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# An aquaporin protein is associated with drought stress tolerance

Jun Li [a, b], Liping Ban [c], Hongyu Wen [a], Zan Wang [a], Nikolay Dzyubenko [d], Vladimir Chapurin [d], Hongwen Gao [a], Xuemin Wang [a, *]

[a] Institute of Animal Science, Chinese Academy of Agricultural Sciences, Beijing 100193, People's Republic of China
[b] Institute of Grassland Research, Chinese Academy of Agricultural Sciences / SharaQin Key Wild Scientific Monitoring Station for Forage Resources of Ministry of Agriculture, 120 Wulanchabu East Street, Saihan District, Hohhot 010010, People's Republic of China
[c] College Animal Science & Technology, China Agricultural University, Beijing 100193, People's Republic of China
[d] N.I.Vavilov All-Russian Research Institute of Plant Industry, St. Petersburg 190000, Russia

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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*.

* Corresponding author. Fax: +86 010 62894560.
*E-mail address:* wangxuemin@caas.cn (X. Wang).

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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



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