Transcriptome analysis of skin color variation during and after overwintering of Malaysian red tilapia

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Abstract The commercial value of red tilapia is hampered by variations in skin color during overwintering. In this study, three types of skin of red tilapia, including the skin remained pink color during and after overwintering (P), the skin changed from pink color to black color during overwintering and remained black color after overwintering (P-B), and the skin changed from pink color to black color during overwintering but recovered to pink color when the temperature rose after overwintering (P-B-P), were used to analyze their molecular mechanisms of color variation. The transcriptome results revealed that the P, P-B, and P-B-P libraries had 43, 42, and 43 million clean reads, respectively. The top 10 abundance mRNAs and specific mRNAs (specificity measure SPM > 0.9) were screened. After comparing intergroup gene expression levels, there were 2528, 1924, and 1939 differentially expressed genes (DEGs) between P-B-P and P-B, P-B-P and P, and P-B and P, respectively. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses of color-related mRNAs showed that a number of DEGs, including tyrp1, tyr, pmel, mitf, mc1r, asip, tat, hpdb, and foxd3, might play a potential role in pigmentation. Additionally, the co-expression patterns of genes were detected within the pigment-related pathways by the PPI network from P-B vs. P group. Furthermore, DEGs from the apoptosis and autophagy pathways, such as baxa, beclin1, and atg7, might be involved in the fading of red tilapia melanocytes. The findings will aid in understanding the molecular mechanism underlying skin color variation in red tilapia during and after overwintering as well as lay a foundation for future research aimed at improving red tilapia skin color characteristics.

Keywords Red tilapia · Skin color · Transcriptome · Overwintering · Differentially expressed transcripts

Introduction

Tilapia is one of the excellent fish species recommended by the Food and Agriculture Organization of the United Nations (Gupta and Acosta 2004). In
recent years, tilapia has been widely accepted and has become an export dominant species of aquaculture in China (Pradeep et al. 2014). Red tilapia was obtained by crossing the mutant *Oreochromis mossambicus* with other tilapia populations such as *Oreochromis niloticus* and *Oreochromis aureus* (Li et al. 2003; Jiang et al. 2019). Red tilapia is a valuable fish due to its uniform red skin, the absence of black peritoneum, fast growth, and adaptability to any culture system, and it has a huge market in many parts of the world, such as China, Malaysia, and Thailand (Pradeep et al. 2014). Therefore, most studies on red tilapia have mainly focused on their growth and development in genetic breeding (Wardani et al. 2020; Zhu et al. 2016). However, the key issue restricting the growth of commercial red tilapia cultures is skin color variation during overwintering. Pavlidis et al. (2008) found that water temperature changed the body color by motility of chromatophore in red porgy (*Pagrus pagrus*), but the molecular mechanism for this change is unclear.

As one of the most diverse phenotypic characteristics in animals, coloration plays numerous adaptive functions such as camouflage, spouse choice, species identification, thermoregulation, and photoreception (Hubbard et al. 2010). Furthermore, in aquaculture species, skin pigmentation patterns can be considered a factor of economic consideration. Therefore, skin colors might play a vital role in quality parameters in certain species. Previous studies have investigated that skin color was affected by many factors, such as genetics, nutrition, physiology, and environmental factors (Jiang et al. 2014; Luo et al. 2021). Water temperature is a major environmental factor for the metabolism, development, and growth of animals (Pavlidis et al. 2008). Many animals are dark under the cold and light under the warm condition (Kats and Van 1986; Sherbrooke and Frost 1989). For example, both the dorsal and ventral skin colors of *Rana chiricahuensis* in low temperatures (5 °C) were significantly darker than those exposed to 25 °C (Fernandez and Bagnara 1991). Red porgy (*Pagrus*) showed a darker dorsal skin area at low (15 °C) water temperatures and lighter skin at 19 °C (Pavlidis et al. 2008). The best pigmentation levels were achieved at temperatures from 26 to 30 °C in goldfish (*Carassius auratus*) (Gouveia and Rema 2005). These researches are all focused on the physical or biochemical level. The molecular and cellular mechanisms of regulating skin color variation in fish, especially color variation during and after overwintering in red tilapia, are still unknown.

In our previous study, Illumina RNA-seq and microRNA-seq analysis were conducted on different color varieties of red tilapia (Zhu et al. 2016; Wang et al. 2018). Wang et al. (2018) indicated that the color variation during the overwintering period of red tilapia might be related to the changes in skin melanocytes and tyrosinase (TYR) activity. In our red tilapia breeding procedure, we found three kinds of changes of color, i.e., the skin remained pink color during and after overwintering (P), the skin changed from pink color to black color during overwintering and remained black color after overwintering (P-B), and the skin changed from pink color to black color during overwintering but recovered to pink color when the temperature rose after overwintering (P-B-P) (Supplementary Fig. S1). In this study, we used RNA-Seq to analyze the transcriptional profiles of P, P-B, and P-B-P skin color of red tilapia during and after overwintering. Particularly, we attempted to screen hundreds of differentially expressed genes (DEGs), which were responsible for skin color variation. Furthermore, the signaling pathways related to color variation during and after overwintering were also examined. Finally, several DEGs were validated by quantitative real-time polymerase chain reaction (qRT-PCR). This study will not only expose the molecular mechanism underlying red tilapia skin color variation during and after overwintering, but also provide valuable genetic information for breeding pure pink color tilapia.

**Materials and methods**

**Sample collection**

The red tilapia used in this study was obtained from the pilot experimental station of Freshwater Fisheries Research Center (FFRC), affiliated with the Chinese Academy of Fishery Sciences. The red tilapia (initial weight: 500 ± 20 g) were cultivated in a 330 m² plastic film shed pond at water temperatures of 18 ± 1 °C and fed twice a day (morning and evening) during the winter. When overwintering ended in April of the next year, a few whole pink tilapias changed from pink color to black color. Then, we classified the red tilapia with body color variation and tagged...
them with passive integrated transponder (PIT) tags. All these fish continue to be cultured in the same pond without a plastic film shed, and the water temperature gradually rose with the ambient temperature (final water temperature: $24 \pm 1 \, ^\circ C$). During the whole period, the fish was cultured under natural light and the range of water pH value was 7.33–7.87. The dissolved oxygen (DO) was more than 6 mg/L, and NH$_4$-N was less than 0.5 mg/L in the water. Some red tilapia reversed body color from black to pink after the water temperature rose.

Skin tissues were collected from four P-B (pink changed to black) red tilapia, four P (pink unchanged) red tilapia, and four P-B-P (black return to pink) red tilapia individuals, respectively. All fresh tissue samples were immediately snap-frozen in liquid nitrogen and then stored at $-80 \, ^\circ C$ until use.

RNA extraction, cDNA library construction, and sequencing

Total RNA was obtained from red tilapia samples using RNA TRIzol (Invitrogen, UK) according to the manufacturer’s protocol, and genomic DNA was removed using DNase (New England Biolabs). RNA purity was assessed using the Nanodrop-2000 (Thermo Scientific, USA). The ratio of A$_{260}$/A$_{280}$ in all RNA samples was higher than 1.9, and that of A$_{260}$/A$_{230}$ was higher than 1.8. Total RNA integrity was then checked using a Bioanalyzer RNA 6000 Pico Kit (Agilent Technologies). Samples with an RNA Integrity Number (RIN) $> 8$ were retained for subsequent analysis.

A total of twelve RNA samples of three different skin colors (four samples per skin color) were prepared and used for library construction. The libraries were constructed by TruSeq RNA Sample Prep Kit v2 (Illumina, San Diego, CA, USA) according to the manufacturer’s instructions. Firstly, mRNA was purified from total RNA by Poly-T oligoattached magnetic beads and then fragmented under elevated temperature. Then first-strand and second-strand cDNA were subsequently synthesized. Secondly, the double-stranded cDNA was purified for end repair, dA tailing, adaptor ligation, and DNA fragment enrichment. Finally, PCR was performed, and aimed products were purified. The final product was assessed for its size distribution using Bioanalyzer DNA High Sensitivity Kit (Agilent Technologies). Each library was conducted on the Illumina X-Ten for 2 × 150 bp pair-end (PE) sequencing.

Quality control and mapping to the reference genome

Quality of all raw reads was conducted by FastQC (Andrews 2014) software. At the initial filtering step, SOAPnuke (Chen et al. 2018b) was used to discard poor quality reads, including adaptor reads and low-quality reads (reads more than 50% bases with a quality value less than 5). Then the clean reads were mapped onto the reference genome independently by HISAT2 version 2.1.0 (Kim et al. 2015) with default values. And RSeQC packages (version 2.6.4) (Wang et al. 2012) were used to make a comprehensive evaluation of RNA-seq data after alignment, including sequencing saturation, mapped reads distribution, coverage uniformity, strand specificity, and transcript level RNA integrity.

Differential expression analysis

Based on the HISAT2 alignment BAM file, featureCounts v1.6.2 (Liao et al. 2014) was used to estimate and quantify gene expression with default parameters so as to generate the raw read count of each RNA gene. Gene expression was normalized by reads per kilobase of exon per million reads mapped (RPKM). Finally, edgeR (Robinson et al. 2010) was used to identify the DEGs by pairwise comparisons. The difference was considered significant if the $|\log_{2} FC| \geq 1$ and FDR (false discovery rate) $\leq 0.05$.

To further understand the mRNA expression of each sample in red tilapia, the specificity measure (SPM) was introduced to analyze all screened mRNA (FPKM value $\geq 1$, at least 3 samples) by PaGeFinder algorithms (Pan et al. 2012). SPM values greater than 0.9 were used as the selection criterion for specific genes. The higher the SPM value, the more the specific gene expression in the sample.

GO and KEGG enrichment analysis of differentially expressed genes

Gene Ontology (GO) term (http://www.geneontology.org/) enrichment and Kyoto Encyclopedia of Genes and Genomes (KEGG, http://www.kegg.jp/) enrichment analysis of DEGs were performed using clusterProfiler package (Yu et al. 2012), KOBAS v3.0 (Xie
et al. 2011), respectively. The calculated $P$-value goes through Bonferroni Correction, taking $P$ adjusted value $\leq 0.05$ as a threshold.

Quantitative real-time PCR analysis

Total RNAs were extracted as described above. Each RNA sample was treated by $5 \times$ PrimeScript™ RT Master Mix (Takara, Japan) to remove residual genomic DNA and reverse transcribed into cDNA. All primer pairs (Supplementary Table S1) were designed based on the unigene sequences and then synthesized by Sangon Biotech. (Shanghai, China). Real-time PCR was performed on a CFX-96 Real-time PCR System (Bio-Rad, CA, USA) in 25 μL reactions containing 12.5 μL SYBR Premix Ex Taq II (Takara Bio), 1 μL each primer (10 μM), 2 μL PCR template (cDNA), and 8.5 μL of nuclease-free water. Amplification was performed with an initial denaturation at 95 °C for 5 min, followed by 40 cycles of 95 °C for 10 s, 60 °C for 30 s, and 72 °C for 30 s. All the reactions were conducted in triplicate, which six biological replicates. At the end of the PCR cycle, the relative expression was calculated using the $2^{-(\Delta \Delta Ct)}$ method with the $\beta$-actin gene as the reference control. The $t$-test and Shapiro–Wilk test in SPSS 25 (IBM, Chicago, IL, USA) were used for homogeneity and normality tests, respectively. Differences in gene expression between P vs. P-B skins were assayed with SPSS 25 (IBM, Chicago, IL, USA) by $t$-test (one-tail). Thresholds for statistical significance were set at $P < 0.05$ (significant) and $P < 0.01$ (highly significant). The values of log2 (fold change) were calculated statistically with Excel sheet software. Positive values indicated up-regulation of genes expression, and negative values represented down-regulation of genes expression. Finally, the expression patterns of genes were compared with the RNA-Seq sequencing results.

Co-expression analysis of protein–protein interaction network analysis

The DEGs were imported into the Search Tool for the Retrieval of Interacting Genes (STRING, https://string-db.org) database to obtain the protein–protein interaction (PPI) information (Szklarczyk et al. 2015). Only validated interactions with a composite score greater than 0.4 were considered significant. Cytoscape 3.6.0 software was used to construct a PPI network and count the number of nodes in DEGs (Franz et al. 2016). The node genes with node degrees above 10 were selected as the key target genes.

Results

Overview of the RNA-Seq data

To better understand the skin color variation of red tilapia during and after overwintering, the mRNA libraries of P, P-B, and P-B-P were determined and analyzed by Illumina sequencing technology. In Table 1, we presented the Q30 percentage,
GC percentage, and the other indexes to describe the libraries. In total, an average of 42,721,496, 43,921,378, and 43,386,254 raw reads were obtained from the P-B-P, P-B, and P libraries, respectively. After filtering the low-quality reads and removing adaptor sequences, the average of 42,664,181, 43,888,258, and 43,347,484 clean reads was retrieved for further analysis. The percentage of G+C content and Q30 ratio was an average of 48.23% and 94.27%, indicating a high-quality sequence. All raw transcriptome data were submitted to the NCBI Short Read Archive (SRA) database with the accession number PRJNA690595.

To assess the quality of sequencing and reassembly, all clean reads were mapped to the Nile tilapia (Oreochromis niloticus) genome within the range of known gene annotations. We found that 90.48–94.62% of the clean reads could be mapped to the Nile tilapia reference genome (Supplementary Table S2). In particular, the percentage of multiple mapped reads and unique mapped reads for all libraries averaged 5.34% and 92.54%. In addition, to consider the total mapping rate of sequencing reads and genomes for transcriptome sequencing, we also need to understand the distribution of mapped reads. The proportion of all reads operation in the CD5 area exceeded 68.14%, and the ratio of matched with the intron area was the lowest, less than 8.71% (Supplementary Table S3).

Analysis of gene expression level of the red tilapia transcriptome

The RPKM method was used to estimate gene expression. The distribution of RPKM values for each sample was shown in Supplementary Table S4. A total of 33,437 genes were identified in the skin of red tilapia, and the expressed genes accounted for more than 63.26% of the total. The number of genes with $0 \leq \text{RPKM} \leq 1$ was the most, while the number of genes with RPKM $\geq 100$ was less than 1%.

Expression profiling of mRNAs

To further understand mRNA expression of different skin colors in red tilapia, SPM analysis was conducted for each sample, in which the expressed mRNAs were filtered. 9755 mRNAs participated in SPM analysis with their mean RPKM value in each group, and 465 specific mRNAs were screened for further analysis (SPM $>0.9$; Supplementary Table S1). In detail, there were 119, 294, and 53 specific mRNAs in P-B-P, P-B, and P skins, respectively. The KEGG results of specific mRNAs showed that metabolic pathways, ribosome biogenesis in eukaryotes, and oxidative phosphorylation were dominant pathways in P-B-P skin, and regulation of actin cytoskeleton, melanogenesis, tight junction, and tyrosine metabolism was dominant in P-B skin (Supplementary Table S2). Furthermore, we also analyzed the top 10 abundance mRNAs of different skin colors in red tilapia. As shown in Table 2, granulin and tat genes were abundant in P skin and other pigment-related genes were highly expressed, including oca2 and slc45a4. The melanin synthesis genes accounted for the largest proportion of abundantly expressed genes, such as tyrp1b, pmelb, tyr, pmela, and tyrp1a, while the tyrp1b gene was the most abundant in P-B skin. In addition, the baxa gene showed dominantly expression in P-B-P skin.

Table 2 Ten most abundant genes of three different colors in red tilapia during and after overwintering

|          | P         | P-B       | P-B-P     |
|----------|-----------|-----------|-----------|
| granulin | tyrp1b    | baxa      |
| tat      | cav2a     | sich73-86n18.1 |
| oca2     | zgc:101810| crtca1a    |
| ENSONG00000007142 | pmelb | ENSONIG00000040753 |
| oni-mir-24a-4 | tyr | sdbb       |
| col10a1a | myh9a     | ppdpfa     |
| ENSONIG00000040391 | ENSONG00000019137 |
| slc45a4  | aqp3      | loxa       |
| map2k7cl | pmela     | ENSONIG0000009288 |
|          | tyrp1a    | ENSONIG00000018504 |

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Differential gene expression (DEGs) identified in different skin patterns

In comparative transcriptome analysis, many genes showed different expression levels in three skin color samples. Under the criteria of FDR ≤ 0.05 and |logFC| ≥ 1, the volcano plots of three pairwise comparisons (P-B-P vs. P-B, P-B-P vs. P, and P-B vs. P) revealed the expression trend of each pair (Fig. 1a). We also constructed a histogram of DEGs in the three skin tissues (Fig. 1b). Compared with the P-B skin, there were 2528 DEGs in P-B-P skin, of which 1420 were up-regulated and 1108 were down-regulated. A total of 1091 DEGs were up-regulated in P-B-P skin compared with P skin, while 833 DEGs were down-regulated. There were 1939 DEGs displaying greater abundance in P-B skin compared with P skin, of which 1387 DEGs were up-regulated and 552 genes were down-regulated. Among these genes, 22 DEGs were detected as shared genes in each comparison group, of which 11 were known DEGs and seemed to play a key role in the color variation process (Fig. 1c). They were st3gal1, si:ch73-86n18.1, plexa4, malb, fabp11a, plcb4, sdhb, si:dkey-65b12.6, si:ch211-157c3.4, agr1, and aqp3, respectively.

To verify the credibility of the sequencing results, we randomly selected 25 DEGs related to pigment biosynthesis for qRT-PCR, including 13 up-regulated genes and 12 down-regulated genes. As shown in Fig. 2, the expression patterns of all down-regulated genes were consistent with the sequencing result, and 12 of the 13 up-regulated genes’ expression patterns were consistent with the sequencing results. Of which, 24 of 25 genes were significantly different by both RNA-Seq and qRT-PCR methods while dctd genes only significantly differed by RNA-Seq method. The results showed that the reliability of the sequencing result was high.

![Fig. 1 DEGs in P-B-P, P and P-B skins. (a) Volcano plot of differential mRNA expression levels among the three pairwise comparisons. The gray, red, and blue dots represent non-significant, up-regulated, and down-regulated transcripts, respectively. (b) Number of DEGs among the three pairwise comparisons. The red and blue colors stand for up-regulated and down-regulated expression, respectively. (c) DEGs number and Venn diagram of the overlap of the different groups.](image-url)
Functional enrichment analysis of DEGs

The top GO function enrichment terms for the pairwise comparisons among three samples were shown in Supplementary Table S3. After GO annotation, all DEGs were classified into different biological processes, molecular functions, and cellular components. In each comparison, the top 50 of GO categories were selected in three different categories. The detailed annotations of each category were depicted in Supplementary Fig. S2a–c. In the molecular function category, binding and catalytic activity were the most mapped terms. In the biological process category, cellular process, metabolic process, biological regulation, and regulation of biological process were the most mapped terms. In the cellular component category, cell, cell part, and membrane were the main mapped terms. Furthermore, a few DEGs were mapped to pigmentation-related terms such as developmental pigmentation (GO:0048066), melanocyte differentiation (GO:0030318), melanosome transport (GO: 0032402), retinal pigment epithelium development (GO:0003406), and pigmentation (GO:0043473). These genes enriched in pigmentation-related processes are informative and worthy of further study.

KEGG analysis of the pathways

To further explore the biological functions of the DEGs, an enrichment analysis based on the KEGG database was performed. A total of 141 KEGG pathways were listed in this study (Supplementary Table S4). The DEGs between the P-B-P and P-B skins involved in ribosome, oxidative phosphorylation, ribosome biogenesis in eukaryotes, cardiac muscle contraction, RNA degradation, RNA polymerase, and DNA replication were significantly enriched (P<0.05). The DEGs were significantly enriched in some genetic information processing between the P-B-P and P skins, including ribosome biogenesis in eukaryotes, spliceosome, RNA degradation, and RNA polymerase. Ten pathways of oxidative phosphorylation, ribosome biogenesis in eukaryotes, tight junction, adrenergic signaling in cardiomyocytes, cardiac muscle contraction, GNRH signaling pathway, mucin-type o-glycan biosynthesis, sphingolipid metabolism, glycosphingolipid...
biosynthesis, and taurine and hypotaurine metabolism were significantly enriched between the P-B and P skins ($P<0.05$). Since fish skin color was mainly correlated with the synthesis of different pigments, we were interested in the pigments biosynthesis pathway. Several pathways including oxidative phosphorylation, ribosome, Wnt (wingless-type MMTV integration site family) signaling pathway, MAPK (mitogen-activated protein kinase) signaling pathway, cell cycle, melanogenesis, tyrosine metabolism, autophagy pathway, and apoptosis pathway were identified, which were related to the skin color regulation and pigmentation (Fig. 3).

**Fig. 3** Pigmentation-related pathways based on Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis. Gene number: number of genes in each pathway; rich factor: ratio of the number of target genes divided by the total number of genes in each pathway.
Candidate genes related to melanophore

The KEGG pathway analysis results showed that 32 candidate genes were involved in pigmentation-related pathways, such as melanogenesis, tyrosine metabolism, Wnt signaling pathway, and MAPK signaling pathway. These genes may play a potential role in the skin color variation of red tilapia during and after overwintering. The heatmap of these genes (Fig. 4) indicated that four genes including agouti signaling protein (asip), tyrosine amino transferase (tat), hydroxyphenylpyruvate hydroxylase (hpdb), and forkhead transcription factor 3 (foxd3) were up-regulated in the P and P-B-P groups compared with the P-B group, while the rest genes including tyrosinase (tyr), tyrosinase-related protein 1 (tyrp1), telanocortin receptor 1 (mc1r), microphthalmia-associated transcription factor (mitf), and premelanosome protein (pemel) were down-regulated in the P and P-B-P groups compared with the P-B group. In addition, some DEGs were involved in autophagy and apoptotic pathways such as baxa, beclin1, and atg7. The expression of these genes in P-B-P skin of red tilapia was significantly higher than that of P-B skin, which plays an important role in black-to-pink skin color transformation in red tilapia.

Correlation of candidate genes at protein levels

Based on the candidate genes in P-B vs. P groups, we identified the mutual correlation of their protein products using the STRING online tool (Fig. 5a). The genes (or proteins) from the pigmentation-related pathway were integrated together, and the relationship between them was extensive and strong. Among them, asip, tat, hpdb, and foxd3 genes were significantly down-regulated, and other genes were significantly up-regulated. In addition, fourteen hub nodes in a PPI network with more than 10 nodes degrees were shown in Fig. 5b. These hub genes included tyr, mc1r, oca2, mitfb, slc45a2, tyrp1b, dct, asip, kit, kitlg, pmela, pmelb, mitfa, and egrfa. Among these genes, the tyr gene showed the highest node degree, which was 18.
Discussion

To explore the different expression patterns among the three types of skins, we performed differential gene expression analysis. When comparing P-B skin to P-B-P skin and P skin, the results showed that more DEGs were up-regulated in P-B skin, indicating that the formation of black skin is complex and that more genes are needed to participate in the process. Combined with the 10 abundance mRNAs result, the genes rich in melanin synthesis were abundantly expressed in P-B skin, while a few of pigment genes were abundantly expressed in P. It was further suggested that melanin genes were involved in the body color variation of red tilapia during overwintering. Eleven known DEGs were shared by P-B-P vs. P-B, P-B-P vs. P, and P-B vs. P comparison groups, of which *st3gal1*, *plxna4*, *fabp11a*, and *aqp3* played a vital role in regulating cell proliferation, migration, and invasion (Wu et al. 2018; Wang et al. 2020). It has been reported that silencing of the *st3gal1* gene suppresses melanoma invasion and significantly reduces the survival ability of aggressive melanoma cells in humans in a metastatic environment (Pietrobono et al. 2020). *Plexin A4* functionally activates tyrosine kinase receptors in mammals, such as MET, RON, HER2, and KDR (Swierzcz et al. 2008). Overexpression of the *aqp3* gene can promote the proliferation and migration of human hepatocytes (Chen et al. 2018a). *AQP3* can reduce the differentiation and inhibit the apoptosis of stem cells in humans through reducing the expressions of related genes in the Wnt/GSK-3 β/β-catenin pathway (Liu et al. 2020). *Fabp11a* were probably involved in cellular uptake and transport of fatty acids, targeting of fatty acids to transport systems, and several signaling pathways in *Oryzias latipes* (Parmar et al. 2012). In this study, all of these genes expression suggested that skin color variation of red tilapia during overwintering might be related to the proliferation, migration, and differentiation of melanocytes.

GO enrichment analysis of DEGs revealed that variations in pigmentation were related to cellular components and biological processes. Most of the DEGs clusters were consistent with previous works on fish, such as zebrafish (Higdon et al. 2013), Midas cichlids (*Amphilophus*) (Henning et al. 2013), and common carp (*Cyprinus carpio*) (Li et al. 2015). KEGG pathway analysis showed that many DEGs were significantly enriched in oxidative phosphorylation, ribosome, ribosome biogenesis in eukaryotes, and cardiac muscle contraction in the P-B-P vs. P-B group and P-B vs. P group. Several studies have shown that high expression of ribosomal protein-related genes was associated with the black color in mice (Skarnes et al. 2011). Four of the five highly expressed genes in pigment cells of zebrafish were ribosomal protein (Higdon et al. 2013). There were many DEGs that participated in oxidative phosphorylation, cardiac muscle contraction signal pathways.

Fig. 5 The mutual protein–protein interactions of candidate genes in P-B vs. P comparison. (a) PPI network. (b) The genes of the PPI according to the node degree over 10
in *Pristella maxillaris* (Bian et al. 2019) and *Lutjanus erythropterus* (Zhang et al. 2015). In addition, the KEGG results of specific mRNAs showed that ribosome biogenesis in eukaryotes, oxidative phosphorylation were dominant pathways in P-B-P skin. Similar results were found in comparative analysis of P-B-P vs. P-B skin and P-B vs. P skin. It was suggested that these pathways may play an important role in color variation in red tilapia.

We also found that the DEGs of three skin colors of tilapia were mainly enriched in the MAPK signaling pathway, Wnt signaling pathway, tyrosine metabolism, and melanogenesis. Tyrosinase metabolism and melanogenesis pathways have been reported in mammals. And both the Wnt and MAPK signaling pathways are involved in melanophore development in vertebrates (Fujimura et al. 2009; Zhang et al. 2017). For specific mRNA, the tyrosinase metabolism and melanogenesis pathways were dominant in P-B skin. It showed that P-B skin required more melanin synthesis than P-B-P and P skins. Meanwhile, we found that some DEGs between the P-B-P and P-B skin expressed abundantly in the process of apoptosis and autophagy pathways. The identification of genes in these pigmentation-related terms and pathways is informative and is worthy of further study.

Studies have suggested that the mRNA expression levels of genes including *tyr*, *tyrp1*, *mc1r*, *mitf*, and *pmel* were higher in P-B skin. TYR carries out tyrosine hydroxylation to L-DOPA, which is the first step in the biosynthetic pathway of melanin. Under the action of dopachrome tautomerase (DCT) and TYP1, the dopaquinone (DOPA) chrome was rapidly oxidized and polymerized to form melanin (Braasch et al. 2010; Simon et al. 2009). Therefore, TYR, TYP1, and DCT are critical enzymes for the formation of melanin. Mutations or dysfunction of *tyr* or *tyrp1* genes lead to melanocyte death or extensive hypopigmentation in zebrafish (Krauss et al. 2015). In our study, compared to P skin samples, the expression levels of *tyr* and *tyrp1* were the highest in P-B skin. This was also consistent with the pigmentation of red crucian carp (Zhang et al. 2017). *Mitf* is a member transcription factor involved in the development of melanocytes, retinal cells, osteoclasts, and mast cells (Minvielle et al. 2010). It has been reported that *mitf* could directly regulate the expression of multiple genes necessary for the development of melanophores, including *tyr*, *tyrp1*, and *det* (Cheli et al. 2010). Compared with P skin colors, P-B skin color was caused by the increase of melanin content, suggesting that *mitf* may play a potential role in regulating the differentiation and development of melanocytes. *Mc1r* gene is a key gene in melanogenesis in animals. Alpha-melanocyte stimulating hormone (α-MSH) binds to *mc1r*, resulting in the decrease of cAMP level. Consequently, the melanin biosynthesis process was triggered (Voisey et al. 2001). Previous studies have shown that *mc1r* mutations were associated with skin color variation in many fish species, such as cavefish (*Astyanax Mexicanus*), guppy (*Poecilia reticulata*), zebrafish, and koi carp (*Cyprinus carpio*) (Gross et al. 2009; Tezuka et al. 2011; Richardson et al. 2008; Dong et al. 2020). Similarly, we observed a significant difference in *mc1r* expression between the red tilapia of the three skin colors used in this study. The *Pmel* gene acts as a scaffold in the melanosome by creating a proteolytic fibrillary matrix where melanin is deposited (Solano et al. 2000). *Pmel* mutations promoted pigment dilution in many animals (Gutierrez et al. 2007). Here, *pmel* was significantly up-regulated in P-B skin when compared to P and P-B-P skin samples. Similarly, we observed the top 10 abundance mRNAs in P-B skin, including *tyrp1b*, *tyr*, *pmelb*, *pmela*, and *tyrp1a*. All genes involved in melanin production, transport, and structural proteins for melanin have been verified in red tilapia. In addition, we noticed that the proteins from pigment-related pathways were distinctly integrated together in PPI networks in P-B vs. P group. It was speculated that those proteins (or genes) could be co-regulated in skin color variation in red tilapia during overwintering. Among them, the most important top 10 genes based on the key nodes in the PPI network, including *tyr*, *mc1r*, *mitfb*, *tyrp1b*, *det*, *pmela*, *pmelb*, and *mitfa*, were consistent with the results of mRNA expression levels.

Regarding the black to pink stage, the mRNA expression levels of *asip*, *tat*, *hpdb*, and *foxd3* were all up-regulated. *Asip* gene product blocks melanogenesis by antagonizing the binding of α-MSH to *mc1r*. *Asip* mutations were associated with skin color variation in *Psetta maxima* (*Scophthalmus maximus*), zebrafish, and medaka (*Oryzias latipes*) (Ceinos et al. 2015, Guillot et al. 2012, José et al. 2005). In our study, we observed higher expression of the *asip* gene in P-B-P skin transcripts and lower expression of the *mc1r* gene, which further establishes the role of *asip* as an
antagonistic of the *mc1r* gene. TAT and HPDB catalyze the substrate tyrosine to form homogeneous acid (HGA). Homogenisate1, 2-dioxygenase catalysis HGA to produce melatonin. A higher level of *tat* and *hpda* gene would directly reduce tyrosine level, thereby inhibiting the synthesis of melanin (Zhang et al. 2008). In addition, the *tat* gene was the most abundant in P skin, suggesting that it affected the skin variation in red tilapia. Foxd3 is a good candidate for the negative regulator of melanophore development. It can affect the lineage between neural or glial and pigment cells by repressing *mitf* at the early phase of neural crest migration (Thomas and Erickson 2009). In addition, overexpression of *foxd3* in melb-a mouse melanoblasts blocked the expression of *mitf* (Lister et al. 2001). Foxd3 was significantly up-regulated in P-B-P skin samples compared to the P-B skin, indicating that *foxd3* might play a significant role in the black-to-pink color transformation in red tilapia.

In addition, autophagy and apoptotic pathways were able to control the transition from black to pink in red tilapia. In detail, the mRNA level of the apoptosis gene, such as *baxa*, was significantly increased in the body color transformation from the P-B-P skin to the P-B and P skins. Meanwhile, the mRNA levels of *beclin1* and autophagy-related genes 7 (*atg7*), as the autophagy genes, were all up-regulated in the P-B-P skin compared with P-B skin. *Baxa*, as one of the homologous proteins of BCL-2, could determine survival or death by an apoptotic stimulus. Overexpression of *baxa* may accelerate cell death (Oltvai et al. 1993). *Beclin1* plays a key role in regulating autophagy and cell death by interacting with either BCL-2 or PI3k class III (Takacs-Vellai et al. 2005). *Atg7* activates the ubiquitin-like protein ATGL2, which binds to *atg5* and extends the autophagic vesicle membrane. Whole-body knock-out of *atg7* in mice led to death within 24 h after birth (Komatsu et al. 2005). *Baxa* and *atg7* gene were among the top 10 abundance mRNAs in P-B-P skin, further confirming that the appearance of autophagy may lead to melanocyte reduction.

Conclusions

In conclusion, we performed a transcriptome study of various skin colors during and after overwintering in red tilapia. We screened the top 10 abundance mRNAs, specific mRNAs and identified significant DEGs by pairwise comparison. These specifically expressed mRNAs provide the basis for further studies to clarify the role in skin variation of red tilapia. GO and KEGG analysis of specific mRNAs and DEGs identified numerous signaling pathways. We elucidated 32 candidate genes involved in skin color variation of red tilapia and constructed a PPI network consisting of these genes for revealing the mechanisms of color variation. These findings will help us learn more about the molecular mechanism of skin pigmentation in red tilapia. More specially, it provides valuable genetic data for breeding improved red tilapia strains with consistent skin color.

Author contribution Zaijie Dong conceived the study; Bingjie Jiang performed the experiments and wrote the paper; Wenbin Zhu provided the experimental materials; Lanmei Wang provided the funding for the experiment; Jianjun Fu and Wei Liu provided technical assistance in the experiments; Mingkun Luo revised the manuscript; Zaijie Dong reviewed the manuscript; all authors read and approved the manuscript.

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Availability of data and material All data generated or analyzed during this study are included in this published article and its supplementary information files.

Code availability Not applicable.

Declarations

Ethics approval The sampling scheme and experimental protocols were subject to approval by the Bioethical Committee of Freshwater Fisheries Research Center (FFRC) of the Chinese Academy of Fishery Sciences (CAFS) (BC 2013863, 9/2013). The methods of samples handled and experimental procedures carried out in accordance with the guidelines for the care and use of animals for scientific purposes issued by the Ministry of Science and Technology, Beijing, China (No.398, 2006).

Consent to participate Not applicable.

Conflict of interest The authors declare no competing interests.

References

Andrews S (2014) FastQC: a quality control tool for high throughput sequence data. http://www.bioinformatics.babraham.ac.uk/projects/fastqc/. Accessed 20 June 2021
Pietrobono S, Anichini G, Sala C et al (2020) ST3GAL1 is a target of the SOX2-GLI1 transcriptional complex and promotes melanoma metastasis through AXL. Nat Commun 11(1):5865

Pradeep PJ, Srijaya TC, Hassan A et al (2014) Optimal conditions for cold-shock induction of triploidy in red tilapia. Aquacult Int 22(3):1163–1174

Richardson J, Lundegaard PR, Reynolds NL, Dorin JR, Porteous DJ, Jackson JJ, Patton EE (2008) Mc1r pathway regulation of zebrafish melanosome dispersion. Zebrafish 5(4):289–295

Robinson MD, McCarthy DJ, Smyth GK (2010) Edger: a bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics 26(1):139–140

Sherbrooke WC, Frost SK (1989) Integumental chromatophores of a color-change, thermoregulating lizard, Phrynosoma modestum (Iguanidae; reptilia). Am Mus Novit 2943(2):1–14

Simon DJ, Peles D, Wakamatsu K et al (2009) Current challenges in understanding melanogenesis: bridging chemistry, biological control, morphology and function. Pigment Cell Melanoma Res 22(5):563–579

Skarnes WC, Rosen B, West AP et al (2011) A conditional knockout resource for the genome-wide study of mouse gene function. Nature 474(7351):337–342

Solano F, Martinez-Esparza M, Jimenez-Cervantes C, Hill SP, Lozano JA, Garcia-Borron JC (2000) New insights on the structure of the mouse silver locus and on the function of the silver protein. Pigment Cell Res 13(8):118–124

Swiercz JM, Worzfeld T, Offermanns S (2008) ErbB-2 and met reciprocally regulate cellular signaling via plexin-B1. J Biol Chem 283(4):1893–1901

Szlarczyk D, Franceschini A, Wyder S et al (2015) STRING v10: protein-protein networks, integrated over the tree of life. Nucleic Acids Res 43:D447–D452

Takacs-Vellai K, Vellai T, Puoti A et al (2005) Inactivation of the autophagy gene bec-1 triggers apoptotic cell death in C. elegans. Curr Biol 15(16):1513–1517

Tezuka A, Yamamoto H, Yokoyama J et al (2011) The mc1r gene in the guppy (Poecilia reticulata): genotypic and phenotypic polymorphisms. BMC Res Notes 4(1):31

Thomas AJ, Erickson CA (2009) Foxd3 regulates the lineage switch between neural crest-derived glial cells and pigment cells by repressing mitf through a non-canonical mechanism. Development 136(11):1849–1858

Voisey J, Box NF, Daal AV (2001) A polymorphism study of the human agouti gene and its association with mc1r. Pigment Cell Res 14(4):264–267

Wang LG, Wang SQ, Li W (2012) RSeQC: quality control of RNA-seq experiments. Bioinformatics 28(16):2184–2185

Wang LM, Song FB, Zhu WB et al (2018) Effects of temperature on body color of Malaysian red tilapia during overwintering period. J Fish China 42(1):72–79

Wang XY, Yang J, Yao Y et al (2020) Aqp3 facilitates proliferation and adipogenic differentiation of porcine intramuscular adipocytes. Genes 11(4):453

Wardani WW, Alimuddin A, Junior MZ et al (2020) Growth performance, robustness against stress, serum insulin, igf-1 and glut4 gene expression of red tilapia (Oreochromis sp.) fed diet containing graded levels of creatine. Aquac Nutr 27(1):274–286

Wu X, Zhao JD, Ruan YY et al (2018) Sialytransferase st3gal1 promotes cell migration, invasion, and TGF-β1-induced EMT and confers paclitaxel resistance in ovarian cancer. Cell Death Dis 9(11):1102

Xie C, Mao XZ, Huang JJ et al (2011) KOBASE 2.0: a web server for annotation and identification of enriched pathways and diseases. Nucleic Acids Res 39(suppl_2):W316-22

Yu GC, Wang LG, Han YY et al (2012) Clusterprofiler: an r package for comparing biological themes among gene clusters. OMICS 16(5):284–287

Zhang Y, Wang L, Zhang S et al (2008) HmgA, transcriptionally activated by hpda, influences the biosynthesis of actinorhodin in Streptomyces Coelicolor. FEMS Microbiol Lett 280(2):219–225

Zhang YP, Wang ZD, Guo YS et al (2015) Morphological characters and transcriptome profiles associated with black skin and red skin in crimson snapper (Lutjanus erythropterus). Int J Mol Sci 16(11):26991–27004

Zhang YQ, Liu JH, Peng LY et al (2017) Comparative transcriptome analysis of molecular mechanism underlying gray-to-red body color formation in red crucian carp (Carassius auratus, red var.). Fish Physiol Biochem 43(6):1387–1398

Zhu WB, Wang LM, Dong ZJ et al (2016) Comparative transcriptome analysis identifies candidate genes related to skin color differentiation in red tilapia. Sci Rep 6(1):31347

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