

# Hydrogen peroxide effects on root hydraulic properties and plasma membrane aquaporin regulation in *Phaseolus vulgaris*

Karim Benabdellah · Juan Manuel Ruiz-Lozano · Ricardo Aroca

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**Abstract** In the last few years, the role of reactive oxygen species as signaling molecules has emerged, and not only as damage-related roles. Here, we analyzed how root hydraulic properties were modified by different hydrogen peroxide ( $H_2O_2$ ) concentrations applied exogenously to the root medium. Two different experimental setups were employed: *Phaseolus vulgaris* plants growing in hydroponic or in potted soils. In both experimental setups, we found an increase of root hydraulic conductance ( $L$ ) in response to  $H_2O_2$  application for the first time. Twenty millimolar was the threshold concentration of  $H_2O_2$  for observing an effect on  $L$  in the soil experiment, while in the hydroponic experiment, a positive effect on  $L$  was observed at 0.25 mM  $H_2O_2$ . In the hydroponic experiment, a correlation between increased  $L$  and plasma membrane aquaporin amount and their root localization was observed. These findings provide new insights to study how several environmental factors modify  $L$ .

**Keywords** Aquaporins · Arbuscular mycorrhizal fungi · Hydrogen peroxide · Immunolocalization · *Phaseolus vulgaris* · Root hydraulic conductance

## Introduction

Plants are exposed to several environmental changes which, in some circumstances, may cause stress. Many environmental stresses induce similar plant responses, such

as tissue dehydration or oxidative damage. These two stress effects are caused by soil water limitation (Aroca et al. 2003), extreme temperatures (Aroca et al. 2003; Wahid et al. 2007), soil salinity (Tattini and Traversi 2008), or flooding (Sairam et al. 2008). Plant tissue dehydration is caused by the imbalance between water loss by leaf transpiration and water uptake by roots. In fact, upon exposure to different stresses, plants reduce their stomatal aperture in order to minimize their water loss (Yang et al. 2005; Schachtman and Goodger 2008). At the same time, root water uptake capacity under different environmental stress conditions is related to tolerance to such stresses (Marulanda et al. 2003; Aroca et al. 2005; del Martínez-Ballesta et al. 2006).

Root water uptake is determined, in part, by root hydraulic conductance ( $L$ ), which is ultimately regulated by plasma membrane aquaporins activity (Kaldenhoff et al. 1998; Javot et al. 2003). Aquaporins are membrane-intrinsic proteins that facilitate the flow of water and other small and neutral solutes through membranes following an osmotic gradient (Maurel 2007; Maurel et al. 2008). In plants, the aquaporin family comprises around 30 different proteins, at least in *Arabidopsis*, maize, and rice (Chaumont et al. 2001; Johanson et al. 2001; Sakurai et al. 2005). Plant aquaporins are subdivided into four subgroups based on their amino acid sequence similarity: plasma membrane intrinsic proteins (PIPs), tonoplast intrinsic proteins (TIPs), nodulin-like intrinsic proteins (NIPs), and small and basic intrinsic proteins (SIPs). PIPs are further subdivided into PIP1 and PIP2 proteins, with PIP2 proteins being more active in terms of water transport capacity (Chaumont et al. 2000; Sakurai et al. 2005). Apart from their different subcellular localization, each aquaporin protein differs in its solute transport selectivity (Katsuhara et al. 2008).

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*L* generally decreases under abiotic stresses conditions (Aroca et al. 2005, 2006; del Martínez-Ballesta et al. 2006; Boursiac et al. 2008). However, a maize cold tolerant genotype and a bean cultivar increased their *L* after some days of cold exposure (Vernieri et al. 2001; Aroca et al. 2005). The decrease of *L* by different abiotic stresses, such as cold or salt, has been linked to the production of reactive oxygen species (ROS) under such conditions (Ktitorova et al. 2002; Lee et al. 2004; Aroca et al. 2005; Rhee et al. 2007; Boursiac et al. 2008). In fact, when exogenous hydrogen peroxide ( $H_2O_2$ ) treatment, commonly used to simulate ROS effects in plants (Desikan et al. 2001; Wan and Liu 2008), is added to solution surrounding roots, a decrease of *L* is commonly observed (Ktitorova et al. 2002; Lee et al. 2004; Aroca et al. 2005; Rhee et al. 2007; Boursiac et al. 2008). However, in some studies, exogenous  $H_2O_2$  caused a slight non significant increase of *L* (Aroca et al. 2005; Boursiac et al. 2008). At the same time, the effect of exogenous application of abscisic acid (ABA) on the root-free exuded sap flow rate is modified by exogenous co-application of catalase ( $H_2O_2$ -scavenging enzyme) or ascorbate (a potent and widespread antioxidant) in *Phaseolus vulgaris* plants, with the effect of antioxidants being different as the exogenous ABA concentration applied rose (Aroca 2006).

The above cited results indicate a possible role of ROS in regulating root hydraulic properties, decreasing or increasing *L* depending on the environmental circumstances. This interpretation fits the dual role of ROS in plants, since they can act as damaging or signaling molecules, depending on the environmental conditions (Hung et al. 2005; Miller et al. 2008; Van Breusegem et al. 2008). In fact, it is well documented how ROS act as signaling molecules during ABA-induced stomatal closure (Pei and Kuchitsu 2005; Wang and Song 2008). At the same time, several mechanisms have been proposed to explain the diminution of *L* caused by ROS. Ye and Steudle (2006) proposed that  $H_2O_2$ , or most likely, hydroxyl radical ( $OH^\bullet$ ) could cause conformational changes on aquaporins, avoiding water transport. Boursiac et al. (2008) found an internalization of plasma membrane vesicles containing a high amount of PIP proteins after  $H_2O_2$  addition to *Arabidopsis* roots, which could cause a diminution of the plasma membrane hydraulic conductivity. The studies of Ye and Steudle (2006) and Boursiac et al. (2008) were carried out in a relatively short period of time, from 15 to 120 min. This time frame fits well with the initial decrease of *L* observed after plant exposure to several stresses (Aroca et al. 2001; del Martínez-Ballesta et al. 2006; Boursiac et al. 2008). However, this time frame is very short when compared to that required by bean plants to increase their *L* upon exposure to cold (1 day; Vernieri et al. 2001), or after ABA application (1 day; Aroca 2006),

or to that needed by a maize cold-tolerant genotype to increase *L* upon exposure to cold (3 days; Aroca et al. 2001, 2005).

Most of the plants in nature are associated with arbuscular mycorrhizal (AM) fungi (Gai et al. 2006). By this association, the fungus obtains carbon compounds from the plant and a niche to complete its life cycle, while the plant obtains access to nutrient and water resources from the fungus, which is inaccessible to the roots (Harrison 2005). At the same time, by the AM symbiosis, the host plant enhances their tolerance to several biotic and abiotic stresses (Wu et al. 2006; Aroca et al. 2007; Liu et al. 2007). This enhancement is, in part, caused by the modulation of the antioxidant capacity of the host plant (Wu et al. 2006), as well as *L* and PIP aquaporin abundance (Aroca et al. 2007). Hence, it is possible that the different responses of *L* and aquaporins to different abiotic stresses between AM and non AM roots (Porcel et al. 2006; Aroca et al. 2007) could be mediated by the different regulation of root cells redox status by the AM symbiosis (Schützendübel and Polle 2002; Wu et al. 2006). Therefore, experiments were carried out with both hydroponic and soil-growing plants, allowing the establishment and development of the AM symbiosis in the second grown medium.

In the present research, we investigated how different exogenous concentrations of  $H_2O_2$  affect *L* in bean plants during different times of exposure (from 1 to 24 h). Also, *L* and PIP aquaporin transcriptional regulation and protein abundance were also studied, as well as their root localization. We found, for the first time, an increase of *L* in response to specific exogenous  $H_2O_2$  concentrations, which correlated with an increase in PIP2 aquaporin amount in the cells from the water-absorbing zone of the roots. These results point out a possible involvement of ROS in the increase of *L* observed in response to some environmental stresses.

## Materials and methods

### Biological materials and experimental design

#### Hydroponic experiments

Seeds of *Phaseolus vulgaris* L. cv. Borlotto were soaked for 3 min in pure ethanol and then rinsed three times with distilled water. Seeds were sown in wet sepiolite (a clay mineral) and, after 9 days, seedlings were transferred to aerate 9-l containers filled with 80% nutrient solution (Aroca 2006). The nutrient solution was replaced every four days. Plants were grown in a controlled growth chamber at 23:21°C (day:night), in a photoperiod of 16:8 h

(day:night), with a photosynthetic photon flux density of  $300 \mu\text{mol m}^{-2} \text{s}^{-1}$ . After 2 weeks of hydroponic growth, the roots were exposed to a final concentration of 0.25, 0.5, 1, or 5 mM  $\text{H}_2\text{O}_2$ . Root samples and  $L$  measurements were taken 1, 6, or 24 h after the  $\text{H}_2\text{O}_2$  addition in both treated and non treated plants. Samples for molecular and biochemical analysis were immersed in liquid nitrogen and stored at  $-80^\circ\text{C}$  until use. The other measurements were immediately carried out after removing the roots from the solution.

#### Soil pot experiments

Bean seeds were treated and germinated as described above, and then transplanted to 500-ml pots filled with a sterilized mixture of sand and soil (1:1, v/v). Half of the pots were inoculated with the AM fungus *Glomus intraradices* (Schenk and Smith) isolate BEG 123. For details about the soil characteristics and inoculation procedure, see Porcel et al. (2006). Five weeks after being transplanted, the soil roots were exposed to a final concentration of 0.25, 0.5, 1, 5, 10, or 20 mM  $\text{H}_2\text{O}_2$  by adding 20 ml of appropriate dilution from a concentrated solution to the pots. Root samples and  $L$  measurements were taken 1, 6, and 24 h after  $\text{H}_2\text{O}_2$  addition in both treated and non treated plants. Samples for molecular and biochemical analysis were immersed in liquid nitrogen and stored at  $-80^\circ\text{C}$  until use. Mycorrhizal development inside the roots was measured after clearing washed roots in 10% KOH and staining with 0.05% trypan blue in lactic acid (w/v), according to Philips and Hayman (1970). The extent of mycorrhizal root colonization was calculated according to the gridline intersect method (Giovannetti and Mosse 1980).

#### Root hydraulic properties measurements

Root hydraulic conductance ( $L$ ) was measured as described previously by Aroca et al. (2001, 2006). Briefly, plants growing hydroponically or in pots (the latter immersed in aerated solution) were cut with a razor blade just below the cotyledons. A pipette connected to a silicon tube was attached to the stem. The liquid exuded in the first 15 min was discarded to avoid phloem contaminations. The exudates of the following 2 h were collected and weighed. The osmolarities of the exuded sap were determined using a cryoscopic osmometer (Osmomat 030, Gonotec GmbH, Berlin, Germany).  $L$  was calculated as:  $L = J_v / \Delta\Psi_s$ , where  $J_v$  is the exuded sap flow rate and  $\Delta\Psi_s$  is the osmotic potential gradient between the exuded sap and the solution.  $L$  was measured in 15 plants from hydroponic pots and nine from soil pots.

#### Reactive oxygen species determination

Hydrogen peroxide contents of root tissues from soil pot plants were determined by Patterson's method (Patterson et al. 1984), with slight modifications as described previously by Aroca et al. (2003). One gram of root fresh weight from four different plants was homogenized in a cold mortar with 5 ml 5% (w/v) TCA containing 0.1 g of activated charcoal and 1% (w/v) PVPP. The homogenate was centrifuged at 18,000g for 10 min. The supernatant was filtered through a Millipore filter (0.22  $\mu\text{m}$ ). A volume of 1.2 ml of 100 mM potassium phosphate buffer (pH = 8.4) and 0.6 ml of the colorimetric reagent were added to 130  $\mu\text{l}$  of the supernatant. The colorimetric reagent was freshly made by mixing 1:1 (v/v) 0.6 mM potassium titanium oxalate and 0.6 mM 4-2 (2-pyridylazo)resorcinol (disodium salt). The samples were incubated at  $45^\circ\text{C}$  for 1 h and the absorbance at 508 nm was recorded. The same procedure was followed to measure the  $\text{H}_2\text{O}_2$  content in the hydroponic solution after 1, 6, or 24 h of  $\text{H}_2\text{O}_2$  application.

The probe 2',7'-dichlorodihydrofluorescein diacetate ( $\text{H}_2\text{DCF-DA}$ ) was used to detect ROS in hydroponic roots, both from untreated or treated with 1 mM  $\text{H}_2\text{O}_2$  plants (Benabdellah et al. 2009). Roots were incubated with 10  $\mu\text{M}$   $\text{H}_2\text{DCF-DA}$  in PBS (137 mM NaCl, 2.7 mM KCl, 1.4 mM  $\text{Na}_2\text{HPO}_4$ , pH = 7.4). The probe can cross the root plasma membrane freely, and is then cleaved to its impermeable counterpart, dichlorofluorescein ( $\text{H}_2\text{DCF}$ ), by endogenous esterases.  $\text{H}_2\text{DCF}$  functions as a reporter of ROS content. After 30 min, the medium was replaced with fresh PBS and observations were made under the fluorescence microscope. The excitation and the emission wavelengths were 450–490 and 520 nm (green), respectively.

#### Root electrolyte leakage

Root electrolyte leakage was measured in hydroponic culture plants as an index of membrane root injury as previously described by Aroca et al. (2005). Briefly, entire root systems from 14 plants were placed individually in tubes containing 50 ml of distilled water. After 3 h of incubation at room temperature in smooth agitation, the conductivity of the solution was measured (conductivity meter, COND 510, XS Instrumentation) and is referred to as  $C_0$ . Then, the tubes were placed at  $-80^\circ\text{C}$  for 1 h and incubated again at room temperature for 2 h. The conductivity of the solution at this time was referred to as  $C_T$ . The conductivity of distilled water before the root immersion was measured and referred to as  $C_W$ . The percentage of electrolyte leakage was calculated as follows:  $\text{EL} = [(C_0 - C_W) / (C_T - C_W)] \times 100$ .

## PIP expression analyses

Expression analyses of the six *PIP* genes described up to now in *P. vulgaris* were carried out by quantitative real-time RT-PCR (Aroca et al. 2007; Zhou et al. 2007). Total RNA was isolated from *P. vulgaris* roots by a phenol/chloroform extraction method followed by precipitation with LiCl (Kay et al. 1987). DNase treatment of total RNA was performed according to Promega's recommendations. Total RNA (2.5 µg) was reversed transcribed to first strand cDNA using AMV-RT enzyme and oligo(dT)<sub>15</sub> primer (Promega, Madison, WI, USA). Quantitative real-time RT-PCR was carried out by using an iCycler (Bio-Rad, Hercules, CA, USA), as described by Porcel et al. (2006). The *PIP* genes analyzed were: *PvPIP1;1* (Acc. No. U97023), *PvPIP1;2* (Acc. No. AY995196), *PvPIP1;3* (Acc. No. DQ855475), *PvPIP2;1* (Acc. No. AY995195), *PvPIP2;2* (Acc. No. EF624001), and *PvPIP2;3* (Acc. No. EF624002). Specific primers for *PvPIP1;2*, *PvPIP2;2*, and *PvPIP2;3* were the same as those described by Aroca et al. (2007) and Zhou et al. (2007). The PCR mix and PCR program were as described by Porcel et al. (2006), except annealing temperature, which was switched to 58°C. The primers for *PvPIP1;1* were forward: ATTAGGTGGTGC ATGTGTCG, and reverse: CAGCCGTACATCACACAT AGAG. Primers for *PvPIP1;3* were forward: GTGGGTAA AAGAACCAGACG, and reverse: CACATCGCTCATGG TCATC. Primers for *PvPIP2;1* were forward: CTCCAAC AAGGCTATGGTGT, and reverse: CCCACAACAACCTG TAGCTGA. Primers were designed to amplify a fragment of the 3' untranslated region of each gene that is specific for each of them. Standardization was carried out by measuring the expression of the *P. vulgaris* actin gene in each sample by using *P. vulgaris* actin-specific primers (Wen et al. 2005). The relative abundances of transcription were calculated by using the  $2^{-\Delta\Delta C_t}$  method (Livak and Schmittgen 2001). Analyses were carried out in three independent RNA samples from three different root samples. Analyses were repeated three times for each RNA sample.

## Western blots

Microsomes were isolated from *P. vulgaris* roots as described by Daniels et al. (1994). Briefly, roots were homogenized in grinding buffer (100 mM Tris-HCl pH = 7.5, 1 mM EDTA, 12% (w/v) sucrose, 0.2 mM aminoethylbenzenesulfonylfluoride, 2 µg ml<sup>-1</sup> aprotinin, 1 µg ml<sup>-1</sup> leupeptin), and the supernatant was collected after centrifugation at 15,000g for 10 min. The supernatant was filtered through a single layer of cheesecloth and centrifuged again at 100,000g for 2 h. The pellet was resuspended in 40 µl of grinding buffer plus 0.1% (w/v)

SDS. Ten micrograms of protein were loaded in each line after incubating for 30 min at 37°C in the presence of denaturing buffer (20 mM Tris-HCl pH = 8.6, 1% (w/v) SDS, 0.3% (v/v) β-mercaptoethanol, 8% (v/v) glycerol, 0.2% (w/v) bromophenol-blue). Proteins were transferred to a PVDF membrane at 100 mA for 1 h. The membrane was blocked for 2 h at room temperature with 5% (w/v) non fat milk in Tris-buffered-saline (TBS) with 0.05% Tween 20. After that, the membrane was incubated overnight at 4°C with the corresponding antibody dilution (1:2,000 for PIP1 and PIP2 antibodies, and 1:1,000 for phosphorylated PIP2 antibody). Goat anti-rabbit Ig coupled to horseradish peroxidase (Sigma) was used as a secondary antibody at a dilution of 1:20,000. The signal was developed using a chemiluminescent substrate (West-Pico, Super Signal, Pierce, Rockford, IL, USA). PIP1 and PIP2 antibodies were kindly provided by Dr. Maurel (INRA, Montpellier, France), and were raised in rabbit against a 42-amino acid N-terminal peptide of AtPIP1;1 or 17-amino acid C-terminal peptide of AtPIP2;1, respectively (Santoni et al. 2003). Phosphorylated PIP2 antibody was kindly provided by Dr. Hermann (Donald Danforth Plant Science Center, St. Louis, MO, USA) and raised against the most conserved sequence among all PIP2 proteins in diverse species with a possible phosphorylation site (Aroca et al. 2005). These antibodies were used previously with *P. vulgaris* root microsomes (Aroca et al. 2006, 2007). Microsomes were isolated from two different root samples. Western blots were developed on both microsome samples of each treatment without significant differences between them.

## PIP2 immunolocalization

PIP2 proteins were localized using the AtPIP2;1 antibody used for Western blots. Roots were fixed for 30 min in freshly prepared 4% (w/v) paraformaldehyde (Sigma) PBS solution, washed two times with PBS for 45 min, and digested at 37°C for 30 min in a mixture of 2.5% (w/v) pectolyase (Sigma) and 2.5% (w/v) cellulose (Sigma) dissolved in PBS in order to facilitate antibody permeation. For thin-section analyses, the roots were cut into 5-mm pieces and fixed in 4% (w/v) formaldehyde in PBS for 2 h at 4°C. Samples were washed twice in PBS and dehydrated through a graded ethanol series and infiltrated in 1:1 100% ethanol and LR White (London Resin Co.) overnight and 100% resin for 24 h. Serial sections, 1-µm thick, were obtained by means of an Ultracut R Ultramicrotome (Leica, Bensheim, Germany).

Sections and roots pieces were incubated with the anti-AtPIP2;1 diluted 1:2,000 in PBS, containing 0.1% w/v bovine serum albumin. Incubation was done in a moist chamber for 90 min at RT. Slides were then washed twice



for 10 min in PBS and incubated for 90 min with secondary antibody, goat anti-rabbit IgG–FITC conjugate (Molecular Probes) diluted to 1:1,000 in the same way as the first antibody. Incubation was performed at the same temperature as with the first antibody in darkness. After washing for 10 min in PBS, the sections and root pieces were mounted under a cover slip using *p*-phenylenediamine anti-fade mounting. Fluorescence was examined with a Nikon Eclipse inverted microscope equipped with epifluorescence and appropriate filter sets (peak excitation, 590 nm; peak emission, 617 nm). Photographs were taken with a DS-Fi1 Nikon camera.

## Results

We first tested how the H<sub>2</sub>O<sub>2</sub> levels were maintained during the experiment in the hydroponic solution. After 1 h of adding 0.25 or 1 mM H<sub>2</sub>O<sub>2</sub>, the H<sub>2</sub>O<sub>2</sub> content was decreased by almost 50%. After 6 h of addition, H<sub>2</sub>O<sub>2</sub> was only detected at 10% of the initial values, and after 24 h, the H<sub>2</sub>O<sub>2</sub> content was almost undetected in both treatments (data not shown).

### Root hydraulic properties

Root hydraulic conductance (*L*) was measured in roots exuding under atmospheric pressure. Under these conditions, water only goes through roots thanks to the osmotic gradient between root solution and root xylem. Hence, water only flowed through the cell-to-cell pathway according to Steudle's model (Steudle and Peterson 1998). Under hydroponic culture, the application of 5 mM exogenous H<sub>2</sub>O<sub>2</sub> reduced root water flux (*J<sub>v</sub>*) and *L* without any recovery after 24 h (Fig. 1a, c). At the same time, the 5 mM H<sub>2</sub>O<sub>2</sub> application caused an increase in the osmotic gradient between the nutrient solution and exuded sap ( $\Delta\Psi_s$ ) after 1 h of exposure (Fig. 1b). A total of 1 mM H<sub>2</sub>O<sub>2</sub> had similar effects to 5 mM in *J<sub>v</sub>* and *L*, but at 1 mM concentration, both parameters recovered after 24 h of exposure (Fig. 1a, c). The two lowest concentrations of H<sub>2</sub>O<sub>2</sub> used (0.25 and 0.5 mM) caused an increase of *J<sub>v</sub>* and *L* after 24 h of exposure (Fig. 1a, c). However, 0.25 mM H<sub>2</sub>O<sub>2</sub> exposure caused an increase of *L* after 6 h, and a decrease of  $\Delta\Psi_s$  after 6 and 24 h (Fig. 1b, c). In summary, lower H<sub>2</sub>O<sub>2</sub> concentrations caused an increase of *L*, more pronounced after 24 h, while higher concentrations caused an inhibition of *L*, beginning just after 1 h. However, a recovery of *L* was observed in 1 mM treated roots after 24 h.

In the plants growing in soil, only the highest H<sub>2</sub>O<sub>2</sub> concentration used (20 mM) had a significant effect on *J<sub>v</sub>* or *L*, and only after 24 h. Therefore, we only show these

results in Fig. 2. Interestingly, 20 mM H<sub>2</sub>O<sub>2</sub> exposure up to 24 h caused an increase of *L* in both AM and non AM plants, but only caused a significant increase of *J<sub>v</sub>* in non AM plants (Fig. 2). Thus, the increase of *L* by H<sub>2</sub>O<sub>2</sub> in AM plants was caused by slight non significant increases or decreases of *J<sub>v</sub>* and  $\Delta\Psi_s$ , respectively (Fig. 2). In other words, the effect of H<sub>2</sub>O<sub>2</sub> was more pronounced in non AM plants.

### Hydrogen peroxide levels in soil roots

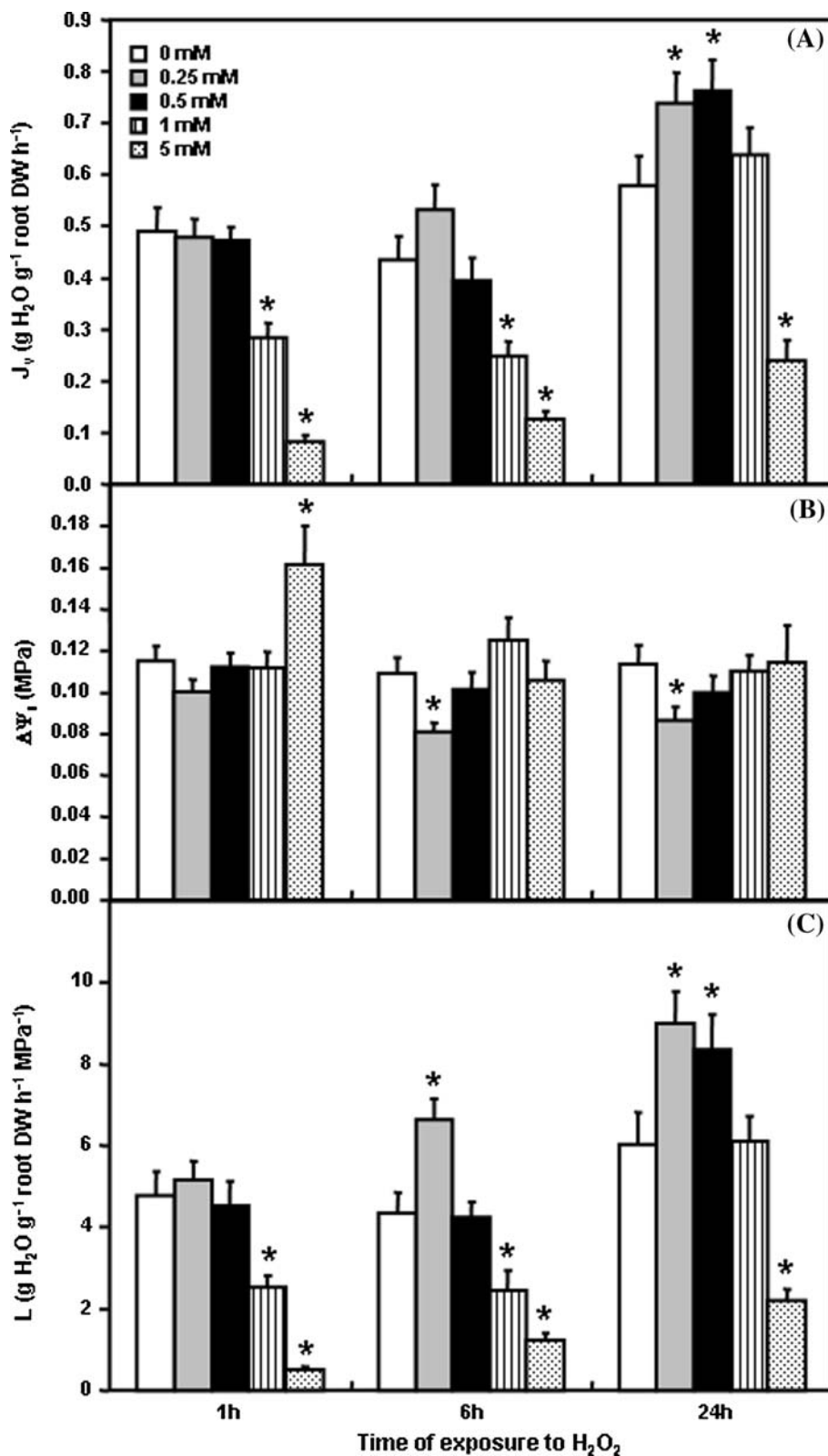
It is well documented that AM and non AM roots differ in their capacity to remove ROS (Wu et al. 2006), thus, we evaluated root H<sub>2</sub>O<sub>2</sub> levels in soil-growing roots in order to clarify the different response of *J<sub>v</sub>* and *L* to H<sub>2</sub>O<sub>2</sub> in AM and non AM plants. Just after 1 h of exogenous H<sub>2</sub>O<sub>2</sub> application, the roots of non AM plants presented 2.5 times higher concentrations of H<sub>2</sub>O<sub>2</sub> than non AM untreated roots, and this difference remained during 6 h of exposure (Fig. 3a). On the contrary, AM roots only increased their H<sub>2</sub>O<sub>2</sub> contents after 6 h of exposure to H<sub>2</sub>O<sub>2</sub> (Fig. 3a). After 24 h of exposure, both kinds of plants returned their root H<sub>2</sub>O<sub>2</sub> content to untreated values (Fig. 3a). Therefore, the roots of non AM plants responded faster and were more prolonged to H<sub>2</sub>O<sub>2</sub> exposure than the roots of AM plants.

### PIP expression and abundance in soil roots

Since *J<sub>v</sub>* and *L* are determined, in part, by PIP aquaporin activity (Kaldenhoff et al. 1998; Javot et al. 2003), we analyzed PIP gene expression and protein abundance in AM and non AM roots after 24 h of exposure to 20 mM H<sub>2</sub>O<sub>2</sub>. H<sub>2</sub>O<sub>2</sub> exposure caused an increase of *PvPIP2;2* and *PvPIP2;3* gene expression in AM roots, while it decreased *PvPIP1;1*, *PvPIP1;2*, *PvPIP2;1*, and *PvPIP2;2* gene expression in non AM roots (Fig. 3b). Thus, the effect of H<sub>2</sub>O<sub>2</sub> exposure on *PIP* gene expression was opposite in both kinds of roots.

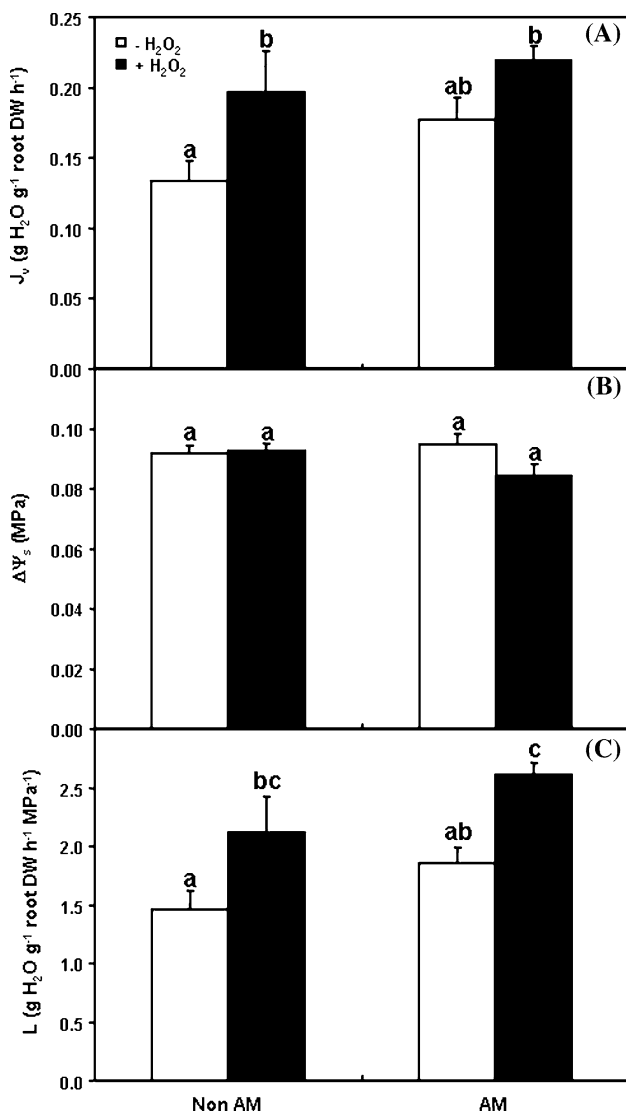
PIPs and PIP2s protein abundance and phosphorylation state at Ser 121 (123) of PIP2s proteins were analyzed by Western blots using antibodies against a 42-amino acid N-terminal peptide of AtPIP1;1, a 17-amino acid C-terminal peptide of AtPIP2;1 (Santoni et al. 2003), and an 8-amino acid peptide surrounding Ser 113 of *Triticum aestivum* PIP2 (Aroca et al. 2005). These same antibodies have been successfully used previously with microsomes from *P. vulgaris* roots (Aroca et al. 2006, 2007). The three antibodies presented the same signaling motif. Without H<sub>2</sub>O<sub>2</sub> treatment, no signal was detected in AM roots (Fig. 3c). After H<sub>2</sub>O<sub>2</sub> treatment, no changes in any of the signal amounts was observed in non AM roots, but a significant increase was observed in AM roots (Fig. 3c). So, a

**Fig. 1** Root-free exuded sap ( $J_v$ , **a**), osmotic potential difference between exuded sap and nutrient solution ( $\Delta\Psi_s$ , **b**), and root hydraulic conductance ( $L$ , **c**) of *Phaseolus vulgaris* plants untreated ( $\square$ ), or treated with 0.25 mM ( $\blacksquare$ ), 0.5 mM ( $\blacksquare$ ), 1 mM ( $\square$ ), or 5 mM ( $\square$ )  $H_2O_2$  for 1, 6, or 24 h, corresponding to 3, 8, or 2 h after the lights turned on. The asterisks indicate significant difference ( $P < 0.05$ ) with the corresponding untreated root after ANOVA and Fisher LSD analysis. The bars represent mean  $\pm$  SD ( $n = 15$ )



certain correlation between an increase of  $L$  and PIP expression and abundance was observed in AM roots, although this correlation was not found in non AM roots.

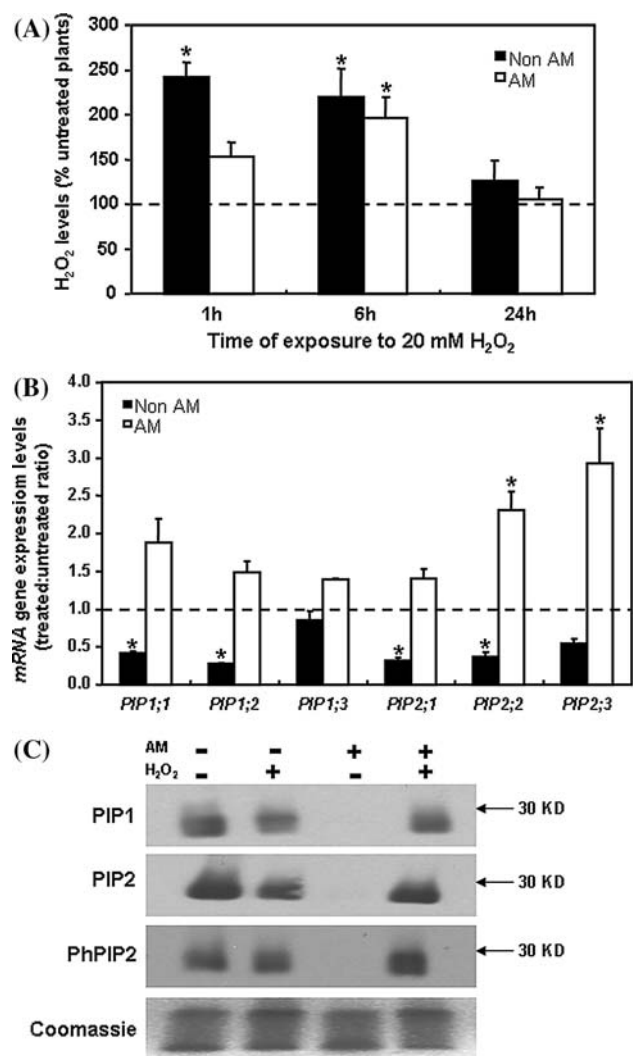
Since PIPs protein abundance changes along the root axes, and between different kinds of root cells (Hachez et al. 2006), we decided to focus on hydroponic experiments.



**Fig. 2** Root-free exuded sap ( $J_v$ , **a**), osmotic potential difference between exuded sap and nutrient solution ( $\Delta\Psi_s$ , **b**), and root hydraulic conductance ( $L$ , **c**) of *P. vulgaris* plants inoculated or not inoculated with the AM fungus *Glomus intraradices*, and exposed (black columns) or not (white columns) to 20 mM  $\text{H}_2\text{O}_2$  for 24 h. The different letters indicate significant differences ( $P < 0.05$ ) among treatments after ANOVA and Fisher LSD analysis. The bars represent mean  $\pm$  SD ( $n = 9$ )

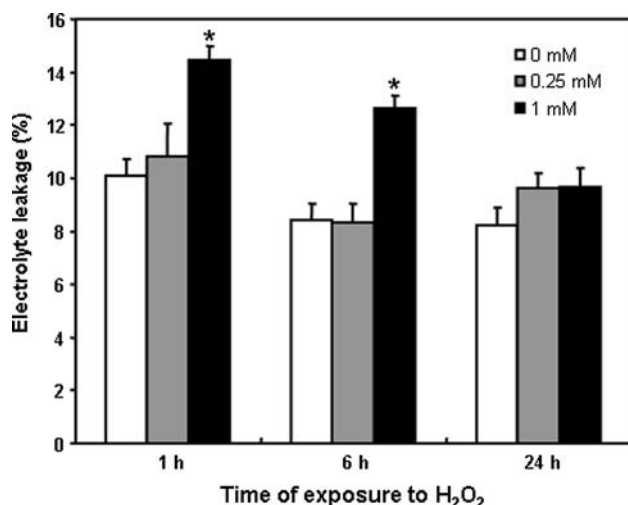
Electrolyte leakage and ROS contents in hydroponic roots

One of the causes of decreasing  $L$  by 1 or 5 mM  $\text{H}_2\text{O}_2$  treatments could be membrane damage. This can be estimated by measuring the root electrolyte leakage (EL) (Aroca et al. 2005; Aroca 2006). So, we decided to measure the EL in roots of untreated or treated with 0.25 or 1 mM  $\text{H}_2\text{O}_2$  plants, selecting the minimal concentrations of  $\text{H}_2\text{O}_2$  that caused an increase and a decrease in  $L$ , respectively (Fig. 1c). EL only increased after 1 and 6 h of root

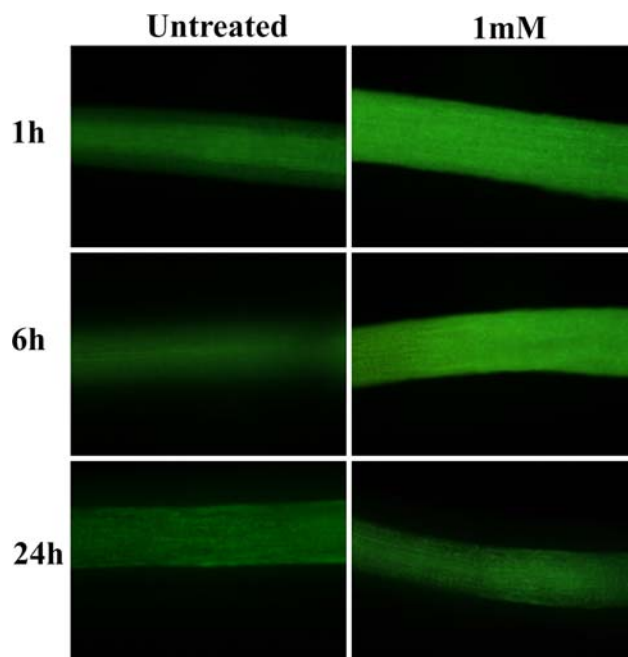


**Fig. 3** **a** Hydrogen peroxide levels of *P. vulgaris* roots inoculated (white columns) or not inoculated (black columns) with the AM fungus *G. intraradices* after 1, 6, or 24 h of exposure to 20 mM  $\text{H}_2\text{O}_2$ . The data are expressed as a percentage of the untreated roots. The asterisks indicate significant difference ( $P < 0.05$ ) with the corresponding untreated root after ANOVA and Fisher LSD analysis. The bars represent mean  $\pm$  SD ( $n = 4$ ). **b** Relative mRNA expression of different PIP genes of *P. vulgaris* roots inoculated (white columns) or not inoculated (black columns) with the AM fungus *G. intraradices* after 24 h of exposure to 20 mM  $\text{H}_2\text{O}_2$ . The asterisks indicate significant difference ( $P < 0.05$ ) with the corresponding untreated root after ANOVA and Fisher LSD analysis. The bars represent mean  $\pm$  SD ( $n = 3$ ). **c** Western blot analysis of microsomes from *P. vulgaris* roots inoculated or not with the AM fungus *G. intraradices* and treated or not with 20 mM  $\text{H}_2\text{O}_2$ . The antibodies used were against AtPIP1;1 (PIP1), AtPIP2;1 (PIP2), or phosphorylated PIP2s at Ser 121 (PhPIP2). The last picture represents the coomassie brilliant blue line of each microsome treatment

exposure to 1 mM  $\text{H}_2\text{O}_2$ , falling back to control values after 24 h (Fig. 4). The same pattern of EL was observed in root ROS content visualized by  $\text{H}_2\text{DCF-DA}$  probe in the roots treated with 1 mM  $\text{H}_2\text{O}_2$  (Fig. 5). The ROS content increased after 1 and 6 h of root exposure to 1 mM  $\text{H}_2\text{O}_2$ ,



**Fig. 4** Electrolyte leakage (EL) of roots grown in hydroponic soil after 1, 6, or 24 h of exposure to 0 (white columns), 0.25 (grey columns), or 1 mM (black columns) H<sub>2</sub>O<sub>2</sub>. The asterisks indicate significant difference ( $P < 0.05$ ) with the corresponding untreated root after ANOVA and Fisher LSD analysis. The bars represent mean  $\pm$  SD ( $n = 14$ )



**Fig. 5** Reactive oxygen species (ROS) detection using dichlorofluorescein (H<sub>2</sub>DCF) dye in roots untreated (left panels) or treated with 1 mM H<sub>2</sub>O<sub>2</sub> (right panels) for 1, 6 or 24 h. More green fluorescence means more ROS amount

but returned back to control levels after 24 h. Therefore, a correlation between an increase of EL and ROS and a decrease of *L* was found in roots exposed to 1 mM H<sub>2</sub>O<sub>2</sub>. At the same time, the same roots recovered their *L* values, while decreasing their EL and ROS levels.

#### *PIP* gene expression and abundance in hydroponic roots

As previously commented, the expression analysis of *PIP* genes was carried out in untreated roots or roots treated with 0.25 or 1 mM H<sub>2</sub>O<sub>2</sub>. In untreated roots, *PvPIP1;1* and *PvPIP1;2* genes did not change their expression during the day (Fig. 6a, c). By contrast, *PvPIP1;3*, *PvPIP2;1*, and *PvPIP2;3* genes decreased their expression after 6 h (8 h after dawn) of H<sub>2</sub>O<sub>2</sub> application (Fig. 6b, e, f). *PvPIP1;3* and *PvPIP2;3* genes recovered to 1-h (3 h after dawn) expression values after 24 h (2 h after dawn) of H<sub>2</sub>O<sub>2</sub> treatment. On the contrary, *PvPIP2;1* gene expression retained 6-h values after 24 h of H<sub>2</sub>O<sub>2</sub> treatment (Fig. 6b, e, f). Finally, *PvPIP2;2* gene expression was lower after 24 h of H<sub>2</sub>O<sub>2</sub> treatment than after 1 or 6 h of treatment (Fig. 6d).

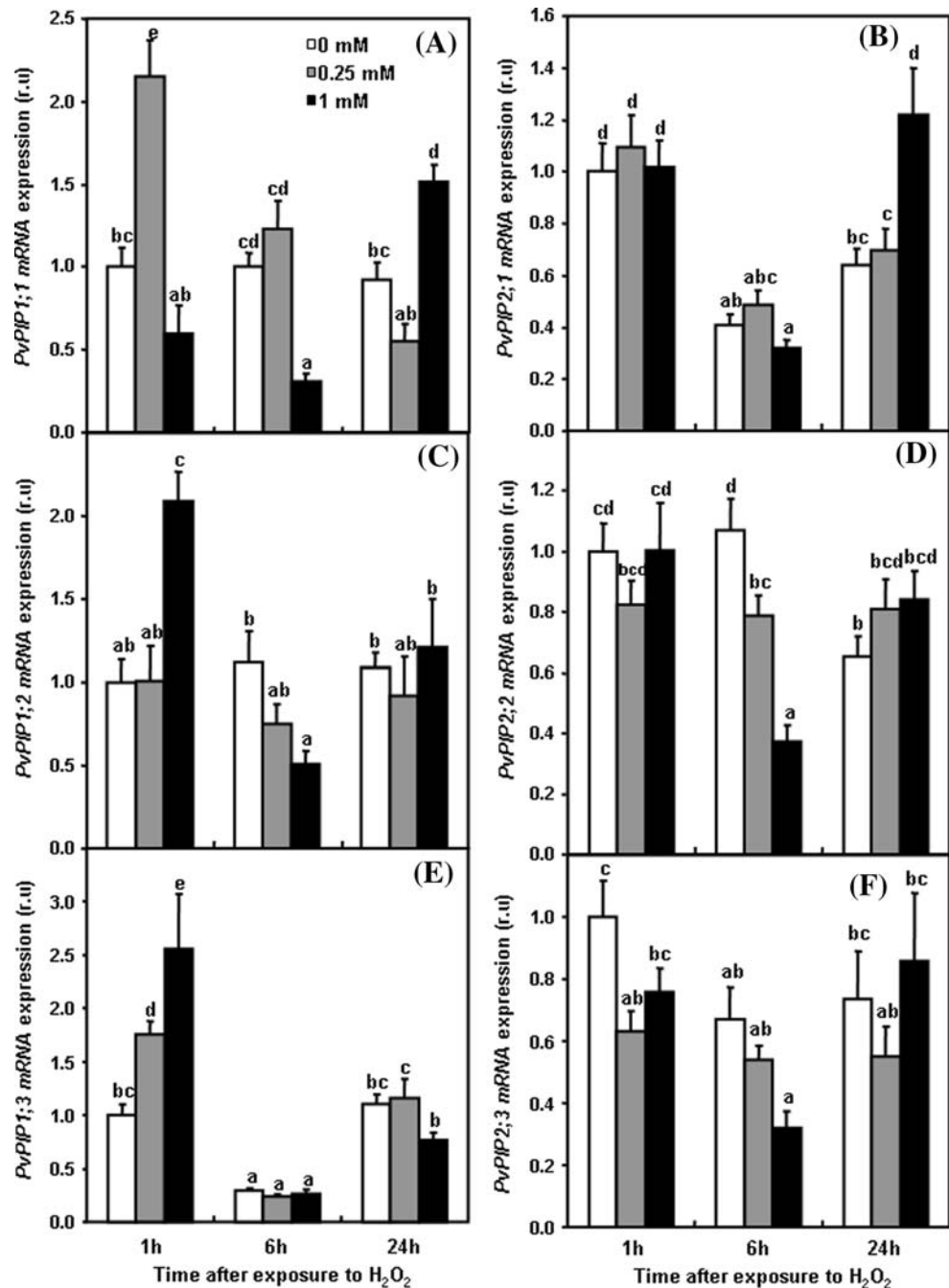
After 1 h of exposure of roots to 0.25 mM H<sub>2</sub>O<sub>2</sub>, *PvPIP1;1* and *PvPIP1;3* genes increased their expression, while the expression of *PvPIP2;3* gene was decreased (Fig. 4a, e, f). At the same time, the expression of *PvPIP2;2* gene also decreased after 6 h of root exposure to 0.25 mM H<sub>2</sub>O<sub>2</sub> (Fig. 6d). On the other hand, root exposure to 1 mM H<sub>2</sub>O<sub>2</sub> caused an increase of *PvPIP1;2* and *PvPIP1;3* gene expression after 1 h of exposure (Fig. 6c, e), while after 24 h of exposure, an increase in expression was observed in *PvPIP1;1* and *PvPIP2;1* genes (Fig. 6a, b). By contrast, after 6 h of root exposure to 1 mM H<sub>2</sub>O<sub>2</sub>, a decrease in the expression of *PvPIP1;1*, *PvPIP1;2*, and *PvPIP2;2* genes was observed (Fig. 6a, c, d). No more significant changes on *PIPs* gene expression by H<sub>2</sub>O<sub>2</sub> treatments were detected.

Thereafter, *PIPs* protein abundance and phosphorylation at Ser 121 was analyzed by Western blots as described above (Fig. 7). No signal was detected with any of the antibodies used in untreated or in 1 mM H<sub>2</sub>O<sub>2</sub> treated root microsomes. However, a strong signal was detected after 1 h of root exposure to 0.25 mM H<sub>2</sub>O<sub>2</sub> with the three antibodies used. Minor signal was detected after 6 h and, again, a strong signal was detected after 24 h of root exposure to 0.25 mM H<sub>2</sub>O<sub>2</sub>. However, the signal at 24 h was slightly lower than that observed at 1 h. It is worthy to mention that these strong signals were detected in the lanes where less protein was loaded according to coomassie staining.

A correlation between *PIP1* protein abundance and *PvPIP1;1* and *PvPIP1;3* gene expression was observed after at least 1 h of exposure to 0.25 mM H<sub>2</sub>O<sub>2</sub> (Figs. 6a, e and 7), but no correlation was found for *PIP2* proteins. So, we decided to analyze *PIP2* genes expression pattern at shorter times (10, 20, and 40 min), but no significant changes by 0.25 mM H<sub>2</sub>O<sub>2</sub> treatment on *PIP2* genes expression were observed (data not shown).



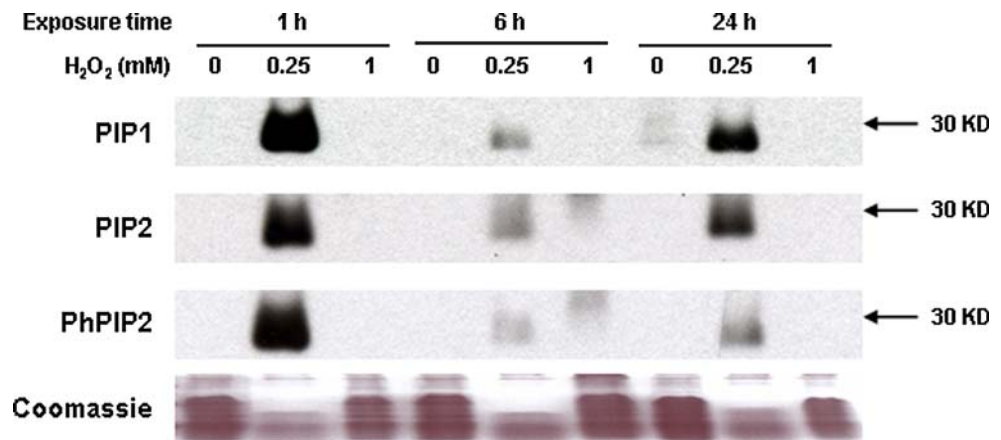
**Fig. 6** Relative mRNA expression of *PvPIP1;1* (a), *PvPIP1;2* (c), *PvPIP1;3* (e), *PvPIP2;1* (b), *PvPIP2;2* (d), and *PvPIP2;3* (f) of roots grown in hydroponic soil after 1, 6, or 24 h of exposure to 0 (white columns), 0.25 (grey columns), or 1 mM (black columns)  $H_2O_2$ . The different letters indicate significant differences ( $P < 0.05$ ) among treatments after ANOVA and Fisher LSD analysis. The bars represent mean  $\pm$  SD ( $n = 3$ )



At the same time, PIPs protein abundance and phosphorylation state increased after 1 and 24 h of root exposure to 0.25 mM  $H_2O_2$  (Fig. 7), but *L* only increased after 24 h (Fig. 1c). Therefore, we decided to carry out the immunolocalization of PIP2 proteins in untreated, 1 and 24 h 0.25 mM  $H_2O_2$  treated roots (Fig. 8). PIP2 was selected instead of PIP1 because PIP2 proteins usually exhibit more water transport activity than PIP1 proteins (Chaumont et al. 2000; Sakurai et al. 2005). Immunolocalization was first carried out in root tips and at 2 cm from

tips, since this is the zone where roots are thought to become more active in terms of water uptake (Hukin et al. 2002; Hachez et al. 2006). After 1 and 24 h of root exposure to 0.25 mM  $H_2O_2$ , PIP2 protein abundance increased in the root tips; the increase at 2 cm above the root tips was only detected after 24 h of exposure (Fig. 8). To observe some differences in the cellular-type localization of PIP2 protein between 1 and 24 h 0.25 mM  $H_2O_2$  treated roots, 1- $\mu$ m sections were carried out at 2 cm above the root tips. The results indicated a general increase of

**Fig. 7** Western blot analysis of microsomes from *P. vulgaris* roots grown in hydroponic soil after 1, 6, or 24 h of exposure to 0, 0.25, or 1 mM H<sub>2</sub>O<sub>2</sub>. The antibodies used were against AtPIP1;1 (PIP1), AtPIP2;1 (PIP2) or phosphorylated PIP2s at Ser 121 (PhPIP2). The last line represents the coomassie brilliant blue line of each microsome treatment



PIP2 protein amount in all kinds of root cells, being more pronounced in stele cells and in the cortical cells surrounding the stele, after 24 h of exposure. A much weaker response was observed after 1 h of exposure (Fig. 8). A very weak signal was detected when PIP2 antibody was omitted (data not shown).

## Discussion

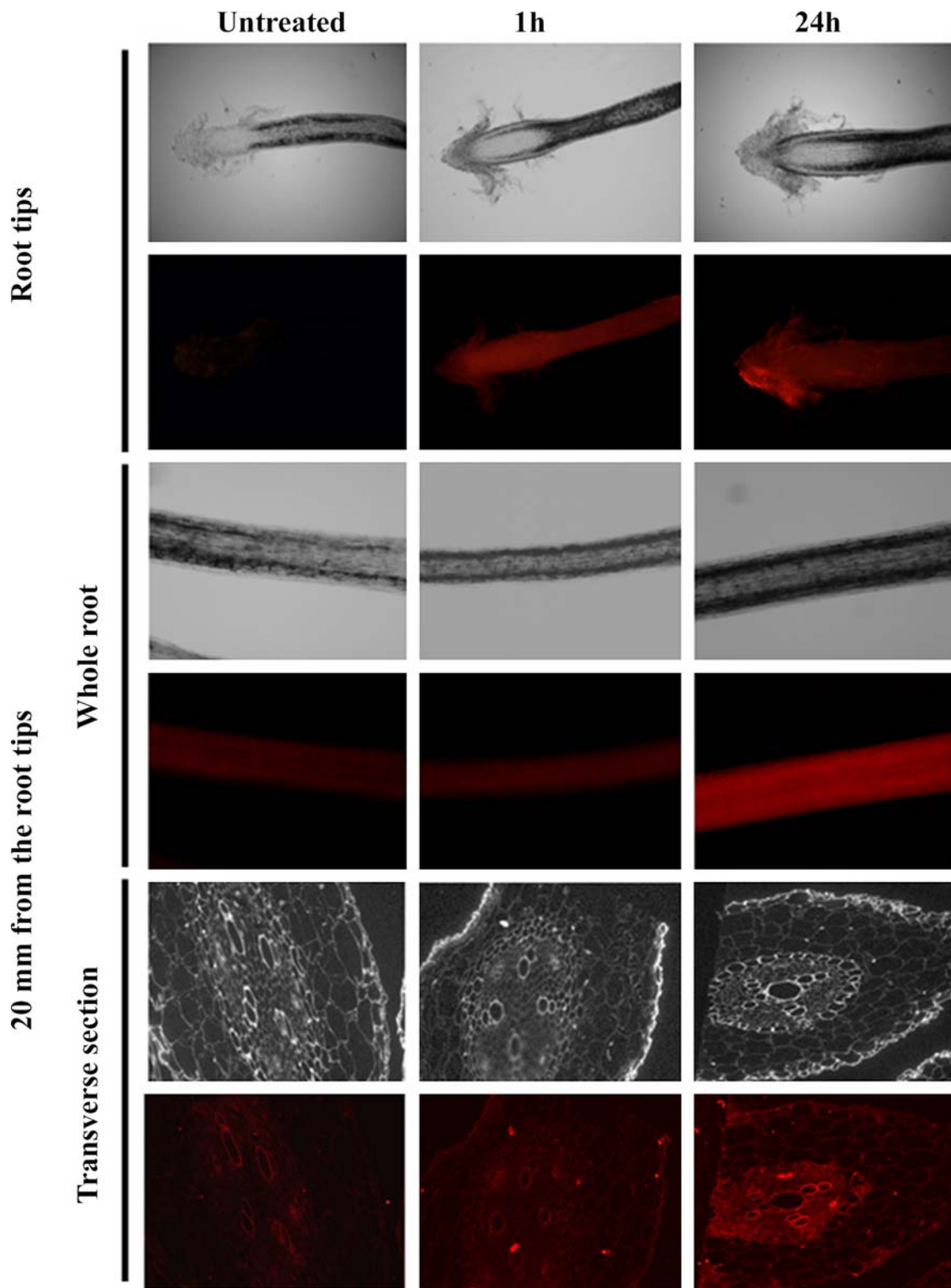
The fact that, after 24 h of adding H<sub>2</sub>O<sub>2</sub>, we could almost not detect H<sub>2</sub>O<sub>2</sub> in the hydroponic solution is in agreement with the half-time of H<sub>2</sub>O<sub>2</sub> in culture medium, ranging from 4 to 15 min when the concentration applied was between 0.1 and 0.2 mM (Dringen and Hamprecht 1997). However, an increase of *J<sub>v</sub>* and *L* was observed here after 24 h of adding 0.25 mM H<sub>2</sub>O<sub>2</sub>. Similarly, Moskova et al. (2009) found that pea leaves sprayed with 2.5 mM H<sub>2</sub>O<sub>2</sub> 24 h before being subjected to the herbicide paraquat were more resistant to it than non pretreated leaves. At the same time, *P. vulgaris* plants needed 24 h to increase their *J<sub>v</sub>* and *L* upon exposure to different ABA concentrations (Aroca 2006). Therefore, it seems that *J<sub>v</sub>* and *L* need a certain period of time to increase their values after the environmental conditions change. It is also known that H<sub>2</sub>O<sub>2</sub> (or other ROS) are intermediate signals that also activate other signals (ion channels, protein kinases, and phosphatases, etc.) before the functional proteins and physiological processes are activated (Van Breusegem et al. 2008; Wang and Song 2008).

Here, an increase of *J<sub>v</sub>* and *L* caused by the exogenous addition of H<sub>2</sub>O<sub>2</sub> was found for the first time. Such an increase was found in both sets of experiments, hydroponic and soil-growing plants. In the hydroponic experiment, when the H<sub>2</sub>O<sub>2</sub> concentration added was equal to or higher than 1 mM, a decrease on *J<sub>v</sub>* and *L* was observed, as found by other researchers (Ktitorova et al. 2002; Lee et al. 2004; Aroca et al. 2005; Rhee et al. 2007; Boursiac et al. 2008)

using different plant species and H<sub>2</sub>O<sub>2</sub> concentrations. It is possible that our finding was related to the plant species (cultivar) used. The same *P. vulgaris* cultivar that has been used here was reported to increase its *L* after 1 day of exposure to 5°C (Vernieri et al. 2001). Since cold stress causes a rise in ROS generation (Hung et al. 2005), it is possible that such ROS were the cause of the *L* increase. On the other hand, Aroca et al. (2005) found that the same concentration of H<sub>2</sub>O<sub>2</sub> (100 μM) applied for 1 h had an opposite effect on *L* depending on the maize cultivar analyzed. Indeed, the cold-tolerant cultivar showed a slight non significant increase of *L*, while the cold-sensitive cultivar showed a strong decrease of *L*. At the same time, Boursiac et al. (2008) linked the reduction of *L* in *Arabidopsis thaliana* caused by salinity or salicylic acid with a generation of ROS, indicating a sensitivity of *A. thaliana* to both treatments. Hence, the results found here open the possibility to consider ROS as signaling molecules for increasing *L* under some environmental stresses, as considered previously for other physiological processes, such as stomatal movements (Pei and Kuchitsu 2005; Wang and Song 2008). We consider ROS and not only H<sub>2</sub>O<sub>2</sub> because other ROS molecules such as OH<sup>•</sup> could be generated by Fenton reaction under our experimental conditions (Ye and Steudle 2006).

## Soil experiment

The pot soil experiment was carried out since most of the plants in nature establish a symbiosis with AM fungi (Gai et al. 2006), by which the fungus obtains carbon compounds and a protective niche, and the plant obtains mineral nutrients (especially phosphorous) and water resources which are not available for the roots (Harrison 2005). At the same time, AM symbiosis confers to the host plant more tolerance to several abiotic stresses, partly due to the higher capacity of AM plants to take water up and to remove ROS (Ruiz-Lozano 2003; Wu et al. 2006). In this



**Fig. 8** Immunolocalization of PIP2 proteins in intact roots or in transversal sections of untreated roots exposed to 0.25 mM H<sub>2</sub>O<sub>2</sub> for 1 or 24 h. The antibody used was against AtPIP2;1. More red

fluorescence means more PIP2 protein amount. Each fluorescence picture is accompanied by its corresponding bright-field picture

study, the percentage of root length colonization by the AM fungi was 21%. Here, soil-growing plants'  $L$  was less than half the average value for hydroponic plants. This difference in  $L$  values could be caused by the different age of the plants (Ruggiero et al. 2003), since soil-growing plants were 3 weeks older than hydroponic plants, or by the different morphology of their roots. In fact, Zimmermann and Steudle (1998) found that hydroponic maize roots'  $L$ , measured as we did in the present research, was higher than that of aeroponic ones (roots growing in a mist culture) because of a development of exodermis in aeroponic roots. Similar development of exodermis could occur in soil-growing roots, but not in the hydroponic roots in our experimental setup.

Besides the above-mentioned differences between hydroponic and soil growing roots, we found an increase of  $L$  and  $J_v$  by  $H_2O_2$  application in both kinds of media. These two results reinforce the validation of our findings. The fact that we needed 20 mM  $H_2O_2$  in soil-growing roots to obtain the same effect as with 0.25 mM in hydroponic roots can be explained because  $H_2O_2$  in soil had a slow rate of diffusion or because  $H_2O_2$  in soil also reacted with several molecules besides roots.

The more pronounced increase of  $L$  in non AM roots than in AM roots could be caused, indeed, by different levels of  $H_2O_2$  inside the roots (lower levels in AM roots). In fact, it is commonly found that there is a higher antioxidant capacity in AM roots than in non AM roots (Ruiz-Lozano 2003; Wu et al. 2006). At the same time, a different *PIPs* gene expression behavior between AM and non AM roots has been extensively found under various environmental conditions (Ouziad et al. 2006; Porcel et al. 2006; Aroca et al. 2007). Here, the patterns of *PIP* gene expression in AM and non AM roots exposed to  $H_2O_2$  changed in opposite directions. While in AM roots two out of six *PIP* genes increased their expression, in non AM roots, four out of six *PIP* genes showed a decrease of their expression. In AM roots, correlation between an increase of *PvPIP2;2* and *PvPIP2;3* gene expression and an increase in PIP2 protein abundance was observed. However, no correlation was found between *PIP1* gene expression and protein abundance in both kinds of roots, or between *PIP2s* gene expression and protein abundance in non AM roots. This lack of correlation between *PIP* gene expression and PIP protein abundance has been seen previously (Aroca et al. 2005; Sakurai et al. 2005; Hachez et al. 2008), and can be explained in different ways. Here, we only analyze the gene expression of three genes out of a potential family of five to eight genes, based on studies in other plant species (Chaumont et al. 2001; Johanson et al. 2001; Sakurai et al. 2005). At the same time, post-transcriptional regulation or different protein degradation could be taking place. A similar lack of correlation was found between PIP

protein amount and the increase of  $L$  observed in non AM roots by the  $H_2O_2$  treatment. This apparent inconsistency could be caused by a different subcellular localization of PIP proteins (Zelazny et al. 2007), or because the antibodies used here did not recognize some of the *PIP* genes involved in the observed  $L$  increase (Aroca et al. 2006). To further continue with the analysis of  $H_2O_2$  effects in PIP behavior, we focused on the hydroponic experiment.

#### Hydroponic experiment

We found a decrease of  $L$  at 1 and 5 mM  $H_2O_2$  application, which recovered after 24 h of exposure only in 1 mM  $H_2O_2$  treated roots. The behavior of  $L$  rising and dropping was inversely matched by the values of EL and ROS contents in the roots, but was independent of *PIP* gene expression. Therefore,  $L$  behavior at 1 mM could be related to membrane damage caused by  $H_2O_2$  application and a subsequent recovery of such damage (Aroca et al. 2005; Aroca 2006). However, a direct blockage of aquaporins (Ye and Steudle 2006) by ROS or a membrane internalization of PIP proteins (Boursiac et al. 2005, 2008) cannot be excluded.

However, the most interesting finding of the present research was the increase of  $L$  caused by 0.25 and 0.5 mM  $H_2O_2$  exposure. Such an increase of  $L$  correlated with an increase of PIP protein abundance, as shown by Western blots. However, an increase of PIP protein abundance was observed after 1 h of exposure to 0.25 mM  $H_2O_2$  without any increase of  $L$ . Therefore, we decided to localize where this PIP protein accumulation was taking place along the root. At both time points (1 and 24 h), an increase of PIP2 protein abundance was observed in the root tips. Since ROS can be involved in promoting root tip growth (Monshausen et al. 2007), and root tip growth needs an active water influx inside the growing cells (Nonami 1998), it is possible that such an accumulation of PIP2 proteins in root tips after  $H_2O_2$  addition was a consequence of the root growth promotion by  $H_2O_2$  application.

On the contrary, when we looked at 2 cm above root tip, where it is the water-absorbing zone of the root is thought to be located (Hukin et al. 2002; Hachez et al. 2006), an increase in PIP2 protein amount was only detected after 24 h of exposure to 0.25 mM  $H_2O_2$ . This different root localization between 1 and 24 h of exposure to 0.25 mM  $H_2O_2$  correlated with the different behavior of  $L$ . At the same time, the highest PIP2 protein amount was localized in the stele cells after 24 h of  $H_2O_2$  application, most probably favoring the flow of water to the xylem. This different localization of PIP2 protein between the two kinds of roots could also explain the results of the Western blots and their apparent discrepancy with  $L$  results.



Zhou et al. (2007) characterized the water transport capacity of *PvPIP1;1*, *PvPIP2;2*, and *PvPIP2;3*, and only the last one was able to transport water in oocytes experiments. Here, no relationship between an increase of *L* and an increase of any of the *PvPIP* genes analyzed was found. Thus, it is possible that other *PvPIP* genes, not yet identified, were involved in such an increase of *L*. To ascertain which specific *PIP* aquaporin gene could be involved in the increase of *L* caused by exogenous  $H_2O_2$ , a complete set of *PIP* aquaporins genes would be needed. At the same time, the use of specific antibodies against each *PIP* gene, or at least some of them, will also be required. These two research tools are already available for maize (Chaumont et al. 2001; Hachez et al. 2006), and the experiments described here could be confirmed in such plant species in future researches.

In conclusion, we reported here for the first time, a significant enhancement of *L* by exogenous application of  $H_2O_2$ . At the same time, a good correlation between *PIP* protein amount and their root localization and *L* has been found in hydroponic experiments. This report provides new insights to study how different environmental factors or endogenous substances modulate *L* in different ways, possibly linked to the different production of ROS. These results fit with the recent discovery by Dynowski et al. (2008), proving the capacity to transport  $H_2O_2$  by some plant *PIPs*. At the same time, the more pronounced response of *L* to  $H_2O_2$  in non AM roots than in AM ones, and the different behavior of *PIP* aquaporins expression and abundance between both kinds of roots, could be caused by the greater capacity of the AM roots to remove ROS. These different behaviors could explain, in part, the different response of *L* and *PIP* aquaporins to abiotic stresses found extensively between both kinds of roots (Ouziad et al. 2006; Porcel et al. 2006; Aroca et al. 2007).

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