Plants in Space: Novel Physiological Challenges and Adaptation Mechanisms

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Abstract Any space exploration initiative, such as the human presence in the Moon and Mars, must incorporate plants for life support. To enable space plant culture we need to understand how plants respond to extraterrestrial conditions, adapt to them, and compensate their deleterious effects at multiple levels. Gravity is a major difference between the terrestrial and the extraterrestrial environment. Gravitropism is the process of establishing a growth direction for plant organs according to the gravity vector. Gravity signals are sensed at specialized tissues by the motion of amyloplasts called statoliths and transduced to produce a cellular polarization capable of influencing the transport of auxin. Gravity alterations eventually result in changes in the lateral balance of auxin in the root, producing deviations of the growth direction. Under microgravity, auxin changes affect the root meristem causing increased cell proliferation and decreased cell growth. The nucleolus, the

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nuclear site of production of ribosomes, is a marker of this unbalance, which could alter plant development. At the molecular level, microgravity induces a reprogramming of gene expression that mostly affects plant defense systems against abiotic stresses, indicating that these categories of genes are involved in the adaptation to extraterrestrial habitats. Nevertheless, no specific genes for plant response to gravitational stress have been identified. Despite this stress, plants survive, developing until the adult stage and reproducing under microgravity conditions. A major research challenge is to identify environmental factors, such as light, which could interact, modulate, or balance the impact of gravity, contributing to the tolerance and survival of plants under spaceflight conditions. Understanding the crosstalk between light and gravity sensing will contribute to the success of the next generation agriculture in human settlements outside the Earth.

1 Plants Are Needed for Space Exploration: Space Plant Biology

> *"Je ne sais pas si les mondes sont habités, et, comme je ne le sais pas, je vais y voir!"* ("I do not know if the worlds are inhabited, and, as I do not know that, I'll go there and see!")
> – Jules Verne, "From the Earth to the Moon" (1867).

This quote, from one of the most famous books by Jules Verne, which was written more than a century and a half ago, reflects an intense human dream, which at the same time is a major challenge for the humankind: to go out of our planet Earth and see how are "the other worlds", in particular, whether we, as living beings and, especially, as intelligent living beings, are alone in the Universe, or we have companions with whom we can interact. Science-fiction literature is full of stories talking about the relationships between humans and aliens, whether they are peaceful or hostile.

In 2019, the entire world has commemorated the 50th anniversary of the first human footprint on the surface of the Moon, and in 2020, the 20 years of the continuous presence of humans as crew members of the International Space Station (ISS). These commemorations have evidenced that space exploration is still considered by most people as a highly exciting and attractive challenge that, additionally, promotes the scientific and technological progress and significantly contributes to a better human life on Earth (Rai et al. 2016). Certainly, some relevant opinions have appeared expressing concerns and doubts, mostly focused on the high costs for citizens – tax payers that this enterprise entails in relation to its effective outcomes (Rinaldi 2016).

As a consequence of this social context, the leading countries of the world, in America, Europe, and Asia, are currently working to promote a manned mission,

first to the Moon, and then to Mars. NASA has made public the "Artemis" program with the explicit goal of the landing of "the first woman and the next man" on the Moon by 2024. Europe, Japan, and Canada have expressed their support to this program as active partners. This objective is considered only as the first step in the run to a human settlement on the Mars surface in the decade of 2030s. On its side, the plans of the Chinese government, though less explicit, are not far from these objectives.

In this exciting story, life sciences must play a relevant role. The environment that space explorers will find in the spaceflight and in nearby planets is very different from Earth in many factors, which are not compatible with terrestrial life. Many of these adverse factors can be counteracted in spaceships, or in Martian or Lunar settlements, by developing specific habitats, but the astronauts, as living beings, and their accompanying living organisms, must adapt to grow and survive under the influence of a completely different gravity level and under radiation doses higher than those existing on the Earth. These environmental factors cannot be currently counteracted by physical means in an affordable and efficient manner. Thus, the main aim of space life science is to understand how the space environment and specifically altered gravity and radiation affect the morphology, physiology, and behavior of living organisms and to design countermeasures to enable terrestrial life, and particularly human life, to develop outside Earth. That is, how they perceive and respond to gravity and radiation and how they adapt to the space environment. This adaptation is the major objective of space life science despite an emerging opinion suggesting that terrestrial gravity should be provided to astronauts as part of their life support, together with, e.g., oxygen or temperature, in view of the severe damages induced by microgravity on the human physiology and the difficulties found in developing effective countermeasures (van Loon et al. 2020).

It is beyond doubt, in any case, that space explorers will need a constant and sustainable supply of oxygen, food, and vitamins, as well as the removal of their waste CO_2 and the regulation of the environmental moisture. Furthermore, their psychological wellbeing should not be neglected. Interestingly, all these resources can be supplied by a single – though varied and diverse – component, namely plants. Therefore, plants must be a key component of any bioregenerative life support system that can be designed for human space exploration.

The culture of plants in spaceflight, or on planets and satellites other than the Earth, such as the Moon or Mars, necessarily requires the creation of a "greenhouse" in which the plant is provided with the necessary environmental elements to enable its development. These elements include light, water, temperature, oxygen, CO_2, aeration, and nutrients, as well as microorganisms, in order to achieve a fully functional and sustainable environment for plants. These factors and conditions should be initially provided through import from the Earth, although, in the case of stable, mid- and long-duration settlements in the Moon or Mars, self-regenerative systems should be implemented to avoid the dependence of terrestrial supplies. For the case of water and light, native sources could be found and effectively used. Once these requirements are met, plant growth should be enabled in the presence of a gravity level different from the Earth value (near-zero g – microgravity – in orbit,

0.17 *g* for the Moon and 0.38 *g* for Mars) and of a certain level of cosmic radiations that escape from shielding devices. This requires that the specimens to be cultivated are adapted to grow and develop in the presence of these two environmental factors, under magnitudes significantly different from the Earth values. The development of technological countermeasures and the use of biological strategies that mitigate the unfavorable impact of gravity and radiation are complementary strategies to consider. These are the major current challenges that space plant biology needs to overcome.

2   Gravity Perception, Transduction, and Response

Gravity is an essential environmental factor for plant growth and development and, in addition, it has been a constant environmental factor throughout the entire history of the biological evolution in the planet Earth (Volkmann and Baluška 2006). The orientation of plant growth is driven by the unchanged direction and magnitude of the gravity vector, which is ultimately responsible for the growth of roots deep in the soil and the growth of stems in the upright direction, in which leaves can be maximally exposed to sunlight, for the most efficient accomplishment of photosynthesis. The process and mechanism by which plants sense and respond to the mechanical stimulus exerted by the gravity vector is termed gravitropism and involves the coordinated activity of different cell types and tissues.

Most of the knowledge acquired on the mechanisms of gravitropism has come from experiments involving the response of plants to a change in the growth orientation. As indicated, the plant growth direction is aligned with the gravity vector. The direction of this vector cannot be changed, but we can turn the plant and uncouple the alignment, such that the axis of the plant does not coincide anymore with the gravity vector. Therefore, the plant must reorient its growth, by re-establishing the alignment according to gravity. This produces a bending or curvature in the root and stem, and the biological mechanism by which this curvature is established illustrates the biological bases of the sensing, transduction and response to the gravity mechanosignal, that is, of the plant gravitropism.

The gravitropic response can be divided into four steps: gravity perception, transduction of the signal within the cell, transmission of the signal from the receptor cells to locations spatially different and separated in the plant, and the growth response (Swarup and Bennett 2018; Gadalla et al. 2018) (Fig. 1). Gravity perception takes place in specialized cells called statocytes. These cells have starch-containing plastids, statoliths, considered as gravity sensors, which sediment in the direction of the gravity vector. In the root, statocytes are located in the root cap (columella), whereas in the shoot, they are found in the endodermis. A change in the gravity direction is sensed by the displacement of statoliths, which re-sediment according to the new direction of the gravity vector (Fig. 1). The discrimination of the precision of the system, in terms of the threshold angle of inclination capable of triggering a response, and of the response time, is an important issue. It could be
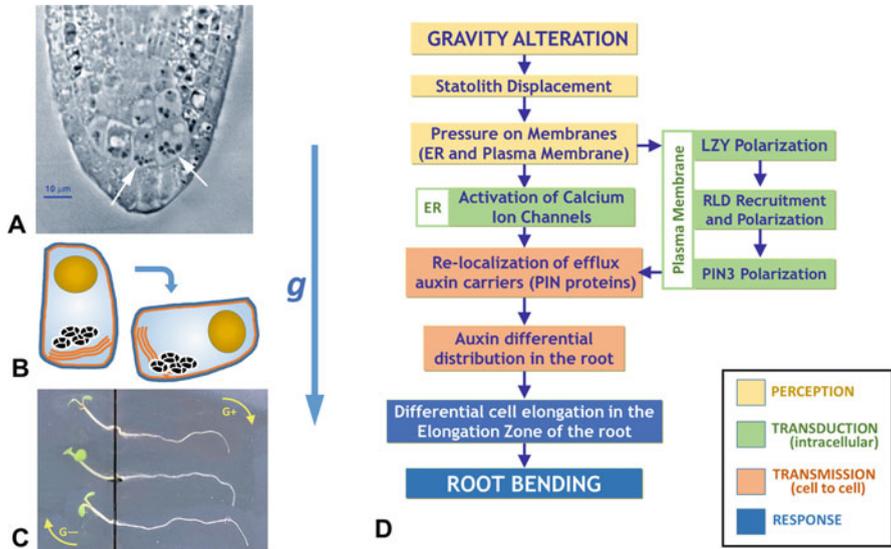
Fig. 1 Root gravitropism: gravity sensing and response in roots. (**a**) Section of an *Arabidopsis thaliana* root tip observed unstained, with phase contrast microscopy. Statoliths (arrows) appear sedimented in the bottom part of columella cells, according to the gravity vector *(g)*. (**b**) A turn of 90° with respect to the gravity vector produces a displacement of statoliths toward the side of the cell that is now at the bottom. (**c**) The results of the turn of 90° in seedlings is the bending of the root and hypocotyl, to reorient their growth direction according to the gravity vector. (**d**) Sequence of events in the gravity sensing and response. The gravitropic signal is sensed and transduced in columella cells, and it is then transmitted throughout the root to produce a change in the lateral distribution of auxin, which results in root bending due to the differential cell elongation occurring in a specific zone of the root

thought that the density of the intracellular milieu and the interactions of amyloplasts with endoplasmic reticulum (ER) and cytoskeleton would restrict the flow of the granules, which would cause the granular system to stop working below a critical angle. However, the statoliths offer surprising precision, as demonstrated by the response of the plant aerial organs to a very weak tilt, allowing the maintenance of the vertical posture of the plant (Moulia et al. 2019). Actually, it has been shown that, in response to an inclination, statoliths do not behave like a classic granular medium, but they move and flow in the cell regardless of the angle of inclination. Like a liquid, the surface of the statolith deposits always recovers horizontally. Using a biomimetic system, consisting of microbeads arranged inside artificial cells of the same size, it was concluded that the joint fluidity of the statoliths derives from the movement of each of them separately. The cellular molecular motors (actin-myosin network) constantly shake them, allowing them not to get stuck and giving to the system, as a whole, properties close to those of a liquid (Bérut et al. 2018). This behavior is essential for the plant, since it allows it to react to the smallest inclinations.

Once the gravitropic signal is sensed by statolith movement, it has to be transduced to produce a physiological response. Although the chain of events in this transduction is not totally and precisely understood, there is experimental evidence that the physical connection of statoliths with the cytoskeleton (especially with actin microfilaments) and with membranes (ER and plasma membrane) is involved in this process. The actin cytoskeleton participates in the statolith motion as part of its general function in the cell as a molecular motor for the intracellular transport of organelles (Kadota et al. 2009). However, an additional role of actin microfilaments in the transmission of the tensions generated by sedimenting statoliths to mechanoreceptors on cell membranes (tensegrity) was reported (Yoder et al. 2001). In apparent contradiction with these roles of actin, experiments involving the treatment of plants with actin inhibitors resulted in an enhancement of the gravitropic response (Yamamoto and Kiss 2002). Actually, actin microfilaments act, at the same time, transmitting the gravitropic stimulus to ER membranes and relieving in part the compressive forces of amyloplasts on membranes. Therefore, a disruption of microfilaments would increase the compressive forces on ER, eventually enhancing gravity sensing and signaling (Leitz et al. 2009). Thus, although an intact actin cystoskeleton is not strictly required for gravitropic signaling and response, it indeed plays a role in the fine-tuning of the early steps of the gravitropic process, as it was additionally suggested by genetic studies indicating the involvement of specific proteins in the interaction between statoliths and actin (Blancaflor 2013).

The statolith motion induces a pressure exerted by these organelles on cell membranes, specifically on ER, which causes membrane deformations, as it has been visualized by electron tomography (Leitz et al. 2009). The consequence of this deformation is the opening of membrane-localized mechanosensitive Ca^{2+} channels that create a Ca^{2+} signal in the cytoplasm (Toyota and Gilroy 2013). This results in a fast alkalization of the cytoplasm of statocytes, which correlates with a change in cellular polarity involving redistribution of auxin efflux carriers (PINs). This correlation of pH change and relocation of PIN proteins is supported by the finding that mutants defective in alkalization of columella cells do not show PIN relocation (Baldwin et al. 2013). The resulting ion current can trigger further signaling cascades (Fig. 1).

Therefore, despite the gaps still existing in our understanding of the precise mechanisms of gravity sensing and signal transduction in the root, the process can be summarized as a message sent by collumella cells to the elongation zone, affecting auxin lateral distribution (Baldwin et al. 2013). The gravitropic signal, perceived by the new location of statoliths, ultimately produces the asymmetric redistribution of the plant hormone, auxin, as a result of auxin signaling events that depend on the coordinated activity of auxin influx (AUX1) and efflux carriers (PIN2, PIN3) within the root apex (Bennett et al. 1996; Friml et al. 2002). Additional support for this mechanism comes from the fact that the *aux1* mutant is totally agravitropic, whereas mutations in various PIN protein genes confer reduced gravicompetence. PIN3 and PIN7, located in the columella cells, change their distribution within minutes after the gravitropic stimuli, and PIN2 transports auxin through epidermis toward the root elongation zone. The mechanism results in an

increased auxin concentration at the lower side of rotated roots that inhibits cell growth in the lower side of the elongation zone, causing a curvature of the root to align itself with the gravity vector (Baldwin et al. 2013). This root bending can be inhibited by a treatment with NPA, an inhibitor of the auxin polar transport (Rashotte et al. 2001).

Recent studies have revealed a fundamental role of the LAZY1 protein family in early phases of gravitropic signal transduction, by acting as intermediates between the relocation of amyloplasts and the gravity-induced change in the directional auxin transport (Nakamura et al. 2019). From the six genes of this family, four of them *(LZY1* to *LZY4)* are expressed in gravity-sensing cells. The involvement of this gene family in gravity signaling was shown in a transcriptomic study carried out in two *Arabidopsis* mutants defective in the gravitropic response. Three members of the family, namely *LZY1, LZY2*, and *LZY3* were identified and characterized. In the triple mutant, the amyloplast relocation was found unaltered, but the formation of the asymmetric PIN3 distribution and auxin flow were reversed. Phenotypically, the mutant plants showed alterations in the growth angle of lateral shoots and roots. Thus, LAZY proteins were suggested to play a key role in controlling lateral auxin flow after the reorientation of statocytes (Taniguchi et al. 2017). The expression pattern of all six genes of the family was analyzed in specific constructs harboring reporter genes. Analysis of single and multiple mutant lines reveals that single mutants show only mild alterations in the growth direction of lateral roots, which are enhanced in the *lzy2 lzy3* double mutant, and the *lzy2 lzy3 lzy4* triple mutant displays reversed root gravitropism, associated to a reversion of the asymmetric auxin distribution (Yoshihara and Spalding 2017). More recently, a model of gravity signaling has been proposed, involving the participation of LZY proteins and their identified interactors, RCC1-like domain (RLD) proteins, in the modulation of auxin flow (Furutani et al. 2020). RLD proteins were shown to act in controlling the abundance and localization of the PIN3 protein. Plasma membrane is likely to be the site of action of LZY proteins in statocytes. Gravistimulation, and the subsequent amyloplasts relocation, induces polarization of LZY3 localization in the direction of gravity in the plasma membrane of columella cells. This polarized LZY recruits RLD proteins from the cytoplasm to the plasma membrane, and RLD would modify PIN3 trafficking, leading to asymmetric auxin flow (Furutani et al. 2020). Therefore, LZY proteins are acting downstream statoliths displacement signal, but upstream auxin transport (Fig. 1).

As it can be inferred from the experiments quoted in the preceding paragraphs, our knowledge of the mechanisms of plant gravity sensing and gravitropic response largely comes from studies performed after induction of a change in the growth direction of the plant with respect to the gravity vector, usually involving a rotation of the seedling or the plant. However, what happens if a gravitropic signal is not sensed by the plant or seedling, because the gravity vector is absent, as it occurs in weightlessness, or under microgravity conditions, e.g. on board of spaceships? And what is the response of the plant to a change in the gravity vector that does not affect to its direction, but to its magnitude, as it occurs in potential habitats in which gravity force is a fraction of the terrestrial value, such as the Moon or Mars? Certainly, we

know significantly less on the plant response in these conditions, simply because experimentation is more constrained. This experimentation was performed either in real microgravity (spaceflight) or using ground-based facilities for simulated microgravity (or fractional gravity), such as the clinostat or the random positioning machine (RPM).

If the morphogenesis of plants under the influence of the terrestrial gravity vector is called "gravimorphogenesis," the mechanisms of response to an environment without the influence of a defined gravity vector (real or simulated microgravity) constitute the so-called automorphogenesis, a process that began to be known and studied as early as in the nineteenth century, using the most classic clinostats. Classical studies carried out in these devices described spontaneous curvatures of roots followed by straight root elongations in random directions (reviewed by Hoson and Soga 2003).

In real microgravity, different studies performed in space experiments also revealed automorphogenesis, but both random and non-random growth directions of roots grown in microgravity were described in different experiments. An experiment on the growth of rice roots in spaceflight showed that in the early phase of growth, most of them elongated in a constant direction, forming a constant angle of about 55° relative to the seed axis, but later the roots grew randomly in various directions, including away from the culture medium (Hoson et al. 2003). In a more recent experiment carried out in the International Space Station (ISS), lentil roots initially curved strongly away from the cotyledons and then slowly straightened out forming a relatively constant angle (Driss-Ecole et al. 2008). A specific phenomenon, termed root skewing was repeatedly observed in seedlings growing in spaceflight (Millar et al. 2011; Paul et al. 2012a). It was defined, under normal ground gravity conditions, as the deviation of the root growth from the gravity direction, caused by a combination of factors, such as the interaction of gravity and touch to a slanted impenetrable medium and the inherent tendency of the root tip to circumnutate with a fixed handedness (Roy and Bassham 2014). The molecular mechanisms underlying such growth phenomenon, and especially its frequent occurrence under microgravity conditions, remain unresolved, although various genes and factors have been proposed to regulate root skewing, involving polar auxin transport and cytoskeletal dynamics (Nakashima et al. 2014; Roy and Bassham 2014). A recent study in the ISS, using two mutants of skewing behavior, affecting different cellular functions, concluded that genes related to skewing could play a prominent role in plant spaceflight adaptation (see later in this chapter) (Califar et al. 2020).

The kinetics of the movement of statoliths in microgravity conditions was studied in spaceflight in an experiment combining 1 g centrifugal acceleration and direct exposure to weightlessness. Amyloplasts were grouped at the distal pole of the statocytes by a centrifuge pulse and then placed in microgravity for increasing periods of time (13, 29, 46, or 122 min) and chemically fixed. Electron microscopical observations showed a gradual displacement of statoliths toward the proximal pole, but this movement was stopped by the nucleus. This position of statoliths, grouped beneath the nucleus, was similar to that observed in roots grown continuously in

microgravity. Treatment with cytochalasin D demonstrated the involvement of the actin microfilaments in the statolith displacement (Driss-École et al. 2000). A similar behavior was observed in simulated microgravity, using the RPM (Kraft et al. 2000).

As a consequence of the changes in the mechanisms of gravity sensing and response in weightlessness, a significant inhibition of the auxin polar transport was reported in early spaceflight experiments (Ueda et al. 1999). Otherwise, when auxin transport was experimentally inhibited, the gravitropic response was suppressed (Muday and Haworth 1994). In a study using different systems of microgravity simulation, namely magnetic levitation and the RPM, the pattern of auxin distribution in the root tip under simulated microgravity, visualized with the reporter gene construction DR5::GUS, was shown to correspond to the inhibition of the auxin polar transport (Herranz et al. 2014). However, in a more recent experiment performed in space, the distribution of auxin in the root was shown to display a "vertical" pattern, similar to the pattern of roots grown under control ground gravity, even though the roots showed a disoriented growth, including numerous bends, coils, and skews (Ferl and Paul 2016). The authors conclude that the auxin transport through the root and the balance of auxin distribution in the root would be independent from gravity sensing. Actually, more research is necessary to explain these findings in the context of the current models of relationships between statolith movement, auxin transport, and root growth direction.

3 Auxin and Meristems: Effects of Microgravity on Meristematic Cells

Auxin fulfills multiple roles in the regulation of plant growth and development, acting in many steps and processes. In the root, auxin establishes the root architecture through its function in initiation and emergence of lateral roots, patterning of the root meristem, and cell expansion in the elongation zone. The configuration of the auxin polar transport throughout the different zones of the root is essential for determining root morphology and anatomy. Under standard environmental conditions for plant growth, in absence of any stress, it has been observed that the auxin maximum accumulation occurs in the proximity of quiescent center in the root tip (Vanneste and Friml 2009).

The function of auxin in plant growth and development is based on the role of this phytohormone in the regulation of organogenesis. This involves the controlled production of new cells in meristems, which are specialized zones of the plant containing a permanent population of undifferentiated totipotent cells whose function is to grow and divide. Thus, regulation of cell proliferation and growth, with further cell expansion and differentiation, in meristems is the basis of plant development. Auxin promotes cell division and drives meristem maintenance, and also plays an important role in the establishment of cellular patterning, according to specific threshold concentrations and cell- or tissue-specific responses

(Perrot-Rechenmann 2010). More precisely, auxin is necessary but not sufficient to stimulate cell division in cultured cells or plant tissues because the presence of cytokinin is also required (Inzé and De Veylder 2006). Conversely, addition of auxin to arrested cells after deprivation of auxin leads to restoration of cell division (Perrot-Rechenmann 2010). The existence of meristems, their functions and mechanisms in relation to differentiation, and their ability of supplying new differentiated cells in any moment of the life of the plant is a basis of the plant plasticity. By means of this plasticity, plants counteract their sessile condition and may adapt to a wide range of environmental changes and conditions.

Unsurprisingly, auxin plays an important role during abiotic stress-induced changes in the root. Through the creation of local auxin maxima and minima, the balance between cell division and cell differentiation, the rate of cell elongation and the emergence of lateral roots can be modulated in response to environmental signals. By these mechanisms, root architecture is ultimately modified in dependence of external stimuli, and auxin becomes an essential player in the plant acclimation to changes in the environmental conditions (Korver et al. 2018). The plant response to gravity changes is not an exception.

3.1   Effects of Microgravity on the Cell Cycle

The influence of environmental gravity on meristematic cell functions, mediated by changes in the levels and distribution of auxin, is only partially known. A relatively small number of experiments have approached this topic, in spaceflight and using microgravity simulation devices on Earth. Most of these experiments were focused on the cell cycle regulation (see Herranz and Medina 2014). Early pioneering studies on spaceflight, using lentils as model species, reported changes in mitotic index of roots grown in microgravity (Darbelley et al. 1986; Driss-École et al. 1994). From data on this parameter it was difficult to draw firm conclusions on the factors and mechanisms affected, but it clearly pointed out that alterations of cell cycle caused by the space environment may occur. Further experiments in real and simulated microgravity showed changes in the proportion of cells in different cell cycle phases, measured by densitometric analysis of nuclear DNA content of root meristematic cells, suggesting that the regulation of the cell cycle progression is somehow modulated by gravity (Legué et al. 1996; Yu et al. 1999).

The first European experiment on plant biology on board the ISS (the "Root" experiment) revealed that one of the most relevant effects of altered gravity was the dissociation of cell proliferation from cell growth in seedling root meristems. A remarkable increase of the cell division rate and a decrease of cell growth were observed. The strict coordination of the rates of these two fundamental processes that characterize meristematic cells was called "meristematic competence" (Mizukami 2001). Consequently, exposure of seedlings to microgravity produces a deep alteration of the normal function of cells of the root apical meristem (Matía et al. 2010). Subsequent experiments performed with seedlings on Earth using different ground

facilities for simulation of altered gravity conditions showed similar trends as the spaceflight experiments. Specifically, the expression levels of cyclin B1 showed a significant decrease in simulated microgravity, as a sign of alterations in the regulation of cell cycle progression (Manzano et al. 2013; Boucheron-Dubuisson et al. 2016). Additional experiments with callus cell cultures in real and simulated microgravity, using genomic and proteomic methods, also demonstrated alterations in the expression of genes and in the levels of proteins involved in cell cycle regulation (Fengler et al. 2015; Manzano et al. 2012; Barjaktarovic et al. 2009; Paul et al. 2012b).

More recent studies have used an *Arabidopsis in vitro* suspension culture immobilized in agar and incubated in the RPM for simulation of microgravity. A suspension culture is a powerful tool in plant cell cycle studies and has the additional advantage of the possibility of synchronizing cells for their progression in the cell cycle (Menges and Murray 2006). This study was complemented with simulation of the Mars gravity (0.38 g) and hypergravity (2 g). Different times of exposure to altered gravity were tested. Whereas 3 and 14 h of growth in experimental conditions produced only mild effects, 24 h of exposure, the approximate duration of an entire cell cycle, produced the most relevant alterations. The most intense effects were found for simulated reduced gravity, whereas hypergravity produced milder effects. Cell proliferation and growth were uncoupled under simulated reduced gravity, similarly as the results obtained in root meristematic cells from seedlings grown in real or simulated microgravity. Alterations in the duration of cell cycle phases were reported, as well as in the tested cell cycle regulators, both affecting the protein levels and the rate of gene transcription (Kamal et al. 2018).

A further study detected variations in the duration of cell cycle phases by flow cytometry, after synchronizing cells in their cell cycle progression by aphidicolin (Menges and Murray 2006). Cell cycle acceleration was demonstrated in cells grown in simulated microgravity, particularly at the cell cycle period comprising G2 and M (mitosis) phases. This period showed a remarkable reduction in time with respect to the 1 g control, outside the RPM. Oppositely, the duration of the G1 period was slightly longer in cells grown in the RPM than in control cells. Alterations in the so-called core cell cycle regulators (Menges et al. 2005), as well as in factors of epigenetic modifications were found in specific cell cycle phases using flow cytometry for protein level evaluation, and qPCR for gene expression. Most of the factors acting at the G2/M cell cycle checkpoint appeared downregulated under simulated microgravity, whereas most of the factors acting at the G1/S checkpoint showed upregulation. Furthermore, in these conditions, nuclear transcription by RNA polymerase II was depleted, while condensed chromatin increased. This was related to the epigenetic regulation of gene expression, including increased DNA methylation and depleted histone acetylation. Therefore, G2/M checkpoint disruption was a significant effect of altered gravity, as well as chromatin remodeling. An additional consequence is the existence of mechanosensors in individual cells, independent from the mechanisms of gravity perception acting on plants, which involve specialized ("professional") cells (Kamal et al. 2019a). This accelerated cell cycle with a reduced subpopulation of cells in G2 and M phases reconciles the

apparent paradox of an increased cell proliferation rate in microgravity (Matía et al. 2010) with a reduced expression of the *CycB1* gene as a marker of G2/M transition (Boucheron-Dubuisson et al. 2016; Manzano et al. 2013).

In a global transcriptomic analysis using the same *Arabidopsis in vitro* cell culture the overall transcriptomic response of cell cultures exposed to simulated microgravity, and, particularly, the differential effects on G1 and G2/M subpopulations of cells were investigated in order to provide new insights into the stress pathways involved in the response to altered gravity conditions. Separate analyses were carried out for different cell populations, namely G2/M- and G1-phase-enriched and an asynchronous culture sample. The gene ontology groups showing the most conspicuous differential expression were cell proliferation, energy/redox and stress responses, plus unknown biological processes. Globally, simulated microgravity produced overall expression inhibition in the three cell populations but the G2/M-phase-enriched cells showed the highest number of downregulated genes and stress response components changed dramatically from G2/M to the G1 subpopulation, suggesting a differential adaptive response to simulated microgravity through the cell cycle (Kamal et al. 2019b). The adaptation of cell cycle regulation, using both known stress mechanisms and unknown function genes, may cope with reduced gravity as a novel evolutionary environment.

3.2   Effects of Microgravity on Ribosome Biogenesis and the Morphofunctional Organization of the Nucleolus

Other than the rate of cell proliferation, the second component of meristematic competence is cell growth. The intuitive concept of cell growth is the increase in size of a cell, but, actually, distinct biological processes may eventually result in an enlarged cell and not all of them should strictly be termed as "cell growth". This concept should be only attributed to the increase in size of proliferating cells, which occurs throughout the interphase of the cell cycle, resulting in the increase in total cellular content of nucleic acids and proteins. No vacuolization is associated to cell growth. In contrast, the process of "cell expansion", also involving the cell enlargement, is associated to the process of differentiation, from a totipotent to a specialized cell and usually involves vacuolization and DNA endoreplication (Perrot-Rechenmann 2010).

Therefore, in highly proliferating cells, such as those of an *in vitro* suspension culture or meristematic tissues of plants, cell growth involves the production of proteins in order to overcome a biomass threshold (or cell size threshold) compatible with the viable size and biomass of daughter cells after mitosis (Doerner 2008). This is the reason for the need of a strict coordination between the rates of cell growth and cell division in proliferating cells, which defines meristematic competence (Mizukami 2001). Consequently, cell growth is largely determined by the activity of biogenesis of ribosomes, the cytoplasmic factories of proteins, which occurs in a

prominent nuclear domain, the nucleolus (Baserga 2007; Bernstein and Baserga 2004; Bernstein et al. 2007).

Ribosome biogenesis is a complex multi-step process. It starts with the transcription of ribosomal genes, which are present in multiple copies of the 45S rRNA transcription unit, arranged in tandem and clustered in one or more specific chromosomal region(s) called nucleolar organizer regions (NOR). This is followed by the cleavage of 45S pre-rRNA to produce the mature rRNAs, which form cytoplasmic ribosomes in association with 5S rRNA and ribosomal proteins (Sáez-Vásquez and Medina 2008).

In addition to ribosomal proteins, hundreds of non-ribosomal proteins, or nucleolar proteins, play specialized roles either as enzymes, or regulating the rate of ribosome production in concert with snoRNAs, or even assuring the structural arrangement of the process (Sáez-Vásquez and Delseny 2019). Some of them interact with factors or mechanisms controlling cell proliferation and cell cycle progression (Medina and González-Camacho 2003), thus linking these crucial cellular processes with ribosome biogenesis and assuring meristematic competence.

Among nucleolar proteins, the multifunctional nucleolin is the most abundant protein of the nucleolus in actively proliferating cells, where it plays a key role in different steps, including rRNA gene transcription, processing of pre-rRNA, and assembly and nucleocytoplasmic transport of ribosome particles. It has also been implicated in other functions, with or without collateral relationship with ribosome biogenesis. For these functional roles, nucleolin activity is controlled at transcriptional, post-transcriptional, and post-translational levels during cell growth and differentiation, as well as in response to cellular stresses. In particular, nucleolin phosphorylation is mediated by kinases involved in cell cycle control (Durut and Sáez-Vásquez 2015; Tajrishi et al. 2011; Medina et al. 2010). While animal and yeast genomes encode a single nucleolin gene, plants show gene multiplicity. In *A. thaliana*, two genes encoding nucleolin proteins have been described: *NUC1* and *NUC2* (Pontvianne et al. 2007). The *NUC1* gene is highly and ubiquitously expressed in normal growth conditions. *NUC2* is a functional protein-coding gene developmentally controlled in most plant tissues and organs, and it contains several cis-acting elements related to biotic and abiotic stress responses (Durut et al. 2014). NUC1 and NUC2 proteins localize in nucleolus. Specifically, NUC1 localizes preferentially in the dense fibrillar component (DFC) and NUC2 colocalizes with peri-nucleolar chromatin, but only NUC1 is able to assist nucleosome remodeling *in vitro* (Durut and Sáez-Vásquez 2015; Medina et al. 2010; Pontvianne et al. 2007; Durut et al. 2014).

Fibrillarin is another nucleolar protein, which functions in the first pre-rRNA processing step, guided by pre-rRNA methylation, in association with snoRNAs and other nucleolar proteins, including nucleolin. Both fibrillarin and nucleolin have been reported to be co-purified in a snoRNP complex and to mostly share their *in situ* localization in the DFC of the nucleolus (Sáez-Vásquez et al. 2004; Medina and González-Camacho 2003).

As a fundamental cellular process, regulation of ribosome biogenesis is modulated by the environmental conditions and coordinated with other cellular processes,

such as cell division and differentiation. Therefore, this process is a major and very useful stress sensor, and many stressors such as DNA damage, temperature changes, hypoxia, osmotic stress, oxidative stress, viral infection, and lack of nutrients have been shown to dramatically alter the rate of ribosome production (Boulon et al. 2010; Mayer and Grummt 2005; Kalinina et al. 2018). Gravity alteration is also capable of producing deep changes in this parameter. This is particularly relevant in highly proliferating cells, such as those constituting the plant meristems and *in vitro* cell cultures. As indicated above, in these cells the rate of ribosome biogenesis is a marker of cell growth and hence of the status of meristematic competence, in concert with the rate of cell division. A significant decrease in the efficiency of the biosynthetic machinery of ribosomes has been detected under real and simulated microgravity in plant proliferating cells, either *in planta* (root meristems from seedlings) or in culture *in vitro*. Nucleolar proteins, particularly nucleolin and fibrillarin have been chosen as reliable markers to detect and evaluate these alterations. The first results were obtained in a pioneering experiment in the ISS, already mentioned, in which the levels of nucleolin were quantified *in situ* by ultrastructural immunolabeling in meristematic cells. A significant reduction of the nucleolin labeling in nucleoli, of around one third of the levels of control 1 *g* samples, was found in samples grown in spaceflight, and also in samples grown under simulated microgravity conditions (Matía et al. 2010). These results were confirmed in successive experiments, using alternative methods of microgravity simulation (Boucheron-Dubuisson et al. 2016; Manzano et al. 2013). The exposure to simulated microgravity of the *nuc1* mutant, characterized by a disorganized nucleolus in ground gravity conditions, produced an intensified nucleolar disorganization and the appearance of nucleolar particles identified as corresponding to wrongly or incompletely processed preribosomal precursors (Boucheron-Dubuisson et al. 2016). A recent detailed analysis of pre-rRNA processing steps in seedlings grown in simulated microgravity has confirmed these alterations (Manzano et al. 2020b).

A deeper study on the effects of altered gravity on ribosome biogenesis through the alterations found on nucleolar proteins was carried out using an *in vitro* plant cell culture exposed to simulated microgravity. This experimental model offered some advantages with respect to meristems. Firstly, no limitations in the amount of biological material, necessary for molecular biology experiments, and, secondly, the possibility of using cell populations synchronized in their progression through the cell cycle. The decrease in the levels of fibrillarin and nucleolin caused by simulated microgravity was confirmed using immunolabeling quantified by flow cytometry and a downregulation in the expression of the *NUC1* gene was revealed by qPCR in cells grown in the microgravity simulator (Fig. 2) (Kamal et al. 2018, 2019a).

An additional reliable and useful estimation of the effects of gravity alteration on ribosome biogenesis in relation to cell proliferation and cell cycle was obtained by a thorough analysis of the structure of the nucleolus. The nucleolus is a polymorphic structure, extremely sensitive to functional changes involving different rates of ribosome production, such as those occurring throughout the different periods of the cell cycle in proliferating cells. Thus, structural features of the nucleolus are a
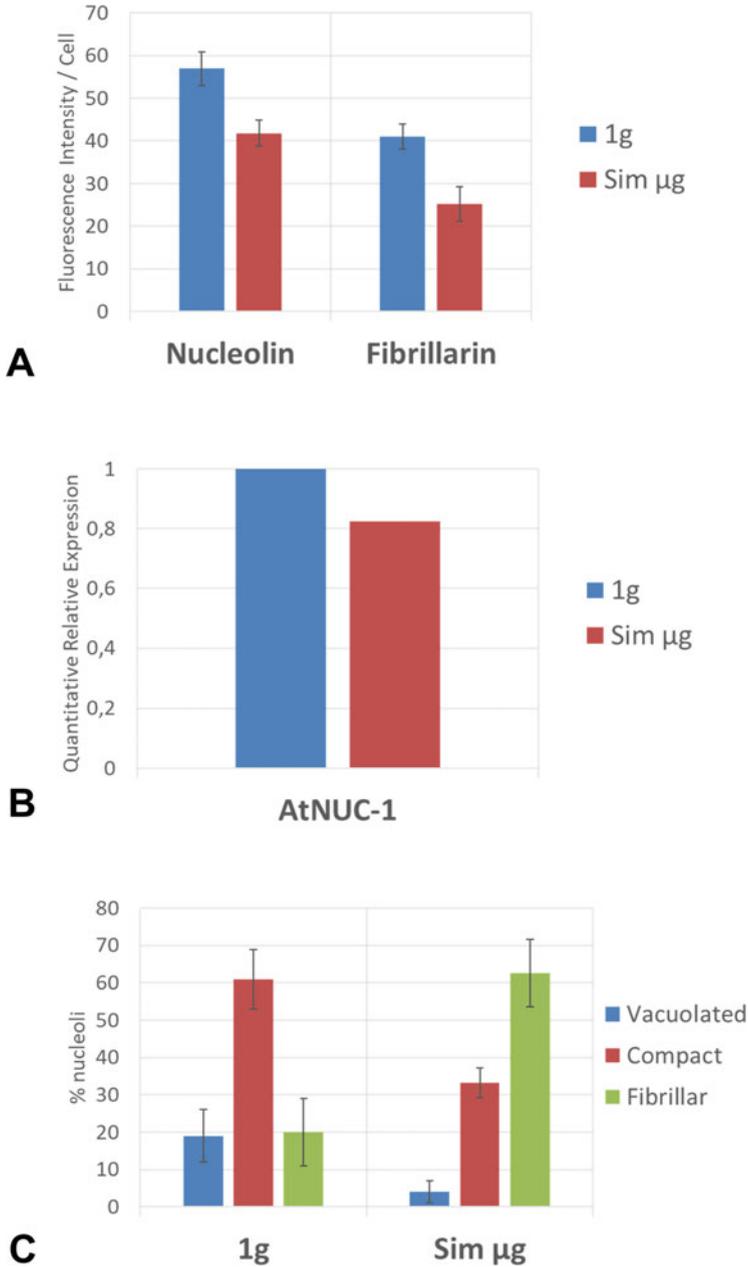
Fig. 2 Quantification of different markers of nucleolar activity and ribosome biogenesis after exposure of an *A. thaliana* cell culture to simulated microgravity. (**a**) Levels of two nucleolar proteins, nucleolin and fibrillarin, estimated by flow cytometry. (**b**) Expression of the nucleolin-1 (*Nuc1*) gene, estimated by qPCR. (**c**) Distribution of the different nucleolar types with functional significance in the cell culture. In all cases, the exposure to simulated microgravity results in a significant depletion of the nucleolar activity and, consequently, of the rate of production of ribosomes

reliable marker of the functional state of the cell and, in particular, of the cell cycle phases (Sáez-Vásquez and Medina 2008). Moreover, the various effects on ribosome production and cell growth induced by the different types of cellular stresses are often accompanied by significant changes in the structural organization and distribution of the nucleolar components (Raska et al. 2006; Srivastava and Pollard 1999). These findings can be used as an efficient tool for the study of the cellular effects of environmental gravity alteration.

Based on previous knowledge on the molecular architecture of the nucleolus and on the distribution of nucleolar subcomponents in dependence of the activity of ribosome biogenesis in plant meristematic cells (Medina et al. 2000; González-Camacho and Medina 2006; Sáez-Vásquez and Medina 2008), three nucleolar structural types or models were defined in *Arabidopsis* proliferating cells with the purpose of that they could serve as indicators of different functional states of the cell, in relation to potential cellular stresses and also to cell cycle phases. These models, identified under the transmission electron microscope were called, according to their morphology, as "vacuolated," "compact," and "fibrillar," arranged from the most to the less active in ribosome production (Fig. 3). To clarify nomenclature, nucleolar "vacuoles" refer to large and clear intranucleolar spaces that contain granules similar to the granular component (GC), that are made of nearly mature preribosomal particles. Their relationship with cytoplasmic vacuoles is only structural resemblance, and not functional analogy. Vacuolated nucleoli were characterized four decades ago in meristematic cells, as the expression of highly active nucleoli (Moreno Díaz de la Espina et al. 1980).

Moreover, a correlation between the structural features and the size of the nucleolus exists, so that the structural arrangement also corresponds to the size arrangement, form larger to smaller. Based on these features, these models can be easily identified on 2 μm semithin sections at the light microscope level (Manzano et al. 2016). These nucleolar models have a potential applicability as environmental stress sensors or as pathological markers.

The relative abundance of each of these nucleolar types, as an estimation of the status of the rate of ribosome biogenesis, and hence of cell growth, was statistically assessed in different conditions of gravity in an *in vitro Arabidopsis* cell callus culture exposed to magnetic levitation as a method of microgravity simulation. Samples exposed to simulated microgravity for 200 min showed a significant decrease in the nucleolar "active" type and an increase in the "inactive" type, compared to 1 g controls. The functional significance of the structural data was validated by several different complementary cellular and molecular indicators (Manzano et al. 2016). These results were confirmed using a suspension cell culture (Kamal et al. 2018) (Fig. 2c).

Otherwise, as previously indicated, and described in several plant model systems, a link exists between nucleolar morphology and cell cycle phases (González-Camacho and Medina 2006). The use of an *Arabidopsis* suspension cell culture synchronized in the cell cycle progression with a pulse of aphidicolin (Menges and Murray 2006) has allowed the unequivocal association between cell cycle phases and nucleolar models at the ultrastructural level. The same analysis performed after
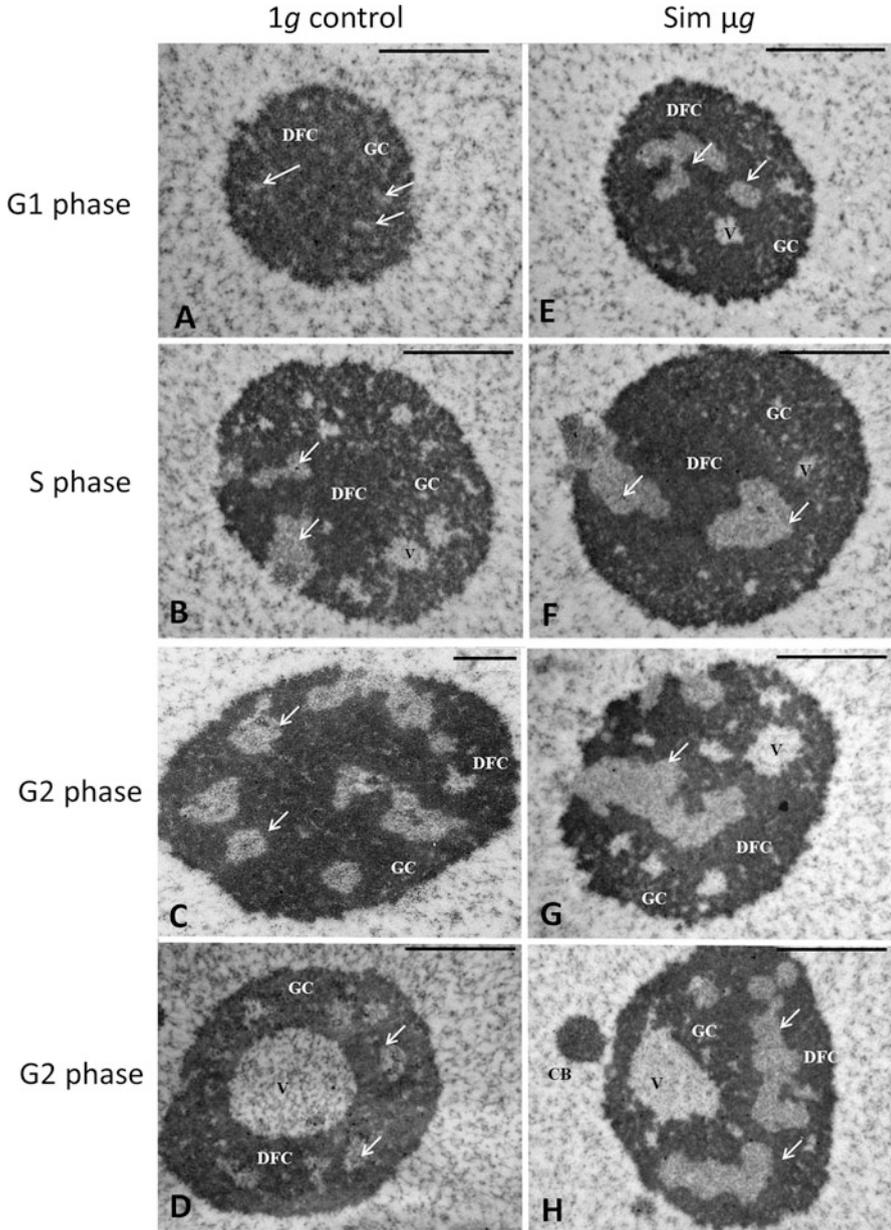
Fig. 3 Ultrastructural study of the nucleolus throughout the different phases of the cell cycle in *A. thaliana* cultured cells, synchronized with aphidicolin and exposed to simulated microgravity, compared to the same cell culture grown under 1 *g* control conditions. The structural and morphometric features of the nucleolus show dramatic changes in the different cell cycle phases, even under control conditions. In G1, the nucleolus is small, compact, and mostly fibrillar in structure (**a**). In S-phase, the size increases, granular component (GC) appears surrounding the dense fibrillar component (DFC) and several fibrillar centers (arrows) distribute scattered throughout the DFC (**b**). In G2, the nucleolus reaches the largest size and two structural types can be identified, namely

the incubation of the synchronized cell culture in a microgravity simulator has provided valuable information of the morphofunctional changes induced by altered gravity on the nucleolus in each phase of the cell cycle (Fig. 3). In parallel, under the light microscope, the quantitative distribution of the nucleolar types in the different phases was statistically assessed.

Under normal ground gravity, in the G1 phase, nucleoli appeared compacted, constituted practically only by dense fibrillar component (DFC) (Fig. 3a). Interestingly, G1 nucleoli did not show any type of nucleolar "vacuolar" space in *Arabidopsis*, such that it was observed in onion (González-Camacho and Medina 2006). G1 compact nucleoli were the smallest among the different nucleolar models which characterized cell cycle phases. This nucleolar structure and organization in G1 phase was altered by simulated microgravity; under these conditions, small nucleolar vacuoles appeared and fibrillar centers (FCs) slightly enlarged. Furthermore, this gravitational alteration did not produce significant differences in the nucleolar size (Fig. 3e).

In the S phase, at 1 *g*, the nucleolus was double-sized, compared to the G1 phase. This increment was accompanied by conspicuous changes in the ultrastructural features. The granular component (GC) appeared in the S phase as an abundant component, giving account of 60% of the nucleolar volume and clearly segregated from DFC, which was reduced in proportion. Small vacuolar areas and a few enlarged FCs were observed (Fig. 3b). S-phase-associated nucleolus structure was altered by simulated microgravity. Although the nucleolar size was not significantly affected, the distribution of the nucleolar components was different from the 1 *g* control. The abundance of DFC significantly increased, GC diminished, and the size of FCs was observed to increment, appearing interconnected in ultrathin sections (Fig. 3f).

Finally, in the G2 phase, two types of the nucleolar models could be distinguished in samples grown at the 1 *g* terrestrial gravity, depending on the presence or the absence of a large central nucleolar vacuole. The "non-vacuolated" model was similar in many aspects to the one in S phase; differences were the larger size, the intermingled distribution of DFC and GC and the multiplicity of smaller FCs (Fig. 3c). The "vacuolated" model shows a large central space, or "vacuole" containing loosely distributed granules, surrounded by a cortex formed by intermingled DFC and GC and small FCs (Fig. 3d). Simulated microgravity produced substantial effects in the nucleolar structure during G2 phase. Both models showed a larger proportion of DFC and FCs, these latter appearing conspicuously enlarged, at the expenses of GC. Furthermore, G2 nucleoli under simulated

Fig. 3 (continued) compact (**c**), with many small fibriilar centers, and vacuolated (**d**), with a large central space, or "vacuole" containing granules. In both types, DFC and GC appear intermingled. Under simulated microgravity, the structural types are conserved, but the nucleolar size is smaller, fibrillar centers are always fewer and larger and the proportion of GC is lower than in the 1 *g* control (**e–h**). Bars indicate 1 μm

microgravity were significantly smaller than in the 1 *g* control. Cajal bodies were found near the nucleolus in the G2 phase (Fig. 3g, h).

When using synchronic cultures and phase-specific cell cycle subpopulations, the characterization of the cell cycle phases in 1 *g* control conditions confirmed that G2 is the most active phase of ribosome production, and consequently of cell growth (González-Camacho and Medina 2006; Ginisty et al. 1999; Medina et al. 2000), and that simulated microgravity produces a substantial depletion effect on the nucleolar activity, which becomes apparent by changes in the nucleolar structure in all phases, but especially during the G2 phase. The evidence of this reduction was also supported by the data obtained in G2/M phase from 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 morphofunctional models in these phases showed an increase in the number of inactive nucleoli under simulated microgravity (Kamal et al. 2019a).

Collectively, all these results show that the cell cycle, ribosome biogenesis, and the nucleolus are sensitive cellular targets of the environmental gravity alteration occurring in spaceflight, particularly detected in meristematic cells. Changes in the auxin transport and distribution can be identified at the origin of these cellular alterations, whose consequences may seriously affect the growth and development of the entire plant.

4 Mechanisms of Adaptation to Spaceflight

It is clear that the spaceflight environment, devoid of the gravitational cue, produces a serious alteration of the biological processes and mechanisms sustaining the life of plants. Gravity is responsible for mechanical signals generated in different organs of the plant due to their weight, and for the production of all-pervasive specific directional cues. In fact, these signals and cues are major drivers of the normal growth and development of plants on Earth. In addition, essential physical processes greatly influencing the mechanisms of biochemical reactions, e.g. gas and liquid flow, are conditioned by the gravity force. As a result, water and gas movements are altered in microgravity, which may cause disturbances in the concentrations of key substances in metabolically active tissues (Porterfield 2002).

This unequivocally confronts plants in space with a suite of environmental conditions with which they are not accustomed to live on Earth, whose consequence is that the spaceflight conditions (microgravity, radiation, confinement, and other factors) are a cause of stress for terrestrial plants. In principle, plants have an enormous plasticity and are capable of surviving and adapting to a great variety of environmental conditions. However, the gravitational stress has some specific features that could result in an adaptive response profoundly different from the responses to terrestrial stresses. Drought, salt, heat, or cold are environmental

conditions that have produced a "memory" in plants throughout evolution. Therefore, plants have had the opportunity of developing appropriate responsive mechanisms that remain stored and silent under normal conditions, but emerge and operate when needed. However, gravity is an all-pervasive condition throughout space and time in the evolutionary story of plants, so that no memory of microgravity exists in the plants that currently populate our planet and they must develop totally new adaptive strategies to new environmental signals.

An interesting collateral discussion relative to gravitational stress is to consider whether the real stress-generating condition for plants is the lack of gravity, or the cause of the stress is actually the very existence of the gravity vector. To briefly comment this consideration, we should first realize that, from an evolutionary perspective, the conquest of mainland by aquatic plants around 470 million years ago, when a multicellular species of green algae left the ocean, was a fundamental milestone in the history of life. It indeed made possible a burst of biological diversity, by dramatically increasing the oxygen atmospheric levels and providing a potential source of food which allowed the appearance of new organisms and helped their diversification and expansion across the terrestrial world. Gravity played a chief role in this process. For succeeding in this decisive step, land plants (embryophytes) indeed had to overcome a new environmental stress, which was mostly caused by the existence of the gravity vector, and trigger an adaptive response. They had to develop a mechanism of graviresistance, leading to the built of a rigid body capable of withstanding the force of gravity, as well as the systems necessary to transport nutrients and water against the pull of gravity (Plackett and Coates 2016; Hoson and Soga 2003). No doubt exists that, at that time, the gravity vector was a profound stress condition for plants. Nevertheless, the story of evolution continued with the successful adaptation of plants and the result is that gravity is a fundamental driver of plant growth and development as we know them today, and its removal certainly causes important alterations and triggers the establishment of new adaptation mechanisms.

4.1 Transcriptomic Changes and Adaptation

In recent years, we have experienced an increasing availability of -omic methods, more and more robust, for determining global changes in gene expression at the level of either gene transcription (transcriptomics), or protein mapping in quantity and quality (proteomics), or even detection of epigenetic changes (epigenomics), among others. This has made possible a substantial number of studies dealing with these kinds of changes undergone by plants when they grow in a microgravity environment, either real (spaceflight) or simulated. Transcriptomic studies, using microarray or, more recently, microsequencing (RNAseq) techniques are, by far, the most frequently performed.

Transcriptomic studies have produced a great deal of information on the plant responses to microgravity and spaceflight environment. The processing and

interpretation of this information is, however, an arduous task, for different reasons. Spaceflight experiments are much less frequent and are subjected to much more constraints than experiments performed in ground laboratories, and this affects the reproducibility of the experiments and the statistical treatment of data. Furthermore, genotypes, growth conditions, hardware used and developmental periods of experimental samples show a great dispersion among different experiments, seriously compromising the harmonization of data. An important effort of data sharing and harmonizing has been undertaken in the project called GeneLab, under the leadership of NASA and the participation of laboratories from different countries. The project is organized into different analysis working groups (AWGs), one of them specifically devoted to plants (Barker et al. 2020; Ray et al. 2018).

Regarding interpretation of data, some studies emphasize the detection of alterations induced in the space environment, with the objective of accurately defining the gravitational stress (Choi et al. 2019; Johnson et al. 2017), whereas in other cases it is claimed that the transcriptional changes are actually reflecting the mechanism of physiological adaptation of plants to spaceflight, since they have been obtained from viable samples (Paul et al. 2012b, 2017). The fact that plants eventually adapt to survive in space is becoming undisputed, since apparently normal adult plants and flowers can be produced in the ISS, and plant full life cycle (seed-to-seed) has been shown to occur in space (Massa et al. 2013; Musgrave et al. 2000; Link et al. 2014). This is the final and successful plant response to the serious alterations in some processes, essential to plant development, that are induced by the microgravity environment, as described in the preceding sections of this chapter. However, most probably, the adaptive response is more complex than a mere change in the pattern of gene expression occurring as soon as the samples experience the new environmental conditions, as it can be detected in transcriptomic studies performed on young seedlings grown for a few days in spaceflight. It is conceivable that plants could trigger an early and primary acclimation response to the environmental change from Earth to space, to overcome the early alterations that could then be extended throughout successive developmental stages. Further, this acclimation would be followed by later adaptive responses involving more stable genetic and epigenetic changes that would be transmitted to the offspring, allowing the survival of plants in the space environment throughout successive generations. The investigation of these complex acclimation and adaptation processes and mechanisms has not yet been undertaken in depth, and it is one of the most important and decisive challenges of space plant biology, now and for the coming years.

The problem is that, most frequently, analyses were done at a single point of the plant development, preferentially on young seedlings, whereas we lack sequential studies on the transcriptional response to microgravity in which the pattern of gene expression of, e.g., young seedlings and mature plants, is compared. Even, these comparative studies should be extended to consecutive generations. In the very few sequential studies performed, the increased duration of the exposure to simulated microgravity resulted in some attenuation of the alterations produced in the root meristem in young seedlings (Boucheron-Dubuisson et al. 2016). Certainly, only

cellular methods were used in this work and the analysis was restricted to early developmental stages.

With all these limitations and uncertainties, it is true that the number of space-flight experiments performed on plants and analyzed with transcriptomic tools is overcoming a certain threshold, allowing us to get some conclusions that can be considered robust and significant. Some genes or groups of genes have emerged from these studies as representing a core set of functions appearing affected by spaceflight environment. The most prominent constituents of this core set are the oxidative stress pathways, involving the production of reactive oxygen species (ROS) (Choi et al. 2019; Kruse et al. 2020; Correll et al. 2013), the system of heat shock response genes, producing heat shock proteins (HSP), which are molecular chaperones acting to protect and refold proteins in response to cellular damage (Choi et al. 2019; Johnson et al. 2017; Zupanska et al. 2013), and the cell wall remodeling system (Kruse et al. 2020; Johnson et al. 2017; Correll et al. 2013; Kwon et al. 2015). In none of these transcriptomic studies, specific genes for the response of plants to microgravity, or to the spaceflight environment, have been identified, although it is noteworthy that the "unknown biological processes" gene ontology group is a major group enriched in differentially expressed genes (DEGs) after incubation of a plant cell culture in simulated microgravity (Kamal et al. 2019b).

Different genotypes, mostly from the model species *Arabidopsis thaliana,* and even different ecotypes of this species have been analyzed in their response to spaceflight. No phenotypical differences were appreciated between them after growing in the space environment, but the transcriptomic results showed highly significant differences in the number and the identity of genes showing altered regulation in comparison with the respective ground controls. This would mean that each genotype, either mutant or ecotype, used a different strategy – a different set of genes – to physiologically adapt to the environmental change (Paul et al. 2017; Johnson et al. 2017). It is very difficult to discriminate which of the found changes are actually adaptive and which are circumstantial, unnecessary for adaptation and therefore dispensable. The aforementioned definition of an affected "core set of functions," though still incomplete, may help in this discrimination, but the specificities of each genotype, organ (root or aerial part) and even each cell type, in the adaptive response, should be taken into account, thus complicating the task.

A further step in understanding the transcriptional adaptive response of plants to spaceflight is the use of mutational analysis for the identification of key elements required for the adaptation. A candidate gene to play a role in the process is the *Altered response to gravity 1 (Arg1),* which is known to participate in the plant gravity responses on Earth through the relocation of auxin efflux carrier proteins PIN2 and PIN3 upon root gravistimulation and the establishment of the auxin lateral gradient in the root (Blancaflor 2013). An experiment in ISS using the wild-type and an ARG1 KO line allowed the identification of DEGs between the two genotypes and the two environments, spaceflight and ground. The data indicate that a high proportion of genes involved in the adaptation of the wild-type to spaceflight are *Arg1*-dependent. The role of this gene in the adaptation mechanism appears to be related to the endomembrane system and cell wall remodeling (Zupanska et al.

2017). Another suitable subject of mutational analysis of the adaptive mechanisms is the network of heat shock factors (HSFs), a member of the core set of genes involved in the plant response to spaceflight. HSFs are transcription factors that regulate the expression of heat shock proteins (HSPs), evolutionarily conserved, that play major roles in the general mechanisms of stress response of almost all organisms. Since cellular stress is characterized by the accumulation of denatured proteins, HSPs are molecular chaperones that control a correct protein folding (Guo et al. 2016). Among HSFs, the *HsfA2* gene was the highest upregulated gene in an *Arabidopsis* cell culture grown for 12 days in space (Zupanska et al. 2013). Therefore, in a further experiment in ISS, cell lines from wild-type and HSFA2 KO were grown and analyzed for their gene expression profiles in search for the reaction to the space environment of a cell line deprived of a putative basic regulator of their response to environmental stresses. The analysis of the space flown samples of the HSFA2 KO line showed that the number of DEGs under spaceflight conditions, compared to the ground control, was much higher than in the wild-type. Plants deprived of this factor appeared affected by endoplasmic reticulum stress, they had to trigger the unfolded protein response (UPR) pathway as a consequence of protein misfolding events, and, consequently, their adaptation and survival in space required a more intense gene reprogramming, thus confirming the key role of HSFs in these processes. This role is played through regulation of cell wall remodeling, plasma membrane signaling and starch production, which appeared as the functional groups with a higher proportion of DEGs exhibiting the most intense changes (Zupanska et al. 2019).

The third and last example of mutational analysis to be mentioned here concerns the use of two lines, respectively, deficient in two genes known to play a role in root skewing, a phenomenon of deviation of the root directional growth with respect to the gravity vector which has been described and discussed in a precedent section of this chapter. The primary functions of the two genes assayed, namely *Spiral1 (Spr1)* and *Sku5,* are, respectively, the regulation of cortical microtubule dynamics and a copper oxidase activity, anchored to glycosylphosphatidylinositol, acting at the interphase between plasma membrane and cell wall. Both SPR1 and SKU5 proteins are associated with cell wall remodeling, one of the processes commonly identified to take part in the plant response to spaceflight conditions. Furthermore, their skewing pathways are different (see Califar et al. 2020 and the references therein). After growing in space for either 4 days or 8 days, the two mutants showed great differences in their transcriptomic response: *spr1* mutant showed fewer DEGs than the Col-0 wild-type, whereas the number of DEGs in the *sku5* mutant was much higher than its corresponding WS wild-type. The interpretation emphasizes the existence of genotypes potentially better prepared to live in the microgravity environment, thus showing an easier adaptation to spaceflight conditions, as indicated by the number of DEGs involved in the response. If this reasoning is correct, the loss of function of the SPIRAL1 protein positively impacts adaptation, whereas, on the contrary, SKU5 protein plays a role in physiological adaptation and the loss of function of this protein initiates a complex and extensive adaptive response involving the participation of mechanisms of response to deep stresses, such as ABA signaling and pathways affecting membrane stabilization and remodeling (Califar

et al. 2020). Interestingly, the number of DEGs in *sku5* is dramatically reduced in 8-day-old seedlings with respect to younger 4-day-old ones. As previously indicated, this sequential study could help to differentiate between the early primary response, mostly giving account of the stress suffered by plants, and the later response, which includes genes involved in the adaptive process.

Proteomic studies, intended to establish quantitative and qualitative differences in the protein map of samples grown in microgravity with respect to 1 *g* ground controls, are fewer than transcriptomic analyses. Membrane proteins associated to stress responses (Mazars et al. 2014) and cell wall remodeling factors (Ferl et al. 2015) have been identified in these studies as the most affected functional groups. A recent study combining RNA-seq analysis (transcriptomics) and protein mass spectrometry (proteomics) provided simultaneous data on protein (peptide) abundance and transcript differential expression, as well as of changes in post-translational modifications of etiolated seedlings grown for 3 days on board of the ISS. Soluble and membrane-bound proteins were analyzed separately (Kruse et al. 2020). The study evidenced that gene expression alone is not enough to draw the full picture of the changes induced by space environment. Post-transcriptional regulatory alterations, especially phosphorylation, constitute a highly significant response, which is not revealed by the mere analysis of gene expression. From a functional perspective, cell wall synthesis and remodeling, microtubule dynamics and its interaction with redox homeostasis, and plastid functions appear as the categories most affected by the spaceflight environment (Kruse et al. 2020).

4.2   The Role of Light in Promoting Adaptation

With the objective of enabling a successful culture of plants beyond Earth, the optimization of plant growth in the microgravity environment of spaceflight, as well as in any other condition of reduced gravity could greatly benefit from the substitution of gravity by another external cue, which could play the same or a similar role in driving plant growth and development as gravity does on Earth. Light is a good candidate to be one of these cues, since it is indeed a tropic stimulus. Phototropism complements gravitropism under normal ground conditions with the objective of optimizing the efficiency of the capture of nutrients. In addition, illumination, especially by red light, is sensed and mediated by phytochromes to produce changes in the regulation of auxin responsive genes and many growth coordinators (Vandenbrink et al. 2014). A specific effect of red light in the activation of cell proliferation and ribosome biogenesis had been previously reported (Reichler et al. 2001). Interestingly, enhancement of light signaling to compensate for the absence of gravity was found to be a spontaneous response of plants to the lack of gravitropic stimuli in spaceflight, as shown by the upregulation of genes associated to plastid functioning, some of them closely related to photosynthesis (Kruse et al. 2020). This response took place even in etiolated seedlings, in the absence of an effective light signal. The culture of seedlings in simulated microgravity under a

photoperiod regime was capable of reverting many of the alterations found on etiolated seedlings incubated in the same facility for microgravity simulation (Manzano et al. 2020b).

In this context, the series of experiments termed the Seedling Growth (SG) Project was conducted in the ISS. Among other objectives, the project intended to investigate to what extent light can act as a signal capable of counteracting the effects caused by the lack of gravity and to determine the combined influence of light and gravity on plant development by paying special attention to the effect of these cues on the root meristem. The project was the result of the cooperation of NASA and ESA, using a European incubator (European Modular Cultivation System, EMCS) (Brinckmann 2005) combined with an American culture chamber for incubation of seeds and growth of seedlings ("Tropi" cassettes) (Kiss et al. 2009). Different collections of mutants of *Arabidopsis thaliana,* affecting the phytochromes, nucleolar proteins and auxin responsive genes were used. Seeds germinated in flight and grew for 6 days under different regimes of illumination and gravity (Fig. 4). In addition to microgravity existing in space, seedlings were subjected to different levels of gravity between 0 g and 1 g, including the Moon and Mars gravity levels, which were produced by a centrifuge installed in the incubator.

The experiments have identified new phototropic responses to blue light in space, which complement former findings obtained in the previous Tropi I and II experiments (Vandenbrink et al. 2016). Actually, blue light is a known source of phototropic stimulus in the Earth (Briggs 2014). In samples grown in space under phototropic stimulation with blue light and under different gravity levels obtained with a centrifuge installed in the EMCS facility, a global transcriptomic study provided a very clear differential transcriptional response to each gravity level from microgravity to 1 g. In the case of the microgravity exposed plants, functions associated with light sensing and response, such as photosynthesis and related factors, appeared downregulated with respect to 1 g controls, suggesting that the growth is not following the phototropic environmental cue in the absence of the gravitropic one. This would mean that gravity responses have an influence on plant development under exposure to directional blue light (Vandenbrink et al. 2019). A similar analysis performed under different levels of gravity, including those corresponding to the Moon and Mars, showed that the effects induced by microgravity were gradually removed by increasing g-load, and that different functions appeared affected at different g-levels. A strong general abiotic stress response was detected at levels lower than the Moon gravity (lower than 0.1 g), probably due to the confluence of different altered stimuli just at the detection threshold of photo- and gravi-sensing mechanisms, which could originate conflicting responses. At higher g-levels, the alteration became progressively weaker; membrane- and cell wall-related genes were the most significant DEGs at the Moon g-level, and similar gene ontologies were observed, but were statistically less relevant at increased g-levels, such as that of Mars (0.38 g). This allowed a discrimination between the differential contribution of the statolith-based gravitropism and other responses based on cell tensegrity that might require a higher g-threshold to operate. In general, blue light
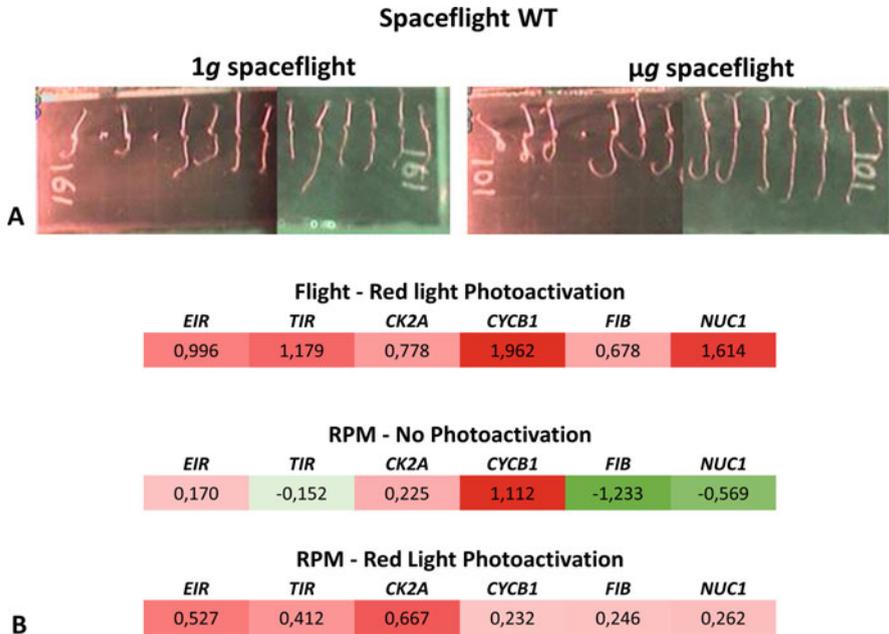
Fig. 4 (**a**) Images of *Arabidopsis thaliana* seedlings, wild-type Ler ecotype, grown for 6 days in the International Space Station (ISS) in the NASA-ESA experiment "Seedling Growth." Seedlings were grown for 4 days under continuous white light and 1 *g*, followed by two days under lateral red light photoactivation (light source at the left side) and either microgravity (μ*g*) or 1 *g*. (**b**) Transcriptomic study, by qPCR analysis from root RNA extracted from samples that were frozen on-board and recovered from the ISS, and from a successive experiment performed on ground, in simulated microgravity, using a Random Positioning Machine (RPM) (Valbuena et al. 2018). "No photoactivation" means that seedlings were in darkness for the two last days of growth. Six genes from three sets were selected as markers of different functions: *EIR* and *TIR* are genes involved in auxin polar transport and perception; Casein Kinase 2 *(CK2A)* and Cyclin B *(CYCB1)* are involved in cell cycle regulation/cell proliferation rate; Fibrillarin *(FIB)*, and Nucleolin 1 *(NUC1)* are involved in the regulation of ribosome biogenesis, indicative of cell growth. Differential expression of these genes was measured in comparison with their respective 1 *g* control (log2Ratio), either in flight or in ground, and expressed with a color code in which red represents upregulation and green downregulation. With red light photoactivation, genes responsible for cell proliferation and cell growth are all upregulated, whereas in darkness, cell proliferation is upregulated and cell growth downregulated. This means that red light photoactivation was capable of restoring meristematic competence, which appeared disrupted in simulated microgravity and darkness, the same as it had been previously found in a former experiment in ISS (Matía et al. 2010). Adapted from Valbuena et al. (2018)

phototropism was found to be capable of reducing the gravitational stress response on orbit (Herranz et al. 2019).

Regarding the effect of red light, a positive effect of photoactivation with this wavelength in counteracting the stress caused by spaceflight on cell growth and proliferation in the root meristem has been found in wild-type samples (Valbuena et al. 2018) (Fig. 4). Unilateral illumination with red light during the last 2 days of

culture, after 4 days of growth under white light photoperiod in the ISS, was capable of reverting (totally or partially) the alterations caused by microgravity on the root meristem. This included re-establishing meristematic competence and auxin transport. The analysis was performed by selecting marker genes for these processes and analyzing by qPCR their differential expression under different conditions of gravity and light. A parallel study on ground using simulated microgravity (RPM) confirmed the spaceflight data (Valbuena et al. 2018) (Fig. 4). In addition, the localization of nuclear proteins and auxin distribution were analyzed by the confocal and electron microscopy. It should be noted that the *in situ* results have been obtained with the plant tissue fixed in aldehyde fixatives on board of the ISS. This experiment has provided a substantial advance, firstly in the technology development with the design of a device to chemically fix samples – Fixbox (Manzano et al. 2020a), and secondly in the understanding of the *in situ* plant response to the microgravity conditions. An auxin accumulation in the root tip was observed, indicating some alterations in the auxin polar transport, confirming previous experiments on simulated microgravity (Herranz et al. 2014).

The use of nucleolin mutants could be integrated in the knowledge of the plant response to spaceflight environment by means of mutational analysis discussed in the preceding section. As previously discussed in this paper, ribosome biogenesis is a cellular process that plays an essential role in plant growth and development, it is a complex stress-sensitive process and it has been found to be seriously altered under conditions of spaceflight and microgravity, both real and simulated. In the process of ribosome biogenesis, nucleolin is a multifunctional nucleolar protein that occupies a central position in its regulation, acting at different steps. Furthermore, two specific features of nucleolin in plants, and particularly in *Arabidopsis thaliana,* provide additional advantages to the use of nucleolin mutants in the SG series of spaceflight experiments. Firstly, plant nucleolin gene expression is known to be upregulated by red light on Earth (Reichler et al. 2001). Secondly, from the two nucleolin genes of *A. thaliana,* one of them, *Nuc2,* is known to participate in the mechanisms of adaptive responses to different stresses (Durut et al. 2019). A global transcriptomic study performed on ground using WT and both *nuc1* and *nuc2* mutant lines, comparing red light photoactivation conditions for 2 days *versus* darkness, has revealed that the capability of the *Nuc2* gene of replacing the functions of *Nuc1* in ribosome biogenesis and cell cycle, when this gene is not present, is enhanced by red light. In addition, photoactivation induces in the *nuc2* mutant an increase in DEGs belonging to functional groups associated to stress response. This means that the *Nuc2* gene may counteract the environmental stress produced by darkness in *nuc1* plants and *nuc2* plants cannot develop a full response to red light (Manzano et al. 2020c).

A full-genome global transcriptomic analysis of red-light-photostimulated plants *versus* plants grown in darkness, in the SG spaceflight experiment, showed that, in photostimulated plants of WT, *nuc1* and *nuc2*, a lower number of genes whose expression is dysregulated by microgravity were obtained, in comparison with plants grown in darkness during the same period. Specifically, the *nuc2* mutant adapted to the microgravity environment by changing the expression of a smaller number of

genes than the wild type. Therefore, a mutant line with this attenuated response may constitute an advantage to be taken into account when selecting the most productive plant varieties for Life Support Systems. The study at the cellular level of the cell cycle and ribosome biogenesis in root meristematic cells resulted in values closer to the 1 *g* control in those samples stimulated with red light.

5   Future Prospects

Most of the plant biology research on ISS has been performed on model plants and there is a lack of fundamental understanding of cultivation and adaptation of crop plants to the space conditions. Nowadays, there is a higher demand for fundamental research that goes beyond the demonstration of plants ability to adapt to the reduced gravity. Bridging the gaps in the knowledge of the acclimation/adaptation mechanisms and effects on the plant development in the space environment through the whole life cycle of a plant, including crop plants, is currently only possible using the ISS research platform and this knowledge is required to ensure reliable and predictable food supplies in future human space exploration. To understand the adaptive processes, long-term responses have to be investigated through sequential studies of plants after different times of exposure to altered gravity and at different phases of the plant development.

Some of the pending basic questions whose answer is necessary to understand how plants acclimate and then adapt to the space conditions refer to our still incomplete knowledge on the signal mechanisms, cell proliferation, cell cycle, cell differentiation, plant tissue, organogenesis and whole plant functioning after the exposure to the spaceflight environment over time during different developmental phases. Together with these topics, other fundamentals aspects of plant biology have been very little or no investigated, such as the maintenance of the plant posture in space and the contribution of proprioception, in comparison with light sensing, to the plant posture in space, the effects on the water transport system and photosynthetic organs and the photosynthetic machinery, the nutrient uptake and transport of water and solutes, the reproductive biology (*e.g.,* sporogenesis, gametogenesis, fertilization, embryogenesis) and the effects of chronic radiation exposure on the plant response to altered gravity.

Different -omics research experiments will play an increasingly chief role in approaching these objectives, but establishing cross-comparisons between the transcriptomic data from different plant spaceflight experiments is now mandatory. New consortia are appearing at the European and International levels (Madrigal et al. 2020; Rutter et al. 2020) to support GeneLab database curation of the data, providing more insight and common criteria for the exploitation and comparison of the datasets. There is also a need for the spaceflight community to be aware of the constraints of the spaceflight research, including a particular care in the preparation and analysis of ground-based reference experiments and controls (Manzano et al. 2020c). Recent reviews present to the scientific community the current opportunities

for space -omics research and discuss how to give to space biology research the opportunity to meet good practice standards similar to those of other plant biology studies, in the attempt to avoid the criticisms that are used from researchers outside of the research field (Overbey et al. 2020; Afshinnekoo et al. 2020).

From an applied point of view, a better understanding of all these topics will pave the road toward bioregenerative life support systems, allowing plants to be used as food for crew and as part of regenerative processes in future missions deeper into space. Additionally, understanding how plants are able to grow in space will have strong synergies with food security and circular economy on Earth.

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