



## Research article

Regulation of water transport in *Arabidopsis* by methyl jasmonate

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## ABSTRACT

Following a stress event, jasmonate-dependent signaling pathway triggers a shift from growth to defense responses that are accompanied by the cessation of growth in many plants. However, the processes leading to this growth inhibition remain obscure. In this study, we provide evidence for a rapid inhibition of cell hydraulic conductivity ( $L_p$ ) by methyl jasmonate (MeJA) in the roots of wild-type *Arabidopsis* within 0.5 h of 20 and 50  $\mu$ M MeJA treatments. We also demonstrate that MeJA did not affect  $L_p$  in *fad3-2* and *fad7-2* *Arabidopsis* mutants that are deficient in jasmonate precursor, linolenic acid. The reductions of  $L_p$  in wild-type plants were accompanied by the down-regulation of several plasma membrane intrinsic protein (PIP) isoforms, and dephosphorylation. Treatments with  $HgCl_2$  did not further reduce  $L_p$  in the wild-type plants, but significantly reduced  $L_p$  in the *fad3-2* and *fad7-2* that had been first treated with MeJA. Continuous prolonged exposure to exogenous 50  $\mu$ M MeJA inhibited the relative growth rates (RGR) of shoots and net photosynthesis ( $P_n$ ) in the *Arabidopsis* wild-type and *fad7-2* plants, but had no effect on the RGR of roots. The results demonstrated that a reduction of aquaporin (AQP)-mediated water transport was the initial target of MeJA exposure, and may contribute to the processes of growth inhibition by MeJA.

## 1. Introduction

Stress responses in plants involve a shift from growth to secondary metabolism as a general stress defense strategy. The synthesis and subsequent accumulation of jasmonic acid (JA) and its volatile derivative methyl jasmonate (MeJA) in plant tissues is usually associated with wounding responses of plants following herbivory and pathogen invasion (Seo et al., 2001; Steppuhn and Baldwin, 2007). However, there is also growing evidence that JA and MeJA act as general stress signaling molecules in plants to trigger the synthesis of proteins involved in stress defenses against various biotic and abiotic factors (Seo et al., 2001; Kim et al., 2009). In addition to activating specific genes involved in plant defenses against stresses, jasmonates including JA and MeJA inhibit plant growth, which precedes a shift to secondary metabolism (Farmer and Ryan, 1992). Jasmonates also directly or indirectly affect a wide range of enzymes and processes in plants including proteinase inhibitors and polyphenol oxidase (Steppuhn and Baldwin, 2007), photosynthesis (Popova et al., 2003), and chlorophyll degradation (Popova et al., 2003; Jung et al., 2007).

Loss of water balance in plants and the resulting inhibition of cell elongation are among the most commonly observed responses in plants

exposed to various environmental stresses (Potters et al., 2009). Since cell elongation is highly sensitive to changes in water supply (Potters et al., 2009), control of water delivery to cells could provide an effective mechanism for plants to rapidly respond to stress factors. Regulation of water relations in plants to balance water loss and water uptake involves stomatal control and changes in the hydraulic conductivities of roots, stems, and leaves (Steudle and Peterson, 1998). Since the radial water transport across the root tissues is the site of greatest resistance to water flow in plants (Steudle and Peterson, 1998), root hydraulic conductivity is plays an important role in the adjustments of leaf function (Liu et al., 2014). Root hydraulic conductivity is defined as an intrinsic ability of root water uptake, which is related to the intensity of water flow and to the nature of forces applied to drive water across the root (Steudle and Peterson, 1998). Jasmonates serve as signaling molecules producing a shift to secondary metabolism and, therefore, it is plausible that they may also serve as signal molecules for altering hydraulic properties of plants, similarly to their role in stomatal closure (Hossain et al., 2011). Since stress responses involve growth inhibition and conservation of water resources, the reported increase in root hydraulic conductivity by MeJA in tomato and *Arabidopsis* (Sánchez-Romera et al., 2014) is intriguing. Higher root conductivities are

**Abbreviations:** A, cell surface area; AQP, aquaporin; DW, dry weight; E, transpiration;  $L_p$ , hydraulic conductivity of root cortical cells; JA, jasmonic acid; MeJA, methyl jasmonate; OA, okadaic acid; OE, overexpression of the *Arabidopsis* jasmonic acid carboxyl methyltransferase gene in *Arabidopsis*; PIP, plasma membrane intrinsic protein; P, turgor pressure;  $P_n$ , net photosynthesis; RGR, relative growth rate

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commonly associated with growth increases unlike the decreases in growth (Ueda and Kato, 1982) and development (Kim et al., 2009) that are usually associated with MeJA. Therefore, in the present study, we focused on hydraulic events taking place in the root cells of *Arabidopsis* plants following MeJA treatments.

Since contribution of the cell-to-cell pathway (symplastic and transmembrane) is relatively greater than that of the apoplastic pathway for the overall water flow of *Arabidopsis* (Javot et al., 2003; Ranathunge and Schreiber, 2011), transmembrane water movement, which is controlled by the abundance and activity of aquaporins (AQPs), is thought to play a significant role in regulating root hydraulic conductivity. AQPs rapidly respond to various environmental stresses including salinity (Lee and Zwiazek, 2015) and low temperature (Aroca et al., 2005). The regulation of AQP-mediated water transport involves gating regulation (Törnroth-Horsefield et al., 2006) and/or changes in processes such as protein phosphorylation/dephosphorylation (Johansson et al., 1998), and distribution of AQPs (Aroca et al., 2005). Due to their cellular localization, plasma membrane intrinsic proteins (PIPs) are the key AQPs involved in transcellular water exchange between cells (Aroca et al., 2005; Lee and Zwiazek, 2015). PIPs are divided into two groups: PIP1 (PIP1; 1 to PIP1; 5) and PIP2 (PIP2; 1 to PIP2; 8). Although the similarities between the homologs of PIP sub-family are high, their water transport properties are quite different and their expression rapidly changes in response to environmental factors (Fetter et al., 2004).

Jasmonates are synthesized in plants from linolenic acid via the octadecanoid pathway (Farmer and Ryan, 1992; Creelman and Mullet, 1997). Linolenic acid is oxygenated by lipoxygenase, and then converted to 12-oxo-phytodienoic acid by allene oxide synthase and allene oxide cyclase (Creelman and Mullet, 1997). Once synthesized, JA may be methylated by the JA carboxyl methyltransferase activity to form volatile MeJA (Seo et al., 2001). Jasmonates interact with membrane receptors, which trigger the production of 13-hydroperoxylinolenic acid to activate the signaling pathway (Creelman and Mullet, 1997). In *Arabidopsis*, the three desaturase enzymes responsible for the  $\omega$ -3 fatty acid desaturation include the microsomal FAD3 and plastid membranes FAD7 and FAD8 (McConn et al., 1994). The FAD3 gene encodes an endoplasmic reticulum desaturase and the FAD7 and FAD8 genes both encode chloroplast desaturase isozymes (Browse et al., 1993; Iba et al., 1993). The FAD3 and FAD7 genes have high homology (Iba et al., 1993). Wounding triggers rapid increases in the mRNA levels of FAD7 in *Arabidopsis*, pointing to its importance in JA signal transduction pathway (Nishiuchi et al., 1997). The fad single mutants including fad3-2 and fad7-2 contain less than 30% of the wild-type levels of linolenic acid (Lemieux et al., 1990; McConn et al., 1994). The fad3-2 and fad7-2 are the mutants deficient in linolenic acid in which the ability to convert 18:2 to 18:3 has been disrupted at the FAD3 and FAD7 loci (Browse et al., 1993). The fad7-1 and fad7-2 have similar fatty acid profiles, and the fad7-1 plants accumulated low levels of basal and pathogen-induced JA, which correlated with reduced levels of the JA precursor 16:3 and 18:3 fatty acid (McConn et al., 1994; Xia et al., 2010). The fad triple mutant fad3-2fad7-2fad8, that has very low levels of linolenic acid, was unable to accumulate jasmonate (McConn et al., 1997), while overexpression of the *Arabidopsis* jasmonic acid carboxyl methyltransferase gene (OE) increased jasmonate levels in *Arabidopsis* plants (Seo et al., 2001).

In the present study, we subjected *Arabidopsis* roots to various concentrations of exogenous MeJA to understand the effects of MeJA on cell hydraulic conductivity of individual cortical cells ( $L_p$ ) and plant growth using the wild-type and genetically transformed *Arabidopsis* plants (OE, fad3-2 and fad7-2). The measurements of  $L_p$  were used to measure water permeability across the cell membranes. The OE plants were chosen due to their reported increase in MeJA levels and fad3-2 and fad7-2 because they are deficient in jasmonate precursor, linolenic acid (Lemieux et al., 1990; McConn et al., 1994; Seo et al., 2001). In this study, we compared the growth, gas exchange, AQP transcript levels

and phosphorylated PIP2 protein expression between the fad3-2 and fad7-2 single mutants, OE plants, and wild-type *Arabidopsis*. We also used protein phosphatase inhibitor, okadaic acid (OA) and AQPs blocker, HgCl<sub>2</sub>, to determine whether these processes may be involved in the responses of  $L_p$  to MeJA. We hypothesized that (1) the initial targets of MeJA action on wild type plants would include a decrease of  $L_p$  through the down-regulation of AQP gene expression and AQP dephosphorylation processes, (2) MeJA overexpression and application of exogenous MeJA in fad plants would result in low  $L_p$  and AQP dephosphorylation, and (3) the inhibition of AQP-mediated  $L_p$  would be associated with growth reductions that are part of the MeJA-induced defense responses in plants.

## 2. Materials and methods

### 2.1. Plant material and MeJA treatment

Seeds of the wild-type (*Arabidopsis thaliana*, Columbia-0) and the fad3-2 (CS 8034) and fad7-2 (CS8042) mutants were obtained from the *Arabidopsis* Biological Resource Center (ABRC), and the seeds of overexpressing *Arabidopsis* jasmonic acid carboxyl methyltransferase in *Arabidopsis* (OE) were kindly provided by Dr. Yang Do Choi (Seo et al., 2001). The fad3-2 is a mutant deficient in linolenic acid in which the ability to convert 18:2 to 18:3 has been disrupted by a nuclear mutation at the FAD3 locus (Browse et al., 1993). The fad7-2 mutant has no FAD7 function resulting in reduced linolenic acid levels (McConn et al., 1994; Xia et al., 2010; Avila et al., 2012). MeJA levels in OE *Arabidopsis* using the 35S promoter has been reported to increase leaf MeJA levels by three-fold (Seo et al., 2001; Kim et al., 2009).

The seeds were sown in Jiffy pots (Jiffy Company, Shippigan, NB, Canada) for 4 d at 4 °C before being transferred to the controlled-environment growth room set to the 16-h photoperiod, 350  $\mu\text{mol m}^{-2} \text{s}^{-1}$  photosynthetic photon flux density, and 23/21 °C (day/night) temperature. After 12 d, the roots were gently washed and the plants were transferred to the mineral solution culture (Lee and Zwiazek, 2015). The plants were grown for 3–4 weeks before treatments in 5-L containers with aerated nutrient solution containing 1.25 mM KNO<sub>3</sub>, 1.5 mM Ca(NO<sub>3</sub>)<sub>2</sub>, 0.75 mM MgSO<sub>4</sub>, 0.5 mM KH<sub>2</sub>PO<sub>4</sub>, 50  $\mu\text{M}$  H<sub>3</sub>BO<sub>3</sub>, 10  $\mu\text{M}$  MnCl<sub>2</sub>, 2  $\mu\text{M}$  ZnSO<sub>4</sub>, 1.5  $\mu\text{M}$  CuSO<sub>4</sub>, 0.075  $\mu\text{M}$  NH<sub>4</sub>Mo<sub>7</sub>O<sub>24</sub> and 74  $\mu\text{M}$  Fe-EDTA. Stock solution of 1 mM MeJA in 0.1% ethanol was added to the nutrient solution to produce the treatment concentrations of 20 and 50  $\mu\text{M}$  MeJA.

### 2.2. Dry weights, relative growth rate, gas exchange measurements and root cortex cell length determinations

The plants of wild-type, fad3-2, fad7-2 and OE were divided into four groups following root application with 0 (control), 20, and 50  $\mu\text{M}$  MeJA for 3 and 6 d. For dry weight determinations, shoots and roots were separated after 3 and 6 d of treatments and dried in an oven at 70 °C for 48 h. The experiment was repeated four times, each time eight plants per treatment were taken for the measurements. The relative growth rate (RGR) of shoots and roots was calculated from the initial and final dry weight (Hoffmann and Poorter, 2002). Net photosynthesis ( $P_n$ ) and transpiration (E) rates were measured under ambient conditions in the growth chamber with an infrared gas analyzer (LI-6400, LICOR, Lincoln, NE, USA) as previously described (Liu et al., 2014) on fully expanded individual leaves of control plants and plants treated with 50  $\mu\text{M}$  MeJA for 0 h, 1 h, 1 d and 3 d. The lengths of root cortical cells were examined at the distance of about 25–30 mm from the root apex after 3 d of treatments with 0 and 20  $\mu\text{M}$  MeJA using a light microscope (Carl Zeiss, Göttingen, Germany).

### 2.3. Cell-pressure probe measurements

A distal root segment of wild-type, fad3-2, fad7-2 and OE plants was

fixed by a small magnetic bar on a metal sledge which was covered with a layer of paper tissue (Lee and Zwiazek, 2015). Nutrient solution of the same composition as for solution culture flowed along the roots during the measurements. A single cortical cell was punctured at a distance of about 25- to 30-mm from the root tip with a silicon oil-filled microcapillary (6–8  $\mu\text{m}$  tip diameter). A meniscus was formed between cell sap and oil. Cell turgor ( $P$ ) was rebuilt by gently pushing the meniscus to a position close to the root surface. Once  $P$  became steady, hydrostatic hydraulic parameters such as half-times of water exchange and cell elastic modulus were determined (Steudle, 1993). Hydraulic parameters were measured in roots exposed to the nutrient solution (0  $\mu\text{M}$  MeJA), and then MeJA were added for 1 h to the circulating medium at a final concentration of 20 or 50  $\mu\text{M}$  MeJA.  $P$  was between 0.3 and 0.5 MPa, and remained constant during the measurements which lasted for 1 h. The roots of plants subjected to the continuous MeJA treatments for 1 h and in control plants were taken for the measurements with cell-pressure probe. The dimensions of the root cells obtained from cross and longitudinal sections were used to determine the cell volume and cell surface area for hydraulic conductivity of root cortical cells ( $L_p$ ) (Steudle, 1993).

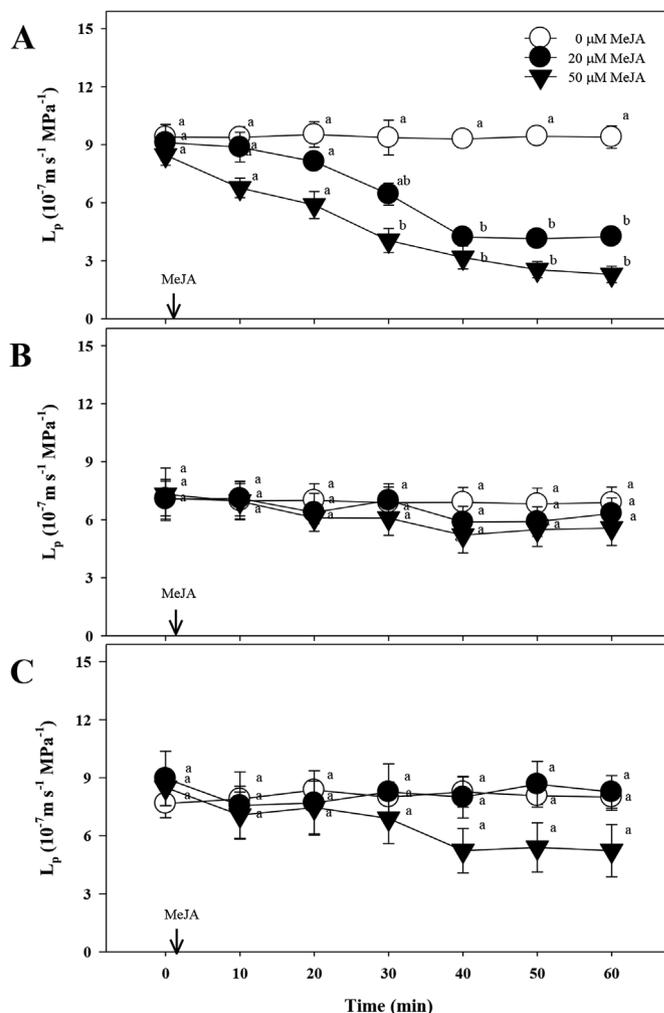
#### 2.4. RNA extraction and real time RT-PCR

The transcript levels of 13 PIP genes were measured in roots of wild-type, *fad3-2* and *fad7-2* plants treated with 0 and 50  $\mu\text{M}$  MeJA for 0.5 and 1 h in solution culture. Total RNA was extracted from the frozen root samples using the Plant RNeasy extraction kit (Qiagen, Valencia, CA, USA). The concentration of RNA was quantified spectrophotometrically using a NanoDrop 1000 apparatus, (NanoDrop, Wilmington, DE, USA). Real-time RT-PCR was performed using a Biosystems 7500 real-time thermal cycling system (Applied Biosystems, Foster City, CA, USA) with the QuantiTect SYBR Green RT-PCR kit (Qiagen). After normalization of the RNA content using actin gene expression pattern in each sample, the expression levels of each of the 13 PIP genes in *Arabidopsis* plants which were grown at 0  $\mu\text{M}$  MeJA (control) were compared with the plants treated with 50  $\mu\text{M}$  MeJA. These experiments were repeated at least three times, and the histograms represent the mean values and standard errors bars of different experiments conducted with different RNA preparations.

#### 2.5. Microsomal isolation, protein and western blot analysis

To extract the microsomal proteins, about 1 g of frozen roots of wild-type, *fad3-2* and *fad7-2* was ground with liquid nitrogen and added to the extraction buffer (50 mM Tris-HCl pH 7.5, 250 mM Sucrose, 1 mM EDTA, 5  $\mu\text{g ml}^{-1}$  of Leupeptin, 1 mM phenylmethylsulfonyl fluoride, 5 mM dithiothreitol). The mixture was stirred for 30 min and centrifuged at 2,700 g for 5 min and the pellet was discarded. The supernatant was centrifuged at 10,000 g for 10 min and the pellet was discarded. The supernatant was then centrifuged at 100,000 g for 30 min. The pellet was dissolved in the suspension buffer (50 mM  $\text{KH}_2\text{PO}_4$ , 330 mM Sucrose, 3 mM KCl pH 7.8) and stored at  $-80^\circ\text{C}$ . All steps in the procedure were carried out at  $4^\circ\text{C}$  to minimize protein degradation.

For Western blotting, 40- $\mu\text{g}$  protein were loaded per lane in a 12% SDS-PAGE, and transferred to a polyvinylidene difluoride (PVDF) membrane. The membrane was blocked for 2 h at room temperature with 0.5% bovine serum albumin in TBS (Tris-buffered-saline), and washed three times for 30 min with TTBS (Tris-buffered-saline with 0.05% Tween 20). The membrane was incubated overnight at  $4^\circ\text{C}$  with the phosphorylated PIP2 primary antibody at 1:1000 dilution (by volume). Antibody against *Phaseolus vulgaris* phosphorylated PIP2 was kindly donated by Prof. R. Aroca (Estación Experimental del Zaidin (CSIC), Spain). After the membrane was washed three times for 30 min with TTBS, secondary antibody (1:10000) raised in goat against rabbit IgG and conjugated to alkaline phosphatase (Sigma-Aldrich) was used



**Fig. 1.** Effects of MeJA on hydraulic conductivity of root cortical cells ( $L_p$ ) in wild type (A), *fad3-2* (B) and *fad7-2* (C) *Arabidopsis* plants that were subjected to 0 (control), 20 and 50  $\mu\text{M}$  MeJA treatments for 60 min. The arrow indicates addition of MeJA to root treatment solution (A, B, C). Means  $\pm$  SE ( $n = 6$ ) are shown. Different letters indicate statistically significant differences between treatments ( $p \leq 0.05$ ). The data were analyzed by ANOVA with repeated-measures.

to detect immunoreactive bands.

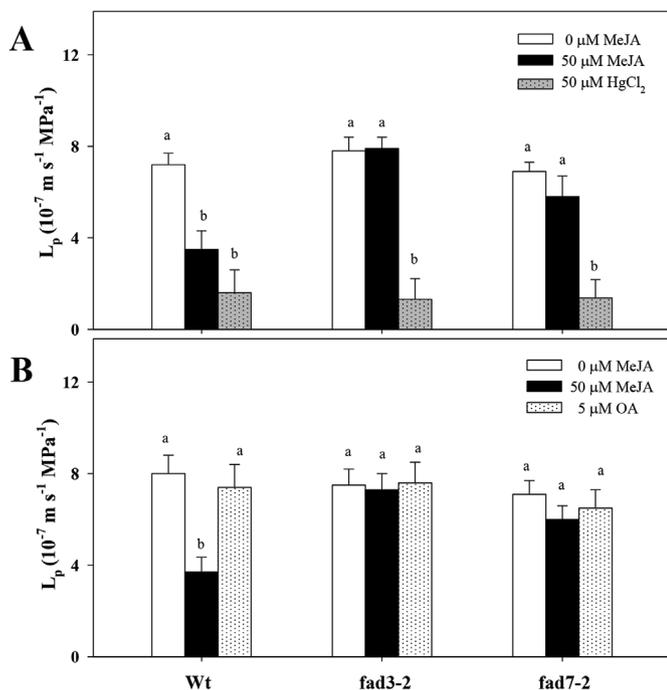
#### 2.6. Statistical analyses

The data were analyzed using unpaired  $t$ -test to compare the AQP transcript levels. Analysis of variance (ANOVA) with Tukey's multiple comparison was used for transpiration, photosynthesis, plant dry weight and hydraulic conductivity of root cortical cells. The data were analyzed by ANOVA followed by the Fisher least significant differences (LSD) comparison test to compare the relative growth rate and root cortical cell lengths. Statistical analyses were performed using SigmaPlot version 11.0 (Systat Software Inc, California, USA).

### 3. Results

#### 3.1. Cell hydraulic conductivity ( $L_p$ ) on MeJA

Hydraulic conductivity of root cortical cells ( $L_p$ ) had similar values in control (untreated) roots of the wild-type, *fad3-2* and *fad7-2* plants and remained relatively stable during the 1 h of the cell pressure probe measurements (Fig. 1A–C). However, the  $L_p$  in OE plants was lower by



**Fig. 2.** Effect of HgCl<sub>2</sub> and okadaic acid (OA) on hydraulic conductivity of root cortical cells ( $L_p$ ). The wild-type, *fad3-2* and *fad7-2* plants that were subjected to 0 (control) and 50  $\mu\text{M}$  MeJA treatment for 0.5 h followed by 50  $\mu\text{M}$  HgCl<sub>2</sub> (A) or 5  $\mu\text{M}$  OA (B). Means  $\pm$  SE ( $n = 6$ ) are shown. Different letters indicate statistically significant differences between treatments ( $p \leq 0.05$ ). The data were analyzed by ANOVA followed by Tukey's multiple comparison test.

approximately two-fold compared to the wild-type, *fad3-2* and *fad7-2* plants (Supplementary Fig. 1). The  $L_p$  values rapidly decreased in the wild-type plants after the application of 20 and 50  $\mu\text{M}$  MeJA (Fig. 1A). The reductions were greater in 50 compared with 20  $\mu\text{M}$  MeJA treatment and progressed over time in the wild-type plants (Fig. 1A). After 10 min of 50  $\mu\text{M}$  MeJA treatment,  $L_p$  was reduced to  $6.7 \times 10^{-7} \text{ m s}^{-1} \text{ MPa}^{-1}$  compared with  $8.5 \times 10^{-7} \text{ m s}^{-1} \text{ MPa}^{-1}$  measured in control (0  $\mu\text{M}$  MeJA) and after 0.5 h of 50  $\mu\text{M}$  MeJA treatment,  $L_p$  was reduced by two-fold compared with control (Fig. 1A). The  $L_p$  was reduced by approximately two- and four-fold compared with control plants after 1 h of the 20 and 50  $\mu\text{M}$  MeJA treatments, respectively (Fig. 1A). During the 1 h of measurements, treatments with 20 and 50  $\mu\text{M}$  MeJA did not significantly alter the  $L_p$  values in *fad3-2* and *fad7-2* plants compared with untreated control (Fig. 1B and C).

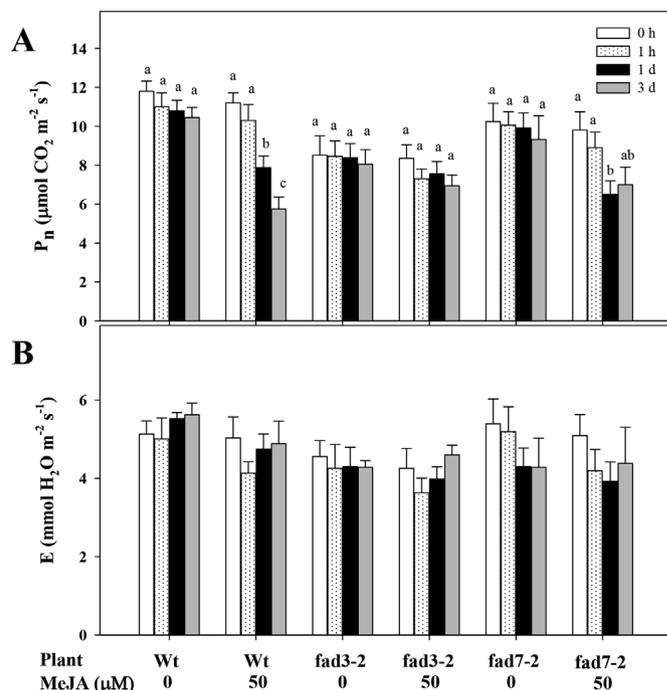
### 3.2. Effects of HgCl<sub>2</sub> and okadaic acid (OA) on $L_p$

The treatment with 50  $\mu\text{M}$  HgCl<sub>2</sub> did not further reduce  $L_p$  in the wild-type plants that had been subjected to 50  $\mu\text{M}$  MeJA (Fig. 2A). However, in *fad3-2* and *fad7-2* plants,  $L_p$  was reduced by four- to six-fold when 50  $\mu\text{M}$  HgCl<sub>2</sub> was added following MeJA treatment (Fig. 2A).

The application of the protein phosphatase inhibitor, 5  $\mu\text{M}$  OA following the 50  $\mu\text{M}$  MeJA treatment, resulted in the return of  $L_p$  to the levels measured prior to the MeJA treatment in the wild-type (Fig. 2B) and OE plants (Supplementary Fig. 1). The application of OA had no effect on  $L_p$  values in *fad3-2* and *fad7-2* plants (Fig. 2B).

### 3.3. Net photosynthesis ( $P_n$ ) and transpiration ( $E$ )

Net photosynthesis ( $P_n$ ) was not significantly affected after 1 h of the 50  $\mu\text{M}$  MeJA treatment in the wild-type, *fad3-2* and *fad7-2* plants (Fig. 3A). The significant  $P_n$  reduction was measured after 1 d following the 50  $\mu\text{M}$  MeJA treatment in the wild-type and *fad7-2* plants, but no reduction was observed in *fad3-2* plants for up to 3 d (Fig. 3A). The

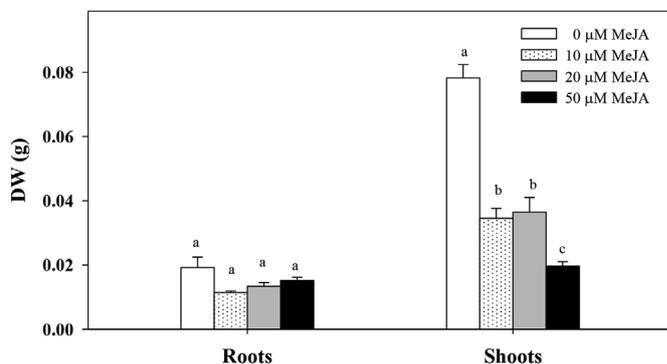


**Fig. 3.** Effects of MeJA on net photosynthesis ( $P_n$ ) and transpiration ( $E$ ) rates. The  $P_n$  (A) and  $E$  (B) were measured in the wild-type, *fad3-2* and *fad7-2* *Arabidopsis* plants treated with 0 (control) and 50  $\mu\text{M}$  MeJA treatment for 1 h, 1 d and 3 d. Means  $\pm$  SE ( $n = 7$ ) are shown. Different letters indicate statistically significant differences between treatments ( $p \leq 0.05$ ). The data were analyzed by ANOVA followed by Tukey's multiple comparison test.

50  $\mu\text{M}$  MeJA treatment did not alter the  $E$  for up to 3 d in plants (Fig. 3B).

### 3.4. Root and shoot dry weights (DW), relative growth rate (RGR) of roots and shoots and root cortical cell length

The shoot growth of the wild-type plants was visibly reduced after 3 d of treatments with 10, 20, and 50  $\mu\text{M}$  MeJA, but the effect was less pronounced in the roots (Fig. 4 and Supplementary Fig. 2A). Shoot DW of treated plants were lower compared with control plants by about two-fold in the 10 and 20  $\mu\text{M}$  MeJA treatments and by about four-fold in the 50  $\mu\text{M}$  MeJA treatment (Fig. 4). The RGR of shoots and roots had similar values in control (untreated) plants of the wild-type, *fad3-2* and *fad7-2* plants (Fig. 5). The application of 20  $\mu\text{M}$  MeJA for 3 d and 50  $\mu\text{M}$  MeJA treatment for 6 d significantly inhibited RGR of shoots in the



**Fig. 4.** Root and shoot dry weights (DW) in the wild-type *Arabidopsis* seedlings treated with 0 (control), 10, 20, and 50  $\mu\text{M}$  MeJA for 3 d. Means  $\pm$  SE ( $n = 8$ ) are shown. Different letters indicate significant differences between the treatments ( $p \leq 0.05$ ) as determined by ANOVA followed by Tukey's multiple comparison test.

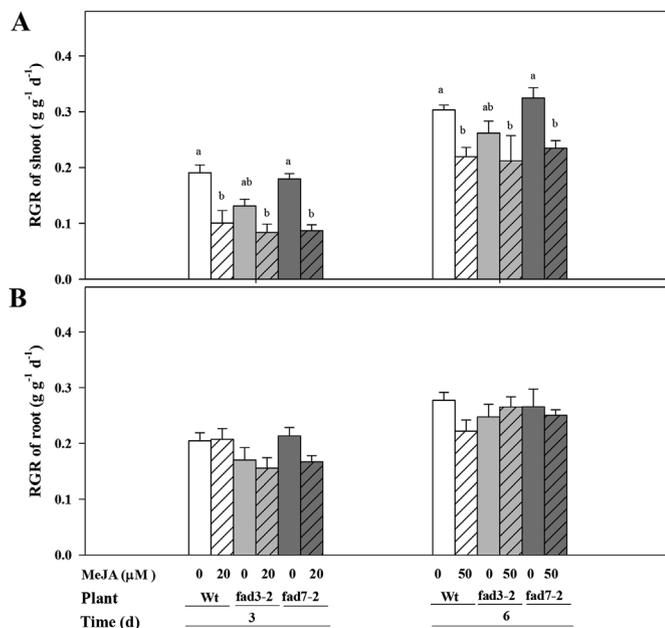


Fig. 5. Effects of MeJA on relative growth rate (RGR) of shoots (A) and roots (B). The wild-type, fad3-2 and fad7-2 *Arabidopsis* plants were subjected to 0 (control), 20 μM MeJA for 3 d, and 50 μM MeJA for 6 d. Means ± SE (n = 8) are shown. Different letters indicate statistically significant differences between treatments (p ≤ 0.05). The data were analyzed by ANOVA followed by Fisher LSD comparison test.

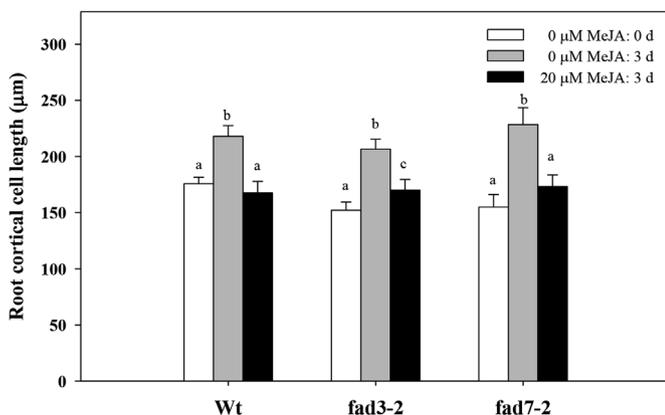


Fig. 6. Effects of 20 μM MeJA on root cortical cell length. The wild-type, fad3-2 and fad7-2 *Arabidopsis* plants were subjected to 0 (control), 0 μM MeJA for 3 d, and 20 μM MeJA for 3 d. Means ± SE (n = 6) are shown. Different letters indicate statistically significant differences between treatments (p ≤ 0.05). The data were analyzed by ANOVA followed by Fisher LSD comparison test.

wild-type and fad7-2 plants, but did not alter RGR of shoots in fad3-2 plants (Fig. 5A and Supplementary Fig. 2B). The 20 and 50 μM MeJA treatments did not significantly affect RGR of roots in any of the plant lines during the treatment durations (Fig. 5B). The length of cortical cells was reduced by 30–32% after 20 μM MeJA treatment for 3 d in the wild-type and fad7-2 plants and by 20% in the fad3-2 plants compared with control roots (0 μM MeJA for 3 d) (Fig. 6).

### 3.5. Expression profiles of PIPs

The transcript levels of PIP1; 3, 1; 4, 1; 5, 2; 2, 2; 4, 2; 5 and 2; 8 were significantly reduced in roots of the wild-type *Arabidopsis* but little changed in the fad3-2 and fad7-2 plants treated for 0.5 h with 50 μM MeJA compared with untreated control (Fig. 7A–C). However, the levels of PIP1; 5, 2; 4, 2; 7 and 2; 8 in the fad3-2 plants and of PIP1; 4, 2;

5, 2; 6 and 2; 8 in the fad7-2 plants sharply increased (Fig. 7B and C). After 1 h of 50 μM MeJA treatment, the transcript levels of PIP1; 4, 2; 4 and 2; 5 returned to the control levels in the wild-type plants (Fig. 7A). The transcript levels of PIP1; 4, 1; 5, 2; 4, 2; 5, 2; 7 and 2; 8, and of PIP1; 4, 2; 4 and 2; 6 were highly up-regulated in fad3-2 and fad7-2 plants treated for 1 h with 50 μM MeJA, respectively (Fig. 7B and C). MeJA treatment did not affect the transcript levels of PIP1; 1 and PIP1; 2 in all plant lines (Fig. 7A–C). Also, the transcript levels of PIP1; 3, 2; 1, 2; 2 and 2; 3 in the fad3-2 and fad7-2 plants remained unaffected by the 50 μM MeJA treatment (Fig. 7B and C).

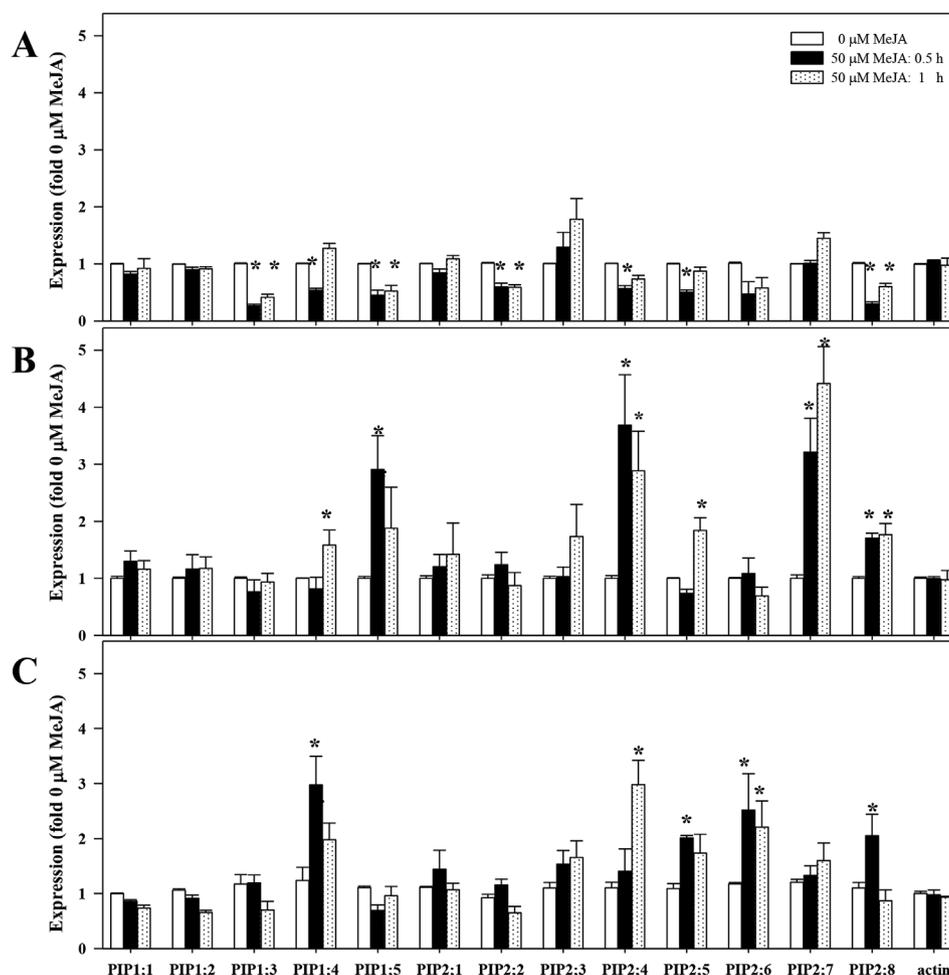
### 3.6. Immunoblotting of phosphorylated PIPs

Immunoblotting of separated microsomal proteins using the antibody against phosphorylated *Phaseolus vulgaris* PIP2 revealed the immunoreactive band of about 29–35 kDa (Fig. 8). The immunostaining intensity and size of the immunoreactive band were clearly greater in control (untreated) wild-type plants compared with control fad3-2 and fad7-2 plants (Fig. 8). After 1 h of treatment with 50 μM MeJA, the intensity and size of the immunoreactive band sharply decreased in the wild-type plants but increased in the fad3-2 and fad7-2 plants (Fig. 8).

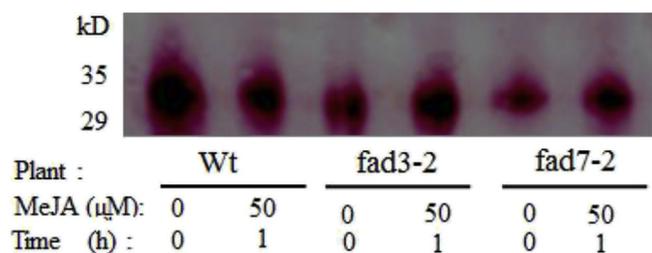
## 4. Discussion

Our data provide evidence for a rapid and concentration-dependent decrease of cell hydraulic conductivity ( $L_p$ ) in *Arabidopsis* roots treated with MeJA. The results indicated that MeJA significantly inhibited the  $L_p$  by two- to four-fold in the wild-type and OE plants, but did not alter  $L_p$  in fad3-2 and fad7-2 mutants. The addition of HgCl<sub>2</sub> following the MeJA treatment clearly demonstrated that  $L_p$  did not further decline in the wild-type *Arabidopsis* roots, but the remarkable reduction of  $L_p$  was shown in the fad3-2 and fad7-2 lines. Since HgCl<sub>2</sub> is a strong AQP blocker (Wan and Zwiazek, 1999), these results strengthen the evidence that AQP-mediated transport is among the initial targets of MeJA in *Arabidopsis*. However, in other studies, hydraulic conductivity of roots that was measured with the hydrostatic method in *Phaseolus vulgaris*, *Solanum lycopersicum* and *Arabidopsis* was increased by MeJA, which was explained as a possible effect of MeJA on calcium- and ABA-dependent and independent signaling pathways (Sánchez-Romera et al., 2014). Enhancement of water flux by JA was also reported during anther dehiscence in *Arabidopsis* and was proposed to be due to the induction of AtSUC1 and its effect on cell-specific modulation of water potentials (Stadler et al., 1999). These discrepancies between these results are not clear, but in our earlier study (Lee et al., 2009), we also observed a similar lack of correlation between the cell and leaf hydraulic conductivities in tobacco exposed to high irradiance, which we attributed to the effect on apoplastic water transport.

Regulation of transmembrane water transport is accomplished through changes in the expression and activity of AQPs (Aroca et al., 2005; Lee and Zwiazek, 2015). In the wild-type *Arabidopsis* roots, the link between  $L_p$  reduction and AQP expression was clear in plants treated with 50 μM MeJA for 0.5 and 1 h with several down-regulated PIPs including PIP1; 3, 1; 5, 2; 2 and 2; 8. PIP2; 2 was demonstrated to be one of the principal water-transporting and abundantly expressed AQPs in *Arabidopsis* roots linked to  $L_p$  regulation (Javot et al., 2003). The down-regulation of PIP 2; 2 by MeJA had likely significant consequences to root water transport. Up-regulation of PIP1; 5, 2; 4, 2; 7 and 2; 8 in the fad3-2, and PIP 1; 4 and 2; 6 in the fad7-2 mutants following exposure to MeJA suggests a possible involvement of these PIPs in maintaining  $L_p$  in these plants. The rapid responses of PIPs which took place within 0.5–1 h of MeJA treatments, demonstrate that PIP gene expression could be rapidly targeted by MeJA. The different effects of MeJA on transcript levels in the wild-type and fad plants signify the complexity of secondary physiological responses triggered by the initial MeJA treatments. The dynamic responses of plants to progressing stress and differences in functions of different AQPs in



**Fig. 7.** Transcript levels of 13 PIP genes in the roots of wild-type (A), *fad3-2* (B) and *fad7-2* (C) *Arabidopsis* seedlings treated with 50  $\mu\text{M}$  MeJA for 0.5 and 1 h. The transcript levels of the 13 PIP genes are plotted as the relative expression (fold) of control (0  $\mu\text{M}$  MeJA). Bars are means  $\pm$  SE from three independent experiments ( $n = 3$ ). The data were analyzed by the unpaired *t*-test and asterisks show statistically significant ( $p \leq 0.05$ ) differences from control.



**Fig. 8.** Western blot of microsomal proteins showing protein bands immunoreactive with the anti-PvPIP2 antibody. *Arabidopsis* wild-type, *fad3-2* and *fad7-2* plants were subjected to 0 and 50  $\mu\text{M}$  MeJA treatment for 1 h. The positions of the molecular mass marker are indicated.

plants may also explain some of the reported discrepancies in AQP gene regulation. Microarray screening of JA-responsive genes in *Arabidopsis* reported up-regulation of PIP1; 1 and down-regulation of two other putative AQPs (Jung et al., 2007). PatPIP1 was also upregulated by JA and several other plant growth regulators as well as osmotic stress, NaCl, and low temperature in *Populus alba*  $\times$  *P. tremula* var. *glandulosa* (Bae et al., 2011).

When separated by SDS PAGE, AQPs have been usually detected as 24–35 kDa protein bands in plants (Johansson et al., 1998; Horie et al., 2011). In our study, the immunoreactive single protein band of approximately 29–35 kDa was detected in Western blots with the antibody raised against phosphorylated PIP2 from *Phaseolus vulgaris*. The activity

of many AQPs is affected by phosphorylation and dephosphorylation events (Johansson et al., 1998; Horie et al., 2011). In these AQPs, phosphorylation facilitates water transport while the dephosphorylation triggers pore closure (Johansson et al., 1998; Horie et al., 2011; Lee and Zwiazek, 2015). In the present study, AQP dephosphorylation was also likely a factor in the inhibition of  $L_p$  by MeJA in the wild-type *Arabidopsis* and OE plants as evidenced by the reversion of the inhibition by 5  $\mu\text{M}$  OA, the protein phosphatase inhibitor (Johansson et al., 1998; Horie et al., 2011; Lee and Zwiazek, 2015) and immunostaining with the anti-PvPIP2 antibody. In contrast, AQP dephosphorylation appeared not to be targeted by MeJA in *fad3-2* and *fad7-2* plants. This was confirmed by both immunostaining and no response of  $L_p$  to OA following the MeJA treatment.

The  $L_p$  reduction in OE plants also supports the notion of the inhibitory effects of MeJA. Overexpression of the *Arabidopsis* *ap2c1* mutant that produced higher amounts of JA upon wounding led to the inactivation of stress-responsive mitogen-activated protein kinase, indicating that the increased JA level can be modulated by dephosphorylation (Schweighofer et al., 2007). Our results also suggest that higher endogenous MeJA level in OE plants resulted in lower  $L_p$  compared with the wild-type and *fad* mutants (Supplementary Fig. 1), suggesting that higher MeJA level may induce the closure of water channel. However, the wild-type and *fad* mutants had similar  $L_p$  values in untreated roots. In contrast, it has been reported that JA defective biosynthesis tomato mutants *def-1* had lower hydraulic conductivity of the whole root system compared with the wild-type, and the

conductivity was restored with the application of MeJA indicating the phosphorylation state of AQP PIP2 (Sánchez-Romera et al., 2014). Earlier studies reported that *fad7-1* plants accumulated very low levels of pathogen-induced JA and *fad* triple mutant *fad3-2fad7-2fad8* is unable to accumulate JA, but the application of linolenic acid induced effective anti-insect defenses in plants (Farmer and Ryan, 1992; McConn et al., 1997). Therefore, we expected that the application of exogenous 20 and 50  $\mu\text{M}$  MeJA for 1 h would decrease  $L_p$  in *fad* mutants for defense and stress resistance processes as well as further decrease of  $L_p$  in OE plants by high endogenous MeJA levels. However, unlike in the wild-type and OE plants, exogenous applications of 20 and 50  $\mu\text{M}$  MeJA for 1 h did not alter  $L_p$  in *fad* mutants, suggesting that  $L_p$  may not be directly affected by the jasmonate signaling pathways or by the MeJA-induced increase in AQP activity.

Our results demonstrated strong inhibitory effects of MeJA treatments on relative growth rate (RGR) of shoots. Continuous prolonged higher exogenous 20  $\mu\text{M}$  MeJA for 3 d and 50  $\mu\text{M}$  MeJA treatment for 6 d decreased RGR of shoot in the *Arabidopsis* wild-type and *fad7-2* plants. However, *fad3-2* plants showed no shoot RGR reduction when treated with 20  $\mu\text{M}$  MeJA for 3 d and 50  $\mu\text{M}$  MeJA for 6 d, suggesting that water balance was maintained at the whole plant level. The results also indicate that jasmonate deficiency is not the principal factor contributing to the inhibition of shoot RGR in *fad3-2*, unlike the *fad7-2* plants. This may be due to the differences in functions and cell localizations since the *FAD3* gene is thought to encode an integral membrane protein in the endoplasmic reticulum whereas the *FAD7* gene encodes a chloroplast membrane protein (Iba et al., 1993). Jasmonates have been frequently reported to regulate defense gene expression (Seo et al., 2001), inhibit plant growth (Ueda and Kato, 1982) and plant development (Kim et al., 2009). Increased MeJA levels triggered a significant decline in plant growth and yield in transgenic overexpression of the *Arabidopsis* jasmonic acid carboxyl methyltransferase in *Arabidopsis* plants and in rice plants (Seo et al., 2001; Kim et al., 2009). In contrast, jasmonate promoted root and shoot development in cabbage (*Brassica oleracea*) under ultralow jasmonate concentrations (2–50 nM) (Toro et al., 2003). These differences in the effects on growth are likely due to different MeJA treatment concentrations and developmental stages of the plants or plant organs (Toro et al., 2003). Growth inhibition allows for a redirection of resources to defense and stress resistance processes, however, the events leading to growth inhibition in response to JA remain to be elucidated (Kim et al., 2009). Although a JASMONATE-ASSOCIATED1 (*JAS1*) gene acting as a repressor of JA-regulated growth inhibition has been reported (Yan et al., 2007), its function and the processes responsible for growth inhibition by JA remains obscure. The stress responses in plants are coordinated by JA with other hormones including ethylene, abscisic acid and cytokinins (Ueda and Kato, 1982). In the present study, leaf angle increased after the MeJA treatments (data not shown), suggesting that ethylene may also be involved in the responses of plants to MeJA. The inhibitory effects of MeJA on leaf growth and bud development could be prevented by co-treatment with benzyladenine (Saniewski, 1988) while the stimulation of plant growth by kinetin was inhibited by JA and MeJA (Ueda and Kato, 1982) indicating that the growth inhibition by MeJA may also involve cell division and differentiation. However, in zucchini (*Cucurbita pepo*) cotyledons, MeJA stimulated cell expansion and inhibited cell division while benzyladenine stimulated both expansion and division (Stoyanova-Bakalova et al., 2008). In our study, the length of root cortical cells in MeJA-treated plants was reduced by almost one-third in the wild-type and *fad7-2* plants, suggesting that cell elongation could be a factor contributing to growth reduction in *Arabidopsis* roots. Since cell elongation is dependent on hydrostatic pressure, it is among the most sensitive processes rapidly leading to growth inhibition in stressed plants.

Maintenance of plant water balance involves stomatal regulation and adjustments of tissue hydraulic conductivity, the processes which are commonly synchronized in response to environmental stimuli

(Steudle and Peterson, 1998). MeJA has been previously reported to reduce photosynthesis through decreases in the rate of  $\text{CO}_2$  fixation and the activity of ribulose-1,5 bisphosphate carboxylase (Rubisco) (Popova et al., 2003), likely to result in further growth inhibitions in a longer term. In our study,  $P_n$  reductions were measured after 1 d of 50  $\mu\text{M}$  MeJA treatments in the wild-type and *fad7-2* plants. However, 50  $\mu\text{M}$  MeJA did not affect  $P_n$  for at least 3 d in the *fad3-2* plant, suggesting that the plant growth was not reduced. Since  $E$  and photochemical efficiency of PSII (data not shown) were not affected as dramatically as the  $P_n$  after 3 d of 50  $\mu\text{M}$  MeJA treatment, they were not likely the primary factors responsible for the  $P_n$  inhibition. However, decreased  $L_p$  by MeJA may not be sufficient to affect the hydraulic conductance along the plant's hydraulic circuit since 50  $\mu\text{M}$  MeJA did not affect  $E$ . The reduction of  $P_n$  after exposure to exogenous MeJA is likely a factor contributing to the inhibition of plant growth in wild type plant, however, further research is needed to better understand the links between  $L_p$ ,  $E$ , and  $P_n$  in plants exposed to MeJA.

In conclusion, MeJA applied to the roots of wild-type *Arabidopsis* plants strongly and rapidly reduced  $L_p$ ,  $P_n$  and shoot growth. MeJA had no effect on  $E$ , and the expression of PIP isoforms responded differently depending on the duration of MeJA treatments in the wild-type, *fad3-2* and *fad7-2* plants. The MeJA-induced reductions of  $L_p$  were accompanied by reduced AQP phosphorylation and PIP transcript levels in the wild-type plants, but the application of MeJA had little effects on  $L_p$  in *fad3-2* and *fad7-2* plants. The application of  $\text{HgCl}_2$  did not further aggravate the  $L_p$  in the wild-type plants that were subjected to the MeJA, but significantly inhibited  $L_p$  in the *fad3-2* and *fad7-2* plants. The effect of MeJA on  $L_p$  was reversed by exposing the roots to 5  $\mu\text{M}$  OA. The results demonstrate that (i) MeJA-induced transmembrane water transport was among the sensitive processes early affected by MeJA in the wild-type *Arabidopsis* plants and its reduction by dephosphorylation and down-regulation of PIP gene expression may have resulted in the decrease in  $L_p$ , (ii) application of exogenous MeJA in *fad* plants did not alter  $L_p$  and AQP phosphorylation, but there were further reductions in  $L_p$  in MeJA overexpression plants, and (iii) the inhibition of AQP-mediated water transport was accompanied by growth reductions in MeJA-treated plants.

#### Declaration of interest

The authors have no conflicts of interest to declare.

#### Author contributions

SL & JZ designed and carried out the experiments. All authors contributed to writing and editing the manuscript.

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#### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.plaphy.2019.04.023>.

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