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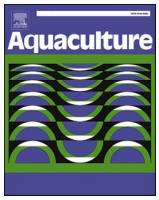

# Integrated volatile metabolomics and transcriptomics provide insights into the formation of characteristic flavor in gynogenetic grass carp and its offspring

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ABSTRACT

To investigate the muscle quality differences and their underlying mechanisms among grass carp with different genetic backgrounds, this study analyzed muscle quality traits, volatile metabolomics, and transcriptomic of gynogenetic grass carp (GGC), disease-resistant grass carp (DRGC) and grass carp (GC). Compared to GC, the muscle of GGC exhibited higher water-holding capacity, hardness, and chewiness, while DRGC displayed greater gumminess in its muscle ($P < 0.05$). Using GC × GC-TOFMS analysis, a total of 2826 volatile flavor compounds (VFCs) were identified, with significant differences observed in VFCs types and relative abundances among the three varieties. Through multivariate statistical analysis, 296 differential VFCs were screened (VIP > 1, $P < 0.05$). By combining relative odor activity values (ROAVs), seven characteristic VFCs (VIP > 1, ROAV >0.1) were further identified as potential markers for variety discrimination and quality evaluation. Transcriptomic analysis, including differential expression analysis and weighted gene co-expression network analysis, identified several potential key genes, including sirt1, hsdl2, capzb, nmnat1, atic, stbd1, and arfgap3, which may influence the formation of characteristic flavor through regulating target metabolic pathways. This study revealed the flavor profiles of GGC and DRGC, and explored the potential mechanisms of characteristic flavor formation, providing scientific insights for improving muscle flavor through breeding.

## 1. Introduction

Grass carp (*Ctenopharyngodon idella*) is a pivotal species in Chinese freshwater aquaculture. In recent years, its annual production has increased rapidly, reaching 5,941,315 tons, which constitutes 21.4 % of China's total freshwater aquaculture production (Bureau of Fisheries, Ministry of Agriculture and Rural Affairs, 2024). This achievement is largely attributed to the unique advantages of grass carp, including its ability to efficiently convert plant material into high-quality animal protein, low cultivation costs, and rapid growth rate (Lin et al., 2022; Wang et al., 2024b). Nevertheless, with the expansion of farming scale and the increase in intensification, the grass carp industry is facing new challenges. Due to continuous artificial inbreeding and limited genetic diversity in breeding populations, grass carp genetic resources have significantly deteriorated, manifesting as reduced growth rates and increased disease susceptibility (Tan et al., 2023; Wang et al., 2022b). Furthermore, the intensive farming model characterized by high stocking density has led to water quality deterioration, further compromising the health condition and product quality of fish (Liu et al., 2018). Therefore, improving grass carp germplasm has become a critical task to promote the sustainable development of aquaculture.

Artificial gynogenesis, as an efficient breeding technique, has been widely applied in aquaculture to develop new varieties with superior traits (Liu et al., 2022; Wang et al., 2024b). It has been shown that heterologous sperm not only activate egg for gynogenesis, but also insert its genetic material into the genome, leading to significant heterosis in

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artificially gynogenetic offspring (Fu et al., 2022; Mao et al., 2019). This includes accelerated growth rate (Wang et al., 2022b), increased survival rate (Ocalewicz et al., 2020), enhanced hypoxia tolerance (Fu et al., 2022), or increased disease resistance (Tan et al., 2023). Using UV-inactivated sperm from koi carp (*Cyprinus carpio haematopterus*) to activate mature grass carp eggs, our laboratory successfully obtained gynogenetic grass carp (GGC). As a result of the insertion of DNA fragments from heterologous sperm, GGC exhibits greater disease resistance and is considere invaluable genetic resource (Mao et al., 2020; Mao et al., 2019). Subsequently, we developed a new improved variety, the disease-resistant grass carp (DRGC), by backcrossing GGC with the grass carp (GC). Aquaculture data indicate that the DRGC exhibit a 16.31 % faster growth rate and a 22.18 % higher survival rate compared to GC, highlighting its significant economic value and development potential in the aquaculture (Wang et al., 2022b). Currently, research on GGC and DRGC primarily focuses on their biological characteristics, disease resistance, and genetic mechanisms (Mao et al., 2020; Mao et al., 2019; Tan et al., 2023; Tan et al., 2024). Nonetheless, it remains unclear whether there are differences in muscle quality between GGC, DRGC, and sexually reproduced grass carp. Therefore, to assess the potential and impact of GGC and DRGC in aquaculture, further studies on muscle quality and the mechanisms of flavor formation are required.

Flesh quality traits and flavor are fundamental attributes of fish quality, directly influencing consumer satisfaction and repeat purchase intentions (Cai et al., 2021; Jiang et al., 2016; Tejerina et al., 2012). Flesh quality traits involve key characteristics such as texture and water-holding capacity. Texture includes the physical properties of the muscle, which are important indicators for assessing the tactile and processing performance of the flesh (Cheng et al., 2014; Tang et al., 2021). The water-holding capacity, which significantly influences the juiciness and tenderness of flesh, reflects its ability to retain moisture (Tejerina et al., 2012). The flavor profile of flesh is primarily shaped by a diverse range of volatile flavor compounds (VFCs), such as aldehydes, alcohols, ketones, and heterocyclic compounds. These compounds result from intricate biochemical processes, including lipid oxidation, thiamin degradation, the Strecker degradation, and the Maillard reaction (Khan et al., 2015). The distinctive flavor of fish products emerges from the sophisticated interplay among these VFCs and the relative proportions of these components. Flavor formation is an exceptionally complex process influenced by multiple factors, with genetics often recognized as a key factor affecting the flavor of fish products (Cai et al., 2021).

Recently, the integration of volatile metabolomics with transcriptomics has provided powerful tools for in-depth research on the composition and formation mechanisms of volatile flavor compounds in meat. Among these techniques, comprehensive two-dimensional gas chromatography-time-of-flight mass spectrometry (GC × GC-TOF MS) has emerged as the preferred technique for characterizing volatile flavor compounds in meat due to its superior resolution, high sensitivity, excellent peak capacity, and rapid analysis speed (Wang et al., 2022c). Transcriptome sequencing technology (RNA-seq) allows for comprehensive analysis of all transcripts within cells under specific physiological conditions (Zhao et al., 2024), providing new avenues for uncovering key genes and their complex regulatory mechanisms involved in the biosynthesis of flavor compounds. These multi-omics techniques have been used extensively in the study of livestock and poultry muscle tissues and has made significant progress in identifying key genes associated with the synthesis of volatile flavor compounds (Wang et al., 2024c; Wang et al., 2022c; Zhang et al., 2022b; Zhao et al., 2024). For example, Zhao et al. (2024) revealed that the differences in meat quality and flavor between Cobb broilers and Daweishan mini chickens may be primarily attributed to amino acid and lipid metabolic processes. Wang et al. (2024c) constructed a molecular network regulating the flavor and lipid composition of pork by using a multi-omics technique and identified six important regulatory genes related to lipolysis, which may lead to flavor differences in different varieties of pork. In the field of aquatic products, metabolomics has gradually become a key technology for quality assessment and monitoring (Ciampa et al., 2023). Ciampa and Picone (2023) and Shumilina et al. (2015) used NMR spectroscopy to study the changes in flesh quality of red mullet (*Mullus barbatus*), bogue (*Boops boops*), and salmon (*Salmo salar*) under different storage temperatures, identifying several metabolite biomarkers associated with freshness. Additionally, using GC–MS technology, Cai et al. (2021) found significant differences in the content of odor-active volatile compounds between diploid and triploid common carp (*Cyprinus carpio* L). However, the application of integrated metabolomics and transcriptomics in fish flavor research remains limited, especially when investigating flavor differences in fish with different genetic backgrounds.

To address this knowledge gap, this study first compared differences in muscle quality traits and volatile flavor compounds among GGC, DRGC, and GC. Subsequently, weighted gene co-expression network analysis (WGCNA) was employed to integrate volatile metabolomics and transcriptomic to elucidate the molecular mechanisms of characteristic flavor formation. This comprehensive analysis revealed the unique muscle quality traits and flavor profiles of DRGC and GGC, providing novel insights into the underlying mechanisms of flavor formation in grass carp. These findings offer a scientific basis for improving grass carp muscle flavor through breeding strategies and hold promise for enhancing the product quality and commercial value of grass carp.

## 2. Materials and methods

### 2.1. Animals and sample collection

The gynogenetic grass carp (GGC), disease-resistant grass carp (DRGC), and grass carp (GC) used in this study were provided by the State Key Laboratory of Developmental Biology of Freshwater Fish, Hunan Normal University, China. All experimental fish were raised in identical aquatic environments and fed the same commercial feed. Eight individuals with similar body weights (mean body weight 509 ± 50.85 g), uniform morphology, and good health were selected from each group of GC, GGC, and DRGC for the experiments. A 24-h fasting period preceded the experiment. Afterward, fish were anesthetized with a 100 mg/L solution of MS-222 (Sigma-Aldrich, USA). After anesthesia, the fish were rapidly euthanized and placed on ice for dissection. The left-side muscle from each fish ($n = 8$) was collected and trimmed into 1 cm × 1 cm × 0.5 cm samples for muscle texture analysis. Additionally, muscle tissue samples (n = 8) were collected to determine water-holding capacity. All samples were analyzed within 24 h of collection. Furthermore, right-side muscle tissues from two fish per group were pooled into a single sample ($n = 4$) and stored at −80 °C for later detection of volatile flavor compounds and transcriptomic profiles.

### 2.2. Measurement of flesh quality traits

#### 2.2.1. Muscle texture measurement

Texture profile analysis (TPA) was conducted on muscle samples using a TMS-PRO device (Food Technology Corporation, USA) equipped with a 250 N load cell and a flat-bottomed cylindrical probe (8 cm diameter). Samples were compressed twice to 60 % of their original height at a constant speed of 30 mm/min with an initial force of 0.1 N, as described by Tang et al. (2021). This method assessed textural properties, including hardness, springiness, chewiness, gumminess, adhesiveness, and cohesiveness.

#### 2.2.2. Centrifugal water loss and cooking loss

To determine centrifugal water loss, muscle samples (approximate 2 *g*) were centrifuged at 1000 ×*g* for 30 min in centrifugal tubes. Then, surface moisture was removed, and the samples were reweighed. Centrifugal weight loss (%) was calculated using the formula:

$$\text{Centrifugal weight loss (\%)} = (\text{Initial weight–Post} - \text{centrifugation weight})/\text{Initial weight} \times 100.$$

A comparable method was employed to determine cooking loss. Muscle samples were placed in perforated centrifugal tubes and heated in a water bath at 70 °C for 15 min. After the removal of excess liquid, the samples were reweighed. Cooking loss (%) was calculated as:

$$\text{Cooking loss (\%)} = (\text{Initial weight–Post} - \text{cooking weight})/\text{Initial weight} \times 100.$$

### 2.3. Volatile flavor compounds analysis

#### 2.3.1. Extraction of volatile compounds by HS-SPME

An appropriate amount of N-hexyl-d13 alcohol standard was dissolved in a 50 % ethanol-water solution to prepare a 10 mg/L solution. Muscle samples were quickly frozen in liquid nitrogen and homogenized using a grinder. The homogenized muscle sample (0.6 g) was transferred to a headspace vial, supplemented with 10 μL of internal standard solution (N-hexyl-d13 alcohol, 10 mg/L). After sealing with a screw cap, the headspace vial was incubated at 80 °C for 10 min. Volatile compounds were extracted using a SPME fiber coated with DVB/CAR/PDMS (50/30 μm × 1 cm). Before adsorption, the SPME fiber was conditioned at 270 °C for 10 min. The fiber was then exposed to the headspace vial, and adsorption was carried out at 80 °C for 25 min. Finally, the fiber was placed into the GC injector for thermal desorption at 250 °C for 5 min.

#### 2.3.2. GC × GC-TOFMS analysis

Volatile compound analysis was performed using a LECO Pegasus® 4D instrument (LECO, USA), with reference to the detection conditions of Wang et al. (2022c). High-purity helium functioned as the carrier gas, maintained at a constant 1.0 mL/min flow rate. The first-dimension column (DB-Heavy Wax, 30 m × 250 μm I.D., 0.5 μm, Agilent, USA) was programmed to start at 50 °C (held for 2 min), then heat up to 230 °C at 5 °C/min and hold for 5 min. The second-dimension column (Rxi-5Sil MS, 2.0 m × 150 μm I.D., 0.15 μm, Restek, USA) was programmed 5 °C above the first column's temperature. The modulator was maintained 15 °C higher than the second column, with a 6.0 s modulation cycle. The mass spectrometer operated in EI mode at 70 eV, scanning $m/z$ 35–550, with an acquisition frequency of 200 spectra/s and a detector voltage set to 2011 V.

### 2.4. Identification of differential volatile flavor compounds

Raw data processing including peak detection and mass spectral deconvolution was carried out using LECO ChromaTOF® software (v 4.5). The signal-to-noise ratio (S/N) was configured to 50, and peak areas were determined from ion chromatograms. Volatile compounds were identified by matching their mass spectra against entries in the NIST 2020 database. Retention indices (RI) were calculated using n-alkanes (C7–C30) and cross-referenced with database values for further validation. Chemical classification of the identified volatile compounds was carried out using the PubChem database (https://pubchem.ncbi.nlm.nih.gov/) and ClassyFire software, categorizing compounds and quantifying their relative content. The sensory odor analysis and comparison of these compounds were performed using the FlavorDB database (https://cosylab.iiitd.edu.in/flavordb/). To compare data of different magnitudes, raw data were normalized by internal standard (Chen et al., 2024; Xiong et al., 2023). The analyses performed included hierarchical clustering and multivariate statistical analysis. The contribution of volatile compounds to muscle flavor was evaluated using relative odor activity values (ROAVs), calculated based on threshold methods (Xu et al., 2017).

### 2.5. RNA extraction and RNA-Seq analysis

Total RNA extraction from muscle tissues was accomplished via the RNA 6000 Nano Kit (Agilent, USA). Subsequent analysis was performed using a NanoDrop 2000 spectrophotometer (Thermo Scientific, USA) to assess the concentration and purity of RNA, complemented by gel electrophoresis to verify RNA integrity. Sequencing libraries were constructed using the TruSeq® RNA Sample Preparation Kit (Illumina, USA) and sequenced in paired-end (PE) mode on Illumina platform utilizing next-generation sequencing (NGS) technology. For bioinformatic processing, raw data were pre-processed with Cutadapt (v1.15) to remove low-quality reads, followed by quality control with FastQC. The clean reads were mapped to the grass carp reference genome using HISAT2 (v2.0.5). Read counts were quantified with HTSeq (v0.9.1) to obtain raw expression levels, which were then normalized to FPKM values. Differentially expressed genes (DEGs) were identified using the R package DESeq2 (v1.30.0), with the following criteria: $|\log_2\text{FoldChange}| > 1$ and a significant $p$-value $<0.05$. Enrichment analysis of DEGs for Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways was conducted using the R package clusterProfiler (v3.4.4).

### 2.6. Weighted gene co-expression network analysis

Weighted gene co-expression network analysis (WGCNA) was conducted on 12 RNA-seq datasets using the WGCNA package (v1.7.1) (Langfelder and Horvath, 2008). To construct a similarity matrix, Pearson correlation coefficients were calculated. The PickSoftThreshold function was employed to determine an optimal soft-thresholding power (β) for constructing a scale-free topology network. Subsequently, the adjacency matrix was converted into a topological overlap matrix, which underwent hierarchical clustering. Gene modules were divided using the dynamic tree-cutting implemented. To explore the relationship between module eigengenes and characteristic volatile flavor compounds, correlation analysis was performed. Significant consensus modules were identified based on the criteria of $|r| > 0.7$ and $p < 0.05$.

Module Membership (MM) was calculated to determine the correlation between module eigengenes and gene expression profiles, while Gene Significance (GS) reflected the correlation between target traits and gene expression levels (Liu et al., 2019). Hub genes within key modules were selected based on thresholds of GS > 0.8 and MM > 0.9 and were further cross-referenced with differentially expressed genes (DEGs) to identify overlapping genes. To evaluate the interactions among these gene, we constructed a protein-protein interaction (PPI) network utilizing the STRING database (https://string-db.org/). The top 30 genes in the network were determined based on their MCC values using the CytoHubba plugin in Cytoscape software. Genes identified through DEGs analysis, WGCNA analysis, and PPI network analysis were defined as potential key genes.

### 2.7. Quantitative real-time PCR verification

To verify the reliability of the results, we selected 12 genes from the candidate genes for qRT-PCR analysis. Total RNA was transformed into cDNA through reverse transcription utilizing the HiScript III RT SuperMix (Vazyme, China). Gene expression analysis was conducted on a QuantStudio 5 Real-Time PCR System (Thermo Fisher Scientific, USA). Each 10 μL reaction consisted of 5 μL ChamQ Universal SYBR qPCR Master Mix (Vazyme, China), 3 μL cDNA template, 0.4 μL of both forward and reverse primers (10 μmol/mL), and sterile water. The thermal protocol included 40 amplification cycles, followed by a melt curve analysis to verify product specificity. Primers were designed using Primer 5.0 software, with full sequences detailed in Supplementary Table S1. Relative mRNA expression was quantified using the $2^{-\Delta\Delta CT}$ method (Winer et al., 1999), with β-actin functioning as the internal reference gene for data normalization.

### 2.8. Statistical analysis

Statistical results were characterized by mean ± standard error of mean (SEM). One-way analysis of variance (ANOVA) was applied to analyze flesh quality traits and qRT-PCR data using SPSS 26 (SPSS Inc., USA). A *p*-value of less than 0.05 was considered to indicate statistical significance. To explore the underlying patterns and differential VFCs in the volatile metabolome, multivariate analyses, including principal component analysis (PCA) and orthogonal partial least squares-discriminant analysis (OPLS-DA), were conducted using SIMCA software (Umetrics AB, Sweden).

## 3. Results and discussion

### 3.1. Flesh quality traits and differences among three varieties of grass carp

Texture characteristics are crucial indicators of the edible quality of flesh products, with hardness and chewiness being key parameters reflecting the mouthfeel of flesh products (Cheng et al., 2014; Jiang et al., 2016). The results showed that the muscle hardness and chewiness of GGC was significantly higher than those of GC (Fig. 1A, D; $p < 0.05$), while the gumminess of DRGC muscle were significantly higher than that of GC (Fig. 1E; $p < 0.05$). For grass carp, higher muscle hardness and chewiness are generally associated with greater sensory crispness, which is preferred by consumers (Lin et al., 2009). Our findings align with those presented by Wu et al. (2022) in their comparative study of gynogenetic and common *Megalobrama amblycephala*, suggesting that heterosis may positively influence the textural characteristics of fish muscle.

Water-holding capacity (WHC) is another critical indicator in the evaluation of flesh quality (Tejerina et al., 2012). In this study, the cooking loss of GGC muscle was significantly lower than that of GC (Fig. 1H; $p < 0.05$), indicating better water retention during heat treatment. This improvement has practical significance, as moisture loss in flesh not only leads to weight reduction but also results in the loss of nutrients and changes in flesh color, thereby affecting consumer satisfaction (Chan et al., 2022). Protein denaturation and myofibrillar structure contraction are two main factors affecting the WHC of cooked meat (Hughes et al., 2014). Further studies on the morphological and chemical changes in GGC muscle tissue are needed to elucidate the mechanisms underlying the improved WHC.

### 3.2. Comparison of volatile flavor compounds in flesh from three varieties of grass carp

#### 3.2.1. The overview of volatile flavor compounds in three varieties of grass carp muscles

In this study, GC × GC-TOF-MS was employed to analyze the volatile flavor compounds (VFCs) in the muscle tissues of GGC, DRGC, and GC. The results were presented in the form of three-dimensional chromatograms (Supplementary Fig. S1). Across the three grass carp varieties, 2826 volatile compounds were identified in the muscle tissues (Supplementary Table S2). Specifically, 1667, 1392, and 1708 compounds were identified in GGC, DRGC, and GC muscle, respectively, with 695 compounds common to all three varieties (Fig. 2A, B). The number of compounds identified in this study significantly exceeds the 119 volatile compounds detected by Chen et al. (2022) using GC–MS in grass carp. This substantial difference can be attributed to the superior resolution and detection sensitivity of the GC × GC-TOF MS employed in this study, which allows for a more comprehensive detection and identification of complex VFCs (Wang et al., 2022c).

The characteristic aroma of flesh is typically determined by the proportional relationships of various volatile flavor components (Cai et al., 2021; Wang et al., 2022c). The identified VFCs were chemically categorized, and Fig. 2C presents a stacked bar chart illustrating the relative proportions of volatile compound classes across different grass carp varieties. The results demonstrate that alcohols, ketones, and esters were predominant in the muscle tissues of all three grass carp varieties. This result differs from those reported in previous studies, and the discrepancy may be attributed to differences in the aquaculture water conditions, feed nutritional composition, and the sensitivity of detection methods employed (Chen et al., 2022; Fu et al., 2024).

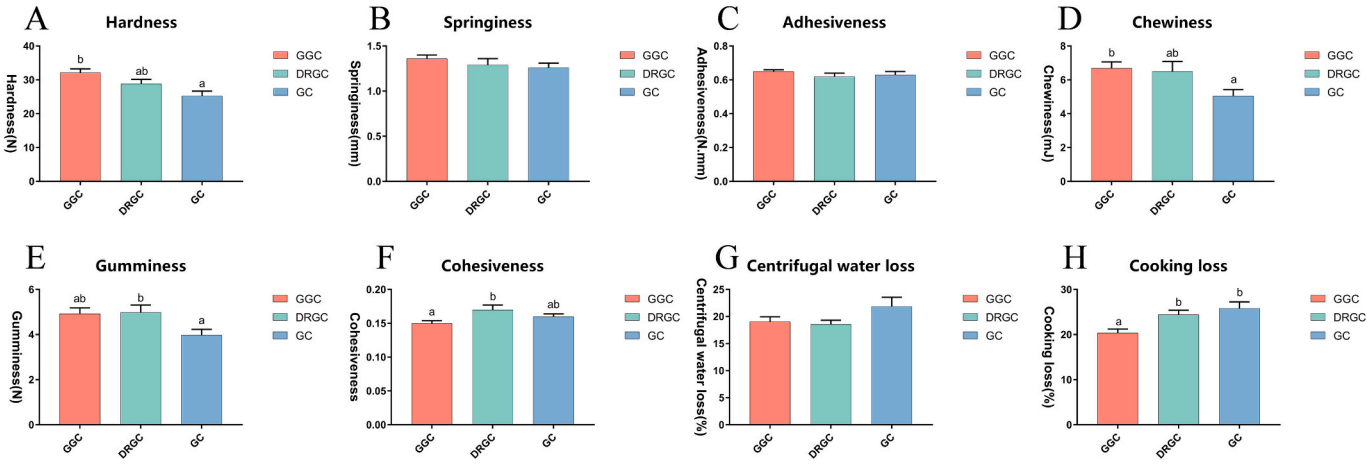


**Fig. 1.** Texture parameters and water-holding capacity of muscle among three varieties of grass carp. (A) hardness; (B) springiness; (C) adhesiveness; (D) chewiness; (E) gumminess; (F) cohesiveness; (G) centrifugal water loss; (H) cooking loss. Different letters indicate significant differences ($p < 0.05$, $n = 8$).

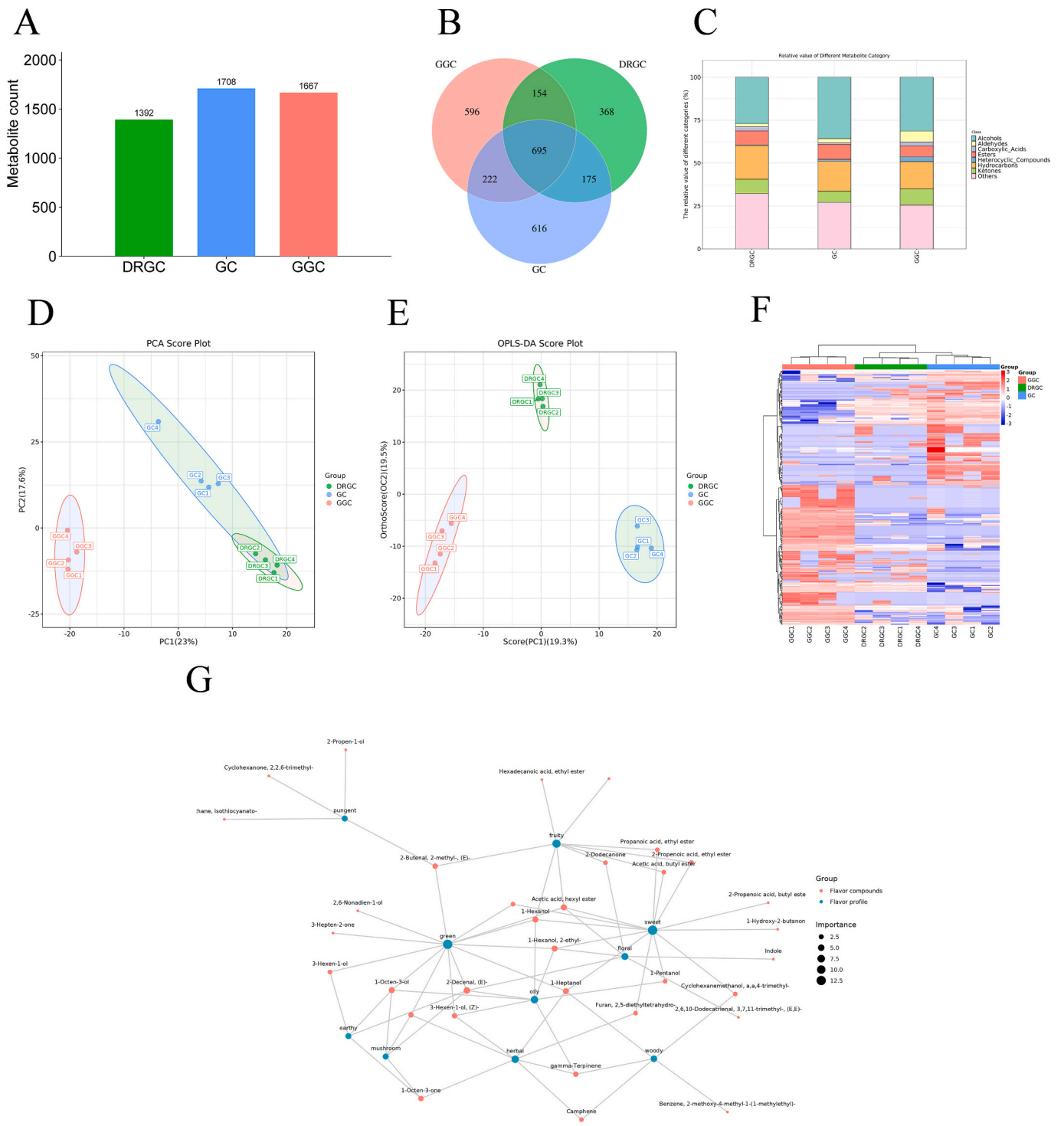


**Fig. 2.** The characteristic analysis and multivariate statistical analysis of volatile flavor compounds in muscle from three varieties of grass carp. (A) Quantities of volatile flavor compounds in muscle from three varieties of Grass Carp; (B) Venn diagrams of volatile flavor compounds in muscle from three varieties of Grass Carp; (C) Relative contents of different compound classes; (D) PCA analysis of the three groups of samples; (E) OPLS-DA analysis of the three groups of samples; (F) Heatmap of differential volatile flavor compounds in muscle from three varieties of Grass Carp (VIP >1, $p < 0.05$); (G) Correlation network for the sensory characteristics and volatile flavor compounds.

Differences in the relative abundance of specific VFC classes were observed among the different grass carp varieties. As shown in Fig. 2C, the relative content of carboxylic acids and ketones in GGC and DRGC muscle was higher than in GC muscle. Ketones are primarily generated through the oxidation and degradation of unsaturated fatty acids, as well as the degradation of amino acids. Most ketone compounds have sweet floral and fruity aromas, while diketones typically have a creamy aroma (Wang et al., 2024c; Wang et al., 2024a). Carboxylic acids, derived mainly from small-molecule fatty acids during lipid hydrolysis and fatty acid oxidation (Pugliese et al., 2015), generally possess high aroma thresholds and have a minimal direct impact on flesh flavor. The relative content of alcohols in GC muscle was higher than in GGC and DRGC muscle. Alcohols, a major class of volatile flavor compounds, are predominantly formed via the oxidation of unsaturated fatty acids and

amino acid metabolism. Among these, unsaturated alcohols have lower thresholds and typically present mushroom and earthy flavors (Cai et al., 2021; Xiong et al., 2023). Furthermore, the relative content of aldehydes and heterocyclic compounds in GGC muscle was higher than in DRGC and GC muscle. Aldehydes, generated from the oxidation of unsaturated fatty acids and Strecker degradation, are essential contributors to flesh flavor, owing to their high volatility and low odor thresholds (Chen et al., 2024; Wang et al., 2024c). Their aroma characteristics often include grassy, fruity, and fatty notes. Heterocyclic compounds, including pyrroles and pyrazines, are known to impart roasted and nutty flavors (Li et al., 2023b). In summary, the types and proportions of VFCs in different grass carp muscles vary greatly, resulting in different flavor profiles. Subsequent analysis is required to identify the core differential volatile compounds and their contributions to the overall flavor.

### 3.2.2. *Analysis of differential volatile flavor compounds in three varieties of grass carp muscles*

To mitigate the impact of missing data on the analysis, compounds with an identification rate below 50 % across all samples were excluded. Ultimately, 1112 volatile compounds were identified and subjected to internal standard normalization for quantitative analysis (Supplementary Table S3) (Chen et al., 2024; Wang et al., 2024a). Principal Component Analysis (PCA) revealed that the first two principal components (PC1 and PC2) explained 23 % and 17.6 % of total variance, respectively. The PCA score plot demonstrated that the samples within each group are tightly clustered, indicating good reproducibility (Fig. 2D). Meanwhile, a distinct separation trend was observed between the GGC group and the DRGC and GC groups, suggesting that the volatile compounds in the GGC muscle had changed considerably.

To further distinguish the differences between groups and reduce within-group errors, supervised orthogonal partial least squares discriminant analysis (OPLS-DA) was employed to analyze the volatile substances (Huang et al., 2018; Yang et al., 2022). The cross-validation parameters of the model were $R^2Y = 0.996$ and $Q^2 = 0.8$, indicating that the model demonstrates strong fit and predictive ability with a low probability of overfitting (Huang et al., 2018). The OPLS-DA score plot indicated that GGC, DRGC, and GC were separated into three distinct categories, exhibiting clear differentiation (Fig. 2E). On the PC1 axis, the DRGC group was located between the GGC and GC groups, a distribution pattern consistent with their genetic relationship. Variable Importance in the Projection (VIP) score serves as critical indicators for assessing the significance of model variables. Typically, a VIP value exceeding 1 is considered as one of the criteria for screening potential biomarkers (Yang et al., 2022). In this study, 296 differential VFCs were identified under the criteria of VIP $>$ 1 in OPLS-DA and $p < 0.05$ in one-way ANOVA (Supplementary Table S4). Compared to GC, the levels of 35 VFCs were significantly increased and 224 VFCs were significantly decreased in GGC muscle. Compared to GC, the levels of 27 VFCs were significantly increased and 206 VFCs were significantly decreased in DRGC muscle. When comparing DRGC with GGC, 65 VFCs were significantly higher, while 116 VFCs were significantly lower. The heatmap (Fig. 2F) clearly demonstrated the differences in VFCs composition in the muscle of three grass carp varieties. Furthermore, using the FlavorDB database, we developed a correlation network diagram depicting the relationships between sensory characteristics and VFCs (Fig. 2G). The overall flavor differences among the three grass carp varieties were primarily manifested in sweet, floral, and fruity notes, which were attributed to differences in VFCs.

The overall sensory characteristics of fish flesh are determined by the combined effects of the odor thresholds and concentrations of various volatile flavor compounds (Xu et al., 2017). Therefore, the Relative Odor Activity Value (ROAV) of VFCs is used to evaluate their contribution to the overall flavor profile. Compounds with ROAV $\geq$1 are regarded as key flavor contributors, while those with 0.1 $\leq$ ROAV $<$1 are considered important modifiers of the overall flavor (Xu et al., 2017). We identified seven characteristic VFCs with ROAV values exceeding 0.1 from 296 differential VFCs. Table 1 lists the ROAV values and odor character of the characteristic volatile compounds in grass carp muscle.

Among them, 1-Hexanol (VIP $>$ 1; ROAV $>$1) was identified as a key volatile compound contributing to the characteristic flavor of GGC. 1-Hexanol derived from the oxidative cleavage of palmitic and oleic acids, has a grassy aroma. It is present in relatively high levels in the muscle tissue of wild Yellow River carp (*Cyprinus carpio*) and Bigeye Tuna (*Thunnus obesus*) (Wang et al., 2022a; Wang and Xie, 2019). Xiong et al. (2023) discovered that 1-hexanol significantly accumulates in Ningdu yellow chicken, serving as a key biomarker for distinguishing between chicken breeds. Zang et al. (2022) found that 1-hexanol is one of the characteristic aroma substances in Hexi Corridor red wine, positively correlated with floral characteristics. 1-Hexanol, 2-ethyl- (VIP $>$ 1; ROAV $>$1) was identified as a key volatile compound contributing to the characteristic flavor of DRGC. 1-Hexanol, 2-ethyl-, with its sweet and floral notes, is one of the most abundant volatile flavor compounds in fresh tilapia (*Oreochromis mossambicus*) fillets (Cheng et al., 2023). 2-Nonenal, (*E*)-, Hexadecanoic acid, ethyl ester, and 1-octen-3-one (VIP $>$ 1; ROAV $>$1) were identified as key volatile compounds contributing to the characteristic flavor of GC. Additionally, 1-octen-3-ol and 2-Propenoic acid, ethyl ester (VIP $>$ 1; ROAV $>$0.1) were volatile compounds that modified the flavor of GC. Hexadecanoic acid, ethyl ester exhibits significant waxy notes (Fan et al., 2021). 2-Nonenal, (E)-, 1-octen-3-ol, and 1-octen-3-one are all products of linoleic acid oxidative degradation. Among these compounds, 2-Nonenal, (E)-, characterized by grassy, earthy and fatty odors (Xu et al., 2023), has been recognized as an odor-active component in Chinese mitten crab (*Eriocheir sinensis*) and triploid rainbow trout (*Oncorhynchus mykiss*) (Gu et al., 2013; Ma et al., 2020). 1-octen-3-ol has a typical mushroom and earthy aroma (Zhang et al., 2022a), which can reflect the degree of lipid oxidation in meat to some extent. Iglesias and Medina (2008) confirmed a high correlation between the levels of 1-octen-3-ol and indicators of lipid oxidation in fish, such as thiobarbituric acid reactive substances and peroxide value. 1-octen-3-one exhibits mushroom or metallic flavors (Lubran et al., 2005; Xu et al., 2023). Notably, 1-octen-3-one may also become a potent off-flavor substance in fish products, depending on

**Table 1**
ROAV values and odor character of characteristic volatile flavor compounds in the muscles of three varieties of grass carp.

| Name | Class | CAS | Formula | Range of odor min | Odor character | GGC_ROAV | DRGC_ROAV | GC_ROAV |
|---|---|---|---|---|---|---|---|---|
| 1-Octen-3-ol | Alcohols | 3391–86-4 | $C_8H_{16}O$ | 11 | Mushroom, Earthy | 0.02 | 0.09 | 0.19 |
| 1-Hexanol, 2-ethyl- | Alcohols | 104–76-7 | $C_8H_{18}O$ | 0.198 | Sweet, Floral | 0.22 | 2.15 | 1.96 |
| 1-Hexanol | Alcohols | 111–27-3 | $C_6H_{14}O$ | 2.4 | Green grass | 1.48 | 0.60 | 0.60 |
| 2-Nonenal, (*E*)- | Aldehydes | 18,829–56-6 | $C_9H_{16}O$ | 0.0002 | Fatty, Earthy, Green | 0.38 | 0.03 | 2.34 |
| 2-Propenoic acid, ethyl ester | Esters | 140–88-5 | $C_5H_8O_2$ | 0.0066 | Sweet, Ester, Plastic, Alcohol, Ammoniacal | 0.00 | 0.13 | 0.36 |
| Hexadecanoic acid, ethyl ester | Esters | 628–97-7 | $C_{18}H_{36}O_2$ | 2 | Wax | 0.23 | 0.87 | 1.13 |
| 1-Octen-3-one | Ketones | 4312-99-6 | $C_8H_{14}O$ | 0.005 | Mushroom-Like, Metallic | 25.89 | 9.35 | 100.00 |

its concentration and relative proportion (Mahmoud and Buettner, 2017). Zhang et al. (2022a) analyzed volatile flavor compounds in fish soup prepared from fish scrap and bone, finding that 1-Octen-3-one may be a key compound responsible for the fishy odor in fish soup. 2-Propenoic acid, ethyl ester, widely used as a food flavoring substance, naturally occurs in various fruits, including passion fruit, pineapple, and durian (EFSA Panel on Food Contact Materials et al., 2017).

Previous studies have suggested that mild "fish-like", "seafood" and "sweet" flavors are generally considered desirable flavor characteristics for aquatic products. However, pronounced "fish-like", "fatty" and "earthy" flavors are viewed as quality defects that may lead to poor product palatability (Jones et al., 2022). In light of our findings, we suggested that the characteristic flavors of GGC and DRGC muscle tend toward a fresh aroma, which may be more appealing to consumers, whereas the characteristic flavors of GC muscle are more associated with fatty, mushroom and earthy odors. This difference might be related to factors such as fat content and lipid oxidation level in different grass carp muscles.

### 3.3. Transcriptomic analysis of flesh from three varieties of grass carp

To investigate the genetic basis underlying the differences in muscle quality among the three grass carp varieties, transcriptome sequencing was performed on their muscle tissues. As detailed in Supplementary Table S5, the analysis generated 76.29 Gb of clean data from 12 samples, with Q30 values exceeding 96.73 % and Q20 values above 99.95 %, indicating high sequencing accuracy and data quality. These clean reads were aligned to the grass carp reference genome, achieving alignment rates between 95.97 % and 98.15 %, which are sufficient for further analyses.

Using DESeq for differential gene expression analysis, a total of 2222 differentially expressed genes (DEGs) were identified in pairwise comparisons among the three grass carp varieties. Specifically, 594 genes were upregulated and 488 downregulated in GGC compared to GC, while 546 genes were upregulated and 237 downregulated in DRGC compared to GC. In the DRGC vs. GGC comparison, 563 genes were upregulated and 509 downregulated (Fig. 3A). An intersection analysis of DEGs across the three comparison groups revealed 22 common DEGs, as shown in Fig. 3B.

KEGG pathway enrichment analysis of DEGs showed that 16, 9, and 5 pathways were significantly enriched in GGC vs. GC, DRGC vs. GC, and DRGC vs. GGC comparisons, respectively (Supplementary Tables S6–S8). Fig. 3C, D, and E present the top 20 enriched pathways for each comparison group. Notably, pathways such as the Calcium signaling pathway, Regulation of actin cytoskeleton, and Arginine and proline metabolism, all associated with muscle quality, were enriched in at least two of the comparisons. The calcium signaling pathway is integral to numerous cellular physiological processes. As a key mediator of both intracellular and extracellular signal transduction, the dynamic regulation of $Ca^{2+}$ directly influences muscle development and metabolic processes. Studies have shown that elevated intracellular $Ca^{2+}$ concentrations activate the calcineurin-NFAT pathway, which is known to regulate the formation of oxidative muscle fibers (Luo et al., 2019). After an animal's death, calcium-binding proteins can lower free calcium

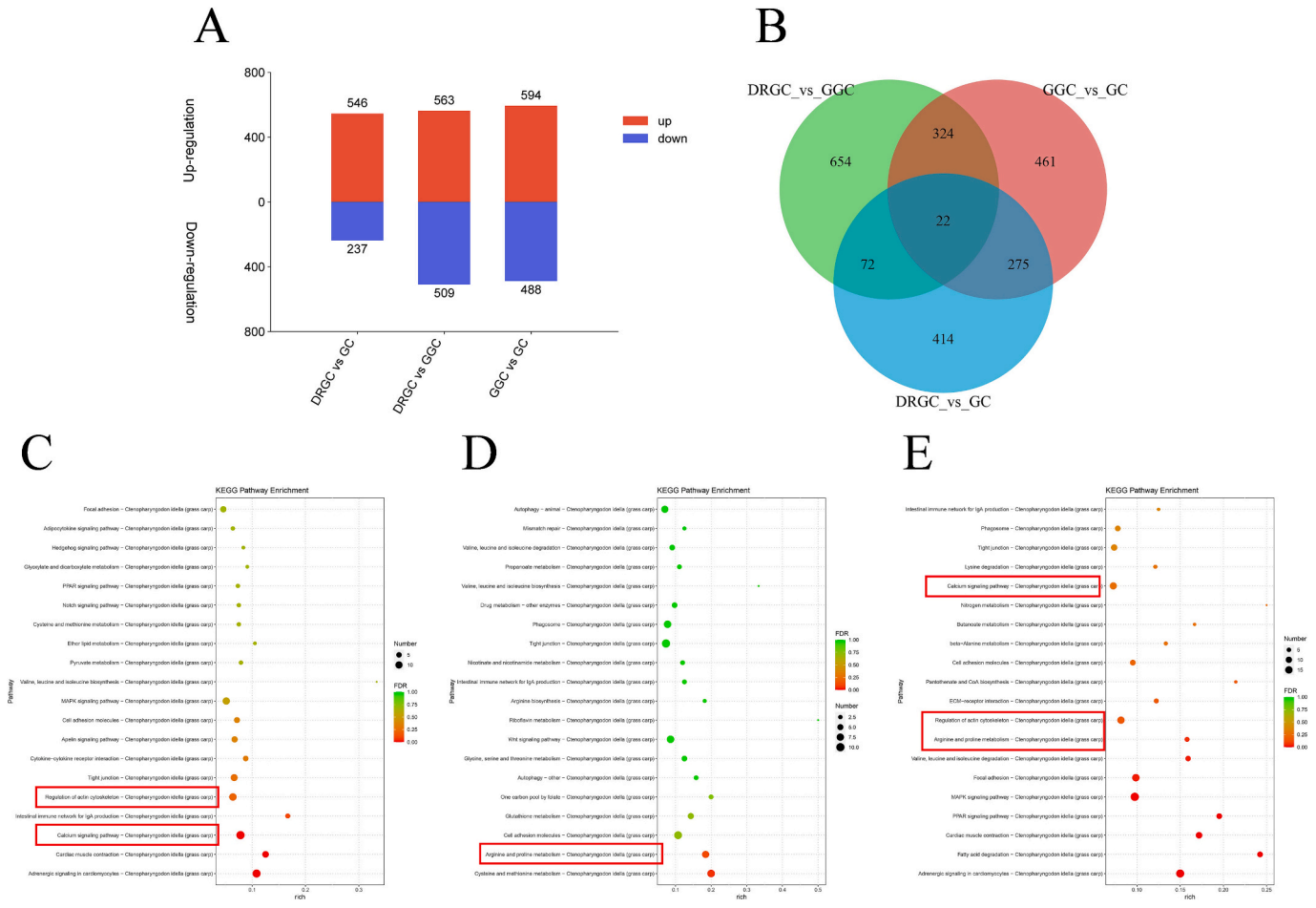


**Fig. 3.** Transcriptome analysis in muscle from three varieties of grass carp. (A) Differentially expressed genes (DEGs) counts in different comparison groups; (B) Overlapping results of DEGs from three comparison groups; (C) Top 20 KEGG enrichment pathways in the DRGC vs GC comparison group; (D) Top 20 KEGG enrichment pathways in the DRGC vs GGC comparison group; (E) Top 20 KEGG enrichment pathways in the GGC vs GC comparison group.

levels, leading to diminished activity of calcium-dependent proteases (e.g., calpain), glycolytic enzymes, and other metabolic enzymes, thereby affecting quality of meat (Purslow et al., 2021). Furthermore, studies indicate that calcium supplementation, both in vivo and in vitro, can promote muscle fat accumulation by inhibiting fatty acid oxidation, which, in turn, influences the texture and flavor of meat (Zhang et al., 2021). The regulation of actin cytoskeleton pathway involves a dynamic network of actin polymers and associated actin-binding proteins. Studies on pigs (Gao et al., 2011), Beijing-You chickens (Liu et al., 2016), and chickens (Xue et al., 2017) have shown that this pathway is critical for muscle development and intramuscular fat accumulation, and that these factors significantly influence meat quality and flavor profiles. Arginine and proline metabolism serves as a central pathway for synthesizing arginine and proline using glutamate (Majumdar et al., 2016). As a conditionally essential amino acid, arginine not only participates in protein synthesis but also plays a regulatory role in physiological metabolism through itself and its metabolites (Jobgen et al., 2009; Li et al., 2022). Studies in mice have shown that arginine can regulate energy metabolism, promote lipid oxidation, reduce white adipose tissue accumulation, and enhance muscle protein deposition (Jobgen et al., 2009). Lipids, as critical precursors of volatile flavor compounds, significantly influence flavor formation through their metabolism. Furthermore, Ma et al. (2024) found that dietary arginine supplementation can promote muscle fiber proliferation and differentiation by regulating the WNT signaling pathway, thereby improving muscle hardness in grass carp. On the other hand, heterocyclic amino acids such as proline and sulfur-containing amino acids undergo thermal degradation during the heat processing of meat products, generating volatile flavor compounds such as thiazoles and pyrroles (Yu et al., 2019). Additionally, amino acids can participate in Maillard reactions with sugars, producing a series of volatile compounds, including aldehydes, ketones, and heterocyclic compounds, which further enhance the flavor characteristics of meat (Khan et al., 2015).

In summary, these results reflect the genetic and molecular basis for differences in muscle quality traits and volatile compounds among different grass carp varieties. Calcium signaling pathway and Regulation of actin cytoskeleton play key roles in lipid metabolism and muscle development, closely correlated to the quality traits and flavor of flesh. Moreover, Arginine and proline metabolism pathway influences flavor formation through multiple mechanisms. On one hand, these amino acids directly generate volatile flavor compounds via thermal degradation and Maillard reactions. On the other hand, they indirectly affect the production of other flavor precursors by modulating lipid metabolism and muscle composition.

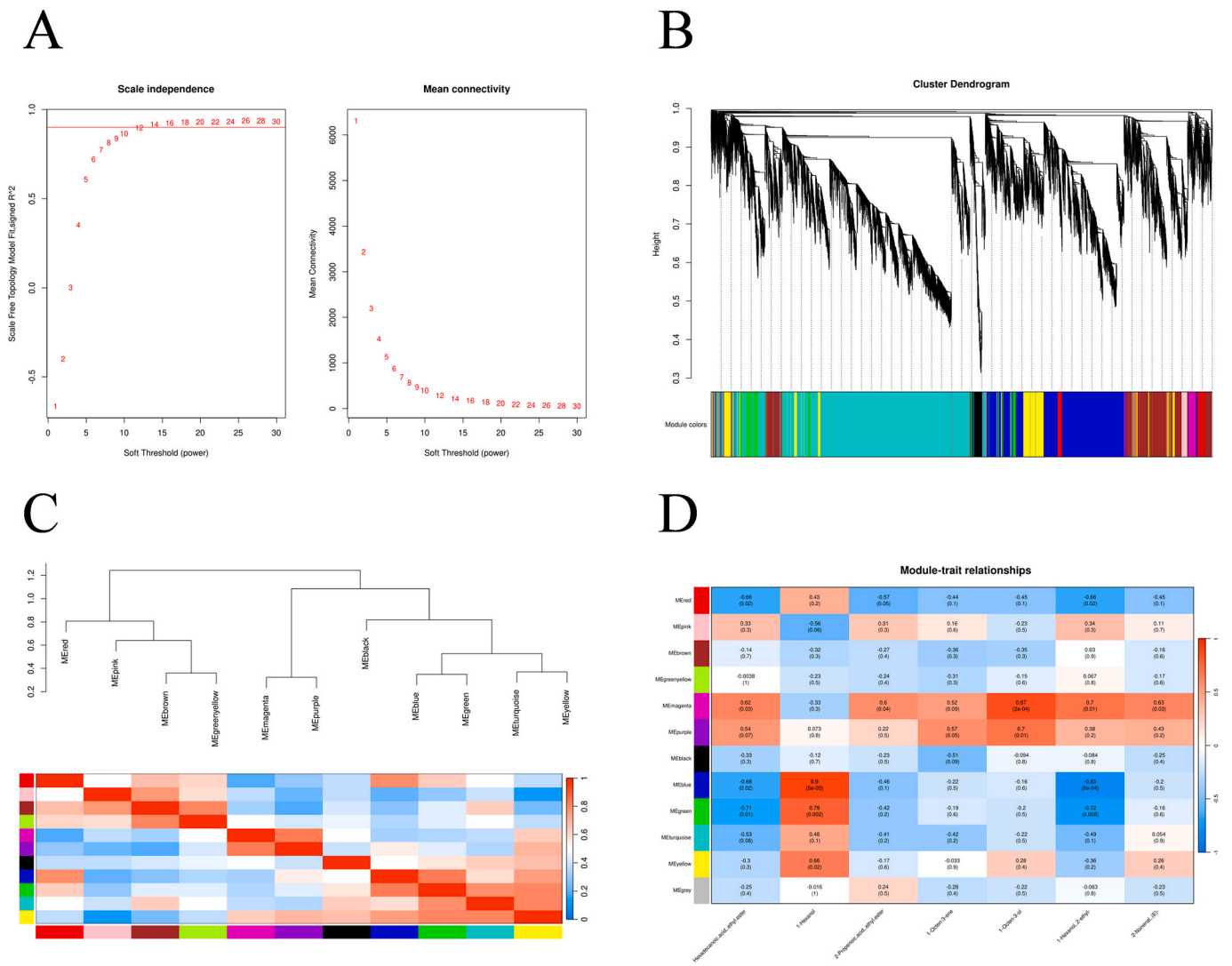


**Fig. 4.** Weighted gene co-expression network construction and module identification. (A) Scale-free index analysis of various soft threshold powers (β); (B) Gene expression clustering tree and recognition module; (C) Correlation analysis between modules; (D) Correlation analysis of 12 modules and characteristic volatile flavor compounds.

### 3.4. Weighted gene co-expression network analysis to screen key genes

To explore the intricate molecular mechanisms contributing to the formation of characteristic VFCs in grass carp muscle, we conducted a Weighted Gene Co-expression Network Analysis (WGCNA) using transcriptomic data. WGCNA is a systems biology approach that clusters genes with similar expression patterns into modules and associates these modules with specific traits or phenotypes to identify potential key genes (Langfelder and Horvath, 2008). In this study, we established a scale-free network by setting the correlation coefficient threshold at 0.9 and the soft threshold power at 12 (Fig. 4A). Hierarchical clustering revealed 12 gene modules with distinct expression patterns (Fig. 4B). The distribution of gene numbers across modules is provided in Supplementary Fig. S2. The Turquoise module contained the most genes (6882), while the greenyellow module had the fewest (68 genes). Additionally, 14 uncorrelated genes were assigned to the gray module, which was excluded from further analysis. The gene co-expression network heatmap revealed interconnections among multiple modules (Fig. 4C).

To explore the relationship between gene modules and characteristic VFCs, seven characteristic VFCs were selected from the volatile metabolomics data, including 1-Octen-3-ol, 1-Hexanol, 2-ethyl-, 1-Hexanol, 2-Nonenal (*E*)-, 2-Propenoic acid, ethyl ester, Hexadecanoic acid, ethyl ester, and 1-Octen-3-one. Correlation analysis revealed significant associations between the gene modules and these VFCs. The correlation heatmap (Fig. 4D) indicated that the blue and green modules were significantly positively correlated with 1-Hexanol content and significantly negatively correlated with 1-Hexanol, 2-ethyl- content ($|r| > 0.7$, $p < 0.05$). Furthermore, the green module was significantly negatively correlated with Hexadecanoic acid, ethyl ester content ($|r| > 0.7$, $p < 0.05$). The purple module showed a significant positive correlation with 1-Octen-3-ol content ($|r| > 0.7$, $p < 0.05$), while the magenta module was significantly positively correlated with both 1-Octen-3-ol and 1-Hexanol, 2-ethyl- content ($|r| > 0.7$, $p < 0.05$). Based on these results, we selected the blue, green, purple, and magenta modules as key modules for subsequent analysis.

To identify key regulatory genes within the key modules, we selected hub genes based on stringent criteria: Gene Significance (GS) exceeding 0.8 and Module Membership (MM) greater than 0.9 (Fig. 5A-H). The hub genes in each key module are listed in Supplementary Table S9. By comparing the hub genes from these four key modules with differentially expressed genes (DEGs) across the groups, we identified 179 overlapping genes. Specifically, 147, 15, 2, and 15 DEGs were found among the hub genes in the blue, green, purple, and magenta modules, respectively (Fig. 6A). These genes were not only differentially expressed among various grass carp muscle samples but may also be key regulators influencing the formation of volatile flavor compounds. To investigate potential interactions between these genes, a protein-protein interaction (PPI) network analysis was conducted using the STRING 11.0 database, applying a confidence threshold greater than 0.4. The network visualization was carried out with CYTOSCAPE 3.6.1 software, and the top 30 genes in the network were determined based on their MCC scores using the CytoHubba plugin (Fig. 6B, C). Based on literature reports, we identified seven potential key genes (sirt1, hsdl2, capzb, nmnat1, atic, stbd1, and arfgap3). Additionally, other genes identified in this study may serve as novel candidates for further research.

These potential key genes may influence the formation of characteristic VFCs through distinct mechanisms. For example, Sirtuin 1 (SIRT1) is a NAD + -dependent deacetylase that controls critical metabolic functions by deacetylating various substrates (Rahman and Islam, 2011). Studies in both cellular and animal models have highlighted the critical role of SIRT1 in regulating lipid metabolism. SIRT1 inhibits lipid accumulation by repressing PPARγ (Picard et al., 2004) and regulating PGC-1α to induce the transcription of genes associated with mitochondrial fatty acid oxidation (Gerhart-Hines et al., 2007). Additionally, changes in sirt1 expression have been shown to influence the regulation

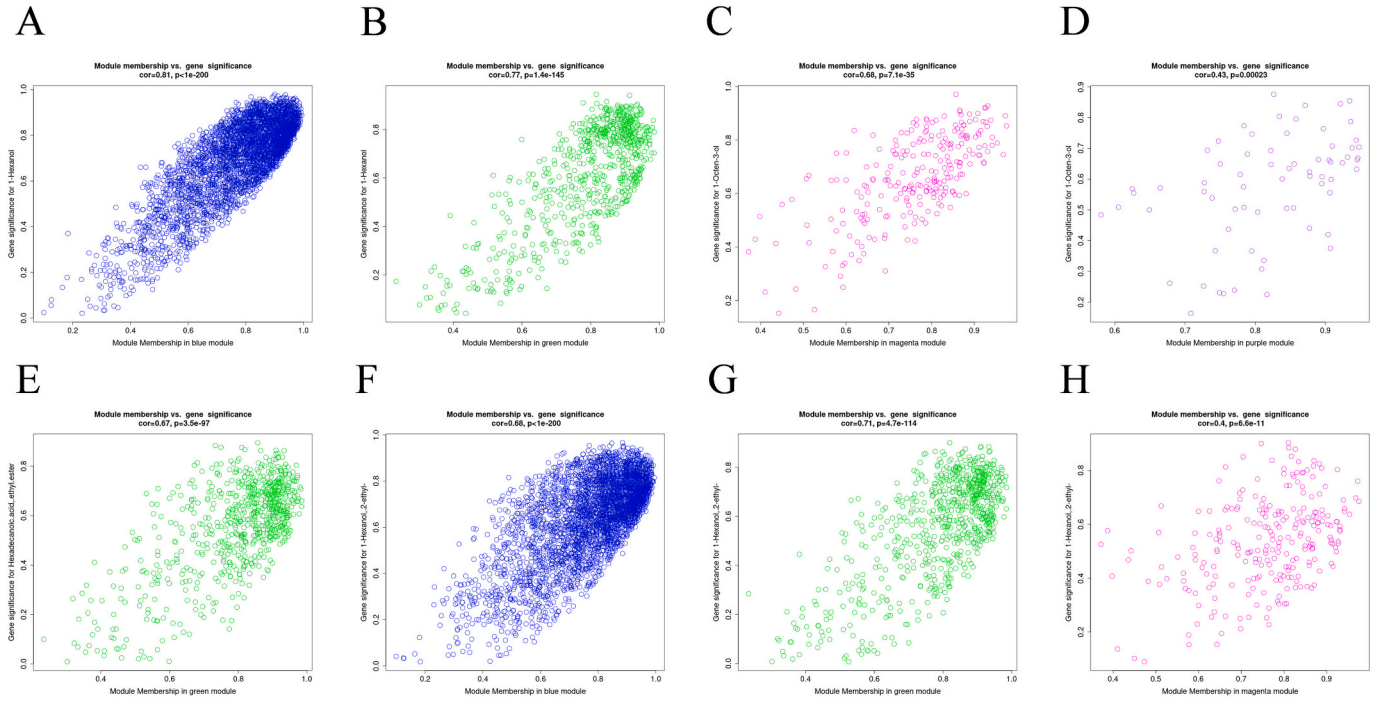


**Fig. 5.** Scatter plot of GS and MM. Abscissa represents the absolute value of correlation value (MM value) between genes and module eigengenes, ordinate represents the absolute value of correlation coefficient between genes and key volatile substances, meaning the significance of the gene. Each point in the map represents a gene. (A) is the blue module related to 1-Hexanol; (B) is the green module related to 1-Hexanol; (C) is the magenta module associated with 1-Octen-3-ol; (D) is the purple module associated with 1-Octen-3-ol; (E) is the green module related to Hexadecanoic acid, ethyl ester; (F) is the blue module related to 1-Hexanol, 2-ethyl-; (G) is the green module related to 1-Hexanol, 2-ethyl-; (H) is the magenta module related to 1-Hexanol, 2-ethyl-. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

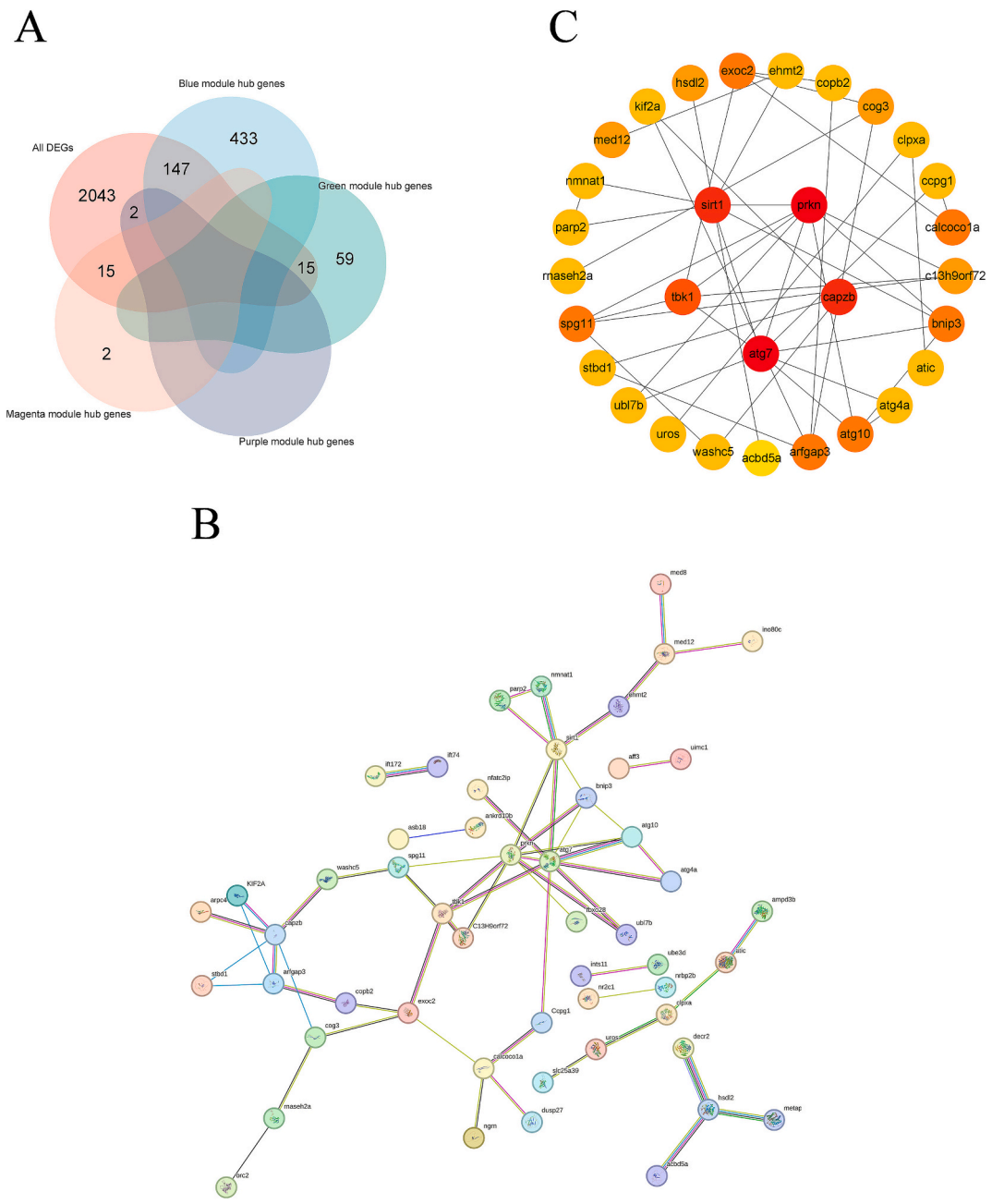


**Fig. 6.** PPI network construction and key gene screening. (A) Overlap results of key module hub genes with all DEGs; (B) The protein-protein interaction network of key genes. Genes without protein-protein interactions with other genes are hidden; (C) Top 30 key genes in the PPI network determined by MCC score.

of multiple genes associated with glucose and lipid metabolism (Rodgers and Puigserver, 2007). SIRT1 activity is dependent on NAD+, and the synthesis of NAD+ requires the catalytic action of nicotinamide mononucleotide adenylyltransferase (NMNAT), which converts nicotinamide mononucleotide (NMN) into NAD+. Thus, changes in nmnat1 expression can alter NAD+ levels, thereby regulating multiple SIRT1-mediated metabolic pathways (Majeed et al., 2021).

HSDL2 functions as an oxidoreductase that catalyzes the oxidation and reduction of various substrates, including steroids, carbohydrates, vitamins, bile acids, and fatty acids (Kavanagh et al., 2008). HSDL2, possessing a sterol carrier protein 2 domain, is believed to be involved in fatty acid metabolism (Kowalik et al., 2009). Research by Samson et al. (2024) revealed that knocking out hsdl2 impairs fatty acid oxidation, mitochondrial respiration, and tricarboxylic acid (TCA) cycle in hepatocytes. These findings suggest that HSDL2 may indirectly affect meat flavor by modulating fatty acid metabolism within muscle cells.

The CAPZB gene encodes the β subunit of the barbed-end actin binding protein, which is part of the F-actin capping protein family (Taye et al., 2017). This gene is vital for skeletal muscle development and growth, regulating cellular signal transduction and participating in actin-mediated myofilament contraction (Xu et al., 2012). Furthermore, CAPZB is essential in muscle metabolism, structural characteristics, and proteolysis, serving as a key connection point between different functional networks (Ponsuksili et al., 2009). In studies on livestock such as pigs and cattle, capzb has been recognized as an important candidate gene influencing meat quality traits, particularly muscle tenderness (Ponsuksili et al., 2009; Taye et al., 2017).

STBD1 is crucial for glycogen transport and metabolism and is highly conserved across species (Jiang et al., 2010). Recent research by Zhu et al. (2023) identified STBD1 as a novel biomarker for beef quality

using label-free proteomics. Their results showed that STBD1 is associated with key sensory attributes of beef, including tenderness, chewiness, stickiness, and flavor, possibly due to its impact on energy metabolism pathways in muscle tissue (Zhu et al., 2023).

ArfGAP3 is a GTPase-activating protein that regulates the hydrolysis of GTP bound to Arf1, a protein associated with the Golgi apparatus (Weimer et al., 2008). ArfGAP3 is essential for regulating vesicular trafficking pathways. Earlier research has demonstrated that ArfGAP3 regulates GLUT4 storage vesicles (GSV) transport and influences muscle cell proliferation and differentiation by affecting glucose uptake (Li et al., 2023a). Additionally, genome-wide association studies in cattle have found that the arfgap3 gene is linked to meat quality (Li et al., 2024; Santana et al., 2015).

Inosine monophosphate (IMP) is an essential flavor compound in meat products. Studies have demonstrated that IMP not only significantly enhances the umami taste of meat but also produces ribose during its degradation, which can participate in the Maillard reaction with amino acids, further enriching the meat flavor (Li et al., 2019). IMP synthesis in vivo involves a ten-step enzymatic process, with the last two steps catalyzed by 5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase/IMP cyclohydrolase (ATIC) (Jeannotte, 2014). Zhu et al. (2017) demonstrated that IMP levels in Dapulian pig muscle were significantly higher than in hybrid pigs and positively correlated with atic mRNA expression in muscle. This finding highlights the role of atic in IMP synthesis and its influence on meat flavor formation.

In conclusion, based on differential expression gene (DEG) analysis, WGCNA, and supporting literature, we identified seven potential key genes (sirt1, hsdl2, capzb, nmnat1, atic, stbd1, and arfgap3) that may indirectly influence flavor compound generation through the regulation of muscle growth, development, and glucose-lipid metabolism. These results offer invaluable insights into the molecular mechanisms responsible for flavor formation in grass carp muscle and suggest potential molecular markers for enhancing grass carp quality. Future research should prioritize functional validation and mechanistic studies of these genes to comprehensively elucidate their contributions to muscle flavor formation in grass carp.

### 3.5. Gene expression verified by quantitative real-time PCR

We performed qRT-PCR analysis on candidate genes associated with characteristic VFCs in order to validate the reliability and accuracy of the RNA-seq data. The expression trends observed in the qRT-PCR analysis were in line with those obtained from the RNA-seq results (Fig. 7), further confirming the reliability of the identified genes.

## 4. Conclusion

In conclusion, the present study revealed changes in muscle traits and volatile flavor compounds in gynogenetic grass carp (GGC) and its backcross progeny (disease-resistant grass carp, DRGC). The findings showed that GGC and DRGC exhibited enhanced muscle texture compared to grass carp (GC). Significant differences were also observed in the type and content of volatile flavor compounds among the three grass carp varieties. Through OPLS-DA, 296 differential volatile flavor compounds were screened across the three varieties (VIP $> 1$, $p < 0.05$). Combining relative odor activity values (ROAV), seven characteristic volatile flavor compounds associated with the varieties were further identified (VIP $> 1$, ROAV $>0.1$). Furthermore, candidate genes including sirt1, hsdl2, capzb, nmnat1, atic, stbd1, and arfgap3, which were associated with the production of characteristic volatile compounds, were identified by DEG analysis, WGCNA, and PPI network analysis. These findings provide invaluable reference for further investigation into the regulatory mechanisms of volatile compound metabolism and the underlying causes of flavor differences among varieties. Future research should focus on functional studies to validate the roles of these key genes. This study not only enhance our understanding of

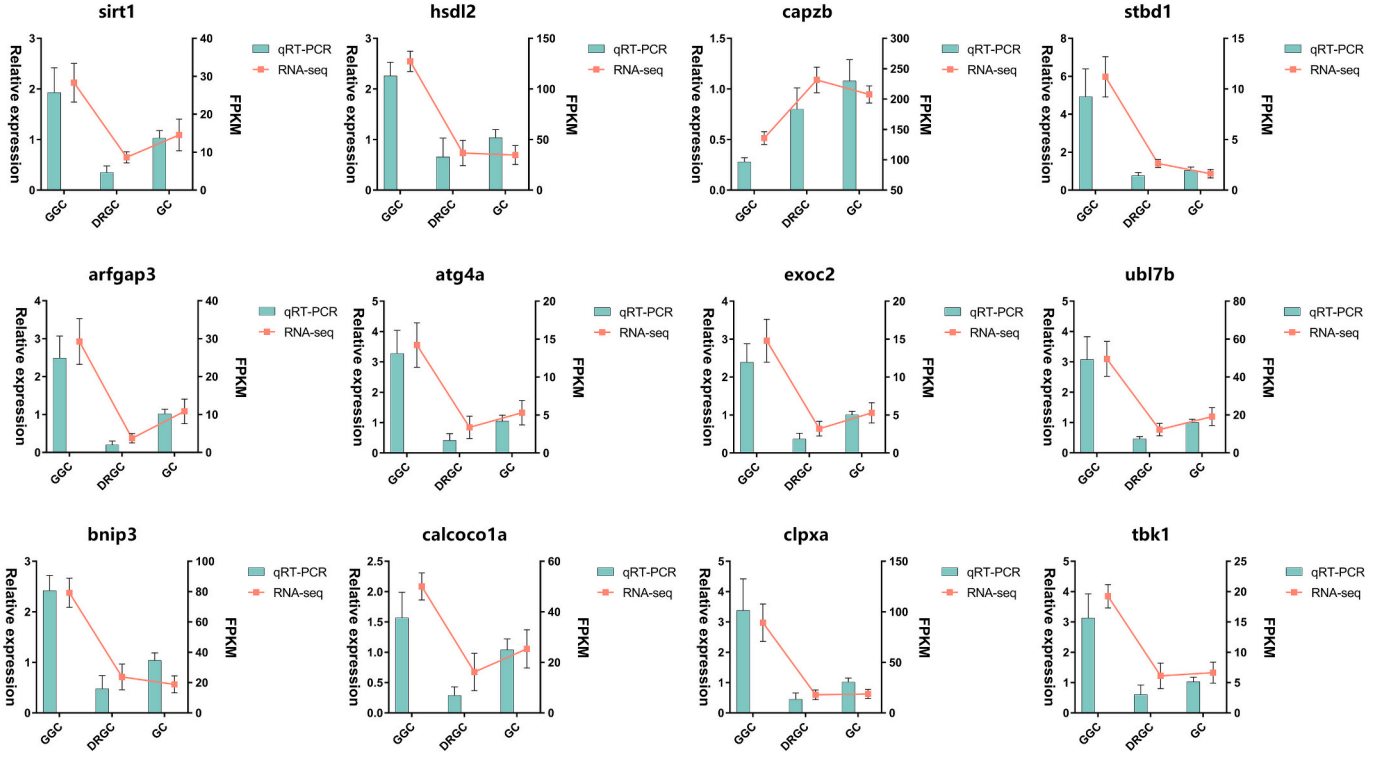


**Fig. 7.** qRT-PCR validation of candidate genes, including sirtuin 1 (sirt1); hydroxysteroid dehydrogenase like 2 (hsdl2); capping actin protein of muscle *Z*-line subunit beta (capzb); starch binding domain 1 (stbd1); ADP-ribosylation factor GTPase activating protein 3 (arfgap3); autophagy related 4 A, cysteine peptidase (atg4a); exocyst complex component 2 (exoc2); ubiquitin-like 7b (ubl7b); BCL2 interacting protein 3 (bnip3); calcium binding and coiled-coil domain 1a (calcoco1a); caseinolytic mitochondrial matrix peptidase chaperone subunit Xa (clpxa); TANK-binding kinase 1 (tbk1).

flavor formation in grass carp, but also offers potential applications for improving flavor quality of grass carp through breeding techniques.

## Ethical statement

All experiments were conducted in compliance with the Guide for the Care and Use of Laboratory Animals and authorized by the Experimental Animal Management Committee of Hunan Normal University.

## CRediT authorship contribution statement

**Jinhai Bai:** Writing – original draft, Investigation, Formal analysis, Data curation, Visualization. **Yan Tang:** Writing – original draft, Investigation, Formal analysis. **Xinyi Deng:** Investigation, Formal analysis, Writing – original draft. **Zhengkun Liu:** Investigation, Formal analysis, Visualization. **Siyang Li:** Investigation, Formal analysis. **Enkui Hu:** Investigation, Formal analysis. **Ling Xiong:** Investigation, Visualization. **Wanjing Peng:** Investigation, Visualization. **Xu Huang:** Project administration, Writing – review & editing. **Chongqing Wang:** Project administration, Writing – review & editing. **Xidan Xu:** Methodology, Writing – review & editing. **Xiaowei Xu:** Methodology, Writing – review & editing. **Kun Zhang:** Writing – review & editing. **Yue Zhou:** Writing – review & editing. **Qinbo Qin:** Funding acquisition, Project administration, Methodology, Conceptualization, Writing – review & editing. **Shaojun Liu:** Conceptualization, Methodology.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2025.742313.

## Data availability

Data will be made available on request.

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