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Molecular Characterization of Metallothionein Gene of the Korean Bitterling *Acheilognathus signifer* (Cyprinidae)

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ABSTRACT Genetic determinant for metallothionein (MT), a cysteine-rich protein playing essential roles in metal detoxification and homeostasis, was characterized in the Korean bitterling (*Acheilognathus signifer*, Cyprinidae), an endemic fish species. The full-length *A. signifer MT* (*AsMT*) cDNA (551 bp) is composed of a single open-reading frame (ORF) to encode a polypeptide of 60 amino acids containing 20 cysteine residues whose positions are conserved in most cypriniform MTs. At the genomic level, the *AsMT* (2,593 bp spanning the 5′-flanking region to the 3′-untranslated region) represented a conserved tripartite (three exons interrupted by two introns) structure with AT-rich introns. The upstream regulatory region (-1,914 bp from the ATG initiation codon) of *AsMT* displayed various sites and motifs for transcription factors involved in the metal-mediated regulation and stress/immune responses. The *AsMT* transcript was ubiquitously detected in various organs with variable expression levels, where the ovary and intestine showed the highest expression, while the heart and skeletal muscle represented the lowest level. During an exposure to copper (immersion in 0.5 μM Cu for 48 h), the levels of *AsMT* transcripts were significantly elevated in the liver (more than 3.5-fold), moderately in the gill, kidney, and spleen (ranging from 1.5- to 2.5-fold), and barely in the brain and intestine. Results of this study could form a useful basis to explore the metal-related stress physiology of this endangered fish species.

Key words : Gene structure and expression, Korean bitterling, *Acheilognathus signifer*, metallothionein

INTRODUCTION

The Korean bitterling, *Acheilognathus signifer* was first recorded by Berg (1907) as an endemic species to the Korean peninsula, with the habitat distributions in the Han, Imjin, Daedong and Abrok Rivers (Baek and Song, 2005a; Oh *et al.*, 2008). Like other bitterling species belonging to genus *Acheilognathus*, this species displayed a unique spawning behavior where the female bitterling lays the fertilized embryos inside the freshwater bivalve (e.g., into the gill cavity of the mussel usually belonging to Unionidae). On the other hand, the recipient mussel tries to attach its larvae (i.e., glochidium) on the gills or fins of the approaching female bitterling in order to make the larvae to be dispersed by the fish. This interesting communication between the two ecological members has been known as a good model for studying the host-parasite interaction in the aquatic ecosystem (Kim and Kim, 1989; Baek *et al.*, 2003; Baek and Song, 2005b). For this reason, the reproductive and developmental characteristics of the bitterling species including the host-specificity for spawning and reproductive behaviors have been important research subjects not only for better understanding their symbiotic relationship but also for providing a fundamental basis to develop a conservation strategy for this endemic species (Baek *et al.*, 2003; Baek and Song, 2005c; Baek and Song, 2006). In addition, the bitterlings species have been reported to represent a considerably flexible karyotype change during their evolution, as evidenced by the scattering of the interstitial teleomeric sequences in many heterochromatin regions, which may also be of importance for addressing the genetic structure or genetic drift of the local bitterling populations (Ueda *et al.*, 2001).

However, despite such a great scientific and/or ecological interest, natural habitats of this bitterling species have been destroyed mainly caused by anthropogenic

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and industrial activities. During last decade the local populations of *A. signifer* have been gradually decreased and now this endemic species is one of critically endangered freshwater species (Oh *et al.*, 2008). Understanding the stress physiology and adaptive capacity of a given endangered species is essential for developing an effective conservation plan, and hence the mining the information on the structures and functions of stress-relevant genes would be valuable to investigate their stress responses at both cellular and organismal levels (Snape *et al.*, 2004; Miracle and Ankley, 2005; Cho *et al.*, 2008).

Metallothionein (MT), a low molecular weight (6~7 kDa) and cysteine-rich protein, is commonly found in most animal species including teleosts. This metal-binding protein has been known to play critical roles in host protection, particularly with respect to the detoxification of excess heavy metals and homeostatic reservation of essential metal ions (Andrews, 2000; Thirumoorthy *et al.*, 2007). Furthermore, several studies on the vertebrate metallothioneins have suggested undoubtedly that MT should be a multiplayer involved in various cellular pathways including antioxidant function and innate immunity. For these reasons, a number of previous studies has proposed the use of MTs as versatile biomarkers to detect not only the health risks of the animals but also the environmental problems arisen from toxic pollutants (Coyle *et al.*, 2002; Haq *et al.*, 2003; Atif *et al.*, 2006).

In line with our long-term goal to provide fine molecular biomarkers in order to aid the conservation activities for *A. signifer*, the objective of this study was to isolate and characterize the genetic determinant of the metallothionein biomarker from this fish species.

MATERIALS AND METHODS

1. Experimental fish and laboratory rearing conditions

Fish specimens used in this study was a laboratory stock maintained in the Department of Marine Biotechnology, Soonchunhyang University. Fish were acclimated to the tank conditions (60 L of water recirculating tanks) for 2 weeks prior to experimental exposure to heavy metal (copper). Fish were fed with commercial diet pellet (40% crude protein; Woosung Feed Cor., Daejeon, Korea) twice per day on an *ad libitum* basis, and the daily water exchange rate was 30%. Water temperature was held at 24±1°C throughout the experiment and dissolved oxygen was adjusted to 5 to 6 ppm.

2. Nucleic acid preparation and cDNA library construction

Total RNA from various somatic tissues and gonads were performed by using the RNeasy Midi Kit (Qiagen, Hilden, Germany) according to the manufacturer's instruction, including DNase treatment step. The integrity and concentration of total RNA sample were checked by electrophoresis and spectrophotometry, respectively. On the other hand, genomic DNA was purified from the caudal fins using the conventional proteinase K/SDS digestion followed by organic extraction and alcohol precipitation.

A cDNA library was constructed with the total RNA prepared from the whole body of *A. signifer* fry. Total RNA was extracted as described above, and the poly(A)+ RNA fraction was purified using the mRNA Isolation Kit (Qiagen). Five μg of mRNA was used as template to synthesize the first strand of cDNA using the Lambda Zap cDNA synthesis Kit (Stratagene; La Jolla, CA, USA) according to the manufacturer's instruction. All the subsequent steps including the manipulation of cDNA ends, *in vitro* packaging and tittering were also followed in line with the guidelines by manufacturer. The final titer of the primary library was 1.3×10^6 pfu/mL and the excised library size was 6.1×10^6 cfu/mL.

3. Isolation of metallothionein cDNA

To isolate the *MT* cDNA from the *A. signifer* cDNA library, vectorette PCR was performed. Vector primers included in the PCR amplifications were SK and T7 primers (Stratagene) of which binding sites were complementary to the multiple cloning site (MCS) of the phagemid vector, pBluescript SK (−) that had been used for the construction of the *A. signifer* cDNA library. On the other hand, *MT*-specific primers that were paired with vector primers in the PCR were two degeneracy primers (CyMT-1F and CyMT-1R) designated based on the conserved region in *MT* sequences from cypriniform species. All the primers and thermal cycling conditions used in this study are provided in Table 1. A phagemid DNA prepared from the excised stock of the *A. signifer* cDNA library was used as a template for vectorette PCR. Each PCR-amplified band was gel-purified using the Gel Extraction Kit (Bioneer, Daejeon, Korea) and cloned into the pGEM T-Easy vector (Promega, Madison, WI, USA). Six-randomly-chosen recombinant clones per each PCR band were subjected to sequencing analysis at both directions. A continuous sequence of *A. signifer MT* cDNA containing the full-length open reading frame (ORF) was assembled in a contig using the sequence editing software, Sequencher (Gene Codes Cor., Ann Arbor, MI, USA), and the sequence was confirmed again by the RT-PCR isolation of full-length ORF from the whole body total RNA using AsMTc FW and AsMTc RV primers.

4. Isolation of metallothionein genomic gene and 5′-flanking upstream region

Based on the cDNA sequence, genomic gene fragment containing the complete coding region of the *A. signifer*

Table 1. Oligonucleotide primers used in this study

Primer	Sequence (5′ to 3′)	Thermal cycling conditions[1]	Purpose
CyMT-1F CyMT-1R	AARACTGGARCTTGCAACTG TTRGTRCACTTGCAGGTGGCA	30 cycles at 94°C for 30 s, 56°C for 30 s and 72°C for 30 s	Isolation of *AsMT* cDNA fragment using vectorette PCR
AsMTc FW AsMTc RV	CCAGTGAAACGATATTTCAAGGG GACAAGGTACACAAGAACCGTAC	30 cycles at 94°C for 30 s, 57°C for 30 s and 72°C for 30 s	Isolation of full-length *AsMT* open reading frame (cDNA)
AsMTg-1F AsMTg-1R	AGGGATTTTCCGACGCCTTAAAG GTAGACAAGGTACACAAGAACCG	30 cycles at 94°C for 45 s, 58°C for 45 s and 72°C for 1 min	Isolation of genomic *AsMT* sequence
AsMT-GW1 AsMT-GW2 AsMT-GW3 AsMT-GW4	TCTTGGCGCAATCGCAAGGATCCATT TTCCTCAGGAGTATCTTTAAGGCGTC AGGCTACTTTTGTTCTGCTAAACCCGA GTAGGAAGAAAGTCATACAGGTTTGGA	7 (the first PCR) or 5 (nest PCR) cycles at 94°C for 24 s and 68°C for 3 min, followed by 32 (the first PCR) or 20 (nest PCR) cycles at 94°C for 24 s and 72°C for 3 min, followed by a final elongation at 72°C for 7 min	Genome walking to 5′-upstream region of *AsMT*
ASMTp-1F ASMTp-1R	CTGTTCATGTGATTCTTGGAGAG CCTCAGGAGTATCTTTAAGGCGTC	30 cycles at 94°C for 45 s, 58°C for 45 s and 72°C for 1.5 min followed by 5 min at 72°C	Isolation of 5′-upstream region of *AsMT* for sequence confirmation
Fi18S rRNA RV	CAAGAATTTCACCTCTAGCGGC	–	Preparation of normalized control (18S rRNA) in RT reaction
qASMT-1F qASMT-1R	ACTCCTGAGGAAAATGGATC TACAGACGACCAGATTCACA	45 cycles at 94°C for 20 s, 58°C for 20 s and 72°C for 20 s	Real-time RT-PCR assay of *AsMT* transcripts
qAS18S-1F qAS18S-1R	TAACGGGGAATCAGGGTTCGAT CAAGAATTTCACCTCTAGCGGC	45 cycles at 94°C for 15 s, 58°C for 15 s and 72°C for 15 s	Real-time RT-PCR of 18S rRNA (normalization control)

[1]Each PCR amplification reaction was performed with an initial denaturation step at 94°C for 2 min.

MT was PCR-amplified using a pair of primers, AsMTg-1F and AsMTg-1R. PCR product was purified and TA-cloned as described above and eight-randomly-chosen clones were sequenced. From the genomic *MT* sequence, genome walking to 5′-upstream flanking region was performed using the GenomeWalker™ Universal Kit (BD Biosciences Clontech, Mountain View, CA, USA) according to the manufacturer's instructions. Four kinds of genome walking libraries were constructed with *Dra*I-, *Eco*RV-, *Pvu*II- or *Stu*I-digested genomic DNA. Two successive PCR amplifications were performed with two reverse primers, AsMT-GW1 and AsMT-GW2, which were respectively paired with AP1 and AP2 forward adaptor primers provided in the kit (Clontech). Based on the contiguous sequence from the first genome walking, the second round of walking was conducted with two primer pairs, AP1/AsMT-GW3 and AP2/AsMT-GW4, in order to obtain the distal part of the 5′-flanking upstream region. A continuous fragment of the 5′-upstream region was amplified again from the genomic DNA using the two PCR primers ASMTp-1F and ASMTp-1R, and its sequence was confirmed.

5. Sequence characterization

Sequence homology of *A. signifer* metallothionein with other vertebrate orthologs was searched against the NCBI GenBank (http://ncbi.nlm.nih.gov) using the BLASTx or BLASTp options. Multiple sequence alignment of deduced amino acid sequence with representative orthologs from Cypriniformes was performed using the ClustalW (http://align.genome.jp/). Predicted molecular weight (Mw) with the theoretical isoelectric point (pI) value was estimated using the ProtParam tool (http://www.expasy.org/tools/protparam.html). Potential transcription factor binding sites or motifs were predicted using the Transcription Element Search System (http://www.cbil.upenn.edu/cgi-bin/tess/tess?RQ=WELCOME) and TFSEARCH (http://www.cbrc.jp/research/db/TFSEARCH.html).

6. Assay of metallothionein transcripts

In order to examine the tissue distribution and basal expression level of *A. signifer MT* mRNA, real-time quantitative RT-PCR was performed. Total RNA was prepared with brain, eye, gill, heart, intestine, kidney, liver, skeletal muscle, ovary and testis pooled from six individuals (average body weights=14.5±3.5 g) as described above. Two μg of total RNA from each tissue was converted to cDNA using the Omniscript Reverse Transcription Kit (Qiagen) according to the instructions by the manufacturer. For preparing the normalizing control, a reverse oligonucleotide primer (Fi18S rRNA RV) of which binding site was conserved in the teleostean 18S rRNA genes was also included at 0.01 μM of the final concentration in the reverse transcription (RT) reaction. The RT product (cDNA) was diluted four-fold (for *MT*) and sixteen-fold (for 18S rRNA), and two μL of the diluted cDNA was subjected to real-time PCR amplifica-

tion. PCR primers were qASMT-1F and qASMT-1R in order to amplify 243 bp of the *A. signifer MT* segment. On the other hand, two oligonucleotide primers (qAS18S-1F/1R) were used to amplify 573 bp of *A. signifer* 18S rRNA segment. Real-time PCR amplification was conducted using the iCycler Real-Time Detection Module (Bio-Rad, Hercules, CA, USA) with a default setting. Based on the standard curves from *MT* and 18S rRNA genes, PCR efficiency for each gene was calculated. The relative expressions of *MT* transcripts across tissues were estimated based on the normalization against its own level of 18S rRNA expression using the comparative CT method (Schmittgen and Livak, 2008). Triplicate assays were carried out in an independent fashion.

In order to survey the candidate tissues showing the transcriptional induction of *MT* in responsive to metal exposure, an acute exposure experiment was conducted with copper (Cu). Healthy *A. signifer* individuals (n=8) were immersed in a 60 L-tank (60 L) containing 50 L of Cu-added water. Dose level was 0.5 μM and treatment duration was 48 h (Cho *et al.*, 2008). No feed was supplied during exposure. At the same time, a control group (n=8) was also prepared identically except the Cu. At 24 h post exposure, one-fifth volume of water was exchanged and Cu was refreshed accordingly. Forty-eight hr after immersion treatment, six selected tissues including brain, intestine, gill, kidney, liver and spleen were surgically removed from each fish belonging to either exposed or non-exposed group. Total RNA and cDNA samples were prepared as above and real-time RT-PCR analysis was performed in duplicates using the same primers used in the tissue distribution assay as described above. The *MT* transcript level in each sample was normalized against 18S rRNA and the *MT* gene expression in responsive to Cu exposure was expressed as fold change in exposed group relative to the control levels of non-exposed control using the comparative CT method (Schmittgen and Livak, 2008).

7. Statistics

Difference in the basal expression levels and the tissue-dependent inducibility of *MT* transcripts among tissues were tested with ANOVA followed by Duncan's multiple range tests using the SPSS software (ver. 10.1.3). Difference was considered to be significant when $P<0.05$.

RESULTS AND DISCUSSION

1. Characteristics of *AsMT* cDNA and genomic sequences

Complementary DNA of *A. signifer MT* (*AsMT*) contained 64 bp of 5′-untranslated region (UTR), 180 bp of single open reading frame (ORF) excluding the TGA stop codon and 289 bp of 3′-UTR excluding the 18 bp of poly (A)+ tail. The *AsMT* cDNA represented the putative polyadenylation signal (AATATA) at 21 bp prior to poly (A)+ tail, however, one additional, more consensus signal (AATAAA) was also found at 194 bp prior to the poly (A)+ tail, suggesting the possible processing of the *MT* transcripts with different 3′-UTR lengths (Chen *et al.*, 2004; Ren *et al.*, 2006). The *AsMT* ORF was deduced to encode 60 amino acids (Mw=5961.9 Da and pI=8.24), in which 20 cysteines (33.3%) were detected in a Cys-X-Cys or Cys-Cys form, which is one of typical features in most vertebrate MTs (Lin *et al.*, 2004; Cho *et al.*, 2005, 2008; Scudiero *et al.*, 2005). From the multiple sequence alignment with seventeen representative orthologs from ten cypriniform species [*A. signifer* (abbreviation AC in Fig. 1), *Cyprinus carpio* (CC), *Rutilus rutilus* (RR), *Gobio gobio* (GG), *Carassius cuvieri* (CCu), *Danio rerio* (DR), *Carassius auratus* (CA), *Barbatula barbatula* (BB), *Hemibarbus mylodon* (HM) and *Misgurnus mizolepis* (MM)], nineteen of twenty Cys residues were clearly conserved in all other cypriniform species examined (Fig. 1). Only the exception was substitution of the 9th Cys residue to glycine (Gly), which was found in the zebrafish MT-IIB. The *AsMT* exhibited a considerable sequence identity (ranging from 93 to 100%) with its orthologs at both α- and β-domains, in which the highest sequence homology at protein level was found with the common carp (*Cyprinus carpio*) MT-2.

PCR amplification isolated successfully the *AsMT* genomic sequence (696 bp) consisting of three exons interrupted by two introns (Fig. 1). The common AG/GT exon-intron splicing rule was well conserved in all the boundary regions. The coding (i.e., exons) sequences in the genomic copy was clearly matched with those in the cDNA counterpart. The tripartite structure was a common feature of the vertebrate *MT*s and the lengths of the three exons (25, 66 and 92 bp, respectively for exon I, II and III) were also quite similar with other fish *MT*s (Knapen *et al.*, 2005; Cho *et al.*, 2009). Further as similarly with many other fish *MT*s, the two introns of *AsMT* were AT-rich (66.4 and 69.7% for intron I and intron II, respectively) (Chen *et al.*, 2004; Lin *et al.*, 2004; Cho *et al.*, 2008).

2. Promoter characteristics of *AsMT*

From the genome walking to 5′-flanking region, a total of 1,914 bp sequence upstream from the translation start site (ATG codon) was characterized. This regulatory region represented various transcription factor (TF) binding sites and/or motifs as well as a canonical *TATA* box (TATAAA) (Fig. 2). Many of these TF binding sites were known to be associated with the metal-homeostasis and/or stress-relevant modulation. The *AsMT* promoter was proven to possess seven metal responsive element (*MRE*) copies in either forward (consensus sequence= TGCRCNC) or reverse (GNGYGCA) orientation. Of the seven *MRE* copes, three copies were found at proxi-

(A)

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cactaaaccagtgaaacgatatttcaagggattttccgacgccttaaagatactcctgaggaaaATGGATCCT  Exon I
                                                                 M  D  P

TGCGATTGCGCCAAGAgtgagtgttttagagcctgagtttacaattgttgacttttctattggaatcaagtta
C  D  C  A  K  T

gttacatgctgccggtaaattagcagtagtaattttcattgtgttgttgttgttgttgttgttgttgttttt

gtagCTGGAACTTGCAACTGTGGTGCCACCTGCAAGTGCACCAACTGCCAGTGTACAACATGCAAGAAGAgta  Exon II
     G  T  C  N  C  G  A  T  C  K  C  T  N  C  Q  C  T  T  C  K  K  S
                              ß-domain (30 aa; pI = 8.11)

cgtgaacttagtaccggtaatattttgtttaaatttctttctccaacgactaactttatttcttttgttacag

GTTGCTGTTCCTGCTGCCCCTCCGGCTGCAGCAAATGTGCCTCTGGCTGCGTCTGTAAGGGCAATTCCTGCGG  Exon III
 C  C  S  C  C  P  S  G  C  S  K  C  A  S  G  C  V  C  K  G  N  S  C  G
                              α-domain (30 aa; pI = 8.02)

CTCCAGCTGCTGTCAAtgaggtcaacgtgatgtcttgccacaacaatgtgaatctggtcgtctgtaccagtca
 S  S  C  C  Q  *

tcgttttgcattgcataaatgttaccattctatgaaataataatgacctccagttctctccaaatctttgac
tgtttaatgttgctgatttgtttaacaaaggctaaagagggaaactgcactgattttgtactgttataatcaa
gactataacatgtctctcctcttctaaattgaccttttgtacggttcttgtgtaccttgtctacaatatatt
aatgtcagcattt poly(A)+ tail
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(B)

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Species      (Accession number)   β-domain                    α-domain
                                  * *     * *   * *   * *   o   ** **   *   *   * *   *   **
AC  MT       (JF419524)           MDPCDCAKTGTCNCGATCKCTNCQCTTCKKSCCSCCPSGCSKCASGCVCKGNSCGSSCCQ   (% identity)
CC  MT-2     (AF249875)           ...A                                                            (100)
RR  MT       (P80593)             ...A                                T                           (98)
GG  MT-1     (AAX55635)           ...A                                P                           (98)
CCu MT-A     (AAN85819)           ...A   P                                                        (98)
DR  MTIIA    (ACG50794)           .  E A                                                          (98)
CA  MT2      (CAA65926)           ...A   A                                                        (98)
BB  MT       (CAA42036)           .  S                                                      D     (97)
HM  MT       (ABS87377)           ...A                                P                 D         (97)
CC  MT-1     (AAV52385)           ...A                         K      P                           (97)
GG  MT-2     (AAX55637)           ...A                                P                 D         (97)
DR  MT-1     (P52722)             .  E A   A                                                  T   (95)
MM  MTIA     (ACH90424)           .  S                   K                                  D     (95)
MM  MTIB     (ACH90423)           .  S                                                     T D     (95)
DR  MTIIB    (ACG50795)           .  E A   A                       G                          T   (93)
CCu MT-B     (AAN85820)           ...A   A                     K      P                 D         (93)
CA  MT1      (AAB32777)           .  E A   A                          F                 N         (93)
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Fig. 1. The nucleotide and deduced amino acid (in singlet code) sequences of *Acheilognathus signifer* metallothionein (A) and multiple amino acid sequence alignment along with representative orthologs from Cypriniformes (B). In (A), coding regions are indicated by bold uppercase letters, while non-coding sequences by lowercase letters. Stop codon (TGA) is noted by an asterisk. Two putative polyadenylation signals (AATAAA and AATATA) are bolded and underlined. In the multiple alignment (B), nineteen cysteine residues conserved in all the cypriniform species examined are indicated by asterisks on the top of the *A. signifer* MT. The Cys residue showing the replacement with Gly only found in the zebrafish MT-IIB is indicated by an open circle. Abbreviations for species can be referred to the main text (results and discussion).

mal region within −200 bp from the ATG start codon while four copies at distal region from −681 to −888 bp. The *MRE* is a *cis*-element to act as the binding target for metal-transcription factor-1 (MTF-1), a typical regulator responsible for the regulation of vertebrate *MT*s under not only basal but also metal-induced conditions (Laity and Andrews, 2007). Presence of multiple *MRE* copies is similar with previous observations on other fish *MT* promoters, in which the *MRE* copies are often detected as a cluster (Chen *et al.*, 2004; He *et al.*, 2007). Other

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-1914 ctgttcatgtgattcttggagagtctttgagactcaaactttcctccttgtcatgcattaagacaatatagacacgtcac
                                                                                  CREB
-1834 cttccagacagatttgtgtcatctttatttgattggaacttcttaaatatttacctgatggtgaaaatgagaattttcaa
                                                                                 HSF
-1754 tactttagctattttcttacatccactttctattttgtgaagctcaacgacctgttgatgcacatcagaactatattctt

-1674 tggttttattcattgtgatggatgattaagggaatttggcttttatgtttcctcatatttctactcctgtgaaacaagaa

-1594 ctcataactagataattgcatgttcctagtcaccctggtgtgctaaaattgtaaatattagcttaataaaaataaaagct
                GATA
-1514 tttatttcatgatgtcattttcctcccactttcaattatttcacttcaatgaaaggttagatttttgtgaatttttatga

-1434 atgaaacatcaaaaggataaactatgcagatttattttgtacagccatctttgatcatatttaacaggggtgccaataat

-1354 tctgtaactgtaacatgtaatggttaaagaccatctttaggtttctgtcgaatgtgaattaaagtccattctgatcacgc
                                                                                  XRE
-1274 accgcttatgaacctttatttaaagagattaaaaaaagaaaagtctgacataatttattcaccattcatttcgttccaa

-1194 acctgtatgactttcttcctactgtacttttggaacatttttcaaaatctcttcttcgggtttagcagaacaaaagtagc
                                                CEBP                          GR
-1114 ctacgtcagaagtttggagcaaatcatgacggaacattcatttctgagtgaaacagtttatatacagtggagttacttca
        CREB                            HSF
-1034 aatagaatataccactaacatgagagtttattcacagactcataacacttccacttgttgagtgaacattgtgttccaaa

 -954 tgtgttcgtttggttattttaatgcttgaaatcatgtatcttcacctccaaaattcaaagacactctgcactcgttacat
                                                                          MRE(+)
 -874 garttcagttgtaatgaagtgactagtcatggaaaaatactacaggcaacagcgagtgagtaaacgagtgcaaatcaatt
                                   NF-AT                          AP1     MRE(−)
 -794 cttctgtttactgtggttccgcccgcatcatcgccgagtgcagacagggggagcacgtgctgtcgcgcacgctcacgcac
                        Sp1            MRE(−)                 USF
 -714 gcacgcagtttgtttcacttcagtgccgtgtgcatagaactacaaactgttgacaaacaaatacagggggaaaaaactt
                                 MRE(−)                          HNF
 -634 taacaaagtcacaaatttcctctccaagaacatattctatgtcttaaacgtcatcgacgggtcaacatctgcataaatta
                                 GR                  CREB
 -554 aacctataacttatttagtctcaaatacgtgttcacattaatatttagtagagtcgaattactttgcctcactcacaaca

 -474 atctaaaagcattaaagagactcagagactgagcaagtatgtcaaagtatgttattttatagacctcgttgattctaaca

 -394 aactactttacgcaatcaaacaaggtctttccacctcccaaaacgacaataacaaacaaaaaacatcggattatataatc

 -314 acataaacaaacagctatcaatattcagtcaatggagtggaacggtagttatataattgttaaaataacacattgatgcg

 -234 acagggtgggaacgatgactcctgattgtttgatagtttgcacccagtgtcattaaggaatcaccgtgtgcagctgaagc
                                   GATA     MRE(+)                        MRE(−)
 -154 gggcgggctttgcacgcggttggtgtgccgtcagagttg[tataaa]agcagcgcgcgctcagttctccagtaccttcgaca
       Sp1        MRE(+)                      TATA box
                                                                                  +1
  -74 tcaactcattcactaaaccagtgaaacgatatttcaagggattttccgacgccttaaagatactcctgaggaaaATGGAT
                                              NF-kB
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Fig. 2. Bioinformatic analysis of the 5′-flanking region for the *Acheilognathus signifer* metallothionein gene. Consensus *TATA* sequence is boxed, and other putative transcription factor (TF) binding sites are underlined along with the indication of the relevant TFs. Forward and reverse orientations of seven metal responsive elements (*MRE*s) are noted by (+) and (−), respectively. Abbreviations for TFs or elements can be referred to the main text (results and discussion).

TF binding sites predicted in the *AsMT* gene include the motifs/elements for activating protein-1 (AP-1; consensus binding motif=TGASTMA), Sp1 (GC box=GGCGGG), GATA factor (WGATAR) and several hepatocyte nuclear factors (HNFs; TRTTKRYTY or RCAAAYA) (Haq *et al.*, 2003; Ren *et al.*, 2006). The *AsMT* promoter revealed a copy of xenobiotic response element (*XRE*=CACGCW), a potential target site recognized by the aryl hydrocarbon receptor (AhR), suggesting the *AsMT* gene could be modulated by xenobiotic inducers (Emi *et al.*, 1996). Various genes involved in the detoxification and antioxidant defense process have been known to be often activated by *XRE*-mediated regulation (Park and Rho, 2002; Kurose *et al.*, 2005). Presence of *XRE* in *MT* promoter has also been reported in a fish species (*Hemibarbus mylodon*) belonging to the same family Cyprinidae (Cho *et al.*, 2008). In addition, the upstream regulatory region of *AsMT* exhibited binding sites for TFs associated with immune-responses. They are nuclear factor kappa B (NF-κB/c-Rel; consensus sequence=GGGRNNYYCC), cAMP-response element binding protein (CREB; consensus sequence, TGACGY), upstream stimulatory factor (USF; CANNTG), nuclear factor for activated T-cells (NF-AT; WGGAAAA) and CCAAT-enhancer binding protein (C/EBP; TTDNGNAA), which suggests the *MT* could be involved in the innate immunity and/or antioxidant defense of the fish (Thirumoorthy *et al.*, 2007; Cho *et al.*, 2009). Finally, the presences of glucocorticoid response element (*GRE*-half site; AGAACA recognized by glucocorticoid receptor, GR) and heat shock element (*HSE*; GAAKKTTC by heat shock factor, HSF) in the *AsMT* promoter suggest the possible use of this *MT* as a general stress indicator, although numerous other biotic and abiotic factors affecting *MT* transcription should be examined prior to versatile use (Cho *et al.*, 2009).

3. Tissue distribution and basal expression levels of *AsMT* transcripts

Among the *A. signifer* tissues examined, *MT* transcripts were the most abundantly expressed in the ovary and intestine under basal conditions ($P<0.05$). The highest expression level in the two tissues was followed by the kidney, and then brain, eye and liver. Gill, spleen and testis showed only a moderate level of *MT* transcripts whereas the heart and skeletal muscle showed the least level of *MT* mRNA ($P<0.05$) (Fig. 3). It is not surprising that the ubiquitous distribution of *MT* transcripts in a wide array of tissue types, considering the multivalent and housekeeping roles of MT proteins in vertebrate tissues (Thirumoorthy *et al.*, 2007). The similar tissue distribution pattern of *MT* transcripts was observed in several fish species especially including *H. mylodon* in terms of the predominant expression of *MT* transcripts in ovary as well as the very low expression in muscular tissues (i.e., heart and skeletal muscles) (Cho *et al.*, 2008).

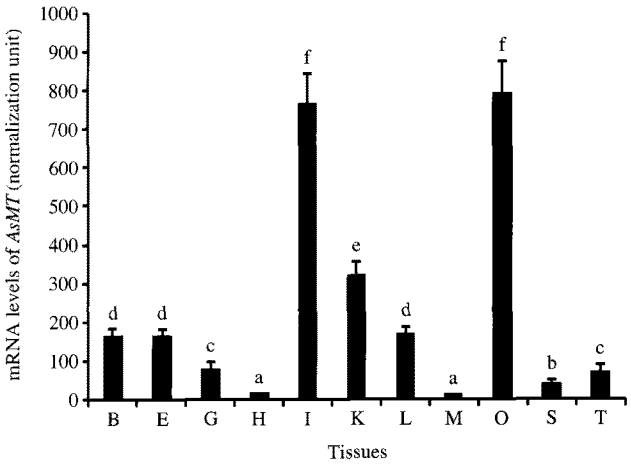

Fig. 3. Real-time RT-PCR assay to show the basal expression levels of *Acheilognathus signifer* metallothionein transcripts in various tissues including brain (B), eye (E), gill (G), heart (H), intestine (I), kidney (K), liver (L), skeletal muscle (M), ovary (O), spleen (S) and testis (T). The mRNA level in each tissue was normalized against its own 18S rRNA level. Based on the triplicate assays, means with different letters (a-f) are significantly different when assessed by ANOVA ($P<0.05$).

Richness of *MT* transcripts in the ovary suggests that *MT* mRNA could be maternally transmitted to early embryos, which is in accordance with the previous evidences that MT-mediated metal homeostasis should be an essential requirement for the normal embryogenesis and early ontogenesis in fish (Chen *et al.*, 2004).

4. Tissue-dependent modulation of *AsMT* during acute Cu-exposure

Transcriptional response of *AsMT* to the acute experimental exposure to Cu was examined in the six selected tissues, brain, gill, intestine, kidney, liver and spleen. The expression levels of *AsMT* mRNA in the six tissues from the non-exposed control group were quite similar with those observed earlier in the tissue distribution assay. However the Cu exposure stimulated the expression of *AsMT* transcripts in several tissues. Of the six tissues tested, brain and intestine didn't show any alteration of the *MT* transcripts in response to the Cu exposure, whereas all other four tissues revealed the significant induction of *MT* transcripts. The induced amount of *MT* transcripts (i.e., fold increase relative to non-exposed control) was the highest in liver (more than 3.5-fold) ($P<0.05$), followed by those in kidney and spleen (up to 2.5-fold). On the other hand, gill displayed only 1.5-fold increase (Fig. 4). Differential responsiveness of *MT* transcription depending upon tissue types to a given inducer has been reported in a number of previous studies on the fish *MT*s (Bi *et al.*, 2006; Woo *et al.*, 2006; Cho *et al.*, 2009). Although the detailed mechanism responsible for the tissue-dependent regulation of *MT* genes under stimulatory con-

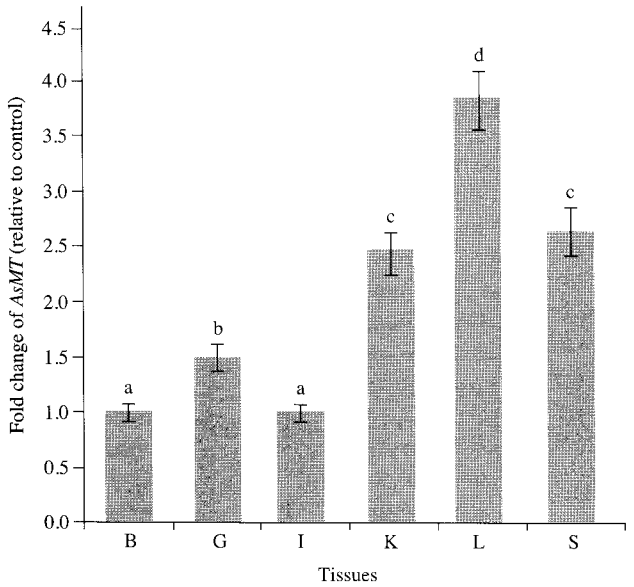

Fig. 4. Tissue-dependent modulation of *Acheilognathus signifer* metallothionein transcripts in response to acute immersion exposure to Cu (0.5 μM for 48 h). Fold change of *MT* mRNA levels in the Cu-exposed group relative to basal levels in non-expoed control group is expressed based on the real-time RT-PCR assay. Abbreviations for tissues can be referred in Fig. 3. Mean ± SDs with different letters (a-d) are significantly different based on the ANOVA ($P < 0.05$).

ditions has not been comprehensively understood yet, it is generally agreed that differential responses among tissues could be in relation with the differential flux rates of the metals depending on tissue types (i.e., different tissue burdens) as well as varying amounts of other metal-binding competitors in tissues (i.e., different levels of other metal-coordinating enzymes or ligands) (Köck *et al.*, 1995; Nam *et al.*, 2006). The most significant response of the hepatic *MT* found in this study is similar with other previous observations (Langston *et al.*, 2002; Lin *et al.*, 2004; Cho *et al.*, 2005, 2008), which is congruent with the general explanation that liver is the central organ responsible for detoxification function in vertebrates. The stimulation of *MT* transcripts in the kidney could be in agreement with that this organ is one of main sites to exhibit the rapid accumulation of heavy metals in fishes especially during the acute phase of exposures (Hollis *et al.*, 2001; Cho *et al.*, 2005). When considering the spleen is the primary organ responsible for the immune response in fishes, the upregulation of *MT* in the spleen suggests that the Cu overload might trigger the inflammation-mediated pathway in the exposed fish, since heavy meal like Cu is a potential agent to cause inflammation (Cerqueira and Fernandes, 2002). At least in part, this explanation is supported also by the prediction of diverse transcription factor binding motifs responsible for the inflammatory responses in the *AsMT* promoter. Gill is the chemophysical interface between internal and external environments in fish, and also the primary organ for the entry of most waterborne pollutants. However unexpectedly, the *A. signifer* gills showed only quite a low level of stimulation of the *MT* transcripts (at most 1.5-fold) during Cu exposure. The responses of metallothioneins in fish gills to metal exposures have been reported to be significantly variable among species, which makes it difficult to interpret clearly the mechanism of *MT* modulations in this organ (Grosell and Wood, 2002; Mazon *et al.*, 2002; Alvarado *et al.*, 2006). The difficultly of interpretation might be closely related with the presence of a variety of cell types in fish gills (e.g., chloride cells, respiratory cells, mucocytes and undifferentiated cells) as well as the cell type-specific turnover rates (i.e., cell renewal) (Dang *et al.*, 2001; Alvarado *et al.*, 2006).

In summary, the genetic determinant of metallothionein was isolated and characterized from an endangered fish species, *A. signifer*. The *AsMT* gene shared conserved structural features with other orthologs at genomic, mRNA and polypeptide levels. Regulatory region of *AsMT* revealed the various motifs for transcription factors involved in the metal-regulation and host defense. The *AsMT* transcript was ubiquitously detected in a variety of tissues with differential levels of basal expression. During an acute exposure experiment using Cu, *AsMT* transcripts were significantly induced in the liver, moderately in the kidney and spleen, and hardly in brain and intestine. Results from this study would be a good start point to provide a useful basis to develop future experiments with MT gene and protein for addressing the metal-caused toxicity and stress in this fish species.

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REFERENCES

Alvarado, N.E., I. Quesada, K. Hylland, I. Marigómez and M. Soto. 2006. Quantitative changes in metallothionein expression in target cell-types in the gills of turbot (*Scophthalmus maximus*) exposed to Cd, Cu, Zn and after a depuration treatment. Aquat. Toxicol., 77: 64-77.

Andrews, G.K. 2000. Regulation of metallothionein gene expression by oxidative stress and metal ions. Biochem. Pharmacol., 59: 95-104.

Atif, F., M. Kaur, S. Yousuf and S. Raisuddin. 2006. In vitro free radical scavenging activity of hepatic metallothionein induced in an Indian freshwater fish, *Channa punctata* Bloch. Chem. Biol. Interact., 162: 172-180.

Baek, H.M., H.B. Song and O.G. Kwon. 2003. Sexual maturation and the spawning season of the Korean bitterling, *Acheilognathus signifer* in upper reaches of the Hongcheon river. Korean J. Ichthyol., 15: 278-288.

Baek, H.M. and H.B. Song. 2005a. Digestive apparatus and food of the Korean bitterling, *Acheilognathus signifer* (Cyprinidae). Korean J. Ichthyol., 17: 57-63.

Baek, H.M. and H.B. Song. 2005b. Habitat selection and environmental characters of *Acheilognathus signifer*. Korean J. Limnol., 38: 352-360.

Baek, H.M. and H.B. Song. 2005c. Spawning in mussel and adaptation strategy of *Acheilognathus signifer* (Cyprinidae: Acheilognathinae). Korean J. Ichthyol., 17: 105-111.

Baek, H.M. and H.B. Song. 2006. Sexual dimorphism and secondary sexual characters of *Acheilognathus signifer* (Cyprinidae: Acheilognathinae). Korean J. Ichthyol., 18: 141-147.

Berg, L.S. 1907. Description of a new cyprinoid fish *Acheilognathus signifer* from Korea with a synopsis of all the Korean Rhodeina. Ann. Mag. Nat. Hist., 19: 159-163.

Bi, Y., G.X. Lin, L. Millecchia and Q. Ma. 2006. Superinduction of metallothionein I by inhibition of protein synthesis: Role of a labile repressor in MTF-1 mediated gene transcription. J. Biochem. Mol. Toxicol., 20: 57-68.

Cerqueira, C.C.C. and M.N. Fernandes. 2002. Gill tissue recovery after copper exposure and blood parameter responses in the tropical fish *Prochilodus scrofa*. Ecotoxicol. Environ. Saf., 52: 83-91.

Chen, W., J.A.C. John, C. Lin, H. Lin, S. Wu, C. Lin and C. Chang. 2004. Expression of metallothionein gene during embryonic and early larval development in zebrafish. Aquat. Toxicol., 69: 215-227.

Cho, Y.S., B.N. Choi, E.M. Ha, K.H. Kim, S.K. Kim, D.S. Kim and Y.K. Nam. 2005. Shark (*Scyliorhinus torazame*) metallothionein: cDNA cloning, genomic sequence, and expression analysis. Mar. Biotechnol., 7: 350-362.

Cho, Y.S., S.Y. Lee, K.-Y. Kim and Y.K. Nam. 2009. Two metallothionein genes from mud loach *Misgurnus mizolepis* (Teleostei; Cypriniformes): gene structure, genomic organization, and mRNA expression analysis. Comp. Biochem. Physiol. B Biochem. Mol. Biol., 153: 317-326.

Cho, Y.S., S.Y. Lee, K.-Y. Kim, I.C. Bang, D.S. Kim and Y.K. Nam. 2008. Gene structure and expression of metallothionein during metal exposures in *Hemibarbus mylodon*. Ecotoxicol. Environ. Saf., 71: 125-137.

Coyle, P., J.C. Philcox, L.C. Carey and A.M. Rofe. 2002. Metallothionein: The multipurpose protein. Cell Mol. Life Sci., 59: 627-647.

Dang, Z.C., M.H.G. Berntssen, A.K. Lundebye, G. Flik, S.E. Wendelaar Bonga and R.A.C. Lock. 2001. Metallothionein and cortisol receptor expression in gills of Atlantic salmon, *Salmo salar*, exposed to dietary cadmium. Aquat. Toxicol., 53: 91-101.

Emi, Y., S. Ikushiro and T. Iyanagi. 1996. Xenobiotic responsive element-mediated transcriptional activation in the UDP-glucuronosyltransferase family 1 gene complex. J. Biol. Chem., 271: 3952-3958.

Grosell, M. and C.M. Wood. 2002. Copper uptake across rainbow trout gills: Mechanisms of apical entry. J. Exp. Biol., 205: 1179-1188.

Haq, F., M. Mahoney and J. Koropatnick. 2003. Signaling events for metallothionein induction. Mutat. Res. Fund. Mol. Mech. Mutagen., 533: 211-226.

He, P., M. Xu and H. Ren. 2007. Cloning and functional characterization of 5′-upstream region of metallothionein-I gene from crucian carp (*Carassius cuvieri*). Int. J. Biochem. Cell Biol., 39: 832-841.

Hollis, L., C. Hogstrand and C.M. Wood. 2001. Tissue-specific cadmium accumulation, metallothionein induction, and tissue zinc and copper levels during chronic sublethal cadmium exposure in juvenile rainbow trout. Arch. Environ. Contam. Toxicol., 41: 468-474.

Kim, I.S. and C.H. Kim. 1989. A study on the egg development and taxonomy of two bitterlings, *Acheilognatus limbata* and *A. signifer* (Pisces, Cyprinidae) from Korea. Korean J. Zool., 32: 22-33.

Knapen, D., E.S. Redeker, I. Inácio, W. De Coen, E. Verheyen and R. Blust. 2005. New metallothionein mRNAs in *Gobio gobio* reveal at least three gene duplication events in cyprinid metallothionein evolution. Comp. Biochem. Physiol. C Pharmacol. Toxicol. Endocrinol., 140: 347-355.

Köck, G., R. Hofer and S. Wögrath, 1995. Accumulation of trace metals (Cd, Pb, Cu, Zn) in arctic char (*Salvelinus alpinus*) from oligotrophic alpine lakes: Relation to alkalinity. Can. J. Fish Aquat. Sci., 52: 2367-2376.

Kurose, K., M. Tohkin and R. Hasegawa. 2005. Transcription factor NF2d9 (LBP-1a) interacts with the positive regulatory element for the xenobiotic responsive element. Biochim. Biophys. Acta. Gene Struct. Expr., 1727: 141-144.

Laity, J.H. and G.K. Andrews. 2007. Understanding the mechanisms of zinc-sensing by metal-response element binding transcription factor-1 (MTF-1). Arch. Biochem. Biophys., 463: 201-210.

Langston, W.J., B.S. Chesman, G.R. Burt, N.D. Pope and J. McEvoy. 2002. Metallothionein in liver of eels *Anguilla anguilla* from the Thames Estuary: an indicator of environmental quality? Mar. Environ. Res., 53: 263-293.

Lin, C., J.A.C. John, L.W. Ou, J. Chen, C. Lin and C. Chang. 2004. Cloning and characterization of metallothionein gene in ayu *Plecoglossus altivelis*. Aquat. Toxicol., 66: 111-124.

Mazon, A.F., C.C.C. Cerqueira and M.N. Fernandes. 2002. Gill cellular changes induced by copper exposure in the South American tropical freshwater fish *Prochilodus scrofa*. Environ. Res., 88: 52-63.

Miracle, A.L. and G.T. Ankley. 2005. Ecotoxicogenomics: Linkages between exposure and effects in assessing risks of aquatic contaminants to fish. Reprod. Toxicol., 19: 321-326.

Nam, Y.K., Y.S. Cho, K.-Y. Kim, I.C. Bang, K.H. Kim, S.K. Kim and D.S. Kim. 2006. Characterization of copper, zinc superoxide dismutase from a cartilaginous shark species, *Scyliorhinus torazame* (Carcharhiniformes). Fish Physiol. Biochem., 32: 305-315.

Oh, M.K., J.Y. Park, M.J. You, E.J. Kang, S.G. Yang, E.O. Kim, Y.C. Jo, I.S. Park, C.H. Kim and I. Toshihiro. 2008. Histological study of oculocutaneous albinism in the Korean bitterling, *Acheilognathus signifier* (Osteichthyes; Cyprinidae). Korean J. Ichthyol., 20: 167-172.

Park, E.Y. and H.M. Rho. 2002. The transcriptional activation of the human copper/zinc superoxide dismutase gene by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin through two different regulator sites, the antioxidant responsive element and xenobiotic responsive element. Mol. Cell Biochem., 240: 47-55.

Ren, H., M. Xu, P. He, N. Muto, N. Itoh, K. Tanaka, J. Xing and M. Chu. 2006. Cloning of crucian carp (*Carassius cuvieri*) metallothionein-II gene and characterization of its gene promoter region. Biochem. Biophys. Res. Commun., 342: 1297-1304.

Schmittgen, T.D. and K.J. Livak. 2008. Analyzing real-time PCR data by the comparative CT method. Nat. Protocols., 3: 1101-1108.

Scudiero, R., P.A. Temussi and E. Parisi. 2005. Fish and mammalian metallothioneins: A comparative study. Gene, 345: 21-26.

Snape, J.R., S.J. Maund, D.B. Pickford and T.H. Hutchinson. 2004. Ecotoxicogenomics: The challenge of integrating genomics into aquatic and terrestrial ecotoxicology. Aquat. Toxicol., 67: 143-154.

Thirumoorthy, N., K.T.M. Kumar, A.S. Sundar, L. Panayappan and M. Chatterjee. 2007. Metallothionein: An overview. World J. Gastroenterol., 13: 993-996.

Ueda, T., H. Naoi and R. Arai. 2001. Flexibility on the karyotype evolution in bitterlings (Pisces, Cyprinidae). Genetica, 111: 423-432.

Woo, S., S. Yum, J.H. Jung, W.J. Shim, C.H. Lee and T.K. Lee. 2006. Heavy metal-induced differential gene expression of metallothionein in Javanese medaka, *Oryzias javanicus*. Mar. Biotechnol., 8: 654-662.

묵납자루(*Acheilognathus signifer*; Cyprinidae) metallothionein 유전자의 클로닝 및 특징 분석

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요 약 : 한반도 고유종인 묵납자루(*Acheilognathus signifer*)로부터 metallothionein (*MT*) 유전자를 분리하고 그 유전자 구조와 발현 특징을 분석하였다. 묵납자루 *MT* cDNA는 20개의 시스테인(cysteines)을 포함한 60개의 아미노산을 암호화하고 있었고, 이들 시스테인 잔기들의 위치는 잉어목 어류에서 잘 보전되어 있었다. 묵납자루 *MT* 유전자는 3개의 exon과 2개의 intron으로 구성되어 있었으며 intron영역은 A/T조성 빈도가 높았다. 생물정보 분석법을 통해 묵납자루 *MT* 유전자의 프로모터 영역은 중금속 조절 및 스트레스/면역관련 조절에 관련한 다양한 전사 조절인자들의 부착 위치들이 보유하고 있는 것으로 예측되었다. Real-time RT-PCR 분석법을 이용한 묵납자루 *MT* mRNA의 조직 별 발현 수준을 조사한 결과, 난소와 장 조직에서의 발현 수준이 가장 높았으며 심장과 근육 조직에서의 발현 수준이 가장 낮은 것으로 확인되었다. 구리를 이용한 중금속 노출 실험(구리 농도 0.5 μM을 이용, 48시간 동안 침지 처리)을 통하여 간 조직에서 *MT* mRNA 발현이 가장 많이 유도되었고(3.5배 이상), 비장, 신장 및 아가미에서도 유의적인 발현양의 증가(1.5~2.5배)가 관찰되었다. 그러나 뇌 및 장 조직에서는 *MT* 발현양의 변화가 없었다. 본 연구 결과는 향후 멸종위기 고유종인 묵납자루의 중금속 관련 스트레스 연구에 유용한 기초 자료를 제공할 수 있으리라 기대된다.

찾아보기 낱말 : 묵납자루, 유전자 구조 및 발현 분석, metallothionein