Transcriptome Analysis of Ovary Tissues From Low- and High-yielding Changshun Green-shell Laying Hens

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Abstract

Background: Changshun green-shell laying hens are unique to Guizhou Province, China, with high egg quality. At this stage, improving egg production performance has become an important breeding task. In recent years, the development of high-throughput sequencing technology provides a fast and exact method for genetic selection. Therefore, this study is based on high-throughput sequencing technology to analyze the differences in the ovarian mRNA transcriptome of low-yield and high-yield Changshun green-shell layer hens, identify critical pathways and candidate genes involved in controlling the egg production rate, and provide basic data for layer breeding.

Results: The egg production rates of the low egg production group (LP) and the high egg production group (HP) were 68.00 ± 5.56% and 93.67 ± 7.09%, with extremely significant differences between the groups ($p < 0.01$). Moreover, the egg weight, shell thickness, strength and layer weight of the LP were significantly larger than those of the HP ($p < 0.05$). More than 41 million clean reads per sample were obtained, and more than 90% of the clean reads were mapped to the *Gallus gallus* genome. Further analysis identified 142 differentially expressed genes (DEGs), and among them, 55 were upregulated and 87 were downregulated in the ovaries. KEGG pathway enrichment analysis identified 9 significantly enriched pathways. Among them, the neuroactive ligand-receptor interaction pathway was the most enriched. GO enrichment analysis indicated that the molecular function GO term: transmembrane receptor protein tyrosine kinase activity, as well as the DEGs identified in this GO term, including PRLR, NRP1, IL15, BANK1, NTRK1, CCK, and HGF may play crucial roles in the regulation of egg production.

Conclusions: The above-mentioned differentially expressed genes should be paid attention to in molecular breeding of Changshun green-shell laying hens. Moreover, enrichment analysis suggested that the neuroactive ligand-receptor interaction pathway and receptor protein tyrosine kinases may play crucial roles in the regulation of ovarian function and egg production.

1. Introduction

Chicken eggs are an important food resource for humans as they contain high-quality protein, essential vitamins and minerals, and they are inexpensive. Global egg consumption has tripled in the past 40 years and this trend is predicted to continue in the future [1]. The question of how to increase egg production has consequently become a critically important question in the egg industry.

Improving the genetic potential of chickens is one of the most important strategies utilized to increase egg production. However, conventional breeding techniques, that are based on long-term selections using egg number and laying rate, are usually laborious and time consuming [2]. Currently, various high-throughput techniques that can identify genes at the genomic and transcriptomic levels have been increasingly employed when studying poultry reproduction. For instance, Kang et al. [3] identified twenty-six genes in goose ovaries that were likely to be related to the egg-laying process by using suppression subtractive hybridization and reverse dot-blot analysis. Using a large-scale transcriptome sequencing technique, Ding et al. [4] identified five genes in the ovarian tissues of *Anser cygnoides* that may play important roles in determining high reproductive performance. Shiue et al. [5] identified nine genes related to high egg production levels in the chicken hypothalamus and pituitary gland using a cDNA chip. High throughput techniques provide a fast and exact method for genetic selection and have the potential to be an appealing alternative to conventional breeding techniques.

Changshun green-shell chickens are native breeds found in Guizhou province, China. They are dual-purpose egg - and meat-type chickens, and their eggs have extremely high economic value, owing to their appearance, higher protein content, better amino acid composition, and lower fat content [6]. In the present study, we conducted high-throughput RNA sequencing in the ovaries of Changshun green-shell chickens, to: 1) determine the differences in the ovary mRNA transcriptomes between the low- and high-yielding Changshun green-shell laying hens, and 2) identify the critical pathways and candidate genes involved in controlling the egg production rate.

2. Materials And Methods

2.1 Ethics statement

This study was carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Ministry of Science and Technology of the People's Republic of China, and followed the Regulations for the Administration of Affairs Concerning Experimental Animals, Qiannan Normal University for Nationalities (Guizhou, China).

2.2 Animal and sample preparation

A total of 80 Changshun green-shell layers raised in the poultry breeding farm of Qiannan Normal University for Nationalities were used in this study. At the beginning of the study, the layers had similar body weights of 1.36 ± 0.14 kg. All layers were housed in individual pens

with the same feeding and management conditions throughout the study period. Egg number and egg weight were recorded every day. At 290 days of age, four high-yield (high egg production group, HP) and four low-yield individuals (low egg production group, LP) were selected from the batch of laying hens according to their laying rates, and measurement was started eggshell thickness and strength. At the age of 299 days, they were fasted overnight. After measuring the final weight in the early morning of the next day, they were anesthetized with sodium pentobarbital, and ovarian samples were obtained after slaughter. All samples were immediately frozen in liquid nitrogen and stored at -80 °C until analysis.

2.3 RNA extraction, cDNA library construction, and mRNA sequencing

Total RNA was extracted from the ovary samples using the Trizol reagent (Takara Bio, Dalian, China), according to the manufacturer's instructions. The concentration and quality of the total RNA was determined using a NANOdrop ND-2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and electrophoresis, and sample integrity were evaluated using a microfluidic assay on a Bioanalyzer system (Agilent Technologies, Inc., Santa Clara, CA, USA). Library construction and RNA sequencing were performed as a fee-for-service by GENEWIZ, Inc. (Suzhou, China). Briefly, mRNAs were enriched using magnetic beads with Oligo (dT) and then randomly fragmented using a fragmentation buffer. The first strand of cDNA was synthesized with a random hexamer-primer using the mRNA fragments as a template. The second strand of cDNA synthesis was then performed using the Buffer, deoxynucleotide triphosphates (dNTPs), ribonuclease H (RNase H), and DNA polymerase I. The cDNA was purified with a QiaQuick PCR extraction kit (Qiagen, Germany) and eluted with EB buffer for end repair and poly(A) addition. Finally, sequencing adapters were ligated to the 5' and 3' ends of the fragments, the fragments were then purified using agarose gel electrophoresis and enriched by PCR amplification to establish a cDNA library. The cDNA libraries were loaded on an Illumina sequencing platform (NovaSeq 6000) for sequencing.

2.4 Data analysis

Quality control checks of the raw reads were performed using FastQC (v0.11.5). Raw reads were then trimmed using the fastx_trimmer (fastx_toolkit-0.0.13.2) to obtain clean reads. Clean reads were then mapped against the chicken reference genome *Gallus gallus* (v6.0) that was available in Ensembl v98 using HiSAT2 (v2.2.1) with default parameters. Raw counts of the genes were obtained using the htseq-count package (v0.12.3) in Python (v3.5). Raw counts were normalized using the DESeq2 package (v1.28.1) in R (v4.0.2) to obtain the gene expression level. The overall similarity between the samples was assessed using principal component analysis (PCA) in R (v4.0.2).

2.5 Identification of differentially expressed genes

The differentially expressed genes (DEGs) were identified using the DESeq2 package (v1.28.1) in R (v4.0.2). Genes with an adjusted P-value ≤ 0.05 and fold change ≥ 2 were assigned as differentially expressed. Hierarchical clustering and heatmaps of the DEGs were performed using the Pheatmap package (v1.0.12) in R (v4.0.2).

2.6 KEGG pathway and GO enrichment analysis

KEGG pathway (Kyoto Encyclopedia of Genes and Genomes; http://www.genome.jp/kegg/) and GO (Gene Ontology; http://geneontology.org) enrichment analysis of the DEGs were carried out using the clusterProfiler package (v3.16.1) in R (v4.0.2), with adjusted $P < 0.05$ as the screening standard.

2.7 Gene expression analysis by qRT-PCR

Of the total RNA, 1 μg was reverse transcribed into cDNA using the Prime Script RT reagent Kit (Takara Bio, Dalian, China). The mRNA expression of six candidate genes, was randomly selected from the DEGs, and analyzed to verify the RNA-sequencing results. β-actin was chosen as an internal control for the normalization of expression levels. The primers used in the qRT-PCR were designed with Primer 5 (Table 1).

Table 1
Primers used for qRT-PCR

Gene Symbol	Gene Name	Primer Sequence (5'-3')	Accession Number
OVA	ovalbumin	F: CACAAGCAATGCCTTTCAGA	NM_205152.2
		R: GACTTCATCAGGCAACAGCA	
OVALX	ovalbumin-related protein X	F: AAGATCCTGGAGCTCCCATT	NM_001276386.1
		R: CTCCATGGTATTGGGATTGG	
OVALY	ovalbumin-related protein Y	F: GCAAACCTGTGCAAATGATG	NM_001031001.1
		R: GTCTTCTCAATCCGCTCCAG	
AMN	amnion associated transmembrane protein	F: GCTCTGGGTTCACAGCTTTC	NM_001277516.1
		R: TGGAAGATGACGTGGTCGTA	
POMC	proopiomelanocortin	F: AAGGCGAGGAGGAAAAGAAG	XM_015285103.2
		R: CTTTTGACGATGGCGTTTTT	
CGA	glycoprotein hormones	F: AGGGTTGTCCAGAGTGCAAG	NM_001278021.1
		R: TCTTGGTGAAAGCCTTTGCT	
β-actin	beta-actin	F: GAGAAATTGTGCGTGACATGA	NM_205518.1
		R: CCTGAACCTCTCATTGCCA	

Gene expression was analyzed using ABI7900 (ABI7900 Applied Biosystems, USA), and the AceQ qPCR SYBR Green Master Mix (Vazyme Biotech Co., Ltd, China). The PCR protocol was initiated at 95 °C for 10 min, followed by 40 cycles of the amplification program: denaturation (95 °C, 15 s) and annealing/extension (60 °C, 60 s). At the end of the last amplification cycle, melt curves were generated to confirm the specificity of the amplification reaction. Each assay was carried out in triplicate and included a negative control. Relative quantification of the gene expression was performed using the $2^{-\Delta\Delta Ct}$ method.

2.8 Statistical analysis

Statistical analyses were performed using R software (v4.0.2, R Development Core Team 2019). Data were analyzed by t-test after testing for the homogeneity of variance with Levene's test. All data are presented as the mean ± SD, and a $p < 0.05$ was considered statistically significant.

3. Results

3.1 Body weight, egg production, and egg quality

Body weight, egg production, and egg quality are shown in Table 2. The laying rates were significantly higher in the HP than LP (93.67 ± 7.09 vs 68.00 ± 5.56, $p < 0.01$). However, egg weight, shell thickness and strength were greater ($p < 0.05$) in the LP than HP. In addition, final body weight was higher ($p < 0.05$) in the LP group than the HP.

Table 2
Body weight, egg production and egg quality of low- and high-yielding Changshun blue-shell laying hen

	Treatment		Sig
	LP	HP	
Initial body weight (g)	1.46 ± 0.08	1.27 ± 0.13	NS
Final body weight (g)	1.54 ± 0.07	1.26 ± 0.15	*
Laying rate (%)	68.00 ± 5.56	93.67 ± 7.09	**
Egg weight (g)	46.91 ± 0.45	45.04 ± 1.02	*
Eggshell thickness (mm)	0.30 ± 0.02	0.29 ± 0.06	*
Shell strength (N/cm^2)	39.22 ± 0.30	37.97 ± 0.69	*
*, P < 0.05; **, P < 0.01; NS, no significant difference.			
LP, low egg production group; HP, high egg production group.			

3.2 RNA sequencing quality assessment

The quality metrics of the transcriptomes are shown in Table 3. A total of 8 cDNA libraries were constructed from the ovaries of the Changshun green-shell laying hens. The raw reads and clean reads of each library were more than 42 and 41 million, respectively, except HP-2, which had approximately 39.2 million raw reads and 39.0 million clean reads. The GC content of all samples ranged from 49.11–52.24%, the base percentage of the Q20 was above 97.79%, and the percentage of the Q30 base was above 93.55%. In summary, the sequencing data could be used for subsequent data analysis.

Table 3
Quality metrics of transcripts in the ovary of Changshun blue-shell laying hen

Samp	Raw reads	Clean reads	Clean bases	Q20 (%)	Q30 (%)	GC (%)	N (ppm)
LP-1	45,772,004	45,672,822	6,793,131,642	97.90	93.84	49.52	4.74
LP-2	45,989,900	45,890,650	6,822,515,847	97.82	93.66	49.61	4.69
LP-3	45,847,818	45,755,138	6,808,443,826	98.15	94.48	49.82	4.77
LP-4	43,101,534	43,023,412	6,400,232,494	98.07	94.20	50.19	5.75
HP-1	47,619,274	47,511,052	7,060,329,437	98.19	94.65	52.24	4.68
HP-2	39,165,330	39,090,800	5,819,349,384	98.13	94.39	49.47	4.72
HP-3	42,022,532	41,943,412	6,246,359,132	97.79	93.55	49.11	5.73
HP-4	44,730,920	44,654,676	6,649,244,662	98.05	94.16	50.24	5.73
Samp, Sample name; Q20, sequencing error rates lower than 1%; Q30, sequencing error rates lower than 0.1%; GC, the percentage of G and C bases in all transcripts; N, unknown base. LP, low egg production group; HP, high egg production group.							

3.3 Transcriptome alignment

The results of the trimming and read mapping are shown in Table 4. The total mapped ratio between the reads and the reference genome of all the samples ranged from 90.30–92.37%. The uniquely mapped ratio ranged from 86.59–88.89%. The results indicated that the transcriptome data were reliable and suitable for subsequent analysis.

Table 4
Summary of trimming and read mapping results

Samp	Total reads	Total mapped	Multiple mapped	Uniquely mapped
LP-1	45,672,822	41,835,034 (91.60%)	1,582,978 (3.47%)	40,252,056 (88.13%)
LP-2	45,890,650	41,787,491 (91.06%)	1,616,242 (3.52%)	40,171,249 (87.54%)
LP-3	45,755,138	41,901,902 (91.58%)	1,585,544 (3.47%)	40,316,358 (88.11%)
LP-4	43,023,412	39,740,455 (92.37%)	1,496,311 (3.48%)	38,244,144 (88.89%)
HP-1	47,511,052	42,941,478 (90.38%)	1,803,134 (3.80%)	41,138,344 (86.59%)
HP-2	39,090,800	35,970,409 (92.02%)	1,342,878 (3.44%)	34,627,531 (88.58%)
HP-3	41,943,412	38,492,966 (91.77%)	1,432,361 (3.41%)	37,060,605 (88.36%)
HP-4	44,654,676	41,115,072 (92.07%)	1,619,095 (3.63%)	39,495,977 (88.45%)

Samp, Sample name; LP, low egg production group; HP, high egg production group.

3.4 Differentially expressed genes

Samples were first analyzed using PCA. In general, the samples from the different groups were divided into 2 parts in the PCA score plots (Fig. 1), indicating an obvious difference between the LP and HP groups. The DEGs were then identified using the DESeq2 package (v1.28.1) with an adjusted P-value ≤ 0.05 and fold change ≥ 2 as the screening criteria. A total of 142 DEGs were identified, including 55 upregulated genes and 87 downregulated genes in the HP group (Fig. 2). The DEGs were subsequently analyzed by hierarchical clustering analysis. Samples from the same group were clustered together, and the heatmap visually reflected the differences in the gene expression patterns between the LP and HP groups (Fig. 3).

3.5 KEGG pathway and GO enrichment analysis

To further elucidate the biochemical functions of the DEGs, we performed KEGG pathway enrichment analysis and GO enrichment analysis. It was found that 53 of the 142 DEGs were annotated by the OrgDb annotation data; consequently, these 53 DEGs were used for enrichment analysis. A total of 9 KEGG pathways were significantly enriched ($p < 0.05$, Fig. 4): neuroactive ligand-receptor interaction, complement and coagulation cascades, *Staphylococcus aureus* infection, ovarian steroidogenesis, prolactin signaling pathway, PI3K – Akt signaling pathway, cAMP signaling pathway, GnRH signaling pathway, and inflammatory mediator regulation of TRP channels. The descriptions of these KEGG pathways are given in Table 5. A total of 220 GO terms were significantly enriched (FDR < 0.05), and most of them belonged to biological processes (BP), followed by molecular functions (MF), and cellular components (CC). The top 25 significantly enriched GO terms for BP as well as all the significantly enriched GO terms for MF and CC are shown in Fig. 5. The descriptions of these GO terms are given in Tables 6, 7, and 8. The top 25 significantly enriched BP GO terms were mainly related to the regulation of peptidase activity and endocrine process, regulation of secretion, and lipid export from cell. The significantly enriched CC GO terms were collagen-containing extracellular matrix, secretory granule lumen, cytoplasmic vesicle lumen, vesicle lumen, collagen trimer, platelet alpha granule, and specific granule. The significantly enriched MF GO terms were mainly involved in peptidase regulator and inhibitor activity, receptor ligand activity, transmembrane receptor protein kinase activity, and growth factor binding.

Table 5
The significantly enriched KEGG pathway

Description	Rich Factor	P value	Q value	Gene
Neuroactive ligand-receptor interaction	0.0235	2.72E-06	1.49E-04	PRLR/FSHB/C3AR1/F2RL1/ CGA/POMC/GALR1/CCK
Complement and coagulation cascades	0.0353	1.74E-03	4.49E-02	FGG/C3AR1/C1QA
Staphylococcus aureus infection	0.0313	2.46E-03	4.49E-02	FGG/C3AR1/C1QA
Ovarian steroidogenesis	0.0392	9.18E-03	1.26E-01	FSHB/CGA
Prolactin signaling pathway	0.0286	1.68E-02	1.54E-01	PRLR/CGA
PI3K-Akt signaling pathway	0.0113	1.69E-02	1.54E-01	PRLR/MYB/NTRK1/HGF
cAMP signaling pathway	0.0139	2.28E-02	1.79E-01	FSHB/CGA/POMC
GnRH signaling pathway	0.0215	2.87E-02	1.96E-01	FSHB/CGA
Inflammatory mediator regulation of TRP channels	0.0200	3.28E-02	1.99E-01	F2RL1/NTRK1

Table 6
The top 25 significantly enriched biological process GO terms

ID	Description	Number of genes	p value	FDR	Gene
GO:0010951	negative regulation of endopeptidase activity	7	3.77E-06	1.96E-03	SPINK7/ITIH5/RARRES1/SERPINB1/SPINK5/CRIM1/HGF
GO:0010466	negative regulation of peptidase activity	7	5.14E-06	1.96E-03	SPINK7/ITIH5/RARRES1/SERPINB1/SPINK5/CRIM1/HGF
GO:0044060	regulation of endocrine process	4	5.26E-06	1.96E-03	F2RL1/POMC/GALR1/GJA5
GO:0052548	regulation of endopeptidase activity	8	1.34E-05	3.75E-03	SPINK7/ITIH5/RARRES1/SERPINB1/CCK/SPINK5/CRIM1/HGF
GO:0052547	regulation of peptidase activity	8	2.09E-05	4.07E-03	SPINK7/ITIH5/RARRES1/SERPINB1/CCK/SPINK5/CRIM1/HGF
GO:0048485	sympathetic nervous system development	3	2.19E-05	4.07E-03	PLXNA4/NRP1/NTRK1
GO:0032098	regulation of appetite	3	2.90E-05	4.63E-03	POMC/CCK/CARTPT
GO:0045861	negative regulation of proteolysis	7	3.86E-05	4.96E-03	SPINK7/ITIH5/RARRES1/SERPINB1/SPINK5/CRIM1/HGF
GO:0018108	peptidyl-tyrosine phosphorylation	7	4.22E-05	4.96E-03	PRLR/NRP1/IL15/BANK1/NTRK1/CCK/HGF
GO:0018212	peptidyl-tyrosine modification	7	4.44E-05	4.96E-03	PRLR/NRP1/IL15/BANK1/NTRK1/CCK/HGF
GO:0050886	endocrine process	4	6.92E-05	6.96E-03	F2RL1/POMC/GALR1/GJA5
GO:1902105	regulation of leukocyte differentiation	6	7.48E-05	6.96E-03	IL15/IHH/FSHB/MYB/SPINK5/CARTPT
GO:1903532	positive regulation of secretion by cell	7	8.15E-05	7.00E-03	ORM1/FGG/MYB/F2RL1/GALR1/CCK/CARTPT
GO:0048771	tissue remodeling	5	1.04E-04	8.14E-03	IL15/IHH/FSHB/CARTPT/GJA5
GO:0051047	positive regulation of secretion	7	1.18E-04	8.14E-03	ORM1/FGG/MYB/F2RL1/GALR1/CCK/CARTPT
GO:0001818	negative regulation of cytokine production	6	1.19E-04	8.14E-03	ORM1/BANK1/F2RL1/POMC/SERPINB1/HGF
GO:0030212	hyaluronan metabolic process	3	1.24E-04	8.14E-03	ITIH5/IL15/HGF
GO:0050731	positive regulation of peptidyl-tyrosine phosphorylation	5	1.44E-04	8.95E-03	NRP1/IL15/BANK1/CCK/HGF
GO:0140353	lipid export from cell	3	1.69E-04	9.92E-03	MYB/POMC/GALR1
GO:0051346	negative regulation of hydrolase activity	7	1.92E-04	1.07E-02	SPINK7/ITIH5/RARRES1/SERPINB1/SPINK5/CRIM1/HGF
GO:1903510	mucopolysaccharide metabolic process	4	2.11E-04	1.08E-02	EGFLAM/ITIH5/IL15/HGF
GO:0050708	regulation of protein secretion	7	2.16E-04	1.08E-02	ORM1/FGG/BANK1/F2RL1/SERPINB1/CARTPT/GJA5

ID	Description	Number of genes	p value	FDR	Gene
GO:0048483	autonomic nervous system development	3	2.23E-04	1.08E-02	PLXNA4/NRP1/NTRK1
GO:2000849	regulation of glucocorticoid secretion	2	3.00E-04	1.20E-02	POMC/GALR1
GO:0002791	regulation of peptide secretion	7	3.07E-04	1.20E-02	ORM1/FGG/BANK1/F2RL1/SERPINB1/CARTPT/GJA5

Table 7
The significantly enriched cellular components GO terms

ID	Description	Number of genes	p value	FDR	Gene
GO:0062023	collagen-containing extracellular matrix	7	6.94E-05	3.10E-03	EGFLAM/ORM1/FREM1/ITIH5/FGG/SERPINB1/C1QA
GO:0034774	secretory granule lumen	6	1.55E-04	3.10E-03	ORM1/FGG/LYZ/POMC/SERPINB1/HGF
GO:0060205	cytoplasmic vesicle lumen	6	1.65E-04	3.10E-03	ORM1/FGG/LYZ/POMC/SERPINB1/HGF
GO:0031983	vesicle lumen	6	1.71E-04	3.10E-03	ORM1/FGG/LYZ/POMC/SERPINB1/HGF
GO:0005581	collagen trimer	3	1.40E-03	1.45E-02	C1QTNF8/C1QA/C1QL2
GO:0031091	platelet alpha granule	3	1.59E-03	1.45E-02	ORM1/FGG/HGF
GO:0042581	specific granule	3	7.78E-03	5.67E-02	ORM1/LYZ/C3AR1

Table 8
The significantly enriched molecular functions GO terms

ID	Description	Number of genes	p value	FDR	Gene
GO:0004867	serine-type endopeptidase inhibitor activity	5	6.02E-06	2.67E-04	SPINK7/ITIH5/SERPINB1/SPINK5/CRIM1
GO:0004866	endopeptidase inhibitor activity	6	8.44E-06	2.67E-04	SPINK7/ITIH5/RARRES1/SERPINB1/SPINK5/CRIM1
GO:0030414	peptidase inhibitor activity	6	1.06E-05	2.67E-04	SPINK7/ITIH5/RARRES1/SERPINB1/SPINK5/CRIM1
GO:0061135	endopeptidase regulator activity	6	1.06E-05	2.67E-04	SPINK7/ITIH5/RARRES1/SERPINB1/SPINK5/CRIM1
GO:0005179	hormone activity	5	2.15E-05	4.34E-04	FSHB/CGA/POMC/CCK/CARTPT
GO:0061134	peptidase regulator activity	6	3.01E-05	5.07E-04	SPINK7/ITIH5/RARRES1/SERPINB1/SPINK5/CRIM1
GO:0048018	receptor ligand activity	7	3.40E-04	4.57E-03	IL15/FSHB/CGA/POMC/CCK/HGF/CARTPT
GO:0030546	signaling receptor activator activity	7	3.62E-04	4.57E-03	IL15/FSHB/CGA/POMC/CCK/HGF/CARTPT
GO:0017154	semaphorin receptor activity	2	4.87E-04	5.47E-03	PLXNA4/NRP1
GO:0004857	enzyme inhibitor activity	6	5.64E-04	5.70E-03	SPINK7/ITIH5/RARRES1/SERPINB1/SPINK5/CRIM1
GO:0004714	transmembrane receptor protein tyrosine kinase activity	3	6.73E-04	6.18E-03	NRP1/NTRK1/CRIM1
GO:0019199	transmembrane receptor protein kinase activity	3	1.36E-03	1.15E-02	NRP1/NTRK1/CRIM1
GO:0005184	neuropeptide hormone activity	2	2.71E-03	2.11E-02	CCK/CARTPT
GO:0004713	protein tyrosine kinase activity	3	6.07E-03	4.34E-02	NRP1/NTRK1/CRIM1
GO:0019838	growth factor binding	3	6.45E-03	4.34E-02	NRP1/NTRK1/CRIM1

3.6 qRT-PCR validation of RNA-Seq results

To validate the RNA-seq results, 6 DEGs were selected for qRT-PCR analysis. These included 3 up-regulated genes (AMN, POMC, and CGA) and 3 downregulated genes (OVA, OVALX, and OVALY). The results showed that the expression trends determined by the qRT-PCR were consistent with the RNA-Seq results (Fig. 6), indicating a high reliability of the RNA-seq results.

4. Discussion

To determine the differences in the ovary transcriptomes of the high - and low-yielding layers, HP and LP groups were assessed. Their laying rates (%) were found to be 93.67 ± 7.09 and 68.00 ± 5.56, respectively, indicating that the animal model was appropriate. We noticed that HP had a lower body weight than LP. It is well known that egg production is positively correlated with energy supply [7, 8]; thus, it is possible that layers of the HP group could utilize more energy for egg production, instead of body weight maintenance. In addition, previous studies have shown that egg production is negatively correlated with egg weight [9, 10], the eggshell thickness is positively correlated with strength [11], and similar results were observed in our study.

Egg production traits are determined by ovarian function and are regulated by the hypothalamic-pituitary-gonadal (HPG) axis [12]. Thus, ovary tissue was selected to carried out the RNA-seq analysis. A total of 142 DEGs were identified in the ovaries of the high and low yielding Changshun green-shell laying hens. The KEGG pathway enrichment analysis identified 9 significantly enriched pathways. Among them, the neuroactive ligand-receptor interaction pathway was the most enriched. This pathway is comprised of multiple receptors that are associated with cell signaling [13, 14]. A previous study in fish found that the neuroactive ligand-receptor interaction pathway could affect steroid hormone synthesis in gonads through the HPG axis [15]. In the present study, the ovarian steroidogenesis pathway was identified as 1 of the 9 significantly enriched pathways, suggesting that the neuroactive ligand-receptor interaction pathway might affect egg production in chickens via a mechanism similar to that found in fish. Similar results were also previously reported for Jinghai yellow chickens [12]. In addition, Tao et al. [16] found that the neuroactive ligand-receptor interaction pathway was also involved in duck egg production. There were 8 DEGs mapped to the neuroactive ligand-receptor interaction pathway (PRLR, FSHB, C3AR1, F2RL1, CGA, POMC, GALR1, and CCK) that may play an important role in the regulation of ovarian function and egg production. The other 7 significantly enriched pathways included the prolactin signaling pathway and GnRH signaling pathway that have already been shown to affect avian egg production performance [17, 18]. The cAMP signaling and PI3K − Akt signaling pathways are reported to be involved in oocyte maturation and ovulation in mammals [19]. Meanwhile, complement and coagulation cascades and inflammatory mediator regulation of the TRP channels that are associated with inflammatory responses [20], have also been shown to be the required processes for ovulation in mammals [21, 22]. Thus, it is likely that these pathways may play similar roles in chicken ovaries, but further research is required to confirm this.

The GO enrichment analysis showed that most of the DEGs were involved in biological processes, and the following were the most enriched: negative regulation of endopeptidase activity, negative regulation of peptidase activity, regulation of endopeptidase activity, and regulation of peptidase activity. Peptidase is the term for any protein capable of catalyzing the hydrolysis of a protein substrate [23]. Enopeptidase is a peptidase that cleaves the interior region of the polypeptide chain. Thus, these results suggest that endopeptidase, but not amino or carboxypeptidase, played a key role in the regulation of chicken ovary functions. The GO terms for the following biological processes were also significantly enriched: peptidyl-tyrosine modification, peptidyl-tyrosine phosphorylation, and positive regulation of peptidyl-tyrosine phosphorylation. The DEGs mapped to these GO terms include PRLR, NRP1, IL15, BANK1, NTRK1, CCK, and HGF. The molecular function of these DEGs was transmembrane receptor protein tyrosine kinase activity. Protein tyrosine phosphorylation is the modification of post-translational proteins, and plays a central role in many signaling pathways leading to cell growth and differentiation in animals [24]. Protein tyrosine phosphorylation is performed by a group of enzymes called protein tyrosine kinases, while receptor protein tyrosine kinases are a subclass of tyrosine kinases [25]. In mouse ovaries, Hess et al. [26] showed that the receptor protein tyrosine kinase Ron was related to the regulation of ovary size and ovulation rate. Meanwhile, many studies have shown that the dysregulation of receptor protein tyrosine kinases is common in ovarian cancer [27–29], implying the importance of receptor protein tyrosine kinases in the maintenance of ovarian function. Thus, the molecular function of transmembrane receptor protein tyrosine kinase activity as well as the DEGs involved in these GO terms, should be further studied in chicken ovary tissues.

In conclusion, the present study characterized and evaluated the ovary transcriptome in low- and high-yielding Changshun green-shell laying hens. A total of 142 differentially expressed genes were identified in the present study, which could be candidate genes for the genetic improvement of egg production. Moreover, enrichment analysis suggested that the neuroactive ligand-receptor interaction pathway and receptor protein tyrosine kinases may play crucial roles in the regulation of ovarian function and egg production.

Abbreviations

DEGs: differentially expressed genes; HP: high egg production group; LP: low egg production group; PCA: principal component analysis; KEGG: Kyoto Encyclopedia of Genes and Genomes; GO: Gene Ontology; PI3K–Akt: Phosphatidylinositol-3-kinase (PI3K) − protein kinase B (Akt); cAMP: Cyclic adenosine monophosphate; GnRH: Gonadotropin-releasing hormone; TRP: Transient receptor potential; FDR: False discovery rate; BP: Biological processes; MF: Molecular functions; CC: Cellular components; AMN: amnion associated transmembrane protein; POMC: Proopiomelanocortin; CGA: glycoprotein hormones; OVA: Ovalbumin; OVALX: ovalbumin-related protein X; OVALY: Ovalbumin-related protein Y; HPG: Hypothalamic-pituitary-gonadal; PRLR: Prolactin receptor; FSHB: Follicle-stimulating hormone subunit beta; C3AR1: complement C3a receptor 1; F2RL1: F2R like trypsin receptor 1; GALR1: Galanin receptor 1; CCK: Cholecystokinin; NRP1: Neuropilin 1; IL15: interleukin-15; BANK1: B-cell scaffold protein with ankyrin repeats 1; NTRK1: Neurotrophic tropomyosin kinase receptor 1; HGF: Hepatocyte growth factor.

Declarations

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Authors' contributions

RM, ZC and WX designed this study, RM and YY conducted animal experiments. WX performed the transcriptome downstream analysis. RM conducted sample analysis and wrote the manuscript. TG, DW and FW assisted with data analysis. All authors approved this manuscript.

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Availability of data and materials

The RNA-Seq datasets are available in the Sequence Read Archive of National Center for Biotechnology Information (https://submit.ncbi.nlm.nih.gov/subs/bioproject/; accession number: PRJNA685318).

Ethics approval and consent to participate

The animal protocol was approved by the Animal Ethics Committee of the Qiannan Normal University for Nationalities.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

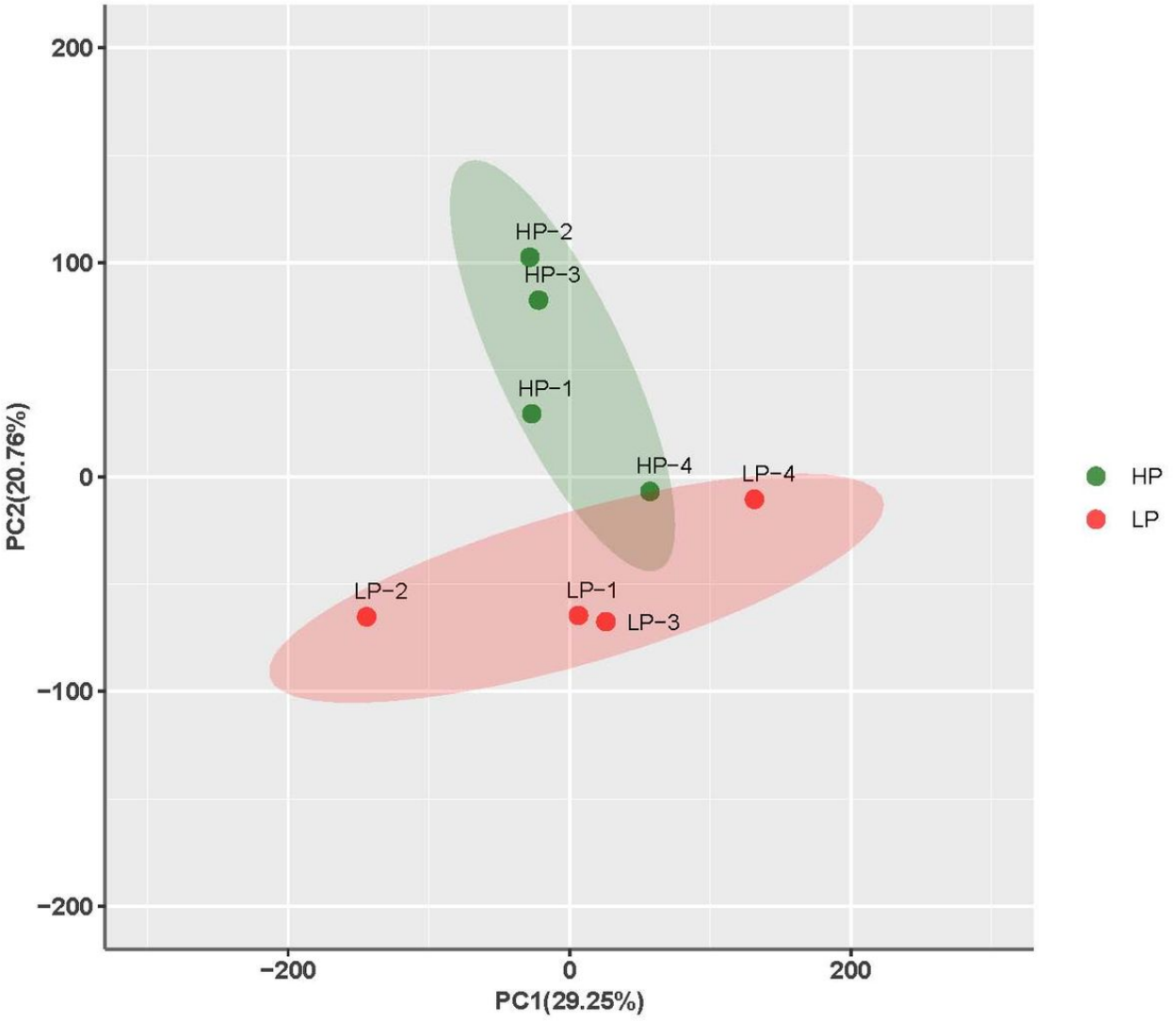

Figure 1

PCA score plot of ovary transcriptomes. HP, high egg production group; LP, low egg production group. Green point, samples from HP; Red point, samples from LP.

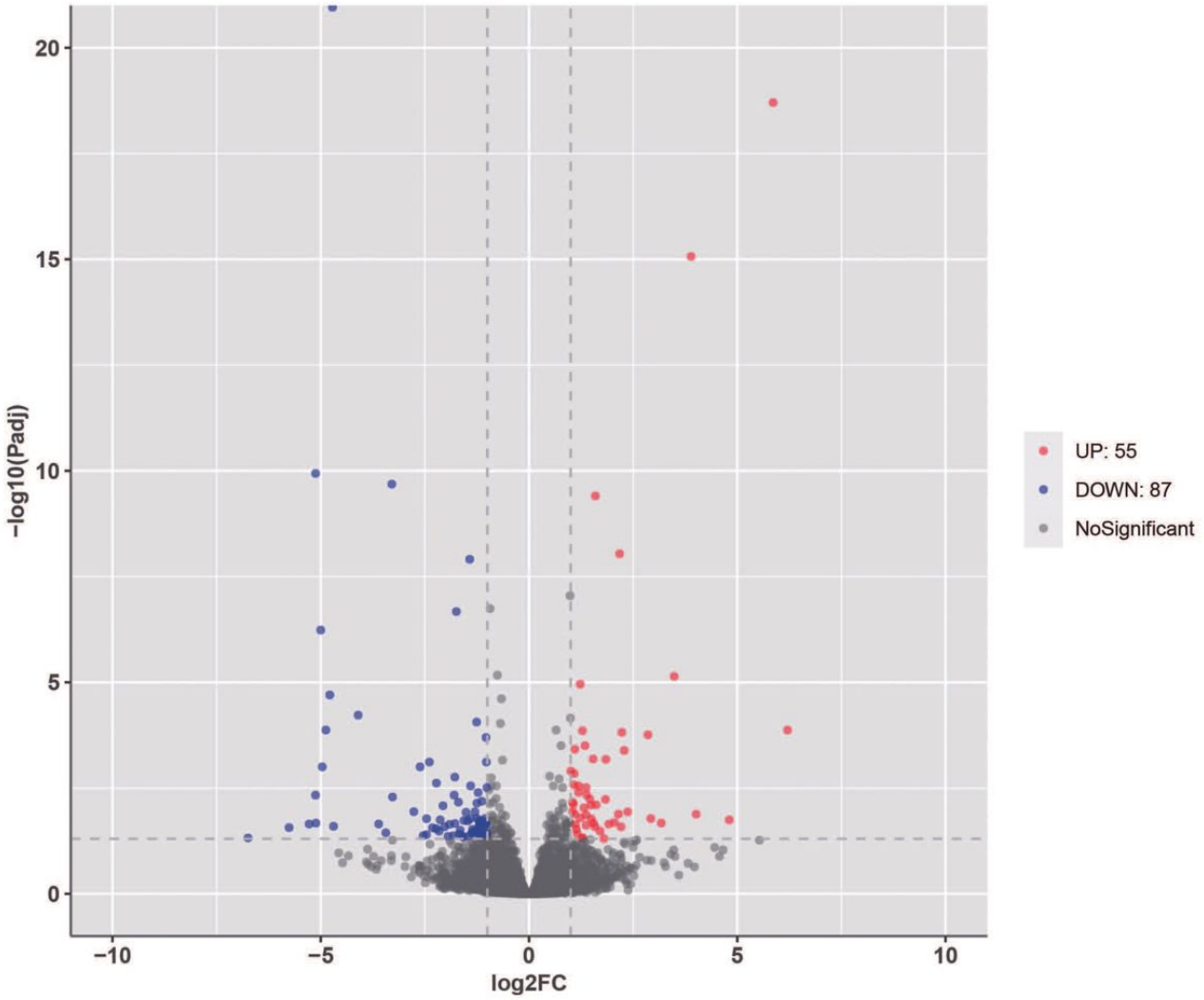

Figure 2

Volcano plot of all expressed genes. The red plots represent significantly upregulated genes; the blue plots represent significantly down−regulated genes; the gray plot represents genes with no significance.

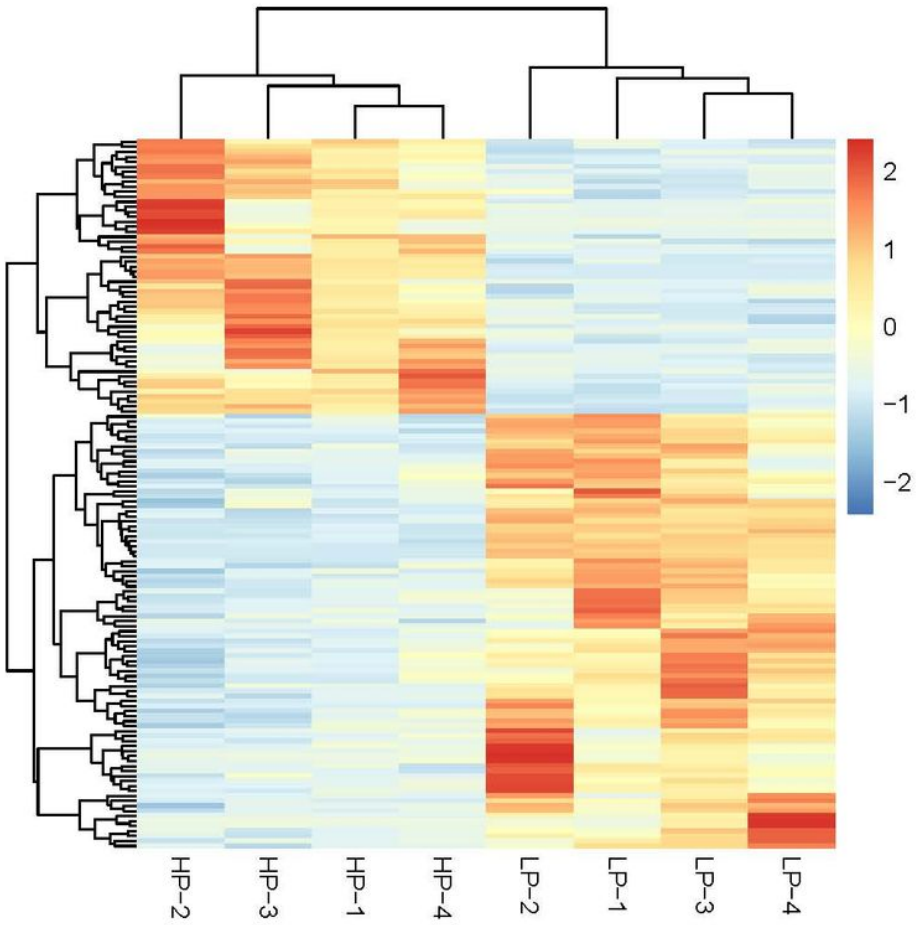

Figure 3

Hierarchical clustering analysis of DEGs. HP, high egg production group; LP, low egg production group.

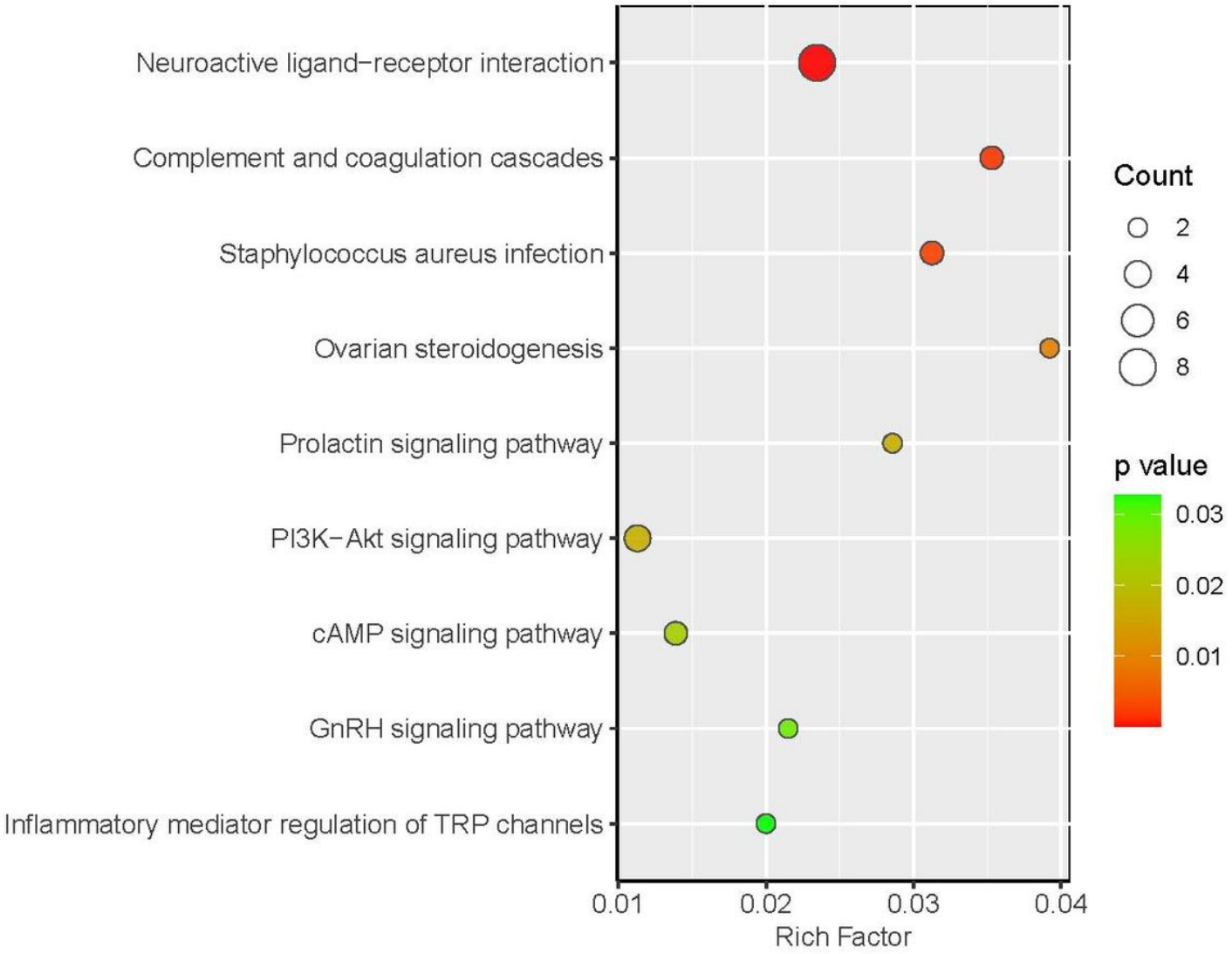

Figure 4

KEGG pathway enrichment analysis of DEGs. Count, number of DEGs enriched in the pathway.

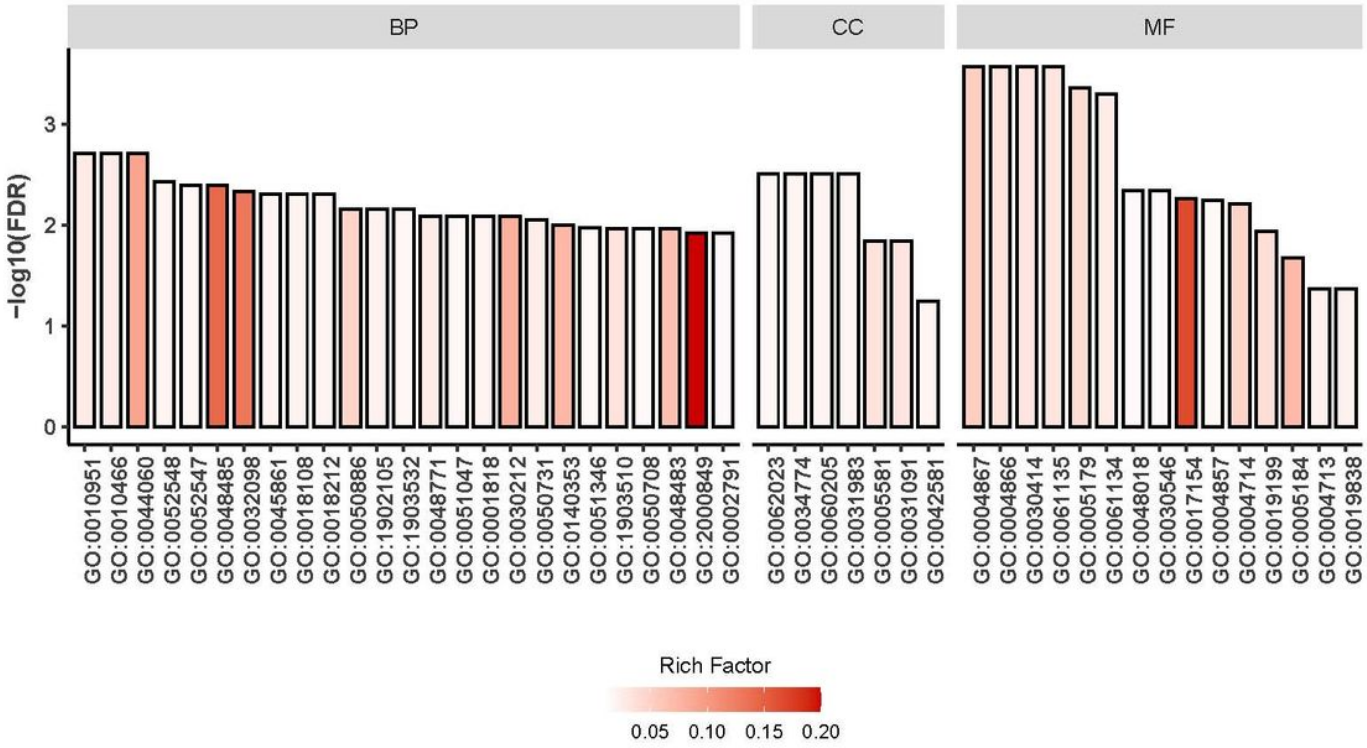

Figure 5

GO enrichment analysis of DEGs. BP, biological processes; CC, cellular components; MF, molecular function.

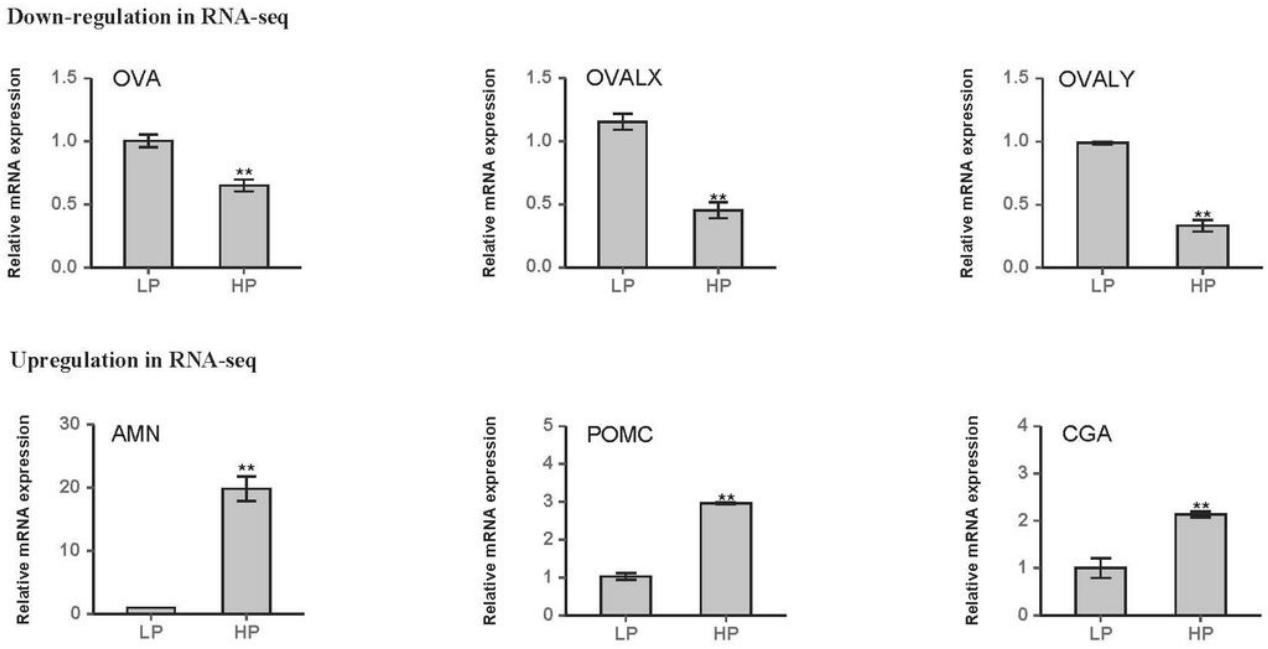

Figure 6

qRT-PCR validation of differentially expressed genes identified in transcriptome sequencing. The relative expression levels of genes were calculated according to the 2-ΔΔCt method using β-actin as an internal reference RNA. **, P <0.01. LP, low rate of egg production group; HP, high rate of egg production group.