

Overexpression of an aquaporin gene *PvPIP2;9* increased biomass yield and protein content, 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 drought tolerance and higher water use efficiency are important for its successful field establishment and production, especially on marginal lands. 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 was studied.

Results: Expression of *PvPIP2;9* was regulated by diurnal oscillation and osmotic stress. Constitutive over-expressing *PvPIP2;9* in switchgrass significantly improved its leaf length, plant height, above-ground biomass, biomass protein contents, and cellulose contents in stressed plants. Under 21 days of drought treatment, transgenic plants showed less electrolyte leakage rates, but higher relative water contents, photochemical efficiencies, and chlorophyll contents, indicating that *PvPIP2;9* positively regulated plant drought tolerance and water use efficiency. Moreover, expression patterns of all 14 switchgrass PIP2 subfamily genes were checked during the drought 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 biomass yield, 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 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 (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 indispensable 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 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 are 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.: Pavr.Ba02478) was the only differentially-expressed and up-regulated aquaporin gene in the transgenic plants (16, 17). Therefore, we hypothesized that *PvPIP2;9* plays 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 (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 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 improved drought tolerance, water use efficiency, higher biomass yield and crude protein contents 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). Three representative transgenic lines (line-1, -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 all *PvPIP2;9*-OX lines showed significantly longer leaf length, taller plant height, and higher above-ground biomass yield (fresh weight and dry weight) 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 (Fig. 5a). During the drought treatment, the pot soil water content dropped from -0.48 MPa to -0.83~-0.85 Mpa, -1.35~-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 -0.48-0.5 MPa (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 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 (Fig. 5c-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 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 above-ground biomass of WT and transgenic lines. As shown in table 2, all transgenic lines had significantly higher crude protein content than WT no matter the plants were grown under the optimum soil water or after 21 days of drought. It is also notable that the tested plants showed varied levels of total sugar, cellulose, hemicelluloses and lignin under the optimum soil water condition; yet after 21 days of drought, transgenic lines had significantly higher cellulose.

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 WT and the three 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 reduced to 20% in WT roots during the drought treatment. While in 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-1 and -7 before drought treatment where they were at similar expression levels to that in WT). Yet, during the drought treatment, expression levels of *PvPIP2;9* decreased in leaves but gradually increased 6-13 folds in roots in the two transgenic lines (Fig. 6).

Secondly, as shown in WT, most *PIP2* subfamily genes were responsive to the drought treatment. For examples, 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 (Fig. 7).

Thirdly, by comparing gene expression patterns in WT and in 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 indicate that *PvPIP2;9* positively regulated switchgrass drought tolerance and WUE.

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 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 (18). And over-expressing a TIP-type aquaporin (*AQUA1*) in white poplar (*Populus alba*) also improved the plant's WUE and RWC (19). 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 drought 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.

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 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, taller plant height and higher above-ground biomass than those of WT. Another interesting finding with the transgenic switchgrass was their higher protein contents 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 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 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 contents in maize cultivars with contrasting drought tolerance has been reported that the drought tolerant cultivar showed higher protein contents than the susceptible one (22). Effects of short-term drought (4 or 7 days of drought) in switchgrass (23) and long-term drought (28 days) in miscanthus (24) 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 (24). The relatively higher cellulose content in 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 CO_2 as well as H_2O_2 (Azad et al., 2016). Further investigation on whether *PvPIP2;9* was involved in transportation of other molecules is warranted in the future.

***PvPIP2;9* and other *PvPIP2* 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 (6). *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 (25), *ZmPIP2;1* and *ZmPIP2;5* in maize (25), and *OsPIP2;4* and *OsPIP2;5* in rice (6). Such an expression pattern suggested that water uptake in leaves during the daytime demand higher transcriptional level of this aquaporin gene. Furthermore, the expression of *PvPIP2;9* in leaves was transiently up-regulated by osmotic stress, but down-regulated by exogenous applied ABA treatment in leaves. 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 (26). 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, indicating that regulation of the *PIP2* gene at the transcription level was important for switchgrass.

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 the 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 (27); yet this ZmPIP1-ZmPIP2 interaction was required for PIP1 trafficking to plasma membrane (28). 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 (27). 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 (29). This big interacting protein mass likely behaved as a "platform" for recruitment of various proteins likely involved in transport activities including those responding to osmotic and oxidative treatments (29). 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 (30). Meanwhile, at the post-transcriptional level, it was reported that microRNAs (miRNAs) were endogenous modulators of multiple aquaporin genes in human (31). Another study in Arabidopsis also reported that salinity treatment invoked a simultaneous transcriptional repression and protein internalization of *PIP2;7* (32). 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 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.

As mentioned earlier, *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, enhanced plant protein contents of above-ground biomass, and significantly improved switchgrass cellulose contents, drought tolerance, and WUE in

stressed plants. Moreover, the result together with previous reports supported that *P/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 *P/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.

Methods

Plant materials and growth condition

Seeds of an elite switchgrass line, HR8, originally selected from the lowland ecotype 'Alamo' was used in this study (33). 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 $\mu\text{mol}\cdot\text{photons m}^{-2}\cdot\text{s}^{-1}$). In other experiments, switchgrass plants were grown in clay loam soil mixed with sand (1:1) in $1.1\times 10^{-2}\text{ m}^3$ pots in the greenhouse at Nanjing Agricultural University (Nanjing, China) with temperatures set at $30/20 \pm 3^\circ\text{C}$ (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. (34), 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 PrimeScriptTM 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 (35). 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 (35) 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 'AGL 1' 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 (33). 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* 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 (36) and were used for drought treatment by withdrawing water. After 28d 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 Corp., Santa Barbara, CA) was used to monitor the soil water content (SWC) in 0-8 cm deep soil layer of each pot. And soil water potential was determined using ERS-water potential and temperature meter (Yibaiyi Mechanical and Electrical Equipment Co., Ltd., Wenzhou, China). The correlation between soil water content and soil water potential was shown in Additional file 2: Figure S1.

Biomass feedstock quality analysis

After 21 d of treatment, the above ground 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 (37) with slight modifications. In brief, 0.05 g samples were added to the mixture of 10 ml ddH₂O and 3 ml HCl and incubated in water bath set at 100°C for 1 h. Then 0.5 ml supernatant was transferred to a 10 ml tube and mixed with 0.5 ml ddH₂O, 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 based on a standard curve generated with known sugar concentrations.

Cellulose content was estimated using the anthrone method (38). Briefly, 0.05 g samples were mixed with 35 ml 60% H₂SO₄ in a 50 ml tube and incubated at ice bath for 30 min. After ten 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 (39). For hemicellulose analysis, 0.1 g sample 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 rinsed three times with ddH₂O, 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 DNS reagent. The mixture was incubated in water bath at 100°C for 5 min and then absorbance of supernatant was measured at 520 nm. For lignin, 0.1 g sample was washed using 10 ml 1% acetic acid and the mixture of ethanol and ether (1:1) and then was dried in a water bath set at 100°C. The sample was incubated with 72% H₂SO₄ for at least for 16 h to remove cellulose. The precipitate was then mixture with 10 ml 10% H₂SO₄ and 0.1 M potassium dichromate and incubated in water bath set at 100°C for 15 min. The lignin content was then quantified after mixed with 5 ml 20% KI and 1 ml 0.5% starch solution by titration using 0.2 M sodium thiosulfate.

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 (40).

Measurement of physiological parameters

Leaf membrane stability was evaluated by measuring the electrolyte leakage (EL) (41) according to a method described before (42). 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 $EL = (C_i/C_{max}) \times 100\%$.

The leaf relative water content (RWC) was determined according to the method described by Hu et al. (43) 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 $RWC = (FW - DW) / (SW - DW) \times 100$.

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 (42). And chlorophyll content were measured using the DMSO extraction method as described before (42).

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, 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.

Tables

Table 1. Over-expression of *PvPIP2;9* affected above-ground growth of WT and transgenic plants. Three independent transgenic lines and tissue culture-regenerated wild-type (WT) plants were propagated by splitting single tillers grown at the optimum condition for two and a half months reached E4 stage. Then the tiller number, leaf width, leaf length, and plant height were measured. The above ground fresh and dry weight were tested from those plants after another 28 days growth. Data are means \pm SE (n=8), and different letters represent significant difference at P <0.05.

	Tiller number	Leaf width (cm)	Leaf length (cm)	Plant height (cm)	Above ground fresh weight (g)	Above ground dry weight (g)
WT	5.00 \pm 0.38b	0.90 \pm 0.011a	43.19 \pm 0.83b	100.54 \pm 0.21c	31.66 \pm 1.41c	6.60 \pm 0.28c
Line 1	6.78 \pm 0.70a	0.90 \pm 0.045a	47.37 \pm 0.64a	110.03 \pm 3.39ab	42.26 \pm 1.64a	9.01 \pm 0.31a
Line 7	5.13 \pm 0.30b	0.88 \pm 0.003a	47.11 \pm 0.79a	113.92 \pm 1.47a	36.14 \pm 1.33b	7.68 \pm 0.30b
Line 9	6.89 \pm 0.32a	0.88 \pm 0.003a	46.78 \pm 0.88a	105.43 \pm 0.30b	40.30 \pm 1.71ab	8.16 \pm 0.23ab

Table 2. Over-expression of *PvPIP2;9* affected biomass feedstock quality. The biomass were harvested from plants after 21 days of drought treatment or under the optimum growth condition (control). Data are means \pm SE (n=3), and different letters represent significant difference at P <0.05.

	Sugars (%)	Cellulose (%)	Hemicellulose (%)	Lignin (%)	Crude protein (g/kg)
Control-WT	59.44 \pm 2.84 a	21.40 \pm 0.25 a	57.06 \pm 4.28 a	20.90 \pm 1.27 b	93.83 \pm 0.61 g
Control-line 1	47.66 \pm 1.41 b	19.96 \pm 0.32 bc	52.01 \pm 0.49 bc	22.71 \pm 1.47 a	114.55 \pm 1.31 f
Control-line 7	56.52 \pm 1.42 a	20.16 \pm 0.19 b	50.70 \pm 0.57 bc	20.72 \pm 0.25 b	110.24 \pm 0.80 d
Control-line 9	51.97 \pm 1.61 ab	19.55 \pm 0.19 cd	48.25 \pm 0.92 bc	20.80 \pm 0.32 b	105.47 \pm 0.48 e
Drought-WT	46.20 \pm 2.16 b	16.40 \pm 0.21 g	50.82 \pm 3.21 bc	18.33 \pm 0.33 c	71.40 \pm 1.25 d
Drought-line 1	48.69 \pm 3.66 b	18.23 \pm 0.29 f	52.36 \pm 5.05 ab	19.35 \pm 0.28 c	82.73 \pm 0.55 a
Drought-line 7	58.03 \pm 5.56 a	19.27 \pm 0.37 de	45.48 \pm 3.11 c	21.27 \pm 0.28 b	92.76 \pm 0.56 b
Drought-line 9	57.72 \pm 8.22 a	18.88 \pm 0.43 e	46.77 \pm 4.15 ab	22.58 \pm 0.32 a	86.88 \pm 0.62 c

Additional Files

Additional file 1: Table S1 Primers used in this study.

Additional file 2: Figure S1 Correlation between soil water content and soil water potential for the potting soil used in this study.

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

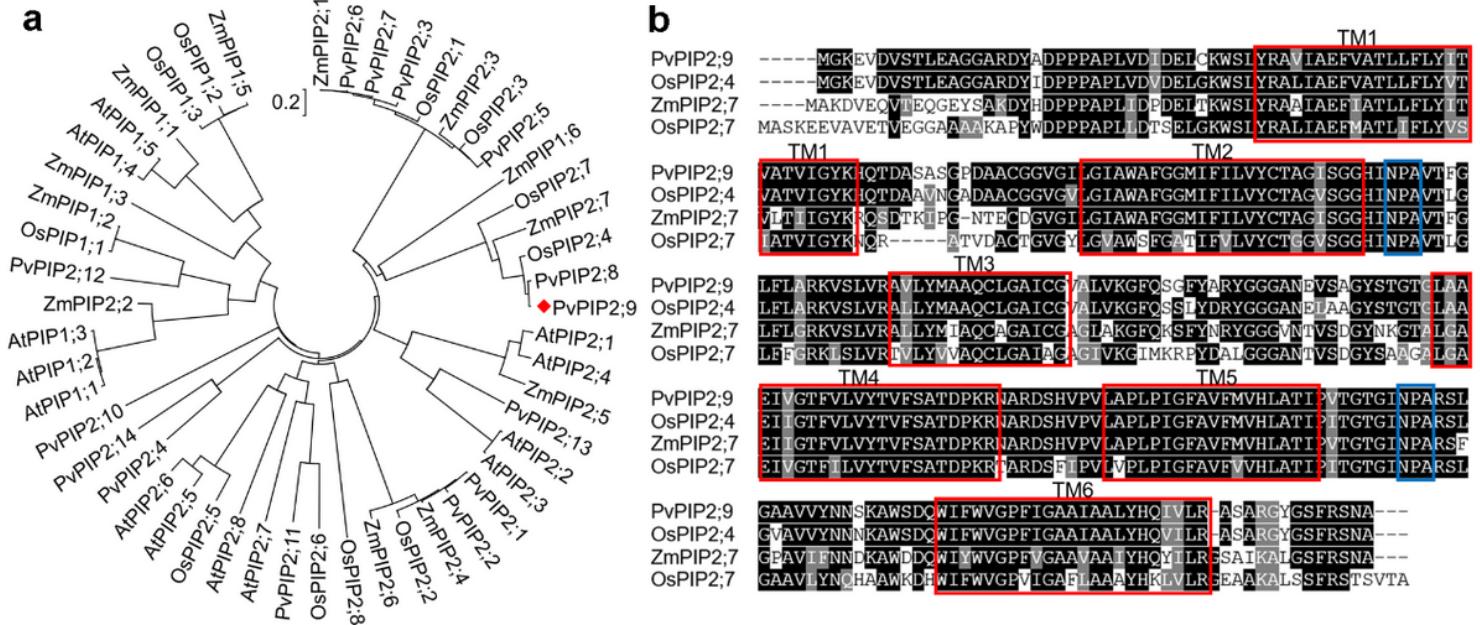


Figure 1

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 (44) 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.

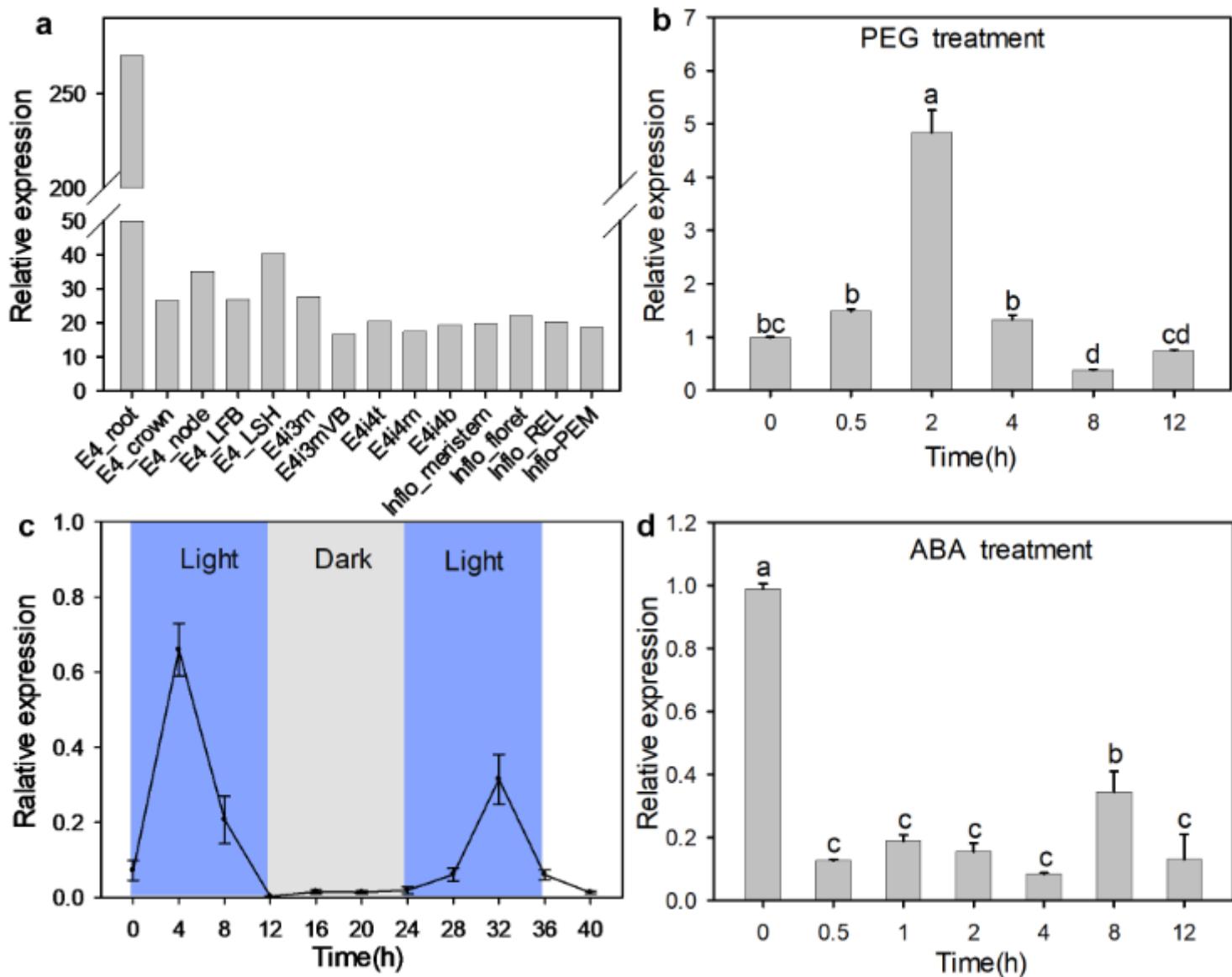


Figure 2

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 μ M 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 \pm SE. Different letters above bars represent significant difference at $P<0.05$. Data in (A) 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; 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.

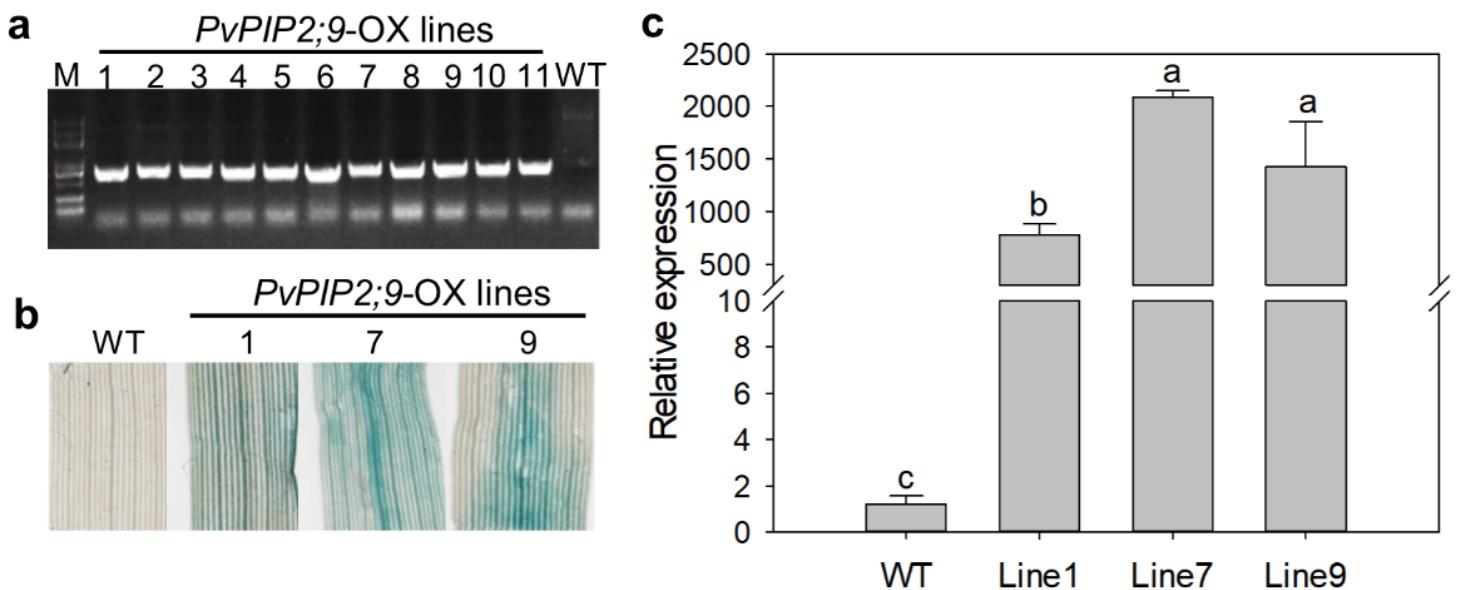


Figure 3

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 and (C) relative expression of *PvPIP2;9* in transgenic lines by qRT-PCR. Data in (C) are means \pm SE, and different letters above bars represent significant difference at $P<0.05$.

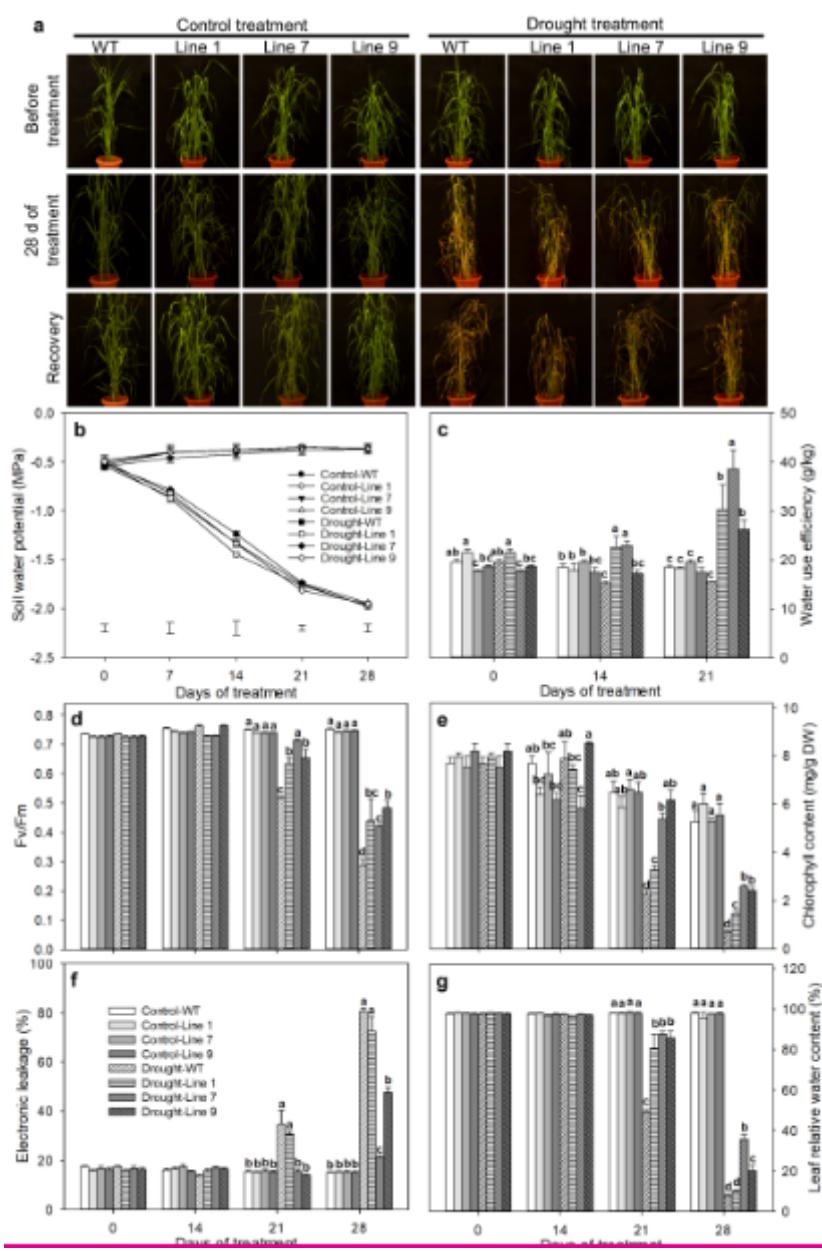


Figure 4

Comparison of drought tolerance between WT and PvPIP2;9 transgenic switchgrass lines. (A) Phenotypes of WT and transgenic lines before and after 28 days of drought 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 \pm SE. Different letters above bars represent significant difference at $P<0.05$.

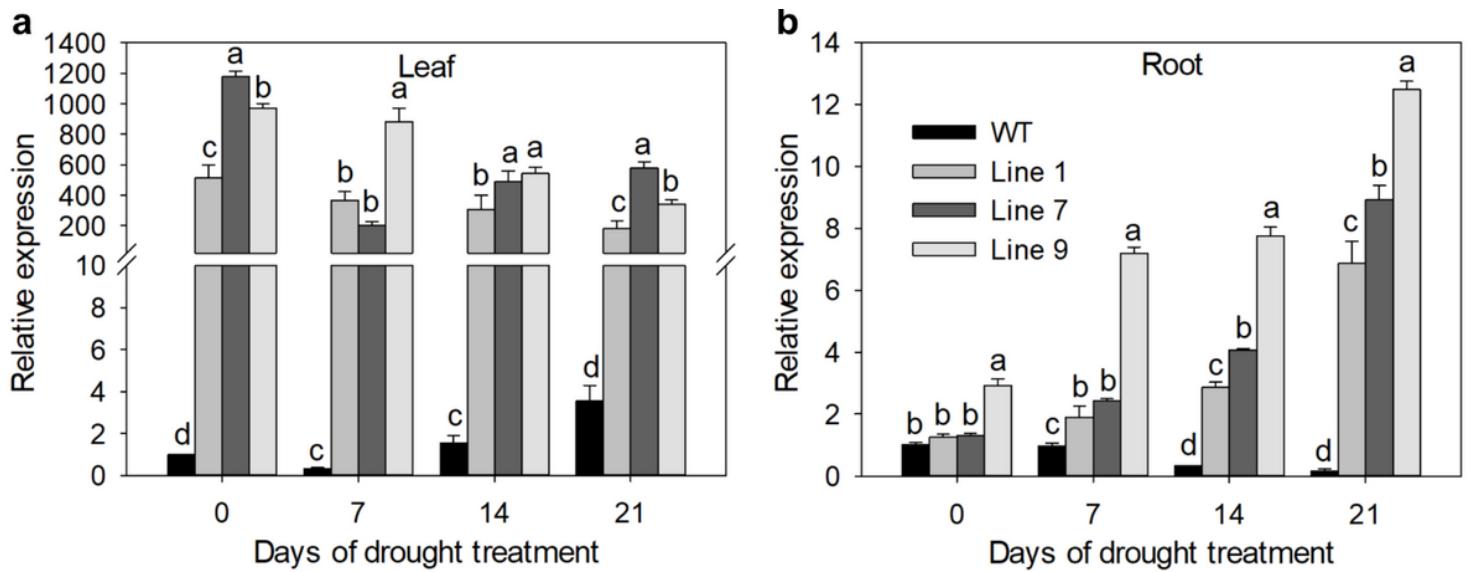


Figure 5

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

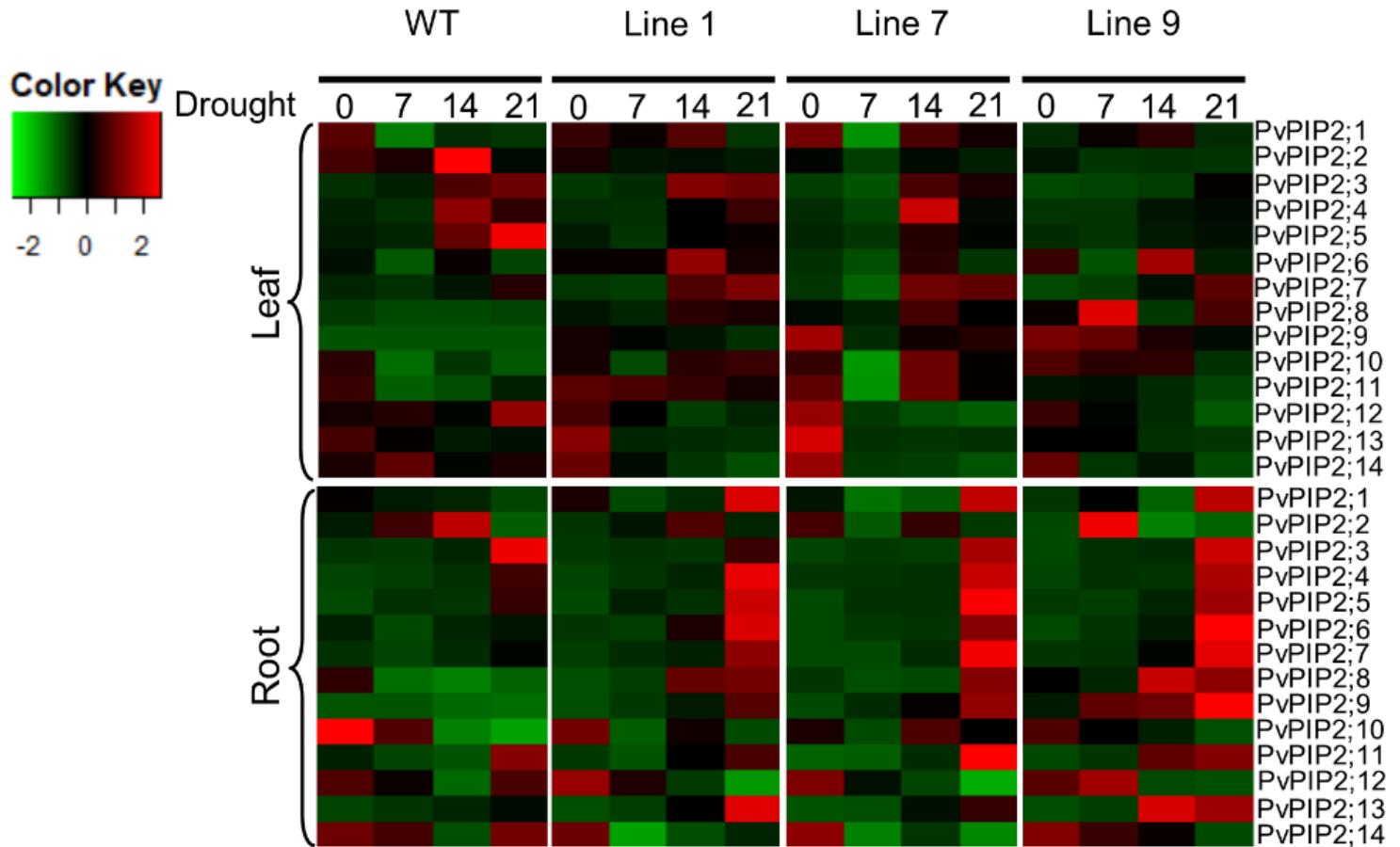


Figure 6

Relative expression of other PIP2 subfamily genes in leaves and roots during 21 days of drought 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](#)
- [Fig.S1.xlsx](#)