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

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ABSTRACT

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 $g = 9.8\ m\ s^{-2}$; 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.

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1. 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 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). 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).

2. Material and methods

2.1. 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.

2.2. 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 $110\,\mu mol\,m^{-2}\,s^{-1}$ photon flux intensity delivered by Biolux tubes