The Physiology and Biophysics of an Aluminum Tolerance Mechanism Based on Root Citrate Exudation in Maize[1]

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Al-induced release of Al-chelating ligands (primarily organic acids) into the rhizosphere from the root apex has been identified as a major Al tolerance mechanism in a number of plant species. In the present study, we conducted physiological investigations to study the spatial and temporal characteristics of Al-activated root organic acid exudation, as well as changes in root organic acid content and Al accumulation, in an Al-tolerant maize (*Zea mays*) single cross (SLP 181/71 × Cateto Colombia 96/71). These investigations were integrated with biophysical studies using the patch-clamp technique to examine Al-activated anion channel activity in protoplasts isolated from different regions of the maize root. Exposure to Al nearly instantaneously activated a concentration-dependent citrate release, which saturated at rates close to 0.5 nmol citrate h^{-1} $root^{-1}$, with the half-maximal rates of citrate release occurring at about 20 μM Al^{3+} activity. Comparison of citrate exudation rates between decapped and capped roots indicated the root cap does not play a major role in perceiving the Al signal or in the exudation process. Spatial analysis indicated that the predominant citrate exudation is not confined to the root apex, but could be found as far as 5 cm beyond the root cap, involving cortex and stelar cells. Patch clamp recordings obtained in whole-cell and outside-out patches confirmed the presence of an Al-inducible plasma membrane anion channel in protoplasts isolated from stelar or cortical tissues. The unitary conductance of this channel was 23 to 55 pS. Our results suggest that this transporter mediates the Al-induced citrate release observed in the intact tissue. In addition to the rapid Al activation of citrate release, a slower, Al-inducible increase in root citrate content was also observed. These findings led us to speculate that in addition to the Al exclusion mechanism based on root citrate exudation, a second internal Al tolerance mechanism may be operating based on Al-inducible changes in organic acid synthesis and compartmentation. We discuss our findings in terms of recent genetic studies of Al tolerance in maize, which suggest that Al tolerance in maize is a complex trait.

Al limits crop production on the acid soils that comprise up to 50% of the world's potentially arable lands (von Uexkull and Mutert, 1995). When the soil pH drops below 5, the rhizotoxic Al species, Al^{3+}, is solubilized into the soil solutions to levels that inhibit root growth and function. However, some plants have evolved Al tolerance mechanisms that enable them to grow in Al-toxic, acid soil environments (for review, see Kochian, 1995; Ma et al., 2001). Earlier work in this field showed that Al tolerance in wheat (*Triticum aestivum*) was associated with a reduced accumulation of Al in the root apex, but not the mature root (see, for example, Rincon and Gonzales, 1992; Tice et al., 1992; Delhaize et al., 1993a). Delhaize et al. (1993a, 1993b) subsequently provided compelling evidence for the existence of an Al tolerance mechanism based on root tip Al exclusion that involved Al-activated release of malate from the root apex of an Al-tolerant near-isogenic line of wheat. It was hypothesized that this enhanced malate release results in the build up of malate levels in the rhizosphere, where it chelates and reduces the activity of toxic Al^{3+} in the rhizosphere. Subsequent studies documented a strong correlation between the degree of Al tolerance and the magnitude of Al-activated root malate release in 36 different wheat cultivars differing in Al tolerance (Ryan et al., 1995a, 1995b).

Considerable evidence has more recently been presented in the literature indicating that Al-activated organic acid release from the root apex may play a role in Al tolerance in a number of different plant species, with the major differences being the identity of the organic acid released, as well as the rapidity of this response (instantaneous versus inducible; for review, see Ma et al., 2001; Ryan et al., 2001b). Al-activated citrate release has been documented in roots of Al-tolerant varieties of maize (*Zea mays*), rye (*Secale cereale*), soybean (*Glycine max*), Cassia tora and Triticale (Pellet et al., 1995; Ma et al., 1997, 2000; Li et al., 2000; Silva et al., 2001). Earlier studies in maize from our laboratory as well as from other researchers indicated that the Al-activated citrate release mecha-

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nism in maize shares similarities with the malate release described for wheat (Pellet et al., 1995; Jorge and Arruda, 1997). In both species, the response has been reported to be localized to the root apex, which is the primary site of Al toxicity (Ryan et al., 1993; Sivaguru and Horst, 1998). However, different time-dependent patterns of organic acid release have also been reported for Al-tolerant genotypes of different crop species (Li et al., 2000; Ma et al., 2001). Al-activated malate efflux in wheat is detectable within minutes after Al exposure without any apparent delay between addition of Al and the onset of malate release (designated Pattern I in Ma, 2000). However, in maize, in addition to this rapidly activated organic acid efflux pattern, there also appears to be a component of Al-activated citrate release that increases with increasing exposure to Al (Pattern II; Pellet et al., 1995).

Recent efforts have been directed toward identifying the cellular mechanisms mediating the transport (i.e. organic acid release) in root tip cells. As organic acids exist primarily as anions in the cytoplasm, the thermodynamics for organic acid efflux indicate that plasma membrane anion channels may mediate the Al-activated organic acid release. The activation (i.e. opening) of plasma membrane anion channels by Al would trigger and mediate the large passive anion efflux (i.e. exudation) down the outwardly directed electrochemical gradient. Anion channels that are specifically activated by extracellular Al^{3+} have recently been reported in protoplasts isolated from root tips of Al-tolerant wheat (Ryan et al., 1997; Zhang et al., 2001) and maize (Kollmeier et al., 2001; Piñeros and Kochian, 2001). Given that these channels are specifically activated by Al and are permeable to organic acids such as citrate and malate, these transport systems are good candidates for mediating the Al-activated root organic acid release in Al-tolerant wheat and maize. There is strong evidence indicating that at least a subset of these Al-activated channels requires extracellular Al^{3+} to maintain channel activity (i.e. opening), and the activation machinery is localized to the plasma membrane (Ryan et al., 1997; Piñeros and Kochian, 2001; Zhang et al., 2001). In addition, evidence has been presented for a second type of Al-activated channel in maize root cortical cells that are activated upon exposure to Al, but do not require continuous Al exposure to remain in their active state (Kollmeier et al., 2001). Although extrapolation from patch-clamp studies with root protoplasts to studies performed with intact roots should be taken cautiously, these electrophysiological studies suggest that the different citrate exudation patterns (I and II) observed in intact root studies might be mediated by different transporters and signaling pathways.

In the present study, we were interested in localizing and characterizing the Al-activated organic acid release that takes place in roots from a very Al-tolerant maize single cross (SLP 181/71 × Cateto Colombia 96/71) by integrating physiologically based root exudation experiments with patch clamp studies to survey the plasma membrane anion transporters in cells from the specific root tissues where the Al stimulation of organic acid release takes place.

RESULTS

As Al exclusion via Al-activated organic acid efflux from the root apex has been suggested to be an Al tolerance mechanism in maize, we investigated root apical Al accumulation in detail in the Al-tolerant single cross cv Cateto-Colombia. This was done by visualizing Al accumulated in the root apex with the Al-binding stain hematoxylin and the fluorescent Al-binding dye morin. As shown in Figure 1, even after a 5-d exposure to nutrient solution containing 222 μM Al (Al^{3+} activity of 39 μM), the root tip of cv Cateto-Colombia showed only a low degree of Al accumulation, with the accumulation being confined to the first few millimeters of the root tip and with little Al accumulation in the root cap. By comparison, an Al-sensitive maize cultivar, Mo17, exhibited significant root damage and dramatic Al accumulation under these conditions (Fig. 1A, right). The Al exclusion seen in the root tip of cv Cateto-Colombia correlates with the high degree of Al tolerance as measured by the ability to maintain near normal root growth in this fairly Al-toxic growth solution. The more detailed examination of the root tip in Figure 1, B and C, indicates that the external cell layers of apical zone adjacent to the root cap showed the most pronounced staining. Cross sections of this region showed that most of the Al accumulation took place in the outer cortical region, although the epidermal layers as well as the first two to three layers of cortical cells were not heavily stained. No evidence of Al accumulation was detected in the endodermis, stelar cells, or vascular tissue (Fig. 1D).

A further indication of the high degree of Al tolerance exhibited by cv Cateto-Colombia was that there were no signs of physical damage to the root (e.g. epidermal degradation) even after 5 d of exposure to Al concentrations as high as 450 μM Al (80 μM Al^{3+} activity). Exposure of the seedlings to solutions containing Al^{3+} activities ranging from 0 to 80 μM in full nutrient solution only caused a modest inhibition of root growth (Fig. 2A), generally causing no more than 20% inhibition (relative to the Al control) at an Al^{3+} activity of 39 μM, and only 30% root growth inhibition in solutions with an Al^{3+} activity of 80 μM. These results established that the maize single cross (SLP 181/71 × Cateto Colombia 96/71) was extremely Al tolerant compared with other maize and other crop species reported in the literature (for comparisons, see Wenzl et al., 2001). The same Al exposures triggered a significant activation of citrate exudation by the primary intact root (Fig. 2A). The rates

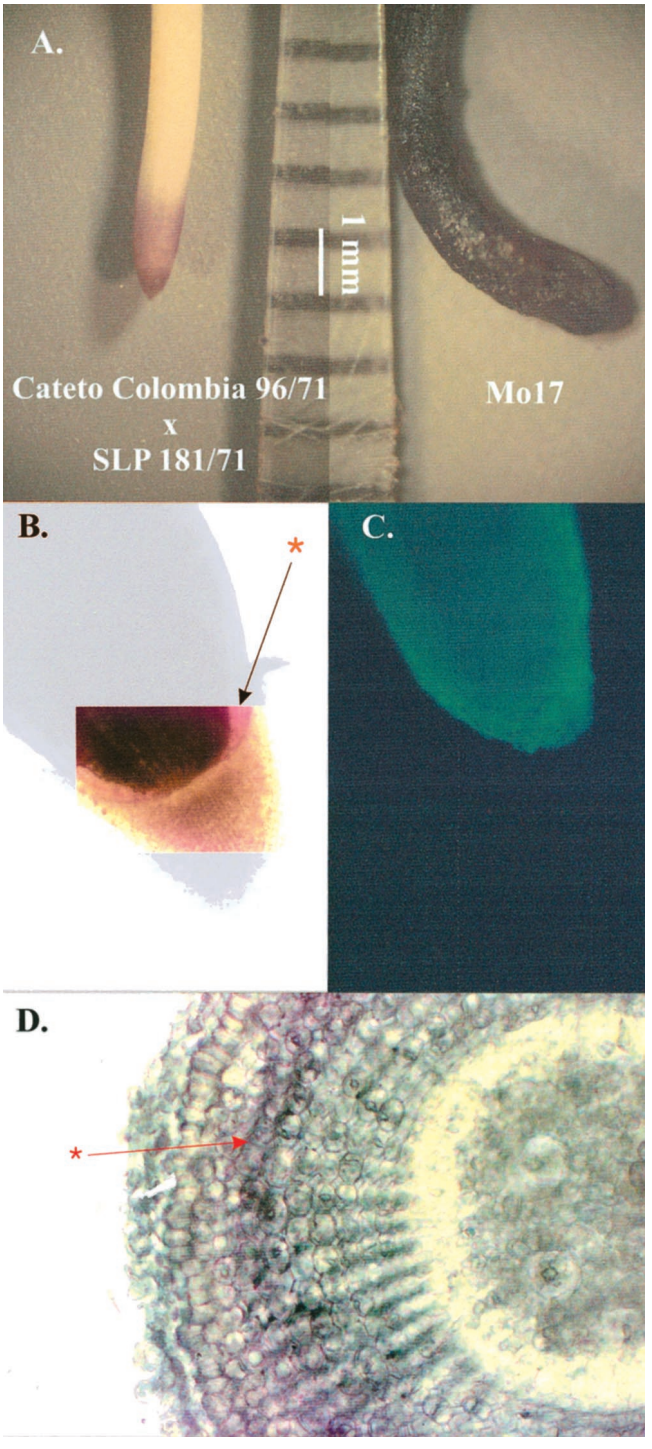

Figure 1. Patterns of Al accumulation in roots from the Al-tolerant maize single cross (SLP 181/71 × Cateto-Colombia 96/71). The root tips shown were exposed to 222 μM Al (Al^{3+} activity of 39 μM) in nutrient solution for 5 d. A, Al accumulation in the root tip of cv Cateto-Colombia as indicated by hematoxylin staining. The Al-sensitive maize cv Mo17 is shown on the right for comparison. Controls in the absence of Al showed no staining (data not shown). B, Closer view of the Al staining on a longitudinal section of the root apical region in cv Cateto-Colombia. The photograph (color) shows the root section containing the root meristem and root cap (magnification ×25). For reference purpose, the remainder of the root tip is shown of as a gray transparency. The red asterisk and arrow indicate the pronounced staining in the outer cell layers of the root section adjacent to the root cap. C, Distribution of Al accumulation at the root tip of cv Cateto-Colombia as indicated by morin staining (magnification ×6.25). D, Transverse section of the hematoxylin-stained cv Cateto-Colombia root depicted in A. The red arrow and asterisk indicate the penetration of the dye to the inner cortical cell layers.

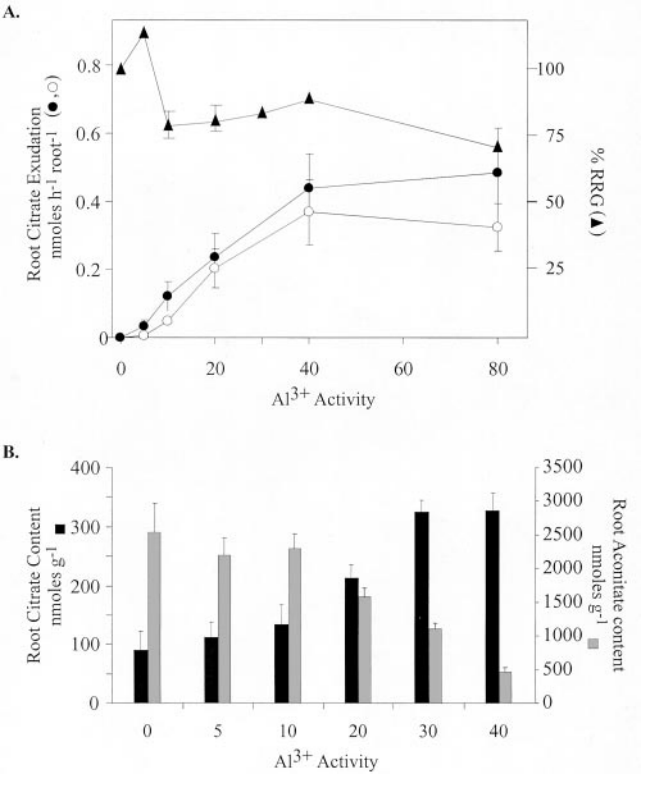

Figure 2. Relationship between Al tolerance (based on Al inhibition of root growth), root citrate exudation, and root citrate and aconitate content in response to increasing activities of Al^{3+} in the growth solution. Vertical bars indicate the SE of the mean. A, Effect of external Al on root growth (right *y* axis) and root citrate exudation (left *y* axis) measured at two time periods (2nd and 4th days). The percentage of relative root growth (RRG) was calculated over a 3-d period as described in "Materials and Methods." Root exudates were collected over 24 h in 4.3 mM $CaCl_2$ (pH 4.0) and $AlCl_3$ at the activities indicated. The figure shows exudation rates calculated after 2 (○) and 4 d of Al exposure (●). B, Root citrate (left *y* axis and black bars) and aconitate (right *y* axis and gray bars) content in the apical 2 cm of the root tip after 5 d of Al treatment.

of citrate efflux were relatively constant over a 4-d period, and were dependent on the external Al activity. Citrate exudation saturated when Al^{3+} activities were increased to between 40 and 80 μM, at exudation rates of 0.37 ± 0.10 and 0.48 ± 0.13 nmol citrate h^{-1} $root^{-1}$ at the end of the 2nd and 4th d of Al exposure, respectively. Half-maximal rates of citrate release occurred at about 20 μM Al^{3+}. The roots also maintained significant levels of malate and phosphate release; however, in contrast to citrate efflux, malate and phosphate exudation were constitutive,

Table I. *Root malate and phosphate exudation rates and root tip content for seedlings of the Al-tolerant maize cv Cateto-Colombia, exposed to different Al^{3+} activities*

Al^{3+} Activity	Exudation Rate		Root Tip Content
	Malate	Phosphate	Malate+Phosphate
μM	*nmol h^{-1} $root^{-1}$*		*nmol g^{-1}*
0	0.15 ± 0.04	0.07 ± 0.02	9,140 ± 1,346
5	0.04 ± 0.01	0.06 ± 0.02	6,243 ± 1,228
10	0.15 ± 0.07	0.04 ± 0.00	5,955 ± 803
20	0.28 ± 0.07	0.05 ± 0.01	6,425 ± 130
40	0.27 ± 0.21	0.13 ± 0.08	5,648 ± 215
80	0.08 ± 0.07	0.17 ± 0.12	4,038 ± 397

and were not dependent on the presence or absence of external Al (Table I).

The organic acid content of root tips was also investigated in response to Al exposure, as internal levels of Al-chelating organic acids have also been implicated in an internal Al tolerance mechanism in buckwheat (*Fagopyrum esculentum*) and hydrangea (*Hydrangea macrophylla*; Ma et al., 1997a, 1997b). As shown in Figure 2B, exposure to increasing levels of Al elicited a strong (over 3-fold) increase in root tip citrate content. In contrast, aconitate content in the root tip showed an inverse relationship with increasing Al exposure, decreasing by 4- to 5-fold as the Al activities were increased (Fig. 2B). In addition, root tip content of malate and phosphate decreased in response to increasing Al (Table I), although the correlation was not as significant as described above for citrate and aconitate. The Al content in root tips also increased with exposure to increasing external Al^{3+} activities (Table II). In contrast to the organic acid content, the increase in root tip Al content was linear ($r^2 = 0.995$), with no signs of saturation.

Next, the spatial localization of the Al-activated citrate exudation was studied by measuring the organic acid release from apical and mature root regions that were spatially isolated from each other (Fig. 3). The terminal 2 cm of the roots initially were spatially isolated from the rest of the root using the specially designed Plexiglas chamber depicted in Figure 3A. During the 24-h period of the exudation experiment, root growth resulted in the terminal 5 cm of the roots being isolated from the rest of the root system. The rates of Al-activated citrate exudation obtained for the tip region of the root were similar in magnitude (0.6 nM citrate h^{-1} $root^{-1}$) to those obtained for the whole root (i.e. Fig. 2A), indicating that most of the citrate exudation is localized to the first 2 to 4 cm of the root. However, as shown in Figure 2B, we did measure a small but significant Al-activated citrate release from the mature root regions.

This spatial distribution of Al-stimulated citrate release is significantly less localized to that previously described in Al-tolerant maize roots, where the Al-stimulated organic acid release was reported to be localized to the first 1 cm of the root (Pellet et al., 1995). As a consequence, we proceeded to investigate the spatial distribution of Al-stimulated citrate release in more detail. This portion of the research first involved determining the role of the root cap in the Al stimulation response. We compared the Al-

Table II. *Al content in root tips of the Al-tolerant maize cv Cateto Colombia after 5 d of A1 treatment*

Al^{3+} Activity	Al Concentration	Al Root Tip Content
μM	μM	$\mu g\ g^{-1}DW$
0	0	0 ± 0
5	27	153 ± 58
10	54	571 ± 119
20	113	1,339 ± 152
30	169	2,199 ± 62
40	222	2,951 ± 140

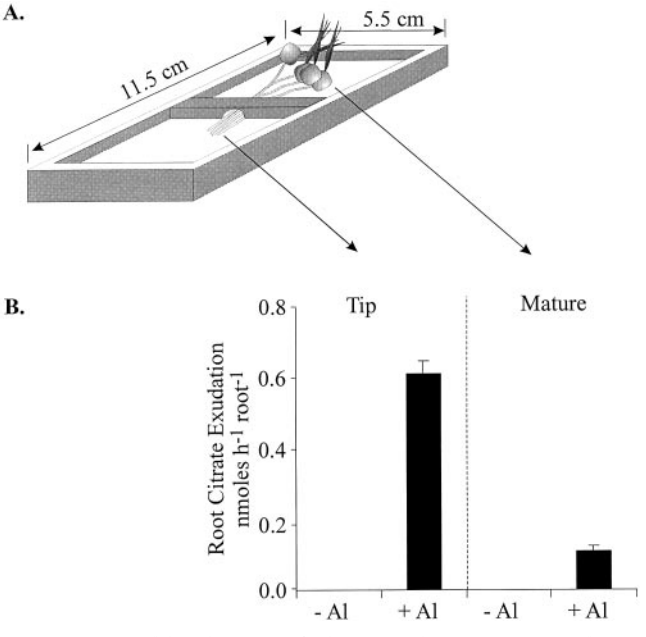

Figure 3. Spatial localization of Al-activated root citrate exudation over a 24-h period from the apical or mature regions of intact roots. A, Diagram of the divided Plexiglas chamber used for the experiment (see "Materials and Methods" for a description). B, Al activation of root citrate exudation from the mature and apical root regions. At the beginning of the experiment, the length of root tip exposed into the chamber was 2 cm, and by the end of the experiment, the root tip in this chamber had an average length of 5 cm. Exudates were collected in 4.3 mM $CaCl_2$ (pH 4.0) and 39 μM Al^{3+} activity.

activated citrate exudation from intact roots and roots that had been decapped, using surgical methods we previously employed to investigate the role of the root cap in Al toxicity (Ryan et al., 1993). Root cap removal did not appear to damage the root, as rates of root growth were the same in intact or decapped roots. As depicted in Figure 4, there were no significant differences between the citrate exudation rates of intact and decapped roots, indicating that the root cap does not play an important role in the Al-stimulated citrate exudation response. These experiments indicated that the root cap was not the site of citrate exudation, and it was not involved in perception of the Al signal that triggers the response. As with the previous intact root experiments, a constitutive malate and phosphate exudation was also observed in decapped and intact roots (Table III). External Al did not have any effect on the exudation of these compounds, and removal of the root cap caused a significant reduction in malate/phosphate exudation, suggesting that cells of the root cap do participate in this constitutive malate and phosphate release.

We also investigated Al-stimulated citrate release in root tips (the terminal 2 cm) that were excised from the seminal (primary) or secondary roots (Fig. 4). The Al-citrate release rates for primary and secondary excised root tips were not significantly different, implying that both types of roots can contribute to the whole plant Al-triggered exudation. The rate of Al-stimulated citrate exudation observed in excised root tips was only about 30% of that measured in intact roots. In contrast, the rates of constitutive malate/phosphate exudation observed in excised roots were of a similar magnitude to those observed in intact roots (Table III). An additional difference between intact and excised roots was the presence a small background of citrate exudation (about 0.02 nM citrate h^{-1} $root^{-1}$) in the absence of Al. Because this Al-independent citrate release was not observed in intact roots, it was presumed that it was the result of a wound-induced leakage from the excised segment.

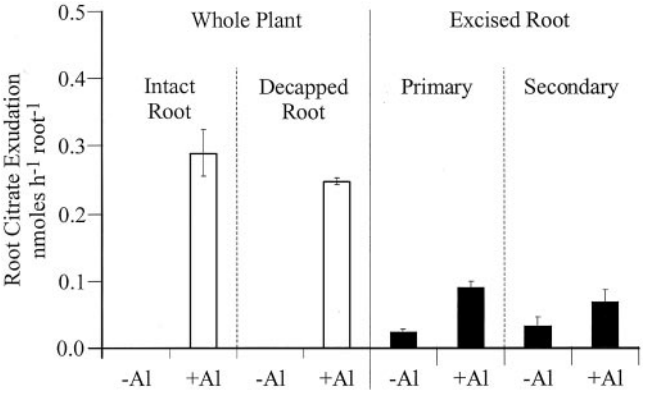

Figure 4. Comparison of the Al activation of root citrate exudation between intact and decapped roots (left), and excised primary and secondary root tips (right). Exudates were collected in 4.3 mM $CaCl_2$ (pH 4.0) and 39 μM Al^{3+} activity. Vertical bars indicate SE.

Table III. *Malate/phosphate exudation rates (nmol h^{-1} $root^{-1}$) for intact (with or without root cap) and excised root tips of cv Cateto-Colombia in the presence and absence of Al*

Al^{3+} Activity	Whole Plant		Excised Root Tips	
	Capped	Decapped	Primary	Secondary
μM				
0	0.30 ± 0.08	0.13 ± 0.08	0.30 ± 0.02	0.27 ± 0.05
20	0.31 ± 0.07	0.15 ± 0.07	0.32 ± 0.03	0.33 ± 0.03

The reduction in the rate of Al-activated citrate exudation in excised root tips (compared with that obtained for intact roots) could potentially be due to two factors. First, because the exudates were collected from root tips that had been excised from the whole root for a total of 10 h (4-h recovery period plus a 6-h exudation), the exudation rates could well be declining during the exudation period, as the metabolic reserves of the excised segments were depleted. However, time course experiments with excised root tips indicated that the exudation rates remained relatively constant over this 10-h period (data not shown). These experiments also indicated that the Al-stimulated citrate release occurred rapidly, and was detectable at the earliest time point used (30 min after initiating Al exposure; data not shown). Second, as the divided chamber experiments showed that mature root tissues well back from the root apex also exhibit an Al-activated citrate release, the exudation measured with excised 2-cm root tips could represent an underestimation of those observed for the whole root.

Nevertheless, because the excised root tip tissues exhibited qualitatively the same Al-activated response seen in intact roots, we proceeded to study the spatial localization of citrate exudation along the root in sequential root segments excised from the following root regions: 0 to 0.5, 0.5 to 1, 1 to 2, 2 to 3, and 5 to 6 cm back from the root tip. As seen in Figure 5, Al-activated citrate exudation averaged approximately 60 pmol citrate h^{-1} for each 1-cm region, with the rates being fairly constant up to 6 cm back from the root apex. The smaller rates observed for the apical 0.5-cm sections are likely due to the lack of significant exudation from the large root cap. When the exudation rates for each root segment were summed together, a total exudation rate of approximately 240 to 300 pmol citrate h^{-1} was obtained, which is within the range for citrate exudation observed for intact roots. Also depicted in Figure 5 is the spatial localization for the constitutive, Al-independent malate and phosphate release, which appears to be constant over the entire range of the root studied. As in the intact root experiments, external Al did not stimulate the malate and phosphate release in excised root segments.

Given that the Al-stimulated citrate exudation was fairly delocalized over the longitudinal axis of the

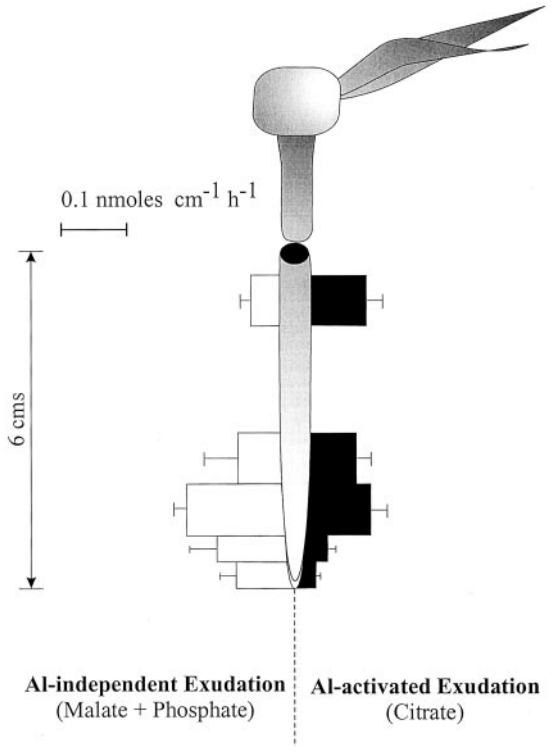

Figure 5. Diagram illustrating the spatial localization of Al-activated citrate exudation (black bars) and Al-independent malate + phosphate exudation (white bars) along the seminal root of the maize seedling. Exudation was collected from 0.5-cm segments (for the first 1 cm of root) and then 1.0-cm root segments for subsequent root regions (as indicated by the width of the bar). Exudation was carried out in 4.3 mM $CaCl_2$ plus 39 μM Al^{3+} activity. The magnitude of the citrate exudation shown represents the difference between the exudation obtained in Al-treated and −Al-treated root segments. As malate and phosphate rates were not Al stimulated, rates shown correspond to rates obtained in ±Al-treated plants. The magnitude and the position of the fluxes are indicated by bars. Scaling bars correspond to the distance along the root and the flux magnitude.

root, we proceeded to see if there was a radial localization to this Al-activated response. Maize roots are amenable to surgical dissection of the stelar tissue from the root epidermis/cortex, and we took advantage of this to investigate Al-activated citrate release in both of these root tissues (Fig. 6A). Although the total fresh weight of the stellar tissue was significantly smaller than that of the cortical/epidermal tissue, the rates of Al-stimulated citrate exudation for the stellar tissue were considerably higher than those observed for the cortical/epidermal tissue (on a grams per fresh weight basis). The citrate exudation rates for both tissues were similar in magnitude to those measured for excised root segments (6 ± 1 and 5 ± 2 to 13 ± 3 nmol citrate h^{-1} g^{-1}, respectively; calculated from the data in Figs. 4 and 5). We also measured the root tissue citrate content in stelar and cortical root tissue. As seen in Figure 6B, there was a strong Al-stimulated increase in citrate content in both tissues, which correlates with what was observed for intact root tips in Figure 2. These results highlight the possible role of the cortical and stellar root tissues in this Al-activated organic acid exudation response.

The patch clamp technique was used to gain an understanding of the plasma membrane anion transporters that could be involved in the Al-activated organic acid release detailed above. We had previously described an Al-activated anion channel from cortical cells in the root tip of another Al-tolerant maize cultivar (SA3), and we proposed that this transporter mediated the Al-activated citrate release in epidermal and outer cortical cells of the maize root tip (Piñeros and Kochian, 2001). However, the present study suggests that this mechanism is not localized exclusively to cells of the maize root periphery, and it is not confined to a localized region of the root tip. Therefore, we proceeded to correlate the physiological data for Al-activated exudation described in the above experiments with the distribution of Al-activated as well as Al-independent anion

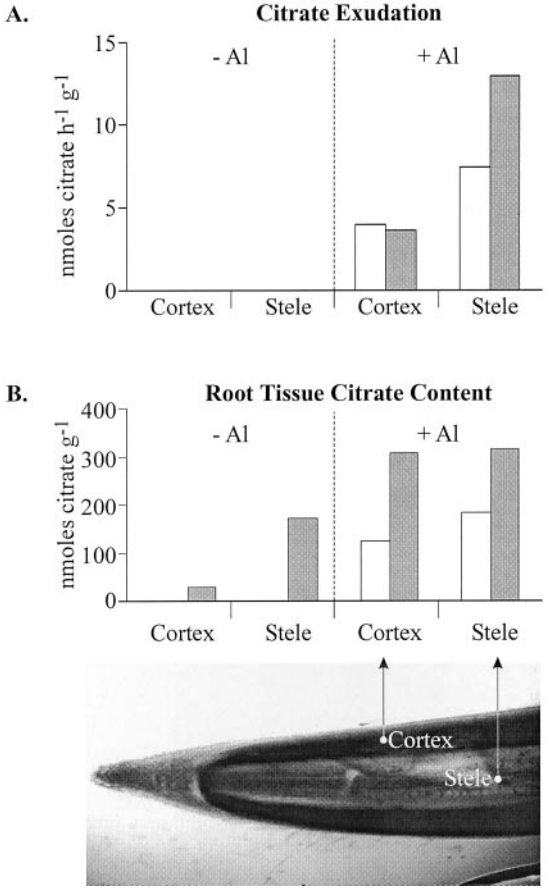

Figure 6. Effect of Al treatment on root citrate exudation (A) and root tissue citrate content (B) for epidermal/cortical and stellar tissue surgically isolated from the root tip. Exudates were collected over a 6-h period in 4.3 mM $CaCl_2$ plus 39 μM Al^{3+} activity after 2 d of Al treatment in full nutrient solution. Tissue content was determined at the end of the exudation experiment. The white and gray histograms represent two replicate experiments.

channels in protoplasts isolated from different root regions. Protoplast for patch-clamp studies were isolated from the terminal 1 to 2 mm or the first 2 cm of primary maize roots, using roots that were not exposed to Al or had been exposed to Al for 24 h. When these two root regions were digested to release protoplasts, the cortical and stelar protoplasts were morphologically distinguishable (see "Methods and Materials").

In the patch-clamp experiments, Cl^- was used as an analog for organic anions. Stelar and cortical protoplasts (isolated from ±Al-exposed roots) did not show any significant whole cell Cl^- conductance under ionic conditions (i.e. tetraethylammonium chloride [TEA]-based solutions lacking Al) designed to maximize Al-independent Cl^- transport (Fig. 7A, left panel). This lack of significant plasma membrane anion conductances was observed in cortical and stellar cells isolated from the first 1 to 2 mm or the terminal 2 cm of root. However, exposure of these protoplasts to extracellular Al^{3+} caused a shift in inward current reversed (E_{rev}) to more positive membrane potentials and simultaneously activated a significant inward current (anion efflux) in 22% ($n = 4$ of 18) of the cortical and 19% ($n = 4$ of 21) of the stellar cells examined (Fig. 7A, right panel). Similar Al^{3+}-activated currents were obtained in protoplasts from the terminal 1 to 2 mm and terminal 2 cm of the root apex. The close relationship between the holding potential at which the Al^{3+}-activated inward current reversed (E_{rev}) and the theoretical reversal potential for Cl^- (E_{Cl}^-) indicated that this current was being selectively carried by Cl^- (Fig. 7A, current-voltage [I/V] curve). Outside-out patches excised under the above ionic conditions (i.e. in the presence of extracellular Al^{3+}) often contained one class of Cl^- channel (five patches of 14; Fig. 7B). The I/V relationships for this channel indicated a unitary conductance between 23 and 55 pS (in 11 and 103 mM extracellular Cl^-, respectively) and a high selectivity for anions over cations, as indicated by the close relationship between the reversal potential and the electrochemical equilibrium for Cl^- (E_{Cl}^-) for the different ionic conditions imposed. The conductivity, selectivity, and kinetic features of this channel were very similar to those of the Al^{3+}-activated large conductance anion channel described previously for the Al-tolerant maize cultivar, SA3 (Piñeros and Kochian, 2001). Furthermore, based on the data from our current studies with cv Cateto-Colombia and the earlier study with cv SA3, the Al-activated channels from the two Al-tolerant cultivars are likely to share a similar type of activation mechanism. Figure 7C indicates that as was previously found in cv SA3, the mechanism required for triggering channel activation in cv Cateto-Colombia is also localized to the plasma membrane. Exposure of "electrically quiet" (i.e. lack of any channel activity as shown in Fig. 7C, I and II) outside-out patches of plasma membrane to extracel-

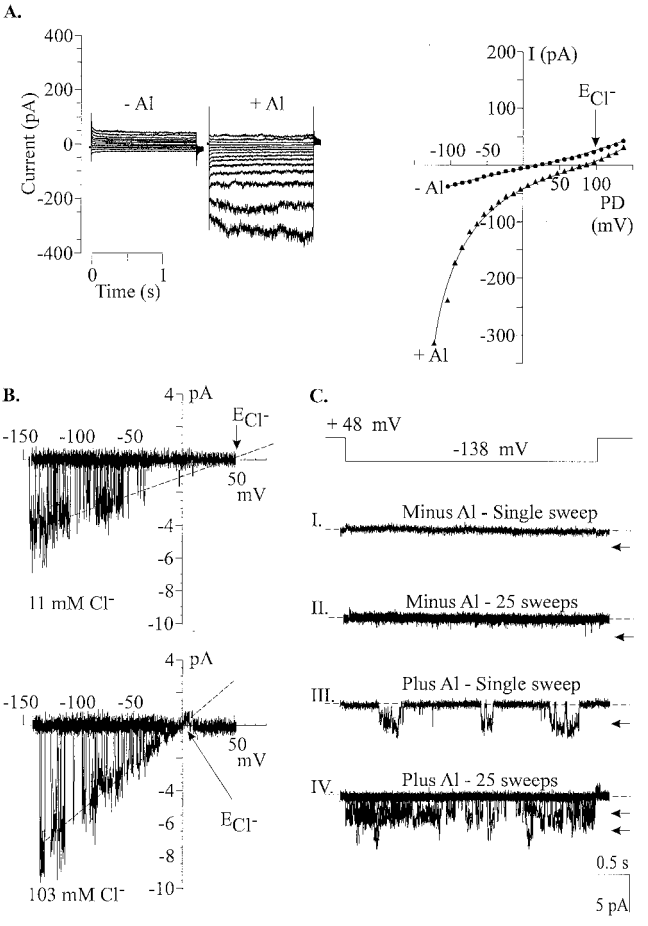

Figure 7. Al-activated anion channels for cortical and stellar protoplasts. A, Example of whole-cell currents recorded with the bath solutions containing 1 mM Cl^- (pH 4.0) minus (left traces) or plus (right traces) 50 μM Al^{3+}, following the voltage protocol described in "Materials and Methods." This particular example was taken from a cortical protoplast with a diameter of 52 μm. Right panel, I/V relationships from the currents shown on the left. The arrow indicates the Cl^- theoretical reversal potential. B, I/V relationships for a single channel in an outside-out patch excised from a stellar protoplasts (diameter of 20 μm) in the presence of extracellular Al^{3+}. The I/V relationships were constructed from slow voltage ramps (see "Materials and Methods"). The bath solution contained 50 μM $AlCl_3$ in 11 mM Cl^- (pH 4.0; top) or 103 mM Cl^- (bottom). The unitary conductances under these conditions were 23 and 55 pS, respectively. The arrows indicate the Cl^- theoretical reversal potential for each ionic condition. Similar channel activity and selectivity was recorded in a total of five different patches. C, A repetitive voltage protocol (sweep shown on the top of the figure; see "Materials and Methods" for further details) was used to test for single-channel activity in solutions lacking and containing Al^{3+}. The resulting trace of one single repetition (single sweep) in the absence of Al^{3+} is shown in trace I. Trace II shows the 25 repetitions of individual sweeps superimposed. Trace III shows a single sweep after 50 μM Al^{3+} was added to the bath solution. Trace IV shows 25 sweeps superimposed in the presence of Al^{3+}. Arrows and dashed lines on the right of each trace represent the open (O) and closed (C) and states of the channel, respectively. The time and current scales are shown at the bottom right corner of trace IV. Similar results were obtained in three separate excised patches.

lular Al^{3+} resulted in the activation of a channel mediating inward currents (Fig. 7C, downward deflections in traces III and IV), with similar biophysical characteristics to those described in Figure 7B. Under the electrical conventions used in the present work, these Al^{3+}-activated inward currents correspond to a Cl^- efflux. As a consequence, this channel is likely to mediate the Al-stimulated efflux of organic anions described in the above experiments.

It was interesting to note that protoplasts isolated from roots exposed to ±Al exhibited no significant inward conductance in the absence of extracellular Al^{3+} that could account for the Al-independent anion (malate or phosphate) exudation observed in the experiments with intact or excised roots. However, membrane patches excised from cortical protoplasts occasionally revealed another type of channel that could mediate anion flux across the plasma membrane of cv Cateto-Colombia root cells in the absence of extracellular Al^{3+}. Under the ionic conditions imposed, this type of channel mediated mainly outward currents (Fig. 8). At steady-state depolarizing potentials, the channel remained active, with no signs of inactivation (Fig. 8A). At extremely negative potentials, this channel could also mediate a small but strongly rectified inward current (i.e. anion efflux; Fig. 8, B and C). However, at these negative membrane potentials, the channel quickly inactivates with time (Fig. 8B). The unitary conductance of the inward current carried by this channel was about 1 to 3 pS in the different Cl^- treatments imposed, whereas the unitary conductance of the outward current increased from 6 to 20 pS as the extracellular Cl^- activity was increased. Likewise, the shifts in the reversal potentials (E_{rev}) following the theoretical reversal potential for Cl^- (E_{Cl^-}) under the different Cl^- activities indicated that this current was selectively being carried by anions (Cl^- in these experiments). The biophysical characteristics of this Al-independent anion channel resemble those described previously for the small conductance anion channel in cv SA3 maize (Piñeros and Kochian, 2001). The lack of any significant whole-cell currents (in solutions lacking Al^{3+}) that would exhibit the characteristics of this channel suggests that in the whole-cell configuration, the channel is in an inactive state, or simply that this type of channels is found in low abundance in the root cell plasma membrane.

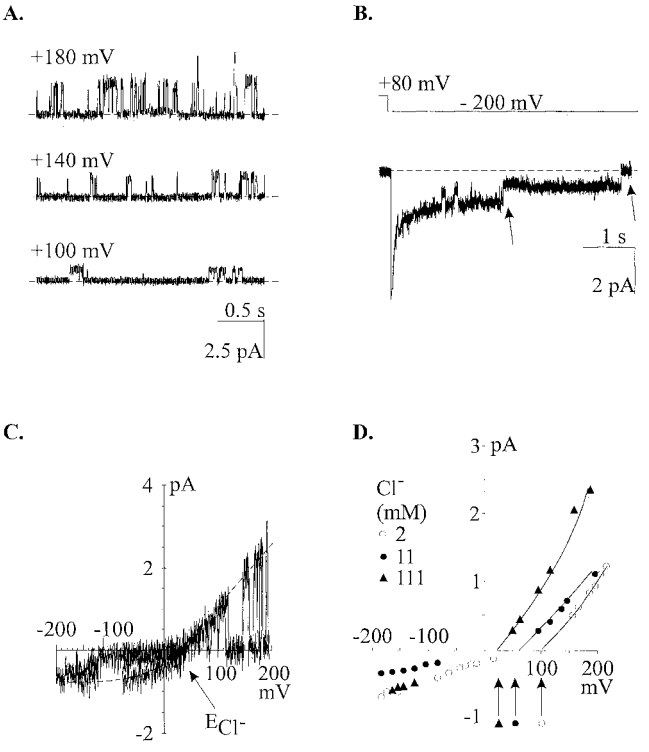

Figure 8. Al-independent anion channel mediates inward and outward currents. Single-channel activity was recorded from outside-out patches in the absence of Al^{3+}. The broken lines in A and B indicate the closed state of the channel. A, Example of single anion channel activity at three positive potentials. Membrane potentials were stepped from 0 mV to the voltage indicated in the left margin of each trace. The bath contained 111 mM Cl^-. B, Example of channel deactivation at hyperpolarizing membrane potentials. This particular patch contained two channels of the same type bathed in 111 mM Cl^-. The voltage protocol is described at the top of the trace. The arrows indicate the closing and inactivation of each one of the two channels in the patch. C, I/V relationship derived from slow voltage ramps from an outside-out patch with 11 mM Cl^- in the bath solution. The arrow indicates the theoretical reversal potential for Cl^-. D, Single-channel I/V relationship for the channel described in A and B when the bath solution contained 2 (○), 11 (●), or 111 (▲) mM extracellular Cl^-. Arrows at the bottom indicate the theoretical reversal potential for each ionic condition. In 2 and 11 mM Cl^-, the unitary conductance of the inward current was between 1 and 3 pS. The unitary conductances of the outward currents in 2, 11, and 111 mM Cl^- were 6 ± 0.1, 13 ± 0.2, and 20 ± 1 pS ($r^2 > 0.950$), respectively. Similar results were obtained in a total of three patches.

DISCUSSION

Al Tolerance and the Spatial Distribution of the Al-Activated Citrate Exudation

In the present study, we correlated the spatial localization of Al accumulation, Al-stimulated citrate release, and the plasma membrane anion transporters that presumably mediate the organic acid exudation in maize roots. RRG and hematoxylin staining have proved to be suitable phenotypic criteria for assessing Al tolerance in maize in full nutrient solution (Magnavaca et al., 1987; Cançado et al., 1999). The South American maize single cross (SLP 181/71 × Cateto Colombia 96/71) used in the present study showed a high level of Al tolerance, as indicated by the small (about 30%) root growth inhibition observed in solutions containing up to 450 μM Al (80 μM Al^{3+} activity). This high degree of Al tolerance also correlated with a low level of Al accumulation (as indicated by the low degree of Al-specific hematoxylin staining in Fig. 1) that was localized to the first 2 mm of the root tip excluding the root cap, and

tended to be more localized to the cells of the outer cortex. Although the degree of Al tolerance displayed by cv Cateto-Colombia (as indicated by the root growth measurements) was higher than that reported for most other Al-tolerant plant species (Wenzl et al., 2001), comparisons should be made with caution, as there could be some degree of amelioration of Al toxicity by the high-ionic-strength full nutrient solutions used in this study (in contrast to some other studies where tolerance has been assessed in less physiological simple-salt solutions).

The high degree of Al tolerance in cv Cateto-Colombia correlated with the onset of a rapid (detectable within 30 min) Al-activated citrate exudation where the rate of exudation was positively correlated with the level of Al in the nutrient solution. The citrate exudation rates reported here were similar in magnitude to those reported for other Al-tolerant maize cultivars (SA3 [Pellet et al., 1995] and ATP-Y [Kollmeier et al., 2001]). Previous intact root studies conducted with Al-tolerant maize (Pellet et al., 1995) and wheat (Ryan et al., 1995) cultivars have suggested that the Al-stimulated organic acid release is localized to the first few to 10 mm of the root tip. Electrophysiological studies have also indicated that the root tip is the root region enriched in Al-activated anion channels, the membrane transporters that presumably mediate the organic acid exudation (Ryan et al., 1997; Kollmeier et al., 2001; Piñeros and Kochian, 2001; Zhang et al., 2001). Furthermore, it has been suggested that within this root region, the root cap (Bennet et al., 1987; Bennet and Breen, 1991) and the distal transition zone between the 1st and 2nd mm of the root apex where cells are transitioning from cell division to elongation (Sivaguru and Horst, 1998; Kollmeier et al., 2000) may play an important role in organic acid exudation and perception of the Al "signal."

However, in the current study, the lack of differences in the Al-stimulated citrate release between intact and decapped maize roots suggests that the root cap is not the site involved in the perception of the Al signal and it does not contribute significantly to the Al-activated citrate release (Fig. 4). Furthermore, our detailed spatial analysis indicates that, at least for the cv Cateto-Colombia, the Al-stimulated citrate exudation is not tightly localized to the root tip, and results from exudation from stellar and cortical root cells of a significant region of the root apex (as much as the terminal 5 cm of root; Fig. 5).

Differences in Al-Activated Organic Acid Transport and Root Organic Acid Metabolism among Al-Tolerant Maize Cultivars

The present and previous electrophysiological studies in maize indicate that Al-activated anion channels are present in the plasma membrane of root cortical cells (Kollmeier et al., 2001; Piñeros and Kochian, 2001). In the present study, we used Cl^- as an organic anion analog. Other studies have also indicated that in addition to Cl^-, Al-activated anion channels in the plasma membrane of root cells are permeable to organic and inorganic anions (Kollmeier et al., 2001; Zhang et al., 2001). As a consequence, it is likely that in addition to catalyzing a Cl^- selective efflux, the plasma membrane channels described in the present work are also capable of mediating (i.e. being permeable to) the organic acid efflux. However, currently we can only speculate on the magnitude of the organic anion permeability relative to that observed for Cl^-. Future assessment of the permeability and pharmacological properties of this channel will allow us to correlate the observed channel-mediated anion fluxes with those observed in vivo. Nevertheless, when the current results from the biophysical and physiological investigations of Al-activated root organic acid exudation are integrated, it appears that root stelar and cortical cells have the machinery (i.e. Al-activated anion channels) to contribute to the overall citrate exudation response. The plasma membrane Al-activated anion channels described here for the maize cultivar Cateto-Colombia share similar biophysical characteristics with those described previously for the Al-tolerant maize cultivar SA3 (Piñeros and Kochian, 2001). These characteristics include a "noisy" open state, a high selectivity for anions over cations, and a unitary conductance of 18 to 55 pS that is dependent on the extracellular Cl^- concentration. However, differences in channel regulation are also evident among the different Al-activated anion channels reported on in the literature. Although the Al-activated whole-cell anion currents in cv SA3 partially deactivated with time, these currents showed no signs of inactivation in cv Cateto-Colombia. Furthermore, the presence of extracellular Al^{3+} was shown to be required to maintain channel activation in the SA3 (Piñeros and Kochian, 2001) and Cateto-Colombia (present study) Al-tolerant maize cultivars, as well as in the Al-tolerant wheat genotypes (Ryan et al., 1997; Zhang et al., 2001). In contrast, although the anion channels investigated in the maize cultivar ATP-Y require Al exposure to activate, removal of extracellular Al^{3+} does not result in channel inactivation (Kollmeier et al., 2001). The root tip cortical cells in cv SA3 and cv ATP-Y also exhibited significant differences with regards to the sensitivity of other ion transporters to extracellular Al^{3+} (e.g. blockade of the K^+ outward rectifier).

These differences in Al responses, as well as reported differences in the lag in time between Al exposure and channel activation, suggest that there is variability in the regulation of these responses, or that somewhat different types of transporters are involved in this Al tolerance response in different genotypes. There are additional differences in the Al-stimulated organic release in the cv Cateto-

Colombia (present study), cv SA3 (Pellet et al., 1995), and cv ATP-Y (Kollmeier et al., 2001) Al-tolerant maize cultivars that support the latter idea. These differences include: Citrate exudation in cv Cateto-Colombia was exclusively Al dependent (i.e. no constitutive citrate release), whereas cv SA3 and cv ATP-Y exhibited a significant constitutive citrate release (i.e. in the absence of Al); citrate exudation rates in cv Cateto-Colombia saturated at higher Al concentrations, whereas citrate exudation rates in cv SA3 and cv ATP-Y were significantly reduced at higher Al concentrations; citrate exudation could be detected within 30 min in cv Cateto-Colombia at rates that remained constant over a 4-d period of Al exposure; this contrasts with cv SA3 where there is a lag phase after the initial Al exposure before citrate efflux reaches maximal values; and cv Cateto-Colombia exhibited constitutive malate and phosphate exudation rates that did not correlate with the presence or absence of external Al, whereas malate and phosphate exudation in cv SA3 and cv ATP-Y was stimulated by the presence of external Al (Pellet et al., 1995; Kollmeier et al., 2001).

Differences in changes in root tip organic acid content (presumably involving organic acid metabolism and transport) in response to Al are also evident between the maize cultivars. The increase in citrate content in cv Cateto-Colombia upon exposure to Al was at least 5-fold higher that that reported for cv SA3 (Pellet et al., 1995). Increases in root tip citrate content and citrate synthase activity have also been reported in Al-tolerant rye and soybean cultivars where Al stimulation of citrate release also takes place (Li et al., 2000; Silva et al., 2001). Levels of root tip aconitate and malate in cv Cateto-Colombia decreased in response to exposure to increasing levels of Al, which contrasts with the Al-induced increase in root malate content reported for cv SA3. This difference is interesting as malate exudation in cv SA3 and cv Cateto-Colombia are Al dependent and Al independent, respectively. It appears that the exudation of those organic acids that are activated by Al correlates with Al-induced increases in the internal tissue content of that organic acid. This leads to the speculation that Al-activated transport and synthesis may be coupled in Al-tolerant maize. Furthermore, it appears that Al tolerance in maize is a genetically complex trait (Magnavaca et al., 1987) that could involve several different Al tolerance mechanisms. These data lead us to speculate that there could be an Al exclusion tolerance mechanism based on citrate exudation, and a second internal tolerance mechanism using increases in internal levels of Al-chelating citrate.

It is interesting that although root citrate content and citrate release saturate at higher Al concentrations, root Al accumulation increases in a linear fashion in response to increasing Al exposure, at least in the first 1 cm of the root tip. This Al accumulation pattern differs from that reported previously for another Al-tolerant maize genotype (L1143), where Al absorption was localized to a constricted area behind the root apex (Cançado et al., 1999). The authors attributed such a pattern to a minimal Al absorption in the developing root during the initial stages of the Al treatment. In contrast, Al accumulation in the cultivar used in the present study was only detected in the growing root tip, even after several days of Al treatment, suggesting again that there are genotype-based differences in Al exclusion and thus, possibly genotypic differences in some aspects of the Al tolerance mechanisms in maize.

Are Multiple Al-Tolerant Mechanisms Operating in Maize?

Although direct comparisons between studies from different laboratories in crop Al tolerance mechanisms should be made with caution, as the experimental conditions used (e.g. composition of the nutrient solutions and Al^{3+} activities) vary significantly, observed differences in the time-dependent kinetics for Al-activated organic acid exudation in different Al-tolerant plant species are currently leading some researchers to suggest that two distinct Al tolerance mechanisms may be functioning in different Al-tolerant crop species (Ma et al., 2000).

Pattern I involves the rapid secretion of organic acids via Al-activated anion channels, whereas pattern II involves a slower Al-activated response that may involve induction of genes implicated in organic acid transport and metabolism. Within the context of this hypothesis with regard to differences in physiological aspects of Al tolerance discussed above for different maize genotypes, it is possible to speculate that in cv Cateto-Colombia, there may be multiple mechanisms for Al tolerance. These could include a constitutive, Al-independent malate/phosphate release (via Al-independent anion channels), which may provide a basal level of tolerance. Upon exposure to Al, several other mechanisms may operate. A rapid citrate release mechanism is activated (via Al-activated channels), allowing a more effective exclusion given that citrate chelates Al^{3+} much more strongly than malate. This tolerance and stress response is accompanied by a slower adaptation that involves regulation of the synthesis, metabolism, and compartmentation of organic acids. Likewise, as discussed above, differences in the patterns for root Al accumulation in different maize genotypes may also suggest that, at least in cv Cateto-Colombia, an additional Al tolerance mechanism (e.g. internal detoxification) may operate to allow the root tip to cope with the ongoing Al accumulation. The findings reported here, as well as other recent reports, are leading us to begin to think that in contrast to Al-tolerant crops such as wheat, Al resistance in maize may be a more genetically and physiologically complex trait.

As a consequence, to clarify the similarities and difference among Al-tolerant cultivars, there is an urgent need to consolidate the experimental conditions under which Al tolerance is assessed. Comparative studies among diverse Al-tolerant and -sensitive genotypes, under standardized experimental conditions, will in the future help us to further elucidate the contribution and the role of organic acid exudation to the Al tolerance response observed in maize.

MATERIALS AND METHODS

Plant Material and Seedling Growth

The maize (*Zea mays*) single cross (SLP 181/71 × Cateto Colombia 96/71) was supplied by Brazilian Agricultural Research Maize and Sorghum Research Center (Sete Lagoas, Brazil). For clarity, this cross will be referred throughout the text as cv Cateto-Colombia. Seeds were surface sterilized in 0.5% (w/v) NaOCl for 15 min and were then germinated in the dark (25°C) for 3 d on filter paper saturated with deionized water. Secondary roots were trimmed (except for the experiment involving measurements of organic acid exudation in secondary roots), and the seminal roots were sown through the mesh bottoms of polyethylene cups (one or four seeds per cup for root growth and organic acid exudation experiments, respectively). The sown seedlings were covered with black polyethylene beads, and the cups were placed into the precut holes of the cover of a black polyethylene container that held 8 liters of aerated nutrient solution. The nutrient solution contained the following macronutrients (in millimoles): Ca, 3.53; K, 2.35; Mg, 0.85; NH_4, 1.3; NO_3, 10.86; PO_4, 0.04; and SO_4, 0.59, and micronutrients (in micromoles): BO_3, 25; Cl, 596; Cu, 0.63; Fe-HEDTA, 77; MoO_4, 0.83; Mn, 9.1; Zn, 2.3; and Na, 1.74. Seedlings were grown for 24 h in a growth chamber at 26°C/24°C (light/dark, 16/8 h) under a light intensity of 550 μmol photons m^{-2} s^{-1}. Al treatments were initiated after this 24-h period by replacing the control growing solution with an identical solution that contained Al added as $AlK(SO_4)_2$ $12H_2O$ to the final concentrations. The desired Al^{3+} activities were estimated using CHEOCHEM-PC speciation software (Parker et al., 1995). The pH of the control and treatment solutions was adjusted to 4.0 with HCl. Seedlings were grown for an additional 1 to 5 d in the treatment solutions, depending on the type of experiment performed. The time periods of the different treatments are given in the text.

Root Growth Measurements

The polyethylene cups containing the seedlings were gently removed from the nutrient solution for 1 to 2 min, and the seminal root length was measured with a ruler. Root growth measurements were done immediately prior to Al treatments and over the following 5 d at 24-h intervals. Root measurements for at least 12 plants per treatment were averaged for each day. It was established that growth rates (millimeters per day) remained constant during the first 3 d of treatment, after which rates of root growth declines slightly, especially at higher Al concentrations. As a consequence, root growth rates (RGR; expressed in millimeters per day) for the control and the different Al treatments were obtained from the regression coefficients (slopes) estimated from the linear regression of the root length values (millimeters) as a function of time (a 3-d period). RRG was calculated as: RRG = (RGR in Al solution/RGR in control solution) × 100.

Hematoxylin and Morin Staining

Roots grown for 5 d in full nutrient solution containing Al (39 μM activity) were washed in 8 liters of distilled water for 1 h. The water was replaced three times during the rinsing procedure. Roots were then stained by soaking them in 500 mL of a solution containing 0.2% (w/v) hematoxylin and 0.02% (w/v) potassium iodide or 100 μM morin for 1 h. Roots were then washed in 8 liters of distilled water for an additional 1 h. The total volume of this last rinsing step was replaced three to four times until no excess staining could be detected in the rinsing water. Root tips were photographed under a stereoscope (SMZ-10; Nikon, Melville, NY) or a light/epifluorescence microscope (Axiovert 100; Carl Zeiss, Jena, Germany) using a digital camera (N90 DCS 420; Nikon).

Organic Acid Exudation and Root Organic Acid Content Determinations

Organic acids in root exudates and root homogenates were analyzed with a capillary electrophoresis (CE) system (P/ACE 5510; Beckman Instruments, Fullerton, CA) controlled by a Pentium II computer interfaced via PACE 1.2.1 software (Beckman Instruments). The background electrolyte used for separation consisted of 0.5 mM dodecyltrimethylammonium bromide, 7.5 mM salicylic acid, and 15 mM Tris adjusted to pH 9.5 with NaOH. Organic acids were separated in a 67-cm capillary (75 μm I.D.) with a constant separation voltage of −28.5 kV at 25°C. Prior to use, the capillaries were pretreated by flushing with 0.1 N HCl for 5 min, followed by another 5 min of flushing with 0.1 N NaOH. Peaks were detected with a UV absorbance detector at a wavelength of 232 nm, and were first identified on the basis of their migration time, with subsequent confirmation by spiking samples with organic acid standards. Given the large interference of some anions present in the full nutrient growth solution, root exudates were collected in simple-salt solution (see below). As malate and phosphate peaks had similar migration times, phosphate was also measured as described by Baykov et al. (1988) and was subtracted from the malate CE readings. Those samples for which phosphate was not determined by the later method, but solely by CE analysis, are referred to as malate + phosphate. Prior to loading root exudate samples, samples were passed through a chromatography column (OnGuard-Ag; Dionex, Sunnyvale, CA) to remove excess Cl^-. Root tissue organic acids were extracted by homogenizing the first 1 cm of the root tips in 18 MΩ (0.5 mg μL^{-1}). Samples were centrifuged for 10 min in a microfuge, and the supernatant was analyzed by CE with no further sample processing. Root tip Al content was measured by inductively coupled argon plasma emission spectrometry. The first 1 cm of the root tips were collected and dried in an oven at 55°C overnight. Dry weights were determined using a microgram balance (MT2; Mettler, Greifensee, Switzerland). Dry samples were digested with 100 μL of 70% (w/v) perchloric acid, were resuspended in 2 mL of 0.5% (w/v) nitric acid, and were analyzed using an inductively coupled argon plasma model 51000 (Perkin-Elmer/Sciex, Norwalk, CT).

The simple-salt solution used to collect root exudates consisted of 4.3 mM $CaCl_2$, plus or minus $AlCl_3$ (to the desired activity), and the pH was adjusted to 4.0 with HCl. This Ca^{2+} concentration corresponded to the total divalent cation concentration in the full nutrient solution, and was chosen to maintain a relatively constant Al stress to that found in the full nutrient solution by emulating any possible Al amelioration by divalent cations. The organic acid exudation observed under this particular set of conditions was similar in magnitude to that observed in full nutrient solutions. This was established by comparing the organic acid exudation rates obtained in simple salt solutions using CE determinations with those obtained in full nutrient solution using enzymatic determinations (for a description of the methodology, see Delhaize et al., 1993b). Given the 10-fold increase in sensitivity using CE determinations of organic acids over conventional enzymatic determinations, CE analysis was the methodology of choice to quantify organic acid exudation and content. Three main groups of experiments were designed to localize and characterize the Al-activated organic acid release: those involving intact roots, those involving excised root tips or excised root segments, and those involving isolated root cortex and stellar tissue.

Whole Plant Experiments. Intact and Decapped nmol g^{-1} Root Experiments

Plants used for root exudates experiments consisted of two groups, one for intact root studies and another group that had their root caps removed. The root caps were removed under a dissecting microscope by gently scraping at the contact area between the root cap and epidermis with a scalpel blade until the edge of the cap began to separate from the root. The point of the blade was then used the lift the cap and pull it from the root apex. This procedure was done prior to the initiation of the Al treatment, and did not dramatically perturb root function (measured as rate of root elongation and root cap regeneration). Following the 24-h Al treatment in full nutrient solution, each cup containing four seedlings (with intact or decapped roots) was fitted on top of a plastic centrifuge tube containing 50

mL of the aerated simple-salt solution described above and was returned to the growth chamber. Root exudates were collected from intact plants for 24 h (i.e. 2nd d of Al treatment). At this point, exudation samples were collected and analyzed as described above. The cups containing the seedlings were returned to their original treatment in full nutrient solution for an additional 24 h. To compare exudation rates at different time periods, a second set of exudates were collected in simple-salt solution over the following 24 h (i.e. 4th d of Al treatment). Following the collection of the exudate samples, the root tips were excised and analyzed as described above.

Divided Root Chamber Experiments

Following a 24-h Al treatment in full nutrient solution, four seedlings were transferred to a Plexiglas chamber (see Fig. 3). The first 2 cm of the root tip were isolated from the remaining mature root region by a notched Plexiglas block that straddled the roots and divided the chamber into two compartments. The solutions from the two compartments were isolated by securing the block with silicone grease along all its edges and along the straddled roots. Each compartment was filled with 15 mL of the aerated simple-salt solution described above and was covered with a series of large microscope cover slides (to minimize evaporation). Root exudation proceeded for the next 24 h. After the exudation samples were collected, one compartment of each chamber was filled with colored dye to verify that there had been no leak from one compartment to the other over the 24-h exudation period.

Excised Root Tips, Root Segments, and Isolated Tissue Experiments

Following a 24-h Al treatment in full nutrient solution, root tips, root segments, or isolated cortical and stellar tissue were excised or dissected from the root tip. Five root tips (1st cm) or 10 root segments (0.5- and 1-cm sections) per sample were excised from the seminal root. The stelar tissue was surgically removed from the cortical and epidermal tissue from the first 4 cm of root tips, as described in Kochian and Lucas (1983). The total tissue fresh weights collected from the root tips varied between 0.1 and 0.2, and 0.02 and 0.03 g cortex^{-1}/epidermal and stelar samples, respectively. The root tips, root segments, or isolated tissue samples were placed in petri dishes containing 5 mL of the control or +Al full nutrient solutions. The samples were placed on a slow rotary shaker (50 rpm) and were allowed to recover from wounding over a period of 4 h. Previous studies (Kochian and Lucas, 1983) have shown that within this period of time, excised maize roots recover from wounding and restore normal physiological function with regard to ionic fluxes. Following this period of recovery, the full nutrient solution was replaced with 5 mL of the simple-salt treatment solution, and exudates were collected from excised root tips, root segments, or isolated root tissue for 6 h. The organic acid exudation and tissue organic acid content were determined as described above.

Protoplast Isolation and Patch-Clamp Protocols

Protoplasts used for patch clamp recordings were isolated following the protocol described previously (Piñeros and Kochian, 2001). The root tips used for protoplast isolation were from seedlings grown in ±Al full nutrient solution over a 24-h period. Protoplasts were isolated from the terminal 1 to 2 mm or the terminal 2 cm of the primary root. Cortical and stelar root protoplasts could be easily distinguished based on their morphological characteristics. Cortical protoplasts were typically larger in diameter (50–70 μm) than stelar protoplasts (20–30 μm in diameter). In addition, these cells differed in their internal morphology, as cortical protoplasts contained a large vacuole and relatively little cytoplasm in contrast to stelar protoplasts, which were rich in cytoplasm.

The intracellular solutions used to fill the patch pipette contained 2 mM $MgCl_2$, 4 mM K_2ATP, 2 mM EGTA, 100 mM TEA, and 10 mM HEPES. The pH was adjusted to 7.2 with KOH. The free Ca^{2+} concentration in these solutions, as estimated by GEOCHEM, was 50 nM. The bath solutions used for recordings contained 0.5 mM $CaCl_2$ and TEA-Cl (concentration indicated in the figure legends), plus or minus 50 μM Al^{3+}. Al^{3+} was added from a stock solution of 10 mM $AlCl_3$ made up in 10 mM HCl. The pH of all solutions was adjusted to 4.0 with 10 mM HCl. The pipette filling solution and the bath solutions were adjusted to 720 and 700 mOsm kg^{-1}, respectively, using sorbitol. Ionic activities were calculated using CHEOCHEM-PC (Parker et al., 1995).

Whole cell and single-channel currents from excised outside-out patches were recorded with an amplifier (Axopatch 200A; Axon Instruments, Foster City, CA) and a data acquisition system (Digitada 1320A; Axon Instruments) using the patch-clamp technique as described previously (Piñeros and Kochian, 2001). In whole-cell configuration, the holding potential was set to 0 mV and voltage pulses stepped between −118 to +138 mV (in 10-mV increments), with a 3-s resting phase at the holding potential between each voltage pulse. The I/V relationships were constructed by measuring the current amplitude at the end of the test pulses (i.e. steady state). Outside-out patches were excised in the absence and presence of extracellular Al^{3+}. When excised in the absence of Al^{3+}, a voltage protocol was used to test for single-channel activity in solutions lacking Al^{3+}. The voltage was stepped from a holding potential of +48 mV to −138 and was held at this test potential for 1.4 s before returning to the holding potential. This protocol was repeated consecutively 25 times, with a 5-s resting phase between repetitions.

For patches in which single-channel activity was detected in the absence of Al^{3+}, further characterization of these particular channels was carried out in solutions lacking Al^{3+}. For those excised patches in which the above protocol indicated lack of single-channel activity in the absence of Al^{3+}, the bath solution was replaced with an identical solution containing 50 μM Al^{3+}, and an identical voltage protocol was repeated. All capacitive currents were removed by subtracting a sweep where no channel activity was detected from each individual sweep. Single-channel I/V relationships were derived from slow voltage ramps or steady-state recordings from outside-out patches. Slow voltage ramps (1.0–1.4 s each) were applied between −198 and +198 mV from a holding potential of +78 mV. Between each voltage ramp there was a 5-s resting phase at the holding potential. The I/V relationships were reconstructed by subtracting averaged ramps where no channel activity was observed from individual ramps where channel events were detected. The I/V relationships shown in each case contain at least four to 12 individual superimposed ramps showing the open and close states of the channel. The unitary conductance of the single channel from I/V relationships derived from ramp protocols was estimated from the slope of the linear portion of the open state of the channel. All currents were filtered at 1 kHz unless otherwise specified.

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