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ORIGINAL ARTICLE

# Identification and validation of drought-responsive microRNAs from *Hevea brasiliensis*

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## Abstract

Drought, in combination with high temperature and low humidity affects the productivity of *Hevea brasiliensis*, the natural rubber tree and its expansion to non-traditional regions. The genotypes of *H. brasiliensis* that perform well in traditional regions often failed in non-traditional regions thus necessitating breeding for stress-tolerant genotypes. This can be accomplished by adopting molecular-assisted selection method. Recent developments in identification of drought-responsive transcripts from *H. brasiliensis* and the findings on role of small RNAs indicate the possibility of employing them as markers for identification of suitable genotypes. In this study, we attempted to identify drought-responsive miRNAs from *H. brasiliensis* through next-generation sequencing (Illumina HiSeq) method. The results revealed the expression of 33 conserved and 32 novel drought-responsive miRNAs. Further, validation of differentially expressed miRNAs by quantitative expression analysis indicated the association of two novel miRNAs, viz., HbmiRn_63 and HbmiRn_42 and two conserved miRNAs, viz., miR168 and miR160 miRNAs with drought tolerance. These miRNAs can be employed as markers for drought tolerance after validation in a larger set of genotypes. This study opens up the possibility of employing miRNAs as markers for drought tolerance in *Hevea*.

**Keywords** Abiotic stress · Drought tolerance · *Hevea brasiliensis* · MiRNAs · Quantitative expression analysis

## Abbreviations

| | |
|---|---|
| ARF | Auxin response factors |
| HMGR | HMG-CoA reductase |
| MFE | Minimal folding free energy |
| miRNA | MicroRNA |
| qPCR | Quantitative PCR |
| RRII | Rubber Research Institute of India |

## Introduction

Drought is a major limiting factor that hinders plant growth and development by affecting several metabolic processes including stomatal conductance, nutrient uptake and photosynthetic assimilation often resulting in significant crop loss (Shinozaki et al. 2003; Jaleel et al. 2009). Understanding the mechanisms of drought tolerance in crop plants is a prerequisite to design strategies for the improvement of crop productivity (Lawlor 2013). Drought also restricts the expansion of rubber to newer areas in several rubber growing countries (Devakumar et al. 1998). In India, its cultivation is extended to non-traditional regions such as North Konkan region of Maharashtra, parts of Madhya Pradesh, Orissa, etc., which experience long dry periods, high temperature and low humidity for almost half of the year. The rainfall distribution in these areas is seasonal (June–August) with almost no rainfall between November and May. The daily sunshine hours is longer during summer with the mean daytime temperature ranging between 37 and 42 °C, occasionally reaching as high as 45 °C. These extreme weather conditions increase the evaporative demand of the atmosphere (Mohanakrishna et al. 1991) thus creating atmospheric drought stress too. Many reports have indicated the influence of drought on yield and overall performance of *Hevea* (Sethuraj et al. 1984; Sreelatha et al. 2007, 2011).

Using conventional plant breeding methods, various attempts were made to develop drought-tolerant *Hevea*

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genotypes. More than five thousand wild germplasm accessions and about three hundred bred genotypes are being evaluated by RRII in drought-prone regions to identify potential drought-tolerant genotypes. Selection of drought-tolerant genotypes using conventional methods is an extensive and time-consuming process which can be circumvented by employing molecular markers. Earlier reports have indicated the possibility of using suitable genetic (Thomas et al. 2012a, b; Luke et al. 2015; Kuruvilla et al. 2017; Sathik et al. 2018), physiological and biochemical markers (Sreelatha et al. 2007, 2011; Thomas et al. 2012a) for selecting *Hevea* genotypes with abiotic and biotic stress tolerance apart from superior growth and yield. Reports on miRNAs also indicate that they can be used as functional markers for selection, as they are stable, polymorphic, functional and have higher transferability potential (Fu et al. 2013; Yadav et al. 2014; Wang et al. 2016).

MicroRNAs (miRNAs) are approximately 21-nucleotide long (between 20 and 24 nucleotides), non-coding small RNAs that regulate gene expression under various biotic as well as abiotic stress situations (Sunkar et al. 2012; Zhang 2015; Shriram et al. 2016). They regulate gene expression by binding with the complementary mRNAs to either cleave or to repress translation at post-transcriptional level (Chinnusamy et al. 2007). Apart from regulating many developmental processes like root initiation, leaf, vascular system, flower and seed development, they regulate biotic and abiotic stress-responsive genes. They also target majority of the stress-responsive transcription factors which play important roles in response to different environmental stresses in plants (Kantar et al. 2010; Ding et al. 2013; Ferdous et al. 2015). However, a better understanding of the modulatory role of miRNAs in stress situations is necessary to evolve more effective and reliable strategies to enhance plant stress tolerance using miRNA-mediated gene regulation (Noman et al. 2017). Transgenic plants engineered to express specific miRNAs (constitutive expression) in response to drought (Li et al. 2015; Ferdous et al. 2016), heavy metals (Noman and Ageel 2017), etc., are known to exhibit respective stress tolerance. In *Hevea*, the role of miRNAs in regulating genes associated with various metabolic as well as abiotic stress-responsive pathways have been reported (Lertpanyasampatha et al. 2012; Gebelin et al. 2012, 2013a, b; Kuruvilla et al. 2016, 2017). However, no reports are available on the differential expression of drought stress-responsive miRNAs in various genotypes of *Hevea* on a high-throughput sequencing level. Hence, this study was undertaken to identify miRNAs that are specifically associated with drought tolerance in *Hevea* genotypes. It was also aimed to distinguish miRNAs that can be employed as markers in crop improvement programs for early selection of drought-tolerant genotypes of *H. brasiliensis*.

## Materials and methods

### Plant material and stress induction

For this study, the genotype RRIM 600 of *H. brasiliensis*, a known drought-tolerant genotype (Chandrasekhar et al. 1994) was selected. The plants were generated by bud grafting the scion (buds) on to rootstock seedlings. Successfully bud-grafted plants were uprooted and the budded stumps were planted in polythene bags. The plants were raised in the nursery of Rubber Research Institute of India (RRII), Kottayam, India. Six-month-old plants at two or three whorl stage were subjected to drought treatment. While one set of six plants were subjected to drought treatment for 10 days (withholding irrigation), another set of six plants (control) were watered to field capacity on alternate days. The impact of drought was assessed by measuring net $CO_2$ assimilation rate (A) and stomatal conductance ($g_s$) using portable photosynthesis system (LI-6400), LI-COR, USA. The measurements were made at a fixed $CO_2$ concentration of 400 ppm and 500 µmol $m^{-2}$ $s^{-1}$ light intensity (red LED source with 10% blue light attached with the leaf chamber). Leaf samples (physiologically matured) were collected and stored in − 80 °C freezer until further analysis.

### Small RNA library construction and sequencing

Total RNA was extracted from the leaves of control and drought-stressed plants of genotype RRIM 600 using Spectrum™ Plant Total RNA Kit (Sigma-Aldrich) as per manufacturer's protocol. The quantity and quality of total RNA were determined spectrophotometrically and by resolving on 1% denatured agarose gel, respectively. The paired-end cDNA sequencing libraries for small RNA were prepared for control and drought-stressed samples using Illumina® TruSeq Small RNA Sample Preparation Kit as per the manufacturer's protocol. The library construction involves ligation of 3′ adapter with 1 µg total RNA followed by 5′ adapter ligation. The adapter-ligated RNA was reverse transcribed, PCR amplified, purified and subjected to deep sequencing using Illumina HiSeq 2000 (Xceleris Genomics, Ahmedabad, India).

### Identification of conserved and novel miRNAs of *H. brasiliensis*

The raw reads were imported to CLC genomics workbench for bioinformatics analysis. Further, the reads were filtered for adapter sequences. The reads with 20–24 bp were retained and reads smaller than 20 bp and greater than 24 bp were discarded. To identify the conserved miRNAs,

the data of small RNAs were mapped to the data of mature plant miRNAs registered in the miRBase (Release 21) database using CLC Workbench (version 6) software allowing two maximum mismatches in the annotation. To identify novel miRNAs, draft genome of *H. brasiliensis* was used as reference (accession no: AJJZ01, total number of contigs, 1,223,365), due to the limited size of which, the draft genome sequences of *Ricinus communis* and *Manihot esculenta* were also used as references (ftp://ftp.jgi-psf.org/pub/compgen/phytozome/v9.0). The secondary structures for precursor molecules of novel potential candidate miRNAs were predicted using m-Fold web server after setting all the parameters to default values. The secondary structure of miRNA precursors with minimal free energy (MFE) value equal to or less than − 25 kcal per mol was considered as novel miRNAs.

### Target prediction for miRNAs

Target prediction for known and novel miRNAs was performed using web-based psRNA Target program (Dai and Zhao 2011) with default parameters and TAPIR software (Bonnet et al. 2010). The 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. The TAPIR programme considered score value and free energy ratio for target prediction. 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 was 4.0 and the default value for free energy ratio was 0.7.

### Validation of miRNAs and their potential target genes by qPCR

Drought-tolerant genotypes (RRIM 600, RRII 430 and RRII 208), drought-susceptible genotypes (RRII 105 and RRII 414), drought-tolerant germplasm accessions (RO 3261 AC 612) and drought-susceptible germplasm accessions (RO 3242 and MT 1619) were used for validation (Table 1). Total RNA (2 µg) from each sample was reverse transcribed using Mir-X miRNA first-strand c-DNA synthesis kit (Clontech). Small RNAs were polyadenylated and reverse transcribed using poly (A) polymerase and SMART MMLV Reverse Transcriptase. Expression of selected miRNAs in control and drought-imposed plants was validated by qPCR on Light Cycler 480 II (Roche) using SYBR Advantage qPCR Premix (Takara). The reaction mix consisted of 0.5 µl of 10 times diluted cDNA, 0.1 µM each of forward and reverse primers and 5 µl of 2× SYBR Advantage qPCR Premix in a 10 µl reaction volume. The reaction conditions included an initial denaturation at 95 °C for 30 s followed by 40 cycles of 95 °C for 5 s (denaturation) and 60 °C for 30 s (both annealing and extension). The level of expression was calculated as normalized fold ratios using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001). Statistical analysis was performed with single factor ANOVA using normalized expression data.

## Results

### Identification and analysis of conserved and novel miRNAs of *Hevea*

To evaluate the stress impact on plants, gas exchange parameters were measured after imposing drought stress. Small RNA libraries from control and drought-stressed leaves were constructed and sequenced separately. A total of 12,176,240 and 18,499,616 reads were identified from control and drought-stressed samples, respectively. Among the 324,448 reads in control, 52,420 reads were found unique, whereas from 353,428 reads in drought-stressed sample, 53,280 reads were found unique. These unique reads were treated as small RNAs and were considered for further analysis. The size distribution pattern of small RNA libraries of both the control and drought-treated samples was similar in which 24 nt length was the most abundant, followed by 21 nt (Fig. 1).

64 miRNAs belonging to 29 known miRNA families and 63 miRNAs belonging to 32 known miRNA families were identified from control and drought-imposed samples, respectively (Table 2). Of the 33 conserved miRNA families identified, miR166 and miR482 were found abundant in both the samples. A significant difference in the number of members in each conserved miRNA family was detected among which miR393, the largest family was found to have eight members followed by miR156 with seven members. Of the remaining, 15 miRNAs families were represented by a single member, while others had as few as 6 members (Supplementary data 1–2). Apart from these, 17 and 25 novel miRNAs

**Table 1** Drought-susceptible and-tolerant clones of *H. brasiliensis*

| | | |
|---|---|---|
| Drought-susceptible clones | RRII 414, RRII 105 | Sumesh et al. (2011), Thomas et al. (2012a, 2014, 2015), Krishan (2017) |
| Drought-tolerant clones | RRII 430, RRII 208, RRIM 600 | |
| Drought-tolerant germplasm accessions | RO 3261, AC 612 | |
| Drought-susceptible germplasm accessions | RO 3242 and MT 1619 | |

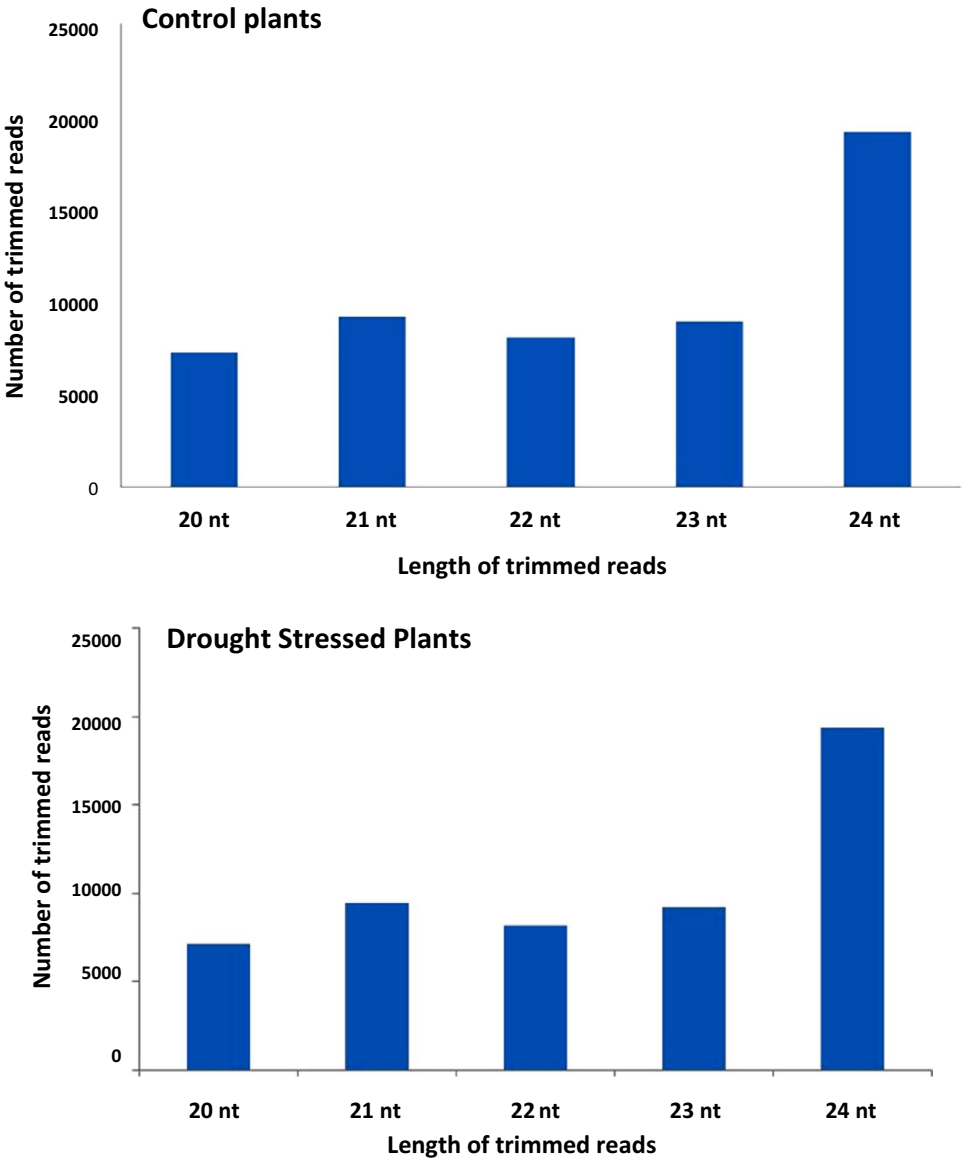


**Fig. 1** Length of small RNA sequences from control and drought-stressed plants of genotype RRIM 600 of *H. brasiliensis*

were identified in control and drought samples, respectively (Table 3 and Supplementary data 3–4), while 10 novel miRNAs were found to be common to both the samples. The secondary structures of novel miRNAs were also predicted and the results are represented in Supplementary data 11.

## Differential expression analyses of miRNAs

Differential expression analysis was performed by DESeq package on the 33 conserved miRNA families obtained by Illumina HiSeq 2000 sequencing (Supplementary Data 11). Among the 33 conserved miRNA families analyzed, 28 miRNA families were found commonly expressed in both the control and drought-stressed samples. miR1432 was found only in control samples, while miR160, miR2118, miR528 and miR6476 were noticed only in drought-stressed samples. While five conserved miRNAs (miR164, miR168, miR3627, miR395 and miR6478) exhibited significant up-regulation in drought-stressed samples, five other miRNAs (miR1310, miR156, miR169, miR393 and miR858) were found significantly down-regulated (Fig. 2).

## Target prediction for conserved and novel miRNAs of *H. brasiliensis*

To understand the functional role of the identified miRNAs, prediction of their target is of prime importance. Target search carried out for all the 33 conserved miRNA families against ESTs or gene sequences revealed 27, 28 and 27 targets in *Hevea brasiliensis, Ricinus communis* and *Manihot esculenta*, respectively (Supplementary data 5–7). These target sequences were further annotated against non-redundant

**Table 2** miRNAs identified from leaves of *H. brasiliensis* and their putative targets

| Sl. No. | miRNA | Sequence (5′–3′) | Target |
|---|---|---|---|
| 1 | MIR166 | TCGGACCAGGCTTCATTCCCCC | Hypothetical protein |
| 2 | MIR482 | AGATGGGTGGCTGGGCAAGAAG | Abscisic acid responsive element |
| 3 | MIR167 | TGAAGCTGCCAGCATGATCTGA | Transmembrane protein |
| 4 | MIR396 | TTCCACAGCTTTCTTGAACTG | Regulatory-associated protein of mTOR |
| 5 | MIR156 | TGACAGAAGATAGAGAGCAC | Nacl-inducible calcium binding |
| 6 | MIR535 | TGACAACGAGAGAGAGCACGT | Leucine carboxyl methyltransferase, putative |
| 7 | MIR397 | ATTGAGTGCAGCGTTGATGAA | Laccase, putative |
| 8 | MIR393 | TCCAAAGGGATCGCATTGATCT | Hypothetical protein |
| 9 | MIR390 | AAGCTCAGGAGGGATAGCGCC | Zinc finger protein |
| 10 | MIR2916 | TGGGGACTCGAAGACGATCATAT | Kinesin, putative |
| 11 | MIR858 | TTCGTTGTCTGTTCGACCTGA | Myb domain protein 13 |
| 12 | MIR4995 | AGGCAGTGGCTTGGTTAAGGG | Guanosine-3′,5′-bis (diphosphate) 3′-pyrophosphohydrolase |
| 13 | MIR1310 | AGGCATCGGGGGCGCAACGCCC | Ribulose-5-phosphate-3-epimerase |
| 14 | MIR7767 | CCCCAAGCTGAGAGCTCTCCC | Cell wall-associated hydrolase |
| 15 | MIR6445 | TTCATTCCTCTTCCTAAAATGG | Hypothetical protein |
| 16 | MIR6478 | CCGACCTTAGCTCAGTTGGTG | Hypothetical protein |
| 17 | MIR157 | TTGACAGAAGATAGAGAGCAC | Myosin-9, putative |
| 18 | MIR159 | TTTGGATTGAAGGGAGCTCTG | MYB transcription factor |
| 19 | MIR169 | GAGCCAAGAATGACTTGCCGA | Nuclear transcription factor Y subunit A-1 |
| 20 | MIR399 | TGCCAAAGGAGAGTTGCCCTG | 2-Oxoglutarate/malate translocator, chloroplast precursor, putative |
| 21 | MIR894 | CGTTTCACGTCGGGTTCACC | 40S ribosomal protein S26, putative |
| 22 | MIR171 | TTGAGCCGCGTCAATATCTCC | SCL protein |
| 23 | MIR395 | CTGAAGTGTTTGGGGGAACTC | Homeobox protein LUMINIDEPENDENS |
| 24 | MIR1425 | TAGGATTCAATCCTTGCTGCT | Leucine carboxyl methyltransferase, putative |
| 25 | MIR1432 | ATCAGGAGAGATGACACCGAC | Aminobutyrate aminotransferase |
| 26 | MIR164 | TGGAGAAGCAGGGCACGTGCA | dTDP-glucose 4-6-dehydratase, putative |
| 27 | MIR168 | TCGCTTGGTGCAGATCGGGAC | Predicted protein [*Populus trichocarpa*] |
| 28 | MIR3627 | TCGCAGGAGAGATGGCACTGTC | Conserved hypothetical protein |
| 29 | MIR444 | TGCAGTTGTTGTCTCAAGCTT | Beclin-1, putative |
| 30 | MIR528 | TGGAAGGGGCATGCAGAGGAG | Conserved hypothetical protein |
| 31 | MIR6476 | TCAGTGGAGATGAAACATGA | Photosystem I reaction centre subunit IV A chloroplast precursor |
| 32 | MIR2118 | GAAATGGGTGGATGGGAGTGA | Rhicadhesin receptor precursor putative |
| 33 | MIR160 | TGCCTGGCTCCCTGTATGCCA | Auxin response factor |

**Table 3** Novel miRNAs in both control and drought samples by NGS method

| Species | No. of novel miRNAs in control | No. of novel miRNAs in drought |
|---|---|---|
| *Hevea brasiliensis* | 7 | 13 |
| *Ricinus communis* | 2 | 4 |
| *Manihot esculenta* | 8 | 8 |
| Total | 17 | 25 |

(nr) protein database for functional identification using blastx. Several regulatory proteins such as auxin response factor (ARF), nuclear transcription factor Y subunit A-1 (NFYA-1), MYB transcription factor, zinc finger protein, Homeobox protein LUMINIDEPEDENS and regulatory-associated protein mTOR were found to be the targets of miR160, miR169, miR858, miR390, miR395 and miR396, respectively. Besides, ribulose-5-phosphate-3-epimerase, azetidine-2-carboxylic acid-resistant 1 family protein, Myosin-9, dtdp-glucose 4–6 dehydratase, transmembrane protein, electron transporter, kinesin, hypothetical protein POPTR, Beclin 1, ascorbate peroxidase, protein-binding protein, chloroplast precursor protein, cell wall-associated hydrolase and several hypothetical proteins were found to be targets of miRNAs identified (Table 2).

Among the novel miRNAs identified from control samples, four, two and six miRNA-target pairs were predicted for *H. brasiliensis, Ricinus* and *Manihot*, respectively, while five, three and five miRNA-target pairs were predicted in

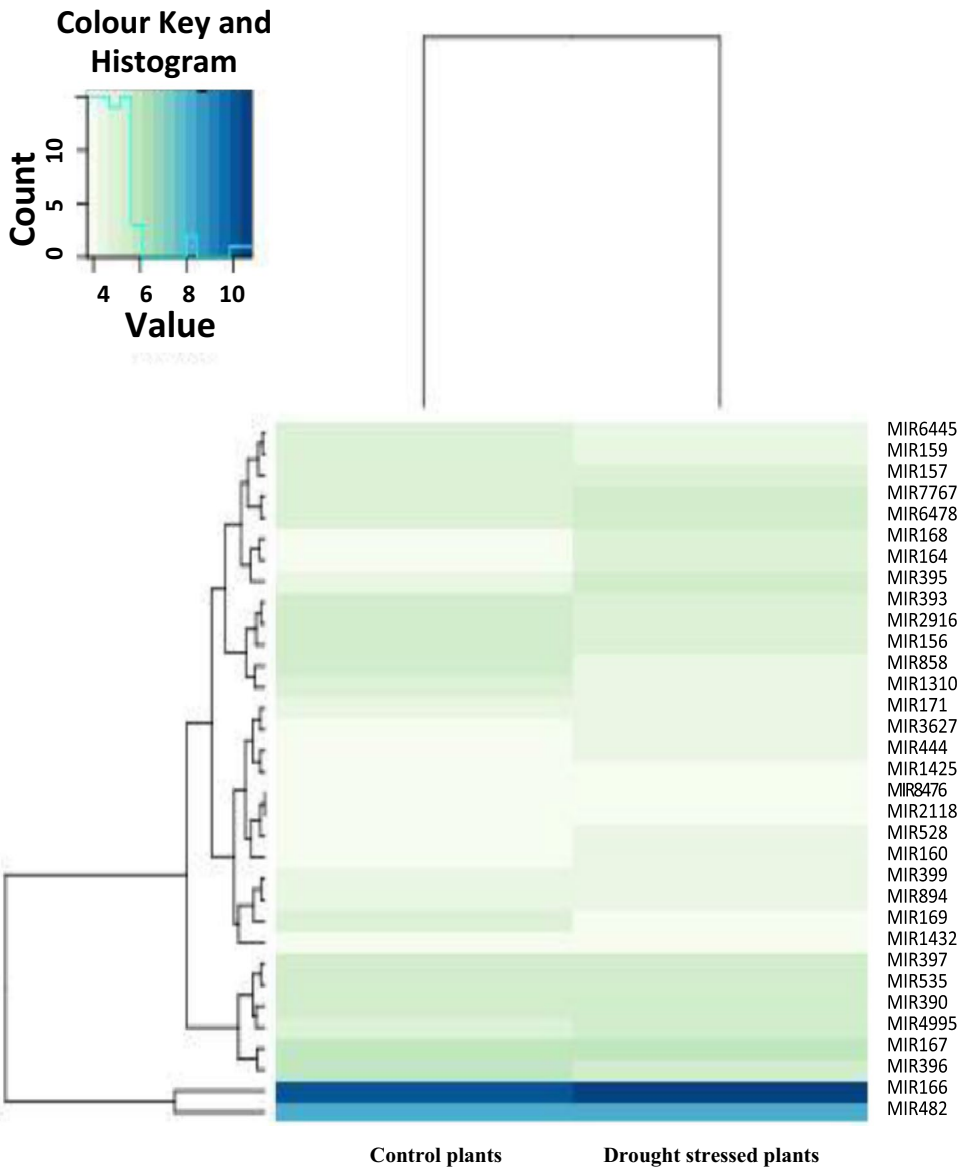


**Fig. 2** Heat map of conserved miRNAs from control and drought-stressed samples

drought-stressed samples (Table 4 and Supplementary data 8–10). HbmiRn_31 and HbmiRn_32 were found to target ubiquitin and WLM domain-containing protein, while HbmiRn_48 and HbmiRn_49 were predicted to target putative DNA-binding protein. HbmiRn_10, HbmiRn_37 and HbmiRn_65 targets ARM repeat superfamily protein, ubiquitin and WLM domain-containing protein and Tar1p, respectively, while both the HbmiRn_60 and HbmiRn_63 target Tubulin beta-7 chain.

## Validation of miRNAs and their potential targets by qPCR

The miRNAs obtained through deep sequencing was further quantified by qPCR. Expression analysis was carried out for ten conserved and three novel miRNAs (Fig. 3). Expression analysis was also carried out for seven previously reported miRNAs (Kuruvilla et al. 2016) in genotype RRII 430 (Fig. 4). All the miRNAs were found differentially expressed under drought stress though their level of expression varied among the genotypes studied. However, expression level differed between deep sequencing and qPCR results. Among them, miR168, miR160 and miR1432 were found up-regulated in tolerant genotypes (RRIM 600, RRII 208 and RRII 430) and down-regulated in susceptible genotypes (RRII 105 and RRII 414). Expression of miR6478 got significantly decreased in susceptible genotypes, while there was no significant change in tolerant genotypes. miR858a was found significantly down-regulated in RRII 105, RRIM 600 and RRII 414, while there was no significant change in clones RRII 208 and RRII 430. However, miR858b got down-regulated in all the five genotypes studied. miR6476 was found to be down-regulated in RRII 105, RRIM 600 and

**Table 4** Novel miRNAs and their predicted targets

| miRNA | miRNA sequence | Target name |
|---|---|---|
| A. Novel miRNAs in *Hevea brasiliensis* | | |
| HbmiRn_10 | CCGAGGAGGGGCTTGCGTCTGAT | ARM repeat superfamily protein |
| HbmiRn_31 | TTCAAATCTGGTTCCTGGCATT | Ubiquitin and WLM domain-containing protein |
| HbmiRn_32 | TTCAAATCTGGTTCCTGGCATT | Ubiquitin and WLM domain-containing protein |
| HbmiRn_37 | TTCAAATCTGGTTCCTGGCAT | Ubiquitin and WLM domain-containing protein |
| HbmiRn_49 | CAGGACTCGAGGAAGAAGCCCC | DNA-binding protein, putative |
| HbmiRn_60 | GAATGACTGGGCGTAAAGGGCA | Tubulin beta-7 chain |
| HbmiRn_63 | GAATGACTGGGCGTAAAGGGCA | Tubulin beta-7 chain |
| HbmiRn_65 | GACACCGCCCGTCGCTCCTACCGA | Tar1p |
| B. Novel miRNAs in *Ricinus communis* | | |
| HbmiRn_1 | TTCAAATCTGGTTCCTGGCACA | Conserved hypothetical protein |
| HbmiRn_5 | ATGGTACTTACTTTCATACAGG | Protein phosphatase 2c |
| HbmiRn_6 | TTATCATTACGATAGGTGTCAAG | Conserved hypothetical protein |
| HbmiRn_12 | AAGGTAGGCTCAAGCTAAGATTC | Hypothetical protein MTR_6g043000 |
| HbmiRn_15 | GAATGACTGGGCGTAAAGGGCA | Protein-binding protein |
| C. Novel miRNAs in *Manihot esculenta* | | |
| HbmiRn_14 | TTCAAATCTGGTTCCTGGCAT | Conserved hypothetical protein |
| HbmiRn_35 | CGAAGCTACTGTGCGCTGGATTAT | Putative senescence-associated protein |
| HbmiRn_43 | GACGGGGTATTGTAAGTGGCAGA | Succinate dehydrogenase |
| HbmiRn_6 | TTATCATTACGATAGGTGTCAAG | Protein phosphatase 2C |
| HbmiRn_18 | TTCAAATCTGGTTCCTGGCATA | Conserved hypothetical protein |
| HbmiRn_20 | GGGATTGTAGTTCAATTGGTCAGA | Conserved hypothetical |
| HbmiRn_26 | AACCGGGACGTGGCGGCTGACGGC | Conserved hypothetical protein |
| HbmiRn_28 | GTCGCGGTTCCACATCCGACCGG | Methylmalonyl-CoA mutase large subunit |
| HbmiRn_46 | GCCAGGCCCCGATGAGTAGGAGG | Solute carrier family 31 (copper transporters) |
| HbmiRn_48 | AGGAGGGCGCGGCGGTCGCTGCA | Ribonucleoprotein, putative |

RRII 414, while it got up-regulated in RRII 208 and RRII 430. Expression of miR3627 got down-regulated significantly in susceptible genotypes and got up-regulated in tolerant genotypes except in RRII 208. Similarly, HbmiRn_63 was also found up-regulated in tolerant genotypes, while it got down-regulated in susceptible genotypes. HbmiRn_48 got down-regulated in all the genotypes except in RRII 430 where it got up-regulated. In contrast, HbmiRn_11 got up-regulated in all the genotypes except RRII 414 which exhibited down-regulation. When seven miRNAs were quantified in tolerant genotype RRII 430, expression of miR482, miR164 and miR398 was found unaltered, whereas, miR167, miR169, miR166 and HbmiRn_42 were found up-regulated under drought.

In continuation with the prediction of miRNA-target pairs obtained, three conserved miRNAs as well as one novel miRNA and their corresponding targets were subjected to miRNA-target pair expression analysis (Fig. 5) in genotypes RRII 105, RRIM 600, RRII 414, RRII 208 and RRII 430. Correlation between target genes MYB (miR858), NFY A-1 (miR169), ARF (miR160) and HMGR3 (HbmiRn_42) and their corresponding miRNAs (given in parenthesis) was examined in drought-stressed samples. Novel miRNA HbmiRn_42 and its target HMGR3 showed a negative correlation in all the genotypes studied. In the case of MYB transcription factor and miR858a, a negative correlation was noticed in all the genotypes except RRII 414. No significant negative correlation could be observed in the case of miR160 and miR169 against their targets.

## Identification of miRNAs associated with drought tolerance

The normalized quantitative expression analysis of miRNAs data was subjected to single factor ANOVA. A significant *F* value could not be obtained when analyzed with the data of both tolerant and susceptible genotypes together at 0.05 and 0.1 level. But, when the analysis was performed in tolerant and susceptible genotypes separately, significant difference at 0.05 levels could be observed in drought-tolerant genotypes. Further, the Fisher's least significant difference analysis in drought-tolerant genotypes indicated a stronger association between HbmiRn_63 and HbmiRn_42 and drought tolerance (Table 5). This analysis also indicated

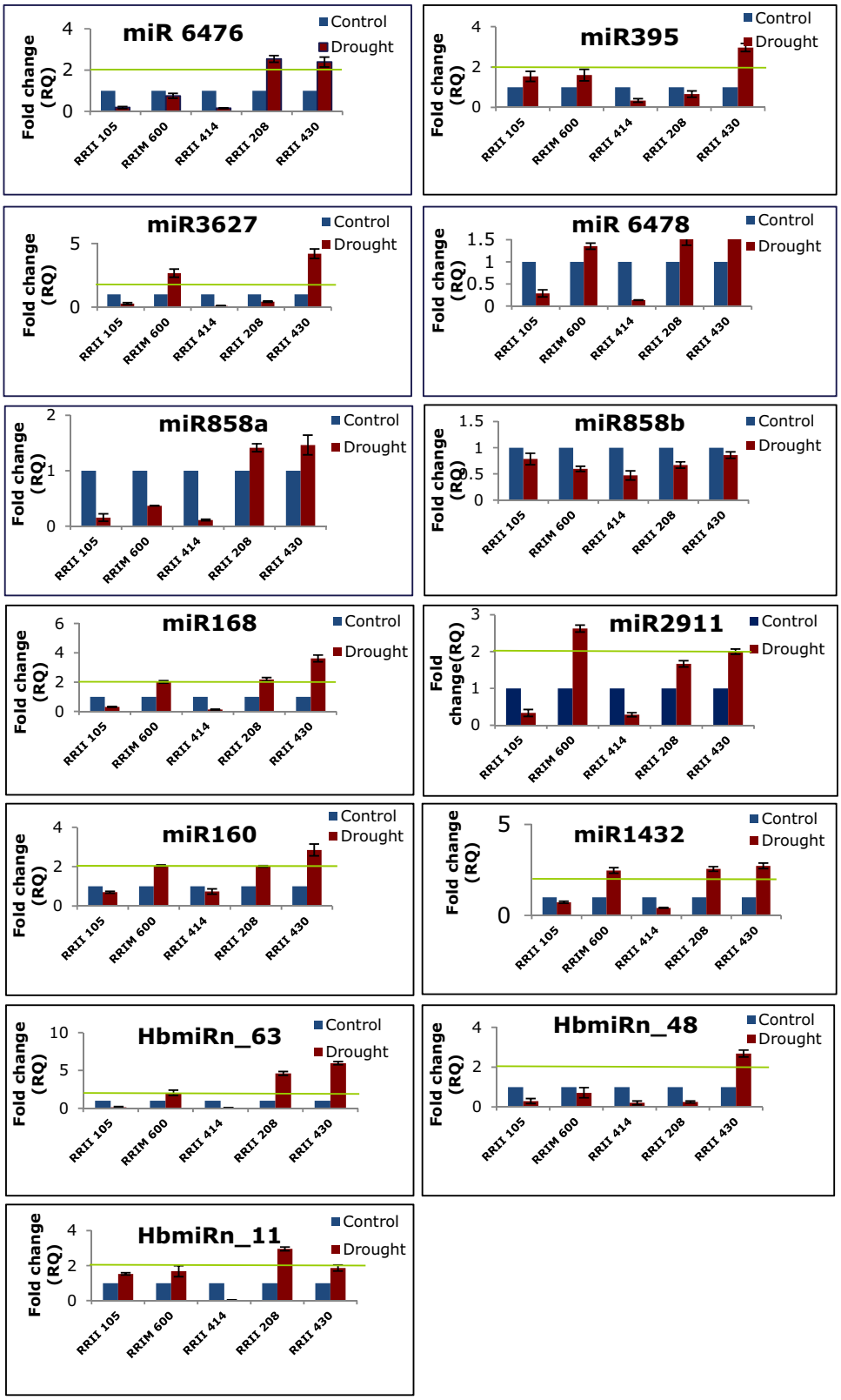


**Fig. 3** Expression analysis of 13 miRNAs in five genotypes of *H. brasiliensis* under drought conditions (error bars indicate standard error of three biological replicates)

up-regulation of other miRNAs such as miR168, miR1432, miR3627, miR160 and HbmiRn_11 which are on par with the HbmiRn_42 thus suggesting their association with drought tolerance.

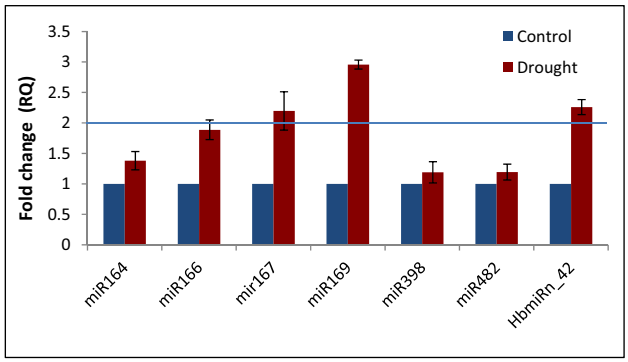


**Fig. 4** Expression analysis of seven miRNAs in genotype RRII 430 under drought conditions (error bars indicate standard error of three biological replicates)

### Validation of miRNAs in germplasm accessions

To ascertain the association of selected miRNAs exhibiting strong association with drought tolerance, expression analyses of two conserved miRNAs (miR160 and miR168) and one novel miRNA (HbmiRn-42) was carried out in two relatively drought-tolerant (RO 3261 and AC 612) and two susceptible (RO 3242 and MT 1619) germplasm accessions. These miRNAs got up-regulated in both the tolerant genotypes (RRIM 600, RRII 208 and RRII 430) and in the tolerant germplasm accessions, while it got down-regulated in susceptible genotypes (RRII 414 and RRII 105). No significant change was observed in susceptible germplasm accessions (Fig. 6).

## Discussion

Under stress situations, expression of genes related to various metabolic pathways in response to stress perception, signal transduction and regulation and synthesis of numerous stress-ameliorating compounds is altered. In recent years, novel molecular biological approaches have been adopted to develop stress-tolerant genotypes of *H. brasiliensis* (Leclercq et al. 2012). Previous reports have established the role of miRNAs in regulating genes associated with abiotic stress-responsive pathways in *H. brasiliensis* (Gebelin et al. 2012, 2013b). Recent studies also reported isolation and cloning of drought-responsive miRNAs (Kuruvilla et al. 2016) by conventional approach as well as cold stress-responsive miRNAs from *H. brasiliensis* using NGS approach (Kuruvilla et al. 2017).

With better understanding on the role of stress-responsive miRNAs, more effective miRNA-mediated gene regulation would be possible to enhance plant stress tolerance (Noman et al. 2017). Ferdous et al. (2016) could successfully demonstrate enhanced drought tolerance in barley by overexpressing Hv-miR827. Overexpression of miR172c (of *Glycine max*) conferred water deficit and salt tolerance in *Arabidopsis* (Li et al. 2015). Such interventions would be possible in *Hevea* only if a large set of data on all possible drought-responsive miRNAs is generated.

miR168 and miR160 were found to be up-regulated in tolerant genotypes of *H. brasiliensis*, while it got down-regulated in susceptible genotypes. miR168, a highly conserved miRNA reported in more than 30 species is known as salt/drought/cold/ABA responsive (Shuai et al. 2013; Liu et al. 2008, 2017; Zhou et al. 2010). It targets ARGONAUTE1 which is a core component of the RNA-induced silencing complex associated with miRNAs to inhibit target genes (Vaucheret 2008; Voinnet 2009). Similarly, miR160 is also known to be associated with drought tolerance. Its association with drought tolerance has been reported in drought-tolerant cowpea genotype (Barrera-Figueroa et al. 2011) and in peach root (Eldem et al. 2012). Various reports indicate the possible link between auxin signaling and miR160 expression (Sunkar et al. 2012). In *H. brasiliensis*, miR160 had been predicted to target one of the Hevamine-encoding genes (Lertpanyasampatha et al. 2012). miR1432 which also exhibited a trend similar as miR168 and miR160 in this study, is predicted to target Poly (ADP-ribose) polymerase and calcium-binding EF hand domains that are involved in activating signal transduction pathways (Kantar et al. 2011).

miR2911, an atypical miRNA derived from ribosomal RNA that does not follow classical miRNA biogenesis (Lee et al. 2003; Gregory et al. 2004; Denli et al. 2004), was found to be significantly down-regulated in susceptible genotypes and up-regulated in tolerant genotypes. Higher levels of miR2911 under drought stress have been earlier reported in cowpea (Barrera-Figueroa et al. 2011), *Populus euphratica, Nicotiana tabacum* and *Helianthus annuus* (Li et al. 2009; Tang et al. 2012; Barozai et al. 2012). It targets cytochrome p450 like tbp (TATA box binding protein) which is involved in stress response (Zhu and Luo 2013). Up-regulation of miR2911 in tolerant genotypes and down-regulation in susceptible genotypes indicate its direct role in drought tolerance. miR3627, a highly conserved family reported in fruit trees, poplar and in other non-woody plant species (Solofoharivelo et al. 2014), targets amino acid transporter in apple (Xia et al. 2012), and an unknown protein in *Acacia crassicarpa* (Liu et al. 2014). This study predicted a conserved hypothetical protein as its target. Expression analysis of this miRNA did not indicate any association with drought tolerance.

miR6476 was found up-regulated in tolerant genotypes and got significantly reduced in sensitive genotypes. Target prediction analysis in *H. brasiliensis* indicated that photosystem I reaction centre subunit IV A (chloroplast precursor) as its target. Whereas, it targets amino acid transporter and TPR domain-containing protein in tomato (Din et al.

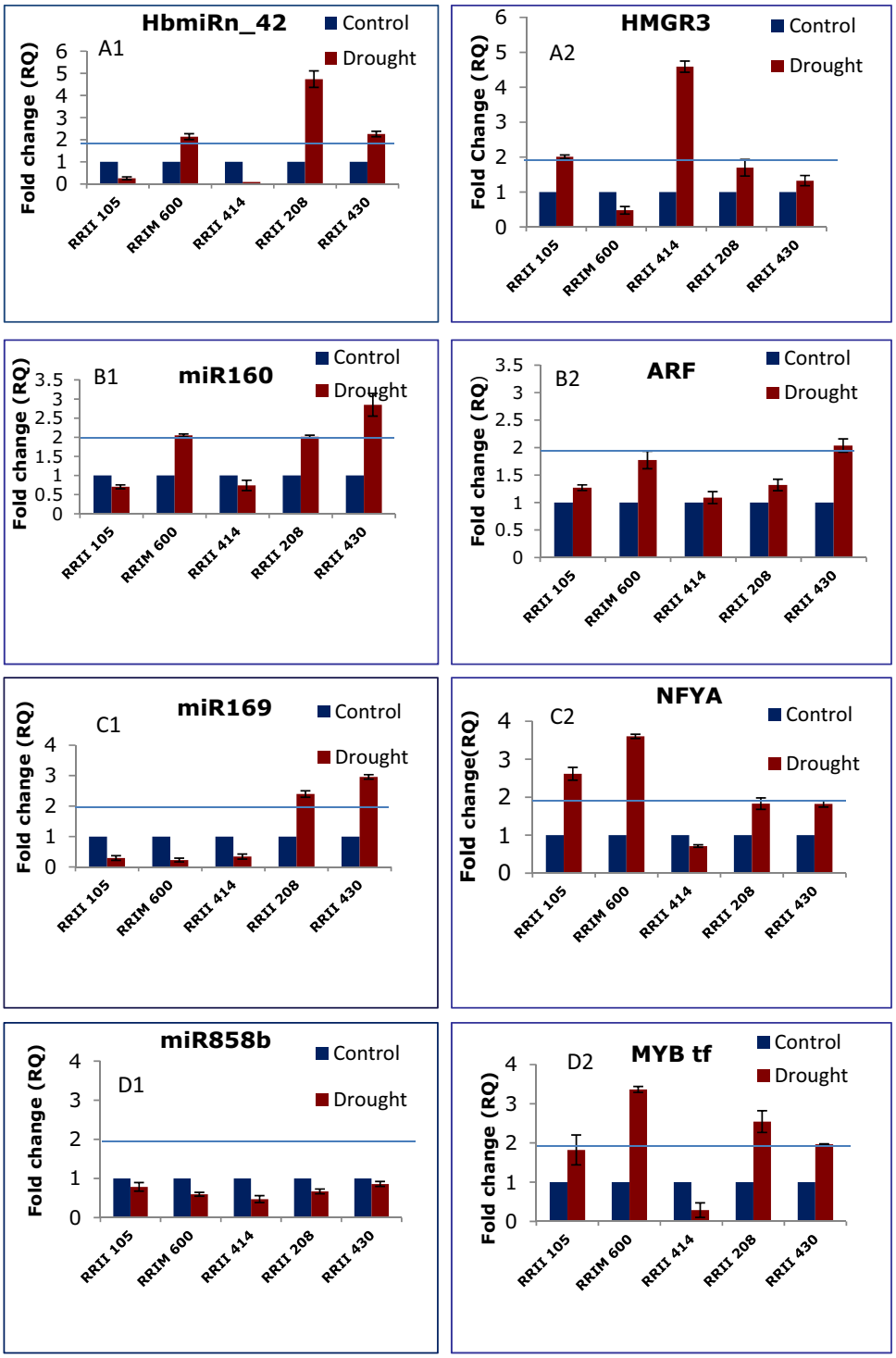


**Fig. 5** Expression analysis of four miRNAs and their corresponding target genes. Expression of HbmiRn_42 (**A1**) and its putative target HMGR3 (**A2**); miR160 (**B1**) and its putative target ARF (**B2**); miR169 (**C1**) and its putative target NFYA (**C2**); miR858b (**D1**) and putative target MYB tf (**D2**)

2014). The transcripts of miR395 which target two families of genes, ATP sulfurylases and sulfate transporter 2;1, were found to be significantly reduced in susceptible genotype RRII 414 and were found to be significantly up-regulated in drought-tolerant genotypes RRII 430 and RRIM 600. Similarly, higher levels of miR395 under drought conditions had been reported in *Oryza sativa* (Zhou et al. 2010) and tobacco (Frazier et al. 2011). These findings suggest that both miR6476 and miR395 are associated with drought tolerance.

In this study, expression of miR164, miR482 and miR398 was found to be unaltered under drought in genotype RRII 430. In an earlier study (Kuruvilla et al. 2016), miR164 level was found unaltered, while miR482 got down-regulated

**Table 5** Fisher's least significant difference analysis in relative quantification values of drought-tolerant clones

| miRNA | RQ value |
|---|---|
| HbmiRn_63 | 4.3270a |
| HbmiRn_42 | 3.0457ab |
| miR168 | 2.6047bc |
| miR1432 | 2.5821bc |
| miR3627 | 2.3820bcd |
| miR160 | 2.3691bcd |
| HbmiRn_11 | 2.2024bcde |
| miR2911 | 2.0995bcdef |
| miR 6476 | 1.8946bcdef |
| miR169 | 1.8603bcdef |
| miR395 | 1.7445bcdef |
| mir167 | 1.7418bcdef |
| miR 6478 | 1.6511bcdef |
| miR858a | 1.5453bcdef |
| miR164 | 1.2214cdef |
| miR166 | 1.2120cdef |
| HbmiRn_48 | 1.1872cdef |
| miR398 | 0.8710def |
| miR858b | 0.7073ef |
| mir482 | 0.5432f |

in tolerant genotypes. Up-regulation was observed for HbmiRn_42, miR167 and miR169 in drought-tolerant genotype RRII 430. Results of this study also corroborated with the findings of Kuruvilla et al. (2016) in the case of HbmiRn_42 that got down-regulated in susceptible genotypes and up-regulated in tolerant genotypes. miR858 which targets MYB genes in plants (Xia et al. 2012; Guan et al. 2014) is the largest transcription factor gene family playing vital roles in plants' responses to various biotic and abiotic stresses. However in this study, expression of miR858a and miR858b did not indicate any association with drought tolerance.

The novel miRNAs viz. HbmiRn_42 and HbmiRn_63 were found expressed at a higher level in tolerant genotypes, while they got down-regulated in drought-susceptible genotypes thus indicating their association with drought tolerance. It can be speculated that these miRNAs might be involved in controlling the expression of their target gene that may function as negative regulator of drought tolerance. Expression analysis in germplasm accessions with known tolerance levels also indicated a similar trend. When the quantification of three miRNAs (miR160, miR168 and HbmiRn_42) which exhibited a stronger association with drought tolerance was performed in two tolerant (RO 3261 and AC 612) and two susceptible (RO 3242 and MT 1619) germplasm accessions, they were found up-regulated in tolerant genotype RRIM 600 and germplasm accessions. But in susceptible genotype RRII 414, it got down-regulated while there was no significant change in the susceptible germplasm accessions. These results clearly indicate the association between miR160, miR168 and HbmiRn-42 and drought tolerance thus substantiating the findings of Thomas et al. (2015).

Target prediction made in this study revealed that many of the targets were transcription factors including MYB, NFYA, and ARFs which are mainly involved in regulating stress-responsive genes. Several reports have established beyond doubt that majority of stress-responsive miRNAs target transcription factors (Zhang and Wang 2015). Transcription factors play an important role during plants' response to different environmental stresses. Stress-induced miRNAs generally down-regulate their target mRNAs, which in turn results in accumulation and function of positive regulators (Chinnusamy et al. 2007). Apart from this, stress responsive and stress amelioration-related genes, hypothetical proteins and cell wall-associated and signaling-related proteins were also found as targets of these miRNAs. Target prediction carried out for the novel miRNAs revealed four miRNA-target pairs from control samples and five miRNA-target pairs from *Hevea brasiliensis* database. Among the five miRNA-target pairs predicted in drought samples, HbmiRn_10 was found to target ARM repeat superfamily protein which is known

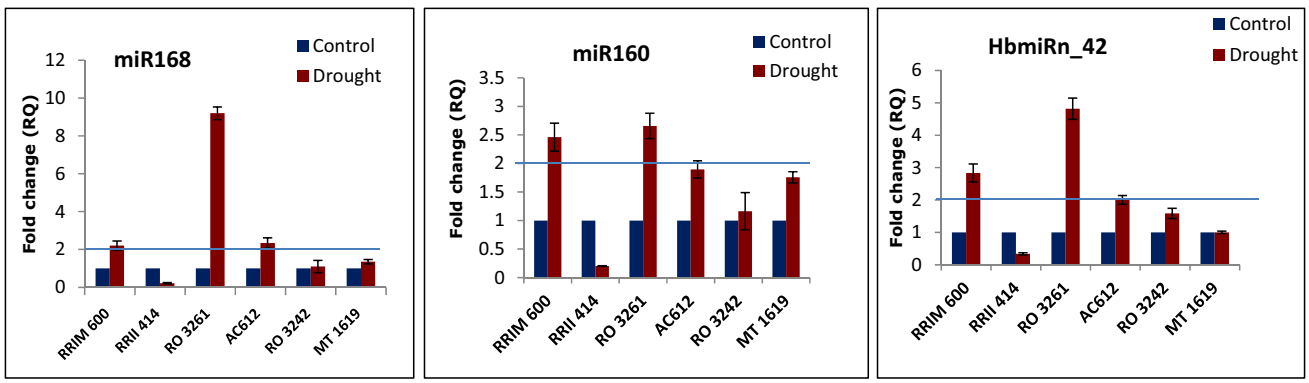


**Fig. 6** Quantification of miR168, miR160 and HbmiRn_42 in germplasm accessions of *H. brasiliensis*

to interact with numerous other proteins and to regulate a set of cellular processes (Mudgil et al. 2004). ARM repeat superfamily proteins are also involved in protein degradation pathways as E3 ubiquitin. HbmiRn_37, HbmiRn_31 and HbmiRn_32 were predicted to target the ubiquitin and WLM domain-containing protein. The WLM (WSS1-like metalloprotease) domain belonging to the zincin-like superfamily of Zn-dependent peptidase functions as a specific de-SUMOylating domain of distinct protein complexes in the nucleus and cytoplasm (Iyer et al. 2004). HbmiRn_65 was predicted to target Tar1p (Transcript Antisense to Ribosomal RNA), while both the HbmiRn_60 and HbmiRn_63 target Tubulin beta-7 chain. HbmiRn_48 and HbmiRn_49 target a putative DNA binding protein.

When the expression patterns of four target genes, viz. MYB, NFYA3, ARF and HMGR3 and their corresponding miRNAs were quantified, MYB was found up-regulated in all the drought exposed genotypes except RRII 414, while its corresponding miRNA (miR858) got reduced in all the genotypes. Expression of MYB has been reported to be significantly higher in tolerant genotypes like RRII 208 and RRIM 600 and was found moderately up-regulated in RRII 105, while it got significantly down-regulated in drought-susceptible genotype RRII 414 (Luke et al. 2015). Though expression of miR858 in genotype RRII 414 was at minimal in this study, the level of its corresponding target MYB was also found to be down-regulated indicating no correlation between them. This warrants more investigations on influence of this miRNA on various subfamilies of MYB.

Novel miRNA HbmiRn_42 and its target gene HMGR3 showed a negative correlation in all the genotypes studied. Its expression in tolerant genotypes resulted in down-regulation of its target protein HMGR3. Down-regulation of this miRNA in drought-susceptible genotypes under drought conditions led to up-regulation of its corresponding protein. It appears that the target of HbmiRn_42 might probably be a negative regulator of drought tolerance. Plant HMGR is a key regulatory enzyme of mevalonate pathway involved in isoprenoid biosynthesis which is controlled by various endogenous signals and environmental factors (Antolín-Llovera et al. 2011). HMGR3's up-regulation in susceptible genotypes under drought conditions indicates the possibility of continued metabolic activity that might restrict diverting the resources for stress amelioration thus resulting in susceptibility when compared to the tolerant genotypes.

miR160 got up-regulated in tolerant genotypes under drought stress, while there was no significant reduction in the expression levels of its target ARF. ARF genes are key regulators of physiological and morphological processes mediated by auxins by binding to specific *cis*-element in the upstream regions of auxin-inducible genes associated with stress adaptation (Guilfoyle and Hagen 2007). Though Gebelin et al. (2012) reported miR160 to target ARF in *H. brasiliensis*, the results of this study did not indicate any correlation. Nuclear factor Y (NF-Y) is a ubiquitous transcription factor which is induced by drought stress at both transcriptional and post-transcriptional levels. Overexpression of NFYA5 and NFYB1 in *Arabidopsis* was reported to impart drought tolerance (Li et al. 2008). miR169 has been reported to direct NFYA3 mRNA cleavage in *Glycine max* (Ni et al. 2013). Overexpression of GmNFYA3 in *Arabidopsis* resulted in reduced leaf water loss and enhanced drought tolerance (Ni et al. 2013). Down-regulation of miR169a under drought conditions resulted in increased levels of *NFYA5* in *Arabidopsis* (Li et al. 2008). A study in *H. brasiliensis* (Luke et al. 2015) also confirmed the abundant expression of NFYA in tolerant genotype (RRIM 600), while it was at minimal in susceptible genotype (RRII 414). In this study, miR169 was found down-regulated in genotypes RRII 105, RRII 414 and RRIM 600, while its target gene got up-regulated significantly in genotypes RRII 105 and RRIM 600.

The ANOVA and LSD test results indicated few miRNAs (HbmiRn_63 and HbmiRn_42 miR168, miR1432, miR3627, miR160 and HbmiRn_11) to have a stronger association with drought tolerance. This study on drought-responsive miRNAs of *H. brasiliensis* opens up the possibility of identification of miRNAs associated with drought tolerance and subsequently employing them as marker for drought tolerance in the crop improvement programs. Though previously identified DNA-based molecular markers have been explored and implemented successfully in crop improvement programs of *H. brasiliensis*, recent investigations on miRNA-based molecular markers have demonstrated its use as novel functional markers because of their higher level of conservation (Razna et al. 2015; Wang et al. 2016). miRNA-based markers have also been demonstrated to facilitate genotyping with low costs, high efficiency, stability and good transferability (Fu et al. 2013). Several reports have also demonstrated the possibility of miRNA-mediated gene regulations to achieve enhanced abiotic stress tolerance in plants (Zhang and Wang 2015; Ni et al. 2013; Song et al. 2016; Li et al. 2016). Investigations on miRNAs of *Hevea* have also led to the identification of several stress-responsive/tolerant miRNAs. Perhaps, it is high time that these data are effectively utilized to achieve crop improvement through genetic manipulations or by employing miRNAs as functional markers (Fu et al. 2013; Yadav et al. 2014; Wang et al. 2016). Findings from this study can further be evaluated in a larger set of genotypes with high variability in terms of drought tolerance/sensitivity to determine candidate miRNAs that can be employed in the crop improvement programs of *H. brasiliensis*.

**Author contribution statement** LK: Basic execution of research project, data analysis, manuscript preparation, etc. MS: designing of the project/experiments, wet lab

experiments and data analysis, manuscript preparation, etc. LL: involved in wet lab experiments, quantitative PCR, etc. MT: involved in raising the required plant materials, drought imposition, measuring the physiological parameters and wet lab experiments.

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