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Electrophysiological characterization of pathways for K^+ uptake into growing and non-growing leaf cells of barley

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ABSTRACT

Potassium is a major osmolyte used by plant cells. The accumulation rates of K^+ in cells may limit the rate of expansion. In the present study, we investigated the involvement of ion channels in K^+ uptake using patch clamp technique. Ion currents were quantified in protoplasts of the elongation and emerged blade zone of the developing leaf 3 of barley (*Hordeum vulgare* L.). A time-dependent inward-rectifying K^+-selective current was observed almost exclusively in elongation zone protoplasts. The current showed characteristics typical of Shaker-type channels. Instantaneous inward current was highest in the epidermis of the emerged blade and selective for Na^+ over K^+. Selectivity disappeared, and currents decreased or remained the same, depending on tissue, in response to salt treatment. Net accumulation rates of K^+ in cells calculated from patch clamp current–voltage curves exceeded rates calculated from membrane potential and K^+ concentrations of cells measured *in planta* by factor 2.5–2.7 at physiological apoplastic K^+ concentrations (10–100 mM). It is concluded that under these conditions, K^+ accumulation in growing barley leaf cells is not limited by transport properties of cells. Under saline conditions, down-regulation of voltage-independent channels may reduce the capacity for growth-related K^+ accumulation.

Key-words: *Hordeum vulgare*; leaf cell elongation; membrane potential; patch clamp; potassium channel; salinity; sodium.

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INTRODUCTION

Plants grow in size through the expansion of individual cells. As cells expand to a multiple of their original volume (e.g. 50- to 100-fold in grass leaf cells), cell contents and dissolved solutes are diluted progressively. Without concomitant accumulation of solutes, cell osmotic pressure and turgor decrease continuously until water can no longer be taken up from a low water potential source (root medium or xylem), or until turgor is too low to force walls to yield. To maintain growth, solute accumulation must accompany cell expansion (Van Volkenburgh 1999; Fricke 2002).

Few studies have measured osmotic pressure throughout growing tissues at cell level. These studies suggest that cell osmotic pressure remains constant during elongation, at between 300 and 400 mosmol kg^{-1} (Pritchard, Fricke & Tomos 1996; Fricke, McDonald & Mattson-Djos 1997; Martre, Bogeat-Triboulot & Durand 1999). A cell of, for example leaf 3 of barley, which expands at a maximum relative rate of 14% h^{-1} (Fricke *et al.* 1997) has to accumulate solutes at a rate equivalent to 42–56 mM h^{-1} (increase of solute concentration if the cell volume remained constant). It is not known whether solute accumulation rates of this magnitude require growth-specific cellular transport systems or membrane energization, and to which degree solute accumulation is limited by the capacity of cells to transport these solutes. Detailed studies for roots or leaves are missing.

Potassium is the major inorganic osmolyte used by most plant tissues. In some cells, such as leaf epidermal cells of barley, K^+ accounts for almost 50% of cell osmotic pressure (Fricke, Leigh & Tomos 1994). Several studies point to the significance of K^+ for elongation growth at organ and cell level. In single-cell cotton fibres, high expression of K^+ and sucrose transporters has been reported (Ruan, Llewellyn & Furbank 2001). In synchronized cell suspension cultures of tobacco, time-dependent inward currents of K^+ were particularly high in elongating cells, and this coincided with more negative membrane potentials (increased driving force for K^+ uptake) as compared to non-elongating cells (Sano *et al.* 2007). In poplar, tobacco and maize, light-stimulated leaf expansion depended on ion transport, and it was postulated that different mechanisms of transport operate in younger and older tissues (Stiles & Van Volkenburgh 2002; Stiles,

McClintick & Van Volkenburgh 2003; Zivanovic, Pang & Shabala 2005). The most detailed study at molecular level exists for coleoptiles of maize, where auxin-induced growth was not observed in the absence of K^+ from bath solution (Claussen *et al.* 1997), and auxin-induced expression of the inward-rectifying K^+ channel *ZmK1* correlated positively with coleoptile growth kinematics (Philippar *et al.* 1999; Fuchs *et al.* 2003).

Grasses offer a convenient experimental system to study growth-related processes in leaves (Fricke 2002). Developmental stages occur in spatially well-defined zones. Cells, particularly epidermal cells, are large enough to be analysed for water and solute relations at cell level (Fricke *et al.* 1994). We have determined previously concentrations of K^+ and Na^+ in growing and non-growing cells of the developing leaf 3 of barley, and observed differences in ion relations, in particular between epidermis and mesophyll, and in relation to salt (Fricke *et al.* 2006). Using solute concentrations and relative growth rates of cells, it was possible to calculate net rates of solute accumulation *in planta* (Fricke & Flowers 1998; Fricke 2002). It is not known to which degree these rates reflect a limitation by associated transport processes at the plasma membrane.

Karley, Leigh & Sanders (2000a,b) used the patch clamp technique to measure ion currents in protoplasts of barley leaves isolated from leaf epidermis and mesophyll. Ion currents between these two tissues did not differ despite considerable differences in ion accumulation, with the exception of a higher $Na^+ : K^+$ selectivity of currents in the epidermis, which coincided with higher accumulation of Na^+ in epidermis compared to mesophyll in response to salt. In the present study, a similar approach was taken to measure K^+ and Na^+ currents in protoplasts isolated from growing and non-growing leaf tissues of barley plants grown under control conditions or exposed to salt (100 mM NaCl). The information gained from patch clamp experiments was combined with membrane potentials of leaf cells measured at various external K^+ concentrations to calculate rates of channel-mediated K^+ (net) uptake, and compare these with actual rates determined *in planta*. Our results show that in most conditions, ion channels in the plasma membrane of elongating leaf cells can provide sufficient rates of uptake of K^+ to support leaf growth. The analysis also reveals what types of channels and transporters are likely to be crucial for K^+-driven growth of barley leaves under low K^+ and high Na^+, thereby providing important background knowledge for future studies into the regulation of these transporters under growth-limiting conditions, as well as gene manipulation for growth enhancement. Cloning and functional characterization of barley genes that may encode these transport pathways are described in a separate paper (Boscari *et al.* 2009).

MATERIALS AND METHODS

Plant material and leaf regions analysed

Barley (*Hordeum vulgare* L. cv. Golf; Svalöf Weibull AB, Svalöv, Sweden) was grown hydroponically on modified Hoagland solution as described previously (Fricke & Peters 2002; see also Boscari *et al.* 2009). The plants were grown in a growth room with 16/8 h day/night cycle at 22/18 °C, relative humidity of 60–70% and photosynthetically active radiation of 250–300 μmol m^{-2} s^{-1}. The plants were analysed when they were 14–15 days old, and leaf 3 was elongating at a near-maximum velocity (2.5–3 mm h^{-1}). For salt treatments, NaCl (final concentration 100 mM) was added to plants up to 3 d prior to harvest; the timing of salt addition was chosen so that the length of leaf 3 at harvest was comparable to that in unstressed, control plants.

A scheme detailing regions of interest along the developing leaf 3 of barley is presented in the accompanying paper (Boscari *et al.* 2009). The developing leaf 3 was analysed either within the (basal) elongation zone or within the portion which has emerged from the sheath of leaf 2 ('emerged blade').

Membrane potential measurements

For measurements of membrane potential in cells of the emerged blade, the midpart of this leaf region was fixed to the bottom of a purpose-built chamber (ca. 5 mL in volume; the leaf was still intact and attached to the plant) such that the lower surface was placed in a reservoir filled with the bathing solution and the upper surface was in the air and illuminated by a cold-light lamp. The bathing solution was in contact with the lower leaf surface in the vicinity of the region where cells were analysed, but it was not in direct contact with these cells.The bathing solution contained the reference electrode, and the chamber was perfused at a rate of 1.5 mL min^{-1} by gravity-suction forces. The leaf was allowed to adapt to this set-up for a minimum of 30 min prior to impalement. The potential of the measuring electrode was zeroed in the bath solution, then a cell was impaled and the membrane potential was recorded.

For measurements of membrane potential in cells of the elongation zone of leaf 3, the elongation zone was exposed by peeling back the sheaths of leaves 1 and 2. The elongation zone was covered with filter paper saturated with 0.1 mM KCl solution (unless stated otherwise), and left to adapt for over 30 min. For measurements, the filter paper was removed from a small spot, leaving an area of about 1 mm^2 exposed; the reference electrode was positioned in contact with the wet filter paper covering the rest of the elongation zone. A small droplet of bath solution was placed onto the exposed surface of the elongation zone, the measuring electrode was zeroed in the droplet, moved into the tissue to impale a cell and the membrane potential was recorded for several minutes. Following this first measurement, the electrode was again zeroed in the same droplet, and the next cell was impaled. The bathing solution was not changed during the impalement. Membrane potential measurements at different bathing medium K^+ were often performed on the same leaf, which was exposed subsequently to increasing K^+ concentrations for at least 30 min prior to measurements.

Table 1. Dimensions and electrophysiological features of protoplasts isolated from the elongation zone and emerged blade portion of the developing leaf 3 of barley (*Hordeum vulgare* L.)

	Elongation zone[a]	Emerged blade	
Variable	Mesophyll/epidermis	Mesophyll	Epidermis
Average protoplast diameter in μm (protoplast number, c, control; s, salt treated)	25.6 ± 8.5 (54, c) 25.0 ± 5.4 (10, s)	30.8 ± 6.7 (26, c) 29.5 ± 6.7 (11, s)	27.8 ± 10.7 (23, c) 23.4 ± 9.6 (8, s)
Average protoplast volume in picolitres	11.8 ± 10.7 (54, c) 9.3 ± 6.3 (10, s)	17.4 ± 10 (26, c) 15.2 ± 8.7 (11, s)	16.6 ± 23.1 (23, c) 10.3 ± 16.0 (8, s)
Occurrence of time-dependent inward current	15 of 42 analysed (36%) (c) 3 of 10 analysed (30%) (s)	1 of 14 analysed (7%) (c) 0 of 11 analysed (0%) (s)	1 of 15 analysed (7%) (c) 0 of 8 analysed (0%) (s)
Occurrence of time-dependent outward current	39 of 42 analysed (93%) (c) 9 of 10 analysed (90%) (s)	7 of 13 analysed (54%) (c) 8 of 11 analysed (73%) (s)	17 of 20 analysed (85%) (c) 6 of 8 analysed (75%) (s)
Size (at –100 mV in 10 mM KCl) and cation selectivity of instantaneous current in control (c) and salt treatment (s)	–10 mA m^{-2} (c) –3 mA m^{-2} (s) Slightly selective for K^+ over Na^+ (c) Non-selective (s)	–4 mA m^{-2} (c) –2 mA m^{-2} (s) Non-selective (c) Slightly selective for K^+ over Na^+ (s)	–45 mA m^{-2} (c) –8 mA m^{-2} (s) Selective for Na^+ over K^+ (c) Non-selective (s)

[a]May apply to both mesophyll and epidermis.

Long-term recordings (>30 min) in the same cell were not possible in the elongation zone because growth displaced the cells and resulted in electrode bending and leakage. For reasons not known to us, it was not possible to obtain reproducible measurements in the elongation zone of plants which had been exposed to salt (100 mM NaCl).

Unless stated otherwise, the bathing solution contained 0.1 mM KCl and 1 mM $CaCl_2$. Calcium was included in the bath solution, because it was also present in the patch clamp experiments. The reference electrode was filled with 100 mM KCl.

Protoplast isolation

Protoplasts were isolated following a procedure developed for *Arabidopsis* roots (Demidchik & Tester 2002). About 100 mg of chopped leaf pieces (1–2 mm long) were digested in 1.2–1.5 mL of enzyme solution containing 1.5% Cellulase Onozuka RS (Yakult Honsha, Tokyo, Japan), 1% Cellulysin (CN Biosciences, Nottingham, UK), 0.1% Pectolyase Y-23 (Kikkoman Co., Noda City, Japan), 0.1% bovine serum albumin (Sigma, St Louis, MO, USA), 10 mM KCl, 10 mM $CaCl_2$, 2 mM $MgCl_2$ and 2 mM 2-(*N*-morpholino)-ethanesulphonic acid (MES) at pH 5.7 (Tris/MES). The osmolality of solutions and of sap extracted from leaf zones (Fricke & Peters 2002) was determined with a vapour pressure osmometer (Wescor 5520, Wescor Inc., Logan, UT, USA). The osmolality of the enzyme solution was adjusted with sorbitol to 350–380 mosmol kg^{-1} (depending on leaf zone and leaf length) for protoplasts from control plants, and to 550–580 mosmol kg^{-1} for protoplasts from salt-treated plants. Leaf pieces were incubated on a shaker (60 rpm) for 90–150 min at 28 °C. The digested tissue was gently washed several times with ice-cold storage solution containing 10 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, pH 5.7 (2 mM MES/Tris) adjusted to the same osmolality as the previously used enzyme solution. Protoplasts were extracted by filtering the digested tissue through 20 μm mesh into 1–2 mL of storage solution. The protoplasts were kept on ice, in the dark and analysed within 8 h following isolation.

Tissue origin of the protoplasts from the emerged blade was clearly indicated by the presence (mesophyll) or absence (epidermis) of numerous chloroplasts (see also Volkov *et al.* 2007). Protoplasts from the elongation zone could not be assigned to any particular tissue, because chloroplasts were much smaller and less differentiated, and the experimental set-up (microscope) did not allow to view those protoplasts which were analysed by patch clamping simultaneously under fluorescence (to look for chlorophyll autofluorescence, Volkov *et al.* 2007). Average diameter and volume of protoplasts used in patch clamp experiments are summarized in Table 1.

Patch clamp

The protoplasts were patch clamped using standard techniques (Amtmann *et al.* 1997). Patch clamp pipettes were pulled on a vertical electrode puller (PP-83; Narashige, Tokyo, Japan) from glass capillaries (Kimax 51, Kimble Products, Vineland, NJ, USA). The pipettes were filled with the 'pipette solution' containing 100 mM KCl, 10 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 2.5 mM EGTA, 2 mM Mg–ATP, 2 mM HEPES, pH 7.0 (Tris). Final pipette resistances were around 10 MΩ. The protoplasts were allowed to settle to the bottom of a measurement chamber (ca. 150 μL in volume), attached to the patch clamp pipette by gentle suction. Once the seal was formed, additional suction was applied to

disrupt the patch and obtain a whole-cell configuration, in which the pipette solution represents the cytoplasmic compartment. The chamber initially contained the 'sealing solution' [20 mM $CaCl_2$, 2 mM MES, pH 5.7 (Tris)], which after seal formation was replaced by differing 'bathing media' containing 1 mM $CaCl_2$, 1 mM $MgCl_2$, 2 mM MES, pH 5.7 (Tris), and varying concentrations of KCl, NaCl and other ions (see figure legends). Bath perfusion rate was 0.14 mL min^{-1}. Osmolality of solutions was adjusted with sorbitol to 380 and 580 mosmol kg^{-1} for control and salt-treated plants, respectively. All solutions were sterile filtered. Experiments were carried out at room temperature (22–25 °C). The reference agar bridge contained 100 mM KCl. Liquid junction potentials (generally below 10 mV) were measured, and the current–voltage (*I–V*) curves corrected accordingly (Amtmann & Sanders 1997).

Ion currents were recorded during application of square voltage pulses from a holding potential (for specifications, see figure legends) and analysed as previously described (Volkov & Amtmann 2006). Instantaneous current was measured at 40–60 ms after change of voltage, and subtracted from total current to obtain time-dependent current. The relative permeability of a current for two ions was calculated from the reversal potentials (E_{rev}, voltage where the current is zero) according to the Goldman–Hodgkin–Katz (GHK) equation (Hille 2001). To account for the fact that ion channel selectivity is not always fully described by the GHK equation, we also determined the relative conductance calculated as the ratio of the chord conductance at a given voltage (slopes of the lines linking the currents at this voltage with 0). Statistical analysis was carried out using standard software packages (Excel 2000 for Windows, Microsoft, and SigmaPlot for Windows; SPSS Science, Chicago, IL, USA). Correlation analysis showed that K^+/Na^+ selectivity was independent of cell size in all protoplasts ($R^2 = 0.10$–0.11).

K^+ flux calculations, and bulk leaf K^+ and Na^+ analyses

Ion currents (mA m^{-2} of protoplast surface) were converted into ion fluxes (mol K^+ min^{-1}) according to Volkov & Amtmann (2006). A current of 1 mA corresponds to a flux of 1 mC s^{-1}, which for a monovalent ion is equal to (Faraday constant)$^{-1}$ or 0.624 μmol min^{-1}. A protoplast with a diameter D of 30 μm will have a surface S ($= \pi \times D^2$) of 2.83×10^{-9} m^2, and a volume V [$= 4/3 \times \pi \times (D/2)^3$] of 1.41×10^{-14} m^3 or 14.1 pL. For this protoplast, a current of 1 mA m^{-2} will be equivalent to an ion flux of 0.624 μmol min^{-1} $m^{-2} \times 2.83 \times 10^{-9}$ $m^2 = 1.77 \times 10^{-15}$ mol min^{-1}. Ion fluxes required to support growth were estimated from the maximal growth rate (relative elongation growth per hour), cell dimension and cell K^+ concentration of 150 mM (see Results). For example, at a maximum relative growth rate of 14% h^{-1} (Fricke *et al.* 1997), net uptake of K^+ required to maintain K^+ concentration in an expanding protoplast of diameter 30 μm would be $14 \text{ pL} \times 0.14 \text{ h}^{-1} \times 150 \text{ mmol L}^{-1} = 4.9 \times 10^{-15}$ mol min^{-1}. For this particular protoplast, a current of 3 mA m^{-2} would suffice to maintain intracellular K^+ constant during elongation.

Bulk leaf extracts were obtained through a centrifugation technique (Fricke & Peters 2002), and analysed for K^+ and Na^+ using inductively coupled plasma atomic emission spectrometry (ICP). Values are given as averages ± SD of four samples, each containing pooled segments of a particular leaf region from three to four plants.

RESULTS

Membrane potentials

We measured the free-running membrane potential (voltage difference across the plasma membrane) in 46 cells (21 different plants) of the emerged blade portion, and in 45 cells (12 plants) of the elongation zone of the developing leaf 3 of barley plants grown on control solution. The average membrane potential was –76.2 ± 7.6 mV in the emerged blade, and –88.7 ± 12.6 mV in the elongation zone (means ± SD; Table 2; values for 0.1 mM K^+ in bath medium). The difference in membrane potential between the two leaf regions was highly significant ($P < 0.001$; Table 2). To evaluate the effect of the apoplastic K^+ environment on the membrane potential, we tested different K^+ concentrations in the bathing solution. Changing K^+ from 0.1 to 1 to 10 mM failed to produce reproducible effects on membrane potentials in the emerged blade portion. In the elongation zone, effects of changes in the bathing medium were reproducible and statistically significant. Here, increasing K^+ concentration in the bathing solution resulted in a decrease of the membrane potential from –88.7 mV in 0.1 mM KCl to –77.6 mV in 1 mM KCl, –67.1 mV in 10 mM KCl and –25 mV in 100 mM KCl (Table 2).

Exposure of the plants to 100 mM NaCl in the growth medium prior to the experiments had little effect on membrane potentials in the emerged blade during the first 60 h,

Table 2. Membrane potential of cells in the elongation zone and emerged blade portion of the developing leaf 3 of barley (*Hordeum vulgare* L.)

K^+ in bath medium (mM)	Membrane potential (mV)	
	Elongation zone	Emerged blade[a]
0.1	–88.7 ± 12.6 (45)	–76.2 ± 7.6*** (46)
1	–77.6 ± 9.6* (9)	
10	–67.1 ± 4.5* (9)	
100	–25.0 ± 3.9*** (4)	

*$P < 0.05$; ***$P < 0.001$.

[a]Successive impalement of epidermal and underlying mesophyll cells did not reveal any significant difference in membrane potential, and values apply to both cell types.

Statistical significance (*t*-test) of difference in values was tested between: (1) elongation zone and emerged blade at 0.1 mM bathing medium K^+; and (2) between successively higher bathing medium K^+ concentrations for the elongation zone.

Table 3. Membrane potential in cells of the emerged blade portion of the developing leaf 3 of barley (*Hordeum vulgare* L.) which was exposed to 100 mM NaCl in the root medium prior to analysis for the times indicated

Duration of NaCl (100 mM) treatment (d)	Membrane potential (mV)
0 (control)	–76.2 ± 7.6 (46)
1.5	–80.7 ± 8.0 (10)
2.5	–71.4 ± 7.2* (9)
3.5	–64.3 ± 2.1*** (3)

The number of cells analysed is shown in parentheses. The difference in membrane potential between plants exposed to salt for 1.5 d and control plants ('0 d') was statistically non-significant (*t*-test). At 2.5 d of NaCl treatment, membrane potential had decreased significantly at $P < 0.05$ (*), and at 3.5 d at $P < 0.001$ (***; compared to 0 d, control).

but caused a depolarization by 12 mV after 84 h of treatment ($P < 0.001$; Table 3).

Ion currents

To identify and characterize the main pathways for K^+ flux across the plasma membrane, we isolated protoplasts from the different leaf zones, and measured electric currents across the plasma membrane during voltage clamp in the whole-cell configuration (patch clamp). Square voltage pulses hyperpolarizing or depolarizing the membrane from a holding potential (usually between –50 and –70 mV) evoked instantaneous changes in the measured current (so-called instantaneous current), and in some but not all protoplasts, an additional current that required some time to reach a new steady-state value (so-called time-dependent current). Time-dependent currents are indicative for voltage-gated channels that open or close in response to a change in voltage, while instantaneous currents are mediated by channels or transporters that are not gated by voltage. Occurrence and features of different types of currents observed in protoplasts from the third leaf of barley are summarized in Table 1.

Currents in mesophyll protoplasts from the emerged blade

Fourteen protoplasts from this tissue were analysed at voltages more negative than –120 mV, and out of these only one (7%) displayed time-dependent inward (negative) current. This current was relatively small (–20 pA at –120 mV, and –40 pA at –180 mV, i.e. –5.5 mA m^{-2} and –11 mA m^{-2}) compared to the one recorded in protoplasts from the elongation zone (see below), and selective for K^+ over Na^+ and Cl^- (data not shown). Time-dependent outward (positive) current at +60 mV or above was found in seven of thirteen protoplasts (54%). Tail currents (currents recorded immediately after changing the voltage from a holding potential at which the channels are open) demonstrated low selectivity of this current for K^+ over Na^+ or Cl^- (data not shown).

All protoplasts exhibited a small instantaneous current (Fig. 1a). The relative permeability of this current for K^+ over Cl^- was at least 7.5 as judged from a shift of reversal potential by –38 ± 9 mV (means ± SD, $n = 7$ protoplasts) when the bathing solution was changed from 100 mM KCl to 10 mM KCl (Fig. 1b). The current was only slightly selective for K^+ over Na^+ with a relative permeability of 1.7 ± 0.47 (means ± SD, $n = 9$) and a relative conductance of 1.32 ± 0.32 (means ± SD, $n = 9$).

Currents in epidermal protoplasts from the emerged blade

Fifteen protoplasts from this tissue were analysed at voltages more negative than –120 mV, and only one (7%) displayed time-dependent inward current. As in mesophyll cells, the current was small (–50 pA at –120 mV equivalent to –7.6 mA m^{-2}) and selective for K^+ over Na^+ and Cl^- (data not shown). A time-dependent outward current was found at voltages of +60 mV and above in 17 out of 20 protoplasts analysed (85%). The current activated slowly, and tail currents demonstrated very low selectivity for K^+ over Na^+ or Cl^- (data not shown).

All epidermal protoplasts produced instantaneous current (Fig. 2a,b). The current was approximately 10 times larger than the instantaneous current in mesophyll protoplasts. Replacing K^+ in the bath with Na^+ caused only small shifts of E_{rev} (1 ± 12.8 mV, $n = 7$ protoplasts), indicating similar permeability for the two ions (Fig. 2c). However, in eight out of twelve protoplasts (67%), currents in 100 mM NaCl were larger than in 100 mM KCl, and showed inward rectification (e.g. the average conductance between –140 and –100 mV was 1.86 ± 0.59 times higher than the average conductance between –60 and –100 mV). Figure 2d shows that the ratio between the averaged currents in 100 mM NaCl and 100 mM KCl was voltage dependent, being approximately 1 at positive voltages and 2.5 at negative voltages. This observation indicates that the instantaneous inward increase in current is related to permeability rather than the number of channels (transporters). An increase in current was also observed when Na^+ was supplied as gluconate salt, showing that it was not because of Na^+ activation of a chloride current (data not shown).

Currents in protoplasts from the leaf elongation zone

Fifteen out of forty-two (36%) protoplasts analysed at voltages more negative than –120 mV displayed time-dependent inward currents (Fig. 3a). In 100 mM KCl, the current had a half-activation potential, V_{50}, of –115 mV ± 6 mV ($n = 4$), and a shallow voltage dependence with a gating charge, z, of 1.34 ± 0.11 ($n = 4$). While time-dependent inward currents of 100 pA and more were recorded in 10 mM KCl, no time-dependent current could be resolved in 100 mM NaCl (Fig. 3b; note that the instantaneous current component increases). K^+ selectivity of the

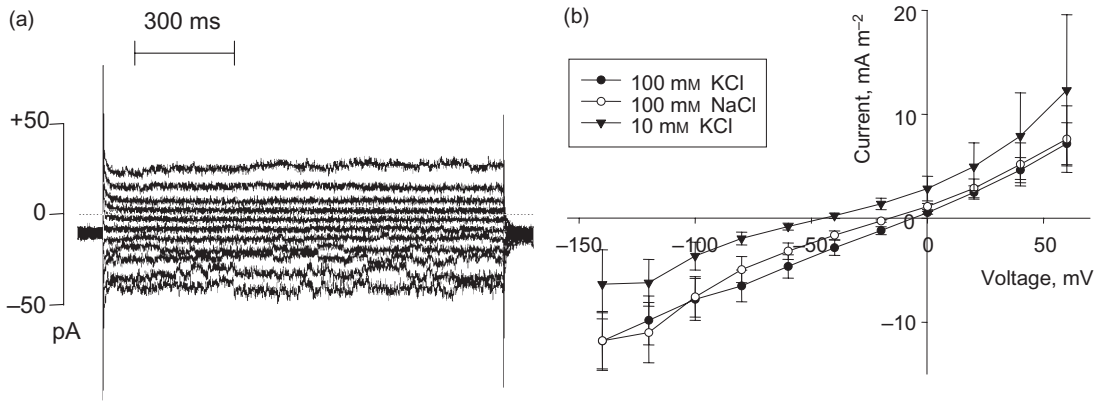

Figure 1. Whole-cell current in mesophyll protoplasts from the emerged blade portion of the developing leaf 3 of barley (*Hordeum vulgare* L.). (a) Typical recording. Protoplast diameter was 38 μm; the bath solution contained 100 mM KCl. The voltage was clamped in steps of 20 mV between –140 and +60 mV from a holding potential of –50 mV. (b) Averaged current–voltage (*I–V*) relationship of the instantaneous current (n = 6–8 protoplasts; error bars are standard errors) at indicated concentrations of KCl and NaCl in the bath. The pipette solution was always 100 mM KCl.

underlying voltage-gated channel was confirmed by the fact that the E_{rev} of tail currents did not change when 90 mM NaCl was added to 10 mM KCl (data not shown), indicating a K^+/Na^+ selectivity of at least 10. The current was reversibly inhibited by 5 mM CsCl (n = 3 protoplasts, Fig. 3c).

Time-dependent outward current at +60 mV or above was found in 39 of 42 protoplasts analysed (93%) (Supporting Information Fig. S1). Tail currents demonstrated that the time-dependent outward current was not selective for K^+ over Na^+ (relative permeability of 1.26 ± 0.14; n = 12

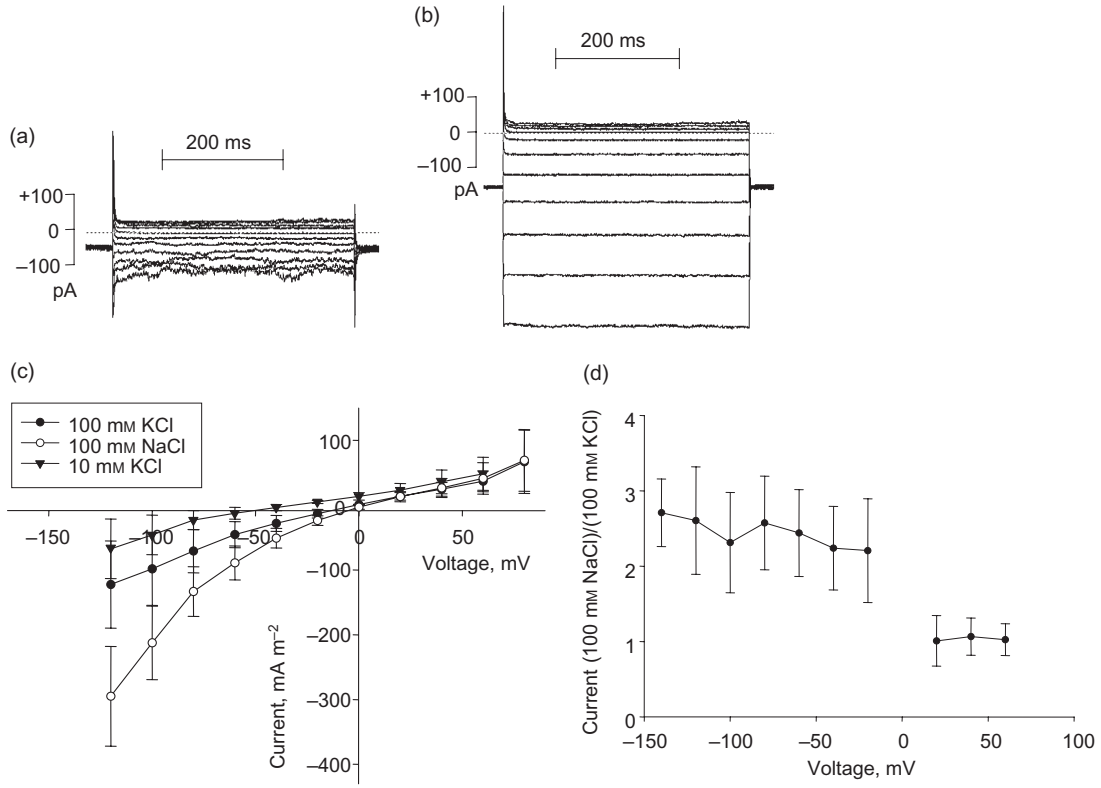

Figure 2. Whole-cell currents in epidermal protoplasts from the emerged blade portion of the developing leaf 3 of barley (*Hordeum vulgare* L.). (a,b) Typical current recordings with (a) 100 mM KCl or (b) 100 mM NaCl in the bath. Protoplast diameter was 29 μm. The voltage was clamped in steps of 20 mV between –120 and +80 mV from a holding potential of –50 mV. (c) Average current–voltage (*I–V*) relationship of the instantaneous current (n = 6–8 protoplasts; error bars are standard errors) at indicated concentrations of KCl and NaCl in the bath. (d) Ratio of instantaneous current measured in 100 mM NaCl and 100 mM KCl at different voltages. The pipette solution was always 100 mM KCl.

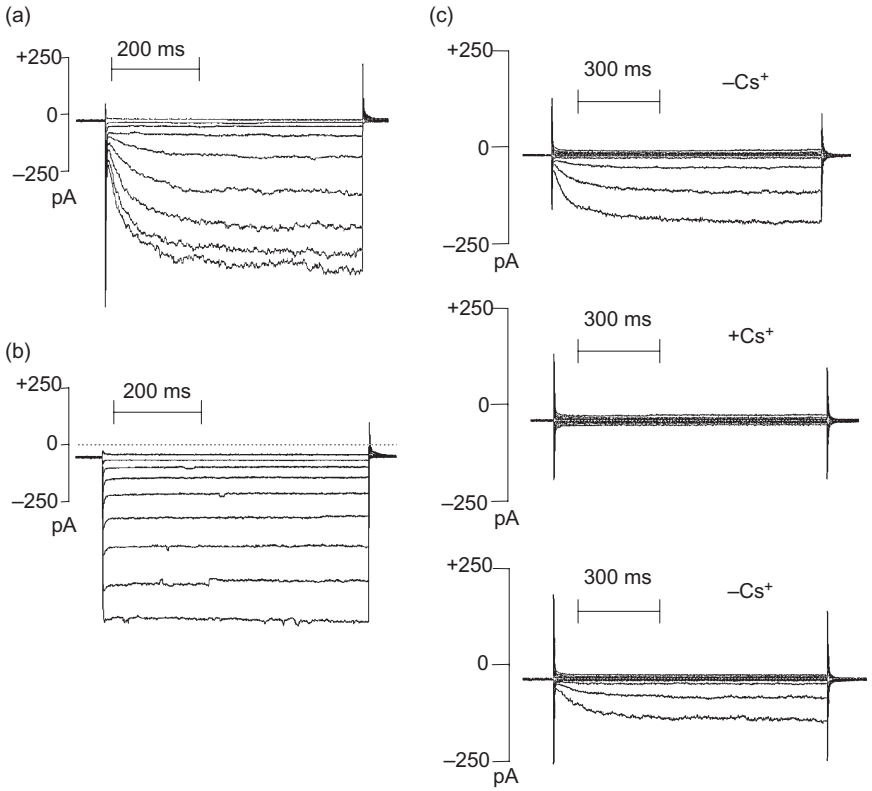

Figure 3. Time-dependent inward current in protoplasts from the elongation zone of the developing leaf 3 of barley (*Hordeum vulgare* L.). (a,b) Typical current recordings at (a) 10 mM KCl or (b) 100 mM NaCl in the bath. The pipette solution contained 100 mM KCl. Protoplast diameter was 34 μm. Holding potential was –50 mV; the voltage was clamped in steps of 20 mV between –200 and –40 mV. Note the absence of time-dependent inward current and the large instantaneous current in 100 mM NaCl. (c) Reversible inhibition of time-dependent inward current by Cs^+. Protoplast diameter was 26 μm; pipette and bath solution contained 100 mM KCl. Current before (top) and after (middle) addition of 5 mM CsCl, and following wash-out (bottom panel) is shown.

protoplasts), and only weakly selective for K^+ over Cl^- (2.46 ± 0.45, $n = 7$). Despite the lack of selectivity, outward-rectifying channels did not produce a significant Na^+ inward current (see Fig. 3b), probably because the activation potential was not negative enough.

The averaged instantaneous current in protoplasts from the elongation zone was larger than in mesophyll, but smaller than in epidermal protoplasts of the emerged blade (Fig. 4a). The current was approximately four times more selective for K^+ than for Cl^- according to a shift of reversal potential by $–30 \pm 7$ mV ($n = 7$ protoplasts), when 100 mM KCl in bath was substituted with 10 mM KCl (Fig. 4b). On average, the current was slightly selective for K^+ over Na^+ with a relative permeability of 1.42 ± 0.22 (calculated from E_{rev} in 100 mM KCl and NaCl), and a relative conductance of 2.22 ± 1.29 ($n = 6$ protoplasts). Combined with the high K^+ selectivity of the time-dependent inward current, this results in an intermediate K^+/Na^+ selectivity of the total inward current (Fig. 4c). However, the selectivity of the instantaneous current differed considerable between individual protoplasts. In some protoplasts, it was slightly selective for K^+ over Na^+, and in this respect resembled the instantaneous current in mesophyll protoplasts of the emerged blade; in other protoplasts, the current was (1.5–4 times) more selective for Na^+ over K^+, thus resembling the current in epidermal protoplasts of the emerged blade. Selectivity for Na^+ over K^+ of the instantaneous current was found in only two of the fourteen protoplasts from the elongation zone that showed no time-dependent inward current, and in two of the eight protoplasts that displayed large time-dependent inward currents. If the cation selectivity of the instantaneous current is a marker that distinguishes epidermal from mesophyll cells, these data suggest that the majority of protoplasts isolated from the elongation zone originated from the mesophyll. This would also mean that the occurrence of time-dependent inward current is not linked to tissue origin, but may differ between growth zones or preparations.

The *I–V* curves determined here allowed us to estimate the amount of K^+ influx into leaf cells of the elongation zone at a given membrane potential. At –67 and –25 mV, the membrane potentials measured *in planta* with 10 mM and 100 mM K^+ in the bathing medium, the average total inward current was 8.7 and 8.1 mA m^{-2}, respectively (Fig. 4c and Table 4). The average instantaneous current at these membrane potentials was 4.5 (in 10 mM KCl) or 8.0 mA m^{-2} (in 100 mM KCl, Fig. 4b), and hence accounted for 52% (in 10 mM KCl) or 99% (in 100 mM KCl) of the total current. Conversely, the time-dependent current constituted 48% (in 10 mM KCl) or 1% (in 100 mM KCl) of the total current (Table 4).

Ion currents in protoplasts of salt-treated plants

Instantaneous current in protoplasts from salt-treated plants differed from that of control plants (Fig. 5). In mesophyll protoplasts from the emerged blade of salt-treated plants, the relative K^+/Na^+ conductance of the instantaneous currents was slightly higher than in the

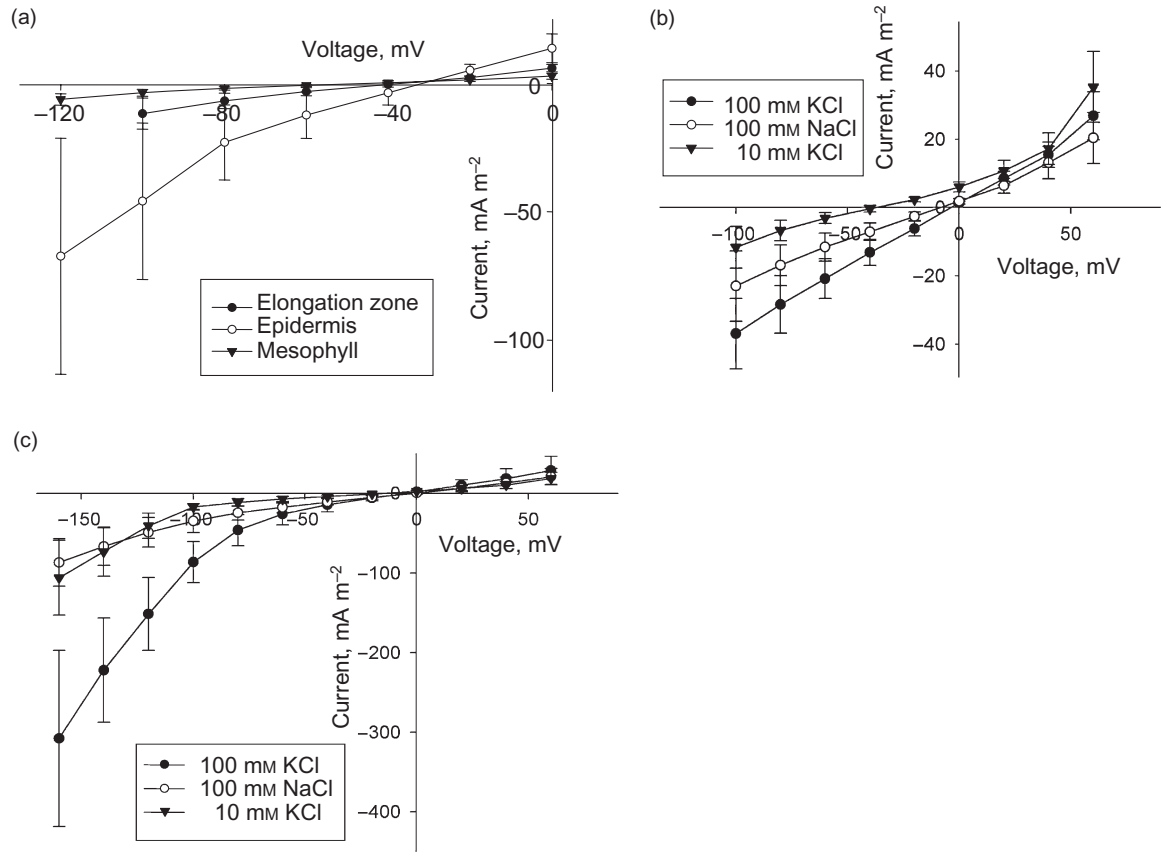

Figure 4. Instantaneous and total current in protoplasts from the developing leaf 3 of barley (*Hordeum vulgare* L.). (a) Comparison of instantaneous current in protoplasts from the elongation zone and emerged blade (mesophyll, epidermis). Bath solution contained 10 mM KCl. (b,c) Averaged whole-cell current–voltage (*I–V*) curves for (b) instantaneous and (c) total currents in protoplasts from the elongation zone at indicated concentrations of KCl and NaCl in the bath (n = 5–7 protoplasts; error bars are standard errors). The pipette solution was always 100 mM KCl.

respective protoplasts of control plants (Fig. 5a compared to Fig. 1b). In epidermal protoplasts from the emerged blade of salt-treated plants, the instantaneous current was considerably smaller than in the respective protoplasts of control plants (threefold lower in 100 mM KCl, and fivefold lower in 100 mM NaCl), and no longer selective for Na^+ over K^+ (Fig. 5b compared to Fig. 2c). In protoplasts from the elongation zone of salt-treated plants, the instantaneous current showed the same relative permeability and conductance for K^+ and Na^+, whereas the corresponding current in control plants was K^+ selective (Fig. 5c compared to Fig. 4b). In 100 mM KCl, the current was less than half of that recorded in control plants.

DISCUSSION

Origin of protoplasts

The protoplasts isolated from the emerged blade portion of leaf 3 could be identified visually as epidermal or mesophyll

Table 4. Size of total, instantaneous and time-dependent inward current in protoplasts isolated from the elongation zone of leaf 3 of barley (*Hordeum vulgare* L.) at the membrane potentials measured with 10 and 100 mM KCl in the bath

K^+ in bath medium (mM)	Membrane potential (mV)	Total protoplast current (mA m^{-2})	Instantaneous current (mA m^{-2})	Time-dependent inward current	
				mA m^{-2}	% of total current
10	–67.1	–8.7	–4.5	–4.2	48
100	–25.0	–8.1	–8.0	–0.1	1

Pipette solution representing the cytoplasm was 100 mM KCl. Values are based on current–voltage (*I–V*) curves shown in Fig. 4 and membrane potentials listed in Table 2.

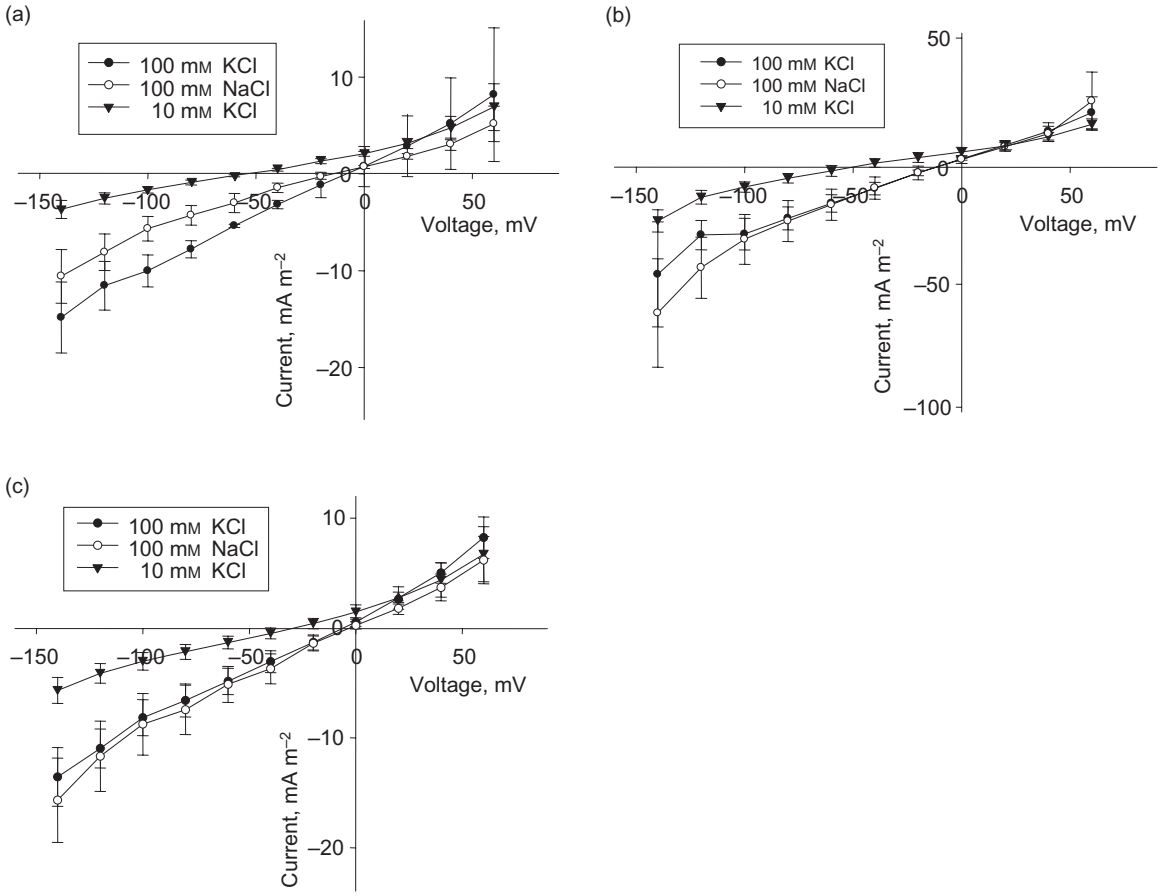

Figure 5. Effect of salt stress on instantaneous current in protoplasts from the elongation zone and emerged blade portion of the developing leaf 3 of barley (*Hordeum vulgare* L.). (a–c) Averaged current–voltage (*I–V*) relationship in protoplasts from (a) mesophyll and (b) epidermis of the emerged blade, and from (c) protoplasts of the elongation zone ($n = 3$–6 protoplasts; error bars are standard errors). Concentrations of KCl and NaCl in the bath are indicated. The pipette solution was always 100 mM KCl. Plants had been exposed to 100 mM NaCl for 3 d prior to protoplast isolation.

in origin (for details, see Volkov *et al.* 2007). The average volume of epidermal protoplasts was 16.6 μL and similar to the volume of mesophyll protoplasts. This implies that epidermal protoplasts did not originate from the much larger epidermal cells which overlie ridges or are located within troughs (ridge and trough cells). Instead, epidermal protoplasts must have originated from the much smaller cells located at the transition between ridge and trough regions, close to stomata (near and interstomatal cells; Fricke, Leigh & Tomos 1996). These cells have K^+ concentrations similar to ridge and trough cells, and respond similar to salinity (Fricke *et al.* 1996); they are therefore representative of the K^+ relations of the epidermis.

In the elongation zone, mesophyll and epidermal protoplasts were less differentiated than in the emerged zone, and it was not possible to visually distinguish between them. The protoplasts were isolated from a 20-mm-long region midway along the elongation zone, where cells had reached 25–75% their final length. In accordance, the average volume of these protoplasts was 69% the volume of protoplasts isolated from the fully expanded emerged blade.

Membrane potentials and driving forces

Changing K^+ from 0.1 to 1 to 10 mM in the external solution failed to produce reproducible effects on membrane potentials in the emerged blade portion. It is possible that the cuticle prevents the perfusion solution from reaching the cells. This explanation is supported by the finding that reproducible changes in membrane potentials were observed in the elongation zone, where the cuticle is not yet fully developed and highly permeable (Richardson *et al.* 2007). The observed difference in membrane potential between cells of the two leaf zones is therefore likely to reflect a difference in apoplastic K^+ concentration rather than a difference in membrane permeability. The membrane potentials measured in the emerged blade were also slightly more depolarized than previously reported values for barley (–83 mV in epidermal cells and –91 mV in mesophyll cells; Cuin *et al.* 2003), probably again because of different apoplastic ionic compositions of the plants used in the studies.

Membrane potentials in the elongation zone readily responded to changes in the bath medium, and membrane

potentials can therefore be used to consider electrochemical gradients for K^+ movement across the plasma membrane. There is generally little information on the cytosolic concentration of K^+ in leaf cells, but triple-barreled H^+- and K^+-selective microelectrodes have been used to determine cytosolic K^+ in the emerged leaf 3 of barley (68 and 79 mM in epidermis and mesophyll, respectively; Cuin *et al.* 2003). Assuming similar cytosolic K^+ concentrations in growing leaf cells, the measured membrane potentials are more negative than the equilibrium potentials of K^+, E_K, for 10 and 100 mM external K^+ (and as low as about 5 mM external K^+), but more positive than E_K for 1 and 0.1 mM K^+. This means that at physiological apoplastic K^+ concentrations (Felle *et al.* 2005), the electrochemical driving force for K^+ is directed into the cell; K^+ uptake is passive and can occur through ion channels (although other types of transporters may also contribute). If, however, the apoplastic K^+ concentration drops significantly below 10 mM (e.g. during K^+ starvation or salt stress; see below) K^+ uptake will require active transport systems to overcome the outward directed electrochemical gradient. A recently cloned putative barley K^+ transporter of the HAK/KUP/KT family, HvHAK4, which is expressed particularly in the elongation zone of leaves, could be an interesting candidate for this function.

Time-dependent K^+-selective inward current

The time-dependent inward currents recorded here in protoplasts from barley leaf 3 have properties that resemble those measured in protoplasts from barley aleurone (van Duijn *et al.* 1996) and leaf 1 (Karley *et al.* 2000a). However, in contrast to the latter study, which reported the occurrence of time-dependent inward currents in all protoplasts analysed (epidermis and mesophyll), we only detected these currents in a small proportion of protoplasts (7% in the emerged blade, and 37% in the elongation zone). Leaves used by Karley *et al.* (2000a) were close to full expansion, and protoplasts of the mature region must have been included based on volume of protoplasts reported and by-chance selection. We grew barley plants under the same conditions as detailed by Karley *et al.* (2000a), and isolated and analysed protoplasts of leaf 1 using the same procedures as for leaf 3, but failed to detect time-dependent inward currents. Similarly, in patch clamp studies on leaf protoplasts from other species, some authors observed time-dependent K^+-selective inward current [e.g. Kourie & Goldsmith 1992 (*Avena sativa*)], whereas others did not [e.g. Li & Assmann 1993 (*Vicia faba*); Bei & Luan 1998 (*Nicotiana tabacum*)], but no systematic difference is apparent when comparing experimental protocols between these studies. No correlation was found in our study between the occurrence of the time-dependent inward current and the characteristics of any other current (e.g. $Na^+ : K^+$ selectivity of instantaneous current) or protoplast size.

In an accompanying study (Boscari *et al.* 2009), we have cloned and functionally characterized barley homologs of Shaker-type channels that typically mediate time-dependent K^+ inward currents (Véry & Sentenac 2002). Expression analysis of *HvAKT1* and *HvAKT2* by PCR and *in situ* hybridization showed that both channels are expressed in growing and emerged blade tissue (Boscari *et al.* 2009). This suggests that absence of time-dependent K^+-inward current in protoplasts does not reflect lack of expression *in planta*. However, as is the case for *AtAKT1* (Li *et al.* 2006; Xu *et al.* 2006), functional expression of *HvAKT1* in *Xenopus laevis* oocytes required simultaneous expression of a calcium-binding protein (CBL) and a CBL-dependent kinase (CIPK, Boscari *et al.* 2009). It is possible that these and/or other regulatory factors are sensitive to unknown (and uncontrolled) parameters of the protoplast isolation procedure, or indeed determine the physiological state of a cell. Whatever factor is responsible for the retention of time-dependent currents in protoplasts, it appears to be better preserved in protoplasts isolated from growing than in protoplasts isolated from mature leaf tissue.

Flux of K^+ associated with leaf growth

The overall K^+ concentration in a typical cell (epidermis or mesophyll) of the elongation zone of leaf 3 of barley is 150 mM (Fricke 2004; Fricke *et al.* 2006). Cells expand at maximum relative rates of up to 14% h^{-1} (Fricke *et al.* 1997). This means that *in planta*, K^+ accumulates at a net rate of up to ($0.14\ h^{-1} \times 150$ mM =) 21 mM h^{-1}. The water potential of an elongating leaf cell is –0.25 MPa (Fricke & Peters 2002). The protoplast of a cell is in local water potential equilibrium with the apoplast because of very short (<1 s) half times of water exchange (Volkov *et al.* 2007). If most of the apoplastic water potential is caused by osmotic potential, rather than tension (plants in the cited studies were transpiring little during analyses) or matrix potential, the apoplast solute concentration of a growing leaf cell is close to 100 mM (equivalent to 0.25 MPa of osmotic pressure). Half or two-thirds (if counterbalanced by mono- or divalent anion, respectively) of apoplast osmotic pressure can be caused by 50–66 mM K^+, which is within the range of external K^+ concentrations used in the bathing medium during patch clamp analyses.

Clearly, the ion currents measured in patch clamp experiments do not reflect *in vivo* ion fluxes in leaves. The cell wall free protoplasts lack the apoplastic environment encountered in the intact plant, and the whole-cell configuration removes potentially important regulators from the cytoplasmic side of the membrane. In addition, growth rates of protoplasts may differ from growth rates of cells (of the leaf elongation zone) from which these protoplasts were isolated. Nevertheless, comparison of the measured currents with fluxes measured *in planta* can be useful to assess whether ion channels contribute to these fluxes, and whether their regulation could account for growth inhibition in different environmental conditions. Based on membrane potentials (Table 2) and *I–V* curves determined at corresponding external K^+ concentrations (Fig. 4), the total inward current in protoplasts of the elongation zone was

–8.1 to –8.7 mA m^{-2} when a time-dependent current was present (Table 4). Considering an average surface of protoplasts of the elongation zone of 2.06×10^{-9} m^2 and an average volume of 11.8 pL, this current could provide a net K^+ accumulation rate of 52–56 mM h^{-1}, which is 2.5–2.7 times the net rate *in planta* (21 mM h^{-1}). Kourie & Goldsmith (1992), using patch clamp, obtained a slightly higher value (120 mM h^{-1}) for whole-cell K^+ current in (mature) oat mesophyll at 10 mM K^+ in bathing medium.

The data suggest that expansion growth of barley leaf cells is not limited by the capacity of K^+-uptake pathways in their plasma membranes, similar to expansion in leaves of the dicot tobacco (Stiles & Van Volkenburgh 2004). However, the data also suggest that any significant (factor 2 and larger) reduction in current (e.g low apoplastic K^+ under K^+ deprivation or channel inhibition by salt stress-induced signals) is likely to impact on growth. Furthermore, the presence of the time-dependent inward current could be critical for growth-related K^+ uptake when the apoplastic concentration in the leaves is as low as 10 mM (the contribution of time-dependent current to the total inward current is 48% in 10 mM KCl). Hence, the regulation of inward-rectifying K^+ channels under growth-inhibiting conditions will require particular attention in future research.

Cellular K^+ : Na^+ ratios in relation to K^+ and Na^+ currents in protoplasts

The developing leaf 3 of barley is a sink for K^+ and Na^+, and does not export these ions at significant quantities. The concentration of K^+ and Na^+ in the elongation zone is 167 ± 20 mM and 5.4 ± 0.7 mM, respectively, at a ratio of K^+ : Na^+ of 31; most of this K^+ and Na^+ is contained within the protoplasmic space of cells. This is almost eight times the ratio in the nutrient solution (K^+ : Na^+ ratio 4:1) and results from the supply of K^+ through phloem, which has a K^+ : Na^+ ratio exceeding 40 (Wolf & Jeschke 1987), and possibly from K^+ selectivity of root xylem loading. Transport properties for K^+ and Na^+ of cells may contribute to the tissue-specific accumulation of these ions in epidermis and mesophyll (Leigh & Tomos 1993; Karley *et al.* 2000b), but can they facilitate an accumulation of K^+ and Na^+ at a bulk ratio of 31?

At –25 mV, the average membrane potential of (epidermal and mesophyll) cells in the elongation zone at 100 mM K^+ in bath medium, the total measured inward current was –8.1 mA m^{-2} with 100 mM KCl, and –6.6 mA m^{-2} with 100 mM NaCl in bathing medium. Assuming independent movement of K^+ and Na^+ across the plasma membrane, this would result in an accumulation ratio of K^+ : Na^+ close to 1.0 at equal concentrations of K^+ and Na^+ in the apoplast. Thus, the actual ratio of K^+ : Na^+ in cells (31) cannot be explained through ion channel properties of the plasma membrane. Instead, this ratio could be caused by a high K^+ : Na^+ ratio in the apoplast and/or differences in the K^+/Na^+ selectivity of tonoplast currents (Czempinski *et al.* 2002; Gobert *et al.* 2007; for review, see Maathuis 2007).

K^+ and Na^+ currents, and accumulation in NaCl-treated plants

During plant exposure to 100 mM NaCl, the K^+ : Na^+ ratio in nutrient solution was at most 1:50. The ratio at which these two ions accumulate in the emerged blade (those cells that were after 3–5 d of stress in the emerged blade had been in the elongation zone at start of stress) is about 1:4.5 (bulk K^+ and Na^+ increase by 25 and 110 mM, respectively; Fricke *et al.* 2006). There is a combined selectivity of around 11 (50/4.5) for K^+ over Na^+ along the transport paths between external (nutrient solution) or internal (leaf 2) ion source and the sink leaf 3 – a selectivity similar to that in unstressed plants.

Epidermal and mesophyll cells of the emerged blade of 3 d NaCl-treated plants accumulate similar concentrations of Na^+ and K^+ (after 3 d of stress; Fricke *et al.* 2006). Nevertheless, we found cell-specific responses of ion currents to salt treatment. In epidermal cells, the instantaneous current decreased by a factor of 3–4 in response to salt (but was still two to three times higher than in mesophyll cells) and lost its selectivity for Na^+ over K^+. In mesophyll cells, the instantaneous current retained its selectivity for K^+ over Na^+ in salt-treated plants. Both responses could be a means to limit Na^+ accumulation and retain K^+.

In the elongation zone, the instantaneous current decreased in response to salt, by factor of 3 (Na^+) to 4 (K^+) which may aid this tissue to prevent overloading with Na^+. However, the current was also less selective for K^+ over Na^+ compared to control conditions. The tissue origin of these protoplasts is not known, and the decrease in K^+ selectivity of instantaneous current could reflect a higher proportion of epidermal protoplasts analysed in salt-treated plants.

CONCLUSIONS

The capacity of the plasma membrane transport systems that contribute to accumulation of K^+ in elongating barley leaf cells is unlikely to limit leaf growth under K-replete conditions. At physiological apoplastic K^+ concentrations around 10 mM, the ion channels identified in the plasma membrane of protoplasts from growing barley leaves provide sufficient capacity for net K^+ uptake with voltage-independent and inward-rectifying K^+ channels equally contributing to the K^+ inward current. At higher apoplastic K^+ concentrations, voltage-independent K^+ uptake pathways become relatively more important.

While our experiments show that ion channels have the basic capacity for growth-sustaining K^+ uptake, net uptake rates could be reduced *in vivo* by intra- and extracellular regulators inhibiting channel activity, or by electroneutral K^+ efflux (invisible in patch clamp experiments) counteracting K^+ inward current. For example, it has been suggested that K^+/H^+ antiport could be a means for cytoplasmic K^+ and pH regulation in plant cells (Britto & Kronzucker 2006; Szczerba, Britto & Kronzucker 2006). In addition, our data indicate that at apoplastic K^+ concentrations of 1 mM or less, channels have to be replaced by active K^+ uptake systems [e.g. K^+/H^+ symport (Maathuis & Sanders 1994)].

It is also important to note that unidirectional K^+ fluxes across the plasma membrane of leaf cells are likely to be much, much larger than the net inward current measured here. Radioactive tracer flux analysis have provided evidence for a considerable rate of electroneutral K^+/K^+ exchange in roots of barley seedlings (Britto & Kronzucker 2006; Szczerba *et al.* 2006). Some of this 'futile cycling' can be accounted for by bidirectional K^+ movement through ion channels, but K^+ flux through non-channel type transporters such as KUP/HAK/KT or HKT-type transporters is likely to make an important contribution. While K^+/K^+ exchange does not contribute to growth-related net K^+ uptake itself, it could become an important factor for growth because of its considerable drain on energy resources.

Future experiments will have to address these issues. Most importantly, *in vivo* fluxes, and apoplastic K^+ concentrations and plasma membrane electrical potential should be measured in different environmental conditions, and correlated with growth rates. The occurrence of potential regulators and their action on the ion channels presented here should be characterised to evaluate their potential role in linking environmental stress factors to growth inhibition.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Time-dependent outward current in a protoplast from the elongation zone of the developing leaf 3 of barley (*Hordeum vulgare* L.). The current shows slow activation kinetics and has no significant selectivity between potassium and sodium (as shown by reversal potential of tail currents of –4 mV – would be –60 mV for a $K^+ : Na^+$ selectivity of 10). Protoplast diameter was 17 μm; bath solution contained 100 NaCl; pipette solution was 100 mM KCl. The protoplast was kept at +80 mV; the tail currents are obtained by clamping the voltage from –24 to +21 mV in successive steps by 5 mV.

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