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A Genome-Wide Association Study of Survival to Unexpected Acute Heat Stress in a F_2 Chicken Population

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ABSTRACT

Heat stress, or hyperthermia, can have a serious effect on chicken performance in poultry industry in many parts of the world. Both genetics and environment play key role in the performance of a chicken and, therefore, it is important to consider both factors in addressing heat stress. On genetics level, genome-wide association studies have become a popular method for studying heat stress in recent years. A population of 202 F_2 chickens was reared for 84 days to find genes and genomic regions affecting growth traits and immune system. But, due to unexpected acute increase in temperature at day 83, 182 birds died (case) and 20 birds remained alive (control). At the age of 70 days, blood sample of all birds was collected to extract their DNA, using modified salting out method. All samples were genotyped by a 60 K Single Nucleotide Polymorphism (SNP) chip. Genome-wide association study was carried out by GCTA to identify gene and genomic regions associated with heat stress tolerance. Results indicated a close relationship between 28 SNPs, located on chromosomes 2, 3, 5, 6, 7, 12, 19, 20, and 21 and heat stress tolerance at the level of suggestive significance. Two suggestively significant markers on chromosome 5, namely, GGaluGA273356 and Gga_rs16479429, were located within and 52 Kb downstream of two genes, including *MAPKBP1*and *SPON1*, respectively. Gene ontology analysis indicated that the resistance of chickens to acute increase of temperature might be linked to the function of *MAPKBP1* and *SPON1* genes and their biological pathways. These results will be useful for understanding the molecular mechanisms of SNPs and candidate genes for heat stress tolerance in chickens and provide a basis for increasing genetic resistance in breeding programs.

Keywords: Gene ontology analysis, GWAS, Hyperthermia, Single nucleotide polymorphism.

INTRODUCTION

In many parts of the world, heat stress, or hyperthermia, can have a serious effect on chicken performance in poultry industry (Lin *et al*., 2006). Heat stress is a condition in chickens that occurs at high temperatures and will be acute when combined with high relative humidity and low air speed (Ahmad and Sarwar, 2006). Two predisposing factors that affect heat stress include genetics and environment. Literature results indicate that continuous increase in growth rate and feed efficiency in modern broiler breeds coincide with a reduced heat stress tolerance (Lara and Rostango, 2013). In an experiment, muscle damage in fast growing broilers was reported to be associated with an ambient temperature (Zahoor *et al*., 2016). Additionally, it has been indicated that much of the variation in response to heat stress has apparently a genetic base (Lu *et al*., 2007). Soleimani *et al*. (2011) found that commercial broilers were more susceptible to heat stress than their Red Jungle fowl counterparts. Also, Mack *et al*. (2013) provided evidence that genetic selection is a useful strategy for reducing heat stress response in laying hens.

Evidences demonstrated that genetic progress of heat stress tolerance can be

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accelerated through application of genomic information in breeding populations. In recent years, several methods such as genome-wide association studies (Van Goor *et al*., 2015) and transcriptome comparison (Coble *et al*., 2014) have been suggested to implement for studying heat stress tolerance. The Genome Wide Association Study (GWAS) has been proposed to be a suitable method for identifying genomic regions affecting the resistance of birds to heat shock (Lamont *et al.*, 2014).

Several Quantitative Trait Loci (QTLs) have been identified to be associated with heat stress tolerance in Holstein cattle (Dikmen *et al*., 2013) and catfish (Jin *et al.*, 2017). For chickens, few results have been found in literatures and several candidate genes were reported to be associated with heat stress tolerance (Van Goor *et al*., 2015). Therefore, the objective of the present study was to identify candidate genes related to survival to an unexpected acute increase in temperature, namely, heat stress tolerance, using GWAS in an F_2 chicken population.

MATERIALS AND METHODS

Experimental Design and Management

All procedures were conducted under the protocols approved by the Laboratory Animal Care Advisory Committee of Tarbiat Modares University. From a reciprocal crosses between fast-growing Arian broiler line (A) and an Iranian Native fowl (N), F_1 chickens were generated from mating of A♂×N♀ and N♂×A♀ birds. The F_1 male birds from each reciprocal cross were each mated to four to eight females from non-related families, resulting in a total of 202 F_2 chickens of eight half-sib families from three consecutive hatches. Day-old F_2 chickens were individually identified with wing tags, and immediately weighed after hatch. The birds were reared on the floor for 7 days under 24 hours light and a brooding temperature of 33°C. This temperature was decreased to 30°C on day 7. On day 8, birds were weighed and moved to individual cages with a temperature of 30°C, which was gradually decreased to reach a final temperature of 22°C, and a 22 hours light and 2 hours dark cycle throughout the experimental period. Chickens did not receive vaccines during rearing period. Feed and water were provided ad libitum. This experiment was conducted to measure chickens body weight and carcass compositions at 84 days of age, but due to unexpected acute increase in the room temperature at the eighty-third night, 182 birds died (case) and 20 birds remained alive (control).

Genotyping and Quality Control

At the age of 70 days, DNA from blood samples of all 202 chickens was extracted using the Salting out method and stored at -20°C. These DNA were used to identify Single Nucleotide Polymorphism (SNP) of each bird, using the Illumina chicken 60 K Beadchip containing 54,340 SNP markers provided by Cobb Vantress with cooperation of Arhus University of Denmark. The PLINK (v1.07) software package was used to determine the quality control of the markers (Purcell *et al*., 2007). After deleting SNPs with a minor allele frequency less than 5%, a Hardy-Weinberg equilibrium test *P*-value less than 1×10^{-6}, and a call rate less than 95%, a total of 47,730 SNP markers and 201 individuals remained for final analysis. A multidimensional scaling (MDS) method, which explains observed genetic distance among individuals (Wang *et al*., 2012), was implemented in PLINK software to evaluate the population structure. In this software, MDS-plot option in conjunction with cluster was used to perform MDS analysis on $N\times N$ matrix of genome-wide identity by state pair wise distance. The results of MDS-plot option was used to extract the number of MDS dimensions and it was plotted by R software.

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Statistical Analysis

The GWAS analysis for heat stress tolerance was performed by the Mixed Linear Model (MLM), using GCTA software program (Yang *et al*., 2011) with the 47,730 SNPs passing quality control. The MLM in this software fits the effects of all the SNPs as random (Goddard *et al*., 2009). The resultant model was as follows:

$$\boldsymbol{y} = \boldsymbol{Xb} + \boldsymbol{Wq} + \boldsymbol{Sa} + \boldsymbol{e} \quad (1)$$

Where, $\boldsymbol{y}$ is a vector of discrete binary observations as 0 for the cases (died) and 1 for controls (alive), b is a vector of fixed effects of overall mean, sex (male and female) and hatch of birth (3 hatches), q is a vector of fixed effects of the first dimensions from MDS analysis, a is a vector of random effects of SNP markers with $\boldsymbol{a} \sim N(0, \boldsymbol{I}\sigma_a^2)$, I is identity matrix, and e is a vector of random residual effects with $\boldsymbol{e} \sim N(0, \boldsymbol{I}\sigma_e^2)$. The X, W,and $\boldsymbol{S}$ are design matrices relating y to b, q, and a, respectively.

For each SNP, an adjusted P-value was computed using the False Discovery Rate (FDR) procedure as

$$P_{FDR} = (i/m) \times Q \quad (2)$$

Where, i is the rank of P-value, m is the number of total SNPs and Q is the false discovery rate (Benjamini and Hochberg, 1995). Based on this procedure, an adjusted threshold value of $P_{FDR} = 4.99 \times 10^{-5}$ $((238/47730) \times 0.01)$, with $-\log(P_{FDR}) = 4.30$, (3) and

$P_{FDR} = 1.83 \times 10^{-3}$ $((1747/47730) \times 0.05)$, with $-\log(P_{FDR}) = 2.74$, (4) was applied for genome-wide and suggestive levels of significance, respectively, to declare significant association between heat stress tolerance and marker.

Manhattan plots of genome-wide association analyses were produced using R 3.2.2 software with the QQMAN package (Turner, 2014). The positions of significant or suggestive SNPs, were identified by NCBI in chicken reference genome database (*Gallus-gallus*-4). Names of the genes with or adjacent to the associated SNPs (in 1-Mb segments surrounding each significant SNP) were detected from NCBI (http://www.ncbi.nlm.nih.gov) and Ensembl (ftp://ftp.ensembl.org/pub/release-73/fasta/gallus_gallus/dna/).The Database for Annotation, Visualization and Integrated Discovery (DAVID: https://david.ncifcrf.gov/) was used to analyze gene ontology (Huang *et al*., 2009). This database provides a comprehensive set of functional annotation tools for understanding the biological meaning behind large list of genes.

RESULTS

The population structure of F_2 chickens was identified through MDS analysis. The first and second components of MDS were used to create two-dimensional plot for individuals. The result is shown in Figure 1. Six different subpopulation groups with different dispersions were identified, indicating the chickens within each half-sib family were clustered together. These results suggest that MDS analysis largely support the consensus clustering outcome in this study.

The Manhattan plot of the P values calculated from the genome-wide association study for heat stress tolerance for all SNPs is presented in Figure 2. No significant association was found between heat stress tolerance and SNP markers at the genome-wide significance level ($P > 4.99 \times 10^{-5}$). A close relationship was found between 28 SNPs, located on chromosomes 2, 3, 5, 6, 7, 12, 19, 20, and 21 and heat stress tolerance at the level of suggestive significance ($P < 1.83 \times 10^{-3}$).

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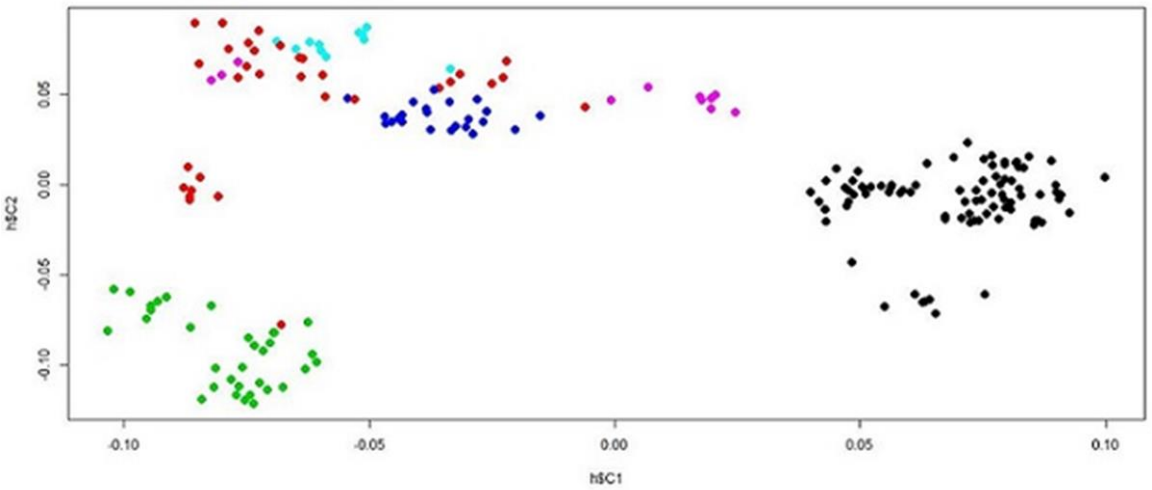

Figure 1. Population structure identified by multidimensional scaling analysis. The scale on the X-axis is the first MDS compound, and the scale on the Y axis is the second MDS compound. Each color identifies a half-sib family.

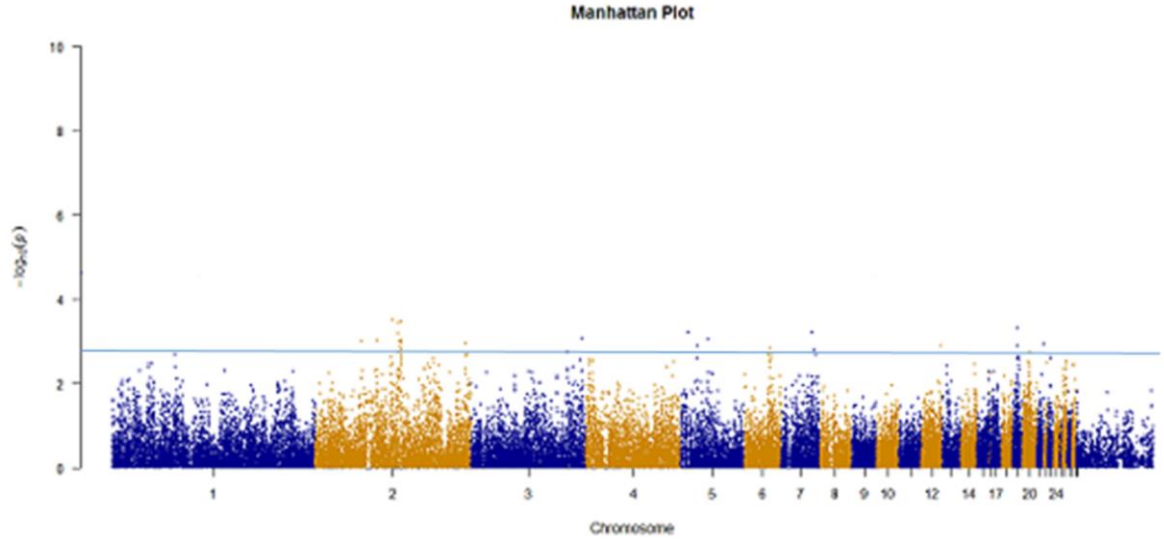

Figure 2. Manhattan chart heat resistance Thermometer for chicken chromosomes. The X axis, the marker position on the chromosome, and the Y axis represent the negative logarithm of the *P*-value 10 base.

The 10 more significant SNPs with *P* values$\leq 9.5 \times 10^{-4}$, the name of chromosome and the gene surrounding each SNP, the position of each marker on the chromosome, and the closest gene to the desired marker are presented in Table 1.

Among the identified markers, the SNP position of Gga_rs16039304 was not detected on chromosome 2. On these chromosomes, four SNPs (GGaluGA155695, GGaluGA155135, GGaluGA155136 and GGaluGA149469) were located within a 22.77 Mb region. These SNPs were clustered in a region between 60.98 and 83.75 Mb and located 39 Kb downstream, 4 Kb upstream of *FHOD3*, *IKZF1* genes, and within *IKZF1* and *LOC107051657* genes, respectively. The GGaluGA238855 SNP located at 106.87 Mb on chromosome 3 was 8 Kb upstream of

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Table 1. Location of and gene information for 10 SNPs association with heat stress tolerance.

SNP	Chr.[a]	Position (bp)	*P*-value	Gene	Distance (Kb)[b]
Gga_rs16039304	2	-	0.000304981	-	-
GGaluGA155695	2	83749423	0.000332619	*FHOD3*	39 kb^{D}
GGaluGA155135	2	80910368	0.000360726	*IKZF1*	4 kb^{U}
GGaluGA155136	2	80918742	0.000634819	*IKZF1*	within
GGaluGA149469	2	60975377	0.000945898	*LOC107051657*	within
GGaluGA238855	3	106874244	0.000849221	*MSRA*	8 kb^{U}
GGaluGA273356	5	6474615	0.000593381	*SPON1*	52 kb^{D}
Gga_rs16479429	5	25109295	0.000878839	*MAPKBP1*	within
Gga_rs14623035	7	28801482	0.000615910	*EN1*	127 kb^{D}
Gga_rs14119331	19	3792047	0.000472838	*MYL10*	35 kb^{D}

[a] Chr. is chromosome. [b] ^{U} and ^{D}: Represent Upstream and Downstream of gene, respectively.

MSRA gene. Two SNPS, namely, GGaluGA273356 and Gga_rs16479429, located within a 18.63 Mb region between 6.47 and 25.11 Mb on chromosome 5 were 52 Kb downstream and within *SPON1* and *MAPKBP1* genes, respectively. Two SNPs, one on chromosome 7 (Gga_rs14623035) and the other on chromosome 19 (Gga_rs14119331), were located at 28.80 and 3.79 Mb and were found to be 127 and 35 Kb downstream of *EN1* and *MYL10* genes, respectively.

DISCUSSION

In the case of heat stress, the physiological responses of different organisms to the increase in ambient temperature is heat gain of body and increment in the blood flow of the skin (Sandercock *et al*., 2001). These can be regarded as indicators of the reaction of body to regulate its production through elimination of excess body heat (Hocking *et al*., 1994). Genes regulation related to activation of Heat Shock Factors (HSFs) and their transcription activators have been identified as cellular responses to different types of stresses (Sorger, 1991). Heat shock proteins have been suggested to be the key cellular defense mechanisms in chickens during exposure to hot environments (Cedraz *et al.*, 2017). Also, the c-Jun amino-terminal Kinases (JNK) and P38 Mitogen-Activated Protein Kinase (MAPK), which are primarily activated in heat shocks in cells, regulate cellular activities such as gene expression, mitosis, differentiation, and cell survival (Tibbles and Woodgett, 1999; Bogoyevitch *et al*., 2010). These two kinase proteins, which are of the subgroups of the MAPK family (Koul *et al*., 2013), are involved in various complex biological processes and strongly respond to stress signals, including osmotic stress (Vassalli *et al*., 2012). Hao *et al.* (2018) suggested that the MAPK signaling was involved in the protective role of HSP70 against heat stress-induce injury in the small intestine of rats.

In the present study, several SNPs were identified to be suggestively associated with heat stress tolerance using information from a population of F_2 chickens. Gene ontology indicated that these SNPs were within or nearby *FHOD3*, *IKZF1*, *LOC107051657*, *MSRA*, *SPON1*, *MAPKBP1*, *EN1* and *MYL10* genes. Two SNPs, Gga_rs16479426 and GGaluGA273356, located on chromosome 5, were found to be within and close to the *MAPKBP1* and *SPON1* genes, respectively. The *MAPKBP1* gene plays an important role in initiating and activating the JNK enzymes and, therefore, protein kinases of MAPK in heat stress environment (Fan *et al*., 2014). The prevention of oxidative stress by the c-Jun N-terminal Kinase due to heat shocks has been reported by Courtial *et al.* (2017) in corals and human cells. Also, a key transcription factor in the Nuclear Factor kappa B (NF-κB) signaling pathway, which regulates cell cycle survival, apoptosis, and cell transformation, has been

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suggested for *MAPKBP1* gene in literature (Fu *et al.*, 2015). Several studies indicated that NF-κB signaling is essential for resistance to heat stress-induced early stage apoptosis in human (Janus *et al.*, 2011; Liu *et al.*, 2015), chickens (Akbarian *et al.*, 2016), pigs (Ganesan *et al.*, 2017) and cattle (Kumar and Singh, 2019).

Furthermore, several studies have reported more than one member of the MAPK family in phosphorylation and HSF-1 activity (Chu *et al*., 1996), and in some studies, it has been confirmed that part of the HSF-1 activity in the body is regulated by MAPK (He *et al*., 1998). HSF-1 is a monomer inactive in a complex with heat shock proteins, including HSP40, HSP70, and HSP90. With heat increment, the HSF-1 is released from the protective complex and, after being transferred to the nucleus, it is phosphorylated and attached to the DNA containing the heat shock elements. The most important HSF-1 target genes are also HSP genes (Shamovsky and Nudler, 2008). HSF-1 proteins increase the level of HSP-encoded proteins (Kim *et al*., 2005), and are also a molecule that is used to reduce or denature defective proteins, as well as protect the cell against heat stress (Zhang *et al*., 2002). It should be noted that among the heat shock proteins, the HSP70 protein plays a central and key role in cellular repair and protection against heat or other stresses (Lindquist, 1986). It seems that the *MAPKBP1* gene plays an important role in increasing the heat resistance in F_2 populations through regulating the activity of JNK protein kinases, consequently activating HSF-1 and increasing the level of heat shock proteins.

In addition to the *MAPKBP1* gene, *SPON1* gene has been also introduced as an important regulator of response to heat stress during heat increment, and reduces the heat shock stress. In current study, the GGaluGA273356 SNP, located 52 Kb downstream of the *SPOD1* gene, was found to be suggestively associated with heat stress tolerance. *SPOD1* gene encodes Spondin 1 (F-spondin), which is an extracellular matrix protein. It was shown that over-expression of Spondin 1 protein suppresses endogenous levels of amyloid β and reduces Aβ plaque deposition in mice (Hafez *et al.* 2012). The cytotoxic formation of amyloid β fibrils by heat shock proteins on lipid membranes in response to heat stress has been reported by Sakono *et al.* (2013). Coble *et al*. (2014) reported a down-regulation for *SPOD1* in response to the heat stress treatment.

For other genes, no functions related to heat stress tolerance have been reported in chickens. Hamzić *et al*. (2015) reported that the *FHOD* gene was significantly associated with percentage of β2-globulin in blood plasma. The *IKZF1* gene was suggested to be a central regulator of hematopoiesis that contributes to nearly every level of B cell differentiation and function (Sellars *et al*., 2011). For *LOC107051657* gene, no function has been reported in the literatures. Xue *et al*. (2017) have reported *MYL10* gene to be associated with the growth and development of muscle in chickens. For *EN1* and *MSRA* genes, the development of cell organ limbs transcription regulation and the binding of specific DNA sequences (Simon *et al*., 2004) and protein regeneration and response to oxidative stress (Stadtman *et al*., 2002) have been reported.

In general, the results of the present study suggest that the resistance of some of our F_2 chickens population to acute increase of temperature may be linked to the function of *MAPKBP1* and *SPON1* genes and their biological pathways. However, this conclusion is based on suggestive association of the SNPs and heat stress tolerance, and needs more realistic models to identify potential genes in LD with significantly associated SNPs. If the findings are confirmed, the results will be useful for understanding the molecular roles and mechanisms of SNPs and candidate genes to heat stress tolerance in chickens and provide a basis for increasing genetic resistance in breeding programs.

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بررسی ارتباط چند شکلی ماندگاری ناشی از تنش گرمایی در یک جمعیت F_2 مرغ

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چکیده

تنش حرارتی یک مشکل جدی در پرورش صنعتی طیور به شمار می‌رود که تحت تأثیر فاکتورهای محیطی و ژنتیکی قرار دارد. در سطح ژنتیکی، مطالعات پویش ژنوم در سال‌های اخیر به روشی رایج برای مطالعه تنش‌های حرارتی تبدیل شده است. در پژوهش حاضر، در مجموع یک جمعیت با تعداد ۲۰۲ قطعه مرغ نسل F_2 برای یافتن ژن‌ها و مناطق ژنومی مؤثر بر صفات رشد و سیستم ایمنی بدن ایجاد و برای ۸۴ روز پرورش داده شدند، اما به دلیل بروز حادثه‌ی ناشی از افزایش ناخواسته دما در روز ۸۳، تعداد ۱۸۲ قطعه پرنده از بین رفته و ۲۰ پرنده زنده ماندند. از نمونه خون این پرندگان، که در سن ۷۰ روزگی جمع

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آوری شده بود، DNA آن‌ها به روش بهینه شده نمکی استخراج شد. همه نمونه‌های استخراج شده توسط تراشه تجاری ۶۰ هزارتایی چندشکلی تک‌نوکلئوتیدی تعیین ژنوتیپ شدند. پویش ژنومی این داده‌ها برای شناسایی ژن‌ها و مناطق ژنومی مرتبط با مقاومت به تنش گرمایی با استفاده از نرم افزار GCTA انجام شد. نتایج نشان داد که ارتباط معناداری بین ۲۸ چندشکلی تک نوکلئوتیدی با صفت مقاومت به تنش گرمایی در سطح پیشنهادی وجود دارد. دو نشانگر معنادار روی کروموزوم ۵، یعنی GGaluGA273356 و Gga_rs16479429، با فاصله ۵۲ کیلو باز پایین دست ژن *SPON1* و درون ژن *MAPKBP1* قرار داشتند. بررسی هستی‌شناسی ژن نشان داد که مقاومت به تنش گرمایی در جوجه‌ها، با ژن‌های *MAPKBP1* و *SPON1* و مسیرهای بیولوژیکی آن‌ها در ارتباط است. این نتایج برای درک مکانیسم مولکولی چندشکلی‌های تک نوکلئوتیدی و ژن‌های نامزد برای مقاومت به تنش گرمایی مفید بوده و می‌تواند در برنامه‌های اصلاحی برای بهبود مقاومت ژنتیکی استفاده شود.

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