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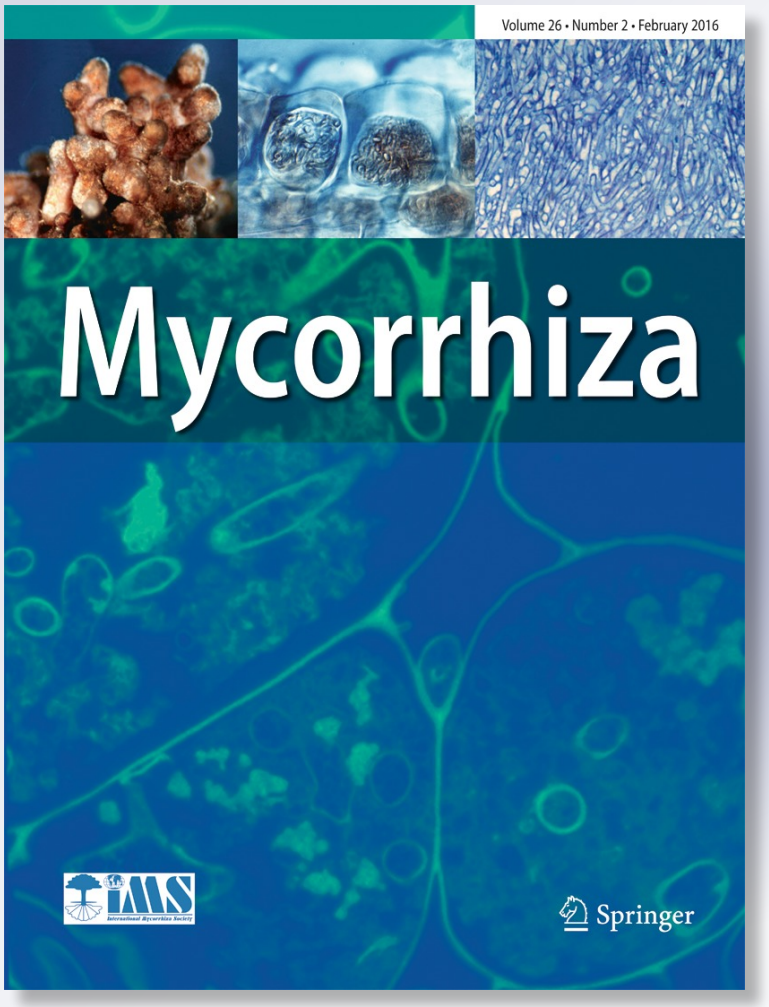

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ORIGINAL PAPER

Arbuscular mycorrhizal symbiosis and methyl jasmonate avoid the inhibition of root hydraulic conductivity caused by drought

Beatriz Sánchez-Romera[1] · **Juan Manuel Ruiz-Lozano**[1] · **Ángel María Zamarreño**[2] · **José María García-Mina**[2] · **Ricardo Aroca**[1]

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Abstract Hormonal regulation and symbiotic relationships provide benefits for plants to overcome stress conditions. The aim of this study was to elucidate the effects of exogenous methyl jasmonate (MeJA) application on root hydraulic conductivity (*L*) of *Phaseolus vulgaris* plants which established arbuscular mycorrhizal (AM) symbiosis under two water regimes (well-watered and drought conditions). The variation in endogenous contents of several hormones (MeJA, JA, abscisic acid (ABA), indol-3-acetic acid (IAA), salicylic acid (SA)) and the changes in aquaporin gene expression, protein abundance and phosphorylation state were analyzed. AM symbiosis decreased *L* under well-watered conditions, which was partially reverted by the MeJA treatment, apparently by a drop in root IAA contents. Also, AM symbiosis and MeJA prevented inhibition of *L* under drought conditions, most probably by a reduction in root SA contents. Additionally, the gene expression of two fungal aquaporins was upregulated under drought conditions, independently of the MeJA treatment. Plant aquaporin gene expression could not explain the behaviour of *L*. Conversely, evidence was found for the control of *L* by phosphorylation of aquaporins. Hence, MeJA addition modified the response of *L* to both AM symbiosis and drought, presumably by regulating the root contents of IAA and SA and the phosphorylation state of aquaporins.

Keywords Aquaporins · Arbuscular mycorrhiza · Drought · Methyl jasmonate · Root hydraulic conductivity

Introduction

The symbiotic association formed by arbuscular mycorrhizal (AM) fungi with most higher plants (Denison and Kiers 2011) results in increased uptake of water and nutrients from the soil, reduction in oxidative damage under stress conditions, and improved regulation of stomatal aperture to get higher water-use efficiency (Ruiz-Lozano 2003; Baslam and Goicoechea 2012). AM fungi can directly influence host plant water absorption through different mechanisms: Hyphae explore soil pores inaccessible to plants since their diameter is tenfold smaller than that of root hairs (Smith et al. 2010); they can facilitate the formation of stable aggregates in the soil thus increasing soil moisture retention properties (Auge et al. 2001), and they can transport 375 to 760 nL of water per hour (Faber et al. 1991), accounting for up to 20 % of the total water absorbed by plant roots (Ruth et al. 2011). On the other hand, root hydraulic conductivity (*L*), which provides information about the plant's capacity to take up water from the soil (Gallardo et al. 1996), usually decreases under drought stress, but this diminution may be partially avoided by AM symbiosis (El-Mesbahi et al. 2012; Bárzana et al. 2012).

It has been assumed that water moves mainly across the apoplastic pathway in plants under well-watered conditions, but under drought conditions, it seems to circulate mainly from cell to cell since transpiration is restricted (Steudle and Peterson 1998), and there is evidence to support the idea that *L* is ultimately governed by aquaporins (AQPs) which are

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✉ Ricardo Aroca
raroca@eez.csic.es

[1] Department of Soil Microbiology and Symbiotic Systems, Estación Experimental del Zaidín (CSIC), C/ Profesor Albareda 1, 18008 Granada, Spain

[2] CIPAV TimacAGRO International—Roullier Group, Polígono Arazuri-Orcoyen, c/C no. 32, 31160 Orcoyen, Navarra, Spain

intrinsic membrane proteins that facilitate water movement across cellular membranes following an osmotic gradient (Postaire et al. 2010; Lee et al. 2012). Of the different kinds of plant AQPs, plasma membrane intrinsic proteins (PIPs) are the most studied (Johanson et al. 2001). PIPs, which are mainly localized in the plasma membrane but can also be located in the membrane of other organelles (Zelazny et al. 2009), have been highly studied because they are supposed to control whole-plant water transport (Postaire et al. 2010) through changes in gene expression, protein abundance, localization or post-translational modifications such as phosphorylation or methylation events (Horie et al. 2011; di Pietro et al. 2013).

Marulanda et al. (2003) analyzed the capacity of six AM fungi to take up water from the soil and found that *Rhizophagus irregularis* and *Funneliformis mosseae* had the highest capacity for taking up water. The strategies followed by each fungus to protect the host plant against water deficit were different. *F. mosseae* downregulated *PIP* gene expression, decreasing the abundance of PIP proteins. In contrast, *R. irregularis* did not change the expression of *PIP* genes under drought stress but was the most efficient fungus in improving plant water uptake (Marulanda et al. 2003; Porcel et al. 2006).

Several AM fungal *AQP* genes have been cloned recently. Aroca et al. (2009) cloned an *AQP* gene called *RiAQP1* (formerly *GintAQP1*) from *R. irregularis*. Its expression varied depending on the host plant species and on environmental conditions, but since *RiAQP1* failed to activate water transport in *Xenopus laevis* oocytes, its function remains unknown (Aroca et al. 2009). Li et al. (2013) have cloned two other fungal *AQP* genes from *R. irregularis*, named *RiAQPF1* and *RiAQPF2* (formerly *GintAQPF1* and *GintAQPF2*, respectively), which exhibited a capacity for water transport in heterologous systems and whose expression increased under drought stress. Thus, the ability of AM plants to increase water uptake under drought conditions may be mediated by such fungal AQPs.

It is known that *L* is governed by plant hormones, e.g., abscisic acid (ABA) usually increases it (see Mahdieh and Mostajeran 2009). Moreover, the combined effects of ABA and AM symbiosis on *L* and *AQP* expression regulation have been studied (Aroca et al. 2008a, b; Ruiz-Lozano et al. 2009). However, the information about the role of other plant hormones in regulating *L* is scarce. Both SA and IAA may decrease *L* (Boursiac et al. 2008; Peret et al. 2012). Data on the role of ethylene in regulating *L* are limited and contradictory. Thus, Kamaluddin and Zwiazek (2002) observed an increase of *L* in aspen trees after application of pure ethylene under both aerated and hypoxic conditions. On the other hand, Li et al. (2009) reported that exogenous application of 1-aminocyclopropane-carboxylic acid, a precursor of ethylene, and ethephone, a chemical compound that liberates ethylene, caused a reduction of *L* in *Medicago falcata* plants. Moreover, these authors found that the reduction of *L* caused by phosphorus deprivation was abolished by inhibiting ethylene synthesis. With respect to MeJA, Sánchez-Romera et al. (2014) reported that exogenous MeJA was able to increase *L* in three different plant species (*Phaseolus vulgaris*, *Solanum lycopersicum* and *Arabidopsis thaliana*) growing hydroponically. However, no information is available about the role of these different hormones in regulating *L* when AM symbiosis is present.

Roots colonized by AM fungi usually have higher JA contents than non-colonized ones (Hause et al. 2002; Meixner et al. 2005), and JA synthesis-deficient plants develop less AM root colonization than wild-type plants (León-Morcillo et al. 2012). However, the addition of exogenous MeJA has been reported to reduce AM root colonization (Ludwig-Muller et al. 2002; Herrera-Medina et al. 2008), so that the role of JA in AM symbiosis is still a matter of debate. Based on the fact that (1) AM symbiosis usually modifies the *L* response to drought stress (Bárzana et al. 2012; El-Mesbahi et al. 2012), (2) AM symbiosis and drought modify the levels of JA in plant roots (De Ollas et al. 2013; Liu et al. 2013), and (3) MeJA application enhances *L* (Sánchez-Romera et al. 2014), the possibility that addition of exogenous MeJA may avoid the decrease in *L* usually caused by drought and thus modify the response of *L* to AM symbiosis was investigated. Since *L* seems to be regulated by several plant hormones, ABA, IAA and SA contents together with PIP AQP gene expression, protein abundance and phosphorylation state were also measured.

Materials and methods

Experimental design

Plants of *P. vulgaris* L. were used in the experiments following a combined factorial design with three factors: (1) plants inoculated or not with the AMF *R. irregularis* (Błaszk., Wubet, Renker & Buscot) C. Walker & A. Schüßler (biological factor), (2) untreated plants and plants treated with 200 μm MeJA (chemical factor), and (3) plants grown under well-watered or drought conditions (abiotic factor). Combinations of the three factors gave eight different treatments with a total of 64 plants (eight replicates per treatment). Four replicates per treatment were used for measurements of *L*, leaf relative water content, growth parameters and AM root colonization, and the other four replicates were frozen in liquid nitrogen immediately after harvest for the molecular and biochemical determinations described below. Physiological measurements and collection of plant samples were carried out 3 h after sunrise for all treatments, to avoid effects of diurnal fluctuations on plant processes. The experiments were repeated twice with similar

results; representative data are presented here from one of them.

Soil and biological materials

Loamy soil was collected from the Granada province (Spain), sieved (5 mm), diluted with quartz sand (0.2 mm) (1:2, soil/sand, *v*/*v*) and sterilized by steaming (100 °C for 1 h on three consecutive days). The original soil had a pH of 8.2 [measured in water 1:5 (*w*/*v*)], 1.5 % organic matter and nutrient concentrations (g kg^{-1}) were as follows: N, 1.9; P, 1.0; K, 6.9.

Seeds of beans (*P. vulgaris*) were soaked for 3 min in pure ethanol and then rinsed three times with distilled water. One seed was sown per pot containing 1 kg of the sterilized 1:2 soil/sand mixture. Mycorrhizal inoculum (*R. irregularis* isolate BEG 121) was bulked in an open-pot culture of *Trifolium repens* L. mixed with *Sorghum vulgare* Pers. X *Sorghum* × *drummondii* (Steud.) Millsp. & Chase plants and consisted of substrate (vermiculite/sepiolite, 1:1) containing spores, mycelia and infected root fragments. Ten grams of inoculum was introduced into each pot at sowing time. Uninoculated plants received the same amount of sterilized inoculum and 2-ml aliquot of a filtrate of the AM inocula in order to provide a general microbial population free of AM fungal propagules.

Growth conditions

Plants were grown 4 weeks from seed germination under greenhouse conditions with temperatures between 19 and 25 °C, 16/8 h light/dark, relative humidity of 50–60 % and maximum photosynthetic photon flux density of 600 μE m^{-2} s^{-1} (measured with a LICOR light meter, model LI-188B; Lincoln, NE, USA). Two weeks after the beginning of the experiment, all plants received a single 10 ml dose of 80 % Hewitt's nutrient solution (Hewitt 1952). MeJA-treated plants received three 10 ml doses of 200 μM MeJA, which were applied to the substrate throughout the last week of the experiment. The first dose coincided with the beginning of drought treatment; the second was applied 2 days later, and the last dose was applied the day before harvest. The MeJA concentration was based on a previous study (Sánchez-Romera et al. 2014), and the same procedure was used as in Aroca et al. (2008b).

Drought treatment was also applied during the last week. Soil moisture was measured each day in all pots with a ML2 ThetaProbe (AT Delta-T Devices Ltd., Cambridge, UK) as previously described (Porcel and Ruiz-Lozano 2004). The ThetaProbe measures volumetric soil moisture content by responding to changes in the apparent dielectric constant of moist soil. The volumetric soil moisture is the ratio between the volume of water present and the total volume of the sample. It is a dimensionless parameter, and it is expressed as a percentage (%). The volumetric soil moisture for well-watered pots was 20 % (corresponding to 150 mg H_2O g^{-1} soil) and 11 % for droughted pots (corresponding to 83 mg H_2O mg^{-1} soil). Water was supplied daily to maintain soil at field capacity during the first 3 weeks after germination, then half of the plants were subjected to drought (55 % field soil capacity) while the other half was maintained at field capacity. Plants took 2 days to reach 55 % field soil capacity and were maintained under drought and non-drought conditions for 7 days before harvesting.

Biomass production and water status

At harvest, four shoots and washed roots were separated per treatment and weighed independently to obtain fresh weights (FWs). Samples were then dried in an oven (75 °C) for 2 days to measure the dry weights (DWs). Leaf relative water content (RWC) was determined as follows: a piece of leaf from four different plants in each treatment was weighed immediately (FW) and then immersed in a closed tube with a wet piece of cotton tissue and incubated 24 h at 4 °C in darkness. After that, the piece of leaf was weighed again (turgid weight (TW)) and dried in an oven (75 °C) for 2 days (DW). RWC was calculated as (FW−DW)/(TW−DW)×100.

Mycorrhiza development

Percentage mycorrhizal root length was estimated in four root systems per treatment 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).

Root hydraulic conductivity (*L*)

L of four bean plants from each treatment was measured using the free exudation method, as described previously (Benabdellah et al. 2009). Under these conditions, water circulates through roots following the osmotic gradient between a root bathing solution and the root xylem. Therefore, according to Steudle's model (Steudle and Peterson 1998), water mostly flows through the cell-to-cell pathway. Four pots of each treatment were immersed in an aerated nutrient solution. Then, stems were cut with a razor blade just below the cotyledons, and a pipette connected to a silicon tube was attached to the stem. The liquid exuding up from the roots during the first 15 min was discarded to avoid phloem contaminations. Plants were maintained under exuding conditions for 2 h, and the stem exudates were collected and weighed. The osmolarity of the exuded sap was determined using a cryoscopic osmometer (Osmomat 030, Gonotec Gmbh, Berlin, Germany). *L* was

calculated as $L=Jv/\Delta\Psi s$, where Jv is the exuded sap flow rate and $\Delta\Psi s$ is the osmotic potential gradient between the exuded sap and the nutrient solution. According to Fiscus (1986), it was assumed that the reflection coefficient of bean plants was very close to 1 (0.98±0.01), and so, it was not included in the calculation of *L* (Sanchez-Romera et al. 2014).

Quantitative real-time RT-PCR

Expression analyses of the six PIP genes of bean known so far were carried out by quantitative real-time RT-PCR, using an iCycler (Bio-Rad, Hercules, CA, USA) (Benabdellah et al. 2009). Total RNA was isolated from bean roots by a phenol/chloroform extraction method followed by precipitation with LiCl (Kay et al. 1987). DNase treatment of total RNA and reverse transcription were done following Qiagen's protocol (Quantitect Reverse Transcription Kit Cat#205311, Qiagen, CA). The *PIP* genes analyzed were *PvPIP1;1* (Acc. No. U97023), *PvPIP1;2* (Acc. No. AY995196), *PvPIP1;3* (Acc. No. DQ855475), *PvPIP2;1* (Acc. No. AY995195), *PvPIP2;2* (Acc. No. EF624001) and *PvPIP2;3* (Acc. No. EF624002). Specific primers are described in Benabdellah et al. (2009). The PCR mix contained 1 μl cDNA, 10.5 μl Master Mix (Bio-Rad Laboratories S.A, Madrid), 8.6 μl deionized water, and 0.45 μl of each primer pair. The PCR program consisted in a 3-min incubation at 95 °C to activate the hot-start recombinant Taq DNA polymerase, followed by 32 cycles of 30 s at 94 °C, 30 s at annealing temperature and 30 s at 72 °C. Annealing temperatures were switched to 58 °C for *PvPIP1;1*, *PvPIP1;3*, *PvPIP2;1* and *PvPIP2;3* and to 60 °C (50 s) for *PvPIP1;2* and *PvPIP2;2*. In order to corroborate the hormone content data, the expression of some hormone-responsive genes was analyzed: allene oxide cyclase (*PvAOC*; Acc. No. XM_007151693.1), late embryogenesis abundant protein (*PvLEA18*; Acc. No. TC17584), Gretchen Hagen 3 gene (*PvGH3*; Acc. No. XM_007162320), and phenylalanine ammonia lyase (*PvPAL*; Acc. No. M11939). The primers used for the quantification of the expression of these genes are given in Table 1S. The PCR program was the same as above, but the annealing temperature was 58 °C. Standardization was carried out based on the expression of the *P. vulgaris* ubiquitin gene in each sample, as measured using the *P. vulgaris* ubiquitin-specific primers forward: CCAAGGAACTTCAGATTG CTG and reverse: GTCATCACCATCATCCATTCC. In relation to fungal AQPs, the genes analyzed were *RiAQP1* (Aroca et al. 2009) and *RiAQPF1* and *RiAQPF2* (Li et al. 2013). The primers used to amplify fungal genes are described in Aroca et al. (2009) and Li et al. (2013). The PCR program consisted in a 3-min incubation at 95 °C to activate the hot-start recombinant Taq DNA polymerase, followed by 40 cycles of 30 s at 94 °C, following of annealing temperature and 30 s at 72 °C. Annealing temperatures were 60 °C (30 s) for *RiAQP1*, 55 °C (50 s) for *RiAQPF1* and 58 °C (30 s) for *RiAQPF2*. Standardization was carried out based on the expression of the ribosomal *18S* gene in each sample (Aroca et al. 2009). The relative abundance of all transcripts was calculated using the $2^{-\Delta\Delta ct}$ method (Livak and Schmittgen 2001). Expression analyses were carried out in three independent RNA samples coming from root samples of three different plants and were repeated twice for each RNA sample.

Microsome isolation

Microsomal membranes were isolated from bean roots of four different plants, essentially as described by Benabdellah et al. (2009). Briefly, roots were homogenized in a grinding buffer (50 mM Tris–HCl pH=8.0, 2 mM EDTA, 250 mM sorbitol, 0.2 mM aminoethylbenzenesulfonylfluoride, 2 μg ml^{-1} aprotinin, 1 μg ml^{-1} leupeptin, 1 μg ml^{-1} chymostatin, 1 μg ml^{-1} pepstatin). A supernatant was collected after centrifugation at 15,000×*g* for 10 min, filtered through a double layer of cheesecloth and centrifuged again at 100,000×*g* for 2 h. The pellet was resuspended in 40 μl 5 mM KH_2PO_4, 330 mM sucrose and 3 mM KCl, pH 7.8. Finally, the samples were sonicated for 15 s.

Enzyme-linked immunosorbent assay

Plates containing 2 μg of microsomes in each well were incubated overnight at 4 °C with coating buffer (0.05 M carbonate/bicarbonate, pH 9.6). The plates were then washed three times for 15 min with Tris-buffered saline with 0.05 % Tween 20 (TTBS), blocked for 1 h at room temperature with 1 % bovine serum albumin (BSA) in TTBS and then washed again three times for 10 min with TTBS. After that, the plates were incubated with 1:2000 dilutions of antibodies raised against a N-terminal peptide of *PvPIP1;3* or a C-terminal peptide of *PvPIP2;1* (Marulanda et al. 2010). An antibody against phosphorylated PIP2 at the C-terminal end of *PvPIP2;1* (CAIKALG{pSER}FRSNA) was also used. A previous check for the specificity of each antibody (Sanchez-Romera et al. 2014) showed that each antibody was very specific to its target protein, phosphorylated or not. These antibodies were developed in rat (anti-PIP1) and in rabbits (anti-PIP2 and anti-phosphorylated PIP2) by Abyntek Company (Bilbao, Spain). The quantification of AQP protein was done by means of standard curves, using specific peptides as standards. The total amount of protein was quantified by the Bradford method using BSA as a standard (Bradford and Williams 1976). Goat anti-rat Ig for anti-PIP1 and goat anti-rabbit Ig for anti-PIP2 and anti-phosphorylated PIP2 coupled to horseradish peroxidase (Sigma) were used as secondary antibodies at a 1:10,000 dilution. The signal was developed using a TMB substrate (Sigma), which detects horseradish peroxidase (HRP) activity, yielding a blue colour (Amax=370 nm and 652 nm) that turns to yellow (Amax=450 nm) upon addition

of a 2 M sulphuric acid solution. For more details, see Sánchez-Romera et al. (2014).

Root ABA, JA, MeJA, IAA and SA accumulation

ABA accumulation in roots was quantified following the protocol described by Aroca et al. (2013). MeJA and JA contents in roots were measured as detailed by Sanchez-Romera et al. (2014) and IAA contents as explained by Bacaicoa et al. (2011). Extraction, purification and further analytical determination of SA were carried out in roots from four plants per treatment combination (n=4) as follows: 0.2 g of frozen plant tissues was homogenized with 50 μl of 1000 ng ml^{-1} d4 salicylic acid in methanol and 2 ml of MeOH/H_2O/HCOOH (90/9/1, $v/v/v$). The mixture was vortexed at 2000 rpm for 1 h and centrifuged at 15,000×g for 15 min at room temperature. For zero point, 5 ml of the supernatants was added to 0.3 ml acetic acid (0.2 %) and centrifuged at 15,000×g for 10 min. The supernatant was later injected into a LC/MS system. SA was quantified by HPLC linked to a 3200 QTRAP LC/MS/MS system (Applied Biosystems/MDS Sciex, Ontario, Canada), equipped with a turbo ion spray interface. Detection and quantification were performed by multiple reaction monitoring (MRM) in the negative-ion mode, employing a multilevel calibration graph with deuterated d4 salicylic acid as internal standards. The source parameters were curtain gas, 20.00 psi; ion spray voltage, 4000 V; GS1, 45.00 psi; GS2, 50.00 psi; scan mode, MRM; CAD gas, medium; temperature, 500 °C.

Statistical analysis

All experimental data were subjected to analysis of variance (ANOVA) with treatments as sources of variation (biological, chemical and abiotic factors). Post hoc comparisons with the least-significant difference (LSD) tests were used to investigate differences between groups. Linear regressions of all parameters were also made with respect to L.

Results

Mycorrhizal colonization, plant growth and water status

After 4-week growth, uninoculated bean roots did not show mycorrhizal colonization. The percentage of AM root length colonization was over 60 % for all AM plants, without significant effects caused by drought treatment or MeJA application (Fig. 1S). Also, no differences in shoot or root fresh and dry weights were observed among treatments (Fig. 2S and 3S).

Leaf relative water content (RWC) was enhanced by AM symbiosis in all plants, except those subjected to drought and MeJA at the same time (Fig. 1b). On the other hand, drought treatment had no effect on RWC except in AM plants treated with MeJA (Fig. 1b), although drought treatment globally diminished RWC (Table 1). Also, MeJA treatment only increased RWC in AM plants not subjected to drought (Fig. 1b).

Root hydraulic conductivity (L)

When plants were grown under well-watered conditions, L was unaffected by the application of exogenous MeJA, but it was decreased by AM symbiosis (Fig. 1a). Drought treatment diminished L in non-AM plants, and both MeJA application or AM symbiosis counteracted this inhibition (Fig. 1a). Also, drought treatment caused an increase of L in AM plants (Fig. 1a). Overall, MeJA treatment had a positive effect on L according to the ANOVA analysis (Table 1).

Plant PIP gene expression

Analysis of *P. vulgaris PIP* gene expression in root tissues, by q-RT-PCR, showed that expression of *PvPIP1;1* and *PvPIP2;3* was not altered by any treatment (Fig. 2; Table 1) whilst *PvPIP1;2* gene expression decreased in AM plants under well-watered conditions (Fig. 2). The combination of AM symbiosis and MeJA increased the expression of *PvPIP2;1* and *PvPIP2;2* genes under drought conditions (Fig. 2), while MeJA treatment also increased *PvPIP1;2* expression in non-AM plants under well-watered conditions. Under drought conditions, MeJA treatment increased *PvPIP1;3* and *PvPIP2;1* expression in non-AM and AM plants, respectively, and decreased *PvPIP2;2* expression in non-AM plants (Fig. 2).

Plant PIP protein abundance and phosphorylation state

Abundance of PIP1, PIP2 and PIP2 phosphorylated proteins (PIP2Ph) at Ser-280, analyzed by ELISA, showed significant changes only for PIP2Ph (Fig. 3a; Table 1). The presence of the AM fungus or the application of MeJA decreased PIP2Ph abundance under well-watered conditions. Drought reduced amounts of PIP2Ph protein in non-AM plants not treated with MeJA, and the exogenous MeJA application counteracted this effect (Fig. 3a). On the contrary, drought treatment caused an increase in the amount of PIP2Ph protein in AM plants in the absence of MeJA (Fig. 3a). A significantly (p<0.05) positive correlation was found between L values and PIP2Ph protein accumulation (Fig. 3b).

Expression of fungal AQPs

Gene expression analysis of *R. irregularis* AQPs (Fig. 4) showed that expression of *RiAQP1* and *RiAQPF2* was upregulated by drought treatment, without any effect of MeJA treatment. The expression of *RiAQPF1* gene did not change significantly (p>0.05) under any treatment.

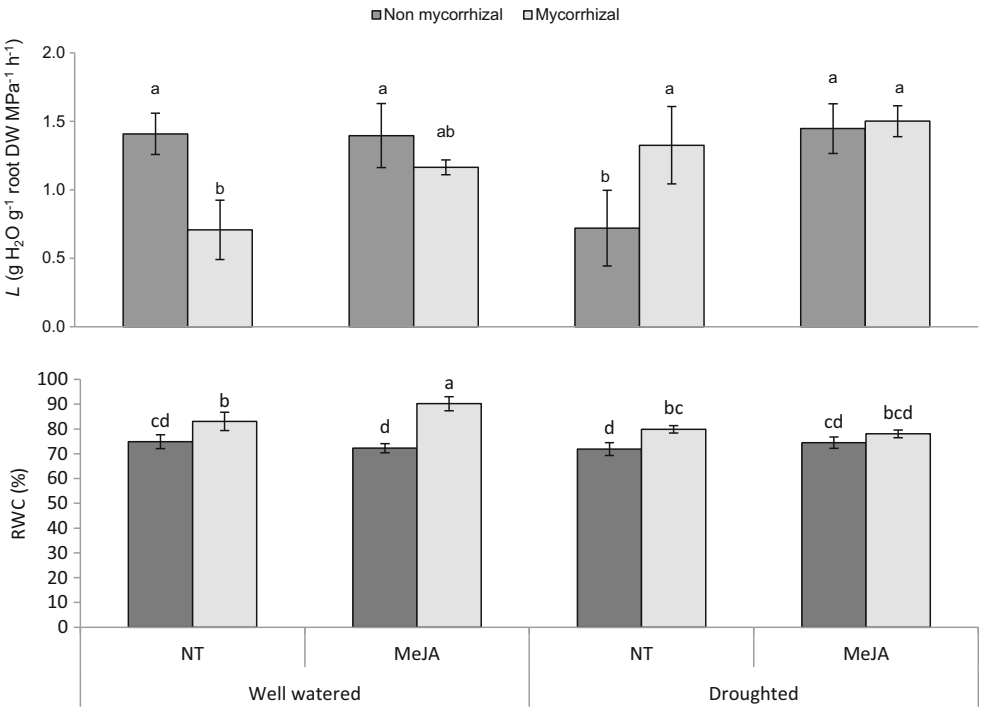

Fig. 1 Osmotic root hydraulic conductivity (*L*) in *P. vulgaris* plants, uninoculated (*dark grey columns*) or inoculated with *Rhizophagus irregularis* (*light grey columns*). Plants were treated or not (NT) with 200 μM MeJA and cultivated under well-watered or drought conditions for 7 days. *Columns* represent means±SE (n=4). *Different letters* indicate significant differences among treatments (p<0.05) after ANOVA and LSD tests

Hormone contents

The exogenous application of MeJA increased MeJA content in roots except in AM plants under well-watered conditions (Table 1; Fig. 5a). Under drought conditions, the increase of MeJA content in roots by MeJA addition was lower in AM plants than in non-AM plants (Fig. 5a). Water regime had no effect on endogenous MeJA contents (Table 1). Plants not treated with MeJA had the same JA content as plants treated with MeJA (Table 1; Fig. 5b). However, AM plants showed lower JA contents than non-AM plants under well-watered conditions (Fig. 5b). Also, drought treatment diminished JA

Table 1 Significance of sources of variation after three-way ANOVA analyses for each parameter

	AM	WR	MeJA	AMxWR	AMxMeJA	WRxMeJA	AMxWRxMeJA
L	ns	ns	*	**	ns	ns	ns
RWC	***	*	ns	*	ns	ns	*
PvPIP1;1	ns	ns	ns	ns	*	ns	ns
PvPIP1;2	***	ns	ns	**	ns	ns	ns
PvPIP1;3	***	ns	ns	ns	ns	**	*
PvPIP2;1	ns	**	ns	**	*	ns	ns
PvPIP2;2	**	*	ns	*	*	ns	ns
PvPIP2;3	ns	ns	ns	ns	*	ns	ns
PIP1	ns	ns	ns	ns	ns	ns	ns
PIP2	ns	ns	ns	ns	ns	ns	ns
PIP2Ph	**	ns	ns	**	ns	ns	**
MeJA	***	ns	***	ns	***	ns	*
JA	***	***	ns	***	ns	ns	ns
AIA	***	***	ns	**	**	***	***
ABA	ns	***	ns	ns	ns	ns	ns
SA	ns	ns	ns	**	*	*	**

The sources of variance were AM symbiosis (AM), water regime (WR) and MeJA addition (MeJA), as well as their interactions

ns not significant effect

*p<0.05, **p<0.01, ***p<0.001

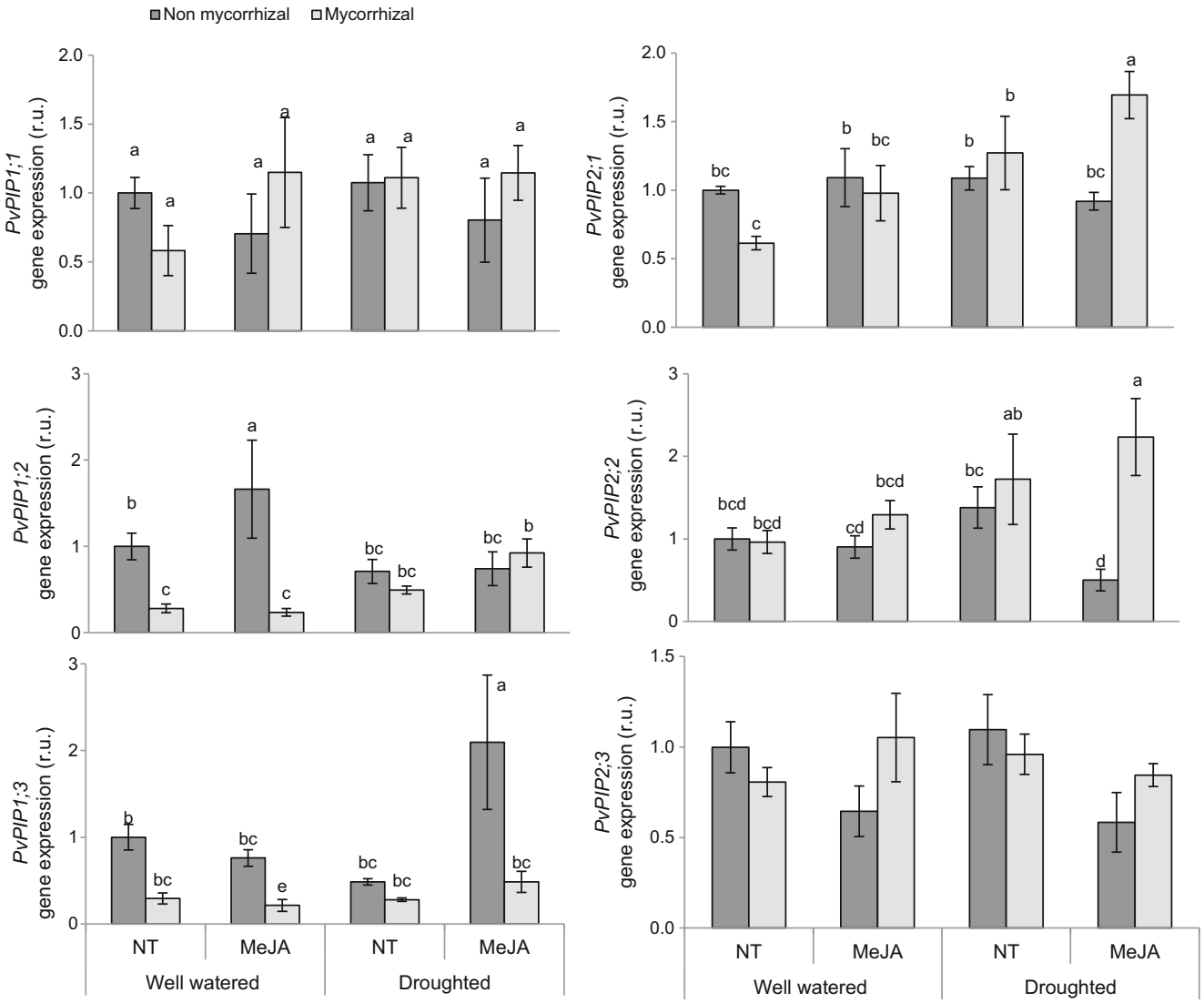

Fig. 2 Relative expression of *PvPIP1;1*, *PvPIP1;2*, *PvPIP1;3*, *PvPIP2;1*, *PvPIP2;2* and *PvPIP2;3* genes determined by q-RT-PCR in roots of *P. vulgaris*, uninoculated (*dark grey columns*) or inoculated with *Rhizophagus irregularis* (*light grey columns*). Plants were treated or not (NT) with 200 μM MeJA and cultivated under well-watered or drought conditions for 7 days. *Columns* represent means±SE (n=3). *Different letters* indicate significant differences among treatments (p<0.05) after ANOVA and LSD tests

contents in non-AM plants to the values of AM plants (Fig. 5b).

AM plants contained more IAA than non-AM plants, regardless of the treatment. However, MeJA application diminished IAA content in AM plants under well-watered conditions while the opposite was observed under drought conditions (Fig. 5c). Non-AM plants did not change their IAA contents under any treatment (Fig. 5c). Additionally, drought stress enhanced ABA content in all roots, but AM symbiosis or MeJA application had no further effects (Table 1; Fig. 5d). Drought treatment increased SA content only in non-AM plants without MeJA treatment (Fig. 5e), and the root content of SA correlated negatively (p<0.05) with L values (Fig. 5f). No more significant correlations between L and other hormones were found.

Expression analysis of some hormonal responsive genes (Fig. 4S) showed that the responses of the JA responsive gene *PvAOC* and the ABA responsive gene *PvLEA18* to different treatments were similar to that of JA and ABA root contents (Fig. 5 and Fig. 4S). Also, a significant positive correlation between the expression of these genes and the root contents of the corresponding hormone was found (Fig. 4S). For the other two genes (*PvGH3* and *PvPAL*), no relationship was found between their expression and corresponding hormone contents (*GH3* expression regarding IAA and *PAL* expression regarding SA). In fact, *PvGH3* expression was not changed by any treatment, and *PvPAL* expression was only increased by MeJA addition in non-AM plants under well-watered conditions (Fig. 4S).

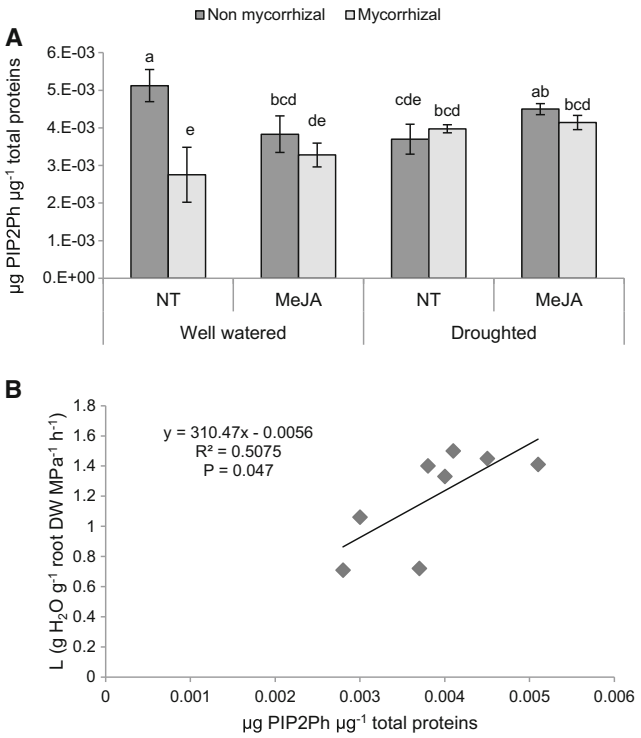

Fig. 3 **a** Abundance of phosphorylated PIP2s at Ser280 (PIP2Ph) proteins in roots of *P. vulgaris*, uninoculated (*dark grey columns*) or inoculated with *Rhizophagus irregularis* (*light grey columns*). Plants were treated or not (NT) with 200 µM MeJA and cultivated under well-watered or drought conditions for 7 days. *Columns* represent means±SE (n=4). *Different letters* indicate significant differences among treatments (p<0.05) after ANOVA and LSD tests. **b** Representation of the linear model established between *L* and PIP2Ph

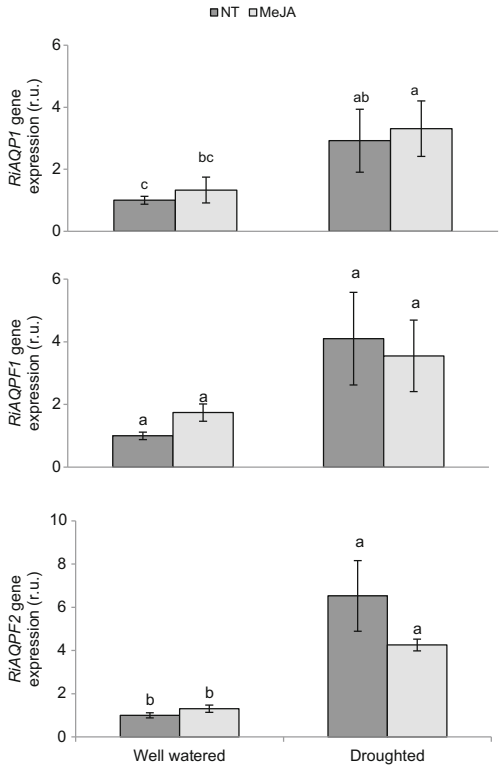

Fig. 4 Expression of *GintAQP1*, *GintAQPF1* and *GintAQPF2* genes in AM *P. vulgaris* roots, treated (*light grey columns*) or not (*dark grey columns*) with 200 µM MeJA under well-watered or drought conditions. *Columns* represent means±SE (n=3). *Different letters* indicate significant differences among treatments (p<0.05) after ANOVA and LSD tests

Discussion

It is known that a fine regulation of root hydraulic properties is crucial to tolerate drought stress (Silva et al. 2004) and that root hydraulic properties can be controlled by AM symbiosis (El-Mesbahi et al. 2012) and MeJA (Sánchez-Romera et al. 2014). The aim of the present study was to elucidate how exogenous MeJA affects root hydraulic properties and contents of endogenous hormones depending on the presence of an AM fungus and on the water regime. The drought treatment applied can be defined as a mild one, since no obvious physiological effects were found other than a reduction of *L* in non-AM plants without MeJA treatment and an increase of ABA contents in roots of all plants. Other authors have found an increase of ABA contents under drought conditions before any physiological parameter changed (Qaderi et al. 2012). So, results from the present study can be explained as initial or early responses of plants to drought. Also, the RWC of bean plants remained almost unchanged by the drought treatment, and root growth was not affected by any treatment so that a possible soil compaction effect can be ruled out.

The only compound of the jasmonate family for which a bioactive action has been proved is (+)-7-iso-jasmonyl-L-isoleucine (JA-Ile; Fonseca et al. 2009). However, it is known that other jasmonate compounds can be converted to JA-Ile, including Me-JA (Tamogami et al. 2008). Moreover, the jasmonate family comprises more than 15 different compounds, JA-Ile being one of the less represented and around ten times lower than JA (Mithöfer et al. 2013; Stitz et al. 2014). In addition, a good correlation between JA and JA-Ile levels has been reported (Mithöfer et al. 2013). The effect of Me-JA addition observed in the present work could be direct or via JA-Ile; future studies will be needed to solve this question.

Drought treatment caused a decrease in root JA content in non-AM plants, while that in AM plants remained unaltered. JA reaction against osmotic stresses seems to be time dependent, being reduced right after stress application and increasing after a few days. Thus, Zhang and Huang (2013) reported a reduction in JA contents in tomato leaves after 48 h of exposure to an osmotic stress, while Asensi-Fabado et al. (2012) found an enhancement of JA contents only after 12 days of salt stress in *Arabidopsis* leaves. Here, AM symbiosis seemed to have an effect on root JA only under well-watered conditions. Several authors found an increase of root JA levels as a consequence of AM development (Isayenkov et al. 2005; Meixner et al. 2005; Liu et al. 2013). On the

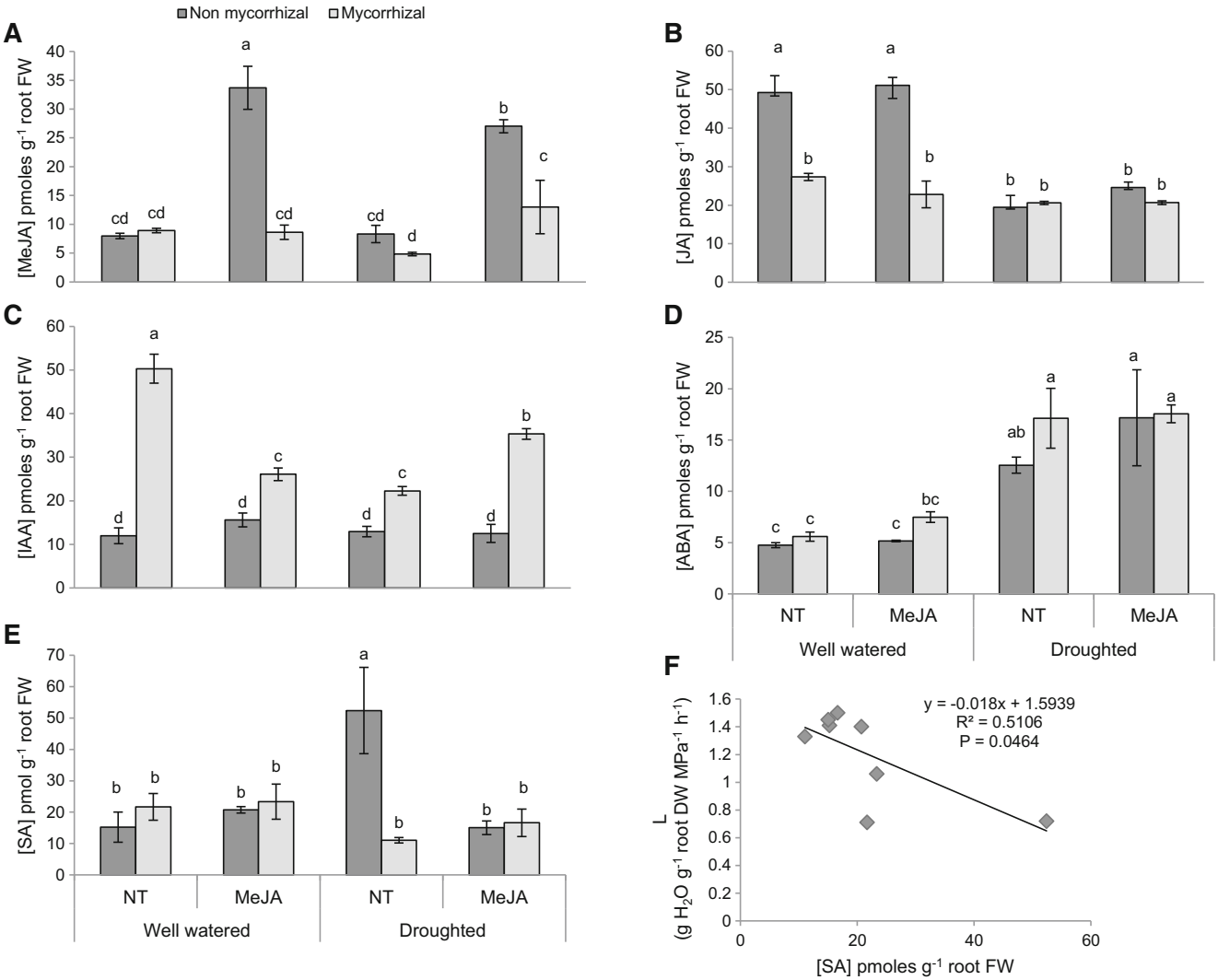

Fig. 5 Concentrations of **a** methyl jasmonate (MeJA), **b** jasmonic acid (JA), **c** indole-3-acetic acid (IAA), **d** abscisic acid (ABA) and **e** salicylic acid (SA) in roots of *P. vulgaris*, uninoculated (*dark grey columns*) or inoculated with *Rhizophagus irregularis* (*light grey columns*). Plants were treated or not (NT) with 200 μM MeJA and cultivated under well-watered or drought conditions for 7 days. *Columns* represent means±SE ($n=4$). *Different letters* indicate significant differences between treatments ($p<0.05$) after ANOVA and LSD tests. **f** Representation of the linear model established between *L* and SA

contrary, other authors found no changes in root JA contents after AM fungal inoculation (Riedel et al. 2008; López-Ráez et al. 2010), as was found here under drought conditions. Unfortunately, the soil water content was not controlled in the studies cited above, so it is possible that such discrepancies could be caused by different soil water availability. Also, changes in root JA levels induced by AM symbiosis may vary during time, decreasing at initial stages of symbiosis and increasing at later stages (Stumpe et al. 2005).

Expression levels of the PvAOC-responsive gene involved in the synthesis of JA (López-Ráez et al. 2010) validated the quantification of JA since the same behaviour was found for JA contents and *PvAOC* gene expression. Likewise, verification based on *PvLEA18* gene expression was positive for ABA contents but failed with *GH3* and *PAL* gene expression for IAA and SA levels. Although the responsiveness of the *GH3* gene to IAA has been documented in several plant species (Feng et al. 2015), its responsiveness in *P. vulgaris* plants was tested here for the first time. At the same time, other *GH3* or *PAL* genes not tested here may be responsive to IAA or SA, respectively.

Sanchez-Romera et al. (2014) found that plants treated with MeJA increased their *L* under hydroponic conditions. In the present study, *L* did not vary in plants grown in pots under well-watered conditions after MeJA application, regardless of the presence or not of the AM fungus. However, MeJA did increase *L* in treated plants under drought conditions. Thus, as it is documented for stomatal closure (Pospíšolová 2003), the effect of MeJA on *L* depends on the water regime. Regardless of the *PIP* gene expression, the abundance of PIP2Ph proteins increased in non-AM plants treated with MeJA under drought conditions. Phosphorylation at the C-terminal tail activates the

PIP2 aquaporin, increasing the transport of water by the cell-to-cell pathway (Prado et al. 2013). The *L* determination method used here only measures this pathway (Steudle and Peterson 1998). Moreover, a positive correlation was found between the amount of PIP2Ph and *L* values, which supports an active role of the phosphorylation on Ser 280. However, since the correlation analysis was done from data taken at only one time point, more studies are needed to confirm this relationship between *L* and PIP2Ph values.

It is known that JA and SA signalling pathways are antagonists (Proietti et al. 2013). Non-AM bean plants which had the lowest *L* values under drought conditions presented higher SA content in their roots, thus supporting findings by Boursiac et al. (2008) in *Arabidopsis*. SA production could play an important role in the reduction of *L* under drought conditions, this effect being counteracted by AM symbiosis or MeJA application. The negative correlation between SA contents and *L* suggests that the reduction of SA levels could be a mechanism to avoid *L* diminutions under drought conditions in AM plants (Bárzana et al. 2012), keeping in mind that this needs to be confirmed by data from additional experiments. Peret et al. (2012) have reported that IAA diminished *L* and aquaporin gene expression. Here, the highest IAA content of AM bean plants under well-watered conditions coincided with lowest *L* values and amount of PIP2Ph proteins. Drought treatment increased *L* in AM plants in the absence of MeJA addition. Siemens and Zwiazek (2004) similarly found a slight increase of *L* in trembling aspen trees under moderate drought stress conditions. The increase of *L* in AM bean plants under drought conditions was matched by an increase in the expression of *PvPIP2;1* and the two fungal AQP genes, as well an increase in the amount of PIP2Ph proteins. Conversely, where IAA levels decreased, this could be inhibiting *L* (Peret et al. 2012).

In conclusion, the response of *L* to AM symbiosis in bean plants was modified by addition of MeJA to the growth substrate so that the decrease in *L* caused by AM symbiosis was prevented by MeJA application under well-watered conditions. Both treatments (AM symbiosis and MeJA application) counteract the decrease in *L* caused by drought treatment, most probably because of a diminution of the SA content increased by drought. At the same time, phosphorylation of PIP2 proteins on Ser 280 may regulate *L* responses to environmental changes. Finally, it is proposed that root IAA contents could be implicated in the regulation of *L* caused by AM symbiosis.

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