Planta (1995) 197: 672–680

Planta

Role of calcium and other ions in directing root hair tip growth in *Limnobium stoloniferum*

I. Inhibition of tip growth by aluminum

David L. Jones, Jon E. Shaff, Leon V. Kochian

U.S. Plant, Soil and Nutrition Laboratory, USDA-ARS, Cornell University, Ithaca, NY 14853, USA

Received: 19th January 1995/Accepted: 23rd February 1995

Abstract. The magnitude and spatial localization of Ca^{2+}, K^+ and H^+ fluxes in growing and non-growing *Limnobium stoloniferum* root hairs was determined using non-invasive, ion-selective vibrating microelectrodes. Both the spatial pattern and magnitude of the ionic flux was dependent on the particular ion in question. Both H^+ and Ca^{2+} influx was localized almost exclusively to the tips of growing root hairs, suggesting that these fluxes may be involved in directing growth. Influx of K^+ showed no distinct localization and uptake appeared uniform along the length of the root hair. Competitive inhibition of Ca^{2+} influx using a range of Mg^{2+} concentrations indicated that the magnitude of the Ca^{2+} flux entering the root hair tip did not determine growth rate; however, the presence of Ca^{2+} on the external face of the membrane was implicit for root hair integrity. Aluminum proved to be a potent inhibitor of root hair growth. At an exogenous Al concentration of 20 μM a complete blockage of Ca^{2+} influx into root hair tips was observed, suggesting that Al blockage of Ca^{2+} influx could be involved in Al toxicity. However, at a lower Al concentration (2 μM), Ca^{2+} fluxes were unaffected while inhibition of growth was still observed along with a distinct swelling of the root hair tip. The swelling at the root hair tips was identical in appearance to that seen in the presence of microtubule inhibitors, suggesting that Al could influence a number of different sites at the plasma-membrane surface and within the cell. The possible role(s) of Ca^{2+} and H^+ fluxes in directing tip growth are discussed.

Key words: Aluminum – Growth – Tip growth – Ion transport – *Limnobium* – Root hair

Introduction

The polarized expansion of leaf hairs, root hairs and pollen tubes occurs predominantly at the cell tip leading to the production of long cylindrically shaped cells. Typically, growth is rapid (5–100 μm·min^{-1}) with a high degree of cellular activity apparent within the region of the tip (e.g. 5000 exocytotic fusions·min^{-1}; Heath 1990). Physiological analysis of the mechanism underlying tip growth has received great attention with particular interest focused on the role of electric fields (Harold and Caldwell 1990), cytoskeleton development (Emons 1987) and calcium fluxes and gradients (Steer and Steer 1989) in controlling polarized tip growth. All of these topics are still open to considerable debate.

In pollen tubes, it has been demonstrated that the presence of an intracellular Ca^{2+} gradient along the pollen tube is associated with growth (Steer and Steer 1989; Miller et al. 1992). Despite many authors advocating the involvement of a Ca^{2+} gradient in polar growth, a lack of quantitative measurements of cytosolic Ca^{2+} activity and difficulty in knowing that only cytoplasmic free Ca^{2+} is being measured, and not organellar or membrane-associated Ca^{2+}, has prevented accurate interpretation of the data (Miller et al. 1992). Recently, Miller et al. (1992), using dextran-conjugated Ca^{2+}-fluorescent dyes estimated that the free cytoplasmic Ca^{2+} concentration at the pollen tube tip was >3 μM, falling to a basal level of 0.2 μM within a distance of 20 μm from the tip. The higher levels of cytoplasmic Ca^{2+} at the growing tip are also associated with a localized Ca^{2+} influx, which presumably mediates the formation of the cytoplasmic Ca^{2+} gradient (Pierson et al. 1994). The relationship between Ca^{2+} influx and growth rate will be directly addressed in this paper.

It has been assumed for many years that the proliferation of root hairs along root axes facilitates the uptake of nutrients from the soil by greatly increasing the surface area for absorption. Apart from phosphate uptake (Barley and Rovira 1970), however, there is very little evidence to support this hypothesis. Recent advances in electrophysiology have for the first time allowed specific ion-flux measurements to be made non-invasively on small regions of membrane (< 5 μm^2) in predetermined cell types (Smith et al. 1994). Using non-invasive, vibrating ion-selective microelectrodes, ion fluxes have now been measured in a number of cell types, for a variety of elements under a wide range of conditions (e.g. H^+, K^+, Mg^{2+},

Correspondence to: D.L. Jones; FAX: 1 (607) 255 2459; Tel.: 1 (607) 255 24 54; E-mail: djl1@cornell.edu

Ca^{2+}, NO_3^-; Kochian et al. 1992; Smith et al. 1994). This technique is therefore ideally suited to investigating the magnitude and patterns of ionic fluxes around root hairs and to assessing the potential role these ion fluxes play in root hair growth and development.

Aluminum has long been recognized as a primary factor limiting plant growth in acid environments (Foy et al. 1978). Although some genetically controlled tolerance mechanisms allowing roots to overcome the toxic effects of Al^{3+} in solution have recently been described (Delhaize et al. 1993; Pellet et al. 1995; Kochian 1995), there is little information, and much less agreement on the primary cause of Al toxicity. Indeed, Al may, and probably does, affect many cellular functions. An understanding of the toxic effects of Al has been hampered by the complexity of cell types and developmental stages in the root apex where toxicity becomes manifest (Ryan et al. 1993). Root hair growth has also been shown to be inhibited by exposure to Al, thus providing a simpler single-cell system for examining Al toxicity effects.

Therefore, the aim of this paper is to identify and quantify H^+, Ca^{2+} and K^+ ion fluxes along growing *Limnobium stoloniferum* root hairs. Secondarily, we hope to assess the potential role of these fluxes in directing polarized tip growth. Finally, we hope to clarify some of the controversy surrounding the causes of Al toxicity in plants.

Materials and methods

Growth conditions and solutions. An aquatic pondweed, *Limnobium stoloniferum*, was grown in the greenhouse in large plastic containers containing 10 L of water overlying 10 cm of potting soil. Fourteen days prior to experimentation, small plantlets (with approximately four leaves) were excised and removed from the containers, washed, and all existing roots excised to allow regeneration of clean roots. The plantlets were then floated in black polypropylene containers containing 2 L of a complete nutrient solution. Concentrations of macronutrients (mM) in the nutrient solution were: $Ca(NO_3)_2$, 0.5; $NH_4H_2PO_4$, 0.1; $MgSO_4$, 0.1; KCl, 0.1; whilst micronutrient concentrations (μM) were: H_3BO_4, 12.5; $MnSO_4$, 1; $ZnSO_4$, 1; $CuSO_4$, 0.5; H_2MoO_4, 0.1; $NiSO_4$, 0.1 and Fe(III) EDTA, 20. Nutrient solutions were changed every 5 d and excess roots and stolon growth removed where necessary. On the evening before the experiment, plantlets were transferred to a solution containing 0.2 mM $CaCl_2$ (pH 5.8). On the day of the experiment, individual plantlets were placed in 14-cm-diameter Petri dishes containing 60 mL of 0.2 mM $CaCl_2$ (pH 4.5) for 2–3 h before making growth or nutrient-flux measurements.

Measurement of root hair growth. Individual roots (8–10 cm long) of the *Limnobium* plantlets were attached to the base of the Petri dishes using Plexiglas blocks smeared with silicone grease. A notch was cut into one side of the Plexiglas block so that each block could straddle the root without causing injury. Indeed, root hairs trapped by the block continued streaming and grew to a limited extent throughout the experiment period. Growth rates of individual root hairs was determined every 20–30 min using an eye-piece micrometer (× 200 magnification). All measurements of root hair length were made in 80 mL of a solution containing 200 μM $CaCl_2$, 100 μM KCl (pH 4.5) ± 20 μM $AlCl_3$. Estimates of free Al^{3+} activities in the root-bathing solutions were made using GEOGHEM Version 2.0 (Parker et al. 1995).

Measurement of root hair K^+, H^+ and Ca^{2+} fluxes with vibrating ion-selective microelectrodes. Measurements of ion fluxes in the unstirred layer at the root-solution interface were made using vibrating microelectrodes containing ion sensor for the particular ion in question. Borosilicate glass capillaries were made using a two-stage pull with a Flaming/Brown MicroPippette Puller to a tip diameter of 2–6 μm, baked (200 °C, 24 h), silanized with tributylchlorosilane (200 °C, 30 min), cooled and stored under vacuum until required. Microelectrodes were backfilled with either 100 mM KCl, 40 mM KH_2PO_4/15 mM NaCl or 100 mM $CaCl_2$ for K^+, H^+ and Ca^{2+} microelectrodes, respectively. A small column of ion sensor, 20–80 μm in length, (Fluka Chemical Company; K^+, #60031; H^+, #95291; Ca^{2+}, #21048) was then introduced into the tip of the electrode and the backfilling buffer reduced to a column length of 10 mm. Electrical contact was made between the electrode and the head stage by introducing a Ag: $AgCl_2$ wire into the back of the microelectrode until the wire was immersed in the backfilling buffer.

Microelectrodes were calibrated in either KCl or $CaCl_2$ (10, 100 and 1000 μM) for K^+ and Ca^{2+} microelectrodes or in 10 mM 2-(N-morpholino)ethanesulfonic acid (Mes)-Tris buffer solutions (pH 4, 5.0 and 6.0) for H^+ electrodes. If a Nernstian response was observed (95% of electrodes tested) the microelectrode was lowered into a Petri dish containing a *Limnobium* plantlet which was attached to the base of the Petri plate as described previously. The microelectrode was then moved to a distance 30 μm from the root hair surface using a series of stepper motors. Microelectrodes were vibrated over a distance of 30 μm (K^+ and H^+) or 50 μm (Ca^{2+}), from the root hair surface at a frequency of 0.3 Hz and angle of 90° to the root surface. Measurement of the ion concentration difference between the two vibrating extremes (e.g. 30 and 80 μm from the root hair surface for Ca^{2+}) allowed the net ion fluxes to be calculated using Fick's first law of diffusion, assuming planar diffusion at the root hair tip and cylindrical diffusion for all other root hair locations (Kochian et al. 1992; Ryan and Kochian 1993). Further details of electrode construction, preparation and an explanation of the data-acquisition process can be found in Huang et al. (1992), Kochian et al. (1992), Kühtreiber and Jaffe (1990) and Smith et al. (1994). All flux experiments were performed at 24 °C ± 2 °C.

Influence of Al^{3+} on Ca^{2+}, K^+ and H^+ fluxes. Net ion fluxes were measured on growing root hairs 600–1000 μm in length at positions of 0, 100, 250, 400, 550 and 700 μm back from the root hair tip. In the K^+-, H^+- and Ca^{2+}-flux experiments, ion fluxes were measured in a background solution of 100 μM KCl and 200 μM $CaCl_2$ adjusted to pH 4.5 with 0.1 M HCl. Flux measurements were serially made at each of the root hair locations over 1- to 2-min periods and the procedure repeated three times over a period of 1 h. The root-bathing solution was then removed via vacuum withdrawal and replaced with an identical solution containing 20 μM $AlCl_3$ (free Al^{3+} activity ≈4 μM). The roots remained firmly in place during this solution interchange. After allowing an equilibration period of 10 min, ion fluxes were again measured in triplicate at each of the root hair locations over a period of 1 h. The original solution containing no Al^{3+} was then replaced and again ion fluxes measured at each of the root hair positions. At the end of the experimental period microelectrodes were again placed in the calibration solutions to confirm the stability of the electrodes over the experimental period. In further H^+- and Ca^{2+}-flux experiments, ionic conditions were kept identical to those described above, except that roots were kept in the $AlCl_3$ solution for up to 4 h, allowing the root hair tips to swell in response to the presence of Al^{3+}.

Calcium and H^+ fluxes were also mapped within the region of the root hair tip at intervals of 10 μm over a range of 150 μm from the root hair tip. An angle of 90° normal to the root hair surface was maintained throughout by modifying the vibration angle of the microelectrode.

The presence of Al did not significantly affect the Nernstian response of any of the microelectrodes.

Uncoupling of Ca^{2+} flux and growth. In order to assess the role of Ca^{2+} transport in directing root hair growth, Ca^{2+} influx into the root hair tip was measured in a solution containing 20 μM $CaCl_2$

and varying amounts of $MgCl_2$ (ranging from 0 to 10 mM) at pH 5.8. At each Ca/Mg concentration, ten Ca^{2+}-flux measurements were made over a 1-h period using the Ca^{2+} vibrating microelectrode set-up described above. The growth rate of root hairs was determined every 10 min during the experimental period.

Uncoupling of Ca^{2+} flux and Al^{3+} rhizotoxicity. In order to assess the involvement of Ca^{2+} transport in the inhibition of root hair growth by Al^{3+}, Ca^{2+} uptake into the root hair tip was determined in a root-bathing solution containing 20 μM $CaCl_2$ and 100 μM KCl ± 2 μM $AlCl_3$. Calcium influx was measured over a period of 1 h, first in a solution containing no Al and then in a solution containing 2 μM Al (free Al^{3+} activity ≈ 1.3 μM). Root hair growth rate was also monitored during this period.

Measurement of membrane potential (E_m). An excised *Limnobium* root was placed in a Plexiglas chamber and fastened down with Plexiglas blocks as described above. Roots were then allowed to equilibrate in the chamber for 30 min in 200 μM $CaCl_2$ (pH 4.5). Root hair E_m values were measured using single-barrelled 3 M KCl (pH 2.0) filled borosilicate microelectrodes (tip diameter = 0.5 μm) impaled close to the root hair tip using a hydraulically driven Narashige micromanipulator. After continuously measuring E_m over a period of 20 min, the root-bathing solution was slowly replaced with a 200 μM $CaCl_2$ solution containing 20 μM $AlCl_3$ (pH 4.5) and E_m further monitored over a 30-min period. This was then again replaced with a 200 μM $CaCl_2$ solution containing no $AlCl_3$ and E_m monitored over time. Confirmation of the validity of the measurement was made by adding a 200 μM $CaCl_2$ (pH 4.5) ± 100 μM KCl solution to the chamber in order to obtain a K^+-induced E_m depolarization and repolarization (upon removal of K^+).

Involvement of Ca^{2+} in the integrity of the root hair tip. To assess whether the presence of Ca^{2+} in solution was necessary for root hair integrity, plantlets were changed from an initial solution (0.2 mM $CaCl_2$, pH 4.5) into solutions containing $CaCl_2$ (0.2 mM, pH 4.5) and either KCl (0.2 mM), $MgCl_2$ (2 mM), EGTA (2 mM) or $AlCl_3$ (0.2 mM). After 20 min the plantlets were then transferred to pH 4.5 solutions containing either KCl, $MgCl_2$, EGTA, $AlCl_3$ or deionized water/HCl containing no Ca^{2+}. The integrity of the root hairs was monitored throughout at a magnification of × 100.

Results

Measurement of root hair growth. Preliminary measurements of root hair growth made in a solution containing a range of $AlCl_3$ concentrations indicated that root hair growth rate was negatively correlated with external Al^{3+} concentration, with a complete inhibition of growth observed at concentrations ≥ 20 μM (Fig. 1). It must be noted, however, that the critical Al^{3+} concentration which causes a complete inhibition of growth is highly dependent on the Al/cation ratio in solution and the time of contact between the root hair and Al^{3+}. In contrast to the root hairs, growth of the main root axis was not inhibited at concentrations ≤ 20 μM indicating that root hairs are highly sensitive to Al^{3+}. Further characterization of root hair growth at external Al^{3+} concentrations of 20 μM indicated that the cessation of root hair growth occurred within 30 min of the addition of Al^{3+} to the root-bathing medium (Fig. 2). Again, this is in contrast to root apices where strong inhibition of root growth occurs after a number of hours of exposure to Al (Ryan et al. 1993). Upon removal of Al^{3+} from the root-bathing solution, root hair growth almost instantaneously began again at

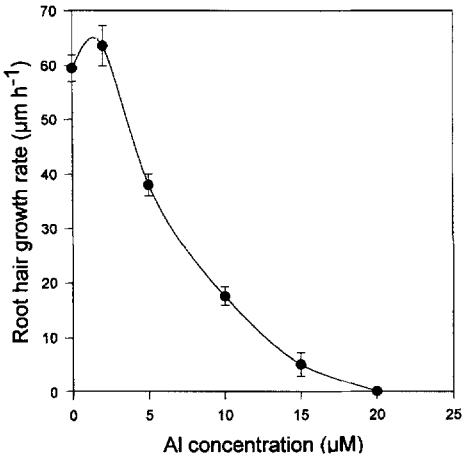

Fig. 1. Growth response of *Limnobium* root hairs over a 30-min period in different external concentrations of $AlCl_3$ at an external pH of 4.5 and in presence of 200 μM $CaCl_2$. All data points are expressed as mean ± SE

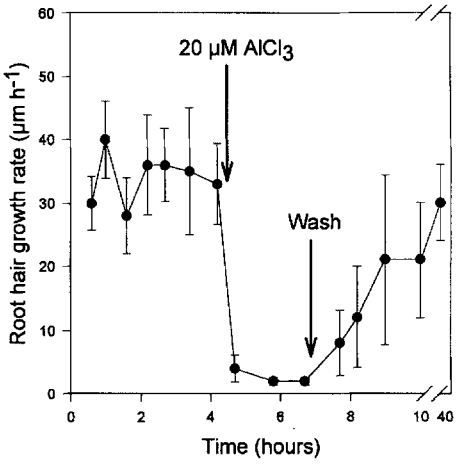

Fig. 2. Time course showing inhibition of *Limnobium* root hair growth after addition of exogenous $AlCl_3$ (20 μM; pH 4.5) to the root-bathing medium. All data points are expressed as means ± SE

growth rates lower than those measured under control conditions Only after > 5 h following the removal of Al^{3+} from the root bathing solution was the growth rate restored to that observed before the addition of Al. Cytoplasmic streaming in the root hairs was maintained throughout the experimental period.

Localization of H^+, K^+ and Ca^{2+} fluxes along root hairs. Ion fluxes were mapped along growing (800 μm in length; ≤ 1 d old) and non-growing (3000 μm in length; ≥ 4 d old) root hairs using non-invasive, vibrating ion-selective microelectrodes. As can be seen from Fig. 3 for growing root hairs, K^+ influx was measured at all positions along the root hair with the magnitude of the flux appearing similar at all locations. This is compared with Ca^{2+} fluxes where influx was only observed within a zone 0–100 μm from the root hair tip. All other areas of the root hair had

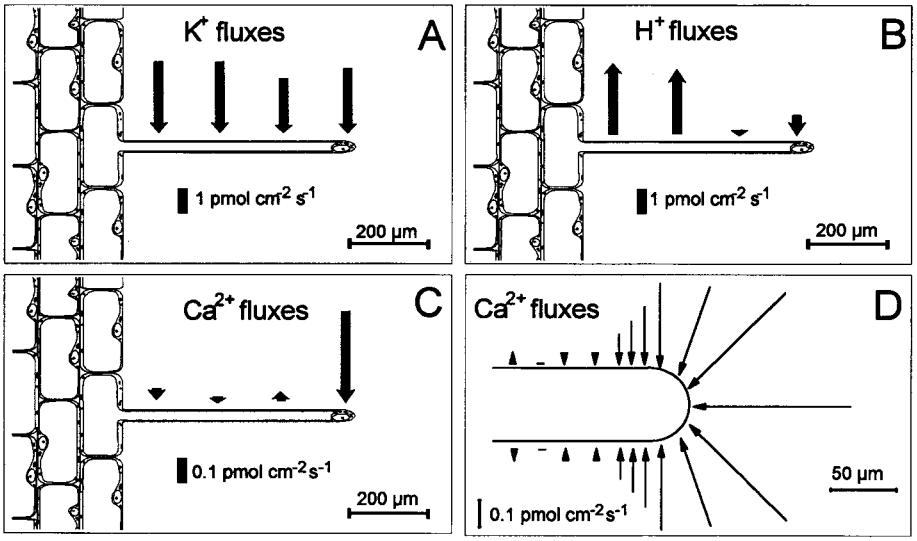

Fig. 3A–D. Diagram illustrating the magnitude of ionic fluxes (K^+, H^+ and Ca^{2+}) around growing *Limnobium* root hairs made using non-invasive, ion-selective vibrating microelectrodes. *Arrows* directed towards the root hair denote influx whilst those directed away denote efflux. Panel **D** shows a close-up of the Ca^{2+} fluxes around a tip of a growing root hair. Background solute concentrations were $CaCl_2$, 200 µM, KCl 100 µM adjusted to pH 4.5 using HCl

small Ca^{2+} fluxes which often oscillated around a net zero flux. In contrast, proton efflux was seen along the entire root hair except for the root hair tip, where a net proton influx was observed. Only in one root hair, from 15 examined, was proton efflux from the tip observed. In older non-growing root hairs, Ca^{2+} influx at the root hair tip was not apparent, indicating that the large Ca^{2+} influx at the tip of growing root hairs may be integrally linked with the growth process. The pattern of H^+ fluxes in older root hairs was also markedly different from those observed in growing root hairs as a net H^+ influx (0.61 ± 0.07 pmol·cm^{-2}·s^{-1}) was seen over 90% of the root hair length. Fluxes of K^+ were not examined in older root hairs. All measured ionic fluxes were within the range 0–4 pmol·cm^{-2}·s^{-1}.

Detailed mapping of the Ca^{2+} fluxes around the root hair tip indicated that the influx of calcium was highly localized to the tip, with Ca^{2+} influx steadily decreasing with distance away from the site of growth (Fig. 3D). This is in agreement with results presented by Schiefelbein et al. (1992). A similar pattern of ion fluxes was also observed for H^+ influx into the root hair tip.

Effect of Al^{3+} on root hair ion fluxes. Inclusion of 20 µM Al^{3+} in the root-bathing solution did not significantly affect either the magnitude or spatial pattern of K^+ influx along the root hair (Fig. 4). It was estimated from analysis of homogenized root tissue that the internal root K^+ concentration was ≈95 mM. It can be expected therefore from thermodynamic predictions (where the E_m is − 185 mV) that K^+ uptake into root hairs can potentially utilize both high-(thermodynamically active) and low-affinity (passive) K^+-transport systems.

In contrast, the presence of Al^{3+} in the root-bathing solution significantly altered the pattern and magnitude of Ca^{2+} fluxes along the root hair, resulting in a near complete inhibition of Ca^{2+} influx at all root hair locations (Fig. 5). After removal of Al^{3+} from the root-bathing solution, the rate of Ca^{2+} influx at the root hair tip was

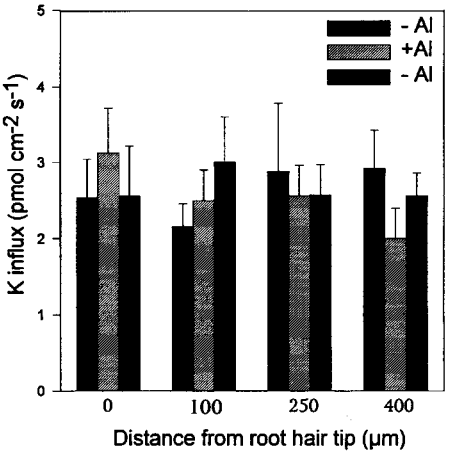

Fig. 4. Effect of exogenously applied Al (20 µM) on K^+ influx into growing *Limnobium* root hairs. Measurements of K^+ flux were initially made from a 100 µM KCl solution at four root hair locations over a period of 1 h (*left-hand column*). The root bathing solution was then replaced wth a 100 µM KCl solution containing 20 µM $AlCl_3$ and again K^+ fluxes measured at each of the root hair locations (*center column*). Finally, the Al was washed away from the root hair and K^+ fluxes again measured (*right-hand column*). All columns represent influx of K^+ into the root hair. Data are means + SE

again restored very rapidly to that observed before the addition of Al^{3+}, suggesting that Al^{3+} is acting at the outer face of the root hair plasma membrane.

As with K^+ fluxes, the immediate addition of Al to the root bathing medium did not significantly affect H^+ fluxes at any of the root hair locations over short periods of time (data not presented). Over longer periods of time, however, an apparent increase in H^+ influx was seen at the root hair tip which increased with time (Fig. 6). This stimulation of H^+ influx correlated with a swelling at the root hair tip (Fig. 7), which eventually resulted in the

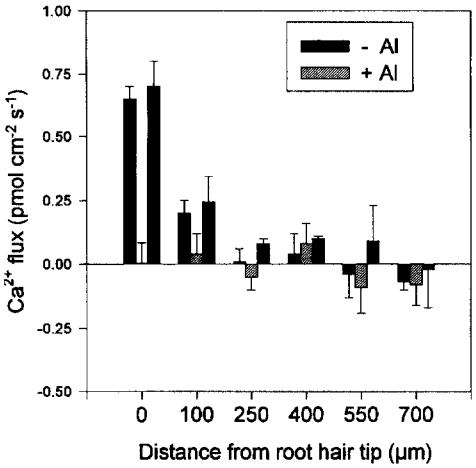

Fig. 5. Effect of exogenously applied Al (20 μM) on Ca^{2+} fluxes around growing *Limnobium* root hairs. Measurements of Ca^{2+} flux were initially made from a 200 μM $CaCl_2$ solution at six root hair locations over a period of 1 h (*left-hand column*). The root bathing solution was then replaced wth a 200 μM $CaCl_2$ solution containing 20 μM $AlCl_3$ and again Ca^{2+} fluxes measured at each of the root hair locations (*center column*). Finally, the Al was washed away from the root hair and Ca^{2+} fluxes again measured (*right-hand column*). Columns above the zero line denote influx whilst those beneath represent efflux. Data are means + SE

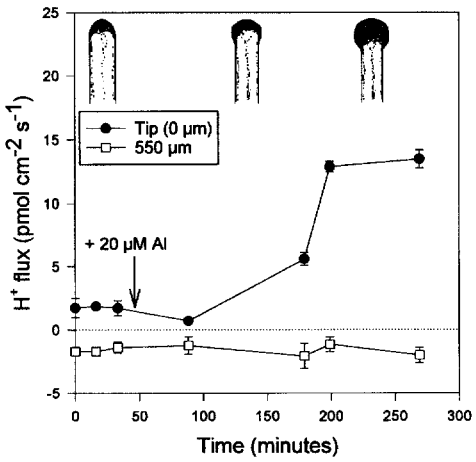

Fig. 6. Effect of exogenously applied Al (20 μM) on H^+ fluxes around growing *Limnobium* root hairs at two locations (0 and 550 μm back from the hair tip). Measurements of H^+ flux were made in a solution containing 100 μM $CaCl_2$, 100 μM KCl ± 20 μM $AlCl_3$ adjusted to pH 4.5 with HCl. Points above the zero line denote influx whilst those below denote efflux. Deformation changes in root hair appearance after the addition of Al are also shown at the top of the figure. All points are means ± SE

bursting of the root hair and a loss of cellular contents. No morphological or H^+ flux changes were seen at any other of the root hair locations (Fig. 6.).

Uncoupling of Ca^{2+} flux and growth. In order to investigate whether root hair growth rate was regulated by the magnitude of the Ca^{2+} flux into the root hair tip, Ca^{2+}

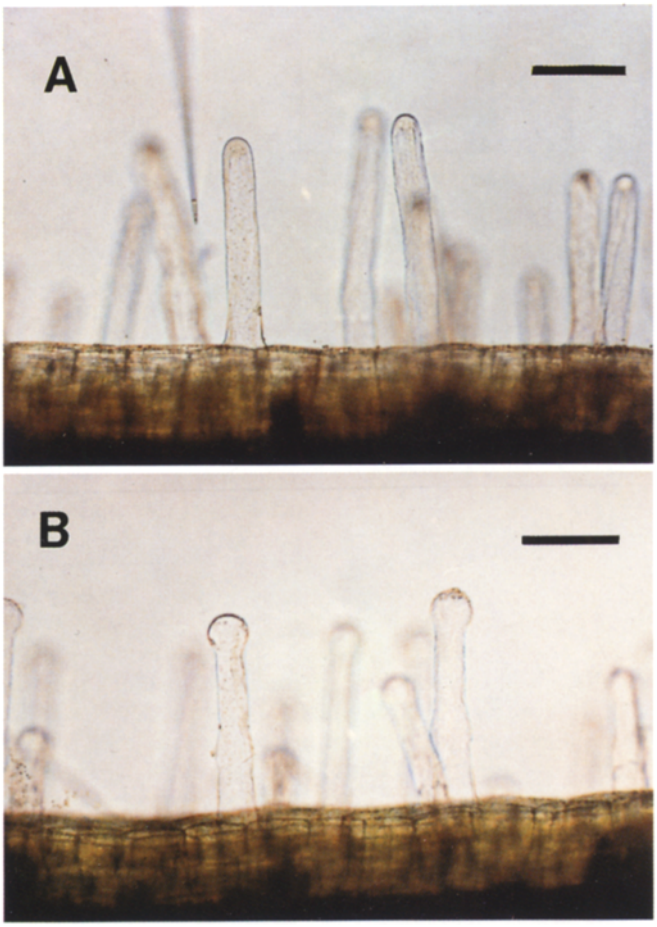

Fig. 7A, B. Photograph showing (**A**) normal *Limnobium* root hairs, and (**B**) root hairs exposed to aluminum (20 μM) for 6 h. The tip of the non-invasive vibrating microelectrode can be seen in **A**. The scale bar in the right-hand corner of both photographs represents a distance of 200 μm

influx was inhibited by increasing concentrations of Mg^{2+} in the bathing solution and root hair growth was simultaneously monitored. The results shown in Fig. 8 show that as expected, increasing Mg^{2+} concentrations significantly reduced the flux of Ca^{2+} into the root hair tip. In agreement with results presented by Huang et al. (1994), root cell plasma-membrane Ca^{2+} channels appeared to have a high selectivity for Ca^{2+}, as 50% inhibition of Ca^{2+} influx could only be achieved when Mg^{2+} was present at concentrations tenfold higher than that of Ca^{2+}. Despite a 90% inhibition of the root hair Ca^{2+} flux by 5 mM Mg^{2+}, the rate of root hair growth did not significantly change, clearly indicating that the magnitude of the Ca^{2+} flux does not directly determine growth rate. At elevated Mg^{2+} levels (20 mM) Ca^{2+} influx was totally abolished. This zero Ca^{2+} influx was accompanied by a complete cessation in growth and an accumulation of cellular components and vesicles at the root hair tip, leading eventually to a gelling of the cytoplasm or bursting of the root hair.

Uncoupling of Ca^{2+} flux and Al^{3+} rhizotoxicity. In order to assess whether Al^{3+}-induced inhibition of root hair

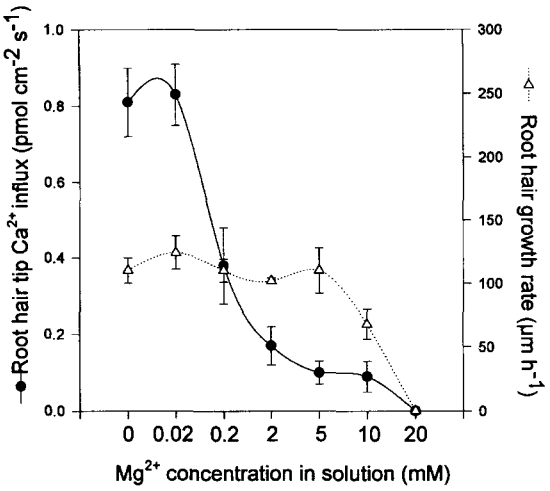

Fig. 8. Uncoupling of the root hair growth rate from the magnitude of the Ca^{2+} influx into the apex of growing *Limnobium* root hairs. The Ca^{2+} flux into the root hair was modified by the addition of various levels of Mg^{2+} (0–20 mM) to the root bathing solution which contained 100 μM KCl, 200 μM $CaCl_2$ at pH 5.8. All points are means ± SE

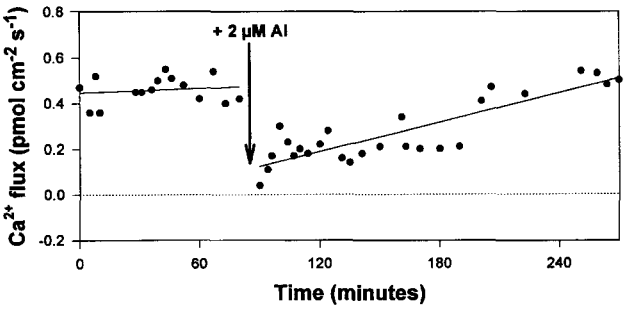

Fig. 9. Magnitude of the Ca^{2+} influx into *Limnobium* root hairs tips after exposure to low levels of Al (2 μM). A complete inhibition of growth occurred after 1 h of exposure to Al; however, Ca^{2+} influx into the tip recovered during this period, indicating that blockage of Ca^{2+} channels by Al is not the only site of Al toxicity. The background solution contained 20 μM $CaCl_2$ and 100 μM KCl at pH 4.5. A representative trace of only one experiment is shown here

growth was solely due to blockage of Ca^{2+} influx, lower Ca/Al concentrations (20 μM Ca^{2+} and 2 μM Al^{3+}) were used as this combination was also found to inhibit root growth. Monitoring of Ca^{2+} fluxes at the root hair tip before the application of Al^{3+} indicated that Ca^{2+} influx was relatively stable (0.45 ± 0.02 pmol·cm^{-2}·s^{-1}) over an initial 80-min period (Fig. 9). After the application of Al^{3+}, uptake of Ca^{2+} was immediately reduced; however, root hair growth did not cease completely for up to 1 h after Al^{3+} exposure. During the following 3 h in Al^{3+}, Ca^{2+} influx gradually recovered to a level comparable to that observed before the addition of Al^{3+}. No growth occurred during this final 2 h. The morphological changes in the root hair tip induced by Al^{3+} exposure over the course of this experiment were similar to those shown in Fig. 6.

Influence of Al exposure on root hair membrane potential (E_m). Root hair cells had a mean E_m of − 185 mV (range − 175 to − 230 mV) which remained stable throughout the experimental period. The presence of Al^{3+} had no affect on E_m (data not presented). Addition of K^+ to the bathing solution induced a depolarization of the E_m to − 135 mV, however, this did not affect the growth rate of root hairs.

Involvement of Ca^{2+} in the integrity of root hair tips. As the presence of Al^{3+} in solution often resulted in the swelling (and occasional bursting after long exposure) of the tips of young growing root hairs, the involvement of Ca^{2+} in root hair integrity was investigated. The results of transferring roots from solutions containing Ca^{2+} (200 μM, pH 4.5), into solutions containing either a mixture of cations or into solutions containing no Ca^{2+} are shown in Table 1. The results clearly indicate that at low pH, Ca^{2+} is required to maintain the integrity of root hair tips. On transferral of roots to solutions containing zero Ca^{2+}, root hairs burst almost instantaneously, with bursting always occurring within 50 μm of the root hair tip.

Table 1. Effect of the presence and absence of calcium in the external root bathing solution on the integrity and growth of *Limnobium* root hairs. All solutions were adjusted to pH 4.5 with HCl. Bursting was only observed at root hair tips

Solution composition (μM)					Root hair growth	Root hair tip bursting	Time to burst (min)
Ca	K	Mg	EGTA	Al			
200					Yes	No	
200	200				Yes	No	
200	200	2000			Yes	No	
200	200		2000		No	Yes	< 1
200				200	No	No	
0					No	Yes	< 1
0	200				No	Yes	≈ 10
0	200	2000			No	Yes	< 1
0	200		2000		No	Yes	< 1
0				200	No	No	

Surprisingly, it was found that $AlCl_3$ could substitute for Ca^{2+}, in that inclusion of Al^{3+} in a Ca^{2+}-free solution prevented the bursting of root hairs, thus maintaining tip integrity. As blebbing of the plasmalemma was not seen when the root tips burst (as occurs during cell wall disintegration; Taylor and Brownlee 1992), we speculate that loss of plasma-membrane integrity (and not cell wall integrity) was occurring. Non-growing root hairs and epidermal cells were never seen to swell or burst in response to low Ca^{2+} or to long exposure to Al^{3+}.

Discussion

The results presented here, clearly indicate that root hairs are capable of ion uptake, with the spatial and temporal pattern of uptake dependent on the particular ion in question. This is further supported by work of Toulemonde and Kochian (unpublished data) which showed an influx of NO_3^- along the entire length of tomato root hairs, using vibrating NO_3^--sensitive microelectrodes. The magnitude of the H^+, Ca^{2+} and K^+ fluxes observed here in *Limnobium* root hairs (0–4 pmol·cm^{-2}·s^{-1}) are similar to those observed at the root surface of *Limnobium*, maize and wheat roots (0–25 pmol·cm^{-2}·s^{-1}, Ryan et al. 1993; Kochian et al. 1992), indicating that root hairs do not possess heightened levels of transport activity at least under solution culture conditions.

An influx of protons (or efflux of OH^-/organic anions) was seen in >90% of the tips of growing root hairs in agreement with results presented for the root apices of many plant species (Miller and Gow 1989). Despite the fact that this H^+ influx could result in the formation of a transcellular electric field, there is still little evidence in plants that this H^+ influx is actually involved in tip growth and not simply a response to tip growth or enhanced metabolic activity in this region (e.g. loss of cytosolic solution during fusion of vesicles to the plasma membrane or efflux of CO_2). It has been postulated that a reduced H^+-ATPase activity in this newly formed region may be causing the apparent H^+ influx; however, there are no data to support this (Harold and Caldwell 1990). It is possible that H^+ influx at the tip produces a localized voltage gradient which may help to redistribute plasmalemma channel proteins (e.g. Ca^{2+}) to the growing tip region (Nuccitelli 1983). Hopefully, immunolocalization of ATPases and Ca^{2+}-channel proteins may help to finally answer this controversial topic. Alternatively, the localized proton influx may be involved in the generation of a cytosolic pH gradient along the root hair similar to that recently observed in *Pelvetia* embryos (gradient of 0.3 pH units tip to base; Gibbon and Kropf 1994). Although cytosolic acidification has been shown to be associated with increased cytosolic free Ca^{2+} in *Zea mays* root hairs (drop of 0.3 pH units associated with an increase in cytosolic Ca^{2+} from $\approx$100 nM to 130 nM; Felle 1988) the changes are small and are probably of little consequence. It is more likely that pH domains in the cytosol can regulate growth-related cellular processes such as microtubule assembly which is known to be highly pH dependent (Tiwari and Suprenant 1994).

It has been postulated that localized Ca^{2+} influx and an associated intracellular Ca^{2+} gradient are prerequisites for tip growth (see, for example, Picton and Steer 1983; Pierson et al. 1994). The observation that removal of Ca^{2+} from the root-bathing solution affects tip growth is not compelling evidence for this, since Ca^{2+} is obviously required for normal cell function and the removal of most macronutrients will inhibit growth over a period of days. As yet, the only evidence presented to suggest that Ca^{2+} influx and a subsequent Ca^{2+} gradient are required for growth, and not simply a symptom of growth (i.e. Ca^{2+} required for new cytosol), is that microinjection of 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid (BAPTA) buffers, which abolishes internal Ca^{2+} gradients, also prevents growth and inhibits tip-localized Ca^{2+} influx (Miller et al. 1992; Pierson et al. 1994). However, injected Ca^{2+} buffers such as BAPTA may also inhibit other cellular processes, including blockage of inositol(1,4,5)triphosphate (IP_3) receptors (Combettes and Champeil 1994), making the conclusions drawn from such studies more difficult to interpret. Although it is clear from Fig. 8 that the magnitude of the Ca^{2+} flux into the root hair tip does not control growth rate, the fact that growth stopped when Ca^{2+} influx was completely abolished, may indicate that a nominal Ca^{2+} flux is required for growth. Further, observations made in our laboratory on root hair proliferation in wheat suggest that hairs often only form after root exposure to the air, where reserves of external calcium are almost negligible.

If it is considered in quantitative terms, the net Ca^{2+} flux at growing root hair tips (0.8 pmol·cm^{-2}·s^{-1}; Fig. 8) can be estimated to be 7.4 fmol Ca^{2+}·tip^{-1}·min^{-1} assuming a tip surface area of 15400 μm^2 and that all Ca^{2+} influx occurs within 35 μm from the apex. If the background cytoplasmic free-Ca^{2+} concentration is assumed to be in the range of 170 nM (Miller et al. 1992), and the tip volume where Ca^{2+} influx is occurring to be 1.8×10^5 μm^3, then the amount of background free Ca^{2+} contained within the tip is 0.031 fmol·tip^{-1}. This estimate of tip Ca^{2+} content is almost certainly higher than the actual value, as the cytosolic volume is overestimated in this calculation due to a lack of consideration of the volume of endomembrane-enclosed compartments (e.g. vacuole and vesicles). Therefore, at least 240 times more Ca^{2+} enters the tip every minute than exists normally. Thus it can be expected that Ca^{2+} concentrations in the unstirred layer of cytoplasm close to the plasma membrane will be extremely high compared with those existing in the bulk cytoplasm which is both mixed by rapid cytoplasmic streaming (starts 30 μm back from tip at a rate of $\approx$240 μm·min^{-1}) and is regulated by Ca^{2+} influx into cellular organelles. As suggested by calculations made here, and as stated by Miller et al. (1992) and Pierson et al. (1994) for pollen tubes, estimates of Ca^{2+} concentrations at the terminal end of tip-growing cells made with fluorescent dyes may underestimate the actual values due to technical limitations.

It has been clearly shown that vesicle fusion is often enhanced in the presence of high calcium (Zorec and Tester 1992; Wollheim and Lang 1994) indicating that calcium influx at the root hair tip may provide a Ca^{2+}

'hot spot' for vesicle fusion. However, the situation is possibly more complex in that other authors have identified a myriad of cytoplasmic factors and proteins which are also required for fusion, many of which are Ca^{2+} dependent (Gomperts 1990; Creutz 1992; White 1992). Thus, Ca^{2+} may directly or indirectly mediate membrane fusion. To further complicate this situation, in some cases, cytosolic Ca^{2+} appears to have no role in vesicle fusion. For example, Knoll et al. (1993), Fray et al. (1987) and White (1992) have demonstrated that in some animal systems, no Ca^{2+} influx is required for vesicle fusion.

Probably, Ca^{2+} is entering the cell via voltage-gated Ca^{2+} channels (Huang et al. 1994), but the significance of this flux in directing hair growth is still far from clear. It is known that microtubule disassembly can be stimulated by high cytoplasmic [Ca^{2+}] due to Ca^{2+}-activated proteases (>100 μM; Kiehart 1981). Influx may therefore allow both unhindered vesicle passage to the plasma membrane and also temporally inhibit microtubule assembly. This is further supported by immunoflourescence studies of *Limnobium* root hairs which show a random array of microtubules at the hair tip which become progressively more ordered with distance from the fusion site (Emons 1987). Thus, the Ca^{2+} gradient per se may not be important in determining growth but there may be an obligatory requirement for high Ca^{2+} at the internal membrane face, with the gradient just a symptom of cytosolic regulation and diffusion away from the fusion site.

That the presence of Ca^{2+} at the external face of the membrane is needed for tip growth was clearly demonstrated here, as removal of Ca^{2+} from the external face by either addition of EGTA or by washing with other cations resulted in rapid bursting of root hairs (Table 1). As bursting was only seen at the tips of growing root hairs, it suggests that a nominal amount of external Ca^{2+} is required to mediate and stabilize vesicle fusion, probably through the stabilization of phospholipid bonds between newly fused vesicles (Deleers et al. 1986). Additionally, external Ca^{2+} may be needed at the tip for proper formation of new cell wall material. The fact that Ca^{2+} could effectively be replaced by Al^{3+}, which is known to significantly increase membrane rigidity (Vierstra and Haug 1978), supports this hypothesis. A recent study by Przybylski et al. (1994) in chick embryo pectoral muscle also showed that external Ca^{2+} concentrations ≥ 200 μM were required on the external face of the membrane in order to promote normal rates of vesicle fusion, whilst studies in *Paramecium* by Plattner et al. (1985) and Kerboeuf and Cohen (1990) indicated that external Ca^{2+} concentrations >0.3 μM were required to stimulate fusion.

With regards to aluminum toxicity in roots, it is clear from these studies with non-dividing cells that Al^{3+} can inhibit cell growth by blocking elongation. The mechanism through which Al^{3+} is acting, however, is still unclear. It can be expected that at high external Al^{3+} concentrations, blockage of the voltage-gated Ca^{2+} channels will prevent Ca^{2+} uptake and thereby dissipate the cytoplasmic Ca^{2+} gradient which may be important in influencing vesicle fusion and microtubule structure. The fact that Al^{3+} applied at low concentrations permits Ca^{2+} influx to proceed, but prevents cell elongation, indicates that growth inhibition is not caused solely by inhibition of Ca^{2+} influx and the associated cytoplasmic Ca^{2+} gradient. The swelling of the root hair tips following exposure to low levels of Al^{3+} (2 μM) is visually identical to swelling of root hairs exposed to chemical agents that disrupt microtubule structure suggesting, quite speculatively, that Al^{3+}-mediated microtubule depolymerization may be involved in growth inhibition.

Evidence now suggests that Al^{3+} can enter cells within minutes (Lazof et al. 1994; Kochian 1995); however, the subsequent chemical changes that Al^{3+} undergoes after entering a high-pH environment such as the cytosol can only be speculated upon. From chemical equilibria studies considering inorganic salts only, it would be expected that Al^{3+}, if that is indeed the form which enters the cell, would immediately precipitate to $Al(OH)_3$ leaving very low levels of free Al^{3+} in solution ($<10^{-10}$ M). In reality, however, Al^{3+} will probably become chelated to organic ligands such as citrate which are present in the cytoplasm at high concentrations and have been shown to readily chelate Al^{3+} in blood serum (Van Ginkel et al. 1990). Studies using carcimoma cells suggests that lactate-bound Al, which is stable at pH 7.4, allows the reaction of Al^{3+} with internal membranes to occur, thus changing the Ca^{2+}-transport properties of the cell (Anghileri 1992). This potential reaction of cytosolic organically bound-Al^{3+} with internal plant membranes and Ca^{2+} stores in plants requires further investigation. It is also possible that Al in the cytoplasm may irreversibly bind to critical sites in proteins, thus disrupting functionality of these proteins. The most likely candidates for disruption include protein kinases and components of the phosphoinositide (IP_3) pathway (Biffen and Hanke 1990; Shi et al. 1993). It had been speculated that Al^{3+}/calmodulin interactions could be important in Al toxicity (Siegel and Haug 1983), but this has been shown more recently to be of little consequence (You and Nelson 1991). Further sites of internal disruption may also include interactions between compounds containing exposed $-PO_4$ groups and Al^{3+} (Deleers et al. 1986).

Despite recent evidence by Gassman and Schroeder (1994) suggesting that Al^{3+} toxicity may be due to blockage of K^+ channels, evidence presented here clearly suggests that roots can bypass the need for K^+ channels through the operation of a high-affinity transport system. It is likely that K^+-channel blockage forms a very minor component of Al phytotoxicity.

The field of Al phytotoxicity is awash with theories for the cause and site of toxicity. The fact that Al^{3+} appears to be able to mimic to some extent Ca^{2+}, which appears to be involved in most cellular events at some level, clearly complicates the issue. Evidence presented here indicates that Al^{3+} is acting at multiple levels (i.e. by blockage of Ca^{2+} channels at higher [Al^{3+}] and possibly by interacting with microtubule assembly at low [Al^{3+}]), clearly suggesting that there is no exclusive answer to the nature of Al toxicity and that the higher the external Al^{3+} concentration and longer the exposure, the greater the number of cellular functions affected.

References

Anghileri LJ (1992) Ehrlich tumour cells: Ca^{2+} -uptake modification by aluminium lactate. Cell Calcium 13: 277–279

Barley KP, Rovira AD (1970) The influence of root hairs on the uptake of phosphate. Soil Sci Plant Anal 1: 287–292

Biffen M, Hanke DE (1990) Polyphosphoinositidase activity in soybean membranes is Ca^{2+} dependent and shows no requirement for guanine-nucleotides. Plant Sci 69: 147–155

Combettes L, Champeil P (1994) Calcium and inositol 1,4,5-triphosphate-induced Ca^{2+} release. Science 265: 813

Creutz CE (1992) The annexins and exocytosis. Science 258: 924–931

Deleers M, Servais JP, Wülfert E (1986) Neurotoxic cations induce membrane rigidification and membrane fusion at micromolar concentrations. Biochim Biophys Acta 855: 271–276

Delhaize E, Ryan PR, Randall PJ (1993) Aluminum tolerance in wheat (*Triticum aestivum* L.). II. Aluminum-stimulated excretion of malic acid from root apices. Plant Physiol 103: 695–702

Emons AM (1987) The cytoskeleton and secretory vesicles in root hairs of *Equisetum* and *Limnobium* and cytoplasmic streaming in root hairs of *Equisetum*. Ann Bot 60: 625–632

Felle H (1988) Cytoplasmic free calcium in *Riccia fluitans* L. and *Zea mays* L.: Interaction of Ca^{2+} and pH? Planta 176: 248–255

Foy CD, Chaney RL, White MC (1978) The physiology of metal toxicity in plants. Annu Rev Plant Physiol 29: 511–566

Fray JCS, Park CS, Valentine AND (1987) Calcium and the control of renin secretion. Endocrinol Rev 8: 53–93

Gassman W, Schroeder JI (1994) Inward-rectifying K^+ channels in root hairs of wheat: A mechanism for aluminum-sensitive low-affinity K^+ uptake and membrane potential control. Plant Physiol 105: 1399–1408

Gibbon BC, Kropf DL (1994) Cytosolic pH gradients associated with tip growth. Science 263: 1419–1421

Gomperts BD (1990) GE: a GTP-binding protein mediating exocytosis. Annu Rev Physiol 52: 591–606

Harold FM, Caldwell JH (1990) Tips and currents: Electrobiology of apical growth. In: Heath IB (ed) Tip growth in plant and fungal cells. Academic Press, Orlando, Fla, pp 59–90

Heath IB (1990) Tip growth in plant and fungal cells. Academic Press, Orlando, Fla

Huang JW, Shaff JE, Grunes DL, Kochian LV (1992) Aluminum effects on calcium fluxes at the root apex of aluminum-tolerant and aluminum-sensitive wheat cultivars. Plant Physiol 98: 230–237

Huang JW, Grunes DL, Kochian LV (1994) Voltage-dependent Ca^{2+} influx into right-side-out plasma membrane vesicles isolated from wheat roots: Characterization of a putative Ca^{2+} channel. Proc Natl Acad Sci USA 91: 3473–3477

Kerboeuf D, Cohen J (1990) A Ca^{2+} influx associated with exocytosis is specifically abolished in a *Paramecium* exocytotic mutant. J Cell Biol 111: 2527–2535

Kiehart DP (1981) Studies on the in vivo sensitivity of spindle microtubles to calcium ions and evidence for a vesicular calcium-sequestering system. J Cell Biol 88: 604–617

Knoll G, Grässle A, Braun C, Probst W, Höhne-Zell B, Plattner H (1993) A calcium influx is neither strictly associated with nor necessary for exocytotic membrane fusion in *Paramecium* cells. Cell Calcium 14: 173–185

Kochian LV (1995) Cellular mechanisms of aluminum toxicity and resistance in plants. Annu Rev Plant Physiol Plant Mol Biol 46: 237–260

Kochian LV, Shaff JE, Kühtreiber WM, Jaffe LF, Lucas WJ (1992) Use of an extracellular ion-selective, vibrating microelectrode system for the quantification of K^+, H^+, and Ca^{2+} fluxes in maize roots and maize suspension cells. Planta 188: 601–610

Kühtreiber WM, Jaffe LF (1990) Detection of extracellular calcium gradients with a calcium-specific vibrating electrode. J Cell Biol 110: 1563–1573

Lazof DB, Goldsmith JG, Rufty TW, Linton RW (1994) Rapid uptake of aluminum into cells of intact soybean root tips: A microanalytical study using secondary ion mass spectrometry. Plant Physiol 106: 1107–1114

Miller AL, Gow NAR (1989) Correlation between profile of ion-current circulation and root development. Physiol Plant 75: 102–108

Miller DD, Callaham DA, Gross DJ, Hepler PK (1992) Free Ca^{2+} gradient in growing pollen tubes of *Lilium*. J Cell Sci 101: 7–12

Nuccitelli R (1983) Transcellular ion currents: Signals and effectors of polarity. Mod Cell Biol 2: 451–481

Parker DR, Chaney RL, Norvell WA (1995) GEOCHEM-PC: A chemical speciation program for IBM and compatible computers. In: Loeppert RH (ed) Chemical equilibria and reaction models. Soil Science Society of America, Madison, Wis, pp 253–270

Pellet DM, Grunes DL, Kochian LV (1995) Organic acid exudation as an aluminum-tolerance mechanism in maize (*Zea mays* L.). Planta 196: 788–795

Picton JM, Steer MW (1983) Evidence for the role of Ca^{2+} ions in tip extension in pollen tubes. Protoplasma 115: 11–17

Pierson ES, Miller DD, Callaham DA, Shipley AM, Rivers BA, Cresti M, Hepler PK (1994) Pollen tube growth is coupled to extracellular calcium gradient: Effect of BAPTA-type buffers and hypertonic media. Plant Cell 6: 1815–1828

Plattner H, Stürzl R, Matt H (1985) Synchronous exocytosis in *Paramecium* cells. IV. Polyamino compounds as potent trigger agents for repeatable trigger-redocking sites. Eur J Cell Biol 36: 32–37

Przybylski RJ, Szigeti V, Davidheiser S, Kirby AC (1994) Calcium regulation of skeletal myogenesis. II. Extracellular and cell surface effects. Cell Calcium 15: 132–142

Ryan PR, Kochian LV (1993) Interaction between aluminum toxicity and calcium uptake at the root apex in near-isogenic lines of wheat (*Triticum aestivum* L.) differing in aluminum tolerance. Plant Physiol 102: 975–982

Ryan PR, Di Tomaso JM, Kochian LV (1993) Aluminum toxicity in roots: An investigation of spatial sensitivity and the role of the root cap. J Exp Bot 44: 437–446

Schiefelbein JW, Shipley A, Rowse P (1992) Calcium influx at the tip of growing root-hair cells of *Arabidopsis thaliana*. Planta 187: 455–459

Shi B, Chou K, Haug A (1993) Aluminum impacts elements of the phosphoinositol signalling pathway in neuroblastoma cells. Mol Cell Biochem 121: 109–118

Smith JS, Sanger RH, Jaffe LF (1994) The vibrating Ca^{2+} electrode: A new technique for detecting plasma membrane regions of Ca^{2+} influx and efflux. Methods Cell Biol 40: 115–134

Steer MW (1988) The role of calcium in exocytosis and endocytosis in plant cells. Physiol Plant 72: 213–220

Steer MW, Steer JM (1989) Pollen tube tip growth. New Phytol 111: 323–358

Taylor AR, Brownlee C (1992) Localised patch clamping of plasma membrane of a polarized plant cell: Laser microsurgery of the *Fucus spiralis* rhizoid cell wall. Plant Physiol 99: 1686–1688

Van Ginkel MF, Van der Voet GB, Van Eijk HG, Wolff FA (1990) Aluminum binding to serum constituents: A role for transferrin and for citrate. J Clin Chem Clin Biochem 28: 459–464

Vierstra R, Haug A (1978) The effects of Al^{3+} on the physical properties of membrane lipids in *Thermoplasma acidophilum*. Biochem Biophys Res Commun 84: 138–143

White JM (1992) Membrane fusion. Science 258: 917–924

Wollheim CB, Lang J (1994) A game plan for exocytosis. Trend Cell Biol 4: 339–341

You G, Nelson DJ (1991) Aluminum ion vs. calcium ion binding to methionine and tyrosine spin-labelled bovine brain calmodulin. J Inorg Biochem 41: 283–292

Zorec R, Tester M (1992) Cytoplasmic calcium stimulates exocytosis in a plant secretory cell. Biophys J 63: 864–867