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Calcium influx at the tip of growing root-hair cells of *Arabidopsis thaliana*

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Abstract. The role of extracellular Ca^{2+} in root-hair tip growth has been investigated in *Arabidopsis thaliana* (L.) Heynh. Root-hair length was found to be dependent on the concentration of Ca^{2+} in the growth medium, with maximum length achieved at $[Ca^{2+}]$ of 0.3–3.0 mM. Using a non-intrusive calcium-specific vibrating microelectrode, an extracellular Ca^{2+} gradient was detected at the tips of individual growing root-hair cells. The direction of the gradient indicated a net influx of Ca^{2+} into root-hair cells. No gradient was detected near the sides of the root hairs or at the tips of non-growing root hairs. When root hairs were exposed to the Ca^{2+}-channel blocker nifedipine, tip growth stopped and the extracellular Ca^{2+} gradient was abolished. These results indicate that Ca^{2+} influx through plasma-membrane Ca^{2+} channels is required for normal root-hair tip growth.

Key words: *Arabidopsis* (calcium and root-hair growth) – Calcium channel – Root hair – Tip growth – Vibrating probe, Ca^{2+}-specific

Introduction

Calcium ions play a key role in many cellular processes in higher plants, including cell division, cell expansion, and cytoplasmic streaming (for reviews, see Hepler and Wayne 1985; Kauss 1987). The importance of Ca^{2+} in plant cell expansion has been particularly well-documented in studies of tip-growing cells, such as pollen tubes and root hairs. These cells are suitable for studying cell expansion because they are easily accessible, grow rapidly, and expansion is limited to a specific region of the cell (for reviews, see Schnepf 1986; Heath 1990).

The importance of Ca^{2+} in pollen-tube tip growth has been demonstrated by several lines of evidence. Growth of pollen tubes in vitro requires Ca^{2+} in the medium at a concentration of 10^{-2}–10^{-6} M (Brewbaker and Kwack 1963; Picton and Steer 1983). When growing pollen tubes are subjected to a sudden decrease in the external Ca^{2+} concentration or to Ca^{2+}-channel blockers (e.g. nifedipine), the tubes display a sharply reduced growth rate and form swellings at the tip of the tube (Reiss and Herth 1985). In addition, growing pollen tubes rapidly accumulate extracellular ^{45}Ca at the tip region (Jaffe et al. 1975). A variety of methods have been used to detect and measure an internal Ca^{2+} gradient in growing pollen tubes, with the highest Ca^{2+} concentration at the tip (Reiss and Herth 1978; Reiss et al. 1983, 1985; Reiss and Nobiling 1986; Nobiling and Reiss 1987). Electrophysiological studies with an extracellular vibrating microelectrode have shown that ion currents flow into the tip of the growing pollen tube (Jaffe and Nuccitelli 1974; Weisenseel et al. 1975), and the recent development of a calcium-specific vibrating probe has led to the demonstration that a large portion of the inward ion current is carried by Ca^{2+} (Kuhtreiber and Jaffe 1990).

Although root-hair elongation is morphologically similar to pollen-tube growth, relatively little is known about the physiology of root-hair growth or the role of Ca^{2+} in this process. Root-hair length has been shown to be sensitive to the concentration of Ca^{2+} in the growth medium, indicating that extracellular Ca^{2+} is necessary for elongation (Cormack 1949; Burström 1952; Tanaka and Woods 1972, 1973; Ewens and Leigh 1985). In addition, an internal tip-to-base Ca^{2+} gradient has been detected in growing root hairs by using the fluorescent Ca^{2+} indicators chlorotetracycline (for membrane-bound calcium; Reiss and Herth 1979) and fura-2 AM (for cytoplasmic calcium; Clarkson et al. 1988). Finally, ion currents entering growing root-hair tips have been detected (Weisenseel et al. 1979; Miller et al. 1986), although the contribution of Ca^{2+} to the currents is not known.

Recently, root-hair development has attracted attention as a potentially useful model system for studying plant cell expansion. This has been, in part, a conse-

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Abbreviation: APW = artificial pond water

quence of the realization that molecular-genetic approaches can be applied to this process (Schiefelbein and Somerville 1990). However, in order to interpret the results of molecular-genetic experiments fully, there is a need for a better understanding of the physiology of root-hair development. With this goal in mind, we have examined the role of external Ca^{2+} on root-hair growth in *Arabidopsis thaliana*. In this paper, we show that root-hair elongation in *Arabidopsis* is sensitive to the concentration of external Ca^{2+} and to the Ca^{2+}-channel blocker nifedipine. In addition, we have used a calcium-specific vibrating microelectrode (Kuhtreiber and Jaffe 1990) to detect extracellular Ca^{2+} gradients around individual root-hair cells.

Material and methods

Plant material and growth conditions. Seeds of *Arabidopsis thaliana* (L.) Heynh. (ecotype Columbia; source: G. Redei, University of Missouri, Columbia, USA) were surface-sterilized, and grown on agarose-solidified media under continuous illumination, as described in Schiefelbein and Somerville (1990). The growth medium used in these studies, artificial pond water (APW), consisted of 0.1 mM KCl, 0.1 mM $CaCl_2$, 0.1 mM $MgCl_2$, 0.5 mM NaCl, 1 mM 2(N-morpholino)ethanesulfonic acid (pH 5.6), and 0.2 mM Na_2SO_4.

To examine the effect of various external Ca^{2+} concentrations, seeds were distributed on modified APW media containing different amounts of $CaCl_2$ and solidified with 0.6% agarose. External Ca^{2+} concentrations below 0.1 mM were controlled by using a Ca^{2+}-EGTA (ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraaceticacid) buffer system (Chang et al. 1988). Agarose was used as the solidifying agent rather than agar, because agar was found to contain appreciable levels of Ca^{2+} as well as an unknown contaminant that inhibits root-hair growth (data not shown). Root-hair length was measured 4 d after seed germination, using hairs that developed from mature portions of the root in contact with the agarose surface.

Vibrating-probe experiments. The vibrating-microelectrode studies were conducted at the National Vibrating Probe Facility, Marine Biological Laboratories, Woods Hole, Mass., USA. The construction and use of the calcium-specific electrode has been described by Kuhtreiber and Jaffe (1990). In the present experiments, seeds were placed on APW medium containing 0.3 mM $CaCl_2$ solidified with 1.5% agarose and incubated in a horizontal orientation. At this agarose concentration, most roots grow along the surface of the medium and do not penetrate into the latter. After 4–6 d of growth, 30 ml of APW liquid medium was applied over the surface of the solid medium. After 1 h, the entire Petri dish was placed on the microscope stage and subjected to vibrating-probe analyses. In the standard experiments, the calcium-specific microelectrode was vibrated perpendicular to the cell surface and positioned such that it was approx. 3 μm away from the root-hair surface at its nearest vibration point. Since the excursion value used in these experiments was 9.6 μm, the probe vibrated between a point approx. 3 μm from the surface of the root hair to a point approx. 12.6 μm from the surface of the root hair. The reference values were obtained by vibrating the probe at positions at least 100 μm away from the root-hair tip. The growth of the root hairs during the vibrating-probe measurements was monitored by time-lapse video-tape recordings.

Nifedipine treatments. A 10 mM nifedipine (Sigma Chemical Co., St. Louis, Mo., USA) stock solution was diluted into the APW liquid medium in the Petri dishes to a final concentration of 100 μM. The growth-rate and Ca^{2+}-gradient measurements were made on individual root hairs at various timepoints from 20 min before to 120 min after the introduction of nifedipine. In the intervals between measurements, the Petri dishes were covered with aluminum foil to protect the nifedipine, which is light-sensitive. Some of the Petri plates containing nifedipine-treated plants were subsequently uncovered, placed under the growth lights for 6 h and then compared with covered, control plants.

Results

Effect of external Ca^{2+} on root-hair length. To examine the effect of external Ca^{2+} on the growth of root hairs of *Arabidopsis*, seedlings were grown on agarose-solidified APW media containing free-Ca^{2+} concentrations from 0.001 to 10 mM. The results of this dose-response experiment are shown in Fig. 1. Root-hair length increased as the external Ca^{2+} concentration was increased from 0.003 to 0.3 mM. Maximum root-hair length of approx. 0.8 mm was obtained at Ca^{2+} concentrations between 0.3 and 3 mM.

The effects of low Ca^{2+} concentrations on root-hair length were shown to be attributable to the reduction in Ca^{2+} and not Cl^-. No change in root-hair length was observed when the Cl^- concentration was reduced by changing the NaCl concentration in the APW medium from 0.5 mM to 0.3 mM (data not shown). Furthermore, similar reductions in root-hair length have been observed when *Arabidopsis* plants were grown on other plant growth media deprived of Ca^{2+} (data not shown).

Although the length of root hairs was affected by changes in the external Ca^{2+} concentration, there was no noticeable effect on root-hair cell shape. However, root growth was affected by the Ca^{2+} treatments of 0.010, 0.003, and 0.001 mM. Under these conditions, the roots were shorter than normal and appeared to be unable to respond normally to gravity (data not shown).

Vibrating-probe analyses. The studies described above indicated that the elongation of *Arabidopsis* root hairs requires extracellular Ca^{2+}. To examine the possibility that external Ca^{2+} is taken up by growing root hairs, we used a calcium-specific vibrating microelectrode (Kuh-

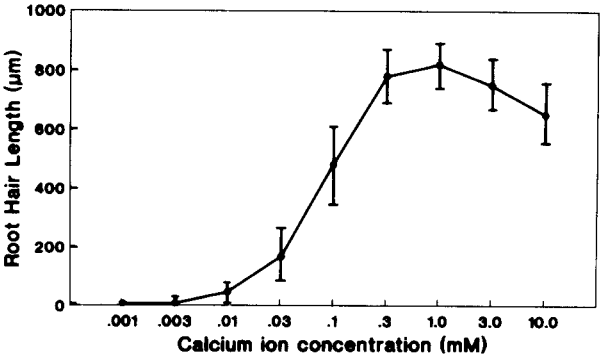

Fig. 1. Effect of external Ca^{2+} on root-hair length in *Arabidopsis*. Seedlings were grown in modified APW medium containing various concentrations of $CaCl_2$, and root-hair length was measured after 4 d of growth. A Ca^{2+}-EGTA buffer system was used for Ca^{2+} concentrations below 0.1 mM. For each datum point, $n = 40$

Table 1. Calcium-specific ion flux at the tips of growing and non-growing root hairs of *Arabidopsis*[a]

Root-hair type	Growth rate ($\mu m \cdot min^{-1}$)	Voltage (μV)	Ca^{2+} flux[b] ($pmol \cdot cm^{-2} \cdot s^{-1}$)
Growing hairs ($n=28$)	1.3 ± 0.3	11.2 ± 1.2	4.4 ± 0.5
Fully mature hairs ($n=21$)	0.1 ± 0.2	0.8 ± 0.8	0.3 ± 0.3
Hairs that stopped growing during examination ($n=8$)	0.2 ± 0.2	0.7 ± 0.6	0.2 ± 0.2

[a] Measurements include ± SE
[b] Vibrating-probe measurements were made by positioning the probe 3 μm in front of the root-hair tip and vibrating parallel to the growth axis

treiber and Jaffe 1990) in attempts to detect extracellular Ca^{2+} gradients around individual root hairs. Because the probe is noninvasive, it was possible to measure the root-hair growth rate and Ca^{2+} gradients simultaneously. The Ca^{2+} concentration used in these studies (0.3 mM) was chosen as a compromise between the desire to allow maximum root-hair elongation and the need to maintain the Ca^{2+} concentration as low as possible to obtain maximum sensitivity of the calcium-probe measurements (for further discussion, see Kuhtreiber and Jaffe 1990).

In the first set of experiments, the calcium probe was positioned directly in front of tips of individual root hairs and vibrated parallel to the axis of growth. When growing root hairs were examined in this manner, a Ca^{2+}-specific voltage difference was detected, indicating a difference in the concentration of Ca^{2+}, and therefore a Ca^{2+} concentration gradient, between the two points of vibration (Table 1). The voltage difference reflected a lower Ca^{2+} concentration at the vibration point closer to the root-hair tip, implying that Ca^{2+} was removed from the liquid media by the growing cells.

When growing root hairs of different lengths (from 50 to 500 μm) were examined, no significant difference was detected in the magnitude of the Ca^{2+} gradients or in the rate of hair elongation. Under these experimental conditions, root hairs of *Arabidopsis* were found to elongate at a relatively constant rate of approx. 1.3 $\mu m \cdot min^{-1}$ (Table 1).

A Ca^{2+} gradient was not detected when the tips of non-growing root hairs (mature hairs) were examined with the vibrating probe (Table 1). In addition, there were several hairs that had stopped growing (reached their mature length) during the period of time they were being examined. In each of these cases, the cessation of growth was accompanied by a loss of the extracellular Ca^{2+} gradient (Table 1).

Extracellular Ca^{2+} gradients were examined in more detail by mapping the gradients around the tip and side walls of individual growing root hairs. The position of the probe varied in each of these measurements, but the direction of vibration was always perpendicular to the nearest cell surface. The results of this analysis showed that the Ca^{2+} gradient is steepest at the root-hair tip and is not detectable at the root-hair side walls (Fig. 2). The same results were obtained from a different set of experiments in which the calcium probe was vibrated in a single position and the root hair was allowed to "grow" past it (Fig. 3). In these experiments, the probe was vibrated perpendicular to the axis of growth and positioned approx. 8 μm from the center of the tip (at its nearest vibration point) to prevent the growing hair from colliding with the probe. Because of this arrangement, the distance between the calcium probe and different sites on the root-hair cell surface was not constant. Therefore, the magnitude of the Ca^{2+} currents detected near the root-hair tip (position 0) was an underestimate of the true relative value. Nevertheless, the results of these two sets of experiments indicate that Ca^{2+} is removed from the

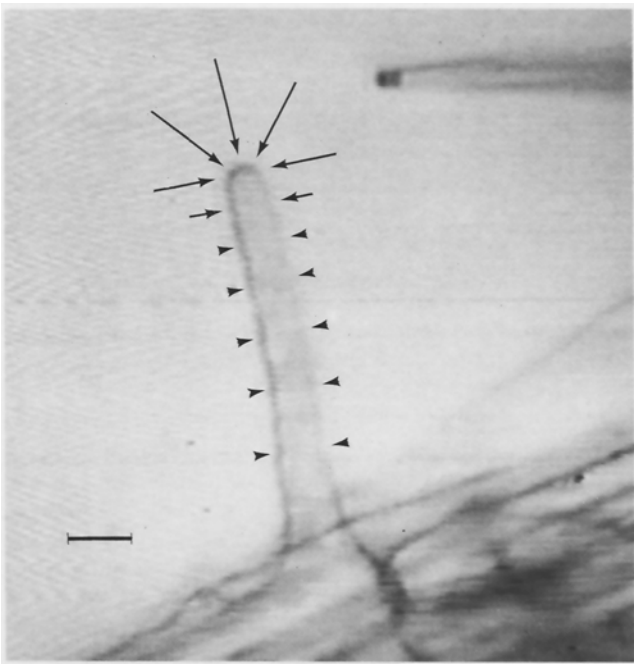

Fig. 2. Calcium gradients around growing root hairs of *Arabidopsis*. The relative Ca^{2+}-flux measurements at various positions around growing root hairs are indicated by the lengths of the arrows. The direction of the Ca^{2+} gradient is indicated by the direction of the arrow (from a site of high Ca^{2+} to low Ca^{2+}). Arrowheads mean that no significant Ca^{2+} flux was detected. At each position, the calcium-specific probe was vibrated perpendicular to the cell surface and at a distance of 3–12.6 μm from the surface. For each probe position, $n=12$. The probe is shown at the upper right portion of the figure. X400; bar = 10 μm

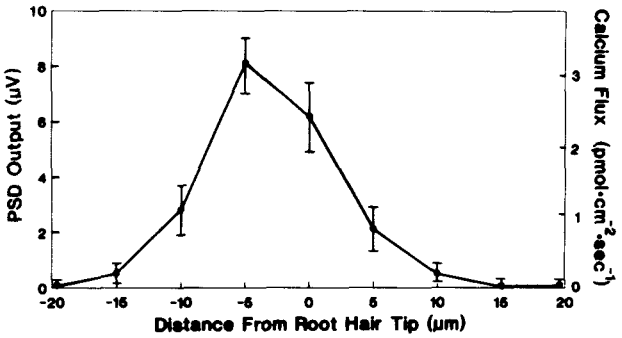

Fig. 3. Calcium-ion flux at various positions relative to the tip of an *Arabidopsis* root hair. The calcium probe was vibrated at a fixed position and the root hair was allowed to grow "past" the probe. The distances with a positive value are in front of the hair tip and those with a negative value are behind the tip (along the side wall of the cell). Because of the design of this experiment, the distance from the probe to the root-hair cell wall is not constant at all positions. *PSD* = phase-sensitive detection

media by the tips of growing root-hair cells. In neither of these experiments was a Ca^{2+} gradient detected along the root-hair surface that might indicate a net Ca^{2+} efflux from the root-hair.

The vibrating probe was also positioned near epidermal cells in the hair-forming zone of the root to try to detect Ca^{2+} gradients that might precede the emergence of root hairs. No marked Ca^{2+} gradient was detected along the length of these epidermal cells. Calcium-ion gradients were only detected when the probe was placed near the portion of an epidermal cell that had already produced a noticeable "bulge", i.e. had initiated a root hair.

Effect of the Ca^{2+}-channel blocker nifedipine. To examine the effect of an organic Ca^{2+}-channel blocker on root-hair growth and the observed Ca^{2+} gradients, *Arabidopsis* seedlings were treated with nifedipine (100 μM). Nifedipine acts by binding to plasma-membrane Ca^{2+} channels to prevent Ca^{2+} entry into cells (Reiss and Herth 1985). One hour after the introduction of nifedipine, root-hair elongation ceased and the extracellular Ca^{2+} gradient was abolished (Table 2).

The nifedipine treatments also caused changes in the shape of the root hairs; the most common abnormality was the formation of an enlarged tip region (Fig. 4). After longer exposures to nifedipine, some hairs also formed branches. These morphological changes were

Table 2. Effect of nifedipine on growth and calcium-specific ion flux in root hairs of *Arabidopsis*[a]

Time after nifedipine treatment (min)	Growth rate (μm · min^{-1})	Ca^{2+} flux[b] (pmol · cm^{-2} · s^{-1})
0	1.4 ± 0.3	4.1 ± 0.3
5	1.2 ± 0.3	3.8 ± 0.4
60	0.1 ± 0.2	0.2 ± 0.4

[a] Measurements were made on the same root hairs before and after treatment with 100 μM nifedipine ($n = 14$) and include ± SE

[b] Calcium flux was determined by positioning the probe 3 μm in front of the root-hair tip and vibrating parallel to the growth axis

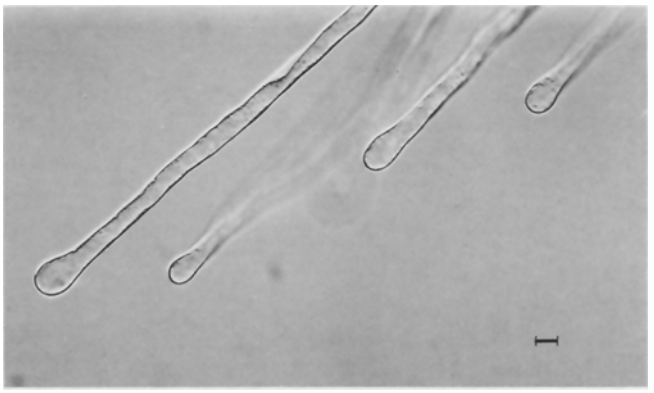

Fig. 4. Morphology of root hairs of *Arabidopsis* following nifedipine treatment. Seedlings were exposed to 100 μM nifedipine in APW for 1 h. Note the bulging root-hair tips. X400; bar = 10 μm

only observed in hairs that had been actively growing; mature hairs were not affected by the nifedipine treatments. In addition, when treated plants were exposed to light, root-hair growth resumed in some of the hairs, implying that inactivation of the nifedipine reversed the growth inhibition.

Discussion

The results of this study show that external Ca^{2+} is required for normal root-hair elongation and that an extracellular Ca^{2+} gradient exists at the tips of growing root hairs. A Ca^{2+} gradient was not detected at the tips of non-growing root hairs nor was one detected near the side walls of growing root hairs. The presence of this gradient indicates that the growing root-hair tip acts as a "sink" for external Ca^{2+}. Because treatment with the Ca^{2+}-channel blocker nifedipine abolished the Ca^{2+} gradient, it follows that the gradient is the result of Ca^{2+} uptake through plasma-membrane Ca^{2+} channels localized at the root-hair tip. This Ca^{2+} influx may be maintained by the continual fusion of Ca^{2+}-channel-containing vesicles at the tip, assuming that the Ca^{2+} channels have a short functional lifetime, as has been suggested to account for similar observations in other tip-growing systems (Schnepf 1986; Heath 1990).

The precise role of the Ca^{2+} taken up by the growing root hairs is not understood. It is possible that some of the Ca^{2+} serves to bind to polysaccharides in the growing cell wall. However, the observation of enlarged and branched tips in nifedipine-treated root hairs indicates that Ca^{2+} is taken up into the cytoplasm and is required, in some way, to maintain polarized growth. Some of its possible intracellular roles include: Ca^{2+} regulation of vesicle secretion; directed organelle transport; cytoskeleton arrangement; and Ca^{2+}- and calmodulin-dependent enzyme activities.

A related unresolved issue is the ultimate fate of the Ca^{2+} taken up by the root-hair cells. Since no outward-directed Ca^{2+} flux was detected at any point along the surface of growing root hairs, the root hair must deal with the excess Ca^{2+} in some other way, perhaps by sequestering it within a cellular compartment (Steer 1989). However, we cannot rule out an alternative possi-

bility, namely that Ca^{2+} efflux occurs over a large portion of the root-hair cell membrane and the resulting diffuse, outward-directed Ca^{2+} flux was too small to be detected by the calcium probe.

The Ca^{2+} gradient detected in this study indicates a net influx of Ca^{2+} into the growing root-hair tip. However, it is likely that Ca^{2+} is also secreted during vesicle fusion with the plasma membrane (Njus et al. 1986). Therefore, the magnitude of the inward-directed Ca^{2+} flux detected in our experiments is probably an underestimate of the actual magnitude of the Ca^{2+} influx.

While the present study provides strong evidence for a role for external Ca^{2+} in root-hair elongation, the importance of external Ca^{2+} in root-hair initiation is not as clear. At external Ca^{2+} concentrations below 0.010 mM, root hairs did not form on most epidermal cells, indicating that external Ca^{2+} is required for initiation. However, extracellular Ca^{2+} currents were not detected prior to the formation of a root hair primordium. It may be that a relatively small Ca^{2+} influx is required for root-hair initiation, and this was not detectable in the present experiments.

In general, the results obtained in our study are similar to the observations of Ca^{2+} influx during pollen-tube growth (see *Introduction*). In each cell type, growth is affected by suboptimal levels of Ca^{2+} and by nifedipine. In addition, the extracellular Ca^{2+} gradient discovered at the tips of growing root hairs has also been reported at growing pollen-tube tips (Kuhtreiber and Jaffe 1990), although the pollen-tube gradients have not been mapped in as much detail. Interestingly, the magnitude of the Ca^{2+} flux at the root-hair tip (approx. 4.4 pmol · $cm^{-1} \cdot s^{-1}$) is similar to that measured at the pollen-tube tip (approx. 4.0 pmol · cm-1 · s-1; Kuhtreiber and Jaffe 1990).

Some of the Ca^{2+}-related alterations in root-hair morphology observed in our study are similar to the phenotypes of root-hair mutants of *Arabidopsis* reported by Schiefelbein and Somerville (1990). For example, some of these mutants produce short hairs and hairs with swellings and branches, which are similar to the hairs produced by plants grown in low concentrations of Ca^{2+} or treated with nifedipine. It is therefore possible that some of these root-hair mutants possess defects in Ca^{2+} uptake or in Ca^{2+}-dependent processes at the root-hair tip. The examination of extracellular Ca^{2+} gradients in these mutant root hairs may serve to define the defect in root hair development further.

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