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Changes in Root Cap pH Are Required for the Gravity Response of the Arabidopsis Root

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Although the columella cells of the root cap have been identified as the site of gravity perception, the cellular events that mediate gravity signaling remain poorly understood. To determine if cytoplasmic and/or wall pH mediates the initial stages of root gravitropism, we combined a novel cell wall pH sensor (a cellulose binding domain peptide–Oregon green conjugate) and a cytoplasmic pH sensor (plants expressing pH-sensitive green fluorescent protein) to monitor pH dynamics throughout the graviresponding Arabidopsis root. The root cap apoplast acidified from pH 5.5 to 4.5 within 2 min of gravistimulation. Concomitantly, cytoplasmic pH increased in columella cells from 7.2 to 7.6 but was unchanged elsewhere in the root. These changes in cap pH preceded detectable tropic growth or growth-related pH changes in the elongation zone cell wall by 10 min. Altering the gravity-related columella cytoplasmic pH shift with caged protons delayed the gravitropic response. Together, these results suggest that alterations in root cap pH likely are involved in the initial events that mediate root gravity perception or signal transduction.

INTRODUCTION

Root gravitropism requires a coordination and interaction of cells responsible for gravity perception, signal transduction, signal transmission, and the growth response. The gravity perception step occurs in the columella cells of the root cap (Sack, 1997; Blancaflor et al., 1998), whereas the growth response (i.e., curvature) is initiated in the distal elongation zone (DEZ) and fully expressed in the central elongation zone (CEZ) (Ishikawa et al., 1991; Ishikawa and Evans, 1993). Thus, for curvature to occur, a signal must move from the gravity-sensing columella cells to the graviresponding cells of the elongation zone. Although there is a wealth of literature implicating the sedimentation of amyloplasts in the columella cells as one of the initial gravity-sensing events (Sack, 1997) and auxin as a factor mediating the growth response (reviewed by Chen et al., 1999; Rosen et al., 1999), the nature of the signal transduction events in the root cap that lead to the growth response have remained elusive.

The molecular mechanisms that mediate the growth response in the elongating cells of the root also are largely unknown. Indeed, growth control in different regions of the root may be very different. The DEZ is defined as being centered on the region of the root elongation zone showing 30% maximal growth rate, and the CEZ is defined as being centered on the region of maximal growth rate (Ishikawa and Evans, 1993). The current evidence suggests that the growth of the CEZ, but not the DEZ, is regulated in part by auxin (Ishikawa and Evans, 1993; Evans et al., 1994; Mullen et al., 1998) and likely is mediated, at least in part, via acid growth phenomena (Edwards and Scott, 1974; Evans, 1976; O'Neill and Scott, 1983; Collings et al., 1992; Taylor et al., 1996; Büntemeyer et al., 1998; Felle, 1998; Peters and Felle, 1999). For example, both auxins and auxin antagonists cause root growth and the pH of the medium around the root to change in a correlated manner, implying a role for changes in wall pH in growth control (Evans et al., 1980; Moloney et al., 1981; Mulkey and Evans, 1982). In addition, changes in the proton fluxes upon reorientation of the root have been proposed to reflect modulation of the acid growth phenomenon during the gravitropic response (Mulkey and Evans, 1981; Mulkey et al., 1982; Pilet et al., 1983; Versel and Pilet, 1986; Zieschang et al., 1993; Monshausen et al., 1996; Taylor et al., 1996). However, whether the altered spatial and temporal patterns of pH

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fluxes induced by gravistimulation translate to steady state changes in wall pH, and whether similar patterns of pH-related growth regulation occur within the CEZ and DEZ, remain to be determined.

In addition to apoplastic proton fluxes and wall pH, recent data have correlated cytoplasmic pH increases and decreases in root cap cells to reorientation of the root (Scott and Allen, 1999). However, it is unclear if these pH changes reflect gravitropic signaling phenomena. For example, treatments that blocked the cytoplasmic pH increase reported upon gravity stimulation of the root cap cells promoted gravitropism, whereas treatments mimicking the gravity-related pH increase blocked gravitropic responses (Scott and Allen, 1999). These apparent contradictions may reflect the multiple roles of pH in plant cell function, the complex interactions between different regions of the root required for a gravitropic response, and the multiple cellular compartments (such as wall, cytoplasm, and vacuole) in which pH may play a regulatory role.

Therefore, to define the role(s) of pH in root gravitropic signaling and growth systems, we developed approaches to monitor apoplastic and cytoplasmic pH in cells throughout the Arabidopsis root. We then followed the changes in pH associated with these compartments during the graviresponse in the wild type and the gravitropically impaired, starch-deficient *pgm1-1* mutant (Caspar and Pickard, 1989; Sack and Kiss, 1989). In addition, we manipulated pH in specific root cap cells to determine the effect of altering potential gravity-related, pH-dependent signaling events on the gravitropic response of the root.

RESULTS

Mapping Cellular Growth Rates and Graviresponse Kinetics

We first characterized the growth response of the Arabidopsis root to gravistimulation at the cellular level under our growth conditions to more closely correlate any observed pH changes to known cellular behavior. We mapped cellular elongation rates along the root by following the growth of individual cells from time-lapse movies made on a vertical stage microscope using a 40× objective (Figure 1A). Under our growth conditions, we determined that the DEZ and CEZ were 410 and 700 μm from the tip, respectively. We also monitored the gravitropic growth kinetics of the whole root in the wild type and the starchless *pgm1-1* mutant (Figures 1B and 1C) to define the kinetics of the reduced gravitropism of *pgm1-1* under our experimental conditions. Finally, from time-lapse movies of the graviresponse, we were able to define when a detectable tropic growth response (>3° deviation from horizontal growth on our microscope system) was initiated in either the wild type (15 min) or *pgm1-1* (45 min) (Figure 1C).

Cellulose Binding Domain Peptide–Oregon Green Allows Measurement of Apoplastic pH in the Arabidopsis Root

After defining the regions of the root involved in the gravitropic growth response, we next characterized wall pH changes associated with gravitropism in these regions. To measure wall pH, we initially used ratiometric analysis of the pH-sensitive fluorescent dye Nerf 1–dextran and the pH-insensitive dye Texas red–dextran, which were infiltrated simultaneously into the root apoplast (Bibikova et al., 1998). However, these indicators tended to leak from the wall during experiments longer than 5 to 10 min, precluding any extended analysis of wall pH responses (data not shown). To circumvent this problem, we generated a novel cell wall pH probe consisting of the fluorescent pH sensor Oregon green (OG) covalently attached to the cellulose binding domain (CBD) peptide from the cellulase of *Clostridium cellulovarans*. Because the CBD peptide binds to cell walls, the attached pH sensor stayed anchored in the apoplast during the several hours of the Arabidopsis gravitropic response. The CBD-OG showed no evidence of uptake to regions other than the cell wall. For example, Figure 2A shows that CBD-OG injected into the root cap was localized to the wall and excluded from the cytoplasm except in a single cell that was intentionally damaged using a micropipette, allowing the wall probe to enter the cytoplasm (arrowhead). Such cytoplasmic staining was not observed under normal conditions. Once loaded into the cell wall, CBD-OG fluorescence remained detectable for at least 24 hr, the longest period we have studied the CBD-OG–loaded roots.

In vitro calibration showed that CBD-OG had pH-responsive fluorescence emission (>520 nm) with 480-nm excitation and pH-independent fluorescence with 440-nm excitation, making it suitable for ratio analysis (Figures 2B to 2D). A similar pH dependence to the in vitro calibration was observed in vivo (Figure 2C). These calibrations showed that CBD-OG could resolve pH changes over the pH range 3.5 to 5.5 (Figure 2D). CBD-OG had no detectable effect on root morphology, growth rate, the kinetics of the gravitropic growth response, or the kinetics of amyloplast sedimentation in the root cap (data not shown). These observations indicated that CBD-OG would be applicable for monitoring wall pH during the gravity response of the root.

Changes in Apoplastic pH in the Root Cap of Gravistimulated Roots

In vertically growing roots, the wall pH in the columella and the meristematic zone of wild-type plants was maintained at a uniform 5.3 to 5.5 (−20 to 0 min; Figures 3A and 3B). Upon gravistimulation, a small apparent acidification was observed in the meristematic zone, but it was not significant ($P > 0.05$, t test, $n = 32$ cells; Figure 3B). In contrast, gravistimulation was associated with a rapid (within 1 min) and uniform cell wall acidification to pH 4.6 throughout the cell

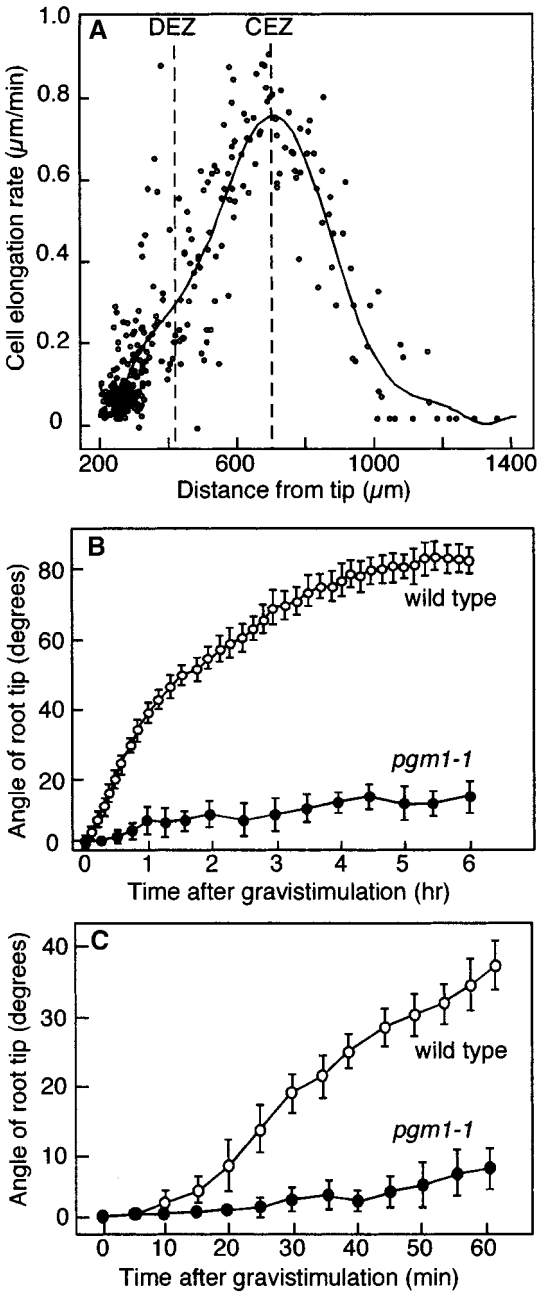

Figure 1. The Kinetics of Root Gravitropism of the Wild Type and the *pgm1-1* Mutant of Arabidopsis.

(A) Epidermal cell elongation rates in vertically growing wild-type roots were determined from movies of root growth monitored at 40× on a vertical stage microscope. The center of the DEZ is defined as the point of 30% maximal elongation rate, and CEZ is defined as maximal elongation rate according to Ishikawa and Evans (1993). $n = 30$ cells from eight separate roots.

(B) Kinetics of the graviresponse of roots of wild-type Arabidopsis and the starch-deficient *pgm1-1* mutant. Roots were grown vertically and then rotated through 90° to gravistimulate. The kinetics of the growth response were monitored for 6 hr. Values are means ±SE, $n = 33$.

(C) High-resolution time course of the first hour of the graviresponse shown in **(B)**.

walls of the cap (Figure 3A and data not shown). There were no detectable differences in either the timing or the magnitude of the wall pH change between the upper and lower sides of the root cap apoplast (data not shown).

To more closely associate these changes in pH with the gravity response system, we repeated these experiments using starch-deficient *pgm1-1* plants. In the vertically growing *pgm1-1* root, patterns of wall pH in the cap and meristem were identical to those in the wild type (Figures 3C and 3D). In addition, as seen in the wild type, wall pH in the meristematic region of the *pgm1-1* root showed no change in response to gravistimulation (Figure 3D). However, although the cap apoplast in *pgm1-1* roots did show a statistically significant acidification after gravistimulation ($P < 0.05$, *t* test, $n = 14$ separate roots) compared with the wild type, this pH decrease was delayed by at least 10 min and reduced in magnitude from 0.6 pH units to ~0.2 units (Figure 3C).

Changes in Apoplastic pH in the Elongation Zone of Gravistimulated Roots

CBD-OG pH measurements in the elongation zone, the zone of root hair differentiation, and the mature zone (>50 μm beyond the last elongating root hair; Figure 4A) indicated that before gravistimulation, wall pH in a vertically growing root was 5.3 ± 0.08 in the DEZ (Figure 4B), 4.8 ± 0.11 in the CEZ (Figure 4C), and 5.5 ± 0.2 in the zone of root hair formation ($n = 40$ cells on eight separate roots). In the mature region of the root, wall pH was uniformly 6.0 ± 0.3 ($n = 38$ cells on eight separate roots).

Upon gravistimulation, the upper side of the DEZ acidified, showing a significant pH decrease ($P < 0.05$, *t* test) from 5.3 ± 0.04 to 4.9 ± 0.1 that was detectable ~10 min after reorientation (Figure 4B). The pH of the lower side remained unchanged at any time after gravistimulation ($P > 0.05$, *t* test, prestimulation versus all times after gravistimulation; Figure 4B). The walls of the CEZ also acidified upon gravistimulation. On the lower flank, gravistimulation was associated with a small but reproducible acidification (4.8 ± 0.07 to 4.65 ± 0.06; $0.05 > P < 0.1$, *t* test). However, the pH decreased on the upper flank of the CEZ from 4.8 ± 0.06 to a minimum of 4.3 ± 0.08 at 20 min after gravistimulation. This pH decrease was detectable after 7.5 min of gravistimulation ($P < 0.05$, *t* test; Figure 4C). The pH of the upper side of the elongation zone (both DEZ and CEZ) returned to

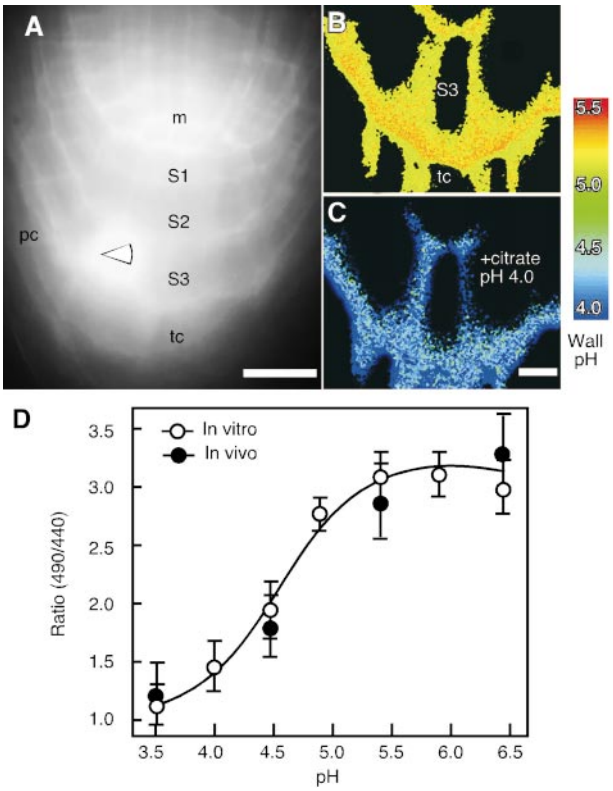

Figure 2. Calibration of the Wall pH Indicator CBD-OG in the Root Cap Apoplast.

(A) Fluorescence from a root tip microinjected with CBD-OG. The indicator was microinjected into the wall by carefully positioning the micropipette on the tip cells of the cap. Note the differential pattern of staining in the cell purposely killed by impalement with a micropipette (arrowhead). m, meristem; S1, story 1 columella; S2, story 2 columella; S3, story 3 columella; pc, peripheral cells; tc, tip cells. Bar = 40 μm.

(B) Ratio image of CBD-OG fluorescence in the apex of a vertically growing wild-type root. The image was pseudocolor coded according to the scale at right.

(C) Ratio image of in vivo calibration of CBD-OG in which wall pH was clamped to pH 4.0 using 50 mM citrate. Note the homogeneous change in wall pH with citrate buffering. Bar = 20 μm for **(B)** and **(C)**.

(D) In vitro and in vivo calibration of CBD-OG. The in vitro pH dependence of the CBD-OG conjugate was determined using 10 μM dye and 50 mM pH buffer (citric acid or Mes/KOH). In vivo calibration was performed by injecting the dye into the columella apoplast as described above followed by incubation in 50 mM citrate or 100 mM Mes or dimethylglutarate buffer, pH 3.5 to 6.0, for 30 min. Values are means ±SE; $n = 25$ for in vitro calibrations, and $n = 9$ for in vivo calibrations.

the pregravistimulated value once the tropic growth response had reoriented the root tip back to vertical growth (Figures 4B and 4C).

The roots of *pgm1-1* plants exhibited a decrease in wall pH in the upper flank of the DEZ that was similar to that observed in wild-type plants after gravistimulation, although possibly slightly delayed (Figure 4D). However, the maximum pH change induced by gravistimulation in the CEZ upper flank was smaller relative to that in the wild type (to 4.6 ± 0.07) and the initiation of acidification was delayed, being maximal at 60 min rather than 20 min after gravistimulation (Figure 4E). Wall pH in the root hair and mature regions of both wild-type and *pgm1-1* roots showed no change in response to gravistimulation (data not shown).

We attempted to determine the functional role of these apoplastic pH changes by setting wall pH in the elongation zone using Mes, dimethylglutarate, or citrate buffers, as described previously for altering pH-dependent root hair formation (Bibikova et al., 1998). Unfortunately, incubating the root in a buffer strong enough (50 to 100 mM) to alter elongation zone wall pH (monitored directly by CBD-OG) arrested root growth, irrespective of the wall pH that was set (data not shown). This apparently nonspecific inhibitory effect on growth precluded the use of this buffering approach to manipulate wall pH during the root graviresponse.

Changes in Cytoplasmic pH upon Gravistimulation

The rapid, gravity-related changes in apoplastic pH led us to investigate any potential effects of gravity stimulation on cytoplasmic pH. Cells in the elongation and root cap regions were microinjected with the fluorescent pH indicator 2,7-bis-(2-carboxyethyl)-5-(and 6) carboxyfluorescein (BCECF)–dextran, and cytoplasmic pH was monitored using a vertical stage epifluorescence or confocal microscope and ratio imaging. We demonstrated previously that dextran-conjugated dyes remain in the cytoplasm and are not obviously accumulated in organelles when microinjected throughout the Arabidopsis root (Bibikova et al., 1997, 1998; Legué et al., 1997). Confocal imaging revealed that microinjected, dextran-conjugated BCECF also remained in the cytoplasm of cells of the Arabidopsis root (Figure 5A and data not shown). There was no evidence of vacuolar accumulation or other punctate fluorescence indicative of accumulation in other organelles. These results suggested that microinjected BCECF-dextran should be usable to report cytosolic pH.

Using microinjection of BCECF-dextran, we observed no differences in cytosolic pH levels between the DEZ (7.2 ± 0.1, $n = 20$) and CEZ (7.17 ± 0.08, $n = 23$) during vertical growth or upon gravistimulation (data not shown). Peripheral and tip cells of the root cap also showed no change in cytoplasmic pH during vertical growth and only a small, insignificant acidification upon gravistimulation ($P > 0.05$, *t* test, $n = 21$; Figure 5B and data not shown). In contrast, cytoplasmic pH in the central columella root cap cells showed rapid

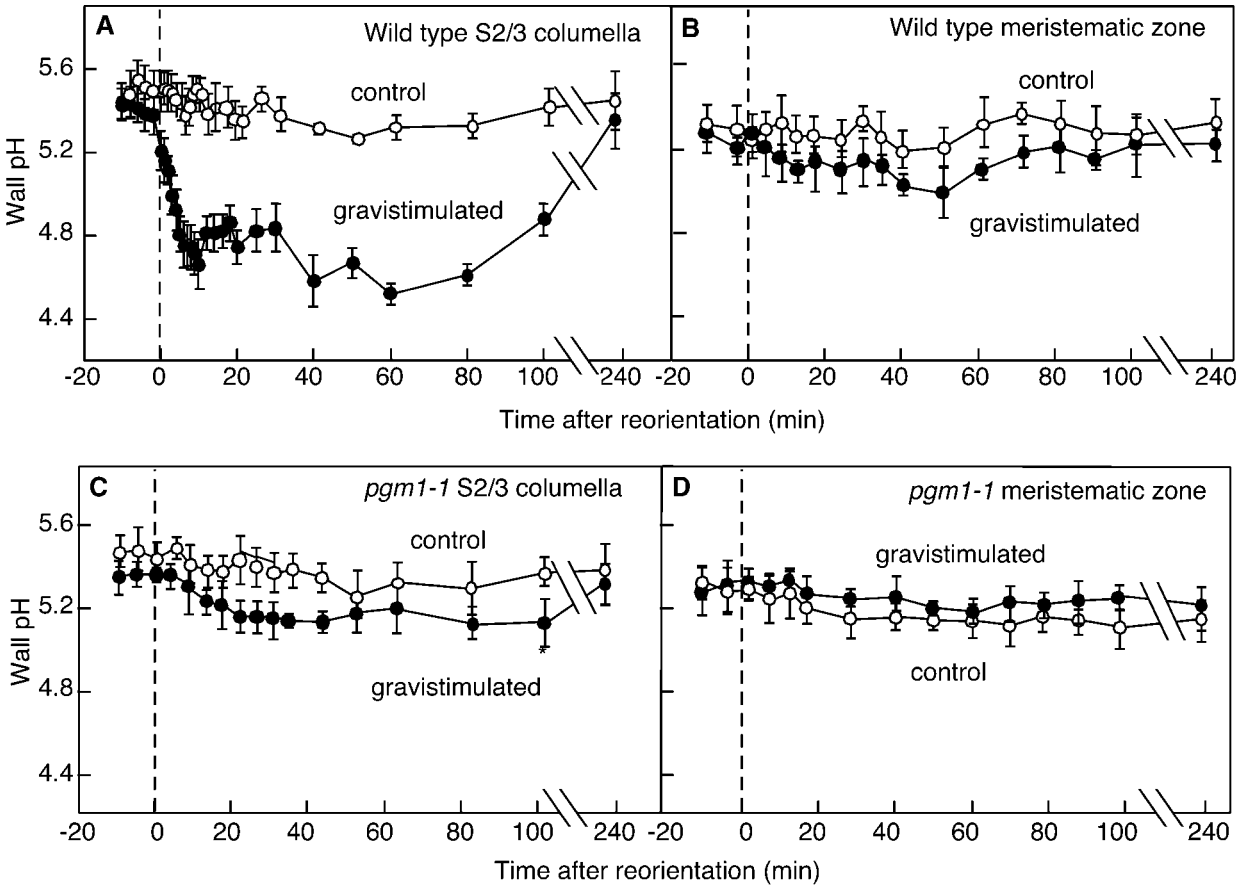

Figure 3. Changes in Cell Wall pH upon Gravistimulation in the Columella and Meristematic Regions of Wild-Type and *pgm1-1* Roots.

(A) Wall pH in root cap around wild-type S2 and S3 columella cells before and after gravistimulation.
(B) Wall pH in wild-type meristematic region before and after gravistimulation.
(C) Wall pH in root cap around *pgm1-1* S2 and S3 columella cells before and after gravistimulation.
(D) Wall pH in *pgm1-1* meristematic region before and after gravistimulation.
Roots were infiltrated with CBD-OG and mounted vertically on the rotatable stage of a vertical stage epifluorescence microscope. Wall pH was then monitored by analyzing 50-μm^2 regions from ratio images of roots either grown vertically (control) or rotated through 90° (gravistimulation occurring at dotted line). Values are mean ±SE; $n \geq 10$ separate roots.

changes upon gravity stimulation. Cells of story 3 (S3) of the columella underwent a rapid and transient alkalinization from pH 7.2 ± 0.1 to 7.5 ± 0.2 ($P < 0.05$, *t* test, $n = 21$) within 30 sec of reorienting the root (Figure 5C). There was no indication of a difference between the upper and lower sides with respect to the timing or magnitude of the pH change in the S3 columella after gravistimulation (Figure 5D).

Similar to the S3 columella, cells of story 2 (S2) showed a cytoplasmic pH increase after gravistimulation, although this transient alkalinization was sustained longer in the S2, lasting 8 to 10 min compared with 6 min for the S3 (Figure 5E). As with the S3, there was no clear difference in the pH change between the upper and lower flanks of the S2 columella (Figure 5F). Because S2 cells were deeper in the tissue than S3 cells, fewer S2 cells ($n = 9$) than S3 cells ($n = 21$) were injected successfully with pH-sensitive dye. Story 1 (S1) cells were never injected successfully (as assessed by a failure to maintain cell structure or a disruption of amyloplast sedimentation kinetics after microinjection) because of their even deeper position within the root tip.

To confirm that the columella cytoplasmic pH changes we observed were likely a component of the gravity response system of the root, we made cytoplasmic pH measurements in the root cap cells of *pgm1-1*. This mutant showed a comparable basal pH (7.2 to 7.3) to the wild type throughout the root (data not shown). Also, as seen in the wild type, the elongation zone ($n = 23$), the peripheral cap ($n = 10$), and the tip cells of the root cap ($n = 12$) of *pgm1-1* roots showed

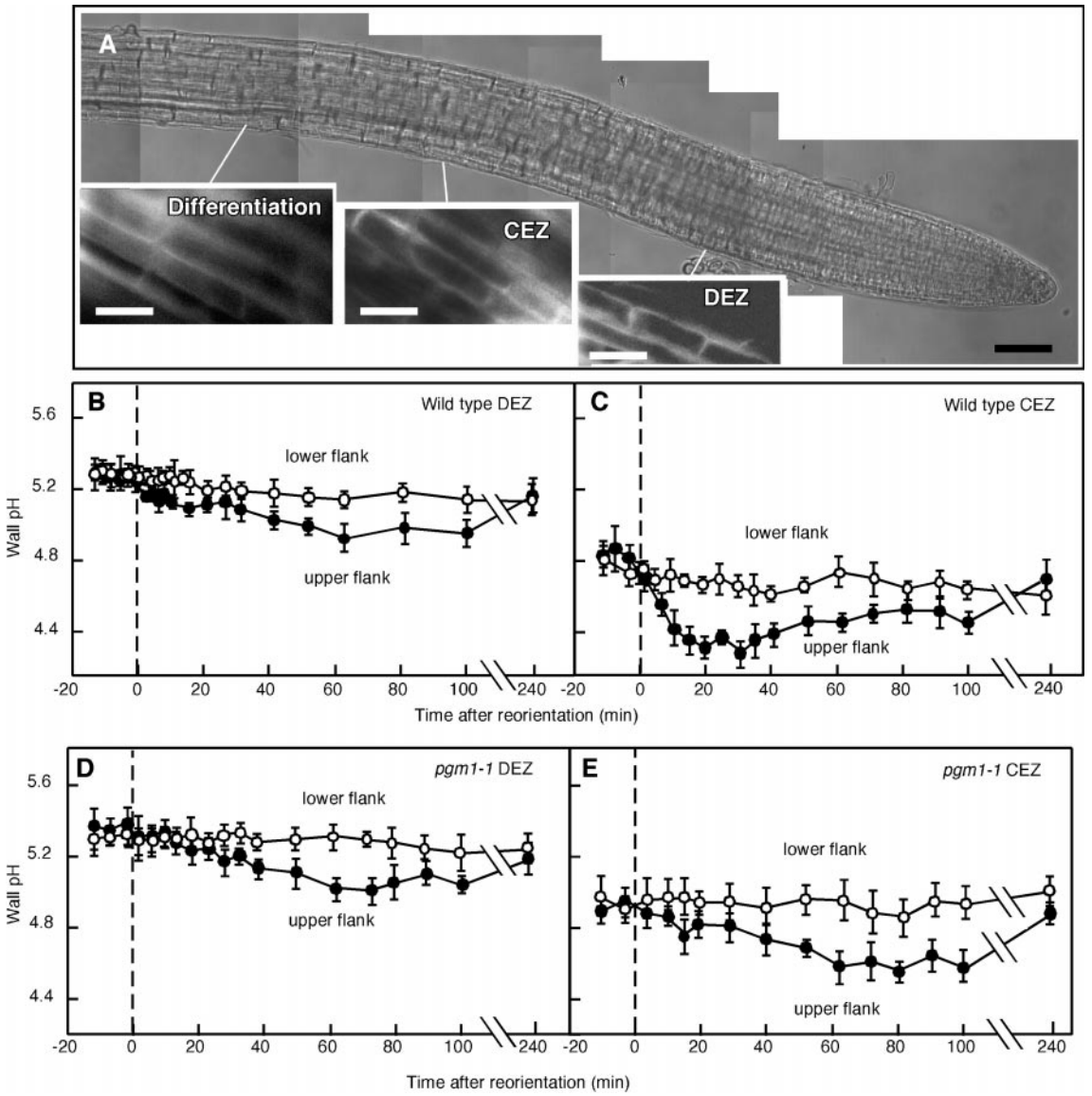

Figure 4. Changes in Cell Wall pH upon Gravistimulation in the Zone of Differentiation, the CEZ, and the DEZ.

(A) A typical Arabidopsis root indicating locations of different growth zones, and fluorescence images of CBD-OG loaded into the zone of root hair formation (differentiation), the DEZ, and the CEZ. Black bar = 100 μm; white bars = 50 μm.
(B) Cell wall pH in the DEZ of wild-type roots during gravistimulation. Note that no statistically significant pH changes occur in the lower flank ($P > 0.05$, *t* test, time = −5 min versus all times after gravistimulation).
(C) Cell wall pH in the CEZ of wild-type roots during gravistimulation.
(D) Cell wall pH in the DEZ of *pgm 1-1* roots during gravistimulation.
(E) Cell wall pH in the CEZ of *pgm 1-1* roots during gravistimulation. Note the delay in pH decrease in the upper flank relative to the wild type.
For **(B)** to **(E)**, roots were infiltrated with CBD-OG and mounted vertically on the rotatable stage of a vertical stage epifluorescence microscope. Wall pH was then monitored by analyzing 50-μm^2 regions from ratio images of roots either grown vertically (control) or rotated through 90° (with gravistimulation occurring at the dotted line). Values are means ±SE, $n \geq 10$ separate roots.

no significant cytosolic pH change upon gravistimulation ($P > 0.05$, *t* test; data not shown). Although cells in the S3 ($n = 11$; Figure 5G) and S2 ($n = 7$; Figure 5H) columella of *pgm1-1* showed a significant alkalinization upon gravistimulation ($P < 0.05$, *t* test), these pH increases were smaller and delayed relative to the responses of the wild-type cells (cf. Figures 5C and 5E with 5G and 5H).

Due to the technical difficulty of microinjecting cells in the S2 and S1 columella, we sought to confirm the microinjection data with a less invasive approach. Therefore, we generated transgenic Arabidopsis expressing a pH-sensitive green fluorescent protein that is amenable to the ratio imaging technique (*GFP H148D*; Elsliger et al., 1999) driven by the cauliflower mosaic virus 35S promoter. A confocal image of the root apex of a plant expressing the pH-sensitive GFP transgene is shown in Figure 6A and confirms that *GFP H148D* is expressed throughout the root and is localized to the cytoplasm. In vivo calibration showed that *GFP H148D* could resolve pH 6.8 to 8.4 (Figure 6B) and that its pH response in planta is comparable to its characteristics in vitro (Elsliger et al., 1999). The presence of cytoplasmic *GFP H148D* had no detectable effect on root morphology, growth rate, or the kinetics of the gravitropic growth response (data not shown), indicating that this construct would be usable to monitor cytoplasmic pH during the graviresponse.

The cytoplasmic pH increase seen in S2 and S3 columella cells using microinjected BCECF-dextran (Figures 5C and 5E) also was observed in plants expressing the pH-sensitive GFP (Figures 6C and 6D). These measurements were made using the optical sectioning abilities of the vertical stage confocal microscope to minimize contributions to the columella measurements from the fluorescence signal of overlying peripheral cells. These peripheral cells did not experience changes in cytoplasmic pH upon gravistimulation and tended to mask the responses of the columella when imaging was conducted using a standard epifluorescence microscope. Vertical stage confocal imaging of roots with cytoplasmic *GFP H148D* allowed us to determine that the S1 columella cytoplasm, which is unreachable by microinjection, also showed a transient alkalinization after gravistimulation (Figure 6C).

Caged Protons Alter Columella pH and the Graviresponse

To determine the significance of the cytoplasmic pH changes elicited by gravistimulation, we attempted to alter the pH dynamics of columella cells. Local application of effectors, such as fusicoccin, via a microinjection pipette was found to be ineffective at localizing the molecules to the cap. Therefore, we adopted a UV light–activated caged probe approach. Roots were loaded with 25 μM caged protons (nitrophenyl ether) for 20 min. Columella or tip cells were then irradiated using the UV laser of the confocal microscope or the epiilluminator of the vertical stage microscope to photoactivate the nitrophenyl ether and release protons to the cytoplasm. Measurements of wall, cytoplasmic, or vacuolar pH with concomitant proton release indicated that the caged protons detectably altered only root cap cytoplasmic pH, leading to a transient cytoplasmic acidification lasting ~12 min (Figure 7A and data not shown). This treatment blocked any detectable gravity-induced cytosolic alkalinization in the cap (Figure 7A, first 10 min after UV activation), although longer term pH increases (Figure 7A, 10 to 50 min, described below) comparable in magnitude to those elicited by gravistimulation (cf. Figures 7A and 7B) were detectable.

Because the caged proton release inhibited the initial gravity-related pH increase in the root cap, we predicted that the rate of gravitropic growth response would be delayed by this treatment. However, proton release in the columella region did not significantly affect the rate of root curvature monitored during 2 hr of subsequent gravistimulation (Figure 7D). We reasoned that the kinetics of root curvature may have been unchanged in these experiments because any effects of the transient changes in cap pH caused by caged proton release would have been masked by the subsequent 2 hr of continuous gravistimulation. This would be especially true if differences occurred only in the initial phases of the response, when angles of tropic curvature are small and differences would be difficult to measure accurately.

To test the possibility of such a transient perturbation of gravitropic response, we released caged protons into the columella cells, gravistimulated the root for up to 20 min, and then observed the roots after 4 hr of growth on a clinostat. The clinostat was used to randomize the gravity vector during the growth period. Thus, tropic bending occurring on the clinostat should represent a response to a signal generated during the initial period of gravistimulation. Therefore, this clinostat analysis likely would reveal any initial, transient disruption of gravity-sensing events by the caged proton release. Uncaging protons in the columella followed by 5 min of gravistimulation almost completely blocked subsequent tropic growth on the clinostat (final angle, 0.19° deviation from straight growth), whereas equivalent controls bent to 7.48° (Figure 7E). Equivalent uncaging experiments with 10 min of gravistimulation significantly inhibited subsequent bending ($P < 0.05$, *t* test, $n = 79$). However, uncaging protons in the columella followed by 20 min of gravistimulation led to significantly greater curvature ($P < 0.05$, *t* test, $n = 114$; Figure 7E). Release of caged protons in tip or peripheral cells (cap cells known not to be involved in gravisensing; Blancaflor et al., 1998) did not alter the graviresponse relative to control roots for any period of gravistimulation ($P > 0.05$, *t* test, $n = 63$; Figure 7E and data not shown).

After caged proton release, cells remained viable, as assessed by staining with vital dyes (continued exclusion of propidium iodide and positive fluorescein diacetate staining; Blancaflor et al., 1998). Also, cells in which protons were

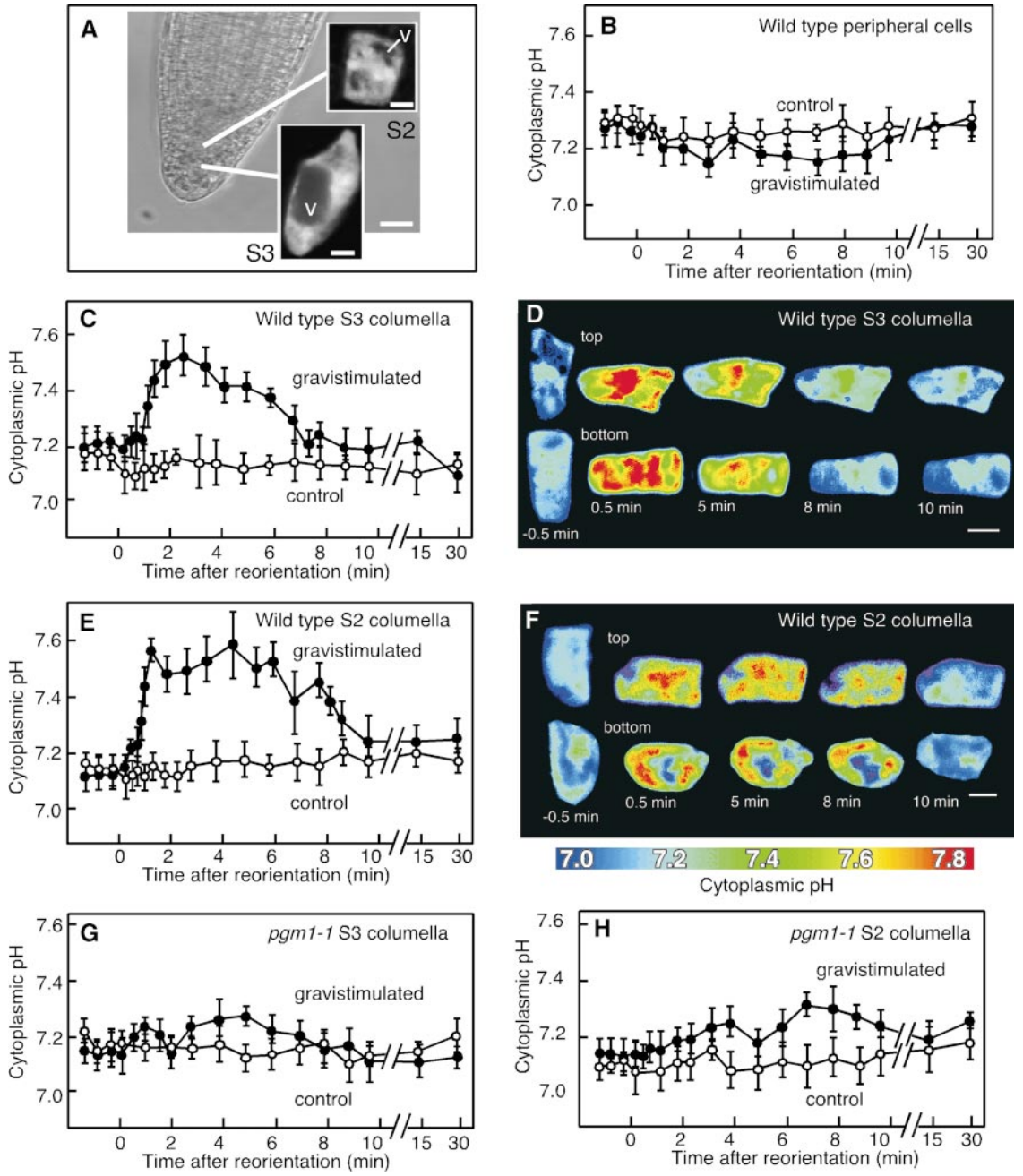

Figure 5. Changes in Root Cap Cytoplasmic pH during Gravitropism Monitored by Microinjected BCECF-Dextran.

(A) Fluorescence images showing S2 (upper inset) and S3 (lower inset) columella cells microinjected with BCECF-dextran. Main bar = 40 μm; inset bars = 10 μm. v, vacuole.

(B) Cytoplasmic pH of peripheral cap cells after gravistimulation.

(C) Cytoplasmic alkalinization of S3 columella cells after gravistimulation.

(D) Representative images of the time course of S3 columella alkalinization after gravistimulation. Pseudocolor coding is according to the scale below **(F)**. Bar = 10 μm.

(E) Cytoplasmic alkalinization of S2 columella cells after gravistimulation.

(F) Representative images of the time course of S2 columella alkalinization after gravistimulation. Bar = 10 μm.

(G) Cytoplasmic pH of *pgm 1-1* S3 columella cells after gravistimulation.

(H) Cytoplasmic pH of *pgm 1-1* S2 columella cells after gravistimulation.

Values in **(B)**, **(C)**, **(E)**, **(G)**, and **(H)** are means ±SE.

released did not show the cytoplasmic condensation characteristic of columella cell death (data not shown). In addition, caged proton release in the central S2 and S3 columella cells did not alter amyloplast sedimentation kinetics (S2, 1.16 ± 0.04 μm/min with and 1.2 ± 0.05 μm/min without caged protons, $n = 56$; S3, 1.00 ± 0.05 μm/min with and 0.9 ± 0.06 μm/min without caged protons, $n = 45$). Similarly, the characteristic vigorous saltation of the sedimenting amyloplasts of the columella cells was not disrupted by caged probe activation (data not shown). Finally, the growth rates of roots after caged probe release were unaltered (210 ± 11 μm/hr with caged probe release to the cap, 205 ± 7 μm/hr in controls, $n = 9$). Controls using UV irradiation alone, incubation in caged probe without UV irradiation, or incubation in prephotolyzed probe all showed no effect on the kinetics or sensitivity of the graviresponse or on cytoplasmic pH (Figure 7C and data not shown). Thus, we think it unlikely that the caged proton–related changes we observed in the kinetics of the graviresponse and root cap pH dynamics reflected nonspecific cytotoxic action of caged probe release.

DISCUSSION

Wall pH but Not Cytoplasmic pH Changes in the Gravitropic Growth Response

To determine the involvement of pH in the gravitropic response of the Arabidopsis root, we have developed a novel wall pH sensor (CBD-OG) that has allowed us to make extended measurements of wall pH throughout the root. The wall of the elongation zone was maintained at a lower pH in vertically growing roots compared with the root hair and the mature and meristematic regions, consistent with a role for acid growth in the elongation process. This acidification of the elongating region is in agreement with the observations of Peters and Felle (1999) that maximal surface acidification occurred in the elongation zone (CEZ) of the maize root. However, those authors also noted a short region of acidic surface pH around the meristematic zone not predicted from our wall pH measurements. These differences may reflect variation between maize and Arabidopsis in pH profile along the root, or, as noted by several authors (Sentenac and Grignon, 1987; Felle, 1998; Peters and Felle, 1999), external pH, even when monitored close to the root surface, may not provide a reliable indicator of steady state wall pH in the root body.

Indeed, within the elongation zone, we found regional differences in wall pH profiles. The CEZ elongated faster and had a lower sustained wall pH than did the DEZ. Upon gravistimulation, this acidification was enhanced on the upper side of the root at a time that preceded detectable asymmetrical growth by ~5 min (Figure 4). Thus, the wall pH changes provided us with an early marker for the initiation of tropic responses in the elongation zone, before detectable growth responses. We also observed that the DEZ and CEZ responded differently to gravistimulation. The DEZ exhibited a slightly smaller and slower wall pH decrease than did the CEZ. Thus, the DEZ showed a maximal wall pH decrease of 0.4 pH units at 60 min after gravistimulation, compared with a 0.5 pH unit decrease by 20 min after gravistimulation for the CEZ (Figure 4). This is consistent with the DEZ showing different growth kinetics, and presumably regulation, than the CEZ (Ishikawa and Evans, 1993; Evans et al., 1994; Mullen et al., 1998). Taylor et al. (1996) used Nerf 1–Texas red wall pH imaging to observe a pH change from 4.9 to 4.5 on the upper flank of the maize elongation zone after 45 min of gravistimulation. These wall pH values correlate well with the decrease in pH from 4.8 to 4.4 that we measured in the CEZ of Arabidopsis after sustained gravistimulation.

We also observed that the wall pH response of the DEZ and CEZ to gravistimulation was different in roots of *pgm1-1* plants. *pgm1-1* is not impaired in growth but shows reduced gravity responses. Consistent with this idea, the CEZ of roots of *pgm1-1* plants had a wild-type–like wall pH profile under vertical growth conditions but had reduced pH changes upon gravistimulation. However, the gravistimulation-related pH changes in the wall of the DEZ were unchanged in *pgm1-1* compared with the wild type (Figure 4). Again, these results highlight a different growth control mechanism in the DEZ than in the CEZ. Defining this mechanism of DEZ growth control should yield important insight into the control of the initial phases of the graviresponse.

In our experiments, there was no obvious sustained cytoplasmic pH change associated with either straight growth or the gravitropic response in the elongation zone cells of the wild type or *pgm1-1* (data not shown). In several roots, apparently spontaneous changes in cytoplasmic pH were noted in the DEZ and CEZ during vertical growth (data not shown). However, these changes were relatively rare (<10% of roots) and showed no obvious correlation to gravistimulation or growth. Although we cannot discount the possibility that cytoplasmic pH changes below the temporal (<1 sec) and/or spatial (<1 μm^2) resolution of our imaging approaches may have occurred during the graviresponse, our results suggest that the signaling systems modulating the growth response of the elongation region of the Arabidopsis root likely are not mediated by changes in cytoplasmic pH.

Gravity Induces Changes in Root Cap Cell Wall and Cytoplasmic pH

We observed rapid changes in root cap wall and cytoplasmic pH after gravistimulation (Figures 3, 5, and 6). Although these alterations in wall and cytoplasmic pH may not be directly linked, it is tempting to speculate that fluxes of protons from cytoplasm to wall mediate both changes. It is important to note that the change in cytoplasmic pH is transient, whereas the reduction in wall pH is sustained. Thus,

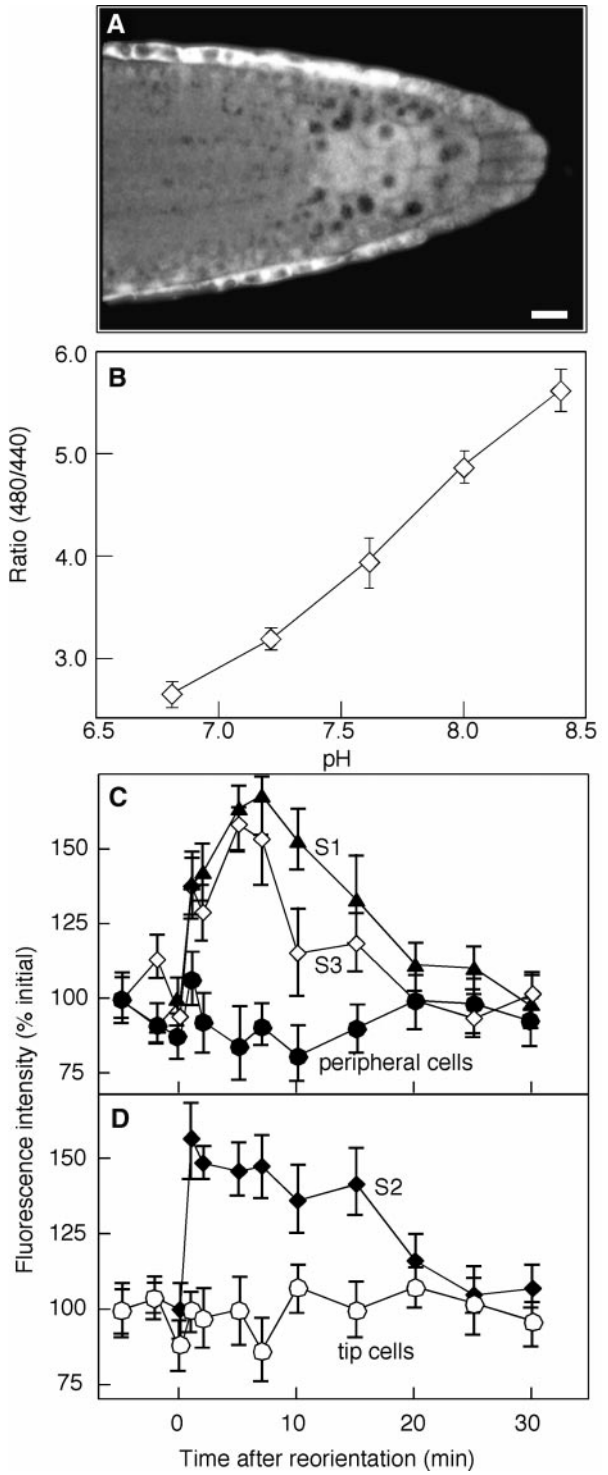

Figure 6. Root Cap Cytoplasmic pH during Gravitropism Measured Using pH-Sensitive *GFP H148D*.

(A) Confocal fluorescence image of an Arabidopsis root expressing the ratiometric pH-sensitive *GFP H148D*. Bar = 25 μm.

(B) In vivo calibration of *GFP H148D*. Note that pH sensitivity covers the expected cytosolic pH range (6.8 to 8.4). Values are means ±SE, $n = 23$.

(C) Changes in *GFP H148D* fluorescence intensity demonstrated by pH increases (as indicated by signal increases) in S1 and S3 columella but not peripheral cells after gravistimulation. Values are means ±SE, $n = 11$ separate roots.

(D) Changes in *GFP H148D* fluorescence intensity demonstrated by pH increases (as indicated by signal increases) in S2 columella but not tip cells after gravistimulation. Values are means ±SE, $n = 10$ separate roots.

For **(C)** and **(D)**, columella changes were resolved in roots expressing *35S-GFP H148D* by analysis of optical sections of the columella using a vertical stage confocal microscope. Contaminating signal from overlying peripheral cap cells masked these columella pH changes in standard epifluorescence analysis. Areas of 50 μm^2 were analyzed from the confocal images to monitor pH in defined regions of the cap, but because the confocal microscope lacks the 440-nm excitation wavelength necessary for ratio analysis of *GFP H148D*, these images use only the pH-sensitive 488-nm excitation wavelength. In this single-wavelength mode, an increase in *GFP H148D* signal (510- to 565-nm emission) reflects an increase in pH but provides only a qualitative measure of pH changes.

for the same proton transport process to support changes in both compartments, a recovery of cytoplasmic pH homeostasis to pregravistimulated levels would have to mask the sustained efflux to the wall. Such a recovery of prestimulated cytoplasmic pH levels might help prevent potential cytotoxic effects of sustained elevated pH. Activation of the columella cell plasma membrane H^+-ATPase and/or modulation of other H^+ transport processes at the plasma membrane, such as organic acid secretion or proton antiporter/symporter systems (Zheng et al., 1998; Delrot et al., 2000) could support these coordinated changes in cytoplasmic and wall pH. Alternatively, the wall and cytoplasmic pH changes may not be directly linked. Thus, proton transport to other compartments, such as proton accumulation in the vacuole via the tonoplast H^+-pyrophosphatase or H^+-ATPase (Maeshima, 2000; Ratajczak, 2000), could well be involved in mediating the cytoplasmic changes. In addition, alterations in wall structure, for example, the methylation state of pectins in the columella cell wall, could contribute to changes in apoplastic pH independently of cytoplasmic changes (Cosgrove, 1997). Clearly, defining the cellular source of the cytoplasmic and wall pH changes will be a critical goal to help generate a model for how gravity might regulate these processes.

Rapid changes in ionic fluxes around the gravistimulated root cap have been reported previously (Bjorkman and Leopold, 1987; Bjorkman and Cleland, 1991; Zieschang et al., 1993). Additionally, Behrens et al. (1985) reported rapid changes (within 8 sec) in the membrane potential of *Lepidium sativum* columella cells when the roots were gravistimulated. Sievers et al. (1995) suggested that the bulk of these potential changes may be due to ionic changes in the colu-

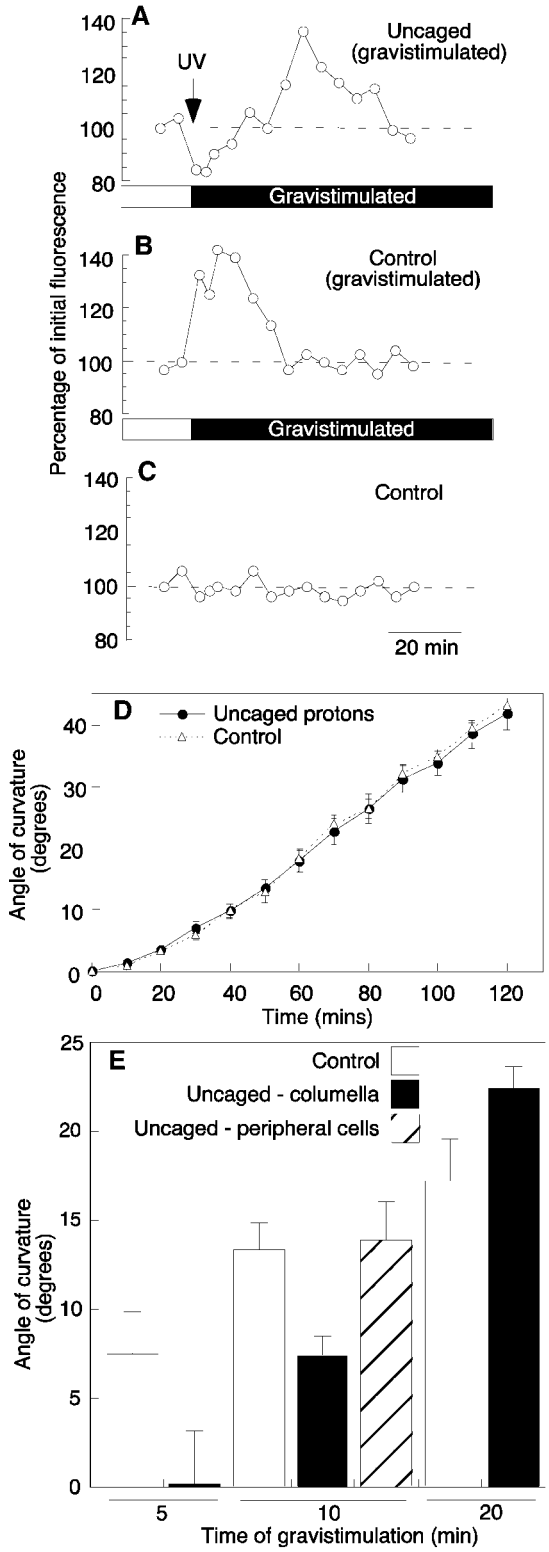

Figure 7. Effects of UV Light Activation of Caged Protons on Cap Cytoplasmic pH and Root Curvature Kinetics after Gravistimulation.

mella apoplast. Our results on wall and cytoplasmic pH suggest that such changes are closely related to alterations in proton fluxes in and around the columella cells of the root cap. Bjorkman and Cleland (1991) have shown that the graviresponse was not inhibited by treating the tips of maize roots with a strong pH buffer (50 mM Mes, pH 6.5), implying that changes in columella wall pH may not be an important factor in gravisignaling. However, we found that our in vivo calibration of CBD-OG required the use of 50 mM citrate, 100 mM Mes, or 100 mM dimethylglutaric acid to overcome the strong buffering of the cell. Thus, the 50 mM Mes used by Bjorkman and Cleland (1991) may not have induced an effective and sustained alteration in wall pH of the columella or the elongation zone, especially in the more massive maize root.

Our detection of rapid cytoplasmic pH increases in the cap upon gravistimulation supports the hypothesis that pH changes either are directly involved in early signaling events in gravisensing or are generated by these events. These changes are not seen in cap cells that are not required for gravisensing, such as peripheral cap cells, and the pH changes are larger and more sustained in S2 compared with

(A) Proton uncaging in the columella followed by gravistimulation produces a mild cytoplasmic acidification of cap cells followed by sustained alkalinization. The trace represents the signal from *GFP H148D* from the columella region (S1 to S3) of a root loaded with caged protons. UV light activation and gravistimulation occurred at 0 min.

(B) and **(C)** The pH-sensitive GFP signal from the columella region (S1 to S3) of roots loaded with caged protons but without UV activation. These roots were either gravistimulated (gravistimulated control **[B]**) or left to grow vertically (control **[C]**).

(A) to **(C)** show representative traces of $n \geq 14$ separate roots for each treatment. Areas of 50 μm^2 were analyzed from the confocal images to monitor pH in defined regions of the cap. Because the vertical stage confocal microscope lacks the 440-nm excitation wavelength necessary for true ratio analysis of *GFP H148D*, these images use only the pH-sensitive 488-nm excitation wavelength. In this single-wavelength mode, an increase in *GFP H148D* signal (510- to 565-nm emission) reflects an increase in pH but provides only a qualitative measure of pH changes.

(D) Release of caged protons in the columella does not affect the long-term kinetics of gravity-induced curvature. Control roots were incubated with caged protons but not irradiated with UV light. Values are means ±SE, $n = 16$ separate roots.

(E) Effect of proton uncaging on gravitropic growth on the clinostat after gravistimulation. Roots were loaded with caged protons and mounted on the microscope, and the specific regions of the cap indicated were irradiated with UV light to locally activate proton release. The roots were then gravistimulated for the indicated times, and the final angles of the root apex were determined after 4 hr of subsequent growth on the clinostat. Any bending occurring during clinostat growth should reflect a response to the initial gravistimulation period before placement on the clinostat. Values are means ±SE, $n > 48$ separate roots.

S3 columella cells (Figures 5 and 6), correlating with the proposed differential contributions of the peripheral cells and S2 and S3 columella regions to gravisensing (Blancaflor et al., 1998). These cytoplasmic pH changes also are reduced in the starchless *pgm1-1* mutant (Figure 5), suggesting that starch-filled amyloplasts are required for columella cell cytoplasmic alkalinization. These root cap pH changes are seen only upon reorientation of the root, supporting a model in which the vertically oriented root is not continuously exporting gravitropic information from the cap but generates a signal only when reorientation of growth is required. In addition, the columella cell cytoplasmic alkalinization and wall acidification occur 5 to 10 min before gravistimulation-related wall acidification in the elongation zone, the earliest detectable marker for gravitropic response in these cells that we could measure.

Cytoplasmic pH Increases in the Columella Are Required for Gravitropism

Using caged protons to acidify the cytosol of the columella cells, we were able to block the transient gravity-induced alkalinization of the columella cells and subsequent gravitropic growth on a clinostat (Figure 7). This treatment blocked the graviresponse after 5 min of gravistimulation and significantly inhibited tropic growth after 10 min of gravistimulation (Figure 7), implying that the transient gravity-induced pH increase normally seen in the columella either is a required part of the gravity-sensing machinery of the cap or is needed to transport a gravitropic signal from the cap cells once it has been generated.

Unexpectedly, we also noted that releasing caged protons to the columella followed by 20 min of gravistimulation significantly enhanced subsequent gravitropic growth on the clinostat (Figure 7E). However, in addition to an initial acidification of the columella cell cytoplasm, caged proton release also led to a delayed (evident after 15 min), sustained increase in cytoplasmic pH that may reflect "overshoot" in homeostatic pH regulation to the mild pH depression induced by uncaging. Twenty minutes of gravistimulation after caged proton release would be sufficient to induce this sustained pH increase (Figure 7A). Thus, the enhanced curvature on the clinostat with 20 min of gravistimulation after caged proton release may reflect the action of this sustained pH increase, mimicking and extending the normal gravity-related pH increase in the columella cells.

Our results broadly agree with the gravity-related cytosolic alkalinization in the columella reported by Scott and Allen (1999). However, we could not detect the differences in the timing of pH changes between the upper and lower flanks of the cap or the acidification in the S3 cells they reported. We confirmed our microinjection measurements using Arabidopsis plants stably expressing a ratiometric, pH-sensitive GFP. This approach does not suffer from the potential cytotoxicity-related artifacts associated with mechanically impaling cells for microinjection and allows simultaneous data collection from a range of cells in the cap. The pH-sensitive GFP therefore has allowed us to map pH changes noninvasively throughout the cap. These GFP imaging experiments reinforce the microinjection results that the columella as a whole shows a uniform cytoplasmic pH increase within 30 sec of gravistimulation, including the innermost S1 columella cells.

Our results indicate that pH changes in the wall and cytoplasm of the columella cells of the root cap occur rapidly in response to gravistimulation and that these cytosolic pH changes appear essential for generating a graviresponse signal. However, the temporal and spatial uniformity throughout the cap suggests that these pH changes do not convey vectorial information regarding the direction of gravity. We speculate that these alterations in pH may reflect the activation of proton pumping at the plasma membrane as part of the machinery that facilitates the transport of the molecule encoding the direction of the gravity stimulus. Our results highlight the plasma membrane H^+-ATPase of the columella cell as one obvious candidate for mediating gravisignaling-related pH changes. Defining how this enzyme is regulated specifically in the root cap columella cells may therefore yield significant insight into the initial events of gravity signal transduction.

METHODS

Plant Material and Growth Conditions

Growth conditions for seedlings of *Arabidopsis thaliana* ecotype Columbia, analysis of growth kinetics, growth on the clinostat, and measurement of amyloplast movements were as described previously (Blancaflor et al., 1998). The *pgm1-1* mutant and wild-type seedlings microinjected with 2,7-bis-(2-carboxyethyl)-5-(and 6) carboxyfluorescein (BCECF), loaded with cellulose binding domain peptide–Oregon green (CBD-OG), or expressing green fluorescent protein (GFP) showed growth rates of 190 to 220 μm/hr, with no significant difference between treatments ($P > 0.05$, *t* test, $n \geq 10$ for each treatment).

Vertical Stage Microscopy

For vertical stage epifluorescence microscopy, intact seedlings were mounted on the stage of a Nikon Optiphot or Diaphot 300 epifluorescence microscope (Nikon, Melville, NY) with a Lambda 10-c filter wheel (Sutter, Novato, CA) in the excitation path. The microscope was mounted on its back so that the rotatable stage was vertical (Legué et al., 1997; Blancaflor et al., 1998). Each root was imaged while growing vertically, and its gravitropic response was elicited by rotating the stage through 90° until the root was horizontal. The root was imaged before, during, and after this stimulation period. Roots were observed with a 100×, 1.2 numerical aperture, a 40×, 0.7 numerical aperture, or a 10×, 0.3 numerical aperture Nikon fluor objective. This imaging setup allowed us to image individual cells and

make growth measurements to a resolution of 2.5-μm changes in cell length. Image processing was performed on a G4 computer (Apple Computer, Cupertino, CA) using IPLabs Spectrum image analysis software (Scanalytics, Fairfax, VA). Bright-field and fluorescence images were collected using either a CH250A or a SenSys cooled charge-coupled device camera (Photometrics, Austin, TX) running under IPLabs Spectrum software.

Confocal microscopy was performed using a Zeiss Axiovert attached to an LSM 410 confocal microscope (Zeiss, Thornwood, NY) and a 40×, 0.7 numerical aperture fluor objective and appropriate filtration as described for each dye used (see below). For vertical stage confocal microscopy, the 100% confocal laser mirror directing light up onto the sample of the inverted microscope was replaced by an equivalent UV light–reflective mirror (Edmund Scientific, Barrington, NJ) mounted at 45°, directing the confocal lasers sideways to a horizontally mounted 40×, 0.7 numerical aperture fluor objective. The sample was mounted on a custom-built, vertical, rotatable stage attached to Narashige coarse manipulators (Nikon), which allowed for stage focusing. Gravistimulation was performed by rotating the root through 90°.

Measurement of Apoplastic pH Using CBD-OG

One hundred micrograms of the CBD peptide of *Clostridium cellulovarans* cellulase (Sigma) was incubated with 10 mM OG 488 succinimidyl ester (Molecular Probes, Eugene, OR) in 50 mM potassium phosphate buffer, pH 8.0, for 2 hr at room temperature. The unconjugated OG was removed by dialysis versus fresh buffer at 4°C for 18 hr. The conjugated peptide was frozen in liquid nitrogen and stored at −80°C until use. The CBD-OG peptide was loaded into the apoplast by pressure injection into the apoplastic space by positioning the tip of a micropipette containing 1 mg/mL CBD-OG next to the wall to be analyzed and pressure injecting with 5 to 10 1-sec, 0.14-MPa pulses of pressure delivered from a PV830 pneumatic pressure regulator (World Precision Instruments, Sarasota, FL). Roots were mounted on the vertical stage microscope and allowed to recover for 1 hr. The pH-dependent (480 nm) and pH-independent (440 nm) excitation wavelengths were selected using interference filters (±20 nm), and emission was monitored at >520 nm (long pass) using a 510-nm dichroic mirror. Autofluorescence represented <5% of the 440- or 480-nm excitation signals. Single-wavelength confocal imaging was performed using a 488-nm excitation laser and 488-nm primary dichroic and 515- to 560-nm emission interference filters.

There was no significant difference ($P > 0.05$, Student's *t* test) between the wall pH on the left versus the right sides of vertically growing roots in any of the regions of the root analyzed (data not shown). These left and right sides of the root became the upper and lower flanks after the root had been rotated 90° for gravistimulation. This uniformity in pH indicated that there was no predetermined lateral asymmetry in wall pH before gravistimulation.

Measurement of Cytoplasmic pH

For measurement of cytoplasmic pH, root cells were pressure microinjected with BCECF linked to 10,000 molecular weight dextran (Molecular Probes), and fluorescence ratio images were analyzed and calibrated as described previously (Bibikova et al., 1998). Root cells were classed as being successfully microinjected if they maintained turgor, cytoplasmic streaming, and cytoplasmic structure during the experiment. One-third of the roots in which cells had been microinjected successfully by the criteria described above showed an inhibition of root growth and/or graviresponse. These were excluded from analysis. Using these criteria for viability, it was possible to successfully inject epidermal and cortical cells in the mature, elongation, and root cap zones.

In addition, we generated transgenic Arabidopsis plants expressing a cytoplasmic pH sensor that is amenable to the ratio imaging technique (*GFP H148D*; Elsliger et al., 1999) under the control of the cauliflower mosaic virus 35S promoter. *35S-GFP H148D* was introduced to a binary plant transformation vector (pBI101), and *Agrobacterium tumefaciens*–mediated transformation of Arabidopsis ecotype Columbia was performed as described by Granger and Cyr (2001). GFP expression in T1 plants (emission at 520 nm, excitation at 490 nm) was quantified using the SenSys charge-coupled device camera and IPLabs Spectrum software. Moderately expressing lines from the T2 and T3 generations were used for experiments. Ratio imaging was performed on the vertical stage microscope with 520-nm (long pass) emission, 440- and 480-nm excitation filters (band pass ±20 nm), and a 510-nm dichroic mirror. Autofluorescence represented <10% of the emission at each excitation wavelength. The pH dependence of the ratio was calibrated by equilibrating intracellular and medium pH by incubating roots in 50 mM Tris or 50 mM Hepes plus 50 mM ammonium citrate at a range of pH values (6.8 to 8.4) for 30 min. Eight or more roots were used for each calibration point. Single-wavelength confocal imaging of GFP-expressing roots was performed using a 488-nm excitation laser and 488-nm primary dichroic and 515- to 560-nm emission interference filters.

There was no significant difference ($P > 0.05$, Student's *t* test) between the cytoplasmic pH on the left versus the right sides of vertically growing roots in any of the regions of the root analyzed (data not shown). Thus, there was no predetermined lateral asymmetry in cytoplasmic pH before gravistimulation.

Caged Probe Release Experiments

The cells of the root cap were loaded with caged protons by incubation in 20 μM nitrophenyl ethyl ester (Molecular Probes), which is nonpolar and cell-permeant, and the seedlings were then mounted on either the vertical stage epifluorescence microscope (Nikon Diaphot 300) or the stage of an LSM 410 confocal microscope. The caged protons were then released in defined regions of the root using a 5-sec irradiation of 360 ± 10 nm light (epifluorescence) or a 1-sec irradiation using the 364-nm laser of the confocal microscope. The region illuminated was restricted either by a variable diameter circular aperture placed in the epifluorescence excitation light path or by using the scan controller of the confocal UV laser (Bibikova et al., 1997); it was confirmed by imaging the region of caged fluorescein activation in samples to which 20 μM caged fluorescein had been added (Bibikova et al., 1997). Because the uncaging protocols involved more handling of roots than normal microscopy experiments, in all experiments involving proton uncaging we exposed controls to the same handling procedures as the experimental material to circumvent potential touch-related alterations in the kinetics of gravitropism (Mullen et al., 2000).

To measure the effect of caged proton release on brief periods of gravistimulation, nitrophenyl ether was photoactivated in the root cap and the root was then gravistimulated by rotating from vertical to 90° for 5, 10, or 20 min. The roots were then mounted on a 1-RPM clinostat with the root axis parallel to the axis of rotation. Root curvature was assessed after 4 hr of growth according to Blancaflor et al. (1998).

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