Characterisation and validation of novel heat-responsive microRNAs identified from miRNome analysis

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Journal of Livestock Science (ISSN online 2277-6214) 16: 1-14
Received on 2/7/24; Accepted on 30/11/24; Published on 2/1/25
doi. 10.33259/JLivestSci.2024.1-14

Abstract

MicroRNAs (miRNAs) are non-coding single stranded small RNAs which regulate the animal growth and development. RNA-RNA interaction is a vital mechanism of gene regulation during biotic and abiotic stress. Heat stress (HS) is one of the most significant abiotic factors which affect the development, milk production, reproductive and feed intake of the dairy cattle. In the present investigation, we have identified novel miRNAs from Tharparkar (TH) and Karan Fries (KF) cattle using de novo assembly. The identified miRNA were mapped for *in silico* characterisation and predicted to have more than two thousand target genes. Most of the predicted target genes were involved in stress metabolic network. Gene ontology revealed that target genes were associated in signal transduction, protein transport and glucose homeostasis. Heat map was generated for most up and downregulated novel miRNA based on differential gene expression and read count value. Eight novel miRNA were validated in four cattle breed by quantitative real time PCR. This study provides comprehensive overview of novel miRNAs and their target genes expression under HS towards enhancing thermotolerance mechanism.

Keywords: Heat stress; MiRNA; Illumina HiSeq; Small RNA analysis; Target genes; Differential gene expression.

Introduction

The abiotic stressors mainly include cold stress, heat stress, poor housing, social stress, loud noise and transportation particularly during noon hours cause stress, also reduction in feed quality and quantity lead stressful conditions (Gadzhiev et al, 2021). High ambient temperatures trigger HS, a condition that occurs when an animal's body temperature rises above the thermoneutral zone's upper limit (Belhadj et al., 2016). HS is one of the most important abiotic factors affecting livestock productivity, especially in the Asian subcontinent, where summer temperatures can reach at very high levels. The animal biology system is affected by HS. It has an effect on physiological and molecular processes as well as productivity. As a result, HS has become a major source of concern because it results in significant financial losses for dairy farms (Polsky et al., 2017; Tao et al., 2018;). Acute and chronic HS have different effects on carbohydrate metabolism and the development of heat shock protein (HSP) (Bharati et al., 2017; Deb et al., 2014; Vaidya,et al., 2023). Some of the physiological, metabolic, cellular, and molecular responses to HS have been discovered in recent years through research. Many studies have recently documented gene changes in cows subjected to HS (Sengar et al., 2018; Li et al., 2018; Srikanth et al., 2017), but few have concentrated on the molecular differences between chronic heat-stress and thermoneutral environments. Comparing cow gene expression profiles under chronic heat stress and thermoneutral conditions may help researchers better understand the mechanisms that control the heat-stress response.

Micro RNAs (miRNAs) are noncoding RNA molecules that regulate gene expression post-transcriptionally by directing Argonaute (AGO) proteins to target sites in the 3'UTR of mRNAs (Tesfaye et al., 2017). MiRNAs regulate gene expression by forming an imperfect base-pairing with the target mRNAs' 5'UTR, 3'UTR, and CDS regions, resulting in mRNA destabilisation or translational repression (Hossain et al., 2012). MiRNAs are crucial for animal growth, cell differentiation, and homeostasis because they are involved in almost every cellular and biological phase (Gebert et al., 2019). Micro RNAs make up 1-5 percent of the total mammalian genome, and in-silico research showed that a single miRNA could theoretically target more than 60% of mammalian genes (Sohel, 2016). MiRNAs, which are both intracellular and extracellular, have been discovered to play a role in controlling gene expression in mammalian cells and tissues (Tahiri et al., 2014). In cattle, altered miRNA expression profiles have been published, with 154 differentially controlled miRNAs sequences identified from fat tissue and mammary glands, 54 of which are fat tissue-specific (Gu et al., 2007). MiRNA-199b, miR-199a-5p, and miR-126 were found to be expressed in bovine mammary glands in other studies (Ogorevc et al., 2009).

To investigate the effects of HS on dairy cows, we used profiles of novel miRNAs identified through *de-novo* assembly approach from our lab. Recently some studies reported the role of miRNAs in defence mechanism under different abiotic stresses in cattle and bovine. In the present investigation we have identified, and characterised novel miRNAs from two indigenous TH and KF cattle breed under HS condition by small RNA sequencing. The selection criteria of these two breeds were based on their response to the thermal stress. TH is Thermotolerant and KF is Thermosusceptible cattle breed. The identified novel miRNAs was validated for its heat responsive nature using quantitative real time PCR. *In-silico* characterisation was done for the target identification and pathway analysis through different bioinformatics software.

Materials and methods

Novel miRNA prediction

This investigation was carried out at ICAR-National Dairy Research Institute (NDRI), Karnal (Haryana). This institute is located at an altitude of 120 meters above the mean sea level. The maximum ambient temperature goes up to 45 °C during summer and minimum of about 2 °C during winter.

An experiment was executed in our lab for the identification of novel and conserved miRNAs in the indigenous cattle (TH and KF) using *de-novo* assembly approach (Kumar et al., 2021). The control and HS treated PBMC samples of both the breed were used for small RNA library construction followed by sequencing using Illumina HiSeq (Illumina, USA). The raw reads obtained after sequencing and trimming were mapped on the reference genome of *Bos taurus*.

In order to identify the novel miRNAs the reads which were not aligning to mirBase as well as filtered due to the length and 3' adapter prior to aligning to mirBase were taken. These reads were given as input along with mapping (BED) file and genome to MIREAP. MIREAP identified novel miRNAs based on alignment, secondary structure, free energy and location on the precursor arm. Sequences not showing hits with known miRNA's were extracted and were considered for novel miRNA prediction. MIREAP integrates miRNA biogenesis, sequencing depth, and structural features to identify miRNAs and their expression level from deep sequenced small RNA libraries. Stem-loop hairpins were retained only when they comply with:

1. The mature miRNAs-associated reads are mapped in the arm region of the precursors.
2. The free energy of the secondary structure calculated by RNAfold (Zuker, 2003) is lower than -18 kcal/mol.

In detail, the potential mature miRNA is defined as the most abundant read sequence that aligns to the potential precursor sequence; the expression level of a miRNA is then specified as the sum of an ensemble of reads that align with the potential mature molecules, allowing three nucleotides sliding beyond the position of the potential mature miRNA at the 5' end. These reads were aligned to the reference genome using bowtie. Novel miRNAs were predicted from the aligned data using Mireap_0.22b**5**. To identify true novel miRNAs predicted candidate miRNAs were matched against *Bos taurus* miRNAs predicted in the genomes but not present in miRBase. Unaligned predicted miRNAs were considered as potential novel miRNAs [ftp://mirbase.org/pub/mirbase/CURRENT/genomes/bta.gff3] if the predicted secondary structure is a proper stem-loop structure defined for a miRNA.

Animal selection, (HS) treatment, and sample collection

For validation of miRNAs blood sample was collected from the four different cattle breed (TH, KF, Karan Swiss and Sahiwal) during the summer season in the month of June and control sample in the month of March of uniform age (4-5 years) from cattle yard. The animals were maintained at experimental animal sheds under well-ventilated and proper hygienic conditions. The physiological parameters like rectal temperature, respiration rate and pulse rate of the animals were also taken to confirm the HS. Blood samples were collected from the jugular vein of the animals from the control and HS group by using the EDTA containing vacutainer tubes. Peripheral blood mononuclear cells (PBMCs) were separated from whole blood sample using Histopaque 1077 density gradient medium and stored in -80°C for further analysis.

Total RNA isolation and quality analysis

Total RNA was isolated from PBMCs cells stored in RNA later using mirVana RNA isolation kit (Invitrogen, UK) according to the manufacture protocol. In brief, the PBMCs cells were washed with 1X PBS. The PBMCs cells were re-suspended by low-speed centrifugation. The cells were disrupted in 600 µL Lysis buffer followed by phase separation using acid phenol-chloroform in the same volume. In the upper aqueous phase, 100% ethanol was added and passed through filter cartridge followed by subsequent washing with wash solution. Finally, the RNA was eluted in 30 µL nuclease free water. The quality and quantity of RNA was checked by Bio-analyzer (Agilent, UK) and stored at −80 °C for downstream application.

In-silico characterization

miRNAs target prediction by TargetScan custom

The target genes of differentially expressed miRNAs in cattle were predicted through the miRanda7 tool (http://cbio.mskcc.org/microrna_data/miRanda-aug2010.tar.gz). For the prediction of novel miRNAs binding site, custom online server was used. This is based on the heptamer seed region of miRNAs and start from two to eight nucleotides of novel miRNAs from 5'UTR or start site. It compares seed sequence of novel miRNAs with known and conserved miRNAs seed sequence of other mammalian species.

Gene Ontology and pathway analysis

Determined target genes (identified by above database) were used for the functional analysis, where the predicted target gene candidates of novel miRNAs were annotated to predict the number of genes involved in different molecular, cellular, biological and signaling functions using the Gene Ontology (GO) database (http://www.geneontology.org) (Ashburner et al., 2000). All potential target genes of novel miRNAs were classified into three enriched Gene Ontology (GO) terms (cellular component, molecular function and biological process). This was conducted by mapping target gene candidates to GO terms using its database (http://www.geneontology.org/). Furthermore, metabolic pathways of the target gene candidates were determined using Kyoto Encyclopedia of Genes and Genomes (KEGG) (http://www.genome.jp/kegg/). KEGG PATHWAY database is a collection of manually drawn pathway maps representing the knowledge on the molecular interaction and reaction networks for metabolism, genetic information processing, environmental information processing, cellular processes, organismal systems and human diseases (Kanehisa et al., 2006).

Secondary structures prediction

Secondary structures were predicted and generated using the Zucker folding algorithm with mFold 3.1 (Zuker, 2003). All mFold outputs including information about the number of structures, free energy (ΔG kcal/mol), miRNA-like helicity, and the number of arms per structure, size of helices within arms, and size and symmetry of internal loops within arms were analyzed. The predicted secondary fold-back structures were manually inspected by comparing to the characteristics of previously known miRNAs. The secondary structure of hairpin stem-loop was assigned a score according to the strategies used by Lai (Lai, et al., 2003**)**. Briefly, free energy (ΔG kcal/mol) and miRNA-like helicity for each individual arm were evaluated and assigned a score. Helicity was calculated +1 for each paired helicity, −1 for each one-nucleotide symmetric loop and −2 for each two-nucleotide symmetric loop, and an overall score was calculated as (helical score + (ABS(ΔG)/2)/2. If there was more than one hairpin stem-loop structure for the ESTs containing the miRNAs, each was scored and the hairpin structure with the highest score was considered the miRNA hairpin stem-loop structure.

Chromosomal Localization of novel miRNAs

Ensemble is a genome browser for vertebrate genomes that supports research in comparative genomics, evolution, sequence variation and transcriptional regulation. Ensemble annotates genes, computes multiple alignments, predicts regulatory function and collects disease data. Ensemble tools include BLAST, BLAT, BioMart and the Variant Effect Predictor (VEP) for all supported species.

Protein-Protein interaction of targeted genes of novel miRNAs

Targets proteins of novel miRNA were used for Protein-protein interactions using Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) database v9.05 (Szklarczyk et al., 2021). The Swiss-Prot identifier for the genes in multiple proteins by names / identifiers were used to search against the STRING database. Network analysis was set at medium stringency (STRING score = 0.4). The interaction map was generated using default settings (Medium confidence of 0.4 and 7 criteria for linkage: neighborhood, gene fusion, co-occurrence, co-expression, experimental evidences, existing databases and text mining). Stronger associations are represented by thicker lines or edges. Proteins are represented as nodes.

Differential gene expression (DGE) analysis

The read count table for all the samples was generated. DGE analysis was carried out using DESeq**8** tool. Variations in the reads are normalized by the library normalization method opted from DESeq library. DESeq calculates size factor**,** each read count is normalized by dividing with size factor. Mean normalized read counts of the samples in a given condition are used for DGE calculation and heatmap. For regulation calculation between the comparisons, log2fold of 1 was used as cutoff. miRNAs >1 were considered as up regulated, miRNAs <-1 were considered as down and those between 1 and -1 were flagged as neutral.

Validation of miRNAs and using quantitative real-time PCR

Designing of primers

Mature miRNA-specific forward primer was designed using Genefisher2 primer designing software (http://bibiserv.techfak.uni-bielefeld.de/genefisher2/), and quality was checked using Oligo Analyzer (Integrated DNA Technologies, USA). Based on read count and differential gene expression data ten forward primers were designed for novel mature miRNA sequences. In case Tm of a mature miRNA was <60 °C, it was adjusted by adding Gs or Cs to the 5′ end and/or As to the 3′ end of the miRNA sequence (Table 1). U6 snRNA (Clontech, USA) was used as an endogenous control to normalize the data.

Quantitative real-time PCR

The validation of identified miRNAs in the present investigation was carried out using the quantitative real time PCR expression analysis. Blood sample collected from large population of different cattle breeds (TH, KF, KS, and Sahiwal) were used for the miRNAs validation. Blood sample collected during the summer season in the month of June was used as HS sample and the sample collected in the month of March was used as control. miRNAs were isolated from the control and HS-treated samples using mirVana RNA isolation kit (Invitrogen, UK) according to the manufacture protocol as described earlier. The quality of isolated miRNA was checked on Bio-analyzer (Agilent), and we observed OD 260/280 ratio of >2.0. The isolated miRNAs were also analyzed on the 12 % TBE acrylamide gels, and prominent RNA bands were observed with ethidium bromide staining. The first-strand cDNA synthesis was carried out using the miScript II RT kit (Qiagen, Germany). 5X miScript HiFlex buffer was used for the quantification of mature and precursor miRNAs.Quantitative RT-PCR analysis was carried out by using miScript PCR Kit (Qiagen) on an ABI 7500 series PCR machine Applied Biosystems. All reactions were performed in triplicate for each sample, and U6 SnRNA (Clontech, USA) was used as the internal control gene. Relative expression levels of miRNAs were quantified by using the $2^{-\Delta\Delta Ct}$ method (Pfaffl, 2002).

Statistical analysis

Statistical analysis was performed by analysis of variance (ANOVA) followed by *post hoc* Tukey's multiple comparison test, using GraphPad Prism version 5.01 for Windows (GraphPad Software, San Diego, CA, USA). Data with different superscript letters are significantly different at $p<0.05$, $p<0.001$, $p<0.0001$ according to *post hoc* ANOVA statistical analysis. Data are expressed as mean ± SEM. The results were considered statistically significant when $p<0.0001$.

Table 1. List of the primers used for the expression profiling of identified novel miRNAs under HS condition.

Mirna ID	Forward Primer sequence	Tm
m0006-3p	ACC ATC GAC CGT TGA TTG TAC CCT A	59.5
m0031-5p	CGC AAA GTT CTG AGA CAC TCC GAC TA	60
m0017-3p	GCG CTT GGA TTT CGT TCC CTG A	60.2
m0027-5p	GCG GCC CCT GTC GGT TTA A	60.5
m0073-5p	GCA AAG TTC TGA GAC ACT CCG ACT CA	60.3
m0045-3p	CGC TTG GAT TTC GTT CCC TGC AA	60.4
m0002-3p	CAC CGA CCG TTG ACT GTA CCT	59
m0011-3p	GAA CCA TCG ACC GTT GAT TGT ACC C	59.6
m0050-5p	GGC AAA GTT CTG AGA CAC TCC GAC TA	60
m0013-3p	GAA CCA TCG ACC GTT GAT TGT ACC CA	59.6

Results

HS is one of the major problems in the livestock sector which significantly reduces the production and productivity. Several works has been initiated on this particular aspect all around the world to decipher the thermotolerance mechanism in dairy cattle by different omics technique. miRNA being one of the most important players of the gene regulation at transcriptional and post transcriptional level inside the living system need to be exploited in the indigenous cattle to explore the tolerance procedure. De novo assembly approach was used for the identification of novel miRNAs in our lab (Kumar et al., 2021). An average of 77 novel miRNAs was reported in TH and KF cattle breed under control and HS condition.

Target gene identification, Gene ontology and KEGG pathway analysis

A range of different bioinformatics tools was used for the target gene prediction, gene ontology and metabolic pathway analysis. Target genes were predicted for the novel miRNAs by target scan and miRanda7 tool. The novel miRNA sequence was used as input along with reference cDNA sequence to miRanda tool and targets were identified based on strong/weak bonding between miRNAs and cDNA sequence. We could able to identify more than ten thousand target genes for the candidate novel miRNAs in both the cattle breed (Fig.1). Most of the miRNA target genes involved in different metabolic pathway and stress related networking. The metabolic pathway was predicted for the novel miRNA target genes using Kyoto Encyclopedia of Genes and Genomes (KEGG). Maximum number of target genes was involved in mitogen activated protein kinase (MAPK) and cyclic AMP signaling pathway (Fig.2). Transcriptional mis-regulation, jak STAT signaling, RNA transport, ribosome biosynthesis and p53 signaling are the other important metabolic pathways predicted for the novel miRNA. The potential target genes for novel miRNAs were mapped for the gene ontology classification. GO description for the biological process showed maximum number of predicted target genes were involved in signal transduction, intracellular protein transport, glucose homeostasis and regulation of cell migration (Fig. 3). More than one thousand predicted target genes were involved in nucleus, cytosol, cytoplasm and extracellular space as described in GO for cellular component (Fig. 4). We have also identified the molecular function for the target genes in both the cattle breed. Most of the genes were related to thermodynamics process (Fig. 5).

Secondary structure analysis and protein-protein interaction

The nucleotide sequences of novel miRNAs were used for the secondary structure prediction using MFold server. We observed different major and minor grooves in the secondary structure of miRNA m006 (Fig.6a). The fold energy analysis was also carried out using the same software (Fig. 6b).The dots represent the superposition of all possible folding within p % of DGmfe, the minimum free energy, where p is the maximum percent deviation from DGmfe. Different colors (two to eight) indicate the varying levels of sub-optimality. The optimal base pairs have been colored in red and black colors base pairs and the optimal energy observed was -25.6 kcal/mol. The sequence of novel miRNA was mapped on the latest survey sequence of *Bos taurus* downloaded from Ensemble genome. The candidate miRNA m006 was observed on the large arm of chromosome one (Fig. 6c). STRING network analysis of protein-protein interactions was performed to identify functionally linked proteins targeted by novel miRNA during heat conditions and determine the potential biological processes affected. The network is presented under a confidence view, whereby stronger associations are represented by thicker lines or edges and vice versa. Proteins are represented as nodes. Ten additional interacting proteins were added to provide a more comprehensive view of the interactions. Protein-protein interaction network of genes, targeted by miRNA m006 in KF is given in Fig. 7.

Differential gene expression

Differential gene expression was analysed by using DESeq software. The FPKM value was calculated for each mature miRNAs and based on the p value. The identified novel miRNAs were categorized as upregulated and downregulated based on log fold change value. Heat map was generated for six most up and downregulated mature miRNAs based log fold change in DGE for KF and TH sample. Mean normalized read counts of the samples in a given condition are used for DGE calculation and heat map. For regulation calculation between the comparisons, log2fold of 1 was used as cutoff. miRNAs >1 were considered as up regulated, miRNAs <-1 were considered as down and those between 1 and -1 were flagged as neutral. Heat maps were generated for each set of DGE using top six miRNAs (Figure. 8a & 8b).

Validation of selected miRNA by quantitative real time PCR

To validate the small RNA sequencing data quantitative real time PCR was conducted to calculate the expression level for the selected novel miRNAs. Eight novel miRNAs were selected for real time PCR validation based on the read count value and differential gene expression. Relative expression analysis was calculated by pfaffl method in four different cattle breed under HS. Novel miRNA m006, m0017, m0025, m0011 and m0027 showed upregulation in all the cattle breed under HS as compared to control. We observed significant decrease in miRNA m0019 and m0031 under HS in all the cattle breed. miRNA m007 showed significant decrease in only KS breed under HS condition, other breed did not show significant response under HS condition (Fig. 9).

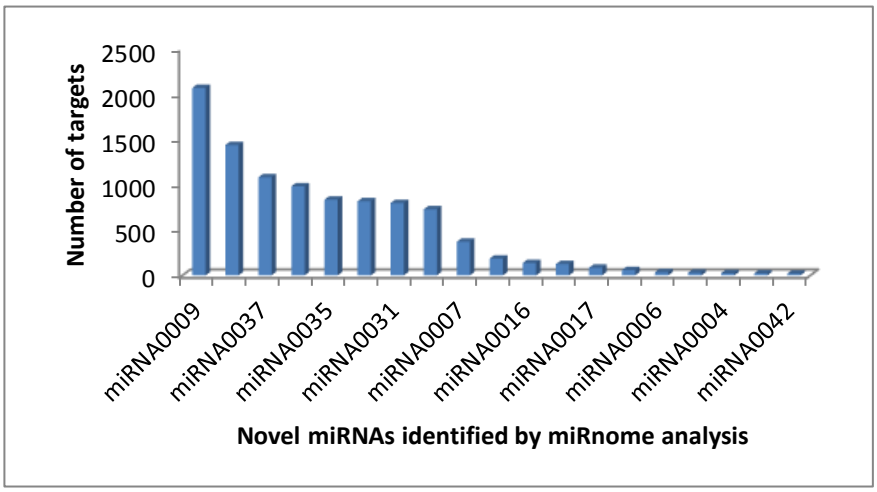


Fig 1. Number of predicted targets identified for the differentially expressed novel miRNAs by miRanda7 tool (http://cbio.mskcc.org/microrna_data/miRanda-aug2010.tar.gz).

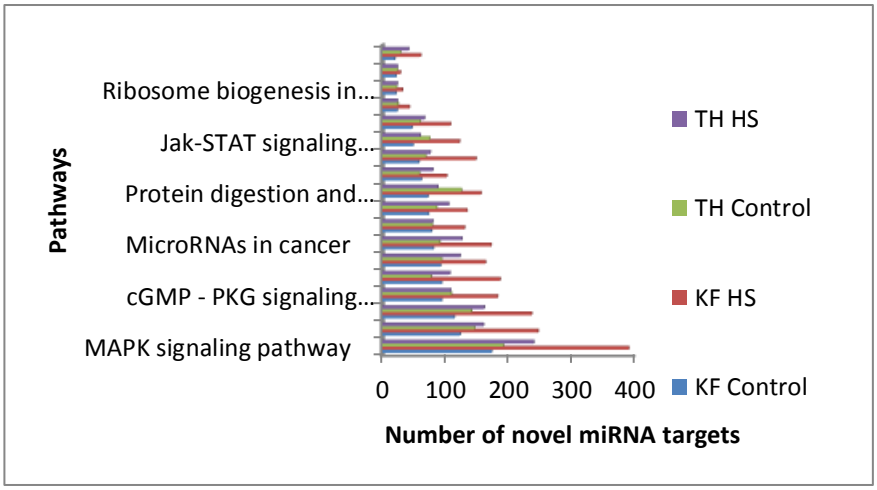


Fig 2. Metabolic pathway prediction for novel miRNA targets genes using Kyoto Encyclopedia of Genes and Genomes (KEGG) (http://www.genome.jp/kegg/).

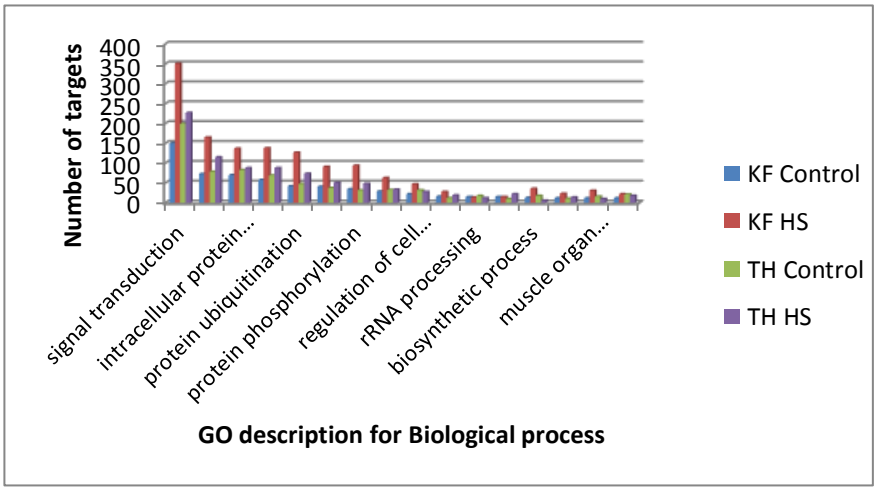


Fig. 3. Gene ontology description for the predicted target gene candidates of novel miRNAs involved in biological process (http://www.geneontology.org)

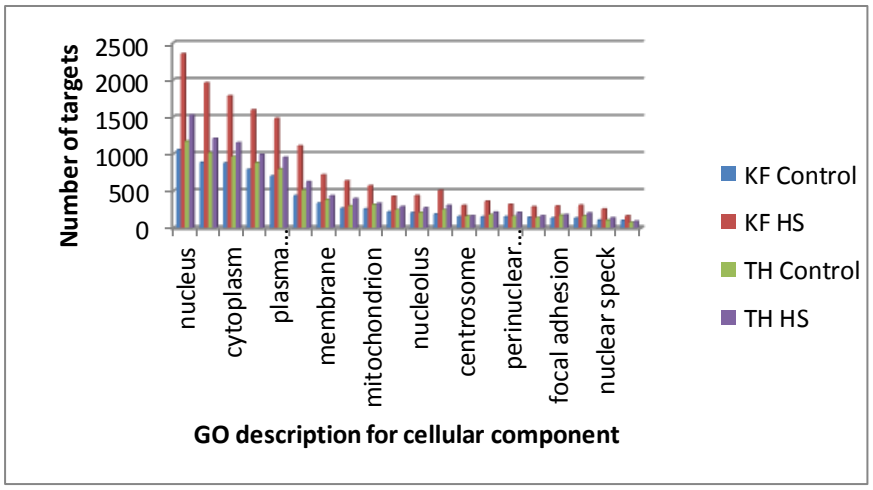


Fig 4. Gene ontology description for the predicted target gene candidates of novel miRNAs involved in cellular component (http://www.geneontology.org).

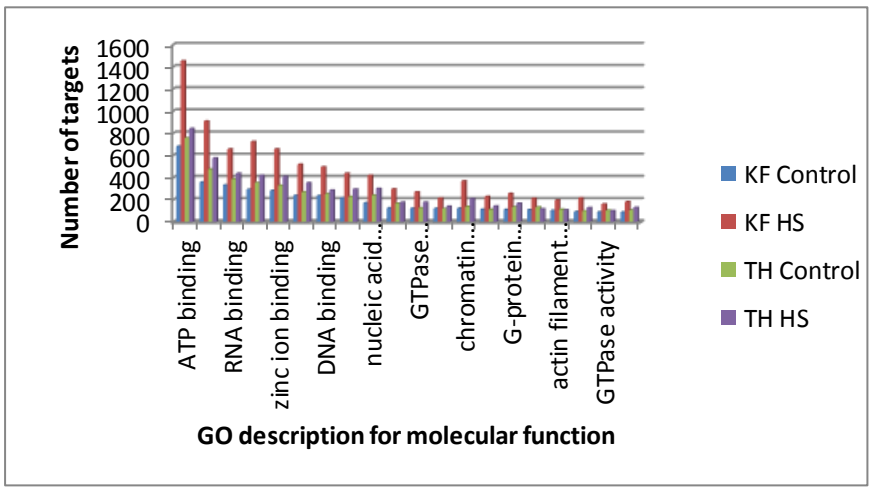


Fig 5. Gene ontology description for the predicted target gene candidates of novel miRNAs involved in molecular function (http://www.geneontology.org).

Discussion

Climate change is one of the biggest challenges for agriculture and livestock production sector in future prospective. HS causes negative effect on the dairy cattle as it reduces feed intake, reproductive health and milk production. The impact of HS results oxidative damage and cell apoptosis (Li et al., 2018). New breed and more climate tolerant animals can be selected using new techniques of biotechnology and biochemistry along with the rules of genetics. miRNAs are the master player of gene regulation in the mammalian thermal stress response (Islam et al., 2013; Place et al., 2014). The information regarding the role of heat stressed novel miRNAs in indigenous cattle is very limited. In the present investigation we have reported novel miRNAs identified by using de novo assembly approach in TH and KF breed under elevated temperature. The novel miRNAs were characterised by integrative systemic approach through different bioinformatics tools. In our previous study we have already reported novel and known miRNAs in the indigenous cattle (Kumar et al., 2021).

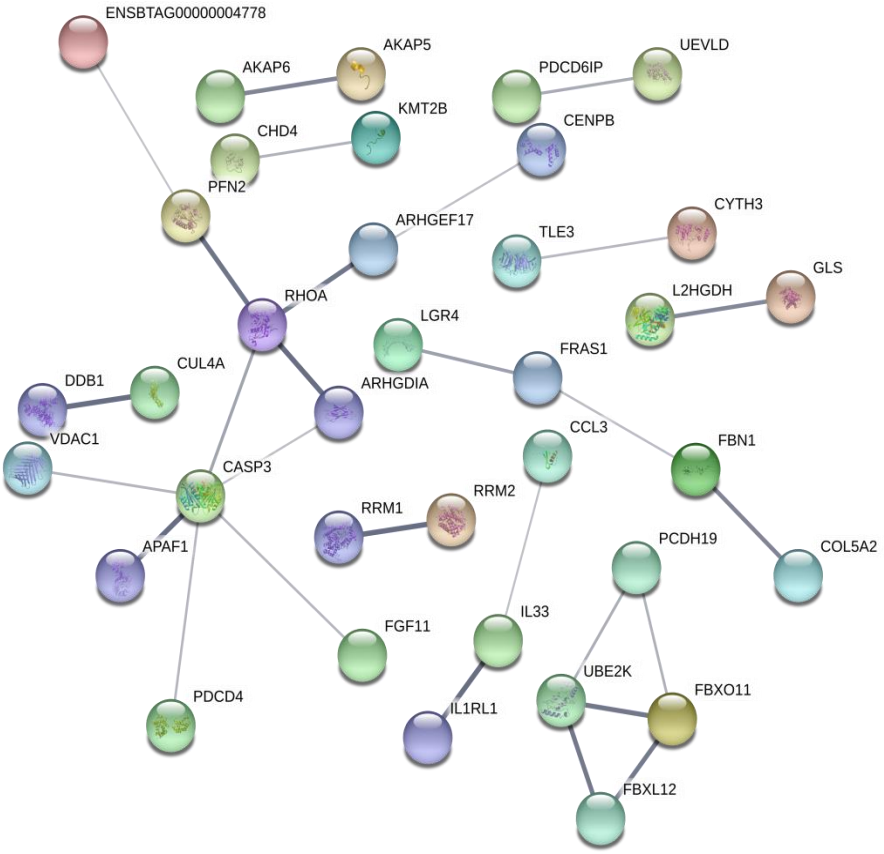
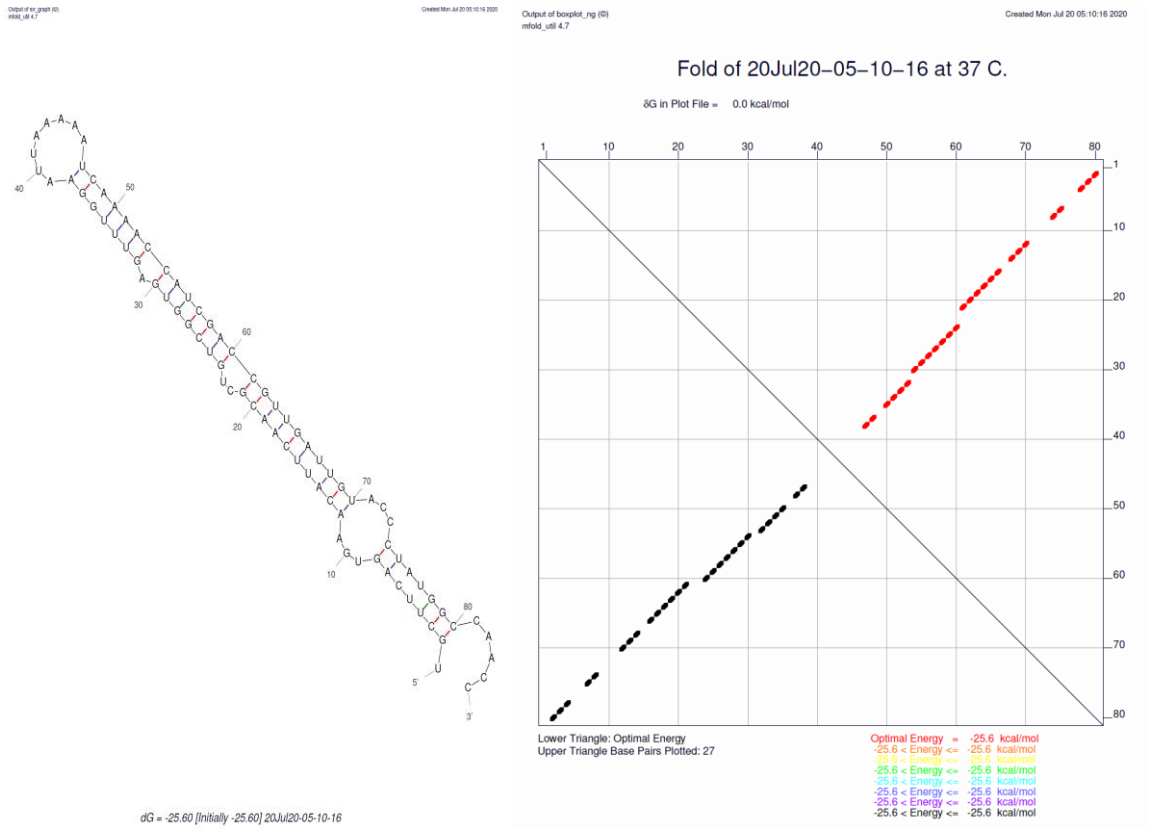


Figure. 6. In-silico characterisation of novel miRNA m006 identified from miRNome analysis, (**a)** secondary structure prediction for the novel miRNA using MFold software, **(b)** folding behaviour of identified miRNA at RT, **(c)** chromosomal localisation of miRNA m006; sequence was mapped on the latest survey sequence of *Bos taurus* downloaded from ensemble

Chaurasiya et al, 2022 identified miRNAs targeting the genes that regulate protein transport and translocation in cattle in heat stress response. Here we focused on novel miRNAs and these miRNAs were characterised by integrative systemic approach through different bioinformatics tools. Liu et al, 2020 identified differentially expressed mRNA and miRNAs in blood from dairy cattle. Recently Lee and his co-worker, 2020 analysed the profiling of circulating miRNA using small RNA sequencing.

Identification of miRNA depends on cloning and sequencing of small RNA libraries which is experimental approach and computational prediction of conserved miRNA (Zhang et al., 2005). Target prediction of novel miRNAs showed more than ten thousand genes were targeted by these miRNAs. Maximum number of target genes was predicted for miRNA m009 followed by m0012, m0037, m008 and m0035. Further we have classified the target genes on the basis of cellular, biological and molecular function with GO description. The MAPK signalling pathway, cellular senescence, circadian entrainment, aldosterone synthesis and secretion, and cancer pathways were all enriched for differently expressed mRNAs, according to the KEGG findings. These pathways were found to be involved in cell survival, apoptosis, and stress response. The MAPK signalling pathway is thought to play a key role in the response to stress stimuli, including mediation of heat stress-induced cell death (Sui et al., 2014; Niaudet et al., 2017; Liu et al., 2020). Heat stress-induced ROS generation and cell apoptosis were reduced when the p38-MAPK signalling pathway was inhibited, according to a report (Li et al., 2018). The KEGG analyses results showed that the following pathways: MAPK signaling pathway, cellular senescence, circadian entrainment, aldosterone synthesis and secretion, and pathways in cancer were enriched for differently expressed mRNAs.



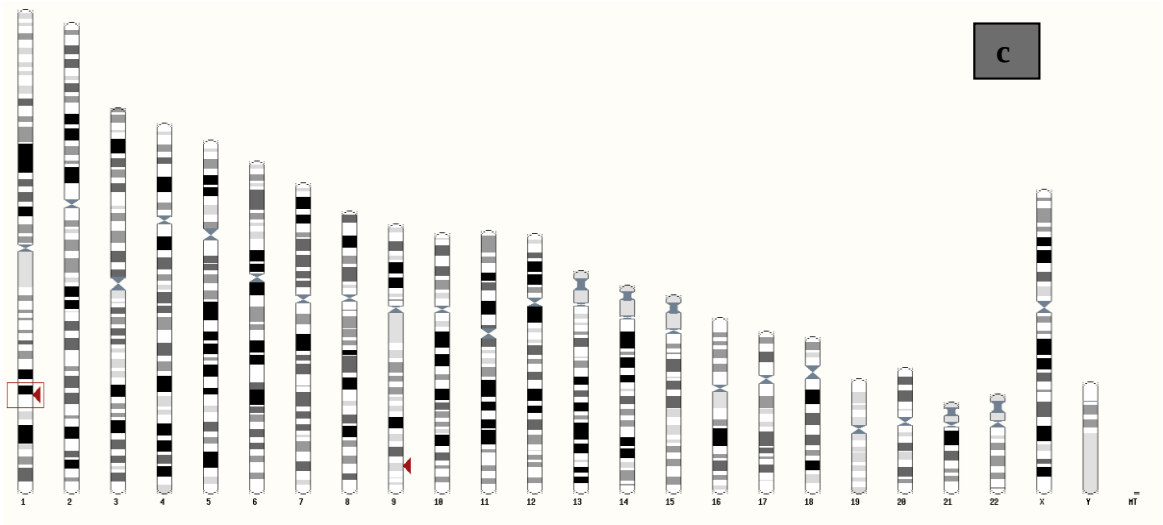


Fig 7. Protein-protein interaction network of genes, targeted by miRNA m006 in KF by STRING. Protein interaction network of the differential proteins from mammary gland (predicted with STRING software. Each node presents a protein; line colours present the types of evidence: pink lines from experimental study, the blue lines from databases, and the yellow lines from abstracts of articles published in PubMed.

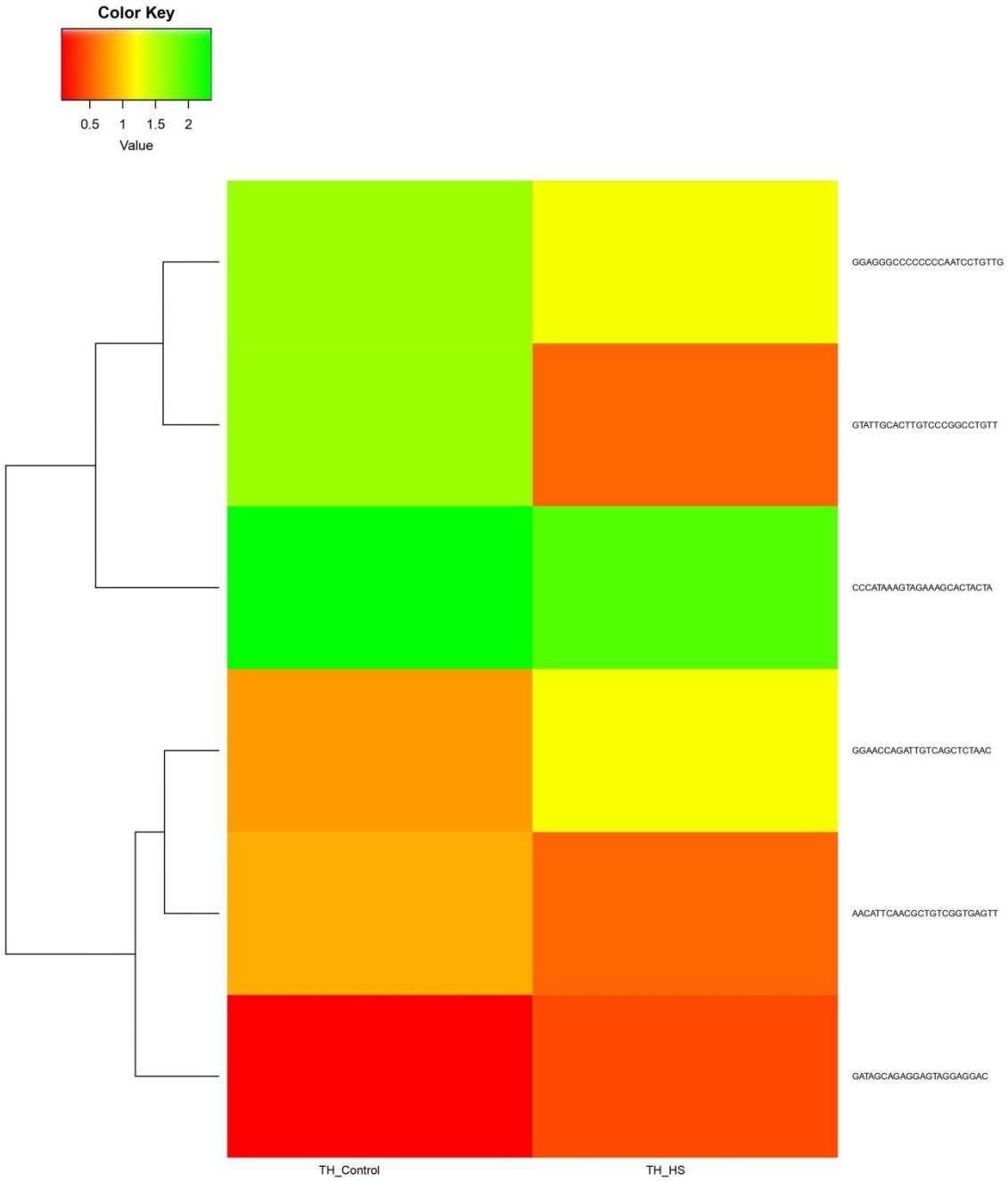


Fig. 8a. Heat map of top six up and down regulated miRNAs in TH control and HS sample. For regulation calculation between the comparisons, log2fold of 1 was used as cutoff. miRNAs >1 were considered as up regulated, miRNAs <-1 were considered as down and those between 1 and -1 were flagged as neutral.

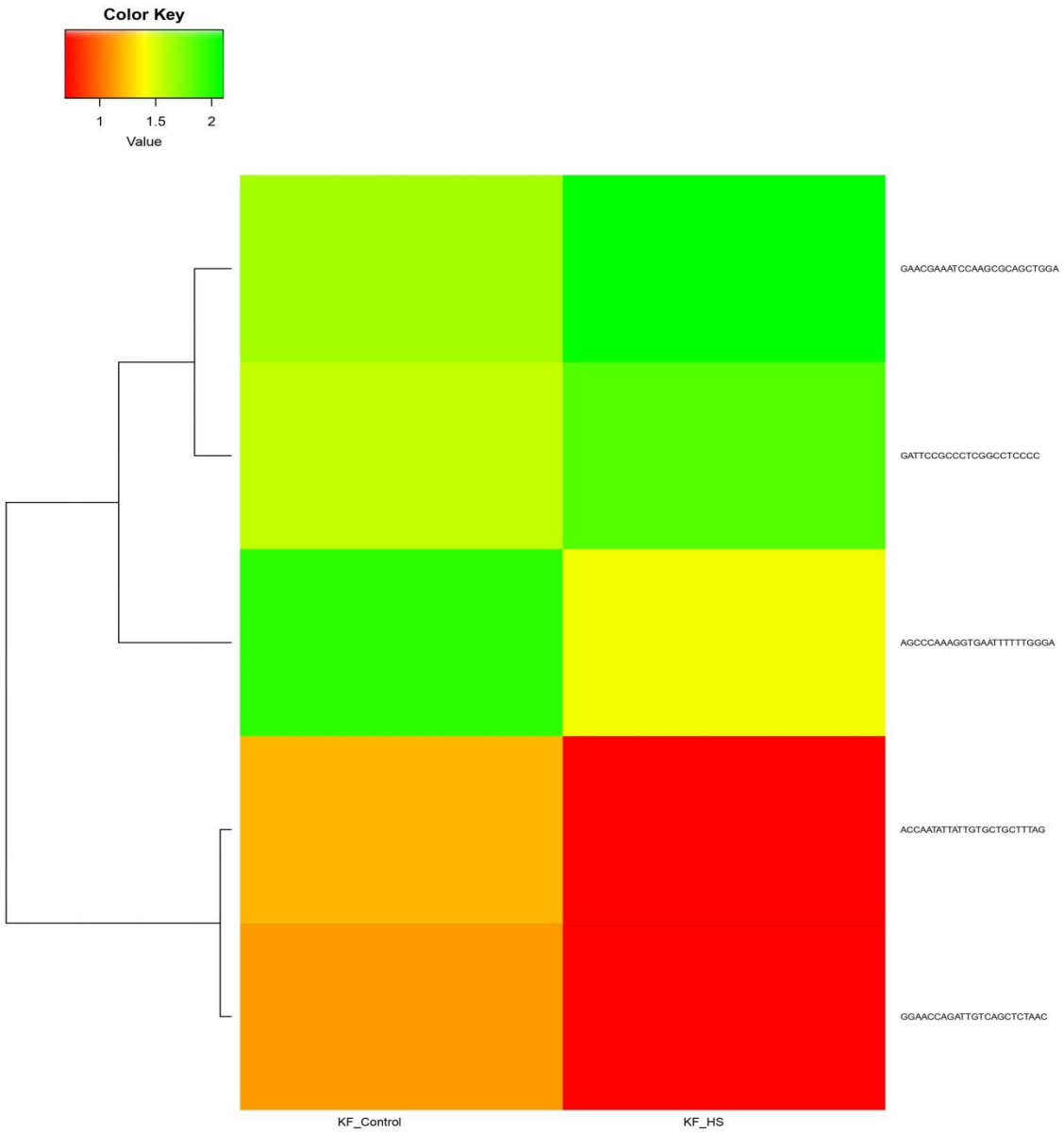


Fig. 8b. Heat map of top six up and down regulated miRNAs KF control and HS treated sample. For regulation calculation between the comparisons, log2fold of 1 was used as cutoff. miRNAs >1 were considered as up regulated, miRNAs <-1 were considered as down and those between 1 and -1 were flagged as neutral.

The functions of these pathways were related to cell survival, apoptosis, and stress response. Of these, the MAPK signalling pathway is believed to play a critical role in the response to stress stimuli (Sui et al., 2014; Niaudet et al., 2017), including mediation of heat stress-induced cell death (Liu et al., 2020). A report showed that the inhibition of the p38-MAPK signalling pathway decreased heat stress-induced ROS generation and cell apoptosis (Li et al., 2018). Secondary structure and fold energy analysis described the stability and optimum energy of the identified miRNAs. Minimum fold energy and minimum fold energy index are the two important parameters for the stability of miRNA. Protein-protein interaction of functionally linked protein targeted by novel miRNA revealed the stress associated protein networking. Heat map was generated for the most up and downregulated novel miRNAs on the basis of differential gene expression. Validation of eight novel miRNAs through quantitative real time PCR showed significant change in the relative expression of the miRNA under HS condition.

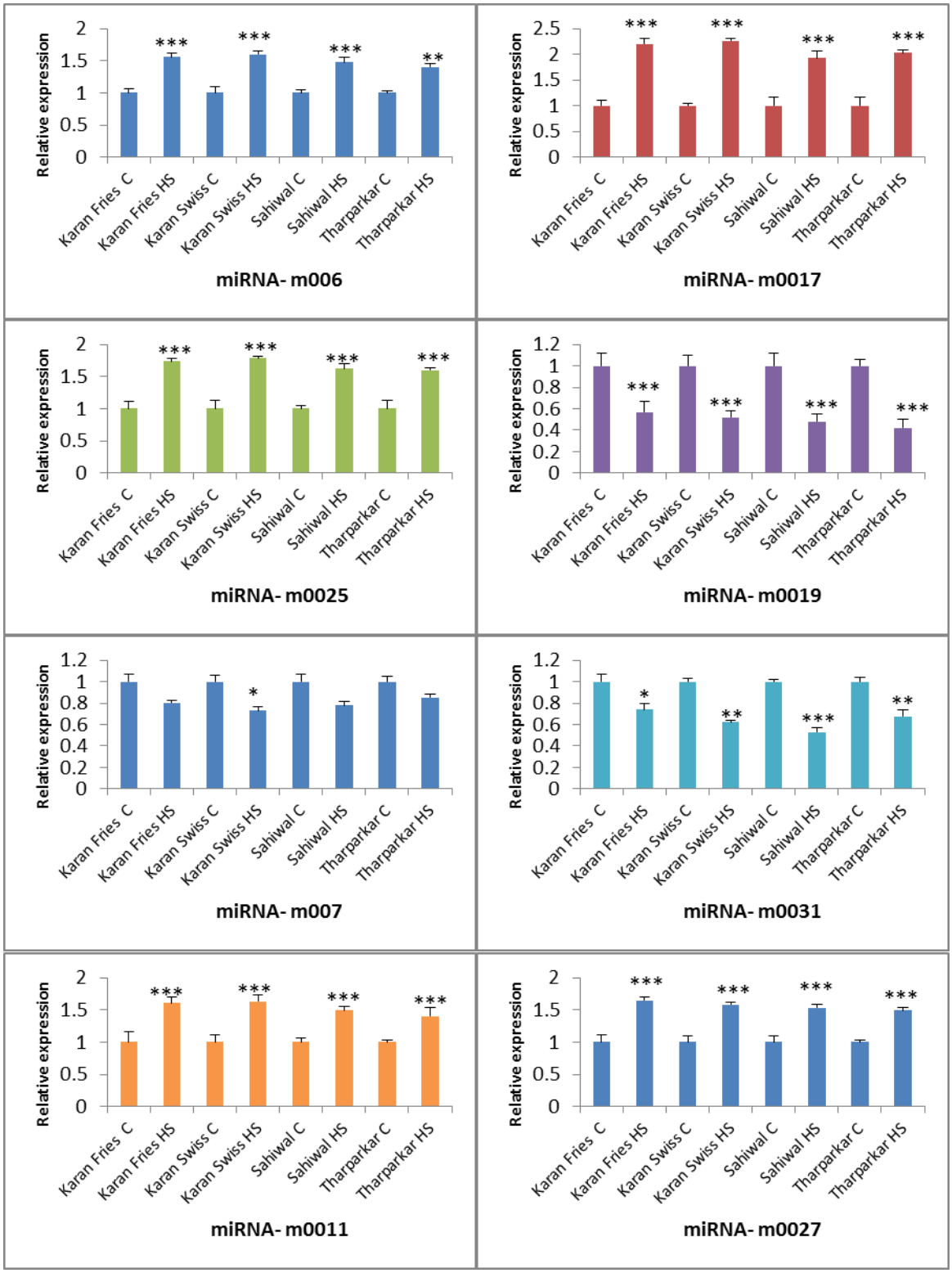


Fig. 9. Validation of identified novel miRNAs in different cattle breed under heat stress, Relative expression of novel miRNAs in Tharparkar, Karan Fries, Sahiwal and Karan Swiss under heat stress condition. U6 snRNA gene was used as endogenous control for normalizing the Ct values, Pfaffl (2001) method was used for the calculation. * symbol indicates a significant difference ($p < 0.05$) between treatments (one-way ANOVA); vertical bars indicate s.e (n = 3).

Conclusion

In conclusion, HS has severe effect on dairy animals. miRNA play important role in defence and metabolic pathway as a master gene regulator. Here, we have identified 77 novel miRNA from TH and KF cattle breed exposed to HS using de novo assembly approach. Validation of selected identified novel miRNAs

showed eight of them to be heat responsive. Target prediction and metabolic pathway analysis revealed that maximum target genes involved in stress networking. Secondary structure and protein-protein interaction was done for selected miRNA. On the basis of differential gene expression heat map was generated for most up and downregulated miRNA. Our data provide valuable information about the role of miRNAs during HS. These new gene resources may be used as miRNAs based biomarkers and to decipher the thermo tolerance.

Author's contribution

MK, GK—conceived and designed the experiments. MK—involved in isolation of miRNA and qRTPCR analysis. MK, AG—performed transcript profiling. MK, AG, GK—wrote the paper and edited the manuscript.

Acknowledgment

Financial assistance provided by the Indian Council of Agriculture Research (ICAR) under the National Innovation in Climate Resilient Agriculture (NICRA) project (Grant no. 2049/303) is highly acknowledged. We also thank Director, ICAR- National Dairy Research Institute (NDRI) for providing all the support required for executing the research work.

Conflict of interest

The authors declare that they have no conflict of interest.

Ethical standards

Experiments comply with the current laws of the country in which they were performed.

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