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How does arbuscular mycorrhizal symbiosis regulate root hydraulic properties and plasma membrane aquaporins in *Phaseolus vulgaris* under drought, cold or salinity stresses?

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Summary

• Here, we evaluated how the arbuscular mycorrhizal (AM) symbiosis regulates root hydraulic properties and root plasma membrane aquaporins (PIP) under different stresses sharing a common osmotic component.

• *Phaseolus vulgaris* plants were inoculated or not with the AM fungus *Glomus intraradices*, and subjected to drought, cold or salinity. Stress effects on root hydraulic conductance (*L*), *PIP* gene expression and protein abundance were evaluated.

• Under control conditions, *L* in AM plants was about half that in nonAM plants. However, *L* was decreased as a result of the three stresses in nonAM plants, while it was almost unchanged in AM plants. At the same time, PIP2 protein abundance and phosphorylation state presented the same trend as *L*. Finally, the expression of each *PIP* gene responded differently to each stress and was dependent on the AM fungal presence.

• Differential expression of the *PIP* genes studied under each stress depending on the AM fungal presence may indicate a specific function and regulation by the AM symbiosis of each gene under the specific conditions of each stress tested.

Key words: arbuscular mycorrhizal fungi, *Glomus intraradices*, *Phaseolus vulgaris*, plasma membrane aquaporins, root hydraulic conductance.

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Introduction

Plants in nature are exposed to several stress conditions that retard their development and diminish their production (Seki *et al.*, 2003). Several of those stresses cause a dehydration of plant tissues, the commonest being drought, salinity and cold (Seki *et al.*, 2003). The dehydration caused by those stresses is a consequence of the imbalance between the water lost in the leaves and the water taken up by roots (Aroca *et al.*, 2001). Although there is much information about how drought, salinity or cold regulate transpiration (Robinson *et al.*, 1997; Comstock, 2002; Pei & Kuchitsu, 2005), much less is known about how these stresses regulate root water uptake (Luu & Maurel, 2005).

Root water uptake depends on root hydraulic conductance, which is ultimately governed by aquaporins (Luu & Maurel, 2005). Aquaporins are membrane intrinsic proteins that form a pore in all cell membranes of living organisms, facilitating the passive water flow through membranes following an osmotic gradient (Kruse *et al.*, 2006). In plants, aquaporins are subdivided in four groups: plasma-membrane intrinsic proteins (PIPs), tonoplast intrinsic proteins (TIPs), nodulin-like intrinsic proteins (NIPs) and small basic intrinsic proteins (SIPs) (Luu & Maurel, 2005). There is some evidence that PIP proteins could regulate the whole water transport through plant tissues, since isolated plasma membranes usually have less water permeability than isolated membranes from vacuoles (Maurel *et al.*, 1997), although these results could be affected by the isolation procedure (Gerbeau *et al.*, 2002). At the same time, plants overexpressing or lacking one or more *PIP* genes have more or less root water uptake capacity, respectively (Aharon *et al.*, 2003; Javot *et al.*, 2003).

Most plants in natural conditions form a symbiosis with arbuscular mycorrhizal (AM) fungi, which are obligate symbionts of plants. This means the fungus occupies a protected ecological niche and receives plant photosynthates, while plants improve their nutrient uptake and their tolerance to biotic and abiotic stresses (Ruiz-Lozano, 2003; Harrison, 2005; Ruiz-Lozano *et al.*, 2006). In fact, AM plants are frequently more tolerant to drought and salt stresses than nonAM plants (Rosendahl & Rosendahl, 1991; Giri & Mukerji, 2004; Khalvati *et al.*, 2005; Al-Karaki, 2006; Porcel *et al.*, 2006), with only few exceptions (Bryla & Duniway, 1997; Ouziad *et al.*, 2006). There is little information about AM fungi effects on plant cold tolerance, mostly because of the inherent low tolerance to low temperatures of the fungi themselves (Liu *et al.*, 2004; Kytöviita, 2005).

Arbuscular mycorrhizal plants are able to take up more water from the soil than nonAM plants under water deficit conditions (Marulanda *et al.*, 2003; Khalvati *et al.*, 2005). However, this capacity of AM fungi depends on the fungal species, *Glomus intraradices* being one of the most efficient AM fungi in enhancing plant water uptake from the soil among six fungi tested (Marulanda *et al.*, 2003). Unfortunately, to our knowledge there is no information about how root hydraulic properties are influenced by AM symbiosis under salt or cold stresses. However, Muhsin & Zwiazek (2002a,b) found that the ectomycorrhizal fungus *Hebeloma crustuliniforme* enhanced root hydraulic conductance of *Picea glauca* and *Ulmus americana* trees under salt or low temperature conditions, respectively.

Porcel *et al.* (2006) found that, under water deficit conditions, the AM fungus *Glomus mosseae* accelerated the decrease of a *PIP* gene expression in roots of *Glycine max* plants and diminished the expression of two *PIP* genes in roots of *Lactuca sativa* plants. On the other hand, Ouziad *et al.* (2006) found that inoculation with a mixture of AM fungi decreased the expression of one *PIP* gene in the roots of tomato plants under saline conditions. To our knowledge, these two are the only studies in which the effects of AM symbiosis on *PIP* gene expression under water deficit or saline conditions have been evaluated. Unfortunately, no measurements of root hydraulic properties were taken in either study. Finally, no data on how AM symbiosis affects *PIP* gene expression under cold conditions are available.

Therefore, the aim of the present work was to evaluate how AM symbiosis influences root hydraulic properties, aquaporin expression and abundance in roots of *Phaseolus vulgaris* plants under drought, cold or salinity conditions. *P. vulgaris* was chosen as it is the most important grain legume for direct human consumption in the world, especially in Latin America and East Africa, and therefore physiological and molecular studies about stress tolerance in *P. vulgaris* are needed (Broughton *et al.*, 2003). At the same time, *P. vulgaris* is a species sensitive to drought, cold and salinity (Vernieri *et al.*, 2001; Bayuelo-Jiménez *et al.*, 2003; Aroca *et al.*, 2006), in which a beneficial effect of AM symbiosis can be observed. *P. vulgaris* plants were inoculated with the AM fungus *Glomus intraradices* and root hydraulic properties and aquaporin expression and abundance were examined after drought, salt or cold stresses.

Materials and Methods

Biological material and experimental design

Seeds of *Phaseolus vulgaris* L. cv. Borlotto were washed for 3 min in pure ethanol and rinsed three times with distilled water. Seeds were then sowed in wet sepiolite (a clay mineral) and after 9 d, seedlings were transferred to 500 ml pots. Pots were filled with a sterilized mixture of soil/sand (1 : 1, v/v). For details of soil characteristics, see Porcel *et al.* (2006). Mycorrhizal inoculum of *Glomus intraradices* (Schenck and Smith) isolate BEG 123 was prepared as described by Porcel *et al.* (2006), and 10 g of the inoculum was added to half of the pots. Noninoculated pots received the same amount of autoclaved mycorrhizal inoculum together with 2 ml of a filtrate of the AM inoculum in order to provide a general microbial population free of AM propagules (Porcel *et al.*, 2006). Inoculated and noninoculated plants were grown in a growth chamber at 23 : 21°C (day : night), in a photoperiod of 16 : 8 h (day : night) with a photosynthetic photon flux density (PPFD) of 300 μmol m^{-2} s^{-1} and keeping the soil to full water capacity. After 4 wk of transplanting (37-d-old plants), plants were divided in four groups: 23°C and full soil water capacity (control); 23°C and no water for 4 d (drought); 4°C with a constant PPFD of 150 μmol m^{-2} s^{-1} for 2 d (cold); and 23°C and watered three times on alternate days with 10 ml 0.5 M NaCl and harvested 2 d after the last application (salinity). The soil electrical conductivities were 0.12 ± 0.02 and 3.06 ± 0.27 dS m^{-1} in control and salinity pots, respectively. The four groups of plants were harvested in the same day, when the plants were 45 d old.

Mycorrhizal development

The percentage of mycorrhizal root infection 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 Philips & Hayman (1970). The extent of mycorrhizal colonization was calculated according to the gridline intersect method (Giovannetti & Mosse, 1980).

Leaf relative water content and leaf transpiration rate

Leaf relative water content (RWC) was measured as described by Aroca *et al.* (2001), while leaf transpiration rate was determined using a gravimetric method (Aroca *et al.*, 2001, 2006). Surfaces of the pots were covered with aluminium foil. The pot-plant system was weighed immediately (W_0) and

again after 2 h (W_f). Leaf transpiration rate was calculated as: $(W_0 - W_f)/(t \times A)$, where t is the time (s) and A is the leaf area (m^2). Leaf area was calculated by scanning detached leaves of a whole plant (HP Scanjet 5550c, Hewlett Packard, Palo Alto, CA, USA). The corresponding images were analysed with Adobe Photoshop CS (Adobe Systems, Inc., San Jose, CA, USA).

Osmotic root hydraulic conductance (*L*)

In the present work, root hydraulic conductance was measured on detached roots exuding under atmospheric pressure. Under these conditions, water is moving only because of an osmotic gradient between the soil solution and the root tissue. Therefore, according to Steudle (2000), the water would be moving mainly via the cell-to-cell path. This path predominates under conditions where the transpiration stream is restricted, compared with the apoplastic path (Steudle, 2000).

Pots were immersed in aerated nutrient solution. Plants were cut below the cotyledons 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 contamination. 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 $L = J_v/\Delta\Psi_s$, where J_v is the exuded sap flow rate and $\Delta\Psi_s$ is the osmotic potential difference between the exuded sap and nutrient solution.

Western and Northern blot analysis

Since osmotic root hydraulic conductance (L) depends on PIP activity (Javot *et al.*, 2003), we analysed PIP protein amount using Western blot (Aroca *et al.*, 2005). The PIP protein family is subdivided in PIP1 and PIP2 subfamilies, so we used specific antibodies for each (Daniels *et al.*, 1994; Kammerloher *et al.*, 1994). Both antibodies were successfully used in *P. vulgaris* roots before (Aroca *et al.*, 2006). At the same time, as aquaporin activity is up-regulated by phosphorylation (Maurel *et al.*, 1995), a specific antibody against PIP2 phosphorylated proteins at Ser-113 was used as well (Aroca *et al.*, 2005), since this residue is also present in the *PvPIP2;1* gene (Aroca *et al.*, 2006). Antibody against *Arabidopsis thaliana* PIP1 protein (Kammerloher *et al.*, 1994) was kindly provided by Prof. A. R. Schäffner (National Research Centre for Environment and Health, Neuherberg, Germany), antibody against *Arabidopsis thaliana* PIP2 protein (Daniels *et al.*, 1994) was kindly provided by Prof. M. J. Chrispeels (University of California San Diego, CA, USA), and antibody against phosphorylated PIP2 proteins (Aroca *et al.*, 2005) was kindly provided by Dr E. M. Herman (Donald Danforth Plant Science Centre, St Louis, MO, USA). Western blots were repeated twice with a different set of plants. To quantify the inmunoblot signal, the intensity of each band was measured using Adobe PhotoShop 5.5, corrected for the background and normalized against the intensity of the corresponding whole Coomassie brillant blue line (Aroca *et al.*, 2005).

We also evaluated the expression of *PIP* genes in the roots of *P. vulgaris* plants inoculated or not with *G. intraradices* under the four experimental conditions using Northern blot analyses. The *PIP* genes studied were *PvPIP1;1*, *PvPIP1;2*, *PvPIP1;3* and *PvPIP2;1*, previously cloned from total RNA of *P. vulgaris* roots (Aroca *et al.*, 2006). Northern blots were carried out as described previously by Porcel *et al.* (2006), and repeated twice with a different set of plants. The 3′ untranslated regions of four *PIP* genes of *P. vulgaris* were used as probes in the Northern blots. The *PIP* genes analysed were *PvPIP1;1* (accession number U97023), *PvPIP1;2* (accession number AY995196), *PvPIP1;3* (accession number DQ855475) and *PvPIP2;1* (accession number AY995195).

Quantitative real-time RT-PCR

Quantitative real-time PCR was used to analyse the expression of the *PvPIP1;2* gene, since it was not detected by Northern blots. The analyses were carried out essentially as described by Porcel *et al.* (2006), using iCycler (Bio-Rad, Hercules, CA, USA). cDNAs were obtained as described by Porcel *et al.* (2006) except that the random hexamer primer was replaced by a oligo$(dT)_{12-18}$ primer. The PCR reaction mixture was identical to that used by Porcel *et al.* (2006), but with the specific primers for the *PvPIP1;2* gene (PvPIP1;2For: GCTCTTCATTCTCCACGTCCTTG; PvPIP1;2Rev: ATGAAATGAAAGAATAAATAAAAGTAACC). These primers amplified a 196 bp DNA product corresponding to the 3′ untranslated region. The PCR programme used was the same as that described by Porcel *et al.* (2006). Standardization was carried out by measuring the expression of the *P. vulgaris* actin gene in each sample, using *P. vulgaris* actin-specific primers (Wen *et al.*, 2005). The relative abundances of transcription were calculated by using the $2^{-\Delta\Delta c_t}$ method (Livak & Schmittgen, 2001). For more details see Porcel *et al.* (2006).

Results

Mycorrhizal development

The percentage of root colonized by the AM fungi ranged from 34.1 ± 6.7% in cold-treated roots to 43.0 ± 5.4% in salt-treated roots, but differences among the four treatments were not significant ($P > 0.05$).

Relative water content

First we evaluated if AM colonization improved leaf water status under the different stresses, and if the applied stresses

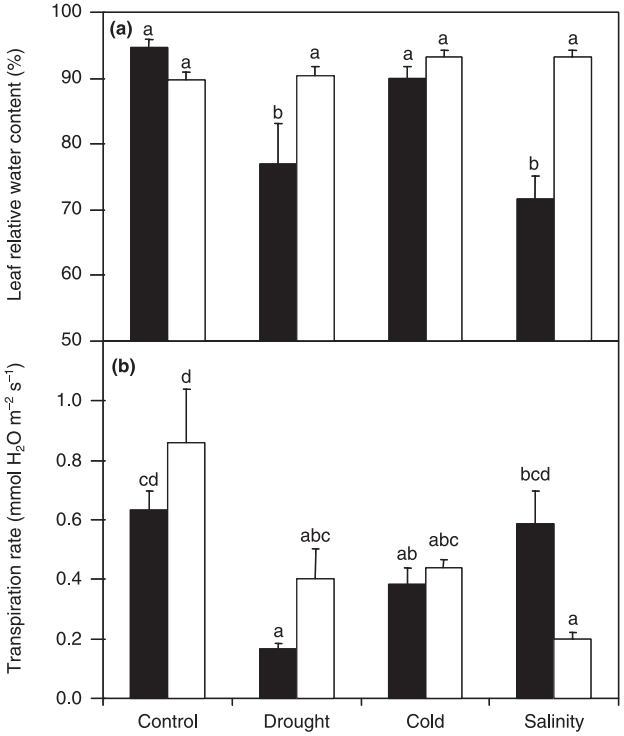

Fig. 1 Leaf relative water content (a) and leaf transpiration rate (b) of *Phaseolus vulgaris* plants not inoculated (nonAM, closed columns) or inoculated with *Glomus intraradices* (AM, open columns). Treatments: control, plants kept at 23°C and watered at full capacity with tap water; drought, plants kept at 23°C and not watered for 4 d; cold, plants transferred to 4°C for 2 d and watered at full capacity with tap water; salinity, plants kept at 23°C and watered every 2 d during a 6 d period with 10 ml of 0.5 M NaCl solution. Columns with different letters are significantly different ($P < 0.05$) after ANOVA and Fisher LSD tests. Columns represent mean ± SE ($n = 7$).

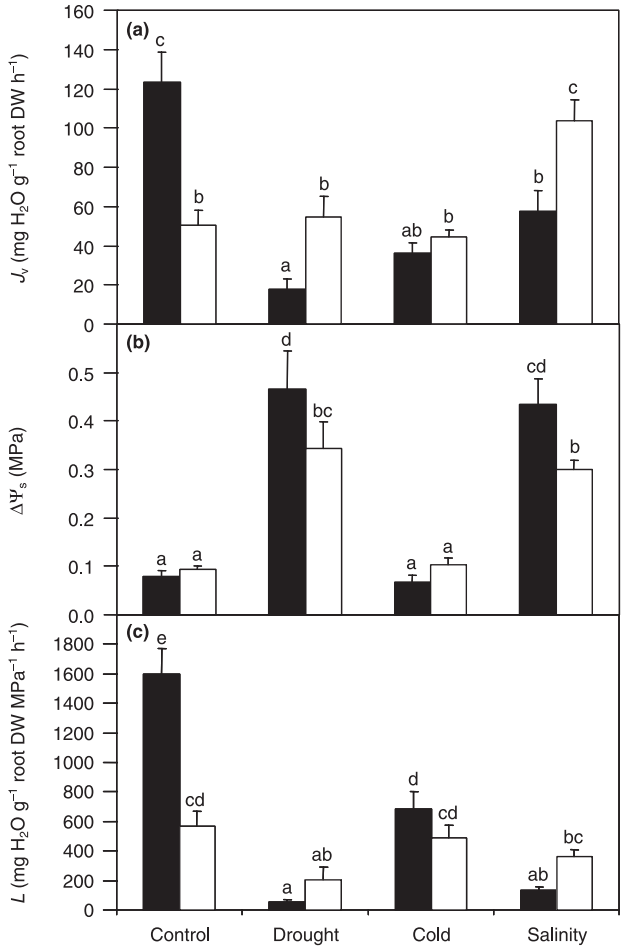

Fig. 2 Free exuded sap flow rate, J_v (a), osmotic potential gradient between free exuded sap and nutrient solution, $\Delta\Psi_s$ (b), and osmotic root hydraulic conductance, L (c), of *Phaseolus vulgaris* plants not inoculated (nonAM, closed columns) or inoculated (AM, open columns) with *Glomus intraradices*. Treatments: control, plants kept at 23°C and watered at full capacity with tap water; drought, plants kept at 23°C and not watered for 4 d; cold, plants transferred to 4°C for 2 d and watered at full capacity with tap water; salinity, plants kept at 23°C and watered every 2 d during a 6 d period with 10 ml of 0.5 M NaCl solution. Columns with different letters are significant different ($P < 0.05$) after ANOVA and Fisher LSD tests. Columns represent mean ± SE ($n = 5$).

caused leaf dehydration. In control plants, no significant differences ($P > 0.05$) in leaf RWC were observed between AM and nonAM plants (Fig. 1a). Cold treatment did not cause any change in RWC in either AM or nonAM plants (Fig. 1a). However, drought and salinity both diminished RWC in nonAM plants but not in AM plants (Fig. 1). Therefore, AM infection improved leaf water status under drought and salinity conditions, while cold treatment had no effect on leaf water content.

Leaf transpiration rate (*E*)

In control plants, no significant differences ($P > 0.05$) between AM and nonAM plants were detected (Fig. 1b). Drought and cold treatments diminished E to the same degree in both AM and nonAM plants (Fig. 1b). By contrast, after salinity treatment, E only decreased in AM plants, while the E of nonAM plants remained within control values (Fig. 1b).

Osmotic root hydraulic properties

The free exuded sap flow (J_v) of control plants was 2.4 times higher in nonAM plants than in AM plants (Fig. 2a). The three stress treatments (drought, cold and salinity) diminished J_v in nonAM plants, the highest inhibition being (85%) seen in drought plants, and the lowest (54%) in salinity plants (Fig. 2a). By contrast, none of the stress treatments diminished J_v in AM plants, and the salinity treatment increased J_v about twofold (Fig. 2a). Therefore, after drought and salinity treatments, AM plants had greater J_v than did nonAM plants, and after cold treatment both kinds of plants had the same J_v-values (Fig. 2a).

Control plants showed no differences in the osmotic potential gradient between soil solution and exuded sap ($\Delta\Psi_s$) (Fig. 2b). At the same time, cold treatment had no effect on $\Delta\Psi_s$ (Fig. 2b). By contrast, both drought and salinity treatments raised $\Delta\Psi_s$ in both AM and nonAM plants, although the increase was lower in AM plants (Fig. 2b). Since osmotic root hydraulic conductance (L) is the ratio between J_v and $\Delta\Psi_s$, under control conditions, L was 2.8 times higher for nonAM plants than for AM plants (Fig. 2c). At the same time, the three stress treatments decreased L in nonAM plants: the highest decrease (97%) was seen in drought plants, and the lowest (57%) in cold plants (Fig. 2c). However, only drought treatment inhibited L in AM plants (63%), while the other two treatments had no significant effect on it (Fig. 2c).

PIP gene expression

Under control conditions, *PvPIP1;1* gene expression was lower in AM than in nonAM roots (Fig. 3a). By contrast, *PvPIP1;2* expression was 1.5 times higher in AM than in nonAM roots (Fig. 4). No differences were found in the expression of *PvPIP1;3* or *PvPIP2;1* genes between AM and nonAM roots under control conditions (Fig. 3b,c). Drought treatment led to the elevation of *PvPIP1;3* and *PvPIP2;1* gene expression by factors of 2.2 and 1.5, respectively, in nonAM roots, but did not change the expression of the *PvPIP1;1* gene and decreased drastically the expression of the *PvPIP1;2* gene (Figs 3, 4). In AM roots, drought treatment increased the expression of the *PvPIP1;1* gene slightly, drastically diminished the expression of the *PvPIP1;2* and *PvPIP1;3* genes, and did not change the expression of the *PvPIP2;1* gene (Figs 3, 4). Moreover, except for the *PvPIP1;2* gene, nonAM roots showed higher gene expression of the other three *PIP* genes than did AM roots under drought conditions, the difference being more pronounced for *PvPIP1;3* and *PvPIP2;1* genes. Furthermore, each gene, again except for *PvPIP1;2*, responded differently to drought depending on the AM fungal presence (Fig. 3).

Cold treatment decreased the expression of *PvPIP1;1* and *PvPIP1;3* genes, and did not change the expression of *PvPIP1;2* and *PvPIP2;1* genes in nonAM roots (Figs 3, 4). In AM roots, cold treatment diminished the expression of *PvPIP1;1* and *PvPIP1;2* genes and did not change the expression of *PvPIP1;3* and *PvPIP2;1* genes (Figs 3, 4). Therefore, *PvPIP1;1* and *PvPIP2;1* responded in the same way to cold treatment in both AM and nonAM roots, while *PvPIP1;2* and *PvPIP1;3* responded to cold differently depending on the AM fungal presence.

Finally, salt treatment elevated the expression of the all *PIP* genes tested, except for *PvPIP1;2*, in both AM and nonAM roots, although the rise was higher in AM roots (Fig. 3). By contrast, *PvPIP1;2* gene decreased its expression in AM roots after salt treatment, while in nonAM roots it did not change (Fig. 4).

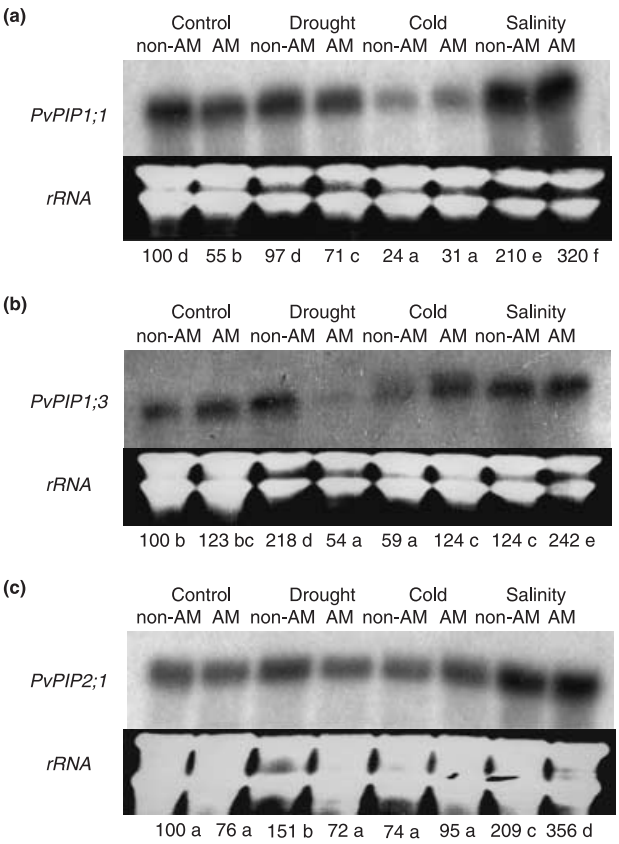

Fig. 3 Northern blot analyses using 3′ UTR as probes of *PvPIP1;1* (a), *PvPIP1;3* (b) and *PvPIP2;1* (c) in total RNA of *Phaseolus vulgaris* roots not inoculated (nonAM) or inoculated (AM) with the arbuscular mycorrhizal fungus *Glomus intraradices*. Treatments: control, plants kept at 23°C and watered at full capacity with tap water; drought, plants kept at 23°C and not watered for 4 d; cold, plants transferred to 4°C for 2 d and watered at full capacity with tap water; salinity, plants kept at 23°C and watered every 2 d during a 6 d period with 10 ml of 0.5 M NaCl solution. Quantification of the gene expression was performed by dividing the intensity value of each band by the intensity of corresponding rRNA stained with ethidium bromide. Control value of nonAM roots was referred as 100. Treatments with different letters are significantly different ($P < 0.05$) after ANOVA and Fisher LSD tests. $n = 3$.

PIP protein abundance

PIP1 proteins recognized by the antibody used were almost undetected in all treatments, except in drought roots, in which an increase in PIP1 protein abundance was observed (Fig. 5). An increase in PIP1 protein abundance was also observed in AM roots subjected to salinity, but not in the nonAM roots (Fig. 5).

Under control conditions, nonAM roots had a greater amount of PIP2 proteins than did AM roots (Fig. 5). However, all the stress treatments decreased the PIP2 protein amount in nonAM roots, while no changes were observed in AM roots (Fig. 5). These results resembled those of J_v and L (Fig. 2). Similar results were found when using specific antibody against phosphorylated PIP2 proteins (Fig. 5).

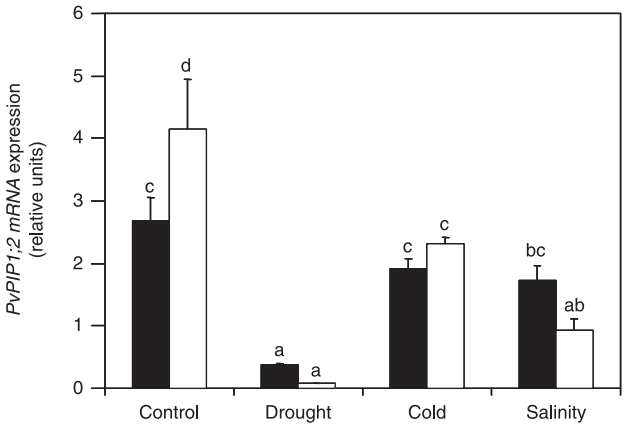

Fig. 4 mRNA levels of the *PvPIP1;2* gene determined by quantitative real-time PCR in *Phaseolus vulgaris* roots not inoculated (nonAM, closed columns) or inoculated (AM, open columns) with the arbuscular mycorrhizal fungus *Glomus intraradices*. Treatments: control, plants kept at 23°C and watered at full capacity with tap water; drought, plants kept at 23°C and not watered for 4 d; cold, plants transferred to 4°C for 2 d and watered at full capacity with tap water; salinity, plants kept at 23°C and watered every 2 d during a 6 d period with 10 ml of 0.5 M NaCl solution. The mRNA levels were calculated using the $2^{-\Delta\Delta}c_t$ method. Columns with different letters are significantly different ($P < 0.05$) after ANOVA and Fisher LSD tests. Columns represent mean ± SE ($n = 6$).

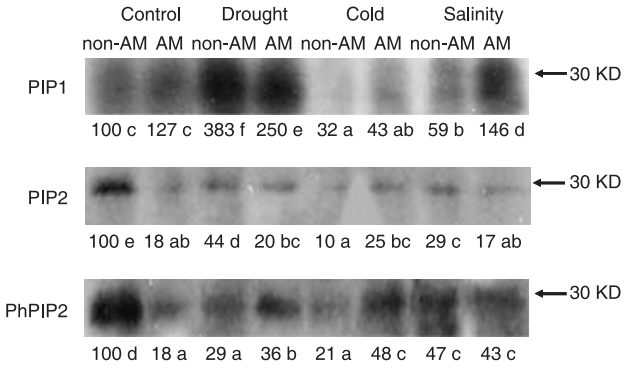

Fig. 5 Western blot analyses in total microsomes of *Phaseolus vulgaris* roots not inoculated (nonAM) or inoculated (AM) with the arbuscular mycorrhizal fungus *Glomus intraradices*, using antibodies against PIP1, PIP2, or phosphorylated PIP2 proteins. Treatments: control, plants kept at 23°C and watered at full capacity with tap water; drought, plants kept at 23°C and not watered for 4 d; cold, plants transferred to 4°C for 2 d and watered at full capacity with tap water; salinity, plants kept at 23°C and watered every 2 d during a 6 d period with 10 ml of 0.5 M NaCl solution. To quantify the inmunoblot signal, the intensity of each band was corrected for the background and normalized against the intensity of the corresponding whole Coomassie brillant blue line.

Discussion

Water relations

Colonization of *P. vulgaris* roots by the AM fungus *G. intraradices* prevented leaf dehydration caused by drought and salinity treatments as revealed by the higher RWC of AM leaves compared with nonAM leaves. These results confirm the beneficial effect of AM fungi in host plant water status under these two stresses (Rosendahl & Rosendahl, 1991; Porcel *et al.*, 2006). However, cold treatment did not cause any leaf dehydration in either of the two plant groups (AM or nonAM). Vernieri *et al.* (2001) found that *P. vulgaris* plants of the same cultivar used here recovered from cold-induced water stress after 48 h exposure to 5°C at a low constant irradiance (conditions also used here), in accordance with our results. However, AM symbiosis was also effective in protecting *L* from cold stress, as *L* decreased in nonAM roots while remaining unchanged in AM roots.

Leaf dehydration is caused by the imbalance between transpiration stream and root water uptake (Pardossi *et al.*, 1992). NonAM plants subjected to salinity did not change their transpiration stream (*E*), but J_v and *L* values were decreased, while AM plants subjected to salinity showed decreased *E* and increased J_v. This different behaviour seen in AM and nonAM plants under salinity conditions could explain why nonAM plants suffered from dehydration while AM plants did not. Under drought conditions, both kinds of plants showed a decrease in *E*, but only nonAM plants also showed a decreased J_v, this decrease in J_v being higher than that of *E* (85 vs 73%, respectively). These results support the finding that AM plants are able to take up more water from the soil than nonAM plants under drought conditions (Marulanda *et al.*, 2003; Khalvati *et al.*, 2005).

Under control conditions, nonAM plants had higher J_v and *L*-values than AM plants, but both kinds of plants had the same *E*. J_v and *L* were measured under osmotic conditions, and most probably the water was following the cell-to-cell path (Steudle, 2000). Therefore, as the whole water flow across the plant was the same in both groups of plants (equal *E* in both AM and nonAM plants), but J_v was lower in AM plants, it is plausible to assume that AM symbiosis enhanced apoplastic water flow in *P. vulgaris* plants. Indeed, Muhsin & Zwiazek (2002b) found that the ectomycorrhizal fungus *Hebeloma crustuliniforme* enhanced apoplastic water flow in *Ulmus americana* seedlings.

Both drought and salinity treatments caused a rise in the osmotic gradient between nutrient solution and xylem-exuded sap. In nonAM plants, such a rise could be caused, in part, by a concentration mechanism, since J_v decreased. However, in AM plants, J_v did not change as a result of drought and was even increased by salinity. Thus, an active transport of solutes should be taking place, in order to keep water flow across the root unchanged. Plassard *et al.* (2002) found an increase in nitrate and potassium ion fluxes in *Pinus pinaster* roots colonized by the ectomycorrhizal fungus *Rhizopogon roseolus*. In addition, Hohnjec *et al.* (2005) found an increase in the mRNA accumulation of several nitrate, phosphate and manganese transporter genes in *Medicago truncatula* roots colonized by *G. intraradices* and *G. mosseae*. However, these studies were

carried out under optimal conditions. Therefore, in future studies we would need to evaluate how drought and salinity modify the ion transport and ion transporter gene expression in AM and nonAM roots.

Aquaporin expression

PIP aquaporins are essential in regulating root hydraulic conductance (Javot *et al.*, 2003). However, their role in drought, cold or salinity tolerance is not clear. Thus, rice and tobacco plants overexpressing a *PIP1* gene increased their drought tolerance (Lian *et al.*, 2004; Yu *et al.*, 2005), whereas tobacco plants overexpressing a different *PIP1* gene decreased their drought tolerance (Aharon *et al.*, 2003). Overexpressing a *PIP1* gene did not change salt tolerance in tobacco plants (Aharon *et al.*, 2003), but overexpressing *PIP1* or *PIP2* aquaporins in *Arabidopsis* enhances salt tolerance at 100 mM NaCl, but not at 150 mM (Guo *et al.*, 2006). Finally, no differences in PIP expression or protein abundance were found when comparing two maize varieties differing in cold tolerance (Aroca *et al.*, 2005).

The most interesting finding of our results is that each *PIP* gene responded differently to each stress, depending on the AM fungal presence. Hence, *PvPIP1;3* and *PvPIP2;1* gene expression increased by drought in nonAM roots, but *PvPIP1;3* almost disappeared and *PvPIP2;1* did not change in AM roots. Also, except for *PvPIP1;2* gene expression, AM roots had lower *PIP* gene expression than did nonAM roots under drought conditions. This response of AM roots can be interpreted as a water conservation mechanism, as has been proposed by Porcel *et al.* (2006). On the other hand, *PvPIP1;3* gene expression decreased in nonAM roots but did not change in AM roots as a result of cold treatment. By contrast, *PvPIP1;2* gene expression decreased in AM roots but did not change in nonAM roots after cold treatment. All *PIP* genes in maize roots decreased in expression after 1 or 3 d at 5°C (Aroca *et al.*, 2005). Furthermore, in rice roots, all *PIP* genes decreased in expression after exposure to cold, except for *OsPIP1;3* (Sakurai *et al.*, 2005). However, there seems to be no correlation between a decrease in *PIP* gene expression and cold tolerance (Aroca *et al.*, 2005).

On the other hand, mycorrhiza formation by itself diminished expression of the *PvPIP1;1* gene, but increased expression of the *PvPIP1;2* gene. Valot *et al.* (2005) found that several plasma membrane proteins were differently regulated by inoculation with *G. intraradices*; some of them were down- or up-regulated, and others were induced. Since *G. intraradices* has the capacity to alter root hydraulic properties (Marulanda *et al.*, 2003; Khalvati *et al.*, 2005; Fig. 3), it is not strange that the fungus also changes *PIP* gene expression. The ectomycorrhizal *Amanita muscaria* down- and up-regulated the expression of several *PIP* genes in *Populus tremula* × *tremuloides* roots under optimal conditions (Marjanović *et al.*, 2005), but no stress treatments were included in this work.

In short experiments of salt stress (no more than 48 h) it has been found that almost all *PIP* genes decrease their root expression (Martínez-Ballesta *et al.*, 2003; Boursiac *et al.*, 2005; Guo *et al.*, 2006), although some specific *PIP* genes can increase their expression, as happened with lower NaCl concentrations (Boursiac *et al.*, 2005; Zhu *et al.*, 2005). However, Kawasaki *et al.* (2001) found that rice roots aquaporins that were initially down-regulated during salt stress were then up-regulated after 7 d. In the present work, *P. vulgaris* plants were subjected to salinity during 7 d, and we found an increase in three of the four *PIP* genes analysed, in accordance with Kawasaki *et al.* (2001).

Aquaporin abundance

When we compared PIP2 and phosphorylated PIP2 protein abundance, recognized by the antibodies used, to J_v or L, we found a strong correlation. PIP2 and phosphorylated PIP2 protein abundance was higher in control nonAM roots than in AM roots, as were L and J_v. Moreover, PIP2 and phosphorylated PIP2 protein abundance decreased as a result of the three stresses in nonAM roots, but remained constant in AM roots. It is known that PIP2 proteins have more water transport capacity than PIP1 proteins when they are expressed in *Xenopus* oocytes, although PIP2 activity can be enhanced by PIP1 proteins (Fetter *et al.*, 2004). Thus, the rise of PIP1 protein abundance induced by salinity in AM roots could explain the rise in J_v observed in AM roots under salinity conditions.

PIP1 protein abundance increased after drought treatment in both nonAM and AM roots, but L decreased in both kinds of roots. A decrease in L in nonAM roots can be explained by the decrease of PIP2 proteins, as has been mentioned previously. On the other hand, Ye *et al.* (2004) found that aquaporins can also close their pores in the presence of high osmotic potentials in the medium. Such a mechanism could be acting in *P. vulgaris* roots under drought conditions.

Conclusion

Here we found that AM symbiosis regulates root hydraulic properties and enhances L tolerance to drought, cold and salinity stresses. Regulation of root hydraulic properties by AM symbiosis was strongly correlated with the regulation of PIP2 protein amount and phosphorylation state. At the same time, gene expression of each *PIP* gene analysed responded differently to each stress, and this response also depended on the AM fungal presence. These results point to the possibility that each *PIP* gene analysed could have a different function and regulation by AM symbiosis under the specific conditions of each stress studied.

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