Citrate-Permeable Channels in the Plasma Membrane of Cluster Roots from White Lupin

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White lupin (Lupinus albus) is well adapted to phosphorus deficiency by developing cluster roots that release large amounts of citrate into the rhizosphere to mobilize the sparingly soluble phosphorus. To determine the mechanism underlying citrate release from cluster roots, we isolated protoplasts from different types of roots of white lupin plants grown in phosphorus-replete (+P) and phosphorus-deficient (−P) conditions and used the patch-clamp technique to measure the whole-cell currents flowing across plasma membrane of these protoplasts. Two main types of anion conductance were observed in protoplasts prepared from cluster root tissue: (1) an inwardly rectifying anion conductance (IRAC) activated by membrane hyperpolarization, and (2) an outwardly rectifying anion conductance (ORAC) that became more activated with membrane depolarization. Although ORAC was an outward rectifier, it did allow substantial inward current (anion efflux) to occur. Both conductances showed citrate permeability, with IRAC being more selective for citrate$^+$ than Cl$^-$ (P$_{Cl}$/P$_{Cit}$ = 26.3), while ORAC was selective for Cl$^-$ over citrate (P$_{Cl}$/P$_{Cit}$ = 3.7). Both IRAC and ORAC were sensitive to the anion channel blocker anthracene-9-carboxylic acid. These currents were also detected in protoplasts derived from noncluster roots of −P plants, as well as from normal (noncluster) roots of plants grown with 25 μM phosphorus (+P). No differences were observed in the magnitude or frequency of IRAC and ORAC currents between the cluster roots and noncluster roots of −P plants. However, the IRAC current from +P plants occurred less frequently than in the −P plants. IRAC was unaffected by external phosphate, but ORAC had reduced inward current (anion efflux) when phosphate was present in the external medium. Our data suggest that IRAC is the main pathway for citrate efflux from white lupin roots, but ORAC may also contribute to citrate efflux.

The development of cluster roots is an important strategy used by some plant species to adapt to infertile soils (Dinkelaker et al., 1995; Neumann and Martinoa, 2002). These bottlebrush-like roots were initially called proteoid roots because they were first characterized in members of the Proteaceae (Purnell, 1960). Cluster roots are groups of short, secondary lateral rootlets that are covered with a dense mat of root hairs (Lamont et al., 1984). In white lupin (Lupinus albus), the development of cluster roots is greatly enhanced under phosphorus (P) deficiency, and this species has been widely used as a model system for studying the morphology and physiology of these specialized root structures (Dinkelaker et al., 1995; Keerthisinghe et al., 1998; Neumann et al., 1999; Watt and Evans, 1999a, 1999b).

Cluster roots are characterized by specific metabolic and physiological changes that help plants overcome the nutrient deficiency that induces their development (Neumann and Martinoa, 2002). One well-described feature of this tissue is their capacity to release large amounts of organic anions, such as malate and citrate (Neumann et al., 1999; Watt and Evans, 1999b; Roelofs et al., 2001; Neumann and Martinoa, 2002; Vance et al., 2003). These organic anions, and citrate in particular, enhance the availability of phosphorus to the plant by solubilizing phosphorus that is bound with cations or other mineral ligands in the soil. There is also evidence that citrate can enhance the mineralization of some organic phosphorus compounds as well (Hayes et al., 2000). The amount of citrate released from white lupin is related to the phosphorus concentration in the growth medium and therefore the phosphorus status of the plants. For instance, the citrate released from plants grown in the absence of phosphorus was 3-fold greater than the release from cluster roots grown in 10 μM phosphorus (Keerthisinghe et al., 1998). The composition of the exudates is dependent on the developmental stage of the clusters, with mature clusters releasing more citrate than malate, juvenile clusters releasing mostly malate, and senescent clusters releasing little of either (Keerthisinghe et al., 1998; Neumann et al., 1999). Citrate exudation from white lupin also shows diurnal variation, with maximum rates occurring during the photoperiod (Watt and Evans, 1999a).

Exudation of organic acids by cluster roots could result from altered organic acid metabolism, as indicated...
by the enhanced in vitro activities of phosphoenolpyruvate carboxylase and malate dehydrogenase (Johnson et al., 1994, 1996; Neumann et al., 1999), reduced activity of aconitase (Neumann et al., 1999; Kihara et al., 2003), and down-regulated ATP-citrate lyase (Langlade et al., 2002). However, other studies have shown little correlation between the activities of these enzymes and citrate exudation (Keerthisinghe et al., 1998; Watt and Evans, 1999a). The correlation between the internal citrate concentration and citrate efflux from these roots is also weak (Keerthisinghe et al., 1998; Neumann et al., 1999). For example, mature cluster roots and senescent cluster roots of white lupin contain similar concentrations of citrate, but only the mature cluster roots release citrate (Neumann et al., 1999). These findings suggest that exudation of organic acids from the cluster roots is unlikely to be controlled solely by their synthesis and, more likely, a coordinated mechanism involving several factors is likely to be operating. Therefore, the transporters that facilitate organic anion movement across the plasma membrane are also likely to play an important role in regulating the exudation of these organic anions (Ryan et al., 2001).

Since citrate exists predominantly as a trivalent anion in the cytoplasm, its movement out of the root cells is an energetically passive process due to the large negative potential difference across the plasma membrane. Therefore, citrate efflux could feasibly occur through a citrate-permeable anion channel in the plasma membrane. The observation that citrate efflux from the cluster roots of white lupin is sensitive to anion channel blockers, such as anthracene-9-carboxylic acid (A-9-C) and ethacrynic acid, is consistent with this proposition (Neumann and Römheld, 1999; Neumann et al., 1999).

To investigate the mechanism underlying the exudation of citrate anions from cluster roots of white lupin, we used the patch-clamp technique to measure the whole-cell currents in protoplasts isolated from mature cluster roots (Fig. 1) of P-deficient (−P) plants. We compared these currents with those in noncluster roots of −P plants, as well as those in noncluster roots from P-replete (+P) plants.

**RESULTS**

**Whole-Cell Currents in Protoplasts Prepared from Mature Cluster Roots**

According to convention, the efflux of anions across the plasma membrane is defined as an inward or negative current. To detect inward currents associated with citrate efflux from protoplasts of white lupin roots, we included citrate$^{3-}$ as the main anion in the patch-pipette solution and tetraethylammonium as the main cation, due to its low permeability across biological membranes. When the holding potential was near 0 mV, two main types of current were detected in the protoplasts: (1) an inwardly rectifying current that activated rapidly and inactivated slowly (Fig. 2A and inset), and (2) an outwardly rectifying current that only showed deactivating currents at hyperpolarized potentials (Fig. 2C and inset). The reversal potentials for the two types of current were 16.7 ± 6.0 mV ($n = 12$) and −7.9 ± 3.7 mV ($n = 26$), respectively (compare Fig. 2, B with D). The outwardly rectifying current also displayed slow current relaxation (tail currents) following depolarizing pulses (Fig. 2C). The inwardly rectifying current was observed in 34% of protoplasts ($n = 65$) and the outwardly rectifying current was observed in 45% of protoplasts (Table I). The remaining protoplasts contained small inward and outward currents (Fig. 2E), which often displayed reversal potentials less than −40 mV and more negative than the equilibrium potentials for the ions present in the system (Fig. 2F). This implies that the H$^+$ pump may be contributing to these small conductances, but this was not investigated further. On a few occasions, the inwardly rectifying current and the outwardly rectifying current were observed in the same protoplast, but not at the same time. The current appeared to switch from one type to the other along with the expected shift in the reversal potential. This switch occurred in a matter of minutes, even though the experimental conditions remained unchanged (Fig. 3).
The Inwardly Rectifying Current Is Carried by Citrate Efflux

Inward currents can be caused by the influx of cations (i.e. Ca$^{2+}$) or the efflux of anions (i.e. citrate$^{3-}$, Cl$^{-}$, or even HEPES). To determine whether Ca$^{2+}$ influx was responsible for the inwardly rectifying current, we tested the effect of the Ca$^{2+}$ channel blocker Gd$^{3+}$. Figure 4A shows that the inward current was not inhibited by the addition of 100 μM GdCl$_3$ to the bath. Furthermore, both the current magnitude and reversal potential of the inwardly rectifying current were independent of CaCl$_2$ concentrations in the bath.
Table I. Characteristics of whole-cell current in protoplasts prepared from mature cluster roots and noncluster roots of −P plants and lateral roots of +P plants

<table>
<thead>
<tr>
<th>Root Cells</th>
<th>IRAC</th>
<th>ORAC</th>
<th>Small Conductance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Frequency</td>
<td>E\textsubscript{rev} (mV)</td>
<td>I\textsubscript{m} (mA m\textsuperscript{-2})</td>
</tr>
<tr>
<td>−P cluster roots</td>
<td>34 (65)</td>
<td>16.7 ± 6.0 (12)</td>
<td>-283.4 ± 46.4 (10)</td>
</tr>
<tr>
<td>−P noncluster roots</td>
<td>40 (15)</td>
<td>8.4 ± 5.4 (6)</td>
<td>-263.8 ± 76.4 (6)</td>
</tr>
<tr>
<td>+P noncluster roots</td>
<td>16 (37)</td>
<td>14.7 ± 5.6 (6)</td>
<td>-182.3 ± 50.6 (6)</td>
</tr>
</tbody>
</table>

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bath (Fig. 4B). These results suggest that the inwardly rectifying current is likely to result from anion efflux rather than Ca\textsuperscript{2+} influx and was accordingly termed inwardly rectifying anion conductance (IRAC). The contributions of HEPES efflux to the observed inward current appeared to be small, as the substitution of external solution from 10 mM CaCl\textsubscript{2}, 5 mM MES, pH 6.0, to 10 mM CaCl\textsubscript{2}, 5 mM HEPES, pH 7.0, did not shift the reversal potential or the current magnitude (data not shown). Therefore, the inward current could result from the efflux of citrate\textsuperscript{3–} and/or Cl\textsuperscript{–}, since a small amount of Cl\textsuperscript{–} was required in the pipette solution to reduce the junction potentials associated with the Ag/AgCl half-cell. The relative permeability of citrate\textsuperscript{3–} and Cl\textsuperscript{–} through the underlying channels can usually be calculated from the reversal potential under biionic conditions (same solution either side of the membrane) because the reversal potential will track closer to the equilibrium potential of the more permeable ions. Attempts to perform these experiments were unsuccessful because the inclusion of citrate in the bath solution caused the protoplast membranes to become unstable and collapse. Therefore, the following two experiments were conducted to examine whether the observed inward current was mainly caused by Cl\textsuperscript{–} or citrate\textsuperscript{3–} efflux.

The first method monitored the shift in reversal potential when the Cl\textsuperscript{–} concentration in the bath solution was changed 10-fold. Reducing CaCl\textsubscript{2} from 10 to 1 mM CaCl\textsubscript{2} shifted the theoretical equilibrium potential for Cl\textsuperscript{–} (E\textsubscript{Cl}) from −40 mV to 17 mV (Fig. 4B). The reversal potential of the IRAC current was 21.6 ± 8.9 mV in 10 mM CaCl\textsubscript{2} and 24.6 ± 6.8 mV (n = 3) in 1 mM CaCl\textsubscript{2}, respectively. Since the reversal potential was unaffected by Cl\textsuperscript{–} concentration in the bath, we can conclude that Cl\textsuperscript{–} efflux does not contribute significantly to the IRAC.

In the second method, Cl\textsuperscript{–} was removed from the pipette solution altogether and the Ag/AgCl half-cell was replaced with a platinum electrode. A rapidly activating, slowly inactivating inward current, typical of IRAC, was still observed under these experimental conditions (Fig. 5A). The reversal potential of this current was 15.4 ± 4.4 mV (n = 3; Fig. 5B), which is comparable to the reversal potential measured with 4 mM Cl\textsuperscript{–} in the pipette (E\textsubscript{rev} = 16.7 ± 6.0; n = 12), confirming that Cl\textsuperscript{–} efflux contributes little to the observed inward current. Since Ca\textsuperscript{2+} influx and Cl\textsuperscript{–} efflux are not responsible for IRAC, we deduce that this inward current is carried predominantly by citrate\textsuperscript{3–} efflux. The relative permeability of the IRAC channels to citrate and Cl\textsuperscript{–} is determined later. Identical currents were also observed when SO\textsubscript{4}\textsuperscript{2–} was removed from the pipette solution (type II pipette solution; see Fig. 5), which discounts any role for SO\textsubscript{4}\textsuperscript{2–} in activating this anion conductance—a phenomenon previously described for a different anion channel in Arabidopsis (Arabidopsis thaliana; Frachisse et al., 1999).

To determine whether the IRAC was permeable to malate\textsuperscript{2–}, the 20 mM citrate salt usually included in the pipette solution was substituted with an osmotically equivalent concentration of malate salt (30 mM). An IRAC-like inward current was observed using malate\textsuperscript{2–} as the main permeant anion in the pipette (data not shown). Current densities at −150 mV were −189.7 ± 35.4 mA m\textsuperscript{-2} (n = 4) with malate in the pipette, and −216.5 ± 32.2 (n = 8) with citrate\textsuperscript{3–} in the pipette. The reversal potential of the current with malate\textsuperscript{2–} in the pipette was 14.4 ± 4.6 mV (n = 4), which is comparable to the reversal potential of 16.7 ± 6.0 (n = 12) measured with citrate\textsuperscript{3–} in the pipette using the same bath solution. These findings suggest that the IRAC is permeable to both citrate\textsuperscript{3–} and malate\textsuperscript{2–} anions.

Pharmacology and Selectivity of IRAC

Citrate efflux from the cluster roots of white lupin is reportedly correlated with rhizosphere acidification via enhancement of H\textsuperscript{+}-ATPase activity (Yan et al., 2002). To test whether citrate efflux is modulated by external pH, we investigated the effect of external pH on the IRAC current. The inward current was unaffected by pH between 4.5 and 8 (n = 4; data not shown).
Since citrate efflux from intact cluster roots of white lupin is inhibited by the anion channel blockers A-9-C and ethacrynic acid (Neumann et al., 1999), we investigated the effects of these blockers on IRAC. Figure 6 shows that 100 μM A-9-C caused a large and reversible decrease on the inward current. The current was inhibited by 89 ± 4.6% (n = 5) at −151 mV. By contrast, 100 μM ethacrynic acid had no effect on IRAC (n = 4; data not shown).

We used the reversal potential of this A-9-C-sensitive current in a modified Goldman-Hodgkin-Katz (GHK) equation (Eq. 2; see “Materials and Methods”) to estimate the relative permeability of the IRAC channels to citrate\(^3\) and Cl\(^−\) (\(P_{\text{Cit}}/P_{\text{Cl}}\)) by assuming A-9-C inhibits the anion conductance only. The A-9-C-sensitive current-voltage curve is generated by subtracting the currents measured after A-9-C addition from the currents measured before A-9-C addition. The reversal potential of the resulting curve was 20 mV (Fig. 6C), which predicts a permeability ratio, \(P_{\text{Cit}}/P_{\text{Cl}}\), of 26.3. Note that the calculation has accounted for the effect of ionic strength on activities of the ions. If the effect of ionic strength on activities of the ions is not taken into account, a much lower value for \(P_{\text{Cit}}/P_{\text{Cl}}\) (1.7) can be obtained.

**The Outwardly Rectifying Current Is Carried by Cl\(^−\) Influx**

The second type of current observed in about 45% of protoplasts was an outwardly rectifying current that exhibited deactivating inward currents during hyperpolarizing pulses (Fig. 2C). When the holding voltage was shifted from near 0 mV to −171 mV, the character of these currents changed. The deactivating inward currents disappeared, but the magnitude of the tail currents increased (Fig. 7A). The amplitude of the tail current began to saturate as the membrane voltage was returned from increasingly more positive potentials. The relative conductance of the tail currents was plotted against voltage (Fig. 7B) and the resulting curve was fitted with a Boltzmann function (Eq. 1):
where $V_{50}$ is the voltage at which the channels have attained one-half their maximal open probability, and $S$ is the slope factor ($RT/zF$, where $R$, $T$, and $F$ have their usual values, and $z$ is the minimal gating charge). The values of $V_{50}$ and $S$ were 35 mV and 37.3 mV, suggesting that activation of the channel was weakly dependent on the voltage. The $z$ value calculated from the slope factor was 0.74. The weak voltage dependence shown in Figure 7B also demonstrates that some inward current was passed at relatively negative voltages. This is also evident in the current-voltage curve (Fig. 2D) and from inhibition of inward current by A-9-C (Fig. 9C).

The reversal potential of protoplasts showing the outwardly rectifying conductance in 10 mM CaCl$_2$ was more positive than the equilibrium potential for Cl$^-$ ($E_{Cl}$), but more negative than the equilibrium potentials for citrate and Ca$^{2+}$ (which are very positive; Fig. 8C). The magnitude of the outward currents and tail currents was reduced when external CaCl$_2$ was reduced from 10 to 1 mM (Fig. 8, A–C) and the reversal potential shifted from $-20.7 \pm 7.4$ mV to $-5.3 \pm 1.2$ mV ($n = 3$), which follows $E_{Cl}$ (Fig. 8C). An identical shift in the reversal potential with changes in external CaCl$_2$ was observed in the presence of the Ca$^{2+}$ channel blocker, Gd$^{3+}$ (data not shown). These results suggest that Cl$^-$ carries this outward current and that the deactivating inward currents observed at negative potentials (see Fig. 2C) are caused by the partial closure of the channels. This conductance was designated outwardly rectifying anion conductance (ORAC).

The effect of external H$_2$PO$_4^-$ (with removal of Cl$^-$) on both IRAC and ORAC was tested by substituting the 10 mM KCl in the bath solution with 10 mM KH$_2$PO$_4$ in a background of 1 mM Ca-(gluconate)$_2$. As shown in Figure 8, D to F, the outward current through ORAC was markedly reduced and the reversal potential shifted from $-15.3 \pm 2.8$ mV to $32.6 \pm 3.7$ mV ($n = 3$) with this substitution. If we assume the contribution of cations to this current is negligible, then the relative permeability of the channel to H$_2$PO$_4^-$ and Cl$^-$ ($P_{H_2PO_4^-}/P_{Cl^-}$) can be estimated to be 0.15 using the GHK equation (Goldman, 1943). More interesting was the inhibition of inward current (Cl$^-$...
and citrate\(^3\)-efflux) through ORAC despite there being a much larger electrochemical gradient for Cl\(^-\)efflux. This effect could be observed both in the initial and transient inward currents, the steady-state inward currents, and the tail currents \((n = 3)\). No effect of external H\(_2\)PO\(_4\) on IRAC was observed (data not shown).

Pharmacology and Selectivity of ORAC

The current through ORAC was almost completely inhibited when 100 \(\mu M\) A-9-C were added to the bath solution (Fig. 9). This inhibition was largely reversible if the blocker was washed out of the bath (data not shown). The inhibition demonstrated that ORAC could carry significant inward current (anion efflux). By subtracting the currents before and after A-9-C addition, we constructed a current-voltage curve for the A-9-C-sensitive current (see above). The reversal potential of this curve was applied to the modified GHK equation (Eq. 2) and the relative permeability of citrate\(^3\)-toCl\(^-\) \((P_{\text{Cit}}/P_{\text{Cl}})\) was estimated to be 0.27. Like the IRAC, ORAC was relatively insensitive to 100 \(\mu M\) ethacrynic acid \((n = 3);\) data not shown.

To identify the single channels responsible for the observed whole-cell IRAC and ORAC currents, inside-out and outside-out patches were obtained. However, we were unable to observe any single-channel activity that was sensitive to A-9-C, whether the channel...
blocker was added to the cytoplasmic or the extracellular side of the plasma membranes (data not shown). The lack of single-channel activity implies that critical components essential for activation of the IRAC and ORAC are lost upon excising the patches from the whole cell.

Whole-Cell Current in Protoplasts Derived from Noncluster Roots of −P and +P Plants

Mature cluster roots of white lupin release more citrate than noncluster roots from the same P-deficient plants (Keerthisinghe et al., 1998; Neumann et al., 1999). Furthermore, the amount of citrate released is inversely related to the phosphorus status of the plants (Keerthisinghe et al., 1998; Neumann et al., 1999; Sas et al., 2001). Given that the efflux of citrate anions from cluster roots is likely to be mediated by the IRAC, we investigated whether this current occurred less frequently or was functionally different in the noncluster root cells. To test this hypothesis, we measured whole-cell currents in protoplasts prepared from the noncluster roots of −P and +P white lupin plants. Typical IRAC, ORAC, and pump currents were observed in protoplasts from all these tissues. Table I shows that the IRAC, ORAC, and small currents in protoplasts from cluster and noncluster roots of −P plants displayed similar frequencies, current densities, and reversal potentials. However, some differences were detected in protoplasts from the roots of +P plants. In these roots, IRAC occurred less frequently and the current density tended to be smaller, albeit insignificantly so (P = 0.062), than in cluster and noncluster roots of −P plants (Table I). In addition to the above currents, an instantaneously activated, noisy inward current was also observed in protoplasts derived from +P noncluster roots in 10 mM CaCl2 solution (data not shown). This type of current remains to be fully characterized, but it was sensitive to Gd3+ and reminiscent of nonselective cation current characterized in wheat (Triticum aestivum; Tyerman et al., 1997) and maize (Zea mays; Roberts and Tester, 1997) root cells. Moreover, we were unable to isolate protoplasts from −P senescent cluster roots that have previously been shown to release very little organic anions (Neumann et al., 1999).

Outwardly Rectifying K+ Conductance

We investigated whether K+ currents could be detected in the cluster roots of white lupin. The KCl in the bath was replaced with either K2-malate, K-Glu, or K2SO4 to avoid confusion with the ORAC-mediated Cl− currents. With 100 mM K+ in the pipette solution, a small, rapidly activating outward current and a noisy inward current were elicited by depolarizing and

Figure 8. Effects of external Cl− and KH2PO4− concentrations on the outwardly rectifying current. Data in A and B show the superimposed current traces from a single cluster root protoplast elicited by voltage pulses between −171 to 59 mV in 20-mV intervals from a holding potential of −1 mV. External solutions contained 10 mM CaCl2 (A) and 1 mM CaCl2 (B). Steady current-voltage curves from A and B are presented in C. Data in D and E show the superimposed current traces from a single −P cluster root protoplast in response to voltage pulses ranging from −173 to 57 mV in 20-mV intervals from a holding potential of −3 mV. External solutions contained 1 mM Ca-(gluconate)2 together with either 10 mM KCl (D) or 10 mM KH2PO4 (E). Steady current-voltage curves from D and E are presented in F. Pipette solution was type I.
hyperpolarizing pulses, respectively (Fig. 10A). Identical currents were found with all three bath solutions (data not shown), suggesting that the outward currents were due to K$^+$ efflux and not anion influx. Support for this conclusion was obtained by increasing the external K$^+$ concentration from 5 to 50 mM, which increased the magnitude of inward current and shifted the reversal potential in a positive direction, toward $E_K$ (Fig. 10B). It is noteworthy that the time-dependent K$^+$ inwardly rectifying channels, often reported in root cells (Maathuis et al., 1997), were not observed in any protoplast tested ($n = 7$; Fig. 10A).

**DISCUSSION**

Cluster roots form on the lateral roots of white lupin during the onset of phosphorus deficiency. The special physiology exhibited by these roots assists lupin plants to extract phosphorus from sparingly soluble pools in the soil that are not accessible to most other species. To investigate the mechanism underlying citrate efflux from the roots of white lupin, protoplasts were isolated from the cluster and noncluster roots of −P plants and from the normal (noncluster) roots of +P plants. Whole-cell currents in these protoplasts were then measured using the patch-clamp technique. Unlike most studies of this kind, in which protoplasts were isolated by digesting finely chopped root tissue

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Figure 9. Effects of A-9-C on the ORAC in protoplasts from cluster roots of −P plants. Data show the superimposed current traces in response to voltage pulses ranging from −151 to 49 mV in 20-mV intervals from a holding potential of −1 mV before (A) and 5 min after (B) addition of 100 μM A-9-C. Current-voltage curves from the steady currents recorded before and after the treatment are shown in (C). Data show the mean ± SEM from four protoplasts.

Figure 10. K$^+$-dependent currents in lupin roots. Superimposed current traces collected from a single cluster root protoplast elicited by voltage pulses ranging from −183 to 37 mV in 20-mV intervals from a holding potential of −43 mV (A). The external solution (mM) was 5 K$_2$-malate, 1 Ca-(gluconate)$_2$, 5 MES, pH 6.0. B, Steady-state current-voltage curves measured with 5 and 50 mM K$_2$-malate in the bath solutions. Data show the mean ± SEM for four to five protoplasts. Pipette solution (mM), 10 KCl, 90 K-Glu, 2 CaCl$_2$, 2 Na$_2$ATP, 2 MgCl$_2$, 10 EGTA, 10 HEPES, adjusted to pH 7.2 with Tris, and osmolality adjusted to 720 mosmol kg$^{-1}$ with Suc.
for several hours (Zhang et al., 2001), we prepared protoplasts by treating intact root tissue with digestive enzymes for relatively short periods, which preferentially digested epidermal cells (see “Materials and Methods”). With citrate\textsuperscript{3−} as the main permeant anion in the cytoplasm, 10 mM Ca\textsubscript{Cl\textsubscript{2}} in the bath solution, and a holding potential near 0 mV, we found two main anion conductances: an IRAC that displayed a fast activation and slow inactivation in response to membrane hyperpolarization (Fig. 2A), and an ORAC with deactivating inward currents (Fig. 2C). However, ORAC could carry significant inward current in the steady state. We also detected proton pump-like conductances in some protoplasts, but these were not investigated in detail (Fig. 2, E and F). On occasion, more than one of these conductances occurred in the same protoplasts, but not at the same time. Instead, it appeared that one type of conductance was replaced by another within several minutes (Fig. 3). Similar changes in conductance have been reported in protoplasts derived from wheat root cells (Tyerman et al., 2001), as were the fast and slow anion conductances in guard cells, which were suggested to be different modes of action for the same channel (Dietrich and Hedrich, 1994). We did not investigate this further, but it remains possible that IRAC and ORAC represent different conductance states of the same transporter. Activation kinetics for the IRAC are reminiscent of hyperpolarization-activated anion channels in carrot suspension cells (Barbara et al., 1994), as well as the aluminum-activated malate channel in wheat roots (Zhang et al., 2001), whereas ORAC shared some features with the slow anion channel in guard cells (Schroeder and Keller, 1992).

IRAC Mediates Citrate Efflux

We conclude that IRAC is caused by citrate\textsuperscript{3−} efflux rather than Cl\textsuperscript{−} efflux or Ca\textsuperscript{2+} influx, and several results support this position. First, IRAC was insensitive to the Ca channel blocker Gd\textsuperscript{3+} (Fig. 4A), and the reversal potential and magnitude of IRAC current were relatively unaffected by changes in Ca\textsuperscript{2+} concentrations in the bath (Fig. 4B). These results indicate that Ca\textsuperscript{2+} fluxes are unlikely to make substantial contributions to the current mediated by IRAC. IRAC was similarly independent of Cl\textsuperscript{−} concentrations in the bath (Fig. 4B), and identical currents were detected in experiments when Cl\textsuperscript{−} was removed from the pipette solution (Fig. 5). This is strong evidence that Cl\textsuperscript{−} fluxes do not contribute significantly to IRAC. Last, the disappearance of IRAC when citrate was removed from the pipette solution (Fig. 9) also supports the conclusion that citrate\textsuperscript{3−} anions are the main carriers of charge for the IRAC currents.

 Attempts to determine the relative permeability of IRAC to citrate\textsuperscript{−} and Cl\textsuperscript{−} in bi-ionic conditions were hampered by the tendency for protoplasts to collapse after citrate was added to the external solution. This injury was probably related to the formation of Ca-citrate complexes that would reduce the free Ca\textsuperscript{2+} activity and destabilize the membranes (Bertl and Slayman, 1990). Instead, we used the reversal potential of the A-9-C-sensitive current to calculate a relative permeability of IRAC for citrate\textsuperscript{3−} and Cl\textsuperscript{−} by assuming A-9-C inhibits the anion conductances only. This assumption seems reasonable in view of the minimal number of permeable ions in our solutions. Our estimate for $P_{\text{Cit/Cl}}$ of 26.3 indicates that IRAC is highly selective for citrate\textsuperscript{3−} and explains why the reversal potential was relatively unaffected by changes in Cl\textsuperscript{−} concentrations in the bath (Fig. 4B).

 Several types of anion channels have been identified in the plasma membrane of root cells, including an outwardly rectifying anion channel in wheat (Skerrett and Tyerman, 1994), an aluminum-activated inwardly rectifying channel in wheat and maize (Ryan et al., 1997; Kollmeier et al., 2001; Piñeros and Kochian, 2001, 2002; Zhang et al., 2001) and three types of anion channels in barley (Hordeum vulgare) xylem parenchyma cells (Kohler and Raschke, 2000). While most plasma membrane anion channels described so far show a higher selectivity for Cl\textsuperscript{−} or NO\textsubscript{3}− than physiologically important organic anions, such as malate and citrate (Barbier-Brygoo et al., 2000), some anion channels do show a degree of permeability to organic anions. For instance, in the quickly activating anion channel in barley xylem parenchyma, $P_{\text{mal/Cl}}$ was 0.32 (Kohler and Raschke, 2000), and in the aluminum-activated anion channel of wheat and maize, $P_{\text{mal/Cl}}$ was 2.6 and 0.25, respectively (Kollmeier et al., 2001; Zhang et al., 2001). For citrate, values for $P_{\text{Cit/Cl}}$ of 0.18 or less have been estimated in the aluminum-activated channel in maize roots (Kollmeier et al., 2001) and a channel on the tonoplast of Kalanchee diuramontiana cells (Hafke et al., 2003). IRAC is notable for its significantly greater selectivity for citrate\textsuperscript{3−} over Cl\textsuperscript{−}.

 Citrate efflux from cluster roots of white lupin is inhibited by A-9-C and, to a lesser extent, ethacrynic acid (Neumann et al., 1999). The observation here that IRAC is sensitive to the anion channel blocker A-9-C is consistent with the hypothesis that IRAC is the pathway for citrate efflux from these roots. Our results also indicate that IRAC is permeable to malate as well as citrate, suggesting that a single transporter could be responsible for the efflux of both of these anions. Previous reports have suggested that malate and citrate transport across the tonoplast may occur via a common transport pathway (Rentsch and Martinoia, 1997; Kollmeier et al., 2001; Zhang et al., 2001) and a channel on the tonoplast of Kalanchee diuramontiana cells (Hafke et al., 2003). The insensitivity of IRAC to ethacrynic acid is inconsistent with this hypothesis, but it remains possible that ethacrynic acid affects several aspects of cell physiology and that channel activity is inhibited indirectly in intact tissue.

ORAC May Also Account for Some Citrate Efflux

ORAC was also frequently observed in protoplasts derived from cluster and noncluster roots of −P as
Activity of IRAC in Different Types of Roots

Previous studies have shown that there are considerable variations in citrate efflux from different types of roots and from different stages of cluster root development. For example, citrate efflux from the mature segments of the cluster roots is approximately 5 times greater than that from noncluster root tissue on a fresh-weight basis (Keerthisinghe et al., 1998; Neumann et al., 1999). An interesting finding in the present study is that IRAC activity, which we propose is a likely pathway for citrate efflux, is similar in cluster root and noncluster root tissues of −P plants (Table I). These observations are consistent with the findings of Kania et al. (2003a, 2003b), which showed that citrate exudation from noncluster roots of +P and −P white lupin plants could be induced by inhibiting the aconitase with monofluoroacetic acid and by acidifying the cytoplasm by weak acid accumulation. Several explanations may account for the apparent discrepancy between the citrate efflux from intact root tissues and the activities of IRAC and ORAC presented here. Most importantly, cluster roots have a significantly greater surface area per unit fresh weight (more than 20-fold) than normal roots due to the density of short, lateral roots covered with root hairs (Lamont et al., 1984). Therefore, measurements of citrate efflux on a root fresh-weight basis would be much greater from cluster roots than noncluster roots due to the difference in surface areas. Additionally, the H+–ATPase activity is greater in cluster roots than in noncluster roots (Yan et al., 2002). As a consequence, cluster root cells may be more hyperpolarized, creating a greater driving force for citrate efflux through IRAC, as well as more acidified cytoplasmic pH inducing citrate efflux through IRAC (Kania et al., 2003a). Finally, the greater efflux of citrate from −P cluster roots than noncluster roots may reflect the higher citrate concentrations in the cluster root tissues (Neumann et al., 1999; Kihara et al., 2003). Some studies have reported that noncluster roots predominantly release malate, while mature cluster roots mainly release citrate (Neumann et al., 1999). Given that the channels underlying the IRAC were found to be permeable to citrate and malate, these differences could reflect the relative concentrations of these organic anions in those tissues (Neumann et al., 1999).

The IRAC and ORAC current types were also observed in protoplasts derived from noncluster roots of plants grown in the presence of 25 μM KH2PO4 (Table I). However, the currents tended to be smaller (P0.062) and occurred less frequently in the noncluster roots of +P plants compared to the roots of −P plants (Table I). These differences, while small, combined with the effect of H2PO4– on ORAC may partly explain why citrate efflux from white lupin roots is dependent upon the phosphorus status of the plants (Keerthisinghe et al., 1998; Sas et al., 2001).

Charge Balance during Citrate Efflux

It is likely that H+–ATPase activity would balance citrate efflux from cluster roots (Neumann and Martinoina, 2002) despite the low occurrence of pump signatures observed for protoplasts in our study (Table I). Indeed, Sas et al. (2001) concluded that the moles of H+ released from the roots of white lupin exceeded the moles of organic acid released. However, this conclusion neglected the valency of the organic anions and, when this is taken into account (citrate3– and malate2–), their data indicate that H+ efflux is unable to balance the loss of the organic anions. Interestingly, the exudation of organic anions from cluster roots of Hakea sp. appears to be accompanied by a concomitant efflux of K+ rather than H+ (Roelofs et al., 2001; see also Dinkelaker et al., 1997). Similarly, the aluminum-activated efflux of malate from wheat roots is accompanied by K+ efflux through a K+ outward rectifier, and this is likely to be the main mechanism maintaining electroneutrality in that system (Ryan et al., 1995; Zhang et al., 2001). The K+ outward current from cluster root cells was about 5-fold smaller than from the Cl– outward current through the ORAC (compare with Figs. 2C and 10), but the K+ outward current would activate at more physiological voltages, allowing for K+ efflux to balance anion efflux when the pump is not active.

In summary, we have identified two anion conductances in the plasma membranes of cells from the cluster and noncluster roots of white lupin that are permeable to citrate3–. These conductances differ with respect to rectification, activation, and selectivity. The hyperpolarization-activated IRAC was highly selective for citrate over Cl–, while the depolarization-activated ORAC was selective for Cl– over citrate3–.
Several results suggest that IRAC is a likely pathway for citrate efflux at hyperpolarized membrane potentials, but the ORAC may become significant at more depolarized membrane potentials.

**MATERIALS AND METHODS**

**Plant Materials**

Seeds of white lupin (*Lupinus albus* L. cv Kive mutant) were germinated in damp sand for 6 d and transferred to black 10-L hydroponic tanks. The composition of the nutrient solution used is described by Neumann and Römheld (1999). 
P + P plants were grown by including or omitting 25 μM KH2PO4 from the hydroponic solution. The plants were grown in a glasshouse maintained at 22°C/15°C on a 12-h day/12-h night cycle. The nutrient solution was continuously aerated and renewed weekly. Every 2 d the pH of the solution was checked and adjusted to pH 6.0. Experiments were performed on root tissues excised from 3- to 4-week-old plants.

**Isolation of Protoplasts**

Mature cluster roots and noncluster lateral roots (Fig. 1) were excised from P plants (grown without KH2PO4) and lateral roots (noncluster) were excised from P plants (grown with 25 μM KH2PO4). The noncluster root tissues included the apical 2 to 4 cm of lateral roots that were devoid of any emerging laterals that could be incipient cluster roots (Fig. 1). Tissues from the P plants were incubated in the following enzyme solution for 1 h at 30°C: 1.6% cellulase (Onozuka RS; Yakult Honsha, Tokyo), 0.12% pectolyase (Sigma, St. Louis), 0.5% (w/v) polyvinylpyrrolidone, 0.5% bovine serum albumin, 1 mM CaCl2, 500 mM sorbitol, 2 mM ascorbic acid, and 10 mM MES/Tris, pH 6.0. Lateral roots from P plants were incubated in a slightly different enzymatic solution that consisted of 0.8% cellulase (Onozuka RS; Yakult Honsha), 0.08% pectolyase (Sigma-Aldrich), 0.5% (w/v) polyvinylpyrrolidone, 0.5% bovine serum albumin, 1 mM CaCl2, 500 mM sorbitol, 2 mM ascorbic acid, and 10 mM MES/Tris, pH 6.0. The digest was filtered through fine muslin, centrifuged at 60 g for 5 min, and the pellet resuspended in 2 to 3 mL of standard bath solution. The protoplasts were kept on ice until experiments were started. The mean diameter of protoplasts derived from the mature cluster roots, noncluster roots of P plants, and lateral roots of P plants was 15.5 ± 0.3 μm (n = 31), 16.4 ± 0.7 μm (n = 15), and 15.2 ± 0.4 μm (n = 15), respectively, which suggests they were derived from the epidermal cells of the root tissue (see Watt and Evans, 1999a).

**Electrophysiology**

The patching pipettes were pulled from borosilicate glass blanks (Clark Electromedical, Reading, UK) and coated with Sylgard (184 silicone elastomer kit; Dow Corning, Midland, MI). The resistance of pipettes containing solution (see below) ranged from 20 to 40 MΩ in 10 mM CaCl2. The voltage across the plasma membrane was controlled and monitored with the Axopatch 200B amplifier (Axion Instruments, Foster City, CA). Pulse protocols with sampling frequencies of 2 kHz and corresponding filtering frequencies of 0.5 kHz were performed on root tissues excised from 3- to 4-week-old plants.

**Experimental Solutions**

Three types of pipette solutions were used. Type I pipette solution (mM) was composed of 20 citric acid, 2 CaCl2, 2 MgSO4, 2 NaH2ATP, 2 EGTA, and 10 HEPES. Type II pipette solution (mM) was composed of 20 citric acid, 2 CaCl2, 2 MgATP, 2 EGTA, and 10 HEPES. Type III solution was the same as that of type II, but without CaCl2. All pipette solutions were adjusted to pH 7.2 and osmolality of 720 mosmol kg−1 with tetraethylammonium hydroxyl and Suc, respectively. Free calcium concentrations of the above pipette solutions were approximately 50 nM, calculated using the chemical speciation program GEOCHEM (Parker et al., 1987). Details of pipette solutions were given in the appropriate figure legends. Unless stated otherwise, bath solutions contained 5 mM MES, pH 6.0 (adjusted with Tris), and had an osmolality of 700 mosmol kg−1 by adding Suc. All solutions were kept at 4°C until used and filtered through a 0.2-μm Millipore (Bedford, MA) filter prior to use. When required, the chemical speciation program GEOCHEM (Parker et al., 1987) was used to compute the free activities of ions. All chemicals used in this study were purchased from Sigma-Aldrich; A-9-C was dissolved in ethanol.

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