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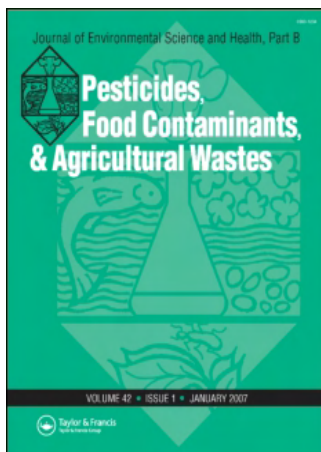
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Identification of potential biomarkers for diazinon exposure to Japanese Medaka (*Oryzias latipes*) using annealing control primers

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A new differential display-polymerase chain reaction (PCR) method based on annealing control primers was used to screen and identify potential biomarkers from differentially expressed genes (DEGs) in medaka exposed to sub-lethal concentration of diazinon (100 ppb). Among the differentially expressed genes identified, the majority were in functional categories of protein biosynthesis, transport and metabolism according to the gene ontology classification. The differential expression of ribosomal protein genes was quantified by real time PCR. The genes encoding ribosomal proteins including L3 and S17 were selected as potential biomarkers for diazinon exposure in medaka fish.

Keywords: Medaka; diazinon; biomarker; differentially expressed genes; real time PCR; annealing control primers; gene ontology.

Introduction

Organophosphorus insecticides are one of the most widely used insect control agrochemicals throughout the world. They are moderately persistent in the environment but frequent application in intensive farming areas can cause persistent surface water contamination. Their contamination of stream systems can cause toxicity to aquatic organisms.^[1] Diazinon (*O,O*-diethyl *O*-[6-methyl-2-(1-methylethyl)-4-pyrimidinyl] phosphorothioate), an organophosphorus insecticide, causes a high toxicity to organisms, especially fish and aquatic invertebrates, although it has relatively low toxic effects on mammals and humans.^[2,3] It is also a neurotoxic chemical and well-known to cause vertebral malformation and behavioral change in fish at lower concentrations.^[4]

A biomarker is defined as a change in a biological response (ranging from molecular, cellular and physiological responses to behavioral changes) which can reflect exposure to or toxic effects of environmental chemicals. Biomarkers are measurements of these responses or changes in a whole organism, body fluids, cells or tissues that could indicate the presence of contaminants or the magnitude of host response.^[5] The most intensively studied biomarkers used in the context of sentinel fish are hepatic cytochrome P4501A induced by several types of hydrophobic recalcitrant pollutants Polyaromatic hydrocarbons (PAHs), Polychlorinated Biphenyls (PCBs), and Polychlorinated dibenzo-dioxins (PCDDs), metallothionein induced by several heavy metals (Zn, Cu, Cd, and Hg), vitellogenin and choriogenin induced by xenoestrogens (PCBs, pthalates, and DDT), acetylcholine esterase (AChE) inhibition induced by neurotoxic chemicals (organophosphorus and carbamate). Extensive investigations on inhibition of cholinesterase activity by neurotoxic chemicals have clearly showed that it could be used as a tool to diagnose organophosphorus pesticide contamination in clam and fish.^[6,7] However, only a few studies have examined the effects of organophosphate insecticides on aquatic invertebrates^[8] and mussels,^[9] which are extensively used in pollution monitoring schemes^[10] and fish.^[11]

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Therefore, it is necessary to develop various biomarkers for environmental monitoring in aquatic ecosystems and a foundation of ecotoxicogenomics.

Medaka has been frequently used to identify mutagenesis in fish. Some phenotypic traits of the fish could not be found in zebrafish mutants obtained by mutagenesis.^[12] Medaka is also widely used for carcinogenesis studies^[13] and for testing endocrine disrupters in ecotoxicology.^[14] This suggests that it is useful to use medaka as a biological system that allows testing of differential expression of specific genes affected by hazardous chemical induced environmental stresses. In our previous studies, behavioral responses or their relevant enzymatic activities of medaka exposed to diazinon were used as biomarkers in an aquatic system.^[15,16]

In this report, we have tried to identify and characterize the differentially expressed genes (DEGs) whose expressions were affected by diazinon exposure at sublethal doses using a new differential display technique that is based on annealing control primers (ACPs).^[17] This technique provides a dramatic improvement of annealing specificity of primers via ACP linker and has been proven useful in identifying DEGs in various developmental stages of organisms.^[18,19] We were able to analyze specific mRNA expression patterns and to isolate differentially expressed transcripts cost-effectively. Several ribosomal RNA protein genes were selected as potential biomarker genes based on a quantitative real-time reverse transcriptase polymerase chain reaction (RT-PCR).

Materials and methods

Experimental fish and chemicals

Medaka fish were obtained from the Toxicology Research Center, Korea Research Institute of Chemical Technology (KRICT, Daejeon, Korea). The adult fish (6–12 months old; body length 3.2–3.6 cm; body weight 2.9–3.0 g) were held in a square glass chamber (40 × 22 × 40 cm) containing 30 liters of dechlorinated water (pH 6.5–7.3) and were raised with an artificial dry diet (Tetramin) under the light regime of 10 hrs of light and 14 hrs of darkness at a temperature of 25 ± 1°C. Before the experiment, tap water in the test aquarium was sufficiently dechlorinated by bubbling air under sunlight for three days. Diazinon (purity: 93.9%) was obtained from Dongyang Chemical Co., Ltd. (Seoul, South Korea). The medaka fish being starved for 48 hours were treated with 100 ppb of diazinon dissolved in dimethylsulfoxide (DMSO; 10 mg/L) along with non-treated control.^[15] The LC50 for diazinon against medaka was 5 mg/L.^[20]

Total RNA extraction

For total RNA extraction, the treated fish were immediately frozen in liquid nitrogen and preserved in a deep freezer (−70°C). Total RNA was extracted from head of the

medaka fish using acid-buffered phenol.^[21] Briefly, 20 mg of tissues were homogenized and suspended in 1 ml of phosphate buffer (pH 8.0, 0.1 M) and extracted with the acid-buffered phenol. Total RNA was then treated with RQ1 DNase (1U of DNase/5° of RNA; Promega) for 60 min at 37°C and extracted twice with phenol/chloroform, and precipitated with ethanol. Total RNA bands were identified as 18S and 28S RNA on 1.2% agarose gels.

Annealing control primer RT-PCR (ACP RT-PCR)

Annealing control primer (ACP) RT-PCR was performed in the following manner. The first step was a first-strand cDNA synthesis, which was performed using the dT-ACP1 primer (5'-CTGTGAATGCTGCGACTACGATXXXXX (T)₁₈-3') (GeneFishing™ DEG Kits, Seegene, South Korea). For amplification, purified total RNA was incubated at 80°C for 3 min after which the RT reaction was performed in 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 6 mM MgCl₂, 2 mM DTT, 1 mM of each dNTP, 20 U of RNase inhibitor, and 200 U of M-MLV (Promega, Madison, WI). The reaction mixture was incubated at 42°C for 90 min and then at 94°C for 2 min. The cDNAs were then subjected to second-strand cDNA synthesis by random PCR amplification using dT-ACP2 and one of 40 arbitrary ACPs (GeneFishing™ DEG kits, Seegene, South Korea) as primers. The polymerase chain reactions were performed using 10 pM of each primer and AccuPower PCR PreMix (Bioneer, South Korea) in a final volume of 20 µl. After incubation at 94°C for 40 sec, 65°C for 40 sec, and 72°C for 40 sec, a post-extension was performed at 72°C for 5 min.

Analysis of cDNA and real-time quantitative PCR

The amplified products were cloned into pGEM-T Easy vector of the pGEM-T Easy Vector System I (Promega Co., Madison, WI, USA) and transformed into JM109 competent cells (Promega Co., Madison, WI, USA). The plasmids were extracted, and the inserts were subjected to dideoxy chain termination sequencing (Applied Biosystems, Model 377). The identity of each insert was confirmed by sequence homology analysis using the Basic Local Alignment Search Tool at The National Center for Biotechnology Information (NCBI).

The Rapid Amplification of cDNA Ends (RACE) PCR using CapFishing™ Full-length cDNA Kit (Seegene, South Korea) was conducted using the primer pairs (5'-primer, 5'-GTC TACCAGGCATTCGCTTCAT-3'; 3'-primer, 5'-CTGTG AATGCTGCGACTACGAT-3') by following the manufacturer's instruction. Equal numbers of mRNAs of both control and treatment specimens were prepared and subjected to reverse transcription as described above. Universal 18S rRNA primer (Ambion) was used as an internal standard. The cDNAs of target genes and 18S rRNA were detected by real-time quantitative PCR

Table 1. DNA sequences of primers used for real-time polymerase chain reaction (PCR) of ribosomal protein genes

Ribosomal proteins	Sequences of forward (F) and reverse (R) primers	Melting point
L3	5'-CGTGGTCTGCGTAAAGTGGCCT-3' 5'-GGGAACCAAGAAGAGGGGGTGA-3'	60°C
S17	5'-CGAGCAAGAAGCTTCGCAACAA-3' 5'-ACTTCAAGCAGCCCCGAGGAGT-3'	60°C
S18	5'-ATGCAGAATCCCCGGCAGTACA-3' 5'-TCGCACCGTCAGTGTGTCCAAG-3'	60°C
S19	5'-AAGCTGGGAGGTGCTGGTGTG-3' 5'-TCCAGCAATTCGGTCAAGGTCC-3'	60°C
S29	5'-ACAAGATGGGCCACCAACAGC-3' 5'-CGGTCCACAGGGATCTCTGGAA-3'	60°C

(qPCR) using the target primer pairs (Table 1). The real-time qPCR was performed according to the manufacturer's real-time PCR system instructions (Mx3000P, Stratagene). The reactions were conducted according to the protocol of the DyNAmo SYBR green PCR kit (Finnzyme Oy, Espoo, Finland). The PCR protocol involved a denaturation procedure (95°C for 15 min) followed by an amplification and quantification procedures to be repeated 40 times (94°C for 20 sec, 61°C for 20 sec, and 72°C for 30 sec with a single fluorescence measurement), a melting curve procedure (65–90°C, with a heating rate of 0.2°C/sec and continuous fluorescence measurement), and finally a cooling step to 4°C. The threshold cycle (Ct) value represents the cycle number at which sample fluorescence rises statistically above the background. To obtain more quantitative PCR data from the differential expression study, the PCR efficiencies of the target and reference genes were validated with similarity. The ddCt calculation ($ddCt = dCt_{\text{Target}} - dCt_{\text{Reference}}$) for the relative quantification of target gene was used, where the reference gene was universal 18S rRNA.^[22] The real-time PCR data were analyzed by using the 2^{-ddCt} method, as explained by Livak and Schmittgen,^[22] where $ddCt = (dCt_{\text{Treatmenttarget}} - dCt_{18\text{SrRNA}}) - (dCt_{\text{Controltarget}} - dCt_{18\text{SrRNA}})$. The quantification of gene expression was expressed as mean and standard errors.

Statistical analysis

Each quantitative RT-PCR data set (12 replicates) was statistically analyzed by the two sample t-test. All *p*-values were compared to α -value of 0.05 or 0.001 to determine their significance. If *p*-values of the test samples were less than 0.05, they were considered significant. The program SPSS (version 11.0) was used for statistical analyses.

Results and discussion

Differential expressed genes in medaka exposed to diazinon

To identify genes that are differentially expressed, we compared the mRNA expression profiles of the medaka un-

treated and treated with diazinon (100 ppb). We extracted mRNAs from head portions of treated and untreated medaka fish and subjected them to ACP RT-PCR analysis using a combination of 40 arbitrary primers and two anchored oligo(dT) primers (dT-ACP 1 and dT-ACP 2) (Fig. 1). We isolated and identified 83 DEGs from the samples of head and body. Sixty-one of the 83 DEGs were known genes and the other 22 DEGs were unknown. Some representative DEGs in the head portion are shown in Table 2. These differential display patterns of control and diazinon-treated medaka as assessed by ACP RT-PCR turned out to be reproducible.

Gene ontology of DEGs

We isolated and identified 83 differentially expressed genes, 32 of which are known for their biological functions identified by biological processes of the gene ontology (Table 2). The gene ontology method in which DEGs are grouped according to a functional class as a biological process is widely used to interpret microarray data.^[23] It allows a customized database application for an effective biological interpretation of gene lists that result from an analysis of a number of DEGs data. Accordingly, 32 DEGs were grouped by several biological process categories in this study. Thirty-one percent of the DEGs were assigned to protein biosynthesis of those categories, while the others belonged to transport, metabolism, development, protein folding, homeostasis, cell adhesion, phototransduction, immune response, cell differentiation, protein modification, and fatty acid biosynthesis. Some interesting DEGs were vitellogenins, choriogenin H, peroxiredoxin, and major histocompatibility complex (MHC). Vitellogenins have been widely used as a biomarker for analyses of estrogenic effects in the environment,^[24,25] and Lee et al.^[26] has shown that mRNAs of choriogenin L and H in male medaka were induced by estrogenic chemicals and the choriogenin proteins could be used as a biomarker for endocrine disrupting chemicals. Peroxiredoxin was demonstrated to have antioxidant functions in some mammalian tissues such as lung, cartilage, and brain,^[27–29] but it remains unknown whether antioxidant defense is the only function of this protein. The major histocompatibility complex (MHC) was used to compare genomics as one of the best characterized regions of the vertebrate genome.^[30] As shown in the Table 2, the major DEGs had a higher sequence similarity to those of medaka and zebrafish. Hence, these genes were highly likely to be DEGs induced by diazinon exposure and were worthy of further investigation to develop biomarkers.

Ribosomal protein genes as potential biomarkers for toxic effects of diazinon

Interestingly, we have found several ribosomal protein genes differentially expressed: genes coding for ribosomal proteins L3, S17, S18, S19, and S29 in

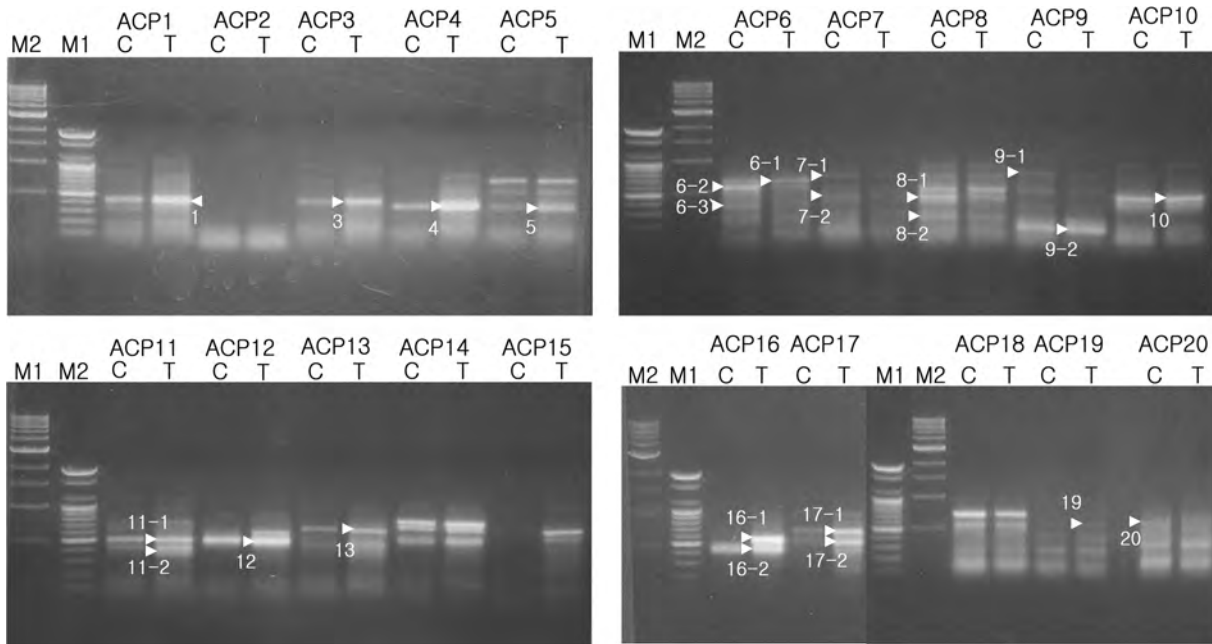


Fig. 1. Agarose gel pictures showing the amplified cDNA products obtained from head portions from control and treated samples with dT-ACP2/arbitrary 1-40ACPs in differentially expressed genes (DEGs) kit. The numbers with arrows indicate differential expressed bands between control and treated samples (C: control; T: treatment; M1: 100 bp DNA ladder; M2: 1 kb DNA ladder).

medaka (numbers of PCR products cloned: 9-1, 11-1, 3, 10, and 4 in order; Fig. 1). We obtained full-length sequences to further study their gene functions in the future. These sequences were confirmed from “The Institute for Genomic Research (TIGR) *Oryzias latipes* Gene Index (OLGI)” (http://www.tigr.org/tigr-scripts/tgi/T_index.cgi?species=o_latipes). OLGI TC Reports of ribosomal proteins L3, S17, S18, S19 and S29 were TC30644, TC36841, TC36969, TC37181, and TC37242, respectively.

Amsterdam et al.^[31] reported that the proteins S18 and S29 were related to protein biosynthesis and carcinogenesis in zebrafish. This research group showed lines with mutations in ribosomal proteins L13, L23a, S7, S18, and S29 as high-cancer lines. Significant changes in expression of ribosomal proteins were observed in human colorectal cancer cells but correlations between the expression levels and disease severity were not found.^[32] Yet abnormal patterns with additional larger transcripts were observed for rpL5, rpL28 and rpS10.

Gene expression levels of all five ribosomal protein genes were higher than the control level (1.0) that was based on ddCt (= 0). Genes for proteins L3 and S17 showed the highest level (Fig. 2). According to the result of *p*-values of significant levels, five ribosomal proteins demonstrated a potential usefulness as molecular biomarkers for fish affected by toxic chemicals such as diazinon in the aquatic ecosystem.

It has been known that ribosomal biogenesis and translation are regulated at multiple levels and are associated with accurate cell growth and proliferation. The loss of

key checkpoints during protein synthesis may contribute to the initiation and progression of cancer.^[33] Moreover, alterations in the expression of ribosomal proteins seem to be consistently associated with tumorigenesis. Mutations

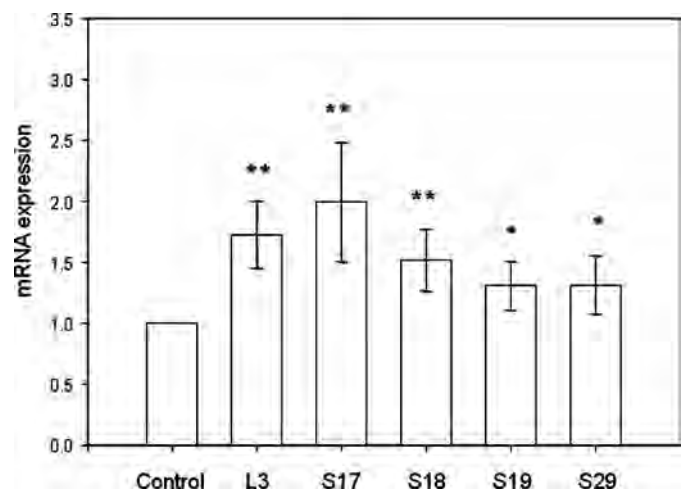


Fig. 2. The mRNA expression level measured by real time quantitative PCR of the ribosomal protein genes compared to the control (equivalent to 1.0; calculated based on Livak and Schmittgen,^[22]). Each expression level was a mean (\pm standard deviation) for the 12 independent measurements of each treatment target gene. All *p*-values derived from t-test were compared to α -values 0.05 and 0.001 to determine the significance of diazinon treatment. If *p*-values were less than 0.05, they were considered significant: **p* < 0.05 and ***p* < 0.001.

Table 2. Ontology relations of some representative differentially expressed genes (DEGs) in head portions of medaka by BLASTN search.

Identity	Clone number ¹	Base pairs sequenced	GenBank Acc. No.	Homology (%)	Gene ontology
<i>Pagrus major</i> 40S ribosomal protein S18 mRNA	3	358	AY190738	295/318 (92%)	Protein biosynthesis
<i>Hippocampus comes</i> 40S ribosomal protein S29 mRNA	4	288	AY357068	159/175 (90%)	
<i>Danio rerio</i> cDNA clone MGC:66406 IMAGE:5915478	5	322	BC096865	160/184 (86%)	
<i>Pagrus major</i> ribosomal protein L8 mRNA	6–1	563	AY190734	360/398 (90%)	
<i>Ictalurus punctatus</i> ribosomal protein L3 mRNA	9–1	768	AF401554	313/366 (85%)	
<i>Danio rerio</i> ribosomal protein L3 (rpl3) mRNA	13	577	AY561514	414/475 (87%)	
<i>Pagrus major</i> ribosomal protein S19 mRNA	10	324	AY190739	214/245 (87%)	
<i>Ictalurus punctatus</i> 40S ribosomal protein S17 mRNA	11–1	429	AF402826	182/202 (90%)	
<i>Oryzias latipes</i> mRNA for embryonic alpha-type globin	30–3	535	AB026052	522/525 (99%)	Transport
<i>Oryzias latipes</i> mRNA for fructose-bisphosphate aldolase A	38–1	636	AB111381	563/564 (99%)	Metabolism
<i>Mus musculus</i> pyruvate dehydrogenase kinase, isoenzyme 2 (Pdk2)	20	1065	AY414728	109/131 (83%)	
<i>Oryzias latipes</i> mRNA for vitellogenin II	16–1	513	AB074891	497/498 (99%)	
<i>Verasper moseri</i> gene for preproinsulin	7–2	385	AB029318	103/114 (90%)	Development
<i>Oryzias latipes</i> mRNA for choriogenin H	9–3	106	D89609	100/102 (98%)	
<i>Mus musculus</i> peptidylprolyl isomerase (cyclophilin)-like 2 (Ppil2)	16–2	364	NM_001017383	29/30 (96%)	Protein folding
<i>Dicentrarchus labrax</i> HSP-90 mRNA	35–1	651	AY395632	219/253 (86%)	
<i>Danio rerio</i> chaperonin containing TCP1, subunit 2 (beta) (cct2)	17–4	328	BC050953	106/121 (87%)	
<i>Branchiostoma belcheri</i> tsingtaunese peroxiredoxin V protein (AmphiPrxV) mRNA	8–1	466	AF498232	64/74 (86%)	Homeostasis
<i>Oryzias latipes</i> mRNA for ferritin H3	11–2	353	AJ238012	336/351 (95%)	Homeostasis, transport
<i>Oryzias latipes</i> OIGC6 gene for guanylyl cyclase C	12–3	324	AB016081	59/68 (86%)	Photo-transduction
<i>Oryzias latipes</i> olgc3 gene for membrane guanylyl cyclase OIGC3	17–3	382	AB049126	90/102 (88%)	
<i>Oryzias latipes</i> MHC Class 1 Region	6–2	486	BA000027	430/455 (94%)	Immune response

¹Number of each clone is derived from the number of each annealing control primer set.

in genes directly responsible for encoding S19 ribosomal protein have been found in the cancer susceptibility syndromes, Diamond–Blackfan anemia.^[34] It appears to be evident that the regulation of ribosome function could be lost in cancer cells. In fact, many ribosomal proteins that belong to the small (S) and the large (L) ribosomal subunits are overexpressed in cancer cell lines, as well as in primary tumors.^[35–36] Microquantity differential display analysis of gene expression profiles between benign (PNT2) and malignant (PC3M) human prostate cell lines identified the

gene encoding ribosomal protein L19 (RPL19) to be over-expressed in the malignant cells.^[37] A differential display technique was recently used to identify new molecular indicators for cadmium exposure to the freshwater bivalve *Corbicula*.^[38] In this study, one differentially expressed gene, ribosomal protein S9 (rpS9), was identified using real-time reverse transcription-polymerase chain reaction. This gene was shown to be up-regulated by cadmium (15 µg/L) and down-regulated by zinc (1 mg/L) while a similar result was obtained in the field test conditions tested. Moreover,

down- and up-regulated expressions of ribosomal proteins were observed in the liver of mouse treated with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin:^[39] ribosomal proteins L9, S3 and S6 for down-regulation; ribosomal proteins L7a and S8 for up-regulation. They were involved in RNA processing, splicing and stability. Numerous other extraribosomal functions of ribosomal proteins were reported such as DNA repair/replication and regulation of development.^[40] Biological significances of the ribosomal protein genes found in our study have yet to be demonstrated in detail. However, little attempt has been made to develop a biomarker from ribosomal protein genes of fish to monitor the exposure of diazinon as a toxicant in an aquatic environment.

Conclusion

A new differential display-PCR method using annealing control primers and gene ontology grouping has enabled identification of potential diagnostic biomarker genes related to functional activities such as protein biosynthesis, development, homeostasis, cell adhesion, and immune response. Real-time quantitative PCR has further confirmed a significant level of differential expressions of ribosomal protein genes. Genes encoding the five ribosomal proteins demonstrated a possibility of biomarkers for fish affected by toxic chemicals including diazinon in aquatic environment and potentially ecosystems. In addition, some genes involved in the medaka development (genes for vitellogenin II, choriogenin H, and preproinsulin) could be good potential biomarkers. Once specific functions of all these DEGs are known, they could contribute to a further development of more effective biomarkers for monitoring an aquatic ecosystem contaminated with organic pollutants including diazinon and other organophosphate toxicants.

Acknowledgments

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