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RESEARCH ARTICLE

Identification and Validation of Cold Responsive MicroRNAs of *Hevea brasiliensis* Using High Throughput Sequencing

Linu Kuruvilla[1], MB Mohamed Sathik[1]*, Molly Thomas[1], Lisha P Luke[1], Sumesh KV[1]

[1]*Rubber Research Institute of India Rubber Board, Kottayam 686009, India*

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Abstract

Cold stress is one of the major abiotic factors that influence the productivity and geographical distribution of many agriculturally important crops like *Hevea brasiliensis*. Cultivation of *H. brasiliensis* in India is being extended to northeastern regions, where low temperature during winter adversely affects its survival, growth, and productivity. Developing cold-tolerant genotypes is a primary requisite to maximize the productivity under such challenging environmental conditions. However, lack of methods for early evaluation of cold tolerance in the newly developed clones and the extensive time required for assessing their tolerance in the field are major constraints for clonal selection. The present study was initiated with an objective to identify and characterize cold stress responsive miRNAs from *H. brasiliensis* that show stronger association with cold tolerance. Next generation sequencing using Illumina HiSeq method revealed the expression of 21 and 29 conserved miRNA (from clone RRIM 600) families in cold-stressed and control samples, respectively. Forty-two novel miRNAs were identified from this study. Upon differential expression analysis, eight conserved miRNAs were found commonly expressed in both the samples. When expression analyses were performed subsequently with six selected miRNAs in two *Hevea clones* (*viz.* RRII 105 and RRIM 600), miR169 showed a strong association with cold tolerance. miRNAs such as miR482 and miR159 also exhibited association with cold tolerance. This study suggests the possibility of employing these miRNAs as markers for cold tolerance after validation in more number of genotypes with varying levels of cold tolerance.

Key words : Cold tolerance, expression analysis, *Hevea brasiliensis*, high throughput sequencing, miRNAs, qPCR

Introduction

Hevea brasiliensis Muell. Arg., a tropical tree native to Amazon rain forests of South America, is the major source of natural rubber. The ideal temperature for rubber cultivation is between a maximum temperature in the range of 29 to 34°C and a minimum temperature not lesser than 20°C. As there are constraints in the availability of cultivable land area in the traditional rubber growing regions of India, cultivation of rubber is being extended to regions having suboptimal environments like northeastern regions where the temperature during winter is too low for its survival and optimum productivity and has been reported to affect the development and latex biosynthesis (Jacob et al. 1999; Priyadarshan et al. 2005). Cold damage to rubber trees is a complex phenomenon which involves differential response of clones, age, and vigor of the plant. Hence, it is imperative to select clones/varieties with enhanced tolerance to low temperature stress in order to achieve sustainable productivity in the cold-prone regions. However, factors like lack of suitable techniques for early evaluation for cold tolerance in the breeding population and the time required to assess their tolerance in field conditions are the real constraints in selecting clones for such abiotic stress-prone regions. In order to maximize the productivity of *Hevea* and to identify the best performing clones for stress-prone agroclimatic zones, attempts have to be made to breed and select suitable clones for such regions.

In general, plants respond to cold stress by adjusting their metabolism and by effecting various physiological and molecular changes in order to acquire enhanced cold tolerance (Thomashow et al. 1999). Cold stress induces changes in membrane fluidity and protein conformation. The plants respond to cold stress by re-arranging its cytoskeleton followed by activation of Ca^{2+} channels which lead to increased cytosolic Ca^{2+} levels eventually triggering the ex-

MB Mohamed Sathik (✉)
Email: mohamed.sathik@gmail.com

pression of cold-responsive (COR) genes. The COR genes are known to be involved in altering the metabolism, protein stability, and cell structure by regulating hundreds of COR genes related to signal transduction, defence against pathogens, and transcription factors. Cold stress also induces the expression of C-repeat binding transcription factors (CBF) which play vital role in regulation of genes such as late-embryogenesis abundant (LEA) type-protein and osmoprotectant biosynthesis in plants. In Arabidopsis, CBF genes are required for enhancing cold tolerance. CBFs are positively regulated by Inducer of CBF Expression 1 (ICE1) and negatively by MYB15. Under cold stress, regulation of cold signalling is effected by MAP (mitogen activated protein) Kinase cascade. MAP Kinase Kinase (MAPKK) is involved in phosphorylation of MPKs under cold stress which further triggers the cold signalling pathway (Chinnusamy 2006). A recent study on cold signalling emphasizes the role of MPK6 mediated regulation of MYB15 in cold stress signalling in Arabidopsis (Kim et al. 2017).

Gene expression studies carried out in low temperature exposed *Hevea* clones RRII 105 and RRIM 600 revealed the existence of stronger association between genes such as NAC transcription factor, LEA 5 protein, and peroxidase with cold tolerance (Sathik et al. 2012). Among them, processes such as repression of genes, mRNA export, and mRNA degradation have been found to be of central importance for the cold-stress response (Zhu et al. 2007). Various reports on a wide range of species have proven that gene regulation by microRNAs is essential for coordinating the plant's responses to cold stress. miRNAs have been found to play a main role in regulating the cold responsive genes and are directly associated with cold tolerance. Cold responsive miRNAs have been reported in *Arabidopsis* (Liu et al. 2008; Sunkar and Zhu 2004; Zhou et al. 2008), poplar (Chen et al. 2012; Lu et al. 2008), *Brachypodium distachyon* (Zhang et al. 2009), rice (Lv et al. 2010), wheat (Tang et al. 2012), tomato (Cao et al. 2014), and potato (Ou et al. 2015). The effect of cold on miRNA expression is species, tissue, or developmental stage dependent (Sunkar et al. 2012). Gebelin et al. (2013b) identified eight cold-specific *MIR* genes in *Hevea*, of which seven *MIR* genes were found significantly down-regulated under cold-stress conditions in clone PB 260. As reports were not available on cold responsive miRNAs of *Hevea* in different clones with varying levels of cold tolerance, finding miRNAs strongly associated with cold tolerance is not possible. Hence, in this study, attempts were made to identify cold-responsive miRNAs and to find miRNAs having stronger association with cold tolerance by validating in two clones of *Hevea* with contrasting levels of cold tolerance.

Materials and Methods

Plant material and stress induction

Six-months-old polybag plants of clone RRII 105 (cold susceptible) and RRIM 600 (cold tolerant) were acclimatized in a growth chamber for three days with a minimum temperature of 15°C during the night (for 3 h) and a gradual rise in maximum temperature up to 25°C in the daytime. From the fourth day onwards, cold treatment was imposed by reducing the temperature to 8°C during night followed by a gradual increase in maximum temperature up to 15°C in the daytime for five consecutive days. As the northeastern regions of India experiences as low as 8°C during winter, the low temperature treatment was restricted to 8°C for this study. Light intensity regime ranging between a minimum of 200 to a maximum of 800 μ mol m^{-2} s^{-1} with RH in the range of 60 to 70% were provided. Control plants were allowed to grow at stress-free and ambient weather conditions.

Gas exchange measurements

Leaf samples were harvested after assessing the stress response of the plants by measuring the net CO_2 assimilation rate (A) and stomatal conductance (gs) using a portable photosynthesis system (LI-6400), LI-COR, USA. All the gas exchange parameters were measured at a constant CO_2 concentration of 360 ppm using a CO_2 injector and at 500 μ mol m^{-2} s^{-1} of light intensity using red LED source (with 10% blue light) attached with the leaf chamber (LI-6400). On the same leaves, chlorophyll fluorescence was also measured using a fluorescence monitoring System (Hansatech, UK). Leaves were dark adapted for 20 min by clamping aluminium leaf clips over the leaf for measuring the maximum potential quantum yield (F_v/F_m). Minimal fluorescence (F_o) and maximum fluorescence (F_m) were measured in dark adapted leaves by giving a saturating flash of light. The flash of light allowed transient closure of PSII reaction centres. Variable fluorescence (F_v) is the difference between F_o and F_m. The ratio (F_v/F_m) = (F_m–F_o)/F_m reflected the maximum potential quantum efficiency of PSII (Maxwell and Johnson 2000).

Small RNA library construction and sequencing

Total RNAs were extracted from leaves of cold- stressed plants of clone RRIM 600 using Spectrum™ Plant Total RNA Kit (Sigma-Aldrich) according to the manufacturer's instructions. The quantity and quality of total RNA was determined using Nanodrop-1000 and by resolving on 1% denatured agarose gel. The pair-end cDNA sequencing library for small RNA were prepared for control and cold-stressed samples using Illumina® TruSeq Small RNA Sample Preparation Kit (Illumina) as per manufacturer's instructions. For the library preparation, 1 μg total RNA was first ligated with 3' adapter followed by 5' adapter ligation. Reverse transcription followed by PCR was performed to create cDNA constructs based on the small RNAs ligated with 3' and 5' adapters. The final PCR products were purified and subjected to deep sequencing by employing Illumina HiSeq 2000 at Xcelris Genomics, Ahemedabad, India.

Identification of conserved and novel miRNAs

To identify the conserved miRNAs, small RNAs were annotated against miRBase database (version 21) by using CLC Workbench (Version 6). A maximum of two mismatches

were allowed in the annotation. To identify novel miRNAs from *Hevea,* sequences ranging from 20 to 24 nt were used for further analysis using stringent criteria for miRNA prediction. The small RNAs were mapped to the draft genome of *Hevea brasiliensis* ((http://www4a.biotec.or.th/rubber/Search) *Ricinus communis* and *Manihot esculenta* to identify novel miRNAs using miRanalyzer (Version 3) with default parameters and the precursor molecules were extracted from their genome sequences.

Target prediction for miRNAs

Target prediction for known and novel miRNAs was performed using web-based psRNA Target program (http://plantgrn.noble.org/psRNATarget) and TAPIR software (http://bioinformatics.psb.ugent.be/webtools/ tapir). Following parameters were used for psRNA Target program *viz* (1) a maximum expectation value of 3.0; (2) a complementarity scoring length of (hsp size) 20; (3) a target accessibility of 25 or less; and (4) no mismatch at positions 9-11. For target prediction, TAPIR score and the free energy ratio were considered. Mismatches and gaps were given a score of 1 and G:U pairs were given a score of 0.5 while these scores were doubled within the seed region. The default score cutoff value of 4.0 and the default value for free energy ratio was 0.7.

Validation of miRNAs by qPCR

Total RNAs were extracted from control and cold-stressed samples of *Hevea* clones RRII 105 and RRIM 600 using Spectrum™ Plant Total RNA Kit (Sigma-Aldrich) according to the manufacturer's instructions. Total RNA (2 µg) from each sample was then reverse transcribed using Mir-X miRNA first strand c-DNA synthesis kit (Clontech). In a single reaction, small RNAs were poly-adenylated and reverse transcribed using poly(A) polymerase and SMART MMLV Reverse Transcriptase. Validation of six conserved miRNAs (Table 1) in control and cold-stressed plants was performed by qPCR on Light Cycler 480 II (Roche) using SYBR Advantage qPCR Premix (Takara). The reaction consisted of 0.5 µl of 10 times diluted cDNA, 0.1 µM of each forward and reverse primers and 5 µl of 2x SYBR Advantage qPCR Premix in a 10 µl reaction volume. The reaction conditions included an initial denaturation step of 95°C for 30 s, followed by 40 cycles of 95°C for 5 s, and 60°C for 30 s. Changes in expressions were calculated as normalized fold ratios using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001).

Table 1. List of miRNAs and their sequences for qPCR analysis.

miRNA	Sequence (5'-3')
miR169	GAGCCAAGAATGACTTGCCGA
miR482	AGATGGGTGGCTGGGCAAGAAG
miR858	TTTCGTTGTCTGTTCGACCTT
miR171	TCTATAATCACGCCAAGTTAG
miR159	AAACCTAACTTCCCTCGAGAC
miR166	AGCCTGGTCCGAAGTAAGGAG

Results

The plants grown under growth chamber conditions showed cold-stress responsive syndromes after cold treatment at 8°C during night and at 15°C during daytime for five days. The physiological parameters such as stomatal conductance (gs), net CO_2 assimilation rate (A) and quantum efficiency of PS II indicated the incidence of stress in both the clones. The stomatal conductance in the susceptible clone RRII 105 got inhibited significantly under cold stress while the tolerant clone RRIM 600 could maintain conductance. While both the clones maintained similar CO_2 assimilation rate under control conditions, RRIM 600 maintained better A than RRII 105 under cold stress (Fig. 1B). Similarly, the F_v/F_m ratio which indicates photochemical efficiency of PS II also was found better in RRIM 600 than RRII 105 under low temperature conditions (Fig. 1C).

Small RNA libraries from cold treated leaf samples and control samples (of clone RRIM 600) were constructed and sequenced using Illumina HiSeq2000 platform. A total number of 11,383,272 reads were generated from the cold-treated library. After removing the 5' and 3' adaptor, the reads smaller than 20 bp and greater than 24 bp were avoided. A total number of 1,162,006 clean reads corresponding to 170,743 unique reads were obtained. Among the unique sequences, 22 nt small RNA was found abundant (Fig. 2).

Identification of conserved and novel miRNAs

Among the 218 miRNAs belonging to 21 conserved miRNA families identified, miR166 got highly expressed with an expression value of 17295 followed by miR159 and miR9386 with expression value of 1410 and 1377, respectively. The number of members varied among miRNA families with largest being the miR166 with 141 members followed by the miR159 with 45 members. Thirteen miRNA families were represented by only one member (Supplementary Data 2). This study could identify a set of 13 conserved miRNAs which were not reported previously in miRBAse (Release 21). The miRNAs identified and reported in this study (Table 3) are *viz.* miR2275, miR3630, niR399, miR4995, miR5021, miR535, miR5368, miR5658, miR7760, miR7782, miR827, miR858, and miR8175.

A large group of novel miRNAs were also identified based on database of *Hevea brasiliensis*, *Ricinus communis,* and *Manihot esculenta* in the cold-treated samples. Consequently, secondary structures were predicted for precursors of such candidate novel miRNAs by using m-Fold web server (mfold.rit.albany.edu) with default parameters. miRNA precursors possessing secondary structure (Table 2) with a free energy of equal or less than -25 kcal per mol were considered as novel miRNAs (Supplementary Data 1).

Targets for miRNAs

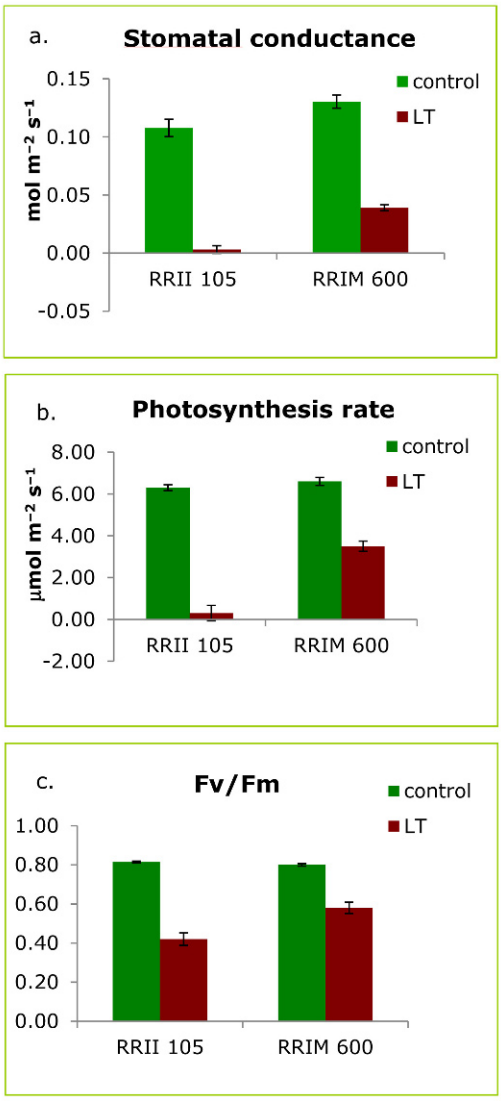

Fig. 1. (a) Stomatal conductance (g_s), (b) CO_2 assimilation rate (A), (c) Fv/Fm of control and cold stressed (LT) plants of RRII 105 and RRIM 600.

All 218 conserved miRNAs were searched for targets against ESTs or genes of *Ricinus*, *Hevea,* and *Manihot*. Among the 218 conserved miRNAs, 203 miRNAs had 399 targets in *Ricinus*, 165 miRNA had 739 targets in *Hevea,* and 14 miRNAs had 16 targets in *Manihot* (Supplementary Data 3). Twenty-six miRNA-target pairs were obtained for six novel miRNAs out of 18 in *Hevea brasiliensis* and five miRNA-target pairs were obtained for four novel miRNAs out of seven in *Ricinus communis* while 27 miRNA-target pairs were obtained for six novel miRNAs out of 17 in *Manihot esculenta* (Supplementary Data 4).

Differential expression analysis of cold stressed and control samples

A total of 29 and 21 miRNA families were identified in control and cold-stressed samples (of clone RRIM 600),

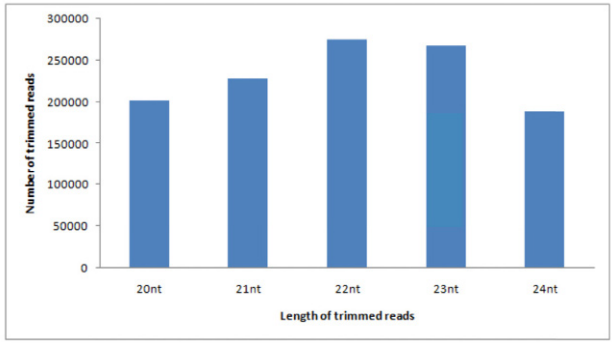

Fig. 2. Length of small RNA sequences in cold treated plants of *H. brasiliensis* (clone RRIM 600).

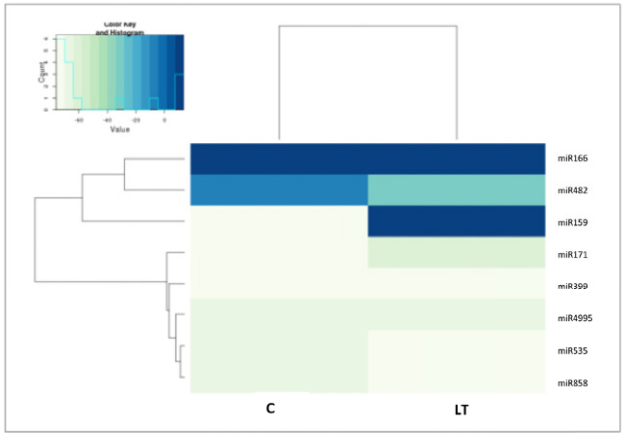

Fig. 3. Digital gene expression analysis of eight miRNAs in control (C) and cold stressed (LT) plants of clone RRIM 600. Darker the shade higher the expression level of miRNA.

Table 2. Novel miRNAs identified from cold stressed *Hevea brasiliensis.*

Species	No. of novel miRNAs
Hevea brasiliensis	18
Ricinus communis	7
Manihot esculenta	17

respectively. From the differential expression analysis, carried out by digital gene expression (DGE) method, eight miRNAs were found commonly expressed in both the control and the cold-stressed samples (Fig. 3). From this analysis, miR166 was the only one miRNA found highly expressed in both the samples. miR159 and miR171 were found highly up-regulated in cold stressed than in control samples while miR482 were found more in control than cold-stressed samples.

Validation of miRNAs by qPCR

In order to reconfirm the DGE results of miRNAs, cold stress-treated plants of clones RRII 105 and RRIM 600 were used for qPCR analysis of six conserved miRNAs (Fig. 4). The qPCR results were found matching with the deep se-

Table 3. Cold-responsive miRNAs of *Hevea brasiliensis* and their putative targets.

miRNA	Expression Value	Sequence	Target ID	Target name
miR9386	1377	UUUGCAGUUCGAAAGUGGAAGC	gi|164375933|gb|DB925992.1|DB925992	Phospholipase C 4 precursor, putative [*Ricinus communis*]
			41674.m000014	Aldehyde dehydrogenase family
miR159	1410	UUUGGAUUGAAGGGAGCUCUG		Not identified
miR166	17295	UCGGACCAGGCUUCAUUCCUC	isotig04051	DNA binding protein,
			contig10287	Homeobox-leucine zipper family protein / lipid-binding START domain-containing protein isoform 6 [*Theobroma cacao*]
miR171	96	AGAUAUUAGUGCGGUUCAAUC	contig509309	beta-1,3-galactosyltransferase 2 [*Jatropha curcas*]
miR2275	25	AGGAUUAGAGGGACUUGAACC		
miR3630	44	UGCAAGUGACGAUAUCAGACA	gi|164377982|gb|DB948752.1|DB948752	phospholipase d delta, putative [*Ricinus communis*]
miR399	132	UGCCAAAGGAGAUUUGCCCUG		Not identified
miR476	67	UAAUCCUUCUUUGCAAAGUC		Not identified
miR482	442	UCUUCCCUACUCCUCCCAUUCC	isotig03914	Conserved hypothetical protein
			gi|164397563|gb|DB933070.1|DB933070	arsenical pump-driving ATPase, putative [Ricinus communis]
miR4995	78	AGGCAGUGGCUUGGUUAAGGG		Not identified
miR5021	23	UGAGAAGAAGAAGAAGAAAA		Not identified
miR535	31	UGACAACGAGAGAGAGCACGC		Not identified
miR5368	601	GGACAGUCUCAGGUAGACA		Not identified
miR5658	14	AUGAUGAUGAUGAUGAUGAAA		Not identified
miR6173	164	AGCCGUAAACGAUGGAUACU		hypothetical protein [*Populus*]
miR7760	18	CAGCGGACAGAAUGGAGCAAGCAG		Not identified
miR7782	11	ACCUGCUCUGAUACCAUGUUGUGA		Not identified
miR8175	13	GAUCCCCGGCAACGGCGCCA		Not identified
miR827	204	UUAGAUGACCAUCAACAAACU		Not identified
miR858	36	UUCGUUGUCUGUUCGACCUUG	gi|164397853|gb|DB937467.1|DB937467	Myb domain protein 7

quencing results. miR166, miR159 and miR171 were found up-regulated in tolerant clone while no significant change could be noticed in the susceptible one. Expression of miR858 in RRII 105 was reduced while it did not show any significant change in RRIM 600. Contrary to this, expression of miR482 was found significantly reduced in RRIM 600 when no significant change could be observed in clone RRII 105. Interestingly, expression of miR169 was found significantly reduced in the tolerant clone while it exhibited significant up-regulation in the susceptible clone RRII 105.

Discussion

The plants respond to cold stress by altering different metabolic pathways and by regulating stress-alleviating genes. During cold stress, photosynthetic processes are often primarily inhibited. In tropical trees, photo assimilation occurs at an optimum level when the ambient temperature is between 15 and 45°C (Sage and Kubien 2007). At temperatures below 15°C, with high light intensity, the major components of photosynthetic apparatus are damaged due to the increase in the levels of reactive oxygen species (ROS). The plants that do not produce sufficient ROS scavenging enzymes may succumb to cold stress in the absence of a protective mechanism to save the plants from photodamage (Foyer and Harbinson 1994). Cold stress or cold damage to the plants or the cold tolerance of the cultivar is in general assessed by analyzing chlorophyll fluorescence parameters along with net gas exchange data (Maxwell and Johnson 2000).

In general, clone RRIM 600 is known as cold tolerant and is being widely cultivated in the cold-prone regions of North East India (Meti et al. 2003; Reju et al. 2003). High light during daytime combined with cold stress in the previous nights during the winter season lead to severe inhibition of

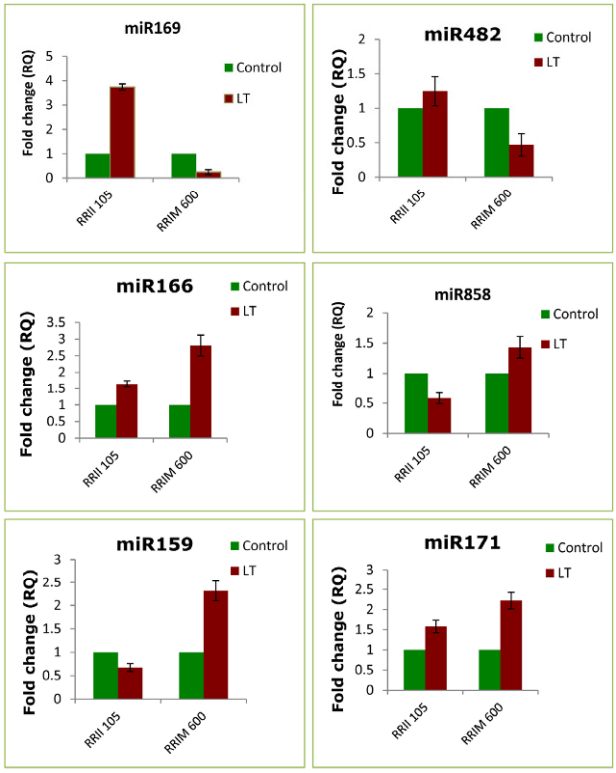

Fig. 4. Relative quantification of six miRNAs in cold stressed plants of *Hevea brasiliensis*. Error bars indicate standard error of three biological replicates.

photosynthesis and chlorophyll bleaching (Devakumar et al. 2002; Jacob et al. 1999; Ray et al. 2004) in cold susceptible clones like RRII 105. RRIM 600 had been reported to have lesser membrane permeability when compared to RRII 105 (Sathik et al. 1998). Alam et al. (2003) reported that the percentage reduction in yield due to winter stress was lesser in clone RRIM 600 than PB 235. Mai et al. (2010) compared eight *Hevea* clones for their tolerance towards cold stress and found clone RRIM 600 as most tolerant one. The results of the physiological parameters recorded in this study also indicated the effect of cold stress by way of reduction in stomatal conductance (gs), net CO_2 assimilation rate (A), and quantum efficiency of PS II (Fv/Fm) in both the clones. The severe reduction in gs and A observed in clone RRII 105 indicated its susceptible nature while the reduction was minimal in RRIM 600, indicating its inherent tolerance. The higher F_v/F_m ratio found in clone RRIM 600 under cold stress also supported its tolerant trait. The results obtained in this study are also in conformity with the previous reports of its field performance (Meti et al. 2003; Reju et al. 2003).

It is now an established fact that genes are regulated by microRNAs as a response to abiotic stress (Khraiwesh et al. 2012; Miura and Furumoto 2013; Sunkar et al. 2012). This study resulted in the identification of 218 miRNAs belonging to 21 conserved miRNA families. The DGE analysis revealed the common expression of eight miRNAs in both the cold-stressed and control samples. miR159, miR171 and miR166 were found expressing significantly at higher levels in cold-stressed than control samples. Expression of miR482 and miR535 were found significantly down-regulated under cold stress in clone RRIM 600. The expression analyses performed subsequently with six selected miRNAs in two contrasting *Hevea* genotypes *viz.* RRII 105 and RRIM 600, up-regulation of miR166 was noticed in tolerant clone RRIM 600 under cold stress. In *Solanum lycopersicum,* miR166 was found up-regulated while its target HD-Zip III got suppressed under cold stress (Valiollahi et al. 2014). Similarly in cotton too, under cold conditions (4°C) the miR166 was found expressed at higher levels (Wang et al. 2016) which have been predicted to negatively regulate its target HD-Zip III transcription factor. In this study also, HD-ZIP III transcription factor was predicted as its target. Probably, in *Hevea* also it might negatively regulate HD-ZipIII transcription factor under cold stress.

miR171 is a widely distributed and highly conserved miRNA family in plants which is known to play an important role in plant growth and development by regulating the expression of *SCARECROW-LIKE* (SCL) transcription factors. In the present study, the expression of miR171 got induced in tolerant clone RRIM 600 while there was no significant change in RRII 105. A similar trend had been reported in tea (*Camellia sinensis*) in which miR171 family was found significantly up-regulated in cold tolerant cultivar whereas it was found down-regulated in cold-sensitive cultivar (Zhang et al. 2014). In Arabidopsis, miR171 has also been reported to target *SCL6-II*, *SCL6-III*, and *SCL6-IV* (*SCL6*) which play important roles in plant root and leaf development, gibberellin response, photochrome signalling, lateral organ polarity, meristem formation, vascular development, and stress response (Lee et al. 2008; Llave et al. 2002a, 2002b; Wang et al. 2010). SCLs play an important role in suppressing chloroplast development in dividing cells during early leaf growth (Ma et al. 2014). In *Hevea*, the target prediction performed using TAPIR, indicated the probability of β-1,3-galactosyltransferase 2 as its target. In *Arabidopsis*, it has been reported to be involved in synthesis of hemicellulose which are basic components of cell wall synthesis and had been reported to be down-regulated under water deficit conditions (Bray 2004). It may be presumed that the up-regulation of miR171 in the tolerant clone might be directly involved in suppression of its target β-1,3-galactosyl transferase.

miR169 regulates the expression of sub-unit A of Nuclear Factor-Y (NF-Y) in many plants (Ni et al. 2013; Rhoades et al. 2002) which in turn play key roles in development and is expressed in response to adverse environmental conditions like drought, cold, salinity, ABA, etc. (Lee et al. 2003). miR169 which had been found associated with drought tolerance from our previous study was also included in this analysis. Its expression was significantly reduced in tolerant clone RRIM 600 while there was a significant up-regulation in the susceptible clone RRII 105, thus confirming its role in cold tolerance also. Expression of miR169 has also been reported to be at higher levels under cold stress in other plants like *Arabidopsis* (Sunkar and Zhu 2004), *Brachypodium* (Zhang

et al. 2009), etc. Over-accumulation of miR169 under cold stress was found correlated with reduction in Nuclear Factor-YA (NF-YA) transcripts in Arabidopsis (Lee et al. 2010; Zhou et al. 2008). The lower levels of miR169 found in tolerant clone RRIM 600 in this study might be involved in positively regulating the accumulation of its target NF-YA thus contributing for cold tolerance.

MIR482 is a highly diverse miRNA gene that has been found ubiquitously distributed across gymnosperm, monocot, and dicot plants (Zhao et al. 2012). miR482 has been reported to suppress the expression of Nucleotide Binding Site-Leucine-Rich-Repeat Receptor protein (NBS-LRR protein) in *Solanum lycopersicum* (Shivaprasad et al. 2012: Zhu et al. 2013) and in *Hevea*, it has been reported to target Abscisic Acid Responsive Element Binding Protein 2 (AREB2) which is abiotic stress responsive (Arenas-Huertero et al. 2009; Lertpanyasampatha et al. 2012). The expression of miR482 was found significantly reduced in tolerant clone RRIM 600 while there was not much change in RRII 105. The down-regulation in tolerant clone might be indirectly promoting the function of its target gene AREB2 which in turn might be imparting cold tolerance in clone RRIM 600.

miR159 is one of the most conserved miRNAs in land plants (Reinhart et al. 2002). In this study, miR159 was found expressed at higher levels in RRIM 600 under cold conditions while there was no change in RRII 105. In tea, miR159 was reported to be down-regulated in cold-sensitive cultivar (Zhang et al. 2014). In *Hevea*, miR159 was predicted to target genes involved in rubber biosynthesis, antioxidant activity and transcription regulation (Gebelin et al. 2012, 2013b). Expression of *HbMIR159a* in leaves and roots was found antagonistic. In leaves, *HbMIR159* genes displayed a significant up-regulation while in root it displayed a significant down-regulation in response to cold stress (Gebelin et al. 2013a). Hence, based on the available reports, it could be presumed that up-regulation of miR159 under cold stress conditions might possibly suppress its target genes associated with rubber biosynthesis, antioxidant activity, and transcription regulation.

A reduction in the levels of miR858 was found in clone RRII 105 while there was no significant change in RRIM 600. In this study, its target was predicted as MYB transcription factor. miR858 has been found to regulate the homologous *MYB2* gene during both Arabidopsis trichome and cotton fibre development (Guan 2014). However, the results of this study did not show any consistent trend with either cold susceptibility or tolerance.

Conclusion

The cold-responsive small RNA data of *Hevea* generated on Illumina platform revealed the expression of 21 conserved miRNA families and 42 novel miRNAs. Through gene expression analysis, miR169 was found to have a distinct association with cold tolerance. miR169 has been found to regulate its target NF-YA which is known to play main role in imparting abiotic stress tolerance in many plants. miR482, which targets AREB2, a known stress-responsive factor and miR159, which targets a set of cold stress and rubber biosynthesis related genes were also found associated with cold tolerance. Though this study could identify miRNAs associated with cold tolerance from two contrasting clones, more clones with wide range of tolerance/susceptibility levels have to be validated in order to confirm these findings and to arrive at a meaningful selection of miRNA markers for cold tolerance.

Abbreviations

miRNA	microRNA
qPCR	quantitative PCR
DGE	digital gene expression
COR	cold-responsive
bp	base pair
nt	nucleotide
PS II	Photosystem II

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