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Original Article

Enhancement of root hydraulic conductivity by methyl jasmonate and the role of calcium and abscisic acid in this process

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ABSTRACT

The role of jasmonic acid in the induction of stomatal closure is well known. However, its role in regulating root hydraulic conductivity (*L*) has not yet been explored. The objectives of the present research were to evaluate how JA regulates *L* and how calcium and abscisic acid (ABA) could be involved in such regulation. We found that exogenous methyl jasmonate (MeJA) increased *L* of *Phaseolus vulgaris*, *Solanum lycopersicum* and *Arabidopsis thaliana* roots. Tomato plants defective in JA biosynthesis had lower values of *L* than wild-type plants, and that *L* was restored by addition of MeJA. The increase of *L* by MeJA was accompanied by an increase of the phosphorylation state of the aquaporin PIP2. We observed that MeJA addition increased the concentration of cytosolic calcium and that calcium channel blockers inhibited the rise of *L* caused by MeJA. Treatment with fluoridone, an inhibitor of ABA biosynthesis, partially inhibited the increase of *L* caused by MeJA, and tomato plants defective in ABA biosynthesis increased their *L* after application of MeJA. It is concluded that JA enhances *L* and that this enhancement is linked to calcium and ABA dependent and independent signalling pathways.

Key-words: *Arabidopsis thaliana*; *Phaseolus vulgaris*; *Solanum lycopersicum*; aquaporins.

INTRODUCTION

Most abiotic stress conditions cause plant tissue dehydration. To avoid such dehydration, plants may reduce water loss from the leaves by closing stomata or trying to absorb more water from the soil. Thus, to regulate their water status, plants control the opening and closing of their stomata, change their root architecture and modify their root water absorption capacity. The regulation of stomatal closure has been extensively studied and it is known that abscisic acid (ABA), reactive oxygen species, salicylic acid and methyl jasmonate (MeJA) are involved in this process (Wang & Song 2008; Hossain *et al*. 2011). Also, ABA is involved in enhancing root water transport capacity by increasing root hydraulic conductivity (*L*). Such involvement of ABA in regulating *L* has been proven by exogenous addition of ABA (Aroca *et al*. 2008; Mahdieh & Mostajeran 2009; Ruiz-Lozano *et al*. 2009; Kudoyarova *et al*. 2011), and by analysing plants with altered endogenous contents of ABA (Thompson *et al*. 2007; Parent *et al*. 2009). Some studies did not show that ABA enhanced *L* (Beaudette *et al*. 2007). It has also been observed that salicylic acid and auxins can cause *L* to decrease (Boursiac *et al*. 2008; Peret *et al*. 2012). Moreover, reactive oxygen species also regulate *L* although their effect can be to reduce *L* (Aroca *et al*. 2005; Boursiac *et al*. 2008; Parent *et al*. 2009) or to enhance *L* (Benabdellah *et al*. 2009). The role of the MeJA in regulating *L* has not been explored in detail, although Lee *et al*. (1996) observed an enhancement of root bleeding rate after application of exogenous MeJA to rice plants.

Jasmonic acid (JA) is a plant hormone that belongs to the octadecanoid family. Its main functions are related to growth processes, including inhibition, senescence and leaf abscission. JA's role in plant defence against pathogens has been studied in detail because it induces the expression of biotic stress-responsive genes (Farmer *et al*. 2003; Boter *et al*. 2004; Wasternack 2007; Wasternack & Hause 2013). JA can be converted into numerous conjugates and derivatives, some of which have a well-described biological activity, such as MeJA, cis-jasmonane and JA-amino acid conjugates (Kramell *et al*. 1995; Wasternack 2007; Pauwels *et al*. 2008; Fonseca *et al*. 2009; Wasternack & Hause 2013). The role of MeJA in regulating stomatal aperture has been well studied (Munemasa *et al*. 2007; Islam *et al*. 2010; Hossain *et al*. 2011; Akter *et al*. 2013). Thus, it is known that MeJA induces calcium concentration oscillations in guard cells (Munemasa

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et al. 2011), and its action is partially mediated by ABA enhancing biosynthesis (Hossain *et al*. 2011).

Water transport capacity of roots can be estimated by measuring *L*, which is determined by root architecture and intrinsic water permeability properties of the root system (Sutka *et al*. 2011). Root system water permeability depends on the paths that water follow through the root: the apoplastic and the cell-to-cell paths (see Steudle 1997). Aquaporins can affect the permeability of the cell-to-cell pathway because they are highly regulated integral membrane protein channels that facilitate the flow of water. These small proteins (23–34 KD) belong to the family of major intrinsic proteins (Maurel *et al*. 2008) and in higher plants; they can be divided into five clades depending on their amino acid sequence (Johanson *et al*. 2001; Sade *et al*. 2009). Aquaporins normally form tetramers where each monomer is a water channel.

Plasma membrane aquaporins (PIP) are divided into two phylogenic groups, PIP1s and PIP2s. The PIP2 proteins have shorter N- terminal extension and a longer C-terminal tail containing phosphorylation sites (Chaumont *et al*. 2000, 2001; Johansson *et al*. 2000; Johanson *et al*. 2001; Prak *et al*. 2008). Fetter *et al*. (2004) discovered that plant PIP2 proteins have a higher water transport capacity than PIP1 proteins. However, although PIP1 aquaporins often are inactive or have low activity, it is also known that co-expression of PIP1s and PIP2s in *Xenopus laevis* oocytes led to an increase in the osmotic water permeability coefficient (Pf) of such oocytes that was dependent on the amount of the PIP1 cRNA injected. This increase in Pf was attributable to the formation of tetramers containing both PIP1and PIP2 proteins (Fetter *et al*. 2004). Zelazny *et al*. (2007) reported an *in vivo* interaction between PIP1 and PIP2 proteins in mesophyll cells of maize. Moreover, most recently, Li *et al*. (2013) found a cooperative interaction also between PIP2 proteins in cotton. Regulation of aquaporin activity by intracellular pH, phosphorylation and divalent cation concentration has been reported (Maurel *et al*. 1995; Gerbeau *et al*. 2002; Tournaire-Roux *et al*. 2003; Törnroth-Horsefield *et al*. 2006; Azad *et al*. 2008; Wu *et al*. 2012b).

Some environmental stimuli related to biotic or abiotic stresses cause increases in cytosolic calcium concentration ($[Ca^{2+}]_{cyt}$). Oscillations of cytosolic calcium can induce the expression of new genes or calcium may bind to and activate other proteins like calmodulin or calcium-dependent protein kinases (Reddy *et al*. 2011; Sarwat *et al*. 2013). Different pharmacological agents have been used to understand the role of changes in $[Ca^{2+}]_{cyt}$ in this process. These include lanthanum chloride ($LaCl_3$) and heparin that can inactivate or block different calcium channels (Poutrain *et al*. 2009; Sun *et al*. 2009; Klaas *et al*. 2011; Amelot *et al*. 2012) or chemicals that can remove the calcium from the medium like ethylene glycol tetraacetic acid (EGTA) (Li *et al*. 2012; Yang *et al*. 2013). EGTA binds calcium ions in the apoplastic space to prevent their entry into the cells (Schwartz *et al*. 1988). $LaCl_3$ is a calcium channel blocker that prevents the flow of calcium ions in/out of the cells (Tester & Macrobbie 1990). Heparin inhibits only calcium channels that need inositol-3 phosphate to open. When this type of channel is inactivated, the calcium ions are kept inside the endoplasmic reticulum and vacuole (Yamamoto *et al*. 1990).

The aim of this study was to investigate the effects of MeJA on *L*, and to elucidate if these effects were mediated by calcium and ABA. The plant species chosen for this purpose was common bean (*Phaseolus vulgaris*) because it has been used extensively in our laboratory to study root hydraulic properties (Aroca 2006; Aroca *et al*. 2006, 2007; Benabdellah *et al*. 2009). Consequently, *P. vulgaris* plants were treated with MeJA together with $LaCl_3$, heparin or EGTA or with the ABA biosynthesis inhibitor fluridone (Chae *et al*. 2004; Hossain *et al*. 2011). However, we also took advantage of *sitiens*, a tomato mutant defective in ABA synthesis (Taylor *et al*. 1988) and *def-1*, a mutant defective in JA (Howe & Ryan 1999) to analyse the involvement of endogenous levels of these two plant hormones in the response to MeJA. Moreover, different Arabidopsis lines expressing several PIP proteins linked to the green fluorescence protein (Boursiac *et al*. 2005) were used to visualize *in vivo* the accumulation of PIP proteins in the roots in response to MeJA treatment.

MATERIAL AND METHODS

Experimental design

In this study, several experiments were carried out and three different plant species were used.

Experiments with P. vulgaris *(bean)*

Seeds of bean (cv Borlotto) were germinated in moist perlite for 1 week under dark conditions. After that, seedlings (7 days old) were transferred to aerated 8-L containers filled with 80% nutrient solution (Aroca *et al*. 2006). Plants were grown in a controlled growth chamber at 23:20 °C (day:night), in a photoperiod of 16:8 h (day:night), with a photosynthetic photon flux density of 300 μmol m^{-2} s^{-1}. After an additional 1 week of growth, the roots were exposed to different chemical treatments:

1 Dose-dependent effects of MeJA. We applied different concentrations of MeJA (0, 1, 10, 100, 500 and 1000 μM MeJA) and *L* measurements were taken 1 or 24 h after MeJA addition in both treated and non-treated plants. Root samples were immersed in liquid nitrogen and stored at –80 °C until use. One and 24 h corresponded to 6 and 5 h after lights turned on, respectively.

2 Effects of calcium channel blockers and chelators of calcium. The plants were supplied with MeJA (100 μM), two calcium channel blockers (1 mM heparin, 1 mM $LaCl_3$), an extracellular calcium chelator (1 mM EGTA) or a mixture of MeJA with each one of the above compounds. Heparin, $LaCl_3$ and EGTA were applied 24 h before MeJA addition. Root samples and *L* measurements were taken 24 h after MeJA addition in both treated and non treated plants.

3 Effects of fluridone, an inhibitor of ABA biosynthesis (Chae *et al*. 2004). Fluridone (10 μM) was applied 24 h

prior to MeJA addition (100 μM). We analysed ABA concentration and measured *L* at 1 and 24 h after MeJA application.

Experiments with Solanum lycopersicum *(tomato)*

Tomato seeds were sown in sterile vermiculite for 1 week and then seedlings were transplanted to aerated 8 L containers filled with a 80% nutrient solution (Aroca 2006). The nutrient solution was replaced every 7 days. The plants were grown in the same conditions as bean plants but for 2 months. Measurements of *L* were done 24 h after MeJA (100 μM) application. We did two different experiments with two different tomato mutant lines:

1. Tomato mutant plants that do not accumulate JA. In this experiment, the mutant *def-1,* which is deficient in JA accumulation (Howe & Ryan 1999) and the corresponding wild-type *Castle mart* were used.
2. Tomato mutant plants that do not accumulate ABA. The tomato mutant plant *sitiens* is deficient in the enzyme activity required for the final step of ABA biosynthesis (Taylor *et al*. 1988). Thus, *sitiens* plants and the corresponding wild type *Rheinlands Rhum* were used.

Experiments with Arabidopsis thaliana *(Arabidopsis)*

The Arabidopsis seeds were sterilized for 7 min in a solution containing 3.4 g L^{-1} Bayrochlore and 86% ethanol, followed by three rinses with absolute ethanol. The seeds were then dried under sterilized conditions and kept at 4 °C for 24 h in darkness before sowing.

Two different experiments were undertaken:

1. Effects of MeJA (100 μM) on root water transport. We grew Arabidopsis sterilized seeds in half strength Murashige and Skoog (1/2 MS) medium supplemented with sucrose (10 g L^{-1}) and agar (7 g L^{-1}; 1/2 MS agar) for 10 days. Plants were then transferred to hydroponic conditions for 2 weeks in the following nutrient solution [1 mM KNO_3, 0.5 mM $MgSO_4$, 2 mM $Ca(NO_3)_2$, 0.5 mM NaH_2PO_4, 50 mM Fe EDTA, 50 mM H_3BO_3, 12 mM $MnSO_4$, 0.7 mM $CuSO_4$, 1 mM $ZnSO_4$, 0.24 mM MoO_4Na_2, 100 mM Na_2SiO_3, 0.5 mM $CaCl_2$]. Finally, *L* was measured with a pressure chamber as described by Boursiac *et al*. (2005) and Javot *et al*. (2003).
2. Detection of aquaporins labelled with green fluorescent protein (GFP). We used transgenic Arabidopsis seeds expressing a specific aquaporin fused to GFP (Cutler *et al*. 2000; Boursiac *et al*. 2005). We checked lines expressing three PIPs (p35S::PIP1.1-GFP, p35S::GFP-PIP1.2, p35S::GFP-PIP2.2) or a control membrane protein (p35S::GFP-LTi6a), all under the control of the 35S CaMV promoter (p35S). These plants were grown in 1/2 MS medium for 6 days. They were then moved to fresh plates 1 day prior to 100 μM MeJA application to the medium. Since leaves never touched the medium, MeJA was only applied to the roots.

Parameters measured

*Root hydraulic conductivity (*L*)*

L was measured by two methods:

Both methods include a stem cutting to collect xylem sap exudation. Such cutting could induce the production of MeJA and derivates, but as it is shown in Supporting Information Fig. S1, MeJA treated roots had almost 100 times more MeJA content than untreated roots.

1. Free exudation method. *L* of beans plants was measured as described previously (Aroca 2006). Under these conditions, water circulates through roots following the osmotic gradient between the root bathing solution and the root xylem. Therefore, according to Steudle's model (Steudle & Peterson 1998), water mostly flows through the cell-to-cell pathway. Plants grown under hydroponic conditions were decapitated with a razor blade just below the cotyledons. A pipette connected to a silicon tube was attached to the stem. The liquid exuded from the root in the first 15 min was discarded to avoid phloem contaminations. Plants were maintained under exuding conditions for 90 min, and the exudates were collected and weighed. Also, the root dry weight of each plant was determined after incubation during 2 days at 70 °C. 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 expressed in a root dry weight basis and $\Delta\Psi s$ is the osmotic potential gradient between the exuded sap and the solution. *L* was measured in 15 plants. According to Fiscus (1986), we assume that reflection coefficient of bean plants was very close to 1 (0.98 ± 0.01), so we did not include it in the calculation of *L*.

2. Pressure chamber method. *L* of bean roots untreated and treated with 100 μM MeJA, ABA- and JA-deficient mutant tomato plants was measured by pressurizing the roots in a pressure chamber (SF-Press-Root, SolFranc, Tarragona, Spain). The stems of tomato and bean plants were cut with a blade, 3 cm above the root. Plastic tubes were put in the stems to collect the exudates. The root systems were placed inside pressure chamber with the nutrient solution and chemical of the different treatments. Finally, the roots were subjected to different pressures (0.2, 0.3, 0.4, 0.5 MPa) for 1 min at each pressure. The exuded sap was collected and weighted. For Arabidopsis, pressure chamber measurements were performed as described by Boursiac *et al*. (2005) and Javot *et al*. (2003). In all cases, sap flow was expressed in mg g (root dry weight)$^{-1}$ h^{-1} and plotted against pressure (MPa), with the slope being the *L* value in mg g (root dry weight)$^{-1}$ h^{-1} MPa^{-1}.

Quantitative real-time RT-PCR

Expression analyses of the six *PIP* genes of bean known until now were carried out by quantitative real-time RT-PCR (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). Treatment of total

RNA with DNAse (Promega, Madison, WI, USA) was performed according to the manufacturer's recommendations. Total RNA (2.5 μg) was reversed transcribed to first strand cDNA using AMV-RT enzyme and oligo(dT)$_{15}$ primer (Promega). Quantitative real-time RT-PCR was carried out by using an iCycler (Bio-Rad, Hercules, CA, USA), as described (Porcel *et al*. 2006). The *PIP* genes analysed 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 and PCR programme were as described (Porcel *et al*. 2006), except annealing temperatures, which were switched to 58 °C for *PvPIP1;1*, *PvPIP1;3*, *PvPIP2;1* and *PvPIP2;3*, and to 60 °C for *PvPIP1;2* and *PvPIP2;2*. Primers were designed to amplify, within each gene, a gene-specific fragment of the 3' untranslated region. Standardization was carried out based on the expression of the *P. vulgaris* ubiquitin gene in each sample, as measured by using *P. vulgaris* ubiquitin-specific primers (Wen *et al*. 2005). The relative abundances of transcripts were calculated by using the $2^{-\Delta\Delta ct}$ method (Livak & Schmittgen 2001). Expression analyses were carried out in three independent RNA samples coming from a pool of three different root samples, and were repeated three times for each RNA sample (Benabdellah *et al*. 2009). The Ct values for the ubiquitin reference gene ranged among the four treatments from 22.41 for untreated roots at 24 h to 23.24 for MeJA treated roots at 1 h.

To determine the JA deficiency of *def-1* tomato plants, the gene expression analyses of JA biosynthetic enzyme allene oxide synthase 2 (AOS2) and multicystatin (MC) genes, both genes being responsiveness to MeJA (López-Ráez *et al*. 2010), were analysed in wild-type (WT) and *def-1* roots. The primers used and the PCR protocol were the same as described López-Ráez *et al*. (2010).

Microsome isolation

Microsomal membranes were isolated from bean roots essentially as described Hachez *et al*. (2006). 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. It was filtered through a double layer of cheesecloth and centrifuged again at 100 000 *g* for 2 h. The pellet was resuspended in 40 μL of 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 (ELISA)

Plates containing 1 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 TTBS (Tris-buffered-saline with 0.05% Tween 20) and blocked for 1 h at room temperature with 1% bovine serum albumin (BSA) in TTBS, 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, and a C-terminal peptide of PvPIP2;1, (Marulanda *et al*. 2010). Also, an antibody against phosphorylated PIP2 at the C-terminal end of PvPIP2;1 (CAIKALG{pSER}FRSNA) was used. To check each PIP2 antibody specificity, we made an ELISA test where we cross reacted each one of the target peptides with different antibodies (Supporting Information Table S1). The results showed that each antibody was very specific to its target protein. These antibodies were developed in rabbits by Abyntek Company (Bilbao, Spain). The quantification of aquaporin amounts was done by means of standard curves, using specific peptides as standards. Total protein amount was quantified by the Bradford method using BSA as a standard (Bradford & Williams 1976). Goat anti-rabbit Ig coupled to horseradish peroxidase (Sigma-Aldrich, St. Louis, MO, USA) was used as secondary antibody at a 1:10 000 dilution. The signal was developed using a TMB substrate (Sigma-Aldrich), 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.

Calcium concentration in the xylem

A pressure chamber was used to collect the sap exudates of four plants of each treatment (control plants and plants treated for 24 h with 1 mM EGTA). For this, bean plants were grown under hydroponic conditions as described above, placed inside a pressure chamber at 0.25 MPa until 1 mL of sap was obtained. Calcium content was determined by the Ionomic Service of Estación Experimental del Zaidin, CSIC, Granada, Spain by means of ICP-OES technique (ICP-OES 720-ES Agilent Technologies, Santa Clara, CA, USA).

Cytosolic Ca^{2+} accumulation

We got small root pieces from control and MeJA (100 μM) treated bean plants. Measurements were taken 1 and 24 h after application of MeJA. Pieces taken 2 cm from the root apex and also root apexes were analysed. The pieces of root were incubated in a solution containing 20 μM Fluo-4/AM dye, 50 mM sorbitol and 5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (pH 7) and 0.03% (v/v) Pluronic, at room temperature for 90 min in the dark, followed by a washing with 50 mM sorbitol and 5 mM HEPES. Before measurements, samples were kept in dark at room temperature for 25 min. Fluorescence was examined with a Nikon Eclipse inverted microscope equipped with epifluorescence and appropriate filter sets (peak excitation, 590 nm; peak emission, 617 nm; Nikon Instruments, Amsterdam, Netherlands). Photographs were taken with a DS-Fi1 Nikon camera (Nikon Instruments). Root samples were mounted in a chamber with a clean cover slip attached to the bottom. The roots were observed with green fluorescence (520 nm). The recovery of fluorescence was quantified by

means of ImageJ software (Rasband W.S., NIH, http://imagej.nih.gov/ij/) which allows the measurement of the mean grey value within a region of interest.

Root ABA and MeJA accumulation

Root ABA accumulation of different bean and tomato plants was quantified by a high performance liquid chromatography tandem mass spectrometry (HPLC/MS/MS) system as described by Aroca *et al.* (2013). MeJA in bean and tomato roots was quantified as follow. MeJA and the internal standard d_2-MeJA were purchased from Sigma-Aldrich. A total of 0.2 g of frozen plant tissue (previously ground to a powder in a mortar with liquid N) was homogenized with 2 mL of methanol:water:formic acid (90:9:1, v/v/v). The deuterium-labelled internal standard (d_2-MeJA) was added (50 μL of a stock solution of 1000 ng mL^{-1} in methanol) to the extraction medium. After 1 h mixing, the samples at 2000 rpm using the Multi Reax shaker (Heidolph, Schwabach, Germany), the solids were separated by centrifugation at 12 000 *g* for 15 min. A total of 0.5 mL of supernatants was separated and 0.3 mL of 0.2% acetic acid was added. Before its injection in the HPLC-electrospary ionization-MS/MS system the samples were centrifuged at 12 000 *g* for 10 min and the supernatants were introduced into chromatographic vials. The MeJA was quantified by HPLC (2795 Alliance HT; Waters, Milford, MA, USA) linked to a 3200 Q TRAP LC/MS/MS system (Applied Biosystems/MDS Sciex, Ontario, Canada), equipped with an electrospray interface, using an reverse-phase column (Synergi 4 μ Hidro-RP 80A, 150 × 2 mm, Phenomenex, Torrance, CA, USA). A linear gradient of methanol (A) and 0.2% acetic acid in water (B) was used: 60% A for 3 min, 60% A to 85% A in 9 min, 85% A for 1 min and 85% to 60% A in 1 min, followed by a stabilization time of 4 min. The flow rate was 0.2 mL min^{-1}, the injection volume was 40 μL, and the column and sample temperatures were 30 °C and 20 °C, respectively.

The detection and quantification were performed by multiple reaction monitoring (MRM) in the positive-ion mode, employing a multilevel calibration graph with d_2-MeJA as internal standard. Compounds dependents parameters are listed in Supporting Information Table S2.

The source parameters are: curtain gas: 20.0 psi, GS1: 45 psi, GS2: 50 psi, ion spray voltage: 5000 V and temperature: 500 °C. Data samples were processed using Analyst 1.4.2 (Applied Biosystems/MDS Sciex).

Cell image analysis of Arabidopsis GFP seedlings

Images were captured at 1 cm from root tip with an inverted confocal laser-scanning microscope (LSM 510 AX70, Zeiss, Göttingen, Germany) with a 40 × water immersion or a 63 × oil immersion objective, essentially as described by Boursiac *et al.* (2005). Excitation wave length (as provided by an argon laser) was 488 nm and the fluorescence emitted by the GFP constructs was detected with a filter set for fluorescein isothiocyanate (BP 500 to 530). Fluorescence was quantified by means of ImageJ software as described (Boursiac *et al.* 2008).

Statistical analysis

All experimental data were subjected to analysis of variance (ANOVA) with treatments as sources of variation. Post hoc comparisons with the least significant difference (LSD) tests were used to investigate differences between groups. In the case of dye roots, aquaporin expression and abundance and xylem calcium concentration, Student's *t*-student was used to check differences between the two groups (treated or not treated).

RESULTS

MeJA dose-response experiment

In a preliminary experiment, treatment of bean roots with different concentrations of MeJA (0, 1, 10, 100 μM MeJA) during 1 or 24 h were performed to inquire which MeJA dose was optimal to get an effect on root hydraulic conductivity (*L*; Fig. 1a). Increases in exogenous MeJA concentration induced a progressive rise of *L*. The most significant effect was observed after application of 100 μM MeJA. Therefore, we chose exposure to 100 μM MeJA for our experiments. Plants treated with 100 μM MeJA had around 100 times higher MeJA concentration in their roots than untreated roots (Supporting Information Fig. S1). The MeJA effects on *L* could not be caused by an increase in the osmotic potential of the nutrient solution since it was 63 ± 3 KPa and 70 ± 5 KPa for 100 μM MeJA treated and untreated solutions, with no significant differences after a *t*-test ($P > 0.05$). In other studies, it has also been found that MeJA effects saturate above 100–200 μM, and that doses higher than 1 mM may cause toxicity (Kim *et al.* 2007; Gould *et al.* 2009; Yu *et al.* 2011). So, we further checked the effects of 0.5 and 1 mM MeJA addition on *L*. When we added 500 μM MeJA, we observed the same effect on *L* as with 100 μM JA (Supporting Information Fig. S2). However, addition of 1 mM JA caused an inhibition of *L* after 24 h of exposure (Supporting Information Fig. S2). Therefore, MeJA doses higher than 100 μM were not applied in further experiments. The enhancement of *L* by 100 μM MeJA determined by the free exudation method was confirmed using the pressure chamber technique (Supporting Information Fig. S3). It should be mentioned that using the free exudation method water only circulates through the cell-to-cell pathway, but when the pressure chamber was used, water circulates through both pathways, cell-to-cell and apoplastic ones. So, MeJA increased the water flow through both pathways. Therefore, for the next experiment with bean plants only the free exudation method was used, keeping in mind that this method only quantifies the cell-to-cell water flow. Furthermore, we calculated *L* of 100 μM MeJA and untreated bean roots at 30 min intervals (Supporting Information Fig. S4), and at all intervals MeJA treated roots had higher *L* than untreated roots. Additionally, we tested if the low levels of JA in the tomato mutant *def-1* resulted in low levels of *L* (Fig. 1b). *L* was indeed lower in *def-1* than in WT plants, and application of 100 μM MeJA during 24 h partially restored *L* values in the mutant. In these particular tomato plants, the MeJA levels were below the

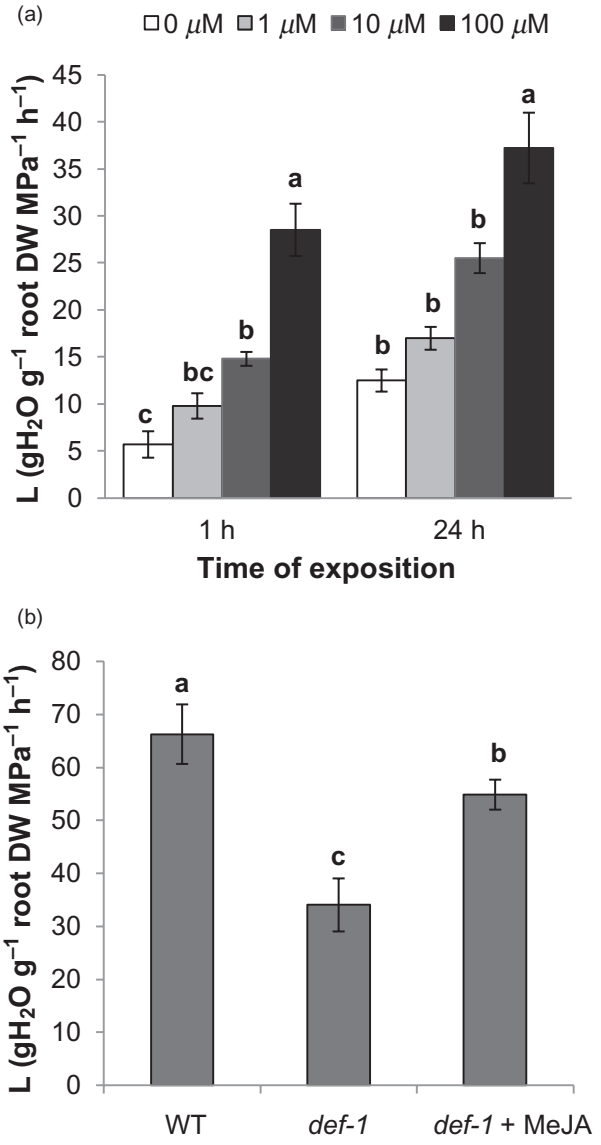

Figure 1. Root hydraulic conductivity (L) of *Phaseolus vulgaris* (a) and *Solanum lycopersicum* (b) plants. (a) *P. vulgaris* plants were subjected to 0, 1, 10 or 100 μM MeJA during 1 and 24 h. L was measured by the free exudation method. Bars represent mean ± SE. Within each time period different letters mean significant differences ($P < 0.05$) after analysis of variance (ANOVA) and least significant difference (LSD) test ($n = 5$). (b) L values of wild type (WT) and *def-1* mutant line deficient in JA biosynthesis, either untreated (*def-1*) or after 24 h of 100 μM MeJA application (*def-1* + MeJA). L was measured with a pressure chamber. Bars represent mean ± SE. Different letters mean significant differences ($P < 0.05$) after ANOVA and LSD test ($n = 8$).

detection limit (4 pmol g^{-1} FW) in both WT and *def-1* plants. However, the impairment of accumulating JA by the *def-1* plants is well supported by the literature (Howe *et al.* 1996; Howe & Ryan 1999; Li *et al.* 2002; O'Donnell *et al.* 2003). Also, we checked the gene expression levels of two genes regulated by MeJA (AOS2 and MC, López-Ráez *et al.* 2010), and both genes were down-regulated in *def-1* roots (Supporting Information Fig. S5). The overall results establish that exogenous MeJA increased *L* in both bean and tomato plants, and that lower endogenous levels of JA result in lower values of *L*.

MeJA effects on *PIP* expression and PIP abundance in *P. vulgaris* roots

Gene expression of the six different PIP aquaporins of *P. vulgaris* known so far (*PvPIP1;1*, *PvPIP1;2*, *PvPIP1;3*, *PvPIP2;1*, *PvPIP2.2*, *PvPIP2;3*) was analysed (Fig. 2). One hour after the addition of 100 μM MeJA (Fig. 2a), gene expression of *PvPIP1;2* and *PvPIP2;1* aquaporins was found to be decreased, whereas gene expression of *PvPIP2;2* was

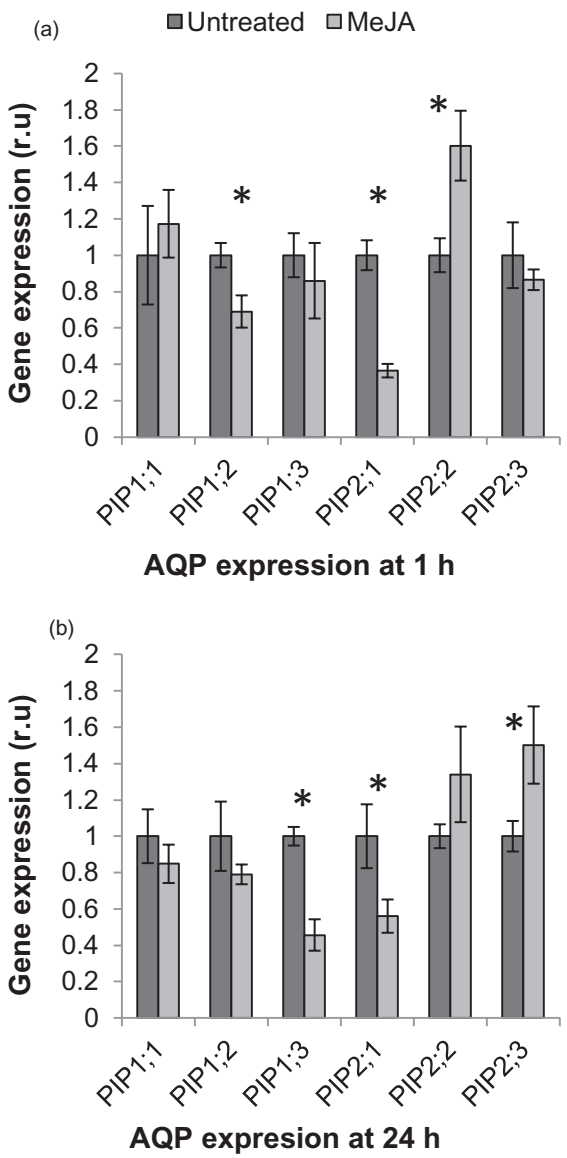

Figure 2. Relative expression of *PvPIP1;1*, *PvPIP1;2*, *PvPIP1;3*, *PvPIP2;1*, *PvPIP2;2*, *PvPIP2;3* genes of *Phaseolus vulgaris* in untreated roots or roots treated with 100 μM MeJA for 1 h (a) or 24 h (b). Asterisks mean significant differences ($P < 0.05$) between untreated and treated roots after Student's *t*-test ($n = 5$).

enhanced. Expression of the other three genes did not change. After 24 h of application of MeJA (Fig. 2b), gene expression of *PvPIP1;3* and *PvPIP2;1* was down-regulated, while that of *PvPIP2;3* was up-regulated. Expression of the other three aquaporin genes did not change. It should be mentioned that the nomenclature for PIP genes between different species does not mean homology, as shown in Supporting Information Fig. S6. The abundance of PIP1 and PIP2 proteins and of the PIP2 proteins phosphorylated at the Ser 280 was analysed in control roots and roots treated with 100 μM MeJA for 1 or 24 h. We used ELISA assays with specific antibodies: anti-PIP1, anti-PIP2 (Marulanda *et al*. 2010), and anti-PIP2 phosphorylated at Ser 280 (anti-PIP2PH). The number of the Ser residue refers to the PvPIP2;1 protein, but these residues are conserved among all PIP2 proteins (Prak *et al*. 2008). Combinations of anti-PIP2 and anti PIP2PH antibodies and their antigen peptides established that each antibody only recognizes its corresponding peptide (Supporting Information Table S1). Figure 3 shows that there was a ~2-fold increase in the phosphorylation of Ser 280 of PIP2 proteins (PIP2PH) after 1 and 24 h of exposure to MeJA. After 24 h, the total amount of PIP2 proteins without phosphorylation in their C-terminal region was also increased by 2.5-fold (Fig. 3b). In contrast, PIP1 protein levels did not increase in response to MeJA treatment.

Effects of MeJA on PIP abundance in intact roots of *A. thaliana*

To visualize how PIP aquaporins responded *in vivo* to MeJA, we used transgenic *A. thaliana* plants that constitutively express fusions of GFP with three of the most highly expressed PIP isoforms, *At*PIP1;1 *At*PIP1;2 and *At*PIP2;2. Similar to bean and tomato, *L* of *A. thaliana* plants is responsive to MeJA and was increased by ~50% after a treatment with 100 μM MeJA for 24 h (Fig. 4a). Laser scanning confocal microscopy of root cells revealed that plants treated with MeJA in the same conditions had a higher fluorescence of GFP-PIP1;2 (+109%) and GFP-PIP2;2 (+30%) than plants grown under standard conditions (Fig. 4b). However, the opposite effect was observed in plants expressing a PIP1;1-GFP fusion protein, with a 34% decrease in abundance after MeJA treatment. As a control, we also analysed another membrane-associated protein called LTi6 (Boursiac *et al*. 2005). MeJA had no effect on the abundance of a GFP-LTI6a reporter protein.

Calcium accumulation inside the cells

Since calcium is implicated in MeJA signalling, we analysed several root sections stained with the calcium dye FLuo 4 AM, to track possible variations in $[Ca^{2+}]_{cyt}$ owing to MeJA treatment. We took image samples at the root tip and 2 cm above the tip. The recordings showed that at both root sites, MeJA-treated roots had a more intense fluorescent signal than control roots after 24 h of exposure, but not after 1 h (Fig. 5). These results indicate that $[Ca^{2+}]_{cyt}$ increased after 24 h of addition of MeJA.

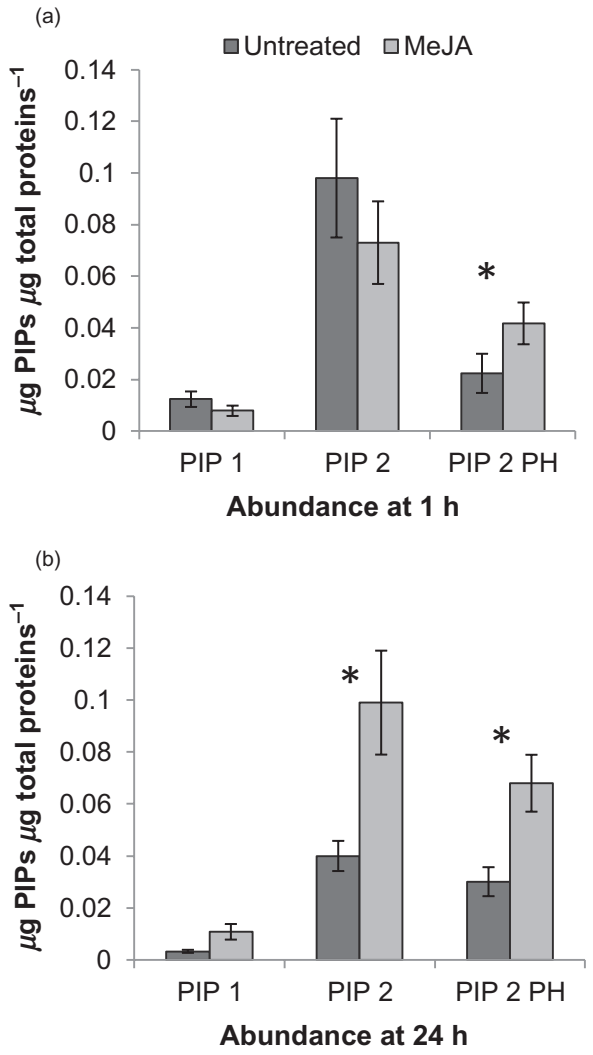

Figure 3. Abundance of PIP1s, PIP2s, phosphorylated at Ser280 PIP2s (PIP2PH), proteins in roots of *Phaseolus vulgaris* untreated or treated with 100 μM MeJA after 1 h (a) or 24 h (b). Bars represent mean ± SE. Asterisks mean significant differences ($P < 0.05$) between untreated and treated roots after Student's *t*-test ($n = 6$).

Calcium channel blockers and calcium chelator effect on MeJA enhances *L*

Since we observed that intracellular calcium ions only increased in root cells after 24 h of exposure to MeJA, the following experiments were done only after 24 h of exposure to MeJA. We used chemicals that either reduce the external concentration of calcium (EGTA), block several calcium channels located in cell membranes ($LaCl_3$) or block IP_3-dependent Ca channels (heparin) (Yamamoto *et al*. 1990; White 2000; Poutrain *et al*. 2009; Klaas *et al*. 2011; Amelot *et al*. 2012; Yang *et al*. 2013).

EGTA experiment

We first checked the capacity of EGTA to eliminate calcium ions from the medium (Supporting Information Fig S7). For

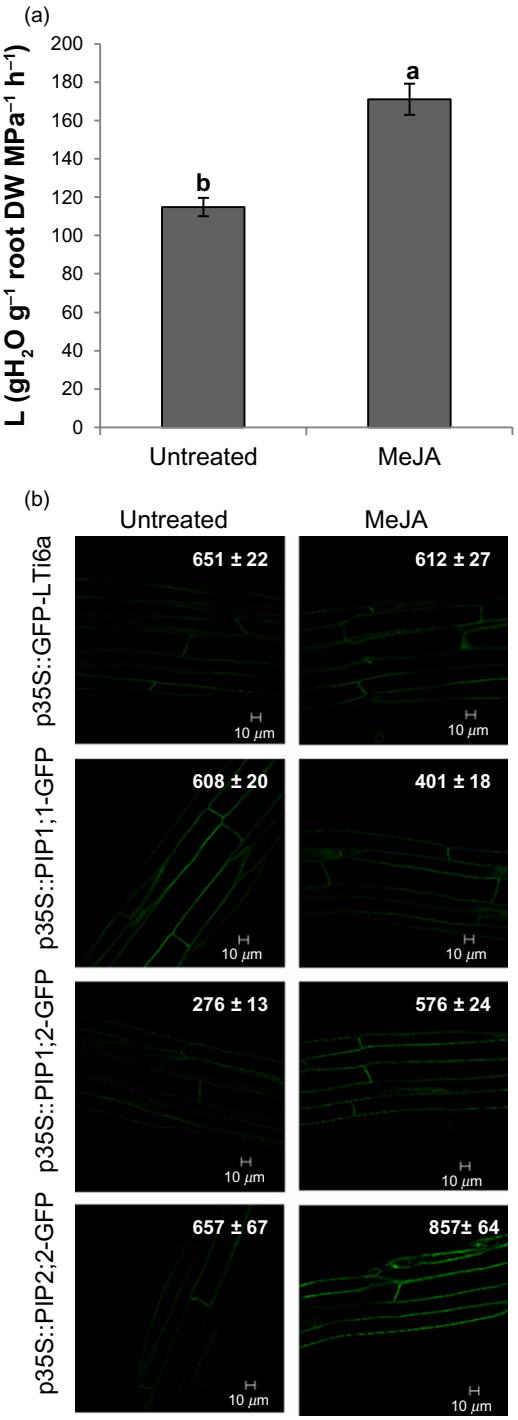

Figure 4 (a) Root hydraulic conductivity (L) of wild type Arabidopsis plants either untreated or exposed to 100 μM MeJA for 24 h. L was measured by a pressure chamber technique, as described in the *Materials and Methods*. Bars represent mean ± SE. Different letters mean significant differences ($P < 0.05$) between untreated and treated roots after Student *t*-test ($n = 55$). (b) Representative confocal images of Arabidopsis root cells expressing the indicated fusions with GFP, all under the control of a p35S promoter. The p35S::GFP-LTi6a line was used as a control. Plants were either untreated or treated with 100 μM MeJA for 24 h. Numbers inside the figures represent the mean ± SE green intensity of at least 30 different roots.

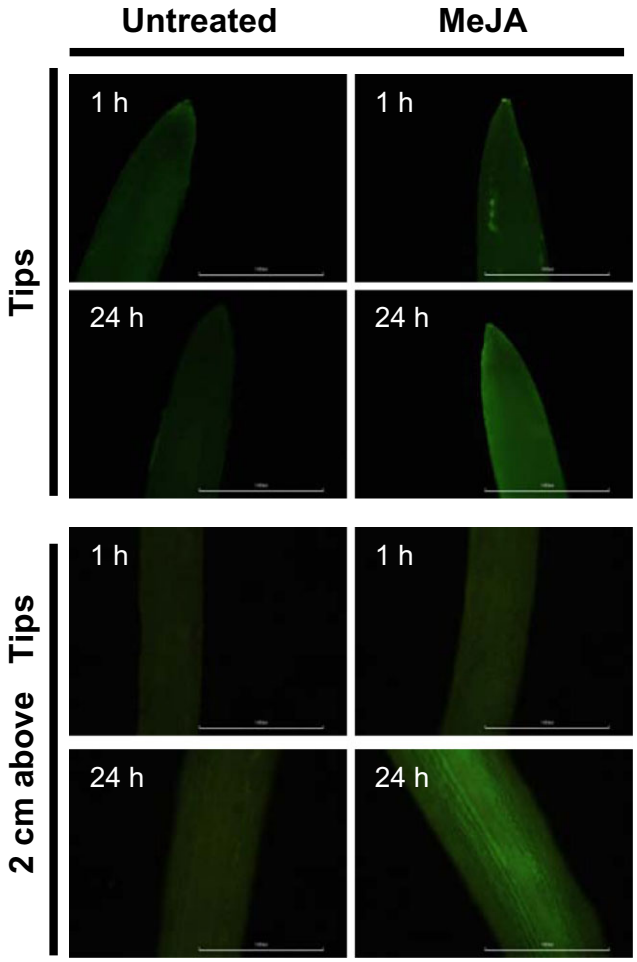

Figure 5. Staining of *Phaseolus vulgaris* roots with Fluo-4/AM dye to determine $[Ca^{2+}]_{cyt}$. Images were taken at root tips and at sections 2 cm above tips, in untreated roots and roots treated with 100 μM MeJA for 1 or 24 h. Representative images are shown.

this, calcium exudation was measured in untreated and EGTA-treated plants. A reduction of calcium exudation rate in plants treated with EGTA was observed, indicating that EGTA is an effective chelator of apoplastic calcium. Its effects on MeJA-dependent regulation were then investigated. After treatments for 24 h with MeJA or EGTA+ MeJA, plants had similar *L* values, both higher than in untreated control plants (Fig. 6a). We also checked that EGTA alone had no significant effect on *L*.

Lanthanum chloride and heparin experiments

Secondly, we used $LaCl_3$ and heparin. These compounds block the calcium channels of plant membranes. MeJA+ $LaCl_3$ treatment partially inhibited the increase of *L* caused by MeJA (Fig. 6b). So, when calcium movement inside the root cells was impeded, the increase of *L* caused by MeJA was reduced. A heparin treatment had even more pronounced effects (Fig. 6c), and completely abolished the enhancement of *L* caused by MeJA treatment.

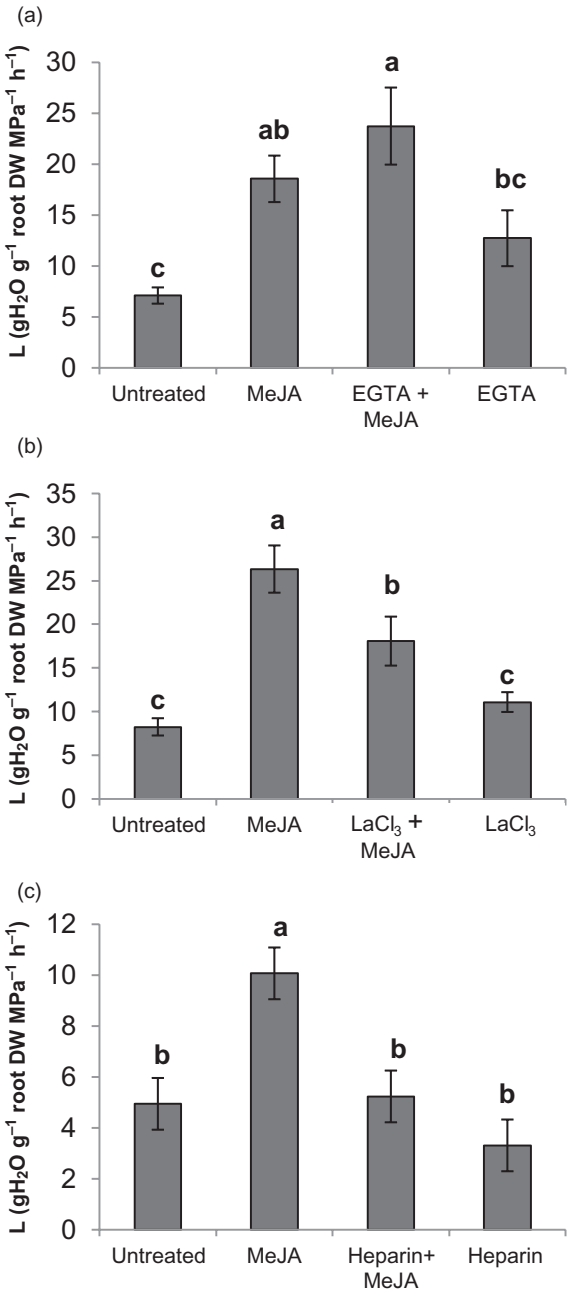

Figure 6. Root hydraulic conductivity (L) measured by the free exudation method in *Phaseolus vulgaris* roots treated with 100 μM MeJA during 24 h plus 1 mM EGTA (a), 1 mM $LaCl_3$ (b) or 1 mM heparin (c). Chemicals were added 24 h before MeJA addition. Bars represent mean ± SE (n = 15). Different letters mean significant differences ($P < 0.05$) after analysis of variance and least significant difference test.

Involvement of ABA on the MeJA action on *L*

MeJA can induce the synthesis of ABA (Adie *et al*. 2007), which in turn can increase *L* (Aroca 2006). To investigate this relationship, we designed experiments using beans and fluridone, an inhibitor of ABA biosynthesis.

We first checked whether MeJA can indeed induce an ABA accumulation and whether such increase can be abolished by a fluridone treatment. The data showed that MeJA application induced an increase of ABA concentration which after 1 h of fluridone treatment was completely inhibited, but after 24 h of exposure to MeJA inhibition was only partial (Fig. 7b).

Water transport was then investigated in control bean plants or plants treated with fluridone, MeJA or fluridone+ MeJA. Similar to its effect on ABA levels, fluridone inhibited partially or completely the increase in *L* caused by treatments with MeJA for 1 h or 24 h, respectively (Fig. 7a). Fluridone alone did not cause any effect on *L* at any time point (Fig. 7a).

We also investigated the regulation of *L* by MeJA in *sitiens* plants, a tomato mutant plant that is deficient in ABA synthesis. After 24 h application of MeJA, *sitiens* plants showed an increase of *L*, similar to wild type plants (Fig. 8a). However, since MeJA did not increase ABA contents in

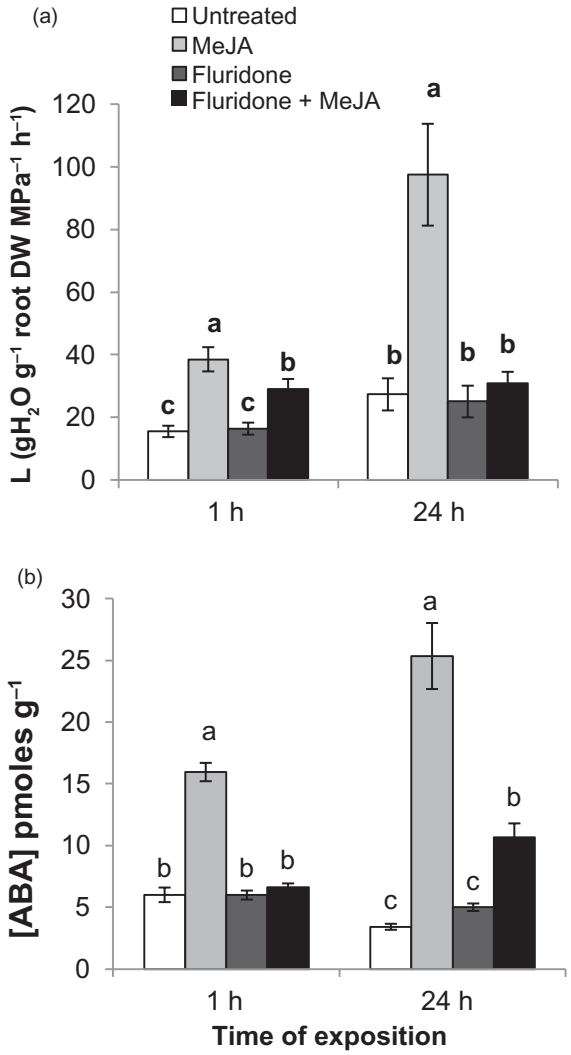

Figure 7. Root hydraulic conductivity (L) measured by the free exudation method (a) and ABA contents (b) in roots of *Phaseolus vulgaris* plants after 1 or 24 h of exposure to 100 μM MeJA plus 10 μM of fluridone. Fluridone was applied 24 h before MeJA addition. Bars represent mean ± SE (n = 8). Different letters mean significant differences ($P < 0.05$) after analysis of variance and least significant difference test.

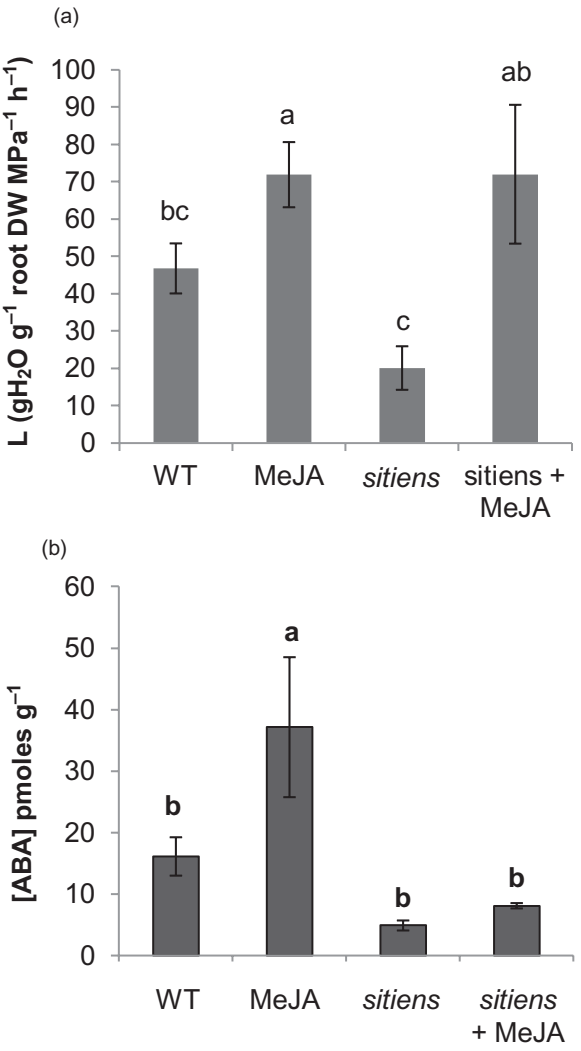

Figure 8. Root hydraulic conductivity (L) measured with a pressure chamber (a) and ABA contents (b) of roots of wild type (WT) tomato and ABA-deficient (*sitiens*) plants after 24 h of exposure to 100 μM MeJA. Bars represent mean ± SE (n = 5). Different letters mean significant differences ($P < 0.05$) after analysis of variance and least significant difference test.

sitiens roots (Fig. 8b), it is clear that MeJA does not need to enhance ABA concentration in roots to get an increase of L in this particular mutant.

DISCUSSION

Effect of MeJA on *L* and PIP aquaporins

There is already much information about the role of MeJA under stress conditions and in regulating stomatal opening (Raghavendra & Reddy 1987; Gehring *et al*. 1997; Suhita *et al*. 2004; Munemasa *et al*. 2007; Hossain *et al*. 2011). However, few investigations have been made to elucidate the role of MeJA on the regulation of root water transport (Lee *et al*. 1996).

Our study establishes that L increased when MeJA was applied to plant roots. This effect was observed in three different plant species, *P. vulgaris*, *A. thaliana* and *S. lycopersicum*. So, exogenous MeJA increases L, similar to ABA (Aroca *et al*. 2008; Mahdieh & Mostajeran 2009; Ruiz-Lozano *et al*. 2009; Kudoyarova *et al*. 2011). Moreover, we confirmed that the free exudation method is valid to measure L in bean plants. Using MeJA deficient tomato plants, we corroborated that plant with lower amounts of MeJA (*def-1* plants; Howe *et al*. 1996; Howe & Ryan 1999; Li *et al*. 2002; O'Donnell *et al*. 2003) have lower L than WT plants, with L being partially restored by exogenous MeJA. Thus, endogenous levels of MeJA also contribute to regulate L. Similar results were found in tomato and maize plants with lower endogenous levels of ABA, which showed lower values of L than wild type or untransformed plants (Nagel *et al*. 1994; Parent *et al*. 2009).

Since PIP aquaporins are known to significantly contribute to L (Javot *et al*. 2003; Postaire *et al*. 2010; Perrone *et al*. 2012), we studied how MeJA regulates PIP expression, abundance and phosphorylation state. Changes in *PIP* gene expression may not explain the increase in L observed after MeJA treatment in *P. vulgaris* plants. In fact, the expression of three *PIPs* was down-regulated (*PvPIP1;2*, *PvPIP1;3* and *PvPIP2;1*), and only the expression of *PvPIP2;2* and *PvPIP2;3* was up-regulated after 1 and 24 h of exposure to MeJA. This apparent lack of relationship between *PIP* expression and L could be because other *P. vulgaris PIP* genes that are not yet identified contribute significantly to L enhancement. The PvPIP2;3 protein has a high capacity for transporting water compared to PvPIP2;2 protein (Zhou *et al*. 2007), so its enhanced expression by MeJA after 24 h could be related to the enhancement of L. Also, a collaborative interaction between PvPIP2;2 and PvPIP2;3 proteins could take place as recently reported Li *et al*. (2013) for PIP2 proteins of cotton. Obviously, a reverse genetic approach is needed to corroborate these possibilities. At the same time, we cannot discard the possibility that an interaction between PIP1 and PIP2 monomers could take place to enhance L in MeJA treated roots (Fetter *et al*. 2004; Zelazny *et al*. 2007).

Our next step was to analyse PIP protein abundance and phosphorylation state. Here, we found a relationship between increase in L by MeJA and the increase in the amount of PIP2 proteins phosphorylated at Ser 280. The phosphorylation of this Ser residue is responsible for the water transport activity of several PIPs proteins (Johansson *et al*. 1998; Törnroth-Horsefield *et al*. 2006; Azad *et al*. 2008). Also, after 24 h of MeJA exposure, an increase in PIP2 protein amount took place, which may be related to the increase in *PvPIP2;3* gene expression.

Altered subcellular localization of PIPs can also lead to changes in L values (Boursiac *et al*. 2005, 2008). Thus, taking advantage of *A. thaliana* lines that constitutively express PIPs proteins fused to GFP (Boursiac *et al*. 2008), we found that MeJA treatment increased the amount in the plasma membrane of two of the three major PIP proteins. These effects may be responsible for the increase of L. However, it remains to be determined if this phenomenon is due to enhanced trafficking of PIPs from intracellular membranes to the cell surface or to enhanced stability of the proteins at the plasma membrane.

Role of calcium in the regulation of *L* by MeJA

Based on the finding that MeJA regulates *L*, we were interested in elucidating how calcium may be involved in this process. Calcium is a highly mobile element, whose apoplastic and cytosolic concentrations can change in response to external stimuli. Previous work showed that MeJA can move calcium ions into the cytosol from apoplastic spaces or from intracellular stores such as the vacuole or the endoplasmic reticulum (Sun *et al*. 2006a; Sun *et al*. 2006b; Islam *et al*. 2010).

By using a fluorescent dye, we first confirmed that MeJA treatment induced an increase in $[Ca^{2+}]_{cyt}$ in *P. vulgaris* roots after 24 h of application, but not after 1 h. Hence, in contrast to short-term (1 h) effects, the long-term increase of *L* observed after 24 h of exposure to MeJA could be linked to changes in $[Ca^{2+}]_{cyt}$. This idea was further explored by using calcium chelators (EGTA) and calcium channel blockers ($LaCl_3$ and heparin). The lack of effects of EGTA on MeJA-induced *L* after 24 h showed that apoplastic calcium may not be involved in this process. However, it is possible that minimal entrance of calcium ions could cause the response to MeJA. When we applied some Ca channel blockers ($LaCl_3$ and heparin) the *L* value was lower than with MeJA alone, especially after heparin application. Hence, the enhancement of *L* caused by MeJA could be mediated by mobilization of internal calcium stores, particularly from those dependent of IP_3 calcium channels, which are localized in endoplasmic reticulum and tonoplast and are inhibited by heparin (White 2000; Poutrain *et al*. 2009; Sun *et al*. 2009). The involvement of calcium in the induction of stomatal closure by MeJA has been also documented (Gehring *et al*. 1997; Herde *et al*. 1997; Munemasa *et al*. 2011).

Involvement of ABA on MeJA-enhanced *L*

Many studies have demonstrated a role for ABA and MeJA in transpiration and defense against pathogens (Herde *et al*. 1997; Suhita *et al*. 2004; Adie *et al*. 2007; Hossain *et al*. 2011). The relationship between the two signalling molecules is still unclear. For instance, Lorenzo & Solano (2005) found that ABA activates the expression of genes by the MeJA signalling pathway, and others found, that it is MeJA that activates the synthesis of ABA (Adie *et al*. 2007; Hossain *et al*. 2011). It is also known that exogenous ABA application increased *L* (Adie *et al*. 2007; Mahdieh & Mostajeran 2009; Ruiz-Lozano *et al*. 2009; Kudoyarova *et al*. 2011).

Here, we found that the initial *L* response to MeJA is partially dependent on ABA, since fluridone treatment did not abolish completely the effect of MeJA. However, after 24 h of exposure to MeJA the increase of *L* is totally dependent on the rise of ABA induced by MeJA. In fact, ABA contents also increased in fluridone treated roots after 24 h of exposure to MeJA, but *L* did not increase. So, we could distinguish two ways of action of MeJA on *L*, a short-term one, partially independent of ABA and calcium (1 h of exposure) and a long-term one, dependent on calcium and ABA (24 h of exposure). In fact, the increase of cytosolic calcium in guard cells induced by MeJA is mediated by ABA (Munemasa *et al*. 2011). Similar ABA-dependent and ABA-independent pathways for MeJA signalling have been previously found for other MeJA effects (Suhita *et al*. 2004; Munemasa *et al*. 2007). To confirm the MeJA ABA-independent signalling pathway, we analysed the response of *L* to MeJA addition in the ABA-deficient tomato mutant *sitiens* (Taylor *et al*. 1988). This mutant is more susceptible to water deficit induce embolism, most probably because overall low hydraulic properties (Secchi *et al*. 2013). Results confirmed the existence of an ABA-independent MeJA signalling pathway in the regulation of *L* by MeJA. The same occurs for stomatal regulation (Suhita *et al*. 2004; Islam *et al*. 2010).

In summary, we report here for the first time, and in three different plant species, that exogenous MeJA causes an increase of *L*. We postulate both calcium-dependent and independent, as well as, ABA-dependent and independent pathways for this effect of MeJA. The regulation of *L* by MeJA could be important under abiotic stress conditions such as drought or salinity, where soil water availability is restricted (Aroca *et al*. 2012), since it is known that MeJA improves water status of plants subjected to these kind of stresses (Anjum *et al*. 2011; Wu *et al*. 2012a). In future research, the involvement of MeJA in controlling *L* under water deficit and salt stress conditions will be studied.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1. MeJA contents of bean roots.
Figure S2. L 0.1, 0.5 and 1 mM MeJA treated roots.
Figure S3. L of MeJA treated beans measured by the pressure chamber.
Figure S4. L measured by free exudation method at 30 min intervals.
Figure S5. Gene expression analyses of AOS2 and MC genes in WT and *def-1* roots.
Figure S6. Phylogenetic tree of *PIP* genes from *P. vulgaris* and Arabidopsis.
Figure S7. EGTA effects on removing calcium ions from the medium.
Table S1. Specificity of the PIP2 antibodies used.
Table S2. Compounds dependent parameters for methyl jasmonate.