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Gene Structure and Altered mRNA Expression of Metallothionein in Response to Metal Exposure and Thermal Stress in Miho Spine Loach *Cobitis choii* (Cobitidae; Cypriniformes)

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ABSTRACT Gene and promoter structures of metallothionein (*MT*) from Miho spine loach (*Cobitis choii*; Cypriniformes) were characterized, and the transcriptional responses to experimental exposures to heavy metals and heat stress were examined. The *C. choii* metallothionein displayed well-conserved features of teleostean metallothioneins at gDNA, mRNA and amino acid levels. Bioinformatic analysis predicted that the *C. choii MT* regulatory region potentially possessed various motifs or elements targeted by various transcription factors associated with metal-coordinating regulation (e.g., metal transcription factor-1), immune responses (e.g., nuclear factor kappa B), and thermal modulations (e.g., heat shock factor). Acute heavy-metal exposures to 0.5 or 1.0 μM of cadmium (Cd), copper (Cu), manganese (Mn), nickel (Ni) or zinc (Zn) showed that *MT* transcription was significantly stimulated by Cd (9.6-fold relative to non-exposed control) and Cu (10.4-fold), only moderately by Mn (2.4-fold), but hardly by Ni and Zn. Elevation of water temperature from 25°C to 31°C caused a rapid modulation of *MT* mRNAs toward upregulation to 9.5-fold; however, afterward the elevated mRNA level slightly decreased during further incubation at 31°C for 6 h. Results from this study suggest that *MT*-based expression assay could be a useful basis for better understanding the metal- and/or heat-caused stresses in this endangered fish species.

Key words : *Cobitis choii*, gene structure, heat stress, heavy metals, metallothionein, mRNA expression

INTRODUCTION

The Miho spine loach *Cobitis choii* (Cypriniformes; Cobitidae), a synonym of *Iksookimia choii*, is one of the critically threatened freshwater species in the Korean peninsula. This endemic species has also been designated as a Natural Monument (No. 454; Cultural Properties Administration, Republic of Korea) (Kim *et al.*, 2008; Song *et al.*, 2008). However during the last decades, they have lost, at least in part, their natural habitats possibly caused by anthropogenic and industrial activities (Son and Byeon, 2005). Considering they often occupy a narrow range of habitats in natural water body, careful efforts for the conservation of this species are urgently needed. Moreover, recent molecular studies have claimed the currently resident *C. choii* populations have a very low level of genetic diversity irrespective of geographic locations of their habitats (Lee *et al.*, 2008; Bang *et al.*, 2009). Conservation projects for *C. choii* are underway with respect to the stock assessment, artificial propagations and *ex situ* and *in situ* restorations. Exploitation of functional genes relevant to stress/toxicity responses and host defense could be fundamental for better understanding its stress physiology, consequently providing useful means for effective designation of the conservation plan.

Metallothionein (MT), a low molecular weight (6~7 kDa) and cysteine-rich protein, is a metal-binding housekeeper, which plays central role in the homeostatic regulation of metals (Klaassen *et al.*, 2009). This protein is responsible for not only the reservation of essential metals but also the detoxification of excess metals. Owing to its high inducibility upon exogenous administration of various heavy metal ions, the *MT*-based expression assay has been one of versatile ways to notice the risks associated with metal pollution in aquatic environments (Bour-

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dineaud *et al.*, 2006; Cho *et al.*, 2009c). It has not been yet clarified whether or not the pollutants particularly including heavy metals have played certain roles in the loss of *Cobitis choii* populations in natural streams. However, the functional evaluation of this multivalent detoxifying protein could be helpful for build-up of basic knowledge on the toxicant-related stress physiology of this endangered species, since MTs have also been proven to be related with many signaling pathways involved in aging, antioxidant defense and immune response (Guo *et al.*, 2009; Fu *et al.*, 2010; Swindell, 2011). Furthermore the information on the *MT* modulation in response to metal toxicants could be a useful basis for the post-monitoring plan (i.e., evaluation of general stress or health status of re-introduced stocks) along with the examination of the possible changes of water quality in the recipient site.

The objective of this study is to isolate and characterize the genetic determinant of *C. choii* metallothionein at both mRNA and genomic levels in order to provide a basis for further development of *MT*-based biomarker. For this, we characterized the full-length cDNA and genomic gene sequences of the *C. choii MT*, highlighted the structural characteristics of the 5′-flanking upstream regulatory region and examined the transcriptional alterations of *MT* in response to acute metal exposures and heat stress.

MATERIALS AND METHODS

1. Molecular cloning of cDNA and genomic gene for *C. choii MT*

The specimens (*Cobitis choii*) used in this study were laboratory-bred individuals produced using the captive broodfish, which had been sampled from the Baekgok stream under the authorized permission. To identify the *MT* cDNA sequence, expressed sequence tag (EST) database constructed with liver, intestine, ovary and whole body of *C. choii* was surveyed (unpublished data). From the database search, an EST clone showing the significant homology with previously known teleost metallothioneins was selected and further sequenced at both 5′- and 3′-directions. A continuous fragment of the *MT* cDNA from 5′-untranslated region (UTR) to 3′-UTR was isolated by RT-PCR using oligonucleotide primers ICMT-1F and ICMT-1R. Information on the oligonucleotide primers used in this study was provided in Table 1. RT-PCR was carried out with the liver cDNA that had been used for the construction of the cDNA library. Amplified fragment was cloned into pGEM®-T easy vector (Promega, Madison, WI, USA), and insert sequence was confirmed at both directions. Based on the cDNA sequence, genomic *MT* sequence was PCR-isolated. Preparation of *C. choii* genomic DNA from the whole blood was carried out using the conventional proteinase K/SDS method and an aliquot (500 ng) of the purified genomic DNA template was subjected to a PCR reaction using the Expand High Fidelity PCR System (Roche Applied Science, Mannheim, Germany). The primer pair was the same as described above. PCR product was gel-purified, TA cloned and six-randomly-chosen recombinant clones were sequenced in order to obtain the representative sequence. All the raw sequence data was edited using the Sequencher® 4.9 (Gene Codes, Ann Arbor, MI, USA). Genomic organization (exon/intron organization) was compared to other teleost *MT* genes. Nucleotide composition analysis of each intron was done using the GENE BOY tool (http://www.dnai.org/geneboy/index.html). Homology search against GenBank was performed using BLASTx

Table 1. Sequences of oligonucleotide primers and thermal cycling conditions used in this study

Primer	Sequence (5′ to 3′)	Thermal cycling conditions	Purpose
ICMT-1F ICMT-1R	TCGGGAACTTTAAAGGCTAC CGTCGACCTCTTCATTGACA	94°C for 2 min, 35 cycles at 94°C for 30 s, 58°C for 30 s and 72°C for 30 s (cDNA/gDNA)	Isolation of *C. choii MT* cDNA/gDNA fragments
ICMT-GW1 ICMT-GW2 ICMT-GW3 ICMT-GW4	GTTGTACACTGGCAGTTAGTGCACTT AGCAATCACAAGGATCCATATTGCCT TCCAAATGCACACTCCACTCACAATA TCCCTGCAAGTATGGTTGTCAAATGA	7 (the first PCR) or 5 (nest PCR) cycles at 94°C for 25 s and 67°C for 3 min, followed by 32 (the first PCR) or 20 (nest PCR) cycles at 94°C for 25 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 *C. choii MT* gene
ICMTp-FW ICMTp-RV	TCTCCTAGATTTCCAAGGAC ATCTGATAACTGGGAGGTCA	94°C for 2 min, 35 cycles at 94°C for 30 s, 58°C for 30 s and 72°C for 5 min	Confirmation of *C. choii MT* gene from the 5′-upstream region to 3′-UTR
qIC18S-1F qIC18S-1R	TGACGGGGAATCAGGGTTCGAT CAAGAATTTCACCTCTAGCGGC	94°C for 2 min, 20 cycles at 94°C for 30 s, 58°C for 30 s and 72°C for 30 s	Semi-quantitative RT-PCR of 18S rRNA (normalization control)
IC18S rRNA RV	CAAGAATTTCACCTCTAGCGGC	–	Preparation of normalized control (18S rRNA) in RT reaction
qICMT-1F qICMT-1R	ATGGATCCTTGTGATTGCTC GAAGACACGTAGTTCGGACA	94°C for 2 min, 21 cycles at 94°C for 20 s, 58°C for 20 s and 72°C for 20 s	Semi-quantitative RT-PCR assay of *C. choii MT* transcripts

or BLASTp option. Amino acid sequence identities between *C. choii* MT and other MTs were compared using the GeneDoc software (http://www.psc.edu/biomed/genedoc).

2. Characterization of 5′-upstream region by genome walking

The 5′-flanking region of the *C. choii MT* was cloned in order to characterize the putative transcription factor binding motifs. Genome walking libraries were constructed using the GenomeWalker™ Universal Kit (BD Biosciences Clontech, Mountain View, CA, USA). PCR amplifications were carried out using oligonucleotide primers, ICMT-GW1 to ICMT-GW4. PCR products were TA cloned and insert DNA was sequenced. Each fragment sequence was assembled into a contig to generate a contiguous sequence. From the contig sequence, a genomic DNA fragment from the 5′-upstream region to 3′-UTR was PCR-amplified again by using the primers ICMTp-FW and ICMTp-RV, and then the sequence was confirmed. Putative transcription factor binding motifs was predicted using the Transcription Element Search System (TESS; http://www.cbil.upenn.edu/cgi-bin/tess/tess?RQ=WELCOME) and Transcriptional Factor Search (TFSEARCH; http://www.cbrc.jp/research/db/TFSEARCH.html).

3. Experimental exposures to heavy metals and thermal stress

To examine the transcriptional response of *MT* to heavy metal exposure, acute immersion exposures of *C. choii* fry to various heavy metals were performed. Eight *C. choii* fry (average body weight=1.1±0.2 g) were allocated into one of two replicate tanks (10 L) filled with 8 L of well-aerated tap water (5.0 μm-filtered) containing 0.5 or 1.0 μM of cadmium (Cd), copper (Cu), manganese (Mn), nickel (Ni) or zinc (Zn). A non-exposed control group was also prepared identically except the administration of the heavy metals. During exposure, no feed was provided. Water temperature was adjusted to 22±0.5°C throughout exposure period. At 24 h post exposure, whole body total RNA was extracted from the six-randomly-chosen fry from each tank (two replicate tanks per group).

In order to examine the modulation of *MT* gene in response to heat stress, a thermal treatment was performed. Forty fry (same-sized as above) were allocated into one of two identical tanks (50 L) containing 40 L of tap water at 25°C, and incubated further for 24 h in order to make the fish to acclimate to the starting temperature. Water temperature for one of the two tanks was gradually elevated with a rate of 1°C/h until 31°C. Eight randomly-chosen fry were sampled at 0 h (25°C; starting point), 3 h (reaching 28°C), 6 h (reaching 31°C) and 12 h (kept constant at 31°C for 6 h after reaching 31°C) post thermal elevation, respectively. At the same time, a random sample of eight individuals was also obtained from the control group, which was maintained at the constant temperature (25°C). The temperature was controlled to be ranged within ±0.5°C and the level of dissolved oxygen was also kept from 5 to 6 ppm.

4. *MT* mRNA assay

Extraction of total RNA from whole body fry was carried out using TriPure reagent (Roche) and the total RNA extracted was purified again with RNeasy Mini Kit including DNase treatment step (Qiagen, Hilden, Germany). In order to prepare an invariant normalization control, a partial segment of *C. choii* 18S rRNA was cloned by PCR isolation using the primers conserved in fish 18S rRNA (unpublished data). An aliquot (2 μg) of the purified total RNA was reverse transcribed into cDNA using the Omniscript Reverse Transcription Kit (Qiagen). During the reverse transcription (RT), a reverse primer complementary to the *C. choii* 18S rRNA (IC18S rRNA RV; 0.1 μM) was also included in the RT reaction. The cDNA pool synthesized was four-fold (for *MT*) or eight-fold (for 18S rRNA) diluted and two μL of the diluted cDNA was used as the template for thermal cycling reaction. Primer pairs for *C. choii MT* (qICMT-1F/1R; amplicon=240 bp) and 18S rRNA (qIC18S-1F/1R; amplicon=569 bp) were included in the PCR reaction independently. Six μL of amplified product was separated on a 1.5% agarose gel, visualized by ethidium-bromide (EtBr) staining and analyzed by the Quantity-One™ image analysis software implemented in the VersaDoc 4000 (Bio-Rad, Hercules, CA, USA). Triplicate assays were performed in an independent fashion. Based on the scanning densitometry, differences in the relative expression levels among groups were assessed by analysis of variance (ANOVA) followed by Duncan's multiple range tests. On the other hand, significant difference from the control levels was tested by Student's *t*-test. All the statistics were performed using the SPSS software (ver. 10.1.3) and differences were considered to be significant when $P<0.05$.

RESULTS AND DISCUSSION

The *C. choii MT* cDNA was composed of 56 bp of 5′-UTR, 180 bp of single open reading frame (ORF) encoding 60 amino acids and 243 bp of 3′-UTR excluding the poly(A)+ tail (Fig. 1; GenBank accession no. JF419523). Two putative polyadenylation signals (AATATA and AATAAA) were found at 153 bp and 16 bp prior to the poly(A)+ tail, respectively, suggesting the potential processing of *MT* transcript variants with different 3′-UTR lengths (Cho *et al.*, 2008). The *C. choii* MT poly-

peptide consisted of twenty cysteine residues at conserved positions when aligned with other representative orthologs from teleosts (alignment not shown). The richness of Cys residues in the Cys-X-Cys or Cys-Cys form at both β- and α-domains of MT proteins has already been reported widely in other vertebrate orthologs (Cho *et al.*, 2005; Knapen *et al.*, 2005; Gao *et al.*, 2009). From the genomic PCR isolation, the *C. choii MT* was proven to possess a tripartite organization which is typical for most known vertebrate *MT*s (Fig. 2). The conserved exon/intron splicing rule (GT-AG) was also found in each junction site. Like many teleost *MT*s, *C. choii MT* showed a high proportion of adenine and thymine in its two introns (Chen *et al.*, 2004; Lin *et al.*, 2004; Cho *et al.*, 2008). Most teleosts are known to have at least two copies of *MT* genes in their genomes, although the chromosomal organization as well as chromosomal synteny between species has not been studied yet in most fish species so far (Knapen *et al.*, 2005; Cho *et al.*, 2009c). Unfortunately, the classification of the *C. choii MT* into *MT-I* (or *MT-*

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  1     gtgaaacgatacagcaaaggaaccttcgggaactttaaaggctactctaagggaaa
 57 ATGGATCCTTGTGATTGCTCAAAAACTGGAACTTGCAACTGTGGTGCCACCTGCAAGTGC
     M  D  P  C  D  C  S  K  T  G  T  C  N  C  G  A  T  C  K  C   20
117 ACTAACTGCCAGTGTACAACCTGCAAGAAGAGTTGCTGTTCTTGCTGCCCATCCGGTTGC
     T  N  C  Q  C  T  T  C  K  K  S  C  C  S  C  C  P  S  G  C   40
177 AGCAAGTGCGCCTCTGGGTGCGTATGTAAGGGCAATTCCTGTGACTCCAGCTGTTGTCAA
     S  K  C  A  S  G  C  V  C  K  G  N  S  C  D  S  S  C  C  Q   60
237 tgaagaggtcgacgcgccgtttgctacaatgtgaactctttgtccgaactacgtgtcttc
297 attgtacatttaatgttgtcttttgagataataaatgacctcccagttatcagatttgt
357 ttcatgttgccaagatcacttttggggggtggggttcacttatttgtactgataaagac
417 tattatgtatctttatgaatgtaaaattgtaacttcactatgtgtcaaatatattaatta
477 agcaaaaaaaaaaaaaaaaaaaaa
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Fig. 1. Complementary DNA and deduced amino acid (single code) sequences of *Cobitis choii* metallothionein. Coding region is indicated by boldface uppercase letters while non-coding region by lowercase letters. Twenty conserved cysteine residues are bolded. Two putative polyadenylation signals are underlined.

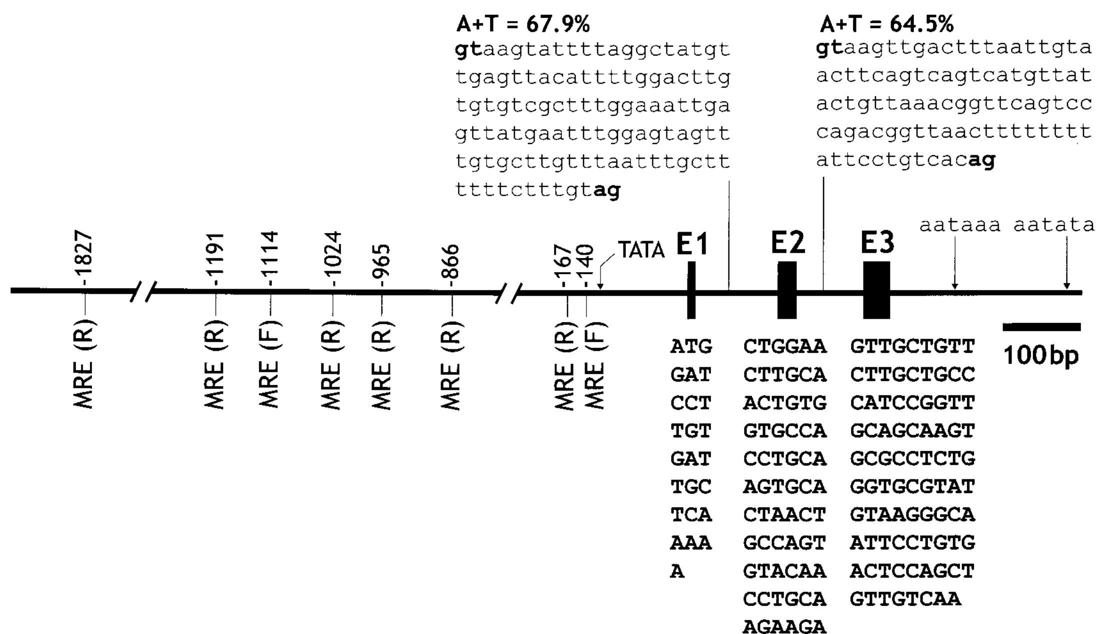

Fig. 2. Genomic organization of *Cobitis choii MT*. In a tripartite structure, three exons (E1 to E3) are indicated by thickened vertical lines and the sequence of each exon is provided as boldface, uppercase letters. Nucleotide sequences of the two A+T rich introns are indicated by lowercase letters in which gt-ag splicing sites are bolded. Consensus *TATA* box and two putative polyadenylation signals are indicated arrows. In the 5′-flanking region, relative positions of eight metal responsive elements (*MRE*s) from the translation initiation site (ATG) are indicated in a forward (F) or reverse (R) orientation. For complete nucleotide sequence of the *C. choii MT* gene and its promoter, refer to GenBank (accession no. JF419523).

A) or *MT-II* (or *MT-B*) has been difficult currently due to the limited information on *MT* orthologs from Cobitidae. Moreover, the present *C. choii* MT showed a higher amino acid sequence identity (100%) with MT from the stone loach (*Barbatula barbatula*; GenBank no. CAA4 2036) belonging to different family (Balitoridae) than MT isoforms (98%) from the mud loach (*Misgurnus mizolepis*: GenBank nos. ACH90423 and ACH90424) belonging to the same family (Cobitidae). Gene duplication of *MT* isoforms in fish genomes has been suggested to occur frequently in a taxon-specific manner (Cho *et al.*, 2009c), indicating that further exploitation of *MT* paralog(s) from the *C. choii* genome should be crucial for more comprehensive comparison between the *MT* isoforms from Cobitidae.

Genome walking to the 5′-direction of *C. choii MT* recovered a 4,090 bp of upstream sequence from the translation initiation codon (ATG) (GenBank no. JF419523) and essential sites or motifs targeted by various transcription factors were predicted (Table 2; Fig. 2). Firstly, eight copies of metal responsive elements (*MRE*s), which are known to be essential for the binding of metal transcription factor-1 (MTF-1), were found in both proximal and distal parts of the regulatory region. MTF-1 is the key factor to regulate both basal and induced expression of the genes to code the metal-coordinating proteins such as MT and superoxide dismutase (SOD) (Laity and Andrews, 2007; Cho *et al.*, 2009a; Ferencz and Hermesz, 2009). The presence pattern of *MRE* copies in the *C. choii MT* regulatory region was similar with those found in other fish *MT* promoters in terms of the clustering of multiple *MRE*s in proximal and/or distal regions rather than random or even distribution (Ren *et al.*, 2006; He *et al.*, 2007; Cho *et al.*, 2008). Other transcription factors identified in the *C. choii MT* promoter included GATA factor, activator protein1 (AP-1) and hepatocyte nuclear factor (HNF), which are known to be often found in most fish *MT* promoters, although their specific roles in the modulation of fish *MT* have not been clearly clarified yet (Yan and Chan, 2004; Cheung *et al.*, 2005; Cho *et al.*, 2008). In addition, several noteworthy transcription factors predicted in the *C. choii MT* promoter were the factors that have been known to be closely involved in the immune responses of vertebrates. They included the nuclear factor kappa B (NF-κB/c-Rel), CAAT-enhancer binding protein (C/EBP) and upstream stimulatory factor (USF), which all have been usually found in the antimicrobial or antioxidant genes, suggesting the proposed employment of MTs in host defense mechanism (Atif *et al.*, 2006; Thirumoorthy *et al.*, 2007; Wang *et al.*, 2009). Finally, the present *C. choii MT* promoter represented two binding sites for heat shock factor (HSF), suggesting the possible modulation of the *MT* gene under thermally stressed conditions (Cho *et al.*, 2009c).

Table 2. Putative transcription factor binding sites predicted in the 5′-regulatory region of *Cobitis choii MT*

Transcription factor	Consensus sequence[a]	Position[b]	Sequence
TFIID	TATAAA	-116	TATAAA
STAT	TTCNNNGAA	-3345	TTCAGTGAA
		-3036	TTCTCTGAA
USF	CANNTG	-540	CACGTG
HNF-3	TRTTKRYTY	-3934	AAACAAATA*
		-3773	AAACAAATA*
		-2794	TGTTTGTTT
		-2146	GAGTAAATA*
		-1779	TATTTATTT
		-330	AAACAAATA*
AP-1	TGASTMA	-3514	TGACTCA
		-850	TGACACA
C/EBP	TTDNGNAA	-2889	TTTCAAAA*
		-2097	TTATGCAA
GATA	WGATAR	-1546	TGATAG
		-1305	CTATCC*
MTF-1	TGCRCNC	-1827	GTGTGCA*
		-1191	GTGTGCA*
		-1114	TGCACCC
		-1024	GAGTGCA*
		-965	GAGTGCA*
		-866	GTGTGCA*
		-167	GTGTGCA*
		-140	TGCACTC
AhR	CACGCW	-1425	TGCGTG*
		-947	CACGCA
NF-κB/c-Rel	GGGRNNYYCC	-400	GGGACCTTCC
NF-AT	WGGAAAA	-4038	AGGAAAA
		-1061	TGGAAAA
GR-*h*	TGTTCT	-2915	AGAACA*
		-2763	TGTTCT
		-987	TGTTCT
HSF	GAAKKTTC	-3027	GAATTTTC
		-37	GAACCTTC*

[a]N=any base; R=A or G; Y=C or T; W=A or T; K=G or T; S=C or G; D=A or G or T; M=A or C
[b]Upstream from the translation start (ATG) site
*Reverse orientation to the consensus sequence

As expected, the *C. choii MT* transcripts were actively induced in response to the acute heavy metal exposures, although the inducibility was highly variable depending upon metal inducers (i.e., Cd, Cu, Mn, Ni and Zn) (Fig. 3). Of the five metals tested, most potent metals were Cd (8.2- and 9.6-fold induction of *MT* mRNA, respectively for 0.5 and 1.0 μM-treated groups when compared to basal level of non-exposed control) and Cu (5.1- and 10.4-fold), while fish exposed to Mn showed only the moderate activation of *MT* at both 0.5 and 1.0 μM concentrations (2.4- and 2.2-fold, respectively) ($P<0.05$). On the other hand, significant alteration was detected in neither Ni nor Zn irrespective of the metal concentrations ($P>0.05$). Differential responses of *MT* expression to different metal inducers have been commonly observed in a number of other fish species (Hermesz *et al.*, 2001; Woo *et al.*, 2006; Lee *et al.*, 2010). However, it could not be

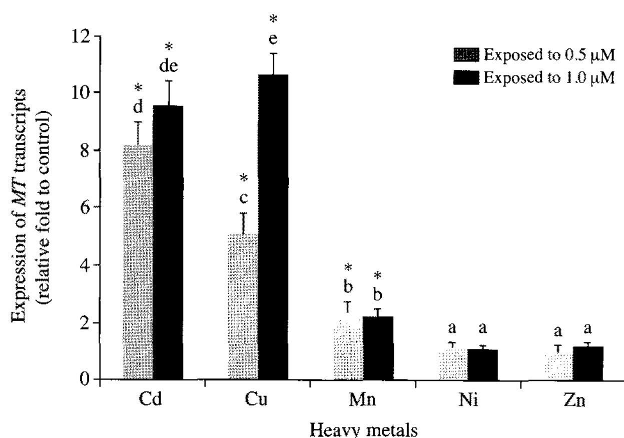

Fig. 3. Transcriptional modulation (fold-induction) of *Cobitis choii* *MT* in response to acute experimental exposure (0.5 or 1.0 μM for 24 h) to Cd, Cu, Mn, Ni or Zn, as assessed by semi-quantitative RT-PCR analysis. Mean ± SDs with different letters (a-e) are significantly different based on ANOVA followed by Duncan's multiple range tests at $P<0.05$. Significant elevation from the basal level observed in the non-exposed control group is indicated by asterisks based on Student's *t*-test ($P<0.05$).

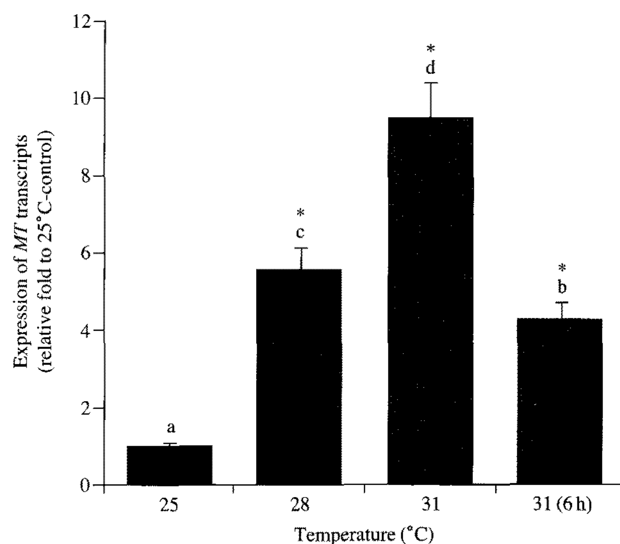

Fig. 4. Transcriptional modulation (fold-induction) of *Cobitis choii* *MT* during thermal elevation from 25°C to 31°C (1°C/h), as assessed by semi-quantitative RT-PCR analysis. Mean ± SDs with different letters (a-d) are significantly different based on ANOVA followed by Duncan's multiple range tests at $P<0.05$. Significant elevations of *MT* transcript levels from the basal level observed in the control group kept at the constant temperature of 25°C are indicated by asterisks based on Student's *t*-test ($P<0.05$). When the temperature reached 31°C, fish were further incubated for 6 h at the temperature [labeled as '31(6 h)'].

ruled out that Ni and Zn may stimulate the transcription of the *C. choii MT* with an extended duration since many previous studies indicated the importance of treatment duration as well as the dose strength (Cho *et al.*, 2008; Rhee *et al.*, 2009). In addition, the possibility that tissue-specific modulation of the *MT* transcripts might be masked under the present assay condition because we performed the analysis with the total RNA samples from whole fry rather than independent adult tissues, which should be addressed in future experiment. Nevertheless, result from this study could support, at least in part, the recent claim on the need of revision of the classic dogma for the *MT* induction, in the sense of that Zn is not always an inducer for activating the *MT* transcription (Bi *et al.*, 2006; Bourdineaud *et al.*, 2006; Cho *et al.*, 2009c). Metal toxicity and modulation of *MT* in response to a given heavy metal exposure have also been known to vary among fish species, and the response patterns are generally in relation with the taxonomic position of the species (Kalman *et al.*, 2010).

Thermal stress could alter significantly the mRNA expression of *C. choii MT* (Fig. 4). Basal expression level observed at the starting temperature (25°C) was sharply elevated up to 5.6-fold at 28°C and then more increased to 9.5-fold at 31°C. This highest level observed at 31°C was slightly decreased down when the fish were kept further at the constant 31°C for 6 h, although the level remained still significantly higher than that observed in the 25°C-control (4.3-fold relative to non-treated control). The modulation of the *C. choii MT* by thermal treatment was well in congruent with our finding the presence of binding motifs for HSFs in its regulatory region. Regulatory mechanism of fish *MT* gene in response to acute thermal change has been little explored. However, external temperature is one of the primary abiotic factors to affect the regulation of various physiological pathways in poikilothermal animals, and heat stress has been known to often cause the formation of reactive oxygen species (ROS) in those animals (Buckley *et al.*, 2006; Cho *et al.*, 2006; Cho *et al.*, 2009b). Although we haven't yet examined the expression pattern of antioxidant enzyme genes together with *MT* gene, the temperature-dependent upregulation of *MT* may be in accordance with its potential involvement in the antioxidant pathway to relieve the prooxidant stress arisen from the thermal elevation. On the other hand, the decline of *MT* transcript levels after reaching peak at 31°C could be explained, in general, by the stabilized or acclimated process after the initial shock-phase, indicating future experiments are needed to examine if the *MT* mRNA levels could return to the basal level or not. It is widely agreed that metallothionein is a useful early warning indicator of the risks associated with heavy metal-mediated toxicity (Rodríguez-Ortega *et al.*, 2009; Falfushynska *et al.*, 2010). However, it has been claimed that the transcription (both basal and induced expressions) of *MT* genes could be greatly modulated by various biotic (e.g., age, sex and health status etc.) and abiotic (e.g., temperature, salinity, and seasons, etc.) factors (Ringwood *et al.*,

1999; Amiard *et al.*, 2006; Dragun *et al.*, 2009; Lee *et al.*, 2010). As evidenced in this study, thermal condition should be carefully considered for all the *MT*-based expression assays in this species.

In summary, gene and promoter structures of the *C. choii* were determined and transcriptional modulations of *C. choii MT* in response to experimental exposures with different heavy metals and heat stress were examined. Result of this study proposes that *MT* could be a potential indicator to address the various cellular stresses in this endangered fish species, however also suggests that careful and extensive analyses should be followed in order to employ the *MT* as an environment-realistic biomarker to aid the conservation of this species. In particular, the assessment of *MT* modulation with more realistic doses of the heavy metals, which are expected potentially to be present in candidate recipient sites for the restoration of this species (i.e. the sites for re-introduction of the fishes), would be a valuable subject in future study. However for this assessment also, it is recommended that seasonal variation of *MT* gene expression caused by the change of water temperature should be taken together into account.

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미호종개 metallothionein 유전자의 구조 및 중금속 노출과 고온 자극에 대한 *MT* mRNA의 발현 특징 분석

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요 약 : 멸종위기 어류 미호종개 (*Cobitis choii*)로부터 중금속해독 단백질 (metallothionein) 유전자를 분리, 클로닝하고 중금속 및 고온 스트레스에 대한 전사 발현 특징을 분석하였다. 미호종개 metallothionein는 gDNA, mRNA 및 아미노산 서열 모두에서 경골 어류 MT들의 구조적 특징을 잘 보전하고 있었으며, 생물정보분석을 통해 미호종개 *MT* 유전자 5′-upstream 영역은 중금속 조절, 면역 반응 및 온도 반응에 관여하는 다양한 전사 조절 인자들의 부착 위치들을 포함하는 것으로 관찰되었다. 카드뮴 (Cd), 구리 (Cu), 니켈 (Ni), 망간 (Mn) 및 아연 (Zn)을 이용한 침지 노출 실험 (0.5 및 1.0 μM; 24시간)에서 미호종개 *MT* mRNA 발현은 구리 및 카드뮴 처리군에서 가장 많이 유도되었고 (1.0 μM Cu 처리군에서 최대 10배), 망간 처리군에서는 비교적 적은 양의 *MT* 발현이 유도된 반면 (2배), 아연 및 니켈 노출 군에서는 유의적인 *MT* 발현의 증감이 관찰되지 않았다. 또한 미호종개 *MT* 전사 발현은 고온 자극 (25°C로부터 31°C까지 증가)에도 민감하게 반응하는 것으로 나타나, 31°C 도달시점에서 25°C 초기 *MT* mRNA 발현 수준보다 9배 높은 mRNA 발현이 관찰되었다. 본 연구 결과는 *MT* 기반의 유전자 발현 분석을 이용함으로써, 향후 멸종위기 어류 미호종개의 스트레스 반응 연구에 유용한 기초 자료를 제공할 수 있다고 기대된다.

찾아보기 낱말 : 미호종개, metallothionein 유전자, 프로모터 구조, 중금속, 고온 자극