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Heat stress-mediated gene expression in the body wall of the Japanese sea cucumber *Apostichopus japonicus*

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ABSTRACT: Suppression subtractive hybridization was used to analyze the differences in gene expression in the body wall of the Japanese sea cucumber *Apostichopus japonicus*, induced by heat stress (28°C), in order to understand the response at the molecular level. A total of 737 clones was selected and analyzed, and 165 distinct sequences were obtained, with 65 sharing similarity to sequences in public databases. The sequenced genes were classified into 3 functional categories. Increased expression of some genes in response to stress was concomitant with decreased expression of genes concerned with energy production and metabolism. The results (1) suggest that heat stress has significant effects on gene expression in *A. japonicus*, and (2) provide the basis for further study of molecular regulation mechanisms underlying the response to heat stress and estivation in *A. japonicus*.

KEY WORDS: *Apostichopus japonicus* · Body wall · Heat stress · Suppression subtractive hybridization · Stress response

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INTRODUCTION

The Japanese sea cucumber *Apostichopus japonicus*, a marine poikilotherm found along Asian coasts, has been exploited as an important economic resource in Russia, China, Japan, and Korea (Liao 1997). In response to bottom temperatures >20°C, adult *A. japonicus* stop feeding and reduce oxygen consumption rate, entering a dormant state called estivation (Choe 1963, Li et al. 1996, Liu et al. 1996). Earlier studies showed that estivation is temperature-dependent and that *A. japonicus* in the estivation state can be reactivated by culturing at a reduced temperature (Li et al. 1996, Liu et al. 1996, Yang et al. 2006).

Heat stress influences physiological and biochemical mechanisms and has a significant effect on growth and energy metabolism in *Apostichopus japonicus* (Li et al. 2002, Yang et al. 2006, Dong et al. 2007, 2008). In response to heat stress, the activity of antioxidases (e.g. superoxide dismutase and catalase) is enhanced and the content of catechol hormones is increased (Wang et al. 2008). Changes in physiological processes under environmental stress are the result of changing gene expression (Gracey et al. 2001, De la Vega et al. 2007, Lü & Wan 2008). A temperature increase is accompanied by increased expression of heat-shock protein 70 (Hsp70) and activation of the DNA repair system in *A. japonicus* (Dong et al. 2007, 2008), but studies have been focused on molecular chaperones and there are few reports of the expression of other genes related to heat stress. There is a need to elucidate details of the molecular regulatory mechanism in *A. japonicus* in response to heat stress.

Suppression subtractive hybridization (SSH) has been used in many marine animals to identify genes that are expressed differentially after exposure to sev-

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eral biotic and abiotic factors. This method has identified such genes in black tiger shrimp *Penaeus monodon* exposed to a range of environmental stressors (De la Vega et al. 2007). In addition, SSH has been used to study the effects of hypoxia on the mudsucker fish *Gillichthys mirabilis* (Gracey et al. 2001) and in blue crab *Callinectes sapidus* (Brouwer et al. 2004), and to analyze expression of genes in response to lipopolysaccharide challenge in mud crab *Scylla paramamosain* (Chen et al. 2010) and in giant freshwater prawn *Macrobrachium rosenbergii* (Lu et al. 2009). In the present study, SSH was used to isolate and identify the genes expressed differentially in the body wall of *Apostichopus japonicus* in response to an acute heat shock (2 h at 28°C). The aim was to find more candidate genes involved in the response to heat stress and to gather information to aid further understanding of estivation.

MATERIALS AND METHODS

Experimental sea cucumbers. Specimens of *Apostichopus japonicus* (average weight: 53.8 ± 5.7 g) were collected at 19°C from the Yellow Sea in Jiaozhou Bay, China, on 2 June 2009 and transferred to the laboratory. The holothurians were acclimated in seawater (salinity 30) at 18°C in a 500 l tank for 1 wk with continuous aeration. Half of this water was exchanged for fresh seawater daily, and the holothurians were fed twice daily with a laboratory-made formula: 36.36 ± 0.39% (w/w) water, plus 63.64 ± 0.26% (w/w) dry matter, which included 5.04 ± 0.19% (w/w) crude protein, 0.26 ± 0.05% (w/w) fat, and 72.20 ± 0.19% (w/w) ash.

Heat stress test. The threshold temperature for estivation in *Apostichopus japonicus* is 28°C (Liu et al. 1996, Yang et al. 2006), and this temperature was used to examine the relationship between heat stress and expression of the *hsp70* gene in *A. japonicus* (Liu 2008). In the present study the same temperature was used to examine the molecular regulation mechanism underlying the heat stress response. Thirty specimens were divided randomly into 2 groups of 15. The heat-treated group was put into seawater at 28°C for 2 h and then allowed to recover for 2 h at 18°C. The other group was kept at 18°C as the control group. After recovery, a sample of the body wall (mid-dorsal body wall near the anus) of each individual was collected, frozen with liquid nitrogen, and stored at –80°C.

RNA extraction. Total RNA was extracted from the frozen tissue using TRIzol® reagent (Invitrogen) following the manufacturer's instructions. Total RNA was pooled for each group (control and heat-treated), and mRNA was purified with an Oligotex mRNA kit (QIAGEN) according to the manufacturer's instructions.

SSH and construction of an SSH library. Forward and reverse SSH libraries were constructed for analysis of gene expression induced by heat treatment. The forward SSH library used the cDNA from the heat-treated group as the tester and the cDNA from the control as the driver in order to find the up-regulated genes. The reverse SSH library was used to identify the down-regulated genes, using the cDNA from the control as a tester and the cDNA from the heat-treated group as a driver (Diatchenko et al. 1996, 1999). SSH was done with the PCR-Select™ cDNA Subtraction Kit (Clontech) according to the manufacturer's instructions. Briefly, double-stranded cDNA was generated from 2 µg of extracted poly(A)$^+$ RNA. The cDNA samples were precipitated in ethanol, digested with 15 U of *Rsa*I at 37°C for 2 h, then extracted with phenol/chloroform/isoamyl alcohol (25:24:1, by vol.), followed by extraction with chloroform/isoamyl alcohol (24:1, v/v), then extracted with 4 M ammonium acetate/95% alcohol (1:7.5, v/v), and twice precipitated in ethanol. Two different adaptors, Adaptor 1 and Adaptor 2R (Table 1), were ligated to the 5′ end of each strand of tester cDNA with 400 U of T4 bacteriophage DNA ligase. The tester cDNA samples were each mixed with driver cDNA, denatured at 98°C for 90 s, and hybridized at 68°C for 8 h. After the first hybridization, the 2 mixtures were combined and hybridized again with freshly heat-denatured driver cDNAs overnight at 68°C. The cDNAs containing both adaptors were identified by 2 rounds of PCR. The first PCR was done with Primer 1 (Table 1) against Adaptors 1 and 2R at 94°C for 25 s, then 27 cycles of 94°C for 10 s, 66°C for 30 s, and 72°C for 90 s. The amplified products were used as a template in the second PCR with Nested primers 1 and 2R (Table 1). The reaction was 12 cycles at 94°C for 10 s, 68°C for 30 s, 72°C for 90 s, and a final extension at 72°C for 5 min.

The PCR products were purified after precipitation in ethanol as follows: 50 ng of insert was ligated to pGEM®-T easy vector (Promega) and used to transform DH5α-competent cells according to the manufacturer's instructions. Transformed cells were grown overnight at 37°C on an Luria-Bertani (LB) agar plate containing 100 µg ml^{-1} ampicillin.

Identification of positive clones. White colonies were picked at random from each SSH library and inserted into 1.5 ml of LB medium containing 100 µg ml^{-1} ampicillin and incubated overnight at 37°C. The PCR reaction was done with 0.2 µl of culture with Nested primers 1 and 2R (Table 1). The PCR reaction profile was: 10 min at 94°C, then 25 cycles of 94°C for 10 s, 68°C for 30 s, 72°C for 90 s, and a final extension at 72°C for 5 min. The inserted fragment size of the chosen clones was identified by 1.5% (w/v) agarose gel electrophoresis and the selected clones were iden-

Table 1. Oligonucleotide primer and adaptor sequences. F: forward; R: reverse

Primer	Target gene	Sequence	Product size (bp)
Hsp20.8 (F) Hsp20.8 (R)	HSP20.8	5′-AGG GAA GAA GGA GGA GGT-3′ 5′-TGC ATA AAC AGC GTC ATA G-3′	191
Hsp90 (F) Hsp90 (R)	HSP90	5′-CCA TGT CTT CTT TAA GGG TCA-3′ 5′-CCA GTT TGG TGT TGG CT-3′	170
Hsp70 (F) Hsp70 (R)	HSP70	5′-CAC CAA GGA CGC AGG AGT-3′ 5′-CAC CGT TAT CAA TGG TCA GG-3′	173
Ferritin (F) Ferritin (R)	Ferritin	5′-CGC TTC AGA TTG GTG ATG TGG T-3′ 5′-GAA GAA CGT GAA CAT GCC GAG A-3′	290
Zinc finger (F) Zinc finger (R)	Zinc finger protein	5′-GTC GCT GAA CCA AGA TAG CC-3′ 5′-CCA AAC CGG TAG CAC CTA AG-3′	165
COX (F) COX (R)	Cytochrome *c* oxidase	5′- AGC AGG AAT GGT TGG AAC AG -3′ 5′- GCC ATG TCT GGA GCA CCT AT -3′	204
CoA (F) CoA (R)	Succinyl-CoA synthetase	5′-AAG CCT GGT CAG TGC AAG AT-3′ 5′-TGT CCA GGC AGT CAA CAA AG-3′	196
Histone (F) Histone (R)	Histone	5′-CTG CCT GTT CAA TCA AAG CA-3′ 5′-CAA GCG TGT AAC CAT CAT GC-3′	234
Actin (F) Actin (R)	β-actin	5′-CAT TCA ACC CTA AAG CCA ACA-3′ 5′-TGG CGT GAG GAA GAG CAT-3′	198
NADH (F) NADH (R)	NADH-ubiquinone oxidoreductase	5′-GTC CTA CGA CCC AAT CTG GA-3′ 5′-ATG AGC CTT GGT TAC GTT GG-3′	196
Adaptor 1		5′-CTA ATA CGA CTC ACT ATA GGG CTC GAG CGG CCG CCC GGG CAG GT-3′	
Adaptor 2R		5′-CTA ATA CGA CTC ACT ATA GGG CAG CGT GGT CGC GGC CGA GGT-3′	
Primer 1		5′-CTA ATA CGA CTC ACT ATA GGG C-3′	
Nested primer 1		5′-TCG AGC GGC CGC CCG GGC AGG T-3′	
Nested primer 2R		5′-AGC GTG GTC GCG GCC GAG GT-3′	

tified by dot hybridization; the forward probe was the 2-round PCR products of the forward library labeled with [α-^{32}P]dATP, and the reverse probe was the 2-round PCR products of the reverse library. Preparation of the probe was done with the Amersham Megaprime DNA Labeling System (GE Healthcare) according to the manufacturer's instructions: the products of 2-round PCRs in both libraries were each allowed to react separately with primer at 95°C for 10 min, then [α-^{32}P]dATP was added and the mixture was incubated at 37°C for 1 h. The reaction was terminated with 2 µl of 500 mM EDTA and the products were stored at –20°C. The denatured PCR product (1 µl) was spotted onto nylon membrane and hybridized at 68°C for 3 h. A [α-^{32}P]dATP-labeled probe was added for hybridization at 68°C overnight. Each clone was done in triplicate. An average ratio (intensity of signal with forward probe divided by intensity of signal with reverse probe) of at least 2 (in the forward library) or at most 0.05 (in the reverse library) was considered positive and was selected for further analysis.

Clone sequence and analysis. Positive clones were sequenced using ABI 3730 automated sequencers (Applied Biosystems) at Shanghai United Gene Group. The sequences obtained were analyzed by DNAstar for alignment. Nucleotide homology comparison was done with the basic local alignment search tool (BLAST), using BLASTn, BLASTx, and BLASTp (www.ncbi.nlm.nih.gov/BLAST). For each comparison, a BLAST E-value < 10^{-5} was regarded as a positive result. Gene ontology (GO) annotation was analyzed in the GO database (http://amigo.geneontology.org/cgi-bin/amigo/go.cgi) using AmiGO.

Real-time PCR analysis. ***Reference gene selection:*** Two reference genes (those coding for NADH-ubiquinone oxidoreductase and β-actin) were selected for analysis. The specific primers for these 2 genes were designed using sequences in GenBank (accession no. EU668024) for β-actin, and the *Apostichopus japonicus* expressed sequence tag (EST) database, which was constructed in our laboratory using the 454 sequencing system, for NADH-ubiquinone oxidoreductase (Table 1). For comparison of control and heat-treated individuals, the expression of the gene coding for β-actin in *A. japonicus* was the same irrespective of conditions and therefore was selected as the reference gene for this species.

Confirmation of subtraction efficiency: To evaluate subtraction efficiency, real-time PCR was used to analyze the depletion of transcript abundance of the gene coding for β-actin in the subtracted tester cDNA compared to the unsubtracted cDNA. The primer informa-

tion is given in Table 1 and each reaction was run in triplicate. The reaction profile was denaturation at 95°C for 10 s, then 40 cycles of 95°C for 5 s, 59°C for 20 s, 72°C for 30 s, followed by a melt curve step in Mastercycler® ep realplex (Eppendorf). The data were analyzed with realplex software version 2.2 (Eppendorf).

Confirmation of differential expression: Eight different clones were selected and the different levels of gene expression in the sea cucumber body wall in response to heat stress were analyzed by real-time quantitative PCR (qPCR). RNA extraction and cDNA synthesis were done as described in 'Materials and methods — RNA extraction'. Briefly, 2 µg of total RNA was reverse-transcribed using 200 U of M-MLV reverse transcriptase (Promega), 40 U of cloned ribonuclease inhibitor (Takara), and a 5 µM dNTP mixture in a reaction volume of 25 µl. cDNAs were diluted in nuclease-free water and stored at –20°C.

Real-time qPCR was done in a 25 µl reaction mixture containing 2 µl of cDNA, 0.2 µmol of each gene-specific primer (Table 1), and 12.5 µl of SYBR Green PCR Master Mix (Takara). Each reaction was run in triplicate. The reaction profile was 95°C for 10 s, followed by 40 cycles of 95°C for 5 s, 59°C for 20 s, and 72°C for 30 s. After PCR, melting curve analysis was used to demonstrate the specificity of the PCR products. All products generated a single, discrete peak in the melting curve analysis. The average C_t value of each triplicate reaction was calculated using realplex software version 2.2 with the gene coding for β-actin as the reference gene. Statistical analysis was done by ANOVA using SPSS software (version 11.5), and the level of statistical significance was set at $p < 0.05$.

RESULTS

Sequencing and analysis of clones in the SSH library

Forward and reverse SSH libraries were constructed to identify *Apostichopus japonicus* genes in the body wall that demonstrated a change of expression in response to heat stress. Due to the lack of molecular information about this holothurian, we selected 768 cDNA clones at random from both libraries and amplified them by PCR; an average insert size of 478 bp (range: 200 to 1000 bp) was observed. Using dot hybridization, 737 clones with a ratio of at least 2 (in the up-regulated library) or at most 0.05 (in the down-regulated library) were selected, it was found that in the forward library 63.8% (231/362) clones represented the gene encoding HSP70. PCR products were assembled and edited. Finally, 165 ESTs were obtained: 117 in the down-regulated library and 48 in the up-regulated library.

Comparison of the 165 distinct EST sequences to those in GenBank revealed that 65 had a high level of similarity to genes with known function (BLAST *E*-value < 10^{-5}) and 100 sequences had no significant similarity to any gene (BLAST *E*-value > 10^{-5}). After assembly of the 165 sequences, 46 unique genes were identified: 14 were found only in the up-regulated library, 29 only in the down-regulated library, and 3 in both libraries (Table 2). The functions of ESTs with significant similarity to sequences in the databases were predicted by AmiGO. All genes were clustered by function (Fig. 1), and in the up-regulated library, genes related to a response to stimulus showed changed expression, but expression of some genes participating in cell component or biological processes (growth, organelle part, etc.) was not observed.

Subtraction efficiency

The subtraction efficiency was determined by real-time qPCR amplification of a housekeeping gene (that coding for β-actin) in subtracted and unsubtracted cDNAs. The amount of β-*actin* template in unsubtracted cDNAs was 10^3- to 10^4-fold greater than the amount in subtracted cDNAs (Fig. 2). Expression of the housekeeping gene was clearly decreased after SSH.

Levels of gene expression

Using real-time qPCR, the expression of 8 genes with different functions in *Apostichopus japonicus* was determined (Fig. 3). All of these genes showed a changed level of expression in response to heat stress. Expression of the mRNA of the heat-shock protein family was significantly different after the heat shock, and expression of the genes encoding the zinc finger protein and ferritin was also increased significantly after the heat shock. The expression of the genes coding for histone, succinyl-CoA synthetase, and cytochrome *c* oxidase (COX) was decreased in response to heat stress. Following the heat shock, the level of expression changed significantly ($p < 0.05$) for 7 of the 8 genes but not for the gene encoding histone.

DISCUSSION

Change of gene expression under heat stress in *Apostichopus japonicus*

Water temperature has an important influence on the activity and survival of marine animals (Fisher 1988, Wang & Li 2002, Monari et al. 2007, Dong et al.

Table 2. Different expression clones in *Apostichopus japonicus* heat-shock suppression subtractive hybridization libraries with BLASTp (BLAST *E*-value < 10^{-5}). Down: reverse library; up: forward library

Clone ID	Redundancy	Gene coding for	Species with homology to	GenBank accession no.	Library	*E*-value
HO054974	7	Heat shock protein 70	*Apostichopus japonicus*	ACJ54702.1	Up	1×10^{-123}
HO054975	1	Ferritin	*Apostichopus japonicus*	AAY89589.1	Up	1×10^{-63}
HO054976	3	Heat shock protein 90-alpha	*Salmo salar*	ACN10985.1	Up	3×10^{-74}
HO054977	3	Heat shock protein 20.8	*Liriomyza sativae*	ABE57141.1	Up	3×10^{-10}
HO054978	2	Zinc finger protein	*Strongylocentrotus purpuratus*	XP_785663.2	Up	8×10^{-69}
HO054981	1	Ribosomal proteinL32	*Strongylocentrotus purpuratus*	XP_782126.1	Up	2×10^{-43}
HO054982	1	Dna J (hsp40) homolog	*Danio rerio*	NP_956067.1	Up	1×10^{-41}
HO054983	1	Putative 60S ribosomal protein	*Novocrania anomala*	ACD65123.1	Up	2×10^{-34}
HO054984	1	Predicted protein	*Nematostella vectensis*	XP_001625396.1	Up	1×10^{-30}
HO054985	1	Ribosomal protein rpl7a	*Arenicola marina*	ABW23163.1	Up	1×10^{-18}
HO054986	1	Similar to Protein lethal (2) essential for life	*Apis mellifera*	XP_001120194.1	Up	2×10^{-14}
HO054987	1	Alpha B crystallin	*Homo sapiens*	ACP18852.1	Up	5×10^{-10}
HO054988	1	Pherophorin-dz1 protein	*Volvox carteri* f. *nagariensis*	CAD22154.1	Up	2×10^{-9}
HO054989	1	Unnamed protein product	*Tetraodon nigroviridis*	CAF90222.1	Up	2×10^{-13}
HO054979	2	Myosin heavy chain	*Strongylocentrotus purpuratus*	XP_785810.2	Both	1×10^{-80}
HO054980	3	Major yolk protein 2	*Apostichopus japonicus*	BAH79577.1	Both	4×10^{-34}
HO055005	3	Alpha-5 collagen	*Strongylocentrotus purpuratus*	XP_795694.2	Both	2×10^{-15}
HO056151	1	Major yolk protein 1	*Apostichopus japonicus*	BAH79576.1	Down	1×10^{-117}
HO054990	1	Succinyl-CoA synthetase alpha subunit	*Strongylocentrotus purpuratus*	XP_786544.2	Down	3×10^{-83}
HO054991	3	Cytochrome *c* oxidase, subunit Va	*Strongylocentrotus purpuratus*	XP_784558.1	Down	2×10^{-58}
HO054992	1	Scavenger receptor cysteine-rich protein type 12	*Strongylocentrotus purpuratus*	NP_999762.1	Down	4×10^{-79}
HO054993	1	Elongation factor	*Caenorhabditis elegans*	AAD03339.1	Down	3×10^{-57}
HO054994	2	NADH dehydrogenase subunit 1	*Apostichopus japonicus*	ACM66296.1	Down	1×10^{-55}
HO054995	1	Ribosomal protein rps12	*Eurythoe complanata*	ABW23206.1	Down	3×10^{-55}
HO054996	1	60S ribosomal protein L9	*Ictalurus punctatus*	Q90YW0.1	Down	4×10^{-46}
HO054997	1	Annexin	*Strongylocentrotus purpuratus*	XP_795341.2	Down	3×10^{-38}
HO054998	1	Ribosomal protein L30	*Strongylocentrotus purpuratus*	XP_783150.1	Down	4×10^{-38}
HO054999	1	Complement component C3	*Strongylocentrotus purpuratus*	XP_001185680.1	Down	3×10^{-37}
HO055000	1	Predicted protein	*Nematostella vectensis*	XP_001625568.1	Down	2×10^{-36}
HO055001	1	H3.3 histone	*Strongylocentrotus purpuratus*	XP_791401.1	Down	5×10^{-35}
HO055002	1	Ribosomal protein L44	*Strongylocentrotus purpuratus*	XP_797556.2	Down	1×10^{-34}
HO055003	1	Hypothetical protein BRAFLDRAFT_88602	*Branchiostoma floridae*	XP_002604312.1	Down	2×10^{-31}
HO055004	1	Neurotrypsin	*Danio rerio*	XP_690649.2	Down	3×10^{-30}
HO055006	1	Hypothetical protein BRAFLDRAFT_74514	*Branchiostoma floridae*	XP_002598809.1	Down	3×10^{-21}
HO055007	1	Septin 7b isoform 2	*Danio rerio*	NP_001119922.1	Down	8×10^{-21}
HO055008	1	Complement C4	*Oncorhynchus mykiss*	NP_001117857.1	Down	4×10^{-7}
HO055009	1	C14orf147 homolog	*Oncorhynchus mykiss*	ACO08325.1	Down	1×10^{-6}
HO055010	1	MEGF10 protein	*Gallus gallus*	XP_424719.2	Down	1×10^{-21}
HO055011	1	60S ribosomal protein L35	*Lycosa singoriensis*	ABX75380.1	Down	1×10^{-16}
HO055012	1	AMPA receptor subunit GluR3B, partial	*Strongylocentrotus purpuratus*	XP_001188377.1	Down	4×10^{-12}
HO055013	1	GD11645	*Drosophila simulans*	XP_002082584.1	Down	1×10^{-6}
HO055014	1	Brain RPTPmam4 iso-form II	*Strongylocentrotus purpuratus*	XP_783327.1	Down	3×10^{-6}
HO055015	1	MGC97760 protein	*Xenopus* (*Silurana*) *tropicalis*	NP_001015831.1	Down	1×10^{-5}
HO055016	1	Predicted protein	*Trichoplax adhaerens*	XP_002109456.1	Down	3×10^{-5}
HO055017	1	Hypothetical protein PANDA_006529	*Ailuropoda melanoleuca*	EFB29313.1	Down	7×10^{-25}
HO055018	1	Ribosomal protein L23a	*Strongylocentrotus purpuratus*	XP_001201900.1	Down	7×10^{-9}

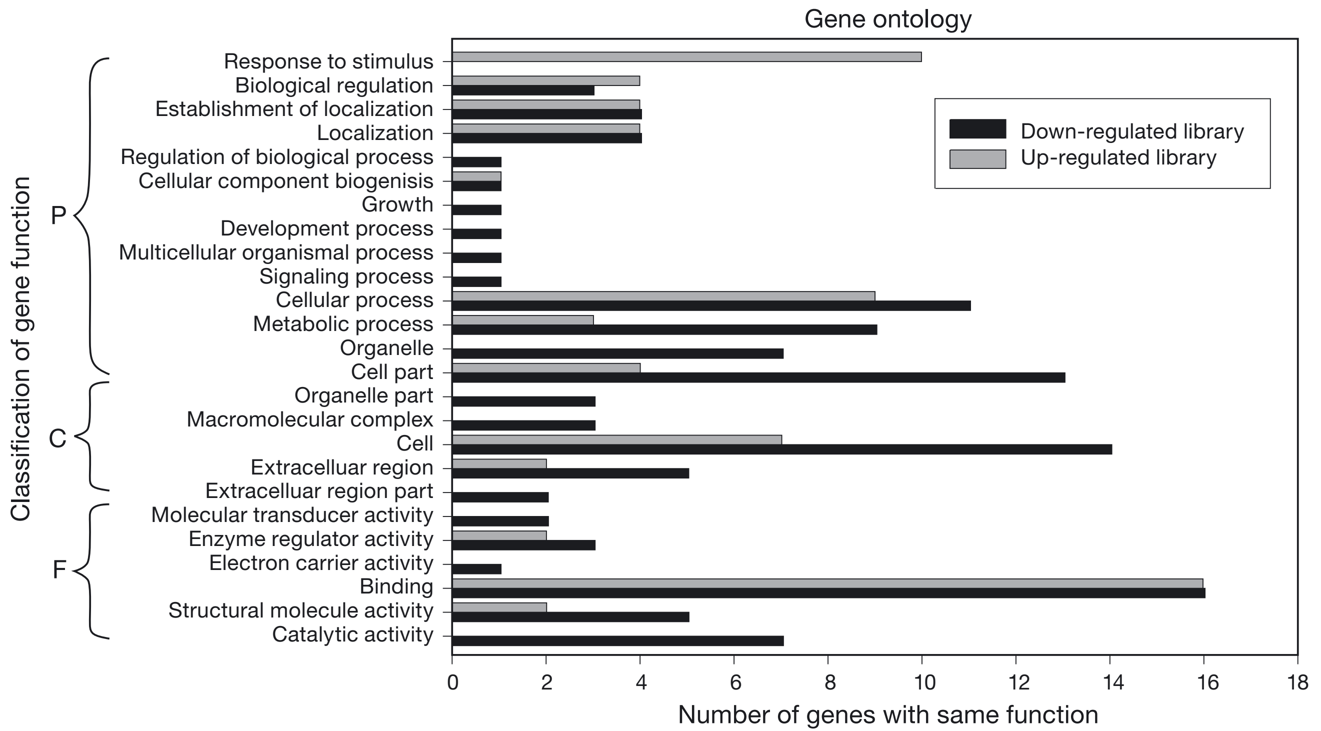

Fig. 1. Distribution of genes screened from suppression subtractive hybridization library from heat-shocked *Apostichopus japonicus* by functional class. P: biological process; C: cell component; F: molecular function

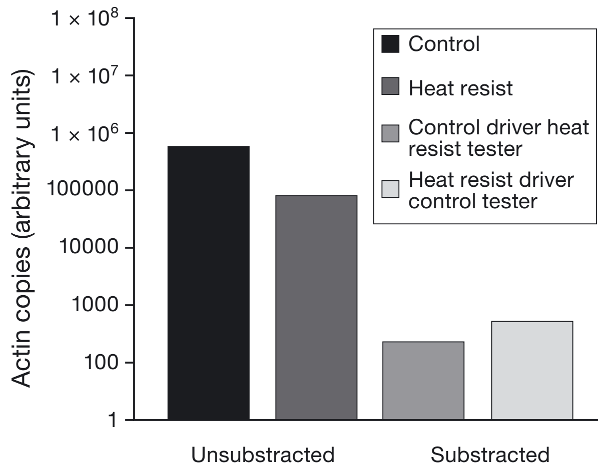

Fig. 2. Real-time quantitative PCR analysis of transcript abundance of the gene coding for β-actin during suppression subtractive hybridization (SSH). Left 2 vertical bars represent concentration of β-actin gene in unsubtracted control nested PCR reactions; right 2 bars represent concentration of β-actin gene in nested PCR reactions following SSH

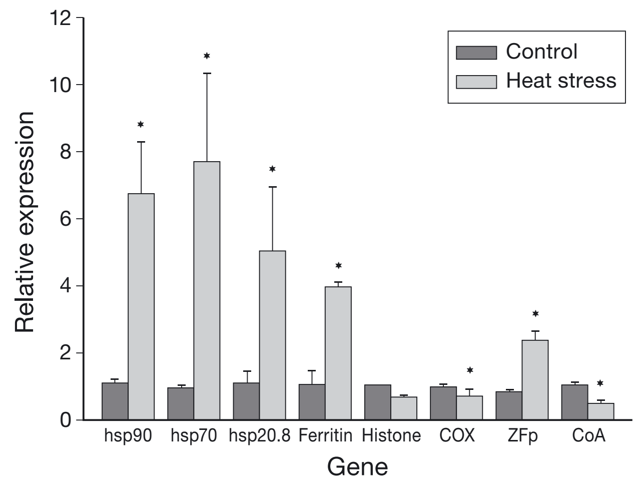

Fig. 3. Expression analysis of different genes in heat-shocked *Apostichopus japonicus* with real-time quantitative PCR. *Significant difference between treated and control groups ($p < 0.05$). COX: cytochrome *c* oxidase; CoA: succinyl-CoA synthetase; ZFp: zinc finger protein. All data are mean ± SD. n = 15 sea cucumbers treatment^{-1}

2008). In the present study, forward and reverse suppression subtraction libraries were constructed from the *Apostichopus japonicus* body wall in order to analyze the changed levels of gene expression in response to heat stress. The genes potentially involved were sequenced and analyzed. Because of the lack of related information, most of the ESTs were unknown on the basis of the BLASTn and BLASTp results. Some of the known ESTs belonged to heat-shock proteins and genes involved in immunity, and binding molecules such as the genes coding for ferritin and zinc finger protein were identified in the forward library. Genes functional in cell component and molecular function were verified in the reverse library, but were not

expressed in the forward library. These results suggest that these libraries reveal a response to heat stress in *A. japonicus*.

Identification of the same contig in both libraries (e.g. genes coding for collagen and myosin) revealed false-positive clones and is likely to be the result of an incomplete subtraction of some genes. But differential expression of 8 randomly selected genes suggests that the rate of false-positive clones was very low.

Chaperone

Heat-shock proteins are chaperones that allow biological processes to work well in a stressful environment. Hsp70, which has been studied widely owing to its sensitivity to stress (Boone & Vijayan 2002, Hamer et al. 2004, Lund et al. 2006), is an important non-specific protein that protects enzymes from heat damage and supervises the accuracy of protein folding and the allocation of new polypeptides, and it speeds the recovery of protein synthesis. Hsp70 repairs protein misfolding and accelerates the degradation of protein that cannot be repaired. Therefore, increased expression of Hsp70 increases the tolerance of organisms to stress and improves the rate of cell survival (Nakano & Iwama 2002). In the present study, 63.8% (231/362) of the clones in the forward library represented the gene encoding Hsp70. The results of real-time qPCR confirmed that the expression of *hsp70* gene transcripts was up-regulated significantly in response to heat stress in *Apostichopus japonicus*. This indicates that the acute heat shock severely damaged cell function but the negative effect was eliminated because the organism expressed large amounts of *hsp70*, which helped to maintain physiological balance.

Transcripts for Hsp90 and the genes coding for small heat-shock proteins (sHSPs) were identified in the forward library, and this had not been reported previously for this holothurian. It has been reported that sHSP is an energy-dependent molecular chaperone in other species, where it has an important role in protecting the cell from stress-induced damage by preventing irreversible aggregation or by promoting correct substrate folding (Sun et al. 2002, Sun & MacRae 2005). HSP90 contributes to the correct folding that maintains the structural integrity of proteins and regulates cytosolic proteins and works with other client proteins to take advantage of its function (Picard 2002, Queitsch et al. 2002, Chen et al. 2006). In the present study, different levels of expression of genes coding for Hsp90, Hsp70, and sHSP transcripts in response to heat stress were observed simultaneously, which indicates that the contributions of these genes were not identical. We suggest that they interact and enhance the heat tolerance in *Apostichopus japonicus*, limiting heat stress-induced damage to the organism and maintaining physiological balance. The relationship among these proteins was not revealed in the present study and it should be the subject of further research.

Genes related to immunity

Zinc finger protein is a DNA-binding protein that is part of the stress-response element (STRE) containing promoters. The amount of zinc finger protein in cells appears to be small under favorable conditions; however, transcription is increased and this protein is among the STREs when unfavorable conditions are encountered. Therefore, STRE-dependent transcriptional induction normally occurs rapidly (Kim et al. 2005, Jang et al. 2007). In the present study, expression of the gene coding for the zinc finger protein increased 2-fold after heat stress and this increase, together with the enhancement of some regulated factors, strengthened the defense of the organism and reduced the damage to cell function.

Ferritin is an iron-chelator protein involved in cellular protection from oxidative stress and integrated stress responses, also known as the acute phase response. Expression of gene coding for ferritin in the edible periwinkle *Littorina littorea* is increased in response to anoxic stress (Beck et al. 2002, Larade & Storey 2004). The presence of a ferritin-like clone in the forward library suggests an increase in mRNA expression in response to heat stress. This result is in accord with the increased activity of other immune enzymes (superoxide dismutase and catalase) under heat stress in *Apostichopus japonicus* (Dong et al. 2007). This suggests that, in order to maintain cell structure and function under heat stress, transcription of genes related to immunity is enhanced, which removes the oxidative products.

In the present study, some EST sequences categorized as recognition receptors, complement components, and growth factors were found in the reverse library. These genes are involved in the innate defense of the brown rock sea cucumber *Holothuria glaberrima* (Ramírez-Gómez et al. 2008) and in the purple sea urchin *Strongylocentrotus purpuratus* (Gross et al. 1999, Rast et al. 2000, Hibino et al. 2006). We concluded that heat stress can influence the innate defense of *Apostichopus japonicus*.

Genes related to energy production and metabolism

After heat stress, the concentration of oxygen radicals can increase and damage the structure and function of the cell membrane; as a result, the rate of elec-

tron transport decreases, which frees more energy for defense against an environmental stress. COX plays a key role in the electron transport chain, where it catalyses the reduction of molecular oxygen to water concomitant with the oxidation of reduced cytochrome *c*, and couples this redox reaction with the electrogenic transfer of protons across the inner mitochondrial membrane (Parker et al. 1994, Shigenaga et al. 1994). The sea cucumber body wall has a respiratory function and requires oxygen. In the present study, there was a decrease in the expression of genes coding for COX, which can be related to an increase in reactive oxygen species and ATP synthesis after heat stress, which is necessary for the survival of this holothurian.

Both the electron transport chain and the tricarboxylic acid (TCA) cycle produce energy for metabolic processes. Some studies found that the rate of the TCA cycle was decreased in response to environmental stress; the activity of enzymes in the TCA cycle decreased significantly in snail, frog, and lungfish during seasonal hibernation (St-Pierre & Boutilier 2001, Bishop et al. 2002, Frick et al. 2008). The activity of these enzymes in *Apostichopus japonicus* has not been analyzed. In the present study, however, several genes involved in the TCA cycle were found in the reverse SSH library, which indicates that expression of these genes was depressed. An analysis of enzyme activity is needed to determine whether heat stress induces depression of the metabolic rate and suppression of ATP utilization in cellular processes.

Sequences of unknown function

Due to the paucity of information available for *Apostichopus japonicus*, there were 100 EST sequences in our 2 libraries that had no significant similarity to any genes (BLAST E-value > 10^{-5}). Some sequences were either too short or were part of an mRNA untranslated region (UTR), but some sequences were long enough to contain a significant open reading frame (Ortiz-Pineda et al. 2009). The relatively long sequences with significant differential expression were probably associated with the regulatory and metabolic responses to heat stress. Further analysis using RNA interference and other techniques is needed to verify the function of these sequences.

Changing water temperature is one of the important environmental factors affecting biological processes in *Apostichopus japonicus*. The present study confirms that heat stress significantly affects the molecular mechanisms in the *A. japonicus* body wall with increased expression of most chaperones and immune genes and decreased expression of genes related to metabolism. The results are consistent with the phenomenon of estivation, when biological metabolic processes are slowed and the state of the immune system is different from that of 'normal' *A. japonicus.* The present study provides information for further understanding of estivation in *A. japonicus*, and the relationship between heat stress and *A. japonicus* health, and will aid in the development of sea-cucumber aquaculture.

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