Differential Ion Accumulation and Ion Fluxes in the Mesophyll and Epidermis of Barley[1]

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In barley (*Hordeum vulgare* L.) leaves, differential ion accumulation commonly results in inorganic phosphate (Pi) being confined to the mesophyll and Ca^{2+} to the epidermis, with preferential epidermal accumulation of Cl^-, Na^+, and some other ions. The pattern was confirmed in this study for major inorganic anions and cations by analysis of barley leaf protoplasts. The work focused on the extent to which differences in plasma membrane ion transport processes underlie these observations. Ion transport across the plasma membrane of barley epidermal and mesophyll protoplasts was investigated electrophysiologically (by microelectrode impalement and patch clamping) and radiometrically. Data from both approaches suggested that similar types of ion-selective channels and membrane transporters, which catalyze the transport of Ca^{2+}, K^+, Na^+, and Pi, exist in the plasma membrane of the two cell types. In general, the simple presence or absence of ion transporters could not explain cell-type-specific differences in ion accumulation. However, patch-clamp data suggested that differential regulation of instantaneously activating ion channels in the plasma membrane could explain the preferential accumulation of Na^+ in the epidermis.

Inorganic ions commonly referred to as "macronutrients" (Marschner, 1995) play central roles in several aspects of plant cell biology as substrates for metabolic assimilation (inorganic phosphate [Pi], NH_4^+, NO_3^-, and SO_4^{2-}), as a generator of intracellular osmotic pressure (K^+), or as a component of signaling pathways (Ca^{2+}). Ions acquired in excess of immediate requirements are accumulated and stored in vacuoles, alongside some "micronutrients" such as Na^+ and Cl^-, which can provide a significant additional component to the intracellular osmotic pressure. Due to their large dimensions in mature cells, vacuoles normally account for the dominant proportion of the total intracellular ionic content. However, it is well documented for a number of species that ions are not uniformly distributed between different plant tissues. The processes leading to the accumulation of ions in specific cell types are largely unexplained, particularly in terms of the underlying membrane transport processes.

Differential compartmentation of inorganic ions between the mesophyll and the upper and lower epidermis of plant leaves has been reported in a range of monocotyledonous and dicotyledonous species, including sorghum (Boursier and Läuchli, 1989), lupin (Treeby et al., 1987), fava bean (Outlaw et al., 1984), *Commelina communis* (Willmer et al., 1974), wheat (Malone et al., 1991), and barley. In barley, the pattern of accumulation of inorganic ions in the mesophyll and epidermis has been particularly well documented by measuring intracellular ion concentrations using x-ray microanalysis (Leigh et al., 1986; Leigh and Storey, 1993; Williams et al., 1993), protoplast analysis (Dietz et al., 1992b), and by extraction and analysis of sap from single cells (Fricke et al., 1994a). The basic pattern of ion accumulation revealed by the three techniques is remarkably consistent, with Ca^{2+} and Cl^- preferentially accumulated in the epidermis, and Pi dominantly in the mesophyll. Na^+ is also preferentially accumulated by the epidermis (Fricke et al., 1994a, 1996). The observation that this pattern of ion accumulation is preserved in the face of a fluctuating ion supply (Dietz et al., 1992b; Fricke et al., 1996) suggests that cell-type-specific ion accumulation is maintained actively by the leaf, perhaps reflecting cell-specific solute requirements or protection of biochemical pathways from interfering ions.

The mechanisms underlying differential ion accumulation are unknown. The membrane transport processes and pathways for solute movement through the leaf have been discussed with respect to their potential involvement in differential ion accumulation by mesophyll and epidermal cells (Leigh and Tomos, 1993). The movement of ions from xylem apoplast to specific leaf cell vacuoles might be controlled at multiple sites. One site at which differential control of ion accumulation might be exerted is the plasma membrane. Although the basic characteristics of ion transport at the plasma membranes of plant cells are now well understood (Sanders and Slayman, 1989), only one study has attempted to relate transport system activity in specific leaf tissues to their differential solute accumulation profiles (Dietz et al., 1992b). Furthermore, this study limited itself to investigation of Cl^- and SO_4^{2-} uptake. In contrast intriguing tissue-specific differences in plasma membrane transport activity are emerging that are postulated to play a role in ion transfer across the cortex and stele of roots (Roberts and Tester, 1995; Maathuis et al., 1998).

To assess the role of plasma membrane ion transport in the control of differential ion accumulation, we aimed to

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elucidate the presence and activity of transport systems involved in the uptake of some ions. Using as our model the primary leaves of barley, which are relatively well characterized in terms of differential ion accumulation, transport of Ca^{2+}, Pi, K^+, and Na^+ across the plasma membrane of mesophyll and epidermal cells was examined.

MATERIALS AND METHODS

Growth of Plant Material

Seeds of barley (*Hordeum vulgare* L. cv Puffin) were grown in washed grit-sand at 22°C/18°C (day/night) with a 14-h photoperiod and 200 to 350 mmol m^{-2} s^{-1} photosynthetically active radiation. Relative humidity was maintained at 60% to 80%, and the pots were watered daily with sufficient nutrient solution to wet the gravel. The nutrient solution contained 9 mM KNO_3, 1 mM KH_2PO_4, 1 mM $CaCl_2$, 1 mM $Ca(NO_3)_2$, 1.5 mM $MgSO_4$, 0.1 mM FeNaEDTA, 0.1 mM NaCl, plus trace elements (23 μM H_3BO_3, 10 μM $MnSO_4$, 0.7 μM $ZnSO_4$, 0.25 μM $CuSO_4$, and 0.65 nM $[NH_4]_6Mo_7O_{24}$), pH 5.5. Plants were harvested after 8 d of growth, by which time the primary leaf was fully expanded.

Chemicals

All chemicals were obtained from Sigma (Poole, Dorset, UK) unless otherwise stated.

Protoplast Isolation

Protoplasts were released by complete enzymatic digestion of primary barley leaves (harvested at the leaf base and stripped of their lower epidermis), in isolation medium (0.5 M sorbitol, 10 mM 2-[N-morpholino]-ethanesulfonic acid [MES] [BDH, Poole, Dorset, UK], 1 mM $CaCl_2$, brought to pH 5.5 with NaOH) containing cellulysin (0.7% [w/v]), macerozyme (0.4% [w/v]; both supplied by Calbiochem, Beeston, Nottingham, UK), pectolyase (0.025% [w/v]; Yakult Pharmaceutical, Tokyo), and bovine serum albumin (1% [w/v]). Digestion was carried out at 28°C for 2.5 h. Mesophyll protoplasts (heavier) were separated from epidermal protoplasts (lighter) by flotation using the method described by Dietz et al. (1992a), with slight modifications in the composition of the sorbitol isolation medium (described above) and glycinebetaine solution (0.5 M glycinebetaine, 20 mM MES, 1 mM $CaCl_2$, brought to pH 5.5 with NaOH), and by underlayering the protoplast suspension with a Percoll solution (0.5 M sorbitol and 20 mM MES dissolved in Percoll, approximately pH 6.0). The protoplast fractions were collected and further purified separately on step gradients according to the method of Martinoia et al. (1986) for the mesophyll, and Dietz et al. (1992a) for the epidermis. The purified protoplast fractions were diluted, if necessary, to a protoplast density of 10^6 mL^{-1}. The membrane integrity and viability of the isolated protoplasts were confirmed using several different stains, including the cytoplasmic stain fluorescein diacetate.

Analysis of Protoplast Ion Contents

Protoplast suspensions were prepared for ion analysis by sonication (mesophyll) or by freeze/thawing and the addition of Triton X-100 (1% [v/v] Triton X-100:epidermis). Success of extraction was determined by viability staining. Aliquots of the disrupted protoplast suspension were diluted in 5% (v/v) HCl for cation analysis or in de-ionized water for anion analysis. Protoplast extracts were analyzed for cations (K^+, Na^+, and Ca^{2+}) by inductively coupled plasma emission spectroscopy, and for anions (Cl^-, NO_3^-, and Pi). The Pi concentration was determined by spectrophotometric detection after reaction with Mo^{VI} and Sb^{III} in acidic medium, followed by reduction with ascorbic acid using a continuous-flow air-segmented system (AAII, Technicon, Autoanalyzer, Pulse Instrumentation, Saskatchewan, Canada). The Cl^- concentration was measured by the detection of the colored $[Fe(SCN)]^{2+}$ complex formed when Cl^- displaces SCN^- from its mercuric compound in the presence of ferric ammonium sulfate. The NO_3^- concentration was determined by reduction to nitrite (using a cadmium coil), followed by diazotization with sulfanilamide and coupling with N-1-naphthylethylenediamine to produce a pink azo dye. The latter two ions were detected using a rapid flow analyzer (Alpkem RFA/2, Advanced Medical Supplies, Hants, UK).

Protoplast Volume Determination

Ion content was related to protoplast volume, which was determined by a method after Werdan and Heldt (1972), using [U-^{14}C]sorbitol as an impermeant extracellular marker and tritiated water (^3H_2O) to label uniformly both intracellular and extracellular water pools. Different methods were used for mesophyll and epidermal protoplasts because of the differences in their densities.

Mesophyll

Mesophyll protoplast suspension (700 μL) was mixed with an equal volume of radiolabeled incubation medium (0.75 M mannitol [Fisons, Loughborough, Leics, UK], 12.5 mM $CaCl_2$, 12.5 mM MES, 1.25 mM mercaptoethanol, brought to pH 6.0 with NaOH, ^3H_2O [Du Pont, Hounslow, Kent, UK: final activity of 11.93 kBq mL^{-1}], and [U-^{14}C]sorbitol [final activity of 0.66 kBq mL^{-1}]). Following a 3.5-min incubation, the protoplasts were separated from the radiolabeled medium; aliquots of the protoplast suspension were pelleted through 100 μL of dibutylphthalate oil in 400-μL Eppendorf tubes by centrifugation in a Microfuge B (Beckman Instruments, Fullerton, CA) for 30 s. Supernatant (20 μL) was transferred to scintillation vials to enable calculation of the specific activity of ^3H_2O and [U-^{14}C]sorbitol per unit volume of the incubation medium. The Eppendorf tube was frozen in liquid N_2 and the tip portion containing the frozen protoplast pellet was cut off using a razor blade and placed in a 1.5-mL Eppendorf tube. The protoplast pellet was dissolved in 0.25 mL of 5% (w/v) SDS and decolorized with 100 μL of sodium hypochlorite and 10 μL of glacial acetic acid. The dissolved and decolo-

rized pellet was transferred to a 20-mL scintillation vial together with 0.75 mL of de-ionized water washings.

Epidermis

The protoplast suspension (700 μL) was mixed with an equal volume of radiolabeled incubation medium (80% [v/v] Percoll solution diluted with sorbitol isolation medium, ^3H_2O with a final activity of 12.85 kBq mL^{-1}, and [U-^{14}C]sorbitol with a final activity of 0.71 kBq mL^{-1}). Aliquots were pipetted into 400-μL Eppendorf tubes, overlayered with 200 μL of silicone oil (AR200, Fluka, Gillingham, Dorset, UK) and 60 μL of de-ionized water. After a 3.5-min incubation, the tubes were centrifuged as above for 20 to 30 s, causing the protoplasts to float up through the oil into the de-ionized water and 50 μL of the latter was transferred to a 4-mL scintillation vial.

Scintillation fluid (Ultima-Gold, Packard Instruments, Meriden, CT) was added to the mesophyll and epidermal extracts (10- and 4-mL aliquots, respectively), and both types of samples were assayed for ^3H and ^{14}C by dual channel liquid scintillation counting. Chemical quenching of radioactivity was corrected for.

Estimation of Cellular Ion Concentrations

The osmolalities of leaf sap (extracted from fresh leaf material using the technique of Tomos et al. [1984]) and experimental solutions were determined using a vapor pressure osmometer (Wescor 5100C, Chemlab Instruments Ltd., London). To facilitate comparison of ion concentrations in the two cell types, osmotic shrinkage of protoplasts during the isolation and volume determination process (leading to artificially high ion concentrations) was taken into account. Protoplast or cell volume was assumed to be (inversely) linearly related to their osmolality, such that a decrease in osmolality led to an equivalent increase in volume. The volume of the protoplasts in a given sample and the osmolality of their volume determination medium (described above) were known, together with the osmolality of the leaf sap, which was the best available reference value for cell osmolality in the intact leaf. The ratiometric difference in osmolality was used to estimate, from protoplast volume, the volume of cells in the intact leaf. Thus, all concentrations are reported relative to these calculated in vivo volumes of leaf cells, not to the volumes of the isolated protoplasts.

Membrane Potential Measurements

Barley seedlings were grown as described above. Entire 5-d-old seedlings were used. The roots were wrapped in moistened tissue while the emergent leaf was fixed in a chamber with the adaxial surface perpendicular to the bottom of the chamber. The chamber was perfused with a standard solution (1 mM CaCl$_2$, 1 mM MgCl$_2$, and 1 mM MES-Tris, pH 6) at a rate of 15 mL min^{-1}. Ag/AgCl half-cells were connected to an impalement micropipette filled with a 100 mM KCl solution, and to an agar bridge containing 100 mM KCl. The tip potential measured when an electrode containing 100 mM KCl was bathed in a solution of 100 mM K gluconate was approximately 10 mV. Thus, the tip potential between the pipette solution and cell contents was anticipated to be similar, and a compensatory voltage offset of -10 mV was set before cells were impaled with the micropipette using micromanipulation. The preparation was visualized with bright-field microscopy. Steady-state membrane potentials were measured in epidermal and mesophyll cells in the upper cell layers of illuminated leaves perfused with standard solution. Response of the membrane potential to changes in external phosphate concentration were measured by adding MgHPO$_4$ to standard solution; changes were measured relative to the steady-state membrane potential before any phosphate was added to standard solution and/or after phosphate was removed from standard solution. At a solution pH of 6.0, at least 90% of the phosphate was present as $H_2PO_4^-$.

Uptake of $^{45}Ca^{2+}$

Protoplasts were isolated as described above, except that the Ca^{2+} concentration of the isolation medium was 0.1 mM. Protoplasts were suspended in sorbitol isolation medium at a density of 10^5 to 10^6 mL^{-1}. Uptake of radiolabel was measured using an adaptation of the method of Marshall et al. (1994). Glass fiber filters (pore size 0.2 μm; GF/C, Whatman, Clifton, NJ) were pre-soaked for several hours in ice-cold wash medium (0.5 M sorbitol, 10 mM MES, 1 mM CaCl$_2$, and 10 mM LaCl$_3$ adjusted to pH 5.5 with NaOH). The Ca^{2+} concentration of the uptake medium (0.5 M sorbitol, 10 mM MES, and 10 mM Glc, pH 5.5 with NaOH) was adjusted with a $^{45}Ca^{2+}$-containing (Amersham, St. Albans, Herts, UK) 1 mM CaCl$_2$ stock solution to give the desired final Ca^{2+} concentration, with a final $^{45}Ca^{2+}$ activity of 40 to 100 kBq mL^{-1} (specific activity 50–500 MBq mmol^{-1}). In some experiments, the uptake medium included 4 mM LaCl$_3$.

Radiotracer uptake was initiated by the addition of 300 μL of protoplast suspension (10^5–10^6 protoplasts mL^{-1}) to 200 μL of uptake medium, and the protoplast suspension was regularly mixed by gentle pipetting. Wide-bore pipette tips were used throughout to minimize shearing of protoplasts. Aliquots (70 μL) of the protoplast uptake suspension were removed at defined intervals, the first sample being taken approximately 15 s after uptake initiation. The protoplast samples were pipetted onto the filters, together with 5 to 10 mL of ice-cold unlabeled wash solution. Vacuum suction was applied to separate the protoplasts from the wash solution, and the uptake time course was carried out in duplicate or triplicate. Following the addition of scintillation fluid (4 mL) to vials containing filters carrying the protoplasts, $^{45}Ca^{2+}$ was detected by liquid scintillation counting. Intactness of protoplasts on the filters was confirmed by fluorescein diacetate staining. Total uptake over the initial part of the time course was fitted by linear or second-order regression using Fig.P software (BIOSOFT, Cambridge, UK) to obtain an initial rate of uptake and an ordinate-intercept value. The latter value was subtracted

from all points to give an uptake time course corrected for extracellular tracer contaminating the samples.

Patch-Clamp Experiments

Protoplasts were isolated using a modified version of the above protocol to reduce leaf digestion times and the amount of leaf material required. Enzyme concentrations were 0.7% (w/v) cellulase and 0.025% (w/v) pectolyase, and the leaves were incubated at 28°C for approximately 1 h, by which time sufficient protoplasts of both cell types had been released to supply material for experiments. Pipette tip resistances were typically 10 to 15 MΩ, depending on the ionic composition of the experimental solutions. The Ag/AgCl reference half-cell was attached to a 100-mM KCl agar bridge. Clamp voltages were controlled with an amplifier (L/M-EPC7, List-Medical Electronic, Darmstadt, Germany), and currents were low-pass-filtered at 1 kHz with an 8-pole Bessel filter and sampled at 0.1 kHz (whole cell currents) by the computer via a CED 1401 interface and related patch clamp software (CED, Cambridge, UK). Seals of GΩ resistance were obtained by applying suction and a small negative clamp voltage (-20 to -30 mV) at the protoplast membrane. Capacitance currents were measured and corrected for using the amplifier. Access resistances were measured with the amplifier and corrected for either with the amplifier or during data analysis; measurements were generally discarded when a high-access resistance introduced an error greater than 5% into the clamp voltage.

Protoplasts in the suspension were allowed to settle onto a glass coverslip attached to the bottom of a Teflon well. Using bath perfusion (0.25 mL min^{-1}), the isolation medium was replaced with an external solution of 100 mM KCl, 1 mM MgCl$_2$, 1 mM CaCl$_2$, 2 mM MES-Tris, pH 5.7, osmolality adjusted to 630 mosmol kg^{-1} with sorbitol. Initial experiments were carried out in symmetrical KCl solutions, with the pipette solution containing 100 mM KCl, 1 mM MgCl$_2$, 1 mM CaCl$_2$ adjusted to 130 nM free Ca with 1.4 mM K-EGTA, 2 mM Mg$_2$ATP, and 6 mM Tris-HEPES, pH 7.5, osmolality adjusted to 650 mosmol kg^{-1} with sorbitol. In experiments to determine the ionic selectivities of currents, the basal composition of the bathing medium was retained in all solutions as 1 mM CaCl$_2$, 1 mM MgCl$_2$, and 2 mM MES-Tris, pH 5.5, osmolality adjusted to 630 mosmol kg^{-1} with sorbitol, but the major ion composition was altered in different experiments to give concentrations indicated in the text. Measurements were analyzed using patch clamp software (CED), with instantaneous currents measured at a fixed time point (50 ms) into the pulse, and time-dependent currents obtained by subtracting the instantaneous current from the total current. Liquid junction potentials were calculated according to the method of Barry and Lynch (1991) or measured according to the method of Amtmann and Sanders (1997), and liquid junction potentials were subtracted from the final current to voltage (I-V) relationships. Ionic reversal potentials were calculated from ionic strength and ion activities in solution. Ca^{2+} concentrations in the buffered pipette solutions were calculated using the program CALCIUM (K.-J. Foehr and

W. Warchol, unpublished data). Membrane voltages are given as the potential of the cytoplasmic side with respect to the extracellular side.

Statistics

Results are reported in the form of means ± SE, with values of n indicated in figure legends.

RESULTS

Protoplast Material

Following isolation, contamination of purified mesophyll protoplast preparations by epidermal protoplasts was low (0.2% ± 0.2%), and vice versa (4.7% ± 0.7%). Mesophyll protoplasts were of a fairly consistent size (average diameter range 30–45 μm) at the osmolality of the isolation medium. Epidermal protoplasts showed a much greater range in size (average diameter range 20–80 μm). Protoplast sizes were similar in patch clamping solutions. Average diameter of patch clamped protoplasts was 32.7 ± 3.7 μm ($n = 20$, mesophyll) and 39.3 ± 7.6 μm ($n = 20$, epidermis), with membrane capacitance equal to 0.96 ± 0.22 ($n = 20$, mesophyll) and 0.94 ± 0.14 ($n = 20$, epidermis) μF cm^{-2}.

Protoplast Ion Accumulation

Differences in ion concentrations observed between the mesophyll and epidermis were consistent and reproducible between plant batches (Fig. 1). Under the growth conditions, the principal cation K$^+$ accumulated to approximately 130 mM in the mesophyll, but to more than 250 mM in the epidermis. Partitioning of excess K$^+$ to the epidermis has been demonstrated by single-cell sap sampling (Fricke et al., 1994a), although other studies have shown slight preferential K$^+$ accumulation in the mesophyll (Dietz et al., 1992b; Leigh and Storey, 1993). The difference in K$^+$ concentration between the two cell types was mirrored by the
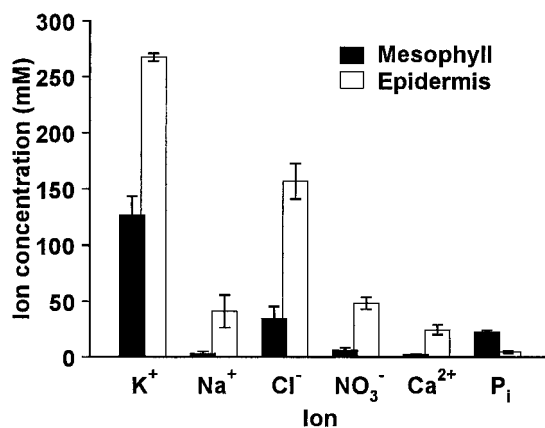

Figure 1. Ion concentrations in mesophyll and epidermal cells isolated from primary barley leaves, based on estimated volumes of cells in vivo (see "Materials and Methods"). Values are the means ± SE of four separate experiments.
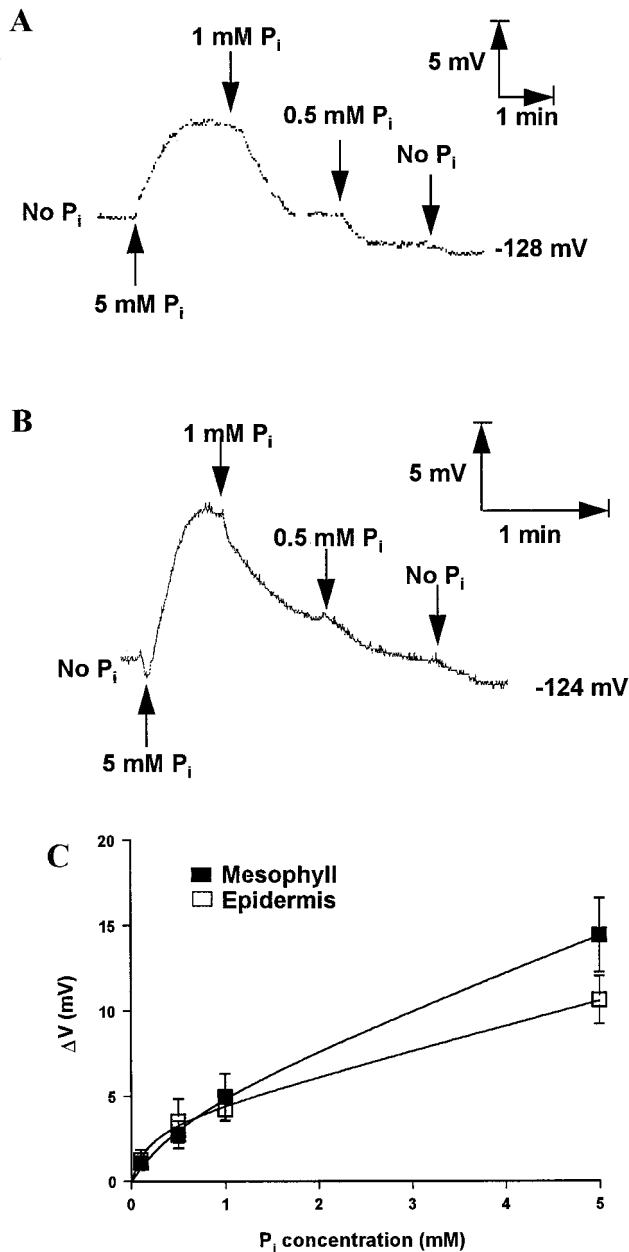
A

B

C

Figure 2. Membrane depolarization in mesophyll (A) and epidermal cells (B) in intact seedlings in response to the addition of 5 mM Pi to the perfusion medium, and subsequent repolarization as the external Pi concentration is sequentially reduced. C, The relationship between the membrane depolarization and the Pi concentration for the two cell types (values are the means ± SE of between four and 16 observations).

the epidermis appears to contradict data showing a fairly equal distribution of this ion between the two tissues (Dietz et al., 1992b; Fricke et al., 1994a), NO_3^- accumulation in the epidermis sometimes represents up to 55% of bulk leaf concentrations (Dietz et al., 1994). Na^+ and Ca^{2+} concentrations were both low in the mesophyll (≤ 10 mM), whereas the epidermis accumulated considerably higher concentrations of both of these ions. In contrast, Pi accumulated preferentially in the mesophyll (20–30 mM, on average), with levels in epidermal cells being at the limit of detection for this ion. These results were largely consistent with those of Dietz et al. (1992b), Leigh and Storey (1993), Williams et al. (1993), and Fricke et al. (1994a).

Membrane Potential Measurements and Pi-Induced Membrane Depolarization

The resting membrane potential (E_m) of intact cells was measured by microelectrode impalement of mesophyll and epidermal cells to determine whether voltage regulation of membrane currents might underlie the differences in ion accumulation. In situ measurements in intact seedlings gave similar values of resting E_m for the two cell types: -120.3 (± 10.6) mV in mesophyll cells ($n = 28$) and -122.3 (± 11.9) mV in epidermal cells ($n = 24$).

Microelectrode impalement of leaf cells of 5-d-old intact seedlings was also employed to monitor changes in the E_m in response to Pi. The addition of 0.1 to 5 mM Pi to the apoplast perfusion medium resulted in membrane depolarization in both cell types (Fig. 2, A and B), suggesting that, as in other species (Ullrich-Eberius et al., 1981; Sakano, 1990), Pi uptake is energized by cation (probably H^+) symport. Depolarization was small (ranging from 0.5–15 mV) and concentration dependent. Removal of Pi from the bathing medium generally led to membrane repolarization, and often hyperpolarization beyond the original membrane potential following exposure to higher (1–5 mM)
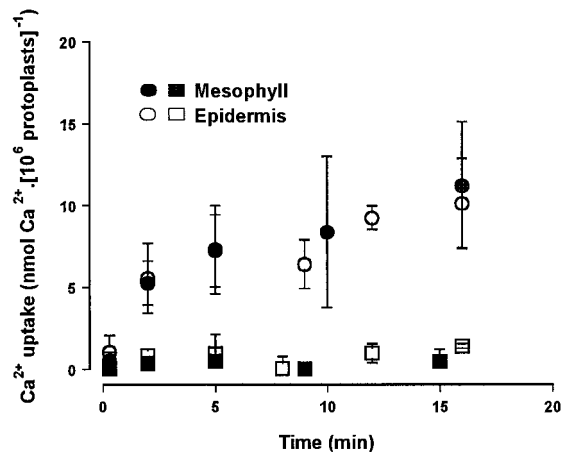

Figure 3. Ca^{2+} uptake from an external concentration of 1 mM Ca^{2+} by mesophyll (●, ■) and epidermal (○, □) protoplasts in the absence (●, ○) or presence (■, □) of 4 mM $LaCl_3$. Each point is the mean ± SE of three to five samples. Before subtraction, zero-time intercepts were (in nmol Ca^{2+} [10^6 protoplasts]$^{-1}$): 8.74 (●), 8.76 (○), 6.29 (■), and 6.93 (□).

differences in Cl^- concentration. While only 35 mM in the mesophyll, the Cl^- concentration in the epidermis exceeded this by a factor of four. The presence of Cl^- in the mesophyll contradicted x-ray microanalysis data (Huang and van Steveninck, 1989; Leigh and Storey, 1993), but confirmed the findings of other studies (Dietz et al., 1992b; Fricke et al., 1994a). NO_3^- concentrations were low relative to Cl^-, and although preferential NO_3^- accumulation in

Figure 4. Inward currents in response to membrane hyperpolarization in a mesophyll (30-μm diameter) (A) and an epidermal (42-μm diameter) protoplast (B). C, The steady-state I-V relationships for time-dependent currents in symmetric 100 mM KCl. Voltage protocols and composition of media are shown at the top of the figure, with pipette medium on the left; values give the added ion concentrations, except for pipette Mg and Ca, for which the free ion activity is given.
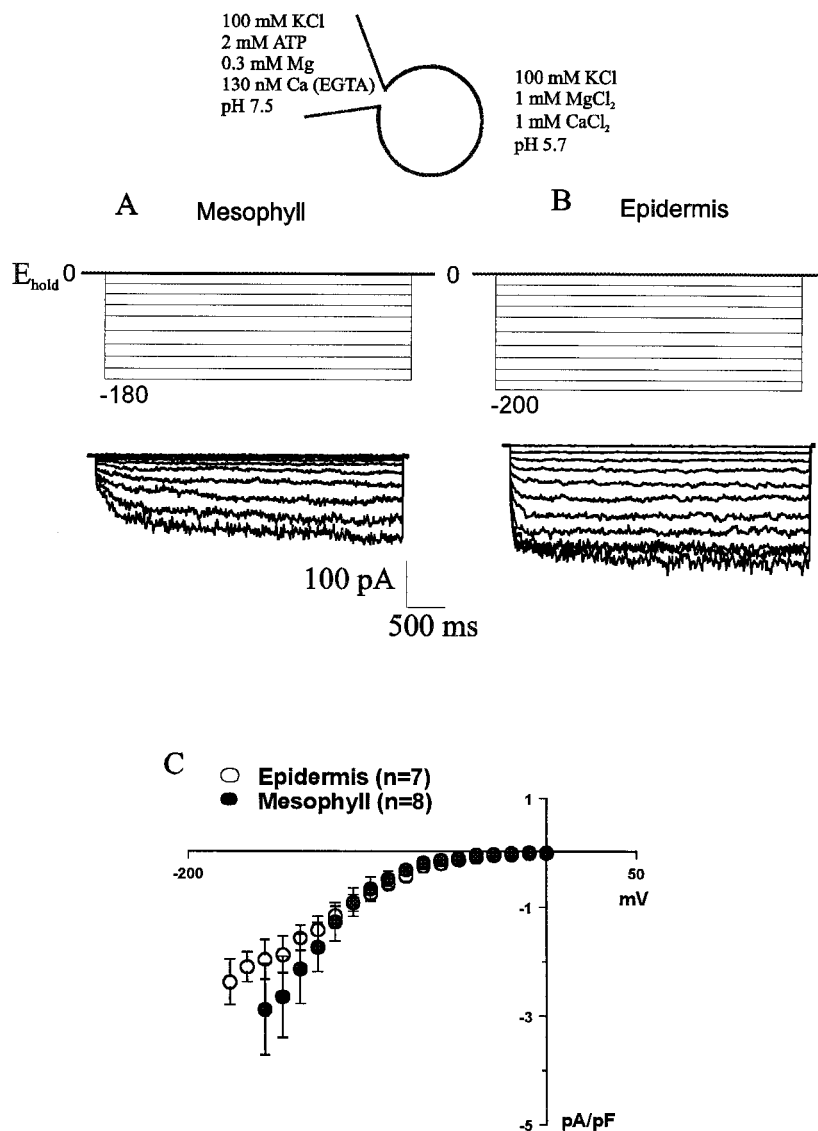
apoplastic Pi concentrations (Fig. 2, A and B). The concentration dependence of changes in membrane potential is shown in Figure 2C. For both cell types, the relationship can be fitted as the sum of a saturable component, which predominates at low concentrations, and a linear component, which better describes the response at higher concentrations. The fits yielded K_m estimates for the saturable components of 0.67 mM (mesophyll) and 0.19 mM (epidermis), and the respective V_{max} values were 4.6 mV and 3.5 mV. For the linear components, $\Delta V = 2.08[Pi]$ for the mesophyll and $1.45[Pi]$ for the epidermis. The depolarization response to Pi was not significantly different between the mesophyll (where Pi accumulates) and the epidermis.

Influx of $^{45}Ca^{2+}$

To compare initial rates of Ca^{2+} uptake in the two cell types, influx was expressed on a per protoplast basis. Figure 3 shows that no difference in Ca^{2+} uptake was discern-ible between the two cell types, and this was also the case at submillimolar external Ca^{2+} concentrations (data not shown). Inclusion of $LaCl_3$ in the incubation medium substantially reduced $^{45}Ca^{2+}$ uptake, with little or no time-dependent uptake above the zero-time intercept, confirming that the increase in radioactivity in the absence of La^{3+} represented bona fide Ca^{2+} uptake. The initial rates of Ca^{2+} uptake in nmol $[10^6$ protoplasts$]^{-1}$ min^{-1} were 2.28 (mesophyll) and 2.64 (epidermis), and were similar to those for tobacco suspension-cultured cells (Mettler and Leonard, 1979a) and amaranth cotyledon protoplasts (Rengel and Elliott, 1992).

Channel-Mediated K$^+$ Influx

K$^+$ is accumulated by both cell types, and in symmetric 100 mM KCl, an exponentially activating, time-dependent inward (TDI) current was observed at voltages negative of -50 mV in most patch-clamped mesophyll (80%; $n = 51$)
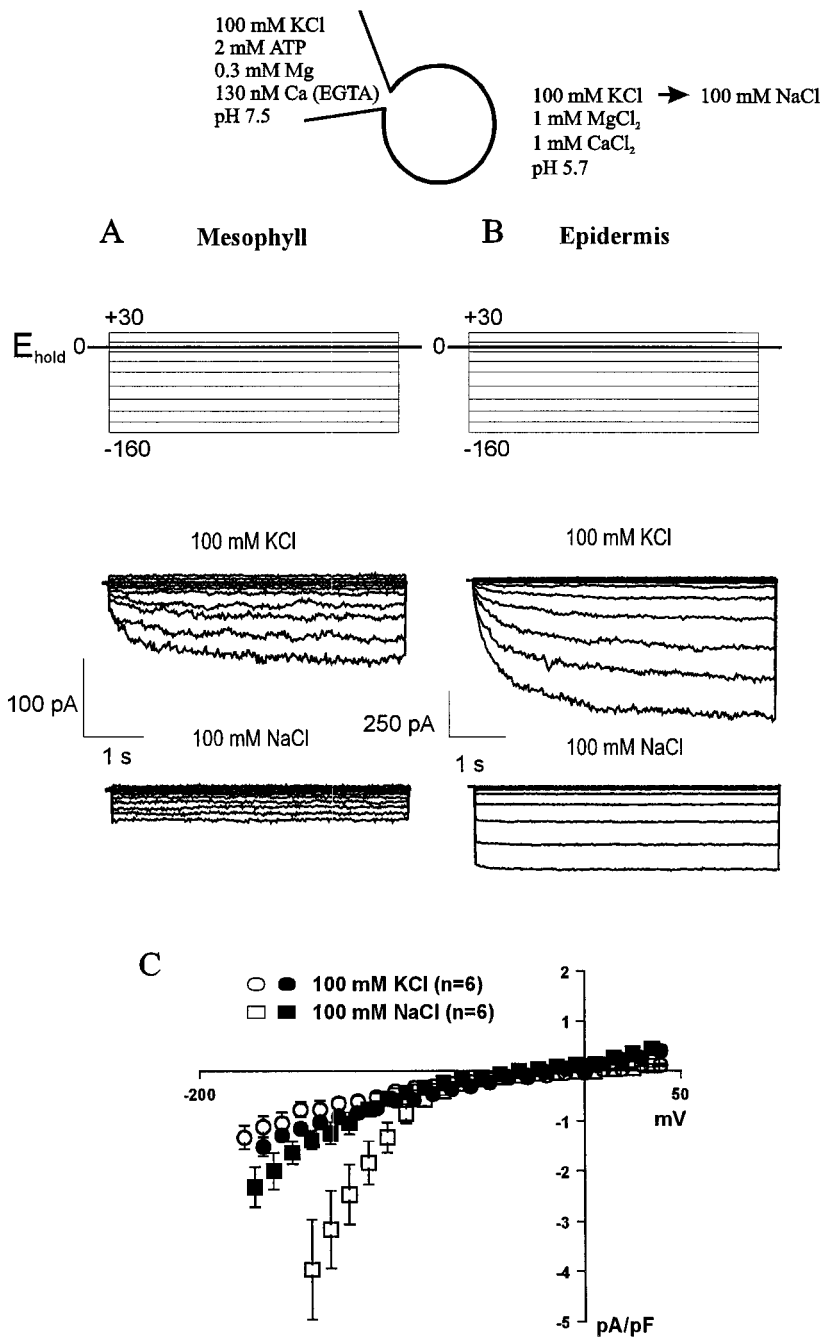
Figure 5. Currents in the presence of 100 mM KCl and 100 mM NaCl, in a mesophyll (34-μm diameter) (A) and an epidermal (44-μm diameter) protoplast (B). Note different scales for the current traces. C, The I to V relationships for instantaneous currents for mesophyll ($\bullet$, $\blacksquare$) and epidermal protoplasts ($\bigcirc$, $\square$). Voltage protocols and composition of media are shown at the top of the figure, with pipette medium on the left; values give the added ion concentrations, except for pipette Mg and Ca, for which the free ion activity is given.

and epidermal (71%; $n = 34$) protoplasts (Fig. 4). Currents were present in both cell types when ATP was omitted from the pipette solution (data not shown), but experiments were performed with ATP in the pipette. Steady-state currents elicited at comparable voltages were larger in epidermal protoplasts, but when expressed in terms of current density, the differences between the two cell types were small, and current density was even slightly higher in mesophyll protoplasts (Fig. 4C). Current activation appeared faster in epidermal protoplasts than in mesophyll protoplasts (Fig. 4, A and B). However, this observation could not be rigorously analyzed by comparison of activa-

tion time constants because currents could not be fitted with a single exponential relationship, and a double exponential relationship gave too many degrees of freedom for a meaningful fit. The I-V relationship for the steady-state TDI current was non-linear (rectifying) in both cell types (Fig. 4C).

The identity of the TDI current was determined by tail current analysis and by ion substitution experiments. Analysis of tail current reversal potentials (E_{rev}) showed, as anticipated, that in the presence of symmetric 100 mM KCl, the value of E_{rev} was 0 mV. In asymmetric conditions (with 10 mM KCl externally: not shown), the amplitude of the

inward current was reduced, and tail current analyses yielded estimates of $E_{rev} = -32.5 \pm 3.6$ mV ($n = 6$, mesophyll) and -37.5 ± 6.3 mV ($n = 4$, epidermis). By comparison with the values of E_K (-52 mV) and E_{Cl} ($+47$ mV) in these conditions, the reversal potentials of the mesophyll and epidermal TDI currents were closer to E_K, indicating that a substantial proportion of the current was carried by K$^+$. Furthermore, replacement of K$^+$ in the external solution with choline$^+$ led to the complete loss of the TDI current in both cell types (data not shown), and the TDI current was reversibly inhibited by the K$^+$-channel blocker tetraethylammonium (20 mM TEA-Cl) and by 1 mM CsCl applied externally (data not shown).

Channel-Mediated Na$^+$ Influx

Instantaneous currents were observed in all mesophyll and epidermal cells in symmetric 100 mM KCl. These currents rectified slightly at extreme potentials (negative of -150 mV) and reversed around 0 mV (Fig. 5). Current density was not significantly different in the two cell types. Replacement of 100 mM KCl with 100 mM NaCl in the external solution led to an increase in the instantaneous inward current that was surprisingly dramatic in epidermal protoplasts (Fig. 5, A–C). Comparison of the two cell types (Fig. 5C) showed that the increase in current density in epidermal protoplasts (where Na$^+$ accumulates; Fig. 1) was at least twice that observed in mesophyll protoplasts over a range of voltages. The current observed in the presence of Na$^+$ was strongly inwardly rectifying, suggesting that internal cations (K$^+$, Ca^{2+}, and Mg^{2+}) did not pass through these channels. Current magnitude was dependent upon external NaCl concentration, and the Na$^+$-induced increase in current was fully reversible upon return to 100 mM KCl in the external solution (not shown), indicating that the increase in instantaneous current was not simply an effect of Na$^+$ on seal stability.

In accordance with the notion that Na$^+$ rather than K$^+$ carries the current, the currents in 100 mM NaCl reversed positive of zero (approximately $+25$ mV, as obtained from individual I-V curves, which are not shown). However, accurate E_{rev} determination was not possible in these conditions because the currents were strongly rectifying. The reversal potential was approximately $+5$ mV when 30 mM NaCl was present externally. The dependence of current magnitude and reversal potential on the external Na$^+$ concentration suggested that this current was indeed carried by Na$^+$.

When 100 mM KCl was replaced with 100 mM NaCl externally, the TDI current virtually disappeared in most (90%) protoplasts, confirming that the TDI currents were carried principally by K$^+$ and that the principal route for Na$^+$ moving into the cells is via the instantaneous current.

DISCUSSION

Differential Ion Accumulation

Partitioning of inorganic solutes between different cell types, observed here in the primary leaves of barley, was similar to that observed in previous studies (Dietz et al., 1992b; Fricke et al., 1994a). The basic pattern is one of accumulation of Ca^{2+} in the epidermis and Pi in the mesophyll, with preferential epidermal accumulation of Na$^+$, K$^+$, and some other inorganic ions. If mesophyll vacuoles, on average, occupy 75% cell volume and epidermal vacuoles occupy 99% cell volume (Winter et al., 1993), it might be postulated that the differences in ion concentration arise from the disparity in relative vacuolar volumes between the two cell types. However, recalculation of data presented in Figure 1, assuming that all ions are located in the vacuole (i.e. mesophyll ion concentrations would increase by 25%; epidermal ion concentrations would increase by 1%) confirms that the measured ion concentrations would still reflect different tissue locations of Ca^{2+} and Pi, and preferential accumulation of Na$^+$, K$^+$, Cl$^-$, and NO$_3^-$ in the upper epidermis. Given that mesophyll and epidermal vacuoles occupy a similar proportion of aqueous leaf space (36% and 35%, respectively; values derived from data in Winter et al., 1993), the ion concentration data are likely to reflect different tissue locations for these ion pools in intact leaves.

Ion Transport Processes

This study attempted to uncover some of the transport processes that might underlie differential ion accumulation in mesophyll and epidermal cells of barley leaves. In situ measurements of the membrane potential demonstrated that the plasma membrane was hyperpolarized to -120 mV in resting conditions in both cell types. If this reflects the situation in vivo, it suggests that differential control of the plasma membrane E_m does not explain cell-type-specific ion accumulation. However, it is possible that the E_m also depends on the apoplastic ionic milieu, which might not be uniform in a transpiring leaf (e.g. Mühling and Sattelmacher, 1997).

Increasing the apoplastic Pi concentration around leaf cells led to plasma membrane depolarization (Fig. 2, A and B), suggesting that transport of Pi was associated with influx of positive charge. This probably indicated symport of Pi with H$^+$, as has been postulated previously for plasma membrane Pi uptake in *Lemna gibba* fronds and *Catharanthus roseus* suspension-cultured cells, with stoichiometric ratios in the range of two to four H$^+$ per phosphate (Ullrich-Eberius et al., 1984; Sakano, 1990). Pi-induced membrane depolarization is concentration dependent and saturable and falls into the low-affinity range for Pi uptake systems described in leaf, root, and suspension-cultured cells (e.g. Mettler and Leonard, 1979b; Ullrich-Eberius et al., 1984; Mimura et al., 1990, 1998; Furihata et al., 1992; Liu et al., 1998). There were no discernible differences in kinetic parameters between mesophyll cells (where Pi accumulates; Fig. 1) and epidermal cells, although the size of the depolarization will of course be determined not only by the current flowing through Pi transport systems, but also by the magnitude of the membrane conductance. Although Ca^{2+} is preferentially located in the epidermis (Fig. 1), Ca^{2+} influx to epidermal protoplasts was also similar to that of the mesophyll (Fig. 3).

The influx of K^+ across the plasma membrane is probably via the TDI channels, which were highly selective for K^+ over other monovalent and divalent cations. Although inward-rectifying currents are absent from the leaf mesophyll cells of several species (Li and Assmann, 1993; Bei and Luan, 1998; Piñeros and Kochian, 1998), the ionic selectivity and inhibitory characteristics observed here have been demonstrated for the TDI current in other types of plant cells, including fava bean guard cells (Schroeder et al., 1987), root cells of wheat cortex (Findlay et al., 1994), and Arabidopsis (Maathuis and Sanders, 1995), oat mesophyll cells (Kourie and Goldsmith, 1992), and barley suspension-cultured cells (Amtmann et al., 1997). The channels responsible for the TDI current are likely to be active at the values of resting E_m measured in situ, although the extent of activation is likely to depend on E_K, which would be more negative in vivo than in patch clamp conditions because the apoplastic K^+ concentrations are lower (e.g. 5–26 mM in fava bean leaf apoplast: Mühling and Sattelmacher, 1997). The higher concentrations of K^+ measured in epidermal cells could not be explained by differences between the mesophyll and epidermis in the ionic selectivity of their TDI currents, and, if anything, current density was larger in mesophyll protoplasts.

The presence of 100 mM NaCl in the external solution led, in both cell types, to an increase in the instantaneous current, which was particularly dramatic in the epidermal cells and might explain the preferential accumulation of Na^+ in this cell type. The instantaneous current has been proposed as a route for Na^+ transport across the plasma membrane in root and guard cells of several plant species (White and Lemtiri-Chlieh, 1995; Roberts and Tester, 1997; White, 1997; Véry et al., 1998), with permeation by Na^+ sometimes exceeding that of K^+ (P_{Na}/P_K ranging from 0.37 in rye roots to >1 in pea leaves; for review, see Amtmann and Sanders, 1999).

Mechanisms Underlying Differential Ion Accumulation

While the work of Dietz et al. (1992b) hinted that differential uptake of ions might partly explain cell type-specific ion accumulation, this study found no evidence for differential uptake of K^+, Pi, or Ca^{2+} at the plasma membrane of isolated barley leaf protoplasts. However, the larger instantaneous currents in epidermal protoplasts in the presence of extracellular Na^+ could underlie the higher intracellular Na^+ concentrations in this tissue. Differences between the two cell types in factors such as plasma membrane ion efflux or vacuolar uptake mechanisms could also allow cell-type-specific ion accumulation to be achieved with a similar complement of plasma membrane ion transport systems.

A number of studies (Tanton and Crowdy, 1972; Canny, 1990a, 1990b) point to the importance of the vascular bundle sheath plasma membrane in controlling the subsequent movement of solutes through the leaf. Certain ionic species imported to barley leaves in the xylem sap might be transported into the mesophyll symplast at the vascular bundle, while other ionic species are taken up by cells as the ions diffuse through the apoplast (Leigh and Tomos, 1993).

Thus, the processes underlying differential accumulation of ions by leaf cells can include regulation of supply of certain ions (possibly Ca^{2+}, Pi, K) or capacity for the uptake of ions (possibly Na^+), both mechanisms leading to the effective filtration of ions as they move through the leaf.

Maintenance of ion partitioning in conditions of variable ion supply (Dietz et al., 1992b; Fricke et al., 1996) suggests that cell-specific ion acquisition and storage is an active process required to satisfy cell-specific ion requirements (e.g. Pi required in mesophyll cells for photosynthesis), osmotic demand (e.g. in epidermal cells, where organic solutes are virtually absent, Ca^{2+} acts as an epidermal osmoticum; Fricke et al., 1994b), chemical restrictions (e.g. accumulation of Ca^{2+} and Pi in the same cell would lead to co-precipitation; Dietz et al., 1992b), or biochemical necessity (e.g. vacuolar accumulation of ions or molecules that would be toxic in excess in the cytosol; Fricke et al., 1996). Further studies of the way in which the leaf achieves this remarkable sorting and storage of solutes will improve our understanding of how the plant manages to control the uptake, targeting, and retranslocation of solutes in the face of environmental fluctuations in supply.

LITERATURE CITED

Amtmann A, Laurie S, Leigh R, Sanders D (1997) Multiple inward channels provide flexibility in Na^+/K^+ discrimination at the plasma membrane of barley suspension culture cells. J Exp Bot **48**: 481–497

Amtmann A, Sanders D (1997) A unified procedure for the correction of liquid junction potentials in patch clamp experiments on endo- and plasma membranes. J Exp Bot **48**: 361–364

Amtmann A, Sanders D (1999) Mechanisms of Na^+ uptake by plant cells. Adv Bot Res **29**: 75–112

Barry PH, Lynch JW (1991) Liquid junction potentials and small cell effects in a patch-clamp analysis. J Membr Biol **121**: 101–117

Bei Q, Luan S (1998) Functional expression and characterisation of a plant K^+ channel gene in a plant cell model. Plant J **13**: 857–865

Boursier P, Läuchli A (1989) Mechanisms of chloride partitioning in the leaves of salt-stressed *Sorghum bicolor* L. Physiol Plant **77**: 537–544

Canny MJ (1990a) What becomes of the transpiration stream? New Phytol **114**: 341–368

Canny MJ (1990b) Rates of apoplastic diffusion in wheat leaves. New Phytol **116**: 263–268

Dietz K-J, Hollenbach B, Hellwege E (1994) The epidermis of barley leaves is a dynamic intermediary storage compartment of carbohydrates, amino acids and nitrate. Physiol Plant **92**: 31–36

Dietz K-J, Schramm M, Lang B, Lanzl-Schramm A, Dürr C, Martinoia E (1992a) Characterization of the epidermis from barley primary leaves: I. Isolation of epidermal protoplasts. Planta **187**: 425–430

Dietz K-J, Schramm M, Lang B, Lanzl-Schramm A, Dürr C, Martinoia E (1992b) Characterization of the epidermis from barley primary leaves: II. The role of the epidermis in ion compartmentation. Planta **187**: 431–437

Findlay GP, Tyerman SD, Garrill A, Skerrett M (1994) Pump and K⁺ inward rectifiers in the plasmalemma of wheat root protoplasts. J Membr Biol **139:** 103–116

Fricke W, Leigh RA, Tomos AD (1994a) Concentrations of inorganic and organic solutes in extracts from individual epidermal, mesophyll and bundle-sheath cells of barley leaves. Planta **192:** 310–316

Fricke W, Leigh RA, Tomos AD (1994b) Epidermal solute concentrations and osmolality in barley leaves studied at the single-cell level. Planta **192:** 317–323

Fricke W, Leigh RA, Tomos AD (1996) The intercellular distribution of vacuolar solutes in the epidermis and mesophyll of barley leaves changes in response to NaCl. J Exp Bot **47:** 1413–1426

Furihata T, Suzuki M, Sakurai H (1992) Kinetic characterization of two phosphate uptake systems with different affinities in suspension-cultured *Catharanthus roseus* protoplasts. Plant Cell Physiol **33:** 1151–1157

Huang CX, van Steveninck RFM (1989) Maintenance of low chloride concentrations in mesophyll cells of leaf blades of barley seedlings exposed to salt stress. Plant Physiol **90:** 1440–1443

Kourie J, Goldsmith MHM (1992) K⁺ channels are responsible for an inwardly rectifying current in the plasma membrane of mesophyll protoplasts of *Avena sativa*. Plant Physiol **98:** 1087–1097

Leigh RA, Chater M, Storey R, Johnston AE (1986) Accumulation and subcellular distribution of cations in relation to the growth of potassium-deficient barley. Plant Cell Environ **9:** 595–604

Leigh RA, Storey R (1993) Intercellular compartmentation of ions in barley leaves in relation to potassium nutrition and salinity. J Exp Bot **44:** 755–762

Leigh RA, Tomos AD (1993) Ion distribution in cereal leaves: pathways and mechanisms. Philos Trans R Soc Lond B **341:** 75–86

Li W, Assmann SM (1993) Characterization of a G-protein-regulated outward K⁺ current in mesophyll cells of *Vicia faba* L. Proc Natl Acad Sci USA **90:** 262–266

Liu H, Trieu AT, Blaylock LA, Harrison MJ (1998) Cloning and characterization of two phosphate transporters from *Medicago truncatula* roots: regulation in response to phosphate and to colonization by arbuscular mycorrhizal (AM) fungi. Mol Plant-Microbe Interact **11:** 14–22

Maathuis FJM, May ST, Graham NS, Bowen HC, Jelitto TC, Trimmer P, Bennett MJ, Sanders D, White PJ (1998) Cell marking in *Arabidopsis thaliana* and its application to patch clamp studies. Plant J **15:** 843–851

Maathuis FJM, Sanders D (1995) Contrasting roles in ion transport of two K⁺-channel types in root cells of *Arabidopsis thaliana*. Planta **197:** 456–464

Malone M, Leigh RA, Tomos AD (1991) Concentrations of vacuolar inorganic ions in individual cells of intact wheat leaf epidermis. J Exp Bot **42:** 305–309

Marschner H (1995) Mineral Nutrition of Higher Plants, Ed 2. Academic Press, London

Marshall J, Corzo A, Leigh RA, Sanders D (1994) Membrane potential-dependent calcium transport in right-side-out plasma membrane vesicles from *Zea mays* L. roots. Plant J **5:** 683–694

Martinoia E, Schramm MJ, Kaiser G, Kaiser WM, Heber U (1986) Transport of anions in isolated barley vacuoles: I. Permeability to anions and evidence for a Cl⁻ uptake system. Plant Physiol **80:** 895–901

Mettler IJ, Leonard RT (1979a) Ion transport in isolated protoplasts from tobacco suspension cells: I. General characteristics. Plant Physiol **63:** 183–190

Mettler IJ, Leonard RT (1979b) Ion transport in isolated protoplasts from tobacco suspension cells: II. Selectivity and kinetics. Plant Physiol **63:** 191–194

Mimura T, Dietz K-J, Kaiser W, Schramm MJ, Kaiser G, Heber U (1990) Phosphate transport across biomembranes and cytosolic phosphate homeostasis in barley leaves. Planta **180:** 139–146

Mimura T, Reid RJ, Smith FA (1998) Control of phosphate transport across the plasma membrane of *Chara corallina*. J Exp Bot **49:** 13–19

Mühling KH, Sattelmacher B (1997) Determination of apoplastic K⁺ in intact leaves by ratio imaging of PBFI fluorescence. J Exp Bot **48:** 1609–1614

Outlaw WH, Tarczynski MC, Miller WI (1984) Histological compartmentation of phosphate in *Vicia faba* L. leaflet. Plant Physiol **74:** 430–433

Piñeros M, Kochian L (1998) Differences in whole cell K⁺ currents across the plasma membrane of leaf protoplasts from two *Thlaspi* species: possible roles in heavy metal accumulation. 11th International Workshop on Plant Membrane Biology, August 9–14, 1998, Cambridge, UK. Experimental Biology Online http://link.spriyer.de/link/service/journals/00898/meeting/cambridge98/sess11.htm#10sess 3

Rengel Z, Elliott DC (1992) Mechanism of aluminium inhibition of net ⁴⁵Ca²⁺ uptake by *Amaranthus* protoplasts. Plant Physiol **98:** 632–638

Roberts SK, Tester M (1995) Inward and outward K⁺-selective currents in the plasma membrane of protoplasts from maize root cortex and stele. Plant J **8:** 811–825

Roberts SK, Tester M (1997) A patch clamp study of Na⁺ transport in maize roots. J Exp Bot **48:** 431–440

Sakano K (1990) Proton/phosphate stoichiometry in uptake of inorganic phosphate by cultured cells of *Catharanthus roseus* (L.) G. Don. Plant Physiol **93:** 479–483

Sanders D, Slayman CL (1989) Transport at the plasma membrane of plant cells: a review. *In* J Dainty, MI De Michaelis, E Marré, F Rasi-Caldogno, eds, Plant Membrane Transport: The Current Position. Elsevier Science Publishers, Amsterdam, pp 3–11

Schroeder JI, Raschke K, Neher E (1987) Voltage dependence of K⁺ channels in guard cell protoplasts. Proc Natl Acad Sci USA **84:** 4108–4112

Tanton TW, Crowdy SH (1972) Water pathways in higher plants: III. The transpiration stream within leaves. J Exp Bot **23:** 619–625

Tomos AD, Leigh RA, Shaw CA, Wyn Jones RG (1984) A comparison of methods for measuring turgor pressures and osmotic pressures of cells of red beet storage tissue. J Exp Bot **35:** 1675–1683

Treeby MT, van Steveninck RFM, De Vries HM (1987) Quantitative estimates of phosphorus concentrations within *Lupinus luteus* leaflets by means of electron probe x-ray microanalysis. Plant Physiol **85:** 331–334

Ullrich-Eberius CI, Novacky A, Fischer E, Lüttge U (1981) Relationship between energy-dependent phosphate uptake and the electrical membrane potential in *Lemna gibba* G1. Plant Physiol **61:** 797–801

Ullrich-Eberius CI, Novacky A, Van Bel AJE (1984) Phosphate uptake in *Lemna gibba* G1: energetics and kinetics. Planta **161:** 46–52

Véry A-A, Robinson MF, Mansfield TA, Sanders D (1998) Guard cell cation channels are involved in Na⁺-induced stomatal closure in a halophyte. Plant J **14:** 509–521

Werdan K, Heldt HW (1972) Accumulation of bicarbonate in intact chloroplasts following a pH gradient. Biochim Biophys Acta **283:** 430–441

White PJ (1997) Cation channels in the plasma membrane of rye roots. J Exp Bot **48:** 499–514

White PJ, Lemtiri-Chlieh F (1995) Potassium currents across the plasma membrane of protoplasts derived from rye roots: a patch clamp study. J Exp Bot **46:** 497–511

Williams ML, Thomas BJ, Farrar JF, Pollock CJ (1993) Visualizing the distribution of elements within barley leaves by energy dispersive x-ray image maps (EDX Maps). New Phytol **125:** 367–372

Willmer CM, Pallas JE, Jackson WA (1974) Major element composition of epidermal and mesophyll tissues of *Commelina communis* L. and *Vicia faba* L.: some further considerations of the role of ions in stomatal functioning. J Exp Bot **25:** 973–980

Winter H, Robinson DG, Heldt HW (1993) Subcellular volumes and metabolite concentrations in barley leaves. Planta **191:** 180–190