Ecotoxicology (2007) 16:525–532
DOI 10.1007/s10646-007-0159-8

Identification of differentially expressed genes from contaminant and thermal exposed goldfish *Carassius auratus* in Gaobeidian Lake in Beijing, China

Jianshe Wang · Yanhong Wei · Xuemei Li · Muqi Xu · Jiayin Dai

Accepted: 13 June 2007 / Published online: 11 July 2007

Abstract Gaobeidian Lake, located in Beijing, China, derives its water mainly from the effluent of the Gaobeidian Wastewater Treatment Plant, which is moderately polluted. Additionally, as this water is used as a coolant in the nearby thermal power plant, the water of this lake has an elevated temperature. To screen differential gene expression in Gaobeidian Lake, suppressive subtractive hybridization (SSH) methodology was performed on RNA in goldfish *Carassius auratus* hepatic tissues from the lake, using Huairou Reservoir as reference site. A total of 768 candidate clones were selected to perform differential screening. Of these, 264 clones were differentially expressed between the two sites, 124 of which were then subjected to DNA sequencing. Consequently, 36 different genes with known functions were obtained, and some of these differential genes were further confirmed by semi-quantitative RT-PCR experiments. Many genes related to detoxification, stress and immune response, and metabolism, such as glutathione S-transferase (GST), cytochrome P450, family 2, subfamily b, polypeptide 10 (CYP2B10), CYP2X10, α-1-antitrypsin, and apolipoprotein A-I (Apo-AI), had higher expression levels in goldfish hepatic tissue from Gaobeidian Lake than those from the reference site. A set of nine genes with known functions were downregulated in Gaobeidian Lake compared to the reference site. The results provided evidence that organisms inhabiting Gaobeidian Lake were suffering a complex stress process and showing metabolism changes and disturbance of homeostasis.

J. Wang · Y. Wei · X. Li · M. Xu · J. Dai (✉)
Key Laboratory of Animal Ecology and Conservation Biology, Institute of Zoology, Chinese Academy of Sciences, Beijing 100080, P.R. China
e-mail: daijy@ioz.ac.cn

Keywords SSH · Goldfish · Acute phase reaction · Pollution · Thermal stress · Metabolism

Introduction

Functional genomics techniques are playing an increasing role in mechanistic and screening studies in environmental toxicology (Bartosiewicz et al. 2001; Hanlon et al. 2005). However, of these techniques, the application to non-model species, such as goldfish *Carassius auratus*, has to date been limited by the comparative lack of sequence data for these animals. Suppressive subtractive hybridization (SSH) is a useful technique in dealing with these types of situations, and involves hybridization of cDNA from one sample population (tester) to an excess of mRNA from another sample population (driver), followed by separation of the unhybridized fraction (target transcripts differently expressed between two transcriptomes) from hybridized common sequences (Diatchenko et al. 1996).

Gaobeidian Lake has a catchment area of approximately 0.15 km^2, and its water source is mainly the effluent of the Gaobeidian Wastewater Treatment Plant. Some physico-chemical water quality data in Gaobeidian Lake are as follows: dissolved oxygen: 3.1 ± 0.6 mg/l; suspended solids: 16.0 ± 3.4 mg/l; total phosphorus: 2.3 ± 0.7 mg/l; total nitrogen: 27.8 ± 4.4 mg/l; chemical oxygen demand: 46.3 ± 6.3 mg/l; temperature: 29.7 ± 10.6°C, and pH 7.7 ± 0.1, indicating that the lake water is moderately polluted. Water in Gaobeidian Lake is also used as a coolant by the nearby Beijing Guohua Thermal Power Plant, and then returned to the aquatic environment at a higher temperature than it was originally. Water temperatures in the lake are between 12 and 41°C, corresponding to seasonal changes, which is approximately 5–10°C higher than the ambient temperature.

Using the SSH method, our present study investigated the effects of the complex environment in Gaobeidian Lake on the global gene expression in goldfish living there. Huairou Reservoir, a drinking water source to the north of Beijing, was used as the reference site. Some physico-chemical water quality data in Huairou Reservoir are as follows: dissolved oxygen: 6.1 ± 0.2 mg/l; total phosphorus: 0.013 ± 0.005 mg/l; total nitrogen: 0.60 ± 0.09 mg/l; chemical oxygen demand: 3.0 ± 0.6 mg/l. The locations of Gaobeidian Lake and HuaiRou Reservoir in Beijing were shown in Fig. 1.

Materials and methods

Experimental animals

Goldfish (body weight: 50.4 ± 4.5 g, body length: 11.9 ± 0.6 cm) were caught from Gaobeidian Lake and Huairou Reservoir on November 23, 2005. The temperatures for the day of collection at the Gaobeidian Lake and Huairou Reservoir were 20 and 4°C, respectively. Fish were killed by a sharp blow to the head followed by severing the spinal cord. Hepatic tissues were dissected on ice, snap-frozen in liquid nitrogen, and stored at –80°C until further analysis.

Total RNA extraction

Total RNA was isolated using Trizol reagent (Invitrogen, USA) and treated with RNase-free DNase I (Qiagen, USA) to remove any remaining genomic DNA according to the manufacturer's instructions. Briefly, 10 μl of DNase I buffer and 1 μl of DNase I (2 U) were added for every 100 μl of RNA sample and the sample was incubated at 25°C for 10 min. RNA quality and quantity were assessed using agarose gel electrophoresis and spectrophotometric absorbency at 260/280 nm. Total RNA concentrations averaged from 700 to 3,300 ng/μl with 260/280 ratios of 2.0.

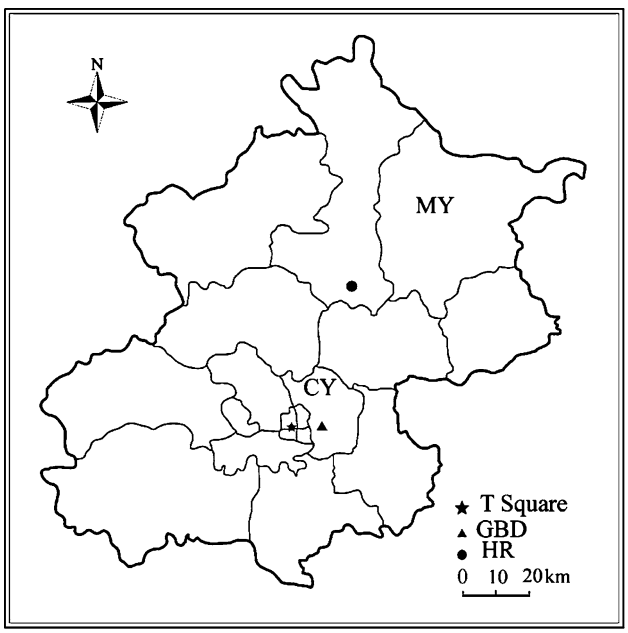

Fig. 1 The locations of Gaobeidian Lake (GBD) and Huairou Reservoir (HR) in Beijing. T Square: Tiananmen Square; MY: Miyun District; CY: Chaoyang District

Generation of subtracted cDNA libraries

Hepatic mRNA was isolated from the total RNA using an Oligotex mRNA Mini Kit (Qiagen, USA). For each group, equal amounts of mRNA were then pooled from each fish (20 fish in each group) to minimize false positives arising from the isolation of differentially expressed genes confined to individuals. Double-stranded cDNA was synthesized using a cDNA synthesis kit (Takara, Japan). SSH in forward and reverse directions was performed using the PCR-Select cDNA Subtraction Kit (Clontech, USA) according to the manufacturer's protocol. The differential PCR products obtained after two rounds of subtraction were inserted into a pGEM-T vector (Promega, USA) and cloned using DH5 alpha bacterial cells.

Differential screening of the subtracted library

A total of 384 individual bacterial colonies each for forward- and reverse-subtracted libraries (768 total colonies) were individually selected with sterile toothpicks and transferred to 96-well microplates containing LB media with ampicillin (100 μg/ml). After overnight growth, clones were amplified with PCR using SP6 and T7 primers, and screened for false positives with a PCR-Select Differential Screening Kit (Clontech, USA). Briefly, the PCR products were denatured and spotted identically on two positive-charge nylon membranes (Promega, USA). Spotted membranes were neutralized in 0.5 M Tris–HCl (pH 7.5), rinsed in water, and UV cross-linked. Forward- and reverse-subtracted cDNA were labeled with ^{32}P-dATP at 37°C for 40 min and purified using Microspin G-25 columns (Amersham, USA) according to manufacturer's direction, and probe radioactivity was quantified in a scintillation counter. ^{32}P-labeled library probes were then hybridized to the subtracted clones arrayed on each duplicate set of membranes. After washing, hybridized membranes were exposed to X-ray film, and the differentially expressed clones were selected and individually transferred to new 96-well microplates containing LB medium with ampicillin. These colonies were re-grown overnight at 37°C with constant agitation at 250 rpm. Plasmids from the selected clones were then isolated and purified from transformed DH5 alpha

competent cells, and sequenced on a commercial ABI 3730 capillary sequencer.

Database and sequence analyses

A total of 124 subtracted clones were sequenced. Obtained sequence data were compared to National Center for Biotechnology Information (NCBI) nucleotide and protein databases using the Basic Local Alignment Search Tool (BLAST) program. Sequence reads with $E < 0.05$ were assigned putative identities, all others were assumed to have no significant match with known sequences in NCBI databases.

Reverse transcription and semiquantitative PCR

Hepatic tissue samples from 20 fish from each site were randomly pooled into three groups ($n = 7, 7, 6$). RNA isolation was performed as described in section "Total RNA extraction." Aliquots of 1 μg of total isolated RNA were used to synthesize the first-strand cDNA with M-MuLV reverse transcriptase (New England Biolabs, UK) and oligo $(dT)_{15}$ primer (Promega, USA). Conditions for reverse transcription were as follows: 60 min at 42°C, followed by 5 min at 98°C. To confirm the differential screening result, nine candidate genes were selected to perform semiquantitative PCR, including warm temperature acclimation-related 65 kDa protein (wap65), glutathione S-transferase (GST), cytochrome P450, family 2, subfamily b, polypeptide 10 (CYP2B10), Kallikrein-like gene, α-1-antitrypsin, apolipoprotein C-I precursor (Apo-CI), fetuin-B, apolipoprotein A-I (Apo-AI), and fibrinogen B beta polypeptide. β-actin served as an internal RNA control. Nucleotide sequences of all the primers and the amplified sizes are listed in Table 1. PCR was performed using 1 U of Taq DNA Polymerase (New England Biolabs,

Table 1 PCR primers for amplification of candidate differential expressed genes

Gene name	Sequence	Product size (bp)
Wap65		
Sense primer	5′-AGATGCTGCCTTTGTGTGTG-3′	
Antisense primer	5′-CCTGTGCTGTTCAGGCATTA-3′	247
GST		
Sense primer	5′-TTTTCTGCTGCAGGTTGTTG-3′	
Antisense primer	5′-ACATGGAGACATCGTCGTGA-3′	155
CYP2B10		
Sense primer	5′-AAATGTGGAGTCCTCGTTGG-3′	
Antisense primer	5′-TTGCTTCCCATCATCAAACA-3′	192
Kallikrein-like gene		
Sense primer	5′-GGCCAAAGTCCCTTAGTGTG-3′	
Antisense primer	5′-CAGCAGTGGTGTTGACAGGT-3′	192
α-1-antitrypsin		
Sense primer	5′-ATCTGCTGCGTCACTGAATG-3′	
Antisense primer	5′-GGTGCCCTACAAAGGCAATA-3′	229
Apo-CI		
Sense primer	5′-GAAGACCAAGACCACCTTCG-3′	
Antisense primer	5′-TGACATGATTGAAGGGACGA-3′	164
Fetuin-B		
Sense primer	5′-AGGACGAGACCCATGAACAC-3′	
Antisense primer	5′-CTTGTCAGGGAAGGATGGAA-3′	194
Apo-AI		
Sense primer	5′-TCAACTGGGAGGAGACCAAG-3′	
Antisense primer	5′-TGCCCAACTCTTCCATCTTC-3′	201
Fibrinogen, B beta polypeptide		
Sense primer	5′-GTTACCGGGGAAGACCAACT-3′	
Antisense primer	5′-GGGTCAGCTCTTCCACAGAT-3′	209
β-actin		
Sense primer	5′-GGCCTCCCTGTCTATCTTCC-3′	
Antisense primer	5′-TTGAGAGGTTTGGGTTGGTC-3′	156

wap65: warm temperature acclimation-related 65 kDa protein; GST: glutathione S-transferase; CYP2B10: cytochrome P450, family 2, subfamily b, polypeptide 10; Apo-CI: apolipoprotein C-I precursor; Apo-AI: apolipoprotein A-I

UK) at a final concentration of 1× PCR buffer as formulated by New England Biolabs, with 250 μM of dNTP and 0.5 μM of each primer set in a total volume of 20 μl. Different cycles were performed, and a cycle number within the exponential phase of the amplification curve was chosen for quantifying the expression of each gene in subsequent experiments. The amplification cycles for wap65, GST, CYP2B10, Kallikrein-like gene, α-1-antitrypsin, Apo-CI, Fetuin-B, Apo-AI, Fibrinogen, B beta polypeptide, and β-actin were 28, 30, 30, 30, 28, 30, 30, 30, 28, and 25, respectively. PCR conditions consisted of 94°C for 2 min, followed by 25–30 cycles (according to linear phase of genes) of 94°C for 30 s, 58°C for 30 s, and 72°C for 30 s, with a final extension period of 72°C for 5 min. PCR products were run on a 2% agarose gel containing ethidium bromide. Band densities of the resulting products were measured using Bandleader Software, and all values are expressed as mean ± SE of 3 independent experiments. The significance of differences between two groups was calculated by a two-tailed Student's t test, and $P < 0.05$ was considered significant.

Results and discussion

Although some physicochemical and thermal data are already known in Gaobeidian Lake, the physiological and biological influences of the complex environmental stresses on the aquatic species living in the lake need further study. This study detected the global transcriptional changes of goldfish hepatic tissue in Gaobeidian Lake by SSH, using goldfish in the Huairou Reservoir as a reference.

Subtractive hybridization is an attractive method for cloning enriched differentially expressed genes. After two rounds of subtraction, the equalized cDNA was ligated to plasmid DNA and used in transformations with DH5 alpha competent cells. In order to reduce the false positive results of SSH, following transformation, 768 candidate bacterial clones were isolated and subjected to the differential screening method. Based on hybridization results using forward- and reverse-subtracted probes, 264 clones were shown to be differently expressed between the two groups (either >3-fold increase or <33% decrease). Of those, 124 clones were subjected to DNA sequencing, and some of these sequenced clones represented fragments of the same genes. Consequently, the total number of differential genes represented by these clones was 45. Of those, 36 could be tentatively matched to genes with known functions. Among this set of genes, 27 are more prominent in the Gaobeidian Lake (Table 2) and 9 in the reference site, Huairou Reservoir (Table 3).

To further confirm the differential screen results, nine genes were selected to perform semiquantitative RT-PCR experiments. β-actin was used as internal control in each PCR experiment. As shown in Fig. 2, 8 of the 9 candidate genes showed a clear differential expression between the two groups by semiquantitative PCR, and the changes were in agreement with the differential screening results, while gene Apo-CI precursor showed no statistical difference between the two groups by this method.

Goldfish, a kind of eurythermal fish, inhabit environments in which temperature varies widely and fluctuates seasonally from near 0 to 30°C (Hirayama et al. 2004; Kikuchi et al. 1995). Goldfish living in Gaobeidian Lake can tolerance higher temperature, and this heat tolerance is a complex trait. Many researches have focused on the mechanisms of thermotolerance which organisms acquire when subjected to a non-lethal high temperature for a few hours before encountering heat shock conditions (King et al. 2002; Weibezahn et al. 2004). Our study may characterize genes responsive to acclimation to long-term elevated temperatures and will be helpful in understanding the molecular mechanisms of thermotolerance and the complex interactions between the rigorous environment and the organisms living there.

Our SSH and RT-PCR results showed that wap65 mRNA was dominantly expressed in Gaobeidian goldfish. cDNAs encoding wap65 were first cloned from warm temperature acclimated goldfish (Kikuchi et al. 1993), and its up-regulated expression in thermal stress was confirmed in other teleost fishes (Hirayama et al. 2003; 2004). The isolation of wap65 from goldfish exposed to elevated temperature with forward subtraction can be seen as additional evidence for the success of the SSH technique. The deduced amino acid sequence of wap65 showed about 30% homology to mammalian hemopexins, a serum glycoprotein that transports heme from hemolysis to the liver, however, its function is not yet clear (Kikuchi et al. 1995; Delanghe and Langlois 2001). Interestingly, wap65 was also expressed in goldfish following intra-administration of the bacterial pathogen, lipopolysaccharide (LPS) (Kikuchi et al. 1997), a potent inducer of immune responses, implying that wap65 may have functions in self defense mechanisms as well as acclimation to warm temperature.

Enzymes involved in hepatic biotransformation play a key role in elimination and detoxification of foreign compounds. Genes encoding GST, CYP2B10, and CYP2X10 were also isolated by SSH. GSTs, distributed in all eukaryotes and in many prokaryotes, are a family of multifunctional enzymes involved in the cellular detoxification and excretion of a variety of xenobiotic substances (Hayes and Pulford 1995; Leaver et al. 1997; Eaton and Bammler 1999; Pham et al. 2004). In mammals, GST enzymes have been well characterized, and are known to catalyze S-conjugation between the thiol group of GSH and an electrophilic moiety in hydrophobic toxicants. Although

Table 2 The upregulated sequences in goldfish hepatic tissues from Gaobeidian Lake compared with that from reference site (Huairou reservoir) confirmed by differential screening and sequencing (sequences showing no similarity to genes with known functions are not included)

GenBank No.	Organism	Definition
XM_694143	*Danio rerio*	PREDICTED: similar to Apolipoprotein C-I precursor (Apo-CI) (ApoC-I) (LOC570638), mRNA
AF274054	*Pimephales promelas*	Glutathione S-transferase mRNA, partial cds
BC049404	*Danio rerio*	Phosphoglycerate mutase 1, mRNA (cDNA clone IMAGE:5604254), partial cds
BC067645	*Danio rerio*	Ribosomal protein S2, mRNA (cDNA clone MGC:85824 IMAGE:6962406), complete cds
BC062289	*Danio rerio*	Ribosomal protein S18, mRNA (cDNA clone MGC:77944 IMAGE:7000924), complete cds
BC083473	*Danio rerio*	Apolipoprotein A-I, mRNA (cDNA clone MGC:103718 IMAGE:7265411), complete cds
AY789109	*Homo sapiens*	Interleukin 3 receptor, alpha (low affinity) (IL3RA) gene, complete cds
AF457150	*Carassius auratus*	Transferrin variant B mRNA, complete cds
NM_001002307	*Danio rerio*	Retinol binding protein 2b, cellular (rbp2b), mRNA
AY461434	*Cyprinus carpio*	Uncoupling protein 1 (UCP1) mRNA, complete cds
AY561513	*Danio rerio*	Ribosomal protein S29 (rps29) mRNA, complete cds
XM_681934	*Danio rerio*	PREDICTED: similar to Ferritin heavy chain (Ferritin H subunit) (Proliferation-inducing gene 15 protein) (LOC558688), mRNA
D50437	*Carassius auratus*	mRNA for warm temperature acclimation-related 65-kDa protein, complete cds
BC098550	*Danio rerio*	Ribosomal protein L23a, mRNA (cDNA clone MGC:110139 IMAGE:7288150), complete cds
XM_703004	*Danio rerio*	PREDICTED: similar to Ribosomal protein L13a, transcript variant 2 (LOC560828), mRNA
AY773183	*Carassius auratus*	14 kDa apolipoprotein mRNA, complete cds
AY254171	*Cyprinus carpio*	Kallikrein-like gene, partial sequence
NM_005659	*Homo sapiens*	Ubiquitin fusion degradation 1 like (yeast) (UFD1L), transcript variant 1, mRNA
L08689	*Cyprinus carpio*	α-1-antitrypsin mRNA, complete cds
AY117540	*Cyprinus carpio*	Clone cL41a ribosomal protein L41 mRNA, complete cds
XM_684858	*Danio rerio*	PREDICTED: similar to LOC402847 protein (LOC561454), mRNA
XM_678035	*Danio rerio*	PREDICTED: similar to CYP2B10 protein (LOC555510), mRNA
AB007005	*Cyprinus carpio*	Bf/C2B mRNA for complement factor B/C2B, complete cds
AB016211	*Cyprinus carpio*	mRNA for complement C3-H1, complete cds
U57388	*Carassius auratus*	Cytochrome C oxidase subunit III mRNA, mitochondrial gene encoding mitochondrial protein, partial cds
AY704452	*Carassius auratus*	Cytochrome oxidase subunit II gene, partial cds; mitochondrial
AY825256	*Danio rerio*	Cytochrome P450 CYP2X10 mRNA, complete cds

Table 3 Downregulated sequences in goldfish hepatic tissues from Gaobeidian Lake compared with those from Huairou reservoir, confirmed by differential screening and sequencing (sequences showing no similarity to genes with known functions are not included)

GenBank No.	Organism	Definition
BC064000	*Danio rerio*	Ceruloplasmin, mRNA (cDNA clone MGC:77935 IMAGE:7000725), complete cds
AJ843092	*Platichthys flesus*	mRNA for 40S ribosomal protein S18 (rps18 gene)
BC044525	*Danio rerio*	Uridine phosphorylase, like, mRNA (cDNA clone MGC:55883 IMAGE:3818682), complete cds
NM_212618	*Danio rerio*	Chymotrypsinogen B1 (ctrb1), mRNA
DQ193534	*Carassius auratus*	Fetuin-B mRNA, complete cds
XM_683888	*Danio rerio*	PREDICTED: hypothetical protein LOC560483 (LOC560483), mRNA
AY561516	*Danio rerio*	Ribosomal protein L13 (rpl13) mRNA, complete cds
DQ020100	*Ctenopharyngodon idella*	Intelectin mRNA, complete cds
NM_212774	*Danio rerio*	Fibrinogen, B beta polypeptide (fgb), mRNA

GSTs in fish have not been characterized to the extent of their mammalian counterparts, all fish species examined to date have been shown to have GST catalytic activity and express soluble hepatic GST isoforms with some structural similarity to the rodent GSTs (Trute et al. 2006). The cytochrome P450s (P450s) in mammals constitute a

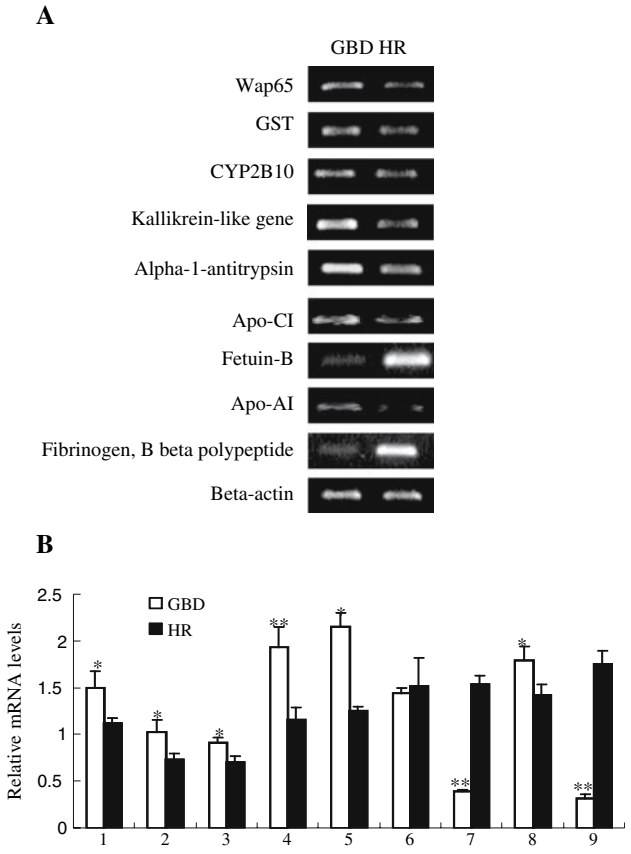

Fig. 2 RT-PCR screen for differential expression of candidate genes. (**A**) Semi-quantitative PCR was performed on cDNA from goldfish hepatic tissues in Gaobeidian Lake (GBD) and in the reference site, Huairou Reservoir (HR). PCR products were run on a 2% agarose gel containing ethidium bromide. The representative ethidium bromide stained gels are shown. (**B**) The density of electrophoretically separated PCR products was determined using Bandleader Software and normalized for β-actin (25 cycles) levels. Each value represents the mean ± SE of 3 independent experiments. The "*" and "**" denote statistical significance ($P < 0.05$ and $P < 0.01$ respectively). 1: warm temperature acclimation-related 65 kDa protein (wap65); 2: glutathione S-transferase (GST); 3: cytochrome P450, family 2, subfamily b, polypeptide 10 (CYP2B10); 4: Kallikrein-like gene; 5: α-1-antitrypsin; 6: apolipoprotein C-I precursor (Apo-CI); 7: Fetuin-B; 8: apolipoprotein A-I (Apo-AI); 9: Fibrinogen, B beta polypeptide

superfamily of heme-containing mono-oxygenases that play a central role in the detoxification of xenobiotics, as well as in the metabolism of endogenous compounds such as steroids, fatty acids, and prostaglandins (Nelson et al. 1996, 2004; Nebert and Russell 2002). The up-regulation of GST, CYP2B10, and CYP2X10 mRNA expression can be considered as an adaptive response to xenobiotic exposure. The up-regulation of those genes in Gaobeidian Lake indicate that goldfish living in the lake are suffering pollutant stresses, and the functional studies of these enzymes will allow a more detailed characterization of the effects of toxic stresses.

Many different expression genes with known functions in our SSH library were metabolism-related, such as Apo-AI, phosphoglycerate mutase 1, and retinol binding protein 2b. It is well known that an increase in environmental temperature seriously reorganizes intermediary metabolism and changes chemical composition, particularly the relative levels of different kinds of lipids (Rau et al. 2004). In our subtractive library, several lipid metabolism-related genes, such as Apo-AI, Apo-CI precursor, and 14 kDa apolipoprotein, were found to be significantly up-regulated in Gaobeidian goldfish as compared with Huairou goldfish. The up-regulation of these transcripts indicates that under the complex environment in Gaobeidian Lake, the animals may have higher lipid metabolism levels. Phosphoglycerate mutase 1, a glycolysis related gene, also had an elevated expression level in goldfish from Gaobeidian Lake, however, other metabolism related genes, such as nucleotide metabolism related gene, uridine phosphorylase, and proteolysis related gene, chymotrypsinogen B1 had lower expression levels in this environment.

Another group of differentially expressed genes with known functions were "acute phase response" genes, such as ceruloplasmin, transferrin variant B, ferritin heavy chain, fibrinogen gamma polypeptide, α-1-antitrypsin, and complement components. The acute-phase response is the reaction of the animal to disturbances in its homeostasis caused by tissue injury and inflammation, and is highly conserved in evolution (Molmenti et al. 1993; Pfeffer et al. 1993). One of its characteristics is the alteration of the concentration of a variety of hepatocyte-derived acute-phase proteins in blood. These proteins function as transport molecules, participate in tissue repair, mediate or inhibit inflammatory processes, and are part of the mechanism that controls homeostasis (Steel and Whitehead 1994). The altered expression of these genes in Gaobeidian Lake goldfish may function as an indicator of stress reactions which the organisms inhabiting the lake are suffering. Although the induction of acute-phase proteins is generally considered an adaptive response that restores homeostasis, persistent exposure to elevated water temperature or xenobiotics may lead to discordant changes of this response and alter the immune system of the fish (Bly and Clem 1992; Prophete et al. 2006). In our study, this phenomenon seemed to be appearing. Generally, when suffering acute stresses, the expression of transferrin is downregulated, while ceruloplasmin transcripts are elevated. However, in our present study, the mRNA expression changes of these two genes were opposite of the ordinary reactions, therefore, we speculate that persistent thermal and toxic stresses may impact the immune systems of animals living in Gaobeidian Lake, and thereby lower their resistance.

We isolated many genes differentially expressed in goldfish living in Gaobeidian Lake compared with the

reference site, and these results are crucial in understanding the biological and physiological changes of aquatic species in this complex environment. However, the enrichment of a target gene by SSH is greatly influenced by its concentration and the concentration ratio between tester and driver (Ji et al. 2002). Due to this methodological limitation, low abundance genes such as transcription factors, cytokines, and receptors which have low expression levels but may be key regulators of many pathological processes were seldom detected in this study. Additionally, though many hundreds of clones were isolated, only a subset were chosen for sequencing, therefore, a number of potentially important products may have been missed.

Conclusion

In summary, to examine the physiological and biological changes occurring in the complex environment of Gaobeidian Lake, the global gene expression of goldfish inhabiting the lake were investigated by SSH and differential screening. About 36 genes with known functions were identified to be differentially expressed in goldfish hepatic tissue in Gaobeidian Lake compared with the reference site, Huairou Reservoir. Many of these genes were associated with stress and immune response (such as acute phase response proteins ceruloplasmin, complement components, transferrin, and α-1-antitrypsin), detoxification (GST and CYP450 family proteins), and metabolism (Apo-AI, Apo-CI precursor, phosphoglycerate mutase 1, uridine phosphorylase, and chymotrypsinogen B1). The results provide evidence that organisms inhabiting Gaobeidian Lake are suffering a complex stress process. The persistent stresses may lead to alterations in metabolism, disturbance of homeostasis, and impacting the immune systems of aquatic species living in this environment.

Acknowledgements This work was funded by 973 Program (2006CB403306) and the Innovation Project of the Chinese Academy of Sciences (No: SCX2-SW-128 to Dai).

References

Bartosiewicz M, Penn S, Buckpitt A (2001) Applications of gene arrays in environmental toxicology: fingerprints of gene regulation associated with cadmium chloride, benzo(a)pyrene, and trichloroethylene. Environ Health Perspect 109:71–74

Bly JE, Clem LW (1992) Temperature and teleost immune functions. Fish Shellfish Immunol 2:159–171

Delanghe JR, Langlois MR (2001) Hemopexin: a review of biological aspects and the role in laboratory medicine. Clin Chim Acta 312:13–23

Diatchenko L, Lau YF, Campbell AP, Chenchik A, Moqadam F, Huang B, Lukyanov S, Lukyanov K, Gurskaya N, Sverdlov ED, Siebert PD (1996) Suppression subtractive hybridization: a method for generating differentially regulated or tissue-specific cDNA probes and libraries. Proc Natl Acad Sci USA 93:6025–6030

Eaton DL, Bammler TK (1999) Concise review of the glutathione S-transferases and their significance to toxicology. Toxicol Sci 49:156–164

Hanlon PR, Zheng W, Ko AY, Jefcoate CR (2005) Identification of novel TCDD-regulated genes by microarray analysis. Toxicol Appl Pharmacol 202:215–228

Hayes JD, Pulford DJ (1995) The glutathione S-transferase supergene family: regulation of GST and the contribution of the isoenzymes to cancer chemoprotection and drug resistance. Crit Rev Biochem Mol Biol 30:445–600

Hirayama M, Kobiyama A, Kinoshita S, Watabe S (2004) The occurrence of two types of hemopexin-like protein in medaka and differences in their affinity to heme. J Exp Biol 207:1387–1398

Hirayama M, Nakaniwa M, Ikeda D, Hirazawa N, Otaka T, Mitsuboshi T, Shirasu K, Watabe S (2003) Primary structures and gene organizations of two types of Wap65 from the pufferfish *Takifugu rubripes*. Fish Physiol Biochem 29:211–224

Ji W, Wright MB, Cai L, Flament A, Lindpaintner K (2002) Efficacy of SSH PCR in isolating differentially expressed genes. BMC Genomics, 3:12

Kikuchi K, Watabe S, Aida K (1997) The Wap65 gene expression of goldfish (*Carassius auratus*) in association with warm water temperature as well as bacterial lipopolysaccharide (LPS). Fish Physiol Biochem 17:423–432

Kikuchi K, Watabe S, Suzuki Y, Aida K, Nakajima H (1993) The 65-kDa cytosolic protein associated with warm temperature acclimation in goldfish, *Carassius auratus*. J Comp Physiol B 163:349–354

Kikuchi K, Yamashita M, Watabe S, Aida K (1995) The warm temperature acclimation-related 65-kDa protein, Wap65, in goldfish and its gene expression. J Biol Chem 270:17087–17092

King YT, Lin CS, Lin JH, Lee WC (2002) Whole-body hyperthermia-induced thermotolerance is associated with the induction of heat shock protein 70 in mice. J Exp Biol 205:273–278

Leaver MJ, Wright J, George SG (1997) Structure and expression of a cluster of glutathione S-transferase genes from a marine fish, the plaice (*Pleuronectes plastessa*). Biochem J 321:405–412

Molmenti EP, Ziambaras T, Perlmutter DH (1993) Evidence for an acute phase response in human intestinal epithelial cells. J Biol Chem 268:14116–14124

Nebert DW, Russell DW (2002) Clinical importance of the cytochromes P450. Lancet 360:1155–1162

Nelson DR, Koymans L, Kamataki T, Stegeman JJ, Feyereisen R, Waxman DJ, Waterman MR, Gotoh O, Coon MJ, Estabrook RW, Gunsalus IC, Nebert DW (1996) P450 superfamily: update on new sequences, gene mapping, accession numbers and nomenclature. Pharmacogenetics 6:1–42

Nelson DR, Zeldin DC, Hoffman SM, Maltais LJ, Wain HM, Nebert DW (2004) Comparison of cytochrome P450 (CYP) genes from the mouse and human genomes, including nomenclature recommendations for genes, pseudogenes and alternative-splice variants. Pharmacogenetics 14:1–18

Pfeffer A, Rogers KM, O'Keeffe L, Osborn PJ (1993) Acute phase protein response, food intake, liveweight change and lesions following intrathoracic injection of yeast in sheep. Res Vet Sci 55:360–366

Pham RT, Barber DS, Gallagher EP (2004) GSTA is a major glutathione S-transferase gene responsible for 4-hydroxynonenal conjugation in largemouth bass liver. Mar Environ Res 58:485–488

Prophete C, Carlson EA, Li Y, Duffy J, Steinetz B, Lasano S, Zelikoff JT (2006) Effects of elevated temperature and nickel pollution

on the immune status of Japanese medaka. Fish Shellfish Immunol 21:325–334

Rau MA, Whitaker J, Freedman JH, Di Giulio RT (2004) Differential susceptibility of fish and rat liver cells to oxidative stress and cytotoxicity upon exposure to prooxidants. Comp Biochem Physiol C Toxicol Pharmacol 137:335–342

Steel DM, Whitehead AS (1994) The major acute phase reactants: C-reactive protein, serum amyloid P component and serum amyloid A protein. Immunol Today 15:81–88

Trute M, Gallis B, Doneanu C, Shaffer S, Goodlett D, Gallagher E (2006) Characterization of hepatic glutathione S-transferases in coho salmon (*Oncorhynchus kisutch*). Aquat Toxicol (Epub ahead of print)

Weibezahn J, Tessarz P, Schlieker C, Zahn R, Maglica Z, Lee S, Zentgraf H, Weber-Ban EU, Dougan DA, Tsai FT, Mogk A, Bukau B (2004) Thermotolerance requires refolding of aggregated proteins by substrate translocation through the central pore of ClpB. Cell 119:653–665