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CYTOCHALASIN D DOES NOT INHIBIT GRAVITROPISM IN ROOTS[1]

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It is generally thought that sedimenting plastids are responsible for gravity sensing in higher plants. We directly tested the model generated by the current statolith hypothesis that the gravity sensing that leads to gravitropism results from an interaction between the plastids and actin microfilaments. We find that the primary roots of rice, corn, and cress undergo normal gravitropism and growth even when exposed to cytochalasin D, a disruptor of actin microfilaments. These results indicate that an interaction between amyloplasts and the actin cytoskeleton is not critical for gravity sensing in higher plants and weaken the current statolith hypothesis.

Key words: actin; cytochalasin D; gravitropism; *Oryza sativa*; rice; roots; statoliths.

It is widely held that amyloplasts are the gravisensor in the cells of higher plants (Audus, 1962, 1979; Juniper, 1976; Volkmann and Sievers, 1979; Sack, Suyemoto, and Leopold, 1984, 1985; Sack and Leopold, 1985; Moore and Evans, 1986; Björkman, 1988; Hensel, 1989; Sack, 1991; Pickard and Ding, 1992; Barlow, 1995). This hypothesis, proposed by Berthold (1886) and Noll (1892), was further developed at the end of the last century by Haberlandt (1900) and Nemec (1900), who noticed that the presence of cells containing sedimenting starch grains (statoliths) was associated with the ability of an organ to sense gravity. Hawker (1932) demonstrated that sedimenting starch grains were correlated with gravitropism in ~80 species, which provided support for the generality of the statolith theory. With the advent of electron microscopy, it became clear that starch grains were not simple, but clustered and surrounded by amyloplast membranes (Audus, 1962). Thus amyloplasts assumed the role of the statoliths.

Originally, it was thought that the statoliths had to sediment to the bottom of the cell in order to achieve a sufficient velocity to effect a graviresponse (Hawker, 1932). However electron microscopy revealed that sedimentation to the bottom of the cell is not required for gravitropism (Perbal, 1978; Hensel, 1984). Subsequently it was suggested that statoliths sediment onto the endoplasmic reticulum (ER), perhaps causing a release of Ca^{2+}, which may be important for gravity sensing by causing a change in the membrane potential and the ionic current pattern around the root (Sievers and Volkmann, 1972, 1977; Volkmann, 1974; Rodriguez-Garcia and Sievers, 1977; Behrens et al., 1982, 1985; Chandra et al., 1982; Sievers et al., 1984, 1995; Björkman and Leopold, 1987; Sievers and Busch, 1992). While Sack and Kiss (1989) do in fact observe numerous contacts between the amyloplasts and the ER in *Arabidopsis,* others find that contacts between the amyloplasts and ER are rarely observed, and thus their significance has been questioned (Perbal, 1978; Marty, 1980; Barlow, Hawes, and Horne, 1984; Wendt, Kuo-Huang, and Sievers, 1987).

Pickard and Thimann (1966) demonstrated that starch-depleted wheat coleoptiles remained responsive to gravistimulation and argued that "amyloplast starch grains are not critical for the geotropic response." They suggested that Czapek's (1898) idea that gravity perception may be achieved by the cell sensing the weight of its own cytoplasm should be reinvestigated. While this suggestion was dismissed by Audus (1979) as being unlikely, Caspar and Pickard (1989) again concluded that neither starch nor plastid sedimentation are required for gravity sensing, following their study of gravitropism in starch-deficient mutants (TC7) of *Arabidopsis,* which are capable of gravitropism. After investigating the same starch-deficient mutants, Sack and Kiss (1989) concluded, however, that while plastids are necessary for full gravitropic sensitivity (see Kiss, Hertel, and Sack, 1989), the "absence of substantial plastid movement following inversion of the gravitropic TC7 mutant (Caspar and Pickard, 1989) indicates that significant plastid sedimentation is not necessary for perception." Sack (1991) concluded that amyloplasts are not necessary for gravitropism, but plastids are, and thus suggested that "the term 'plastid-statolith' hypothesis should replace the 'starch-statolith' hypothesis in our thinking. . . ."

Since gravitropic curvature can take place without much contact between plastids and the ER, or without significant sedimentation, some researchers have asked the question: Which other cellular structures may interact with plastids to achieve gravisensitivity? It was discovered that actin microfilaments are found in root columella cells and are always closely associated with plastids (Hensel, 1986; Sack and Kiss, 1989; Sievers et al., 1989, 1991; White and Sack, 1990) and it was then hypothesized that "statoliths interact with AFs [actin microfilaments], and that a gravity-caused displacement of statoliths is transmitted to the plasma membrane and/or cortical ER. . . ." (Sievers et al., 1995). This hypothesis is supported by the observations that cytochalasins, agents that specifically inhibit actin-mediated processes: (1) disturb the polarity of cells involved in the sensing of gravity (Hensel and Sievers, 1981; Sievers and Heyder-Cas-

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pers, 1983; Hensel, 1985; Wendt and Sievers, 1986; Hilaire et al., 1995); (2) increase the sedimentation rate of plastids (Sievers et al., 1989); and (3) may affect the membrane potentials in gravistimulated roots (Sievers et al., 1995).

Wendt, Kuo-Huang, and Sievers (1987) report that CD treatment of *Lepidium* roots during centrifugation, followed by a washout of the CD, had no effect on gravicurvature following subsequent gravistimulation. However, the effect of cytochalasin on gravitropism per se has never been reported. Here we report that cytochalasin D has no effect on gravitropism in roots, a result that is incompatible with the proposed role of actin filaments in gravisensing, and we suggest an alternate mechanism for gravity sensing in higher plants.

MATERIALS AND METHODS

Rice (*Oryza sativa* cv. La Belle) seeds were allowed to imbibe in tap water for 30 min, after which they were surface sterilized in ~1% commercial sodium hypochlorite for 30 min. The seeds were then placed on germination paper (Anchor Paper Products, St Paul, MN), which was rolled up and placed vertically in a plastic beaker containing water to a height of ~2 cm, and allowed to germinate in darkness. We chose rice because they naturally grow in water. Thus throughout the experiments, the roots can be in constant contact with solutions of cytochalasin D.

Seedlings with straight, 1.5–2.5 cm roots were selected and sandwiched between two layers of cheesecloth placed between two glass microscope slides, such that the seeds were held by the cheesecloth and the roots protruded perpendicular to the long axis of the glass slides. The slides were placed in a chamber containing the experimental medium such that the roots were immersed in the aerated test solution, parallel to the vector of gravity (90°). Unless stated otherwise, after 45 min the slides were reoriented so the roots were perpendicular to the vector of gravity (0°). Gravicurvature was then followed for 240 min.

Experiments were also performed on garden cress (*Lepidium sativum*) and corn (*Zea mays*) because these are popular organisms for the study of gravitropism. These seedlings were treated identically to those of rice, except that the seeds were not surface sterilized prior to germination and cress seedlings were used when their roots were ~0.5 cm long.

Root growth and curvature were monitored with a video camera (Model CCD72; Dage MTI, Michigan City, IN) and images were collected every 15 min by an Image 1/AT image processor (Universal Imaging, Inc., West Chester, PA) using a time-lapse program. Curvature and growth were determined from measurements taken with a goniometer from a video monitor (Trinitron; Sony, Ichinomiya, Japan). The growth rates were compared using an analysis of variance program (Minitab Inc., State College, PA). Data are presented as the mean ± 1 SE.

Cytochalasin D (CD; Sigma, St. Louis, MO; Lots 59F4008, 32H4037, 25H4049, 16H4030) was dissolved in dimethyl sulfoxide (DMSO) and diluted with artificial pond water (APW; 0.1 mmol/L NaCl, 0.1 mmol/L KCl, 0.1 mmol/L $CaCl_2$) to a final concentration of 20 μmol/L CD and 0.1% DMSO. We chose to use CD since cytochalasin B has been shown to inhibit root growth (Wendt, Kuo-Huang, and Sievers, 1987; Baskin and Bivens, 1995). Due to the expense of using large volumes of cytochalasin D, we used the solutions for 2 d. The working solutions were kept at room temperature. We find that after 2 d at room temperature, or after refrigeration, the working solutions lose their potency. Therefore we routinely checked their efficacy for inhibiting cytoplasmic streaming in the root hairs of the species being tested and on *Chara* internodal cells.

Cytoplasmic streaming was observed with an Olympus BH-2 microscope equipped with a 20× SPLAN objective (numerical aperture = 0.46) and Nomarski optics (final magnification = 313×). The roots were placed in a solution of APW, containing either 0.1% DMSO or 20 μmol/L CD on a glass slide. A coverslip was placed above the root, supported by four dabs of high vacuum grease. When we compared proportions from independent samples, we tested the significance of the differences with the chi-squared test, using the continuity correction as described by Snedecor and Cochran (1967).

In preliminary experiments, actin microfilaments were observed in rice using a method slightly modified from Vaughan and Vaughn (1987). Roots were fixed for 60 min, while shaking (800 rpm), in freshly prepared 4% (w/v) buffered paraformaldehyde. The buffer included 50 mmol/L PIPES, 5 mmol/L EGTA (ethylene glycol-bis (β-aminoethyl ether) N,N,N',N'-tetraacetic acid) and 5 mmol/L $MgSO_4$ and was titrated to pH 6.9 with NaOH. After fixation, the roots were washed with the buffer for 30 min and subsequently digested for 30 min in 1% (w/v) cellulysin (Calbiochem, La Jolla, CA) and 0.05% (w/v) Bovine serum albumin (Sigma) dissolved in the above buffer. The roots were again washed in the buffer alone, macerated with a glass rod and air dried. The cells were then stained for 30 min at room temperature with a 1:10 dilution of rhodamine-phalloidin (Molecular Probes, Junction City, OR) dissolved in phosphate-buffered saline (PBS: 8.5 g/L NaCl; 0.39 g/L $NaH_2PO_4 \cdot H_2O$; 1.93 g/L $Na_2HPO_4 7H_20$). The root caps were washed with PBS and mounted in PBS containing 0.05% (w/v) n-propyl gallate (Sigma). The cells were then observed with green excitation, epifluorescence optics using an Olympus BH-2 microscope equipped with a 100× SPLAN APO objective (numerical aperture = 1.4). A shortcoming of this method is that the individual cells are separated and thus their identity is ambiguous.

In order to observe actin microfilaments in cells, which could be definitely identified by their position, we developed an alternate procedure. We used corn, instead of rice, since the roots are larger and easier to section. First, the mucilage was removed from the root caps with lens tissue (Ross, Huntington Station, Long Island, NY). Then longitudinal and medial hand sections of corn root tips were cut with a pair of razor blades screwed together. Although sectioning with a vibratome (Series 1000; Lancer, St Louis, MO) produced well-preserved thin medial sections at the tissue level of organization, it was inadequate since vibratoming (at all amplitudes) seems to degrade the actin microfilaments. Hand sections were then fixed for 30 min, while shaking (800 rpm), in freshly prepared 4% (w/v) buffered paraformaldehyde plus 0.075% Triton X-100. The buffer included 50 mmol/L PIPES, 5 mmol/L EGTA, and 5 mmol/L $MgSO_4$ and was titrated to pH 6.9 with NaOH.

In order to visualize actin in the elongation zone and the meristematic zone, the cells were then stained for 20 min while shaking at room temperature with a 1:100 dilution of rhodamine-phalloidin (Molecular Probes, Junction City, OR), dissolved in phosphate-buffered saline and 0.075% Triton X-100. In order to visualize actin in the columella and peripheral cells, the cells were then stained for 60 min while shaking at room temperature with a 1:20 dilution of rhodamine-phalloidin dissolved in phosphate-buffered saline and 0.075% Triton X-100.

The root caps were washed with PBS (2 mL) containing 0.075% Triton X-100 while shaking for 2 h and then mounted in PBS containing 0.05% (w/v) n-propyl gallate (Sigma).

The cells were then observed with green excitation, epifluorescence optics using an Olympus BH-2 microscope equipped with a 100× SPLAN APO objective (numerical aperture = 1.4) two 1.25× intermediate pieces, and either 20× eyepieces or a 3.3× photoeyepiece (final magnification = 3125×, or 516×, respectively). Images were captured by a video camera (Model CCD72), digitized by the Image 1/AT image processing system and enhanced using the sum (16 frames), background subtract, auto enhance, and digital contrast functions.

RESULTS

Rice seedlings exposed to cytochalasin D (20 μmol/L, 45 min–4 h) showed no change in their gravitropic cur-

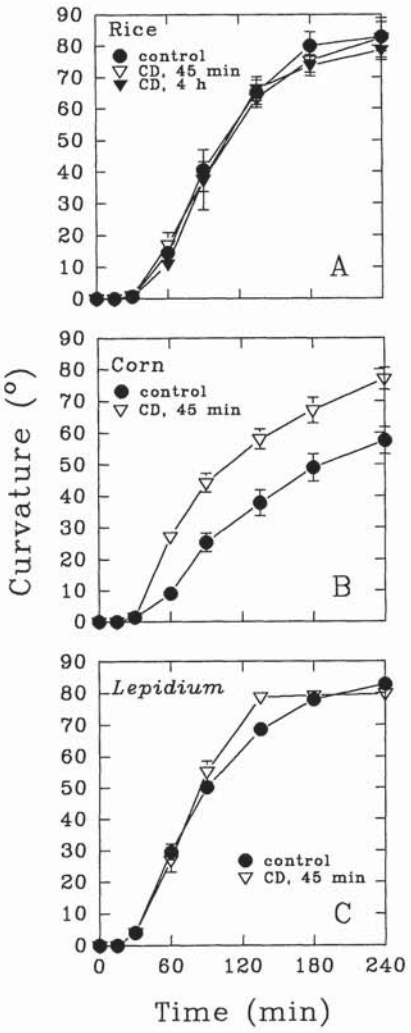


Fig. 1. Time course of gravistimulated curvature of (A) rice, (B) corn, and (C) *Lepidium* roots growing in APW containing 0.1% DMSO with or without 20 μmol/L CD. CD has no significant effect on the growth rates of roots. The growth rates of rice roots pretreated for 45 min were 0.52 ± 0.04 and 0.49 ± 0.02 mm/h for control and CD-treated roots, respectively. The growth rates for rice roots pretreated for 4 h were 0.54 ± 0.02 and 0.54 ± 0.02 mm/h for control and CD-treated, respectively. The growth rates of corn roots (0.71 ± 0.03 and 0.79 ± 0.05 mm/h) and *Lepidium* roots (0.61 ± 0.05 and 0.65 ± 0.03 mm/h) also did not vary significantly between control and CD-treated roots. Means ± 1 SE are presented (N=14–23).

vature (Fig. 1A) or root growth compared with controls. The growth rates for roots pretreated for 45 min were 0.52 ± 0.04 and 0.49 ± 0.02 mm/h for control roots and roots treated with CD, respectively. Likewise, the growth rates for roots pretreated for 4h were 0.54 ± 0.02 and 0.54 ± 0.02 mm/h for control and CD-treated roots, respectively. An analysis of variance shows that the growth rates were not significantly different (P = 0.81 for 45 min; P = 0.36 for 4 h).

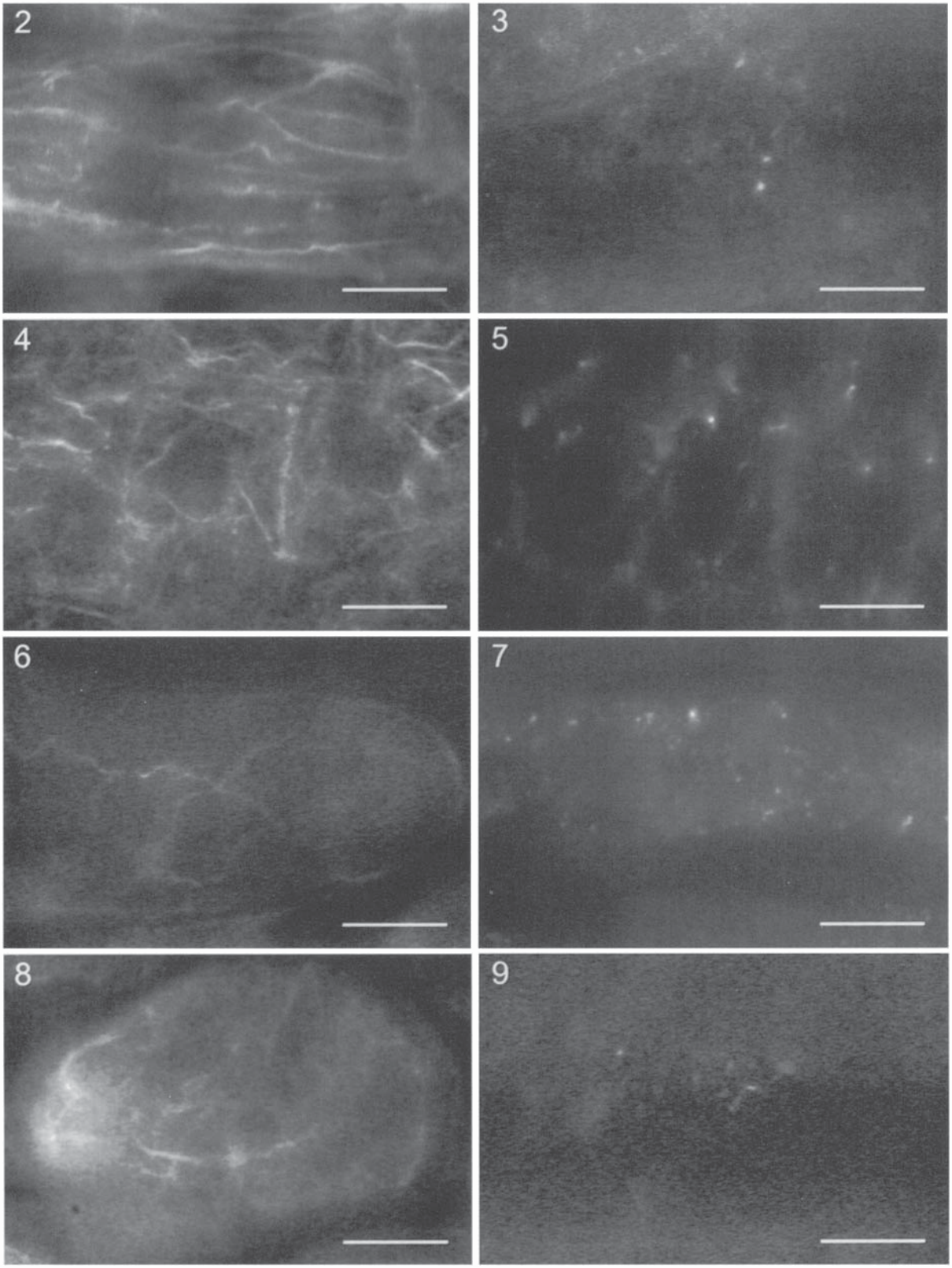
We considered the possibility that CD has no effect on gravitropism because rice roots are resistant to CD. In order to test this possibility, we fixed and stained rice roots, both CD-treated and controls, with rhodamine-phalloidin and examined cells in the root caps. We find that CD treatment (20 μmol/L, 45 min) destroys the organization of the actin microfilaments in rice roots (data not shown), as it does in corn roots (Vaughan and Vaughn, 1987). In addition, we observed the effect of CD on the rotational streaming of the root hairs. This bioassay also indicates that CD affects actin-dependent processes in rice roots since CD (20 μmol/L) instantly reduces the percentage of root hair cells showing a vigorous cytoplasmic streaming from 91% in the control root hairs (N=500) to only 2.2% (N=500; χ^2 = 393). Thus CD inhibits actin-dependent processes in rice roots without affecting gravitropism.

We also find that cytochalasin D (20 μmol/L, 45 min) has no effect on root gravitropism of *Lepidium* or, in agreement with Björkman (1987) corn seedlings (Fig. 1b, c). We confirm the observations of Vaughan and Vaughn (1987) that in corn roots, CD (20 μmol/L, 45 min, the pretreatment time for the gravitropism experiments) perturbs the structure of the actin cytoskeleton in ~95% of the cells. Specifically, CD causes the actin in elongation zone cells (Figs. 2,3), meristematic cells (Figs. 4,5), peripheral cells (Figs. 6,7), and columella cells (Figs. 8,9) to appear fragmented, thickened, or punctate, indicating that it is likely that CD exerts its effect on roots by disrupting the actin cytoskeleton. Stained sections from roots maintained in CD for up to 4 h were indistinguishable from roots incubated in CD for 45 min. In total, these data indicate that while actin microfilaments are an important regulator of columella cell polarity (Hensel and Sievers, 1981; Sievers and Heyder-Caspers, 1983; Hensel, 1985; Wendt and Sievers, 1986; Hilaire et al., 1995), rate of amyloplast sedimentation (Sievers et al., 1989), and gravity-induced membrane potential changes (Sievers et al., 1995), actin microfilaments, and perhaps by extension, the phenomena they regulate, are not essential components of gravitropism in roots of rice, cress, or corn.

DISCUSSION

The sedimentation of statoliths composed of $BaSO_4$ crystals is important for gravitropism in *Chara* rhizoids (Sievers, 1965, 1967a, b; Hejnowicz and Sievers, 1971; Sievers and Schröter, 1971; Schröter, Läuchli, and Sievers, 1975; Kiss, 1994; Gerber, Bisson, and Chau, 1995; Wang-Cahill and Kiss, 1995; Leitz, Schnepf, and Greulich, 1995; Braun, 1996). In this case statoliths affect gravitropism directly by physically displacing the Golgi-derived vesicles necessary for wall growth from the lower side of a gravistimulated rhizoid. The unequal deposition of Golgi-derived vesicles results in positive gravicurvature.

In higher plants, however, it seems that statoliths may not directly affect gravitropism, since sedimentation of statoliths is not required for gravitropism in some instances. Thus an interaction between statoliths and actin has been postulated. We believe that the statolith–actin hypothesis is considerably weakened by the data presented in the present paper, combined with the results of numerous workers. Given the observations that: (1) significant sedimentation of plastids is not necessary for gravitropism (Casper and Pickard, 1989; Kiss and Sack, 1989; Sack and Kiss, 1989); (2) a clear relationship be-



Figs. 2–9. Photomicrographs of elongation zone cells (**2**), meristematic zone cells (**4**), peripheral cells (**6**), and columella cells (**8**) of control roots (treated with 0.1% DMSO, 45 min); and elongation zone cells (**3**), meristematic zone cells (**5**), peripheral cells (**7**), and columella cells (**9**) of roots treated with cytochalasin D (20 μmol/L, 45 min). Sections were stained with rhodamine-phalloidin and viewed with epifluorescence optics. Scale bars = 10 μm.

tween the plastids and any other cellular membranes has not been established (Perbal, 1978; Marty, 1980; Barlow, Hawes, and Horne, 1984; Wendt, Kuo-Huang, and Sievers, 1987); and (3) while a connection between plastids and the actin cytoskeleton has been reported (Hensel and Sievers, 1981; Sievers and Heyder-Caspers, 1983; Hensel, 1985, 1989; Wendt and Sievers, 1986; Sievers et al., 1989, 1995), CD does not inhibit gravisensing (present results); it has not escaped our notice that there is room for an alternative hypothesis for gravisensing in higher plants that does not require a sensing function for statoliths. Such a mechanism has been presented before ("The Gravitational Pressure Model"; Wayne, Staves, and Leopold, 1990; Wayne and Staves, 1996). In brief, we suggest that the gravireceptors reside at the plasma membrane-extracellular matrix junction and these are activated at the top and bottom of the cell by the tensile and compressive stresses, respectively, that result from the settling of the protoplast as a whole within the extracellular matrix (Staves, Wayne, and Leopold, 1992; Wayne, Staves, and Leopold, 1992). In characean internodal cells, the sensing of gravitational pressure results in a polarity of actin-mediated cytoplasmic streaming. The present results, which reveal that disruption of the actin network has no effect on root gravitropism, indicate that the polarity of cytoplasmic streaming is a consequence, not a cause of gravisensing, and that the gravity-sensing mechanism must be independent of any polarity of cytoplasmic streaming (Kessler, 1979).

In conclusion, while actin microfilaments are an important regulator of columella cell polarity, rate of amyloplast sedimentation, and gravity-induced membrane potential changes, actin microfilaments, and perhaps by extension, the phenomena they regulate, are not essential components of gravitropism in roots.

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