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Isoform-specific response of two GAPDH paralogs during bacterial challenge and metal exposure in mud loach (*Misgurnus mizolepis*; Cypriniformes) kidney and spleen

Young Sun Cho, Dong Soo Kim and Yoon Kwon Nam†

Institute of Marine Living Modified Organisms, Pukyong National University, Busan 608-737, Korea

Gene expression of two glyceraldehyde-3-phosphate dehydrogenase (GAPDH) paralogs was examined during *Edwardsiella tarda* challenge and heavy metal exposures in mud loach (*Misgurnus mizolepis*; Cypriniformes) kidney and spleen. Transcription of the two mud loach *GAPDH* paralogs (*mlGAPDH-1* and *mlGAPDH-2*) was significantly modulated by these stimulatory challenges in an isoform-dependent manner. Based on the real-time RT-PCR analysis, the *mlGAPDH-2* transcripts were more preferentially induced by *E. tarda* challenge, whereas the *mlGAPDH-1* transcripts were proven to show more inducibility in response to heavy metal exposure using Cd, Cu, Mn and Zn at 5 μM. Their isoform-specific response patterns were closely in accordance with the TF binding profiles in promoter and intron-1 of the two *mlGAPDH* isoforms, in which the *mlGAPDH-2* has more binding sites for immune-related transcription factors than *mlGAPDH-1* while the *mlGAPDH-1* possesses exclusively metal responsive elements in its intron. Collectively, the *mlGAPDH*s are potentially involved in cellular pathways independent of glycolysis and the two GAPDH paralogs might undergo functional diversification or subfunctionalization at least at the transcription level.

Key words : Bacterial challenge, GAPDH isoforms, Heavy metals, *Misgurnus mizolepis*

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH; EC 1.2.1.12) catalyzes the oxidative phosphorylation of glyceraldehyde-3-phosphate to 1,3-bisphosphoglycerate, which is an essential step of the catabolic carbohydrate metabolism in energy production. GAPDHs are found in most aerobic organisms and ubiquitously detectable in most cell types. However, previous studies on mammalian species highlighted that this classic, glycolytic protein should be recognized as a multiplayer involved in various cellular pathways that are independent of its essential role in energy production (Sirover, 2005). Mammalian GAPDHs have been proven to function in as a modulator of apoptosis (Ishitani *et al*., 2003), nuclear transporter of tRNA (Singh and Green, 1993) and protector of telomeric DNA (Demarse *et al*., 2009). They are also known to be closely related with DNA repair and membrane fusion of the mammalian cells (Robbins *et al*., 1995; Azam *et al*., 2008). To date, most of nonglycolytic functions of GAPDHs in mammals have been turned out to be based on their post-translational modifications that are frequently followed by subcellular translocation and localization, rather than transcriptional regulation of the mRNA levels (Mazzola and Sirover, 2003). Mammalian species express a major isoform (GAPDH-1) in diverse tissues wherein this

†Corresponding author : Yoon Kwon Nam
Tel : +82-51-629-5918
E-mail : yoonknam@pknu.ac.kr

isoform displays multivalent functions as described above and also a second, minor isoform (GAPDH-2) that is transcribed only in spermatogenic cells (so-called also "GAPDH-S") (Welch *et al*., 2000 and 2006).

Teleost species also express two functional GAPDH isoforms, GAPDH-1 and GAPDH-2. However unlike mammals, the second isoform GAPDH-2 is not sperm-specific, instead, its transcripts are reported to be widely distributed in most fish tissues (Manchado *et al*., 2007; Cho *et al*., 2008). Also fish GAPDH-2s lack the long N-terminal proline-rich domain that is typically found in their mammalian orthologs (GAPDH-S). Based on these critical differences between fish and mammalian GPADH-2s, the fish GAPDHs have been proposed to experience different evolutionary history from that common to mammalian counterparts (Kim and Nam, 2008). However, in contrast to the richness of information on the mammalian GAPDHs, cellular functions independent of glycolysis have not been extensively studied in fish, although fish GAPDHs might represent basically similar functions seen in mammalian orthologs. Unlike mammalian GAPDH-1s of which diverse functions are mainly controlled at post-translational levels rather than at transcriptional levels, a few previous studies have shown that fish GAPDHs exhibited a significant regulation at transcription level in response to different stimulatory treatments (Cho *et al*., 2008). Although the post-translational regulation of fish GAPDH isoforms have not been yet characterized, the transcriptional alterations of fish GAPDH genes under stimulated conditions were reported to be isoform-specific depending on the kinds of stimulations, suggesting the possibility that the two fish GAPDH isoforms might experience subfunctionalization in nonglycolytic roles (Manchado *et al*., 2007; Cho *et al*., 2008).

In this study, we aimed to evaluate potential diversification between the two GAPDH isoforms in their mRNA expression in response to two different stimulatory treatments (bacterial challenge and heavy metal exposure) in mud loach (*Misgurnus mizolepis*; Cypriniformes), a candidate experimental model organism. For this we challenged mud loach individuals either with *Edwardsiella tarda* or four different heavy metal ions, and then examined if the transcriptional alterations of the two GAPDHs would be isoform-specific or not.

Materials and Methods

Fish specimens and GAPDH genes

The experimental mud loach individuals were a laboratory-bred stock maintained at the Institute of Marine Living Modified Organisms, Pukyong National University, Busan, Korea. Fish culture and management were performed according to methods previously established in the institute. From our local expressed sequence tag (EST) database constructed from various tissues of mud loaches, two full-length paralog GAPDH mRNA sequences (*mlGAPDH-1* and *mlGAPDH-2*) were identified, confirmed by direct sequencing of RT-PCR product and deposited in GenBank under the accession numbers, **JN230712** (*mlGAPDH-1*) and **JN230713** (*mlGAPDH-2*). Based on the cDNA sequence, genomic sequence of each isoform was PCR-isolated and its representative sequence was determined (unpublished data). To obtain a 5'-flanking region of each GAPDH isoform, genome walking was conducted using Universal

GenomeWalker Kit (Clontech Laboratories Inc., Mountain View, CA, USA) followed by contig assembly. Continuous or overlapped genomic fragments were re-isolated by PCR and representative sequences were determined. The gene structure and exon-intron organization for each GAPDH isoform gene can be referred to the GenBank accession numbers, **JN230716** and **JN230717**, respectively. Potential transcription factor binding motifs related with metal responsiveness or inflammation were bioinformatically predicted in 5'-upstream regions and/or intron 1 following the non-translated exon 1 of each isoform using Transcription Element Search System (TESS; http://www.cbil.upenn.edu/cgi-bin/tess/tess?RQ=WELCOME) and TFSEARCH (ver. 1.3; http://www.cbrc.jp/research/db/TFSEARCH.html).

Bacterial challenge

The bacterial challenge was performed using a Gram negative bacterial species, *Edwardsiella tarda* (FSW910410 strain) that is known as a causative agent for edwardsiellosis in this species. The *E. tarda* was freshly grown in LB broth to an optical density at 600 nm of 0.8, washed twice with phosphate buffered saline (PBS; pH 7.4) and suspended in the PBS. Healthy mud loach individuals (n = 8) with similar average body weights (15 ± 1 g) were given an intraperitoneal injection of an aliquot (injection volume = 200 μL) of bacterial suspension containing 1×10^7 *E. tarda* cells (determined by estimating the colony forming units). For preparing the non-challenged control group, same-sized fish (n = 8) were injected with an equal volume of PBS alone. Challenged and control groups were maintained in two separate 50 L-tanks to prevent cross-contamination of *E. tarda*. Water temperature was adjusted to be 25 ± 1 °C and dissolved oxygen was 4-5 ppm throughout the experiment. No feed was supplied after the injection. At 48 h post injection, randomly chosen six individuals from each tank were sacrificed, kidney and spleen tissues were obtained individually, and stored at -80 °C until used.

Heavy metal exposure

For metal exposures, the mud loach individuals (n = 6) with the same body weight range as above were allocated into one of four experimental tanks (50 L) containing cadmium (Cd), copper (Cu), manganese (Mn) or zinc (Zn) solution each at 5 μM of the nominal concentration. The non-exposed control group was prepared identically except the heavy metal. Forty-eight hours after immersion exposure, kidney and spleen were surgically removed from each fish belonging to metal-exposed or non-exposed control group for expression assay. All other tank conditions were the same with those used in the bacterial challenge experiment.

Real-time RT-PCR assay of *mlGAPDH* transcripts

Total RNA was purified from the kidney and spleen using the RNeasy Midi Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Reverse transcription of an aliquot (3 μg) of total RNA was performed with the Omniscript® Reverse Transcription System (Qiagen) based on the oligo-d$(T)_{20}$ priming method. All the reaction conditions were made according to the manufacturer's protocol, except an inclusion of a mud loach 18S rRNA primer (ML18S-RV; 5′-CAAGAATTTCACCTCTAGCGGC-3′; 0.05 μM final concentration) in order to prepare normalization control

during RT reaction. The RT product (cDNA sample) was diluted twofold (for the *mlGAPDH* cDNAs) or tenfold (for 18S rRNA) with sterile distilled water and then 1 μL of the diluted cDNA sample was subjected to thermal cycling reaction. Based on the standard curves for *mlGAPDH-1*, *mlGAPDH-2*, and 18S rRNA, PCR efficiencies at least higher than 90% were confirmed before the assays. Real-time PCR amplification was performed using the 2× iQ™ SYBR® Green Supermix (Bio-Rad, Hercules, CA, USA). Primer pairs for *mlGAPDH-1* and *mlGAPDH-2* were mlGAPDH1q1F (5′-GATCTGACCGTCCGTCTTGA-3′) / mlGAPDH1q1R (5′-ATGTGTGCCATCAGGTCGCA-3′) and mlGAPDH2q1F (5′-CTACAGCCATCGTGTTGCA-3′) / mlGAPDH2q1R (5′-CGGTTACACCCAGAATGGAA-3′), respectively. The 18S rRNA control gene was amplified with qML18S 1F (5′-ACCCATTGGAGGGCAAGTCT-3′) and qML18S 1R (5′-CCTAGCTGAGATATTCAGGC-3′). Expected sizes of PCR products were 263, 273, 274 bp for *mlGAPDH-1*, *mlGAPDH-2* and 18S rRNA, respectively. Differential expression of *mlGAPDH* isoforms in response to stimulatory treatments (*i.e., E. tarda*-injection and metal exposures) was addressed as the fold change relative to the level of the unstimulated (*i.e.,* PBS-injected or non-exposed control), based on the prenormalization against 18S rRNA level (Schmittgen and Livak, 2008). Triplicate assays were carried out for each sample. Differences in the relative expression levels among samples were assessed with analysis of variance (ANOVA) followed by Duncan's multiple range test and/or Student's *t* test, with a significance level of $P = 0.05$, using the SPSS software v. 10.1.3 (SPSS Inc., Chicago, IL, USA).

Results and Discussion

Immune-and metal-responsive transcription factor binding sites in GADPH promoters

The two mud loach GAPDH isoform genes have 12 (*mlGAPDH-1*; **JN230716**) and 11 (*mlGAPDH-2*; **JN230717**) exons interrupted respectively 11 and 10 introns. Each of the two *mlGAPDH* genes possessed a non-translated exon (NTE1). The ATG translation initiation sites in the exon 2 of *mlGAPDH-1* and *mlGAPDH-2* were found to locate at 23 bp and 32 bp from the 5′ end of the exon 2 of each isoform. The intron 1 (between NTE1 and exon 2) in *mlGAPDH-2* is markedly longer (6.32 kb) than that in *mlGAPDH-1* (0.37 kb). Based on the transcription factor (TF)-binding motifs/elements analysis, both isoforms showed the canonical TATA box (-49 bp and -38 bp from the putative transcription start site) and also represented various TF binding sites related with immune or stress responses (Fig. 1). They including motifs for hypoxia inducible factor 1 (HIF-1), CAAT-enhancer binding protein (C/EBP), nuclear factor kappa B (NF-κB), signal transducer and activator of transcription (STAT), and the interleukin 6 (IL6) response element binding protein (IL-6 REBP) (Murray, 2007; Sehgal, 2008; Rebl *et al*., 2010). In a comparison of the two *mlGAPDH* isoforms, *mlGAPDH-1* has more copies of C/EBP motifs and proximal CAAT boxes than *mlGAPDH-2*. On the other hand, the *mlGAPDH-2* has revealed more copies of immune-related TF binding sites such as HIF-1, NF-κB and STAT sites. Furthermore, *mlGAPDH-2* has multiple binding motifs for interferon regulatory factor 1 (IRF-1) and the cAMP response element binding protein (CREBP), which are not clearly

seen in the *mlGAPDH-1* promoter. In addition, a bioinformatic TF motif search showed that many of the immune-relevant TF-binding sites (*e.g.,* IL-6 REBP, NF-κB, and C/EBP sites) were also predicted in intron 1 of the *mlGAPDH-2* isoform. More interestingly, a canonical TATA signal was also found in intron 1 of the *mlGAPDH-2* (37 bp upstream from the first translated exon 2), which does not occur in intron 1 of *mlGAPDH-1*, suggesting the possibility of the alternative transcription in *mlGAPDH-2* (Mezquita *et al*., 1998). On the other hand, the intron 1 of the *mlGAPDH-1* revealed two putative metal-responsive elements (MREs) targeted by metal-transcription factor-1 (MTF-1) that has been known as a crucial modulator for metal-coordinating expression of the target genes (Andrews, 2000). The MRE is observable in neither promoter nor intron 1 of the *mlGAPDH-1*. The different profiles of TF-binding motifs in *mlGAPDH-1* and *mlGAPDH-2* suggest the functional differentiation or subfunctionalization of the two isoforms, although the pinpointing the TFs' involvements in the differential modulation of fish GAPDH isoform genes.

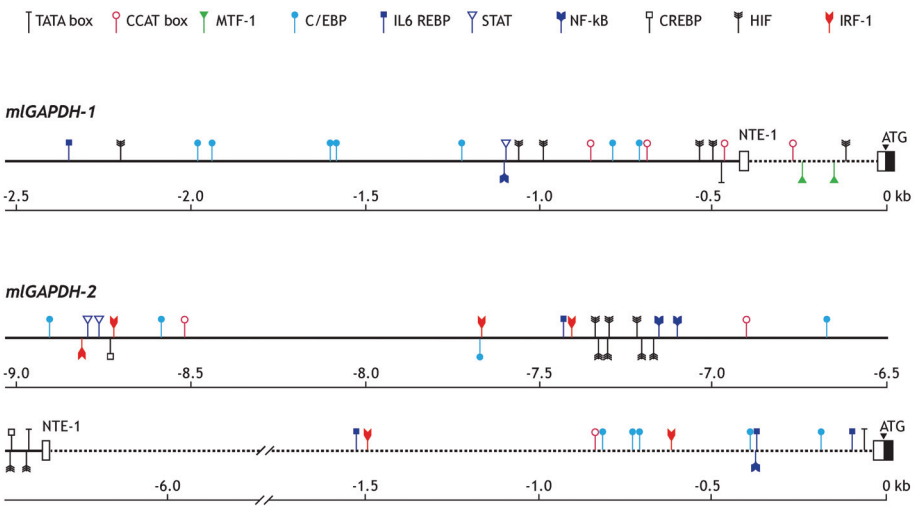

Fig. 1. Distribution of transcription factor binding motifs in 5′-flanking region (solid horizontal line) and intron 1 (dashed horizontal line) of *mlGAPDH-1* and *mlGAPDH-2* isoforms. Non-translated exon 1 and non-translated region in exon 2 in each isoform are indicated by open vertical box, while coding region in exon 2 by closed vertical box. Relative distance from the translation start site (ATG) of each isoform is indicated with a 500-bp interval. For abbreviations for transcription factors and elements, refer to main text.

Transcriptional response of mlGAPDH isoforms during *E. tarda* challenge

During the *E. tarda* challenge, the mRNA expression pattern of the two *mlGAPDH* isoforms was different in the kidney and spleen (Fig. 2). The *mlGAPDH-1* isoform was not responsive to the bacterial injection in these two tissues, in which there was not significant different in the transcript level as compared to the PBS-injected control ($p > 0.05$). However in contrast to the *mlGAPDH-1*, the transcription of *mlGAPDH-2* was significantly

stimulated by the same challenge. In kidney, the *mlGAPDH-2* mRNA level was elevated up to 4.8-fold relative to the PBS-injected control ($p < 0.05$). As similarly, the transcript level of *mlGAPDH-2* in the spleen was increased up to 4-fold ($p < 0.05$). Several studies have reported the possible modulation of GAPDH transcripts by different stimulatory factors, undertaken mainly to evaluate the utility of GAPDH mRNA expression as an internal control for gene expression studies in fish (Tang *et al*., 2007; Mitter *et al*., 2009; Dang and Sun, 2011). However there are only a few studies on the quantitative comparison of the expression of the GAPDH isoforms in response to a given immune challenge in fish. The *mlGAPDH-2*-activation during bacterial challenge might also be in concordance with the different patterns of TF-binding motifs in 5′-flanking regulatory regions. The 5′-flanking region of *mlGAPDH-2* has more copies of several immunity-or stress-related motifs/elements than that of *mlGAPDH-1*, particularly including the motifs for HIF-1, NF-κB, CREBP and IRF-1, which are also known to be closely related to the acute immune responses and often found in regulatory regions of a battery of genes activated during the acute phase of inflammation. Further, *mlGAPDH-2* also displayed various relevant TF binding sites in the intron 1 region close to the translation start ATG site. It may suggest that those motifs may also be involved in the regulation of *mlGAPDH-2* in response to the immune stimulation, although functional typing of these sites should be performed in future. In addition, a large scale screening of *mlGAPDH-2* transcripts would be needed as a future subject in order to examine if there are differentially processed *mlGAPDH-2* mRNAs in the kidney and spleen through the alternative recognition of TATA signals in either 5′-flanking region or intron 1 (see also Mezquita *et al*., 1998). To date, molecular mechanism behind the differential regulation of fish GAPDH-2 genes by immune stimulation has not been clearly understood yet, although inflammation-induced GAPDH proteins could be proposed to be involved in triggering the apoptosis of the damaged cells based on the examinations in mammalian GAPDHs (Bannerman and Goldblum, 2003; Li *et al*., 2005). In fish, similar finding on the differential induction of GAPDH mRNAs during infectious treatments has been reported only in a couple of species previously (Cho *et al*., 2008; Booth and Bilodeau-Bourgeois, 2009). Nevertheless, previous and present data may support the possibility that the GAPDH isoforms in teleosts may undergo functional diversification in their nonglycolytic roles particularly including the innate immunity.

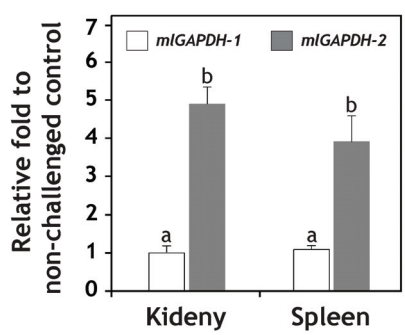

Fig. 2. Differential expression of *mlGAPDH-1* and *mlGAPDH-2* isoforms in kidney and spleen during *Edwardsiella tarda* challenge, as determined by real-time RT-PCR analysis. Based on the normalization against 18S rRNA control, mean ± SDs (from triplicate assays) with different letters were significantly different based on student's *t*-test at $p = 0.05$.

Transcriptional response of GAPDH isoforms during heavy metal exposures

Heavy metal exposures also modulated the mRNA expression of both *mlGAPDH* isoforms and the expression pattern was different between the two isoforms in which the overall inducibility of the mRNA was more significant in *mlGAPDH-1* than in *mlGAPDH-2* (Fig. 3). In the kidney, the highest induction was observed in the Cu-exposed group regardless the isoforms (14.2-fold in *mlGAPDH-1* and 4-fold in *mlGAPDH-2*). Other three metals (Cd, Mn and Zn) also activated the transcription of *mlGAPDH-1*, consequently resulting in the elevated mRNA levels ranging 5-to 11-fold relative to non-exposed control ($P < 0.05$). However unlike *mlGAPDH-1*, the exposure with Cd, Mn or Zn did not elevate the *mlGAPDH-2* mRNAs in the kidney ($p > 0.05$). Similar, but not identical, pattern was observable in the spleen. Again, both isoforms were significantly activated by the Cu-exposure in which the elevated mRNA level of each isoform was higher when compared to those achieved by other three heavy metals ($p < 0.05$). Exposure with Cd, Mn or Zn stimulated significantly the *mlGAPDH-1* mRNAs with the range from 1.4-to 4-fold ($p < 0.05$). However in *mlGAPDH-2*, only the Cd-exposure could elevate the transcripts of *mlGAPDH-2* in a small quantity but remaining other two metals resulted in no alteration of GAPDH-2 in the spleen ($p > 0.05$). Meta-induced expression of GAPDH mRNAs in fish tissues has been firstly identified, possibly extending the functional involvement of GAPDHs in metal-caused stress physiology of fish. Preferential induction of *mlGAPDH-1* is well in agreement with our finding of the presence of MRE copies only in the intron-1 of *mlGAPDH-1*, although it has not been yet empirically determined whether these MRE could be actually targeted by MTF-1 or not. In this study, Cu showed the highest fold induction for both *mlGAPDH-1* and *mlGAPDH-2* isoforms. The rapid induction of *mlGAPDH-1* by Cu exposure is in accordance with our previous finding from the metal exposure experiment with this species where Cu would be one of the most potent inducers to activate the metallothionein genes of which metal-related transcription is regulated also by MTF-1 (Cho *et al*., 2009). Based on this, the induction of *mlGAPDH-1* mRNA during Cu exposure would be, at least in part, possibly associated with the regulatory involvement of MTF-1. However, the mechanism for induction of *mlGAPDH-2* that has MRE in neither promoter nor intron 1 has not been clearly proposed yet. One possible, but untested, explanation is that the Cu exposure may cause a significant damage and/or proinflammatory response in this species, since the heavy metals including Cu have already been known to be able to elicit significantly inflammation and oxidative stress in fish tissues (Bols *et al*., 2001; Valavanidis *et al*., 2006). Severe damage or inflammation might trigger the induced transcription of *mlGAPDH-2* having various immune-and stress motifs in its regulatory region. Hence, the induced expression of *mlGAPDH-2* might be associated with inflammation-mediated process rather than direction modulation by MTF-1. Our previous finding that the viability of mud loach is more severely damaged by exposure to Cu than other metal ions (Lee *et al*., 2010) might also be supportive of the present observation on the upregulation of the *mlGAPDH-2* by Cu.

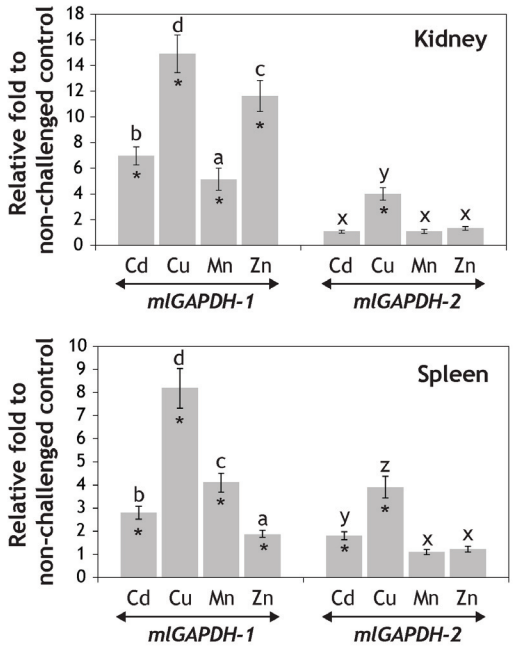

Fig. 3. Altered mRNA levels of *mlGAPDH-1* and *mlGAPDH-2* isoforms during exposure to 5 μM of either cadmium (Cd), copper (Cu), manganese (Mn) or zinc (Zn), as assessed by real-time RT-PCR analysis. The same letters on histograms (*a-d* for *mlGAPDH-1* and *x-z* for *mlGAPDH-2*) indicate that means were not statistically different based on ANOVA followed by Duncan's multiple ranged tests ($P > 0.05$). Asterisks indicate the significant difference from the control level observed in non-exposed group based on the student's *t*-test ($p < 0.05$).

In summary, the mRNA expression of two GAPDH paralogs from mud loach was significantly modulated by challenges with bacterial pathogen and heavy metals. However, the response patterns of the *mlGAPDH* isoforms to these stimulatory treatments were different from each other. The *mlGAPDH-2* has more potential in the response to immune challenge than *mlGAPDH-1*, whereas the *mlGAPDH-1* is more preferentially activated by heavy metal-mediated stimulation than *mlGAPDH-2* is. Such an isoform-dependent response pattern is in accordance with the TF binding profiles in their promoters and intron-1s. Collectively, the *mlGAPDH*s are potentially involved in cellular pathways independent of glycolysis and the two paralogs might undergo functional diversification or subfunctionalization at least at transcription level.

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