Plant Molecular Biology (2006) 60:389–404
DOI 10.1007/s11103-005-4210-y

PIP aquaporin gene expression in arbuscular mycorrhizal *Glycine max* and *Lactuca sativa* plants in relation to drought stress tolerance

Rosa Porcel, Ricardo Aroca, Rosario Azcón and Juan Manuel Ruiz-Lozano*
*Departamento de Microbiología del Suelo y Sistemas Simbióticos, Estación Experimental del Zaidín (CSIC), Profesor Albareda, no. 1, Granada 18008, Spain (*author for correspondence; e-mail juanmanuel.ruiz@eez.csic.es)*

Received 23 July 2005; accepted in revised form 17 October 2005

Key words: arbuscular mycorrhizal symbiosis, drought tolerance, PIP aquaporin, water deficit

Abstract

Although the discovery of aquaporins in plants has resulted in a paradigm shift in the understanding of plant water relations, the relationship between aquaporins and plant responses to drought still remains elusive. Moreover, the contribution of aquaporin genes to the enhanced tolerance to drought in arbuscular mycorrhisal (AM) plants has never been investigated. Therefore, we studied, at a molecular level, whether the expression of aquaporin-encoding genes in roots is altered by the AM symbiosis as a mechanism to enhance host plant tolerance to water deficit. In this study, genes encoding plasma membrane aquaporins (PIPs) from soybean and lettuce were cloned and their expression pattern studied in AM and nonAM plants cultivated under well-watered or drought stressed conditions. Results showed that AM plants responded to drought stress by down-regulating the expression of the *PIP* genes studied and anticipating its down-regulation as compared to nonAM plants. The possible physiological implications of this down-regulation of *PIP* genes as a mechanism to decrease membrane water permeability and to allow cellular water conservation is further discussed.

Introduction

The arbuscular mycorrhizal (AM) symbiosis is the visible result of the interaction between a plant root and a fungus. By this, the AM fungus occupies a protected ecological niche and receives plant photosynthates, while plants improve their ability for nutrients uptake and their tolerance to biotic and abiotic stresses (Smith and Read, 1997). Among the abiotic stresses, water deficit is one of the most common environmental stress factors experienced by soil plants. It interferes with both normal development and growth and has a major adverse effect on plant survival and productivity (Kramer and Boyer, 1997; Sheng *et al.*, 2004). Several eco-physiological studies investigating the role of AM symbiosis in protection against drought stress have demonstrated that the symbiosis often results in altered rates of water movement into, through and out of the host plants, with consequences on tissue hydration and plant physiology (for reviews see Augé, 2001, 2004; Ruiz-Lozano, 2003).

The AM system is an excellent example for the extensive morphological alterations that plant root cells undergo in order to accommodate the presence of symbionts. Cytoskeleton elements are rearranged, the nucleus increases in size, amyloplasts lose their starch content and changes occur in the membrane systems of arbuscule-containing cells. The plant plasma membrane extends to form a novel periarbuscular membrane, which closely surrounds the fungal hyphae resulting in an estimated 3- to 10-fold increase in the outer plant cell

surface (Bonfante and Perotto, 1995; Gianinazzi-Pearson, 1996). Since most of the mycorrhiza-induced changes in plant root cells concern cytoplasmic or vacuolar membrane systems, a variation of expression patterns concerning genes that encode membrane-associated proteins can be expected (Krajinski *et al*., 2000). Accordingly, it has been shown that in mycorrhizal roots a gene encoding for a plant aquaporin is up-regulated and the expression is localized in the highly compartmented vacuole of arbuscule-containing cells (Roussel *et al*., 1997; Krajinski *et al*., 2000). Several aquaporin-encoding genes have also been shown to be up-regulated in ectomycorrhizal poplar plants, and this was correlated with an increased water transport capacity of mycorrhizal poplar roots (Marjanovic *et al*., 2005). Finally, it has been also shown that the impairment of a PIP gene in an antisense tobacco mutant reduced the symbiotic efficiency of two AM fungi under drought stress conditions (Porcel *et al*., 2005a).

Aquaporins are water channel proteins that facilitate and regulate the passive movement of water molecules down a water potential gradient. These proteins belong to the large major intrinsic protein (MIP) family of transmembrane proteins and are represented in all kingdoms (Chrispeels and Agre, 1994; Maurel, 1997; Tyerman *et al*., 2002). Two major classes of plant aquaporins, located in the plasma membrane (PIPs) or tonoplast (TIPs), respectively, have been identified so far (Johnson *et al*., 1990; Kammerloher *et al*., 1994). Other two classes of plant aquaporins are the homologues to the soybean Nodulin-26 aquaporin (NIPs) and the small basic intrinsic proteins (SIPs). The localization and function of SIPs are unknown at the moment (Johanson *et al*., 2001; Luu and Maurel, 2005).

The rate of water flux into or out of a cell is determined by the water potential gradient that acts as the driving force for transport and by the water permeability of the membrane. Aquaporin proteins facilitate osmosis by forming water-specific pores as an alternative to water diffusion through the lipid bilayer, thus increasing the water permeability of the membrane (Schäffner, 1998; Kjelbom *et al*., 1999; Smart *et al*., 2001). It has been suggested that vacuolar and plasma membrane aquaporins, acting in concert, are responsible for the cytosolic osmoregulation that is necessary for maintaining normal metabolic processes (Kjelbom, 1999). Moreover, inhibition studies of aquaporins *in vivo* and antisense transgenic studies have also suggested that aquaporins are crucial for the bulk flow of water in plants (Grote *et al*., 1998; Kjelbom *et al*., 1999; Martre *et al*., 2002; Siefritz *et al*., 2002; Javot *et al*., 2003).

The discovery of aquaporins in plants has caused a significant change in the understanding of plant water relations. However, the relationship that exists between aquaporins and plant responses to drought still remains elusive and with contradictory results (Aharon *et al*., 2003; Lian *et al*., 2004). Moreover, the contribution of aquaporin genes to the enhanced tolerance to drought in AM plants has never been investigated. Krajinski *et al*. (2000) proposed that the up-regulation of aquaporins by the AM symbiosis probably optimizes nutrient and water exchange between both symbiotic partners. They may also permit efficient osmoregulation of the highly compartmented root cells (Maurel *et al*., 2002). However, the studies by Roussel *et al*. (1997) and Krajinski *et al*. (2000) were carried out under well-watered conditions and they did not test the expression of the aquaporin gene in AM plants under drought stress conditions.

Many studies have suggested that aquaporins contribute significantly to the hydraulic conductivity of cells and that they have a role in cellular osmoregulation (Kjelbom *et al*., 1999; Martre *et al*., 2002; Javot *et al*., 2003). In recent years, much effort has been concentrated on investigating the function and regulation of PIP aquaporins. These aquaporins seem to play a specifically important role in controlling transcellular water transport. For instance, they are abundantly expressed in roots where they mediate most of soil water uptake (Javot and Maurel, 2002) and transgenic plants down-regulting one or more PIP genes had lower root water uptake capacity (Siefritz *et al*., 2002; Javot *et al*., 2003). Since aquaporins are regulated both at transcriptional and activity levels (Martre *et al*., 2002), we have considered of interest to study whether the expression of aquaporin-encoding genes in roots is altered by the AM symbiosis as a mechanisms to enhance host plant tolerance to water deficit. To achieve this, genes encoding PIPs from soybean and lettuce were cloned and their expression pattern studied, in AM and nonAM plants cultivated under well-watered or drought stress conditions by using northern blot and quantitative real-time PCR. At the same

time, PIP protein abundance was studied by western blot.

Materials and methods

Experimental designs and statistical analysis

First experiment with Glycine max

The experiment consisted of a randomized complete block design with three inoculation treatments: (1) non-inoculated control plants (NI), (2) plants inoculated with the nitrogen-fixing bacteria *Bradyrhizobium japonicum*, strain USDA 110 (Br) and (3) plants inoculated with the arbuscular mycorrhizal (AM) fungus *Glomus mosseae* (Nicol. and Gerd.) Gerd. and Trappe and *B. japonicum* (Gm + Br). The use of these three inoculation treatments was decided because legume plants, in their natural state, are nodulated. Hence, the nonAM control plants were those inoculated only with *B. japonicum* (Br treatment). However, we also included an absolute non-inoculated control treatment for comparison. Twelve replicates of each microbial treatment were done, totalling 36 pots (one plant per pot) so that half of them was cultivated under well-watered conditions throughout the entire experiment, while the other half was drought-stressed for 10 days before harvest (35 days after inoculation).

Second experiment with Glycine max

The experiment consisted of a randomized complete block design with the same inoculation treatments described for the first experiment: For each treatment plants were harvested at four time intervals: 5, 12, 20 or 35 days after inoculation (dai). There were different number of replicates for each treatment, ranging from 12 replicates for plants harvested after only 5 days, to 6 replicates for plant harvested after 35 days, totalling 108 pots (one plant per pot). Half of the plants were cultivated under well-watered conditions throughout the entire experiment, while the other half were drought-stressed for 5 days (for plants harvested 5 dai) or for 10 days (for the rest of treatments) before harvest.

Experiment with Lactuca sativa

The experiment consisted of a randomized complete block design with three inoculation treatments: (1) non-inoculated control plants (NI), (2) plants inoculated with the AM fungus *Glomus mosseae* (Nicol. and Gerd.) Gerd. and Trappe (Gm) and (3) plants inoculated with the AM fungus *Glomus intraradices* (Schenck and Smith) (Gi). There were 10 replicates of each treatment, totalling 30 pots (one plant per pot), so that half of them were cultivated under well-watered conditions throughout the entire experiment, while the other half were drought-stressed for 10 days before harvest.

Data were subjected to analysis of variance (ANOVA) with microbial treatment, water supply and microbial treatment-water supply interaction as sources of variation, and followed by Duncańs multiple range test (Duncan, 1955). Percentage values were arcsin transformed before statistical analysis.

Soil and biological materials

Loamy soil was collected from the Zaidin Experimental Station (Granada, Spain), sieved (2 mm), diluted with quartz-sand (< 1 mm) (1:1, soil:sand, v/v) and sterilized by steaming (100 °C for 1 h on 3 consecutive days). The soil had a pH of 8.1 (water); 1.81% organic matter, nutrient concentrations (mg kg^{-1}): N, 2.5; P, 6.2 ($NaHCO_3$-extractable P); K, 132.0. The soil texture was made up of 35.8% sand, 43.6% silt and 20.5% clay.

Soybean (*Glycine max* L. cv Williams) seeds were sterilized in a 15% H_2O_2 solution for 8 min, then washed several times with sterile water to remove any trace of chemical that could interfere in seed germination, and placed on sterile vermiculite at 25 °C to germinate. Three-day-old seedlings were transferred to plastic pots containing 600 g of sterilized soil/sand mixture (1:1, v/v). When appropriate, a suspension (2 ml seed) of the diazotrophic bacterium *Bradyrhizobium japonicum*, strain USDA 110 (10^8 cell ml^{-1}) was sprinkled over the seedling at the time of planting.

Three seeds of lettuce (*Lactuca sativa* L. cv. Romana) were sown in pots containing 500 g of the same soil/sand mixture as described above and thinned to one seedling per pot after emergence.

Mycorrhizal inoculum for each endophyte was bulked in an open-pot culture of *Zea mays* L. and consisted of soil, spores, mycelia and infected root fragments. The AM species were *Glomus mosseae* (Nicol. and Gerd.) Gerd. and Trappe, isolate BEG 122 and *Glomus intraradices* (Schenck and Smith) isolate BEG 121. Ten grams of inoculum of the

two *Glomus* isolates, possessing similar infection characteristics (about 80 infective propagules per gram, according to the most probable number test), were added to appropriate pots at sowing time just below soybean seedlings or lettuce seeds.

Uninoculated control plants for each microbial treatment received the same amount of autoclaved rhizobial and/or mycorrhizal inoculum together with a 2-ml aliquot of a filtrate (< 20 μm) of the AM inoculum in order to provide a general microbial population free of AM propagules.

Choice of plant and fungal species

The choice of *Glycine max* and *Lactuca sativa* is based on the fact that both species are highly mycotrophic and responsive to drought stress, thus representing a good system to study the effects of the AM symbiosis when coping with drought stress.

The choice of *G. mosseae* and *G. intraradices* as mycorrhizal fungi is based on the fact that they have a clearly different physiological behaviour, including symbiotic efficiency and colonization pattern of the host root. In addition, these fungi have a clearly different ability to improve plant water uptake under drought stress, as has been evidenced though a number of studies (Ruiz-Lozano and Azcón, 1995; Tobar *et al.*, 1994a, b; Marulanda *et al.*, 2003).

Growth conditions

Plants were grown in a controlled environmental chamber with 70–80% RH, day/night temperatures of 25/15 °C, and a photoperiod of 16 h at a Photosynthetic photon flux density (PPFD) of 460 μmol m^{-2} s^{-1} (Licor, Lincoln, NE, USA, model LI−188B).

Soil moisture was measured with a ML2 ThetaProbe (AT Delta-T Devices Ltd., Cambridge, UK) as previously described (Porcel and Ruiz-Lozano, 2004; Porcel *et al.*, 2004, 2005b). Four weeks after planting half of the plants were allowed to dry until soil water content reached 70% field capacity, while the other half were maintained at field capacity. Plants were maintained under such conditions for 10 days. The soil water content was daily measured with the ThetaProbe ML2 (at the end of the afternoon) and the amount of water lost was added to each pot in order to keep the soil water content at the desired level (Porcel and Ruiz-Lozano, 2004; Porcel *et al.*, 2004, 2005b). For the second experiment, half of the plants were maintained at field capacity during the entire experiment, while the other half were drought stressed as indicated above for 5 days (plants harvested 5 dai) or for 10 days for the rest of harvests.

Each week throughout the experiment, soybean plants received 10 ml of Hewitt́s nutrient solution lacking N and P (Hewitt, 1952). Three weeks after planting, plants received nutrient solution amended with N and/or P as follows (Goicoechea *et al.*, 1997): 0.18 mM K_2HPO_4 and 2 mM NH_4NO_3 (NI plants), 0.35 mM K_2HPO_4 (Br plants). Nutrient concentrations were chosen in an attempt to obtain well-watered plants of similar size and nutrient contents in all the microbial treatments.

Each week throughout the experiment, uninoculated control lettuce plants received 10 ml of Hewitt́s nutrient solution (Hewitt, 1952), modified to contain 4 mM N + 1 mM P. Mycorrhizal plants did not receive nutrient solution. The use of such fertilization level for nonmycorrhizal plants was meant to obtain well-watered control plants of similar size and nutrient contents to the AM plants tested in this assay.

Symbiotic development

The percentage of mycorrhizal root infection in soybean and lettuce plants was estimated by visual observation of fungal colonization after clearing washed roots in 10% KOH and staining with 0.05% trypan blue in lactic acid (v/v), according to Phillips and Hayman (1970). The extent of mycorrhizal colonization was calculated according to the gridline intersect method (Giovannetti and Mosse, 1980). Nodule number in soybean roots was determined using a dissecting microscope.

Relative water content

The relative water content (RWC) in plant shoots was determined at the harvest time as previously described by Ruiz-Lozano and Azcón (1997).

Leaf water potential

The leaf water potential (Ψ) was determined one day before harvest with a C−52 thermocouple psychrometer chamber and a HR−33T dew point microvoltmeter (Wescor Inc, Logan, UT, USA). Leaf discs were cut, placed inside the psychrometer chamber and allowed to reach temperature and water vapour equilibrium for 30 min before measurements were made by the dew point method.

RNA isolation and synthesis of first strand cDNA
Total RNA was isolated from soybean or lettuce roots by phenol/chloroform extraction (Kay *et al.*, 1987). DNase treatment of total RNA was performed according to Promega's recommendations. Total RNAs (2.5 μg) from soybean and from lettuce roots subjected to drought stress were reverse transcribed to first strand cDNA using AMV-RT enzyme (Finnzymes, Espoo, Finland) and oligo(dT)$_{15}$ primer (Promega, Madison, WI), in a final volume of 25 μl with the buffer and temperature recommended by the enzyme supplier.

Cloning the GmPIP *and* LsPIP *genes*
Several stretches of conserved amino acids were apparent from the compilations of sequences for aquaporins in plants and fungi. Two stretches were used to design degenerate oligonucleotide primers as described by Numberg *et al.* (1989): primer forward 5′-CA(CT) AT(CA) AAC CC(AG) GC(GA) GTG AC−3′ and primer reverse 5′-C CAT GAA (AC)AC (AC)GC AAA (TA)CC (AG) AT−3′. Using cDNA from either soybean or from lettuce roots subjected to drought stress as template, a cDNA fragment of about 335 bp was amplified with these primers and the polymerase chain reaction (PCR). PCR was carried out as described previously (Porcel *et al.*, 2004, 2005b). The amplified cDNA was purified following electrophoresis through a 1.2% agarose gel with the QIAEX II Gel Extraction Kit (Qiagen, Hilden, Germany) and cloned into pGEM plasmid (Promega). Recombinant plasmids were used to transform competent *E. coli* DH−5α cells. Positive clones screened by PCR were subcultured and plasmid DNA isolated using QIAprep® Spin Miniprep kit (Qiagen).

Sequencing the cloned cDNA and analyses
Sequencing was performed by the dideoxy-sequencing method (Sanger *et al.*, 1977) using fluorescent dye-linked universal M13 primers and a Perkin–Elmer ABI Prism model 373 DNA sequencer. Similarity searches were carried out using the BLAST software or the FASTA program, available on-line from the National Centre for Biotechnology Information (NCBI).

Northern blot analysis
Northern blot with *GmPIP1*, *GmPIP2*, *LsPIP1* or *LsPIP2* probes were carried out as previously described (Ruiz-Lozano *et al.*, 2002; Porcel *et al.*, 2004, 2005b). Hybridizations were carried out overnight at 65 °C under standard conditions (Sambrook *et al.*, 1989). After washing twice for 5 min at room temperature in 2× SSC and 0.1% SDS, and twice for 15 min at 65 °C with 0.5× SSC and 0.1% SDS, membranes were exposed overnight to Kodak X-RAY-OMAT at −70 °C. Before performing the northern blot, equal RNA loading and transfer to the nylon membranes were verified by methylene blue staining of the membranes (Herrin and Schmidt, 1988). The amount of rRNA in these membranes was quantified using Quantity One software (BioRad, Hemel Hempstead, UK). After the northern blot the signals on autoradiograms were analyzed and quantified using the same software. Transcript accumulation levels for each gene probe (in arbitrary units) were divided by the corresponding amount of rRNA in the membrane (also in arbitrary units). Each quantification of signals on autoradiograms and of rRNA in the membranes was repeated three times and the average value for each was used for normalization. Northern blot analyses were repeated two times with different set of plants.

Quantitative real-time RT-PCR
GmPIP2 and LsPIP2 gene expression was studied by real-time PCR by using iCycler (Bio-Rad, Hercules, California, USA). cDNAs were obtained from 2.5 μg of total DNase-treated RNA in a 20 μl reaction containing 500 ng random hexamer primer, 0.5 mM each dNTP, 10 mM DTT, 40 U of RNase inhibitor, 1× first strand buffer (Invitrogen, Carlsbad, California, USA) and 200 U of Superscript II Reverse Transcriptase (Invitrogen). The primer sets used to amplify *GmPIP2 and LsPIP2 genes* in the synthesized cDNAs are shown in Table 1. Each 25 μl reaction contained 1 μl of a dilution 1:10 of the cDNA, 200 nM dNTPs, 400 nM each primer, 3 mM $MgCl_2$, 2.5 μl of 1× SyBR Green (Molecular Probes, Eugene, Oregon, USA), and 0.5 U Platinum *Taq* DNA polymerase (Invitrogen) in 1× PCR buffer (20 mM Tris–HCl, pH 8.4, 50 mM KCl).

The PCR program consisted in a 4 min incubation at 95 °C to activate the hot-start recombinant *Taq* DNA polymerase, followed by 30 cycles of 45 s at 95 °C, 45 s at 60 °C, and 45 s at 72 °C, where the fluorescence signal was measured. The

Table 1. Primers used in this study.

Primer	
AQPGmFor	5′-TTGGCGAGGAAGTTGTCGTTGC-3′
AQPGmRev	5′-AGATCCAGTGTTCATCCCAACC-3′
18SgmFor	5′-CGATCAGATACCGTCCTAGTC-3′
18SGmRev	5′-CCAACTAAGAACGGCCATGCACC-3′
AQPLsFor	5′-CAAATGGTCCTTCTACAGAGC-3′
AQPLsRev	5′-CAAACACTGTGCAATCATGTATCC-3′
18SLsFor	5′-CAGGTCCAGACATAGTAAGA-3′
18SLsRev	5′-GACCATTCAATCGGTAGGAGC-3′

The annealing temperature used for all primer sets from soybean was 60 °C and 56 °C for primers used with lettuce.

specificity of the PCR amplification procedure was checked with a heat dissociation protocol (from 70–100 °C) after the final cycle of the PCR. The efficiency of the primer set was evaluated by performing real-time PCR on several dilutions of plasmid DNA. The results obtained on the different treatments were standardized to the 18S rRNA levels, which were amplified with the primers 18S shown in Table 1.

Real-time PCR experiments were carried out at least five times, with the threshold cycle (C_T) determined in triplicate. The relative levels of transcription were calculated by using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Negative controls without cDNA were used in all PCR reactions.

Western blot analysis

The microsome purification, SDS-PAGE gel, transferring proteins to nitrocellulose membrane, and blocking were all carried out as described by Aroca *et al*. (2005). The membranes were incubated in Tris-buffered-saline buffer (TBS) with 0.05% Tween 20 in presence of PIP1 or PIP2 antibodies from *Arabidopsis thaliana* (Daniels *et al*., 1994; Kammerloher *et al*., 1994). Each antibody was incubated 1:500 dilution overnight at 4 °C and the secondary antibody (Mouse anti-chicken IgG coupled to horseradish peroxidase; Sigma) 1:5000 1 h at room temperature. The signal was developed using a chemiluminescent substrate (West-Pico, Super Signal; Pierce, Rockford, IL). Quantification of inmunoblots was carried out as described previously (Aroca *et al*., 2005).

Nucleotide sequence accession numbers

The nucleotide sequences corresponding to *GmPIP1, GmPIP2, LsPIP1* and *LsPIP2* cDNAs have been deposited in the EMBL database under accession numbers AJ937960, AJ937961, AJ937962 and AJ937963, respectively.

Results

Symbiotic development in soybean and lettuce plants

No AM colonization or nodules were observed in non-inoculated soybean plants. In the first experiment with soybean the percentage of AM infection was near 65% (Br + Gm plants), with no significant differences between well-watered and drought stressed conditions. The number of nodules in *B. japonicum*-inoculated plants ranged from 30 to 50 (data not shown). In the second experiment with soybean (time-course experiment) mycorrhizal infection inside roots and nodule formation were visible 12 dai and both symbioses were progressing until the last harvest (35 dai). The AM colonization at 12 dai was 17% (ww) and 15% (ds) of mycorrhizal root length, at 20 dai it reached 30% (ww) and 25% (ds) and at 35 dai it was 55% (ww) and 47% (ds), while the number of nodules ranged from 20 to 30 in *B. japonicum*-inoculated plants (data not shown).

In lettuce (third experiment), the percentage of AM colonization was near 70% for *G. mosseae*-inoculated plants and near 84% for *G. intraradices* -inoculated plants. The AM infection resulted unaffected by drought stress (data not shown).

Relative water content

Soybean plants from the first experiment showed no significant differences in RWC when cultivated under well-watered conditions (Figure 1B). Drought stress decreased the RWC in all the treatments, but AM plants maintained a significantly higher RWC than both nonAM treatments.

Soybean plants from the time-course experiment also showed no significant differences in their relative water content when cultivated under well-watered conditions (Figure 2B). Drought decreased their RWC in all treatments but at 20 and 35 dai AM plants showed higher RWC than nonAM plants.

Lettuce plants showed a similar trend, with no significant differences in RWC under well-watered conditions and higher RWC of AM plants than

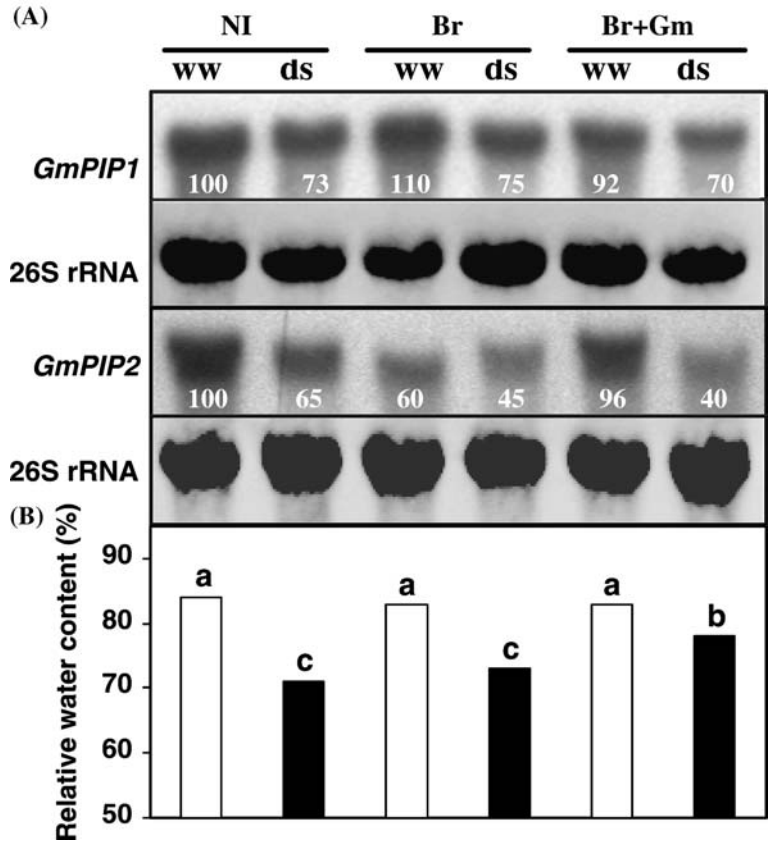

Figure 1. (A) Northern blot of total RNA (15 μg) from soybean roots using *GmPIP1* and *GmPIP2* gene probes. Treatments are designed as NI, noninoculated controls; Br, *Bradyrhizobium japonicum*; Br + Gm, *B. japonicum* plus *G. mosseae*. Plants were either well-watered (ww) or drought stressed (ds) for 10 days. The lower panels show the amount of 26S rRNA loaded for each treatment (methylene blue staining). Numbers close to each northern represent the relative gene expression (after normalization to rRNA) as a percentage of the value for well-watered noninoculated plants. (B) Relative water content of soybean plants grown under well-watered conditions (white column) or subjected to drought stress (black column).

nonAM plants when subjected to drought (Figure 3B).

Leaf water potential (Ψ)

The leaf water potential of soybean (first experiment) and lettuce plants (third experiment) was unaffected by the microbial treatment when cultivated under well-watered conditions (Table 2). In contrast, under drought stress conditions Ψ was higher (less negative) in AM plants than in nonAM plants.

Cloning GmPIP *and* LsPIP *genes*

The use of the degenerate primers for aquaporins allowed us to obtain several clones, which contained inserts of the expected size using cDNA from soybean and from lettuce roots. The sequencing of four of the clones obtained from soybean cDNA showed that all of them contained a cDNA insert putatively encoding for aquaporins. These four clones corresponded to two different sequences named *GmPIP1* and *GmPIP2*. The first clone (*GmPIP1*) contained a cDNA fragment of 335 bp encoding for a putative protein of 88% identity with PIP1 from *Medicago truncatula* (accession Q946J9, $e = 1\ e^{-109}$). The second clone (*GmPIP2*) contained a cDNA fragment of 331 bp encoding for a putative protein of 83% identity with a PIP2 from *Zea mays* (accession Q9ATM4, $e = 7\ e^{-46}$). The homology between *GmPIP1 and GmPIP2* nucleotide sequences was 68%.

In the case of lettuce, another four clones were sequenced, that corresponded to two different

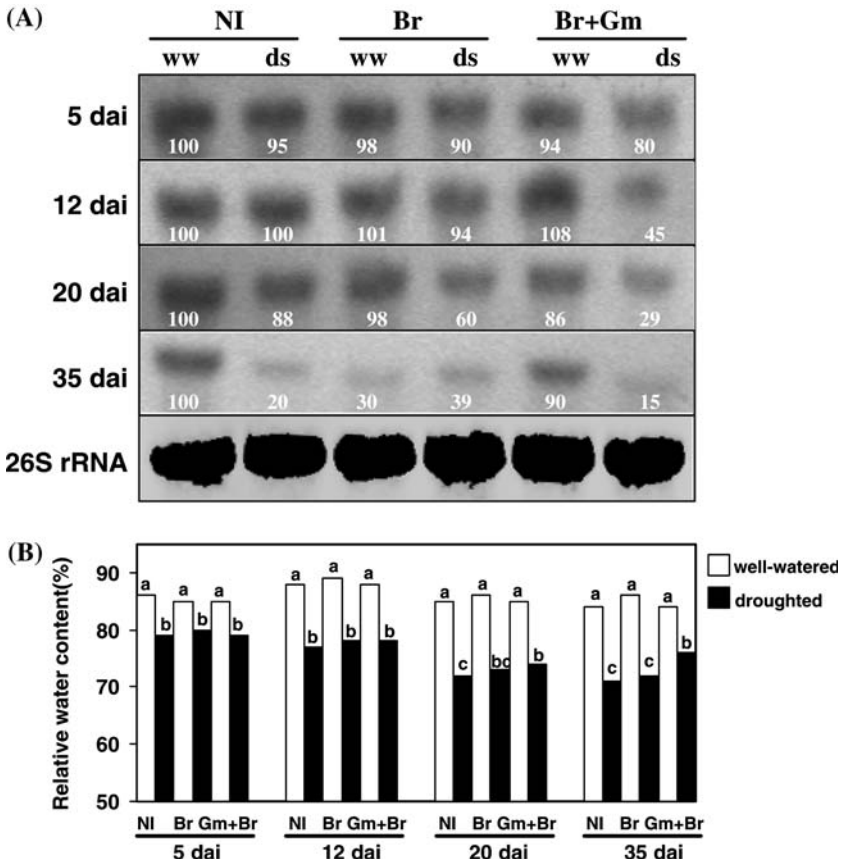

Figure 2. (A) Northern blot of total RNA (15 μg) from soybean roots using *GmPIP2* gene probe. Plants were harvested 5, 12, 20 or 35 days after inoculation (dai). Treatments are designed as NI, noninoculated controls; Br, *Bradyrhizobium japonicum*; Br + Gm, *B. japonicum* plus *G. mosseae*. Plants were either well-watered (ww) or drought stressed (ds) for 10 days. The lower panel shows a representative example of the amount of 26S rRNA loaded for each treatment (methylene blue staining). Numbers close to each northern represent the relative gene expression (after normalization to rRNA) as a percentage of the value for well-watered noninoculated plants. (B) Relative water content of soybean plants grown under well-watered conditions (white column) or subjected to drought stress (black column).

sequences putatively encoding for aquaporins. Such clones were named *LsPIP1* and *LsPIP2*. The first clone (*LsPIP1*) contained a cDNA fragment of 334 bp. The putative protein encoded by this cDNA gave 92% identity with a PIP1 from *Vitis berlandieri* (accession Q9M7B2, $e = 4\ e^{-52}$). The second clone (*LsPIP2*) contained a cDNA fragment of 331 bp. The putative protein encoded by this cDNA gave 93% identity with a PIP2 from *Vitis vinifera* (accession Q5PXH0, $e = 2\ e^{-54}$). The homology between *LsPIP1* and *LsPIP2* nucleotide sequences was 75%.

Northern blot analysis with soybean RNAs

Both cDNA inserts from soybean (*GmPIP1* and *GmPIP2*) were used as probes in northern blot analyses with soybean root RNA from non mycorrhizal and mycorrhizal treatments (see experimental design). In the first experiment, the expression of both genes resulted in a down-regulation by drought stress (Figure 1A). *GmPIP1* showed the highest expression level in plants cultivated under well-watered conditions. The gene expression corresponding to non-inoculated control plants was set as 100% in arbitrary units after normalization according to the amount of ribosomal RNA loaded in the blots. The three treatments decreased in a similar way (by 25–30%) *GmPIP1* gene expression when cultivated under drought stress conditions. A similar trend was found for *GmPIP2* gene expression (Figure 1A), with the exception of nodulated control plants (Br) that exhibited a decreased gene expression also

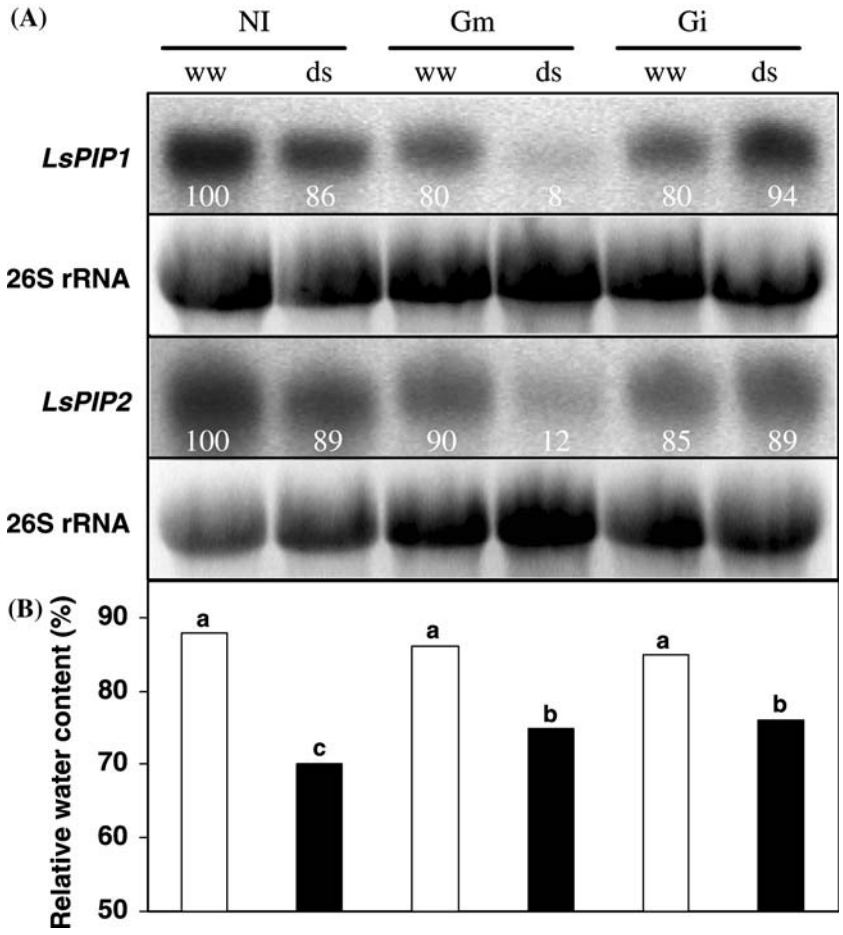

Figure 3. (A) Northern blot of total RNA (15 μg) from lettuce roots, using *LsPIP1* and *LsPIP2* gene probes. Treatments are designed as NI, noninoculated controls; Gm, *Glomus mosseae* and Gi, *Glomus intraradices*. Plants were either well-watered (ww) or drought stressed (ds) for 10 days. The lower panels show the amount of 26S rRNA loaded for each treatment (methylene blue staining). Numbers close to each northern represent the relative gene expression (after normalization to rRNA) as a percentage of the value for well-watered noninoculated plants. (B) Relative water content of soybean plants grown under well-watered conditions (white column) or subjected to drought stress (black column).

under well-watered conditions. Drought stress decreased *GmPIP2* transcript accumulation in a higher extent than for *GmPIP1*, ranging from 45% decrease in NI plants to 60% decrease in AM plants.

Quantitative real-time RT-PCR in soybean

As both genes showed a similar pattern of gene expression in AM vs. nonAM plants and also in drought stressed vs. well-watered plants, we designed specific primers only for one of the genes in order to follow its expression pattern also by quantitative real-time PCR. We selected *GmPIP2* since there are evidences that PIP2 are more active in water flow across plasma membranes than PIP1 (Chaumont *et al*., 2000; Fetter *et al*., 2004; Bots *et al*., 2005). The data on gene expression obtained for plants from the first experiment (Figure 4) corroborated the pattern of gene expression found with northern blot. In fact, non-inoculated plants showed the highest gene expression under well-watered conditions and an important decrease under drought stress. Nodulated nonAM plants (Br) showed again a reduced gene expression level even under well-watered conditions, while AM plants also showed higher *GmPIP2* gene expression under well-watered conditions and significant down-regulation under drought stress conditions.

Table 2. Leaf water potential (Ψ, MPa) in soybean and lettuce plants. Treatments for soybean are designed as NI, noninoculated controls; Br, *Bradyrhizobium japonicum*; Br + Gm, *B. japonicum* plus *G. mosseae* and for lettuce as NI, noninoculated controls; Gm, *Glomus mosseae* and Gi, *Glomus intraradices*. Plants were either well-watered or drought stressed for 10 days.

	Ψ	
Treatment	Well-watered	Droughted
Soybean		
NI	−1.4c	−2.4a
Br	−1.6c	−2.3a
Gm + Br	−1.5c	−1.9b
Lettuce		
NI	−1.1c	−2.0a
Gm	−1.1c	−1.6b
Gi	−1.3c	−1.7b

Within each plant species, means followed by the same latter are not significantly different ($P < 0.05$) as determined by Duncan's multiple range test ($n = 4$).

Time-course analysis of GmPIP2

Based on the results obtained by northern blot and by quantitative PCR which showed a down-regulation of PIP genes under drought stress conditions, but no important differences between nonAM and AM plants, we planned a time-course experiment in order to study the expression level of *GmPIP2* gene at different time intervals of the symbioses in soybean roots. Plants harvested at 5 dai showed little variation in *GmPIP2* transcript accumulation under any condition (Figure 2A). Only the AM plants exhibited a higher down-regulation (by 20%) of *GmPIP2* gene expression under drought stress conditions than the rest of treatments. At 12 dai, the two nonAM treatments continued showing little variation in gene expression under all conditions. AM plants showed again a significant down-regulation (by 55%) of *GmPIP2* gene expression under drought stress conditions than the rest of treatments. At 20 dai, all the treatments showed down-regulation of *GmPIP2* gene under drought, but this down-regulation was lower for the two nonAM plants (the decrease ranged from 12% for NI plants to 40% for Br plants) than for the AM plants (the decrease was 71%). Finally, at 35 dai the patter of *GmPIP2* gene expression was similar to that obtained in the first northern blot (Figure 1A) or by means of quantitative PCR (Figure 4). In fact, in all these cases plants were of the same age and at the same developmental stage. The most remarkable result was that under drought stress conditions non-inoculated plants down-regulated *GmPIP2* gene expression to a similar extent as AM plants. Nodulated control plants (Br) showed reduced gene expression even under well-watered conditions.

Northern blot analysis with lettuce RNA

In order to test the behaviour of AQP genes in a non-legume plant and to avoid the interference of

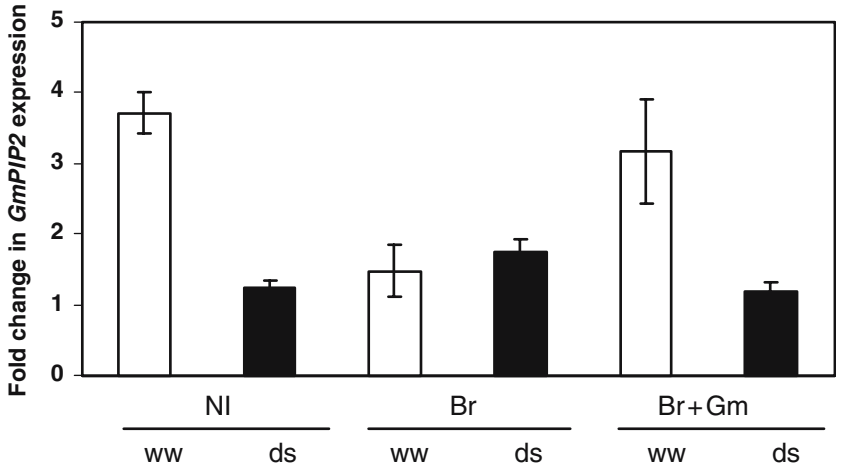

Figure 4. Fold change in *GmPIP2* gene expression determined by quantitative real-time PCR using gene-specific primers for *GmPIP2* and 18S rRNA. The fold change in *GmPIP2* gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method. Treatments are designed as NI, non-inoculated controls; Br, *Bradyrhizobium japonicum*; Br + Gm, *B. japonicum* plus *G. mosseae*. Plants were either well-watered (ww) or drought stressed (ds) for 10 days. Data represent the mean of five replicates. Bars represent SE.

the AM symbiosis with the *Bradyrhizobium* symbiosis, the cDNAs cloned from lettuce (*LsPIP1* and *LsPIP2*) were used for northern blot analysis with RNA from non-inoculated or AM lettuce roots cultivated either under well-watered or drought stressed conditions. Two AM fungi (*G. mosseae* and *G. intraradices*) with clearly different ability to improve plant water uptake under drought stress (Marulanda *et al.*, 2003) were used. Results showed that both lettuce *PIP* genes behave similarly (Figure 3A). The highest gene expression was found in non-inoculated plants under well-watered conditions that was set as 100% in arbitrary units. Gene expression in these non-inoculated plants was slightly decreased by drought stress (14% decrease for *LsPIP1* and 11% decrease for *LsPIP2*). Both AM treatments showed a reduced transcript accumulation for both PIP genes under well-watered conditions, as compared to non-inoculated plants. While *G. mosseae*-inoculated plants decreased drastically (by near 90%) the level of gene expression under drought stress conditions, *G. intraradices*-inoculated plants did not show such a decrease.

Quantitative real-time RT-PCR in lettuce

We designed specific primers only for one of the PIP genes in order to follow its expression pattern also by quantitative real-time PCR. As in the case of soybean, we selected *LsPIP2*.

Data from quantitative PCR experiments showed a similar pattern of *LsPIP2* gene expression to northern blot analysis, with the highest transcript accumulation in non-inoculated plants under well-watered conditions and down-regulation in these plants under drought stress conditions (Figure 5). Both AM treatments showed a lower transcript accumulation than nonAM plants and, again, *G. mosseae*-colonized plants showed a drastic down-regulation of *LsPIP2* gene expression under drought stress conditions. *G. intraradices*-colonized plants did not show such a down-regulation of gene expression under drought stress as compared to well-watered conditions.

Western blot

We did western blot analyses on soybean and lettuce roots using PIP1 and PIP2 antibodies from *Arabidopsis thaliana*. Unfortunately, none of the antibodies recognized the corresponding protein in soybean. In lettuce, only PIP1 antibody recognized the corresponding protein, while no results were obtained for PIP2 antibody. The gene expression study has focussed more on PIP2 but, unfortunately, we only can correlate the gene expression study with data on protein levels for *LsPIP1* gene. Results in lettuce are shown in Figure 6 and paralleled results from northern blot shown in Figure 3B, demonstrating the important decrease of PIP1 protein accumulation in lettuce plants

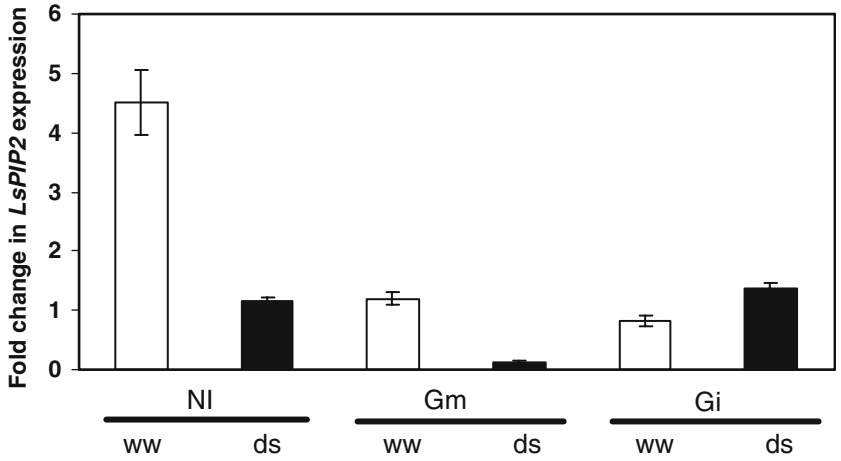

Figure 5. Fold change in *LsPIP2* gene expression determined by quantitative real-time PCR in non-inoculated control plants (NI) or in plants inoculated with *G. mosseae* (Gm) or with *G. intraradices* (Gi). Plants were either well-watered (ww) or drought stressed (ds). Data represent the mean of five replicates. Bars represent SE.

colonized by *G. mosseae* under drought stress conditions.

Discussion

Tolerance to drought stress in plants is a complex phenomenon and involves many changes at both biochemical and physiological levels (Ingram and Bartels, 1996). Mechanisms of osmotic adjustment and modulation of tissue hydraulic conductivity are required to maintain tissue water potential (Bohnert *et al*., 1995). Such mechanisms, which regulate water flux, are likely to be mediated, in part, by aquaporins (Maurel, 1997).

The discovery of aquaporins has lead to the realization that water flow across membranes may be regulated not only by osmotic pressure differences, but also by modulating the abundance and/or the activity of aquaporins (Martre *et al*., 2002). Water that is lost from the leaves through transpiration is replenished by an apoplastic and transcellular water flow from cells that have a higher water potential. Transcellular flow requires the movement of water across the tonoplast and the plasma membrane and such transmembrane water movement is facilitated by aquaporins (Maurel, 1997; Schäffner, 1998; Luu and Maurel, 2005). It has been proposed that regulation of PIPs in the plasma membrane of root cells may play a key role in controlling radial water uptake, whereas TIPs may rather determine a general role of the vacuole in buffering osmotic fluctuations in the cytoplasm (Maurel *et al*., 2002).

PIP aquaporins have been shown to be regulated by drought at the transcript level (Mariaux *et al*., 1998). In this study, all the genes studied showed the highest sequence homology to PIP aquaporins. We aimed to investigate if the AM symbiosis alters the pattern of PIP gene expression as a mechanism to improve plant tolerance to drought stress. Previous studies have shown that the AM fungi can take up water from soil and transfer such water to the host plant. This has been proposed as a mechanism that can help the plant to cope with drought stress (Hardie, 1985; Faber *et al*., 1991; Ruiz-Lozano and Azcón, 1995; Marulanda *et al*., 2003; Porcel *et al*., 2003). If AM fungi are transferring water to the root of the host plants, it is expected that the plant must increase its permeability for water and that aquaporin genes should be up-regulated in order to allow a higher rate of transcellular water flow (Javot and Maurel, 2002). In fact, aquaporins are enriched in zones of fast cell division and expansion, or in areas where water flow or solute flux density would be expected to be high. This included biotrophic interfaces between plants and symbiotic bacteria or fungi, as is the case of the AM symbiosis (Tyerman *et al*., 2002).

In contrast, to the above hypothesis, our results show that the genes studied here are down-regulated both in soybean and lettuce under drought stress and that such down-regulation is even more severe in AM plants than in nonAM plants. A similar result has been obtained very recently by Ouziad *et al*. (2005) regarding the expression of PIP and TIP genes in roots of AM tomato plants subjected to salt stress. The down regulation of the aquaporin genes is not as evident in soybean plants from the first experiment, since the down-regulation of both *GmPIP* genes in AM and in noninoculated plants is of a similar magnitude (Figures 1A and 4). However, when the expression of *GmPIP2* is analyzed in a time-course, it is clearly visible that AM plants already down-regulated that gene significantly at 5 and 12 dai, while both nonAM control plants still maintained *GmPIP2* gene expression almost unaltered. At 20 dai, the more intense down-regulation of that gene in AM plants than in both nonAM plants was still clearly visible. Finally, at 35 days

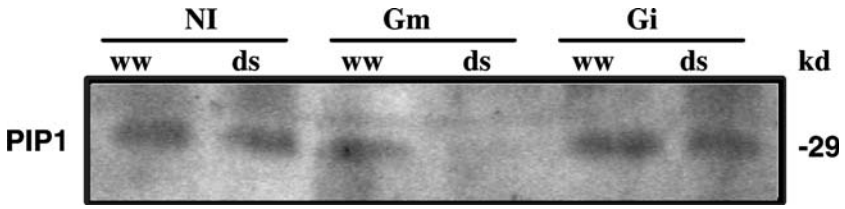

Figure 6. Western blot analysis of PIP1 protein accumulation in lettuce roots. Treatments are designed as NI, noninoculated controls; Gm, *Glomus mosseae* and Gi, *Glomus intraradices*. Plants were either well-watered (ww) or drought stressed (ds) for 10 days.

all treatments had the same level of *GmPIP2* gene expression. This effect of the AM symbiosis anticipating the down-regulation of *GmPIP2* gene may have a physiological importance to help AM plants to cope with drought stress. In fact, according to Aharon *et al.* (2003), the over-expression of a PIP aquaporin in transgenic tobacco improves plant vigor under favorable growth conditions, but the over-expression of such PIP gene has no beneficial effect under salt stress, and has even negative effect during drought stress, causing fast wilting. Hence, the decreased expression of plasma membrane aquaporin genes during drought stress in AM plants can be a regulatory mechanism to limit the water lost from the cells (Barrieu *et al.*, 1999). In support of this hypothesis, data on leaf Ψ and RWC show that AM plants (soybean and lettuce) had a less negative Ψ and also higher water content than nonAM plants.

The up- or down-regulation by drought stress of mRNAs encoding aquaporins homologues has been described in the roots of many plant species (Javot and Maurel, 2002). There are currently two opposite descriptions of the role of aquaporins in response to dehydration stress (Smart *et al.*, 2001). The first is based on evidence that expression of some aquaporins is induced under dehydration stress (Fray *et al.*, 1994; Yamada *et al.*, 1997; Barrieu *et al.*, 1999; Jang *et al.*, 2004), which is predicted to result in greater membrane water permeability and facilitated water transport. The second is based on the fact that aquaporin activity is down-regulated under dehydration stress, which should result in decreased membrane water permeability and may allow cellular water conservation (Yamada *et al.*, 1995; Johansson *et al.*, 1998; Smart *et al.*, 2001) during periods of dehydration stress.

Data obtained with lettuce plants also colonized by *G. mosseae* point in the same direction, namely that under drought stress conditions there is a higher down-regulation of the PIP genes studied (and also at the protein level, as revealed by western blot) in AM plants than in nonAM plants. In contrast to *G. mosseae*, plants colonized by *G. intraradices* do not exhibit such down-regulation of PIP gene expression or protein accumulation. The expression of PIP genes under drought stress in these plants is similar to control nonAM plants. However, functional diversity among different AM fungi has been widely observed in several aspects of the symbiosis. Burleigh *et al.* (2002) showed that the functional diversity between AM fungal species occurs not only at the level of mycorrhiza formation, plant nutrient uptake or plant growth, but also at the molecular level. These authors studied seven AM fungal species and found that the seven species widely varied in their influence on the root expression of *MtPT2* and *Mt4* genes from *Medicago truncatula* and also of *LePT1* and *TPSI1* genes from *Lycopersicon esculentum* involved in plant P nutrition. In the same way, previous studies from our research group showed a differential regulation by both AM fungi of the expression of genes encoding late embryogenesis abundant proteins (Porcel *et al.*, 2005b).

The exact reason for the different influence of *G. mosseae* and *G. intraradices* on lettuce *PIP* gene expression is not known. However, in a previous study, also with lettuce, we evaluated the ability of six AM fungal species, including *G. mosseae* and *G. intraradices*, to enhance the amount of soil water uptake by these plants (Marulanda *et al.*, 2003). The study demonstrated that there were substantial differences among the six AM fungi used. One of the most efficient fungi stimulating water uptake by plants was *G. intraradices*, while *G. mosseae* showed a reduced ability to improve plant water uptake. This may suggest that the strategy of both fungi to protect the host plant against water deficit is different. *G. intraradices* seems to have an important capacity to enhance the rate of water uptake by lettuce roots. This means that the water movement in these roots must be enhanced and thus, the root water permeability must also increase, maybe by maintaining high levels of PIP aquaporin gene expression as we observe in this study. Contrarily, *G. mosseae* seems to direct its strategy for plant protection against water deficit toward the conservation of the water existing in the plant and by that reason down-regulates the expression of PIP genes. Such down-regulation of PIP genes has been interpreted as a mechanism to decrease membrane water permeability and to allow cellular water conservation (Yamada *et al.*, 1995; Johansson *et al.*, 1998; Smart *et al.*, 2001). In any case, both strategies seems to protect the host plant in a similar way since lettuce plants had similar RWC and leaf Ψ regardless of the fungus colonizing their roots.

A curious result obtained in this study concerns the reduced *GmPIP2* gene expression in Br plants under well-watered conditions. Apart from the studies describing the soybean Nodulin−26 aquaporin (Dean *et al.*, 1999; Niemietz and Tyerman, 2000), we do not know of any other study describing an effect of *Rhizobia* on the expression of aquaporins or its meaning for the biological nitrogen fixation. In any case, it is likely that this symbiosis can affect the expression of aquaporin genes as consequence of the changes that the root cells must undergo to accommodate the nodules. In addition, it must be considered that aquaporins not only are water channels, but they also allow passage to small neutral molecules such as glycerol or urea, or small gases such as ammonia or CO_2 (Luu and Maurel, 2005). Hence, it cannot be discarded that the rhizobial symbiosis can be also regulating the *PIP* gene expression under well-watered conditions.

In conclusion, results from this study suggest that AM plants respond to drought stress by down-regulating the expression of the two *PIP* genes studied and anticipating its down-regulation as compared to nonAM plants, rather than by maintaining high levels of these PIP genes expression. This down-regulation of *PIP* genes is likely to be a mechanism to decrease membrane water permeability and to allow cellular water conservation. It must be considered, however, that as *PIP* are members of a multi-gene family, other *PIP* isoforms in soybean and lettuce plants may be regulated differently.

Acknowledgements

This work and R. Porcel were financed by CICYT-FEDER (Project AGL2002–03952). The authors thank Dr A.R. Schäffner (Ludwig-Maximilians Universität) and Dr M.J. Chrispeels (University of California, San Diego) for the generous gift of PIP1 and PIP2 antibodies, respectively.

References

Aharon, R., Shahak, Y., Wininger, S., Bendov, R., Kapulnik, Y. and Galili, G. 2003. Overexpression of a plasma membrane aquaporins in transgenic tobacco improves plant vigour under favourable growth conditions but not under drought or salt stress. Plant Cell 15: 439–447.

Aroca, R., Amodeo, G., Fernández-Illescas, S., Herman, E.M., Chaumont, F. and Chrispeels, M.J. 2005. The role of aquaporins and membrane damage in chilling and hydrogen peroxide induced changes in the hydraulic conductance of maize roots. Plant Physiol. 137: 341–353.

Augé, R.M. 2001. Water relations, drought and vesicular-arbuscular mycorrhizal symbiosis. Mycorrhiza 11: 3–42.

Augé, R.M. 2004. Arbuscular mycorrhizae and soil/plant water relations. Can. J. Soil Sci. 84: 373–381.

Barrieu, F., Marty-Mazars, D., Thomas, D., Chaumont, F., Charbonnier, M. and Marty, F. 1999. Desiccation and osmotic stress increase the abundance of mRNA of the tonoplast aquaporin BobTIP26–1 in cauliflower cells. Planta 209: 77–86.

Bohnert, H.J., Nelson, D.E. and Jensen, R.G. 1995. Adaptations to environmental stresses. Plant Cell 7: 1099–1111.

Bonfante, P. and Perotto, S. 1995. Strategies of arbuscular mycorrhizal fungi when infecting host plants. New Phytol. 130: 3–21.

Bots, M., Feron, R., Uehlein, N., Weterings, K., Kaldenhoff, R. and Mariani, T. 2005. PIP1 and PIP2 aquaporins are differentially expressed during tobacco anther and stigma development. J. Exp. Bot. 56: 113–121.

Burleigh, S.H., Cavagnaro, T. and Jakobsen, I. 2002. Functional diversity of arbuscular mycorrhizas extends to the expression of plant genes involved in P nutrition. J. Exp. Bot. 53: 1593–1601.

Chaumont, F., Barrieu, F., Jung, R. and Chrispeels, M.J. 2000. Plasma membrane intrinsic proteins from maize cluster in two sequence subgroups with differential aquaporin activity. Plant Physiol. 122: 1025–1034.

Chrispeels, M.J. and Agre, P. 1994. Aquaporins: water channel proteins of plant and animal cells. Trends Biochem. Sci. 19: 421–425.

Daniels, M.J., Mirkov, T.E. and Chrispeels, M.J. 1994. The plasma membrane of *Arabidopsis thaliana* contains a mercury-insensitive aquaporin that is a homolog of the tonoplast water channel protein TIP. Plant Physiol. 106: 1325–1333.

Dean, R.M., Rivers, R.L., Zeidel, M.L. and Roberts, D.M. 1999. Purification and functional reconstitution of soybean Nodulin 26. An aquaporin with water and glycerol transport properties. Biochemistry 38: 347–353.

Duncan, D.B. 1955. Multiple range and multiple *F*-tests. Biometrics 11: 1–42.

Faber, B.A., Zasoski, R.J. and Munns, D.N. 1991. A method for measuring hyphal nutrient and water uptake in mycorrhizal plants. Can. J. Bot. 69: 87–94.

Fetter, K., Van Wilder, V., Moshelion, M. and Chaumont, F. 2004. Interactions between plasma membrane aquaporins modulate their water channel activity. Plant Cell 16: 215–228.

Fray, R.G., Wallace, A., Grierson, D. and Lycett, G.W. 1994. Nucleotide sequence and expression of a ripening and water stress-related cDNA from tomato with homology to the MIP class of membrane channel proteins. Plant Mol. Biol. 24: 539–543.

Gianinazzi-Pearson, V. 1996. Plant cell response to arbuscular mycorrhizal fungi: getting to the roots of the symbiosis. Plant Cell 8: 1871–1883.

Giovannetti, M. and Mosse, B. 1980. An evaluation of techniques for measuring vesicular-arbuscular infection in roots. New Phytol. 84: 489–500.

Goicoechea, N., Antolin, M.C. and Sánchez-Díaz, M. 1997. Gas exchange is related to the hormone balance in

mycorrhizal or nitrogen-fixing alfalfa subjected to drought. Physiol. Plant. 100: 989–997.
Grote, K., Trzebiatovski, P. and Kaldenhoff, R. 1998. RNA levels of plasma membrane aquaporins in *Arabidopsis thaliana*. Protoplasma 204: 139–144.
Hardie, K. 1985. The effect of removal of extraradical hyphae on water uptake by vesicular-arbuscular mycorrhizal plants. New Phytol. 101: 677–684.
Herrin, D.L. and Schmidt, G.W. 1988. Rapid, reversible staining of northern blot prior to hybridization. Biotechniques 6: 196.
Hewitt, E.J. 1952. Sand and water culture methods used in the study of plant nutrition. Technical Communication 22, Farnham Royal, Commonwealth Agricultural Bureaux, Bucks.
Ingram, J. and Bartels, D. 1996. The molecular basis of dehydration tolerance in plants. Annu. Rev. Plant Physiol. Plant Mol. Biol. 47: 377–403.
Jang, J.Y., Kim, D.G., Kim, Y.O., Kim, J.S. and Kang, H. 2004. An expression analysis of a gene family encoding plasma membrane aquaporins in response to abiotic stresses in *Arabidopsis thaliana*. Plant Mol. Biol. 54: 713–725.
Javot, H., Lauvergeat, V., Santoni, V., Martin-Laurent, F., Guclu, J., Vinh, J., Heyes, J., Franck, K.I., Schaffner, A.R., Bouchez, D. and Maurel, C. 2003. Role of a single aquaporin isoform in root water uptake. Plant Cell 15: 509–522.
Javot, H. and Maurel, C. 2002. The role of aquaporins in root water uptake. Ann. Bot. 90: 301–313.
Johanson, I., Karlsson, M., Shukla, V.K., Chrispeels, M.J., Larsson, C. and Kjellbom, P. 1998. Water transport activity of the plasma membrane aquaporin PM28A is regulated by phosphorylation. Plant Cell 10: 451–459.
Johanson, U., Karlsson, M., Johanson, I., Gustavsson, S., Sjövall, S., Fraysse, L., Weigh, A.R. and Kjellbom, P. 2001. The complete set of genes encoding major intrinsic proteins in *Arabidopsis* provides a framework for a new nomenclature for major intrinsic proteins in plants. Plant Physiol. 126: 1358–1369.
Johnson, K.D., Höftem, H. and Chrispeels, M.J. 1990. An intrinsic tonoplast protein of proteins storage vacuoles in seeds is structurally related to a bacterial solute transporter (GlpF). Plant Cell 2: 525–532.
Kammerloher, W., Fischer, U., Pienchottka, G.P. and Schäffner, A.R. 1994. Water channels in the plant plasma membrane cloned by immunoselection from a mammalian expression system. Plant J. 6: 187–199.
Kay, R., Chau, A. and Daly, M. 1987. Duplication of CaMV 35S promoter sequences creates a strong enhancer for plants genes. Science 236: 1299–1302.
Kjelbom, P., Larsson, C., Johansson, I., Karlsson, M. and Johanson, U. 1999. Aquaporins and water homeostasis in plants. Trends Plant Sci. 4: 308–314.
Krajinski, F., Biela, A., Schubert, D., Gianinazzi-Pearson, V., Kaldenhoff, R. and Franken, P. 2000. Arbuscular mycorrhiza development regulates the mRNA abundance of *Mtaqp1* encoding a mercury-insensitive aquaporin of *Medicago truncatula*. Planta 211: 85–90.
Kramer, P.J. and Boyer, J.S. 1997. Water Relations of Plants and Soils. Academic Press, San Diego.
Lian, H.L., Yu, X., Ye, Q., Ding, X.S., Kitagawa, Y., Swak, S.S., Su, W.A. and Tang, Z.C. 2004. The role of aquaporin RWC3 in drought avoidance in rice. Plant Cell Physiol. 15: 481–489.
Livak, K.J. and Schmittgen, T.D. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta Ct}$ method. Methods 25: 402–408.
Luu, D.T. and Maurel, C. 2005. Aquaporins in a challenging environment: molecular gears for adjusting plant water status. Plant Cell Environ. 28: 85–96.
Mariaux, J.B., Bockel, C., Salamini, F. and Bartels, D. 1998. Desiccation- and abscisic acid-responsive genes encoding major intrinsic proteins (MIPs) from the resurrection plant *Craterostigma plantagineum*. Plant Mol. Biol. 38: 1089–1099.
Marjanovic, Z., Uehlein, N., Kaldenhoff, R., Zwiazek, J.J., Weiß, M., Hampp, R. and Nehls, U. 2005. Aquaporins in poplar: what a difference a simbiont makes! Planta (DOI: 10.1007/s00425–005–1539-z).
Martre, P., Morillon, R., Barrieu, F., North, G.B., Nobel, P.S. and Chrispeels, M.J. 2002. Plasma membrane aquaporins play a significant role during recovery from water deficit. Plant Physiol. 130: 2101–2110.
Marulanda, A., Azcón, R. and Ruiz-Lozano, J.M. 2003. Contribution of six arbuscular mycorrhizal fungal isolates to water uptake by *Lactuca sativa* L. plants under drought stress. Physiol. Plant. 119: 526–533.
Maurel, C. 1997. Aquaporins and water permeability of plant membranes. Annu. Rev. Plant Physiol. Plant Mol. Biol. 48: 399–429.
Maurel, C., Javot, H., Lauvergeat, V., Gerbeau, P., Tournaire, C., Santoni, V. and Heyes, J. 2002. Molecular physiology of aquaporins in plants. Inter. Revi. Cytol. 215: 105–148.
Niemietz, C.M. and Tyerman, S.D. 2000. Channel-mediated permeation of ammonia gas through the peribacteroid membrane of soybean nodules. FEBS Lett. 465: 110–114.
Numberg, J.H., Wright, D.K., Cole, G.E., Petrovskis, E.A., Post, L.E., Compton, T. and Gilbert, J.H. 1989. Identification of the thymidine kinase gene of feline herpesvirus: use of degenerate oligonucleotides in the polymerase chain reaction to isolate herpesvirus gene homologs. J. Virol. 63: 3240–3249.
Ouziad, F., Wilde, P., Schmelzer, E., Hildebrandt, U. and Bothe, H. 2005. Analysis of expression of aquaporins and Na^+/H^+ transporters in tomato colonized by arbuscular mycorrhizal fungi and affected by salt stress. Environ. Exp. Bot. (doi: 10.1016/j.envexpbot.2005.05.011).
Phillips, J.M. and Hayman, D.S. 1970. Improved procedure of clearing roots and staining parasitic and vesicular-arbuscular mycorrhizal fungi for rapid assessment of infection. Trans. Brit. Mycol. Soc. 55: 159–161.
Porcel, R., Azcón, R. and Ruiz-Lozano, J.M. 2004. Evaluation of the role of genes encoding for Δ^1-pyrroline−5-carboxylate synthetase (P5CS) during drought stress in arbuscular mycorrhizal *Glycine max* and *Lactuca sativa* plants. Physiol. Mol. Plant Pathol. 65: 211–221.
Porcel, R., Azcón, R. and Ruiz-Lozano, J.M. 2005b. Evaluation of the role of genes encoding for dehydrin proteins (LEA D−11) during drought stress in arbuscular mycorrhizal *Glycine max* and *Lactuca sativa* plants. J. Exp. Bot. 56: 1933–1942.
Porcel, R., Barea, J.M. and Ruiz-Lozano, J.M. 2003. Antioxidant activities in mycorrhizal soybean plants under drought stress and their possible relationship to the process of nodule senescence. New Phytol. 157: 135–143.
Porcel, R., Gómez, M., Kaldenhoff, R. and Ruiz-Lozano, J.M. 2005a. Impairment of *NtAQP1* gene expression in tobacco plants does not affect root colonization pattern by arbuscular mycorrhizal fungi but decreases their symbiotic efficiency under drought. Mycorrhiza 15: 417–423.
Porcel, R. and Ruiz-Lozano, J.M. 2004. Arbuscular mycorrhizal influence on leaf water potential, solute accumulation

and oxidative stress in soybean plants subjected to drought stress. J. Exp. Bot. 55: 1743–1750.

Roussel, H., Bruns, S., Gianinazzi-Pearson, V., Hahlbrock, K. and Franken, P. 1997. Induction of a membrane intrinsic protein-encoding mRNA in arbuscular mycorrhiza and elicitor-stimulated cell suspension cultures of parsley. Plant Sci. 126: 203–210.

Ruiz-Lozano, J.M. 2003. Arbuscular mycorrhizal symbiosis and alleviation of osmotic stress. New perspectives for molecular studies. Mycorrhiza 13: 309–317.

Ruiz-Lozano, J.M. and Azcón, R. 1995. Hyphal contribution to water uptake in mycorrhizal plants as affected by the fungal species and water status. Physiol. Plant. 95: 472–478.

Ruiz-Lozano, J.M. and Azcón, R. 1997. Effect of calcium application on the tolerance of mycorrhizal lettuce plants to polyethylene glycol-induced water stress. Symbiosis 23: 9–22.

Ruiz-Lozano, J.M., Collados, C., Porcel, R., Azcón, R. and Barea, J.M. 2002. Identification of a cDNA from the arbuscular mycorrhizal fungus *Glomus intraradices* which is expressed during symbiosis and up-regulated by N fertilization. Mol. Plant-Microbe Interact. 15: 360–367.

Sambrook, J., Fritsch, E.F. and Maniatis, T.A. 1989. Molecular Cloning: A Laboratory Manual., 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sanger, F., Nicklen, S. and Coulsen, A.R. 1977. DNA sequencing with chain terminating inhibitors. Proc. Natl. Acad. Sci. USA 74: 5463–5467.

Schäffner, A.R. 1998. Aquaporin function, structure and expression: are there more surprises to surface in water relations?. Planta 204: 131–139.

Sheng, G., Sagi, M., Weining, S., Krugman, T., Fahima Korol, T. A.B. and Nevo, E. 2004. Wild barley *eibi1* mutation identifies a gene essential for leaf water conservation. Planta 219: 684–693.

Siefritz, F., Tyree, M.T., Lovisolo, C., Schubert, A. and Kaldenhoff, R. 2002. PIP1 plasma membrane aquaporins in tobacco: from cellular effects to function in plants. Plant Cell 14: 869–876.

Smart, L.B., Moskal, W.A., Cameron, K.D. and Bennett, A.B. 2001. Mip genes are down-regulated under drought stress in *Nicotiana glauca*. Plant Cell Physiol. 42: 686–693.

Smith, S.E. and Read, D.J. 1997. Mycorrhizal Symbiosis. Academic Press, San Diego, CA.

Tobar, R.M., Azcón, R. and Barea, J.M. 1994a. The improvement of plant N acquisition from an ammonium-treated, drought-stressed soil by the fungal symbiont in arbuscular mycorrhizae. Mycorrhiza 4: 105–108.

Tobar, R.M., Azcón, R. and Barea, J.M. 1994b. Improved nitrogen uptake and transport from ^{15}N-labelled nitrate by external hyphae of arbuscular mycorrhiza under water-stressed conditions. New Phytol. 126: 119–122.

Tyerman, S.D., Niemietz, C.M. and Bramley, H. 2002. Plant aquaporins: multifunctional water and solute channels with expanding roles. Plant Cell Environ. 25: 173–194.

Yamada, S., Katsuhara, M., Kelly, W.B., Michalowski, C.B. and Bonhert, H.J. 1995. A family of transcripts encoding water channel proteins: tissue-specific expression in common ice plant. Plant Cell 7: 1129–1142.

Yamada, S., Komori, T., Myers, P.N., Kuwata, S., Kubo, T. and Imaschi, H. 1997. Expression of plasma membrane water channel genes under water stress in *Nicotiana excelsior*. Plant Cell Physiol. 38: 1226–1231.