Fish Physiol Biochem (2015) 41:819–832
DOI 10.1007/s10695-015-0049-6

Effects of dietary glucose and dextrin on activity and gene expression of glucokinase and fructose-1,6-bisphosphatase in liver of turbot *Scophthalmus maximus*

Qin Nie · Huijun Miao · Shuyan Miao · Huihui Zhou · Yanjiao Zhang · Wenbing Zhang · Kangsen Mai

Received: 4 November 2014 / Accepted: 31 March 2015 / Published online: 17 April 2015

Abstract Glucokinase (GK) and fructose-1,6-bisphosphatase (FBPase) play crucial role in glucose metabolism. In the present study, the cDNA encoding GK and FBPase was cloned from the liver of turbot *Scophthalmus maximus* by rapid amplification of cDNA end technique. Effects of dietary glucose and dextrin on the activities and gene expressions of these two enzymes were also studied. Results showed that the full length of GK cDNA was 2226 bp, consisting of an open reading frame (ORF) of 1434 bp. The full-length cDNA coding FBPase was 1314 bp with a 1014 bp ORF encoding 337 amino acids. Analyses of gene expression of GK and FBPase were conducted in gill, liver, the whole intestine, the whole kidney, heart, the dorsal white muscle and brain. The highest expression of GK was found in liver, followed by muscle. The expression of FBPase was found higher in liver than heart and gill. Both hepatic GK activity and mRNA expression were highly induced in turbot after being fed with dietary carbohydrates ($p < 0.05$). However, the GK activity and mRNA expression in the group with dietary glucose did not significantly differ from those in the group with dietary dextrin ($p > 0.05$). Compared with the control group, there were no significant differences in FBPase activity and mRNA expression in the glucose as well as dextrin group ($p > 0.05$). The increased hepatic GK activity and gene expression indicated that the first step of glycolysis was activated in turbot by dietary carbohydrates.

Keywords Turbot · Glucokinase · Fructose-1, 6-bisphosphatase · Carbohydrate · Nutrition

Q. Nie · H. Miao · S. Miao · H. Zhou · Y. Zhang · W. Zhang (✉) · K. Mai
The Key Laboratory of Aquaculture Nutrition and Feeds, Ministry of Agriculture, The Key Laboratory of Mariculture (Ministry of Education), Ocean University of China, Qingdao 266003, People's Republic of China
e-mail: wzhang@ouc.edu.cn

Introduction

Feed nutrition for intensively farmed fish still relies heavily on high protein contents. Efforts toward partial replacement of feed protein by lipid or carbohydrate sources have been undertaken for many years. Many studies have shown that it is possible to minimize the amount of protein in diets and cover energy requirements using carbohydrates without affecting growth or feed utilization (Caseras et al. 2002; Jafri 1995; Kirchner et al. 2003; Metón et al. 2004). However, excess levels of carbohydrate can reduce the growth rate of fish (Hemre et al. 2002).

Glycolysis and gluconeogenesis act as the physiological function of carbohydrate decomposition and synthesis. Although most enzymes are shared by the two pathways, glucokinase (GK), 6-phosphofructo-1-kinase (PFK) and pyruvate kinase (PK) catalyze unidirectional reactions and play a key role in glycolysis.

Phosphoenolpyruvate carboxykinase (PEPCK), fructose-1,6-bisphosphatase (FBPase) and glucose-6-phosphatase (G6Pase) catalyze the reverse reactions and act as the key enzymes in gluconeogenesis. Genes coding these enzymes have been cloned and characterized in some fish species, such as GK and PK in rainbow trout *Oncorhynchus mykiss* (Panserat et al. 2001), G6Pase in gilthead sea bream *Sparus aurata* (Metón et al. 2004), FBPase and PEPCK in Siberian sturgeons *Acipenser baerii* (Gong et al. 2013). Glucokinase is the initial key enzyme of the glycolytic pathway and catalyzes the phosphorylation of glucose into glucose-6-phosphate, which is also the substrate for glycogen synthesis reaction. Therefore, GK has an important role in both the decomposition of glucose and glycogen biosynthesis pathway. Meanwhile, some studies have been performed to analyze the nutritional regulation of the GK enzyme in humans and rodents (having high levels of dietary carbohydrates in their diets), in the chicken (moderate level of carbohydrates in its diet) and rainbow trout (no carbohydrate intake in its diet). These data illustrated the nutritional importance of the GK enzyme irrespective of feeding habits, even in animals known to poorly use dietary carbohydrates (carnivorous species) (Panserat et al. 2014). Fructose-1,6-bisphosphatase, one of the key enzymes in gluconeogenesis, catalyzes the hydrolysis of fructose-1,6-bisphosphate to fructose-6-phosphate.

Turbot *Scophthalmus maximus* is a carnivorous fish species with high economic value, delicious meat and rapid growth. It is widely cultured in Europe and Asia. Nutritional studies in this species are mainly focused on the nutrients of protein (Regost et al. 2001; Van Ham et al. 2003), amino acids (Gouillou-Coustans et al. 2002) and lipid (Regost et al. 2001). There is little information available on the metabolism and utilization of carbohydrate in turbot. To date, studies on the carbohydrate utilization of this species showed that turbot have a moderate tolerance to utilize glucose (Garcia-Riera and Hemre 1996). Nie et al. (2013) found that both the dietary carbohydrate sources and levels influenced the activities of carbohydrate metabolic enzymes in turbot. Dextrin and glucose are two kinds of carbohydrates with different molecular complexity. Miao et al. (2013) suggested that turbot can utilize dextrin more efficiently than glucose. No adverse effects of 15 % of dietary dextrin were found on the growth of juvenile turbot. Meanwhile, 15 % of dietary glucose or higher dietary dextrin level (28 %) resulted in significantly lower growth rate (Miao et al. 2013). However, how these two carbohydrates influence the growth rate, feed utilization and glucose metabolism in turbot is still unclear.

The aim of this study is to clone and analyze the GK and FBPase genes, and then to comparatively study the effects of dietary glucose and dextrin on activity and gene expression of these two enzymes in liver of turbot *S. maximus*. This study will be helpful for understanding the carbohydrate metabolism in this fish species.

Materials and methods

Cloning, characterization and phylogenetic analysis of GK and FBPase

Three adult turbots (body weight 215–235 g) were used for GK and FBPase gene cloning and tissue-specific expression detection. Total RNA was extracted from liver, using Trizol reagent (Invitrogen, USA). They were treated with recombinant DNase I (RNase-free) (Takara, Japan) to avoid genomic DNA amplification. The integrity of isolated RNA was assessed by 1.2 % agarose gel electrophoresis, and its quantity and purity were determined by absorbance measures at 260 and 280 nm with the Nano-Drop® ND-1000 spectrophotometer (Nano-Drop Technologies, Wilmington, DE, USA). Hepatic cDNA was obtained by annealing 1 μg of total RNA with 1 μl of random primers and incubating with 1 μl of PrimeScript Reverse Transcriptase (Takara, Japan).

The partial sequence of turbot GK was amplified by the degenerate primers (GK-1 and GK-2). All primer sequences used in this study are listed in Table 1. They were designed based on the conserved regions of its counterparts in gilthead sea bream (GenBank accession no. AAC33585.2), rainbow trout (GenBank accession no. AAC33586.2), common carp *Cyprinus carpio* (GenBank accession no. AAC33587.2), Nile tilapia *Oreochromis niloticus* (GenBank accession no. XP_003451068.1) and Japanese sea perch *Lateolabrax japonicus* (GenBank accession no. AEZ36053.1). The degenerate primer pair of FBPase (FBPase-1 and FBPase-2) were designed according to the conserved coding regions of FBPase sequences in chicken *Gallus gallus* (GenBank accession no. XP_425040.1), African clawed frog *Xenopus laevis* (GenBank accession no. NP_001080528.1), Japanese sea perch (GenBank

Table 1 Primers used for GK and FBPase gene cloning

Primer	Sequence (5′–3′)	Sequence information
GK-1 (forward)	GAGATGGAKMGRGGACTGCGT	GK RT primer
GK-2 (reverse)	ACGYACAATGTCACAGTCCAG	GK RT primer
GK-3 (forward)	GACTTTGAGATGGACGTGGTTGC	GK 3′ RACE outer primer
GK-4 (forward)	ATCCTGTCCTCCCTGGGTGTTCT	GK 3′ RACE inner primer
GK-5 (reverse)	GCCTTGAAGCCTTTGGTCCAGTTGAGC	GK 5′ RACE outer primer
GK-6 (reverse)	CCCGTCATGGCATCCTCAGGAATGG	GK 5′ RACE inner primer
FBPase-1 (forward)	CTGGATATCCTGTCCAATGMYCT	FBPase RT primer
FBPase-2 (reverse)	CAGCAGCCTCAGCTTGCCWKT	FBPase RT primer
FBPase-3 (forward)	TCCTCTGGGTTTCACCTTCT	FBPase 3′ RACE outer primer
FBPase-4 (forward)	CAAGGGAATCCTGCTCAACT	FBPase 3′ RACE inner primer
FBPase-5 (reverse)	AGCCTCAGCTTGCCATTAGGACTCTT	FBPase 5′ RACE outer primer
FBPase-6 (reverse)	CGGATCAAGCATGAAGCAGTTGACTC	FBPase 5′ RACE inner primer
3′ UPM	TACCGTCGTTCCACTAGTGATTT	3′ RACE outer primer
3′ NUP	CGCGGATCCTCCACTAGTGATTTCACTATAGG	3′ RACE inner primer
5′ UPM	CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT	5′ RACE outer primer
5′ NUP	AAGCAGTGGTATCAACGCAGAGT	5′ RACE inner primer
M13 (forward)	GAGCGGATAACAATTTCACACAGG	Vector primer
M13 (reverse)	CGCCAGGGTTTTCCCAGTCACGAC	Vector primer
Oligo (dT)-adaptor	AAGCAGTGGTATCAACGCAGAGTACT$_{25}$	

G/T = K; A/C = M; A/G = R; C/T = Y; A/T = W

accession no. AFC36525.1), Nile tilapia (GenBank accession no. XP_003449698.1), zebrafish *Danio rerio* (GenBank accession no. NP_998297.1) and gibel carp *Carassius auratus gibelio* (GenBank accession no. ADD91325.1) (Table 1). The reaction mixture of cDNA PCR amplification contained 2.5 μl 10× Ex Taq Buffer (Mg^{2+} Plus), 2 μl dNTP mixture (2.5 mM), 16.5 μl dH_2O, 1 μl each primer (10 mM) and 0.125 units Ex Taq (5 U μl^{-1}) (Takara, Japan). The PCR amplification program is pre-denatured at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s, extension at 72 °C for 1 min 30 s and post-extension at 72 °C for 5 min. PCR products were selected by 1.2 % agarose gel electrophoresis, and fragments of the expected size were purified by Gel Extraction Mini Kit (Watson Biotechnologies Inc, China). The purified DNA fragments were inserted into the *pEASY*-T1 Simple Cloning Vector (TransGen Biotech, China) and used for transformation of *Trans1*-T1 Phage Resistant Chemically Competent Cell (TransGen Biotech, China). Clones with inserts were sequenced in both forward and reverse directions using the universal M13 primers (Huada Genomics Co. Ltd., China).

Subsequently, 3′ and 5′ rapid amplification of cDNA ends (RACE) was performed to obtain the full-length cDNA sequences of GK and FBPase. The SMART™ RACE cDNA Amplification Kit (Clontech, USA) was used for the 5′ ends, and the 3′-Full RACE Core Set Ver. 2.0 (Takara, Japan) was used for the 3′ ends. Turbot-specific GK and FBPase primers for gene cloning (shown in Table 1) were designed by Primer Premier 5 based on the two partial sequences we had obtained. For 3′ RACE, the first-strand cDNA was synthesized using 3′ RACE Adaptor and Reverse Transcriptase M-MLV (Takara, Japan). The 3′ end of GK and FBPase was amplified by the method of nested PCR using the primers GK-3/GK-4 and FBPase-3/FBPase-4 with 3′ UPM and 3′ NUP, respectively. For 5′ RACE, the first-strand cDNA was synthesized by using 5′-CDS Primer A, the SMARTer IIA oligo and SMART Scribe Reverse Transcriptase (Clontech, USA). The 5′ end of GK and FBPase was also obtained by two rounds of nested PCR with the use of the primers GK-5/GK-6 and FBPase-5/FBPase-6 with 5′ UPM and 5′ NUP, respectively. PCR products were subjected to electrophoresis in 1.2 % agarose gels, and the relevant fragments were purified, cloned and sequenced as described above.

The cDNA sequence of GK and FBPase was compared with DNA sequences from the GenBank database using the basic local alignment search tool (BLAST) algorithm (http://www.ncbi.nlm.nih.gov/BLAST/). Sequence alignments and percentage conservation of amino acid sequences were assessed with the Clustal-W multiple-alignment algorithm (http://www.ebi.ac.uk/clustalw/). The search for a peptide signal in turbot GK and FBPase cDNA sequence was assessed using the SignalP 4.1 algorithm (http://www.cbs.dtu.dk/services/SignalP). The phylogenetic tree was produced by the neighbor-joining (NJ) method (Kimura 2-parameter model, 10,000 replicates, bootstrap phylogeny test) based on GK and FBPase amino acid sequences using the MEGA software version 3.1.

Tissue expression of GK and FBPase mRNA

Analysis of gene expression of GK and FBPase was conducted in gill, liver, the whole intestine, the whole kidney, heart, the dorsal white muscle and brain. Real-time PCR was performed in the quantitative thermal cycler (Mastercycler ep realplex, Eppendorf, Germany) using UltraSYBR Mixture (Cwbiotech, China). Total RNA was extracted from these selected tissues. The first-strand cDNA was synthesized as described above. The primers used for GK and FBPase mRNA quantification were designed (shown in Table 2) for detecting the expression of GK and FBPase in these tissues. The total volume of the PCR was 25 μl, containing 1 μl primers (10 mM), 1 μl cDNA template, 12.5 μl UltraSYBR Mixture and 9.5 μl dH_2O. Thermal cycling was initiated with the incubation at 95 °C for 10 min to activate GoldStar Taq DNA Polymerase. A total of 40 steps of PCR were performed. Each step consists of heating at 95 °C for 15 s for denaturing, annealing at the primer's hybridization temperature for 15, and 20 s for extension (Table 2). Following the final cycle of the PCR, melting curves were systematically monitored (95 °C for 15 s, 55 °C for 15 s, then the temperature rose from 55 to 95 °C in 20 min).

In this study, the gene of α-elongation factor 1 (EF1α, AF467776.1) was found to be stably expressed and used as an endogenous reference to normalize the template amount. Before the detection, the primer amplification efficiency (the E value) was optimized for each pair of primers, which resulted in: 0.9794 for EF1α, 1.0635 for GK and 0.9947 for FBPase (Table 2). The absolute ΔC_T value of GK C_T − EF1α C_T and FBPase C_T-EF1α C_T of the slope is 0.0201 and 0.0062, respectively, which both <0.1 indicated that the $\Delta\Delta C_T$ calculation for the relative quantification of target genes might be used. The expression level of GK and FBPase was calculated by $2^{-\Delta\Delta C_T}$ method, and the value stood for an n-fold difference relative to the calibrator (Livak and Schmittgen 2001). Since the lowest expressions of GK and FBPase were found in the gill, the expression levels of these two enzymes in the other tissues were normalized to that of gill.

Feeding trial and sample analysis

Three experimental diets were designed in this study (Table 3). The high protein content (50.22 %) and low carbohydrate content (1.91 %) diet was used as the control (diet-0). The other two low-protein-content (40.26 and 40.44 %, respectively) diets were designed to have relatively high carbohydrate contents (16.68 and 15.86 %, respectively) from glucose (diet-G) and dextrin (diet-D), respectively.

Table 2 Primers used for GK and FBPase mRNA quantification by RT-PCR

Gene (accession number)	Forward primer (5′–3′)	Reverse primer (5′–3′)	Target size (bp)	Annealing temperature (°C)	*E* value
EF1α (no. AF467776.1)	AGACCGGTATCCTGAAGCCT	GGATCTCCTTGACGGACACG	159	58	0.9794
GK (no. JX678944)	CGACACGAGGACATTGACAAG	CCAACAATCATCCCGACTTCAC	218	60	1.0635
FBPase (no. KC184130)	CAGGAAGGCTGGGATCGCTAAC	CTCATCTTCCTCCGACACAAG	157	60	0.9947

Table 3 Composition and proximate analyses of the experimental diets

	Diets		
	Diet-0	Diet-G	Diet-D
Ingredients (g/100 g dry weight basis)			
White fish meal	36.0	36.0	36.0
Casein	21.2	12.8	12.8
Gelatin	5.3	3.2	3.2
Glucose	0.0	15.0	0.0
Dextrin	0.0	0.0	15.0
Microcrystalline cellulose	20.6	16.1	16.1
Sodium alginate	1.0	1.0	1.0
Soybean lecithin	2.0	2.0	2.0
Fish oil	11.0	11.0	11.0
Attractant[a]	0.5	0.5	0.5
Vitamin premix[b,d]	0.5	0.5	0.5
Mineral premix[c,e]	1.0	1.0	1.0
Choline chloride	0.25	0.25	0.25
Ethoxyquin	0.05	0.05	0.05
$Ca(H_2PO_4)_2$	0.5	0.5	0.5
Calcium propionate	0.1	0.1	0.1
Proximate analysis (dry matter basis)			
Crude protein (%)	50.22	40.26	40.44
Crude lipid (%)	12.36	12.05	12.39
Carbohydrate (%)	1.91	16.68	15.86
Ash (%)	11.23	11.73	11.72
Gross energy (kJ/g)	17.09	17.14	17.18

[a] Attractants: taurine/glycine/betaine = 1/3/3

[b] Kindly provided by Qingdao Great Seven Bio-Tech. Co., Ltd., China

[c] Kindly provided by Qingdao Master Bio-Tech. Co., Ltd., China

[d] Vitamin premix (mg/kg diet): vitamin A, 32 mg; vitamin D, 5 mg; vitamin E, 240 mg; vitamin K, 10 mg; vitamin B_1, 25 mg; vitamin B_2, 45 mg; nicotinic acid, 200 mg; vitamin B_6, 20 mg; biotin, 60 mg; inositol, 800 mg; calcium pantothenate, 60 mg; folic acid, 20 mg; vitamin B_{12}, 10 mg; vitamin C, 2000 mg; microcrystalline cellulose, 4292.54 mg

[e] Mineral premix (mg/kg diet): $CuSO_4 \cdot 5H_2O$, 10 mg; Na_2SeO_3, 20 mg; $MnSO_4 \cdot H_2O$, 45 mg; $CoCl_2 \cdot 6H_2O$ (1 %), 50 mg; $ZnSO_4 \cdot H_2O$, 50 mg; $Ca(IO_3)_2$, 60 mg; $FeSO_4 \cdot H_2O$, 80 mg; $MgSO_4 \cdot 7H_2O$, 1200 mg; zeolite powder, 18,485 mg

Juvenile turbot (initial body weight 8.06 ± 0.08 g) were purchased from a local commercial farm in Qingdao, China. Fish were acclimated to the laboratory conditions for 2 weeks before being randomly distributed into nine experimental tanks in a re-circulating water system. There were three treatments (diet-0, diet-G and diet-D). Each treatment has three replicates. Each tank (500 l) with 28 fish was used as a replicate. Fish were hand-fed to apparent satiation twice daily (07:00 and 18:00). During the 9-week feeding trial, the water temperature was maintained at 19.0 ± 1.0 °C, pH 7.7 ± 0.1 and the salinity 29.2 ± 1.0 ‰.

At the end of the feeding trial, six fish per tank were sampled 24 h after the last meal. Three livers were pooled into a 1.5-ml tube (RNAase-Free, Axygen, USA) for total RNA extraction, and the other three livers were pooled together for the analysis of the activities of hepatic GK and FBPase. All liver samples were frozen in liquid nitrogen and then kept at the − 80 °C for measurements of mRNA expression or enzyme analysis.

Total RNA extraction, first-strand cDNA synthesis and real-time PCR were performed as above. The expression levels of GK and FBPase in glucose (diet-G) and dextrin (diet-D) group were normalized to that in the control group (diet-0). The analyses of the activities of hepatic GK and FBPase were conducted by the methods of Tranulis et al. (1996) and Polakof et al. (2008) with some modifications. Briefly, a frozen sample of liver (500 mg) was homogenized (dilution 1/10) in ice-cold buffer (pH 7.6) consisting of 50 mmol l^{-1} Tris, 5 mmol l^{-1} EDTA, 2 mmol l^{-1}, 1,4-dithiothreitol and 1 % protease inhibitor cocktail (Sigma, USA). The homogenate was centrifuged, and the supernatant was used immediately for enzyme assays. GK and FBPase enzyme activities were expressed in per milligram of total protein (specific activity). The total protein content in crude extracts was determined at 30 °C using bovine serum albumin as a standard based on the method of Bradford (1976). One unit of enzyme activity was defined as the amount of enzyme that catalyzed the hydrolysis of 1 μmol of substrate per mg protein per minute at 30 °C.

Statistical analysis

All data were presented as mean ± SEM (standard error of the mean) and analyzed by the one-way analysis of variance (ANOVA) using SPSS (version 17.0) software. When overall differences were significant at <5 % level, Tukey's test was used to compare the mean values between individual treatments.

Results

The growth performance and feed utilization data after this feeding trial were published in previous study (Miao et al. 2013). Briefly, there was no significant difference in survival of turbot, which ranges from 95.24 to 96.43 %. Turbot fed with 15 % of dietary glucose had significantly lower weight gain rate (216.37 %) than those in treatment with 15 % of dietary dextrin (267.51 %) or without carbohydrate supplementation (255.45 %). There were no significant differences in feed efficiency between these three treatments (diet-0, 1.15; diet-G, 1.04; diet-D, 1.11) (Miao et al. 2013).

Cloning and sequence analysis of turbot hepatic GK and FBPase genes

Degenerate primer pairs GK-1/2 were used for GK gene PCR amplification, and a fragment of 971 bp was obtained, which had a high degree of consistency with GK nucleotide sequence of other species. Based on the sequence above, four specific primers were designed to clone the full-length cDNA of GK. For 3′ end, nested PCR was carried out by using primers GK-3/4, and two fragments of 1504 and 1034 bp were obtained, respectively. And two cDNA fragments of 745 and 492 bp were amplified with the primers GK-5/6 by 5′ RACE technology, respectively.

The full-length cDNA coding GK was completed by assembling the core fragment, 3′ and 5′ end sequences, and submitted to GenBank (accession no. JX678944). It is 2226 bp, consisting of a 1434 bp open reading frame (ORF), a 5′ untranslated region (UTR) of 106 bp and a 3′ UTR of 683 bp (Fig. 1). The deduced amino acid sequence of the ORF predicts a polypeptide of 478 amino acids with a calculated molecular mass of 53.53 kDa, a theory isoelectric point of 4.88. In the termination codon (TAA) downstream, consensus polyadenylation signal sequences ATTAAA (2161th to 2165th) and a poly(A) tail are found. The BLAST result showed that the turbot GK amino acid sequence is highly homologous to other species. The turbot GK showed the highest identity with gilthead sea bream (AAC33585.2, 94 %), followed by Japanese sea perch (AEZ36053.1, 93 %) and Nile tilapia (XP_003451068.1, 91 %). Structural analysis of the turbot GK sequence revealed some conserved functional sites, including one conserved hexokinase signature sequence L^{158}–F^{183}, two N-linked glycosylation sites N^{178} and N^{216}, two ATP-binding sites D^{90}–K^{102} and K^{116}, one cell attachment sequence R^{202}–D^{204} and one glycosaminoglycan attachment site S^{457}–G^{460}.

The PCR product amplified by the degenerate primers FBPase-1/2 was 751 bp, and its nucleotide sequence was significantly homologous to other known FBPase. Based on the sequence above, four specific primers were designed to clone the full-length cDNA of FBPase. For 3′ end, nested PCR was carried out by using primers FBPase-3/4, and two fragments of 1504 and 1034 bp were obtained, respectively. Two cDNA fragments of 745 and 492 bp were amplified with the primers FBPase-5/6 by 5′ RACE technology, respectively. The full-length cDNA coding FBPase was completed by assembling the core fragment, 3′ and 5′ end sequences, and submitted to GenBank (accession no. KC184130).

The 1314 bp FBPase cDNA sequence includes an open reading frame (ORF) of 1014 bp that began with the first ATG codon and ended with a TAA stop codon at position 1070 bp (Fig. 2). The open reading frame encodes 337 amino acid residues and having a molecular weight of 10.67 kDa and a theory isoelectric point of 5.01. The 3′ untranslated region (UTR) covered 244 bp containing a AATAAA polyadenylation signal and a poly(A) tail. The BLAST result showed that the turbot FBPase sequence shares about 96 % amino acids with Japanese sea perch liver FBPase sequences (AFC36525.1), next Nile tilapia liver FBPase about 94 % (XP_003449698.1). Structural analysis of turbot FBPase amino acid sequence revealed some conserved functional sites, including the FBPase active site (G^{274}–A^{286}), the nuclear localization sequence (NLS) (K^{203}–K^{207}), the substrate/Fru-1,6-P_2ase-binding site (K^{275}) and the metal ion-binding site (D^{119}, D^{122}).

Phylogenetic analysis of GK and FBPase

The evolutionary relationship between the GK was investigated by the construction of a phylogenetic tree using Clustal X 1.83 and MEGA 4.0. As shown in Fig. 3, all the fish species were in the same group, which was distinguished from endotherms and amphibian. Within the fish group, turbot, Japanese sea perch, gilthead sea bream, Nile tilapia, spotted green puffer fish *Tetraodon nigroviridis* and rainbow trout were clustered into one subgroup, in which these fish

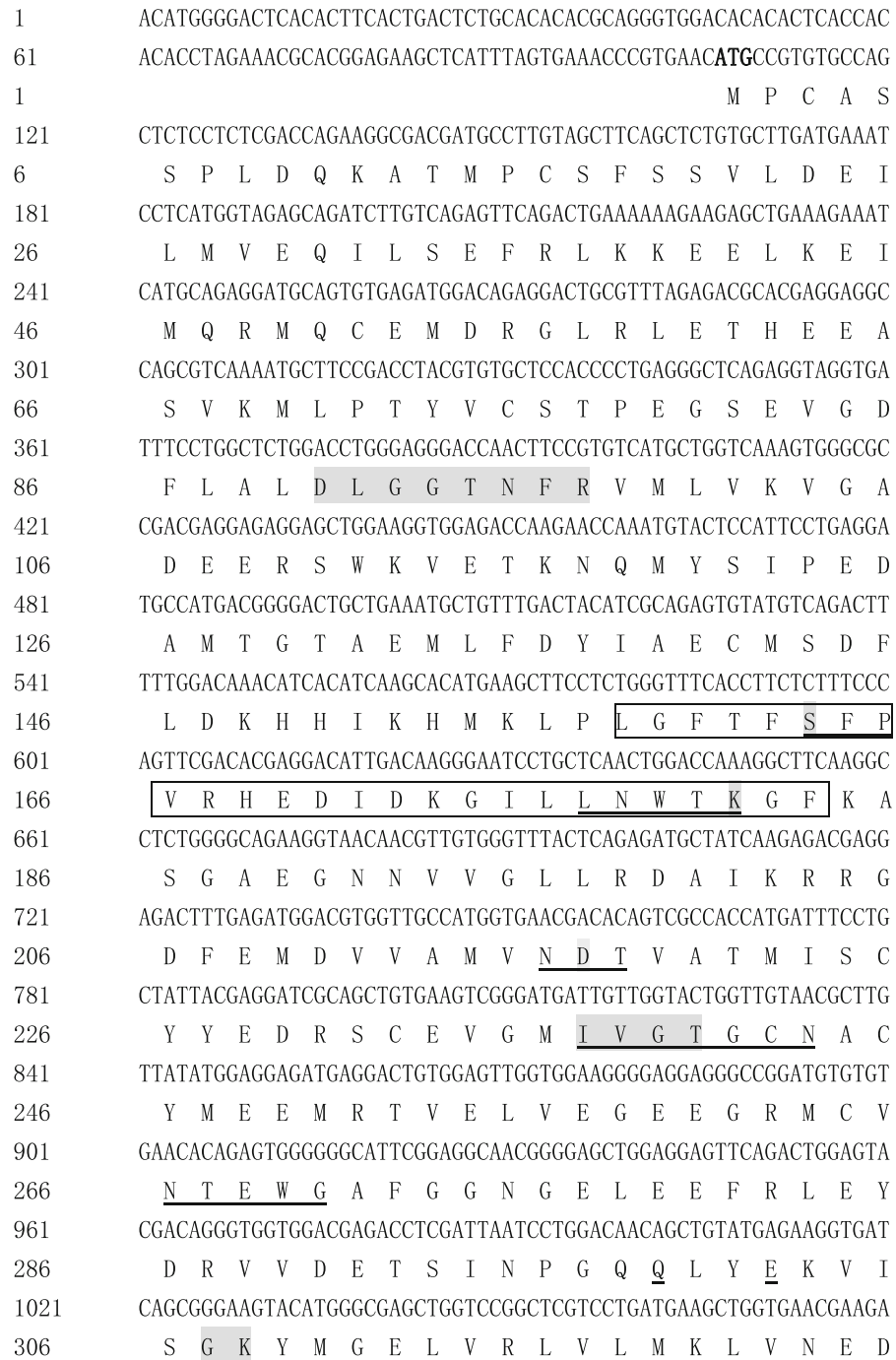

Fig. 1 Complete nucleotide and deduced amino acid sequences of glucokinase (GK) from turbot. Both the nucleotides and amino acids are *numbered along the left margin*. The start (ATG) and stop (TAA) codons are marked in *bold*. The polyadenylation signal (AATAAA) is *underlined in bold*, and the poly(A) tail is in *italic*. The hexokinase signature is in a *box*. The *gray amino acids* indicate MgATP-binding site. The *underlined amino acids* indicate glucose-binding site

species were carnivorous except for the Nile tilapia. Common carp, grass carp *Ctenopharyngodon idella*, zebrafish and topmouth culter *Culter alburnus*, which belong to the freshwater fish, are clustered to the other subgroup, with a 100 % bootstrap value.

At present, two mammalian genes coding FBPase are known. In order to characterize the evolutionary relationships of the two FBPase isoenzymes, a phylogenetic analysis was performed using the amino acid sequences of liver and muscle FBPase from GeneBank

Fig. 1 continued

```
1081  CCTGCTGTTTGATGGCGAAGCCTCGGAGCTGCTGAAGACACGTGGCAGCTTTGAGGCGCG
326     L  L  F  D  G  E  A  S  E  L  L  K  T  R  G  S  F  E  A  R
1141  TTACGTCTCACAGGTTGAGAGTGACACTGGGGACAGAAAACAGATCTACAACATCCTGTC
346     Y  V  S  Q  V  E  S  D  T  G  D  R  K  Q  I  Y  N  I  L  S
1201  CTCCCTGGGTGTTCTGCCGTCCGAGCTGGACTGTGATATTGTGCGTCTGGTCTGTGAGAG
366     S  L  G  V  L  P  S  E  L  D  C  D  I  V  R  L  V  C  E  S
1261  CGTCTCCACCCGCTCCGCTCACATGTGCGGCGCAGGGCTCGCATGTGTGATCAACATAAT
386     V  S  T  R  S  A  H  M  C  G  A  G  L  A  C  V  I  N  I  M
1321  GCGGGAGCGACGCGGCCAGGAAGCCCTGAAGATCACAGTGGGGGTGGACGGCTCTGTCTA
406     R  E  R  R  G  Q  E  A  L  K  I  T  V  G  V  D  G  S  V  Y
1381  CAAGCTGCACCCATGCTTCCGTGACAGGTTCCACAAAATTGTCAGGGACCTCACGCCTCA
426     K  L  H  P  C  F  R  D  R  F  H  K  I  V  R  D  L  T  P  H
1441  CTGTGACATCACCTTCATCCAGTCGGAGGAGGGGAGCGGTCGTGGCGCAGCCCTTATCTC
446     C  D  I  T  F  I  Q  S  E  E  G  S  G  R  G  A  A  L  I  S
1501  AGCGGTGGCCTGTAAGATGGCCGCATGCATGCTGACACAATAAACGGAGCTGCGCACTGA
466     A  V  A  C  K  M  A  A  C  M  L  T  Q  *
1561  GCTGTCTCCCTGCAGGACAGCGGCGAACTCTGAGCTCAATACACTGATGACGACGACGAT
1621  GACGATGACGATCATCATCATCCACAGACGTGCAGCTCTGGTCTTCTCCAGGCTCGGAGG
1681  GAGGTGTGTCAGTGAGCTGGATATCACTGGAACGATGTACAGACGAGAAAGCAGCAGTTT
1741  GGATTATAGACTATATTTGGTTGTAAACTGAACCAACTTGTAGCAATATCAGTGTTATTG
1801  AACTTTAATATTTGGTTTACTGTGGCACCCGGAACATTAGGTGGTTCATTTCATATACAT
1861  TCGCCACCACGGCCTGTTGATAGTGTTTTGAAGTGGGCTGCTCCATTTCAAAAATGGCTT
1921  GTGTATTATTGATGATAGTGTATTATATTACCAGTGAAAAGGATTCTTCTTGTATATGCT
1981  GTTGTAAACTATGTGACTACATCGAATCCAATGCTTTGTCGTGAAGTGATTCCTGTTTGT
2041  GAACGATGACAAATTTCACTGGCATCTTATATTTCCATTTAAATGTTTATAACTCCATGT
2101  GTCTTAGCCAGTAGGTTGTAATTTCTTCACATGTGCTTGATGAAATGTACTCTGGATGTG
2161  AATAAGATTAACTGTAAATAAAAAAAAGCTCAATTATGATAAAAGCTGAACTCTAAAAAA
2221  AAAAAA
```

by the software of Clustal X 1.83 and MEGA 4.0. The consensus tree shows two main branches (Fig. 4). The one clustered all FBPase liver type of teleost. The other contained FBPase muscle type of teleosts. The turbot FBPase sequence bears high similarity with Japanese sea perch FBPase liver type sequences, and the positioning of the nematode (CAB69047.1) sequence in the FBPase tree remains alone.

Tissue distribution of GK and FBPase mRNA

Tissue distribution of GK and FBPase mRNA in the gill, liver, intestine, kidney, heart, muscle and brain of adult turbot was determined by the real-time PCR method (Fig. 5). The result showed that GK mRNA was expressed at high level in the liver and moderate level in the muscle. The low levels were found in the intestine, kidney and brain. The lowest level was found in the gill and heart. The highest level of FBPase mRNA expression was also detected in the liver, moderate levels in intestine, kidney and brain, and the lowest level in gill, heart and muscle.

GK and FBPase expression responding to dietary carbohydrates

After the feeding trial, fish fed diet-G or diet-D had significantly higher GK mRNA level in liver than those fed the control diet-0 ($p < 0.05$) (Fig. 6A). Meanwhile, there was no significant difference in GK

Fig. 2 Complete nucleotide and deduced amino acid sequences of fructose-1,6-bisphosphatase (FBPase) from turbot. Both the nucleotides and amino acids are *numbered along the left margin*. The start (ATG) and stop (TAA) codons are marked in *bold*. The polyadenylation signal (AATAAA) is *underlined in bold*, and the poly(A) tail is in *italic*. The FBPase active site is in a *box*. The nuclear localization sequence (NLS) is *underlined*. The *gray amino acid* (K^{275}) indicates substrate/Fru-1,6-P_2ase-binding site. The active site which Mg^{2+} or Mn^{2+} is predicted to bind (D^{119}, D^{122}) is marked in *bold* and *box*

```
1     ACATGGGGTCACTTACGGCCTCAGGTTCCTGTCCCAGTCGCACACCTGACAGCAGAATGT
1                                                              M
61    CTGACAAGGGAACCTTCGATACCAACGTGCTGACCCTCACCAGGTTTGTTCTGGAGGAGG
2     S  D  K  G  T  F  D  T  N  V  L  T  L  T  R  F  V  L  E  E
121   GGAGGAAAGCACAGGGAACAGGTGAGCTGACCAACCTGCTCAACTCCATCTGCACTGCTG
22    G  R  K  A  Q  G  T  G  E  L  T  N  L  L  N  S  I  C  T  A
181   TCAAAGCCATTTCCACTGCTGTCAGGAAGGCTGGGATCGCTAACCTATATGGCATTGCTG
42    V  K  A  I  S  T  A  V  R  K  A  G  I  A  N  L  Y  G  I  A
241   GAAGCACCAATGTGACGGGGGACCAGGTGAAGAAGCTGGATATCCTGTCCAATGACCTGG
62    G  S  T  N  V  T  G  D  Q  V  K  K  L  D  I  L  S  N  D  L
301   TCATCAACATGATCAAGTCCTCCTTCACCTCCTGCGTGCTTGTGTCGGAGGAAGATGAGA
82    V  I  N  M  I  K  S  S  F  T  S  C  V  L  V  S  E  E  D  E
361   AGGCCATCATTGTGGACCCAGACATCAGAGGAAAATACATTGTGTGCTTTGATCCACTGG
102   K  A  I  I  V  D  P  D  I  R  G  K  Y  I  V  C  F  D  P  L
421   ATGGTTCCTCAAACATCGACTGTCTTGTCTCCATCGGAACCATTTTTGCCATCTACAGAA
122   D  G  S  S  N  I  D  C  L  V  S  I  G  T  I  F  A  I  Y  R
481   AGACCACAGACGATGAGCCTGCAGAGAATGATGCTTTGCAACCAGGAAGAAATATTGTTG
142   K  T  T  D  D  E  P  A  E  N  D  A  L  Q  P  G  R  N  I  V
541   CTGCTGGTTATGCTCTGTATGGCAGTGCCACCATGATGGTCCTCTCCACTGGTCAGGGAG
162   A  A  G  Y  A  L  Y  G  S  A  T  M  M  V  L  S  T  G  Q  G
601   TCAACTGCTTCATGCTTGATCCGGCAATTGGTGAGTTCATTTTAGTGGATCGAGATGTAA
182   V  N  C  F  M  L  D  P  A  I  G  E  F  I  L  V  D  R  D  V
661   AGATCAAGAAAAAGGGAAAAATCTACAGTTTGAATGAGGGATATGCACAGCACTTTTATC
202   K  I  K  K  K  G  K  I  Y  S  L  N  E  G  Y  A  Q  H  F  Y
721   CAGATGTGACAGAATACCTTCAAAAGAAGAAATACCCAGAGGATGGTTCTGCTCCGTATG
222   P  D  V  T  E  Y  L  Q  K  K  K  Y  P  E  D  G  S  A  P  Y
781   GCAGTCGATATGTTGGCTCAATGGTAGCCGATGTTCATCGTACTTTGGTGTATGGAGGAA
242   G  S  R  Y  V  G  S  M  V  A  D  V  H  R  T  L  V  Y  G  G
841   TCTTTTTATATCCTGCTAATGTCAAGAGTCCTAATGGCAAGCTGAGGCTGCTGTATGAAT
262   I  F  L  Y  P  A  N  V  K  S  P  N  G  K  L  R  L  L  Y  E
901   GCAACCCCATGGCCTTCATCATGGTGCAGGCAGGAGGCATGGCCACCACAGGATCCATGA
282   C  N  P  M  A  F  I  M  V  Q  A  G  G  M  A  T  T  G  S  M
961   ACGTTCTGGACATCCAGCCCACCTCTATCCACCAGCGAGTCCCTGTGGTCCTTGGATCCC
302   N  V  L  D  I  Q  P  T  S  I  H  Q  R  V  P  V  V  L  G  S
1021  CTGATGATGTGCAAGAATATATTTCCATTTACAAGAAGCATAACAAATGAGCAGGCGGAG
322   P  D  D  V  Q  E  Y  I  S  I  Y  K  K  H  N  K  *
1081  TCTGGCAGTGAGCAGAGCTAAACACAATCAGAAGACTCAGTCAGACGTTGCTTTCTACTC
1141  ATTCCTGCTGCACTGATGAACGAAGCAAGCGCTTCACTGGAACCTCACACTGTTTTCATT
1201  ACGCACAAGCATTGCGCTGTGTGTTATTTGAAGGGACATACCCTGTATTTTTTAAATAAA
1261  GACAAATGAAACAAAAAAAAAAACCTATAGTGAAAAAAAAAAAAAAAAAAAAA
```

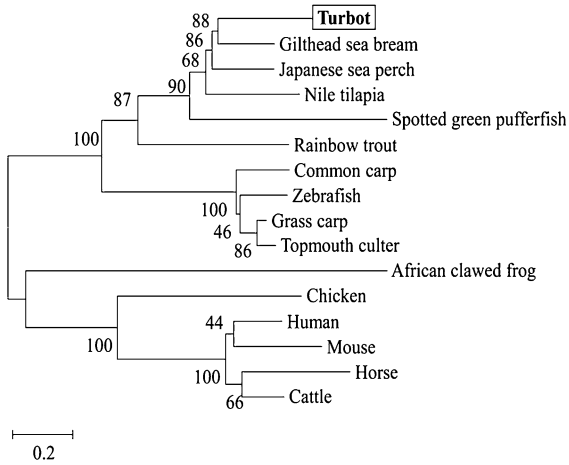

Fig. 3 Phylogenetic tree based on GK amino acid sequences made with MEGA 4.0 software using neighbor-joining (NJ) method. Figures represent percentage values from 1000 bootstrapping trials. The other GK sequences were selected from the GenBank, and the accession numbers were as follows: turbot (*Scophthatmus maximus*, JX678944) (*marked in a box*), gilthead seabream (*Sparus aurata*, AAC33585.2), Japanese sea perch (*Lateolabrax japonicus*, AEZ36053.1), Nile tilapia (*Oreochromis niloticus*, XP_003451068.1), rainbow trout (*Oncorhynchus mykiss*, NP_001117721.1), common carp (*Cyprinus carpio*, ACD37722.1), African clawed frog (*Xenopus laevis*, CAA63761.1), Grass carp (*Ctenopharyngodon idella*, ADD52460.1), zebrafish (*Danio rerio*, NP_001038850.2), topmouth culter (*Chanodichthys ilishaeformis*, ABA41457.1), human (*Homo sapiens*, ABS31137.1), mouse (*Mus musculus*, AAB00360.1), chicken (*Gallus gallus*, AAM83106.4), horse (*Equus caballus*, XP_001495888.2), spotted green puffer fish (*Tetraodon nigroviridis*, CAG08582.1), cattle (*Bos taurus*, NP_001095772.1)

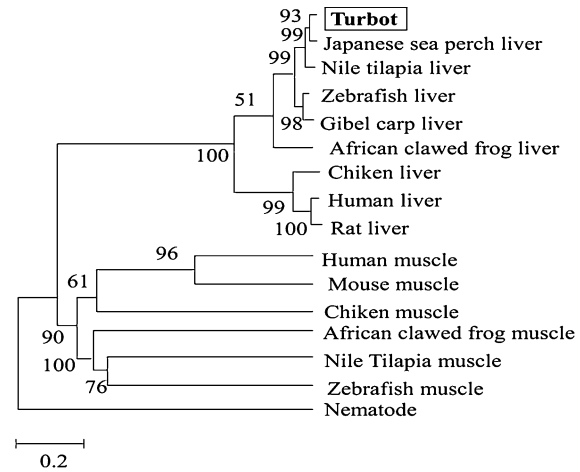

Fig. 4 Phylogenetic tree based on FBPase amino acid sequences made with MEGA 4.0 software using neighbor-joining (NJ) method. The figures represent percentage values from 1000 bootstrapping trials. The other FBPase sequences were selected from the GenBank, and the accession numbers were as follows: turbot (*Scophthalmus maximus*, KC184130) (*marked in a box*), human liver (*Homo sapiens*, AAA35817.1), rat liver (*Rattus norvegicus*, NP_036690.2), chicken liver (*Gallus gallus*, XP_425040.1), African clawed frog liver (*Xenopus laevis*, NP_001080528.1), Japanese sea perch liver (*Lateolabrax japonicus*, AFC36525.1), Nile tilapia liver (*Oreochromis niloticus*, XP_003449698.1), zebrafish liver (*Danio rerio*, NP_998297.1), gibel carp liver (*Carassius gibelio*, ADD91325.1), nematode (*Caenorhabditis elegans*, CAB69047.1), human muscle (*Homo sapiens*, NP_003828.2), mouse muscle (*Mus musculus*, AAH12720.1), chicken muscle (*Gallus gallus*, XP_425039.1), African clawed frog muscle (*Xenopus laevis*, NP_001167494.1), Nile Tilapia muscle (*Oreochromis niloticus*, XP_003444530.1), zebrafish muscle (*Danio rerio*, AAH80232.1)

mRNA levels between the glucose group and the dextrin group.

There was no significant difference in FBPase mRNA levels between all treatments ($p > 0.05$) (Fig. 6B).

Activities of GK and FBPase in liver

Activities of GK and FBPase in liver are presented in Fig. 7. Activities of GK in fish fed with glucose diet or dextrin diet were significantly higher than those in the control ($p < 0.05$). There was no significant difference in GK activity between the two carbohydrate treatments (diet-G and diet-D) ($p > 0.05$). The FBPase activities in liver were not significantly affected by different dietary treatments ($p > 0.05$).

Discussion

The GK and FBPase gene cloning and analysis

The present study showed that the turbot GK encoded a protein of 478 amino acids, which displayed 81–94 % identity to gilthead sea bream, perch, zebra fish, mouse, frog, chicken and human. Some conserved functional domains were found in turbot GK amino acids, including one conserved hexokinase signature sequence L^{158}–F^{183}. In human, the glucose-binding sites include S^{151}–P^{153}, N^{166}–K^{169}, N^{204}–T^{206}, I^{225}–N^{231}, N^{254}–G^{258}, Q^{287} and E^{290}. The residues T^{168}, K^{169}, N^{204}, D^{205}, N^{231}, E^{256} and E^{290} also contribute to the formation of hydrogen bonds with all the oxygen atoms of glucose (Mahalingam et al. 1999). These sites were also existed in turbot GK

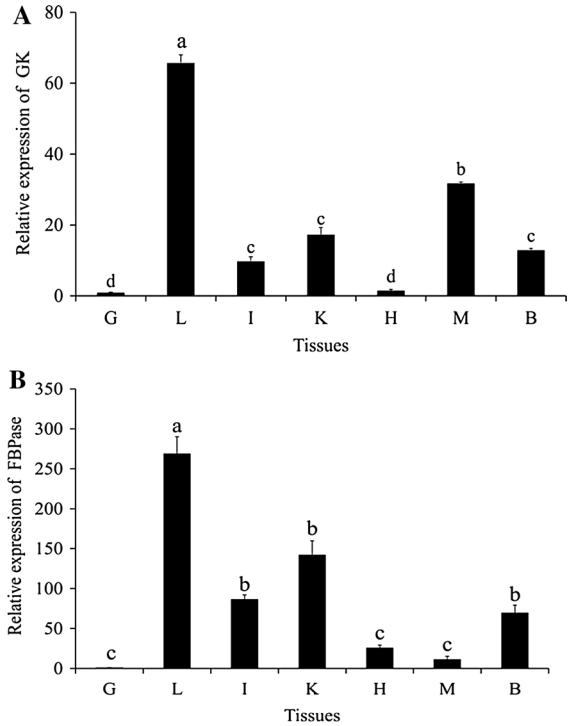

Fig. 5 Tissue-specific expression levels of glucokinase (GK) (**A**) and fructose-1,6-bisphosphatase (FBPase) (**B**) mRNA in turbot adult as detected by real-time PCR. GK (**A**) and FBPase (**B**) mRNA levels in liver (L), intestine (I), kidney (K), heart (H), muscle (M) and brain (B) were normalized to that of gill (G). A housekeeping gene α-elongation factor 1 (EF1α) was used as an endogenous reference. All values represent the mean ± SE ($n = 3$). *Bars* bearing *different letters* are significantly different ($p < 0.05$, Tukey's test)

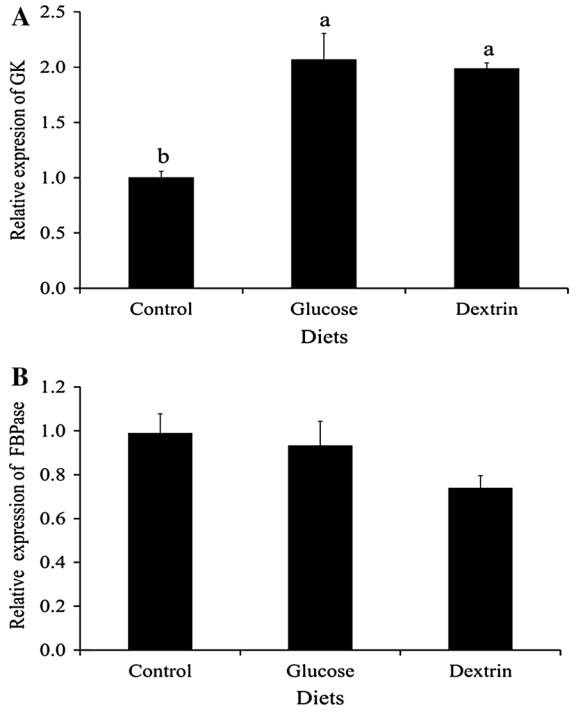

Fig. 6 Relative glucokinase (GK) (**A**) and fructose-1,6-bisphosphatase (FBPase) (**B**) mRNA levels after fed with different diets in the liver of turbot. GK and FBPase mRNA levels were evaluated by real-time quantitative PCR and normalized to that of control group. All values represent the mean ± SE ($n = 3$ replicates, and three fish per replicate). Significant differences between the diets are indicated by *different letters* ($p < 0.05$, Tukey's test)

amino acids. The ATP-binding site is formed by residues D^{78}–R^{85}, S^{151}, K^{169}, D^{205}, I^{225}–T^{228}, G^{295}–K^{296}, E^{331}–R^{333}, S^{336} and G^{410}–H^{416} in human GK amino acid; in particular, residues S^{151}, K^{169}, D^{205}, I^{225}–T^{228} are also involved in the formation of the glucose-binding site (Mahalingam et al. 1999). All of these residues are conserved and in similar locations in all species except that residue T^{332} was replaced by the residue A^{344} in turbot. In African clawed frog GK amino acid, residues H^{141}–L^{144}, E^{51} and E^{52} participate in the binding of the regulatory protein (Veiga-da-Cunha et al. 1996). In these residues of turbot, K^{142} was replaced by M^{154}, and other sites were conservatively existed.

At present, two mammalian genes coding FBPase are known. They are the liver isoenzyme and the muscle isoenzyme (Tillmann et al. 2002). The former mainly expresses in liver, kidney and monocytes. The latter is isolated in the skeletal muscle. The two isoenzymes differ in their kinetics, immunological properties and the amino acid composition (Stein et al. 2001). The amino acid sequence of turbot FBPase was highly conserved with the sequence of other species. It had a typical nuclear localization structure (K^{203}–K^{207}) and an FBPase active site (G^{274}–A^{286}). In human and rat liver, K^{275} is involved in binding with substrate or fructose-2,6-phosphate (El-Maghrabi et al. 1991). Turbot also has this site. Residues D^{119} and D^{122} were conserved in human liver and pig kidney, which contribute to the combine of the enzyme with the ions. The mutations occurred in these two residues significantly reduce the catalytic activity of FBPase (El-Maghrabi et al. 1993). These residues were conserved in turbot FBPase. In the FBPase sequence of human, residues E^{98}, E^{99} and D^{119} are predicted to bind metal ions, and residues of L^{31}, A^{25}, M^{178}, T^{32}, Y^{114} and R^{141} are predicted to

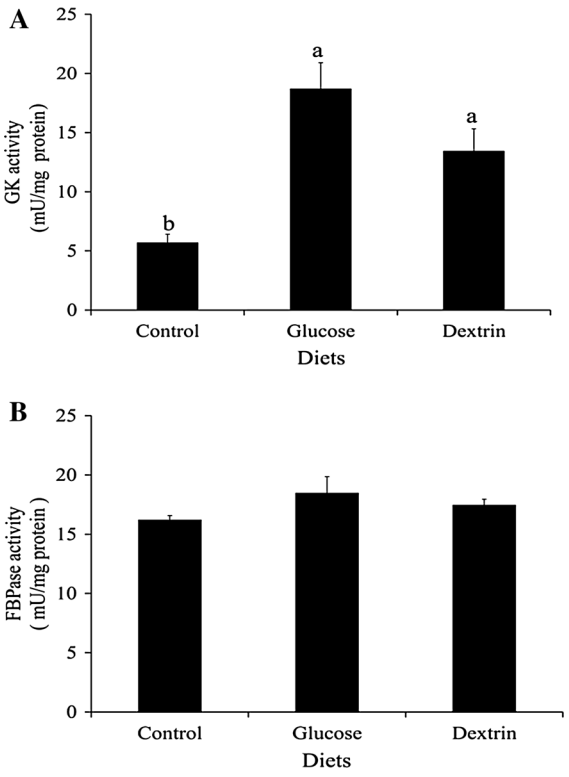

Fig. 7 Effects of dietary carbohydrates on the activities of glucokinase (GK) (**A**) and fructose-1,6-bisphosphatase (FBPase) (**B**) in the liver of turbot. All values represent the mean ± SE ($n = 3$ replicates, and three fish per replicate). Significant differences between the diets are indicated by *different letters* ($p < 0.05$, Tukey's test)

interact with the allosteric inhibitor AMP. In these key sites, residue M^{178} was replaced by T^{178} in turbot. Four metal ion-binding sites (E^{97}, D^{118}, E^{280} and L^{120}) are located at the pig kidney FBPase enzyme active site. And a hypothesis predicts that mutation of either D^{118} or D^{121} would greatly decrease Fru-l,6-P_2ase catalysis (Zhang et al. 1993). These residues were all conserved in turbot FBPase.

The results of neighbor-joining phylogenetic tree revealed that turbot GK was closer to gilthead sea bream than any other vertebrates. The present data revealed that turbot GK is mainly presented in the liver cells, which proves that the liver is the main organ to form GK. The tissue distribution of the GK in turbot is similar to those in mammals. In humans, GK has been discovered in specific cells in four types of tissue: liver, pancreas, small intestine and brain. However, it is mainly expressed in glucose-sensitive tissues, such as liver and pancreatic islet beta cells under the control of insulin (Tanizawa et al. 1991).

According to the results of clustering analysis, the turbot FBPase sequence cloned in this study belonged to the liver type. The results of tissue distribution of FBPase showed that the highest level was found in liver, the lower level in the kidney and intestine and the lowest level in the heart and gill. This result is consistent with the tissue distribution of FBPase in other fish species.

Influences of dietary carbohydrates on the GK and FBPase activities and gene expressions

The GK, as one of the hexokinase (HK) isoenzymes, is the initial enzyme of the glycolytic pathway and catalyzes the phosphorylation of glucose into glucose-6-phosphate. In the present study, the enhanced activity and gene expression of hepatic GK in turbot were found after dietary carbohydrate intake. In mammals, high-carbohydrate diet could effectively modulate GK activity (Iynedjian 2009). Previous studies on fish, such as rainbow trout, gilthead sea bream and common carp, also found that the activity as well as the gene expression of the hepatic GK was highly induced by dietary carbohydrates (Capilla et al. 2003; Caseras et al. 2002; Metón et al. 2004; Panserat et al. 2000, 2001, 2002, 2014). In the present study, the main differences between the control diet and the carbohydrate-supplemented diets were the contents of protein and carbohydrates (Table 1). The diet-0 had relatively high protein and low carbohydrate levels. The diet-G and diet-D had relatively low protein and high carbohydrate contents. Combined the data on GK activity and gene expression, in the present study, it was suggested that turbot could utilize the carbohydrates to meet part of the energy demands in the case of low dietary protein content.

There was no significant difference in the activity or mRNA level of hepatic GK between the treatments with glucose- and dextrin-supplemented diets. However, they were both significantly higher than those in the diet-0 without carbohydrate supplementation. Nevertheless, previous studies on European sea bass and gilthead sea bream showed that dietary glucose induced significantly higher hepatic GK activity than dietary starch (Enes et al. 2006b, 2008). In the liver of gilthead sea bream, both GK mRNA levels and activity reached a peak at a postprandial time of

4–8 h (Caseras et al. 2000). Garcia-Riera and Hemre (1996) observed that turbot was able to restore alterations in carbohydrate metabolism efficiently within 24 h when injected intraperitoneally with exactly 1 g glucose per kg body weight. Miao et al. (2013) reported that there were no significant differences in blood glucose levels in turbot fed 15 % of dietary glucose (diet-G), dextrin (diet-D) or diet without carbohydrate supplementation (diet-0). The blood glucose concentrations were 0.27, 0.23 and 0.29 mmol l^{-1}, respectively. The lack of consistency between GK activity, gene expression and blood glucose level of turbot cannot be explained at the moment, partly due to that the samples were taken 24 h after meal. Further studies are therefore necessary to elucidate the regulation of GK activity in turbot, especially at the different time points after meal.

As one of the key enzymes in gluconeogenesis, FBPase plays an important role in catalyzing the hydrolysis of fructose-1,6-bisphosphate to fructose-6-phosphate. In mammals, FBPase gene expression is regulated through both transcriptional and posttranscriptional mechanisms (Okar and Lange 1999). The results in this study confirmed that both expression and enzyme activity of FBPase were not influenced by dietary carbohydrates. In accordance, previous study found that in carnivorous species like the perch (Borrebaek and Christophersen 2000), rainbow trout (Kirchner et al. 2008), European sea bass (Enes et al. 2006a; Moreira et al. 2008) and gilthead sea bream (Enes et al. 2008; Fernández et al. 2007), both the FBPase gene expression and activity in liver could not be regulated by dietary carbohydrates. In omnivores like common carp, Panserat et al. (2002) also reported no differences in FBPase gene expression between fish fed with or without carbohydrates. It is suggested that the gene expression or activity of FBPase was irrespective to the carbohydrate intake.

In conclusion, results from the molecular sequence similarities and phylogenetic analysis revealed that the deduced amino acid sequence of GK and FBPase shared high identity with other species. The increased hepatic GK activity and gene expression indicated that the first step of glucose utilization was activated in turbot by dietary carbohydrates. There is no significant effect on gene expression and/or activity of FBPase by dietary carbohydrates. However, this study only offers information on the regulation of these enzymes at the fasting state. Further studies during the postprandial period are needed.

Acknowledgments This research was financially supported by the National Basic Research Program (973 Program, No. 2014CB138600) and the National Natural Science Foundation of China (No. 31072219).

References

Borrebaek B, Christophersen B (2000) Hepatic glucose phosphorylating activities in perch (*Perca fluviatilis*) after different dietary treatments. Comp Biochem Phys B 125:387–393

Bradford M (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle dye binding. Anal Biochem 72:248–254

Capilla E, Médale F, Navarro I, Panserat S, Vachot C, Kaushik S, Gutiérrez J (2003) Muscle insulin binding and plasma levels in relation to liver glucokinase activity, glucose metabolism and dietary carbohydrates in rainbow trout. Regul Pept 110:123–132

Caseras A, Metón I, Fernández F, Baanante IV (2000) Glucokinase gene expression is nutritionally regulated in liver of gilthead sea bream (*Sparus aurata*). BBA Gene Struct Expr 1493:135–141

Caseras A, Metón I, Vives C, Egea M, Fernández F, Baanante IV (2002) Nutritional regulation of glucose-6-phosphatase gene expression in liver of the gilthead sea bream (*Sparus aurata*). Br J Nutr 88:607–614

El-Maghrabi MR, Lange AJ, Kümmel L, Pilkis SJ (1991) The rat fructose-1,6-bisphosphatase gene. J Boil Chem 266:2115–2120

El-Maghrabi MR, Gidh-Jain M, Austin LR, Pilkis SJ (1993) Isolation of a human liver fructose-1,6-bisphosphatase cDNA and expression of the protein in *Escherichia coli*. J Boil Chem 268:9466–9472

Enes P, Panserat S, Kaushik S, Oliva-Teles A (2006a) Effect of normal and waxy maize starch on growth, food utilization and hepatic glucose metabolism in European sea bass (*Dicentrarchus labrax*) juveniles. Comp Biochem Phys A 143:89–96

Enes P, Panserat S, Kaushik S, Oliva-Teles A (2006b) Rapid metabolic adaptation in European sea bass (*Dicentrarchus labrax*) juveniles fed different carbohydrate sources after heat shock stress. Comp Biochem Phys A 145:73–81

Enes P, Panserat S, Kaushik S, Oliva-Teles A (2008) Growth performance and metabolic utilization of diets with native and waxy maize starch by gilthead sea bream (*Sparus aurata*) juveniles. Aquaculture 274:101–108

Fernández F, Miquel AG, Córdoba M, Varas M, Metón I, Caseras A, Baanante IV (2007) Effects of diets with distinct protein-to-carbohydrate ratios on nutrient digestibility, growth performance, body composition and liver inter-mediary enzyme activities in gilthead sea bream (*Sparus aurata*, L.) fingerlings. J Exp Mar Biol Ecol 343:1–10

Garcia-Riera MP, Hemre GI (1996) Glucose tolerance in turbot, *Scophthalmus maximus* (L.). Aquac Nutr 2:117–120

Gong G, Xue M, Wang J, Su X O, Wu X F, Zheng Y H, Han F (2013) Cloning and sequence analysis of full length cDNA of key enzymes of gluconeogenesis in Siberian Sturgeon (*Acipenser baerii*). Chin J Anim Nutr 25(7):1504–1518 (in Chinese with English abstract)

Gouillou-Coustans MF, Fournier V, Métailler R, Vachot C, Desbruyères E, Huelvan C, Moriceau J, Delliou HL, Kaushik SJ (2002) Dietary arginine degradation is a major pathway in ureagenesis in juvenile turbot (*Psetta maxima*). Comp Biochem Phys A 132:305–319

Hemre GI, Mommsen TP, Krogdahl Å (2002) Carbohydrates in fish nutrition: effects on growth, glucose metabolism and hepatic enzymes. Aquac Nutr 8:175–194

Iynedjian PB (2009) Molecular physiology of mammalian glucokinase. Cell Mol Life Sci 66:27–42

Jafri AK (1995) Protein-sparing effect of dietary carbohydrate in diets for fingerling *Labeo rohita*. Aquaculture 136(3):331–339

Kirchner S, Kaushik S, Panserat S (2003) Low protein intake is associated with reduced hepatic gluconeogenic enzyme expression in rainbow trout (*Oncorhynchus mykiss*). J Nutr 133:2561–2564

Kirchner S, Panserat S, Lim PL, Kaushik S, Ferraris RP (2008) The role of hepatic, renal and intestinal gluconeogenic enzymes in glucose homeostasis of juvenile rainbow trout. J Comp Physiol B 178:429–438

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods 25:402–408

Mahalingam B, Cuesta-Munoz A, Davis EA, Matschinsky FM, Harrison RW, Weber IT (1999) Structural model of human glucokinase in complex with glucose and ATP. Diabetes 48:1698–1705

Metón I, Caseras A, Fernández F, Baanante IV (2004) Molecular cloning of hepatic glucose-6-phosphatase catalytic subunit from gilthead sea bream (*Sparus aurata*): response of its mRNA levels and glucokinase expression to refeeding and diet composition. Comp Biochem Phys B 138:145–153

Miao SY, Nie Q, Miao HJ, Zhang WB, Mai KS (2013) Effects of different carbohydrate sources and levels on growth performance and metabolic response of juvenile turbot *Scophthalmus maximus*. J Fish China 37(6):910–919 (in Chinese with English abstract)

Moreira IS, Peres H, Couto A, Enes P, Oliva-Teles A (2008) Temperature and dietary carbohydrate level effects on performance and metabolic utilisation of diets in European sea bass (*Dicentrarchus labrax*) juveniles. Aquaculture 274:153–160

Nie Q, Miao HJ, Miao SY, Chen CY, Li J, Zhang WB, Mai KS (2013) Effects of dietary carbohydrate sources and levels on the activities of carbohydrate metabolic enzymes in turbot *Scophthalmus maximus* L. Acta Hydrobiol Sin 37(3):425–433 (in Chinese with English abstract)

Okar DA, Lange AJ (1999) Fructose-2, 6-bisphosphate and control of carbohydrate metabolism in eukaryotes. Biofactors 10(1):1–14

Panserat S, Médale F, Blin C, Brèque J, Vachot C, Plagnes-Juan E, Gomes E, Krishnamoorthy R, Kaushik S (2000) Hepatic glucokinase is induced by dietary carbohydrates in rainbow trout, gilthead seabream, and common carp. Am J Physiol Regul Integr Comp Physiol 278:R1164–R1170

Panserat S, Capilla E, Gutierrez J, Frappart PO, Vachot C, Plagnes Juan E, Aguirre P, Breque J, Kaushik S (2001) Glucokinase is highly induced and glucose-6-phosphatase poorly repressed in liver of rainbow trout (*Oncorhynchus mykiss*) by a single meal with glucose. Comp Biochem Physiol B 128:275–283

Panserat S, Plagnes-Juan E, Kaushik S (2002) Gluconeogenic enzyme gene expression is decreased by dietary carbohydrates in common carp (*Cyprinus carpio*) and gilthead seabream (*Sparus aurata*). BBA Gene Struct Expr 1579:35–42

Panserat S, Rideau N, Polakof S (2014) Nutritional regulation of glucokinase: a cross-species story. Nutr Res Rev 27:21–47

Polakof S, Míguez JM, Soengas J (2008) Dietary carbohydrates induce changes in glucosensing capacity and food intake of rainbow trout. Am J Physiol Reg I 295:478–489

Regost C, Arzel J, Cardinal M, Robin J, Laroche M, Kaushik SJ (2001) Dietary lipid level, hepatic lipogenesis and flesh quality in turbot (*Psetta maxima*). Aquaculture 193:291–309

Stein S, Liehr T, Eschrich K (2001) Characterization of the mouse liver fructose-1,6-bisphosphatase gene. Gene 264:215–224

Tanizawa Y, Koranyi LI, Welling CM, Permutt MA (1991) Human liver glucokinase gene: cloning and sequence determination of two alternatively spliced cDNAs. Proc Natl Acad Sci USA 88(16):7294–7297

Tillmann H, Bernhard D, Eschrich K (2002) Fructose-1,6-bisphosphatase genes in animals. Gene 291:57–66

Tranulis MA, Dregni O, Christophersen B, Krogdahl A, Borrebaek B (1996) A glucokinase-like-enzyme in the liver of Atlantic salmon (*Salmo salar*). Comp Biochem Physiol B 114:35–39

Van Ham EH, Berntssen MHG, Imsland AK, Parpoura AC, Wendelaar Bonga SE, Stefansson SO (2003) The influence of temperature and ration on growth, feed conversion, body composition and nutrient retention of juvenile turbot (*Scophthalmus maximus*). Aquaculture 217:547–558

Veiga-da-Cunha M, Courtois S, Michel A, Gosselain E, Schaftingen EV (1996) Amino acid conservation in animal glucokinases. J Biol Chem 27:6292–6297

Zhang Y, Liang JY, Huang S, Ke H, Lipscomb WN (1993) Crystallographic studies of the catalytic mechanism of the neutral form of fructose-1, 6-bisphosphatase. Biochemistry 32:1844–1857