Transcriptome analysis reveal alterations in hepatic glycan biosynthesis and metabolism of grass carp (Ctenopharyngodon idellus) fed with broad beans

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Research Article

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Transcriptome analysis reveal alterations in hepatic glycan biosynthesis and metabolism of grass carp (*Ctenopharyngodon idellus*) fed with broad beans

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Abstract: The meat of grass carp (*Ctenopharyngodon idellus*) fed broad beans is crispy, called crisp grass carp. In order to better understand the changes mechanistic in liver tissue of crisp grass carp, gene expression profiles and pathways of liver tissues were performed by using RNA-seq. As a result of the transcriptome analysis, the total number of reads produced for each liver sample ranged from 35,914,404 to 42,460,834. A total of 2519 differentially expressed genes (DEGs) were identified. Among them, 1156 genes were up-regulated and 1363 genes were down-regulated. Gene Ontology (GO) annotations indicated that DEGs were mainly enriched in biological processes of ribosome and structural constituent of ribosome. Kyoto encyclopedia of genes and genomes (KEGG) pathway analysis revealed that DEGs were mainly enriched in metabolism of energy, amino acid, carbohydrate, and lipid acid, and the genes in these pathways were up-regulated. The protein-protein interaction (PPI) network with 260 nodes and 249 edges was constructed and 3 modules were extracted from the entire network. *ITML, STT3B, SEL1L, UGGT1, MLEC, IL1B, ALG5, KRTCAP2, NFKB2, IRAK3* genes were the top 10 hub genes with the closest connections to other nodes. In summary, this study identified several candidate
genes and focused on glycan biosynthesis and metabolism pathways, providing a reference for further investigation into the mechanism of liver metabolism in grass carp fed with broad beans.

**Keywords:** grass carp; broad bean; liver; transcriptomics

### 1 Introduction

Grass carp is one of the freshwater fish with the largest amount of cultivation in China, which also is the most consumed fish due to its delicious taste and rich protein content (Yu et al., 2017). With the continuous improvement of the living standards of Chinese residents, the cultivation of higher quality grass carp products has become a key issue. Currently in fish feed, fishmeal and soybean meal are the main protein sources for grass carp (Peng et al., 2020). With the rapid development of the aquaculture industry, fishmeal and soybean meal became scarce and their prices increased (Sarker et al., 2018). Therefore, it is essential to find an alternative plant protein feed for grass carp.

The broad bean is a herbaceous plant belonging to the subfamily Papilionaceae of the family Fabaceae of the order Rosaceae, and is widely planted all over the world. Broad beans are rich in protein, carbohydrates and trace elements (Mejri et al., 2018). It is commonly used as a nutritional feed for animals such as pigs, poultry, ruminants and fish (Shi et al., 2022). In the early 1970s, the technicians of the May 7th Cadre School in Guangdong Province accidentally discovered that in the adult grass carp breeding stage, after feeding a single feed of broad beans for 90-120 days, the muscle hardness increased, the meat was firm, and the taste was crisp (Yu et al., 2014; Fu et al., 2022). The modified grass carp is called crisp grass carp. Crisp grass carp shows higher muscle hardness and crispness (Fu et al., 2022; Yu et al., 2017), which is extremely popular among consumers.

Compared to common grass carp, crisped grass carp showed significant increase in muscle hardness, elasticity, chewing power and adhesion, collagen content, myofibril length and density, and reduction in myofibril diameter (Zhang
et al., 2021; Fu et al., 2020). In addition, broad beans affect the fatty acid content of fish and increase fat deposition in viscera (Tian et al., 2019). It has been shown that continuous consumption of broad beans by grass carp leads to permanent inflammation-induced intestinal mucosal damage and hepatic steatosis (Li et al., 2018; Lin et al., 2012), which seriously affects the health and quality of grass carp. The liver plays an important role in maintaining metabolic homeostasis as the main site of synthesis, metabolism and storage of carbohydrates, proteins and lipids (Trefts et al., 2017).

Therefore, in order to explore the effect of broad bean on the growth and metabolism of grass carp, this study took grass carp as the research object, and set up two groups of experiments, namely the group fed with broad bean and the group without fed with broad bean. Transcriptome sequencing analysis was carried out on the grass carp livers of the two groups of experiments, and the effect of broad beans on the liver tissue metabolism of grass carp was clarified from the perspective of molecular biology, so as to provide a reference for the healthy breeding and quality improvement of grass carp.

2 Materials and Methods

2.1 Animal and Sample Collection

Healthy grass carp were purchased from an aquaculture farm in Zunyi, Guizhou Province, China. The fish were first temporarily cultured in a cement pond (5 m × 5 m × 1.5 m) for 1 week and the feed amount for each day was 2-3% of fish weight. A total of 180 fish with initial weight of 768 ± 75 g were randomly divided into crisp grass carp (Group A) and ordinary grass carp groups (Group B), with three replicates each group. They were cultured in six cement ponds (2 × 2 × 1.5 m), with 30 fish in each pond. Crisp grass carp were fed solely with whole faba beans, the feed was soaked in about 0.15% salt water for 24 h, and then soaked in water for 12 h until the broad beans were opened after the germ. The single feeding amount of broad bean accounted for 2%-3% of the body weight of fish. The ordinary grass carp were fed with commercial diet (crude protein: 329.9 g kg⁻¹; crude lipid: 43.8 g kg⁻¹; Tongwei Company, China). The fish were
fed twice per day (at 8:00 and 17:00). The water temperature was kept at
25-30 °C, pH was 6.5-7.5, and dissolved oxygen was above 5.0 mg/L. The final
weights of crisp grass carp and ordinary grass carp were 1,992 ± 125 g and
2,457 ± 132 g after 120 days, respectively. One fish was randomly selected from
each pond to collect its muscle tissue, which was placed at −80 °C until RNA
extraction. The procedure in this experiment was approved by the Ethics
Committee of Experimental Animal of Zunyi Normal College.

2.2 RNA Preparation

Total RNA was isolated from liver tissue of grass carps and crisp grass carps
using the TRIzol reagent (Takara, Dalian, China) according to the manufacturer’s
instruction. The concentration of the isolated RNA was determined by measuring
absorbance at 260 nm. The integrity of the RNA was determined by agarose gel
electrophoresis and Agilent BioAnalyzer 2100 (Agilent Technologies, San Jose,
CA, USA). The RNA was used for transcriptomics analysis.

2.3 Library Construction and Sequencing

Six RNA samples with high quality (RIN > 8.7) and concentration (average
concentration: 477.4 ng/µL) were used to construct the sequencing libraries and
high-throughput sequencing was performed on the Illumina novaseq 6000
platform (San Diego, CA, USA) following the manufacturer’s recommendations,
generating 150 bp paired-end reads (Table S1). The high-quality clean reads
were obtained by filtering the raw reads and removing: (1) the sequences
containing adapters; (2) the sequences with more than 10% of N bases; (3) the
sequences with more than 50% base quality values less than 10.

2.4 Differential Expression Analysis

Illumina HiSeq 4000 sequencer reads were paired-end and quality controlled
by Q30. Amplification of the 30 adaptor and the removal of low-quality reads
were performed by cutadapt software (v1.9.3), followed by alignment with the
reference genome (C_idella_female_scaffolds. fasta V1) using hisat2 software
(v2.0.4). Guided by the Ensembl gtf gene annotation file, cuffdiff software was
then used to get the gene level fragments per kilobase per million (FPKM) as the
expression profiles of mRNA and fold change. The number of clean reads for each gene was calculated and FPKM was used to estimate the expression abundance of transcripts from different samples (Roberts et al., 2011). Differential expression analyses of the A and B groups were performed using the DESeq R package (Wang et al., 2009; Love et al., 2014) and genes with a p-value $\leq 0.05$ and an expression $|\log_2 \text{Fold}| \geq 1$ were identified as DEGs.

2.5 GO and KEGG Analysis

To annotate the function of these DEGs, Gene Ontology (GO) analysis was conducted by using the GOseq software for each of the three main categories: biological process, cellular component and molecular function. Biological pathways enriched for the identified DEGs through Kyoto encyclopedia of genes and genomes (KEGG) pathway analyses were carried out using the KOBAS software.

2.6 Statistical Analysis

Data are expressed as mean ± standard mean of error (SEM). The statistical significance of the difference between the two groups were conducted using one-way ANOVA with Dunnet’s t-test at $p < 0.05$ probability levels in SPSS 25.0. “*” was considered significant difference ($p < 0.05$); “**” was considered an extremely significant difference ($p < 0.01$).

3 Results

3.1 High-Throughput Sequencing and Read Mapping

In this study, a total of six libraries in liver tissue were established by high-throughput RNA sequencing. The clean reads of each sample ranged from 35 million to 43 million, the mapped reads and unique mapped reads were more than 92.13% and 94.16%, respectively. In addition, more than 84.12% mapped to gene were detected in each sample (Table 1). According to the correlation analysis of 6 samples, the differences between biological replicates were small and the repeatability was high, which indicated that the selection of experimental samples was consistent and reliable (Figure 1).
3.2 Analysis of Differentially Expressed Genes

A comparison of the liver transcriptomes of A and B groups revealed 2519 DEGs, with criteria of $|\log_{2}\text{FoldChange}| > 1$, a $p$-value of less than 0.05, including 1156 up-regulated and 1363 down-regulated DEGs (Figure 2). However, with an adjusted $p$-adjust less than 0.05, 1399 genes were detected in liver tissues. A number of differentially expressed genes were highly expressed in the liver tissues of both groups.

3.3 Gene ontology enrichment for functional analysis of differentially expressed genes

To dissect the functional categories of DEGs, GO enrichment analysis were performed. GO enrichment revealed that most of the DEGs were classified into three major functional categories, including cellular component, biological process, and molecular function (Figure 3A). In the cellular component category, most genes were enriched in nuclear outer membrane-endoplasmic reticulum membrane network (GO:0042175, 53 genes), endoplasmic reticulum membrane (GO:0005789, 52 genes), endomembrane system (GO:0012505, 118 genes), endoplasmic reticulum (GO:0005783, 40 genes) and organelle membrane (GO:0031090, 96 genes). In the molecular function category, DEGs were enriched in iron ion binding (GO:0005506, 28 genes), oxidoreductase activity (GO:0016491, 72 genes), heme binding (GO:0020037, 24 genes), tetrapyrrole binding (GO:0046906, 24 genes) and catalytic activity (GO:0003824, 339 genes). Meanwhile, the DEGs involved in fatty acid metabolic process (GO:0006631, 21 genes), small molecule metabolic process (GO:0044281, 78 genes), lipid metabolic process (GO:0006629, 54 genes), and oxidation-reduction process (GO:0055114, 69 genes) were enriched in the biological process category (Table S2).

GO enrichment showed that up-regulated genes were significantly enriched for cellular components and biological process (Figure 3B). In the molecular function category, up-regulated genes were enriched in iron ion binding.
(GO:0005506, 19 genes), oxidoreductase activity (GO:0016491, 42 genes),
monooxygenase activity (GO:0004497, 16 genes), heme binding (GO:0020037, 16
genes), tetrapyrrole binding (GO:0046906, 16 genes), and oxidoreductase
activity, acting on paired donors, with incorporation or reduction of molecular
oxygen (GO:0016705, 17 genes). Meanwhile, in the biological process category,
up-regulated genes were involved in oxidation-reduction process (GO:0055114,
38 genes) mainly (Table S3). GO enrichment showed that down-regulated genes
were significantly enriched for cellular components (Figure 3C). In the molecular
function category, down-regulated genes were enriched in endomembrane
system (GO:0012505, 95 genes), nuclear outer membrane-endoplasmic reticulum
membrane network (GO:0042175, 45 genes), endoplasmic reticulum membrane
(GO:0005789, 44 genes), endoplasmic reticulum (GO:0005783, 37 genes), and
organelle membrane (GO:0031090, 74 genes) (Table S4).

3.4 Kyoto encyclopedia of genes and genomes enrichment for functional
analysis of differentially expressed genes

Kyoto encyclopedia of genes and genomes pathway classification and
functional enrichment for DEGs were performed to determine the main
biochemical metabolic pathways and signal transduction pathways. KEGG
pathway enrichment analysis showed that the DEGs were statistically enriched in
20 pathways (Figure 4A), and 10 of the pathways were related to metabolism of
energy, amino acid, carbohydrate, and lipid acid metabolism, including steroid
biosynthesis, glutathione metabolism, metabolism of xenobiotics by cytochrome
P450, fatty acid degradation, drug metabolism - cytochrome P450, terpenoid
backbone biosynthesis, N-Glycan biosynthesis, and steroid hormone biosynthesis.
The genes in these pathways were up-regulated (Table S5).

KEGG pathway enrichment analysis showed that the up-regulated genes
were statistically enriched in 20 pathways (Figure 4B), and 17 of the pathways
were related to metabolism and organismal systems. Metabolism pathways
included metabolism of xenobiotics by cytochrome P450, drug metabolism -
cytochrome P450, fatty acid degradation, arachidonic acid metabolism, drug metabolism - other enzymes, linoleic acid metabolism, tryptophan metabolism. Organismal systems pathways included protein digestion and absorption, longevity regulating pathway - worm, cholesterol metabolism, ovarian steroidogenesis, vitamin digestion and absorption, and carbohydrate digestion and absorption (Table S6).

KEGG pathway enrichment analysis showed that the down-regulated genes were statistically enriched in 20 pathways (Figure 4C), and 13 of the pathways were related to metabolism and organismal systems. Metabolism pathways included amino sugar and nucleotide sugar metabolism, steroid biosynthesis, terpenoid backbone biosynthesis, N-Glycan biosynthesis, various types of N-glycan biosynthesis, fatty acid elongation, and fatty acid biosynthesis. Organismal systems pathways included IL-17 signaling pathway, fat on and absorption, adipocytokine signaling pathway, osteoclast differentiation, and C-type lectin receptor signaling pathway (Table S7). This result, in consistence with GO analysis, further indicating metabolic and immune enhancement in grass carp fed broad bean.

3.5 PPI network construction and hub gene identification

After STRING analysis of the DEGs, the PPI network was constructed and visualized by Cytoscape with 260 nodes and 249 interactions (Figure 5), and the whole PPI network was analyzed by cytoHubba. After the connectivity degree of each node was calculated, **ITML, STT3B, SEL1L, UGGT1, MLEC, IL1B, ALG5, KRTCAP2, NFKB2, IRAK3** were the top 10 hub genes with the closest connections to other nodes.

The whole PPI network was analyzed by MCODE (Figure 6). A total of 8 modules were mined from the PPI network. Among these, three modules (Modules 1-3) with both MCODE score >3 and nodes >3 were further selected for functional analysis. The pathway enrichment analysis revealed that DEGs in module 1 were mostly enriched in glycan biosynthesis and metabolism. The hub
genes ITM1, STT3B, SEL1L, UGGT1 and MLEC participated in the pathway. Module 2 was mainly associated with immunity, and included the hub genes IL1B, NFkB2, BCL3, and IRAK3. Module 3, including RAB7, STX8, RAB20, and TBC1D2, exhibited a close relationship with intrinsic transport and secretion processes of the cell.

3.6 Hub gene identification of metabolism

The metabolic pathway of differential gene enrichment was further analyzed. Metabolism pathway enrichment analysis of the up-regulated genes including Amino acid metabolism, Lipid metabolism, Metabolism of cofactors and vitamins and Xenobiotics biodegradation and metabolism (Table 2). After STRING analysis of the up-regulated genes, the PPI network was constructed and analyzed by cytoHubba (Figure 7A). After the connectivity degree of each node was calculated, BBOX1 were the hub gene with the closest connections to other nodes.

Metabolism pathway enrichment analysis of the down-regulated genes including Amino acid metabolism, Carbohydrate metabolism, and Lipid metabolism (Table 3). After STRING analysis of the down-regulated genes, the PPI network was constructed and analyzed by cytoHubba (Figure 7B). After the connectivity degree of each node was calculated, NANSA, ALG5, NSDH1 were the hub genes with the closest connections to other nodes.

4 Discussion

Aquaculture provides people with high-quality protein (Fiorella et al., 2021). Grass carp is one of the economically important and widely farmed freshwater fish species in China (Lu et al., 2020). The crisp grass carp is very popular among consumers because of its improved taste, and some products are exported to Southeast Asia and North America (Lin et al., 2009; Fu et al., 2020). Here, transcriptome sequencing was performed on liver tissues of grass carp using Illumina platform, and the metabolic changes of grass carp fed broad beans were comprehensively analyzed.

In this study, transcriptome databases were constructed using liver tissues of
grass carp. One reason was that the liver plays an important role in maintaining the metabolic stability of fish (Trefts et al., 2017). Another reason was that dietary nutrient levels or dietary changes have significant effects on the liver of fish. Studies have shown that broad bean affects the lipid and fatty acid content of grass carp liver, and further affects the meat quality of grass carp (Yu et al., 2017).

The clean reads of each sample ranged from 35 million to 43 million, the mapped reads and unique mapped reads were more than 92.13% and 94.16%, respectively. In addition, more than 84.12% mapped to gene were detected in each sample. Consistent with most studies (Wang et al., 2009; Soneson et al., 2015), our results also showed that the sequencing depth is sufficient and the sequencing quality is high enough to meet the requirements of later analysis. The identified 2519 DEGs help to illustrate the underlying differences between A and B groups, which exhibit significant different metabolism, and will be valuable for future studies on the mechanism of liver metabolism in grass carp fed with broad bean.

In this study, GO and KEGG analysis results of DEGs showed that metabolism capacity of the liver of crisp grass carp were enhanced. Among them, lipid acid metabolism, amino acid metabolism, glycan biosynthesis and metabolism were significantly enriched. This may also further explain the characteristics of crisp grass carp. The liver mainly performs metabolic functions in the body (Madrigal et al., 2014). Previous studies have shown that crisp grass carp has hard meat and crisp taste, which may be caused by the enhancement of liver lipid acid metabolism and amino acid metabolism (Xu et al., 2020; Coda et al., 2015). Studies have shown that feeding broad beans enhances muscle stiffness and liver metabolism in fish, which is consistent with the results of this study (Smith et al., 2013).

In this work, the construction of the PPI network and the identification of the hub genes were carried out for the differential genes. Ten hub genes were screened, including ITML, STT3B, SEL1L, UGGT1, MLEC, IL1B, ALG5, KRTCAP2,
NFkB2, IRAK3 genes. The hub genes ITM1, STT3B, SEL1L, UGGT1 and MLEC genes are mainly involved in the upregulation of glycan biosynthesis and metabolism. These glycosyl biosynthetic and metabolic pathways play critical roles in the normal function of cells and organisms (Mikolajczyk et al., 2020). For example, the synthesis and modification of glycoproteins and glycolipids are critical for cellular signaling, cell adhesion, and cell-cell interactions.

UGGT1 (UDP-glucose: glycoprotein glucosyltransferase 1) is an enzyme in the endoplasmic reticulum (ER), which is involved in the glycosylation modification process of proteins (Adeva et al., 2016). ALG5 (Asparagine-linked glycosylation 5 homolog) is a glycosyltransferase involved in the synthesis of N-glycan chains of proteins. ALG5 and UGGT1 genes play an important role in the protein quality control of the endoplasmic reticulum and the maintenance of cellular homeostasis. Adams found that UGGT1 gene is central hubs in the chaperone network of the endoplasmic reticulum (ER), acting as gatekeepers to the early secretory pathway. IL1B (Interleukin-1 beta) is a cytokine (cytokine), which plays an important regulatory role in the immune system and inflammatory response. In this study, IL1B and IRAK3 genes down-regulated in liver tissue of the cripe grass carp. A series of inflammatory reactions in the body will lead to excessive levels of IL1B (Lopez et al., 2011). Previous studies have shown Interleukin-1 receptor-associated kinase 3 (IRAK3) is a pseudokinase mediator in the human inflammatory pathway, and ablation of its function is associated with enhanced antitumor immunity (Rowley et al., 2022). The decline in IL1B transcripts of cripe grass carp in this study implied that broad bean diet might inhibit the inflammatory response (Zhong et al., 2022).

KRTCAP2 (Keratinocyte associated protein 2) is an intracellular structural protein that is related to keratin and may play a role in cellular structures such as the cytoskeleton and intercellular connections. Sun et al. findings suggest that KRTCAP2 is a prognostic marker for hepatic carcinoma patients with potential clinical implications for predicting immunotherapeutic responsiveness (Sun et al., 2022; Ito et al., 2015). NFkB (nuclear factor kappa B) is the important gene in
the nuclear factor kappa B pathway. The nuclear factor kappa B pathway is an
important cell signaling pathway that plays a key role in cellular immune and
inflammatory responses (De et al., 2020; Shen et al., 2022; Adams et al., 2020).
These hub genes were significantly up-regulated in the liver of crisp grass carp,
confirming the results of the GO and KEGG pathways. Feeding broad beans
enhanced the metabolic capacity of grass carp liver, especially glycan
biosynthesis and metabolism. However, some of these candidate genes are
related to immunity, and more changes in their functions and immune
mechanisms still need to be further investigated.

5 Conclusions

In summary, this study did a transcriptomic analysis in the liver tissue of
grass carps and crisp grass carps. A substantial number of DEGs have been
identified, which are associated with crucial metabolic processes including lipid
acid metabolism, amino acid metabolism, and glycan biosynthesis and
metabolism. These hub genes are significantly up-regulated in the liver of crisp
grass carp, which further indicates that broad bean diet enhances liver
metabolism, especially glycan biosynthesis and metabolism. These findings could
serve as a reference for further investigation into the liver metabolic changes in
animals fed with a broad bean diet.

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Author contribution: Meilin Hao participated in the design of the study. Wenjie Cheng, Lanlan Yi and Yuxiao Xie did the experiments and did the data analysis. Meilin Hao, Sumei Zhao and Junhong Zhu drafted the manuscript and all authors contributed to finalizing the writing.

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Data availability: The data that support the findings of this study are available from the corresponding author upon reasonable request.

Declarations

Ethics approval: The experiment was carried out in accordance with the
research plan of the Institutional Animal Care and Use Committee of Zunyi Normal College.

Consent to participate: All authors agree to participate in this study.

Consent for publication: All authors agree to participate in the publication of this article.

Competing interests: The authors declare no competing interests.
Figures

Figure 1
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Figure 2
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Figure 6

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Figure 7
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