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ORIGINAL ARTICLE

High-throughput miRNA deep sequencing in response to drought stress in sugarcane

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Abstract

Drought is a major factor which reduces cane growth and productivity. In the present study, we sequenced drought susceptible (V1) and drought tolerant (V2) sugarcane varieties using high-throughput miRNA deep sequencing method to study the regulation of gene expression by miRNAs during drought stress in sugarcane. A total of 1224 conserved miRNAs which belong to 89 miRNA families were identified and 38% of the differentially regulated miRNAs were common for both varieties. Additionally 435 novel miRNAs were also identified from four small RNA libraries. We identified 145 miRNAs that were differentially expressed in susceptible variety (V1–31) and 143 miRNAs differentially expressed in the tolerant variety (V2–31). Target prediction revealed that the genes mainly encoded transcription factors, proteins, phosphatase and kinases involved in signal transduction pathways, integral component of membrane and inorganic ion transport metabolism, enzymes involved in carbohydrate transport and metabolism and drought-stress-related proteins involved in defense mechanisms. Pathway analysis of targets revealed that "General function prediction only" was the most significant pathway observed in both tolerant and susceptible genotypes followed by "signal transduction mechanisms". Functional annotation of the transcripts revealed genes like calcium-dependent protein kinase, respiratory burst oxidase, caffeic acid 3-O-methyltransferase, peroxidase, calmodulin, glutathione S-transferase and transcription factors like MYB, WRKY that are involved in drought tolerant pathways. qRT-PCR was used to verify the expression levels of miRNAs and their potential targets obtained from RNA sequencing results.

Keywords Sugarcane · Drought stress · miRNAseq · Target regulation · Differential expression

Introduction

Sugarcane is an important crop in the tropical and subtropical regions and it is responsible for almost 86% of sugar production in the world (OECD-FAO 2019). Among the various abiotic stress affecting sugarcane drought is considered to be the most detrimental stress, affecting crop production worldwide (Wang et al. 2003; Rampino et al. 2006). In recent years it has been the major limitation for sugarcane production and sugarcane growth is highly sensitive to water deficit (Companhia Nacional de Abastecimento) (Lakshmanan and Robinson 2014; CONAB 2017). Drought stress decreases stomatal conductivity reducing water loss in the leaf and reduction in photosynthesis takes place leading to low sugarcane yields (Namwongsa et al. 2019). Yield losses due to drought stress goes up to 60% (Gentile et al. 2015), depending on the severity and duration of water stress (Machado et al. 2009). Development of sugarcane varieties

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with increased drought resistance has been regarded as an efficient strategy to stabilize and improve production. Studies have demonstrated that the morphological, physiological and biochemical responses of plants to drought stress are controlled by various molecular mechanisms that are regulated by gene expression (Farooq et al. 2009; Osakabe et al. 2014). Plant responses to stress are modulated by relative expression of genes as well as their regulation at different levels, post transcriptional or post translational.

Among the eukaryotes RNA silencing, which is facilitated by small non-coding RNAs of 20–35 nucleotides is an important form of gene regulation apart from transcriptional factors. In recent years, regulatory small RNAs (sRNAs) have received wide attention for their roles in the post-transcriptional and translational regulation of gene expression and form an universal component of endogenous plant transcriptome (Axtell 2013). SmallRNAs can be classified into four major categories based on their origin, processing mode and effector protein association. They are microRNA (miRNA), small interfering RNA (siRNA), transfer RNA-derived small RNAs (tsRNAs) and PIWI-interacting RNA (piRNA, animals only) (Borges and Martienssen 2015; Zhu et al. 2018; Czech et al. 2018). miRNAs are small non-coding RNAs of 18–25 nucleotides that occur in most eukaryotic genomes. Mature microRNAs generated from primary miRNAs with the aid of dicer-like enzymes (DCL1, RNA III enzymes) could inactivate the expression of target RNAs possessing complementary sequences in plants by binding and guiding their effector proteins (Rogers and Chen 2013). They play significant roles by negatively regulating target genes in many biological processes such as growth and hormone signal transduction of the organism (Bartel 2004; Jin et al. 2008; Sunkar 2010; Sunkar et al. 2012).

There are several miRNAs that have been identified in a wide array of species which play important role in response to biotic and abiotic stresses (Khraiwesh et al. 2012; Pei et al. 2013; Sailaja et al. 2014; Shriram et al. 2016).Very few studies are reported in identifying mature miRNA sequences and their expression during drought stress in sugarcane plants (Ferreira et al. 2012; Thiebaut et al. 2012; Gentile et al. 2013, 2015). In the present study, high-throughput miRNA deep sequencing method was used to study the regulation of gene expression by miRNAs and validate their expression during drought stress in sugarcane. We sequenced sRNA from leaf tissues of sugarcane varieties Co 8021 (drought susceptible) and Co 06022 (drought tolerant) which were subjected to drought treatment to identify differentially expressed miRNAs and their interaction with predicted target mRNAs. The results suggested the differential regulation of miRNAs that are associated with drought and the varied roles of their target mRNAs that will help to establish the molecular basis of drought stress tolerance in sugarcane plants.

Materials and methods

Selection of plant samples and RNA isolation

Two sugarcane varieties Co 8021 (Variety1-V1) and Co 06022 (Variety2-V2) were selected based on differential drought tolerance potential (Devi et al. 2018) from the fields of ICAR-Sugarcane Breeding Institute, Coimbatore and were planted in pots under normal irrigation for 2 months. After 60 days of planting, gradual drought stress was imposed by withholding water for 10 days, while the control plants were normally irrigated throughout the experiment (Devi et al. 2019). Tissue was collected (Leaf + 1, the highest unfolded leaf with a visible dewlap) in triplicates after 10 days drought treatment along with the respective controls. The samples were flash-frozen and total RNA was extracted using TRI reagent (Sigma-Aldrich, USA) and purified using Spectrum™ Plant Total RNA kit (Sigma-Aldrich, USA). The quantification of the total RNA samples was done using NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). The quality and quantity of RNA was also analysed using Agilent RNA bioanalyzer chip. The samples were named as V1–11 (Co 8021-control), V1–31 (Co 8021-treated) and V2–11 (Co 06022-control) and V2–31 (Co 06022-treated).

Analysis of small RNAs

Small RNA libraries were prepared from total RNA of four selected samples using the Illumina Truseq small RNA library prep (Illumina, USA). The libraries were sequenced using IlluminaNextSeq500.751platforms (Illumina, Inc. USA) (according to manufacturer's instructions). The libraries were sequenced by the Genotypic Technology Pvt. Ltd., Bangalore. The raw reads from the libraries were first cleaned by removing 5′ and 3′ adaptors, low quality reads and reads with length < 16 or length > 35 bp were filtered using the srna-workbenchV3.0_ALPHA1 (University of East Anglia (UEA)). Only trimmed reads 16–35 nt in length was considered for further analysis.

Known miRNA and novel miRNA prediction

The clean reads were used for BLASTn search against Sanger RNA database (Rfam) (http://www.sanger.ac.uk/software/Rfam). All reads were checked for ncRNA (rRNA, tRNA, snRNA and snoRNA) contamination. The unaligned reads to ncRNAs were clustered based on the 95% coverage and 90% similarity to generate the read count. To obtain known miRNAs, clustered reads were used for homology search of the miRNAs against all matured Viridiplantae

miRNA sequences retrieved from miRbase-21 database using ncbi-blast-2.2.30. To predict novel miRNAs in the samples, sRNAseq reads not aligning to miRBase were given as input to MIREAP v0.2 software (http://sourceforge.net/projects/mireap/). MIREAP identified novel microRNA based on alignment, secondary structure, free energy and location on the precursor arm. Stem-loop hairpins were retained only when 1. The mature miRNAs-associated reads, mapped in the arm region of the precursors and 2. The free energy of the secondary structure calculated by RNAfold (http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi) was lower than − 18 kcal/mol. Other parameters that were set included a minimal miRNA length of 18, maximal miRNA length of 26; minimal reference miRNA length of 20; maximal reference miRNA length of 24; uniqueness of miRNA was 20; maximal free energy for the miRNA precursor was − 25 kcal/mol; maximal space between miRNA and miRNA* was 300, minimal base pairs of miRNA and miRNA* was 14, maximal bulge of miRNA and miRNA* was 4, maximal asymmetry of miRNA/miRNA* duplex was 4 and flank sequence length of miRNA precursor was 20. The free energy of the secondary structure of the miRNAs calculated by RNAfold is lower than − 5 kcal/mol.

Differential gene expression (DGE) analysis

Read count table for all the samples were generated. Read counts of each miRNAs were normalized to calculate the normalized miRNA abundance values using following formula RPM = RmiR/Rall*10^6 where: RmiR—number of reads mapped to a particular miRNA reference in the sample, Rall—total number of reads mapped in the sample. DGE analysis was carried out using DESeq5 tool and list of miRNAs expressed only in control, both in control and stress and only in stress were generated. miRNAs that were significantly expressed under drought stress were screened with p value < 0.05 and fold change > 1 or < -1.

Target prediction for miRNA

Differentially expressed miRNA's with copy number ≥ 10 were considered for target prediction. The miRNA target sequences were predicted using miRanda-3.3 (http://www.microrna.org/microrna/getGeneForm.do) using the "strict" option. This option ensures strict alignment in the seed region in position between 2 and 8 nucleotides. This option prevents the detection of target sites which contain gaps or non-canonical base pairing in this region. Other criteria were conservation, free energy, and site accessibility. miRNA hits having minimum free energy ≤ − 25 were assumed to be the targets for reported miRNA.

KOG annotation and KEGG pathway analysis of miRNA target

All the miRNA targets were searched against KOG protein sequences. KOG classification analysis was conducted to reveal the biological functions of the miRNA targets. Pathway analysis was performed using KEGG pathway database (https://www.Genome.jp/kegg).

Stem-loop reverse transcription and RT-qPCR validation

Reverse Transcription stem-loop primers (STL), sequence-specific forward PCR primers and universal reverse primers were designed following Chen et al. (2005) for reverse transcription and qPCR amplification of sugarcane miRNAs. miRNA-cDNA synthesis was carried out using the TaqMan microRNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, USA) and reverse transcription reactions were performed as described by Varkonyi-Gasic et al. (2007). Each reaction contained 10 ng of DNA-free total RNA and 1 µl of each RT-loop primer (1 µM). The mix was incubated for 10 min at 65 °C and then placed on ice for 2 min. Subsequently, 2.5 µl of 10 × Reverse transcription buffer, 0.25 µl of $dNTP_s$ (100 mM), 0.3 µl of RNAse inhibitor (40 U/µl), and 1 µl of Multiscribe™ Reverse Transcriptase (100 U/µl) enzyme (Taqman® MicroRNA Reverse Transcription Kit, Thermo Fisher Scientific) and 13.95 µl of nuclease free water were added. This reaction was incubated in a Thermal Cycler (MJ Research Inc. model: PTC 100) for 30 min at 16 °C, followed by 42 °C for 30 min and finally at 85 °C for 5 min at for the enzyme inactivation. To validate the expression levels of miRNA, 25 s rRNA was used as internal control. qRT-PCR amplification of the miRNAs was performed using SYBR Premix Ex Taq II (Takara, Dalian, China) in Rotor gene Q real time PCR machine (Qiagen, Germany). The reaction mixture included 8 µl of SYBR green master mix (1 ×), 2 µl of cDNA, 1 µl of specific forward primer (10 mM), 1 µl of universal reverse primer (10 mM). The volume was made upto 20 µl with 8 µl of nuclease free water. The reactions were performed at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min with a final dissociation curve analysis. All reactions were run in triplicates. The relative expression values were calculated using the $2^{-\Delta\Delta CT}$ method as described by Livak and Schmittgen (2001). qRT-PCR for selected miRNA and their targets was also performed to check the miRNA target regulation. Ten miRNAs and their target genes were used for expression analysis with cDNA synthesized from leaf samples of 10 days stress of both varieties.

Results

Sequencing results of small RNA libraries

Four sRNA libraries were constructed and sequenced from control and drought stressed samples of the susceptible variety Co 8021 (V1–11, V1–31) and tolerant variety (V2–11, V2–31) to identify drought responsive miRNAs in sugarcane plants. Each library generated 11–18 million raw reads (Additional file 1: Table S1).The data has been submitted in the National Center for Biotechnology Information (NCBI) with accession number PRJNA593909 (https://www.ncbi.nlm.nih.gov/sra/PRJNA593909). Clean reads were obtained by removing adaptors, junk and low-quality reads and small RNAs within 16–35 nt were subjected to further analysis. The lengths of miRNA were mainly distributed between 21 and 25 nt, which account for an average of 88% of the small RNAs. However, there was a considerable difference for the length distribution among different small RNA libraries. Among all sRNAs, 24 nt miRNAs had the highest number of reads in all of the four libraries (Fig. 1).

Identification of known miRNAs in sugarcane and their response to drought stress

To identify the known miRNAs, the clean reads were aligned with miRNAs in miRBase. A total of 1224 known miRNAs were identified, which belong to 89 miRNA families. There were 313, 296, 331 and 284 known miRNAs identified in the V1–11, V1–31, V2–11 and V2–31 small RNA libraries, respectively (Additional file 1: Table S2). The distribution of the miRNAs revealed that 293 miRNAs were common in all libraries. The numbers of miRNAs specific to each library was 41, 30, 65 and 30 in V1–11, V1–31, V2–11 and V2–31 respectively (Fig. 2a). The numbers of miRNAs varied in different miRNA families, with the maximum number of 34 in MIR166 followed by 23 miRNAs in the family of MIR159 and 21 miRNAs in the family of MIR396 in V2–31, when compared to other libraries (Fig. 3). The expression of the miRNAs in the different libraries varied to a great extent. miRNAs that showed higher expression levels (more than 10,000 reads) in the control and drought stressed libraries of Co 8021 were *bdi-miR166e-3p*, *osa-miR168A-5p* and *zma-miR396g-3p* respectively and in Co 06022 were *pta-miR166a*, *osa-miR168a-5p* and *sbi-miR396d* respectively. Around 14 and 11 miRNAs had more than 1000 reads in Co 8021 and Co 06022 libraries respectively, which belonged to ten miRNA families. Differentially regulated miRNAs were identified across different comparison sets to obtain up and down regulated miRNAs. Comparison of drought susceptible Co 8021 (V1–11, V1–31) revealed 14 miRNAs (log2 ratio ≥ 1 up and ≤ 1 down, $p \leq 0.05$) that were differentially expressed including seven upregulated and seven downregulated. Similar comparison in the tolerant cultivar (V2–11, V2–31) revealed 12 miRNAs that

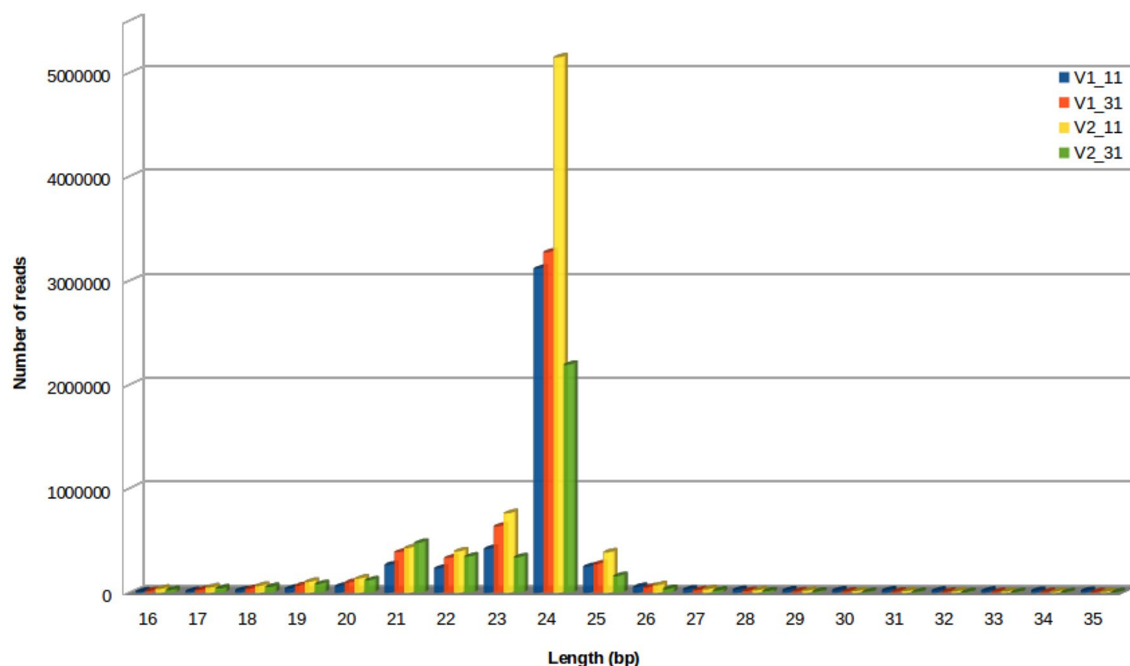

Fig. 1 Read length distribution of small RNAs in four libraries of sugarcane cultivars. The most abundant class of sRNA sequence length was 24 nt. V1_11—Co 8021 control, V1_31—Co 8021 subjected to 10 days drought, V2_11—Co 06022 control and V2_31—Co 06022 subjected to 10 days drought

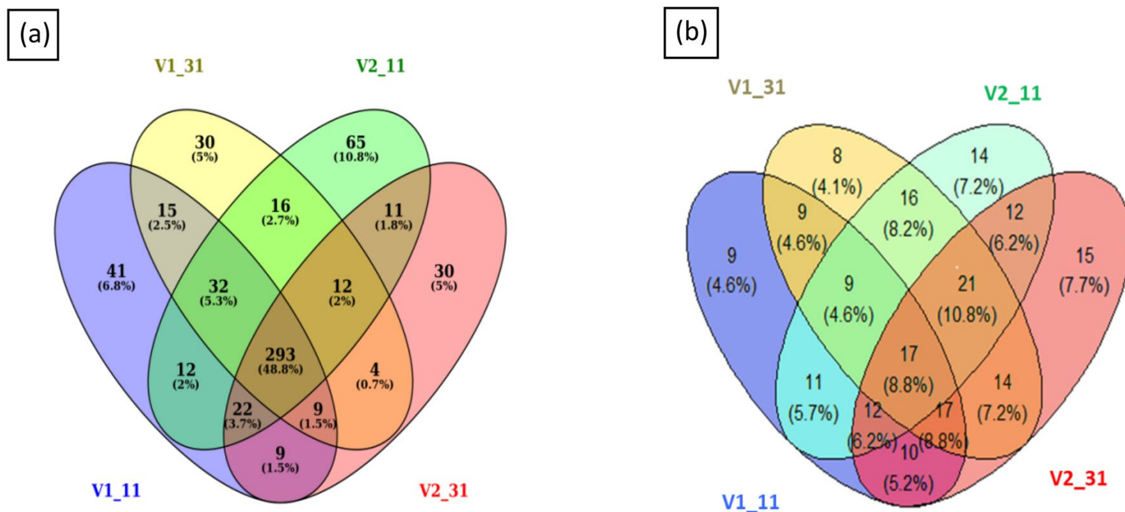

Fig. 2 Venn diagram showing number of overlapping **a** known and **b** novel miRNAs in the four libraries of sugarcane cultivars. V1_11—Co 8021 control, V1_31—Co 8021 subjected to 10 days drought, V2_11—Co 06022 control V2_31—Co 06022 subjected to 10 days drought. The numbers within the intersection are the number of miRNAs common between the libraries compared and the numbers outside the intersection are the miRNAs specific to control and water stressed libraries of the two cultivars

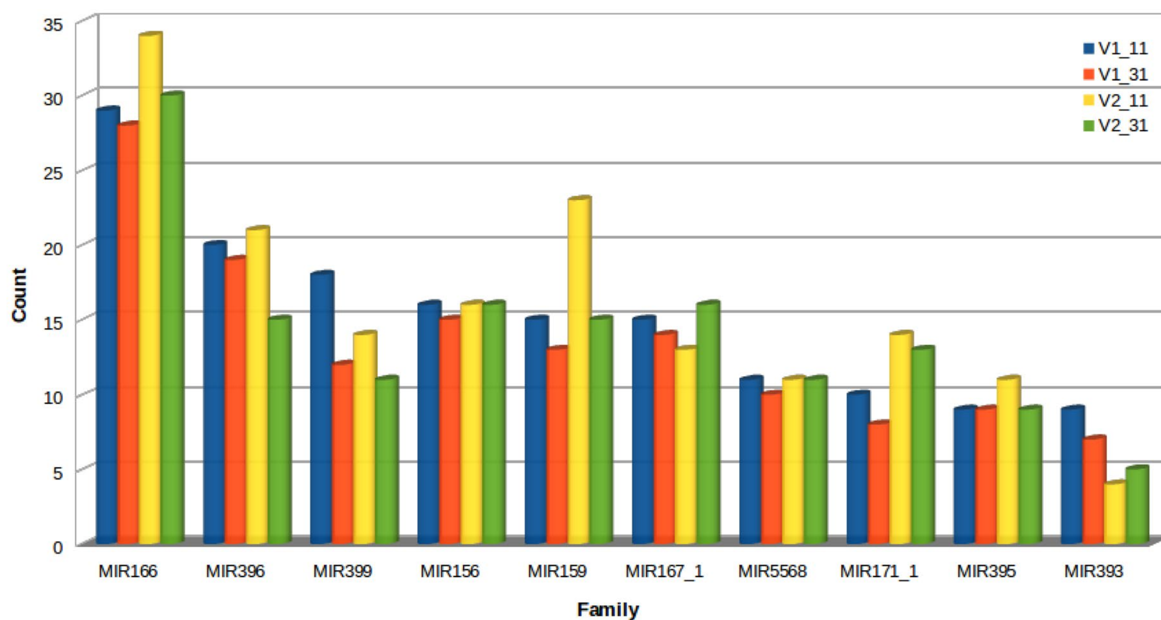

Fig. 3 Top 10 abundant miRNA families in the four libraries of sugarcane cultivars. V1_11—Co 8021 control, V1_31—Co 8021 subjected to 10 days drought, V2_11—Co 06022 control, V2_31—Co 06022 subjected to 10 days drought

were differentially expressed including seven upregulated and five downregulated. Further comparison of drought stressed libraries of both varieties identified six differentially expressed including five up and one downregulated (Additional file 2: Tables S1, S2, S3, S4). Among the different comparison sets (Fig. 4a) of the differentially regulated miRNAs no miRNA was common among different comparisons. Seven were specific to V1–11 vs V1–31 comparisons of which 5 (*sbi-miR5568g-3p*, *bna-miR156a*, *zma-miR393c-3p*, *tae-miR5384-3p* and *zma-miR393a-3p*) were up regulated and two (*hvu-miR444b*, *smo-miR408*) were downregulated. Similarly six miRNAs were found specific to V2–11 vs V2–31 of which four (*ath-miR845a*, *gma-miR166k*, *ppt-miR390c-5p*, *sbi-miR6235-5p*) were upregulated and two (*ath-miR394b-5p*, *bdi-miR166e-3p*) were downregulated. Common miRNAs that were differentially expressed in both

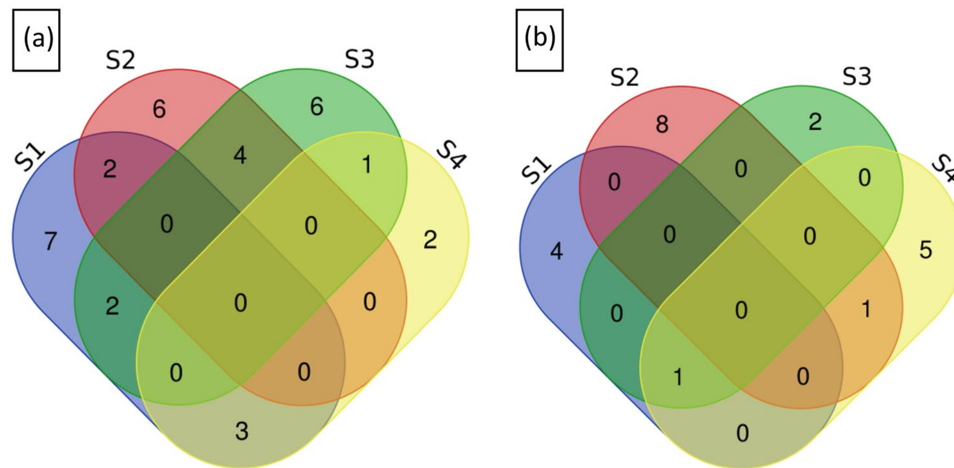

Fig. 4 Venn diagram showing number of overlapping differentially expressed mature miRNAs (p value < 0.05 and fold change > 1 or < −1) between comparisons **a** known miRNAs. **b** Novel miRNAs. The comparisons are S1—Co 8021 control vs treated (V1_11 vs V1_31), S2—Co 06022 control vs treated (V2_11 vs V2_31), S3—Co 8021 control vs Co 06022 control (V1_11 vs V2_11), S4—Co 8021 treated vs Co 06022 treated (V1–31 vs V2–31). The numbers within the intersection are the number of differentially expressed miRNAs common between the different comparisons and the numbers outside the intersection are the differentially expressed miRNAs specific to each comparison

the varieties were *sbi-miR397-3p* that was downregulated in both V1–11 vs V1–31 and V2–11 vs V2–31 and *csi-miR166a* that was downregulated in V1–11 vs V1–31 and upregulated in V2–11 vs V2–31. Two miRNAs, *vvi-miR394c* and *bdi-miR159b-3p.3* were uniquely expressed when the treated libraries (V1–31 and V2–31) of both varieties were compared.

Identification of novel miRNAs and their expression under drought stress

The putative novel miRNAs detected in the present study using MIREAP software ((http://sourceforge.net/projects/mireap/), Li et al. 2012) were evaluated for their secondary structure. The length of the novel miRNAs precursor identified ranged from 62 to 224 nt that folded to form hairpin structures with ≤ 4 symmetrical mismatches in the duplex. The free energy of the secondary structure ranged from − 20 to − 134.9 kcal/mol as calculated by RNAfold from ViennaRNA Package 2.0 (Lorenz et al. 2011). Also the mature miRNA reads mapped onto the arm region of the stemloop hairpins. The free energy of the mature miRNAs were less than − 5 kcal/mol as calculated by RNAfold. Example of secondary structure of four precursor miRNAs along with their minimum free energy values are given in Additional file 3: Fig. S1.

The number of novel miRNAs detected varied among the four libraries (Additional file 4: Tables S1, S2, S3, S4). In the drought susceptible variety Co 8021, 94 and 111 novel miRNAs were discovered in V1–11 and V1–31 libraries and in the drought tolerant variety Co 06022, 112 and 118 novel miRNAs were discovered in V2–11and V2–31 libraries. Seventeen miRNAs were common among the different libraries (Fig. 2b). Most novel miRNAs had a relatively low expression and only mireap-m0040-5p had reads over 1000 in the library V1–11. A total of 11, 9, 14 and 10 novel miRNAs were detected with reads count over 50 in the V1–11, V1–31, V2–11and V2–31 libraries respectively. Differential expressed novel miRNAs (log2 ratio ≥ 1 up and ≤ 1 down, $p \leq 0.05$) were identified in this study (Additional file 4: Tables S5, S6, S7, S8). In the drought-susceptible variety Co 8021, five novel miRNAs were found to be differentially expressed between V1–11 and V1–31, of them, four were upregulated (*mireap-m0095-5p*, *mireap-m0049-5p*, *mireap-m0099-3p*, *mireap-m0024-3p*) and one was downregulated (*mireap-m0045-5p*). In the drought-tolerant variety Co 06022, nine novel miRNAs were found to be differentially expressed between V2–11 and V2–31, of them, three (*mireap-m0017-3p*, *mireap-m0087-5p*, *mireap-m0082-3p*) were upregulated while six (*mireap-m0021-3p*, *mireap-m0016-5p*, *mireap-m0086-5p*, *mireap-m0049-5p*, *mireap-m0104-3p*, *mireap-m0061-3p*) were downregulated. Comparisons of the stressed libraries of the two varieties V1–31 vs V2–31, revealed five downregulated (*mireap-m0043-3p*, *mireap-m0103-3p*, *mireap-m0020-5p*, *mireap-m0062-3p*, *mireap-m0073-3p*) and two upregulated novel miRNAs (*mireap-m0011-3p*, *mireap-m0067-5p*). There were no common miRNAs that was differentially expressed in the two varieties (Fig. 4b).

Functional analysis of target genes of the drought induced conserved miRNAs in sugarcane

A total of 10,312 target genes were found to be regulated by the 145 known miRNAs expressed in the susceptible variety and 11,357 target genes were regulated by 143 known miRNAs expressed in the tolerant variety (Additional file 5: Tables S1, S2). The predicted target genes mainly encoded transcription factors, proteins, phosphatase and kinases involved in signal transduction pathways, integral component of membrane and inorganic ion transport metabolism, enzymes involved in carbohydrate transport and metabolism, drought-stress related proteins involved in defense mechanisms, and cell wall/membrane biogenesis. For instance, the target of *sbi-miR397-3p*, *tae-miR5384-3p*, *hvu-miR444b* and *sbi-miR5568g-3p* were genes encoding protein phosphatase type 2-C, calmodulin-dependent protein kinase phosphatase, calcium-dependent protein kinase, mitogen-activated protein kinase, receptor protein kinase CRINKLY4, Histidine kinase 3, and serine/threonine-protein kinase which are involved in signal transduction pathways. Some targets encode drought responsive transcription factors such as MYB transcription factor, AP2-EREBP, Dof-type zinc finger protein, DRE-binding protein, GNAT, GATA-4/5/6, ALFIN-like transcription factor, GRF transcription factor, MADS-box transcription factor, heat shock factor protein 7 predicted by miRNAs *tae-miR5384-3p*, *hvu-miR444b*, *smo-miR408* and *sbi-miR396d*. Some of the miRNAs like *csi-miR166a*, *sbi-miR397-3p*, *osa-miR393b-3p*, *zma-miR393c-3p* and *cme-miR156j* have targets that are involved in integral component of membrane modification. The targets of *tae-miR5384-3p* encode several genes like phenylalanine ammonia-lyase, amine oxidase and laccase gene which are involved in secondary metabolites biosynthesis, transport, catabolism and genes encoding calcium-transporting ATPase, catalase, zinc transporter 4, potassium channel which function in inorganic ion transport and metabolism.

The target genes of the conserved miRNAs were further subjected to GO and KEGG pathway analysis. Target genes of known miRNAs of V1–31 were classified into 1092, 264 and 676 GO terms and targets of V2–31 were classified into 1105, 264 and 688 GO terms for biological process, cellular component and molecular function ontology, respectively (Additional file 6: Tables S1, S2, S3). GO analysis showed that the target genes of many differentially-expressed known miRNAs enriched in the GO term cellular component, were related to integral component of membrane and nucleus. The GO term biological process was enriched with targets for transcription, DNA integration and carbohydrate metabolic processes and the GO term molecular function was enriched with targets for ATP binding, DNA binding, zinc ion binding, protein kinase activity and transcription factor activity (Fig. 5a). To identify the pathways in which the target genes were involved, they were mapped on KEGG pathway database. The top enriched pathways of both genotypes were studied to identify genes involved in major metabolic activities (Table 1). "General function prediction only" was the most significant category observed in both tolerant and susceptible genotypes followed by signal transduction mechanisms and posttranslational modification, protein turnover and chaperones (Fig. 6a). Pathways such as secondary metabolite biosynthesis, transport and catabolism, inorganic ion transport and metabolism, defence mechanisms and cell wall/membrane/envelope biogenesis were other pathways that are involved in sugarcane stress tolerance.

Functional analysis of target genes of the drought induced novel miRNAs in sugarcane

A total of 5308 targets were identified by 50 novel miRNAs expressed upon drought stress in susceptible variety (V1–31) and 5979 targets were identified for 49 novel miRNAs in the tolerant variety (V2–31) (Additional file 7: Tables S1, S2). The novel target genes mainly encoded drought-stress related genes involved in plant defense mechanisms. For instance, the novel miRNAs *mireap-m0020-5p*, *mireap-m0104-3p*, *mireap-m0086-5p*, *mireap-m0043-3p*, *mireap-m0049-5p*, *mireap-m0017-3p* targeted genes encoding, ALDH (aldehyde dehydrogenase), calmodulin binding protein, carboxypeptidase, cytochrome P450, disease resistance protein RPM1, peroxidase, sucrose-phosphate synthase, heat shock 70 kDa protein, HSP20 family protein and xyloglucan endotransglucosylase/hydrolase, respectively. Some targets of novel miRNAs, were drought responsive transcription factors like MYB, TGA transcription factor, putative bZIP transcription factor super family protein encoded by *mireap-m0043-3p*, *mireap-m0020-5p* and *mireap-m0067-5p* respectively.

The target genes of the novel miRNAs were further classified into GO terms (Fig. 5b). Novel miRNAs of V1–31 identified 790, 179 and 475 GO terms and of V2–31 identified 853, 201 and 520 GO terms for the biological process, cellular component and molecular function ontology, respectively (Additional file 8: Tables S1, S2, S3). In biological process, more number of targets were involved in transcription, regulation of transcription, DNA integration and carbohydrate metabolic process. However, cellular component had more targets that were related to stress response such as integral component of membrane and nucleus. Furthermore, molecular function had many novel miRNAs related to ATP binding, DNA binding, zinc ion binding, protein kinase activity and transcription factor activity. The targets were enriched in stress response pathways such as plant pathogen interaction, plant hormone signal transduction, MAPK signaling pathway, phenylpropanoid biosynthesis etc. (Fig. 6b). Other pathways such as

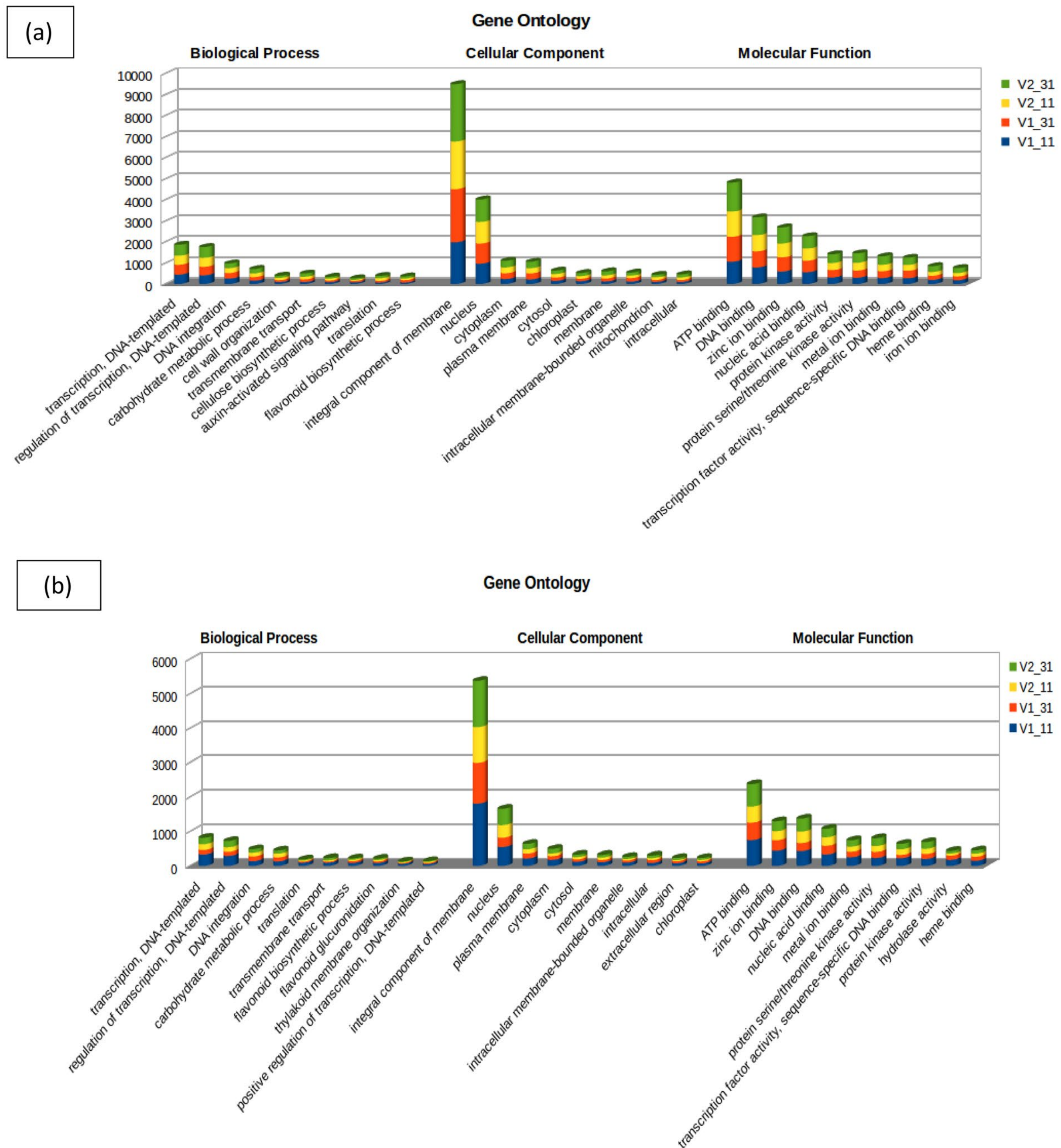

Fig. 5 Gene ontology classification of predicted targets of **a** known and **b** novel miRNAs. The results are summarized in three main categories: biological process, cellular component and molecular function. V1_11—Co 8021 control, V1_31—Co 8021 subjected to 10 days drought, V2_11—Co 06022 control V2_31—Co 06022 subjected to 10 days drought

starch and sucrose metabolism, glycan metabolism; pectin degradation, glutathione metabolism, carotenoid biosynthesis, fatty acid biosynthesis, glycerolipid metabolism, zeatin biosynthesis, biosynthesis of unsaturated fatty acids, glyoxylate and dicarboxylate metabolism, cyanoamino acid metabolism and brassinosteroid biosynthesis were also identified.

Network analysis of drought responsive miRNA and their targets

The network analysis of drought responsive miRNA and their targets upon stress in sugarcane was investigated. Drought tolerant variety (V2–31) had 144 conserved miRNAs belonging to 32 families encoding 28 genes and 89

Table 1 Predicted target genes of miRNAs involved in top enriched pathways of drought tolerance in sugarcane

S. No.	Pathway	miRNAs	Target genes	Gene ID
1	Plant-pathogen interaction	Smo-miR408 Mireap-m0077-3p Mireap-m0073-3p Mireap-m0049-5p Mireap-m0016-5p Mireap-m0016-5p Mireap-m0073-3p	Serine/threonine protein kinase transcription factor MYB CERK1; chitin elicitor receptor kinase 1 RPM1; disease resistance protein RPM1 CPK; calcium-dependent protein kinase RBOH; respiratory burst oxidase Carboxypeptidase	31_c37860_g1_i2 41_c56809_g2_i6 31_c35993_g1_i3 51_c41883_g1_i4 51_c45744_g2_i2 41_c55871_g2_i1 41_c57068_g1_i4
2	Plant hormone signal transduction	Tae-miR5384-3p Smo-miR408 Tae-miR5384-3p Tae-miR5384-3p Tae-miR5384-3p Tae-miR5384-3p Tae-miR5384-3p Tae-miR5384-3p Tae-miR5384-3p Sbi-miR396d Sbi-miR397-3p Tae-miR5384-3p Mireap-m0020-5p Mireap-m0043-3p Mireap-m0020-5p Mireap-m0067-5p Mireap-m0049-5p Mireap-m0020-5p Mireap-m0049-5p Mireap-m0049-5p Mireap-m0049-5p	Mitogen-activated protein kinase Phosphoribulokinase Pyruvate dehydrogenase 2C-type protein phosphatase protein (uncharacterized protein) ACC synthase 2 Rhomboid-like protein Putative CRINKLY4-like receptor protein kinase family protein (uncharacterized protein) Protein kinase domain containing protein Calmodulin (uncharacterized protein) Histidine-containing phosphotransfer protein 4 ATP binding protein (uncharacterized protein) 1-Aminocyclopropane-1-carboxylate synthase Jasmonate ZIM domain-containing protein GH3; auxin responsive GH3 gene family TGA; transcription factor TGA putative bZIP transcription factor superfamily protein ARR-B; two-component response regulator ARR-B family JAZ; jasmonate ZIM domain-containing protein Putative snRK/SAPK family protein kinase SNRK2; serine/threonine-protein kinase SRK2 Serine/threonine-protein kinase SAPK6	41_c61679_g1_i3 51_c35422_g1_i2 21_c47811_g1_i4 41_c61886_g1_i2 51_c31538_g1_i1 21_c46681_g2_i1 51_c40570_g1_i2 21_c44921_g3_i1 11_c45593_g1_i2 21_c33465_g1_i2 51_c34821_g1_i2 11_c50950_g2_i1 51_c31421_g1_i1 11_c35859_g1_i5 11_c40163_g1_i1 31_c22010_g1_i2 41_c57376_g1_i1 21_c39304_g1_i2 51_c47267_g3_i5 21_c46764_g1_i2 51_c47267_g3_i4
3	MAPK signaling pathway—plant	Tae-miR5384-3p Mireap-m0075-5p Mireap-m0011-3p Mireap-m0020-5p Mireap-m0067-5p Mireap-m0043-3p Mireap-m0020-5p Mireap-m0016-5p	Mitogen-activated protein kinase WRKY transcription factor 33 ERECTA-like 2 Chitinase 1 ACC synthase 1 PREDICTED: abscisic acid receptor PYL4-like RBOH; respiratory burst oxidase peroxidase activity	41_c89678_g1_i1 41_c34210_g1_i1 21_c51388_g2_i1 41_c49938_g1_i3 31_c40137_g1_i3 31_c25419_g2_i1 41_c47344_g2_i1 41_c55871_g2_i1
4	Phenylpropanoid biosynthesis	Tae-miR5384-3p Tae-miR5384-3p Mireap-m0077-3p Mireap-m0043-3p Mireap-m0049-5p	Peroxidase Glycosyltransferase Peroxidase Peroxidase Laccase	41_c55109_g2_i1 11_c45559_g1_i2 21_c52014_g1_i5 11_c33778_g2_i6 11_c35815_g1_i1
5	General function prediction only	Sbi-miR397-3p Sbi-miR397-3p Osa-miR393b-3p Osa-miR393b-3p Sbi-miR5564c-5p Tae-miR5384-3p Tae-miR5384-3p	Putative polyprotein Caffeic acid 3-*O*-methyltransferase (EC 2.1.1.6) (caffeic acid-*O*-methyltransferase) Putative RING zinc finger domain superfamily protein (uncharacterized protein) *S*-acyltransferase (EC 2.3.1.225) (palmitoyl-transferase) Putative gag-pol polyprotein NAD dependent epimerase/dehydratase family protein (uncharacterized protein) Carboxypeptidase	41_c50099_g1_i1 31_c31301_g1_i1 51_c28098_g1_i1 41_c62565_g1_i2 21_c53598_g1_i2 41_c59268_g5_i4 41_c57369_g4_i1

Table 1 (continued)

S. No.	Pathway	miRNAs	Target genes	Gene ID
6	Posttranslational modification, protein turnover, chaperones	Sbi-miR397-3p Sbi-miR397-3p Tae-miR5384-3p Tae-miR5384-3p Mireap-m0020-5p Mireap-m0043-3p	Heat shock protein MYB-like transcription factor DIVARICATA Carboxypeptidase (EC 3.4.16.-) Thioredoxin M-type HSP20; HSP20 family protein HSP90B; heat shock protein 90 kDa beta	51_c29439_g1_i4 21_c49879_g1_i2 41_c62042_g1_i4 51_c25511_g1_i1 21_c36141_g1_i1 41_c54140_g1_i3
7	Carbohydrate transport and metabolism	Tae-miR5384-3p Sbi-miR397-3p Sbi-miR397-3p Tae-miR5384-3p Mireap-m0073-3p	Trehalose-6-phosphate synthase component TPS1 and related subunits Glycosyltransferase (EC 2.4.1.-) Fructose-6-phosphate 2-kinase/fructose-2,6-biphosphatase Alpha-galactosidase (EC 3.2.1.22) (melibiase) GAPDH; glyceraldehyde 3-phosphate dehydrogenase [EC:1.2.1.12]	21_c4416_g1_i1 11_c18089_g1_i1 11_c53370_g1_i1 31_c37634_g2_i2 21_c47885_g2_i1
8	Secondary metabolite biosynthesis transport and catabolism	Sbi-miR397-3p Tae-miR5384-3p Tae-miR5384-3p Mireap-m0052-3p	Cytochrome P450 Acc oxidase *S*-glutathione dehydrogenase/class III alcohol dehydrogenase Cytochrome P450	31_c38488_g1_i2 11_c49592_g2_i2 11_c48315_g1_i1 51_c36473_g1_i1
9	Glutathione metabolism	Tae-miR5384-3p Csi-miR166a Tae-miR5384-3p Mireap-m0086-5p	Multicopper oxidases Glutathione S transferase Glutathione S transferase Glutathione *S*-transferase	21_c49637_g2_i3 51_c29924_g1_i2 41_c51347_g1_i1 51_c34512_g1_i1

Pathway: pathway in which protein is involved; miRNAs: differentially expressed miRNAs with log2 ratio ≥ 1 up and ≤ 1 down, $p \leq 0.05$; target genes: description of genes obtained from GenBank by *BLAST* search; gene ID: target mRNA ID (sugarcane transcript list available in NCBI BioProject ID—PRJNA590595) to which miRNA binds

novel miRNAs belonging to 14 families encoding 19 genes for stress tolerance. Drought susceptible variety (V1–31) had 128 conserved miRNAs belonging to 34 families encoding 27 genes and 89 novel miRNAs belonging to 17 families encoding 20 genes involved in stress tolerance. The genes were mostly encoding stress responsive transcription factors, plant hormone signal transduction and phenylpropanoid biosynthesis. Combined network analysis of most abundant miRNAs revealed that 9 novel miRNAs and 19 known miRNAs interacted with 36 genes in V1–31, and in V2–31, 10 novel miRNAs and 20 known miRNAs interacted with 32 genes (Fig. 7a, b). It was found that different miRNAs targeted different number of stress-responsive genes. For instance, the conserved miRNA *osa-miR396f-5p* targeted only one gene (heat shock protein 90), while *bdi-miR5054* targeted five genes (peroxidase, phospholipase D1/2, poly(A)-specific ribonuclease, mitochondrial phosphate carrier protein 3 and cleavage and polyadenylation specificity factor subunit 4). Similarly, the novel *mireap-m0100-5p* targeted only one gene (thiazole biosynthetic enzyme), *mireap-m0087-3p* targeted two genes (L-ascorbate peroxidase and WRKY transcription factor 26) but *mireap-m0077-3p* targeted seven genes (aconitatehydratase, catalase, peroxidase, WRKY, heat shock 70 kDa protein 5, lipoxygenase and malonate-semialdehyde dehydrogenase). Among the different targets, peroxidase gene was targeted by most of the known and novel miRNAs.

Expression analysis of miRNAs and their targets by qRT-PCR

qRT-PCR was used to verify the expression levels of miRNAs obtained from sRNA sequencing results. Eight known miRNAs (*sbi-miR396d*, *csi-miR166a*, *cme-miR156j*, *osa-miR393b-3p*, *sbi-miR397-3p*, *sly-miR169e-5p*, *osa-miR444c.1* and *mtr-miR166c*) and seven novel miRNAs (*mireap-m0052-3p*, *mireap-m0061-3p*, *mireap-m0066-3p*, *mireap-m0026-3p*, *mireap-m0034-5p*, *mireap-m0100-5p* and *mireap-m0077-3p*) were selected for the validation (Additional file 9: Table S1). Among the 15 miRNAs, 11 miRNAs were found to be consistent with the expression pattern obtained through small RNA deep sequencing data (Fig. 8). miRNAs can regulate post-transcriptional gene expression by targeting mRNAs for degradation. To explore the potential extent of miRNA-directed regulation of mRNA levels, qRT-PCR expression analysis of the targets of differentially expressed miRNAs was done (Additional file 9: Table S2). The results of the qRT-PCR analysis revealed that all miRNAs negatively regulate their target genes except *hvu-miR444* which positively regulated defensin gene in both V1 and V2. Similarly miRNAs,

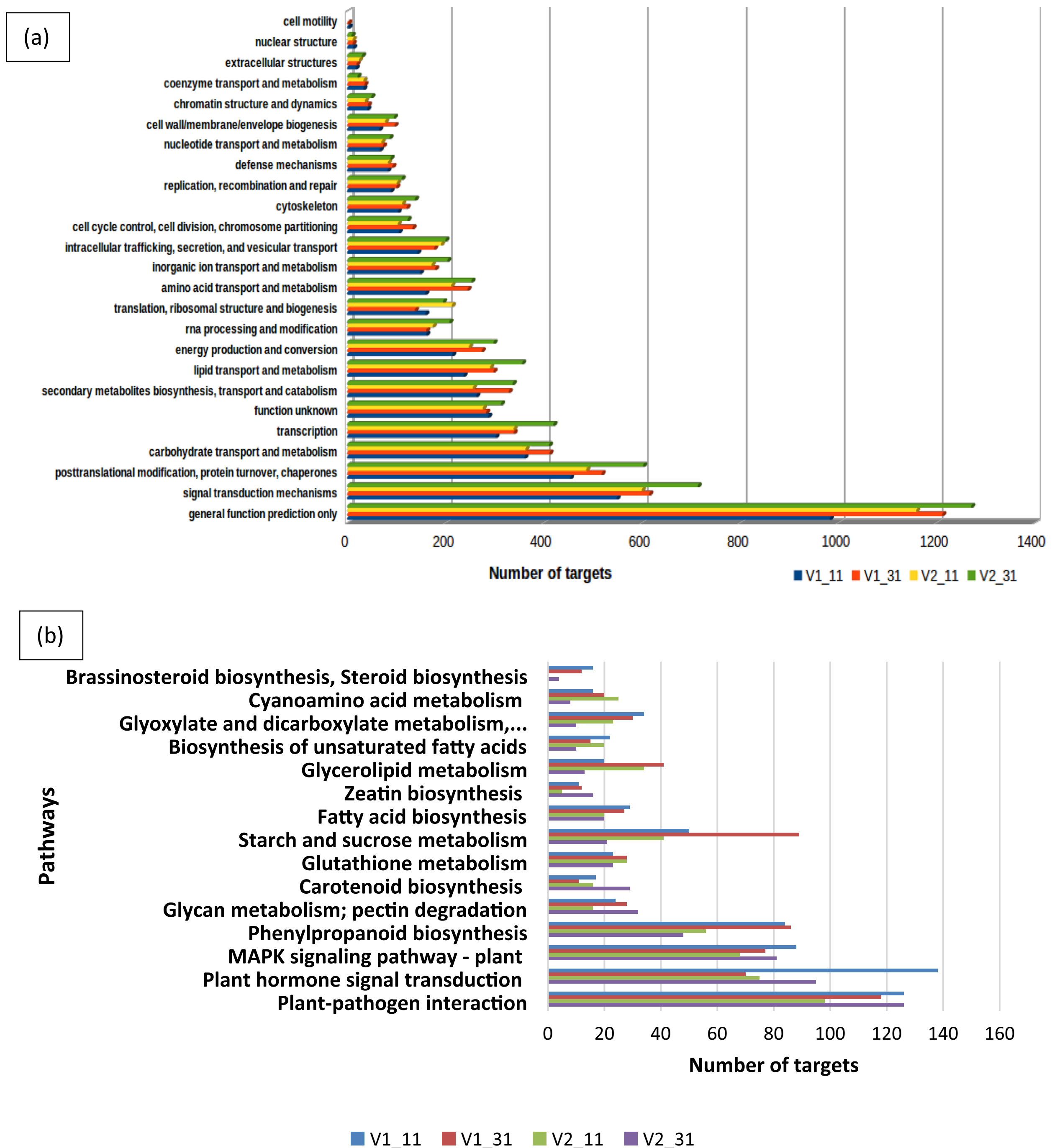

Fig. 6 KEGG enrichment analysis of predicted target genes of **a** known and **b** novel miRNAs. The *x*-axis shows the number of unigenes involved in each pathway and the y axis denotes the different metabolic pathways. V1_11—Co 8021 control, V1_31—Co 8021 subjected to 10 days drought, V2_11—Co 06022 control V2_31—Co 06022 subjected to 10 days drought

gma-miR166k and *cpa-miR167d* revealed positive regulation of peroxidase and WRKY transcription factor in V1 and V2 respectively (Fig. 9). The target gene expression of putative cellulose synthase, disease resistant protein RGA2, AP2-EREBP transcription factor, xyloglucan endotransglucosylase hydrolase, heat shock protein, zinc finger protein and abscisic acid showed consistent up and down regulation by the concomitant expression of miRNA in both V1 and V2 showing that these are highly regulated by the miRNA.

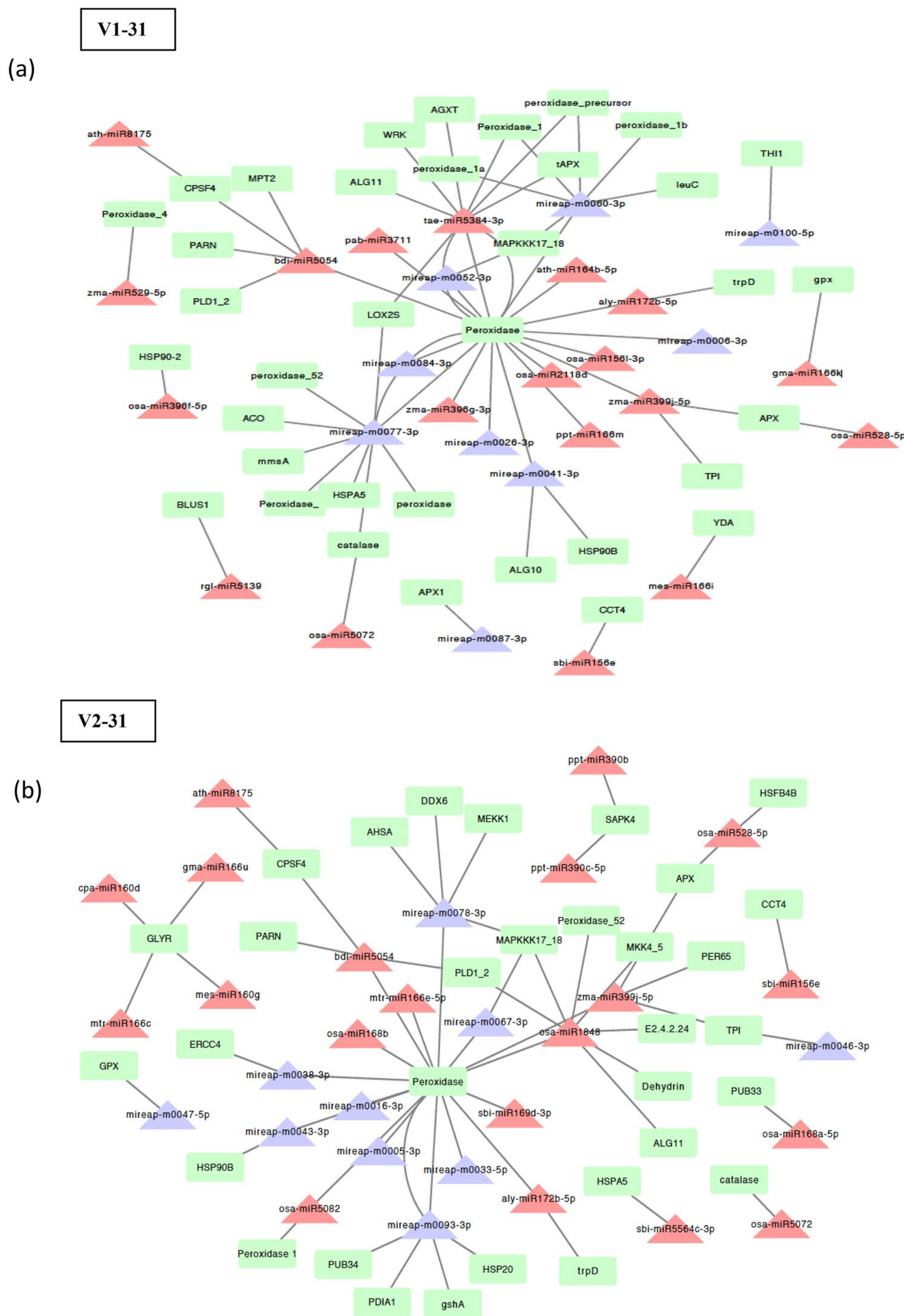

Fig. 7 Network analysis of abundant conserved (in red triangle) and novel miRNAs (in blue triangle) and predicted target genes (in green box) of **a** drought susceptible Co 8021 (V1–31) and **b** drought tolerant Co 06022 (V2–31)

Discussion

During drought stress, plant activates several defense mechanisms to withstand environmental challenge, which includes physiological, biochemical and molecular changes (Valliyodan and Nguyen 2006). microRNAs play an important role in the regulation of plant responses to numerous environmental stimuli (Bartel 2004). Several studies proved that "omic" technologies have greatly enhanced our knowledge to interpret the mechanisms of stress tolerance in plants (Zhuang et al. 2014). High throughput deep sequencing approaches have been used to discover small RNAs and is a most effective method for miRNA detection in plants (Fahlgren et al. 2007). In this study, we performed high throughput deep sequencing for drought tolerant and susceptible sugarcane varieties to study the regulation of gene expression by miRNAs during drought stress.

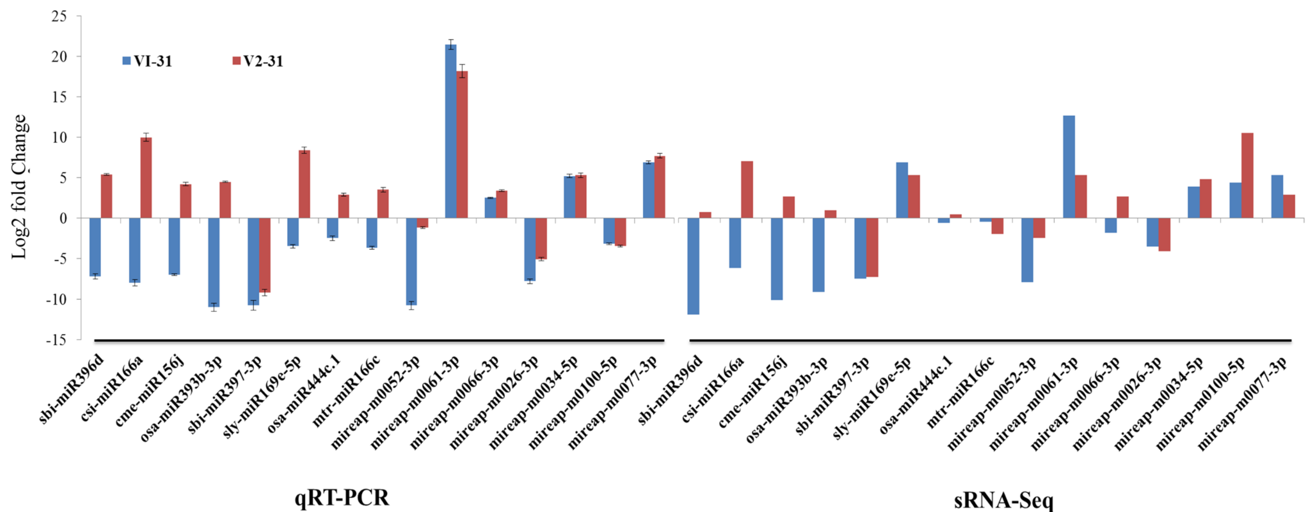

Fig. 8 Expression profiling of 15 randomly selected miRNAs by qRT-PCR and comparison with sRNA-Seq data of 10 days drought stress in drought susceptible Co 8021 (V1–31) and drought tolerant Co 06022 (V2–31). For qRT-PCR each bar represents the average of three replicates and error bars indicate SE

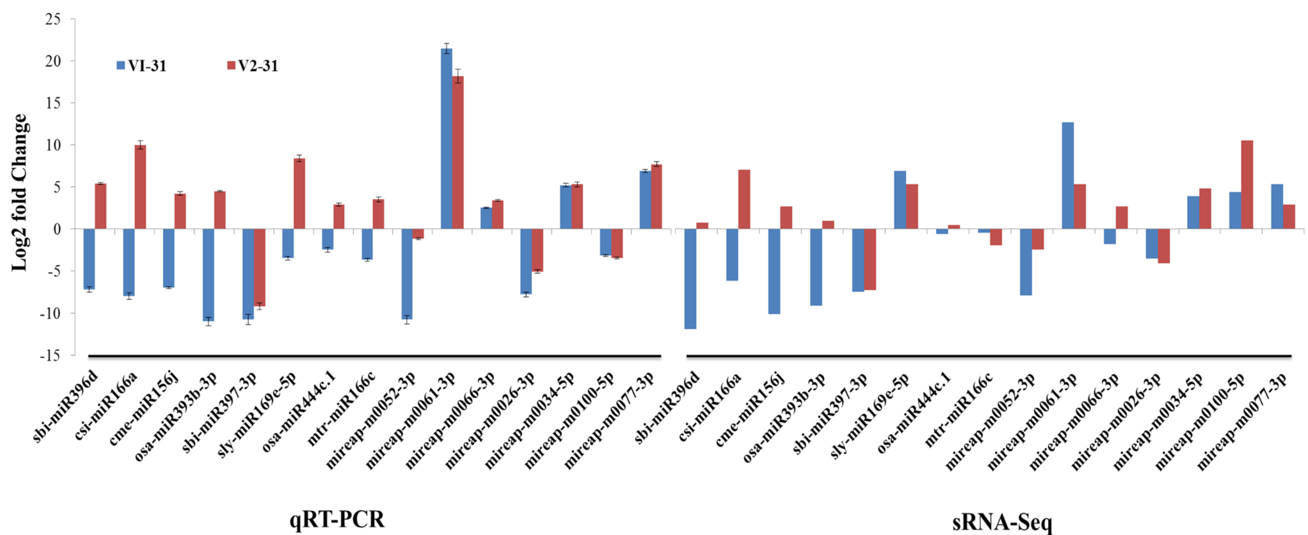

Fig. 9 Expression profiling of miRNAs and predicted target genes of drought susceptible Co 8021 (V1) and drought tolerant Co 06022 (V2) sugarcane cultivars subjected to 10 days drought stress. The target genes are cellulose synthase, *RGA2* disease resistance protein RGA2, *AP2-EREBP* AP2-EREBP transcription factor, peroxidase, *XET* xyloglucan endotransglucosylase hydrolase, *HSP 70* heat shock protein 70-kDa, *ZFP* zinc finger domain superfamily protein, defensin, *WRKY* WRKY transcription factor and *ABA* abscisic acid. In qRT-PCR each bar represents the average of three replicates and error bars indicate SE

In the present study, the length of small RNAs was mainly distributed between 21 and 25 nt, which account for an average of 88% of the small RNAs. Among all nucleotides, 24 nt small RNAs had the highest number of reads in all of the four libraries. Ferreira et al. (2012) reported that the majority of the reads were 21–24 nt in length, with 21 nt being the most redundant species, followed by 24 nt in the drought stressed and irrigated libraries of sugarcane cultivars. Findings of Yang et al. (2017) indicate that 21 nt and 24 nt small RNAs were the most abundant under low temperature stress in sugarcane genotypes. Su et al. (2017) reported sRNA sequence length in four libraries within the range of 20–24 nt, under *Sporisorium scitamineum* treatment in sugarcane genotypes.

A total of 313, 296, 331 and 284 known miRNAs were identified in the V1–11, V1–31, V2–11 and V2–31 small RNA libraries, respectively. Comparison of V1–11 and V1–31 revealed 14 differentially expressed miRNAs (log2

ratio ≥ 1 up and ≤ 1 down, $p \leq 0.05$) including seven upregulated and seven downregulated. Furthermore, comparison of drought stressed libraries of both varieties (V1–31 and V2–31) revealed six known differentially expressed miRNAs including five upregulated and one downregulated. Ferreira et al. (2012) detected a total of 21 miRNA candidates belonging to 18 miRNAs families in sugarcane drought stressed libraries obtained at different time points of stress. Most of the miRNAs that were differentially expressed varied between the two varieties showing varietal differences in drought responses. Differences in expression pattern of miRNAs depending on the cultivar, growth conditions, duration of stress has been previously observed in sugarcane (Gentile et al. 2015). Among the differentially expressed known miRNAs, only two miRNAs, *sbi-miR397-3p* and *csi-miR166a* were common in both varieties, where *sbi-miR397* was downregulated in both varieties and *csi-miR166a* was upregulated in the tolerant cultivar and downregulated in the susceptible cultivar. The role of *miR397-3p* in drought stress has been commonly observed in several crops (Ferreira et al. 2012; Chen et al. 2020; Hamza et al. 2016). Differential expression of *miR397-3p* was observed in all stages of drought stress in switch grass and sorghum (Chen et al. 2020; Hamza et al. 2016) whereas in sugarcane *mir397* was upregulated at 2 days stress in both tolerant and susceptible cultivars and when drought progressed to 4 days it was downregulated in both types. It is also notable that in our study *sbi-miR397-3p* targeted around 407 genes including several drought responsive genes like molecular chaperone HSP90 family, cytochrome P450, Myb superfamily, Ca2+/calmodulin-dependent protein kinase, Zn-finger protein among others. Similarly differential regulation of *csi-miR166a* was observed in tea plants subjected to drought stress (Guo et al. 2017). *miR166* has been identified as a drought responsive miRNA that is conserved among several plant species (Chen et al. 2017; Aravind et al. 2017; Akdogan et al. 2016; Zhu et al. 2011) and plays an important role in post transcriptional changes related to root architecture during drought stress. Comparison of drought stressed libraries of both varieties revealed two miRNAs, *vvi-miR394c* and *bdi-miR159b-3p.3* that were not shared in other comparisons. Drought stress studies in desert plant *Ammopiptanthaus mongolicus* showed that miR159 family had the highest members of miRNA that were expressed during drought stress (Gao et al. 2016). Oxidative stress responses in crops like rice, *Brachypodium distachyon* have also reported upregulation of *bdi-miR159a/b* during stress (Zhou et al. 2010; Bertolini et al. 2013) indicating involvement of *bdi-miR159a/b* in abiotic stress responses. Similarly miR394 family is conserved among plants and is known to regulate plant growth and biotic and abiotic stress responses. Predicted targets of *vvi-miR394c* include dehydration responsive proteins, MYB super family proteins, CBS domain containing protein, glycosyltransferase, which play important roles in drought and salt responsive pathways (Yang et al. 2017; Dong et al. 2020; Singh et al. 2012; Pagliarani et al. 2017). High levels of down regulation of *sbi-miR396d*, *cme-miR156j*, *osa-miR393b-3p* (log2 fold change − 11, − 10, − 9, respectively) in Co 8021 and *osa-miR171h*, *osa-miR444b.2*, (log2 fold change − 9.5, − 9.5, respectively) in Co 06022 was observed. MicroRNA *sbi-miR396d* targets growth regulating factors coordinating cell division and differentiation that affects leaf growth and orientation, conferred drought tolerance in *Arabidopsis* upon induction (Jones-Rhoades and Bartel 2004). Monocot abundant *miRNA156g-h* targeting squamosa-binding protein which positively regulates stem and internode growth was identified to be downregulated in sensitive sorghum cultivars during drought stress (Katiyar et al. 2015). Similarly upregulation of *osa-miR393b-3p* and down regulation of *osa-miR171* has shown to improve drought tolerance in rice (Zhang et al. 2017; Cheah et al. 2015). MicroRNAs *gma-miR166k*, *ppt-miR390c-5p* were highly upregulated in Co 06022 (log2 fold change 10, 9 respectively). Enhanced expression of *gma-miR166k* which targets genes for root growth and elongation and *ppt-miR390c* targeting stress responsive leucine-rich repeat receptor like kinase imparts tolerance to several biotic and abiotic stresses (Guirao et al. 2018; Huang et al. 2018; Li et al. 2017; Ding et al. 2016).

In present study, 94 and 111 novel miRNAs were detected in the library of the drought-susceptible variety V1–11 and V1–31 respectively and in the drought-tolerant variety, 112 and 118 novel miRNAs were detected in V2–11 and V2–31 libraries respectively. Most novel miRNAs had a relatively low expression and only *mireap-m0040-5p* had reads over 1000 in the library V1–11. In the drought-susceptible variety Co 8021, five novel miRNAs were found to be differentially expressed (log2 ratio ≥ 1 up and ≤ 1 down, $p \leq 0.05$) and in drought-tolerant variety Co 06022, nine novel miRNAs were found to be differentially expressed. All novel miRNAs expressed were cultivar specific. More number of novel miRNAs were upregulated in Co 8021 whereas in Co 06022 many were downregulated. Novel miRNAs *mireap-m0016* and *mireap-m0020* downregulated in Co 06022 was also observed to be differentially expressed in flag leaf and anthesis spikelet and mature root of drought tolerant Nagina 22 rice (Mutum et al. 2016). Upregulation of *mireap-m0017* and *mireap-m0082* was observed in Co 06022 and similar observations were made in wild rice Dongxiang during drought stress (Fantao et al. 2018). Overall novel miRNAs implicated in drought stress in this study have also been found to play important roles in crops like tomato, barley, rice etc. (Liu et al. 2017; Smoczynska et al. 2020; Mutum et al. 2016).

The predicted target genes of known and novel miRNAs mainly encode transcription factors, proteins, phosphatase

and kinases involved in signal transduction pathways, integral component of membrane and inorganic ion transport metabolism, enzymes involved in carbohydrate transport and metabolism, drought-stress related proteins involved in defense mechanisms and cell wall/membrane biogenesis. Hua et al. (2019) reported that a number of target genes regulated by differentially expressed miRNAs for drought stress in wheat, included signal transducer, response to stress and antioxidant pathways. Furthermore, Li et al. (2017) reported that most of the drought-responsive miRNAs were involved in development or disease resistance, substance synthesis and transportation indicating these miRNAs play important roles during drought stress in alfalfa leaves.

According to previous reports, GO and KEGG analysis indicated that miRNAs were intricate in stress-responsive biological pathways. In our study, *bdi-miR159*, *cpa-miR167d* and *sbi-miR397-3p* targeting auxin responsive factors were regulated in both V1–31 and V2–31 varieties under drought stress. Studies in *Ipomea* indicated that miR160 and miR167 were involved in auxin response by targeting of auxin response factors (ARF) (Glazinska et al. 2013). The up-regulation of miR167 was identified in wheat genotypes, suggesting that miR167 and the trans-acting short-interfering RNA-auxin response factor had an important role in the auxin signaling pathway and developmental response to cold stress (Tang et al. 2012). Among the miRNA targets, transcription factors such as the ARF, TIR, MYB and NF-Y were identified to be involved in the response of sugarcane to abiotic stresses and these factors control a series of downstream genes by binding to cis-elements in promoter regions (Singh et al. 2002). In our study, *tae-miR5384-3p*, *hvu-miR444b*, *smo-miR408* and *sbi-miR396d* targeted drought responsive transcription factors like heat shock factor protein 7, ScMYB32 protein, GNAT, NAC, GRAS, WRKY, DRE-binding protein 3, Dof-type zinc finger protein, AP2-EREBP and BZIP respectively. Among them HSP7, DREB3, AP2-EREBP and NAC were upregulated in both varieties and ScMYB32, GNAT, GRAS, WRKY and BZIP were downregulated in both sugarcane genotypes in response to drought stress. According to Silva et al. (2019) some miRNAs, such as miR159, miR160, miR319, and miR396 were differentially regulated in sugarcane seedlings upon aluminum stress and their targets were transcription factors associated with seed germination, embryo development, cold and drought responses.

Several phosphatases (*sspmiR394* and *ssp-miR397*), kinases (*ssp-miR399-seq 1* and *ssp-miR528*), and oxidases (*sspmiR1432)* have been involved in signal transduction pathways and few others like glyceraldehyde-3-phosphate dehydrogenase (*ssp-miR394*) was involved in carbohydrate metabolism during drought stress in sugarcane genotypes (Ferreira et al. 2012). In present study, the targets of *sbi-miR397-3p*, *hvu-miR444b* and *tae-miR5384-3p*, *sbi-miR5568g-3p* which were down and upregulated respectively in Co 8021 were genes encoding protein phosphatase type 2-C, calmodulin-dependent protein kinase phosphatase, calcium-dependent protein kinase, mitogen-activated protein kinase and receptor protein kinase CRINKLY4, respectively, that are involved in signal transduction pathways. Target genes of *tae-miR5384-3p*, *sbi-miR397-3p* and *hvu-miR444b* which are all downregulated encode glyceraldehyde 3-phosphate dehydrogenase, invertases and alpha-galactosidase respectively, which are involved in carbohydrate transport and metabolism.

The target genes of novel miRNAs mainly encode drought-stress related genes involved in plant defense mechanisms such as *mireap-m0077-3p* (upregulated), *mireap-m0086-5p* (downregulated), *mireap-m0073-3p* (downregulated), *mireap-m0061-3p* (upregulated), *mireap-m0049-5p* (upregulated), *mireap-m0043-3p* (downregulated), *mireap-m0067-5p* (upregulated), *mireap-m0020-5p* (downregulated) and *mireap-m0104-3p* (downregulated) targeted genes encoding ABA responsive element binding factor (Ferreira et al. 2012), calmodulin binding protein (Liu et al. 2017), carboxypeptidase (Selvi et al. 2020), cytochrome P450 (Li et al. 2017), disease resistance protein RPM1 (Hua et al. 2019), peroxidase (Wei et al. 2009), glutathione S-transferase 4 (Devi et al. 2019), HSP20 family protein (Zhao et al. 2018) and calcium-dependent protein kinase (Selvi et al. 2020), respectively during drought stress.

GO analysis showed that the target genes of many differentially-expressed miRNAs were related to integral component of membrane and nucleus in cellular component category Yang et al. (2014) and Liu et al. (2017) reported that several differentially-expressed genes were involved in physiological, metabolic and biochemical pathways, however, most genes were involved in plant adaptation during abiotic stress by cellular metabolism. The enrichment of biological process GO term, response to abiotic stimulus and response to water were identified in tomato plants during drought stress (Liu et al. 2017). In our study, enrichment of biological process GO terms was related to stress tolerance, such as transcription factors, DNA integration and carbohydrate metabolic processes. Significantly enriched molecular function GO terms were ATP binding, DNA binding, zinc ion binding, protein kinase activity and transcription factor activity. The top enriched category "General function prediction only" was observed in both tolerant and susceptible genotypes with 1212 and 1271 genes respectively, followed by signal transduction mechanisms with 615 and 714 genes respectively and posttranslational modification, protein turnover, chaperones with 518 and 603 genes respectively. Pathways such as secondary metabolites biosynthesis, transport and catabolism, inorganic ion transport and metabolism, defense mechanisms and cell wall/membrane/envelope

biogenesis were also involved in plant stress tolerance. Golldack et al. (2014) and Xia et al. (2015) reported that many differentially-expressed genes were involved in various stress-related metabolic pathways such as plant hormone signal transduction, phosphatidylinositol signalling system and oxidative phosphorylation.

Several factors may explain the regulation of miRNA and their targets. Although not widely reported, studies have suggested that miRNAs could also act as positive regulators of transcription (Vasudevan et al. 2007). Further, not all miRNAs-targets are identified by the algorithms nor the complexity of miRNA-target gene regulation is fully studied. Expression analysis done in our study indicated that most miRNAs negatively regulate their targets. However miRNAs like *miR166* showed positive regulation of peroxidases in V1 and negative regulation in V2 while cellulose synthase was negatively regulated in both varieties. Abundance of *miR166* was observed in the tolerant sugarcane genotype and are known to be involved in ABA dependent stress response by targeting class III homeodomain-leucine zipper genes which play a major role in growth and development (Yan et al. 2016). Similarly xyloglucan endotransglycosylase a prominent cell wall modifying enzyme that causes cell expansion, loosening/reinforcing cell walls, particularly in response to environmental stress was negatively regulated by *miR6235* in both varieties. Thus miRNA:mRNA regulation is a complex phenomenon owing to the fact that each miRNA could regulate the expression of several target genes and the expression of each target gene could be potentially regulated by several miRNAs.

Conclusions

Our study was the first study that provided an insight into microRNA gene regulation of Indian tropical sugarcane cultivars that are tolerant and susceptible to drought stress at formative stage. The study provided valuable information on differentially expressed known and novel microRNAs that contribute to varietal variations in mitigating sugarcane drought. It was interesting to note that most miRNAs significantly expressed during drought were specific to the tolerant and susceptible cultivars indicating the various mechanisms of drought tolerance adopted by the varieties. The study also led to identifying target genes that are regulated by the microRNAs and their possible functions in overcoming drought. Major pathways that attributed to stress tolerance like plant hormone signal transduction, post-translational modification protein turn over and chaperones, carbohydrate metabolism, MAPK signalling pathway, plant plant-pathogen interaction, phenyl propanoid biosynthesis were identified.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s13205-021-02857-x.

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Author contributions AS: conceptualization, project administration, data analysis and interpretation of the data, supervision, writing, review and editing. KD: laboratory experiments, validation, writing and review. RM: methodology for RNA Seq, analysis and interpretation of the data. PTP: data analysis, methodology for validation, review and editing. VPR: methodology for validation and expression analysis. KL: data analysis.

Data availability All the data supporting the results of this article are given in the paper and in additional files. The sequencing reads have been submitted as sequence read archive (SRA) in NCBI with the BioProject ID—PRJNA593909.

Declarations

Conflict of interest The authors declare that they have no conflict of interest in this publication.

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