RESEARCH PAPER

Water permeability differs between growing and non-growing barley leaf tissues

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Abstract

A pressure probe technique and an osmotic swelling assay were used to compare water transport properties between growing and non-growing tissues of leaf three of barley. The epidermis was analysed in planta by pressure probe, whereas (predominantly) mesophyll protoplasts were analysed by osmotic swelling. Hydraulic conductivity (Lp) and, by implication, water permeability (Pf) of epidermal cells was 31% higher in the leaf elongation zone (Lp=0.5±0.2 μm s⁻¹ MPa⁻¹; Pf=65±25 μm s⁻¹; means ±SD of n=17 cells) than in the, non-growing, emerged leaf zone (Lp=0.4±0.1 μm s⁻¹ MPa⁻¹; Pf=50±15 μm s⁻¹; n=24; P<0.05). Similarly, water permeability of mesophyll protoplasts was by 55% higher in the elongation compared with emerged leaf zone (Pf=13±1 μm s⁻¹ compared with 8±1 μm s⁻¹; n=57 and 36 protoplasts, respectively; P<0.01). Within the leaf elongation zone, a small population of larger-sized protoplasts could be distinguished. These protoplasts, which originated most likely from parenchymatous bundle sheath or midrib parenchyma cells, had a three-fold higher water permeability (P<0.001) as mesophyll protoplasts. The effect on Lp and Pf of known aquaporin inhibitors was tested with the pressure probe (Au⁺, Ag⁺, Hg²⁺, phloretin) and the osmotic swelling assay (phloretin). Only phloretin, when applied to protoplasts in the

swelling assay caused an average decrease in Pf, but the effect varied between isolations. Technical approaches and cell-type and growth-specific differences in water transport properties are discussed.

Key words: Aquaporin, bundle sheath, epidermis, Hordeum vulgare, leaf cell elongation, mesophyll, pressure probe, protoplast, water permeability.

Introduction

Plant organs grow in size through the irreversible expansion of cells. Cell expansion requires the continuous uptake of water, and it is possible that water transport properties of cells hydraulically limit the expansion rate of growing tissues. There exists information in support and against a hydraulic limitation of growth (Steudle, 1985; Cosgrove, 1987; Nonami et al., 1997; Fricke and Flowers, 1998; Tang and Boyer, 2002; Boyer and Silk, 2004). On the one hand, higher plant cells have generally short half-times of water exchange and, by implication, high hydraulic conductivity; this makes a hydraulic limitation of growth unlikely. On the other hand, significant growth-induced water potential gradients exist between plant internal water source (xylem) and peripheral, growing cells; this supports the idea of a hydraulic limitation of growth. These two lines of evidence do not have to be

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contradictory. If water has to pass (through) many cells of high hydraulic conductivity before reaching peripheral tissues, the overall hydraulic conductance can be low, depending on the pathway of water movement. In addition, growing tissues are composed of various cell types, and it is possible that one cell type has particularly low hydraulic conductivity and presents a hydraulic bottle-neck for growth.

Water can move along three major pathways between plant internal water source (xylem) and peripheral elongating cells (epidermis) (Steudle and Peterson, 1998); it can move along the apoplastic and bypass path along its path; it can move from cell-to-cell through plasmodesmata (symplast); or it can move from cell-to-cell by crossing membranes. The latter pathway predominates when the driving force for water movement is osmotic rather than hydrostatic (pressure gradients), and water movement is facilitated through aquaporins. If water transport occurs predominantly through membranes, transport properties of cells and aquaporin function (Kjellbom et al., 1999; Tyerman et al., 1999; Johansson et al., 2000; Luu and Maurel, 2005) become central to the overall rate of water movement and organ growth. There exists no information on water transport properties specific to growing as compared to non-growing leaf cells. The existing information on roots and hypocotyls suggests that both the flow path and hydraulic properties change as cells and tissues develop (Nonami et al., 1997; Ramahaleo et al., 1999; Hukin et al., 2002; Eisenbarth and Weig, 2005).

In barley leaves, growth-induced water potential gradients are significant and growth appears to be limited by hydraulic properties of tissues, at least under certain growth conditions (Fricke and Flowers, 1998). In leaf three, the first leaf, which is entirely dependent on nutrient supply from external sources (rather than endosperm), the elongation zone stretches from about 2 mm to 40 mm from the point of leaf insertion. Maximum cell elongation rates occur between 10–30 mm (Fricke et al., 1997; Fricke, 2004). The portion of the growing leaf which has emerged from the sheaths of older leaves has ceased cell elongation and is, unlike to the elongation zone, transpiring. Of 100 water molecules entering the growing leaf, between 98–99 molecules pass through (xylem) the elongation zone and are lost through transpiration, while only one or two water molecules are used for elongation growth (Fricke, 2002). A water channel belonging to the subgroup of plasma membrane intrinsic proteins, PIP1s, is specifically expressed in the epidermis of the leaf elongation zone. In addition, further, putative water channels are differentially expressed between elongation and emerged zone (W Wei, E Alexandersson, T Miller, D Golldack, PO Kjellbom, W Fricke, unpublished data). Together, these observations suggest that water relations of growing and non-growing barley leaf cells differ from each other or are at least regulated through different mechanisms.

In the present study, two approaches were used to determine the hydraulic conductivity and water permeability of cells of the elongation and emerged leaf zone. (The term ‘water permeability’ is used here in preference to ‘osmotic water permeability’ since it cannot be ruled out that, during pressure probe measurements, some of the water transport across membranes was pressure-driven. Water transport during osmotic swelling assays of protoplasts was driven by osmotic forces, even though this water transport is also referred to as ‘water permeability’). Firstly, the pressure probe technique was used to determine Lp in epidermal cells in the intact, growing plant (Steudle, 1993). This technique is easier to apply to surface tissues and to cells which are highly vacuolated and contain little cytosol which could clog the microcapillary during cell impalement. Secondly, osmotic swelling assays were used to determine Pf in protoplasts isolated from the two leaf zones. The bulk of protoplasts originated from the mesophyll, but a small population of larger-sized protoplasts, which were of different origin, could be distinguished and analysed in the leaf elongation zone. To the best of our knowledge, this is the first study in which protoplasts were isolated from growing leaf tissues.

**Materials and methods**

**Plant material and growth conditions**

All experiments were carried out with barley (Hordeum vulgare L. cv. Golf (Svalöf Weibull AB, Svalöv, Sweden)), on the elongating leaf three. Plants were grown hydroponically, on modified half-strength Hoagland nutrient solution (Fricke and Peters, 2002). Pressure probe experiments were carried out at Paisley, while osmotic swelling assays were carried out at Louvain-la-Neuve. At Paisley, plants grew in a growth chamber (Microclima MC1000HE, CEC Technology, Glasgow, UK). Photosynthetically active radiation at third-leaf level was 350–400 µmol photons m⁻² s⁻¹, relative humidity was 70%, temperature was 21/15 °C (light/dark) and the light/dark period lasted 16/8 h. At Louvain-la-Neuve, plants were grown under similar growth conditions in a growth room. Plant development (time between seed germination and harvest), visual appearance of plants, and final length and elongation velocity of leaf three were comparable at the two locations.

Leaf three was analysed at a developmental stage when it elongated at a (near-) maximum and steady velocity (2.5–3.0 mm h⁻¹). At this developmental stage, plants were 13–15-d-old.

**Pressure probe measurements**

Pressure probe measurements were performed on the elongating leaf three as detailed previously (Fricke and Peters, 2002). Plants were analysed either within the elongation zone, between 10–30 mm from the point of leaf insertion, or half-way along the portion of leaf three which had emerged from the sheath of leaf two. It takes between 1.5 d and 2 d for cells to be displaced from the elongation zone into the emerged leaf zone. The emerged zone is also referred to as ‘mature zone’: although cells are less mature than, for example, cells of a fully-expanded blade, they are in contrast to elongating cells, photosynthesizing and not growing. They may also have secondary walls. Six different batches of plants were analysed for each leaf region.
To access the elongation zone of leaf three, sheaths (and blades) of leaf one and two were removed and the exposed elongation zone was fixed onto a specially-designed holder and covered with moist tissue paper to protect it from drying out. After 10–15 min, pressure probe measurements started. For analysis of the emerged zone, no manipulations on plants were required except for fixing the growing leaf three into a Perspex sample holder. Roots were kept in nutrient solution during measurements. Abaxial epidermal cells were analysed in both leaf zones since these cells were easier to access in the elongation zone. The bulk of volume of the abaxial epidermis is composed of rather large cells, which are either bulliform- or tube-like in shape. The latter cells were analysed in the present study. Typical cell dimensions were: length (L), 500–1000 μm; and width (W) and cross-sectional depth/thickness (D), 20–30 μm. Cell volumes (V) were in the upper picolitre to nanolitre range.

The main determinant of cell volume and potential source of error during volume and surface (S) determinations and calculation of volumetric elastic modulus (e) for the cell-type studied was cell length. The length of cells, which were analysed by pressure probing for elastic modulus, was measured prior to impalement, by using a graticule fixed to a stereomicroscope at ×100 magnification. Width of cells was determined at slightly higher magnifications. Cell depth (cross-sectional ‘thickness’) was deduced from free-hand cross-sections of leaf regions. The depth:width ratio was 0.90±0.06 and 0.90±0.04 in the elongation and emerged zones, respectively (means ± SD of five plants, with 10 cells analysed from each plant). This value was used to deduce depth from measured width of pressure-probed cells.

To calculate volumes, it was assumed that cells were cylinders, with an ellipsoid cross-section of diameters D and W:

\[ V = L \times W \times D \times (\pi/4) \]

(1)

Cell surface, S, was calculated as:

\[ S = \pi \times (D + W) \times L \]

(2)

For simplification, bottom and top areas of cylindrical cells were neglected since these accounted for less than 0.2% of total cell surface. As a result, the volume:surface ratio of cells was effectively dependent on cell width:

\[ (V/S) = 0.118 \times W \]

(3)

Half-times of water exchange (T1/2) were measured by pressure relaxation as described previously (Fricke, 2000; for a detailed description of the method see Fig. 1 and Steudle et al., 1980). Three consecutive cycles of endosmotic and exosmotic water flow were induced. There was no consistent difference between exosmotic and endosmotic water flow, and half-times were combined to calculate the average T1/2 of a particular cell. Only small pressure changes were induced to avoid sudden increases in T1/2 (Cosgrove and Steudle, 1981; Wan et al., 2004). Turgor, T1/2, cell length, and cell width and changes in pressure in response to known changes in cell volume (ΔP/ΔV) were determined for the same cell and used together with inferred cell depth (see above) and previously determined values of cell osmolality (Fricke, 2004), to calculate cell elastic modulus (e=VΔP/ΔV), cell surface and cell hydraulic conductivity (Lp). Changes in cell volume, ΔV, were induced by moving the meniscus of the cell-sap/silicon-oil boundary forward and backward between two marked positions along the microcapillary. The volume of liquid between these two positions was determined repeatedly by sucking in water into the capillary, expressing the equivalent of one filling between marks into a small container of liquid paraffin and measuring (stereomicroscope) the diameter of droplet to calculate droplet volume.

A comparison of turgor and T1/2 between the adaxial and abaxial epidermis of the emerged zone showed that both leaf surfaces had similar turgor and T1/2 (not shown).

The half-time of water exchange of cells was in the second- to sub-second range. Water equilibrated so fast across membranes that this interfered with determination of ΔP/ΔV and e; the pressure change induced by an imposed volume change through the pressure probe was underestimated, since water started to move across membranes before the volume change was completed. It was necessary to correct e-values for fast water flow as described by Steudle et al. (1980). Using equation 13 of Steudle et al. (1980) and substituting (ln2/T1/2) for k (the rate constant of pressure relaxation) the corrected e was calculated as:

\[ \varepsilon_{\text{corr}} = \varepsilon_{\text{meas}} \times \ln(2) \times (t/T1/2) \times (1 - \exp[-\ln(2) \times (t/T1/2)])^{-1} \]

(5)

where t denotes the time required to complete a pressure change prior to the pressure relaxation used to determine T1/2.

Hydraulic conductivity, Lp, was calculated as:

\[ Lp = (V/A) \times \ln(2) \times (T1/2 \times (\varepsilon_{\text{corr}} + OP))^{-1} \]

(6)

where OP is the osmotic pressure of cells (elongation zone, 0.947 MPa; emerged zone, 1.038 MPa; Fricke, 2004).

Hydraulic conductivity was converted into (osmotic) water permeability, Pw, according to Maurel (1997):

\[ Pw = Lp \times R \times T/Vw \]

(7)

where Lp is given in μm s⁻¹ MPa⁻¹, R (general gas constant) is 8.314 K J⁻¹ mol⁻¹ or 0.0082 1 MPa (K mol⁻¹), Vw (partial molal volume of water) is 18 cm³ mol⁻¹, and T (absolute temperature) is 294 K at 21 °C. Using these values, Pt calculated to

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*Fig. 1. Example of a recording of pressure relaxation of leaf epidermal cells (elongated blade) of barley, which was used to determine T1/2, the half-time of water exchange, and ΔP/ΔV for calculation of cell elastic modulus and hydraulic conductivity. Following impalement of the cell, turgor stabilizes quickly (at 1.17 MPa). The pressure–relaxation recording starts with a reduction in cell pressure, which is induced by the pressure probe through moving the cell-sap/silicon-oil meniscus by a known volume (ΔV); this is followed by an endosmotic relaxation (recovery) of turgor. Turgor pressure stabilizes at a value slightly below the original turgor since inflow of water dilutes cell contents. Once turgor has stabilized, cell pressure is increased by moving the sap/silicon-oil meniscus to its original position; this is followed by an exosmotic relaxation of turgor. This procedure is repeated several times, and individual half-times for endosmotic (here: 0.6, 0.7, and 0.8 s) and exosmotic water flow (here: 0.5, 0.6, and 0.6 s) are averaged. The T1/2 of cells is short and it is impossible to change turgor fast enough (‘t’) to obtain a correct ΔP. Instead, ΔP is underestimated and needs to be corrected for (see equation 5). Original traces (oscillating pattern) were fitted by hand to exponential curves for calculation of T1/2.*
$P_f = 133.9 \times Lp \times MPa (\mu m s^{-1})$

Several potential inhibitors of aquaporin activity were tested in their effect on $P_f$. A 2-cm long segment of the emerged-leaf zone was excised (in attempts with low Ag, a 6–7 cm long segment was excised), vacuum-infiltrated with the respective inhibitor solution and analysed after 10–50 min. Inhibitors were tested at the following concentrations: Ag (as AgNO₃), 50 μM, 0.5 mM, and 1 mM; Au (as HAuCl₄), 50 μM, 100 μM, and 1 mM; Hg (as HgCl₂), 0.1–0.9 mM; and phloretin (dissolved in 0.06% DMSO and sonicated), 1.25 mM. Water-infiltrated segments were taken as controls (or 0.06% DMSO in phloretin experiments). ABA at 20 μM was also tested, and leaf tissue analysed within 15–60 min following infiltration.

**Protoplast isolation and osmotic swelling assays**

Protoplasts were isolated according to Moshelion et al. (2004), with some minor modifications. The elongation zone was harvested between 10–30 mm from the point of leaf insertion, the region of highest relative elemental growth rates. Leaf tissue from elongation and emerged leaf zone was cut into 0.1-mm wide sections and transferred into 1 ml of BMS medium (Moshelion et al., 2004) containing 1% cellulose (CEL, Worthington, NJ), 0.5% Driselase (Fluka Biochemika, Buch, CH), 0.05% Pectolyase (Aspergillus japonicus; Sigma), 0.1% BSA, and 0.025% polyvinylpyrrolidone K30 (Fluka). Sorbitol was used to adjust the osmolarity of the protoplast isolation and iso-osmotic medium to between 420 to 470 mOsm kg⁻¹. The small variation in medium osmolality between experiments accounted for 0.5–1 d differences in leaf developmental stage. Bulk osmolality differs by around 20–40 mOsm kg⁻¹ between elongation and emerged zone (Fricke, 2004), and the same isolation medium was used for the two leaf zones, using the (higher) osmolality of the emerged leaf zone as guidance; epidermis and mesophyll osmolality differ little from bulk osmolality (Fricke et al., 1994; Fricke 2004). A small metal rod was used to push leaf tissue underneath the surface of the solution and remove air from intercellular air spaces. Leaf material from 2–3 plants was pooled for each leaf region. Tissue was incubated on a rotary shaker (90 rpm) in the dark at 30 °C for approximately 1 h. The protoplast solution was filtered through a 30 μm mesh and the filtrate pelleted gently by centrifugation (30 s at 90 g) using an Eppendorf bench centrifuge. The supernatant was removed and the pellet resuspended in fresh BMS medium and used as protoplast preparation. Protoplasts were analysed within 6 h of preparation.

Swelling assays and calculation of (osmotic) water permeability were carried out essentially as described by Moshelion et al. (2004). In short, protoplasts were allowed to settle and immobilized in a small chamber that was perfused with solution at a volume flow rate of 4 ml min⁻¹ and viewed under an inverted microscope at magnifications ×200 to ×400; immobilization of protoplasts was achieved by coating the bottom of the chamber with protamine sulphate (Sigma). One swelling run lasted 90 s. During the first 15 s, iso-osmotic medium run through the chamber; at 15 s, media were switched to hypo-osmotic solution for the following 60 s to induce protoplast swelling; at 75 s (15 s+60 s), media were switched back to iso-osmotic solution for another 15 s (15 s+60 s+15 s=90 s=1 run). The system was set up that way that hypo-osmotic solution reached the protoplast location within 2 s following exchange of solutions. Hypo-osmotic media had osmolalities which were by approximately 200 mOsm kg⁻¹ lower than those of the respective iso-osmotic medium. Water permeability, $P_w$, was calculated using the method described in Moshelion et al. (2004) slightly modified to be fully automated and compatible with measuring several protoplasts simultaneously. The image sequence was loaded in the ImageJ software environment (http://rsb.info.nih.gov/ij) and processed with a plugin designed to ease the tracking of protoplasts (‘Protoplast analyzer’ program available from Xavier Draye). Protoplasts area was determined following automated detection and vectorization of their edges. Using the Visual Basic for Applications programming language several macro command files were created that enabled us automatically to import the result files (from ‘Protoplast analyzer’), sort, convert them into average volume time-course graphs, and calculate the $P_f$ through computer fitting of data. Two types of $P_f$ were calculated, $P_f$ at the very beginning of the hypo-osmotic swelling response (referred to as ‘initial $P_f$’ or $P_f$-initial) and $P_f$ towards the end of the hypo-osmotic treatment (referred to as ‘final $P_f$’ or $P_f$-final’). Since $P_f$-final considers the increase in $P_f$ during the swelling, it reflects more the potential hydraulic properties of the membrane (Moshelion et al., 2004). Therefore, this value was used in particular to compare $P_f$ between leaf zones and treatments and is simply referred to as ‘$P_f$’.

In some experiments, $P_f$ was compared between elongation and emerged leaf zone, while in other experiments the effect on $P_f$ of the aquaporin inhibitor phloretin was tested. Phloretin experiments were conducted only on protoplasts of the elongation zone since these stuck better to the assay chamber and gave more measurements per run. Phloretin (Fluka) was dissolved in DMSO at a concentration of 2 M and this solution further diluted with iso-osmotic solution to 1.25 mM phloretin and 0.06% DMSO. This suspension was sonicated for 10 min (on ice) to redissolve (some) phloretin. Undissolved residue was removed by centrifugation (10–15 s at 13 000 g) and the supernatant used as phloretin stock. It is not known how much phloretin was redissolved and 1.25 mM is therefore an upper figure for phloretin concentration. Prior to analyses, protoplasts were incubated in iso-osmotic medium containing, at a final concentration, either 1.25 mM phloretin and 0.06% DMSO (phloretin treatment) or 0.06% DMSO (DMSO control). After 15–30 min, protoplasts were transferred to the swelling chamber. Iso- and hypo-osmotic media which were used to flush the chamber or induce osmotic swelling were devoid of phloretin and DMSO. Protoplasts from ten separate isolations were analysed, from four independent batches of plants (two batches for elongation-zone/ emerged-blade comparison and two batches for phloretin study).

**Statistics**

Statistical significance of differences between treatments was assessed by $t$ test (Excel). The number of replicates and the level of significance is given in tables and text.

**Results**

**Pressure probe analyses**

The elongating leaf three of barley was analysed during a developmental stage when leaf elongation was mainly due to elongation of blade tissue. Cells were analysed in the central portion of the elongation zone and halfway along that portion of the blade which had emerged from the sheath of leaf two.

Almost two-thirds of epidermal cells analysed in the leaf elongation zone had volumes between 100–400 pl, while one third had volumes larger than 600 pl (Fig. 2A). In the emerged leaf zone, most cells had volumes of between 300–1200 pl, and only four cells volumes larger than 2400 pl (Fig. 2B). The average volume of cells was 485 and 1094 pl, emerged-blade comparison and two batches for phloretin study).
through 50% of their elongation, it would have been expected that their size was about half the final size in the emerged blade. 

Cell width differed less between the elongation zone (22 μm) and the emerged zone (28.7 μm) than cell length (Table 1). The volume:surface ratios of cells was 2.59±0.81 μm in the elongation zone compared with 3.39±0.64 μm in the emerged blade (average ±SD of 17 and 24 cells, respectively; P<0.001).

Turgor in the elongation zone was 0.46 MPa, compared with 0.80 MPa in the emerged portion of the leaf (Table 1). Osmolalities of cells were 0.95 MPa (385 mOsm kg⁻¹) and 1.04 MPa (422 mOsm kg⁻¹), respectively, as determined in a previous study (Fricke, 2004). This calculated to cell water potentials of −0.49 MPa and −0.24 MPa in the epidermis of the elongation and the emerged leaf zones, respectively, and confirms earlier reports for the same barley cultivar (Fricke and Peters, 2002).

The half-time of water exchange was in the second- to sub-second range and significantly larger in the elongation compared with the emerged leaf zone (Table 1). It took almost 50% longer in the elongation zone for water to equilibrate across cell membranes following a perturbation of local water potential equilibrium through a sudden change in cell pressure. Part of the difference in $T_{1/2}$ between the elongation and emerged zones was due to differences in volume:surface ratios of cells; the largest part was due to an almost 3-fold difference in cell elastic modulus (3.2 MPa and 9.3 MPa in elongation and emerged leaf zone, respectively; Table 1). Despite the slower half-time in the elongation zone, cell hydraulic conductivity ($L_p$) was by 31% higher than in the emerged zone ($P=0.016$; Table 1). Values were normally distributed in both leaf zones (Fig. 2C).

The short $T_{1/2}$ of cells required correction of $\epsilon$ for rapid water flow during determination of $\Delta P/\Delta V$. Without this correction, $\epsilon$ would have been significantly underestimated, particularly in the emerged leaf zone (Table 1).

Cell elastic modulus increased with cell volume in a linear way (Fig. 3). The relationship was similar for the elongation and emerged leaf zones, but the correlation was higher in the emerged zone ($r^2=0.944$, compared with $r^2=0.669$ in the elongation zone). When the two measurements for cell volumes larger than 1000 pl were not considered in regression analysis of the elongation zone, the regression (slope and intercept) was little affected.

None of the potential inhibitors of aquaporin activity that were tested, Ag, Au, Hg, and phloretin, showed any significant effect on $T_{1/2}$. Abscisic acid, which has been shown to shorten $T_{1/2}$ in maize root cells (Hose et al., 2000), had no effect on $T_{1/2}$ either (not shown).

Osmotic swelling assays of leaf protoplasts

Protoplasts were isolated enzymatically from the elongation and emerged zones of leaf three. In the elongation
Table 1. Summary of pressure-probe and respective anatomical data

Results are given as means ± SD. Seventeen cells were analysed in the elongation zone and 24 cells in the emerged blade. \( Lp \) and \( P_t \) values were rounded off to two digits. The statistical significance of difference in value between elongation zone and emerged blade is indicated by * \((P < 0.05)\), ** \((P < 0.01)\) and *** \((P < 0.001)\).

| Variable                  | Leaf region          |
|---------------------------|----------------------|
|                           | Elongation zone (EZ) | Emerged blade (EmBl) |
| **Turgor (MPa)**          | 0.46±0.10            | 0.80±0.10***          |
| **\( T_{1/2} \)** (s)    | 1.0±0.2              | 0.7±0.3***            |
| **Cell volume (pl)**      | 485±514              | 1094±827**            |
| **Cell surface area (mm\(^2\))** | 0.159±0.097          | 0.324±0.198***         |
| **Cell length (µm)**      | 1139±318             | 1822±913**            |
| **Cell width (µm)**       | 22.0±6.8             | 28.7±5.4***           |
| **\( \varepsilon \) (meas) (MPa)** | 2.0±1.5              | 4.5±2.6***            |
| **\( \varepsilon \) (corr) (MPa)** | 3.2±2.3              | 9.3±4.9***            |
| **\( Lp \) (µm s\(^{-1}\) MPa\(^{-1}\))** | 0.5±0.2              | 0.4±0.1*               |
| **\( P_t \) (µm s\(^{-1}\))** | 65±25                | 50±15*                |

Fig. 3. Dependence on cell volume of cell elastic modulus in epidermal cells of leaf three of barley. Cell elastic modulus was determined using the cell pressure probe and related to volumes of cells. Epidermal cells were analysed in the elongation zone (EZ, filled circles) and emerged blade (EmBl, open circles). Analyses were carried out in planta, and each point represents one cell.

Parenchymateous bundle sheath (PBS) cells also contain chloroplasts. In barley, three types of PBS cells have been distinguished depending on their location within the vascular bundle (Williams et al., 1989). Two of these PBS cell types have fewer chloroplasts than mesophyll cells, whereas the third type, termed L-type by Williams et al. (1989) has chloroplasts similar in size and concentration to mesophyll cells. However, these L-type PBS cells are particularly large, and the difference in volume compared with mesophyll cells is greater than the three-fold difference using average volumes of PBS cell types (Williams et al., 1989; see also Fig. 4D, E). Epidermal protoplasts appear translucent (Fig. 4F, G) and are even less dense than PBS cells and float towards the surface of the isolation medium (not shown). Only those protoplasts which sedimented at 90 g during the isolation procedure were further analysed (see Materials and methods) and this might explain why predominantly mesophyll protoplasts were obtained. In addition, the 30 µm mesh used to filter debris from protoplasts will have restricted the passage of large-sized epidermal and PBS protoplasts.

A total of 36 protoplasts were analysed from the emerged leaf zone. The size distribution of these protoplasts was normal, except for a small number (7) of protoplasts, which had volumes larger than 32 pl (32.2–37 pl; Fig. 5A). The average volume of all 36 protoplasts was 24.4 pl (Table 2). The appearance of protoplasts was uniform and typical of that of mesophyll protoplasts. By contrast, in the leaf elongation zone, the size distribution tailed off significantly towards larger volumes, in particular at volumes >20 pl (Fig. 5A). A total of 63 protoplasts were analysed in the leaf elongation zone, and six protoplasts had volumes larger than 20 pl, including two protoplasts with volumes >40 pl (44 pl and 46 pl). The appearance of these six protoplasts was not uniform and differed from that of the remaining 57 ones: apart from being much larger in volume (average of 31.4 pl compared to 6.5 pl; Table 2), the cytoplasm was more homogenous and chloroplasts were either less abundant or not easy to identify in black-and-white recordings (Fig. 4D, E). Some of these larger-sized protoplasts showed chlorophyll auto-fluorescence (Fig. 4E) while others failed to do so (not shown); those protoplasts without chlorophyll auto-fluorescence were not epidermal since they were not as translucent. Therefore, the most likely origin of the six larger-sized protoplasts was PBS and large-sized cells which are located at the adaxial side of the vascular bundle in midrib parenchyma (MP) tissue (Fig. 4H; see also study by Westgate and Steudle, 1985).

For comparison between the elongation and emerged zones, the 57 normal-distributed protoplasts of the elongation zone were considered since they represented mesophyll, similar to the protoplasts isolated from the emerged zone. The average volume of mesophyll protoplasts analysed from the elongation zone was 6.5 pl and therefore
Protoplasts were isolated from the elongation and emerged leaf zone. The majority of protoplasts used for swelling assays originated from mesophyll (A, B, C, smaller protoplasts in D), but some protoplasts originated from either parenchymateous bundle sheath (PBS) or midrib-parenchyma (MP) tissue (see the two larger protoplasts in D, together with protoplasts in E and voluminous cells in H). Epidermal protoplasts are shown in (F) and (G). Protoplasts were viewed with different optical set-ups: (A, B, D), set-up used for swelling assays (black and white recordings; inverted microscope [Louvain-la-Neuve]); (C, E), light microscope with (C, lower part of E) or without UV-excitation to show chlorophyll autofluorescence (red-yellow); (F, G) inverted microscope used at Paisley for further identification of protoplasts (here: epidermis); and (H), same microscope as in (F) and (G), but cross-section of midrib region of elongation zone of leaf three. Pictures were resized to fit the composed figure. Leaf regions: protoplast volumes were: (A) Elongation zone: 6.5 pl, 5.1 pl, and 4.4 pl; (B) Emerged zone: 23.3 pl and 24.7 pl; (C) Emerged zone: 24 pl; (D) Elongation zone: 46.3 pl and 27.5 pl (the two bigger protoplasts) and 6.1 pl and 8.5 pl (two smaller protoplasts at top left); (E), Elongation zone: 36 pl – the two pictures depict the same PBS or MP protoplast, with or without UV-excitation to visualize chlorophyll autofluorescence; (F) Elongation zone: 291 pl and 246 pl; (G) Emerged blade: 579 pl; and (H) Elongation zone: parenchyma cells [right half of picture] with volumes ranging from around 35 pl to 200 pl.

Fig. 4. Visual appearance of protoplasts isolated from different developmental regions and tissues of the developing leaf three of barley. Protoplasts were isolated from the elongation and emerged leaf zone. Water transport in growing grass leaf cells

only 26% the volume of protoplasts in the emerged zone (Table 2). A value of 50% would have been expected if protoplasts had been isolated from about 20 mm from the point of leaf insertion, halfway through the elongation zone. Leaf segments used for protoplast isolation were cut from 10–30 mm from the point of leaf insertion, and the volume data suggest that most protoplasts, which were analysed from the elongation zone originated from between 10–20 mm, possibly because of less-mature and more easy-to-digest cell walls. It is also possible that there was some bias towards easier attachment of smaller elongation-zone protoplasts to the chamber used for swelling assays.

The (osmotic) water permeability towards the end of the 45 s swelling period of (mesophyll) protoplasts isolated from the elongation zone was 13 \( \mu \text{m s}^{-1} \) and by 55% larger than the water permeability in the emerged zone (\( P <0.01; \) Table 2; \( P_1 \) values rounded off to two digits). A similar difference was observed for \( P_{f} \) between the two leaf zones was due to a larger proportion of (mesophyll) protoplasts with \( P_{f} \) higher than 12.5 \( \mu \text{m s}^{-1} \) in the elongation zone (Fig. 5B). There was no apparent dependency on cell volume of \( P_{f} \) (Fig. 5C; the six largest protoplasts in the elongation zone were not mesophyll but PBS or MP protoplasts). Protoplasts from both leaf zones responded in swelling with the same delay (c. 15 s) to hypo-osmotic environment (Table 2).

Five independent protoplast isolations were carried out, and the difference in \( P_{f} \) between leaf zones was apparent from swelling curves (Fig. 6A–D; one isolation is not shown since only two protoplasts of the emerged-leaf region survived the switch back, from hypo- to iso-osmotic medium). Elongation-zone protoplasts increased in volume more than emerged-zone protoplasts (see also Table 2) and behaved more like perfect osmometers. After the switch back to iso-osmotic solution (at 60 s), protoplasts started to shrink, at a rate similar to or lower than that during swelling. The shrinking response of protoplasts of the emerged zone was less consistent and protoplasts often burst as hypo-osmotic solution was changed back to iso-osmotic solution.

In the elongation zone, water permeability of PBS and MP protoplasts averaged 35 \( \mu \text{m s}^{-1} \) (Table 2). This was almost three times the permeability of mesophyll protoplasts (Table 2). Water permeability of PBS and MP protoplasts varied considerably, ranging from 10 \( \mu \text{m s}^{-1} \) to 83 \( \mu \text{m s}^{-1} \) (Fig. 5B). The delay in swelling was similar for the different types of protoplasts (Table 2).

The effect on \( P_{f} \) of the potential aquaporin inhibitor phloretin (Moshelion et al., 2004) was tested. Three independent experiments were carried out. Since DMSO had to be used to solubilize phloretin, an appropriate DMSO-control was run in parallel. This control gave similar results as the minus-DMSO control (not shown). Incubation of protoplasts with phloretin prior to swelling produced variable results (Table 3), with an average
reduction in $P_f$-final of 25%. By contrast, initial $P_f$ was reduced by more than 50% in all isolations. Phloretin reduced $P_f$ on average through an increase in the number of low-$P_f$ ($P_f$-final) and decrease of high $P_f$ ($P_f$-initial) protoplasts (Fig. 7).

**Discussion**

The present study provides, for the first time, a comparison of (osmotic) water permeability between growing and non-growing leaf tissues. Two methods were employed, the pressure probe and an osmotic swelling assay; two ‘organizational’ levels were studied, cells *in planta* and protoplasts *in vitro*; and between three to four different leaf tissues were analysed, mesophyll, epidermis, and PBS and/or MP cells. In addition, the effect on $P_f$ of potential aquaporin inhibitors, in particular phloretin, was tested.

The data lead to several conclusions. (i) Water permeability is significantly higher in growing compared with non-growing leaf tissues. The difference amounts from 31% to 55%. (ii) Both epidermis and mesophyll show higher $P_f$ in the elongation compared with the emerged leaf zone. (iii) The difference in $P_f$ between the epidermis and the mesophyll either reflects a true difference in $P_f$ between tissues or results from differences in experimental approach (pressure probe versus osmotic swelling assay); and (iv) barley leaf protoplasts respond to a hypo-osmotic (200 mOsm kg$^{-1}$) environment with a delay in swelling, whereas cells *in planta* respond to local perturbations in water potential instantaneously.

**Growing versus non-growing leaf tissues**

The elongation zone of leaves was analysed, but does this imply that cells or protoplasts were actually growing during analyses? During pressure probe measurements, the growing leaf three kept elongating while on the probe stage as shown by the bending of the inserted microcapillary or by displacement of marks while viewing under the stereomicroscope (not shown; see also Fricke and Peters, 2002). Although this shows that at least some cells were elongating, it does not necessarily mean that the cell which was probed was elongating. Following impalement of a cell, pressure in the probe chamber is raised within seconds to match turgor. The speed of adjustment is a reflection *per se* of a short $T_{1/2}$. It is difficult to envisage how within the space of a few seconds, $T_{1/2}$ changes significantly from a value in

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Fig. 5. Volume and (osmotic) water permeability ($P_f$) of barley leaf protoplasts analysed by swelling assays. (A) Frequency distribution of volume of protoplasts of the elongation zone (EZ, black bars) and emerged-blade (EmBl, grey bars) of leaf three of barley. (B) Frequency distribution of $P_f$ of protoplasts of the elongation zone (EZ 57), 57 mesophyll protoplasts, open bars; EZ 6, six bundle sheath or midrib-parenchyma protoplasts, hatched bars, and emerged-blade (EmBl, crossed bars). (C) Dependence on protoplast volume of $P_f$ in elongation zone (EZ, filled circles) and emerged blade (EmBl, open circles). Data from five independent experiments were pooled. A total of 36 protoplasts were analysed in the emerged blade. In the elongation zone, 63 protoplasts were analysed; based on visual appearance, 57 protoplasts (referred to as ‘EZ(57)’ in (B)) were classified as mesophyll, while the remaining six protoplasts originated either from parenchymatous bundle sheath or midrib-parenchyma tissue (referred to as ‘EZ(6)’ in (B)). These bundle-sheath and midrib protoplasts were larger in volume than the remaining 57 protoplasts and are the six protoplasts of the EZ with largest volume shown in (A) and (C).
a previously growing cell. Therefore, it appears justified to relate pressure-probe data of the elongation zone to growth.

For osmotic swelling assays, the situation was different. Firstly, protoplasts rather than cells were analysed and, secondly, this was done at least 1 h following dissection of growing tissue. It is not known whether protoplasts, which were isolated from the elongation zone were still growing, nor is it known whether these protoplasts had retained (some) growth potential and their metabolic activity. Regardless of this uncertainty, the swelling experiments clearly show that protoplasts isolated from the elongation zone of leaves retain some characteristics during the isolation procedure that renders their osmotic water permeability higher than that of protoplasts isolated from non-growing, emerged, leaf tissue.

Both epidermal cells and mesophyll protoplasts had higher water permeability in the elongation compared with the emerged leaf zone. In epidermal cells, the half-time of water exchange in response to pressure-induced flows was slower, not faster, in the elongation compared with the emerged leaf zone. This was due to a three-times lower cell wall elastic modulus in the elongation zone ('looser' walls). Therefore, disturbances in local water potential equilibrated faster in the emerged zone, despite a less water-conductive plasma membrane.

### Table 2. Summary of osmotic swelling data for elongation zone, EZ, and emerged blade, EmBl

Results from five protoplasts isolations, each consisting of an EZ and EmBl fraction isolated from the same leaves, were pooled; a total of 63 and 36 protoplasts were analysed of the EZ and EmBl, respectively; for the elongation zone, only the 57 normal distributed (in size) protoplasts were considered for statistical comparison with the EmBl; protoplasts originated from mesophyll. Protoplasts of the elongation zone were classified into two groups according to size and visual appearance: an average-sized fraction (protoplast volume <20 pl; *n*=57 protoplasts) and a larger-sized protoplast fraction (protoplast volume >20 pl; *n*=6 protoplasts). The former fraction represents mesophyll and the latter fraction most likely parenchymatous bundle-sheath and midrib-parenchyma protoplasts. The terms 'P_f-initial' and 'P_f-final' refer to the (osmotic) water permeability of protoplasts at the beginning and end of swelling induced by hypo-osmotic media. *P_f* values were rounded off to one digit. Results are given as means±SE.

| Variable                              | Leaf region                  | Elongation zone (EZ) | Emerged blade (EmBl) |
|---------------------------------------|------------------------------|----------------------|----------------------|
| Volume <20 pl                         | 13±1                         | 35±11                | 8±1**                |
| Volume >20 pl                         | 3±0                          | 2±1                  | 2±0*                 |
| Delay in swelling (s)                 | 15.0±0.8                     | 17.9±1.2             | 14.8±1.0             |
| Protoplast volume (pl)                | 6.5±0.4                      | 31.4±4.5***          | 24.4±1.1***          |
| Volume increase of initial (%)        | 16.0±1.2                     | Not                  | 7.2±0.7***           |

**Fig. 6.** Osmotic swelling and shrinking curves of protoplasts isolated from leaf three of barley. The elongation zone (EZ, filled circles) and emerged-blade (EmBl, open circles) of the growing leaf three was analysed. Data from four independent experiments (A–D) are shown, and between 6 to 20 protoplasts were recorded during an experiment for one particular leaf zone; error bars represent standard errors. Protoplasts were immobilized in a Perspex chamber and viewed under an inverted microscope while being bathed in isotonic solution, which run through the chamber at a cross-sectional volume flux rate of 4 mm s⁻¹. At time zero, recordings started. At 15 s, the solution was switched to hypo-osmotic, and it took about 2 s for this solution to reach protoplasts. Protoplasts were exposed to hypo-osmotic medium until 60 s, when the solution was switched back to iso-osmotic. At 90 s, recordings terminated. Regressions which were fitted to curves between 40 s and 60 s and which were subjected to covariance analyses gave statistical differences in slopes (and *P_f*) between EZ and EmBl for each experiment at *P* <0.0001.
Table 3. Summary of osmotic swelling data where the effect of the putative aquaporin inhibitor phloretin was tested

Protoplasts were isolated from the elongation zone of leaf three of barley. The stock solution of phloretin was dissolved in DMSO, sonicated and tested at a final concentration of 1.25 mM phloretin and 0.06% DMSO. There was no apparent effect of DMSO on $P_f$ (not shown). Three independent protoplast isolations were carried out. The number of protoplasts analysed of each protoplast preparation and treatment is given in parenthesis. $P_f$ values were rounded off to one digit. Results are given as means ±SE.

| Variable | Isolation | Treatment |
|----------|-----------|-----------|
| $P_f$-final (µm s⁻¹) |            | DMSO control | Phloretin |
| 1        | 16±4 (11)  | 15±4 (19)   |
| 2        | 17±2 (12)  | 15±3 (11)   |
| 3        | 14±5 (8)   | 2±1* (10)   |
| 1–3 pooled | 16±2 (31)  | 12±2 (40)   |
| $P_f$-initial (µm s⁻¹) | 1        | 6±3 (11)   | 3±1 (19)   |
| 2        | 9±2 (12)   | 3±1* (11)   |
| 3        | 2±1 (8)    | 0±0 (10)    |
| 1–3 pooled | 6±1 (31)  | 2±0** (40) |

The values for cell elastic modulus reported here for barley leaf epidermal cells are within the range of values reported previously for plant tissues (e.g. roots; Jones et al., 1983; Miyamoto et al., 2002; leaves; Steudle et al., 1980; Tomos et al., 1981; Westgate and Steudle, 1985; Thomas et al., 1989; and hypocotyls; Cosgrove and Steudle, 1981). Differences in cell elastic modulus between elongating and mature tissues have been reported for corn roots (Hukin et al., 2002), pea roots (Miyamoto et al., 2002), castor bean hypocotyls (Eisenbarth and Weig, 2005), and leaves of Lolium temulentum (Thomas et al., 1989). The data support the idea that walls of growing cells are less rigid than walls of mature cells and also show that growing tissues have a higher storage capacity for water ([=V/(ɛ+OP)], Steudle et al., 1980) than mature tissues. This aids growth and response to stress in two ways: less driving force (MPa, water potential gradient) is consumed per unit uptake of water into cells; and growth becomes partially buffered to sudden changes in external water availability, since more water needs to be lost from cells per unit water potential change.

Cells versus protoplasts

Using the pressure probe, cells forming part of a tissue (epidermis) were analysed as opposed to protoplasmic units in isolation. There exists no information as to the symplastic continuity or discontinuity of the leaf epidermis of grasses. Indirect evidence suggests that in the mature blade, symplastic water transport contributes insignificantly to cellular water exchange (Fricke, 2000).

When iso-osmotic media were replaced by hypo-osmotic media, protoplasts started to swell with a delay of about 15 s, yet it took only 2 s for hypo-osmotic solution to reach the protoplast chamber. It is puzzling why protoplasts failed to swell for at least 13 s in a hypo-osmotic environment. This phenomenon has been discussed in detail recently (Mosshelion et al., 2004), and the most favoured explanation for the delay in swelling is a transition from a low- to a highly water permeable plasma membrane. Notably, when media were switched back from hypo-osmotic to iso-osmotic, the rate of protoplast swelling decreased and shrinking commenced with a much shorter delay (typically 2–3 s; Fig. 8). This appears to rule out unstirred-layer (USL) effects as cause...
for the 15 s delay, in particular, since USL would have been expected to interfere through sweep-away effects (Ye et al., 2006) particularly during the shrinkage response. Also, interference by USL has been ruled out previously for protoplasts showing $P_t$ values less than 400 $\mu$m s$^{-1}$ (Ramahaleo et al., 1999), like the present ones. The following consideration further supports this conclusion. The thickness of the USL can be calculated, based on water volume flux rates (m$^3$ m$^{-2}$ s$^{-1}$), osmolyte concentrations in bulk solution and close to the membrane, and diffusion coefficient of the (main) osmolyte (equation 1 of Ye et al., 2006). The diffusion coefficient of sorbitol is in the range of that of small molecular weight sugars ($c. 10^{-9}$ m$^2$ s$^{-1}$). If we take a protoplast of 20 pl volume (with radius 16.8 $\mu$m and surface area $3.55 \times 10^3$ $\mu$m$^2$) and assume that a 5% increase in volume during 5 s following change in bulk osmolality at protoplast location would have remained non-detectable by the imaging techniques used (the resolution was far better than 5%; see Fig. 8), the volume flux would have amounted to 0.056 $\mu$l ms$^{-1}$. If sweep away effects caused osmolyte concentrations next to the external surface of plasmamembrane to remain near iso-osmotic after the switch to hypo-osmotic medium (and cancel out entirely any osmotic driving force), the USL would have had to be 9.7 mm thick. That is more than the depth of the protoplast chamber. Similarly, if we take the above scenario and assume that the thickness of the USL was in the range of protoplast dimensions, 20 $\mu$m (a more realistic value for USL would be an upper limit of 1 $\mu$m), the water volume flux necessary to build up near iso-osmotic concentrations next to the external surface of plasmamembrane would have amounted to 506 pl over 5 s. This would have been a 25-fold increase in protoplast volume and, apart from surely being detectable, would have burst even the most resilient protoplast.

An alternative explanation for the presence of a large delay in swelling response (but not in shrinking response) could be that switch from iso-osmotic to hypo-osmotic medium does not transmit sufficiently rapid to the bottom of the protoplast chamber (where the protoplasts stuck to) due to the lower density of the hypo-osmotic medium. However, this explanation can be ruled out. It was regularly observed during the switch of media that particles and protoplasts were flushed along the bottom of the chamber, and at predicted wash rates (4 mm s$^{-1}$).

There is a noticeable difference between the $T_{1/2}$ response of pressure-probed cells and the osmotic-swelling response of protoplast location at 62 s (dashed line in C). At 90 s, recordings terminated. Data of five representative protoplasts are shown and each symbol represents a different protoplast. Note also, that the ‘baseline’ of protoplast volume prior to commencement of swelling in (B) varied by less than 1%, which reflects the ‘noise’ of method used for volume determination.

![Fig. 8. Osmotic swelling and shrinking curves, and associated delays in volume changes, of five protoplasts isolated from the elongation zone of leaf three of barley. The entire time-course of swelling and shrinking is shown in (A), whereas (B) and (C) show the swelling and shrinkage response at higher graphic resolution to highlight the difference in delay between swelling (B; c. 15 s delay) and shrinking (C; c. 2–3 s delay). Protoplasts were immobilized in a Perspex chamber and viewed under an inverted microscope while being bathed in isotonic solution, which run through the chamber at a cross-sectional volume flux rate of 4 mm s$^{-1}$. At time zero, recordings started. At 15 s, the solution was switched to hypo-osmotic, and it took about 2 s for this solution to reach protoplast location (as indicated by dashed line in B). Protoplasts were exposed to hypo-osmotic medium until 60 s, when the solution was switched back to iso-osmotic, and the change in osmolality reached](https://academic.oup.com/jxb/article-abstract/58/3/377/555201)
of protoplasts: although $T_{1/2}$ may increase greatly when inflicted pressure changes are too high (through gating of aquaporins, Wan et al., 2004), water movement commences instantaneously, without delay. The three properties that differ between cells and protoplasts, and may have most relevance in explaining the difference in delay, are membrane tension (higher in protoplasts), interface between plasma membrane and cell wall (absent in protoplasts), and metabolic activity (likely to be affected by slightly hypoxic conditions in protoplasts).

**Aquaporin inhibitors**

None of the potential inhibitors of aquaporin activity tested produced a significant effect on epidermal cells *in planta*. It is possible that water transport through barley leaf epidermal cells does not occur through aquaporins, or at least not through aquaporins which can be inhibited by the chemicals applied. Notably, aquaporin inhibitors have been tested successfully only on organs (roots; Zhang and Tyerman, 1999; Hose et al., 2000; Hukin et al., 2002) or cells (e.g. giant algae; Ye et al., 2004) submerged in liquid medium, avoiding any difficulties arising from vacuum-infiltration of tissues (e.g. formation of local precipitates, flooding of intercellular airspace causing hypoxic conditions, and binding to wall matrix) as attempted here.

Phloretin was the only potential aquaporin inhibitor tested on isolated protoplasts. There was a clear tendency towards inhibition of water transport, in particular concerning $P_f$-initial (see also Moshelion et al., 2004). $P_f$-initial reflects the activity of aquaporins present in the plasma membrane at the beginning of experiment. Possibly, these aquaporins were better accessed (and inhibited) during pre-incubation with phloretin. The variation in response may be due partly to difficulties in applying an inhibitor that is poorly soluble in aqueous media and at unknown concentration.

**Accuracy and resolution of osmotic-swelling assays**

The main factor contributing to systematic errors in osmotic swelling assays and calculation of $P_f$ is accuracy of measurements of (changes in) volume (see also Ramahaleo et al., 1999). The accuracy of the ‘protoplast analyser’ program was determined by measuring the radius of eight glass beads with known radius 7.5 μm (see also Moshelion et al., 2004). (The beads, which have been designed for confocal laser scanning microscopy, in particular for confirming optical sectioning thickness, were purchased from Molecular Probes, Eugene, OR, catalog no. F7235; the company’s official statement concerning the accuracy of bead diameters is ‘The nominal bead diameters are 15 μm. The bead diameters have very small coefficients of variation, typically <3%). The measured radius averaged 7.68 μm, which deviates 0.18 μm (2%) from the predicted value, and the standard deviation was 0.02 μm, less than 0.5% of the mean. The radius of protoplasts analysed of the elongation zone was in the range 9–15 μm, averaging 11.4 μm. The average percentage increase in protoplast volume was 16%. For an average protoplast of 11.4 μm radius, this corresponds to an increase in volume by 0.99 pl, from 6.20 pl to 7.19 pl. An error in the determination of radius of 0.18 μm (see above) amounts to an error in determination of volume increase of 0.37 pl. Therefore, the relative error in determination of $P_f$ was $(100\times(0.37)/(0.99))=37\%$. Similar reasoning can be applied to protoplasts of the emerged leaf zone (mean radius, 17.8 μm; relative increase in volume, 7.2%; increase in volume 1.7 pl; error in determination of increase in volume, 0.68 pl), and the relative error in determination of $P_f$ was 40%. These errors are smaller than the 55% difference in $P_f$ between emerged blade (lower $P_f$) and elongation zone (higher $P_f$) and therefore do not interfere with the basic conclusion of the present study. (If the error in bead diameter determination by the supplier is taken into consideration, leading to possible true bead radii of 7.3 μm and 7.7 μm, respectively, this would have meant that our determination of bead radius deviated by 0.38 μm and 0.02 μm, respectively, from the true value. Even if there had been a systematic overestimation in radii by 0.38 μm, water permeability was calculated from slopes of relative-volume versus time curves, not from end-point volume values, this overestimation would have led to a larger overestimation of volume increase (and water permeability) in the larger-sized protoplasts of the mature zone than for the smaller-sized protoplasts of the elongation zone. In that case, the ‘true’ difference in swelling rates (relative volume increase with time) between elongation zone (higher swelling rate) and emerged blade (lower swelling rate) would have been even larger than reported here).

Based on Rayleigh’s criterion, the spatial resolution of optical instruments is limited to about 0.5–1.0 μm, due to the wavelength of light and aperture. Rayleigh’s criterion deals with the discrimination between two neighbouring features in the image signal when these features become so close that the effect of diffraction in the image signal makes it impossible to deconvolute the signal and extract each feature’s signal. However, in the present study, discrimination was not an issue because the object (protoplast image on screen) was a single, isolated object which contrasted clearly with the background of the image. For determination of the (change in) area (and volume) of the protoplast image, it was necessary to estimate the position of a feature, namely the border of the protoplast. The protoplast (image) was split into 16 sectors (like a pie; each sector with 22.5° angle). In each sector, the most peripheral 5–10 pixels were considered (for a protoplast with a radius of 10 μm, this makes 170–340 pixels) to estimate the average intensity level as a function of distance from the centre. Using a spline function, the highest peak signal was located and was considered as the border (in that sector). The standard error (‘noise’) in estimating the position of the border (protoplast edge) for a protoplast of, for example,
10 μm radius 0.01 μm (0.01%) and negligible compared to the error in determination of the radius of the protoplast (see above). The borders of the 16 sectors were connected with a spline to estimate the area of the protoplast, from which an equivalent radius (and volume) could be computed. Therefore, the resolution which was achieved in locating the border was much better than Rayleigh’s criterion.

**Differences in Pf between leaf cell types**

The present study provides data on water permeability for different cell types of barley leaves. More protoplasts of the parenchymateous bundle sheath (PBS) and midrib parenchyma (MP) need to be analysed, and isolated from well-defined leaf tissue (e.g. midrib) to verify these preliminary data. The much higher Pf in epidermal cells compared with mesophyll protoplasts either represents a true difference between tissue types or results from comparison of Pf between intact cells (epidermis) and isolated protoplasts. Zhang and Tyerman (1999) compared hydraulic conductivity of maize root cells, which was determined with the pressure probe, to that predicted from Lp of isolated plasma membrane and tonoplast vesicles. The authors concluded that Lp of isolated membranes is 3–10 times lower than in intact cells and hypothesized that this may be due to metabolic regulation (of water channels). The same may apply to protoplasts and it is possible that study of vesicles and protoplasts leads to a general underestimation of Lp in intact cells. Similarly, the use of different organizational systems could also explain why there was no delay in osmotic response in cells but there was in protoplasts.

Differences in water permeability between cell types have been reported for epidermal and cortex tissue of growing pea epicotyl segments (Cosgrove and Steudle, 1981) and for midrib tissue of mature maize leaves (Westgate and Steudle, 1985). In the latter study, cells similar to MP cells were analysed and almost 10-fold difference in Lp (and water permeability) were observed between cells located closer to the adaxial or abaxial leaf surface. This could, in part, explain the large variation in Pf observed for PBS or MP protoplast in the present study. It remains to be shown to which degree the difference in Pf between tissues is reflected in cell-type specific expression or regulation of water channels (Chaumont et al., 2001; Johanson et al., 2001) and relates to cell function.

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