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Screening and Analysis of Pigment-Related Genes in Liver Tissue of Changshun Blue-Shelled Chicken Based on Transcriptome Sequencing Technology

Zhenyang Wu*, Jinzhu Meng, Xiaozhen Yang and Qingming An*

Tongren University, Tongren 554300, Guizhou, PR China

ABSTRACT

To study the molecular mechanism of pigmentation in blue-shelled hens, transcriptome sequencing technology was used to analyze differences in the liver transcript expression profiles of Changshun blue-shelled chicken and Roman Pink laying hens. Three 10-month-old laying hens from each of the Changshun blue-shelled chicken and Roman Pink breeds were slaughtered, and their liver tissues were collected for transcriptome sequencing. A total of 342 differentially expressed genes (DEGs) were obtained by high-throughput sequencing, among which 192 were upregulated and 150 were downregulated in blue-shelled chickens. The gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) signaling pathways of the DEGs were analyzed. The GO analysis results were divided into 20 groups across three main categories, among which 40.0% were involved in biological processes (BP), 20.0% were involved in cellular components (CC), and 40.0% were involved in molecular functions (MF). KEGG signaling pathway analysis identified 10 pathways, among which the pathway involved in metabolism was the most enriched. The DEGs were verified by fluorescence quantitative PCR. A total of 6 genes possibly related to pigmentation were screened. The results of fluorescence quantitative PCR showed low expression of the *CYP27A1*, *FAM136A* and *SLC6A9* genes were low expressed in the liver of Changshun blue-shelled chickens, while the *ERVK25*, *EDN3* and *SLC34A2* genes were highly expressed. In summary, this study enriches our knowledge of the genetic basis of pigmentation in blue-shelled laying hens and provides a theoretical basis for the breeding of these chickens.

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Z-YW: Data analysis, prepared the manuscript and provided funding. Q-MA: Sample collection and some experiments. J-ZM: Helped with data analysis and produce some figures. X-ZY: Conceived and designed the experiment.

Key words
Transcriptome sequencing, Pigmentation, Blue eggshell phenotype, Liver tissue, Changshun blue-shelled chicken, Roman Pink laying hens

INTRODUCTION

The most common eggshell colors are pink, brown, white and blue, and studies on eggshell color carried out in the early 20th century showed that eggshell color is mainly determined by three pigments, namely protoporphyrin IX, biliverdin, and biliverdin zinc chelate (Kennedy and Vevers, 1976; Poole, 1965). The blue eggshell phenotype is caused by the deposition of biliverdin and its zinc chelates in the eggshell during egg formation (Iwasaki *et al*., 2022). Although the eggshell formation site is in the chicken's uterus, the site where biliverdin is synthesized and secreted remains unclear. By using high-performance liquid chromatography and ultraviolet spectrophotometry, Zhao *et al*. (2006) determined the concentrations of biliverdin in the blood, eggshell, bile and uterus (shell gland) within the oviduct of brown-shelled laying hens and blue-shelled laying hens. It was found that the concentration of bilivertin in uterus of blue shelled laying hens was significantly higher than that of brown shelled laying hens, while the concentration of biliverdin in the blood and bile were not significantly different between the two breeds. Therefore, it is speculated that the secretion site of biliverdin occurs in the uterus (shell gland) within the chicken oviduct. However, the biosynthesis pathway of biliverdin, which is synthesized from heme present in the body by heme oxygenase, is relatively complex (Santos *et al*., 2021). Under the action of a series of enzymes, heme is converted into biliverdin via the intermediate of hemoglobin biliverdin and releases free iron ions (Salim *et al*., 2021; Zhang *et al*., 2020). Biliverdin is a blue-green pigment that can be further reduced to bilirubin by biliverdin reductase. However, the activity of biliverdin

* Corresponding author: wuzhenyang0724@163.com, 741073485@qq.com
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reductase in poultry is very low. The biliverdin pigments found in blue eggshells and bile are the same, and their production is catalyzed by heme oxygenase. Heme oxygenase is the key enzyme involved in biliverdin synthesis, and changes in its expression are related to changes in biliverdin contents in tissues. A study by Bu (2017) showed that the expression of the heme oxygenase (*HO-1*) gene was highest in the liver, and the authors speculated that the liver may be the main site of biliverdin synthesis. Another possibility regarding the sites of biliverdin synthesis and secretion is that heme in aged red blood cells enters the liver, where heme oxygenase catalyzes the synthesis of biliverdin. Thereafter, a portion of that biliverdin is transported to the shell gland via an unknown some mechanism and is finally deposited in the eggshell.

In this study, transcriptome sequencing was performed in the liver tissues of Changshun blue-shelled and Roman Pink laying hens. Bioinformatics was used to screen differentially expressed genes (DEGs) related to pigmentation and to analyze the mechanism of eggshell pigment deposition in blue-shelled laying hens. This work deepens our understanding of the molecular mechanism underlying the genetic phenotype of blue eggshells.

MATERIALS AND METHODS

Experimental animals and sample collection

Laying hens of two chicken breeds, Changshun blue-shelled and Roman Pink, were used in the present study. Changshun chicken comes from Changshun County, Guizhou Province, China. It is characterized by blue eggshells and black and red feathers. Historically, Changshun chickens were selected to have blue eggshells, but some of their appearance traits and feather coloration are not homogeneous. Roman Pink laying hens are a well-known, high-yielding laying hen breed characterized by pink eggshells and white feathers.

To eliminate any effect of the genetic background, liver tissue samples were collected from three Changshun blue-shelled laying hens and three Roman pink laying hens at the peak of egg production, when they were 10 months old. The laying hens were quickly slaughtered, after which a 1 cm×1 cm liver tissue specimen was quickly cut with a scalpel, placed in a tube, and frozen in liquid nitrogen, then transferred to a -80°C refrigerator for permanent storage.

RNA extraction and library construction

Total RNA was isolated from the livers of the six chickens using TRIzol Reagent (AmbionH, Life Technologies Ltd.) following the manufacturer's recommended approach. The concentration and purity of all RNA samples were determined with a NanoDropH ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies). RNA integrity was detected using agarose gel electrophoresis. Six RNA samples were sent to Genergy Biotechnology Co., Ltd. (Shanghai, China) for RNA library construction and sequencing. Sequencing was performed using an Illumina HiSeq 2000 Genome Analyzer (Illumina Inc., Santiago, CA, USA). Libraries were constructed using a TruSeq Small RNA Sample Preparation kit (Illumina Inc., Santiago, CA, USA).

Data preprocessing

The quality of the original raw sequencing data obtained by deep sequencing was assessed using FastQC software. Clean reads were obtained by trimming low-quality reads and eliminating reads with contaminants. Prior to mapping reads to the reference database, we filtered all sequences as follows: (i) remove adaptor sequences and low-quality sequences (a percentage of low-quality bases with a quality value ≤5 within a read >50%); (ii) remove N >10% in a read (N represents unidentified bases); (iii) remove 5' adaptor-contaminated reads; (iv) remove reads without 3' adaptor sequences and inserts; (v) remove 3' adaptor sequences and polyA/T/G/C reads.

Mapping reads to the reference genome

In this study, TopHat 2 (Kim *et al*., 2013) was applied to align the clean reads to the chicken genome (chicken genomic DNA from the ensemble genome database, http://www.ensembl.org/) using a spliced mapping algorithm. The algorithm could segment samples without full-length matching. In addition, TopHat 2 was employed to align the whole sequence to genomic exons. Two mismatches were allowed in matching, and <= 1 multi hit was allowed in each read.

Normalization of gene expression levels and DEGs screening

The results of the Tophat 2 alignment were quantitatively analyzed using Cufflinks (Trapnell *et al*., 2013), and the fragments per kilobase of transcript per million mapped reads (FPKM) values of each gene were calculated. The mapped read counts for each gene were normalized for RNA length and for the total read number in the lane according to reads per kilobase of exon model per million mapped reads (RPKM), which facilitates the comparison of transcript levels between samples (Mortazavi *et al*., 2008). The cuffdiff function of cufflinks was used to normalize the resulting FPKM values for the analysis of differences between the samples.

Table I. The information of gene primer.

Gene names	Primer sequences	Amplicon length (bp)	Annealing temperature (°C)
ERVK-25	F: 5'-GTGCCGGACATGCTAGAACT-3'	108	60
	R: 5'-CTGTGCTGCTAGAGGGATGG-3'		
EDN3	F: 5'-CGTCTACTACTGCCACCTC-3'	138	60
	R: 5'-CTGACTGAACCCTCCAAG-3'		
SLC34A2	F: 5'-TCGGTCCGTTCACTCTGTTG-3'	164	60
	R: 5'-GCCACGTTGCCTTTGTGATT-3'		
CYP27A1	F: 5'-AGGACTTTCGTCTGGCTCT-3'	185	58
	R: 5'-CTCCGCATCGGGTATTT-3'		
FAM136A	F: 5'-GCACGCTTCACTGCTCTGACA-3'	153	56
	R: 5'-ACCCGTCCCGCATCCTCTT-3'		
SLC6A9	F: 5'-CGTACCTCTGCTACCGCAAT-3'	261	60
	R: 5'-CACGCGTCATGGACACAAAG-3'		
β-actin	F: 5'-CAGCAAGCAGGAGTACGATG-3'	145	60
	R: 5'-ATAAAGCCATGCCAATCTCG-3'		

Gene ontology (GO), pathway enrichment analysis and validation of DEGs

GO functional enrichment analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses were performed using DAVID software (david.ncifcrf.gov). DEGs were confirmed by qPCR. All six samples (three blue-shelled and three pink-shelled chicken samples) were included in the qPCR analysis. The primers employed are shown in Table I. One microgram of total RNA from each sample was reverse-transcribed into cDNA using the thermo scientific revert aid first strand cDNA synthesis kit (Thermo Fisher Scientific Inc., Waltham, MA, USA). β-actin was used as the internal control. qPCR was performed using standard protocols on a Roche LightCycler 480 Real-Time PCR Detection System (Hoffmann-La Roche Ltd, Basel, Switzerland). The $2^{-\Delta\Delta Ct}$ method was used to analyze expression levels (Livak *et al*., 2001).

RESULTS

Overview of sequencing data

To identify DEGs in the two chicken breeds (one is Changshun blue-shelled breed and other is Roman Pink laying hens), six mRNA libraries were constructed for Illumina sequencing. A total of 279,446,145 raw reads and 252,284,522 clean reads were obtained after eliminating the low-quality reads and reads containing N sequences or adaptor sequences. The basic information of the sequencing data is provided in Table II.

Table II. The information of raw data filtering.

Sample name	Total reads	Clean reads	Clean ratio	Q20	Q30
1B	47,026,229	42,459,982	90.29%	97.28%	89.98%
2B	45,863,515	41,382,650	90.23%	97.95%	91.56%
3B	47,617,562	42,974,850	90.25%	98.30%	93.10%
1P	47,340,921	42,758,320	90.32%	97.02%	85.87%
2P	47,063,616	42,503,152	90.31%	97.17%	89.01%
3P	44,534,302	40,205,568	90.28%	98.17%	90.53%

Gene expression level analysis and clustering of DEGs between the samples

The genetic quantification of the TopHat alignment results was performed using Cufflinks, and the FPKM values for each pair of genes were calculated. A total of 14,036 annotated genes were obtained. We list the 10 genes with the highest expression levels in Table III. These genes were found to mediate the cellular uptake of organic ions in the liver, the sodium-dependent uptake of thyroid hormones, and receptor recognition and membrane fusion mediated by retroviral envelope proteins.

Cuffdiff was used to analyze the normalized FPKM values between the samples. A total of 342 DEGs were obtained. Among these genes, Changshun blue-shelled laying hens showed 192 upregulated genes and 150 downregulated genes compared with Roman pink laying hens. Table IV lists only the top 10 upregulated and downregulated genes.

Table III. The most abundantly expressed gene in the liver tissue of laying hens (top 10).

Gene names	Base-mean	P value	Gene function
SSC5D	374,224	2.7×10^{-4}	Scavenger receptor activity and laminin binding; Binds to extracellular matrix proteins. Binds to pathogen-associated molecular patterns (PAMPs), behaving as a pattern recognition receptor (PRR).
Gpx3	273,292	9.07×10^{-2}	Transcription factor binding and selenium binding; Protects cells and enzymes from oxidative damage, by catalyzing the reduction of hydrogen peroxide, lipid peroxides and organic hydroperoxide.
SLCO1A2	23,082	5.1×10^{-4}	Transporter activity and organic anion transmembrane transporter activity; Encode a sodium-independent transporter which mediates cellular uptake of organic ions in the liver. Its substrates include bile acids, bromosulphophthalein, and some steroidal compounds.
SLCO1C1	15,870	5.1×10^{-22}	Transporter activity and transmembrane transporter activity. The encoded protein is a transmembrane receptor that mediates the sodium-independent uptake of thyroid hormones in brain tissues.
ADH1	4,422	9.6×10^{-5}	oxidoreductase activity and alcohol dehydrogenase (NAD+) activity.
ERVK-25	2,846	5.7×10^{-13}	Structural molecule activity. Retroviral envelope proteins mediate receptor recognition and membrane fusion.
ACSS2	2,752	5.1×10^{-4}	AMP binding and acetate-CoA ligase activity, catalyzes the activation of acetate for use in lipid synthesis and energy generation.
ERVK-6	2,712	5.1×10^{-4}	Nucleic acid binding and structural molecule activity; Retroviral envelope proteins mediate receptor recognition and membrane fusion.
SGK1	1,732	3.2×10^{-6}	Transferase activity, transferring phosphorus-containing groups and protein tyrosine kinase activity. This gene encodes a serine/threonine protein kinase that activates certain potassium, sodium, and chloride channels.
SOD3	1,233	6.9×10^{-4}	Heparin binding and superoxide dismutase activity; Catalyze the conversion of superoxide radicals into hydrogen peroxide and oxygen.

Table IV. The information of differentially expressed gene in liver of different laying hen breeds.

Gene names	Log_2 (fold change)	P value	Gene function
ERVK-25	8.9	1.0×10^{-9}	Structural molecule activity; retroviral envelope proteins mediate receptor recognition and membrane fusion.
ERVK-6	8.3	4.3×10^{-9}	Nucleic acid binding and structural molecule activity; Retroviral envelope proteins mediate receptor recognition and membrane fusion.
MMP1	7.4	1.0×10^{-9}	Calcium ion binding and metallopeptidase activity; Proteins in this family are involved in the breakdown of extracellular matrix in normal physiological processes.
EDN3	5.7	1.1×10^{-4}	signaling receptor binding and hormone activity; Endothelins are endothelium-derived vasoactive peptides involved in a variety of biological functions.
CACNA2D4	4.6	1.8×10^{-2}	Voltage-gated calcium channel activity and calcium channel regulator activity; Regulate calcium current density and activation/inactivation kinetics of the calcium channel.
TNNI2	4.2	1.7×10^{-2}	Actin binding and troponin T binding; The troponin complex, along with tropomyosin, is responsible for the calcium-dependent regulation of striated muscle contraction.
ERVK-11	4.1	2.0×10^{-2}	RNA-DNA hybrid ribonuclease activity and RNA-directed DNA polymerase activity; Endogenous Pol proteins may have kept, lost or modified their original function during evolution
ADGB	2.8	2.3×10^{-2}	Iron ion binding and oxygen binding; Predicted to enable calcium-dependent cysteine-type endopeptidase activity; heme binding activity; and oxygen binding activity.
SLC34A2	2.8	2.5×10^{-2}	Protein domain specific binding and phosphate ion binding; Involved in actively transporting phosphate into cells via Na(+) cotransport.
SLC35F1	2.0	4.5×10^{-2}	Predicted to enable transmembrane transporter activity; Predicted to be involved in transmembrane transport.

Table continued on next page.....

Gene names	Log_2 (fold change)	P value	Gene function
CYP27A1	-8.1	1.0×10^{-9}	iron ion binding and oxidoreductase activity; Its related pathways are Synthesis of bile acids and bile salts and Oxidation by cytochrome P450.
IGHV3-23	-7.2	3.1×10^{-4}	Antigen binding; V region of the variable domain of immunoglobulin heavy chains that participates in the antigen recognition.
FAM136A	-6.2	1.3×10^{-4}	This gene encodes a mitochondrially localized protein that is highly conserved across species.
SLC6A9	-6.1	2.2×10^{-4}	Neurotransmitter:sodium symporter activity and amino acid:sodium symporter activity; Stop glycine signaling by removing it from the synaptic cleft.
MYO15A	-5.1	4.5×10^{-4}	Actin binding and cytoskeletal motor activity; Myosins are actin-based motor molecules with ATPase activity.
POL	-4.8	5.7×10^{-4}	Retrovirus-related Pol polyprotein Reverse transcriptase
MKX	-4.4	1.0×10^{-2}	Sequence-specific DNA binding and DNA-binding transcription activator activity; May act as a morphogenetic regulator of cell adhesion.
CABP7	-4.2	2.3×10^{-2}	Calcium ion binding; Negatively regulates Golgi-to-plasma membrane trafficking by interacting with PI4KB and inhibiting its activity.
ANXA4	-4.2	1.3×10^{-2}	Calcium ion binding and calcium-dependent protein binding; Calcium/phospholipid-binding protein which promotes membrane fusion and is involved in exocytosis.
ERVK-8	-4.0	2.7×10^{-2}	Endogenous Retrovirus Group K Member 8; Mediate receptor recognition and membrane fusion during early infection.

We further conducted a heatmap analysis of the DEGs (Fig. 1). The heat map obtained via cluster analysis showed that the identified DEGs were reproducible among the samples.

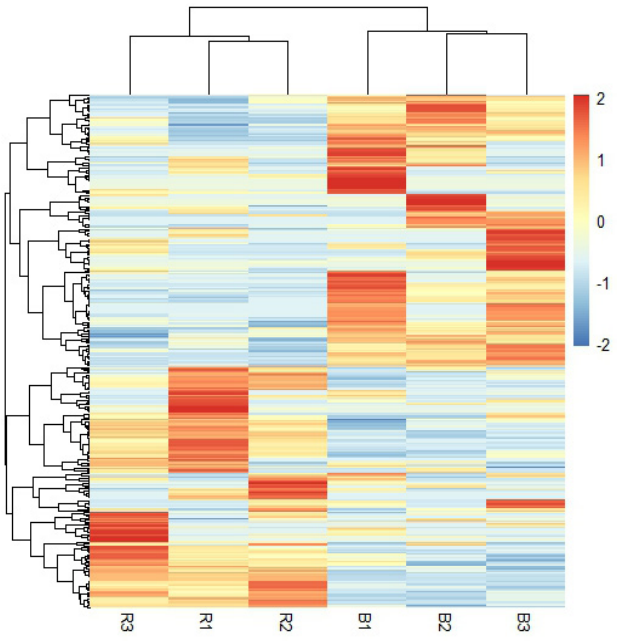

Fig. 1. The hierarchical clustering based on differentially expressed genes. B1, B2 and B3 are the liver samples from blue-shelled chickens; R1, R 2 and R3 are the liver samples from pink-shelled chickens.

Analysis of differential gene function

Genes that might affect pigmentation were classified through the GO functional enrichment analysis of the DEGs identified in the livers of Changshun blue-shelled and Roman Pink laying hens. The 342 obtained DEGs were divided into 3 major categories and 20 groups. Genes involved in biological processes (BP), including lipid metabolic process, viral process, small molecule biosynthetic process, organic hydroxy compound biosynthetic, accounted for 40.0% of these genes; genes related to cellular components (CC), including mitochondrial proton-transporting ATP synthase complex and virion component, accounted for 20.0%, and genes related to molecular function (MF), including enzyme regulator activity, lipid binding, sulfuric ester hydrolase activity, accounted for 40.0% (Fig. 2).

KEGG enrichment analysis was used to identify the major biochemical pathways and signaling pathways involving the DEGs. The results showed that the DEGs were significantly enriched in 10 KEGG pathways ($P < 0.05$) (Fig. 3), including metabolism, lipid biosynthesis proteins, and biosynthesis of unsaturated fatty acids.

Confirmation of differential gene expression by qPCR

We selected the six most important genes, *CYP27A1, FAM136A1, SLC6A9, ERVK25, EDN3* and *SLC34A2* genes, for validation of the expression profiles obtained by RNA-Seq. *ERVK25, EDN3* and *SLC34A2* were relatively highly expressed genes in the liver of blue-shelled laying hens (Fig. 4), with *ERVK25* showing extremely significant differences ($P<0.01$), while *EDN3* and *SLC34A2* showed

significant differences (P<0.05). The genes with relatively low expression in the livers of blue-shelled laying hens were *CYP27A1, FAM136A* and *SLC6A9*. The most significant differences were found for *CYP27A1* and *FAM136A* (P< 0.01), while *SLC6A9* (P<0.05) showed significant differences (Fig. 4).

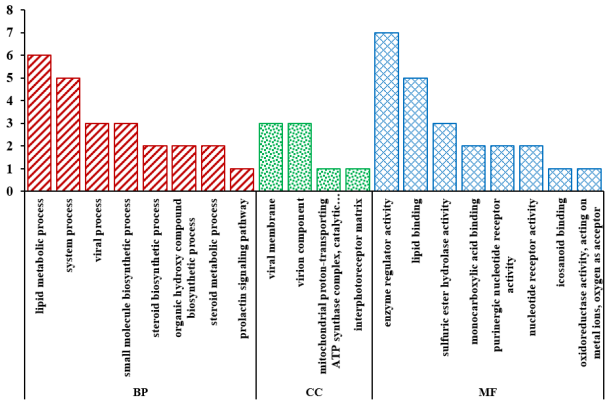

Fig. 2. GO analysis of differentially expressed genes in different chickens breed.

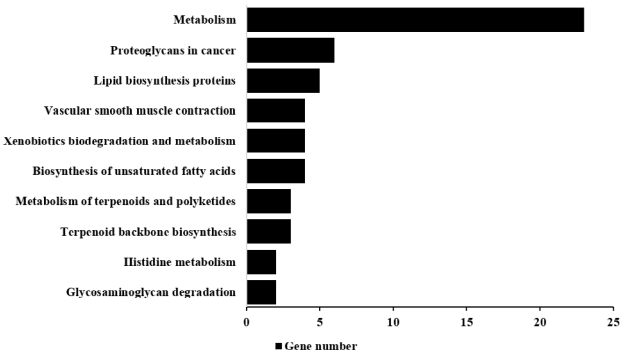

Fig. 3. Analysis of KEGG signaling pathway of differentially expressed genes.

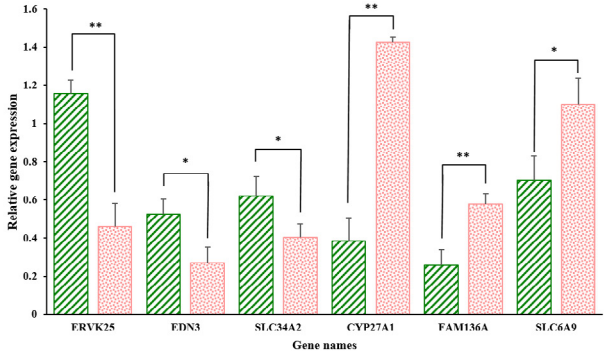

Fig. 4. Relative expression of six differentially expressed genes. * p < 0.05, ** p < 0.01.

DISCUSSION

In this study, 342 DEGs were obtained by sequencing analysis of the livers of laying hens with different eggshell colors. GO functional enrichment and KEGG signaling pathway analyses were performed on the DEGs, and the results suggested that the ion binding and transport and mineral absorption signaling pathways may regulate the depth of eggshell color. In this study, 6 candidate genes related to pigmentation were selected, and the results of real-time fluorescence quantitative PCR verification showed that the *ERVK25, EDN3* and *SLC34A2* genes were highly expressed in the liver of Changshun blue-shelled laying hens (P < 0.01 or P < 0.05). However, the *CYP27A1, FAM136A* and *SLC6A9* genes showed low expression (P < 0.01 or P < 0.05), which was basically consistent with the results obtained by Illumina sequencing. These findings further demonstrate the authenticity and reproducibility of the RNA-Seq analysis results and gene expression data.

The *SLCO1B3* gene, which is widely recognized as being related to the blue shell trait, is located on chromosome 1 in chickens; its product contributes to the transmembrane transport of bile salts to the oocyst gland, which secretes biliverdin into the eggshell (Altgilbers *et al*., 2022). In this study, the selected *SLC6A9* gene was downregulated in the blue shell group, while the *SLC34A2* gene was significantly upregulated. Both genes belong to the solute transporter (SLC) gene family. The *SLC34* gene family is involved in phosphate transport (Forster *et al*., 2013) and plays an important role in the transport of Na^{+}, Ca^{2+} and Fe^{3+} on the cell membrane (Fredriksson *et al*., 2008). One of its members, *SLC34A2,* has a regulatory effect on calcification in organisms (Xiao, 2022). Although the regulatory mechanism of *SLC6A9* is still unclear, Wang *et al*. (2019) preliminarily demonstrated that the *SLC6A9* gene may be involved in melanin synthesis in mouse B16 cells. Studies have shown that ion transport plays a key role in eggshell formation (Fan *et al*., 2013) and may regulate the eggshell calcification process through the transport of SLC family genes during red blood cell cleavage to produce eggshell pigment, thus affecting the deposition of biliverdin. This is similar to the results of a study on blue-shelled duck eggs whose authors speculated that some genes of the SLC family affect differences in eggshell color through small molecule transport (Xu *et al*., 2018).

Promoters and enhancers in the LTR region of ERV can influence the transcription of neighboring genes in the cell and alter the epigenetic status of neighboring regions. Wang *et al*. (2013) and Wragg *et al*. (2013) found that endogenous retrovirus EAV-HP elements had integrated into the 5- non-coding region of the *SLCO1B3* gene

through reverse insertion and enhanced the function of the *SLCO1B3* gene promoter. Thus, the specific expression of the gene in the egg shell gland is activated, resulting in the transportation of biliverdin and its deposition in the shell egg. Chang *et al*. (2007) and Sato *et al*. (2007) showed that the endogenous retrovirus ALV was inserted and integrated into the 4th intron of the *TYR* gene, resulting in the abnormal expression of the gene transcript and, thus, the recessive white feather trait. Therefore, it is speculated that high expression of the *ERVK25* gene can lead to the expression of SLC family genes and thereby affect the deposition of pigment on the eggshell surface.

This study showed that *CYP27A1,* a member of the cytochrome P450 family, was downregulated in the blue shelled group. The cytochrome P450 protease superfamily is composed of several functionally related genes encoding heme-mercaptan proteins (Dam-on *et al*., 2024), which are a monooxygenases that can oxidize many inert compounds under favorable conditions, directly affecting the binding of heme and Fe (Edwards *et al*., 2019; Brignac-Huber *et al*., 2016). Therefore, it is speculated that *CYP27A1* of the P450 family may play a role in the formation of biliverdin through oxidation reaction. Mihaljevi *et al*. (2020) reported that the expression of metabolically related cytochrome P450 gene family members may lead to changes in the downstream α-linolenic acid metabolism and arachidonic acid metabolism pathways. Yang *et al*. (2020) have showed that the α-linolenic acid metabolism and arachidonic acid metabolism pathways can regulate cytochrome production in a feedback-mediated manner. Considering these past findings with the results of this study, it is speculated that the biosynthesis of unsaturated fatty acids with significant enrichment of DEGs may be an important regulatory signal in the process of eggshell color formation.

The significantly downregulated *FAM136A* and upregulated *EDN3* genes identified in the liver tissue of blue-shelled laying hens in this study have not been previously reported to play a role in eggshell pigmentation. However, the *FAM210B* gene, another member of the FAM family, which is regulated by the transcription factor KatA-L and is abnormally expressed in mitochondria, is an important candidate gene for the blue-shelled trait of Dongxiang blue-shelled laying hens. In cells with a defective *FAM210B* gene, Yien *et al*. (2018) found that heme synthesis and iron input were reduced and *Alas2* gene transcription and porphyrin accumulation were inhibited. The *EDN3* gene product can increase the expression of pigmentation-related genes and the content of melanin in vertebrates such as red tilapia (Fang *et al*., 2022) and sheep (Li *et al*., 2017), and an appropriate concentration of exogenous EDN3 can promote the proliferation and differentiation of melanocytes. Bovo *et al*. (2021) also showed that *EDN3* plays an obvious regulatory role in the pigmentation of different parts of various species.

CONCLUSION

The blue eggshell color is mainly caused by the precipitation of biliverdin on the eggshell during the eggshell formation process, and biliverdin is converted from heme through a certain metabolic pathway in the biological body, and the liver may play a crucial role in this metabolic pathway. The functions of 6 candidate genes were analyzed, including CYP450 family genes, SLC family genes, FAM family genes, and ion transport signaling pathways involved in eggshell pigment synthesis, transport and deposition. The above genes and signaling pathways may be candidate genes and key signaling pathways affecting the eggshell of Changshun laying hens.

DECLARATIONS

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Statement of conflict of interest

The authors have declared no conflict of interest.

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