Pearl Millet Aquaporin Gene PgPIP2;6 Improves Abiotic Stress Tolerance in Transgenic Tobacco

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Pearl millet [Pennisetum glaucum (L) R. Br.] is an important cereal crop of the semiarid tropics, which can withstand prolonged drought and heat stress. Considering an active involvement of the aquaporin (AQP) genes in water transport and desiccation tolerance besides several basic functions, their potential role in abiotic stress tolerance was systematically characterized and functionally validated. A total of 34 AQP genes from P. glaucum were identified and categorized into four subfamilies, viz., plasma membrane intrinsic proteins (PIPs), tonoplast intrinsic proteins (TIPs), nodulin-26-like intrinsic proteins (NIPs), and small basic intrinsic proteins (SIPs). Sequence analysis revealed that PgAQPs have conserved characters of AQP genes with a closer relationship to sorghum. The PgAQPs were expressed differentially under high vapor pressure deficit (VPD) and progressive drought stresses where the PgPIP2;6 gene showed significant expression under high VPD and drought stress. Transgenic tobacco plants were developed by heterologous expression of the PgPIP2;6 gene and functionally characterized under different abiotic stresses to further unravel their role. Transgenic tobacco plants in the T2 generations displayed restricted transpiration and low root exudation rates in low- and high-VPD conditions. Under progressive drought stress, wild-type (WT) plants showed a quick or faster decline of soil moisture than transgenics. While under heat stress, PgPIP2;6 transgenics showed better adaptation to heat (40°C) with high canopy temperature depression (CTD) and low transpiration; under low-temperature stress, they displayed lower transpiration than their non-transgenic counterparts. Cumulatively, lower transpiration rate (Tr), low root exudation rate, declined transpiration, elevated CTD, and lower transpiration indicate that PgPIP2;6 plays a role under abiotic stress tolerance. Since the PgPIP2;6 transgenic plants exhibited better adaptation against major abiotic stresses such as drought, high VPD, heat, and cold stresses by virtue of enhanced transpiration efficiency, it has the potential to engineer abiotic stress tolerance for sustained growth and productivity of crops.

Keywords: canopy temperature depression (CTD), exudation rate, Pennisetum glaucum, PgPIP2;6, progressive drought stress, transpiration rate, transpiration efficiency, vapor pressure deficit
INTRODUCTION

Pearl millet [Pennisetum glaucum (L) R. Br.], which is a diploid (2n = 14) cereal crop from the Poaceae family, is the sixth most important cereal crop providing food security to over 500 million people across Africa and Asia. It is known to be a versatile dryland cereal used as multi-purpose crop for food, feed, fuel, construction material, and forage, especially in the arid and semiarid regions (Nambiar et al., 2011; Vadez et al., 2012). Its germplasm displays a high degree of genetic diversity and variable tolerance to most of the abiotic stresses, thereby providing better grain yield in areas that are too harsh for profitable production of other cereals like rice, wheat, sorghum, and maize. Pearl millet possesses abiotic stress tolerance due to efficient water conservation mechanisms (Kholová et al., 2010a; Reddy et al., 2017, 2021), which is considered as an efficient way of enhancing crop yield under terminal drought stress (Kholová et al., 2010a; Zaman-Allah et al., 2011b; Belko et al., 2012). Enhanced water conservation traits (genes that relate to hydraulic processes in the plant would be highly desirable for better drought adaptation of pearl millet cultivars (Kholová et al., 2010a,b, 2012; Reddy et al., 2017, 2021; Tharanya et al., 2018). The rapid response of the transpiration rate (Tr) to increased vapor pressure deficit (VPD) suggests a possible role of hydraulic signals mediating the Tr response upon rising VPD. These hydraulic signals are regulated by aquaporins (AQPs), the specialized membrane transporters that play a pivotal role in conducting water at the cellular level (Reddy et al., 2017; Shekoofa and Sinclair, 2018; Tharanya et al., 2018).

Aquaporins (AQPs) are members of the Major Intrinsic Proteins (MIP) family, which is represented in all kingdoms from archaea, animals to plants (Gomes et al., 2009; Maurel et al., 2015; Deshmukh et al., 2016). They are 23–31 kDa proteins having several specific characteristic features, including transmembrane helices (TM) linked by five inter-helical loops (LA-LE) and conserved dual aspargine-proline-alanine (NPA) motifs (Maurel et al., 2008). A number of AQPs have been identified in diverse plant species, including 35 in Arabidopsis (Johanson et al., 2001), 43 in maize (Chaumont et al., 2001), 34 in rice (Sakurai et al., 2005), 58 in Populus (Gupta and Sankaramakrishnan, 2009), 72 in soybean (Zhang et al., 2013), 41 in Sorghum (Reddy et al., 2015a), 57 in Brassica rapa (Sonah et al., 2017), 28 in Beta vulgaris (Kong et al., 2017), 65 in wheat (Madrid-Espinoza et al., 2018), 41 in Setaria italica (Singh et al., 2019), 39 in cucumber (Zhu et al., 2019), 76 in tobacco (De Rosa et al., 2020), and 38 in pomegranate (Liu et al., 2021). Plant AQPs show a high degree of gene multiplicity and assembled as tetramers that are typically divided into seven subfamilies, including plasma membrane intrinsic proteins (PIPs), tonoplast intrinsic proteins (TIPs), nodulin-26-like intrinsic proteins (NIPs), and small basic intrinsic proteins (SIPs) that have been identified in both primitive and higher plants (Anderberg et al., 2012). Hybrid intrinsic proteins (Hips), GlpF-like intrinsic proteins (GIPs) (Danielson and Johanson, 2008), and X-intrinsic proteins (XIPs) have only been identified in mosses as well as several dicotyledonous plants (Bienert et al., 2011), including soybean (Zhang et al., 2013), but were not identified in monocots. These subclasses have been defined based on sequence similarity as well as intracellular localization (Maurel et al., 2015). AQPs received special attention due to their association with water deficit conditions and correlated with the abiotic stress tolerance mechanism (Sreedharan et al., 2013; Afzal et al., 2016). Although a few AQPs have been identified and functionally characterized from crop plants, except for some preliminary reports, there is no detailed investigation on the functional validation of the AQP gene family in millets, including pearl millet (Reddy et al., 2017; Grondin et al., 2020).

Studies on AQPs have been demonstrated in both model and crop plants under different abiotic stresses, where thePIP family has been shown to play a major role in abiotic stress tolerance by modulating the water transport pathway (Fetter et al., 2004; Yaneff et al., 2014). The overexpression of the RsPIP2;1 gene has been shown to enhance drought and salt tolerance in transgenic Eucalyptus (Tsuchihira et al., 2010), while TdPIP1;1 and TdPIP2;1 from durum wheat and HvPIP2;5 from barley have been shown to improve salinity and osmotic stress tolerance in tobacco and Arabidopsis, respectively (Ayadi et al., 2011; Alavilli et al., 2016). Upon overexpression of AQPs from tomato (SIPIP2;1, SIPIP2;7, or SIPIP2;5) and rice (OsPIP1 and OsPIP2) in Arabidopsis, higher hydraulic conductivity levels and survival rates were displayed under drought stress conditions (Mosa et al., 2012; Li et al., 2016). Specific AQP inhibitors, such as HgCl2, AgNO3, and H2O2, have rapidly reduced the root hydraulic conductivity in chickpea and pearl millet (Sivasankthi et al., 2017a, 2020; Tharanya et al., 2018). Grondin et al. (2020) reported that pearl millet genotypes contrasting for water use efficiency differed in AQP gene expression. PgPIP1;3 and PgPIP1;4 were significantly more expressed in the low-water-use efficiency line than the high-water-use efficiency line. In addition, the low-water-use efficiency line has more reduction of root hydraulic conductivity by azide (AQP inhibitor) treatment than the high-water-use efficiency line. Our earlier report, PgPIP2;3, showed higher transcript abundance in a high transpiration rate (TR) than the low TR line under high-VPD conditions (Reddy et al., 2017).

The overexpression of the StPPI1 gene was shown to ameliorate drought tolerance in potato where it improved water use efficiency, increased non-structural carbohydrates, thereby minimizing carbon starvation, and increasing biomass yield (Wang et al., 2017). The ectopic expression of MaPPIP1;1 and MaPPIP2;7 was shown to display multiple abiotic stress tolerance in transgenic banana (Xu et al., 2020, 2021), while transgenic rice overexpressing OsPPI1;3 promoted plant growth and water uptake (Liu et al., 2019). More recently, PePPIP2;7 from bamboo has been demonstrated to confer abiotic stress tolerance in yeast and Arabidopsis (Sun et al., 2021).

Since pearl millet is a robust cereal crop of the semiarid tropics, it is important to understand different components of the abiotic stress tolerance mechanism. We systematically analyzed the sequences and relative expression of all PgAQPs under high VPD and progressive drought stress, besides the functional characterization of the PgPIP2;6 gene in tobacco. This work...
provides insights on the molecular basis of water conservation mechanisms in pearl millet.

MATERIALS AND METHODS

Identification of Pearl Millet Aquaporin Genes
Pearl millet AQP gene sequences were retrieved using maize, rice, and sorghum AQP genes as a search query and scanned against the pearl millet genome by NCBI blast N, X, and P databases. Chromosomal locations of PgAQPs were determined with the information obtained from the pearl millet genome database. The intron-exon structures were predicted using the Gene Structure Display Server (Hu et al., 2015), and the MOTIF search was used to retrieve the conserved domains. Sequence analysis, including the open reading frame (ORF) and amino acid (AA) translations, molecular weight, and isoelectric point (pI) of PgAQPs genes, was analyzed using the MacVector software (V16.09). The GRAVY (grand average of hydropathicity) instability and aliphatic indices were predicted by employing ProtParam of Expasy tools (Bailey et al., 2009). PAL2NAL software was used to calculate the set in-between 3 and 60, while parameters were set as default parameters such as the number of motifs 10, width of the motifs 4, and motifs 12. The intron-exon structures were predicted using the Gene Structure Display Server (Guo et al., 2015), while the TMHMM server was used to screen the putative trans-membrane helices (Moller et al., 2001). Phosphorylation sites (serine, threonine, and tyrosine) of the PgAQPs were searched using the NetPhos 3.1 program (Bloom et al., 2004). Multiple sequence alignment (MSA) and phylogenetic analysis were carried out with full-length protein sequences of closely related AQPs from monocots and dicots using the ClustalW program of MacVector and MEGA 6.0 (Tamura et al., 2013). Bootstrap analysis was performed using 1,000 replicates, and the branch lengths corresponded to phylogenetic distances in units of the number of amino acid substitutions per site. Conserved motifs were identified with the help of the MEME suite with parameters such as the number of motifs 10, width of the motifs set in-between 3 and 60, while parameters were set as default (Bailey et al., 2009). PAL2NAL software was used to calculate the synonymous and non-synonymous sites, their substitution rates, and dN/dS for the PgAQPs orthologs and paralogs (Suyama et al., 2010a) and high VPD (Reddy et al., 2017, 2021) where the controls were maintained at 26°C for high-VPD treatment. Leaf and root tissues from the respective plants were collected and immediately snap frozen in liquid nitrogen for total RNA extraction and qRT-PCR analysis. Three biological replicates were maintained and collected for each stress treatment.

Cloning and Genetic Transformation of PgPIP2;6 Gene
Based on the expression profile, PgPIP2;6 gene was selected and PCR amplified from the pearl millet cDNA and cloned between the CaMV35S - Nos terminator in the Gateway-compatible entry vector (pL12R34H-Ap) by digesting with NdeI and NotI restriction enzymes. The expression cassette of PgPIP2;6 gene (CaMV35S:PgPIP2;6:NosT) from the entry vector was further cloned into the Gateway-based plant transformation vector pMDC100 using LR Gateway cloning. The recombinant plasmid was transformed into Agrobacterium tumefaciens strain EHA105 by electroporation and used for tobacco transformation as reported previously (Divya et al., 2019); the selected rooted shoots were transferred to pots containing autoclaved sand and soil (1:1) mixture and maintained in a containment greenhouse until flowering and the seed set.

Growth of Tobacco Transgenic Plants
The untransformed wild type (WT) plants were used as negative control and a recombinant plasmid containing the PgPIP2;6 expression cassette as positive control. Fully grown transgenic tobacco plants (T1 generation) were self-pollinated using paper bags to produce T2 seeds. Progeny plants (T2) selected with kanamycin (50 mg L⁻¹) on an agar medium were grown in a growth chamber at 25°C at a photoperiod of a 16-h light and 8-h dark cycle, followed by their advancement to T2 generation for further analysis. The T2 generation plants and their untransformed wild types were maintained at 25–27°C with a 16-h light and 8-h dark cycle with 300 μmol m⁻² s⁻¹

\[1\] http://www.gigadb.org/dataset/100192
\[2\] http://gsds.cbi.pku.edu.cn
\[3\] http://www.gnome.jp/tools/motif/
\[4\] http://web.expasy.org/protparam
\[5\] http://www.genscript.com/wolf-psort.html
\[6\] http://www.cbs.dtu.dk/services/TMHMM/
\[7\] http://www.cbs.dtu.dk/services/NetPhos/
\[8\] http://meme-suite.org/tools/meme
\[9\] http://bioinformatics.psb.ugent.be/webtools/plantcare/html/
\[10\] http://swissmodel.expasy.org

\[11\] https://bioinfo3d.cs.tau.ac.il/patchdock
\[12\] http://bioinfo3d.cs.tau.ac.il/firedock
light and 70-80% relative humidity (RH) in a biosafe glasshouse and used for physiological and molecular analysis. The genomic DNA of the putative Tₒ tobacco transgenic plants (20 days) was isolated using the DNeasy Plant Mini Kit (Qiagen, Germany). Primer sets for CaMV35S_F (GATATCGTACCCTACTCCCA)-PgPIP2;6_R (AGAAGACGGTGTAGACGAGCA) and NptII_F - NptII_R were used to confirm the transgene integration.

**Measurement of Vapor Pressure Deficient**

The T₂ generation transgenic tobacco plants and the WT plants were grown in 8-inch plastic pots filled with 5.5 kg of Alfisol where 16 replicate pots of each genotype were grown and the 10 most uniform plants were selected to design the experiments in controlled environment growth chambers. One day before the experiment, the pots were well watered, allowed to drain to a field capacity, and bagged with a transparent plastic bag wrapped around the plant stem to prevent the evaporation of soil water during the evaluation of plant transpiration. In controlled growth chambers, the transpiration rate response to stable and increasing VPD is carried out; for stable VPD, the chamber was maintained at 1.2 kPa throughout the experiment, whereas, for increasing VPD or treatment, the chamber VPD was raised from 0.6 to 3.80 kPa gradually (Supplementary Table 5). Ten replications of wild-type and transgenic tobacco plants were used in both stable and increasing VPD chambers to measure the plant transpiration response throughout the day. An equal number of WT plants were also maintained in two chambers where one chamber was maintained at 1.2 kPa throughout the experiment (a low-VPD chamber), while the other had a ladder of VPD ranging from 0.69 to 3.8 kPa (a high-VPD chamber) with the VPD stress imposed as described earlier (Reddy et al., 2017, 2021) (Supplementary Table 5). Within each VPD level, the plants were weighed gravimetrically every 60 min with 0.01-g precision scales (KERN 3600-2N, Kern & Sohn GmbH, Balingen, Germany) to derive transpiration values from the control and heat-stressed plants were collected, snap frozen in liquid nitrogen and stored at −80°C until RNA extraction.

**Heat Stress**

The plants grown in a glasshouse for 45 days were exposed to heat stress at 40°C for 4 h and RH of 35%. Other set of plants was kept in control conditions in the growth chamber (28°C and 70% RH). Transpiration in both heat-stressed and control-chamber-grown plants was measured by weighing the pots before and after heat stress treatments. In addition, thermal images (infra-red) were taken in both heat-stressed and control plants in the chamber using infrared (IR) FlexCam S (Infrared Solutions, Plymouth, MN, United States) with a sensitivity of 0.09°C with an accuracy of ± 2%. SmartView 2.1.0.10 software (Fluke Thermography Everett, WA, United States) was used for the analysis of thermal images and estimation of canopy temperatures (Zaman-Allah et al., 2011a; Sivasakthi et al., 2017b). Leaf and root tissues from the control and heat stressed plants were collected, snap frozen in liquid nitrogen, and stored at −80°C for RNA extraction.

**Cold Stress**

One set of 45-day-old plants was exposed to 4°C for 4 h in the cold room, while the other set of plants was maintained in a growth chamber (28°C and 70% RH) as controls. Transpiration in both cold-stressed and control plants was measured before and after stress treatment. Leaf and root tissues from the control and cold-stressed plants were collected, snap frozen in liquid nitrogen, and preserved at −80°C for RNA extraction.

**Quantitative Real-Time PCR Analysis**

Total RNA was isolated from pearl millet plants (VPD sensitive and insensitive genotypes treated with high VPD and progressive drought stress) and transgenic tobacco plants (control and
treated) using 100 mg of tissue with RNEasy Plant Mini kit (Qiagen, Germany). The quantity and the quality of RNA were analyzed using a NanoVue plus spectrophotometer (GE Health Care, United States) and the ratio of the absorbance measured at 260 and 280 nm (260/280) of the samples ranging from 1.8 to 2. The integrity of the RNA was further verified through denaturing 1.4% agarose gel and Bioanalyzer. The total RNA isolated from control and treated samples was diluted to 30 ng/μl concentrations for the direct use in qRT-PCR reactions performed in the optical 96-well plates on a Realplex (Eppendorf, Germany). Reactions were performed in a total volume of 10 μl, containing 1 μl of RNA (30 ng), 400 nM of each primer, 5 μl of 2 × one step SYBR RT-PCR buffer 4 (Takara, Japan), and 0.5 μl of Prime Script One Step Enzyme Mix 2 (Takara, Japan), and final volume made up to 10 μl with RNase-free H2O. qRT-PCR reactions of all the samples were carried out by following a standard thermal profile: 42°C for 5 min and 95°C for 10 s (reverse transcription), followed by 40 cycles of 15 s at 95°C, 15 s at 62°C with a fluorescent signal recording and 15 s at 72°C. Amplicon dissociation curves were recorded after 40 cycles by heating from 58 to 95°C with fluorescence measured within 20 min. The experiments were independently repeated three times, and the data from these experiments were averaged. The relative expression levels of PgAQP genes in response to high VPD and progressive drought stress in Tr contrasting genotypes were estimated using qBase software (Hellemans et al., 2007) by normalizing with corresponding control samples as well as with reference genes (Reddy et al., 2015b). Relative expression levels of the PgPIP2;6 gene in response to different tissues of transgenic tobacco and abiotic stress treatments were estimated using the 2−ΔΔCt method (Livak and Schmittgen, 2001) by normalizing with L25 and EF-1 alpha reference genes (Schmidt and Delaney, 2010).

**Statistical Analysis**

Transpiration response to increasing VPD (a high-VPD chamber) was analyzed with non-linear regression of Graph pad Prism version 6 (Graph Pad software, Inc., CA, United States), which provides r2, a break point, and slope values. For the progressive drought stress experiment, the relationship between NTR and FTSW was analyzed with non-linear regression of Graph pad Prism version 6 (Graph pad software, Inc., CA, United States), which provides breakpoint values (Kholová et al., 2010b). Software SmartView 2.1.0.10 (Fluke Thermography) was used for analysis of the thermal images and the estimation of canopy temperatures (Zaman-Allah et al., 2011a). CTD was calculated as the difference between the air temperature (T) and leaf temperature (CTD = T air – T leaf). The relative expression of the PgPIP2;6 gene under different abiotic stresses was analyzed according to Livak and Schmittgen (2001). All the data from stress experiments (VPD, drought, heat, cold) and relative expressions were analyzed with statistical program package CoStat version 6.204 (Cohort Software, Monterey, CA, United States). One-way ANOVA was carried out to test for genotypic difference among the transgenic events and WT. The means were compared using Tukey-Kramer test and least significant difference (LSD) (at p ≤ 0.05).

**RESULTS**

**Identification of Aquaporin Gene Family and Sequence Analysis**

A phylogeny of AQPs was carried out to understand the evolutionary relationships among pearl millet AQPs within *Pennisetum* and with that of sorghum, maize, and rice (Figure 1A and Supplementary Figure 1). PgAQPs were grouped into four major subfamilies based on their conserved and high similarity index, including PgPIPs, PgTIPs, PgNIPs, and PgSIPs (Figure 1A). A total of 34 *PgAQP* genes were identified in pearl millet and named based on their relationship with their counterparts in the related species (Table 1 and Supplementary Figure 1). Among all classes, the PgPIP subfamily occupied the highest number of AQP genes with 11 members. Exon/intron structure displayed a range of 1–19 (PgSIP2;1) introns, the highest number present in the PgNIP subfamily and the lowest in the PgTIP subfamily (Table 1 and Figure 1B). The identified AQPs were distributed on six chromosomes of pearl millet. Of the 34, 3 were located on Chromosome 1, 7, 2, 15 on 3, 5, 4, 2 on 5, and 1 on 6. While Chromosome 3 was the hot spot with a maximum number of AQP genes with most PIPs and TIPs, Chromosome 7 did not show any AQP genes (Table 1). PgAQPs ORFs ranged from 636 bp (PgPIP2;8) to 2,823 bp (PgNIP4;1) with an average size of 528 bp. PgAQP proteins consisted of amino acids, ranging from 211 (PgPIP2;8) to 940 (PgNIP4;1), majority of them being basic, where the pI values varied between 4.9 (PgNIP1;4) and 9.7 (PgPIP2;2). The MWs ranged from 21K Da to 99.79K Da with an instability index varying from 20.68 (PgTIP2;2) to 41.75 (PgNIP4;1), which was the only unstable one. The GRAVY values indicated that most of them were hydrophobic except PgSIP2;1, the only hydrophilic AQP, and all exhibiting the highest aliphatic index (Table 1). Most of the PgAQPs showed the presence of serine, threonine, and tyrosine, where serine was the most dominating phosphorylation site (Supplementary Table 1). The highest number of serine residues was observed in the PgNIP subfamily, followed by PIPs and TIPs. Protein kinase C (PKC) and an unspecific kinase were the major types present in all the proteins. The common kinases in all subfamilies were next to PKC, cdc2, PKA, CDK1, DNAPK, P38MAPK, and PKG (Supplementary Table 1). Localization of PgAQPs indicated that, while they were abundant in the plasma membranes with an average of six transmembrane helices, only NIP3 subfamily members were found in the vacuole (Table 1). Motif organization of the four subfamilies of PgAQP was deduced by submitting protein sequences to MEME31. A total of 10 conserved motif sequences were identified in 34 PgAQP proteins, showing group-specific distribution. Motifs 1 and 5 were the most conserved across all PgAQPs subfamilies positioned at C-terminus (Supplementary Figure 2). While motifs 6 and 7 were conserved in all the PgTIPs and PgNIPs, motifs 2 and 8 were noticed at the PgPIP subfamily’s N-terminus and Motif 9 in TIP and NIP subfamilies. Motif 3 was common in all the groups situated in the middle (Supplementary Figures 3, 4).

https://meme-suite.org/meme/tools/meme
All PgAQP proteins had the inherent conserved NPA motif (Motifs 1 and 3) and the aromatic/arginine (ar/R) selectivity filter (Supplementary Figures 2, 3).

A total of 13 paralogs were identified where five were from NIPs, four from PIPs, three from TIPs, and one from SIP. The paralog’s duplication events were grouped into regional and segmental gene duplications based on their chromosomal localization and phylogenetic analysis. Of the 13 events, five were regional duplications; PgPIP1;1/PgPIP1;2, PIP2;3/PIP2;5, and PIP2;6/PIP2;8 were noticed on Chromosome 3 and NIP3;2/NIP3;5, and NIP3;3/NIP3;4 on Chromosome 4. The remaining eight were segmental duplication events (Supplementary Table 1). Furthermore, among the non-synonymous to synonymous substitutions, out of 13, 9 events exhibited purifying/stabilizing selection (<1), while the remaining four were positive/Darwinian selection (>1). The evolutionary relationship of the pearl millet with others showed a class-specific, group-specific relationship (Supplementary Table 1). Of the 12 orthologous events, six had an orthologous relationship with sorghum while three each with maize and rice. There were only three orthologous duplication events (PgPIP2;7/SbPIP2;7, PgTIP5;1/SbTIP5;1, and PgNIP3;2/SbNIP3;2) that showed positive selection, while the remaining nine events exhibited Darwinian selection pressure, purifying/stabilizing selection during evolution. While sorghum and maize showed 16 orthologous events, sorghum and rice exhibited four events, rice and maize exhibited one orthologous event (Supplementary Figure 1).

**Promoter Analysis**

To understand the regulatory mechanism of PgAQP genes of pearl millet, cis-acting elements specific to tissue and abiotic stress within the upstream regions of PgAQP genes were identified (Supplementary Table 4). Up to seven Skn1 elements specific to endosperm expression were detected in majority of the PgAQP genes. GCN4 was another endosperm specific element present only in the PgPIP genes (PgPIP2;1, PgPIP2;2, PgPIP1;1, and PgPIP1;2). CAT-box and CCGTCC-box related to meristem expression were noticed in the promoter regions of all the PgAQP genes. Along with tissue-specific cis-elements, several abiotic stress-inducible cis-elements were detected in the promoter regions of the PgAQP genes. HSE cis-element involved in heat stress was observed in the promoter regions of 6 PgAQP genes (PgPIP1;1, PgPIP2;1, PgTIP1;1, PgTIP2;2, PgNIP1;3, and PgNIP3;4). The cold-responsive LTR element was noticed in 11 PgAQP genes (PgPIP1;1, PgPIP1;6, PgPIP2;4, PgPIP2;7, PgPIP2;8, PgTIP2;1, PgTIP2;2, PgTIP2;3, PgTIP4;1, PgNIP3;1, and PgSIP1;1). While over 50% of the PgAQP genes had drought-inducible MBS elements, the ABRE cis-acting element involved in the abscisic acid (ABA)-inducibility was detected in the promoter regions of 15 PgAQP genes. The TCA cis-element involved in salicylic acid responsiveness was noticed in the promoter regions of the four PgAQP genes. CGTCA and TGACG-motifs involved in the MeJA responsiveness were recognized in most of the upstream regions of PgAQP genes. Several core promoter regions were observed in PgAQP promoters, including TATA-box, CCAAT-motif, and TC-rich repeats (Supplementary Table 4).

**Expression of PgAQP Genes Under Progressive Drought and High Vapor Pressure Deficit Stress in Pearl Millet**

**Progressive Drought Stress**

The expression profiling of PgAQPs was investigated among VPD-insensitive - ICMR 1122 and VPD-sensitive - ICMR 1152 genotypes in leaf and root tissues under progressive drought and high-VPD stresses using qRT-PCR. To understand the function of AQP genes in drought stress adaptation/tolerance of pearl millet, progressive drought stress was imposed in pearl millet genotypes (ICMR 1122 and ICMR 1152), contrasting for...
### TABLE 1 | Physico-chemical characteristics of PgAQPs.

| S. no | Accession number | Gene Role in Abiotic Stress Tolerance |
|-------|------------------|--------------------------------------|
| 1     | Pgl_GLEAN_10010809 | PgPIP1;1, PgTIP1;1, PgNIP4;6, PgSIP1;1, PgNIP3;3, PgNIP3;5, PgPSP1;1, PgPSP1;2 |
| 2     | Pgl_GLEAN_10001520 | PgPIP1;2, PgTIP2;1, PgNIP1;3, PgNIP2;1, PgNIP3;4, PgNIP3;5, PgPSP1;1, PgPSP1;2 |
| 3     | Pgl_GLEAN_10005724 | PgPIP1;6, PgTIP2;2, PgNIP1;4, PgNIP2;2, PgNIP3;3, PgNIP3;5, PgPSP1;1, PgPSP1;2 |
| 4     | Pgl_GLEAN_10028064 | PgPIP2;1, PgTIP2;3, PgNIP1;5, PgNIP2;3, PgNIP3;4, PgNIP3;5, PgPSP1;1, PgPSP1;2 |
| 5     | Pgl_GLEAN_10009812 | PgPIP2;2, PgTIP2;4, PgNIP1;6, PgNIP2;4, PgNIP3;4, PgNIP3;5, PgPSP1;1, PgPSP1;2 |
| 6     | Pgl_GLEAN_10028056 | PgPIP2;3, PgTIP2;5, PgNIP2;3, PgNIP2;5, PgNIP3;4, PgNIP3;5, PgPSP1;1, PgPSP1;2 |
| 7     | Pgl_GLEAN_10005765 | PgPIP2;6, PgTIP2;6, PgNIP2;6, PgNIP2;7, PgNIP3;5, PgPSP1;1, PgPSP1;2 |
| 8     | Pgl_GLEAN_10010255 | PgPIP2;7, PgTIP2;7, PgNIP2;7, PgNIP3;5, PgPSP1;1, PgPSP1;2 |
| 9     | Pgl_GLEAN_10028060 | PgPIP2;8, PgTIP3;1, PgNIP2;1, PgNIP3;2, PgNIP3;3, PgPSP1;1, PgPSP1;2 |
| 10    | Pgl_GLEAN_10000671 | PgTIP1;1, PgTIP2;2, PgNIP1;3, PgNIP2;1, PgNIP3;3, PgPSP1;1, PgPSP1;2 |
| 11    | Pgl_GLEAN_10005761 | PgTIP1;2, PgTIP2;3, PgNIP1;4, PgNIP2;2, PgNIP3;3, PgPSP1;1, PgPSP1;2 |
| 12    | Pgl_GLEAN_10003011 | PgTIP2;4, PgNIP1;5, PgNIP2;3, PgNIP3;4, PgPSP1;1, PgPSP1;2 |
| 13    | Pgl_GLEAN_10009813 | PgTIP3;1, PgNIP2;4, PgNIP3;5, PgPSP1;1, PgPSP1;2 |
| 14    | Pgl_GLEAN_10000673 | PgTIP4;1, PgNIP1;6, PgNIP2;5, PgNIP3;5, PgPSP1;1, PgPSP1;2 |
| 15    | Pgl_GLEAN_10012175 | PgTIP4;3, PgNIP2;6, PgNIP3;6, PgNIP3;7, PgPSP1;1, PgPSP1;2 |
| 16    | Pgl_GLEAN_10009814 | PgNIP1;7, PgNIP2;7, PgNIP3;8, PgPSP1;1, PgPSP1;2 |
| 17    | Pgl_GLEAN_10005762 | PgNIP3;9, PgNIP4;7, PgPSP1;1, PgPSP1;2 |
| 18    | Pgl_GLEAN_10003012 | PgNIP4;8, PgPSP1;1, PgPSP1;2 |

**Note:** ORFs, number of amino acids (bp), proteins (amino acids), chromosomal localization, isoelectric point (pI), molecular weight (MW), DNA binding domains (DBD), number of introns, localization, GRAVY, instability index, aliphatic index, and transmembrane helices.
transpiration rate response to increasing VPD. Many PgAQP genes showed upregulation in both leaf and root tissues upon drought imposition (Figure 2A). PgPIP2;6 of ICMR 1152 and NIP1;3 and PgNIP1;3 of ICMR 1122 exhibited the highest level of expression in leaf as well as root tissues. Similarly, drought stress caused upregulation of six PgAQP genes like PgPIP2;5, PgPIP2;6, PgNIP3;2, PgNIP3;3, PgNIP3;5, PgNIP4;1, in both the tissues of the genotype ICMR 1152. PgTIP2;3, PgPIP2;8, and the PgTIPs, such as PgNIP1;1, PgNIP1;3, PgNIP3;1, PgNIP3;2, and PgSIP1;1, were upregulated only in leaf tissues. Notably, PgPIP1;2, PgPIP2;5, PgPIP2;6, PgTIP2;3, PgTIP3;1, PgTIP4;1, PgTIP4;3, PgTIP4;4, PgNIP1;1, PgNIP1;4, PgNIP1;3, PgNIP2;2, PgNIP3;3, PgNIP3;4, PgNIP3;5, PgNIP4;1, PgSIP1;1, PgSIP1;2, and PgSIP2;1 exhibited higher expressions in root tissues of the genotype ICMR 1122. In contrast, only one gene, PgNIP3;3, showed high transcript levels in the leaf tissues of same genotype. All genes recorded higher expressions in the ICMR 1152 genotype compared to ICMR 1122. The least expression/downregulation was noticed in almost all AQPs except a few in the leaf tissues of the ICMR 1122 genotype. In summary, differential expression patterns were observed in root tissues of ICMR 1122 and leaf and root tissues of ICMR 1152 (Figure 2A).

High Vapor Pressure Deficit
Under high-VPD conditions, PgAQP genes showed differential expressions where the AQP genes, viz., PgPIP1;6, PgPIP2;6, PgTIP4;1, PgTIP5;1, PgNIP1;1, PgNIP1;3, PgNIP1;4, PgNIP3;2, and PgNIP3;3, were upregulated in the root tissues of ICMR 1152 where expressions were highest among all the 34 tested PgAQPs. Interestingly, PgTIP3;1, PgTIP4;4, and PgNIP3;4 showed moderate or high expressions in roots and leaf tissues of ICMR 112 in comparison with ICMR 1152 (Figure 2B). Apart from these, 10 genes, namely, PgPIP1;1, PgPIP1;2, PgPIP2;2, PgPIP2;7, PgPIP2;8, PgTIP1;1, PgTIP2;1, PgTIP2;2, PgTIP4;1, and PgNIP3;2, displayed higher expression levels in the leaf tissues of ICMR 1152. Among them, PgPIP1;1, PgTIP1;1, PgTIP2;1, PgTIP2;2 and PgNIP3;2 exhibited expressions in the leaf tissues of ICMR 1122. Genes, such as PgNIP1;1, PgNIP1;3, PgNIP1;4, PgNIP3;2, PgNIP3;3, PgNIP3;4, and PgNIP5;1 exhibited significant upregulation in root tissues of both ICMR 1152 and ICMR 1122. Genes, such as PgTIP4;1, PgNIP1;3, and PgNIP3;3 in ICMR 1152 and PgTIP3;1, PgTIP4;4, PgNIP1;1, NIP1;3, and PgNIP4;1 in ICMR 1122 exhibited significant expressions in root tissues (Figure 2B). Other PgAQP genes revealed the lowest expression in both the tissues of ICMR 1122 upon high-VPD stress treatment. In general, the PgAQP genes were highly induced in root tissues than leaf tissues of both the genotypes in response to high-VPD stress.

Cloning and Transformation of Tobacco With PgPIP2;6
Based on the gene expression profile, the gene PgPIP2;6 was cloned into the PMDC100 vector under the control of CaMV35S promoter using the Gateway-cloning system. Plant expression
cassette harboring CaMV35s-PgPIP2;6-NosT (Figure 3) was transformed into tobacco and the putative transgenic plants confirmed by PCR with CaMV35S trans_F - PgPIP2;6 trans_R and nptII F- nptII R primers for integration of the transgenes. Out of the 41 T₀ transgenic plants, nine events in T₁ were PCR positive that were analyzed through Mendelian segregation, and three T₂ transgenic events (E 1–20, E 11–10, and E 28–9) were selected for further analysis.

**Physiological Performance of the PgPIP2;6 Transgenic Tobacco Plants**

**Transpiration Response to Increasing and Stable Vapor Pressure Deficit Conditions**

Transpiration rate (Tr) was gradually increased under a ladder or a high-VPD (0.6–3.8 kPa) chamber in both transgenic as well as WT plants (Figure 4A). By contrast, Tr was stable or parallel response in a low-VPD (1.2 kPa) chamber in both transgenic and WT plants (data not shown). Under high-VPD conditions, while Tr increased in all the tested genotypes, the slope of Tr decreased beyond the VPD breakpoint (2.4 kPa), thereby indicating limited transpiration response in most of the genotypes (Figure 4A). WT plants displayed higher Tr than the transgenic events (except in the transgenic event E 1–20). Transgenic events, such as E 11–10 and E 28–9, showed lower Tr at high VPD, indicating that they might have water-conserving character. Water conservation mechanisms were highly expressed under high evaporative demand. Mean Tr comparison between transgenic and WT plants in low-VPD and high-VPD-chamber conditions displayed statistically significant genotypic differences at p ≤ 0.05 (Figure 4B and Table 2). The root exudation rate was slightly higher when exposed to high VPD than in the low-VPD-exposed plants. Under low- and high-VPD (1.2 and 3.8 kPa) conditions, transgenic plants exhibited slightly lower root exudation rates than WT plants (Figure 4C), thereby implying lower Tr.

**Progressive Drought Stress**

Imposition of progressive drought stress on transgenic and WT tobacco plants revealed that WT plants utilized soil moisture more quickly than the transgenics that also showed genotypic differences with declined transpiration between WT and transgenic plants (Figure 5A). Segmental regression analysis revealed a slightly higher FTSW breakpoint value in WT plants than the transgenic events (except the event E 28–9), although the events E 1–20 and E 11–10 exhibited a slower decline in transpiration than the WT plants (Figure 5A). Total transpiration was measured in WW as well as in WS plants where a threefold total transpiration difference was recorded (Figure 5B). While under WW conditions, events E 1–20 and E 28–9 were significantly different from WT (Figure 5B and Table 2). Similarly, event E 28–9 was significantly different from WT under WS conditions (Figure 5B and Table 2).
**Temperature Stress**

Transpiration was measured in both control and heat-stressed plants where significant differences were noticed in their transpiration rates (Figure 6A), transgenics exhibiting lower transpiration than the WT plants (Figure 6A). Thermal image analysis revealed lower canopy temperature (CT) in transgenics than WT plants under high-temperature (40°C) stress (Figure 6B). Similarly, transgenics showed lower CT than the WT plants under optimum (28°C) and high-temperature (40°C) conditions (Figure 6C). Canopy temperature depression (CTD) analysis demonstrated higher CTD in transgenics than WT plants under high-temperature stress conditions (Figure 6D), which reverses the CT measurements. A comparison of the mean CT, CTD, and transpiration data among the transgenic and WT plants in both heat-stressed and control plants (optimum temperature, 28°C) revealed statistically significant \((p \leq 0.05)\) genotypic differences (Table 2). Under cold stress (4°C), there was no significant difference between transgenic and WT plants. By contrast, transgenic events E 1–20, E 11–10, and E 28–9 significantly lower transpiration than WT plants (Figure 7 and Table 2).

**Transgenic Tobacco Plants Have Higher Expression of the \textit{PgPIP2;6} Gene Under Abiotic Stresses**

Expression levels of the \textit{PgPIP2;6} gene in leaf and root tissues, subjected to different abiotic stress treatments, were compared with their corresponding control samples using the \(\Delta\Delta^{CT}\) method where a steady upregulation was recorded in response to high VPD stress. Expression of \textit{PgPIP2;6} gene increased significantly in all the tested transgenic events (Figure 8A). Its expression in leaves was higher than that in root tissues in all the three transgenic events except for E 1–20 in which the expression levels were comparatively less in both the tissues. Overall, the transgenic event E 28–9 showed the highest expression levels in both leaf and root tissues in comparison with other transgenic events (Figure 8A). These expression results were
closely related to Tr data where the event E 1–20 displayed higher Tr than the other event under high-VPD conditions (Figures 4A, 8A). The relative expression of \( \text{PgPIP2;6} \) was investigated under drought vis-à-vis WW conditions for each transgenic event. Under WS conditions, significant variation in the abundance of the \( \text{PgPIP2;6} \) transcript was observed among diverse transgenic events (Figure 8B). \( \text{PgPIP2;6} \) transgene expression was upregulated under WS in root as well as in the leaf tissues. Overall, the transgenic event E 11–10 showed significantly higher transcript abundance in root tissues than other transgenic events (Figure 8B). A correlation was noticed between physiological characteristics, the level of expression of \( \text{PgPIP2;6} \), and drought tolerance. Under heat stress, the \( \text{PgPIP2;6} \) transgene showed significant upregulation in all the transgenic events. Higher expression levels were observed in the leaf compared to root tissues in all transgenics (Figure 8C). Among all the tested transgenic events, E 28–9 showed higher expression in leaf as well as root tissues. These expression results agree with the physiological data (Figure 6) where the events with lower \( \text{PgPIP2;6} \) gene expression showed a higher transpiration rate under high-temperature conditions. Under cold stress, root tissues exhibited comparatively higher gene expression than the leaf where the transgenic event E 1–20 showed the highest expression in root tissues than the other two events (Figure 8D). \( \text{PgPIP2;6} \) expression levels were upregulated in all the transgenic events under cold stress conditions that are similar to the Tr data obtained from the physiological experiments (Figure 7).

### TABLE 2 | Summary of different physiological traits that contribute to different abiotic stress tolerance in \( \text{PgPIP2;6} \) transgenic and WT tobacco plants.

| S.no | Trait name | E 1–20 | E 11–10 | E 28–9 | WT | L.S.D. (0.05) | \( P \)-value significance | Stress |
|------|------------|--------|---------|--------|----|--------------|-----------------------------|--------|
| 1    | Transpiration rate – Low VPD (mg H\(_2\)O cm\(^{-2}\) min\(^{-1}\)) | 0.18a | 0.13bc | 0.11c  | 0.15b | 0.019 | *** | VPD  |
| 2    | Transpiration rate – High VPD (mg H\(_2\)O cm\(^{-2}\) min\(^{-1}\)) | 0.32a | 0.19c | 0.16c  | 0.27b | 0.024 | *** |  |
| 3    | Root exudation rate – Low VPD (mg H\(_2\)O cm\(^{-2}\) min\(^{-1}\)) | 0.00121ab | 0.001006b | 0.00121ab | 0.00151a | 0.00028 | * |  |
| 4    | Root exudation rate – High VPD (mg H\(_2\)O cm\(^{-2}\) min\(^{-1}\)) | 0.001715ab | 0.001268b | 0.00129b | 0.00232a | 0.00073 | * |  |
| 5    | Leaf area VPD (cm\(^{-2}\)) | 501c | 843b | 1091a  | 1086a | 83.709 | ** |  |
| 6    | Total chlorophyll content (\(\mu\)mol per m\(^2\) of leaf) | 27.26b | 28.03ab | 30.33a  | 25.90b | 2.060 | * |  |
| 7    | Total Biomass (g) | 3.31c | 7.07b | 9.46a  | 6.0415b | 1.189 | ** |  |
| 8    | Total transpiration-Well watered (g) | 1177.11b | 1238ab | 1217.88b | 1370.44a | 103.72 | ** | Progressive drought stress |
| 9    | Total transpiration-Well stress (g) | 309.41ab | 309.5ab | 297.58b | 325.16a | 13.68 | ** |  |
| 10   | Total Biomass-Well watered (g) | 8.8216c | 13.6266ab | 15.7433a | 11.7933a | 2.147 | ** |  |
| 11   | Total Biomass-Water stress (g) | 5.39c | 9.8244ab | 11.333a | 8.4133b | 1.822 | ** |  |
| 12   | Relative water content (%)-Well watered | 95.63bc | 94.30c | 96.39b | 98.96a | 1.479 | *** |  |
| 13   | Relative water content (%)-Water stress | 88.40b | 93.39a | 91.90ab | 93.24a | 2.045 | ** |  |
| 14   | Transpiration- Control at 28°C (g H\(_2\)O in 3 h) | 18.46d | 28.46b | 22.2c  | 34.26a | 2.77 | *** | Heat |
| 15   | Transpiration-Heat stress at 40°C (g H\(_2\)O in 3 h) | 46b | 56.33ab | 62.33ab | 74a | 19.101 | ns |  |
| 16   | Canopy temperature-Control at 28°C | 29.402b | 29.163b | 28.500c | 31.157a | 0.307 | *** |  |
| 17   | Canopy temperature-Heat stress at 40°C | 35.218c | 36.230b | 36.560b | 38.090a | 0.482 | *** |  |
| 18   | Canopy temperature depression (CTD)-Control at 28°C | - | - | -0.500a | -3.156c | 0.309 | *** |  |
| 19   | Canopy temperature depression (CTD)-Heat stress at 40°C | 4.781a | 3.769b | 3.439b | 1.909c | 0.483 | *** |  |
| 20   | Transpiration- Control at 28°C (g H\(_2\)O in 3 h) | 18.46d | 28.46b | 22.2c  | 34.26a | 1.28 | *** | Cold |
| 21   | Transpiration-Cold stress at 4°C (g H\(_2\)O in 3 h) | 15a | 15a | 16a    | 21a | 4.416 | * |  |

Means with different letters of alphabets are significantly different (\( P < 0.05 \)) and the same letters represent the absence of significant difference. The asterisk *, **, and *** symbols are significantly different at \( P < 0.05, P < 0.01, P < 0.001 \), and ns: non-significant, respectively.
**DISCUSSION**

Maintaining water budget levels under different abiotic stress conditions is crucial for the plants to survive and to employ several adaptive mechanisms to cope with the environmental stresses (Mahdieh et al., 2008). *AQP* genes play critical roles in water transport, homeostasis, growth, and development of plants, including other organisms (Luu and Maurel, 2005; Galmés et al., 2007; Li et al., 2015; Afzal et al., 2016; Ranganathan et al., 2017). Functional characterization of AQPs in different crop species showed improved tolerance against abiotic stress conditions (Ayadi et al., 2011; Zhou et al., 2012; Liu et al., 2013; Ranganathan et al., 2016; Xu et al., 2020, 2021; Sun et al., 2021). An adaptive mechanism of pearl millet to most of the abiotic stresses also involves this important class of proteins through the regulation of *AQP* genes. The *AQP* gene family has been characterized in diverse taxa, such as *Oryza sativa*, *Zea mays*, *Triticum aestivum*, *Hordeum vulgare*, *Sorghum bicolor*, *Panicum virgatum*, and *Brachypodium distachyon* (Johanson et al., 2001; Forrest and Bhave, 2008; Nguyen et al., 2013; Hove et al., 2015; Azad et al., 2016). However, systematic characterization and functional validation of the *AQP* genes have not been demonstrated in *Pennisetum glaucum*. The present study carried out expression profiles of *AQP* genes in leaf and root tissues of contrasting genotypes under high VPD and progressive drought stresses with the primary objective of elucidating the essential roles being played by AQPs. In all, 34 *AQP* genes were identified in the *P. glaucum* genome that are closer to those from other monocots like rice, *S. bicolor*, and *Z. mays*. Pearl millet AQP proteins contained similar structural motifs like MIP domains, NPA, LE1, LE2, H1, H2, and P1-P5, thereby indicating similar functional roles in pearl millet also. The presence of cis-elements in the promoter regions infer that they might play a vital role in developmental and abiotic stress regulations. *PgAQP* genes displayed different expression patterns in different tissues (leaf and root) of the VPD-sensitive and insensitive genotypes, indicating their tissue specific roles during abiotic stress tolerance. Based on the expression data of all *PgAQP* genes under high VPD and drought stress to VPD-sensitive and insensitive genotypes, the *PgPIP2;6* gene of the PIP family could be a probable candidate gene that plays a pivotal role during abiotic stress tolerance, particularly drought. This result has been well aligned with our earlier study in pearl millet (Reddy et al., 2017). *PgPIP1;3*, *PgPIP1;4*, and *PgPIP2;3* showed higher transcript abundance in high TR genotype than low TR genotype under high VPD conditions (Reddy et al., 2017; Grondin et al., 2020).

The PIP proteins belong to the AQP family, which are known to play critical roles during water transport in plasma membranes of many plant species (Martre et al., 2002; Tyerman et al., 2002; Song et al., 2016; Zargar et al., 2017) toward abiotic stress adaptation. In our pursuit to identify a functional role of the pearl millet *PgPIP2;6* AQP gene in transgenic tobacco plants, sequence analysis of the protein with AQPs isolated from other monocot and dicot species revealed that *PgPIP2;6* protein shares high sequence homology with ZmPIP2;6 and ShPIP from maize and sorghum where the predicted structure resembles that of a
classic AQP. Homology-based modeling revealed the orientation of NPA motifs similar to that of other characterized AQPs, besides the capacity to form homotetramers that are typical of AQPs. The $PgPIP2;6$ gene sequence is similar to other well-characterized AQP sequences with all the essential components for its function. Transgenic lines with the $PgPIP2;6$ gene under the control of the constitutive CaMV35S promoter thus generated were advanced to T2 generation where three events (E 1–20, E 11–10, and E 28–9) were selected based on initial molecular characterization for elucidation of the function of the $PgPIP2;6$ gene under different abiotic stress conditions.

In the present study, $PgPIP2;6$ transgenic tobacco plants exhibited lower $Tr$ at high VPD than WT plants, indicating that the transgene enabled the plants with water-conserving properties. Genotypic differences for the transpiration response to increasing VPD have also been reported in other crop species like soybean (Fletcher et al., 2007; Gilbert et al., 2011), sorghum (Gholipoor et al., 2010), peanut (Devi et al., 2010), pearl millet (Kholová et al., 2010b; Reddy et al., 2017, 2021), cowpea (Belko et al., 2012), maize (Yang et al., 2012; Gholipoor et al., 2013), and chickpea (Zaman-Allah et al., 2011a; Sivasakthi et al., 2017b). The $Tr$ response of transgenic chickpea plants was shown to be associated with lower $Tr$ slopes than WT control plants under increasing VPD conditions (Anbazhagan et al., 2015). The root exudation rate also showed a clear genotypic difference between WT and transgenics where the transgenic plants showed a lower exudation rate than the WT plants. Such a water conservation strategy has been shown to be directly linked to increased crop production under water-limited conditions (Zaman-Allah et al., 2011b; Vadez et al., 2013, 2014; Kholová et al., 2014). These results are in accordance with the previous reports in pearl millet, sorghum, and chickpea (Choudhary et al., 2013; Sivasakthi et al., 2017a,b; Tharanya et al., 2017). $PgPIP2;6$ transcript levels in transgenic tobacco were closely related with the $Tr$ data where transgenic lines displayed lower $Tr$ under high-VPD conditions. Exposure of plants to high VPD is known to reduce the stomatal apertures to control water losses (Kholová and Vadez, 2013). Since AQPs play a critical role in stomatal movements and
regulation (Li et al., 2004), tobacco transgenics overexpressing *PgPIP2;6* exhibited lower Tr in high-VPD conditions when compared to the WT controls. This suggests that these plants may have better water-conserving mechanisms by enhancing their transpiration efficiency, thereby leading to better adaptation to water-limiting conditions.

Controlled progressive drought stress imposition is an important methodology for assessing crop performance under water-limiting environments (Kholová et al., 2010a) where the FTSW is an important parameter for drought adaptation. In the present study, progressive drought stress imposed on transgenic tobacco plants resulted in a lower FTSW threshold value than the WT plants, resulting in a slower decline of soil moisture in transgenic plants, possibly due to their water-saving characteristics as also shown in chickpea (Anbazhagan et al., 2015). Genotypic differences were also found for transpiration response to progressive water-deﬁcit stress in several other crops (Vadez and Sinclair, 2001; Bhatnagar-Mathur et al., 2007; Hufteller et al., 2007; Devi et al., 2010; Anbazhagan et al., 2015). A correlation between physiological characteristics and expression of *PgPIP2;6* transgene seems to be responsible for drought tolerance in our study where the physiological mechanisms linked to drought stress adaptation/tolerance are closely related to the expression of drought-responsive genes (Kiani et al., 2007). Expression of the *PgPIP2;6* gene in root and leaf tissues under water-deﬁcit conditions further supports the role in stress tolerance. Water transport through roots depends on the anatomical structure of roots regulated by the complex phenomenon. Plants growing under adverse conditions, including drought, reduce the water permeability of their root membrane cells to avoid possible loss of water from the roots to the soil, as has been proposed earlier (Stedule, 2000). Hence, the lower Tr found in *PgPIP2;6* transgenic tobacco plants compared to WT plants presumably developed the adaptive mechanism. These results are consistent with the earlier reports on chickpea and pearl millet (Sivasakthi et al., 2017b; Tharanya et al., 2018).

Heat stress lowers the relative air humidity if water is supplemented to the ambient atmosphere (Prasch and Sonnewald, 2013), a scenario frequently occurring under natural climatic conditions and exerted in regular heat-stress studies. Consequently, plants suffer from heat stress and additional ambient water stress (Georgii et al., 2017). High temperature enhances transpiration to minimize heat damage by transpiration-mediated leaf cooling (Crawford et al., 2012). For any given environmental conditions, the canopy temperature (CT) is closely related to the rate of transpiration from the canopy surface, while the canopy temperature depression (CTD) indicates the capacity of stomata to regulate leaf water loss under stressed conditions (Isoda and Wang, 2002). Therefore, the differences in CT could possibly be used to estimate the differences in Tr, providing a more relevant trait proxy for Tr (Jones et al., 2002). Our results of thermal image analysis revealed that transgenic plants displayed lower CT than WT plants under high-temperature (40°C) conditions and optimum temperatures (28°C). Transgenics exhibited more CTD than WT plants under high-temperature stress conditions, inferring the adaptation of a possible water-saving strategy. During heat stress, plant-water relations are regulated through changes in abundance of AQPs (Maurel et al., 2008). AQP genes are expressed differentially in diverse tissues under various abiotic stress conditions in higher plants. Accordingly, it is noticed that *PgPIP2;6* gene expression levels were signiﬁcantly upregulated under high-temperature conditions in transgenic tobacco lines. This leads us to speculate that the upregulation of *PgPIP2;6* might be a spontaneous response to heat stress and might play a critical role in speeding up the water homeostasis, following the stress.

Aquaporins have also been shown to respond to various environmental stresses, including cold (Jang et al., 2004; Aroca et al., 2005; Guo et al., 2006; Peng et al., 2008; Sade et al., 2010) that may be directly related to their function in the transport of water across membranes. In the present study, *PgPIP2;6* transgenic lines of tobacco displayed slightly lower transpiration than the WT control plants under cold stress conditions. *PgPIP2;6* expression levels were signiﬁcantly upregulated in transgenics under cold stress except in the line E 11–10, which displayed comparatively lower expression and higher transpiration than the other two transgenics. Similarly, overexpression of *OsPIP2;7* has been shown to enhance the Tr, leading to improved tolerance against low temperature in rice (Li et al., 2008). Leaf dehydration is caused by the imbalance between water lost by leaf transpiration and water taken up by the roots (Pardossi et al., 1992; Vernieri et al., 2001), resulting in a decrease in VPD between the atmosphere and the leaf surface, and leading to a decline in the transpiration rate (Aroca et al., 2003). Hence, under low-temperature stress, stomata of the tolerant transgenic plants close more rapidly (Aroca et al., 2001, 2003; Bloom et al., 2004). Besides, the recovery of plants from cold stress has been linked with the expression of AQPs, particularly PIPs (Afzal et al., 2016).

In conclusion, our study identiﬁed 34 AQP genes in pearl millet, which are grouped into four subfamilies, including 11
PIPs, 9 TIPs, 11 NIPs, and 3 SIPs. Phylogenetic relationship within the subfamilies of PgAQPs and between AQPs of related species shows their evolutionary relationship and possible functions with their localizations, mainly in the plasma membrane. Cis-motif analysis showed the presence of several tissue and abiotic stress-specific cis-elements, indicating their possible role during abiotic stress adaptation. Expression analysis of PgAQPs in Tr-contrasting genotypes of pearl millet in leaf and root tissues in VPD and progressive drought stress revealed their critical roles during abiotic stress conditions. Tobacco transgenics constitutively expressing PgPIP2;6 gene showed differences in response to diverse abiotic stresses. Tr under heat and drought stresses recorded marked differences between transgenics and the non-transgenic WT plants, where the transgenics exhibited better tolerance due to lower Tr when compared to WT plants. Constitutive expression of pearl millet aquaporin PgPIP2;6 in tobacco seemed to impart abiotic stress tolerance with reduced transpiration, thereby helping in water conservation. Further improvement in stress tolerance is possible by using tissue-specific or stress-inducible promoters for more specific AQP expression levels. Thus, these studies provide a point of reference for the functional studies and molecular breeding of a pearl millet crop for enhanced adaptation and resilience to abiotic stresses, especially under uncertainties due to the ensuing climate change.

**DATA AVAILABILITY STATEMENT**

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.
AUTHOR CONTRIBUTIONS

PR, PB-M, VV, and KKS conceived the idea and designed the project outline. PR, MD, KS, KD, KSC, and PK performed the experiments. PR, VV, PB-M, KS, PK, MN, and KKS analyzed the data. PR, MD, KS, KD, KKS, and PK prepared and refined the manuscript. All authors read and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.820996/full#supplementary-material

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