

## Diurnal variations in hydraulic conductivity and root pressure can be correlated with the expression of putative aquaporins in the roots of *Lotus japonicus*

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**Abstract.** The hydraulic conductivity of excised roots ( $L_{p_r}$ ) of the legume *Lotus japonicus* (Regel) K. Larsen grown in mist (aeroponic) and sand cultures, was found to vary over a 5-fold range during a day/night cycle. This behaviour was seen when  $L_{p_r}$  was measured in roots exuding, either under root pressure (osmotic driving force), or under an applied hydrostatic pressure of 0.4 MPa which produced a rate of water flow similar to that in a transpiring plant. A similar daily pattern of variation was seen in plants grown in natural daylight or in controlled-environment rooms, in plants transpiring at ambient rates or at greatly reduced rates, and in plants grown in either aeroponic or sand culture. When detached root systems were connected to a root pressure probe, a marked diurnal variation was seen in the root pressure generated. After excision, this circadian rhythm continued for some days. The hydraulic conductivity of the plasma membrane of individual root cells was measured during the diurnal cycle using a cell pressure probe. Measurements were made on the first four cell layers of the cortex, but no evidence of any diurnal fluctuation could be found. It was concluded that the conductance of membranes of endodermal and stelar cells may be responsible for the observed diurnal rhythm in root  $L_{p_r}$ . When mRNAs from roots were probed with cDNA from the *Arabidopsis* aquaporin *AthPIP1a* gene, an abundant transcript was found to vary in abundance diurnally under high-stringency conditions. The pattern of fluctuations resembled closely the diurnal pattern of

variation in root  $L_{p_r}$ . The plasma membranes of root cells were found to contain an abundant hydrophobic protein with a molecular weight of about 31 kDa which cross-reacted strongly to an antibody raised against the evolutionarily conserved N-terminal amino acid sequence of *AthPIP1a*.

**Key words:** Aquaporin – Diurnal cycle – Hydraulic conductivity – *Lotus* (aquaporin) – Plasma membrane – Pressure probe

### Introduction

Water and mineral nutrients are vital resources acquired through the roots of land plants. In their passage through the plant, these resources share a number of common pathways and there can be linkages between their fluxes. In the plasma membrane, there are at least two pathways along which water is believed to move. One is diffusion through the lipid bilayer, a process which depends on the thermal motion of the membrane lipids, and the other is conduction through water channels which are established by polypeptides of the aquaporin type. Flow through these two pathways may be independently regulated (Henzler and Steudle 1995; Steudle and Henzler 1995). When considering water flow across a structure as complex as a root, the principal resistance in the pathway is often envisaged as being due to plasma membranes of cells in the pathway. A common view is that the plasma membrane of the endodermis may contribute the major part of this resistance (Weatherley 1982), but there are different opinions about this matter (Newman 1973; Kramer and Boyer 1995; Steudle and Peterson 1998).

In classic experiments, it was observed that the root hydraulic conductivity ( $L_{p_r}$ ) declined towards the end of the light period and rose again at the end of the dark period (Parsons and Kramer 1974). Such effects have been taken as an indication that root resistance to water

Abbreviations and symbols:  $A_{(\text{root})}$  = cell (root) surface area; (Ath)PIP = plasma intrinsic protein (from *A. thaliana*);  $C_{(x/o)}$  = concentration (in xylem/medium);  $d_{\text{cell/layer}}$  = cell diameter/distance of cell or layer to root surface;  $\epsilon$  = cell elastic modulus;  $J_{vr}$  = rate of water flow through root system;  $l_{\text{cell}}$  = cell length;  $L_{p_r}$  = cell (root) hydraulic conductivity; PIP = plasma-membrane intrinsic protein;  $P_{(r/\text{bomb})}$  = cell (root/bomb) pressure;  $\sigma_{sr}$  = root reflection coefficient;  $T_{1/2}^w$  = half-time of water exchange;  $t_{1/2}$  = half-time of exponential kinetic;  $V_{(x/\text{xylem}/\text{root})}$  = cell (exuded/xylem/root) volume

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flow can decrease as flow increases (Mees and Weatherley 1957; Passioura and Tanner 1985), although this view has been challenged by Fiscus (1975). Alternatively, a variation in root  $L_p$  can be also interpreted in terms of relative changes in flows of water through several pathways across the root (Steudle and Peterson 1998; Tyerman et al. 1999).

An unexplained interaction between cell and root hydraulic conductivity (cell:  $L_p$ , root:  $L_{p_r}$ ) and the supply of certain mineral nutrients has been described in many species. At the cellular level,  $\text{NO}_3^-$  deprivation decreased  $L_p$  of cortical cells in cotton roots to less than 20% of control values over a period of 4 d (Radin and Matthews 1989). At the whole-plant level, root  $L_{p_r}$  decreased by a similar extent in cotton plants grown in P-deficient conditions (Radin and Eidenbock 1984). In  $\text{SO}_4^{2-}$ -deprived barley roots,  $L_{p_r}$  decreased to 20% of controls over a 4-d period (Karmoker et al. 1991) and in N-, or P-deficient wheat roots a similar proportional decrease was observed in plants examined in either the light or dark phases of the photoperiod. In many studies, nutrient deprivation has been shown to lead to stomatal closure and lowered transpiration (Clarkson and Scattergood 1982; Chapin et al. 1988). This raised the possibility that the observed decline in root  $L_{p_r}$  is a consequence of stomatal closure and might, therefore, be explained in a similar way to diurnal and transpirational variation in  $L_{p_r}$  mentioned above. However, in experiments using a split-root design, it was shown that N- or P-deprived roots had lowered  $L_{p_r}$  in conditions where stomatal conductance and transpiration were similar to those of control plants (Carvajal et al. 1996).

Water-channel proteins or aquaporins belong to an ancient family of membrane intrinsic proteins (MIPs) and are present in a wide variety of animal, microbial and plant membranes. They are encoded by genes which display a remarkable degree of conservation across taxa and kingdoms, the most obvious homology stems from two loops that both harbour the signature amino acid motif Asn-Pro-Ala (NPA) (Maurel 1997; Schäffner 1998; Tyerman et al. 1999). Based on structural studies with mammalian aquaporins, the NPA loops are presumed to form the pore which confers permeability to water driven by osmotic or hydraulic gradients across the membrane (Jung et al. 1994). There are observations that individual plant aquaporins may be permeable to other small molecules like glycerol although the physiological relevance is not yet clear (Biela and Kaldenhoff 1998); similar dual or multiple permeabilities have been shown for mammalian aquaporins (Yang and Verkman 1997). In addition, mercurial inhibition and permeability studies on whole algal cells indicate slippage of non-water molecules assuming that mercury-sensitive aquaporins with multiple permeabilities exist in these cells (Steudle and Henzler 1995; Hertel and Steudle 1997). Mercurial inhibition of aquaporins is due to non-specific interactions with cysteine residues at various positions (Preston et al. 1992; Jung et al. 1994; Kammerloher et al. 1994; Daniels et al. 1996; Shi and Verkman 1996). However, there is no unambiguous relation between the presence of certain cysteine residues and mercurial

sensitivity because plasma-membrane intrinsic proteins (PIPs) bearing homologous cysteines may either show sensitivity (e.g. AthPIP2a, AthPIP2b) or resistance (e.g. AthPIP2c/RD28) (Kammerloher et al. 1994). Therefore, it seems likely that plant plasma membranes possess a mixture of Hg-sensitive and -insensitive types of aquaporins. The functional significance of this diversity is not understood.

In the present paper we demonstrate a well marked diurnal cycle of  $L_{p_r}$  in the roots of *Lotus japonicus* which can be observed independently of the rate of water flow across the root. The cycle is matched by fluctuations in the abundance of an mRNA which hybridises with cDNA probes from *A. thaliana* AthPIP1a. We show that the plasma membrane of *L. japonicus* contains an abundant hydrophobic protein which is recognised by an antibody raised against a part of the N-terminus of AthPIP1a.

## Materials and methods

**Plant culture.** Plants of *Lotus japonicus* (Regel) K. Larsen cv. Gifu were grown from cuttings taken from vegetatively cloned 'mother' plants grown originally from seeds kindly provided by Dr. Judith Webb of IGER Aberystwyth, UK (Waterhouse et al. 1996). At an appropriate stage, when adventitious roots were 10–20 mm long, cuttings were transferred either to aeroponic cultures in large mist tanks or transplanted into sand/vermiculite medium. Plants were grown in both Long Ashton and Bayreuth using standardised conditions.

**Mist culture.** Aeroponic tanks were located in both a temperature-controlled greenhouse or controlled environment. In the former, they were provided by supplementary illumination to simulate a 16-h day. The temperature of the mist around the plant roots was controlled by cooling the nutrient reservoir. It was maintained at 17 °C while the air temperature varied from 18 to 24 °C. The nutrient solution contained  $\text{NO}_3^-$  at 5 mM and  $\text{H}_2\text{PO}_4^-$  at 0.75 mM with a pH of 6.5 (see Waterhouse et al. 1996). In the controlled environment, plants were grown at a constant temperature of 20 °C with roots at 17 °C, and were subjected to a 16-h day with light provided by fluorescent and incandescent lamps giving approx.  $250 \mu\text{mol m}^{-2} \text{s}^{-1}$  photosynthetically active radiation at plant height.

**Sand culture.** Plants were grown in sand to minimise artefacts which might have arisen from mechanical disturbance of roots during exudation experiments. Rooted cuttings were placed in 80-mm-diameter plastic pots filled with a 1:1 (w/w) mixture of coarse sand/vermiculite and, on alternate days, were provided with a nutrient solution sufficient to saturate the medium. Pots were placed on capillary matting (polyethylene coated) in a greenhouse and experienced natural daylength in Long Ashton and Bayreuth during spring and summer 1997.

**Anatomy.** Transverse sections were prepared from root segments embedded in 10% (w/v) gelatin, chilled on ice, and cut into cubes with a razor blade. Cubes were fixed with 5% (v/v) glutaraldehyde for 3 h. Cross-sections were made using an Oxford vibratome (Microinstruments, Oxford, UK). They were briefly stained with 0.001% (w/v) Toluidine Blue O in water. Bright-field micrographs were photographed using a Zeiss Axiophot microscope. Cell diameters ( $d_{\text{cell}}$ ) and distances to the root surface ( $d_{\text{layer}}$ ) were measured using a digitising tablet. According to absolute values of  $d_{\text{cell}}$  and  $d_{\text{layer}}$ , cells were grouped into different layers. Longitudinal sections were obtained from roots fixed in FAA (95%

ethanol:40% formaldehyde:glacial acetic acid:water; 15:6:1:30, by vol) and embedded in paraffin wax. After removing the wax, the tissue was lightly stained with Toluidine Blue O, as above. Lengths of cells in the outer and inner cortical layers were measured using a microscope with an eyepiece graticule.

*Measurement of steady volume flow using pressure chambers and free exudation.* Two approaches have been used in Bayreuth to measure the hydraulic conductivity of root systems ( $L_{pr}$ ) grown in sand/vermiculite in steady-state conditions: (i) a pressure-chamber technique and (ii) the conventional technique using free exudation. With the pressure chamber the flow driving force was largely hydrostatic (hydraulic) whereas during exudation flow was driven by osmotic forces alone.

(i) In the pressure chamber-technique, the stem was cut below the first node and the root system sealed into a pressure chamber using a rubber seal. A pressure of 0.4 MPa was applied to the root medium ( $P_{bomb}$ ). To prevent the loss of xylem sap, which emerged as drop from the cut end, a short collar of rubber tube was connected to the remaining root stump. For given periods of time ( $\Delta t$ ) xylem sap was collected into Eppendorf tubes using a micropipette. The volume of exuded sap ( $\Delta V_x$ ) was determined by weighing. Xylem sap concentration ( $C_x$ ) was measured with the aid of a freezing-point osmometer (Gonotec, Berlin, Germany). The hydraulic conductivity of the root system was calculated according to Eq. 1:

$$L_{pr} = \frac{J_{vr}}{P_{bomb} + \sigma_{sr} \cdot RT \cdot (C_x - C_o)} = \frac{\Delta V_x}{\Delta t} \cdot \frac{1}{A_{root}} \cdot \frac{1}{P_{bomb} + \sigma_{sr} \cdot RT \cdot (C_x - C_o)}, \quad (\text{Eq. 1})$$

where R denotes the gas constant, T the absolute temperature,  $A_{root}$  the surface area of the root system,  $J_{vr}$  the flow rate, and  $C_o = 10$  mM the concentration of the solution used to irrigate the sand. Mean reflection coefficients of the root ( $\sigma_{sr}$ ) for the solutes in the xylem sap were estimated from root pressure ( $P_r$ ) and the concentration difference ( $\Delta C = C_x - C_o$ ) at steady state ( $J_{vr} = \text{const.}$ ), according to Eq. 2:

$$\sigma_{sr} = \left( \frac{P_r}{RT \cdot \Delta C} \right)_{J_{vr}=\text{const.}} \quad (\text{Eq. 2})$$

The parameter  $A_{root}$  was estimated by first determining the ratio between root surface area (from morphometric analysis of the main axes and all root branches) and fresh weight (volume) for seven adventitious roots. The conversion factor obtained from these measurements,  $73 \cdot 10^{-6}$  m (volume/surface area), was then used to evaluate the surface area of the other root systems.

(ii) During ordinary root exudation, the same procedure was used except that no pressure was applied in these experiments ( $P_{bomb} = 0$  MPa).

*Measurements from roots grown in aeroponic culture.* These measurements were made at Long Ashton and differed only slightly from those described above. Plants were removed from the mist culture and the aerial parts of plants removed leaving a 10-mm long-piece of stem. Roots were introduced into a wide-necked tube which was placed inside a pressure vessel. The short piece of stem was attached to a coupling device which fitted into the lid of a pressure chamber (Else et al. 1995). A pneumatic pressure of 0.4 MPa was applied to the solution surrounding the roots. The solution had the same composition as that used during the growth of the plants. Sap emerging from the coupling device was collected, weighed, frozen for storage, and then used to measure its concentration (see above).

*Cell pressure-probe measurements.* A cell pressure probe was used to measure  $L_p$  of the root cortical cells (Azaizeh et al. 1992). An oil-filled capillary (outer tip diameter: 4–7  $\mu\text{m}$ ) was attached to a

pressure chamber which contained an electronic transducer (silicon chip). The probe was fixed on a micromanipulator (Huxley-Goodfellow, Cambridge, UK) which allowed insertion of the tip into individual cells. By measuring the depth of insertion of the tip into the cortex, the location of punctured cells could be measured. Prior to insertion into root tissue, the pressure probe was completely filled with silicone oil (type AS4 from Wacker, München, Germany). When a cell was punctured, a meniscus formed between oil and cell sap, and this meniscus was kept at a certain position. The pressure transducer converted the pressure signal into a proportional voltage. Pressure/time curves were recorded on a chart recorder. For processing the data (turgor, hydraulic conductivity, elastic modulus), recorder strips were digitised using a digitising tablet. Cell elastic moduli ( $\epsilon$  in MPa) were evaluated from cell volumes and from changes in cell volume ( $\Delta V$ ) which caused changes in cell turgor pressure ( $\Delta P$ ), since (Azaizeh et al. 1992):

$$\epsilon = V \cdot \frac{\Delta P}{\Delta V} \quad (\text{Eq. 3})$$

Cell volumes were estimated from cross-sections and longitudinal sections (see above) assuming a cylindrical shape of cells ( $V = \pi (d_{cell}/2)^2 \cdot l_{cell}$ ;  $l_{cell}$ : cell length). For a proper determination of  $\epsilon$ , changes in volume had to be rapidly executed to avoid errors due to a water flow.

Hydrostatic pressure relaxations were performed by rapidly moving the meniscus to a new position and keeping it there until a steady pressure was re-attained. During these experiments, pressure returned exponentially to a value which was close to the initial one. From the half-time ( $T_{1/2}^w$ ) of the relaxation, the  $L_p$  was calculated according to Eq. 4 (Azaizeh et al. 1992):

$$L_p = \frac{V \cdot \ln(2)}{A \cdot T_{1/2}^w \cdot (\epsilon + \pi_0^i)} \quad (\text{Eq. 4})$$

where A denotes the cell surface area ( $A = \pi \cdot d_{cell} \cdot l_{cell}$ , neglecting top and bottom areas of cylindrical cells) and  $\pi_0^i$  the osmotic pressure of the cell. Usually it holds that  $\epsilon \gg \pi_0^i$ . Under this condition, Eq. 4 can be re-written as:

$$L_p = \frac{\ln(2)}{A \cdot T_{1/2}^w \cdot \frac{\Delta P}{\Delta V}} \quad (\text{Eq. 5})$$

Cells were probed at distances between 30 and 100 mm from the apex of roots which had been grown in mist culture

*Root pressure-probe measurements.* A root pressure probe was used to measure root pressure in roots (Steudle et al. 1993). After cutting off the stem just below the first leaves, the remaining stump was fixed to the probe with a rubber seal. The pot with the root system was supplied continuously with nutrient solution. With some root systems, root pressure could be recorded for several days. Curves were digitised using a digitising tablet.

*Expression of aquaporin homologous genes.* A modified version of the procedure of Verwoerd et al. (1989) was used to isolate RNA from roots of plants grown in mist cultures. Typically, a ratio of 8 ml of extraction buffer/phenol and 4 ml chloroform/isoamyl alcohol (24:1, v/v) was used to extract RNA from 2 g of ground, frozen root tissue. Concentrations of RNA were estimated spectrophotometrically. An aliquot of 10  $\mu\text{g}$  RNA used for northern blots was denatured using glyoxal and subjected to electrophoresis (0.9% agarose) using N,N-bis[2-hydroxyethyl]-2-aminoethane sulphonic acid (BES; Sigma) buffer (Grundemann and Koepsell 1994). The RNA was transferred to Zetaprobe GT membranes (BioRad) by capillary transfer and fixed by baking for 2 h at 80 °C. To visualise rRNA and to size hybridising messages, filters were stained with methylene blue, just before hybridisation (Sambrook et al. 1989). Confirmation of rRNA and transcript sizes was obtained using standard RNA markers. Filters were hybridised overnight with  $^{32}\text{P}$ -labelled *AthPIP1a* full-length cDNA in 250 mM  $\text{Na}_2\text{HPO}_4$  (pH 7.2) and 7% SDS at 65 °C. Filters were also

hybridised to  $^{32}\text{P}$ -labelled cDNAs to *Lotus japonicus* root-enhanced (cytosolic) glutamine synthetase GS1, *L. japonicus* nitrate reductase and *L. japonicus* high-affinity nitrate-transporter homologue, Ig7. After exposure, filters were washed for 10 min at 65 °C (high stringency) in each of the following solutions: (i) 250 mM  $\text{Na}_2\text{HPO}_4$  plus 5% SDS; (ii) double-strength sodium chloride/sodium citrate buffer (SSC;  $1\times$  SSC = 15 mM  $\text{Na}_3\text{-citrate}$ , 150 mM NaCl; pH 7.0) plus 1% SDS; (iii) SSC plus 0.5% SDS; (iv) 0.2-strength SSC plus 0.1% SDS. Washed filters were dried and exposed to Kodak Safety fine grained X-ray film for 2–6 d prior to development of the autoradiographs.

**Isolation of plasma-membrane proteins and western blots.** Batches of root material of approx 15 g FW were taken from plants growing in mist cultures. This material was used for the isolation of a plasma-membrane-enriched fraction using the aqueous two-phase technique (Larsson et al. 1987). The root material was chopped and vacuum-infiltrated with 60 ml 50 mM Hepes and 0.5 M sucrose, adjusted to pH 7.5 with NaOH, plus 1 mM DTT (dithiothreitol), 5 mM ascorbic acid and 0.6% (w/v) insoluble polyvinylpyrrolidone. The buffer-saturated material was homogenised, filtered through a 240- $\mu\text{m}$  nylon cloth, the filtrate centrifuged at 10 000 g for 15 min and the supernatant further centrifuged at 100 000 g for 30 min to yield a microsomal pellet which was resuspended in 3 ml 0.33 M sucrose, 5 mM phosphate buffer (pH 7.8). The suspension (3 ml) was added to 9 g of an aqueous two-phase mixture to produce a 12-g two-phase system with a final composition of 6.5% (w/w) Dextran T500 (Pharmacia), 6.5% (w/w) polyethylene-glycol 3350 (Sigma), 3 mM KCl, 5 mM phosphate buffer (pH 7.8) and 0.33 M sucrose.

The phase system was centrifuged for 3 min at 4000 g and the resulting plasma membranes (upper phase) purified using a batch procedure (Larsson et al. 1987). The third upper phase was diluted with phosphate buffer (pH 7.8) and centrifuged at 100 000 g for 30 min. The resulting pellet was resuspended in 0.9 ml of 5 mM Mes-Tris buffer (pH 6.5) plus 0.33  $\mu\text{M}$  sucrose, and was stored at  $-20$  °C. Protein samples were denatured in Laemmli buffer supplemented with 100 mM DTT and 2% SDS for 20 min at 56 °C. After separation on 12% denaturing polyacrylamide gels according to Laemmli (1970), the gel was transferred on to Fluorotrans membrane (Pall-Gelman Sciences, Roßdorf, Germany) using a semi-dry blotting apparatus (Schleicher & Schüll, Dassel, Germany). The membrane was incubated with a rabbit polyclonal antiserum raised against 42 N-terminal amino acids of *Arabidopsis thaliana* AthPIP1a expressed as a fusion peptide to glutathione *S*-transferase in *Escherichia coli*. This stretch of amino acids is highly conserved among *A. thaliana* AthPIP1 members. In addition, a homologous region is present in PIPs from other species; therefore, the antiserum may cross-react with heterologous PIP1 members. To visualise the immunoreactive bands, a cy5-fluorochrome-labelled secondary antibody (Pharmacia-Amersham) was used. Signals were recorded using a Molecular Dynamics (Krefeld, Germany) Storm 860 phosphoimager.

## Results

### Anatomy

**Root cortex cells.** Anatomical measurements were made on cortical cells from transverse and longitudinal root sections taken at distances between 30 and 80 mm from the root apex (Table 1). To relate the position of a cell to a mean cell diameter ( $d_{\text{cell}}$ ), distances of the outer boundaries of distinct cell layers from the root surface ( $d_{\text{layer}}$ ) have been computed. For the rhizodermis, (and also for the first and second cell layers), a clear distribution could be seen from sections (data not

**Table 1.** Summary of anatomical data from root cortical cells of *Lotus japonicus*. Data of cell diameters ( $d_{\text{cell}}$ ) and lengths ( $l_{\text{cell}}$ ) were evaluated from longitudinal and transverse sections at distances of 30–80 mm behind the root tip. Distinct cell layers were computed and mean values of  $d_{\text{cell}}$  calculated ( $\pm$  SD;  $n$  = number of cells). Layers start at a position of  $d_{\text{layer}}$  from the surface. The outermost layer is the rhizodermis (rhiz.). Data of cell lengths were grouped into outer cortex (rhiz. to 3rd layer) and inner cortex (4th to 7th layer). Cell volumes ( $V \pm$  SD, whereby SD denotes a propagated error,  $10^{-15} \text{ m}^3 = 1 \text{ pl}$ ) were also calculated, assuming a cylindrical cell shape

Layer	$d_{\text{layer}}$ [ $\mu\text{m}$ ]	$d_{\text{cell}} \pm$ SD ( $n$ ) [ $\mu\text{m}$ ]	$l_{\text{cell}} \pm$ SD ( $n$ ) [ $\mu\text{m}$ ]	$V \pm$ SD [ $10^{-15} \text{ m}^3$ ]
rhiz.	0	$17 \pm 4$ (222)	$100 \pm 14$ (15)	$23 \pm 11$
1st	17	$30 \pm 6$ (188)	↓	$71 \pm 30$
2nd	47	$38 \pm 5$ (90)	↓	$113 \pm 34$
3rd	85	$36 \pm 6$ (79)	↓	$102 \pm 37$
4th	121	$34 \pm 6$ (73)	$157 \pm 20$ (24)	$143 \pm 53$
5th	155	$28 \pm 9$ (59)	↓	$97 \pm 63$
6th	183	$20 \pm 8$ (98)	↓	$49 \pm 40$
7th	203	$16 \pm 5$ (43)	↓	$32 \pm 20$

shown). For the sake of completeness, boundaries for the other cell layers were calculated. To calculate cell volumes and surface areas of a given cell penetrated by the cell pressure probe (see below), the distance from the root surface was measured and the cell assigned to a cell layer. Cells up to the fourth layer were probed.

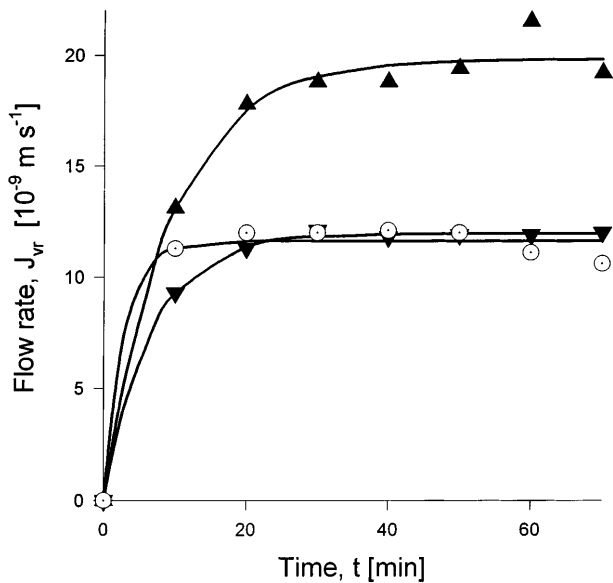
**Root systems.** The mean volume of the root systems used for measurements of flow rates was  $V_{\text{root}} = (0.86 \pm 0.3) 10^{-6} \text{ m}^3$  (range: 0.26 to 1.56;  $n = 31$  root systems) and the mean root surface area was  $A_{\text{root}} = (0.0118 \pm 0.004) \text{ m}^2$  ( $n = 31$  root systems). A comparison of cross-sectional areas of roots and their conducting xylem vessels (visualised by staining the lignified vessels with Toluidine Blue O) as determined from prints yielded a ratio of  $V_{\text{xylem}}/V_{\text{root}} = 1.4\%$  which is similar to data obtained with other species.

### Root hydraulic conductivity

**Preliminary note.** At an early stage in the work it was noticed that some plants failed to produce root exudate when transferred from mist cultures to smaller containers of culture solution. This has been recognised in previous reports (Miller 1987) and may be explained by mechanical disruption of the junctions between lateral roots and the axes from which they are derived. For this reason, we made a comparison of root  $L_{\text{p}}$  through undisturbed root systems, grown in sand, and those transferred from mist to solution culture. Under applied hydrostatic pressure the range of  $L_{\text{p}}$  values overlapped in root systems from the two growth systems, but the mean and variance recorded during the daily maximum was somewhat greater in roots transferred from mist culture (data not shown). Variation was even greater if such roots were left to exude sap under root pressure alone (data not shown), and we report exudation results mainly from sand/vermiculite-grown plants.

**Pressure-chamber experiments.** To test whether or not a stationary flow was attained during the experiments, three different root systems were subjected to an applied hydrostatic pressure of 0.4 MPa for 70 min. The results indicated that about 10 min were required to attain a constant flow of  $(15 \pm 5) 10^{-9} \text{ m s}^{-1}$  (mean  $\pm$  SD;  $n = 3$  roots; Fig. 1) irrespective of the absolute rate of water flow in individual plants. An exponential fit of the kinetics during the initial adjustment of  $J_{\text{vr}}$  gave a half-time of  $t_{1/2} = (4 \pm 2) \text{ min}$ . The rate of exchanging the whole xylem volume for the three plants was  $(75 \pm 20)\% \text{ min}^{-1}$  ( $n = 22$  samples of 10 min each). The osmotic concentration of collected xylem sap decreased exponentially with time to a mean concentration of  $C_x = (11 \pm 5) \text{ mM}$  which was similar to that of the medium ( $C_o = 10 \text{ mM}$ ).

In another experiment, tests were made to see if water flow through pressurised root systems was comparable to that of intact transpiring plants. Shoots of three intact plants with their roots enclosed in pots wrapped with Parafilm, were exposed to natural light and weighed at intervals of 90 min to determine the water loss by transpiration. In parallel, one additional plant was excised, placed in a pressure bomb, as described above and pressurised (0.4 MPa). Sap exuded from the root stump was collected and weighed at intervals of 0.5 h. All volume flows were normalised to the surface areas of the root systems as described above. Pressurised, excised roots and intact transpiring plants showed similar flow rates that slightly decreased towards the end of the photoperiod. Mean flow rates were  $(19 \pm 2) 10^{-9} \text{ m s}^{-1}$  for two pressurised excised roots and  $(14 \pm 3) 10^{-9} \text{ m s}^{-1}$  for six intact transpiring plants (mean  $\pm$  SD,  $n = 2$  and 6, respectively).

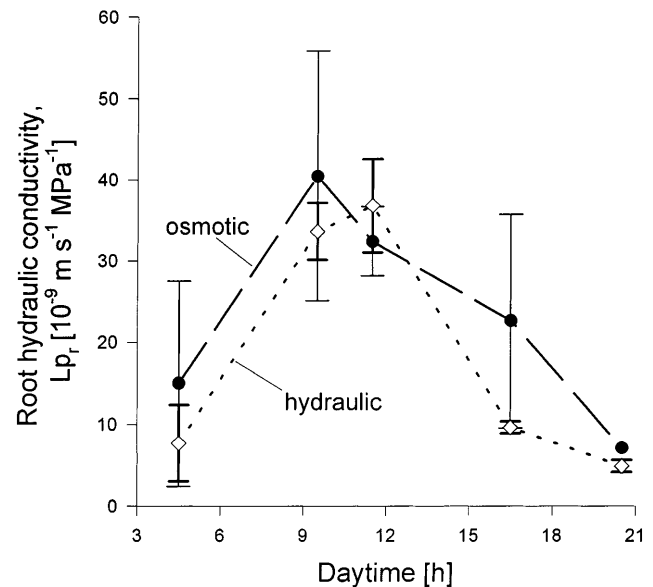


**Fig. 1.** Time dependences of water flow ( $J_{\text{vr}}$ ) across excised root systems from three different plants of *Lotus japonicus*. Xylem sap was sampled at a pneumatic pressure of  $P_{\text{bomb}} = 0.4 \text{ MPa}$  grown in sand/vermiculite applied to the root systems at  $t = 0$ . Samples were taken every 10 min and its volume determined by weighing. Flow rates exponentially approached a steady state ( $t_{1/2} = (4 \pm 2) \text{ min}$ ; mean  $\pm$  SD,  $n = 3$  roots)

A diurnal change in  $L_{\text{pr}}$  was observed (Fig. 2). For each time interval, two pressurised root systems were placed in pressure chambers and two to four freely exuding root systems were examined. Sap collected during the first 10 min was discarded to guarantee that a steady state had been established. Then, for pressurised roots, two sub-samples were collected at intervals of 15 min. For freely exuding roots, only one sample collected over 30 min was taken since the exudation rate was much slower than with pressurised roots. In the morning, there was a strong increase in  $L_{\text{pr}}$  which reached a maximum 5–7 h after dawn (4:30 a.m.). In the afternoon, the hydraulic conductivity decreased and regained its initial value 16 h after dawn.

For free exudation under only an osmotic driving force, the absolute value of  $L_{\text{pr}}$  is strongly dependent on the value of the reflection coefficient ( $\sigma_{\text{sr}}$ ). However, values required to calculate  $\sigma_{\text{sr}}$  (Eq. 2) could not be measured for the same plants at the same time. Therefore,  $\sigma_{\text{sr}}$  was estimated by means of Eq. 2, using root pressure values of  $P_r = 0.02 \text{ MPa}$  obtained with the root pressure probe (see below), and a mean xylem sap concentration of 33 mM (from free exudation experiments). This yielded an estimate for the reflection coefficient of  $\sigma_{\text{sr}} = 0.25$  which was used to calculate the osmotic root  $L_{\text{pr}}$ . Due to uncertainties in estimating root  $\sigma_{\text{sr}}$ , osmotic  $L_{\text{pr}}$  values should be regarded as a upper limit (see *Discussion*).

**Influence of transpiration on diurnal cycle of  $L_{\text{pr}}$ .** Mist-cultured plants, grown in a greenhouse at Long Ashton during sunny spring weather, were allowed to transpire at rates determined by ambient conditions or were



**Fig. 2.** Diurnal rhythm of hydraulic conductivity ( $L_{\text{pr}}$ ) of pressurised ( $P_{\text{bomb}} = 0.4 \text{ MPa}$ , open symbols) and freely exuding (filled symbols) excised roots of *Lotus* plants grown in sand/vermiculite. For each time, a set of two (hydraulic) or two to four (osmotic) plants was measured. With pressurised plants, two sub-samples were measured every 15 min. A clear diurnal rhythm of  $L_{\text{pr}}$  was found, with a maximum at 5–7 h after dawn (4:30 a.m.) in both experiments. Means are given  $\pm$  SD with  $n = 2$ –4 roots

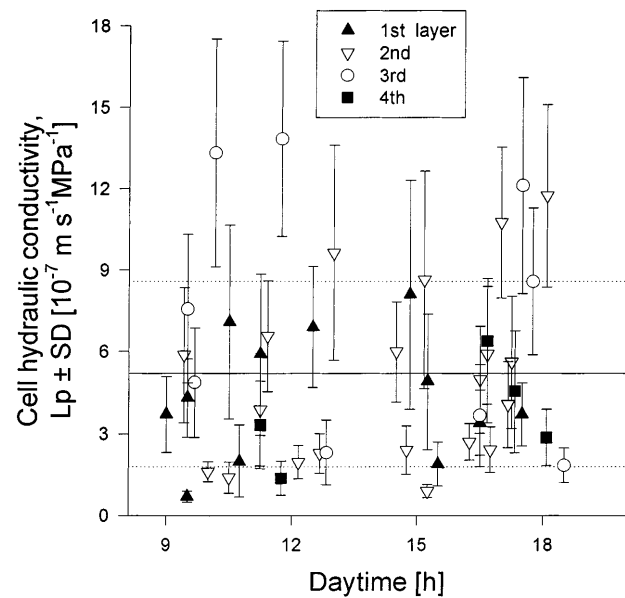
**Table 2.** Effect of transpiration on the root  $L_{p_r}$  of *Lotus japonicus* at different times of the day. Plants were grown in mist culture. Transpiration rates refer to ambient or reduced conditions whereby the reduction was achieved by placing transparent covers over the plants. After 4 d of adjusting to the new conditions, four plants from each treatment were used for measurements at each time of the day. Excised roots were placed in a pressure bomb and pressurised for 40–60 min ( $P_{\text{bomb}} = 0.4$  MPa; see *Materials and methods*). Reduced transpiration did not change the diurnal cycles in root  $L_{p_r}$ . Although plants with reduced transpiration usually showed a higher hydraulic conductivity, values were only significantly different during mid-morning ( $P$  values from  $t$ -test at  $\alpha = 0.05$ ; homogeneity of variance tested with Levene's test at  $\alpha = 0.05$ ; s = significant; non = not significant)

Point in cycle	Hydraulic conductivity $L_{p_r} \pm \text{SD}$ [ $10^{-9} \text{ m s}^{-1} \text{ MPa}^{-1}$ ]		Difference
	Ambient	Reduced	
Pre-dawn	$14.4 \pm 8.6$	$21.3 \pm 6.8$	non $P = 0.25$
Mid-morning	$27.9 \pm 5.1$	$48.0 \pm 8.7$	s $P = 0.007$
Noon	$42.4 \pm 12$	$52.3 \pm 17$	non $P = 0.37$
Mid-afternoon	$36.5 \pm 13$	$47.1 \pm 9.0$	non $P = 0.22$
End of day	$9.8 \pm 5.1$	$13.5 \pm 9.1$	non $P = 0.51$

covered with glass vessels to minimise transpiration. At different times of day, four plants from each treatment were transferred to pressure vessels and  $J_{\text{vr}}$  recorded over a period of 40–65 min (applied pneumatic pressure of 0.4 MPa). The osmotic driving force due to solutes in the sap was added to the hydrostatic pressure to derive a total driving force, again assuming a root  $\sigma_{\text{sr}} = 0.25$  (Eq. 1). The rate of transpiration had no significant effect on the general features of the diurnal rhythm of root  $L_{p_r}$  (Table 2). Although mean values of  $L_{p_r}$  were generally greater for plants grown at reduced transpiration, differences were significant only at mid-morning (Table 2).

**Cell hydraulic conductivity.** In total, 45 cortical cells from root segments of 15-d-old *L. japonicus* plants grown in mist culture were probed. There was a considerable scatter in the physical properties of cells (elastic modulus, turgor pressure, hydraulic conductivity). No significant differences in these properties could be detected for the first four cell layers. The wide scatter of measurements shown in Fig. 3 documents that neither the position of a cell nor the time of day had a significant effect on its hydraulic conductivity. Therefore, the data were pooled and mean values  $\pm \text{SD}$  (standard deviation;  $n = 45$  cells) are given for all cells measured. Mean turgor pressure was  $P = (0.41 \pm 0.05)$  MPa (range: 0.3–0.5). The mean elastic modulus was  $\epsilon = (7.1 \pm 2.4)$  MPa (range: 2.5–13.6 MPa). Therefore, the assumption did hold that  $\epsilon \gg \pi_0^1 \approx P$ . The mean hydraulic conductivity was  $L_p = (5.2 \pm 3.4) 10^{-7} \text{ m s}^{-1} \text{ MPa}^{-1}$  (range: 0.68–14). Within the limits of variation, no diurnal changes could be detected (Fig. 3). Absolute values were similar to those given in the literature for  $L_p$  of root cells of different herbaceous species (Steudle and Peterson 1998).

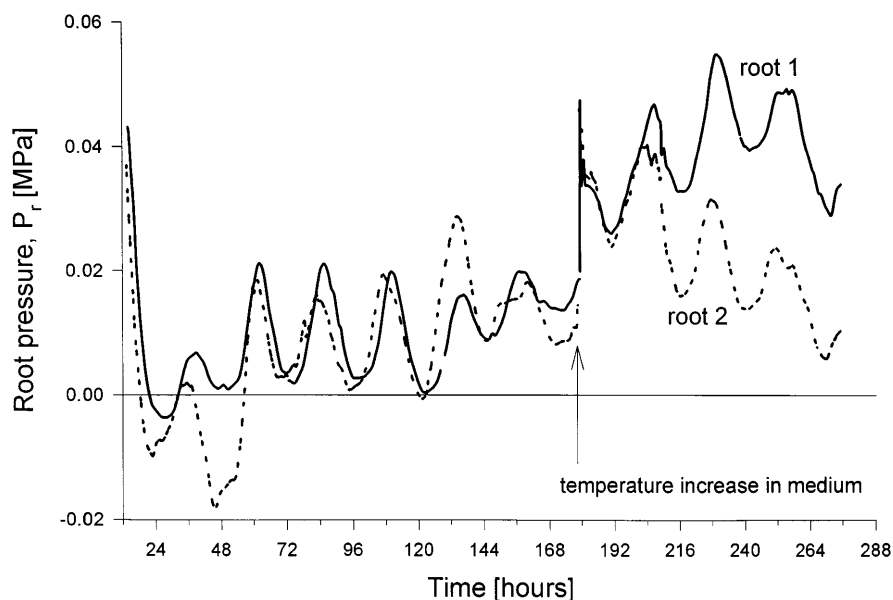
**Circadian rhythms in root pressure.** A root pressure probe was used to monitor root pressure ( $P_r$ ) in excised



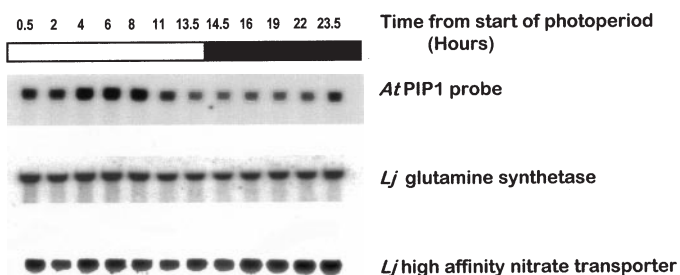
**Fig. 3.** Diurnal course of hydraulic conductivity ( $L_p$ ) of root cortical cells of young plants of *Lotus japonicus* as measured with the cell pressure probe. Plants were grown in mist culture (aeroponics) for about 15 d. Roots were probed at a distance of 40–80 mm from the root tip. Measurements were performed at different distances from the root surface (see Table 1). Mean  $L_p$  values of single cells are given  $\pm$  propagated SD which includes errors in the measurement of  $T^{w/2}$ ,  $\epsilon$ , and cell dimensions. No diurnal variation in  $L_p$  could be detected in the root cortex. The mean  $L_p$  of all 45 cells (solid line) was  $(5.2 \pm 3.4) 10^{-7} \text{ m s}^{-1} \text{ MPa}^{-1}$  (dotted lines:  $\pm \text{SD}$ )

root systems. Absolute values of root pressure were low (0.02–0.06 MPa). In Fig. 4, typical time courses are shown for two root systems grown in sand/vermiculite. After a period of adjustment of 36 h to changed conditions, both roots developed a circadian rhythm in root pressure that reached its maximum at noon. At midnight, root pressure nearly vanished. Roots tended to lose their ability to perform endogenous rhythms in root pressure after the fourth cycle (about 144 h after starting the experiments). By increasing the temperature of the medium, rhythms could be induced again. However, after two more cycles, the ability to perform rhythms was lost completely.

**Diurnal variation in mRNA homologous to aquaporin gene transcripts.** Plants were grown in mist chambers in a controlled environment with a 16-h photoperiod. At intervals, groups of five plants were taken and the mRNA in the root extracted as described in *Materials and methods*. Transcripts hybridising strongly to an AthPIP1 cDNA probe were abundant (Fig. 5). Northern probes with AthPIP2b cDNA showed exactly similar diurnal patterns of expression (data not shown). There is much sequence homology between members of the large aquaporin gene family. It is probable that the *Arabidopsis* aquaporin probes will hybridise to several homologues in the *L. japonicus* mRNA pool. Hybridisation cannot be taken, therefore, as evidence for the specific existence of PIP1 or PIP2 members of the gene

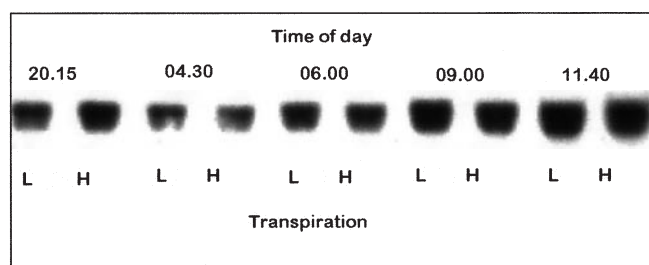


**Fig. 4.** Diurnal rhythm of root pressure ( $P_r$  in MPa) in excised root systems of young plants (15 d) of *Lotus japonicus* as measured with the root pressure probe. Root pressure was measured simultaneously on two root systems grown in sand/vermiculite. Roots were set up in the early afternoon (24 h on the time axis denotes midnight of the first day). After 36 h of adjusting to the new conditions, roots developed a circadian rhythm in root pressure for about 4 d. The  $P_r$  reached its maximum of 0.02 MPa at noon, and was nearly absent at midnight. After 4 d, roots tended to lose the circadian rhythm which could be stimulated again by transiently increasing the temperature of the medium (+ 10 K)



**Fig. 5.** Northern blots of mRNA extracted from roots of *Lotus japonicus* at different times in the day/night cycle. Plants were grown in aeroponic (mist) culture tanks (see *Materials and methods*). Sources of cDNA probes: *Arabidopsis thaliana* At(h)PIP1, *Lotus japonicus* (*Lj*) glutamine synthetase (GS1 cytosolic) and the high-affinity nitrate transporter (Ig7, homologue of barley HvNPT2; Trueman et al. 1996). The mRNA of the aquaporin built up to a maximum value 4–9 h into the photoperiod followed by a marked decrease in its abundance. Note that all blots were made from the same gel and mRNA extract

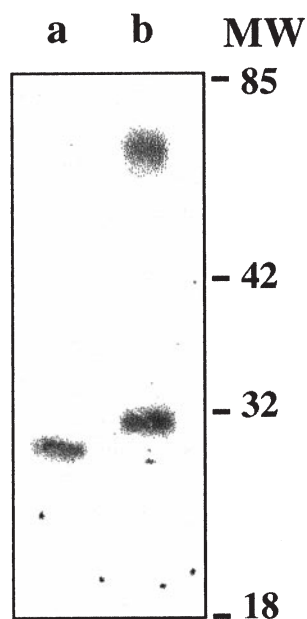
family. Aquaporin homologues varied in abundance during the diurnal cycle; distinct maxima were evident 6–8 h into the photoperiod followed by a marked decline to a minimum value at, or shortly after the beginning of the dark period. There was an indication that the abundance of the hybridising transcript increased towards the end of the night period, thus anticipating the beginning of the photoperiod. It obviously correlated with the diurnal variation in  $Lp_r$  (Fig. 2) but the cycle was offset towards the dark period. Figure 5 includes results for transcripts of glutamine synthetase *GS1* (cytosolic) and an *L. japonicus* homologue of the high-affinity nitrate transporter *HvNTR2*. With these transcripts, there was only a slight indication of diurnal fluctuations. Similar patterns of diurnal expression of putative aquaporin mRNA were seen in four additional, separate experiments in roots of plants growing in a greenhouse and experiencing supplemented natural daylight (see e.g. Fig. 6).



**Fig. 6.** Northern blot of mRNA extracted from roots of *Lotus japonicus*. Plants were grown in mist culture under conditions of ambient (*H*) or strongly reduced (*L*) transpiration. Transparent covers were placed over shoots of the *L* treatment at the start of the photoperiod. The root systems of three plants were combined for RNA extraction after differential transpiration treatments had been imposed. The mRNA was probed with cDNA from AthPIP1. In plants with both high and low transpiration, transcript levels increased markedly during the first 6 h of the photoperiod

Figure 6 shows Northern blots of mRNA from roots which were companion plants to those used for  $Lp_r$  measurements (Table 2). In plants with both high and low transpiration, transcript levels increased markedly during the first 6 h of the photoperiod.

Western blots of solubilised *Lotus* plasma-membrane protein (see *Materials and methods*), along with *A. thaliana* microsomal fraction as a control were challenged with an antiserum raised against 42 amino acid residues from the N-terminus of *A. thaliana* AthPIP1a. This N-terminal peptide shows a considerable conservation among plant PIP1 members and has been shown to cross-react with membrane protein preparations of several plant species, such as pea (*Pisum sativum* L.), tomato (*Lycopersicon esculentum* L.) and grape (*Vitis vinifera* L.) (Schäffner 1998; and data not shown). As expected, a single band of PIP1 antigens at an apparent molecular mass of 28 kDa was recognised in the *A. thaliana* lane (Fig. 7). A cross-reacting band



**Fig. 7.** Western blot of *Lotus* plasma membrane and *Arabidopsis thaliana* microsomal fraction probed with anti-AthPIP1a antiserum. An aliquot of 1.5  $\mu\text{g}$  *Arabidopsis thaliana* microsomal fraction (lane a) and 2  $\mu\text{g}$  *Lotus* plasma membrane (lane b) were separated on an SDS polyacrylamide gel (Materials and methods). Immunoreactive bands detected by the anti-AthPIP1a antiserum are shown. Molecular weight markers (MW, kDa) are given at the right

with a somewhat slower electrophoretic mobility of 31 kDa was detected in the *Lotus* plasma membrane. Although known PIP1 members from different species do not differ that much in size it is not uncommon to find unusual electrophoretic behaviour in hydrophobic membrane proteins. An additional signal migrating at 58 kDa may indicate typical persistent dimeric forms of membrane intrinsic proteins (MIPs) that are frequently observed (Kammerloher et al. 1994; Qi et al. 1995). These results demonstrate the presence of PIP1-type proteins in *Lotus* roots bearing an N-terminus homologous to that of *Arabidopsis* AthPIP1 proteins.

## Discussion

The results presented in this paper show a diurnal variation in root hydraulic conductivity ( $L_{p_r}$ ) of *L. japonicus*. This variation was coincident with a diurnal rhythm of the expression of mRNA encoding putative water channels (aquaporins) in the root tissue. The finding suggests that there may be a de-novo synthesis and/or degradation of water channels during the diurnal cycle that affects water flow by changing the root  $L_{p_r}$ . However, before this conclusion can be accepted, it has to be verified that  $L_{p_r}$  rather than components of the driving force are responsible for the changes in  $J_{v_r}$ . According to Eq. 1, the product of  $L_{p_r}$  and the driving force [ $P_{\text{bomb}} + \sigma_{\text{sr}}RT(C_x - C_o)$ ] determines the flow of water through the root. Water flow was driven by differences in hydrostatic ( $P_{\text{bomb}}$ ) and osmotic [ $\sigma_{\text{sr}} \cdot RT \cdot (C_x - C_o)$ ] pressure exerted between medium and root xylem. In the presence of a constant

hydrostatic pressure gradient of  $P_{\text{bomb}} = 0.4$  MPa, the examination of the data for the osmotic pressure of the xylem sap revealed that at no time during the diurnal cycle was there a contribution of more than 2% by  $\sigma_{\text{sr}} \cdot RT \cdot (C_x - C_o)$  to the overall driving force. Hence, there was a diurnal rhythm in root  $L_{p_r}$  under these conditions.

In the other case of an ordinary exudation in the absence of a hydrostatic pressure gradient, only the osmotic driving force was present. Changes in root exudation ( $J_{v_r}$ ) may have been due to a variation in root reflection coefficient ( $\sigma_{\text{sr}}$ ), besides root  $L_{p_r}$ . Unlike the concentration difference between root xylem and medium ( $C_x - C_o$ ) which was measured for each sample of xylem sap,  $\sigma_{\text{sr}}$  was assumed to be constant. This assumption may be questioned. Changes in root  $\sigma_{\text{sr}}$  have been reported in the literature. For another legume *Fiscus* (1986; *Phaseolus vulgaris*), found a diurnal variation of  $\sigma_{\text{sr}}$  of only 1% as compared to an initial value of  $\sigma_{\text{sr}} \approx 0.98$ . Values of  $\sigma_{\text{sr}}$  were obtained from fits of water and solute flows. Absolute values of  $J_{v_r}$  and  $L_{p_r}$  were larger by an order of magnitude than those given here for *Lotus*. At low gradients of hydrostatic pressure ( $P_{\text{bomb}} = 0-0.2$  MPa), the author found a marked maximum in root  $J_{v_r}$  (as found here). This, however, was referred to diurnal changes in solute flow rather than in  $L_{p_r}$ . In another study on water relations of young roots of *Phaseolus coccineus* (Peters and Steudle 1999), there were also no indications of a diurnal rhythm of  $\sigma_{\text{sr}}$ , but  $L_{p_r}$  and solute pumping into the root xylem and root pressure showed a marked rhythm (as found here). The plants exhibited a root  $\sigma_{\text{sr}} = 0.4-0.6$  (measured for electrolytes such as KCl, NaCl and  $\text{NaNO}_3$  at zero water flow) and values of  $L_{p_r}$  similar to those found here for *Lotus* (Peters and Steudle 1999). Substantial variations in the reflection coefficient have been reported for wheat, barley and maize roots by Schneider et al. (1997). These authors measured the root  $\sigma_{\text{sr}}$  of intact plants in the presence of substantial water flows and rather large salt concentrations in the medium. They found an increase of  $\sigma_{\text{sr}}$  with increasing flow across the roots (transpiration rate). Similar effects may have played a role here because transpiration would vary during the light phase. However, the effects of Schneider et al. (1997) should have been largely due to concentration polarisation effects at the root endodermis caused by the high actual water flow in the presence of high salt concentrations of the medium (unstirred-layer effects; Freundl et al. 1999). In our experiments, absolute rates of water flow were small during the experiments and the external medium dilute. Effects of unstirred layers caused by diurnal changes in transpiration and related effects should also have been small. Hence, from our own and other evidence we conclude that changes in the absolute value of root  $\sigma_{\text{sr}}$  should have been too small to explain changes in  $L_{p_r}$  by a factor of as large as five at fairly constant osmotic gradients during the day.

Absolute values of root  $\sigma_{\text{sr}}$  could not be precisely worked out. This was due to the fact that absolute values of root pressure were quite small (0.02 MPa at maximum) and that errors in its absolute value were rather



large. The value of root  $\sigma_{sr} = 0.25$  used to calculate  $L_{pr}$ , was probably an underestimate of the true value. Hence, the osmotic  $L_{pr}$  was probably overestimated. For other legumes, absolute values of reflection coefficients were substantially larger (see above). For *Phaseolus coccineus*, water flow across the root cylinder was from cell to cell due to a high cell  $L_p$ , and diurnal changes in  $L_{pr}$  should have reflected changes in the  $L_p$  of cell membranes (as proposed here for *Lotus*). Diurnal changes in the root reflection coefficient cannot account for the substantial variations in the rates of water flow which, therefore, should be due to a diurnal variation in  $L_{pr}$ .

Rhythms in root pressure were measured on two individual plants for time intervals of up to 9 d. Root systems attached to a root pressure probe had the cut end of the xylem in a sealed compartment of fixed volume, and the water and solute (nutrient) flow across the system was virtually zero. In an excised root, xylem vessels were open and sap (water and nutrients) was continuously leaking out. When the root pressure probe was attached to an excised *Lotus* root, the increase in root pressure in the morning resulted from solute deposition in the xylem which exceeded the leakage from the system. During the night, the leakage was greater than solute deposition. Hence, the diurnal rhythm of solute pumping caused the diurnal rhythm in root pressure measured with the root pressure probe. Due to the high rigidity of xylem vessels, even a small excess of solute deposition over leakage may have increased the root pressure considerably. However, freely exuding plants diluted the solutes in the xylem so that an increased deposition rate in the morning may not be detected in the xylem sap collected during the experiments. The prolonged period over which variations in root pressure occurred in the present and other reports (e.g. Mees and Weatherley 1957; Parsons and Kramer 1974; for other references see Kramer and Boyer 1995) are remarkable, bearing in mind that root pressure depends on metabolically driven ion pumping. Evidently, the energy source for such pumping (ultimately ATP) was not exhausted for several days after supplies of photosynthate and reducing power from the shoot were cut off. The source of ATP must have been from substrate reserves and from tissue degradation. If the latter was significant, it was also remarkable that the integrity of membranes, critical in maintaining the diffusion barrier between the xylem and the outside solution, was preserved for so long.

The results from the cell pressure probe measurements did not resolve the question concerning the location of the conductance which changed diurnally. However, they did seem to exclude the possibility that changes occurred in the cortical cells in a zone 30–80 mm from the apex of adventitious roots. Beyond this, only speculation is possible. It has not yet been possible to measure  $L_p$  in endodermal cells or more deeply located cells. It is difficult to position the probe precisely in these deeply located cells and their small cross-section makes it even more difficult to puncture them without damaging them. Although they successfully measured

radial profiles of cell turgor in wheat root tissue including the stele, Pritchard et al. (1989) did not measure cell  $L_p$ . In the present study, the magnitude of the changes in volume flow lead to an intuitive conclusion that much of the root surface was involved. There is some precedent for this view in studies of transpiring plants, where it was shown that water fluxes across roots increased in old, fully differentiated root zones to as great, or greater extent as in younger, more permeable zones, when transpiration is increased (Brouwer 1953; Sanderson 1983). It is possible that the variable  $L_{pr}$  is located in cell layers internal to the deepest locations we have studied, i.e. deeper than four cortical layers. The endodermis is obviously of great interest in this regard, but also the cells around the vessels in the stele. For young maize roots, Steudle et al. (1993) showed that puncturing the root cortex and even peeling it off did not increase  $L_{pr}$ . Puncturing the endodermis also did not affect  $L_{pr}$ . However, when larger arrays of the endodermis, including stelar tissue, were affected, there was a strong increase in  $L_{pr}$ . This indicated that the hydraulic resistance was concentrated in the stele rather than in the cortex, at least in the young maize roots used, which were lacking an exodermis and suberin lamellae in the endodermis. These findings support the view that changes in the membrane hydraulic properties may have been expressed in the stele. For geometric reasons (cross-sectional area), they have a bigger effect on  $L_{pr}$  there. So, if the plant manipulates water-channel function to regulate the transport of water, it would be most efficient to do this in the membranes of stelar cells around the vessels.

Interestingly, in-situ hybridisations on *Arabidopsis* roots indicated the endodermis and stele to be the root tissues where the expression of plasma-membrane water-channel proteins is highly increased (Schäffner 1998). Barrieu et al. (1998) localised a maize tonoplast aquaporin *ZmTIP1* in root epidermal, endodermal, and xylem parenchyma cells. Therefore, the idea of whether the diurnal fluctuation of  $L_{pr}$  would correlate with the expression of potential *Lotus* aquaporins was examined. Indeed, it was shown, that transcripts with homology to *Arabidopsis* AthPIP1-type aquaporins can be detected in *Lotus*. Transcripts displayed a diurnal variation that preceded the increase in root  $L_{pr}$  by a few hours. The expression of PIP1-type aquaporins in *Lotus* roots was independently confirmed by the identification of a PIP1-cross-reacting band on Western blots. Thus, we present evidence that *Lotus* homologues of PIP1-type water-channel proteins may contribute to  $L_{pr}$  and that their diurnal fluctuation is correlated with the variation in root  $L_{pr}$ .

Evidence from different sources points to the general importance of root  $L_{pr}$  as a component of a signalling system. Short periods of oxygen starvation, variation in the supply of nutrients (N, P, S) and of NaCl (high salinity) are all characterised by marked changes in the  $L_p$  of roots or root cells (Radin and Eidenbock 1984; Radin and Matthews 1989; Karmoker et al. 1991; Azaizeh et al. 1992; Birner and Steudle 1993; Barthes et al. 1995; Else et al. 1995; Carvajal et al. 1996). It has

been shown that the major phenotypic effects of down-regulating the expression of AthPIP1b in *A. thaliana* are a decrease in root  $L_p$  and cell  $L_p$  and a gross increase in relative size (weight and extent) of the root system (Kaldenhoff et al. 1998). Similar increases in root size have been found frequently in plants which are drought- or N- and P-stressed. Evidently, the morphological response is a long way downstream of the initial perception of stressful conditions, but it implicates disturbances in aquaporin function and cell  $L_p$  as early responses in the chain of events. In future, it will be an obvious challenge to find out how the diurnal variation in root  $L_p$  and aquaporin expression fits into this broader picture of variable root  $L_p$ , which has been known for a long time.

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