



## Cell proliferation and plant development under novel altered gravity environments

Journal:	<i>Plant Biology</i>
Manuscript ID:	PlaBio-2013-05-0180-REV
Manuscript Type:	Review Article
Date Submitted by the Author:	09-May-2013
Complete List of Authors:	Herranz, Raúl; Centro de Investigaciones Biológicas (CSIC), F. Javier, Medina; CSIC,
Keyword:	gravitropism, graviresistance, cell proliferation, cell cycle, gene expression, root meristem, cell cultures in vitro

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Review

**Article type:** Review

**Running head:** Cell proliferation and plant development under altered gravity

## **Cell proliferation and plant development under novel altered gravity environments**

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**Keywords:** cell cultures *in vitro*; cell cycle; cell proliferation; gene expression; gravitropism; graviresistance; root meristem

**Accepted:** 2 August 2013

## ABSTRACT

Gravity is a key factor for life on Earth. It is the only environmental factor that has remained constant throughout evolution, and plants use it to modulate important physiological activities; gravity removal or alteration produces substantial changes in essential functions. For root gravitropism, gravity is sensed in specialised cells, which are capable of detecting magnitudes of the  $g$  vector lower than  $10^{-3}$ . Then, the mechanosignal is transduced to upper zones of the root, resulting in changes in the lateral distribution of auxin and in the rate of auxin polar transport. Gravity alteration has consequences for cell growth and proliferation rates in root meristems, which are the basis of the developmental programme of a plant, in which regulation *via* auxin is involved. The effect is disruption of meristematic competence, *i.e.* the strict coordination between cell proliferation and growth, which characterises meristematic cells. This effect can be related to changes in the transport and distribution of auxin throughout the root. However, similar effects of gravity alteration have been found in plant cell cultures *in vitro*, in which neither specialised structures for gravity sensing and signal transduction, nor apparent gravitropism have been described. We postulate that gravity resistance, a general mechanism of cellular origin for developing rigid structures in plants capable of resisting the gravity force, could also be responsible for the changes in cell growth and proliferation parameters detected in non-specialised cells. The mechanisms of gravitropism and graviresistance are complementary, the first being mostly sensitive to the direction of the gravity vector, and the second to its magnitude. At a global molecular level, the consequence of gravity alteration is that the genome should be finely tuned to counteract a type of stress that plants have never encountered before throughout evolution. Multigene families and redundant genes present an advantage in that they can experience changes without the risk of being deleterious and, for this reason, they should play a key role in the response to gravitational stress.

## THE ROLE OF GRAVITY IN PLANT EVOLUTION AND PHYSIOLOGY

Living systems have been evolving on Earth for over a billion years in the presence of a wide range of environmental conditions, and some of their individuals or groups have been able to adapt to extreme environments, including suboptimal temperatures (from polar plants to hyperthermophilic bacteria), hydration/salinity, illumination or nutrient availability. Consequently, organisms have evolved collections of genes and stress pathways able to control the early response, *i.e.* acclimation and adaptation to a wide range of environmental conditions, from suboptimal to adverse.

In contrast, one parameter has remained constant on Earth since life appeared on the surface of our planet, and this parameter is gravity. Certainly, the perception of gravity is not the same depending on the medium in which an organism is placed, *e.g.* it is not the same for an aquatic organism as for a terrestrial organism. With regard to the influence of gravity on biological evolution, a crucial step was the transition from living in the sea milieu to the aerial life, above the Earth's surface. This step comprised the need to develop specific mechanisms of response to resist the new magnitudes of the gravity vector, without the compensation of Archimedes forces. In the case of plants, this step in evolution was decisive to allow the appearance of vascular tissues, and of seeds and flowers as reproductive organs. However, once this challenge was overcome, gravity has become a constant environmental factor, with regard to the direction and magnitude of the vector, which has permanently affected terrestrial living systems and, particularly, plants. Consequently, terrestrial organisms have not experienced the need to produce specific mechanisms to respond or adapt to an ambient altered gravity. Actually, the strategy followed by plants with respect to this environmental factor has consisted of using it to modulate important physiological activities, such as nutrition and growth. In fact, plants have acquired specific organs, tissues and molecular systems capable of detecting the gravity vector, with the objective of orienting their growth according to it (gravitropism), with the final purpose of obtaining valuable or useful environmental resources. For example, plants use root gravitropism to facilitate access to soil nutrients, and shoot gravitropism to place leaves in the most favourable position to capture sunlight for efficient performance of photosynthesis.

## GRAVITY ALTERATION INFLUENCES PLANT RESPONSES TO BIOTIC AND ABIOTIC STRESSES

A consequence of the constant and permanent presence of the gravity vector as a component of the Earth environment is that all biological functions and mechanisms of terrestrial organisms have been developed under its influence, and they proceed taking into account the presence of this mechanical force. Obviously, this includes the strategies and mechanisms of perception, response and adaptation to a wide range of biotic and abiotic environmental stresses in plants. Therefore, loss or alteration of the gravitational cue is not only a stressful event by itself, but will also alter the way in which organisms detect and respond to other environmental factors. Under suboptimal environmental conditions produced by biotic and/or abiotic factors, the result of gravity alteration is a synergistic effect that promotes a complex environmental stress response, combining both abiotic and biotic stress elements (Beckingham 2010; Herranz *et al.* 2010). On the one hand, even the environmental factor itself can be modified through the alteration or loss of gravity (alteration in the distribution, availability or concentration of nutrients in the atmosphere or in the soil); on the other hand, environmental data can be detected differentially by the plant receptors. A nice example of the influence of altered gravity on the perception of other environmental factors is the phototropic response to certain red light wavelengths shown by *Arabidopsis* hypocotyls under microgravity conditions (Millar *et al.* 2010). Interestingly, ancient plant lineages (moss and ferns) show this red light phototropism on Earth, but flowering plants have lost this feature during evolution (obviously, under 1g gravity). It is only removal of the gravity factor that unmasks the capacity for directional red light sensing for phototropism in higher plants.

In addition, both space flight and ground-based simulations of microgravity conditions impose a number of constraints, some of them of a technical character, but more importantly, some others affecting environmental factors. Actually, it is practically impossible to find a scenario of 'pure' gravitational alteration, either in space or in ground simulation conditions. Real weightlessness, as it occurs in space flight, is intimately associated with radiation, confinement and other factors present in the cabin of the spaceship, which are quite different from the typical Earth environment; furthermore, the preparation of material for space flight and its preservation until recovery often require exposure to suboptimal conditions. On the other hand, ground-based alteration of gravity (or

of gravity perception by the experimental subject) is only made possible by certain mechanisms that involve physical factors, such as shearing forces, centrifugation, magnetic fields and others, which are inseparable from the gravity alteration. The consequence is a serious impairment to the advancement of gravitational and space biology, which proceeds at a pace slower than ideal.

### **GRAVITY ALTERATION PRODUCES CHANGES IN ROOT MERISTEMATIC CELL GROWTH AND PROLIFERATION THAT CAN BE RELATED TO GRAVITROPISM**

It was established long time ago in the ‘starch–statolith theory’ that gravity is sensed in plant roots by means of the movement of specialised amyloplasts called statoliths, which are present in certain cells of the columella (a region of the root tip) called statocytes (Perbal 1974). This is corroborated by results showing defective gravitropism in mutant plants lacking starch or having low levels of it (Kiss *et al.* 1998).

Statolith movement is driven by the direction of the gravity vector. Under normal gravity conditions (1g), statoliths sediment in the bottom of the statocytes, indicating the direction of root growth. If the direction of the root axis changes with respect to the gravity vector, statoliths move accordingly and the result is bending of the root to adopt the new direction of the gravity vector. In conditions of absence of gravity, statoliths move towards central regions of the cell (Perbal *et al.* 1987). In order to estimate the minimum threshold values capable of triggering the mechanisms of graviperception in the root, two parameters have been defined, namely the threshold acceleration, which is the minimum value of  $g$  capable of inducing a gravitropic response, and the threshold presentation dose, defined as the minimum product of gravity acceleration  $g$  by time in seconds that stimulates the graviperception mechanisms and induces a response (Perbal *et al.* 2002; Driss-Ecole *et al.* 2008). It has been demonstrated that the quantitative gravitropic response, estimated as the angle of curvature of the root, varies as a hyperbolic function of the dose of stimulation (Perbal *et al.* 2002). In general, it has been demonstrated that roots grown in microgravity before stimulation are more sensitive than roots grown in 1g. In an experiment carried out in the International Space Station (ISS), comprising seedling growth in microgravity followed by stimulation in a centrifuge, it was shown that the threshold acceleration was between 0 and  $2.0 \times 10^{-3}g$ , although it was estimated at  $1.4 \times 10^{-5}g$ , whereas the threshold presentation dose was  $0.67 g \times s$  (Driss-Ecole *et al.* 2008).

The physical information derived from statolith sedimentation should be converted into a chemical signal, capable of being transduced to the site where the gravity response is expressed (Fig. 1). The mechanism of this process of conversion and transduction is not totally elucidated at this moment. The actin cytoskeleton was proposed to be a major regulator of plant gravitropism (Volkman *et al.* 1999; Kiss 2000); however, more recent reports showing that actin inhibitors enhance the gravitropic response have introduced controversy into such statements. Genetic and cell biological studies indicate that the protein degradation machinery is probably involved in the regulation of statolith–actin interactions, and that a ligand–receptor mechanism, similar to that described for the unicellular green alga *Chara* (Limbach *et al.* 2005), might operate in the gravity signal transduction mechanism of higher plants, regulated by actin (Blancaflor 2013).

Furthermore, mechanosensitive ion channels in the plasma membrane or endoplasmic reticulum (ER) were also proposed to contribute to sensing and transduction of the gravity signal. Sedimenting amyloplasts in columella cells could distort membranes (with or without the participation of actin microfilaments) and open these channels, resulting in a change in the concentration of ions, especially  $\text{Ca}^{2+}$ , in that region of the cell (Blancaflor & Masson 2003). The existence of interactions between statoliths and ER, producing membrane distortions capable of opening ion channels, has been experimentally visualised (Leitz *et al.* 2009). Nevertheless, recent experiments, involving treatments with channel inhibitors, attempting detection of changes in  $\text{Ca}^{2+}$  levels in response to gravistimulation or evaluating the effects of direct and indirect inhibitors of transduction of cytosolic  $\text{Ca}^{2+}$  signals, have produced ambiguous results. Otherwise, it has not been possible to attribute a precise role in gravity signal transduction to specific  $\text{Ca}^{2+}$  channels previously identified (Baldwin *et al.* 2013).

Whatever the mechanism, the consequence of transduction of the mechanosignal is the reorientation of auxin efflux carriers and subsequent redistribution of auxin streams in the distal regions of the root. This is in agreement with the classical theory of Cholodny-Went, who stated for the first time in 1928 that root bending induced by a change in the root axis with respect to the gravity vector was caused by asymmetric growth of some parts of the root, which in turn was due to the asymmetric lateral distribution of auxin.

In roots growing vertically, auxin polar transport follows the pattern of a ‘reverse fountain’: auxin is transported acropetally throughout the centre of the root until the root tip, where the flow is divided and redirected basipetally throughout the outer layers of the root (Swarup & Bennett 2003). Subsequent to the gravitropic stimulus, a change in auxin flow is

established in the columella and transmitted through the lateral cap and epidermal meristematic cells. This change is due to the relocation (in columella cells) of two auxin efflux carrier proteins, namely PIN3 and PIN7, to the new bottom side of the cell (Friml *et al.* 2002; Kleine-Vehn *et al.* 2010). Therefore, polarised PIN localisation is critical for establishing and transmitting the auxin gradient in the root. In this process, PIN phosphorylation is important for localisation and activity of these proteins (Baldwin *et al.* 2013).

In addition to the changes in distribution of auxin, growth in microgravity (real or simulated) produces a substantial inhibition of auxin polar transport, as reported in several studies (Oka *et al.* 1995; Ueda *et al.* 1999), with the consequent inhibition of growth and development (Miyamoto *et al.* 1999). In turn, gravitropic response was suppressed when auxin transport was experimentally inhibited (Muday & Haworth 1994). Moreover, in a study on root morphogenesis carried out on a slowly rotating clinostat, meristematic activity and changes in auxin levels were measured in the root tips of rapeseed seedlings. Under these conditions, secondary roots were initiated earlier than in the vertical control. Analysis of meristematic activity and determination of the levels of auxin showed that, after few days of growth on the clinostat, the increased length of the primary root was due to higher cell proliferation and coincided with an increase in auxin content (Aarouf *et al.* 1999a, b).

The ‘Root’ experiment was carried out in the ISS during the course of the ‘Cervantes’ Spanish Soyuz mission. Samples grown in space were analysed by quantitative estimations of seedling length and the number and size of root meristematic cells in order to estimate the cell proliferation rate, and morphometric, ultrastructural and immunocytochemical study of the nucleolus, in order to examine the rate of ribosome biogenesis. The results showed an enhanced rate of cell proliferation accompanied by a reduction in ribosome biogenesis per cell in samples grown in real microgravity, compared to ground 1g controls. This suggests that weightlessness is a serious stress for the plant cell, capable of uncoupling cell proliferation and ribosome biogenesis, two processes that are closely interrelated in ground physiological conditions (Matía *et al.* 2010). Then, on the basis of these space results, a new experiment, sequential and dynamic, was designed using simulated microgravity provided by the random positioning machine (RPM; for an explanation of the term ‘simulated microgravity’, see Herranz *et al.* 2013). This experiment paid special attention to two proteins, cyclin B1 and nucleolin, implicated in cell proliferation/cell cycle and in cell growth/ribosome biogenesis, respectively (see Matía *et al.* 2009 for a preliminary report). In the study, samples obtained from seeds germinated in



the RPM were sequentially taken at 2, 4 and 8 days after sowing in order to allow dynamic analysis of the effects of simulated microgravity.

The evaluation of cell number and size in the root meristem showed enhanced proliferation with shortened cell size in samples taken on day 2, but these parameters stabilised at similar levels as seedling growth proceeded. Therefore, it appears that the phenotypic differences shown by 4- and 8-day-old seedlings have their origin in cellular events occurring in younger plants. The existence of alterations in cell proliferation and cell cycle regulation was confirmed with the data on cyclin B1 expression, which was lower in simulated microgravity-grown samples compared to 1g controls at the three time points, although the differences were much higher in the 2-day-old sample. Regarding parameters related to the rate of ribosome biogenesis, taken as an indirect evaluation of cell growth, they appeared depleted in the RPM samples with respect to the 1g control. In particular, analysis of nucleolar ultrastructure revealed that, in all cases, nucleoli from RPM samples showed typical features of low nucleolar activity.

More recently, a similar experiment has been performed in the Magnetic Levitation Instrument (Manzano *et al.* 2009); this instrument has the additional advantage that samples can be studied simultaneously at  $0g^*$ ,  $1g^*$  and  $2g^*$  ( $g^*$  = effective gravity; Berry & Geim 1997). With these experiments, we have completed a comparative study on seedlings grown in real and simulated microgravity, which, to our knowledge, has not been performed earlier with such an extension. In the Magnetic Levitation Instrument, the results were similar to those obtained with the RPM, but less pronounced, mostly concerning cell proliferation rate and seedling length. Interestingly, even the  $1g^*$  samples in the magnet showed some effects on ribosome production compared to controls outside the magnet. These data, together with the estimation of cyclin B1 expression, suggest that the high magnetic field itself affects cellular activities.

Taken together, these results show that the weightlessness environment is a stress condition for plant proliferating cells, expressed as the uncoupling of cell proliferation and ribosome biogenesis, two cellular events that are closely associated in ground gravity. The effects of the gravitational stress are detected from the very beginning of germination: in 2-day-old seedlings. The enhanced cell proliferation rate is not accompanied by an increase in levels of cyclin B1, as would be normal in ground gravity, but, on the contrary, these levels appear depleted. At the same time, lower cellular growth was observed, since ribosomes, the cellular factories for proteins, were produced at a lower rate. This depletion had already been detected in previous studies (Sobol *et al.* 2006), but it was not put in relation to other

cellular processes. Since cyclin B1 is synthesised in the G2 phase of the cell cycle, and also this period is the most active in ribosome production, a shortening of the G2 phase is compatible with the above-mentioned observed uncoupling. The causes of this shortening could be found in a failure or malfunction of the cell size checkpoint that immediately precedes mitosis (De Schutter *et al.* 2007; González *et al.* 2007).

The most reasonable interpretation of these effects of gravity alteration on specific fundamental features of meristematic cells puts them in relation to the mechanisms of gravity sensing and response in the root, which is responsible for root gravitropism. As indicated in preceding paragraphs, the final effect of the gravitropic response in roots is a change in transport and distribution of auxin. In the specific case of plant reorientation within the gravity field, the visual result is differential cell elongation in the distal elongation zone, producing root bending and readjusting the direction of root growth (Fig. 1). However, the root meristem is an intermediate step in the asymmetric auxin flux between root cap and elongation zone (Heisler & Jönsson 2007), and the role of auxin in regulation of the cell proliferation rate and cell cycle progression in the root meristem can now be considered unquestionable, since it has been demonstrated in many different experiments (Jiang & Feldman 2005; Magyar *et al.* 2005; Jurado *et al.* 2010; Dudits *et al.* 2011). In a previous paper, we already discussed the role of auxin in mediating the effects of gravity alteration between gravity sensing and root meristematic cell proliferation (Medina & Herranz 2010).

#### **GRAVITY ALTERATION IN CELL CULTURES *IN VITRO* PRODUCES SIMILAR EFFECTS AS IN MERISTEMS: THE SYSTEM OF GRAVITY SENSING, TRANSDUCTION AND RESPONSE IN NON-SPECIALISED CELLS**

Experiments on the effects of altered gravity on cell growth and proliferation have recently been extended to the use of *in vitro* plant cell cultures. Similar to root meristematic cells in seedlings, cultures are composed of a population of actively proliferating cells; however, these cultured cells are not integrated in an organism possessing specialised mechanisms for gravity sensing, whereas the response to gravistimulus in them is autonomous in each cell, not being the result of transduction of a signal from a more-or-less distant receptor organ. An important advantage of cell cultures is that they produce abundant biomass of proliferating cells, suitable for biochemical, genomic and proteomic methods, which is not usually possible with root meristems, where (specifically in *Arabidopsis*) the number of

proliferating cells is very limited. The study carried out on plant cell cultures grown for a short period under simulated microgravity (in an RPM), and analysing similar parameters as previously measured in root meristematic cells, gave comparable results to those obtained with seedlings. Therefore, we could conclude that meristematic competence is lost in proliferating cells as a consequence of gravity alteration, both in root meristems and in cell cultures *in vitro*.

As previously indicated, no specialised mechanisms for gravity perception have been described in cell cultures *in vitro*, unlike those present in statocytes of the columella cells of the plant root. Therefore, changes observed in the physiology of these cells as a consequence of gravity alterations cannot be attributed to the transduction of mechanosignals, as occurs in root cells. Thus, what is the graviperception mechanism acting in this kind of cell? There is a mechanism for gravity perception and response that differs from gravitropism and which, unlike gravitropism, is not based on the existence of specialised plant organs for gravisensing. This mechanism is gravity resistance, or graviresistance, a capacity acquired by plants during evolution at the time of the transition from an aquatic environment to a terrestrial environment, more than 400 million years ago, with the purpose of developing rigid structures capable of resisting the gravity force attracting them towards the centre of the Earth in order to keep them in their proper place.

Gravity resistance need not occur in specialised cells, but may operate in several different (if not all) cell types (Hoson *et al.* 2005). The perception of gravity in this mechanism is carried out through mechanoreceptors located on the plasma membrane, consisting of mechanosensitive ion channels. Actually, this type of mechanosensitivity can be put in relation to the gravitational pressure model of gravity sensing, an alternative mechanism of gravitropism that was shown to operate in intermodal cells of the green alga *Chara*, and also postulated to function in higher plant roots to account for experimental results in which cells of the distal elongation zone, lacking statoliths, are capable of sensing gravity (Staves *et al.* 1997). In this model, the entire protoplast is the gravity receptor, because it is based on detection of the total weight of the protoplast on its cell wall. This results in differential tension and compression between the plasma membrane and the cell wall at the top and bottom of the cell, activating the mechanosensitive ion channels located in these positions, which are recognised as triggering the cellular mechanism of graviresistance (Hoson *et al.* 2005).

The gravity signal sensed in this way is then transduced in order to generate a response. Unlike gravitropism, signal transduction in graviresistance does not involve

different tissues or organs of the plant, but instead is intracellular. The first transduction step is the reorientation of cortical microtubules, which are responsible for the structural stability of the cytoplasm and sustain various functions of the cell wall (Hoson *et al.* 2010). Since there is physiological continuity between the plasma membrane, cortical microtubules and the cell wall, the signal transduction leads to an alteration in cell wall rigidity as the final response. This alteration is modulated by the magnitude of the gravity vector, since, under hypergravity conditions, the rigidity of the cell wall increases, whereas under microgravity, tubulin mutants that show low capacity for gravity resistance and a disordered growth pattern on Earth are rescued and can grow and develop normally. In parallel, signal transduction reaches the nucleus and induces the expression of different genes, influencing the structure and function of various membrane components (Hoson *et al.* 2005, 2010).

Although there is no experimental supporting evidence that the mechanisms of gravity resistance are responsible for the alterations in cell growth and proliferation parameters detected in cell cultures *in vitro*, it is true that they are compatible with the results obtained. Actually, gravity resistance is a unique mechanism that is experimentally supported and provides an explanation for the detection, transduction and response to gravity signals of general application to all kinds of plant cells. Therefore, similar effects recorded in both root meristematic and *in vitro* cultured cells could be obtained *via* different mechanisms (Fig. 1). Certainly, if gravity resistance is a mechanism of general occurrence, it should also be present in meristematic cells.

#### **DIFFERENTIAL EFFECT OF THE TWO COMPONENTS OF THE GRAVITY VECTOR ON RESPONSE TO GRAVISTIMULATION**

The alteration of two components of the gravity vector (direction and magnitude) may have different effects on the plant developmental programme (Morita 2010). It is reasonable to argue that, once a threshold value of residual gravity is achieved, sensor organs of gravitropism could promote a response to the direction of the gravity vector and produce similar phenotypes, independently of the magnitude of the gravity vector. In fact, the gravitropic response has been modelled using a hyperbolic function, in which the maximum response is indicated by the asymptotic behaviour of the curve  $\alpha = a \cdot d / (b + d)$ , where  $\alpha$  is the gravitropic response, expressed as the angle of curvature of the root,  $d$  is the dose of stimulation, which is directly proportional to the acceleration of gravity, and  $a$  and  $b$  are constants (Perbal *et al.* 2002). In opposition, other graviresistance mechanisms that involve

tensions on cellular scaffold structures (cytoskeleton, cell wall or membranes) show logarithmic behaviour (Soga *et al.* 2001), *i.e.* producing an effect that is proportional to the magnitude of the gravity vector (Fig. 2). These arguments provide an explanation for the differential, complementary or additive effects driven mainly by the direction of the  $g$  vector in specific organs (gravitropism) and the effects driven by the magnitude of the  $g$  vector (graviresistance), which are detectable in specialised (statocytes containing statoliths) and non-specialised cells.

It is because of the prevalence of gravity acceleration on the Earth surface that the only way to study the effect of gravity on organisms, and to unravel the different contributions of the specific/non-specific or  $g$  vector direction/quantity effects, is to perform experiments in free-fall conditions (in space orbital trajectories, parabolic flights, sounding rockets or drop towers), through magnetic levitation compensation or mechanical randomisation of the  $g$  vector amount or direction, respectively (Herranz *et al.* 2013). Under real or simulated microgravity, both effects should be removed so that we cannot determine which of the two components is more important except by using cell cultures that do not contain specialised organelles to detect gravitropism. In contrast, using partial  $g$  levels (like those of the Moon or Mars), the contribution of the two components could be quite different, especially if seedlings are able to detect the gravity vector direction (gravitropism threshold is achieved), but the quantity of gravity is low enough to avoid most of the graviresistance mechanisms (Fig. 2). Hypergravity environments should therefore become a very interesting research field for similar reasons.

## **GLOBAL STRATEGIES TO COUNTERACT GRAVITY ALTERATION AND GRAVITATIONAL STRESS**

The question is how can organisms adapt themselves to an environment without this essential clue for their existence and survival on our planet. What happens at the molecular level in terms of transcriptional profile? This problem has been addressed in both spaceflight and ground-based simulated microgravity facilities using multiple model systems. In particular, *A. thaliana* seedling and cell culture transcriptomes have been analysed after space flight exposure (Paul *et al.* 2012). In both cases, some fundamental environmental parameters (including absence of lighting, containment and temperature constraints from launch site delivery and spaceflight until arrival at the ISS) have been added to the absence of the gravity signal. The consequence has been that hundreds of genes

have shown gene expression variations, reaching values above seven-fold. This dramatic effect should be related to phenotypic alterations, such as the decoupling of cell proliferation and cell growth observed in early *Arabidopsis* seedlings grown in space (Matía *et al.* 2010). In the case of *Arabidopsis*, space flight transcriptional effects have also been evaluated from two different biological sources – seedlings and cell cultures – thus also obtaining unique transcriptional responses (Paul *et al.* 2012). Our own transcriptomic work using *in vitro* cell cultures in simulated microgravity facilities, carefully designed to maintain optimal culture environmental conditions, produced a less evident gravitational effect, which was significantly enhanced when gravitational and magnetic field environmental stresses were maximised (Manzano *et al.* 2012). In addition, a static magnetic field has an effect on seedlings that is stronger than the effect of a magnetic field of similar intensity, but higher gradient, that we used in cell cultures (Paul *et al.* 2006).

In fact, our hypothesis is that the entire transcriptome needs to be finely tuned in a global way that counteracts multiple gravity-related parameters but, since microgravity is a novel and extremely unusual environment for organisms, the genome lacks particular gene sets evolved to do it. Consequently, genes with fewer limitations in their functions can be up- or down-regulated in certain organs, tissues or cellular compartments in order to respond to the new environmental challenge. For experiments involving the use of whole genome microarray platforms, not only plants (Manzano *et al.* 2012; Paul *et al.* 2012) but also different populations of small animals (Leandro *et al.* 2007; Herranz *et al.* 2009, 2012) have been exposed to microgravity, starting the experiment under different environmental or developmental conditions (reflecting different transcriptional states). Thus, these different systems promoted different final transcriptomic profiles that appeared as unique transcriptional responses to microgravity. Nevertheless, even in different organisms, the groups of genes affected by the treatment belonged to similar gene ontology domains, specifically, those related to biotic and abiotic stress genes. Likewise, when analysing results from the literature obtained using proteomic techniques, similar stress-related proteins appear to be involved in the response of *Arabidopsis* to microgravity or hypergravity conditions (Martizivanou *et al.* 2006; Barjaktarovic *et al.* 2009).

An interesting result of the microarray studies on the global response of the genome to gravitational stress is that redundant genes or multigene families were often found among the differentially expressed genes (Herranz *et al.* 2012; Manzano *et al.* 2012). This is a clear indication that they are the best candidates to play a major role in adaptation to new ecological situations. Redundancy in plants is more important than in animals, due to their

sessile condition: plants cannot escape from suboptimal or adverse environments, so adaptation strategies are mandatory. It is known that plants have undergone extensive duplications (up to four times) of large genomic regions, producing a large amount of duplicated genes, and that during evolution, plants have taken advantage of the existence of these duplications for the benefit of adaptation through multiple mechanisms.

Since we are interested in the cell proliferation and cell growth system, we have focused our attention on two gene families. In the first case, nucleolin, as the major protein of the nucleolus, is a marker of cell growth in actively proliferating cells, and precisely two genes code for this protein in *A. thaliana*. Do these genes have compensating effects, or do they tune their expression in unusual environmental conditions? Moreover, we know that multiple cyclins and CDKs are involved in cell proliferation control. A detailed analysis of our *Arabidopsis* transcriptomic data from simulated microgravity in ground-based facilities leads to the conclusion that multiple genes tune their expression in a concerted way, depending on the gravity change direction (Manzano *et al.* 2012; Table 1).

We think this is an exciting and very promising research line. Mechanisms of gravisensing and graviresponse in non-specialised cells must be experimentally demonstrated, and the differences and similarities with mechanisms supporting gravitropism, as well as the existence of possible synergies and crosstalk between the different pathways, should be investigated. At the level of molecular global strategies to counteract the gravitational stress, our results with cyclins and nucleolin genes, as representative elements of multigene families and redundant genes, should first be completely understood, and second extended to other examples of high functional relevance. In addition to providing answers to fundamental questions of plant biology, this research will make possible the most efficient use of plants in future space exploration missions and in sustainable agriculture on Earth.



## ACKNOWLEDGEMENTS

We thank Drs. Isabel Matía, Ana I. Manzano (CIB-CSIC, Madrid), Jack J.J.W.A. van Loon (Free University of Amsterdam), Eugénie Carnero-Diaz (UPMC, Paris), Veronica Pereda (University of Toulouse), Richard Hill, Laurence Eaves, Oliver Larkin (University of Nottingham), Julio Sáez-Vásquez (University of Perpignan) and Peter Christianen (University of Nijmegen), and our technician Mercedes Carnota, for their contribution to the experiments mentioned in this paper. Especially, we acknowledge the contribution of Dr. Eugénie Carnero-Diaz to the design of Fig. 1. Work performed in the authors' laboratory was supported through the Plan Nacional de I+D+i of the Spanish Government (grant Nos. AYA2009-07952 and AYA2010-11834-E).

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For Peer Review

**Table 1.** Cell cycle (cyclin) genes, as an example of adaptability of expression to unusual environmental conditions in a multigene family. Relative changes (%) in expression of cell cycle regulation genes under altered gravity. A more than 30% change in gene expression *versus* 1g\* or 1g are shaded (in bold when statistically meaningful *versus* 1g, pval- Limma < 0.01). The first four columns correspond to different positions in the magnet in an experiment with magnetic levitation ( $g^*$  = effective gravity within the magnet; LDC: large diameter centrifuge; RPM: random positioning machine).

	0g*	0.1g*	1.9g*	2g*	LDC (2g)	RPM (simulated $\mu g$ )
CKS1	-51%	-37%	-22%	4%	-7%	-12%
CKS2	-32%	-18%	18%	17%	45%	-13%
CYCA1;1	-40%	-12%	-57%	30%	64%	-5%
CYCA1;2	-8%	-21%	20%	-1%	-12%	-8%
CYCB1;2	-52%	-57%	-7%	52%	12%	-22%
CYCB1;3	-47%	-51%	-17%	43%	72%	-12%
CYCB1;4	-49%	-42%	-28%	58%	74%	-21%
CYCB1;5	-54%	-45%	-15%	46%	20%	-30%

**FIGURE LEGENDS**

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**Figure 1.** Comparison of the processes of gravity sensing, transduction and response in the plant root and in an *in vitro* plant cell culture. In the root, the mechanical gravity signal is sensed by statolith movements in statocytes. Mechanical signal is converted into chemical signal through a mechanism not totally understood at present, in which actin and Ca<sup>2+</sup> channels probably take part, which results in the modulation of auxin polar transport and lateral distribution throughout the root. Alterations in these parameters produce effects on the growth of cells in the elongation (transition) zone, which are the immediate precursors of differentiated cells of the root. Effects of gravity alteration on cell proliferation and growth have also been observed in the root meristem, most probably related to changes in auxin transport and distribution, since auxin is an essential regulator of cell proliferation and cell cycle progression. In turn, cultured cells lack statoliths, and no specific mechanism of gravity sensing has been described in these cells, other than the gravity resistance mechanism. Since tensions in peripheral microtubules and the cell wall have been reported to be an effect of gravity alteration in non-differentiated cells of hypocotyls (as part of the mechanism of gravity resistance), it is reasonable to assume that these changes might also occur in proliferating *in vitro* cultured cells. The mechanosignal, sensed by this or another unknown mechanism, can be converted into a chemical signal by Ca<sup>2+</sup> channels, also present in this kind of cells and transduced intracellularly and probably also through cell–cell interactions. Interestingly, the effects of gravity alteration on the two biological systems, at the level of cell growth, proliferation and differentiation, are quite similar.

Author: Please change ‘mechanic’ to ‘mechanical’ in this figure.

**Figure 2.** There are two processes triggered by gravity sensing and response in plants, namely gravitropism and graviresistance. The first uses gravity to spatially orient the growth of the plant; the second allows the survival and development of aerial parts of the plant, producing rigid structures capable of adopting positions not necessarily imposed by the gravity force. The two components of the gravity vector, namely direction and magnitude, are differentially involved in these two processes: gravitropism mostly uses direction,

although magnitude also plays a role, particularly in the determination of thresholds; graviresistance is essentially triggered by the magnitude of the vector, with an almost negligible influence of the vector direction. Consequently, the response curves for both processes in relation to the stimulus are quite different: gravitropism is characterised by a hyperbolic curve (continuous line; 1), whereas the response for graviresistance is logarithmic (dashed line; 2). The two curves must cross each other in a point, which corresponds to the  $g$  level at which the differential contribution of gravitropism and graviresistance to graviresponse are the same. Since the curves plotted in the figure are only theoretical (*i.e.* they have not been plotted with real experimental data), we do not know exactly where (at what  $g$  level) this point is located. For this reason, this point has been marked with a question mark. An additional interesting consequence of these models, when exploration is becoming a chief objective of space research, could be determination of the differential contribution of gravitropism and graviresistance to the gravity response in conditions of fractional gravity, such as those existing on the Moon ( $0.17g$ ) or Mars ( $0.37g$ ).

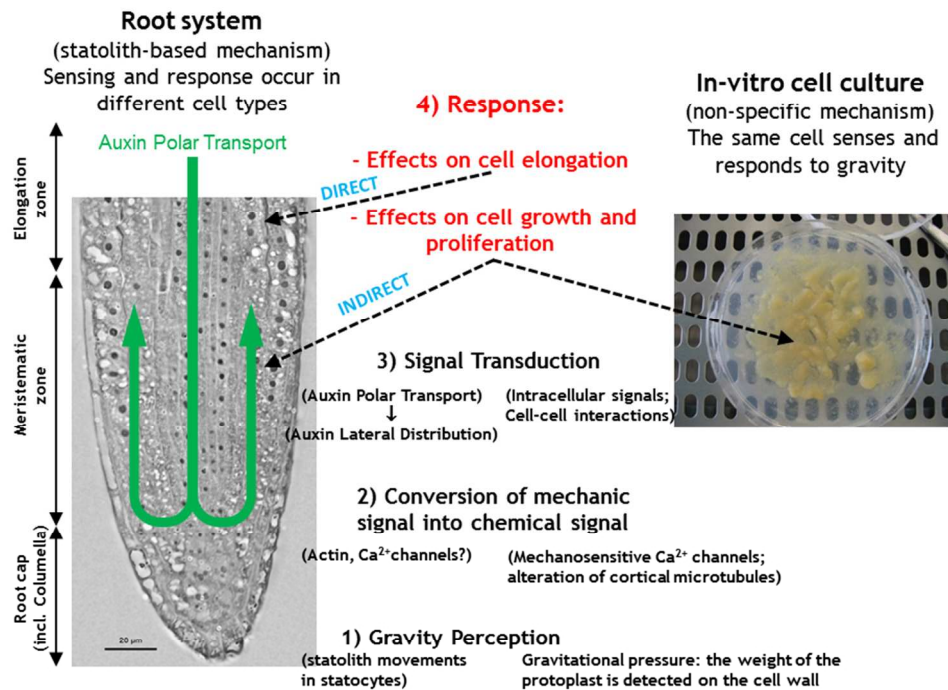


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254x190mm (96 x 96 DPI)

Type of Gravity Response	Sensitive component of $g$ vector	Response curve
Gravitropism	Direction (+++) and Magnitude (+)	Hyperbolic (1)
Graviresistance	Magnitude	Logarithmic (2)

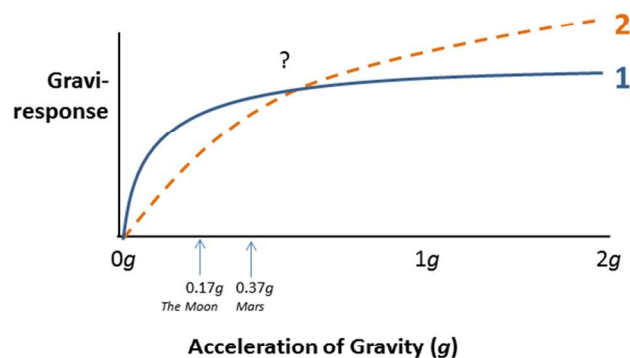


Figure 2. There are two processes triggered by gravity sensing and response in plants, namely gravitropism and graviresistance. The first of them uses gravity to spatially orient the growth of the plant; the second one allows the survival and development of aerial parts of the plant, producing rigid structures capable of adopting positions not necessarily imposed by the gravity force. The two components of the gravity vector, namely direction and magnitude, are differentially involved in these two processes: gravitropism mostly uses direction, although magnitude also plays a role, particularly in the determination of thresholds; graviresistance is essentially triggered by the magnitude of the vector, with an almost negligible influence of the vector direction. Consequently, the response curves for both processes in relation to the stimulus are quite different: gravitropism is characterized by a hyperbolic curve (continuous line; 1), whereas the response for graviresistance is logarithmic (dashed line; 2). The two curves must cross each other in a point, which corresponds to the  $g$  level at which the differential contribution of gravitropism and graviresistance to graviresponse are the same. Since the curves plotted in the figure are only theoretical (i.e., they have not been plotted with real experimental data), we don't know exactly where (at what  $g$  level) this point is located. For this reason, this point has been marked with a question mark. An additional interesting consequence of these models, when exploration is becoming a chief objective of space research, could be the determination of the differential contribution of gravitropism and graviresistance to the gravity response in conditions of fractional gravity, such as those existing on the Moon (0.17g) or Mars (0.37g).

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