유기인산계 농약에 노출된 송사리의 환경위해성 생물마커 개발

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Development of hazardous biomarkers for environment in Medaka, *Oryzias latipes*, Exposured to an organophosphate pesticide

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1. Introduction

The biological system includes biomarkers that are molecular and physiological indicators of chemical stress [1]. In the present study, we focused on developing a variety of hazardous biomarkers of specific expression in medaka exposed to the ecotoxic chemical. Differential display methods, which are based on PCR using short arbitrary primers, are simple and fast [2], but suffer from high rates of false positives. Recent developments in molecular biology offer new possibilities for developing biomarker with the least of false positive. In this work, a new differential display-PCR (DD-PCR) method based on annealing control primers (ACPs) [3] was investigated in order to develop hazardous biomarkers from differentially expressed genes (DEGs) of mdeaka (*Oryzias latipes*) in the exposed to diazinon, an organophosphate pesticide, and in the unexposed areas. This is an easy technique without false positives that only allows real products to be amplified. We used it to analyze specific mRNA expression patterns shown in medaka exposed to diazinon and to isolate differentially expressed transcripts. Furthermore, the biological meaning of the gene expression changes was analyzed using the gene annotation information provided by the Gene Ontology (GO) Consortium (http://www.geneontology.org) and we confirmed the gene expression changes using real-time qPCR.

2. Experimental procedure

2.1. Experimental animal and chemical

Medaka (*Oryzias latipes*) was obtained from Toxicology Research Center, Korea Research Institute of Chemical Technology (KRICT, Daejeon, Korea). The fish were held in a square glass chamber (40 ×22 ×40 cm) containing 30-liter of dechlorinated water (pH 6.5-7.3) with aeration and was reared with artificial dry diet (Tetramin®) under the light regime of L10: D14 at a temperature of 25±1℃. Diazinon (purity: 93.9%) was obtained from DC Chemical Co. Ltd. (Seoul, Korea) and the medaka was treated under appropriate sublethal concentrations of diazinon. The fish were subjected to diazinon treatment after starvation for 48hr [1].

2.2. Annealing Control Primer RT-PCR and Cloning of cDNA

Total RNA was extracted from medaka using acid phenol [4]. The first step is first-strand cDNA synthesis, which is performed using the dT-ACP1 primer (GeneFishing™ DEG kits, Seegene, Korea), 20 U of RNase inhibitor, and 200 U of M-MLV (Promega). For the amplification, the RT reactions were conducted according to the protocol of GeneFishing™ DEG kits. The cDNAs are then subjected to second-strand cDNA synthesis by random PCR amplification using dT-ACP2 and one of 40 arbitrary ACPs (GeneFishing™ DEG kits, Seegene, Korea) as primers. This PCRs were conducted according to the protocol of GeneFishing™ DEG kits. The amplified products were cloned into the pGEM -T Easy vector of the pGEM - T Easy Vector System Ⅰ (Promega) and transformed into JM109 competent cells (Promeg). The colonies were grown for 16-18 hr at 37℃ on Luria broth agar plates containing ampicillin, X-gal (5-bromo 4-chloro 3-indoyl-b-D-galactopyranoside), and isopropyl-b-D-thiogalactopyranoside were used for blue/white colony selection. The plasmids were extracted and the inserts were subjected to dideoxy chain termination sequencing (Applied Biosystems, Model 377). The identity of each product was confirmed by sequence homology analysis using the Basic Local Alignment Search Tool at NCBI.

2.3. Real Time qPCR analysis

The real-time qPCR was performed according to the real-time PCR system manufacturer's instructions (Mx3000P, Stratagene). Universal 18s rRNA primer (Ambion) was used as an internal standard. The cDNAs of target genes and 18s rRNA were then detected by real-time qPCR using the target primer pairs shown in Table 1. The reactions were conducted according to the protocol of the DyNAmo SYBR green PCR kit (Finnzyme Oy, Espoo, Finland).

Table 1. Primer sequences of real-time PCR for ribosomal protein genes.

Gene	Forward (F) and Reverse (R) primer sequences	m.p.
RPL3	(F) 5'-CGTGGTCTGCGTAAAGTGGCCT-3' (R) 5'-GGGAACCAAGAAGAGGGGGTGA-3'	60℃
RPS17	(F) 5'-CGAGCAAGAAGCTTCGCAACAA-3' (R) 5'-ACTTCAAGCAGCCCCGAGGAGT-3'	60℃
RPS18	(F) 5'-ATGCAGAATCCCCGGCAGTACA-3' (R) 5'-TCGCACCGTCAGTGTGTCCAAG-3'	60℃

RPS19	(F) 5'-AAGCTGGGAGGTGCTGGTGTTG-3' (R) 5'-TCCAGCAATTCGGTCAAGGTCC-3'	60℃
RPS29	(F) 5'-AACAAGATGGGCCACCAACAGC-3' (R) 5'-CGGTCCACAGGGATCTCTGGAA-3'	60℃

3. **Results and discussion**

To identify genes that are specifically expressed, we compared the mRNA expression profiles of the medaka untreated and treated with diazinon. To do this, the mRNAs from head and body portions of each of medaka fish exposed and un-exposed to diazinon were extracted and subjected 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. 2). We identified and isolated 83 DEGs. 61 of the 83 DEGs are known genes and the other 22 are unknown genes. These differential display patterns between control and treatment in medaka as assessed by ACP RT-PCR were very reproducible.

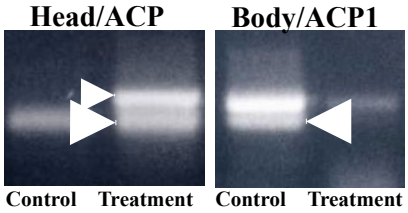

Fig. 2. Examples of gel photographs that show the differential banding patterns identifying DEGs of the medaka untreated (Control) and treated (Treatment) by diazinon. The arrows indicate DEGs that show up-regulated or down-regulated expression of the medaka untreated by diazinon. The left blot shows the identification of head portions of medaka using the ACP 16 primer, while the right one shows the identification of body of medaka using the ACP 16 primer.

We analyzed 41 genes of 83 genes from which function is known using the biological processes of Gene Ontology (Fig. 3).

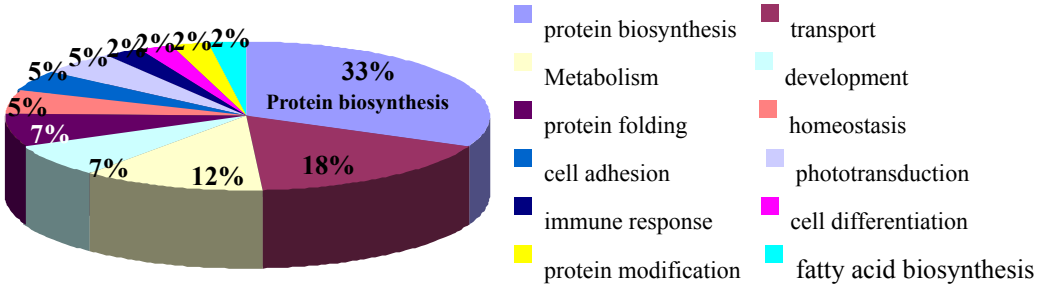

Fig. 3. Overview of 41 gene expression through analysis using the biological process of Gene Ontology.

Among other things, we focused on ribosomal proteins related to protein biosynthesis and known as cancer related genes [5]. Also, we confirmed the substantial gene expression changes using real-time qPCR. qPCR experiments used relative quantification without using standard curves, the efficiencies of the target and reference genes validated similarity and the ddCt calculation for the relative quantification of target was used, where reference gene was universal 18s rRNA. The real-time PCR

data were analyzed by using 2^{-ddCt} method [6]. The results of analyzed data were expressed as vertical bar chart of mean and standard errors and shown only up-regulated relative quantity of treatment target in comparison with control target in figure 5. According to result of *p*-values of considered significant levels, five ribosomal proteins demonstrated possibility as molecular marker in aquatic ecosystem.

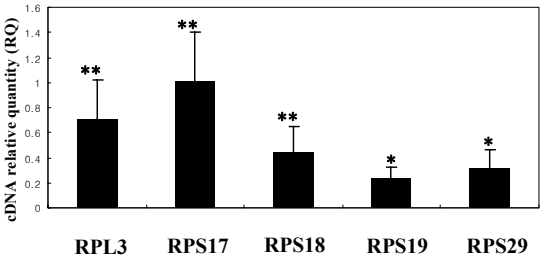

Fig. 5. cDNA relative quantity (RQ) of treatment target in comparison with control target. Each of the error bar represents values (±standard deviation) for mean of twelve measurements in treatment target gene. All *p*-values were compared to an α-values of 0.05 to determine significance, and *p*-values of < 0.05 were considered significant. The program SPSS (version 11.0) was used for all statistical calculations, $*P < 0.05$ and $***P < 0.001$ compared with each of control (t-test).

4. Conclusion

We identified 83 DEGs from medaka treated with diazinon. Among these genes, ribosomal protein genes related cancer were quantified by real-time PCR. As a result, we identified potential possibility as hazardous biomarkers in aquatic ecosystem.

5. References

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