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# Cloning *PIP* genes in drought-tolerant vetiver grass and responses of transgenic *VzPIP2;1* soybean plants to water stress

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## Abstract

Vetiver grass [*Vetiveria zizanioides* (L.) Nash] displays comprehensive abiotic stress tolerance closely related to fine maintenance of plant water relation mediated by plasma membrane intrinsic proteins (PIPs). Two open reading frame sequences of *PIP*s (867 and 873 bp) were cloned from vetiver grass and named as *VzPIP1;1* and *VzPIP2;1*, respectively. Expression of green fluorescent protein revealed only subcellular localization of *VzPIP2;1* in the plasma membrane. *Agrobacterium tumefaciens* mediated transgenic (*VzPIP2;1*) soybean plants had a higher water content in above-ground parts under sufficient water supply through enhancing transpiration as compared to the non-transgenic plants but displayed a more severe drought injury because of a lower photosynthesis and a higher transpiration rate. However, *A. rhizogenes* mediated transgenic soybean plants kept a higher water content in above-ground parts by improving root water transport and kept a more effective photosynthesis under normal and drought conditions.

*Additional key words*: *Agrobacterium*, *Glycine max*, root hydraulic conductivity, transpiration, *Vetiveria zizanioides.*

## Introduction

Vetiver [*Vetiveria zizanioides* (L.) Nash syn. *Chrysopogon zizanioides* L. Roberty], belonging to family *Poaceae*, is perennial $C_4$ plant native to tropical and subtropical areas (Zhou and Yu 2009a,b, Singh *et al*. 2014). It displays comprehensive abiotic stress tolerance and good ecological adaptability for soil and water conservation, highway slope stabilization, and land rehabilitation. It is a densely tufted grass with a well-developed reticular root system and a high biological yield (Zhou and Yu 2010, Lal *et al*. 2013, Abaga *et al*. 2014, Ye *et al*. 2014). Our previous works have suggested that vetiver grass can adopt the strategies of osmotic adjustment and maintenance of total polyamine homeostasis and a higher (spermine + spermidine)/ putrescine ratio to cope with drought and salt stresses (Zhou and Yu, 2009a,b, 2010). In addition, vetiver grass with a high content of cellulose and hemicellulose in leaves is now considered to be a potential bio-fuel (Zhou and Yu 2012).

Aquaporins (AQPs) represent a family of channel proteins that transport water and other small molecules ($CO_2$, urea, $H_2O_2$, $NH_3$, glycerol, formaldehyde, silicon, boron, *etc*.) across cell membranes (Maurel *et al*. 2008, Soto *et al*. 2012). In plants, AQPs have been linked to many functions not only in water relation involved in plant adaptation and tolerance against water deficit, but also in carbon metabolism, oxidative stress responses, and mineral nutrition. Based on localization, plant AQPs have been divided into four subfamilies: plasma membrane intrinsic proteins (PIPs), tonoplast intrinsic proteins (TIPs), nodule-like intrinsic proteins (NIPs), and small basic intrinsic proteins (SIPs) (Mahdieh *et al*. 2008, Maurel *et al*. 2008). Among them PIPs, as the largest category, play a key role in water transport by the

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*Abbreviations*: 1/2 - half strength; ψ - osmotic potential; AQP - aquaporin; $c_i$ – intercellular $CO_2$ concentration; E - transpiration; $F_v/F_m$ - variable to maximum chlorophyll fluorescence ratio; GFP - green fluorescent protein; $g_s$ - stomatal conductance; $K_{leaf}$ - hydraulic conductance from stem to leaf; $K_{plant}$ - hydraulic conductance from soil to leaf; $Lp_h$ - hydrostatic root water conductivity; $Lp_{os}$ - osmotic root water conductivity; $Lp_r$ - root hydraulic conductivity; MS - Murashige and Skoog; NT - non-transgenic; ORF - open reading frame; PEG - polyethylene glycol; PIP - plasma membrane intrinsic protein; PM - plasma membrane; $P_N$ - net photosynthetic rate; REL - relative electrolyte leakage; RT - reverse transcription; RACE - rapid amplification of cDNA ends; RWC - relative water content.

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symplastic route through cytoplasmic continuities and plasmodesmata and by the transcellular path across cell plasma membranes in both well-watered plants and drought- stressed plants (Maurel *et al*. 2008). The PIP subfamily can be further divided into PIP1 and PIP2 isoforms based on sequence homology. When expressed in *Xenopus oocytes*, PIP2 isoforms generally show a more marked water channel activity compared with PIP1 isoforms (Mahdieh *et al*. 2008).

Drought is major abiotic stress for both natural plant populations and agricultural crops (Stolf-Moreira *et al*. 2010, Juenger 2013, Song *et al*. 2013). Among the crops, soybean is oilseed crop important for human food and animal feed (Manavalan *et al*. 2009, Bellaloui 2012, Le *et al*. 2012). Water deficit often limits soybean production and yield and accounts for about a 40 % crop loss (Ohashi *et al*. 2006, Manavalan *et al*. 2009, Neves-Borges *et al*. 2012). At present, transgenic approach is pursued actively to improve crop traits including tolerance to biotic and abiotic stresses, as for soybean, when transgenic soybean cultivars have been planted in the global range (Ashraf 2010, Bonny 2011). Soybean genetic transformation methods, including cotyledonary node tissue transformation mediated by *A. tumefaciens*, embryogenic tissue transformation mediated by particle bombardment, and *A. rhizogenes*-mediated soybean hairy root transformation, are commonly used (Cao *et al.* 2009, Hernandez-Garcia *et al.* 2010).

Up to date, a study on gene cloning and function analysis of vetiver has not yet been reported because of unavailability of its complete genome sequence. Only Singh *et al*. (2014) have analyzed for the first time the genetic diversity among 131 accessions of Indian vetiver germplasm using a combination of rapid amplified polymorhic DNA (RAPD) and simple sequence repeat (SSR) markers as well as cross transferability of rice SSR marker and revealed a high Indian vetiver germplasm diversity but a lower transferability of a rice SSR markers in vetiver. Although the roles of plant AQPs have been extensively studied in the last two decades, the information of AQPs and their correlation to abiotic stress tolerance in vetiver still remains to be solved. Therefore, the aim of this study was to clone vetiver *VzPIP* genes and to investigate the roles of *VzPIP2;1* in drought-stressed transgenic soybean plants.

## Materials and methods

**Plants, growth conditions, and treatments:** Vetiver [*Vetiveria zizanioides* (L.) Nash] plants were cultured as in our previous work (Zhou and Yu 2010). Selected nine-month-old plants were individually fixed in the sheet of foam board, and their roots were dipped into a half strength (1/2) Hoagland's nutrient solution in plastic square boxes. The plants were cultured in a growth chamber at a 16-h photoperiod, a photosynthetic photon flux density of 500 μmol $m^{-2}$ $s^{-1}$, day/night temperatures of 26 ± 2 / 20 ± 2 °C, and a relative humidity of 60 - 70 %. When the plants renewed their growth, about 1/3 of the plants were subjected to 1/2 Hoagland's nutrient solution plus 25 % (m/v) polyethylene glycol (PEG-6000); the other 2/3 of plants were continued to culture in 1/2 Hoagland's solution. After three days, the PEG treated plants were rewatered with 1/2 Hoagland's solution for another three days, at the same time, a half of the other 2/3 plants were treated with 1/2 Hoagland's solution + 25 % PEG-6000 for three days, and the residual half of plants were continued to culture in 1/2 Hoagland's solution for three days and represented the control. All the solutions had pH = 6.0 and were renewed every two days. Finally, all the plants were prepared for measuring water flow rate across roots, root hydraulic conductivity ($Lp_r$) including hydrostatic root water conductivity ($Lp_h$) and osmotic root water conductivity ($Lp_{os}$), hydraulic conductance from soil to leaf ($K_{plant}$) and that from stem to leaf ($K_{leaf}$).

Soybean (*Glycine max* L. Merr. cv. Lee68) seeds were surface-sterilized with 1 g $dm^{-3}$ $HgCl_2$ for 5 min, then fully rinsed in distilled water, soaked in distilled water for 6 h, and finally germinated at 25 °C in the dark. The germinated seeds (with about a 0.5 - 1 cm epicotyl) were planted in plastic pots (25 × 18 cm) containing *Vermiculite* moistened with 1/2 Hoagland's solution in a greenhouse for *VzPIP2:1* transformation by *A. tumefaciens*-mediated adventitious shoot induction (for more detail see below). After the transgenic soybean seeds (*tVzPIP2;1*, $T_1$) were harvested and verified by PCR, the transgenic and non-transgenic seedlings were subjected into 10 % (m/v) PEG-6000 solution (in 1/2 Hoagland's solution) for 5 d, while another group was cultured in 1/2 Hoagland's solution as the control. The transgenic *VzPIP2;1* composite soybean seedlings (*hrVzPIP2;1*) transformed by *A. rhizogenes*-mediated hairy root system, and the vector control (for more detail see below) were treated with a 15 % (m/v) PEG-6000 solution for 4 h and then rewatered for 1 d. The other experimental conditions were the same as above-mentioned.

**Measurement of $Lp_h$ and $Lp_{os}$ in vetiver grass:** Measurements were carried out from 10:00 to 13:00 according to Parent *et al*. (2009). The seminal root system was excised by sectioning the mesocotyl and then fixed tightly to a silicon tube using silica gel. The silicon tube was connected to a vacuum pump equipped with a negative pressure table to trigger a hydrostatically driven xylem sap flow. The water flow across the root system was measured with a water trap made of a 2-$cm^3$ tube filled with dry cotton and inserted onto the tubing between the roots and the vacuum port. Water flow was measured by weighing the sap absorbed by the cotton that was renewed every 10 min. After 30 min stabilization of

exudation rate at a -0.04 MPa pressure, water flux was measured every 10 min. Suction force applied to the root system was changed every 10 min in a standardized way (0, -0.02, -0.04, -0.06, -0.06, -0.04, -0.02, and 0 MPa). Hydrostatic root water conductivity was calculated from the slope of regression between the water flow and the suction applied to the root system (dJ/dP): $Lp_h$ = (dJ/dP)/A, where A represents the area of the root system scanned with an image analyzer. Osmotic potentials of the sap ($\pi_{sap}$) and of the nutrient solution ($\pi_{sol}$) were determined by an automatic freezing-point depression osmometer (*FM-8P*, *Shanghai University's Instrument Co*., Shanghai, China), and $Lp_{os}$ = J/[($\pi_{sap}$-$\pi_{sol}$) × A], where J is water flux through the root system without depressurization.

**Determination of $K_{leaf}$ and $K_{plant}$ in vetiver grass:** $K_{plant}$ and $K_{leaf}$ were calculated with the evaporative flux method under steady-state conditions as described by Tsuda and Tyree (2000) and Martre *et al*. (2002). One leaf per plant was covered before the beginning of photoperiod with a self-adhesive tape and aluminum foil to prevent transpiration (the bagged leaf). Transpiration rate (E) was determined with a portable photosynthesis meter (*LI-6400*, *LI-COR*, Lincoln, USA) on two leaves per plant 5 h after the beginning of photoperiod. One hour before E determination, the plant was immersed to nearly the height of the pot in a nutrient solution. One-half of an hour after E determination, the bagged leaf and the two leaves which transpiration was measured were excised at the base of stem. Pressure potentials of the bagged leaves and transpiring leaves were assumed to provide estimates of osmotic potential of stem ($\psi_{stem}$) and average $\psi_{leaf}$, respectively; $\psi_{sol}$ is osmotic potential of nutrient solution; the potentials were measured by a dew point hygrometr (*HR33T*, *Wescor*, Logan, USA) with leaf disc sample chambers (*C-52*). Hydraulic conductance from soil to leaf ($K_{plant}$) and $K_{leaf}$ were calculated as E/($\psi_{sol}$ - $\psi_{leaf}$) and E/($\psi_{stem}$ - $\psi_{leaf}$), respectively.

**Extraction of the total RNA, cDNA synthesis, and PIP genes cloning in vetiver grass:** Leaf tissue (0.5 g) of vetiver grass was ground in liquid nitrogen and the total RNA was extracted with a *TRIzol* reagent (*Invitrogen*, Carlsbad, USA). After digestion of DNA with RNase-free DNase I, the integrity of RNA was detected by agarose gel electrophoresis. The first-strand cDNA was obtained by reverse transcription (RT)-PCR using the total RNA as template through *M-MLV* reverse transcriptase (*Promega*, Madison, USA) according to the manufacturer's instructions. According to the reported conserved sequences of *PIP1s* and *PIP2s* of *Arabidopsis*, rice, maize, barley, and so on from GenBank, more than 20 degenerate primers were designed and finally the following were selected: primer 1: forward 5'-ATGGAGGGGAAGGCGG-3', reverse 5'-TTAAGA CCTGCTCTTGAATGG-3'; primer 2: forward 5'-ATG GGCAAGGACGACGTGA-3', reverse 5'-TTAGGCGTT GCTCCGGAAGGG-3'. Cloning *PIP* genes in vetiver grass was performed mainly according to Li *et al*. (2014). Using the above cDNA as template and the primers, homologous sequence fragments were amplified by RT-PCR. Its profile was set as follows: an initial denaturation at 94 °C for 4 min followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 58 °C for 30 s, extension at 72 °C for 1 min, and an additional extension at 72 °C for 10 min. The PCR products were separated by agarose gel electrophoresis, and inserted into the *pMD19-T* simple vector (*TaKaRa*, Dalian, China) for sequencing. The full-length cDNA of *VzPIP1* or *VzPIP2* was synthesized from the RNA with a *SMART RACE* cDNA amplification kit (*Clontech*, Palo Alto, USA) following the protocol provided by the manufacturer. One μg of the vetiver grass plant total RNA was used for 5'-end and 3'-end cDNA synthesis. After the 5' and 3' sequences of each cDNAwere synthesized, RT-PCR was performed to isolate the full-length cDNA, accuracy of which was confirmed by further sequencing. Finally, by aligning the sequences of the 5'-end and 3'-end PCR products and the previous partial DNA sequence, a full-length cDNA sequence or open reading frame (ORF) of *VzPIP1* or *VzPIP2* was deduced and obtained by RT-PCR with *KOD-PLUS* (Toyobo, Japan) using specific primers.

**Sequences analysis of VzPIP1;1/VzPIP2;1 and their protein subcellular localization:** Sequence analysis and comparisons with the deduced amino acid sequences were performed using the *NCBI BLAST* server (http://www.ncbi.nlm.gov/BlastP). The *TMHMM v. 2.0* server was used to predict transmembrane helices (http://www.cbs.dtu.dk/services/TMHMM-2.0/).
Molecular masses and theoretical pIs were predicted using the *ProtParam* tool (http://web.expasy.org/ protparam/). *PSORT* (http://psort.ims.utokyo.ac.jp/ form.html) was used to predict subcellular localization of proteins. The *ClustalW2* program (http://www. ebi.ac.uk/Tools/msa/ clustalw2/) was used for sequence alignment, and phylogenetic trees were constructed using *MEGA v. 5.0*. The coding sequence of *VzPIP1;1* or *VzPIP2;1* containing a *Kpn*I/*Spe*I restriction site was amplified by PCR, and subcloned into a *Kpn*I/*Spe*I site of the pCAMBIA1300-GFP expression vector under control of the CMV35S promoter. The pCAMBIA1300-*VzPIP1; 1*-GFP and pCAMBIA1300-*VzPIP2;1*-GFP were transiently expressed in onion epidermal cells (from adaxial epidermis peeled off the fresh onion bulbs) by the biolistic *PDS-1000/He* particle delivery system (*Bio-Rad*, Hercules, USA) according to Fong *et al*. (2006). Fluorescence of the green fluorescent protein (GFP) was observed with a fluorescence microscope (*DP72*, *Olympus*, Tokyo, Japan) after incubation on 1/2 Murashige and Skoog (MS) medium at 25 °C for 24 h.

**Construction of a plant expression vector:** According to the method of Nie *et al*. (2015), the cDNA of *VzPIP2;1*

was subcloned into the pEASY-Blunt Zero cloning vector by replacing the coding region between *Kpn*I and *Spe*I sites, transformed into *Escherichia coli* strain DH 5α, and incubated in Petri dishes containing 50 mg·dm$^{-3}$ kanamycin to grow single colonies, which were selected to expand culturing. Then the plasmids were isolated, PCR, enzyme digestion, and sequence verification were carried out using *VzPIP2;1* specific primers, and pEASY Blunt Zero-*VzPIP2;1* was obtained. Under the role of $T_4$ DNA ligase, the sequence of *VzPIP2;1* obtained from the vector of pEASY-Blunt Zero-*VzPIP2;1*, which was restricted by enzymes *Kpn*I and *Spe*I, was linked to the visible band from the pCAMBIA 1300 vector restricted by *Kpn*I and *Spe*I, and then the resultant recombinant plasmid pCAMBIA 1300-*VzPIP2;1* was constructed. Its verification by PCR (primers: forward 5'-GGGGTACCGCCACGCTGCTGTTCCTCT-3', reverse 5'-GGACTAGTAAGGGGCCCACCCAGAAGAT-3', *Kpn*I and *Spe*I sites underlined, respectively), enzyme restriction, and sequencing were conducted as mentioned before to confirm the right plant expression vector.

**Overexpression of *VzPIP2;1* in transgenic soybean plants transformed by *A. tumefaciens*:** *VzPIP2;1* transformation of soybean plants mediated by *A. tumefaciens*, and adventitious shoot induction were conducted as described Li *et al*. (2012). Briefly, the four-d-old soybean (cv. Lee68) seedlings with unexpanded cotyledons, from which apical and lateral buds were excised, were used. A few scars were made in the cotyledonary nodes using a surgical blade. *A. tumefaciens* (strain EHA105) containing pCAMBIA1300-*VzPIP2;1* were incubated to $A_{600}$ = 0.6 ~ 0.8 and resuspended in ½ MS medium after centrifugation, and then dripped to the scar position of cotyledonary nodes. The soybeans with the treated cotyledonary nodes were covered with moist *Vermiculite* and incubated at 28 °C and a 12-h photoperiod to grow adventitious buds and multiple shoots. The soybean seedlings ($T_1$) were verified by PCR amplification with specific *VzPIP2;1* gene primers. The transgenic (*tVzPIP2;1*) and non-transgenic soybean plants were used to measure angles between two opposite leaves under favourable conditions to observe a plant phenotype and to evaluate drought tolerance under 10 % (m/v) PEG-6000 solution for 5 d. Some physiological parameters in leaves, such as relative electrolyte leakage (REL), net photosynthetic rate ($P_N$), stomatal conductance ($g_s$), intercellular $CO_2$ concentration ($c_i$), E, and variable to maximum chlorophyll fluorescence ratio ($F_v/F_m$) were determined using the methods described by Zhou and Yu (2010). Relative electrolyte leakage was measured using a digital conductivity meter (*DDS-307*, *Shanghai Yidian Scientific Instrument Co*, Shanghai, China) and calculated as: $C_1 - C_w/C_2 - C_w$, where $C_1$ is conductance of leaf tissue before boiling, $C_2$ is conductance after boiling, and $C_w$ is deionized water conductance. A portable photosynthesis meter (*LI-6400*) was used to measure the values of $P_N$, $g_s$, $c_i$, and E. Photosynthetic photon flux density, temperature, and ambient $CO_2$ concentration were 1000 μmol m$^{-2}$ s$^{-1}$, 26 °C, and 390 μmol mol$^{-1}$, respectively. Variable to maximum chlorophyll fluorescence ratio ($F_v/F_m$) was measured at room temperature with a plant efficiency analyzer (*Handy PEA Fluorometer*, *Hansatech Instruments*, Effetrich, Germany). The first pair of unifoliolate leaves was dark-adapted for 30 min using *Handy-PEA* leaf clips and the PPFD flash was 2 000 μmol m$^{-2}$ s$^{-1}$.

**Overexpression of *VzPIP2;1* in transgenic hairy roots of soybean plants transformed by *A. rhizogenes*:** According to the plant transformation method of the soybean hairy root system described by Ali *et al*. (2012), the recombinant plasmid pCAMBIA 1300-*VzPIP2;1* was introduced into *A. rhizogenes* strain K599 by the liquid nitrogen freeze-thaw method. Soybean seeds were surface-sterilized and soaked in distilled water, and then sown in pots to develop seedlings with emerged true leaves. One day before injection, *A. rhizogenes* carrying the recombinant plasmid pCAMBIA 1300-*VzPIP2;1* was grown overnight in 1 cm$^3$ of Lurie-Brot medium containing 50 mg dm$^{-3}$ kanamycin at 28 °C and shaken at 180 rpm. Transformed *A. rhizogenes* was harvested by centrifugation at 10 000 *g* and room temperature for 2 min. The pellet was suspended gently in a 10 mM $MgSO_4$ solution followed by two washings. Absorbance at 600 nm of the final suspension was adjusted to 1.0 with a 10 mM $MgSO_4$. Then 5 ~ 10 mm$^3$ of the inoculum was injected at the junction of two cotyledons for 3 ~ 5 injections per plant. The injection point was covered with *vermiculite* at 28 °C, and water was applied for keeping the sufficient moisture for 5 ~ 7 d. True roots were cut off below the injection point where hairy roots developed, and the seedlings with hairy roots were transplanted to plastic square boxes containing 1/2 Hoagland's solution and cultured in a growth chamber at day/night temperatures of 26/20 ± 2 °C and a 12-h photoperiod. After one week of growth in the chamber, PCR analysis confirmed the positive transgenic hairy roots. Finally, the control plants tranformed only with the vector and *VzPIP2;1*-over-expessing plants were subjected to a drought stress [a 15 % (m/v) PEG-6000 solution for 2 d or for 4 h following rewatering for 1 d], and then the photosynthetic parameters were conducted as before. Relative water content (RWC) in overground parts was determined by the method of Zhou and Yu (2010). The overground parts of the vector and *hrVzPIP2;1* plants were excised and fresh mass (FM) was immediately recorded. Then they were soaked in distilled water at 4 °C in the dark for 4 h and blotted dry for recording water saturated mass (TM). After drying for 15 min at 105 °C and then at 80 °C for 3 ~ 4 d, dry mass (DM) was recorded. Relative water content was calculated as [(FM - DM)/(TM - DM)] × 100.

**Statistical analysis:** Data were expressed as means ± SDs of three replicates, and analyzed using one-way analysis of variance (*ANOVA*) with Tukey's multiple range test using the *SPSS 19.0*.

## Results

Plants tend to control root water uptake and plant water status *via* root growth and $Lp_r$; $Lp_r$ can be divided into $Lp_h$ and $Lp_{os}$. Excised roots of the vetiver grass plants under the control conditions, PEG-6000 stress, and rewatering were placed in 1/2 Hoagland's solution and subjected to a tension of -0.02 MPa. Water flow across roots treated with the 25 % (m/v) PEG-6000 solution for 3 d was by 96.4 % higher than across control roots ($P < 0.05$), and after rewatering for 3 d, water flow continued to rise slightly. The changes in $Lp_h$ of vetiver grass under the control, PEG-6000 stress, and rewatering were very similar to those of the measured water flow across roots, and the rises under the PEG-6000 stress and rewatering were more than 3 times of the control. With regard to the value of $Lp_{os}$, it increased evidently under the PEG-6000 stress and recovered to the control level after rewatering (Table 1). Under treatment with the 25 % (m/v) PEG-6000 solution for 3 d, the values of both $K_{plant}$ and $K_{leaf}$ significantly declined to about 30 % of those of the control plants, and after rewatering for 3 d, the values of $K_{plant}$ and $K_{leaf}$ were restored although still remained lower than in the control ($P < 0.05$). The change in E under the above-mentioned conditions displayed a similar trend as $K_{plant}$ and $K_{leaf}$ (Table 1). Thus, we may deduce that the drought-stressed vetiver grass plants could strengthen water absorption in roots by increasing $Lp_r$, and at the same time, decreased $K_{plant}$ and $K_{leaf}$ and restrained reduced water loss to the atmosphere by E. This is well consistent with drought tolerance performance of vetiver grass.

Table 1. Effects of drought stress [25 % (m/v) PEG-6000 in half-strength Hoagland's nutrient solution for 3 d] and rewatering (half-strength Hoagland's nutrient solution for 3 d) on water flow at 0.02 MPa [$10^{-7}$ m $s^{-1}$], hydrostatic root water conductivity ($Lp_h$) and osmotic root water conductivity ($Lp_{os}$) [$10^{-7}$ m $s^{-1}$ $MPa^{-1}$], hydraulic conductance from soil to leaf ($K_{plant}$) and hydraulic conductance from stem to leaf ($K_{leaf}$) [mmol($H_2O$) $s^{-1}$ $m^{-2}$ $MPa^{-1}$], and transpiration (E) [mmol($H_2O$) $m^{-2}$ $s^{-1}$] in vetiver grass seedlings. Means ± SDs, $n = 3$; different letters show significant differences ($P < 0.05$) according to Tukey's multiple range test.

| Treatments | Water flow | $Lp_h$ | $Lp_{os}$ | $K_{plant}$ | $K_{leaf}$ | E |
|---|---|---|---|---|---|---|
| Control | 0.282±0.030$^a$ | 11.028±0.805$^a$ | 0.315±0.051$^a$ | 3.485±0.305$^a$ | 21.005±1.137$^a$ | 4.063±0.307$^a$ |
| PEG-6000 | 0.552±0.045$^b$ | 31.889±2.829$^b$ | 0.638±0.079$^b$ | 1.393±0.153$^c$ | 7.152±1.650$^c$ | 1.110±0.225$^c$ |
| Rewatering | 0.582±0.060$^b$ | 35.889±4.829$^b$ | 0.385±0.061$^a$ | 2.365±0.140$^b$ | 12.840±0.786$^b$ | 2.770±0.210$^b$ |

Considering the very close relationships among plant AQPs, water uptake, and drought tolerance, we designed the degenerate primers, adopted the rapid amplification of cDNA ends PCR technique, and successfully obtained two ORF sequences of *PIP* genes using mRNA isolated from leaves of the vetiver grass seedlings. The ORF of the *PIP1* cDNA had 867 bp that translated into the VzPIP1 protein with 288 amino acids, a predicted molecular mass of 30.58 kDa, and a theoretical pI of 9.64. The ORF of the *PIP2* cDNA had 873 bp that translated into the VzPIP2 protein with 290 amino acids, a predicted molecular mass of 30.30 kDa, and a theoretical pI of 8.29. Analysis by *BLASTP* shows that VzPIP1 and VzPIP2 shared 95 and 99 % sequence identity with ZmPIP1;1 and ZmPIP2;1, respectivly, from *Zea mays*. Both the predicted VzPIP1 and VzPIP2 proteins contained six transmembrane domains connected by five loops, two NPA (Asn-Pro-Ala) motifs, and one selective ar/R filter pipe, which are conserved features of all aquaporins (Fig. 1 Suppl.). A phylogenetic tree aligned by *Clustal W* was constructed (Fig. 1 Suppl.) based on amino acid sequence alignment of plant AQPs with *Arabidopsis*, maize, and rice sequences obtained from *GenBank*. This confirms that on an evolutionary timescale, VzPIP1 and VzPIP2 are very close to the ZmPIP1;1 and ZmPIP2;1 subfamily in maize (both graminaceous $C_4$ plants), and therefore we named the two new *PIP* genes in vetiver grass as *VzPIP1;1* and *VzPIP2;1*, respectively.

Prediction analysis for subcellular localization of the VzPIP1;1 and VzPIP2;1 proteins indicates that both are positioned on the plasma membrane (PM). For further verification, cDNAs of *VzPIP1;1* and *VzPIP2;1* were connected to the CaMV35S promoter, fused to the C-terminal of GFP and were built into the transient expression vector, which were used as particles for bombardment of onion epidermal cells. Fluorescence was observed with a fluorescence microscope after 24-h dark cultivation. Only VzPIP2;1 was found to be located in the PM, whereas VzPIP1;1 was found diffused throughout the cell (Fig. 1). In view of the clear subcellular localization of plant PIP2s, we constructed the plant expression vector pCAMBIA 1300-*VzPIP2;1* (Fig. 2*A*) and used it for overexpression of *VzPIP2;1* under control of the CaMV35S promoter in the soybean plants **(**Fig. 2*B*,*C***)** and investigated drought stress responses of the transgenic *VzPIP2;1* soybean plants transformed by *A. tumefaciens* or *A. rhizogenes*.

A high regeneration rate of multiple shoots could be obtained by *in situ* induction of soybean adventitious buds (in our experiment it could reach as high as 80.83 % for cv. Lee68). The soybean multiple shoots were infected by *A. tumefaciens* strain EHA105 carrying pCAMBIA 1300-*VzPIP2;1*, and the transgenic soybean seeds (*tVzPIP2;1*, $T_1$) were harvested and verified by PCR (Fig. 3). The angle between the vertical stem and

expanding leaf of growing seedlings can reflect the velocity of water uptake in roots and water content in aboveground parts (Siefritz *et al*. 2002). During the daytime under the control conditions, the angles between two opposite leaves (Fig. 2 Suppl.) of *tVzPIP2;1* and non-transgenic (NT) plants gradually enlarged from 63.41 and 92.25 ° to 113.96 and 143.79 °, respectively, after 7 h. When irradiation continued to 11 h, the angles of *tVzPIP2;1* and the NT plants remained constant. It is worth to notice that the angles for *tVzPIP2;1* were obviously higher than those of the NT plants (Fig. 2 Suppl.). This may be attributed to a higher water uptake or water content in *tVzPIP2;1* soybean when compared to the NT plants.

Under the 10 % PEG-6000 solution for 5 d, growth of the *tVzPIP2;1* and NT plants was inhibited, but the growth inhibition and injury symptom of *tVzPIP2;1* were more severe than of the NT plants (Fig. 4*A*,*B*).

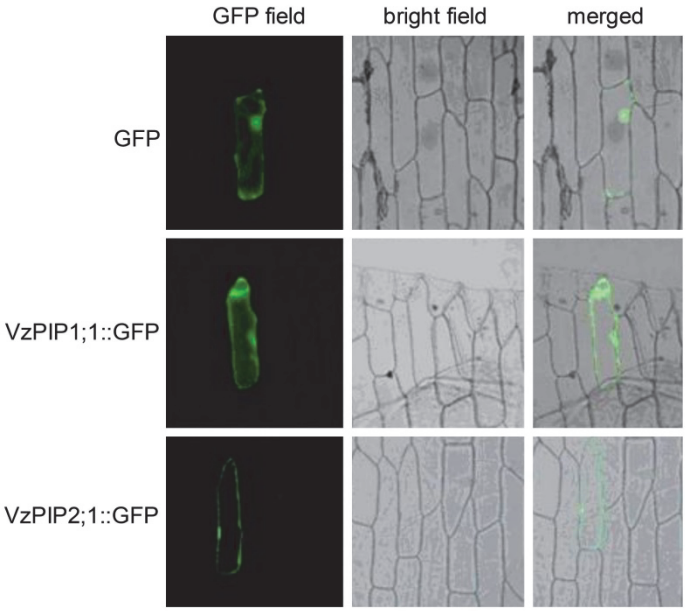


Fig. 1 Subcellular localization analysis of VzPIP1;1::GFP and VzPIP2;1::GFP proteins in onion epidermal cells. GFP - green fluorescent protein.

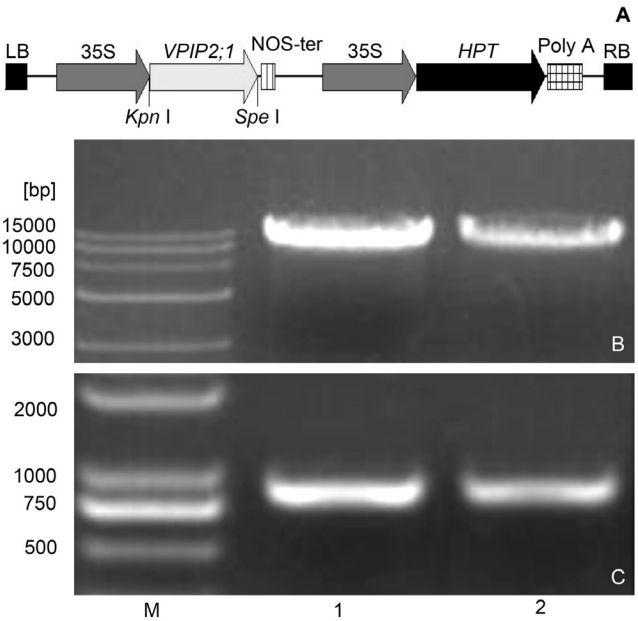


Fig. 2. Step verification of the pCAMBIA 1300-*VzPIP2;1* construction. *A* - Model of the pCAMBIA 1300-*VzPIP2;1* construction. *B* - Plasmid isolation of the pCAMBIA 1300-*VzPIP2;1* cloning vector is shown in *lane 2* (11 720 bp); *lane 1* is pCAMBIA 1300 in a plasmid (10 847 bp). *C* - Colony PCR analysis of pCAMBIA 1300-*VzPIP2;1* transformed *Agrobacterium tumefaciens*; *lane 1* shows a positive colony, *lane 2* shows PCR products of *VzPIP2;1* from transformed *A. tumefaciens*, M represents a DNA marker.

Accordingly, a significant increase in leaf REL was found in *tVzPIP2;1* (Table 2) and when the measured photosynthesis parameters were compared, $P_N$ and $F_v/F_m$ were very close in the *tVzPIP2;1* and NT seedlings under sufficient water supply, but the *tVzPIP2;1* plants displayed a significantly higher E than the NT plants. However, when the *tVzPIP2;1* and NT soybean seedlings were exposed to the 10 % PEG-6000 solution for 5 d, leaf E of *tVzPIP2;1* was significantly lower than under the control conditions but still significantly higher than in the NT plants treated with PEG-6000 ($P < 0.05$). This suggests that the higher E of *tVzPIP2;1* soybean under the drought stress could result in a higher water loss from the leaf. For this reason, a greater decline in $F_v/F_m$ and $P_N$, whereas no obvious changes in $g_s$ and $c_i$, were observed in *tVzPIP2;1* as compared to the NT plants (Table 2). The velocity of water transport from stems to leaves and water content in plants are closely related to the E. The above-mentioned results indicate a more severe damage of the *tVzPIP2;1* soybean seedlings due to the drought stress as compared to the NT plants.

The soybean seedling cuttings without original roots were infected on cotyledons with *A. rhizogenes* strain K599 containing the vector pCAMBIA 1300-*VzPIP2;1*, and the composite soybean plants with transgenic hairy roots (*hrVzPIP2;1*) were verified by PCR analysis (Fig. 5). Two composite soybean plant lines with transgenic hairy roots (*hrVzPIP2;1*) together with a line transformed with an empty vector were examined for responses to the drought treatment. Under the 15 % PEG-6000 solution for 2 d, the phenotype of *hrVzPIP2;1* displayed no obvious damage, whereas the empty vector soybean plants showed serious wilting (Fig. 6). Values of RWC in leaves of the *hrVzPIP2;1* and vector plants were all significantly reduced when compared with those under the control conditions. Relative water content of

Table 2. Comparison of leaf relative electrolyte leakage (REL) [%], net photosynthetic rate ($P_N$) [μmol($CO_2$) $m^{-2}$ $s^{-1}$], stomatal conductance ($g_s$) [mol ($CO_2$) $m^{-2}$ $s^{-1}$], intercellular $CO_2$ concentration ($c_i$) [μmol($CO_2$) $mol^{-1}$], transpiration (E) [mmol($H_2O$) $m^{-2}$ $s^{-1}$], and variable to maximum chlorophyll fluorescence ratio ($F_v/F_m$) between transgenic *VzPIP2;1* soybean seedlings *tVzPIP2;1* transformed by *Agrobacterium tumefaciens* and non-transgenic soybean plants under control conditions or 10 % (m/v) PEG-6000 solution treatment for 5 d. Means ± SDs, $n = 3$; different letters show significant differences ($P < 0.05$) according to Tukey's multiple range test.

| Treatments | Plants | REL | $P_N$ | $g_s$ | $c_i$ | E | $F_v/F_m$ |
|---|---|---|---|---|---|---|---|
| Control | NT | 3.82±0.24$^a$ | 12.933±0.294$^a$ | 0.159±0.013$^a$ | 296.333±5.955$^a$ | 2.638±0.066$^b$ | 0.822±0.004$^a$ |
| | *tVzPIP2;1* | 4.42±0.27$^a$ | 12.867±0.973$^a$ | 0.125±0.007$^b$ | 252.167±10.741$^b$ | 3.113±0.203$^a$ | 0.812±0.003$^a$ |
| PEG | NT | 6.24±0.22$^a$ | 10.340±0.152$^b$ | 0.088±0.009$^c$ | 212.000±12.981$^c$ | 2.266±0.109$^c$ | 0.824±0.008$^a$ |
| | *tVzPIP2;1* | 30.81±2.43$^b$ | 7.117±0.659$^c$ | 0.094±0.006$^c$ | 222.833±12.189$^c$ | 2.457±0.111$^b$ | 0.570±0.040$^b$ |

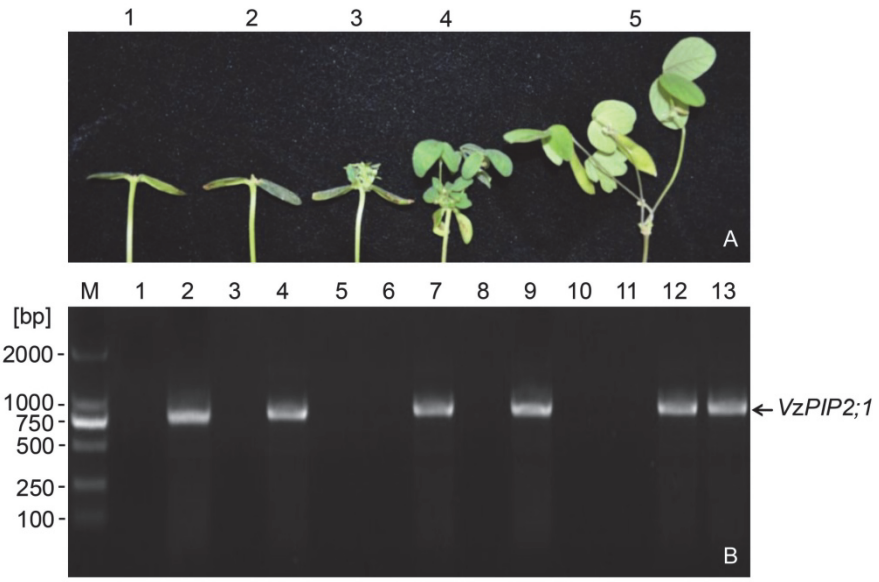


Fig. 3. Transformation of *VzPIP2;1* by *Agrobacterium tumefaciens*-mediated adventitious shoot induction, harvesting soybean $T_1$ seeds, and its PCR analysis. *A*: 1 - induction phase, 2 - adventitious shoot formation, 3 - multiple shoots formation, 4 - shoot elongation, 5 - podding period. *B*: M represents DL2000 DNA marker, *lanes* 1, 3, 5, 6, 8, 10, and 11 are the non-transgenic plants, and *lane*s 2, 4, 7, 9, 12, and 13 are the transgenic plants.

*hrVzPIP2;1* was 66.82 % and higher than RWC of the plants transformed with the vector (41.16 %) after PEG-6000 treatment ($P < 0.05$) (Table 3). We further investigated the effects of PEG-6000 treatment and rewatering on photosynthesis of the *hrVzPIP2;1* and empty vector transgenic plants. Under the control conditions, $P_N$, $g_s$, $c_i$, and E of *hrVzPIP2;1* showed no significant differences from those of the empty vector plants ($P > 0.05$). When the *hrVzPIP2;1* and empty vector plants were subjected to the 15 % PEG-6000 solution for 4 h, in addition to the $c_i$ value with no obvious change, the values of $P_N$, $g_s$, and E declined significantly compared to those under the control conditions, and drops in $P_N$ and E of *hrVzPIP2;1* were obviously smaller than those of the empty vector plants. After rewatering for 1 d, except of the $g_s$ of the empty vector plants which continued falling, $P_N$, $g_s$, and E showed a trend of recovery and the recovery in *hrVzPIP2;1* was faster than in the empty vector plants. Net photosynthetic rate of *hrVzPIP2;1* returned to the level under the control conditions, and its E value was significantly higher than that of the vector plants (Table 3).

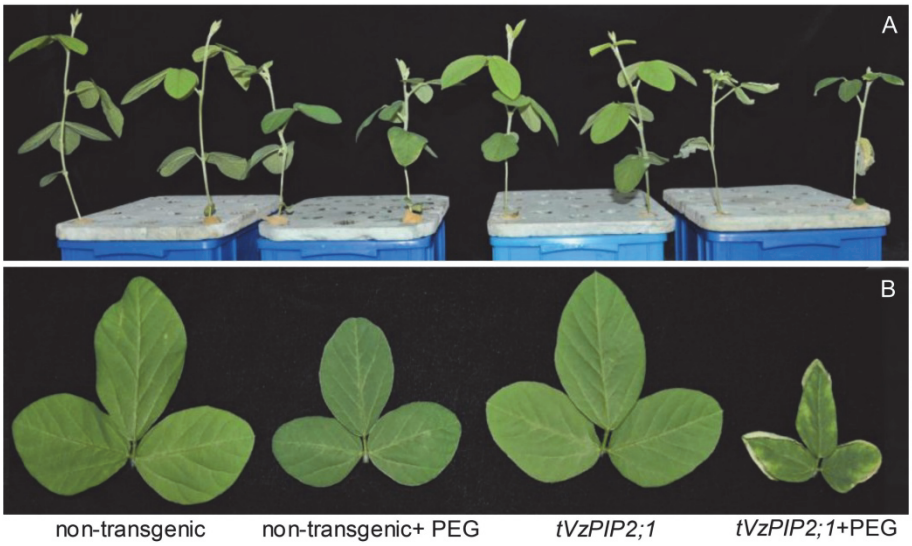


Fig. 4. Comparison of phenotypes of shoots (*A*) and the 1st trifoliate leaves (*B*) between transgenic *VzPIP2;1* soybean seedlings (*tVzPIP2;1*) transformed by *Agrobacterium tumefaciens* and non-transgenic plants under control conditions or 10 % (m/v) PEG-6000 solution treatment for 5 d.

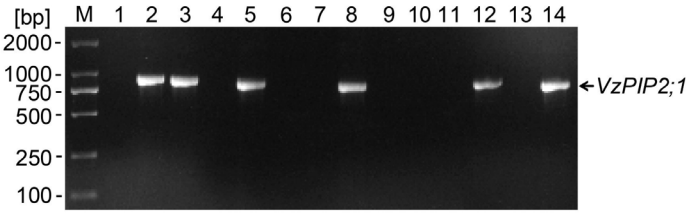


Fig. 5. PCR analysis of transgenic *VzPIP2;1* composite soybean plants transformed by *Agrobacterium rhizogenes*-mediated hairy root system. M represents the DL2000 DNA marker, *lanes* 2, 3, 5, 8, 12 and 14 show transformed plants.

Table 3. Comparison of RWC [%], $P_N$ [μmol($CO_2$) $m^{-2}$ $s^{-1}$], $g_s$ [mol($CO_2$) $m^{-2}$ $s^{-1}$], $c_i$ [μmol($CO_2$) $mol^{-1}$], and E [mmol($H_2O$) $m^{-2}$ $s^{-1}$] (for explaining the abbreviations see Table 2) in control conditions and under 15 % (m/v) PEG-6000 solution treatment for 4 h and then rewatering for 1 d between transgenic *hrVzPIP2;1* seedlings transformed by *Agrobacterium rhizogenes* and those transformed by the empty vector.

| Treatments | Plants | RWC | $P_N$ | $g_s$ | $c_i$ | E |
|---|---|---|---|---|---|---|
| Control | vector | $77.81\pm0.65^{ab}$ | $11.723\pm0.681^{a}$ | $0.155\pm0.005^{a}$ | $376.333\pm13.577^{ab}$ | $2.533\pm0.044^{a}$ |
| | *hrVzPIP2;1* | $81.59\pm0.81^{a}$ | $11.380\pm0.792^{a}$ | $0.160\pm0.016^{a}$ | $321.000\pm15.716^{b}$ | $2.632\pm0.349^{a}$ |
| PEG-6000 | vector | $41.16\pm1.67^{c}$ | $5.133\pm0.696^{d}$ | $0.073\pm0.010^{c}$ | $405.333\pm48.911^{a}$ | $0.515\pm0.085^{d}$ |
| | *hrVzPIP2;1* | $66.82\pm1.55^{b}$ | $8.072\pm1.190^{bc}$ | $0.063\pm0.006^{cd}$ | $364.333\pm33.501^{ab}$ | $1.497\pm0.054^{c}$ |
| Rewatering | vector | - | $5.810\pm1.468^{cd}$ | $0.038\pm0.006^{d}$ | $407.333\pm28.885^{a}$ | $0.863\pm0.084^{d}$ |
| | *hrVzPIP2;1* | - | $9.217\pm0.928^{ab}$ | $0.105\pm0.012^{b}$ | $343.667\pm9.074^{ab}$ | $2.067\pm0.085^{b}$ |

## Discussion

Maintenance of water status is not only vital for growth of plants under favourable conditions, but also crucial for plant adaptation to drought and other abiotic stresses. To adapt to drought stress or mitigate drought damage, stressed plants need to focus on coordinate water uptake and loss (Maurel *et al*. 2008). Generally, drought has a negative effect on $Lp_r$ of crop plants depending on stress level and plant species (Mahdieh *et al*. 2008).

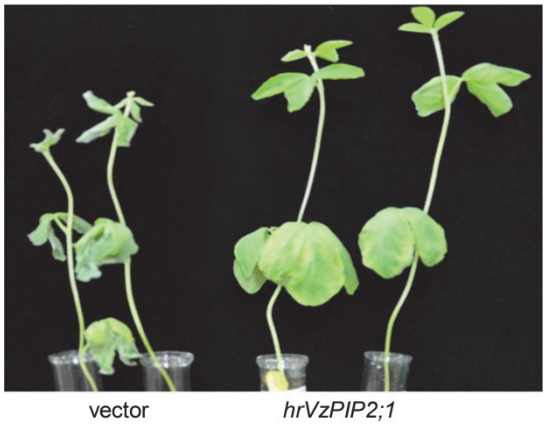


Fig. 6. Comparison of phenotypes of transgenic *hrVzPIP2;1* composite soybean seedlings transformed by *Agrobacterium rhizogenes*-mediated hairy root system and of plants transformed with the empty vector under 15 % (m/v) PEG-6000 solution treatment for 2 d.

The parameters $Lp_r$ (including $Lp_h$ and $Lp_{os}$), $K_{leaf}$, and $K_{plant}$ can reflect water absorption and transport in roots, stems, and leaves (Martre *et al*. 2002, Parent *et al*. 2009). Hydraulic conductance from soil to leaf or $K_{leaf}$ under water deficit may be mainly linked to E and AQPs and affects mesophyll cell water status (Martínez-Ballesta *et al*. 2011). In our previous work (Zhou and Yu 2010), we used 20, 40, and 60 % PEG-6000 for 6 d to evaluate drought tolerance of vetiver grass, and we found that it could survive even under 40 % PEG-6000 treatment. In this study, we adopted 25 % PEG-6000 treatment for 3 d on vetiver grass and subsequent rewatering for measurement plant water relation parameters $Lp_h$, $Lp_{os}$, $K_{leaf}$, $K_{plant}$, and E. Values of water flow rate, $Lp_h$, and $Lp_{os}$ in roots of the drought-stressed vetiver grass significantly increased, and values of $K_{plant}$, $K_{leaf}$, and E significantly decreased ($P < 0.05$). After rewatering, $Lp_{os}$ declined but still remained slightly higher than that of the control ($P > 0.05$), water flow rate and $Lp_h$ remained at a higher level, while the previously decreased $K_{plant}$, $K_{leaf}$, and E recovered (Table 1). Plasma membrane intrinsic proteins play a significant role in recovery of *Arabidopsis* plants from water deficit, and *PIP* antisense mutants have a slower recovery after rewatering (Martre *et al.* 2002). Increased $Lp_r$ is associated with up-regulated *PIP* gene expression especially in plant roots during water stress, and down-regulated *PIP* expression in leaves resulting in decreased $K_{plant}$ and $K_{leaf}$ would be beneficial for limiting loss of cellular water under water stress (Yue *et al*. 2014). This may suggest that under drought stress, ability of water uptake in roots of vetiver grass is increased, but that water transport or water loss *via* transpiration in leaves is remarkably dropped. Such a mechanism should be a key strategy for adaptation of vetiver grass to drought.

Responses of plant *PIPs* to abiotic stresses, such as drought, cold, and salinity, are generally complicated, and *PIP* overexpressions have either beneficial effects or adverse effects on stress tolerance depending on investigated aquaporin genes, plant species, or stress factors (Maurel *et al*. 2008, Zhou *et al*. 2012). Siefritz *et al*. (2002) found that antisense *NtAQP1*-expressing tobacco plants show a reduced root hydraulic conductivity and cellular water permeability, and finally they are more sensitive to drought stress. Sade *et al*. (2010) reported that tobacco plants overexpressing *NtAQP1* display higher $Lp_r$, water use efficiency, and fresh mass, and an enhanced stress resistance under normal and salt stress conditions as compared with wild-type plants. Transgenic *Arabidopsis* plants over-expressing either *OsPIP1;1* or *OsPIP2;2* show an enhanced tolerance to salt and drought stresses (Guo *et al.* 2006). In wheat, *TaAQP7* overexpression confers drought stress tolerance not only by retaining a better water status but also by reducing reactive oxygen species accumulation and membrane damage (Zhou *et al*. 2012). However, some studies have also demonstrated that overexpression of an AQP gene in a homologous or heterologous plant species may disturb its endogenous stress response and increase plant sensitivity to drought stress (Maurel *et al*. 2008). Grapevine plants ove-rexpressing *VvPIP2;4N* (Perrone *et al.* 2012) and *AtPIP1;2* transgenic tobacco plants (Aharon *et al*. 2003) grow well in favourable water conditions, whereas under drought stress both display a more severe damage than control plants. Jang *et al*. (2007) reported that transgenic plants (*Arabidopsis* and tobacco) overexpressing *AtPIP1;4* or *AtPIP2;5* display a rapid water loss and retarded germination and seedling growth under drought stress. In this study, we successfully obtained *VzPIP1;1* and *VzPIP2;1* from vetiver grass and found that only VzPIP2;1 was localized in the PM of onion cells (Fig. 1). The results are not very consistent with those obtained by software analysis. Zelazny *et al*. (2007) reported that ZmPIP2s fused to a monomeric yellow fluorescent protein and/or to a monomeric cyan fluorescent protein could be sublocalized to the PM, whereas ZmPIP1s are retained in the endoplasmic reticulum (ER) and could be relocalized to the PM only when co-expressing with ZmPIP2s and forming a hetero-oligomers between ZmPIP1s and ZmPIP2s. This is the same case for NtPIP1;1 and NtPIP2;1 in tobacco (Mahdieh *et al*. 2008). This was the reason for us to choose only *VzPIP2;1* for soybean transformation.

A low transpiration is generally beneficial for plant adaptation to drought stress (De Paiva Rolla *et al*. 2014, Nir *et al*. 2014,). The *VzPIP2;1* transgenic soybean plants transformed by *A. tumefaciens*, displayed large opening angles between two opposite leaves and a higher E value as compared to its NT plants under the well-watered conditions (Fig. 1 Suppl., Table 2). The *tVzPIP2;1* plants had an excellent capacity for root water absorption coordinated by a strong E although no effects on values of leaf $P_N$ and $F_v/F_m$ were observed (Table 2). Considering that the drought tolerance of soybean was not as strong as of vetiver grass, we reduced the PEG-6000 concentration for water deficit tests on the transgenic soybean seedlings. Under 10 % PEG-6000 for 5 d, growth of the *tVzPIP2;1* and NT plants was markedly suppressed, and the values of $P_N$, $g_s$, $c_i$, and E dropped significantly, but the E value of *tVzPIP2;1* was still significantly higher than that of the NT plants. In addition, the values of REL and $F_v/F_m$ in leaves of *tVzPIP2;1* were remarkably increased and reduced, respectively, and therefore the *tVzPIP2;1* plants suffered a more serious drought injury (Table 2). This may indicate that the more severe injury of *tVzPIP2;1* than the NT plants was mainly related to an abnormal increase of leaf water loss *via* transpiration and water imbalance in the whole plant after *VzPIP2;1* transformation.

*A. rhizogenes*-mediated transformation has become a powerful tool for studying target gene function and root biology due to its quick and simple methodology (Cao *et al*. 2009). In order to further investigate the effect of differential *VzPIP2;1* expression in roots and overground parts of the soybean plants on their drought tolerance, we generated the transgenic *VzPIP2;1* hairy roots of the composite soybean plants (*hrVzPIP2;1*) and found that under 15 % PEG-6000 for 2 d, they displayed a normal phenotype and a higher RWC in overground parts than the empty vector plants, where the latter were more wilted (Fig. 6, Table 3). Under the well-watered conditions, leaf $P_N$, $g_s$, $c_i$, and E of *hrVzPIP2;1* showed no significant differences from those of the empty vector plants, but we should especially notice that the unchanged E value of *hrVzPIP2;1* (Table 3) was different from that of *tVzPIP2;1* transformed by *A. tumefaciens*, and that the latter displayed a significant E rise compared to the NT plants (Table 2). When the *hrVzPIP2;1* and vector plants were subjected to 15 % PEG-6000 solution for 4 h, with the exception of no obvious variation in $c_i$ values and a marked drop in $g_s$ values in both the soybean plant types as compared to those under the control conditions, $P_N$ and E values decreased significantly, but *hrVzPIP2;1* displayed significantly higher $P_N$ and E values than the vector plants (Table 3). In general, reduced transpiration can be attributed to reduced stomatal conductance (Nir *et al*., 2014). But in this work, the markedly dropped $g_s$ showed no difference in the *hrVzPIP2;1* and vector plants under the PEG treatment. The relatively higher E in the *hrVzPIP2;1* than in the vector plants might be beneficial for enhancing water uptake by roots. After rewatering for 1 d, $g_s$ values of the vector plants continued to drop significantly and E and $P_N$ recovered with no significant difference. However, values of $g_s$, E, and $P_N$ in the *hrVzPIP2;1* were all restored significantly, and $P_N$ was even restored to a level higher than under the control conditions (Table 3). The above results suggest that the composite soybean plants with only ectopic expression of *VzPIP2;1* in hair roots could enhance their drought tolerance by improving root water absorption, maintaining higher water content in overground parts, and higher $P_N$ in comparison with the vector plants.

In conclusion, *VzPIP2;1* might play an important role in adaptation of vetiver grass to water deficit by improving root water absorption and controlling leaf water loss. As for ectopic expression of *VzPIP2;1* in soybean, the whole plant gene expression mediated by *A. tumefaciens* transformation caused no profit, but was harmful to drought resistance of the transgenic soybean plants, wheras the composite soybean plants with transgenic hairy roots mediated by *A. rhizogenes* showed the enhanced drought tolerance. This can illustrate that roles of *VzPIP2;1* in response to water stress are closely related to the organ-specific expression in plants. The differential expression of *VzPIP2;1* in roots and leaves of vetiver grass and the transgenic soybean plants can differentially regulate root water absorption and leaf transpiration under normal or unfavourable water conditions. Accordingly, *VzPIP2;1* can be considered as drought tolerance determinant. In addition, *VzPIP2;1* can also be used to construct transgenic plants with a high root water absorption ability, which can make plants fully use a limited water supply and is suitable for plant cultivation or afforestation in arid areas. Indeed, since this is only a preliminary or initial study on AQP family in drought-tolerant vetiver grass, more clear functions of *VzPIP2;1* in plant drought response or adaptation may be further clarified by using *PIP* mutants of various plant species including *Arabidopsis* in the future.

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