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Plastid position in *Arabidopsis* columella cells is similar in microgravity and on a random-positioning machine

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Abstract. In order to study gravity effects on plant structure and function, it may become necessary to remove the *g*-stimulus. On Earth, various instruments such as clinostats have been used by biologists in an attempt to neutralize the effects of gravity. In this study, the position of amyloplasts was assayed in columella cells in the roots of *Arabidopsis thaliana* (L.) Heynh. seedlings grown in the following conditions: on Earth, on a two-dimensional clinostat at 1 rpm, on a three-dimensional clinostat (also called a random-positioning machine, or an RPM), and in space (true microgravity). In addition, the effects of these gravity treatments on columella cell area and plastid area also were measured. In terms of the parameters measured, only amyloplast position was affected by the gravity treatments. Plastid position was not significantly different between spaceflight and RPM conditions but was significantly different between spaceflight and the classical two-dimensional clinostat treatments. Flanking columella cells showed a greater susceptibility to changes in gravity compared to the central columella cells. In addition, columella cells of seedlings that were grown on the RPM did not exhibit deleterious effects in terms of their ultrastructure as has been reported previously for seedlings grown on a two-dimensional clinostat. This study supports the hypothesis that the RPM provides a useful simulation of weightlessness.

Key words: Amyloplast – *Arabidopsis* – Simulated microgravity – Spaceflight experiments – Weightlessness

Introduction

All organisms have originated and evolved in Earth's environment where a gravity stimulus is continually present. Since the processes that regulate and maintain life developed in such an environment, it can be useful to study organisms in microgravity, a near gravity-free setting. One obvious way to achieve a low-gravity environment is by performing spaceflight experiments. However, flight research is expensive and the opportunities have been very limited (Krikorian et al. 1992; Katembe et al. 1998).

Short of actual spaceflight, the following methods have been used to minimize or eliminate 1-*g* conditions (Claassen and Spooner 1994; Mesland 1996): drop towers (seconds of weightlessness) and parabolic flights in aircraft (tens of seconds of weightlessness). In addition, rather than using expensive orbital facilities such as the space shuttle and biosatellites, sounding rockets have been a more cost-effective alternative and provide minutes of weightlessness. Nevertheless, for most biological studies, these methods provide a period of weightlessness that is too short to study most developmental and growth phenomena, and this certainly is the case for research with flowering plants (Dutcher et al. 1994).

Therefore, instruments known as clinostats have been developed in an attempt to neutralize unilateral 1-*g* effects on Earth (reviewed in Salisbury 1993; Claassen and Spooner 1994; Hoson et al. 1997). A clinostat is a device that rotates specimens around an axis or, in some cases, three dimensions. A variety of clinostats have been utilized to study plant growth and development as well as to answer basic questions about sensory physiology. These clinostats can be divided into three types: (i) one-axis slow-rotating (1–4 rpm) clinostats; (ii) one-axis fast-rotating (50–120 rpm) clinostats; and (iii) two-axis or three-dimensional clinostats (also called random-positioning machines; RPMs).

While these devices do not literally eliminate gravity, they can be used to produce more of an omnilateral

Abbreviations: CL = clinostat; FL = spaceflight; GR = ground; RPM = random positioning machine

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gravity stimulus rather than a unilateral 1-*g* stimulus, and, thus, have been utilized in numerous plant biology studies (Hoson et al. 1997 and references therein). The slow-rotating clinostat has been the most extensively used in many types of developmental studies, especially in studies of gravity perception (Kiss et al. 1989; Hilaire et al. 1995). However, a number of papers have demonstrated that this type of clinostat causes undesirable side effects (due to a "chronic dynamic stimulation," Sievers and Hejnowicz 1992; Hoson et al. 1997) such as an increase in ethylene production and/or a destruction of cellular polarity (e.g. Hensel and Iversen 1980; Hensel and Sievers 1980). Fast-rotating clinostats avoid some of these problems, but they can only be used with small specimens (diameter less than 1 mm) since unacceptable centrifugal accelerations may occur with larger samples (Brown et al. 1996).

The RPM has been found to have a great deal of promise in botanical research (Mesland 1996; Hoson et al. 1997, 1999). The RPM was developed as a refinement of the one-axis (or one-plane) clinostat and has been shown to be an effective tool to simulate weightlessness (reviewed in Mesland 1996; Hoson et al. 1997). Recently, two instruments have been described in the scientific literature. The first RPM was developed by a Japanese team (Hoson et al. 1992, 1997), and a European group has developed an RPM that is now commercially available (Mesland 1996). The basic design of the RPM consists of two planar frames that can be rotated independently and randomly (Mesland 1996; Hoson et al. 1997).

Many articles have focused on comparing the biological effects of two-dimensional clinostats to true microgravity (e.g. Brown et al. 1974; Lorenzi and Perbal 1990; Brown et al. 1996; Kordyum 1997). The overwhelming conclusion is that while these clinostats may be effective tools in some instances, there are great differences observed between biological specimens that develop and grow on clinostats and those that develop in true microgravity. In fact, as stated above, in plant cells, rotation on two-dimensional clinostats can have deleterious effects (Hensel and Sievers 1980).

Since the RPM is a relatively new tool, there are fewer comparative studies available on the effects of this instrument versus true microgravity effects. However, in terms of certain biological parameters, such as starch content and endoplasmic reticulum distribution in columella cells of *Lepidium* (Buchen et al. 1993), structural polarity in *Chara* rhizoids (Hoson et al. 1997), and automorphogenesis in *Arabidopsis* and *Oryza* (Hoson et al. 1999), the RPM appears to provide a good simulation of weightlessness conditions achieved during spaceflight.

To further investigate the utility of this instrument, in this paper, we compare results obtained with an RPM to data obtained (i) from spaceflight investigations and (ii) from a classical two-dimensional clinostat. In terms of plastid position in the columella cells, the RPM produced results similar to true microgravity, whereas the results from the two-dimensional clinostat were different as compared to microgravity. This is one of the first reports to directly compare results from a spaceflight experiment to those obtained with both an RPM and a classical clinostat.

Materials and methods

Plant material and culture conditions. Dark-grown seedlings of *Arabidopsis thaliana* (L.) Heynh. (strain Wassilewskija, Ws) were used in these experiments approximately 4 d after hydration of seeds with a growth medium (Kiss et al. 1996). Seedlings were grown in spaceflight hardware (Fig. 1) for most of the studies reported in this paper. This hardware included type-I containers, each with two minicontainers, as described in Katembe et al. (1998). The inside of the minicontainers had two surfaces for plant growth and consisted of a "sandwich" of filter paper and cellulose ester membranes held together by o-rings (Katembe et al. 1998; Kiss et al. 1998). In some cases, for comparative purposes, seedlings were grown on a nutrient agar in Petri dishes (Kiss et al. 1996).

Spaceflight and 1-g control studies. The experiments were flown on the space shuttle *Atlantis* in May 1997 on mission STS-84 in the European Space Agency's (ESA's) Biorack laboratory module. Crew procedures have been summarized in a previous publication (Kiss et al. 1999b), and seedlings from spaceflight conditions are termed flight (FL) specimens. Briefly, dry seeds were placed into the minicontainers prior to launch, and an astronaut mission specialist hydrated the seeds to activate the experiment. At the end of an incubation period of 89 h at 22 °C, seedlings were placed into type-II fixation devices which were filled with 4% (v/v) glutaraldehyde in 100 mM phosphate buffer (pH 7.2). Due to spaceflight constraints, seedlings were kept in glutaraldehyde for a total of 5.5 d. However, preliminary studies (Kiss et al. 1999a) revealed this did not have a deleterious effect on ultrastructure. Processing for microscopy continued on the ground, and, as the final step, the samples were embedded in Quetol 651 resin (Guisinger and Kiss 1999). A parallel experiment at 1 *g* (henceforth termed GR) was performed on the

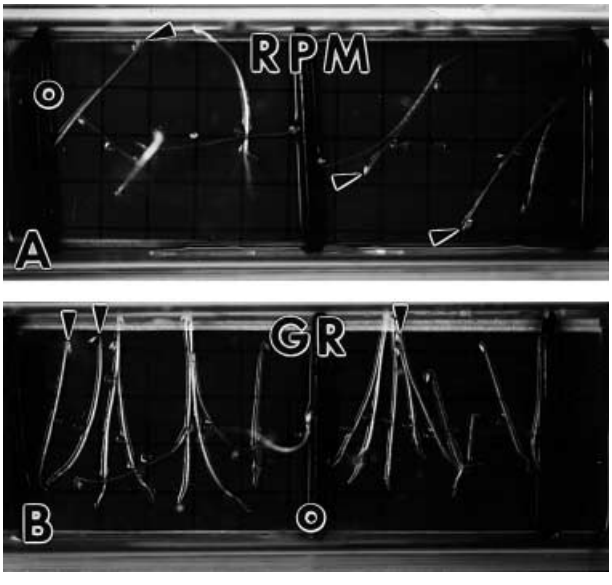

Fig. 1A,B. Photographs of *Arabidopsis* seedlings in minicontainers illustrating growth on the RPM (**A**) and in the GR control (**B**). *Arrowheads* indicate the hypocotyl apices, and *o* indicates o-rings that support the membranes in the minicontainer. The gravity vector is toward the bottom of **B**, and the distance between grid lines on the black membrane is 3 mm

ground with the same hardware, procedures, and time line at the Kennedy Space Center, Fla., USA.

Clinostat and RPM studies. Both a standard, slow-rotating (1 rpm), one-axis clinostat (described in Kiss et al. 1998) and a two-axis clinostat, also referred to as an RPM, were used in these studies which were performed at 1 *g*. In both cases, seedlings were grown in spaceflight hardware and procedures were performed as outlined above. In the case of the one-axis clinostat (henceforth termed CL), experiments were performed at Kennedy Space Center during the time of the spaceflight. In the case of the RPM, experiments were performed later at the Free University of Amsterdam. Samples in the clinostat studies were grown and prepared as described above.

The RPM was built by Fokker Space in Leiden, Netherlands, and its essential features are described in Mesland (1996). This instrument is similar to an earlier Japanese model, which was extensively reviewed in Hoson et al. (1997). The principle of the RPM is that the direction of the gravity vector experienced by an organism continuously changes in three-dimensional space. The sample was mounted at the center of two independent frames, and their rotation is driven by two separate motors. The movement is controlled by software which causes movement along a path of calculated position based on numbers from a random generator.

Microscopy and image analysis. Median longitudinal sections of root tips were cut using a Reichert Ultracut S microtome (at 1 μm in thickness) and were stained with 1% (w/v) toluidine blue in 0.1% (w/v) sodium carbonate. Sections were examined and photographed using brightfield optics with an Olympus BH-2 compound light microscope with Kodak Technical Pan film at an ASA of 50 (Number 2415; Eastman-Kodak, Rochester, N.Y., USA). In some cases, ultrathin sections were stained with uranyl acetate and lead citrate, and examined with a JEOL JEM-100S transmission electron microscope operating at 60 kV. Negatives were scanned, and TIF format images were used in subsequent analyses.

Central and flanking columella cells of the root cap, as illustrated and defined in Fig. 2, were examined in this study. Plastid position, cell area, and plastid area were analyzed using the program Image-Pro Plus (version 4.0; Media Cybernetics, Silver Spring, Md., USA) on a personal computer (PC) (Fig. 3). Prior to analysis of plastid position, images of root tips were rotated, if necessary, so that the long axis of the root was considered as the vertical. The plastid position was defined as the vertical distance (Fig. 3) from the centroid of the plastid to the (nearest) distal cell wall. Cell and plastid areas (Fig. 3) were calculated by the program after the cells and plastids were outlined (by hand). In *Arabidopsis* roots, differences in area correspond to differences in cell and organellar volume as shown by stereology in our previous study (Guisinger and Kiss 1999).

For all measurements, 5–8 different root tips were examined for each treatment. Statistical significance was determined by using the ANOVA/Tukey test ($P < 0.05$), or, where ANOVA criteria were not met (i.e. assumption of normality), a Mann-Whitney rank sum test ($P < 0.05$) was used. All statistical analyses were performed on a PC using Jandel Sigma Stat (version 2.0).

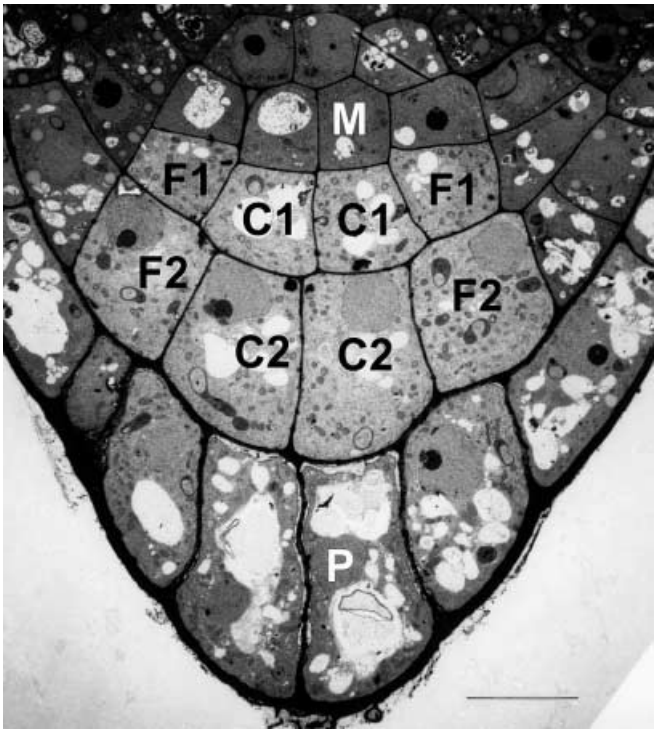

Fig. 2. Electron micrograph of an *Arabidopsis* root tip to illustrate the cell types considered in this study: meristem (*M*), flanking columella cells (*F*), central columella cells (*C*), and peripheral cells (*P*). Additionally, the two types of columella cells can be classified into story-one cells (*1*) and story-two cells (*2*). Bar = 10 μm

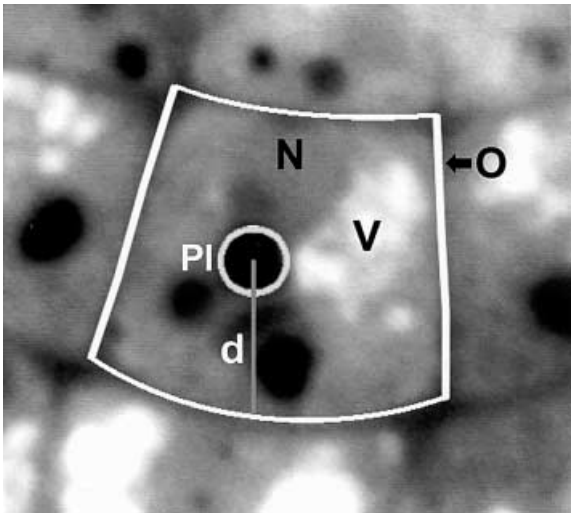

Fig. 3. Light micrograph of a central columella cell which summarizes the parameters measured by image analysis: outline (*O*) of the cell, nucleus (*N*), vacuoles (*V*), and plastids (*Pl*). In addition to the cell area, the distance (*d*) from the center of each plastid to the distal cell wall (wall furthest from the meristem) and the area of each plastid (*circle*) were both measured

Results

Morphology of seedlings grown on the RPM. Dark-grown *Arabidopsis* seedlings that developed on the RPM (Fig. 1A) were disoriented and thus similar to those grown in space (see Kiss et al. 1999b). In contrast, GR control seedlings exhibited oriented growth relative to gravity (Fig. 1B). However, the length of the seedlings did not appear to be different between the two treatments (Fig. 1).

Structural features of roots grown on different substrates. Since most of our research to date has been with *Arabidopsis* seedlings grown on agar, to have a basis for comparison, we studied the morphology of seedlings

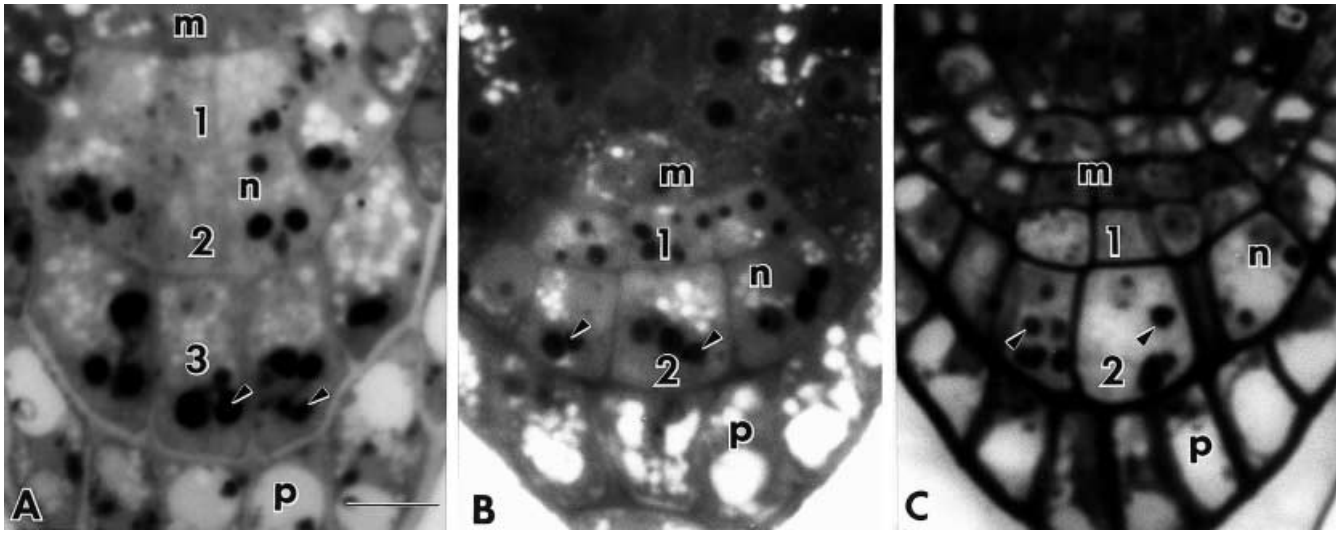

Fig. 4A–C. Light micrographs which compare root tips from, *Arabidopsis* plants grown on agar (**A**) to plants grown on nitrocellulose filter paper (**B, C**). The seedlings grown at constant 1 *g* on agar had developed three stories of columella cells (**A**), but plants grown at constant 1 *g* on nitrocellulose filter paper only had two stories of columella cells (**B**). These results were found for all plants grown on nitrocellulose filter paper, including those that developed on the RPM (**C**). The *numerals* indicate the story of columella cells, and the *arrowheads* indicate amyloplasts in the columella cells. In *A* and *B*, the gravity vector is toward the bottom of the micrographs. *m*, meristem; *n*, nucleus; *p*, peripheral cell. Bar = 10 μm

grown on a cellulose nitrate substrate, which was used in our present space and clinostat studies. The root cap of seedlings grown on agar consists of three rows or stories of columella cells (Fig. 4A). In contrast, when seedlings were grown on cellulose nitrate substrate, only two stories of columella cells developed in the root cap (Fig. 4B). The critical factor in the development of two stories (versus three stories) in the root cap was the use of cellulose nitrate as a substrate. When grown on cellulose nitrate, seedlings from the GR control (Fig. 4B), RPM samples (Fig. 4C), and FL samples (see Guisinger and Kiss 1999) developed two stories of columella cells.

The ultrastructure of columella cells from seedlings that developed on the RPM. Since previous studies have suggested that constant clinorotation has deleterious effects on the ultrastructure of columella cells and on the graviresponse of roots (Hensel and Sievers 1980), we investigated the structural features of these cells in root caps of seedlings grown under a variety of gravity conditions (Fig. 5). In the GR control (Fig. 5A), amyloplasts were localized to the distal part of the cell whereas in the RPM (Fig. 5B) and FL (Fig. 5C) samples, amyloplasts had a more scattered distribution throughout the cell. The ultrastructure of columella cells of seedlings that developed on the RPM (Fig. 5B) was well-preserved (and similar to the GR and FL specimens) as indicated by a dense cytoplasm and characteristic organelle profiles of mitochondria, amyloplasts, the endoplasmic reticulum, and the nucleus.

Comparison of seedlings grown on an RPM and CL to those that developed in true microgravity (FL). In order to better evaluate the treatments, we compared amyloplast distribution in the columella cells of seedlings that developed on the RPM and CL to plastid distribution in seedlings that were grown during the spaceflight (FL) experiment. Image analysis was used to measure the distance of the plastids from the distal cell wall (Fig. 3) in the different cell types in the two stories of the columella region (Fig. 2).

In the central columella cells of story 1, there were no statistical differences ($P > 0.05$) among the three treatments in terms of plastid position relative to the distal cell wall (Fig. 6A). However, in the flanking cells, there were significant differences among the treatments ($P < 0.05$) with the CL specimens having a more exaggerated plastid position compared to the FL or RPM samples (Fig. 6B). The columella cells of story 2 had a very similar plastid distribution to cells of story 1 in that there were no significant differences among the treatments ($P > 0.05$) in the central cells (Fig. 6C). However, in story-2 flanking cells, while there was no difference ($P > 0.05$) between the seedlings from FL and those grown on the RPM, plastid distances in the CL-grown samples were significantly greater ($P < 0.05$) than those from either the FL or RPM treatments (Fig. 6D). In the case of story-2 cells, the plastid distances in the GR control were 2.2 ± 0.3 μm and 2.9 ± 0.2 μm for the central and flanking cells, respectively, and these were significantly smaller compared to plastid distances in specimens from any of the three other gravity treatments.

In addition to investigating plastid position, we also studied the effects of the gravity treatments on the area of columella cells. The cell area was not significantly affected ($P > 0.05$) by any treatment in any of the four types of columella cells (Fig. 7). In addition, for all cell types, the columella cell area in the GR control was not significantly different from these cell areas in the FL,

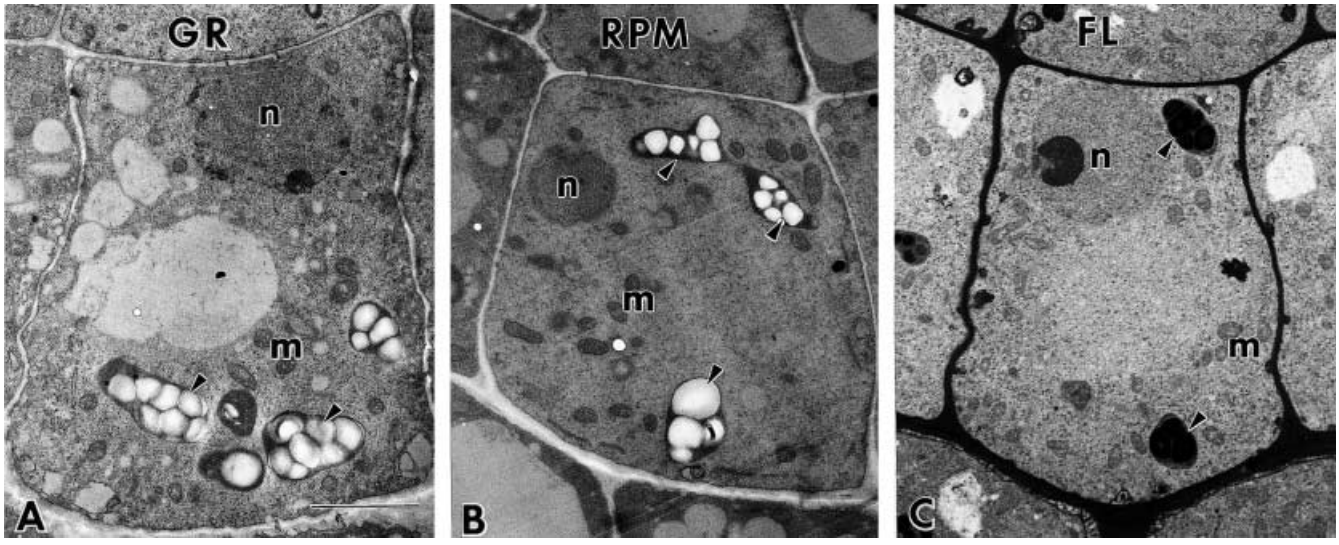

Fig. 5A–C. Electron micrographs of story-2 central columella cells of *Arabidopsis* seedlings grown on the ground (GR; **A**), on the RPM (**B**), and during spaceflight (FL; **C**). Amyloplasts (*arrowheads*) were sedimented toward the distal cell wall in the GR control and were more dispersed in the RPM and FL samples. General ultrastructural preservation of the cells was good under all conditions. *m*, mitochondria; *n*, nucleus. Bar = 5 μm

RPM, and CL samples. For instance, in story-2 cells, the values for cell area in the GR sample were 83.7 ± 18.9 and 88.4 ± 14.1 μm^2 for central and flanking cells, respectively. Finally, we examined the effects of gravity treatments on the area of columella cell plastids (data not shown), and similar to the cell area results, there were no significant differences ($P > 0.05$) among GR, FL, RPM, and CL treatments.

Discussion

Significance of this study. To our knowledge, this is the first report to concurrently compare the effects of the RPM, the CL, and spaceflight on structural parameters in *Arabidopsis* seedlings. Other such comparative studies have included reports on *Chara* rhizoids and *Lepidium* columella cells (reviewed in Hoson et al. 1997). In terms

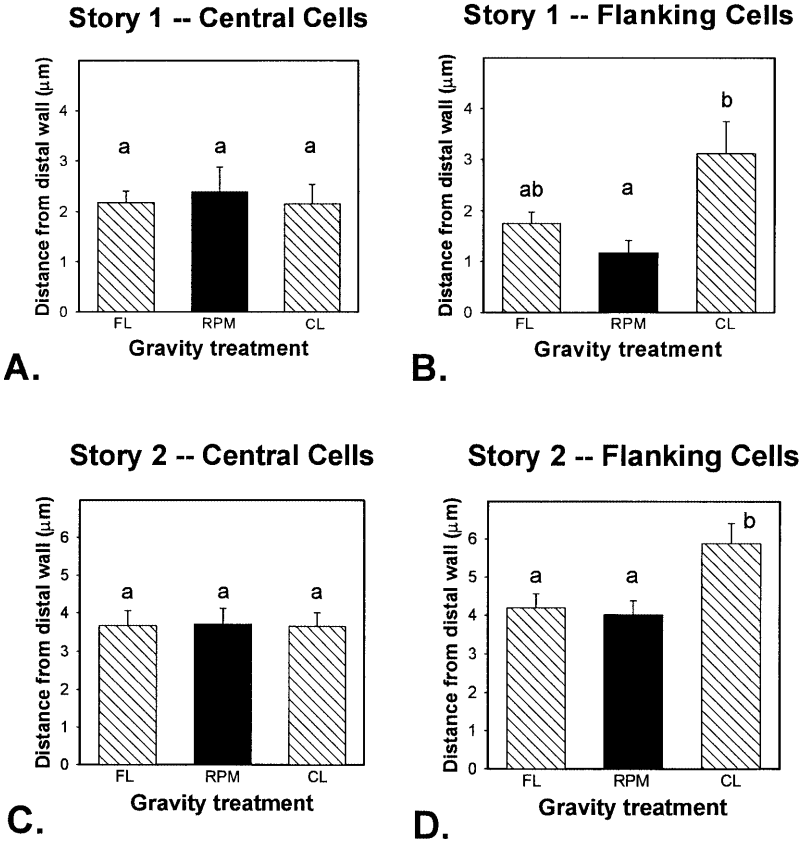

Fig. 6A–D. Plastid sedimentation as measured from the center of each plastid to the distal cell wall under different gravity treatments. Statistical differences ($P < 0.05$) are indicated by different letters above the histogram bars. For story-1 central columella cells (**A**), there was no statistical difference among space flight FL, RPM, and CL treatments. This was also true for story-2 central columella cells (**C**). However, there were statistical differences in plastid sedimentation in story-1 flanking columella cells (**B**). For story-2 flanking cells (**D**), there were no significant differences between the FL and RPM treatments, but the FL and CL treatments were significantly different. Bars represent the standard error of the mean. The number of plastids was $6 < n < 14$ in story 1, and $18 < n < 41$ in story 2

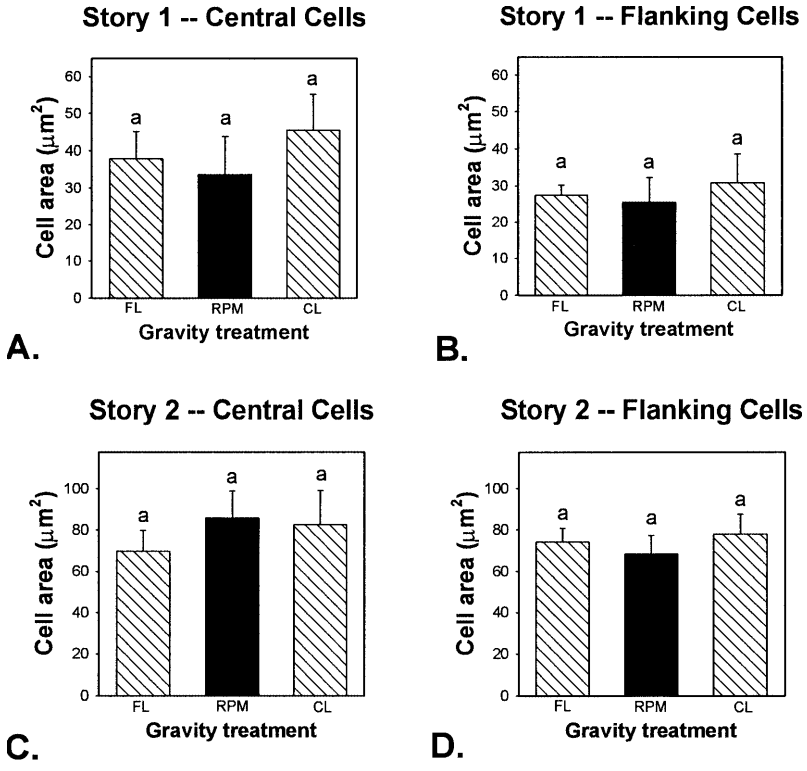

Fig. 7A–D. Histograms of columella cell areas of *Arabidopsis* plants that were exposed to different gravity treatments. For both central and flanking columella cells in stories 1 and 2, there were no statistical differences ($P > 0.05$) among the treatments. Bars represent the standard error of the mean. The number of cells was $4 < n < 6$ in story 1, and $8 < n < 16$ in story 2

of plastid position in columella cells in *Arabidopsis*, the FL results were more similar to the RPM results than they were to the results obtained with a classical, slow-rotating CL. Thus, along with the previous results summarized by Hoson et al. (1997, 1999), our study supports the hypothesis that the RPM provides a good simulation of microgravity for flowering plants.

Comparison to other RPM studies. Previous research evaluating the effects of the RPM on structural polarity in plant cells has been performed with *Lepidium* columella cells and *Chara* rhizoids (Buchen et al. 1993; Hoson et al. 1997). In contrast to results with a classical CL (Hensel and Sievers 1980), the columella cells of roots of *Lepidium* seedlings that were continuously grown on the RPM did not lose their cellular integrity. In addition, *Lepidium* columella cells from RPM-grown seedlings showed several structural features that were comparable to spaceflight results, including displacement of amyloplasts from the distal wall, increase in the total area of the endoplasmic reticulum, and an increase in the diameter of lipid bodies. In *Chara* rhizoids, which are a good unicellular model system in gravitropism studies, the dislocation of statoliths and the polarity of the cell have been tested in space and during growth on the RPM (Hoson et al. 1997). Under both RPM and space conditions, tip growth and the typical polar organization of the rhizoid were maintained, but the statoliths were displaced in the basal direction.

There have also been RPM-based studies with several species of flowering plants on the topic of automorphosis or automorphogenesis, a spontaneous growth response that occurs in a "stimulus-free" environment (Hoson et al. 1992, 1998, 1999). These "curvatures" have been observed in plants grown in space and on the RPM. Curvature from automorphogenesis can be exaggerated by the RPM, and thus this instrument is a good tool to use when trying to understand this developmental phenomenon (Hoson et al. 1992). However, in a recent experiment on the space shuttle, Hoson et al. (1999) demonstrated that the degree of automorphogenesis in *Arabidopsis* and *Oryza* seedlings was similar under true microgravity conditions and on the RPM.

Plastid and cell area are unaffected by the gravity treatments. In this study, of the parameters measured, only plastid position in the columella cells was affected by the different treatments (GR, FL, RPM, and CL). The area of these cells and the area of plastids in the columella region were not significantly different among the treatments. In a recently published study using sterological methods (Guisinger and Kiss 1999), we demonstrated that measuring cell and plastid area yielded results equivalent to measuring the relative volumes of columella cells in spaceflight-grown *Arabidopsis* seedlings. Therefore, we can infer that since the measured areas of the cells and plastids (in this *Arabidopsis* system) were not different among the gravity treatments, the relative volumes of the columella cells and plastids also were not different.

Flanking columella cells are more sensitive to alterations in the gravitational field compared to central columella cells. Gravity perception in roots is hypothesized to occur in the root cap region (reviewed in Sack 1997; Chen et al. 1999). Previous studies have shown that the central columella cells of the cap provide the greatest contribution to gravity sensing in *Arabidopsis* roots

(Blancaflor et al. 1998). Therefore, it was unexpected that the RPM and FL conditions had the greatest effect on plastid position in the flanking columella cells (compared to the central cells).

One possible explanation for the greater effect of altered gravity treatments on the localization of plastids in flanking cells is a difference in cytoskeletal organization in flanking cells versus the central columella cells (Baluska and Hasenstein 1997; Blancaflor et al. 1998). Flanking cells may be more susceptible to perturbations in the gravity field produced by FL, RPM, and CL conditions since they have more of a "restrained" organization of their cytoskeleton (Baluska and Hasenstein 1997). Thus, the cytoskeletal network in these cells may be more readily disrupted and lead to the greater dispersal of amyloplasts that we observed in the cells of the flanking columella. The experiments of Sievers and Heyder-Caspers (1983) support these ideas since they reported that the flanking columella cells, compared to the central cells, recovered quicker from centrifugal accelerations in roots of *Lepidium*. In any case, further research, including additional studies of plastid position under hypergravity conditions (Fitzelle and Kiss 1999), is needed for a better understanding of this phenomenon.

Future uses of the RPM in plant biology studies. In this paper, we have shown that plastid position is similar in columella cells of *Arabidopsis* seedlings grown on the RPM and in seedlings that developed in true microgravity achieved in low Earth orbit by the space shuttle. Therefore, this study, along with the extensive work by Hoson and colleagues (Hoson et al. 1997, 1999) supports the idea that the RPM provides an effective simulation of weightlessness. While these data are intriguing, the hypothesis that the RPM effectively simulates weightlessness needs to be confirmed on greater number of biological parameters in a wider range of plant species. Nevertheless, the RPM appears to be useful in the ground-based preparation for spaceflight experimentation and also as a tool in the study of basic questions in plant developmental biology.

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