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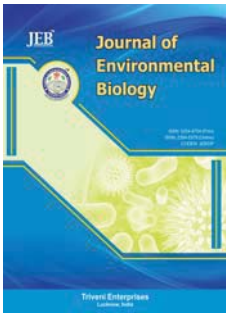

Gene expression profiling and expression analysis of freshwater shrimp (*Neocaridina denticulata denticulata*) using expressed sequence tags and short-term exposure to copper

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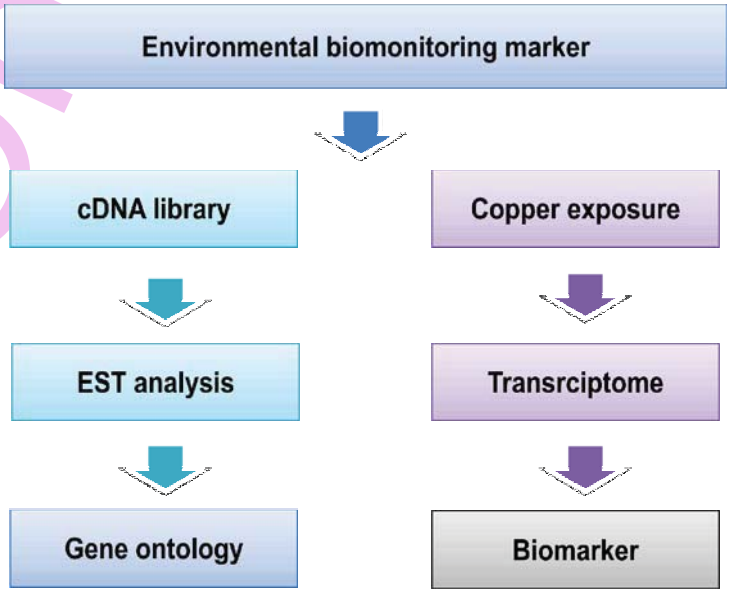
Abstract

Aim : The aim of the current study was to investigate environmental potential biomonitoring marker to exposure the copperfrom freshwater shrimp using expressed sequence tag (EST) analysis.

Methodology : The freshwater shrimp was collected from Lake Chang Pyung, South Korea and made cDNA libraries from whole body. To analyze expression patterns of putative biomarkersaccording to the individuals'exposure to copper in a time-and concentration-dependentmanners, Quantitative PCR (qPCR) was conducted.

Results : The expression levels of 9 potential biomarkers for heavy metal exposure including arginine kinase, acheron, cytochrome c oxidase subunit I, metallothionein, methylene tetrahydrofolatereductase, glutathione peroxidase, cathepsin L, cathepsin A and Cu/Zn superoxide dismutase genes were up-regulated 1-24 hr post-exposure to copper, in an exposure time- and/or dose-dependent manner. Furthermore, the discovery of genes that relate to heavy metal exposure and immune defense enables us to investigate the toxicity mechanisms and ecological effects of heavy metal pollution.

Interpretation : This study contributes to the identification of numerous EST clones that can be applied to further clarify the genetics and biomonitoring markers of freshwater shrimp.



Introduction

In many countries, freshwater shrimp cultivation is an economically important component of aquaculture industry. Freshwater shrimp has commercial value as fishing bait as food. In addition, inappropriate pond management or any event that reduce water quality can induce disease outbreaks among the shrimps and thus, decrease production (Chamberlain, 1997). Changes in certain environment factors, such as amount of dissolved oxygen, salinity and temperature, can affect the environment and increase the susceptibility of shrimp to disease (Lightner, 1996).

Other studies have reported that both heavy metals released from sediments and contamination of shrimp farms by pesticides and pollutants from agriculture or industrial activities may decrease the resistance of these shrimp to disease. Heavy metal pollution and eutrophication are common problems in freshwater ecosystems throughout the industrialized world.

Heavy metals ultimately enter aquatic systems and may cause short-term and long-term negative biological effects. Among these pollutants, copper is a widespread contaminant in coastal and estuarine ecosystems (Wright, 1986; Arun Kumar and Achyuthan, 2007; Munari and Mistri, 2007). Sources of copper in aquatic habitats include antifouling paint and algaecides, runoff of agricultural fertilizers, mine drainage, waste water from metal smelting, and atmospheric fallout from fossil fuel combustion and refuse incineration (IPCS, 1998). Bioavailability of copper is largely dependent on the concentration of free divalent cation but bioaccumulation of copper can also be influenced by salinity (Bidwell and Gorrie, 2006). Copper is an essential trace element that functions, as a cofactor for enzymes involved in oxidation/reduction reactions. Copper is also a component of hemocyanin, the molecule responsible for oxygen transport in the hemolymph of arthropods. However, copper can have toxic effects at concentrations above those that are essential (Bradl, 2005).

Mechanisms of copper toxicity include binding to proteins and DNA at sulfhydryl, carboxylate, and imidazole sites and catalyzing the production of reactive oxygen species. Copper can directly impair protein function, cause peroxidation of lipids, damage DNA and organelles, and ATP depletion (Stohs and Bagchi, 1995). The toxic effects of copper may reduce survival, induce behavioral alterations and delay development in aquatic invertebrates (Lodhi *et al*., 2006). Copper may also inhibit reproduction in aquatic invertebrates by delaying sexual maturation, reducing fertilization success (Reichelt-Brushett and Michalek-Wagner, 2005), decreasing fecundity (Garnacho *et al*., 2001), and reducing the proportion of reproductive females in a population.

In recent years, genomic approaches have been increasingly applied to study toxicology. Genomics helps in understanding the mechanisms that underlie the toxic effects of chemicals on the living tissues of various organisms. The approach may help to identify gene expression profiles that could serve as biomarkers of exposure to toxins (Calzolai *et al*., 2007). Until now, many investigators have used expressed sequence tags (ESTs) to screen potential biomarkers by analyzing expressed transcripts from bioindicator organisms of heavy metal pollution (Auslander *et al*., 2008; Wang *et al*., 2014; Cornu *et al*., 2017).

The freshwater shrimp *(Neocaridina denticulata denticulata)* inhabits lentic and lotic waters of the Indo-West Pacific, China, Japan, and Taiwan. Several characteristics make this species a good aquatic indicator for assessing environmental pollution: small size (2-3 cm), spontaneous interbreeding, and absence of metamorphosis stage during development (Mykles and Hui, 2015).

This is the first report for the profile of the freshwater shrimp transcriptome to investigate environmental potential biomonitoring marker to exposure the copper. We constructed a cDNA library using mRNA extracted from the whole bodies of freshwater shrimp for expressed sequence tag(EST) analysis. The results revealed valuable candidate genes that are potential biomonitoring markers.

Materials and Methods

Animals and sample preparation : A total of 10 freshwater shrimp were collected from the Lake Chang Pyung, Kunwigun, (an area with relatively clean-water), Gyung Sang Buk Do Province, South Korea, and were maintained in aerated natural lake water. In laboratory, the whole body of each specimen was rapidly frozen with liquid nitrogen and ground with a mortar and pestle.

cDNA library construction : Total RNA was isolated from the samples using Trizol reagent (Invitrogen, USA), and mRNA was purified from total RNA using an mRNA purification kit (Stratagene, USA). To construct a cDNA library, a directional λ ZAP cDNA synthesis/Gigapack III gold cloning kit (Stratagene, USA) was used. The reverse transcription of mRNA for first-strand cDNA synthesis was primed from the poly-A tail using an oligo-dT linker primer containing an *Xho*I cloning site. Following second-strand synthesis, *Eco*RI linkers were ligated to the 5′-termini. The fractionated cDNAs (above 500 bp) were then precipitated and ligated to the ZAP Express vector (pBK-CMV). Primary library was produced by *in vitro* packaging of ligation product using a ZAP Express cDNA Gigapack III Gold cloning kit (Stratagene, USA)(Aniello *et al*., 1996). The library contained 5.2×10^5 plaque forming units. Mass excision was performed and the cDNA inserts from the amplified λ ZAP library were rescued as pBK-CMV phagemids in XLOLR *Escherichia coli*. Average insert size was determined by Alpha Digi-Doc analysis and compared with a standard 100 bp Plus DNA ladder. The average insert size

for library was 1.5 kb.

Plasmid isolation and DNA sequencing : Independent colonies were picked randomly, inoculated into individual wells of 96-well deep well plates containing 1 ml of Luria Bertani media broth and incubated at 37°C for 18 hr. Plasmid DNAs was extracted using a Montage Plasmid Miniprep 96 Kit (Millipore, USA) according to the manufacturer's instructions. The cDNA inserts were sequenced once from the 5' end (T3 universal primer) of clones using a BigDye Terminator Sequencing Kit ver. 3.1 (Applied Biosystems, USA) and a 3730XL DNA Analyzer (Applied Biosystems, USA)(Martin *et al*., 2006).

Cleaning, annotation and clustering of ESTs : The ESTs obtained from Suppression subtractive hybridization clones were initially analyzed and annotated using Pipeline for EST Analysis Service (PESTAS), an automated EST analysis platform (unpublished, http://pestas.kribb.re.kr). In the present study, analysis was carried out in two steps. In step 1, *N. denticulata denticulata* EST trace data were base-called using the program Phred with a Phred score of 13 (Ewing and Green, 1998). The sequences were then processed with the cross_match (http://www.phrap.org), RepeatMasker (http://www.repeatmasker.org/) and SeqClean (http://compbio.dfci.harvard.edu/tgi/software/) programs to filter out vector, repetitive element and mitochondrial DNA sequences. Trimmed sequences more than 100 bp in length were clustered and assembled into unique sequences by TIGR Gene Indices clustering tools (TGICL) (Pertea *et al*., 2003) and CAP3 programs (Huang and Madan, 1999) using their default options. In step 2, to assign putative functions to EST, a BLASTX search of nonredundant (NR) protein database was performed at the National Center for Biotechnology Information (NCBI). Descriptions and sequence alignments were taken into account for the best hits with E-values below $1e^{-10}$ and at least 30% identity over 30 amino acids.

Gene ontology (GO) analysis : GO annotations were assigned using the program Blast2GO(cut-off value $<1e^{-10}$) (Conesa *et al*., 2005). BLASTX results were loaded into the program and the default settings were used to assign GO terms to all unique sequences. From these annotations, pie charts were made using 3rd level GO terms based on the biological process, molecular function and cellular component. In order to predict pathways in *N. denticulata denticulata*, we mined enzyme information was mined from the annotated results of the UniProtKB database (Schneider *et al*., 2009) that contained Enzyme Commission (EC)

Table 1 : Real-time polymerase chain reaction primers for the potential biomarkers of *N. denticulata denticulata*

Clone	Putative description	Primer name	5→3	bp
CL1	arginine kinase	AK1-F	CTTTGGTCTGTCCCCTGGTA	183
		AK1-R	TTGGGATCACCAGACATGAA	
CL4	acheron	Aen-F	ACGAAGTTGGAGAGGCAAGA	171
		Aen-R	GCCTTCTTCGCTACCATCAG	
CL7	cytochrome c oxidase subunit I	CytC-F	CGTTCCCTCGAATGAACAAT	204
		CytC-R	CGCCCAGAATCGAAGAAATA	
CL10	heat shock protein 70	HSP70-F	ACTCCCAGCGTCAAGCCACCAA	248
		HSP70-R	GTCAAAATCTTCACCGCCCAAATG	
CL63	glutathione synthetase	GSA-F	TTGAGTTTGGGGTTGGTGAGGTC	215
		GSA-R	TCCGGTAGGTGATCCAGCAGTTC	
CL132	metallothionein	metallo-F	CCGAAGGAGGCTGCAAGAAAAAT	233
		metallo-R	CTCGGGTGAAGGTAAACTGG	
CL155	methylene tetrahydrofolate reductase	MetHydroA-F	GGATTGTCGTGCGCTTGGTATTA	360
		MetHydroA-R	TCCTCTCCGCTTCTCTTGTGATTG	
Sgt10	glutathione peroxidase	GPA-F	GCCTGCTTCCACCACCCTGCTAAT	312
		GPA-R	AGTAAAGTTGGGCTGGAAATCGTT	
Sgt56	NADPH-dependent diflavinoxidoreductase 1	NADPH-F	AAAGAAGAATGGGAGCAGTTAGTG	274
		NADPH-R	CCAGGTCCTTCTTCATGTATTCTT	
Sgt108	cathepsin L	CatheL-F	TCGCCGAAGGAGATGAAAAT	286
		CatheL-R	AAGGCCGCAGGTTATGGTTG	
Sgt126	aquaporin, isoform B	Aquapo-F	ATTATTCCGGTGGTTATTTCA	199
		Aquapo-R	ATTCCTTATTCCTCCTTGGGTTTG	
Sgt145	cathepsin A	CathpA-F	AATGGTGGTCCGGGCTGTTCCT	204
		CathpA-R	TGCCGTTTCGTCATCGTTCGTG	
Sgt215	heat shock 70 protein form 2	HSP70iso-F	AAGCCAGCATTGAGATAGATTCCT	290
		HSP70iso-R	CACAGCAGCACCATAAGCAACAG	
Sgt382	Cu/Zn superoxide dismutase	CuZnSOD-F	TCGTTTTATCTGGCGGTGTTGAGG	222
		CuZnSOD-R	CGTGACGTTCCCGATGTTCTGGT	
Sgt190	β-actin	β-actin-F	CGAGTGGCCCCCGAGGAGTC	249
		β-actin-R	TCGCAGGATGGCATGAGGGAGAG	

numbers in the descriptions of matches with E-values less than or equal to $1e^{-10}$.

Effect of copper exposure : To analyze expression patterns of various genes organisms were exposed to heavy metal stress, three 3 freshwater shrimps were treated with varying concentrations of copper. In brief, the environmental effects of adding copper were tested in a time-dependent (0, 1, 3, 6, 9 or 24 hr) and dose-dependent (0, 1, 2, 5, 10 or 100 g l^{-1} for 24 hr) manner. The exposure concentrations of copper were chosen based on the results of previous studies (Ryu *et al*., 2007). The pH (8.0), salinity (28 PSU, practical salinity unit), temperature (20°C) and Do concentration (>5.6 mg l^{-1}, 60% saturation) were measured and maintained each day. The exposed freshwater shrimp were in a 12 hr light/12 hr darkness photoperiod at 20°C. After exposure period, total RNA was extracted from the whole body of and stored at -80° C prior to use.

Real-time RT-PCR analysis after copper exposure : After the shrimp underwent treatment with different concentrations of copper, gene expression pattern of putative biomarker for copper exposure in freshwater shrimp were verified by semi-quantitative RT-PCR. Specific primers for genes isolated by EST analysis were designed and used in PCR. In brief, total RNA from the shrimp with different concentrations of copper was isolated and the the pooled total RNA was used for cDNA synthesis. cDNA was synthesized from 1 g of total RNA using Superscript II reverse transcriptase and Oligo (dT) 20 primer (Invitrogen, USA) was used in RT-PCR. Real-time RT-PCR was performed using Fast Start SYBR Green Master Mix (Roche, USA) and 100 ng of synthesized cDNA in a 20-µl reaction volume. Quantitative PCR was conducted using an iQ5 Multicolor Real-Time PCR instrument (Bio-Rad, USA), and normalization, β-actin gene was used as internal control. Thermal cycling conditions were 1 cycle of 3 min at 95°C (initial denaturation) followed by 40 cycles of 10 sec at 95°C, 10 sec at 55°C, and 20 sec at 72°C. All data were obtained from triplicate experiments and were presented as fluorescence relative to β-actin gene expression. The fold change in relative gene expression was determined by $2^{-\Delta\Delta Ct}$ method (Giulietti *et al*., 2001). PCR primers for amplification of putative biomarker genes are listed in Table 1.

Statistical analysis : Data were expressed as means ± S.D. Significant differences between the observations of control and

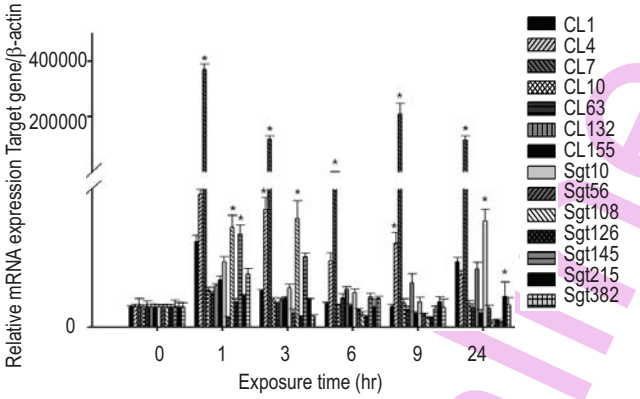


Fig. 1 : Relative mRNA expression of various genes when freshwater shrimp were exposed to copper in a time-dependent (0, 1, 3, 6, 9, and 24 hr) manner by real-time polymerase chain reaction analysis. Vertical error bars represent the standard error of the mean (S.E.M.) (N = 3). * = significant at $p < 0.05$

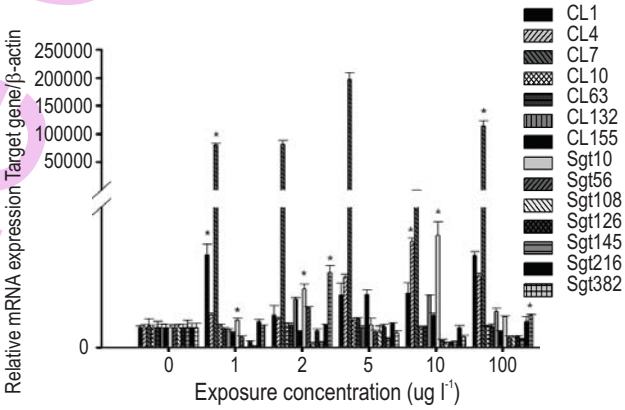


Fig. 2 : Relative mRNA expression of various genes when freshwater shrimp were exposed to copper in dose-dependent (0, 1, 2, 5, 10, and 100 µg l^{-1} for 24 hr) manner by real-time polymerase chain reaction analysis. Vertical error bars represent the standard error of the mean (S.E.M.) (N = 3). * = significant at $p < 0.05$

Table 2 : Summary of EST analysis of *N. denticulata denticulata*

Description	Number
Total number of clones sequenced	1,296
Total number of successful sequenced	1,247
Average EST length	695
EST clusters	
Cluster	185
Number of unique sequenced	603
Contigs	205
Singletons	398
Significant blast hit (NCBI NR database)[a]	319/1,247

[a] NCBI = National Center for Biotechnology Information, NR = non-redundant

Table 3 : The most abundant expressed sequence tags (ESTs) detected from the EST sequencing

Name of cluster	No. of contigs	No. of EST	Putative description	% of Total
CL1	1	49	arginine kinase 1 [*Macrobrachium rosenbergii*]	3.93
CL2	3	38	unknown	3.04
CL3	3	30	unknown	2.41
CL5	9	28	conserved hypothetical protein [*Arthroderma benhamiae* CBS 112371]	2.25
CL4	1	27	acheron [*Manduca sexta*]	2.17
CL7	1	21	cytochrome c oxidase subunit I [*Halocaridina rubra*]	1.68
CL6	3	18	TPA_exp: troponin T isoform 3 [Apismellifera]	1.44
CL8	1	18	unknown	1.44
CL9	2	17	putative ATP-dependent RNA helicase DDX17 [*Acromyrmex echinatior*]	1.36
CL11	1	14	ribosomal protein P1 [*Palaemonetes varians*]	1.12
CL10	1	13	heat shock protein 70 [*Marsuspenaeus japonicus*]	1.04
CL12	2	13	RNA polymerase I transcription factor subunit Spp27	1.04
CL13	1	12	unknown	0.96
CL14	3	12	nesprin-1 [*A. echinatior*]	0.96
CL15	1	11	unknown	0.88
CL16	1	10	putative Y box binding protein [*Pediculus humanus corporis*]	0.80
CL17	1	9	arrestin [*Limulus polyphemus*]	0.72
CL18	1	9	putative Hrp65 protein [*P. humanus corporis*]	0.72
CL19	1	9	unknown	0.72
CL20	1	8	hypothetical protein [*Scylla paramamosain*]	0.64
CL21	1	8	myosin light chain [*M. japonicus*]	0.64
CL22	1	8	RNA granule protein invertebrate [*Strongylocentrotus purpuratus]*	0.64
CL23	1	8	hypothetical protein EAI_04489 [*Harpegnathos saltator*]	0.64
CL25	1	8	translation initiation factor-3 [*Culex quinquefasciatus*]	0.64
CL24	1	7	calmodulin [*Procambarus clarkii*]	0.56
CL26	1	7	unknown	0.56
CL30	1	7	hypothetical protein TcasGA2_TC004915 [*Tribolium castaneum*]	0.56
CL27	1	6	ribosomal protein S2 [*Urechis caupo*]	0.48
CL28	1	6	unknown	0.48
CL29	1	6	unknown	0.48
CL34	1	6	unknown	0.48
CL35	1	6	unknown	0.48
CL36	1	6	unknown	0.48
CL39	1	6	ferritin [*Macrobrachium rosenbergii*]	0.48
CL31	1	5	unknown	0.40
CL32	2	5	alpha-tubulin [*Eriocheir sinensis*]	0.40
CL40	1	5	chromatin subfamily D member 1-like [*Bombus terrestris*]	0.40
CL41	1	5	serine/arginine-rich splicing factor 7-like [*A. mellifera*]	0.40
CL42	1	5	60S ribosomal protein L11 [*Ascaris suum*]	0.40
CL43	1	5	phosrestin-1 [*Culex quinquefasciatus*]	0.40
CL44	1	5	unknown	0.40
CL45	1	5	single VWC domain protein 1 [*Litopenaeus vannamei*]	0.40
CL46	1	5	putative Tob1 protein [*P. humanus corporis*]	0.40

treatment groups were analyzed using Student's paired t-test and one-way and/or multiple-comparison ANOVA followed by Tukey's test. Any difference showing $p< 0.05$ was considered significant. All statistical analyses were performed using SPSS® ver. 11.5 software (SPSS Inc., USA).

Results and Discussion

A total of 1,296 clones from the libraries of freshwater shrimp were sequenced, and 1,247 high-quality ESTs (96.2%) were identified as being with known genes from many organisms by BLAST searches (Table 2). These pre-processed ESTs ranged from 100 bp to 844 bp in length, with a mean length of 695±97 bp. The clustering analysis of 1,247 ESTs yielded 603 unique sequences, of which 205 and 398 were contigs and singletons, respectively, with an average length of 726 bp (Table 2).

The results of EST analysis of abundantly expressed genes from the freshwater shrimp *N. denticulata denticulata* is shown in Table 3, and the most abundantly expressed gene was

Table 4 : The 30 most represented biological process, molecular function, and cellular component gene ontology (GO) terms

	Level	GO ID	GO term	No. of seqs.
Biological process	2	GO:0065007	biological regulation	71
	3	GO:0043170	macromolecule metabolic process	74
	4	GO:0044260	cellular macromolecule metabolic process	65
	4	GO:0044249	cellular biosynthetic process	59
	2	GO:0008152	metabolic process	122
	1	GO:0008150	biological_process	161
	3	GO:0044237	cellular metabolic process	109
	3	GO:0044238	primary metabolic process	103
	2	GO:0009987	cellular process	139
	3	GO:0009058	biosynthetic process	60
Molecular function	5	GO:0032555	purine ribonucleotide binding	51
	4	GO:0032553	ribonucleotide binding	51
	2	GO:0003824	catalytic activity	112
	4	GO:0017076	purine nucleotide binding	52
	1	GO:0003674	molecular_function	223
	3	GO:0000166	nucleotide binding	68
	3	GO:0016787	hydrolase activity	49
	3	GO:0005515	protein binding	91
	3	GO:0003676	nucleic acid binding	50
	2	GO:0005488	binding	186
Cellular component	2	GO:0005623	cell	150
	6	GO:0043231	intracellular membrane-bounded organelle	68
	3	GO:0044464	cell part	150
	3	GO:0043227	membrane-bounded organelle	68
	4	GO:0044424	intracellular part	119
	5	GO:0005737	cytoplasm	86
	4	GO:0005622	intracellular	122
	5	GO:0043229	intracellular organelle	99
	1	GO:0005575	cellular_component	157
	2	GO:0043226	organelle	99

CL1 (3.9%) of all sequenced ESTs. BLASTX searches indicated that this transcript was most similar to anarginine kinase-1 protein from freshwater shrimp, *Macrobrachium rosenbergii* (179E-173). The gene encoding arginine kinase-1 (AK-1, CL1 contig) was of particular interest because this enzyme is thought to play an important role in the coupling of energy production and utilization and the immune response in shrimp (Arockiaraj *et al*., 2011). Recent studies have suggested that arginine kinase might play an important role in many biological events in crustaceans. For example, expression of arginine kinase was correlated closely with hypoxic stress in *M. japonicas* (Abe *et al*., 2007), exposure to lead in yabby, *Cherax destructor* (Morris *et al*., 2005), and acclimation to cadmium in crab, *Eriocheir sinensis* (Silvestre *et al*., 2006).

The unique sequences were compared to annotations by Gene Ontology analysis using the automated software Blast2GO (Conesa *et al*., 2005). GO terms were obtained for 412 unique sequences, including, 146 contigs and 266 singletons. The 30 most highly represented GO terms are shown in Table 4.

In fish, copper toxicity, is one of the most common heavy metal toxicity conditions and is primarily related to structural damage in gills. However, in crustaceans including shrimp, the physiological effect of copper toxicity is not as clearly understood (Wilson and Taylor, 1993). To study the potential biomarkers for heavy metal exposure and toxic effect of exposure to metal ions in freshwater in vertebrate, *N. denticulata denticulata* were exposed to copper of varying concentrations for varying lengths of time.

As shown in Fig. 1 and 2, the expression levels of 14 transcripts varied according to the individuals' exposure to copper in a time-and concentration-dependent manners. A total of 9 putative biomarker genes for heavy metal exposure, including CL1 (arginine kinase), CL4 (acheron), CL7 (cytochrome c oxidase subunit I), CL132 (metallothionein), CL155 (methylene tetrahydrofolate reductase), Sgt10 (glutathione peroxidase), Sgt108 (cathepsin L), Sgt145 (cathepsin A) and Sgt382(Cu/Zn superoxide dismutase) transcripts were significantly increased (over 2-fold) at periods ranging from 1-24 hr post exposure, in a time-and/or dose-dependent manner.

Hence, on the basis of observations it may be concluded that nine potential biomarkers including arginine kinase, acheron, cytochrome c oxidase subunit I, metallothionein, methylene

tetrahydrofolate reductase, glutathione peroxidase, cathepsin L, cathepsin A and Cu/Zn superoxide dismutase genes are potential biomonitoring markers of heavy metal pollution which have different toxicity mechanisms and ecological effects in nature.

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