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Expression of miRNAs of *Hevea brasiliensis* under drought stress is altered in clones with varying levels of drought tolerance

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MicroRNAs (miRNAs) in plants play critical role in regulating gene expression at the post-transcriptional level in a number of biological processes including plant growth, development and defense responses against biotic and abiotic stresses. Recent reports indicate the role of miRNAs in regulating genes associated with various metabolic as well as abiotic stress responsive pathways in *Hevea*. The present study was initiated with an objective to identify drought responsive miRNAs from *Hevea* and to elucidate their role in stress response/tolerance in four clones of *Hevea* with varying levels of drought tolerance. miRNAs were isolated from leaves of *Hevea* and annotated after sequencing. The selected miRNAs were quantified using real time PCR. Four of the miRNAs displayed definite pattern of expression. The miR164, miR167 and the novel miRNA (HbmiRn_42) were down regulated in susceptible clones. HbmiRn_42 got upregulated in tolerant clones, indicating its strong association with stress tolerance. On the contrary, miR482 was down regulated in tolerant clones, while there was no change in its expression level in susceptible clones. This study throws light on stress responsive miRNAs of *Hevea* and their regulation under drought conditions.

Keywords: Drought stress, *Hevea brasiliensis*, qPCR expression analysis, miRNA

Introduction

Hevea brasiliensis Müll. Arg. (Family: Euphorbiaceae), the natural rubber tree, is the most important source of natural rubber (NR). For reasons of high yield, low impurities and convenience of harvesting, *Hevea* accounts for over 99% of the world's natural rubber produced and consumed. During recent years, the global consumption of NR has been found steadily increasing; however, the production does not increase proportionately to meet the demand. To cope up with the increasing global demand for NR, attempts are being made to extend the cultivation to non-traditional areas, which are known for its extreme climatic conditions. NR latex production depends on the genetic make-up of cultivated clones, environmental factors and harvesting conditions[1].

Under drought stress conditions, plants exhibit altered expression of genes related to different metabolic pathways associated with stress perception, signal transduction and regulation and synthesis of a number of compounds[2]. Our previous investigations also indicate the existence of altered level of expression of drought specific genes in *Hevea* clones with varying levels of drought tolerance[3-4]. However, the regulation of gene expression is a complex process, especially at the transcriptional level[5-6]. MicroRNAs (miRNAs) are single-stranded non-coding RNAs, ~20 to 24 nucleotides (nt) in length, that play critical roles in regulating gene expression at the post-transcriptional level by repressing translation or enhancing degradation of specific target mRNAs[7]. They are involved in regulating various developmental and metabolic pathways, signal transduction and respond to environmental stresses, such as, oxidative stress, nutrient stress, dehydration and mechanical-stress[8-9]. In plant, miRNAs are processed in the nucleus from longer stem loop structures called pre-miRNAs (approx 70 nt in length) by a Dicer-like enzyme and are loaded into RNA induced silencing complex (RISC), where the miRNA guides sequence-specific post-transcriptional repression of target mRNA(s) by degradation or translational repression[10]. A large number of miRNA sequences are evolutionarily conserved across species boundaries and have near perfect complementarities with their specific messenger RNA (mRNA) targets[11]. Eventually owing to this complementarity, the plant miRNAs guide the cleavage, degradation or

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translational inhibition of their target mRNA, thereby interfering and regulating the gene expression. Regulation of gene expression through sequence specific interaction between miRNAs and their target mRNAs offers an accurate and inheritable mechanism for plant to respond to environment stimuli[12]. Number of studies show that miRNAs are associated with drought stress response[13-14].

Recent reports have established the role of miRNAs in regulating genes associated with various metabolic as well as abiotic stress responsive pathways in *Hevea*[15-18]. Until now, 31 mature miRNA sequences are available in miRBase of *H. brasiliensis*. The present study was initiated with an objective to identify miRNAs that are expressed under drought condition in *H. brasiliensis* and to find out the miRNAs that are specifically associated with stress tolerance/susceptibility. For this purpose, we quantified expression of seven miRNAs in four clones of *Hevea* with varying levels of drought tolerance.

Materials and Methods

Plant Material and Stress Induction for miRNA Isolation

In order to isolate and clone drought stress specific miRNAs, 6-month-old polybag grown plants of *Hevea* (clone RRIM 600) were exposed to drought condition in the open field of Rubber Research Institute of India (RRII), Kottayam (Kerala), India, during summer season. The clone was generated through budding of seedlings raised from *Hevea* seeds. The clonal buds were collected from *Hevea* budwood nursery maintained at RRII. One set of plants was subjected to water stress by withholding irrigation for 10 d, while the other set was watered on alternate days to maintain field capacity. The leaf samples were harvested after assessing the drought status of the plant by measuring the net CO_2 assimilation rate (A) and stomatal conductance (g_s) using a portable photosynthesis system (LI-6400), LI-COR, USA. The samples were collected in liquid N_2 and stored at −80ºC.

miRNA Isolation

Small RNAs were isolated using mirVana miRNA isolation kit (Ambion, USA). About 2 µg of small RNAs (measured spectrophotometrically; Nanodrop, USA) were resolved on a 12% denaturing (7 M urea) polyacrylamide gel. miRNA marker (NEB) was loaded as size control for the identification of RNAs in 17-25 nt size range. The gel was stained with SybrGold nucleic acid stain and visualized on a UV transilluminator (Fig. 1a). RNA fragment(s) of 20-25 nt size were excised from the gel and purified using DTR columns.

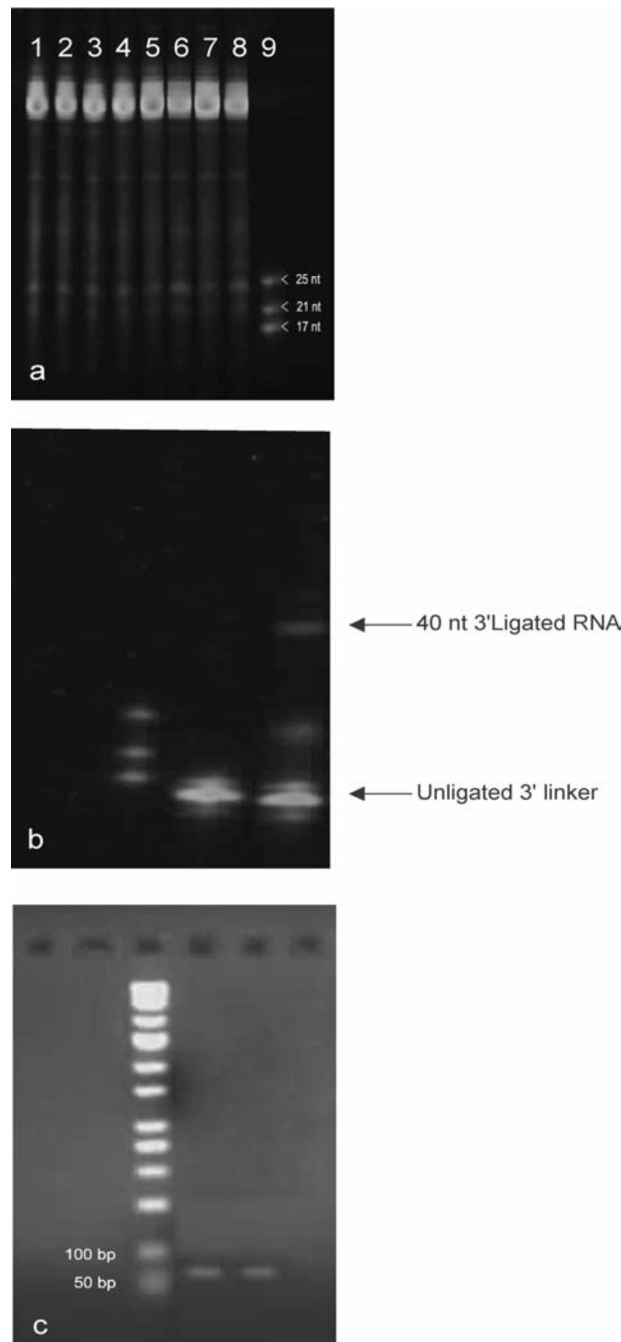

Fig. 1 (a-c)—Gel images of each stage of miRNA isolation from leaf samples of drought stressed *Hevea* clone RRIM 600: a. PAGE profile of small RNAs [Lanes 1-8, miRNA from 8 samples; Lane 9, miRNA marker]; b. PAGE profile of 3′ linkered small RNA species; c, PCR amplified products of 3′ and 5′ linker adapted small RNAs [Lane 1, DNA marker; Lanes 2 & 3, Linker attached small RNAs].

Reverse Transcription and PCR Amplification

The purified small RNAs were ligated with a 3′ and 5′ linker in two separate reactions. Initially, 3′ linker was ligated with the enriched miRNAs. 3′ linkered species was resolved on a 12% denaturing (7 M urea) polyacrylamide gel (Fig. 1b). This step was followed by ligating the 5′ MRS (multiple restriction site) linker to the 3’ linkered small RNAs in the presence of 1.0 mM ATP. The 5′ and 3′ ligated miRNA were converted to cDNA using SuperScript III Reverse Transcriptase (Invitrogen) and RT/REV primer (provided by the IDT miRNA cloning kit). PCR amplification using linker specific primers was carried out (Fig. 1c) and the amplicons were further purified to proceed with cloning and sequencing.

Cloning and Sequencing

PCR products were ligated into the pTZ57R/T cloning vector (PCR Cloning Kit, Fermentas) and were transformed into JM109 cells using TransformAid Bacterial Transformation Kit (Fermentas). The inserts from individual colonies were amplified by adaptor specific forward and reverse primers and were sequenced. After sequencing, the adaptor sequences were trimmed and the small RNA sequences having a length of 18-30 nt were searched against Rfam family database to find if any of the sequences were similar to non-coding RNAs. The sequences were then BLAST analyzed against miRBase database v20.0. Small RNAs that did not show similarity to any miRNAs in miRBase were analyzed for potential novel miRNAs.

Plant Material and Stress Induction for qPCR Analyses

Drought stress was imposed on 4 *Hevea* clones (RRII 105, RRIM 600, RRII 414 & RRII 208) with varying levels of drought tolerance. After growing for 6 months in open field conditions, the polybag plants were transferred to glasshouse conditions before imposing drought. The imposition of drought stress and leaf sample collection was performed as described above. Total RNAs were extracted using Spectrum™ Plant Total RNA Kit (Sigma-Aldrich) according to the manufacturer’s instructions. The total RNA (2 µg) from each sample was then reverse transcribed using Mir-X miRNA first strand c-DNA synthesis kit (Clontech). Validation of six conserved miRNAs (miR164, miR166, miR167, miR398, miR482 & miR169) and one novel miRNA (Table 1) in control and drought imposed plant was done by qPCR on Light Cycler 480 II (Roche) using SYBR Advantage qPCR Premix (Takara). The reaction consisted of 10 times diluted 0.5 µL cDNA, 0.1 µM of each forward and reverse primers and 5 µL 2× SYBR Advantage qPCR Premix in a 10 µL reaction volume. The reaction conditions included an initial denaturation step of 95ºC for 30 sec, followed by 40 cycles of 95ºC for 5 sec and 60ºC for 30 sec. For each treatment, three biological replications were included in the qPCR analysis. Statistical analysis was performed with the relative quantification data using ANOVA. The ratio with a P-value <0.05 was adopted as significant for down or up regulation.

Results

miRNAs were isolated from the leaf samples of drought stressed RRIM 600 plants. The stomatal conductance of stressed plants got reduced to near zero after 10 d of drought compared to control (0.33 mol m^{-2} s^{-1}) plants (Fig. 2a). Water stressed plants exhibited significant reduction in net CO_2 assimilation rate (2.7 µmol m^{-2} s^{-1}) than the control (11.5 µmol m^{-2} s^{-1}) plants (Fig. 2b).

Sequencing and blast analyses of 120 clones of small RNAs showed three families of conserved miRNAs (miR166, miR167 & miR482) and one novel miRNA after excluding redundancy. The miR166 was found repeated thrice and had a length of 20 and 21 nt in length. Only one sequence was obtained for miR167 and miR482 occurred twice. Among the rest of the sequences when mapped to the draft genome of *H. brasiliensis* to identify the precursor molecule, one of them was confirmed as novel (HbmiRn_42). The secondary structure of the precursor molecule was predicted using mFold tool with default parameters and the free energy was found to be (-30.7). The sequence of the novel miRNA (HbmiRn_42) is given in Table 1 and the stem loop structure is given in Fig. 3.

To understand the drought responsiveness of these miRNAs and their differential expression under stress conditions in *Hevea* clones (RRII 105, RRIM 600,

Table 1—The sequence and putative target of miRNAs investigated

miRNA	Sequence (5’-3’)	Putative target
miR164	TGGAGAAGCAGGGCACGTGCA	NAC tf
miR166	UCGGACCAGGCUUCAUUCCCC	HD-ZIP III protein
miR167	CAGAUCAUGCUGGCAGCUUC	auxinresponsefactors
miR482	GGAAUGGGCGGUGUGGGUAAGA	LRR Protein
miR398	GGAGCGACCTGAGATCACATG	Cu/Zn SOD
miR169	GAGCCAAGAATGACTTGCCGA	NFYA-1
Hbmi Rn_42	CCAGGCGTCGGCCAGCGGGCTC	Unknown

RRII 208 & RRII 414) with varying levels of drought tolerance, quantitative expression analyses were made. Reduction in stomatal conductance was noticed in all the clones under drought stress, while it was maximum in RRII 414 (Fig. 4a). Similarly, reduction in photosynthetic assimilation rate was found in all the clones that were under drought stress and it was the maximum in clone RRII 414 (Fig. 4b). Apart from four miRNAs identified from this study, we also included three more miRNAs (miR164, miR398 & miR169) that were already reported to be drought responsive. The miRNA expressions among the treatment were compared using the $2^{-\Delta\Delta C_T}$ method[19]. All the miRNAs showed differential expression under drought stress condition and their level of expression varied among the clones studied (Fig. 5). Among them, miR482 got down regulated in clones RRIM 600 and RRII 208 (relatively drought tolerant), whereas there was no significant change in relatively drought susceptible clones, such as, RRII 105 and RRII 414. In contrast, miR164 and miR167 were down regulated in RRII 105 and RRII 414, while there was not much change in RRIM 600 and RRII 208. The expression of miR169 got down

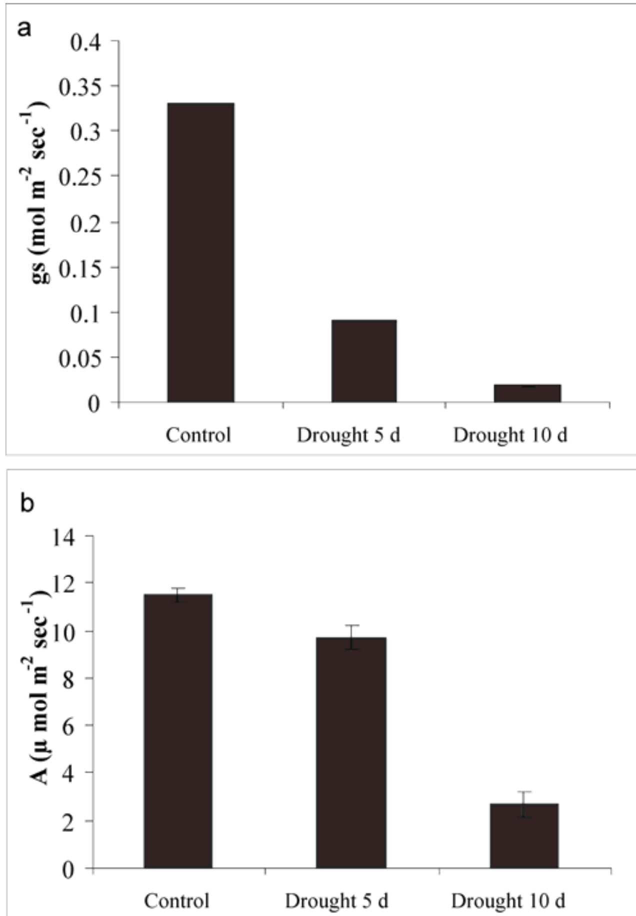

Fig. 2 (a & b)—a. Stomatal conductance (g_s); b. CO_2 assimilation rate (A), of irrigated and drought imposed plants of *Hevea* clone RRIM 600.

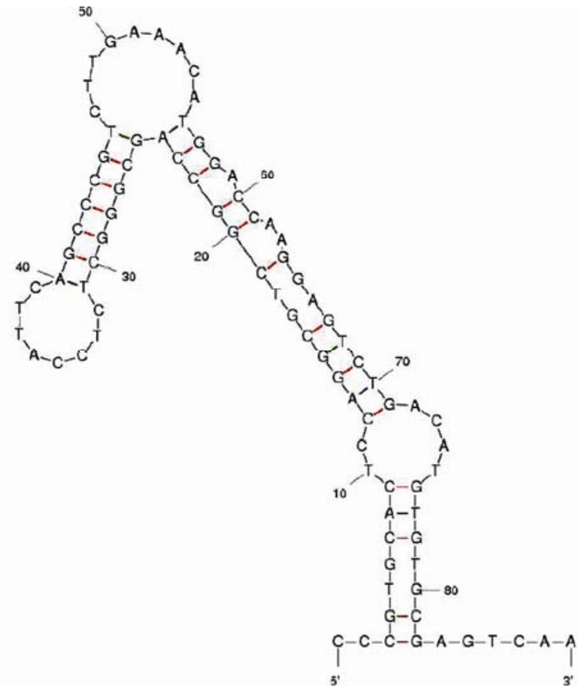

Fig. 3—Stem loop structure of novel miRNA (HbmiRn_42).

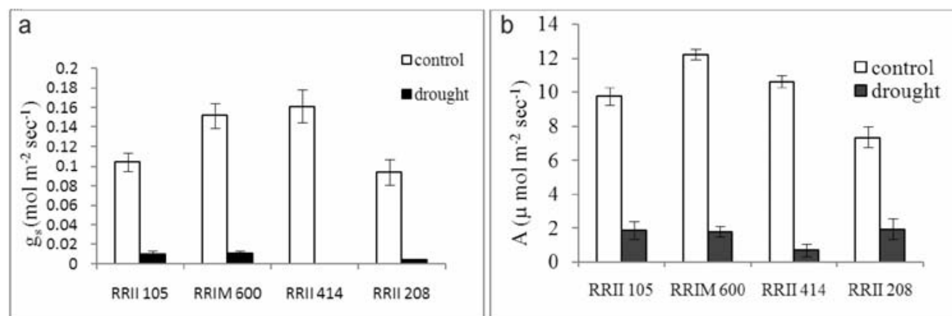

Fig. 4 (a & b)—a. Somatal conductance (g_s); & b. CO_2 assimilation rate (A), of irrigated and drought imposed plants of four *Hevea* clones.

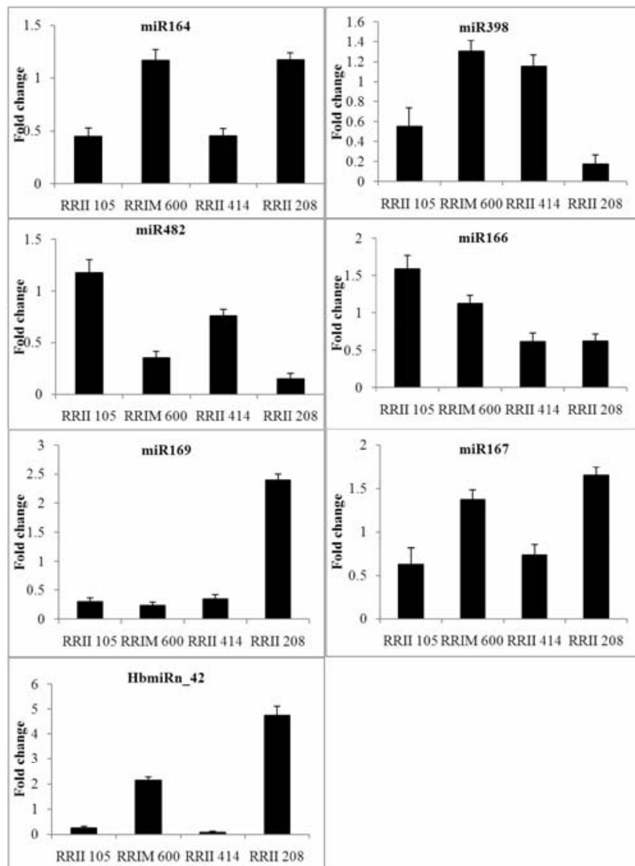

Fig. 5—Expression analysis of seven microRNAs in four clones of *Hevea* under drought condition. [Error bars indicate standard error of three biological replicates]

regulated in clones RRII 105, RRIM 600 and RRII 414, except in clone RRII 208, in which it got upregulated under drought. In the case of miR398, down regulation was much evident in RRII 208, whereas in other clones there was no much change except a slight reduction in RRII 105. In contrast, miR166 did not show any significant change in its expression level among the clones studied. Expression of novel miRNA (HbmiRn_42) was higher in RRIM 600 and RRII 208, while it got down regulated in drought susceptible clones.

Discussion

Cultivation of rubber plant in traditional regions of India has become saturated and there is a need to extend its cultivation to suitable regions in the non-traditional areas. Various clones of *H. brasiliensis* are being evaluated in such region in order to identify a clone with better stress tolerance trait. Among the various approaches made to develop clones with improved stress tolerance, molecular biological approaches have also been attempted in the recent past. Tolerant clones exhibit several inherent adaptive mechanisms to manage or escape the adverse impacts of extreme climate. In the present study, an attempt was made to identify drought stress specific miRNAs from *Hevea* to quantify their expression and also to characterize their role in drought tolerance. We could identify four miRNAs among which one was found novel. Expression of these miRNAs along with another set of three miRNAs (miR164, miR169 & miR398), which were reported to be drought responsive [20-22], was quantified.

In order to understand the role and level of expression of these miRNAs, 4 clones of *Hevea* with varying levels of drought tolerance were exposed to drought condition. CO_2 assimilation rate was found inhibited in drought-imposed plants of all the clones, while it was much severe in susceptible clone RRII 414. Similarly, stomatal conductance also declined significantly in all the clones under drought stress, while it was almost zero in RRII 414. These results indicated that the drought stress was set in and drought responsive genes would have been activated in those plants. The expression of almost all the seven miRNAs quantified was found altered under drought condition.

miR482 are known to suppress the expression of nucleotide binding site-leucine-rich-repeat receptor protein (NBS-LRR protein)[23-24] and to target abscisic acid responsive element binding protein 2 (AREB2), which is abiotic stress responsive[25]. miR482 family is reported to have more variable sequences than other miRNA families[23]. The miR482 obtained from the present study was found similar to csmiR482b[26] and was different from the one (HbmiR482) reported previously in *H. brasiliensis*[16]. When quantified, its expression in tolerant clones (RRIM 600 & RRII 208) got reduced to 0.2 fold, while not much change was observed in the relatively susceptible clones like RRII 105 and RRII 414. This indicates that miR482 must be playing a specific role in imparting drought tolerance in *Hevea*. Probably expression analysis in few other drought tolerant clones would confirm its association with drought tolerance.

The expression of miR167 was slightly higher in drought tolerant clones (RRIM 600 & RRII 208), while it was lesser in RRII 105 and RRII 414, which are relatively susceptible. miR167 has been reported to regulate auxin response factors (ARF), such as, ARF6 and ARF8 under drought condition[27]. ARFs are

important transcription factors involved in relaying auxin signaling at the transcriptional level by binding to specific *cis*-element in the upstream regions of auxin-inducible genes. Auxin is involved in drought stress tolerance as well as in auxin signaling, which is crucial for plant growth and development. Various reports indicate the existence of possible link between auxin signaling and miRNA expression[28]. The miR167 guides the regulation of ARF6 and ARF8, which are reported to negatively regulate free IAA levels by interfering with the GH3-like gene expression[29]. Under drought condition, expression of miR167 was induced in *Arabidopsis*[27]. Slight increase in expression of miR167 was found in relatively tolerant clones in the present study. Whether miR167 is involved in down regulation of ARFs in tolerant clones under drought stress is worth investigating further.

miR398 targets two closely related Cu/Zn SODs (CSD1 & CSD2), which are known to be involved in oxidative stress detoxification[22], and cytochrome C oxidase subunitV (*COX5b*) which is associated in electron transport system of the mitochondrial respiratory pathway[8]. miR398 is down regulated under drought stress in *Medicago truncatula*[30] and in maize[31]. This leads to increased activity of CSDs rendering oxidative stress tolerance. The expression analysis data indicate that its level did not alter in clones RRIM 600 and RRII 414, while it got reduced significantly in RRII 208 and to some extent in RRII 105. Probably the ROS scavenging enzyme Cu/Zn SOD levels would have been up regulated in RRII 208. From the results, it can be presumed that free radical scavenging activities must have been much higher in RRII 208 when compared to other clones studied.

miR169 targets the NFYA5 mRNA, encoding a subunit of the nuclear factor Y (NF-Y) transcription factors[27], which are plant specific transcription factors playing important role in plant development and in coping up with the environmental stresses[32]. It was reported to be down regulated under drought in *Arabidopsis* and *M. truncatula*, while it was up regulated in rice[33] (miR169g) and tomato[34]. miR169 is a conserved miRNA family that regulates a homologous target; it appears to behave in contradictory ways in different plant species, because of differences in plant developmental stages, growth conditions and the duration and strength of the applied stress[35]. In the present study, miR169 was found down-regulated in clones RRII 105, RRIM 600 and RRII 414, whereas it was up regulated only in clone RRII 208. The results indicated the down regulation of miRNA in three clones of both drought tolerant and susceptible nature. In contrast, the clone RRII 208 presumed to be drought tolerant displayed up regulation of miR169.

miR166 is reported to be drought responsive and is known to regulate class III homeodomain-leucine zipper (*HD-Zip III*) transcription factors, which are important for lateral root development, axillary meristem initiation and leaf polarity[36]. In barley and *Triticum dicoccoides*, miR166 has been found to be down regulated in response to drought[37-38]. However, in *M. truncatula*, it was up regulated in roots, while being suppressed in seedlings and shoots under drought stress[39]. In the present study, thus there is no significant difference in miR166 expression among the tolerant and susceptible clones.

miR164 is reported to be involved in regulating the post transcriptional processing of NAC transcription factors[20]. Expression of NAC proteins in response to abiotic stresses in various plants and their possible role have been reported[40]. A rice stress responsive NAC gene, *SNAC1*, confers drought resistance under field drought conditions by promoting stomatal closure[41]. On the contrary, the recent report in rice indicated the association of miR164 targeted NAC genes with drought susceptibility[42]. Under drought stress, expression of miR164 was found down regulated in relatively susceptible clones, while there was not much change in relatively tolerant clones. The expression of novel miRNA identified from the present study (HbmiRn_42) under drought condition was very much interesting as it was found highly down regulated in susceptible clones and upregulated in relatively tolerant clones, indicating its association with drought tolerance. Probably, it might be controlling the expression of its target gene, which might be a negative regulator of drought tolerance. It would be worth investigating to identify its nature and role in imparting drought tolerance.

Thus the present study revealed the existence of differential expression of miRNAs among various *Hevea* clones studied under drought stress conditions. Among the miRNAs identified, four of them displayed a trend with the drought tolerance of clones. The miR482 was found down regulated in tolerant clones, while there was no change in its expression in susceptible clones, indicating its role in imparting

drought tolerance. The miR164, miR167 and the novel one (HbmiRn_42) were found down regulated in susceptible clones under drought stress. HbmiRn _42 was found up regulated in tolerant clones, indicating its strong association with stress tolerance. Further investigations are warranted to identify and characterize this novel miRNA's target gene and its regulation in tolerant and susceptible clones of *Hevea*. Expression analysis of target genes of these miRNAs in clones with varying levels of drought tolerance would further reveal the significant roles of such small RNAs in drought tolerance potential of *Hevea* clones.

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