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Cross talk between heat shock protein 10 and a heat shock factor identified from *Marsupenaeus japonicus*

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ABSTRACT

Heat shock factors (HSFs) and heat shock proteins (HSPs) are crucial regulators and effectors of the heat shock response (HSR). In this study, the full-length cDNA sequences of MjHSP10 and MjHSF1 were cloned by rapid amplification of cDNA ends (RACE). The deduced MjHSP10 and MjHSF1 amino acid (aa) sequences exhibited conserved structures and the functional features of HSP10 and HSF1, respectively. The tissue distributions and mRNA expression profiles of the two genes in response to heat stress were analyzed by quantitative real-time PCR (qRT-PCR). MjHSP10 and MjHSF1 were ubiquitously expressed in various tissues. Heat stress induced a significant increase in MjHSP10 expression that tend to positively correlate with temperature. Additionally, MjHSF1 transcription was up-regulated less than MjHSP10 transcription under heat stress. MjHSF1 expression in the hepatopancreas was up-regulated under only long-term (48 h) heat stress, and MjHSF1 transcription in the gill increased under only acute (34 °C) heat stress. MjHSF1 knockdown by RNA interference (RNAi) down-regulated MjHSP10 expression. Glutathione-S-transferase (GST) pull-down assays showed an interaction between MjHSP10 and the DNA-binding domain (DBD) of MjHSF1. This study provided new insights into cross talk between HSP10 and HSF1 in *Marsupenaeus japonicus*.

1. Introduction

Temperature is one of the most crucial environmental factors for organisms, therefore, a vital issue for organisms is how to address sudden changes in habitat temperature. It has become clear that organisms share a common adaptation mechanism termed the heat shock response (HSR) to cope with temperature-induced stress that results in a dramatic change in gene expression patterns and leads to the elevated synthesis of a battery of molecular chaperones and the induction of other cell-protective pathways [1–4]. The heat shock factor (HSF) and heat shock protein (HSP) mediated regulation pathway plays crucial roles in the HSR, and has received a great deal of attention in studies on HSR mechanisms and the thermotolerance of organisms [4–7].

HSPs are important effector molecules in the HSR, that act as molecular chaperones to maintain protein homeostasis via promoting the degradation of abnormal or damaged proteins, preventing protein aggregation and denaturation, repairing and refolding denatured or misfolded proteins [8–10]. Based on their sequence homology and molecular mass, HSPs are classified into several families: the HSP110, HSP100, HSP90, HSP70, and HSP60 families and the small HSPs (sHSPs) [11]. Members of the sHSP family, which have a molecular mass ranging from 15 to 30 kDa, are virtually ubiquitous molecular chaperones that prevent the irreversible aggregation of denatured or misfolded proteins as well as serve as co-chaperonins for other HSPs, forming a first line of defense against protein aggregation and maintaining protein homeostasis [12–16]. HSP10, also known as chaperonin 10 (cpn10), the eukaryotic homolog of phage growth E small (GroES) in *Escherichia coli*, acts as a co-chaperone for HSP60 in protein folding as well as the assembly and disassembly of protein complexes involved in various biological processes and plays a pivotal role in the organismal defense mechanism [17–20].

HSFs are crucial regulatory molecules in the HSR that mediate transcriptional regulation of the HSR [21–24]. Among HSF family members, HSF1 is the principal transcriptional factor required for regulation of the HSR and plays an indispensable role in the HSR [6,7]. Heat stress induces the increased expression of HSF1 as well as assembly of inactive HSF1 monomers into the active, functional HSF1 homotrimer [25]. The HSF1 trimer can bind specifically to the

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heat shock element (HSE) in the promoters of various HSPs and initiate the transcription of these genes. Meanwhile, HSF1 activity is feedback controlled by HSPs via direct interactions between HSF1 and HSPs, which block the DNA-binding and transactivation activities of HSF1 [26–30].

The kuruma shrimp *Marsupenaeus japonicus* is an important commercial species widely distributed throughout the Indo-West Pacific [31,32] and mainly cultured in China [33]. *M. japonicus* exhibits relatively poorer thermotolerance compared to other penaeids [34], consequently, the mass mortality of *M. japonicus* frequently occurs during summer months, resulting in severe economic losses. Hence, there is an urgent desire to obtain a better understanding of the HSR in *M. japonicus*. Although studies to elucidate the mechanisms underlying the HSR in *M. japonicus* have been conducted [35–37], the transcriptional regulatory mechanism of the HSR in this thermally sensitive taxon remains largely unclear. Herein, the full-length cDNA sequences of HSP10 and HSF1 in *M. japonicus* were cloned and characterized. Furthermore, changes in the transcription of these two genes in response to heat stress and crosstalk between the two genes were investigated. This study enriches our knowledge on the bidirectional regulatory mechanism between HSF1 and HSP and provides new insights into transcriptional regulation of the HSR in *M. japonicus*.

2. Materials and methods

2.1. Animals

All *M. japonicus* prawns were obtained from an aquaculture farm in Dongshang (Zhangzhou, Fujian, China) and acclimated in environmentally controlled rectangular tanks (70 cm × 50 cm × 40 cm) in aerated seawater at a salinity of 28‰ before experimentation. The seawater was renewed daily, and the prawns were fed twice daily with commercial pellets.

2.2. Cloning of the full-length cDNA sequences of HSP10 and HSF1 in *M. japonicus*

Total RNA was extracted using RNAiso Plus (TaKaRa, Japan) following the manufacturer's instructions. The purity of the total RNA was detected by a Quawell UV–vis spectrophotometer Q5000 (Quawell, USA) at A260 nm and A280 nm, and RNA integrity was determined by analysis on a 1.0% (w/v) agarose gel. Reverse transcription to synthesize first-strand cDNA was conducted using EasyScript One-Step gDNA Removal and cDNA Synthesis SuperMix (TransGen Biotech, Beijing, China) following the manufacturer's instructions. MjHSP10 and MjHSF1 cDNA fragments were PCR-amplified with primers designed based on the partial cDNA sequences of the two genes identified from the transcriptome of *M. japonicus* by Primer Premier 5.0 software. PCR amplification was performed using the following procedure: one initial denaturing step of 5 min at 95 °C, followed by 35 cycles of 95 °C for 30 s, 58 °C for 30 s, and 72 °C for 2 min, and a final extension step at 72 °C for 10 min. The amplified products were ligated into the pMD19-T vector (TaKaRa) and sequenced in both directions. The sequences were identified using the BLAST program (http://www.ncbi.nlm.gov/blast).

After determination of the partial cDNA sequences, the full-length MjHSP10 and MjHSF1 cDNA sequences were obtained by rapid amplification of cDNA ends (RACE) using a SMARTer™ RACE cDNA Amplification Kit (TaKaRa) according to the manufacturer's protocols. Sequences of the primers used for various PCR experiments are listed in Table 1. The first round of the 3′ RACE and 5′ RACE reactions was conducted under the following conditions: 95 °C for 5 min, followed by 30 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min 30 s, and a final extension step at 72 °C for 10 min. Secondary nested PCR was then conducted using the primary PCR product as the template with the following amplification conditions: 95 °C for 5 min, followed by 30 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min, and a final extension step at 72 °C for 10 min. The amplified products were cloned into the pMD19-T vector (TaKaRa) and identified as described above.

2.3. Bioinformatics analysis

The open reading frames (ORFs) of the MjHSP10 and MjHSF1 cDNA sequences were predicted using ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The amino acid (aa) sequences were deduced by DNAMAN software. Protein molecular masses and isoelectric points (pIs) were predicted using the ProtParam program (http://web.expasy.org/protparam/). The deduced aa sequences were determined using BioEdit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Functional domains were predicted by InterPro (http://www.ebi.ac.uk/interpro/) and SMART (http://smart.embl-heidelberg.de/). Multiple sequence alignment was performed using ClustalW and the multiple alignment display program ESPript3.0 (http://espript.ibcp.fr/ESPript/ESPript/). Neighbor-joining phylogenetic trees were constructed by MEGA 5.05 with 1000 bootstrap replicates.

2.4. Analysis of MjHSP10 and MjHSF1 tissue expression

The expression profiles of MjHSP10 and MjHSF1 in various tissues (hepatopancreas, gill, hemocytes, muscle, heart, stomach, intestine and eyestalk) from six prawns were individually tested with three technical replicates by quantitative real-time RT-PCR (qRT-PCR). QRT-PCR was performed on an Applied Biosystems QuantStudio 6 Flex Real-time PCR System (Applied Biosystems, USA) using TB Green® Premix DimerEraser™ (Perfect Real Time) (2×) (TaKaRa) according to the manufacturer's instructions. *M. japonicus* elongation factor 1-α (EF1-α) served as the reference for internal standardization. Sequences of the specific primers used for qRT-PCR are listed in Table 1. QRT-PCR was conducted in a total volume of 20 μL containing 10 μL of TB Green® Premix DimerEraser™ (2×) (TaKaRa), 0.6 μL of each primers (10 mM), 0.4 μL of Rox Reference Dye II (50×), 2 μL of cDNA, and 6.4 μL of DNase/RNase-Free water. The PCR cycling conditions were as follows: 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s, 60 °C for 30 s and 72 °C for 30 s. Melting curve analysis was performed at the end of the reaction to confirm that only one PCR product had been amplified and was detected. The slopes and regression curves of the standard curves were calculated to determine the PCR efficiency (E) with 7 tenfold template dilutions. E-values were calculated according to the equation $E = 10^{(-1/\text{slope})} - 1$. The $2^{-\Delta\Delta Ct}$ method was used to calculate fold-changes in gene expression relative to the control [38]. All data are presented as the mean ± standard deviation (SD). Statistical significance between groups was determined using one-way analysis of variance (ANOVA) with SPSS 17.0 software. A p-value<0.05 indicated a statistically significant difference.

2.5. Expression patterns of MjHSP10 and MjHSF1 in *M. japonicus* under heat stress

M. japonicus weighing 4.45 ± 1.18 g were used in the heat stress experiment. A gradient of four temperatures (28 °C, 30 °C, 32 °C and 34 °C) was used with fifty shrimp per group. Shrimp were transferred from the acclimation tanks (28 °C) to tanks containing seawater preheated to 28 °C (control group), or 30 °C, 32 °C or 34 °C

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Table 1
Primer sequences used in the present study.

Primer name	Primer sequence (5′-3′)	Target
MjHSP10-F	AAATGGCTGGTGCTCTGA	cDNA fragment
MjHSP10-R	ACTCGCTCTTCATCTTGG	cDNA fragment
MjHSF1-F	TGTATGGGTTTCACAAGGTA	cDNA fragment
MjHSF1-R	ATCCGTATTGAACATCGTAA	cDNA fragment
MjHSP10-5′-Out	TCCCTCTCCTACAGCAACCAC	5′-RACE PCR
MjHSP10-5′-In	GGACCTTGGCTACCGACTTCT	5′-RACE PCR
MjHSP10-3′-Out	AGGACCGATGCTGGCACTAC	3′-RACE PCR
MjHSP10-3′-In	TGATGCTCCCTGAGTTTGGC	3′-RACE PCR
MjHSF1-5′-Out	GTGCGGCTCGTTGGTATCT	5′-RACE PCR
MjHSF1-5′-In	GAAAGTGTGGGTGGGCGAA	5′-RACE PCR
MjHSF1-3′-Out	AAGAGCCAGAAAACCCCAC	3′-RACE PCR
MjHSF1-3′-In	ACCACCATTGACTCGCTGC	3′-RACE PCR
MjHSP10-RT-F	TGAGTTGAAAATGGCTGGTGC	qRT-PCR
MjHSP10-RT-R	GTCCTGGCTCCCTCTCCTACA	qRT-PCR
MjHSF1-RT-F	ATGGCAAGTTTTGTGAGGCAG	qRT-PCR
MjHSF1-RT-R	GAAAGTGTGGGTGGGCGAA	qRT-PCR
EF1-α-F	GGAACTGGAGGCAGGACC	qRT-PCR
EF1-α-R	AGCCACCGTTTGCTTCAT	qRT-PCR
His-DBD-F	AATGTCCCGGCGTTTCTGA	protein expression
His-DBD-R	TTACTTTCTCTTTATGTTCTCAATGAGG	protein expression
His-TAD-F	ACTGAAGAAAACACAGAATTCCCA	protein expression
His-TAD-R	TTAATCCGTATTGAACATCGTAAGCAG	protein expression
GST-HSP10-F	CGCGGATCCATGGCTGGTGCTCTGAAGAGGT	protein expression
GST-HSP10-R	CCGGAATTCTTACTCGCTCTTCATCTTGGCCA	protein expression
dsMjHSF1-T7-F	GATCACTAATACGACTCACTATAGGGACATCTTCACCGTGTCCGAA	RNAi
dsMjHSF1-T7-R	GATCACTAATACGACTCACTATAGGGGGTGGGGTTTTCTGGCTCTT	RNAi
dsEGFP-T7-F	GATCACTAATACGACTCACTATAGGGACCCTCGTGACCACCCTGAC	RNAi
dsEGFP-T7-R	GATCACTAATACGACTCACTATAGGGTCTCGTTGGGGTCTTTGCTC	RNAi

(heat stress groups), respectively. During the heat stress period, the hepatopancreases and gills of five individual shrimp were collected at 0 h (prior to heat stress treatment, unstressed), 3 h, 6 h, 12 h, 24 h, 48 h, 72 h and 96 h. All samples were immediately frozen at −80 °C until the extraction of total RNA for analysis. The expression patterns of MjHSP10 and MjHSF1 in the hepatopancreases and gills of *M. japonicus* under heat stress were analyzed by qRT-PCR as described above.

2.6. Knockdown of MjHSF1 and its effect on MjHSP10 expression

2.6.1. Preparation of double-stranded RNAs (dsRNAs)

The DNA templates of MjHSF1 dsRNA (dsMjHSF1) and enhanced green fluorescent protein (EGFP) dsRNA (dsEGFP) were amplified by PCR with primers containing the T7 RNA polymerase binding site at the 5′ terminus (Table 1), and products with the T7 promoter were confirmed by sequencing. The dsRNAs were generated using an in vitro Transcription T7 Kit (for siRNA Synthesis) (TaKaRa) following the manufacturer's protocols. The in vitro transcriptional products were incubated with RNase-free DNase I (5 U/μL) and RNase T1 (4 U/μL) for 2 h at 37 °C and subjected to phenol/chloroform extraction and isopropanol precipitation. The purified dsRNAs were quantified, and their concentrations were adjusted to 200 ng/μL.

2.6.2. Efficiency of in vivo MjHSF1 knockdown by RNA interference (RNAi)

M. japonicus weighing 10.45 ± 1.25 g were used for the RNAi experiment. Shrimp from three groups of fifteen shrimp each, the dsMjHSF1 injection group, dsEGFP injection group (negative control) and saline injection group (blank control), were intramuscularly injected with 25 μL of dsMjHSF1, dsEGFP and saline, respectively. The hepatopancreases, hemocytes, and hearts of six randomly sampled shrimp from each group were collected at 24 h and 48 h post injection (hpi), and the gene silencing efficiency was examined by qRT-PCR as described above.

2.6.3. Expression of MjHSP10 in MjHSF1 silenced M. japonicus

Based on the RNAi silencing efficiency, the levels of MjHSP10 transcripts in hemocytes at 24 hpi and 48 hpi were analyzed to investigate the effect of MjHSF1 knockdown on MjHSP10 expression.

2.7. Expression of recombinant proteins

The cDNA fragment encoding MjHSP10 peptide was amplified using primers with the BamH I and EcoR I restriction sites (Table 1). The target cDNA fragment was ligated into the pGEX-4T-1 expression vector with a glutathione-S-transferase (GST)-tag. cDNA fragments encoding the DNA-binding domain (DBD) and transactivation domain (TAD) of MjHSF1 were amplified using TransStart® FastPfu DNA polymerase (TransGen Biotech, China) and ligated into the pEASY®-Blunt E1 expression vector (TransGen Biotech, China) with a hexahistidine-tag. The recombinant plasmids were confirmed by sequencing, transformed into *E. coli* BL21 (DE3) and induced at 18 °C for 12 h with isopropyl-β-thioga lactopyranoside (IPTG) at a final concentration of 0.1 mM. The induced bacteria were collected by centrifugation (10,000*g*) for 10 min at 4 °C, and then suspended in 0.1 M phosphate buffered saline (PBS) and lysed. The supernatants were collected by centrifugation (10,000*g*) for 30 min at 4 °C. The recombinant GST-HSP10, His-DBD and His-TAD proteins in the supernatants were detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analysis. Briefly, proteins in the supernatants were separated via SDS-PAGE and then transferred onto polyvinylidene fluoride (PVDF) membranes. The PVDF membranes were blocked with 5% skimmed milk dissolved in PBST (0.1 M PBS, 0.1% Tween 20, pH 7.4) for 3 h at room temperature, followed by incubation with mouse anti-GST primary antibody (1:1000, TransGen Biotech, China) or mouse anti-His primary antibody (1:1000, TransGen Biotech, China) for 2 h. The PVDF membranes were then washed three times with PBST and incubated with HRP-linked goat anti-mouse secondary antibody (1:1000, TransGen Biotech, China) for 2 h, and immunoreactive bands were

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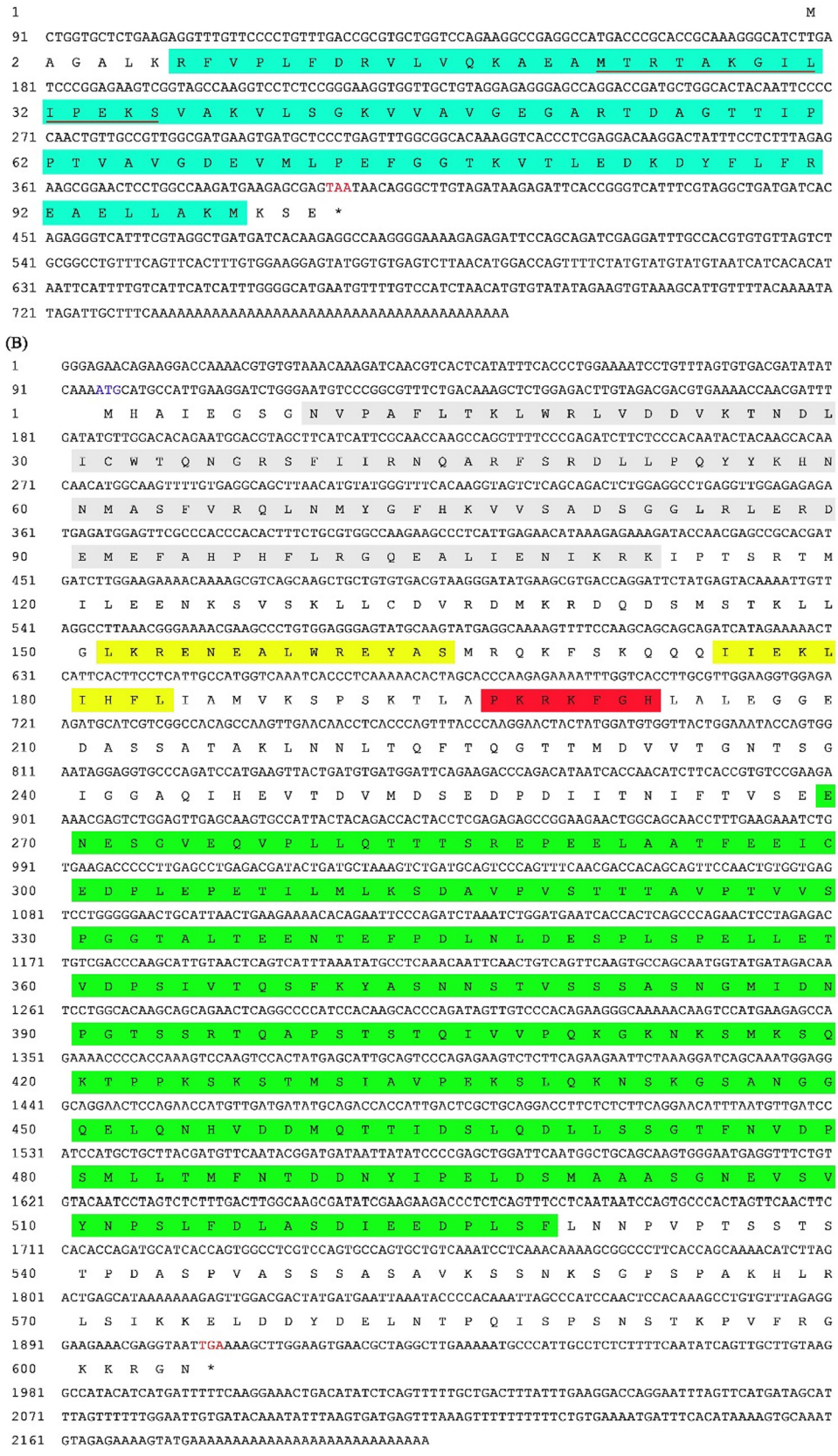

Fig. 1. Complete nucleotide and deduced aa sequences of MjHSP10 (A) and MjHSF1 (B) cDNA in *M. japonicus*. Blue letters indicate the start site, while red letters represent the stop codon. The cpn10 domain is shaded in cyan and the mobile loop is underlined in red. The functional domains in HSF1 (DBD, OD, NLS and TAD) are highlighted in gray, yellow, red and green, respectively.

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visualized using an EasySee® Western Blot Kit (TransGen Biotech, China) and a Tanon 5200S chemiluminescence imaging system (Tanon Science & Technology Co., Ltd., China).

2.8. GST pull-down assay

A GST pull-down assay was performed as previously described with slight modification [39]. The lysate supernatants of induced bacteria that expressed GST-HSP10 and the negative control protein GST were incubated with glutathione-coupled sepharose beads (TransGen Biotech, China) for 2 h at 4 °C with gentle rocking. Then, the beads were washed 7 times with 1 mL of wash solution (0.1 M PBS and 1‰ Triton X-100) to remove unbound proteins. The lysate supernatants of induced bacteria that expressed His-DBD and His-TAD were incubated with beads coupled with GST-HSP10 or GST, respectively, at 4 °C for 4 h with gentle rotation. Then, the beads were washed 7 times with 1 mL of wash solution (0.1 M PBS and 1‰ Triton X-100), resuspended in 2 × SDS-PAGE loading buffer and analyzed by SDS-PAGE and western blotting as described above.

3. Results

3.1. Sequence analysis of MjHSP10 and MjHSF1

The full-length cDNA of MjHSP10 (GenBank accession number: MK628599) was 765 bp long and contained a 5′-untranslated region (UTR) of 86 bp, a 3′-UTR of 370 bp and an ORF of 309 bp. The ORF encoded a polypeptide of 102 aa with a predicted molecular weight of 10.95 kDa and a theoretical pI of 8.42. The deduced aa sequence of MjHSP10 contained the conserved features of HSP10: a cpn10 domain (R^{7}-M^{99}) and a mobile loop structure (^{23}-MTRTAKGILIPEKS^{36}) (Fig. 1A). The full-length cDNA of MjHSF1 (GenBank accession number: MK628600) spanned 2203 bp and comprised a 5′-UTR of 94 bp, a 3′-UTR of 294 bp and an ORF of 1815 bp encoding a polypeptide of 604 aa. The predicted molecular weight of MjHSF1 was 66.19 kDa, and its theoretical pI was 5.11. The deduced aa sequence of MjHSP10 exhibited a typical HSF structure containing a DBD, oligomerization domain (OD) composed of hydrophobic heptad repeats (HR-A/B), nuclear localization signal (NLS) and TAD (Fig. 1B).

3.2. Multiple sequence alignment

Homology comparisons showed that the deduced aa sequences of MjHSP10 and MjHSF1 are highly similar to their counterparts from other species (Fig. 2). MjHSP10 shared high aa sequence identity with HSP10 proteins from other crustaceans, such as *Litopenaeus vannamei* (92.1%), *Penaeus monodon* (90.1%) and *Scylla paramamosain* (79.4%). Multiple sequence alignment showed that the cpn10 domain of MjHSP10 is well conserved among various species (Fig. 2A). MjHSF1 shared high aa sequence identity with HSF1 from *L. vannamei* (87.4%) and relatively lower identity (19.8%~24.1%) with that from *Daphnia magna*, *Crassostrea gigas*, *Haliotis diversicolor*, *Apostichopus japonicus* and *Danio rerio*. Multiple sequence alignment revealed that the DBD and OD of HSF1 proteins from different species are well conserved, while the NLS and TAD are more varied (Fig. 2B).

3.3. Phylogenetic analysis

Phylogenetic analysis showed that MjHSP10 clustered with HSP10 proteins from other crustaceans and was closely phylogenetically related to HSP10 from *P. monodon* and *L. vannamei* (Fig. 3A). MjHSF1 was closely phylogenetically related to HSF1 from *L. vannamei*, as supported by a bootstrapping value of 100.

3.4. Tissue distribution of MjHSP10 and MjHSF1

Tissue distribution analysis showed that MjHSP10 and MjHSF1 were ubiquitously expressed in the hepatopancreas, gill, hemocytes, stomach, intestine, heart, muscle and eyestalk (Fig. 4). MjHSP10 exhibited relatively higher expression levels in the hepatopancreas, gill, stomach and heart compared to those in hemocytes, the intestine, muscle and eyestalk (Fig. 4A). MjHSF1 was mainly expressed in the heart, intestine, hemocytes and stomach (Fig. 4B).

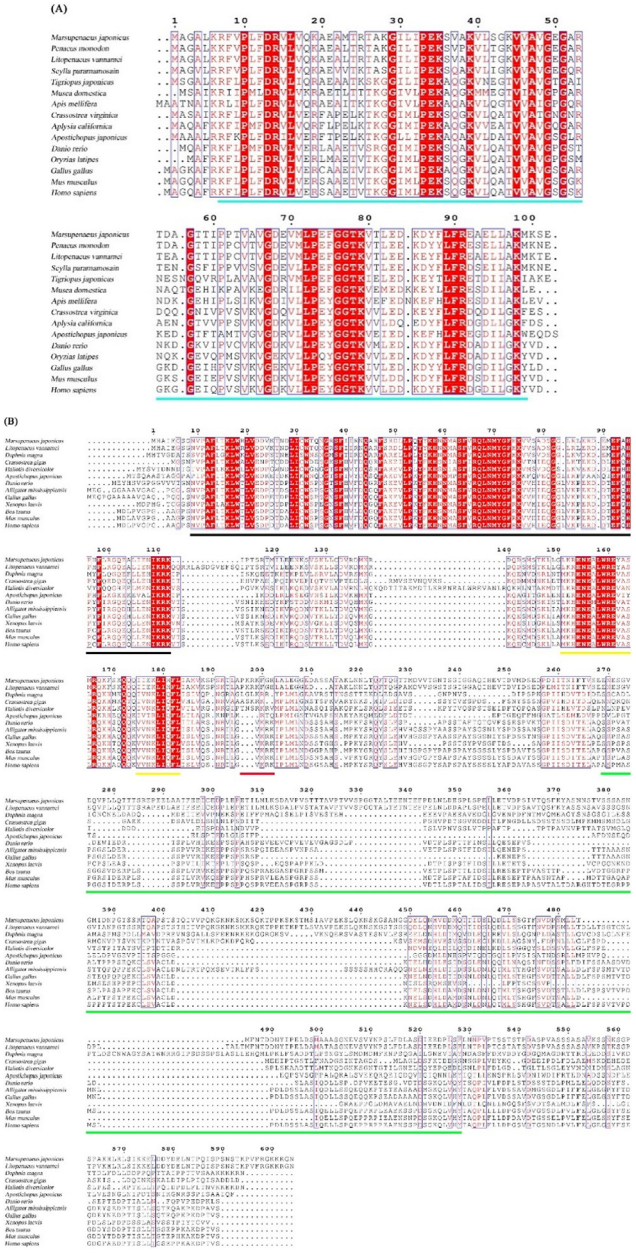

Fig. 2. Multiple sequence alignment of the deduced aa sequences of MjHSP10 (A) and MjHSF1(B) with other known HSP10 and HSF1 sequences. Highly conserved aa residues are indicated by blue boxes, and identical residues are shaded red. The cpn10 domain is underlined in cyan, the DBD is underlined in black, the OD is underlined in yellow, the NLS is underlined in red, and the TAD is underlined in green.

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3.5. Expression profiles of MjHSP10 and MjHSF1 in response to heat stress

Significant increases in MjHSP10 expression levels were observed in both the hepatopancreas and gill of *M. japonicus* under heat stress, and this up-regulated expression tended to positively correlate with temperature (Fig. 5). The mRNA expression levels of MjHSP10 in the hepatopancreases of shrimp exposed to water temperatures of 32 °C and 34 °C both peaked at 3 h and were increased by 4.87-fold and 9.83-fold, respectively ($p < 0.05$) (Fig. 5A). The transcriptional level of MjHSP10 in the hepatopancreases of shrimp at 32 °C gradually returned back to basal levels after peaking, however, it was increased throughout the heat stress period and tended to be decreased in hepatopancreases of shrimp exposed to 34 °C (Fig. 5A). MjHSP10 transcription in the gills of shrimp at 32 °C and 34 °C was significantly up-regulated at most time points and peaked at 96 h and 3 h with 3.09-fold and 3.60-fold increases ($p < 0.05$), respectively. The mRNA expression levels of MjHSF1 in the hepatopancreases of shrimp exposed to water temperatures of 30 °C, 32 °C and 34 °C were significantly elevated at only 48 h for all heat stress groups and increased by 2.43-fold, 2.55-fold and 4.08-fold, respectively, compared with the control

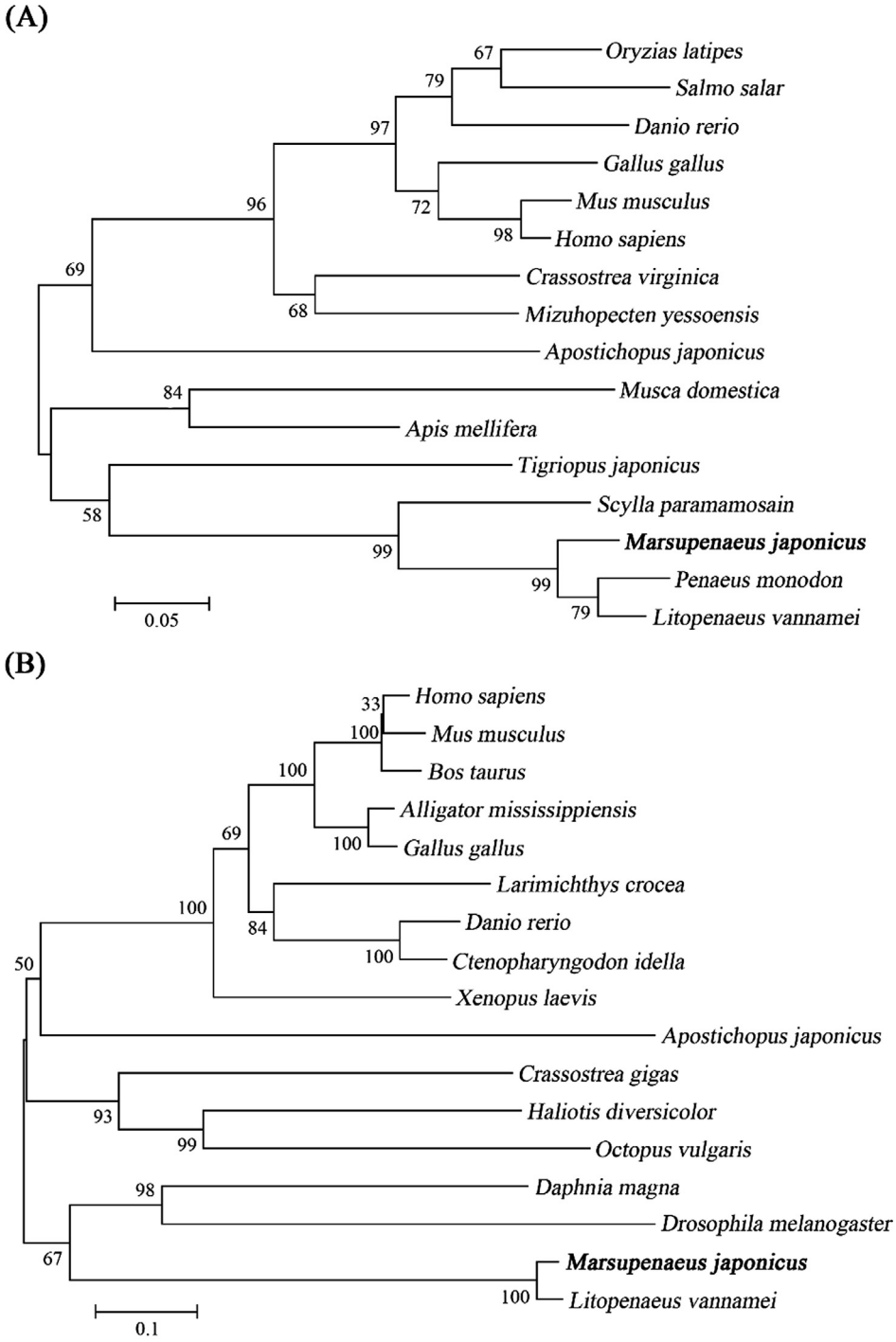

Fig. 3. Phylogenetic analysis of MjHSP10 (A) and MjHSF1 (B). The tree topology was constructed by the neighbor-joining method and evaluated by 1000 replication bootstraps with MEGA 5.0 based on deduced aa sequences alignments with ClustalW. The numbers at the forks indicate the bootstrap proportions.

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group ($p < 0.05$). The mRNA expression levels of MjHSF1 in the gills were up-regulated when shrimp were exposed to water temperatures of only 34 °C for only 3 h and 24 h and increased by 2.18-fold and 1.96-fold ($p < 0.05$), respectively.

3.6. RNAi-mediated repression of MjHSF1 transcription and its effect on MjHSP10 expression

MjHSF1 transcription in hemocytes was highly repressed by 5.40-fold ($p < 0.05$) at 24 hpi after RNAi, however, MjHSF1 transcription was not suppressed in the hepatopancreas or heart (Fig. 6). MjHSF1 transcription in hemocytes was inhibited and decreased by 2.05-fold ($p < 0.05$) at 48 hpi after RNAi, while transcription of MjHSF1 in the heart was also repressed by 2.23-fold ($p < 0.05$) (Fig. 6). In the hemocytes of MjHSF1-silenced *M. japonicus*, the expression level of MjHSP10 was significantly decreased by 2.30-fold at 48 hpi after RNAi ($p < 0.05$) (Fig. 7).

3.7. Analysis of MjHSP10 and MjHSF1 protein interaction by GST pull-down assay

To investigate the interaction between the MjHSP10 and MjHSF1 proteins, the GST, GST-HSP10, His-DBD and His-TAD recombinant proteins were produced in an *E. coli* expression system. SDS-PAGE and western blot analysis showed that the recombinant proteins exhibited the expected sizes of approximately 26 kDa (GST), 37 kDa (GST-HSP10), 12 kDa (His-DBD) and 17 kDa (His-TAD) (Fig. 8). The interactions between MjHSP10 and the DBD or TAD were assessed by an in vitro pull-down assay. The His-DBD protein was specifically bound to GST-HSP10 but not to the GST protein, however, the His-TAD protein was bound to neither the GST-HSP10 nor GST protein (Fig. 9). These results indicated that the GST-HSP10 protein can specifically bind to the DBD of the MjHSF1 protein in vitro.

4. Discussion

HSPs and HSFs are vital effectors and regulatory molecules in the HSR, and the basis for organismal thermotolerance. In the current study, the full-length cDNA sequences of MjHSP10 and MjHSF1 were identified and characterized. The deduced aa sequence of MjHSP10 contains a well conserved cpn10 domain (R^{7}-M^{99}) and mobile loop structure (^{23}MTRTAKGILIPEKS^{36}) that interacts with HSP60 to form a protein folding machine [19,40]. The deduced aa sequence of MjHSF1 contains a highly conserved N-terminal DBD that binds specifically to HSEs in HSP promoters and a C-terminal TAD. Additionally, MjHSF1 contains an OD reported to be essential for trimerization and an NLS indispensable for nuclear entry [3,41,42]. Homology analysis suggested that MjHSP10 and MjHSF1 share the highest aa identity with their counterparts from *L. vannamei*, which is closely phylogenetically related to *M. japonicus*. Dendrograms constructed by phylogenetic analysis of MjHSP10 and MjHSF1 were basically in agreement with the concept of traditional taxonomy.

In previous studies, HSP10 and HSF1 were found to be expressed in various tissues in marine invertebrate species and exhibit species-specific tissue distribution profiles [43–47]. In the present study, tissue distribution analysis showed that MjHSP10 and MjHSF1 are ubiquitously expressed in all examined tissues, indicating that MjHSP10 and MjHSF1 could be involved in various biological processes and play important roles in supporting basic life activities.

As major stress proteins in cells, HSPs function as molecular chaperones to maintain protein homeostasis and assist organisms in dealing with internal or external stress that are constitutively expressed in normal conditions and over-expressed upon various environmental stresses [48]. In this study, MjHSP10 was significantly up-regulated at the mRNA level, and the transcription of MjHSP10 in both the hepatopancreas and gill rapidly responded to heat stress within 3 h, indicating its potential biological function in resisting heat stress. Notably, the response of MjHSP10 was much more pronounced than that of other HSPs (MjHSP60, MjHSP70 and MjHSP90) in *M. japonicus*, as we previously reported [37]. The fast and robust increase in MjHSP10 implies the significant role of MjHSP10 in providing cellular protection under heat stress. HSP10 acts as not only a chaperone to prevent the irreversible aggregation of proteins but also a co-chaperonin with HSP60 that forms a protein folding machine, which may lead to heavy demand for HSP10 under stress. Due to their inducible expression and sensitivities to a wide range of environmental stressors, HSPs are biomarkers for environmental monitoring and assessment [49–52]. Our results revealed that the transcription of MjHSP10 in the hepatopancreas quickly and dramatically increased with increasing temperature, and MjHSP10 up-regulated tend to positively correlate with temperature. The rapid and temperature-dependent induction of MjHSP10 in the hepatopancreas under heat stress indicated that MjHSP10 may be a promising candidate indicator gene to assess the heat stress status of *M. japonicus*.

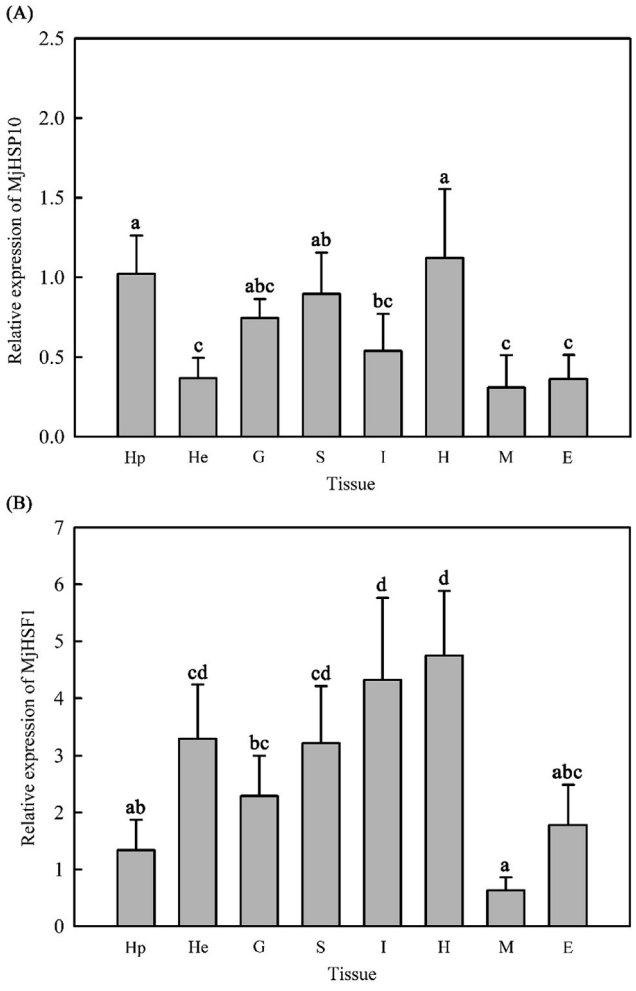

Fig. 4. Tissue distributions of MjHSP10 (A) and MjHSF1 (B) mRNA in *M. japonicas*. Expression levels in hemocytes (He), the gill (G), stomach (S), intestine (I), heart (H), muscle (M) and eyestalk (E) are normalized to those in the hepatopancreas (Hp). Each bar represents the mean ± S.D (n = 5). Significant difference between groups at $p < 0.05$ (n = 5, ANOVA) are indicated by different letters above the bars.

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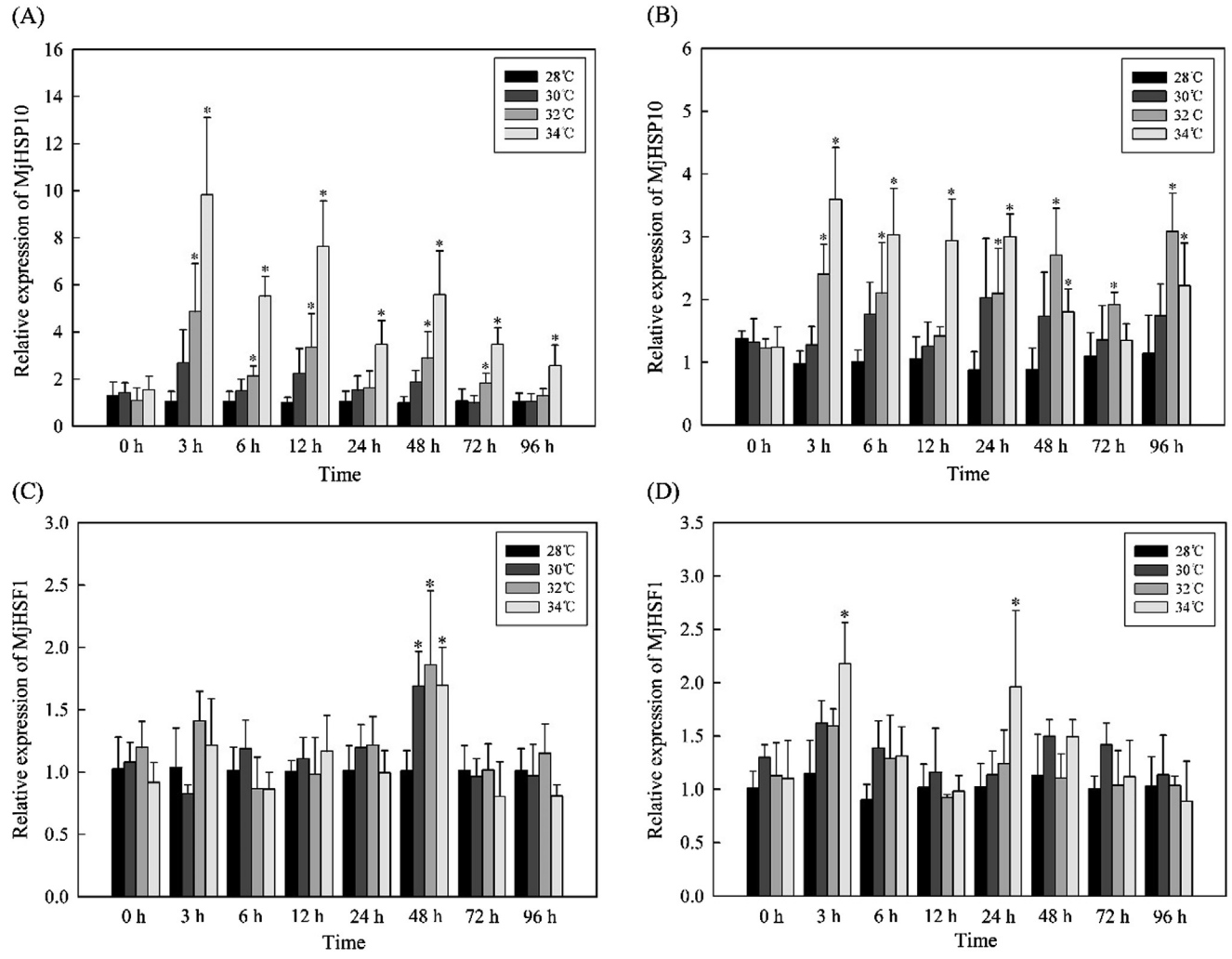

Fig. 5. Expression profiles of MjHSP10 and MjHSF1 in the hepatopancreas (A and C) and gill (B and D) of *M. japonicus* under heat stress. Each bar represents the mean ± SD (n = 5). Significant differences between the control group and the high temperature stress groups are indicated by * above the bars (ANOVA, $p < 0.05$).

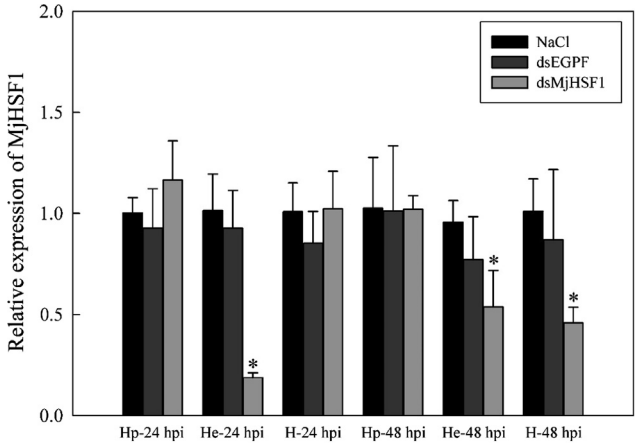

Fig. 6. Efficiency of MjHSF1 silencing mediated by dsMjHSF1. Hp: hepatopancreas; He: hemocytes; H: heart. Each bar represents the mean ± SD (n = 6). Significant differences between the control group and the dsMjHSF1 injection group are indicated by * above the bars (ANOVA, $p < 0.05$).

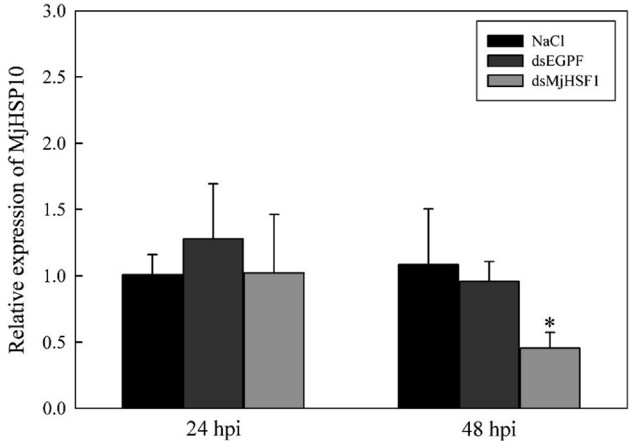

Fig. 7. Expression of MjHSP10 in *M. japonicus* hemocytes after in vivo gene silencing of MjHSF1. Each bar represents the mean ± SD (n = 6). A significant difference between the control group and the dsMjHSF1 injection group is indicated by * above the bars (ANOVA, $p < 0.05$).

HSF1 is an essential member of the HSF family that undergoes increased transcription or post-transcriptional regulation under heat stress. In several aquatic organisms (the tiger shrimp (*Penaeus monodon*) [53], the abalone (*Haliotis diversicolor*) [43], the common octopus (*Octopus vulgaris*) [54] and the sea cucumber (*A. japonicus*) [44]), HSF1 transcription was significantly induced by heat stress,

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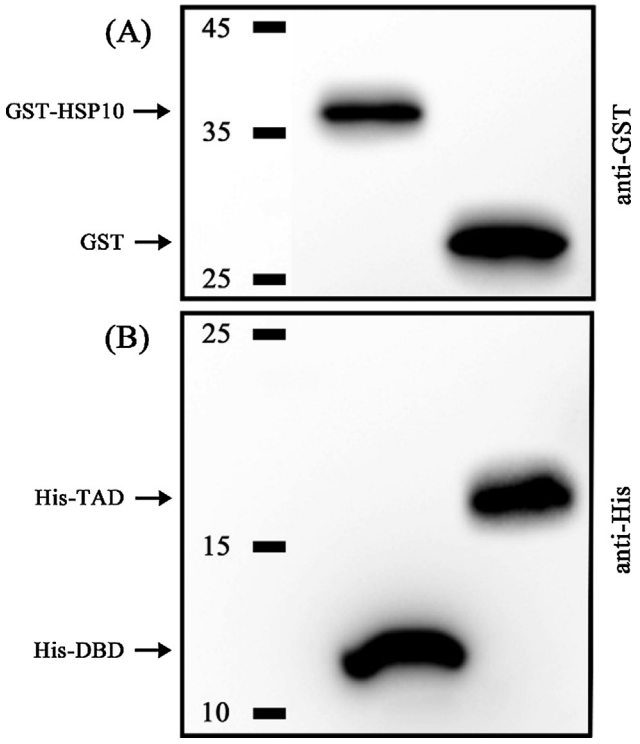

Fig. 8. Western blot analysis of the recombinant proteins.

suggesting the critical roles of HSF1 in coping with heat stress in aquatic organisms. In the current study, MjHSF1 transcription remained at its basal level during heat stress and was up-regulated to a lesser extent than MjHSP10 transcription, which may be because the regulation of HSF1 under moderate/short-term heat stress primarily involves a conversion that affects its activity, such as its conversion from a monomer-to-trimer or phosphorylation, rather than a change in its synthesis [22]. In contrast, HSF1 expression was increased under acute/long-term heat stress (Fig. 10A). These results indicated that distinct regulatory strategies might be employed by *M. japonicus* to cope with different degrees of heat stress.

HSF1 is a master transcriptional regulator in the HSR that induces the transcription of HSP genes that function as molecular chaperones to protect organisms from the deleterious consequences of insults that induce protein damage [55,56]. In the current study, the potential function of MjHSF1 in regulating MjHSP10 expression was investigated by RNAi-mediated gene silencing. RNAi is a sequence-specific posttranscriptional gene silencing technology using double-stranded RNA that has been widely applied in gene function studies [57–59]. Previous studies have revealed that HSF1 knockdown by RNAi-mediated gene silencing leads to the down-regulation of various HSPs in *L. vannamei* and *P. monodon* [45,53]. In this study, injection of sequence-specific dsMjHSF1 resulted in the significant repression of MjHSF1 at the transcriptional level in hemocytes at 24 hpi and 48 hpi, however, MjHSF1 transcription in the heart was inhibited at only 48 hpi, and MjHSF1 in the hepatopancreas was not inhibited. The dsRNA injected into shrimp may have entered the circulatory system, where it directly and frequently contacted hemocytes, thus, RNAi-mediated silencing was remarkably efficient in hemocytes. The RNAi-mediated knockdown of MjHSF1 expression significantly suppressed the transcription of MjHSP10, indicating that MjHSF1 plays an essential role in regulating MjHSP10 expression.

Interestingly, HSPs have also been shown to act as negative feedback regulators of HSF1 and play key roles in the auto-regulation of the HSR. The discovery of interactions between HSF1 and HSPs, such as Hsp70 and HSP90, indicates a negative-feedback loop, by which excess HSPs under non-stress conditions keep HSF1 inactive. Earlier studies found that the molecular chap-

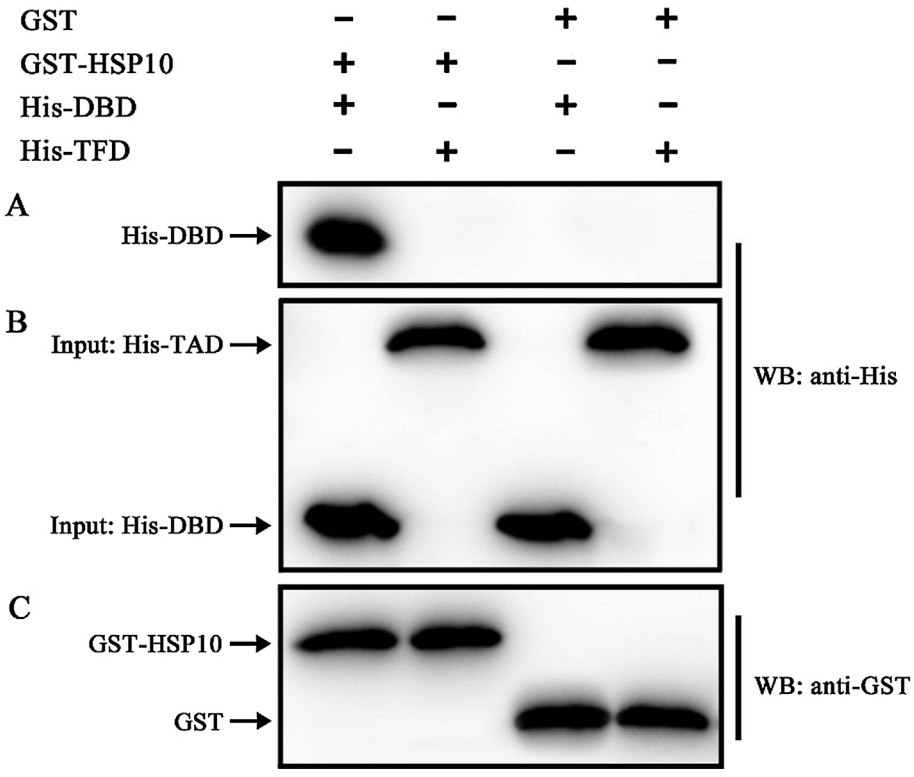

Fig. 9. GST pull-down assay between the MjHSP10 and MjHSF1 proteins.

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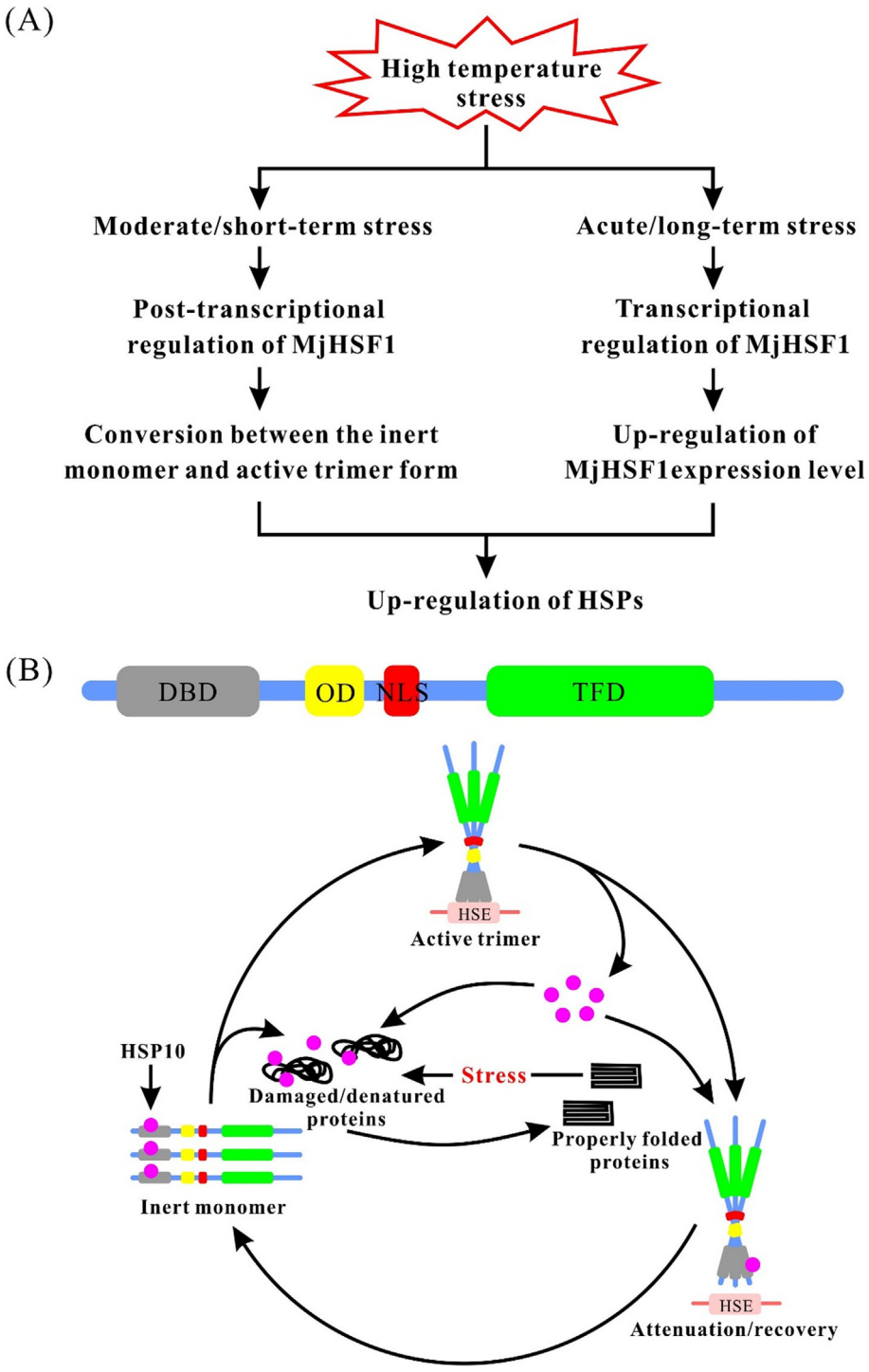

Fig. 10. Proposed schematic of the heat shock transcriptional response under heat stress (A) and cross talk between MjHSP10 and MjHSF1 (B) in *M. japonicus*.

erone HSP70 can interact directly with the TAD and DBD of HSF1 and negatively regulate the transactivation and DNA-binding activity of HSF1, consequently attenuating the HSR [26–28]. HSP90, a cellular chaperone known to regulate several signal transduction molecules and transcription factors [60–62], has also been shown to participate in modulating the HSR via influencing the interconversion of HSF1 between monomeric and trimeric forms as well as the transcriptional activation of HSF1 [29,30]. To investigate the interaction between MjHSP10 and MjHSF1 and verify the manner by which MjHSP10 blocks the activity of MjHSF1 in the negative feedback regulatory loop in the HSR, the recombinant GST-HSP10, His-DBD and His-TAD proteins were successfully produced, and the interactions between MjHSP10 and the two functional domains of MjHSF1, the DBD and TAD, were analyzed by GST pull-down assay. In this study, MjHSP10 was found to interact specifically with the DBD of MjHSF1, which may impede MjHSF1 binding to HSEs in HSP promoters. However, no interaction between MjHSP10 and the TAD of MjHSF1 was detected. Thus, it

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is reasonable to infer that MjHSP10 may function as a negative regulator in the auto-regulatory loop of the HSR via blocking the DNA-binding activity of MjHSF1 rather than repressing the transactivation activity of MjHSF1.

Based on these findings, we propose a reversible and multistep regulatory regime involving MjHSP10 and MjHSF1 for control of the HSR in *M. japonicus*. Under non-stress conditions, MjHSP10 binds to the DBD of MjHSF1 and blocks the DNA-binding activity of MjHSF1. Upon exposure to heat stress, proteins that are damaged and denatured by stress compete with monomeric MjHSF1 for MjHSP10 binding. MjHSP10 is sequestered to damaged and denatured proteins, resulting in dissociation of the MjHSF1-MjHSP10 complex and the release of DNA binding-competent MjHSF1. Trimeric MjHSF1 binds specifically to the HSE of MjHSP10 and induces its transcription and synthesis. With the accumulation of MjHSP10 and saturation of the MjHSP10 pool, excess MjHSP10 again binds to the DBD of MjHSF1 and shuts down the transcription of MjHSP10 (Fig. 10B).

5. Conclusion

In conclusion, two vital molecules involved in the HSR, MjHSP10 and MjHSF1, were identified from *M. japonicus* in the current study. The expression patterns of these two genes in response to heat stress and their interactions were elucidated for the first time. MjHSP10 and MjHSF1 exhibited different expression profiles in response to heat stress. The transcription of MjHSP10 quickly and robustly increased under heat stress, while MjHSF1 was up-regulated to a lesser extent. MjHSF1 knockdown by dsRNA-mediated gene silencing resulted in the down-regulation of MjHSP10, which indicated that MjHSF1 plays a crucial role in regulating the transcription of MjHSP10. GST pull-down assays showed an interaction between MjHSP10 and the DBD of MjHSF1, which may block the DNA binding activity of MjHSF1.

Acknowledgments

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijbiomac.2019.10.072.

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