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# The role of extracellular free-calcium gradients in gravitropic signalling in maize roots

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**Abstract.** Gravitropism in roots has been proposed to depend on a downward redistribution of calcium across the root cap. However, because of the many calcium-binding sites in the apoplast, redistribution might not result in a physiologically effective change in the apoplasmic calcium activity. To test whether there is such a change, we measured the effect of gravistimulation on the calcium activity of statocyte cell walls with calcium-specific microelectrodes. Such a measurement must be made on a tissue with gravity sensing cells at the surface. To obtain such a tissue, decapped maize roots (*Zea mays* L. cv. Golden Cross Bantam) were grown for 31 h to regenerate gravitropic sensitivity, but not root caps. The calcium activity in the apoplasm surrounding the gravity-sensing cells could then be measured. The initial pCa was $2.60 \pm 0.28$ (approx 2.5 mM). The calcium activity on the upper side of the root tip remained constant for 10 min after gravistimulation, then decreased 1.7-fold. On the lower side, after a similar lag the calcium activity increased 1.6-fold. Control roots, which were decapped but measured before recovering gravisensitivity (19 h), showed no change in calcium activity. To test whether this gradient is necessary for gravitropic curvature, we eliminated the calcium activity gradient during gravitropism by applying a mobile calcium-binding site (dinitro-BAPTA; 1,2-bis(2-amino-5-nitro-phenoxy)ethane-N,N,N′,N′-tetraacetic acid) to the root cap; this treatment eliminated gravicurvature. A calcium gradient may be formed by proton-induced calcium desorption if there is a proton gradient. Preventing the formation of apoplastic pH gradients, using 10 and 50 mM 2-(N-morpholino)ethanesulfonic acid (Mes) buffer or 10 mM fusicoccin to stimulate proton excretion maximally, did not inhibit curvature; therefore the calcium gradient is not a secondary effect of a proton gradient. We have found a distinct and rapid differential in the apoplasmic calcium activity between the upper and lower sides of gravistimulated maize root tips which is necessary for gravitropism.

**Key words:** Calcium and gravitropism – Gravitropism (root) – Root gravitropism – *Zea* (root gravitropism)

## Introduction

This paper reports a test of the hypothesis that in the gravitropic curvature of maize roots, gradients of extracellular calcium are generated across the root tip and are required for the response. Redistribution of total calcium and of applied $^{45}Ca^{2+}$ during gravitropic curvature has been reported in coleoptiles (Slocum and Roux 1983), stems (Arslan-Çerim 1966) and root tips (Lee and Evans 1985; Moore et al. 1987). It has been assumed that this redistributed calcium accumulates in the apoplast, and that the redistribution is essential for the signal-transmission phase of gravitropism (Evans et al. 1986). Because of the large number of calcium-binding sites in the wall, most of the apoplastic calcium will be bound (Sentenac and Grignon 1981; Virk and Cleland 1988). However, to be physiologically important in signalling, calcium ions must be free in solution. Since there is a complex interrelation between bound calcium, free calcium and wall pH (Sentenac and Grignon 1981), gradients of total calcium do not indicate that a gradient of free calcium has been established. The amount of free calcium in the extracellular solution is measured as the calcium activity, essentially the effective concentration in a non-ideal system. In this study, the calcium activity in the apoplast of maize root tips was measured directly, using calcium microelectrodes, to show that a gradient of calcium activity does indeed develop in response to gravistimulation.

Calcium gradients have been considered a potential signal in gravitropism because asymmetric application of calcium produces curvature (Lee et al. 1983a). However,

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*Abbreviations:* BAPTA = 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid; FC = fusicoccin; Mes = 2-(N-morpholino)ethanesulfonic acid.

the appearance of a gradient of calcium activity does not mean that the gradient is required for gravitropism. Migliaccio and Galston (1987) suggested that the gradient was a consequence rather than a cause of gravitropism. The addition of a mobile calcium buffer to the root tip to equalize the calcium activity across the tip has been used here to show that the calcium gradient is required for gravitropic curvature.

A gradient in calcium activity could arise in at least two ways: there could be transport of calcium across the tissue, or there could be differential solubilization of bound calcium as a consequence of a gradient in pH across the root tip. If this calcium gradient is established by a pH gradient, bathing the whole tip in either a neutral-pH buffer or in fusicoccin to cause uniform proton excretion should block gravitropic curvature.

## Material and methods

*Plant growing conditions.* Seeds (caryopses) of *Zea mays* L. cv. Golden Cross Bantam (Olds Seed Co., Madison, Wis., USA) were surface-sterilized with 0.5% NaOCl (prepared from commercial bleach) for 5 min then grown in germination paper (Anchor Paper, St. Paul, Minn., USA) wetted with 10 mM KCl, 0.1 mM $CaCl_2$, 1 mM 2-(N-morpholino)ethanesulfonic acid (Mes) KOH, pH 6.0 (hereafter referred to as growth medium) and loosely covered with a plastic bag to prevent evaporative cooling. The seedlings were grown at 21° C, with red or white light to induce gravitropic competency, for 42–48 h at which time straight roots 10–25 mm long were selected for decapping. After wiping off superficial mucilage, the root cap was removed by pulling the cap with a scalpel, taking care not to damage the root body. For calcium measurements, roots were used at 30 – 31 h after decapping. At that time nearly all were graviresponsive but they did not yet produce sufficient amounts of mucilage to foul the microelectrode. For nonresponsive controls, roots were used 19 h after decapping, the longest time when none were graviresponsive. For measurements of the recovery of gravisensitivity, the apical 300 μm of the cap were cut off the control roots so that a similar amount of wounding was incurred, but the gravity sensing cells were not removed. The seedlings were rewrapped in germination paper until used.

*Curvature measurements.* The angle of the root tip relative to a reference mark on the mount was measured with a protractor either directly or from a projected photograph of the roots. To determine the time of initial curvature in decapped roots (Fig. 2) the roots were recorded on a time-lapse videotape recorder, and the tape played backward to determine when first curvature occurred. To determine the capacity of roots to curve after decapping, after various times the roots were mounted vertically in plastic boxes ($30 \cdot 15 \cdot 8$ $cm^3$) for 1 h then horizontally for 4 h, at which time the amount of curvature was measured. The presentation time of seedlings that had recovered from decapping for 28 h was determined by mounting sets of 10–20 seedlings in the plastic boxes; after 1 h in the vertical position they were held horizontally for various times, then rotated at 1 rpm on a clinostat for 30 min to allow curvature to develop. The mean of each set was used as a datum to produce a relationship between gravistimulation time and curvature.

*Total calcium.* Root sections were dried at 70° C, weighed, digested with 0.5 ml concentrated $HNO_3$, decolorized with $H_2O_2$, redried, and dissolved in a measuring solution containing 14 mM $La^{3+}$, 3.8 mM $Cs^{2+}$ and 0.167 N HCl. The calcium concentration was measured with an atomic absorption spectrophotometer (Model 303; Perkin Elmer, Norwalk, Conn., USA).

*Calcium-specific microelectrodes.* Microelectrodes were prepared as described by Amman (1986). Briefly, glass microelectrodes were pulled to a tip outside diameter of 1.5–2 μm. The glass was silanized with tributyl silane (Fluka, Happague, N.Y., USA), filled with electrolyte, and the tip filled with calcium-selective resin to a length of about 250 μm. The electrolyte was 0.5 M KCl, 0.1 M $CaCl_2$. The ETH1001-based calcium resin contained 86% calcium "cocktail" (Fluka; No. 21048), 14% polyvinyl chloride (Fluka; No. 81392) that was diluted with three volumes of distilled tetrahydrofuran. The resistance of the electrodes was approx. $5 \cdot 10^{10}$ Ω. Unsilanized micropipettes filled with 0.1 M KCl served as reference electrodes, the ground electrode was Teflon tubing (1 mm inner diameter) filled with 10 mM KCl and 0.5% agarose.

The electrodes were calibrated in $CaCl_2$ solutions containing 10 mM KCl so the ionic strength would be comparable to that of the solution with which the roots were wetted. The calcium activity was calculated from the Debye-Hückel equation (Daniels and Alberty 1976). For calibration solutions with pCa 4, 3, 2.5 and 2.2, the total calcium concentrations were, in this order, $1.614 \cdot 10^{-4}$, $1.786 \cdot 10^{-3}$, $7.28 \cdot 10^{-3}$, and $2.415 \cdot 10^{-2}$ M. The slope of the calibration was generally 28 mV/decade; electrodes under 24 mV/decade were discarded. The electrodes were calibrated before and after each run.

*Measurements of apoplasmic calcium.* Roots were mounted on an acrylic slide and held in place with loops of monofilament fishing line. The root was swathed in small pieces of paper tissue (Kimwipe; Kimberly Clark, Roswell, Ga., USA) wetted with 10 mM KCl leaving only the apical 2 mm exposed. The slide was mounted on the microscope so that the root was vertical. A ground electrode was in contact with the Kimwipe. The calcium and reference electrodes contacted the tip close to each other with the calcium electrode at the end of the region containing gravity-sensing cells (see Fig. 1). The region of gravity-sensing cells was assumed to correspond to the region having sedimenting amyloplasts, described for this material by Hillman and Wilkins (1982). When the measured calcium activity had been stable at least 5 min, the apparatus, including microscope, electrodes and tissue, were rotated 90°. Measurements were recorded each min. The rapid growth rate (approx. 20 μm · $min^{-1}$) necessitated frequent repositioning of the electrodes so that the same small region (approx. 5 μm) of the root was consistently sampled.

*Solution application.* The effect of applying solutions only to the root cap was measured by attaching a small tube to the apex of the root and filling it with various solutions. Root tips were wetted with 10 mM KCl to hydrate the mucilage which could then be wiped off

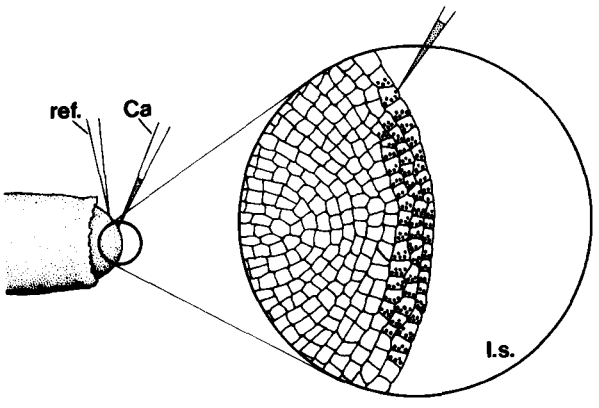


**Fig. 1.** Diagram of electrode placement on maize root tip for measuring the extracellular calcium activity. The calcium-sensitive electrode was placed at the upper or lower edge of the zone where sedimenting plastids develop, hence the location where the chage in calcium activity would be expected to be the greatest. The reference electrode was placed a few cells away. Drawing by Phyllis Woolwine

so that it would not interfere with solution getting to the cap. A 2.5-mm-long piece of 1.5 mm outer diameter glass tubing (TW150; World Precision Instruments, New Haven, Conn., USA) was put on the end of each root so that it covered the apical 1 mm, consisting primarily of the root cap. These roots were mounted vertically in plastic boxes for 1 h, then solutions were added to each tube. After an additional 1 h, the boxes were rotated. Solutions were replenished hourly during growth. Roots with empty tubes or tubes with only KCl or neutral pH buffer (10 mM Mes-KOH, pH 6.5) all curved the same as roots without any tubes.

Each treatment was applied to 12 roots, and each experiment was repeated three times, with representative results shown in Figs. 7 and 8.

The calcium-buffer solution contained 10 mM 1,2-bis(2-amino-5-nitro-phenoxy)ethane-N,N,N′,N′-tetraacetic acid (dinitro-BAPTA; Molecular Probes, Eugene, Ore., USA; stock No. B–3001), 7.5 mM $CaCl_2$, 10 mM Mes-KOH, pH 6.5. The measured pCa was 3.4. The affinity of this buffer for calcium depends on the ionic strength of the solution. In 100 mM KCl it is 7 mM (Pethig et al. 1989), in this solution it is apparently somewhat less than 1 mM as expected from the response of other BAPTA buffers to ionic strength (Harrison and Bers 1987). Root tips killed by freezing and thawing did not significantly change the pCa of this solution during incubation.

*Statistical analysis.* For the presentation time, a regression line was calculated for the relationship between the logarithm of stimulation time and curvature after growth on the clinostat. Each point used in this analysis was the mean of a set of 10 to 20 roots which were treated together. The regression line was used to estimate the x intercept. A confidence interval for this estimate was found by calculating the x intercepts after adding and subtracting the standard deviation of the slope:

$$\text{x intercept} = (\bar{x} - (\bar{y}/(\beta \pm S_\beta)) \ .$$

In Figs. 5 and 6, the mean pCa at each time point for the different runs is shown. For time series with varied initial pCa and different onsets of a change in pCa, the variation in the response is not described by the standard deviation of the mean value for each time point. For this we used a parametric procedure, expectation maximization (Dempster et al. 1976), to find the initial pCa, the time when the pCa changes and the magnitude of the change, assuming for simplicity that it was a step change. The values of these parameters for each treatment were then compared using Student's t-test (Tables 1, 2).

## Results

*Recovery of gravitropism.* The roots began to recover gravitropic competence about 20 h after removal of the cap, and all had recovered by 40 h. At intermediate times, only a portion of the roots were graviresponsive (Fig. 2) and they did not respond to the same extent as intact roots (Fig. 3). Removing the apical half of the root cap did not cause an inhibitory wound response (Fig. 3). The presentation time, a measure of the early steps of gravity sensing, was similar in roots 28 h after decapping (7.8 min; confidence interval = 7.2–8.3 min) and intact roots (6.1 min; confidence interval = 5.4–6.7 min) (Fig. 4).

*Extracellular calcium activity.* The initial calcium activity of vertical root tips was variable (mean pCa = 2.60 ± 0.28; $n = 41$) and higher than the growth medium (pCa 3.8). The total calcium content was

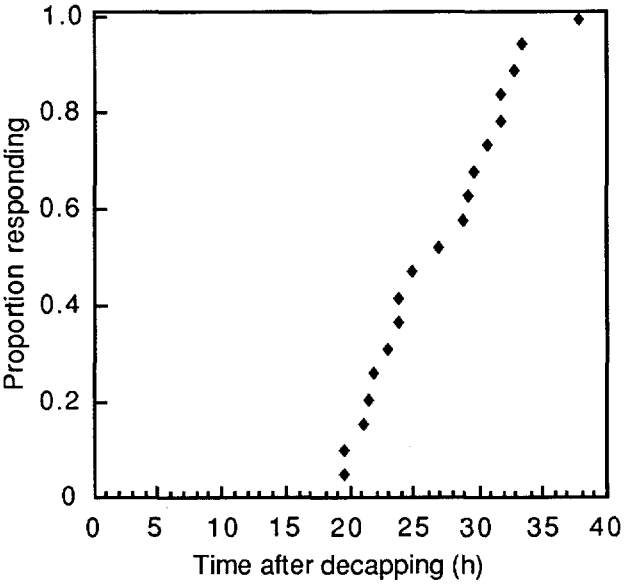


**Fig. 2.** Proportion of maize roots showing graviresponsiveness during recovery from decapping. We selected roots which were graviresponsive, as well as similar roots which were not responsive as a control. From this figure we determined that all roots at 32 h would be responsive, and none by 19 h. Roots were held horizontally and the time of initial curvature was recorded on video tape

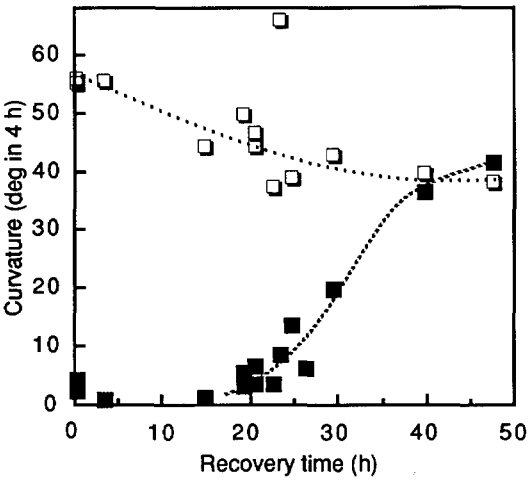


**Fig. 3.** Recovery of graviresponsiveness in maize roots. As roots recovered from decapping, not only did more roots respond, but the strength of the response increased. The lack of curvature was not a generic wound response, because half the cap could be removed with no inhibition of curvature (···□··· detipped: apical half of cap removed; ···■···, decapped: whole cap removed)

$1.7 \pm 0.2$ mg · (g DW)$^{-1}$ in the root cap ($n = 4$ samples of 18–20 caps) and $0.9 \pm 0.1$ mg · (g DW)$^{-1}$ in the first millimeter of the root body (6 samples of 12–20 sections). Assuming that the weight of the extracellular solution equals the dry weight, less than 6% of the total calcium in the cap would be apoplastic free calcium.

*Response of calcium activity to gravistimulation.* The calcium activity on the upper and lower side of gravistimulated root tips was measured continuously for 25 min. In roots which had not yet recovered gravisensitivity

**Table 1.** Estimates of the changes in pCa after gravistimulation on the upper and lower sides of the gravity-sensitive portion of maize root tips. Time series for individual roots were fitted to a model which estimated the change in pCa using the EM algorithm. The mean initial pCa was 2.60

| Side | Recovery time (h) | ΔpCa Mean | ΔpCa SE | n | P(ΔpCa ≠ 0)[a] | P(trt > cont)[b] |
|---|---|---|---|---|---|---|
| Upper | 31 | 0.223 | 0.015 | 7 | 0.9999 | 0.998 |
| | 19 | 0.027 | 0.027 | 4 | NS | |
| Lower | 31 | −0.203 | 0.067 | 4 | 0.944 | 0.953 |
| | 19 | −0.034 | 0.021 | 4 | NS | |

[a] Probability that the change in pCa is not zero
[b] Probability that the change in pCa is greater in the graviresponsive roots than in the corresponding non-responsive controls

**Table 2.** Time after beginning of gravistimulation when pCa changed as calculated by the EM algorithm. The model estimates the midpoint of the change rather than the onset

| Side | Mean | SE[a] |
|---|---|---|
| Upper | 9.4 | 1.4 |
| Lower | 11.3 | 1.3 |
| Pooled[b] | 10.1 | 1.0 |

[a] SE of time change
[b] Both groups combined because there was no significant difference between them

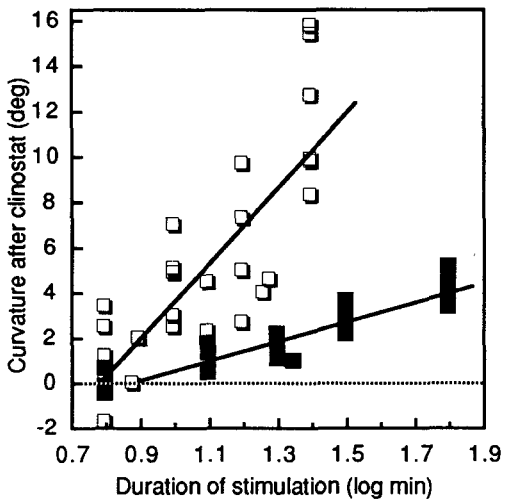


**Fig. 4.** Presentation time of recovered decapped maize roots. Although the curvature was weaker in recovered roots (28 h after decapping), the presentation time was essentially the same for the newly differentiated statocytes in the root tip as in the columella statocytes of the intact roots (—□— intact; —■— decapped). The similarity of the presentation times suggests that, though the location of the statocytes is different, the mechanism of sensing is the same. The presentation time also provides a benchmark for indicating when a gravitropically-relevant change in the calcium activity might be expected

(19 h), there was no change in calcium activity (Fig. 5). In roots that had recovered gravisensitivity (31 h), the calcium activity increased on the lower side (pCa decreased) and decreased on the upper side (Fig. 6). This observation that only gravisensitive roots produce a gradient in calcium activity indicates that the gradient is a consequence of gravity sensing.

The magnitude of the change (Table 1) is equivalent to a 1.6-fold change of the calcium activity, increasing

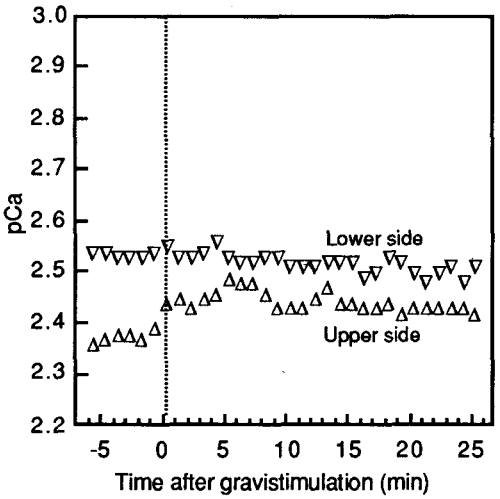


**Fig. 5.** The calcium activity of statocytes in non-responsive maize roots (19 h recovery) did not change during gravistimulation after these roots were turned, indicating that processes unrelated to gravitropism did not produce any effect on extracellular calcium activity. Calcium was measured on the upper or lower side of the gravity-sensitive region as shown in Fig. 1; $n=4$ for each set

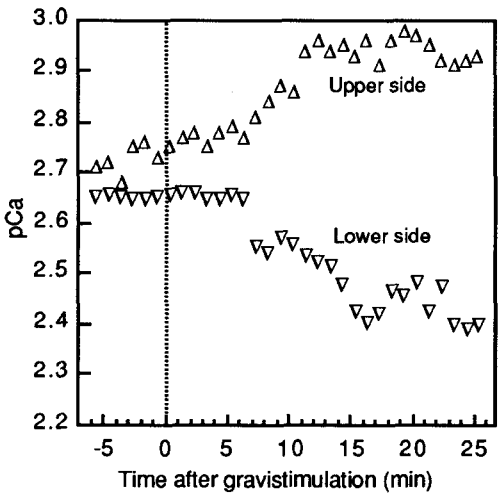


**Fig. 6.** The extracellular calcium activity of statocytes changed in graviresponsive maize roots (31 h recovery) during gravistimulation. The change was in opposite directions on the two sides as would be expected for a gradient produced by gravity sensing. The magnitude of the change was about 1.6-fold. Calcium was measured on the upper or lower side of the gravity-sensitive region as shown in Fig. 1; $n=7$ on upper side, $n=4$ on lower side). A statistical treatment of these data is in Tables 1 and 2

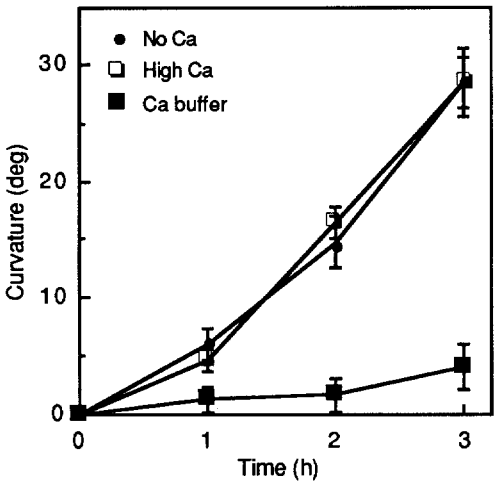


**Fig. 7.** Inhibition of curvature in maize roots when the calcium gradient is eliminated. The calcium buffer dinitro-BAPTA maintains the calcium activity at a normal level and also makes calcium diffuse faster by providing a mobile binding site; it should be highly effective in destroying calcium gradients while having no other effect. Solutions were applied only to the root cap, 1 h before and continuously during gravistimulation. All solutions included 10 mM KCl and 1 mM Mes-KOH, pH 6.5. In addition, the treatments contained no calcium (—●—); high calcium, 30 mM $CaCl_2$ (—□—); or calcium buffer, 10 mM dinitro-BAPTA–$CaCl_2$, pCa 3.4 (—■—). Each point is the mean of 12 roots; the error bars represent the SE

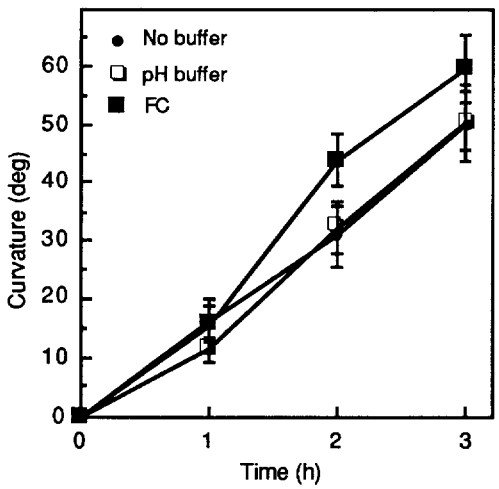


**Fig. 8.** No inhibition of curvature in maize roots when pH gradient is prevented. A pH gradient can produce a calcium gradient in the wall solution when protons displace wall-bound calcium. Two approaches were used to eliminate pH gradients: buffering and uniform proton excretion. All treatments contained 10 mM KCl and 1 mM $CaCl_2$. In addition, the treatments contained no buffer (—●—); strong pH buffer, 50 mM Mes-KOH, pH 6.0 (—□—); or a proton-pumping stimulator, 10 μM FC (—■—). Each point is the mean of 12 roots; the error bars represent the SE

from 2.5 mM to 4.0 mM on the lower side, and decreasing from 2.5 mM to 1.6 mM on the upper side, with the gradient produced being 2.5:1. The midpoint of the change in calcium activity occurred at 10 min (Table 2), only slightly longer than the presentation time of 7.8 min (Fig. 4), as expected if the change in calcium activity is a consequence of gravistimulation.

*Importance of calcium and proton gradients.* In order to determine whether the calcium gradient in the root tip is essential for gravicurvature, we mobilized the calcium ions with the newly-synthesized calcium buffer dinitro-BAPTA, which has a binding constant similar to the measured pCa in the cell wall (Pethig et al. 1989). The root cap, but not the growing zone, was immersed in a 10 mm dinitro-BAPTA, pCa 3.4, and 10 mM Mes-KOH, pH 6.5, buffer. In addition to acting as a buffer, this calcium chelator creates a mobile binding site for calcium and allows calcium to redistribute and neutralize gradients. This treatment effectively eliminated curvature. High calcium alone (30 mM $CaCl_2$) did not block gravicurvature, however (Fig. 7).

A calcium gradient could be formed by differentially displacing wall-bound calcium, with protons of the gravity-sensing cells producing a pH gradient. This gradient was prevented with both a stronger Mes buffer (50 mM), as well as 10 μM FC, which should maximally and symmetrically stimulate proton pumping. Neither of these inhibited curvature (Fig. 8).

## Discussion

An extracellular calcium gradient in the root cap has been suggested as a mediator of gravitropic transduction in roots because applied radioactive calcium is polarly distributed during gravitropism (Lee et al. 1983b) and because asymmetrically applied calcium produces root curvature (Lee et al. 1983a). For such a gradient to be a signal, a difference in the chemical activity of calcium along the gradient must be demonstrated. The very large pH-dependent cation-binding capacity of the wall means there may be little relationship between total calcium and calcium activity (Sentenac and Grignon 1981). In this paper, direct measurement of calcium activity has demonstrated the presence of such an activity gradient, which begins to develop with a lag time similar to the presentation time for curvature.

If extracellular calcium is a physiological signal, it is likely to act on cell function locally, not diffuse to the growing zone because of its low diffusion constant in the apoplast. The experiments in this paper were designed to study the effects of the treatments on the development of a physiological gradient in the cap without being complicated by effects on growth-related processes in the elongating zone. The proposed physiological events apply only to the response of statocytes to gravistimulation, not to growth.

The initial calcium activity of the root apoplasm was high relative to the growth medium, implying that calcium is leaking from these young seedlings, rather than being taken up from the growing medium. There is no barrier and the activity difference is a direct measure of the driving force for diffusion. The activity is 10-fold higher than in soybean hypocotyls (Cleland et al. 1990), the only other tissue analyzed in this respect, and the total calcium is about 2.5-fold higher. Calcium appears to be more abundant relative to the binding capacity in these seedling roots than in the soybean hypocotyl.

During gravistimulation, there was a clear change in the calcium activity in the apoplasm of gravity-sensing cells at 10 min, a little longer than the presentation time of 8 min as expected from a physiological response to

gravity sensing. This change is polar: the activity on the upper decreased by a factor of 1.6 and on the lower half increased by the same factor. Therefore, gravity sensing rapidly produced a extracellular gradient along the axis of the gravity vector. This gradient is consistent with results for calcium diffusion between agar blocks on the root tip (Lee et al. 1983b). The 2.5-fold gradient is comparable to the 1.5-fold auxin gradient following gravistimulation reported by Young et al. (1990), but without the reverse transient observed for auxin.

The gradient in calcium activity across the root tip is apparently required for gravitropism, because elimination of this gradient by making calcium ions mobile eliminated curvature. Dinitro-BAPTA binds calcium and the complex is highly diffusible, but its binding constant is such that the calcium activity is constant throughout the apoplasm and only slightly below the activity that exists in its absence. On the other hand, addition of 30 mM calcium did not block curvature. This is presumably because of its immobility in the wall; the large number of fixed binding sites will make diffusion of these ions slow.

The mechanism responsible for the generation of the gradient of apoplastic calcium activity is unknown. One possibility is that it is the consequence a difference in apoplastic pH on the two sides. Since protons can displace bound calcium from the wall and increase the calcium activity, enhanced proton transport from the cells on the lower side relative to the upper side of the root could be responsible for the calcium gradient. Direct measurements of a pH gradient across the corn root tips, using a similar experimental design, were unsuccessful, apparently because of interference by fixed charges in the mucilage. However, the fact that neutral buffers, which should prevent such a pH gradient, and FC, which should cause maximal proton efflux on both sides of the tip, both failed to block gravicurvature indicates that a pH gradient is not the cause.

A second possibility is that there is differential efflux of calcium from the cells on the two sides of the root tip. The amount of calcium required to increase the apoplastic calcium activity from 2.5 to 4.0 mM seems to be more than what the cell can export, if one considers the large buffering capacity of the wall. In addition, if the ATP-driven calcium efflux is a $Ca^{2+}$-$2H^{+}$ exchange (Rasi-Caldogno et al. 1987) the local alkalinization of the wall solution accompanying calcium efflux would increase the binding of $Ca^{2+}$ to the walls, negating any increase in calcium activity.

A final possibility is that there is diffusion of extracellular calcium from the upper to the lower side of the root tip. Direct diffusion of free ions seems unlikely in view of the large number of calcium-binding sites in the wall, but the diffusion of calcium coupled with some mobilizing agent is a possibility.

*In conclusion,* the physiological response of maize root-cap statocytes to gravistimulation produces a gradient in the activity of calcium in the apoplasm. This activity gradient is necessary for subsequent gravitropic curvature.

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