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Identification and characterization of expressed sequence tags from the liver of rare minnow (*Gobiocypris rarus*)

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Abstract

Rare minnow (*Gobiocypris rarus*) is a newly developed aquatic test organism that has been widely used in a range of studies of toxicological risk assessment by virtue of its higher sensitivity to xenobiotics. To describe extensively the transcripts expressed in the livers of adult rare minnow, we generated 6919 high-quality expressed sequence tags (ESTs) from a non-normalized cDNA library. After processing, a total of 1773 unigenes (unique genes) comprising 771 contigs (consensus sequences) and 1002 singlets were acquired. Based on the analysis by BLAST, 1512 unigenes (85%) had been identified and annotated. The result of functional classification reveals that the genes involved in the processes of general metabolism prevail in liver expressed genes. In addition, we compiled a potentially toxicology-related catalog comprising 262 unigenes that associated with metabolism of xenobiotics and adaptive responses. There are eleven groups referring to diverse functions in the catalog. This report provides the first set of genetic data for rare minnow which is of great value for further exploitation of this species in functional genomics and toxicogenomics, and sets a basis for the discovery of new molecular markers of exposure and for the production of the function-focused microarray.

Keywords: cDNA library; Transcriptomics; Teleosts; Toxicogenomics

1. Introduction

Rare minnow (*Gobiocypris rarus*), a newly developed aquatic test organism, has been reported to be an ideal fish for toxicological risk assessment of a wide range of chemicals (Zhou et al., 2002). Compared with fathead minnow (*Pimephales promelas*), rainbow trout (*Oncorhynchus mykiss*), zebrafish (*Danio rerio*), medaka (*Oryzias latipes*) and some other aquatic test teleosts recommended by the International Organization for Standardization (ISO), rare minnow has many attractive characteristics that make it suitable for aquatic toxicity tests. The characteristics include sensitivity to chemicals, small size, wide temperature range, ease of laboratory culture, and short embryonic development period (Zhou et al., 1995; Lu and Shen, 2002). To date, a variety of studies have been conducted utilizing rare minnow to evaluate the impact of many chemicals (Yang and Feng, 2003; Ma et al., 2004; Zhong et al., 2004, 2005; Zha et al., 2007).

Although it has been widely recognized and utilized in broader fields of research, there are few genetic resources or any transcriptional data about this organism available to the public. This forms a formidable obstacle to exploiting this organism for further study. Many results could not be clearly elucidated due to the lack of knowledge of the alteration induced by xenobiotics on the level of gene transcription and protein expression prior to the endpoint effects, on account of the scarcity of genetic resources.

Here, we applied expressed sequences tag (EST) analysis to characterize the gene expression profile of liver of rare minnow. Besides, given that rare minnow is a newly developed species for chemical toxic test, the genes involved in absorption, transportation, biotransformation, metabolism of xenobiotics and adaptive responses elicited by chemicals are of great value. Thus, we attempted to compile a potential toxicological catalog recruiting the genes that are involved in the toxicological

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processes stimulated by chemicals exposure. The catalog will facilitate the better use of the rare minnow liver EST for toxicogenomic researches in teleosts.

This work will not only help to enhance gene discovery and functional genomic research in rare minnow, but also set a basis for toxicogenomics in rare minnow, for the identification of molecular biomarkers of exposure, for elucidating the molecular mechanism of action of various chemicals and for the construction of the function-focused microarray (Chini et al., 2006).

2. Materials and methods

2.1. cDNA library construction

The adult rare minnows (about 9 months old) were obtained from a laboratory hatchery. The sex ratio was bout 1:1 and the gender was determined by observing the shape of the abdomen and the distance between the abdomen fin and the stern fin. After anesthetized on ice, the livers were dissected and immediately frozen in liquid nitrogen. Total RNA was isolated with TRIzol reagent (Invitrogen, CA, USA), and mRNA was purified with Oligotex mRNA Isolation Kits (Qiagen, Valencia, CA, USA) according to the supplier's method. The cDNAs were synthesized with Xho I primer and SuperScript II-RT (Invitrogen) and DNA polymerase I (Promega) following the manufacturer's instruction. All cDNAs were flanked by EcoR I (Stratagene, TX, USA) and cut by Xho I (Stratagene). The cDNAs with two adaptors were extracted by electrophoretic separation with three fractions (0.5–1 kb, 1–2 kb, >2 kb) and ligated to EcoR I and Xho I digested pBluescript II SK (+) vector. The plasmid was transformed into *E. coli* (DH10B) to amplify the cDNA using a MicroPulser™ electroporation system (Bio-Rad) under the standard condition. The cDNA clones were picked randomly. Sequence messages were acquired through Megabase 1000 DNA sequencer (Amersham Pharmacia) from the 5′-end using the T3 primer.

2.2. Bioinformatics

Firstly, the chromatogram files were processed as raw sequencing data for base-calling and quality assessing by PHRED (Ewing et al., 1998). The low-quality sequences were trimmed off with Q20 (99% accuracy). The vector sequences were screened with CROSS_MATCH program. All sequences were then compared to linker sequences and interrupted on the locus of the linker to generate high-quality ESTs for later analysis, after eliminating the ESTs shorter than 100 bp. The sequence assembly software Phrap was used to build the consensus sequences from all high-quality and clean ESTs with 30 minmatch and 0.98 repeat stringency, and to view the result for each cluster. As assembled results, any singlet (not grouped) or contig (consensus sequence) was said to be a unigene, that is, a unique gene.

Unigenes were annotated by sequence similarity comparison against the non-redundant (nr) protein database, nucleic acid (nt) database and dbEST database in GenBank with the BLAST algorithm (BLASTX and BLASTN) (Altschul et al., 1997). Assignment of putative identities with the nr protein database required maximal *E*-values of $1E^{-5}$. Any unigenes failing to match the nr protein database were successively searched with nt database and dbEST database at *E*-values $<1E^{-10}$ as recognized putative identities. The standards of exact choice of most related entries in each group of alignments depended not only on the best hit values but also on the detailed information of matched sequences (Liu et al., 2006). To identify the function of each unigene, all unigenes were firstly annotated by BLASTX with minimum *E*-values of $1E^{-5}$ against the UNIPROT (UniProtKB/Swiss-Prot, UniProtKB/TrEMBL, and PIR) protein database followed by classifying the matched known genes into different functional categories according to Gene Ontology (GO) (Ashburner et al., 2000).

3. Results and discussion

3.1. Overview of ESTs from rare minnow liver cDNA library

A non-normalized cDNA library was constructed from adult rare minnow liver. A total of 7386 random clones were partially sequenced from cDNA 5′ends to generate ESTs. After screening, processing and eliminating the low-quality ESTs, we obtained 6919 high-quality ESTs ranging from 100 bp to 808 bp. A large portion of ESTs lie between 400 and 700 bp and the average read length is 555 bp. The sequences were deposited in the NCBI Expressed Sequence Tags database (www.ncbi.nlm.nih.gov/projects/dbEST) and are available with the following accession numbers: from EE392478 to EE399396.

The 6919 high-quality ESTs were processed with the Phrap assembly program to estimate the number of unique transcripts represented in the rare minnow liver library. As a result, 771 contigs composed of 5917 ESTs (85% of the total ESTs) were generated, whereas the remaining 1002 ESTs (15% of the total ESTs) did not cluster, and are called singlets. The number of unigenes identified from the rare minnow liver EST set was therefore estimated at 1773.

The cDNA library constructed is a non-normalized primary library without amplification, so the clone abundance or the cluster size will reflect the relative mRNA population (Zhang et al., 2006). Groups of singlets and contigs (2–9), containing fewer ESTs for each unigene, account for the larger fraction of unigenes or ESTs, and approximately one-fourth of ESTs fit into clusters that are comprised of more than 50 ESTs, reflecting the complexity and specificity of the transcript population of the rare minnow liver (Fig. 1).

3.2. Gene identification and annotation

All the unigenes generated were searched for homologies in the non-redundant (nr) protein database and nucleic acid (nt) database in GenBank by BLAST to acquire their annotations. As a result, 1355 unigenes (76% of the total unigenes) produced BLAST match against the nr protein database under the *E*-value of $1E^{-5}$. The subsequent search, without hits to nr protein database against nt database, revealed merely 157 genes (9% of the total unigenes) matches with *E*-value $<1E^{-10}$. Another 59

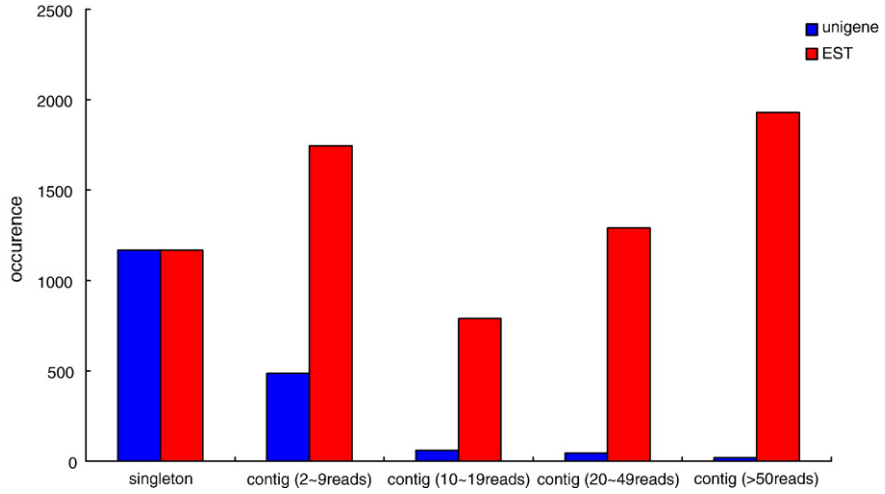

Fig. 1. Prevalence distribution of unigene cluster sizes. The majority of generated unigenes consist of 2 to 9 ESTs and singletons, and approximately one-fourth of ESTs fit into clusters that are comprised of more than 50 ESTs, 'Singleton' represents the ESTs that have not been grouped, while 'contig' refers to the consensus sequences.

ESTs representing 49 unigenes matched the existing ESTs in the dbEST database. The unigenes matched to the dbEST together with the unmatched ones made up the still unknown gene clusters consisting of 261 unigenes. This probably suggested that there are a few identified genes that are novel or particularly expressed in rare minnow liver. In addition, the average length of 261 unidentifiable unigenes was 465 bp that was shorter than that of known unigenes which was 533 bp in average. This suggested that some of unknown unigenes may be the UTR sequences outside the ORF (open reading frame). Therefore, the number of known unigenes identified from liver cDNA library of rare minnow was 1512, representing 85% of the total unigenes.

Among all the gene annotations from the matches to the nr protein database, genes from teleosts account for the largest group (90% or 1227 of 1355 unigenes). Within this group, zebrafish derived genes constitute the absolute majority (74% or 1003 of 1355 unigenes). The result can be attributed mostly to the same taxonomy (the Rasborinae) of these two fishes rather than the availability of the considerable amount of zebrafish genetic data. The result of a larger number of zebrafish gene hits helps confirm the good sequence similarity in expressed genes between the rare minnow and zebrafish. Accordingly, considering the great number of sequences resources from zebrafish that are available to the public, we may further exploit the genetic resources from rare minnow on the ground of their higher similarity in sequences.

The highly expressed genes (more than 50 reads) in rare minnow liver are listed in Table 1. The most abundant gene detected 203 clones and encoded a protein similar to Mid1 interacting protein, a thyroid hormone-inducible hepatic protein spot 14 that is thought to be required for induction of hepatic lipogenesis (Zhu et al., 2001). In general, it can easily be seen from Table 1 that the genes involved in metabolic processes predominate in rare minnow liver. 14 kDa apolipoprotein (140 clones), apolipoprotein A-I (102 clones), delta-9-desaturase (79 clones), and fatty acid binding protein 10 (75 clones) belong to an enzyme group correlated with fatty acid metabolism; fructose-bisphosphate aldolase b (64 clones) belongs to the enzyme group involved in carbohydrate catabolism; and serpina1 protein (102 clones) are related to protein metabolism. This is consistent with the high catabolic activity of the liver, the essential tissue for lipid, protein and

Table 1
Identification of the highly abundant genes (>50 reads) in rare minnow liver

Unigene [a]	Reads [b]	*E*-value	Annotation [c]	Species [d]
uni68397228	203	$8.00E^{-35}$	Similar to Mid1 interacting protein	*Danio rerio*
uni13359451	181	$6.00E^{-65}$	Putative senescence-associated protein	*Pisum sativum*
uni55610734	144	$1.00E^{-17}$	Similar to rRNA intron-encoded homingendonuclease	*Pan troglodytes*
uni58802481	140	$2.00E^{-30}$	14 kDa apolipoprotein	*Carassius auratus gibelio*
uni29570700	111	0	Cytochrome c oxidase subunit I	*Sarcocheilichthys variegatus microoculus*
uni49900376	102	0	Serpina1 protein	*Danio rerio*
uni68443519	102	$1.00E^{-110}$	Similar to apolipoprotein A-I	*Danio rerio*
uni4572552	98	0	Vitellogenin precursor	*Pimephales promelas*
uni801738	92	0	Warm temperature acclimation-related 65-kDa protein	*Carassius auratus*
uni5738564	79	$1.00E^{-173}$	delta-9-desaturase	*Ctenopharyngodon idella*
uni51980432	75	$3.00E^{-58}$	Fatty acid binding protein 10, liver basic	*Danio rerio*
uni42734425	64	0	Aldolase b, fructose-bisphosphate	*Danio rerio*
uni15778562	63	$1.00E^{-165}$	Vitellogenin	*Cyprinus carpio*
uni20385167	52	$2.00E^{-20}$	Toxin-1	*Oncorhynchus mykiss*
uni15293929	51	$3.00E^{-59}$	60S ribosomal protein L30	*Ictalurus punctatus*

[a] Unigenes were acquired by Phrap software and clustered manually.
[b] Number of sequenced clones in unigenes.
[c] The best match from BLASTX.
[d] The original organism of annotation.

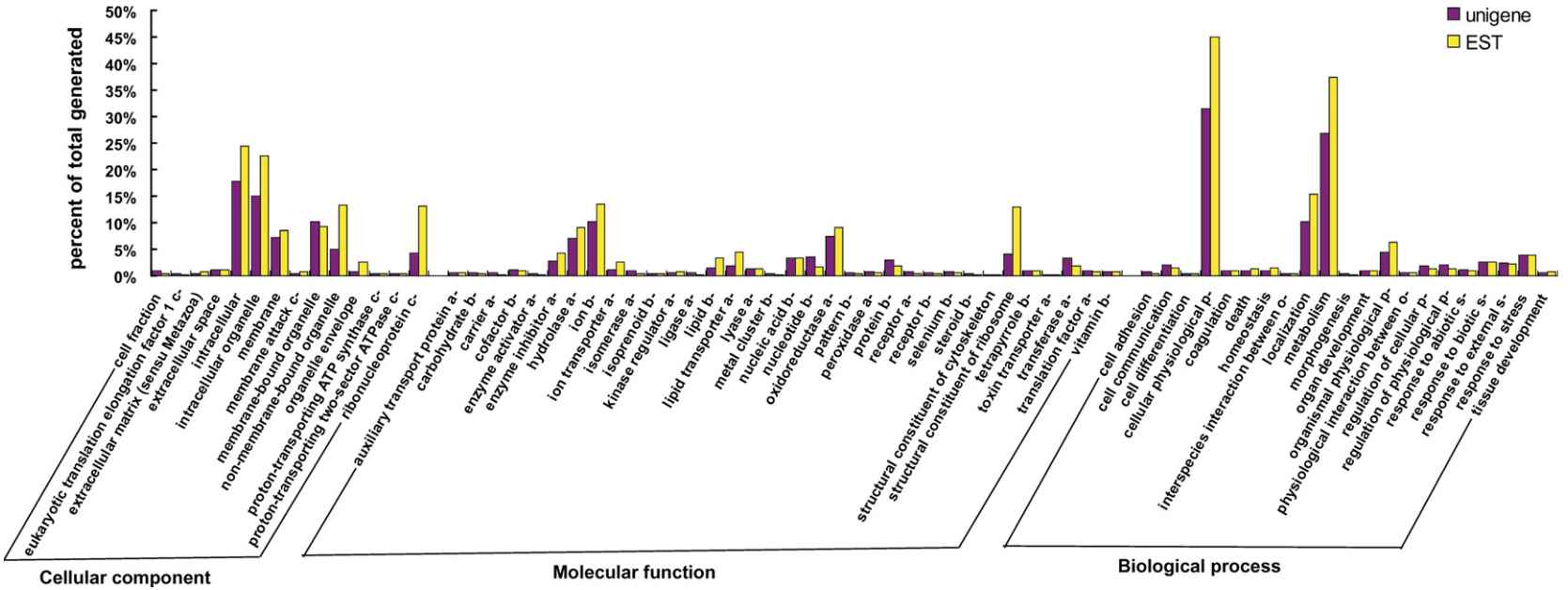

Fig. 2. GO classification. A total of 731 genes from rare minnow liver cDNA library were assigned to the three main groups in GO: cellular components, molecular function and biological process that were not exclusive to or within these groups. Genes involved in intracellular components comprise the largest group among all cellular components. According to the molecular function, many expressed genes have hydrolase and oxidoruductase activity. In terms of the biological process group, a majority of genes in the liver are associated with metabolism, consisting of 35% of all ESTs, which strongly verifies the vigorous catabolic activity of the liver. Unigenes having fewer than seven ESTs (about 0.1% of total ESTs) are not included. In the GO functional categories, a-, b-, c-, p-, and s- stand for activity, binding, complex, process and stimulus, respectively.

carbohydrate metabolism (Feldmann, 1994). In addition, genes involved in the mitochondrial respiratory chain, such as cytochrome c oxidase subunit I (111 clones), and ribosomal protein like 60 S ribosomal protein L30 (51 clones), also take up a large portion of expressed genes in rare minnow liver. Besides these, warm temperature acclimation-related 65-kDa protein whose expression increases with temperature (Picard and Schulte, 2004) have been identified in many transcripts. Remarkably, another intriguing highly abundant gene is Vtg, a vital index and biomarker for monitoring endocrine-disruption of chemicals in the aquatic environment (Liao et al., 2006). Although rare minnow has been widely used in assessment of chemical endocrine-disruptions (Zhong et al., 2005), few sequences of Vtg from this species has been acquired in public databases. Here we obtained 63 and 98 ESTs of Vtg and its precursor, respectively. As many as 63 clones are sufficient to build a consensus sequence of more than 2000 bp of Vtg.

3.3. Known gene expression functional profile

A total of 731 genes were assigned to the three main groups in GO: cellular components, molecular function and biological process that were not exclusive to or within these groups. As far as the cellular components are concerned, the intracellular group is the biggest, suggesting that almost all ESTs are from intracellular components except for no more than about 1% of the total ESTs from the extracellular space. According to the molecular function, many expressed genes have hydrolase and oxidoruductase activity. These are consistent with the great number of enzymes responsible for the major metabolism in liver. Besides, the genes involved in structural constituents of ribosome expressed abundantly, account for 13% of the total ESTs. The similar results had been reported for liver of sea bass (Chini et al., 2006). In terms of the biological process group, a majority of genes in the liver are involved in metabolism, consisting of 35% of all ESTs, which strongly verifies the vigorous catabolic activity of the liver (Fig. 2).

3.4. The potential toxicology-related clusters

In order to compile a catalog of potential toxicology-related genes, we conducted a comparative analysis between the putative identities of rare minnow liver ESTs annotated in GenBank and data from the rat toxicology U34 array. The rat toxicology U34 array is a gene expression array designed by Affymetrix (www.affymetrix.com). The array, embracing more than 850 collaboratively selected mRNA transcripts and EST clusters, correlated with the response to toxicants, and has been widely (successfully) used in many predictive toxicology studies. The comparative analysis was performed by matching the annotations of rare minnow unigenes to the genes names of rat toxicology U34 array. Combining the results of comparison with the toxicology U34 array and the function described by GO, the toxicology-related transcripts were picked out and assigned to the corresponding group in the toxicological catalog.

This catalog involved eleven groups of genes of diverse functions: (1) xenobiotic metabolic enzyme; (2) stress and defense; (3) signal transduction (4) growth, apoptosis and cell cycle regulation; (5) immune response; (6) mitochondrial energy metabolism; (7) kinase and phosphatase; (8) gene expression and regulation; (9) structure and motility; (10) related to growth factors and receptors; and (11) channel and transport (Table 2). As many as 262 unigenes from rare minnow liver cDNA library had been enrolled in the catalog. Since some genes have multiple functions, they would be assigned to different groups simultaneously. The genes appear distinct expressed levels among the groups and a total of 1030 ESTs are involved in this catalog (Table 2).

The biggest group comprises 61 genes associated with channel and transport. Many genes responsible for transportation of lipid, protein, electron, ion, and etc. have been identified in our library, such as apolipoprotein A, protein transport protein, potassium large conductance calcium-activated channel, transferrin variant and multidrug resistance-associated protein, etc. This kind of genes is essential for organism to maintain the metabolism and keep the internal balance under the normal condition. Meanwhile, these genes, when insulted by xenobiotics, play an important role in absorption and transportation of xenobiotics to the target organs or cells (Bleasby et al., 2006; Lickteig et al., 2007; Hagos et al., 2007), like Multidrug resistance-associated protein (Hoffmann and Kroemer, 2004), or as the target molecular impaired directly or indirectly by toxicant (Fleck et al., 2003; Hitzl et al., 2003; Cermak and Wolffram, 2006) (details see supplementary data).

The number of genes involved in stress and defense, immune response and gene expression and regulation are approximate 30 for each group. The group of stress and defense contains the stress-responsive and protective genes involved in response to oxidative stress, superoxide metabolism, killing the cells of another organism, defense responses to pathogens, acute-phase response and DNA repair. Besides, we identified many transcripts which take part in the humoral immune response and inflammatory response, like complement C3, C1, C9, macrophage inflammatory protein, inteleukin and so on. In the group of gene expression and regulation, some transcription factors, enhancer binding protein, zinc finger protein and some other transcriptional regulating sequences have been identified in many transcripts.

Table 2
Overview of potential toxicology-related clusters identified from rare minnow liver cDNA library

	Number of unigenes	Number of ESTs
Xenobiotic metabolic enzyme	19	31
Stress and defense	34	119
Signal transduction	21	61
Growth, apoptosis and cell cycle regulation	12	18
Immune response	34	128
Mitochondrial energy metabolism	12	204
Kinase and phosphatase	14	17
Gene expression and regulation	37	128
Structure and motility	10	18
Related to growth factors and receptors	8	15
Channel and transport	61	291
Total	262	1030

These genes can help elucidate the mechanism of chemical action in terms of the alteration in gene expression (details see supplementary data).

The group of signal transduction which includes 21 genes enrolls many important components in six signaling pathways, including the G-protein coupled receptor signaling pathway, insulin receptor signaling pathway, Wnt receptor signaling pathway, enzyme linked receptor protein signaling pathway, small GTPase mediated signal transduction, and the stress-activated protein kinase signaling pathway. It has been shown that these signal pathways regulate a good many of processes including organogenesis, angiogenesis, stem cell proliferation, carcinogenesis (Blitzer and Nusse, 2006). Additionly, a great number of studies confirm that apoptosis and misregulation of the cell cycle are largely involved in carcinogenesis (Barbacid et al., 2005; Chen et al., 2006; Wright and Deshmukh, 2006). Thus, these sequences of the groups responsible for signaling transduction and growth, apoptosis and cell cycle regulation will allow us to use rare minnow in the study of carcinoma and development. In addition, xenobiotics metabolism enzymes, the key role in toxic response, comprise 19 members from our library. The number of genes from groups of mitochondrial energy metabolism, kinase and phosphatase, structure and motility and related to growth factors and receptors are 12, 14, 10 and 8, respectively (details see supplementary data).

This catalog of genes is potentially involved in the absorption, transportation, biotransformation, metabolism of xenobiotics and adaptive responses elicited by chemicals. No doubt, a good number of toxicology-related genes would not be expressed or up-expressed to the levels that are detectable unless the insult by chemical exposure. Accordingly, due to the un-stimulated fish samples we used, we could not get access to the good abundant toxicology-related genes associated with the complex processes induced by a variety of chemicals. Nevertheless, it is evident that some genes involved in the adaptive response to xenobiotics also take part in this biological process un-stimulated. Meanwhile, there are also many toxicology-related genes displaying the lowest basal level or constitutive expression prior to stress (Tseng et al., 2005). The knowledge of sequences of toxicology-related genes will facilitate the better use of the rare minnow liver EST in the toxic tests of chemicals.

This report provides the first view of the genetic programs in the liver of rare minnow and is of great value for further exploitation of this species in functional genomic studies. In addition, the toxicological catalog will facilitate the better use of the rare minnow liver ESTs for the toxicogenomic researches in teleosts.

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