Genome-wide miRNAs profiles of pearl millet under contrasting high vapor pressure deficit reveal their functional roles in drought stress adaptations

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Abstract
Pearl millet (Pennisetum glaucum [L.] R. Br.) is an important crop capable of growing in harsh and marginal environments, with the highest degree of tolerance to drought and heat stresses among cereals. Diverse germplasm of pearl millet shows a significant phenotypic variation in response to abiotic stresses, making it a unique model to study the mechanisms responsible for stress mitigation. The present study focuses on identifying the physiological response of two pearl millet high-resolution cross (HRC) genotypes, ICMR 1122 and ICMR 1152, in response to low and high vapor pressure deficit (VPD). Under high VPD conditions, ICMR 1152 exhibited a lower transpiration rate (Tr), higher transpiration efficiency, and lower root sap exudation than ICMR 1122. Further, Pg-miRNAs expressed in the contrasting genotypes under low and high VPD conditions were identified by deep sequencing analysis. A total of 116 known and 61 novel Pg-miRNAs were identified from ICMR 1152, while 26 known and six novel Pg-miRNAs were identified from ICMR 1122 genotypes, respectively. While Pg-miR165, 168, 170, and 319 families exhibited significant differential expression under low and high VPD conditions in both genotypes, ICMR 1152 showed abundant expression of Pg-miR167, Pg-miR172, Pg-miR396 Pg-miR399, Pg-miR862, Pg-miR868, Pg-miR950, Pg-miR5054, and Pg-miR7527 indicating their direct and indirect role in root physiology and abiotic stress responses. Drought responsive Pg-miRNA targets showed upregulation in response to high VPD stress, further narrowing down the miRNAs involved in regulation of drought tolerance in pearl millet.

1 | INTRODUCTION
Pearl millet (Pennisetum glaucum [L.] R. Br.) is a highly nutritious, short-duration, annual C4 cereal belonging to the Poaceae subfamily. It is widely cultivated for staple food, feed, fuel, building material, and forage by farmers of Sub-Saharan Africa and the Indian subcontinent. It holds sixth position among the most economically important cereals consumed by over 500 million people (Satyavathi, 2017). Being grown in adverse climatic conditions of the arid and semi-arid tropical regions, known for their stochastic environments with low rainfall and...
moisture, extreme temperatures, and poor soil conditions, the crop is highly resilient to abiotic stress and thrives in conditions, where other cereals such as rice, wheat, maize, and sorghum tend to fail in producing economic yields (Vadez et al., 2012; Yadav et al., 2012). Nevertheless, although quite well adapted to dry conditions, extreme drought stress proves deleterious to the crop’s productivity (Yadav et al., 2002). Thus, genetic improvement of pearl millet with respect to terminal drought stress tolerance is a major breeding objective. Water saving mechanisms, such as lowering of transpiration rate (Tr) at higher fractions of transpirable soil water content and high vapor pressure deficit (VPD), reduction in leaf area, and improved transpiration efficiency (TE) have been studied in detail in this crop using the near isogenic lines introgressed with drought-tolerance quantitative trait loci (QTLs) (Kholova et al., 2012; Yadav et al., 2002). Several studies have elucidated the role of microRNAs in plant development and stress tolerance (Ayubov et al., 2019; de Lima et al., 2012; Gupta, Kumari, et al., 2017; Li & Zhang, 2016; Sunkar et al., 2012; Wen et al., 2020). miRNAs are 20–22 nt long small regulatory RNAs that regulate gene expression post-transcriptionally, either through guided Dicer-mediated degradation or by translational repression (Jones-Rhoades et al., 2006). A number of miRNAs across a range of plant species have revealed enhanced expression under abiotic stress conditions such as drought, salinity, and temperature fluctuations (Sunkar et al., 2012). Besides, transgenic plants overexpressing stress responsive miRNAs have shown enhanced abiotic tolerance in crops such as creeping bentgrass (Agrostis stolonifera) for salinity and drought tolerance (Zhou & Luo, 2013), tomato for drought tolerance (Zhang et al., 2011), and regulating lateral root development under different conditions in rice (Lu et al., 2018). Root-specific expression of miR393 has shown modulation of auxin-signaling, resulting in the adaptation to drought stress in Arabidopsis thaliana (Chen et al., 2012). In pearl millet, the performance of various genotypes in response to various stress conditions such as drought, heat, and high VPD has been studied in detail, to reveal various physiological parameters such as lowered Tr, leaf area, increased relative water content, and root sap exudation rates that contribute to drought stress adaptation (Aparna et al., 2014; Gupta, Yadav, et al., 2017; Kholova et al., 2010; Reddy, Divya, et al., 2017; Reddy, Tharanya, et al., 2017; Vadez et al., 2012). Furthermore, various genes such as Hsp10 and aquaporins have been identified and linked to these water saving mechanisms (Grondin et al., 2020; Nitnavare et al., 2016; Reddy, Divya, et al., 2017; Reddy, Tharanya, et al., 2017). However, limited information is available about the miRNAs in this crop and how these regulate gene expression in response to abiotic stresses.

In the present study, we evaluated the physiological performance of two genotypes (ICMR 1122 and ICMR 1152) that show a contrasting Tr response to increasing VPD conditions. Root specific miRNAs expressed under low and high VPD conditions were identified from both genotypes by deep sequencing. The study reports conserved and novel miRNAs from pearl millet and generates a vast novel resource of Pg-miRNAs. Comparison of miRNA expression from two contrasting genotypes narrows down the miRNA candidates involved in adaptation to high VPD conditions experienced during drought stress. Ontologies of Pg-miRNAs target genes have been studied to understand their possible role in stress tolerance.

2. MATERIALS AND METHODS

2.1 Plant material and abiotic stress imposition

Two pearl millet genotypes (ICMR 1152 and ICMR 1122) selected for the present study belong to high-resolution crosses (HRCs) developed earlier by crossing a promising near-isogenic line, ICMR 01029, containing a terminal drought tolerance QTL, to a drought-sensitive line H77/833-2 (Serraj et al., 2005). ICMR 01029 is a BC4F3 introgression of a terminal drought tolerance QTL from the tolerant donor PRLT-2/89-33 into drought sensitive H77/833-2 (Kholova et al., 2010; Reddy, Divya, et al., 2017; Reddy, Tharanya, et al., 2017). ICMR 1152 and ICMR 1122 genotypes were selected based on the Tr response to increasing VPDs. Plants were grown in a glasshouse in 8” plastic pots filled with 5.5 kg of sand (to facilitate root tissue collection) collected from the ICRISAT farm and routinely irrigated with water. Each pot was sown with four seeds and thinned to two healthy seedlings after a week and grown under well-watered conditions up to 28 days (8th leaf fully developed) with natural day-light oscillations around 22/22°C and relative humidity of 72/88%. Nine most uniform plants of each genotype were selected from nine replica pots of each genotype for experiments in the controlled growth chambers. A thermo-hygrograph sensor (Tinytag Ultra 2 TGU-4500 Gemini Data loggers Ltd) was positioned within the plant canopies in the growth chamber for a regular record of the air temperature and relative humidity during the measurement period. The plants were moved to another growth chamber a day before the experiment during the late afternoon for night acclimatization. The plants were acclimatized to a day regime VPD of 1.8 kPa (15:00–18:30) and night regime (19:30–05:30 h) and VPD of 0.67 kPa. The transition period for the day and night regimes occurred between 05:30 and 06:30 h and 1830–1930 h, respectively. At 08:00 h, the VPD was increased progressively in the treatment chamber (typically covering a range of 0.69–4.21 kPa), whereas the control chamber was kept constantly under low VPD (1.2 kPa) conditions. The plants were weighed gravimetrically using a bench electronic 10 kg balance with a resolution of 0.1 g (FBK, Kern & Sohn GmbH) to measure the transpiration values. At the end of the VPD ladder at 4.21 kPa at 15:00 pm, leaf and root samples were collected from both control (1.2 kPa) and treatment chambers (4.21 kPa), immediately frozen in liquid nitrogen and stored in –80°C for future RNA isolation.

2.2 Physiological analysis related to root traits

Six replicates maintained for physiological measurements were used to measure leaf surface area, using a leaf area meter (LI-3100, Li-Cor). The transpiration was normalized with respect to leaf area and the
time to calculate Tr. Leaf and stem samples were dried in the oven at 65°C for 48 h to estimate the leaf dry weight (LDW) and stem dry weight (StDW). At the end of the VPD experiment, plant shoots from low and high VPD chambers were cut using a razor blade. The root exudate (xylem sap) was collected for 45 min at the cut surface using preweighed eppendorf cones stuffed with tissue paper (Kimtech Science). The root sap exudation rate was calculated by normalizing the amount of sap exuded by the root surface area (RSA) and time. The RSA (cm²) and root volume (RV; cm³) were calculated by scanning the roots with a Shimadzu scanner and analyzing them with the WinRhizo software (WinRhizo, Regent Ltd). Later these were dried in an oven (65°C for 48 h) to estimate their dry weight (RDW). Dry weight parameters like LDW, StDW, shoot dry weight (LDW + StDW), and total dry matter (LDW + StDW + RDW) were also calculated. Other parameters such as root:shoot ratio (R/S; Richard 1992), shoot:root ratio (Bolinder et al., 2002), and root dry weight (RDW)/total plant dry weight ratio were analyzed (Varshney et al., 2014).

2.3 | Small RNA library construction and deep sequencing

Total RNA was extracted from root tissues sampled from pearl millet plants subjected to high and low VPD conditions using the RNeasy Plant mini kit (Qiagen) according to the manufacturer’s instructions. The integrity of the RNA was checked by performing agarose gel electrophoresis and using the BioAnalyzer (Agilent). sRNA fractions (~18–30 nt) were purified by running total RNA on a 15% polyacrylamide gel electrophoresis and eluting the portion corresponding to small RNAs (sRNAs). The obtained sRNAs were sequentially ligated to 3’ and 5’ adapters using T4 RNA ligase 1 (NEB). The adapter-ligated sRNAs were used for cDNA synthesis using the ProtoScript kit (NEB). The cDNA was further purified and subjected to sequencing using the Illumina MiSeq platform. Adaptor trimming was performed, and low-quality reads were removed, and high-quality reads were further subjected to downstream analysis.

2.4 | Identification and characterization of miRNA

High quality reads of all samples were mapped to the reference genome of Pearl millet v1.1 (Varshney et al., 2017) using the Bowtie program v1.1.2 (Langmead et al., 2009). Further reads corresponding to different types of sRNAs such as rRNA, siRNA, piRNA, snRNA, snoRNA, trRNA, and TasiRNA were removed. Pre-miRNAs and mature-miRNAs of all plant species were obtained from miRBase (release 22). Unaligned unique reads were further used for the prediction of novel and known miRNAs by mapping them with the PM reference genome by using miRDeep2. Mature and precursor miRNAs from miRBase were used as training sets (Friedländer et al., 2011). Aligned reads that were more than 17 nt long, with ≤3 mismatches were selected for prediction, other parameters such as miRNA sequence length corresponding to 18–25 nt, maximum free energy (MFE) of –18 kcal/mol for miRNA precursor, and maximum free energy index (MFEI) were used to filter the data.

2.5 | Differential expression analysis of miRNAs

A highly expressed miRNA will likely have a higher number of read support. Based on this, we performed differential expression analysis of miRNAs (DEMs) in both genotypes by comparing Low VPD root (LR) and High VPD root (HR) samples. DEMs were determined for LRVs HR comparison by using fold changes (FC >2). Further, targets for each miRNA were predicted by aligning Pg-miRNA sequences with gene sequences from the pearl millet reference genome by using psRNATarget (Dai et al., 2018). The Gene Ontology (GO) database was used to determine the annotations of all predicted target genes with respect to their cellular process, subcellular localization, and molecular function. The pathways in which target genes are involved were identified using the Plant Metabolic Network (PMN) pathways database (Schläpfer et al., 2017). Further, the miRNA-target interaction network was constructed using Cytoscape v3.6.1 (https://cytoscape.org/).

2.6 | Validation of functionally important genes using qRT-PCR analysis

Exactly 1.5 μg of total RNA was used for cDNA synthesis and qRT-PCR analysis. qRT-PCR was carried out in 96-well optical reaction plates within a total volume of 10 μl containing 0.5 μM of each primer (0.8 μl), cDNA (1.0 μl), Sensi Master Mix (2X), and dH₂O up to 3.2 μl. The PCR primers were designed using Primer3 (bioinfo.ut.ee/primer3-0.4.0/) and had a GC content of 40–60%, a Tₘ of 62°C, primer length 20–25 nucleotides, and an expected product size of 90–180 bp. The qRT-PCR reactions were carried out by following standard thermal profile: 95°C for 10 s and then 40 cycles of 15 s at 95°C, 15 s at 61°C with fluorescent signal recording and 15 s at 72°C. After 40th cycle, amplicon dissociation curves were measured by heating from 58 to 95°C with fluorescence measured within 20 min. All qRT-PCR data were obtained from three biological replicates with three technical replicates. Relative expression was calculated with the qBase+ software (Schmidt & Delaney, 2010) by normalizing with corresponding control samples and as well as with PgELF4a and PgMDH as reference genes (Reddy et al., 2015).

2.7 | Statistical analysis

Statistical analysis of Tr and root traits (root exudation rate [RHC], RSA, RV, R/S, and RDW) of ICMR 1122 and ICMR 1152 were analyzed with the statistical program package CoStat version 6.204 (Cohort Software). One-way ANOVA was carried out to test for genotypic differences between the ICMR 1122 and ICMR 1152 genotypes.
Means were compared using the Tukey–Kramer test and LSD at $P \leq 0.05$ (5% level).

3 | RESULTS

3.1 | Pearl millet genotypes show varying physiological responses to different VPD conditions

Genotypes ICMR 1122 and ICMR 1152 are known to show different phenotypic responses under high VPD conditions (Reddy, Divya, et al., 2017; Reddy, Tharanya, et al., 2017). In the present study, these genotypes showed contrasting $Tr$ ($\text{mg cm}^{-2} \text{ min}^{-1}$) upon progressive increase in VPD from 1.2 to 4.21 kPa (Figure 1A). While under increasing VPD conditions, ICMR 1152 showed significantly lower $Tr$ compared to ICMR 1122 ($P \leq 0.05$). Root sap exudation rate (also termed as “bleeding rate”) was higher in the ICMR 1122 genotype compared to ICMR 1152 under high VPD conditions (Figure 1B). Moreover, morphological traits such as RSA (Figure 1C), RV (Figure 1D), and root biomass (Figure 1E) showed clear genotypic differences between the two tested genotypes. ICMR 1122 had higher RDWs compared to ICMR 1152, which may be attributed to the genotypic differences (Figure 1F). Overall, the analysis of physiological parameters relevant to water saving traits such as lowered transpiration and root sap exudation rates indicated that ICMR 1152 was better adapted to high VPD stress than ICMR 1122.

3.2 | Genome wide mapping and identification of conserved and novel miRNAs

Four sRNA libraries generated from the roots of ICMR 1152 and ICMR 1122, under low (1.2 kPa) and high VPD (4.21 kPa) conditions, denoted as LR-ICMR 1152, HR-ICMR 1152, LR-ICMR 1122, and HR-ICMR 1122, respectively, were used to identify miRNAs. In total, 84.33, 60.04, 26.40, and 98.09 million adapter trimmed reads were obtained from LR-ICMR 1152, HR-ICMR 1152, LR-ICMR 1122, and HR-ICMR 1122, respectively (Table 1). Following filtration of the low-quality sequences and adaptors, the unique reads from each sample were further mapped to available siRNA, piRNA, snRNA, snoRNA, tRNA, tasiRNA, and Rfam rRNAs to remove redundancies (Table 1). Length distribution of cleaned sequenced sRNAs varied from 17 to 50 nt. miRNAs expressed in roots of ICMR 1122 and ICMR 1152 under low and high VPD conditions were predicted and categorized into the following classes: novel (no similar mature miRNAs found in plant and vertebrates), known (similar mature miRNA sequence present in other plant species), and conserved (similar mature miRNA sequence present in vertebrates; Figure 2). A total of 116 known, 61 novel, and 352 conserved Pg-miRNAs were identified from ICMR 1152. Similarly, in ICMR 1122, 26 known, six novel, and 46 conserved Pg-miRNAs were identified (Figure 2, Table S6).

Higher number of miRNAs were expressed in ICMR 1152 as compared to ICMR 1122 under both high and low VPD conditions. Fourteen known, eight novel, and 22 conserved miRNAs were found to be differentially expressed in ICMR 1152. In ICMR 1122, only two
conserved miRNAs were found to be common for both low and high VPD conditions (Figure 3). Among the known miRNAs, differential expression was observed in both genotypes for Pg-miR165a-3p, Pg-miR319a-5p, Pg-miR395c-5p, Pg-miR399a, Pg-miR5054, Pg-miR1223a, Pg-miR5225, Pg-miR5368, Pg-miR7527, Pg-miR7769-5p, Pg-miR7780-3p, Pg-miR862-3p, Pg-miR868-5p, and Pg-miR950b (Figure 4). These miRNAs were highly abundant in root samples under low VPD conditions (Tables S3 and S5).

Prediction of novel Pg-miRNAs determined 44 miRNAs from HR-ICMR 1152, 17 miRNAs from LR-ICMR 1152, 55 miRNAs from HR-ICMR 1122, and one miRNA from LR-ICMR 1122 (Figure 2; Table S6). Highly expressed novel Pg-miRNAs (having estimated probability >45% along with higher miRdeep score and read support) were identified with respect to specific cultivar and treatment namely, LR-ICMR 1152 (1), HR 1152 (4), LR-ICMR 1122 (1), and HR-ICMR 1122 (1) along with their 2D structures (Figure 5). In addition, precursor sequences of predicted Pg-miRNAs were identified based on hairpin secondary structure with ≤ 4 symmetrical mismatches and stable MFE, as described previously by Meyers et al. (2008) and (Gupta, Kumari, et al., (2017); Tables S2, S3, S4, and S5).

In-depth analysis indicated that the relative abundance of Pg-miRNAs members within one family varied greatly in pearl millet, suggesting the functional divergence within families. For instance, miR156 family abundance varied from 34 reads (miR-156j) to 13,360 reads (miR-156c). In addition, sequence polymorphisms or multiple clones in Pg-miRNAs families were observed. For example, miR156a-5p, has a mature sequence 5’-UGACAGAAGAGAGUGAGCAC-3’ and had four different precursor sequences expressed from different chromosomes of pearl millet (Table S3). The prediction of the two-dimensional structure of novel Pg-miRNAs identified in low and high VPD conditions (Figure 5) revealed that different members within a given miRNA family could have different hairpin structures, which is a function of miRNA concentration and biological conditions at a given time.

### 3.3 | Pg-miRNA targets and metabolic pathways analysis

To illustrate the biological processes and physiological functions of DEMs, the target genes were predicted using the psRNA target tool along with the reference genome. Hits with expectation value of ≤ 4.0 and energy require to unpair the target site (UPE) ≥ 25 were selected as target genes. Over 493 and 157 unique potential target genes were identified in ICMR 1152 (Table S7A) and ICMR 1122 (Table S8A), respectively. GO analysis of the targets showed that most targets were involved in oxidation-reduction processes, followed by transmembrane transport (Figure 6; Tables S7A and S8A). It was intriguing that most Pg-miRNAs targets showed membrane, plasma membrane or integral membrane component localizations, mapping to a variety of GO biological processes (Tables S7 and S8). This included genes such as Pgl_GLEAN_10025099, involved in lipid biosynthesis, Pgl_GLEAN_10022868 involved in ceramide biosynthesis and Pgl_GLEAN_10019070 functioning in glutathione metabolism. Various transporters such as Pgl_GLEAN_10026510, a malate transporter,
FIGURE 2  Number of small RNA identified in pearl millet samples. Number of total, known, and novel miRNAs identified in roots of ICMR 1152 and ICMR 1122 genotypes under low and high VPD conditions.

FIGURE 3  Distribution of PgmiRNAs identified from LR-ICMR 1152, HR-ICMR 1152, LR-ICMR 1122, and HR-ICMR 1122. Venn diagrams for identified (A), known (B), novel, and (C) conserved miRNAs showing common and unique Pg-miRNAs from each sample.

FIGURE 4  Differential expression of known PgmiRNA under contrasting VPD conditions. (A) HR-ICMR 1152 versus LR-ICMR 1152 (B) HR-ICMR 1122 versus LR-ICMR 1122.
Pgl_GLEAN_10036572 functioning in lipid transport, and four genes are with transmembrane transporter activities (Pgl_GLEAN_10005703, Pgl_GLEAN_10015952, Pgl_GLEAN_10015952, and Pgl_GLEAN_10019667). Two genes, Pgl_GLEAN_10025832 and Pgl_GLEAN_10025832 showed polysaccharide binding activity. At least 20 genes regulated by Pg-miR862 and Pg-miR399a mapped to protein kinase activity that are known to regulate other proteins through phosphorylation. This indicates the complex nature of the gene regulatory network pertaining to drought response.

Further the miRNA-target interaction network of ICMR 1152 (Figure 7A) and ICMR 1122 (Figure 7B) showed that novel and known Pg-miRNAs directed inhibition of different targets. For instance, Pg-miR-156a-5p, Pg-miR159a, Pg-miR160, Pg-miR165, Pg-miR170, Pg-miR171, Pg-miR319, and Pg-miR395 directed the cleavage of 16, 11, 3, 21, 9, 18, 10, and 12 targets of both genotypes, respectively. Further GO annotation and PMN metabolic pathway analysis revealed that out of 493 genes from ICMR 1152, only 226 showed participation in different metabolic pathways (Table S7B), while in ICMR 1122, out of 157, only 63 were involved in different pathways (Table S8B). The metabolic pathway analysis showed that under high VPD biosynthesis of adenosine ribonucleotides, cytokinin, cutin, jasmonic acid, and formaldehyde oxidation II were severely affected in ICMR 1152, while in ICMR 1122, biosynthesis of UDP- and beta-L-rhamnose, homogalacturonan, caffeoylglucarate, and glycolysis were impacted.

3.4 Validation of the Pg-miRNAs inhibited target genes

Under high VPD conditions, during high evaporative demand from the atmosphere, the expression of selected target genes (Table 2) regulated by high VPD stress miRNAs showed a significant increase in

![FIGURE 5](image-url) Secondary structure of the novel Pg-miR family identified from pearl millet root samples. Pearl millet plants subjected to contrasting VPD regimes under low VPD. (A) Pg-miR1_LR-ICMR 1152, (B) Pg-miR1_LR-ICMR 1122 and high VPD conditions, (C) Pg-miR1_HR-ICMR 1152, (D) Pg-miR2_HR-ICMR 1152, (E) Pg-miR3_HR-ICMR 1152, (F) Pg-miR4_HR-ICMR 1152, and (G) Pg-miR1_HR-ICMR 1122
roots with relatively higher expression in drought-tolerant ICMR 1152, as compared to drought-sensitive ICMR 1122 (Figure 8). In ICMR 1122, expression levels of targets genes corresponding to Pg-miR8051-3p were downregulated during low VPD conditions, while these were upregulated during high VPD. Specifically, upregulation of PgL_GLEAN_10022920, homologous to A. thaliana disproportioning enzyme (DPE2), and PgL_GLEAN_10016491, homologous to L-type lectin-domain receptor kinase-like protein (AT3G45390). DPE2 is a transglucosidase with an essential role in the pathway from starch to sucrose, and metabolizing maltose in leaves at night (Chia

**FIGURE 6** Gene ontology enrichment analysis of Pg-miRNA target genes identified in pearl millet samples. Functional annotation of predicted miRNA target genes of for known and novel Pg-miRNAs of ICMR1152 and ICMR 1122 of top gene ontology terms in each biological process, cellular component and molecular function categories.
et al., 2004), suggesting a role in governing leaf osmolarity. DPE2 has also been found to be upregulated in transgenic rice plants expressing PSARK::IPT, a stress induced promoter that resulted in drought tolerance (Peleg et al., 2011). Similarly, LOW is a root-specific gene that was found to be highly downregulated in a drought-sensitive variety of barley (Harb et al., 2020). While the other class of target genes such as autophagy related, DNA domain binding, transcription activators (Pgl_GLEAN_10003760, Pgl_GLEAN_10016561, Pgl_GLEAN_10016930, Pgl_GLEAN_10022817, Pgl_GLEAN_10032938, and Pgl_GLEAN_10022225) associated with Pg-miR156a-5p, Pg-miR3633a-5p, and Pg-miR159a, showed moderate levels of expression in roots, indicative of inherent repair mechanisms functioning under starved conditions. Furthermore, the Pgl_GLEAN_10012226 gene, a homolog of mediator of RNA polymerase II transcription subunit (MED3/AT3G09180), an integral component of the stress responsive mediator complex, was also upregulated in ICMR 1122 under high VPD (Figure 8). The mediator complex is well known for relaying information from DNA-bound transcription factors to the RNA polymerase II, thereby regulating gene expression during plant development and abiotic stress response (Mathur et al., 2011). This indicates that miRNAs indeed play a complex role of master regulating gene expression in response to abiotic stress conditions.

In contrast, ICMR 1152 under high VPD conditions, had higher expression of Pgl_GLEAN_10014608, a homolog to transcription factor HYS(O24646), that is responsible for reprogramming of leaf growth during drought stress, explaining negative regulation of Pg-miR5185a-3p linked with the gene under stress. Calcium chloride activated channel related anoctamine like gene and electron transfer reaction related genes such as NAD(P)H dehydrogenase (Pgl_GLEAN_10005258, Pgl_GLEAN_10012279, and Pgl_GLEAN_10017815) showed higher expression levels in the leaves of ICMR 1152. In addition to the leaves, the class of genes related to cell morphogenesis, cell differentiation, and several transcription factors such as topoisomerases, cytochrome subunit encoding genes (Pgl_GLEAN_10020911, Pgl_GLEAN_10012226, Pgl_GLEAN_10020056, Pgl_GLEAN_10030892, Pgl_GLEAN_10037842, Pgl_GLEAN_10012279) showed remarkable expression levels in the roots of this genotype (Figure 8). Expression levels of the genes belonging to the calcium chloride channels (Pgl_GLEAN_100122279 and Pgl_GLEAN_10017815) were comparable in roots demonstrating the maintenance of water uptake movement throughout the plant. In summary, most of the target genes were significantly expressed in the root tissues of the ICMR 1152 and ICMR 1122 with a higher expression in ICMR 1152 (Figure 8).

4 | DISCUSSION

4.1 | Physiological performance

Pearl millet has been a crop of significant interest to molecular physiologists because of its adaptation to very harsh conditions in the semi-arid tropics (Ghatak et al., 2020). Various studies evaluating the morphological and physiological aspects of drought tolerance in this crop have generated insights for water-saving mechanisms in drought-adapted germplasm (Kholova et al., 2012; Vadez et al., 2012). Constitutive water-saving mechanisms during vegetative stages ensure water availability during the postanthesis period, resulting in better seed setting and higher yield (Kholova &
It is known that processes governing plant water use are affected by VPD (Kholova et al., 2016; Reddy, Divya, et al., 2017; Reddy, Tharanya, et al., 2017).

Thus, we evaluated traits such as Tr (mg cm⁻² min⁻¹) and root exudation rates under low and high VPD conditions. In our study, although both ICMR 1122 and ICMR 1152 demonstrated a gradual increase in Tr with increasing VPD, ICMR 1152 showed lower Tr than ICMR 1122, under both low and high VPD conditions, indicating a higher TE (Figure 1). Limited transpiration at increasing VPD allows crops to save water (Kholova et al., 2010; Messina et al., 2015; Vadez et al., 2013) ultimately improving the daily TE (Sinclair et al., 1984). Additionally, ICMR 1152 showed traits such as lowered root sap exudation rates with lower Tr indicative of a better adaptation to high VPD conditions than ICMR 1122. These observations are in line with recent reports in pearl millet (Tharanya et al., 2018) and chickpea (Sivasakthi et al., 2017; Sivasakthi et al., 2020) where low Tr genotypes also had a lower RHC than high Tr under high VPD conditions. Root exudation rates play a crucial role in plant water transport pathways and substantial variation in root exudation rate have been found in crops like sorghum, pearl millet, and chickpea (Medina et al., 2017; Sivasakthi et al., 2020; Tharanya et al., 2018). It also suggested that drought-adapted genotypes have a lower root exudation rate than drought-sensitive genotypes.

### Table 2

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<tr>
<td>Pgl_GLEAN_10020056</td>
<td>DNA topoisomerase 3-alpha [Setaria italica]</td>
</tr>
<tr>
<td>Pgl_GLEAN_10030892</td>
<td>Cytochrome P450 89A-like</td>
</tr>
<tr>
<td>Pgl_GLEAN_10014608</td>
<td>Transcription factor HY5</td>
</tr>
<tr>
<td>Pgl_GLEAN_10037842</td>
<td>Myosin-2 heavy chain isoform X1</td>
</tr>
<tr>
<td>Pgl_GLEAN_10005258</td>
<td>Hypothetical protein HU200_033634</td>
</tr>
<tr>
<td>Pgl_GLEAN_10012279</td>
<td>Anoctamin-like protein Os01g0706700</td>
</tr>
<tr>
<td>Pgl_GLEAN_10017815</td>
<td>Probable NAD(P)H dehydrogenase subunit CRR3, chloroplastic</td>
</tr>
</tbody>
</table>

In recent years, many studies have reported the contribution of miRNAs in response to drought, extreme temperatures, osmotic pressure, and other abiotic stress conditions (Ferdous & Hussain, 2015; Lotfi et al., 2015; Nehammer et al., 2015; Zhang, 2015). In the present study, various miRNAs were found to be highly expressed in the root tissues of ICMR 1152 compared to ICMR 1122 subjected to differential VPD conditions (Figures 2–4) indicating their involvement in extenuating abiotic stress conditions and could help elucidate the underlying mechanisms of the stress responses. For example, we found no differential expression of the Pg-miR156 family in ICMR 1122 plants subjected to low VPD and high VPD conditions, whereas...
when compared to the former, a 1.5- and 7.5-fold upregulation was observed in LR-ICMR 1152 and HR-ICMR 1152, respectively (Figure 4). miR156 has been previously reported to regulate SPL genes, repressing adventitious root development in response to stress (drought, nutrient deprivation, and wounding; Gupta, Kumari, et al., 2017; Steffens & Rasmussen, 2016; Xu et al., 2016). In addition, the upregulation of the Pg-miR170, Pg-miR171, and Pg-miR319 family was observed in both genotypes in our study. miRNAs belonging to miR170 and miR171 families are known to play a role in signaling and development by silencing the GRAS domain or SCARECROW-like proteins that are essential for radial patterning in roots, signaling by the phytohormone gibberellin, and light signaling (Helariutta et al., 2000).

Similarly, conserved Pg-miR319 exhibited higher expression in ICMR 1152 as compared to ICMR 1122. miR319 regulates the TCP transcription factors that are involved in multiple developmental pathways, including leaf development and senescence, organ curvature, hormone biosynthesis, and signaling (Schommer et al., 2014). During low and high VPD conditions, the expression of Pg-miR160 and Pg-miR165 showed an increase in ICMR 1152 (Figure 3A). Higher expression of Pg-miR165 in ICMR 1152 as compared to ICMR 1122 showed a more drought-tolerant nature of the former (Yang et al., 2019). Similarly, expression of Pg-miR168 in the ICMR 1122 genotype corroborated with characteristics of stress tolerance previously reported in

<table>
<thead>
<tr>
<th>No</th>
<th>Assigned miRNA IDs</th>
<th>miR family</th>
<th>Function reported</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Pg-miR165a-3p</td>
<td>miR165</td>
<td>Root development in drought response</td>
<td>Candar-Cakir et al. (2016)</td>
</tr>
<tr>
<td>2</td>
<td>Pg-miR1076-3p</td>
<td>miR1076</td>
<td>ROS and apoptosis</td>
<td>Sharma et al. (2012)</td>
</tr>
<tr>
<td>3</td>
<td>Pg-miR1223a</td>
<td>miR1223</td>
<td>Plant growth, development, hormone signaling, and stress responses</td>
<td>Mishra et al. (2019)</td>
</tr>
<tr>
<td>4</td>
<td>Pg-miR1513a-5p</td>
<td>miR1513</td>
<td>Regulate cell death and defense, involved in abscisic acid (ABA) signaling</td>
<td>Djami-Tchatchou et al., (2017)</td>
</tr>
<tr>
<td>5</td>
<td>Pg-miR1524</td>
<td>miR1524</td>
<td>Defense response</td>
<td>Pelaez et al., (2012)</td>
</tr>
<tr>
<td>6</td>
<td>Pg-miR1534</td>
<td>miR1534</td>
<td>Involved in hormone synthesis as a defense</td>
<td>Zhang et al. (2018)</td>
</tr>
<tr>
<td>7</td>
<td>Pg-miR159a</td>
<td>miR159</td>
<td>Promoting the programmed cell death of these tissues</td>
<td>Millar et al. (2019)</td>
</tr>
<tr>
<td>8</td>
<td>Pg-miR160a-5p</td>
<td>miR160</td>
<td>Control root cap formation, involved in adventitious rooting</td>
<td>Yang et al., (2019)</td>
</tr>
<tr>
<td>9</td>
<td>Pg-miR165a-3p</td>
<td>miR165</td>
<td>Organ polarity establishment and vascular development</td>
<td>Zhou et al. (2007)</td>
</tr>
<tr>
<td>10</td>
<td>Pg-miR167a-5p</td>
<td>miR167</td>
<td>Various aspects of plant development</td>
<td>Barik et al. (2015)</td>
</tr>
<tr>
<td>11</td>
<td>Pg-miR169a-5p</td>
<td>miR169</td>
<td>Key role in stress induced flowering</td>
<td>Xu et al. (2014)</td>
</tr>
<tr>
<td>12</td>
<td>Pg-miR170-3p</td>
<td>miR170</td>
<td>Floral development</td>
<td>Sunkar and Zhu (2004)</td>
</tr>
<tr>
<td>13</td>
<td>Pg-miR2107</td>
<td>miR2107</td>
<td>Nutrient acquisition and plant development</td>
<td>Kulcheski et al., (2015)</td>
</tr>
<tr>
<td>14</td>
<td>Pg-miR319a-3p</td>
<td>miR319</td>
<td>Regulates transcription factors of the TCP family</td>
<td>Koyama et al., (2017)</td>
</tr>
<tr>
<td>16</td>
<td>Pg-miR3635-3p</td>
<td>miR3635</td>
<td>Plant development and stress responses</td>
<td>Sun et al. (2015)</td>
</tr>
<tr>
<td>17</td>
<td>Pg-miR3710g</td>
<td>miR3710</td>
<td>Involved in leaf senescence</td>
<td>Niu et al., (2015)</td>
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<tr>
<td>18</td>
<td>Pg-miR393a-5p</td>
<td>miR393</td>
<td>Involved in root elongation</td>
<td>Bai et al. (2017)</td>
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<tr>
<td>19</td>
<td>Pg-miR394a</td>
<td>miR394</td>
<td>Participates in the regulation of plant development</td>
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</tr>
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<td>20</td>
<td>Pg-miR395c-5p</td>
<td>miR395</td>
<td>Involved in sulfur metabolism</td>
<td>Al et al. (2016)</td>
</tr>
<tr>
<td>21</td>
<td>Pg-miR396e-5p</td>
<td>miR396</td>
<td>Known to control cell proliferation</td>
<td>Debernardi et al. (2012)</td>
</tr>
<tr>
<td>22</td>
<td>Pg-miR399a</td>
<td>miR399</td>
<td>Affects plant physiology including flowering time</td>
<td>Kim et al. (2011)</td>
</tr>
<tr>
<td>23</td>
<td>Pg-miR4379</td>
<td>miR4379</td>
<td>Promotes cell growth and cell cycle</td>
<td>Mantri et al., (2013)</td>
</tr>
<tr>
<td>24</td>
<td>Pg-miR5054</td>
<td>miR5054</td>
<td>MAPK signaling pathway and plant hormone signal transduction</td>
<td>Su et al. (2017)</td>
</tr>
<tr>
<td>25</td>
<td>Pg-miR6191</td>
<td>miR6191</td>
<td>Anthocyanin biosynthesis</td>
<td>Sun et al. (2017)</td>
</tr>
<tr>
<td>26</td>
<td>Pg-miR7708b-3p</td>
<td>miR7708</td>
<td>Root signal transduction</td>
<td>Liang et al., (2017)</td>
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<td>27</td>
<td>Pg-miR7737-5p</td>
<td>miR7737</td>
<td>Leaf growth in drought</td>
<td>Bertolini et al. (2013)</td>
</tr>
<tr>
<td>28</td>
<td>Pg-miR7780-3p</td>
<td>miR7780</td>
<td>Leaf growth in drought</td>
<td>Bertolini et al. (2013)</td>
</tr>
<tr>
<td>29</td>
<td>Pg-miR8050-5p</td>
<td>miR8050</td>
<td>ribosome biosynthesis and lipid synthesis</td>
<td>Deng et al., (2021)</td>
</tr>
<tr>
<td>30</td>
<td>Pg-miR8170-5p</td>
<td>miR8170</td>
<td>Involved in MAPK signaling pathway</td>
<td>Jatan et al. (2019)</td>
</tr>
<tr>
<td>31</td>
<td>Pg-miR950a-3p</td>
<td>miR950</td>
<td>Promote lateral root development</td>
<td>Zhang et al. (2019)</td>
</tr>
</tbody>
</table>
Abelmoschus moschatus, Arabidopsis (Li et al., 2012), ICMR 1152 showed abundant expression of Pg-miR167, Pg-miR172, Pg-miR396, Pg-miR399, Pg-miR862, Pg-miR868, Pg-miR950, Pg-miR5054, and Pg-miR7527 indicating that these directly and indirectly participate in root physiology and abiotic stress responses (Pan et al., 2016; Wang et al., 2015). Annotated functions of Pg-miRNAs based on their family reported in literature were listed in Table 3.

4.3 Molecular mechanisms underlying drought tolerance response in pearl millet

Numerous genes having implications on drought tolerance have been identified (Dudhat et al., 2021; Shinozaki & Yamaguchi-Shinozaki, 2007). These genes mainly code for proteins involved in osmolyte biosynthesis (Umezawa et al., 2006), protective proteins (such as LEA, heat shock proteins, and dehydrins; Reddy et al., 2012; Reddy et al., 2016; Singh et al., 2015), ROS scavenging proteins (Chakradhar et al., 2017; Reddy et al., 2009), ABA homeostasis (Seiler et al., 2011), and water and ion transport proteins (Jarzyniak & Jasinski, 2014). In addition, post-transcriptional regulation of genes by microRNAs and its implications on drought resistance has been extensively studied (Martin et al., 2010; Sunkar et al., 2010). miRNAs function by downregulating target mRNAs, which might have a negative function associated with drought response. miRNAs also influence other miRNAs’ expression, leading to the accumulation of their target mRNAs that might contribute positively to stress adaptation (Shirram et al., 2016). Studying the role of miRNAs, which are essentially at the center of gene regulatory networks, it is essential to decipher the molecular mechanisms in plants under drought conditions (Jones-Rhoades et al., 2006).

It has been suggested that the regulation of plant development and drought tolerance by miRNAs is tightly linked, as miRNAs such as miR156, miR168, miR171, miR172, miR396, miR397, and miR518 involved in various developmental stages are often upregulated in response to drought stress (Ferdous & Hussain, 2015). In this study, using expression analyses we were able to validate this in pearl millet, as a homolog of HYS transcription factor (Pgl_GLEAN_10014608) corresponding to miR518 was found upregulated under high VPD (Figure 8). Prolonged stress conditions may lead to failure in scavenging ROS causing extensive cell damage and eventually death. This possibly indicates increased binding of this transcription factor to the G-box present in the promoter region of ROS responsive genes, thereby indicating a limited Tr (Chen et al., 2013). Interestingly, the upregulation of the putative homolog of cytochrome450 (P_gLEAN_10030892) depicted the gene’s response to regulation of secondary metabolites under drought. For example, expression of miR169 is induced remarkably in rice roots compared to the shoots under drought stress (Li et al., 2008). miR169 is linked with the expression of most probable homolog of Mediator of RNA polymerase II transcription subunit 23 (MED23; Pgl_GLEAN_10012226). MED has multiple functions such as regulation of plant immunity and stress tolerance. MED23 is induced by abscisic acid (ABA) under abiotic stress conditions, particularly drought stress (Ni et al., 2013). This gene was upregulated in roots in the case of both these genotypes (Figure 8). Thus, it is important to study miRNAs’ tissue-specific expression to decipher their link with stress-responsive processes. In this study, we chose to study miRNA expression in pearl millet roots to understand which miRNAs may influence water uptake during mid-season drought. Additionally, it is essential to deliberate that although miRNAs are conserved across different plant species, their targets might not be (Lu et al., 2005). Thus, the targets of Pg-miRNAs were identified from the drought-responsive pearl millet transcriptome (Reddy et al., unpublished).

During low VPD conditions, that is, conditions amenable for plant growth, miR-160 was found to have higher transcript abundance in ICMR 1152 than ICMR 1122. At high VPD conditions, miR-160 showed significant upregulation in ICMR 1152 whereas its expression remained unchanged in ICMR 1122. miR160 negatively regulates the expression of auxin response factor (ARF). ARFs are essential for primary and lateral root growth; thus, miR160 indirectly regulates root growth. It also modulates the ABA response which is an important mechanism for drought and osmotic stress tolerance (Bustos-Sanmamed et al., 2013). Other miRNAs namely, miR-159, miR167, and miR169, which affect the ABA response, were also found to show altered expressions. Pgl_GLEAN_10032938, regulated by miR159, involved in the crocetin biosynthesis pathway is expressed at a higher level in both genotypes under high VPD conditions (Table S7A). These results are similar to Moldovan et al. (2010), where miRNA159 target genes were highly expressed in roots when assayed under stress conditions. miR159 has been reported to inhibit the MYB transcription factors that regulate ABA-dependent signaling pathways, thus affecting stress adaptation (Reyes & Chua, 2007) and is responsive in hypoxia conditions (Moldovan et al., 2010). We observed similar results for miR169, that modulates the ABA response by negatively regulating NFYA transcription factors (Li et al., 2008). It is known that miRNAs also regulate genes involved in cell and organ development to help them adapt to stress conditions.

In addition, differential expression of miR396, miR166, and miR168 in ICMR 1122 and ICMR 1152 roots, revealed targets linked to plant development (Figure 4). While, miR396 targets the growth response factors (GRFs), affecting cell proliferation and elongation in roots (Ercoli et al., 2016), miR166, which is essential for cell development, targets the class III homeodomain-leucine zipper (HD-Zip III) transcription factors, which in turn influence lateral root formation (Li et al., 2016). Similarly, miR168 targets the argonautes (AGO) involved in miRNA processing and shows upregulation during abiotic stress conditions (Liu et al., 2008). The Pg-miRNA targets included genes involved in various functions ranging from metabolism, transcription regulation, to membrane transport. Most genes got mapped to the “integral membrane component.” This did not come as surprise as plasma membrane and cell wall proteins are important in sensing, signaling, and regulating drought responses. For example, proteins such as ABA-responsive elements (ABREs) at the membrane are known to initiate or suppress a signaling cascade to generate a molecular response to drought stress (Chaves et al., 2003). The ABRE mediated
pathway contributes to the regulation of pathways essential for stomatal opening and guard cell movement, in order to prevent water loss (Wu et al., 2019). Additionally, membrane proteins such as aquaporins are known to regulate water transport and contribute to drought tolerance (Reddy, Divya, et al., 2017; Reddy, Tharanya, et al., 2017). Membrane proteins such as sodium, potassium, and chloride transporters have also been reported to be involved in ion homeostasis and nutrient uptake (Nieves-Cordones et al., 2019) which severely gets affected during drought stress (Figure 6). Moreover, this signaling at the plasma membrane initiates a cascade of other regulatory processes such as phytohormone-mediated signaling, ROS scavenging, and controlling of transcription factors involved in growth and metabolism. These results suggest that regulation by miRNAs could be one of the initiating steps in the long cascade of gene regulation associated with drought tolerance.

The identification of miRNAs and their putative target genes from pearl millet genotypes showing a contrasting response to high VPD and drought has generated an important resource that could be tapped for more insights into underlying molecular mechanisms. The identification of root specific and high-VPD induced novel Pg-miRNAs warrants further studies to characterize and validate their functions. The Pg-miRNAs identified from this study could be used as markers to screen drought tolerant pearl millet varieties and genotypes. While highly expressed miRNAs such as Pg-miR169 and Pg-miR1_HR-ICMR 1122 from ICMR 1122 could be used as markers for drought tolerance traits, miRNAs such as Pg-miR319a, Pg-miR862, Pg-miR399a, Pg-miR4_LR-ICMR 1152, and Pg-miR5_LR-ICMR 1152 could very well be used to screen for drought sensitivity. Furthermore, the knowledge from this study could be used to design miRNA-SSR markers, thereby contributing to the marker-assisted breeding programs in pearl millet.

5 | CONCLUSIONS

In this study, we investigated the morpho-physiological response of two pearl millet genotypes, ICMR 1122 and ICMR 1152 in response to low and high VPD conditions. ICMR 1152 showed lowered transpiration, root sap exudation rates, and higher TE as compared to ICMR 1122, under high VPD. Furthermore, we identified root specific miRNAs from these genotypes that showed contrasting physiological responses when subjected to low and high VPD. The identified miRNAs included novel, known, and conserved Pg-miRNAs from both genotypes, that showed differential expression in response to contrasting VPD conditions. The GO and PMN pathway analysis of predicted target genes of the differentially expressed miRNAs indicated their contribution to drought tolerance through various mechanisms such as modulation of lateral root development, ROS scavenging, ABA response, and water/nutrient uptake. This study has generated a new pearl millet miRNA resource that could be tapped into to advance our understanding of high VPD tolerance mechanisms in pearl millet.

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AUTHOR CONTRIBUTIONS

Palakolanu Sudhakar Reddy and Pooja Bhatnagar-Mathur conceived the work and designed the experiments. Saurabh Gupta, Richa K Yeshvekar, Sivasakthi Kaliamoorthy, Sivarama Prasad Lekkala, Aishwarya Rajesh Shankhapal, Ashwini Soumya Vempati, Navajeet Chakravarty, and Rajadurai Chinnaasamy Perumal performed in silico, in vitro, and wet lab experiments. Palakolanu Sudhakar Reddy, Navajeet Chakravarty, Saurabh Gupta, Pooja Bhatnagar-Mathur, Manuel Philip, Boney Kuriakose, and Vijaya Bhaskar Reddy Lachagari analyzed the results. Palakolanu Sudhakar Reddy, Saurabh Gupta, Richa K Yeshvekar, Vijaya Bhaskar Reddy Lachagari, and Pooja Bhatnagar-Mathur contributed to writing the manuscript, discussed the results, and commented on the manuscript.

DATA AVAILABILITY STATEMENT

Raw sequence reads of small RNA sequencing data are available in the NCBI SRA under accession no PRJNA594828.

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