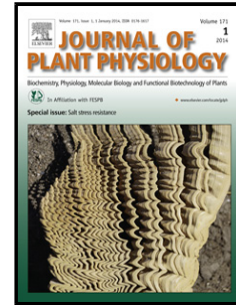# Accepted Manuscript

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Please cite this article as: Boucheron-Dubuisson Elodie, Manzano Ana I, Disquet Isabel Le, Matía Isabel, Sáez-Vasquez Julio, van Loon Jack JWA, Herranz Raúl, Carnero-Diaz Eugénie, Medina F.Javier.Functional alterations of root meristematic cells of Arabidopsis thaliana induced by a simulated microgravity environment.*Journal of Plant Physiology* http://dx.doi.org/10.1016/j.jplph.2016.09.011

Functional alterations of root meristematic cells of *Arabidopsis thaliana* induced by a simulated microgravity environment

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Summary

Environmental gravity modulates plant growth and development, and these processes are influenced by the balance between cell proliferation and differentiation in meristems. Meristematic cells are characterized by the coordination between cell proliferation and cell growth, that is, by the accurate regulation of cell cycle progression and the optimal production of biomass for the viability of daughter cells after division. Thus, cell growth is correlated with the rate of ribosome biogenesis and protein synthesis. We investigated the effects of simulated microgravity on cellular functions of the root meristem in a sequential study. Seedlings were grown in a clinostat, a device producing simulated microgravity, for periods between 3 and 10 days. In a complementary study, seedlings were grown in a Random Positioning Machine (RPM) and sampled sequentially after similar periods of growth. Under these conditions, the cell proliferation rate and the regulation of cell cycle progression showed significant alterations, accompanied by a reduction of cell growth. However, the overall size of the root meristem did not change. Analysis of cell cycle phases by flow cytometry showed changes in their proportion and duration, and the expression of the cyclin B1 gene, a marker of entry in mitosis, was decreased, indicating altered cell cycle regulation. With respect to cell growth, the rate of ribosome biogenesis was reduced under simulated microgravity, as shown by morphological and morphometric nucleolar changes and variations in the levels of the nucleolar protein nucleolin. Furthermore, in a nucleolin mutant characterized by disorganized nucleolar structure, the microgravity treatment intensified disorganization. These results show that, regardless of the simulated microgravity device used, a great disruption of meristematic competence was the first response to the environmental alteration detected at early developmental stages. However, longer periods of exposure to simulated microgravity do not produce an intensification of the cellular damages or a detectable developmental alteration in seedlings analyzed at further stages of their growth. This suggests that the secondary response to the gravity alteration is a process of adaptation, whose mechanism is still unknown, which eventually results in viable adult plants.

Abbreviations:

EDZ: Elongation/Differentiation Zone
EMCS: European Modular Cultivation System
g: Acceleration of gravity. In the Earth, $g = 9.8 \ \text{m} \cdot \text{s}^{-2}$
GC: Granular component of the nucleolus

GUS: β-glucuronidase

ISS: International Space Station

MS: Murashige and Skoog

NPG: Nucleolar perichromatin-like granules

PBS: Phosphate buffered saline

PM: Proximal Meristem

RPM: Random Positioning Machine

RT: Room temperature

STN: Stem Cell Niche

TZ: Transition Zone

Keywords:

Gravity; cell cycle; cell proliferation; nucleolus; ribosome biogenesis; flow cytometry.

Introduction

Plants have the capacity to respond rapidly to changes in the environment. Among the environmental factors that influence plant growth, development, survival and evolution, gravity is the only one with a constant presence on Earth throughout the entire history of life. Plants establish the direction of root and shoot growth according to the gravity vector (gravitropism) (Baldwin et al., 2013), and gravity influences the length of roots and shoots and the angle of emergence of secondary or lateral organs (gravimorphism) (Millar et al., 2011).

One of the strategies of response to environmental changes is based on the existence of meristematic tissues in adult plants. They are composed of undifferentiated, totipotent cells with a high capacity for cell proliferation and cell growth, capable of producing any specialized tissue at any time in the life of the plant. This is possible because, in these cells, cell proliferation and growth are strictly coordinated, being regulated simultaneously by the same factors. This coordination is called "meristematic competence" (Mizukami, 2001). In

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general, meristems are the source of cells for plant development. Indeed, this process greatly depends on the balance between cell proliferation and cell differentiation that exists in meristems, which is controlled, in turn, by the phytohormone auxin (Perrot-Rechenmann, 2010). Furthermore, it is widely known that environmental conditions modulate meristematic activities, directly or indirectly, at different levels of regulation (Komaki and Sugimoto, 2012).

These findings make it of interest to investigate the influence of environmental gravity on meristematic cell functions, which is the objective of this paper. The results obtained in space experiments showed alterations in the progression of the cell cycle, although there is not agreement regarding the specific changed parameters, probably due to differences in the experimental setup (Darbelley et al., 1989, Driss-École et al., 1994, Matía et al., 2010). Actually, little is known about the specific cell cycle regulatory processes that may be affected by a change in the environmental gravity.

Long-term exposure to real microgravity (gravity $< 10^{-6}g$) is only possible in outer space. However, since access to space experiments is expensive and subject to many constraints, devices capable of counteracting the perception of the Earth gravity vector by living beings, such as clinostats or the Random Positioning Machine (RPM) were designed and constructed (reviewed by Herranz et al., 2013). It is important to stress, however, that these devices do not suppress the gravity vector, but only act at the level of the mechanism by which living beings might perceive it. Other devices frequently used in gravitational research, such as free-fall towers, sounding rockets or parabolic flights, provide short-term and/or transitory periods of microgravity.

This paper reports a sequential study of seed germination and seedling growth using the clinostat, a reliable device for simulated microgravity. The clinostat study was complemented by a parallel experiment carried out in a different simulated microgravity facility, the Random Positioning Machine (RPM) (van Loon, 2007), with a similar general setup and experimental approach. Parameters related to cell proliferation and cell growth were evaluated. Cell proliferation in meristems unequivocally refers to cell cycle progression, which is regulated in specific checkpoints in order to allow cell division at a certain rate (De Veylder et al., 2007, Van Leene et al., 2010). With respect to meristematic cell growth, this expression has a univocal meaning, different from other processes of cell enlargement that may occur in other cell types. Meristematic cell growth means the production of cell biomass, essentially proteins, exceeding a threshold necessary to assure the viability of daughter cells after mitosis. There is a specific cell cycle checkpoint for this purpose (Mizukami, 2001).

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Therefore, specifically in these cells, cell growth is determined largely by the activity of the ribosome biogenesis and the protein synthesis (Baserga, 2007, Bernstein et al., 2007). Ribosome biogenesis occurs in a well-defined nuclear domain, the nucleolus, whose structural features are a reliable marker of the rate of ribosome production (Sáez-Vásquez and Medina, 2008). It should be stressed that an increase of the cell size may occur in other cell types at the expense of the formation of vacuoles. This process, which is not associated with cell proliferation but with cell differentiation, is not strictly cell growth but cell elongation (or enlargement) and, consequently, it is not related to ribosome biogenesis and nucleolar activity. The difference is not semantic, since it reflects two different functional processes with different purposes, driven by different factors (for a detailed explanation of these concepts see Baserga, 2007, Doerner, 2007, Li et al., 2005, Sablowski and Carnier Dornelas, 2014).

A difference between the two experiments of simulated microgravity in the two facilities was the illumination regime. In the clinostat, seedlings grew under a continuous photoperiod, whereas seedling growth in the RPM occurred under full darkness, giving rise to etiolated seedlings in which light was not necessary for growth. The reasons were, first, that the use of etiolated seedlings in the RPM allowed the validation of the results obtained with simulated microgravity against a similar experiment performed in space, under real microgravity, during the "Cervantes" Soyuz Mission to the International Space Station (ISS), in which seedlings had to grow in total darkness due to experimental constraints (Matía et al., 2010). Furthermore, these conditions allowed better discrimination of the effects of altered gravity on plant growth and development, without the influence of light, which not only was a source of energy, but also affected the growth direction of seedlings due to phototropism (Hohm et al., 2013, Wyatt and Kiss, 2013). The comparison of the results obtained with and without photoperiod has allowed us to discriminate how light is capable of modulating the gravitational stress. This can be useful for future experiments in the ISS in which the implementation of new advanced hardware, such as the European Modular Cultivation System (EMCS), has strongly reduced the experimental constraints and is making possible the use in space of methods considered standard on ground (reviewed by Kittang et al., 2014).

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Material and methods

1. Material and growth conditions

Seeds of *Arabidopsis thaliana* wild type, of the transgenic line *CYCB1;1::uidA* (Colon-Carmona et al., 1999)*,* and of the mutant *AtnucL1* (Pontvianne et al., 2007), all of them in a Col0 background, were sterilized with 70% ethanol for 2 min and 5% calcium hydrochloride for 5 min, then washed in sterile water before being sown on Petri dishes that contained half strength MS medium (Murashige and Skoog, 1962) (Duchefa Biochemie B.V., Haarlem, The Netherlands) supplemented with 10 g L^{-1} sucrose and 0.8% agar (Duchefa). The pH was adjusted to 5.7 with 1 M KOH. The prepared dishes were cold-treated for 72 h at 4°C before transfer to clinostat or RPM.

2. Two-dimensional (2D) clinostat experiment

The 2D clinostat is a microgravity simulator that is based on the principle of "gravity-vector-averaging." During an experiment run, the sample experiences a zero-gravity-simulated stimulus for two dimensions. Two 2D clinostats, one of them with horizontal axis for microgravity simulation, and the second one with vertical axis for control conditions, both of them rotating at 1 rpm, were placed in a growth chamber under a 16 h light / 8 h dark cycle at 25°± 1°C with 110 μmol m^{-2} sec^{-1} photon flux intensity delivered by Biolux tubes L58W/840 (Osram, Molsheim, France). In the 2D clinostat with axis perpendicular to gravity, the maximum centrifugal force reaches 1.1×10^{-4} *g* at the outer perimeter of the observed area (10 cm).

Petri dishes containing seeds were mounted on the clinostat. Seed germination and seedling growth occurred under clinorotation. Samples were obtained after 3 days, 7 days and 10 days of incubation.

3. Random positioning machine (RPM) experiment

This experiment was performed in the RPM located in the Dutch Experiment Support Center (DESC), Vrije Universiteit, Amsterdam, The Netherlands. This machine does not remove gravity, but it eliminates the effect of gravity on biological samples located in a certain position of its architecture by means of rotation around two independent axes to change their orientation in space in complex ways (van Loon, 2007). The rotational velocity of the RPM frames was randomized with a maximum of $\pm 60°$ s^{-1}; the interval and direction was set at random. The experimental samples were positioned in the center of the inner frame, and the largest radius was, at maximum, 5 cm to the outermost sample container. This

resulted in a maximum residual gravity of less than $10^{-4}g$ (van Loon, 2007). At the same time, a complementary set of samples was attached to the RPM scaffold to perceive the apparatus vibrations but remains at a 1g control into the same temperature controlled chamber.

Seed germination and seedling growth occurred in the RPM. Samples were obtained after 2 days, 4 days and 8 days of incubation.

4. Morphometry from clinorotated samples

Quickly after removal from the clinostat, seedlings were photographed and then fixed in 4% paraformaldehyde in Phosphate Buffered Saline (PBS) and mounted on slides for microscopic observation.

Root length was measured from digital images of the plates using NeuronJ, which is a plugin of National Institutes of Health ImageJ 1.34S software (http://rsb.info.nih.gov/ij). Measurements were done on 10 roots for each stage, taken from two different biological replicates.

Cell measurements were performed on optical slices of the roots made with a "Zeiss ApoTome" microscope (Carl Zeiss AG, Oberkochen, Germany), using a DIC filter.

5. Microscopy, immunocytochemistry and quantification from RPM samples

Samples grown on the RPM were quickly fixed in 4% paraformaldehyde and 0.5% glutaraldehyde, washed in PBS and photographed. Then, root tips were excised and dehydrated in a methanol series, until a concentration of 70% methanol in water. At this stage, samples were stored at 4ºC and transported to Madrid. Sample processing continued by dehydration until 100% methanol followed by the methylation-acetylation procedure, consisting in an overnight treatment at 25ºC with acetic anhydride and methanol (1:5, v/v). This technique allows a better resolution of the nucleolar ultrastructure and nucleolar subcomponents (Testillano et al., 1995). Finally, all root tips were embedded in LR White acrylic resin (London Resin Company, London, UK).

From resin blocks, semithin sections (2 μm thick) were cut and observed unstained under a Leica DM2500 microscope (Leica Microsystems, Wetzlar, Germany) equipped with phase contrast. Images were digitally recorded with a Leica DFC320 CCD camera (Leica Microsystems, Wetzlar, Germany). For ultrastructural studies, ultrathin sections (around 60 nm thin) were mounted on Formvar-coated nickel grids, stained with 5% uranyl acetate and 0.3% lead citrate and observed in a Jeol 1230 electron microscope, operating at 100 kV. For immunogold labeling, ultrathin sections were incubated with an anti-nucleolin antibody (Sáez-Vásquez et al., 2004) diluted 1:100, for 90 min at room temperature (RT), washed

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repeatedly and incubated with goat anti-rabbit IgG secondary antibody coupled with 10 nm colloidal gold particles (Sigma-Aldrich, St Louis MO, USA), diluted 1:50, for 1 h, at RT. Prior to observation, grids were counterstained with uranyl acetate, either alone or followed by lead citrate.

Quantitative measurements were carried out on digital images using the quantitation software "QWin Standard" (Leica Microsystems). Parameters measured were root length, number of cells per mm in root meristem cell files, cross-sectional area of meristematic cell nucleoli, and density of gold particles, which is the number of particles per μm^2 in the nucleolar dense fibrillar component. Measurements of cell parameters were performed in a zone of 50 ± 30 μm above the quiescent center (approx. 100 ± 30 μm from the root tip), corresponding to the highest rate of cell division, according to the available literature (Beemster and Baskin, 1998). In this zone, epidermal, cortical and endodermal cells were selected.

The number of samples measured was: 20 seedlings for root length, 10 roots for number of cells per mm, 60 nucleoli (light microscopy) for cross-sectional area and 15 nucleoli (electron microscopy) for density of gold particles, all of them taken from two different biological replicates. Statistical analysis of data was performed using SPSS *v* 13.0 software (IBM Corporation, Armonk, New York, USA). The description of quantitative variables was performed using means and standard deviations after checking normality with the Kolmogorov–Smirnov test. Mean values were compared using the Student's *t*-test for independent samples; differences were considered significant for $p \leq 0.05$.

6. Meristem size analysis

Since the transition zone between the meristem and the elongation zone in the root meristem were difficult to be delimited with precision, we have measured the size of the meristem according to two techniques.

In the first method, the meristem size has been measured using the *CYCB1::uidA* reporter gene *uidA* (Colon-Carmona et al., 1999). Cyclin B1 is a protein encoded by the gene *CYCB1; 1,* and is involved in regulating the cell cycle at the G2 / M transition. It is then destroyed at the end of mitosis. To determine the size of the meristem, we delineated the zone between the stem cells around the quiescent center and the farthest cell from this center which was in the process of dividing. Root meristem size was measured with IMAGE J software (http://rsb.info.nih.gov/ij). Mean values were compared using the Student's *t*-test for independent samples; differences were considered significant for $p \leq 0.05$.

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In the second method, meristem size was expressed as the number of meristematic cortex cells proximal to the quiescent center that did not yet rapidly elongate, following the procedure described by Casamitjana-Martinez et al. (2003). Root apices were observed on optical slices obtained with the ZEISS ApoTome microscope and the measurements were performed with AxioVision Rel.4.8 software from Zeiss (http://zeiss.fr/microscopy/software/axiovision-pour-la-biologie.html). Mean values were compared using the Student's t-test for independent samples; differences were considered significant for $p \leq 0.05$.

7. Flow cytometry

For flow cytometry, 20 root apices were collected and were chopped with a razor blade in 0.1 M citric acid with 0.1 M Tween 20. Nuclei were filtered through a 33-µm-pore mesh and, before analysis, acidity was neutralized by adding 1.25 mL of 0.4M Na_2HPO_4. Nuclear DNA was labeled with propidium iodide (50 µg ml^{-1}) after an RNase (50 µg ml^{-1}) treatment. The analysis was performed in a MoFlo Astrios cytometer (Beckman-Coulter Life Sciences, Indianapolis, USA).

8. Histochemical assay (GUS)

Histochemical GUS assays were performed on samples which were incubated for 18 h in 40 mM 5-bromo-4-chloro-3-indolyl-b-D-glucuronide (X-Gluc; Duchefa, Haarlem, Netherlands), and 5 mM K$^+$- ferricyanide (in order to limit the diffusion of the intermediary products of the reaction) in 100 mM phosphate buffer (pH 7). Reactions were stopped by several washings of samples in 100 mM phosphate buffer (pH 7) before being mounted in phosphate buffer with glycerol; then the sections were photographed using either a Zeiss Axioscop (Carl Zeiss AG, Oberkochen, Germany) or a Leica DM2500 photomicroscope (Leica Microsystems, Wetzlar, Germany).

Results

1. Clinostat Experiment

Seeds were germinated in the clinostat and seedlings grew under the conditions of simulated microgravity. The sequential study consisted of taking samples at three stages of early seedling development, namely at 3, 7 and 10 days after seed hydration.

Root length of seedlings cultivated in simulated microgravity under photoperiod (2D clinostat) for 3 days was similar to the vertical control. However, growth of primary root was

greater for seedlings grown in the clinostat than in 1*g* control at 7 days and 10 days of culture (Fig. 1). We also observed an increase of the quantity of secondary roots in simulated microgravity (data not shown). The whole seedling showed the same type of changes induced by simulated microgravity as the primary root in the three points of sampling (data not shown).

1.1. Size of the root meristem

The *Arabidopsis* root meristem (proximal meristem, PM) can be divided into three developmental zones (Fig. 2): the stem-cell niche (STN) around the quiescent center, the proximal meristem (PM) and the elongation/differentiation zone (EDZ). The boundary between the PM and the EDZ is marked by a transition zone (TZ) where cells stop dividing and rapidly elongate. The TZ is different for each cell type, giving a jagged shape to the boundary between dividing and expanding cells. For that reason, the size of the meristem was determined following two different methods.

The first method determined the size of the meristem by visualizing dividing cells present in the PM. We used a specific cell cycle regulator protein considered a marker of the G2/M transition, namely cyclin B1. The expression of the *CYCB1;1* gene (Ferreira et al., 1994), was visualized *in situ* in transgenic plants in which the promoter of this gene was fused with the coding sequence of the *uidA* gene, encoding β-glucuronidase (Colon-Carmona et al., 1999) (Fig. 2A and B). The distance between the nearest and the farthest stained spots with respect to the root tip was measured in order to evaluate the size of the meristematic zone of the primary root. It was observed that the size of meristematic zone was quite similar in plants grown in simulated microgravity and in the 1*g* control (no significant difference with Student's *t*-test). Nevertheless, we could note that the size of the meristematic zone was gradually decreasing at 7 and 10 days compared to 3 days, irrespective of the gravity conditions of growth. In particular, a significant difference was observed between 3 and 10 days (with a p value of 0.04 with Student's *t*-test for the control and a p value of 0.015 for the 2D clinostat) (Fig. 2C).

The second method used to evaluate the meristem size was to determine the number of cortex cells in the cortex file extending from the STN to the first elongated cell observed in the TZ (Casamitjana-Martínez et al., 2003, Dello Ioio et al., 2007). Measurements where performed on optical slices obtained with the ZEISS ApoTome microscope (Fig. 2D and E). No changes in root meristem size were observed in clinorotated plants compared to 1*g* control (Fig. 2F). The data confirmed those obtained from *CYCB1::uidA* transgenic plants. Note that

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the size of meristem remains constant with time, regardless of the experimental condition (Fig. 2F), while it tends to decrease when the measurements were performed on samples visualizing the expression of the transgene *CYCB1::uidA* (Fig. 2C).

1.2. Alteration of cell cycle phases: flow cytometry

The relative frequency of cell cycle phases in conditions of simulated microgravity, compared to the 1*g* control, was assessed by flow cytometry on samples grown in the clinostat for 3 and 10 days. Data from the nuclear DNA content were obtained and ascribed to 2C, 4C, 8C and 16C level respectively, and the relative proportion of each one of these classes was estimated in both clinostat and 1*g* control samples and in both 3- and 10-day sampling points. The 2C and 4C DNA content classes correspond to the G1 and G2 periods of the cell cycle, respectively, whereas the 8C and 16C classes correspond to cells which have escaped from the cell cycle, entering in a process of endoreduplication, associated to cell differentiation. (Fig. 3). Actually, it is possible that a few cells having 4C DNA content would correspond to cells at the G1 period after having experienced endoreduplication, since the exit from the cell cycle towards the differentiation pathway may have occurred at different phases. Unfortunately, we have no means to discriminate the precise amount of these cells, but their proportion with respect to meristematic cycling cells should not be significant to change the interpretation of the quantitative data.

A common result for both clinostat and control samples was that, as expected, differentiation markedly increased at 10 days compared to the level shown at 3 days, irrespective of the gravity conditions of growth (Fig. 3). Looking to the effects of gravity alteration at both time points of seedling growth, we observed that there was a little effect of simulated microgravity on cell cycle phases at 3 days (Fig. 3A). However, at 10 days, apart from the fact that a significant proportion of cells were differentiated (near 50%), this cell differentiation was increased by clinorotation (Fig. 3B). From cells remaining in cycle at this time point, G1 cells were significantly reduced in the clinostat and G2 cells were slightly increased with respect to 1*g* control (Fig. 3B). This would represent a shorter cell cycle (and shorter cells), if the duration of individual phases would not be greatly affected.

Furthermore, the overall increase of differentiation at 10 days was accompanied by a balanced decrease of cycling cells, affecting equally to both G1 and G2 periods under control conditions. However, in the clinostat, the increase of differentiation at 10 days (more intense than in control) was accompanied by an unbalanced decrease of cycling cells, affecting differently to either G1 or G2 cells (Fig. 3, compare values for each period and each condition

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in A and B). These observations could correspond to the effect of a change in the regulation of cell cycle progression, induced by gravity alteration.

2. Random Positioning Machine (RPM) Experiment

A parallel complementary experiment was performed using a different facility for simulated microgravity, namely the Random Positioning Machine (RPM) (van Loon, 2007). The samples used and the conditions for seed germination and seedling growth were similar to those used in the clinostat experiment except for the illumination regime. Instead of applying a photoperiod, samples were grown in the RPM under darkness, the same as in the previous "Root" space experiment and associated ground controls (Matía et al., 2010). Like the clinostat experiment, the RPM study was sequential, using three successive stages of the early seedling development for taking samples, but here the first sampling was performed even earlier than in the clinostat, at only two days from the seed hydration, in order to detect the first response of the root meristematic cells to the simulated microgravity environment, just at the time of the root sprouting.

As observed in the clinostat, the early response to simulated microgravity at the first sampling point was not detected by significant differences in the root length, but longer roots for RPM-treated seedlings were observed in later growth stages (4 and 8 days) (Supplemental Fig. 1).

2.1. Cell proliferation in the root meristem

The rate of cell proliferation was evaluated on samples grown in the RPM by determining the "rate of local cell production," i.e. the variation of the number of cells per unit of length (100 µm) in the cell files of the root meristem, namely epidermis, cortex and endodermis (Beemster and Baskin, 1998) throughout the time of seedling growth. Counts were performed on 2-µm semithin sections from resin-embedded roots, observed under phase contrast.

A very early increase in the rate of local cell production in samples grown in the RPM with respect to the 1g control was recorded at day 2, with high differences (Fig. 4A), which can be easily detected from the mere observation of micrographs (Fig. 4B). Interestingly, this significant difference at the cellular level was not extended to the level of the whole root, as described above. However, as seedling growth proceeded, in later sampling times (4 and 8 days), there were no significant differences in this parameter between RPM and control samples (Fig. 4A).

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2.2. Expression of cyclin B1

Cell cycle alterations in roots grown in simulated microgravity conditions were identified using a reliable marker of the control of cell cycle progression, namely the cyclin B1 gene detected by microscopical methods. The identity of this gene and the use of transgenic *Arabidopsis* plants harboring the *CYCB1;1:uidA* construct have already been described in a precedent paragraph, dealing with the clinostat experiment.

In the RPM, using the same transgenic plants and the same staining method, the expression of cyclin B1 was observed at the microscope, showing a reduction in the level of *CYCB1;1* gene expression throughout the seedling growth period, irrespective of the gravity level (Fig. 5). The comparison of samples grown in simulated microgravity with their corresponding 1g control samples clearly showed a lower level of expression of the gene at the three sampling points in seedlings grown in the RPM (Fig. 5). The difference in staining intensity was greater in very young seedlings (2 days) and it was attenuated after longer periods of growth. Actually, the stained spots observed with this method, corresponding to the cells expressing *CYCB1;1:uidA,* represent cells in the pre-mitotic (G2) or mitotic stage of the cell cycle. Only these cells were marked, as the construct presents mitotic degradation signal (Colon-Carmona et al., 1999).

In the clinostat, no significant difference was observed at 3 and 7 days of culture between simulated microgravity and 1g control, although the trend was for a reduction of the number of stained cells in simulated microgravity after 7 days of growth. However, at 10 days, we could observe a significant decrease of cells entering into mitosis in plants grown in the clinostat, compared to 1g control (Supplemental Fig. 2).

2.3. Cell growth: the nucleolus and ribosome biogenesis

The activity of cell proliferation depends on a continuous supply of proteins as the main requirement to reach the critical size allowing cell division. Ribosomes are the cellular factories of proteins, and the rate of ribosome biogenesis is directly correlated, in proliferating meristematic cells, with the cell growth necessary for cell division (Baserga, 2007, Bernstein et al., 2007). In fact, the so-called "meristematic competence" involves a strict coupling of cell proliferation with cell growth (Mizukami, 2001). The nucleolus is a nuclear structure in which ribosome biogenesis takes place up to the production of ribosome subunits which are exported to the cytoplasm where they are assembled into functional ribosomes. The nucleolus is formed as a result of the expression of the multiple copies of the pre-rRNA gene, producing the precursor RNA of mature rRNAs. Structurally, the nucleolus is formed by a few basic

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components, common to all eukaryotic cell types, whose organization and relative proportion, also including the size of the organelle, depends on the cellular type, on the cell activity and on the cell cycle period in which the cell is found (Medina et al., 2000, Sáez-Vásquez and Medina, 2008).

We observed changes in the nucleolar size and ultrastructure in meristematic cells of roots grown in $1g$ and simulated microgravity in the RPM. Some features were similar in both conditions and reflected a high nucleolar activity, such as well-developed nucleoli, with abundant granular component and multiple small fibrillar centers. However, samples grown in simulated microgravity showed smaller nucleoli than control nucleoli in all three points of seedling growth (Fig. 6A and B). They contained less granular component (data not shown).

Nucleolin is the major nucleolar protein of actively proliferating eukaryotic cells, as it has been shown in different biological model systems, and it is a multifunctional protein, acting at different levels of pre-rRNA transcription and processing, actively regulating the efficiency of these processes (Sáez-Vásquez and Medina, 2008). The levels of nucleolin have been demonstrated to be correlated with the proliferative status of the cell. Therefore, nucleolin can be used as a direct marker for cell growth and as an indirect marker of cell proliferation. Consequently, we examined nucleolin immunolocalization in the RPM experiment and compared the levels of the protein, detectable by immunogold at the transmission electron microscopical level. The density of gold particles (number of particles per μm^2 in the nucleolar dense fibrillar component) was significantly reduced in simulated microgravity conditions at the three growth time points analyzed (Fig. 6C).

In order to know in more detail the effects of altered gravity on nucleolin and ribosome biogenesis, we incubated the nucleolin mutant *AtnucL1* in the RPM for 4 days. *AtnucL1* is defective in the *AtNuc-L1* gene, the major of the two genes coding for nucleolin in *Arabidopsis*. In this mutant, the expression of the *AtNuc-L1* gene is replaced by the expression of *AtNuc-L2*, a gene which is not usually expressed in normal plants grown under normal standard conditions (Pontvianne et al., 2010, Pontvianne et al., 2007). Under normal $1g$ conditions, mutant plants are viable, but they contain root meristematic cells with a highly disorganized nucleolus. Unlike the nucleolus of these cells in wild-type plants, the mutant plant nucleolus is mostly granular in structure, the dense fibrillar component is reduced to small territories, and the so-called "nucleolar perichromatin-like granules" (NPG), different from the granules of the standard nucleolar granular component, can be identified in it (Fig. 7A, C and E) (Pontvianne et al., 2007). The nucleolar disorganization reveals serious alterations in the process of ribosome biogenesis, which can be related to the alteration of pre-

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rRNA transcription previously shown in the mutant (Pontvianne et al., 2010), and to the original identification of NPG as particles containing incompletely or wrongly processed pre-ribosomal precursors (Puvion-Dutilleul et al., 1983). In *Atnuc-L1* samples grown in the RPM, the nucleolar disorganization is even greater than in the parallel mutant samples grown under normal gravity conditions (compare Fig. 7B with 7A). The nucleolus appears as a uniform mass of loosely organized granules without any traces of fibrillar centers or dense fibrillar component (Fig. 7B). The number of NPG increases (compare Fig. 7D with 7C), and this is a sign of important alterations of the ribosome-producing machinery.

Discussion

The present paper reports the effect of simulated microgravity on the root apical meristem of very young seedlings, which were sequentially analyzed during a period extending up to 10 days from seed hydration and germination. The focus was placed on the cellular functions that characterize the meristematic tissue, namely cell proliferation and cell growth.

1. Simulated microgravity affects meristematic competence

Two parallel experiments were performed in two different devices for microgravity simulation, incorporating some methodological differences. Collectively, they provide an informative picture of the primary cellular response of plant meristematic cells to gravity alteration during the earliest period of plant development. The most relevant effect of altered gravity that we found was the disruption of the "meristematic competence" (Mizukami, 2001) in root meristematic cells. This disruption or disorganization means that, under the altered environmental conditions, cell proliferation and cell growth appeared to lose their coordinated progress which is characteristic of these cells under normal ground gravity conditions, resulting in the uncoupling of these cellular functions.

This alteration of proliferating cells was first found in samples grown in space, in the course of an experiment performed in the ISS (Matía et al., 2010). More recently, similar effects have been described in seedlings grown under magnetic levitation (Manzano et al., 2013) and in *in vitro* cultured callus cells incubated in different devices of microgravity simulation (Manzano et al., 2016, Manzano et al., 2012). However, this is the first time in which a sequential study, including three sampling periods, is performed and in which seedlings are analyzed as early as two days after seed hydration. This means that we are able of reporting just the earliest response of meristematic cells to the altered gravity condition, at

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the time of the root sprouting, when cells are undergoing the first cell cycle; furthermore, our study gives account of the degree of persistence of these alterations through the process of seedling development. This developmental perspective had never been considered in previous studies, as well as the comparison between different devices of simulated microgravity.

We indeed observed in our sequential study that a significant cell cycle alteration could be detected as early as two days after germination. In relation to this, an effect of microgravity, either real or simulated, on the proliferation rate of root meristematic cells was previously described. Mitotic index was the parameter reported to change in early pioneering studies on Plant Space Biology using lentil seedlings grown in microgravity, but the results of different experiments were not coincident (Darbelley et al., 1986, Driss-École et al., 1994). An interpretation was that real microgravity promoted the arrest in the G2 phase of the cell cycle (Driss-École et al., 1994).

In connection with the alteration of cell cycle phases, the process of ribosome biogenesis was reduced in simulated microgravity-grown samples according to the measured parameters. This reduction of ribosome biogenesis was already observed in meristematic root cells grown in real or simulated microgravity (Matía et al., 2010, Shen-Miller and Hinchman, 1995, Sobol et al., 2005). In general, in dividing cells a decrease in the rate of ribosome biogenesis is closely correlated to a decrease in the synthesis of proteins, whose factories are cytoplasmic ribosomes, and, consequently, to cell growth depletion (Baserga, 2007). Furthermore, it is known that in these cells the peak of nucleolar activity/ribosome biogenesis occurs in G2, immediately preceding mitosis (Sáez-Vásquez and Medina, 2008). Therefore, a shortening of this phase induced by microgravity may lead to a reduction in the rate of ribosome production. Different elements of the machinery of pre-rRNA synthesis and processing in the nucleolus are affected by this environmental change, as shown by the results obtained on the mutant defective in the major gene of nucleolin *(AtNucL1)*. Under microgravity conditions, meristematic cells of this mutant showed a serious damage of the nucleolar structural organization and assembly, as well as an increase in the amount of nucleolar perichromatin-like granules, compared to the same samples grown under normal ground gravity. However, we did not observe any conspicuous alteration of the nucleolar morphological organization in wild-type samples grown in microgravity, as it had been previously reported (Shen-Miller and Hinchman, 1995). This indicates that the gravitational stress *per se* has no direct effects on nucleolar assembly, but it has a synergistic action, contributing to the enhancement of disruptive effects originated by other causes, such as the lack of expression of the major nucleolin gene.

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These changes in specific functions of meristematic cells should be integrated in a general strategy of adaptation to the environmental change (see below, heading 4); the adaptation of cells involves gene expression reprogramming, compared to living on the ground, which results in changes to their metabolism (Correll et al., 2013, Fengler et al., 2015, Manzano et al., 2012, Paul et al., 2012). The modification of cell cycle regulation and of ribosome biogenesis occurring in root meristematic cells is a fundamental part of this general strategy.

The factor triggering the cascade of functional events that eventually result in the alteration of meristematic cell proliferation and growth remains to be elucidated. According to previously published data, a change in the hormonal signaling pathway mediated by the auxin polar transport could be a consistent candidate to play this triggering role (Medina and Herranz, 2010). This hypothesis is supported by experimental data showing changes in auxin distribution in the root as the primary effect of the gravitropic response (Friml et al., 2002, Kleine-Vehn et al., 2010) and the known role of this hormone in the regulation of cell cycle progression and the coordination between cell growth and cell division (Jurado et al., 2010, Perrot-Rechenmann, 2010).

2. Reliability of the results: real *vs* simulated microgravity; clinostat *vs* RPM; photoperiod *vs* darkness.

Since our study was performed in ground-based facilities for microgravity simulation, and not in real microgravity (which can only be obtained in space, given the duration of the treatments applied to our biological samples) a fundamental question is whether or not the data obtained from these facilities reliably reproduce the effects obtained in space. In general, the use of these simulation devices has received support from a recent work in which different facilities are analyzed in a wide range of biological models (Herranz et al., 2013), but, in addition, the results of our work are particularly consistent with those obtained in a previous spaceflight experiment (Matía et al., 2010). In order to facilitate the comparison, the RPM experiment was performed in darkness, the same as it was done in space, suppressing the usual illumination regime (photoperiod) which was applied to growing seedlings in the clinostat experiment. This was an additional support to the validation of the simulated microgravity facilities for these kinds of studies.

The results of the spaceflight experiment were affected by constraints imposed by the duration of the experiment and the conditions of seedling growth. Furthermore, the analysis of the space-grown seedlings was necessarily restricted to a single point of their development,

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namely four days. Now, the results reported in this paper confirm that meristematic competence is disturbed, not only as a general effect of the spaceflight environment, but, specifically, by the lack of perception of a definite gravity vector by the plant, which characterizes the so-called "simulated microgravity" (Herranz et al., 2013). This is true for the two different devices that have been used, and throughout the period of early seedling development that have been analyzed, namely from 2 days to 10 days. Actually, some differences were appreciated between the two simulated microgravity experiments affecting parameters related to cell proliferation in some sampling points (e.g. the level of expression of cyclin B1 detected by the reporter gene GUS). These differences, which are compatible with a similar general trend of the functional alteration observed for the two devices, may be associated with the illumination conditions during seedling growth, which vary in each experiment. Light modulates plant development by playing a role in the regulation of cell proliferation (Vandenbrink et al., 2014) and the exposure to microgravity specifically alters the sensitivity of plants to the light (Kiss et al., 2012, Vandenbrink et al., 2014). Since gravity and light are closely related in the control of plant growth it is likely that light, in turn, acts on the sensitivity of plants to gravity. The differences in response that we observed between the experiments carried on in light and those made in the dark would support this effect.

3. Root development: gravimorphic effects.

Considering the overall aspects of the root development, in the early phases investigated in this study, we observed that primary roots of plants grown on 2D clinostat or RPM were longer than those of plants grown in 1g control. As previously indicated, the same gravimorphic response to real microgravity was also obtained by other authors in different species (Levine and Krikorian, 1996, Matía et al., 2010), although this effect was not commonly reported in all experiments. Actually, since the meristem only accounts for a minor proportion of the root length, this effect can hardly be attributable to the alterations in cell growth and proliferation which have been found in root meristematic cells, even though it is compatible with a new organization of root apical meristem in an altered gravity environment (Herranz et al., 2014). The change observed in the entire root can be attributed to the phenomenon called "automorphogenesis", responsible for the new sizes and shapes appearing in plants grown in space or in simulated microgravity. In these conditions, it has been reported that elongation is stimulated and lateral expansion suppressed in plant organs (Driss-Ecole et al., 2008, Hoson, 2014). Other morphological alterations of the root, such as a skewed growth and an abnormal number of adventitious roots were also reported to occur in

space-grown seedlings (Millar et al., 2011). Interestingly, a "seed-to-seed" experiment in space has shown that, even though the entire life cycle of plants can be achieved in real microgravity without serious apparent damages to the health and viability of daughter plants, noticeable changes in the shape of plant organs were identified (Link et al., 2014).

With respect to the root apical meristem, its size was not affected by the simulated microgravity treatment whatever the measurement mode, even though it showed a shortening along the time irrespective of the gravity level. It should be noted that the meristem size measurements made with the genetic construct *CYCB1::uidA* showed a decrease in the size of meristem between 3 and 10 days, whereas the cell counting in the cortex file showed that meristem size remains constant during the period of observation. These two results clearly show the difficulty of accurately delimit the meristem and thus to measure its size exactly According to the point of reference used, results may vary. In this work, we consider only the results confirmed by the two techniques; i.e. that simulated microgravity on clinostat does not change the size of the root meristem.

4. Gravitational stress and adaptation

The response to gravity alteration at the level of the root meristem is intense enough to consider the microgravity environment as a source of abiotic stress for the plant. The existence of a true "gravitational stress" in plants as a consequence of gravity changes has been previously suggested in view of the effects found in other plant components and functions (Volkmann and Baluška, 2006). Interestingly, the plant response to gravity alteration has some common and some specific features regarding the functions of meristematic cells, compared to other environmental changes, according to the results of this and previous papers (Manzano et al., 2016, Manzano et al., 2013, Manzano et al., 2012, Matía et al., 2010). It is widely known that abiotic stresses, such as thermal shock (heat or cold), drought or saline stress, usually produce the cell cycle arrest of actively proliferating cells (De Veylder et al., 2007, Doerner, 2007, Komaki and Sugimoto, 2012). In the case of gravitational stress, the cycle is not arrested but the regulation is severely affected, as in the case of the failure of the G2/M checkpoint detected by the altered expression of the cyclin B1 gene. This failure would allow cells to enter mitosis before a critical size is reached.

The cellular alterations found in this work to occur in root meristematic cells of seedlings as the early response to the simulated microgravity treatment would seriously endanger plant development and even plant survival. However, longer periods of exposure to simulated microgravity do not produce either an intensification of the cellular damages or a

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detectable developmental alteration in seedlings analyzed at further stages of their growth. On the contrary, alterations found in some parameters, such as the cell proliferation rate or the expression of the cyclin B1 gene, in very young seedlings become progressively attenuated with time progression. This suggests that the secondary response to the gravity alteration is a process of adaptation, whose mechanism is still unknown, which eventually results in viable adult plants. This is in agreement with recent experiments in space which are showing with increasing certainty that plants grown in the real microgravity environment are capable of surviving without major alterations, even though adult plants often show some abnormalities (De Micco et al., 2014, Link et al., 2014, Massa et al., 2013). The apparent paradox of the finding of important modifications at the cellular and molecular level which do not result in later substantial changes at the developmental level affecting the full organism, is not exclusive of plants (Herranz et al., 2010, Marco et al., 2003). Nowadays, this paradox remains unresolved. Therefore, one of the most attractive challenges of space plant biology for the near future is to investigate the molecular processes and mechanisms involved in enabling plants to counteract the gravitational stress and to adapt to the space environment, surviving and developing in it. Specifically regarding the essential functions of meristematic cells, dedicated transcriptomic/proteomic studies using isolated meristems and/or proliferating *in vitro* cultured cells will be necessary to find accurate solutions to this problem.

5. Concluding remarks

This work provides evidence of a serious disruption of essential parameters of plant meristematic cells induced by the alteration of the environmental gravity. If the suppression of the gravity vector is capable of producing such these effects, it results that gravity is essential for sustaining a normal body plan and weightlessness is a major stress condition for plant development and growth. Numerous questions have been raised from these novel results, many of them remaining open to be answered in future experiments. These answers will be necessary for achieving a successful culture of plants on board of spaceships and, in general outside the Earth environment, which is essential to make possible the coming enterprises of space exploration by the human being.

Acknowledgements

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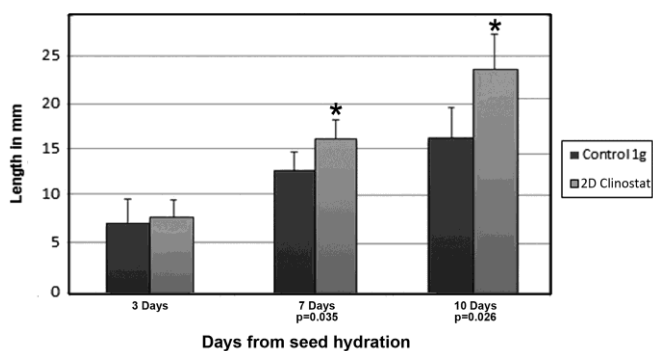
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Figure captions

Fig. 1

Figure 1.

Length of roots for samples grown in the clinostat . The root is practically unchanged, with regards to the 1g control, at the beginning of the treatment (3 days), but in further sampling points roots of samples grown in simulated microgravity are longer than the corresponding controls. Asterisks indicate statistical significance obtained with t test; p values are shown. Error bars represent StdDev.
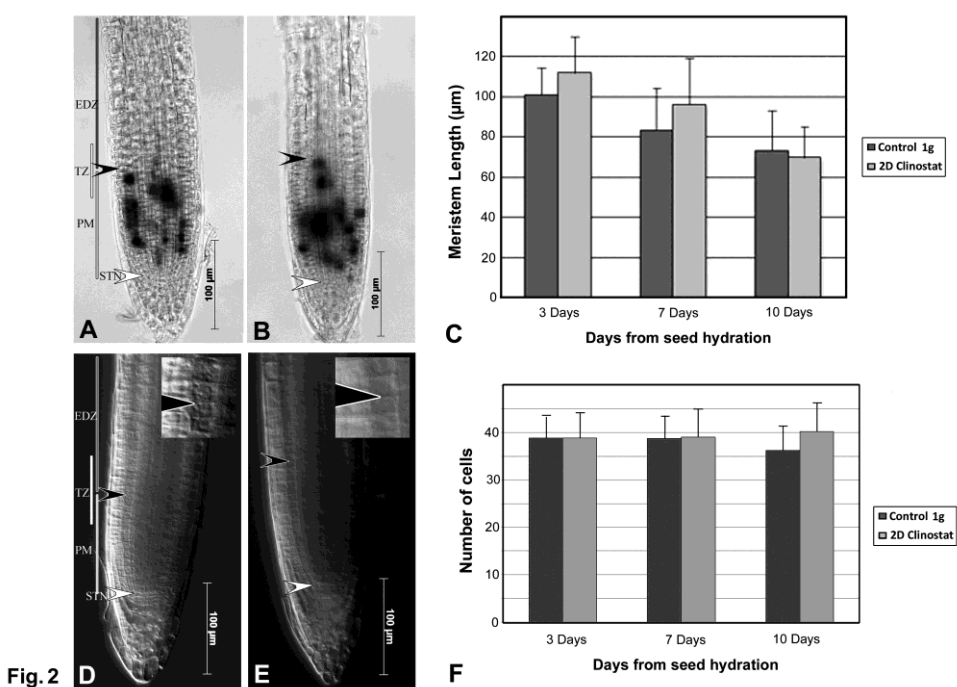

Fig. 2

Figure 2.

Estimation of the size of the root meristem. Upper row (A, B, C): Meristem size (length), estimated from the microscopic visualization of dividing cells (meristematic cells) in the root tip of seedlings of the transgenic line *CYCB1::uidA,* showing the expression of the

specific G2/M transition marker gene *CYCB1;1* by means of GUS staining. Observations were done after 3, 7 and 10 days of culture in 1*g* (A) and in clinorotated samples (B). Images show root tips of 3-day seedlings. Root zones are indicated: Stem-Cell Niche (STN), Proximal Meristem (PM), Transition Zone (TZ), Elongation/Differentiation Zone (EDZ). C: Quantitative data on the meristem length, determined by the distance between the nearest and the farthest stained spots in all samples. The size of meristems was quite similar in plants grown in simulated microgravity and in the 1*g* control in the three sampling points. Note that the size of the meristematic zone was gradually decreasing at 7 and 10 days compared to 3 days, irrespective of the gravity conditions of growth. Lower row (D, E, F): Meristem size, estimated by the number of cortex cells in the cortex file extending from the Stem-cell Niche (STN, white arrows) to the Transition Zone (TZ, black arrows). D, E: Root tips of 3-day seedlings in 1*g* (D) or clinorotated (E). The inserts show a blow-up of elongation cells exiting from the meristem at the cortex, indicating the onset of the TZ. F: Quantitative data on the root meristem cell number. No changes in root meristem size were observed in clinorotated seedlings compared to 1*g* control and the size of meristem remained constant throughout the time of culture, regardless of the experimental condition. P values for statistical significance are indicated in (C) and (F). Error bars represent StdDev.
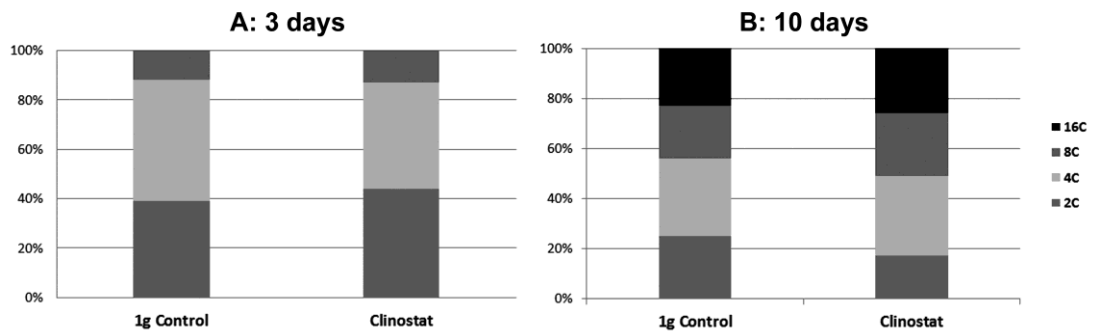
Fig. 3

Figure 3.

Study by flow cytometry of the changes in cell cycle phases in samples grown in the clinostat for 3 days (A) and 10 days (B) compared with the 1g control. The content of DNA was measured and classified in four different categories: 2C, corresponding to the G1 period, 4C, corresponding to the G2 period, 8C and 16 C, these latter two corresponding to cells that have experienced endoreduplication, having escaped from the cell cycle and entered in the process of differentiation. Since the exit from the cell cycle may occur at different phases, it could be possible that a few cells having 4C DNA content would correspond to endoreduplicated cells at the G1 period.
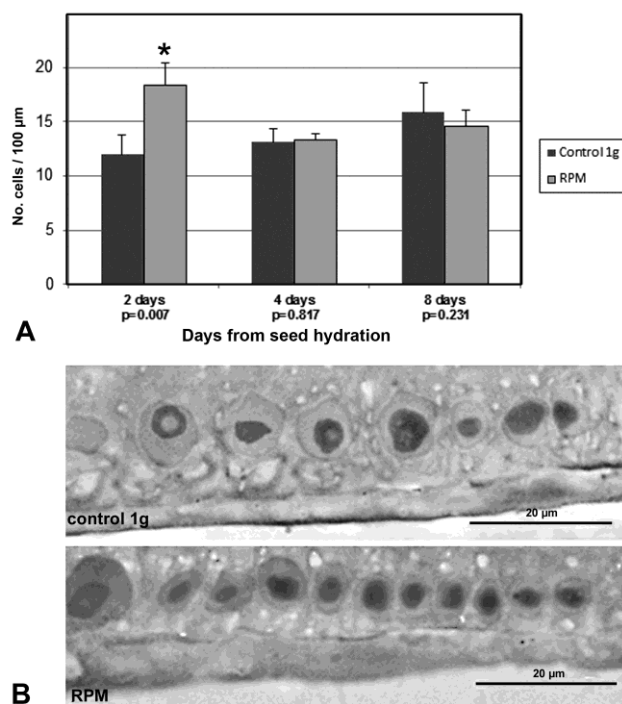
A

B RPM

Fig. 4

Figure 4.

Rate of cell proliferation in the RPM, estimated by counting the number of cells per unit of length (100 µm) in the different cellular rows of the root meristem (A). The rate of cell proliferation is much higher in samples grown in simulated microgravity, compared to the 1g control, at the very early stages of germination (2-day seedlings). However, at the two later sampling points (4 and 8 days), the cell proliferation rates do not differ with the changing gravity conditions. The microscopical image (B) shows a part of a cellular row from a sample of 2 days grown in control 1g conditions and in the RPM, showing smaller and more numerous cells as an effect of the simulated microgravity environment. P values for statistical significance are indicated. Error bars represent StdDev.

2 days   **4 days**   **8 days**

Control 1g

50 µm

RPM
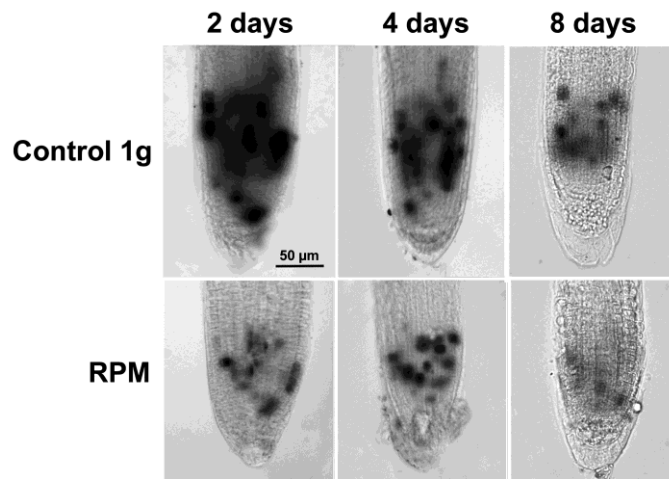
Fig. 5

Figure 5.

Expression of the cyclin B1 gene, a regulator of the G2/M transition in the cell cycle, evaluated after *in situ* localization, using the same line and the same staining procedure as shown in Fig. 2. Microscopical images of samples grown in the RPM, compared with those grown under 1*g* control conditions, stained to reveal the GUS reaction, showing the expression of the cyclin B1 gene as blue dots. Control samples clearly show a heavier staining than those grown in the RPM, at the three sampling points. The difference is maximum at the beginning of development (day 2 samples) and is attenuated in successive sampling points. In general, there is a reduction in the level of cyclin B1 expression throughout the seedling development.
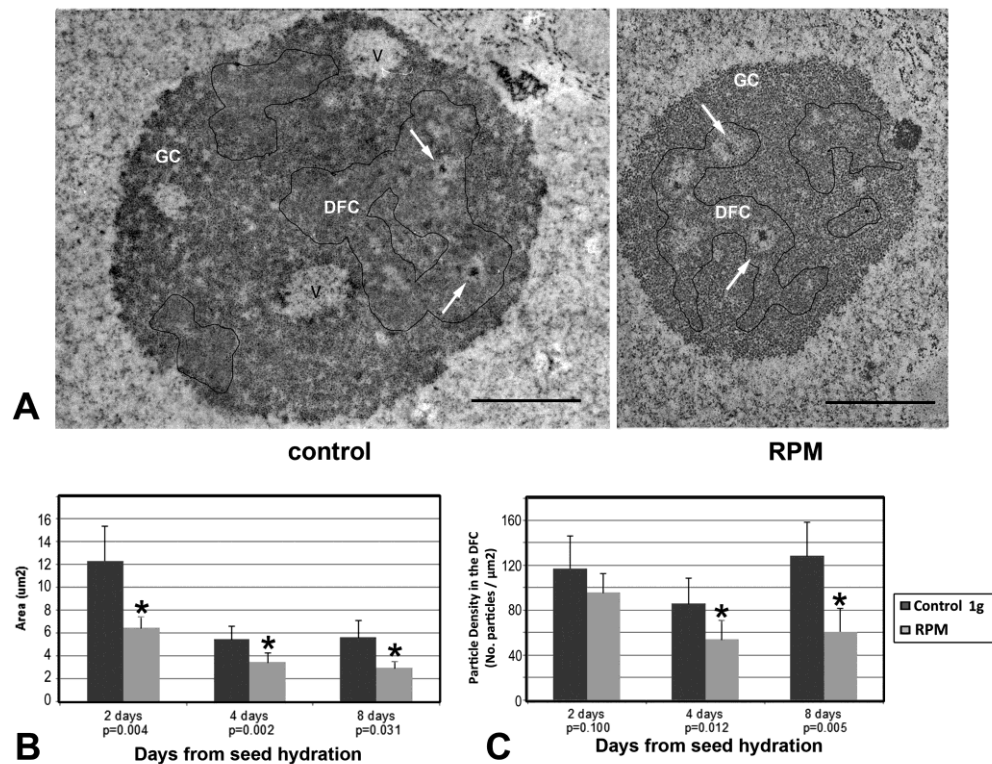
Fig. 6

Figure 6.

Nucleolar activity and ribosome biogenesis in RPM-treated samples. A: Electron microscopic images of meristematic cell nucleoli from 2-day-old seedlings corresponding to control 1*g* samples and RPM-treated samples. The size and structure of the nucleolus reveals a depletion of nucleolar activity as a result of the growth under simulated microgravity, in the RPM. Thus, the nucleolus is smaller, it shows a reduced amount of granular component (GC) and the fibrillar centers (arrows) are larger, less numerous and some of them of the heterogeneous type. DFC: Dense Fibrillar Component. The nucleolar area occupied by DFC is outlined. V: Nucleolar Vacuole. Bars indicate 1μm. B: Quantitative study of the nucleolar size throughout the seedling development in control and RPM conditions determined from the cross-sectional area of the nucleolus. C: Quantitative determination of the levels of the nucleolar protein nucleolin, an active regulator of different steps of pre-rRNA transcription and processing, detected *in situ* by the immunogold procedure, throughout the seedling development. The estimation of the levels of the protein was done by measuring the particle density, i.e. the number of particles per μm^2 in the nucleolar dense fibrillar component. The lower levels of nucleolin in RPM-treated samples indicate a lower level of nucleolar activities and, consequently, of the production of ribosomes (p values for statistical significance are indicated).
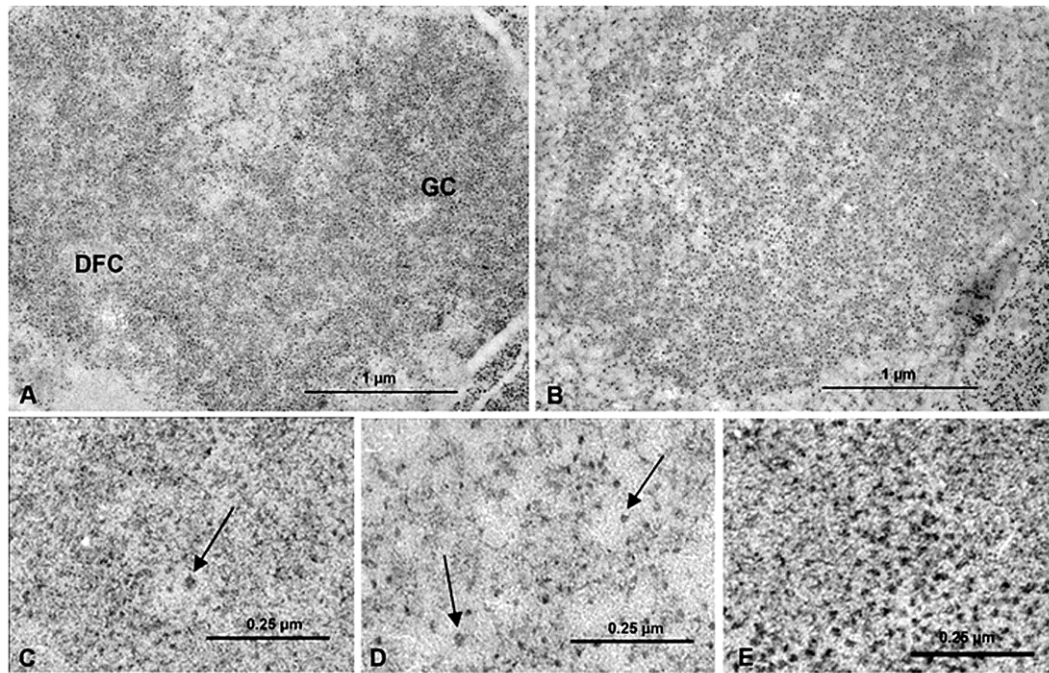
Fig. 7

Figure 7.

Nucleolar activity and ribosome biogenesis in RPM-treated samples: effects on the nucleolin mutant *AtnucL1*. A: Mutant plant nucleolus under control conditions. B: The nucleolus of a mutant plant grown in RPM. The structure of the nucleolus, which is seriously disorganized in the mutant in control conditions, appears even more de-structured in the RPM, being formed by a loose mass of granules without any other discrimination of regular nucleolar subcomponents. C, D: Higher magnification of the control (C) and RPM (D). Arrows point to nucleolar perichromatin-like granules, which are particles containing badly processed preribosomal precursors. For comparison, E: granular component of a wild type plant cell nucleolus.

Supplemental Material

Supplemental Figure 1.

Length of roots for samples grown in the RPM. The results are similar to those obtained in the clinostat, showing the root practically unchanged, with regards to the $1g$ control, at the beginning of the treatments (2 days), but longer than the control in further sampling points. Asterisks indicate statistical significance obtained with *t* test; p values are shown. Error bars represent StdDev.

Supplemental Figure 2.

Expression of the cyclin B1 gene, a regulator of the G2/M transition in the cell cycle, evaluated after *in situ* localization, using the same line and the same staining procedure as shown in Fig. 2. Number of GUS-stained cells per optical section, estimated by counting the number of blue spots, in samples grown in the 2D clinostat, compared to the $1g$ reference control samples. The number decreases in clinostat-treated samples, 7 and 10 days after germination. At 10 days, the decrease is statistically significant (p =0.005). Error bars represent StdDev.