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Relationship between Differential Expression of Estrogen Receptor and Follicle Stimulating Hormone Receptor Genes in Ovary and Heterosis of Egg Number Traits in Chickens*

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ABSTRACT : In order to understand the molecular mechanism of heterosis of reproduction traits in chickens, we used the quantitative real-time reverse transcriptional polymerase chain reaction (Quantitative real-time RT-PCR) technique to investigate the differential expression of estrogen receptor (*ESR*) and follicle stimulating hormone receptor (*FSHR*) genes in 32-week-old ovaries of inbred chickens and their hybrid offspring in 4×4 diallel crosses, which involved White Plymouth Rock (E), CAU Brown (D), Silkies (C) and White Leghorn (A). We found that there were significant differences in mRNA expression of *ESR* and *FSHR* genes not only between hybrids and their parental lines ($p<0.01$), but also among different crosses ($p<0.01$). Furthermore, positive correlations between differential expression of both *ESR* and *FHSR* in hybrids and heterosis percentages of 32-week-old and 42-week-old egg number traits were significant at $p<0.05$. Our results suggested that differential expression of *ESR* and *FSHR* genes in the ovaries of inbred chickens and their hybrids could play roles in the formation of heterosis of egg number traits to some extent. (**Key Words** : *ESR*, *FHSR*, Heterosis, Chicken, Gene Expression)

INTRODUCTION

Heterosis has been exploited in domestic animal production with significant contributions to the world's egg and meat supplies, and study on its genetic basis has been one of the most attractive and difficult studies of life science over the past years. Three hypotheses exist for explaining heterosis: dominance (Davenport, 1908), over-dominance (Shull, 1908) and epistasis (Wright, 1951). More recently, some achievements on the molecular mechanism of heterosis have been obtained. With the mRNA differential display reverse transcription-polymerase chain reaction (DDRT-PCR) technique, many studies demonstrated that there was significant differential gene expression between F1 hybrids and their parents in rice (Cheng et al., 1996; 1997), wheat (Sun et al., 1999), maize (Cheng et al., 1997) and chickens (Wang et al., 2005), which was classified into various patterns by comparing gene expression of hybrids to their parents. Furthermore, patterns of differential gene expression were found to be correlated with heterosis percentages of some traits of rice (Xiong et al., 1998), wheat (Xie et al., 2003), maize (Tian et al., 2002; 2003) and chicken (Wang et al., 2004; Sun et al., 2005; Sun et al., 2005). However, all of these reports focused only on qualitative differential gene expression of hybrids and cannot completely explain the genetic basis of heterosis, because those traits responsible for significant heterosis appear to be complex quantitative traits that are controlled by multiple minor-effect genes coupled with environmental effects. It is therefore necessary to investigate quantitative differential gene expression between hybrids and their parents and its relationship with heterosis.

Our previous study showed that gene expression altered

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significantly between inbred chickens and their hybrid offspring in 32-week-old ovaries and classified it into seven patterns of differential gene expression (T1-T7) using the DDRT-PCR method (Wang et al., 2005); the patterns of T3 (over-expression) and T7 (hybrid-specific expressed) were correlated with the heterosis percentages of egg number of 32-week-old and 42-week-old chickens ($p<0.01$, $p<0.01$). Based on these results, we proposed to investigate the relationships between the differentially expressed important candidate genes and heterosis of reproduction traits in chickens.

Steroid hormones perform many essential roles in vertebrates during embryonic development, reproduction, growth, water balance and responses to stress (Sutherland et al., 1998), of which estrogen and follicle stimulating hormone are the most important hormones relating to reproduction. Estrogen plays a key role in regulation of the neuroendocrine system and control of reproductive behavior, and it is essential for normal reproductive activity and has direct actions during sex determination in some vertebrates (Maxwell et al., 1987; Griffin et al., 2001) through activation and binding to its estrogen receptor (ESR) (Griffin et al., 1999; Katsu et al., 2004). So the *ESR* gene was considered as one of the candidates for litter size and determined to be associated with litter size in pigs (Rothschild et al., 1996; 1997). Follicle stimulating hormone (FSH) accelerates the maturation of germ cells and ovulation through stimulating and binding to the FSH receptor (FSHR) (Dankbar, 1995). Also, previous study has indicated that the *FSHR* gene is related to litter size in pigs (Chen et al., 2002).

In the present study, the quantitative real-time reverse transcriptional polymerase chain reaction (Quantitative real-time RT-PCR) technique was used to investigate the differential mRNA expression of *ESR* and *FSHR* genes in 32-week-old ovaries between chicken hybrids and their parents in 4×4 diallel crosses, which involved White Plymouth Rock (E), CAU Brown (D), Silkies (C) and White Leghorn (A), and the correlations between differential expression of these two genes and heterosis percentages of 32-week-old and 42-week-old egg number traits were further analyzed.

MATERIALS AND METHODS

Animals

A 4×4 diallel cross was developed using four chicken breeds which were White Leghorn (A), Silkies (C), CAU Brown (D) and White Plymouth Rock (E). Sixteen crosses including a total of 3084 female individuals were obtained, namely, AA, CC, DD, EE, AC, CA, AD, DA, AE, EA, CD, DC, CE, EC, DE and ED, with about 200 (more than 192) females included in each cross. Individual 32-week-old and 42-week-old egg numbers were measured. To ensure that traits of hybrids were measured under the same condition as their parents and to eliminate systematic environmental error, phenotypic values for 32-week-old and 42-week-old egg number traits of four parents (A, C, D and E) were measured using about 200 female offspring from the four respective purebred crosses (AA, CC, DD and EE). The heterosis percentage of egg number traits for the 12 hybrids was calculated as:

$$\frac{F_1-(P_1+P_2)/2}{(P_1+P_2)/2}\times 100$$

Fresh ovaries of eight healthy females were randomly collected from 12 F1 hybrids and four purebred cross at 32 weeks old and stored in liquid nitrogen.

Total RNA extraction and synthesis of cDNA

Total RNA was extracted from each sample using Trizol reagent (Gibco-BRL, Gaithersburg, MD, USA). Minor DNA amongst the total RNA was digested using DNase (Promega, Madison, WI, USA) (Liang and Pardee, 1992).

Reverse transcription was performed in a total volume of 40 μl containing 0.4 μg pooled RNA, 50 mM Tris-HCl (pH 8.3), 40 mM KCl, 7 mM $MgCl_2$, 10 mM DTT, 20 mM dNTP and 200 pM of each 3′-end anchored primer (H-T11G: 5′-AAGCTTTTTTTTTTTG-3′, H-T11C: 5′-AAGCT TTTTTTTTTC-3′, H-T11A: 5′-AAGCTTTTTTTTTTTA-3′ and T18: 5′-TTTTTTTTTTTTTTTTT-3′) to which was added 100 U M-MLV reverse transcriptase (Promega, Madison, WI, USA) after denaturation at 65°C for 5 min. The mixture was incubated at 37°C for 1 h, followed by at 75°C for 5 min.

Quantitative real-time polymerase chain reaction

The primers were designed according to the cDNA sequences of chicken ESR, FSHR and GAPDH genes using Oligo 6.0 software and synthesized. Forward and reverse primer sequences were 5′-GCCGTGGTGAGGACAAACT-3′and 5′-GGAAGCGGGAGGTGAAGTA-3′ for *ESR*, 5′-CGGAGAACGAATTTGACTATG-3′and 5′-TTGCACATT AGAAAACGAGGT-3′ for *FSHR* and 5′-TCACAAGTT TCCCGTTCTCA-3′ and 5′-GGAACACTATAAAGGCGA GAT-3′ for *GAPDH* with the expected PCR product size of 192 bp for *ESR*, 202 bp for *FSHR* and 220 bp for *GAPDH*. In the total volume of 20 μl, the PCR mixture contained 12 μl the SYBR Green Master Mix (ABI), containing 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 1.5 mM MgCl B_2, 20 mM dNTPs and 1 U Taq DNA polymerase, 200 pM of each primer and 4 μl cDNA. The PCR mixture was programmed at 50°C for 2 min and then at 95°C for 10 min for the first cycle followed by 40 cycles of 92°C for 15 s, 59°C for 50 s, and 72°C for 50 s, and then extension at 72°C for 7 min. *GAPDH* was used as housekeeping gene for generation of a

Table 1. Heterosis percentages of egg number traits (%)

Traits	AC	AD	AE	CA	CD	CE	DA	DC	DE	EA	EC	ED
32-week-old egg number	12.20	7.90	24.04	21.61	11.49	23.07	15.85	36.16	25.75	12.47	33.61	11.87
42-week-old egg number	11.55	5.08	13.78	14.9	9.11	15.18	11.28	24.77	17.93	12.18	26.82	9.03

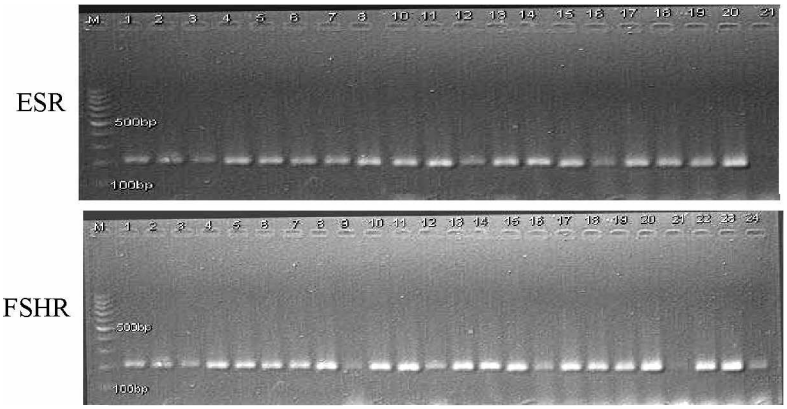

Figure 1. RT-PCR identification of the *ESR* and *FSHR*. Lane 1-20: Partial individuals of AA and BB; M: 100 bp DNA Ladder marker.

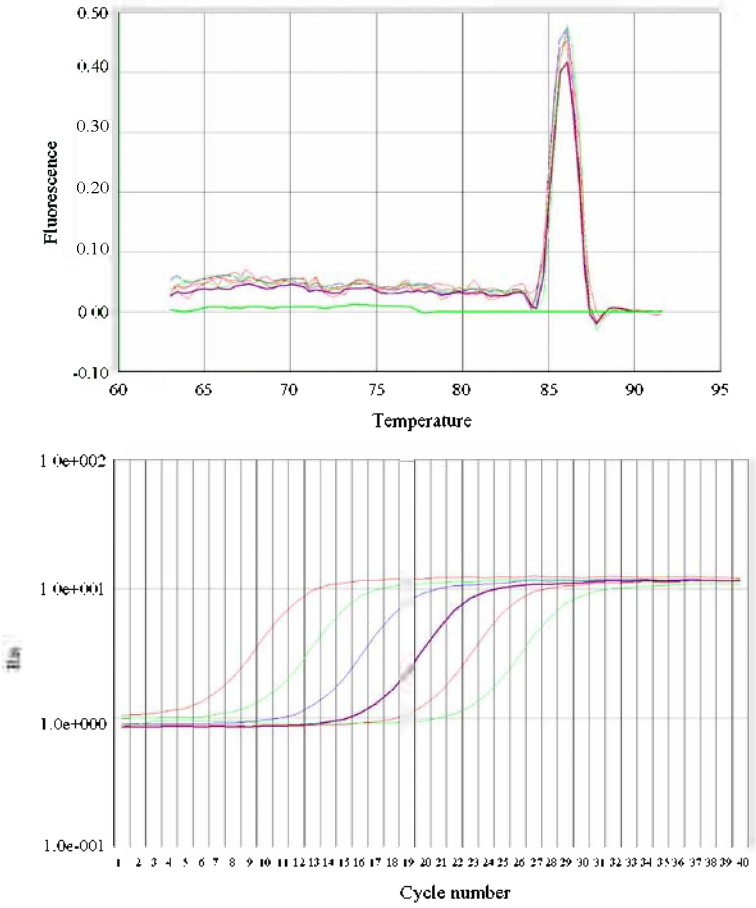

Figure 2. Precision and accuracy of quantitative real-time RT-PCR of ER amplification profiles and standard curves generated using SYBR Green.

standard curve. A series of 10^7-fold dilutions of cDNA synthesis reaction (10^7 fold ranges) were performed and amplified, and then detected by SYBR Green to generate the standard curve. The mRNA level of *ESR* and *FSHR* was determined based on the standard curve and normalized to that of *GAPDH* in each sample.

Statistical analysis

Using SAS (v. 8.02) software, Duncan's Multiple Range Test was performed to test the differences of mRNA

expression of *ER* and *FSHR* among the 16 crosses. In addition, correlations were calculated betwen the mRNA level of *ESR* or *FSHR* gene of each hybrid, as X variable, and heterosis percentage of each trait of each cross, as Y variable.

RESULTS

Heterosis percentage of egg number traits

The heterosis percentages of 32-week-old and 42-week-old egg number traits of 12 F1 hybrids were calculated and are listed in Table 1. Results indicated that each hybrid showed significant heterosis for 32-week-old and 42-week-old egg number traits, suggesting the experimental chickens employed in this study were suitable for investigation of the genetic basis of heterosis for reproduction traits.

Differential expression of ESR and FSHR between crosses

To determine if there were differences in mRNA expression of *ESR* and *FSHR* between F1 hybrids and their parents, quantitative RT-PCR analysis was employed (Figures 1 and 2). After normalization with the corresponding *GAPDH* mRNA level of each sample followed by Duncan's Multiple Range Test, as expected, we found there were significant differences of *ESR* expression between hybrids and their parents (DC, CD vs. DD, CC; EC, CE vs. EE, CC; AE, EA vs. AA, EE; CA, AC vs. CC, AA; AD, DA vs. AA, DD; DE, ED vs. DD, EE), eg., the mRNA level of *ESR* in DC of 0.948 was higher than that of its parents DD and CC at 0.931 and 0.88 ($p<0.01$), respectively. Also, significant differences of *ESR* mRNA expression were observed among some crosses. The mRNA level of *ESR* in combinations DD, DA and EE was significantly different from all other hybrids ($p<0.01$). There was no difference in *ESR* expression in combinations DD, DE and AA ($p>0.05$), but these were significantly different from crosses DC, CE,CA, AD, AC, EA, ED, CD, CC, DA and EE ($p<0.01$). Table 2 shows all the comparisons of mRNA levels of *ESR* in various crosses.

Similarly, the *FSHR* gene was significantly differentially expressed not only between hybrids and their parents ($p<0.01$), but also among some crosses ($p<0.01$, Table 2).

These findings could be related to the different degree of heterosis for 32-week-old and 42-week-old egg number traits among different hybrids.

Table 3. Correlation coefficients between differential expression of *ESR* and *FSHR* and heterosis percentages of egg number traits

Genes	32-week-old egg number	42-week-old egg number
ESR	0.6543*	0.5904*
FSHR	0.5842*	0.5230*

* $p<0.05$.

Correlations between differential expression of ESR and FSHR and heterosis percentages

Based on the results of mRNA expression levels of *ESR* and *FSHR*, the differential expression of these two genes between each hybrid and their parents was calculated through subtracting the mean expression level of parent from that of the hybrid. Having obtained the differential expression of *ESR* and *FSHR* genes of 12 F1 hybrids, then correlations were analyzed between the differential expression of *ESR* gene of hybrids and heterosis percentages of 32 week-old and 42 week-old egg number traits as well as between the differential expression of *FSHR* gene and heterosis percentages of these two reproduction traits. Correlation coefficients are presented in Table 3.

Table 2. mRNA expression of *ESR* and *FSHR* in various crosses (Concentration of cDNA: copy numbers/ml)

Crosses	ESR (Mean±SD)	Significance[1]	FSHR (Mean±SD)	Significance[1]
DC	0.948±0.0026	A	0.689±0.0065	D
EC	0.933±0.0015	B	0.736±0.0014	BC
DD	0.931±0.0021	BC	0.773±0.0024	BC
DE	0.931±0.0020	BC	0.746±0.0027	BCD
AA	0.930±0.0042	BC	0.825±0.0023	B
AE	0.926±0.0043	C	0.789±0.0034	BC
CE	0.921±0.0040	D	0.811±0.0036	B
CA	0.916±0.0043	E	0.820±0.0031	B
AD	0.915±0.0097	E	0.806±0.0047	B
AC	0.907±0.0053	F	0.850±0.0039	B
EA	0.906±0.0029	F	0.845±0.0015	B
ED	0.903±0.0027	F	0.578±0.0029	E
CD	0.884±0.0036	G	0.575±0.0033	E
CC	0.880±0.0058	G	0.650±0.0050	ED
DA	0.865±0.0059	H	0.548±0.0068	E
EE	0.830±0.0044	I	1.342±0.0050	A

[1] Different letter means significant differences at $p<0.01$; same letter means no significant differences.

Statistical analysis of the results showed that positive correlations between the differential expression of both *ESR* and *FSHR* genes and heterosis percentages of 32-week and 42-week old egg number traits were significant at $p<0.05$.

DISCUSSION

In the present study, we first demonstrated that *ESR* and *FSHR* were differentially expressed in the ovary between chicken hybrids and their parents using the quantitative real-time RT-PCR technique ($p<0.01$). The results of statistical tests showed that positive correlations between the differential expression of both *ESR* and *FSHR* genes and heterosis percentages of 32-week and 42-week old egg number traits were significant at $p<0.05$. Our finding is coincident with previous reports in rice, wheat and chickens, which showed that there was differential gene expression between F1 hybrids and their parents (Cheng et al., 1996; 1997; Sun et al., 1999; Xiong et al., 1998; Tian et al., 2002; 2003; Xie et al., 2003; Wang et al., 2004; Sun et al., 2005; Wang et al., 2005). Taken together, it may be concluded that it is the differences of gene expression that result in the heterosis of hybrids. Although all the genes in hybrid F_1 were derived from their parental lines, the hybrid's genetic performance is not simply the additive product of genetic material from both parents, but the result of variations in quantitative and qualitative expression of two sets of genes within the hybrid by interactions resulting in the occurrence of heterosis.

The FSH receptor belongs to the family of G protein-coupled receptors, complex transmembrane proteins characterized by seven hydrophobic hrlices inserted in the plasmalemma and by intracellular and extracellular domains of variable dimensions depending on the type of ligand. The intracellular portion of the FSH receptor is coupled to a G_s protein and, upon receptor activation by the hormonal interaction with the extracellular domain, initiates the cascade of events that ultimately leads to the specific biological effects of the gonadotropin.

Correlation analysis showed that there was significant correlation between the differential expression of *ESR* and *FSHR* and heterosis percentages of 32-week and 42-week old egg number traits ($p<0.05$). It is suggested that the differential expression of *ESR* and *FSHR* genes between hybrids and their parents is related to the formation of heterosis of egg number traits, at least partly explaining its genetic mechanism. This could be due to the important roles of ESR and FSH in the regulation of ovulation rate and follicle development through activation and binding to their receptor, ESR and FSHR, respectively. In addition, our finding indicated that *ESR* and *FSHR* genes were over-expressed in some hybrids than in their parents, which was consistent with our previous research indicating the gene differential expression patterns of T3 (over-expression) was correlated with the heterosis percentages of egg number of 32-week and 42-week old chickens at $p<0.01$ and $p<0.01$, respectively (Wang et al., 2005).

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