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Exogenous ABA accentuates the differences in root hydraulic properties between mycorrhizal and non mycorrhizal maize plants through regulation of PIP aquaporins

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Abstract The arbuscular mycorrhizal (AM) symbiosis has been shown to modulate the same physiological processes as the phytohormone abscisic acid (ABA) and to improve plant tolerance to water deficit. The aim of the present research was to evaluate the combined influence of AM symbiosis and exogenous ABA application on plant root hydraulic properties and on plasma-membrane intrinsic proteins (PIP) aquaporin gene expression and protein accumulation after both a drought and a recovery period. Results obtained showed that the application of exogenous ABA enhanced osmotic root hydraulic conductivity (*L*) in all plants, regardless of water conditions, and that AM plants showed lower *L* values than nonAM plants, a difference that was especially accentuated when plants were supplied with exogenous ABA. This effect was clearly correlated with the accumulation pattern of the different PIPs analyzed, since most showed reduced expression and protein levels in AM plants fed with ABA as compared to their nonAM counterparts. The possible involvement of plant PIP aquaporins in the differential regulation of *L* by ABA in AM and nonAM plants is further discussed.

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Introduction

Plant water relationships are disturbed by drought as the lowered water potential in the soil hampers water uptake or even favors water loss from plant roots (Jones 2007). Plant adaptation to drought is regulated through multiple physiological mechanisms at the cellular, tissue, and whole-plant levels, which are controlled by changes in gene expression (Yamaguchi-Shinozaki and Shinozaki 2005; Ito et al. 2006). Traditional explanations for the drought-induced regulation of plant responses emphasize the importance of the decline of the shoot water status, which commonly accompanies severe soil dehydration. It is now accepted, however, that many of the plant's responses to soil dehydration can occur in the absence of changes in the shoot water status, via chemical signals such as abscisic acid (ABA; Wilkinson and Davies 2002). ABA regulates the plant water status by regulating important plant processes such as root hydraulic conductivity (*L*) (Quintero et al. 1999; Hose et al. 2000; Wan et al. 2004; Schraut et al. 2005; Aroca 2006) and transpiration rate (Netting 2000; Holbrook et al. 2002; Wilkinson and Davies 2002; Zhang et al. 2006), as well as, by inducing genes that encode enzymes and other proteins involved in cellular dehydration tolerance (Bray 2002; Zhang et al. 2006; Hirayama and Shinozaki 2007).

In nature, most plants can establish a symbiotic association with the arbuscular mycorrhizal (AM) fungi. When the AM symbiosis is established the fungus receives carbon molecules from the plant, while the plant receives nutrients (especially phosphorus) and water from the fungus

(Harrison 2005; Gosling et al. 2006). In this way, AM plants are usually more tolerant to several stresses, including drought, than nonAM plants (Augé 2001, 2004; Ruiz-Lozano 2003, Ruiz-Lozano et al. 2006). The beneficial effect of AM symbiosis under drought stress conditions has been studied largely at the physiological level including the regulation of transpiration rate or increasing root water absorption (Augé 2001, 2004). More recently, it has also been noted that, under drought stress conditions, AM and nonAM plants regulate differently the expression of several stress related genes in root tissues (Ruiz-Lozano et al. 2006). These changes have been postulated to be caused (among others) by different contents of ABA between AM and nonAM plants (Ruiz-Lozano et al. 2006). Among the genes regulated by the AM symbiosis during drought, aquaporin genes have been described (Porcel et al. 2006; Ruiz-Lozano et al. 2006; Aroca et al. 2007, 2008a, b). Aquaporins are membrane intrinsic proteins that facilitate water and small neutral solutes flow, always following an osmotic gradient (Chrispeels and Agre 1994; Maurel 1997). The activity of aquaporins has been related to cellular and whole-plant water movement (Javot and Maurel 2002; Javot et al. 2003, for recent reviews see Maurel et al. 2008; Zhao et al. 2008).

ABA has been shown to regulate the expression of aquaporin genes in a variety of plants (Mariaux et al. 1998; Seki et al. 2002, Suga et al. 2002; Jang et al. 2004; Zhu et al. 2005). Hose et al. (2000) have shown that ABA transiently increased the water conductivity of roots and the water permeability of cortex cells in maize. In any case, Jang et al. (2004) showed that the regulation of certain aquaporin genes by drought, salt or cold stresses in *Arabidopsis* occurred by an ABA-dependent signaling pathway. In contrast, other aquaporin genes were regulated by these stresses but did not show any response to ABA treatment, indicating an ABA-independent regulation pathway and demonstrating the complexity of the regulation of aquaporin expression.

ABA is, thus, considered the most important signal transduction pathway among all the plant responses to stresses (Wilkinson and Davies 2002; Zhang et al. 2006; Hirayama and Shinozaki 2007). At the same time, the AM symbiosis has been shown to modulate the same physiological processes as ABA and to improve plant tolerance to water deficit (For reviews see Augé 2001, 2004; Ruiz-Lozano 2003). Indeed, improved *L* and transpiration rate have been observed in AM plants during drought stress (Augé 1989; Ruiz-Lozano et al. 1995; Green et al. 1998; Sánchez-Blanco et al. 2004; Aroca et al. 2007, 2008b). Modulation of aquaporin genes by the AM symbiosis during salt, cold and drought stresses has also been described (Ouziad et al. 2006; Porcel et al. 2006; Aroca et al. 2007; Jahromi et al. 2008). In previous studies dealing with the combined effects of ABA and the AM symbiosis on plant drought tolerance our research group has evaluated the influence of AM symbiosis and exogenous ABA application on the responses of lettuce plants during drought (Aroca et al. 2008b). The results showed that the application of exogenous ABA had contrasting effects on the physiological responses of AM and nonAM plants and on the expression of several stress-related genes (*lea*, *p5cs* or *nced*). The results obtained in that study suggested that AM plants regulate better and faster their ABA levels than nonAM plants, allowing a more adequate balance between leaf transpiration and root water movement during drought and recovery. Another study was carried out with the ABA-deficient tomato mutant *sitiens* and its near isogenic wild-type parental line (Aroca et al. 2008a). Results showed different regulation patterns of four PIP aquaporin genes in wild-type and *sitiens* plants, suggesting that their expression is modulated by the plant ABA phenotype (endogenous plant ABA content). The mycorrhization of the two tomato plant lines with *G. intraradices* differently regulated the expression of several drought-induced genes in wild-type and in *sitiens* plants, which suggests that the effects of the AM symbiosis on plant responses to water deficit are also mediated by the plant ABA phenotype (endogenous plant ABA content).

In contrast to the above described studies, little information is available dealing with the combined effects of exogenous ABA on the root hydraulic properties and the regulation of aquaporins in roots by the AM symbiosis. This is why, the aim of the present research was to evaluate the combined influence of AM symbiosis and exogenous ABA application on plant root hydraulic properties and on PIP aquaporin gene expression and protein levels after both a drought and a recovery period. The starting hypothesis are: (1) the application of exogenous ABA to plants will change the root hydraulic properties and plant responses to drought and (2) the responses to exogenous ABA of AM and nonAM plants will differ. To achieve our objective, maize plants were inoculated or not with the AM fungus *Glomus intraradices* and subjected to a 4-day water deficit cycle followed by a 3-day recovery period. Plants were also treated or not exogenously with an ABA solution just before and during the water deficit treatment.

Materials and methods

Experimental design and statistical analysis

The experiment consisted of a factorial design with two inoculation treatments: (1) non-inoculated control plants (NI), (2) plants inoculated with the AM fungus *Glomus intraradices* (Schenck and Smith) BEG 121 (Gi). There

were sixty replicates of each microbial treatment, totalling 120 pots (one plant per pot), so that half of the plants were supplied with exogenous ABA while the other half remained ABA-free. In addition, one-third of the plants were cultivated under well watered conditions throughout the entire experiment and two-third of the plants were subjected to drought stress for 4 days. Then, one half of these drought-stressed plants were harvested and the remaining plants were allowed to recover from drought for three additional days under well watered conditions. The experiment had a total of 12 treatments with 10 replicates for each treatment. Five replicates were used for the measurement of transpiration rate and root hydraulic conductivity and the other five replicates were frozen in liquid nitrogen immediately after harvest and then used for the remaining determinations described in this section. For all the treatments, physiological measurements and the collection of plant samples were carried out 3 h after light turned on, in order to avoid diurnal fluctuations in plant processes.

Data were subjected to analysis of variance (ANOVA) with inoculation treatment, ABA treatment and water regime as sources of variation. Post Hoc comparisons with the Tukey's test were used to find out differences between groups. 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.

Two seeds of maize (*Zea mays* L. cv. Potro) were sown in pots containing 750 g of the same soil/sand mixture as described above and thinned to one seedling per pot after emergence.

Mycorrhizal inoculum was bulked in an open-pot culture of *Zea mays* L. and consisted of soil, spores, mycelia and infected root fragments. The AM species was *Glomus intraradices* (Schenck and Smith) isolate BEG 121. Ten grams of inoculum with about 60 infective propagules per gram (according to the most probable number test), were added to appropriate pots at sowing time.

Uninoculated control plants received the same amount of autoclaved 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.

Growth conditions

The experiment was carried out under greenhouse conditions with temperatures ranging from 19 to 25°C, 16/8 light/dark period, a relative humidity of 50–70% and a photosynthetic photon flux density of 800 μE m^{-2} s^{-1}, as measured with a light meter (LICOR, Lincoln, NE, USA, model LI-188B).

At the middle of the experiment, nonAM plants received an application (10 ml per pot) of Hewitt's nutrient solution (Hewitt 1952) in order to enhance plant growth and obtain AM and nonAM plants of comparable size before starting the drought and recovery treatments. One day before starting the drought stress treatment and 3 days after starting the drought stress (just 1 day before harvesting), half of plants received 10 ml/pot of an aqueous ABA solution (100 μM). The group of plants allowed to recover from drought stress for three additional days, received an additional application of ABA (10 ml per pot) the day before their harvest (sixth day after starting the treatments). The concentration of ABA and the systemic application were selected as the most convenient in preliminary experiments in which we tested from 10 μM to 1 mM ABA, and is the same dose we used in previous studied (Aroca et al. 2008a, b).

Soil moisture was measured with a ML2 ThetaProbe (AT Delta-T Devices Ltd., Cambridge, UK) as previously described (Porcel and Ruiz-Lozano 2004). Water was supplied daily to maintain soil at field capacity during the first 7 weeks after planting. Then, two-thirds of the plants were allowed to dry by withholding water irrigation for 4 days, while the other third were maintained at field capacity. Plants were maintained under such conditions for 4 days before harvesting or before being re-watered again to field capacity for three additional days (treatments recovered from drought).

Parameters measured

Biomass production

At harvest (53 or 56 days after planting), the shoot and root system were separated and the shoot dry weight (DW) measured after drying in a forced hot-air oven at 70°C for 2 days.

Symbiotic development

The percentage of mycorrhizal root infection in maize 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).

Leaf transpiration rate

Leaf transpiration rate was determined by a gravimetric method (Aroca et al. 2007). Surfaces of the pots were covered with aluminum foil. The pot-plant system was weighed and referred as W_0. The pot-plant system was weighed again after 2 h and referred as W_f. Leaf transpiration rate was calculated as: $(W_0–W_f)/t \times A$, where t is the time in seconds, and A is the leaf area in m^2. Leaf area was calculated as follows: leaves of a whole plant were detached and scanned (hp scanjet 5550c, Hewlett Packard, Palo Alto, CA). The corresponding images were analyzed with Adobe Photoshop CS (Adobe Systems Incorporated, San Jose, CA).

Sap flow rate and osmotic root hydraulic conductivity

In the present work, sap flow rate (Jv) and hydraulic conductivity (L) were measured on detached roots exuding under atmospheric pressure (Aroca et al. 2007). Under these conditions, water is moving only due to an osmotic gradient between the soil solution and the root tissue. Therefore, according to Steudle (2000), the water would be moving mainly by the cell-to-cell path. This path predominates under conditions where the transpiration stream is restricted, contrary to the apoplastic path (Steudle 2000).

Pots were immersed in aerated nutrient solution. The aerial part was separated from the root and a pipette with a silicone tube was attached to the stem. We discarded the liquid exuded in the first 15 min to avoid phloem contaminations. The exudate of the following 2 h was collected with a syringe and weighed. The osmolarities of the exuded sap and the nutrient solution were determined using a cryoscopic osmometer (Osmomat 030, Gonotec Gmbh, Berlin, Germany). Osmotic root hydraulic conductance (L) was calculated as $L = Jv/\Delta\Psi$, where Jv is the exuded sap flow rate and $\Delta\Psi$ the osmotic potential difference between the exuded sap and nutrient solution. These measurements were carried out 3 h after light turned on.

Relative water content

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

Proline content

Free proline was extracted from 1 g of fresh leaves (Bligh and Dyer 1959). Proline was estimated by spectrophotometric analysis at 515 nm of the ninhydrin reaction, according to Bates et al. (1973).

ABA content

ABA was determined by an indirect ELISA based on the use of DBPA1 monoclonal antibody, raised against S(+)-ABA (Vernieri et al. 1989). The ELISA was performed according to the method described by Walker-Simmons (1987), with minor modifications. The ability of DBPA1 monoclonal antibody to give precise quantitation of ABA in crude aqueous extracts of maize tissues was previously confirmed by validation experiments (Bochicchio et al. 1994; Sturaro et al. 1996; Aroca et al. 2003).

ABA was measured on lyophilized shoot samples (150 mg DW) after extraction in distilled water (water:tissue ratio = 100:1 v:w) overnight at 4°C. Plates were coated with 200 µl per well ABA-4'-BSA conjugate and incubated overnight at 4°C, then washed three times with 75 mM PBS buffer, pH 7.0, containing 1 g l^{-1} BSA and 1 ml l^{-1} Tween 20, keeping the third washing solution for 30 min at 37°C. Then 100 µl ABA standard solution or sample and, subsequently, 100 µl DBPA1 solution were added to each well and competition allowed to occur at 37°C for 30 min. Plates were then washed again as described above and 200 µl per well of secondary antibody (Alkaline phosphatase-conjugated rabbit anti-mouse (Sigma cat. N. A4312) in PBS buffer containing 10 g l^{-1} BSA and 0.5 ml l^{-1} Tween 20 at a final dilution of 1:2,000) was added and incubated for 30 min at 37°C. Plates were washed again and 200 µl per well *p*-Nitrophenyl phosphate were added and incubated for 30 min at 37°C. Absorbance readings at 415 nm were obtained using a MDL 680 Perkin–Elmer microplate reader. Three independent samples were assayed for each treatment. All sample results were the average of three serial dilutions within the linear range of the ABA standard curve.

Quantitative real-time RT-PCR

Total RNA was isolated from maize roots by a phenol/chloroform extraction method followed by precipitation with LiCl (Kay et al. 1987). The expression of seven PIP aquaporin genes (the most expressed in maize roots, according to Hachez et al. 2006) was studied by real-time PCR by using iCycler (Bio-Rad, Hercules, CA, USA). cDNAs were obtained from 2.5 µg of total DNase-treated RNA in a 20 µl reaction containing 500 ng oligo dT(18) primer, 0.5 mM each dNTP, 10 mM DTT, 40 U of RNase inhibitor, 1× first strand buffer (Invitrogen, Carlsbad, CA, USA) and 200 U of Superscript II Reverse Transcriptase (Invitrogen). The primer sets used to amplify each PIP gene in the synthesized cDNAs were designed in the 3′ and 5′

untranslated regions of each gene (the less conserved regions) in order to avoid unspecific amplification of the different PIP genes (Hachez et al. 2006). Standardization was carried out by measuring the expression levels of the α-tubulin gene in each sample, using specific primers *for Zea mays* α-tubulin gene (Hachez et al. 2006). Samples for RNA extraction were taken 3 h after light turned on. Samples were kept immediately in liquid nitrogen and stored at −80°C until use.

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, OR, 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 35 cycles of 30 s at 95°C and 60 s at 60°C, where the fluorescence signal was measured. The specificity of the PCR amplification procedure was checked with a heat dissociation protocol (from 60 to 100°C) after the final cycle of the PCR. The efficiency of each primer set was evaluated by performing real-time PCR on several dilutions of plasmid DNA.

Real-time PCR experiments were carried out in two independent RNA samples and at least three times for each sample, 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

Microsomes were isolated from maize roots as described by Daniels et al. (1994). Briefly, roots were homogenized in grinding buffer (100 mM Tris–HCl pH = 7.5, 1 mM EDTA, 12% (w/v) sucrose, 0.2 mM aminoethylbenzenesulfonylfluoride, 2 µg ml^{-1} aprotinin, 1 µg ml^{-1} leupeptin), and collecting the supernatant after centrifugation at 15,000*g* for 10 min. The supernatant was filtered through a single layer of cheesecloth and centrifuged again at 100,000*g* for 2 h. The pellet was resuspended in 40 µl of grinding buffer plus 0.1% (w/v) SDS. Ten micrograms of protein were loaded in each line after incubating for 30 min at 37°C in presence of denaturing buffer (20 mM Tris–HCl pH = 8.6, 1% (w/v) SDS, 0.3% (v/v) β-mercaptoethanol, 8% (v/v) glycerol, 0.2% (w/v) bromophenol-blue). Proteins were transferred to a PVDF membrane at 100 mA for 1 h. The membranes were blocked during 2 h at room temperature with 5% (w/v) non fat milk in Tris-buffered-saline (TBS) with 0.05% Tween 20. After that, the membranes were incubated overnight at 4°C with 1:2,000 dilutions of antibodies raised against the amino-terminal peptides of ZmPIP1;2, ZmPIP2;1, ZmPIP2;5 or ZmPIP2;6 (Hachez et al. 2006). Goat anti-rabbit Ig coupled to horseradish peroxidase (Sigma) was used as secondary antibody at a 1:20,000 dilution. The signal was developed using a chemiluminescent substrate (West-Pico, Super Signal, Pierce, Rockford, IL, USA). ZmPIP1;2, ZmPIP2;1, ZmPIP2;5 and ZmPIP2;6 antibodies were kindly provided by Dr. F. Chaumont (Université Catholique de Louvain, Louvain-la-Neuve, Belgium). Microsomes were isolated from two different root samples. Western blots were developed on both microsomes samples of each treatment without significant differences between them. The equal loading of the proteins in the different lines was confirmed by staining the gel with coomassie brilliant blue. To quantify the immunoblot signal, the intensity of each band was measured using Adobe PhotoShop 8.0.1 (Adobe Systems, Mountain View, CA), corrected for the background and normalized against the intensity of the corresponding coomassie brilliant blue band (Aroca et al. 2005).

Results

Mycorrhizal colonization

Uninoculated control plants did not show mycorrhizal colonization. Plants inoculated with *G. intraradices* showed a percentage of root colonization ranging from 82 to 86% of root length (Table 1). Neither the application of ABA nor the drought stress significantly affected the colonization of roots by *G. intraradices*.

Plant growth

In order to obtain AM and nonAM plants of similar size before starting the drought and recovery treatments, nonAM plants received an application of nutrient solution. Although under well-watered conditions the application of ABA decreased shoot dry weight and under drought stress conditions it enhanced shoot dry weight the differences were not statistically significant (Table 1). In contrast, the root dry weight showed a significant decrease by ABA application under well-watered conditions and a significant increase by ABA application for nonAM plants under drought stress conditions. Finally, the recovery from drought for 3 days did not significantly affect shoot dry weight, while root dry weight was also reduced by exogenous ABA application.

Sap flow rate and osmotic root hydraulic conductivity

When plants were cultivated under well-watered conditions, Jv was enhanced by the application of exogenous ABA in AM and nonAM maize plants (Fig. 1A).

Table 1 Shoot and root dry weights, percentage of root AM colonization and shoot and root relative water contents in maize plants

Treatment	SDW (g plant^{-1})	RDW (g plant^{-1})	AM (%)	SRWC (%)	RRWC (%)
Well-watered					
Control	1.24 ± 0.2ab	2.7 ± 0.4ab	0b	80 ± 1.6abc	87 ± 2.8ab
G. intraradices	1.09 ± 0.1ab	2.8 ± 0.7ab	86 ± 4a	81 ± 7.8abc	88 ± 2.8a
Control + ABA	1.01 ± 0.1ab	1.9 ± 0.1c	0b	76 ± 8.2bc	87 ± 1.1ab
G. intraradices + ABA	0.83 ± 0.1b	1.8 ± 0.2c	82 ± 2a	94 ± 1.1a	87 ± 0.8ab
Drought					
Control	1.04 ± 0.3ab	2.0 ± 0.3bc	0b	80 ± 9.9abc	82 ± 1.5c
G. intraradices	0.94 ± 0.1b	2.1 ± 0.1bc	83 ± 3a	81 ± 2.4abc	86 ± 0.4ab
Control + ABA	1.26 ± 0.1ab	3.0 ± 0.3a	0b	67 ± 4.0c	84 ± 0.8bc
G. intraradices + ABA	1.09 ± 0.1ab	2.3 ± 0.2bc	84 ± 1a	91 ± 1.3a	85 ± 1.4ab
Drought + Rec					
Control	1.42 ± 0.2a	2.4 ± 0.2b	0b	71 ± 2.3bc	83 ± 1.3bc
G. intraradices	1.17 ± 0.1ab	2.2 ± 0.3bc	85 ± 2a	76 ± 2.1bc	83 ± 0.8bc
Control + ABA	1.22 ± 0.1ab	1.6 ± 0.1c	0b	81 ± 0.8abc	86 ± 1.4ab
G. intraradices + ABA	1.11 ± 0.2ab	1.7 ± 0.1c	86 ± 2a	84 ± 4.2ab	86 ± 1.1ab

Plants were inoculated with the AM fungus *G. intraradices* or remained as uninoculated controls. Plants received exogenous ABA or remained ABA-free. Plants were cultivated under well-watered conditions for the entire experiment or were subjected to a 4-day drought episode followed or not of a 3-day recovery period

Means are followed by the standard deviation. Within each column, means followed by the same latter are not significantly different ($P < 0.05$) as determined by the Tukey's LSD test ($n = 5$)

However, there were no significant differences in Jv between AM and nonAM plants. Drought stress decreased Jv, in both plant treatments, but this decrease was statistically significant only in plants supplied with exogenous ABA. In fact, plants treated with exogenous ABA always had higher Jv values than the corresponding treatments without ABA. After recovery from the stress AM and nonAM plants exhibited similar (nonAM plants) or higher (AM plants) Jv values than under well-watered conditions, and the positive effect of ABA on this parameters was observed again for nonAM plants. NonAM plants which had recovered from drought and were supplied with exogenous ABA had 56% higher Jv values than the corresponding AM plants.

Well-watered plants enhanced *L* after ABA application (increase by 238 and 207% for nonAM and AM plants, respectively; Fig. 1B). In any case, after the addition of ABA, nonAM plants exhibited a significantly higher *L* than AM plants (increase by 46%). Drought stress decreased significantly *L* in AM plants with this decrease being more evident in AM and nonAM plants when treated with exogenous ABA. Again, the values of *L* were higher (increase by 110%) in nonAM plants supplied with ABA than in their AM counterparts. Plants that had been subjected to drought stress and recovered for 3 days also recovered their *L* to values similar to or better than well-watered plants. As observed under well-watered and drought stress conditions, the application of ABA enhanced *L* values in nonAM and AM plants (increase by 154 and 70%, respectively). Indeed, nonAM plants supplied with ABA exhibited the highest *L* in this study. After the recovery from drought, nonAM plants supplied with ABA had 58% higher *L* than the corresponding AM plants. These data illustrate that the application of exogenous ABA accentuated the differences in *L* between AM and nonAM plants.

Transpiration rate

Under well-watered conditions and in the absence of ABA, the inoculation with the AM fungus decreased the transpiration rate by 56%. The application of exogenous ABA decreased this parameter by 22% only in nonAM plants (Fig. 1C). Under drought stress conditions the transpiration rate of nonAM plants decreased when compared to well-watered conditions, while the AM plants exhibited a similar rate of transpiration as under well-watered conditions. Under drought conditions, the application of ABA to AM plants enhanced the transpiration rate by 86% while in nonAM plants the enhancement was not significant. When plants were recovered from drought for three additional days, the transpiration rate of AM plants reached the highest values in this study (77% increase over nonAM plants). In contrast, the application of ABA to plants recovered from drought prevented an increase of the transpiration rate in AM plants and did not affect the transpiration rate of nonAM plants.

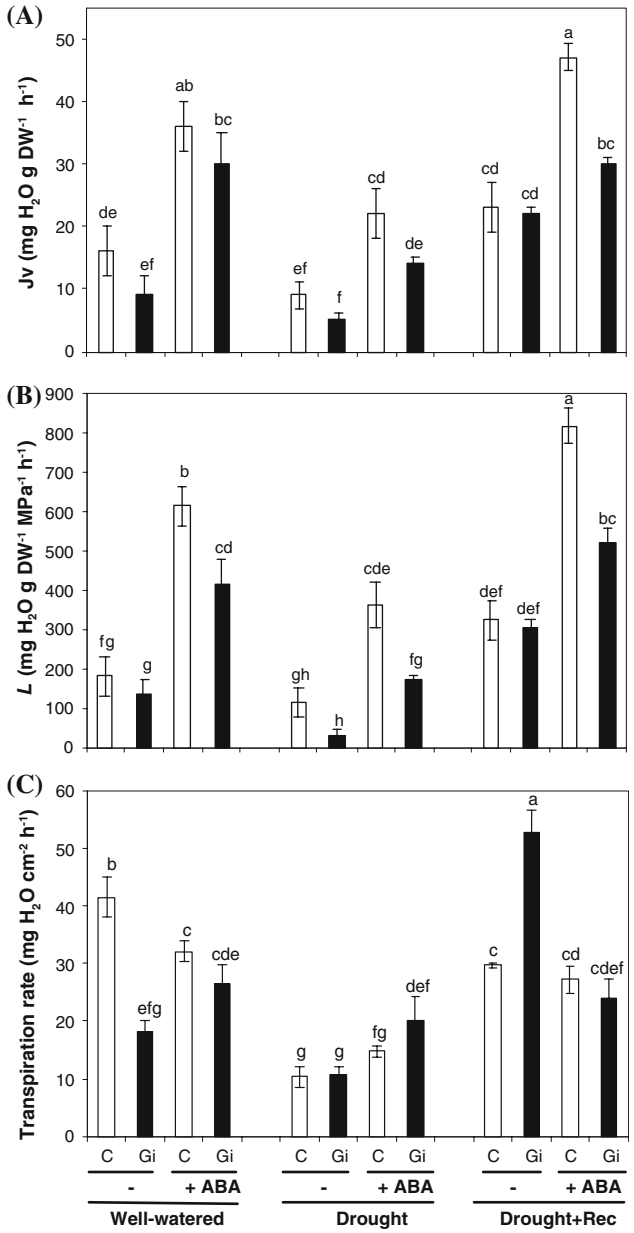

Fig. 1 **A** Free exuded sap flow rate (Jv), **B** osmotic root hydraulic conductivity (*L*) and **C** transpiration rate of maize plants. Plants were either inoculated with *Glomus intraradices* (Gi, *black bars*) or remained as uninoculated controls (C, *open bars*). Plants were cultivated under well-watered conditions or subjected to a 4-day drought stress period. A group of plants were allowed to recover from drought for an additional 3-day period under well-watered conditions. Finally, plants were applied of exogenous ABA just before and during the drought stress or recovery periods (+ABA) or did not receive ABA (−). Means followed by the *same letter* are not significantly different ($P < 0.05$) as determined by Tukey's LSD test ($n = 5$)

Shoot and root relative water content

Little changes were observed in the shoot or root relative water content (RWC) of the different plant treatments (Table 1). Indeed, these parameters did not show significant differences due to the mycorrhization or to the application of ABA. Only the combination of both treatments had a significant effect under well-watered and under drought stress conditions. In fact, under these conditions, the application of exogenous ABA had a positive effect on the shoot RWC of AM plants that was higher than the shoot RWC of nonAM plants.

Table 2 Shoot proline accumulation and shoot ABA content in maize plants

Treatment	Proline (nmol g DW^{-1})	ABA (ng g DW^{-1})
Well-watered		
Control	185 ± 9b	247 ± 9hi
G. intraradices	174 ± 22b	211 ± 20hi
Control + ABA	182 ± 1b	567 ± 95ef
G. intraradices + ABA	175 ± 30b	320 ± 40gh
Drought		
Control	304 ± 60a	2,920 ± 150b
G. intraradices	203 ± 1b	754 ± 98de
Control + ABA	204 ± 30b	3,654 ± 175a
G. intraradices + ABA	226 ± 30b	800 ± 70 cd
Drought + Rec		
Control	183 ± 4b	615 ± 40ef
G. intraradices	166 ± 10b	145 ± 15i
Control + ABA	164 ± 20b	894 ± 94 cd
G. intraradices + ABA	177 ± 19b	302 ± 24gh

Plants were inoculated with the AM fungus *G. intraradices* or remained as uninoculated controls. Plants received exogenous ABA or remained ABA-free. Plants were cultivated under well-watered conditions for the entire experiment or were subjected to a 4-day drought episode followed or not of a 3-day recovery period

Means are followed by the standard deviation. Within each column, means followed by the same latter are not significantly different ($P < 0.05$) as determined by the Tukey's LSD test ($n = 5$)

Proline content

When plants were cultivated under well-watered conditions or were recovered from drought stress, the proline content in shoots of maize plants was not affected by mycorrhization or by the application of exogenous ABA (Table 2). Only under drought stress and in the absence of exogenous ABA, nonAM plants enhanced their proline accumulation by 64% as compared to well-watered conditions, while AM plants did not change their proline content.

Shoot ABA content

The shoot ABA content was similar in AM and nonAM plants under well-watered conditions (Table 2). The application of exogenous ABA to these plants enhanced the ABA content of nonAM plants by 129% but no significant

change was observed in AM plants. Drought enhanced the ABA accumulation of all treatments, especially in nonAM plants. In these plants, the combination of drought plus exogenous ABA application produced the highest ABA content. AM plants also enhanced their ABA content as a consequence of drought, but their levels remained considerably lower than those of nonAM plants (decrease by about 75%). The recovery from drought reduced the ABA content in maize plants to levels similar to those found under well-watered conditions in the case of plants colonized by *G. intraradices* (both fed and non fed with ABA), while nonAM plants maintained enhanced ABA levels over those found under well-watered conditions. After recovery from drought, the nonAM plants exhibited again higher ABA contents than their AM counterparts.

PIP gene expression

The mRNA accumulation pattern of seven PIP genes previously shown to be highly expressed in maize roots (Hachez et al. 2006) was studied. The most important findings are remarked here.

Gene ZmPIP1;1

Drought enhanced the expression of this gene, especially in the AM plants (383% increase) and the application of exogenous ABA had a contrasting effect in AM and in nonAM plants (Fig. 2A). Indeed, ABA further enhanced the expression of *ZmPIP1;1* in droughted nonAM plants, but decreased gene expression by 78% in the droughted AM plants, which returned to the expression level found under well-watered conditions. After recovery from drought, nonAM plants fed with ABA maintained the highest expression levels for this gene.

Gene ZmPIP1;2

When maize plants were subjected to drought, AM plants exhibited a higher expression of *ZmPIP1;2* than nonAM plants, but the application of exogenous ABA had again a contrasting effect in AM and in nonAM plants (Fig. 2B). ABA enhanced the expression of this gene in droughted nonAM plants and decreased the expression in droughted AM plants. NonAM plants fed with ABA which had recovered from drought showed a considerable increase in *ZmPIP1;2* gene expression.

Gene ZmPIP1;5

The mRNA accumulation pattern of *ZmPIP1;5* did not show significant changes when plants were cultivated under well-watered or drought stress conditions (Fig. 2C). After the recovery from drought only the nonAM plants fed with ABA enhanced the expression of this gene.

Gene ZmPIP2;1

As already noticed for the previous PIP1 genes, the only treatment that affected significantly the expression of *ZmPIP2;1* was the application of exogenous ABA to nonAM plants which had recovered from drought which considerably enhanced the expression of this gene (Fig. 3A). Under drought and after the recovery from drought AM plants supplied with exogenous ABA showed inhibition of *ZmPIP2;1* gene expression as compared to their nonAM counterparts.

Gene ZmPIP2;2

Under well-watered conditions AM plants showed a lower expression rate for *ZmPIP2;2* than nonAM plants (inhibition by about 70% both in presence and in absence of ABA; Fig. 3B). Under drought stress conditions, the lower expression of the *ZmPIP2;2* gene in AM plants than in nonAM plants was significant when plants were fed with exogenous ABA. Again, after the recovery from drought, nonAM plants supplied with exogenous ABA reached the highest expression rate for this gene.

Gene ZmPIP2;5

Under well-watered conditions AM plants showed reduced *ZmPIP2;5* gene expression as compared to nonAM plants both in the presence and absence of exogenous ABA (Fig. 3C). In the absence of exogenous ABA, drought stress enhanced the expression of *ZmPIP2;5* by 366% in AM plants, while nonAM plants reduced the expression of this gene by 33%. In contrast, the application of exogenous ABA to droughted plants resulted in an inhibition of *ZmPIP2;5* gene expression by 77% in AM plants and enhanced by 83% the expression of this gene in nonAM plants. After recovery from drought the *ZmPIP2;5* gene was also induced considerably in nonAM plants supplied with exogenous ABA.

Gene ZmPIP2;6

Under well-watered conditions the expression of the *ZmPIP2;6* gene was inhibited by about 80% in AM plants as compared to nonAM plants, but no effect of exogenous ABA was observed (Fig. 3D). Under drought stress nonAM plants reduced the expression of this gene by 84%, while AM plants enhanced the expression of *ZmPIP2;6* by 430%. However, the application of exogenous ABA to droughted plants prevented the effect of drought on the expression of

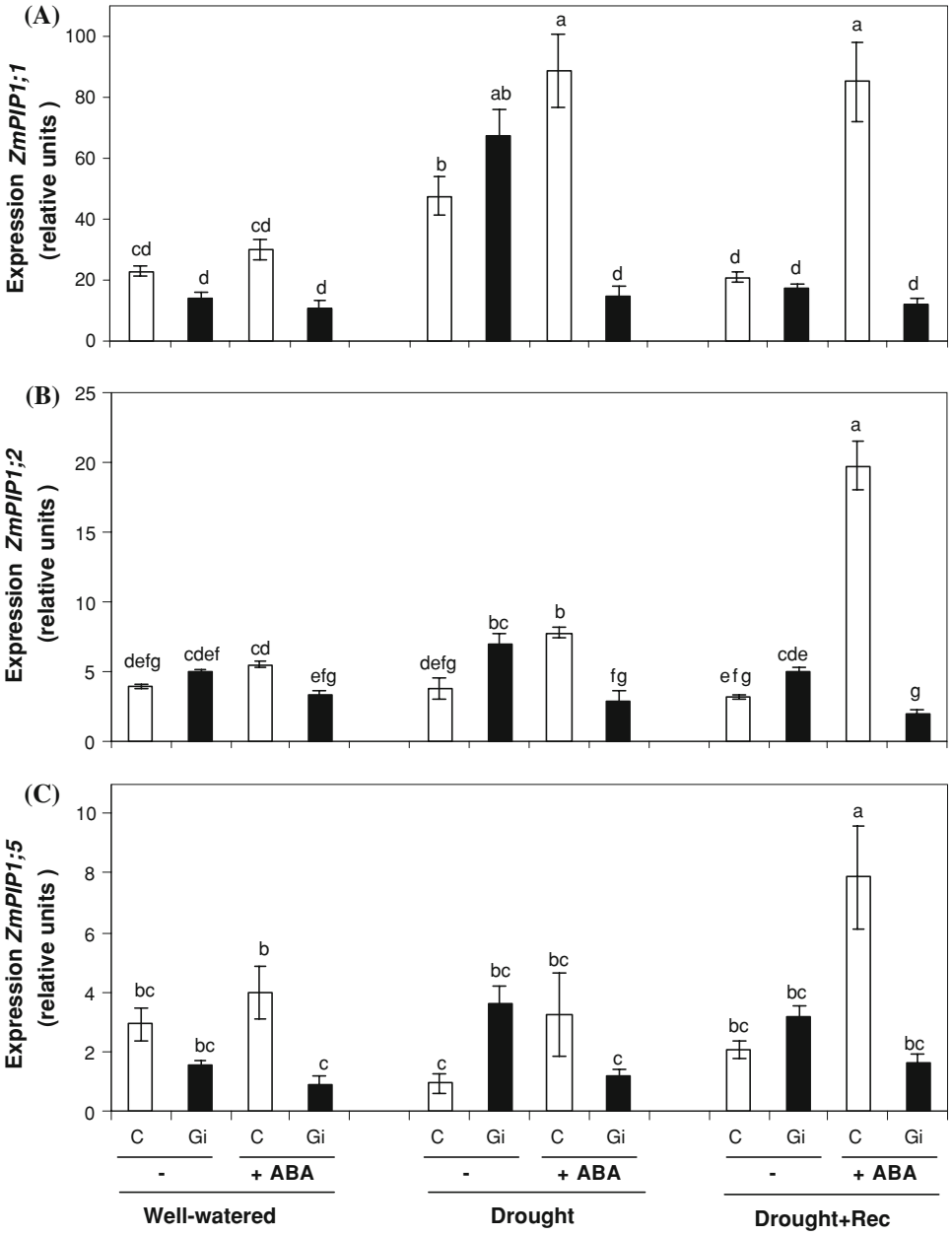

Fig. 2 Expression levels of the *ZmPIP1;1* (**A**), *ZmPIP1;2* (**B**) and *ZmPIP1;5* (**C**) genes determined by quantitative real-time PCR in maize roots. Plants were either inoculated with *Glomus intraradices* (Gi, *black bars*) or remained as uninoculated controls (C, *open bars*). Plants were cultivated under well-watered conditions or subjected to a 4-day drought stress period. A group of plants were allowed to recover from drought for an additional 3-day period under well-watered conditions. Finally, plants were applied of exogenous ABA just before and during the drought stress or recovery periods (+ABA) or did not receive ABA (−). Means followed by the *same letter* are not significantly different ($P < 0.05$) as determined by Tukey's LSD test ($n = 5$, with two independent biological samples and two or three qRT-PCR reactions for each sample)

this gene. It is remarkable that under drought stress, the application of ABA inhibited the expression of the *ZmPIP2;6* gene by 75% in AM plants only. Finally, as we have observed for all the other PIP genes, the expression of *ZmPIP2;6* was also induced considerably in nonAM plants supplied with ABA after recovery from drought.

PIP protein accumulation

The pattern of ZmPIP1;2, ZmPIP2;1, ZmPIP2;5 and ZmPIP2;6 protein accumulation in maize roots was analyzed by Western blot. However, the latter protein could not be detected in the root extracts. Thus, only results for ZmPIP1;2, ZmPIP2;1 and ZmPIP2;5 are presented in Fig. 4. No perfect match between protein accumulation and the gene expression patterns was found. In fact, ZmPIP1;2 showed correlation between mRNA and protein levels only under drought or after recovery from drought, but not under well-watered conditions. The opposite was found under well-watered conditions for ZmPIP2;1 and ZmPIP2;5 that accumulated more in the nonAM roots than in those of AM plants, showing a clear correlation with gene expression. Under drought stress or after recovery from drought the correlation between protein and gene expression levels was less evident. However, all three proteins showed induced accumulation in nonAM roots supplied with ABA and which had recovered from drought, in agreement with the mRNA patterns for these three genes.

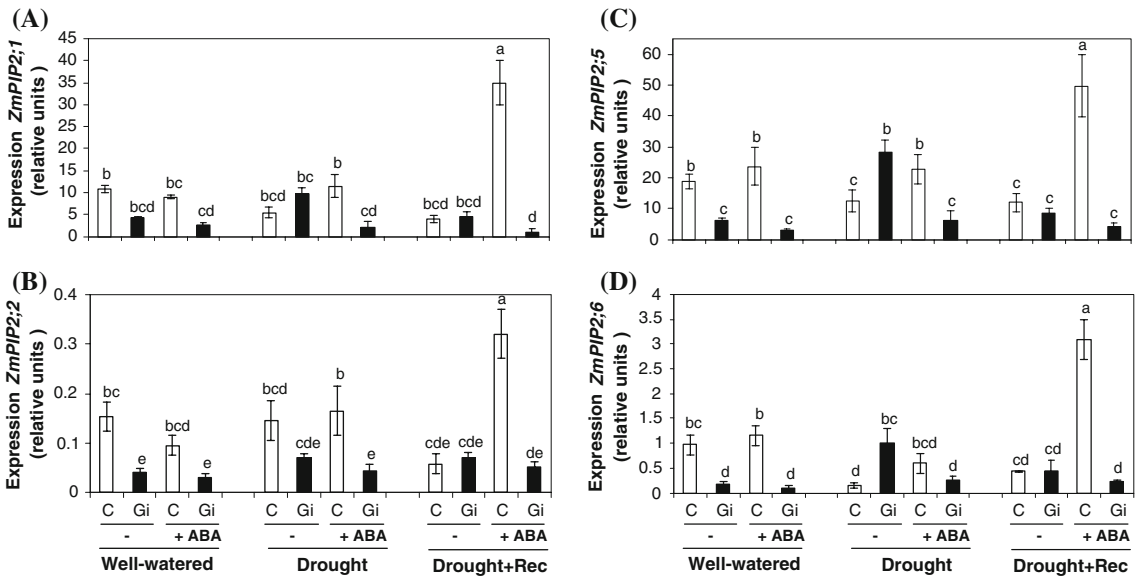

Fig. 3 Expression levels of the *ZmPIP2;1* (**A**), *ZmPIP2;2* (**B**), *ZmPIP2;5* (**C**) and *ZmPIP2;6* (**D**) genes determined by quantitative real-time PCR in maize roots. Plants were either inoculated with *Glomus intraradices* (Gi, *black bars*) or remained as uninoculated controls (C, *open bars*). Plants were cultivated under well-watered conditions or subjected to a 4-day drought stress period. A group of plants were allowed to recover from drought for an additional 3-day period under well-watered conditions. Finally, plants were applied of exogenous ABA just before and during the drought stress or recovery periods (+ABA) or did not receive ABA (−). Means followed by the *same letter* are not significantly different ($P < 0.05$) as determined by Tukey's LSD test ($n = 5$, with two independent biological samples and two or three qRT-PCR reactions for each sample)

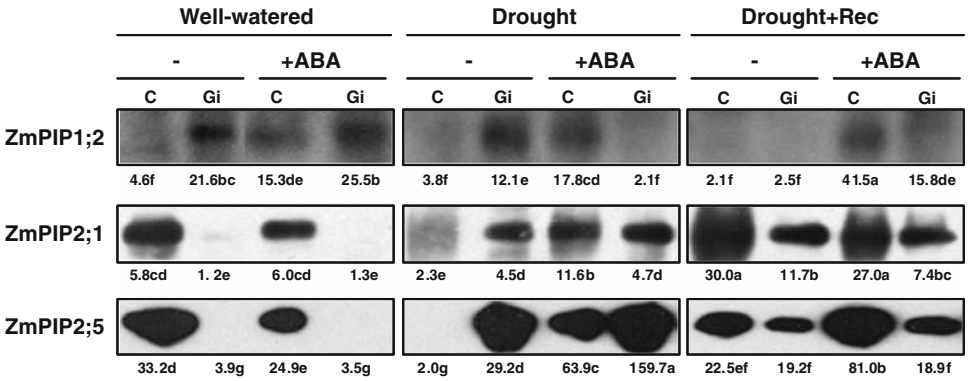

Fig. 4 Western blot analysis of ZmPIP1;2, ZmPIP2;1 and ZmPIP2;5 protein accumulation in maize roots. Plants were either inoculated with *Glomus intraradices* (Gi) or remained as uninoculated controls (C). Plants were cultivated under well-watered conditions or subjected to a 4-day drought stress period. A group of plants were allowed to recover from drought for an additional 3-day period under well-watered conditions. Finally, plants were applied of exogenous ABA just before and during the drought stress or recovery periods (+ABA) or did not receive ABA (−). To quantify the immunoblot signal, the intensity of each band was corrected for the background and normalized against the intensity of the corresponding whole coomassie brilliant blue line. Means followed by the *same letter* are not significantly different ($P < 0.05$) as determined by Tukey's LSD test ($n = 4$, with two independent biological samples)

Discussion

Plant growth and physiology

Drought is one of the most important abiotic stresses which reduce agricultural productivity. Plant tolerance to drought stress is a complex phenomenon that involves several changes at physiological and biochemical levels (Ingram and Bartels 1996). In this respect, it is well known that ABA plays a major role in plant responses to a range of stresses and that ABA levels in vegetative plant tissues rise in response to stresses causing plant water deficit (Bray 2002; Zhang et al. 2006). The protective effect of ABA is explained by the fact that ABA primarily promotes stomatal closure to minimize transpirational water loss and then mitigates stress damage through the activation of

stress-responsive genes, which collectively increase plant stress tolerance (Bray 2002; Zhang et al. 2006; Hirayama and Shinozaki 2007).

Mycorrhizal symbiosis is a key component in helping plants to cope with adverse environmental conditions. The AM symbiosis generally increases host plant growth due to improved plant nutrition (Smith and Read 1997). In studies on water relationship in plants confined to containers, it is often difficult to compare treatments if plants are not of comparable size since unequal size causes different degrees of soil water depletion, plant transpiration and, consequently, unequal stress (Goicoechea et al. 1997). In this study, nonAM plants received a single application of nutrient solution at the middle of the experiment in order to obtain AM and nonAM plants of similar sizes before starting the drought and recovery treatments. Furthermore, the application of exogenous ABA did not significantly affect plant growth. Therefore, the effects of the different treatments on transpiration and root hydraulic properties can be seen as a direct effect not mediated by plant size.

To counter drought stress many plants decrease the osmotic potential of their cells by synthesizing and accumulating compatible osmolytes such as proline that participate in the osmotic adjustment (Yoshiba et al. 1997). In this study, this effect is clearly visible only in nonAM plants subjected to drought, which enhanced significantly the accumulation of proline, while both the mycorrhization and the application of exogenous ABA prevented the accumulation of proline in maize plants under drought. Although the precise reason for such an effect is currently not known, the regulation of proline accumulation by an ABA-dependent and an ABA-independent (plant dehydration) pathway has been described (Savoure et al. 1997), which could explain the effect of ABA. In addition, the AM symbiosis has been reported to protect host plants from dehydration stress and these plants may need to accumulate less proline than their nonAM counterparts (Porcel et al. 2004).

Under drought stress, ABA is synthesized in the roots and released by root tissues to the xylem for its translocation to the shoots (Hartung et al. 2005). Here we found that the ABA content in nonAM maize plants under drought stress conditions or after recovery was higher than in AM plants. Moreover, AM plants subjected to drought did not enhance their ABA levels after the addition of exogenous ABA, while nonAM plants showed the highest ABA levels in this study. The exact reason for the lower ABA content in AM plants is not known. However, differences between AM and nonAM plants in the rate of ABA metabolism, recirculation and exudation to the rhizosphere or changes in its internal localization could account for this effect (Wilkinson and Davies 2002; Hartung et al. 2005). Previous studies have also shown reduced ABA content in AM than in nonAM plants under drought (Duan et al. 1996; Goicoechea et al. 1997; Ludwig-Müller 2000; Estrada-Luna and Davies 2003) or under salt stress (Jahromi et al. 2008), and it has been proposed that AM plants could be accumulating less ABA than nonAM plants as a consequence of primary drought avoidance mechanisms (Porcel et al. 2005; Ruiz-Lozano et al. 2006).

Root hydraulic properties and PIP aquaporin gene expression and protein accumulation

The effects of ABA on PIP aquaporin gene expression are not fully understood, with the effect being either positive or negative depending on the gene analyzed and the plant genotype (Lian et al. 2006). So far, few studies have been conducted dealing with the combined influence of AM symbiosis and exogenous ABA application on plant root hydraulic properties and on PIP aquaporin gene expression under drought conditions. However, it has been described that under drought conditions AM symbiosis regulates ABA content (Goicoechea et al. 1997; Ludwig-Müller 2000; Estrada-Luna and Davies 2003) and expression of some aquaporin genes (Porcel et al. 2006; Ruiz-Lozano et al. 2006; Aroca et al. 2007, 2008a, b) in the host plant.

Our study shows two consistent results: first, the application of exogenous ABA enhanced Jv and L in AM and nonAM plants, regardless of the water regime, and second, when plants received exogenous ABA AM plants always exhibited lower L values than nonAM plants. In fact, the application of exogenous ABA accentuated the differences in L between AM and nonAM plants. The enhancement of L by exogenous ABA application has previously been shown (Quintero et al. 1999; Hose et al. 2000; Wan et al. 2004; Schraut et al. 2005: Aroca 2006). The intimate mechanisms involved in the ABA promotion of root water transport still remain barely explored (Aroca 2006). However, the regulation of L has been related to changes in aquaporin activity and/or abundance (Javot and Maurel 2002; Luu and Maurel 2005; Beaudette et al. 2007) and it has been proposed that aquaporin function and ABA signal transduction are interconnected (Kaldenhoff et al. 2008). Indeed, enhanced transcellular water flow by ABA through the induction of aquaporin accumulation has been proposed recently (Kaldenhoff et al. 2008; Lovisolo et al. 2008).

The values of L measured in this study are only due to the osmotic gradient existing between the root tissues and the soil solution (no transpiration in detached plants). Hence, according to the composite water flux model proposed by Steudle (2000), increases or decreases of L mean reciprocal increases or decreases of water movement through the cell-to-cell pathway, a pathway in which aquaporins are supposed to participate (Javot and Maurel 2002; Javot et al. 2003; Luu and Maurel 2005). According

to this model, it is thus expected that the role played by aquaporins in water transport may become crucial under water stress, when conditions of reduced transpiration do not allow high driving forces derived from significant water potential gradients. On the other hand, it is also known that ABA modulates the expression of some PIP genes (Suga et al. 2002; Jang et al. 2004; Zhu et al. 2005; Aroca et al. 2006). Thus, the regulation of *L* by ABA may be linked to modulation of aquaporins. In fact, the enhanced *L* in plants fed with exogenous ABA (especially the nonAM plants) and the reduced *L* in AM plants as compared to nonAM plants was clearly correlated with the expression of most of the PIPs analyzed, that were also lower in AM plants than in nonAM plants. It is also remarkable the important increase of *L* and of the expression of the seven PIPs analyzed after the recovery from the stress in nonAM plants fed with exogenous ABA, while their AM counterparts showed a clear reduction of *L* in parallel with reduced PIPs gene expression.

In this study, we also show that the effects of ABA on PIP gene expression depend on the growing conditions and on the presence or absence of the AM fungus. Indeed, under well-watered conditions, exogenous ABA did not regulate significantly any of the PIP genes analyzed. After recovery from drought the effect of ABA was only remarkable in nonAM plants, which enhanced considerably the expression of all the PIP genes analyzed. In contrast, under drought stress conditions *ZmPIP1;1*, *ZmPIP1;2* and *ZmPIP2;5* were up-regulated by ABA in nonAM plants but resulted down-regulated by ABA in AM plants. A down-regulation of *ZmPIP2;6* by ABA was also observed in AM plants, while in nonAM plants not significant changes were observed. These findings suggest that the water permeability of plants under drought stress is governed by complex regulation of aquaporins, depending on isoform-specific roles in the plant (Alexandersson et al. 2005; Luu and Maurel 2005).

The changes of *L* due to the two other treatments included in this study (drought and AM symbiosis) do not show a perfect correlation with changes in the expression or protein accumulation of the different aquaporins analyzed. The lack of complete correlation between the expression of aquaporin genes and *L* has also been described by Galmés et al. (2007). It may be due to the fact that regulation of aquaporin activity is not only restricted to the transcriptional level. Post-transcriptional regulation via phosphorylation, methylation, re-localization or changes in cytosolic pH has been described (Maurel et al. 2008). Besides, aquaporins are not the unique way to control *L*, symplastic movement of water via plasmodesmata may contribute significantly to *L* depending on the exact environmental circumstances (Galmés et al. 2007). Finally, it must be considered that aquaporins constitute a multiple gene family in plants (Maurel 2007) and in this study we only analyzed seven PIP aquaporin genes. Thus, differential effect of others aquaporins not analyzed here can account for the changes observed in *L*.

The western blot analysis showed that some important hints found for gene expression were also confirmed at the protein level. For instance, the lower *ZmPIP2;1* and *ZmPIP2;5* gene expression in AM plants under well watered conditions correlated with the protein pattern. Also, an important increase in protein accumulation was found for ZmPIP1;2, ZmPIP2;1 and ZmPIP2;5 in nonAM plants recovered from drought and supplied with exogenous ABA, while AM plants did not show this enhanced protein accumulation, as happened with gene expression. However, for some treatments there was no perfect match between the level of gene expression and the level of protein accumulated. The lack of mRNA-protein correlation in the case of aquaporins has also been observed in previous studies (Lopez et al. 2003; Aroca et al. 2005; Boursiac et al. 2005) and suggests that post-transcriptional and post-translational regulation or a different rate of protein degradation could be taken place in these treatments (Lopez et al. 2003). In addition, in the case of ZmPIP2;1, it must be taken into account that the antibody used also recognizes ZmPIP2;2 (Hachez et al., 2006), and thus, both proteins account for the result obtained.

The lower values of *L* in AM plants than in nonAM plants disagree with previous reports that have showed enhanced *L* in droughted tomato, rosemary and lettuce AM plants as compared to nonAM plants (Dell'amico et al. 2002; Sánchez-Blanco et al. 2004; Aroca et al. 2008b). Nevertheless, Augé (2001) reported that root hydraulic conductivity is generally not improved by the AM symbiosis in the absence of AM-induced growth or P effects, as we found for plant growth in this study. In addition, Kyllo et al. (2003) showed that the effect of the AM symbiosis on *L* depended of the plant species and of light intensity, and very recently, Siemens and Zwiazek (2008) have proposed that the hyphae of ectomycorrhizal fungi may have little influence on root hydraulic properties of balsam poplar. Reduction of *L* by arbuscular mycorrhization has also been observed in citrus plants subjected to drought (Levy et al. 1983) or in bean plants when cultivated under optimal conditions (Aroca et al. 2007).

In conclusion, our data show two consistent results: first that the application of exogenous ABA enhanced *L* in all plant, regardless of water conditions, and second that AM plants showed lower *L* values than nonAM plants, especially when plants were supplied with exogenous ABA, which accentuated the differences in *L* between AM and nonAM plants. This effect was clearly correlated with the accumulation pattern of the different PIPs analyzed, since most of them reduced their expression and

protein levels in AM plants fed with ABA as compared to their nonAM counterparts. As a whole, results suggest that the combination of exogenous ABA and AM symbiosis inhibited the expression of PIP aquaporins as a strategy of water conservation in the host plant, which allowed these plants to maintain higher shoot RWC, although this hypothesis needs further studies to be corroborated.

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