Cell cycle acceleration and changes in essential nuclear functions induced by simulated microgravity in a synchronized *Arabidopsis* cell culture

Short Running Title: Cell cycle and nuclear changes under microgravity

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ABSTRACT

Zero-gravity is an environmental challenge unknown to organisms throughout evolution on Earth. Nevertheless, plants are sensitive to altered gravity, as exemplified by changes in meristematic cell proliferation and growth. We found that synchronized *Arabidopsis* cultured cells exposed to simulated microgravity showed a shortened cell cycle, caused by a shorter G2/M phase and a slightly longer G1 phase. The analysis of selected marker genes and proteins by qPCR and flow cytometry in synchronic G1 and G2 subpopulations indicated changes in gene expression of core cell cycle regulators and chromatin-modifying factors, confirming that microgravity induced misregulation of G2/M and G1/S checkpoints and chromatin remodeling. Changes in chromatin-based regulation included higher DNA methylation and lower histone acetylation, increased chromatin condensation and overall depletion of nuclear transcription. Estimation of ribosome biogenesis rate using nucleolar parameters and selected nucleolar genes and proteins indicated reduced nucleolar activity under simulated microgravity, especially at G2/M. These results expand our knowledge of how meristematic cells are affected by real and simulated microgravity. Counteracting this cellular stress is necessary for plant culture in space exploration.

Keywords:

Altered Gravity; Cell proliferation; Ribosome Biogenesis; Nucleolus; Chromatin remodeling; Flow cytometry; Transcription; Immunofluorescence microscopy; qPCR.

INTRODUCTION

Gravity is a key cue for life, the only factor of the Earth environment that has remained constant through the evolution of living organisms. It is of high interest to know how gravity modulates plant cellular and molecular functions, as well as to determine whether plants may eventually respond and adapt to changing levels of gravity. These questions remained at an exclusively theoretical and speculative level until the advent of spaceflight, when the exposure of living beings to gravity conditions different from the Earth, including weightlessness (strictly, microgravity) became a real fact. In the last few decades, understanding the physiological processes occurring in plant cells under the space environment has become crucial to successfully undertake space exploration strategies. At the same time, gravitational and space research has revealed as a useful tool to solve some pending biological questions related to the life on Earth of plants, animals and even human beings (Schlacht et al., 2016). Just from the beginning of the “space race”, ideas about the mechanical stress induced on cells by altered gravity, the existence of a mechanical sensor in cells and the possibility of simulating on ground the gravitational conditions present in space, were put forward (Pollard, 1965).

Research in the near-Earth orbit and access to International Space Station (ISS) are still today severely constrained by a limited number of flight opportunities. Ground-based facilities (GBFs) for altered gravity are valuable tools providing additional cost-efficient platforms for gravitational research (Herranz et al., 2013). Microgravity can be simulated using the 3D clinorotation in a Random Positioning Machine (RPM, Dutch Space, Leiden, The Netherlands) (van Loon, 2007) and it has been shown that the simulation is effective and reliable with regard to the biological effects of microgravity, specifically on plants (Kraft, van Loon & Kiss, 2000, Medina, Herranz, Arena, Aronne & De Micco, 2015).

The alteration of the gravity vector has an influence on cell proliferation and cell growth, as it has been shown in a variety of biological systems (Manie, Konstantinova, Breittmayer, Ferrua & Schaffar, 1991, Manzano, Herranz, Manzano, Van Loon & Medina, 2016, Medina & Herranz, 2010, Perbal, 2001, Thiel et al., 2012). Actually, while eukaryotes (human, animal and plants) rely on cell proliferation control by essentially similar mechanisms (Cross, Buchler & Skotheim, 2011, Harashima, Dissmeyer & Schnittger, 2013), they apparently respond differently to microgravity by changing the

71 regulation of specific mechanisms involved in this cellular process. In plants,
72 microgravity environment seems to dissociate cell proliferation from cell growth in
73 seedling root meristems, notably increasing cell division rates and decreasing cell growth.
74 Significant alterations in cell cycle progression were reported on lentils, suggesting
75 modifications in the regulatory mechanisms of the cell cycle (Legué, Yu, Driss-École &
76 Perbal, 1996, Merkys, Laurinavicius & Svegzdiene, 1984, Yu, Driss-École, Rembur,
77 Legué & Perbal, 1999). More recently, in our previous experiments on *Arabidopsis*
78 seedlings grown in the ISS and RPM, we reported that microgravity increased the cell
79 proliferation rate while the cell growth rate was decreased, compared to 1g controls
80 (Manzano et al., 2013, Matía et al., 2010). A reduction of the rate of ribosome biogenesis
81 (strictly correlated to cell growth in this system) was reported on meristematic cells from
82 seedlings grown in simulated microgravity (Shen-Miller & Hinchman, 1995, Sobol,
83 Gonzalez-Camacho, Rodriguez-Vilariño, Kordyum & Medina, 2006). However, many
84 points remained unresolved after these studies, especially dealing with the effects of
85 altered gravity on the regulation of these cellular processes.

86 The cell cycle (its progression and its regulation) is one of the most comprehensively
87 studied biological processes. In plants, the role of cell cycle machinery in growth and
88 development has been especially taken into account (Inzé & De Veylder, 2006). The
89 regulation of cell cycle progression in the known checkpoints determines the rate of cell
90 division and modulates meristematic cell proliferation, an essential parameter for plant
91 development (De Veylder, Beeckman & Inze, 2007, Inzé & De Veylder, 2006, Van Leene
92 et al., 2010).

93 Cell cycle studies under gravitational alteration may greatly benefit from the use of an *in*
94 *vitro* cell culture system. It may reveal the pure response of individual cells to the gravity
95 change, in comparison to the statolith system existing in the root and shoot tips,
96 responsible for gravitropism, which is not present in this system (Hashiguchi, Tasaka &
97 Morita, 2013, Herranz, Valbuena, Youssef & Medina, 2014). Furthermore, it enables the
98 achievement of different cellular and molecular biological analyses without the
99 restrictions and constraints of the studies *in planta*. Finally, a unique advantageous feature
100 of cell cultures (cell culture suspensions) is their ability to be synchronized in their
101 progression through the cell cycle, using e.g. the aphidicolin block/release approach
102 (Menges & Murray, 2002). Cell cycle synchronization may provide us with precise data
103 on the cell cycle timing (acceleration/deceleration) which determines the cell proliferation

rate, in addition to a deep analysis of the cell cycle phases under the gravitational alteration, allowing the characterization of functional processes in each phase and their regulation.

An additional point of interest in the cellular effects of altered gravity is the occurrence of changes in the regulation of gene expression involving chromatin-remodeling mechanisms (Bird, 2007, Goldberg, Allis & Bernstein, 2007). It is known that two major modifications are the basis of chromatin remodeling, namely DNA methylation and histone acetylation, which affect the chromatin structure by modifying its condensation state. These chromatin changes could have either positive or adverse impact on gene expression and they play a role in the adaptive response of plants to stress (Jerzmanowski, 2007, Kouzarides, 2007, Zilberman, 2008). Whereas the alteration of chromatin features by environmental stress and the effect of these alterations on gene expression have been demonstrated, the causal relationship between chromatin remodeling and transcriptional response has not been discriminated until now (Asensi-Fabado, Amtmann & Perrella, 2017). Another point of interest, still unresolved, is the heritable condition of stress-induced changes in chromatin structure (Asensi-Fabado et al., 2017). Specifically regarding cell cycle, chromatin is a highly dynamic and major player in cell cycle regulation, since some specific chromatin modifications are crucial to moving across the cell cycle (Costas, Desvoyes & Gutierrez, 2011, Sanchez, Caro, Desvoyes, Ramirez-Parra & Gutierrez, 2008). Furthermore, it was reported recently that core cell cycle regulators control gene expression by modifying histone patterns (Raynaud et al., 2014).

Here in this paper, we describe the first successful use of synchronized cells to characterize different cell cycle phases under simulated microgravity obtained in an RPM, a ground-based facility capable of averaging the gravity vector, suppressing in practice its effects on the living organisms. Samples enriched in cell cycle phases were obtained under the gravitational alteration with the purpose of identifying the specific alterations in cell growth and cell proliferation, including transcriptomic changes and chromatin remodeling in each cell cycle phase. Implications of our results studying cell cycle in altered gravity have the potential to be used on Earth in applications related to plant response and adaptation to stresses through control of the cell cycle regulation (Burssens et al., 2000, Kitsios & Doonan, 2011, West, Inzé & Beemster, 2004). The need of building efficient systems of bioregenerative life support for space exploration, in which plants

must occupy a prominent position, may contribute to improve the efficiency of plant cultures of agricultural interest on Earth.

MATERIAL AND METHODS

1. Biological material

Synchronization of immobilized *Arabidopsis* cell suspension culture

Arabidopsis thaliana (L.) (Ecotype *Landsberg erecta*) cell suspension culture (MM2d) was grown in Murashige and Skoog medium supplemented with 3% w/v sucrose, 0.5mg/L NAA, 0.05mg/L kinetin, with pH adjusted to 5.8 using 1N NaOH (MMS medium). MM2d cells were maintained by weekly sub-culturing (1:20 dilution) every 7 days in 250mL Pyrex flasks, with continuous shaking at 120 rpm in an incubator shaker at 27° C in darkness (Menges & Murray, 2006). Aphidicolin synchronization method, described by Menges and Murray (2006) in this biological system to arrest cell cycle progression at the G1/S transition to obtain synchronic subpopulation of cells (aphidicolin block/release, named T0), was used in these experiments.

Before incubating synchronous cells (T0) under simulated microgravity and 1g control, the synchronous suspension culture was immobilized. A procedure for immobilizing cells in agarose, initially designed to be used in spaceflight, and modified in our laboratory, was followed (Kamal, van Loon, Medina & Herranz, 2017). Briefly, low melting agarose (2% (w/v); gelling at 26-30°C; SeaPlaque™ Agarose, BDH, Pool, UK) was dissolved in MSS medium and allowed to cool down to 28-27°C. The agarose solution was then mixed with an equal volume of the prepared cell suspension, grown as described above, resulting in a final concentration of 1% (w/v) agarose and a 1:40 dilution of cells in MSS medium. After gentle mixing, 10 ml of the agarose-cell mixture were poured into Petri dishes. After the agarose had solidified, Petri dishes were sealed with Micropore™ tape. All steps were carried out at room temperature and under sterile conditions. The immobilized culture was then subjected to the simulated microgravity and 1g control treatments and cells, either fixed or frozen, were recovered at the end of each experiment. Agarose-embedded cells were fixed by adding 1 ml of 4% (w/v) paraformaldehyde (PFA, Electron Microscopy Sciences, Hatfield, PA) in phosphate-buffered saline (PBS buffer) onto the surface of the plate for 1 hour and recovered by centrifugation after dissolving the agarose

by immersion in a water bath at 63°C. Samples to be recovered frozen were first gently fixed with 1 ml 1% (w/v) PFA for 15 min, to arrest the biological activity. Then, cells were successively dissolved in a water bath, concentrated by centrifugation and directly frozen by immersion in liquid nitrogen. The time lapse to collect frozen samples was always less than 1 hour after the end of the experiment.

2. Microgravity simulator and experimental design

In this study, we applied the Random Positioning Machine, RPM (Airbus Defence & Space, Leiden, the Netherlands) (Borst & van Loon, 2009), to generate simulated microgravity for the immobilized cell cultures accommodated in standard 9 cm diameter Petri dishes. The RPM was set to the real random mode and random direction with a maximum speed of 60°/s. The samples were affixed to the center of the inner frame with a resulting largest radius of 5 cm for the outermost sample, resulting in a maximum residual g due to rotation of less than $10^{-4}g$ (van Loon, 2007). RPM controls (1g) were placed outside the rotating platform, but in the same environment as the RPM samples.

Arabidopsis thaliana cell suspension cultures seven days old were incubated with 4.16 µg/ml aphidicolin for 24 hours at 27°C, in a shaking incubator working at 120 rpm, in dark conditions, and were washed as described in the synchronization protocol (Menges & Murray, 2006). Samples collected just after drug removal by washing out were called T0 samples. Then cells were embedded in agarose and exposed to simulated microgravity using the RPM (or kept at 1g for the control). Time-course experiments were performed for 72 hours in three replicates, taking samples at time intervals (T0, T1, T2, T4, T6, T8, T10, T12, T14, T16, T18, T20, T22, T24, T26, T28, T30, T32, T34, T36, T40, T44, T48, T52, T56, T60, T64, T68, T72). Cells were recovered either fixed or frozen, as described above. From cells recovered from frozen samples, nuclei were isolated for cell cycle analysis using flow cytometry. A reference time was determined for each cell cycle phase during the first cell cycle (S, G2/M, and G1 phase).

3. Flow cytometry and other cell cycle analysis

3.1 Determination of individual cell DNA content (% of cells in G phases)

Frozen cell pellet (500 mg) was treated with the High Resolution Kit for plant ploidy level analysis (Kit Cystain UV precise P), containing solution A (Nuclei extraction buffer) and solution B (Staining buffer containing DAPI: 4, 6, diamino-2-phenyl-indol) (Partec

GmbH, Munster, Germany) to determine the DNA content. For nuclei release, the cells were rinsed in solution A, carefully chopped with a sharp razor blade, and then cells were filtered and rinsed in solution B (Menges & Murray, 2006). On average, 10000 particles in three replicates were counted by flow cytometry (Cell sorter FACS Vantage, Becton–Dickinson, San Diego, California), using ion laser tuned at 360 nm and detection of emission using a blue fluorescence emission filter (bandpass filter of 424/44 nm Bandpass). FACS analysis results were analyzed using BD CellQuest™ software for Windows XP to determine the ratios of cell cycle phases according to the DNA content of individual cells (2n for phase G1, 2<n<4 for S, 4n for G2/M phases).

3.2. Specific protein quantification in single cells using flow cytometry

The levels of relevant nuclear proteins in the cell cycle phases were quantified by a flow cytometry protocol previously used in our laboratory with onion (González-Camacho & Medina, 2006) and adapted by us for *Arabidopsis* cells. Isolated nuclei were incubated in isolation buffer after filtration on ice for 2 min. After centrifugation, they were fixed on ice for 15 min with 1% PFA. Fixed nuclei were washed twice with 0.01% Triton X-100 containing PBS. Stained nuclei were blocked in 500 µl of PBS blocking solution for 30 min at 4°C. The nuclei were incubated with the first antibody (Supplemental Table S1) in blocking solution for 45 min at 4°C followed by incubation with Alexa Fluor®-labeled secondary antibody (Supplemental Table S1), for 30 min at 4°C, and finally washed with PBS (3 × 5 min) using centrifugations at 1500 rpm. Nuclei were counterstained with the staining solution B of the kit, containing DAPI labeling. A control experiment was run, in which the first antibody was omitted. Two fluorescence detectors have been used with the standard 360 nm laser. Alexa Fluor®-protein fluorescence intensity was detected between 530/30 nm (FL1 channel). For detection of DAPI intensity (DNA content) 424/44 nm blue fluorescence emission range was used at FL2 channel. Data were analyzed using BD CellQuest™ software for protein fluorescence intensity detected by flow cytometry. Side scatter *versus* forward scatter diagrams were used to locate and gate nuclear populations by particle size. The fluorescence intensity level was normalized by subtracting the value obtained for the control.

4. Fixation and processing for immunofluorescence

Embedded fixed cells were additionally fixed in 1 ml of 4% PFA for 1 hour, at RT and washed in PBS (3×10min). Then the cell wall was digested using 1ml of an enzyme

cocktail [2% (w/v) cellulose, 1% (w/v) pectinase, 0.05% (w/v) macerozyme, 0.4% (w/v) mannitol, 1% (v/v) glycerol and 0.2% (v/v) Triton X-100, 30 min at 37°C]. Finally, samples were washed with 1% (v/v) glycerol and 0.2% (v/v) Triton X-100 in PBS (3 × 10 min). A drop of the cell pellet was placed on a microscope slide covered with poly-lysine and blocked with 2% (w/v) BSA and 0.05% (v/v) Tween in PBS blocking solution, for 30 min at RT. Samples were incubated with the first antibody (Supplemental Table 2) for 12 h at 37°C. Then, they were washed with PBS (3 × 5 min) and incubated with the secondary antibody (Supplemental Table S1), for 3 h at 37°C, followed by washing with PBS (2 × 5 min) and counterstaining with 5 µg/µl DAPI in PBS, for 5 min. After washing with PBS (2 × 5 min) and with double-distilled water (2 × 5 min), coverslips were placed under PVA-DABCO™ (a glycerol-based mounting medium containing an anti-fading reagent for use with immunofluorescence preparations) and inverted onto glass slides. A control experiment was run, in which the first antibody was omitted. Confocal laser scanning microscopy was performed using Leica TCS SP5 with AOBS (Acousto Optical Beam Splitter, Mannheim, Germany) with 63× oil immersion optics. Laser lines at 488 nm (Green) and 561 nm (Red) for excitation of GFP and TxR (x-Ray system) were provided by an Ar laser and a DPSS laser. Detection ranges were set to eliminate cross-talk between fluorophores. Images were analyzed using Leica LAS AF, image analysis software v2.4 for measurement of the intensity of the fluorescence signal. The intensity of the signal was normalized against the values obtained for the control experiment.

5. Sample processing for ultrastructural analyses

Samples were fixed using 3% (v/v) glutaraldehyde in PBS for 2 hours at RT. Cells were pipetted and rinsed in the fixative during the fixation period. They were then washed three times with phosphate-buffered saline (PBS), 10 min each, dehydrated in an ethanol series, treated for the methylation-acetylation procedure (Testillano, González-Melendi, Ahmadian & Risueño, 1995) and finally embedded in LR White resin (London Resin, EMA, UK). From resin-embedded materials, semi-thin 2 µm thick sections were obtained from the LR White blocks, visualized by phase contrast in a Leica DFC320 microscope equipped with a Leica DM2500 CCD digital camera. Ultrathin sections were stained for 30 min with 5% (w/v) uranyl acetate and for 90 seconds with 0.3% (w/v) lead citrate, separated by a wash in distilled water. Samples were observed using a JEOL 1230 transmission electron microscope operating at 100 kV acceleration voltage. The images

obtained were processed for quantitative studies using QWin Standard image analysis (Leica Microsystems) and Image J 2.0 (*imagejdev.org*) software.

6. RNA extraction and quantitative real-time PCR (Rt-qPCR)

Total RNA was extracted from the frozen samples using the “REAL Total RNA Spin Plants and Fungi” kit (REAL, Durviz S. L. Lot/21015). RNA concentration and quality were estimated by measuring the absorbance (absorbance ratio 260 nm/ 280 nm should be between 1.6 and 2.0) using a spectrophotometer (NanoDrop ND-1000, Thermo, USA). Four µg of RNA samples incubated with 1µl DNase enzyme (Turbo DNA-free; Ambion AM1907) and 5 µl of 10× enzyme buffer at 37°C for 1 hour. DNase enzyme was inactivated with 5 µl stopped DNase for 2 min at room temperature. The sample was centrifuged at 13000 rpm for 2 min, and then the supernatant containing the purified RNA sample was collected. RT-qPCR was performed using DNA amplification and quantification kit (SYBR Green QRT-PCR, Agilent, USA) according to the instructions of the manufacturer, using gene-specific primers (Supplemental Table 3). RT-qPCR running was carried out using the iQ™5 Multicolor Real-Time PCR Detection System, BIO-RAD, by the Genomics Service at CIB-CSIC, Madrid. The RT-qPCR data for each target gene were presented as average expression levels over three biological replicates, with two technical replicates per reaction, about the expression standards of the *Actin* reference gene (Supplemental Table S2). Data analyses were performed using iQ™5 optical system software v2.1.

7. Statistical analyses

Data were collected from different analyses after each experiment in an Excel datasheet (Microsoft Office 2010). The data range, mean and standard deviation in each experiment were estimated. Data were shown as mean ± SE of different replicates. Standard Error (SE) was estimated using the values of the Standard Deviation (SD) using this formula

$$(SE = \frac{SD}{\sqrt{n}}).$$

In quantitative studies involving data comparison between different experimental means, data were analyzed according to Steel (1980). SPSS v.22 program was used for statistical analysis of the variance of differences using ANOVA test. The degree of freedom was followed as $p \leq 0.05$ (95%) considered statistically significant (represented with an asterisk [*] in the figures).

RESULTS

Arabidopsis cell cycle progression through 24 hours after aphidicolin block/release under different gravitational conditions

Cell cycle progression was analyzed on MM2d cell cultures synchronized by treatment with aphidicolin and immobilized in agarose. The distribution of cell cycle phases was determined by flow cytometry analysis of samples stained for DNA with DAPI. Samples incubated in simulated microgravity conditions (RPM) for 72 h and 1g control samples, were used.

The effect of simulated microgravity on the duration of the cell cycle was estimated by comparing the duration of the cell cycle phases of the simulated microgravity (Sim μ g) and the 1g control experiments throughout a period extending for 24 hours from the release of the aphidicolin block (Figure 1). The comparison shows that cell cycle progression rate was higher under Sim μ g, compared with the 1g control. Precisely, according to our data, the cell cycle was two hours shorter under Sim μ g than under 1g control conditions (Figure 1).

The reliability of these results was validated by obtaining prior evidence that, after the release of the aphidicolin block (T0), cell cycle progressed synchronously over a 72 h period in the immobilized culture (Supplemental Figure S1). Aphidicolin synchronized the cell cycle by arresting approximately 2 out of 3 cells in the late G1/S phase. Samples taken immediately after the release of the drug treatment were labeled as T0. This degree of synchrony of the cell population was kept unaltered for more than one entire cell cycle (24 h). Thus, specific reference times were defined as representative of the successive cell cycle phases. The peak of synchronized cells corresponding to S phase was obtained after 2 hours (T2=65.2%). Through 10 hours, T10 sample showed the maximum peak for G2/M subpopulation of cells reaching 69%. These cells needed 6 hours more to divide, going through a G2/M phase and entering a new cell cycle beginning with G1 phase (T16 sample represents G1 subpopulation peak, with 68.5% of cells in G1).

A similar approach was performed under simulated microgravity using the RPM (Sim μ g) after aphidicolin block (Supplemental Figure S2). First S-phase peak was shown after 2 hours (T2 = 65.9%) as expected, but the cell cycle progression and entry into G2/M

phase occurred at T7 (65.9% of cells in G2/M), three hours earlier than in the 1g control. G1 phase population peak of the new cell cycle after mitosis was observed after 14 hours (T14 = 61.4%), two hours earlier than in the 1g control. This means a deceleration of one hour in G1.

Subpopulations of the different cell cycle phases were defined and confirmed through the previous results using different cellular biological techniques (Supplemental Figure S3) for a better characterization of the gravitational effect on each cell cycle phase.

Gene expression changes of cell cycle regulators in synchronous *Arabidopsis in vitro* cell culture under simulated microgravity.

A preliminary transcriptomic study using a full genome microarray was carried out on two synchronic cell culture subpopulations enriched in G1 and G2/M phases of the cell cycle, respectively, comparing the effects of the simulated microgravity treatment with the 1g control conditions. Microarray data were deposited at CATdb (Project Au13-11_Gravity) according to the 'Minimum Information About a Microarray Experiment' standards. Data are also accessible from Gene Expression Omnibus database with the reference GSE60473 and GENELAB NASA database with the reference GLDS-144. The purpose was to detect specific changes in gene expression induced by the gravity alteration associated to either of these phases of the cell cycle. It is worth mentioning that the chosen periods for synchronized cells include the three known checkpoints acting in the regulation of cell cycle progression (G1/S transition, G2/M transition, and anaphase).

Heat map visualization (R software) was used to represent the expression changes of the whole families of CDK, CKL, and cyclins (Supplemental Figure S4).

Furthermore, we have selected a few relevant nuclear proteins, which are commonly recognized as markers of the investigated functions. We have carried out an estimation of the effects of simulated microgravity by measuring the levels of these proteins in isolated nuclei by flow cytometry and the changes in the expression of genes coding for them by qPCR.

Effects on markers of cell cycle regulation (Cyclin B1 and Prolifera)

Flow cytometry analyses confirmed that cyclin B1 is an accurate G2/M phase-expressed protein, compared with G1 and S phases (Figure 2). Under simulated microgravity, the

increase in the cyclin B1 levels in G2/M phase was significantly lower in comparison with 1g control. Contrary, the level of cyclin B1 mRNA was shown to peak significantly on our S-phase samples, compared with G1 and G2 phases, in both 1g control and simulated microgravity. This level was conspicuously decreased in G2/M phase subpopulation (Figure 3). In turn, we confirmed the feature of the Prolifera antigen as G1 phase marker, along with a significant reduction of the level of this protein in S and G2/M phases (Figure 2). This observation was supported by the gene expression analysis in which an up-regulation was shown under simulated microgravity (Figure 3). Prolifera mRNA levels were altered in the experimental conditions, indicating a significant upregulation in all phases.

Analysis of gene expression changes of factors affecting chromatin dynamics and remodeling in synchronous *Arabidopsis in vitro* cell culture under simulated microgravity

As performed for the cell cycle regulatory genes, a preliminary global analysis of the expression patterns of the chromatin remodeling factors was carried out. The same as with cell cycle regulatory genes, heat map visualization (R software) was used to represent the expression changes of the different gene families, namely DNA methylation, histone methylation and histone acetylation. Results revealed significant changes in several of these factors (Supplemental Figure S5).

The same type of analysis based on selected markers, evaluated by flow cytometry and qPCR, has been used in this case. Methylated cytidine (5mdc) fluorescent intensity, measured by flow cytometry, suggests that DNA was significantly more methylated in S and G2/M phases compared with G1, in which a lower level of DNA methylation was observed under 1g control conditions. This substantial difference between phases was attenuated under simulated microgravity since the DNA methylation was shown significantly increased in G1 in comparison to the levels of the 1g control. In S and G2/M phases, the gravity alteration did not cause changes in this parameter (Figure 2). This alteration was supported by the data on DNA methyltransferase gene expression (Figure 3), which increased significantly in simulated microgravity conditions, not only in G1 phase, but in all the cell cycle phases, compared with 1g control. The MET1 gene was significantly more expressed in S phase compared with G1 and G2/M in both 1g control and under simulated microgravity. On the other hand, acetylated Histone H4 was

inversely correlated with the methylation marker. G1 phase peak, observed under 1g control, was not detected under simulated microgravity conditions (Figure 2).

Simulated microgravity modifies the levels of the chromatin remodeling markers *in situ*, as well as the chromatin organization and RNA polymerase II activity through the cell cycle phases

The detected changes in factors of chromatin remodeling induced by simulated microgravity have been further validated by evaluating their amount and distribution and getting complementary data using an *in situ* approach by confocal immunofluorescence microscopy. Antibodies for *in situ* labeling of DNA methylation (5mdc) and histone H4 acetylation were used for immunofluorescence staining analysis, together with DAPI counterstaining, to observe alterations in chromatin masses as well as to evaluate the level of the intensity of each one in different cell cycle phases under 1g and simulated microgravity conditions (Supplemental Figures S6 and S7). Quantification of the fluorescence intensity showed an increase of the DNA methylation level in G2/M phase, compared with the G1 and S phase. Exposure to simulated microgravity reinforced the DNA methylation dynamics, since significant increases were observed in all cell cycle phases *versus* 1g control conditions (Figure 4A).

In turn, immunofluorescence image analysis for acetylated histone H4 revealed a similar but inverted effect as for methylation. Under 1g control conditions, the level of the histone acetylation fluorescence intensity was reduced in G2, compared with G1 and S phases. Under simulated microgravity, histone H4 acetylation was reduced in all phases in comparison to control, being the reduction particularly severe in G2 and G1 phases (Figure 4B).

We have also evaluated the relationship between the changes detected in chromatin remodeling markers, the chromatin structural pattern *in situ* and the overall transcriptional state of the cell nucleus. Since RNA polymerase II is responsible for the synthesis of mRNA from protein-coding genes, it has been the focus of most studies on transcription in eukaryotes (Roeder, 1996). For these reasons, we have used RNA pol II as a global marker of the transcriptional activity during cell cycle progression in our experimental conditions. Samples were analyzed by confocal immunofluorescence using anti-RNA pol II and DAPI (DNA staining) (Supplemental Figure S8). Under 1g control conditions, the quantitative level of the fluorescent signal due to RNA pol II transcription changed with

the cell cycle progression (Figure 5). There was a gradual increase in the intensity levels from the point of release of the aphidicolin treatment, through S-phase, until becoming significantly increased in G2-phase samples and then reduced again gradually until returning to original S-phase values at G1-phase samples (Figure 5C). However, the spatial arrangement of the chromatin masses and of the RNA pol II transcription signals in nuclei was similar in all the cell cycle phases (Figure 5B).

Under simulated microgravity, a remarkable difference was shown in the structural pattern of chromatin and the structural organization of transcription in the nucleus. Unlike control samples, which showed a diffuse pattern evenly distributed throughout the nucleus, the RPM samples showed a reticulated/punctuated pattern of chromatin and transcription, appearing a few small foci with more intense staining by the RNA pol II antibody (Figure 5A, B). This pattern was always associated with a lower level of transcription (Jordan, Timmis & Trewavas, 1980, Yelagandula et al., 2014); the reduction of the intensity of the extranucleolar transcription fluorescence was observed in all phases of the cell cycle and it was particularly severe in G2-phase samples under simulated microgravity compared with the control conditions (Figure 5C).

Simulated microgravity effects on the process of ribosome biogenesis using molecular markers and nucleolar structure and activity in different cell cycle subpopulations

A function closely associated to the cell cycle progression in proliferating cells is cell growth. The cell cycle checkpoint immediately preceding cell division (G2/M checkpoint) includes a strict checking of cell size, which is essential for assuring the viability of daughter cells. Therefore, cell proliferation must be closely coordinated with cell growth, such that the coupling of these two processes is called “meristematic competence” (Mizukami, 2001). In proliferating cells, cell growth is correlated with the synthesis of proteins and hence of ribosomes, the protein factories. The production of ribosomes takes place in the nucleolus, the most prominent nuclear organelle. Therefore, a fully reliable estimation of the cell growth rate in these cells is the evaluation of the status of ribosome biogenesis, in other words, of the nucleolar activity (Bernstein, Bleichert, Bean, Cross & Baserga, 2007). For the evaluation of this process, we have used a similar experimental approach as used for the biological validation of the microarray

study, by selecting relevant markers and getting data on their gene expression and on their protein levels in the different cell cycle phases.

By means of flow cytometry approach, the level of the major nucleolar protein nucleolin was increased significantly (doubled) in S-phase compared with G1, irrespective of the gravity conditions (Figure 6A). This level appeared increased even more (almost triplicated) in the G2/M phase, but this occurred only under 1g control conditions, whereas under simulated microgravity the level of the protein was similar as in S-phase (Figure 6A). In G1 and S, the impact of simulated microgravity was reduced. Nucleolin gene quantitative expression analysis showed a highly significant increase (triple amount) from G1 to S, which was sustained in G2/M phase under 1g control conditions. However, under simulated microgravity, the first increase was even higher, but the expression of the nucleolin gene dropped in G2/M until the rates of G1 (Figure 6B). The level of another relevant nucleolar protein, fibrillarin, showed a significant increase in G2/M phase compared with G1 and S phase in both, 1g control and simulated microgravity (Figure 6A). Similar to nucleolin, the G2/M phase increment in the case of the simulated microgravity was smaller than in 1g control conditions. Fibrillarin gene expression followed a similar trend as the protein level analysis. A significant increment was found in G2/M phase, compared with G1 and S-phase, under control 1g conditions but, as in the case of the nucleolin gene, there was a significant drop in G2/M, induced by simulated microgravity, compared with 1g control (Figure 6B).

Apart from the assessment of the consequences of the experimental treatment on each step of ribosome biogenesis by using relevant proteins or genes, the overall effects on the efficiency of this fundamental process can be estimated by evaluating nucleolar activity using a morpho-functional approach. The nucleolar size and the nucleolar ultrastructure are highly sensitive parameters whose change reflects with high accuracy the functional changes in the process of ribosome production (Farley, Surovtseva, Merkel & Baserga, 2015, Sáez-Vásquez & Medina, 2008). We have measured the changes in nucleolar area, in each cell cycle phase, using two different approaches: on immunofluorescent images obtained after the application of the anti-nucleolin antibody and on electron microscopical images of nucleolar ultrastructure (Figure 6C). Considering cell cycle progression, the nucleolar size remained constant through G1 and S phases (anti-nucleolin labeling) and was significantly doubled in G2/M phase in both 1g control and simulated microgravity conditions. On another hand, the increase in size observed for G2/M nucleoli in 1g control

conditions was significantly reduced under simulated microgravity, while any microgravity effect was barely observed on the nucleolar area in G1 and S phases. (Figure 6C).

DISCUSSION

Cell cycle progression rate is increased under simulated microgravity conditions

Cell cultures represent an advantageous system to investigate the cell cycle and its regulation under environmental cues, and their advantages are long known (Gould, 1984). Their capability of synchronization allows us to better understanding this process (Menges & Murray, 2002, Menges & Murray, 2006). We have used this powerful tool to investigate alterations in the regulation of the cell cycle progression caused by the simulated microgravity treatment. Specifically, we paid attention to changes in the duration of the different phases and in the expression of regulatory factors acting at the different cell cycle checkpoints. Cell synchronization was performed by arresting cells in late G1/S by a 24 h treatment with aphidicolin. Sampling at different times during 72 hours after the release of the drug allowed the identification and isolation of various cell populations enriched in particular cell cycle phases.

In these synchronized cultures, increased cell cycle progression rate under simulated microgravity conditions was confirmed in detail. The time required to reach the G2/M subpopulation peak was reduced in three hours (7 hours under simulated microgravity *versus* 10 hours under 1g control), causing a shorter cell cycle. Close examination of the gene expression data obtained for cell cycle core regulators of G2/M phase showed apparent disruptions. B-type cyclins, which are known to play a fundamental role in the G2/M checkpoint (Inzé & De Veylder, 2006, Menges & Murray, 2002, Vandepoele et al., 2002), showed a general downregulation, while the alterations in cyclins of the A and D types was not certain. Cyclins A/B/D, associated with both CDKA and CDKB, are part of the G2/M transition regulatory mechanism (Inzé & De Veylder, 2006). Downregulation of elements of the CDK-activating kinase pathway produces effects in controlling the activity of the distinct CDK/CYC complexes (Shimotohno, Umeda-Hara, Bisova, Uchimiya & Umeda, 2004, Umeda, Shimotohno & Yamaguchi, 2005, Umeda, Umeda-Hara & Uchimiya, 2000). Down-regulation of the WEE1 kinase, which is

putatively involved in the inhibitory phosphorylation of CDKs (Sorrell et al., 2002, Vandepoele et al., 2002), promotes the acceleration of the G2/M transition. Alternatively, the overexpression of WEE1 genes causes cell cycle arrest (Sorrell et al., 2002, Sun et al., 1999). Although cyclin B expression decrease is usually associated with a lower proliferation rate, our results showing enhanced proliferation rate with reduced levels of cyclin B can be interpreted by assuming that a shorter G2/M period will lead to less accumulation of the cyclin B messenger. Together with WEE1 expression, these data are supporting an early entry into M phase.

On the other hand, an opposite alteration was observed in the G1 regulatory mechanism, leading to a delay of one hour compared with the control, eventually producing a shortening of two hours in the entire duration of the cell cycle caused by simulated microgravity conditions (T14 *versus* T16 for G1 cells), according to flow cytometry data. Global transcriptomic analysis of the cell cycle regulators in G1/S transition reflects a general upregulation of the D-type cyclins (CYCD) and of the CDKA/CYCD complex through the G1/S transition (Inzé & De Veylder, 2006, Menges & Murray, 2002, Vandepoele et al., 2002), in contrast with the downregulation observed in the G2/M subpopulation. The activation of the CDKA/CYCD complex requires the phosphorylation of CYCD (Inzé & De Veylder, 2006). All factors participating in this mechanism appear upregulated in simulated microgravity, supporting the change in the G1/S transition checkpoint. This interpretation of the activation of the G1/S transition is also supported by the upregulation of the E2F/DEL families, which are associated with DNA replication at the S-phase (del Pozo, Boniotti & Gutierrez, 2002, Menges, De Jager, Grissem & Murray, 2005). However, KRPs which inhibit the activated CDK/CYCD complexes under stress (De Veylder et al., 2001, Verkest et al., 2005a, Verkest, Weinl, Inze, De Veylder & Schnittger, 2005b, Zhou, Wang, Gilmer, Whitwill & Fowke, 2003), do not appear altered under simulated microgravity, in contrast with observations reported for other types of abiotic stresses such as ABA and cold (Wang, Fowke & Crosby, 1997).

A schematic representation of the primary data collected at the level of gene expression during the two cell cycle checkpoints, summarizing the general downregulation of regulatory factors in G2/M and the general upregulation of regulatory factors in G1/S is shown in Figure 7. These changes in gene expression coincide with an acceleration of the cell cycle in conditions of simulated microgravity, which is the result of a stronger acceleration in G2 and a slighter deceleration in G1.

Under simulated microgravity, changes in chromatin remodeling factors are detected through cell cycle progression

Chromatin changes drive cell cycle transitions, as it has been shown in plant (Costas et al., 2011, Sanchez et al., 2008) and animal systems (Liu et al., 2010, Probst, Dunleavy & Almouzni, 2009). We have investigated the chromatin patterns through the cell cycle by pooling together the data from the immunofluorescence studies on DNA methylation (5mdc), Histone H4 acetylation (AcH4), chromatin staining (DAPI) and the RNA polymerase II staining, in addition to gene expression and protein levels. Under control 1g conditions, chromatin becomes locally decondensed in S phase, highly condensed during the G2/M phase, and again decondensed before reentry into G1, as expected for the requirements of cell cycle phases. Chromatin condensation during G2/M is temporally associated with a low level of histone H4 acetylation, whereas the increments in the acetylation in G1 and G1/S transition correlate to chromatin decondensation (Costas et al., 2011, Xu, Bai, Duan, Costa & Dai, 2009). Otherwise, heavily methylated regions of DNA are associated with chromatin condensation. This hypermethylation was noticed in G2/M and S phases, with a reduction in G1. Consistent with this, the MET1 expression is upregulated at G1/S transition (Menges, Hennig, Gruissem & Murray, 2003). In an interestingly coordinated process, proteins that bind methylated DNA also form complexes with proteins involved in deacetylation of histones (Aufsatz, Stoiber, Rakic & Naumann, 2007, Mutskov, Farrell, Wade, Wolffe & Felsenfeld, 2002, Vaissière, Sawan & Herceg, 2008).

Under simulated microgravity, chromatin organization was severely affected by the cell cycle phases, increasing its condensation (Figure 8). Immunofluorescence analysis, used to visualize and quantify the distribution pattern of extranucleolar transcription by RNA pol II, showed an overall inhibition of transcription. This inhibition is compatible with a change in the nuclear chromatin pattern, detected by chromatin staining using DAPI fluorescence, involving an increment of chromatin condensation. Thus, our results clearly support the association of chromatin remodeling with variations in transcriptional activity (Fedorova & Zink, 2008, Wang, Maharana, Wang & Shivashankar, 2014). This chromatin remodeling, leading to condensation under microgravity conditions, is consistent with the observed modifications in specific factors, namely an increase of DNA methylation and histone deacetylation. In fact, MET1 expression is upregulated through the cell cycle progression, accompanied by downregulation of the histone H4 acetylation.

The results of the genome-wide microarray experiment indicate that MET1, as well as the DNA methylation-related CMT3 genes, are upregulated in both G1 and G2/M, in conditions of simulated microgravity.

Whereas our results have shown the existence of changes in chromatin remodeling factors, in the state of condensation of chromatin and in the overall status of nuclear transcription, the identity of genes affected in their transcription by these mechanisms has not been established. In fact, the existence of these alterations cannot be interpreted as an evidence of causal relationships of them with transcriptional regulation, in the debate currently open on this matter (Asensi-Fabado et al., 2017). The discrimination of this causal relationship between gravitational alteration, chromatin remodeling and transcriptional response is one of the main pending questions for understanding the mechanism of adaptation of plants to the microgravity environment. As proposed for other abiotic stresses (Asensi-Fabado et al., 2017), a combination of the most recent molecular biology approaches with more traditional biochemical and cell biological techniques will allow the mechanistic characterization of the process.

Simulated microgravity conditions reduce cell growth: Revisiting the relations between nucleolus morphology, ribosome biogenesis and cell growth under gravitational alterations

In highly proliferating cells, such as those of an *in vitro* suspension culture or meristematic tissues of plants, cell growth is strictly correlated to the production of proteins in order to overcome a biomass threshold compatible with cell division (Doerner, 2008, Mizukami, 2001). Consequently, cell growth is largely determined by the activity of biogenesis of ribosomes, the protein factories, which occurs in a nuclear domain, the nucleolus (Baserga, 2007, Bernstein & Baserga, 2004, Bernstein et al., 2007). The structural features of the nucleolus are a reliable marker of the rate of ribosome production, and they conspicuously change throughout the cell cycle as a reflect of functional changes (Sáez-Vásquez & Medina, 2008).

When using synchronic cultures and phase-specific cell cycle subpopulations, the characterization of the cell cycle phases in 1g control conditions confirmed that G2 is the most active phase of ribosome production, and consequently of cell growth. Our results have shown that simulated microgravity produces a substantial effect on the nucleolar structure and activity during the G2 phase. Morphological and morphometrical features

of the nucleolus indicate a depletion of nucleolar activity, which means a reduction of the rate of ribosome biogenesis (Ginisty, Sicard, Roger & Bouvet, 1999, González-Camacho & Medina, 2006, Pontvianne et al., 2007). The evidence of this reduction was also supported by the data obtained in G2/M phase from two essential nucleolar proteins, namely nucleolin and fibrillarin, which appeared downregulated in their gene expression and showed lower protein amounts in simulated microgravity. On the contrary, neither S phase nor G1 phase subpopulations showed clear variations in these parameters of ribosome biogenesis, even though quantification of the different nucleolar morpho-functional models detected in these phases an increase in the number of inactive nucleoli under simulated microgravity.

The fast and detectable response of the nucleolus to changes in cell conditions of growth is related to the key role played by the nucleolus in the functional activities of the eucaryotic cell (Raska, Shaw & Cmarko, 2006, Srivastava & Pollard, 1999). In fact, the nucleolus and nucleolar activity have been identified as efficient and reliable indicators of cellular stress (Boulon, Westman, Hutten, Boisvert & Lamond, 2010, Mayer & Grummt, 2005). It is suggestive that the various effects on ribosome production and cell growth induced by the different types of cellular stresses, such as microgravity conditions, are often accompanied by dramatic changes in the organization and composition of the nucleolus.

Concluding remarks

The cellular mechanisms that have been revealed in this work to be altered under simulated microgravity in cell cultures may give account of most of the disturbances producing an anomalous early development in *Arabidopsis* plants in spaceflight conditions due to the loss of meristematic competence. In the synchronized culture, cell growth and cell proliferation were uncoupled after simulated microgravity treatment to a similar extent as previously described in meristematic cells from seedlings grown in space (real microgravity) or under microgravity simulation. Moreover, the advantages of this biological model have allowed us to get a detailed knowledge of the genes and proteins involved as well as of side effects of the alteration, e.g. at the level of chromatin remodeling. An additional consequence revealed in this work is that the loss of the cell cycle inhibition in the G2/M phase should be connected with the existence of mechanosensors in individual cells, independent from the mechanisms of gravity

643 perception acting on plants, which involve specialized cells. Finally, the existence of
644 severe effects of microgravity on a crucial cellular process for establishing the plant
645 developmental patterns implies the need to counteract them, as a prerequisite for the
646 objective of successful culture of plants in space, in the context of space exploration.

ACKNOWLEDGEMENTS

The skillful technical assistance of Mrs. Mercedes Carnota (CIB-CSIC) with different lab procedures is gratefully acknowledged, as well as the Technical Services of Genomics, Flow Cytometry and Confocal Microscopy of CIB-CSIC for the assistance with the procedures and analyses of their respective specialities. We also thank Mr. Alan Dowson for his support at the TEC-MMG LIS lab (ESA-ESTEC), and Dr. Crisanto Gutierrez, at CBM (UAM-CSIC) and Dr. Julio Sáez-Vásquez (CNRS-University of Perpignan-Via Domitia, France) for the generous supply of MM2d cultures and anti-nucleolin antibody, respectively. This work was supported by the Spanish “Plan Estatal de Investigación Científica y Técnica y de Innovación” of the Ministry of Economy, Industry and Competitiveness [Grant numbers AYA2012-33982 and ESP2015-64323-R, co-funded by ERDF], by a predoctoral fellowship to [Kh.Y.K.] from CSIC, Spain [JAE-PreDoc Program, Ref. JAEPRe_2010_01894] the ESA-ELIPS Program [ESA SEGMGSPE_Ph1 Project, contract number 4200022650], and ESA support via contract TEC-MMG / 2012/263. This work is part of a PhD Thesis dissertation presented by Kh.Y.K. at the Complutense University of Madrid.

FIGURE LEGENDS

Figure 1. Synchronic progression of the first cell cycle after aphidicolin release in the *Arabidopsis* cell line MM2d. A) 1g control. B) Sim μ g. Distribution of cell cycle phases by flow cytometry analysis using 10000 cells regarding the DNA content detected by DAPI ($n = 10000$). The first peak ($2n$) corresponds to G1 phase and the second peak ($4n$) corresponds to G2/M phase. Each colored block represents a sample taken at a certain time after aphidicolin release, Red color = T0, blue color = T2, pink color = T7, lime color = T10, cyan color = T14, orange color = T16.

Figure 2. Flow cytometry-based quantification of the levels of relevant *Arabidopsis* proteins, used as markers of cell cycle regulation (Cyclin B1 and Prolifera) and indicators of chromatin modifications (DNA methylation and Histone-4 acetylation). Data on the protein levels are shown for the cell cycle phases, in 1g control and simulated microgravity (Sim μ g RPM) conditions. Fluorescence intensity using flow cytometry per each cell was quantified on 10000 cells labeled with each specific antibody. Data shown mean $\pm$ SE of the three replicates ($n = 10000$, $r = 3$). P-Value <0.05 is shown for significant differences calculated within cell cycle phases (without changing g level, *) or simulated microgravity *versus* 1g control (without changing cell cycle phase, #).

Figure 3. Quantitative PCR gene expression in *Arabidopsis* cells of the genes coding for the selected relevant proteins, markers of cellular functions, previously analyzed at the protein level. Genes coding for cell cycle regulators (Cyclin B1 [CycB1;1] and Prolifera [PRL]) and chromatin remodeling factors (DNA methylation [MET1]) were measured through the cell cycle phases, on synchronic cell subpopulations, in 1g control and simulated microgravity (Sim μ g RPM) conditions. Relative gene transcription was evaluated using qPCR analysis. Data shown mean $\pm$ SE of the three replicates ($r = 3$). P-Value <0.05 is shown for significant differences calculated within cell cycle phases (without changing g level, *) or simulated microgravity *versus* 1g control (without changing cell cycle phase, #).

Figure 4. Confocal microscopy-based quantification of chromatin modifications (DNA methylation (5mdc) and Histone H4 Acetylation (AcH4) fluorescence intensity levels) through *Arabidopsis* synchronic cell cycle phases under 1g control and simulated microgravity (Sim μ g). Quantitative fluorescence intensity level was

determined using Leica confocal software in each cell to detect the green signal intensity. An average of 50 cells were measured using only one replicate. Data shown as mean $\pm$ SE (n = 50, r = 1). P-Value <0.05 is shown for significant differences calculated within cell cycle phases (without changing g level, *) or simulated microgravity versus 1g control (without changing cell cycle phase, #).

Figure 5. pre-mRNA (extranucleolar) transcription. Immuno-staining by anti-RNA polymerase II through *Arabidopsis* cell cycle progression under simulated microgravity exposure in the reference cell cycle time course after synchronization.

A) Comparison of the structural patterns of chromatin masses and of pre-mRNA transcription under 1g control conditions and simulated microgravity. The structural patterns show remarkable differences, appearing diffuse and evenly distributed in the control samples and reticulated/punctuated in the RPM samples, showing a few small foci with brighter staining. This is associated to a lower level of transcription. The figure shows the chromatin structure pattern detected by DAPI staining (blue), the transcriptional pattern estimated by anti-RNA pol II immuno-staining of nuclei (green), and confocal merge of fluorescence images. **B)** Pre-mRNA transcriptional levels estimated by anti-RNA pol II immuno-staining of nuclei through different cell cycle phases. Bar indicates 10 μ m. **C)** Quantitative fluorescence intensity level was determined using Leica confocal software in each individual cell to detect the green signal intensity. An average of 100 cells was measured in three different biological replicates. Data shown mean $\pm$ SE (n = 100, r = 3). P-Value < 0.05 is shown for significant differences calculated between simulated microgravity versus 1g control (without changing cell cycle phase, *).

Figure 6. Effect of simulated microgravity on ribosome biogenesis throughout the cell cycle.

A) Flow cytometry-based quantification of the levels of relevant *Arabidopsis* proteins, used as markers of ribosome biogenesis (Nucleolin and Fibrillarin). **B)** Relative gene expression (qPCR) in *Arabidopsis* cells of the genes coding for these selected proteins (AtNucL1 and AtFib1). **C)** Quantification of the nucleolar area measured *in situ* in the different cell cycle phases after anti-nucleolin immunofluorescence (left graph), and on electron microscopical images (right graph). All data are shown for the cell cycle phases, in 1g control and simulated microgravity (Sim μ g RPM) conditions. Data on the Nucleolar area were measured using 50 cells in three different replicates. P-Value <0.05 using three replicates (n= 50, r= 3) is shown for significant differences calculated within

cell cycle phases (without changing g level, *) or simulated microgravity *versus* 1g control (without changing cell cycle phase, #).

Figure 7. Current working model of the Arabidopsis cell cycle regulation under the simulated microgravity conditions. Based on analysis of marker genes we propose that genes participating in the regulation of both the G2/M and the G1/S transitions in the cell cycle appear altered in their expression under microgravity conditions. Most of the genes expressed at the G2/M checkpoint would appear downregulated. During the G2 phase of the cell cycle, cyclins of the A, B, and D types (CYCA, CYCB, and CYCD), which associate with CDKs of the A and B types (CDKA and CDKB), would be downregulated. The whole CDK-activating kinase pathway would be negatively regulated, disrupting the activity of the distinct CDK/CYC complexes. CDK activity can be negatively regulated by the WEE1 kinase, which would be triggered upon loss of DNA integrity, and also would appear downregulated in our experimental conditions. On the contrary, positive regulation was found for most of the G1/S transition regulators, such as D type cyclins (CYCD), which associate with A-type CDK (CDKA) forming an inactive CDKA/CYCD complex. This complex is activated through phosphorylation by the CDK-activating pathway, which involves CDKF and CDKD associated with CYCH. KRPs, which can inhibit the activated CDK/CYD complexes, would appear slightly downregulated. Green color reflects the downregulation change, while the red color reflects upregulated gene expression. These changes in gene expression are coincident with the acceleration of the G2 period (green arrows) and the (less intense) deceleration of the G1 period (red arrows), according to flow cytometry data. The overall result is a shortened cell cycle under simulated microgravity, compared with the 1g control.

Figure 8. Schematic model for the mechanism of chromatin condensation and decondensation under simulated microgravity. Models for condensation and decondensation of chromatin, a determinant of transcriptional activity, involves enzymes for histone acetylation (HAT) and histone deacetylases (HDAC), DNA methylation, and RNA polymerase activity. Under simulated microgravity, DNA is hypermethylated and histone deacetylated in the presence of the low activity of the transcription through RNA polymerase II activity and altered with the nucleolin depletion lead to the chromatin condensation. This chromatin remodeling is reversible under changing environmental conditions.

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A) Ig Control

B) Sim μ g RPM

Figure 1

Figure 2

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Figure 5

Figure 6

Figure 7

Figure 8

Supplemental Table S1. List of specific antibodies used in the immunofluorescence-based analyses. Dilutions used (in blocking solution) are indicated. Dilution is the same for all primary antibodies (1:1000), except 5mdc (1:25) for DNA methylation and AcH4 (1:50) for histone acetylation. Thus, the number of washes after incubation was increased to 5 times for these two antibodies, compared with 3 times for the rest of the antibodies. For secondary antibodies, the dilution was 1:100 in all cases. The source of antibodies (either commercial or supplied by a colleague) is also indicated.

Protein of interest	Specific antibodies	
	Primary (1:1000)	Secondary (1:100)
Nucleolin L1	Rabbit polyclonal Ani-AtNUC-L1 (1:1000) Provided by Dr. Julio Sáez Vásquez	Anti-Rabbit (Alexa 488-Green)
Fibrillarin	Mouse monoclonal Anti-Fibrillarin (1:1000) Abcam, ab4566, Cambridge, UK	Anti-Mouse (Alexa 488-Green) (Alexa 568-Red)
Cyclin B1	Goat polyclonal anti B-like cyclin (1:1000) Santa cruz, sc-12859, Texas, USA	Anti-Goat (Alexa 488-Green)
Prolifera	Goat polyclonal anti Prolifera (1:1000) Santa cruz, sc-12853, Texas, USA	Anti-Goat (Alexa 488-Green)
RNA polymerase II	Rabbit monoclonal anti RNA polymerase II (1:1000) Abcam, ab5408, Cambridge, UK	Anti-Mouse (Alexa 488-Green)
DNA methylation	Mouse Monoclonal Anti 5-Methylcytidine (1:25) Eurogentec , BI-MECY-0500, Belgium	Anti-Mouse (Alexa 488-Green)
Histone Acetylation	Rabbit Polyclonal Anti acetyl-Histone H4 (1:50) Milipore, Cat.# 06-866, Temecula, USA	Anti-Mouse (Alexa 488-Green)

Supplemental Table S2. Sequence of the different primers (5'-3') used to follow the expression of key genes used as markers of cell functions.

Functions	Genes	Primer (Forward)	Primer (Reverse)
Ribosome biogenesis	<i>AtNUC-L1</i> (At1g48920)	ATGGGAAAGTCTAAATCCGC	TCCACGACCACGATCACTT
	<i>AtFIB1</i> (At5g52470)	CGTCTTTCGTTCTTCACTTTTAGACAA G	GCCCACTACGGCCTCTGTCA
Cell cycle	<i>CYCB1;1</i> (At4g37490)	CAGCAATGGAAGCAACAAGA	ATGVAGTGTTTGGGATTGAA
	<i>ProliferaPRL</i> (At4g02060)	TGGGTGGAAGAGGAAAATTG	CTGGCTCCTTCATCCTTCAG
Epigenetics	<i>MET1</i> (At5g49160)	GCTTAATCCAGCCCAGCATA	CACCTTTACCAGCAGCCTTC
Reference	<i>Actin</i> (At3g18780)	GCACCCTGTTCTTCTTACCG	ATCCAGCACAAATACCGGTTGTA

Supplemental Figure S1. Cell cycle progression for 72h after aphidicolin block/release of *Arabidopsis* cell line MM2d immobilized in 1g control condition. (A, B): Distribution of cell cycle phases by flow cytometry analysis. The DNA content (DAPI staining) is represented *versus* the relative number of cells. Samples were taken at different times after the release of aphidicolin block in late G1/early S phase (T0). Each box represents a sample taken at a certain time from t=0 to t=72 hours. Sampling was performed every 1, 2, and 4 hours in the first, second, and the third day respectively. (C): Histogram of flow cytometry analysis, showing the distribution of cells according to their DNA content, corresponding to cell cycle phases: G1 (Black), S-phase (White) and G2/M (Brown). Under 1g control conditions, the different cell cycle subpopulation peaks at T2, T10, and T16 so they were referenced to S, G2/M, and G1 phases, in which more than two thirds of the cells were synchronized.

Supplemental Figure S2. Cell cycle progression for 72h after aphidicolin block/release of *Arabidopsis* cell line MM2d immobilized in simulated microgravity (RPM) condition. (A, B): Distribution of cell cycle phases by flow cytometry analysis. The DNA content (DAPI staining) is represented *versus* the relative number of cells. Samples were taken at different times after the release of aphidicolin block in late G1/early S phase (T0). Each box represents a sample taken at a certain time from t=0 to t=72 hours. Sampling was performed every 1, 2, and 4 hours in the first, second, and the third day respectively. (C): Histogram of flow cytometry analysis, showing the distribution of cells according to their DNA content, corresponding to cell cycle phases: G1 (Black), S-phase (White) and G2/M (Brown). Under simulated microgravity conditions, the different cell cycle subpopulation peaks at T2, T7, and T14, so they were referenced to S, G2/M and G1 phases, in which more than two thirds of the cells were synchronized.

Supplemental Figure S3. Cell cycle regulators (Cyclin B1 and Prolifera) used as cell cycle phase markers to verify cell cycle subpopulation enrichment. Cells from the six reference sampling times exposed to different gravity levels (T0, T2 as common points and the 4 specific G2/M and G1 points from Sup. Fig. S1 and S2) were compared. Protein expression level was evaluated by flow cytometry by quantifying fluorescence intensity in 10000 cells using specific antibodies against Cyclin B1 or Prolifera revealed by immunofluorescence. Cyclin B1 is known to play a role in the control of G2/M transition (Mironov et al., 1999; Breyne and Zabeau, 2001; Potuschak and Doerner, 2001), and “Prolifera” (Mcm7) protein is known to accumulate in the nucleus of recently divided cells during G1 phase (Springer et al., 2000). This check was necessary due to the partial (2/3) synchronization level. Frozen aliquots of the same samples were used to validate the cell cycle reference samples. Cyclin B1 (fluorescence intensity), used as G2/M Marker, reached the maximum values at T10 and T7 under 1g control and simulated microgravity respectively, confirming subpopulation assignments for G2/M (T10 and T7 reference samples under 1g control and simulated microgravity, respectively). Prolifera level (fluorescence intensity), used as G1 marker, peaks at T16 and T14, under 1g control and simulated microgravity respectively, correlating with the G1 phase subpopulations as expected (T16 and T14 reference samples under 1g control and simulated microgravity, respectively).

Supplemental Figure S4. Microarray-based transcriptomic analysis of the expression of core cell cycle regulators in *Arabidopsis* synchronous cultures under simulated microgravity versus 1g control. Cell subpopulations corresponding to G2/M and G1 phases were analyzed. Heat maps generated using R software show the differential expression of gene families: **A)** CDK and CKL kinases gene families **B)** Cyclin (A, B, C, D) and CYL. **C)** Other core cell cycle regulators. Colors represent gene expression change in $\log_2\text{ratio}$ scale, as indicated at the bottom of the figure (black color indicates low-statistical confidence variations with a FDR $p > 0.05$). CDK genes were unevenly affected by altered gravity in the different cell cycle phases; CDKB2;1 and CDKB2;2 were downregulated in G2/M and upregulated in G1, while CDKE;1 showed the opposite trend and CDKD;1 was downregulated in all the conditions under simulated microgravity. On the other hand, CKL genes were upregulated in all the conditions under simulated microgravity, but CKL3 was downregulated in G2/M and CKL10 in G1. Moreover, the expression of the CYCA family was altered by the simulated microgravity and upregulated in all the experimental conditions, except a high down-regulation of CYCA;1;1 in G2/M. In turn, CYCBs were altered by the simulated microgravity and the cell cycle progression. General downregulation of CYCB in the synchronous G2/M and upregulation in G1 was observed. Induction of WEE1 was noticed in G1, while it was slightly downregulated in G2/M. In G1, KRP CDK inhibitors showed downregulation, while CKS family (CKS1-2) and E2F/DEL families showed upregulation.

Supplemental Figure S5. Microarray-based transcriptomic analysis of the expression of genes producing changes in DNA and histones in *Arabidopsis* synchronous cultures under simulated microgravity versus 1g control. Cell subpopulations corresponding to G2/M and G1 phases were analyzed. Heat maps generated using R software show the differential expression of gene families: Chromatin remodeling/DNA methylation (MET1 methyltransferase, CMT3 chromomethylase and Deficient in DNA Methylation 1 DDM1), histone kinases (AUR1-3, Aurora kinases 1-3), histone methylation (SUVH1-3) and histone acetylation (HAG1-3, histone acetylases 1-3 of the GNAT family) and deacetylation (HDT1-4, histone deacetylases 1-4 of the HD2 family; HDA18-19, histone deacetylases of the RPD3/HDA1 family). The pattern of H4 expression has been included as a reference for a well-characterized gene that is upregulated at the G2/M transition under simulated microgravity. Colors represent gene expression change in $\log_2\text{ratio}$ scale, as indicated at the bottom of the figure (black color indicates low-statistical confidence variations with a FDR $p > 0.05$). The DNA methylation gene MET1 was significantly upregulated in both G2/M and G1 phases, while CMT3 appeared less affected (Figure 3). Histone kinase (AUR1-3 kinase family) activity was clearly upregulated in G1 but repressed in G2/M, while the activity of histone methylases was generally repressed (SUVH1-3). These changes could correlate with the activity of histone acetylases (slightly upregulated) and deacetylases (mostly downregulated in G2/M) despite the variations in these gene families were in general small.

Supplemental Figure S6. DNA methylation (5mdc) immunofluorescence patterns through *Arabidopsis* synchronic cell cycle phases under 1g control and simulated microgravity (μg RPM). Confocal images showing immunolocalization of 5mdc (green), the chromatin pattern (DAPI) and the merged images. Original magnification was 63x (bars indicate 10 μm).

Supplemental Figure S7. Histone H4 Acetylation (AcH4) immunofluorescence patterns through *Arabidopsis* synchronic cell cycle phases under 1g control and simulated microgravity (μg RPM). Confocal images showing immunolocalization of AcH4 (green), the chromatin pattern (DAPI) and the merged images. Original magnification was 10x (bars indicate 50 μm).

Supplemental Figure S8. pre-mRNA (extranucleolar) transcription. Immunostaining by anti-RNA pol II through *Arabidopsis* synchronic cell cycle phases under 1g control and simulated microgravity (μ g RPM). **A) Chromatin structure pattern, detected by DAPI staining (blue). **B**) Transcriptional levels estimated by anti-RNA polymerase II immuno- staining of nuclei (green). **C**) Merge of confocal immunofluorescence images.**

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