Changes in the expression of four heat shock proteins during the aging process in *Brachionus calyciflorus* (rotifera)

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Abstract Heat shock proteins (HSPs) are molecular chaperones and have an important role in the refolding and degradation of misfolded proteins, and these functions are related to aging. Rotifer is a useful model organism in aging research, owing to small body size (0.1–1 mm), short lifespan (6–14 days), and senescence phenotypes that can be measured relatively easily. Therefore, we used rotifer as a model to determine the role of four typical *hsp* genes on the aging process in order to provide a better understanding of rotifer aging. We cloned cDNA encoding *hsp* genes (*hsp40*, *hsp60*, *hsp70*, and *hsp90*) from the rotifer *Brachionus calyciflorus* Pallas, analyzed their molecular characteristics, determined its modulatory response under different temperatures and H₂O₂ concentrations and investigated the changes in expression of these genes during the aging process. We found that *Bchsp70* mRNA expression significantly decreased with aging. In addition, we also studied the effects of dietary restriction (DR) and vitamin E on rotifer lifespan and reproduction and analyzed the changes in expression of these four *Bchsp* genes in rotifers treated with DR and vitamin E. The results showed that DR extended the lifespan of rotifers and reduced their fecundity, whereas vitamin E had no significant effect on rotifer lifespan or reproduction. Real-time PCR indicated that DR increased the expression of these four *Bchsp*s. However,

vitamin E only improved the expression of *Bchsp60*, and reduced the expression of *Bchsp40*, *Bchsp70*, and *Bchsp90*. DR pretreatment also increased rotifer survival rate under paraquat-induced oxidative stress. These results indicated that *hsp* genes had an important role in the anti-aging process.

Keywords Aging · *Brachionus calyciflorus* · Dietary restriction · Heat shock proteins · Paraquat · Vitamin E

Abbreviations

ANOVA	Analysis of variance
bp	Base pair
DR	Dietary restriction
ATP/GTP	Adenosine triphosphate/guanosine triphosphate
EPA	Artificial freshwater medium
ER	Endoplasmic reticulum
H ₂ O ₂	Hydrogen peroxide
HSF	Heat shock factor
HSP	Heat shock protein
HSR	Heat shock response
O ₂ [−]	Superoxide anion
ORF	Open reading frame
PCR	Polymerase chain reaction
RACE	Rapid amplification of cDNA ends
ROS	Reactive oxygen species
RT-PCR	Real-time PCR
SOD	Superoxide dismutase
UTR	Untranslated region

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Introduction

Heat shock proteins (HSPs) are a highly conserved, ubiquitously expressed family of stress-response proteins (Hartl

1996) and are classified into six subfamilies according to their molecular weight, such as small HSPs (16–30 kDa), HSP40, HSP60, HSP70, HSP90, and the large HSP110 (Becker and CRAIG 1994; Kalmar and Greensmith 2009). HSPs can act as molecular chaperones facilitating protein folding, preventing protein aggregation, and targeting improperly folded proteins to specific degradation pathways (Kalmar and Greensmith 2009), and most of these functions are related to aging. Reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2) and superoxide anion (O_2^-), which are mainly by-products of aerobic respiration, can damage many cell macromolecules, including lipids, nucleic acids, and proteins (Finkel and Holbrook 2000; Kalmar and Greensmith 2009). The proteins damaged by ROS can easily lose their native folded conformation to become misfolded leading to functional abnormalities (Kalmar and Greensmith 2009). Some HSPs facilitate the refolding of damaged proteins and improve their impaired functions (Heikkilä 2010). If the damaged proteins cannot recover, some HSPs, mainly members of the HSP70 family and its co-chaperones, play a crucial role in protein sorting and quality control by selecting and directing aberrant proteins to proteasomes or lysosomes for degradation (Mayer and Bukau 2005). Thus, HSPs regulate the quality of proteins and have an important role in the aging process. Indeed, *hsp70* induced by moderate, nonlethal heat treatment is a candidate protein for longevity in the yeast *Saccharomyces cerevisiae* (Shama et al. 1998). More directly, the life expectancy of *hsp70* transgenic *Drosophila melanogaster* increased by 30 % compared with the wild type when its gene expression was induced by heat treatment (Tatar et al. 1997). However, changes in *hsps* gene expression in rotifer aging process is very rarely reported. The rotifer, *Brachionus calyciflorus*, has a short lifespan (5–7 days), and it is easy to detect the changes in gene expression during the aging process. Therefore, we analyzed the changes in *hsps* gene expression in *B. calyciflorus* at different ages to explore the role of *hsps* genes in aging.

Dietary restriction (DR) is usually defined as a moderate (normally 20–40 %) reduction in available nutrients without causing malnutrition. It was first noted in the 1930s that food restriction significantly extended the lifespan of rodents (McCay et al. 1935). In the laboratory, DR is one of the most common lifespan-extending methods in evolutionarily distinct eukaryotes from single cell to multi-cellular organisms (Fanestil and Barrows 1965; Klass 1977; Lin et al. 2002; Lints 1985; Walker et al. 2005). It was reported by Kaneko et al. that the mRNA levels of *hsp70* and glucose-regulated protein 94 (GRP94) were significantly higher in the exponential growth phase than in the stationary phase (Kaneko et al. 2002) and that rotifers in the stationary phase lived longer than those in the exponential phase (Yoshinaga et

al. 2000). Nutritional deprivation also potentiates HSP60 protein induction (Wheelock et al. 2002). Therefore, we also analyzed the effects of DR on the expression of *hsps* genes to explore the reasons why DR extends life.

Vitamin E is an antioxidant and acts as a H_2O_2 radical scavenger, preventing the propagation of ROS in tissues and can affect lifespan (Traber and Stevens 2011). Previous studies have shown that vitamin E extended the lifespan of several types of rotifers (Litton 1987; Sawada and Enesco 1984; Snell et al. 2012). Thus, we hypothesized that the antioxidant effects produced by vitamin E may affect the expression of *hsp* genes.

The aims of this study were to: (1) clone the *hsp40*, *hsp60*, *hsp70*, and *hsp90* cDNAs in the freshwater rotifer *B. calyciflorus*; (2) detect the expression of these four *hsps* under temperature stress and oxidative stress (produced by H_2O_2) conditions; (3) examine the mRNA expression levels of these four *hsps* during the aging process using highly sensitive quantitative real-time PCR; (4) investigate the effect of DR and vitamin E on the expression of the four *hsps*. These studies were undertaken to provide a better understanding of the role of *hsps* in rotifer aging.

Materials and methods

Rotifers

The animals used during the study, *B. calyciflorus*, were female neonates (0–2 h old) hatched from resting eggs in artificial freshwater medium (EPA medium, pH ~7.8) composed of 96 mg $NaHCO_3$, 60 mg $CaSO_4 \cdot H_2O$, 123 mg $MgSO_4$, and 4 mg KCl in 1 L deionized water at 25 °C (ASTM 2001). This species was originally collected in Gainesville, Florida in 1983 (Snell et al. 1991) and since then, has been cultured in the laboratory continuously with periodic collection and storage of resting eggs. The females were fed by suspending the green alga *Chlorella pyrenoidosa* (Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan, China) in the culture media at $\sim 3 \times 10^6$ cells/mL. The feeding alga was cultured in Bold's basal medium at 25 °C in 3-L flasks under constant fluorescent illumination at 2,000 lux.

*Bch*sp cloning

To clone the *hsps* from *B. calyciflorus* Pallas, the degenerate primers were designed with reference to DNA nucleotide sequences of the *hsps* gene reported for the rotifer *Brachionus plicatilis*, *Brachionus ibericus*, *Brachionus manjavacas*, the yeast *S. cerevisiae*, the nematode *Caenorhabditis elegans*, and humans.

The extraction of total RNAs from rotifer was conducted using the Trizol (Invitrogen, China) technique following the

manufacturer's instructions. The total RNA concentration was determined by measuring the absorbance at OD₂₆₀. RNA integrity was checked by electrophoresis. The total RNA was reverse transcribed into cDNA using the aM-MLV RTase cDNA Synthesis Kit (TaKaRa, Japan).

Polymerase chain reaction (PCR) was conducted for 3 min at 94 °C followed by 35 cycles at 94 °C for 10 s, 48 °C for 40 s, and 72 °C for 30 s. The final extension step was performed at 72 °C for 10 min. Each of the 20 µL reaction mixtures contained 1.5 mM of MgCl₂, 0.2 mM of dNTP, 0.2 mM of each of the primers, 1 U of Taq DNA polymerase (TaKaRa, Japan), and 5 ng of cDNA. Amplicons of expected sizes were purified using an Agarose Gel DNA Purification Kit (TaKaRa, Japan), and then subcloned into the pMD-19T cloning vector (TaKaRa, Japan). Positive clones containing inserts of an expected size were sequenced using M13 primers and sequenced at BGI (Shanghai, China). The *Bchsp*s partial cDNA sequence was extended using the 5' and 3' rapid amplification of cDNA ends (RACE; SMART™ cDNA Kit) technique following the manufacturer's instructions. A total of five gene-specific primers were designed based on the *Bchsp*s partial cDNA sequence.

Bchsp40, *Bchsp60*, *Bchsp70*, and *Bchsp90* were cloned respectively via the procedure described above. The degenerate primers for PCR and the specific primers for RACE are shown in Table 1.

Sequence analysis of *Bchsp*s

Nucleotide and deduced amino acid sequences of *hsps* cDNA were analyzed using the software Vector NTI Advance 11. HSPs sequences from different organisms were obtained using the NCBI BLAST search program. The cDNA sequence and deduced amino acid sequence from *B. calyciflorus* were analyzed using the BLAST algorithm (<http://www.ncbi.nlm.nih.gov/blast>). Translation and protein analyses were performed using ExPASy tools (<http://us.expasy.org/tools/>). A multiple sequence alignment was created with ClustalW (<http://www.ebi.ac.uk/clustalw/>). The calculated molecular mass and the theoretical isoelectric point were predicted using the Protein Mol Wt and AA Composition Calculator (http://www.proteomics.com.cn/proteomics/pi_tool.asp). An unrooted phylogenetic tree based on the amino acid sequence alignment was constructed using the Bayesian method. A total of 39 sequences were retrieved from the GenBank databases. Multiple sequence alignment results were first converted to Nexus format and analyzed using MrBayes v3.1.2 for Windows (Huelsenbeck and Ronquist 2001). Four parallel Monte Carlo Markov chains were run for 100,000 generations to approximate the posterior probabilities, and trees were sampled every 100 generations (Rhee et al. 2011). The analysis results were visualized using the Fig Tree v1.3.1 software.

Experimental design

The effects of temperature, vitamin E and H₂O₂ on hsp expression

Experiments were conducted to test the effects of temperature, H₂O₂, and vitamin E on the expression of *hsps* in *B. calyciflorus*. In the temperature test, 100 rotifers were cultured in 50 mL EPA medium at 15, 25 (control), 30, and 37 °C for 0, 0.5, 1, 2, 3, and 6 h, respectively. The rotifers were then collected and total RNA was extracted. Three replicates were prepared for each treatment. In the H₂O₂ test, 100 rotifers were placed into 50 mL H₂O₂ (H₂O₂ concentration was 0.1 and 0.2 mM) medium and cultured for 0, 1, 2, 4, 6, and 12 h, respectively. The rotifers were then collected and total RNA was extracted. Three replicates were prepared for each treatment. In the vitamin E test (vitamin E concentration was 40 µM), rotifers were treated using the same procedure as in the H₂O₂ test described above. Vitamin E was obtained from the Sigma Chemical Company (St. Louis, MO), dissolved in DMSO and stored at −20 °C.

Age-related changes in hsp gene expression

In total, 600 neonates (0–2 h old) were collected and divided into six groups of 100 individuals, which were then cultured in 50 mL of prepared media and maintained at 25 °C for 0.5, 1, 2, 3, 4, and 5 days. Three replicates were prepared for each treatment. Rotifers were transferred into new medium every 24 h and checked every 12 h to record the number of neonates, and the neonates were removed each day. Any surviving rotifers were then collected after 0.5, 1, 2, 3, 4, and 5 days. The total RNA was then extracted and reverse transcribed into cDNA. Real-time PCR (RT-PCR) was carried out to measure the mRNA expression levels of *hsps* at the different time points described above. The real-time PCR method was as described below.

The effects of DR on lifespan and reproduction

Six rotifers (0–2 h old) were transferred into 12-well culture plates, which contained 3 mL culture media under different feeding regimes (AL, DR12, DR24, and DR36). In the AL group, rotifers were transferred to a newly prepared culture medium containing microalgae every 12 h. In the DR group, rotifers were alternately placed in a culture medium with and without microalgae (alternated 12, 24, and 36 h in DR12, DR24, and DR36, respectively). Six replicates were prepared for each treatment. The rotifers were checked every 12 h to record the number of neonates and remove the neonates until all the experimental animals had died.

Table 1 Degenerate and specific primers used in the experiment

Primers	Sequence (5'-3')	Application
Hsp40F	TCTAGTNTGCCAGGTGAGAT	Hsp40 amplification
Hsp40R	TTCTNTCATAAGCTGGATCC	Hsp40 amplification
Hsp40-5'1	CACCCCTTCCACTACA	Hsp40 RACE
Hsp40-5'2	CACCACCACCGCCGAAGA	Hsp40 RACE
Hsp40-5'3	GAAAATATCCATTGGTGAAC	Hsp40 RACE
Hsp40-3'1	TCACCAGGTCTAGAACCA	Hsp40 RACE
Hsp40-3'2	TGAGGGTATGCCAATG	Hsp40 RACE
Hsp60F	TTYGAYCGHGGMTAYATYTCNCC	Hsp60 amplification
Hsp60R	TCHACYTCRCKTGADCCRCDDAC	Hsp60 amplification
Hsp60-5'1	TTACGTTGAGCATTGG	Hsp60 RACE
Hsp60-5'2	TGGCTAATTCTAAAGCTGGT	Hsp60 RACE
Hsp60-5'3	CTCTGAGATTAAGACAAAAGC	Hsp60 RACE
Hsp60-3'1	GGAGCTACTGTATTCGGCGATGAAGCT	Hsp60 RACE
Hsp60-3'2	GGGTAGCCGTTTTGAAAGTCGGTGGTT	Hsp60 RACE
Hsp70F	GTCGANCCGCCNACCANNACAAT	Hsp70 amplification
Hsp70R	TGGGNGGNGGNACNTTYGACGT	Hsp70 amplification
Hsp70-5'1	TTCTGATGTCTTTACCA	Hsp70 RACE
Hsp70-5'2	TGTTGTCAAAATCTTCACCACC	Hsp70 RACE
Hsp70-5'3	CACCATTTGTGGCTACTACTTCA	Hsp70 RACE
Hsp70-3'1	ATTTGGGTGGTGAAGATTTTGACAACAGAG	Hsp70 RACE
Hsp70-3'2	AAACCAGTTCAAAAGGTCTTGGAAGATGCT	Hsp70 RACE
Hsp70-3'3	TCCACCAGCCCCAAGAGGTGTACCAC	Hsp70 RACE
Hsp70-3'4	CCGCTGAAGACAAAGGTACTGGTAACAAA	Hsp70 RACE
Hsp90F	TKGTNGARTCYTCRTGDATNGC	Hsp90 amplification
Hsp90R	TCHGGMACBAARGCNTTYATGGA	Hsp90 amplification
Hsp90-5'1	CAACCGTATTGTGAAG	Hsp90 RACE
Hsp90-5'2	CCAAACGAGTTGAAACAGTGAC	Hsp90 RACE
Hsp90-5'3	CGTATTTTTCAGCATCGGATTC	Hsp90 RACE
Hsp90-3'1	GTCACGTGTTCAACTCGTTTGGTCTCATC	Hsp90 RACE
Hsp90-3'2	CACAATACGGTTGGTCCGCCAATATGG	Hsp90 RACE
Hsp40RTF	AATGGATATTTTCGAGATG	Real-time PCR
Hsp40RTR	TGCCTTGACAAGTTTTAC	Real-time PCR
Hsp60RTF	CTACTGTATTCGGCGATGAA	Real-time PCR
Hsp60RTR	CGGCTACACCATTACTCAAT	Real-time PCR
Hsp70RTF	GCCACAAATGGTGATACTCAT	Real-time PCR
Hsp70RTR	TGGAAGATGCTGGTTTGA	Real-time PCR
Hsp90RTF	CTGCTTACTTAGTCGCTGAT	Real-time PCR
Hsp90RTR	CTTCTTCTTCTGTCTTATCG	Real-time PCR
β -actinRTF	GAAATTGTGCGCGACATCAAGGA	Real-time PCR
β -actinRTR	GCAATGCCCCGGGTACATGGTGGT	Real-time PCR

The effects of DR on hsp expression

Rotifers hatched from resting eggs were cultured using different feeding regimes (AL, DR12, DR24, and DR36) as previously mentioned. Each treatment contained 100 rotifers in 50 mL medium. Three replicates were prepared for each treatment. Rotifers were transferred into newly prepared culture media every 12 h and checked every 12 h to remove

neonates, which were then collected and the total RNA extracted and reverse transcribed into cDNA after 3 days.

The effects of vitamin E on lifespan and reproduction

Six rotifers (0–2 h old) were transferred into 24-well culture plates, which contained 3 mL of test solution and *C. pyrenoidosa* at 3×10^6 cells/mL. The concentration of

vitamin E was 40 μ M. Rotifers cultured in normal medium acted as controls. Six replicates were prepared for each treatment. The rotifers were checked every 12 h to record the number of neonates and remove the neonates until all the experimental animals had died.

Survival test

One hundred twenty individuals were collected and divided into three groups, AL, DR12, and DR24. The AL group was transferred to a newly prepared culture media containing the feeding alga every 12 h, whereas the DR12 and DR24 groups were alternately placed in a culture media with and without the feeding alga every 12 and 24 h, respectively. After 2 days, 30 rotifers were transferred from each of the AL, DR12, and DR24 groups to the culture medium containing paraquat at a concentration of 15.25 mg/L. The number of surviving rotifers was recorded every 12 h after exposure. Three replicates were prepared for each treatment. Paraquat (DMSO stock) was obtained from the Jiangsu Academy of Agricultural Sciences (Nanjing, China).

Quantitative analysis of *hsps* gene transcription

Total RNA was extracted using the procedures described above. Total RNA was reverse transcribed into cDNA using the PrimeScript RT Reagent Kit (TaKaRa, Japan). Real-time PCR was performed on a Mastercyclerrealplex (Eppendorf, Germany) to study gene expression. The PCR reaction was performed in a 25 μ L volume with a SYBR Premix Ex TaqTMKit (TaKaRa, Japan), 2 μ M of each specific primer, and 1 μ L of cDNA using the following procedure: initial denaturation at 95 $^{\circ}$ C for 2 min followed by 40 cycles of amplification (95 $^{\circ}$ C for 10 s and 55 $^{\circ}$ C for 30 s). Table 1 shows the primers used in quantitative real-time PCR. B-actin was used as the internal control for quantitative real-time PCR. The relative expression levels of different genes were calculated using the $2^{-\Delta\Delta CT}$ method (Kenneth and Thomas 2001).

Statistical analysis

All the statistical analyses were carried out using the SPSS 16.0 analytic package. Results from the lifespan experiments were analyzed using survival analysis (Kaplan–Meier log-rank test); results from the DR and vitamin E reproduction experiments were analyzed using one-way analysis of variance (ANOVA), Duncan test, and Student's *t* test, respectively. The mRNA expression level was determined by one-way ANOVA with the Tukey test. Differences were considered significant when *P* value was <0.05. All figures were produced using Sigma Plot version 11.0.

Results

Characterization of *Bchsp40*, *Bchsp60*, *Bchsp70*, and *Bchsp90* genes in *B. calyciflorus*

Bchsp40

The full-length *hsp40* cDNA of *B. calyciflorus* (*Bchsp40*) was 1,496 bp long, and contained 128 bp in the 5' untranslated region (UTR), an open reading frame (ORF) of 1,212 bp that encoded 403 amino acids, and 156 bp in the 3' UTR with a poly A signal (ATTTAA; data not shown). In a GenBank Blastx search, the cDNA sequence of BcHSP40 showed three DnaJ domains. The calculated molecular mass of the deduced mature BcHSP40 was 45.1005 kDa, and the theoretical isoelectric point was 7.09. The deduced amino acid sequence of *Bchsp40* contained an N-terminal conserved domain (J domain, aa7–67), a central domain containing four highly conserved cysteine-rich repeats with a consensus sequence of CxxCxGxG where *x* is any amino acid (CRR domain, aa139–205), and a C-terminal domain (C domain, aa262–341) (Fig. 1a). The primary structure of BcHSP40 showed high levels of similarity to HSP40s in four other animals. It showed 80.15, 62.62, 60.11, and 58.66 % identity with orthologs of the marine rotifer *B. plicatilis* (GenBank accession number ADR79278), *Mus musculus* (NP_032324), *Dugesia japonica* (AES12470), and *Xenopus laevis* (NP_001079772), respectively. Furthermore, there were eight cysteine residues (cys-140, 142, 155, 158, 182, 185, 198, and 201) that are essential for BcHSP40 binding to zinc.

Bchsp60

Full-length *Bchsp60* cDNA consisted of 1940 nucleotides with a 1,740-bp ORF encoding 579 amino acids. The calculated molecular mass of the deduced mature BcHSP40 was 61.7469 kDa, and the theoretical isoelectric point was 6.66. The deduced sequence contained a presequence of 26 amino acids at the N terminus that is required for import into the mitochondria. BcHSP60 has a conserved ATP-binding/Mg²⁺ binding site, a hinge region, and stacking interaction sites (Marchler-Bauer et al. 2007) (Fig. 1b). BcHSP60 displayed very high homology with shrimp HSP60s from *B. ibericus* (ADR79280, 90.78 %), *D. melanogaster* (NP_511115, 70.06 %), *Daphnia pulex* (EFX84424, 72.09 %), and *X. laevis* (NP_001083970, 71.11 %).

Bchsp70

Full-length *Bchsp70* cDNA consisted of 2,155 nucleotides with a 1,971-bp ORF encoding 656 amino acids. The calculated molecular mass of the deduced mature BcHSP70 was 72.3939 kDa, and the theoretical isoelectric point was 5.23.

The *Bchsp90* cDNA contained a 2,166-bp ORF that encoded 721 amino acids with a calculated molecular weight of 82.7858 kDa and a theoretical isoelectric point of 5.07. The deduced amino acid sequence of *Bchsp90* also contained HSP90 family motifs and signatures, including

An unrooted phylogenetic tree based on the amino acid sequence alignment was constructed using the Bayesian method (Fig. 2). A total of 39 sequences were retrieved from the GenBank databases (Table 2). All HSPs can be classified into four distinct clusters: HSP40 family, HSP60 family, HSP70 family, and HSP90 family. HSP40, HSP60, HSP70, and HSP90 from *B. calyciflorus* clustered together

Fig. 1 (continued)

with other HSP40s, HSP60s, HSP70s, and HSP90s as a subgroup, respectively.

Time course of *hsp*s expression following exposure to different temperatures

To study the modulatory effect of different temperatures on the expression of *hsp*s, we measured expression at 15, 30, and 37 °C relative to the control (25 °C) over 6 h (Fig. 3). High temperature (37 °C) significantly increased the expression of *Bchsp40*, *Bchsp60*, and *Bchsp70* at 0.5 h after exposure and showed a steady upregulation over time. However, the expression of *Bchsp90* was temporarily increased after exposure to 37 °C, and then returned to normal levels (Fig. 3d). At 30 °C, the expression of *Bchsp40* increased immediately ($P < 0.05$) (Fig. 3a). The expression of *Bchsp90* increased when

exposed to 30 °C for 1 h and showed a steady upregulation over time (Fig. 3d). The expression of *Bchsp60* and *Bchsp70* steadily increased after exposure to 30 °C. At low temperature (15 °C), the expression of *Bchsp40*, *Bchsp60*, and *Bchsp70* increased significantly after 1 h of exposure, and the expression level returned to normal after 6 h of exposure. The expression of *Bchsp90* was not significantly different after exposure to 15 °C (Fig. 3d).

Effect of H_2O_2 on *hsp*s mRNA expression

Figures 4 and 5 illustrate the effect of H_2O_2 on the expression of *hsp*s mRNA. Following H_2O_2 (0.1 or 0.2 mM) exposure, the mRNA expression of the four *hsp*s was upregulated to different extents. In both the 0.1 and 0.2 mM groups, the expression of the four *hsp*s significantly

Fig. 2 Phylogenetic analysis of HSPs constructed by Bayesian method. The abbreviation of HSPs and the GenBank accession No. used to construct phylogenetic tree are given in Table 2. Numbers at branch nodes represent the confidence level of posterior probability

increased (30- to 90-fold) immediately after exposure to H_2O_2 for 1 h, and even though expression decreased slightly after 2 h, the mRNA expression levels were still significantly higher (5- to 10-fold) than the normal level ($P < 0.05$).

Effect of DR and vitamin E on rotifer lifespan and reproduction

Figure 6 illustrates the effect of DR on the lifespan of *B. calyciflorus*. The average lifespan in the DR12 group was 120.4 ± 4.3 h, which was significantly longer (log-rank test, $P < 0.0001$) than that in the control group (95.0 ± 3.7 h) (Fig. 6c). However, the average lifespan in the DR24 and DR36 groups was 61.9 ± 2.3 and 66.0 ± 1.4 h, respectively and were significantly less ($P < 0.05$) than that in the control group (Fig. 6c). Figure 7a illustrates the fecundity of rotifers in the AL and DR groups at each time point. The average number of offspring produced per rotifer in the DR12, DR24 and DR36 groups was 5.9 ± 2.4 , 2.9 ± 0.4 , and 5.4 ± 0.3 , respectively, significantly less ($P < 0.05$) than that in the control group 13.6 ± 5.5 (Fig. 7b). Figure 7c illustrates the number of egg carried by rotifers in the AL and DR groups at each time

point. We also studied the effect of vitamin E on the lifespan and reproduction of *B. calyciflorus* and found that vitamin E had no effect on lifespan or reproduction (Fig. 8).

Age-related changes in *hsp*s mRNA expression

Figure 9 shows *Bchsp*s mRNA expression in *B. calyciflorus* at different ages. The expression level of *Bchsp70* mRNA significantly decreased with age ($P < 0.05$) (Fig. 9c), whereas a small but significant ($P < 0.05$) increase in the mRNA levels of *Bchsp40* was only observed in 4-day-old individuals (Fig. 9a). The expression of *Bchsp60* mRNA significantly decreased ($P < 0.05$) in 1-, 2-, and 3-day-old individuals and significant decreases ($P < 0.05$) in the mRNA levels of *Bchsp90* were observed in 2-, 3-, and 4-day-old individuals (Fig. 9b, d).

The effects DR on *hsp*s mRNA expression

Qualitative real-time PCR showed that DR12 and DR24 treatment significantly improved ($P < 0.05$) the mRNA expression of *Bchsp40*, *Bchsp60*, *Bchsp70*, and *Bchsp90* (Fig. 10), whereas DR36 treatment significantly improved

Table 2 Species and GenBank accession numbers of HSPs sequences used for phylogenetic analysis

Species	GenBank No.	Abbreviation type	Size (aa)
<i>Alligator mississippiensis</i>	BAF94139	<i>A. mississippiensis</i> Hsp40	398
<i>Brachionus ibericus</i>	ADR79278	<i>B. ibericus</i> Hsp40	368
<i>Dugesia japonica</i>	AES12470	<i>D. japonica</i> Hsp40	411
<i>Anolis carolinensis</i>	XP_003224682	<i>A. carolinensis</i> Hsp40	396
<i>Ictalurus punctatus</i>	NP_001187713	<i>I. punctatus</i> Hsp40	408
<i>Monodelphis domestica</i>	XP_001362945	<i>M. domestica</i> Hsp40	423
<i>Xenopus laevis</i>	NP_001079772	<i>X. laevis</i> Hsp40	397
<i>Paralichthys olivaceus</i>	ABA54277	<i>P. olivaceus</i> Hsp40	395
<i>Brachionus calyciflorus</i>	KC176712	<i>B. calyciflorus</i> Hsp40	403
<i>B. ibericus</i>	ADR79280	<i>B. ibericus</i> Hsp60	581
<i>Cricetulus griseus</i>	XP_003504389	<i>C. griseus</i> Hsp60	573
<i>Danio rerio</i>	NP_851847	<i>D. rerio</i> Hsp60	575
<i>Daphnia pulex</i>	EFX84424	<i>D. pulex</i> Hsp60	576
<i>Drosophila melanogaster</i>	NP_511115	<i>D. melanogaster</i> Hsp60	573
<i>Gallus gallus</i>	NP_001012934	<i>G. gallus</i> Hsp60	573
<i>Harpegnathos saltator</i>	EFN79769	<i>H. saltator</i> Hsp60	608
<i>Oreochromis niloticus</i>	XP_003456322	<i>O. niloticus</i> Hsp60	575
<i>X. laevis</i>	NP_001083970	<i>X. laevis</i> Hsp60	579
<i>B. calyciflorus</i>	KC176713	<i>B. calyciflorus</i> Hsp60	579
<i>Ascaris suum</i>	ADY43887	<i>A. suum</i> Hsp70	655
<i>B. ibericus</i>	ADR79282	<i>B. ibericus</i> Hsp70	653
<i>Coturnix japonica</i>	BAF38391	<i>C. japonica</i> Hsp70	652
<i>C. griseus</i>	NP_001233668	<i>C. griseus</i> Hsp70	654
<i>Homo sapiens</i>	NP_005338	<i>H. sapiens</i> Hsp70	654
<i>Mus musculus</i>	NP_071705	<i>M. musculus</i> Hsp70	655
<i>P. olivaceus</i>	ABG56392	<i>P. olivaceus</i> Hsp70	654
<i>Salmo salar</i>	NP_001135114	<i>S. salar</i> Hsp70	657
<i>Xenopus tropicalis</i>	XP_002941690	<i>X. tropicalis</i> Hsp70	655
<i>B. calyciflorus</i>	KC176714	<i>B. calyciflorus</i> Hsp70	656
<i>B. calyciflorus</i>	KC176715	<i>B. calyciflorus</i> Hsp90	721
<i>Apis florea</i>	XP_003694932	<i>A. florea</i> Hsp90	717
<i>B. ibericus</i>	ADR79283	<i>B. ibericus</i> Hsp90	720
<i>D. rerio</i>	AAC21566	<i>D. rerio</i> Hsp90	724
<i>Eriocheir sinensis</i>	ADE60732	<i>E. sinensis</i> Hsp90	718
<i>H. saltator</i>	EFN88374	<i>H. saltator</i> Hsp90	723
<i>Philodina roseola</i>	ACC43981	<i>P. roseola</i> Hsp90	739
<i>Portunus trituberculatus</i>	ACQ90225	<i>P. trituberculatus</i> Hsp90	721
<i>Quadrastichus erythrinae</i>	AFC76152	<i>Q. erythrinae</i> Hsp90	721
<i>Tigriopus japonicus</i>	ACA03524	<i>T. japonicus</i> Hsp90	721

($P < 0.05$) the mRNA expression of *Bchsp40*, *Bchsp60*, and *Bchsp90* but not *Bchsp70* mRNA ($P > 0.05$) (Fig. 10c). The expression level of *Bchsp60*, *Bchsp70*, and *Bchsp90* mRNA reached maximum levels in the DR12 group.

Effect of vitamin E on *hsps* mRNA expression

Real-time quantitative RT-PCR demonstrated that vitamin E (40 μ M) significantly decreased the expression of

Bchsp40 (Fig. 11a). However, the expression of *Bchsp70* and *Bchsp90* was temporarily increased after exposure to vitamin E for 1 h, and after exposure for 4 h, the expression levels of *Bchsp70* and *Bchsp90* were significantly decreased compared with the control (Fig. 11c, d). The expression of *Bchsp60* was significantly increased after exposure to vitamin E, and the expression level was maximal after 4 h of exposure (Fig. 11b).

Fig. 3 Hsps mRNA expression of *B. calyciflorus* at different temperature exposures of 10, 30, and 37 °C with control (25 °C). The expression levels of hsp mRNA was statistically analyzed by one-

way ANOVA with the Tukey test (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$): **a** hsp40, **b** hsp60, **c** hsp70, and **d** hsp90

Effect of DR pretreatment on oxidative stress resistance

Figure 12 illustrates the effects of DR pretreatment on the survival rate of rotifers under oxidative stress conditions. After exposure to oxidative stress for 12 h, survival rate in the DR12 pretreatment group was 0.78 ± 0.02 which was significantly higher ($P < 0.05$) than that in the control group (0.60 ± 0.25). In the 24-h-exposed group, the survival rates in the DR12 pretreatment groups and control groups were 0.55 ± 0.02 and 0.38 ± 0.02 , respectively ($P < 0.05$). However, the survival rate in the DR24 pretreatment groups were 0.39

± 0.04 which was significantly lower ($P < 0.05$) than that in the control groups (0.60 ± 0.25). These results indicate that DR12 pretreatment increased rotifer survival rate under paraquat-induced oxidative stress.

Discussion

HSPs are a highly conserved, ubiquitously expressed family of stress response proteins which are expressed at low levels under normal physiological conditions (Hartl 1996). In the

Fig. 4 Relative mRNA levels of *hsps* of *B. calyciflorus* treated under H_2O_2 (0.1 mM) stress condition. The expression levels of *hsps* mRNA were statistically analyzed by one-way ANOVA with the Tukey test.

The different letters indicate a significant difference for each time point with control ($p < 0.05$): **a** *hsp40*, **b** *hsp60*, **c** *hsp70*, and **d** *hsp90*

present study, we cloned cDNAs encoding four typical *hsp* genes (*Bchsp40*, *Bchsp60*, *Bchsp70*, and *Bchsp90*) from the rotifer *B. calyciflorus*. Multiple sequence alignment revealed that HSPs from *B. calyciflorus* were highly homologous with the HSPs from other animals, which indicated that they may have a similar function to the HSPs in other species. HSPs can be induced by a variety of stressful stimuli, such as temperature, cellular energy depletion, physical injury, and various toxic substances (Feder and Hofmann 1999; Ritossa 1962), therefore, HSPs are also called “stress proteins” (Kalmar and Greensmith 2009; Wheelock et al. 2002).

In general, coordinated activation of HSP expression, called the heat shock response (HSR), is a ubiquitous adaptation mechanism in organisms ranging from bacteria to mammals, which helps them to survive and adapt to a wide range of environmental challenges (Lindquist and Craig 1988). To

study the modulatory effect of different temperatures on the expression of *Bchsp*s genes, we measured expression at 15, 30, and 37 °C relative to the control (25 °C) over 6 h and found that temperature stress (15 and 37 °C) significantly induced the expression of *Bchsp40*, *Bchsp60*, and *Bchsp70* genes, indicating that *Bchsp40*, *Bchsp60*, and *Bchsp70* genes were involved in the chaperoning process to protect proteins under temperature stress in rotifers. However, temperature stress (15 and 37 °C) had no effect on the expression of *Bchsp90*. Our findings are in accordance with previous studies where *hsp40*, *hsp60*, and *hsp70* were required for rotifer thermotolerance; however, *hsp90* was not essential (Smith et al. 2012). Increased *hsps* expression under high and low temperatures can protect cells from temperature stress (Dangi et al. 2012).

In addition to typical heat stress, oxidative stress can also induce the expression of HSPs. H_2O_2 is the most stable

Fig. 5 Relative mRNA levels of *hsps* of *B. calyciflorus* treated under H_2O_2 (0.2 mM) stress condition. The expression levels of *hsps* mRNA was statistically analyzed by one-way ANOVA with the Tukey test.

The different letters indicate a significant difference for each time point with control ($p < 0.05$): **a** *hsp40*, **b** *hsp60*, **c** *hsp70*, and **d** *hsp90*

intermediate that generates intracellular nonradical ROS (Rosa et al. 2008). It can easily diffuse through cellular membranes into the cell, react with transition metals, and generate hydroxyl radicals ($HO\cdot$) (Abele-Oeschger et al. 1997). As an oxidant, H_2O_2 can regulate the expression of many genes, for example superoxide dismutase (SOD) and the *hsp20* gene in the rotifer *Brachionus* sp. (Rhee et al. 2011). In the present study, to analyze the modulatory effect of oxidative stress on *Bchsp*s genes, we initially exposed rotifers to H_2O_2 for various time periods and found that H_2O_2 significantly increased the mRNA expression of these four *Bchsp*s. The high expression levels of *Bchsp*s contributed to activation of the HSR, and protected cells from oxidative stress. Previous studies have also shown that environmental stress (i.e., heavy metals, osmotic stress, and various chemicals) increased the expression of *hsps* (Rios-Arana et al. 2005; Seo et al. 2006; Ventura et al.

2007; Wheelock et al. 1999). The expression of *hsps* was significantly induced by typical temperature pressure and oxidative stress and reflected the characteristics of the *hsps* as “stress proteins.”

Aging is likely regulated by low affinity, low specificity interactions, and mediated through a complex network of overlapping pathways (Snell et al. 2012). The quality of proteins declines during the aging process, for example, in 80-year-old humans, half of all proteins may become oxidized (Stadtman and Berlett 1998). Due to these degradation defects, damaged proteins accumulate in the cells of aging organisms, and this may cause a variety of protein folding diseases (Söti and Csermely 2002). HSPs are present in the cells of all living organisms; they act as molecular chaperones and can facilitate protein folding, prevent protein aggregation, and target improperly folded proteins to specific degradation pathways (Kalmar and Greensmith 2009). For

Fig. 6 Effect of DR on rotifer lifespan. **a** Feeding regimes. **b** Survival rate of *B. calyciflorus* cultured in different feeding regimes. **c** Mean lifespan of *B. calyciflorus* cultured in different feeding regimes

example, HSP40s are important for protein homeostasis because they stimulate the ATPase activity of the HSP70 proteins that are involved in protein translation, folding, unfolding, translocation, and degradation (Qiu et al. 2006). HSP60s is a mitochondrial chaperonin that is typically held responsible for the transportation and refolding of proteins from the cytoplasm into the mitochondrial matrix (Koll et al. 1992). HSP70s play a role in protein synthesis under normal cellular conditions, fixing denatured proteins and preventing the misfolding or aggregation of proteins (Danwattanasorn et al. 2011). HSP90 participates in the folding, maintenance of

Fig. 7 Effect of DR on rotifer reproduction. **a** The fecundity of *B. calyciflorus* in the AL and DR groups at each time point. **b** Absolute fecundity of *B. calyciflorus* cultured in different feeding regimes. **c** The number of eggs carried by *B. calyciflorus* in the AL and DR groups at each time point

structural integrity, and the proper regulation of a subset of cytosolic proteins (Picard 2002). HSPs contribute to protein quality control and are related to aging owing to their functions mentioned above (Becker and CRAIG 1994;

Fig. 8 Lifespan and fecundity of *B. calyciflorus* cultured in vitamin E. y-axis (left) is the survival rate. y-axis (right) is the number of daughters. x-axis is survival (in hours)

Fig. 9 *Hsps* mRNA expression of *B. calyciflorus* of different age classes. The expression levels of *hsps* mRNA was statistically analyzed by one-way ANOVA with the Tukey test: **a** *hsp40*, **b** *hsp60*, **c** *hsp70*, and **d** *hsp90*

Fig. 10 *Hsps* mRNA expression of *B. calyciflorus* of different feeding regimes. The expression levels of *hsps* mRNA was statistically analyzed by one-way ANOVA with the Tukey test. The different letters

indicate a significant difference for each time point with control ($p < 0.05$): **a** *hsp40*, **b** *hsp60*, **c** *hsp70*, and **d** *hsp90*

Calderwood et al. 2009; Nardai et al. 2002; Soti and Csermely 2003). The induced expression of HSPs in response to various stresses is dependent on the activation of specific members of a family of transcription factors, the heat shock factors (HSFs), which bind to the heat shock elements in the promoters of the genes encoding HSPs (Åkerfelt et al. 2010). Previous research has recently shown that increased protein damage during aging may be exacerbated by reduced levels of HSPs, and the resultant loss of protein quality control (Calderwood et al. 2009). In this study, we examined the effects of aging on the gene expression of four rotifer *Bchsp*s genes to determine the relationship between aging and these four *Bchsp*s genes. We found that *Bchsp70* mRNA expression was significantly decreased with aging. HSP70 is mostly found in the cytosol and nucleus and is used for protein folding and cytoprotection as well as a molecular chaperone (Becker and CRAIG 1994). The cloned BcHSP70 contains a

highly conserved typical C-terminal retention sequence (HDEL in mammalian and KDEL in yeast cells) that is common for soluble ER-localized proteins and inhibits its exit from the ER (MUNRO and PELHAM 1987). This ER-localized HSP70 protein, initially described as the mammalian glucose-regulated protein GRP78 (Lee et al. 1981; Munro and Pelham 1986) is induced by the accumulation of misfolded proteins inside the ER (Kozutsumi et al. 1988; Rose et al. 1989). Evidence has indicated that HSP70s in the ER not only aid protein folding and assembly but also may be directly involved in the import of secretory proteins (Vogel et al. 1990). Thus, the decrease in expression of *Bchsp70* mRNA means that accumulation of damaged proteins is accelerated. In other organisms, the increased expression of *hsp70* gene can extend the lifespan of *S. cerevisiae* and *D. melanogaster* (Shama et al. 1998; Tatar et al. 1997). On the other hand, the presence of misfolded proteins (damaged proteins) can induce

Fig. 11 Relative mRNA levels of *hsps* of *B. calyciflorus* treated with vitamin E (40 μ M). The expression levels of *hsps* mRNA was statistically analyzed by one-way ANOVA with the Tukey test. The different

letters indicate a significant difference for each time point with control ($p < 0.05$): **a** *hsp40*, **b** *hsp60*, **c** *hsp70*, and **d** *hsp90*

activation of the HSR by activating the transcription factors, HSFs (Abravaya et al. 1992; Morimoto 1992). Age-dependent waning of the HSR is a general effect found in neuronal tissues (Hands et al. 2008; Sherman and Goldberg 2001; Winklhofer et al. 2008), skeletal muscle, cardiac muscle (Kayani et al. 2008), and liver (Gagliano et al. 2007). The decline in HSR in aging cells indicated that the capacity of the organism to resist stress diminished. The decline in the HSR may be one of the factors which cause aging.

As a natural stress, moderate starvation has been reported to enhance the expression of many genes, including SOD (Kaneko et al. 2005; Kaneko et al. 2011), catalase (CAT) (Kaneko et al. 2011), *hsp70*, and GRP94 (Kaneko et al. 2002). DR is the most common starvation method for prolonging life in the laboratory, and it has been reported to extend the lifespan of different organisms (Fanestil and Barrows 1965; Klass 1977; Lin et al. 2002; Walker et al.

2005). In rotifers, previous studies have also suggested that DR can extend the lifespan of different types of rotifers (Ozdemir 2009; Weithoff 2007; Yoshinaga et al. 2000). Interestingly, DR-induced longevity and other beneficial effects can be transmitted to subsequent generations in *B. plicatilis* (Kaneko et al. 2011). In the present study, we found that DR significantly extended the lifespan of the rotifer, *B. calyciflorus*, and reduced fecundity. Our findings are in accordance with previous studies where DR extended the lifespan accompanied by a reduction of infertility in *D. melanogaster* (Chapman and Partridge 1996; Chippindale et al. 1993; Partridge et al. 2005) and in rotifers (Ozdemir 2009; Yoshinaga et al. 2000). We also found that DR significantly increased the expression of *Bchsp40*, *Bchsp60*, *Bchsp70*, and *Bchsp90* mRNA. The expression of HSPs is not only induced by heat stressors but also by other stressors (Feder and Hofmann 1999), and upregulated HSPs are generally

Fig. 12 Effects of DR pretreatments on the *B. calyciflorus* survival rate under oxidative stress conditions (paraquat). **a** Experimental designs. **b** The survival rate of *B. calyciflorus* in the AL and DR pretreatment groups at each time point. The survival rate was statistically analyzed by one-way ANOVA with the Duncan test

described as part of the cellular stress response (Roberts et al. 2010). The increased expression of HSPs may contribute to diminish the accumulation of damaged proteins and extend the lifespan (Tatar et al. 1997). These examples confirm the hypothesis that HSPs have a better adaptation capacity to various stresses which greatly increases the chances of longevity.

Vitamin E is known to function as an antioxidant, and it has long been investigated for its effects on rotifer aging. Therefore, it was necessary for us to further determine the relationship between HSPs and aging through the anti-oxidative and anti-aging effects of vitamin E. It was reported by Sawada and Enesco that when the rotifer *Asplanchna brightwellii* was exposed to 25 $\mu\text{g/mL}$ (58 μM) of vitamin E, its lifespan was extended by 21 % due to lengthening of the pre-reproductive and reproductive periods (Sawada and Enesco 1984). Litton reported that exposure to α -tocopherol extended the lifespan of four other rotifer species by about 10 % (Litton 1987). However, in the present study, we found that vitamin E had no effects on rotifer lifespan or reproduction. These inconsistent results may be caused by the differences in rotifer species. In recent research, Snell et al found that the lifespan of the rotifer, *B. manjavacas*, was extended only when antioxidants were paired with trolox, *N*-acetyl cysteine, or L-carnosine. Trolox

treatment alone did not prolong the lifespan of rotifers (Snell et al. 2012). This also suggested that antioxidant treatment could not always extend the lifespan of rotifers. Actually, the anti-aging effect of vitamin E is also controversial in worms. For example, vitamin E (α -tocopherol) increased lifespan in the first two studies (Harrington and Harley 1988; Ishii et al. 2004) but not in the third (Adachi and Ishii 2000). Likewise, trolox, an α -tocopherol derivative, increased lifespan in Benedetti's study (Benedetti et al. 2008) but not in Schulz's (Schulz et al. 2007). We analyzed the expression of *Bchsp*s in rotifers after exposure to vitamin E and found that vitamin E significantly increased the mRNA expression of *Bchsp60* only and not the other three *hsp*s. This suggests that vitamin E may not improve the HSR in rotifers. To some extent, this also explains why vitamin E does not extend the lifespan of rotifers.

Aging has been associated with a loss of stress resistance, and treatments that extend lifespan are often associated with increased stress resistance (Kirkwood and Austad 2000; Vermeulen and Loeschcke 2007). Previous studies showed that there was a close correlation between stress resistance and longevity in several long-lived *C. elegans* and *Drosophila* mutants (Lithgow and Kirkwood 1996). To assess resistance to oxidative stress in rotifers, we used paraquat, a

widely used compound, to generate ROS inside the cells (Kaneko et al. 2011). Under paraquat-induced oxidative stress, moderate DR pretreatment increased rotifer survival rate. Our findings are in accordance with previous studies which showed that treatments which extended lifespan increased stress resistance. *Rosa damascena* is a rose hybrid commonly harvested for its oil used in perfumery and for rose water used to flavor food. It was reported by Schriner et al. that *R. damascena* extended the lifespan of *D. melanogaster* through a DR-associated mechanism (Schriner et al. 2012). The authors also found that *R. damascena* could improve *D. melanogaster* resistance to paraquat and H₂O₂. The same study also showed that the natural product, black tea (*Camellia sinensis*), blocked the age-dependent accumulation of iron, improved iron tolerance, and extended the lifespan of *D. melanogaster* (MASSIE et al. 1993). Enhanced stress resistance is likely to benefit from the upregulated expression of *hsps*. In fact, overexpression of *hsp22* has been shown to increase fly lifespan, protect flies against paraquat, and improve their locomotor ability (Morrow et al. 2004). In rotifers, DR pretreatment was reported to benefit paraquat and juglone tolerance (Kailasam et al. 2011; Ozaki et al. 2010). A previous study indicated that the beneficial effects of DR may contribute to the improvement in antioxidant gene expression (SOD and CAT) (Yang et al. 2013). In the present study, we also found that inducing the expression of *hsps* by DR may be helpful for DR-induced extended lifespan. DR also had other beneficial effects, such as resistance to oxidative stress.

Conclusions

In this study, we successfully cloned four typical *hsp* genes (*hsp40*, *hsp60*, *hsp70*, and *hsp90*) from the rotifer *B. calyciflorus* Pallas, analyzed the molecular characteristics, and found that *Bchsp*s was significantly induced by temperature and H₂O₂ stress. The mRNA expression of *Bchsp70* decreased with age which indicated waning of the HSR with aging in rotifers. DR extended the lifespan and improved resistance to paraquat, and these findings may have benefitted from the upregulated expression of *hsps*.

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