Characterization of Anion Channels in the Plasma Membrane of Arabidopsis Epidermal Root Cells and the Identification of a Citrate-Permeable Channel Induced by Phosphate Starvation

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Organic-acid secretion from higher plant roots into the rhizosphere plays an important role in nutrient acquisition and metal detoxification. In this study we report the electrophysiological characterization of anion channels in Arabidopsis (Arabidopsis thaliana) root epidermal cells and show that anion channels represent a pathway for citrate efflux to the soil solution. Plants were grown in nutrient-replete conditions and the patch clamp technique was applied to protoplasts isolated from the root epidermal cells of the elongation zone and young root hairs. Using SO$_4^{2-}$ as the dominant anion in the pipette, voltage-dependent whole-cell inward currents were activated at membrane potentials positive of $-180$ mV exhibiting a maximum peak inward current ($I_{\text{peak}}$) at approximately $-130$ mV. These currents reversed at potentials close to the equilibrium potential for SO$_4^{2-}$, indicating that the inward currents represented SO$_4^{2-}$ efflux. Replacing intracellular SO$_4^{2-}$ with Cl$^-\text{ or NO}_3^-$ resulted in inward currents exhibiting similar properties to the SO$_4^{2-}$ efflux currents, suggesting that these channels were also permeable to a range of inorganic anions; however when intracellular SO$_4^{2-}$ was replaced with citrate or malate, no inward currents were ever observed. Outside-out patches were used to characterize a 12.4-picoSiemens channel responsible for these whole-cell currents. Citrate efflux from Arabidopsis roots is induced by phosphate starvation. Thus, we investigated anion channel activity from root epidermal protoplasts isolated from Arabidopsis plants deprived of phosphate for up to 7 d after being grown for 10 d on phosphate-replete media (1.25 mM). In contrast to phosphate-replete plants, protoplasts from phosphate-starved roots exhibited depolarization-activated voltage-dependent citrate and malate efflux currents. Furthermore, phosphate starvation did not regulate inorganic anion efflux, suggesting that citrate efflux is probably mediated by novel anion channel activity, which could have a role in phosphate acquisition.

Anion channels in the plasma membrane of plant cells catalyze anion fluxes both into and out of the cell and serve a variety of functions. They have been implicated in stomatal function, where their activation is thought to be one of the rate-limiting steps in the loss of salts (and thus cell turgor) from guard cells leading to stomatal pore closure (Roelfsema et al., 2004). In less specialized cells, anion channel activation is a likely step in the transduction of signals modulating hypocotyl growth, including blue light (Cho and Spalding, 1996) and possibly auxin (Thomine et al., 1997); these signal transduction events arise from depolarizations resulting from anion channel activation. Anion channels are also thought to facilitate the release of organic acids from higher plant roots. Al$^{3+}$-activated anion channels (ALAACs) in the tips of wheat (Triticum aestivum) and maize (Zea mays) roots have been shown to be permeable to malate and/or citrate (Ryan et al., 1997; Kollmeier et al., 2001; Pineros and Kochian, 2001; Zhang et al., 2001), a function of which is thought to reduce Al$^{3+}$ stress by chelating this cation. Thus activation of ALAACs by Al$^{3+}$ and their pharmacological profile, which resembles that for Al$^{3+}$-induced organic-acid efflux from cereal roots, makes ALAACs likely candidates for mediating Al$^{3+}$-induced organic-acid secretion from roots.

The biophysical properties of plant anion channels have been best characterized in guard cells. Two types of anion channels have been extensively investigated: rapidly activating (R-type) and slowly activating (S-type) anion channels (e.g. Hedrich et al., 1990; Schroeder and Keller, 1992; Schmidt and Schroeder, 1994). The R-type anion channel exhibits activation/deactivation kinetics in the millisecond range and inactivates in response to prolonged membrane depolarization. Thus, R-type channels in guard cells are thought to mediate transient anion efflux and membrane depolarization. In contrast, S-type channels activate and deactivate slowly (with a time constant of seconds), and they do not inactivate. S-type channels may possibly mediate prolonged anion efflux. R- and S-type anion channels also have distinct gating
properties. The typical R-type whole-cell current voltage relationship is exemplified by a pronounced peak current magnitude at relatively positive voltages and complete deactivation at relatively negative (resting) membrane voltages; in contrast, S-type whole-cell current voltage relationships display a less pronounced peak current and exhibit inward rectification in hyperpolarized conditions (Schroeder and Keller, 1992; Roelfsema et al., 2004). Similar R- and S-type anion channels have also been characterized in the plasma membrane of hypocotyl cells (Thomine et al., 1995, 1997; Frachisse et al., 2000).

Anion channels in roots have not been well characterized compared to those in guard cells, despite their potential importance in regulating acquisition from soil solution. The ALAACs of wheat and maize roots (see above) resemble S-type channels in that they display slow activation kinetics (Pineros and Kochian, 2001; Zhang et al., 2001) and exhibit inwardly rectifying current voltage relationships. Other reports of anion channel activity in roots are limited to an outwardly rectifying anion-selective channel in wheat and maize, which allows anion influx from the soil solution (Skerrett and Tyerman, 1994; Pineros and Kochian, 2001) and three different anion conductances in the xylem parenchyma cells of barley (Hordeum vulgare) roots, which are thought to mediate anion efflux to the xylem vessels during salt delivery to the shoot (Kohler and Raschke, 2000; Kohler et al., 2002).

In particular, there has been no systematic study of anion channels in roots with respect to root soil interaction and nutrient acquisition.

In this study we address this dearth of knowledge and use the patch clamp technique to investigate anion channel activity in the epidermis of Arabidopsis (Arabidopsis thaliana) roots. We show two types of voltage-dependent channel activity, which resemble the R-type anion channel activity described in guard cells and hypocotyls. One of these channels was ubiquitously expressed in the epidermal cells and was impermeable to the inorganic anions, \( \text{SO}_4^{2-} \), \( \text{NO}_3^- \), and \( \text{Cl}^- \); but was impermeable to organic-acid anions, citrate and malate. The second anion channel was less frequently observed, was induced by phosphate starvation, and mediated the efflux of organic-acid anions. It is suggested that the phosphate-regulated anion channel mediates organic-acid anion efflux from Arabidopsis roots, which is thought to be an important strategy for efficient phosphate acquisition by higher plants (Narang et al., 2000).

**RESULTS**

The Arabidopsis line J0841 showed green fluorescent protein (GFP) expression specifically only in root peripheral cells, namely the epidermal cells (Fig. 1). These included cells of the elongation zone as well as young emerging root hair (trichoblast) and atrichoblast cells. However, GFP expression was not observed in the root tip, or in some of the older root hair or atrichoblast cells. Only cells expressing GFP were used in this study.

The whole-cell configuration of the patch clamp technique and standard bath solution (SBS) was used to record anion currents across the plasma membrane of GFP-expressing protoplasts isolated from the epidermis of Arabidopsis roots. SBS contained 5 mM LaCl\(_3\); La\(^{3+}\) is an established broad-spectrum cation blocker and, as illustrated in Figure 2, was...
effective in blocking inward and outward currents at negative and positive potentials, respectively. Although the La\(^{3+}\)-sensitive currents illustrated in Figure 2 were not investigated further, they most likely represent cation currents through nonselective and Ca\(^{2+}\)-permeable channels, which have been reported previously in Arabidopsis root hairs and epidermal cells (Kiegle et al., 2000; Very and Davies, 2000; Demidchik et al., 2002). Pipette solutions were based on the use of caesium salt; although Cs\(^{+}\) permeates nonselective channels (Demidchik et al., 2002), Cs\(^{+}\) does not significantly permeate K\(^{+}\) channels (Tester, 1988), and thus currents in this study were recorded without interference from this channel type.

**Anion Currents in Arabidopsis Root Epidermal Cells**

Using SBS and standard pipette solution (in which 25 mM SO\(_4^{2-}\) was the main anion), all root epidermal cells exhibited inward currents with strong voltage dependence. SO\(_4^{2-}\) was employed as the main anion because intracellular SO\(_4^{2-}\) has been shown to be both a substrate for and an activator of plasma membrane anion channels in Arabidopsis hypocotyl cells (Frachisse et al., 1999). Polarization of the membrane to potentials more positive than −180 mV activated an inward current, which peaked at approximately −130 mV (Fig. 2). At potentials more positive than the peak inward current \(I_{\text{peak}}\), the current decreased with a pronounced rectification at voltages close to the reversal potential \(E_{\text{rev}}\). Using SBS containing 1 mM Cs\(_2\)SO\(_4\), whole-cell currents reversed close to the equilibrium potential of SO\(_4^{2-}\) (+41 mV), which is distinct from the equilibrium potentials for Cl\(^{−}\) (−62 mV) and Cs\(^{+}\) (−82 mV) and indicates that the inward currents were likely to be mainly carried by SO\(_4^{2-}\) efflux (Fig. 3). To investigate further the nature of the ion responsible for the inward current, reversal potentials were recorded in varying extracellular Cs\(_2\)SO\(_4\) concentrations. Increasing extracellular SO\(_4^{2-}\) to 10 and 25 mM shifted \(E_{\text{rev}}\) of the whole-cell currents to 13.2 ± 2.6 and 2.4 ± 0.9 mV, respectively (Fig. 3, A and B); thus, \(E_{\text{rev}}\) followed predicted changes in the equilibrium potential for SO\(_4^{2-}\). Furthermore, lowering pipette SO\(_4^{2-}\) concentration from 25 to 1 mM reduced the mean current density from 32.7 ± 5.99 to 1.27 ± 0.34 pA/pF \(n = 13\). These data are consistent with the inward currents being carried by SO\(_4^{2-}\) efflux. It is also apparent that increasing extracellular SO\(_4^{2-}\) induced a shift in the \(I_{\text{peak}}\) potential to more negative voltages illustrating that extracellular SO\(_4^{2-}\) was able to modulate the gating of the inward current such that the activation potential was shifted to more negative potentials. Similar gating by extracellular anions has been reported for anion channels in a variety of plant cells including guard cells (Dietrich and Hedrich, 1998) and hypocotyl cells in Arabidopsis (Colcombet et al., 2001).

In experiments using SBS supplemented with 1 mM SO\(_4^{2-}\), the inward current reversed between the equilibrium potentials for SO\(_4^{2-}\) and Cl\(^{−}\) (Fig. 3B), indicating that the channels mediating the inward current are likely to be permeable to other anions. To investigate further the selectivity of the channels that underlie the inward current, intracellular SO\(_4^{2-}\) was substituted by Cl\(^{−}\), NO\(_3^{−}\), malate, and citrate (supplied as cesium salts). The substitution of cyto-

![Figure 2](https://example.com/figure2.png)

**Figure 2.** Isolation of whole-cell voltage-dependent inward currents from epidermal root cells using extracellular La\(^{3+}\). A. Whole-cell currents measured across the plasma membrane of a GFP-expressing protoplast. Currents resulted from voltage pulses ranging from −216 mV to +64 mV in 10-mV steps (for clarity only currents from 40-mV intervals are shown). Holding voltage was −16 mV. Standard pipette solution was used. Extracellular solution was as for SBS but with LaCl\(_3\) omitted and supplemented with 1 mM Cs\(_2\)SO\(_4\). B. As A except extracellular solution was SBS supplemented with 1 mM Cs\(_2\)SO\(_4\). C. Current voltage relationship of steady-state whole-cell currents shown in A (●) and B (▲).
Figure 3. Anion efflux underlies the whole-cell voltage-dependent inward currents from the root epidermis. A, Current voltage relationships of steady-state voltage-dependent currents using standard pipette solution and SBS supplemented with 1 mM (●), 10 mM (▲), and 25 mM (■) Cs₂SO₄. Inset, An expanded view of the region corresponding to the current reversal potentials ($E_{\text{rev}}$). Arrows indicate calculated values for $E_{\text{sulfate}}$. B, $E_{\text{rev}}$ of whole-cell currents plotted as a function of extracellular SO₄²⁻. Extracellular sulfate was varied using Cs₂SO₄ added to SBS. Data represent the mean (±SEM) for three separate experiments. Standard pipette solution was used. The dashed lines represent values where $E_{\text{rev}} = E_{\text{sulfate}}$, $E_{\text{Cs}}$, or $E_{\text{Cl}}$. C, Current voltage relationship of steady-state whole-cell currents using standard pipette solution but with 25 mM Cs₂SO₄ replaced with 50 mM CsCl. Currents shown are representative of 26 independent experiments. Currents were recorded 1 (●) and 7 (▲) min after obtaining the whole-cell configuration. Inset, Whole-cell currents (used for the construction of the current voltage relationship) result from voltage pulses ranging from −204 to +76 mV in 10-mV steps (for clarity only currents every 40-mV interval are shown). Holding voltage was +36 mV. D, Current voltage relationship of steady-state whole-cell currents using standard pipette solution but with 25 mM Cs₂SO₄ replaced with 50 mM CsNO₃. Currents shown are representative of 21 independent experiments. Currents were recorded 1 (●) and 11 (▲) min after obtaining the whole-cell configuration. Inset, Whole-cell currents (used for the construction of the current voltage relationship) result from voltage pulses ranging from −209 to +71 mV in 10-mV steps (for clarity only currents every 40-mV interval are shown). Holding voltage was +31 mV.
solic rather than extracellular anions was favored because it represented a more physiological condition; that is, the negative potential that usually exists across the plasma membrane of plant cells tends to drive the passive flow of anions from the cells. Furthermore, addition of citrate and malate to the bath solution resulted in an increase in membrane conductance (i.e. leak conductance), consistent with a loss of integrity of the membrane and/or the seal between the membrane and the glass pipette. We suspect that this was the result of cation chelation by citrate and malate.

Replacing pipette SO$_4^{2-}$ with Cl$^-$ or NO$_3^-$ resulted in inward currents, which exhibited similar voltage dependence to that observed for SO$_4^{2-}$ efflux currents (Fig. 3, C and D). Furthermore, decreasing pipette Cl$^-$ or NO$_3^-$ reduced the mean current density of the inward currents consistent with them representing anion efflux (Table I). However, no inward currents were observed when the pipette SO$_4^{2-}$ was replaced with citrate or malate, indicating that the anion channels were not significantly permeable to organic-acid anions. It is also significant that the whole-cell currents reversed at $E_{Cl}$ when pipette SO$_4^{2-}$ was replaced with Cl$^-$ (Fig. 3C); i.e. Cl$^-$ was the principal anion in both the bath and pipette solution. Taken together, these sets of data show that the channels underlying the voltage-gated inward currents in Arabidopsis root epidermal cells are anion selective and mediate at least SO$_4^{2-}$, Cl$^-$, and NO$_3^-$ efflux but not that of malate and citrate.

**Regulation of Anion Currents in Arabidopsis Root Epidermis**

The following observations indicated that intracellular SO$_4^{2-}$ was a potent activator of the voltage-dependent anion efflux currents. The magnitude of the Cl$^-$ and NO$_3^-$ efflux currents decreased with time and had usually completely disappeared within 15 min (Fig. 3, C and D); in contrast, SO$_4^{2-}$ efflux current magnitudes remained stable for at least 2 h. In experiments using standard pipette solution (i.e. with SO$_4^{2-}$ as the charge-carrying anion), the inward currents exhibited a marked increase in magnitude with time. Specifically, the magnitude of $I_{peak}$ increased 733% ± 247% (within a mean time of 8.1 ± 1.8 min; $n = 19$) after obtaining the whole-cell configuration. Indeed, in four experiments, inward currents were absent immediately after obtaining the whole-cell configuration but developed and increased in magnitude over several minutes—a typical example is shown in Figure 4. It is unlikely that this gradual increase in the inward current reflected a gradual and slow equilibration of the cytosol with SO$_4^{2-}$, because the equilibration of small inorganic anions between the pipette media and cytosol would be expected to be relatively immediate and complete within a minute. Rather, these observations are consistent with the activation of the anion efflux currents by intracellular SO$_4^{2-}$ and are similar to those previously reported by Frachisse et al. (1999) in Arabidopsis hypocotyl epidermal cells.

It is noteworthy that an instantaneously activating, outwardly rectifying current was evident in some root epidermal cells. From Figures 3, C and D, and 4, it is apparent that at least some of this outward current does not run down or increase with time, illustrating that the outward current was mediated, at least in part, by a channel type distinct from the channel responsible for the voltage-dependent inward current. Although the outwardly rectifying current was not investigated further in this study, it is interesting that after the complete rundown of the inward current in Figure 3 and before the runup in Figure 4, the remaining outwardly rectifying current reversed close to $E_{Cl}$, consistent with this current being carried by anion influx. Outwardly rectifying anion-selective channels have also been characterized in the roots of maize and wheat (Skerrett and Tyerman, 1994; Pineros and Kochian, 2001) where they are thought to mediate anion influx in high-salt concentration (e.g. during salinity stress).

**Table I. Frequency of occurrence and mean (±SEM) peak-current density of whole-cell inward currents with varying intracellular anions and anion concentrations**

All measurements were in SBS. Pipette solution was based on standard pipette solution and modified by replacing Cs$_2$SO$_4$ with appropriate anion (as a cesium salt) and adjusted to 700 mosmol kg$^{-1}$ using sorbitol when necessary.

<table>
<thead>
<tr>
<th>Internal Anion</th>
<th>Percentage of Cells with Current (Total Number of Whole-Cell Measurements)</th>
<th>Current Density ($\mu A$/pF)</th>
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<tbody>
<tr>
<td>25 mm SO$_4$</td>
<td>100 (20)</td>
<td>32.7 ± 6.0</td>
</tr>
<tr>
<td>25 mm Cl</td>
<td>42 (19)</td>
<td>1.8 ± 0.7</td>
</tr>
<tr>
<td>50 mm Cl</td>
<td>56 (26)</td>
<td>6.7 ± 2.0</td>
</tr>
<tr>
<td>25 mm NO$_3$</td>
<td>49 (37)</td>
<td>4.0 ± 1.2</td>
</tr>
<tr>
<td>50 mm NO$_3$</td>
<td>38 (21)</td>
<td>13.8 ± 4.9</td>
</tr>
<tr>
<td>60 mm Citrate</td>
<td>0 (35)</td>
<td>–</td>
</tr>
<tr>
<td>60 mm Malate</td>
<td>0 (12)</td>
<td>–</td>
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*Thirteen of these experiments used pipette solution that was the same as that detailed in Figure 7A.*

**Activation/Deactivation Kinetics**

Figure 5 shows typical currents resulting from stepping the voltage from values either more negative (−216 mV) or more positive (+64 mV) than the $I_{peak}$ voltages. Upon stepping the potential from −216 mV, inward currents at potentials negative of −96 mV were characterized by a fast time-dependent activation, which could be roughly fitted by a single exponential, whereas inward currents at potentials positive of −96 mV exhibited instantaneous activation. In contrast, stepping from holding potentials positive of the $I_{peak}$ voltage, whole-cell inward currents instantaneously increased with the driving force for SO$_4^{2-}$ efflux before decreasing to reach a new steady-state value. The time-dependent decrease of the whole currents reflected...
a fast deactivation, which could be roughly fitted by a single exponential (note that deactivation of the inward currents was apparent only at voltages negative of the $I_{\text{peak}}$ voltage). The time constants for exponential activation and deactivation were in the millisecond range and were voltage dependent (Fig. 5B).

A slight decay (or inactivation) of the anion efflux current was evident at activating voltages of more than 2 s in duration (see currents at $-136$ mV in Fig. 5A). To determine the extent of inactivation in response to prolonged stimulation, voltage protocols were applied for up to 7 min. During prolonged depolarizations to $-136$ mV, inward-current magnitude decayed to approximately 50% of the initial peak-current amplitude (Fig. 5C) within 1 min, after which current magnitude remained stable or decayed slightly over 6 min. Note the fast (millisecond) time-dependent activation of the inward current when the time scale is expanded to a higher resolution (Fig. 5C inset).

**Single Channels**

To characterize further the channel activity underlying the whole-cell inward-anion currents, we recorded single-channel activity in the outside-out patch clamp configuration. Figure 6 shows standard single-channel activity. These channels did not exhibit rundown in their activity; however, many patches contained up to 50 channels (e.g. Fig. 6A) making single-channel analysis difficult. As a consequence, data analysis of single-anion channel activity was restricted to three patches from which single-channel currents could be resolved. Figure 6B illustrates the single-channel activity of the anion channel using SBS containing 1 mM SO$_4^{2-}$. Plotting single-channel current amplitudes as a function of voltage revealed a single-channel conductance of $12.4 \pm 0.1$ picoSiemens (pS; $n = 3$; Fig. 6C). The following observations suggest that the single-channel activity shown in Figure 6B underlay the whole-cell inward currents. First, averaging single-channel recordings resulted in current traces that displayed similar deactivation kinetics to that observed for whole-cell currents (compare traces in Figs. 6D with whole-cell currents). Second, increasing extracellular SO$_4^{2-}$ from 1 to 25 mM significantly increased channel activity at potentials negative of the $I_{\text{peak}}$ voltage (Fig. 6E). This increase in channel activity is consistent with the SO$_4^{2-}$-dependent shift of the activation potential to more negative voltages observed for the whole-cell recordings (Fig. 3A).

**Phosphate Starvation Induces an Anion Channel-Mediated Citrate Efflux**

It is well established that Arabidopsis roots secrete citrate in soils depleted in phosphate. To investigate further citrate efflux from Arabidopsis roots, we applied the whole-cell patch clamp technique to epidermal protoplasts isolated from Arabidopsis roots that had been exposed to phosphate-free media (see “Materials and Methods” for growth conditions). Experiments were conducted using 60 mM citrate in the pipette as the dominant anion. Voltage-dependent inward currents (Fig. 7A) were observed in 11 out of 78 cells (14%) with a mean current density of $2.6 \pm 0.56$ pA/pF; notably, these currents were not observed in protoplasts derived from Arabidopsis roots cultured in phosphate-replete media (Table I). Unfortunately, the inward currents displayed rapid rundown and they were completely abolished within 10 min after...
obtaining the whole-cell configuration (Fig. 7, A and B). As a consequence of this rapid rundown, analysis of the single channels that underlie the currents shown in Figure 7 was not possible. However, we generated a difference current voltage ($\Delta I-V$) relationship by subtracting the current measured before rundown from that remaining after complete rundown (see Fig. 7A inset). The $\Delta I$ represented citrate efflux currents (assuming no cation permeation), which reversed at +20 mV (Fig. 7A inset), predicting a permeability ratio ($P_{\text{cit}}/P_{\text{Cl}}$) of 26 (see Kohler and Raschke, 2000, equation 4). The identity of the inward currents was further confirmed in separate experiments using citrate-containing pipette media supplemented with 4 mM Cl$^-$ and a bath solution containing 10 mM HEPES. In these experiments $E_{\text{Cl}}$ and $E_{\text{HEPES}}$ were set at -62 mV and -76 mV, respectively. Figure 7C shows a representative example from three independent recordings in which $I_{\text{peak}}$ voltage was close to $E_{\text{Cl}}$ and $E_{\text{HEPES}}$, demonstrating that the inward currents were not the result of Cl$^-$ or HEPES efflux but rather represented citrate efflux. Similar experiments, in which intracellular citrate was replaced with equimolar malate, showed 5 out of 27 epidermal root protoplasts isolated from phosphate-starved roots also exhibited small anion efflux currents. However, due to their small current magnitude ($1.06 \pm 0.7 \text{ pA/pF}$), high variability, and low frequency of occurrence, these currents were not analyzed further (data not shown).

Finally, we investigated the possibility that the citrate efflux currents were mediated by the anion channel activity responsible for SO$_4^{2-}$, Cl$^-$, and NO$_3^-$ efflux. To investigate this possibility, we compared the magnitudes of whole-cell currents (i.e. $I_{\text{peak}}$) for SO$_4^{2-}$ and Cl$^-$ effluxes from epidermal protoplasts isolated from the roots of Arabidopsis grown in phosphate-replete and phosphate-free media. In experiments using standard pipette solution (containing 25 mM SO$_4^{2-}$ or with the SO$_4^{2-}$ replaced with 50 mM Cl$^-$), phosphate starvation did not significantly affect either the frequency of occurrence or current density of SO$_4^{2-}$ or Cl$^-$ efflux currents (Fig. 7D). This suggests that organic-acid efflux is mediated by a distinct and novel channel activity, which is induced by phosphate starvation.

Figure 5. Kinetic properties of whole-cell anion efflux currents using standard pipette and bath solutions. A, Activation and deactivation of whole-cell currents in response to the voltage protocol as detailed in the bottom of the figure. Inset, Expanded time scale for current trace at -136 mV illustrating time dependent (exponential) activation. B, Time constants for deactivation ($\tau$) and activation (d) of whole-cell currents plotted as a function of voltage. Current deactivation was recorded by pulsing to test voltages from a holding voltage of +26 mV, and current activation was recorded by pulsing to test voltages from a holding voltage of -216 mV. Values for activation time constants are the mean (±SEM) from 5 independent experiments, and values for deactivation time constant are the mean (±SEM) from 10 independent experiments. C, Inward currents in response to depolarization for 7 min to -136 mV from a holding voltage of -216 mV. The currents are representative of three independent experiments. Note that the expansion of the time scale immediately following depolarization reveals time-dependent activation (and slight decay) as shown for equivalent depolarization in A.
DISCUSSION

Epidermal cells are in direct contact with the soil solution and are most likely to be involved in nutrient absorption and modification of the rhizosphere (see below). Arabidopsis line N9093 showed GFP expression exclusively in the root epidermal cells (Fig. 1; see also Kiegle et al., 2000; Diatloff et al., 2004), ensuring that protoplasts isolated from the root epidermis were used in this study.
Figure 7. Voltage-dependent citrate efflux currents from epidermal root protoplasts isolated from phosphate-starved roots. A, Current voltage relationships of whole-cell steady-state currents from the cell shown in B. Currents are representative of 11 independent experiments. Currents were recorded 3 (○), 4 (△), 5 (▼), 6 (●), and 10 (■) min after obtaining the whole-cell configuration. Extracellular solution was SBS. Pipette solution was as standard pipette solution except 25 mM Cs\textsubscript{2}SO\textsubscript{4} was replaced with 60 mM Cs\textsubscript{3}citrate and 2 mM MgCl\textsubscript{2} was omitted. Inset, Difference current voltage relationship obtained by subtracting current values at 3 min from current values at 10 min after obtaining whole-cell configuration. Reversal potential of the difference current was +20 mV. B, Example of whole-cell currents used to construct the current voltage relationship shown in A. Currents were recorded 3 and 10 min after obtaining whole-cell configuration and result from voltage pulses ranging from −226 to +54 mV in 10-mV steps (for clarity only currents every 40-mV interval are shown) from a holding voltage of +24 mV. C, Current voltage relationship of steady-state whole-cell currents using pipette solution containing 60 mM Cs\textsubscript{3}citrate, 0.5 mM HEPES, 2 mM MgCl\textsubscript{2}, 1 mM MgATP, 5 mM EGTA, pH 7.2, and adjusted to 700 mosmol kg\textsuperscript{−1} using sorbitol. Extracellular solution was SBS supplemented with 10 mM HEPES. Arrows denote values for $E_{\text{Cl}}$ and $E_{\text{HEPES}}$. Inset, Whole-cell currents used for the construction of current voltage relationship resulting from voltage pulses ranging from −226 to +54 mV in 10-mV steps (for clarity only currents every 40-mV interval are shown). Holding voltage was +24 mV. Currents are representative of three independent experiments. D, Peak inward-current densities from protoplasts isolated from roots growing in phosphate replete (+P) and phosphate-free (−P) media (see “Materials and Methods” for details). SO\textsubscript{4}\textsuperscript{2−} efflux currents (black bars) were recorded using standard pipette solution, chloride efflux currents (white bars) were recorded using standard pipette solution with 25 mM Cs\textsubscript{5}SO\textsubscript{4} replaced with 50 mM CsCl, and citrate efflux currents (hatched bars) were recorded using pipette media as in A. Current densities were calculated only from cells exhibiting a voltage dependent inward current with a defined current peak. Numbers in parentheses represent the number of cells exhibiting voltage-dependent efflux current/total number of cells tested.
Whole-Cell Currents

Using the whole-cell configuration of the patch-clamp technique, we have identified a voltage-dependent inward-rectifying anion channel activity in the epidermal cells of Arabidopsis roots, which we will refer to hereafter as Arabidopsis root anion channel (ARAC). \(SO_4^{2-}\) was chosen as the permeable intracellular anion to investigate ARAC activity because it has been shown to prevent anion channel rundown (Frachisse et al., 1999); consequently, the use of intracellular \(SO_4^{2-}\) permitted prolonged investigation of ARAC activity for up to 3 h. ARAC activity was also recorded free from the interference from cation-permeable channels as a result of using the established cation channel blockers, La\(^{3+}\) and Cs\(^+\). Surprisingly, extracellular La\(^{3+}\) has been reported to block the blue-light activated anion channel activity in the hypocotyls of Arabidopsis (Lewis and Spalding, 1998); however, increasing extracellular La\(^{3+}\) from 0.5 to 5 mM had no appreciable effect on ARAC activity (data not shown).

ARAC possessed properties characteristic for R-type anion channels, which have been previously reported in guard cells (Keller et al., 1989; Hedrich et al., 1990), Arabidopsis hypocotyls (Thomine et al., 1995, 1997), and suspension cells of tobacco (Nicotiana tabacum; Zimmerman et al., 1994), and carrot (Daucus carota; Barbara et al., 1994). Specifically, activation of ARAC is tightly controlled by transmembrane voltage, being deactivated at negative (resting) membrane potentials and activated by subsequent depolarization. The activation/deactivation kinetics are rapid, being in the millisecond range, and ARAC is permeable to other inorganic anions, including NO\(^3\) and Cl\(^-\) (for review of R-type anion channel properties, see White and Broadly, 2001). However, ARAC differed from R-type channels in guard cells in that ARAC was only partially inactivated (i.e. by 50%) during prolonged application of activating voltages compared to 90% inactivation of R-type channels in guard cells. Thus, whereas guard cell R-type anion channels are thought to mediate a transient anion efflux, ARACs are likely to mediate sustained anion efflux. It is also noteworthy that ARAC was not appreciably permeable to the organic-acid anions, malate and citrate. This is in contrast to that reported for guard cells and hypocotyls in which malate is reported to permeate R-type channels (e.g. Hedrich and Marten, 1993; Frachisse et al., 1999).

ARAC Activity: Comparisons with Other Root Anion Channels

There have been only a few studies of anion channel activity in higher plant roots. Kiegle et al. (2000), in a preliminary study, identified inward currents, which were probably carried by \(SO_4^{2-}\) efflux and exhibiting similar voltage dependence to ARAC; however, no rigorous investigation of their gating, selectivity, or possible physiological function was performed. More rigorous characterizations of anion channels have been performed on cells from cereal plant roots. From the epidermal and cortical cells of maize and barley root tips, inwardly rectifying anion channels, activated specifically by extracellular Al\(^{3+}\) and permeable to both inorganic (Ryan et al., 1997; Pineros and Kochian, 2001) and organic (Kollmeier et al., 2001; Zhang et al., 2001) anions have been reported. As a consequence of their activation by Al\(^{3+}\), ALAACs are thought to represent the pathway for organic-acid efflux from cereal roots, which is responsible for chelating and detoxifying extracellular Al\(^{3+}\). ARAC activity is distinct from the ALAACs in that it is not modulated by Al\(^{3+}\), La\(^{3+}\), or Cu\(^{2+}\) (data not shown) and is not permeable to organic-acid anions. Furthermore, ALAACs display S-type anion channel characteristics, i.e. slow deactivation kinetics and inwardly rectifying current voltage relationship. Finally, ALAACs have relatively large conductance of between 27 and 144 pS compared to the small unitary conductance values recorded for ARAC.

Anion efflux channels have also been characterized in the xylem parenchyma cells of barley roots (Kohler and Raschke, 2000; Kohler et al., 2002) where they are proposed to mediate anion transport into the xylem vessels. In these studies, three anion efflux channel types were identified. X-SLAC was found in only 7% of cells and displayed R-type current voltage relationships but displayed slow (S-type) deactivation kinetics (in the range of tens of seconds). The more prevalent channel types, X-QUAC and X-IRAC, exhibited S-type current voltage relationships. Thus to date, this study represents the only record, to our knowledge, of an R-type anion channel reported in higher plant roots.

Regulation of ARAC

ARAC is regulated by voltage (see above) and cytosolic factors. This study indicates that \(SO_4^{2-}\) is a potent activator of ARAC and, in the absence of intracellular \(SO_4^{2-}\), ARAC exhibited rundown. Thus, the loss of cytosolic factors necessary for ARAC activity could be compensated for by intracellular \(SO_4^{2-}\). The basis of this channel regulation is unknown, but it is proposed to reflect the binding of \(SO_4^{2-}\) to an intracellular regulatory site (Frachisse et al., 1999). In this study, stable \(SO_4^{2-}\) efflux currents could be recorded using 1 mM intracellular sulfate, suggesting that the binding constant of such a site would be less than 1 mM. Furthermore, ARAC activity recorded in outside-out patches was also stable, suggesting that the binding site could be an integral part of the channel.

We also observed that ARAC whole-cell current magnitude increased by approximately 7-fold within 10 min after obtaining the whole-cell configuration, indicating that intracellular \(SO_4^{2-}\)-activated or recruited inactive plasma membrane anion channels in Arabidopsis epidermal root cells. Interestingly, Table I shows that all cells exhibited \(SO_4^{2-}\) efflux currents,
but only approximately 50% of cells exhibited NO$_3^-$ or Cl$^-$ efflux. This result was initially surprising because it was expected that the same channel type was responsible for SO$_4^{2-}$, NO$_3^-$, and Cl$^-$ efflux (which is likely; see review by White and Broadly, 2001) and therefore the proportion of cells exhibiting anion efflux currents should be independent of the intracellular anion chosen as the charge carrier. The explanation for this anomaly is that ARACs are present in all epidermal root cells, but a significant proportion of these cells are in a quiescent state in which ARAC is inactive. This is consistent with Figure 4 in which ARAC activity is absent immediately after obtaining the whole-cell configuration (i.e. ARAC is inactive) but becomes apparent over several minutes. Thus, cells exhibiting Cl$^-$ or NO$_3^-$ efflux currents probably reflect the proportion of cells in which ARAC is active in planta.

The above observations indicate that ARAC is under strong posttranslational regulation in planta. The physiological significance of this is unknown, but it probably reflects the fact that the epidermal root cells used in this study represent a variety of cell types (e.g. atrichoblast and trichoblast cells and cells from the root elongation zone) that have different physiological roles and demands as influenced by the range of different extracellular environments individual root cell types will encounter (see below). The basis of this posttranslational regulation was not investigated in this study but could reflect the absence of a cytosolic factor on which channel activity is dependent (see above); candidates could include cytosolic calcium, kinases or phosphatases, pH or nucleotides—all of which have been shown to regulate anion channel activity in plant cells (for review, see White and Broadly, 2001).

Physiological Significance of ARAC

Plasma membrane anion efflux channels in plants play a number of fundamental roles in plant cell biology and root cell physiology. Osmoregulation has been best studied in guard cells, in which stomatal pore closure is initiated by salt loss effected principally by the opening of anion channels. Epidermal root cells are likely to experience large variations in the osmotic potential of the soil solution requiring cells to osmoregulate. Using Arabidopsis suspension cell cultures, Teodoro et al. (1998) showed that hypoosmotic shock induced a Cl$^-$ efflux, which was inhibited by the anion channel blocker, A-9-C. By analogy with anion channels in the guard cells, ARAC could initiate anion efflux in response to hypoosmotic conditions. Anion channels are also thought to play a pivotal role in cytosolic pH regulation in which they provide a shunt conductance to facilitate activation of H$^+$ pumping (and hence removal of cytosolic H$^+$) in response to cytosolic acidification (Johannes et al., 1998). Indeed, cytosolic pH is known to regulate Cl$^-$ efflux in higher plants (Beffagna et al., 1997). Although the regulation of ARAC by cytosolic pH has not been investigated in this study, ARAC could mediate an anion efflux shunt conductance during acid stress in root cells.

Organic-acid efflux has been well documented from higher plant root cells, and in Arabidopsis, malate and citrate efflux from the roots has been shown to be necessary for efficient phosphate acquisition (Narang et al., 2000) and the detoxification of Cu$^{2+}$ (Murphy et al., 1999). However, in the absence of measurable ARAC-mediated citrate or malate efflux in this study and the absence of ARAC regulation by phosphate supply (see below) or by extracellular Cu$^{2+}$ (data not shown), it is unlikely that ARAC is responsible for organic-acid efflux from Arabidopsis roots. Although it has received far less attention than organic-acid anions, inorganic anion efflux has also been recorded from higher plant roots, for example Cl$^-$ efflux from barley (Jackson and Edwards, 1966) and Arabidopsis (Lorenzen et al., 2004) and SO$_4^{2-}$ efflux from carrot (Cram, 1983) and tomato roots (Lopez et al., 2002). The physiological significance of these fluxes is not always clear, but they may be important in preventing the toxic accumulation of anions in the cytosol of root cells, particularly when extracellular anion concentration is high. ARAC probably mediates inorganic anion efflux; indeed, the gating properties of ARAC are well suited to preventing intracellular accumulation of SO$_4^{2-}$ in that (1) increasing extracellular SO$_4^{2-}$ modulates the gating of ARAC such that it activates at more negative membrane voltages (and thus increases the dynamic voltage range at which ARAC is active), and (2) accumulation of intracellular SO$_4^{2-}$ would further enhance ARAC activity.

Phosphate Starvation and Citrate Efflux

Phosphate is a major mineral nutrient required by plants, but it is one of the most immobile and inaccessible nutrients present in soils (Holford, 1997). Plant have evolved a variety of strategies to increase the availability of phosphate from the soil solution, a key one being the secretion of organic acids to mobilize sparingly soluble forms of phosphate from soil solutions (Hoffland et al., 1992; Jones, 1998). The roots of Arabidopsis and the closely related members of the Brassica family have been shown to enhance malate and citrate efflux in response to phosphate deficiency (Narang et al., 2000). Since citrate and malate exist predominately as tri- and divalent anions in the cytoplasm, their movement out of root cells is an energetically passive process due the large negative potential difference across the plasma membrane. Thus, it is likely that citrate- and malate-permeable channels mediate organic-acid efflux.

Root epidermal cells from Arabidopsis plants grown in phosphate-free conditions possessed citrate (and malate) efflux currents, which exhibited channel-like activation/deactivation kinetics and voltage dependence (i.e. similar to that exhibited by ARAC-medi-
ated anion efflux currents). This raised the possibility that either (1) phosphate starvation induced a citrate- and malate-permeable channel activity distinct from ARAC, or (2) citrate and malate conductance through ARAC is small and beyond the resolution of the patch clamp technique but that phosphate starvation up-regulated the ARAC activity to levels that allow citrate and malate efflux currents to be resolved. However, the absence of any significant regulation of ARAC activity by phosphate supply (Fig. 7D) suggests that phosphate starvation induces a novel channel activity distinct from ARAC activity and permeable to organic-acid anions. Thus, we refer to the phosphate-regulated ARAC as PR-ARAC.

In some experiments, Cl\(^-\) was omitted from the pipette media to avoid the contamination of citrate efflux currents by Cl\(^-\) efflux. From a technical viewpoint, Ag/AgCl electrodes (which were used in this study) ideally require the presence of high millimolar amounts of Cl\(^-\) in the pipette solution. However, in this study, junction potentials could be offset by the amplifier, and no drift in the junction potential during the experiment was evident, suggesting that the Ag/AgCl electrodes were stable in this study. Possible reasons for this are that only small currents (\(<50\, \text{pA}\)) were recorded using citrate-containing pipette solutions and that there was sufficient contaminant Cl\(^-\) (calculated to be at least 0.3 \(\mu\text{M}\) from the reported impurities in the citric acid) for reliable stable operation of the Ag/AgCl electrodes. Taking intracellular Cl\(^-\) to be 0.3 \(\mu\text{M}\), \(E_{\text{Cl}}\) was set at \(-305\, \text{mV}\); thus, the inward currents shown in Figure 7A could not be the result of Cl\(^-\) efflux.

PR-ARAC differs significantly from other channels from higher plant roots that show a degree of permeability to organic-acid anions. First, PR-ARAC has significantly greater selectivity for organic-acid anions over Cl\(^-\) compared to that reported for ALAACs from maize root tips (\(P_{\text{cit}}/P_{\text{Cl}} = 0.18\) and \(P_{\text{mal}}/P_{\text{Cl}} = 0.25\); Kollmeier et al., 2001) and wheat roots (\(P_{\text{mal}}/P_{\text{Cl}} = 2.6\); Zhang et al., 1991). Second, the ALAACs from maize and wheat exhibit S-type voltage dependence (i.e. inwardly rectifying current voltage relationships), whereas PR-ARAC exhibited R-type voltage dependence. Finally, ALAAC activity is dependent on the presence of extracellular \(\text{A}\text{t}^{3+}\) and does not exhibit rundown, even in excised outside-out patches (Pineros and Kochian, 2001). However, the rapid rundown of PR-ARAC indicates that its activity is probably dependent on cytosolic factors that are washed out during the patch clamp experiment.

The low frequency of occurrence for PR-ARAC currents indicates that relatively few epidermal root cells were active in organic-acid anion efflux in response to phosphate starvation. It is expected that the sites of organic-acid efflux in the root system will probably reflect sites of active phosphate uptake. Indeed, organic-acid efflux has been shown to be a key factor influencing the phosphate acquisition efficiency of Arabidopsis (Narang et al., 2000). The completion of the Arabidopsis genome has revealed nine members of the phosphate transport family, Pht1, which are thought to mediate the uptake of phosphate from the soil solution (Mudge et al., 2002). Promoter analysis of Pht1 family members revealed complex and cell-specific expression in response to phosphate starvation (Mudge et al., 2002). For example, Pht1:2 phosphate-induced expression was limited to mainly the root hairs (or trichloblast cells of the root epidermis) but was absent in the epidermal atrichloblast cells and the root elongation zones and root tip. Thus, the pattern of Pht1:2 expression only partially overlaps with the cell-specific GFP expression of the N9093 line used in this study; hence, many of the cells used in this study may not have been active in phosphate uptake. Therefore, it is possible that the low frequency of occurrence of PR-ARAC reflects the isolation of protoplasts from root epidermal cells, which were not directly mediating phosphate uptake.

In summary, we have characterized a novel anion conductance in the plasma membrane of epidermal cells from Arabidopsis roots, which is permeable to citrate and malate and regulated by extracellular phosphate supply. It is likely that the channels underlying this conductance are involved in phosphate acquisition and represent the pathway for organic-acid anion efflux associated with phosphate nutrition in Arabidopsis roots.

**MATERIALS AND METHODS**

**Plant Material and Growth Conditions**

Seeds of a GFP-expressing Arabidopsis (Arabidopsis thaliana) line, Haseloff donor number J0841 (Nottingham Stock no. N0993), were surface sterilized first for 10 min with 80% (v/v) ethanol, then for 10 min with 1.2% (v/v) active chlorine bleach (NaOCl), and finally thoroughly rinsed with sterile water. These seeds were planted onto the surface of sterile agar plates (90-mm diameter) containing 0.8% phytagel (Sigma-Aldrich, St. Louis), full-strength Murashige and Skoog basal medium (Sigma-Aldrich), and 2% Suc, pH 5.5. The plates were placed vertically in a Sanyo (Sanyo Electric Biomedical, Sakata, Japan) MLR-350 environmental chamber with a light intensity of 100 \(\mu\text{mol}\, \text{m}^2\, \text{s}^{-1}\) for 16 h at a constant 22°C. Roots were harvested after 7- to 20-d growth. For phosphate starvation, 7- to 10-d-old plants were transferred to phosphate-free agar plates containing full-strength Murashige and Skoog basal medium without phosphate, 0.8% purified agarose (MBI Fermentas, Vilnius, Lithuania), and 2% Suc, pH 5.5.

**Confocal Microscopy**

Roots were imaged live in situ on the agar plates using a laser-scanning confocal microscope (TCS SP2, Leica Microsystems, Wetzlar, Germany). Imaging was performed using an excitation wavelength of 488 nm and emission window of 510 to 525 nm. Images are presented without manipulation.

**Protoplast Isolation**

Roots were removed from the agar plants and finely chopped in a solution (10 mM CaCl\(_2\), 10 mM KCl, 2 mM MgCl\(_2\), 2 mM MES/KOH, pH 6.0) containing (w/v) 1.5% cellulase (Onozuka RS, Yakult Honsha, Tokyo), 0.1% pectolyase Y-23 (Kikkoman, Japan), and 1% cellulase (Calbiochem, UK), 0.1% bovine serum albumin, and adjusted to 500 mosmol kg\(^{-1}\) with sorbitol. The chopped

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tissue was agitated at 30°C for 90 min. The digest was filtered using 50-μm nylon mesh and centrifuged at 60g for 5 min. The pellet was resuspended in 5 mL of ice-cold Solution A (500 mM sorbitol, 1 mM CaCl₂, 5 mM MES/KOH, pH 6.0) and protoplasts were isolated using a Suc gradient as previously described (Roberts and Tester, 1995). After the Suc gradient step, clean protoplasts were resuspended in 5 mL Solution A and centrifuged at 60g for 5 min. Protoplasts were resuspended in 1 mL of Solution A and stored on ice.

Electrophysiology

GFP fluorescence in root protoplasts was detected using a fluorescence microscope equipped with an excitation filter of 460 to 500 nm and an emission filter at 510 to 560 nm. Whole-cell currents from GFP-fluorescent protoplasts were recorded at approximately 20°C with an Axopatch 200A amplifier (Axon Instruments, CA) using conventional patch clamp techniques (Hamill et al., 1981). Cells were perfused in a chamber consisting of a thin glass base to which protoplasts adhered loosely. Electrodes were pulled from borosilicate-glass capillaries (Kimax 51, Kimax Products, NJ) to give resistance of less than 25 MΩ. Borosilicate-glass base to which protoplasts adhered loosely. Electrodes were pulled from borosilicate-glass capillaries (Kimax 51, Kimax Products, NJ) to give resistances of less than 25 MΩ in sealing solution (see below). Using sealing solution, seals >10 GΩ were regularly achieved. To reduce pipette capacitance, electrodes were coated by dipping the pipette tip into a 30% (w/w) mixture of mineral oil and Paraffilm (American National Can, Greenwich, CT). An Ag/AgCl reference electrode was connected to the bath via a 3-M KCl/agar salt bridge. Whole-cell capacitance and series resistance were compensated for by the amplifier. Access resistance was measured during experiments and was less than 20 MΩ. Before analog-to-digital conversion, the voltage signals representing clamp currents were low-pass filtered at 5 kHz. Outside-out patches were obtained from the whole-cell configuration by pulling away the pipette from the protoplast. Data were digitized at 2 kHz and filtered at 500 Hz for analysis. All data were acquired using either pClamp 8.0 or FigP (version 2.2, Biosoft, Cambridge, UK). Liquid-junction potential was corrected for in all experiments as described by Neher (1992). Tip potentials were measured at the end of an experiment by measuring the potential change when the pipette tip was broken. Only experiments in which the tip potential was less than 4 mV were used. Ion equilibrium potentials were calculated after correction for ionic activities (as calculated by GEOCHEM-PC; Parker et al., 1995). Variation in this solution was replaced by SBS, which contained 5 mM LaCl₃, 10 mM CaCl₂, 5 mM MgCl₂, 10 mM MES, adjusted to pH 6.0 with Tris-base and adjusted to 720 mosmol kg⁻¹ using sorbitol; all currents were recorded in this unless otherwise stated. Standard intracellular (pipette-filling) solution (25 mM CsSO₄, 2 mM MgCl₂, 10 mM HEPES, 1 mM MgATP, 2 mM EGTA adjusted to pH 7.2 with Tris base and 720 mosmol kg⁻¹ using sorbitol) was used in all experiments unless otherwise stated.

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LITERATURE CITED


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