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***Original Research Paper***

# Genome wide analysis of heat responsive microRNAs in banana during acquired thermo tolerance

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## ABSTRACT

**MicroRNAs are a class of small regulatory RNAs in plants, which play vital roles during various abiotic and abiotic stress conditions including plant processes. In this present study, we examined the expression of miRNAs and their predicted target expression levels during heat stress in banana. Out of 235 miRNA found in *Musa*, 40 miRNA showed homology to heat responsive miRNAs from other plants. Further, 14 targets for miRNA were predicted that are potentially regulated by their cognate miRNAs and were monitored under three stages of stress viz, induction, induction + lethal alone using qPCR analysis. The results suggest that generally, there is a negative relationship in the expression patterns of miRNA and their predicted cognate targets - *HSP70, HSP90,* SAP, DNAj genes. These were highly up regulated and their respective miRNAs showed lower expression. This is the first report in banana, which demonstrated that during induction stress, various thermo-protective genes are activated at initial stages of stress to achieve thermotolerance through altered miRNA expression. The results will help in broadening our understanding acquired thermotolerance and their regulation by miRNAs in plants.**

**Key words:**Acquired thermo tolerance, banana, heat stress, miRNA,thermo protective genes.

## INTRODUCTION

MicroRNAs (miRNAs) are a class of small nonprotein-coding RNAs previously was considered as junk, with a nucleotide length of 20–24 and are involved in regulation ofa broad range of metabolic and physiological processes (Bartel, 2004; Mallory and Vaucheret, 2006; Ruiz-Ferrer and Voinnet 2009) indicating that miRNAs also play an important role in plant response to abiotic and biotic stresses.The miR393 and other miRNAs are induced by cold stress in *Arabidopsis* and miR169g and miR393 are up-regulated under drought stress in rice (Zhao and Srivastav, 2007). Sunkar and Zhu (2004) reported that expression of miR393 to be strongly up-regulated by cold, dehydration, and NaCl treatments in radish, miR397b and miR402 are slightly up-regulated by all the stress treatments, whereas miR319c appears to be up-regulated by the cold. Thus, miRNAs can be used as a promising tool to improve our understanding plant response to environmental stresses.

Differential expression of miRNAs was seen in response to heat stress in wheat and banana revealed through high-throughput transcriptome sequencing (Xin *et al*., 2010). They cloned small RNAs from heat-stressedwheat leaves. Among the 32 miRNA families identified in wheat, nine conserved miRNAs were putatively heat-responsive. In a study on banana roots under salt stress, miRNAs and their targets that responded were identified using transcriptome sequencing. Results indicate that several of the differentially expressed genes (DEG) were majorly down-regulated in miRNAs, and increased the expression of predicted target-genes. The targets were found to participate in diverse signaling and stress defense pathways (Lee *et al*., 2015).

Plants have an inherent ability to survive at high temperatures (basal thermotolerance) and to acquire thermotolerance (Senthil Kumar *et al*., 2003). Acquired thermotolerance may be induced by either exposure to short but sub-lethal high temperatures (De Klerk

and Pumisutapon *et al*., 2012), or,by a gradual temperature increase to lethally high levels experienced under natural conditions (Larkindale and Vierling, 2008), therefore reflecting a natural mechanism contributing to thermotolerance in plants.

In bananas (*Musa* spp.), heat stress is a serious threat to yield and affects productivity. In our study, heat-stress related miRNA and their targets were predicted; subsequently, their conservation and expression patterns during acquired thermotolerance were determined.

## MATERIAL AND METHODS

### Plant material and stress treatment

Five to six week old uniform banana seedlings (Grand Naine) were used for temperature induction responses (TIR) experiment. Three different stages of stress viz, control (C), induction stress (I), induction + lethal stress (I+L), were employed for heat stress treatment. Control plants were maintained at room temperature around 30°C. Heat treatment was given in the temperature controlled growth chamber. The plants were kept at 32°C and the temperature was slowly increased to 42°C for 2.5 hrs called "induction" stress, and later the plants were shifted to 55°C for 2 hrs (referred as "induction + lethal" or I+L). After each stress treatment leaf samples were immediately harvested and frozen in liquid nitrogen and stored in -80°C until use (Vidya *et al*., 2017).

### miRNA prediction

Initial identification of miRNAs involved in heat stress, in plants was based on previous studies (Reference). miRNAs from different plants such as *A. thaliana* (Fujii*et al*., 2005) wheat (Xin *et al*., 2010), maize (Gong*et al*., 1997) and cotton (He *et al*., 2014), which were involved in heat stress were selected in this study. They were individually examined through homology search using BLAST software (NCBI) against 235 miRNAs reported in Banana (D'Hont *et al*., 2012). Finally, 40 miRNA from Banana sequences, which showed homology to heat responsive miRNAs were chosen for experimental **(Supplementary Table 1).**

Primers were designed using miRprimer software (Balcells*et al*., 2011), where a primer and primer pair are assigned a score for each of the features that are relevant for the performance in qPCR. The output consists of a list of primer pairs ranked according to score. The best possible $3^{O}$- end sequence of the primer and then make the primer longer towards the $5^{O}$ - end until a Tm of 59°C to 60°C reached were chosen **(SupplementaryTable 1).**

### Target gene prediction

A web based plant small RNA target analysis software psRNATarget (http://plantgrn.noble.org/psRNATarget; Dai and Zhao, 2011) was used to predict the mRNA target genes for selected heat responsive miRNAs **(Supplementary Table 2)** with the following default parameters: maximum expectation of 3.0, length for complementarity scoring of 20 bp, target accessibility-allowed maximum energy to unpair the target site (UPE) of 25.0, flanking length around target site for target accessibility analysis of 17 bp in upstream and 13 bp in downstream, and a range of central mismatch leading to translation inhibition of 9 to11 nt. In order to validate the target gene association with the miRNA, fourteen-target genes expression was examined using quantitative real-time PCR analysis.

### RNA extraction

Total RNA was extracted from the leaf samples following the "Pine tree method" (Chang, 1993), from three biological replicates for each treatment. RNA quantification was done using the Nano Drop (Spectramax M2, Molecular devices) and 10µg of total RNA was treated with RNase free DNase (Cat no #AM1907, Ambion) following the manufacturer's instructions.RNA integrity was examined on 1.2% agarose gel prior and after DNase treatments.

### miRNA specific cDNA synthesis

First strand cDNA synthesis was performed by using a miScript II RT kit (Qiagen) following the manufacturer's protocol. Further, cDNA was diluted to 1:10 concentration and used for further study, qRT-PCR was performed using DyNAmo Flash SYBR Green qPCR master mix (Thermo Scientific, #218161) using ABI7500 (Applied Biosystem).

### Expression analysis of miRNA and targets

The total RNA was converted to cDNA. For miRNA, the cDNA synthesis kit used was miScript II RT kit and for target genes cDNA was prepared using

the Revert Aid RT Kit (Thermo Scientific, #K1622) following manufacturer's instructions. qPCR for miRNA was done using primers listed in **Supplementary Table 1** and target genes primers are listed in **Supplementary Table 2.**

qPCR was performed using SYBR Green Master mix (ROX) using ABI7500 (Applied Biosystem). 2µl of cDNA template was added to 10µl of SYBR Green Master mix (ROX) and $H_2O$ to a final volume of 20µl reaction. Thermal profile for qPCR was: 50°C for 2 min, 95°C for 10 min, followed by 39 cycles each consisting of 95°C for 15 sec and 60°C for 1 min, 72°C for 30sec. 25S gene was used as an endogenous control (Ling *et al*., 2014).The Comparative Ct method was used to calculate the fold change of transcript between an experiment and calibrator sample (Schefe *et al*., 2006).

### Statistical analysis

Data from different treatments were statistically analyzed using one-way analysis of variance (ANOVA) method using MS-excel.The data were presented as mean values [mean ± standard error (SE)].

## RESULTS

### Identification of heat stress responsive miRNA

Using previously reported heat responsive miRNAs from plants wheat (Xin *et al*., 2010), maize (Singletary *et al*., 1994), cotton (He *et al*., 2014), *A. thaliana*(Sunkar*et al*., 2007) a comparison was done with banana genome. These miRNAs were compared with miRNAs of banana through homology search using BLASTn with the cut off value of E value $<1e^{-10}$ was employed. We found 40 miRNAs that were identified as heat responsive in banana genome. The identified miRNAs were analyzed through, qPCR in heat stress treatments.

### Validation of heat-responsive miRNA

The predicted 40 miRNAs were initially standardized, with 25S gene as an endogenous reference control by qPCR. Later, only14 miRNAs were selected for further studies. Under heat stress (HS), the variation in the expression of these identified miRNA was observed in cultivar Grand Naine. In order to validate the identified conserved miRNAs, all the 14 miRNAs sequences (4 up-regulated and 10 down-regulated) were subjected to quantitative real time-PCR under the differential heat stress conditions.

In cultivar Grand Naine, higher levels of expression were observed in case of miR399, where fold change was 2.1 during Induction (I), 4.0 fold in Induction + lethal (I+L). Similarly, miR156, and miR169 were also up-regulation greater than the reference 2.2, 2.2 folds, and 2.1, 2.0 folds respectively in I, I+L respectively. The majority of miRNAs showed down regulation under heat stress, the significant ones such as miR171 having 0.9, 0.7 fold change, miR397- 0.5,0.6, miR398-0.5,0.4and miR414- 0.7,0.6, miR854-0.1,0.1 fold change in I and I+L respectively **(Fig. 1).**

### Identification of miRNA specific target genes

The differentially expressed miRNAs were used for the target prediction analysis. In order to identify miRNA specific targets, a plant small RNA target analysis server psRNATarget software, (http:// plantgrn.noble.org/psRNATarget/;Dai and Zhao, 2011) was used to predict target genes. Plant miRNAs usually bind to the protein-coding region of their target genes with nearly perfect sequence complementarities, which results in either degradation of their target mRNAs or translation (Rhoades *et al*., 2002). Based on analysis, we have selected 14 target genes (mRNA) for each of the miRNAs.The predicted targets were found to be involved in diverse metabolic and physiological processes, transcription factors, signal transduction, disease resistance proteins, cell differentiation and growth.

### Validation of target genes by quantitative real time PCR

We have examined the expression of the target genes, using qPCR. The transcript profiling of Heat shock protein DNAj was found to have highest level of expression (Induction 4.9, I+L 4.8) followed by *HSP90* (4.6, 4.8), *HSP70* (3.2, 3.8), Stress associated protein (3.7, 3.1) respectively. Highly down-regulated targets were Glutathione synthase (0.1, 0.2), Ribosomal Protein S8 (0.18, 0.2).

## DISCUSSION

Heat stress affects growth and development, thus reducing the productivity of crops. Plant miRNAs

**Supplementary Table 1:List of primer sequences used in qPCR for miRNA**

| Sr. No. | Primer name | Primer Sequences (5'-3') |
|---|---|---|
| 1 | miR399F | GGG GAA AAT GGC AGG GCA ATT CTC |
| | R | CCA GTT TTT TTTTTTTTT TGC CAA AGG CCA AAG |
| 2 | miR169F | GCC AAG GAT GAC TTG CCT GTG TC |
| | R | GGT CCA GTT TTT TTTTTTTTT TAG GAG AGG AGA GG |
| 3 | miR400F | ACG CAG CGC AGT ATG AGA GTA TTA TAA GT |
| | R | GGT CCA GTT TTT TTTTTTTTT TGT GAG TGA GTG AG |
| 4 | miR854F | CGC CGC AGG ATG AGG ATA GG |
| | R | GGT CCA GTT TTT TTTTTTTTT TCT CCT CCTCCT C |
| 5 | miR390F | ATG GGT AAG TAG GAA CTT GTG TTC TGT TTG TCT AGA G |
| | R | CCA GTT TTT TTTTTTTTT TGA GTA GGA TGA GTA GGA TGA G |
| 6 | miR414F | CGC CGC AGT CAT CTT CAT CATCAT C |
| | R | GTC CAG TTT TTTTTTTTTTT GAC GGA CGG AC |
| 7 | miR397F | GCC CAG CGC TGC ACT CA |
| | R | GGT CCA GTT TTT TTTTTTTTT TCG TCGTCG T |
| 8 | miR156F | TTG ACA GAA GAG AGT GAG CAC ACA G |
| | R | AGG TCC AGT TTT TTTTTTTTT TTA CCA CCA CC |
| 9 | miR159F | CGC GCG CAG TTT GGA TTG AAG |
| | R | CAG GTC CAG TTT TTTTTTTTTTT AAG AAGAAGAAGAAG AA |
| 10 | miR393F | CAA AGG GAT CGC ATT GAT CCA CAC |
| | R | GTC CAG TTT TTTTTTTTTTT GAG AGG AGA GGA G |
| 11 | miR529F | GCG CAG CTG TAC CCT CTC TC |
| | R | GTC CAG TTT TTTTTTTTTTT GAA GAA GGA AGA AGG AA |
| 12 | miR398F | CCA AAG GTA GCC AAG GAC AAA CTT GC |
| | R | GGT CCA GTT TTT TTTTTTTTT TCT GCT GCT GC |
| 13 | miR171F | ACC TTT TTT CTG ATT GAG CCG TGC CAA TAT CTT AG |
| | R | CAG GTC CAG TTT TTTTTTTTTTT ATG ATGATGATGATGATG |

Note: miR156F/R primers were used for two targets.

**Supplementary Table 2: Sequences for primers used for target genes in qPCR analysis**

| Sr. No. | Predicted Targets | Forward primer (5'-3') | Reverse primer (5'-3') |
|---|---|---|---|
| 1 | Outer envelope protein of 80 kDa | TGGGACAAACAGCGTAAGAG | TCCATAGTCTCCAAACAGCAC |
| 2 | Glutamate synthase | GGATGAAGTGGAACCTGCTAG | ACCAGTGTTAGATTTGCCTCC |
| 3 | Putative Pentatricopeptide repeat-containing protein. | TGGATAGGTTTGGCGATGTG | TCCCTCAACTTTAATGCCTCTG |
| 4 | Stress-induced protein, | CTCGTCTATATCACTGCCATCTG | GTCATGTATCGTCACAGTCCAG |
| 5 | CAMK includes calcium/calmodulinde-pedent protein kinases, expressed | GTAGATATGTGGAGTCTTGGCG | GAGAATGGACTGCGAAAATGG |
| 6 | Heat shock protein DnaJ | CATCGTCTCCTTTTGTCCTCG | TCAGCATCCCTTTCCAGTTC |
| 7 | Heat shock 70 kDa | GATGAGAAGGATGTGAGAGGG | ATATTCTCCACACTCAACCCTG |
| 8 | Tropinonereductase. | TCGCCTACCCTCATCTCAAG | CCATGAAGCCTACGACAGAAG |
| 9 | GAMYB | CCTGGTCGCACTGATAATGAG | GCTGACAATCTGAGTTTGCTG |
| 10 | Serine/threonine-protein kinase receptor. | TTGCGACCAGTTCTACCTTG | TGGCTGTAGTCAACGAAGTG |
| 11 | Putative Squamosa promoter-binding-like protein 12 | GATCTGTATGTTCGTCTGGTCG | TGATTTTCTCTTTCCTGCCCC |
| 12 | SOD | CGTTGATGGAGTAGCTGAGG | AGTGGTAAGGCTGAGTTCATG |
| 13 | Ribosomal Protein S8 | AAGACCCGTATCCTTGATGTG | TCCAATCTCAACCCCGTAATG |
| 14 | HSP90 | CCTGAGTCTCTAGTTGTGGAAATC | TCGCCGTAAAGAACACCAAC |

function as a gene regulator through binding to the protein-coding region of their target genes, further to initiate degradation or translational repression of the transcripts (Palatnik*et al*., 2003).

In case of *Musa* species, 37 miRNA families in *Musa*A genome (D'Hont*et al*., 2012) and 42 miRNA families in *Musa* B genome (Davey *et al*., 2013) were identified. In this study, through homology search we identified 40 heat responsive miRNAs, of which 14 miRNAs, and their targets expression were analyzed during various stages of heat stress treatment.

The induction stress treated banana plants (32$^{o}$ C- 42$^{o}$ C for 2 hrs and 30 min), which were later challenged with high temperature (55$^{o}$ C for 2 hrs), showed better recovery. This showed that induced plants, show better tolerance than the non-induced ones through acquired thermotolerance (Vidya *et al*., 2017).

In order to investigate the regulation of miRNAs and their targets, during thermotolerance, differential expression analysis was done. The miR397 was less expressed under HS, compared to control, in case of cv. Grand Naine, the miR397 was observed to be down regulated due to heat stress at the induction stage **(Figure 1A).** The down-regulation of miR397, and higher expression of the its predicted target gene *HSP70* which is a thermoprotective gene (Vidya *et al*, 2016b). We have also recorded higher expression of *HSP70* at induction and I+L stages(Yu *et al*., 2010, Vidya et al., 2018). Similarly, miR414, miR854, and miR171 expression were down regulated during induction stages. The predicted targets are DNAj**(Figure 1B),** Stress associated proteins (SAPs) **(Figure 1C)** and Heat shock protein 90 **(Figure 1D)** respectively was up regulated during heat stress. In one of the studies, carried out on stress associated protein gene (SAP's) on banana, the expression profiles of *Musa*SAP1 also showed up regulation at 24 and 48hrs of drought, heat stress which is also in agreement with the results we have observed here (Shekhawat *et al*., 2015). From the earlier studies, it is well known that *HSP70*, *HSP90*,DNAj, SAP acts as thermoprotective genes. The alteration in expression pattern of heat responsive miRNAs (miR397, miR414, miR854, miR171) thus regulating the expression levels of thermoprotective genes (*HSP70*, *HSP90*,DNAj, SAP) during heat stress indicates their direct role in tolerance to heat stress.

Under heat stress, miR398 expression was decreased, and its target superoxide dismutase (SOD) (Beauclair*et al*., 2010) expression was up-regulated **(Figure 1E).** miR398 has been reported to be involved in tolerance. Heat stress triggers the accumulation of ROS, up-regulating the antioxidant activity. Similar

pattern was observed in Arabidopsis (Yan *et al*., 2012) for miR398, under oxidative stress where down regulation of miR398 enhanced the expression of CSD1 and CSD2 (Sunkar *et al*., 2006). Similarly, miR400 was also down regulated during HS. The miR400 regulates Pentatricopeptide repeat (PPR) genes **(Figure 1F).** However, the miR400 target pentatricopeptide repeat (PPR), which is involved in plant development and abiotic stress, was up-regulated by heat stress in Arabidopsis (Park *et al*., 2014) miR393 targets serine/ threonine-like receptor kinases called receptor-like kinases (RLKs) **(Figure 1G),** which are the major class of cell surface receptor. The relative expression of the target gene was down regulated suggesting that the RLKs play a vital role under heat stress responses (Bhargava *et al*., 2013). These observations were supported by earlier studies. In a transgenic study on Arabidopsis where down regulation of miRNA miR398 enhanced the expression of targets, *HSP70*. The plants were heat tolerant when compared to the wild type (Guan *et al*., 2013).

For miR399, miR529 and miR169, the target genes are outer envelope 80kDa protein **(Figure 1H),** ribosomal protein **(Figure 1I)** and glutamate synthase **(Figure 1J)** respectively. These miRNAs were up-regulated by heat stress, correspondingly the target genes were down regulated. The target genes are majorly involved in protein synthesis. The ribosomal protein, membrane proteins are involved in protein synthesis, since it is a highly energy demanding process and protein synthesis is at low level during HS (Pillai *et al*., 2007). It is also observed that the expressions of these miRNAs were significantly altered during the induction stress treatment, suggesting that they respond to mild stress. miR169 and miR399 which were most frequently found heat responsive micro RNA's in plant system and were up regulated with their respective targets being down regulated in response to heat stress (Chai *et al*., 2015). The results observed here were consistent with the previous reports that they are responsive to heat stress in both rice and wheat (Xin *et al*., 2010; Yu *et al*., 2010).

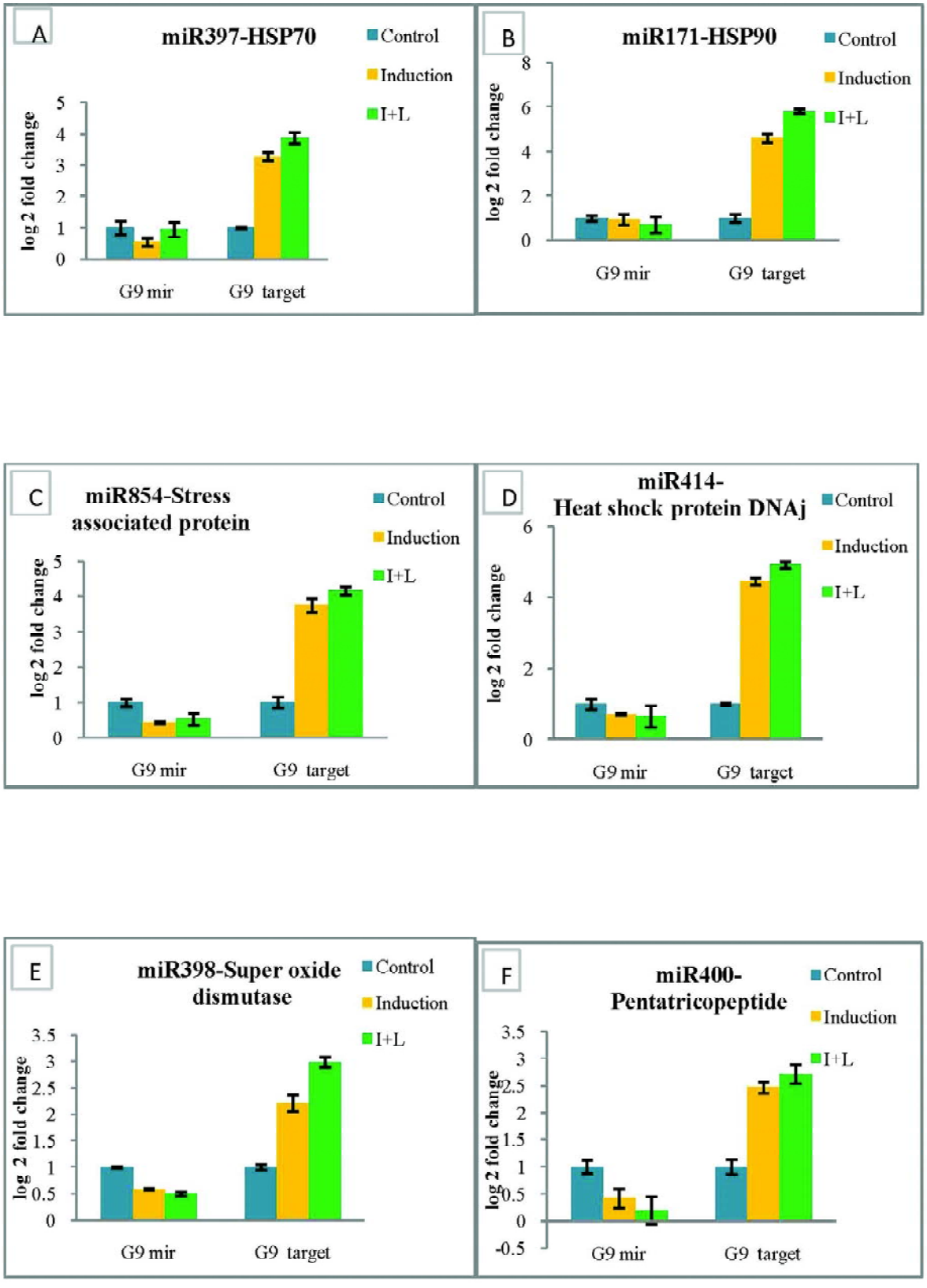
The remaining miRNAs (miR159, miR390) expressions are not altered much in our study. These miRNAs have targets GAMYB for miR159 **(Figure 1K),** Calmodulin for miR390 **(Figure 1L)** and SPL for miR156 **(Figure 1M).** Calmodulin was up-regulated only in induction stress. However, MYB was up-regulated in all the stress treatments. In one of the studies on *Triticum aestivum L,* in contrasting cultivars the *TaGAMYB* was found to be decreased initially and increased during 2 hrs of heat stress treatment. Therefore, we speculate that MYB plays an important role in the aspect of plant growth and development, stem elongation and the up-regulation of these genes confirms its role in heat stress.The remaining miRNAs studied was miR156, target being *Squamosapromoter-binding like* (SPL) and Tropinonereductase (TR) **(Figure 1N)** gene was highly down regulated during lethal conditions compared to mild heat stress i.e induction stress **(Figure 1M).** The SPL gene in rice, was observed to have complementarity to miR156, was expressed in the leaves and shoots (Xie*et al*., 2006).

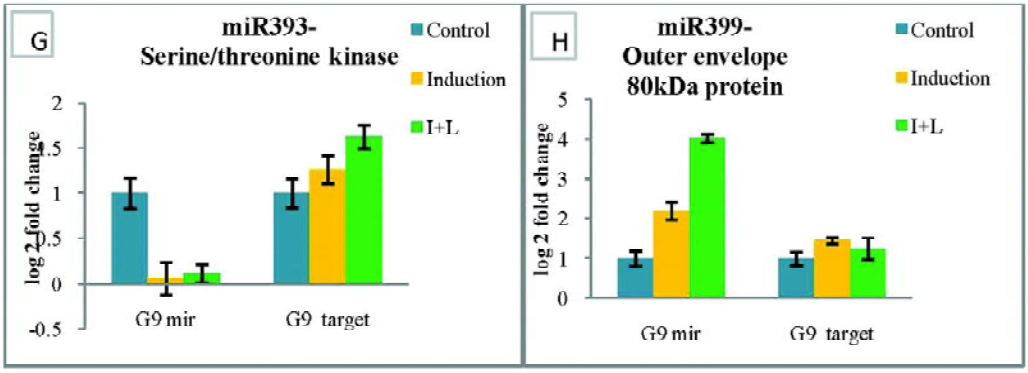
miR390 was observed to have no alteration in I and I+L due to heat stress suggesting that the corresponding targets Calmodulin-binding proteins which are involved in calcium signaling are regulated **(Figure 1L).**However, there was reduction in expression if thestress levels increased when compared to control conditions where the fold change was 0.9. The probable reason for this might be $Ca^{2+}$ signaling was not altered by heat stress in the tolerant cultivar Grand Naine. It has been demonstrated that involvement of calmodulin in on heat shock induced thermotolerance in maize seedlings (Gong *et al*., 1997) and possibly similar pathway could be operating in banana

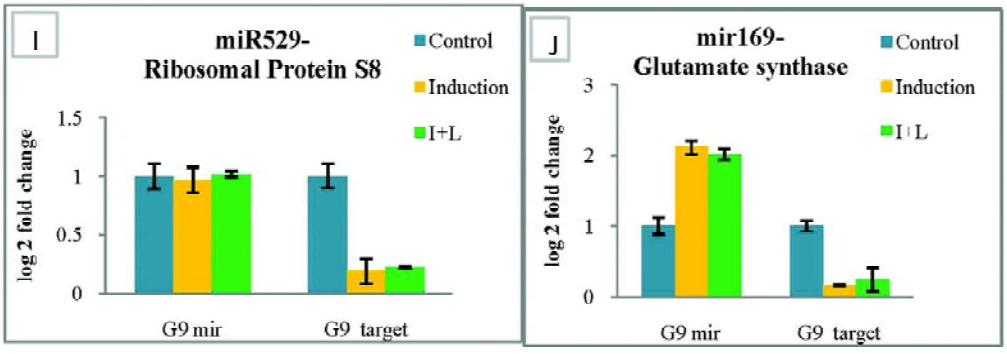
These findings indicate that relationships between miRNAs and their target genes expression vary and not always one-to-one. The expression patterns of miRNAs and corresponding target genes had negative relation as well. A negative correlation between the expression of miRNAs and their target genes was also observed that the correspondence between the expression pattern of miRNA and their targets can vary between mRNAs belonging to the same gene family and even for the same target mRNA at different developmental stages (Lopez-Gomollon*et al*., 2012).

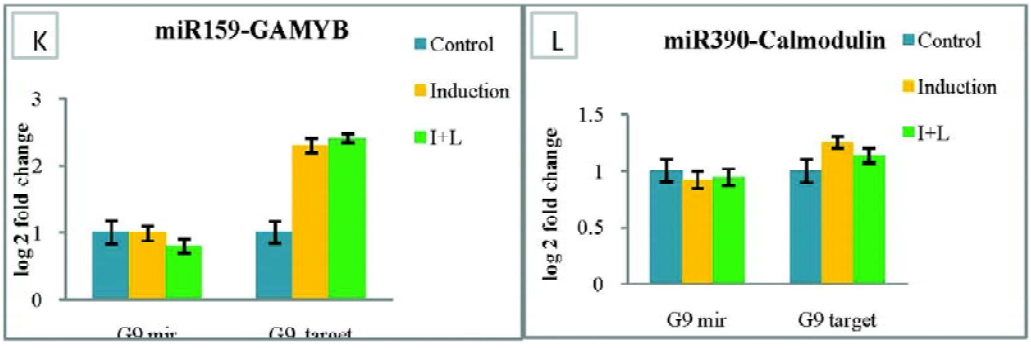
Plants have ability to adopt various strategies to optimize their response to thermotolerance. One of them might be the change in pattern of miRNAexpression, thus, altering expression of thermoprotective genes is achieved. For example,

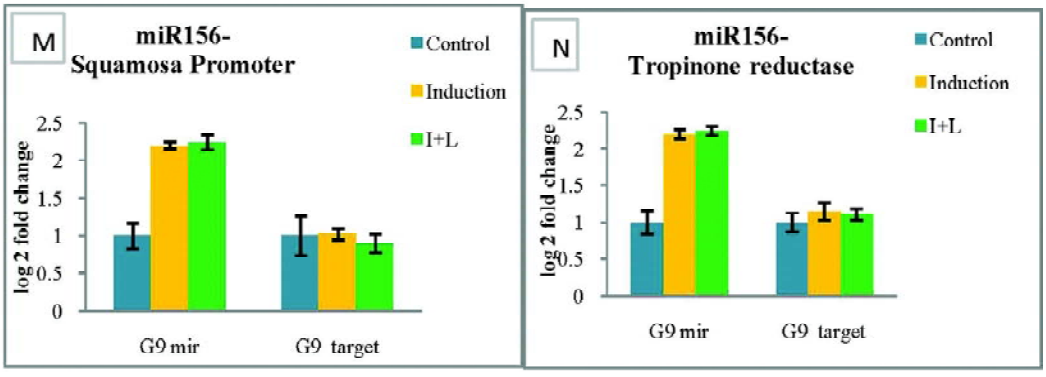
**Figure 1 : Expression analysis of miRNA and their respective target genes under different heat stress conditions. Each column represents the mean±SE of three biological replicates.**




G
miR393-
Serine/threonine kinase
Control
Induction
I+L
log 2 fold change
G9 mir
G9 target
H
miR399-
Outer envelope
80kDa protein
Control
Induction
I+L
log 2 fold change
G9 mir
G9 target


I
miR529-
Ribosomal Protein S8
Control
Induction
I+L
log 2 fold change
G9 mir
G9 target
J
mir169-
Glutamate synthase
Control
Induction
I+L
log 2 fold change
G9 mir
G9 target


K
miR159-GAMYB
Control
Induction
I+L
log 2 fold change
G9 mir
G9 target
L
miR390-Calmodulin
Control
Induction
I+L
log 2 fold change
G9 mir
G9 target


M
miR156-
Squamosa Promoter
Control
Induction
I+L
log 2 fold change
G9 mir
G9 target
N
miR156-
Tropinone reductase
Control
Induction
I+L
log 2 fold change
G9 mir
G9 target

*HSP70, HSP90,*DNAj, SAP has a direct relevance during thermotolerance and are regulated by miRNAs. Induction stress activates an array of signal transduction pathways, which helps in adaptation to severe stress (Srikanthbabu *et al*., 2002; Lindquist,1986). The results of the present study indicate that changes in expression levels of miRNA, which further alters the expression of many thermoprotective genes during induction and I+L. This is further expected to confer tolerance to high temperature their-by acquired thermotolerance.

## CONCLUSIONS

The role of heat stress related miRNAin banana has been demonstrated through this study. Significant changes in the pattern of miRNA expression in banana indicated the key role of miRNAs in the heat stress-response. An increase in the expression of thermoprotective genes regulated by their respective miRNAs was evident. miRNAs have been reported to be master regulator of plant growth and development, though it constitutes only 1 per cent of the total protein-coding genes of an organism. An increase in the expression of HSPs like *HSP70, HSP90,* DNAj and other stress associated proteins and lower expression of respective miRNAs indicated their roles in the heat tolerance. Therefore, it appeared that miRNAs are involved in the regulation of expression of stress associated genes and metabolic pathway associated genes which can impart the tolerance to heat stress in banana.

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