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GENE EXPRESSION STUDY OF BIO-MARKERS FOR STRESS RESPONSE IN RABBITS FED GENETICALLY MODIFIED COTTON SEED MEAL.

Salisu, I.B[1 3]*, Ibrahim, A.A[1]. Abdurrahaman, S.L[1]. Umar, U.A[2], and Shahid, A.A[3]

[1]*Department of Animal Science, Faculty of Agriculture, Federal University Dutse, PMB 7156 Dutse, Jigawa State-Nigeria*

[2]*National Animal Production Research Institute, Ahmadu Bello University, Shika, Zaria Nigeria*

[3]*Centre of Excellence in Molecular Biology, University of the Punjab, 87-West Canal Bank Road Lahore-53700, Pakistan*

Corresponding Author email address: abuyasir212@gmail.com, salisu.ib@fud.edu,ng

Abstract

The response of rabbits at mRNA level to genetically modified feed ingredients was studied using a nutrigenomics approach. A total of forty-eight (48) rabbits were randomly divided into four groups and fed a diets containing varying levels of GM-cottonseeds at 0%, 20% 30%, and 40% inclusion levels. The examined genes expression patterns in the liver of both male and female rabbits of major genes involved in stress response were comparable regardless of diets fed. No significant ($p > 0.05$) variations were detected in the relative expressions of these genes (i.e Hsp60, Hsp70 and Hsp90) in all the dietary groups except for the relative expression level of Hsp90 in the liver of male rabbits fed 30% GM cotton seed meal, where significant up-regulation was detected as compared to the rest of the dietary groups. Our findings demonstrated that consumption of GM-cottonseeds meals could not significantly affect the hepatic expression patterns of stress related genes in rabbits.

Key words: Nutrigenomic, GM cottonseed meal, Rabbits, Liver, gene expression

INTRODUCTION

Diet is a potent mechanism for altering the cells` environment of most organs and considered as one of the key environmental factors affecting lifespan. Genetically modified (GM) crops, expressing insect resistance or herbicide tolerance are currently receiving attention all over the world and are frequently becoming part of human and animal diets (Nadal et al., 2018). The fact that GM food may affect human or animal health is debated. Even though, several studies revealed that GM food/feed are safe for both human and animal consumption, however, it cannot be ignored that some scientific studies have reported the structural and molecular modifications in different organs and tissues of GM-fed animals (Malatesta et al., 2008). These observations suggested that the risk of GM crops cannot be ignored and deserves further investigations (El-Shamei et al., 2012). Nutrigenomic, is an emerging area which study the interactions of food and its components with the genome at the molecular, cellular and systemic levels. This novel approaches highlight how dietary nutrients can interact with genes affecting transcription factors, RNA and protein expression, cellular homeostasis and metabolite production (Benítez, Núñez and Óvilo, 2017). Heat shock proteins (HSPs) are molecular chaperones for nascent and stress accumulated misfolded proteins expressed in reaction to a number of factors including the physiological as well as the environmental factors (Sathiyaa and Vijayan, 2003). Studies have shown that HSPs are significantly involved in mediating immunological functions, including cytoprotection and cell survival (Multhoff 2006, Schmitt et al., 2007, Basu et al. 2002, Aneja *et al*., 2006).The liver is one of the most sensitive organs used for monitoring the changes in gene expression with respect to the dietary treatments, as it plays a significant role in metabolism as well as detoxification of noxious substances (Malatesta *et al.* 2008). This study was therefore designed to evaluate the potential unintended effects of GM cottonseeds meal on hepatic expression level of genes significantly involved in stress response in rabbits.

MATERIAL AND METHODS

Study area, test material, animals, management and sampling procedure

The study was conducted at National Centre of Excellence in Molecular Biology (CEMB), University of the Punjab, Lahore, Pakistan, to examine the gene expression patterns of biomarkers for stress response in rabbits Fed GM cotton seed expressing binary insect resistance genes (*Cry1AC + Cry2A*) and herbicide tolerance trait (*CP4 EPSPS*). The site is located between latitude 31° 34' 55.3620"N and longitudes 74° 19' 45.7536" E, at an altitude of 213 m above sea level. A total of Forty-eight New Zealand white male and female (24 pairs) rabbits weighing averagely 570.50 ± 0.50 g were obtained and allowed to acclimatize for seven day before being assigned randomly to four dietary treatment groups namely: G1, G2, G3, and G4 with 6 pairs per group, for six-month feeding trial. The rabbits in G1 were raised on a normal diet without GM cottonseed. Rabbits in G2, G3, and G4 were raised on diet containing 20%, 30% and 40% of GM cottonseeds respectively. At the termination of the study, rabbits were anaesthetized with chloroform and sacrificed. For the purpose mRNA study, the liver samples were carefully excised using a sterile surgical blade and washed with 1X PBS.The samples were collected in sterile tubes each containing RANlater™ (Invitrogen, Cat # 00465894) to maintain the RNA integrity. Tubes were then instantly stored at -20°C before being proceed for the total RNA isolation.

RNA extraction and qPCR Analysis

For the total RNA isolation, all liver samples which were previous kept in RANlater™ (Invitrogen, Cat # 00465894) to maintain the RNA integrity, were Lysed and homogenized in TRIzol™ Reagent (Invitrogen, Cat # 15596026) with the help of homogenizer. One hundred milligram of tissue was used per one milliliter of TRIzol™ Reagent (i.e. 100 mg of tissue per 1000 µL TRIzol™ Reagent) for total RNA extraction. After extraction with chloroform, precipitation with isopropanol and washings with 70% (v/v) ethanol, the extracted RNA was then resuspended in RNase-Free Water. The quality and integrity of RNA were evaluated using the NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific). 1µg of RNA was used for reverse transcription with RevertAid first-strand cDNA synthesis kit (Thermo Scientific, Cat #K1621) according to the manufacturer instructions. Primer specific for *Hsp60, Hsp70, Hsp90* as well as GAPDH (reference gene) were designed using online NCBI primer design software (Primer3, http:// http://bioinfo.ut.ee/primer3-0.4.0/). The primers were synthesized from GeneLink™ USA in lyophilized form. The quality of the primers for the real time PCR analysis was assessed through amplification efficiency and melting curve. The primers details are presented in Table 1.Quantitative Real-time polymerase chain reaction (qPCR) analysis was performed by SYBR Green Master Mix (Applied Biosystems™ Cat # 1306409) using PIKOREAL 96 real-time PCR instrument (Thermo scientific) to assay the relatively quantitative mRNA expression of Hsp60, Hsp70and Hsp90 in the liver of rabbits. Fifty Nano gram (50 ng) of the cDNA template and 1 mM primer concentration were used in a total of 10 µL reaction volume. The qPCR reaction conditions were 95 °C (5 min), 40 cycles of 94 °C (20 sec), and 30 second at annealing temperature shown in Table 1, and 72 °C (30 sec). Data for the relative gene expression were analyzed using $2^{-\Delta\Delta CT}$ (Ct = cycle threshold) method (Livak and Schmittgen 2001).The results were expressed in fold change as compared to untreated control (control =1 fold).

Table 1. Primers details used for the analysis of hepatic gene expression study

Gene	Primers Sequence (5` –3`)	Tm (oC)	PA (bp)	Accession number
GAPDH	F: ACGACATCAAGAAGGTGGTG R: GCATCGAAGGTAGAGGAGTG	60	124	NM_001082253.1
Hsp60	F: CCAATCCATCGTACCTGCTC R: GCTACAACCTGAAGACCAAC	60	135	XM_002721722.3
Hsp70	F: AAGATGCTACGGTCTGCTTG R: TTGGATAACGCGATCTGGAC	60	129	XM_008255002.2

Hsp90	F: TATGCCTTGGTGTTACGTGG R: TGGAGAGTTCCTCCGTCAAG	60	134	XM_002712368.3

F; forward, R; reverse, Tm; annealing temperature, P_A; amplicon size

Statistical analysis

Data analyses were done with Graph pad prims (Version 8) for windows. Results on gene expression studies were all analyzed by one-way ANOVA. Results were analyzed separately for male and female rabbits. To determine any significant differences among treatments, "Turkey`s multiple comparison test" was applied. Significant differences were considered when $p < 0.05$. All data in figure were reported as mean ± standard error ($\bar{x} \pm SEM$).

Results

Figure 1, described the effects of dietary incorporation of GM cottonseed meal on the relative expression of genes related to stress responses in the liver of rabbits. The examined genes (i.e *Hsp60*, *Hsp70* and *Hsp90*) expression patterns in the liver of both male and female rabbits were comparable in most of the dietary groups following 180 days of consumption of diet containing different concentrations of GM cottonseeds meal. No significant ($p > 0.05$) variations were detected in the relative expressions of these genes (i.e *Hsp60*, *Hsp70* and *Hsp90*) in all the dietary groups except for the relative expression of *Hsp90* in the liver of male rabbits fed G3 diets, where significant up-regulation was detected as compared to the rest of the dietary groups.

Discussion

The potential changes in gene (*Hsp60, Hsp70 and Hsp90*) expression pattern due to feeding GM cotton were analyzed in the liver of rabbits using qPCR. No significant changes were recorded in the relative expression of these genes except for *Hsp90* in the liver of male rabbits fed G4 diet where significant up-regulation was observed. The finding of this study agreed with previous report in which no substantial changes were detected in the expression patterns of genes significantly involved in apoptosis, cell cycle and inflammatory response in the intestine and liver of dairy cow when a diet containing up to 41.9% of GM maize was fed to the cows for a period of 25 month (Guertler *et al*. 2012). Contrarily, Malatesta et al. (2008), reported that significant down-regulation of several *HSPs* were detected in the liver of female mice when GM soybean meal was fed to the mice for 24 months (Malatesta et al. 2008).These results variation in our studies could be attributed to the differences in the animal species used in the studies, differences in study duration or could be due to the animals` physiological differences among others.

Conclusion

In conclusion, the findings of our study, revealed that dietary substitution of GM cottonseeds in rabbits' diet could not significantly change the mRNA expression pattern of biomarkers for stress response following 180-day ingestion.

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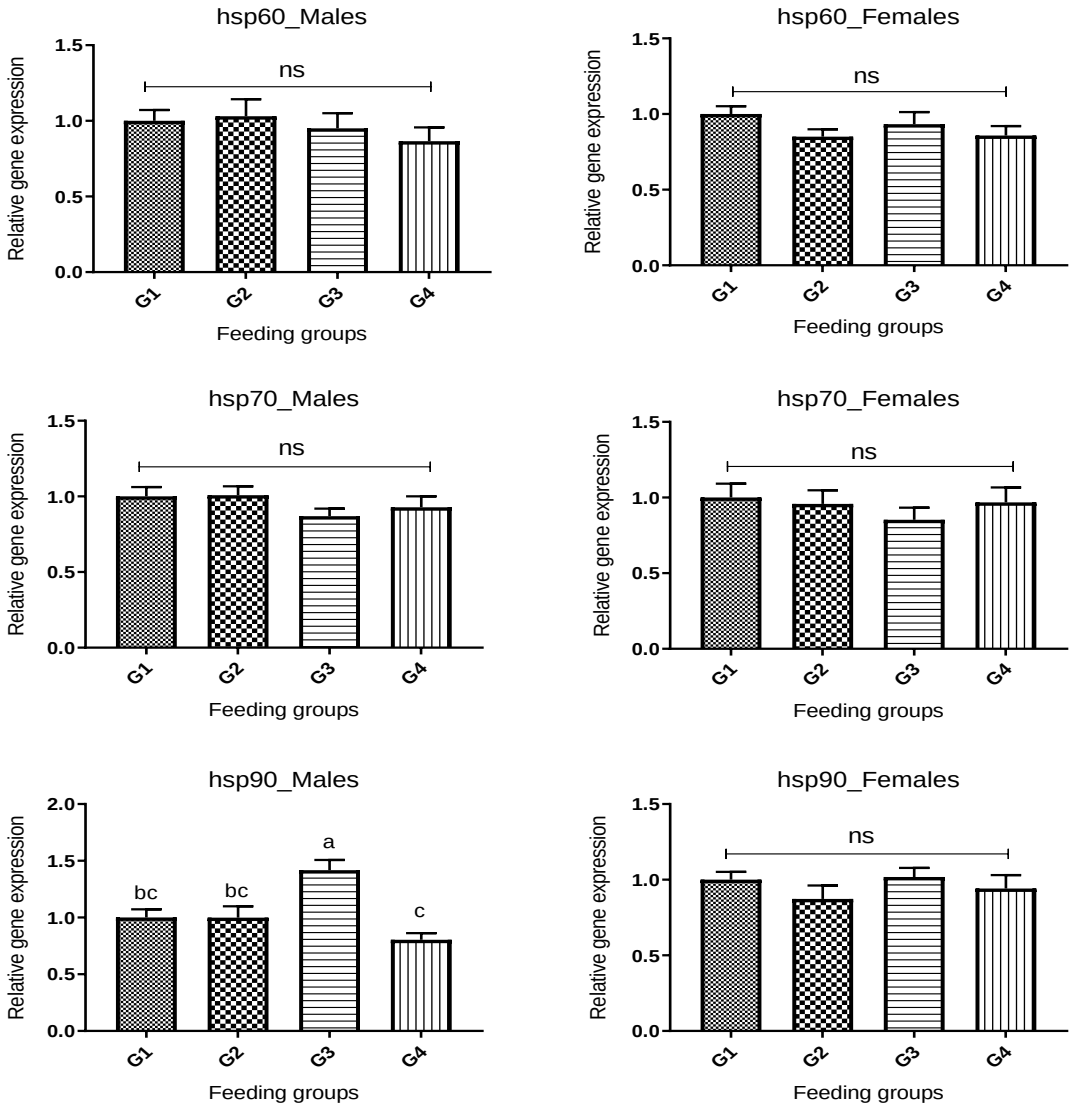

Figure 1. Relative expression of genes involved in stress response in the liver of male and female rabbits. Relative expression of genes was presented as mean ± SE. G1 represent the control groups, G2, G3 and G4 represent 20%, 30% and 40% transgenic cottonseeds fed groups. Ns; no significant ($p > 0.05$) difference, while bars bearing different letter are significantly ($p < 0.05$) different.