INTRODUCTION

Switchgrass (*Panicum virgatum* L.) is a warm season tall perennial grass originated in North America and has been used as non-grain-based bioenergy (Sanderson, Read, & Reed, 1999) and forage feedstock (Anderson et al., 1988). A previous study showed that all tested switchgrass ecotypes suffered severe biomass reduction (75%–80%) with...
water stress at −4 MPa (Barney et al., 2009). On the other hand, drought stress was also known to affect the biomass feedstock quality with reduced protein and altered cell wall composition in several grasses (Jiang, Yao, & Wang, 2012; Thakur & Rai, 1982; van der Weijde et al., 2016). To minimize competition with primary food crop production for land use, much of switchgrass production are on less productive marginal lands where irrigation is often limited or unavailable during prolonged drought periods. Therefore, improved drought tolerance and higher water use efficiency (WUE) are important targeting traits for switchgrass molecular breeding.

Aquaporin family genes play important regulatory roles in water movement through the symplastic pathway and maintenance of cellular water homeostasis in plants (Zargar et al., 2017). Based on their sequence compositions, aquaporins can be divided into five types, including tonoplast intrinsic proteins (TIPs), nodulin 26-like intrinsic proteins, small basic intrinsic proteins, X-intrinsic proteins (XIPs) and plasma membrane intrinsic proteins (PIPs), among which PIPs might be the main gateways controlling water permeability (Yaneff, Vitali, & Amodeo, 2015). Furthermore, PIPs can be categorized into two phylogenetic subgroups, PIP1 and PIP2, according to their main structural differences. A number of PIP2s were found possessing high water channel activities and played indispensable roles in water transport (Kammerloher, Fischer, Piechotta, & Schöffner, 1994; Sakurai, Ishikawa, Yamaguchi, & Schäffner, 1994; Sakurai, Ishikawa, Yamaguchi, & Schäffner, 1994; Sakurai, Ishikawa, Yamaguchi, & Schäffner, 1994). Several individual studies have reported that over-expressing PIP2 subfamily genes improved plant drought tolerance, including FaPIP2;1 in tall fescue (Festuca arundinacea; Zhuang, Liu, Yuan, Yang, & Huang, 2015), PIP2;5 in Populus (Populus tremula × Populus alba; Ranganathan et al., 2017), TaAQP7 in wheat (Triticum aestivum; Zhou et al., 2012) and HvPIP2;2 in barley (Hordeum vulgare; Hanba et al., 2004). These results suggested that some PIP2 genes functioned as positive regulators in plant drought tolerance. Yet, in some other cases, over-expressing PIP2 genes compromised drought tolerance. For examples, over-overexpression of a soybean (Glycine soja) stress-inducible PIP2 gene (GsPIP2;1) in transgenic Arabidopsis (Arabidopsis thaliana) increased plant sensitivity to dehydration (Wang et al., 2015). Over-expression of PIP1;4 and PIP2;5 in both Arabidopsis and tobacco (Nicotiana tabacum) resulted in rapid water loss under dehydration stress as well as retarded seed germination and seedling growth under drought stress (Jang et al., 2007). Lee et al. (2009) reported that activating an E3 ligase led to degradation of PIP2;1 and improved drought tolerance in Arabidopsis. In short, some PIP2s were functional in water transport, yet their roles in plant drought tolerance diverged from each other, and how these PIP2 family genes interacted and cooperated in maintaining plant water status was still unclear.

There are 68 aquaporin genes in switchgrass, including 7 PIP1 and 14 PIP2 subfamily members (Azad et al., 2016), and none of their functions has been characterized so far. In our previous study, over-expressing an Arabidopsis NAC transcriptional factor gene, LONG VEGETATIVE PHASE ONE (LOV1) in switchgrass resulted in smaller leaf angles, improved drought tolerance and higher WUE (Xu, 2011; Xu et al., 2012). Comparative microarray analysis revealed that there were 105 significantly differentially expressed genes, among which PvPIP2;9 (microarray probe ID: KanlowCTG00810_at; Phytozyme accession No.: Pavir. Ba02478) was the only differentially expressed and upregulated aquaporin gene in the transgenic plants (Xu, 2011). Therefore, we hypothesized that PvPIP2;9 plays an important regulatory role in switchgrass drought tolerance and WUE. In the current work, the expression pattern and functional role of PvPIP2;9 in switchgrass drought tolerance were studied, and the effects of drought and over-expression of PvPIP2;9 on other PIP2 family genes were also measured.

2 | MATERIALS AND METHODS

2.1 | Plant materials and growth condition

Seeds of an elite switchgrass line, HR8, originally selected from the lowland ecotype “Alamo” was used in this study (Xu, Huang, et al., 2011). In the qPCR experiment to study PvPIP2;9 expression pattern, 4-week-old plants were cultured in 1/2 Hoagland solution and grown in a growth chamber with a 12-hr light/dark cycle and accurately controlled temperature (30/25°C [day/night]) and light intensity (photosynthetically active radiation at 750 µmol photons m⁻² s⁻¹). In other experiments, switchgrass plants were grown in clay loam soil mixed with sand (1:1) in 1.1 × 10⁻² m³ pots in the greenhouse at Nanjing Agricultural University (Nanjing, China) with temperatures set at 30/20 ± 3°C (day/night) and the photoperiod set at 14/10 hr (day/night).

2.2 | Gene expression analysis using the PviGEA database and qRT-PCR

The corresponding Unitranscript ID for PvPIP2;9 was retrieved from the PviUTs database and then used to search against the integrated transcript sequence database PviGEAs in which the expression atlas was from a single line “AP13” that was selected from “Alamo” (Zhang et al., 2013).
The second fully expanded leaves from the top were sampled for relative gene expression level and physiological parameter analyses. To detect the diurnal oscillation of \textit{PvPIP2;9}, the first sampling time was set at the dawn for consecutive 40 hr with 4 hr internals in-between. For stress treatments, plants were grown in 1/2 Hoagland solution containing 20% polyethylene glycol (PEG) 6000 (Huada) and 100 μM ABA according to Yuan et al. (2015), and sampled 0, 0.5, 1, 2, 4, 8 and 12 hr after the treatment.

The total RNA was isolated using OMEGA E.Z.N.A.® plant RNA Kit (Omega Bio-Tek). The first strand cDNA was synthesized with 1 μg RNA using the PrimeScript™ RT reagent Kit (TaKaRa) with the Perfect Real Time gDNA Eraser (TaKaRa). The qRT-PCR was performed using SYBR Green Master Mixes on a Roche LightCycler®480 II machine. The correlation between soil water content and soil water potential is shown in Figure S1.

### 2.3 Gene cloning and vector construction

According to the switchgrass genomic sequence information (\textit{P. virgatum} v4.1, DOE-JGI, http://phytozome.jgi.doe.gov), we cloned the gene from gDNA for its functional characterization. In brief, the gene was amplified from switchgrass genomic DNA using PCR, cloned into the vector pENTR/D and sequenced. Then we sub-cloned the gene into the Gateway-compatible binary vector pVT1629 (Xu, Escamilla-Treviño, et al., 2011) using LR reaction (Invitrogen). The resultant vector, pVT1629-PvPIP2;9, harboring the \textit{PvPIP2;9} driven under maize ubiquitin promoter and the \\
\textit{uidA} (GUS) reporter gene under CaMV 35S promoter, was transformed into the \textit{Agrobacterium tumefaciens} strain “AGLI” through electroporation.

### 2.4 Switchgrass genetic transformation

Switchgrass line “HR8” was used for \textit{Agrobacterium}-mediated genetic transformation and the transformation procedure was the same as reported before (Xu, Huang, et al., 2011). Hygromycin B (Sigma) at 50 mg/L was used to select against the non-transformed calli. Regenerated plants from independent calli were regarded as putative transgenic lines which were further verified by GUS staining and PCR for the detection of \textit{HPTII} gene present in the T-DNA.

### 2.5 Drought treatment of wild-type and transgenic lines

Three independent transgenic lines and tissue culture-regenerated wild-type (WT) lines were generated. These lines were propagated by splitting single tillers. Plants grown from a single tiller for 2.5 months reached E4 stage (Hardin et al., 2013) and were used for drought treatment by withdrawing water. After 28 days of drought, the treated plants were re-watered to observe their re-growth status. At the same time period, normally watered plants were used as controls. A soil water content detector (Mini Trase Kit 6050X3; Soil Moisture Equipment Co., Ltd.) was used to monitor the soil water content (SWC) in the 0–8 cm deep soil layer of each pot. And soil water potential was determined using ERS-II water potential and temperature meter (Yibaiyi Mechanical and Electrical Equipment Co., Ltd.). The correlation between soil water content and soil water potential is shown in Figure S1.

### 2.6 Biomass feedstock quality analysis

After 21 days of treatment, the aboveground of WT and transgenic plants were collected and dried in a 70°C oven and then ground for feedstock quality analysis. The amount of total sugar was determined by the phenol sulfuric acid reagent method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956) with slight modifications. In brief, 0.05 g of samples was added to the mixture of 10 ml ddH$_2$O and 3 ml HCl and incubated in 100°C for 1 hr. Then 0.5 ml supernatant was transferred to a 10 ml tube and mixed with 0.5 ml ddH$_2$O, 1 ml 5% phenol and 5 ml sulfuric acid. The reaction mixture was incubated at 30°C for 20 min in a water bath and then the absorbance was quantified at 490 nm. The quantity of total sugar was evaluated based on a standard curve generated with known sugar concentrations.

Cellulose content was estimated using the anthrone method (Viles & Silverman, 1949). Briefly, 0.05 g of samples was mixed with 35 ml 60% H$_2$SO$_4$ in a 50 ml tube and incubated at ice bath for 30 min. After 10 times dilution, the absorbance of supernatant was quantified at 620 nm. Microcrystalline cellulose (Avicel) was used as a standard for the standard curve generation.

Hemicellulose and lignin content were measured by hydrochloric acid hydrolysis method and sulfuric acid method, respectively (Xiong, Zuo, & Zhu, 2005). For hemicellulose analysis, 0.1 g of samples was mixed with 10 ml 80% calcium nitrate and boiled on a heater for 5 min. The mixture was then centrifuged and the supernatant was discarded. After rinsing three times with ddH$_2$O, 10 ml 2 M HCl was added to the mixture and boiled for another 45 min. The supernatant was neutralized using NaOH and mixed with 3,5-dinitrosalicylic acid reagent. The mixture
was incubated in a water bath at 100°C for 5 min and then the absorbance of the supernatant was measured at 520 nm. For lignin, 0.1 g of samples was washed using 10 ml 1% acetic acid in a mixture of ethanol and ether (1:1) and the mixture was then dried in a water bath set at 100°C. The sample was incubated with 72% H₂SO₄ for at least 16 hr to remove cellulose. The precipitate was then mixed with 10 ml 10% H₂SO₄ and 0.1 M potassium dichromate and incubated in a water bath set at 100°C for 15 min. The lignin content was quantified after mixing with 5 ml 20% KI and 1 ml 0.5% starch solution by titration using 0.2 M sodium thiosulfate (Xiong et al., 2005).

The Kjeldahl procedure was used to determine the total nitrogen (TN) content, and the crude protein content was calculated by multiplying TN by 6.25 (Krishnamoorthy, Muscato, Sniffen, & Van Soest, 1982).

2.7 | Measurement of physiological parameters

The middle section of the fourth leaves from the top was used for the physiological measurement. Leaf membrane stability was evaluated by measuring the electrolyte leakage (EL; Blum & Ebercon, 1981) according to a method described before (Zhang et al., 2016). In brief, leaves were excised and cut into 3 cm segments. Then the leaves were incubated in 35 ml distilled deionized water. Centrifuge tubes were shaken on a shaker for 24 hr at room temperature, and the initial level of EL (C₁) was measured using a conductance meter (Thermo Scientific). Then the leaf tissue was killed by autoclaving at 121°C for 15 min, and then incubated for 24 hr on a shaker for measuring the maximum conductance (Cₘₜₐₓ) of the solution. Relative EL was calculated as EL = (C₁/Cₘₜₐₓ) × 100%.

The leaf relative water content (RWC) was determined according to the method described by Hu, Wang, Du, and Huang (2010) with modifications. In brief, RWC was determined using fresh fully expanded leaves. About ~0.2 g of leaf samples was detached from the plants and immediately weighed to determine the fresh weight (FW). Samples were placed into covered centrifuge tubes filled with water for leaves to reach full hydration. After approximately 24 hr at 4°C, leaf samples were blotted dry with paper towels and weighed to determine the saturated weight (SW). Leaf tissue was then dried in an oven at 65°C for 72 hr to determine dry weight (DW). Leaf RWC was calculated as RWC = (FW – DW)/(SW – DW) × 100.

The ratio of the variable fluorescence (Fᵥ) to the maximal fluorescence (Fₘ) (Fᵥ/Fₘ) was used to represent leaf photosynthetic efficiency (Oxborough & Baker, 1997). The Fᵥ/Fₘ ratio was determined using a fluorescence meter (Dynamax) as described before (Zhang et al., 2016).

Chlorophyll (Chl) content was measured using the dimethylsulfoxide (DMSO) extraction method. In brief, leaves were soaked in DMSO for 48 hr, the extracts were measured at 663 and 645 nm using a spectrophotometer for their absorbance values (Spectronic Instruments), and the Chl contents were calculated using equations reported by Barnes, Balaguér, Manrique, Elvira, and Davison (1992).

Leaf instantaneous WUE was calculated by measuring leaf net photosynthetic rate (Pn) and transpiration rate (Tr) using the LI-6400 portable photosynthesis system (LI-COR). The area of leaves enclosed in the leaf chamber was determined on a scanner, which was then used to calculate the Pn and Tr values. The WUE was calculated as Pn/Tr.

2.8 | Statistical analysis

Data in this study were statistically analyzed using one-way ANOVA, and their means were compared by the Duncan test at the significance level of 0.05 using SPSS20.0.

3 | RESULTS

3.1 | Phylogenetic analysis and expression pattern of PvPIP2;9

According to the switchgrass genomic sequence information (P. virgatum v4.1, DOE-JGI, http://phytozome.jgi.doe.gov/), we cloned PvPIP2;9 from switchgrass. PvPIP2;9 had 286 amino acids with a predicted molecular mass of 29.87 kDa. As a typical aquaporin protein, PvPIP2;9 had six conserved transmembrane domains and two nucleosome assembly protein domains (Figure 1a). A phylogenetic tree comprising of PvPIP2;9 and its orthologues in rice (Oryza sativa), maize (Zea mays), and Arabidopsis showed that PvPIP2;9 was most closely related to OsPIP2;4 with 97% of amino acid similarity (Figure 1b).

At the organ/tissue level, the transcript level of PvPIP2;9 in roots was the highest, which was ~6 to 10 times higher than those in leaves and leaf sheaths, and was ~10 to 20 times higher than those in inflorescence of rachis, florets, vascular bundle and internodes (Figure 2a). In leaves, the expression of PvPIP2;9 showed a clear diurnal change that increased after dawn, reached to its maximum level in the middle of daytime and declined thereafter to its basal level at the end of the daytime (Figure 2b). Furthermore, we measured its relative expression level in plants under 20% PEG6000 (water potential: −0.735 MPa) and 100 μM ABA treatments compared with the control sampled at the same time point. Treating switchgrass plants with PEG6000-induced osmotic stress resulted in significantly increased expression of the gene within 2 hr, and such an activated expression was
transient that returned to its initial levels after 4 hr of treatment and remained at its basal level thereafter (Figure 2c). However, ABA treatment did not activate but suppressed the expression of \( \text{PvPIP2;9} \) (Figure 2d), indicating that the osmotic stress-induced expression of \( \text{PvPIP2;9} \) was not due to the ABA signal.

**FIGURE 1** Phylogenetic tree and multiple alignment of \( \text{PvPIP2;9} \) and other PIP2s. (a) The neighbor-joining evolutionary tree was built with PIP1 and PIP2 proteins in switchgrass (PvPIPs), Arabidopsis (AtPIPs), rice (OsPIPs) and maize (ZmPIPs) using MAGA6 (Tamura, Stecher, Peterson, Filipski, & Kumar, 2013) with the sum of branch length = 24.55096375. The tree is drawn to scale and there was a total of 264 positions in the final dataset. (b) Multiple sequence alignment of \( \text{PvPIP2;9} \) with its closest orthologues, showing that these PIP2s shared conserved transmembrane domains and NPA residues.

**FIGURE 2** Expression pattern of \( \text{PvPIP2;9} \). (a) Transcripts levels of \( \text{PvPIP2;9} \) in different organ/tissues according to PviGEA database. (b–d) qRT-PCR measurement of \( \text{PvPIP2;9} \) relative expression levels during 40 hr, and relative expression of \( \text{PvPIP2;9} \) after treatment with 20% PEG6000 or 100 μM ABA. The relative expression of the gene was calculated by normalizing the relative expression of \( \text{PvPIP2;9} \) under treatment against those under normal growth condition at the same time point. Data are means ± SE. Different letters above bars represent significant difference at \( p < .05 \). Data in (a) were adopted from PviGEA database and the abbreviations are as follows. E4i4b: Bottom 1/5 fragment of the fourth internode; E4i4t: Top 1/5 fragment of the fourth internode; E4i4m: Middle 1/5 fragment of the fourth internode; E4-LFB: Pooled leaf blade from plant; E4-LSH: Pooled leaf sheath; Inflo-REL: Rachis and branch elongation of inflorescence (50–150 mm); Inflo-PEM: Panicle emergence of inflorescence (>200 mm); E4i3m: Middle 1/5 fragment of the third internode; E4i3m-VB: Vascular bundle isolated from 1/5 fragment of the third internode; E4-root: Whole root system; E4-crown: Whole crown; E4-node: Pooled nodes; Inflo-meristem: Inflorescence meristem (0.5–3.0 mm); Inflo-floret: Floret of inflorescence when glumes are 10–20 mm.
3.2 Over-expressing *PvPIP2;9* led to improved drought tolerance, WUE, higher biomass yield and crude protein contents in switchgrass

The *PvPIP2;9* over-expressed in switchgrass driven by the maize ubiquitin promoter by *Agrobacterium*-mediated genetic transformation. A total of 11 putative transgenic lines were generated. The presence of T-DNA in these putative lines was confirmed by PCR amplifying a fragment of *HPTII* gene (conferring hygromycin resistance; Figure 3a). Three representative transgenic lines (lines: −1, −7 and −9) with over-expressed *PvPIP2;9* and positive GUS staining signals (Figure 3b,c) were chosen for further phenotypic analyses.

Transgenic lines and tissue culture-regenerated WT plants of the same age were vegetatively propagated by splitting and growing single tillers under the optimum growth condition. After 2.5 months of growth, single tillers of WT and the transgenic lines proliferated into five to seven tillers, and all *PvPIP2;9*-OX lines showed significantly longer leaf length, taller plant height and higher aboveground biomass yield (FW and DW) than those of WT (Table 1).

These switchgrass plants were treated by withdrawing water to evaluate whether over-expressing *PvPIP2;9* affected drought tolerance. After 28 days of drought period, WT became severely wilted that even the newly emerged leaves at the top withered and turned yellow, while plants of all transgenic lines still remained 1–2 green leaves at the top in each tiller. After re-watering, WT plants did not recover but completely died off, while all *PvPIP2;9*-OX lines recovered back with new green expanding leaves (Figure 4a).

During the drought treatment, the pot soil water content dropped from −0.48 MPa to −0.83 to −0.85 MPa, −1.35 to −1.20 MPa, and −1.65 MPa after 7, 14, 21 and 28 days of water withdrawal, respectively, while those under well-watered condition had a relative constant soil water content of −0.48 to −0.5 MPa (Figure 4b). Five physiological parameters, including photochemical efficiency (*Fv/Fm*), chlorophyll (Chl) content, EL, leaf RWC and WUE were measured in WT and transgenic lines during the drought treatment. As shown in Figure 4c–g, there was no significant difference among these physiological parameters between WT and *PvPIP2;9*-OX lines when under the well-watered condition. Yet, under the drought treatment, significant differences were observed after 21 days of treatment for all five physiological parameters that transgenic plants had significantly lower EL, but higher RWC, *Fv/Fm*, Chl contents and WUE than those of the WT (Figure 4c–g). WUE of plants after 28 days of water holding was not measured because leaves of WT were completely wilted already. Furthermore, values of WUE, EL and RWC of the transgenic lines were also related to their relative expression of *PvPIP2;9*. For example, transgenic

| Tiller number | Leaf width (cm) | Leaf length (cm) | Plant height (cm) | Aboveground fresh weight (g) | Aboveground dry weight (g) |
|---------------|----------------|-----------------|------------------|-----------------------------|----------------------------|
| WT            | 5.00 ± 0.38b   | 0.90 ± 0.011a   | 43.19 ± 0.83b    | 100.54 ± 0.21c              | 31.66 ± 1.41c             |
| Line 1        | 6.78 ± 0.70a   | 0.90 ± 0.045a   | 47.37 ± 0.64a    | 110.03 ± 3.39ab             | 42.26 ± 1.64a             |
| Line 7        | 5.13 ± 0.30b   | 0.88 ± 0.003a   | 47.11 ± 0.79a    | 113.92 ± 1.47a              | 36.14 ± 1.33b             |
| Line 9        | 6.89 ± 0.32a   | 0.88 ± 0.003a   | 46.78 ± 0.88a    | 105.43 ± 0.30b              | 40.30 ± 1.71ab             |

Note: Data are means ± SE (n = 8), and different letters represent significant difference at p < .05.
FIGURE 4  Comparison of drought tolerance between wild-type (WT) and *PvPIP2;9* transgenic switchgrass lines. (a) Phenotypes of WT and transgenic lines before and after 28 days of drought and non-stress treatment, and after 10 days of re-watering (recovery). (b) Soil water potential in pots for the well-watered control and for those under the drought treatment. (c–g) Dynamic changes of WUE (c), *Fv/Fm* (d), Chl contents (e), EL (f) and RWC (g) of the WT and transgenic lines during the drought treatment. The second fully expanded leaves from the top were used for the data analysis. Data are means ± SE. Different letters above bars represent significant difference at *p* < .05.
line-7 had the highest expression level of *PvPIP2;9* in leaves, while line-1 had the least. After 28 days of drought treatment, line-7 showed the lowest EL and the highest RWC, while the opposite was true for line-1. These results supported that over-expressing *PvPIP2;9* significantly improved switchgrass drought tolerance and WUE associated with higher photochemical efficiency, higher membrane stability, higher Chl content and better leaf water status under this prolonged stress.

To understand whether over-expressing *PvPIP2;9* also affected biomass feedstock quality, we measured total sugar, cellulose, hemicellulose, lignin and crude protein contents in aboveground biomass of WT and transgenic lines. As shown in Table 2, all transgenic lines had significantly 12.5%–30% higher crude protein content than WT no matter the plants were grown under the optimum soil water or after 21 days of drought. Before drought treatment, there were slightly varied levels of other cell wall compositions. For example, cellulose content in WT was 5.8%–8.6% higher than those of the transgenic lines likely due to natural variation within this half-sib switchgrass population. Drought stress caused decreased protein, total sugar and cellulose contents (e.g., 23.9%, 22.3% and 23.4% off in WT, respectively). Notably, after drought stress, cellulose contents in transgenic plants turned to be 11.1%–17.5% higher than that in WT.

![Table 2](image)

**Table 2** Over-expression of *PvPIP2;9* affected biomass feedstock quality. The biomass was harvested from plants after 21 days of drought treatment or under the optimum growth condition (control)

|                      | Total sugar (%) | Cellulose (%) | Hemicellulose (%) | Lignin (%) | Crude protein (g/kg) |
|----------------------|-----------------|---------------|-------------------|------------|---------------------|
| Control-wild-type (WT) | 59.44 ± 2.84a   | 32.10 ± 0.25a | 16.82 ± 1.08a    | 20.90 ± 1.27b | 93.83 ± 0.61g        |
| Control-line 1       | 47.66 ± 1.41b   | 29.95 ± 0.32bc| 15.60 ± 0.15bc   | 22.71 ± 1.47a | 114.55 ± 1.31f       |
| Control-line 7       | 56.52 ± 1.42a   | 30.24 ± 0.19b | 15.21 ± 0.17bc   | 20.72 ± 0.25b | 110.24 ± 0.80d       |
| Control-line 9       | 51.97 ± 1.61ab  | 29.33 ± 0.19cd| 14.47 ± 0.28bc   | 20.80 ± 0.32b | 105.47 ± 0.48e       |
| Drought-WT           | 46.20 ± 2.16b   | 24.60 ± 0.21g | 15.25 ± 0.96bc   | 18.33 ± 0.33c | 71.40 ± 1.25d        |
| Drought-line 1       | 48.69 ± 3.66b   | 27.35 ± 0.29f | 15.71 ± 1.51ab   | 19.35 ± 0.28c | 82.73 ± 0.55a        |
| Drought-line 7       | 58.03 ± 5.56a   | 28.91 ± 0.37de| 13.64 ± 0.93c    | 21.27 ± 0.28b | 92.76 ± 0.56b        |
| Drought-line 9       | 57.72 ± 8.22a   | 28.32 ± 0.43c | 14.03 ± 1.24ab   | 22.58 ± 0.32a | 86.88 ± 0.62c        |

*Note:* Data are means ± SE (*n* = 3), and different letters represent significant difference at *p* < .05.

![Figure 5](image)

**Figure 5** Relative expression of *PvPIP2;9* in (a) leaves and (b) roots during 21 days of drought using qRT-PCR. Data are means ± SE. Different letters above bars represent significant difference at *p* < .05.

### 3.3 Over-expression of *PvPIP2;9* affected expression patterns of other *PIP2* genes during drought treatment

To understand whether over-expressing *PvPIP2;9* also affected other *PIP2* subfamily genes, we further measured relative expression levels of all *PvPIP2* genes during the 21 days of water-withdrawal treatment in WT and the three transgenic lines.

First, it was notable that expression of *PvPIP2;9* per se was responsive to the long-term drought stress but in distinctively different patterns in leaves and roots. For example, after the 21 days of drought treatment, the relative expression of *PvPIP2;9* increased ~3 times in WT leaves, but reduced to 20% in WT roots. Yet, in the three transgenic lines, expression levels of *PvPIP2;9* slightly decreased in leaves but gradually increased 6–13 folds in roots during the drought treatment (Figure 5). Overall, relative expression levels of *PvPIP2;9* in transgenic lines were ≥200 times higher in leaves and ≥2 times higher in roots than those of WT.

Second, as shown in WT, most *PIP2* subfamily genes were responsive to the drought treatment. For example, transcriptional levels of *PvPIP2;3*, *PvPIP2;4* and *PvPIP2;5* increased in response to decreasing soil water content in both leaves and roots while those of *PvPIP2;11* and *PvPIP2;13*
showed increased expression pattern only in roots but not in leaves (Figure 6).

Third, by comparing gene expression patterns in WT and in transgenic lines, we found that expression of most PIP2 subfamily genes was affected by the over-expression of PvPIP2;9. For example, in leaves of transgenic lines, one (PvPIP2;8) showed higher expression levels and five (PvPIP2;2, PvPIP2;5, PvPIP2;12, PvPIP2;13 and PvPIP2;14) showed lower expression levels than those in WT. While in roots of transgenic lines, five PvPIP2 genes (PvPIP2;1, PvPIP2;4, PvPIP2;6, PvPIP2;7 and PvPIP2;8) showed significantly higher and one (PvPIP2;14) showed lower expression patterns along with the decreasing soil water content (Figure 6).

4 | DISCUSSION

There are 68 aquaporin genes in switchgrass (Azad et al., 2016). Yet, none of these genes was functionally characterized so far. Based on our previous study, we predicted that PvPIP2;9 might be an important aquaporin contributing to the plant water status in switchgrass. As mentioned earlier, PvPIP2;9 was the only aquaporin gene with significantly increased expression in the LOV1 transgenic plants that showed improved drought tolerance (Xu, 2011). We also tested whether or not the LOV1 transcription factor could directly bind to the −2 kb promoter region of PvPIP2;9 using the yeast one-hybrid system. However, our results showed that there was no transactivation effect of LOV1 on the PvPIP2;9 promoter (data not shown), suggesting that LOV1 indirectly activated the expression of PvPIP2;9 in switchgrass. Current results in this study indicate that PvPIP2;9 positively regulated switchgrass drought tolerance and WUE as well as other important biomass feedstock traits including yield, protein and cellulose contents.

4.1 | PvPIP2;9 is an important aquaporin regulating plant water status in switchgrass

Under drought treatment, transgenic switchgrass demonstrated significantly improved drought tolerance and WUE under this prolonged drought stress. The association between aquaporin genes’ expression and WUE has been documented before. For example, over-expressing a stress-inducible aquaporin gene (NaAQP1) in tobacco, tomato (Lycopersicon esculentum) and Arabidopsis all increased their WUE and photosynthesis under both optimal and salt stress conditions (Sade et al., 2010). And over-expressing a TIP-type aquaporin (AQUA1) in white poplar (P. alba) also improved the plant’s WUE and RWC (Ariani, Francini, Andreucci, & Sebastiani, 2016). In this study, we found transgenic plants had significantly higher WUE associated with lower EL, higher \( F_v/F_m \), Chl content, and RWC than WT after prolonged drought treatment (Figure 5). Lower EL value indicated better cell membrane integrity, higher \( F_v/F_m \) and Chl content further corroborated that leaves of transgenic plants were bringing their functions into better play, and the higher RWC in transgenic plants confirmed that PvPIP2;9 positively contributed to cellular water status of leaves when the soil water content was low. We reasoned that the higher WUE in transgenic plants should be another important reason for the improved drought tolerance that saved water loss from evapotranspiration, which, in turn, contributed to better cellular water status as reflected in leaf RWC and the maintenance of integral cell membrane system and photosynthesis system.

4.2 | Expression level of PvPIP2;9 affected plant growth and biomass feedstock quality in switchgrass

Under the well-watered condition, constitutive over-expression of PvPIP2;9 in switchgrass did not significantly
alter the WUE and leaf RWC though, suggesting that over-expressing of this aquaporin gene did not significantly affect water transportation or water status when there was sufficient soil water supply. Yet, transgenic lines did show significantly longer leaf length, taller plant height and higher aboveground biomass than those of WT. Another interesting finding with the transgenic switchgrass was their higher protein content no matter they were grown under the optimum or drought condition, and showed significantly higher cellulose content after 21 days of drought. The higher biomass protein content was not essential for lignocellulosic biofuel feedstock but was a highly desirable trait when switchgrass was used as forage feedstock. To our knowledge, such an effect on biomass protein and cellulose contents was not well emphasized or recorded for a PIP gene before.

It was reported that leaf elongation rate responded to rapid changes in evaporation and soil water availability much quicker than transpiration and leaf water potential (e.g. 30 min vs. 1–2 hr), and small flux of water potentials could cause rapid decline of simulated leaf elongation rate (Caldeira et al., 2014). In fact, it was found in rice that it could encounter temporary “water shortage” with decreased leaf WUE and RWC values which were associated with inadequate expression of aquaporin genes even in well-watered paddy field (Nada & Abogadallah, 2014). We reasoned that these WT and transgenic plants be challenged by temporary water deficit (e.g. at noon time) even though they were regularly watered and grown under optimum condition in greenhouse. While due to the effect of PvPIP2;9 over-expression, transgenic plants were less challenged with temporary fluxes of unfavorable leaf water potential. Decades earlier, effect of water stress on protein content in maize cultivars with contrasting drought tolerance has been reported that the drought-tolerant cultivar showed higher protein contents than the susceptible one (Thakur & Rai, 1982). Effects of short-term drought (4 or 7 days of drought) in switchgrass (Jiang et al., 2012) and long-term drought (28 days) in miscanthus (van der Weijde et al., 2016) were reported that their cell wall components were affected by drought stress to various degrees. For example, cellulose content was significantly lower in drought treated miscanthus (van der Weijde et al., 2016). The relatively higher cellulose content in drought-stressed PvPIP2;9 transgenic switchgrass was at least partially explained by the better plant water status during the drought treatment.

Another possible reason for the plant growth effect of the transgene could be due to other beneficial growth-promoting effect of PvPIP2;9. It was reported that OsPIP2;4 contributed to plant boron tolerance (Kumar et al., 2014) and might have the capability of transporting non-aqua substrates CO2 as well as H2O2 (Azad et al., 2016). Further investigation on whether PvPIP2;9 was involved in transportation of other molecules will help our understanding the function of this protein and the associated phenotypes in OE plants.

### 4.3 PvPIP2;9 and other PVP2 subfamily genes were fine-tuned and interacted at the transcriptional level

OsPIP2;4 was the closest rice orthologous gene of PvPIP2;9. Although it is still unclear whether OsPIP2:4 plays a regulatory role in plant drought tolerance or not, it was reported that the expression of OsPIP2;4 showed a clear diurnal expression pattern and OsPIP2;4 had high water channel activity (Sakurai et al., 2005). PvPIP2;9 also had a similar expression pattern to OsPIP2;4 that its diurnal transcription level was in conjunction with the activity of plant diurnal water transport. The diurnal changes of aquaporin genes have also been reported for a few other PIP2 genes in other plant species, such as HvPIP2;1 in barley (Lopez et al., 2003), ZmPIP2;1 and ZmPIP2;5 in maize (Lopez et al., 2003), and OsPIP2;4 and OsPIP2;5 in rice (Sakurai et al., 2005). Such an expression pattern suggested that water uptake in leaves during the daytime demand higher transcriptional level of some aquaporin genes. Dehydration caused difficulty in root water absorption with lower osmotic potential in the first place, and caused less water evapotranspiration rate by inducing stomata closure and induced ABA production at a later stage (Zhang et al., 2018). The transiently increased expression of PvPIP2;9 upon PEG treatment could be a direct response to the water deficit due to PEG-induced shortage of root water absorption. And the ABA-induced suppression of PvPIP2;9 in leaves could be due to the quick response of stomata closure that, in turn, helped balancing leaf water status. In short, the expression pattern of PvPIP2;9 was highly responsive to plant water status, which was in agreement with the gene’s function in the regulation of plant water status, indicating that regulation of the PIP2 gene family at the transcription level was important for switchgrass.

It was also interesting to note that during drought stress, expression of PvPIP2;9 in all transgenic plants also changed even though the transgene was driven under maize ubiquitin promoter. Although being regarded as a constitutive promoter, the maize ubiquitin promoter contains stress-responsive cis-element and was found upregulated under heat and other stress conditions (Streatfield et al., 2004), which might be one explanation. Another possibility for such a drought-responsive expression pattern in the transgenic plants is that expression of PvPIP2;9 be regulated at the post-transcriptional level, for example, through unidentified small RNAs similar to the cases of multiple human
aquaporin genes (Gomes, da Silva, Rodrigues, Castro, & Soveral, 2018).

Upon the recognition of PIPs’ contributions to water transport and cellular water homeostasis, there is an interest to understand the full picture of how these PIPs interacted and coordinated with each other in these cellular processes. First, from the perspective of the protein–protein interactome, previous studies on maize PIP1 and PIP2 subfamily genes showed that ZmPIP1–ZmPIP2 interaction was required for PIP1 trafficking to plasma membrane (Zelazny et al., 2007), and such physical interaction between PIP1 and PIP2 (e.g., ZmPIP1;2-ZmPIP2;1) was required for their functions to form consolidated water channels (Fetter, Van Wilder, Moshelion, & Chaumont, 2004). A more recent work studying on interactomes of PIP1;2 and PIP2;1 protein using immunoprecipitation and quantification by mass spectrometry (IP-MS) revealed that these two proteins interacted with a total of 481 proteins among which 343 interacted with both PIP1;2 and PIP2;1 (Bellati et al., 2016). This big interacting protein mass likely behaved as a “platform” for recruitment of various proteins involved in transport activities including those responding to osmotic and oxidative treatments (Bellati et al., 2016). Second, at the post-translational level, 12 out of 13 Arabidopsis PIPs were found to have varied types of post-translational modifications including phosphorylation, methylation, deamidation and acetylation in response to environmental stresses (di Pietro et al., 2013). Third, at the post-transcriptional level, it was reported that microRNAs (miRNAs) were endogenous modulators of multiple aquaporin genes in human (Gomes et al., 2018). Another study in Arabidopsis also reported that salinity treatment invoked a simultaneous transcriptional repression and protein internalization of PIP2;7 (Pou et al., 2016).

Yet, it was less recognized that, at transcriptional level, expression of these PIP2 genes was also inter-affected. There are 14 PvPIP2 genes in switchgrass in reference to the current switchgrass genome database (“P. virgatum v4.1, DOE-JGI, http://phytozome.jgi.doe.gov/”). In this study, we found that over-expressing PvPIP2;9 in switchgrass significantly affected expression of many other PIP2 genes (Figure 6). We reasoned these changes of other PIP2 genes at the transcriptional level might be due to feedback effect of cellular status in the transgenic plants because of potential functional redundancy between these PIP2 genes, or due to post-transcriptional regulation of PIP2 genes (e.g. targeted by miRNA on certain common sequence among these PIP2 genes), which was yet to be studied in the future. Overall, our current results indicate that there was a complicated interacting network of PIP2s at the transcriptional level as well. Together with previous findings, PIPs likely responded to environmental constraints at multiple levels of gene regulation to adjust plant water status.

A PIP2 gene was cloned and functionally characterized for the first time in switchgrass in this study. PvPIP2;9 positively contributed to plant water status and its over-expression lead to significantly improved drought tolerance, WUE, biomass yield and feedstock quality in switchgrass when under the drought stress condition. Moreover, the result together with previous reports supported that PIP2s were coordinately regulated at multiple levels, including transcriptional, post-transcriptional and post-translational levels, to adjust plant water status. Results of this study highlight the importance of the aquaporin gene, PvPIP2;9, and the complexity of PIP2 family genes in the regulation of switchgrass water status. Such information will be useful for switchgrass molecular breeding toward improved drought tolerance and higher WUE.

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

AUTHORS’ CONTRIBUTIONS

B.X., J.Z. and B.H. designed the experimental studies; B.X. and J.Z. wrote the manuscript; J.Z., W.W., H.L. and Q.L. conducted the experiments; J.Z., W.W., H.L. and B.X. analyzed the data. All authors read and approved the final manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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