Overexpression of an aquaporin gene PvPIP2;9 increased leaf length, improved drought tolerance and water use efficiency and affected other PIP2 genes' expression in switchgrass (Panicum virgatum L.)

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Abstract

Background: Switchgrass (*Panicum virgatum* L.) is a prime candidate for non-grain-based bioenergy feedstock production. Improved the drought tolerance and higher water use efficiency are important traits for its successful field establishment and production especially in marginal land. Aquaporins are key channels and regulators for water transportation and maintenance of cellular water status. In this study, the functional role of an aquaporin gene, *PvPIP2;9*, in switchgrass drought tolerance was studied.

Results: Expression of *PvPIP2;9* was regulated by the diurnal oscillation and osmotic stress. Constitutive over-expressing *PvPIP2;9* in switchgrass significantly improved its drought tolerance and water use efficiency with less electrolyte leakage rates but higher relative water contents, photochemical efficiencies, and chlorophyll contents. Moreover, expression patterns of all 14 switchgrass *PIP2* subfamily genes were checked in wildtype and transgenic plants during the water-withdrawal treatment, and the result showed that over-expressing *PvPIP2;9* also affected transcript levels of most other *PIP2* genes.

Conclusions: Together, this study showed that improved drought tolerance and higher water use efficiency can be achieved by manipulating the expression level of *PvPIP2;9* and also suggested *PIP2* subfamily genes were transcriptionally regulated in a coordinated manner.

Introduction

Switchgrass (*Panicum virgatum* L.) is a warm season tall perennial grass originated in North America and has been used as a pasture forage (1) and a non-grain-based bioenergy feedstock (2). A previous study showed that all tested switchgrass ecotypes suffered severe biomass reduction (75–80%) with water stress at −4 MPa (3). To minimize competition with primary food crop production for land use, much of switchgrass production are on less productive marginal land 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 (4). Based on their sequence compositions, aquaporins can be divided into five types, including tonoplast intrinsic proteins (TIPs), nodulin 26-like intrinsic proteins (NIPs), small basic intrinsic proteins (SIPs), X-intrinsic proteins (XIPs), and plasma membrane intrinsic proteins (PIPs), among which PIPs might be the main gateways controlling water permeability (5). 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 indispensible roles in water transport (6, 7). Several individual studies have reported that over-expressing *PIP2* subfamily genes improved plant drought tolerance, including *FaPIP2;1* in tall fescue (*Festuca arundinacea)* (8), *PIP2;5* in Populus (Populus tremula × Populus alba) (9), *TaAQP7* in wheat (*Triticum aestivum)* (10), and *HvPIP2;1* in barley (*Hordeum vulgare)* (11). 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 (12). 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 (13). Lee et al. (14) reported that activating a 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 might be diversified, 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 (15), 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. 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 up-regulated aquaporin gene in the transgenic plants (16, 17). Therefore, we hypothesized that *PvPIP2;9* might play an important role in the regulation of switchgrass drought tolerance. In the current work, the expression pattern and functional role of *PvPIP2;9* in switchgrass drought tolerance were studied, and effects of drought and over-expression of *PvPIP2;9* on other *PIP2* family genes were also measured.

Results

Phylogenetic analysis and expression pattern of *PvPIP2;9*

According to the switchgrass genomic sequence information (*Panicum 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 kD. As a typical aquaporin protein, PvPIP2;9 had six conserved transmembrane (TM) domains and two nucleosome assembly protein (NAP) domains (Fig.1a). A phylogenetic tree comprising of PvPIP2;9 and its orthologs 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 (Fig. 1b).

At the organ/tissue level, the transcript level of *PvPIP2;9* in roots was the highest, which was ~6–10 times higher than those in leaves and leaf sheaths, and was ~10–20 times higher than those in inflorescence of rachis, florets, vascular bundle, and internodes (Fig. 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 (Fig. 2b). Furthermore, we measured its relative expression level in plants under 20% PEG6000 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 hrs of treatment and remained at its basal level thereafter (Fig. 2c). However, ABA treatment did not activate but suppressed the expression of *PvPIP2;9 (*Fig. 2d), indicating that the osmotic stress-induced expression of *PvPIP2;9* was likely independent of the ABA signal.

Over-expressing *PvPIP2;9* led to longer leaf length, improved drought tolerance and water use efficiency in switchgrass

The *PvPIP2;9* was over-expressed in switchgrass under driven of 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) (Fig. 3a). Two representative transgenic lines (line–7 and –9) with over-expressed *PvPIP2;9* and positive GUS staining signals (Fig. 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 two and a half months of growth, single tillers of WT and the transgenic lines proliferated into five to seven tillers, and both *PvPIP2;9*-OX lines showed significantly longer leaf length and taller plant height than those of WT (Fig. 4).

These switchgrass plants were treated by withdrawing water to evaluate whether over-expressing *PvPIP2;9* affected drought tolerance. After 28 days of water-withdrawal period, WT became severely wilted that even the newly emerged leaves at the top withered and turned yellow, while plants of both 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 both *PvPIP2;9*-OX lines recovered back with new green expanding leaves (Fig. 5a). During the water-withdrawal treatment, the pot soil water content dropped from 40% to 23–26%, 11–15%, 5–6%, and 4% 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 40–50% (Fig. 5b). Five physiological parameters, including photochemical efficiency (Fv/Fm), chlorophyll (Chl) content, electrolyte leakage (EL), leaf relative water content (RWC), and WUE were measured in WT and transgenic lines during the drought treatment. As shown in figure 5c-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 water-withdrawal 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 (Fig. 5c-g). WUE of plants after 28 days of water holding was not measured because leaves of WT were completely wilted already. 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.

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 water-withdrawal treatment in both WT and transgenic plants.

Firstly, it was notable that expression of *PvPIP2;9 per se* was responsive to the long-term drought stress but was distinctively different in leaves and roots: the relative expression of *PvPIP2;9* increased ~3 times in WT leaves, but decreased ~5 times in WT roots during the drought treatment. While in both transgenic lines, relative expression levels of *PvPIP2;9* were ≥200 times higher than those of WT in leaves, and ≥2 times higher than those of WT in roots (the only exception was that in line–7 before water-withdrawal treatment where it was at similar expression level to that in WT). Yet, during the water-withdrawal treatment, expression levels of *PvPIP2;9* decreased in leaves but gradually increased 9–13 folds in roots in the two transgenic lines (Fig. 6).

Secondly, as shown in WT, most *PIP2* subfamily genes were responsive to the water-withdrawal treatment. For examples, transcriptional levels of *PvPIP2;3,* P*vPIP2;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 (Fig. 7).

Thirdly, by comparing gene expression patterns in WT and in two transgenic lines, we found that expression of most PIP2 family genes were affected by the over-expression of *PvPIP2;9.* For examples, 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 (Fig. 7).

Discussion

There are 68 aquaporin genes in switchgrass (15). 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 leaf water status in switchgrass. Current results in this study clearly proved that *PvPIP2;9* positively regulated switchgrass drought tolerance and WUE.

OsPIP2;4 was the closest rice orthologous gene of PvPIP2;9 with 97% amino acid similarity and the same conserved domains (Fig. 1). 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 (6). *PvPIP2;9* also had a similar expression pattern to *OsPIP2;4* that increased after dawn, decreased after the noon time, and then maintained at minimum expression level at night which was in conjunction with the activity of plant diurnal water transport (Fig. 2). 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 (18), *ZmPIP2;1* and *ZmPIP2;5* in maize (18), and *OsPIP2;4* and *OsPIP2;5* in rice (6). Such an expression pattern suggested that water uptake in leaves during the daytime might demand higher transcriptional level of this aquaporin gene. Furthermore, the expression of *PvPIP2;9* in leaves was transiently up-regulated by osmotic stress (PEG6000), but down-regulated by exogenous applied ABA treatment in leaves (Fig. 2). 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 (19). 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 congruent with the gene's function in the regulation of plant water status.

Under the well-watered condition, constitutive over-expression of *PvPIP2;9* in switchgrass did not significantly alter the WUE and leaf relative water content 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 and taller plant height than those of WT. It was reported that leaf elongation rate responded to rapid changes in evaporation and soil water availability in an even considerably quicker manner than transpiration and leaf water potential (e.g. 30 min vs. 1–2 h), and small flux of water potentials could cause rapid decline of simulated leaf elongation rate (20). Nada & Abogadallah (21) reported that reduced WUE and decreased RWC at mid-day in rice even under well-watered paddy field which was associated with inadequate expression of aquaporin genes, and such an suppression could be eliminated upon removal of radial barriers to water flow in roots. We reasoned that these WT and transgenic plants might 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, leaves of transgenic plants were less challenged with temporary fluxes of unfavorable leaf water potential, which lead to the fact that transgenic plants had longer leaves and taller plant height than WT. Another possible reason for such an improvement in transgenic plants 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 CO_2 as well as H_2O_2 (Azad et al., 2016). It deserved to be further investigated on whether PvPIP2;9 was involved in transportation of other molecules in the future.

Under water-withdrawal 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 examples, 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 (22). And over-expressing a TIP-type aquaporin (*AQUA1)* in populous (*Populus alba)* also improved the plant's WUE and RWC (23). In this study, we found transgenic plants had significantly higher WUE associated with lower EL, higher Fv/Fm, Chl content, and RWC than WT after prolonged water-withdrawal treatment (Fig. 5). Lower EL value indicated better cell membrane integrity, higher Fv/Fm 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.

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. From the perspective of protein-protein interactome, previous studies on two maize *PIP1* and three *PIP2* subfamily genes showed that ZmPIP1;1 and ZmPIP1;2 could form heterodimers but showed no activity of osmotic water permeability (24); yet this ZmPIP1-ZmPIP2 interaction was required for PIP1 trafficking to plasma membrane (25). At the same time, such physical interaction between PIP1-PIP2 (e.g. ZmPIP1;2-ZmPIP2;1) was required for their functions to form consolidated water channels (24). A more recent work studying on interactomes of PIP1;2 and PIP2;1 proteins, using the approach of immunoprecipitation and quantification by mass spectrometry (IP-MS), revealed that these two proteins shared about 400 interacting proteins (26). This big interacting protein mass likely behaved as a "platform" for recruitment of varied proteins likely involved in transport activities including those responding to osmotic and oxidative treatments (26). 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 stress (27). Meanwhile, at the post-transcriptional level, it was reported that microRNAs (miRNAs) were endogenous modulators of multiple aquaporin genes in human (28). Another study in Arabidopsis also reported that salinity treatment invoked a simultaneous transcriptional repression and protein internalization of *PIP2;7* (29). Yet, it was less recognized that, at transcriptional level, expression of these *PIP2* genes were also inter-affected. There are 14 *PvPIP2* genes in switchgrass in reference to the current switchgrass genome database ("*Panicum 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 (Fig. 7). 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 potentially 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 result manifested 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.

As mentioned in the part of Introduction, *PvPIP2;9* was the only aquaporin gene with significantly increased expression in the *LOV1* transgenic plants that showed improved drought tolerance (16, 17). We also tested whether or not the LOV1 transcription factor could directly bind to the –2 Kb promoter region of *PvPIP2;9* using yeast one-hybrid test. 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.

Conclusion

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 significantly improved switchgrass drought tolerance and WUE when over-expressed. Moreover, the fact that over-expressing *PvPIP2;9* also affected expression of other *PIP2* genes suggested that *PIP2*s were coordinately regulated at multiple levels (including transcriptional, post-transcriptional and post-translational levels) to adjust plant water status. Results of this study highlighted 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.

Experiemental Procedures

Plant materials and growth condition

Seeds of an elite switchgrass line, HR8, originally selected from the lowland ecotype ‘Alamo’ was used in this study (30). In the qPCR experiment to study *PvPIP2;9* expression pattern, 4-wk-old plants were cultured in 1/2 Hoagland solution and grown in a growth chamber with a 12-hour (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 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).

qRT-PCR analysis

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 *PvPIP2;9,* the first sampling time was set at the dawn for consecutive 40 hr with four hr internals in-between. For stress treatments, plants were grown in 1/2 Hoagland solution containing 20% polyethylene glycol (PEG) 6000 (Huada, Shantou,

China) and 100 μM ABA according to Yuan et al. (31), and sampled after 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. The first strand cDNA was synthesized with 1 μg RNA using the PrimeScript™ RT reagent Kit (Takara, Dalian, China) 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 qRT-PCR was performed with three biological replicates and two technical replicates, and the qPCR program set as follows: 10 min at 95 °C for initial denaturation, and 40 cycles (95 °C for 15s, 58 °C for 15 s, and 72 °C for 20 s). Relative expression levels of *PvPIP2;9* were calculated using the $2^{-\Delta\Delta CT}$ method with *PvACTIN* as the reference gene (32). Primers used in this study were shown in Additional file 1: Table S1.

Gene cloning and vector construction

According to the switchgrass genomic sequence information (*Panicum 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 (32) using LR reaction (Invitrogen). The resultant vector, pVT1629-PvPIP2;9, harboring the *PvPIP2;9* driven under maize ubiquitin promoter and the *UidA* (GUS) reporter gene under CaMV 35S promoter, was transformed into the *Agrobacterium tumefaciens* strain '*AGL1'* through electroporation.

Switchgrass genetic transformation

Switchgrass line 'HR8' was used for *Agrobacterium*-mediated genetic transformation and the transformation procedure was the same as reported before (30). 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 *HPT*Ⅱ gene present in the T-DNA.

Drought treatment of WT and transgenic plants

Two independent transgenic lines and tissue culture-regenerated wild-type (WT) plants were propagated by splitting single tillers grown at the optimum condition. Plants grown from a single tiller for two and a half months reached E4 stage (33) and were used for drought treatment by withdrawing water. After 28d of water-withdrawal, 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 Corp., Santa Barbara, CA) was used to monitor the soil water content (SWC) in 0–8 cm deep soil layer of each pot.

Measurement of physiological parameters

Leaf membrane stability was evaluated by measuring the electrolyte leakage (EL) (34) according to a method described before (35). 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 (C_i) was measured using a conductance meter (Thermo Scientific, Beverly, USA).Then the leaf tissue was killed by autoclaving at 121°C for 15 min, and then incubated for 24 h on a shaker for measuring the maximum conductance (C_{max}) of the solution. Relative EL was calculated as EL = (C_i/C_{max}) × 100%.

The leaf relative water content (RWC) was determined according to the method described by Hu et al. (36) with modifications. In brief, RWC was determined using fresh fully expanded leaves (~0.2 g). Leaf samples were 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 h 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 (*F*v) to the maximal fluorescence (*F*m) (*F*v/*F*m) was used to represent leaf photochemical efficiency (Oxborough and Baker, 1997). The *F*v/*F*m ratio was determined using a fluorescence meter (Dynamax, Houston, TX, USA) as described before (35). And chlorophyll content were measured using the DMSO extraction method as described before (35).

Leaf WUE was calculated thought measuring leaf net photosynthetic rate (Pn) and transpiration rate (Tr) using the LI–6400 portable photosynthesis system (LI-COR, Lincoln, NE). 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.

Statistical analysis

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

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

All authors consent for publication.

Availability of data and materials

All data generated or analyzed in the present study are included in this published article and in additional information.

Competing interests

The authors have no conflict of interest to declare.

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Authors' contributions

BX, ZJ and BH designed the experimental studies; BX and ZJ wrote the manuscript; ZJ, WW, HL, and QL conducted experiments; ZJ, WW, HL and BX analyzed data. All authors read and approved the final manuscript.

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Figures

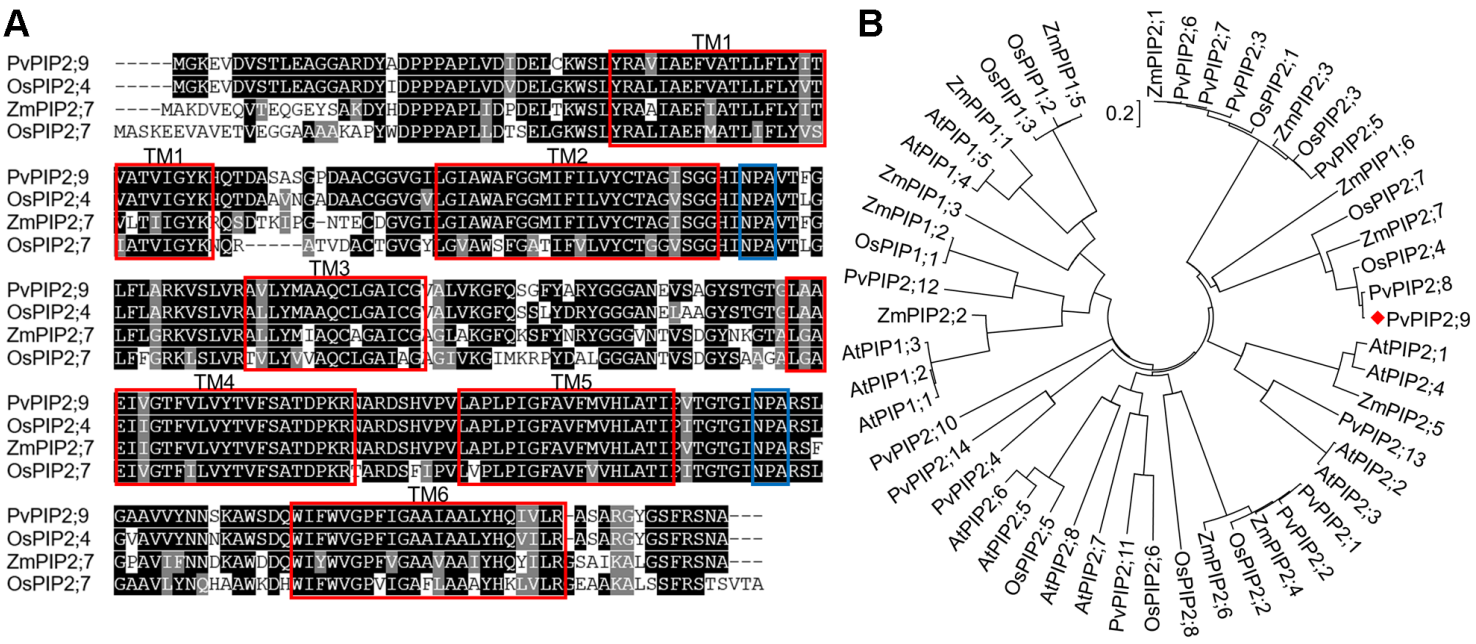

Figure 1

Phylogenetic tree and multiple alignment of 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 (37) with the sum of branch length = 24.55096375. The tree is drawn to scale and there were a total of 264 positions in the final dataset. (B) Multiple sequence alignment of PvPIP2;9 with its closest orthologues, showing that these PIP2s shared conserved TM domains and NPA residues.

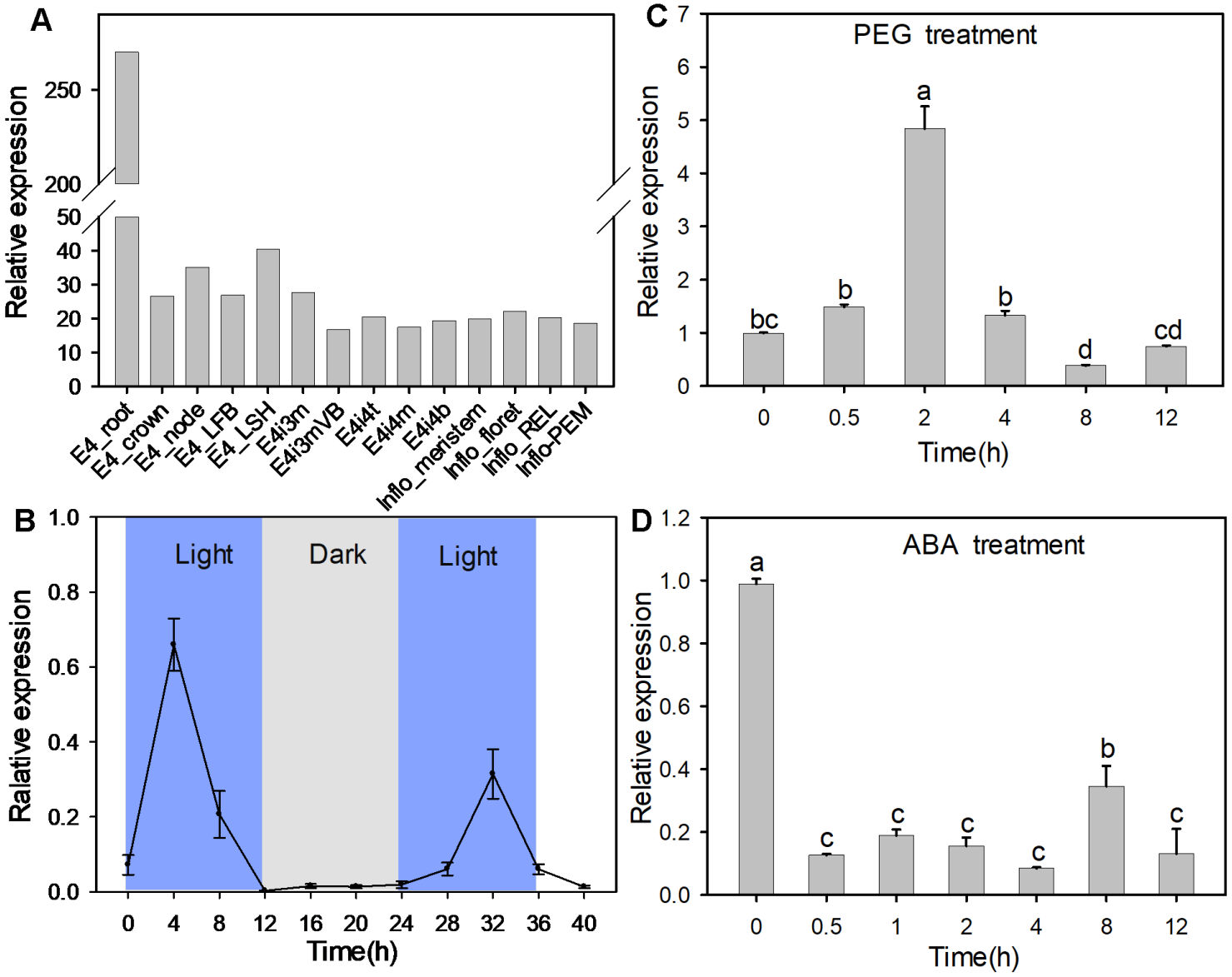

Figure 2

Expression pattern of PvPIP2;9. (A) Transcripts levels of PvPIP2;9 in different organ/tissues. (B-D) qRT-PCR measurement of PvPIP2;9 relative expression levels during 40 hrs, and relative expression of 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 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 $P<0.05$. Data in (A) was were adopted from PviGEA database and the abbreviations were as follows. E4i4b: Bottom 1/5 fragment of the 4th internode; E4i4t: Top 1/5 fragment of the 4th internode; E4i4m: Middle 1/5 fragment of the 4th internode 4; 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 3rd internode; E4i3m-VB: Vascular bundle isolated from 1/5 fragment of the 3rd 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.

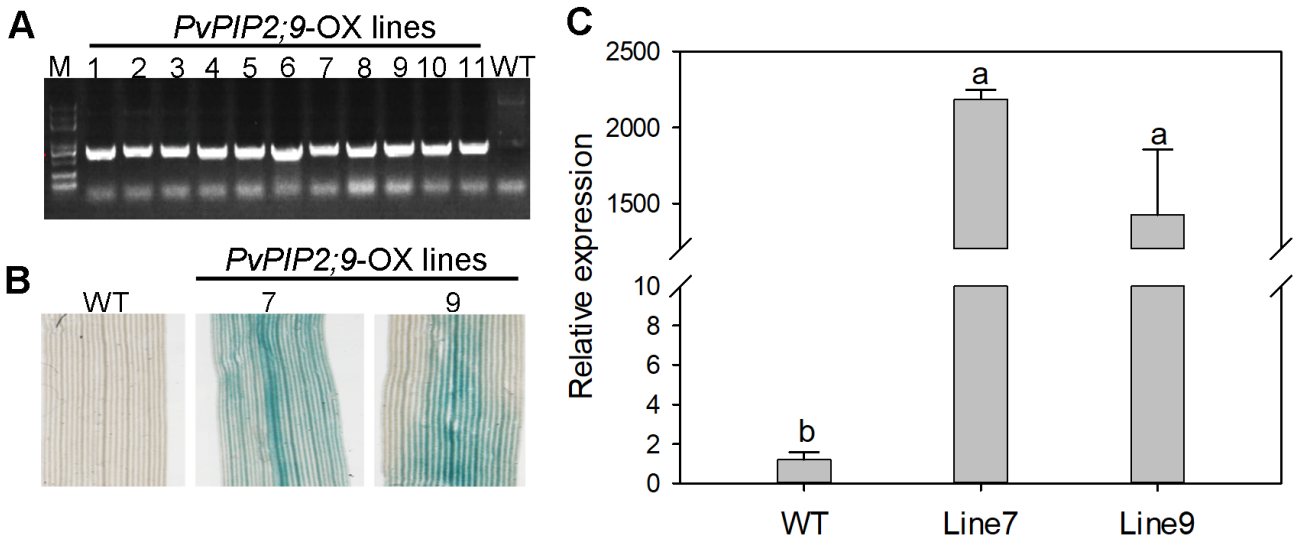

Figure 3

Identification of PvPIP2;9 transgenic switchgrass lines. (A) Confirmation of the T-DNA insertion with PCR for the presence of HPTII gene. (B) GUS staining of two transgenic lines. (C) Relative expression of PvPIP2;9 in two transgenic lines by qRT-PCR. Data in (C) are means ± SE, and different letters above bars represent significant difference at $P<0.05$.

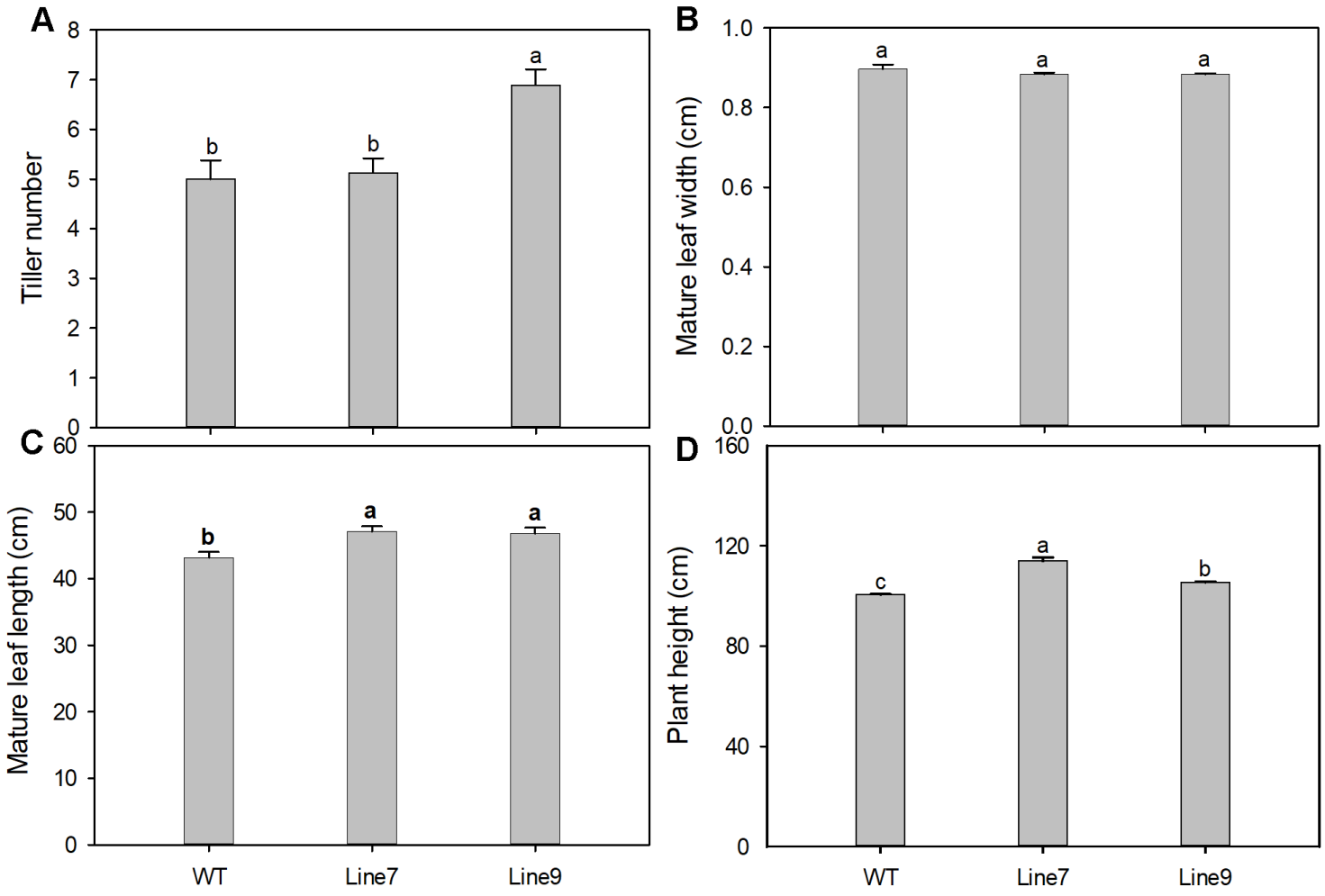

Figure 4

Phenotypic comparison between WT and transgenic plants, including tiller number (A), leaf width (B), leaf length (C) and plant height (D). Single tillers of WT and the transgenic lines were grown in separate pots for two and a half months of growth. The data were from eight individual plants for WT and each transgenic line (n=8). Data are means ± SE, and different letters above bars represent significant difference at $P<0.05$.

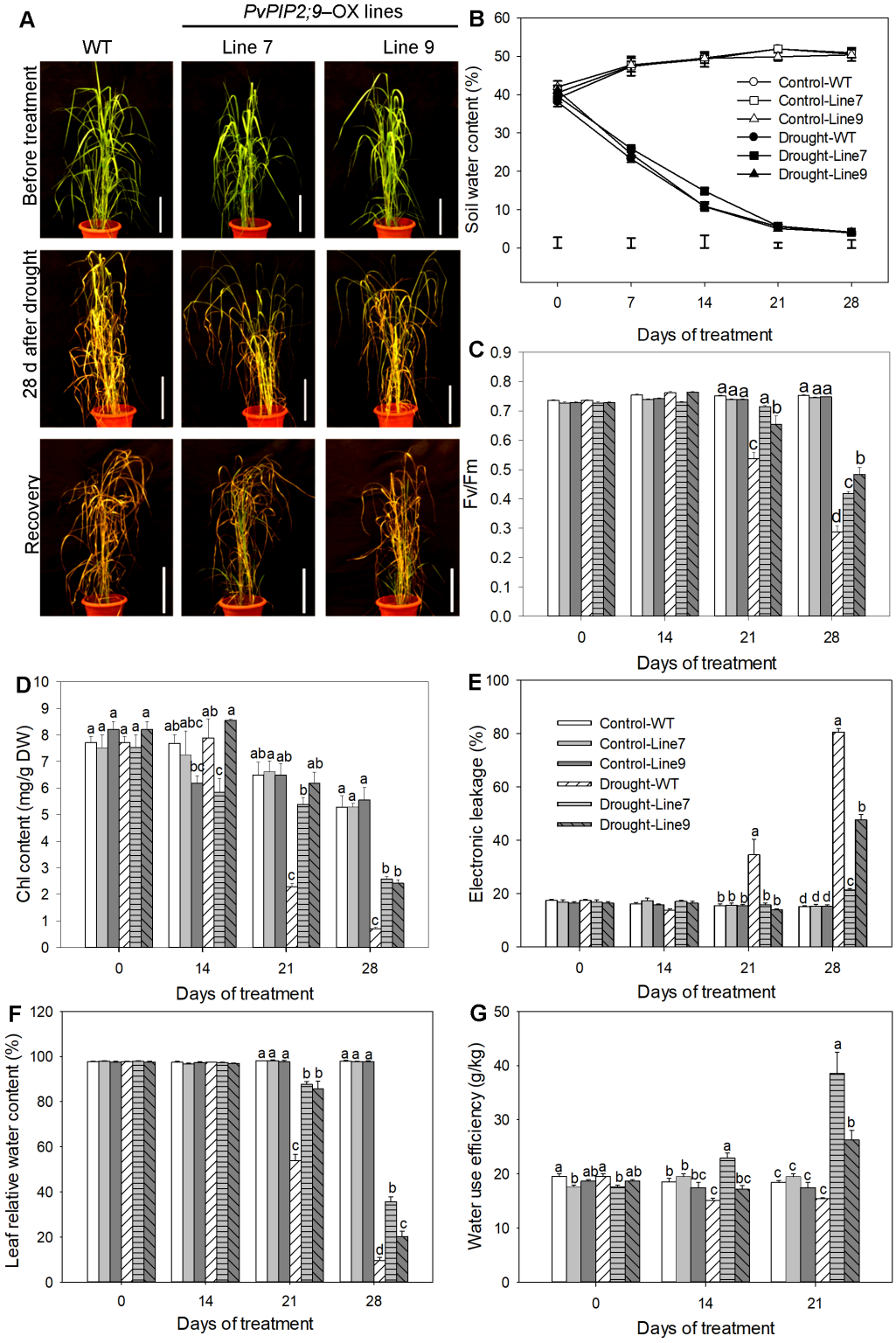

Figure 5

Comparison of drought tolerance between WT and PvPIP2;9 transgenic switchgrass lines. (A) Phenotypes of WT and two transgenic lines before and after 28 days of water-withdrawal treatment, and after 10 days of re-watering (recovery). (B) Soil water content in pots for the well-watered control and for those under the water-withdrawal treatment. (C-G) Dynamic changes of Fv/Fm (C), Chl contents (D), EL (E), RWC (F), and WUE (G) of the WT and two transgenic lines during the water withdrawing period. 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<0.05.

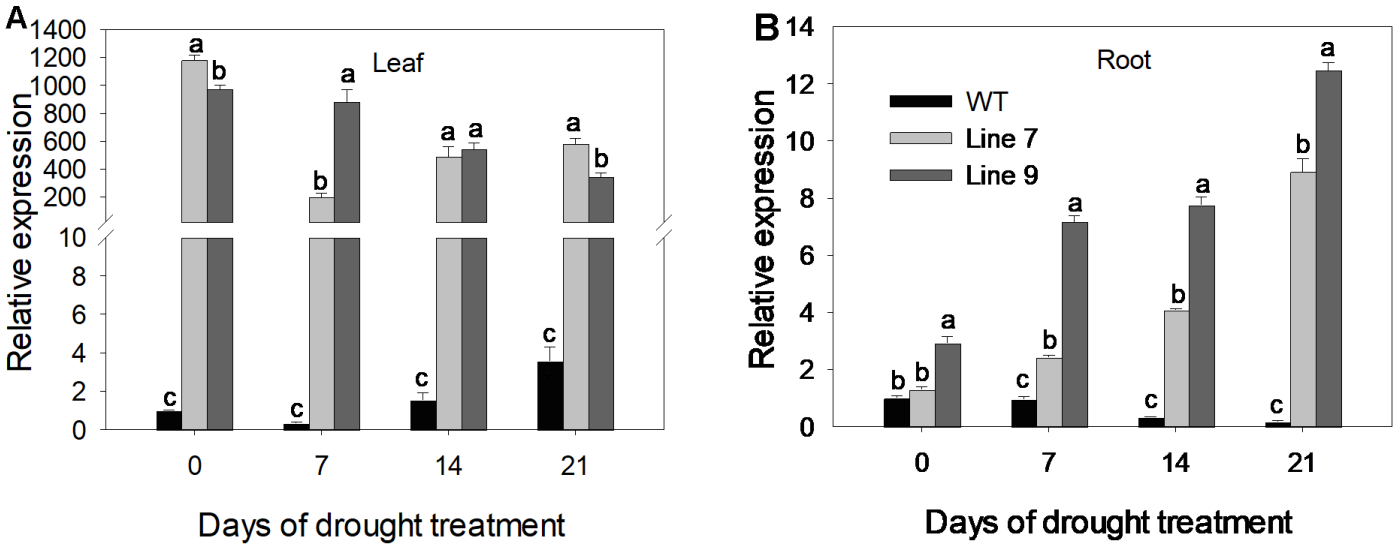

Figure 6

Relative expression of PvPIP2;9 in leaves and roots during 21 days of water-withdrawal using qRT-PCR. Data are means ± SE. Different letters above bars represent significant difference at P<0.05.

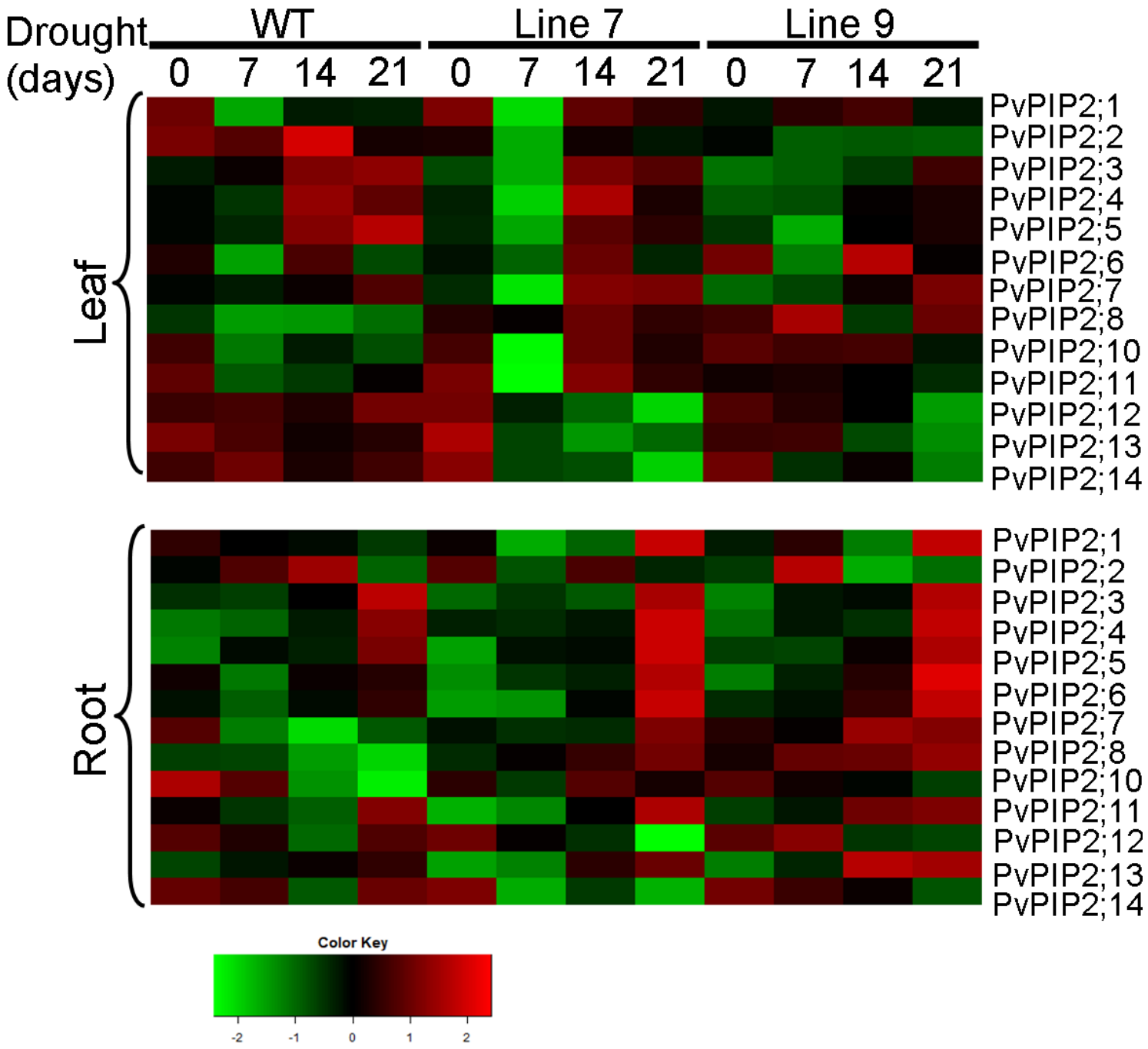

Figure 7

Relative expression of other PIP2 subfamily genes in leaves and roots during 21 days of water-withdrawal using qRT-PCR. Data of qRT-PCR were converted into the heatmap using R- package software (version 3.3.1) and the quantitative color scheme was based on log2 of each PIP2 subfamily genes' relative expression levels.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- TableS1.docx