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An aquaporin protein is associated with drought stress tolerance

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ABSTRACT

Water channel proteins known as aquaporins (AQPs) regulate the movement of water and other small molecules across plant vacuolar and plasma membranes; they are associated with plant tolerance of biotic and abiotic stresses. In this study, a PIP type AQPs gene, designated as *GoPIP1*, was cloned from *Galega orientalis*, a high value leguminous forage crop. The *GoPIP1* gene consists of an 870 bp open reading frame encoding a protein of 289 amino acids, and belongs to the PIP1 subgroup of the PIP subfamily. The transcript level of *GoPIP1* was higher in the root of *G. orientalis* than in the leaf and stem. The level of *GoPIP1* transcript increased significantly when treated with 200 mM NaCl or 20% polyethylene glycol (PEG) 6000. Transient expression of GoPIP1 in onion epidermal cells revealed that the GoPIP1 protein was localized to the plasma membrane. Over-expression of *GoPIP1* increased the rosette/root ratio and increased sensitivity to drought in transgenic *Arabidopsis* plants. However, *GoPIP1* over-expression in *Arabidopsis* had no significant effect under saline condition. The present data provides a gene resource that contributes to furthering our understanding of water channel protein and their application in plant stress tolerance.

1. Introduction

Abiotic stresses such as salinity and drought cause significant losses in plant production throughout the world [1]. Water is vital for plant production [2]. The movement of water through cellular membranes is required for all forms of water transportation, including osmoregulation, single cell expansion, and long-distance transport [3]. Water permeability in both vacuolar and plasma membranes can be enhanced by a class of water channel proteins known as aquaporins (AQPs) [4]. Plant AQPs are involved in the opening and closing of cellular gates [5], which play important roles in the physiology of both water balance and water use efficiency [6–10].

Plant AQPs comprise a large and highly diverse protein family [4,11]. Plasma membrane intrinsic proteins (PIP) is one of the subfamilies in AQPs, and the PIPs are further subdivided into two phylogenetic subgroups: PIP1 and PIP2 [12]. Considerable progress has been made in understanding the functions of PIPs. A large number of studies have shown that over-expressing certain PIP1 genes is beneficial for plants under favorable and/or conditions with abiotic stresses [13–15]. For example, *TdPIP1;1* (*Triticum turgidum* L. subsp. *durum*), Ta*AQP*, *OsPIP1;1* each overexpression increased salt-stress tolerance [13–15]. Concurrently, over-expression of some AQP genes have shown opposite effects in response to abiotic stresses [10,16]. Although an increasing number of studies have demonstrated that PIPs are responsive to environmental stresses in plants, the functions of PIPs seem to conflict and our knowledge and understanding of these proteins remain limited.

The *Galega orientalis* is a promising perennial forage legume for both forage production and soil improvement [17,18]. This forage legume has been introduced widely in northwest China in recent years. In the current study, we characterized a *G. orientalis* PIP protein and investigated the response of *GoPIP1* to abiotic stresses. The results indicated that *GoPIP1* responds differently to various environmental conditions when ectopically expressed in *Arabidopsis*.

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2. Materials and methods

2.1. Plant materials and growth conditions

Mature seeds of *G. orientalis* were surface-sterilized and germinated on petri dishes (in water) at 24 °C under 12 h light/12 h dark condition. After 2–3 days, seeds were transplanted into pots with vermiculite and perlite (3:1, v/v) and grown in a growth chamber at 28 °C with a 16 h light/8 h dark photoperiod. *Arabidopsis* (Col-0) plants grown in a 24 °C constant temperature greenhouse with 16 h light/8 h dark conditions were used for plant transformation.

2.2. Cloning of the GoPIP1 gene

Total RNA was isolated from leaf samples with TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions. Based on a known expressed sequence tag (EST), rapid amplification of cDNA ends (RACE) was performed to amplify the uncharacterized 3′ and 5′cDNA ends of *GoPIP1* according to the manufacturer's protocol (Clontech, USA). The primers GSP1 and GSP2 (Supplementary Table S1) were used to amplify the 5′-cDNA end and the 3′-cDNA end of *GoPIP1* gene, respectively.

2.3. Bioinformatics analysis

Sequence similarity analysis was performed using BLAST tools (http://blast.ncbi.nlm.nih.gov/). DNAMAN v.6.0 software was used for multiple alignments of the deduced amino acid sequences. A search for open reading frames (ORF) and translation of the nucleotide sequences were performed using ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). A phylogenetic tree was constructed using the neighbor-joining method with MEGA 5.0 software.

2.4. Q-RT-PCR analysis

Total RNA was extracted from 5-week-old *G. orientalis* seedlings, which were grown under 200 mM NaCl, 20% PEG-6000 for 0, 2, 4, 8, 12, or 24 h. First strand cDNA was synthesized from total RNA (5 μg) and stored at −80 °C. Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) was used to measure the expression levels of the *GoPIP1* gene. A SYBR Premix *Ex Taq*™ kit (TAKARA, Japan) was used for qRT-PCR assay in ABI 7500 Real-Time PCR instrument (ABI, USA). The relative expression of genes was calculated using the $2^{-\Delta\Delta Ct}$ formula [19].

2.5. Subcellular localization of the GoPIP1

The cloned *GoPIP1* sequence was sub-cloned into the expression vector pCAMBIA1302. The correct *GoPIP1*-GFP fusion construct was confirmed by sequencing. The empty vector pCAMBIA1302 (used as control) and GoPIP1-GFP fusion construct were transformed into onion epidermal cells using a Model PDS-1000/He Biolistic Particle Delivery System according to the manufacturers' protocol (Bio-Rad, USA). Cells were incubated at 25 °C for 1 d and then observed for transient expression of GFP protein using a confocal laser scanning microscope (Olympus FV500, Japan).

2.6. Genetic transformation of Arabidopsis

The open reading frame of *GoPIP1* was amplified with specific primers modified to include *Xba*I and *Bam*HI restriction sites (Supplementary Table S1). The amplicon was digested with *Xba*I and *Bam*HI and ligated into the pBI121 vector (Clontech, Japan). This vector was then transformed into *Agrobacterium tumefaciens* strain GV3101 using the freezing/heat shock method. Transgenic *Arabidopsis* plants were produced using the floral dip method [20]. The seeds were selected on half-strength Murashige and Skoog (MS) plates containing 50 mg/L kanamycin. The plants were examined by genomic PCR and reverse transcriptase PCR (RT-PCR) analysis. Homozygous T_2 lines were self-pollinated to produce the T_3 generation. Transgenic homozygous and control T_3 plants were also identified by RT-PCR and southern hybridization and protein blot analysis. Southern hybridization and detection were performed with the DIG High Prime DNA Labeling and Detection Starter Kit I according to the manufacturers' instructions (Roche, Switzerland). Protein blot was analyzed as previously described [21].

2.7. Stress treatments

T_3 generation transgenic plants and wild type (WT) plants were surface-sterilized and germinated on 1/2 MS agar plates in a controlled-growth chamber with 16 h photoperiod, 25 °C. Five days after germination, 14 small seedlings were transferred to 1/2 MS agar plates with or without either 100 mM NaCl or 100 mM mannitol, in a controlled-growth chamber. The following measurements were taken after 20 days: rosette weight, rosette dry weight, and root dry weight. To observe visual phenotypes, 5 transgenic plants and 5 WT plants were grown vertically on MS agar plates containing 100 mM NaCl or 100 mM mannitol. All experiments were repeated three times.

For the ion content analysis, the shoots of the transgenic *Arabidopsis* and the WT seedlings plants were harvested after 20 days on 1/2 MS medium containing 100 mM NaCl, and the Na^+ and K^+ content were analyzed by atomic absorption as previously described [22].

Fresh weight loss from detached leaves (FWLDL) measured as described by Zhang et al. [23]. For the drought stress, well-rooted seedlings were transplanted into soil in 15-cm diameter pots, and the same amount of water was added to each sample. After 20 days, drought stress was applied by stopping irrigation for 18 days. Malondialdehyde (MDA) and proline content was assessed as described by Bai et al. [24] and Troll et al. [25].

Leaf osmolality and water potential were measured as described by Ruggiero et al. [26] and Brini et al. [27]. The plant growth conditions were on 1/2 MS agar plates with or without 100 mM mannitol 20 days after germination in a controlled-growth chamber. Measurement of leaf transpiration was performed on four plants per line at 25 °C and a relative humidity of 70% using a GFS-3000 gas exchange system (WALZ, Germany). The light-saturated photosynthetic rate was determined at PPFD = 1200 μmol m^{-2} sec^{-1} with 400 ppm CO_2.

2.8. Expression of stress-related genes in transgenic Arabidopsis under drought conditions

To study the mechanism of *GoPIP1*-mediated signaling transduction under drought conditions, the expression of 16 genes involved in specific stress signaling pathways were analyzed by qRT-PCR in WT and *GoPIP1*-expressing *Arabidopsis* plants. The plant growth conditions and drought treatments of the samples analyzed by qRT-PCR were the same as described above (15-cm diameter pots). The primers used were listed in Supplementary Table S1.

3. Results

3.1. Isolation and sequence analysis of full-length GoPIP1 cDNA

The length of the *GoPIP1* cDNA was 1274 bp, including an 870 bp open reading frame (encoding 289 amino acids) (Genbank

accession no. HM991477). The putative protein contains the AQP family signature sequence 'HINPAVTFG' and two 'NPA' motifs, and was highly similar to other PIPs, sharing 97.58% identity with the *Medicago truncatula* MtPIP (Fig. S1). Phylogenetic tree analysis showed that the deduced amino acid sequence belongs to the PIP1 subfamily (Fig. S2).

3.2. GoPIP1 gene expression pattern analysis

The Quantitative RT-PCR showed that *GoPIP1* was higher in the roots than in leaves or stems (Fig. 1A). The Salt and PEG stress significantly and rapidly induced *GoPIP1* expression (Fig. 1 B, C). The expression level of *GoPIP1* were markedly induced by PEG treatment, reaching a peak at 4 h, then declining prior to peaking again at 24 h (Fig. 1C).

3.3. Subcellular localization of GoPIP1

The ProtComp online v.9.0 program (www.softberry.com) was used and the result predicted that GoPIP1 was predominately localized to the plasma membrane. The subcellular localization in a transient transfection assay showed that the pCAMBIA1302-GoPIP1-GFP fusion protein was exclusively localized to the plasma membrane (Fig. S3 D, E, F). In contrast, the GFP signal from cells transformed with the pCAMBIA1302-GFP vector was distributed throughout the onion epidermal cells (Fig. S3 A, B, C), indicated a cytosolic localization of GFP.

3.4. Over-expression of GoPIP1 in Arabidopsis increased the rosette/root ratio under favorable growth conditions

The transgenic *Arabidopsis* were verified by southern hybridization, RT-PCR and western blot, respectively (Fig. S4 A, B, C). Under normal growth conditions, the transgenic plants had no difference in rosette weight, rosette water content, or total dry weight compared with the WT plants (Table S2). There were also no differences in leaf water potential and osmolality between transgenic and WT plants (Fig. S5). The transgenic plants did, however, exhibit a significantly higher rosette/root ratio than WT plants.

3.5. Analysis of transgenic Arabidopsis under high salt conditions

No difference was observed between transgenic lines and WT plants after treatment with 100 mM NaCl on 1/2 MS medium or 300 mM NaCl treatment in small pots (Table S3, Fig. S6A). There was also no difference in ion content (Na^+, K^+, K^+/Na^+ ratio) between the transgenic and WT plants (Fig. S6 B, C, D).

3.6. Analysis of transgenic Arabidopsis under dehydration treatment

There was no significant difference in germination rates between the transgenic lines and the WT plants under 0, 50, 100, 150, or 200 mM mannitol treatment (data not shown). After growth on 1/2 MS medium with 100 mM mannitol, however, the transgenic plants had a lower rosette weight, rosette water content, and root dry weight compared with WT plants (Fig. 2 A–C). The difference in rosette/root ratio was not significant with this treatment (Fig. 2 D). Additionally, transgenic plants exhibited faded leaves compared with WT plants following vertical growth (Fig. 3A).

The rate of FWLDL from the transgenic lines was significantly higher than that of the WT plants, particularly in the first 6 h of treatment (Fig. 3B). The transgenic lines had slight increases (0.17- to 0.27 fold) in transpiration rate compared to WT, indicating that over-expression of *GoPIP1* increases water loss through transpiration in *Arabidopsis*. The transgenic plants had decreased water potential but increased osmolality under drought conditions, as compared to WT plants. The MDA contents from transgenic lines 2, 25, and 37 were 68%, 117%, and 115% respectively, and higher than WT (Fig. 3F). Proline contents from same lines were 33.6%, 37.1%, and 60.3% lower ($P < 0.01$), respectively, than that of the WT plants (Fig. 3G).

The expression of sixteen genes that are closely associated with drought tolerance in *Arabidopsis* was selected for expression analysis. The expression levels of the ABA-biosynthesized gene *NCED3* [28] and *AAO3* [29] showed 2.0- and 1.7-fold increases, respectively, in *GoPIP1* transgenic plants (Fig. 4A). Both *RD29A* [30,31] and *RD19a* [32] in the transgenic plants showed a 1.3-fold increase in gene expression level as compared with the WT plants (Fig. 4A). The expression level of a detoxification gene, *ALDH7B4* [33,34] was increased by 1.5-fold in transgenic plants (Fig. 4B). No detectable differences were found between the transgenic and the WT plants in the expression levels of salt overly sensitive (SOS) signaling pathway genes (*SOS1*, *SOS2* and *SOS3*), cation exchanger genes (*SOS1* and *CAX1*), or other tested genes.

4. Discussions

Plants have a large number of aquaporin homologs. The PIP proteins belong to the AQP family, which is known to play an important role in water transport in the plasma membrane of many plant species [6–8]. A number of PIP family proteins have been reported to participate in abiotic stress responses, with some of them playing positive roles [13,14]. In the present study, under favorable conditions, the transgenic plants had a higher rosette/root ratio compared to the WT, indicating that *GoPIP1* gene did affect the morphology in transgenic plants under normal condition.

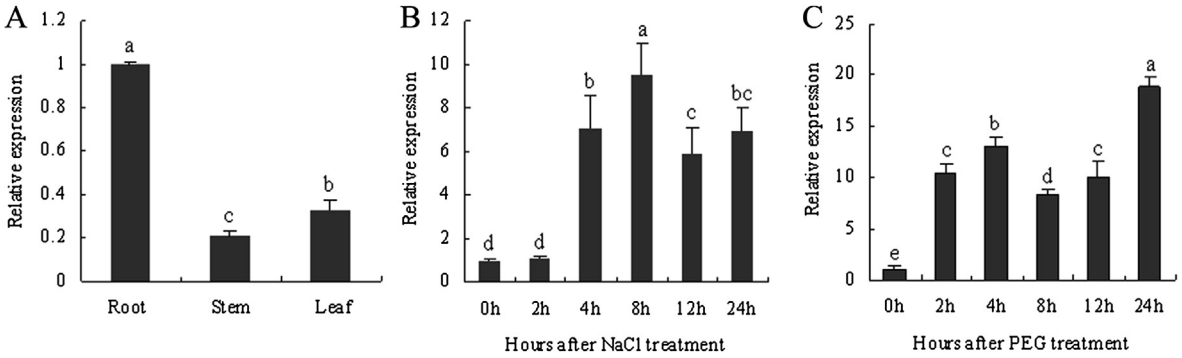

Fig. 1. Expression patterns of *GoPIP1* in *G. orientalis*. (A) Root, stem, and leaf of 5-week-old plants under non-stress conditions. (B) Seedling leaf (5-week-old) under high-salt treatment (200 mM NaCl). (C) Seeding leaf (5-week-old) under 20% PEG treatment. Values are the means of three replicates (SE), and statistically significant differences at the 0.05 level are labeled with different letters.

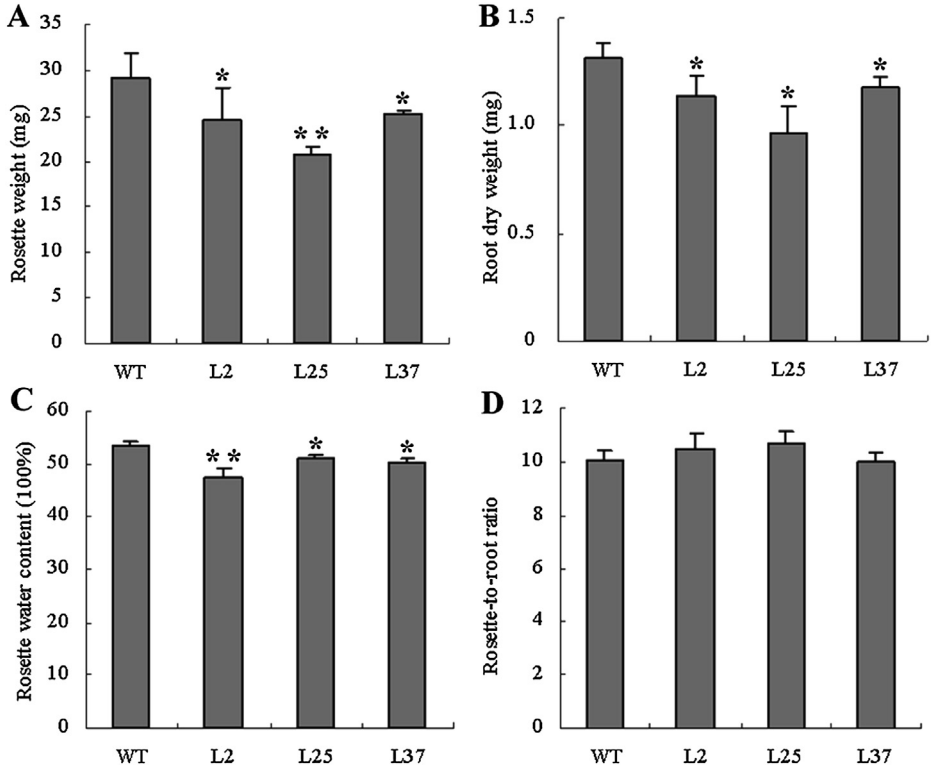

Fig. 2. Effects of drought stress on transgenic and WT plants growth state. (A–D) Rosette weight, root dry weight, rosette water content, rosette-to-root ratio of transgenic plants and control growth 20 days under 100 mM mannitol. Values are the means of three replicates (SE, n = 3). One or two asterisks indicate significance corresponding to $P < 0.01$ or $P < 0.05$, respectively (one way ANOVA, Dunnett's test).

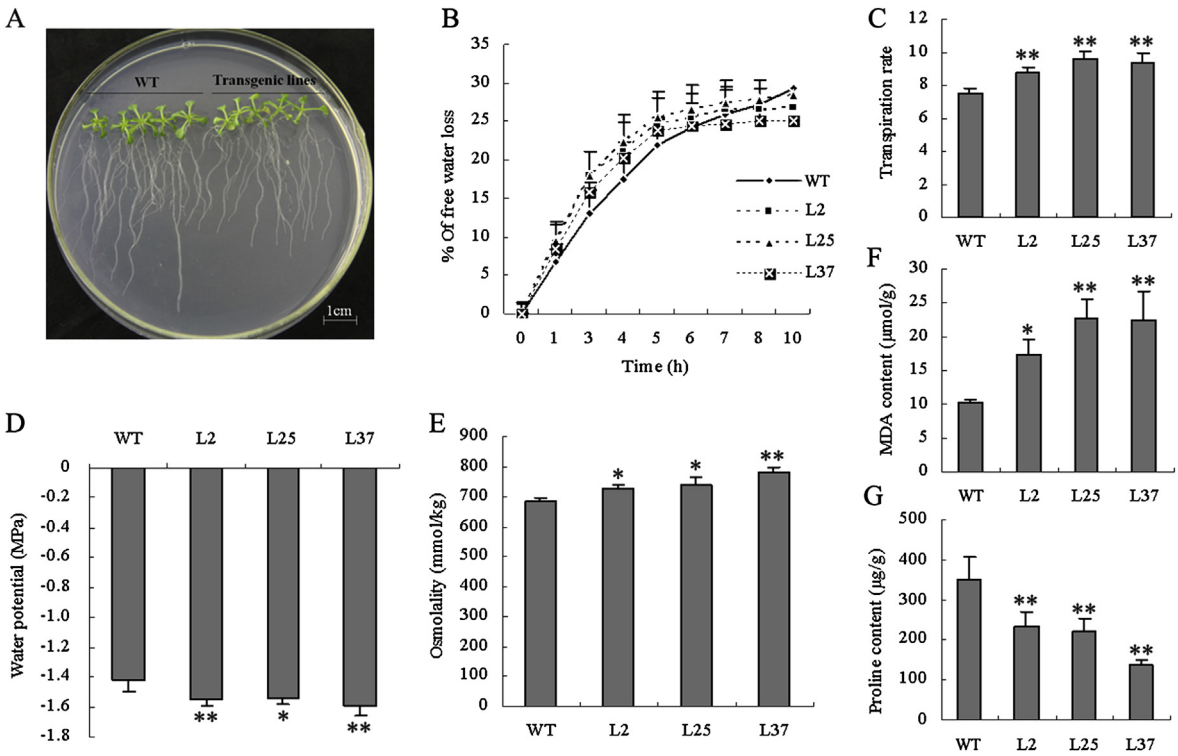

Fig. 3. Effects of drought stress on transgenic and WT plants phenotype and physiological index. (A) Representative phenotypes of transgenic plants with 100 mM mannitol treatment. Scale bar, 1 cm. (B) Water loss rate of detached leaves from transgenic and WT plants, each point is mean ± SE (n = 3). (C) Transpiration rate (mmol m^{-2} sec^{-1}) of detached leaves from transgenic and WT plants. (D–E) Leaf water potential and osmolality of transgenic lines and WT under drought conditions. (F–G) Malondialdehyde (MDA) and proline content after drought treatment for 18 days in small pots (n = 3). One or two asterisks indicate significance corresponding to $P < 0.01$ or $P < 0.05$, respectively (one way ANOVA, Dunnett's test).

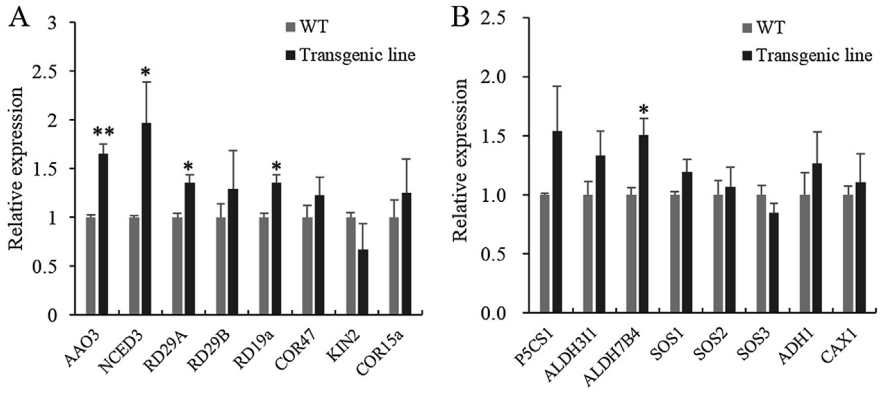

Fig. 4. Expression pattern of marker genes. (A–B) Expression level of stress induced or related marker genes in leaves of the WT and the transgenic lines. Error bars indicate SE (n = 3). One or two asterisks indicate significance corresponding to P < 0.01 or P < 0.05, respectively (one way ANOVA, Dunnett's test).

Similar phenotypes have been observed in transgenic rice over-expressing barley *HvPIP2;1* and in transgenic tobacco over-expressing *AtPIP1b* [10,35]. This is most likely a consequence of a compensation mechanism that decreases the root/shoot ratio. It may also be the case that over-expression of *GoPIP1* enhanced water transport in such a way that the morphological change, as a result of a plant feedback reaction, occurred at a cellular level.

Under salt stress treatment, there was no significant gross phenotype or ion content difference between transgenic and WT plants. These results are consistent with previous studies by Jang [16], which differed from previous studies [10,14,35]. As AQPs transports water and other small molecules (eg. Na^+, K^+) through biological membranes [14], it is speculated that *GoPIP1* did not largely facilitate ion transport or at least Na^+ and K^+ based on the ion content analysis.

In addition to salt treatment, clear differences were observed with respect to water stress. The lower rosette weight and the mildly faded leaf phenotype in transgenic *Arabidopsis* plants over-expressing *GoPIP1* under drought conditions were closely associated with drought sensitivity. Additionally, the transgenic plants over-expressing *GoPIP1* had lower leaf water content and lower water potential, but higher osmolality (Fig. 3). The leaf water content, water potential and osmolality are all associated with water movement. The transgenic plants had faster water loss through leaves to the atmosphere, indicating that *GoPIP1* might affect stomatal aperture thus modifying water movement. Previous studies have demonstrated an important role for AQPs in the maintenance of water balance in plants. Transgenic plants over-expressing *PIP1;4* or *PIP2;5* showed rapid water loss under dehydration stress, and displayed retarded seedling growth under drought stress [16]. Aharon et al. [10] reported that over-expressing *AtPIP1b* in transgenic tobacco had a negative impact during drought stress, with the transgenic plants wilting more rapidly than the WT plants. In contrast, Siefritz et al. [6] reported that reduction of *PIP* expression accelerated wilting under water stress. In the current study, over-expression of *GoPIP1* had a negative impact on plant growth under drought stress. Our result supports the previous proposition that a general increase in water transport is harmful in most plant tissues and cells under drought stress [10,16]. It was also interesting that the rosette/root ratio (dry matter) under stress conditions differed from that of plants grown under favorable conditions. This may be because the transgenic lines are sensitive to the stressed environment and accumulate less photosynthetic products in rosettes.

The ABA biosynthesis-related genes *NCED3* [28] and *AAO3* (aldehyde oxidase 3) [36] had more abundant transcripts in the transgenic plants than in the WT plants. The expression of *RD29A* and *RD19a* was higher in transgenic plants than in the WT plants (P < 0.05). It appears that *GoPIP1* may activate the ABA biosynthesis pathway, thus inducing higher expression level of *RD29A* and *RD19a*. Under drought conditions, abscisic acid content is typically increased; this is responsible for the alterations in the expression of drought-stress-induced genes that play important roles in plant responses to drought tolerance [37]. ABA has long-lasting effects on plant hydraulic properties *via* aquaporin activity which contributes to the maintenance of favorable plant water status [38]. It is apparent that the changes of ABA signals play positive roles for the drought sensitivity in *GoPIP1* over-expressing *Arabidopsis* plants.

In conclusion, an *AQP* gene *GoPIP1* was functionally characterized from *G. orientalis*. Constitutive over-expression of *GoPIP1* in *Arabidopsis* led to an increased rosette/root ratio and decreased drought resistance. However, over-expression of *GoPIP1* had no significant impact on plant resistance to salt stress. Drought sensitivity may be a result of increased transpiration, changes in organic osmolyte accumulation and ABA signals in the *GoPIP1* transgenic plants. The present work enhances our knowledge of the relationship between AQPs proteins and abiotic stress.

Conflict of interest

None.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.02.052.

Transparency document

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