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RNA-sequencing Reveals Differentially Expressed Genes of Laying Hens Fed Baihu Decoction Under Heat Shock

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Egg production, an important economic trait in the poultry industry, is sensitive to heat stress. The hypothalamus is a crucial center for thermoregulation by detecting temperature changes and regulating the autonomic nervous system in poultry. Baihu decoction (BH), which contains four ingredients (*Rhizoma Anemarrhenae*, *Gypsum Fibrosum*, *Radix Glycyrrhizae*, and *Semen Oryzae Nonglutinosae*), is a traditional Chinese medicinal formula for clearing heat. Our study aimed to investigate the changes in gene transcription levels in the hypothalamus of laying hens treated with heat stress with and without BH using RNA sequencing. A total of 223 differentially expressed genes (DEGs) were identified in the heat-treated group compared with the control group and 613 DEGs were identified in the BH group compared with the heat-treated group. Heat shock led to significant changes in the expression of multiple genes involved in the "neuroactive ligand-receptor interaction" pathway. Moreover, feeding BH led to significant upregulation in the expression of eight genes encoding heat shock proteins (HSPs), which were highlighted as candidates to control the "protein processing in the endoplasmic reticulum (ER)" pathway. These results provide the novel insight that BH responds to heat stress by participating in regulation of the ER signaling pathway and HSPs expression.

Key words: Baihu decoction, chicken, heat stress, hypothalamus, RNA-seq

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Introduction

Egg production in laying hens plays an essential role in the global agricultural economy (Vandana *et al*., 2021). However, heat shock adversely affects laying hen performance by influencing hen mortality, egg production and features, yolk pigmentation, shell solidity, nutritional rate, and expiry dates (Mashaly *et al*., 2004; Lara and Rostagno, 2013).

Baihu decoction (BH) is a traditional Chinese medicine documented in the book "Shang Han Lun." One of the main functions of BH is to alleviate high fever (Zhang 1993). Studies have shown that BH exerts antipyretic effects in rabbits and mice (Yang and Xu 2015; Wu *et al*., 2018). However, few studies have explained this phenomenon at the genetic level in laying hens, particularly focusing on the hypothalamus. The hypothalamus is a crucial site in the brain that regulates body temperature and controls tolerance to high temperatures (Yossifoff *et al*., 2008). The hypothalamus helps to dissipate heat through the hypothalamic-pituitary-adrenal axis, and regulates the sympathetic nervous system and other neuroendocrine processes (Miller and O'Callaghan, 2002).

With global warming, hyperthermia has become an important environmental factor affecting the laying hen industry. This ecological damage impedes the ability of proteins to fold correctly, thus affecting the functions of transmembrane proteins in the endoplasmic reticulum (ER). ER stress is characterized by the accumulation of unfolded proteins, resulting in the formation of large protein aggregates and significant disruption of normal cellular functions (Oakes and Papa, 2015). The accumulation of excessive misfolded proteins leads to an unfolded protein response (UPR), which triggers cell death (Korennykh and Walter, 2012). To maintain the normal physiological activities of cells, protein folding ability must be quickly restored. Heat shock proteins (HSPs) play major roles in protein three-dimensional structuring and remodeling, as they serve as molecular chaperones.

There are seven main HSP families that are categorized according to their size: HSP110, HSP100, HSP90, HSP70, HSP60, HSP40, and small HSPs (Rappa *et al*., 2012). Among the HSPs,

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HSP70 and HSP90 are remarkably conserved ATP-dependent families (Mayer and Kityk, 2015; Schopf *et al*., 2017) that perform their molecular chaperone activities either alone or synergistically. HSP70 is the most common chaperone with many homologous proteins in multiple cell cavities. HSP70 performs various functions under the influence of different co-factors, such as protein structuring, transmembrane transport, and degradation of misfolded proteins (Mayer, 2013). HSP70 has two domains: an ATPase domain and a substrate-binding domain. The activity of HSP70 is driven by dynamic interactions between the two domains and co-chaperones such as HSP40 (Kampinga and Craig, 2010; Mayer, 2010; Zuiderweg *et al*., 2013). HSP40 is the primary substrate recruiter of HSP70 and stimulates its ATPase activity. In typical protein structuring and restructuring pathways, HSP40 is a lengthened V-shaped dimer comprising a distinctive helical J-part that triggers the ATPase activity of HSP70 (Jiang *et al*., 2007; Ahmad *et al*., 2011). HSP90, another common molecular chaperone with a more specific function, represents a greatly changeable protein involved in regulating multiple pathways by interacting with a variety of different interactors (Didenko *et al*., 2012; Johnson, 2012; Li *et al*., 2012). HSP90 prevents protein aggregation by binding to unnatural polypeptides, thereby maintaining the normal functions of proteins and cells (Saibil, 2013). HSP90 cooperates with HSP70 to participate in protein recombination (Genest *et al*., 2019).

Hy-Line Brown is considered the most balanced brown egg layer because of its high production performance, feed efficiency, good internal egg quality, and excellent survivability. The purpose of our study was to identify specific differentially expressed genes (DEGs) in the hypothalamus of Hy-Line Brown laying eggs that respond rapidly to feeding BH under 3 h of heat shock, and to further investigate the signaling pathways that these DEGs participate in to elucidate the underlying mechanism.

Materials and methods

Animal treatments

Laying hens at 51 weeks old exhibited vigorous vitality and good production performance. Therefore, laying hens of this age were selected as the research subjects. Healthy Hy-Line Brown hens were obtained from a Farm in Xuchang, China. The breeding experiments were conducted in August. Forty-five hens with good health status and similar body weight and production performance were selected. Laying hens were raised in a three-layer ladder-type cage with four laying hens raised in each layer. The hens were fed twice daily with a normal supply of water and food. The first step in the feeding scheme was to pre-feed the hens for 7 days, marked as the observation adaptation period. The 45 hens were then randomly divided into three groups (15 hens per group): hens reared at 25°C (Control group), nurtured at 25°C for 7 days and then kept at a high temperature of 36°C for 3 h (Heat group), fed BH for 7 days and then reared at a high temperature of 36°C for 3 h (BH group). Each kilogram of fodder in BH contains 21 g *Rhizoma Anemarrhenae*, 56 g *Gypsum Fibrosum*, 7 g *Radix Glycyrrhizae*, and 26 g *Semen Oryzae Non-glutinosae*. All the traditional Chinese medicinal materials were purchased from Bozhou Traditional Chinese Medicine Market in Anhui Province.

The health of the flocks was monitored daily and dead hens exposed to heat shock were simultaneously removed from the group. Finally, three laying hens with similar status were screened as triplicate samples in each group and slaughtered by stunning and exsanguination simultaneously as soon as possible after 3 h of heat shock to obtain the hypothalamic tissues. All experimental methods and management procedures were approved by the Experimental Animal Ethics Committee of Henan University of Animal Husbandry and Economy (Approval number: MJK202108B012).

RNA extraction and sequencing

The hypothalamus was extracted and stored at –80°C until RNA extraction. Total RNA was isolated from 500 mg of each sample using TRNzol Universal Reagent (TIANGEN, Beijing, China). RNA quantity and quality were analyzed spectrophotometrically on a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). RNA stability was tested using the Agilent 2100 system (Agilent Technologies, Foster City, CA, USA) and LabChip GX kits (PerkinElmer, Waltham, MA, USA). The mRNA of each sample was enriched using mRNA Capture Beads and used to construct cDNA libraries. The effective concentration of the library was measured by quantitative polymerase chain reaction (qPCR). To ensure product quality, an effective concentration > 2 nM was used as the standard. An Illumina NovaSeq 6000 platform (Illumina, San Diego, CA, USA) was used for RNA sequencing (RNA-seq) and cDNA library construction.

Data quality control

Raw sequencing data were saved in FASTQ format and filtered by removing adapter sequences and low-quality reads. The remaining clean data were aligned to the reference genome using the HISAT2 system (Kim *et al*., 2015). The mapped reads were assembled and the transcripts were quantified using the StringTie method (Pertea *et al*., 2015).

Correlation assessment of biological replicates and DEGs analysis

The Pearson correlation coefficient (r) was used to assess the reproducibility of the three biological replicates (Liu *et al*., 2018). Differential gene expression analysis was performed using DEseq2 (Love *et al*., 2014). The selection parameters were a fold change equal to or above 2 and a false discovery rate less than 0.05.

Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis

Functional enrichment analysis of the DEGs was performed and compared with the genome background. Significantly enriched GO terms in the Gene Ontology database (Ashburner *et al*., 2000) of DEGs were defined using a hypergeometric test. To further understand the gene functions, the KEGG pathway-related database was used. KOBAS software (version 3.0; http://kobas.cbi.pku.edu.cn) was used to evaluate the statistical enrichment of DEGs among the KEGG pathways.

Reverse transcription (RT)-qPCR confirmation of DEGs

To confirm the accuracy and reliability of the RNA-seq data, 12 genes involved in the "protein processing in the endoplasmic reticulum" pathway were chosen for RT-qPCR verification. The primers for these genes were designed using Primer Premier 5.0 software (www.premierbiosoft.com) and are listed in Table S1; *GAPDH* was used as an internal standard. The extracted RNA was reverse-transcribed to cDNA using HiScript II Reverse Transcriptase (Vazyme, Nanjing, China), and qPCR was performed using qPCR SYBR Green Master Mix (Vazyme, China) on a LightCycler480II instrument (Roche Diagnostic, Indianapolis, IN, USA). The following PCR parameters were used: 95°C for 30 s, followed by 40 cycles of 95°C for 10 s, 60°C for 30 s, and 95°C for 15 s, 60°C for 60 s, 95°C for 15s. Experiments were performed in triplicate.

Results

Transcriptome changes in the hypothalamus of laying hens

RNA-seq was performed to investigate changes in the hypothalamic transcriptome of laying hens exposed to heat shock. Total RNA was extracted from the hypothalamic tissue samples and subjected to transcriptome analysis, resulting in a total 57.19 Gb of clean data with an average of 5.94 Gb reads per sample. The proportion of Q30 bases exceeded 93.55%. The percentage of clean reads plotted on the reference genome ranged from 86.69% to 93.09% (Table S2).

Furthermore, the correlations between the transcriptional expression of each gene among the three groups were evaluated. The three replicates from each group clustered together, indicating that the experimental error was minimal (Fig 1).

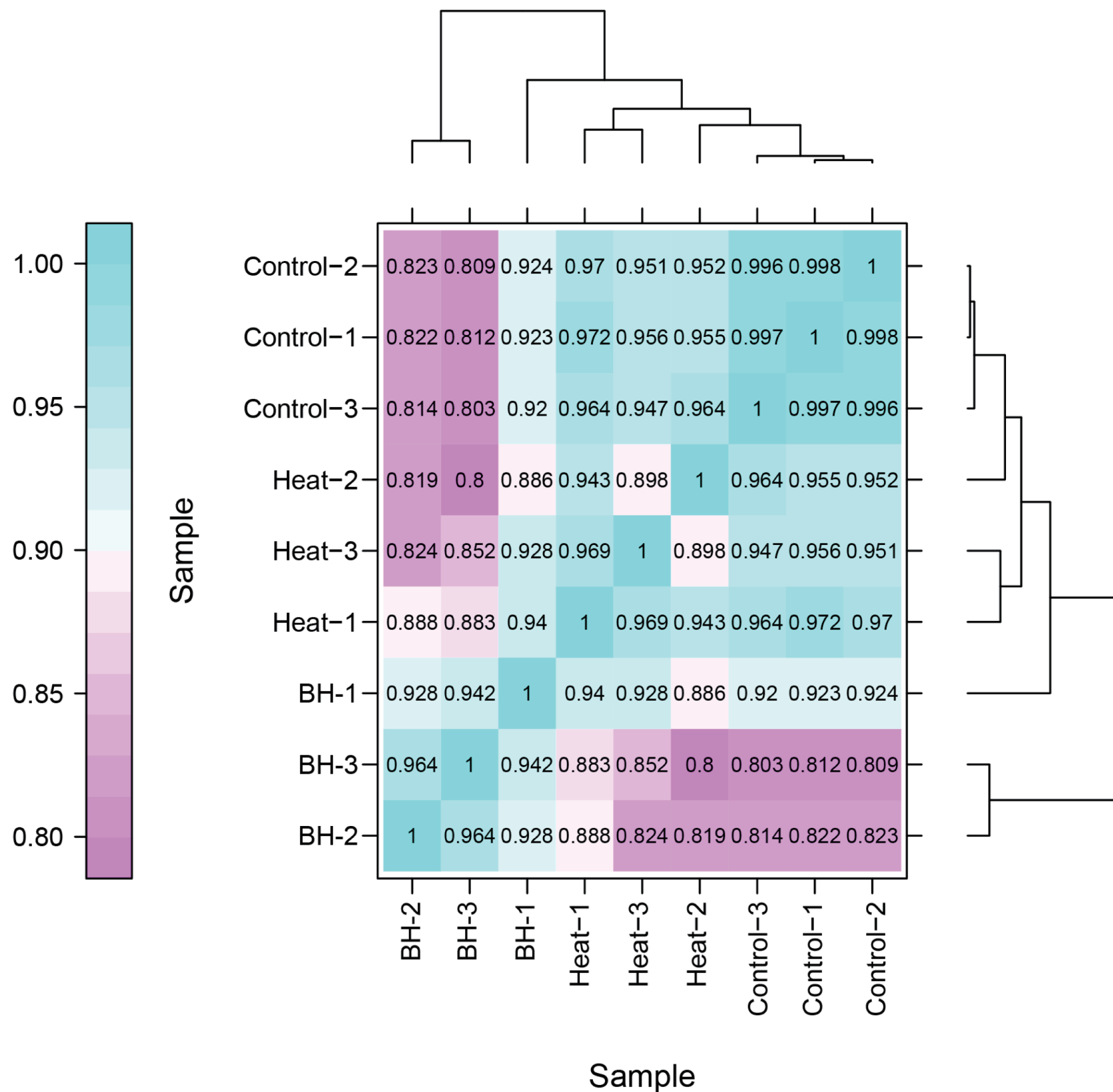

Fig. 1. **Heatmap of correlation in gene expression between hypothalamus samples.**
Statistical evaluations were performed using Pearson's correlation analysis; a coefficient closer to 1 indicates a stronger correlation between two replicates.

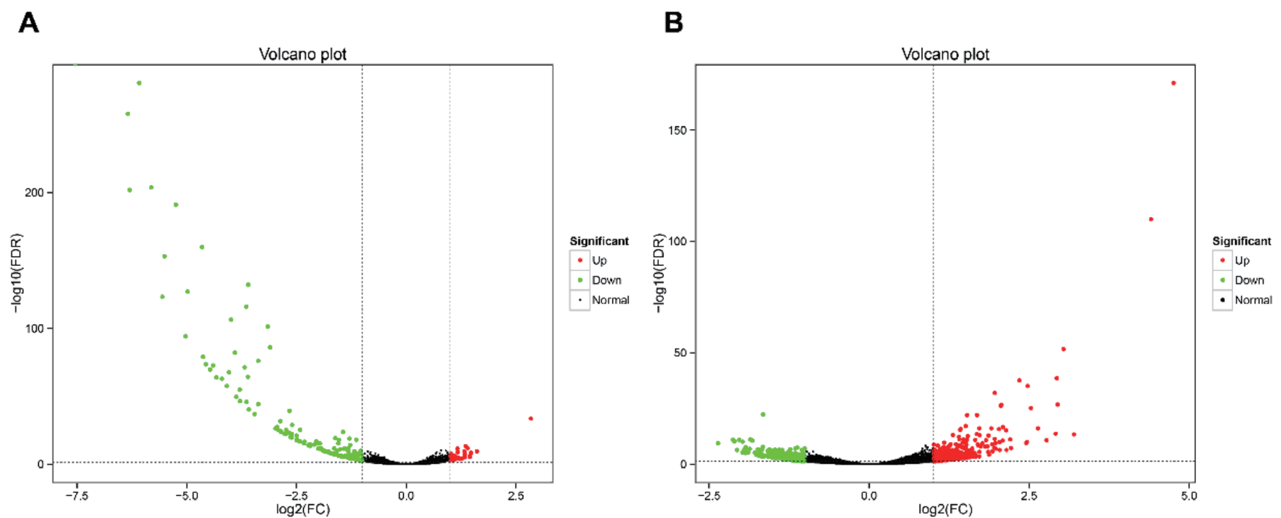

Fig. 2. **Volcano plot analysis of differential gene expression. (A) Heat vs Control, (B) Baihu decoction vs Heat.** The color code signifies the following: green dots in the figure represent downregulated differentially expressed genes, red dots represent upregulated differentially expressed genes, and black dots represent non-differentially expressed genes.

Identification of DEGs under heat shock and BH treatment

Analysis of transcriptional changes in chicken hypothalamus samples revealed that 39 genes were overexpressed and 184 genes were underexpressed in response to heat shock stress (Fig 2A). In the BH group, 335 genes were overexpressed, whereas 278 genes had lower expression levels compared to those in the Heat group (Fig 2B).

Heat-induced DEGs were involved in multiple signaling pathways

To identify the molecular and cellular pathways of DEGs affected by heat stress, the enriched KEGG pathways were analyzed. The DEGs comprised four major pathway categories: (1) metabolism, (2) cellular processes, (3) environmental information processing, and (4) organismal systems. Among these, the majority of DEGs were enriched in metabolic pathways. Of note, the pathway with the highest enrichment ratio of DEGs was the "neuroactive ligand-receptor interaction" pathway, which belongs to the category "environmental information processing" (Fig 3A). This indicates that high temperatures cause significant changes in the nervous system of the chicken brain.

KEGG maps were constructed to identify the DEGs involved in the "neuroactive ligand-receptor interaction" pathway (Fig 3B). Eleven neuroactive ligand genes were differentially expressed under heat shock stress, with nine of these down-regulated: *ADRA1B*, *KNG1*, *C3*, *C5AR2*, *SST*, *PLAU*, *F2*, *PLGL,* and *GHRHR*. In addition, the two upregulated genes (*ADCYAP1R1* and *GRM2)* were also involved in this pathway (Fig 3C). Detailed information regarding these genes is presented in Table S3.

GO and KEGG enrichment analysis of BH-induced DEGs

GO and KEGG enrichment analyses were performed to understand the functional enrichment of the DEGs induced by BH under heat stress. The primary biological processes and molecular functions of the DEGs detected by BH were analyzed using a GO enrichment network map. The top five GO terms under biological process were "erythrocyte differentiation" (8 DEGs), "regulation of B cell differentiation" (4 DEGs), "T-cell-mediated cytotoxicity" (3 DEGs), "regulation of cytokine secretion" (5 DEGs), and "negative regulation of inclusion bodies assembly" (3 DEGs) (Fig 4A). The top five GO terms in the molecular function category were in the subcategories "unfolded protein binding" (10 DEGs), "R-SMAD binding" (5 DEGs), "G-protein-coupled purinergic nucleotide receptor activity" (4 DEGs), "protein heterodimerization activity" (18 DEGs), and "inositol-1,4,5-trisphosphate 3-kinase action" (2 DEGs) (Fig 4B).

KEGG pathway enrichment analysis was used to predict the significantly enriched pathways associated with the identified DEGs (Fig 5). The KEGG annotation results categorized the detected DEGs into five classes: "protein processing in endoplasmic reticulum" in genetic information processing, "endocytosis" in cellular processes, "neuroactive ligand-receptor interaction" in environmental information processing, "glycerophospholipid metabolism" in metabolism, and "adrenergic signaling in cardiomyocytes" in organismal systems (Fig 5).

BH induced DEGs involved in protein processing in the ER

We further focused on the DEGs associated with the KEGG category "protein processing in the endoplasmic reticulum." The results showed that all 12 DEGs were upregulated under BH treatment, including eight HSP family members: *HSPA5*, *HSPA8*, *HSPA4L*, *HSPH1*, *HSP90AA1*, *Bip*, *HSP90B1*, and *DNAJB1*. Among these HSP genes, five belong to the HSP70 family (*HSPA5*, *HSPA8*, *HSPA4L*, *HSPH1*, *Bip*), two belong to the HSP90 family (*HSP90AA1* and *HSP90B1*), and one belongs to

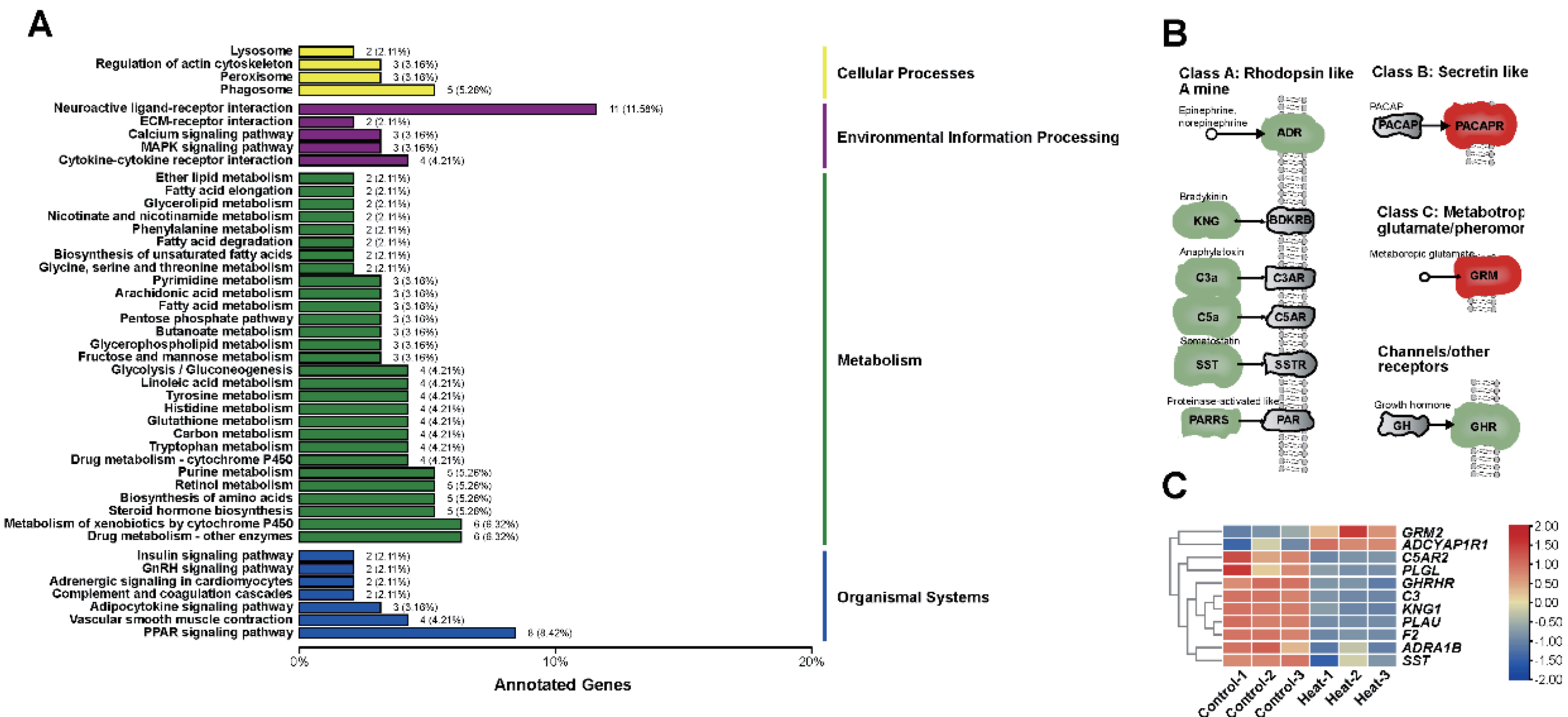

Fig. 3. **Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis of differentially expressed genes (DEGs) induced by heat.** (A) KEGG scheme with all DEGs. (B) Alterations in the neuroactive ligand-receptor communication pathway. Red color marks DEGs that are overexpressed, while the green color marks down-regulated DEGs. (C) Heatmap and hierarchical clustering analysis of DEGs that were annotated in the neuroactive ligand-receptor interaction pathway.

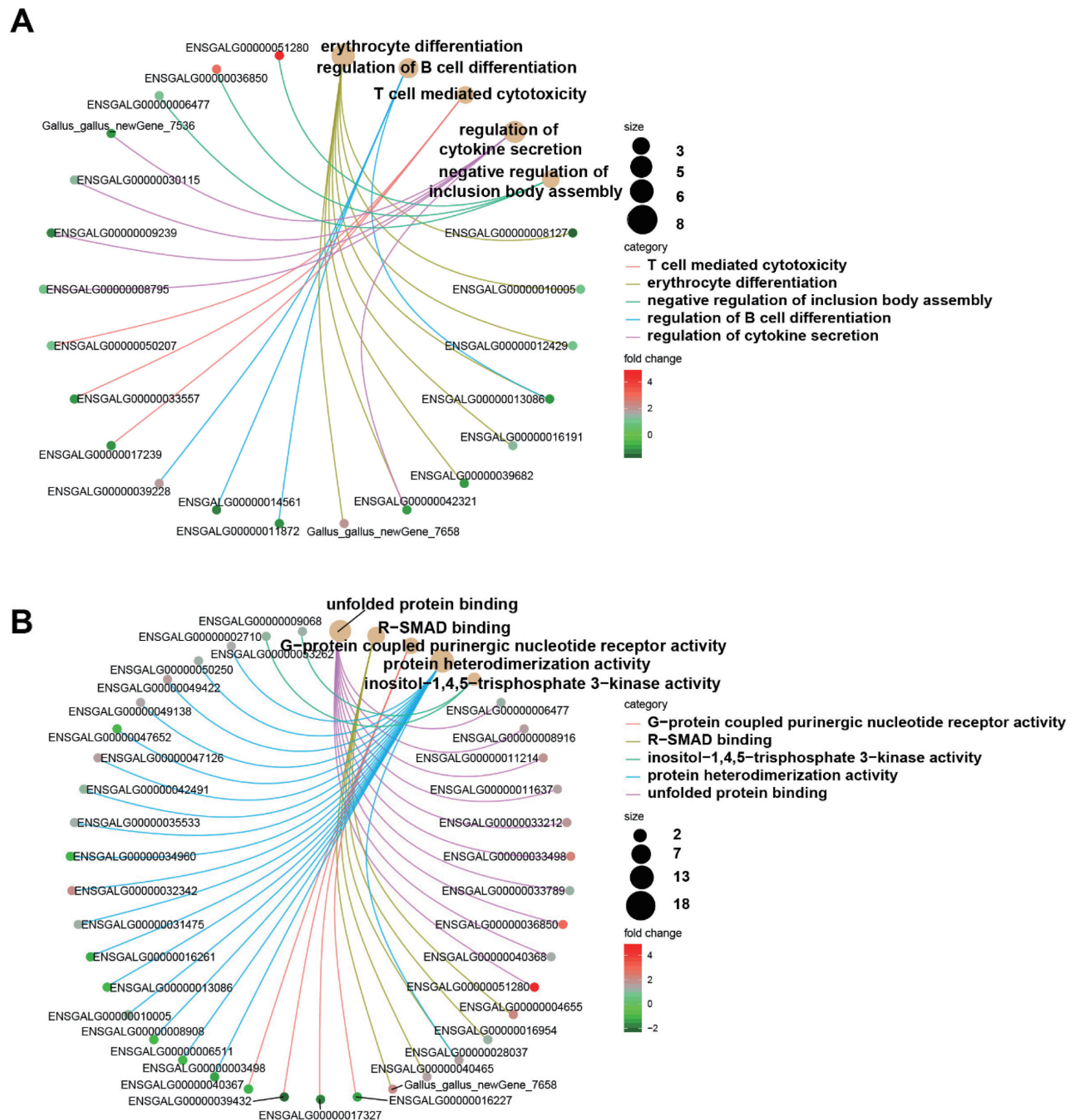

Fig. 4. **Gene Ontology (GO) enrichment network map of differentially expressed genes induced by Baihu decoction (BH).** (A) Top five GO terms under biological process. (B) Top five GO terms under molecular function. The colors of the edges represent different pathways and the colors of the gene nodes represent the multiples of differences. The larger the pathway node, the greater the number of genes enriched in the pathway.

the HSP40 family (*DNAJB1*) (Fig 6, Table S4).

Given that BH increased the expression levels of multiple HSP genes in Hy-Line Brown laying hens, our results indicate that BH regulates protein assembly by regulating HSP expression. The other four upregulated DEGs were *HERPUD1*, *CALR*, *MAP2K7,* and *DP71L* (Fig 6). These DEGs, together with HSPs, appear to regulate the ER pathway.

RT-qPCR confirmation of the detected DEGs

Twelve DEGs involved in the "protein processing in endoplasmic reticulum" pathway were additionally validated by RT-qPCR. The results showed that variations in their relative expression levels presented analogous tendencies to the RNA-seq

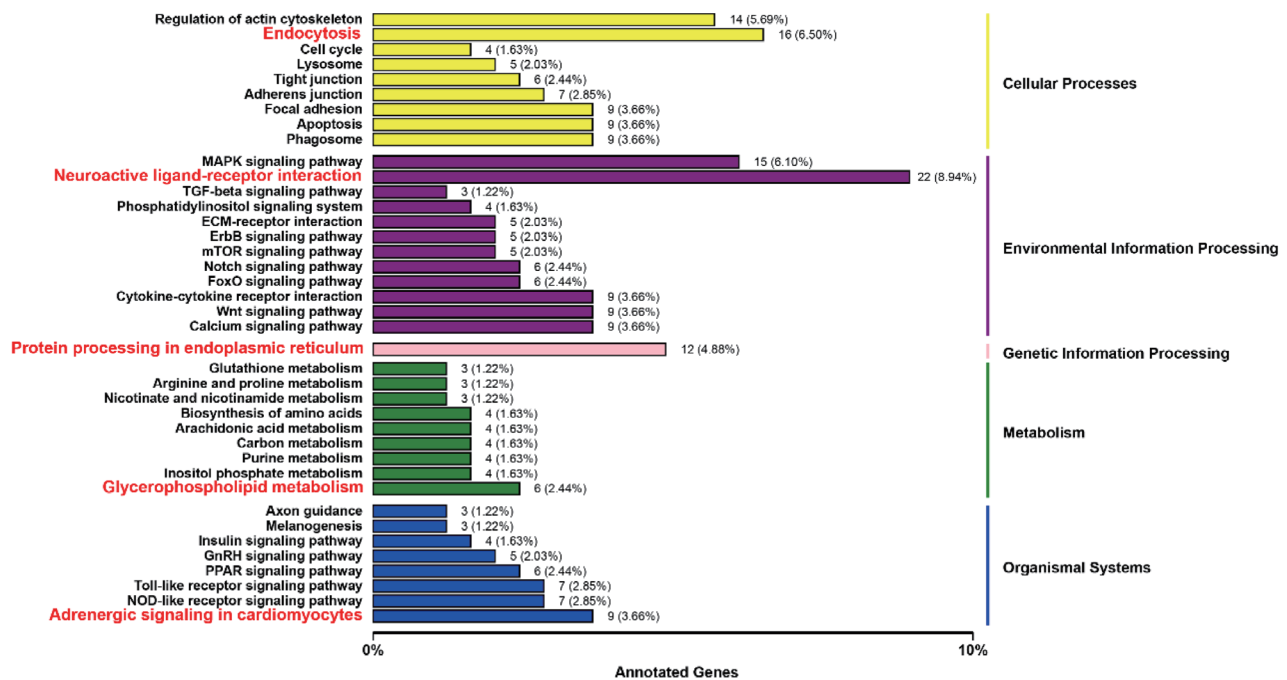

Fig. 5. **Kyoto Encyclopedia of Genes and Genomes pathway enrichment annotation of differentially expressed genes induced by Baihu decoction.**

results, except for the gene *CALR*, whose increasing trend was not apparent at the transcriptional level with RT-qPCR. These findings show that heat treatment did not significantly alter the expression of these 12 genes, whereas BH treatment increased their expression. Notably, the expression levels of *HSPH1* and *DNAJB1* increased multiple-fold after BH treatment (Fig 7). Overall, these results confirmed the reliability of our RNA-seq data.

Discussion

High temperatures are a significant challenge for the poultry industry, a major producer of food of animal origin (Nawab *et al*., 2018). For many years, the consumption of chicken eggs has increased worldwide owing to their high nutritional value and low price. Egg production and the broodiness of laying hens are influenced by decreased progesterone, prolactin, and estrogen levels induced by heat stress (Vandana *et al*., 2021).

In this study, we investigated transcriptome changes in the hypothalamus of laying hens exposed to heat. Surprisingly, the data showed that the expression of HSP genes was not significantly different after 3 h of heat shock treatment, but was significantly upregulated after BH treatment. We compared our transcriptome data to the results available in the National Center for Biotechnology Information database (GSE202003). Similarly, the data showed no significant change in the expression of HSP genes in the hypothalamus 6 h after heat shock. By contrast, Sun *et al*. (2015) compared all of the DEGs of meat chickens exposed to 34°C for 24 h using microarray data and found that heat-shock treatment resulted in significant changes in HSP genes, including *HSP40* and *HSP90*. The difference between these studies may be due to the different timing of the heat stress applied.

DEGs under heat stress

Among the 223 DEGs identified after exposure to heat shock, 11 were neuroactive ligand genes related to the nervous system. Four categories of receptors are involved in this pathway: class A (rhodopsin-like), class B (secretin-like), class C (metabotropic glutamate/pheromone), and channels/others. We focused on three of these genes, including *KNG1*, which showed a substantial decrease in expression under heat stress, reaching nearly zero. In contrast, other authors have reported significantly increased expression of *KNG1* in the serum of rats with Yin-deficiency-heat syndrome compared to that of control rats. This syndrome is a model used in classical Chinese medicine to describe health complications caused by "Yin deficiency" (Liu *et al*., 2016). These previous results confirmed that the expression of *KNG1* is closely related to internal heat, and the decrease in *KNG1* expression during heat shock detected in our study further demonstrates its association with heat. Another downregulated gene under heat stress was *GHRHR*. Numerous new loci have been identified for heat tolerance involved in neuroactive ligand–receptor interactions (Cheruiyot *et al*., 2021). *GHR* expression was reported to be reduced under heat-shock stress in dairy cows and other birds, which is consistent with our results. Remarkably, our results showed that the expression of *ADCYAP1R1*, which encodes the

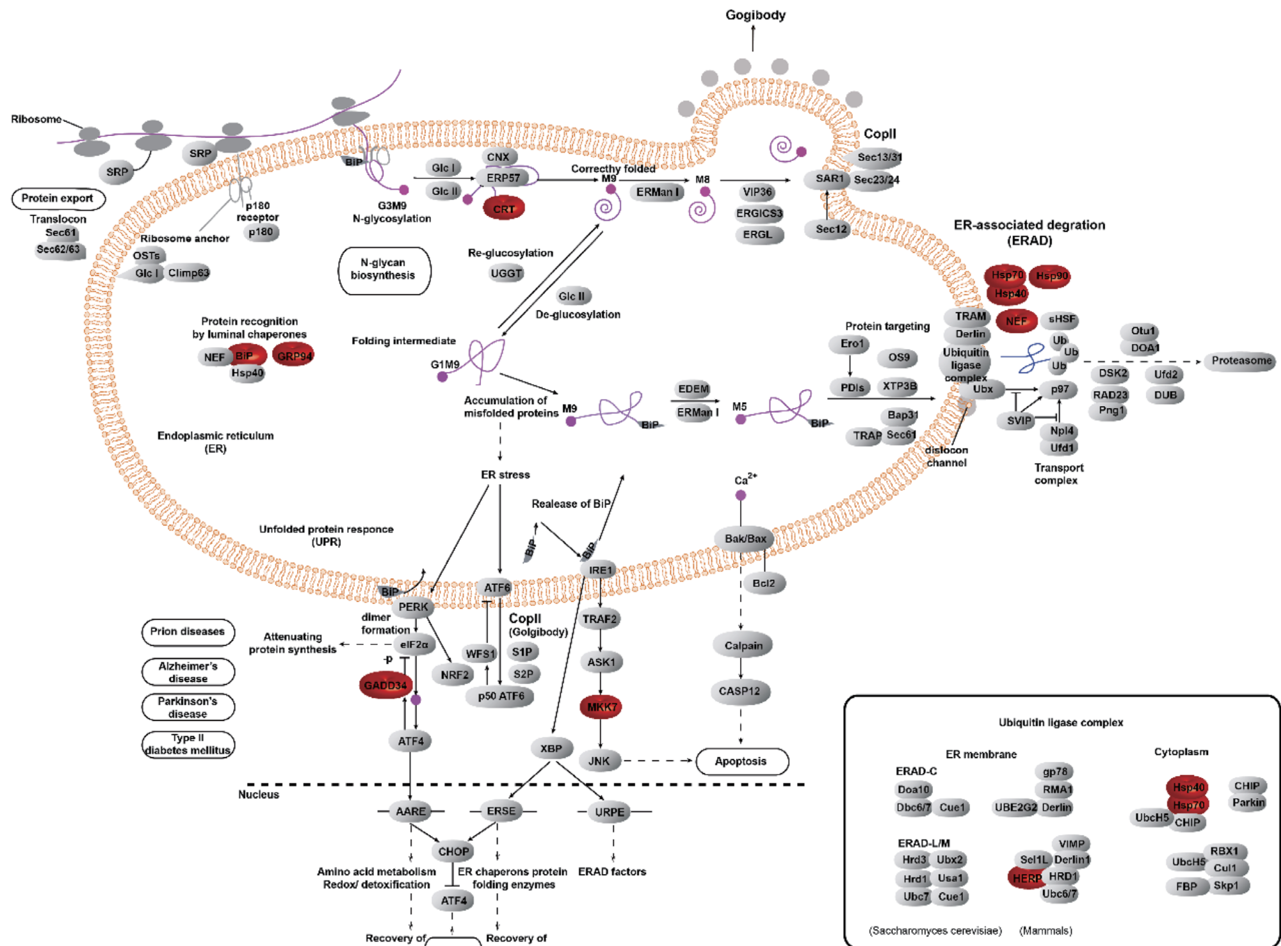

Fig. 6. **Kyoto Encyclopedia of Genes of Genomes pathway map of differentially expressed genes (DEGs) involved in the endoplasmic reticulum pathway.** Red signifies overexpressed DEGs and gray represents background DEGs.

pituitary adenylate-cyclase-activating polypeptide (PACAP) receptor (PACAPR), was significantly increased under heat stress. Injection of PACAP38 in mice could reverse reserpine-induced hypothermia, which was regulated by PACAPR, providing new evidence that PACAP might be a central regulator of thermogenesis (Masuo *et al*., 1995). Subsequently, Gray *et al*. (2002) provided evidence that the survival of PACAP-null mouse lines depended on temperature, which again proved that PACAP is essential for body temperature defense. The same study further showed that the body temperatures of PACAP-null mice were lower than those of littermate control mice under cold exposure. Other authors have provided further evidence that the administration of PACAP increases body temperature in rats (Pataki *et al*., 2000), chickens (Tachibana *et al*., 2007), and mice (Hawke *et al*., 2009; Banki *et al*., 2014). In contrast, PACAPR expression was found to be downregulated in response to cold acclimation (Cline *et al*., 2019). Our results also suggest that *PACAPR* might respond to heat stress by increasing its expression, which is consistent with the results of previous studies. The present study provides additional knowledge on the involvement of other genes in this thermotolerance mechanism, such as *ADRA1B*, *C3*, *C5AR2*, *SST*, *PLAU*, *F2*, *PLGL*, and *GRM2*, for which no related data were found in the literature.

Eight HSP genes were up-regulated under BH feeding

Environmental stress affects protein metabolism. This prevents proteins from folding correctly, impedes their modification, and transports them through the membranes into the ER. Consequently, large amounts of misfolded proteins accumulate in the ER, leading to ER stress. The HSP family plays a key role in protein remodeling and assembly, which is critical for coping with ER stress (Doyle *et al*., 2011; Mishiba *et al*., 2013; Higgins *et al*., 2015). HSPs act as molecular chaperones to regulate cellular homeostasis and promote cell survival. Generally, HSPs are constitutively expressed without stress or are induced by stressors. Among all HSPs, HSP70 and HSP90 are highly conserved ATP-dependent molecules that can assemble and modify proteins.

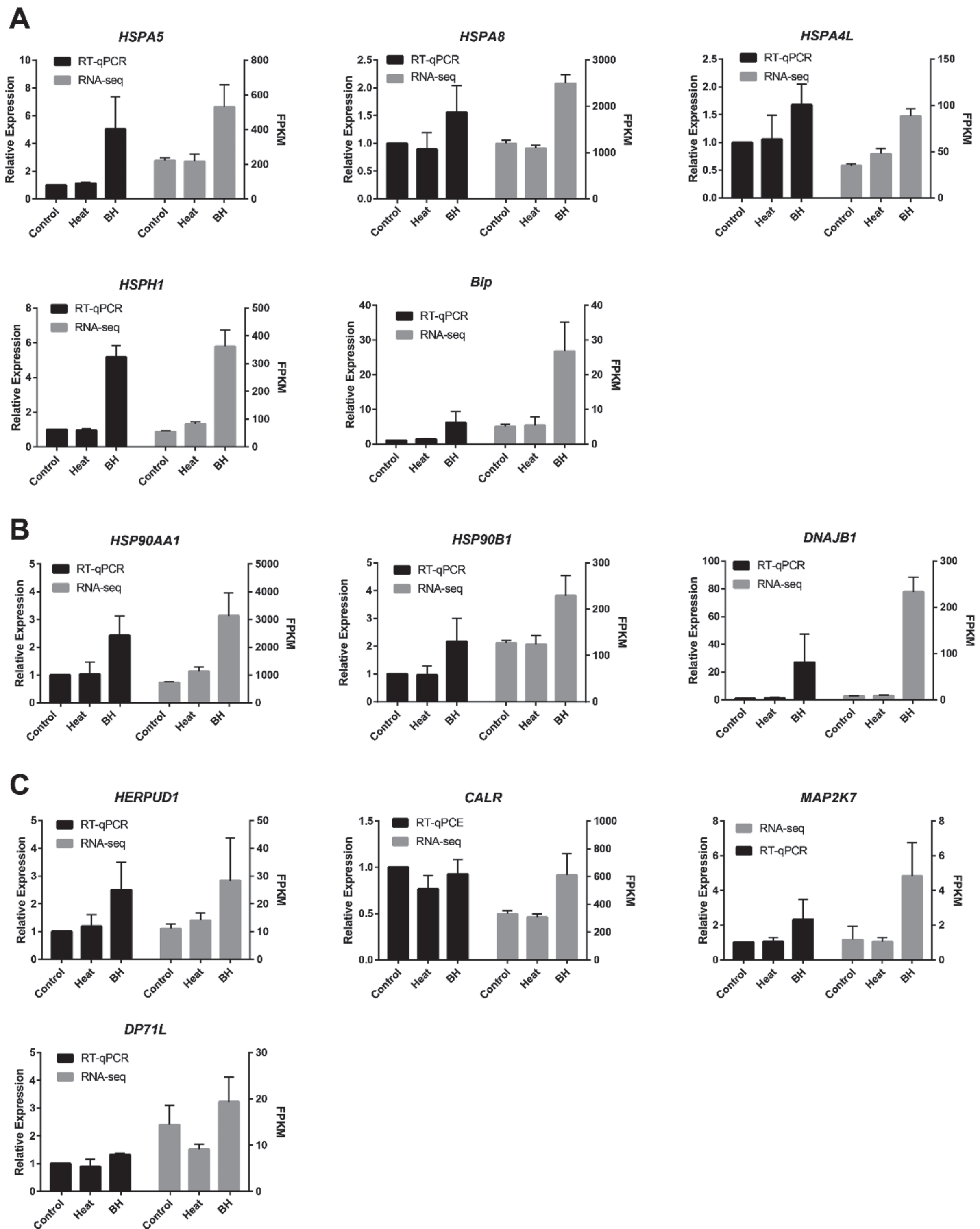

Fig. 7. **Comparison of expression levels of 12 differentially expressed genes determined by RNA-seq and RT-qPCR.** The housekeeping gene *GAPDH* was used as an internal standard.

They are major constituents of the cellular apparatus that drives cellular homeostasis and are involved in almost all physiological processes. Moreover, HSP70 and HSP90 cooperate to complete protein remodeling (Saibil, 2013; Genest *et al*., 2019).

Through KEGG analysis of the DEGs induced by BH, we found that multiple DEGs clustered in the pathway "protein processing in endoplasmic reticulum" in the "genetic information processing" category. Among these DEGs, the expression of five genes encoding HSP70s increased with BH feeding under heat stress in hens. One of the upregulated genes was *HSPA5*, which is a vital regulator of the ER stress response. Similarly, *BiP* expression was upregulated. Both *HSPA5* and *BiP* encode the function-rich protein BiP, which participates in the protein folding process (Pobre *et al*., 2019) and plays an important role in delivering proteins to the ER. This potentiates UPR initiation and decreases the levels of unfolded/misfolded proteins (Wang *et al*., 2017). Accordingly, the increased expression of *HSPA5* and *BiP* identified in the BH group suggests that BH may regulate the ER signaling pathway by promoting the expression of these two genes, thereby maintaining protein homeostasis under heat shock.

Another DEG of interest in this study was *HSPA8*. Our RNA-seq data showed that *HSPA8* had steadily high expression, which doubled in the BH-fed laying hens. These results are not surprising because the protein product HSP70/HSC70 encoded by *HSPA8* is involved in numerous cellular functions such as uncoiling clathrin-coated ATPase during endocytosis (Stricher *et al*., 2013) and assisting in protein folding. HSP70 interacts with newly synthesized polypeptides and remodels them during ATP hydrolysis (Beckmann *et al*., 1990; Frydman and Hartl, 1996). Notably, HSP70 not only interacts with misfolded and newly synthesized proteins but also eliminates many protein aggregates (Stricher *et al*., 2013). Moreover, HSP70 controls protein degradation, especially via the ubiquitin–proteasome pathway (Ciechanover, 1998). HSP70 binds to its signal sequence by forming a polymer with HSP40, which enables nascent protein molecules to enter the ER (Chirico *et al*., 1988; Abell *et al*., 2007; Rabu *et al*., 2008). HSP40 binds to unfolded or non-native polypeptides to prevent their aggregation or binds to folded proteins to affect protein complex remodeling (Rabu *et al*., 2008; Kampinga and Craig, 2010). Furthermore, we found that the expression of *DNAJB1* increased approximately 20 times after BH feeding, indicating that this decoction maintained protein homeostasis by upregulating the expression of HSP40.

In conclusion, two hundred and twenty-three DEGs were detected in heat-exposed chickens. Thirty-nine of these DEGs genes were overexpressed, while one hundred and eighty-four had lower expression level under heat treatment. The KEGG pathway analysis showed that the highest enrichment among these DEGs belonged to the "neuroactive ligand-receptor interaction" pathway. Six hundred and thirteen DEGs were detected between the hens in the BH group and the heat-shock one. Three hundred and thirty-five of these DEGs were overexpressed while two hundred and seventy-eight genes had a lower expression. The KEGG pathway enrichment examination showed that DEGs with the highest enrichment ratio were those associated with "endocytosis", "neuroactive ligand-receptor interaction", "protein processing in the endoplasmic reticulum (ER)", "glycerophospholipid metabolism" and "adrenergic signaling in cardiomyocytes". Furthermore, twelve DEGs including eight *HSP* genes involved in the ER pathway were identified, including five *HSP70s* (*HSPA5*, *HSPA8*, *HSPA4L*, *HSPH1*, *Bip*), 2 *HSP90s* (*HSP90AA1*, *HSP90B1*) and 1 *HSP* (*DNAJB1*).

Sequence Data Availability

All raw Illumina data are freely available from the College of Animal Veterinary Medicine at Henan University of Animal Husbandry and Economy. The reference genome of *Gallus gallus* (Gallus gallus. GRCg6a) was downloaded from http://asia.ensembl.org/index.html.

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Author Contributions

Lihong Li designed the experiments, Tingting Lu analyzed the data and wrote the paper, Xianghui Li performed the experiments, and Yuwei Li raised the animals.

Conflicts of Interest

The authors declare no conflict of interest.

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