Cation currents in protoplasts from the roots of a Na\(^+\) hyperaccumulating mutant of \textit{Capsicum annuum}

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Abstract

A wiltly mutant (scabrous diminutive, \textit{sd}) of \textit{Capsicum annuum} L. hyperaccumulates Na\(^+\) in all tissues and has a lower K\(^+\) content in the roots. This has been shown to be due to a greater efflux of \(^{86}\text{Rb}^+\) (K\(^+\)) and influx of \(^{22}\text{Na}^+\) in the mutant. In this study, the transporters responsible for these fluxes were investigated by applying patch clamp techniques to protoplasts derived from root cortical cells. Inwardly rectifying K\(^+\) currents were comparable in the two genotypes, but a characteristically bigger outward K\(^+\) current was observed in protoplasts from mutant roots, correlating with a bigger efflux of \(^{86}\text{Rb}^+\) from mutant plants. Whole-cell currents due to the movement of Na\(^+\) have also been studied in both genotypes. The magnitude of the time-independent inward currents that conduct Na\(^+\) at hyperpolarizing voltages were comparable in both genotypes. However, microelectrode measurements of membrane potentials in cortical cells of roots in high Na\(^+\) conditions revealed that the membrane potentials of the root cells in the mutants were approximately 60 mV more negative than in wild-type root cells. Quantitatively, this hyperpolarization is calculated to be sufficient to account for the increased Na\(^+\) influx in the mutants.

Key words: K\(^+\) channels, Na\(^+\) transport, patch clamp, protoplasts, root cation uptake, salinity tolerance.

Introduction

Soil salinity (due mostly to high concentrations of Na\(^+\) salts) is one of the environmental stresses that most affects plant productivity globally (Boyer, 1982). Analysis of the basis for salinity toxicity has often shown specific toxic effects of Na\(^+\) ions (Kingsbury and Epstein, 1986; Munns, 1993). Excessive Na\(^+\) influx results in toxic levels of Na\(^+\) building up in the cytoplasm, bringing about a range of detrimental cellular effects (Munns, 2002; Volkmar et al., 1999; Tester and Davenport, 2003).

The electrochemical potential difference for Na\(^+\) is such that Na\(^+\) influx across the plasma membrane of plant cells is passive and efflux is active at most physiological Na\(^+\) concentrations and membrane potentials (Maathuis and Amtmann, 1999). \textit{AtHKT1} encodes, in heterologous systems, a Na\(^+\) influx pathway, although, \textit{in planta}, it appears to have more of a role in root-to-shoot allocation than in initial root influx (Berthomieu et al., 2003; Essah et al., 2003). From electrophysiological and radioactive tracer studies on plant material, evidence has recently been accumulating to suggest that weakly voltage-dependent non-selective cation channels (NSCCs) are a major pathway for Na\(^+\) entry (Amtmann and Sanders, 1999; Davenport and Tester, 2000; Tester and Davenport, 2003). Na\(^+\) permeable NSCCs were first described at the single channel level in plants after incorporation of plasma membrane-enriched vesicles from rye roots into planar lipid bilayers (White and Tester, 1992), work confirmed using patch clamp techniques by White and Lemtiri-Chlieh (1995), and reviewed by Demidchik et al. (2002). Further patch clamp studies on maize and wheat roots confirmed the presence of NSCCs that are selective for K\(^+\) but are also significantly permeable to Na\(^+\) (Roberts and Tester, 1997; Tyerman et al., 1997). A comparison of the contribution of K\(^+\) inward rectifying channels and NSCCs towards K\(^+\) and Na\(^+\) influx suggested that the transport of K\(^+\) through NSCCs is negligible due to their presence in small numbers. Conversely, in saline conditions where the Na\(^+\)/K\(^+\) ratio is high, the NSCCs would allow a large influx of Na\(^+\)
over a wide range of voltages (Maathuis and Amtmann, 1999). Further support for the likely importance of these channels for toxic Na⁺ uptake has been provided by work with wheat, in which remarkable similarities were seen in the Ca²⁺ inhibition of Na⁺ toxicity, ²²Na⁺ influx into intact roots and Na⁺ currents through single NSCCs (Davenport and Tester, 2000). To try to improve current understanding of the possible mechanisms involved in Na⁺ influx, the Na⁺ hyper-accumulating sd mutant of Capsicum annuum was studied.

A spontaneous monogenic mutation of pepper, Capsicum annuum L. scabrous diminutive (sd), originally described by Bergh and Lippert (1964), has a wide range of effects on the phenotype. One of the most notable of these is the excessive accumulation of Na⁺ in the shoots (Tal et al., 1976). Flux studies on excised roots of these plants showed that the influx of ²²Na⁺ into the roots of mutant plants was much higher than into the roots of wild-type plants (Tal and Benzioni, 1977). The rate of Na⁺ efflux from mutant roots was twice as high as from wild-type roots (Tal and Benzioni, 1977). These results imply that there is some alteration in the mechanism of Na⁺ transport in the mutant plants, causing an accumulation of this ion, which makes it an interesting system to investigate the mechanism and control of Na⁺ entry into plants.

Benzioni and Tal (1978) also studied K⁺ fluxes in the wild type and mutant of C. annuum, using ⁸⁶Rb⁺ as an analogue. The unidirectional ⁸⁶Rb⁺ influx was similar in both the genotypes in the range where the low affinity K⁺ transporters dominate, but ⁸⁶Rb⁺ influx was slower in the mutants in conditions where the high affinity K⁺ transport system was dominant. The rate of ⁸⁶Rb⁺ efflux from mutant roots was twice as high as from wild-type roots. Furthermore, JR Morton, DG Lurie, Z Cohen, and M Tal (unpublished results) found that 25 mM TEA inhibited Rb⁺ efflux by 14% in the wild-type roots, whereas an inhibition of 31% was seen in the mutants. Thus, there is evidence for at least four distinct ion transport processes being affected by the mutation: elevated low affinity Na⁺ influx, inhibited Na⁺ efflux, inhibited high affinity K⁺ influx, and elevated K⁺ efflux. These may be due to effects on four transport proteins, namely, NSCCs or HKT1 (for Na⁺ influx), a Na⁺/H⁺ antiporter (for Na⁺ efflux), a K⁺/H⁺ symporter (for high affinity K⁺ influx), and an outward rectifying (OR) K⁺ channel (for K⁺ efflux). In this study, the currents due to K⁺ and Na⁺ have been characterized by patch clamping protoplasts isolated from the roots of wild-type and sd mutant C. annuum to elucidate some of the differences in K⁺ and Na⁺ transport between the two genotypes.

Materials and methods

Plant growth and protoplast preparation

Seeds of Capsicum annuum L. were a generous gift from Professor M Tal (Ben Gurion University of the Negev, Israel) and Dr D Garvin (Cornell University, USA). Wild-type and mutant plants of C. annuum were grown in vermiculite which was watered daily to field capacity with deionized water and given 1/10th Hoagland’s solution twice a week. They were grown in large walk-in growth rooms with a photon irradiance of approximately 300 µmol m⁻² s⁻¹ PAR and a 12/12 h day/night cycle (30/15 °C).

Roots from about 25–30-d-old seedlings were used for the protoplast isolation. Roots were washed briefly in running tap water after removal from the vermiculite. About 1 g of whole lateral roots was finely chopped in 5 ml of solution containing 1 mM CaCl₂, 0.26% (w/v) BSA, 1.7% (w/v) cellulose (Onozuka RS; Yakult Honsha, Tokyo), 0.026% (w/v) pectolyase (Sigma, Poole, UK), 1.7% (w/v) cellulysin (Calbiochem, UK), 285 mM sorbitol, and titrated to pH 5.5 with KOH. The tissue was incubated for 90 min at 30 °C, shaking at approximately 30 rpm. The digest was filtered through nylon mesh (30 µm diameter) to remove most debris and centrifuged at 60 g for 5 min. The pellet was resuspended in 1 ml of ice-cold 500 mM sucrose, 1 mM CaCl₂, 5 mM MES/KOH, pH 6.0, and stored on ice.

Protoplasts isolated from both the wild-type and mutant roots were typically 15–20 µm in diameter and contained a large vacuole and granular cytoplasm. It is probable that the protoplasts mainly originated from the mature root cortex because light microscopic examination of the enzyme-digested root tissue by safranin and fast green staining, revealed that all parts of the root except the epidermis and cortex were intact, and the cortex contributes by far the most root volume.

Patch clamp electrophysiology

Whole-cell currents from protoplasts were recorded at room temperature (approximately 20 °C) with an Axopatch 200A amplifier (Axon Instruments, USA) using conventional patch clamp techniques (Hamill et al., 1981).

Cells were held in a flowing chamber of less than 0.5 ml volume, allowing fast solution changes with perfusion at a rate of 0.4 ml min⁻¹. The chamber had a thin glass base to which protoplasts adhered loosely. Electrodes from borosilicate glass capillaries (Kimax 51, Kimax Products, USA) were pulled and fire polished using a Zeitz Instrumente Universal Puller (Augsburg, Germany) to give resistances of 10–11 MΩ in the ‘standard bath solution’ (which is described below). A Ag/AgCl reference electrode was connected to the bath via a 3 M KCl/agar salt bridge. The whole-cell configuration of the patch clamp technique was obtained by gentle suction leading to gigohm seals, followed by a strong suction pulse applied to the interior of the pipette to obtain access to the cytosol. Whole-cell capacitance was partially compensated for by the amplifier. Access resistance was usually less than 30 MΩ. Before analogue-to-digital conversion, the voltage signals representing clamp currents were low-pass filtered at 2 kHz, unless stated otherwise. The generation of voltage test pulses, recording of whole-cell currents and storage of data were controlled by the software package pClamp 6.0 (Axon Instruments) and a personal computer. Analysis of data was done using pClamp software and Fig.P (version 2.2, Biosoft, Cambridge, UK).

Liquid-junction potentials ranged from +0.5 mV to −6 mV and were corrected whenever they were more than 2 mV, as described by Neher (1992). Corrections were also made for tip potentials. Ion equilibrium potentials were calculated after correction for ionic activities (as calculated by GEOCHEM-PC: Parker et al., 1995). The magnitudes of the time-dependent (TD) outward rectifying (OR) and inward rectifying (IR) currents were calculated by subtraction of the instantaneous currents from steady-state currents, unless stated otherwise. Variation in data is presented as the standard error of the mean followed by the number of replicates in parentheses.

Experimental solutions

All solutions were filtered (0.22 µm, Millipore) before use. Gigaohm seals were formed in the standard bath solution containing 30 mM
KCl, 2.1 mM CaCl₂, 5 mM MES, 5.5 mM KOH, pH 6.0 and an osmotic potential of 700 mOsm kg⁻¹ using 592 mM sorbitol. Osmotic pressure was measured using a Wescor 5520 vapour pressure osmometer. After obtaining a whole-cell configuration, all currents were recorded in this solution. For experiments where external K⁺ concentrations were varied, the other components in the bath solution, including the osmolality were kept the same as in the standard bath solution. In experiments where the Cl⁻ concentration in the bath was lowered to 4 mM, 30 mM K-glucuronate replaced equimolar KCl.

A standard intracellular (pipette-filling) solution containing 100 mM K-glucuronate, 3 mM MgCl₂, 3 mM K₂ATP, 4 mM EGTA, 10 mM HEPES (adjusted to pH 7.2 with 17 mM KOH and 720 mOsm kg⁻¹ using 609 mM sorbitol) was used in the experiments. In experiments excluding intracellular Cl⁻, no MgCl₂ was added, and the K₂ATP was replaced with MgATP. When this latter solution was used, about 4 cm of a 5.5 cm pipette was filled, then, to maintain a functional half cell, 10 mM KCl was back-filled into the top 1 cm or so of the pipette. No drift in electrical properties attributable to diffusion of salts from this backfilling was ever observed.

Membrane potential measurements

Roots from about 25–30-d-old seedlings were briefly washed in running tap water after removal from the vermiculite. Lateral roots were secured in a small Perspex chamber (volume 4 ml) using Blu-tac. All impalements were made in a bathing solution containing 0.1 mM KCl, 1 mM CaCl₂, and 5 mM MES/BTP, pH 6. Solution was added at the base of one side of the Perspex chamber and removed from the opposite end of the chamber by a Gilson Minipuls 3 peristaltic pump, at a rate of 0.5 ml min⁻¹.

Triangular cross-section glass pipettes (1.2 mm height and 0.25 mm wall thickness) were pulled using a Narishige vertical pipette puller and filled with 150 mM KCl. Both electrodes were placed in the impalement solution and the potential difference between them was zeroed. One microelectrode was then inserted into the root using earthed Prior micro-manipulators, visualized with a horizontal Wild dissecting microscope at ×50 magnification. A voltage follower (designed by Mr Peter Joyce, Department of Physiology, University of Cambridge) and Tekman TE 850 pen recorder were used to measure and record the membrane voltage.

Capsicum roots are thin and thus were difficult to immobilize perfectly; combined with the optics of our impalement set-up, it was not possible confidently to identify the cell type being impaled for each membrane potential measurement. It is highly likely that a mixture of epidermal and cortical cells were impaled. If there were significant differences in membrane potential between the two cell types, a disjunction in the measurements would have been observed. As no such disjunction was evident, it seems likely that the measurements made reflected those of cortical cells.

Results

Similar inward K⁺ currents in the two genotypes

Plasma membrane K⁺ channels have been characterized from various cell types, including root hairs, cortex, and stele in a range of plants including wheat, maize, and Arabidopsis (reviewed in White, 1997). As found in many plants and tissues, in the plasma membrane of protoplasts isolated from Capsicum annuum roots, a time- and voltage-dependent inward K⁺ current was measured upon hyper-polarization negative of −100 mV. This was observed at high frequency in both genotypes (Fig. 1). It was observed that inward TD current densities fell into two distinct populations that could be separated by 30 mA m⁻². The means of the current density of these two populations were different by a factor of about three and were significantly different using a single factor Anova test (P <0.01 for wild type and P <0.1 for mutant). The frequency of protoplasts with small inward currents and their average current densities were similar in wild-type and mutant plants (Table 1). Since these protoplasts would contribute relatively much less to total K⁺ uptake into roots compared with the protoplasts with bigger TD inward currents, and as there were no differences in these smaller currents between genotypes, they were excluded from further analysis.

The larger TD inward currents had slightly different activation kinetics, with the mutant having an additional fast component (Table 1). In both genotypes, the extracellular K⁺ concentration was varied from 30 to 100 mM and the reversal potential from tail current analysis showed that the currents were selective for K⁺ (Murthy, 1999).

Different outward K⁺ currents in the two genotypes

The TD OR K⁺ currents in protoplasts from the wild-type roots in standard conditions were small, with plasma membrane depolarization to +100 mV resulting in only small, slowly activating TD outward currents (Fig. 2A, C). By contrast, in the mutants, depolarization positive of +5 mV resulted in large TD outward currents in most protoplasts (Fig. 2B, D) (P <0.0001). In the currents from the wild type, the signal–noise ratios were very small, precluding quantitative analysis. However, in the mutants, the TD outward currents activated sigmoidally in all protoplasts (Table 1).

In both the wild type and mutants, the tail current analysis showed that the TD outward currents were selective for K⁺. Furthermore, reduction of external Cl⁻ had no effect on the characteristics of the inward currents. With 4 mM external Cl⁻, the mean current densities at +100 mV were 7.5±3 mA m⁻² (n=6) in the wild type and 143±61 mA m⁻² (n=5) in the mutant (cf similar values with 34 mM Cl⁻ in standard bath solution; Table 1).

When wild-type protoplasts were clamped at membrane potentials positive of +100 mV, a transient TD outward current was observed in 43% of protoplasts. These currents were initially large and inactivated slowly (Fig. 3). Such inactivating currents were seen only in those protoplasts where the small TD outward currents were not seen and they were never observed in protoplasts from mutant plants. The mean current density at +160 mV in the protoplasts showing these currents was 742±200 mA m⁻² (n=20). The TD inactivation of these outward currents appeared similar to transient outward K⁺ currents described in Arabidopsis thaliana guard cells by Pei et al. (1998) and outward K⁺ currents conducted by the channel encoded by the mRNA shal (a sister gene of the Drosophila Shaker gene), when injected in Xenopus oocytes (Wei et al., 1990). However,
### Table 1. Characteristics of the TD inward and outward currents in the plasma membrane of protoplasts isolated from the roots of wild-type and mutant *Capsicum annuum* with standard bath and pipette solutions

<table>
<thead>
<tr>
<th>Type of current</th>
<th>Inward currents at −160 mV</th>
<th>Outward currents at +100 mV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genotype</td>
<td>Wild type</td>
<td>Mutant</td>
</tr>
<tr>
<td><strong>Small currents</strong>&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Current density (mA m&lt;sup&gt;−2&lt;/sup&gt;)</td>
<td>−29±6 (9)</td>
<td>−28±5 (5)</td>
</tr>
<tr>
<td>% of protoplasts</td>
<td>38%</td>
<td>31%</td>
</tr>
<tr>
<td><strong>Large currents</strong>&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Current density (mA m&lt;sup&gt;−2&lt;/sup&gt;)</td>
<td>−109±30 (10)</td>
<td>−81±41 (6)</td>
</tr>
<tr>
<td>% of protoplasts</td>
<td>42%</td>
<td>38%</td>
</tr>
<tr>
<td>Activation kinetics&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1 exponential</td>
<td>2 exponentials</td>
</tr>
<tr>
<td>Time constants (ms)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>τ=858±14 (3)</td>
<td>τ&lt;sub&gt;1&lt;/sub&gt;=791±120 (3)</td>
</tr>
</tbody>
</table>

<sup>a</sup>‘Small currents’ defined as having a density of <40 mA m<sup>−2</sup>. ‘Large currents’ defined as having a density of >70 mA m<sup>−2</sup>. In addition, no TD inward currents were observed in 20% of protoplasts from the wild type and 30% from the mutant.

<sup>b</sup> In addition, a population of wild-type protoplasts (43%) show slowly inactivating outward currents (see text and Fig. 3). No TD outward currents were observed in 40% of protoplasts from the wild type and 13% from the mutant. Sufficient currents were measured in 24% of mutants to enable determination of the current type in the cells, but not enough to obtain quantitative data for this table.

<sup>c</sup> Time-courses of current activation were fitted to a single or a double exponential, or were fitted to a sigmoidal curve, as defined by the Hodgkin–Huxley equation (Hodgkin and Huxley, 1952). The type of curve providing the best fit is given in the Table, as are the time constants, τ (or, for curves with two time exponentials, τ<sub>1</sub> and τ<sub>2</sub>.)
these currents were not analysed in detail since they
activated at very positive membrane potentials which
suggest they are unlikely to contribute significantly to
long-term fluxes of cations in intact roots.

Na⁺-conducting currents in the two genotypes

One possible pathway mediating the influx of Na⁺ into the
roots is via TD inwardly rectifying channels. However,
when external K⁺ was completely replaced with Na⁺
(30 mM), no significant TD inward currents were seen
(Fig. 4A, B). In these experiments, external Ca²⁺ had to be
kept high (2.1 mM) to maintain the stability of the seal,
since replacing external K⁺ with Na⁺ made the seals
unstable at lower external Ca²⁺. In the presence of external
Na⁺, only small instantaneous inward currents remained,
even in protoplasts with substantial TD inward K⁺ currents
prior to replacement of external K⁺ with Na⁺. These
instantaneous currents appear similar to those attributed
to Na⁺ influx in roots of other plants (see Introduction).
They were much smaller than the instantaneous currents
observed with external K⁺ (Fig. 4C), hence the apparent
noisiness of the traces. The sizes of the currents with
external Na⁺ were similar in protoplasts from wild-type and
mutant plants (Fig. 4; Table 2).

Ca²⁺ influx is unlikely to contribute significantly to these
instantaneous currents given the characteristic features of
hyperpolarization-activated Ca²⁺ currents (White, 1998;
Kiegle et al., 2000) were not observed (results not presented). To test the possibility that Cl\(^-\)/C\(_{255}\) efflux formed a component of these instantaneous currents, Cl\(^-\)/C\(_{255}\) was excluded from the pipette (see Materials and methods) and Na\(^+\) was maintained as the main external cation. The instantaneous currents measured in these experiments were similar to those measured when the pipette solution contained Cl\(^-\)/C\(_{255}\), with a current density at C\(_{255}\) 160 mV of C\(_{255}\) 59 6 9 mA m\(^{-2}\) (n=4). This indicates that the instantaneous inward currents are principally due to Na\(^+\) when the bath contains Na\(^+\) as the main cation.

The permeability ratio \(P_{Na}/P_{K}\) for the instantaneous inward currents was calculated using the Nernst equation for bionic potentials (Hille, 1992), and was found to be smaller for the mutant (1.02) than the wild type (5.3). Therefore, to investigate further why there is a greater influx of Na\(^+\) in the mutant, the resting membrane potentials of cells in the intact roots of the two genotypes were measured by microelectrode impalement (see below).

Table 2. Magnitudes of the instantaneous inward and outward currents in the plasma membrane of protoplasts isolated from the roots of wild-type and mutant Capsicum annuum with standard bath and pipette solutions

<table>
<thead>
<tr>
<th>Type of current</th>
<th>Inward currents at (-160) mV</th>
<th>Outward currents at (+100) mV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genotype</td>
<td>Wild type</td>
<td>Mutant</td>
</tr>
<tr>
<td>KCl in bath</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Current density</td>
<td>(-49\pm7) (10)</td>
<td>(-21\pm5) (6)</td>
</tr>
<tr>
<td>NaCl in bath(^a)</td>
<td>(-28\pm10) (6)</td>
<td>(-23\pm11) (4)</td>
</tr>
</tbody>
</table>

\(^a\) For measurements with NaCl in bath, all KCl in the bath was replaced with NaCl and pH was adjusted with NaOH instead of KOH.

Kiegle et al., 2000) were not observed (results not presented). To test the possibility that Cl\(^-\) efflux formed a component of these instantaneous currents, Cl\(^-\) was excluded from the pipette (see Materials and methods) and Na\(^+\) was maintained as the main external cation. The instantaneous currents measured in these experiments were similar to those measured when the pipette solution contained Cl\(^-\), with a current density at \(-160\) mV of \(-59 \pm 9\) mA m\(^{-2}\) (n=4). This indicates that the instantaneous inward currents are principally due to Na\(^+\) when the bath contains Na\(^+\) as the main cation.

Fig. 4. (A, B) Typical instantaneous inward currents across the plasma membrane of protoplasts derived from the cortex of roots of wild-type and mutant C. annuum. In all cases, the pipette solution was the standard solution containing 123 mM K-gluconate; the bath contained standard bath solution but 30 mM KCl replaced with 30 mM NaCl and pH adjusted with NaOH (not KOH). Holding potential was \(-60\) mV. The voltage pulses ranged from \(-100\) mV to \(-200\) mV, in 20 mV steps and each episode lasted for 4 s with a time interval of 20 s between each episode. Currents were low pass filtered at 200 Hz. (A) Wild type. Protoplast diameter: 18 \(\mu\)m. (B) Mutant. Protoplast diameter: 18 \(\mu\)m. (C, D): I–V relationships of instantaneous currents across the plasma membrane of protoplasts from roots of the wild type (filled squares) and the mutant (open squares). (C) K\(^+\) is the main cation in the bath (n=10 for wild type and n=6 for mutant). (D) Na\(^+\) is the main cation in the bath (n=6 for wild type and n=4 for mutant).
specific activation of outward current by external Na\(^+\), this suggests that the outward currents are activated by the difference between the \(E_K\) and the holding potential. By contrast, in protoplasts from mutant roots, this activation of TD outward currents upon replacement of KCl was not observed (Fig. 5B).

**Sensitivity of the K\(^+\) currents to inhibitors**

Extracellular TEA caused a weakly voltage-dependent and dose-dependent block of TD inward currents in both genotypes. At \(-160\) mV, 2–3 mM external TEA reduced the inward current by approximately 50% in both genotypes (Fig. 6). This inhibition could be partially reversed after washing the protoplast with TEA-free bath solution for 10 min. TEA had a less potent effect on the TD outward currents compared with the TD inward currents in the protoplasts derived from roots of both genotypes (data not shown). 15 mM TEA reduced outward currents by about 50% in both genotypes. TEA had no effect on the instantaneous currents.

**Microelectrode impalement of whole roots**

At steady-state, in a solution containing 0.1 mM KCl, membrane potentials (\(E_m\)) were in the range of \(-120\) to \(-130\) mV in the wild type (mean = \(-125\pm2\) mV, \(n=6\)) and \(-150\) to \(-160\) mV in the mutant (mean = \(-157\pm1.5\) mV, \(n=7\)) (significantly different using a single factor Anova test, \(P<0.00015\)). Activity of the plasma membrane H\(^+-\)ATPase could significantly contribute to \(E_m\) because addition of 1 mM KCN rapidly depolarized \(E_m\) to \(-80\) mV in the wild type and \(-54\) mV in the mutant, presumably by inhibiting the plasma membrane H\(^+-\)ATPase.

Addition of high Na\(^+\) solution, the same as used by Tal and colleagues for their Na\(^+\) flux experiments, depolarized \(E_m\) in wild-type plants by \(31\pm5\) mV (\(n=4\)) over a time-course of 5–6 min. In mutant plants, although addition of NaCl caused a similar sized depolarization (32±4 mV, \(n=5\)), this occurred more slowly (over 10–20 min) and was then followed by a repolarization to the original potential over 25–30 min (Fig. 7). In steady-state conditions in high Na\(^+\), this gave the mutants plants a membrane potential over 60 mV more negative than in the wild-type plants.

**Discussion**

**K\(^+\) currents**

In protoplasts derived from the root cortex of wild-type *Capsicum* plants, the plasma membrane current that was most frequently observed with the solutions used was the TD K\(^+\)-selective inwardly rectifying currents. These were similar to the currents found in the cortex of maize (Roberts and
K⁺ (86Rb⁺) efflux, given the negative membrane potentials activated at positive membrane potentials, could increase annuum (Benzioni and Tal, 1978). Efflux studies on the roots of wild-type and mutant C. annuum plant root cortex had a significant TD outward conductance which activated at small positive voltages (i.e. about +5 mV), in addition to a TD inward current which was similar to that found in the wild-type roots.

The magnitude of the TD inward K⁺ currents from the wild type and mutant were comparable, although there was a difference in the kinetics of activation of these currents. Although this suggests some difference in the properties of the K⁺ inward rectifier, it is not one that would be expected to affect long-term steady-state K⁺ influx via these channels. In the mutant, the larger outward K⁺ currents correlate with the bigger K⁺(86Rb⁺) efflux observed from intact roots of mutant plants.

The differences are unlikely to be due to the protoplast isolation technique, since both sets of protoplasts were isolated using the same protocol. Also, upon enzymatic digestion of the roots, the cortex was usually found to be fully digested in both genotypes, suggesting that protoplasts from cortical cells were being used for the patch clamp measurements in both cases. This is an important criterion when comparing currents in different cells because different cell types can have distinct currents (Roberts and Tester, 1995). Thus, the comparable TD inward K⁺ currents in the wild type and mutant and the larger TD outward K⁺ currents in the mutant correlate with the 86Rb⁺ influx and efflux studies on the roots of wild-type and mutant C. annuum (Benzioni and Tal, 1978).

Of course, how increased outward rectifier activity, activated at positive membrane potentials, could increase K⁺(86Rb⁺) efflux, given the negative membrane potentials measured, is uncertain. It is possible that the channels were active in cells other than those in which membrane potential were measured, or perhaps they were activated during oscillations in membrane potential that were not observed in the experimental conditions in which potentials were measured, or over the time periods in which membrane potentials were measured.

However, the TD currents are not the only pathway for significant low-affinity cation uptake into these roots, the instantaneous currents must also be taken into account. Furthermore, the observation that mutant root cells appeared more hyperpolarized will also affect considerations of steady-state K⁺ influx. Adding TD and instantaneous inward K⁺ currents at −94 mV and −157 mV for wild type and mutant plants (taken from Figs 1C, D, 4C) gives average total K⁺ inward currents of 20±15 mA m⁻² (n=10) and 102±46 mA m⁻² (n=6), respectively. This trend reflects early observations of greater K⁺ accumulation in mutant plants when grown in high K⁺ (Tal et al., 1976) and a higher 86Rb⁺ influx from 50 mM Rb⁺ in mutant plants grown in high K⁺ (Benzioni and Tal, 1978).

This simple comparison is complicated by the likelihood that the K⁺:Rb⁺ selectivity of the instantaneous current will be lower than for the TD current (e.g. compare selectivities in Davenport and Tester, 2000, with those in White, 1997), which would give rise to greater inward Rb⁺ currents in the wild type relative to the mutant (see the higher instantaneous currents in the wild type in Fig. 4C). This latter observation is in agreement with the effects of the mutation on 86Rb⁺ influxes measured at 10 mM and lower concentrations by Benzioni and Tal (1978).

**Effects of inhibitors**

The inward and outward K⁺ currents have been further characterized by studying the effect of inhibitors such as TEA. The sensitivity of the inward K⁺ currents to TEA was comparable in both genotypes, reflecting unpublished results of JR Morton, DG Lurie, Z Cohen, and M Tal on TEA inhibition of 86Rb⁺ influx. TEA also appears to weakly inhibit the TD outward K⁺ currents in the wild type (n=3) and mutant (n=2). 86Rb⁺ efflux in the mutant was reported to be inhibited to a greater extent by TEA than in the wild type (JR Morton, DG Lurie, Z Cohen, M Tal, unpublished results). This is likely to be because a greater proportion of the efflux in the mutant is catalysed by the TEA-sensitive TD outward current relative to the TEA-insensitive instantaneous currents.

**Na⁺ currents**

The study of Na⁺ currents in the roots of C. annuum suggests that, as in the case of the roots from rye (White and Tester, 1992), wheat (Tyerman et al., 1997; Davenport and Tester, 2000), maize (Roberts and Tester, 1997) and Arabidopsis (Demidchik and Tester, 2002), Na⁺ could be
mainly transported into these cells by a time-independent current.

The *sd* mutant of *C. annuum* has been shown to exhibit a higher Na\(^+\) influx compared to the wild type (Tal and Benzioni, 1977). However, the comparable instantaneous Na\(^+\) currents in the two genotypes suggests that the difference in the Na\(^+\) transport between the wild type and the mutant was not due to differences in the Na\(^+\) transport capacity of the plasma membrane. It is possible that differences in the instantaneous Na\(^+\) currents were obscured due to the relatively high Ca\(^{2+}\) concentration used (2 mM), but this is unlikely given the phenotype appears in a range of conditions, including being originally isolated in a horticultural soil (Bergh and Lippert, 1964). However, micro-electrode impalements showed that the *E*\(_m\) of cells in intact mutant roots were 60–70 mV more negative compared with the wild type. This hyperpolarization would increase inward Na\(^+\) currents, potentially accounting for the elevated Na\(^+\) influx observed in intact mutant roots. Assuming all cells in the roots contributed equally to Na\(^+\) influx, and that all Na\(^+\) was from the same concentration into intact roots of both genotypes, at the corresponding external Ca\(^{2+}\) concentrations, it is unlikely that the mutants had a more negative membrane potential. It is possible that the Na\(^+\) hyperpolarization of –30 mV could be accounted for by the greater Na\(^+\) influx in the mutant, with no other alterations necessary in membrane characteristics (notably, of Na\(^-\) conductance).

It is unsure why this membrane hyperpolarization does not also increase \(\text{\textsuperscript{86}Rb}\) influx in the mutants. It is possible that \(\text{\textsuperscript{86}Rb}\) influx is not primarily catalysed by either of the transporters responsible for influx, or that there could be kinetic controls of the transporters studied in this work that are disrupted by the invasive patch clamp technique. It seems clear that the patch clamp measurements, combined with membrane potential measurements, fail to describe all the differences in cation fluxes measured using radioactive tracers, especially for K\(^+\) fluxes. However, it seems likely that the elevated Na\(^+\) influx in mutant plants, the main focus of this work, is due to a more negative membrane potential. The basis for this hyperpolarization remains the subject of future study.

It is clear that the *sd* mutation in *C. annuum* affects a range of plasma membrane transport processes, most notably instantaneous and TD outward K\(^+\) currents. This correlates with increased unidirectional K\(^+\) efflux measured in intact roots. A more negative membrane potential was also observed across the plasma membrane of cells in mutant roots, which may be due to the higher ATPase activity observed in mutant root membrane preparations by JR Morton, DG Lurie, Z Cohen, and M Tal (unpublished results). This more negative membrane potential is large enough to explain the increased unidirectional Na\(^+\) influx observed in intact mutant roots. However, there are also other subtle effects on membrane transporters, such as differences in the activation kinetics of the TD inward K\(^+\) currents. Furthermore, effects of the mutation on high affinity K\(^+\) uptake were beyond the scope of this study, as were investigations of effects on both the H\(^+\)ATPase and tonoplast transporters. The Na\(^+\) hyperaccumulation of the *sd* wilt mutation of *C. annuum* is, therefore, unlikely to be due simply to the mutation of one ion transporter, but could be due to a controlling element that can affect a wide range of transporters.

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**References**


