A THESIS

FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Exploration of Stress Biomarkers in Disk Abalone by cDNA Microarray Technique

Qiang Wan

Department of Biotechnology

GRADUATE SCHOOL

JEJU NATIONAL UNIVESITY

2011.02

Exploration of Stress Biomarkers in Disk Abalone by cDNA Microarray Technique

Qiang Wan

(Supervised by Professor Jehee Lee)

A thesis submitted in partial fulfillment of the requirement of the degree of DOCTOR OF PHILOSOPHY

2011.02

The thesis has been examined and approved by

Thesis director, Moon-Soo Heo, Professor of Marine Life Science

Associated director, In-Kyu Yeo, Professor of Marine Life Science

Sung-Ju Jung, Professor of Aqualife Medicine, Chonnam National University

You-Jin Jeon, Professor of Marine Life Science

Jehee Lee, Professor of Marine Life Science

2011-02

Date

Department of Biotechnology

GRADUATE SCHOOL

JEJU NATIONAL UNIVERSITY

CONTENTS

국문요약

전복은 복족류로써 고부가가치를 가지는 동물입니다. 다른 동물에 비하여 늦게 성장하고 주위의 환경적 변화에 대단히 민감합니다. 최근 몇십년에 있어서, 전세계적으로 전복의 수는 과잉수확, 기후변화, 환경오염 및 급작스런 병으로 인하여 급격히 감소하였습니다. 그동안 전복의 양식 과정에서 pH, 온도, 염분, 용존산소, 병원균등 이 같은 환경적 요인으로부터 전복의 체력과 생산 효율을 유지하는 것이 중요한 문제로 대두 되고 있습니다. 따라서, 이러한 스트레스 반응의 메커니즘을 이해하고, 그 후에 전복에 사용될 수 있는 적절한 스트레스바이오마커에 의한 조기경계 시스템을 개발하는 것은 매우 중요합니다.

이 논문에서는 이러한 연구를 3 개의 파트로 나누었으며 파트 I 에서는 cDNA microarray 를 통한 스트레스 반응에 뛰어난 유전자의 식별, 파트 II 에서는 바이오마커로서 스트레스 반응에 좋은 유전자의 기능적인 설명, 파트 III 에서는 바이오마커로써 활용이 가능한 housekeeping 유전자의 선택으로 이루어져 있습니다.

파트 I 에서는 까막전복 (*Haliotis discus discus*)으로부터 부분적인 유전적 정보를 얻어 이를 기초로 하여 cDNA 칩을 만들었습니다. 이렇게 만들어진 cDNA 칩은 4188 개의 까막전복의 유전자를 갖고 있었습니다. cDNA 칩을 통하여 까막전복의 유전적 발현 실험을 하였으며 다음과 같이 각각의 다른 환경적 요인을 적용하여 실험을 하였습니다. 열(30 °C), 냉기(10 °C), 저염분(25 psu), 고염분(40 psu)카드뮴(20ppm $CdCl_2$), 구리(20ppm $CuSO_4$), 수은(5ppm $HgCl_2$), PAHs (25ppmβ-NF), PCBs (50ppm Aroclor),및 TBT (2ppm TBT-Cl). 결과적으로 825 개의 유전자에서 유의적인 발현이 나타났으며 이는 전체 4188 개의 유전자 중에 20%정도를 차지합니다. 고온, 저온, 카드뮴

스트레스에서 각각 200 개 이상의 많은 유전자가 반응을 하였습니다. 염분, 구리, 수은, EDC 에서는 69-125 개의 유전자가 발현 변화를 나타내었습니다. 고온 스트레스를 통하여 발현된 유전자는 protein folding, nucleic acid processing, metabolism 과 oxidative stress 등의 기능을 Gene ontology 분석을 통하여 나타내었습니다. 저온 스트레스를 통하여 발현된 유전자는 nucleic acid processing 과 metabolism 기능이 있다는 것을 알 수 있었습니다. 염분 스트레스를 통한 실험에서는 biosynthesis and metabolism 기능을 갖고 있었습니다. 중금속 스트레스를 통한 실험에서는 protein synthesis, molecular chaperone, proteolysis 과 apoptosis regulation 기능을 갖고 있었습니다. 3 가지의 EDC 실험에서는 유전자는 많지 않았으며 transport, apoptosis regulation, innate immunity 의 기능을 갖고 있었습니다. 각각의 실험중에서도 서로 겹치는 동일한 유전자인 small HSP, kruppel factor, programmed cell death 5, insulin-related peptide binding protein and cholinergic receptor 를 찾았으며 이는 매우 중요한 유전자로 인식을 하였습니다. 이유는 다른 환경요인적 스트레스에도 같은 기작을 갖고 있으며 스트레스 반응 네트워크에 중점적인 유전자이기 때문입니다.

파트 II 에서는 저분자 heat shock protein 인 HSP20 를 중심으로 실험을 하였으며 이 유전자는 실험을 한 모든 요인에서 매우 높은 발현을 나타내었기 때문입니다. 이는 스트레스마이오마커로써의 가능성을 내보입니다. 분자유전학적으로 이 유전자를 연구하기 위하여 전형적인 구조적 특징인 α-crystallin domain, Cysteine-free, Glx/Asx-rich 과 compact β-sandwich structure 을 내포하고 있었습니다. HSP20 재조합 단백질을 포함하고 있는 대장균 셀의 열 내성을 강화 할 수 있었습니다. qRT-PCR 의 표현 분석에 의해

까막전복의 HSP20 가 열쇼크에 의해 극단적으로 상승하였으며 최고 2000 배의 차이를 나타내었습니다. 또한 저온, 염분, 중금속 및 다양한 EDCs 에 의해서도 상당히 상승하였습니다. 이러한 결과는 cDNA microarray 분석에 의한 결과와 일치하고 있습니다. 이를 통하여 HSP20 유전자는 고온을 비롯하여 다양한 환경 요인에서도 유전자의 발현량이 상승한다는 것을 알 수 있었습니다. 이는 환경적 요인으로 인해 세포가 입는 데미지를 줄이기 위하여 chaperone 기능을 갖고 있다는 것을 알 수 있었습니다. HSP20 는 환경 스트레스에 매우 민감한 유전자이며 이는 전복의 건강상태를 완전히 반영할 수 있는 이상적인 바이오마커로서 사용이 가능할지도 모르겠습니다.

파트 3 에서는 housekeeping 유전자로 사용이 가능한지에 대해서 실험을 하였습니다. 총 12 개의 유전자를 실험하였으며 qRT-PCR 을 통하여 실험을 하였습니다. EDC 스트레스를 받은 까막전복의 아가미와 간췌장으로 실험을 하였습니다. 기존에 사용된 housekeeping 유전자 중에 18s rRNA, glyceraldehyde-3-phosphate dehydrogenase 과 β-actin 은 각각의 발현수준이 많이 변화하였으며 이는 housekeeping 유전자로 사용을 할 수 없다는 것을 나타내었습니다. 그렇기 때문에 우리는 이러한 housekeeping 유전자로 사용을 할 수 있는 새로운 유전자를 찾아야 했으며 다양한 실험에 사용된 유전자를 분석 한 결과 TBT 스트레스 실험에서는 ribosomal protein L-5, elongation factor 1 유전자가 매우 안정적으로 발현이 되었으며 E2 스트레스 실험에서는 ribosomal protein L-5/ succinate dehydrogenase 유전자가 안정적으로 발현이 되었습니다. 유전자의 발현 정도를 알아보기 위해 비교하는 housekeeping 유전자가 부적절한 경우, 이를 통해 실험의 결과가 부정확하거나 과대 혹은 과소 분석이 될 수 있습니다. 이것은 바이오마커의 정량을 얻기 위해서 안정하며

확인이 가능한 housekeeping 유전자를 확보하는 것이 중요하다는 것을 알 수 있었습니다.

결론적으로 본 논문에서는 DNA microarray 기술을 사용하여 까막전복의 스트레스 반응에 좋은 유전자를 찾기 위하여 전체적인 탐색을 실행 하였으며 많은 유전자들이 환경적 스트레스에 반응을 하여 변화를 하였으며 이는 감시를 위한 바이오마커로서 가능성이 있다는 것을 내비치고 있습니다. 그리고 이 연구에서 까막전복의 스트레스 반응에 따른 메커니즘을 분자유전학적으로 그 증거를 제공하였습니다. 지금까지는 저분자 HSP20 유전자만 기능적 분석을 하였습니다. 또한 이 유전자는 EDCs 를 통한 스트레스에서 housekeeping 유전자로써 사용이 가능하다고 여겨집니다. 전복의 스트레스 감시 시스템을 설립하기 위해서 이 밖에도 다양한 연구가 추가적으로 필요합니다.

Summary

Abalone is a family of gastropod molluscs with great economic values. However, abalone is relatively slow growing and highly sensitive to the change of ambient environment. In recent decades, the worldwide population of wild abalone has been declined steadily due to the overharvesting, climate change, environment pollution and/or disease outbreak. Meanwhile, for abalone in aquaculture process, how to maintain the heath and production efficiency of abalone by avoiding the stress from pathogens and environmental factors such as temperature, salinity, dissolved oxygen and pH is also a critical issue. Therefore, understanding the mechanisms of stress response and subsequently developing an early warning system by appropriate stress biomarkers in abalone is of great significance.

Our experimental works in this present study is consisted of three parts: I, identification of stress-responsive genes by cDNA microarray; II, functional characterization of an import stress-responsive gene as biomarker; III, selection of appropriate housekeeping genes for biomarker application. In Part I, on the basis of the partial transcriptomic information of disk abalone *Haliotis discus discus* obtained by our earlier works, we constructed a cDNA microarray composed of 4188 unique abalone genes. The cDNA microarray was then employed for the expression analysis of abalone genes in response to a set of environmental stressors: heat (30 ^{o}C), cold (10 ^{o}C), low-salinity (25 psu), high-salinity (40 psu), cadmium (20 ppm $CdCl_2$), copper (20 ppm $CuSO_4$), mercury (5 ppm $HgCl_2$), PAHs (25 ppm β-NF), PCBs (50ppm Aroclor) and TBT (2 ppm TBT-Cl). Following microarray assay, a real-time PCR analysis of 10 target genes was also conducted for microarray data validation. Then, in Part II we carried out molecular cloning, characterization and expression analysis for a small heat shock protein, which has shown great inducibility by various environmental stressors in microarray assay. The data accuracy of biomarker expression analysis is highly dependent on the selection of housekeeping gene as reference. In this regard, a systematic comparison of abalone housekeeping genes for biomarker monitoring of EDCs was finally conducted in

Part III.

Upon the challenges of different environmental stressors, a total of 825 genes were shown significant expression changes by cDNA microarray anlysis, over 20% of 4188 analyzed genes. Heat, cold and cadmium stress affected the highest number of genes (>200 in each), whereas the exposures of extreme salinities, copper, mercy and three organic EDCs represented only moderate impact on abalone global gene expression, with 69-125 differentially expressed genes. Gene ontology analysis revealed that genes associated with protein folding, nucleic acid processing, metabolism and oxidative stress were largely regulated by heat stress. While in cold stress, the genes associated with nucleic acid processing and metabolism exhibited as the predominant part of response. Following salinity changes, the genes in the process of biosynthesis and metabolism were highlighted. The genes elicited by heavy metals are mainly associated with the protein synthesis, molecular chaperone and proteolysis as well as the apoptosis regulation. Three organic EDCs significantly affected the least number of genes amongst all the environmental stressors. However, their expression patterns of quite similar where genes associated with transport, apoptosis regulation and innate immunity were commonly regulated. Importantly, we noted that there are considerable overlaps of differentially expressed genes between different stress conditions. Furthermore, we also identified a certain number of genes that commonly respond to various stress, such as small HSP, kruppel factor, programmed cell death 5, insulin-related peptide binding protein and cholinergic receptor. Taken together, the results indicated a crosstalk in the stress response pathways to different environmental stressors. These commonly responsive genes may perform as the key nodes of stress response network in abalone.

In disk abalone genome, we identified two putative small HSPs (HSP20 and HSP26). The HSP20 gene has registered the highest induction levels in several stress conditions by microarray analysis, indicating a potential as sensitive stress biomarker. Thereby, we carried out the further functional characterization for this gene. It exhibited several typical structural

characteristics such as conserved α–crystallin domain, Cysteine-free, Glx/Asx-rich and compact β-sandwich structure in C-terminal region. In addition, the over-expression of recombinant HSP20 protein could enhance the thermotolerance of *E. coli* cells *in vivo*. The expression analysis by qRT-PCR expression showed that abalone HSP20 was dramatically induced by heat shock (up to 2000-fold), but also significantly elevated by cold shock, extreme salinities, heavy metals and organic endocrine disrupting chemicals (EDCs). These data are consistent with the findings by cDNA microarray. Taken together, the data in the present study demonstrate positive correlations between the expression of abalone HSP20 and various environmental stressors. During stress, HSP20 probably plays protective roles against cellular damage as a molecular chaperone in abalone. HSP20 could be ideal as a sensitive biomarker to completely reflect the integrated severity of the environmental stress and the health condition of abalone in field.

In the work of housekeeping gene validation, relative expression levels of twelve candidate housekeeping genes were examined by qRT-PCR in gill and hepatopancreas of abalone following the challenge with tributyltin chloride and 17β-estradiol, respectively. The expression levels of several conventional HKGs, such as 18s rRNA, glyceraldehyde-3-phosphate dehydrogenase and β-actin, were significantly altered by challenges, indicating that they might not be suitable internal controls. Instead, ribosomal protein L-5/ elongation factor 1 and ribosomal protein L-5/ succinate dehydrogenase were shown as the most stable HKGs under TBT and E2 challenges, respectively. When unsuitable HKGs were used for normalization, the influence of two EDCs on biomarker was imprecisely overestimated or underestimated, which strongly emphasized the importance of selecting appropriately validated housekeeping genes for biomarker quantitation.

In conclusion, through the application of DNA microarray technology, we carried out a global exploration of stress-responsive genes in the transcriptome of disk abalone. A large number of genes have shown correlations between their expression and environmental stress, thus indicating potential as biomarkers for environmental monitoring. Our study also

provides the molecular evidences for stress response mechanisms in abalone. So far the functional characterization of these biomarker candidate genes is only carried out in a small HSP (HSP20). Also, the housekeeping validation for biomarker application is limited in the aspect of organic EDCs. Further efforts thereby will be needed to establish the stress monitoring system in abalone.

LIST OF FIGURES

LIST OF TABLES

Chapter I.

Global profiling of gene expression in disk abalone *Haliotis discus discus* challenged with various environmental stressors by cDNA microarray

Abstract

In the present study, a cDNA microarray composed of 4188 uinique genes was constructed and employed to profile gene expression pattern in the disk abalone (*Haliotis discus discus*) challenged with different environmental stressors including heat, cold, low-and high-salinity, heavy metals and endocrine-disrupting chemicals (EDCs). Following bioinformatic analysis, we identified a total of 825 genes responsive to these stress conditions. Approximately 50% of these genes are with no homology to known sequences or with unidentified functions, whereas the rest half of known genes are involved in a number of biological processes. In heat stress, the genes associated with protein folding, nucleic acid processing, metabolism and oxidative stress were largely regulated. In cold stress, the genes associated with nucleic acid processing and metabolism possessed the predominant proportion. In response to the stress due to salinity change, the genes in the process of biosynthesis and metabolism were highlighted. The exposure of heavy metals elicited the response of genes mainly involved in the synthesis, modification and catabolism of proteins as well as the apoptosis regulation. Three organic EDCs commonly affected the expression of genes involved in transport, apoptosis regulation and innate immunity. Additionally, through analyses of the overlapping gene expression pattern in different stress conditions, we identified several genes that commonly respond to various environmental stressors, as the key nodes in stress response network. Overall, data reported in our study provide novel insights into the molecular pathways that could mediate stress responses in abalone. The identified genes with differential expression in response to environmental stressors may be useful as potential novel biomarkers.

Key words: Abalone, Microarray, Environmental stress, Biomarker, Heavy metal, EDCs

1. Introduction

Abalone is an herbivorous mollusc species from the *Haliotidae* family. It is widely fished and cultured along the coastal lines of many countries, bringing great economic values. However, in recent decades, the wild population of abalone worldwide has been steadily declining. As a consequence, several species of abalone family, such as white abalone (*H. sorenseni*), black abalone (*H. cracherodii*) and northern abalone (*H. kamtschatkana*), have been listed on the red list as critically endangered (Hobday and Tegner). The deterioration of marine environment was considered as to virtually attribute the declines of abalones. Similar with other lifes in marine environment, wild abalone is constantly exposed to various biological and abiotic stressors that include the exposure to predators, food deprivation, pollutants, disease pathogens as well as the variations of ambient temperature, salinity and dissolved oxygen. Yet little is currently known about the underlying genes, proteins, molecular pathways, and physiological mechanisms in response to environmental stressors in abalone.

Our most extensive knowledge about stress response comes from studies carried out in other model organisms (Stronach and Perrimon 1999). It is described as an evolutionally high conserved defense mechanism that protects cells and organisms from threatening agents in their environment (Steinberg, Sturzenbaum et al. 2008). Physiologically speaking, stress response is referred to as a immediate cascade of neuroendocrine events that comprise the secretion of the hormones into the blood, the elevation of plasma glucose level and the increase of heart rate and metabolism in all vertebrates and some invertebrates as well (Wendelaar Bonga 1997; Lacoste, Malham et al. 2001). Meanwhile, at the cellular level, a set of stress proteins including molecular chaperones, cell cycle regulators, proteasome regulators, DNA repair proteins and enzymes involved in homeostasis of redox and energy metabolism often are also elicited during stress (Pearce and Humphrey 2001; Kultz 2005). If the stress is too severe and/or is chronic, however, protective mechanisms can be overridden,

resulting in several deleterious effects on individual organisms such as reduced growth rate, decreased disease resistance, altered behavior and reduced survivability. Therefore, to prevent the irreversible impacts of environment stress on wildlife and ecosystem, development of an early warning system based on the sensitively responsive biomarker genes are critically needed.

DNA microarray is a novel technology revolutionizing the basic and applied molecular biology and medical research and diagnosis. It allows scientists to study tens of thousands of genes at once, instead of working on a gene-by-gene basis. In the field of marine environmental monitoring, DNA microarray has become a significant tool for biomarker exploration. Measurements of global change in gene expression by DNA microarray have been widely conducted in several fish, shrimp and mollusc species; and a number of novel stress-specific and common stress-responsive genes have been identified as biomarker candidates (Venier, De Pitta et al. 2006; Evans and Somero 2008; Aoki, Wang et al. 2010). In addition, these experiments also represented the primary level of integration between environmental factors and their genomes, providing a comprehensive view of how an organism is responding to the change of its ambient environment and an ultimate guide to diagnose the major stressors. To identify stress-responsive genes and document widespread transcriptional changes occurring in the stress response of abalone species, we developed a cDNA microarray for disk abalone *H. discus discus* in the present study. To the best of our knowledge, this study describes the first utilization of cDNA microarray for the study of gene expression profiles of abalone exposed to different environmental stressors.

2. Materials and Methods

2.1 Animals, treatments and experimental design

Healthy two-year-old disk abalones (*Haliotis discus discus*) weighing 50-60 g and with well-developed gonads were obtained from the Youngsoo abalone farm on Jeju Island, South

Korea. Abalones were acclimated in laboratory aquaria for 1 week prior to the challenge experiment. The seawater was filtered and aerated continuously, with the salinity and temperature maintained at 32 ± 1 ‰, and 20 ± 1 °C, respectively. During the acclimation, abalones were fed daily with fresh seaweed diet. Thereafter, abalones were divided into three control groups and ten challenge groups, comprised of four abalones in each. To induced heat stress, we challenged abalones by an immersion in the seawater of 30°C for 6 hours; while for cold stress, the immersion was at 10°C for 24 hours. In the groups of high- and low- salinity, the salinity of seawater was adjusted to 40 psu and 25 psu by adding artificial sea salt or distilled water, respectively. For challenge, abalones were maintained in the seawater with the prepared salinities for 24 hours. In heavy metal challenge groups, $CdCl_2 \cdot 2.5\ H_2O$, $CuSO_4 \cdot 5H_2O$ or $HgCl_2$ was dissolved in PBS and intramuscularly injected into abalones at the dose of 20 μg, 20 μg and 4 μg per g body weight, respectively. In EDC challenge groups, beta-naphathoflavone (β-NF), aroclor-1254 (Aroclor) and tributyltin chloride (TBT) were dissolved in DMSO and intramuscularly injected into abalones at the dose of 20 μg, 50 μg and 2 μg per g body weight, respectively. The challenge of heavy metals and EDCs were carried out in duration of 24 hours. The control abalones for physical stress, heavy metal stress and EDC stress challenges were prepared by using untreated, PBS-injected and DMSO-injected animals, respectively. After challenge experiment, the gill tissues from the physical stress groups and the hepatopancreas tissues from the heavy metals and EDCs groups were dissected, frozen immediately in liquid nitrogen and stored at -80 °C.

2.2 cDNA library construction and microarray printing

The disk abalone cDNA library was constructed using mRNA isolated from different tissues of abalone (*H. discus discus*) and cDNA library construction kit (Creator SMART, Clontech, Mountain View, CA, USA). The cDNA library was normalized with a Trimmer-Direct normalization kit according to the manufacturer's protocol (Evrogen,

Moscow, Russia). After the massive sequencing by a Big Dye Terminator sequencing kit and ABI 3700 sequencer (Macrogen, Korea), sequences were further edited to remove vector and adaptor sequences, cleaned and filtered Cleaning involved masking of poor quality bases and low complexity sequences such as poly A tails. Filtering removed contaminating sequences (bacteria, yeast) and only high quality sequences of more than 100 bases in length were retained. Contigs were annotated using the BLASTX program (http://www.ncbi.nlm.nih.gov/BLAST/), and the entry to which they received the highest similarity was assigned as the gene identity. To construct cDNA microarray, the cDNA inserts of 4188 selected clones were amplified by PCR using adaptor-specific primers. The amplicons were examined by 1% agarose gel electrophoresis, purified using Sephadex G-50 columns, air-dried and resuspended in 50% DMSO solution. The purified amplicons were then spotted using an Omnigrid^{TM} Microarrayer (GeneMachine, San carlos, CA) onto silanized glass slides (GAPS-II^{TM}, Corning, Charlotte, NC). Each slide was finally crosslinked with 300 mJ of short wave ultraviolet (UV) irradiation (Stratalinker, Stratagene, La Jolla, CA) and stored in humidity and light-controlled conditions until use.

2.3 RNA isolation, fluorescent labeling and hybridization

The gill or hepatopancrease total RNA from 4 stressed and 4 control animals for each stress challenge was isolated in the method of tissue pools using the Tri Reagent (Sigma,USA) according to the manufacturer's protocol. The quality of all RNA preparations was confirmed prior to microarray hybridization with an Agilent Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA) using the RNA 6000 Nano Labchip kit (Agilent Technologies). For fluorescent cRNA synthesis, high-quality total RNA (150 ng) was labeled with Low RNA Input Fluorescent Linear Amplification Kit (Agilent Technologies) according to the manufacturer's instructions. In this procedure, cyanine 5-dCTP (Cy5) and cyanine 3-dCTP (Cy3) (PerkinElmer, Boston, MA, USA) were used to generate labeled cRNA from the

stress-treated RNA and the control RNA, respectively. Labeled cRNAs (0.75 μg each) from one treatment and the control were combined and fragmented in a hybridization mixture with the In Situ Hybridization Kit Plus (Agilent Technologies) according to the manufacturer's instructions. The mixture was hybridized for 17 h at 60 °C to the printed microarray, which carries probes to 4188 transcripts of abalone. After hybridization, the microarray was washed with SSC buffer, and then scanned in Cy3 and Cy5 channels with the Agilent DNA Microarray Scanner (model G2565BA).

2.4 Microarray data analysis

The signal intensity and local background of each spot generated from the Two channel scanned images with Feature Extraction Software version 7.5 (Agilent Technologies) in the default settings. Spots that did not pass quality control procedures in this software were flagged and removed from further analysis (abiding by the software's default settings). The data obtained were imported to GeneSpring GX 7.3 (Agilent Technologies) to normalize the ratio (Cy5/Cy3) of the signal intensities generated in each microarray in Lowess (locally weighted linear regression curve fit) method. On the basis of the Lowess-normalized ratios, analytical tools in GeneSpring GX were systematically employed to extract differentially expressed genes between all stress-treated samples and the controls. Thereafter, a statistical significance of the difference was examined by One-way analysis of variance (ANOVA) controlling false discovery rate (FDR) at the level of 0.05.The processed data were further analyzed using an integrated function in the GeneSpring GX software version 7.3 for gene tree clustering. Hierarchical cluster classifies samples according to their overall gene expression on the basis of correlations of their expression level pattern in all samples. To analyze the function of each differentially expressed abalone gene present in the microarray, gene ontology (GO) annotation was performed by means of the universal platform Blast2GO with a threshold cutoff at 10^{-3}.

2.5 Real-time PCR validation

Microarray results were validated by real-time PCR of 10 target genes. Primers of 10 target genes were designed by using Primer 3 program with the size of amplicons ranged between 100 and 150 base pairs (Table. 1). Abalones were similarly challenged with different environmental stressors, and the total RNA was isolated as described above. First-strand cDNA was synthesized from 2.5 µg of total RNA and 500 ng of dT primer in a reaction volume of 20 µl, using SuperScript III reverse transcriptase (Invitrogen). PCR reactions were set up as follows: 12.5 µl of SYBR Premix Extaq (Takara), 1 µl of 10 µM sense and antisense gene-specific primers, 4 µl of cDNA template at a dilution of 1:20 and RNase-free water to a final volume of 25 µl. The Takara™ real time PCR detector TP800 was used for performing the amplification in triplicate. The thermal profile was programmed as follows: 3 min at 94°C, and 40 cycles of 20 s at 95°C for, 20 s at 60°C, and 30 s at 72°C. Dissociation curves were examined at the end of the PCR reaction to check for unspecific amplification and primer-dimers. PCR efficiency (E) was estimated for each primer pair by serial dilutions (from 1/20 to 1/640) of cDNA. The primers efficiency was determined by the slope of the standard curves by the following formula: $E=10^{[-1/slope]}$. Real-time PCR Ct values were converted to expression level in the method of $2^{\Delta Ct}$. The obtained data were then compared to the microarray values.

Table 1. List of primers used for Real-time PCR validation of ten abalone genes.

Gene	Forward (5'-3')	Reverse (5'-3')	Amplicon size (bp)
Small heat shock protein	AAGGTCAGCCAGCTGGAGAAACAA	ATTCCCGTGTGAATTCCCTGCTGA	98
Heat shock protein 40-B4	ATGGCAACCCACACGAAACATTCC	AAGTGATGACCAAAGCCTGTCCCT	118
Glutathione synthetase	CACATCCAACCCAACTGCACAACT	TCTCCCACCTCGGTTTGGTTTGAT	85
Glutaredoxin	AGCATTGCTAAATCCGCACCTTGG	GCCTAAACCTGCACAAGTGTATCCT	103
Proteasome ATPase	ACGTCAGTTGGCAGTGAGCACTAT	TCATCTGACAACACTCCCACCACA	125
Ubiquitin activating enzyme E1	TGGTTGTACCTGACTTCAAGCCGA	AGCTGTTCTTGGTCACATCCCTCA	125
Programmed cell-death 5	TCAAGAGGAACAGCAGCAAGCTCA	TTTGTCCAGTCCGCGTCATCTGTA	105
Microtubule-associated protein	ATGCGTGATTACGATCCCGTCCAT	GTTGTTTCCGTCATTGGCTTGGCT	137
2OG-Fe-oxigenase	ACGAGTTCCTGCGAGATATGCAAC	TAAACCTGATCCCAAGGCCAGCAA	92
Glycerol-3-phosphate cytidylyltransferase	ACGATACAGCCAAGGGTTCTGCTT	GGACAACCGTCATCATCTTTCGCA	106

3. Results and Discussions

3.1 Overview of gene expression profiling in abalone by cDNA microarray

A disk abalone cDNA microarray chip containing 4188 spots was produced by printing a series of PCR products from the pre-constructed cDNA library. After microarray assay, the spot quality of each microarray chip was examined to filter the bad spots that are resulted from the poor-quality PCR products or printing have been eliminates. Finally, expression data of 4168 clones for heat, cold, low-salinity and high-salinity treatments, 4184 clones for cadmium, copper, PCBs and TBT treatments, and completely 4188 clones for mercury and PAHs treatment were retained after normalization and filtering. The additional data quality examination for each hybridization was also performed in the way of M-A and scatter plots. To identify the differentially expressed genes in response to different environmental stresssors, we applied two sets of criteria to the microarray data: a fold change cutoff of 2-fold for heat and cold stress treatment or a P value of 0.05 plus fold change cutoff of 1.8 for the other treatment. In total, 825 cDNA clones were identified as differentially expressed. The distribution pattern of these identified cDNA clones under different stress was illustrated in Figure 1. Heat, cold and cadmium stress possessed the highest number of differentially expressed genes (>200 in each), whereas the other seven environmental stressors represented more moderate impact on abalone global gene expression, with significant changes in 69-125 genes. In addition, to explore the relationship among the stress-responsive genes, the microarray data were further examined by hierarchical clustering analysis (Fig 2). Three trees were constructed according to gene expression patterns in physical stress, heavy metals and EDCs, respectively. The branch length of tree represents the similarity between genes.

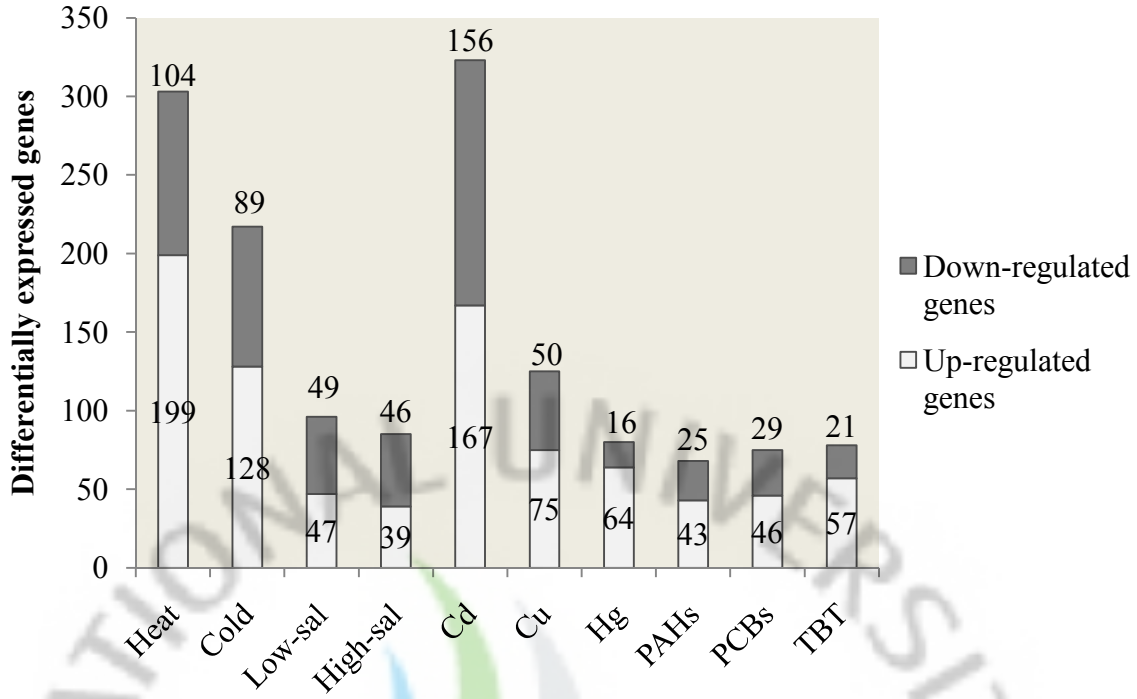


Fig 1. Differentially expressed genes in response to different environmental stressors. The number of significantly down-regulated (black bars) and up-regulated (white bars) genes in response to heat (cutoff of 2-fold), cold (cutoff of 2-fold), low-salinity (cutoff of 1.8-fold and $P<0.05$), high-salinity (cutoff of 1.8-fold and $P<0.05$), cadmium (cutoff of 1.8-fold and $P<0.05$), copper (cutoff of 1.8-fold and $P<0.05$), mercury (cutoff of 1.8-fold and $P<0.05$, PAHs (cutoff of 1.8-fold and $P<0.05$), PCBs (cutoff of 1.8-fold and $P<0.05$) and TBT (cutoff of 1.8-fold and $P<0.05$) are represented.

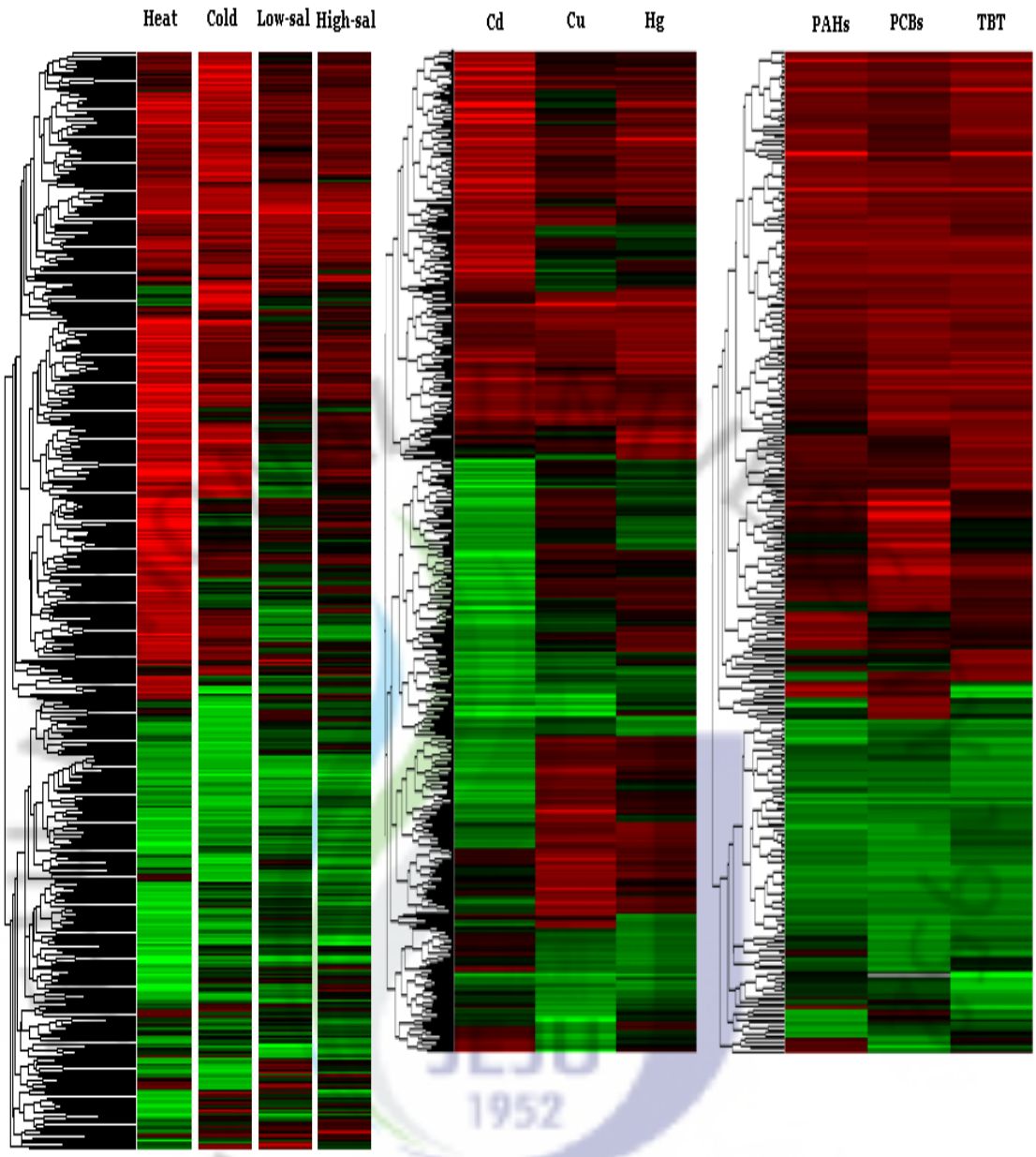


Fig 2. Hierarchical clustering analysis of differentially expressed genes of abalone under different environmental stress. Each row represents a single gene and each column an experimental sample. Genes were linked by the dendrogram shown on the left to illustrate similarity in their expression pattern. Up-regulated genes are red and down-regulated genes are green.

Table 2. Selected genes whose transcription was enhanced after heat stress.

Gene ID	Fold Change	Description	E-value to best hit
cDNA_26-C07	2.8 ↑	Acheron	2E-49
cDNA_32-F02	2.9 ↑	Acheron	2E-14
cDNA_27-H06	3.2 ↑	Adult retina protein	9E-43
cDNA_15-G05	2.9 ↑	Arrestin domain containing 3, partial	8E-24
cDNA_08-G05	4.2 ↑	Arsenic (+3 oxidation state) methyltransferase	6E-14
cDNA_63-G12	2.6 ↑	ATPase, H+ transporting	8E-52
cDNA_50-E06	3.1 ↑	Baculoviral IAP repeat-containing 4 isoform 2	4E-57
cDNA_09-E06	2.4 ↑	Barrier to autointegration factor 1	7E-36
cDNA_04-E07	3.5 ↑	BAZ1B protein	2E-42
cDNA_28-D07	2.8 ↑	Bcl2-like (mitochondrial outer membrane protein) pro	4E-17
cDNA_33-H12	2.4 ↑	Calmodulin	7E-26
cDNA_45-B07	2.4 ↑	Catalase	3E-06
cDNA_44-F09	2.1 ↑	CCAAT /enhancer binding protein gamma	1E-14
cDNA_55-C04	2.1 ↑	Cct5-prov protein	6E-120
cDNA_14-G04	5.1 ↑	Cctq (cytosolic chaperonin)	1E-42
cDNA_69-E09	2.6 ↑	CDC42 (a protein involved in regulation of the cell cycle.)	5E-29
cDNA_14-G05	2.1 ↑	Centrosomal protein 27	2E-40
cDNA_23-F12	4.1 ↑	Chaperonin containing TCP1	4E-117
cDNA_67-E05	2.5 ↑	Chaperonin containing TCP1, subunit 5 (epsilon)	2E-75
cDNA_14-G02	2.2 ↑	Cholinergic receptor, nicotinic, beta polypeptide 4	4E-21
cDNA_23-F01	2.4 ↑	Chst11-prov protein	8E-22
cDNA_40-E11	2.8 ↑	Coiled-coil domain containing protein	1E-36
cDNA_13-G04	2.1 ↑	Cubilin	4E-17
cDNA_25-C10	2.7 ↑	Cubilin	4E-13
cDNA_19-H11	3.5 ↑	Cubilin	1E-16
cDNA_16-F09	2.6 ↑	Cyclic AMP phosphoprotein	3E-19
cDNA_05-B09	2.1 ↑	Cyclin I	1E-51
cDNA_01-H07	2.8 ↑	DEAD/H box 56 RNA helicase/noh61	4E-77
cDNA_58-B12	2.5 ↑	Deleted in malignant brain tumors 1 protein	3E-24
cDNA_68-F04	2.1 ↑	Dimethylaniline monooxygenase	2E-52
cDNA_48-B03	2.6 ↑	Dipeptidyl-peptidase II precursor (DPP II)	9E-71
cDNA_24-D07	2.2 ↑	DnaJ (Hsp40) homolog	8E-78
cDNA_04-B01	2.1 ↑	Dual specificity phosphatase	4E-46
cDNA_07-A11	2.3 ↑	Dual specificity phosphatase 7	6E-25
cDNA_51-F02	2.7 ↑	Endoplasmic reticulum protein	2E-42
cDNA_35-A12	3.9 ↑	Eukaryotic translation initiation factor 3	9E-74
cDNA_36-G02	2.7 ↑	Eukaryotic translation initiation factor eIF3	1E-33
cDNA_14-G12	2.6 ↑	Expansin	2E-06
cDNA_47-C06	2.3 ↑	F-box protein 11	1E-37
cDNA_67-A05	2.0 ↑	GDP dissociation inhibitor	4E-78
cDNA_20-D11	2.3 ↑	Glutathione S-transferase sigma	7E-36
cDNA_27-D03	3.2 ↑	Glycoside hydrolase, family 10	7E-34
cDNA_04-D07	6.9 ↑	Heat shock protein	2E-93
cDNA_14-F06	9.3 ↑	Heat shock protein 90	2E-99
cDNA_56-G10	2.8 ↑	hypoxia up-regulated protein	5E-24
cDNA_52-C07	2.5 ↑	Import inner membrane translocase subunit TIM44	3E-61
cDNA_49-A03	3.0 ↑	Incilarin A	8E-16
cDNA_14-E05	6.3 ↑	Insulin-related peptide binding protein	1E-26

cDNA_16-G04	2.6 ↑	Isocitrate dehydrogenase 2 (NADP+), mitochondrial	2E-130
cDNA_67-A08	2.1 ↑	Isopenicillin N synthase and related dioxygenases	1E-09
cDNA_19-G11	6.9 ↑	Kruppel-like factor	2E-37
cDNA_18-B07	2.1 ↑	Leucine rich repeat containing protein	3E-68
cDNA_53-B06	3.3 ↑	Low density lipoprotein receptor-related protein 3	4E-06
cDNA_52-C04	3.9 ↑	MAP kinase-interacting serine/threonine kinase	2E-65
cDNA_51-A12	4.1 ↑	Mu class glutathione S-transferase	1E-68
cDNA_01-F07	2.7 ↑	Muscle LIM protein	2E-35
cDNA_08-D04	3.6 ↑	MYND domain protein	5E-08
cDNA_61-F11	2.3 ↑	Myophilin-like protein	2E-41
cDNA_05-A09	2.1 ↑	Myosin II heavy chain	7E-79
cDNA_63-F12	2.2 ↑	Myosin regulatory light chain	2E-55
cDNA_13-B09	2.2 ↑	N-acetylserotonin O-methyltransferase-like protein	1E-13
cDNA_53-G01	2.1 ↑	NMD3	2E-80
cDNA_05-C08	2.7 ↑	Nonmuscle myosin essential light chain	5E-52
cDNA_49-F06	2.1 ↑	Novel protein vertebrate aldo-keto reductase family 1	3E-62
cDNA_18-F01	3.3 ↑	Nuclear factor, interleukin	4E-11
cDNA_44-B08	7.2 ↑	Omega class glutathione S-transferase	2E-37
cDNA_38-F03	9.6 ↑	Omega class glutathione S-transferase	3E-62
cDNA_21-F10	2.1 ↑	Oxidoreductase, zinc-binding dehydrogenase	2E-59
cDNA_02-H07	3.5 ↑	P23-like protein	1E-20
cDNA_13-G11	4.0 ↑	Phosphoglyceride transfer protein	2E-27
cDNA_48-H07	2.7 ↑	Phosphoribosylaminoimidazole carboxylase	2E-79
cDNA_35-D05	2.6 ↑	Phosphoribosylaminoimidazole carboxylase	3E-72
cDNA_14-E01	2.3 ↑	Poly(A) polymerase alpha	2E-101
cDNA_66-C09	2.6 ↑	Probable Beta-agarase	1E-09
cDNA_15-A03	2.5 ↑	Programmed cell death 5	8E-22
cDNA_11-G07	4.0 ↑	Proteasome beta	2E-72
cDNA_12-D11	3.0 ↑	Putative ubiquitin-conjugating enzyme	4E-77
cDNA_37-A09	2.1 ↑	pyruvate dehydrogenase complex, component X	6E-43
cDNA_30-E11	2.6 ↑	Scavenger receptor class F	2E-36
cDNA_17-B06	2.2 ↑	Selenoprotein W1	7E-19
cDNA_65-H04	2.0 ↑	SEP15_BRARE 15 kDa selenoprotein precursor	7E-37
cDNA_67-A07	4.1 ↑	Short-chain dehydrogenease/reductase	2E-72
cDNA_26-E09	9.4 ↑	Small heat shock protein	8E-10
cDNA_12-E05	2.8 ↑	Small nuclear ribonucleoprotein F	1E-35
cDNA_54-F10	3.9 ↑	Stress-induced-phosphoprotein (Hsp70/Hsp90-organizing protein)	5E-53
cDNA_17-A09	4.3 ↑	Succinate-Coenzyme A ligase, ADP-forming, beta subunit	7E-60
cDNA_52-A03	3.3 ↑	Sulfatase 1 precursor	1E-86
cDNA_09-F04	2.0 ↑	Sulfotransferase	2E-22
cDNA_07-G03	2.0 ↑	SUMO1/sentrin/SMT3 specific protease 3 isoform 16	4E-175
cDNA_05-D01	3.3 ↑	T-complex polypeptide	0E+00
cDNA_15-C10	5.3 ↑	T-complex protein 1, gamma subunit (TCP-1-gamma)	1E-37
cDNA_39-G03	2.2 ↑	Thioredoxin	6E-18
cDNA_50-B06	3.3 ↑	Thioredoxin peroxidase	1E-86
cDNA_16-F10	2.4 ↑	Transgelin	2E-48
cDNA_18-A06	2.2 ↑	Twitchin	2E-64
cDNA_13-D01	2.6 ↑	Type I iodothyronine deiodinase	2E-11
cDNA_61-B11	2.4 ↑	U2 small nuclear ribonucleoprotein auxiliary factor large subunit	8E-56
cDNA_14-H05	3.8 ↑	Ubiquitin-activating enzyme E1	4E-97

cDNA_54-F04	2.2 ↑	Universal stress protein	4E-11
cDNA_26-A05	5.6 ↑	Unknown	*N/A*
cDNA_47-D02	5.8 ↑	Unknown	*N/A*
cDNA_20-G12	6.0 ↑	Unknown	*N/A*
cDNA_13-G03	7.4 ↑	Unknown	*N/A*
cDNA_09-C05	7.7 ↑	Unknown	*N/A*
cDNA_14-E06	7.8 ↑	Unknown	*N/A*
cDNA_13-H02	9.8 ↑	Unknown	*N/A*
cDNA_47-D06	6.4 ↑	Vitelline envelope sperm lysin receptor	2E-129

Table 3. Selected genes whose transcription was repressed after heat stress.

Gene ID	Fold Change	Description	E-value to best hit
cDNA_50-B01	2.2 ↓	Aldo/keto reductase YtbE	1E-53
cDNA_03-F06	2.0 ↓	Thioredoxin domain-containing proteinisoform 2	9E-61
cDNA_26-A09	2.4 ↓	Axonemal p66.0	3E-76
cDNA_51-C06	2.2 ↓	Calmodulin	1E-16
cDNA_03-F05	2.1 ↓	Calmodulin	2E-16
cDNA_32-F04	2.7 ↓	Calmodulin	8E-77
cDNA_44-H08	2.0 ↓	CLIP-associating protein (Cytoplasmic linker)	4E-48
cDNA_01-A02	2.1 ↓	Crystallin, gamma N2	5E-06
cDNA_52-B10	2.1 ↓	Electron-transfer-flavoprotein, beta polypeptide	9E-96
cDNA_27-D11	2.1 ↓	Endo-1,4-beta-mannanase 1	2E-76
cDNA_38-F10	3.7 ↓	ETS-family transcription factor	8E-51
cDNA_20-C06	2.8 ↓	FAT tumor suppressor homolog	3E-27
cDNA_03-D07	2.1 ↓	Fignl1-prov protein	6E-73
cDNA_37-F04	2.2 ↓	H2A histone family, member Z	3E-50
cDNA_32-E04	2.2 ↓	Histone cell cycle regulation defective homolog A	4E-129
cDNA_62-D06	2.3 ↓	Iduronate 2-sulfatase precursor	3E-30
cDNA_24-H06	2.4 ↓	L-isoaspartyl protein carboxyl methyltransferase, like	3E-68
cDNA_47-D10	2.2 ↓	Macrophage expressed protein	5E-99
cDNA_29-D07	2.9 ↓	Methyltransferase	3E-24
cDNA_03-E02	2.0 ↓	Mitochondrial ribosomal protein	3E-71
cDNA_05-F04	2.0 ↓	PHD finger protein	3E-12
cDNA_17-G02	3.1 ↓	Phytanoyl-CoA hydroxylase interacting protein	2E-25
cDNA_34-E02	2.7 ↓	Pol	8E-13
cDNA_29-A09	2.4 ↓	Pol	1E-07
cDNA_03-A08	4.0 ↓	Poly ADP-ribose Metabolism Enzyme (250.3 kD) (pme-5	5E-14
cDNA_19-F05	2.0 ↓	Putative cytoplasmic protein family member of ancient origin	8E-35
cDNA_06-F07	2.7 ↓	RNA binding motif protein 18	1E-31
cDNA_27-G08	2.1 ↓	Scavenger receptor class F or MEGF	3E-21
cDNA_03-F02	2.3 ↓	Solute carrier	8E-14
cDNA_09-A04	2.0 ↓	S-phase 2 protein	2E-17
cDNA_38-G04	2.0 ↓	Splicing factor, arginine/serine-rich	1E-13
cDNA_30-E09	2.0 ↓	Tu translation elongation factor, mitochondrial	7E-75
cDNA_56-E02	4.3 ↓	Ubiquitin fusion degradaton protein	6E-56
cDNA_16-B12	10.0 ↓	Unknown	*N/A*
cDNA_58-G04	6.7 ↓	Unknown	*N/A*
cDNA_56-F02	5.4 ↓	Unknown	*N/A*
cDNA_42-E09	4.2 ↓	Unknown	*N/A*
cDNA_19-C03	2.2 ↓	Wolf-Hirschhorn syndrome candidate 1 protein isoform 1	*N/A*

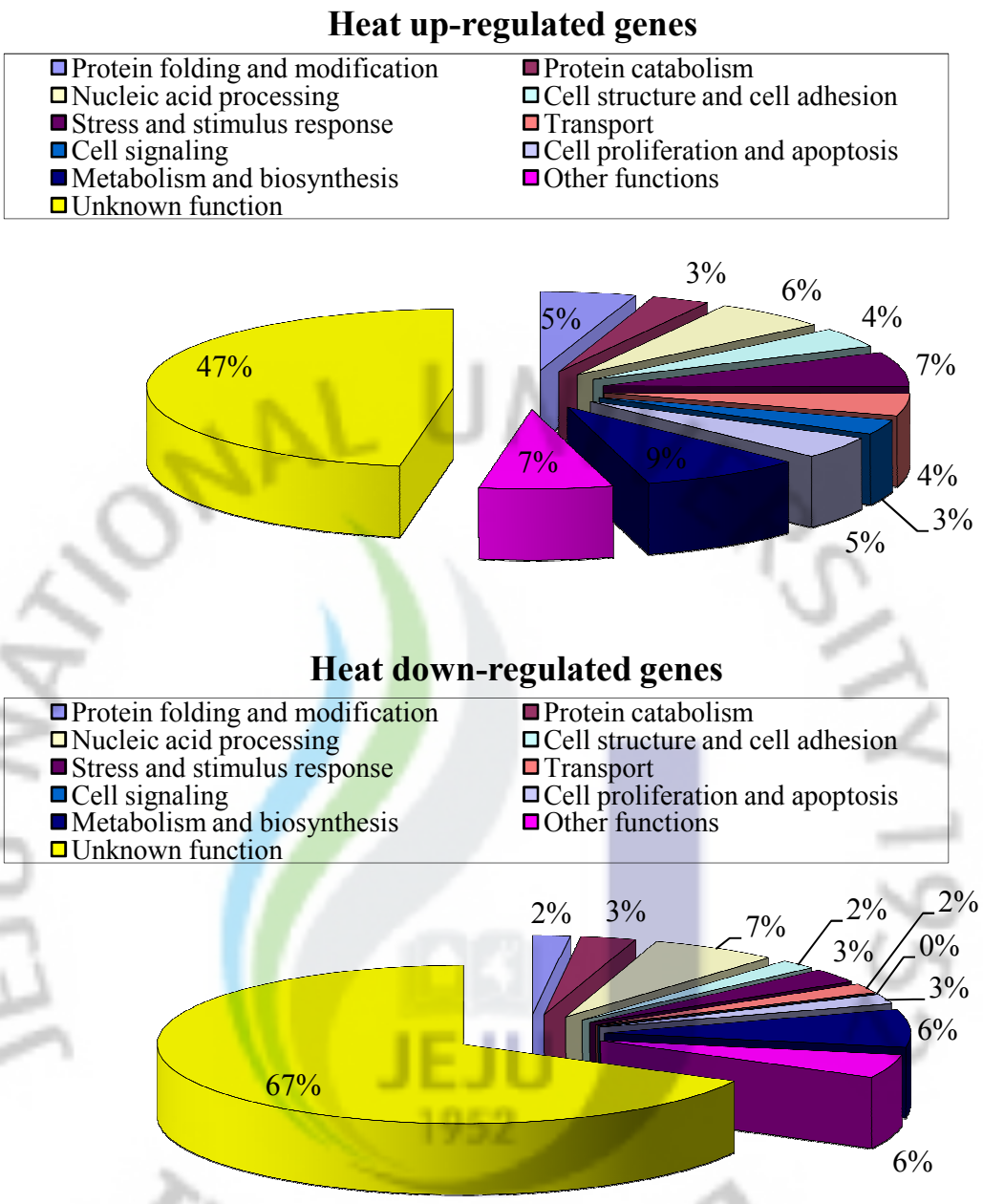


Fig 3. Functional categorization of up- and down-regulated genes under heat stress. The Go annotations of differentially expressed genes are based on Blast2GO analysis for level 3 of the biological process category.

3.2 Microarray analysis of expression response to physical stressors

Heat Temperature is one of the most important environmental factors affecting marine organisms, determining their general metabolism, behavioral responses as well as the population distribution. As poikilotherms, abalone must adjust the body temperature to cope with the thermally variable environments. The transcriptomic analysis of abalone genes under extreme temperatures could be helpful in understanding the heat and cold adaption machinery of these animals. To induced heat stress, we used the sudden upshift in temperature from 20 to 30°C for 6 hours. A total of 303 genes were regulated by heat stress in abalone, including 199 up-regulated genes and 104 down-regulated genes. The genes with great fold change were selected and listed in Table 2 and Table 3. To further analyze and interpret the data, we classified all genes that were significantly regulated into 11 functional classes on the basis of their roles in different biological processes. For purpose of classification and illustration, each gene was assigned to only one primary functional category.

As shown in Fig 3, about half of the heat-induced genes were with no homology to known sequences or with unidentified functions, while the rest half of genes averagely fell into 10 different classes with percentages ranging from 3% to 9%. One of the most important impacts of heat stress on cell is the damage of cellular protein. As expected, we observed 10 genes in protein folding and modification category strongly induced by heat stress. They are comprised of 4 heat shock proteins (HSP) and 6 T-complex peptide (TCP) family proteins and registered the highest fold-change amongst all functional categories. Accumulation of these molecular chaperones could facilitate the fold of newly synthesized proteins and repair the misfolded proteins during heat stress. In addition, the expression of 6 protease or peptidase related genes in protein catabolism category were also enhanced, indicating the need of degradation of the abnormally folded protein. Not surprisingly, 14 genes of general stress and stimulus response were also significantly induced by heat stress. They possessed

the second large cluster of total heat-induced genes and include a set of antioxidant proteins like glutathione S-transferase, thioredoxin and thioredoxin peroxidase. This result is in agreement with the connection between heat shock and oxidative stress proposed by previous studies (Pappolla, Sos et al. 1996). The largest functional category in heat-induced genes is the metabolism and biosynthesis, which is comprised of 17 genes playing roles in macromolecule synthetic and carboharate metabolic processes. Importantly, the genes involved in carboharate metabolism could perform as cellular energy pools to fuel stress response and repair mechanisms. It has been proven that many post-translational mechanisms are important for regulating the proteins in this category. In this regard, they were absent of strong elevation in mRNA levels, generally with about 2-fold change. Similar to the findings in fish, a number of genes that encode abalone cytoskeletal proteins were also moderately induced (Podrabsky and Somero 2004; Buckley, Gracey et al. 2006). However, no such gene in this category was found to be regulated by heat shock in archaeon *M. barkeri* or bacillus *Y. pestis* (Han, Zhou et al. 2005; Zhang, Culley et al. 2006). The underlying mechanism is uncertain, but the accumulation of cytoskeletal proteins may be a general strategy to stabilize the eukaryotic cell structure at high temperature. 30°C is a fatal temperature for disk abalone (*H. discus discus*), leading to severe tissue and cellular injury. As a consequence, 9 genes in cell proliferation and apoptosis category were significantly induced. Of them, incilarin and bcl-2 are considered to positively regulate cell proliferation and inhibit the apoptotic effect caused by heat stress, but programmed cell death 5 (PCD5) has been reported to be able to enhance cell apoptosis by several investigations (Chen, Sun et al. 2001). On the other hand, three genes including one cyclin and two dual specificity phosphastases are the key molecules required for passage through the restriction point in cell cycle. It was demonstrated that exposing mammalian cells to heat stress conditions could result in transient cell cycle arrest at G1/S and thereby increase the cylclin level in a temperature- and time-dependent manner (Nitta, Okamura et al. 1997). Apart from these genes involved in the classical biological processes of stress response, we also noted a group

of genes (7% of total) with the other functions were up-regulated by heat. Similar to those genes with unknown functions, they may represent critical parts of heat stress adaption that not yet been identified and studied.

Compared with the profile of up-regulation, much larger proportion of genes repressed by heat stress (67%) were with no blast hit or with unidentified functions. As shown in Fig 3, the major negative regulation on abalone gene expression occurred in the category of nucleonic acid processing, which includes 7 genes responsible for ribosome modification (poly ADP-ribose Metabolism Enzyme), transcription control (ETS-family transcription factor), post-transcriptional regulation (splicing factor, arginine/serine-rich) and protein translation (Tu translation elongation factor). This finding suggested a generally reduced cellular pool of RNAs and proteins resulted by heat stress. Also, in the protein catabolism category, we found an ubiquitin fusion degradaton protein. The down-regulation of this protein is believed to faciliate the degradation of damaged proteins. In stress and stimulus response category we noted the repression of three putative calmodulin genes. Interestingly, they were reported to play roles in various stress conditions (Phean, Punteeranurak et al. 2005). In heat stress, Ca2+/calmodulin transduction pathways have been implicated in mediating heat shock signal transduction by promoting the DNA-bind activity of HSF (Liu, Li et al. 2003). Similar to the result of up-regulation, there are 6% of down-regulated genes involved in other biological processes. Further investigation is needed to elucidate their roles in stress response.

Table 4. Selected genes whose transcription was enhanced after cold stress.

Gene ID	Fold Change	Description	E-value to best hit
cDNA_66-F04	2.0 ↑	Acetyltransferase	6E-12
cDNA_32-F02	3.8 ↑	Acheron	2E-14
cDNA_26-C07	2.6 ↑	Acheron	2E-49
cDNA_27-E01	2.7 ↑	Actin-depolymerizing factor 2	3E-12
cDNA_52-A11	2.2 ↑	ADP-ribosylation factor-like 2	3E-80
cDNA_27-H06	2.2 ↑	Adult retina protein	9E-43
cDNA_66-G03	2.1 ↑	Alkaline phosphatase	8E-42
cDNA_58-E06	2.3 ↑	Apolipoprotein O	8E-19
cDNA_26-A03	2.3 ↑	ATPase, H+ transporting	9E-15
cDNA_09-E06	2.1 ↑	Barrier to autointegration factor 1	7E-36
cDNA_28-D07	2.8 ↑	Bcl2-like	4E-17
cDNA_33-E07	2.2 ↑	Beta-tubulin	1E-133
cDNA_44-A09	2.6 ↑	BTB (POZ) domain containing 2	3E-10
cDNA_31-E04	2.1 ↑	CD63 antigen	1E-37
cDNA_69-E09	3.2 ↑	CDC42	5E-29
cDNA_50-C03	2.6 ↑	CDC42	3E-41
cDNA_14-G02	2.2 ↑	Cholinergic receptor, nicotinic, beta polypeptide 4	4E-21
cDNA_27-G03	3.4 ↑	Chromatin modifying protein	3E-30
cDNA_68-G04	2.0 ↑	Coatomer protein complex	3E-78
cDNA_19-H11	2.7 ↑	Cubilin	1E-16
cDNA_64-D11	2.1 ↑	Cubilin	9E-15
cDNA_25-C10	2.1 ↑	Cubilin	4E-13
cDNA_13-F04	2.4 ↑	DNA-directed RNA polymerase	6E-09
cDNA_04-B01	3.3 ↑	Dual specificity phosphatase	4E-46
cDNA_07-A11	2.5 ↑	Dual specificity phosphatase 7	6E-25
cDNA_19-A03	3.6 ↑	Fatty acid synthase	5E-08
cDNA_15-G11	2.3 ↑	Fatty-acid-binding protein, FABP	6E-08
cDNA_22-B10	2.3 ↑	Glutaredoxin	1E-44
cDNA_27-D03	2.4 ↑	Glycoside hydrolase	7E-34
cDNA_14-F06	2.1 ↑	Heat shock protein 90	2E-99
cDNA_52-G01	2.5 ↑	Hypoxia induced gene	3E-14
cDNA_14-E05	3.3 ↑	Insulin-related peptide binding protein	1E-26
cDNA_67-A08	2.2 ↑	Isopenicillin N synthase and related dioxygenases	1E-09
cDNA_09-D06	2.2 ↑	Keratin-associated protein	1E-13
cDNA_19-G11	5.1 ↑	Kruppel-like factor	2E-37
cDNA_32-H12	2.0 ↑	Late endosomal/lysosomal Mp1 interacting protein (p14)	7E-47
cDNA_68-D12	3.3 ↑	Lipophorin receptor	9E-16
cDNA_13-E06	2.6 ↑	Long-chain fatty acid--CoA ligase	9E-07
cDNA_52-C04	2.1 ↑	MAP kinase-interacting serine/threonine kinase	2E-65
cDNA_30-D04	2.7 ↑	Meningioma expressed antigen 5 (hyaluronidase)	5E-15
cDNA_13-H08	4.3 ↑	Methyltransferase	1E-49
cDNA_33-C03	2.1 ↑	Myosin regulatory light chain 2	1E-55
cDNA_13-B09	2.3 ↑	N-acetylserotonin O-methyltransferase-like protein	1E-13
cDNA_53-G01	2.2 ↑	NMD3	2E-80
cDNA_18-F01	2.6 ↑	Nuclear factor, interleukin	4E-11
cDNA_14-C01	2.1 ↑	Nucleolar protein	7E-86
cDNA_13-G11	3.0 ↑	Phosphoglyceride transfer protein	2E-27
cDNA_48-H07	2.1 ↑	Phosphoribosylaminoimidazole carboxylase	2E-79

cDNA_14-B04	2.9 ↑	Pim-3 protein	1E-91
cDNA_20-B04	2.1 ↑	Pinx1 protein	8E-62
cDNA_43-C03	2.0 ↑	Proteasome (prosome, macropain) 26S subunit, ATPase 2	2E-59
cDNA_57-D09	2.4 ↑	RAE1 RNA export 1 homolog	4E-113
cDNA_16-E07	2.1 ↑	Rho1 GTPase	1E-45
cDNA_67-A07	2.0 ↑	Short-chain dehydrogenease/reductase	2E-72
cDNA_50-D10	2.2 ↑	Small nuclear ribonucleoprotein D2-like protein	2E-47
cDNA_12-E05	2.3 ↑	Small nuclear ribonucleoprotein F	1E-35
cDNA_15-D06	2.0 ↑	Solute carrier	7E-17
cDNA_19-E02	2.3 ↑	Solute carrier	3E-113
cDNA_67-E06	2.2 ↑	Sorbitol dehydrogenase	2E-84
cDNA_28-F09	2.5 ↑	Stearoyl-CoA desaturase (delta-9-desaturase)	3E-72
cDNA_19-B11	2.1 ↑	Sterile20-like kinase	3E-40
cDNA_43-A06	3.9 ↑	Sterol regulatory element binding transcription factor	5E-58
cDNA_52-A03	3.4 ↑	Sulfatase 1 precursor	1E-86
cDNA_27-F03	3.8 ↑	Sulfotransferase	5E-27
cDNA_07-G03	2.1 ↑	SUMO1/sentrin/SMT3 specific protease 3 isoform 16	4E-175
cDNA_16-H09	3.3 ↑	Suppressor of cytokine signaling 2	4E-27
cDNA_15-C10	2.2 ↑	T-complex protein 1, gamma subunit	1E-37
cDNA_20-E05	2.7 ↑	Topoisomerase (DNA)	1E-45
cDNA_13-D01	2.2 ↑	Type I iodothyronine deiodinase	2E-11
cDNA_14-F04	7.4 ↑	Unknown	*N/A*
cDNA_13-E01	6.8 ↑	Unknown	*N/A*
cDNA_13-A04	5.9 ↑	Unknown	*N/A*
cDNA_26-A05	4.3 ↑	Unknown	*N/A*
cDNA_13-H02	3.7 ↑	Unknown	*N/A*
cDNA_16-G05	2.2 ↑	Vacuolar protein sorting factor	5E-16

Table 5. Selected genes whose transcription was suppressed after cold stress.

Gene ID	Fold Change	Description	E-value to best hit
cDNA_17-F04	2.6 ↓	40S ribosomal protein	4E-65
cDNA_09-G06	2.2 ↓	Aldo-keto reductase	1E-74
cDNA_08-G09	2.4 ↓	Ascorbate oxidase AO4	8E-28
cDNA_03-F06	2.1 ↓	Thioredoxin domain-containing protein	9E-61
cDNA_03-F05	2.3 ↓	Calmodulin	2E-16
cDNA_01-A02	2.1 ↓	Crystallin, gamma N2	5E-06
cDNA_39-G07	2.2 ↓	Dynein light chain-2	4E-45
cDNA_26-E06	2.2 ↓	E-1 enzyme	2E-60
cDNA_52-B10	2.1 ↓	Electron-transfer-flavoprotein, beta polypeptide	9E-96
cDNA_27-D11	2.3 ↓	Endo-1,4-beta-mannanase	2E-76
cDNA_51-F02	2.7 ↓	Endoplasmic reticulum protein	2E-42
cDNA_17-C05	2.4 ↓	FG-GAP repeat family protein	1E-29
cDNA_03-A02	2.1 ↓	Glutathione synthase	2E-30
cDNA_12-D08	2.3 ↓	Hexamethylene bis-acetamide inducible protein	1E-19
cDNA_28-D08	2.6 ↓	Hillarin	2E-24
cDNA_02-A01	3.0 ↓	Histone H1	8E-22
cDNA_62-D06	2.1 ↓	Iduronate 2-sulfatase	3E-30
cDNA_03-F01	2.3 ↓	IMP dehydrogenase/GMP reductase:Thrombospondin type 3 repeat	5E-15
cDNA_02-C01	3.1 ↓	Isopullulanase	5E-06
cDNA_18-B07	2.5 ↓	Leucine rich repeat containing protein	3E-68
cDNA_03-B10	2.6 ↓	MAP kinase	1E-127
cDNA_42-G11	2.5 ↓	Mortality factor 4 like 1 (predicted), partial	7E-49
cDNA_17-D05	2.6 ↓	NADH dehydrogenase	9E-40
cDNA_19-D12	2.6 ↓	Novel protein vertebrate mitochondrial enoyl Coenzyme A hydratase	2E-89
cDNA_68-H05	2.5 ↓	Parkin co-regulated gene	4E-73
cDNA_49-G07	2.7 ↓	Programmed cell death 4a	2E-97
cDNA_23-C10	3.4 ↓	Protein Tyrosine Phosphatase	5E-08
cDNA_06-F04	2.8 ↓	Remodeling and spacing factor 1	2E-50
cDNA_06-F07	4.2 ↓	RNA binding motif protein	1E-31
cDNA_02-B01	2.6 ↓	Scavenger receptor	1E-13
cDNA_20-D01	2.2 ↓	Selenophosphate synthetase	3E-142
cDNA_03-F02	2.3 ↓	Solute carrier	8E-14
cDNA_03-C07	2.5 ↓	Transcription factor 2B	8E-106
cDNA_12-H04	2.5 ↓	Transmembrane 9 superfamily protein	2E-58
cDNA_30-E09	2.4 ↓	Tu translation elongation factor, mitochondrial	7E-75
cDNA_46-H01	2.1 ↓	Transmembrane protein	6E-39
cDNA_18-A07	4.8 ↓	Unknown	N/A

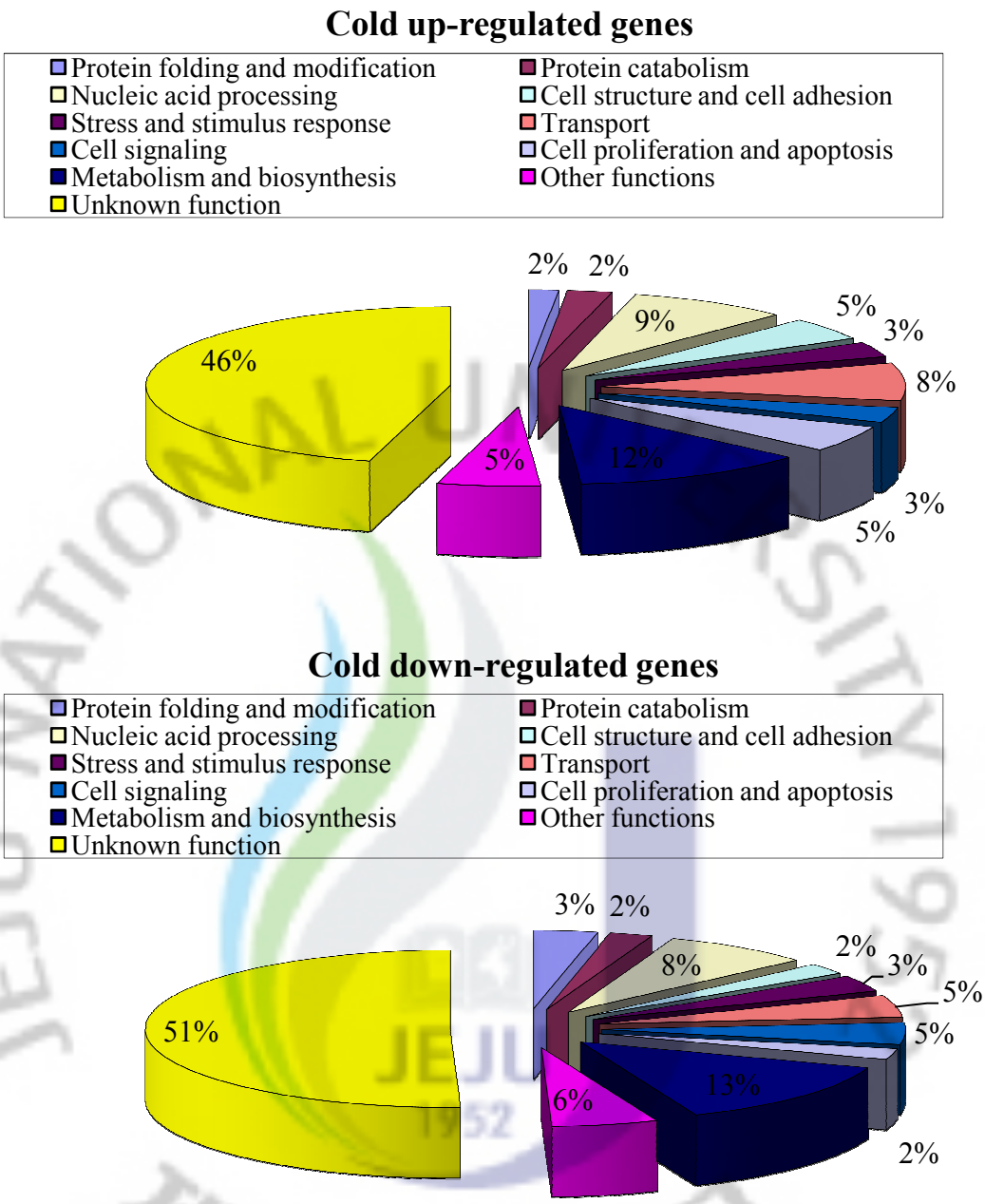


Fig 4. Functional categorization of up- and down-regulated genes under cold stress. The Go annotations of differentially expressed genes are based on Blast2GO analysis for level 3 of the biological process category.

Cold The growth rate of abalones was shown strong dependence on the water temperature. The optimal temperature for the growth of disk abalone (*H. discus disucs*) is about 20°C, while at a water temperature below 10°C abalones tend to grow very slowly. To investigate the impact of cold on abalone gene expression, we treated abalone with a cold shock at 10°C for 24 hours. In response to cold stress, a total of 207 genes were exclusively regulated, including 128 up-regulated genes and 89 down-regulated genes. The genes with great fold change were selected and listed in Table 4 and Table 5. The functional categorization of these differentially expressed genes was displayed in Fig 4. Unlike heat shock, conserved responses to low temperature stress are largely unknown within prokaryotes and eukaryotes. However, it is well known that cold stress can majorly cause formation and stabilization of secondary structures in RNA, which will interfere with efficient ribosomal binding, elongation and translation termination (Weber and Marahiel 2003). As a consequence, a large number of gene involved in transcriptional regulation, RNA splicing and translation have been frequently found to be responsible to cold stress (Gracey, Fraser et al. 2004). In this regard, the functional analysis in our study showed that 9 % of cold-induced genes and 8% of cold-repressed genes are in nucleonic acid processing category, including ribosomal protein genes, transcription factors, translation elongation factor and RNA polymerase. In prokaryotes, scientists have identified a family of cold shock proteins (Csp) which bind to single-stranded nucleic acids and function as RNA chaperones, rescuing RNAs trapped in unproductive folding states (Kim, Khunajakr et al. 1998). In fish, ATP-dependent RNA helicases and cold-inducible RNA-binding protein (CIRBP) have been proven to be robustly up-regulated and able to alter RNA secondary structure during cold stress (Saito, Sugimoto et al. 2000; Gong, Dong et al. 2005). However, neither Csp nor CIRBP is present in abalone genome, and the RNA helicase gene also showed similar expression as control level. The reason of this result is poorly understood. Instead, we observed the significant induction of a stearoyl-CoA delta9-desaturase, which is another key cold-responsive gene. This gene is involved in the introduction of double bonds to saturated

fatty acids (SFA) within biological membranes and thereby plays essential roles in the regulation of cell membrane fluidity at reduced temperature (Tiku, Gracey et al. 1996). Of 128 cold up-regulated genes, 16 genes (11% of total) are in metabolism and biosynthesis category. Besides stearoyl-CoA delta9-desaturase, this group of genes also includes long-chain fatty acid--CoA ligase, fatty-acid-binding protein, short-chain dehydrogenease/reductase, fatty acid synthase and fatty acid methyltransferase, which are mainly involved in the synthetic process of lipid fatty acid. Accordingly, two genes (GPSN2 and mitochondrial enoyl Coenzyme A hydratase) which are involved in the breakdown of fatty acids were found significantly repressed by cold stress. Similar findings were also reported in the cold shock response of bacteria where the transcription of genes encoding enzymes or regulators involved in fatty acid biosynthesis were induced (Han, Zhou et al. 2005). This phenomenon was correlated with the remodeling of cell membrane components in response to drastic downshift of temperature. Taken together, our data lead us to speculate that an important adaptive mechanism of abalone to reduced temperature is to regulate the content of lipid fatty acids. In the list of cold up-regulated genes, we also note two molecular chaperones, TCP-1 and HSP90. TCP-1, also known as HSP60, is a large complex that facilitates the proper folding of cytoskeleton proteins such as tubulin, actin, and centractin. To date, several direct links have been made between TCP-1 and cold adaption of organisms, although protein denaturation is not a major effect caused by cold stress. In yeast, TCP-1 is up-regulated by cold shock, and a cold-sensitive mutant was reported to result from mutation of TCP-1 (Chen, Sullivan et al. 1994). In carp, four genes of the TCP-1 chaperonin complex were induced in different tissues after cold shock (Gracey, Fraser et al. 2004). More recently, the up-regulation of TCP-1 also has been associated with cold hardiness in several insects (Kayukawa, Chen et al. 2005). In contrast, the response of HSP90 to cold stress was found not consistent. Despite up-regulated in most case, also identified is the continued down-regulation following the initial increase (Chen, Kayukawa et al. 2005).

Table 6. Selected genes whose transcription was enhanced after low-salinity stress.

Gene ID	Fold Change	Description	E-value to best hit
cDNA_41-G04	2.1 ↑	Arylsulfatase B	2E-43
cDNA_14-G02	2.1 ↑	Cholinergic receptor, nicotinic, beta polypeptide 4	4E-21
cDNA_13-E07	2.2 ↑	COMM domain containing 2	9E-32
cDNA_05-H02	1.9 ↑	COX4 neighbor	8E-33
cDNA_19-A03	1.9 ↑	Fatty acid synthase	5E-08
cDNA_15-G11	2.9 ↑	Fatty acid-binding protein	6E-08
cDNA_16-A06	2.2 ↑	Glucosephosphate isomerase	1E-89
cDNA_14-E05	1.9 ↑	Insulin-related peptide binding protein	1E-26
cDNA_67-A08	2.0 ↑	Isopenicillin N synthase and related dioxygenases	1E-09
cDNA_41-H09	2.8 ↑	Ketohexokinase	2E-47
cDNA_13-E06	2.1 ↑	Long-chain fatty acid--CoA ligase	9E-07
cDNA_58-C08	3.1 ↑	MLX interacting protein	2E-08
cDNA_14-H02	1.8 ↑	MYOM_LYMST Myomodulin neuropeptides	2E-37
cDNA_13-B09	2.6 ↑	N-acetylserotonin O-methyltransferase-like protein	1E-13
cDNA_15-A01	1.8 ↑	Pancreatic lipase-related protein 2	1E-39
cDNA_64-H06	2.0 ↑	NADP-dependent retinol dehydrogenase;	2E-34
cDNA_26-H10	2.5 ↑	Phenol sulfotransferase	4E-31
cDNA_13-G11	1.9 ↑	Phosphoglyceride transfer protein	2E-27
cDNA_15-A03	2.1 ↑	Programmed cell death 5	8E-22
cDNA_63-E10	1.8 ↑	Solute carrier family 6	1E-36
cDNA_15-H02	1.8 ↑	SRB7 suppressor of RNA polymerase B homolog	3E-40
cDNA_19-B11	2.2 ↑	Sterile20-like kinase	3E-40
cDNA_67-B05	1.8 ↑	Unknown	*N/A*
cDNA_14-F04	4.8 ↑	Unknown	*N/A*
cDNA_13-E11	2.9 ↑	Unknown	*N/A*
cDNA_14-H06	2.8 ↑	Unknown	*N/A*
cDNA_18-G03	2.6 ↑	Unknown	*N/A*
cDNA_16-F07	2.5 ↑	Unknown	*N/A*

Table 7. Selected genes whose transcription was repressed after low-salinity stress.

Gene ID	Fold Change	Description	E-value to best hit
cDNA_09-A01	1.9 ↓	3-hydroxybutyrate dehydrogenase	6E-20
cDNA_29-H10	1.8 ↓	Abhydrolase domain-containing protein 7	7E-76
cDNA_36-H12	2.1 ↓	Angiopoietin-like	2E-33
cDNA_03-F06	2.5 ↓	Thioredoxin domain-containing protein	9E-61
cDNA_02-G08	2.4 ↓	Calmodulin	5E-09
cDNA_03-F05	2.1 ↓	Calmodulin	2E-16
cDNA_26-F11	2.3 ↓	Cat eye syndrome critical region protein	3E-35
cDNA_27-D11	2.0 ↓	Endo-1,4-beta-mannanase	2E-76
cDNA_03-B05	2.1 ↓	Expansin	8E-11
cDNA_02-G09	2.6 ↓	Farnesoic acid o-methyltransferase	4E-06
cDNA_03-D07	2.0 ↓	Fignl1-prov protein	6E-73
cDNA_03-A02	1.9 ↓	Glutathione synthase	2E-30
cDNA_12-G01	1.8 ↓	Hemocyanin type 1	1E-80
cDNA_62-D06	2.4 ↓	Iduronate 2-sulfatase	3E-30
cDNA_03-F01	2.1 ↓	IMP dehydrogenase/GMP reductase:Thrombospondin type 3 repeat	5E-15
cDNA_50-E06	2.6 ↓	Inhibitor of apoptosis protein	4E-57
cDNA_19-G11	1.8 ↓	Kruppel-like factor	2E-37
cDNA_07-C08	2.0 ↓	Mannosidase	7E-17
cDNA_03-B10	2.5 ↓	MAP kinase	1E-127
cDNA_03-E02	1.9 ↓	Mitochondrial ribosomal protein	3E-71
cDNA_02-A12	2.1 ↓	Proteasome 26S non-ATPase	5E-83
cDNA_23-C10	2.1 ↓	Protein Tyrosine Phosphatase	5E-08
cDNA_06-F07	2.3 ↓	RNA binding motif protein	1E-31
cDNA_03-F02	1.9 ↓	Solute carrier	8E-14
cDNA_51-E01	1.8 ↓	Spectrin beta chain	5E-103
cDNA_07-G04	1.9 ↓	Syntaxin-5	2E-60
cDNA_12-H04	2.2 ↓	Transmembrane 9 superfamily protein	2E-58
cDNA_46-H01	100.0 ↓	Transmembrane protein	6E-39
cDNA_56-E02	2.5 ↓	Ubiquitin fusion degradation protein	6E-56
cDNA_53-G07	100.0 ↓	Unknown	*N/A*
cDNA_56-F02	2.8 ↓	Unknown	*N/A*
cDNA_67-H07	2.6 ↓	Unknown	*N/A*
cDNA_58-C07	2.6 ↓	Unknown	*N/A*

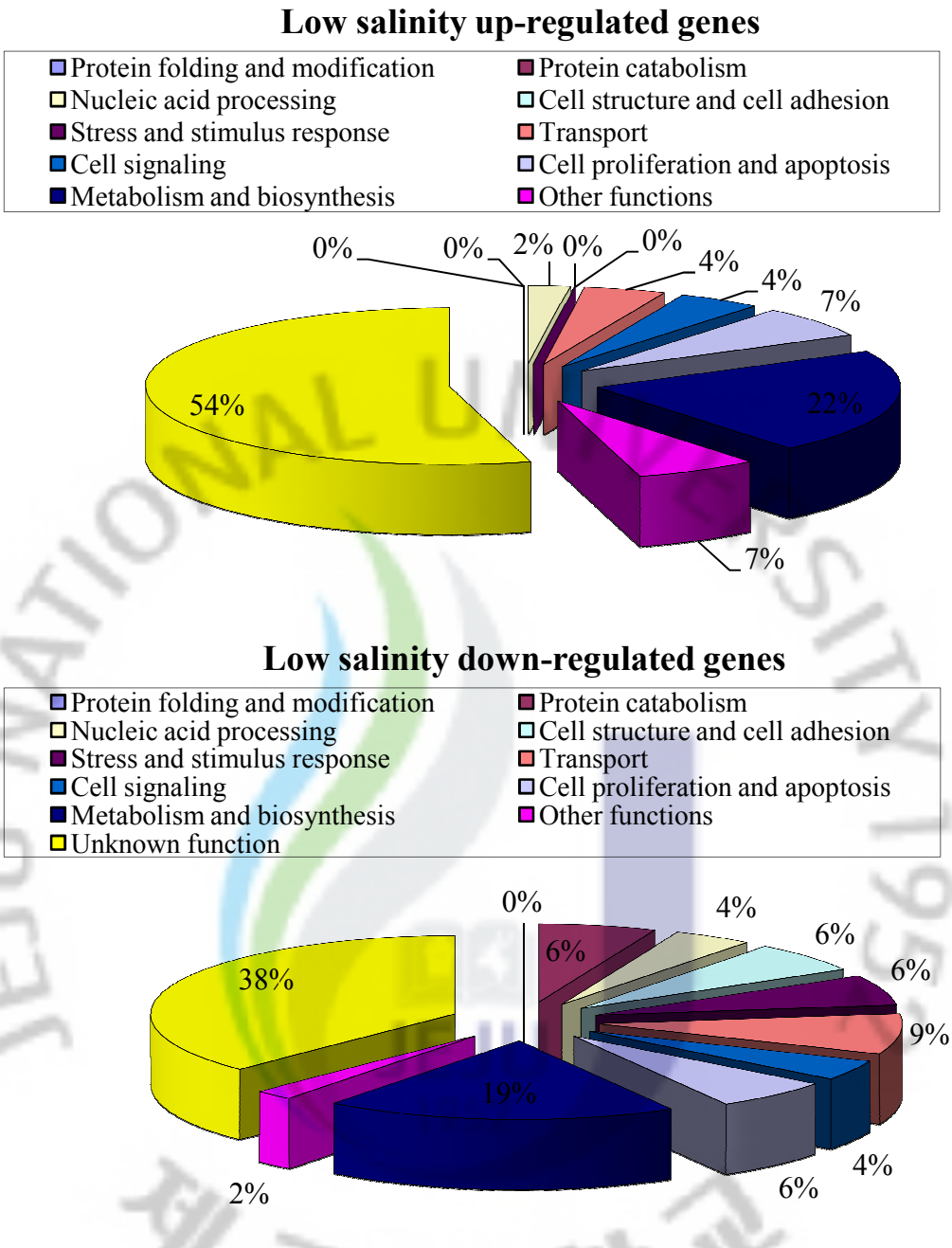


Fig 5. Functional categorization of up- and down-regulated genes under low-salinity stress. The Go annotations of differentially expressed genes are based on Blast2GO analysis for level 3 of the biological process category.

Table 8. Selected genes whose transcription was enhanced after high-salinity stress.

Gene ID	Fold Change	Description	E-value to best hit
cDNA_13-B10	2.3 ↑	Asparagine-linked glycosylation	1E-151
cDNA_14-G02	2.3 ↑	Cholinergic receptor, nicotinic, beta polypeptide 4	4E-21
cDNA_13-E07	1.9 ↑	COMM domain containing 2	9E-32
cDNA_07-A11	2.2 ↑	Dal specificity phosphatase 7	6E-25
cDNA_04-B01	2.3 ↑	Dual specificity phosphatase	4E-46
cDNA_15-G11	2.4 ↑	Fatty-acid-binding protein, FABP	6E-08
cDNA_67-A05	1.8 ↑	GDP dissociation inhibitor	4E-78
cDNA_14-E05	1.9 ↑	Insulin-related peptide binding protein	1E-26
cDNA_19-G11	1.9 ↑	Kruppel-like factor	2E-37
cDNA_13-E06	2.0 ↑	Long-chain fatty acid--CoA ligase	9E-07
cDNA_13-B09	2.5 ↑	N-acetylserotonin O-methyltransferase-like protein	1E-13
cDNA_13-G11	2.1 ↑	Phosphoglyceride transfer protein	2E-27
cDNA_15-A03	2.0 ↑	Programmed cell death 5	8E-22
cDNA_17-B06	2.3 ↑	Selenoprotein W1	7E-19
cDNA_09-F04	1.9 ↑	Sulfotransferase 1B2	2E-22
cDNA_15-C10	2.0 ↑	T-complex protein 1, gamma subunit (TCP-1-gamma)	1E-37
cDNA_14-F04	4.7 ↑	Unknown	*N/A*
cDNA_52-F06	2.8 ↑	Unknown	*N/A*
cDNA_46-F05	2.8 ↑	Unknown	*N/A*
cDNA_16-F07	2.7 ↑	Unknown	*N/A*
cDNA_13-B10	2.3 ↑	Asparagine-linked glycosylation	1E-151

Table 9. Selected genes whose transcription was repressed after high-salinity stress.

Gene ID	Fold Change	Description	E-value to best hit
cDNA_17-F04	2.2 ↓	40S ribosomal protein	4E-65
cDNA_21-B05	1.9 ↓	Ac1147-like protein	1E-28
cDNA_36-H12	2.1 ↓	Angiopoietin	2E-33
cDNA_03-F06	1.9 ↓	Thioredoxin domain-containing protein	9E-61
cDNA_02-G08	2.1 ↓	Calmodulin	5E-09
cDNA_03-F05	1.9 ↓	Calmodulin	2E-16
cDNA_27-D11	2.5 ↓	Endo-1,4-beta-mannanase	2E-76
cDNA_35-A12	1.8 ↓	Eukaryotic translation initiation factor 3	9E-74
cDNA_02-G09	2.1 ↓	Farnesoic acid o-methyltransferase	4E-06
cDNA_48-G03	1.9 ↓	Fibrillar collagen	1E-27
cDNA_07-G09	2.0 ↓	G protein-coupled receptor	3E-11
cDNA_02-A01	2.3 ↓	Histone H1	8E-22
cDNA_03-F01	2.1 ↓	IMP dehydrogenase/GMP reductase:Thrombospondin type 3 repeat	5E-15
cDNA_03-H02	1.9 ↓	Iron-sulfur domain-containing protein	4E-25
cDNA_02-C01	2.0 ↓	Isopullulanase	5E-06
cDNA_34-D11	1.8 ↓	MAK-V/Hunk	5E-40
cDNA_07-C08	1.8 ↓	Mannosidase	7E-17
cDNA_03-B10	2.3 ↓	MAP kinase	1E-127
cDNA_29-D07	2.0 ↓	Methyltransferase	3E-24
cDNA_13-F08	3.2 ↓	Peroxinectin	2E-40
cDNA_16-A12	3.3 ↓	Phage integrase:Phage integrase, N-terminal SAM-like	2E-06
cDNA_03-A08	2.0 ↓	Poly ADP-ribose Metabolism Enzyme (250.3 kD)	5E-14
cDNA_02-F09	1.8 ↓	Prolyl oligopeptidase	2E-36
cDNA_28-E10	1.9 ↓	Protei kinase, cGMP-dependent, type I	6E-71
cDNA_56-D03	1.9 ↓	Ribosomal protein L22	4E-17
cDNA_06-F07	3.4 ↓	RNA binding motif protein 18	1E-31
cDNA_02-B01	2.2 ↓	Scavenger receptor	1E-13
cDNA_17-E04	2.1 ↓	Scavenger receptor class F, member 1 isoform 1	3E-14
cDNA_03-F02	2.1 ↓	Solute carrier	8E-14
cDNA_38-G04	1.8 ↓	Splicing factor, arginine/serine-rich	1E-13
cDNA_12-H04	1.9 ↓	Transmembrane 9 superfamily protein	2E-58
cDNA_56-E02	2.2 ↓	Ubiquitin fusion degradaton protein	6E-56
cDNA_16-B12	15.3 ↓	Unknown	8E-01
cDNA_48-A09	2.9 ↓	Unknown	1E+00
cDNA_18-H01	2.9 ↓	Unknown	2E-02
cDNA_56-F02	2.6 ↓	Unknown	2E+00
cDNA_42-H12	2.5 ↓	Unknown	2E+00

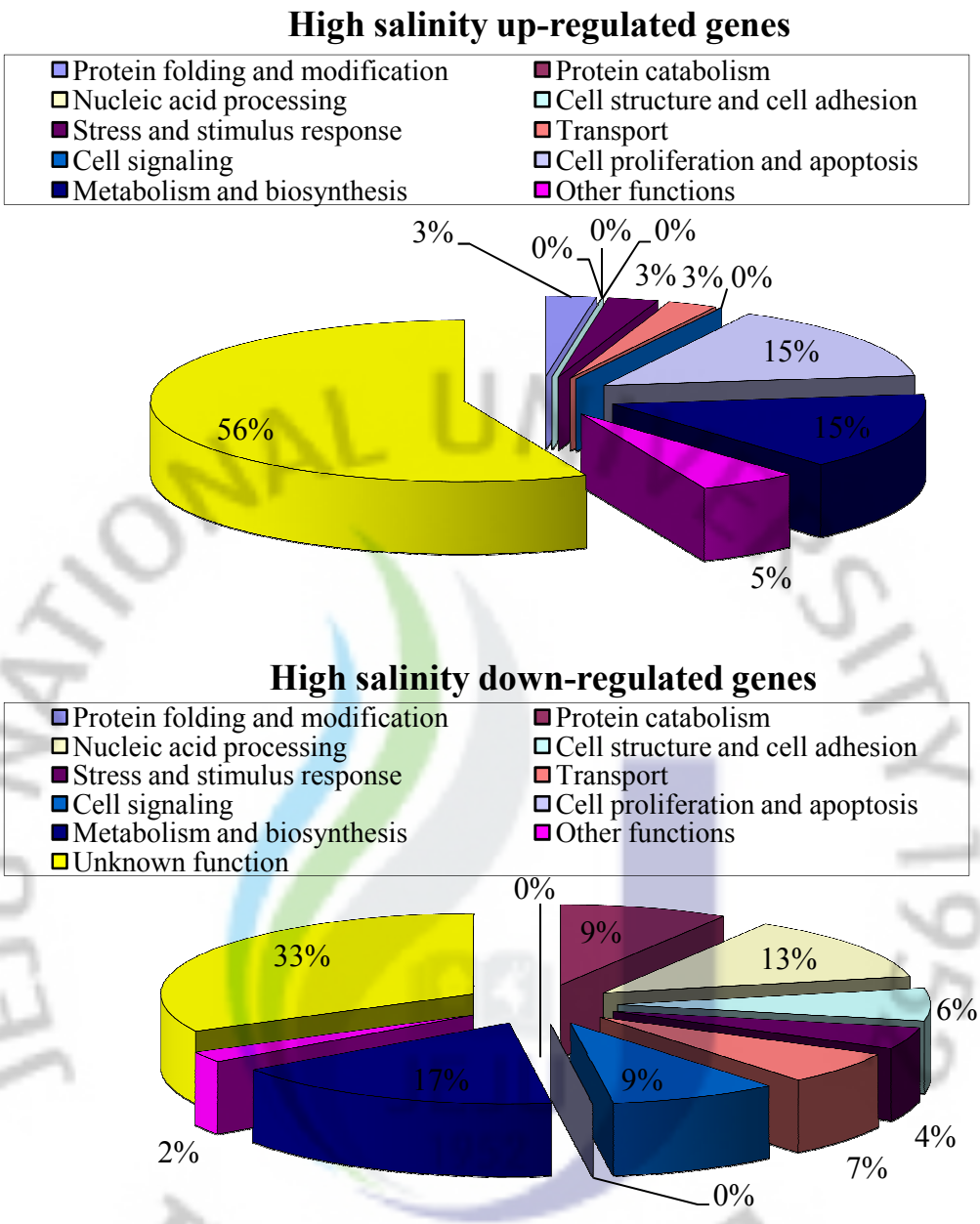


Fig 6. Functional categorization of up- and down-regulated genes under high-salinity stress. The Go annotations of differentially expressed genes are based on Blast2GO analysis for level 3 of the biological process category.

Low-Salinity and high-salinity For all the organisms living in aquatic environment, salinity is a pervasive abiotic factor that has a strong influence on cellular and organismal function. Consequently, regulation of intracellular and extracellular solute and water balance has become a fundamental requirement for survives. In the natural habitat of abalones, salinity can fluctuate with tidal cycles and with rainfall and drainage from adjacent terrestrial sites. The range of salinity tolerance for most abalone species is generally between 25-40 psu. Herein we investigated the response of abalone genes following 48-hour exposure at low-salinity (25 psu) and high-salinity (40 psu), which are close to the limit of their salinity tolerance.

The genes with significant up-regulation and down-regulation (>1.8 fold, $P<0.05$) by salinity stress were listed in Table 6-9. To gain an understanding of the stress response pathway, differentially expressed genes upon low- and high-salinity were subsequently assigned to different functional categories in Fig 5 and Fig 6 as described above. Compared with heat and cold stress, the number of gene responsive to salinity change was apparently much fewer. 47 genes and 49 genes were respectively up- and down-regulated by low-salinity, while 39 genes and 46 genes were respectively up- and down-regulated by high-salinity. In the up-regulation profiles of both low- and high- salinity stress, we interestingly noted a remarkable lack of genes in the category of stress and stimulus response. However, the apoptosis regulation gene PCD5 was consistently induced. The gills of abalone are the dominant site for maintaining ion homeostasis. The salinity change can effect on the activity of water channels and lead to efflux or influx of water from or into the gill cells: high-salinity causes shrinking, low-salinity causes swelling. Consequently, stressed gill cells can undergo cytoskeletal reorganization to modulate cell volume (Erickson, Northrup et al. 2003); however, no induction effect was found for the genes in the cell structure and cell adhesion category by either low- or high-salinity. Additionally, two calmodulin genes in the list of down-regulated genes were repressed by both low- and high-salinity. In the previous study of bivalve mollusc *N. ponderosa*, calmodulin was proven involvement in cell volume

recovery following hypo-osmotic swelling (Pierce, Politis et al. 1989). Therefore, we may speculate that the cells of abalone gill might be able to maintain the cell volume following a short term of salinity-stress and hence cytoskeletal genes and calmodulins were not activated. In contrast, genes involved in metabolism and biosynthesis were shown as the dominant category of differentially expressed genes during salinity stress. In this category, a set of genes functioning in fatty acid synthesis and metabolism were significantly induced by both low- and high-salinity. Fatty acids are the main components of the cell membrane and also are closely related to the regulation of cell-membrane function, such as ion transportation. Indeed, fatty-acid metabolism was found to be involved in the resistance of salinity stress. In *Caenorhabditis elegans*, RNA interference of fatty acid desaturase and fatty acid elongase has remarkably enhanced salinity-stress tolerance (Horikawa and Sakamoto 2009). In the study of fish, insulin-like growth factor has been shown to perform as upstream signaling molecule during the adaption of hyper-osmotic stress, by increasing the number and size of gill chloride cells, and the abundance of Na^+, K^+-ATPases and $Na^+/K^+/Cl^-$ co-transporters (Sakamoto and McCormick 2006). The majority of IGFs in extracellular fluids are complexed with their specific binding proteins (IGFBPs), which mediate the efflux, receptor interaction and degradation of IGFs. In the study of euryhaline fish *G. mirabilis*, IGFBR mRNA level were found to increase in hyper-osmotically stressed gill tissue but not in hypo-osmotically stressed gills (Evans and Somero 2008). In accordance with these findings, we also identified a putative insulin-related peptide binding protein with significant induction upon high-salinity but not upon low-salinity, indicating a evolutionally conserved upstream signal event of salinity stress response.

Common stress response to different physical stressors Through analysis of the gene expression data from cDNA microarray, we interestingly found that there is considerable overlap between the sets of genes regulated by the different physical stressors. As shown in Fig 7, 55 of the 128 cold-up-regulated genes also showed induction upon heat stress.

Approximately 50% of the differentially expressed genes in high-salinity stress were also significantly up- or down-regulated by low-salinity. These data indicate a cross-talk of the stress responses to different physical stressors. Notably, 10 genes including insulin-related peptide binding protein, cholinergic receptor, N-acetylserotonin O-methyltransferase-like protein, phosphoglyceride transfer protein, long-chain fatty acid-CoA ligase and 4 unknwon genes were induced by all four physical stressors. Noteworthy is that these identified genes are all playing critical roles in the general stress response. As discussed above, insulin-related peptide binding protein may mediate with the function IGF and play regulatory roles in different stress responses. Cholinergic receptor with the ability of binding acetylcholine could transmit the stress signal through never system and activate a series of transcription factors like AP-1 signal through never system and activate a series of transcription factors like AP-1 and NFκB to regulate the down-stream gene expression (Li, Song et al. 1996). N-acetylserotonin O-methyltransferase is the key enzyme for the biosynthesis of melatonin, which is considered as a pervasive and powerful antioxidant to protect the nuclear and mitochondrial DNA during stress (Reiter, Acuna-Castroviejo et al. 2001). Phosphoglyceride transfer protein and long-chain fatty acid-CoA ligase are involved in the synthesis and modification of phospholipids of cell membrane. Several studies have proven that the composition of membrane lipid is closely related to the resistance of stress (Swan and Watson 1999). Meanwhile, expression of 7 genes including calmodulin, thioredoxin domain containing protein, RNA binding motif protein, endo-1,4-beta-mannanase, solute carrier and two unknown genes were consistently repressed by four physical stressors. However, the down-regulation of these genes is somewhat rationalized against the know mechanisms of stress response, since they are also involved in the important biological processes of stress response like cellular signal transduction, cellular redox balancing, protein synthesis, energy production and molecular transport. Despite the need of further investigation, identification of these genes with overlapped stress responses provides insights into finding the key signal node regulating gene expression in stress

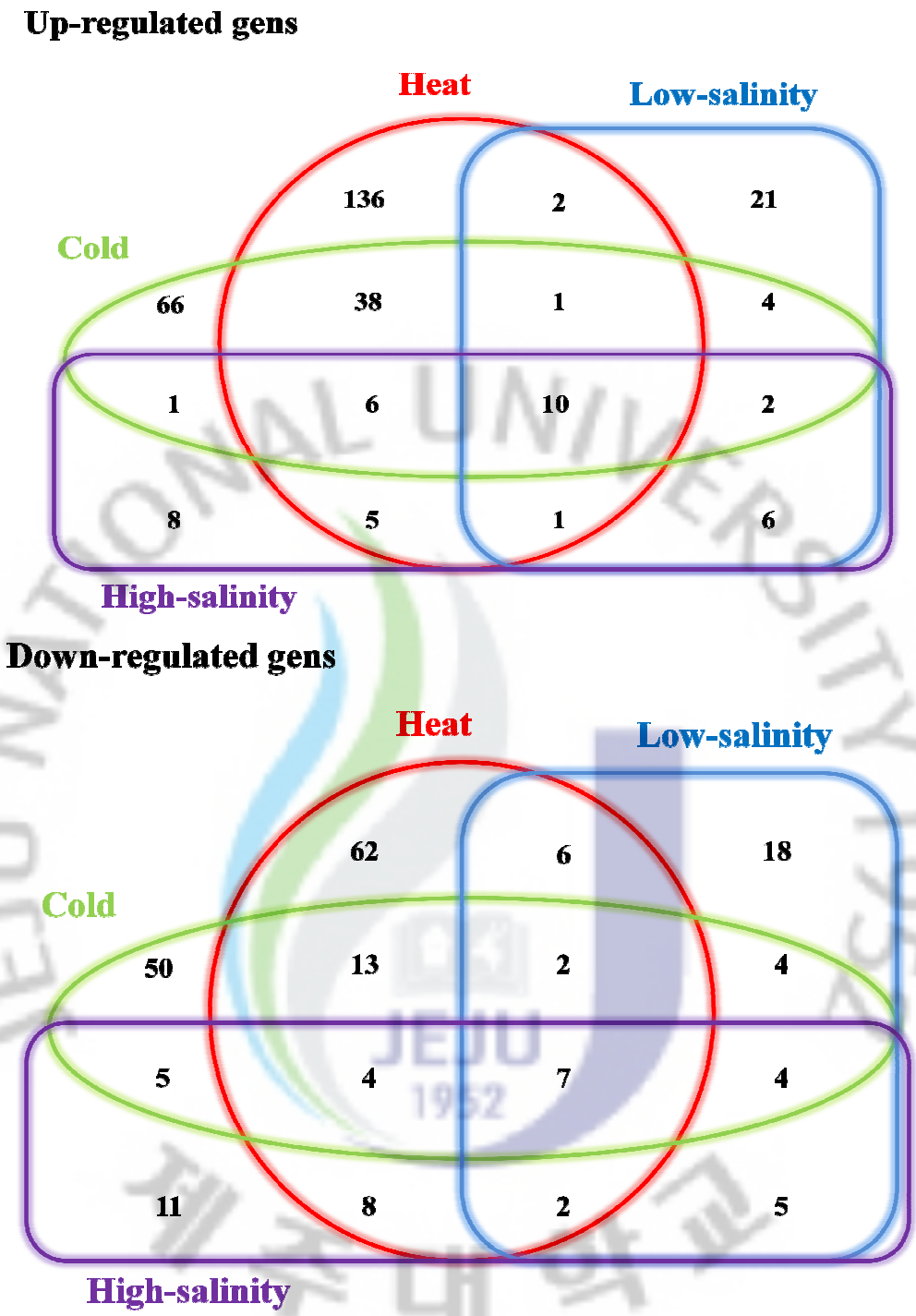


Fig 7. Overlapping of genes up- and down-regulated by physical stresses in abalone. Numbers in the figure indicate the numbers of transcipts exhibiting significant changes in abundance following 4 h heat shock at 30 °C, 24 h cold shock at 4 °C, and 24 h low salinity exposure at 25 psu and high salinity exposure at 45 psu.

response. Additionally, we found a large number of genes sharing the similar expression patterns in more than one stress.

3.3 Microarray analysis of expression response to heavy metal pollutants

Heavy metals are the most commonly detected pollutants in aquatic environment. They can bind to the sulfhydryl groups of proteins and also interfere with redox cycling, cell proliferation, DNA replication and repair, and apoptotic pathways. A few genes with protective functions are well documented to respond to heavy metal, including metallothioneins that chelate metal ions, heat shock proteins that renature the damaged protein and antioxidant enzymes that scavenge the oxidative agents (Koizumi and Yamada 2003). However, the detailed biological effects of heavy metals stress and the cellular protective mechanisms against it are remained unclear, especially in non-model organisms. With this respect, we carried out this investigation of expression pattern of global abalone genes upon three different heavy metals. The admissions of heavy metal were conducted by muscular injection and the hepatopancrease, which is the major organ of heavy metal metabolism and detoxification, was selected as the target site to examine gene expression.

Cadmium Following cadmium exposure, 167 genes were significantly induced and 156 genes were significantly repressed (fold>1.8, $P<0.05$). Of them, the genes with identified functions were selected and listed in Table 10 and Table 11. The large number of differentially expressed gene might be correlated to the strong toxic effect of cadmium. In accordance with it, the functional analysis of cadmium up-regulated genes revealed that 18 genes involved in general stress response have possessed the dominant category (11% of total). These genes include metallothionein (MT), heat shock proteins (HSP), glutathione S-transferase (GST), glutathione synsthetase (GS), cytochrome p450 enzyme (CYP), thioredoxin (TRX) and thioredoxin peroxidase (TRXP). MTs are known to be highly

metal-inducible and play a major role in detoxification of heavy metal by chelating them. In all the known sequence of disk abalone, we identified two putative MT genes. However, only one MT isoform showed induction response upon cadmium exposure. Similar result were also found for the MT-IL and MTIII genes in Hela cells and it was explained due to the function divergence among different MT isoforms (Stennard, Holloway et al. 1994). As expected, a series of abalone HSP genes including HSP90, small HSP and HSP70/HSP90 organization protein were highly induced by cadmium to deal with the production of denatured proteins. Similar to the data in heat stress, small HSP also registered the highest fold-change of all cadmium induced genes. GST is known to play a role in detoxification and is also a crucial antioxidant agent for the resistance of oxidative stress caused by heavy metals. The elevation of GST expression has been widely found in animals exposed to cadmium and other heavy metals (Lee, Lee et al. 2007; Kim, Dahms et al. 2010). In the current study, four members of abalone GST family were strongly induced by cadmium, while the other two were reduced. Our earlier study on abalone GSTs has demonstrated that different members of GST family could may different functions and hence exhibit distinct responses to stimulus (Wan, Whang et al. 2008). The cellular redox state is maintained by the GSH and TRX-dependent systems (Holmgren, Johansson et al. 2005). In this regard, the induction of GS and TRX is believed to cope with imbalanced redox state resulted by the ROS production cadmium exposure. In addition to stress proteins, the genes involved in protein synthesis and catabolic processes were largely induced as well: five genes encoding eukaryotic translation initiation factor, five genes encoding proteasome protein, two valosin-containing proteins and two genes encoding ubiquitin-activating enzyme E1 and ubiquitin-conjugating enzyme. These genes are thought to play roles in the synthesis of nascent protein and degradation of denatured protein. The response of this cluster of genes has been reported to be important for the cadmium resistance in yeast (Jungmann, Reins et al. 1993). We also note that cadmium exposure can significantly affect the cell cycling, proliferation and apoptosis of abalone hepatopancreas cell by regulating the expression of a

Table 10. Selected genes whose transcription was enhanced after cadmium stress.

Gene ID	Fold Change	Description	E-value to best hit
cDNA_56-B11	2.0 ↑	26S protease regulatory subunit 6B	2E-100
cDNA_42-H07	2.4 ↑	2oG-Fe(II) oxygenase	3E-07
cDNA_27-E01	2.6 ↑	Actin-depolymerizing factor 2	3E-12
cDNA_08-G05	6.8 ↑	Arsenic (+3 oxidation state) methyltransferase	6E-14
cDNA_70-F03	2.0 ↑	Aspartyl-tRNA synthetase	3E-32
cDNA_09-E06	1.8 ↑	Barrier to autointegration factor 1	7E-36
cDNA_04-E07	3.1 ↑	BAZ1B protein	2E-42
cDNA_28-D07	2.9 ↑	Bc12-like protein	4E-17
cDNA_47-H03	1.9 ↑	BolA-like 3	9E-19
cDNA_40-E11	2.0 ↑	CCDC58 protein	1E-36
cDNA_14-G04	2.2 ↑	Cctq	1E-42
cDNA_50-C03	2.4 ↑	CDC42	3E-41
cDNA_14-G05	2.0 ↑	Centrosomal protein 27	2E-40
cDNA_14-G02	2.9 ↑	Cholinergic receptor	4E-21
cDNA_68-G04	3.4 ↑	Coatomer protein complex	3E-78
cDNA_16-C09	2.3 ↑	Conopressin	7E-46
cDNA_16-F09	2.4 ↑	Cyclic AMP phosphoprotein	3E-19
cDNA_20-H06	2.0 ↑	CYP27B1	2E-49
cDNA_68-F04	2.8 ↑	Dimethylaniline monooxygenase	2E-52
cDNA_48-B03	2.0 ↑	Dipeptidyl-peptidase II	9E-71
cDNA_32-E03	2.8 ↑	Endothelial differentiation-related factor	1E-40
cDNA_15-A10	2.0 ↑	Eukaryotic translation initiation factor	1E-44
cDNA_25-E05	2.8 ↑	Eukaryotic translation initiation factor 2	2E-75
cDNA_54-D11	2.4 ↑	Eukaryotic translation initiation factor 3	7E-97
cDNA_57-G09	3.4 ↑	Eukaryotic translation initiation factor 5	1E-27
cDNA_36-G02	3.3 ↑	Eukaryotic translation initiation factor eIF3	1E-33
cDNA_47-C06	2.1 ↑	F-box protein 11	1E-37
cDNA_06-A03	2.7 ↑	FK506-binding nuclear protein	2E-15
cDNA_35-D04	2.1 ↑	Gaba(A) receptor associated protein	5E-61
cDNA_31-D04	2.4 ↑	Gamma filamin	6E-60
cDNA_23-F10	2.0 ↑	Gamma-butyrobetaine hydroxylase	4E-13
cDNA_53-C02	2.0 ↑	Glutamine synthetase	7E-42
cDNA_24-F05	2.1 ↑	Glutathione S-transferase	7E-35
cDNA_03-A02	8.3 ↑	Glutathione synthase	2E-30
cDNA_44-F10	1.9 ↑	GTP-binding protein SAR1B	1E-86
cDNA_47-G05	2.4 ↑	H/ACA ribonucleoprotein complex subunit 4	4E-116
cDNA_14-F06	6.7 ↑	Heat shock protein 90	2E-99
cDNA_04-D07	4.5 ↑	Heat shock protein 90	2E-93
cDNA_21-A09	2.5 ↑	Hillarin	5E-21
cDNA_54-F12	2.0 ↑	Hillarin	7E-46
cDNA_12-C11	2.1 ↑	Histone demethylase	3E-66
cDNA_68-F06	2.8 ↑	Hydroxysteroid (17-beta) dehydrogenase	4E-06
cDNA_52-G01	1.8 ↑	Hypoxia induced gene	3E-14
cDNA_52-C07	2.1 ↑	Import inner membrane translocase subunit TIM44	3E-61
cDNA_54-B01	1.9 ↑	Inorganic pyrophosphatase	3E-94
cDNA_19-G11	2.6 ↑	Kruppel-like factor	2E-37
cDNA_53-B06	2.3 ↑	Low density lipoprotein receptor-related protein	4E-06
cDNA_46-G06	1.9 ↑	MEGF11 protein	7E-16

cDNA_40-B10	1.9 ↑	Metallothionein	1E-08
cDNA_13-A10	2.3 ↑	Microtubule-associated protein	2E-76
cDNA_08-H05	2.2 ↑	Mollusk-derived growth factor	2E-43
cDNA_51-A12	6.2 ↑	Mu class glutathione S-transferase	1E-68
cDNA_33-C03	2.1 ↑	Myosin	1E-55
cDNA_65-C07	2.7 ↑	NF-X1 type zinc finger containing protein	6E-41
cDNA_38-F03	13.0 ↑	Omega class glutathione S-transferase	3E-62
cDNA_44-B08	9.9 ↑	Omega class glutathione S-transferase	2E-37
cDNA_56-G10	3.4 ↑	Oxygen regulated protein	5E-24
cDNA_03-A01	2.2 ↑	Pancreatic lipase-related protein	2E-47
cDNA_14-B04	1.8 ↑	Pim-3 protein	1E-91
cDNA_68-D06	2.7 ↑	Poly (ADP-ribose) polymerase	1E-23
cDNA_11-G07	3.3 ↑	Proteasome	2E-72
cDNA_43-C03	2.2 ↑	Proteasome	2E-59
cDNA_36-E02	2.7 ↑	Proteasome	6E-83
cDNA_14-A07	2.1 ↑	Proteasome	5E-86
cDNA_14-F11	1.8 ↑	proteasome 26S subunit subunit 4 ATPase	2E-125
cDNA_35-D06	1.9 ↑	RAB1	2E-108
cDNA_68-G07	2.5 ↑	Ribosomal protein L20	2E-26
cDNA_17-B06	1.8 ↑	Selenoprotein W1	7E-19
cDNA_26-E09	13.8 ↑	Small heat shock protein	8E-10
cDNA_53-A07	1.8 ↑	Soman-Aged Human Butyryl Cholinesterase	9E-12
cDNA_54-F10	2.1 ↑	Stress-induced-phosphoprotein (Hsp70/Hsp90-organizing protein)	5E-53
cDNA_17-A09	2.5 ↑	Succinate-Coenzyme A ligase	7E-60
cDNA_52-A03	1.9 ↑	Sulfatase 1 precursor	1E-86
cDNA_16-H09	2.0 ↑	Suppressor of cytokine signaling 2	4E-27
cDNA_39-G03	2.8 ↑	Thioredoxin	6E-18
cDNA_50-B06	2.9 ↑	Thioredoxin peroxidase	1E-86
cDNA_16-F10	2.3 ↑	Transgelin	2E-48
cDNA_23-C10	2.1 ↑	Tyrosine Phosphatase	5E-08
cDNA_14-H05	6.6 ↑	Ubiquitin-activating enzyme E1	4E-97
cDNA_12-D11	2.1 ↑	Ubiquitin-conjugating enzyme	4E-77
cDNA_03-B12	3.8 ↑	Valosin containing protein	1E-116
cDNA_12-C12	2.2 ↑	Valosin containing protein	9E-115
cDNA_47-D06	6.2 ↑	Vitelline envelope sperm lysin receptor	2E-129
cDNA_48-D08	2.7 ↑	Yellow protein	7E-29

Table 11. Selected genes whose transcription was repressed after cadmium stress.

Gene ID	Fold Change	Description	E-value to best hit
cDNA_29-H10	4.1 ↓	Abhydrolase domain-containing protein 7	7E-76
cDNA_07-B08	3.3 ↓	ADP-dependent glucokinase	2E-51
cDNA_41-B06	2.0 ↓	Alkaline phosphatase	1E-25
cDNA_54-D09	1.9 ↓	Ankyrin-1	9E-12
cDNA_35-C12	1.9 ↓	Apextrin	8E-42
cDNA_36-A08	1.9 ↓	Aryl sulfotransferase	6E-29
cDNA_63-B02	2.3 ↓	Arylsulfatase B precursor	2E-63
cDNA_25-G03	2.2 ↓	ATP synthase D chain	8E-31
cDNA_66-C09	3.2 ↓	Beta-agarase	1E-09
cDNA_26-F11	2.4 ↓	Cat eye syndrome critical region protein 5	3E-35
cDNA_18-A03	2.5 ↓	Cholinesterase 1	1E-71
cDNA_44-H08	2.6 ↓	CLIP-associating protein	4E-48
cDNA_38-D04	2.0 ↓	Crystallin, zeta variant	4E-35
cDNA_30-C12	2.6 ↓	Cubilin	1E-36
cDNA_07-A08	3.9 ↓	Cubilin	8E-46
cDNA_28-E02	2.4 ↓	Cysteine peptidase 2	2E-81
cDNA_41-G01	2.1 ↓	Cytochrome b	1E-117
cDNA_54-C09	1.9 ↓	Cytoplasmic intermediate filament protein A	9E-47
cDNA_42-A04	3.0 ↓	Divalent metal transporter	3E-85
cDNA_58-B12	2.5 ↓	DMBT	3E-24
cDNA_53-H04	1.9 ↓	Ectodermin	7E-25
cDNA_09-H08	2.1 ↓	Elongation factor	5E-54
cDNA_31-A08	2.4 ↓	Endo-1,3-beta-D-glucanase	9E-67
cDNA_17-B10	2.0 ↓	Endonuclease-reverse transcriptase	2E-06
cDNA_25-G05	5.5 ↓	Ependymin related protein	5E-30
cDNA_15-H01	2.3 ↓	Ependymin related protein-1	2E-33
cDNA_43-A04	2.5 ↓	Ferritin	1E-34
cDNA_39-C04	1.9 ↓	Gamma-butyrobetaine hydroxylase	1E-15
cDNA_33-D07	2.0 ↓	Ganglioside GM2 activator precursor	9E-26
cDNA_11-D10	1.8 ↓	Glutathione S-transferase	1E-27
cDNA_19-B03	2.4 ↓	GM2 activator protein	1E-25
cDNA_11-E09	2.2 ↓	Harmonin	2E-45
cDNA_69-B09	2.5 ↓	Headcase protein	3E-46
cDNA_22-F09	2.1 ↓	Heparanase	6E-31
cDNA_16-A05	2.2 ↓	Heparanase	1E-43
cDNA_12-D05	2.2 ↓	Hexokinase	6E-50
cDNA_38-E04	2.6 ↓	Hexokinase	2E-74
cDNA_32-F03	1.9 ↓	Hillarin	2E-12
cDNA_09-H09	2.0 ↓	Histamine H2 receptor	5E-25
cDNA_29-C10	2.0 ↓	Hpgd-prov protein	1E-42
cDNA_49-B10	1.9 ↓	HYA22	3E-71
cDNA_27-B11	1.8 ↓	Hydroxysteroid (17-beta) dehydrogenase 4	1E-80
cDNA_16-B05	2.4 ↓	Iduronate 2-sulfatase	3E-42
cDNA_52-F08	2.3 ↓	Iduronate 2-sulfatase precurso	7E-29
cDNA_50-E06	2.0 ↓	Inhibitor of apoptosis	4E-57
cDNA_16-G04	2.0 ↓	Isocitrate dehydrogenase 2 (NADP+), mitochondrial	2E-130
cDNA_24-H06	2.5 ↓	L-isoaspartyl protein carboxyl methyltransferas	3E-68

cDNA_11-H09	3.4 ↓	Low density lipoprotein receptor-related protein	2E-07
cDNA_25-B06	2.9 ↓	Low-density lipoprotein receptor domain class A containing protein	4E-15
cDNA_24-E11	2.6 ↓	Malate dehydrogenase	6E-78
cDNA_51-B08	1.8 ↓	Mannosidase	3E-50
cDNA_52-C04	3.5 ↓	Map kinase interacting kinase	2E-65
cDNA_09-A08	1.9 ↓	Mariner transposase	1E-05
cDNA_29-D07	2.7 ↓	Methyltransferase	3E-24
cDNA_25-F06	2.3 ↓	Microsomal glutathione S-transferase	2E-42
cDNA_12-A01	2.0 ↓	mitochondrial ATP synthase coupling factor 6	2E-19
cDNA_49-E01	6.6 ↓	Myc	2E-44
cDNA_57-H06	2.4 ↓	Myosinase	3E-47
cDNA_11-F10	1.9 ↓	NADH dehydrogenase	4E-32
cDNA_54-C03	2.8 ↓	Ncotinic acetylcholine receptor	1E-33
cDNA_13-A09	1.9 ↓	Peptidase	3E-15
cDNA_50-G10	2.3 ↓	Placental protein 11	1E-28
cDNA_69-G04	2.0 ↓	PLC_HALLA Perlucin	6E-17
cDNA_03-A08	1.9 ↓	Poly ADP-ribose Metabolism Enzyme (250.3 kD)	5E-14
cDNA_49-G07	4.9 ↓	Programmed cell death 4a	2E-97
cDNA_25-A06	1.9 ↓	Protein transport protein	8E-98
cDNA_40-H05	2.1 ↓	Regucalcin [Danio rerio] >gnl	2E-45
cDNA_17-E12	1.8 ↓	Selenide, water dikinase	1E-11
cDNA_49-D01	2.3 ↓	Serine incorporator 3	1E-39
cDNA_39-G11	1.8 ↓	Solute carrier family 6	3E-74
cDNA_27-E02	1.9 ↓	Sccinate dehydrogenase	5E-18
cDNA_25-B04	2.9 ↓	Sulfatase 1 precursor	2E-96
cDNA_34-A06	2.9 ↓	Tetraspanin-CD63 receptor	7E-14
cDNA_45-D11	1.9 ↓	Thioesterase domain containing 1	4E-39
cDNA_02-H02	2.0 ↓	TRAF	1E-20
cDNA_27-H05	2.0 ↓	Transmembrane protein	1E-54
cDNA_38-F07	1.9 ↓	Transposable element tcb1 transposase	1E-32
cDNA_49-G12	1.8 ↓	Tumor suppressor candidate 3	2E-120
cDNA_03-H05	1.8 ↓	Tmor suppressor TSBF1	1E-17
cDNA_30-F05	1.8 ↓	Uiquitin specific protease 7	1E-121
cDNA_20-E04	2.4 ↓	Universal stress protein	2E-10
cDNA_63-C01	2.5 ↓	Valyl-tRNA synthetase	1E-114
cDNA_03-C04	2.3 ↓	Vitellogenin	3E-27
cDNA_06-B08	2.2 ↓	Voltage-gated potassium channel beta-2	3E-53

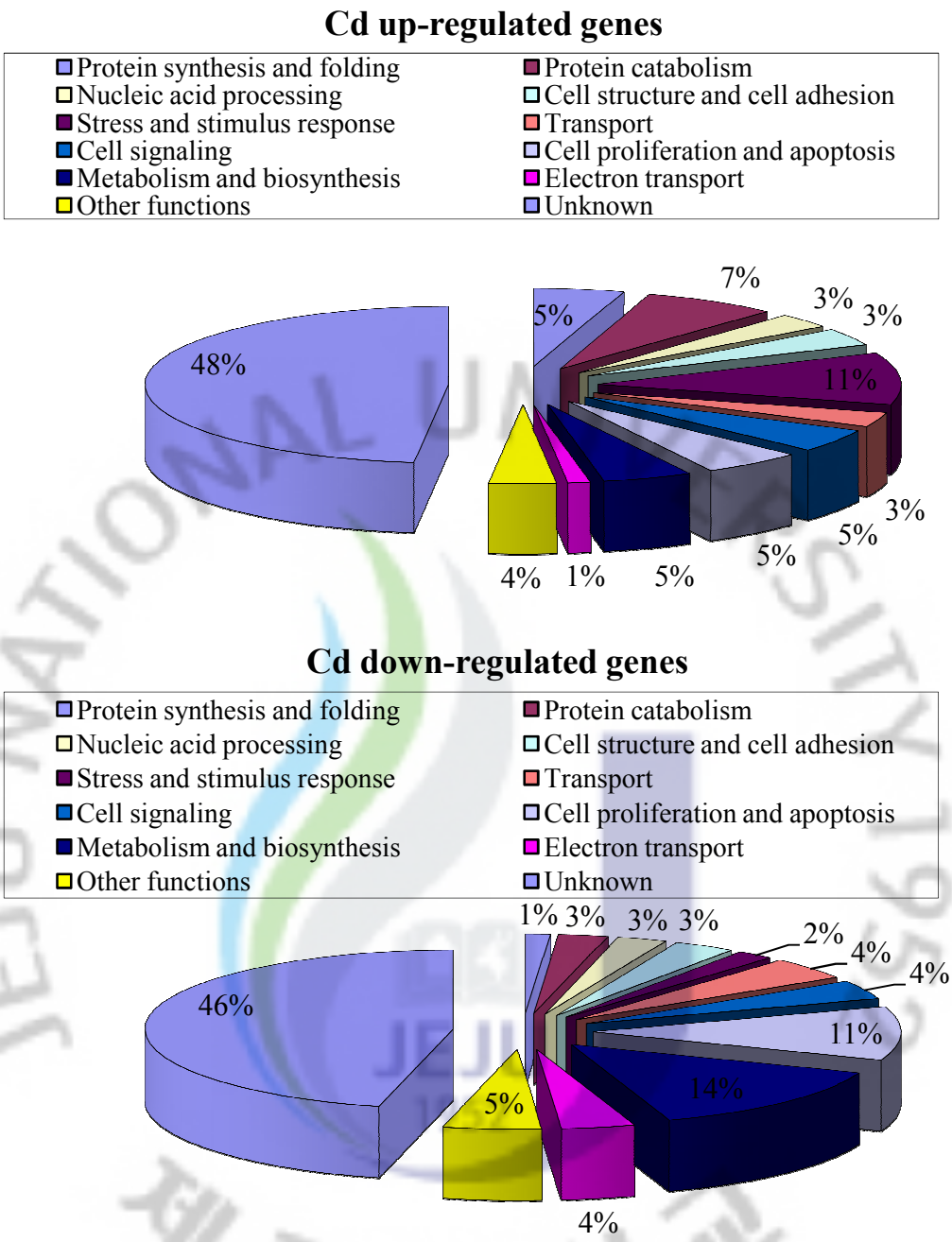


Fig 8. Functional categorization of up- and down-regulated genes under cadmium stress. The Go annotations of differentially expressed genes are based on Blast2GO analysis for level 3 of the biological process category.

large number of the genes involved in these processes. Apart from the direct damages at cell level, cadmium has also shown the effects on the sex hormone receptor genes, suggesting an endocrine-disrupting activity (Menke, Guallar et al. 2008). In agreement with this, we interestingly found that two hydroxysteroid (17-beta) dehydrogenases, which are the important enzymes in sex hormone synthesis, were respectively up- and down-regulated by cadmium.

Copper The exposure of copper resulted in total 125 genes differentially expressed to a degree greater than 1.8-fold with statistical significance $P<0.05$, including 75 up-regulated genes and 50 down-regulated genes (Table 12, 13). This number is much fewer than that of cadmium treatment. Moreover, we also observed much fewer genes in the category of "stress and stimulus response". The maximal induction was observed in an unknown gene with 4.0-fold while the maximal reduction occurred in an acheron gene with 5.6-fold. The small HSP gene, which possessed the highest induction in cadmium treatment, showed only 1.8-fold expression change upon copper. In the functional analysis, a cluster of genes involved in metabolic processes possessed a predominant proportion. These genes include several important enzymes in lipid synthesis and metabolism pathway. This finding is not surprising since the exposure to high concentration of heavy metals can also trigger a subsequent osmotic stress, leading the flux of water and ion in abalone cells. As we discussed above, activation of genes related in lipid metabolism could contribute to the resistance of stress. Furthermore, to modulate the cellular volume change and repair the cell damage, microtubule-associated protein, which is involved in cytoskeleton organization, was strongly induced. Addtionally, we noted that the expression of genes involved in protein folding and protein catabolism was also largely affected, as a general response to heavy metal stress. It is noteworthy that the expression of a glutaredoxin gene has been greatly repressed (4.1-fold). Glutaredoxin is a small enzyme to catalyzing the reduction of disulphide bridges and reverses the glutathionylation of proteins to regulate and/or protect protein activity. It is considered as crucial in cellular redox homeostasis and the prevention

Table 12. Selected genes whose transcription was enhances after copper stress.

Gene ID	Fold Change	Description	E-value to best hit
cDNA_67-A08	2.6 ↑	2OG-Fe(II) oxygenase	1E-09
cDNA_11-B10	2.9 ↑	Abhydrolase domain containing 7	8E-63
cDNA_63-C12	2.1 ↑	Arylsulfatase B	5E-71
cDNA_19-G10	1.8 ↑	Arylsulfatase B precursor (ASB)	
cDNA_67-C05	1.9 ↑	Cat eye syndrome critical region protein	8E-37
cDNA_26-F11	1.9 ↑	Cat eye syndrome critical region protein 5	3E-35
cDNA_16-B08	1.9 ↑	Chaperonin containing TCP1	1E-74
cDNA_14-G02	3.4 ↑	Cholinergic receptor	4E-21
cDNA_18-B11	2.1 ↑	Coiled-coil domain containing 93	5E-83
cDNA_15-F12	2.9 ↑	Collagen	6E-25
cDNA_34-D01	2.5 ↑	Cystathionine beta-synthase	1E-125
cDNA_12-H11	1.8 ↑	Deoxycytidylate deaminase	1E-47
cDNA_57-G09	2.3 ↑	Eukaryotic translation initiation factor 5	1E-27
cDNA_19-A03	3.0 ↑	Fatty acid synthase	5E-08
cDNA_69-H04	1.8 ↑	Glutaminyl cyclase	1E-38
cDNA_14-F06	2.6 ↑	Heat shock protein 90	2E-99
cDNA_16-A05	2.2 ↑	Heparanase	1E-43
cDNA_19-G11	2.4 ↑	Kruppel-like factor	2E-37
cDNA_64-A03	2.3 ↑	Lipoma HMGIC fusion partner	1E-52
cDNA_13-A10	2.8 ↑	Microtubule-associated protein	2E-76
cDNA_69-G10	2.3 ↑	Misexpression suppressor of ras	4E-43
cDNA_25-D10	2.1 ↑	Omega-3 fatty acid desaturase	5E-38
cDNA_56-G10	2.6 ↑	Oxygen regulated protein	5E-24
cDNA_70-F07	2.1 ↑	PDZ domain containing ring finger 3	7E-16
cDNA_16-D05	2.1 ↑	Phosphoserine aminotransferase	4E-132
cDNA_33-A01	2.5 ↑	Phytanoyl-CoA dioxygenase	4E-32
cDNA_15-A03	2.5 ↑	Programmed cell death 5	8E-22
cDNA_18-G08	1.9 ↑	Protein kinase	5E-29
cDNA_26-E09	1.8 ↑	Small heat shock protein	8E-10
cDNA_67-F03	1.9 ↑	Sulfatase 1 precursor	2E-06
cDNA_19-H12	1.9 ↑	Sulfatase 1 precursor	8E-66
cDNA_24-A08	2.1 ↑	Sulfotransferase	3E-33
cDNA_53-D01	2.0 ↑	Surface antigen msp4	4E-42
cDNA_18-D08	1.9 ↑	T-complex protein 1	2E-136
cDNA_15-A01	2.0 ↑	Ubiquitin-like	1E-39
cDNA_23-C01	3.4 ↑	Unknown	*N/A*
cDNA_13-H02	4.0 ↑	Unknown	*N/A*
cDNA_18-A11	3.6 ↑	Unknown	*N/A*
cDNA_67-B09	1.8 ↑	WD repeat-containing protein 18	1E-58
cDNA_21-A05	1.9 ↑	Zn-finger, CCHC type and RNA-directed DNA polymerase and Integrase	1E-110

Table 13. Selected genes whose transcription was repressed after copper stress.

Gene ID	Fold Change	Description	E-value to best hit
cDNA_07-H04	1.8 ↓	5'-nucleotidase domain-containing protein 3	3E-08
cDNA_26-C07	3.0 ↓	Acheron	2E-49
cDNA_32-F02	5.5 ↓	Acheron	2E-14
cDNA_04-E02	2.1 ↓	Component 1, q subcomponent	2E-07
cDNA_01-H07	2.3 ↓	DEAD/H box 56 RNA helicase/noh61	4E-77
cDNA_54-A09	2.0 ↓	DYRK2 protein	6E-147
cDNA_54-B09	1.8 ↓	Fibrinogen C domain containing 1	9E-32
cDNA_07-G09	1.8 ↓	G protein-coupled receptor	3E-11
cDNA_39-C04	2.1 ↓	Gamma-butyrobetaine hydroxylase	1E-15
cDNA_22-B10	4.1 ↓	Glutaredoxin	1E-44
cDNA_12-D05	2.6 ↓	Hexokinase	6E-50
cDNA_38-E04	3.3 ↓	Hexokinase	2E-74
cDNA_02-A01	1.8 ↓	Histone H1	8E-22
cDNA_03-H01	1.8 ↓	HLA-B associated transcript 8	4E-14
cDNA_03-B10	2.1 ↓	MAP kinase	1E-127
cDNA_19-A07	1.9 ↓	Methyltransferase	6E-18
cDNA_03-E02	1.8 ↓	Mitochondrial ribosomal protein	3E-71
cDNA_37-A06	2.8 ↓	Mitochondrial ribosomal protein	2E-06
cDNA_01-F07	2.0 ↓	Muscle LIM protein	2E-35
cDNA_01-H08	1.9 ↓	Mo-inositol oxygenase	1E-83
cDNA_70-H06	1.9 ↓	Solute carrier protein	6E-06
cDNA_03-F10	2.0 ↓	Thioester-containing protein	1E-27
cDNA_46-D07	2.5 ↓	Unknown	*N/A*
cDNA_37-G07	2.6 ↓	Unknown	*N/A*
cDNA_46-B06	3.1 ↓	Unknown	*N/A*
cDNA_40-H08	3.3 ↓	Unknown	*N/A*
cDNA_40-C02	3.3 ↓	Unknown	*N/A*
cDNA_54-E06	3.3 ↓	Unknown	*N/A*
cDNA_07-F05	3.5 ↓	Unknown	*N/A*
cDNA_40-H07	3.9 ↓	Unknown	*N/A*
cDNA_03-C04	2.2 ↓	Vitellogenin	3E-27

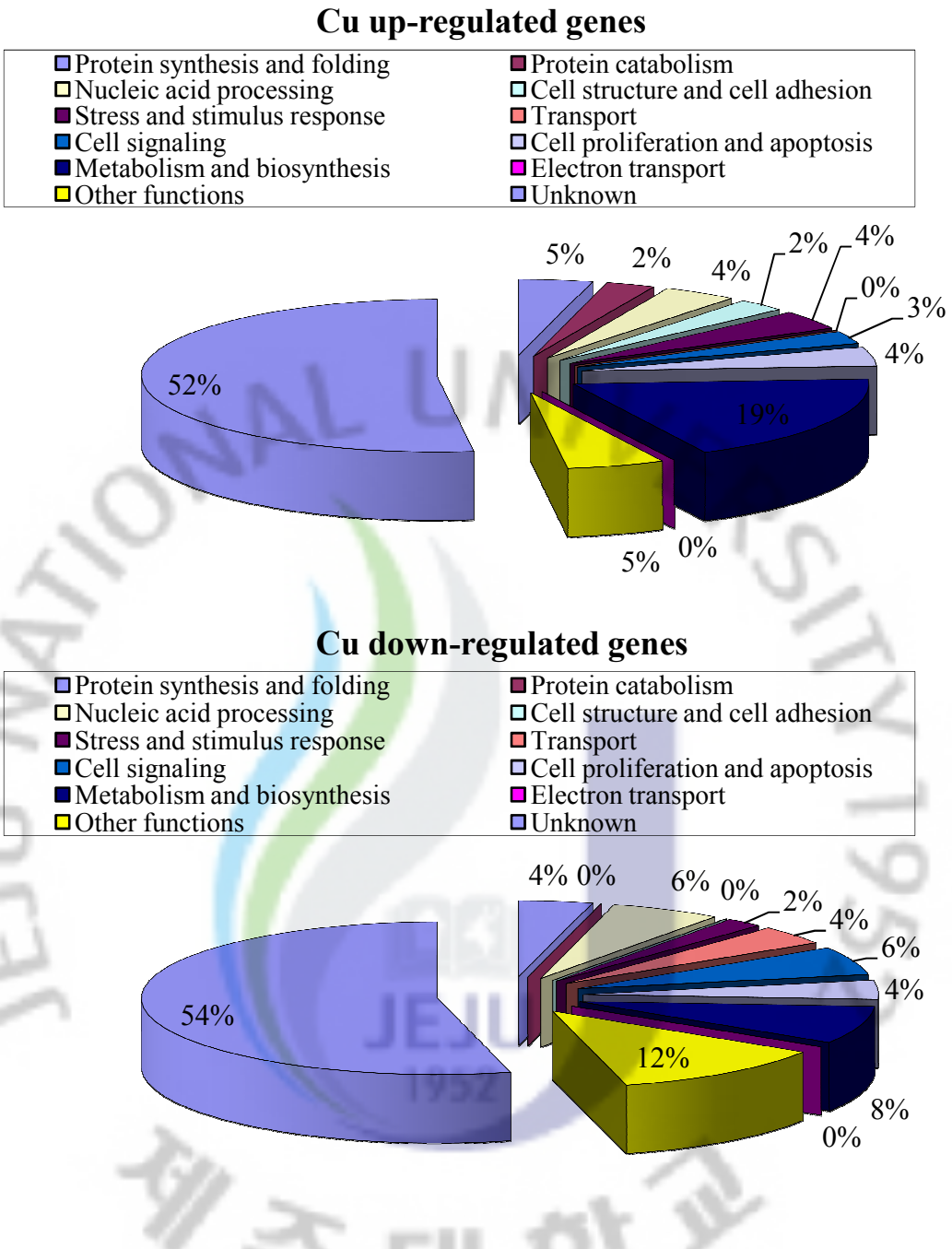


Fig 9. Functional categorization of up- and down-regulated genes under copper stress. The Go annotations of differentially expressed genes are based on Blast2GO analysis for level 3 of the biological process category.

of oxidative stress. However, a study on cadmium toxic effect reported that acute cadmium exposure could inactivate glutaredoxin via interaction with vicinal thiols (Chrestensen, Starke et al. 2000). Therefore, we may speculate the repression of abalone glutaredoxin in our study is similarly due to the toxic effect of copper.

Mercury In response to mercury exposure, a total of 80 genes exhibited significant expression change (>1.8-fold, $P<0.05$), including 64 up-regulated genes and only 16 down-regulated gene (Table 14, 15). The reason causing the big imbalance between the numbers of up- and down-regulated genes is not clear. The functional categorization of up-regulated genes revealed that the category of "stress and stimulus response" is the dominant part of response, which is comprised of three HSPs, three GSTs, a universal stress protein and an oxygen regulated protein. This result is much similar to the patterns of cadmium exposure, implying a similar effect of protein denaturation and oxidative stress by mercury. Small HSP registered the highest fold-change of up-regulation (12.0-fold). The second important group of mercury induced genes includes a series of genes with the function in cytoskeleton organization. As discussed above, these genes are associated with the cell damage and remodeling of cellular volume by mercury. In addition, we also found the induction of proteasome and ubiquitin-activating enzyme that are involved in protein catabolic process, similar to the response in other two heavy metal treatment. In the 17 Hg down-regulated genes, 7 genes are with unknown function and the other 9 genes are distributed into the functions of nucleic acid processing, signal transduction, apoptosis, metabolism and immune response.

Table 14. Selected genes whose transcription was enhances after mercury stress.

Gene ID	Fold Change	Description	E-value to best hit
cDNA_13-A08	1.9 ↑	Actin	9E-69
cDNA_27-E01	2.0 ↑	Actin-depolymerizing factor 2	3E-12
cDNA_36-H12	2.0 ↑	Angiopoietin-like 1 precursor	2E-33
cDNA_04-E07	2.1 ↑	BAZ1B protein	2E-42
cDNA_14-G02	2.3 ↑	Cholinergic receptor	4E-21
cDNA_13-F03	1.9 ↑	Colipase-dependent pancreatic lipase	5E-18
cDNA_39-G07	2.0 ↑	Dynein light chain-2	4E-45
cDNA_35-A12	3.1 ↑	Eukaryotic translation initiation factor	9E-74
cDNA_04-D07	3.8 ↑	Heat shock protein	2E-93
cDNA_14-F06	4.9 ↑	Heat shock protein 90	2E-99
cDNA_13-A10	5.0 ↑	Microtubule-associated protein	2E-76
cDNA_51-A12	1.9 ↑	Mu class glutathione S-transferase	1E-68
cDNA_13-B09	1.8 ↑	N-acetylserotonin O-methyltransferase-like protein	1E-13
cDNA_65-C07	2.1 ↑	NF-X1 type zinc finger containing protein	6E-41
cDNA_16-A08	2.0 ↑	Nudix	8E-43
cDNA_38-F03	1.8 ↑	Omega class glutathione S-transferase	3E-62
cDNA_44-B08	2.1 ↑	Omega class glutathione S-transferase	2E-37
cDNA_15-C11	2.8 ↑	Ornithine decarboxylase	5E-59
cDNA_56-G10	2.8 ↑	Oxygen regulated protein	5E-24
cDNA_66-G10	2.1 ↑	Poly (ADP-ribose) polymerase	4E-14
cDNA_14-E01	1.8 ↑	Poly(A) polymerase alpha	2E-101
cDNA_15-A03	2.0 ↑	Programmed cell death 5	8E-22
cDNA_14-F11	2.1 ↑	Proteasome	0E+00
cDNA_26-E09	12.1 ↑	Small heat shock protein	8E-10
cDNA_17-A09	2.3 ↑	Succinate-Coenzyme A ligase	7E-60
cDNA_14-H05	2.1 ↑	Ubiquitin-activating enzyme E1	4E-97
cDNA_54-F04	2.5 ↑	Universal stress protein	4E-11
cDNA_16-A12	2.6 ↑	Unknown	*N/A*
cDNA_15-F11	2.6 ↑	Unknown	*N/A*
cDNA_13-H02	2.7 ↑	Unknown	*N/A*
cDNA_37-E05	2.8 ↑	Unknown	*N/A*
cDNA_22-G12	2.7 ↑	Unknown	*N/A*
cDNA_14-E06	2.6 ↑	Unknown	*N/A*
cDNA_26-A12	2.8 ↑	Unknown	*N/A*
cDNA_54-H04	2.7 ↑	Unknown	*N/A*
cDNA_13-D10	2.5 ↑	Unknown	*N/A*

Table 15. Selected genes whose transcription was repressed after mercury stress.

Gene ID	Fold Change	Description	E-value to best hit
cDNA_08-B08	1.8 ↓	Alpha-aminoadipate aminotransferase	1E-20
cDNA_50-F10	1.8 ↓	Cytokine induced apoptosis inhibitor 1	2E-32
cDNA_12-H11	2.1 ↓	Deoxycytidylate deaminase	1E-47
cDNA_09-B08	1.9 ↓	Enoyl CoA isomerase	8E-75
cDNA_07-G09	1.8 ↓	G protein-coupled receptor	3E-11
cDNA_03-B10	1.8 ↓	MAP kinase	1E-127
cDNA_09-A08	1.9 ↓	Mariner transposase	1E-05
cDNA_19-A07	1.9 ↓	Methyltransferase	6E-18
cDNA_03-F10	1.8 ↓	Thioester-containing protein	1E-27
cDNA_01-B02	1.8 ↓	Unknown	*N/A*
cDNA_04-H04	1.8 ↓	Unknown	*N/A*
cDNA_33-A10	1.8 ↓	Unknown	*N/A*
cDNA_37-A06	2.7 ↓	Unknown	*N/A*
cDNA_38-C01	2.1 ↓	Unknown	*N/A*
cDNA_61-C11	2.1 ↓	Unknown	*N/A*
cDNA_70-H06	2.0 ↓	Unknown	*N/A*

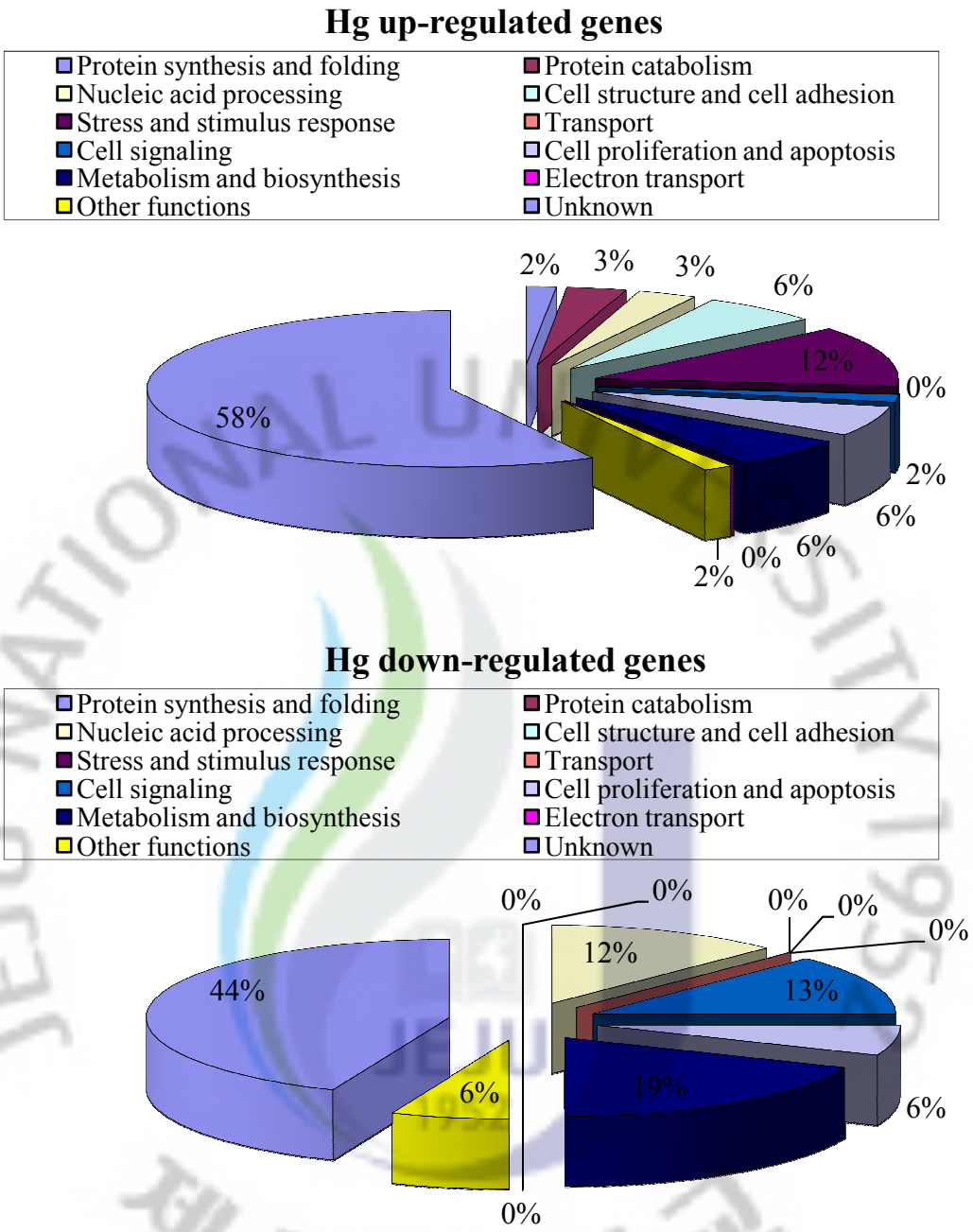


Fig 10. Functional categorization of up- and down-regulated genes under mercury stress. The Go annotations of differentially expressed genes are based on Blast2GO analysis for level 3 of the biological process category.

Common stress response to heavy metals Similar to the data analysis for physical stressors, we also conducted an investigation of the common response among different heavy metal treatments. As shown in the venn diagram of Fig 12, the molecular response in mercury treatment is highly correlated to the responses in cadmium and copper treatments, with over 50% gene overlapped. These overlapped genes with known functions mainly fell into the categories of general stress response and cytoskeleton organization. A total of 10 genes were identified sharing the similar expression pattern in response to all three heavy metal stress. These include small HSP, HSP90, oxygen regulated protein, cholinergic receptor, kruppel-like factor and four genes with unknown function. As important molecular chaperones, HSP90 and small HSPs are responsive to the changes in various environmental factors such as temperature, heavy metal concentrations, active oxygen concentration and salinity, protecting the structure and function of cells from stress and maintaining cellular homeostasis. However, the mRNA level of stress-inducible HSPs could be only transiently increased in response to heavy metal. Following the long-term heavy metal exposures, mRNA expression of HSP70 and HSP90 were reported to level off to the initial levels of control (Singer, Zimmermann et al. 2005; Choi, Jo et al. 2008). This phenomenon can be interpreted as a reduction in the metabolic capacity of the organism due to the sever tissue damage. Noteworthy is that this property may limit the biomarker utility of HSP genes for monitoring the heavy metal pollutions in field, which is possibly associated with chronic stress effect. The oxygen regulated protein, also known as hypoxia up-regulated protein (Hyou1), is well known as a stress protein inducible by hypoxia. Similar to HSPs, Hyou1 works as important chaperone function at the endoplasmic reticulum location, hence some studies also classified it as of the members of HSP family. In addition to the direct hypoxia exposure, Hyou1 were also found to be induced in the human peripheral blood cells exposed to cadmium (Dakeshita, Kawai et al. 2009). Heavy metals may interfere with hypoxia signaling pathway either by interacting with the hydroxylase enzyme of transcription factor hypoxia inducible factor (HIF-1) that direct HIF-1 protein turnover in response to oxygen

levels, or by inducing the generation of ROS and oxidative stress (Galanis, Karapetsas et al. 2009). The up-regulation of cholinergic receptor expression by different heavy metal stress is consistent with our finding for physical stresses. It is believed that cholinergic receptor could build a bridge between the upstream physiological stress response at hormone level and the downstream cellular stress response at the protein level. Abalone kruppel-like factor is another gene that we identified with significant expression change upon all physical stressors and heavy metals. Except for the reduction by low-salinity, its expression was highly induced in other stress conditions. Kruppel-like factor is a family of transcription factors that play essential roles in cell proliferation, differentiation and apoptosis event. The studies carried out in mammalian cells demonstrated that the expression of kruppel-like factors are regulated by the signals of tissue injury and a set of cytokines such as TGF-β and IFNγ (Bieker 2001). Importantly, the gene of suppressor of cytokine signaling was significantly induced by cadmium, indicating a suppressed cytokine signaling pathway under heavy metal stress. With this respect, the damage of hepatopancreas by heavy metals can be mainly responsible to the activation of abalone kruppel-like factor in our study. The induction of kruppel-like factor also could be the explanation for the activation/repression of a large battery of genes associated with cell proliferation and apoptosis as we observed in cadmium stress. Additionally, we noted that the expression responses of a certain number of genes are specific to only one heavy metal, particularly for cadmium stress. It provides us an insight to explore the biomarkers for understanding the pollution of specific heavy metal species in the further work.

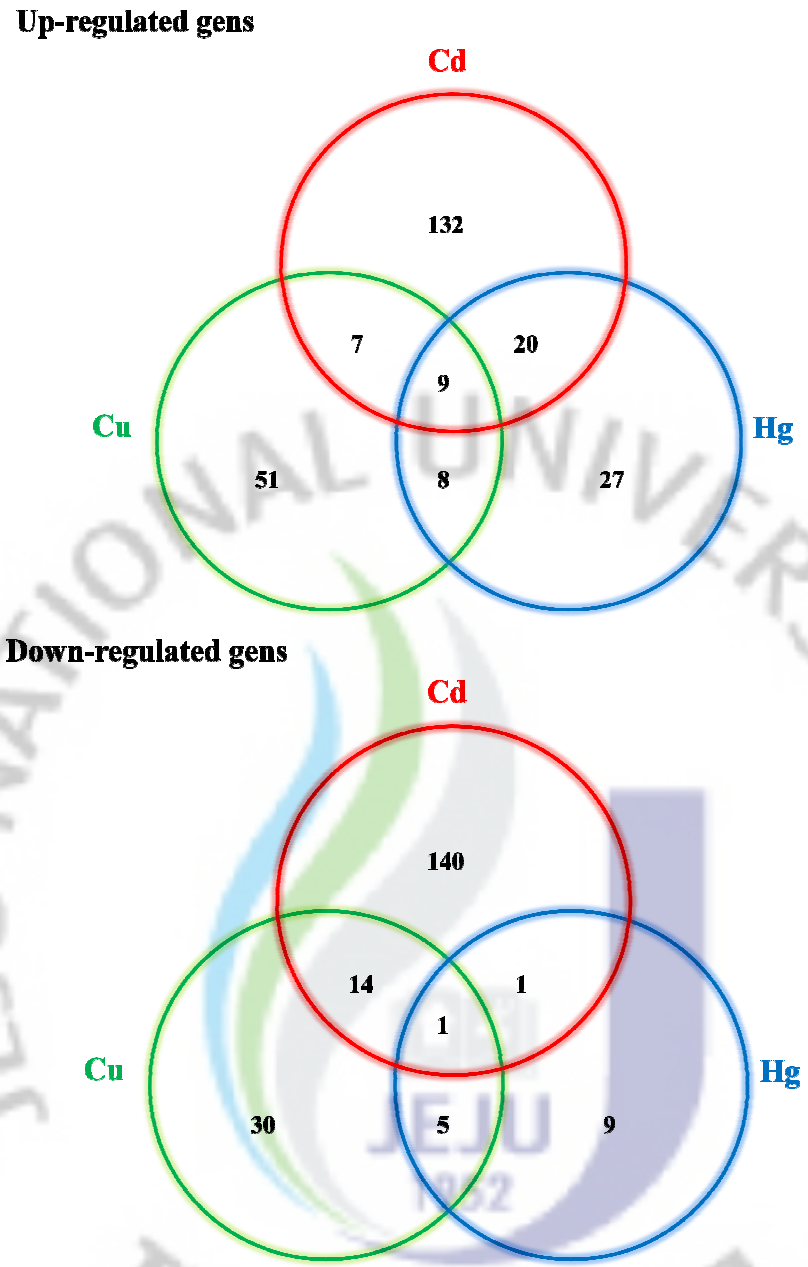


Fig 11. Overlapping of genes up- and down-regulated by different heavy metals in abalone. Numbers in the figure indicate the numbers of transcipts exhibiting significant changes in abundance following 24 h exposure to cadmium, copper and mercury, respectively.

3.4 Microarray analysis of expression response to organic EDCs

EDCs are a group of chemicals with the properties to interfere with the endocrine system of animals by mimicking or block the action of natural hormones in the body. In aquatic environment, endocrine disruption effects of EDCs have been observed in mammals, birds, reptiles, fish, and mollusks, as a significant issue in ecotoxicological study (Porte, Janer et al. 2006). The three chemicals (β-NF, Aroclor and TBT) used in our experiment respectively represented the EDCs of polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs) and tribuyltin (TBT) categories, which are the most commonly present in marine environment. Similar to the treatment of heavy metals, the admissions of three EDCs were also carried out by muscular injection of their DMSO solution.

PAHs In response to PAHs stress, a total of 68 gene exhibited significant expression changed (Fold>1.8, P<0.05), including 43 induced genes and 25 repressed genes. The genes with known function and great fold-change were selected and listed in Table 16-17. Notably, in the cluster of PAHs induced gene, 63% to tatal genes were with no homological sequence in the Genbank of NCBI (Fig 12). Two unknown genes also exhibited the maximal induction and repression levels, respectively. Indeed, the large proportion of unknown gene in the data brought hindrance to understand the molecular mechanism underlying in PAHs stress. For the all PAHs-regulated genes with known functions, we conducted a function categorization analysis based on their roles in biological processes. Surprisingly, the analysis result demonstrated that none of these genes fell in the category of “stress and stimulus response”. It is well known that PAHs require a biotransformation by cellular detoxification enzymes including CYPs and GSTs to exert their toxicity. The reactive metabolites from this process can bound convalently to cellular macromolecule such as proteins, DNA and RNA and caused the cell damage (Villeneuve, Khim et al. 2001). It is possible that our chemical could not be metabolized in hepatopancreas in a short-term treatment or its metabolites may have

lesser effect of protein denaturation and generation of oxidative stress, thus leading to the absence of general stress response. Instead, we noted that the expression of a group of genes associated with apoptosis regulation was significantly affected, including PCD5 (1.8-fold), kruppel-like factor, two acherons (2.8-fold and 3.9-fold), ornithine decarboxylase (2.4-fold), incilarin (2.2-fold), misexpression suppressor of ras (1.9-fold) and tumor suppressor TSBF1 (1.8-fold). In the previous study in mammalian models, it has been demonstrated that the induction of apoptosis by PAHs is mediated with the generation of ROS (Tsai-Turton, Nakamura et al. 2007). In our study, however, it seems that no or only scanty amount of ROS has been produced, indicated by the absence of response of stress proteins. Taken together, we can speculate that effect of PAHs stress in abalone could mediate with the induction cell apoptosis via a ROS independent pathway.

Table 16. Selected genes whose transcription was enhances after PAHs stress.

Gene ID	Fold Change	Description	E-value to best hit
cDNA_26-C07	2.8 ↑	Acheron	2E-49
cDNA_32-F02	3.9 ↑	Acheron	2E-14
cDNA_64-D11	1.8 ↑	Cubilin	9E-15
cDNA_35-A12	2.4 ↑	Eukaryotic translation initiation factor 3	9E-74
cDNA_69-H04	2.8 ↑	Glutaminyl cyclase	1E-38
cDNA_49-A03	2.2 ↑	Incilarin A	8E-16
cDNA_19-G11	1.9 ↑	Kruppel-like factor	2E-37
cDNA_64-A03	2.0 ↑	Lipoma HMGIC fusion partner-like 3	1E-52
cDNA_13-A10	3.8 ↑	Microtubule-associated protein	2E-76
cDNA_69-G10	1.9 ↑	Misexpression suppressor of ras,	4E-43
cDNA_15-C11	2.4 ↑	Ornithine decarboxylase 1	5E-59
cDNA_69-D05	1.8 ↑	Ovoperoxidase	3E-24
cDNA_15-A03	1.8 ↑	programmed cell death 5	8E-22
cDNA_69-F06	1.9 ↑	Retinal homeobox gene 3	4E-11
cDNA_65-A05	1.8 ↑	Solute carrier family 5	3E-45
cDNA_63-B08	1.9 ↑	Translocase of inner mitochondrial membrane	2E-88
cDNA_68-A04	4.8 ↑	Unknown	*N/A*
cDNA_67-B05	3.2 ↑	Unknown	*N/A*
cDNA_15-F11	2.4 ↑	Unknown	*N/A*
cDNA_61-C11	2.3 ↑	Unknown	*N/A*
cDNA_64-A01	2.2 ↑	Unknown	*N/A*
cDNA_15-G08	2.2 ↑	Unknown	*N/A*
cDNA_26-A12	2.2 ↑	Unknown	*N/A*
cDNA_14-F04	2.2 ↑	Unknown	*N/A*
cDNA_58-C07	2.2 ↑	Unknown	*N/A*
cDNA_19-C09	2.1 ↑	Unknown	*N/A*
cDNA_13-D10	2.1 ↑	Unknown	*N/A*
cDNA_67-H07	2.0 ↑	Unknown	*N/A*
cDNA_56-A09	2.0 ↑	Unknown	*N/A*
cDNA_63-F02	2.0 ↑	Unknown	*N/A*
cDNA_67-G07	2.0 ↑	Unknown	*N/A*

Table 17. Selected genes whose transcription was repressed after PAHs stress.

Gene ID	Fold Change	Description	E-value to best hit
cDNA_31-C12	1.8 ↓	Beta 1,3-glucanase	6E-79
cDNA_16-A09	2.2 ↓	Beta-carotene 15	8E-55
cDNA_26-F11	2.1 ↓	Cat eye syndrome critical region protein 5	3E-35
cDNA_38-D04	2.1 ↓	Crystallin, zeta variant	4E-35
cDNA_12-H11	1.8 ↓	Deoxycytidylate deaminase	1E-47
cDNA_07-G09	1.8 ↓	G protein-coupled receptor	3E-11
cDNA_13-A09	1.9 ↓	Peptidase	3E-15
cDNA_03-F02	2.0 ↓	Proton-coupled folate transporter	8E-14
cDNA_56-H06	1.9 ↓	Quinone oxidoreductase	9E-21
cDNA_12-G04	2.0 ↓	Thioester-containing protein	2E-52
cDNA_53-D01	1.9 ↓	Thioester-containing protein	4E-42
cDNA_03-F10	1.8 ↓	Thioester-containing protein	1E-27
cDNA_12-H04	1.8 ↓	Transmembrane 9 superfamily protein	2E-58
cDNA_03-H05	1.8 ↓	Tumor suppressor TSBF1	1E-17
cDNA_16-D05	2.5 ↓	Unknown	*N/A*
cDNA_03-G05	2.2 ↓	Unknown	*N/A*
cDNA_41-H07	2.0 ↓	Unknown	*N/A*
cDNA_19-H08	2.0 ↓	Unknown	*N/A*
cDNA_02-B12	2.0 ↓	Unknown	*N/A*
cDNA_48-G09	2.0 ↓	Unknown	*N/A*
cDNA_64-G04	2.0 ↓	Unknown	*N/A*
cDNA_01-B02	2.0 ↓	Unknown	*N/A*
cDNA_03-D11	1.9 ↓	Unknown	*N/A*

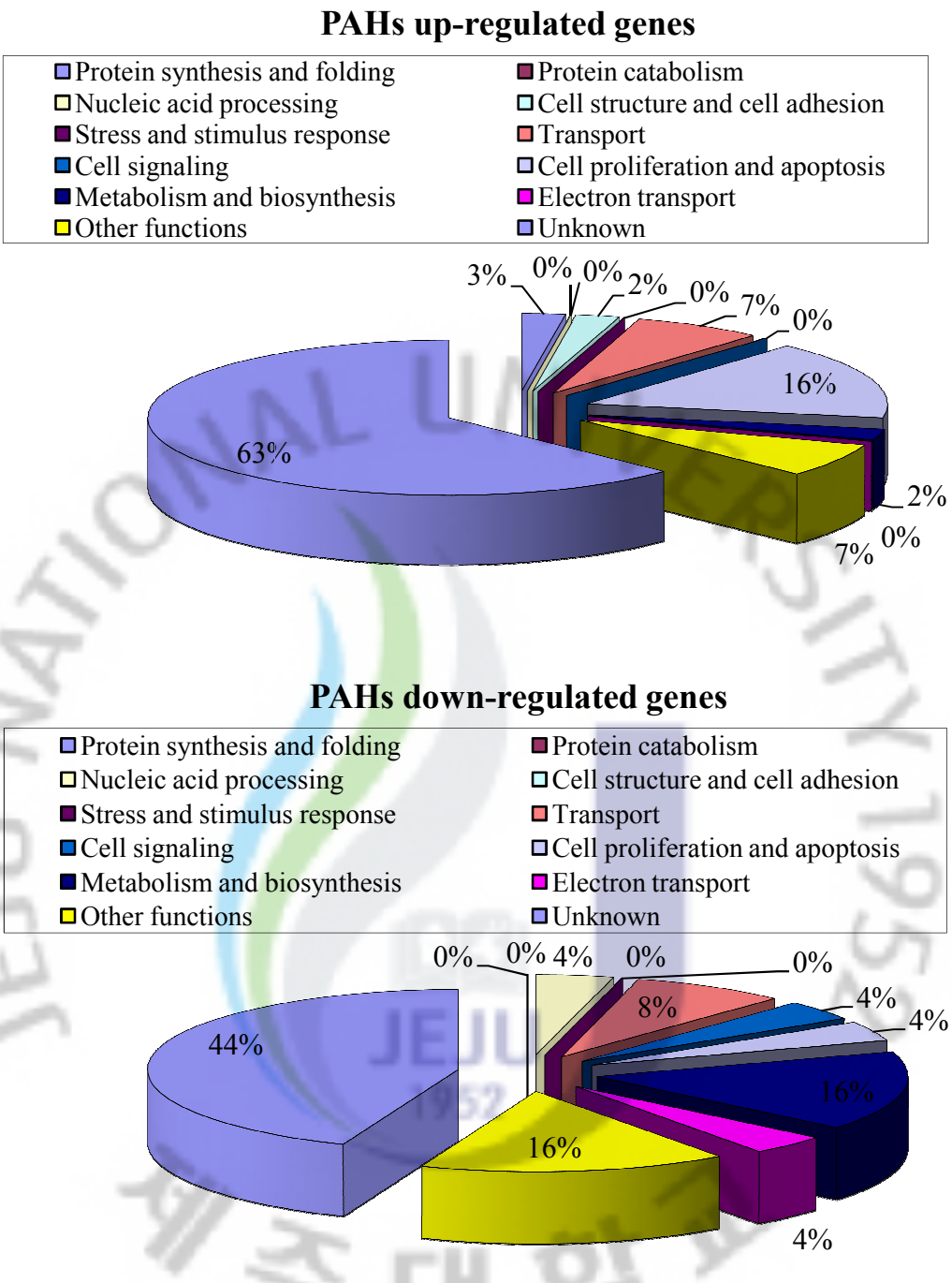


Fig 12. Functional categorization of up- and down-regulated genes under PAHs stress. The Go annotations of differentially expressed genes are based on Blast2GO analysis for level 3 of the biological process category.

PCBs 24-h exposure to PCBs resulted in a total of 75 genes differentially expressed to a degree greater than 1.8-fold with statistical significance $P<0.05$ (Table 18-19). 46 up-regulated genes and 29 down-regulated genes were also submitted to the function analysis as described above. Similar with the response to PAHs stress, none of genes in "stress and stimulus response" was shown significant expression change upon PCBs. However, we identified several genes involved in protein folding and catabolism with significant up-regulation, indicating the formation of denatured protein by PCBs stress. Importantly, a number of genes involved in cell apoptosis regulation were also significantly regulated. In addition to the genes in classic biological processes of stress response, a certain number of genes with other functions were also shown to be responsive to PCBs. Of these genes, we interestingly found molluscan defence molecule, macrophage mannose receptor, immunoglobulin mu binding protein and thioester-containing protein, which are crucial for the innate immune response in abalone. This result is consistent with the large body of reports in other animal models, which have documented the effect of PCBs on the immune functions (Duffy, Carlson et al. 2002; Canesi, Ciacci et al. 2003). In the list of PCBs down-regulated genes, we observed a series of genes involved in cellular signaling transduction, such as MAP kinase, regucalcin, G protein-coupled receptor and angiopoietin. This finding is in agreement with the neurotoxicity of PCBs (Costa 1998).

Table 18. Selected genes whose transcription was enhances after PCBs stress.

Gene ID	Fold Change	Description	E-value to best hit
cDNA_32-F02	3.9 ↑	Acheron	2E-14
cDNA_26-C07	2.4 ↑	Acheron	2E-49
cDNA_64-D11	2.1 ↑	Cubilin	9E-15
cDNA_50-C02	1.8 ↑	Ependymin-related protein	9E-06
cDNA_69-H04	2.1 ↑	Glutaminyl cyclase	1E-38
cDNA_67-A08	1.8 ↑	Isopenicillin N synthase and related dioxygenases	1E-09
cDNA_68-D12	1.8 ↑	Lipophorin receptor	9E-16
cDNA_09-A08	1.9 ↑	Mariner transposase	1E-05
cDNA_13-A10	3.3 ↑	Microtubule-associated protein	2E-76
cDNA_67-A09	2.0 ↑	molluscan defence molecule precursor	2E-18
cDNA_14-H02	1.9 ↑	Myomodulin neuropeptides precursor	2E-37
cDNA_13-B09	1.9 ↑	N-acetylserotonin O-methyltransferase-like protein	1E-13
cDNA_46-C03	1.9 ↑	Nicotinic acetylcholine receptor alpha-5	2E-34
cDNA_16-A08	2.0 ↑	Nudix	8E-43
cDNA_67-C05	1.8 ↑	Organic cation transporter protein	8E-37
cDNA_15-C11	2.0 ↑	Ornithine decarboxylase 1	5E-59
cDNA_15-A03	2.0 ↑	Programmed cell death 5	8E-22
cDNA_14-F11	1.9 ↑	proteasome 26S subunit subunit 4 ATPase	0E+00
cDNA_69-F06	2.3 ↑	Retinal homeobox gene 3	4E-11
cDNA_65-A05	1.8 ↑	Solute carrier family 5	3E-45
cDNA_68-A04	3.3 ↑	Unknown	*N/A*
cDNA_14-F04	3.1 ↑	Unknown	*N/A*
cDNA_15-G08	3.0 ↑	Unknown	*N/A*
cDNA_67-B05	3.0 ↑	Unknown	*N/A*
cDNA_37-H07	2.5 ↑	Unknown	*N/A*
cDNA_15-F11	2.4 ↑	Unknown	*N/A*
cDNA_66-H04	2.4 ↑	Unknown	*N/A*
cDNA_13-D10	2.4 ↑	Unknown	*N/A*
cDNA_46-E04	2.2 ↑	Unknown	*N/A*
cDNA_13-A11	2.2 ↑	Unknown	*N/A*
cDNA_13-E01	2.2 ↑	Unknown	*N/A*
cDNA_55-A08	2.1 ↑	Unknown	*N/A*
cDNA_19-C09	2.1 ↑	Unknown	*N/A*
cDNA_58-C07	2.1 ↑	Unknown	*N/A*
cDNA_59-A12	2.0 ↑	Unknown	*N/A*
cDNA_63-E08	2.0 ↑	Unknown	*N/A*
cDNA_67-A03	2.0 ↑	Unknown	*N/A*
cDNA_67-G03	2.0 ↑	Unknown	*N/A*
cDNA_16-F03	2.0 ↑	Unknown	*N/A*
cDNA_67-B09	1.8 ↑	WD repeat domain 18	*N/A*

Table 19. Selected genes whose transcription was repressed after PCBs stress.

Gene ID	Fold Change	Description	E-value to best hit
cDNA_07-H04	1.9 ↓	5'-nucleotidase domain-containing protein	3E-08
cDNA_08-A02	2.3 ↓	Adenosylhomocysteinase	4E-88
cDNA_36-H12	2.1 ↓	angiopoietin-like 1 precursor isoform 3	2E-33
cDNA_45-A06	1.8 ↓	Beta-glucuronidase	3E-80
cDNA_12-C04	2.3 ↓	D-beta-hydroxybutyrate dehydrogenase	1E-37
cDNA_48-G09	1.9 ↓	ependymin-related protein	5E-06
cDNA_35-A12	2.7 ↓	Eukaryotic translation initiation factor 3	9E-74
cDNA_07-G09	1.8 ↓	G protein-coupled receptor	3E-11
cDNA_03-H03	1.8 ↓	Immunoglobulin mu binding protein	1E-25
cDNA_50-E08	1.8 ↓	Macrophage mannose receptor precursor	8E-08
cDNA_03-B10	2.0 ↓	MAP kinase	1E-127
cDNA_62-B03	100.0 ↓	Methyltransferase	2E-16
cDNA_16-D05	2.0 ↓	Phosphoserine aminotransferase 1	4E-132
cDNA_16-G11	1.9 ↓	Regucalcin	2E-47
cDNA_08-C02	2.0 ↓	Ring finger protein	8E-88
cDNA_03-F02	1.9 ↓	Solute carrier	8E-14
cDNA_03-F10	2.0 ↓	Thioester-containing protein	1E-27
cDNA_12-B04	2.4 ↓	TnpA	6E-158
cDNA_03-H05	1.9 ↓	Tumor suppressor TSBF1	1E-17
cDNA_26-A12	3.6 ↓	Unknown	*N/A*
cDNA_12-A05	2.6 ↓	Unknown	*N/A*
cDNA_54-F04	2.4 ↓	Unknown	*N/A*
cDNA_08-B02	2.1 ↓	Unknown	*N/A*
cDNA_03-G05	2.1 ↓	Unknown	*N/A*
cDNA_70-H06	1.9 ↓	Unknown	*N/A*
cDNA_56-A09	2.0 ↓	Unknown	*N/A*

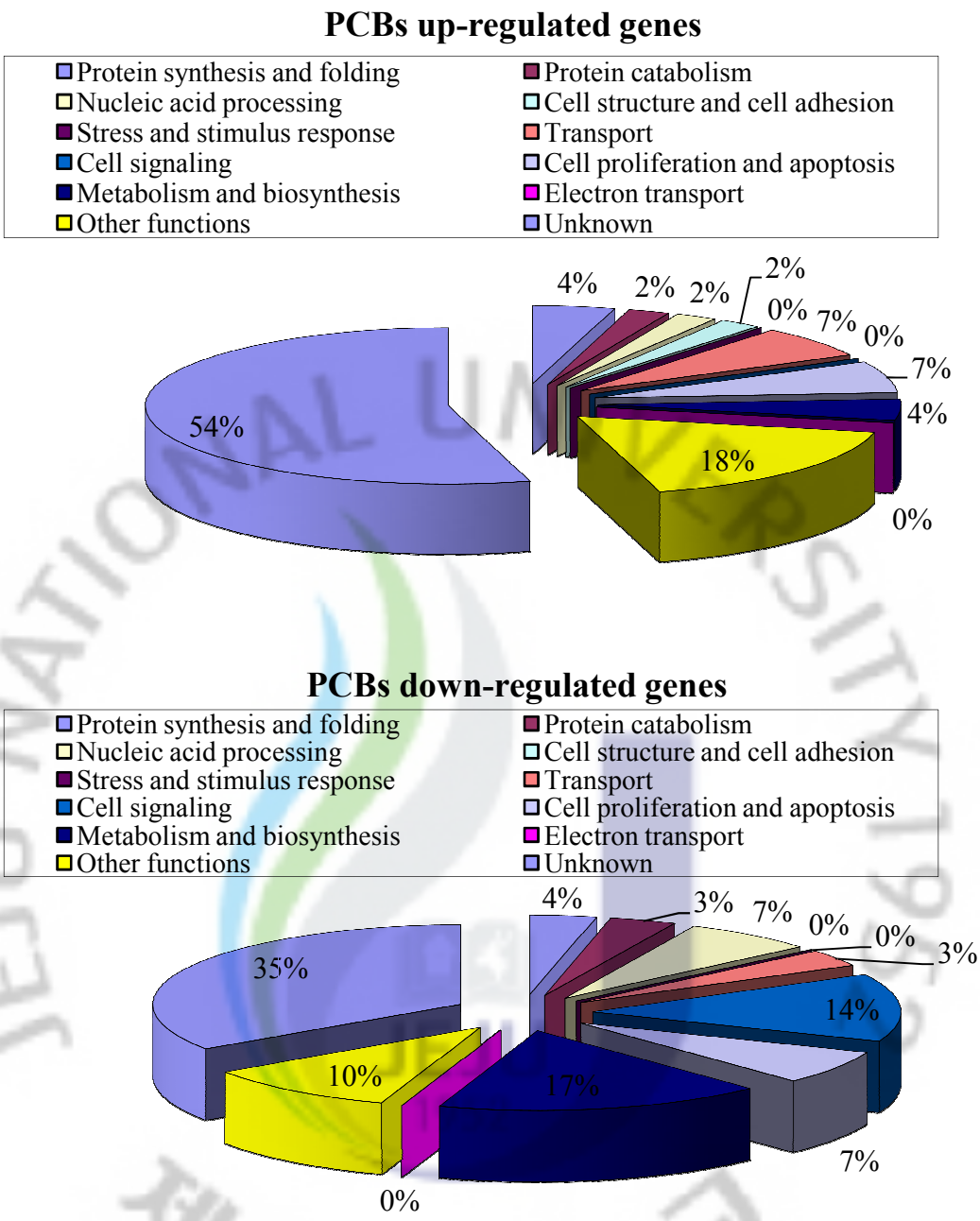


Fig 13. Functional categorization of up- and down-regulated genes under PCBs stress. The Go annotations of differentially expressed genes are based on Blast2GO analysis for level 3 of the biological process category.

TBT TBT represents a group of organotin compounds that have been widely used as biocides in antifouling paints. Mollusc species possess a particular sensitivity to trace level of TBT (10-50 ng/l), thereby are frequently used as indictors of TBT pollution. Herein we investigated the response of abalone genes to TBT stress by cDNA microarray analysis. Following a 24-h exposure of TBT by muscular injection, 57 genes were significantly induced while 21 genes were significantly repressed (>1.8-fold, $P<0.05$). In contrast the expression profiles of PAHs and PCBs, stress proteins including HSPs and GST were highly induced by TBT, indicating a stronger toxic effect. The functional analysis of differentially expressed genes showed that the regulation of genes involved in "cell proliferation and apoptosis" category is the dominant response to TBT exposure (Fig 14). This result is fairly similar to the data in PAHs and PCBs experiment, indicating that apoptosis is the common toxic effect of these EDCs. Additionally, we observed induction of nicotinic acetylcholine receptor, another member of cholinergic receptor family that has exhibited consistent induction upon heavy metals. Taken together with the induction of small HSP and HSP90, which were also commly induced by three heavy metals, these data collectively suggested a crosstalk between the stress response of TBT and heavy metals.

Table 20. Selected genes whose transcription was enhances after TBT stress.

Gene ID	Fold Change	Description	E-value to best hit
cDNA_34-C01	1.9	28S ribosomal protein S23	2E-22
cDNA_32-F02	2.8	Acheron	2E-14
cDNA_26-C07	2.2	Acheron	2E-49
cDNA_08-G05	1.8	Arsenic (+3 oxidation state) methyltransferase	6E-14
cDNA_04-E07	1.9	BAZ1B protein	2E-42
cDNA_64-D11	2.0	Cubilin	9E-15
cDNA_34-D01	5.2	Cystathionine-beta-synthase	1E-125
cDNA_19-A03	2.1	Fatty acid synthase	5E-08
cDNA_04-D07	3.0	Heat shock protein	2E-93
cDNA_14-F06	3.4	Heat shock protein 90	2E-99
cDNA_67-A08	1.9	Isopenicillin N synthase and related dioxygenases	1E-09
cDNA_19-G11	1.8	Kruppel-like factor	2E-37
cDNA_68-D12	2.1	Lipophorin receptor	9E-16
cDNA_52-C04	1.9	Map kinase interacting kinase	2E-65
cDNA_13-A10	3.3	Microtubule-associated protein	2E-76
cDNA_25-C01	2.0	MIS12 homolog	7E-16
cDNA_13-B09	1.8	N-acetylserotonin O-methyltransferase-like protein	1E-13
cDNA_68-A05	1.9	Nanos 2	3E-12
cDNA_46-C03	1.9	Nicotinic acetylcholine receptor	2E-34
cDNA_44-B08	2.0	Omega class glutathione S-transferase	2E-37
cDNA_15-C11	2.3	Ornithine decarboxylase 1	5E-59
cDNA_15-A01	2.0	Pancreatic lipase-related protein 2	1E-39
cDNA_15-A03	1.9	Programmed cell death 5	8E-22
cDNA_43-C03	1.8	Proteasome	2E-59
cDNA_69-F06	1.8	Retinal homeobox gene 3	4E-11
cDNA_52-H12	2.2	Ribosomal protein S13	2E-24
cDNA_57-A04	1.9	RNA-binding protein	4E-26
cDNA_26-E09	10.6	Small heat shock protein	8E-10
cDNA_65-A05	1.9	Solute carrier family 5	3E-45
cDNA_67-B05	3.0	Unknown	*N/A*
cDNA_23-C01	2.8	Unknown	*N/A*
cDNA_57-B04	2.6	Unknown	*N/A*
cDNA_42-B12	2.6	Unknown	*N/A*
cDNA_14-F04	2.5	Unknown	*N/A*
cDNA_33-B11	2.5	Unknown	*N/A*
cDNA_13-D10	2.5	Unknown	*N/A*
cDNA_13-H02	2.5	Unknown	*N/A*
cDNA_13-A11	2.3	Unknown	*N/A*
cDNA_09-C05	2.3	Unknown	*N/A*
cDNA_13-H01	2.1	Unknown	*N/A*

Table 21. Selected genes whose transcription was repressed after TBT stress.

Gene ID	Fold Change	Description	E-value to best hit
cDNA_31-C12	1.8	Beta 1,3-glucanase	6E-79
cDNA_18-A03	1.8	Cholinesterase 1	1E-71
cDNA_12-H11	1.8	Deoxycytidylate deaminase	1E-47
cDNA_58-B12	1.8	DMBT	3E-24
cDNA_07-G09	1.8	G protein-coupled receptor	3E-11
cDNA_02-A01	1.9	Histone H1	8E-22
cDNA_03-E10	1.8	mRNA transport regulator 3	5E-49
cDNA_11-A04	1.8	Myc homolog	2E-08
cDNA_03-A08	1.8	Ply ADP-ribose Metabolism Enzyme (250.3 kD)	5E-14
cDNA_03-F02	2.1	Proton-coupled folate transporter	8E-14
cDNA_40-H05	1.8	Regucalcin [Danio rerio] >gnl	2E-45
cDNA_03-G02	1.8	Selenoprotein	8E-40
cDNA_03-F10	2.3	Thioester-containing protein	1E-27
cDNA_37-E05	3.2	Unknown	*N/A*
cDNA_03-G05	2.2	Unknown	*N/A*
cDNA_03-D11	1.9	Unknown	*N/A*
cDNA_01-B02	1.9	Unknown	*N/A*
cDNA_11-B04	1.8	Unknown	*N/A*
cDNA_12-D04	1.8	Unknown	*N/A*
cDNA_54-E06	1.8	Unknown	*N/A*
cDNA_70-H06	1.8	Unknown	*N/A*

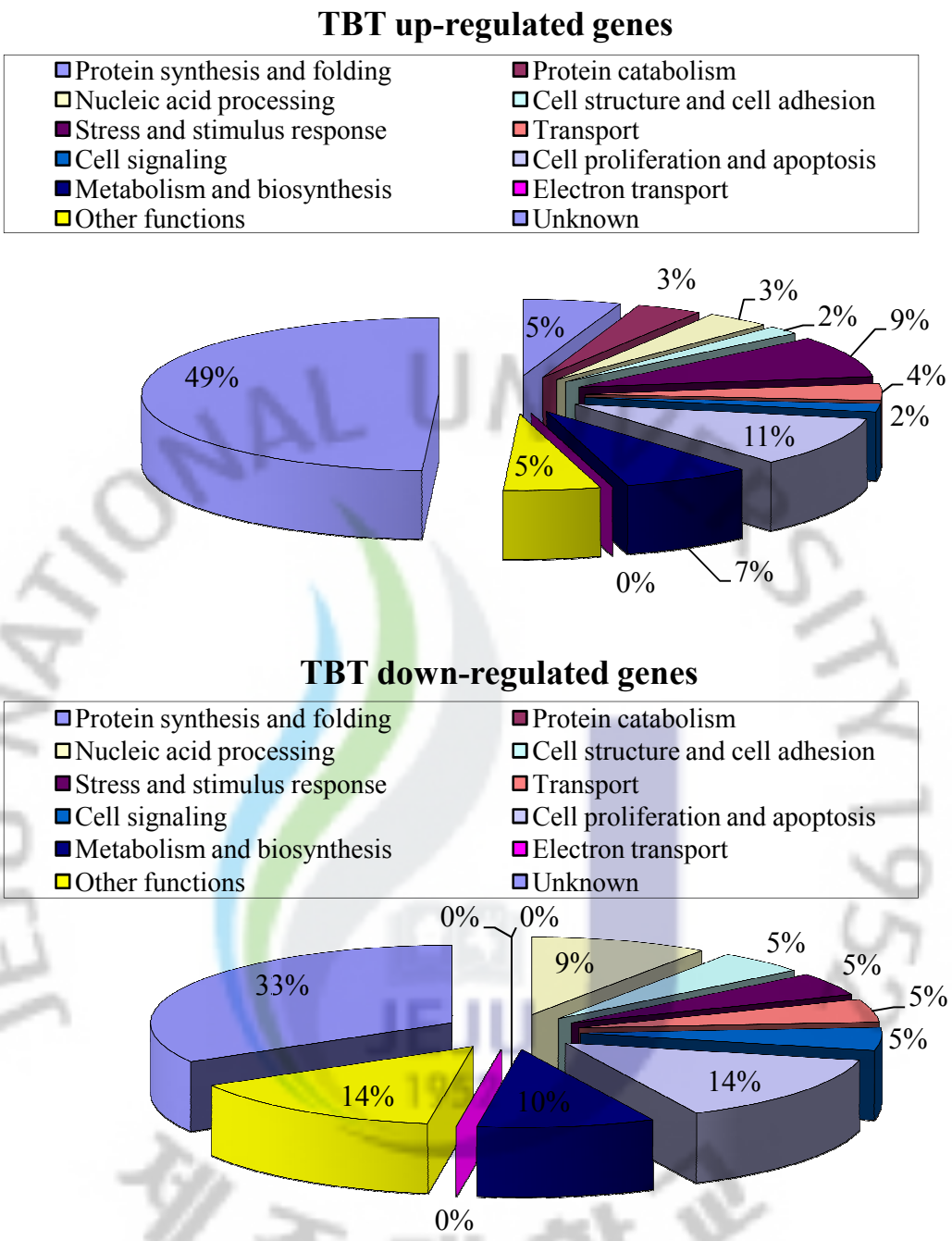


Fig 14. Functional categorization of up- and down-regulated genes under TBT stress. The Go annotations of differentially expressed genes are based on Blast2GO analysis for level 3 of the biological process category.

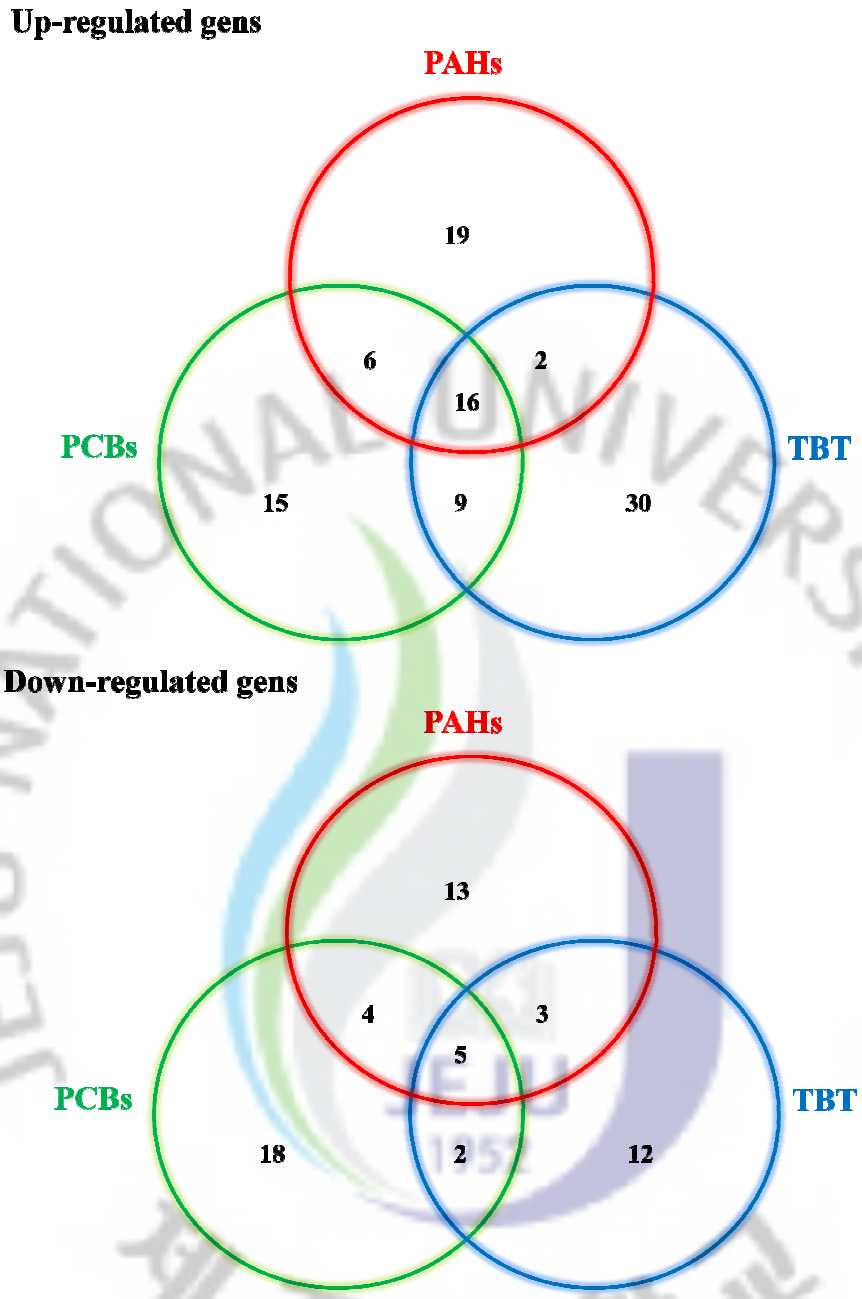


Fig 15. Overlapping of genes up- and down-regulated by different EDCs in abalone. Numbers in the figure indicate the numbers of transcipts exhibiting significant changes in abundance following 24 h exposure to β-NF, Aroclor and TBT, respectively.

Common stress response to EDCs The overlapped response of abalone genes to three EDCs was illustrated in Fig 15. There are considerable overlaps between the sets of genes regulated by the different EDC treatments, with more than 30% of the total 154 differentially expressed genes co-regulated by more than two EDCs. Particularly, over half of the PAHs and PCBs up-regulated genes were overlapped. Finally, we indentified 16 genes (microtubule-associated protein, solute carrier family 5, cubilin, ornithine decarboxylase 1, PCD5, retinal homeobox gene, 2 Acherons and 8 unknown genes) induced and 5 genes (thioester-containing protein, proton-coupled folate transporter, solute carrier, G protein-coupled receptor and an unknown gene) repressed by all three EDCs. Microtubule-associated protein (MAP) functions in cellular cytoskeleton stabilization to maintain the normal shape and function of cells during stress. In a recent research of human breast cancer, scientists found estrogen receptor could influence the expression of MAP (Ikeda, Taira et al. 2010). It suggests us that the induction of MAP in our study might be mediated with the effect of EDCs on abalone hormone system. Similarly, another gene, cubilin, is also implicated in abalone hormone system, since it has been demonstrated as an essential coreceptor in the endocytic pathway that regulates steroids hormone metabolism by several studies in human (Nykjaer, Fyfe et al. 2001). As we discussed earlier, the regulation of the genes related to apoptosis is a crucial strategy to cope with the strong apoptotic effect of EDCs. To our knowledge, the activation of PCD5 and two archrens is considered as the positive regulator of cell death. To cope with it, we identified the induction of another anti-apoptotic gene, ornithine decarboxylase (ODC), which is a rate-limiting enzyme of polyamine biosynthesis and also plays important role as mediator of apoptosis. Inhibition of ODC activity by DFMO can induce apoptosis of HC11 mouse mammary epithelial cells (Ploszaj, Motyl et al. 2000). Cells overexpressing ODC were reported to be resistant to apoptosis by reducing intracellular ROS production (Liu, Hung et al. 2005). We interestingly found that three solute carrier family members were consistently involved in the stress response to three EDCs. The solute carrier (SLC) family includes a group of membrane

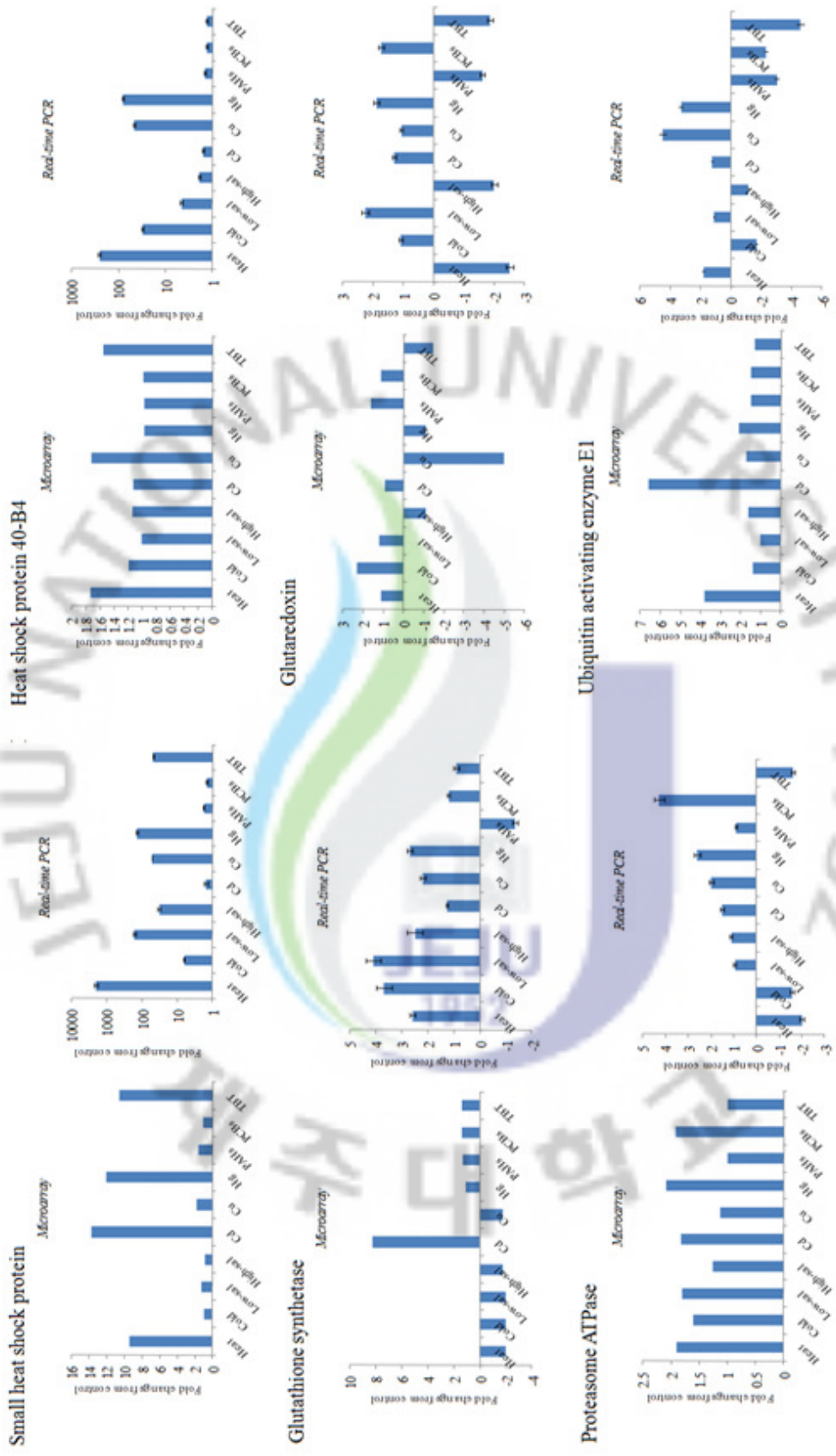
organic anion transporter proteins with over 300 members organized into 47 subfamilies. The functional role of SLCs in stress response has not been completely elucidated, although their expression levels are often significantly regulated as a consequence of different stresses (Papaiahgari, Yerrapureddy et al. 2007). We also noted a retinal homeobox (Rx) gene with induction in response to all three EDCs. Similar up-regulation of its expression was found for the treatment of three heavy metals as well; however, under the stress of heat, cold and high-salinity, its expression was significantly repressed, exhibiting distinct expression patterns between physical and chemical stress. So far, the knowledge about regulation and function of Rx gene is still limited in the aspect of eye development (Strickler, Famuditimi et al. 2002). The universal response of Rx to various environmental stressors in our experiment provides insights into it novel function in the stress response network. In addition, further characterization work also needed to uncover the roles of 9 unknown genes during EDC stress.

3.5 Real-time PCR validation

Microanalysis provides a powerful and rapid tool to identify genes that are differentially expressed in response to environmental stress. However, this technique is also subjected to many sources of experimental variability, thus an alternative means of estimating gene expression is needed to verify the results obtained. Herein we selected the method of real-time PCR for the validation. The 10 target genes that have exhibited strong expression-change in microarray analysis were selected for validation of microarray data, covering the full range of signal intensity and fold-change results. Their expression profiles determined by real-time PCR are represented in Figure 16. In the range of low and intermediate fold-changes, overall data revealed a good correlation between real-time PCR and microarray results. However, in the upper range of down- and up-regulated changes (fold-change > 10), the dynamic range of the microarray is generally lower than that of the

PCR assay, and expression changes of two HSPs and glycerol-3-phosphate cytidylyltransferase were significantly under-estimated by the microarray. Notably, we also observed a few contrary expression patterns between microarray and real-time PCR analysis for several genes, such as the response of GS to physical stress and the response of glycerol-3-phosphate cytidylyltransferase to EDCs exposure. These results might be due to the significant biological variances among different abalone individuals.

In conclusion, our microarray analysis of gene expression in abalone identified a large number of genes responsive to different environmental stressors, which may be useful as potential novel biomarkers. Following the functional categorization, these identified genes have covered a wide range of biological processes, indicating that the effect of environmental stress on abalone and the resistance of abalone to it are a systematic combination of multiple cellular events. We also observed considerable overlaps in the gene expression patterns between different stress conditions. This result suggests a crosstalk in the stress response pathways to different environmental stressors. Furhtermore, the geneswith overlapped patterns are considered as the key nodes in stress response network.



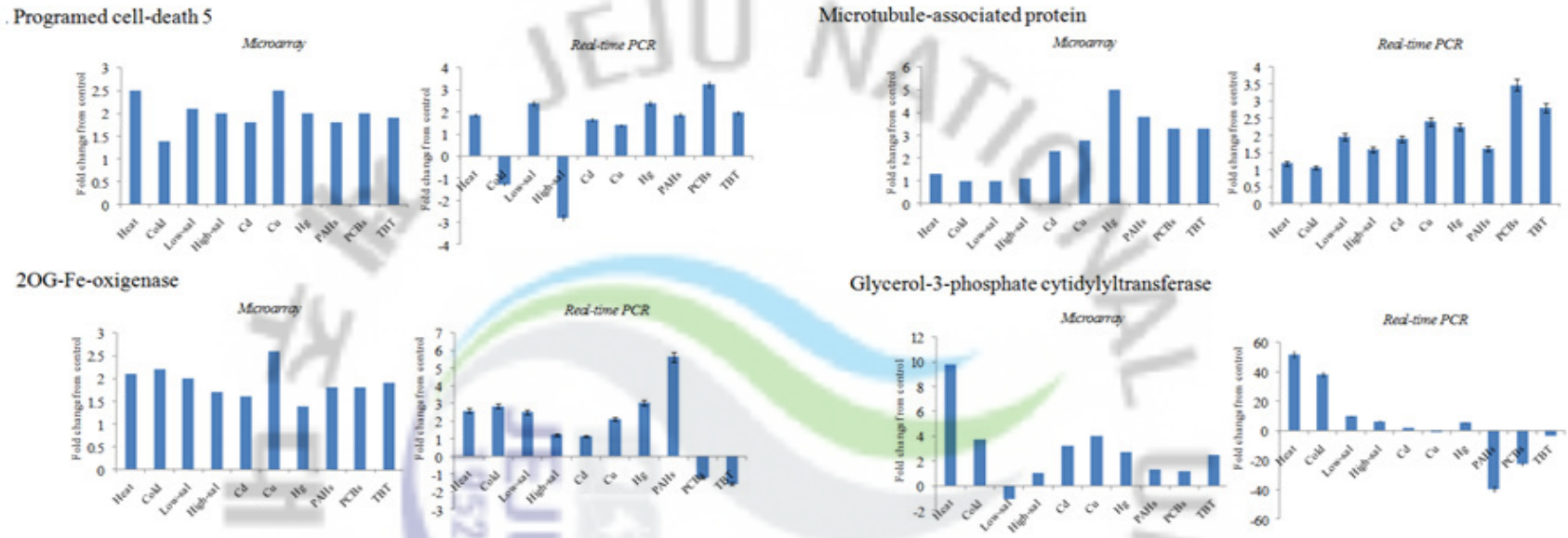


Fig 16. Comparison of the mRNA levels change estimated by cDNA microarray and real-time PCR of ten selected genes. The results are represented as the fold-change upon the control level of each gene under ten different environmental stressors including heat, cold, low-salinity, high-salinity, cadmium, copper, mercury, β-NF, Aroclor and TBT. The Ct values of each gene obtained from real-time PCR were covered into expression level by $2^{-\Delta Ct}$ method without normalization process by reference gene.

Chapter II.

HSP20 of disk abalone *Haliotis discus discus* as a novel universal biomarker of environmental stresses

Abstract

Small heat shock proteins (HSPs) constitute a group of molecular chaperones with low molecular mass and high sequence diversity. They are ubiquitously distributed in almost all existing organisms, playing crucial roles in a number of cellular and physiological processes. Here, we reported the cloning, functional characterization and expression analysis of one novel small HSP gene, HSP20, in disk abalone *H. discus discus*. Abalone HSP20 shares the highest similarity with a group of invertebrate small HSPs of approximately 20 kDa. Through analysis in primary and three-dimensional structure, we identified several signature features of small HSPs in abalone HSP20, including conserved α–crystallin domain, Cysteine-free, Glx/Asx-rich and compact β-sandwich structure in C-terminal region. Additionally, we also observed the ability of abalone HSP20 recombinant protein to enhance the thermotolerance of *E. coli* cells *in vivo*, suggesting its function in the cellular chaperone network. To further understand its function in abalone, the expression patterns of abalone HSP20 under the challenges of different environment stressors were investigated by quantitative RT-PCR. The expression of abalone HSP20 was dramatically induced by heat shock, but also significantly elevated by cold shock, extreme salinities, heavy metals and organic endocrine disrupting chemicals (EDCs). Collectively, the above results strongly suggest abalone HSP20 involvement in general stress response of abalone as a molecular chaperone and hence great potential as a universal biomarker to indicate various environmental stresses.

Keywords: Abalone, Small HSPs, Molecular chaperone, Biomarker, Environmental stress

1. Introduction

Heat shock proteins (HSPs) represent a super family of stress-inducible genes, so called because they were first discovered while observing heat shock response in *Drosophila* (Tissieres et al., 1974). The steady-state levels of heat shock proteins are usually low in nonstressed cells, however, their synthesis can be markedly induced in response to elevated temperatures and other stress factors, acting as molecular chaperones to restore denatured proteins to native conformation and prevent them from irreversible aggregation in stress conditions. On the basis of molecular weight and sequence homology, heat shock proteins are classified into several families, including HSP100, HSP90, HSP70, HSP60, HSP40 and small HSPs (Narberhaus, 2002).

Small HSPs constitute the smallest members of HSPs family, with a low monomeric molecular mass ranging from 9 to 43 kDa (majorly 14-27 kDa). They ubiquitously exist throughout the animal, plant and microbiotic kingdoms except in few bacteria species, e.g., *Mycoplasma genitalium*. Compared with other heat shock protein families, small HSPs are highly diverse in primary sequences. Nevertheless, they share an evolutionally conserved α–crystallin domain of 80-100 amino acid residues in C-terminal part. This domain shows high sequence similarity with the vertebrate eye lens protein and is frequently flanked by a highly variable N-terminal region and a flexible C-terminal tail (Kappe et al., 2003; MacRae, 2000). Furthermore, the most striking features is that small HSPs can oligomerize into large complexes composed of 9-40 subunits, which is a latent state under normal physiological conditions. While upon heat stress, the large oligomeric structure readily dissociates into the basic building blocks, dimmers, accompanied by simultaneous exposure of the hidden hydrophobic sites to convey chaperone function (Haslbeck et al., 2008; Kim et al., 1998). Small HSPs are considered to interact with a wide range of partially denatured proteins and stabilize them against aggregation in an ATP-independent manner, creating a transient reservoir of unfolded protein for subsequent refolding by ATP-dependent chaperones like

HSP90s and HSP70s. In addition to protect organisms from thermal injury as molecular chaperones, small HSPs are also implicated in a number of fundamental cellular processes (Arrigo, 2000; Arrigo et al., 2002; Kostenko and Moens, 2009; Mounier and Arrigo, 2002), diverse physiological functions (Davidson et al., 2002; Huang et al., 2007; Patel et al., 2006) and many human diseases (Dunlop and Muggli, 2000; Sherman and Multhoff, 2007). Comparatively, small HSPs represent the highest induction level of all stress inducible genes in response to various stresses (Ventura et al., 2007).

Abalone is a marine gastropod species from the *Haliotidae* family. There are over 100 different species of abalone inhabiting rocky coastline from the intertidal zone down to 50 m depth throughout the world's oceans. In addition to be a vital component of in marine ecosystems, abalone is also suggested to be extremely valuable as compared with other seafood species, creating billions of dollars worth every year. Noteworthy is that most abalone species grow rather slowly and are highly sensitive to water quality, temperature, salinity, pathogens and other ambient factors, lack of adaptation ability to rapid environment change. Owing to the global climate change, environment pollution and/or the outbreak of diseases, the worldwide populations of wild abalone continue to decline substantially in recent decades so that several abalone species were listed as critically endangered. However, there are scanty reports about efficient biomarkers that could monitor and assess the impact of environmental stress in abalone. Given that, we conducted the present study to clone and characterize a novel small HSP (HSP20) from disk abalone *Haliotis discus discus*, which is one of the most abundant wild and hatchery-reared abalone species in Korea. We profiled the expression pattern of abalone HSP20 under the challenge of physical (extreme temperature and salinity) and chemical (heavy metals and EDCs) stressors. The thermal-tolerance of *E. coli* cells overexpressing abalone HSP20 recombinant protein was also tested.

2. Materials and Methods

2.1 Chemicals and reagents

All the chemicals used in this study were of molecular biology grade and were purchased from Sigma (USA) unless otherwise stated. SYBR TaKaRa Ex Taq, taq polymerase, restriction enzymes, and other reagents for gene manipulation were obtained from TaKaRa Bio (Japan). The kits for plasmid extraction, PCR- and Gel-purification were provided by Bioneer (Korea). All the oligonucleotide synthesis was performed by Integrated DNA technologies, Inc (USA).

2.2 Animals

1-year-old disk abalones (*H. discus discus*) with ~30 g weights were obtained from an abalone farm in Jeju island, South Korea. After purchase, abalones were acclimated in laboratory aquaria for 1 week prior to the challenge experiment. The seawater was filtered and aerated continuously, with the salinity and temperature maintained at 32 psu and 20 ± 1 ℃, respectively. After acclimation, abalones were divided into one control group and twelve groups for different challenges. In heat shock challenge group, abalones were maintained at 30 ^{o}C for different duration time from 0.5 hour to 6 hours. In cold shock challenge group, abalones were maintained at 4 ^{o}C for different duration time from 0.5 hour to 24 hours. In two groups of osmotic shock, abalones were respectively maintained in the seawater with the salinities of 45 psu and 20 psu for different duration time from 0.5 hour to 48 hours. In heavy metals challenge groups, $CdCl_2 \cdot 2.5\ H_2O$, $CuSO_4 \cdot 5H_2O$, $HgCl_2$ and the mixture of three chemicals were respectively used for 12-h waterborne exposure with final concentrations of 10, 50, 200, and 500 μg/L. In endocrine disrupting chemicals (EDCs) challenge groups, beta-naphathoflavone (β-NF), benzo[α]pyrene (B[α]P), aroclor-1254 (Aroclor), and tributyltin chloride (TBT) were dissolved in Dimethyl sulfoxide (DMSO) and used in the treatment for 12-96 hours with final concentration of 1.0 mg/L, 1.0 mg/L, 500 μg/L and 10 μg/L in seawater, respectively. Additionally, six 3-year-old abalones (3 male and

3 female) with mature gonads were also prepared to investigate tissue distribution of transcripts.

2.3 Cloning and recombinant expression of abalone HSP20

We previously constructed and sequenced a normalized cDNA library of disk abalone *H. discus discus* (Helani Munasinghe et al., 2006). From over 4000 expressed sequence tags (ESTs) obtained, we identified one novel small HSP gene (HSP20) by a BLASTX search on NCBI (http://www.ncbi.nlm.nih.gov/BLAST). Two subsequent sequencing reactions were then carried out to gain the complete cDNA sequence. The open reading frames (ORFs) of abalone HSP20 were amplified by PCR using two cloning primers: 5'-gagagaCATATGCGTCCATTTTTCCGATCCAGCATC-3' (NdeI site is underlined), and 5'- gagagaGGATCCTCAATCTGTCTTCTTCTCTCCCTCC -3' (BamHI site is underlined). The PCR product was purified, digested with NdeI and BamHI, and ligated into pET16b vector (Novagen, USA). Afterwards, the constructed vector, pET16b-HSP20, was transformed into *E. coli* strain BL21(DE3). A single colony was picked up on transformation plate and inoculated in 5 ml Luria-Bertani (LB) broth containing 100 μg/ml of ampicillin (Amp) for an overnight incubation at 37 °C. The overnight seed culture was again inoculated into 500 ml LB/Amp medium at a dilution of 1:100, and was grown until the OD_{600} reached to 0.8. Thereafter, isopropyl-b-D-thiogalactoside (IPTG) was added to the medium at a final concentration of 0.5 mM to induce recombinant protein expression at 30 °C. After 4 h IPTG-induction, the cells were cooled on ice for 30 min and harvested by centrifugation at 4°C. The purification of recombinant HSP20 was carried out by His•Bind® Kit (Novagen, USA). The purity and molecular weight of the protein preparation was finally analyzed by SDS polyacrylamide gel electrophoresis (SDS – PAGE).

2.4 Thermotolerance of *E. coli* BL21(DE3) expressing abalone

HSP20

The thermal-tolerance assay was performed as previously reported in the study of shrimp small HSP (Crack et al., 2002). Briefly, *E. coli* BL21(DE3) cells transformed by pET16b or pET16b-HSP20 were incubated in 5 ml LB broth containing 100 μg/ml of Amp at 37 °C until the midlog phase (OD600=0.5), followed by adding IPTG to 0.5 mM and overnight induction at 30 °C. Immediately before heat shock, the overnight cultures were diluted 1:10 with fresh LB/Amp broth. The diluted cultures were incubated at 54 °C in a water bath. After 0, 15, 30, 45, 60 and 75 min of heat shock, 100 μl samples were removed, diluted with cold LB broth, and maintained on ice. Then, the cells were plated on LB/Amp agar in duplicate. The surviving colonies of two types of *E. coli* cells were counted after 16 h incubation at 37 °C. To verify the presence of abalone HSP20 recombinant protein, cells were collected from 1 ml of two IPTG-induced cultures and analyzed by SDS-PAGE assay.

2.5 Multiple-alignment, Phylogenetic analysis and Homology modeling of abalone HSP20

Multiple-alignment was carried out by aligning the amino acid sequences of abalone HSP20 with seven other HSP20 genes that share highest sequence similarities, using the Clustal W method with default parameters setting. The phylogenetic tree containing HSP20 genes from abalone and 21 other species was rebuilt using the neighbor-joining method with 1000 bootstrap replicates on the platform of MEGA v.4.1. Two bacterial HSP20 genes were selected to root the tree. All the sequences for multiple-alignment and phylogenetic tree building were obtained from GenBank of NCBI. To generate the three dimensional structure model, the amino acid sequence of abalone HSP20 was submitted to Swiss-Model (http://swissmodel.expasy.org/) (Schwede et al., 2003). In automate mode, the 2.5A resolution structure of a small HSP from the parasitic flatworm *Taenia saginata* Tsp36 (2bolA) was selected as template. The sequence similarity between template and our target

sequence is 18.8%. The 3D image was generated by Swiss-Pdb viewer version 4.01.

2.6 Quantitative reverse transcription PCR (qRT-PCR) analysis of abalone HSP20 expression

Gill tissues for challenge experiment and eight different tissues for tissue distribution study were dissected from abalones and frozen in liquid nitrogen immediately. Total RNA was then isolated by using TRIzol Reagent. For the samples of hepatopancrease tissue in particular, an additional clean-up procedure by S.N.A.P. total RNA isolation kit (Invitrogen) was subsequently carried out due to the abundance of polysaccharide and pigments which would interfere the following enzymatic processes. Total RNA concentration was obtained by measuring absorbance at 260 nm. The quality of RNA was also verified by formaldehyde agarose gel electrophoresis. First-strand cDNA was synthesized by using PrimeScript™ 1st strand cDNA Synthesis Kit (Takara). Briefly, 1 μg total RNA, RNase free H2O, 1 μl of 50 μM oligo dT primer, and 10 mM dNTP mix were added together to incubate at 65°C for 5 min, followed by an incubation on ice for at least 1 min. Thereafter, 4 μl 5 x PrimeScript™ Buffer, 20 U RNase inhibitor and 200 U PrimeScript™ RTase were added to a final volume of 20 μl and together incubated at 42°C for 60 min. Finally, the reverse transcription reaction was terminated at 70°C for 15 min.

Two pairs of specific primers were designed by Primer 3.0 to amplify abalone HSP20 (Forward: AAGGTCAGCCAGCTGGAGAAACAA, Reverse: ATTCCCGTGTGAATTCCCTGCTGA) and the reference gene, ribosomal protein L5 (Forward: TCACCAACAAGGACATCATTTGTC, Reverse: CAGGAGGAGTCCAGTGCAGTATG). Real-time PCR was performed on Takara™ real time PCR detector TP800 (Takara Inc. Japan). Each PCR reaction consisted of a total volume of 25 μl including 12.5 μl of SYBR Premix Extaq, 4 μl diluted cDNA derived from 25 ng of total RNA and a pair of specific primers. The thermal profile was one cycle of

95 °C for 10 s, followed by 40 cycles of 95 °C for 5 s, 58 °C for 10 s, and 72 °C for 20 s. To verify that the used primer pair produced only a single product, the dissociation curve of product was also investigated by heating from 60 ^{o}C to 95 ^{o}C in the end of reaction. For each cDNA sample, a triple duplication of reaction was performed. Finally, the C_t values obtained from real-time PCR were converted into relative expression levels by using $2^{-\Delta\Delta Ct}$ method.

3. Results

3.1 Cloning and sequence analysis of abalone HSP20

Through BLASTX analysis in ESTs data of disk abalone normalized cDNA library, we isolated a cDNA clone that appeared as a putative small HSP. Its complete cDNA sequence shows a total length of 1834 nucleotides excluding 3' polyadenylic acid tail (Fig. 1). We predicted an open reading frame of 480 base pairs (bp), flanked by a ~300bp 5' UTR and a ~1.1kbp 3'UTR. The predicted ORF encodes a polypeptide of 160 amino acids, with an 18.76 kDa molecular mss and a putative PI of 5.82. The deduced amino acid sequence owns over 30% similarity with a group of invertebrates' small HSPs that have molecular weights of approximately 20 kDa, thereby we recognized this abalone gene as a novel HSP20 protein. In agreement with most small HSPs, no cysteine residue was observed in amino acid composition of abalone HSP20. Instead, it contains 26 glutamine and glutamic acid (Glx) residues which constitute 16.5% of the total amino acids. As shown in multiple-alignment result in Fig. 2, despite of the high variety in N-terminal region and C-terminal tail, abalone HSP20 and other HSP20 homologues share a highly conserved α–crystallin domain of ~80 residues in C-terminal region, which is the signature motif of small HSP family.

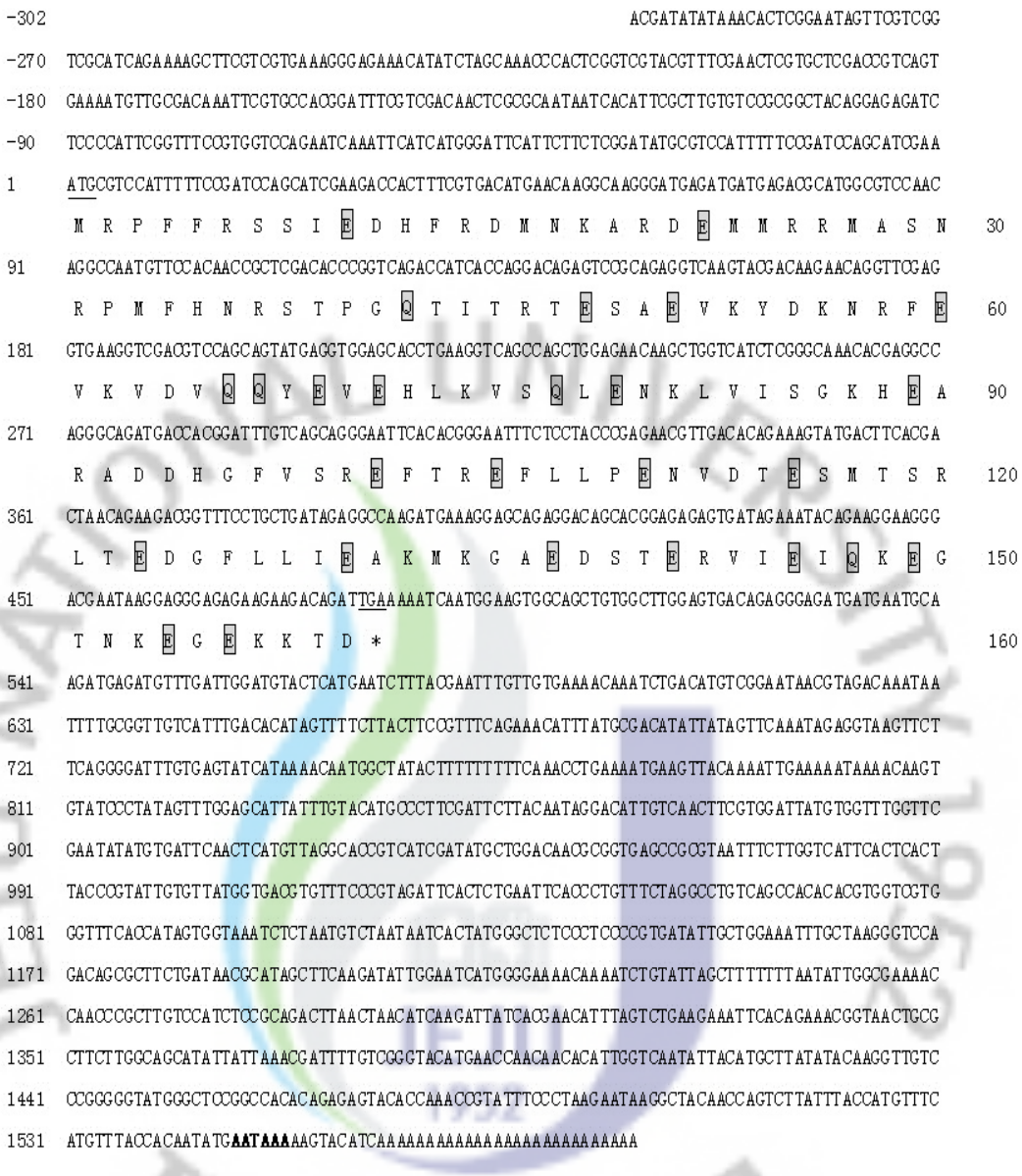


Fig. 17 Nucleotide and deduced amino acid sequences of abalone HSP20. The start and stop codons are marked by underlines. The polyadenylation signal is marked in bold. Glx residues are highlighted in shaded boxes.

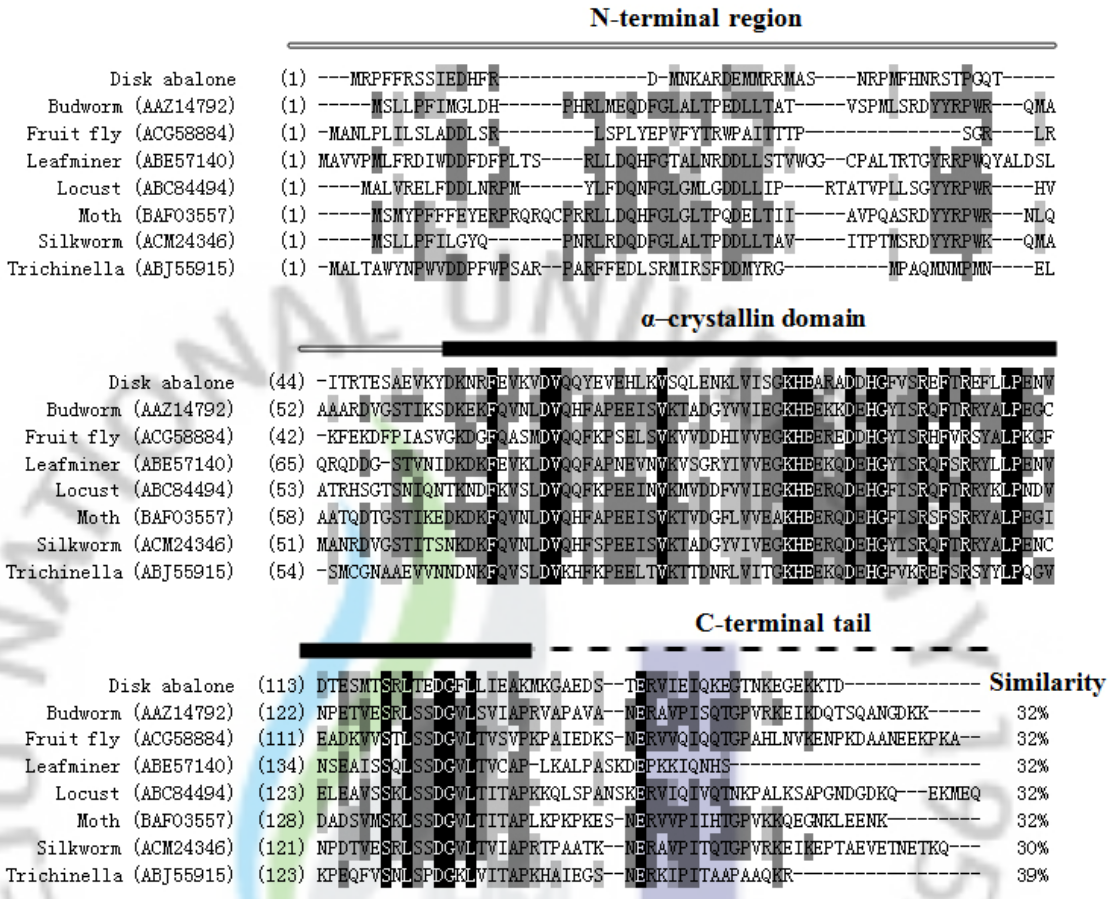


Fig. 18 Multiple-alignment of HSP20s from different species. Deduced amino acid sequence of abalone HSP20 was aligned with the homologues from other invertebrate species using Clustal W alignment program. All the sequences used are obtained from GenBank on NCBI. The sequence similarities are displayed at the end of alignment. Abalone HSP20 shares a highly conserved α-crystallin domain with HSP20s of other invertebrates. However, the N-terminal region and C-terminal tail are much variable.

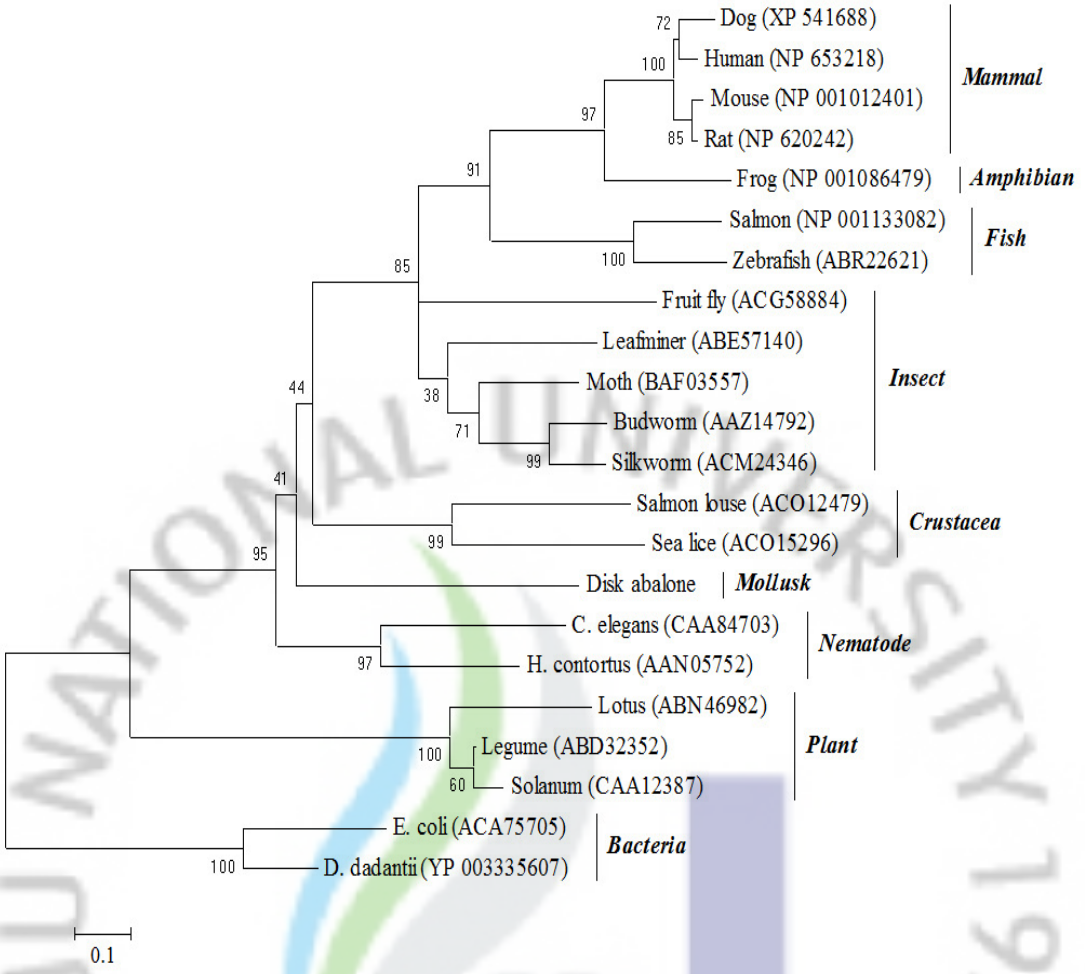


Fig. 19 Neighbor-joining phylogenetic tree of different HSP20 genes. The tree is rebuilt by using MEGA program, comprising of HSP20 genes from 22 different species ranging from mammals to bacteria. All the sequences used are obtained from GenBank on NCBI.

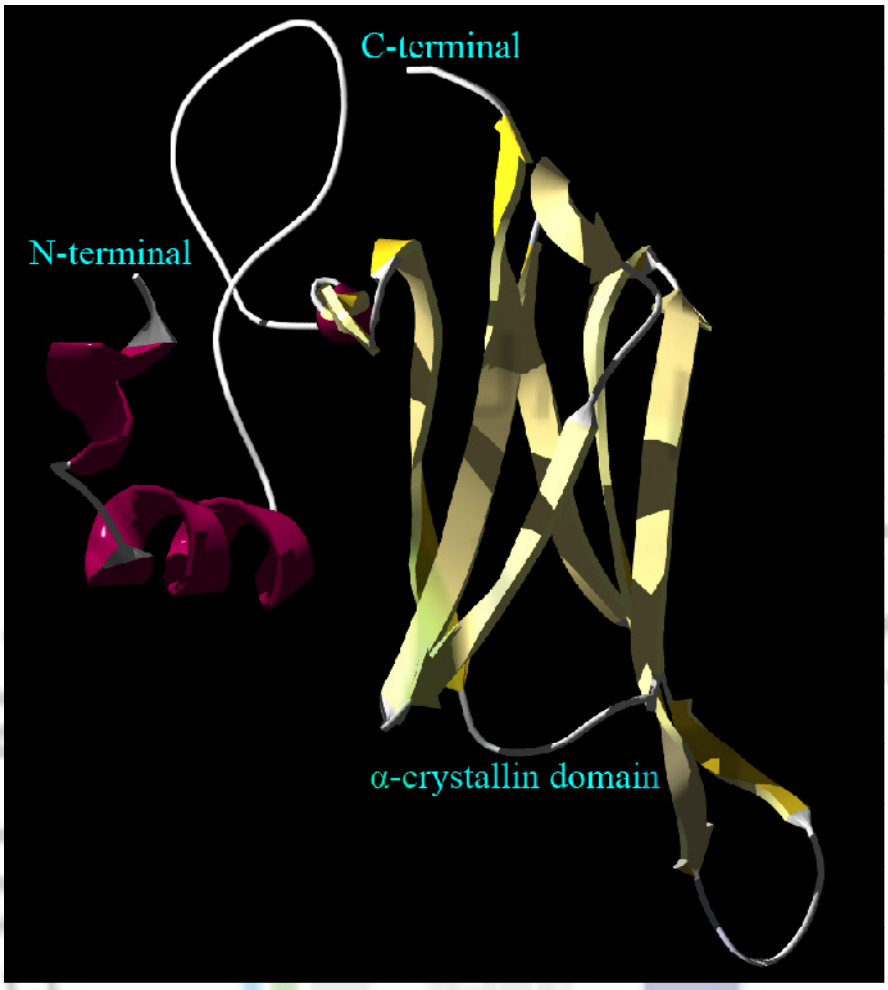


Fig. 20. 3D modeling structure of abalone HSP20.

To uncover the relationship of abalone HSP20 with other HSP20s, we rebuilt a neighbor-joining phylogenetic tree using the genes from a wide range of phyla. As shown in Fig. 19, the tree was automatically separated to different clades according to phylogenetic taxonomy, such as mammal, fish, insect, crustacea, nematode and plant. As the only member in mollusc phylum, abalone HSP20 was placed between nematode and insect, which is similar with the evolutionary relationship as expect.

The three-dimensional structure of abalone HSP20 was predicted on the basis of the X-ray structure of parasitic flatworm *Taenia saginata* Tsp36 (PDB 2bolA). Due to the low sequence similarity (18.8%), the predicted structure model contains amino acid residues only from 4 to 136, lack of C-terminal tail. The α–crystallin domain in C-terminal region of abalone HSP20 forms a compact β-sandwich structure composed of antiparallel β-sheets, while the N-terminal domain is rich in α–helix (Fig. 20). It is in agreement with the structure characteristics of other small HSPs.

3.2 Recombinant expression and purification of abalone HSP20

To avoid the potential hindrance in oligomer structure formation and hence the chaperone function of recombinant abalone HSP20, we chose pET16b vector that introduces only a small 6 x His-tag as a recombinant expression system. As shown in Fig. 21, despite usually low solubility and high inclusion body formation of his-tag fusion proteins, our recombinant abalone HSP20 showed a major distribution in soluble fraction of total bacterial protein after 4h 0.5mM IPTG induction at 30 ℃, indicating a fine solubility. The purity and molecular weight of purified recombinant protein of abalone HSP20 were examined by 12.5% SDS-PAGE. The apparent molecular mass of the recombiant protein was approximately 20 kDa (with a His-tag of ~1kDa molecular mass), consistent with the predicted value.

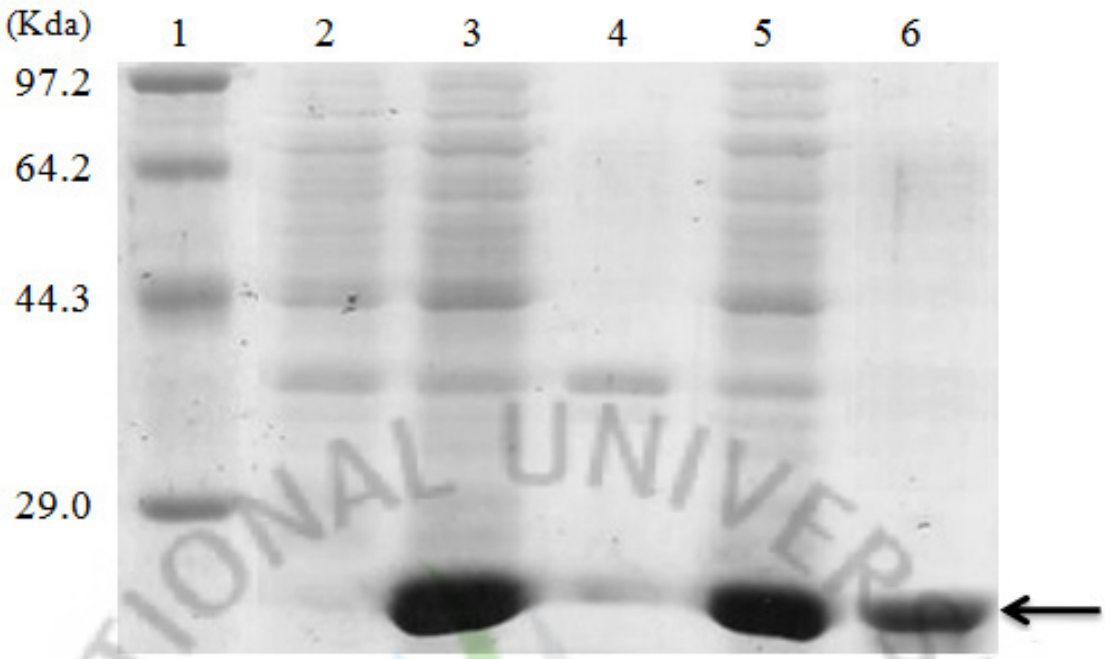


Fig. 21 SDS-PAGE analysis of the recombinant abalone HSP20. Lane 1, molecular mass markers with the sizes shown on the left in kilodaltons; lane 2, total cellular extract of the uninduced *E. coli* BL21 (DE3) pLys; lanes 3 total cellular extracts of the induced *E. coli* containing pET16b-HSP20 plasmid; lanes 4, insoluble protein in the induced *E. coli* cells; 5, soluble protein in the induced *E. coli* cells; 6, purified recombinant abalone HSP20 protein fused with a His-tag at the N-terminal end (arrow marked).

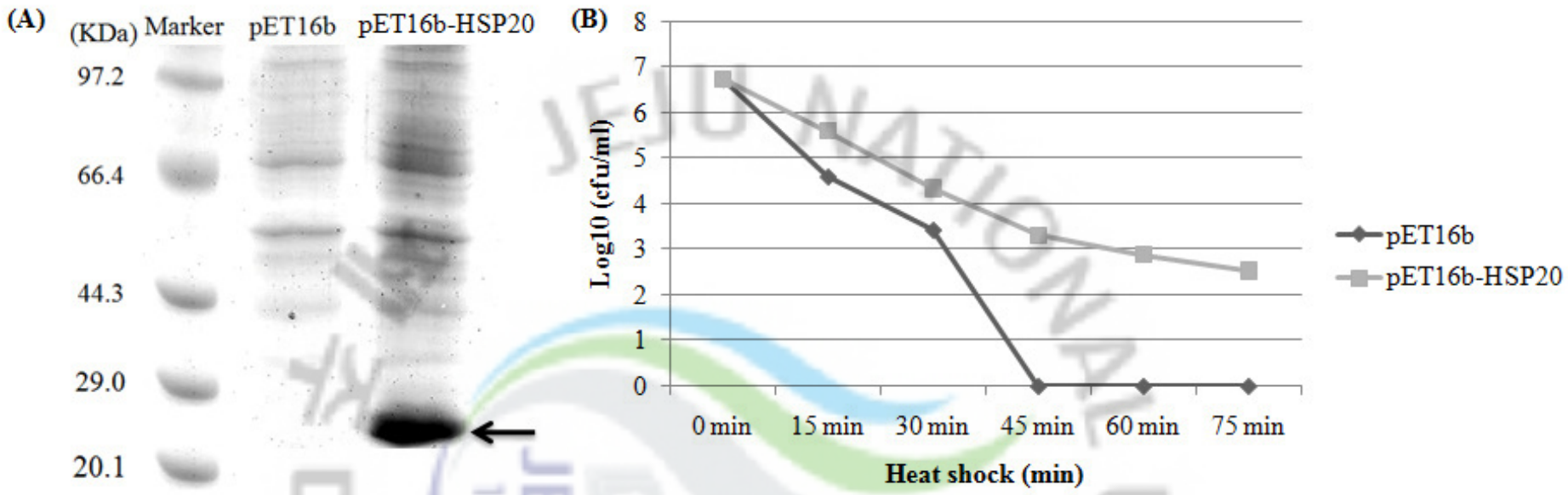


Fig. 22 Thermaltolerance of *E. coli* cells overexpressing abalone HSP20. (A) Prior to heat shock challenge *E. coli* BL21 (DE3) pLys cells respecitively transformed by pET16b (Control) and pET16-HSP20 plasmid were incubated at 30 ^{o}C for 10 h in the present of 0.5 mM IPTG to induce protein overexpression. **(B)** After 0, 15, 30, 45, 60, 75 min of heat shock, the survival colony numbers of two types of transformed *E. coli* cells were quantified as $\log_{10}$ values of colony-foming units (cfu) per milliliter. The experiments were performed in triplicate.

3.3 Enhanced thermotolerance of *E. coli* cells overexpressing abalone HSP20

To determine whether abalone HSP20 could functionally protect cells against thermal injury *in vivo*, we examined the survival rates of two types of *E. coli* BL21 (DE3) cells that are transformed with pET16b (control) and pET16b-HSP20 plasmids after heat shock. As negative control, the bacteria cells transformed with pET16b plasmid exhibited a high sensitivity to 54 °C heat shock, with a dramatic decline in survival rate as the increase of exposure time (Fig. 22-B). No colony could be observed on the plates for the samples after heat shock of 45 min. In contrast, the bacteria cells transformed with plasmids containing abalone HSP20 displayed a significant resistance to heat killing. Even after a heat shock of 75 min, countable colonies were still observed on the plate. Before heat shock, we also ran a SDS-PAGE to verify the content of recombinant abalone HSP20 protein in the cells transformed with pET16b-HSP20 plasmids (Fig. 22-A). Our data indicates that abalone HSP20 protein is able to provide a protection against heat stress and resultant cell damage.

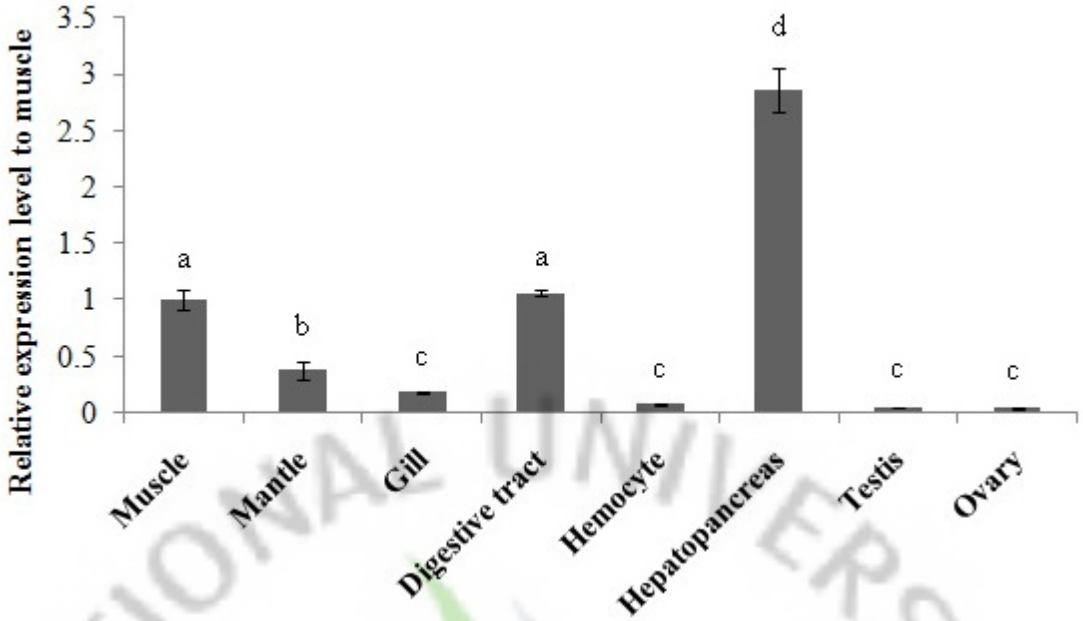


Fig. 23 Tissue distribution of abalone HSP20 transcript. qRT-PCR was performed in the tissues of muscle, mantle, gill, digestive tract, haemocyte, hepatopancreas, testis and ovary. The results are represented as means (n=3) ± S.E.M. Data with different letters are statistically different at $P < 0.05$ based on one-way ANOVA.

3.4 Tissue distribution of HSP20 mRNA in disk abalone

To determine the tissue distribution of abalone HSP20, its mRNA levels in both outer (gills, abduct muscle and mantle) and inner (digestive tract, hepatopancreas and gonad) organs were examined by qRT-PCR. The Ct values of abalone HSP20 ranged from approximately 26 to 33 (data not shown), suggesting a moderate level of constitutive expression when abalone under normal physical condition. As shown in Fig. 23, abalone HSP20 mRNA levels were highly variable in diverse tissues. The highest level was found in hepatopancreas tissue. In contrast, the expression in gill, hemocyte and gonads were comparatively scanty. Hepatopancreas is the digestive organ where a large number of enzymes are synthesized and catalyze reactions, but also is the major organ responsible for detoxification of xenobiotics which could lead to chemical denaturation of proteins. Thus, the hepatopancreas-specific pattern of abalone HSP20 might be implicated in the immediacy requirements for correct proteins folding in hepatopancreas tissue.

3.5 Expression of abalone HSP20 in response to physical stress

We selected gill as a target tissue to investigate the response of abalone HSP20 expression to various environmental stresses since it performs as primary site in response to ambient stress and also possesses an ease of RNA preparation in comparison with other tissues.

Heat The expression pattern of the abalone HSP20 following different lengths of heat shock at 30 °C is illustrated in Fig. 24-A. As the most inducible member of heat shock protein family, abalone HSP20 exhibited a dramatic induction upon heat shock. Its expression elevated rapidly in a time-dependent manner till 2-h when the expression level reached to the peak (approximately 2000-fold of control level). Thereafter, the induction

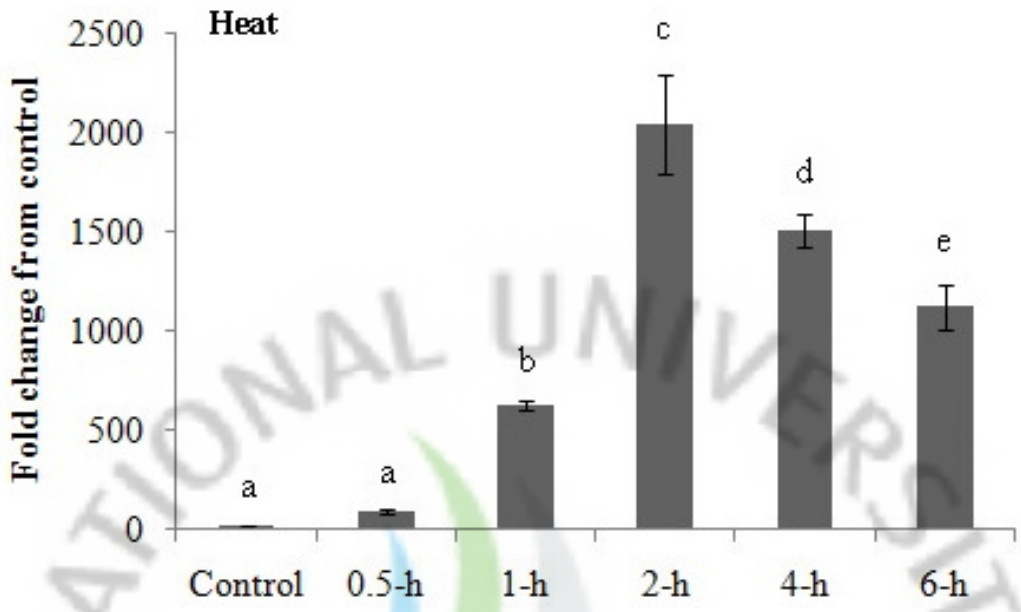


Fig. 24-A Expression profiles of abalone HSP20 in response to heat stress. Abalones were challenged by heat shock at 30 °C. The expression level of abalone HSP20 was analyzed by qRT-PCR with a ribosomal protein as reference. The results are represented as means (n=3) ± S.E.M. Data with different letters are statistically different at $P < 0.05$ based on one-way ANOVA.

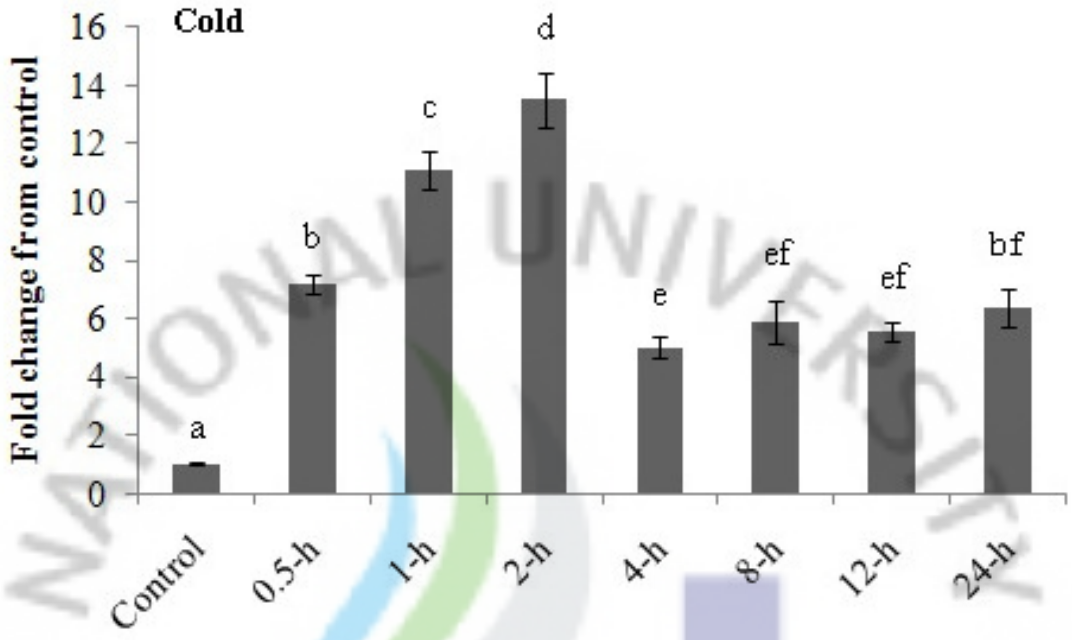


Fig. 24-B Expression profiles of abalone HSP20 in response to cold stress. Abalones were challenged by cold shock at 4 ^{o}C. The gill tissues were sampled after different time durations. The expression level of abalone HSP20 was analyzed by qRT-PCR with a ribosomal protein as reference. The results are represented as means (n=3) ± S.E.M. Data with different letters are statistically different at $P < 0.05$ based on one-way ANOVA.

level was slightly decline but still over 1000-fold. After 6-h exposure at 30 °C, the abalones exhibited a loss of muscle strength and were not able to survive through the recovery process at 20 °C, indicating irreversible damage caused by heat stress.

Cold The challenge of cold stress in abalone was conducted by a cold shock at 4 °C, which could cause significant mortality if over 48 hours exposure. As shown in Fig. 24-B, the expression of abalone HSP20 was only mildly elevated by cold shock in comparison with the thousand-fold induction of heat shock. But similar to heat shock, the expression of abalone HSP20 was also rapidly induced to the peak within 2-h and then subsequently leveled off despite continuous exposure.

Osmotic stress To understand the role of abalone HSP20 in hypo- and hyper-osmatic stress, we challenged abalones by incubating them in seawater with 20 ‰ and 45 ‰ salinity, respectively. These two extreme salinities were proved as fatal conditions that could cause mortality within 7 days in our earlier study. In generally, the expression of abalone HSP20 was induced by both low and high salinities although fluctuations of induction level occurred during the challenge progress (Fig. 24-C, D). In contrast to the rapid induction under extreme temperatures, we note that the response of abalone HSP20 to osmotic stress was much delayed. The highest induction levels in both salinities were together investigated at the longest challenge time. This finding indicates that abalone HSP20 might not be a primary target of osmotic stress but could play an important role against subsequent stress in the late phase.

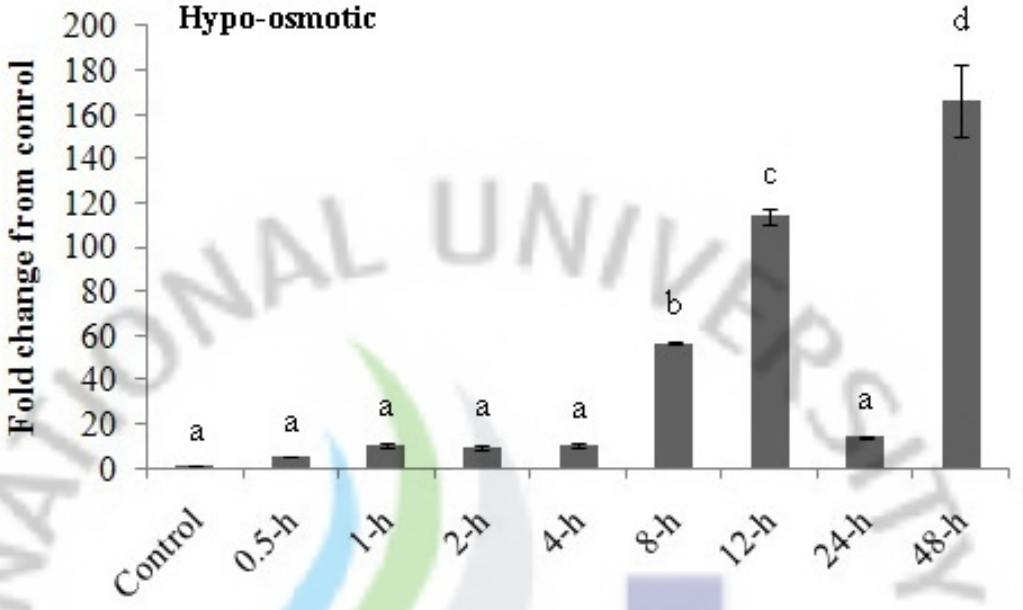


Fig. 24-C Expression profiles of abalone HSP20 in response to hypo-osmotic stress. Abalones were challenged by a low salinity of 20 psu. The gill tissues were sampled after different time durations. The expression level of abalone HSP20 was analyzed by qRT-PCR with a ribosomal protein as reference. The results are represented as means (n=3) ± S.E.M. Data with different letters are statistically different at $P < 0.05$ based on one-way ANOVA.

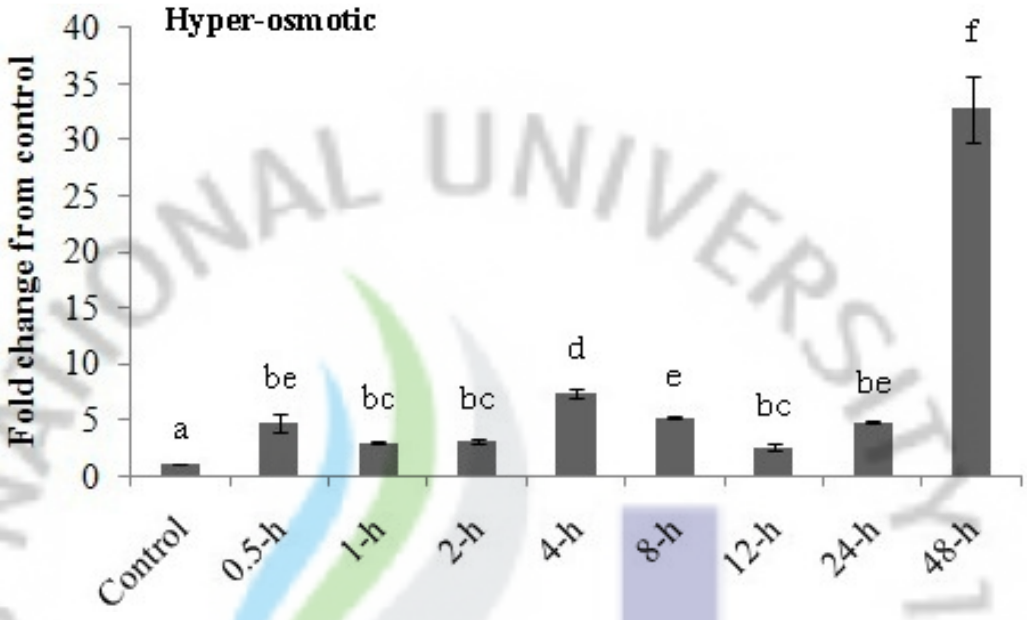


Fig. 24-D Expression profiles of abalone HSP20 in response to hyper-osmotic stress. Abalones were challenged by a low salinity of 45 psu. The gill tissues were sampled after different time durations. The expression level of abalone HSP20 was analyzed by qRT-PCR with a ribosomal protein as reference. The results are represented as means (n=3) ± S.E.M. Data with different letters are statistically different at $P < 0.05$ based on one-way ANOVA.

3.6 Expression of abalone HSP20 in response to marine pollutants exposure

Heavy metal As shown in Fig. 25, the expression of abalone HSP20 increased in a clear dose-dependent manner following the exposure of copper and mercury. These two heavy metals elevated HSP20 expression up to 140-fold at the highest dose (200 μg/L). In contrast, the effect of cadmium is much weaker, with maximally 2-fold induction. Simultaneously, we also challenged abalones with the mixture of three heavy metals at medium-dose. Compared with single heavy metal, the mixture gave a slightly higher induction level, indicating a synergetic effect of different heavy metals on abalone HSP20.

EDCs The time-course profiles of abalone HSP20 expression following β-NF, B[α]P, Aroclor and TBT exposures were illustrated in Fig. 26. In general, abalone HSP20 was significantly induced by all four EDCs ($P < 0.05$). However, we also noted clear variations in the induction levels and time-dependent patterns amongst four EDCs. TBT owned the most drastic fold-changes (up to 50-fold), while other thee EDCs exhibited only mild effects on abalone HSP20 expression (<10-fold). In response to B[α]P and TBT, the expression of abalone HSP20 was continuously elevated in a manner of time-dependent. In contrast, the patterns upon β-NF and Aroclor were reversal that the expression was initially increased to peak and then leveled off to near basal level of control.

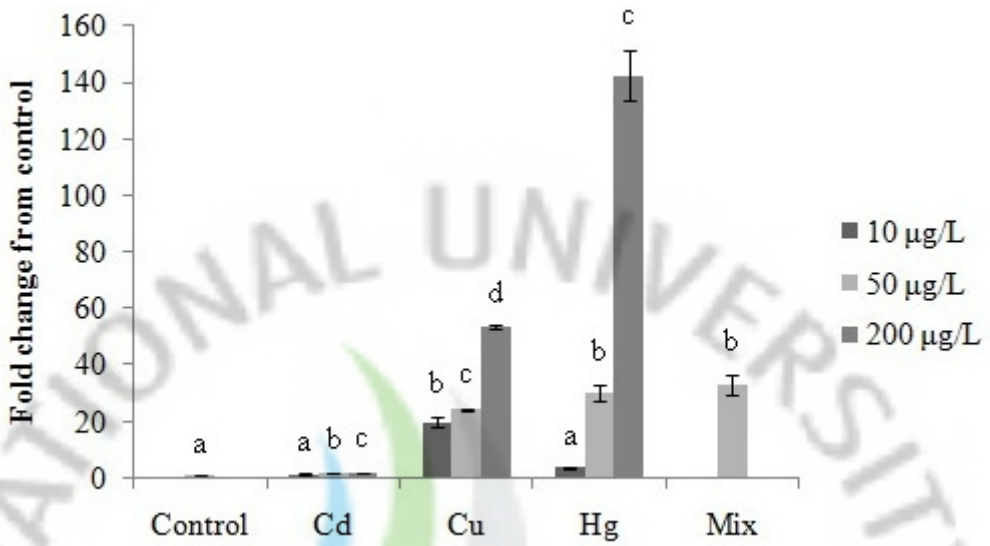


Fig. 25 Expression profiles of abalone HSP20 in response to heavy metal exposure. $CdCl_2 \cdot 2.5\ H_2O$, $CuSO_4 \cdot 5H_2O$ and $HgCl_2$ were used in the treatment with final ion concentrations of 10, 50 and 200 µg/L. Mix represents the mixture of Cd^{2+}, Cu^{2+} and Hg^{2+} each at a concentration of 50 µg/L. After 12-h exposure, the expression level of abalone HSP20 in gill tissue was analyzed by qRT-PCR with a ribosomal protein as reference. The results are represented as means (n=3) ± S.E.M. Data with different letters are statistically different at $P < 0.05$ based on one-way ANOVA.

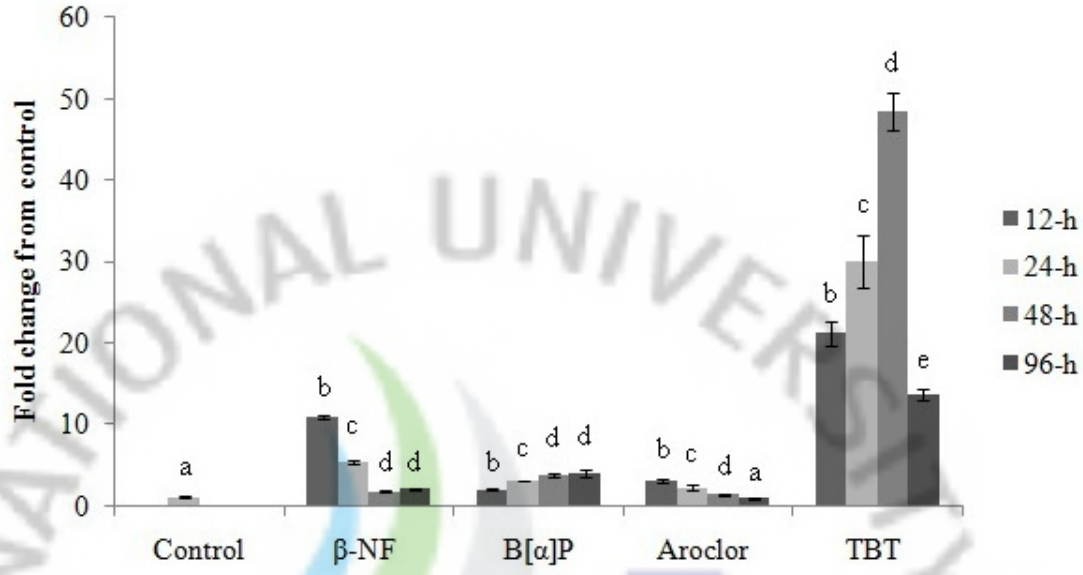


Fig. 26 Expression profiles of abalone abalone HSP20 in response to model EDCs exposure. Abalones were challenged by waterborne exposure of beta-naphathoflavone (β-NF), benzo[α]pyrene (B[α]P), aroclor-1254 (Aroclor), and tributyltin chloride (TBT) with the dose of 1.0 mg/L, 1.0 mg/L, 500 μg/L and 10 μg/L, respectively. Gill tissues of challenged abalones were sampled at 12-h, 24-h, 48-h and 96-h. The expression level of abalone HSP20 was analyzed by qRT-PCR with a ribosomal protein as reference. The results are represented as means (n=3) ± S.E.M. Data with different letters are statistically different at $P < 0.05$ based on one-way ANOVA.

4. Discussions

Small HSPs constitute a diverse group of stress-induced proteins which are highly variable in sequence, size and function. To date, only 5 complete sequences of small HSPs have been reported in mollusc species, which are three HSP22 from clam, one HSP22 from scallop and one HSP26 from abalone. Interestingly, our gene showed low sequence similarity (<20%) with all these molluscan small HSPs, whereas much more identical (>30%) to a group of small HSPs with molecular mass of approximately 20 kD from flat worm and insects. Therefore, we named this novel small HSP as HSP20 and grouped it together with other homologous HSP20s as a subfamily. In eukaryotic organisms, the small HSP family generally possesses multiple genes that might play distinct functional roles. Through exhaustive searches of small HSPs in the available complete genome sequences on NCBI, we found 19 small HSPs in Arabidopsis thaliana, 16 in Caenorhabditis elegans, 12 in Drosophila melanogaster, 12 in Danio rerio, 9 in Xenopus laevis and 10 in human. However, in over 12000 ESTs of *H. discus discus* (unpublished data), only two small HSPs were identified, the HSP20 of the present study and a partial sequence homological to HSP26 that was recently reported from another disk abalone species H. discus hannai (Park, Kim et al. 2008). Yet this missing of corresponding ortho/paralogs could only suggest but not come to a conclusion that abalone or mollusc species might have much fewer small HSP members than other animal phyla, due to the limitation of our search and sequencing efforts which predicatively has not retrieved all the small HSPs present in disk abalone. However, if this hypothesis is correct, we would speculate that the limited number of small HSPs in abalone might possibly play universal functional roles in multiple cell types and biological processes, which are generally responsible by specific members of expansive small HSPs in other eukaryotes.

Ambient temperature is one of the most decisive factors for the normal functioning of multiple physiological processes in animals, especially ectotherms like abalone. In general,

when cells are exposed to an elevated temperature, many proteins would trend to aggregate and lose biological activity, thus leading to a serial of adverse cellular events (Laszlo 1992). To cope with it, organisms have developed a series of mechanisms, in which increased synthesis of HSPs is generally found. In the case of several small HSPs which are not constitutively expressed at non-stress condition, they could be accumulated to over 1% of the total cellular protein after thermal stress and functionally could maintain approximately one-third of the cytosolic protein in a soluble state (Derocher, Helm et al. 1991). Likewise, abalone HSP20 showed a tremendous induction upon heat shock with over 2000-fold increase in mRNA level as compared to the steady-state level (Fig. 24-A). In contrast, the responses of abalone HSP70 and HSP90 genes were reported to be much weaker (only 2-10 fold) by previous studies (Cheng, Liu et al. 2007; Wang, Whang et al. 2010). The elevated expression of HSPs in response to thermal stress is well known to be regulated by heat shock transcription factors (HSFs), which can bind specifically to heat shock elements (HSEs) in the promoter region of HSPs and stimulate expression (Gurley and Key 1991). Accordingly, genes with strong thermo-inducible expression usually present multiple HSE elements in the promoter region. However, one should bear in mind that in addition to HSE-HSF regulatory pathway many other promoter elements such as TATA motif could also determine the transcription activity of HSPs in response to heat shock (Czarnecka, Key et al. 1989; Sung, Vierling et al. 2001). Therefore, to understand molecular mechanisms underlying the strong heat-inducibility of abalone HSP20, further efforts are needed to ascertain its promoter structure. Other than transcriptional-activation, many lines of evidence suggest that the enhanced expression of HSPs in cell could functionally confer a transient resistance to killing by heat stress, also known as thermotolerance (Landry, Chretien et al. 1989). In light of this, we also carried out a thermotolerance assay by evaluating the survival rate of *E. coli* BL21 (DE3) cells at 54 °C, which is higher than the maximum grow temperature but could still retain the natural structure of small HSPs (Shearstone and Baneyx 1999). The results demonstrated a substantial increase in thermotolerance when abalone HSP20 was

overexpressed in *E. coli* cells. Similar findings have been reported in other small HSPs from mammals (Lavoie, Hickey et al. 1993), drosophila (Rollet, Lavoie et al. 1992) and plant (Dafny-Yelin, Tzfira et al. 2008). Despite lack of active refolding properties, small HSPs is believed to perform functions in chaperone network of host cells by stabilizing actin cytoskeleton and preventing the aggregation of cellular proteins, thereby enhancing thermotolerance of cells (Gusev, Bogatcheva et al. 2002). Moreover, because small HSPs bind denaturing substrate proteins in an ATP-independent manner, their function is crucial under intracellular ATP depletion caused by thermal stress where the activity of ATP-dependent chaperones is inhibited.

Alternatively, when ambient temperatures decline, particularly as to below a certain critical point, organisms may experience cold stress. Abalone HSP20 herein exhibited rapid induction upon a cold exposure at 4 ^{o}C (Fig. 24-B). This is consistent with previous findings in which a suite of heat shock proteins including HSP90, HSP70 and small HSPs were accumulated in response to cold stress (Krishna, Sacco et al. 1995; Laios, Rebeyka et al. 1997; Yocum, Joplin et al. 1998). Furthermore, this up-regulation of HSPs was proposed to contribute an increased tolerance to the cold injury in plants, yeast and insects (Burton, Mitchell et al. 1988; Pacheco, Pereira et al. 2009; Ji-hao Sun, Jian-ye Chen et al. 2010). Whereas a certain number of studies also conversely illustrated that HSPs could show no significant change or an actual suppression in response to cold stress (Han, Zhou et al. 2005; Park, Kim et al. 2008). Although the mechanisms for activation and function of HSPs under cold stress remain elusive, many similarities between the net cellular physiological effects of heat and cold stress, such as activation of HSF-1, denaturation of proteins and stimulation of apoptosis, were intriguingly found (Sonna, Fujita et al. 2002). Thus, we could speculate that abalone HSP20 might likewise serve as molecular chaperone and protect abalone from injury during cold stress, similar as the role under heat stress.

Abalones are vulnerable to salinity fluctuation because they are considered as osmoconformers whose haemolymph osmolality is maintained close to the salinity of

surrounding seawater (Hosoi, Takeuchi et al. 2005). The optimal salinity of different abalone species is generally in a narrow range of 30-35 psu (Chen, Zhong et al. 2000); however, the salinity of their inhabited environment in coastal and estuarine/bay area could usually drop down to 20 psu due to the rainfall and water exchange. The mechanisms of osmotic regulation in abalone are not completely understood. In other mollusc species, it has been proposed as accumulation or release of organic and inorganic solutes by regulating the activity of membrane transporters and ionic channels (Hosoi, Takeuchi et al. 2005; Ruiz and Souza 2008). This process occurs very slowly and has to expend a considerable amount of energy, which thereby possibly lead to ATP depletion and enhance the denaturation and aggregation of cellular protein. Clearly, the adverse effect of osmotic stress is quite similar as that under heat stress, implying considerable overlap between heat and non-heat stress regulatory networks of HSPs (Swindell, Huebner et al. 2007). In the present study, the effect of extreme salinities on abalone HSP20 expression was investigated and remarkable induction was detected upon challenge. The overexpression of small HSPs have been demonstrated to play protective roles against the osmotic injury in a variety of organisms (Jiang, Xu et al. 2009). However, compared to the rapid early response upon extreme temperatures, abalone HSP20 responded much more slowly to osmotic stress. In the beginning hours of challenges, only moderate elevation of expression were observed and the expression peak appeared until 48-h. This mild response in the early phase of osmotic stress might be associated with alteration of membrane fluidity or shrinking and swelling of cells, but without protein denaturation (Beck, Neuhofer et al. 2000). However as the osmotic stress progressed, excessive denatured protein might be produced, and thereby largely stimulated the expression of HSP20 to repair the cellular damage.

Despite not yet a model sentinel species as bivalves in environmental biomornitoring programs, abalone and other gastropod species are being gradually accepted by scientists, mainly due to their advantages of high sensitivity to pollutants and broad environmental distribution (Hall, Rhind et al. 2009). However, little is known about the biomarkers in

gastropods. With this respect we challenged abalones with a set of pollutants ubiquitously present in marine environment and investigated the response of abalone HSP20. Following the acute exposure of three different heavy metals, a drastic, dose-dependent increase in abalone HSP20 expression was observed with copper and mercury, but not with cadmium. Similar findings were reported in zebra mussel where cadmium also demonstrated the weakest effect on the HSP70 production (Singer, Zimmermann et al. 2005). In a recent study using clam, two small HSPs were even down-regulated by cadmium (Li, Wang et al. 2010). Noteworthy is that many contrary findings from the studies using fish, mammalian cell line and bivalves have demonstrated cadmium as strong inducer of HSPs (Choi, Jo et al. 2008; Zhang, Wang et al. 2010). It was hypothesized that apoptosis and tissue damage caused by cadmium should be mainly responsible for the lack of HSPs induction in some cases, from the view point of anti-apoptosis role of HSPs. However, studies on comparative toxicities of heavy metals implied that a few marine invertebrates including abalone and shrimp might have higher tolerances to cadmium in comparison with other metal species (Lorenz, Francese et al. 2001; Gorski and Nugegoda 2006). Moreover, similar weak effect of cadmium on abalone antioxidant enzymes was also observed in our earlier study (Wan, Whang et al. 2009). Taken together, another underlying possibility throughout our results is that the dosage of cadmium in use might not reach to the effective concentration and hence failed to induce the expression of stress proteins. Similar to heat stress, heavy metals provoke damages at the cellular or molecular level mainly through direct denaturation of proteins and indirect induction of oxidative stress (Bertin and Averbeck 2006). Therefore, the increase of HSP20 expression under heavy-metal stress is probably implicated in the repair and cytoproection approaches in abalone as well. On the other hand, pollutions of organic EDCs have also addressed growing scientific and public concerns since they may be partly responsible for the population declines of wildlife by adversely affecting their development, endocrine, reproductive, and immune systems (Bernanke and Kohler 2009; Zhou, Cai et al. 2010). In the past few years, researchers have successfully developed a suite of sensitive

biomarker genes for EDCs exposure in mammals and fish, such as vitellogenin, zona radiata protein and cytochrome P4501A (Assuncao, Miller et al. 2007; Maradonna and Carnevali 2007). Nevertheless, owing to the large divergences in endocrine and hormonal system in comparison with vertebrates, many of those well-established biomarkers are currently unavailable, or require further validation in invertebrate models. On the contrary, many stress proteins, notably HSPs, have shown good correlations between their expression and exposure of organic EDCs as potential biomarkers in invertebrates (Kohler, Knodler et al. 1999; Björne Olsson, Brian P. Bradley et al. 2004; Seo, Park et al. 2006). Likewise, our results in the present study revealed significant induction of HSP20 in abalone after the short-term exposure to four model EDCs that respectively belong in polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs) and tributyltin (TBT) categories. TBT is well recognized as the most toxic compound ever for mollusc species. The mechanism of cellular toxicity of TBT has been closely linked to the direct denaturation of membrane and cellular proteins (Massaro, Zucker et al. 1989). In addition, a number of studies noted that TBT exposure could also induce significant production of reactive oxygen species (ROS) and malondialdehyde (MDA) (Liu, Wang et al. 2006). Probably owing to the synergistic effect of denatured protein and oxidative stress, TBT evoked the strongest induction of abalone HSP20 amongst four EDCs. Apart from small HSP, the expressed sequence tag analysis in small abalone (*H. diversicolor*) reported that other members in HSP family such as HSP70 and HSP90 were also responsive to TBT exposure (Jia, Zhang et al. 2009). Furthermore, in the field transplant study using clam, a relationship between TBT body burden and HSP levels was recognized (Sole, Morcillo et al. 2000). These findings indeed implied the possible utilization of molluscan HSPs as biomarker for TBT contamination. Compared with TBT, the responses of abalone HSP20 to three EDCs of PAHs and PCBs categories were much less sensitive despite at doses well above the environmentally relevant concentrations. Moreover, as the increase of exposure time, the induction levels of HSP20 did not increase but steadily decreased to almost control levels

upon β-NF and Aroclor. The lack of effect on HSP expression by PCBs was also reported in the study using Arctic charr (*S. alpinus*) that no response of liver HSP70 and HSP90 was observed following a 40-day treatment of Aroclor (Vijayan, Aluru et al. 2006). The study of another HSP20 in intertidal copepod (*T. japonicas*) observed either insignificant change or down-regulation in level of inducible HSP20 after PAHs exposure (Seo, Park et al. 2006). Additionally, the transient response of abalone HSP20 is also consistent with the findings in isopod (*O. asellus*) exposed to B[a]P and PCB52 where no significant induction of HSP70 could be detected for exposure times greater than 24 hours independent of the concentration of chemicals (Kohler, Knodler et al. 1999). Apart from PAHs and PCBs, an *in vitro* study using mammalian cell lines reported that a certain number of organic EDCs similarly were not able to induce any HSP expression (Ait-Aissa, Porcher et al. 2000). The detailed mechanism underlying this phenomenon is remained uncertain, although several studies suggested that the cytotoxicity and lipophilicity of chemical stressors could be dependent factors for HSP response (Neuhaus-Steinmetz and Rensing 1997).

Taken together, the data in the present study demonstrate positive correlations between the expression of abalone HSP20 and various environmental stressors. During stress, HSP20 probably plays protective roles against cellular damage as a molecular chaperone in abalone. However, the utility of HSP20 as an indicator of pollutions in the field condition is under discussion, since its non-specific responses to multiple physical and chemical stressors might mask the effect of the specific pollutant to be monitored. But still, HSP20 could be ideal as a sensitive biomarker to completely reflect the integrated severity of the environmental stress and the health condition of abalone in field. Yet the experimental period in our study only allowed for the determination of the acute response of abalone HSP20 to stress, further investigation for chronic exposure therefore will be necessary to fully elucidate its function and biomarker potential.

Chapter III.

Validation of housekeeping genes as internal controls for studying biomarkers of endocrine-disrupting chemicals in disk abalone by real-time PCR

Abstract

Our experiments were designed to identify suitable housekeeping genes (HKGs) in disk abalone as internal controls to quantify biomarker expression under endocrine disrupting chemicals (EDCs) challenges. Relative expression levels of twelve candidate HKGs were examined by real-time reverse transcription PCR (qRT-PCR) in gill and hepatopancreas of abalone following 7-day challenge with tributyltin chloride (TBT) and 17β-estradiol (E2), respectively. The expression levels of several conventional HKGs, such as 18s rRNA, glyceraldehyde-3-phosphate dehydrogenase and β-actin, were significantly altered by challenges, indicating that they might not be suitable internal controls. Instead, the geNorm analysis pinpointed ribosomal protein L-5/ elongation factor 1 and ribosomal protein L-5/ succinate dehydrogenase as the most stable HKGs under TBT and E2 challenges, respectively. Moreover, these three HKGs also showed the highest stabilities overall amongst different tissues, genders and EDC challenges. The expression of a biomarker gene, cytochrome P450 4B (CYP4), was also investigated and exhibited significant increase after challenges. Nevertheless, when unsuitable HKGs were used for normalization, the influence of two EDCs on CYP4 expression was imprecisely overestimated or underestimated, which strongly emphasized the importance of selecting appropriately validated HKGs as internal controls in biomarker study.

Key words: Housekeeping genes, internal control, real-time PCR, EDCs, biomarker.

1. Introduction

Endocrine disrupting chemicals (EDCs) are a class of chemicals that can interfere with natural hormone actions in the body (Kavlock, Daston et al. 1996). As a result of exposure to EDCs, humans and wildlife may appear several signs of ill-health including abnormal plasma hormone levels, abnormal reproductive organs, reduced germ cell count, dysfunctional immune system, and increased cancer risk (Sonnenschein and Soto 1998; Mills and Chichester 2005). Every year, however, great quantities of EDCs from various sources are discharged into the ambient environment. As the ultimate pool of all kinds of waste effluents, the marine environment is suffering the most serious contamination with EDCs. The instances of endocrine disruption in marine organisms are also continuously increasing worldwide (Matthiessen, Allen et al. 2002; Ford, Fernandes et al. 2004; Porte, Janer et al. 2006). As a consequence, monitoring, risk assessment and management of marine EDCs pollution have given rise to significant concerns from the scientific community, government agencies and the general public. To date, several biomarker genes, such as vitellogenin (VTG), cytochrome p450 1A (CYP1A), glutathione S-transferase (GST) and metallothionein (MT), have been well established in marine organisms to monitor EDCs contamination (Fossi, Casini et al. 2002; Wan, Whang et al. 2008; Rhee, Raisuddin et al. 2009).

In recent ecotoxicological studies, qRT-PCR may be one of the most widely used methods to evaluate the expression of biomarker genes under environment contaminants. However, scientists showed little concern about selection and validation of suitable HKGs for biomarker quantification. To the best of our knowledge, there are only few published works in this regard, which were respectively conducted in marine mammal (Spinsanti, Panti et al. 2008), fish (Filby and Tyler 2007), crustacean (Heckmann, Connon et al. 2006) and insect (de Boer, de Boer et al. 2009). There is a substantial lack of information in mollusc phylum, although they have been frequently used as sentinel species in pollution monitoring

programs (Valdez Domingos, Azevedo et al. 2007; Hall, Rhind et al. 2009). Most of the studies that quantify biomarker expression in mollusc species seemed to use HKGs randomly, without any validation of their expression stabilities (Puinean and Rotchell 2006; Banni, Dondero et al. 2007; Brooks, Lyons et al. 2009; Zapata, Tanguy et al. 2009). Recently, a large body of evidence has demonstrated that the expression of several commonly used HKGs such as 18s rRNA, β-actin and GAPDH might be considerably variable in certain tissues or experimental conditions (Goidin, Mamessier et al. 2001; Selvey, Thompson et al. 2001; Ruan and Lai 2007). It is well-known that selection of an inappropriate HKG in normalization may increase experimental noise and thus strongly influence the reliability of qRT-PCR analysis. In the present study, therefore, we attempted to identify suitable HKGs in abalone (marine gastropod mollusc) for studying biomarker of EDCs contamination. We selected twelve HKGs of disk abalone (*Haliotis discus discus*), and evaluated their expression stabilities after 7-day waterborne exposure of TBT and E2 in gill and hepatopancreas tissues. Furthermore, the influence of using different HKGs on quantification of biomarker expression was also investigated.

2. Materials and Methods

2.1. Animals

Two-year-old disk abalones with 50-60 g average weight and well-developed gonads were obtained from an abalone farm on Jeju Island, South Korea. Abalones were acclimated in laboratory aquaria for 1 week prior to the challenge experiment. The seawater was filtered and aerated continuously, with salinity and temperature maintained at 32 ± 1 ‰, and 20 ± 1 °C, respectively. Thereafter, abalones were divided into one control group and two challenge groups, having three males and three females in each group. The abalones in two challenge groups were exposed to waterborne 1 μg/L of TBT and 100 μg/L of E2 dissolved in dimethyl sulfoxide (DMSO), respectively. Toxicant burden seawater was renewed every 24 h. In the control group, same quantity of DMSO vehicle was added into the seawater. After 7 days exposure, gill and hepatopancreas tissues were dissected and frozen in liquid

nitrogen immediately for further experiments.

2.2. Total RNA extraction and cDNA synthesis

Total RNA of gill and hepatopancreas was extracted from the tissue pools containing the same weight of tissue from three abalones using TRI Reagent (Sigma). A secondary purification using S.N.A.P. total RNA isolation kit (Invitrogen) was subsequently carried out for hepatopancreas samples due to the abundance of polysaccharides and pigments, which would interfere with the subsequent enzymatic processes. Total RNA concentration was obtained by measuring absorbance at 260 nm. The quality of RNA was also verified by formaldehyde agarose gel electrophoresis. First-strand cDNA synthesis was carried out using the SuperScript III First-Strand Synthesis System (Invitrogen). Briefly, 2.5 µg RNA, 1 µl of 50 µM oligo $(dT)_{20,}$ 1 µl of 50 ng/µl random hexamers and 10 mM dNTP mix were combined to incubate at 65°C for 5 min, then placed on ice for at least 1 min. Then, 2 µl 10 × RT buffer, 2 µl 25 mM $MgCl_2$, 2 µl 0.1 M DTT, 40 U RNaseOUT and 200 U SuperScript III were added and then incubated at 25°C for 10 min and 50°C for 50 min. The reaction was terminated by incubating at 85°C for 5 min and the residual RNA was removed by incubating at 37°C for 20 min with the addition of 1 µl RNaseH. Finally, the cDNA was diluted 1:20 for use in real-time PCR.

2.3. Primer design and qRT-PCR analysis

Sequences of twelve HKGs and one biomarker gene (CYP4) obtained from disk abalone cDNA library were used to design primers using Primer 3.0. The primer pairs were selected using the following criteria: melting temperature (T_m) of approximately 60 ^oC, primer length ranging from 18 bp to 24 bp with 22 bp as the optimum, and amplicon size of approximately 150 bp (Table 22). Real-time PCR was run in triplicate in 0.2 mL 8-strip PCR tubes with a Takara™ real time PCR detector TP800 (Takara Inc., Japan). Reaction consisted of 12.5 µl of SYBR Premix Extaq (Takara), 4 µl cDNA from 25 ng total RNA, and a pair of specific

primers. The thermal profile was programmed as follows: 3 min at 94 °C, and 40 cycles of 20 s at 95 °C for, 20 s at 60 °C, and 30 s at 72 °C. In order to ensure amplification specificity, the dissociation curve of PCR product was investigated by heating from 60 °C to 95 °C at the end of each reaction.

2.4. Data processing and statistics

The PCR results were analyzed by the software platform of Takara TP800. Baseline and threshold values were automatically set by the program. The number of PCR cycles to reach the fluorescence threshold in each sample was defined as the C_t value. C_t values of twelve housekeeping genes were transformed into relative quantities using the $2^{-\Delta Ct}$ method where $\Delta C_t = C_{t\ treated} - C_{t\ control}$ (Livak and Schmittgen 2001). Expression stability values of the 12 HKGs under either E2 or TBT were then calculated by the geNorm VBA applet for Microsoft Excel (Vandesompele, De Preter et al. 2002). To determine the effect of using the different internal controls on biomarker gene quantification, the expression of CYP4 in gill and hepatopancreas after TBT and E2 exposure was normalized by the 12 HKGs. Two-group comparisons of control and experimental groups were carried out using the Student's *t*-test in SPSS 16.0 for Windows (SPSS, Chicago, IL, USA). Statistical significance was assigned at $p < 0.05$. Experimental data are shown as the mean ±S.E.M.

Table. 22. Candidate housekeeping genes, biomarker gene of interest and their specific primers used in the study.

Symbol	Gene name	Function	Forward primer	Reverse primer	Size (bp)
18rRNA	18s ribosomal RNA	Structural constituent of ribosome	AGACTGTCGATGGTAAGTGCTATGC	ACTACCTCCTCGTATCGAGATTGG	153
ACTB	Beta-actin	Cytoskeletal structural protein	GAATCTGCTGGTATCCATGAAACC	GGGATGTGATCTCCTTCTGCAT	151
BGLU	Beta-glucuronidase	Carbohydrate metabolic process	ATAGTCCAGGCCTTTCCCTCGAAT	CCTCTTCAGCTGGAATGGATGTGT	155
CY	Cyclophilin	Protein folding	GATCCAAGGTGGAGACTTCACTAAG	AACTGGGAACCATTGGTGTTG	153
ELF	Elongation factor 1-alpha	Essential component of the eukaryotic translational apparatus	CTGCCACACAGCCCATATTG	CCACACACATGGGCTTGCT	152
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	Glycolytic enzyme	TGGGCGTGAACCACACTAAGTAC	GCGGTGTAAGCATGAACTGTTG	151
HH2A	Histone H2A	Chief structural protein of the nucleosomes	GCAATGCCAGTAAGGATTTGAAAG	TTTCCGATCAGAGACTTGTGGATA	151
HPRT	Hypoxanthine phosphoribosyltransferase 1	Metabolic salvage of purines	ACGACATCTCAACAGGGAACATC	GACTTGGGCTTCACTTCCTTCA	151
RPL5	Ribosomal protein L5	Ribosomal protein in ribosomal large subunit	TCACCAACAAGGACATCATTTGTC	CAGGAGGAGTCCAGTGCAGTATG	152
SDHA	Succinate dehydrogenase	Electron transporter in the TCA cycle and respiratory chain	CTAGCTCTGCCTGTCACATACCA	TACCAAACACAAACCCTGTGGATA	151
TUBB	Beta-tubulin	Member of the tubulin family of structural proteins	AGTTCTGGGAGGTGATTTCAGATG	TCCAAATCGACGAGGATAGCA	151
UBC	Ubiquitin-conjuction enzyme	Protein degradation	CACTGGCAAGCAACAATAATGG	CCATTGCTGTTGATGTTTGGA	152
CYP4	Cytochrome p450 family 4	Phase I detoxification enzyme	AGAAGAAGGACACCTACGCATACA	CGTCCGACTTTGTGGCTTATATC	151
GSTM	Mu class glutathione S-transferase	Phase II detoxification enzyme	CCAATCTGCCCTACTATGTGGAT	CTGAAGTCCATGGCTGTGTCA	151

3. Results

3.1. General expression levels of twelve abalone HKGs

To reduce possible errors due to co-regulation of different genes, we purposely selected twelve HKGs that are involved in different cellular functions: 18s rRNA, ACTB, BGLU, CY, ELF, GAPDH, HH2A, HPRT, RPL5, SDHA, TUBB and UBC (Table. 1). All twelve HKGs exhibited high similarities (>50% in protein sequence) to their respective gene homologues in the NCBI database. The expression levels of twelve HKGs in abalones were investigated by qRT-PCR using gene-specific primers. Each different primer pair used in qRT-PCR analysis had an expected efficiency of 1.9-2.0 and displayed a single dissociation peak at the expected temperature (data not shown), indicating a highly efficient and specific PCR amplification. The C_t values of twelve HKGs obtained are shown in Fig. 27. C_t values are proportional to the negative logarithm of the specific transcript copy number in input cDNA. 18s rRNA registered the highest abundance in both gill and hepatopancreas (C_t<10), over 5 cycles earlier than other HKGs to reach the fluorescent threshold. Following 18s rRNA, the ACTB, ELF and RPL5 were the other three most highly expressed genes (C_t≈15). In contrast, BGLU and HPRT1 were expressed at lowest levels in both gill and hepatopancreas (C_t≈25). It is noteworthy that several HKGs exhibited remarkably different expression levels between two tissues.

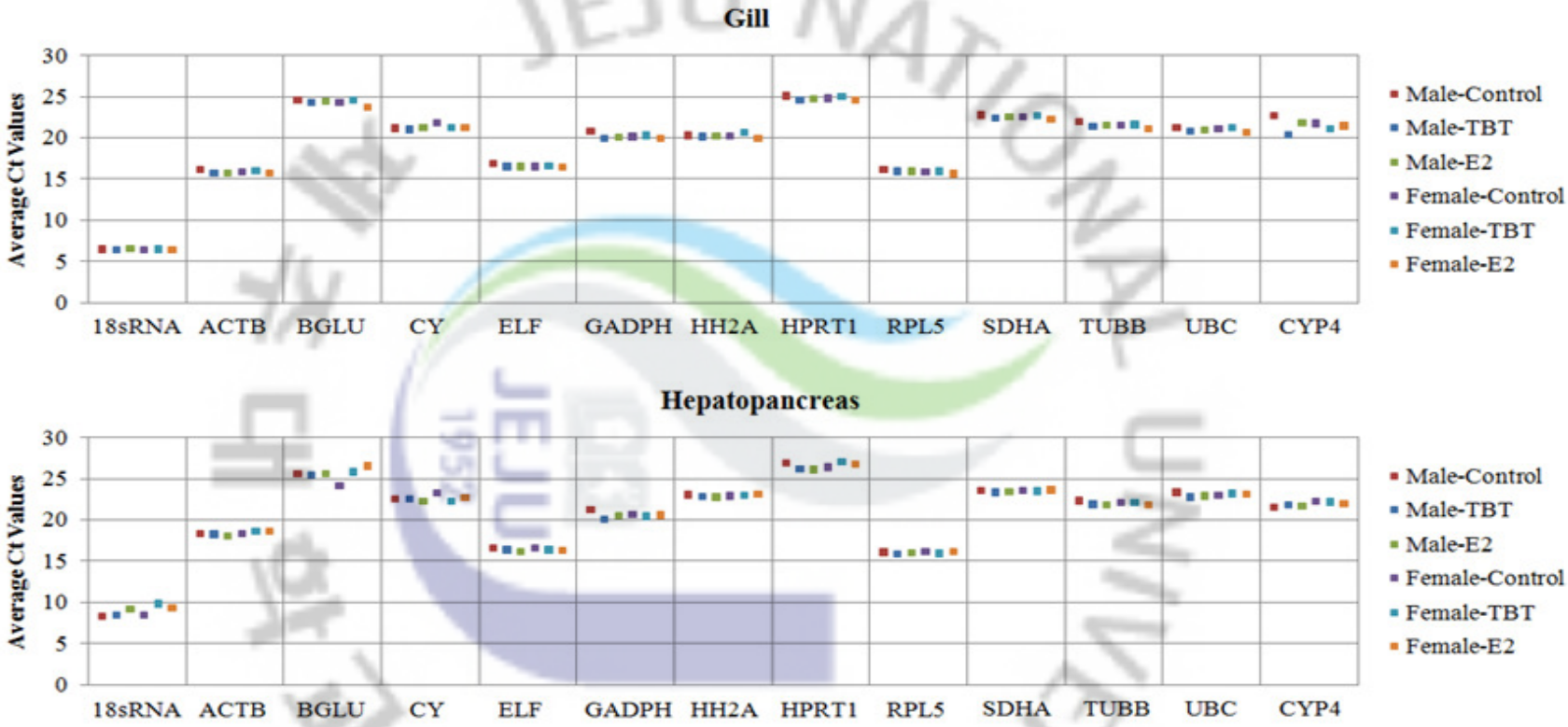


Fig. 27. Raw C_t values of twelve abalone HKGs and one biomarker gene CYP4 for different tissues (gill and hepatopancreas), genders (male and female), and experimental conditions (control, 7-day exposure of 1.0 μg/L TBT and 100 μg/L E2). The results are represented as the arithmetical mean of Ct values obtained from three replicates in the qRT-PCR assay.

3.2. Relative expression levels of twelve abalone HKGs following EDCs exposure.

As shown in Fig. 28, significant expression changes ($P < 0.05$) were found for all twelve HKGs, and these changes were highly associated with tissue type, gender and/or treatment condition. In gill, the expression patterns of twelve HKGs were interestingly similar. All twelve HKGs were induced by TBT in male gill and slightly repressed in female gill, except for CY, which was induced in both genders. While in E2 challenge, significant inductions were investigated in gill of both genders for ten HKGs. Additionally, we detected no statistically significant change in the expression of 18s rRNA and RPL5 for two challenges in gill tissue.

In contrast, the responses of twelve HKGs in hepatopancreas were fairly variable. In male hepatopancreas, TBT significantly induced the expression levels of nine HKGs up to 2-fold; while in female, five HKGs were significantly repressed, three HKGs were significantly induced and four HKGs showed no statistically significant change. Similarly, E2 challenge also caused significant induction of nine HKGs in male hepatopancreas; while in female, five HKGs were significantly repressed, three HKGs were significantly induced and four HKGs showed no statistically significant change. Amongst twelve HKGs, RPL5 was comparatively stable in hepatopancreas, showing significant expression change only in TBT treated male abalone.

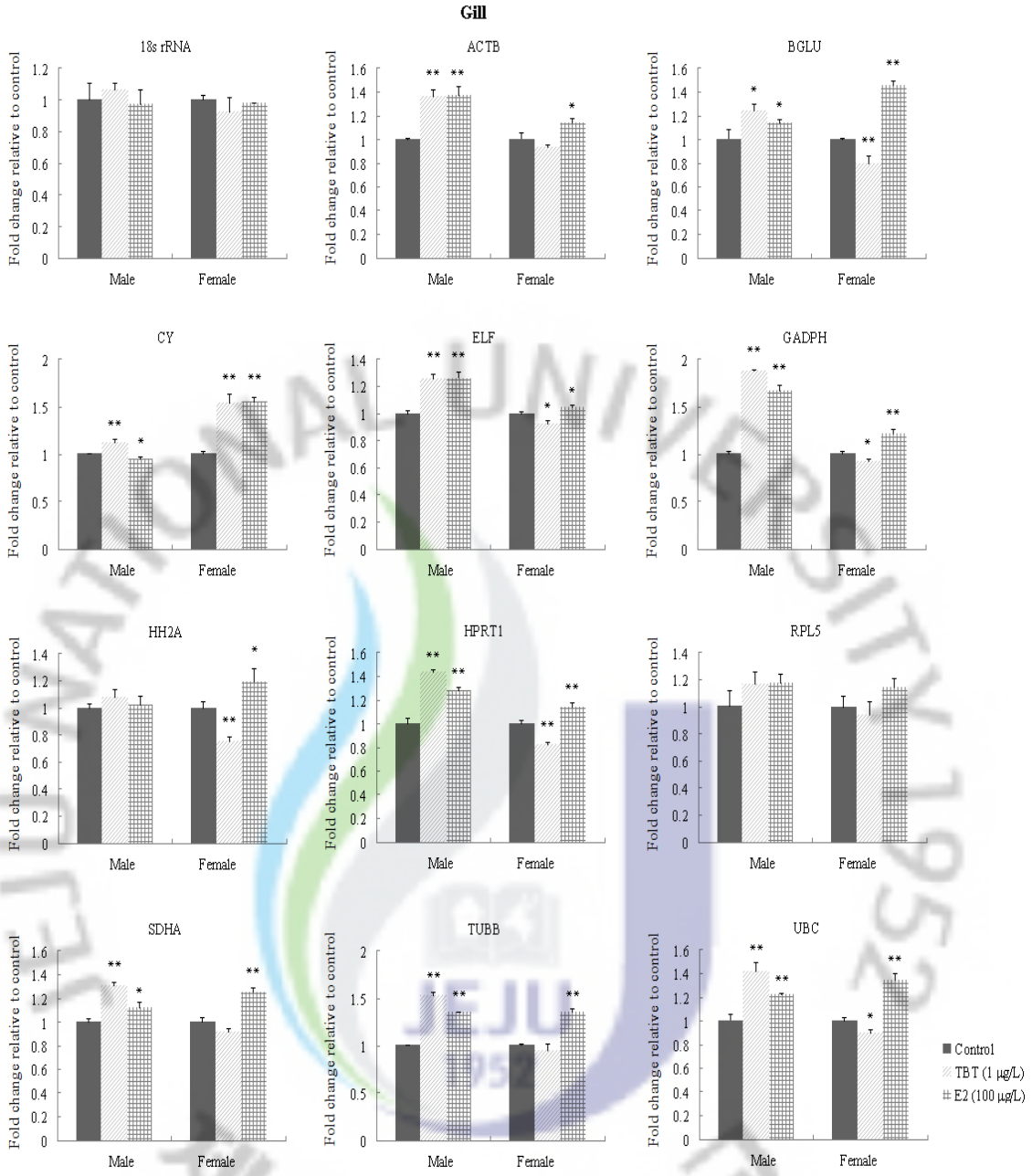


Fig. 28-A. Relative gene expression of twelve abalone HKGs following 7-day TBT and E2 challenges in gill. Relative expression fold of each HKG in gill was calculated based on the Ct values from qRT-PCR in the method of $2^{-\Delta Ct}$. The results are represented as means (n=3) ± S.E.M. Statistically significant differences in gene expression between control and TBT/E2 treated abalone are denoted as follows: * $P < 0.05$, ** $P < 0.01$ (Student's *t*-test).

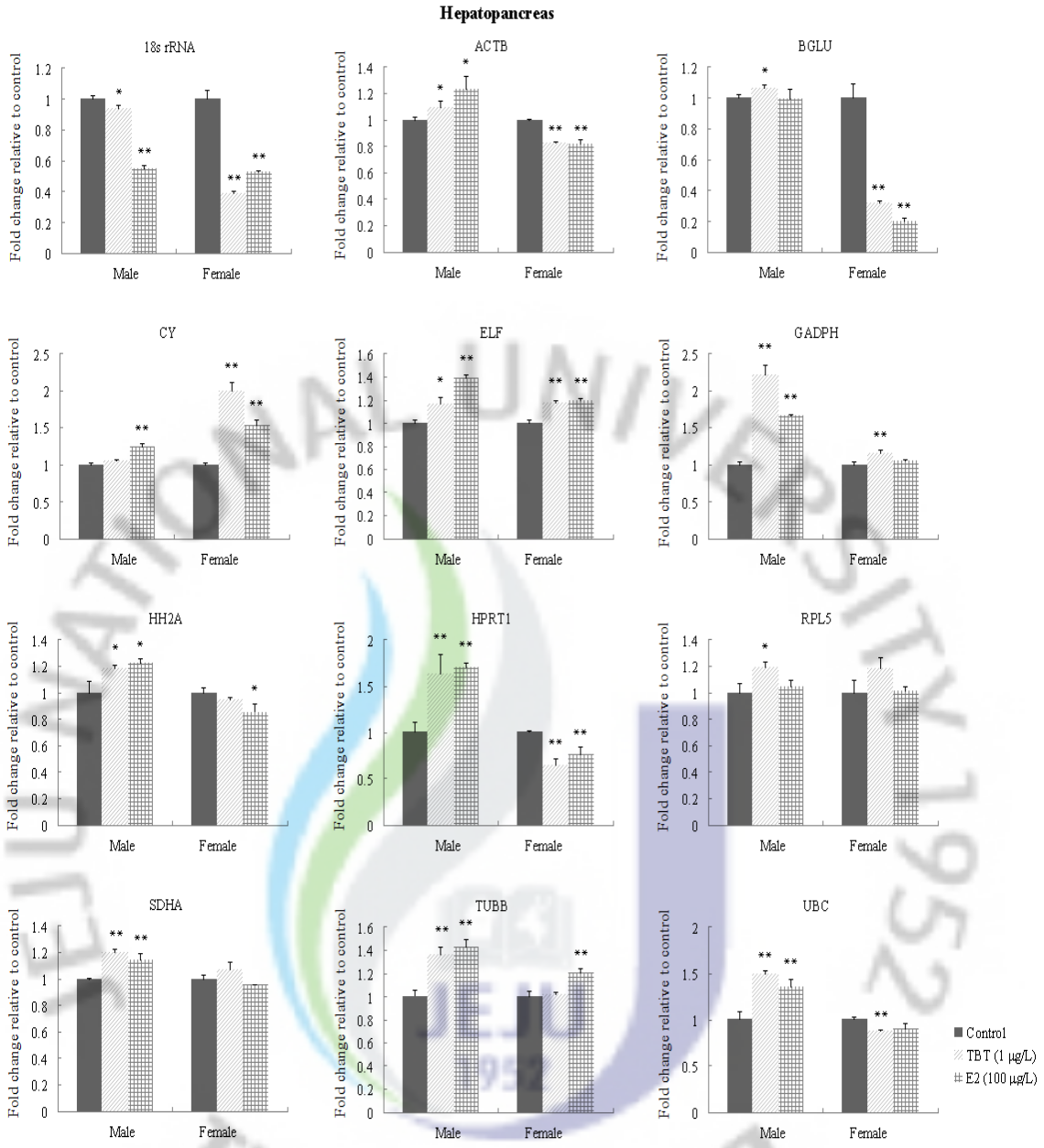


Fig. 28-B. Relative gene expression of twelve abalone HKGs following 7-day TBT and E2 challenges in hepatopancreas. Relative expression fold of each HKG in hepatopacreas was calculated based on the Ct values from qRT-PCR in the method of $2^{-\Delta Ct}$. The results are represented as means (n=3) ± S.E.M. Statistically significant differences in gene expression between control and TBT/E2 treated abalone are denoted as follows: * $P < 0.05$, ** $P < 0.01$ (Student's *t*-test).

3.3 Expression stability analysis and selection of the most suitable HKGs for normalization

As shown in Fig. 29, twelve HKGs were ranked by average expression stability (M) values from the geNorm analysis, starting from the least stable gene at the left (highest M value), and ending with the two most stable genes at the right (lowest M value). All twelve HKGs exhibited general high stability, with the M values lower than 0.35. For TBT challenge, ELF and RPL5 were the two most stable genes (Fig. 29-A); while for E2 challenge, RPL5 and SDHA showed the lowest M values (Fig. 29-B). Finally, RPL5 and SDHA (followed by ELF) were determined as the most stable HKGs in the overall 12 samples from two EDC challenges (Fig. 29-C). In contrast, the 18s rRNA and BGLU were considered as the worst HKG candidates by the geNorm classification, due to their high M values.

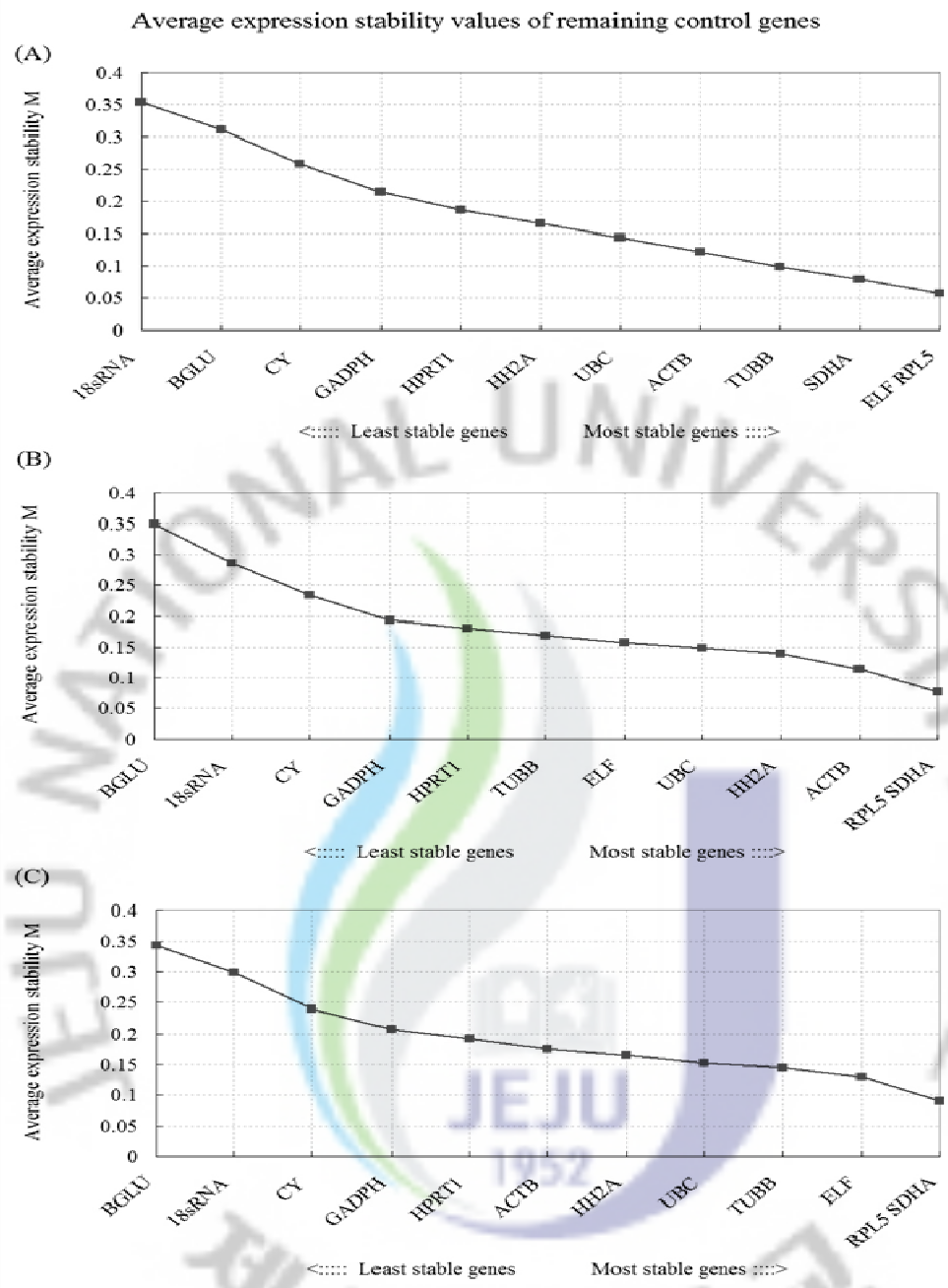


Fig. 29. Average expression stability measure (M) of twelve abalone HKGs following exposure to 1.0 μg/L TBT (A), 100 μg/L E2 (B) and overall (C), respectively. In overall analysis, 12 relative expression values from two tissues, two genders and two challenges were together input into the geNorm program.

3.4. Effect of different HKGs on biomarker gene quantification

The expression of CYP4 in abalone gill and hepatopancreas after TBT and E2 challenges was examined by qRT-PCR (Fig. 30). Without normalization by HKGs, assuming that the same amount of input cDNA has been used for each sample, the expression of CYP4 was significantly induced by TBT challenge ($P < 0.001$) in both gill and hepatopancreas of two genders. Similarly, CYP4 expression following E2 challenge also demonstrated significant induction ($P < 0.001$) in male abalone. However, the E2 effect in female abalone was much less or insignificant, indicating a highly male-specific effect. When the expression data of CYP4 was normalized against different HKGs, the expression patterns in gill were generally similar, although the fold-changes and/or significances were remarkably variable (Fig. 30-A). In contrast, the normalized patterns of CYP4 expression in hepatopancreas against different HKGs were drastically distinct, resulting from the strong influences of EDCs on individual HKGs in the tissue (Fig. 30-B). Through the normalization by different HKGs, we could investigate an either significant induction or significant suppression or inconspicuous of CYP4 expression under TBT and E2 challenges. Finally, we normalized CYP4 expression data with an optimal normalization factor (NF), which was obtained by the geometric mean of two most stably expressed HKGs in geNorm analysis. Abalone CYP4 displayed significant induction by TBT and E2 challenges in male tissues ($P < 0.001$). While in female abalone, significant change in CYP4 expression was investigated only in TBT treated gill ($P < 0.001$).

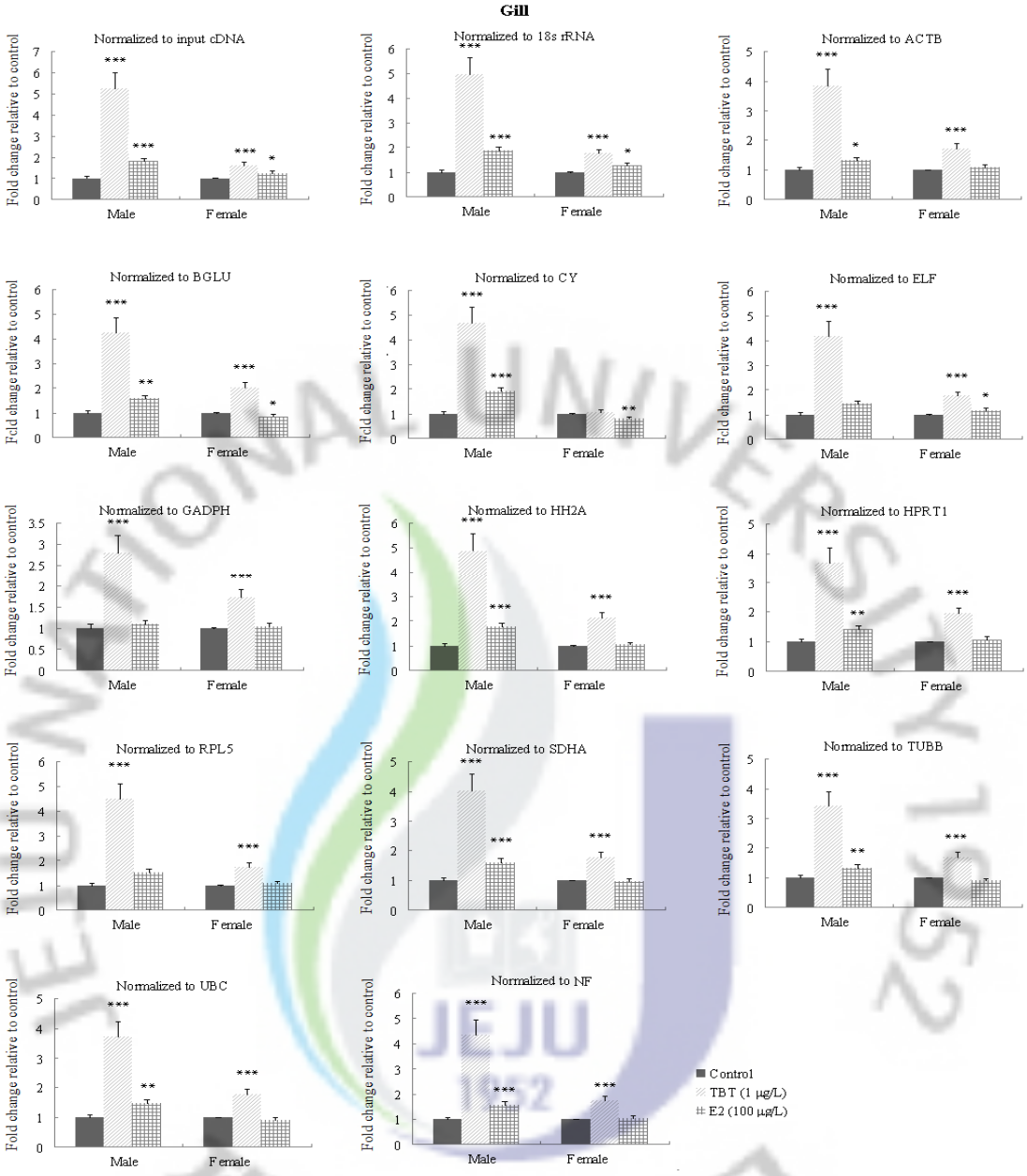


Fig. 30-A Relative gene expression of biomarker gene CYP4 in abalone gill following 7-day TBT and E2 challenges, with or without normalization to twelve housekeeping genes and a normalization factor (NF). The results are represented as means (n=3) ± S.E.M. Statistically significant differences in gene expression between control and TBT/E2 treated abalone are denoted as follows: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (Student's *t*-test).

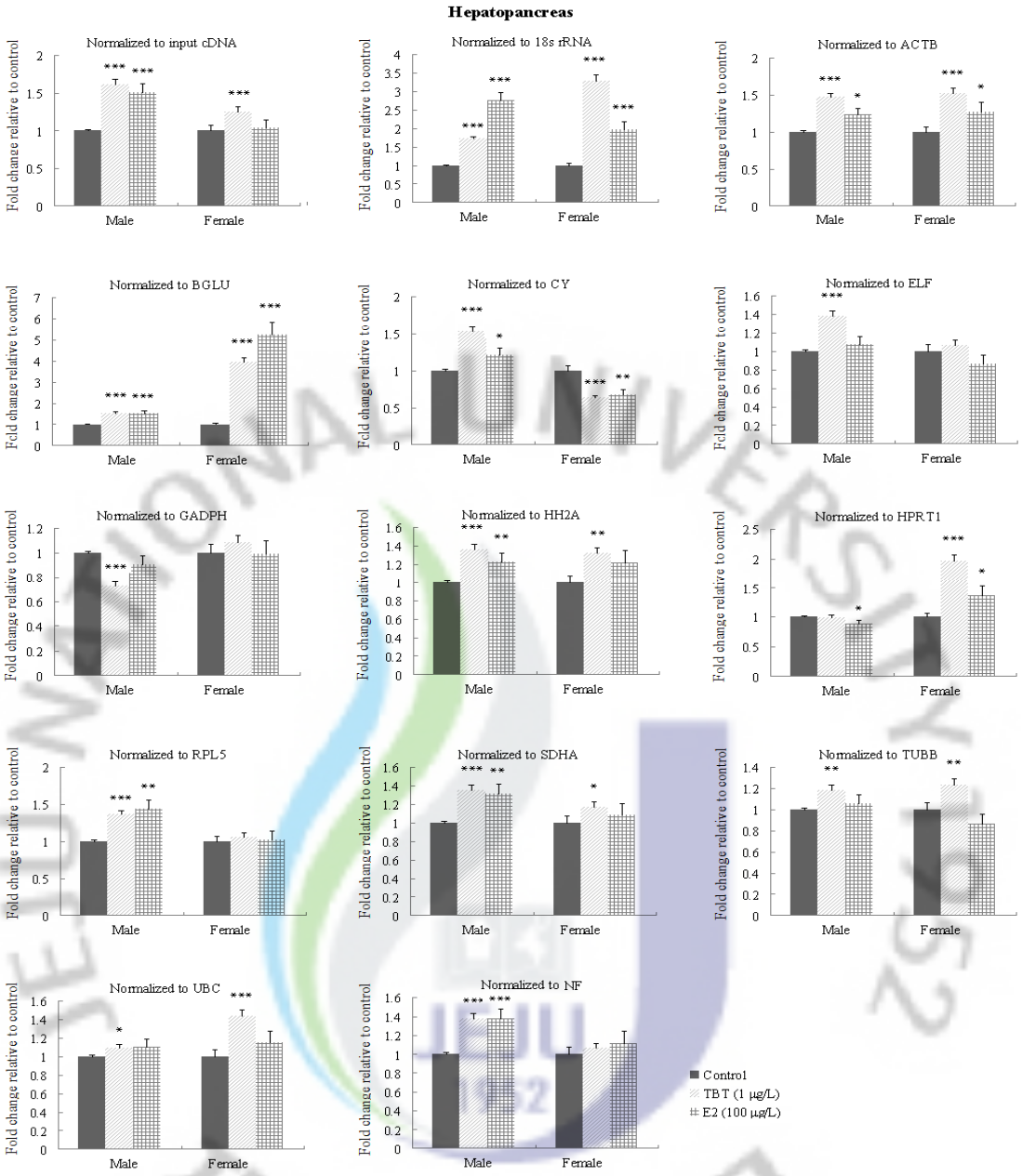


Fig. 30-B. Relative gene expression of biomarker gene CYP4 in abalone hepatopancreas following 7-day TBT and E2 challenges, with or without normalization to twelve housekeeping genes and a normalization factor (NF). The results are represented as means (n=3) ± S.E.M. Statistically significant differences in gene expression between control and TBT/E2 treated abalone are denoted as follows: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (Student's *t*-test).

4. Discussion

Validation of HKGs for accurate normalization of real-time PCR data in specific biological samples or experimental conditions have been extensively carried out in many cell lines and model organisms for the research of different fields. Nevertheless, the relevant work in non-model organisms has received much less attention (Siah, Dohoo et al. 2008). Our study represents the first effort aimed toward the systematic comparison of HKGs for environmental biomarker studies in mollusc species. We selected abalone (a marine gastropod) as an experimental animal, not only because of its high economic value in fisheries, but also due to its important roles in marine ecosystems and biological diversity. Even though no risk of extinction has been perceived yet, the wild populations of abalone in Korea, *H. sieboldii* in particular, have shown a rapid decline over the past few years, as a result of overfishing, climate change, environmental pollution and disease outbreak. Like most mollusc species, abalone appears particularly sensitive to EDCs due to a lack of an efficient system to metabolize and eliminate exogenous organic chemicals. In abalone, TBT and derivatives were reported to disturb the reproductive cycle and cause ovarian spermatogenesis in a female ovary, which contributed to abalone population decline in the examined site (Horiguchi, Takiguchi et al. 2000; Horiguchi, Kojima et al. 2002; Sloan and Gagnon 2004). On the other hand, excessive E2 exposure could evoke a “superfemales” syndrome in female mollusks and massive egg production and elevated mortality (Duft M, Schmitt C et al. 2006). We purposely selected these two model EDCs for the challenge experiment because we wish our findings could be representative for other EDCs with

similar androgenic or estrogenic properties.

The study of biomarkers places strong emphasis on quantitative analysis of gene expression, and hence the qRT-PCR assay undoubtedly is one of the most important tools. In our earlier studies, however, we had met serious problems with the use of a randomly selected actin gene to normalize qRT-PCR data in abalone until we realized the importance of examining other appropriate HKGs. Although actin genes have been largely used as internal controls in quantitative analysis of biomarker expression in mollusc species (Rhee, Raisuddin et al. 2008; Park, Ahn et al. 2009), the expression of our gene was tightly regulated by many challenge conditions, and also showed huge variations among different tissues of abalone (data was not shown), being a great obstruction to obtain accurate analysis of interested biomarker genes. Notably, several similar findings about varying expression of actin genes have been widely reported in other organism models and cell lines, as they were subjected to various stimuli such as temperature, hypoxia, microbial infection, oxidative stress and diseases (Ruan and Lai 2007; Araya, Siah et al. 2008; Chen and Ruan 2009). These evidences strongly challenged the role of actin as the conventional reference gene. Moreover, an association between hormones and regulation of actin expression has been revealed by several studies (Verma and Shapiro 2006; Schroder, Pelch et al. 2009). Thus, the use of β-actin as internal control in a study about the effects of EDCs, which could potentially alter normal hormone levels, especially requires careful examination. In the present study, we have tested another actin gene (ACTB) of disk abalone, which showed highest similarity to cytoplasmic β-actin from other organisms. Its expression was much

more stable than the previously utilized one, and showed moderate expression stability among 12 HKG candidates by geNorm analysis, especially in the exposure of E2 (Fig. 29). However, we noted that this β-actin gene had a significant tissue-specificity with approximately 10-time higher expression in gill than in hepatopancreas (Fig. 27), which would potentially lead to a false tissue distribution pattern of the target gene if used as an internal control. In addition to β-actin, GADPH and 18s rRNA are the two most used HKGs as internal controls in many studies, but they were grouped into the least stable expressed HKGs since their expression was dramatically altered after exposure to the two EDCs (>0.5-fold change). In addition, there is still a debate over whether ribosomal RNA is suitable as an internal control in qRT-PCR mainly due to the high abundances (80-90% of total RNA) compared with target mRNA transcripts within cells, which makes it difficult for accurate baseline subtraction in qRT-PCR analysis (Vandesompele, De Preter et al. 2002). In contrast, some unconventional HKGs exhibited better expression-stability in our study and seemed to be used under the condition of challenge by EDCs.

Nevertheless, we noted that the simple comparison of expression patterns (up- or down-regulation) is not adequate to identify most appropriate HKGs as there are several gene candidates showing similar expression changes. To address this issue, many computer programs such as geNorm, NormFinder and BestKeeper are available. Despite different statistical algorithms used to measure the expression stability, scientists found that the results produced by these programs usually showed only minor deviations (Radonic, Thulke et al. 2005). Therefore, it is recommended to use only one of these tools to pinpoint stable HKGs

under most experimental conditions. In our study, we chose geNorm to evaluate expression stability of 12 HKGs because this program ranks the genes according to the average pairwise variation between a particular gene and all other control genes, and also provides a measure of the minimum optimal number of reference genes (Vandesompele, De Preter et al. 2002). Although the geNorm algorithm is highly dependent on the proposition that the expression of HKG candidates should not be co-regulated in experimental conditions, the 12 HKGs that we selected are involved in different biological processes, and the effect of such co-regulation thus theoretically should be minor. Based on the result of geNorm analysis, ELF, SDHA and especially RPL5 were identified as the most stable genes under TBT and E2 exposure. The high stability of three HKGs in our study is highly consistent with the findings for E2 exposure on cetacean fibroblast culture and 17α-ethinylestradiol (EE2) exposure on fathead minnow, which were the only works regarding validation of HKGs in the challenge of EDCs other than ours. RPL5 is one of the constitutive proteins in large ribosomal subunit that catalyzes mRNA-directed protein synthesis. It binds specifically to 5S rRNA and forms a L5/5S RNA protein complex that is a precursor to ribosome assembly. Moreover, the expression of RPL5 is not coordinated with synthesis of other ribosomal proteins as an exception. As the most stable gene in our study, the expression of RPL5 was altered by less than 0.2-fold in all of the samples, in contrast to over 0.5-fold changes investigated for other HKGs. Although ribosomal proteins were previously recommended for the use in only less sensitive detection methods like Northern blot (Thellin, Zorzi et al. 1999), many recent HKG validation studies have reported that ribosomal proteins showed

remarkable stabilities in different cell lines and tissues of mammals (Brinkhof, Spee et al. 2006; Spinsanti, Panti et al. 2006; Janovick-Guretzky, Dann et al. 2007), fish (Infante, Matsuoka et al. 2008), shellfish (Siah, Dohoo et al. 2008), and plants (Barsalobres-Cavallari, Severino et al. 2009). Overall, we would suggest ribosomal proteins can be a good candidate to substitute the traditional HKGs as an internal control in real time PCR assays.

In addition to 12 HKGs, we also investigated the expression of one biomarker gene in abalone not only to ensure the impact of our experimental conditions on abalone, but also to assess the influences by using inappropriate HKGs as internal controls. In vertebrates like mammals and fish, vitellogenin (Vtg), the serum phospholipoglycoprotein precursor to egg yolk, has been proposed as ideal biomarker for estrogenic contaminations due to its remarkable elevated expression both at the level of transcription and translation in response to the exposure (Matozzo, Gagne et al. 2008). Unfortunately, however, the Vtgs of molluscs have been isolated and characterized in only a few bivalve species, but not in abalone. A similar predicament also happened to selection of a biomarker for TBT, that there is no definitive biomarker gene available other than some general stress proteins like CYP1A and GSTs. Consequently, we chose this CYP4 gene of abalone, which is highly identical to mammalian cytochrome P450 4B enzymes, as the biomarker gene of interest. As the major enzymes in phase I biotransformation, many cytochrome P450s, such as CYP1B1, CYP17 and CYP19, are mediated in biosynthetic and metabolic pathways of estradiol (Tsuchiya, Nakajima et al. 2005). Although the enzymes in cytochrome P450s family 4 are typically related to the cholesterol metabolizing as fatty acid omega hydroxylases, they are also

known to play a prominent role in xenobiotics metabolism and thereby serve as biomarker for xenobiotics exposure (Okita and Okita 2001; Jorgensen, Rasmussen et al. 2005). The expression of abalone CYP4 was highly induced after 7-day waterborne exposure of TBT in gill but only slightly up-regulated in hepatopancreas, indicating that our challenge possibly caused acute oxidative stress in gill tissue that directly contacts with chemicals. On the other hand, if our challenge could be a long term (> 30 days), TBT might be accumulated in hepatopancreas and alter the levels of circulating androgens that would induce the expression of CYP4 as well (Imaoka, Yamazoe et al. 1992). Compared to TBT, the exposure of E2 showed a weak effect on CYP4 expression in both tissues (≤0.5 fold-change). This observation is consistent with the reports in human that mRNA level and omega-hydroxylase activity of CYP4 genes were unaffected by E2 treatment (McCabe, Roman et al. 2001; Savas, Hsu et al. 2005). It is noteworthy that the normalization procedure of CYP4 should be paid more attention since its low induction levels are easily altered by variations in internal control. As shown in Fig.30, through normalization against the unstable HKGs including 18s rRNA, BGLU and CY, we could possibly obtain erroneous results of biomarker quantification and thus mislead the monitoring and environment risk assessment of EDCs.

In conclusion, we analyzed the expression-stability of 12 housekeeping genes in abalone under the conditions of TBT and E2 challenges. Our validation results indicated that ELF, SDHA and especially RPL5 might be suitable internal controls to normalize expression data of EDCs biomarkers in abalones. However, we would strongly recommend further examinations for the application of these genes or their orthologs as internal controls in other

mollusc species and/or with additional chemical challenges of known EDCs in addition to TBT and E2.

References

Ait-Aissa, S., J. Porcher, et al. (2000). "Activation of the hsp70 promoter by environmental inorganic and organic chemicals: relationships with cytotoxicity and lipophilicity." Toxicology **145**(2-3): 147-157.

Aoki, T., H. C. Wang, et al. (2010). "Microarray Analyses of Shrimp Immune Responses." Mar Biotechnol (NY).

Araya, M. T., A. Siah, et al. (2008). "Selection and evaluation of housekeeping genes for haemocytes of soft-shell clams (Mya arenaria) challenged with Vibrio splendidus." J Invertebr Pathol **99**(3): 326-331.

Arrigo, A. P. (2000). "sHsp as novel regulators of programmed cell death and tumorigenicity." Pathol Biol (Paris) **48**(3): 280-288.

Arrigo, A. P., C. Paul, et al. (2002). "Small stress proteins: novel negative modulators of apoptosis induced independently of reactive oxygen species." Prog Mol Subcell Biol **28**: 185-204.

Assuncao, M. G., K. A. Miller, et al. (2007). "Cytochrome P450 1A expression and organochlorine contaminants in harbour seals (Phoca vitulina): evaluating a biopsy approach." Comp Biochem Physiol C Toxicol Pharmacol **145**(2): 256-264.

Banni, M., F. Dondero, et al. (2007). "Assessment of heavy metal contamination using real-time PCR analysis of mussel metallothionein mt10 and mt20 expression: a validation along the Tunisian coast." Biomarkers **12**(4): 369-383.

Barsalobres-Cavallari, C. F., F. E. Severino, et al. (2009). "Identification of suitable internal control genes for expression studies in Coffea arabica under different experimental conditions." BMC Mol Biol **10**: 1.

Beck, F. X., W. Neuhofer, et al. (2000). "Molecular chaperones in the kidney: distribution, putative roles, and regulation." Am J Physiol Renal Physiol **279**(2): F203-215.

Bernanke, J. and H. R. Kohler (2009). "The impact of environmental chemicals on wildlife vertebrates." Rev Environ Contam Toxicol **198**: 1-47.

Bertin, G. and D. Averbeck (2006). "Cadmium: cellular effects, modifications of biomolecules, modulation of DNA repair and genotoxic consequences (a review)." Biochimie **88**(11): 1549-1559.

Bieker, J. J. (2001). "Kruppel-like factors: three fingers in many pies." J Biol Chem **276**(37): 34355-34358.

Björne Olsson, Brian P. Bradley, et al. (2004). "Physiological and proteomic responses in Mytilus edulis exposed to PCBs and PAHs extracted from Baltic Sea sediments " Hydrobiologia **514**(1-3): 15-27.

Brinkhof, B., B. Spee, et al. (2006). "Development and evaluation of canine reference genes for accurate quantification of gene expression." Anal Biochem **356**(1): 36-43.

Brooks, S., B. Lyons, et al. (2009). "Biomarker responses in mussels, an integrated approach to biological effects measurements." J Toxicol Environ Health A **72**(3-4): 196-208.

Buckley, B. A., A. Y. Gracey, et al. (2006). "The cellular response to heat stress in the goby Gillichthys mirabilis: a cDNA microarray and protein-level analysis." J Exp Biol **209**(Pt 14): 2660-2677.

Burton, V., H. K. Mitchell, et al. (1988). "Heat shock protection against cold stress of Drosophila melanogaster." Mol Cell Biol **8**(8): 3550-3552.

Canesi, L., C. Ciacci, et al. (2003). "Effects of PCB congeners on the immune function of Mytilus hemocytes: alterations of tyrosine kinase-mediated cell signaling." Aquat Toxicol **63**(3): 293-306.

Chen, B., T. Kayukawa, et al. (2005). "The expression of the HSP90 gene in response to winter and summer diapauses and thermal-stress in the onion maggot, Delia antiqua." Insect Mol Biol **14**(6): 697-702.

Chen, C., Y. Zhong, et al. (2000). "The effect of salinity on food intake, growth and survival of Haliotis diversicolor supertexta." JOURNAL OF FISHERIES OF CHINA **01**.

Chen, J. and R. Ruan (2009). "Identifying stable reference genes for evaluation of antioxidative enzyme gene expression in auditory cortex and cochlea of young and old Fischer 344 rats." Acta Otolaryngol **129**(6): 644-650.

Chen, X., D. S. Sullivan, et al. (1994). "Two yeast genes with similarity to TCP-1 are required for microtubule and actin function in vivo." Proc Natl Acad Sci U S A **91**(19): 9111-9115.

Chen, Y., R. Sun, et al. (2001). "Nuclear translocation of PDCD5 (TFAR19): an early signal for apoptosis?" FEBS Lett **509**(2): 191-196.

Cheng, P., X. Liu, et al. (2007). "Cloning and expression analysis of a HSP70 gene from Pacific abalone (Haliotis discus hannai)." Fish Shellfish Immunol **22**(1-2): 77-87.

Choi, Y. K., P. G. Jo, et al. (2008). "Cadmium affects the expression of heat shock protein 90 and metallothionein mRNA in the Pacific oyster, Crassostrea gigas." Comp Biochem Physiol C Toxicol Pharmacol **147**(3): 286-292.

Chrestensen, C. A., D. W. Starke, et al. (2000). "Acute cadmium exposure inactivates thioltransferase (Glutaredoxin), inhibits intracellular reduction of protein-glutathionyl-mixed disulfides, and initiates apoptosis." J Biol Chem **275**(34): 26556-26565.

Costa, L. G. (1998). "Signal transduction in environmental neurotoxicity." Annu Rev Pharmacol Toxicol **38**: 21-43.

Crack, J. A., M. Mansour, et al. (2002). "Functional analysis of a small heat shock/alpha-crystallin protein from Artemia franciscana. Oligomerization and thermotolerance." Eur J Biochem **269**(3): 933-942.

Czarnecka, E., J. L. Key, et al. (1989). "Regulatory domains of the Gmhsp17.5-E heat shock promoter of soybean." Mol Cell Biol **9**(8): 3457-3463.

Dafny-Yelin, M., T. Tzfira, et al. (2008). "Non-redundant functions of sHSP-CIs in acquired thermotolerance and their role in early seed development in Arabidopsis." Plant Mol Biol **67**(4): 363-373.

Dakeshita, S., T. Kawai, et al. (2009). "Gene expression signatures in peripheral blood cells from Japanese women exposed to environmental cadmium." Toxicology **257**(1-2): 25-32.

Davidson, S. M., M. T. Loones, et al. (2002). "The developmental expression of small HSP."

Prog Mol Subcell Biol **28**: 103-128.

de Boer, M. E., T. E. de Boer, et al. (2009). "Reference genes for QRT-PCR tested under various stress conditions in Folsomia candida and Orchesella cincta (Insecta, Collembola)." BMC Mol Biol **10**: 54.

Derocher, A. E., K. W. Helm, et al. (1991). "Expression of a Conserved Family of Cytoplasmic Low Molecular Weight Heat Shock Proteins during Heat Stress and Recovery." Plant Physiol **96**(4): 1038-1047.

Duffy, J. E., E. Carlson, et al. (2002). "Impact of polychlorinated biphenyls (PCBs) on the immune function of fish: age as a variable in determining adverse outcome." Mar Environ Res **54**(3-5): 559-563.

Duft M, Schmitt C, et al. (2006). "Prosobranch snails as test organisms for the assessment of endocrine active chemicals—an overview and a guideline proposal for a reproduction test with the freshwater mudsnail Potamopyrgus antipodarum." Ecotoxicology **DOI: 10.1007/s10646-006-0106-0**

Dunlop, M. E. and E. E. Muggli (2000). "Small heat shock protein alteration provides a mechanism to reduce mesangial cell contractility in diabetes and oxidative stress." Kidney Int **57**(2): 464-475.

Erickson, G. R., D. L. Northrup, et al. (2003). "Hypo-osmotic stress induces calcium-dependent actin reorganization in articular chondrocytes." Osteoarthritis Cartilage **11**(3): 187-197.

Evans, T. G. and G. N. Somero (2008). "A microarray-based transcriptomic time-course of hyper- and hypo-osmotic stress signaling events in the euryhaline fish Gillichthys mirabilis: osmosensors to effectors." J Exp Biol **211**(Pt 22): 3636-3649.

Filby, A. L. and C. R. Tyler (2007). "Appropriate 'housekeeping' genes for use in expression profiling the effects of environmental estrogens in fish." BMC Mol Biol **8**: 10.

Ford, A. T., T. F. Fernandes, et al. (2004). "Endocrine disruption in a marine amphipod? Field observations of intersexuality and de-masculinisation." Mar Environ Res **58**(2-5): 169-173.

Fossi, M. C., S. Casini, et al. (2002). "Biomarkers for endocrine disruptors in three species of Mediterranean large pelagic fish." Mar Environ Res **54**(3-5): 667-671.

Galanis, A., A. Karapetsas, et al. (2009). "Metal-induced carcinogenesis, oxidative stress and hypoxia signalling." Mutat Res **674**(1-2): 31-35.

Goidin, D., A. Mamessier, et al. (2001). "Ribosomal 18S RNA prevails over glyceraldehyde-3-phosphate dehydrogenase and beta-actin genes as internal standard for quantitative comparison of mRNA levels in invasive and noninvasive human melanoma cell subpopulations." Anal Biochem **295**(1): 17-21.

Gong, Z., C. H. Dong, et al. (2005). "A DEAD box RNA helicase is essential for mRNA export and important for development and stress responses in Arabidopsis." Plant Cell **17**(1): 256-267.

Gorski, J. and D. Nugegoda (2006). "Sublethal toxicity of trace metals to larvae of the blacklip abalone, Haliotis rubra." Environ Toxicol Chem **25**(5): 1360-1367.

Gracey, A. Y., E. J. Fraser, et al. (2004). "Coping with cold: An integrative, multitissue analysis of the transcriptome of a poikilothermic vertebrate." Proc Natl Acad Sci U S A **101**(48): 16970-16975.

Gurley, W. B. and J. L. Key (1991). "Transcriptional regulation of the heat-shock response: a plant perspective." Biochemistry **30**(1): 1-12.

Gusev, N. B., N. V. Bogatcheva, et al. (2002). "Structure and properties of small heat shock proteins (sHsp) and their interaction with cytoskeleton proteins." Biochemistry (Mosc) **67**(5): 511-519.

Hall, C. M., S. M. Rhind, et al. (2009). "The potential for use of gastropod molluscs as bioindicators of endocrine disrupting compounds in the terrestrial environment." J Environ Monit **11**(3): 491-497.

Han, Y., D. Zhou, et al. (2005). "DNA microarray analysis of the heat- and cold-shock stimulons in Yersinia pestis." Microbes Infect **7**(3): 335-348.

Han, Y., D. Zhou, et al. (2005). "DNA microarray analysis of the heat- and cold-shock stimulons in Yersinia pestis." Microbes Infect **7**(3): 335-348.

Haslbeck, M., A. Kastenmuller, et al. (2008). "Structural dynamics of archaeal small heat shock proteins." J Mol Biol **378**(2): 362-374.

Heckmann, L. H., R. Connon, et al. (2006). "Expression of target and reference genes in Daphnia magna exposed to ibuprofen." BMC Genomics **7**: 175.

Helani Munasinghe, Hyun Sil Kang, et al. (2006). "Analysis of Digestive Gland Expressed Sequence Tag Library from the Disk Abalone, Haliotis discus discus." Journal of the World Aquaculture Society **37**(1).

Hobday, A. J. and M. J. Tegner "Status review of white abalone Haliotis sorensoni throughout its range in California and Mexico." NOAA Technical Memorandum NMFS.

Holmgren, A., C. Johansson, et al. (2005). "Thiol redox control via thioredoxin and glutaredoxin systems." Biochem Soc Trans **33**(Pt 6): 1375-1377.

Horiguchi, T., M. Kojima, et al. (2002). "Tributyltin and triphenyltin induce spermatogenesis in ovary of female abalone, Haliotis gigantea." Mar Environ Res **54**(3-5): 679-684.

Horiguchi, T., N. Takiguchi, et al. (2000). "Ovo-testis and disturbed reproductive cycle in the giant abalone, Haliotis madaka: possible linkage with organotin contamination in a site of population decline." Mar Environ Res **50**(1-5): 223-229.

Horikawa, M. and K. Sakamoto (2009). "Fatty-acid metabolism is involved in stress-resistance mechanisms of Caenorhabditis elegans." Biochem Biophys Res Commun **390**(4): 1402-1407.

Hosoi, M., K. Takeuchi, et al. (2005). "Expression and functional analysis of mussel taurine transporter, as a key molecule in cellular osmoconforming." J Exp Biol **208**(Pt 22): 4203-4211.

Huang, L., J. N. Min, et al. (2007). "Insights into function and regulation of small heat shock protein 25 (HSPB1) in a mouse model with targeted gene disruption." Genesis **45**(8): 487-501.

Ikeda, H., N. Taira, et al. (2010). "The estrogen receptor influences microtubule-associated protein tau (MAPT) expression and the selective estrogen receptor inhibitor

fulvestrant downregulates MAPT and increases the sensitivity to taxane in breast cancer cells." Breast Cancer Res **12**(3): R43.

Imaoka, S., Y. Yamazoe, et al. (1992). "Hormonal regulation of rat renal cytochrome P450s by androgen and the pituitary." Arch Biochem Biophys **299**(1): 179-184.

Infante, C., M. P. Matsuoka, et al. (2008). "Selection of housekeeping genes for gene expression studies in larvae from flatfish using real-time PCR." BMC Mol Biol **9**: 28.

Janovick-Guretzky, N. A., H. M. Dann, et al. (2007). "Housekeeping gene expression in bovine liver is affected by physiological state, feed intake, and dietary treatment." J Dairy Sci **90**(5): 2246-2252.

Ji-hao Sun, Jian-ye Chen, et al. (2010). "Expression of sHSP genes as affected by heat shock and cold acclimation in relation to chilling tolerance in plum fruit." Postharvest Biology and Technology **55**(2).

Jia, X., Z. Zhang, et al. (2009). "Expressed sequence tag analysis for identification and characterization of genes related to Tributyltin (TBT) exposure in the abalone Haliotis diversicolor supertexta." Comp Biochem Physiol Part D Genomics Proteomics.

Jiang, C., J. Xu, et al. (2009). "A cytosolic class I small heat shock protein, RcHSP17.8, of Rosa chinensis confers resistance to a variety of stresses to Escherichia coli, yeast and Arabidopsis thaliana." Plant Cell Environ **32**(8): 1046-1059.

Jorgensen, A., L. J. Rasmussen, et al. (2005). "Characterisation of two novel CYP4 genes from the marine polychaete Nereis virens and their involvement in pyrene hydroxylase activity." Biochem Biophys Res Commun **336**(3): 890-897.

Jungmann, J., H. A. Reins, et al. (1993). "Resistance to cadmium mediated by ubiquitin-dependent proteolysis." Nature **361**(6410): 369-371.

Kappe, G., E. Franck, et al. (2003). "The human genome encodes 10 alpha-crystallin-related small heat shock proteins: HspB1-10." Cell Stress Chaperones **8**(1): 53-61.

Kavlock, R. J., G. P. Daston, et al. (1996). "Research needs for the risk assessment of health

and environmental effects of endocrine disruptors: a report of the U.S. EPA-sponsored workshop." Environ Health Perspect **104 Suppl 4**: 715-740.

Kayukawa, T., B. Chen, et al. (2005). "Expression of mRNA for the t-complex polypeptide-1, a subunit of chaperonin CCT, is upregulated in association with increased cold hardiness in Delia antiqua." Cell Stress Chaperones **10**(3): 204-210.

Kim, J. H., H. U. Dahms, et al. (2010). "Expression profiles of seven glutathione S-transferase (GST) genes in cadmium-exposed river pufferfish (Takifugu obscurus)." Comp Biochem Physiol C Toxicol Pharmacol **151**(1): 99-106.

Kim, K. K., R. Kim, et al. (1998). "Crystal structure of a small heat-shock protein." Nature **394**(6693): 595-599.

Kim, W. S., N. Khunajakr, et al. (1998). "Conservation of the major cold shock protein in lactic acid bacteria." Curr Microbiol **37**(5): 333-336.

Kohler, H., C. Knodler, et al. (1999). "Divergent kinetics of hsp70 induction in Oniscus asellus (Isopoda) in response to four environmentally relevant organic chemicals (B[a]P, PCB52, gamma-HCH, PCP): suitability and limits of a biomarker." Arch Environ Contam Toxicol **36**(2): 179-185.

Koizumi, S. and H. Yamada (2003). "DNA microarray analysis of altered gene expression in cadmium-exposed human cells." J Occup Health **45**(6): 331-334.

Kostenko, S. and U. Moens (2009). "Heat shock protein 27 phosphorylation: kinases, phosphatases, functions and pathology." Cell Mol Life Sci **66**(20): 3289-3307.

Krishna, P., M. Sacco, et al. (1995). "Cold-Induced Accumulation of hsp90 Transcripts in Brassica napus." Plant Physiol **107**(3): 915-923.

Kultz, D. (2005). "Molecular and evolutionary basis of the cellular stress response." Annu Rev Physiol **67**: 225-257.

Lacoste, A., S. K. Malham, et al. (2001). "Evidence for a form of adrenergic response to stress in the mollusc Crassostrea gigas." J Exp Biol **204**(Pt 7): 1247-1255.

Laios, E., I. M. Rebeyka, et al. (1997). "Characterization of cold-induced heat shock protein expression in neonatal rat cardiomyocytes." Mol Cell Biochem **173**(1-2): 153-159.

Landry, J., P. Chretien, et al. (1989). "Heat shock resistance conferred by expression of the human HSP27 gene in rodent cells." J Cell Biol **109**(1): 7-15.

Laszlo, A. (1992). "The effects of hyperthermia on mammalian cell structure and function." Cell Prolif **25**(2): 59-87.

Lavoie, J. N., E. Hickey, et al. (1993). "Modulation of actin microfilament dynamics and fluid phase pinocytosis by phosphorylation of heat shock protein 27." J Biol Chem **268**(32): 24210-24214.

Lee, Y. M., K. W. Lee, et al. (2007). "Sequence, biochemical characteristics and expression of a novel Sigma-class of glutathione S-transferase from the intertidal copepod, Tigriopus japonicus with a possible role in antioxidant defense." Chemosphere **69**(6): 893-902.

Li, C., L. Wang, et al. (2010). "Identification of two small heat shock proteins with different response profile to cadmium and pathogen stresses in Venerupis philippinarum." Cell Stress Chaperones **15**(6): 897-904.

Li, X., L. Song, et al. (1996). "Cholinergic stimulation of AP-1 and NF kappa B transcription factors is differentially sensitive to oxidative stress in SH-SY5Y neuroblastoma: relationship to phosphoinositide hydrolysis." J Neurosci **16**(19): 5914-5922.

Liu, G. Y., Y. C. Hung, et al. (2005). "Ornithine decarboxylase prevents tumor necrosis factor alpha-induced apoptosis by decreasing intracellular reactive oxygen species." Apoptosis **10**(3): 569-581.

Liu, H. G., Y. Wang, et al. (2006). "Tributyltin induces DNA damage as well as oxidative damage in rats." Environ Toxicol **21**(2): 166-171.

Liu, H. T., B. Li, et al. (2003). "Calmodulin is involved in heat shock signal transduction in wheat." Plant Physiol **132**(3): 1186-1195.

Livak, K. J. and T. D. Schmittgen (2001). "Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method." Methods **25**(4): 402-408.

Lorenz, S., M. Francese, et al. (2001). "Heavy metals affect the circulating haemocyte

number in the shrimp Palaemon elegans." Fish Shellfish Immunol **11**(6): 459-472.

MacRae, T. H. (2000). "Structure and function of small heat shock/alpha-crystallin proteins: established concepts and emerging ideas." Cell Mol Life Sci **57**(6): 899-913.

Maradonna, F. and O. Carnevali (2007). "Vitellogenin, zona radiata protein, cathepsin D and heat shock protein 70 as biomarkers of exposure to xenobiotics." Biomarkers **12**(3): 240-255.

Massaro, E. J., R. M. Zucker, et al. (1989). "Fixation of the plasma membrane/cytoplasm complex: a mechanism of toxic interaction of tributyltin with the cell." Biol Trace Elem Res **21**: 305-312.

Matozzo, V., F. Gagne, et al. (2008). "Vitellogenin as a biomarker of exposure to estrogenic compounds in aquatic invertebrates: a review." Environ Int **34**(4): 531-545.

Matthiessen, P., Y. Allen, et al. (2002). "The impact of oestrogenic and androgenic contamination on marine organisms in the United Kingdom--summary of the EDMAR programme. Endocrine Disruption in the Marine Environment." Mar Environ Res **54**(3-5): 645-649.

McCabe, T. J., L. J. Roman, et al. (2001). "Induction of rabbit lung CYP4A4 prostaglandin omega-hydroxylase by various steroid hormones." Arch Biochem Biophys **393**(1): 78-86.

Menke, A., E. Guallar, et al. (2008). "The association of urinary cadmium with sex steroid hormone concentrations in a general population sample of US adult men." BMC Public Health **8**: 72.

Mills, L. J. and C. Chichester (2005). "Review of evidence: are endocrine-disrupting chemicals in the aquatic environment impacting fish populations?" Sci Total Environ **343**(1-3): 1-34.

Mounier, N. and A. P. Arrigo (2002). "Actin cytoskeleton and small heat shock proteins: how do they interact?" Cell Stress Chaperones **7**(2): 167-176.

Narberhaus, F. (2002). "Alpha-crystallin-type heat shock proteins: socializing minichaperones in the context of a multichaperone network." Microbiol Mol Biol

Rev **66**(1): 64-93; table of contents.

Neuhaus-Steinmetz, U. and L. Rensing (1997). "Heat shock protein induction by certain chemical stressors is correlated with their cytotoxicity, lipophilicity and protein-denaturing capacity." Toxicology **123**(3): 185-195.

Nitta, M., H. Okamura, et al. (1997). "Heat shock induces transient p53-dependent cell cycle arrest at G1/S." Oncogene **15**(5): 561-568.

Nykjaer, A., J. C. Fyfe, et al. (2001). "Cubilin dysfunction causes abnormal metabolism of the steroid hormone 25(OH) vitamin D(3)." Proc Natl Acad Sci U S A **98**(24): 13895-13900.

Okita, R. T. and J. R. Okita (2001). "Cytochrome P450 4A fatty acid omega hydroxylases." Curr Drug Metab **2**(3): 265-281.

Pacheco, A., C. Pereira, et al. (2009). "Small heat-shock protein Hsp12 contributes to yeast tolerance to freezing stress." Microbiology **155**(Pt 6): 2021-2028.

Papaiahgari, S., A. Yerrapureddy, et al. (2007). "Genetic and pharmacologic evidence links oxidative stress to ventilator-induced lung injury in mice." Am J Respir Crit Care Med **176**(12): 1222-1235.

Pappolla, M. A., M. Sos, et al. (1996). "The heat shock/oxidative stress connection. Relevance to Alzheimer disease." Mol Chem Neuropathol **28**(1-3): 21-34.

Park, E. M., Y. O. Kim, et al. (2008). "Cloning and expression analysis of a small HSP26 gene of Pacific abalone (Haliotis discus hannai)." J Environ Biol **29**(4): 577-580.

Park, H., I. Y. Ahn, et al. (2009). "Glutathione S-transferase as a biomarker in the Antarctic bivalve Laternula elliptica after exposure to the polychlorinated biphenyl mixture Aroclor 1254." Comp Biochem Physiol C Toxicol Pharmacol **150**(4): 528-536.

Patel, A. B., W. G. Robertson, et al. (2006). "Heat-shock protein 25 ameliorates calcium oxalate crystal-mediated oxidative stress in renal epithelial cells." BJU Int **98**(5): 1094-1099.

Pearce, A. K. and T. C. Humphrey (2001). "Integrating stress-response and cell-cycle checkpoint pathways." Trends Cell Biol **11**(10): 426-433.

Phean, O. P. S., P. Punteeranurak, et al. (2005). "Calcium signaling-mediated and differential induction of calmodulin gene expression by stress in Oryza sativa L." J Biochem Mol Biol **38**(4): 432-439.

Pierce, S. K., A. D. Politis, et al. (1989). "Evidence of calmodulin involvement in cell volume recovery following hypo-osmotic stress." Cell Calcium **10**(3): 159-169.

Ploszaj, T., T. Motyl, et al. (2000). "Inhibition of ornithine decarboxylase by alpha-difluoromethylornithine induces apoptosis of HC11 mouse mammary epithelial cells." Amino Acids **19**(2): 483-496.

Podrabsky, J. E. and G. N. Somero (2004). "Changes in gene expression associated with acclimation to constant temperatures and fluctuating daily temperatures in an annual killifish Austrofundulus limnaeus." J Exp Biol **207**(Pt 13): 2237-2254.

Porte, C., G. Janer, et al. (2006). "Endocrine disruptors in marine organisms: approaches and perspectives." Comp Biochem Physiol C Toxicol Pharmacol **143**(3): 303-315.

Puinean, A. M. and J. M. Rotchell (2006). "Vitellogenin gene expression as a biomarker of endocrine disruption in the invertebrate, Mytilus edulis." Mar Environ Res **62 Suppl**: S211-214.

Radonic, A., S. Thulke, et al. (2005). "Reference gene selection for quantitative real-time PCR analysis in virus infected cells: SARS corona virus, Yellow fever virus, Human Herpesvirus-6, Camelpox virus and Cytomegalovirus infections." Virol J **2**: 7.

Reiter, R. J., D. Acuna-Castroviejo, et al. (2001). "Free radical-mediated molecular damage. Mechanisms for the protective actions of melatonin in the central nervous system." Ann N Y Acad Sci **939**: 200-215.

Rhee, J. S., S. Raisuddin, et al. (2008). "A Mu-class glutathione S-transferase (GSTM) from the rock shell Thais clavigera." Comp Biochem Physiol C Toxicol Pharmacol **148**(3): 195-203.

Rhee, J. S., S. Raisuddin, et al. (2009). "Differential expression of metallothionein (MT) gene by trace metals and endocrine-disrupting chemicals in the hermaphroditic mangrove killifish, Kryptolebias marmoratus." Ecotoxicol Environ Saf **72**(1):

206-212.

Rollet, E., J. N. Lavoie, et al. (1992). "Expression of Drosophila's 27 kDa heat shock protein into rodent cells confers thermal resistance." Biochem Biophys Res Commun **185**(1): 116-120.

Ruan, W. and M. Lai (2007). "Actin, a reliable marker of internal control?" Clin Chim Acta **385**(1-2): 1-5.

Ruiz, J. L. and M. M. Souza (2008). "Osmotic stress and muscle tissue volume response of a freshwater bivalve." Comp Biochem Physiol A Mol Integr Physiol **151**(3): 399-406.

Saito, T., K. Sugimoto, et al. (2000). "Cloning and characterization of amphibian cold inducible RNA-binding protein." Comp Biochem Physiol B Biochem Mol Biol **125**(2): 237-245.

Sakamoto, T. and S. D. McCormick (2006). "Prolactin and growth hormone in fish osmoregulation." Gen Comp Endocrinol **147**(1): 24-30.

Savas, U., M. H. Hsu, et al. (2005). "Conditional regulation of the human CYP4X1 and CYP4Z1 genes." Arch Biochem Biophys **436**(2): 377-385.

Schroder, A. L., K. E. Pelch, et al. (2009). "Estrogen modulates expression of putative housekeeping genes in the mouse uterus." Endocrine **35**(2): 211-219.

Schwede, T., J. Kopp, et al. (2003). "SWISS-MODEL: An automated protein homology-modeling server." Nucleic Acids Res **31**(13): 3381-3385.

Selvey, S., E. W. Thompson, et al. (2001). "Beta-actin--an unsuitable internal control for RT-PCR." Mol Cell Probes **15**(5): 307-311.

Seo, J. S., T. J. Park, et al. (2006). "Small heat shock protein 20 gene (Hsp20) of the intertidal copepod Tigriopus japonicus as a possible biomarker for exposure to endocrine disruptors." Bull Environ Contam Toxicol **76**(4): 566-572.

Shearstone, J. R. and F. Baneyx (1999). "Biochemical characterization of the small heat shock protein IbpB from Escherichia coli." J Biol Chem **274**(15): 9937-9945.

Sherman, M. and G. Multhoff (2007). "Heat shock proteins in cancer." Ann N Y Acad Sci **1113**: 192-201.

Siah, A., C. Dohoo, et al. (2008). "Selecting a set of housekeeping genes for quantitative real-time PCR in normal and tetraploid haemocytes of soft-shell clams, Mya arenaria." Fish Shellfish Immunol **25**(3): 202-207.

Singer, C., S. Zimmermann, et al. (2005). "Induction of heat shock proteins (hsp70) in the zebra mussel (Dreissena polymorpha) following exposure to platinum group metals (platinum, palladium and rhodium): comparison with lead and cadmium exposures." Aquat Toxicol **75**(1): 65-75.

Sloan, S. and M. M. Gagnon (2004). "Intersex in Roe's abalone (Haliotis roei) in Western Australia." Mar Pollut Bull **49**(11-12): 1123-1126.

Sole, M., Y. Morcillo, et al. (2000). "Stress-protein response in tributyltin-exposed clams." Bull Environ Contam Toxicol **64**(6): 852-858.

Sonna, L. A., J. Fujita, et al. (2002). "Invited review: Effects of heat and cold stress on mammalian gene expression." J Appl Physiol **92**(4): 1725-1742.

Sonnenschein, C. and A. M. Soto (1998). "An updated review of environmental estrogen and androgen mimics and antagonists." J Steroid Biochem Mol Biol **65**(1-6): 143-150.

Spinsanti, G., C. Panti, et al. (2008). "Selection of reliable reference genes for qRT-PCR studies on cetacean fibroblast cultures exposed to OCs, PBDEs, and 17beta-estradiol." Aquat Toxicol **87**(3): 178-186.

Spinsanti, G., C. Panti, et al. (2006). "Selection of reference genes for quantitative RT-PCR studies in striped dolphin (Stenella coeruleoalba) skin biopsies." BMC Mol Biol **7**: 32.

Steinberg, C. E., S. R. Sturzenbaum, et al. (2008). "Genes and environment - striking the fine balance between sophisticated biomonitoring and true functional environmental genomics." Sci Total Environ **400**(1-3): 142-161.

Stennard, F. A., A. F. Holloway, et al. (1994). "Characterisation of six additional human metallothionein genes." Biochim Biophys Acta **1218**(3): 357-365.

Strickler, A. G., K. Famuditimi, et al. (2002). "Retinal homeobox genes and the role of cell proliferation in cavefish eye degeneration." Int J Dev Biol **46**(3): 285-294.

Stronach, B. E. and N. Perrimon (1999). "Stress signaling in Drosophila." Oncogene **18**(45): 6172-6182.

Sung, D. Y., E. Vierling, et al. (2001). "Comprehensive expression profile analysis of the Arabidopsis Hsp70 gene family." Plant Physiol **126**(2): 789-800.

Swan, T. M. and K. Watson (1999). "Stress tolerance in a yeast lipid mutant: membrane lipids influence tolerance to heat and ethanol independently of heat shock proteins and trehalose." Can J Microbiol **45**(6): 472-479.

Swindell, W. R., M. Huebner, et al. (2007). "Transcriptional profiling of Arabidopsis heat shock proteins and transcription factors reveals extensive overlap between heat and non-heat stress response pathways." BMC Genomics **8**: 125.

Thellin, O., W. Zorzi, et al. (1999). "Housekeeping genes as internal standards: use and limits." J Biotechnol **75**(2-3): 291-295.

Tiku, P. E., A. Y. Gracey, et al. (1996). "Cold-induced expression of delta 9-desaturase in carp by transcriptional and posttranslational mechanisms." Science **271**(5250): 815-818.

Tissieres, A., H. K. Mitchell, et al. (1974). "Protein synthesis in salivary glands of Drosophila melanogaster: relation to chromosome puffs." J Mol Biol **84**(3): 389-398.

Tsai-Turton, M., B. N. Nakamura, et al. (2007). "Induction of apoptosis by 9,10-dimethyl-1,2-benzanthracene in cultured preovulatory rat follicles is preceded by a rise in reactive oxygen species and is prevented by glutathione." Biol Reprod **77**(3): 442-451.

Tsuchiya, Y., M. Nakajima, et al. (2005). "Cytochrome P450-mediated metabolism of estrogens and its regulation in human." Cancer Lett **227**(2): 115-124.

Valdez Domingos, F. X., M. Azevedo, et al. (2007). "Multibiomarker assessment of three Brazilian estuaries using oysters as bioindicators." Environ Res **105**(3): 350-363.

Vandesompele, J., K. De Preter, et al. (2002). "Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes." Genome Biol **3**(7): RESEARCH0034.

Venier, P., C. De Pitta, et al. (2006). "Development of mussel mRNA profiling: Can gene expression trends reveal coastal water pollution?" Mutat Res **602**(1-2): 121-134.

Ventura, M., C. Canchaya, et al. (2007). "Molecular characterization of hsp20, encoding a small heat shock protein of bifidobacterium breve UCC2003." Appl Environ Microbiol **73**(14): 4695-4703.

Verma, A. S. and B. H. Shapiro (2006). "Sex-dependent expression of seven housekeeping genes in rat liver." J Gastroenterol Hepatol **21**(6): 1004-1008.

Vijayan, M. M., N. Aluru, et al. (2006). "Fasting augments PCB impact on liver metabolism in anadromous arctic char." Toxicol Sci **91**(2): 431-439.

Villeneuve, D. L., J. S. Khim, et al. (2001). "In vitro response of fish and mammalian cells to complex mixtures of polychlorinated naphthalenes, polychlorinated biphenyls, and polycyclic aromatic hydrocarbons." Aquat Toxicol **54**(1-2): 125-141.

Wan, Q., I. Whang, et al. (2008). "Molecular cloning and characterization of three sigma glutathione S-transferases from disk abalone (Haliotis discus discus)." Comp Biochem Physiol B Biochem Mol Biol **151**(3): 257-267.

Wan, Q., I. Whang, et al. (2009). "Novel omega glutathione S-transferases in disk abalone: Characterization and protective roles against environmental stress." Comp Biochem Physiol C Toxicol Pharmacol **150**(4): 558-568.

Wang, N., I. Whang, et al. (2010). "Molecular characterization and expression analysis of a heat shock protein 90 gene from disk abalone (Haliotis discus)." Mol Biol Rep.

Weber, M. H. and M. A. Marahiel (2003). "Bacterial cold shock responses." Sci Prog **86**(Pt 1-2): 9-75.

Wendelaar Bonga, S. E. (1997). "The stress response in fish." Physiol Rev **77**(3): 591-625.

Yocum, G. D., K. H. Joplin, et al. (1998). "Upregulation of a 23 kDa small heat shock protein transcript during pupal diapause in the flesh fly, Sarcophaga, crassipalpis." Insect Biochem Mol Biol **28**(9): 677-682.

Zapata, M., A. Tanguy, et al. (2009). "Transcriptomic response of Argopecten purpuratus post-larvae to copper exposure under experimental conditions." Gene **442**(1-2):

37-46.

Zhang, L., L. Wang, et al. (2010). "The involvement of HSP22 from bay scallop Argopecten irradians in response to heavy metal stress." Mol Biol Rep **37**(4): 1763-1771.

Zhang, W., D. E. Culley, et al. (2006). "DNA microarray analysis of anaerobic Methanosarcina barkeri reveals responses to heat shock and air exposure." J Ind Microbiol Biotechnol **33**(9): 784-790.

Zhou, J., Z. H. Cai, et al. (2010). "Are endocrine disruptors among the causes of the deterioration of aquatic biodiversity?" Integr Environ Assess Manag **6**(3): 492-498.

ACKNOWLEDGEMENT

First and foremost I would like to acknowledge the support and guidance of my supervisor Dr. Jehee Lee. You have provided me this precious opportunity to continue my graduate study in your laboratory in Korea. You were always willing to meet in order to share ideas, give guidance, and address any concerns I may have had.

I would like to thank all other professors in the Department of Marine Life Science including Prof. Choon-Bok Song, Prof. Moon-Soo Heo, Prof. In-Kyo Yeo, Prof. You-Jin Jeon, Prof. Ki-Wan Lee, Prof. Gi-Young Kim, Prof. Kwang-Sik Choi, Prof, Prof. Kyeong-Jun Lee, Prof. Joon-Bum Jeong, and Prof. Young-Dong, Lee for their teaching, helpful discussions, support of laboratory facilities and the guidance in my thesis writing. I am also thankful to Prof. Sung-Ju Jung of Chonnam National University who was one of thesis referees and purposely came to Jeju for attending my thesis defence.

I am grateful to all the past and present members of the Marine Molecular Genetics Lab that I have had the honor of working with, including Dr. Mahanama Dezoysa, Dr. Chamilani Nikapitiya, Dr. Chulhong Oh, Dr. Hyunsil Kang, Dr. Ning Wang, Dr. Ilson Whang, Young-deuk Lee, Yucheol Kim, Sukkyoung Lee Hyowon Kim and Uma.Thanks for lending a helping hand when it was needed and staying out of my way when it was definitely needed.

I also appreciate the support given by all my Chinese, Korean and other foreign friends in Jeju National University, especially for Wang Ning, Li Ying, Tian Ke, Chen Huan, Chamilani, Mahanama and Tushari. I would like to express my special appreciation to Mr Yucheol Kim, my best friend in Korea. He is always a loyal friend and a source of delight in my daily life in Jeju.

To my family, thanks for all your support, for believing in me and being there whenever I needed you, no matter what I needed you for.