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THE VERIFICATION OF DIFFERENTIALLY EXPRESSED GENES RELATED TO FEATHER COLOUR IN QUAIL EMBRYOS BY RT-qPCR BASED ON RNA-seq

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Abstract

Many differentially expressed genes associated with feather colour in quail embryos can be screened using RNA-seq technology. The aim of this study was to verify the accuracy of RNA-seq using RT-qPCR. The transcriptome of embryonic skin tissues of quails were sequenced by Illumina HiSeq TM2000 sequencing platform. As a result, 2512 differentially expressed genes were found. Among them, 950 differentially expressed genes were up-regulated in the skin tissues of white-feather quail embryos, and 1562 differentially expressed genes were down-regulated. The candidate genes were analyzed by RT-qPCR method to verify the accuracy and reliability of the RNA-seq. The results showed that the relative expression levels of ASIP and DDC in white-feather quails were significantly increased, while HOXD1, CTSD, KRT2, MBD2 and MT1 were significantly decreased, which was consistent with the results of RNA-seq. The validation results of the candidate genes can also provide some ideas to explain the difference in feather colour between maroon-feather quails and white-feather quails.

Key words: quail, RT-qPCR, gene expression, RNA-seq, feather colour

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Introduction. In quails, as in most birds, the variability of plumage colour comes from the diversity of pigments, the pattern of pigment deposition in different parts of the feathers, and the modular organization of feather bundles throughout the bird's body [1]. The pigment in maroon-feather quails' feathers was mainly melanin, and the content and distribution of melanin was the main reason for the dark and light patterns of maroon-feather quails [2]. The difference between maroon-feather quails and white-feather quails plumage colour arises from the differential expression of genes related to the plumage colour trait; the high expression of genes favouring melanin synthesis inevitably leads to a deeper colouration of the relevant tissues in the corresponding species, while the opposite occurs in albino [3].

In order to investigate the genetic mechanism of plumage colour difference, we can analyze the differentially expressed genes of white-feather quails and maroon-feather quails in wing tissue of embryonic by RNA-seq technology, analyze the biological functions and signalling pathways of the screened genes, and explore the interaction pattern of differentially expressed genes, in order to understand the mechanism of plumage colour difference between maroon-feather quails and white-feather quails more deeply, and then screen the core control genes.

Materials and methods. ***Sample collection.*** Sixteen fertilized eggs of white-feather quails and sixteen fertilized eggs of maroon-feather quails were selected from the experimental ranch of Henan University of Science and Technology. The embryonic skin tissue was sampled at 8, 10, 12 and 14 days old, and the number of repetitions at each stage was four. The total RNA was extracted by kit method (Takara, TaKaRa MiniBEST Universal RNA Extraction Kit). After passing the NanoDrop D2000 test, the extracted RNA was reverse transcribed into cDNA using the kit method (Takara, TB Green® Premix Ex Taq™ II). Three maroon-feather quails skin samples and three white-feather quails skin samples were taken from 10 days old embryos, and sent to Berry Hutchinson corporation in dry ice for RNA-Seq.

Sequencing data quality assessment and the comparison of sequence. The original image data file obtained by high-throughput sequencing (Illumina platform) was transformed into raw reads by casava base recognition analysis, and then clean reads were obtained. Mapping of clean reads with the quail reference genome (GCA_001577835.2) was done using HISAT2 software. The clean reads of each sample were compared back to the reference genome by bowtie (V1.0.0) software, and the qualified data were analyzed for expression and comparison information statistics. The FPKM value of each unigene in each sample's expression quantity was calculated by RSEM (v1.2.15) software. The differential expression of unigenes in each sample was analyzed by edge R software, and the p value and FDR value of differentially expressed genes were calculated. Screen according to FDR < 0.05 & $\log 2|\text{foldchange}| > 1$.

Real-time fluorescent quantitative PCR verification. The internal reference gene was GAPDH. Primer 5.0 software was used to design primers for

Table 1

Primer information of real-time fluorescent quantitative PCR

Gene	Template sequence	Primer sequences ($5' \rightarrow 3'$)	Product length (bp)	Annealing temperature (°C)
ASIP	XM_015881357.2	F:TCCCACCCATCTCCATCGTA R:GCATTTGCACAAGGCACAGTAG	209	61
KRT2	XM_015887006.2	F:GGCTTTGGCTATGGTGTTGG R:GCGAATTTGTTGTTGAGGGTCT	257	61
CTSD	XM_015863832.2	F:TATGGTGAGATTGGCATTGGG R:CACATAGGTGCTGGATTTGGA	159	60
MBD2	XM_015850434.2	F:CCGACCTCAATACGGCTCTT R:TCAATATCTCCTGCCCCACG	192	61
DDC	XM_015854339.2	F:TAGGTTGTGTGGGCTTCTCTTG R:CCCGTCTGATGGTTTTTGTTCT	207	61
MT1	XM_032447116.1	F:CTCTCTGCCGCCCTGGATAA R:TGCTGGGGATGGAGTTTCAC	66	59
HOXD1	XM_032445884.1	F:CCACAATAGTTCCTGGGTCTC R:CTGCGTCGTCTTTATACTTTCC	214	58
GAPDH	XM_015873412.2	F:TGCCGTCTGGAGAAACC R:CAGCACCCGCATCAAAG	160	56

The reaction system for qPCR was 20 µl, containing cDNA 1 µL, upstream and downstream amplification primers 0.75 µL each, SYBR® Premix Ex Taq™ II (2×) 10 µL, ddH_2O 7.5 µL; reaction conditions were: pre-denaturation at 95 °C for 3 min; denaturation at 95 °C for 30 s, annealing for 30 s (set according to the annealing temperature of each primer), extension at 72 °C 20 s, 40 cycles; the signal was collected during the extension phase; the melting curve was increased by 0.5 °C every 5 s from 65 °C to 95 °C

the candidate genes, and the primers were synthesized by Oakdingsheng (Wuhan) Biotechnology Co. The primers results were shown in Table 1. The relative expression of genes was calculated using $2^{-\Delta\Delta Ct}$, and SPSS 17.0 software was used for t-test analysis, and Graphpad Prism 8.0 software was used for graphing.

Analysis and results. ***Extraction of total RNA from embryonic skin tissue of quails and detection of RNA quality.*** The total amount of RNA in embryonic skin tissue of maroon-feather quails and white-feather quails was greater than 10 µg, and the integrity (RIN value) was greater than 7, and both 18S:28S were greater than 2.2. The quality met the requirements of database construction and can be used for subsequent analysis.

Assembly analysis of unigenes. As presented in Fig. 1 and Table 2 the analysis of the differentially expressed genes in embryonic skin tissues of maroon-feather quails and white-feather quails showed that 2512 differentially expressed unigenes were obtained. There were 950 differentially expressed genes down-regulated in the skin tissue of maroon-feather quail embryos, such as ASIP and

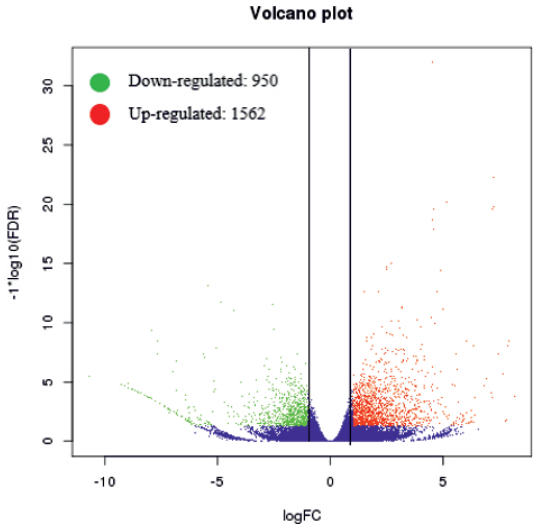

Fig. 1. Differential expression volcano maps. The screening results of differentially expressed genes are shown. Blue colour indicates genes that are not associated with plumage colour, red and green colours are significantly different genes; the horizontal axis is logFC (log2FoldChange), and the vertical axis is $-1 \cdot \log 10$ (FDR)

T a b l e 2

Results of differential expression of unigenes in different samples

Gene name	BF1	BF2	BF3	LF1	LF2	LF3	logFC	FDR
ASIP	42.688	38.213	47.588	21.348	24.511	25.042	−0.8797	4.77E−4
KRT2	191.719	591.72	64.977	1415.147	1241.412	2631.075	2.3416	2.76E−3
MT1	256.158	479.172	124.232	859.063	769.424	956.947	1.5933	5.42E−4
CTSD	0.671	1.416	0.796	5.921	4.688	5.461	2.5084	6.28E−10
MBD2	5.011	6.755	4.935	22.93	18.247	32.691	1.9443	8.84E−10
DDC	1.734	2.11	1.573	0.409	0.711	0.313	−1.9171	4.1E−07
HOXD1	9.369	8.49	13.547	25.469	20.879	36.604	1.4162	9.05E−05

BF1, BF2 and BF3 indicate the FPKM value of sequencing samples of white-feather quails, LF1, LF2 and LF3 indicate the FPKM value of sequencing samples of maroon-feather quails. logFC: $\log 2|$FoldChange$|$ value, log2(sample2_FPKM/sample1_FPKM); FDR: False Discovery Rate, i.e. corrected p value, the smaller the FDR value, the more significant the gene expression difference

DDC and others. And 1562 differentially expressed genes were up-regulated such as KRT2, MT1, CTSD, MBD2 and HOXD1 and others.

qPCR verification results. The result is shown in Fig. 2. Among the candidate genes, ASIP and DDC were down-regulated, HOXD1, KRT2, MT1, CTSD and MBD2 were up-regulated in maroon-feather quail embryos which were consistent with the RNA-seq results of the transcriptome. And it can be considered that the RNA-seq results was reliable. At the same time, the relative expression levels of candidate genes at 8d, 12d, and 14d were the same as the result of 10d. Thus, it can be considered that the expression levels of candidate genes were significantly different in each stage of maroon-feather and white-feather quail embryos.

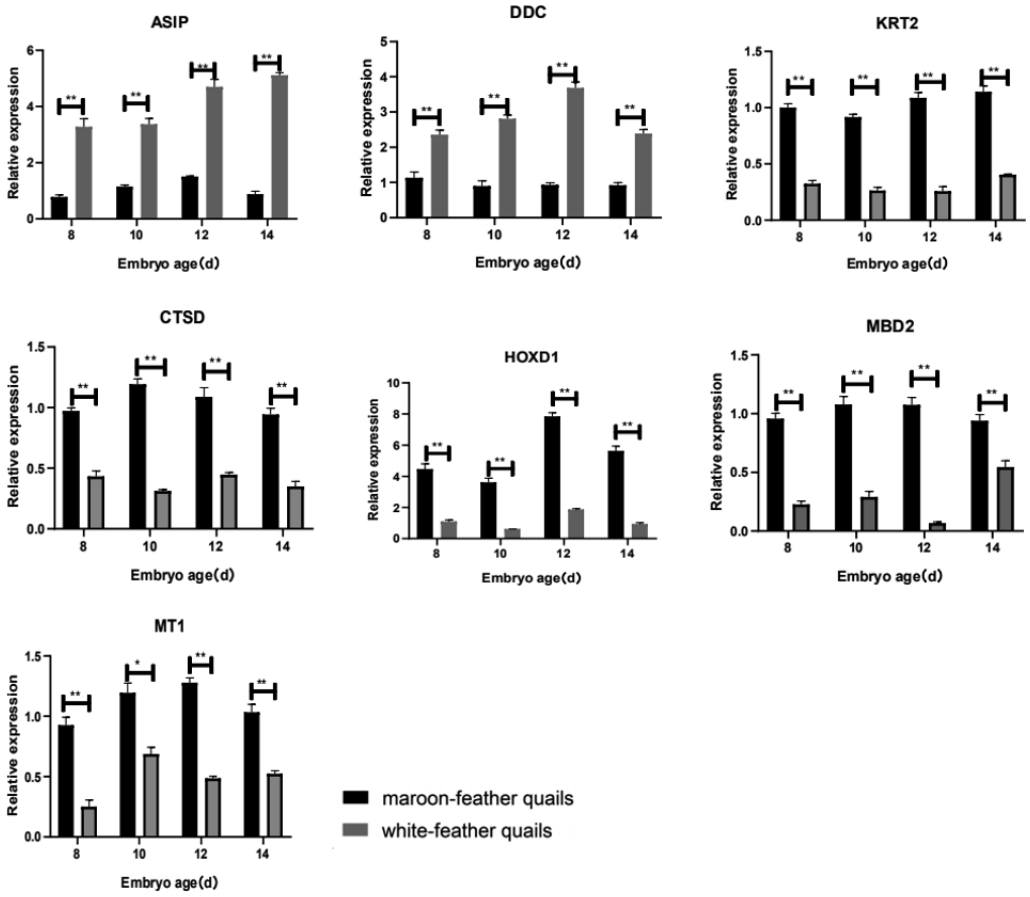

Fig. 2. Relative expression of each candidate gene. * indicates significant difference; ** indicates highly significant difference; Embryo age: Embryonic development days

Discussion. ASIP and DDC can be involved in melanin synthesis and transport in different pathways. In black-bone chickens, the mRNA levels of ASIP were higher in black skin than in white skin [4]. While the mRNA levels of ASIP in duck embryos were higher in the abdomen than in the dorsum [5] and it was similar to the results of the present study that the expression of ASIP in white-feather quails was higher than it in maroon-feather quails. The antagonistic effect of ASIP on MC1R resulted in a black phenotype of hair follicle melanocytes [6], which was not observed in chicken skin, but was somewhat verified in our study. The results of reduced total melanin in all mutant quails compared to wild-type quails except brown quails [7] were consistent with the results of our study. DDC was an important catalase for melanin synthesis in insects and its high expression would promote the formation of dopamine [8], it was deviating from the pathway of melanin synthesis in animals. Our study showed that the increase in DDC may lead to a decrease in the tyrosine used for melanin synthesis and the mRNA expression level of TRY was down-regulated in white-feather quails. DDC was

essential for the development of pigmentation in drosophila, and the regulation of DDC and two other genes may have evolved in concert, co-mapping a complex pattern of abdominal spots indicating the presence of a regulatory effect on pigmentation indeed [9]. In this study, the significant differential expression of DDC in maroon-feather and white-feather quails in the KEGG pathway was mainly enriched in pathways such as tyrosine metabolism pathway and serotonergic synapse pathway, and the variation in its expression level was consistent with the results for both quails. The vertebrate central nervous system was more complex than that of insects and would be part of the reason that DDC affects pigment deposition. However, its specific mechanism needs further study.

HOXD1 plays a crucial role in headgear patterning in higher ruminants, and many studies have shown a blueprinted association in this gene between morphology and colour [10], while the low expression of HOXD1 in white-feather quails may be related to its low complexity of the colour patterning. The study by Fischer et al. [11] found higher pigmentation in mice with high KRT2 than it in control groups, which was similar to the results in the present study that KRT2 in maroon-feather quails was more than it in white-feather quails. It has also been found that mutations in KRT2 in dark skin of mice may impair intermediate filament assembly, leading to lysis of basal keratin-forming cells, secondary hyperkeratosis and melanocytosis [12], which can be deeply studied in maroon-feather quails and white-feather quails. The activation of CTSD was found to occur in the macular region in the study of Rakoczy et al. [14] and the overexpression of CTSD has been researched in several human cancers, including melanoma [14], and it can degrade hormones, peptide precursors, polypeptides, structural and functional proteins. At present, we can only see that the expression of CTSD in white-feather quails was lower than that in maroon-feather quails, its mechanism needs further research. The expression of MBD2 was associated with transcriptional activation/repression, chromatin structural regulation, pluripotency development and differentiation [15]. Pan et al. [16] showed that knock-out MBD2 leads to more deposits accumulating on the BM (Bruch's membrane) and it may reduce the development of melanoma by triggering choroidal endothelial activation and inflammatory response, improved microcirculation, and reduced lipid deposition. This was similar to the findings of MBD2 low expression in white-feather quails in the present study. Melatonin could stimulate the activity of tyrosinase and it would lead to increased melanin content in melatonin-treated cells. The results of Shaverdashvili et al. [17] in which decreased expression of MT1 facilitated the reduction of melanocyte metastasis can provide some evidence for the downregulation of MT1 in white-feather quails in our study. Many effects of MT1 binding to melatonin including the promotion of hair growth were verified by Slominski et al. [18] who indicated that the decrease in MT1 expression may facilitate the formation of white feathers which could provide a direction to explain the destination of melanin in maroon-feather quails.

Conclusion. The results of RNA-seq were accurate. And the up-regulation of ASIP and DDC, the down-regulation of HOXD1, KRT2, MT1, CTSD and MBD2 in candidate genes could provide important information to study the molecular mechanism of the difference in feather colour between maroon-feather quails and white-feather quails.

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