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Analysis of heat, cold or salinity stress-inducible genes in the Pacific abalone, *Haliotis discus hannai*, by suppression subtractive hybridization

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ABSTRACT

In order to investigate environmental stress inducible genes in abalone, we analyzed differentially expressed transcripts from a Pacific abalone, *Haliotis discus hannai*, after exposure to heat-, cold- or hyposalinity-shock by suppression subtractive hybridization (SSH) method. 1,074 unique sequences from SSH libraries were composed to 115 clusters and 986 singletons, the overall redundancy of the library was 16.3%. From the BLAST search, of the 1,316 ESTs, 998 ESTs (75.8%) were identified as known genes, but 318 clones (24.2%) did not match to any previously described genes. From the comparison results of ESTs pattern of three SSH cDNA libraries, the most abundant EST was different in each SSH library: small heat shock protein p26 (sHSP26) in heat-shock, trypsinogen 2 in cold-shock, and actin in hyposalinity SSH cDNA library. Based on sequence similarities, several response-to-stress genes such as heat shock proteins (HSPs) were identified commonly from the abalone SSH libraries. HSP70 gene was induced by environmental stress regardless of temperature-shock or salinity-stress, while the increase of sHSP26 mRNA expression was not detected in cold-shock but in heat-shock condition. These results suggest that the suppression subtractive hybridization method is an efficient way to isolate differentially expressed gene from the invertebrate environmental stress-response transcriptome.

Key words: Pacific abalone, Suppression subtractive hybridization, Expressed sequence tags, Response-to-stress gene

INTRODUCTION

The Pacific abalone, *Haliotis discus hannai*, is distributed alone the coastal waters of East Asia, which is one of the most flavorful species among mollusks and is highly valued species. Although, aquaculture production of the Pacific abalone in Korea has reached about 6,941 ton in 2011 (Korean National Statistical Office 2012), the development of abalone culture has long been hampered by the problems of low growth rate and mass mortality. To resolve these problems, many studies have been performed by several groups (reviewed by Hannon *et al*., 2013). However, those studies on abalone have concentrated mainly on mortality, physiology, and aquaculture practice. Although cDNA microarray was applied to analyze the transcriptional profiles in gills and hemocytes of disk abalone, Haliotis discus discus, after viral hemorrhagic septicemia virus (VHSV) challenge (De Zoysa *et al*., 2012), the understanding at molecular level on stress responses in abalone is very limited yet.

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The stress response has a complex relationship with disease and has been implicated in disease outbreaks in most animals, including abalone (Hooper *et al*., 2007). Especially temperature and salinity are the primary physical factors affecting the life of mollusks. Limited research has been undertaken to date on stress and/or immune responses in abalone. Recently, a link has been established in abalone between increased stress and decreased immune functional capacity (Martello and Tjeerdema, 2001; Malham *et al*., 2003; Cheng *et al*., 2004a; 2004b; 2004c; 2004d; 2004e), leading to increased rates of bacterial infections and increased mortality (Cheng *et al*., 2004b; 2004c; 2004d; 2004e). This link is based on immune function tests carried out after applying stressors such as altered salinity, shaking, decreased dissolved oxygen, increased concentrations of ammonia and nitrate and increased temperature. In mollusks including abalone, both of the stress response and the immune response appear to be centered in the hemocytes, as these cells produce mediators of stress and the main immune responses (Ottaviani and Franceschi, 1997).

In the present study, we have employed suppression subtractive hybridization method to investigate the inducible genes in response to temperature-shock or salinity-stress in the Pacific abalone, *Haliotis discus hannai*.

MATERIALS AND METHODS

1. Animals

Abalones, *Haliotis discus hannai* were supplied form the Jeju Fisheries Research Institute of National Fisheries Research and Development Institute (NFRDI) on Jeju Island and were maintained in 6 tons flow-through tank at 18-20°C under a natural photoperiod. Abalone was exposed heat-shock or cold-shock from ambient sea water from 18°C to 28°C or from 18°C to 4°C, respectively. Hyposalinity was derived from ambient sea water from 35‰ to 20‰. The hemolymph from three abalones were sampled at 0, 0.5, 1, 2, 3, 4 and 5 h in case of heat-shock treatment, or at 0, 3, 6, 9 and 24 h in cold-shock treatment, or at 0, 1, 3, 6, 9 and 24 h in hyposalinity treatment. Hemocytes were isolated from the pooled hemolymph at each time point by centrifugation at 3,000 × rpm for 5 min at 4°C, then were snap-frozen or in excess, tissue homogenization was performed using a mortar and pestle cooled to temperature in a liquid nitrogen bath.

2. RNA Isolation

Total RNA was extracted from each sample using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Genomic DNA from total RNA was removed using DNaseI (Invitrogen) following the manufacturer's protocol. mRNA was isolated using a PolyA Tract mRNA isolation kit (Promega) following the manufacturer's protocol. After total RNA of each sample was adjusted to 0.5 mg, then, placed into 15 ml tube and RNase-free water was added to make 500 μl of final volume, and heated for 10 min at 65°C. Mixture of 3 μl biotinylated-Oligo (dT) probe and 13 μl 20 × SSC was added to the RNA tube. The annealed mixture was hybridized to Streptavidin -Paramagnetic Particles (SM-PMP), then mRNA-bound SM-PMP was captured with magnetic. Hybrid mRNA was dissolved with 250 μl DEPC-water. After addition of 0.1 volume of 3M sodium acetate (NaOAc) and 1 volume of isopropanol the mRNA was, stored at -20°C for cDNA synthesis.

3. Construction and Screening of Subtracted cDNA libraries

The suppression subtractive hybridization technique (SSH) (Diatchenko *et al*., 1996) was used to characterize new genes involved in stress and immune response. Briefly, cDNA was synthesized from 1 μg of each mRNA sample using the SMART PCR cDNA Synthesis Kit (Clontech), which allowed the full-length amplification of cDNA from mRNA transcripts. A SSH assay was then performed using the PCR-Select cDNA Subtraction Kit (Clontech). The cDNA from the tester and from the driver were digested with Rsa I, and the tester cDNA was then ligated to either two different cDNA adaptors. During a first hybridization, excess driver was added to tester cDNA samples, which were then denatured and allowed to anneal. In the second

hybridization, the two primary hybridization samples were mixed without denaturation. For further selection of differentially expressed sequences, denatured driver cDNA was again added to these hybrid samples. As a result, the remaining subtracted, equalized single-stranded tester cDNA reassociated to form hybrids with a different adaptor on each end. This forward-subtracted sample (genes present or up-regulated in stress treated compared with controls) was then used in PCR to amplify the differentially expressed sequences. PCR mixture was ligated using pGEM-T Easy system (Promega) and transformed into *E. coli* competent cells. A reverse subtracted library was also performed following the same protocol identify genes present or up-regulated in controls compared with infected tissues. Selected colonies were amplified by PCR using Nested PCR primer 1R and 2R from PCR-Select cDNA Subtraction Kit. Agarose gel electrophoresis was performed to visualize the amplified fragments and to select by size the samples to be sequenced and arrayed. The PCR profile consisted of: initial denaturation for 5 min at 94°C; 35 cycles of 30 s denaturation at 94°C, 30 s annealing at 65°C and 1.5 min elongation at 72°C; final extension for 7 min at 72°C. Excess of primers and nucleotides was removed by enzymatic digestion using 10 and 1 U of *Exo*I and SAP, respectively (Amersham Biosciences) at 37°C for 1 h followed by inactivation of the enzymes at 80°C for 15 min. DNA sequencing was performed using the ABI 3730 automatic DNA sequencer (PE Applied Biosystems) and the ABI Prism Big Dye Terminatior Cycle Sequencing Ready Reaction kit (PE Applied Biosystems).

4. Bioinformatic Analysis

Bioinformatic analysis was conducted to determine gene identities using GeneMaster 3.0 software (Ensoltek). Briefly, vector sequence was removed and database search was limited to ESTs > 100 bp in length. ESTs were then assembled in clusters of contigous sequences (contig) using ICAtools program (Parsons, 1995). Gene annotation procedures and homology searches of the sequenced ESTs was locally done by BLASTX for amino acid similarity comparisons (Altschul *et al*., 1997). Matches were considered to be significant only when the probability (P) was less than $1 \times e^{-3}$ using BLASTX with all parameters at the defaults. All ESTs that were not identified as orthologues of known genes were designated as unknown EST clones and hypothetical proteins were considered as known EST clones.

5. RT-PCR Verification

Verification of expression levels of target genes was carried out by RT-PCR. Total RNA samples were extracted from the collected samples according to each stress condition using TRIzol reagent (Invitrogen). Subsequently, first-strand cDNA synthesis was carried out using the Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics). The expression levels of selected genes were detected by RT-PCR using specific primers, which were based on the nucleotide sequence of EST clones. As an internal control, beta-actin was amplified using the appropriate primers (Table 1). The PCR conditions were as follows: 95°C for 5 min, followed by 30 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s, with a final step of 72°C for 7 min. The amplified PCR products were analyzed on 1.5% agarose gel containing ethidium bromide (100 ng/ml).

RESULTS AND DISCUSSION

1. Summary of ESTs in the SSH cDNA Libraries

A total of 1,536 ESTs were selected randomly from the heat-shock, cold-shock or hyposalinity SSH cDNA libraries and were partially sequenced; 384 ESTs from the heat-shock SSH library, 672 ESTs from the cold-shock SSH library and 480 clones from hyposalinity SSH library. A summary of the identified genes from the SSH cDNA libraries is shown in Table 2. The initial ESTs were grouped into 1,101 consensus sequences, comprised of 115 clusters (heat-shock: 42, cold-shock: 52 and hyposalinity: 21) and 986 singletons (heat-shock: 217, cold-shock: 491 and hyposalinity: 278). The SSH libraries had an overall redundany of 16.3%. BLASTX search established that 998 (75.8%) of the clones were orthologues of known genes (E-value < $1 \times e^{-3}$), and the remaining 318 (24.2%) clones were

Table 1. Sequences of primers used in this study

Primer	Sequence	Usage
β-actin-RT-F	5'-GCCGCTTGACTCTTGTGTGC-3'	RT-PCR for β-actin
β-actin-RT-R	5'-CTCCTCTGGTGCAACGCGG-3'	
HSC-RT-F	5'-CTGTGCGCTGACCTGTTTCG-3'	RT-PCR for HSC
HSC-RT-R	5'-CGAGGTACGCAACTGCTTC-3'	
HSC70-RT-F	5'-GTCGGCCTTGCTTAGACGAC-3'	RT-PCR for HSC70
HSC70-RT-R	5'-CTTGGCAAGTTTGAGCTCAC-3'	
HSP84-RT-F	5'-GGTTTCCAATCGTCTGGTTAC-3'	RT-PCR for HSP84
HSP84-RT-R	5'-GTCTTCCAGGGAGAAGCC-3'	
HSP26-RT-F	5'-CAGAGTCCTTGGGCGATCTAAC-3'	RT-PCR for HSP26
HSP26-RT-R	5'-GCAGGAGCTTTCTCATCGGCTTC-3'	
cHSP70-RT-F	5'-GGACTTGCCAGTGCTCTTGTC-3'	RT-PCR for cHSP70
cHSP70-RT-R	5'-GGACACGAGCGACGCCATC-3'	
HSP71-RT-F	5'-GCCACGTGGTGTGCCTCAG-3'	RT-PCR for HSP71
HSP71-RT-R	5'-CGGCTTGTCTCACGCTGAAG-3'	

Table 2. A summary of the SSH cDNA libraries from the Pacific abalone

	Stress condition			Sum
	Heat-shock	Cold-shock	Hyposalinity	
Total number of clones sequenced	384	672	480	1,536
Total number of successful sequenced	346	639	331	1,316
Number of contigs	42	52	21	115
Unique sequences	259	543	299	1,101
Redundancy (%)	25.1	15	9.7	16.3
ESTs with E value < $1 \times e^{-3}$ (known) (%)	303 (87.6)	403 (63.1)	292 (88.2)	998 (75.8)
ESTs with E value ≥ $1 \times e^{-3}$ (unknown) (%)	43 (12.4)	236 (36.9)	39 (11.8)	318 (24.2)

not identifiable via similarity comparisons (E-value ≥ $1 \times e^{-3}$). Among the 1,101 EST clones, only 4 unique genes were identified as homologues of previously reported abalone ESTs and 1,097 (99.6%) genes were identified as orthologues of known genes from other organisms. These results suggest that EST analysis constitutes a powerful technique for the rapid discovery of large numbers of useful genes in shellfish.

2. Expression Profile in the SSH cDNA Libraries

ESTs which were sequenced at very high frequencies are summarized in Table 3. The most abundantly expressed gene in the heat-shock SSH cDNA library were as follows: small heat shock protein p26 (2.9%), erythrocyte membrane protein 1 (2.3%), heat shock protein 70 (1.8%) and heat shock protein 90 (1.3%). The most abundantly expressed genes in the cold-shock SSH cDNA library were as follows: trypsinogen 2 (1.3%), chymotrypsinogen 2 (1.0%), hemoglobin beta-A chain (0.6%), and MHC class II A antigen (0.4%). The most abundantly expressed genes in the hyposalinity SSH cDNA library were as follows: actin (1.3%), cytoplasmic heat shock protein 70 (1.0%) and calcium binding protein 1 (0.4%). The expression profile in the heat-shock or cold-shock SSH cDNA library was more polarized than in its hyposalinity SSH cDNA library.

3. Distribution of the Identified ESTs in the SSH cDNA Libraries

Table 3. The most abundant ESTs detected in the SSH cDNA libraries from the Pacific abalone

Libraries	Putative identities	Frequency of Total (%)
Heat-shock	Small heat shock protein p26	2.9
	Erythrocyte membrane protein 1	2.3
	Heat shock protein 70	1.8
	Heat shock protein 90	1.3
Cold-shock	Trypsinogen 2	1.3
	Chymotrypsinogen 2	1.0
	Hemoglobin beta-A chain	0.6
	MHC class II A antigen	0.4
Hyposalinity	Actin	1.3
	Cytoplasmic heat shock protein 70	1.0
	Calcium binding protein 1	0.4

Table 4. Response-to-stress genes in the SSH cDNA libraries from the Pacific abalone

EST no.	Putative identification	Closest species	Identity (%)
ABHSL-3_F06_46_12	heat shock cognate 70	*Tetranychus urticae*	79
ABHSL-3_G04_31_14	84kDa heat shock protein	*Haliotis tuberculata*	91
ABHSL-1_E04_29_10	heat shock protein hsp40	*Homo sapiens*	98
ABHSL-2_D11_84_07	small heat shock protein p26	*Artemia persimilis*	23
ABHSL-4-A9	heat shock protein 71	*Haliotis tuberculata*	96
ABHSL-4-H9	cytoplasmic heat shock protein 70	*Strea edulis*	79
ABHSL-3_A06_41_02	heat shock protein 70	*Bombyx mori*	52
ABHSL-3_H08_64_16	70kDa heat shock protein	*Crassostrea gigas*	61
ABHSL-2_G05_39_13	heat shock protein 70	*Wuchereria bancrofti*	66
ABLSL-1_D12	Heat shock cognate 71 kDa protein	*Paralichthys olivaceus*	99
ABLSL-1_A3	Heat shock protein 90 beta	*Paralichthys olivaceus*	90
ABLSL-4_C10	Hemoglobin beta-A chain	*Siniperca chuatsi*	88
ABLSL-2_H11	Hemoglobin subunit alpha-B	*Seriola quinqueradiata*	66
ABLSL-3_C11	Suppressor of cytokine signaling 3a	*Tetraodon nigroviridis*	77
ABLSL-3_F11	Alpha 1 collagen type 1	*Sparus aurata*	93
ABLSL-4_C9	40S ribosomal protein S3	*Pseudopleuronectes americanus*	95
ABLSL-4_H7	Serum lectin isoform 1	*Verasper variegatus*	67
ABLSL-7_H7	Peroxisome proliferator-activated receptor beta	*Sparus aurata*	96
LSASL-5_D10	Proteasome subunit beta type	*Branchiostoma lanceolatum*	72
LSASL-5_G4	Heat shock protein 70kDa	*Diguetia mojavea*	78
LSASL-5_B10	Fructose-bisphosphate aldolase	*Biomphalaria glabrata*	83
LSASL-5_C11	Ubiquitin/ribosomal fusion protein homologue	*Salmo salar*	98

Based on the major functions of their encoded proteins, the identified clones were classified into sixteen broad categories; biogenesis of cellular components, cell cycle/ DNA processing, cell fate, cell rescue/ defense/ virulence, cell type differentiation, cellular communication/signal transduction mechanism, cellular transport/ transport facilitation/ transport routes, development (Systemic), energy, interaction with the cellular environment, metabolism, protein activity regulation, protein fate (folding, modification, destination), protein synthesis, protein with binding function/ cofactor requirement (structural or catalytic) and transcription. The distribution of identified clones from the three SSH cDNA libraries are shown in Figure 1. No notable differences were detected between the cold-shock and hyposalinity SSH cDNA libraries. However, the distribution of identified clones from the heat-shock subtracted library showed some differences, compared to others. Especially, cell rescue/ defense/ virulence, cellular transport/ transport facilitation/

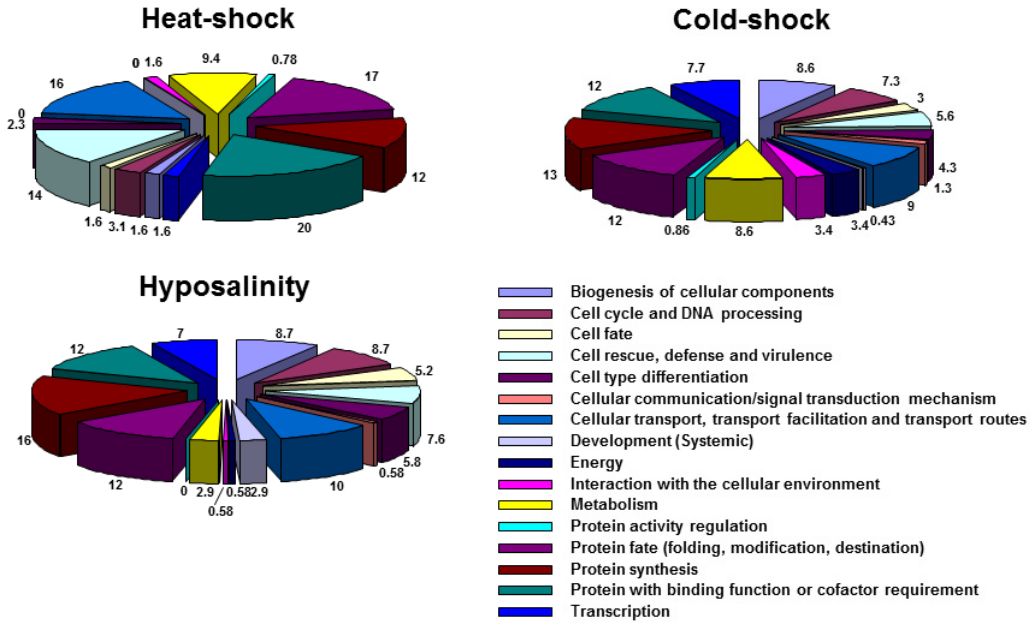

Fig. 1. Functional categorization of the three SSH cDNA libraries taken from the Pacific abalone. Based on the major functions of their encoded proteins, the identified clones were classified into sixteen broad categories; biogenesis of cellular components, cell cycle/ DNA processing, cell fate, cell rescue/ defense/ virulence, cell type differentiation, cellular communication/signal transduction mechanism, cellular transport/ transport facilitation/ transport routes, development (Systemic), energy, interaction with the cellular environment, metabolism, protein activity regulation, protein fate (folding, modification, destination), protein synthesis, protein with binding function/ cofactor requirement (structural or catalytic) and transcription.

transport routes, protein fate (folding, modification, destination) and protein with binding function/ cofactor requirement (structural or catalytic) were more abundant than the others.

4. Isolation of stress-response genes from the SSH cDNA libraries

Database searches identified putative response-to-stress genes that were expressed in the three SSH cDNA libraries (Table 4). The putative amino acid sequence deduced from several cDNA clones were identified as a family of heat shock proteins. Heat shock proteins (HSPs) are well known as stress proteins because various forms of stress enhance their transcriptional activation and biosynthesis in organisms, ranging from bacteria to humans (Lindquist, 1986). RT-PCR was performed using several HSP family genes to confirm the results of our study and transcription of HSPs were regulated by thermal stress conditions. All transcripts of tested HSPs genes were gradually increased under the heat-shock condition (Fig. 2A). In the cold-shock condition, however, the expression of small HSP 26 (sHSP26) was not induced even though other HSPs were strongly induced (Fig. 2B). This result indicates that sHSP26 could be used as a specific molecular marker for heat-shock.

In conclusion, these results suggest that the suppression subtractive hybridization method is an efficient way to isolate differentially expressed gene from the invertebrate environmental stress-response transcriptome.

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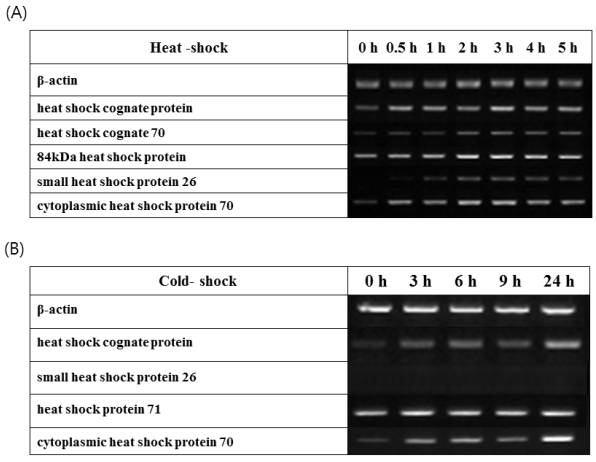

Fig. 2. Expression level of putative stress-related genes. HSP families were analyzed by RT-PCR. As a positive control for RT-PCR, the β-actin gene was amplified to determine template concentration.

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