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Aluminum Rapidly Depolymerizes Cortical Microtubules and Depolarizes the Plasma Membrane: Evidence that these Responses are Mediated by a Glutamate Receptor

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Efforts to understand how plants respond to aluminum have focused on describing the symptoms of toxicity and elucidating mechanisms of tolerance; however, little is known about the signal transduction steps that initiate the plant's response. Here, we image cortical microtubules and quantify plasma-membrane potential in living, root cells of intact *Arabidopsis* seedlings. We show that aluminum depolymerizes microtubules and depolarizes the membrane, and that these responses are prevented by calcium channel blockade. Calcium influx might involve glutamate receptors, which in animals are ligand-gated cation channels and are present in the *Arabidopsis* genome. We show that glutamate depolymerizes microtubules and depolarizes the plasma membrane. These responses, and also the inhibition of root elongation, occur within the first few min of treatment, but are evoked more rapidly by glutamate than by aluminum. Microtubule depolymerization and membrane depolarization, induced by either glutamate or aluminum, are blocked by a specific antagonist of ionotropic glutamate receptors, 2-amino-5-phosphonopentanoate; whereas an antagonist of an aluminum-gated anion channel blocks the two responses to aluminum but not to glutamate. For growth, microtubule integrity, and membrane potential, responses to combined glutamate and aluminum were not greater than to glutamate alone. We propose that signaling in response to aluminum is initiated by efflux of a glutamate-like ligand through an anion channel and the binding of this ligand to a glutamate receptor.

Keywords: Aluminum — *Arabidopsis thaliana* — Cortical microtubules — Membrane potential — Ionotropic glutamate receptors — Roots.

Abbreviations: AP-5, 2-amino-5-phosphonopentanoate; GFP, green fluorescent protein; MBD, microtubule-binding domain; NMDA, *N*-methyl-D-aspartate; NPPB, 5-nitro-2-(3′-phenylpropyl-amino)-benzoate.

Introduction

Aluminum in soil limits agricultural productivity throughout the world. Because aluminum is deleterious and abundant, plants have evolved mechanisms to minimize or avoid damage. Tolerance can be achieved by sequestering aluminum inside the cell in an inert form or by excreting organic anions that chelate the metal and render it harmless (Matsumoto 2000, Ma et al. 2001). While these two strategies for tolerance are probably complementary, the secretion of organic acids has received the most attention, particularly in cereals. The best understood pathway to tolerance is for maize and wheat; in these plants, exposure of roots to aluminum evokes a rapid secretion of citrate (maize) or malate (wheat). Secretion does not require de novo synthesis of the organic acid and is not limited by the activities of the relevant biosynthetic enzymes. The secretion apparently depends on plasma-membrane anion channels with appreciable conductance for organic anions, a conductance that is gated by aluminum (Ryan et al. 1997, Kollmeier et al. 2001, Piñeros and Kochian 2001, Zhang et al. 2001).

In the framework of signal transduction, the secretion of organic acids is an output, a consequence of perceiving an input, namely the presence of aluminum. For aluminum, the rest of the signal transduction pathway is almost totally undefined, although parts of such a pathway are indicated by some reports. For example, in wheat, aluminum inhibits a key signal transduction enzyme, phospholipase C (Jones and Kochian 1995). Also in wheat, aluminum transiently induces a protein kinase within 30 s of exposure (Osawa and Matsumoto 2001). Finally, organic acids are secreted by rye in response to aluminum after a delay of a few h and following induction of biosynthetic enzymes, a timetable implying that a signal heralding aluminum reaches the nucleus (Li et al. 2000).

To delineate the signal transduction pathway, it is necessary to characterize the events in the first min of aluminum treatment. In doing so, we followed a suggestion of Dennison and Spalding (2000) and focused on the glutamate receptor. The *Arabidopsis* genome contains a family of at least 20 genes homologous to animal, ionotropic glutamate receptors (Lam et al. 1998, Chiu et al. 1999, Lacombe et al. 2001). In animals, these receptors are ligand-gated cation channels, some conducting sodium and others calcium (Dingledine et al. 1999). In

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Arabidopsis, at least some of these receptors may be plasma-membrane calcium channels. Symptoms of calcium deficiency were observed in a transgenic plant in which expression of one family member was obliterated (Kim et al. 2001); moreover, cytosolic calcium was elevated transiently by glutamate, apparently following calcium influx across the plasma membrane (Dennison and Spalding 2000). In view of the central relevance of calcium transients in plant signal transduction (Knight 2000), a ligand-gated calcium channel is a good candidate for a governor of signal transduction.

Here, in living root cells of intact *Arabidopsis* seedlings, we image cortical microtubules and quantify plasma-membrane potential. We show that treatment with aluminum or glutamate depolymerizes cortical microtubules and depolarizes the membrane. Both responses are blocked by a specific antagonist of ionotropic glutamate receptors whereas an antagonist of an aluminum-gated anion channel blocks the two responses to aluminum but not to glutamate. As a working model, we hypothesize that endogenous glutamate effluxes through an anion channel and binds its receptor to transduce the cell's response to aluminum.

Results

Aluminum and glutamate elicit similar long-term root growth responses

To study a rapid and cellular response to aluminum, we took advantage of an *Arabidopsis* transgenic line, GFP-MBD (green fluorescent protein–microtubule-binding domain), that allows microtubules to be imaged in living root cells (Marc et al. 1998, Granger and Cyr 2001). First, we determined that the seedling primary root in this line elongated comparably to wild type, and that elongation and root diameter were affected by aluminum comparably in the two genotypes (Fig. 1A). Aluminum requires relatively acidic pH to exert toxic effects on plants, and all media were prepared at pH 4.5, considered optimal for experiments on aluminum (Matsumoto 2000); at this pH, in the absence of aluminum, roots of both genotypes elongated vigorously (the rates for controls shown in Fig. 1 are higher than often reported for *Arabidopsis* at any pH). A concentration of 100 μM aluminum elicited about half maximal response, and this dose, rather than a saturating dose, was chosen for further experiments to minimize potential toxicity. At that concentration in our medium, the activity of the monomeric, trivalent species (presumably the most toxic) cannot be calculated exactly, but has been estimated to be about 10 μM by T.B. Kinraide (U.S. Department of Agriculture, Beaver WV, personal communication) who replicated the preparation of our media and performed ferron assays (Kinraide and Sweeney 2001) over the time period of our experiments.

To determine whether glutamate-receptor homologs may be involved in the response to aluminum, we used exogenous glutamate to stimulate the receptor. Glutamate inhibited root elongation and stimulated radial expansion, and the response

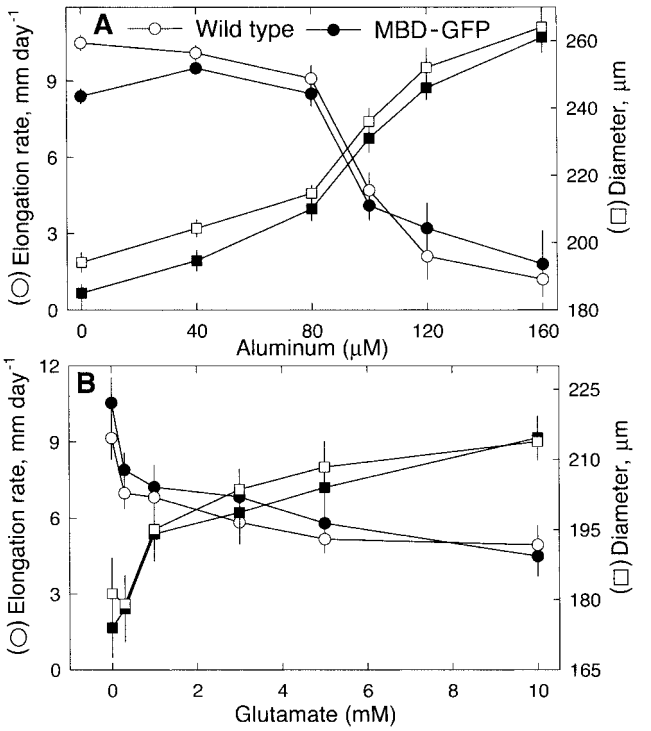

Fig. 1 Growth responses of the primary root of *Arabidopsis* to (A) aluminum and (B) glutamate. Seven-day-old seedlings were transplanted onto the indicated concentration and elongation rate and root diameter measured after 24 h. Data are means ± SE of three replicate plates from a single, representative, experiment.

was similar in both genotypes (Fig. 1B). In contrast, root elongation was not affected by 5 mM aspartate (not shown). Compared with aluminum, glutamate evoked a weaker response and, at the lowest dose, glutamate inhibited elongation without affecting root diameter. Nevertheless in general the growth responses of glutamate-treated roots resembled the responses of roots treated with aluminum, with both compounds inhibiting elongation and stimulating radial expansion. A dose of 5 mM glutamate was chosen for further experiments.

If aluminum and glutamate act in different pathways then both treatments given together should produce an additive effect. Instead, combining 5 mM glutamate with 100 μM aluminum inhibited elongation and increased diameter to the same extent as glutamate alone (Table 1), which is consistent with these compounds acting within a single pathway. In fact, the similarity of the responses to glutamate and to glutamate-plus-aluminum suggests that glutamate is epistatic to aluminum, implying that glutamate is downstream of aluminum, but the similarity may be coincidental. Note that in the medium used here, glutamate has been calculated to chelate monomeric, trivalent aluminum minimally (T.B. Kinraide, personal communication).

Aluminum and glutamate depolymerize cortical microtubules

In the GFP-MBD plants, cortical microtubules were reliably imaged throughout the root growth zone; for consistency,

Table 1 Comparison of long-term growth responses to aluminum and glutamate given singly and in combination

Treatment	Elongation rate (mm d^{-1})	Diameter (μm)
Control	6.9±0.3 [a]	152±0.3 [a]
100 μM aluminum	3.0±0.5 [b]	199±2.0 [b]
5 mM glutamate	5.0±0.1 [c]	165±1.9 [c]
Aluminum + glutamate	4.7±0.3 [c]	165±1.9 [c]

One-week-old seedlings were transplanted onto treatment plates and the elongation rate measured over the following 24 h and root diameter after 24 h. Data are means ± S.E. for three replicate plates with about 10 seedlings per plate. For each column, different superscript letters indicate equivalence of means rejected at $P<0.001$ by Student's t test. The same letter indicates no evidence for rejection.

images were obtained between 150 and 300 μm from the quiescent center, which is in the distal elongation zone of the young seedlings studied here. Microtubule images were clearest for lateral root cap cells, being outermost (Fig. 2A); however, epidermal cells were frequently imaged concurrently, and both cell types responded similarly. Microtubules in roots perfused with control medium remained unaffected on the stage of the confocal for more than 1 h (not shown); in contrast, perfusion of roots with aluminum caused microtubules to depolymerize (Fig. 2B). Depolymerization was evident by there being fewer microtubules and by the appearance of puncta, possibly reflecting oligomers of tubulin in a complex with MBD (Marc et al. 1998). Microtubules were not affected by concentrations of aluminum up to 75 μM (not shown), indicating that the sensitivity of the microtubules to aluminum resembled that of root growth (Fig. 1). Cortical microtubules were also depolymerized by perfusion with glutamate (Fig. 2C). Microtubules were unaffected by aspartate (Fig. 2D) but responded strongly to *N*-methyl-D-aspartate (NMDA) (Fig. 2E), an agonist of some but not all glutamate receptors (Dingledine et al. 1999). As with growth, perfusion with aluminum and glutamate together was no more effective than perfusion with glutamate alone (Fig. 2F).

Ionotropic glutamate receptors in animal neurons are frequently calcium channels; therefore, if homologs of these receptors mediate microtubule depolymerization, then a calcium influx would be an expected intermediary event. To determine whether a calcium influx is required for microtubule depolymerization, prior to perfusion with aluminum or glutamate, we incubated plants for 1 h in 100 μM lanthanum or gadolinium, trivalent cations known to block calcium channels. Note that these compounds at 100 μM affected root elongation only slightly in 24 h of exposure. Gadolinium alone (or lanthanum, not shown) did not affect microtubules (Fig. 2G), showing that trivalent cations per se do not act like aluminum; however, gadolinium (or lanthanum, not shown) prevented aluminum or glutamate from depolymerizing microtubules (Fig. 2H, I). Likewise, neither aluminum nor glutamate depolymerized microtubules when roots were pretreated for 1 h with the

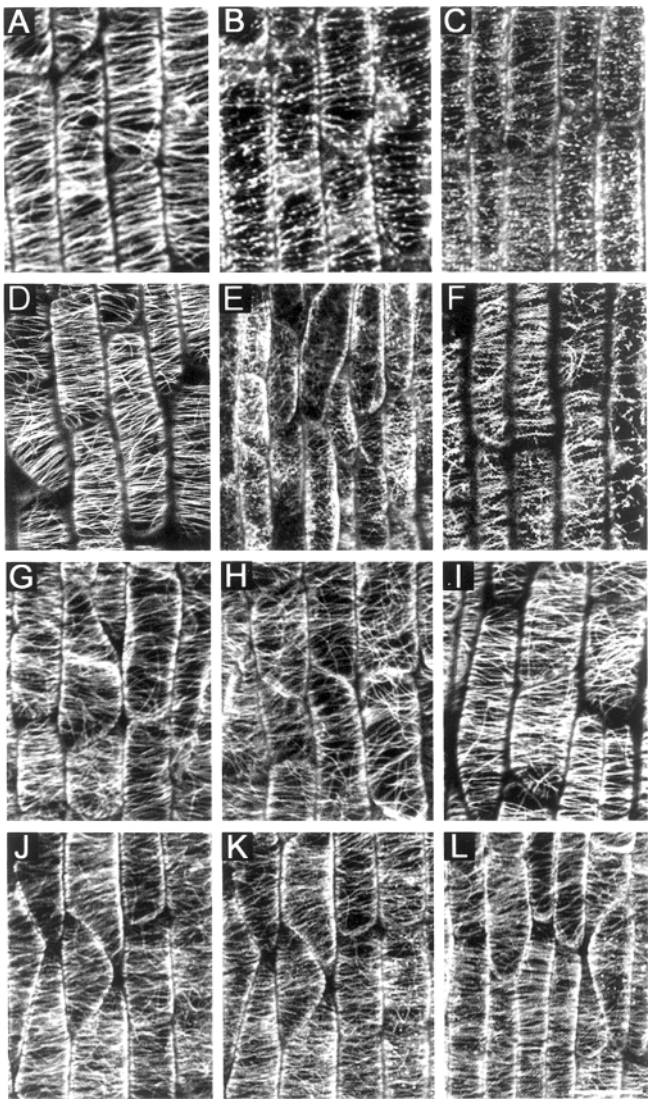

Fig. 2 Confocal micrographs of cortical microtubules imaged in living, lateral root-cap cells of intact seedlings expressing the MBD-GFP construct: (A) control; (B) aluminum (100 μM $AlCl_3$) for 30 min (same root as A); (C) glutamate (5 mM) for 30 min; (D) aspartate (5 mM) for 30 min; (E) NMDA (100 μM) for 15 min; (F) glutamate and aluminum for 32 min; (G) gadolinium (100 μM $GdCl_3$) for 1 h; (H) gadolinium for 1 h followed by aluminum for 32 min; (I) gadolinium for 1 h followed by glutamate for 31 min; (J) AP-5 (100 μM) for 1 h; (K) AP-5 for 1 h followed by aluminum for 35 min (same root as J); (L) AP-5 for 1 h followed by glutamate for 30 min. Scale bar = 10 μm.

calcium chelator, EGTA (ethylene glycol-bis(beta-aminoethyl-ether)-*N*,*N*,*N*′,*N*′-tetra-acetate) (data not shown).

If the response to aluminum requires a glutamate receptor, then the response might be blocked by the specific antagonist of neuronal glutamate receptors, 2-amino-5-phosphonopentanoate (AP-5) (Davies et al. 1981). AP-5 alone for 1 h did not affect microtubules (Fig. 2J) but prevented a subsequent perfusion with either aluminum (Fig. 2K) or glutamate (Fig. 2L)

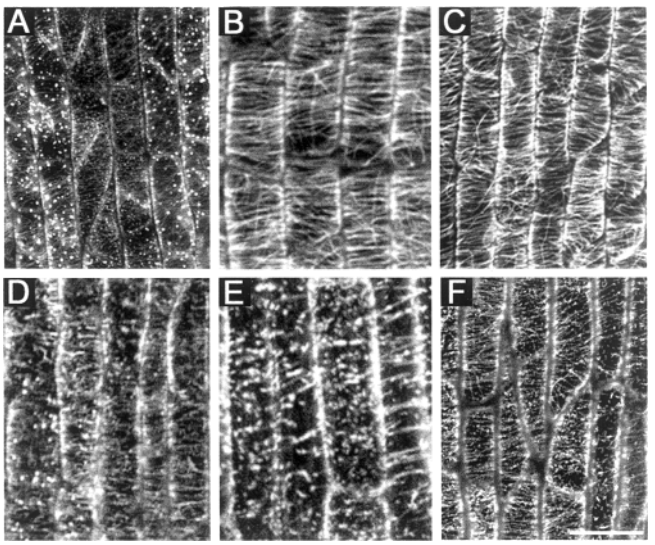

Fig. 3 Confocal micrographs of cortical microtubules in living cells in response to calcium or oryzalin: (A) calcium (10 mM) for 30 min; (B) gadolinium for 1 h followed by calcium for 30 min; (C) AP-5 for 1 h followed by calcium for 30 min; (D) oryzalin (1 μM) for 15 min; (E) gadolinium for 1 h followed by oryzalin for 16 min; (F) AP-5 for 1 h followed by oryzalin for 15 min. Scale bar = 10 μm.

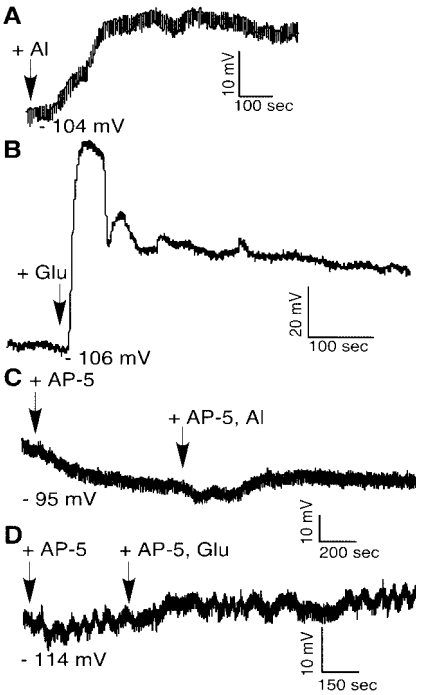

Fig. 4 Recordings of membrane potential from the root apex of intact seedlings in response to aluminum or glutamate: (A) 100 μM aluminum; (B) 5 mM glutamate; (C) AP-5 (100 μM) then AP-5 plus aluminum; (D) AP-5 then AP-5 plus glutamate.

from depolymerizing microtubules. That AP-5 blocked aluminum as well as glutamate from affecting microtubules suggests that the response to aluminum proceeds via a glutamate receptor.

To determine whether the rare earths and AP-5 block calcium channels effectively in the present system, we used calcium as a positive control: perfusing roots with 10 mM calcium depolymerized microtubules dramatically (Fig. 3A), but not if preceded by incubation in either lanthanum (not shown), gadolinium (Fig. 3B) or AP-5 (Fig. 3C). If calcium channel blockade somehow stabilized microtubules, then that would offer a trivial explanation for the observed inhibition of aluminum- or glutamate-induced microtubule depolymerization. To test for such stabilization, we used the microtubule inhibitor, oryzalin, which blocks microtubule assembly but not ongoing disassembly, thus causing depolymerization (Hugdahl and Morejohn 1993). Oryzalin depolymerized microtubules extensively (Fig. 3D), even when roots were pretreated with lanthanum (not shown), gadolinium (Fig. 3E) or AP-5 (Fig. 3F), showing that cortical microtubules under calcium blockade have physiological dynamics.

Aluminum and glutamate depolarize the plasma membrane

Depolarization of the membrane in response to aluminum has been observed frequently (Olivetti et al. 1995, Papernik and Kochian 1997, Takabatake and Shimmen 1997, Sivaguru et al. 1999a) but not always (Kinraide 1993, Lindberg and Strid 1997), and has been reported in response to glutamate for the *Arabidopsis* root (Dennison and Spalding 2000). We compared the effects of glutamate and aluminum further by measuring membrane potential in single root cells of intact seedlings. Impalements were made within the growth zone (within 2 mm

Table 2 Summary of membrane potential parameters for depolarization in response to aluminum, glutamate, or both

Treatment	Lag time (s)	Rate (mV s^{-1})	Depolarization (mV)
Aluminum (100 μM)	158±82	0.1±0.02	24±10
Glutamate (5 mM)	59±23	1.2±0.39	34± 9
Aluminum + glutamate	53± 9	0.9± 0.1	26± 4

Lag time is the time interval between the time of perfusion and the onset of depolarization. Rate is the initial rate of depolarization obtained from a line fitted to the initial membrane potential response. Depolarization is the difference between the membrane potential just before perfusion and after the response stabilized. Data are means ± S.D. for six roots for each treatment, except the combined treatment where $n = 5$. Conditions as described in the legend of Fig. 4.

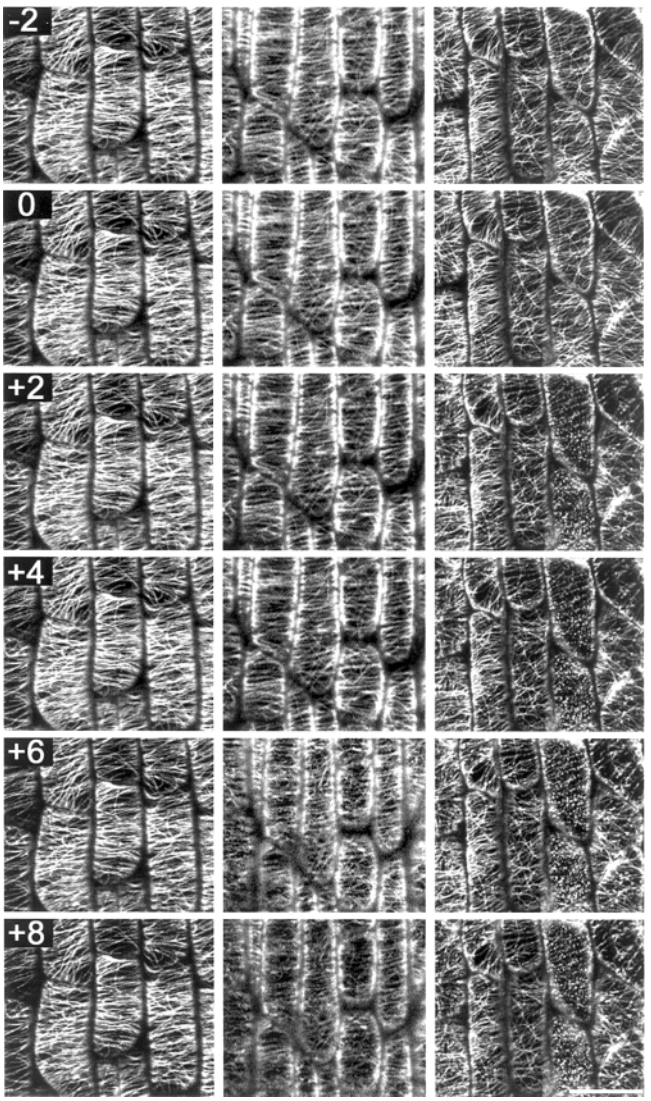

Fig. 5 Confocal micrographs of microtubules in living *Arabidopsis* roots showing the kinetics of the response to aluminum or glutamate. Roots were placed on the stage of the confocal and imaged at 2-min intervals. The left column is for control roots (perfused with growth medium at time zero), the center column for roots perfused with 100 μM aluminum, and the right column for roots perfused with 5 mM glutamate. Scale bar = 10 μm.

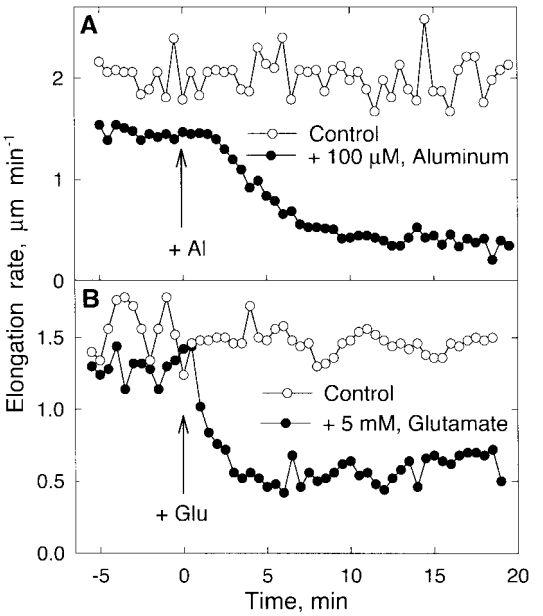

Fig. 6 Kinetics of root elongation measured under the same conditions used to obtain Fig. 5; transmitted light images were obtained at 30-s intervals and used to measure root tip displacement: (A) 100 μM aluminum; (B) 5 mM glutamate. Controls for each figure were perfused with growth medium at time zero.

of the root tip; Beemster and Baskin 1998), 1 h or more after equilibrating roots in a bathing solution (growth medium + 1 mM KCl, pH 4.5). Aluminum or glutamate depolarized the plasma membrane by 20–30 mV (Fig. 4A, B). On average, compared with glutamate, aluminum took longer to evoke depolarization, and caused a slower and less extensive depolarization (Table 2). Membrane potential was more stable after adding aluminum than glutamate (Fig. 4A, B). Depolarization in response to combined aluminum and glutamate was no more severe than for glutamate alone (Table 2). When calcium influx was blocked with gadolinium (not shown) or AP-5 (Fig. 4C, D), membrane potential was largely unaffected by aluminum or glutamate (mean potential change ± SD for AP-5: 7.5±5.9 mV, n = 15; AP-5 + aluminum: 7.3±1.1 mV, n = 8; AP-5 + glutamate: 8.2±1.9 mV, n = 7). This implies that membrane depolarization requires calcium influx.

Kinetics of microtubule and growth responses

Membrane depolarization was faster in response to glutamate than to aluminum; to see whether the kinetics of microtubule depolymerization differ between the two treatments, we imaged cortical microtubules at 2-min intervals, starting before perfusion. Microtubules began to depolymerize within 2 min of adding glutamate but not until 8–10 min of adding aluminum (Fig. 5). To compare response kinetics additionally, we measured root elongation under the same conditions used to image microtubules. Control roots elongated steadily for at least 0.5 h, whereas aluminum and glutamate inhibited elongation within minutes (Fig. 6). Interestingly, the kinetics of elongation rate inhibition were similar to membrane depolarization. Thus, membrane depolarization, microtubule depolymerization, and elongation inhibition each happened faster in response to glutamate than to aluminum. These data are consistent with the hypothesis that aluminum leads to the release of glutamate (or a related compound) that in turn causes the observed responses.

The response to glutamate is downstream of the response to aluminum

Along with kinetics, the results where glutamate and alu-

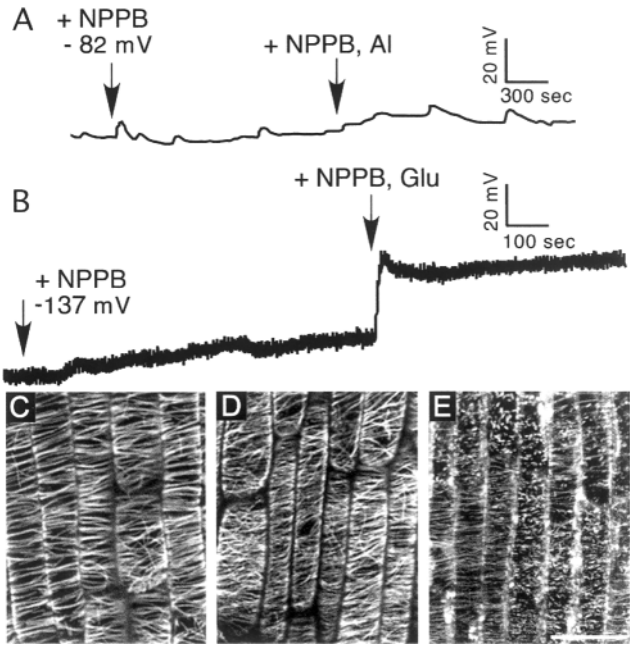

Fig. 7 Membrane potential (A, B) and cortical microtubules (C–E) in the presence of the anion channel blocker, NPPB (10 μM, otherwise conditions as for Fig. 4). (C–E) Imaged as described for Fig. 2: (C) NPPB; (D) NPPB followed by NPPB + aluminum; (E) NPPB followed by NPPB + glutamate.

minum were given together have suggested that the glutamate receptor is downstream of an aluminum receptor. To separate the responses in a different way, we used the anion channel blocker, 5-nitro-2-(3′-phenylpropyl-amino) benzoate (NPPB) (Wangemann et al. 1986), known to block an aluminum-gated anion channel (Ryan et al. 1997). Pretreatment with NPPB for 1 h affected neither membrane potential nor microtubules, but blocked aluminum from depolarizing the membrane and depolymerizing microtubules; however, NPPB did not block the responses to glutamate (Fig. 7) (mean potential change ± SD for NPPB: 6.6±2.7 mV, n = 20; NPPB + aluminum: 5.7±2.8 mV, n = 11; NPPB + glutamate: 34.3±10.4 mV, n = 9). Experiments with other anion channel blockers were inconclusive because these compounds depolarized the membrane considerably. Results with NPPB suggest that anion channel opening is upstream of microtubule depolymerization and membrane depolarization.

Discussion

To discover steps in a signal transduction pathway for the response to aluminum, we studied three distinct processes in intact plants that occur within min of treatment: elongation inhibition, microtubule depolymerization, and membrane depolarization. These responses were mimicked by treatment with exogenous glutamate. Glutamate and aluminum did not act additively, and a specific antagonist of neuronal glutamate receptors, AP-5, blocked the responses. We propose the following model, consistent with the observations though far from proven: aluminum opens a channel through which glutamate effluxes; glutamate binds its receptor, a ligand-gated calcium channel at the plasma membrane, and triggers a calcium influx; calcium influx initiates subsequent downstream events including microtubule depolymerization, membrane depolarization, and elongation inhibition.

Microtubules and membrane potential were studied in different cell types (lateral root cap, epidermis and cortex), but all of them fall within the elongation zone, considered to be the site of greatest sensitivity to aluminum (Ma et al. 2001). This includes the lateral root-cap cells, which, in *Arabidopsis*, surround the distal part of the elongation zone. Elongation presumably reflects the behavior of all cell types and it responded in parallel with depolymerization and depolarization. Cortex and epidermal cells are coupled electrically via plasmodesmata so the depolarization measured in cortical cells almost certainly occurs in epidermis, and probably also in lateral root-cap cells. Microtubules were difficult to image in the root cortex and it is possible that, in response to aluminum, microtubules depolymerize only in epidermal and lateral root-cap cells; but even if so, this would indicate a specialization among response elements and would still support our contention that primary responses to aluminum involve a glutamate receptor.

On calcium influx following aluminum treatment

We have amassed indirect evidence that, within a few minutes of treatment, aluminum increases the rate of calcium influx across the plasma membrane. Both microtubule depolymerization and membrane depolarization in response to aluminum are not only prevented by calcium channel blockade (lanthanum, gadolinium, AP-5), and by calcium chelation, but in addition are mimicked by exogenous calcium and by glutamate, a compound that, in *Arabidopsis*, produces a large and transient rise in cytosolic calcium (Dennison and Spalding 2000).

That exogenous calcium mimics aluminum treatment in causing microtubule depolymerization might lead one to conclude from our model that calcium and aluminum given together should act synergistically, the opposite of what is usually observed; however, added calcium in the apoplast reduces the activity of aluminum and can therefore be understood to dampen responses and ameliorate toxicity without invoking intracellular signals (Rengel 1996).

The inferred calcium influx runs counter to the widespread idea that aluminum blocks plasma-membrane calcium channels (Knight 2000). The calcium conductance of many channels has indeed been reported to be blocked by aluminum but, to our knowledge, all the reports concern voltage or stretch-activated channels. The channel hypothesized here is a ligand-gated channel and consequently may have distinct pharmacology from voltage-gated channels. Also, the default state for plasma membrane calcium channels must be mostly closed, and therefore a transient opening induced by glutamate would still result in a considerable transient calcium influx even if the

channel is slowly blocked thereafter by ambient aluminum.

Calcium concentrations in cells have been measured after aluminum treatment by several groups, with conflicting results. In root hairs of *Arabidopsis* (Jones et al. 1998a) and in the wheat root apex (Zhang and Rengel 1999), cytoplasmic calcium rises gradually, whereas in tobacco tissue culture cells calcium level actually decreases (Jones et al. 1998b). Nevertheless, all of these reports sampled at intervals between 2 and 10 min, too low a temporal frequency to have recorded a calcium transient like the ones recently measured in *Arabidopsis* in response to glutamate (Dennison and Spalding 2000), or in other signal transduction cascades (e.g. Baum et al. 1999). These calcium transients typically have a peak width at half maximum of around 10 s and the calcium concentration returns to baseline after 30–60 s. To our knowledge, in only one study have cellular calcium levels following aluminum treatment been measured with a temporal resolution on the order of seconds, a study reporting that, in protoplasts, aluminum induced a rapid and transient increase in calcium level (Lindberg and Strid 1997), consistent with our model. Clearly, cytosolic calcium level needs to be measured in intact roots with high temporal resolution to confirm the existence of a calcium spike in response to aluminum.

On the role of cortical microtubules in the response to aluminum

We find that aluminum or glutamate depolymerizes cortical microtubules within minutes. Although all the data reported here were obtained with a single transgenic line, we have confirmed our findings in fixed wild-type roots and also in two other transgenic lines, in which either α- or β-tubulin was fused to GFP. Cortical microtubules are well known to function in controlling expansion anisotropy (Green 1980, Baskin 2001). Indeed, aluminum causes aberrant cell and organ shape and disorganizes cortical microtubules in the root growth zone (Blancafluor et al. 1998, Sivaguru et al. 1999a, Sivaguru et al. 1999b) as well as in tissue culture cells (Schwarzerová et al. 2002). However, the earliest time-point examined in those reports was 1 h, and typically several hours were needed for the reported effects to become manifest. Possibly, the depolymerization of the array reported here represents the onset of such disruptions earlier in *Arabidopsis* than reported hitherto for other species. However, although we did not extensively study microtubule behavior during long-term aluminum exposure, after a few h, cortical microtubules re-polymerized, and after 12–24 h they were variously disorganized and disrupted. We suggest that changes in microtubule organization seen after h of aluminum exposure (as in the papers cited above) reflect downstream events, such as redirected expansion, or even symptoms of toxicity; whereas, the partial depolymerization of the cortical array seen here within min of exposure reflects early events in signal transduction.

Apart from a potential role in signaling, rapid microtubule depolymerization supports our inference that aluminum and glutamate elevate cytosolic calcium. Conceivably, elevated calcium depolymerizes microtubules directly; however, it is more likely that calcium acts via calmodulin or a calcium-dependent kinase to change the status of microtubule-associated proteins. Binding between microtubules and associated proteins is an essential regulator of microtubule dynamics and often depends on calmodulin or phosphorylation (Drewes et al. 1998). Although most evidence comes from animal cells, in plant protoplasts, microtubule stability is known to depend on interactions between calcium and proteins other than tubulin (Fisher et al. 1996). Our results suggest that microtubules depolymerize as a consequence of transiently elevated calcium levels. Cortical microtubules associate with the plasma membrane and their transient depolymerization could condition cellular responsiveness. For example, microtubules have been shown recently to bind the enzyme phospholipase D (Gardiner et al. 2001), and depolymerization could alter the enzyme's activity or localization. Calcium and cortical microtubules, along with plasma-membrane potential, may form an integrated response network in plant signal transduction.

On the function of glutamate receptors in the plant's response to aluminum

Surprisingly, the observed responses to aluminum were mimicked by glutamate and were blocked by AP-5. In neurobiology, sensitivity to AP-5 is considered to demonstrate the activity of ionotropic glutamate receptors, conclusively (e.g. Kelley 1999, Canesin et al. 2000, Tao and Auerbach 2000). That glutamate is involved in plant signal transduction must be considered in view of the presence in the plant genome of sequences homologous to the metazoan ionotropic glutamate receptor family (Lacombe et al. 2001). In addition to glutamate, these receptors respond to a variety of other ligands; indeed, the spectrum of response is used to delineate different subtypes of receptors within the family (Dingledine et al. 1999). Some ligands that are well known in neurobiology as antagonists of glutamate receptors are secondary plant metabolites. The customary explanation has been that these metabolites defend against herbivory through their ability to wreak havoc on a herbivore's nervous system; however, the discovery of glutamate receptors in plants led to the suggestion that these metabolites have a positive role in the plant as agonists, and it was shown that at least some of these metabolites can interfere with photomorphogenesis in seedlings (Lam et al. 1998, Brenner et al. 2000).

We suggest these receptors are an important class of calcium channel in the plasma membrane and that glutamate itself is a physiologically relevant ligand. Root exudates contain copious amounts of glutamate, along with other amino acids, and the profile of exuded amino acids changes as a function of environment and development (Curl and Trueglove 1986). In the nervous system, glutamate is delivered to the receptor at high concentration via synaptic vesicles; in the plant, exudation also achieves a high external concentration, but the responsible mechanism is not known. We hypothesize that glutamate

permeates through an aluminum-gated anion channel, because the anion channel blocker, NPPB, prevented responses to aluminum but not to exogenous glutamate. Conceivably, it is the same channel through which organic acids permeate, or perhaps a related, aluminum-gated channel specializing in amino acids. Alternatively, aluminum may act indirectly via binding to a plasma membrane receptor, which in turn interacts with various proteins.

Our model for the role of glutamate in the *Arabidopsis* root's response to aluminum makes novel predictions that can be tested by collecting root exudates, imaging cytosolic calcium, and patch-clamping membranes; this, along with ongoing molecular genetic studies of loci conferring aluminum tolerance, should help to determine the score from which the plant conducts the instruments of its response, including membrane transport, microtubule stability, and cellular expansion.

Materials and Methods

Plant material and growth conditions

Plant material used was *Arabidopsis thaliana* L. (Heynh), background Columbia, and a transgenic line in the same background that harbored a construct fusing green fluorescent protein (GFP) to the microtubule-binding domain of human MAP-4 (MBD), with the expression driven by the 35-S promoter. The GFP-MBD plants were made and characterized by Richard Cyr (Marc et al. 1998, Granger and Cyr 2001) and generously supplied by him. Plants were grown on nutrient-solidified agar in vertical Petri plates in a growth chamber under constant light (100 μmol m^{-2} s^{-1}) and temperature (20°C), as described (Baskin and Wilson 1997). An exception was that the medium composition was slightly modified, as follows: 1% sucrose, 0.5% agar, 2 mM KNO_3, 1 mM $Ca(NO_3)_2$, 2 mM $MgSO_4$, 0.1 mM KH_2PO_4, 5 μM H_3BO_3, 1 μM $MnCl_2$, 0.1 μM $ZnSO_4$, 0.03 μM $CuSO_4$, 0.01 μM MoO_3 (adjusted to pH 4.5 with 0.1 M HCl). Medium containing aluminum was prepared by pouring a solution containing growth medium and aluminum (as $AlCl_3 \cdot 6H_2O$; ICN, Aurora, OH, U.S.A.) over previously solidified growth medium in Petri plates and allowing the aluminum to diffuse into the underlying agar. After 48 h (to ensure diffusion reached equilibrium), the overlying solution was discarded. Care was taken to ensure that the volumes of added solution and solid medium were equal (20 ml per plate), and solution was prepared with aluminum at twice the desired final concentration to account for the dilution.

Root elongation rate and diameter were measured by transplanting 6 or 7-day-old seedlings onto plates with the desired addition and scoring the position of the root tip on the back of the Petri dish at known times. After 1–2 d of growth as noted, Petri plates were photocopied, the growth increment between marks was measured with a digitizing tablet (Baskin and Wilson 1997), and the diameter measured at its maximum or where the root hairs initiated under a compound microscope (Baskin and Wilson 1997).

Imaging cortical microtubules

All test compounds were dissolved in growth medium (without agar). Seedlings were treated as indicated and mounted in the test solution. Fluorescence from GFP-MBD was examined in living roots by placing the slide on an inverted stand (Olympus IX70) interfaced to a confocal microscope (BioRad-Radiance Laser Sharp 2000, Hercules, CA, U.S.A.). Fluorescence from GFP was excited with the 488 nm line of an argon laser, and images were captured through a water immersion objective (60×, 1.2 NA, U-plan apochromat). Observations were repeated at least twice with 6–10 individual roots per session.

Electrophysiology

Three to four seedlings were mounted with elastic bands onto a Plexiglas holder and held vertically during equilibration. After 1–2 h equilibration in aerated growth medium supplemented with 1 mM KCl (pH 4.5), they were placed horizontally in a 5 ml Plexiglas perfusion chamber. To keep potassium concentrations constant, glutamate was given as the Tris salt; incubations with Tris alone had no effect. Microelectrodes consisted of fiberglass-filament-containing 1.0-mm-diameter borosilicate glass capillaries that were pulled to a tip diameter of 0.5 μm with a vertical puller (D. Kopf Inc., Tujunga, CA, U.S.A.) and filled with 3 M KCl. Reference electrodes were 1.6 mm-diameter polyethylene tubes containing 3 M KCl in 2% agar. Microelectrodes and reference electrodes were connected to an electrometer amplifier (FD 223, World Precision Instruments, Sarasota, FL, U.S.A.) with Ag/AgCl wires. A micromanipulator was used to insert the microelectrode into a cortical cell within 2 mm of the root apex (epidermal cells are too small to impale). Measurements of the membrane potential were continuously recorded, while the roots were perfused with growth media. Several min after a stable potential was reached, the growth medium perfusion solution was changed to a treatment solution. Stable membrane potentials usually ranged between –90 and –140 mV; this is less negative than often reported in plant cells and possibly resulted from the acidic growth medium (pH 4.5). Analog output was recorded on a strip chart recorder or logged directly into a computer using digital data acquisition software (Digidata 1322A + Axoscope v. 8.1; Axon Instruments, Foster City, CA, U.S.A.).

High resolution measurements of elongation rate

Root elongation rate was measured at high resolution on the stage of the confocal microscope. Seedlings were handled in the same way as for imaging the microtubules. Transmitted-light images of seedlings were obtained every 30 s for about 5 min and then either control or treatment solution was perfused between the slide and coverslip and image acquisition continued for about 30 min. The series of images obtained were analyzed with NIH Image (U.S. National Institutes of Health, and available on the Internet at http://rsb.info.nih.gov/nih-image/) on a Macintosh computer. The distance between the tip of the root and the frame was measured for each image and the difference between two successive images divided by the time interval was calculated as elongation rate.

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