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Plastid Sedimentation Kinetics in Roots of Wild-Type and Starch-Deficient Mutants of Arabidopsis[1]

Scott A. MacCleery and John Z. Kiss*

Department of Botany, Miami University, Oxford, Ohio 45056

Sedimentation and movement of plastids in columella cells of the root cap were measured in seedlings of wild-type, a reduced starch mutant, and a starchless mutant of Arabidopsis. To assay for sedimentation, we used both linear measurements and the change of angle from the cell center as indices in vertical and reoriented plants with the aid of computer-assisted image analysis. Seedlings were fixed at short periods after reorientation, and plastid sedimentation correlated with starch content in the three strains of Arabidopsis. Amyloplasts of wild-type seedlings showed the greatest sedimentation, whereas plastids of the starchless mutant showed no significant sedimentation in the vertically grown and reoriented seedlings. Because previous research has shown that a full complement of starch is needed for full gravitropic sensitivity, this study correlates increased sensitivity with plastid sedimentation. However, although plastid sedimentation contributed to gravisensitivity, it was not required, because the gravitropic starchless mutant had plastids that did not sediment. This is the first study, to our knowledge, to measure plastid sedimentation in Arabidopsis roots after reorientation of seedlings. Taken together, the results of this study are consistent with the classic plastid-based and protoplast-based models of graviperception and suggest that multiple systems of perception exist in plant cells.

Of the many different environmental cues that any organism uses for orientation, gravity is the most constant and pervasive. All life has evolved in its presence. Since the emergence of plants from an aqueous environment, gravity has been an essential factor in plant development (Barlow, 1995). Gravitropism can be divided into three phases, distinct in time and, in higher plants, space: perception, transduction, and response (Evans et al., 1986; Salisbury, 1993). It has long been hypothesized that the central columella cells of the root cap are the graviperceptive cells, i.e. the statocytes, in plant roots (Volkmann and Sievers, 1979). Direct evidence supporting the role of columella cells as statocytes includes work by Behrens et al. (1985), who found that only the columella cells experienced rapid membrane potential change after reorientation, and work by Blancaflor et al. (1998), who showed that selective laser ablation of columella cells reduced gravitropic sensitivity.

When a plant is reoriented with respect to a gravitational force, the amyloplasts in the columella cells settle on the new "lower" cell wall. This original observation by Haberlandt (1914) implicated amyloplasts in graviperception and still is a good indication of a graviperceptive organ (Volkmann and Sievers, 1979; Sack, 1991; Salisbury, 1993). On theoretical grounds, Björkman (1988) calculated that amyloplasts have the mass and mobility to activate a receptor with enough energy to make it reliable (250 times thermal noise and 15 times activation energy), whereas other organelles, including mitochondria and the entire protoplast, do not. But even with an amyloplast's relatively great mass, some discernible movement is required for any reliable perception of gravity, according to these theoretical calculations (Björkman, 1988).

An alternative hypothesis for the perception of gravity that does not require starch or plastids is the protoplast-pressure model (Wayne et al., 1990). Despite the large turgor pressure between the protoplasm and the wall, Wayne and coworkers implicated the total weight of the protoplast on the cell wall as the susceptor (with integrins as a possible receptor; see Katembe et al., 1997). This model is supported by the absence of sedimenting particles in both unicellular gravitactic organisms such as *Euglena gracilis* (Häder et al., 1995; Häder, 1997) and in internodal cells of *Chara* sp. (Staves et al., 1992; Staves, 1997). Other studies of the effect of the density of the external medium on gravity-dependent processes in *Chara* sp. (Wayne et al., 1992) and rice (Staves et al., 1997) seem to bolster this hypothesis. According to Staves (1997), the unidirectional force of gravity can be perceived through any omnidirectional force of turgor. This perception could be analogous to thermotropism in maize roots or hydrotropism in maize and pea roots, in which small signals could be detected through a much larger background (for discussion, see Staves et al., 1992). According to this view, amyloplasts could be used only as added weight within the protoplast statolith.

The original theory of Haberlandt (1914) suggests that starch is a necessary component of graviperception in plants. Plants without starch would seem to be a good system in which to test this hypothesis. Studies of starch-depleted wheat coleoptiles (Pickard and Thimann, 1966) found continued gravitropism, whereas studies of cress roots (Iversen, 1969) and barley pulvini (Song et al., 1988) showed no response to reorientation. Although it is difficult to reconcile some of these results, it must be emphasized that the methods used to destarch plants, especially the columella cells, are harsh and can affect the plant in

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* Corresponding author; e-mail kissjz@muohio.edu; fax 1–513–529–4243.

Abbreviations: ANOVA, analysis of variance; WT, wild type.

many unintended ways (Sack, 1991). The use of starch-deficient mutants is a more direct means of measuring differences in graviperception.

As starchless mutants became available, some studies of the root curvature showed a markedly different graviresponse compared with that seen in WT plants. Although gravity was perceived, the mutant was much less perceptive than the WT in maize (mutant amylomaize; Hertel et al., 1969), Arabidopsis (mutant TC7; Kiss et al., 1989), and tobacco (mutant NS458; Kiss and Sack, 1989, 1990). These observations raised two important questions: Were the plastids in these starchless mutants sedimenting in response to gravity? And was the absence of starch affecting the plants in ways other than by changing the mass of plastids in the columella cells? The amylomaize mutant had smaller amyloplasts that sedimented less than the WT amyloplasts (Hertel et al., 1969), supporting the starch/gravitropism correlation. In initial tests, the plastidic phosphoglucomutase mutant TC7 showed no statistically significant difference in vertical plastid positioning between vertical and inverted roots (Caspar and Pickard, 1989). Caspar and Pickard concluded that the plastids did not sediment and therefore could not be the ultimate source of graviperception in these starchless mutants. Kiss et al. (1989) proposed that because the plastids in the mutant were the most mobile structure in the columella cells and were relatively dense, they could still function as statoliths. They reasoned that at least some starch was needed for full graviperception. Knowing the positions of the plastids in the early stages of gravitropism would help in clarifying these issues.

Some new starch-deficient mutants of Arabidopsis have been characterized and were generated by T-DNA mutagenesis (Kiss et al., 1996). The ACG 21 mutant is starchless, whereas mutant ACG 20 has 51% of the WT starch complement. Using measures similar to those tested on the TC7 mutant (Kiss et al., 1989), Kiss et al. (1996) found the starchless mutant to be the least responsive to gravity and the reduced starch mutants to have an intermediate level of graviresponsiveness.

There have been few quantitative studies of plastid movement in response to gravistimulation by reorientation (in dandelion [Clifford and Barclay, 1980], in mung bean [Heathcote, 1981], and in corn [Sack et al., 1985]). However, these were performed on relatively large organs, and it has been technically difficult to perform these types of studies with seedlings of small plant species. It is only with advances in computer-based image analysis that the present study of (small) Arabidopsis roots has been made possible.

Previous papers have reported on plastid movement in starchless mutants in a preliminary manner. We (Kiss et al., 1989) performed low-gravity centrifugation studies to show that the starchless plastids are relatively dense and moveable. In addition, Caspar and Pickard (1989) performed some inversion studies that were based on relatively few data points. The present paper presents a more complete study of plastid movement in response to reorientation in roots of WT and starch-deficient mutants of Arabidopsis. The issue of plastid movement in response to gravity is important in the evaluation of theories of gravity perception in plants (Salisbury, 1993; Sack, 1997). Thus, the focus of these experiments is on the early stages of plastid movement (0.5–60 min), because, according to the starch-statolith hypothesis, significant movement must take place within this period. The major aims of this study were to determine if plastid sedimentation is a requirement for gravitropism and how starch deficiency affects plastid kinetics in reoriented roots.

MATERIALS AND METHODS

Origin of Seed Stock and Plant Propagation

The starch-deficient mutants of Arabidopsis were produced by T-DNA insertional mutagenesis. *Agrobacterium tumefaciens* was used to infect and transform Arabidopsis seeds (T_1), and the seeds produced by infected cells of the T_1 plants were collected and germinated for mutational determination (Feldmann and Marks, 1987). All seeds, including the WT (geographic race Wassilewskija), ACG 20, and ACG 21, were propagated from seeds originally obtained from the DuPont culture collection (Kiss et al., 1996, 1997).

All plants for seed stocks were grown in potting soil (Metro-Mix, Scotts-Sierra Horticultural Products, Marysville, OH) at room temperature (21°C) under constant illumination at 80 to 100 μmol m^{-2} s^{-1} PAR from 34-W fluorescent bulbs (Watt Miser, General Electric). They were watered alternately with tap water and nutrient solution (Haughn and Somerville, 1986). To control for seed age among the WT and the two mutants, seeds used in each experiment were grown and harvested concurrently. After harvest, all seeds were stored at 5°C for 3 to 9 months.

Culture Conditions

Seeds were first surface-sterilized with 30% (v/v) commercial bleach (sodium hypochlorite) and 0.01% (w/v) Triton X-100 (Sigma) for 10 min and then washed three times with sterile water and Triton X-100. Using the final wash, the seeds were sown in a pipette onto 1.7% (w/v) agar medium containing nutrients as described by Haughn and Somerville (1986) with 1% (w/v) Suc in sterile Petri dishes (60 mm in diameter, 15 mm in depth). The seeds were sown in two rows with 8 to 10 seeds per row. In reorientation studies, seeds were immediately covered with 0.8% (w/v) agar medium containing nutrients with Suc (temperature <55°C) to a depth of 3 to 5 mm. All Petri dishes were then sealed with laboratory film (Parafilm, American National Can, Greenwich, CT). The dishes were placed on the 15-mm edge with seed rows horizontal, and a line was drawn indicating the gravity vector. The seeds were germinated and grown under continuous light, as described above, at 18°C to 21°C. The illumination was continuous from side to top to side, and the clear Petri dishes were placed on white paper to negate any potential phototropic response. Germination was greater than 95% in all genotypes. The seedlings were grown for approximately 80 h, until they were 10 to 15 mm long.

Fixation and Sectioning

Fixation for the nonreoriented seedlings was performed with the seedlings still adhering to the agar. A row of seedlings was excised from the Petri dish and placed on glass slides. The glass slides were placed on the edge (maintaining the original gravity vector) in a staining dish for 30 min. The transfer process took less than 10 s for each strip of agar (row of seedlings). The primary fixative, 2% (v/v) glutaraldehyde in 50 mM sodium cacodylate buffer and 50 mM $CaCl_2$, pH 7.2, was added to the staining dish, and the dish was kept at 5°C for 2 h. The staining dish was then washed with buffer three times. The seedlings that remained on the agar in an upright position were collected, and their primary roots were cut 10 to 15 mm from the root tip and postfixed in buffered 2% (w/v) OsO_4 at 5°C for 2 h.

For the reoriented seedlings, the Petri dishes containing the seedlings were turned 90°, a hole was melted in the top with a heated needle, and fixative was added (the dishes were still sealed with laboratory film) at the following intervals: 0 (immediately upon reorientation), 0.5, 2, 5, 10, 30, and 60 min after the start of reorientation. Care was taken when the holes were melted to avoid touching the agar or seedlings with the hot metal. A nonreoriented control was also fixed. The fixative consisted of 1% (w/v) *p*-formaldehyde and 2% (v/v) glutaraldehyde in 50 mM sodium cacodylate buffer and 5 mM $CaCl_2$, pH 7.2, at 4°C. The formaldehyde was used to increase the speed of fixation, because the fixative had to penetrate up to 5 mm of agar. After the addition of fixative, the samples remained at room temperature for 30 min and then were kept at 5°C for 120 to 180 min.

After three washings in the cacodylate buffer, the agar-embedded root tips were excised with a no. 11 scalpel (Fisher Scientific). Each specimen was left in this agar block (through fixation, dehydration, and embedding) to allow for orientation during sectioning. The agar block surrounding the specimen was excised from the Petri dish with a no. 2 cork borer (i.d., 4.5 mm) and marked on the reoriented, lower side (see Fig. 1). This allowed for determination of the reorientation direction of the root tip and easier placement of the specimen in the Quetol resin (Electron Microscopy Sciences, Fort Washington, PA).

Both the specimen containing blocks and the roots from the nonreoriented group were washed with buffer three times and postfixed in buffered 2% (w/v) OsO_4 at 5°C for 2 h. The tissue was then washed, dehydrated in a graded ethanol series, and embedded in Quetol resin according to the method of Kushida and Kushida (1982). Semithin sections (1 μm) were cut with a glass knife and stained with 0.1% (w/v) toluidine blue.

Digitization, Stereology, and Image Analysis

Toluidine-blue-stained sections were visualized with a research microscope using bright-field optics (Zeiss) and a CCD (charge-coupled device) camera (Dage-MTI, Michigan City, IN). The images were then captured using image-analysis hardware and software (Image-1, Universal Imaging Corp., Media, PA). In some cases, sections were

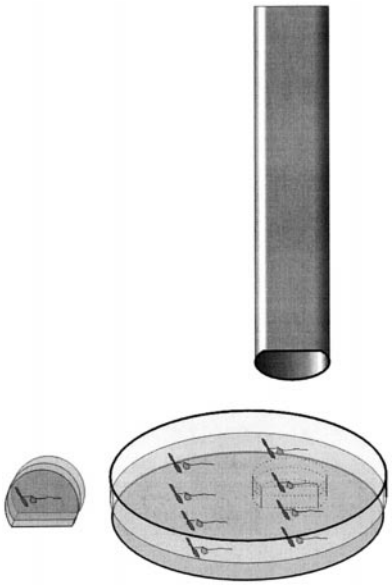

Figure 1. Method used to remove agar-encased seedlings from the Petri dishes in which they were grown. A cork borer was used to excise a block of agar that contained the seedling. Processing of the seedling while it was encased in agar permitted the determination of the plane of reorientation when the roots were sectioned. The block to the left, which had already been excised, would be sectioned along the plane of the page (from front to back).

examined and photographed with bright-field optics using a compound microscope (BH-2, Olympus) with Technical Pan film (no. 2415, Kodak) at ASA (American Standards Association) 50.

An exact median longitudinal section of the Wassilewskija WT (see Fig. 2) shows the arrangement of the root-cap columella cells. All of the mutants used in this study, which were derived from the Wassilewskija strain, had similar root-cap morphology. The lighter staining of the cytoplasm and the presence of darkly staining plastids allowed for fairly easy differentiation of the columella cells.

The distribution of amyloplasts within the columella cells was quantified using stereology to allow the best control over cell-to-cell comparisons. First, the cells were grouped into three stories (as described by Sack and Kiss, 1989), with the first story (S1) being made up of the youngest cells, which were just emerging from the meristem, and the third story (S3) being made up of the oldest cells, which were about to become peripheral cells. In these studies, a maximum of three micrographs per cell was used. To avoid counting the same plastid twice, if a cell was used more then once we determined that the positions of the measured plastids did not coincide. To allow the best stereological control over plastid positioning and comparisons, we used cell micrographs only if the nucleus was visible and none of the walls was sectioned tangentially. These three criteria helped to standardize the data.

The Image-1 computer program, with a Trinitron monitor (Sony Electronics Inc., San Jose, CA), was used to visualize and capture the digitized micrographs and make all of the measurements. After each image was calibrated, the

following data points were determined: each cell's corners (four), the bottom of the plastid, the center of the plastid, and the bottom of the cell wall directly below the plastid (see Fig. 3).

Seedlings were reoriented 90° in the second group, and plastid movement relative to the new, lower cell wall was measured. This implies movement in two dimensions, not just a linear sedimentation. Because root-cap columella cells in Arabidopsis are not exactly rectangular, as some studies seem to suggest, measurement of the radial movement of plastids around the cell permits a more complete picture of plastid motion (Sack et al., 1985). By using the centroid as a relative center of each cell, an angle for each plastid was determined at all times (vertical and 0, 0.5, 2, 5, 10, 30, and 60 min). The centroid was the origin, whereas a line to each plastid and a line from the centroid up the root, parallel with the original gravity (as the plant was grown), made the sides (see Fig. 3). The following data points were recorded for each plastid: (a) the angle from the axis of the root, (b) the distance from the origin, (c) the distance to the distal cell wall, (d) the distance to the new bottom cell side, and (e) the absolute Cartesian coordinates of all points.

From this point in the center of the cell, two fixed reference lines, relative to general cell morphology, were used to determine angular plastid movement. One line was relative to the gravity vector, and the other was relative to the cell corner (by which plastids must pass if they are to sediment to a new lower wall). The former measurement relates the movement of the plastids to gravity and provides an indication of where the plastids might move if there were no obstructions and no other forces acting on them. This measurement also reveals whether the calculated centroid is a valid point on which measurements can be based, because before reorientation, plastids should be along the gravity vector "below" the centroid (approximately 180°). The second measurement, relative to the cell corner between the original and the new, lower cell walls, is important because it accounts for the fact that the columella cells are not rectangular (see Fig. 2). These cells are trapezoidal, but the corners are almost never 90°. Measurement relative to a corner helps correct for the true morphology of the cell and affords yet another measure of movement (Sack et al., 1985). With this measurement, one can determine if and when a plastid ever moves past the corner.

RESULTS

Root-Cap and Columella Cell Morphology

Bright-field microscopy of roots from 4-d-old (vertically grown) Arabidopsis seedlings demonstrated that the root cap consists of four horizontal stories and four vertical files of cells (Fig. 2). The stories are labeled 1 to 4 from the meristem to the root tip according to the method of Sack and Kiss (1989), and usually the first three stories are considered columella cells. Beyond story 3, the columella cells usually lose polarity and develop enlarged vacuoles, and thus become peripheral cells, before being shed from the tip. Cells were determined to be columella cells if they were polarized, had a small vacuolar area, and stained much lighter than the surrounding cells (peripheral cells and the meristematic region). In vertically grown seedlings, a typical columella cell has a polarized morphology, with the nucleus always at or near the proximal end and the plastids near the distal end (Fig. 2). Even in the starchless mutant, the plastids were not near the "top" of the cell, close to the nucleus.

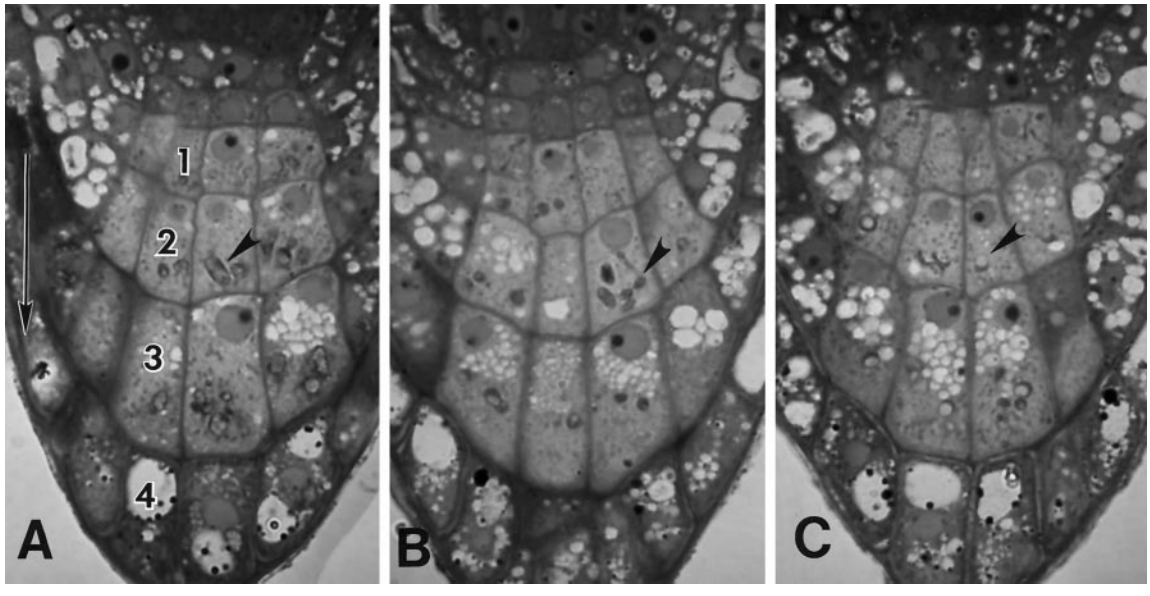

Figure 2. Light micrographs of 1-μm sections of the root cap of Arabidopsis WT (A), ACG 20 (51% starch; B), and ACG 21 (0% starch; C) stained with toluidine blue. Arrowheads indicate plastids in columella cells. Columella cells are in stories 1 through 3, and peripheral cells are in story 4. The WT plastids are somewhat larger than those of the mutants, but otherwise the morphology of the root of all strains is uniform. The arrow indicates the gravity vector and equals 25 μm.

Although only columella cells from stories 2 and 3 were used for this study, there is still variation in size and shape within this cell type. There are two inner and two outer cells in each story that differ in shape. These are referred to as the central and flank columella cells, respectively, according to Blancaflor et al. (1998). As shown in Figure 2, the cells on one side of the center line are mirror images of the cells on the other side. Even though they still have longitudinal running sides, the proximal and distal cell walls are sloped toward the center. An approximately equal number of cells from each side of the center line was used to normalize the data. Also, it was assumed that this morphology also occurs in the Z direction (to and away from the observer). This was compensated for by specifying a certain cell morphology for all cells in which plastid position was analyzed: The nucleus must be sectioned in approximately the middle third, the longitudinal walls must be parallel, and the cytoplasm must be light, continuous, and demarcated abruptly at the cell wall.

Both the absolute and the fractional cell data were quantified to characterize the position and movement of plastids in the columella cells of Arabidopsis. Whereas many studies have measured absolute or fractional cell plastid positional information, almost none has made direct measurements of both parameters.

Vertically Oriented Seedlings

The extent of sedimentation of the plastids was determined in roots of vertically oriented seedlings, and this analysis showed that the cell fractional sedimentation differed significantly (Table I), as determined by ANOVA with Tukey's posttest ($P < 0.05$). The sedimentation of the WT plastids was significantly different and greater in magnitude than that of plastids from both starch-deficient mutants. In addition, plastids of the starchless mutant were approximately one-half as sedimented as the WT plastids.

Reoriented Seedlings

Linear Plastid Sedimentation

Because the plastid-statolith hypothesis suggests that it is the settling of plastids upon some receptor that allows graviperception, an important measurement of plastid movement is linear movement parallel to gravity. Presumably, it is this movement toward the new, lower wall that allows graviperception (e.g. interaction with the ER or the cytoskeleton). In measurements relative to the new, lower cell wall (both absolute distance in micrometers and fraction of cell height), the WT plastids sedimented more than the plastids of the two starch-deficient mutants (Figs. 3 and 4). The positions of plastids of the WT and starch-deficient seedlings became statistically significantly different compared with the positions of the plastids of the starchless mutant at 0.5 and 10 min, respectively (ANOVA with Tukey's posttest, $P < 0.05$; Fig. 4). Once their positions diverged from those of the starchless plastids, the plastids of the other two strains continued to sediment, and the difference (from the starchless mutant) increased.

The WT and reduced-starch plastids sedimented at approximately the same rate, whereas the sedimentation of the starchless mutant was not significantly different (ANOVA with Tukey's posttest, $P > 0.05$). At the WT presentation time of 5.3 min (Kiss et al., 1996), the plastids had sedimented 68% to 87% of the final distance (relative to the 30- and 60-min points).

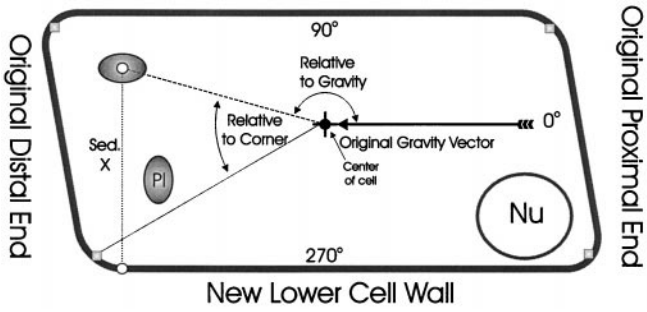

Figure 3. Central columella cell (inside-"right") after reorientation of the root. As the diagram suggests, the distal and proximal cell walls were not perpendicular to the root axis. The plastid angle relative to gravity, the plastid angle relative to the cell corner, and the sedimentation measurements are indicated (the original "up" vector being 0°). Pl, Plastid; Nu, nucleus. Each shape represents a measured point. Black circle, Center of cell (calculated); open circles, measured points for each plastid; gray squares, corners of cell.

Angular Data

Because roots were rotated 90° in the reorientation study, movement in two dimensions, not just linear sedimentation, was measured in these studies. Considering that root-cap columella cells in Arabidopsis are not exactly rectangular, as some studies seem to imply, measurement of the radial movement of plastids around the cell permits a more complete picture of plastid motion (Sack et al., 1985). An angle was determined by first finding the "center" of the cell, which was done by manually tracing the cell and having the software determine the center of area (see "Materials and Methods").

From the center of the cell, two fixed reference lines, compared with general cell morphology, were used to determine angular plastid movement: (a) relative to the gravity vector, and (b) relative to the cell corner by which the plastids must pass if they are to sediment to a new, lower

Table I. *Plastid sedimentation by cell fraction in the root columella*

Seedlings were fixed vertically at 4 d. The sedimentation and total cell height were measured from 1-μm sections stained with toluidine blue. Sedimentation was defined as the ratio of the distance from the center of the plastid to the distal cell wall. The values indicated with different letters are significantly different as determined by ANOVA/Tukey's method ($P < 0.05$). *n*, No. of plastids.

Sample	Mean Plastid Distance Relative to Lower Wall
WT ($n = 87$)	0.1593 ± 0.0191a
ACG 20 ($n = 67$)	0.2397 ± 0.0448b
ACG 21 ($n = 67$)	0.3086 ± 0.0523b

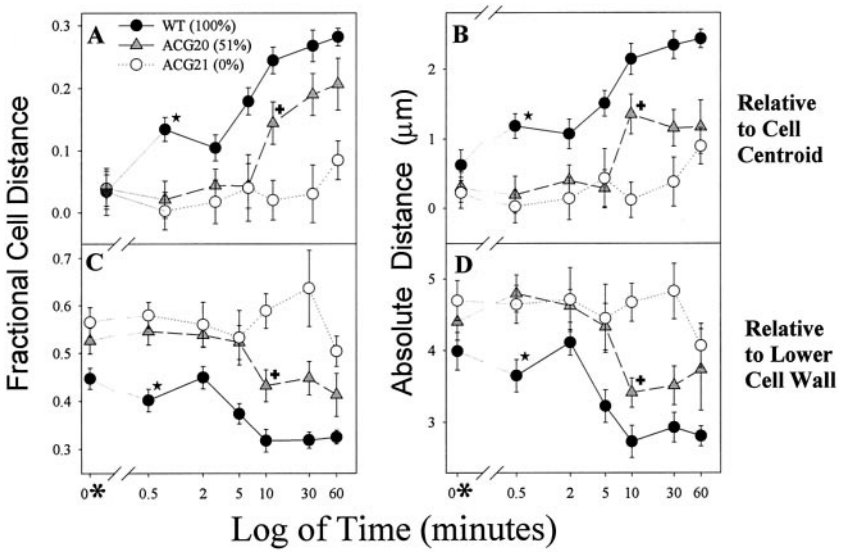

Figure 4. Time-course studies of linear plastid sedimentation in the root-cap columella cells of WT and mutant Arabidopsis. The seedlings were reoriented 90°, and the vertical distance was measured relative to the calculated cell center (A and B) or the new cell bottom (C and D) at the times indicated. Plastids of both starch-deficient mutants sedimented less than those of the WT. Stars and crosses indicate when the WT and 51% starch-deficient mutant, respectively, became statistically significantly different from the starchless mutant (ANOVA with Tukey's posttest, $P < 0.05$). A, Mean vertical plastid position relative to the cell center measured as a fraction of the cell size. Because the seedlings were reoriented 90°, the mean plastid position was almost zero ("middle" of the cell) at time 0. B, Mean vertical plastid position relative to the cell center as absolute distance (μm). The average distance across the cell was 10 μm. C, Mean vertical plastid position relative to the new, lower cell wall measured as a fraction of the cell size. D, Mean vertical plastid position relative to the new, lower cell wall as absolute distance (μm). Values in parentheses indicate the amount of starch relative to the WT, and error bars represent SE. Each data point represents a mean of 25 to 40 plastids. ●, WT; △, ACG 20; ○, ACG 21. Time zero (*) is added to the logarithm time scale for comparison.

wall (see Fig. 3). The former measurement gives some indication of where the plastids might move if there was nothing in their way and no other forces acting on them.

Thus, when the plastid angle relative to gravity was examined, it became apparent that the plastids of all three strains started at approximately the same mean point (≃180° along the gravity vector), but WT plastids had the greatest angular displacement (Fig. 5A). At 2 min and later, the WT plastids had a statistically significantly greater angle than both the starchless and the reduced-starch mutants ($P < 0.05$). In addition, WT plastid displacement was at a faster rate than plastid displacement in the reduced-starch and starchless mutants. The WT plastids had their greatest angular velocity between 2 and 10 min and slowed markedly after 30 min. By 60 min, the WT plastids had a less random positioning, as shown by the relatively small SE. The plastids of both starch-deficient mutants were displaced at about the same rate, which was less than the rate of the WT.

The second angular measurement (Fig. 5B), plastid angle relative to cell corner (i.e. relative to the cell corner between the original and the new, lower cell walls), is important because it accounts for the fact that the columella cells are not rectangular (see Fig. 2).

Although the central cells are approximately rectangular, the flanking cells deviate from this shape. Thus, measurement relative to a corner helps correct for the true morphology of the cell and affords another important measure of movement (Sack et al., 1985). With this measurement, one can determine if and when the plastids ever move past the corner. If, as proposed by many researchers (for review, see Sack, 1991), plastids allow perception by falling onto a sensitive surface, then it is only after they pass the corner that they are able to fully apply pressure to such a surface.

Although plastids of all three strains started at different angles relative to the cell wall corner, WT plastids had the greatest angular displacement (Fig. 5B). Despite the fact that the WT plastids were larger than the plastids of either of the mutants, they moved farther around the cell. The WT amyloplasts, in fact, were the only plastids to pass the corner, after which their movement slowed to a great degree.

A series of light micrographs qualitatively illustrate the plastid-sedimentation patterns (Fig. 6) that were documented in the quantitative time-course studies (Figs. 4 and 5). Amyloplasts in columella cells in roots of WT seedlings showed the most obvious sedimentation (Fig. 6A), whereas plastids in the reduced-starch mutant also appeared to sediment (Fig. 6B). In contrast, plastids in columella cells of the starchless mutant lacked obvious sedimentation (Fig. 6C).

DISCUSSION

Comparison with Other Studies of Plastid Sedimentation

Uncertainties about definite receptors, the original asymmetrical signal, and even the signal transmitted from the

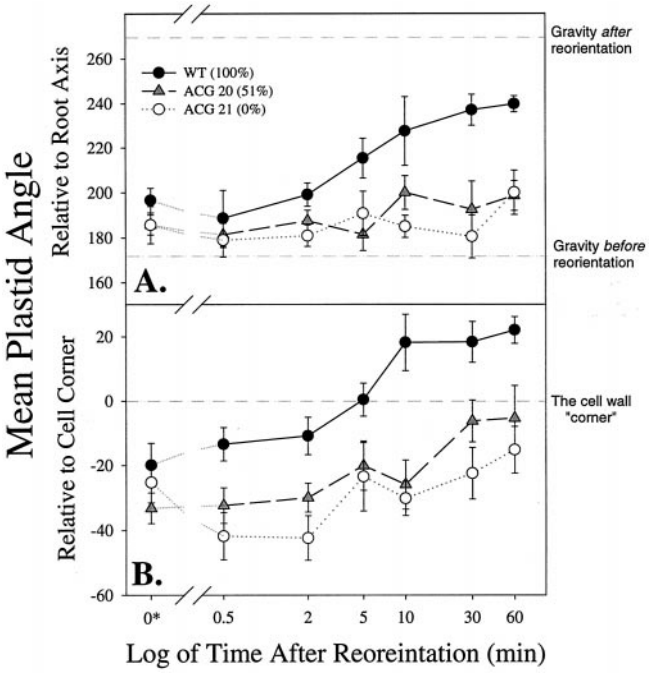

Figure 5. Time-course studies of angular plastid sedimentation in the root-cap columella cells of WT and mutant Arabidopsis. The seedlings were reoriented 90°, and the plastid angle from the calculated cell center was measured relative to the cell corner or the root axis (gravity). By 2 min, WT plastids sedimented significantly more than plastids in both starch-deficient mutants ($P < 0.05$). A, Mean angle of plastids relative to the root axis (gravity). B, Mean angle of plastids relative to the cell corner. Values in parentheses indicate the amount of starch relative to the WT. Each data point represents a mean of 25 to 40 plastids, and error bars represent SE. ●, WT; ▲, ACG 20; ○, ACG 21. Time zero (*) is added to the logarithm time scale for comparison.

root cap to the elongation zone have compounded the problem of finding the original gravity susceptor(s). The aim of this research was to analyze plastid movement from many different and biologically important perspectives. This study continues the preliminary observations of plastid movement in WT and starchless Arabidopsis roots reported by Caspar and Pickard (1989) and Kiss et al. (1989). Thus, a more complete study of plastid movement in response to reorientation in roots of WT and starch-deficient mutants of Arabidopsis is presented here. Furthermore, an accurate study of plastid kinetics in the extremely small roots of Arabidopsis was made possible only by recent advances in computer-based image analysis.

Plastids in the Vertically Oriented Starch-Deficient Seedlings Sediment Less Than WT Plastids

One standard measure of graviresponsiveness is the orientation of seedlings around the gravity vector (Sack, 1991; Kiss et al., 1996, 1997). Although the gravity vector is unchanged, Arabidopsis starch-deficient mutants are not as oriented around gravity as the WT (Kiss et al., 1996). This presumably is a function of the smaller, buoyant mass of the plastids, but to our knowledge, until this study it was not known if there was less sedimentation of plastids. Based on comparison of micrographs, it appears that the cell morphology is unchanged except for the positions and sizes of the plastids. In vertically grown seedlings, the plastids within the columella cells were at different positions relative to the distal cell wall (which here is the cell bottom with respect to gravity) and differed in their arrangement. Plastids of the starchless mutant (ACG 21) were only about one-half as sedimented as WT plastids.

The word "sedimented" is used in a general sense in this discussion, relative to the whole cell, as it has been used by workers in the field (e.g. Sack et al., 1985, 1986). It is important to note that sedimentation is not an absolute measure. Depending on how, and relative to what, sedimentation is defined (available space for sedimentation, relative to which cellular apparatus), many different views can be supported.

The positioning of the starch-deficient plastids also was less regular than that of the WT amyloplasts, as indicated by the greater SE. The plastid positioning in nonreoriented roots agrees with data from inverted studies of the TC7 starchless mutant (Caspar and Pickard, 1989) and qualitative observations of TC7 (Saether and Iversen, 1991) as well as data from the starch-deficient mutant of maize (Hertel et al., 1969). The similar results at the plastid level, along with studies of the kinetics of gravitropic curvature (Kiss et al., 1996), are particularly significant, because these two starchless mutants were isolated from different ecotypes and by different mutational methods (chemical and T-DNA insertion).

Starchless plastids are less than one-half as large as the WT plastids and are more irregularly shaped, as measured by light microscopy. The large size of the WT amyloplasts theoretically would prevent them from packing as tightly as the starch-deficient plastids. Thus, the difference in sedimentation detected here could be somewhat underestimated. However, it was observed that the WT plastids were more tightly packed within a cell (in the reoriented seedlings as well). One simple explanation for the rather regular arrangement of the WT plastids is that they sediment and come to some equilibrium point near the bottom of the cell. The large size and tight grouping of these WT plastids would subdue movement compared with the starch-deficient plastids. It is important to note that the plastids in all genotypes are not at "rest" and are most likely continually moving in one direction or another by limited cytoplasmic streaming and saltations (Sack et al., 1986). These saltations, if of equal strength in the mutants, would likely cause a less concentrated plastid group in the reduced-starch mutants, because the plastids presumably have a smaller mass.

It is also interesting to note the sedimentation when the volume displaced by other organs is taken into account. All columella cells in Arabidopsis have a large (about one-quarter of the cell length), proximally located nucleus (Kiss et al., 1989). Thus, if one were to subtract the proximal vertical distance of the cell that the nucleus occupies, the starchless mutant plastids would be found at the vertical midpoint of the cell.

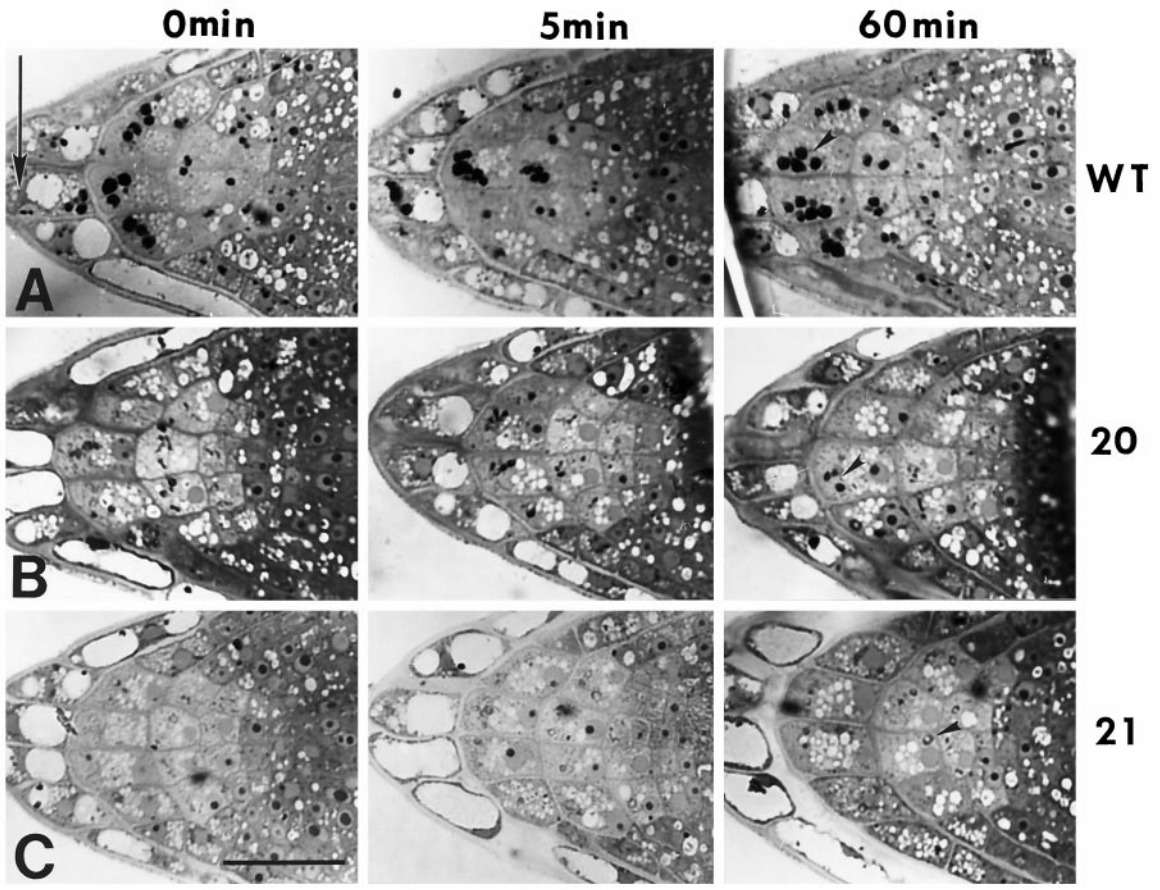

Figure 6. Time-course photomicrographs of root caps of WT and mutant Arabidopsis seedlings after gravistimulation by reorientation. The seedlings were grown for 4 d, reoriented 90°, and then fixed at the times shown. Row A shows WT, row B shows ACG 20 (51% starch), and row C shows ACG 21 (0% starch) at 0, 5, and 60 min after reorientation. Arrowheads indicate plastids in columella cells. WT plastids sedimented in response to reorientation, whereas starchless mutant plastids did not appear to sediment. In the starchless mutant, a plastid can be seen in the proximal end of the cell above the nucleus (bottom arrowhead). This was not common, and it was never observed in the WT. The direction of gravity is toward the bottom of the figure, as indicated by the arrow. Bar = 25 μm.

WT Plastids Sediment Faster and to a Greater Magnitude When the Root Is Reoriented Compared with Plastids of the Starch-Deficient Mutants

Linear measurement in the reoriented seedlings showed the sedimentation of the plastids to the new, lower cell wall. Presumably, it is this movement toward or settling onto the new, lower wall that allows graviperception (interaction with the ER or the cytoskeleton). This traditional measurement of sedimentation shows that the plastids with more starch have a greater degree of sedimentation. Although a seemingly obvious observation, this is an important consideration in the evaluation of possible models of perception. At the WT presentation time of 5.3 min, 68% to 87% of the plastids had sedimented.

It is important to note that only the WT plastids pass the cell corner. If, as claimed by many researchers in the field (for review, see Sack, 1991), plastids allow perception by falling onto a sensitive surface (presumably below the plastids), then it is only here (after they pass the corner) that they first are able to apply full pressure to any supposed sensitive surface.

For the plastid-reorientation study, precision in sectioning is important in both the radial (around the root) and longitudinal (along the axis of the root) directions (Fig. 7). The longitudinal precision was controlled by close observation of the morphology of the cells. The radial imprecision could not be observed and corrected. Although the roots remained encased in agar during the fixation and embedding procedure, some twisting could still take place, causing the section to be out of radial alignment (about one-tenth of the original seedlings were discarded when twisting was observed after root excision but before infiltration of resin). A section not in the correct radial plane would result in measurement of a less sedimented, more random plastid position, because the section would not be 90° from the plastid movement. Therefore, we cannot conclude that the sedimentation of the plastids shown in the reoriented seedlings is a realistic maximum. Rather, the data measured here can be viewed as a slightly low approximation, and, because all genotypes were sectioned identically, the differences observed presumably would still hold.

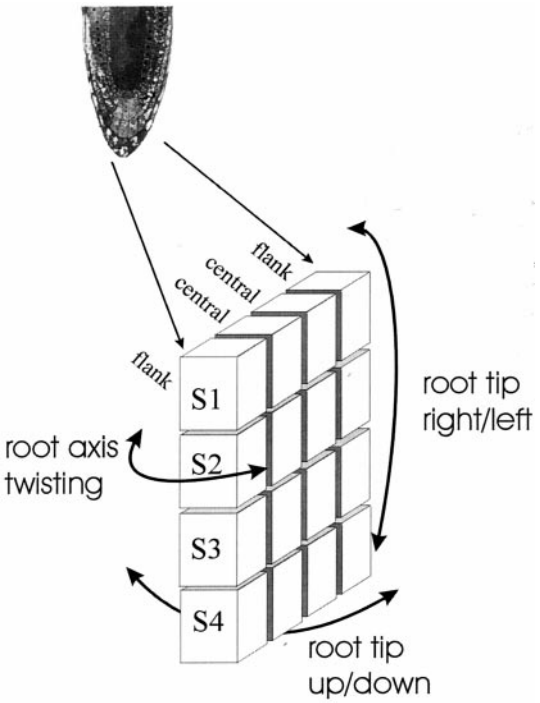

Figure 7. Root-cap cells in Arabidopsis. The four stories (S1–S4) and four files (central and flank) are labeled. The arrows indicate the possible orientational errors when sectioning. Although the left/right and up/down of the root tip could be well accounted for, twisting (a possibility when cutting the seedlings out of the agar) of the root could introduce errors in the absolute measurements. This is one reason why multiple methods to measure plastid sedimentation were used in this study.

A Greater Degree of Plastid Sedimentation Is Required for a Full Gravitropic Response

As measured by the three methods used in this study (i.e. vertical orientation, angular reorientation, and linear reorientation), WT plastids sedimented faster and to a greater degree than the starchless plastids, and the reduced-starch plastids sedimented an intermediate amount. This demonstrates a correlation of starch content with the rate and magnitude of plastid sedimentation, and because previous research (Kiss et al., 1996, 1997, 1998) has shown that starch content is positively correlated with increased gravisensitivity, the present and previous studies demonstrate that graviresponse is correlated with the rate and magnitude of plastid sedimentation. Although not necessarily a cause, sedimentation does seem to be closely involved with perception. However, this does not explain the lack of significant sedimentation in starchless plants that are still able to perceive gravity. This finding seems to indicate that there is a different, less sensitive mechanism that allows the starchless mutant to perceive gravity, possibly a protoplast-based system. Redundancy at various levels seems to be common in the evolution of plant perceptual systems (Barlow, 1995). Alternatively, it is possible that the plastids still perceive gravity but that any gross movement is not required (Nick et al., 1997).

Presentation time, a measure that includes the perception and transduction/transmission steps, and perception time, which includes the perception and perhaps the intracellular statocyte asymmetry transduction, have been used traditionally as measures of relative graviperception (Volkmann and Sievers, 1979). For WT roots (Kiss et al., 1996), the calculated presentation time is 5.3 min, and the present study demonstrates that there is significant movement of plastids toward the new, lower wall after gravistimulation by reorientation.

Perception times can be approximated by using intermittent stimulation, alternating with clinostat rotation. In this manner, stimulation times can be summed to produce an effect (a single application would not cause a response). Kiss et al. (1996) found that with 6:4 intermittent stimulation, the reduced starch mutant had 64% of the WT curvature, whereas the starchless mutant had 20% of the WT curvature. With these estimated measurements, relative gravisensitivity was determined. In the present study, calculations of the percentage of WT plastid sedimentation (Fig. 4) showed an approximate correlation to these values. Thus, the reduced starch mutant had 55% of WT plastid sedimentation and the starchless mutant had 16% of the sedimentation of the WT.

Sedimentation of Plastids Can Play a Role in Graviperception but Is Not Required for Perception

When a columella cell is reoriented with respect to gravity, the plastids sediment to the new, lower cell wall. This is the original observation that implicated plastids in graviperception (Haberlandt, 1914). Even in recent studies mapping the functional role of columella cells, those with the most readily sedimentable plastids were the ones that seemed to contribute the most to graviperception (Blancaflor et al., 1998).

The protoplast model proposes that amyloplasts are used only as added weight within the protoplast statolith (Staves et al., 1992; Staves, 1997). However, it seems probable that these models are not mutually exclusive (Barlow, 1995). Multiple systems could be working at different levels. When starch is present, the sensitive plastid-statolith mechanism would predominate. Yet when the seedling is starch free and the plastids in the columella cells do not sediment appreciably, the protoplast mechanism would still allow a minimum level of graviperception.

One important shortcoming of the starch-statolith theory is the lack of a definitive receptor. This greatly affects any interpretation of plastid positioning and movement. Nevertheless, in this study we examined plastid sedimentation in many different and presumably biologically important ways. The starch-deficient mutants, which are less gravitropic than the WT, have plastids that sediment less than the WT amyloplasts. However, plastids in starchless plants, which can perceive gravity, do not show statistically significant sedimentation, and this observation implies that there is a different, parallel system capable of detecting gravity. Taken together, the results of this study are consistent with the classic plastid-based and protoplast-based

models of graviperception and suggest that multiple systems of perception exist in plant cells (Barlow, 1995).

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