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Molecular Characterization and Expression Profiling of ENOX2 Gene in Response to Heat Stress in Goats

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Abstract

NADPH oxidase (NOX) proteins are membrane-associated, multi-unit enzymes that catalyze the reduction of oxygen using NADPH as an electron donor. The present study was carried out to analyse expression profile of ENOX2 gene in heat stress susceptible (HSS) and heat stress tolerant (HST) individuals in response to heat stress. The genomic DNA was isolated from blood. The total RNA was isolated from different tissues such as liver, spleen and kidney. High Resolution Melting (HRM) genotyping exhibited two different genotypes in four goat breeds. The relative expression pattern of ENOX2 gene in liver, spleen and kidney showed 7.40 10.01 and 3.92 fold higher expression than control. Barbari, Jamunapari, Jakhrana and Sirohi exhibited 8.11, 10.97, 5.65 and 5.87 higher fold expression than control. Heat stress-susceptible (HSS) and heat stress-tolerant (HST) individuals exhibited 12.45 and 5.41 fold ENOX2 gene expressions than control in response to long term heat stress.

Keywords: ENOX2; Goat; Expression; HRM; Heat stress phenotype

Introduction

Heat stress is a major challenge for livestock production. It is necessary to develop different strategies to ameliorate heat stress for maintaining production performance. Regulation of heat stress at cellular level is one of the mechanisms to identify better resilient animals. The activity of NOX gene is linked to wide range of physiological functions at cellular level [1]. NOX proteins regulate different physiological processes such as cell growth, apoptosis and cytoskeleton remodeling. The NOX protein also plays major role in host defense [2]. NOX derived reactive oxygen species (ROS) play significant roles in signal transduction, cell differentiation, gene expression and cell death [3]. NOX family plays regulatory role at cellular level during different stress and disease conditions. The NOX families of NADPH oxidase (NOX2) variants regulate the cellular expression in stressed and malignant cell [4,5]. NOX proteins within a cell and their subcellular localization and coupling to external stimuli are determinants of the response to NOX activation [6]. NOX gene consists of two membrane-bound elements, gp91phox ("phox" stands for phagocyte oxidase) and p22phox; three cytosolic proteins, p40phox, p47phox, p67phox; and a small G-protein, Rac [7]. The misregulation or absence of certain NOX isoforms has been linked to a variety of diseases in organ system [8,9]. NOX2 regulates the innate host defense, both by producing ROS to attack invaders after phagocytosis and by acting as a signaling molecule to initiate number of inflammatory and immune protective responses [2]. NOX2, or the neutrophil NADPH oxidase, have been extensively studied in human and mice [10,11], therefore the present study was designed to analyse genetic variation in ENOX gene. Again to analyse goat expression profiling between heat stress-susceptible (HSS) and heat stress-tolerant (HST) phenotype in goat.

Materials and Methods

Institute Animal Ethics Committee (IAEC), Central Institute for Research on Goats (CIRG), Makhdoom, approved the experimental procedures

Animal selection

The investigation has been carried out in four different breeds of semi-arid region of India exhibiting contrasting coat colours and body size (Table 1). The observations were recorded on Barbari, Jakhrana, Sirohi and Jamunapari goats maintained at the Central Institute for Research on Goats (CIRG). The goats were maintained under semi-intensive management system with 6-7 h of grazing and feeding with seasonally available green fodder, supplemented with concentrate mixtures depending upon the status and age of the animals.

Physiological Parameter & DNA Isolation

The three Physiological parameters such as rectal temperatures (RT), respiration rate (RR) and heart rates (HR) were measured during heat stress period. The animals were exposed to sunlight during the highest temperature of the day ranging from 1330hrs to 1430hrs. The experiment was carried out during the May- June period for 60 days. The study was carried during 45.0°C to 49.4°C temperature and relative humidity (RH) varied from 14.33-27.0%. The animals were exposed to radiation for 4-5 hours for 60 days. Non-lactating females of 1-2 years of age group were included in experiment. The heat stress assessment in goats was carried out in temperature humidity index (THI) ranging from 85.36 – 89.80. The details of climatic variation were presented in Table 2. The blood samples were collected with the anticoagulant-EDTA coated vacutainer tube (BD Biosciences, Franklin Lakes, NJ, USA) under aseptic conditions) during the period and stored at -20°C until analysis. The DNA was extracted from 50 samples in four goat breeds.

Recognition of heat stress-tolerant (HST) and heat stress-susceptible (HSS)

Phenotyping for heat stress susceptibility was carried out based on respiration rate (RR) and heart rate (HR). Based on the distribution of RR and HR over the breeds in the population, it was observed that individuals having RR>=50 and HR>=130 would-be recognized as heat stress-susceptible (HSS) phenotype and RR<=30 and HR<=100

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Breed	Natural Habitat	Coat colour	Body size
Barbari	The home tract of the breed is Agra, Aligarh, Kanpur regions of Uttar Pradesh	white color and beautiful brown patches all around the body	Medium
Jamunapari	Chakarnagar area of Etawah district in Uttar Pradesh State	Tall, white colour, roman nose, pendulous ears	Large
Jakhrana	Alwar region of Rajasthan State	Coat colour of the breed is black with white speckles on the ears	Medium to Large
Sirohi	Udaipur, area of Rajasthan State	brown with light or dark brown patches	Large

Table 1: Body description along with habitat, coat colour and body size.

Mean environmental conditions with range						
Experimental period	**Duration**	**Temperature (ºC)**	**R.H. (%)**	**Rainfall (mm)**	**Sunshine (h)**	**THI**
Heat Stress Period	12	47.50 ± 0.40 (45-49.4)	21.31 ±1.28 (14.33-27)	0	10.30 ± 0.35 (8-12)	87.57 ± 0.44 (85.36-89.80)
Thermo-neutral condition	11	26.32 ± 1.13 (19.5-30)	75.09 ± 4.21 (50.67-93.67)	1.82 ± 0.84 (0-7)	4.77 ± 1.13 (0-8.2)	69.80 ± 0.79 (65.32-73.12)

RH: Relative Humidity in Percentage
THI: Temperature-Humidity Index

Table 2: Mean environmental conditions in semi-arid region during heat stress and thermo-neutral period.

would be recognized as heat stress-tolerant (HST) individual in adult goats [12].

Polymerase chain reaction (PCR)

The genomic DNA was extracted by phenol-chloroform extraction method [13]. The purity, quality and quantity of DNA were checked by Biophotometer (Eppendorf, Germany). The primers were designed by basing on Sequence (NCBI XM_002699541.2) using the Primer 3 (www.ncbi.nlm.nih.gov). Sequence specificity of the primers was confirmed by homology through a BLAST search (http://www.ncbi.nlm.nih.gov/blast/blast.cgi). The primers of ENOX2 were forward 5' CTCCGGCCTTCTGACAAAGT3' and reverse primer ENOX2 5' GCAGGACTTCTGACTGGTCC 3' ranging in the region 1535 base pair to 1798 base pair was used. Gradient PCR was carried out to optimize the annealing temperature using the thermal cycler (My Cycler, Bio-Rad) under the PCR cycling. Again the standardization of annealing temperature and magnesium concentration was carried out on the Light Cycler 480 Real-Time PCR System (Light Cycler 480) followed by 4% agarose gel electrophoresis in order to detect any other (spurious) bands.

RNA extraction and cDNA preparation

Tissue samples were washed twice in PBS followed by RNA later. The tissue samples from different organs such as liver, spleen and kidney were collected. Subsequently, tissue samples were stored at -70°C for future use. The total RNA from different tissues was isolated [12]. A 200mg tissue sample was used for RNA Isolation with TRI reagent method. One microgram of total RNA was loaded in 1.5% agarose gel to check the integrity of RNA. One microgram of total RNA was used for cDNA followed by transcriptor first strand cDNA synthesis kit (www.roche-applied-science.com). The cDNA was stored at -70°C for further use. RNA samples were purified from DNA contaminations, as described previously [12].

Genotyping by High Resolution Melting (HRM) analysis

HRM was carried out in 96-well plate in the Light Cycler 480 (www.roche-applied-science.com) real-time PCR System. Reaction volume was 20 µL. Reaction mix includes genomic DNA (10 ng/µL), 1X HRM Master mix (10 µL), 0.2 µM of each primer, 2.5 mM of $MgCl_2$ and water. The PCR program started with an initial denaturation of 10 min at 95°C, continued with 45 cycles of 10 s at 95°C, 15 s at 56°C for 20s at 72°C. This program also allows one step for heteroduplex formation by heating to 95°C for 1min and cooling down to 40°C for 1 min. For HRM, the plate was heated from 65°C to 95°C performing 25 acquisitions per 1°C. Amplicons were prepared for HRM analysis by heating to 95°C for 1 min, rapid cooling to 40°C and incubation for 1 min. Fluorescence-based data were visualized using fluorescent signal normalization, temperature shifting and difference plotting and then analyzed using the automated grouping functionalities provided by the Light Cycler 480 gene scanning software Module (www.roche-applied-science.com).

All samples were amplified and analyzed in duplicate. The samples with melting curves different from the duplicate were removed from analysis. The HRM curve data (sequence variation in the PCR product) was analysed using the Light-Cycler 480 gene scanning software version 1.5 (www.roche-applied-science.com). The absolute quantification analysis was carried out to prepare a new subset of proper amplified samples for further analysis. Subsequently, the raw melting curve was normalized by setting the pre-melt (initial fluorescence) and post-melt (final fluorescence) signals of all samples to uniform value. The next step was shift the temperature axis of the normalized melting curves at the point where the entire double-stranded DNA was completely denatured. Finally the difference plot was generated by subtracting the curves from a reference curve. The specificity of the amplified PCR products is critical in HRM analysis, the PCR products were electrophoresed on 4% agarose gel to assess whether each primer set generated single PCR products of the expected size. Melting curve analysis was also carried out to check any spurious amplification.

RT-PCR

Real-time PCR was carried out for ENOX2 and housekeeping gene (GADPH). Real-time PCR was performed in the Light Cycler 480 (Roche Applied Science, Indianapolis, IL, USA) using SYBR green detection. Each reaction contained 10 µl of SYBR green I master mix (containing Fast start Taq DNA Polymerase, dNTP mix, SYBR green I dye, $MgCl_2$ (Roche Applied Science, Indianapolis, IL, USA)), 1 µl of the sense and antisense specific primers, and 5 µl of cDNA in a final volume of 20 µl. A negative control without cDNA template was run in each assay.

To allow relative quantification after PCR, absolute quantification was carried out to analyse the whole set of data. The crossing point

(Cp) readings for each unknown samples were then used to calculate the amount of either the target or housekeeping gene using the second derivative maximum method with the Light-Cycler 480 analysis software version 1.5 (Roche Applied Science, Indianapolis, IL, USA). GADPH was used to normalize gene expression. The susceptible individual was used as positive calibrator to obtain normalized gene expression.

Statistical analysis

Gene expression levels were quantified with crossing point (cycle threshold) values, which are the raw data from the real-time PCR, and it is the number of PCR cycles required for the fluorescence signal to cross threshold line. Cp values are inversely proportional to the amount of target nucleic acid, so the greater the Cp value, the lower the amount of target nucleic acid in the sample. All analyses were performed on mean Cp values, which were calculated from the sample replicates used in the real-time PCR. Fold-change in expression levels of the target genes (ENOX 2) were presented using the 2–ΔΔCt method and by E-method [14].

Results and Discussion

Genetic variation and expression pattern of ENOX2 gene in different tissues in response to heat stress

The amplified fragments exhibited 260 base pair in length .The temperature-shifted (temp-shifted) melting peak and the difference plot indicated two different groups in the analysed samples (Figures 1A and 1B). The percentage of distribution of analysed samples in the two groups was 62.50% and 37.50%. The two variants obtained from HRM analysis were subjected to direct DNA sequencing; Sequence analysis did not exhibit any variation in ENOX2 gene in four different breeds (Sequence has been submitted to gene bank accession no.-KJ477040, 41, 42).

The relative expression pattern of ENOX2 in the liver, spleen and

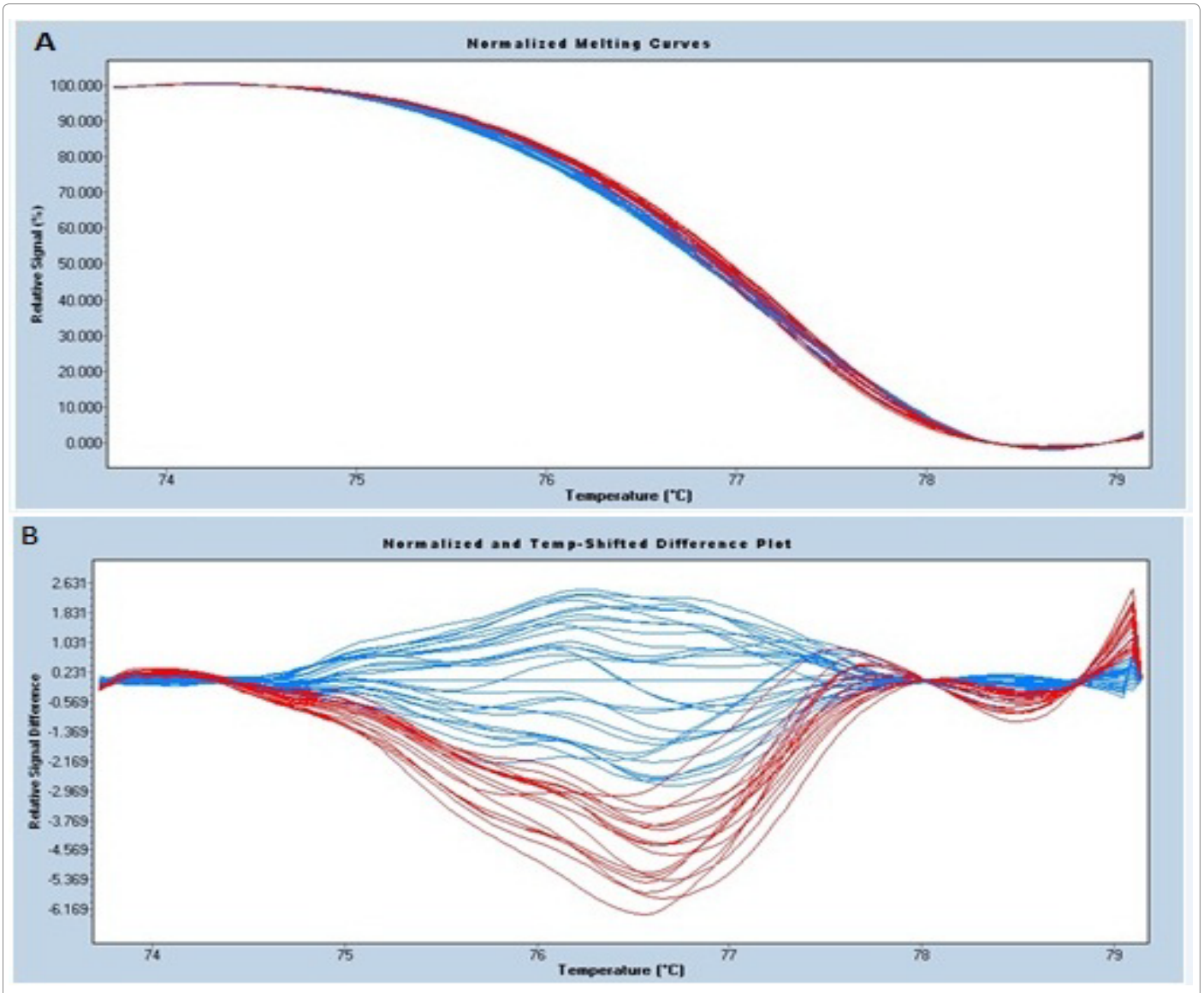

Figure 1A&B: The temperature-shifted melting peak showed two different genotypes in the analysed Samples. The percentage of distribution of analysed samples in the two groups was 57.69% (Blue) % and 34.62% (Red)%.

kidney indicated 7.40, 10.01 and 3.92 fold higher gene expression than control, respectively (Table 3 and Figure 2). Spleen showed highest up regulation of ENOX2 gene in comparison to liver and kidney during heat stress. Spleen showed 1.35 and 2.55 fold higher expression as compared to liver and kidney. The breed expression pattern of ENOX2 gene indicated that the Barbari, Jamunapari, Jakhrana and Sirohi had 8.11, 10.97, 5.65 and 5.87 fold higher gene expressions than control (Table 4 and Figure 3). Jamunapari breed showed highest 10.97 fold expression as compared to other breeds in response to heat stress**.** Again jamunapari showed 1.35, 1.94 and 1.86 fold higher mRNA level than Barbari, Jakharana and Sirohi goats. The phenotypic expression of two different group HSS (heat stress-susceptible) and HST (heat stress-tolerant) showed 12.45 and 5.41 fold expression than control indicating expression pattern was higher in HSS group than HST (Table 5 and Figure 4).

The characterization of ENOX2 protein in livestock in general and goat in particular have remained unexplored. The biological function of NOX proteins in human being has been characterized [15]. Reactive oxygen species (ROS) are important mediator of cell growth, adhesion, differentiation, migration and apoptosis. NADPH oxidases of the Nox family play multifarious role in different biological processes, such as host defense, signal transduction and hormone synthesis [16]. NOX family genes play diverse role in different pathway as signaling molecules in cellular response to growth factor, cytokines and hormone [17]. We did not observe any sequence variation over the breeds in ENOX2 gene indicating conserve region. Again the expression pattern indicated higher expression of ENOX2 gene in heat stress individuals. The Jamunapari goats showed higher expression of ENOX 2 gene as compared to Sirohi breeds. Sirohi is better adapted to heat stress as compared to Jamunapari and others [12]. ENOX 2 gene was also exhibited variable expression in different tissue samples. NOX isoforms have been observed in different vascular tissues [18-20].

Conclusion

ENOX2 gene expression was observed in liver, spleen and kidney and spleen showed higher ENOX2 gene expression in comparison to liver and kidney in response to chronic heat stress. ENOX2 gene expression was higher in heat stress susceptible individual than heat stress tolerant individual.

Organs	No. of Samples	Target/Ref	Relative fold Expression
Control	4	1	1
Liver	8	7.40	7.40
Spleen	8	10.01	10.01
Kidney	6	3.92	3.92

Target gene- ENOX
Reference gene- GAPDH
Secondary derivative and E- method used for analysis

Table 3: Gene expression pattern of ENOX in different organs.

Breed	No. of sample	Target/Ref	Relative fold expression
Control	4	1	1
Barbari	6	8.11	8.11
Jamunapari	6	10.97	10.97
Jakhrana	4	5.65	5.65
Sirohi	6	5.87	5.87

Target gene- ENOX
Reference gene- GAPDH
Secondary derivative and E- method used for analysis

Table 4: Gene expression pattern of ENOX gene in different goat breeds.

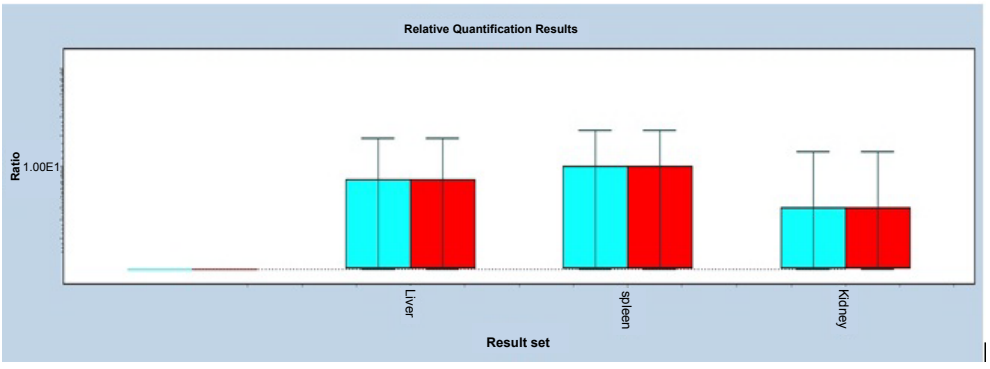

Figure 2: Gene expression pattern of ENOX2 gene in different organs.

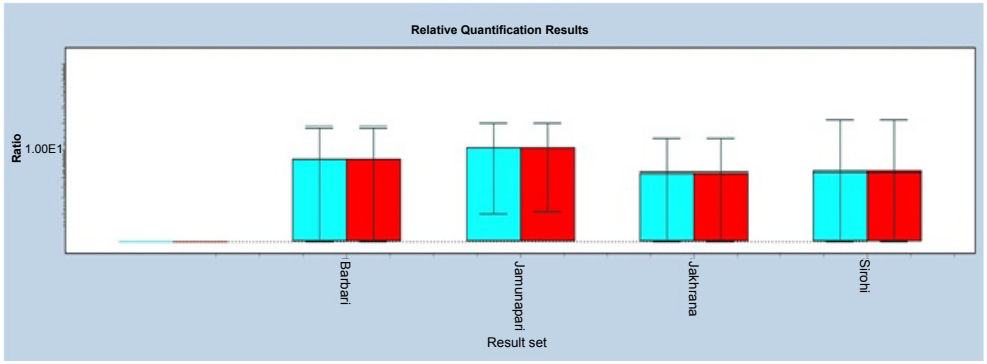

Figure 3: Gene expression pattern of ENOX2 gene in four different goat breeds.

Heat Stress Phenotype	No. of Sample	Target/Ref	Relative fold expression
Control	4	1	1
Susceptible	9	12.45	12.45
Tolerant	9	5.41	5.41

The environmental conditions of the heat stress period were THI (85.36-89.80), temperature (45-49.4), relative humidity (RH) (%) (14.33-27) and sunshine (h) (8-12).
Target gene- ENOX
Reference gene- GAPDH
Secondary derivative and E- method used for analysis

Table 5: Expression pattern of ENOX gene in heat stress-tolerant (HST) and heat stress- susceptible (HSS) phenotypes in response to chronic heat stress.

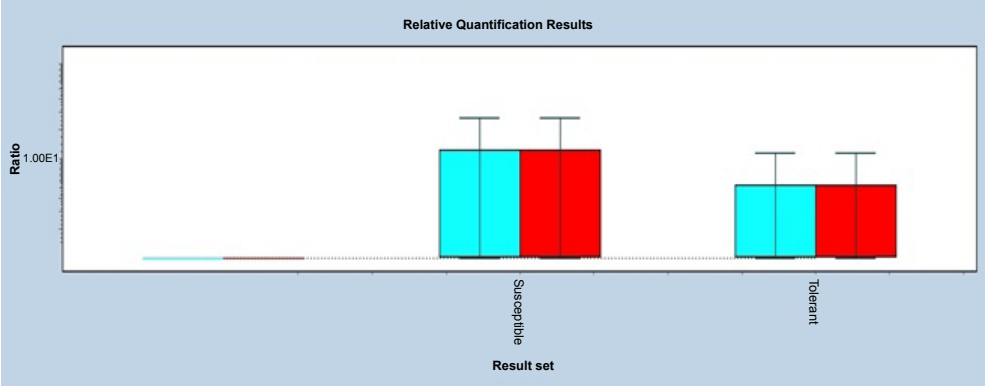

Figure 4: Expression pattern of ENOX2 gene in heat stress-tolerant (HST) and heat stress- susceptible (HSS) phenotypes in response to chronic heat stress.

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