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RESEARCH PAPER

Relationships of root conductivity and aquaporin gene expression in *Pisum sativum:* diurnal patterns and the response to $HgCl_2$ and ABA

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Abstract

Experiments were undertaken to test how aquaporins (AQPs) facilitate the uptake of water by roots of *Pisum sativum*. Changes in *PsPIP2-1* gene expression and root hydraulic conductivity (Lp_r) were measured in response to the time of day as well as treatment of the roots with a compound that reduced Lp_r [i.e. mercuric chloride ($HgCl_2$)] and one that was intended to increase Lp_r [abscisic acid (ABA)]. There was a diurnal rhythm in *PsPIP2-1* expression in lateral roots that was strongly correlated with diurnal changes in Lp_r. Taproots also displayed a rhythm in *PsPIP2-1* expression, but this was offset from that of Lp_r. This suggested that changes in Lp_r were mediated by changes in *PsPIP2-1* mRNA transcript abundance. Reduction of Lp_r by $HgCl_2$ treatment was accompanied by an increase in *PsPIP2-1* expression, implying that *PsPIP2-1* expression may have increased to compensate for AQPs blocked by mercury. ABA usually increased Lp_r, but changes in *PsPIP2-1* were variable and the direction of the response was strongly dependent on the dose of ABA that was applied. Overall, the coincident rhythms in Lp_r and PIP2 expression and response to AQP blockage are consistent with the hypothesis that Lp_r changes are mediated, at least in part, by changes in *PsPIP2-1* expression. Inconsistencies with ABA data may have been due to more complex interactions of ABA with AQP channels.

Key words: ABA, aquaporin, mercuric chloride, *Pisum sativum*, root hydraulic conductivity.

Introduction

Aquaporins (AQPs) are membrane proteins that belong to the large family of major intrinsic proteins (MIPs; Agre *et al*., 1998). The most abundant group of AQPs is that of the plasma membrane (plasma membrane intrinsic proteins or PIPs). They are subdivided into two categories, PIP1 and PIP2 AQPs, the latter of which has been shown to have higher water channel activity (Chaumont *et al*., 2000).

Root water passage involves both radial and axial movement. Due to the absence of membranes, the role of AQPs along the axial pathway is negligible. However, radial water entry involves permeation across several layers of cells. It occurs along a combination of apoplastic, symplastic, and transcellular pathways (Steudle, 2000), and thus membranes must be traversed. This is the result of the endodermal (and often exodermal) Casparian band preventing water from moving directly into the xylem via the apoplast. Therefore, AQPs might facilitate water passage past such barriers by channelling the water through membranes.

A few lines of study have indicated that AQPs are necessary to assist water passage through roots. For example, Kaldenhoff *et al*. (1998) used antisense RNA to block the expression of an AQP gene in *Arabidopsis* and found that the AQP-knockout developed a much larger root system for which water permeability was 20–30% of that in controls. It was concluded that roots developed a larger surface area to compensate for the missing AQP.

The use of mercuric chloride ($HgCl_2$) to inhibit root conductivity (Lp_r) has further implicated a role for AQPs in water uptake. Since $HgCl_2$ blocks most AQPs, it has

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ABA, abscisic acid; ANOVA, analysis of variance; AQP, aquaporin; GAPDH, glyceraldehyde phosphate dehydrogenase; Lp_r, root hydraulic conductivity; LSD, least significant difference.

been frequently used to estimate their contribution to whole root water transport (Javot and Maurel, 2002). Many studies have found that roots exposed to this compound experienced a decline in Lp_r (reviewed in Javot and Maurel, 2002). This evidence indicated that AQPs are critical in bulk water flow and suggested that they are responsible for most of the water permeability of the plasma membrane (reviewed in Steudle, 2000).

In biophysical studies of root water entry, Emery and Salon (2002) and Murphy (2003) detected non-linear flow at high pressures by measurements in pressure chambers. Both studies suggested that this was consistent with a role for plasma membrane AQPs. Owing to their finite capacity to allow water to pass through, it was reasoned that at high pressures the water channels would become saturated and their inability to move an increasing volume of water would limit flow. Furthermore, maximum flow rates at high pressure changed diurnally (Emery and Salon 2002), increasing at 9:00 h, peaking at 11:00 h, and declining thereafter. Changes in conductivity were hypothesized to be correlated to changes in AQP abundance.

Investigations between Lp_r rhythms and AQP gene expression have been undertaken in two studies, which showed that, similarly to Lp_r, AQP expression exhibits a diurnal rhythm. Henzler *et al.* (1999) examined diurnal conductivity in *Lotus japonicus* and correlated this to changes in *PIP1* transcript levels. They reported that Lp_r peaked about midday and declined to a minimum at 20:30 h. Expression of two PIP1 genes showed some overlap with the diurnal Lp_r rhythm, whereby Lp_r peaked 5–7 h after onset of the photoperiod and AQP expression peaked 6–8 h into the photoperiod. Lp_r declined to a minimum at 20:30 h, while AQP expression reached a minimum at either the same time or shortly thereafter (Henzler *et al.*, 1999). In contrast, Lopez *et al.* (2003) found that peak transcript abundance of two *PIP2* genes preceded maximum sap flux by 2–4 h and PIP protein levels peaked at the same time as maximum sap flux (i.e. midday). The discrepancy between these studies makes it uncertain if AQPs affect Lp_r or whether their rhythms are coincidental.

The purpose of this study was to determine what factors contribute to how AQPs regulate the transfer of water from rhizosphere to xylem in pea. To address this, experiments combined measurements of Lp_r and AQP gene expression. Based on the hypothesis that an increase in AQP expression would cause an increase in Lp_r, three predictions were made: (i) diurnal changes in Lp_r would be preceded by, or coincident with, changes in AQP expression; (ii) following manipulation to decrease Lp_r, AQP expression should increase to compensate for the reduction in water flow through roots; and (iii) following manipulation to increase Lp_r, AQP expression would decrease since AQPs would not be as critical for maintaining water flow.

Peas were used as the model system because their Lp_r physiology has previously been described (Emery and Salon, 2002). Additionally, AQP genes in peas have been previously characterized by Schuurmans *et al.* (2003) who identified *PsPIP2-1*, the AQP gene used in this study. *PsPIP2-1* belongs to the PIP2 subcategory of PIPs. PIP2 AQPs are of considerable interest since all plant PIP2 proteins examined in water permeability assays so far have shown higher water channel activity than their PIP1 counterparts, which had either low or no activity in several species (Fetter *et al.*, 2003) and, in particular, peas (Schuurmans *et al.*, 2003).

For the first prediction, an attempt was made to resolve the discrepancy between Henzler *et al.* (1999) and Lopez *et al.* (2003) by analysing the diurnal relationship between Lp_r and *PsPIP2-1* expression in greater detail. This was done by taking root heterogeneity into consideration since Hukin *et al.* (2002) found that AQP expression differs over various regions of the root. Thus, *PsPIP2-1* expression was studied separately for tap and lateral roots. Lateral roots would perform most of the water uptake, based on greater available mass, and surface area and proportion that would be at a maturity conducive to water uptake (Waisel and Eshel, 2002). Moreover, quantitative real-time polymerase chain reaction (PCR) was used for its greater sensitivity for discerning differences in *PsPIP2-1* mRNA levels among sampling time points.

To assess the second prediction, Lp_r was reduced with $HgCl_2$ to observe the influence on *PsPIP2-1* expression. While the physiological effects of $HgCl_2$ have been well described, its influence on AQP gene expression is unknown. It was expected that Hg^{2+} would reduce Lp_r by blocking AQPs and that *PsPIP2-1* expression would increase to compensate for this blockage. Furthermore, as concentrations of Hg^{2+} increased, a corresponding increase in AQP expression was predicted until the concentration of Hg^{2+} reached a toxic level.

To test the third prediction, roots were treated with abscisic acid (ABA), which is known to increase Lp_r in most systems (Freundl *et al.*, 1998; Quintero *et al.*, 1999; Hose *et al.*, 2000; Sauter *et al.*, 2002; Lee *et al.*, 2005; Schraut *et al.*, 2005), although this effect is not thought to involve changes in AQPs (Quintero *et al*, 1999). It was predicted that the plants, which received adequate water, would be acclimated such that they would have appropriate levels of AQPs present in the roots. Thus, as Lp_r increased, *PsPIP2-1* expression would either remain the same or decrease since the same number or even fewer AQPs should be required to conduct the same amount of water. In both the $HgCl_2$ and ABA experiments, a range of concentrations was applied to roots. Given the potential for diurnal fluctuations, the timing of application and root harvest was controlled precisely. Furthermore, the region of the root harvested for AQP expression analysis was the same for each experiment.

Materials and methods

Growth conditions

Peas (*Pisum sativum,* Cutlass cultivar) were grown in a Conviron PGR15 (Winnipeg, MN) growth chamber with a 16 h light/8 h dark cycle.

Peas were grown in Ray Leach RLC-7UV Cone-tainers™ (Stuewe & Sons, Inc. Corvallis, OR, USA). Cone-tainers™ were used because they allow peas to develop uniform root systems, and their small size (115 ml) enabled them to fit inside the pressure chamber. Peas were planted in Fafardä Agro Mix (Saint-Bonaventure, QC, Canada), a sphagnum peat moss growing medium. Seeds were inoculated with Nitragin™ pea inoculant (Milwaukee, WI, USA). Peas were watered regularly with deionized water. Once the shoot appeared on the surface of the soil, peas were watered to saturation on alternate days with nutrient solution (Emery and Salon, 2000). Excess water or nutrient solution drained out through holes in the bottom of pots or Cone-tainers™.

Harvest

To study diurnal *PsPIP2-1* expression, plants were harvested at 12:00, 3:00, 6:00, 9:00, 11:00, 13:00, 16:00, 19:00, and 22:00 h once they had reached the 6–8 node developmental stage. Peas used for conductivity experiments were harvested at the 6–8 node stage at 15:00 h and 17:00 h in addition to the times used for gene expression analysis. Three replicate plants were harvested at each time point for gene expression and conductivity experiments.

Root systems were removed from pots, and growth medium was washed off with water. Lateral roots were separated from taproots and each was cut 1–4 cm below the cotyledonary node and blotted dry with a paper towel. From each type of root, 30–100 mg of tissue was collected and stored inside a microfuge tube in RNAlater stabilization solution (Qiagen, Valencia, CA, USA). Samples were stored in a –80 °C freezer until they were used for RNA extraction.

Root conductivity (Lp_r) analysis

Pressure-flow curves and Lp_r calculations were generated using a pressure chamber as described in Emery and Salon (2002). Plants used for conductivity experiments were cut just below the first node. The cut surface of the shoot was thoroughly rinsed with distilled water. Each root system (still within its Cone-tainer™) was sealed inside the pressure chamber and a piece of supple rubber tubing was fitted snugly around the root stump. The tube came out through the top of the chamber and delivered xylem exudate into pre-weighed microfuge tubes. Pressures of 0.05, 0.10, 0.20, 0.30, 0.40, 0.50, and 0.60 MPa were applied to the chamber using an air compressor (Mastercraft Model No. 820218, Toronto, ON, Canada). Stepwise pressure increments were made every 10 min. Microfuge tubes were re-weighed at the end of the experiment to determine the volume of xylem exudate that flowed at each pressure. Plants were removed from the pressure chamber and their roots washed to remove soil. Roots were weighed after drying them in an oven for 48 h at 50 °C. Once a plant had been subjected to pressure-flow manipulations, it was not used in other experiments.

Mercuric chloride treatment

For gene expression analysis, 50 ml of 1, 10, or 100 μM $HgCl_2$ (Fisher, Fair Lawn, NJ, USA) was poured onto the soil 1 h and 3 h prior to harvesting the roots at 9:00, 11:00, and 16:00 h. Controls received 50 ml of water. All treatments were performed in triplicate.

The same $HgCl_2$ concentrations used in gene expression analysis were used for root conductivity measurements (i.e. 1, 10, and 100 μM). At 1 h prior to harvesting, 30 ml of $HgCl_2$ solution was delivered directly to the soil. The 3 h exposure was not used for conductivity experiments. Controls received 30 ml of water. Plants were harvested at 16:00 h. All treatments were performed in triplicate.

Abscisic acid treatment

For gene expression analysis, roots were treated with 50 ml of 0.01, 1, 10, and 100 μM (±)-2-*cis*-4-*trans* ABA (Lancaster, Pelham, NH, USA) at 24 h prior to harvest, while controls received 50 ml of water. Plants were harvested at 9:00, 11:00, and 16:00 h. All treatments were done in triplicate. ABA was diluted to its final concentration using ultra pure water.

The same ABA concentrations used in gene expression analysis were used for conductivity measurements (i.e. 0.01, 1, 10, and 100 μM). Twenty-four hours prior to harvesting, 30 ml of each ABA solution was applied to the soil, while controls received 30 ml of water. Plants were harvested at 16:00 h. All treatments were done in triplicate.

RNA extraction and quantification

RNA was extracted from roots using the RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA) and stored at –80 °C. The RNA concentration was measured on a Nanodrop® ND-1000 UV-visible spectrophotometer (Wilmington, DE, USA).

Real-time PCR

Diethylpyrocarbonate (DEPC)-treated water was used to dilute RNA to 0.5 ng ml^{-1} prior to its use in real-time PCR. Gene expression analysis was performed on the ABI 7900HT (Applied Biosystems, Foster City, CA, USA).

Reactions were set up in a 96-well plate using the Quantitect™ SYBR® Green RT-PCR Kit (Qiagen, Valencia, CA, USA). A 2 μl aliquot (1 ng) of RNA was added to each well in the plate. A master mix containing reaction buffer, dNTPs, *PIP2* or *GAPDH* primers (to a final concentration of 0.4 μM), ROX (passive reference dye), $MgCl_2$, RNase-free water, HotStarTaq™ polymerase, and Omniscript and Sensiscript reverse transcriptases were added to bring the total reaction volume in each well to 20 μl. Plates were sealed using an optical cover and placed inside the ABI 7900HT. Primers used to amplify *PIP2-1* and glyceraldehyde phosphate dehydrogenase (GAPDH) are given in Table 1.

The ABI 7900HT was programmed as follows: 30 min at 50 °C for reverse transcription; 15 min at 95 °C to inactivate the reverse transcriptase enzymes and activate HotStarTaq™ DNA polymerase; 40 cycles of 15 s at 94 °C, 30 s at 60 °C, and 30 s at 72 °C. Data collection was carried out during the 72 °C extension step. A dissociation curve was constructed following completion of 40 amplification cycles. No-template controls and no-reverse transcriptase controls were included to ensure that reagents and samples were free of contamination. Standard curves were generated for *PsPIP2-1* and *GAPDH* in each experiment. All samples (standards, controls, and unknowns) were run in triplicate to account for variations in pipetting.

Analysis of real-time PCR data

Conversion of cycle threshold (C_T) values into relative RNA quantities was followed according to the instructions outlined in the ABI Prism 7700 Sequence Detection System User Bulletin #2 (Applied Biosystems, 2001). Expression of *PsPIP2-1* was normalized by dividing its mean relative value by the mean relative *GAPDH* value. The smallest normalized *PsPIP2-1* value was designated as the calibrator and all other normalized values were divided by the calibrator's normalized value.

Table 1. *Primer sequences (5′–3′) used to amplify PsPIP2-1 and GAPDH from pea root cDNA*

Primers were designed to span an intron–exon boundary. *PsPIP2-1* and *GAPDH* sense primers have nucleotides that bind upstream of the intron (shown in bold). Nucleotides shown in normal type bind downstream of the intron. Antisense primers do not span an intron–exon boundary. Primers that cross an intron–exon were used to avoid genomic DNA amplification.

Primer	Sequence (5′–3′)	Expected product size (bp)	Gene accession number
PsPIP2-1 sense	**GTTCCTGT**GTTTTGGCACCAT	202	AJ243307
PsPIP2-1 antisense	GGTGGTAAATTGCAGCCACT		
GAPDH sense	**TTACAGCAG**TACCCGTGTGG	80	AA430910
GAPDH antisense	TAGCACTACCAACCGCAGTG		

Transpiration measurements

Transpiration was measured using a LI-1600 Steady-State Porometer (LI-COR, Lincoln, NE, USA). Measurements were taken from one leaf on each plant at 6:00, 9:00, 11:00, 16:00, and 19:00 h. The same leaf was measured at each time point and the leaf chosen for measurement was always located at one of the top two nodes on the pea. This ensured that variation in transpiration between young and old leaves was not a factor.

In the $HgCl_2$ experiments, transpiration was measured at 9:00, 11:00, or 16:00 h the day before $HgCl_2$ was applied to roots. The next day roots were exposed to $HgCl_2$ for 1 h or 3 h. Prior to harvesting plants at 9:00, 11:00, or 16:00 h for gene expression analysis, transpiration was again measured. This enabled data to be expressed as a percentage of transpiration prior to $HgCl_2$ treatment. Transpiration was only measured in roots exposed to $HgCl_2$ for 3 h. As in the diurnal and ABA experiments, the same leaf from each plant was used on both days to ensure that variations between individual leaves on the same plant would not be a factor.

In ABA experiments, transpiration was measured at 9:00, 11:00, and 16:00 h. Since roots were exposed to ABA for 24 h, these data were collected just before treating roots with the hormone. The next day, transpiration was measured again at 9:00, 11:00, and 16:00 h. Following this, roots were harvested for gene expression analysis.

Statistical analysis

Statistical analyses were completed using KaleidaGraph 4.0 software (Synergy Software, Reading, PA, USA). Within each experiment, detection of differences among means was performed using one-way analysis of variance (ANOVA) followed by the Fisher's least significant difference (LSD) *post hoc* test (P <0.05).

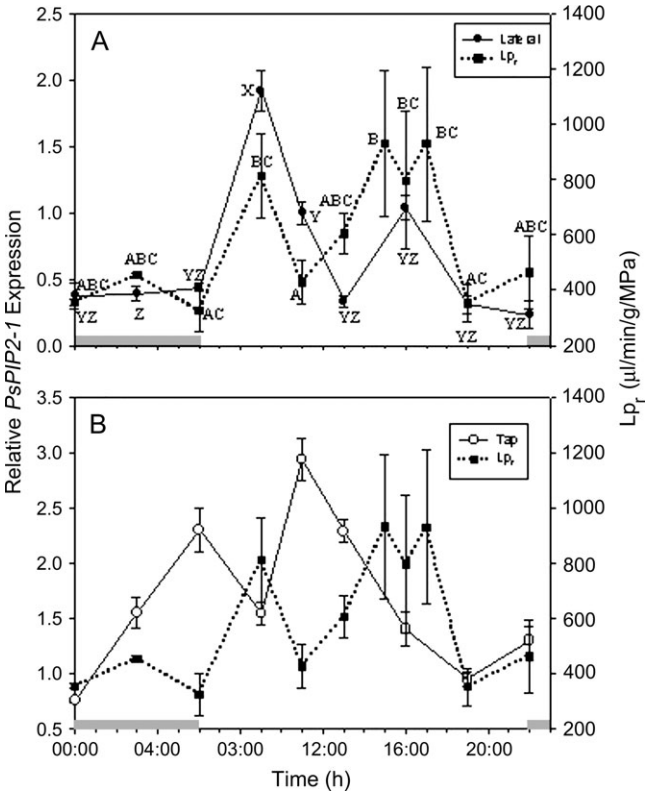

Fig. 1. Rhythm in Lp_r and relative *PsPIP2-1* expression in lateral roots (A) and taproots (B) of *Pisum sativum*. Standard error bars are shown. All data for Lp_r and *PsPIP2-1* expression are the mean values of three replicates. Separate ANOVA and LSD tests were performed for Lp_r and *PsPIP2-1*. Data points with the same letter are not significantly different (P <0.05). Note that the same Lp_r trace is shown in both graphs. Grey bars indicate darkness in the growth chamber.

Results

Diurnal experiment

Hydraulic conductivity was calculated from the slope of pressure-flow curves to measure changes in Lp_r throughout a 24 h period to determine any correlation with changes in *PsPIP2-1* expression.

During a 24 h period, Lp_r displayed two peaks. The first occurred at 9:00 h and the second between 15:00 h and 17:00 h (Fig. 1). Increases in Lp_r relative to those at midnight were ~220% and 250%, respectively. One-way ANOVA and subsequent LSD analysis revealed that the Lp_r peaks at 9:00 h and 15:00–17:00 h were significantly different from Lp_r at 11:00 h (P <0.05).

Real-time PCR was used to examine expression of the *PsPIP2-1* gene in lateral roots and taproots of pea during a 24 h period. Expression of *PsPIP2-1* differed in lateral roots and taproots (Fig. 1). In both tissues, *PsPIP2-1* transcript levels were at a minimum at midnight. In lateral roots, expression of *PsPIP2-1* increased at 6:00 h and reached a maximum at 9:00 h (Fig. 1A). At 9:00 h, *PsPIP2-1* expression was >4-fold greater than at midnight. This declined throughout the remainder of the morning and during the early afternoon. At 16:00 h, transcript abundance increased to almost 3-fold the value at midnight. Following this, expression declined for the

next 6 h and at 22:00 h it approached a value similar to the value observed at midnight. ANOVA and LSD *post hoc* analysis demonstrated that *PsPIP2-1* expression at 9:00 h was significantly different from *PsPIP2-1* levels at all other times of the day ($P < 0.05$).

Taproots followed a different expression rhythm from that of lateral roots. *PsPIP2-1* increased 3-fold from midnight to 6:00 h. After this, expression decreased slightly at 9:00 h but increased again at 11:00 h (Fig. 1B). Transcript levels were almost 4-fold greater at 11:00 h than at midnight. For the remainder of the day, *PsPIP2-1* expression steadily declined and at 22:00 h approached values similar to those at midnight. Increases in taproot *PsPIP2-1* transcript abundance were not significantly different ($P > 0.05$).

The diurnal experiment demonstrated that changes in Lp_r coincided with changes in lateral root *PsPIP2-1* expression. Specifically, when Lp_r increased, so did *PsPIP2-1* expression in the lateral roots. In contrast, the peaks in *PsPIP2-1* expression in taproots preceded the peaks in Lp_r by 3–6 h.

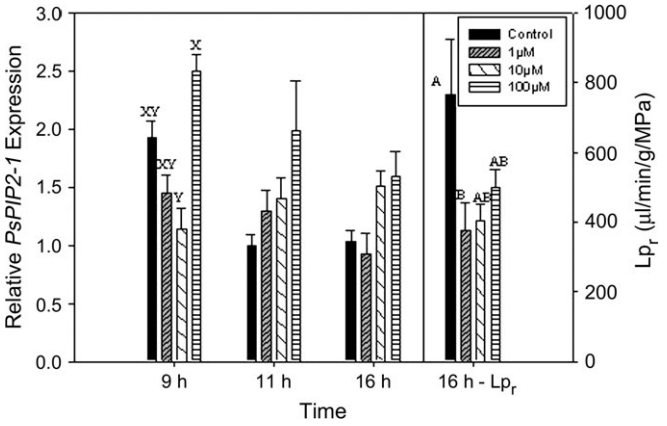

Fig. 2. Changes in relative *PsPIP2-1* expression at 9:00, 11:00, and 16:00 h in lateral roots of *Pisum sativum* treated with increasing concentrations of mercuric chloride ($HgCl_2$) for 1 h. Change in Lp_r at 16:00 h in roots of *P. sativum* treated with increasing concentrations of $HgCl_2$ for 1 hr. Error bars represent 1 SE ($n=3$). ANOVA and LSD tests were performed at each time point separately for Lp_r and *PsPIP2-1*. Data points with the same letter are not significantly different ($P < 0.05$). Time points with no letters indicates there are no significant differences ($P > 0.05$).

Mercuric chloride treatment

Roots were treated with $HgCl_2$ for 1 h to manipulate their hydraulic conductivity. The purpose of this was to decrease Lp_r and then determine if this had an effect on *PsPIP2-1* expression.

Since the diurnal experiment revealed that Lp_r was greatest during the afternoon, 16:00 h was chosen as the time to measure Lp_r changes in roots treated with $HgCl_2$ and ABA. Because gene expression was not quantified at 15:00 h and 17:00 h, these times were not used for Lp_r manipulations. Therefore, the Lp_r changes in $HgCl_2$- and ABA-treated roots were all measured at 16:00 h.

Peas were treated with $HgCl_2$ to reduce their Lp_r. In all plants treated with 1, 10, or 100 µM $HgCl_2$, Lp_r was less than in controls that received only water (Fig. 2). Specifically, Lp_r in controls was significantly different from Lp_r in roots treated with 1 µM $HgCl_2$ ($P < 0.05$). On the whole, $HgCl_2$ reduced Lp_r to ~50% of control values.

Gene expression was measured at 9:00, 11:00, and 16:00 h. In addition, *PsPIP2-1* expression was evaluated in roots exposed to $HgCl_2$ for 3 h. In six of nine treatments, roots exposed to $HgCl_2$ for 1 h experienced an increase in *PsPIP2-1* expression, relative to controls (Fig. 2). Exceptions to this were in 1 µM- and 10 µM-treated roots harvested at 9:00 h and 1 µM-treated roots harvested at 16:00 h. At each time point examined, 100 µM $HgCl_2$ increased *PsPIP2-1* expression more than the lower $HgCl_2$ concentrations. At 9:00 h *PsPIP2-1* expression was significantly different in roots treated with 10 µM versus 100 µM $HgCl_2$ ($P < 0.05$). The next most effective concentration for increasing AQP expression was 10 µM $HgCl_2$ at 11:00 h and 16:00 h.

In contrast to roots treated with $HgCl_2$ for 1 h, 3 h of exposure caused a decline in *PsPIP2-1* expression relative to controls at all times and all concentrations (data not shown). The only exception was in roots exposed to 10 µM $HgCl_2$ and harvested at 11:00 h. In this case, expression was ~20% greater than in the control. In general, the pattern was that *PsPIP2-1* expression increased in roots treated with $HgCl_2$ for 1 h and decreased in roots treated with $HgCl_2$ for 3 h.

ABA treatment

ABA was added in an effort to increase Lp_r and determine if this had any effect on *PsPIP2-1* expression. Gene expression was measured using real-time PCR in roots exposed to 0.01, 1, 10, or 100 µM ABA for 24 h (Fig. 3).

Plants treated with 0.01, 1, and 10 µM ABA for 24 h experienced an increase in Lp_r relative to controls (Fig. 3). This was most pronounced in 0.01 µM and 10 µM treatments that displayed Lp_r values ~2.5 times greater than controls. Lp_r readings in roots treated with 0.01 µM or 10 µM ABA were significantly different from those of controls, 1 µM and 100 µM ABA-exposed roots ($P < 0.05$). In roots treated with 100 µM ABA, Lp_r was reduced to almost 50% of that of controls.

At 9:00 h, 1 µM and 10 µM ABA increased *PsPIP2-1* ~30% and 50% relative to controls. At 11:00 h, these same concentrations increased *PsPIP2-1* expression (~40%), and by 16:00 h very little difference existed between these treatments and controls (Fig. 3). However, at 9:00 h and 11:00 h, the data for 1 µM and 10 µM ABA treatments were quite variable.

In contrast, 0.01 µM and 100 µM ABA generally reduced *PsPIP2-1* transcript abundance. This was most

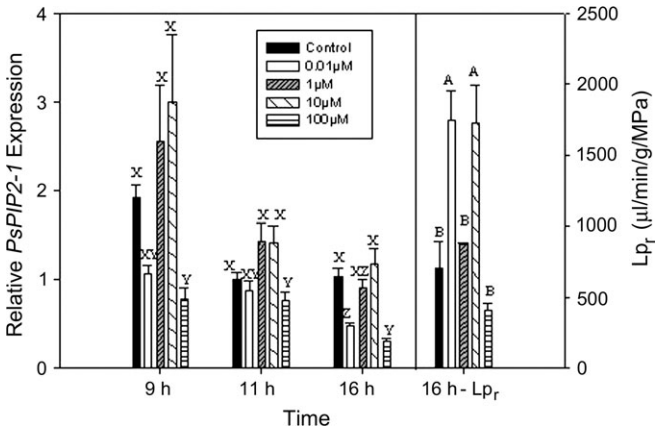

Fig. 3. Changes in relative *PsPIP2-1* expression at 9:00, 11:00, and 16:00 h in lateral roots of *Pisum sativum* treated with increasing concentrations of abscisic acid (ABA) for 24 h. Changes in Lp_r are also shown at 16:00 h. Error bars represent 1 SE (n=3). ANOVAs were performed at each time point for Lp_r and *PsPIP2-1* separately. Data points with the same letter are not significantly different (P <0.05).

pronounced at 9:00 h and 16:00 h when expression decreased relative to controls. At 9:00 h and 11:00 h, *PsPIP2-1* expression levels in 100 µM-exposed roots were significantly different from controls and 1 µM and 10 µM treatments (P <0.05).

For all real-time PCR experiments, no-template (NTC) and no-reverse transcriptase (no-RT) control reactions were included. Negative NTC controls meant reagents were free of contamination; negative no-RT controls indicated that genomic DNA was not amplified.

Transpiration

To determine if Lp_r or *PsPIP2-1* expression was driven by water demand, transpiration was evaluated in 15 replicate plants at 6:00, 9:00, 11:00, 16:00, and 19:00 h. The purpose of this was to establish if there was a diurnal transpiration pattern. Such a rhythm would suggest that water usage changed during the day and could be compared with Lp_r and *PsPIP2-1* expression data to see if there was a correlation. This experiment revealed that transpiration increased steadily after 6:00 h, peaked at 9:00 h, and declined for the remainder of the day (Table 2). Mean transpiration values varied significantly throughout the day (P <0.05). Most notably, the 9:00 h peak was different from that at 6:00, 16:00, and 19:00 h, while that at 11:00 h was significantly different from those at 16:00 h and 19:00 h. The increase in transpiration at 9:00 h coincided with the first peak in Lp_r as well as the rise in lateral root *PsPIP2-1* expression at 9:00 h. However, when lateral root *PsPIP2-1* expression increased again at 16:00 h, transpiration was declining. This indicated that diurnal changes in transpiration were not correlated to changes in Lp_r or *PsPIP2-1* expression.

Roots were treated with $HgCl_2$ for 3 h and transpiration measurements were made at 9:00, 11:00, and 16:00 h. Data in roots treated with 1, 10, or 100 µM $HgCl_2$ (Table 2) were not significantly different from those in controls at 9:00 h and 16:00 h (P >0.05). At 11:00 h, all concentrations of $HgCl_2$ caused a slight decline in transpiration relative to controls, and all of these changes were statistically significant.

With the exception of a single treatment (0.01 µM at 16:00 h), all plants treated with ABA (0.01, 1, 10, or 100 µM) for 24 h experienced a decline in transpiration relative to controls (Table 2). Most of the reductions in transpiration following ABA treatment were statistically significant (P <0.05). The magnitude of the reduction increased as the ABA concentration increased.

Discussion

This study investigated how AQPs are involved in root water uptake from rhizosphere to xylem. To address this question, root conductivity, Lp_r, and expression of an AQP gene, *PsPIP2-1*, were quantified in roots of *P. sativum* over a 24 h period as well as in response to treatment with a compound that decreases Lp_r ($HgCl_2$) and one that was intended to increase Lp_r (ABA).

The majority of the data supported the hypothesis that changes in *PsPIP2-1* expression were related to dynamics of pea root Lp_r. Firstly, diurnal changes in lateral root *PsPIP2-1* expression coincided exactly with the diurnal Lp_r rhythm. Secondly, in roots treated with $HgCl_2$, a reduction in Lp_r coincided with increased *PsPIP2-1* expression. As root conductivity decreased due to $HgCl_2$ exposure, *PsPIP2-1* expression increased, perhaps to compensate for those AQPs blocked by Hg^{2+}. However, roots exposed to increasing concentrations of ABA did not display predictable changes in *PsPIP2-1* transcript abundance. Although changes in *PsPIP2-1* mRNA abundance were not correlated to the application of ABA, this could be the result of an interaction between the protein and hormone that is not yet well understood.

Diurnal Lp_r and PsPIP2-1 rhythm

To test the hypothesis that AQPs increase Lp_r in pea roots, Lp_r and *PsPIP2-1* expression were measured during a 24 h period. It was predicted that changes in *PsPIP2-1* transcript abundance would either just precede, or directly coincide with, changes in Lp_r. In lateral roots, this prediction was supported since there was a strong correlation between *PsPIP2-1* expression in lateral roots and Lp_r. Specifically, *PsPIP2-1* expression displayed two distinct peaks at 9:00 h and 16:00 h. At all other times, *PsPIP2-1* expression was very close to its baseline expression level at midnight. Similarly, Lp_r reached maxima at 9:00 h and 16:00 h. The distinct overlap in

Table 2. *Transpiration rates of* Pisum sativum *exposed to increasing concentrations of $HgCl_2$ for 3 h and to increasing concentrations of ABA for 24 h*

Values are the mean of three replicates (i.e. three separate plants) and are given as a percentage of transpiration prior to treatment with $HgCl_2$ or ABA. Diurnal transpiration is given as a percentage of transpiration at 6:00 h. Values are the means (±SE) of 15 replicates (i.e. 15 separate plants). All concentrations are in μM. 'C' indicates control.

	Diurnal	$HgCl_2$ (3 h)				ABA (24 h)				
		C	1	10	100	C	0.01	1	10	100
6:00 h	100									
9:00 h	127±7.6	74±8.4	62 ±8.1	69±10.1	86±2.6	125±7.9	116±4.8	103±4.9	67±8.9	38±3.5
11:00 h	120±6.8	86±2.3	60±4.7	66±6.0	53±1.2	109±3.3	107±4.7	90±7.8	66±9.3	31±3.2
16:00 h	101±9.5	102±14.0	104±20.2	103±13.8	104±4.5	109±9.7	134±11.7	109±0.2	70±7.2	61±3.3
17:00 h	99±9.6									

their peaks demonstrated that changes in Lp_r were correlated to changes in *PsPIP2-1* transcript abundance. Therefore, conductivity increases may have been mediated by increases in *PsPIP2-1* abundance. This is supported by Schuurmans *et al.* (2003) who found that PsPIP2-1 proteins exhibited the greatest water permeability of all the known AQP proteins in pea.

Increases in taproot *PsPIP2-1* preceded increases in Lp_r by 3–6 h. While *PsPIP2-1* displayed two peaks in taproots at 6:00 h and 11:00 h, Lp_r peaked at 9:00 h, and between 15:00 h and 17:00 h. The lack of a relationship between *PsPIP2-1* expression and Lp_r in taproots suggests that AQPs in taproots may have a less significant role in water uptake than AQPs in lateral roots. The taproot comprises a very small proportion of the total root system area, compared with the lateral roots, which take up the majority of water used by the plant. However, these results emphasize the importance of studying these two types of roots separately since analysis of a mixture of tap and lateral roots may not have revealed any consistent diurnal rhythm of *PsPIP2-1* expression.

A rhythm in AQP gene expression has been identified in other studies that have monitored it over the course of a day (Henzler *et al.*, 1999; Lopez *et al.*, 2003). The present findings are more consistent with the results of Henzler *et al.* (1999) who found an overlap in *PIP1* gene expression and Lp_r. Similar to Henzler *et al.* (1999) who found that AQP expression followed a circadian rhythm, the present data also indicated that *PsPIP2-1* expression exhibits circadian behaviour. In their study though, the increase in Lp_r slightly preceded the increase in PIP1 expression. In contrast, Lopez *et al.* (2003) found that peaks in AQP expression were late and out of phase with water movement. Inconsistencies between *L. japonicus* (Henzler *et al.*, 1999), maize (Lopez *et al.*, 2003), and the present study could be attributed to a number of factors. The most obvious is, firstly, that a different species was used in each study. Secondly, neither Henzler *et al.* (1999) nor Lopez *et al.* (2003) specified whether lateral roots or the taproots were used for gene expression analysis. The present study revealed that the type of tissue sampled is critical since *PsPIP2-1* was differentially expressed in lateral roots and taproots. Moreover, Hukin *et al.* (2002) found that variation in AQP abundance exists at even small intervals along the root-growing zone of maize as the expression of two AQP genes increased in the more mature region of the root. Previous studies also did not specify which region of the root was used to harvest tissue. In the present study, lateral root and taproot tissue were always harvested 1–4 cm below the cotyledonary node. As a more developed part of the root, this region would offer some degree of apoplastic blockage (Schreiber *et al.*, 1999).

Thirdly, the physiological determination of apparent root conductivity by Lopez *et al.* (2003) was measured using spontaneous root system sap exudation upon shoot removal and not Lp_r. In field studies of pea, it was found that there is no correspondence between Lp_r generated from pressure chamber manipulations and spontaneous sap exudation from cut root stumps (Emery and Salon, 2002; Emery *et al.*, 2002). Thus, caution should be used in comparing the results of those two techniques.

$HgCl_2$—effects on PsPIP2-1 and Lp_r

To test the prediction that a reduction in Lp_r would increase *PsPIP2-1* expression, $HgCl_2$ was applied to block AQPs physically. With the exception of 9:00 h, this prediction was not supported by statistical analysis. Many of the roots treated with $HgCl_2$ for 1 h and harvested at 9:00, 11:00, or 16:00 h displayed an increase in *PsPIP2-1* expression but the only significant difference in expression was between roots treated with 10 μM and 100 μM $HgCl_2$ at 9:00 h. The treatments that did not increase *PsPIP2-1* expression over controls mostly occurred in plants exposed to low doses of $HgCl_2$, (mainly 1 μM). Following exposures to higher concentrations, the increased *PsPIP2-1* expression coincided with a significant decrease in Lp_r. The decline in Lp_r occurred regardless of the concentration of $HgCl_2$. These latter data are in agreement with the many studies that have shown that treating roots with $HgCl_2$ causes a reduction in their ability to conduct water, presumably through AQPs

(reviewed in Javot and Maurel, 2002). Therefore, the inverse relationship between 10 μM and 100 μM $HgCl_2$ and *PsPIP2-1* expression supports the idea that blockage by $HgCl_2$ would up-regulate *PsPIP2-1* expression to compensate for restricted water movement.

Although it could be argued that the response to the high $HgCl_2$ concentrations in the present study was due to Hg^{2+} toxicity, other studies have reported the use of even greater concentrations without damaging plant cells. Maggio and Joly (1995) exposed tomato roots to 500 μM $HgCl_2$ for >2 h and measured Lp_r using a pressure chamber. They found that not only did Lp_r decrease by 57%, but there was no significant change in osmotic potential, or K^+ concentration of xylem exudates, and no divergence in pressure flow linear responses. A linear pressure-flow response was also observed in the present study at all concentrations of $HgCl_2$. However, the duration of the exposure of pea roots to $HgCl_2$ influenced *PsPIP2-1* expression. While expression generally increased in roots treated with $HgCl_2$ for 1 h, the opposite effect was seen after 3 h. With the exception of roots exposed to 10 μM $HgCl_2$ for 3 h and harvested at 11:00 h, all other 3 h treatments caused a decline in *PsPIP2-1* expression (data not shown). Therefore, it is thought that a 3 h exposure was toxic to the roots.

ABA—effects on PsPIP2-1 and Lp_r

To test the prediction that increasing Lp_r would decrease the expression of *PsPIP2-1*, ABA was applied as a root drench. Roots were treated with a range of ABA concentrations. The greatest concentration (100 μM) was tested since it has been commonly used in other studies; however, it is so large that it is not likely to be physiologically relevant. The rationale was that AQP expression would decline because ABA is known to increase Lp_r in most systems (Freundl *et al*., 1998; Quintero *et al*., 1999; Hose *et al*., 2000; Sauter *et al*., 2002; Lee *et al*., 2005; Schraut *et al*., 2005). It was assumed the plant would be acclimated with pre-existing AQPs so that as Lp_r increased, the same number of or even fewer AQPs would be required to conduct the same amount of water. With a few exceptions, this prediction was not supported.

Changes in *PsPIP2-1* expression and Lp_r were not correlated in the ABA-treated samples, and the response of these two variables was highly ABA dose dependent. At 16:00 h, the lowest and highest concentrations of ABA (0.01 μM and 100 μM, respectively) lowered *PsPIP2-1* expression, and intermediate concentrations had no effect relative to controls. The effect on Lp_r at 16:00 h was also dependent on the ABA concentration. ABA elicited a typical hormone response curve whereby low to medium concentrations promoted Lp_r but high concentrations led to a decline in Lp_r. Curiously, 1 μM ABA caused very little change in *PsPIP2-1* expression or Lp_r relative to controls. At 9:00 h and 11:00 h, the trend in *PsPIP2-1* expression was almost identical to the pattern at 16:00 h. Normally, ABA has been applied in the range of 0.005–4 μM (Freundl *et al*., 1998; Quintero *et al*., 1999; Hose *et al*., 2000; Sauter *et al*., 2002; Schraut *et al*., 2005). Therefore, 100 μM ABA was too high a concentration to be physiologically relevant and the Lp_r decline at this dose may have been caused by hormone toxicity. Although other physiological studies reported that Lp_r increased at lower ABA concentrations (this study found the same when 0.01, 1, and 10 μM ABA were applied), none examined AQP gene expression at the same time to determine if the two factors were related. Several studies have examined AQP gene expression (although without corresponding Lp_r data) in various plant tissues treated with ABA. All show that the AQP response is complex (Weig *et al*., 1997; Mariaux *et al*., 1998; Jang *et al*., 2004).

The ABA component of this study does not support the hypothesis that PIP2 AQPs are functionally involved in regulating Lp_r changes. However, unlike $HgCl_2$, the effect of ABA on AQPs is largely unknown. Whether or not ABA increases Lp_r in roots through a direct or indirect interaction with AQPs remains unclear. It has been suggested that ABA stabilizes AQPs by binding to and maintaining these channels in an open conformation (Wan *et al*., 2004; Lee *et al*., 2005), although this hypothesis remains speculative.

It has been proposed that ABA increases Lp_r during periods of water stress so the plant can capture all available water remaining in the soil (Javot and Maurel, 2002). However, without understanding how ABA increases Lp_r, one can only speculate that it might occur through an interaction with AQPs. The process could be more complex and involve a cascade of events that include other signalling processes within the cell or an interaction with other hormones. This hypothesis is supported by the complex transcriptional response in a number of AQP genes in ABA-treated roots of *Arabidopsis* and *Craterostigma plantagineum* (Weig *et al*., 1997; Mariaux *et al*., 1998; Jang *et al*., 2004). These studies demonstrated that PIP2s respond differentially to ABA treatment and that such a response may involve both ABA-dependent and independent signalling pathways (Mariaux *et al*., 1998; Jang *et al*., 2004).

Water demand

When $HgCl_2$ was applied to roots, there was often no difference in transpiration between Hg^{2+}-treated roots and controls (Table 2). In contrast, ABA caused a marked decrease in transpiration, an effect that has been known for some time (Dodd and Davies, 2004). However, because diurnal changes in transpiration were not correlated to changes in Lp_r or *PsPIP2-1* gene expression, there

is little evidence to suggest that Lp_r or *PsPIP2-1* expression were driven by water demand. Furthermore, although the simultaneous increase of Lp_r and transpiration rate has been shown previously (Mees and Weatherley, 1957; Passioura and Tanner, 1985), there do not appear to be any studies that have correlated AQP expression with changes in transpiration. Henzler *et al*. (1999) also examined both factors, but found no relationship between *PIP1* transcript abundance and transpiration rate.

Future steps towards understanding the role of pea PIP2 AQPs in root water uptake will be to determine if changes in PsPIP2-1 protein levels are correlated to the *PsPIP2-1* gene expression and Lp_r changes that have been demonstrated. This could be studied using an antibody to monitor changes in PsPIP2-1 protein levels, or by using RNA interference (RNAi) to knock out the expression of the gene. Additionally, a histological approach could be used to determine where along the radial pathway PsPIP2-1 is most prevalent. By establishing its location along the root cylinder, it will be possible to resolve which layer of cells is utilized to facilitate xylem water entry. A further study could involve examining AQP expression in a species in which the path of water movement is largely apoplastic. For example, modelling and the inhibition of AQP activity with $HgCl_2$ has determined that radial water flow in lupins is predominantly apoplastic, whereas that of wheat uses a combination of cell–cell and apoplast pathways (Bramley, 2006). Thus, in the case of a species such as lupin, Lp_r should not be as limiting, and it would be predicted that AQP expression would be unaffected by treatment with $HgCl_2$. All of these approaches would offer further insight into the functional role AQPs play in root water uptake.

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