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Cytoplasmic Free Ca^{2+} in Arabidopsis Roots Changes in Response to Touch but Not Gravity[1]

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Changes in cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$) have been proposed to be involved in signal transduction pathways in response to a number of stimuli, including gravity and touch. The current hypothesis proposes that the development of gravitropic bending is correlated with a redistribution of $[Ca^{2+}]_i$ in gravistimulated roots. However, no study has demonstrated clearly the development of an asymmetry of this ion during root curvature. We tested this hypothesis by quantifying the temporal and spatial changes in $[Ca^{2+}]_i$ in roots of living Arabidopsis seedlings using ultraviolet-confocal Ca^{2+}-ratio imaging and vertical stage fluorescence microscopy to visualize root $[Ca^{2+}]_i$. We observed no changes in $[Ca^{2+}]_i$ associated with the graviresponse whether monitored at the whole organ level or in individual cells in different regions of the root for up to 12 h after gravistimulation. However, touch stimulation led to transient increases in $[Ca^{2+}]_i$ in all cell types monitored. The increases induced in the cap cells were larger and longer-lived than in cells in the meristematic or elongation zone. One millimolar La^{3+} and 100 μM verapamil did not prevent these responses, whereas 5 mM EGTA or 50 μM ruthenium red inhibited the transients, indicating an intracellular origin of the Ca^{2+} increase. These results suggest that, although touch responses of roots may be mediated through a Ca^{2+}-dependent pathway, the gravitropic response is not associated with detectable changes in $[Ca^{2+}]_i$.

When positioned horizontally a root responds by curving downward toward the gravity vector. The positive gravitropic response of roots depends on a series of events: gravity perception, translocation of the gravity stimulus to the site of the response, and the tropic-growth response. The latter is characterized by asymmetric growth across the elongation zone, with reduced growth along the lower side compared with the upper side (Ishikawa et al., 1991). The gravity-sensing cells in the root are thought to be the cells of the starch-containing columella in the cap. A widely used model for root gravitropism is that sedimentation of amyloplasts in the columella (Sack, 1991) leads to asymmetrical auxin redistribution in the cap. Auxin from the cap moves preferentially toward the lower half of a horizontally oriented root, causing inhibition of cell elongation and gravicurvature (Evans et al., 1986). Analysis of growth rate changes during root gravitropism has shown that the graviresponse is initiated in the postmitotic isodiametric growth region between the meristem and the zone of rapid elongation (Baluska et al., 1994), also called the distal elongation zone (Ishikawa and Evans, 1993). Therefore, the site of the response is greatest at a certain distance from the gravity-sensing cells. However, the precise cellular mechanisms of the initial perception, signal transduction, signal translocation, and induction of asymmetrical growth have remained elusive.

Ca^{2+} ions are attractive candidates for regulating each of the phases of the graviresponse because of their widespread role in physiological processes in plants (Hepler and Wayne, 1985; Bush, 1995). In roots several experiments suggest that changes in apoplastic Ca^{2+} are necessary for the gravitropic response. Lee et al. (1983a) demonstrated that the application of Ca^{2+} chelators to the root cap abolished the graviresponse without inhibiting root elongation, and replacement of the chelator with Ca^{2+} restored gravisensitivity. Furthermore, Ca^{2+} transport studies showed that $^{45}Ca^{2+}$ movement was symmetrical in vertical roots but preferentially accumulated along the lower side of graviresponding roots (Lee et al., 1983b). Using Ca^{2+}-specific microelectrodes, Björkman and Cleland (1991) found a distinct and differential gradient in the apoplastic Ca^{2+} activity between the upper and the lower side of gravistimulated maize (*Zea mays* L.) root tips that was required for gravitropism. These observations, in conjunction with the finding that asymmetric application of Ca^{2+} caused curvature toward the source (Ishikawa and Evans, 1992), provide indirect evidence that gravity-induced Ca^{2+} redistribution is necessary for gravitropic curvature. The development of this asymmetry in apoplastic Ca^{2+} may mediate growth control. However, the mechanism of the redistribution is poorly understood (Björkman and Cleland, 1991).

A role for $[Ca^{2+}]_i$ has also been suggested, in which sedimentation of amyloplasts in the columella elicits

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Abbreviations: $[Ca^{2+}]_i$, cytoplasmic Ca^{2+} concentration; CaM, calmodulin; DMGA, dimethyl glutaric acid.

$[Ca^{2+}]_i$ transients because of interaction with the ER or plasma membrane (Poovaiah et al., 1987). Like apoplastic Ca^{2+}, evidence for the role of $[Ca^{2+}]_i$ is also circumstantial. Protoplasts isolated from the cap of maize, potentially from graviperceptive cells, show lower $[Ca^{2+}]_i$ compared with protoplasts from the elongation zone (Kiss et al., 1991). In addition, analytical ion microscopy and energy-filtering microscopy report high Ca^{2+} signals in the amyloplasts and ER of the columella cells (Chandra et al., 1982; Busch et al., 1993). However, the significance of these findings in regard to root gravity perception, and whether alterations occur in $[Ca^{2+}]_i$ in graviresponding roots have not been reported.

The role of Ca^{2+} in root gravitropism is also suggested by the apparent involvement of CaM in this process. Higher CaM levels were observed at root tips (Allan and Trewavas 1985; Stinemetz et al., 1987) and shown by immunocytochemistry to be strongly localized in the columella cells (Dauwalder et al., 1986). In the Merit cv of maize, which requires light to become graviresponsive, CaM activity increases upon illumination (Stinemetz et al., 1987). In Arabidopsis seedlings mRNA of a root-specific CaM (CaM-1) has been shown to increase 3-fold within 30 min of gravistimulation (Sinclair et al., 1996). CaM antagonists have also been shown to inhibit the graviresponse of both maize and Arabidopsis roots (Stinemetz et al., 1992; Sinclair et al., 1996) and to interrupt auxin redistribution in isolated maize root caps (Stinemetz et al., 1992).

It has also been proposed that the graviresponse and the touch response may share an underlying mechanism (Trewavas and Knight, 1994; Haley et al., 1995), because both responses include a proposed role for Ca^{2+} and CaM. Indeed, genes for CaM and CaM-like proteins are induced upon touch stimulation (e.g. Braam and Davies, 1990), and the *CaM-1* gene in Arabidopsis is induced by gravity (Sinclair et al., 1996). There is also extensive evidence that touch stimuli lead to rapid transient increases in $[Ca^{2+}]_i$ levels (Knight et al., 1994; Trewavas and Knight, 1994), and that touch-induced genes may also be induced by elevations in $[Ca^{2+}]_i$ levels (Xu et al., 1995).

Despite the large number of studies implicating $[Ca^{2+}]_i$ in the gravitropic response, there is still no direct evidence of changes in $[Ca^{2+}]_i$ in live, graviresponding roots. Gehring et al. (1990) recorded a slight, sustained increase in $[Ca^{2+}]_i$ from 255 to 370 nM within the first 10 min of horizontal stimulation of maize coleoptiles. However, the physiological relevance of these small changes remains controversial (Firn and Digby, 1990) and those experiments were limited to aerial organs. We therefore decided to investigate whether the graviresponse or the touch response of roots was associated with a change in $[Ca^{2+}]_i$ in intact roots of Arabidopsis using confocal ratio imaging and vertical stage fluorescence microscopy. This approach has allowed us to determine the $[Ca^{2+}]_i$ dynamics at the level of the whole organ down to identified individual cells in various regions of the root. We report that although the touch stimulus elicits $[Ca^{2+}]_i$ transients in root cells, which display cell-type-specific kinetics, the gravity stimulus/response is not accompanied by detectable increases in $[Ca^{2+}]_i$.

MATERIALS AND METHODS

Seeds of *Arabidopsis thaliana* ecotype Columbia were surface-sterilized by immersing them successively in 95% ethanol and 10% bleach for 5 min each followed by five rinses in sterile, distilled water. They were planted on a thin film of medium (2 mL) layered onto 48- × 65-mm no. 1 cover glasses (Clay Adams, Lincoln Park, NJ). The medium consisted of 3 mM KNO_3, 2 mM $Ca(NO_3)_2 \cdot 4H_2O$, 0.5 mM $MgSO_4 \cdot 7H_2O$, 1 mM $(NH_4)_2PO_4$, 1 mg/mL thiamine, 0.5 mg/mL pyridoxine-HCl, 0.5 mg/mL nicotinic acid, 0.56 mM myo-inositol, 2.3 mM Mes, 0.1 g/L Suc, micronutrients, and 1% Phytagel at a pH of 5.7. The micronutrients were 25 μM KCl, 17.5 μM H_3BO_3, 1 μM $MnSO_4 \cdot H_2O$, 1 μM $ZnSO_4 \cdot 7H_2O$, 0.25 μM $CuSO_4 \cdot 5H_20$, 0.25 μM $(NH_4)_6MoO_{24} \cdot 4H_2O$, and 25 μM Fe-Na EDTA. The seedlings and cover glasses were contained within 90-mm plastic Petri dishes wrapped with Parafilm. These were grown at a constant photon flux density of 36 μmol m^{-2} s^{-1} at 22 to 24°C. Plants were used after 4 d, when the root was approximately 2 cm long. Unless otherwise stated, chemicals were obtained from Sigma.

Gravitropic Stimulation and Video Imaging

Root growth and curvature after gravistimulation were monitored using a video camera (model C2400, Hamamatsu, Tokyo) and a 52-mm macro lens (Nikkor, Kogaku, Japan). Images were captured every 20 min for 12 h using a frame grabber (model LG-3, Scion Corporation, Frederick, MD) and computer (Quadra 800, Apple Computer Inc., Cupertino, CA) running image acquisition software (IPLabs Spectrum, Signal Analytics, Vienna, VA). The resolution of this imaging allowed us to detect 50-μm changes in root length.

Alternatively roots were mounted vertically on the stage of an epifluorescence microscope (Optiphot, Nikon) that had been mounted on its back such that the rotatable stage was vertical. Roots were then rotated through 90° to the horizontal while being continuously imaged. Using a 10×, 0.3-numerical aperture fluorescent objective (Nikon), this imaging setup allowed us to detect 5-μm changes in root length.

Measurement of Cytoplasmic Ca^{2+}: Indo-1 Loading and *Confocal Ratio Imaging*

For fluorescence ratio imaging of $[Ca^{2+}]_i$, the roots were acid-loaded with 25 μM indo-1 (Molecular Probes, Eugene, OR). The seedlings were first incubated with 1 mL of growth medium (minus Phytagel) at pH 4.5 for 15 min to ensure that the Phytagel matrix was well hydrated. The medium was then replaced with 0.25 mL of 25 μM indo-1 in 25 mM DMGA, pH 4.5. The plants were incubated in the dye in the dark for 1 h, after which time the excess liquid was removed and rinsed with two washes of growth medium (2 mL each for at least 30 min each). After establishing that acid-loaded indo-1 was in the cytoplasm (see Fig. 1 and below), we also tested how this indicator might affect the growth and gravitropic response of the roots. At con-

centrations below 50 μM in the acid-loading medium, indo-1 did not inhibit the growth rate or affect the kinetics (rate of curvature or latent time) of the graviresponse of Arabidopsis roots (see Fig. 3). Similarly, at 25 mM DMGA, the pH buffer used for acid-loading, the graviresponse was unaffected. Above these concentrations growth rate and gravitropic bending of the roots was inhibited (data not shown). Therefore, all experiments were performed using 25 μM indo-1 and 25 mM DMGA. Seedlings loaded in 25 mM DMGA and 25 μM indo-1 showed normal root growth and development, continued to form root hairs, and exhibited no obvious difference in root morphology for up to 2 d after loading (data not shown). For acid-loaded roots we estimate the cytoplasmic indo-1 to be 1 to 10 μM, calculated as by Gilroy (1996).

Plants that had been successfully acid-loaded were positioned such that the root was vertically oriented. After 2 h of vertical growth, the Petri dishes were rotated 90° so that the roots were horizontally oriented. After various times of gravistimulation, cover slips with seedlings and gel matrix intact were placed on the stage of an inverted microscope (Axiovert, Zeiss) attached to a laser scanning confocal microscope (LSM410, Zeiss), and imaged through the cover slip using a 40×, 0.75-numerical aperture, dry objective (Zeiss). This setup allowed us to take a single optical section that contained both the physiological upper and lower sides of the gravistimulated root. Each root was used for a single time point to ensure the gravitropic response was not compromised by repeated placement of an individual root on the stage of the confocal microscope for the $[Ca^{2+}]$ imaging.

Fluorescence from the dye was excited with the 364-nm line of a UV laser (Enterprise, Coherent Ltd., Auburn, CA) set at approximately 10 mW, using an 80/20 beam splitter. Emitted light was simultaneously detected at 400 to 435 nm and >460 nm using a 460-nm dichroic mirror and the appropriate interference filters (Zeiss) on each of the two photomultiplier detectors. Each frame represents a single 8-s scan of the laser. Photobleaching represented <10% per channel per scan for each ratio image.

Transmission images were also taken for each ratio image using the transmission detector of the confocal microscope and illumination by the 633-nm He/Ne laser of the confocal attenuated to 10% with neutral-density filters. Pseudocolor ratio images of the $[Ca^{2+}]_i$ distribution were calculated as in Gilroy (1996). Image processing was carried out by a computer (PowerMac 8100, Apple) using image analysis software (IPLabs Spectrum, Signal Analytics). Dark current represented less than 5% of the indo-1 fluorescence signal at each detector. Autofluorescence was highly variable between batches of plants. Plants showing autofluorescence more than 15% of the final indo-1 signal were rejected from further analysis.

In these experiments optical sections parallel to the cover slip were taken through three planes of the root (starting 25 μm into the root, with subsequent sections 25 μm apart) to ensure that the view of the root $[Ca^{2+}]_i$ was not affected by the plane of focus. The image taken approximately 25 μm into the root was found to be representative of the distribution of Ca^{2+} within the cytoplasm at all planes observed. This section also showed both epidermal and cortical cells and is therefore the section shown in all of the figures. Sections deeper than 25 μm showed signal degradation and did not provide reliable ratio images (Gilroy, 1997).

As a control for the acid-loaded experiments, equivalent experiments were performed using roots microinjected with indo-1, indo-1 linked to a 10-kD dextran, calcium green-1, or calcium green-1 linked to a 10-kD dextran, essentially the same as used by Gilroy (1996). Dextran-conjugated dyes are not thought to cross organelle membranes, so once they are introduced into the cytoplasm they should report $[Ca^{2+}]_i$ levels. Root cells were classed as being successfully microinjected if they maintained turgor, cytoplasmic streaming, and cytoplasmic structure during the experiment. Cells injected in the postmitotic and elongation zone also had to undergo normal cell elongation to be included in subsequent analysis (e.g. see Fig. 7). Approximately 75% of injected cells showed a loss of turgor and developed granularity in the cytoplasm over the 1-h recovery period after injection, or failed to complete normal elongation growth. These roots were excluded from further analysis. One-third of the roots where cells had been microinjected successfully by the criteria outlined above showed an inhibition of subsequent root growth and/or graviresponse. These were also excluded from analysis. Using these criteria for viability, it was possible to successfully inject epidermal and cortical cells in the elongation, postmitotic, and meristematic zones.

Although the peripheral cells of the root cap could be acid-loaded, the columella cells in the central zone of the cap often acid-loaded poorly and could not be visualized in more than 25% of the roots studied. To overcome this limitation these cells were loaded by microinjection of Ca^{2+} indicator. Injection of cells of the central starchy columella required impalement through the overlying cell layers, leading to the disruption of the overlying one or two cells. Even so, columella cells could be successfully injected with no adverse effects on root growth or graviresponse.

Vertical Stage Microscope Experiments

The fastest confocal imaging obtained was 30 s to 1 min after placing the root on the stage of the microscope, so rapid changes following gravistimulation may have been missed. We therefore employed the vertical stage epifluorescent microscope outlined above to improve our temporal resolution. Cells from the cap, meristematic, postmitotic, and elongation region were impaled and microinjected with micropipettes containing 100 μM indo-1 or calcium green-1, or 1 mM calcium green-1 dextran as outlined above. To ensure minimal stress, only one cell was injected per root. After allowing the roots to recover from the microinjection procedure for 1 h in the vertical position, cover slips containing the seedlings were mounted on the vertical stage epifluorescent microscope. Individual cells were imaged with a 10- or 15-s exposure using a cooled CCD camera (CH250A, Photometrics, Tucson, AZ) running under image acquisition software (IPLabs Spectrum, Signal Analytics). After collecting images in vertically growing roots, the stage was rotated 90° so that

the root was horizontal. An image was collected immediately after rotating the stage and subsequent images were collected in intervals of 30 s for 10 min. Acid-loaded roots were not used for such experiments, because the reduced spatial resolution of the vertical stage microscope relative to the confocal microscope meant that we were unable to obtain clear images of defined cell types.

Imaging was performed using a 40×, 0.7-numerical aperture, fluorescent objective (Nikon), 340 ± 10 nm excitation, 400-nm dichroic, and 405- ± 10-nm/480- ± 10-nm emission filter for indo-1, and 480- ± 10-nm excitation, 500-nm dichroic, >520 nm emission for calcium green. Single-wavelength imaging using calcium green was found to provide the strongest signal and allow for the most rapid temporal resolution, but it is important to stress that this single-wavelength technique provides more qualitative data on $[Ca^{2+}]_i$ levels. Rigorous quantitative measurement was afforded by the indo-1 ratiometric analysis.

Touch Stimulation

Roots that had been successfully acid-loaded or microinjected with indo-1 were placed on the stage of the confocal microscope and ratio-imaged as outlined above. A touch stimulus was applied to the roots as four to five pulses of the growth medium delivered from a micropipette, tip diameter 2 to 3 μm, pulled from 1.5-mm external diameter borosilicate filament electrode glass (World Precision Instruments, New Haven, CT) using a pipette puller (PC-84, Sutter Instruments, Novato, CA). These pulses were 20-p.s.i. each, regulated by a pneumatic picopump (PV830, World Precision Instruments, Sarasota, FL). Confocal ratio images were collected before and immediately after applying the touch stimulus. Subsequent images were collected in intervals of 30 s for 10 min.

Indo-1 and Calcium Green Ca^{2+} Calibration

Ratio images and calcium green measurements were calibrated using Ca^{2+} calibration standards from Molecular Probes (Eugene, OR) and in vitro calibration according to the method of Gilroy (1996). Confirmation of the applicability of this in vitro calibration to in vivo data was made by incubating indicator-loaded roots with 20 μM Ca^{2+}-ionophore Br-A23187 with either 2 mM external Ca^{2+} or 5 mM EGTA. Ratio and calcium green images of these roots showed the expected trends in changes in fluorescence values predicted from the in vitro calibration. When treated with 20 μM Br-A23187 in the presence of 2 mM extracellular Ca^{2+} the entire root showed an elevation in $[Ca^{2+}]_i$ levels to >1 μM. Similarly, the addition of ionophore and 5 mM EGTA lowered the $[Ca^{2+}]_i$ in the whole root to basal levels (approximately 40 nM). The addition of ionophore plus 1 mM $MnCl_2$ led to the expected quenching of intracellular dye. We take these observations as an indication that we can detect changes in $[Ca^{2+}]_i$ levels in the root.

RESULTS

Indo-1 Reports Cytoplasmic Ca^{2+} Levels

Before using indo-1 to monitor the $[Ca^{2+}]_i$ changes occurring during the touch and gravitropic response of Arabidopsis roots, we needed to ensure that this indicator was localized to the cytoplasm. Several criteria were used to ensure that this was the case for indo-1. First, roots that were acid-loaded with indo-1 and then plasmolyzed in growth medium supplemented with 500 mM sorbitol showed that the indo-1 was localized to the plazmolyzed

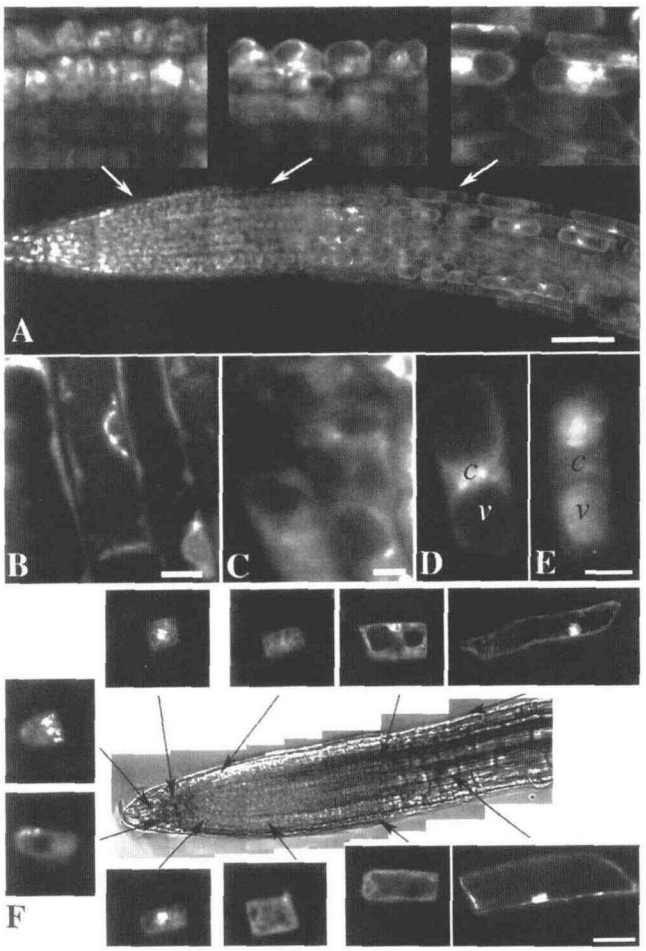

Figure 1. Cytoplasmic indo-1 distribution in acid-loaded and microinjected roots of Arabidopsis. Roots were acid-loaded with indo-1 (A–C) or microinjected with indo-1-dextran (D–F) and distribution of dye was monitored by confocal imaging. A, An acid-loaded root plasmolyzed for 30 min in 500 mM sorbitol, showing that indo-1 was localized to the cytoplasm. Fluorescence images of nonplasmolyzed cells in the elongation zone (B) and peripheral cap (C) acid-loaded with indo-1. Fluorescence images of cells microinjected with indo-1 in the postmitotic zone region indicating cytoplasmic (D) and vacuolar (E) localization of dye. *c*, Cytoplasm; *v*, vacuole. F, Cytoplasmic distribution of microinjected indo-1 dextran in cells located in different regions of the root. The figure is a montage of injected cells from many roots illustrating the appearance of microinjected cells with respect to their location in the root. For actual experiments only one cell was injected per root to minimize stress due to impalement. Scale bars, 100 μm (A); 10 μm (B–F).

protoplast and did not accumulate in the cell wall (Fig. 1A). Confocal imaging of nonplasmolyzed, indo-1-loaded roots also indicated that the dye did not accumulate in the apoplast, the cell walls appearing as dark lines between the brightly fluorescent indo-loaded cells (Fig. 1, B and C). These images also show that indo-1 was excluded from the vacuole. Vacuolar loading appeared as a distinct and easily recognizable feature of dye distribution when it occurred (compare Fig. 1, D and E). Epidermal and cortical cells in the meristematic, elongating, and maturing regions could be effectively acid-loaded with indo-1. The distribution of indo-1 in cells that had been acid-loaded and in cells microinjected with indo-1 or indo-1 conjugated to a 10-kD dextran were also similar (compare Fig. 1, A and F). Punctate regions of fluorescence were noted, but it is unclear whether these reflected organelle uptake or the complex structure of the cytoplasm (Read et al., 1992). However, dextran-conjugated dyes are thought to remain in the cytoplasm, suggesting that the acid-loaded indo-1 was likewise located in the cytoplasm. Indo-1-dextran microinjected into the peripheral root cap or into meristematic or elongation zone cells also indicated the same resting $[Ca^{2+}]_i$ (128 ± 45 nM, $n = 19$) as acid-loaded roots (146 ± 39 nM, $n = 28$), again suggesting that these indicators were in the same subcellular compartment, the cytoplasm. These indicator distributions did not change with gravistimulation of the root for as long as we observed (up to 12 h of gravistimulation). Thus, taken together, these results suggest that acid-loaded indo-1 was taken up into the cytoplasm of the root cells and remained there during the entire experimental period.

The Cytoplasmic Indo-1 Is Responsive to Ca^{2+}

We next established whether acid-loaded and microinjected indo-1 could be used to visualize any $[Ca^{2+}]_i$ gradients across the root that may form upon touch or gravistimulation. At the whole-organ level a detailed in vivo calibration proved difficult to perform. The same problem was reported by Cramer and Jones (1996) and could well reflect problems such as incomplete ionophore penetration or highly active Ca^{2+} homeostasis at the whole-tissue level. However, we were able to confirm the Ca^{2+} responsiveness of acid-loaded indo-1 by treating roots with 20 μM Ca^{2+} ionophore Br-A23187 with 2 mM extracellular Ca^{2+}. This increased the $[Ca^{2+}]_i$ levels to >1 μM from a homogeneous resting $[Ca^{2+}]_i$ of approximately 100 nM (Fig. 2A). We were concerned whether the dye could also resolve increases to intermediate $[Ca^{2+}]_i$ levels, as well as determining the minimum increase from the resting levels that could be resolved. We therefore performed an in vivo calibration in single epidermal cells microinjected with dextran-conjugated indo-1. These single cells proved amenable to an in vivo calibration, and ratio images showed the expected changes in values predicted from the in vitro calibration. This analysis also revealed that we could reliably resolve $[Ca^{2+}]_i$ changes from the resting level of approximately 150 to 300 nM (Fig. 2B).

Roots Do Not Develop a Steady-State Gradient in Cytoplasmic Ca^{2+} in Response to Gravity

Figure 3 shows the time course of the graviresponse of indo-1 acid-loaded and control (unloaded) Arabidopsis roots. Prior to gravistimulation roots were grown vertically for up to 2 h after acid-loading or microinjection. A mean final angle of 80° was reached after 12 h of continuous gravitropic stimulation for both indo-1-loaded and control roots (Fig. 3). The highest rate of curvature was observed during the first 1 h of stimulation with a rate of 20° h^{-1}. Figure 4 shows that vertically oriented roots of wild-type Arabidopsis have a homogeneous distribution of $[Ca^{2+}]_i$ of approximately 100 nM, which does not change with time of vertical growth for as long as we have followed them (up to 12 h). Very occasional spontaneous increases in $[Ca^{2+}]_i$ up to 500 nM in the elongation zone were noted in these vertically entrained plants (3/39 roots). The occurrence of these changes was not predictable, was limited to a small patch of 3 to 4 cells, lasted less than 30 s (the time between each confocal image), and showed no obvious correlation with the duration of growth, growth rate, or gravistimulus. Such spontaneous transients have been noted previously from studies with aequorin transformed plants but are of unknown significance (Sedbrook et al., 1996).

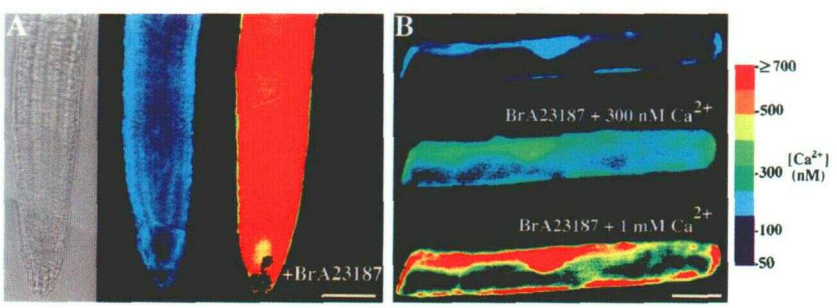

Figure 2. Effect of Br-A23187 on $[Ca^{2+}]_i$ levels in roots acid-loaded or microinjected with indo-1. A, Montage of confocal images of acid-loaded root before and after treatment with 20 μM Br-A23187. B, Elongation zone cell microinjected with indo-1-dextran and imaged before and after treatment with 20 μM Br-A23187 and 300 nM external Ca^{2+} or 20 μM Br-A23187 and 1 mM external Ca^{2+}. Three hundred nanomolar external Ca^{2+} was set using Ca^{2+} standards from Molecular Probes containing 5 mM EGTA. BrA23187 was added from a 1 mM stock in DMSO. Controls using the same final DMSO concentration showed no effect on $[Ca^{2+}]_i$. Ratio images have been pseudocolor coded according to the inset scale. Scale bar, 100 μm (A); 10 μm (B).

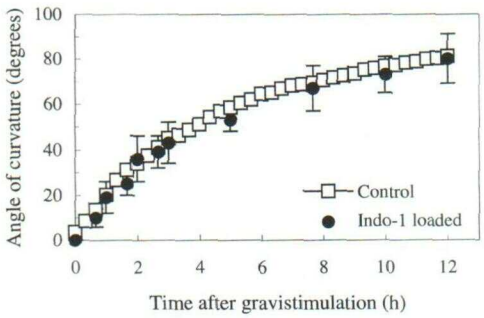

Figure 3. Curvature of control and indo-1-loaded *Arabidopsis* roots continuously gravistimulated for 12 h. The kinetics of graviresponse of indo-1-loaded roots was similar to nonloaded controls. Each data point represents a mean ± SE of at least 10 roots.

Upon horizontal stimulation, the roots showed a gravitropic response (Fig. 3). However, at no time did we observe an asymmetry in $[Ca^{2+}]_i$ using confocal ratio analysis of either acid-loaded or microinjected material in any zone of the root during the initial 1 min, time of fastest bending (1 h after gravistimulation), or up to 8 h of gravistimulation, by which time vertical reorientation of the root was almost complete (Fig. 4). Detailed quantitative analysis comparing the upper and lower sides of the elongation zone, postmitotic zone, and peripheral cap at various times after gravistimulation also failed to reveal changes or an asymmetry in $[Ca^{2+}]_i$ (Fig. 5).

Because of the time required to place the root on the confocal microscope stage and take the first image, the earliest measurement of $[Ca^{2+}]_i$ distribution we were able to make using the confocal approach was between 30 s and 1 min after gravistimulation. Also, the root had to be laid on the horizontal confocal microscope stage to take the images, potentially compromising the original gravity stimulus. Although the confocal approach allowed very high spatial resolution of responses, it was limited to detecting relatively long-term gradients (lasting >1 min) associated with the graviresponse. Very rapid transients would not be detected. To overcome this limitation we constructed an epifluorescent microscope mounted horizontally, such that its microscope stage was vertical. The stage was rotatable and thus we were able to continuously monitor fluorescence from the loaded root as it was rotated from growing vertically to the horizontal, gravistimulated position. This microscope lacked the spatial resolution of the confocal approach but allowed superior temporal resolution (10 s per data point). However, because this vertical stage microscope lacked the spatial resolution to identify individual cells against the fluorescent background of acid-loaded tissue we had to utilize microinjected roots to be able to resolve the cell from which the $[Ca^{2+}]_i$ response was being monitored.

Figure 6 shows the kinetics of curvature and elongation of the upper versus the lower surface of *Arabidopsis* roots mounted in the vertical stage epifluorescence microscope. Differential elongation between the upper and lower flank of the roots could be detected 15 min after horizontal reorientation (Fig. 6A) and by 1 h the angle of curvature was about 15° (Fig. 6B). Using the vertical stage microscope we followed the responses of $[Ca^{2+}]_i$ in the columella, peripheral cap, meristematic cells, postmitotic zone cells (approximately 300 μm from the tip), and cells from the upper and lower sides of the elongation zone (approximately 700–1000 μm from the tip) for up to 1 h after gravistimulation. Initially, images were collected every 10 min for 30 min in vertically growing roots, after which time the stage was rotated so that roots were reoriented by 90°. Images were taken immediately after this gravistimulation and every 30 s thereafter. In agreement with the confocal microscope data, in no case did we observe any changes in $[Ca^{2+}]_i$ associated with the graviresponse in any of the cell types tested (Fig. 7).

To determine whether localized changes in $[Ca^{2+}]_i$ occurred during the graviresponse, or if an unchanging signal from the nucleus biased our whole-cell measurement toward no changes, the signal from fluorescence from different regions of the cytoplasm of each individual cell was measured. Even at this level, we were unable to detect any changes in $[Ca^{2+}]_i$ associated with gravistimulation (Fig. 7, right panels). In addition, to minimize any effects of potential impalement artifacts in the microinjected cells, flu-

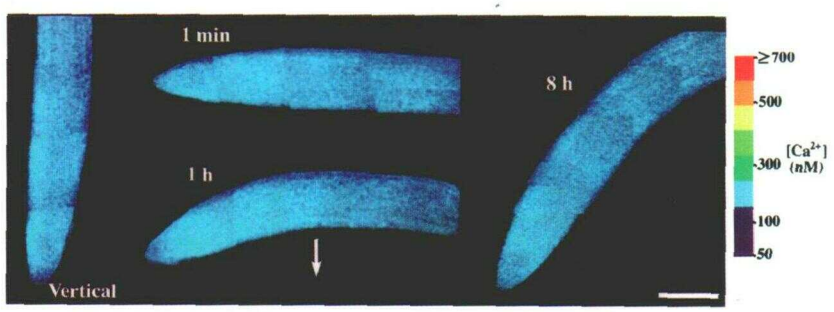

Figure 4. Effect of gravistimulation on $[Ca^{2+}]_i$ levels in roots of *Arabidopsis* acid-loaded with indo-1. Roots were acid-loaded with indo-1 and gravistimulated, and confocal ratio images were taken at the indicated times. No asymmetry in $[Ca^{2+}]_i$ was observed in the cap, meristematic, and elongation zone at any time point during the graviresponse. Montaged images are shown for vertical control roots and different roots imaged at 1 min, 1 h, and 8 h after gravistimulation. These images are representative of the $[Ca^{2+}]_i$ distribution seen during the entire gravistimulus time course (12 h). Ratio images have been pseudocolor coded according to the inset scale. Arrow indicates the gravity vector. Each image is representative of $n \geq 14$. Scale bar, 100 μm.

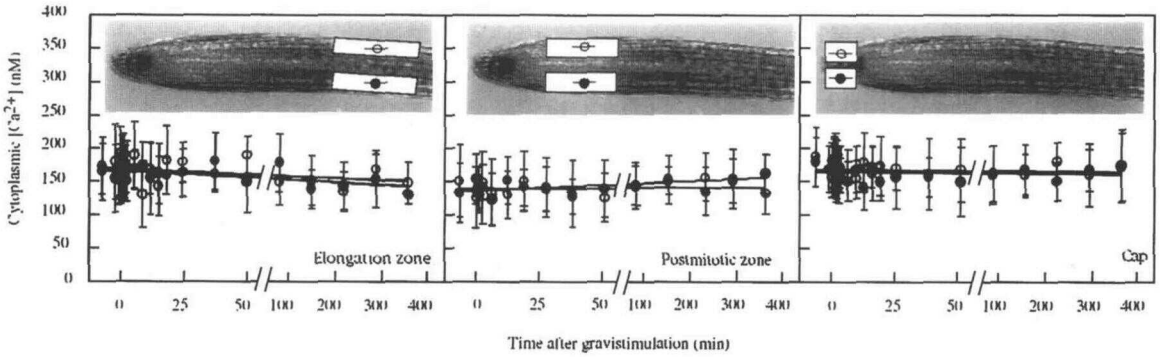

Figure 5. Cytoplasmic Ca^{2+} levels along the upper and lower sides of gravistimulated roots. Roots were acid-loaded with indo-1, and $[Ca^{2+}]_i$ was monitored by confocal ratio imaging. Ca^{2+} concentrations are the mean values calculated from the boxed regions. Results are representative of $n \geq 14 \pm$ SE.

orescence from dye-coupled cells (see insets for meristem and postmitotic cells) was also analyzed. These cells loaded by dye coupling also showed no change in $[Ca^{2+}]_i$ associated with gravistimulation.

Roots Show a Transient Increase in Cytoplasmic Ca^{2+} in Response to Touch Stimulation

Having established the apparent lack of a change in $[Ca^{2+}]_i$ associated with the gravitropic response we used the touch response to determine the sensitivity of our measurement approach. Touch stimulation elicited a relatively prolonged (2–5 min) transient increase in $[Ca^{2+}]_i$ at the cellular level in all regions of the root tested, including the cap and the meristematic, elongation, and differentiated zones (Figs. 8 and 9). The touch stimulus led to elevated $[Ca^{2+}]_i$ that moved away from the site of the stimulus in what may be a propagating wave of Ca^{2+}. The increase in $[Ca^{2+}]_i$ was transient, lasting approximately 2 to 5 min in each cell but appearing at adjacent cells with an apparent speed of 25 to 50 μm min^{-1}, and a maximum range from the point of stimulus of up to 250 μm (Fig. 8B). The transient increases in $[Ca^{2+}]_i$ in the peripheral cap cells (Fig. 8C) were larger and more prolonged relative to the response in the elongating cells (Fig. 8D) and cells in the other zones.

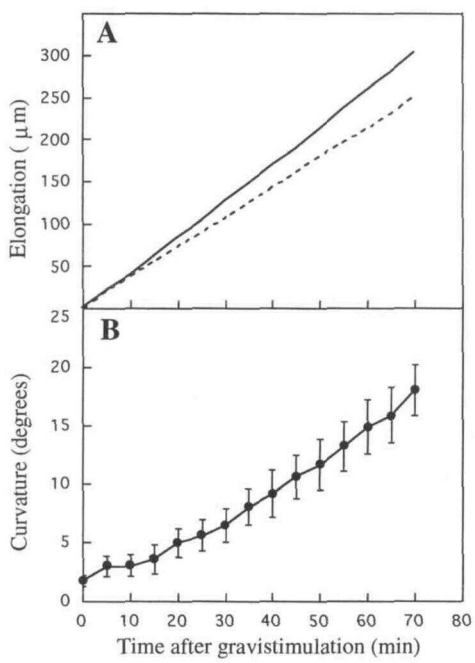

Figure 6. Graviresponse of Arabidopsis roots mounted in the vertical stage epifluorescence microscope. A, Differential elongation between the upper (solid line) and lower (broken line) surface can be detected within 15 min after gravistimulation. B, Curvature of roots mounted in the vertical stage microscope. Each data point represents a mean $\pm$ SE, $n \geq$ seven roots.

The touch stimulus applied was from four to five pulses of growth medium delivered as 20-p.s.i. pulses from a micropipette (3-μm tip diameter) placed approximately 20 μm from the root surface. Higher pressures or more pulses did not elicit a larger response (data not shown). Whereas approximately 50% of cap cells tested (7/12) responded to two pressure pulses with a full Ca^{2+} transient (Fig. 9, A–C), fewer than four pulses failed to elicit any $[Ca^{2+}]_i$ response in 80% of the elongation, meristematic, and differentiated zone cells tested (Fig. 9, D–F).

These touch-related increases in $[Ca^{2+}]_i$ were abolished by 5 mM EGTA and 50 μM ruthenium red, a putative inhibitor of intracellular Ca^{2+} stores (Denton et al., 1980; Knight et al., 1992), but were unaffected by up to 100 μM of the plasma membrane channel blocker verapamil or 1 mM La^{3+} (Fig. 10). Repeated stimuli led to repeated transients, with a refractory period of 3 to 5 min (Fig. 11).

Although these stimuli led to large changes in $[Ca^{2+}]_i$, no detectable thigmotropic curvature away from or toward the stimulus was evident upon subsequent root growth. In addition, there was no detectable change in the rate of root growth after the touch stimulus (data not shown)

DISCUSSION

Ca^{2+} ions have been suggested to play a role in the gravitropic response of roots (for review, see Belyavskaya [1996]). However, the evidence has been indirect and sometimes contradictory. For example, unilateral application of

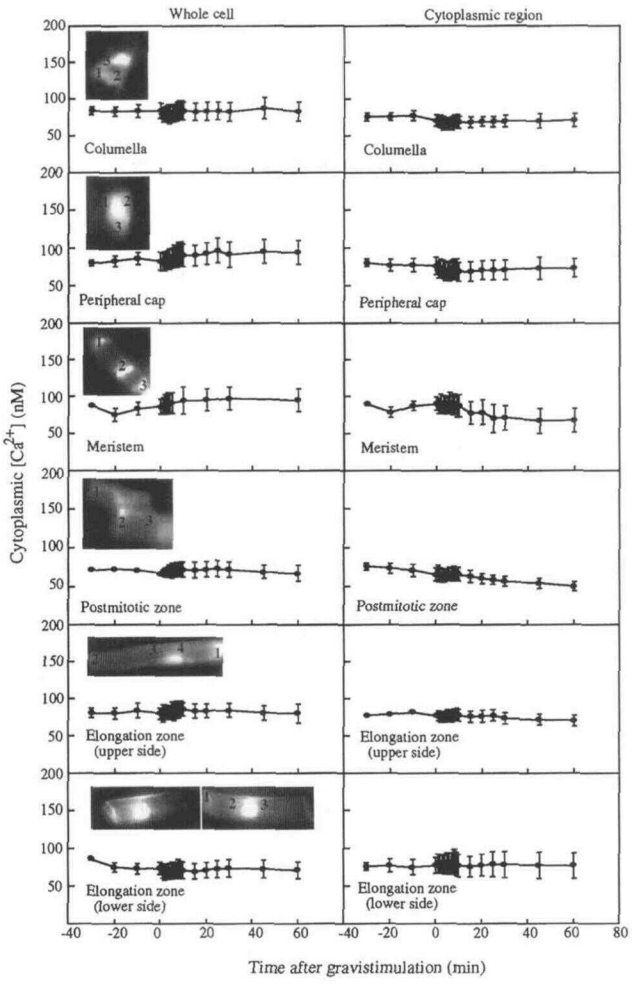

Figure 7. Effect of gravistimulation on $[Ca^{2+}]_i$ levels in individual root cells of Arabidopsis. Cells from the different regions of the root were microinjected with calcium green-1 and imaged in a vertical stage epifluorescence microscope. After microinjection, roots were positioned vertically for 1 h to allow recovery from the microinjections prior to gravistimulation. Insets show examples of fluorescence images of microinjected cells as seen with the vertical stage microscope. Analysis of whole cell fluorescence indicates no changes in $[Ca^{2+}]_i$ in the columella, upper and lower sides of the peripheral cap, and meristematic, postmitotic, and elongation zones (left panel). Numbers in the insets indicate cytoplasmic regions of the cell analyzed for changes in fluorescence which also show no changes in $[Ca^{2+}]_i$ (right panel). Lowermost inset in the left panel shows an elongating cell immediately after microinjection (left) and 25 min after injection (right). In addition to criteria for viability of microinjected cells, fluorescence from dye-coupled cells (see insets for meristem and postmitotic cells) was also analyzed to minimize any artifact as a result of cell impalement. Results are representative of $n \geq 6 \pm$ SE.

Ca^{2+} via agar blocks, which has often been cited as evidence for a role of Ca^{2+} asymmetries in the root graviresponse, do not always lead to the same result (Hasenstein et al., 1988; Ishikawa and Evans, 1992; Takahashi et al., 1992). Ca^{2+} transport studies have shown the preferential polar transport of $^{45}Ca^{2+}$ toward the lower side of graviresponding maize roots (Lee et al., 1983b), but no asymmetry in free Ca^{2+} between upper and lower sides of the roots could be detected (Dauwalder et al., 1985).

Although most of the evidence outlined above could also point to apoplastic Ca^{2+} fluxes as a factor during gravitropism, $[Ca^{2+}]_i$ has often been proposed to be a primary player in the perception/transduction phase of root gravitropism (Evans et al., 1986; Poovaiah et al., 1987; Belyavskaya, 1996). However, there has been no report on $[Ca^{2+}]_i$ dynamics in intact, graviresponding roots. To clarify this issue we applied confocal ratio analysis and vertical stage fluorescence microscopy to image changes in $[Ca^{2+}]_i$ during the graviresponse of roots. Our results show that long-lived (i.e. longer than 5–10 s) asymmetries or localized transients in $[Ca^{2+}]_i$ do not develop in the root cap or in the meristematic and elongation zones upon gravistimulation in roots. Gehring et al. (1990) reported a relatively small increase in $[Ca^{2+}]_i$ from 255 to 370 nM within 3 min of gravistimulation of maize coleoptiles. This increase remained constant for the entire period of their experiment (9 min). The apparent contradictions between this present study and that of Gehring et al. (1990) may be explained by the use of different plant organs (roots versus coleoptiles), with the transduction of the gravistimulus in roots differing from aerial tissues. However, Gehring et al. (1990) were only able to image changes occurring on the lower side of the graviresponding organ. The absence of data on $[Ca^{2+}]_i$ changes along the upper, slower-growing side of the coleoptile makes interpretation of their results complex.

Among all regions of the root we have examined, the region where changes in $[Ca^{2+}]_i$ were most expected to occur was in the columella cells. These cells have been proposed to perceive gravity (Sack, 1991) and have received the greatest speculation as a site of Ca^{2+}-dependent signaling (Evans et al., 1986; Poovaiah et al., 1987; Belaysvkaya, 1996). There have been some attempts to quantify $[Ca^{2+}]_i$ in columella cells. Kiss et al. (1991) showed that isolated protoplasts from cap cells of maize roots have lower $[Ca^{2+}]_i$ levels compared with protoplasts from the elongation zone. They interpreted these findings as a way by which cap cells, because of their lower resting $[Ca^{2+}]_i$ levels, may be more sensitive to an influx of Ca^{2+}. Ca^{2+} signals have also been reported to be high in the amyloplasts of columella cells (Chandra et al., 1982; Busch et al., 1993) and these results have been used to suggest a role for $[Ca^{2+}]_i$ in the perception of the gravity signal (Belyavskaya, 1996). However, like other regions of the root examined, we could detect no changes in $[Ca^{2+}]_i$ in columella cells microinjected with either indo-1 or calcium green-1 after gravistimulation (Fig. 7).

Measurements obtained from the columella cells were the most technically demanding in this study and therefore may not completely rule out changes in $[Ca^{2+}]_i$ being involved in the perception phase of gravitropism. It is possible that $[Ca^{2+}]_i$ changes are very highly localized, e.g. within the immediate area around the amyloplasts and ER. High Ca^{2+} signals have been reported to occur in these regions using ion microscopy (Chandra et al., 1982; Busch et al., 1993). Such highly localized changes may have been beyond the resolution of the techniques we used. Further-

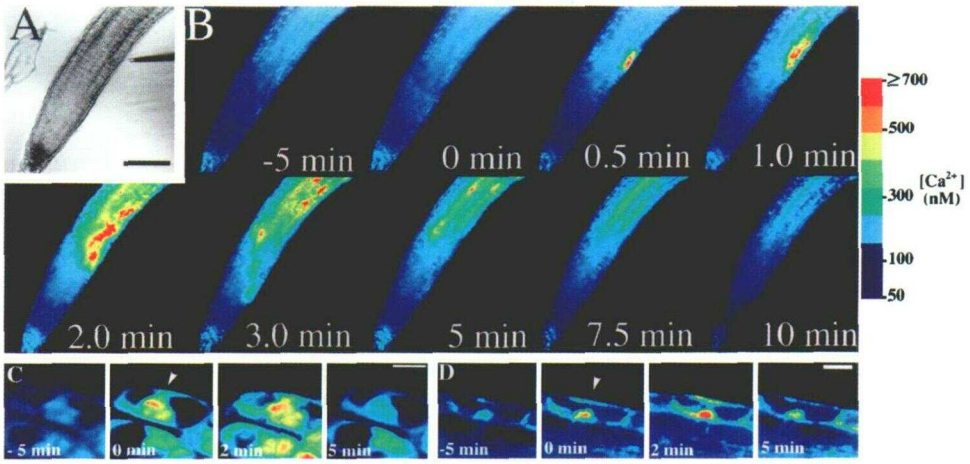

Figure 8. Effect of touch stimulus on $[Ca^{2+}]_i$ levels in Arabidopsis roots. Roots were acid-loaded with indo-1 and $[Ca^{2+}]_i$ was monitored by confocal ratio imaging. A, The touch response was elicited by applying four, 20-p.s.i. pressure pulses from a micropipette adjacent to the root. Confocal ratio images of whole-root response (B), cells in the periphery of the root cap (C), and epidermal cells along the elongation zone (D). Images were taken at the indicated time after the touch stimulus (arrowhead). Ratio images have been pseudocolor coded according to the inset scale. Scale bar, 100 μm (A and B); 10 μm (C and D).

more, very rapid changes (less than a second) would be below the temporal resolution of the techniques employed. The columella cells also represented the most difficult cells to image and load. Microinjection of these cells allowed us to monitor Ca^{2+} levels during the gravistimulus, but to load dye into the cells, the microinjection pipette had to be pushed through the overlying cell layer. Although this treatment did not inhibit the graviresponse of the root, we cannot discount the possibility that a wounding artifact from these cells may have masked any rapid $[Ca^{2+}]_i$ changes in columella cells. However, despite these caveats, the data presented strongly suggest that sustained asymmetries and changes in $[Ca^{2+}]_i$ of longer than 5 to 10 s in duration do not develop in Arabidopsis root cells in response to gravity.

It is possible that changes in the sensitivity of the root may explain the absence of changing $[Ca^{2+}]_i$ that we report in this study. The concept of changing sensitivity has been used to explain the involvement of auxin in plant gravitropism (Salisbury, 1993), since asymmetries in auxin do not always develop during the graviresponse (Firn and Digby, 1980). It is of interest that Sinclair et al. (1996) have noted increases in CaM mRNA levels associated with the graviresponse in Arabidopsis. In addition, the higher CaM levels that have been reported in the cap (Stinemetz et al., 1987) and the strong association of CaM with amyloplasts (Dauwalder et al., 1986) could enhance the sensitivity of the columella to basal levels or even small or highly localized changes in $[Ca^{2+}]_i$. The concept that increases in CaM levels or activity could increase the sensitivity of cells to $[Ca^{2+}]_i$ is

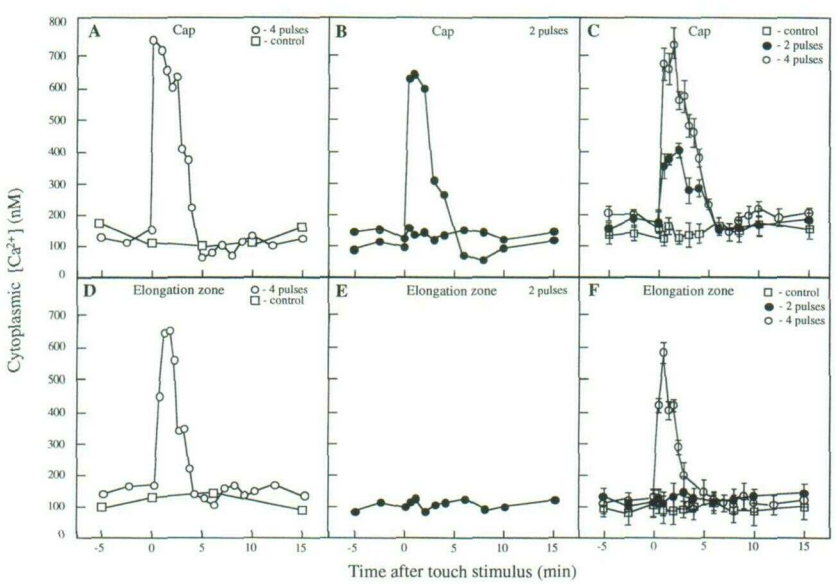

Figure 9. Effect of touch on $[Ca^{2+}]_i$ levels in root cells of Arabidiposis. Roots were acid-loaded with indo-1, and $[Ca^{2+}]_i$ monitored in single peripheral cells of the root cap (A–C) and epidermal cells in the elongation zone (D–F) by confocal ratio imaging. The touch response was elicited by applying two or four, 20-p.s.i. pressure pulses from a micropipette adjacent to the cell. A, B, D, and E, Single-cell responses; C and F, mean cellular response ± SE, $n > 7$. Note some cap cells can be elicited by two pressure pulses.

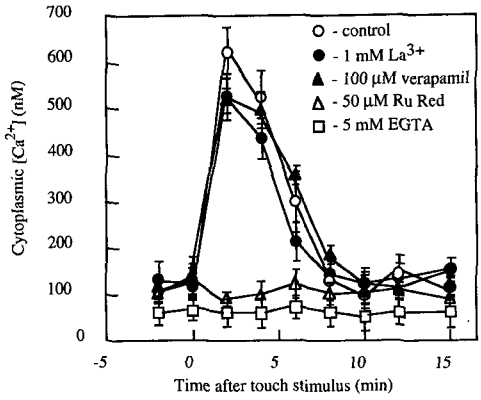

Figure 10. Effect of Ca^{2+} channel inhibitors on touch induced Ca^{2+} transients in root cap cells. Roots were acid-loaded with indo-1, and $[Ca^{2+}]_i$ was monitored in single cells of the root cap periphery by confocal ratio imaging. The touch response was elicited by applying four, 20-p.s.i. pressure pulses from a micropipette adjacent to the cell. Roots were pretreated for 20 min with the relevant inhibitor. Results are representative of $n \geq 5 \pm$ SE.

consistent with reports showing that application of CaM antagonists inhibit polar Ca^{2+} and auxin (Stinemetz et al., 1992) transport across the root as well as the graviresponse (Stinemetz et al., 1992; Sinclair et al., 1996). Elevating CaM levels has also been shown to decrease the Ca^{2+} requirement for enzyme activation in vitro (Cox, 1986). Lu et al. (1996) have identified a Ca^{2+}/CaM-dependent protein kinase in maize roots that may well be involved in light-regulated gravitropism, further implicating CaM in the graviresponse.

It has been proposed that touch and gravity-related responses may be closely linked (Trewavas and Knight, 1994). Thus touching a root can elicit tropic curvature (Ishikawa and Evans, 1992) or even interrupt growth for several hours (Hanson and Trewavas, 1982). Although the precise mechanism whereby touch is transduced to tropic or developmental events is unknown many studies have highlighted the role of $[Ca^{2+}]_i$ in this process (for review, see Trewavas and Knight [1994]; Knight et al., 1994). We therefore characterized $[Ca^{2+}]_i$ changes during the touch response in Arabidopsis roots to compare them with the gravity response. Touch stimulus led to a cell type-specific Ca^{2+} transient signature in all cell types tested. The cap cells seem much more sensitive to touch than other regions of the root (Fig. 8C). This may reflect the important sensory role for the cap, which will be the first region of the root to encounter obstacles and new environments as the root explores the soil.

Touch-induced transients observed in other plants have been monitored at the tissue level using aequorin-transformed plants (Knight et al., 1991, 1992, 1994; Trewavas and Knight, 1994). These transients are generally 10 to 20 s in duration, i.e. faster than those we describe. These differences could be inherent in the different measurements systems, reflect some degree of Ca^{2+} buffering by the dyes we have used, be associated with the different ways the mechanical stress was applied, or be due to the tissue or whole-seedling level of aequorin measurements relative to the cellular resolution afforded by fluorescence ratio imaging.

Even though the precise kinetics of the touch-related transients seen here are different from those seen in aequorin plants, the other characteristics are similar. Thus inhibitors of Ca^{2+} fluxes across the plasma membrane (lanthanum and verapamil) did not prevent these changes, even at concentrations that have physiological effects in the root (inhibition of elongation; S. Gilroy, unpublished observations) or prevent root hair growth (Wymer et al., 1997). However ruthenium red, a putative blocker of intracellular Ca^{2+} fluxes (Denton et al., 1980; Knight et al., 1992) blocked the touch-induced changes. These results suggest an intracellular site for the released Ca^{2+} and that mechanosensitive Ca^{2+} channels in the plasma membrane (Ding and Pickard, 1993) may not be the primary site of mechanoperception in the root cells. This intracellular response system is uncharacterized but cytoskeletal links transmitting forces in the wall to intracellular Ca^{2+} represent attractive candidates for future study.

The touch-elicited $[Ca^{2+}]_i$ transients also show a distinct refractory period of 3 to 5 min, over which time repeated stimulation leads to progressively lower transients until none can be elicited. This refractory period is similar to the

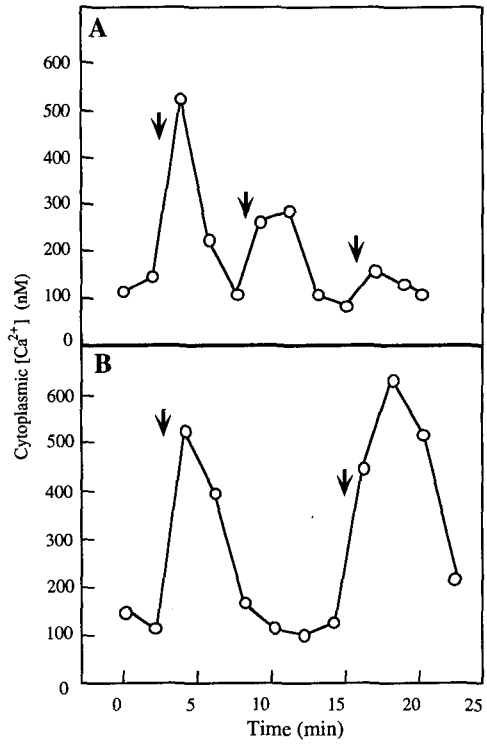

Figure 11. Latent time for Ca^{2+} transients in root cap cells in response to the touch stimulus. Roots were acid-loaded with indo-1, and $[Ca^{2+}]_i$ was monitored in single cells in the cap periphery by confocal ratio imaging. The touch response was elicited by applying four, 20-p.s.i. pressure pulses (arrows) from a micropipette adjacent to the cell. Touch stimulus was applied at 5-min (A) and 10-min (B) intervals. Note that the shorter time between touch stimuli decreases the magnitude of the response with each succeeding stimulus. Results are representative of five independent experiments.

1-min refractory period reported for the seedling response to touch (Knight et al., 1992). Again tissue, stimulus, and measurement technique differences may lead to qualitative differences between these and our observed responses. A refractory period is consistent with the refilling of intracellular stores that are discharged to support the initial transient. The nature of these stores is unknown but ER and vacuole are potential candidates.

CaM (*TCH-1*) also belongs to a class of touch-induced (*TCH*) genes, which also encode CaM-like proteins (*TCH-2* and *TCH-3*) (Braam and Davis, 1990; Ling et al., 1991; Braam, 1992; Perera and Zielinski, 1992; Sistrunk et al., 1994; Takezawa et al., 1995) and a xyloglucan endotransglycosylase (*TCH-4*; Xu et al., 1995). Induction of *TCH-3* is blocked when stimulus-evoked changes in $[Ca^{2+}]_i$ are blocked (Polisensky and Braam, 1996), suggesting one role for touch-induced $[Ca^{2+}]_i$ changes may be the regulation of gene transcription. Touch is also known to have a systemic effect on plant growth and the wave of $[Ca^{2+}]_i$ changes seen in Figure 8 may be showing the propagation of just such a systemic signal. Similar waves of $[Ca^{2+}]_i$ may underlie systemic signaling in cold-shocked plants (Campbell et al., 1996) and cold shock is also known to induce TCH gene transcription (Polisensky and Braam, 1996). In animal cells, propagating waves may be elicited by phosphoinositide-signaling cascades or Ca^{2+}-induced Ca^{2+} release (Jaffe, 1993), with the wave-like propagation reflecting the refractory period for refilling of the Ca^{2+} stores after the initial discharge. The touch-elicited $[Ca^{2+}]_i$ changes we have observed in roots bear some of these features in that the propagation appears to be via mobilization of intracellular stores that show a distinct refractory period after emptying (Fig. 11). Both Ca^{2+}-induced Ca^{2+} release and inositol phosphate-signaling systems have been observed to be active in plants (Gilroy et al., 1990; Drobak, 1993). Further work will be required to define whether one of these systems underlies the propagation of the touch-related $[Ca^{2+}]_i$ changes in roots.

In summary we have characterized the $[Ca^{2+}]_i$ dynamics in Arabidopsis root cells in response to gravity and touch. The graviresponse of roots is not accompanied by any detectable increases in $[Ca^{2+}]_i$. The touch response is accompanied by large increases in $[Ca^{2+}]_i$ that are characteristic of different root cell types and that propagate throughout the root. These results suggest that changes in $[Ca^{2+}]_i$ may not be a central component of the graviresponse, or that if changes do occur they could be so small, rapid, or highly localized as to be undetectable by the techniques we employed. Future work will test this possibility using lipophilic derivatives of Ca^{2+} indicators to monitor Ca^{2+} levels close to the plasma and organellar membrane during the gravity response.

Supplemental Quicktime movie sequences of the touch response are available at www.bio.psu.edu/faculty/gilroy/touch.html.

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