The purified DNAs of the PCR products were ligated into the pGEM-T easy vector (Promega, USA) and were then transformed into *Escherichia coli* strain JM109. The positive clones were screened by colony PCR with M13 forward and reverse primers. The subsequent PCR products were directly sequenced with an ABI 3130xl capillary sequencer using BigDye chemistry (Applied Biosystems, USA).

Sequence data analysis

Nucleotide and amino acid sequences were analyzed using GENETYX WIN (v.7.0.3) software. Homology analysis and cleavage site prediction were accomplished with BLASTP (see http://ncbi.nlm.nih.gov/). Conceptual translation was performed and the characteristics of the protein were predicted using the ExPASy web server (http://www.expasy.ch). The motifs were predicted using SMART (http://smart.embl-heidelberg.de/) [30], and subcellular localization predictions were performed on the PSORT II sever (http://psort.hgc.jp/form2.html). Multiple sequence alignments were created using ClustalW [31]. The neighbor-jointing phylogenetic trees were then generated by MEGA4 software [32].

Expression analysis after heat shock and white spot syndrome virus (WSSV) challenge experiments

In order to study the *Mj*HSP40, *Mj*HSP70 and *Mj*HSP90 expression after heat shock treatment, apparently healthy

Table 1 PCR primers and primer sequences used for the amplification of *Mj*HSP40, *Mj*HSP70 and *Mj*HSP90 cDNAs, and the quantitative real-time PCR and RT-PCR primers used in the experiment

Primer	Primer sequence (5′–3′)	Purpose
HSP40-F1	CAAGATGGTGAAGGAAACTG	Cloning of *Mj*HSP40 partial cDNA sequence
HSP40-R1	ATCTCAAAGATGTCCATGGG	Cloning of *Mj*HSP40 partial cDNA sequence
HSP40-F2	GTTAAGGATGTCATCCATCAA	Cloning of *Mj*HSP40 partial cDNA sequence
HSP40-R2	AATGCTAGTTTTCTGTATGCC	Cloning of *Mj*HSP40 partial cDNA sequence
HSP40-F3	TTCACGCTCTGAGACCCTCG	Cloning of *Mj*HSP40 partial cDNA sequence
HSP40-R3	CCTCATTGCTCAGGACTTCA	Cloning of *Mj*HSP40 partial cDNA sequence
HSP40-F4	TTCCCGGGGTCGACCCACGCGTC	Cloning of *Mj*HSP40 partial cDNA sequence
HSP40-R4	CGTAAGCTTGGATCCTCTAGAG	Cloning of *Mj*HSP40 partial cDNA sequence
HSP40-F5	GTGGATTCCAGAAGCCTATC	Cloning of *Mj*HSP40 partial cDNA sequence
HSP40-F6	GTACAAGAACCCTTTCGAGA	Cloning of *Mj*HSP40 partial cDNA sequence
HSP70-F1	GTATTGATCTGGGAACCACC	Cloning of *Mj*HSP70 partial cDNA sequence
HSP70-R1	AAGGTCTTCTTGTCGCCCTT	Cloning of *Mj*HSP70 partial cDNA sequence
HSP70-F2	GTATCTTCGAAGTAAAGTCCA	Cloning of *Mj*HSP70 partial cDNA sequence
HSP70-R2	TGTAGAAGTCGATACCTTCGA	Cloning of *Mj*HSP70 partial cDNA sequence
HSP70-F3	GAAGTCACCTTCGACATCGAC	Cloning of *Mj*HSP70 partial cDNA sequence
HSP70-R3	TCAACTGTCGACTTCATGTTG	Cloning of *Mj*HSP70 partial cDNA sequence
HSP70-R4	TTAATCGACCTCCTCGATGGT	Cloning of *Mj*HSP70 partial cDNA sequence
HSP90-F1	GTCCCTGATCATCAACACGT	Cloning of *Mj*HSP90 partial cDNA sequence
HSP90-R1	ACTGACCGATCATAGAGATG	Cloning of *Mj*HSP90 partial cDNA sequence
HSP90-F2	ACAGTACATCTGGGAGTCGTC	Cloning of *Mj*HSP90 partial cDNA sequence
HSP90-R2	TCAGGTACTCGGGGATCAGTT	Cloning of *Mj*HSP90 partial cDNA sequence
HSP90-F3	AGCTACAAGAAGTTCTACGA	Cloning of *Mj*HSP90 partial cDNA sequence
HSP90-R3	GCTTCTGGTTCTCCTTCATGC	Cloning of *Mj*HSP90 partial cDNA sequence
HSP90-F4	ACCATGGGCTACATGGCCGCCA	Cloning of *Mj*HSP90 partial cDNA sequence
HSP90-R4	CCTTCACAGACTTGTCGTTCT	Cloning of *Mj*HSP90 partial cDNA sequence
EFl-F	ATGGTTGTCAACTTTGCCCC	Quantitative real-time PCR
EFl-R	TTGACCTCCTTGATCACACC	Quantitative real-time PCR
EFs-F	ATTGCCACACCGCTCACA	Quantitative real-time PCR and RT-PCR
EFs-R	TCGATCTTGGTCAGCAGTTCA	Quantitative real-time PCR and RT-PCR
*Mj*HSP40l-F	TTCACGCTCTGAGACCCTCG	Quantitative real-time PCR
*Mj*HSP40l-R	CCTCATTGCTCAGGACTTCA	Quantitative real-time PCR
*Mj*HSP40s-F	CGGAGAAGTTCTAGACCAAGATGGT	Quantitative real-time PCR and RT-PCR
*Mj*HSP40s-R	GTGGGCTTCACCCCTAGGAT	Quantitative real-time PCR and RT-PCR
*Mj*HSP70l-F	GTATTGATCTGGGAACCACC	Quantitative real-time PCR
*Mj*HSP70l-R	AAGGTCTTCTTGTCGCCCTT	Quantitative real-time PCR
*Mj*HSP70s-F	TCGGCCGCAAATTCGA	Quantitative real-time PCR and RT-PCR
*Mj*HSP70s-R	GAAGGGCCAGTGCTTCATGT	Quantitative real-time PCR and RT-PCR
*Mj*HSP90l-F	AGCTACAAGAAGTTCTACGA	Quantitative real-time PCR
*Mj*HSP90l-R	GTCATGTAGATGACCTCGAA	Quantitative real-time PCR
*Mj*HSP90s-F	TCGCTGAATTCCTCAGATACCA	Quantitative real-time PCR and RT-PCR
*Mj*HSP90s-R	GCACTCCTTGAGAGAAGACATATCG	Quantitative real-time PCR and RT-PCR

kuruma shrimp, each weighing about 10 g, were acclimated to a salinity of 30–32 ppt at 25°C for 7 days before experimentation, and then heat shock treatment was performed at 32°C for 3 h. Gills were dissected from three shrimp sampled before the heat shock treatment as an initial control, and at 1 and 3 h after heat shock. After this, *Mj*HSP40, *Mj*HSP70 and *Mj*HSP90 mRNA expression levels were examined by quantitative real-time PCR. Total RNAs were extracted using the RNAiso reagent (TaKaRa Bio Inc., Japan), as described in the manufacturer's protocol.

The first-strand cDNAs were synthesized from 2 μg of the total RNAs using Moloney murine leukemia virus reverse transcriptase (Invitrogen, USA). Primer sets for *Mj*HSP40, *Mj*HSP70 and *Mj*HSP90 were designed with ABI Primer Express Software v.3.0 (Applied Biosystems, USA), as shown in Table 1. Quantitative real-time PCR assays were done in a 20 μl reaction volume consisting of 5 μl template cDNA (2 μg/ml), 0.4 μl of both forward and reverse primers (10 pM), 10 μl Power SYBR Green Master Mix (Applied Biosystems), and 4.2 μl distilled water. Real-time PCR analysis was performed on an ABI7300 real-time PCR system (Applied Biosystems), following the manufacturer's protocol. Expression levels were measured by the $2^{-\Delta\Delta C_T}$ method [33]. Elongation factor-1 α (EF-1α) mRNA levels were used as an internal control. Differences in expression were measured by one-way analysis of variance followed by the Tukey significant difference test using SPSS 16.0 software. P values of <0.05 were considered significant.

In addition, the *Mj*HSP40, *Mj*HSP70 and *Mj*HSP90 mRNA expression levels were examined after the WSSV challenge experiment. Apparently healthy kuruma shrimp, each weighing about 10 g, were acclimated to laboratory conditions for 7 days and then injected with 50 μl of 10^6 × WSSV stock suspension. This virus stock dilution was used based on an earlier in vivo viral titration assay which suggested that this particular dilution is optimal for use in challenge experiments. Hemocytes, lymphoid organ and hepatopancreas were collected from three shrimp sampled before the WSSV challenge experiment as an initial control and at 1, 3 and 5 days post-WSSV injection, and total RNA was extracted and pooled together at each sampling time. Then the first-strand cDNA was synthesized as described above. RT-PCR was performed to analyze the expression profiles of *Mj*HSP40, *Mj*HSP70 and *Mj*HSP90 using *Mj*HSP40s-F, *Mj*HSP40s-R, *Mj*HSP70s-F, *Mj*HSP70s-R, *Mj*HSP90s-F and *Mj*HSP90s-R (see Table 1). EF-1α was amplified as an internal control using EFs-F and EFs-R (see Table 1). One microliter of the first-strand cDNA was used as the template in the PCR amplification. The RT-PCR reaction was conducted with an initial predenaturation step performed at 95°C for 5 min followed by 25 and 30 cycles of 95°C for 30 s, 55°C for 30 s and 72°C for 30 s, and a final extension at 72°C for 5 min. Ten microliters of the amplified products were separated by electrophoresis with a 1% agarose gel and visualized with ethidium bromide. The mRNA bands were semi-quantitatively assessed for their relative expression following the method described by Lindenstrøm et al. [34] using ImageJ software to measure light intensity [35].

Results

Characterization of *Mj*HSP40, *Mj*HSP70 and *Mj*HSP90 genes in kuruma shrimp

The full-length *Mj*HSP40, *Mj*HSP70 and *Mj*HSP90 cDNA sequences (GenBank accession no. AB520825, AB520826 and AB520827, respectively) from kuruma shrimp were obtained by PCR amplification. For *Mj*HSP40, the sequence consists of 1,191 nucleotides of an open reading frame (ORF) encoding 396 amino acids with a calculated molecular weight of 44.42 kDa and a theoretical p*I* of 6.62. The deduced amino acid sequence of *Mj*HSP40 contains an N-terminal conserved domain (J domain, aa 5–60), a glycine/phenylalanine region (G/F domain, aa 67–96), a central domain containing four highly conserved cysteine-rich repeats with a consensus sequence of CxxCxGxG where x is any amino acid (CRR domain, aa 122–207), and a C-terminal domain (C domain, aa 220–344) (Fig. 1). In a GenBank BLASTP search, the deduced amino acid sequence of *Mj*HSP40 showed high homology with those of other invertebrates: domestic silkworm *Bombyx mori* (NP_001040292, 69% identity), red flour beetle *Tribolium castaneum* (XP_971446, 64%), and jewel wasp *Nasonia vitripennis* (XP_001607240, 64%). *Mj*HSP40 also had high similarities to DnaJ (Hsp40) homolog subfamily A member 1 from human *Homo sapiens* (NP_001530, 66%), heat shock protein 40 from American alligator *Alligator mississippiensis* (BAF94139, 65%), and DnaJ-like subfamily A member 4 from zebrafish *Danio rerio* (XP_689328, 62%) (Fig. 2). The deduced amino acid sequence of *Mj*HSP70 was found to be very similar to *M. japonicus* HSP70s in the GenBank (accession nos. ABF83607 and ABK76338). Since both submissions were unpublished, we first verified the sequences of these genes.

The deduced amino acid sequences of *Mj*HSP70 also contain HSP70 family motifs and signatures, including an adenosine triphosphate/guanosine triphosphate (ATP/GTP)-binding site, a bi-partite nuclear localization signal, a non-organellar motif, and a conserved EEVD motif. *Mj*HSP70 displayed very high homology with shrimp HSP70s from *L. vannamei* (AAT46566, 99%), *P. monodon* (AAQ05768, 99%), and *M. ensis* (ABF20530, 97%). Furthermore, *Mj*HSP70 showed high similarities to HSP70 from American lobster *Homarus americanus* (ABA02165, 96%), marbled crab *Pachygrapsus marmoratus* (ABA02164, 94%), and pearl oyster *Pteria penguin* (ABJ97377, 86%).

The *Mj*HSP90 cDNA contains a 2,172-bp ORF that encodes 723 amino acids with a calculated molecular weight of 83.6 kDa and a theoretical p*I* of 4.92. The deduced amino acid sequence of *Mj*HSP90 also contains

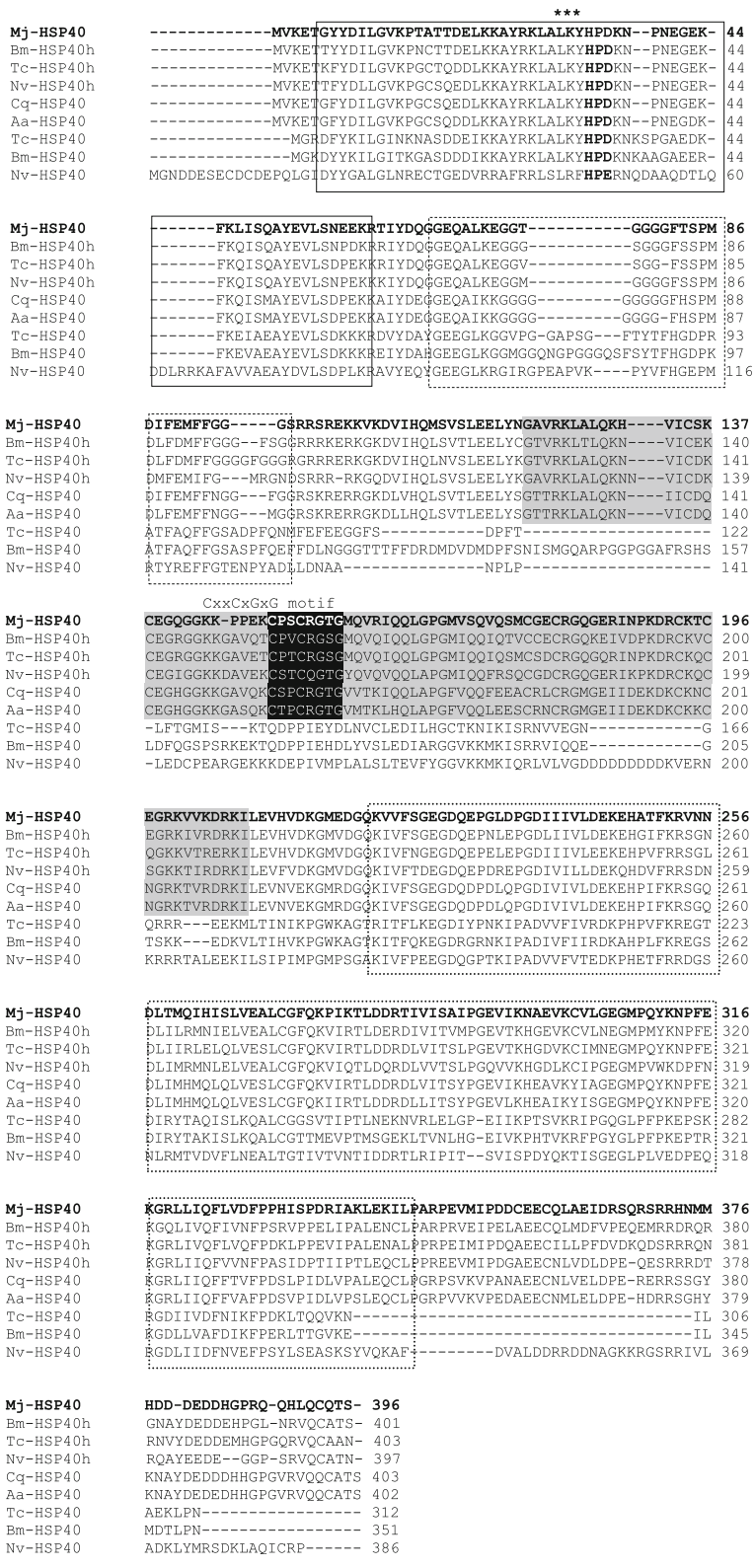

Fig. 1 Multiple alignment of the deduced amino acid sequences of HSP40 from kuruma shrimp and other animals. The amino acid positions are shown on the *right*. The N-terminal conserved domain (J domain) is *boxed with a solid line*, the glycine/phenylalanine-rich region (G/F domain) is *boxed with a dashed line*, and the C-terminal domain (CTD domain) is *boxed with a dotted line*. The central domain containing four repeats of a CxxCxGxG motif (CRR domain) is shown with a *gray background*. The HPD motif is marked with *asterisks*. *Mj-HSP40 M. japonicus* HSP40, *Tc-HSP40h Tribolium castaneus* HSP40 homolog (XP_971446), *Tc-HSP40 T. castaneus* HSP40 (XP_966855), *Bm-HSP40h Bombyx mori* DnaJ homolog subfamily A member 1 (NP_001040292), *Bm-HSP40 B. mori* HSP40 (BAD90846), *Nv-HSP40h Nasonia vitripennis* DnaJ homolog subfamily A member 1 (XP_001607240), *Nv-HSP40 N. vitripennis* HSP40 (XP_001601548), *Cq-HSP40 Culex quinquefasciatus* HSP40 (DnaJ chaperone) (XP_001844792), and *Aa-HSP40 Aedes aegypti* HSP40 (DnaJ chaperone) (ABF18277)

HSP90 family motifs and signatures, including five signatures, a typical histidine kinase-like ATPase (HATPase) domain, a GxxGxG motif, and a conserved MEEVD motif. *Mj*HSP90 exhibited high homology with HSP90s of other invertebrates: *P. monodon* (ABM54577, 96%), *M. enesis* (ABR66910, 94%), Chinese mitten crab *Eriocheir sinensis* (ACJ01642, 90%), honey bee *Apis mellifera* (NP_001153536, 85%); and even with vertebrates: cattle

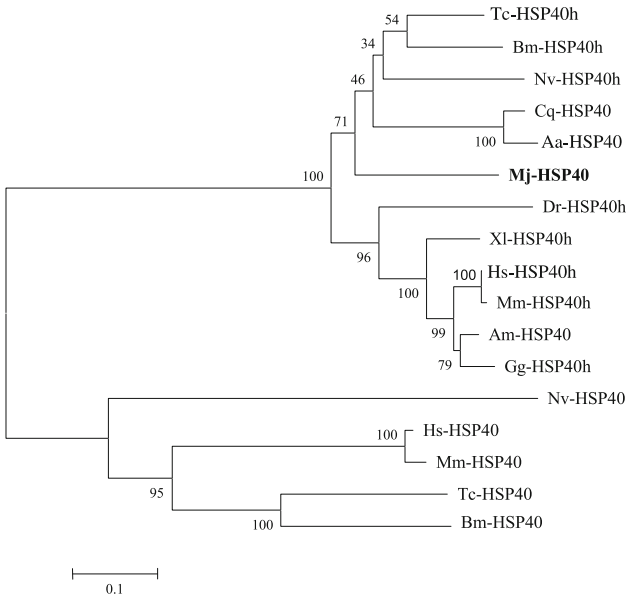

Fig. 2 Phylogenetic analysis of *Mj*HSP40. The neighbor joining (NJ) was done with MEGA4 software. A bootstrap test of the NJ analysis was conducted to evaluate the reliability of each branch in the tree. *Numbers at the branches* indicate the percentage of bootstrap values estimated from 1000 bootstrap replicates. *Mj-HSP40 M. japonicus* HSP40, *Tc-HSP40h T. castaneus* HSP40 homolog (XP_971446), *Tc-HSP40 T. castaneus* HSP40 (XP_966855), *Bm-HSP40h B. mori* DnaJ homolog subfamily A member 1 (NP_001040292), *Bm-HSP40 B. mori* HSP40 (BAD90846), *Nv-HSP40h N. vitripennis* DnaJ homolog subfamily A member 1 (XP_001607240), *Nv-HSP40 N. vitripennis* HSP40 (XP_001601548), *Cq-HSP40 C. quinquefasciatus* HSP40 (DnaJ chaperone) (XP_001844792), *Aa-HSP40 A. aegypti* HSP40 (DnaJ chaperone) (ABF18277), *Dr-HSP40h Danio rerio* DnaJ (HSP40) homolog subfamily A member 1 (NP_955956), *Xl-HSP40h Xenopus laevis* DnaJ (HSP40) homolog subfamily A member 1 (NP_00108365), *Hs-HSP40h Homo sapiens* DnaJ (HSP40) homolog subfamily A member 1 (NP_001530), *Hs-HSP40 H. sapiens* HSP740 (BAA12819), *Mm-HSP40h Mus musculus* DnaJ (HSP40) homolog subfamily A member 1 (NP_032324), *Mm-HSP40 M. musculus* HSP40 (BAA95672), *Am-HSP40 Alligator mississippiensis* HSP40 (BAF94139), and *Gg-HSP40h Gallus gallus* DnaJ (HSP40) homolog subfamily A member 1 (NP_001012963)

Bos taurus (NP_001012688, 84%), African clawed frog *Xenopus laevis* (NP_001086624, 81%), and chicken *Gallus gallus* (NP_996842, 81%).

Expression profiles of *MJ*HSP40, *Mj*HSP70 and *Mj*HSP90 after heat shock and white spot syndrome virus (WSSV) challenge experiments

In a preliminary experiment, we conducted a heat shock experiment at 35°C, and all shrimp died within 10 min. Thereafter, we tried it at 33°C, and we observed that most of the shrimp became moribund. We therefore conducted a heat shock experiment at 32°C. The expression profiles of *Mj*HSP40, *Mj*HSP70 and *Mj*HSP90 mRNA after the shrimp had experienced the heat shock treatment are shown in Fig. 3. At 3 h post heat shock, the expression of

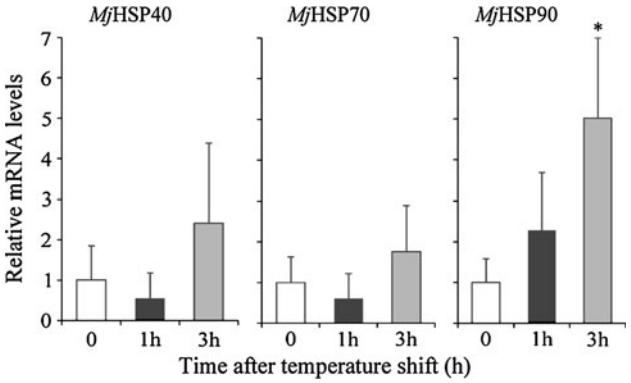

Fig. 3 Relative expressions of *Mj*HSP40, *Mj*HSP70 and *Mj*HSP90 in gills of kuruma shrimp at 0, 1 and 3 h after heat shock treatment, as examined by quantitative real-time PCR. Each group at each time point consists of three samples pooled together. EF-1α mRNA levels were used as an internal control. Data are presented as the mean ± SD of triplicate measurements. *Column bars with asterisks* indicate values that are significantly different from those at time 0 ($P < 0.05$)

*Mj*HSP90 was significantly increased (fourfold) compared with that in shrimp that had not experienced heat shock ($P < 0.05$), whereas the expression of *Mj*HSP40 and *Mj*HSP70 showed no change. Furthermore, *Mj*HSP40, *Mj*HSP70 and *Mj*HSP90 exhibited increased expression in the hemocytes and lymphoid organ after WSSV challenge compared with the initial control (Fig. 4). Both tissues play important roles in the innate immune response in shrimp. However, the gene expressions of the 3 *Mj*HSPs in the hepatopancreas, another major defense-related tissue, showed no noticeable change.

Discussion

Recently, a number of HSPs were identified while extensively annotating the GenBank databases established from many crustaceans. HSPs function prominently in stress tolerance and promoting cell survival, and are especially involved in refolding proteins and preventing their denaturation [1, 19]. They also participate in a variety of normal cellular processes, including protein trafficking, signal transduction, DNA replication, and protein synthesis [14]. HSP genes such as HSP100, HSP90, HSP70, HSP60, HSP40, and small heat shock protein families consist of stress-inducible and constitutively expressed genes. Inducible genes are expressed at low levels under non-stress conditions, but are expressed increasingly in response to stress such as elevated temperatures, nutritional deficiencies, viral infection, ischemia–reperfusion injury, and exposure to oxidative stress, ultraviolet radiation, chemicals, and ethanol [36–42]. In the present study, the full-length cDNA sequences of HSP40, HSP70, and HSP90

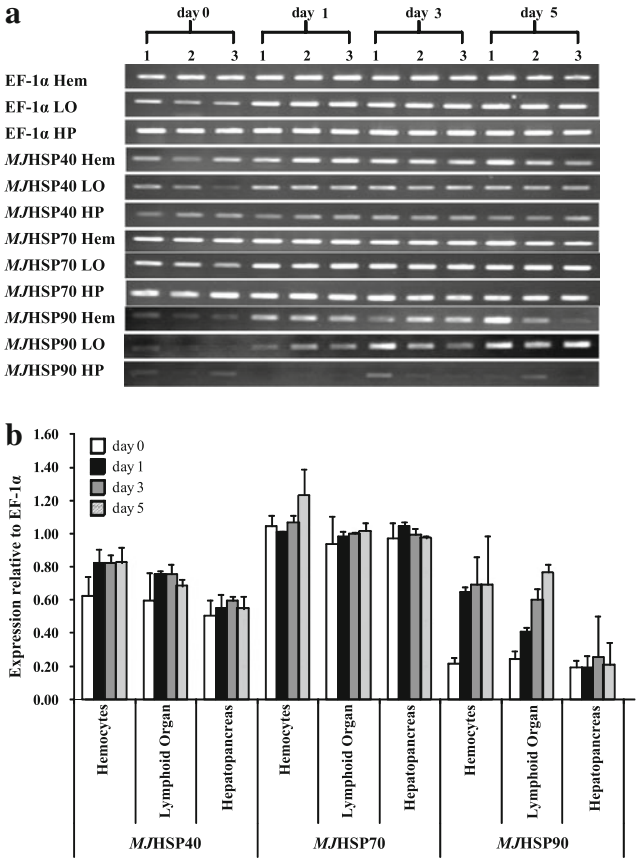

Fig. 4 Expression profiles of *Mj*HSP40, *Mj*HSP70 and *Mj*HSP90 mRNAs in defense-related tissues (i.e., hemocytes, lymphoid organ and hepatopancreas) of kuruma shrimp at 0, 1, 3 and 5 days post-WSSV injection (d.p.i.), as determined by RT-PCR (**a**), and a subsequent semi-quantitative analysis of their expression relative to EF-1α (**b**). Three samples were analyzed independently for each sampling period. EF-1α was used as an internal control. For the semi-quantitative analysis, mean values of the three measurements of specific gene expression relative to the corresponding EF-1α expression are presented. *Bars* represent standard deviations

were isolated from kuruma shrimp. Structural analysis shows that *Mj*HSP40 contains an N-terminal conserved domain, a G/F domain, a central domain containing a CRR domain, and a C-terminal domain (Fig. 1). All DnaJ/HSP40 proteins contain the J domain, through which they bind to HSP70s [43–46], and they can be categorized into three groups depending on the presence of other domains [3]. This domain consists of four helices and a loop region between helices II and III that contains a highly conserved tripeptide of histidine, proline and aspartic acid (the HPD motif, aa 34–36) [8] (Fig. 1). Because the amino acid sequence of *Mj*HSP40 is present in most type I HSP40 homologs [5], we classified it as a type I HSP40 homolog. Type I and type II HSP40s form chaperone pairs with cytosolic HSP70 that fold proteins with different efficiencies and carry out specific cellular functions [47, 48]. Type I DnaJ/HSP40 homolog or HSP40 and type II HSP40 differ in terms of structure, such that the former contains a central domain made up of four highly conserved cysteine-rich repeats with a consensus sequence of CxxCxGxG, and the latter contains only a J and G/F domain.

Our experiments also show that the expression of *Mj*HSP40, *Mj*HSP70 and *Mj*HSP90 respond to heat shock and WSSV infection. In the heat shock treatment, the expression of *Mj*HSP90 was increased (fourfold) compared with that in shrimp without heat shock at 3 h post heat shock (Fig. 3). Recently, although the HSP40 expression after heat shock treatment was not studied in shrimp, in the larvae of the midge *Chironomus riparius*, the expressions of HSP40 and HSP90 were upregulated within 1 h of heat shock treatment [49]. Similarly, in *F. chinensis*, *P. monodon* and freshwater shrimp, *Macrobrachium rosenbergii*, HSP70s and HSP90s appear to be transcriptionally up-regulated when the temperature is increased [22, 27, 28, 50]. Our results show that only the expression of *Mj*HSP90 was increased after heat shock treatment, suggesting that *Mj*HSP90 plays a role during and after thermal stresses by refolding and repairing denatured proteins. Hence, HSP90 can be used as a molecular marker gene for temperature stress conditions in shrimp. Moreover, the expressions of *Mj*HSP40, *Mj*HSP70 and *Mj*HSP90 after WSSV challenge were increased in the hemocytes and lymphoid organ (Fig. 4). The expression profiles of *F. chinensis* HSP70 and HSP90 were also upregulated after infection with WSSV [51]. Therefore, the expressions of *Mj*HSP40, *Mj*HSP70 and *Mj*HSP90 after WSSV challenge suggest that these proteins may play important roles in the immune function of shrimp against viruses. However, the expressions of these three *MJ*HPS did not show any apparent change in the hepatopancreas (Fig. 4). EST analysis of cDNA libraries derived from shrimp hemocytes and hepatopancreas showed differences in the overall patterns of gene expression, including those of HSPs, suggesting that they have different functional roles in shrimp [52], which could explain why the expression patterns of *MJ*HSPs in the two tissues are different.

In conclusion, HSP40 (*Mj*HSP40), HSP70 (*Mj*HSP70) and HSP90 (*Mj*HSP90) from kuruma shrimp were characterized. A J domain, a G/F-rich region, and a central domain containing four repeats of a CxxCxGxG motif were found in the predicted *Mj*HSP40 amino acid sequence. The conserved signature sequences of the HSP70 and HSP90 gene families were also found in the *Mj*HSP70 and *Mj*HSP90 amino acid sequences, respectively. The expression profiles of *Mj*HSP40, *Mj*HSP70 and *Mj*HSP90 mRNAs were also examined after heat shock and WSSV challenge treatment. Our results show that only *Mj*HSP90 mRNA levels were significantly induced by heat shock at 32°C, and that the expressions of *Mj*HSP40, *Mj*HSP70 and *Mj*HSP90 increased after WSSV injection.

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