Transcriptome profiling in Indian Cattle revealed novel insights into response to heat stress

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Abstract

Heat stress induced by high environmental temperature and humidity affects livestock production and health. With global warming on the uprise, indigenous cattle known for their heat tolerance are gaining importance than the Crossbreds. However, systems biology behind this phenotype in indigenous cattle is less known. This study, revealed novel insights in response to heat stress in Indian cattle (Tharparkar breed – Indigenous breed vs Vrindhavani – Crossbred).The number of DEGs in Crossbred were found to be more, than in Tharparkar, suggesting a greater dysregulation in systems biology in Crossbred. A contrast in gene expression was observed with 18.5 % of upregulated genes in Crossbred (Vrindavani cattle) were downregulated in Tharparkar and 17.5% upregulated genes in Tharparkar were downregulated in Crossbred. The increased HSPs levels have been found positively correlated with tolerance in many species. Upregulation of *HSF*, *HSP70*, *HSP90*, and activation of EIF2 signaling pathway in Tharparkar and vice-versa in Crossbred delineates how Tharparkar, withstands heat stress. Unlike Crossbred, Tharparkar is not only endowed with higher expression of the scavengers (*UBE2G1*, *UBE2S*, and *UBE2H*) of misfolded proteins but also with protectors (*VCP*, *Serp1*, and *CALR*) of naïve unfolded proteins. Further, the apoptotic genes that were dysregulated in both genetic groups indicated a relatively higher probability of apoptosis in Crossbred than in Tharparkar. Also, higher expression of the antioxidants in Tharparkar enables it to cope up with higher levels of

free radicals generated as a result of heat stress. In this study we found relevant molecules/genes dysregulated in Tharparkar in the direction that can counter heat stress. To best of our knowledge this is a comprehensive comparison between Tharparkar and Crossbred under heat stress using transcriptome analysis.

Introduction

Cattle being homoeothermic, modulate their internal body temperature in sync to environmental temperature by equilibrating the amount of heat produced within the body and dissipating it to the ambient environment. The stress that arises due to disproportionate thermodynamic behavior between cattle and its surrounding environment is termed as heat stress [1]. Environmental induced hyperthermic stress lowers feed intake, which in turn reduces growth, milk production and reproductive efficiency, thereby negatively affecting the economics of livestock keepers [2-4]. Heat stress has been associated with reduced fertility through its deleterious impact on oocyte maturation and early embryonic development [5]. Increased morbidity and mortality was observed in animals due to immune depressive effect of heat stress [6].

India has a wide variety of indigenous cattle breeds distributed throughout its agro-climatic zones. These are known for their natural tolerance to tropical heat [7,8].To meet the growing demand for milk and to combine the heat tolerance and tick resistance of zebu with the productivity of temperate dairy breeds [9] several crossbreeding programs were taken up in India. Every state had its own crossbreeding policy, which is agro-climatic and breed-specific. Though the zebu crosses with European breeds produced more milk than zebu, they were found not withstanding heat/solar radiation [10]. Crossbreds are susceptible to tropical diseases and require a constant input of good management conditions [8]. Antagonism exists between heat tolerance and milk productivity [11]. The adaptive capacity to heat stress varies between species and genetic groups within species. Among various adaptive mechanisms, physiological adaptability seems to be the primary step in cattle. Sahiwal cows better regulate body temperature in response to heat stress than Karan Fries [8]. It was observed that Ongole cattle rely on the respiration rate to maintain thermal balance, while, Bali cattle rely on rectal temperature [12]. In Brazil, Sindhi and Girolando breeds showed better physiological response to thermal stress than Gir cattle [13]. Increase in respiration rate was reported in Nellore breed when exposed to heat load [14].

In India, Tharparkar is one among the best dairy breeds. It is adapted to the Indian states of Punjab and Haryana. [15,16]. It is considered to be the hardiest, disease resistant, heat tolerant and tick resistant indigenous cattle breed of the country [17]. This breed has also been used in several crossbreeding programs. Currently, percentage of purebreds is exceptionally low in India (Department of Animal Husbandry & Dairying, Govt. of India). Most of the farmer's in India have Crossbreds and the percentage of exotic inheritance in these Crossbreds is unknown. Vrindavani, a Crossbred (synthetic), with 27% of Indigenous blood and 73% of exotic inheritance [18] is a representation of the kind of admixture that prevails in Indian cattle. Therefore, comparing Tharparkar with Vrindavani may establish the lost importance of Indigenous cattle and would further emphasize the need of conserving our indigenous purebreds because of advantageous traits like Heat tolerance.

Studies explaining the difference between the genetic groups (Crossbreds and Indigenous cattle) have been done mainly to address the physiological responses vis – a – vis heat stress and very few studies at the genomic level have been taken up [19,20].Also, there is an increasing need to develop methods by combining the knowledge from -omics technologies to identify heat tolerant animals [11]. Transcriptome profiling / RNA-Sequencing (RNA-seq) is a high throughput omics approach to measure relative global changes in the transcripts under specific condition(s) [21-23] to study the systems biology behind a phenotype [23,24]. RNA - seq allows for analysis of transcriptome in an unbiased way, with, a tremendous dynamic detection range (>8,000 fold), and low background signals [25]. It has been used as an investigating tool in understanding disease pathogenesis [26,27] and differential physiological response to various biotic and abiotic factors [28,29].

In this study, Tharparkar and Crossbred - Vrindavani cattle were subjected to heat stress and blood samples were collected on 0th day and 7th day, as it is known that short term acclimation occurs around 5 - 6 days [30,31]. The transcriptome of 7th day was compared and with 0th day in both the genetic groups to understand their differential response to heat stress.

Results

Physiological Parameters

The overview of the analysis is given in Figure 1. Respiration rate (RR), rectal temperature (RT) and T3 level increased significantly ($p<0.05$) on 7th - day post heat stress in

both the genetic groups (n=5) (Figure 2). However, the increase in RR, RT and T3 level, was found significantly ($P<0.05$) higher in Crossbred than in Tharparkar.

Comparison of DEGs of Crossbred and Tharparkar under heat stress

The differentially expressed genes for each genetic group were obtained on comparing the 0 day and 7th day RNA-seq data using EdgeR after obtaining the gene counts from RSEM. Under heat stress, global expression profiles of Crossbred and Tharparkar were identified with 6042 and 4718 differentially expressed genes (DEGs), respectively (Supplementary Table 1). Among these, 3481 DEGs were found common between the two genetic groups, while 2561 and 1238 DEGs were uniquely found in Crossbred and Tharparkar, respectively (Figure 3a). Additionally, 3132 and 2924 genes were upregulated and downregulated in Crossbred, respectively, while 2367 and 2358 genes were upregulated and downregulated in Tharparkar, respectively (Figure 3b). On comparison of upregulated and downregulated genes, 724 and 1416 genes were found uniquely upregulated and 514 and 1145 genes were found uniquely downregulated in Tharparkar and Crossbred, respectively. The comparison also revealed that 17.5% of upregulated genes (1278) in Tharparkar were downregulated in Crossbred and 18.5% downregulated genes (1344) in Tharparkar were upregulated in Crossbred. However, the number of common upregulated and downregulated genes in both the genetic groups were 357 (4.9%) and 498 (6.8%), respectively (Figure 3c).

Analysis of knowledge-based genes

Under heat stress four major physiological processes are found to be usually associated with heat stress viz. elicitation of unfolded protein response (UPR) in cells; Induction of apoptosis; Ubiquitination and; Imbalance in production of ROS and antioxidants (refer to materials and methods for details). Heat shock genes and its associated genes are involved in elicitation of unfolded protein response (UPR) in cells. Heat shock genes have been found dysregulated under heat stress in both the genetic groups. Most of the genes encoding Heat shock proteins (HSPs) - Heat shock 70 kDa protein 4 (*HSPA4),* Heat shock cognate 71 kDa protein *(HSPB8),* Heat shock 70 kDa protein 1A *(HSPA1A)* ,Heat shock cognate 71 kDa protein *(HSPA8),* Heat shock protein HSP 90-beta *(HSP90AB1)* and Heat shock protein HSP 90-alpha (*HSP90AA1*) and heat shock protein regulating factors- Heat shock factor 1 *(HSF1)* and Eukaryotic Translation Elongation Factor 1 Alpha 1 *(EEF1A1)* have been found to be

downregulated/not-differentially expressed in Crossbred but upregulated in Tharparkar. However, Calcium/Calmodulin Dependent Protein Kinase II Delta *(CAMK2D) that* is involved in the regulation of expression of heat shock genes was upregulated in Crossbred and downregulated in Tharparkar.

Among the apoptotic genes, genes encoding Bcl-2-like protein 11 (*BCL2L11)* , Tumor necrosis factor ligand superfamily member 6 *(FASLG)* , TIR domain-containing adapter molecule 2 *(TICAM2)* , Toll-like receptor 4 *(TLR4),* Adenomatous polyposis coli protein *(APC),* Caspase-3 *(CASP3),* Mitogen-activated protein kinase 8 *(MAPK8),* Mixed lineage kinase domain-like protein *(MLKL),* Late endosomal/lysosomal adaptor and MAPK and MTOR activator 5 *(XIP),* Vimentin *(VIM),* and High mobility group protein B2 *(HMGB2)* were found to be upregulated in Crossbred and downregulated in Tharparkar. The number of upregulated genes involved in achieving the balance of ROS production and antioxidants, were found to be more in Tharparkar than in Crossbred. Among these, genes encoding Glutathione peroxidase 3 (*GPX3),* Nudix Hydrolase 2 *(NUDT2),* Catalase *(CAT),* Cytochrome c *(CYCS),* Copper chaperone for superoxide dismutase *(CCS),* Peroxiredoxin-5 *(PRDX5),* Peroxiredoxin-6 *(PRDX6),* Peroxiredoxin-1 *(PRDX1),* Superoxide dismutase *(SOD1),* and Cytochrome b-245 heavy chain *(CYBB*) were found either downregulated/not-differentially expressed in Crossbred and upregulated in Tharparkar. More number of genes involved in Ubiquitination were differentially expressed in Crossbred than in the Tharparkar. Genes encoding Ubiquitin-conjugating enzyme E2 G1 (*UBE2G1),* Ubiquitin-conjugating enzyme E2 *(UBE2S),* Ubiquitin-conjugating enzyme E2 H *(UBE2H),* Ubiquitin A-52 residue ribosomal protein fusion product 1 *(UBA52),* and Ubiquitin-activating enzyme E1 *(UBA1)* have been found downregulated/not-differentially expressed in Crossbred and upregulated in Tharparkar. However, Valosin-containing protein *(VCP),* RING finger protein 40 *(RNF40),* and Ubiquitin-conjugating enzyme E2 L3 *(UBE2L3)* have been found downregulated in Crossbred but not-differentially expressed in Tharparkar. Among the genes involved in Unfolded Protein folding response (UPR), genes encoding Membrane-bound transcription factor site-1 protease (*MBTPS1),* Cyclic AMP-responsive element-binding protein 3-like protein 1 *(CREB3L1),* Stress-associated endoplasmic reticulum protein 1 *(SERP1)* , Glycogen synthase kinase-3 alpha *(GSK3A),* Eukaryotic translation initiation factor 2 subunit 3 *(EIF2S3),* Calreticulin *(CALR)* , and Stress-associated

endoplasmic reticulum protein 1 (*SERP1)* have been found downregulated in Crossbred and upregulated in Tharparkar (Figure 4).

Protein - protein interaction (PPI) network revealed functional importance of HSP70 (*HSPA8* and *HSPA1A)* and ubiquitin (UBB, UBA52), in coordinating genes involved in heat stress

A total of 246 knowledge-based genes were identified from the reactome database. Out of these 177 and 194 genes were found to be differentially expressed in Tharparkar and Crossbred, respectively. Among these 126 genes were found to be commonly differentially expressed in Tharparkar and Crossbred. PPI network for these common knowledge-based genes between Tharparkar and Crossbred was constructed (Supplementary Figure 1). In PPI networks, hubs define the functional and structural importance of a network. The genes, which act as hubs in PPI networks were found to be *UBB, UBA52, HSPA8*, and *HSPA1A* (Supplementary Figure 1). Among the 4 hubs, *UBB* was downregulated in both genetic groups and the rest were downregulated in Crossbred and upregulated in Tharparkar.

A change in the expression of the hub protein will have a larger effect than change in expression of non-hub proteins [32]. Therefore, UBB, UBA52, HSPA8, and HSPA1A are taken to be critical for coordinating the changes in systems biology under heat stress. The hubs HSPA8 and HSPA1A are connected to genes that are associated with regulation of stress viz. nucleoporins genes - *NUP188, NUP155, NUP210* & *NUP214*; BAG family molecular chaperone regulators - *BAG1, BAG3* & *BAG4*; Heat Shock Protein Family A - *HSPA5, HSPA4, HSPA12B* & *HSPA9*; DnaJ Heat Shock Protein Family i.e. HSP40 - *DNAJA1, DNAJC2* & *DNAJB6*; Heat shock factor - *HSF1*; Ubiquitin - *UBB* & *UBA52* and; Sirtuin - *SIRT1*. The hubs - UBB and UBA52 are connected to molecules of different proteasome subunits viz. α type subunits - *PSMA1* & *PSMA2*; β type subunits - *PSMB4* & *PSMB8*; ATPase subunits - *PSMC2* & *PSMC5* and non-ATPase subunits - *PSMD2* & *PSMD13*. These hubs were also found connected to ubiquitin specific peptidases - *USP9X* and *USP7* and Ubiquitin-conjugating enzyme - *UBE2B, UBE2G1, UBE2Z, UBE2H, UBE2J2, UBE2S* & *UBE2D2*.

Canonical pathway analysis by Ingenuity Pathway Analysis (IPA) revealed contrast in signaling pathways in Crossbred and Tharparkar

Canonical pathways associated with Crossbred and Tharparkar are represented in Figure 5a and 5b. In Crossbred, Oncostatin M Signaling, Phospholipase C Signaling, EIF2 Signaling,

Integrin Signaling, IL-3 Signaling, and CXCR4 Signaling were found to be highly inactivated (Z – score > 2.0) and PTEN signaling was found to be highly activated (Z – score < 2.0). In Tharparkar, EIF2 Signaling, Androgen Signaling, Oncostatin M Signaling, α-Adrenergic Signaling, BMP signaling pathway, and UVC-Induced MAPK Signaling were found to be highly activated and PTEN signaling was found to be inactivated. The canonical pathway Oncostatin M Signaling and EIF2 Signaling were found to have the highest ratio of genes involved vis-a-vis the genes in the database in Crossbred and Tharparkar, respectively.

While carrying out comparative analysis through IPA, Calcium-induced T Lymphocyte Apoptosis, BMP signaling pathway, UVC-Induced MAPK Signaling, Regulation of Cellular Mechanics by Calpain Protease, fMLP Signaling in Neutrophils, Melatonin Signaling, and Leukocyte Extravasation Signaling, were found inactivated in Crossbred and activated in Tharparkar (Supplementary Figure 2). Genes involved in Oncostatin M Signaling- Growth factor receptor-bound protein 2 (*GRB2)*, GTPase HRas *(HRAS)*, Janus kinase 1 *(JAK1)*, Janus kinase 3 *(JAK3)*, Mitogen-activated protein kinase kinase 1 *(MAP2K1)*, Mitogen-activated protein kinase 1 *(MAPK1)*, Oncostatin-M *(OSM)*, Ras-related protein Rap-1b *(RAP1B)*, Ras-related protein Rap-2a *(RAP2A)*, Signal transducer and activator of transcription 1-alpha/beta *(STAT1)*, Signal transducer and activator of transcription 5B *(STAT5B)*, Non-receptor tyrosine-protein kinase *(TYK2)*, and Ras-related protein *(RRAS)* were found downregulated in Crossbred and upregulated in Tharparkar (Figure 6a, b). While the key genes involved in PTEN Signaling pathway – Fas Ligand (*FASLG)*, member of RAS oncogene family *(RAP2A)*, Bcl-2-like protein 11 *(BIM)*,Caspase-3 *(CASP3)* and microspherule protein 1 (*MSP58)* were found upregulated in Crossbred and downregulated in Tharparkar as well (Figure 7a, b).

Variation in microRNAs and Transcription factors

IPA, on evaluating the differentially expression genes predicts miRNAs and Transcription Factors (upstream regulators). In Crossbred, 111 miRNAs were found to be inactivated and 37 activated. In Tharparkar, 205 miRNAs were found to be inactivated and 272 activated. Among them, 52 microRNAs were found common between the two genetic groups. Most of the common miRNAs were found activated in Crossbred and inactivated in Tharparkar (Supplementary Figure 3). miR-4779, miR-4651, miR-1207-5p, miR-6967-5p and miR-504-3p are the top 5 miRNAs that were activated in Crossbred and inactivated in Tharparkar.

Various Transcription factors were found to regulate the expression of the identified DEGs. Transcription factors, 19 in Tharparkar (11 activated and 8 inactivated) and 26 in Crossbred (8 activated and 18 inactivated) were identified in IPA that regulate the expression of DEGs. Among them, *PAX5, MTA3, MYC, PROX1* and *SMAD7* in Crossbred and, *HMGA1, MAF, MAX NOTCH22* and *NCOR1* in Tharparkar are the top 5 upregulated and activated TFs. On comparing the TFs of Tharparkar and Crossbred, it was found that *BHLHE40, HMGA1, HMGB1, IKZF1,* and *TCF7* were found to be common. *BHLHE40, HMGA1,* and *TCF7* were found to be activated in Tharparkar and inactivated in Crossbred and it was vice - versa with *HMGB1* and *IKZF1* (Supplementary Figure 4)

Real-time validation.

Six genes (*HSF1, SOD1, CALR, GSK3A, CAT* & *GPX3*) that were upregulated in Tharparkar but downregulated/not expressed in crossbred and four genes (*CASP3, FASLG, BCL2L11* & *APC*) that were upregulated in crossbred but downregulated in Tharparkar were considered for Real time PCR based on their role in heat stress. Briefly, CALR is involved in unfolded protein response upon Endoplasmic reticulum stress; HSF1 in trimer induces the expression of HSPs; GSK3A, kinase inhibits trimerization of HSF1 regulating the expression of HSPs; SOD1 converts free radical into hydrogen peroxide, which is further converted by GPX3A and/or CAT into water and oxygen molecules in regulatory pathway of ROS scavenging and ; BCL2L11, FASLG, APC and CASP3 induce apoptosis in stressed cell. The expression of genes was in concordance with the RNA- Seq results (Supplementary Figure 5 and Supplementary table 2).

Discussion

Heat stress is a natural phenomenon that affects domestic animals in tropical, sub-tropical and often in temperate regions of the world during summer months. Heat and humidity during the summer months combine to make an uncomfortable environment for dairy cattle. Heat stress negatively impacts a variety of dairy parameters resulting in economic losses [33]. Response to heat stress varies with species and genetic groups within species [5,34,35]. In this study, transcriptome of genetic groups – Crossbred and Tharparkar cattle under heat stress was evaluated to understand their differential response to heat stress.

Animals (n=5) of both the genetic groups were exposed to a temperature of 42 °C for 7 days. Around 5th- 6th day, short term heat acclimation occurs [30,31]. This time point was selected to understand the differences in systems biology to heat stress in the two genetic groups. Initially, heat stress indicators - RR, RT, and T3 level were evaluated. RR was found to increase in both genetic groups under heat treatment and the increase in Crossbred was found to be significantly ($P<0.05$) different from that in Tharparkar. A positive correlation exists between RR and heat treatment [36-38]. This increase is an attempt to dissipate excess body heat by vaporizing more moisture in expired air or response to a greater requirement of oxygen by tissues under heat stress. Also, the physiological response to heat stress includes reduced heat production, which is achieved by lowering feed intake and thyroid hormone secretion [39]. T3 level increases under heat stress [40,41]. A significant increase in T3 level in Crossbred as compared to Tharparkar indicates an effective regulatory mechanism in modulating T3 levels in Tharparkar in response to heat stress. The T3 triggered metabolism may be one of the reasons that increases heat production resulting in high rectal temperature in Crossbred in comparison to Tharparkar as was found in our study. The significant increase in RR, RT and T3 level in Crossbreed than in Tharparkar, suggests the inability of Crossbred to cope up with heat stress in comparison to Tharparkar.

A phenotype is defined by the changes in systems biology. Transcriptome profiling by RNA-seq is the most common methodology to study the changes in systems biology. RNA profiling based on next-generation sequencing enables to measure and compare gene expression patterns[21]. The transcriptome of Tharparkar and Crossbred indicated differential response to heat stress as evident from the DEGs, which were either distinct to both or have a difference in expression. The number of DEGs were higher in Crossbred than in Tharparkar, suggesting a greater dysregulation in systems biology in Crossbred. Among the dysregulated genes, the number of upregulated genes were more than the downregulated genes in both genetic groups. However, a contrast in expression was observed with 18.5 % of upregulated genes in Crossbred, were downregulated in Tharparkar and 17.5% upregulated genes in Tharparkar weredownregulated in Crossbred. Some of the genes were confirmed for their expression by Realtime PCR. The genes selected for Realtime PCR were selected based on their role in the major physiological processes usually associated with heat stress.

IPA revealed activation or inactivation of several pathways in both the genetic groups. It is known that - EIF2 signalling, helps in initiation of global protein translation [42]; MAPK-signalling pathway, induces cell proliferation [43]; androgen signalling, enhances pro-survival and anti-apoptotic activity in cell [44]; a-Adrenergic signalling, maintains immune defence mechanism [45] and, helps in tissue repair upon stress [46] and increases angiogenesis [47]; integrin pathway, resists the cell against apoptosis and other environmental insults [48]; IL-3 signalling, aids in cell survival and haematopoiesis [49]; CXCR4 signalling modulates cell survival and cell motility [50] and ; Phospholipase C signalling aids in cell survival in stress through protein kinase C dependent phosphorylation of BCL-2 [51]. Inactivation of these pathways except MAPK-signalling pathway in Crossbred and activation of α-Adrenergic signalling, Androgen signalling, EIF2 signalling and MAPK signalling in Tharparkar indicates that the systems biology in Tharparkar is moving towards countering the effects due to heat stress.

While exploring the DEGs at a functional level we considered a knowledge-based approach. Under this, four major physiological processes are found to be usually associated with heat stress viz. elicitation of unfolded protein response (UPR) in cells; Ubiquitination; Induction of apoptosis and; Imbalance in production of ROS and antioxidants (Figure 13). Heat shock and its associated genes are involved in elicitation of unfolded protein response (UPR) in cells. Most of the heat shock genes were found upregulated in Tharparkar and downregulated in Crossbred. The increased HSP levels have been found positively correlated with tolerance in many species[52,53]. HSF1, that positively regulates the transcription of *HSP70* and *HSP90* [54,55] was found upregulated in Tharparkar and downregulated in Crossbred. Upregulation of *HSF1, HSP70 and HSP90* in Tharparkar and vice-versa in Crossbred corroborates to state that Tharparkar is better equipped to counter heat stress than Crossbred. Further, to ensure that the HSP70 in Tharparkar is maintained at an optimum level, dysregulation of *CAMK2D* and *GSK3A* seems to act as negative feedback. CAMK2D that induces the transcription of HSP70 via HSF1 [56] was found downregulated in Tharparkar. GSK3A that inhibits the trimerization of HSF1 that is needed for the induction of HSP70 [57] was found upregulated in Tharparkar. The decreased level of HSP70 in Crossbred makes it inevitable that such negative feedbacks would further reduce its level and hence, *GSK3A* was found downregulated and *CAMK*, upregulated (Figure.13).

Ubiquitination is positively correlated with heat tolerance [58,59]. Ubiquitin-Proteasome System (UPS) regulates the levels of proteins and acts by removing the misfolded or damaged proteins that may accumulate as a result of exposure to abiotic stress. Malfunctioning of ubiquitin-proteasome system (UPS) could have negative consequences for protein regulation, including loss of function [60]. In Tharparkar after heat acclimation, HSP70 tends to activate the ubiquitination pathway to minimize the accumulation of the unfolded proteins that can't be refolded by it [61]. This pathway activation is supported by upregulation of E2 ligases - *UBE2G1*, *UBE2S*, and *UBE2H* that catalyze covalent attachment of E2 to E3 [62-65] in Tharparkar. USP7 that deubiquitinates target proteins [66,67] was found upregulated in Crossbred and downregulated in Tharparkar. Further, a group of molecules – *VCP*, *SERP1*, and *CALR* that ensure the protection of naïve proteins during their transport within the cell [68-70] were found upregulated in Tharparkar and downregulated in Crossbred. Unlike Crossbred, Tharparkar is not only endowed with higher expression of the scavengers of misfolded proteins but also with protectors of naïve unfolded proteins.

Activation of apoptosis pathway is one of the major physiological processes linked with heat stress. Among the apoptotic genes, *BCL2L11*, *FASLG*, *MLKL*, *CASP3*, *MAPK8*, and *VIM* have been found upregulated in Crossbred and downregulated in Tharparkar under heat stress. BCL2L11 induces apoptosis by neutralizing key molecules of pro-survival BCL2 sub-family [71,72], FASLG transduces the apoptotic signal into cells[73,74], CASP3 activates caspases and executes apoptosis [75], and MAPK8, MLKL, and VIM also induce apoptosis [76,77]. PTEN signaling pathway that drives apoptosis [78,79] was found inactivated in Tharparkar and activated in Crossbred. This indicates a relatively higher probability of apoptosis in Crossbred than in Tharparkar.

The ability to balance the ROS and antioxidant level, is one of the key factors that would determine the tolerance of an individual to heat stress. The antioxidant triad of GPX, SOD, and CAT that forms the first line of defense against reactive oxygen species [80-82], was found upregulated in Tharparkar and downregulated in Crossbred. Additionally, genes belonging to Peroxiredoxins - *PRDX3*, *PRDX5* and *PRDX6* that catalyze the reduction of hydrogen peroxide and organic hydroperoxides [83-87],were also found upregulated in Tharparkar and were either downregulated or not-differentially expressed in Crossbred. Higher expression of the

antioxidants in Tharparkar enables it to cope up with higher levels of free radicals generated as a result of heat stress while Crossbred is unable to do so.

Conclusion

A contrast in expression was observed with 18.5 % of upregulated genes in Crossbred were downregulated in Tharparkar and 17.5% upregulated genes in Tharparkar were downregulated in Crossbred. Transcripts of molecules that stimulate heat shock response, Ubiquitination, unfolded protein response and antioxidant level were found upregulated in Tharparkar and downregulated in Crossbred. EIF2 Signaling that promotes protein translation and PTEN signaling that drives apoptosis were found activated and inactivated in Tharparkar, respectively and vice-versa in Crossbred. We found relevant molecules/genes dysregulated in Tharparkar in the direction that counters heat stress. A proposed contrasting interplay of molecules in both the two groups is shown in Figure 8. To the best of our knowledge this is a comprehensive comparison between Tharparkar and Crossbred at a global level using transcriptome analysis.

Methods

Experimental condition and Ethical Statement

The animals used for the study were from the Indian Veterinary Research Institute. The permission to conduct the study was granted by Indian Veterinary Research Institutional Animal Ethics Committee (IVRI-IAEC) under the Committee for Control and Supervision of Experiments on Animals (CPCSEA), India, vide letter no 387/CPSCEA. Genetic groups - Tharparkar (Indigenous breeds) and Vrindavani (synthetic Crossbred) were considered in this study. Prior to experiment, the animals – 05 Tharparkar and 05 Crossbred (Vrindavani) cattle, were acclimatized for 15 days outside the Psychometric chamber. The experiment was conducted during October when the environmental Temperature Humidity Index (THI) was 73.0242. These animals were exposed in Psychometric chamber at 42 °C for six hours for 7 days (THI =78.5489). All the animals were fed with wheat straw and concentrate mixture in 60:40 ratios. Respiration rate (RR) and rectal temperature (RT) of animals from each genetic group were measured on 0 day (Control, n=5) before exposure to Psychometric chamber and on 7th day of heat exposure (Treated, n=5). Blood samples were collected from the animals at the mentioned

time points and serum concentration of Triiodothyronine (T3) was estimated by RIA technique using T_3 ^{125}I (Immunotech) as per the manufacturer's instructions.

RNA sequencing (RNA-seq**)**

PBMCs were collected from the blood samples using Ficol histopaque gradient method**.** Total RNA from each of the collected samples (PBMCs) was isolated using the RNeasy Mini kit (Qiagen GmbH, Germany) according to the manufacturer's protocol. The integrity and quantity of isolated RNA were assessed on a Bioanalyzer 2100 (Agilent Technologies, Inc). The library was prepared using NEBNext Ultra RNA Library Prep Kit for Illumina (NewEngland Biolabs Inc.) following the manufacturer's protocol. Approximately, 100ng of RNA from each sample was used for RNA library preparation. The quality of the libraries was assessed on Bioanalyzer. Libraries were quantified using a Qubit 2.0 Fluorometer (Life technologies) and by qPCR. Library (1.3ml, 1.8pM) was denatured, diluted and loaded onto a flow cell for sequencing. cDNA library preparation and Illumina Sequencing was performed at Sandor Life Sciences Pvt. (Hyderabad, India). Finally, the RNA-seq data were provided in FASTQ format.

Data processing

The sequenced reads were paired-end and 150bp in length. Quality control checks on raw sequence data from each sample were performed using FastQC (Babraham Bioinformatics). Processing of the data was performed using prinseq-lite software [88] to remove reads of low quality (mean phred score 25) and short length (< 50) for downstream analysis. The data was submitted to the GEO database with accession number GSE136652.

Identification of Differentially Expressed Genes (DEGs)

Bos taurus reference genome (release 94) and its associated gene transfer file (GTF) were downloaded from Ensembl FTP genome browser [89]. The reference genome was prepared and indexed by RNA-Seq by expectation maximization (RSEM) [90] by rsem-prepare-reference command. Further, the clean reads obtained from filtering of raw data were aligned to the indexed reference genome by Bowtie2 [91] to estimate gene abundance in counts by rsem-calculate-expression command. To compare the gene expression levels among different samples, the aligned reads were used to generate a data matrix by rsem-generate-data-matrix command. In each genetic group, all the samples of day 7 (treated) were compared with the day 0 (Control)

for the calculation of differential gene expression by edgeR [92] package. The Ensemble IDs of the differentially expressed genes (DEGs) were converted to the respective gene ID by g: Convert of g: Profiler [93,94].

Functional Analysis of DEGs

Under heat stress four major physiological processes are found to be usually associated - Induction of apoptosis [95,96]; Ubiquitination [97,98]; elicitation of unfolded protein response (UPR) in cells [99] and ; Imbalance in production of ROS and antioxidants [100,101]. The genes involved in these processes were retrieved from Reactome database [102]. From these genes that are involved in the four major physiological processes, the genes that were differentially expressed in both the genetic groups were extracted to compare and contrast their expression between genetic groups. These genes were referred to as knowledge-based genes. Their protein-protein interaction network was also studied in both the genetic groups.

Predicted protein-protein interaction of the knowledge-based genes

Protein-protein interaction (PPI) network among the knowledge-based DEGs that were found common to both Tharparkar and Crossbred, was retrieved using interactions available in the String database [103]. The degree was calculated using igraph package (https://cran.r-project.org/web/packages/igraph/ index.html). The PPI network was then visualized using Cytoscape software V. 3.7 [104]

Ingenuity Pathway Analysis (IPA) Analysis

QIAGEN's IPA (QIAGEN, Redwood City, USA) [105] is used to quickly visualize and understand complex omics data and perform insightful data analysis and interpretation by placing experimental results within the context of biological systems. Here, IPA was used to analyze the identified DEGs of Crossbred and Tharparkar.The list of DEGs from each genetic group was used to identify the canonical pathways and the most significant biological processes against Ingenuity Pathways Knowledge Base (IKB). Core analysis for each dataset was performed to know activated (Z score > 2) or inactivated (Z score < −2) canonical pathways. Upstream regulators - Transcription factors and microRNAs were also identified.

Validation of reference genes identified

Genes - *BCL2L11, FASLG, CASP3, CAT, SOD1, GSK3A, CALR, HSF1, APC,* and *GPX3* were selected based on their role in heat stress and qRT-PCR was performed on Applied Biosystems 7500 Fast system. *GAPDH* was taken as the internal control. Each of the samples was run in triplicates and relative expression of each gene was calculated using the $2^{-\Delta\Delta CT}$ method with control as the calibrator [106].

Statistical Analysis

Respiration rate, Rectal temperature and T3 level were compared using student's *t*-test in JMP9 (SAS Institute Inc., Cary, USA) to test the significance of the difference between the control (0 day) and treated (7th day). This comparison was done within and between genetic groups. Differences within/between groups were considered significant at $P \leq 0.05$.

Declarations

Ethics approval and consent to participate

The permission to conduct the study was granted by Indian Veterinary Research Institutional Animal Ethics Committee (IVRI-IAEC) under the Committee for Control and Supervision of Experiments on Animals (CPCSEA), India, vide letter no 387/CPSCEA.

Consent for publication

Not applicable.

Availability of data and materials

The data was submitted to the GEO database with accession number GSE136652

Competing interests

None of the authors had a conflict of interest to declare

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in relation to diseases" for the funds to carryout Sampling, Wet Lab experiments and for the procurement of License of IPA.

Authors' contributions

AKT and RG conceived and designed the research. SG, SmS, AS,AV, VV , PK , ShS and GS conducted the wet lab work. RINK, ARS, NH, WAM, MRP, SK , AP and RG analyzed the data. RINK, ARS, MRP, RG , AS and GS helped in manuscript drafting and editing. AKT and RG proofread the manuscript

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Legends

Figure 1: Overview of the work done : Two genetic groups (Tharparkar and Crossbred) of cattle were exposed to a temperature of 42 °C for 7 days. Heat stress indicators - Respiration rate (RR), Rectal temperature and T3 level before exposure to heat (0day – control group) and at 7th day of exposure (treated) were measured to evaluate heat stress. At these time points, RNA was isolated from PBMCs for high throughput sequencing. Transcriptome analysis was done to identify differentially expressed genes (DEGs) under heat treatment in both genetic groups. Genes involved in physiological processes (heat stress response, apoptosis, ubiquitination, unfolded protein response and antioxidant level) that are commonly associated with heat stress were

compared between the two genetic groups. Further, functional annotation of DEGs was done using IPA.

Figure 2: Respiration rate, Rectal Temperature and T3 level measured at 0 day (control) and 7 day post-heat exposure (treated) in Crossbred and Tharparkar (n=5) Levels sharing the same superscript are not significantly ($P > 0.05$) different from each other.

Figure 3: Expression of DEGs in Crossbred and Tharparkar under heat stress: (a) Venn diagrams showing unique/common DEGs between Crossbred and Tharparkar (b) Number of upregulated and downregulated in both genetic groups (c) Contrast in the expression of common DEGs

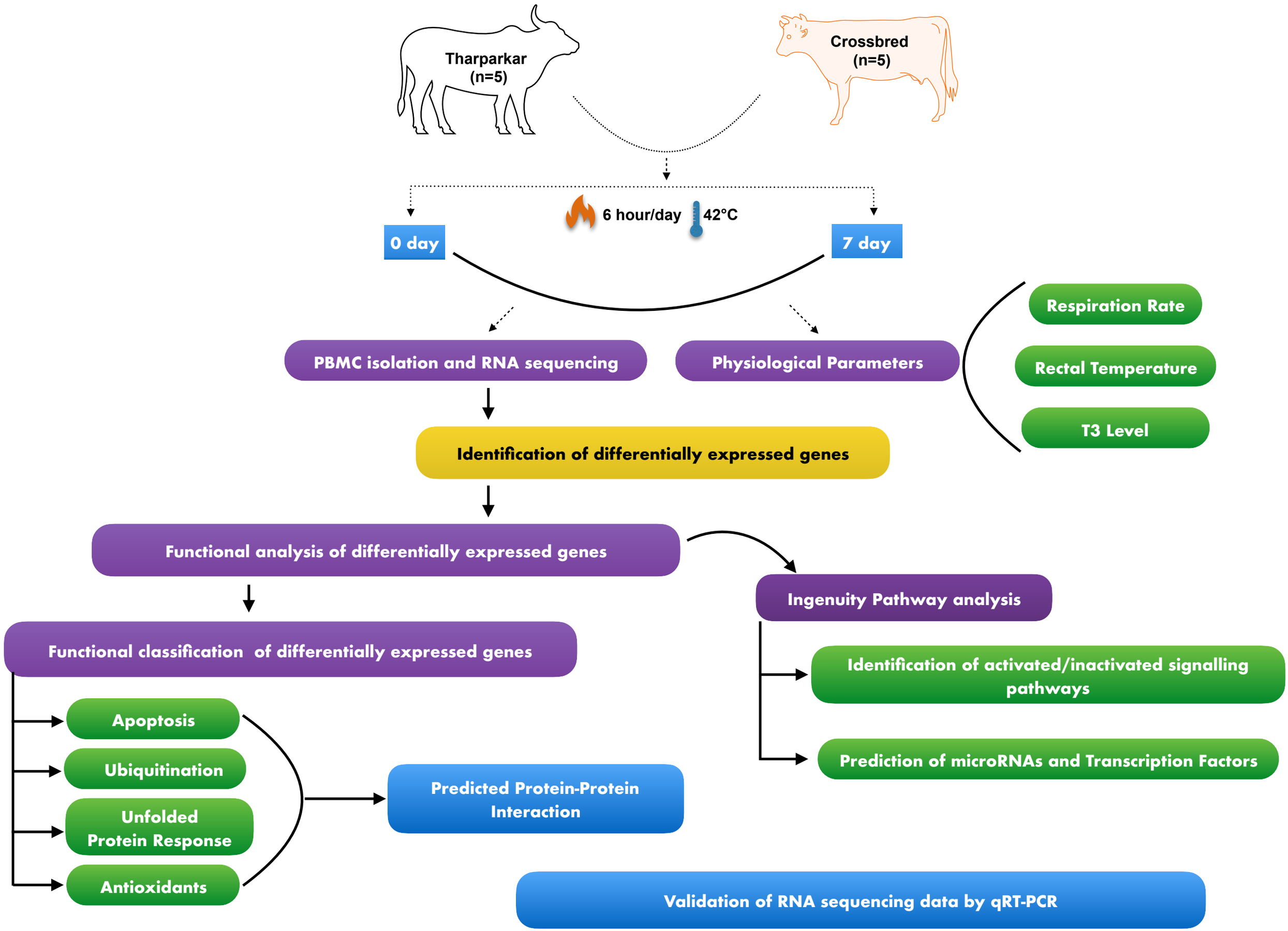
Figure 4: Contrast in the expression of genes involved in heat stress response, apoptosis, ubiquitination, unfolded protein response and balance in the production of ROS and antioxidants between two genetic groups.

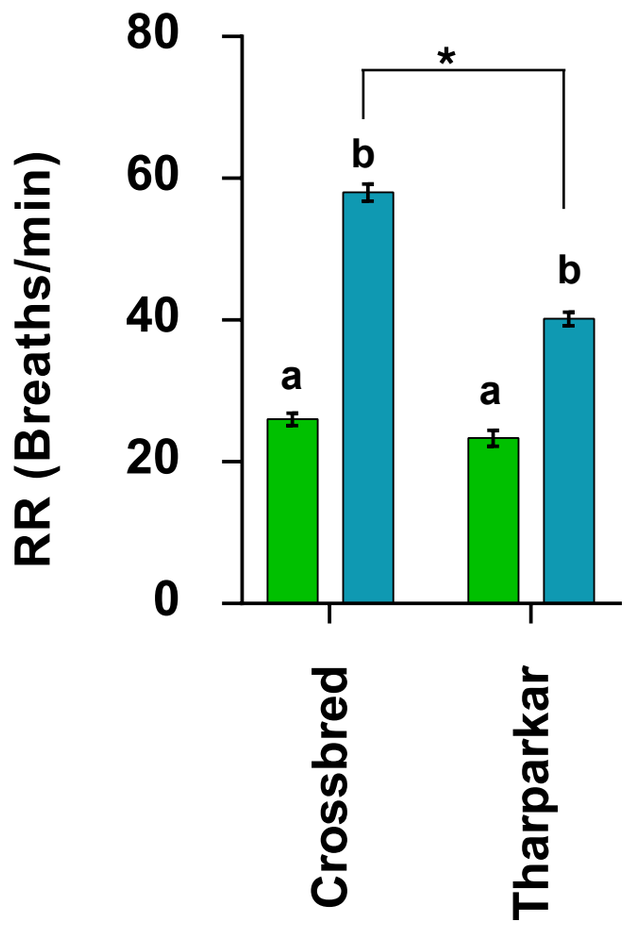
Figure 5 : Canonical pathways activated/inactivated in (a) Crossbred (b) Tharparkar under heat stress generated in the core analysis of Ingenuity pathway analysis tool. Orange color pathways are activated ($Z > 2$) and blue color pathways are inactivated ($Z < -2$). Height of the bar graphs indicates -log (p-value) and line graph showing the ratio of list genes found in each pathway over the total number of genes in that pathway.

Figure 6: Canonical pathways generated in Ingenuity Pathway Analysis of Oncostatin M signaling pathway of DEGs in (A) Crossbred, (B) Tharparkar. Genes that were upregulated are shown in red and downregulated in green. The intensity of red and green corresponds to an increase and decrease, respectively, in Log2 fold change. Genes in grey were not significantly dysregulated and those in white are not present in the dataset but have been incorporated in the network through the relationship with other molecules by IPA.

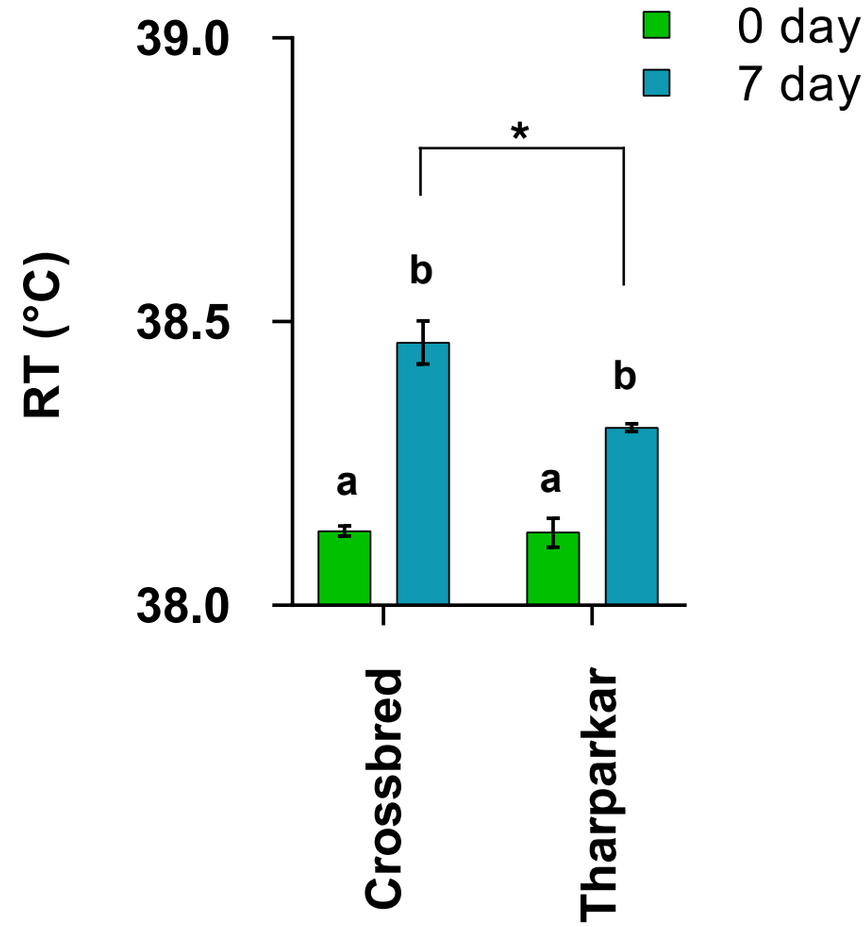
Figure 7: Canonical pathways generated in Ingenuity Pathway Analysis of PTEN signaling pathway of DEGs in (A) Crossbred, (B) Tharparkar. Genes that were upregulated are shown in red and downregulated in green. The intensity of red and green corresponds to an increase and decrease, respectively, in Log2 fold change. Genes in grey were not significantly dysregulated and those in white are not present in the dataset but have been incorporated in the network through the relationship with other molecules by IPA.

Figure 8: Predicted interplay of molecules that is underway during heat stress in both groups : Heat stress causes unfolding of native proteins. HSP70 acts as a chaperone to facilitate refolding to restore the structure of unfolded proteins. Under normal condition, HSP70 is bound to HSF1 thereby preventing HSF1 to promote transcription of HSP70. Under heat stress ATP binds to the HSP70 and HSF1 complex to release HSF1, promoting the binding of the unfolded protein to HSP70 and ATP. CAMK2D that induces the transcription of HSP70 via HSF1 was found downregulated in Tharparkar. GSK3A that inhibits the trimerization of HSF1 that is needed for the induction of HSP70 expression was found upregulated in Tharparkar. The decreased level of HSP70 in Crossbred makes it inevitable that such negative feedbacks would further reduce its level and GSK3A was found downregulated and CAMK2D, upregulated. Further, in Tharparkar, HSP70 tends to activate ubiquitination pathway to decrease the accumulation of unfolded proteins that can't be refolded by it. This pathway activation is supported by upregulation of E3 ligases (UBE2G1, UBE2S, and UBE2H) in Tharparkar. However, the E3 ligase in Crossbred was found downregulated. With HSP70 being upregulated and having cytoprotection activity, Tharparkar shows the decline in apoptosis as compared to Crossbred. This is supported by downregulation of BCL2L11, FASLG, MLKL, CASP3, MAPK8 and VIM in Tharparkar and vice-versa. Besides, higher expression of the antioxidants (SOD, CAT, GPX) in Tharparkar enables it to cope up with higher levels of free radicals generated as a result of heat stress while Crossbred is unable to do so. Green arrow indicates downregulation and Maroon arrow indicates upregulation.

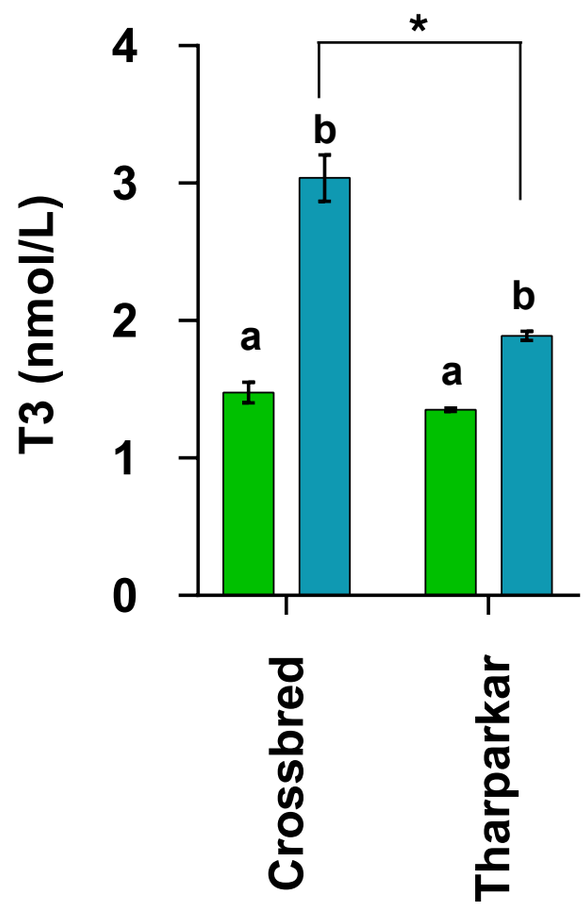

Tharparkar
(n=5)
Crossbred
(n=5)
6 hour/day
42°C
0 day
7 day
PBMC isolation and RNA sequencing
Physiological Parameters
Respiration Rate
Rectal Temperature
T3 Level
Identification of differentially expressed genes
Functional analysis of differentially expressed genes
Ingenuity Pathway analysis
Identification of activated/inactivated signalling pathways
Prediction of microRNAs and Transcription Factors
Functional classification of differentially expressed genes
Apoptosis
Ubiquitination
Unfolded Protein Response
Antioxidants
Predicted Protein-Protein Interaction
Validation of RNA sequencing data by qRT-PCR


RR (Breaths/min)
80
60
40
20
0
*
a
b
a
b
Crossbred
Tharparkar

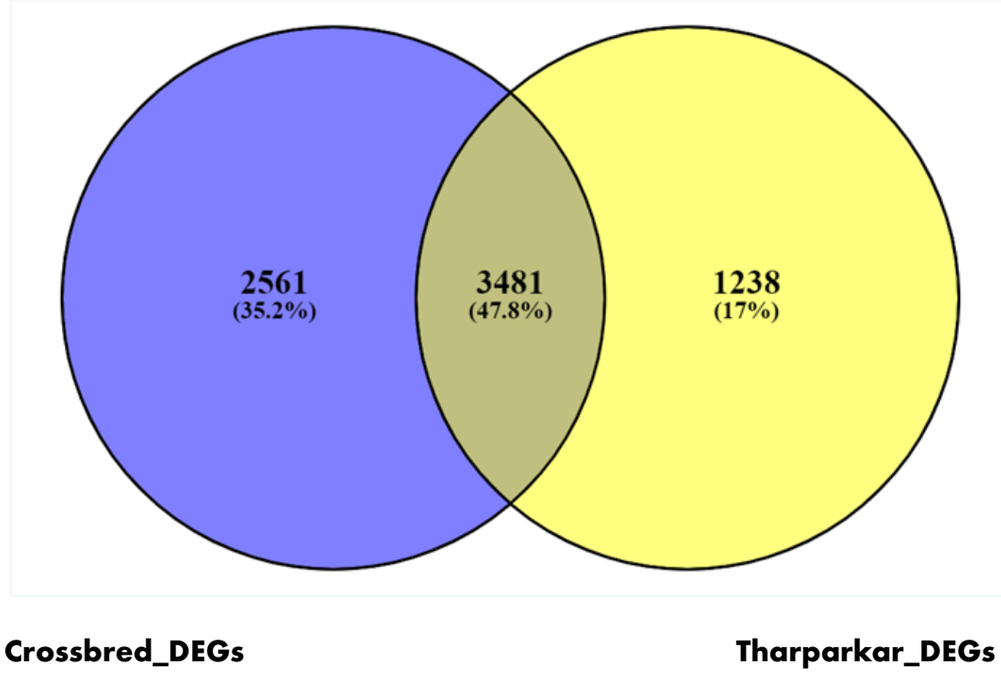
(a)


0 day
7 day
RT (°C)
39.0
38.5
38.0
*
a
b
a
b
Crossbred
Tharparkar

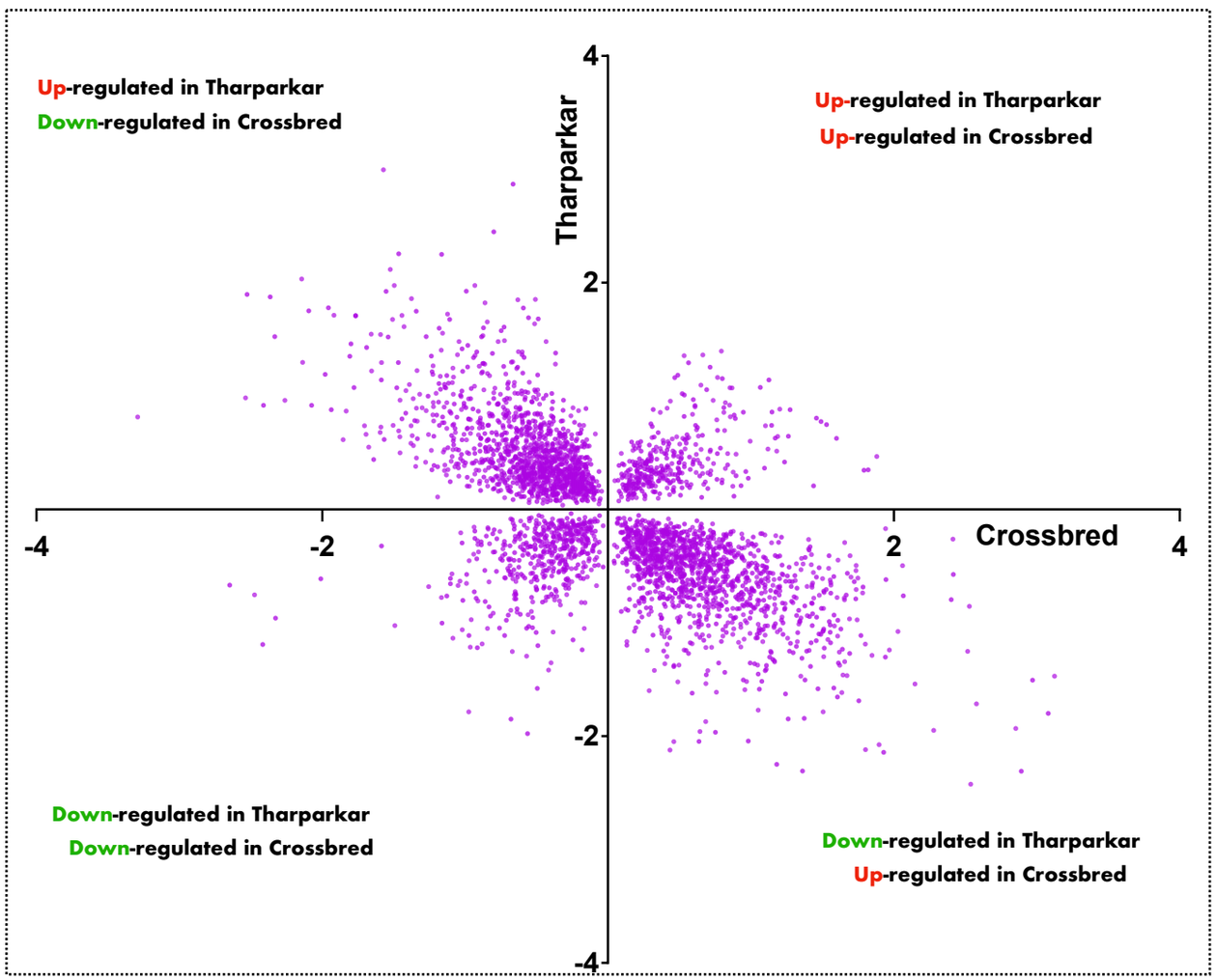
(b)


T3 (nmol/L)
4
3
2
1
0
*
a
b
a
b
Crossbred
Tharparkar

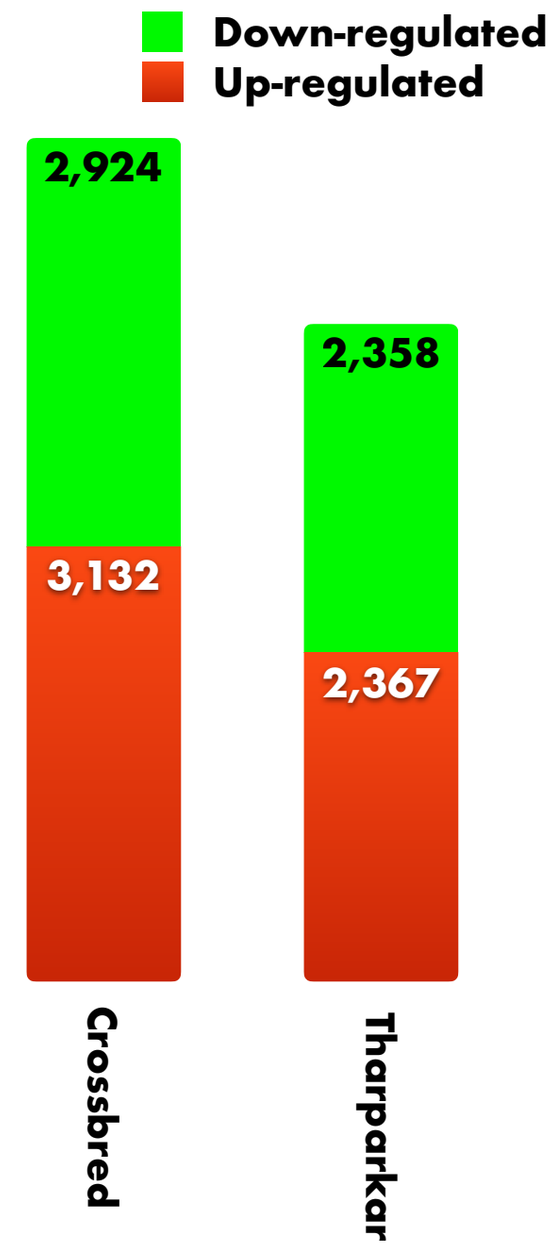
(c)


2561
(35.2%)
3481
(47.8%)
1238
(17%)
Crossbred_DEGs
Tharparkar_DEGs

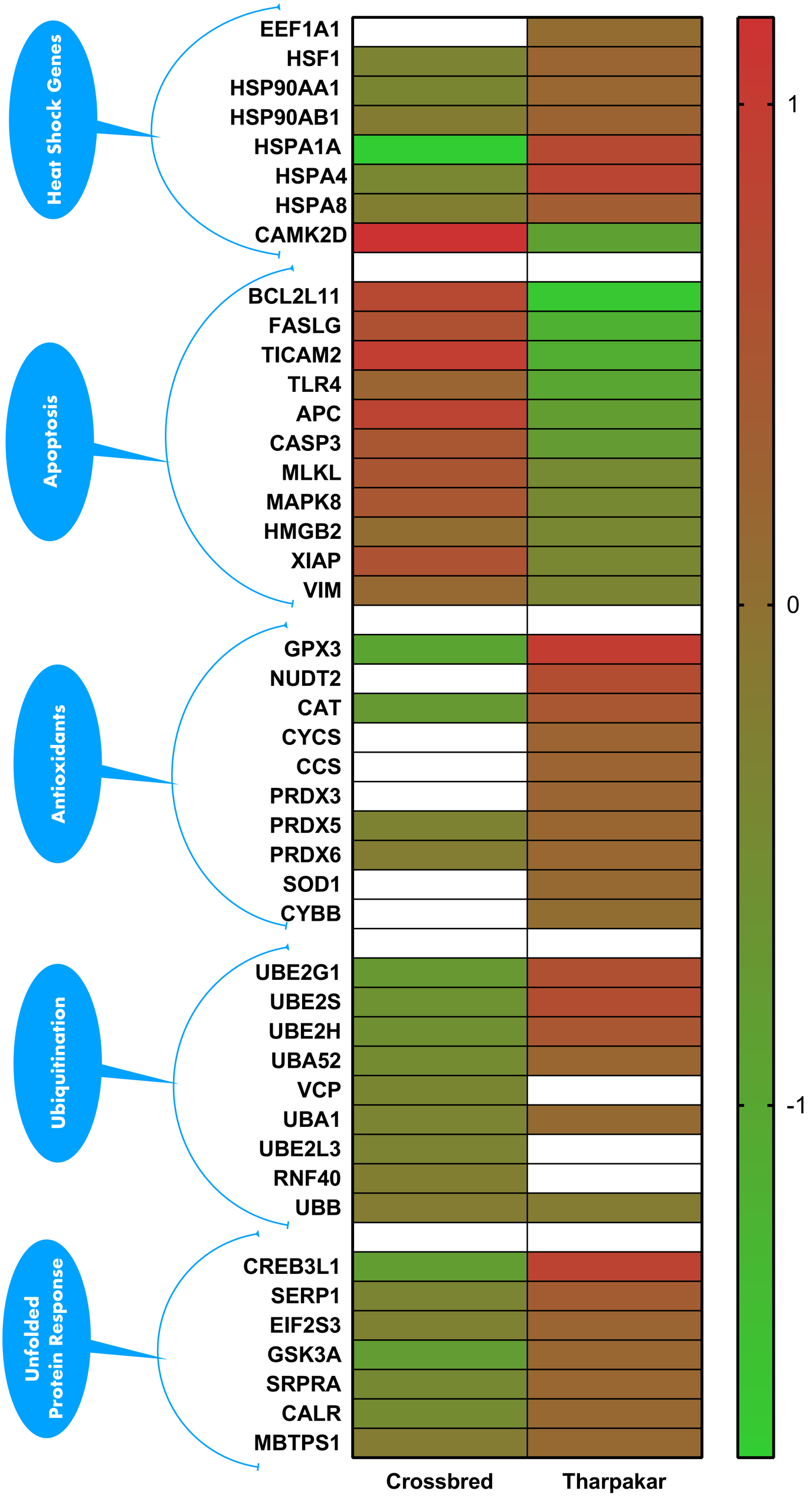
(a)

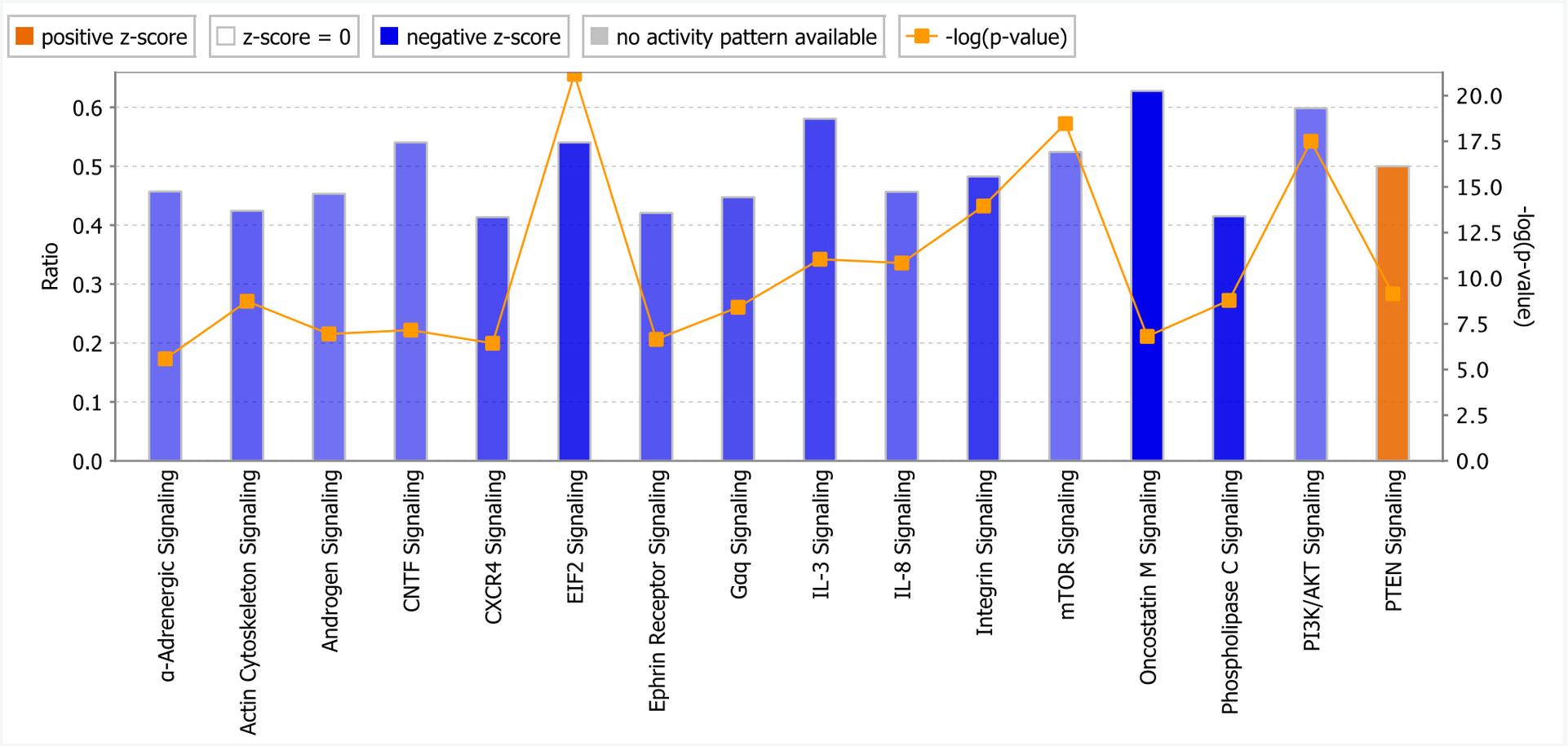

Up-regulated in Tharparkar
Down-regulated in Crossbred
Up-regulated in Tharparkar
Up-regulated in Crossbred
Down-regulated in Tharparkar
Down-regulated in Crossbred
Down-regulated in Tharparkar
Up-regulated in Crossbred
Tharparkar
Crossbred
4
2
-2
-4
-4
-2
2
4

(c)

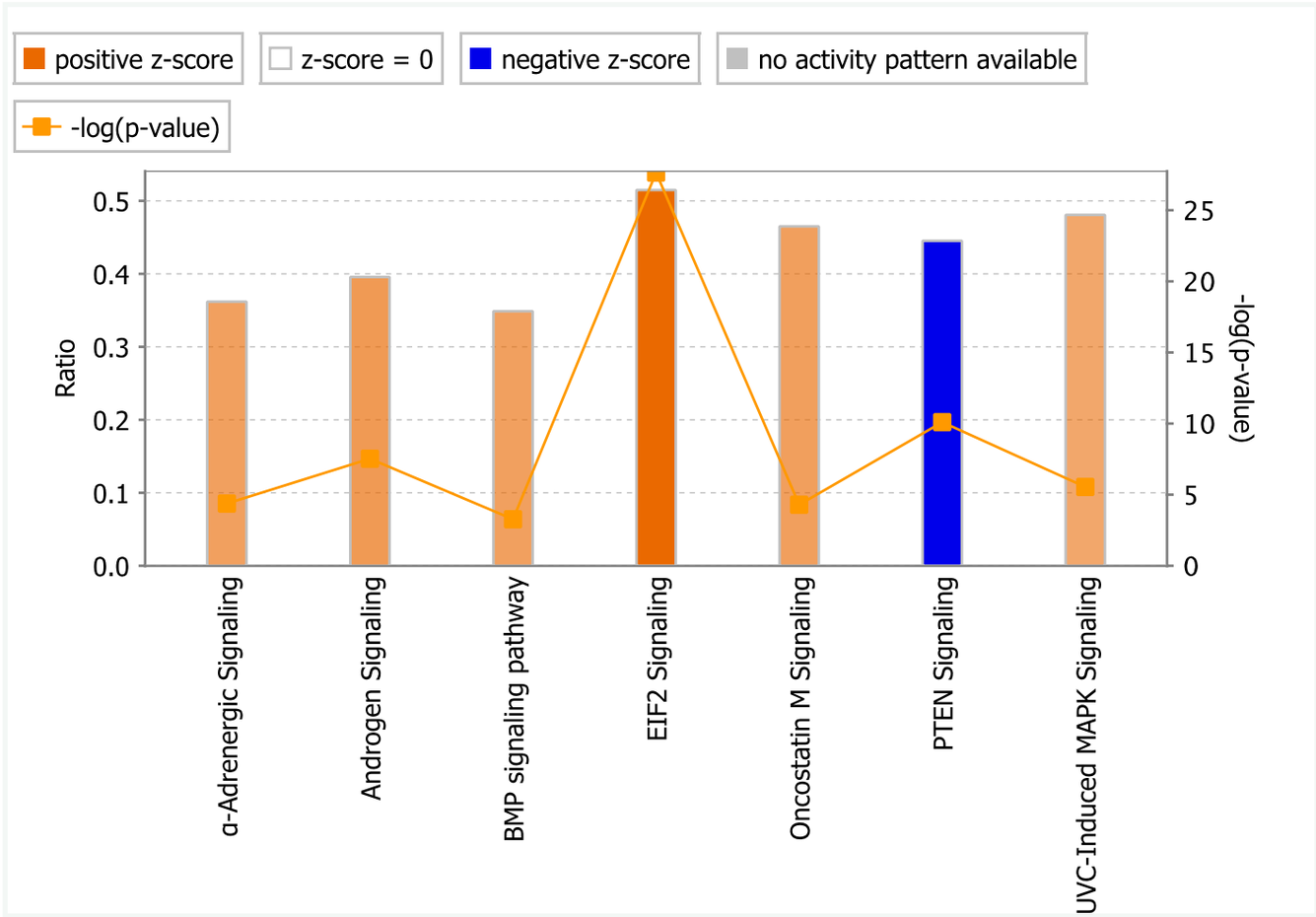

Down-regulated
Up-regulated
2,924
3,132
2,358
2,367
Crossbred
Tharparkar

(b)

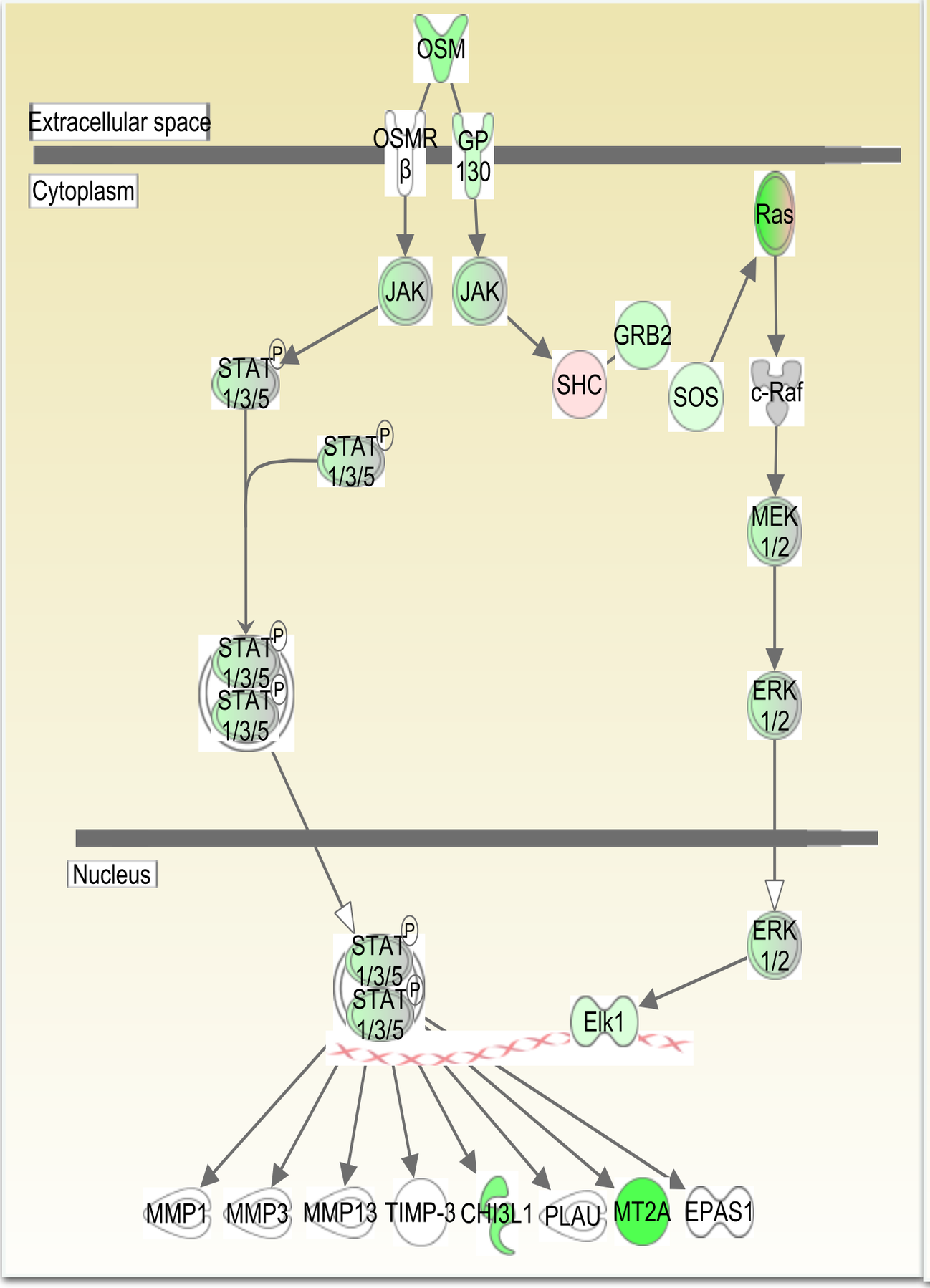

Heat Shock Genes
EEF1A1
HSF1
HSP90AA1
HSP90AB1
HSPA1A
HSPA4
HSPA8
CAMK2D
Apoptosis
BCL2L11
FASLG
TICAM2
TLR4
APC
CASP3
MLKL
MAPK8
HMGB2
XIAP
VIM
Antioxidants
GPX3
NUDT2
CAT
CYCS
CCS
PRDX3
PRDX5
PRDX6
SOD1
CYBB
Ubiquitination
UBE2G1
UBE2S
UBE2H
UBA52
VCP
UBA1
UBE2L3
RNF40
UBB
Unfolded Protein Response
CREB3L1
SERP1
EIF2S3
GSK3A
SRPRA
CALR
MBTPS1
Crossbred
Tharpakar
1
0
-1


positive z-score
z-score = 0
negative z-score
no activity pattern available
-log(p-value)
Ratio
-log(p-value)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.0
2.5
5.0
7.5
10.0
12.5
15.0
17.5
20.0
α-Adrenergic Signaling
Actin Cytoskeleton Signaling
Androgen Signaling
CNTF Signaling
CXCR4 Signaling
EIF2 Signaling
Ephrin Receptor Signaling
Gαq Signaling
IL-3 Signaling
IL-8 Signaling
Integrin Signaling
mTOR Signaling
Oncostatin M Signaling
Phospholipase C Signaling
PI3K/AKT Signaling
PTEN Signaling

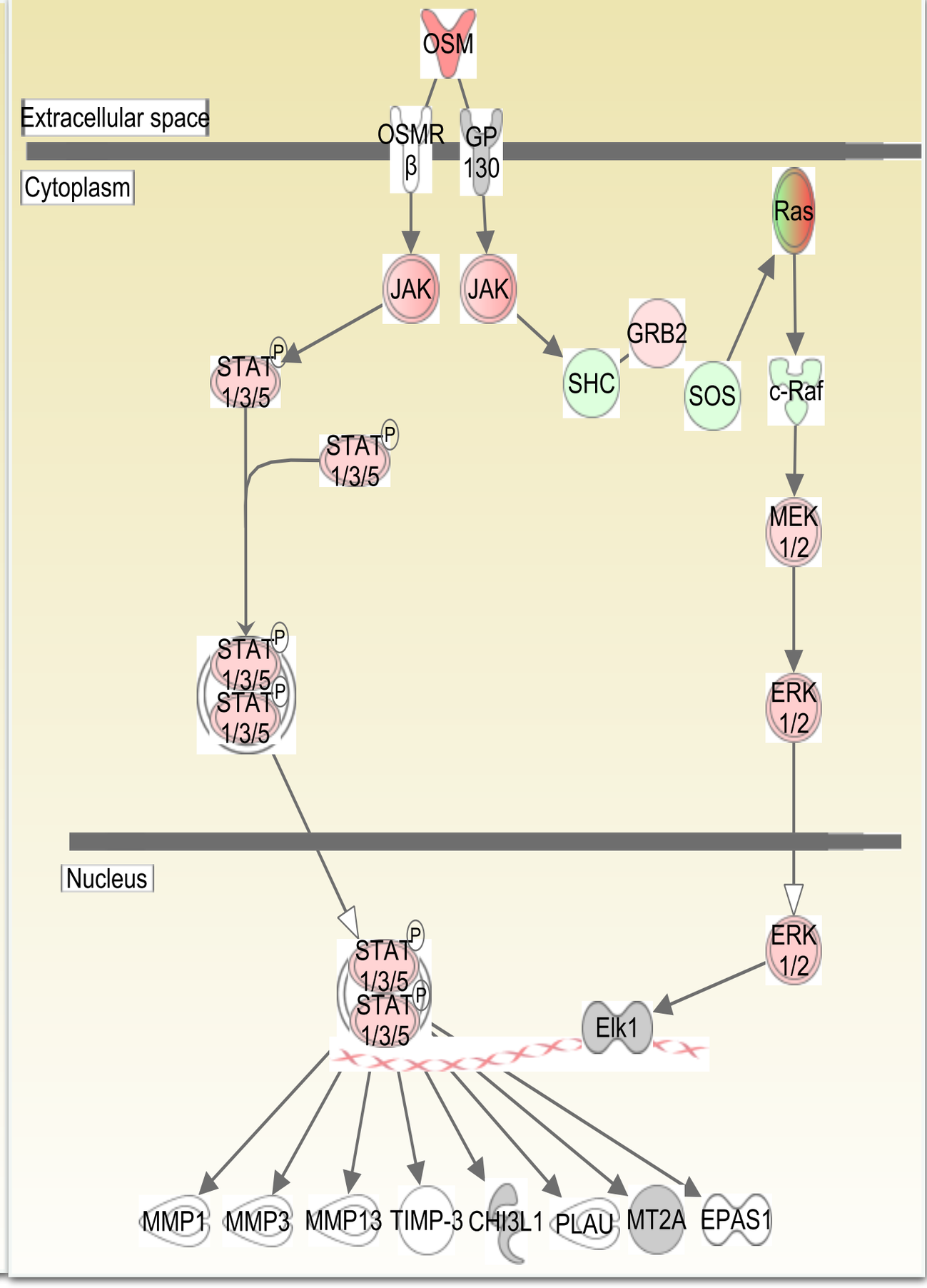
(a)


positive z-score
z-score = 0
negative z-score
no activity pattern available
-log(p-value)
Ratio
-log(p-value)
0.0
0.1
0.2
0.3
0.4
0.5
0
5
10
15
20
25
α-Adrenergic Signaling
Androgen Signaling
BMP signaling pathway
EIF2 Signaling
Oncostatin M Signaling
PTEN Signaling
UVC-Induced MAPK Signaling

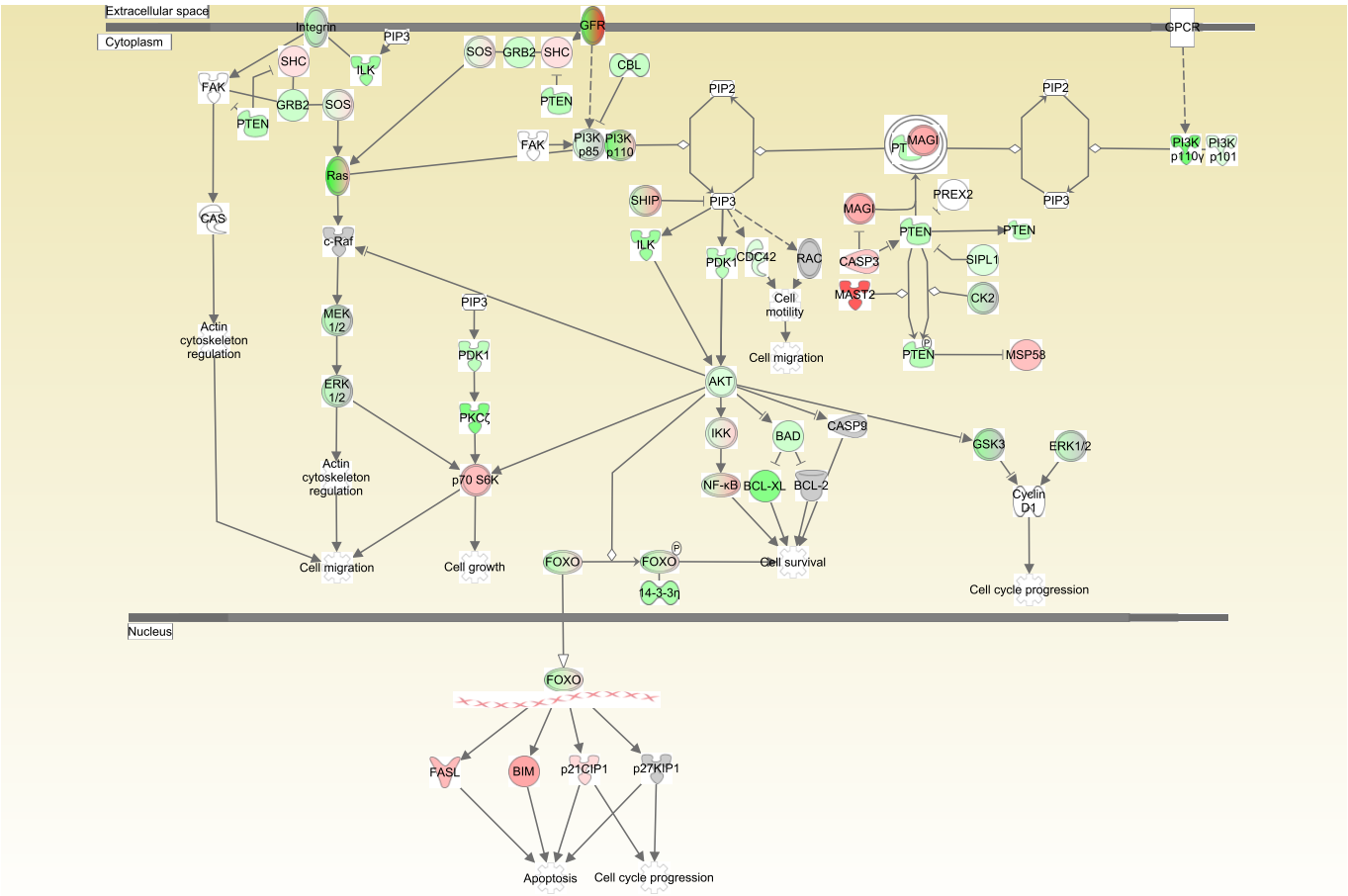
(b)


OSM
Extracellular space
OSMR β
GP 130
Cytoplasm
Ras
JAK
JAK
GRB2
STAT 1/3/5
SHC
SOS
c-Raf
STAT 1/3/5
MEK 1/2
STAT 1/3/5
STAT 1/3/5
ERK 1/2
Nucleus
STAT 1/3/5
STAT 1/3/5
ERK 1/2
Elk1
MMP1
MMP3
MMP13
TIMP-3
CHI3L1
PLAU
MT2A
EPAS1

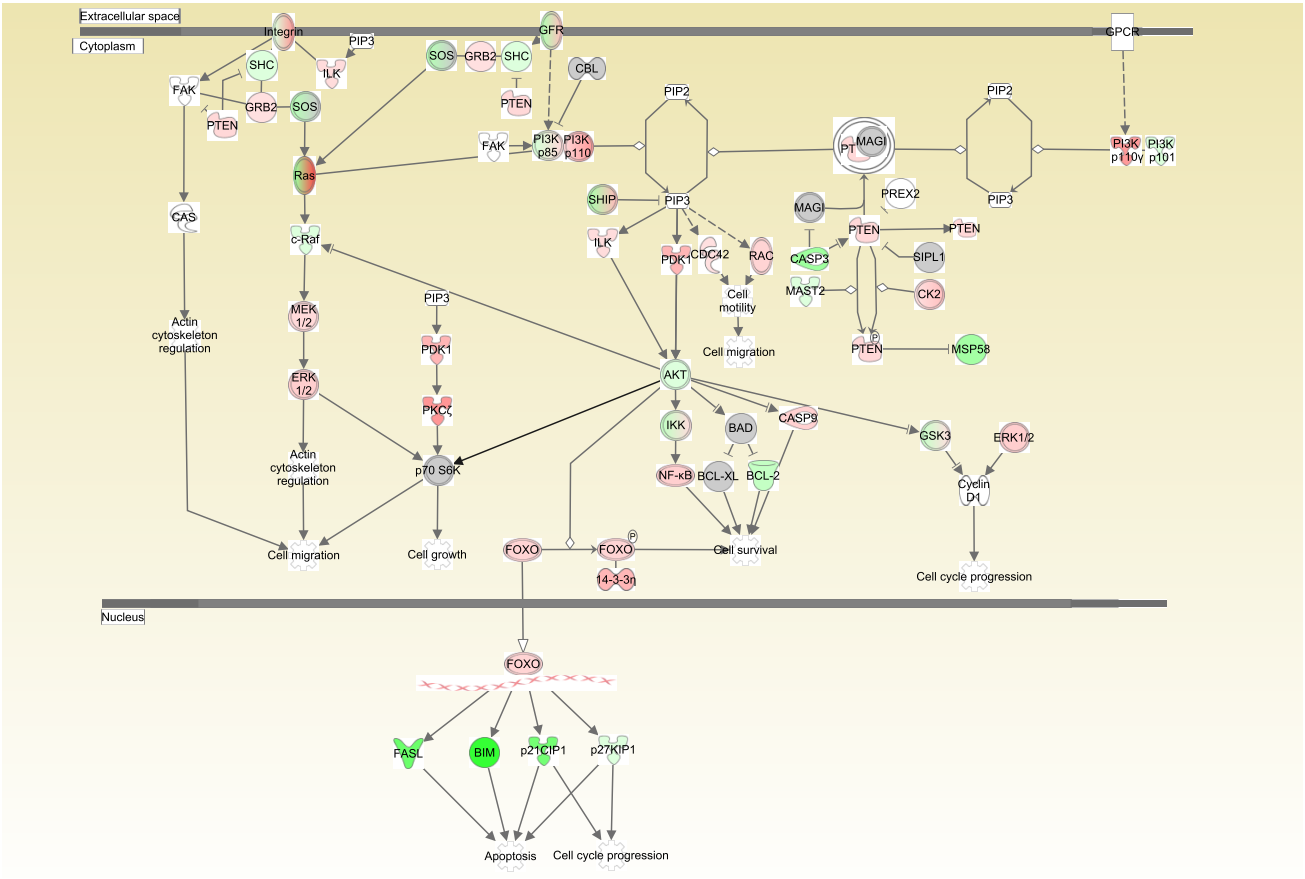
(a)


OSM
Extracellular space
OSMR β
GP 130
Cytoplasm
Ras
JAK
JAK
GRB2
STAT 1/3/5
SHC
SOS
c-Raf
STAT 1/3/5
MEK 1/2
STAT 1/3/5
STAT 1/3/5
ERK 1/2
Nucleus
STAT 1/3/5
STAT 1/3/5
ERK 1/2
Elk1
MMP1
MMP3
MMP13
TIMP-3
CHI3L1
PLAU
MT2A
EPAS1

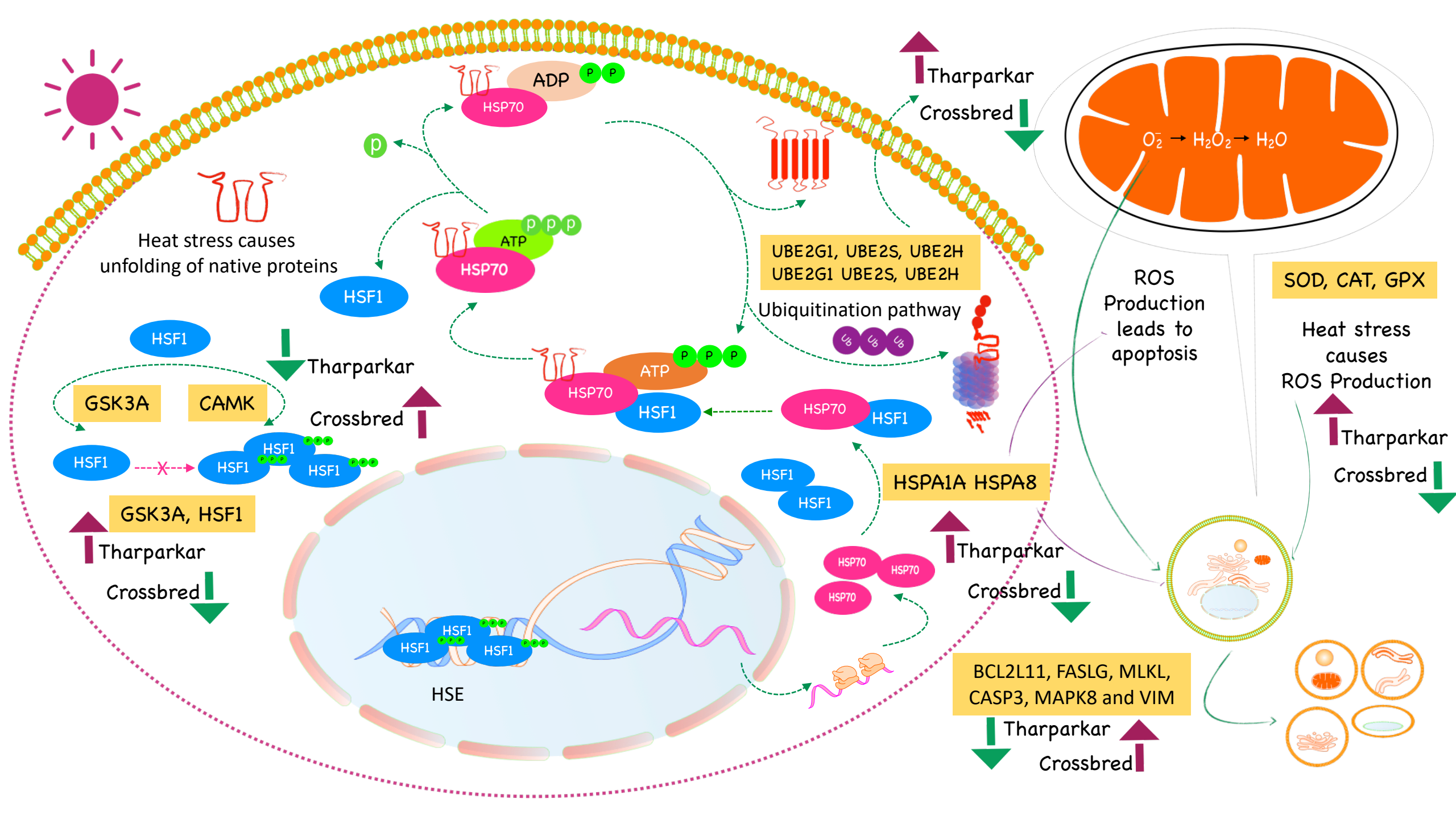
(b)


Extracellular space
Cytoplasm
Integrin
PIP3
GFR
GPCR
SHC
ILK
SOS
GRB2
CBL
FAK
PTEN
PI3K p85
PI3K p110
PIP2
PT
MAGI
PI3K p110γ
PI3K p101
Ras
SHIP
PREX2
CAS
c-Raf
PDK1
CDC42
RAC
CASP3
SIPL1
Cell motility
MAST2
CK2
MEK 1/2
Actin cytoskeleton regulation
Cell migration
MSP58
ERK 1/2
AKT
PKCζ
IKK
BAD
CASP9
GSK3
ERK1/2
NF-κB
BCL-XL
BCL-2
Cyclin D1
p70 S6K
Cell growth
FOXO
14-3-3η
Cell survival
Cell cycle progression
Nucleus
FASL
BIM
p21CIP1
p27KIP1
Apoptosis

(a)


Extracellular space
Cytoplasm
Integrin
PIP3
GFR
GPCR
SHC
ILK
SOS
GRB2
CBL
FAK
PTEN
PI3K p85
PI3K p110
PIP2
PT
MAGI
PI3K p110γ
PI3K p101
Ras
SHIP
PREX2
CAS
c-Raf
PDK1
CDC42
RAC
CASP3
SIPL1
Cell motility
MAST2
CK2
MEK 1/2
Actin cytoskeleton regulation
Cell migration
MSP58
ERK 1/2
AKT
PKCζ
IKK
BAD
CASP9
GSK3
ERK1/2
NF-κB
BCL-XL
BCL-2
Cyclin D1
p70 S6K
Cell growth
FOXO
14-3-3η
Cell survival
Cell cycle progression
Nucleus
FASL
BIM
p21CIP1
p27KIP1
Apoptosis

(b)


ADP
HSP70
Tharparkar
Crossbred
$O_2^- \rightarrow H_2O_2 \rightarrow H_2O$
Heat stress causes
unfolding of native proteins
ATP
HSP70
HSF1
UBE2G1, UBE2S, UBE2H
UBE2G1 UBE2S, UBE2H
Ubiquitination pathway
ROS
Production
leads to
apoptosis
SOD, CAT, GPX
Heat stress
causes
ROS Production
Tharparkar
Crossbred
HSF1
Tharparkar
GSK3A
CAMK
Crossbred
HSF1
HSF1
HSF1
HSF1
GSK3A, HSF1
Tharparkar
Crossbred
HSP70
HSF1
HSP70
HSF1
HSF1
HSF1
HSPA1A HSPA8
Tharparkar
Crossbred
HSP70
HSP70
HSP70
HSF1
HSF1
HSF1
HSE
BCL2L11, FASLG, MLKL,
CASP3, MAPK8 and VIM
Tharparkar
Crossbred