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Root growth direction in simulated microgravity is modulated by a light avoidance mechanism mediated by flavonols

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Abstract

In a microgravity environment, without any gravitropic signal, plants are not able to define and establish a longitudinal growth axis. Consequently, absorption of water and nutrients by the root and exposure of leaves to sunlight for efficient photosynthesis is hindered. In these conditions, other external cues can be explored to guide the direction of organ growth. Providing a unilateral light source can guide the shoot growth, but prolonged root exposure to light causes a stress response, affecting growth and development, and also affecting the response to other environmental factors. Here, we have investigated how the protection of the root from light exposure, while the shoot is illuminated, influences the direction of root growth in microgravity. We report that the light avoidance mechanism existing in roots guides their growth towards diminishing light and helps establish the proper longitudinal seedling axis in simulated microgravity conditions. This process is regulated by flavonols, as shown in the flavonoid-accumulating mutant *transparent testa 3*, which shows an increased correction of the root growth direction in microgravity, when the seedling is grown with the root protected from light. This finding may improve the efficiency of water and nutrient sourcing and photosynthesis under microgravity conditions, as they exist in space, contributing to better plant fitness and biomass production in space farming enterprises, necessary for space exploration by humans.

1 | INTRODUCTION

Plants respond to gravistimulation by perception of the gravity vector in statocytes located in the root tip and in the shoot endodermis. Gravity perception triggers mechanisms that modulate cell expansion, so that the plant organs grow according to the direction of the gravity vector. Root positive gravitropism ensures anchoring in the soil and mechanical support for the whole plant, as well as access to water and nutrient supply. A root constantly perceives the surrounding environment conditions, such as water level, salinity or light, and responds to them by adapting its architecture (Gruber et al., 2013; Kellermeier et al., 2014; Mo et al., 2015; Sun et al., 2008). When environmental conditions change, the root is capable of triggering new mechanisms of response; for example, when cracks in the soil expose roots to light, negative phototropism guides the growth of the root away from the light source (Galen et al., 2007; Rellán-Álvarez et al., 2015). Plants respond not only through tropisms but also by adapting the cell proliferation and differentiation balance; in fact, root growth is the result of the sum of cell proliferation and elongation (cell expansion) (Beemster & Baskin, 1998). A spatial longitudinal organization of the root has been defined, in which different root zones can be distinguished, each one of them characterized by a different major cellular activity, namely cell proliferation (root apical meristem), cell elongation (elongation zone) and cell differentiation (differentiation zone). In addition, a transition zone (TZ) was defined between meristem and elongation (Ivanov & Dubrovsky, 2013). The balance of the relative

contributions of each zone and each cellular activity determines the rate of root growth, being essential for the developmental pattern of the plant. Environmental conditions deeply affect the rates at which the different cellular events occur; consequently, they determine the absolute and relative sizes of the different zones and, eventually, the rate of root growth and development. For example, light and gravity are known to modulate cell proliferation and root meristem size (Matía et al., 2010; Silva-Navas et al., 2016).

The different tropisms, in response to different environmental factors, are mediated and regulated by various signaling molecules, some of them simultaneously involved in multiple tropisms. Factors involved in gravitropic response also participate in photo- and hydrotropisms (Krieger et al., 2016; Zhang et al., 2013). Among these elements, reactive oxygen species (ROS) were identified (Monshausen et al., 2009; Krieger et al., 2016; Ponce et al., 2017), also regulating the transition from proliferation to differentiation in the TZ (Tsukagoshi et al., 2010). Yokawa et al. (2011) reported that illumination of *A. thaliana* roots induced a burst of ROS. Furthermore, upon the illumination, flavonols accumulate at the side closer to the light source in the TZ (Silva-Navas et al., 2016). Light usually does not penetrate deep into the soil (Mo et al., 2015). Nevertheless, heavy rains and cracks during drought may temporarily expose roots to light, which induces the light avoidance tropism. In this context, ROS and flavonols modulate the elongation rate of the roots (García-González et al., 2022; Silva-Navas et al., 2016; reviewed in Cabrera et al., 2022; Lacek et al., 2021), and they take part in the light escape mechanism, which redirects the root towards the dark into the soil (Yokawa et al., 2011).

Plants, like every terrestrial organism, have evolved under the constant level of gravitational acceleration on Earth surface (1 *g*). When exposed to micro- or partial gravity, their physiology is altered and they activate stress response mechanisms (Herranz et al., 2019; Villacampa, Ciska, et al., 2021; Villacampa, Sora, et al., 2021). Understanding the plant response to these environmental conditions is necessary for future space exploration. Microgravity is a minimal gravity environment (below 10^{-3} *g*) (Herranz et al., 2013) that can be obtained in orbit, for example, aboard a spacecraft or a space station, and for a limited period of time during a parabolic flight (20 s) or in drop towers (9 s) (Selig et al., 2010). High costs and logistic constraints highly limit experiments performed in real microgravity. Nevertheless, a number of ground simulators, called Ground-Based Facilities (GBFs), were developed to conduct experiments in simulated microgravity conditions in laboratories. These facilities do not suppress the gravity vector but mitigate the gravity vector effect on the organism by continuously changing its position (reviewed in Herranz et al., 2013). As shown in previous experiments, microgravity not only has a significant impact on plant physiology but also modulates seedling response to other factors, for example light. Specific responses to space environment, such as root positive phototropism to red light (Millar et al., 2010; Vandenbrink et al., 2016) and blue light (Vandenbrink et al., 2016), were reported in real microgravity. At terrestrial gravity level, these root phototropisms were not observed (Millar et al., 2010; Vandenbrink et al., 2016). On the other hand, results from real and simulated microgravity experiments suggest a positive impact of unilateral light exposure on seedling growth and development in comparison to etiolated seedlings. This light condition partly mitigates the negative impact of microgravity on these processes (Manzano et al., 2021; Villacampa, Ciska, et al., 2021; Villacampa, Sora, et al., 2021).

Around half of the plant species present negative root phototropism, while the rest shows no apparent phototropism and a small proportion shows positive root phototropism to unilateral light (Hubert, 1937; Kutschera & Briggs, 2012; Schaefer, 1911). White and blue lights cause negative phototropism and are perceived by the photoreceptor PHOTOTROPIN 1 (Phot1) expressed in the elongation zone of the root (Briggs & Christie, 2002; Liscum & Briggs, 1995; Sakamoto & Briggs, 2002). In addition, a subtle positive red light phototropism mediated by phytochromes present in the root cap was described in *A. thaliana* roots (Kiss et al., 2012; Kiss, Correll, et al., 2003; Kiss, Mullen, et al., 2003). In general, root exposure to light modulates root architecture and response to auxin, as well as certain shoot system features, for example, the flowering time (Silva-Navas et al., 2015).

A key role in the response of roots to light and gravity stimuli is played by flavonols, a subset of molecules belonging to a higher category called flavonoids. Flavonoids are low molecular weight pigments that play multiple functions in plants. Flavonols show a strong relationship with auxin (IAA) (Gayomba et al., 2016; Peer et al., 2013) and abscisic acid (ABA) (Watkins et al., 2014, 2017) and through these relationships, they regulate cell growth, differentiation and adaptation of the plant architecture according to the external stimuli (Brown et al., 2001; Brunetti et al., 2018; Buer & Muday, 2004; Gayomba et al., 2016; Peer et al., 2004). A well-recognized function of all flavonoids is shielding against the UV-B radiation (Bieza & Lois, 2001; Landry et al., 1995; Li et al., 1993), but flavonoid biosynthesis is activated not only by UV-B irradiation but also by blue light (Siipola et al., 2015), salinity (Agati et al., 2011) or copper (Babu et al., 2003). They play a role in defense and are involved in multiple regulatory pathways (reviewed in Falcone Ferreyra et al., 2012). The flavonoid pathway in *A. thaliana* is well characterized and the genes involved are mostly represented in a single copy (Winkel-Shirley, 2001). A set of *transparent testa (tt)* mutants was generated, which include mutations in different enzymes involved in the flavonoid synthesis pathway (Koornneef, 1990; Wisman et al., 1998). The study of *tt* mutants provided a better understanding of different flavonoid functions. Flavonols, such as quercetin, kaempferol and other aglycone molecules synthesized in the early steps of the flavonoid biosynthetic pathway, modulate auxin transport and hence the auxin-dependent tropisms such as root gravitropism, although they are non-essential regulators of these processes (Brown et al., 2001; Jacobs & Rubery, 1988; Lewis et al., 2011). In *tt4* mutant, which does not synthesize flavonoids (Brown et al., 2001; Murphy et al., 2000), auxin transport is elevated and root gravitropism is delayed, an effect that is reversed by complementation with the flavonoid naringenin (Buer et al., 2006; Buer & Muday, 2004). Moreover, quercetin restores the gravitropic response in *pin2* mutant, deficient in the auxin efflux transporter PIN2

(PIN-FORMED 2) involved in basipetal transport (Santelia et al., 2008). MYB Domain Protein 12 (MYB12) regulates flavonol accumulation in the root through two independent pathways; auxin-dependent (TIR-1 [TRANSPORT INHIBITOR RESPONSE 1] mediated) and ethylene-dependent (ETR-1 [ETHYLENE RECEPTOR-1] mediated) (Lewis et al., 2011).

It is a normal procedure of the plant laboratory routine that in vitro seedling cultures are grown in transparent Petri dishes, with roots exposed to light. Thus, it is plausible to assume that this condition alters some aspects of the plant physiology and modulates responses to other stimuli. In fact, two decades ago, Vitha et al. (2000) underlined the impact of root exposure to light on gravitropic responses and stressed that light stimuli should be taken into account in root gravitropism experiments. Subsequently, alternative culture systems, which protect the roots from light, were developed by different researchers (Silva-Navas et al., 2015; Xu et al., 2013; Yokawa et al., 2013). The first attempts to improve the culture system consisted of simply covering the lower part of the Petri dish with light-shielding material (Yokawa et al., 2013). In turn, Xu et al. (2013) developed an improved plant growth system (IPG), in which roots were grown in the dark, but shoots were grown exposed to light outside of the culture plate, which generated different environmental conditions for roots and shoots. Silva-Navas et al. (2015) designed a more advanced Dark-ROOT (D-ROOT) device, which consisted of a methacrylate cover and a comb inserted inside the Petri dish over the seeds to further reduce the exposure of roots to light. In turn, experiments in real or simulated microgravity were generally conducted up to now with seedlings grown either in darkness (etiolated seedlings) or fully exposed to light (white, blue, and red) (Matía et al., 2010; Paul et al., 2017; Valbuena et al., 2018; Villacampa, Ciska, et al., 2021; Villacampa, Sora, et al., 2021). Here, for the first time, we investigate the response to microgravity of the seedlings grown with roots protected from light exposure, similar to the naturally occurring conditions, by using the D-ROOT device. We show that the seedlings grown in these conditions are capable of partly restoring the root growth direction towards the darker section of the plate. Light avoidance, also called escape tropism (negative root phototropism), is the mechanism applied in the restoring of the root growth direction and is mediated by flavonols, as shown in the analysis of the response of *tt* mutants to microgravity in this light condition.

2 | MATERIALS AND METHODS

2.1 | Material and growth conditions

Material: WT Col-0 (sourced from Lehle Seeds, Round Rock, TX); *CYCB1-GUS* (sourced from Nottingham Arabidopsis Stock Centre; NASC, CS68143); *tt3-5* (NASC ID: N799660) and *tt4-11* mutants (NASC ID: N2105573). All lines are in Columbia ecotype background.

Seeds of each genotype were surface-sterilized first with 70% ethanol with 0.01% Triton X-100 for 4 min and then with 95% ethanol for 3 min. A number of 14–15 seeds were positioned at a distance of 7 mm from each other, and 2 cm from the upper edge of a square 12 cm Petri dish. Seedlings were grown on half-strength MS medium (M0221, Duchefa) with 2.56 mM MES (M8250 Sigma–Aldrich), 29.21 mM sucrose (107,651, Merck) and 0.8% plant agar (P1001, Duchefa) for 6 days at 23°C under slow (1 rpm) vertical clinorotation (Villacampa, Ciska, et al., 2021; Villacampa, Sora, et al., 2021) or with the Petri dishes positioned vertically (1 *g* control). Humidity was 65%. Seedlings were grown in three light conditions: whole seedling exposed to light, only shoot exposed to light and root covered from light in D-ROOT device (Silva-Navas et al., 2015), and whole seedling kept in darkness (Petri dish wrapped in aluminum foil). Seedlings exposed to light were grown under a long day photoperiod regime (16 h/8 h). White light was applied with fluorescent light bulbs (Radium Bonalux Super, NL 54 W/840, white, color temperature 4000 K), with luminous flux 4450 lm, and intensity between 80 and 90 μmol s^{-1} m^2. D-ROOT device is a black methacrylate box that fits a 12 cm square Petri dish and a black methacrylate comb that is inserted in the medium over the seeds. It protects the root from light exposure but does not cover the upper part of the Petri dish so that the shoot system is exposed to standard photoperiods. To ensure the germination of the seedlings grown in total darkness, all the seeds were exposed for 30–60 min to white light before the transfer to the germination chamber. Simulated microgravity was obtained by vertical clinorotation of the seedlings on a 2D-clinostat (Villacampa, Ciska, et al., 2021; Villacampa, Sora, et al., 2021) granted to our laboratory by the Zero-Gravity Instrument Project (ZGIP) of the United Nations Office for Outer Space Affairs (UNOOSA).

Pictures of the seedlings were taken immediately at the end of the experiment and samples were processed for corresponding studies.

2.2 | Morphological study

Root and shoot lengths and root gravitropic indexes were measured with ImageJ software (v1.53c). Integral averaged angular declination (Θ) and Gravitropic Index (GI, also called Straightness), Vertical Growth Index (VGI) and Horizontal Growth Index (HGI) were calculated as described by Grabov et al. (2005).

2.3 | GUS staining

For GUS staining, CYCB1-GUS seedlings were fixed in 90% acetone at −20°C for 24–48 h. Next, seedlings were washed three times with 0.1 M sodium phosphate buffer pH 7.2. GUS signal was revealed by enzymatic reaction with 1 mM X-GlcA (X1405, Duchefa), 1 mM potassium ferricyanide (P4066, Sigma–Aldrich), 0.25 mM trihydrate ferricyanide (455,989, Sigma–Aldrich) in 0.05 M sodium phosphate buffer pH 7.2, at 37°C, overnight. Next, samples were washed three times in 0.05 M sodium phosphate buffer pH 7.2, mounted in 85% glycerol and observed under a Leica DM2500 microscope. Staining intensity

was quantified with ImageJ in the meristem area in grayscale (Villacampa et al., 2018).

2.4 | ROS staining (NBT, DAB)

For ROS measurements in the root meristem, we used diaminobenzidine (DAB) and nitro blue tetrazolium (NBT) for staining hydrogen peroxide and superoxide, respectively. In order to measure hydrogen peroxide in the root, seedlings were stained with DAB. Seedlings were incubated with 4.6 mM DAB (D5637, Sigma) in ddH_2O, with pH 3.8. Samples were incubated with vacuum for 10 min in the staining solution and then incubated for 1 h in the dark. Then, samples were incubated in 100, 75, 50 and 25% ethanol, 15 min each. Last, seedlings were incubated 15 min in 25% glycerol and mounted in slides with 85% glycerol.

For superoxide staining, the seedlings were incubated in 2 mM NBT (N6878, Sigma), with 0.1 M sodium chloride in potassium phosphate buffer 20 mM, pH 6.4, in the dark, for 15 min (Dunand et al., 2007). Samples were then processed as for the DAB staining.

2.5 | Confocal microscopy

Briefly, seedlings were fixed in 3% (v/v) formaldehyde for 1 h at room temperature (RT). The fixative was rinsed three times in PBS and then seedlings were digested with digestion solution, containing: 1% (w/v) pectinase, 0.5% (w/v) macerozyme, 0.4% (w/v) mannitol, 10% (v/v) glycerol and 0.2% (v/v) Triton x-100. For cell wall staining, the tissues were stained with SCRI 2200, a cellulose specific stain (Renaissance Chemicals, North Duffield, UK), at 2% with 4 DMSO, for 2 h (Musielak et al., 2015; Robert et al., 2015). Samples were observed in a confocal laser microscope (Leica TCS SP5) with a 40× objective, under UV laser ($\lambda_{em} = 415$ nm, $\lambda_{ex} = 489$ nm).

2.6 | Statistics

Statistical differences were analyzed with SPSS software (v. 25). For normality and homoscedasticity, Kolmogorov-Smirov and Levene tests were used, respectively. ANOVA test was used for statistical differences analysis in normal distributed data, whereas Mann–Whitney *U*-test was used for non-normal distributed data.

3 | RESULTS

3.1 | Seedlings grown in D-ROOT in simulated microgravity partially restored root growth direction

Seedlings were grown in simulated microgravity and control condition (vertically) in three different light conditions: whole seedling exposed to light during photoperiod ("Light"), seedlings grown with the root protected from light exposure ("D-ROOT") or etiolated seedlings ("Darkness"), for 6 days. Representative seedlings are shown in Figure 1A. We have investigated root morphology in detail by measuring four relevant parameters of the "quantification of the root shape" described by Grabov et al. (2005), namely Gravitropic index (GI, also called Straightness, S), vertical growth index (VGI), horizontal growth index (HGI) and integral averaged angular declination (Θ).

VGI is defined as the ratio between the straight-line distance from the base of the root to the root tip (Ly) and the root length (L) (Figure 1B). The higher the VGI value (max 1), the straighter the root. When the root grows straight downward, VGI is equal to $+1$ and, in situations when the root grows upward, VGI is equal to -1. HGI is the result of dividing the distance in the horizontal line between the base of the root and the root tip (Lx) by the root length (L) (Figure 1B). The higher the deviation from the straight line, the closer HGI value is to 1. GI (S) is the ratio between the shortest distance from the base of the root to the root tip (Ld) and the root length (L) (Figure 1B) and, finally, the integral averaged angular declination (Θ) is the angle formed by the vector of gravity and straightened root, with the root tip located at the depth Ly (see Figure 1B).

The indexes described by Grabov et al. (2005) are quality indicators describing a wide range of possible variations in the root morphology. Typical root growth at a wide range of angles (Figure 1A) and the values recorded for the root shape indexes confirmed the lack of gravity-directed root growth in simulated microgravity, as the values of GI and VGI were lower (Figure 1C,D) and the values of HGI (Figure S1) and integral averaged angular declination (Θ) (Figure 1E) were higher than in the control in all light conditions. Additionally, the values of VGI, HGI and Θ were closer to the control values in the D-ROOT grown seedlings than in other light conditions, meaning that the protection of the root from light, while the shoot is exposed to light during the photoperiod, can partly restore the growth of the root towards the darker section of the plate.

3.2 | Light condition influences seedling growth in simulated microgravity

Light conditions, as well as simulated microgravity, are known to influence plant morphology (Manzano et al., 2021; Matía et al., 2010; Silva-Navas et al., 2015; Xu et al., 2013; Yokawa et al., 2013). Nevertheless, the influence of the root exposure to light on the response to simulated microgravity has not been investigated before. We have quantified the root and shoot length of the seedlings exposed to simulated microgravity for 6 days in different light conditions.

The root length was reduced in etiolated seedlings and the length of the shoot highly increased in comparison to samples in other light conditions (Figure 1F,G). Light condition can greatly affect shoot-root communication. In etiolated seedlings, where the shoot is kept in darkness, a developmental program called skotomorphogenesis is activated due to the change in the shoot-root communication. As a consequence, both the root and the shoot lengths are affected, which ultimately leads to root growth arrest (Sassi et al., 2012). Control (1 *g*)

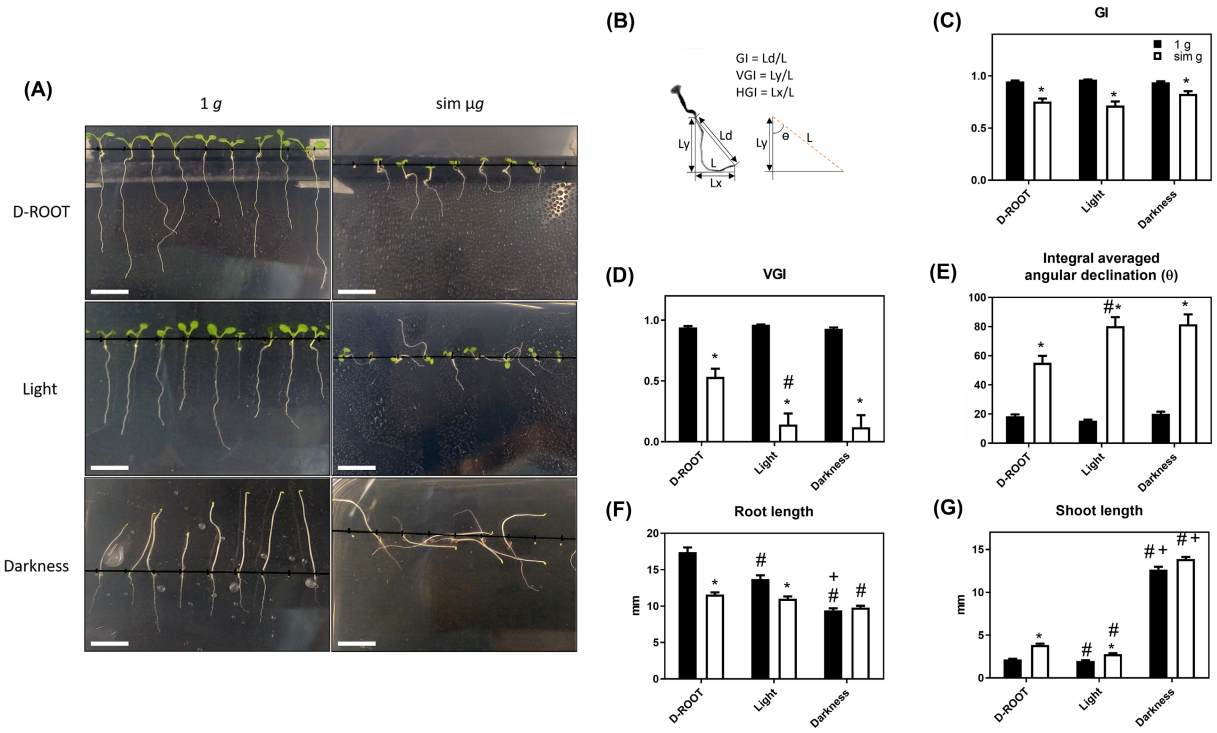

FIGURE 1 Root direction and root and shoot growth in simulated microgravity are influenced by different illumination conditions. (A) Images of 6-day-old seedlings in a Petri dish at the end of the experiment run. Scale bar represents 1 cm. (B) Graphic representation of the different indexes quantified. (C) Gravitropic index (GI) or straightness (S) in the different light and gravity conditions. (D) Vertical growth index (VGI) quantification. (E) Integral averaged angular declination (Θ). (F) Root length quantification in mm. (G) Shoot length in mm.* indicates statistically significant differences compared to the 1 *g* control of the same light condition. # indicates statistically significant differences compared to D-ROOT in the same gravity condition, and + indicates statistically significant differences compared to light in the same gravity condition

D-ROOT grown seedlings presented the longest roots in comparison to other light conditions, which is in agreement with previous reports (Silva-Navas et al., 2015). In the seedlings grown in D-ROOT under simulated microgravity and in the seedlings exposed to light, root length was reduced in comparison to the control (1 *g*, vertical position) (Figure 1F). In etiolated seedlings, the length was already reduced by the absence of light and the difference between simulated microgravity and control was negligible (Figure 1F). These observed reductions in root length could have been even more drastic if a culture medium devoid of exogenous sucrose was used.

Interestingly, in the seedlings grown in D-ROOT and in the seedlings fully exposed to light, the reduction in the root length in simulated microgravity was accompanied by an increase in the shoot length (Figure 1G). This difference was more pronounced in the seedlings grown in D-ROOT. On the other hand, in etiolated seedlings, the shoot elongation caused by the lack of light was also observed in the control group (1 *g*, in comparison to other light conditions) and there was no significant difference between the two gravity conditions.

To investigate the relationship of the decrease in the root length in simulated microgravity in D-ROOT and "Light" conditions with the proliferation activity of root meristematic cells, we measured the size of the root meristem (meristematic zone, MZ), expressed as the total length and the number of cells in the meristematic layer (cortex). To establish the outline of individual cells and determine the size of the meristem and TZ, we have stained the cellular walls with a cellulose-specific stain (SCRI 2200, Renaissance Chemicals; Musielak et al., 2015; Robert et al., 2015) (Figure 2A–C). We have also estimated the proliferation activity in the CYCB1-GUS reporter line exposed to microgravity in different conditions (Figure 2D,E).

Root meristem size and cyclin B1 levels (GUS relative staining) were the lowest in the etiolated seedlings in control and in seedlings exposed to simulated microgravity in comparison to other light conditions. The same trend was observed for the size of the TZ (Figure S2). This suggests that root meristem proliferation activity and, consequently, the rate of root growth were reduced in etiolated seedlings. This is in line with the reduced root length in this light condition. In the D-ROOT grown seedlings, a clear reduction in the proliferation rate was observed in microgravity, as indicated by the decrease in root meristem size (length and the number of meristematic cortex cells Figure 2A–C) and in cyclin B1 levels (GUS stain) (Figure 2E). This is in agreement with the reduced root length in microgravity in this light condition, which suggests that this reduction is caused by the decrease in proliferation activity of the root meristem. In the seedlings fully exposed to light, no significant difference was observed in the meristem and transition zone sizes or in the levels of cyclin B1 (Figure 2E) between the two gravity conditions. It is possible that the reduction of root length in simulated microgravity in this light condition is caused by the reduction in root elongation.

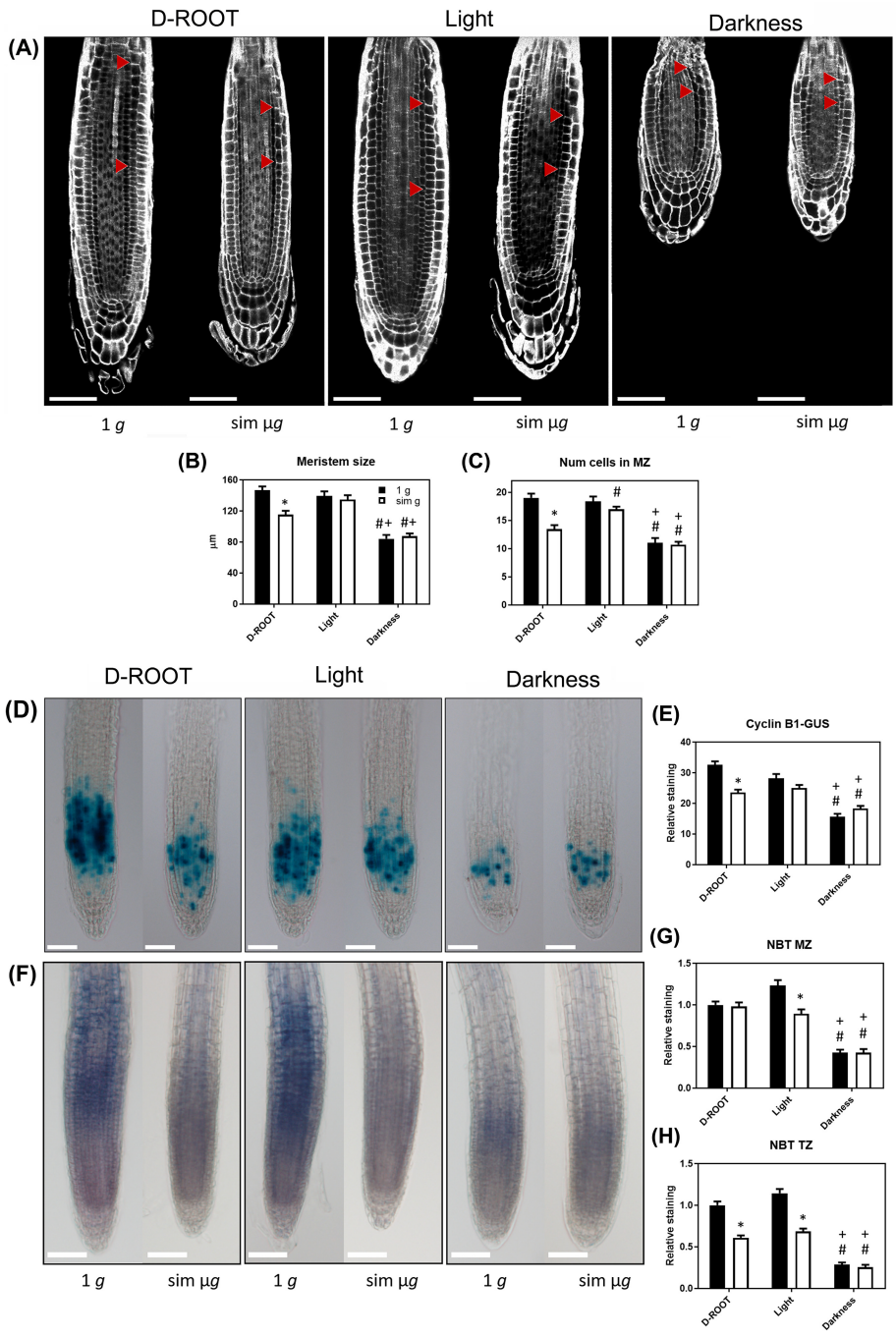

FIGURE 2 Meristem size, B1-cyclin expression and superoxide levels are affected by light conditions. (A) Confocal microscopy images of cell wall-stained roots. Arrows point to the limits of meristems and transition zone (TZ) areas. Scale bar represents 50 µm. (B) Meristem size quantification. (C) Quantification of the number of cells in the meristem row. (D) Optical microscopy images of CYCB1-GUS stained roots. Scale bar represents 50 µm. (E) Relative GUS staining quantification. (F) Optical microscopy images of nitro blue tetrazolium (NBT)-stained roots. Scale bar represents 50 µm. (G) Quantification of relative NBT staining in the meristem zone (MZ). Each run was normalized to D-ROOT 1 *g*. (H) Quantification of relative NBT staining in the TZ. Each run was normalized to D-ROOT 1 *g*. * indicates statistically significant differences compared to the 1 *g* control of the same light condition. # indicates statistically significant differences compared to D-ROOT in the same gravity condition and + indicates statistically significant differences compared to light in the same gravity condition

3.3 | The light condition influences ROS accumulation in simulated microgravity

Peroxidases modulate the transition from proliferation to differentiation in the TZ by regulating the levels of hydrogen peroxide and superoxide (Dunand et al., 2007). Accumulation of superoxide [$O^{2\cdot}$] in the meristem promotes proliferation, and elevated levels of hydrogen peroxide [H_2O_2] promote cellular differentiation (Tsukagoshi et al., 2010). We have measured the levels of $O^{2\cdot}$ by NBT stain and H_2O_2 levels by DAB stain.

The lowest levels of superoxide were found in the meristems (MZ) and TZ of etiolated seedlings in comparison to other light conditions, which is in agreement with reduced meristem size in this light condition. Simulated microgravity caused a decrease in superoxide levels in the MZ and TZ of the seedlings fully exposed to light in comparison to the control (1 *g*) (Figure 2F–H), although a reduction in meristem size was not observed in seedlings exposed to microgravity in this light condition. On the other hand, the levels of the superoxide decreased only in the TZ, but not in MZ, of the D-ROOT grown seedlings exposed to the microgravity (Figure 2F–H), which coincides with the reduction of meristem size observed in microgravity.

No statistically significant difference was observed in peroxide levels in different conditions, except for the decrease in the MZ and

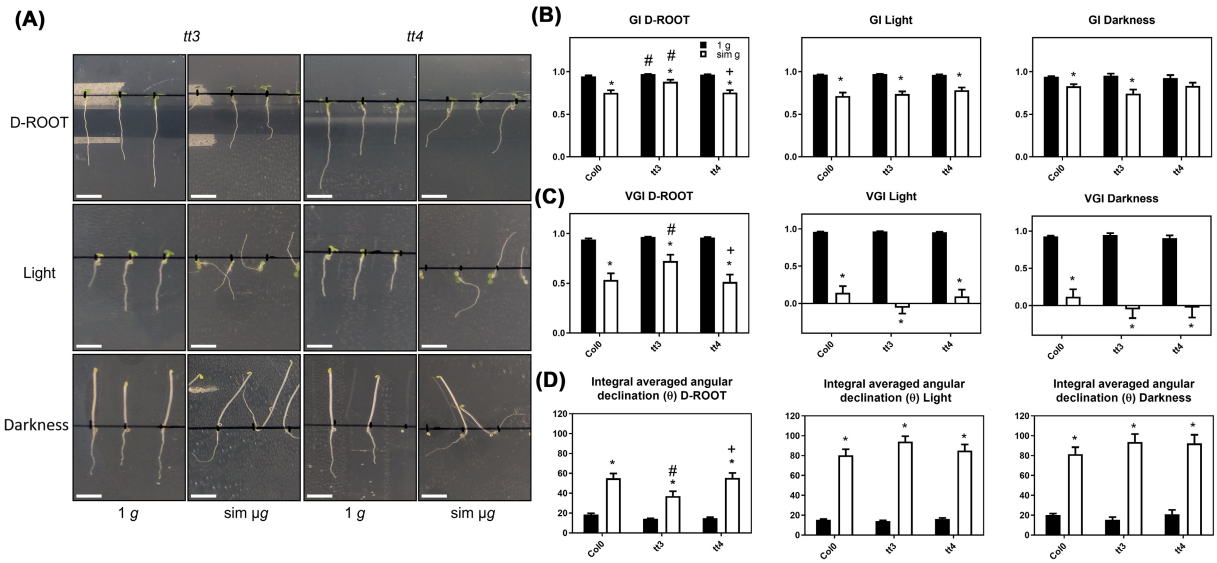

FIGURE 3 Root direction in *tt3* and *tt4* genotypes compared to WT (Col-0). (A) Pictures of seedlings at the end of the experiment run. Scale bar represents 0.5 cm. (B) Gravitropic index (GI) presented by light condition. (C) Vertical growth index (VGI) by light condition. (D) Integral averaged angular declination (Θ) by light condition. * indicates statistically significant differences compared to the 1 *g* control of the same light condition. # indicates statistically significant differences compared to Col-0 in the same gravity condition and + indicates statistically significant differences compared to *tt3* in the same gravity condition

TZ of the etiolated seedlings exposed to microgravity in comparison to the control (1 *g*). In addition, peroxide levels in this sample were lower than in seedlings exposed to microgravity in other light conditions (Figure S3).

3.4 | Flavonoids are implicated in restoring root growth direction in microgravity, as indicated by the growth pattern of *tt3* and *tt4* mutants

Flavonoids modulate multiple processes and tropisms, in particular flavonols through their role as auxin transport inhibitors (Jacobs et al., Jacobs & Rubery, 1988; Brown et al., 2001; Lewis et al., 2011). Flavonoid mutants, *tt3* and *tt4*, indeed display altered auxin transport. The *tt3* mutant contains a mutation in the gene encoding DIHYDROFLAVONOL 4-REDUCTASE (DFR) and accumulates flavonoids. In turn, the *tt4* mutant contains a mutation in the gene encoding CHALCONE SYNTHASE (CHS) and is deficient in flavonoid production (Peer et al., 2004). Furthermore, the *tt4* mutant exhibits delayed gravitropic response in spite of increased auxin transport due to impaired auxin gradient formation (Buer & Muday, 2004; Peer et al., 2004; Peer & Murphy, 2007). To investigate if flavonoids influence the seedling response to microgravity in different light conditions, we have investigated the response of *tt3* and *tt4* mutants to simulated microgravity in three light conditions.

As expected, in simulated gravity, GI presented lower values than controls (1 *g*) in all genotypes and all light conditions, except for etiolated *tt4* seedlings where the difference was not statistically significant (Figure 3B). In the seedlings grown fully exposed to light and in etiolated seedlings, no difference was observed between the *tt* mutants and wild type. Nevertheless, in D-ROOT grown seedlings, an increase in GI value was observed in *tt3* mutant with respect to the wild type in both the control (1 *g*) and in seedlings exposed to microgravity, suggesting that accumulation of flavonoids may mediate the restoration of root growth direction. The value of GI in the D-ROOT grown *tt3* mutant exposed to microgravity was higher and closer to the control value than in other light conditions (Figure S4A), suggesting the positive influence of the root protection from light exposure, together with flavonoid accumulation, on the correction of the root growth direction. This difference between light conditions was not observed in the wild type (Figure 1C) or *tt4* mutant (Figure S4A).

The restoration of the longitudinal axis of the root growth in D-ROOT grown *tt3* mutant was also evident in the VGI values. The VGI value in this sample was higher than in wild type and *tt4* mutant (Figure 3C). In the *tt3* seedlings fully exposed to light and in *tt3* and *tt4* etiolated seedlings, a high number of roots growing upward (negative VGI value) were observed (Figures 3C and S4B). This trend was not observed in the wild type (Figure 1D) or D-ROOT grown *tt* seedlings (Figure S4B).

As expected, we have observed higher values of HGI in all samples exposed to simulated microgravity than in controls and no statistically significant differences were observed between the mutant lines and the wild type or between the light conditions (Figure S5 and S4C).

In line with the trend of correction of root growth direction in D-ROOT grown *tt3* mutant exposed to simulated microgravity, Θ was lower in this sample than in wild type and *tt4* mutant in this light condition (Figure 3D). Nevertheless, this correction was not observed in other light conditions (Figure 3D and S4D), which further suggests that flavonoids in combination with the D-ROOT cultivation system

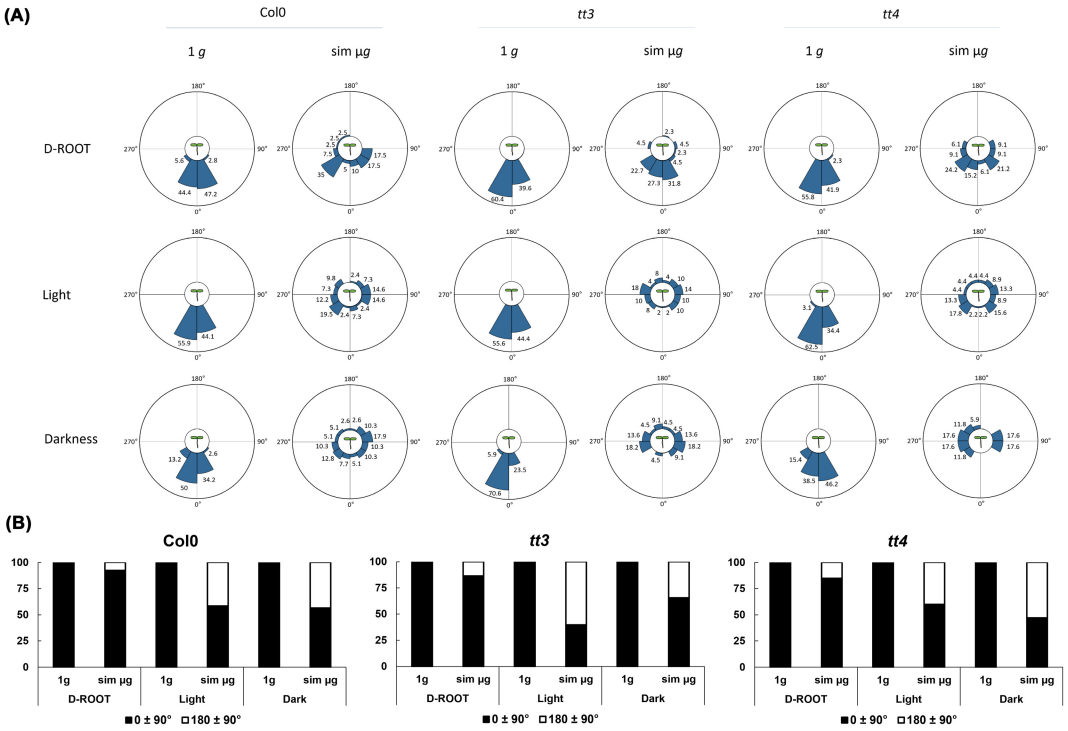

FIGURE 4 Root angle direction. (A) Graphical representation of root direction angle in 30° ranges. (B) Percentage of roots that grew below (0 ± 90°) or above (180 ± 90°) the base of the root

have a positive effect on the root growth direction correction in simulated microgravity.

To better understand how the root growth direction is restored in D-ROOT and the impact of flavonoid accumulation in this process, we have investigated the root angle distribution in each light condition for each genotype. In the control (1 *g*), the distribution of root angle in all light conditions was generally uniform and most of the roots were grouped in the categories 0–30° and 330–360° with respect to the gravity vector. In some genotypes and/or light conditions, a clear preference (generally 0–30° group, except for D-ROOT WT and *tt4* Darkness) was visible. On the other hand, in the samples exposed to simulated microgravity, the angle of the roots presented a wide range of values (Figure 4A). We have observed that light influences the range of root angles in simulated microgravity. In seedlings grown with the root exposed to light and in etiolated seedlings, no clear trend could be distinguished. Nevertheless, in the D-ROOT grown seedlings, some predominant angle categories could be distinguished as representing more than 25% of the total number of roots in the sample. In wild type, 35% of roots are in the 300–330° range, and 35% between 30 and 90°; in the *tt3* mutant, about 32% are between 0 and 30° and 50% between 300 and 360°. However, in *tt4* mutant, no predominant angle categories were found (Figure 4A). On the other hand, the number of roots that grew above the base of the root was much lower in the D-ROOT grown seedlings than in the seedlings grown with the root exposed to light or etiolated seedlings in all genotypes investigated, which is in agreement with the VGI values in D-ROOT samples. We have quantified the percentage of the roots that grow below (0 ± 90°) and above (180 ± 90°) the root base in all the samples (Figure 4B). In genotypes grown in D-ROOT device and exposed to simulated microgravity, the number of roots above the root base was below 20% (7.5% wild type, 13.5% *tt3* and 15.15% *tt4*). In contrast, in the seedlings grown with the root exposed to light (41.5% in wt seedlings, 60.4% in *tt3* and 40% in *tt4*) and etiolated seedlings (43.6% in etiolated wt seedlings, 34.4% in etiolated *tt3* seedlings and up to 52.9% in *tt4* etiolated seedlings), the number of the roots above the root base was higher than 30% (Figure 4B).

Summing up, flavonoid-accumulating *tt3* mutant grown in microgravity in D-ROOT device presents a better correction of the root growth towards the darker side of the plate than WT and flavonoid-deficient *tt4* mutant, as shown by higher GI and lower Θ values. This strongly suggests that flavonoids are involved in restoring root growth direction in this condition.

3.5 | Flavonoids influence the root and shoot growth in simulated microgravity

Root length in the *tt* mutants grown in D-ROOT device and fully exposed to light was significantly reduced in comparison to the wild type in both controls (1 *g*) and samples exposed to microgravity (Figure 5A,B). In addition, the length of the root was reduced in simulated microgravity in wild type and *tt3* D-ROOT-grown seedlings in comparison to the control (1 *g*) (Figure 5A). In the seedlings grown with roots exposed to light, this reduction was only observed in the

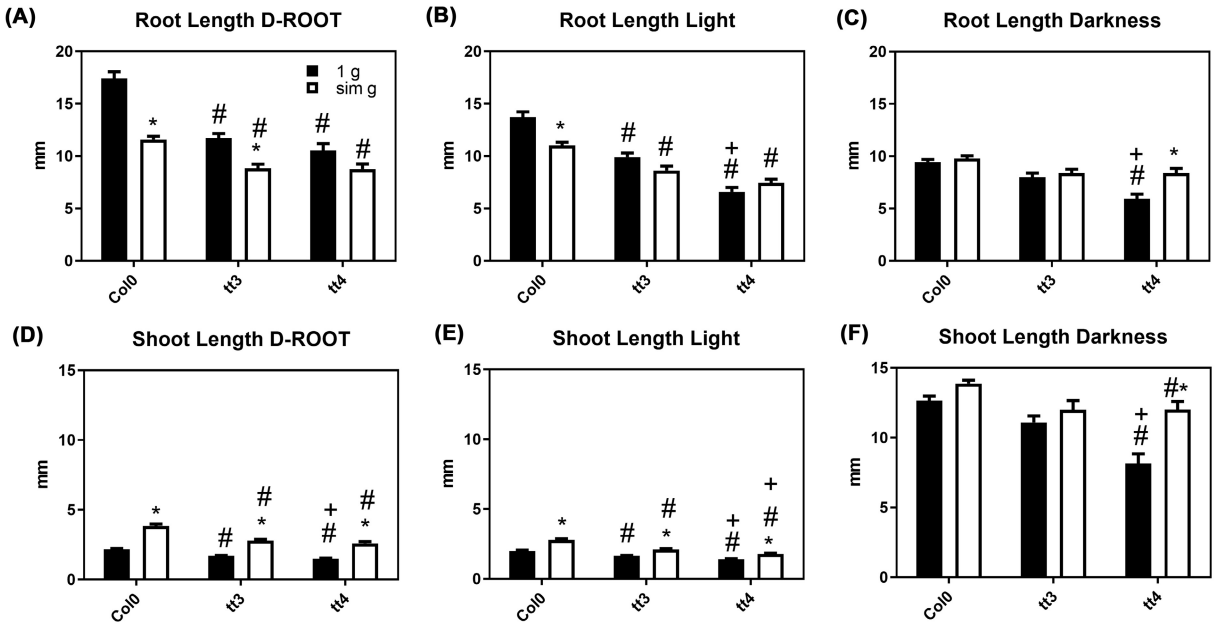

FIGURE 5 Root and shoot length in *tt* mutants compared to WT (Col-0). (A) Root length of the three genotypes in 1 *g* and simulated microgravity grown in the D-ROOT device. (B) Root length in the three genotypes of the seedlings grown with the root exposed to light. (C) Root length in the three genotypes of the etiolated seedlings. (D) Shoot length of the three genotypes in 1 *g* and simulated microgravity grown with the D-ROOT device. (E) Shoot length in the three genotypes of the seedlings grown with the root exposed to light. (F) Shoot length in the three genotypes of the etiolated seedlings* indicates statistically significant differences compared to the 1 *g* control of the same light condition. # indicates statistically significant differences compared to Col-0 in the same gravity condition and + indicates statistically significant differences compared to *tt3* in the same gravity condition

wild type (Figure 5B). In the roots of *tt* mutants exposed to light and D-ROOT-grown *tt4* seedlings, the difference was not statistically significant (Figure 5A,B). On the other hand, in the etiolated seedlings, the root length did not change in simulated microgravity in comparison to the control (1 *g*) in wild type and *tt3* mutant but significantly increased in *tt4* mutant (Figure 5C). Root length in the control sample (1 *g*) in this condition was significantly reduced in comparison to wild type and *tt3* mutant.

Additionally, we have compared the root length between light conditions in each *tt* mutant (Figure S4E,F). The significant root length decrease in microgravity observed in seedlings grown fully exposed to light was observed neither in *tt3* nor in *tt4* (Figure S4E). In the D-ROOT-grown seedlings, the reduction in root length in microgravity was observed in *tt3* but not in *tt4* mutant (not statistically significant) (Figure S4E).

On the other hand, the shoot length increased in microgravity in all light conditions, except for etiolated wild type plants and *tt3* (Figure 5D–F). Similar to the trend observed for the root length, the shoot length in the *tt* mutants was significantly decreased in comparison to the wild type in the D-ROOT-grown seedlings and seedlings fully exposed to light (Figure 5D–F). As in the wild type, etiolated *tt* mutant seedlings (control and simulated microgravity samples) displayed longer shoots than seedlings in other light conditions (Figure S4F).

Summing up, *tt* mutants displayed reduced root and shoot length when exposed to light or grown in the D-ROOT device. As in wild type, the reduction in root length was observed in microgravity in all D-ROOT-grown genotypes. The shoot length increase in microgravity was a clear trend in all genotypes in D-ROOT-grown and fully exposed to light seedlings, and in *tt3* mutant etiolated seedlings.

4 | DISCUSSION

The response of plants to microgravity in early developmental stages has been extensively studied, but exclusively in seedlings grown in full darkness or fully exposed to light. Gravitropism and phototropism guide the root and shoot growth from early embryogenesis to establish the longitudinal axis of the seedling (Kircher & Schopfer, 2012; Abbas et al., 2015; Lee et al., 2017; Zhu and Gallem, Zhu et al., 2019). This enables the penetration of the root deep into the soil, which provides anchor and access to water and nutrient (macro and microelement) supplies. In microgravity, a condition of near-zero gravity, or when the gravity vector is averaged (simulated microgravity), seedlings are not able to establish a well-defined longitudinal axis, which can deeply affect not only the morphology but also the intake of water and nutrients. Phototropism can be applied to guide the establishment of a longitudinal axis if a unilateral light is applied (Manzano et al., 2021). Nevertheless, the exposure of the root to light has a negative effect on the plant's physiology. We have investigated how having root protected from and the shoot exposed to light influences the

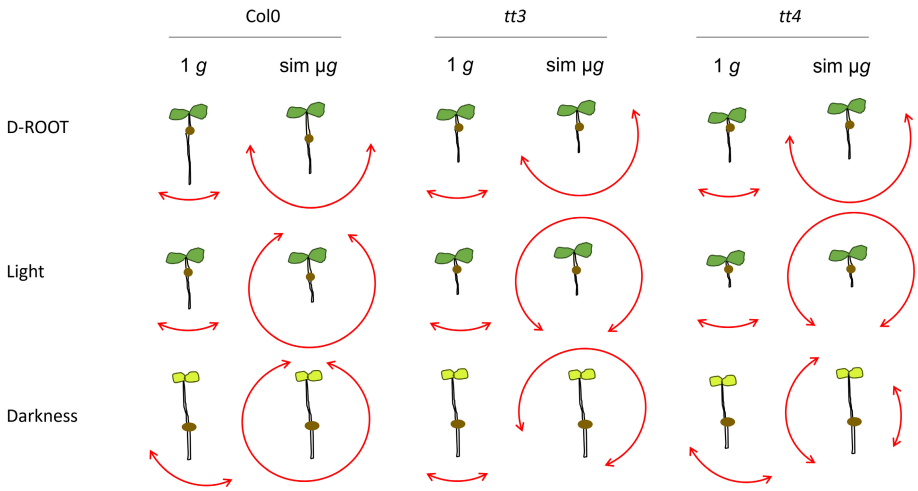

FIGURE 6 Seedling growth patterns in the different light and gravity conditions, and the different genotypes. Growth patterns, shoot and root length, automorphogenesis (green leaves) or skotomorphogenesis (yellow leaves) and the most common root angle are represented for each condition. Red arrows represent >90% of the root angle in each experimental condition and genotype

growth and the response to simulated microgravity of seedlings. The scheme in Figure 6 sums up our results.

The intimate interaction between gravi- and phototropism has been recognized in multiple studies (Kiss, Correll, et al., 2003; Kiss, Mullen, et al., 2003; Manzano et al., 2021; Okada & Shimura, 1992; Vitha et al., 2000). Gravitropism is generally the dominant tropism in the root (Kiss, Correll, et al., 2003; Kiss, Mullen, et al., 2003). Nevertheless, when the root is exposed to strong unilateral blue or white light, negative phototropism takes over to guide the root away from the light source (Darwin, 1880; Kutschera & Briggs, 2012; Okada & Shimura, 1992; Pfeffer, 1904; Strasburger & Karsten, 1911; von Sachs, 1879). Silva-Navas et al. (2015) demonstrated that illuminated roots respond faster to gravitropic stimuli and this is a result of modulation of auxin signaling by light. On the other hand, Kimura et al. (2018) reported that root phototropism in *A. thaliana* does not require asymmetric auxin distribution and that, in agravitropic *phosphoglucomutase-1* (*pgm-1*) mutant, phototropic curvatures of the roots were enhanced.

Still, prolonged root exposure to light has a clear negative effect on growth, as it reduces the primary root length and meristem size (Ha et al., 2018; Silva-Navas et al., 2015, 2016). Root illumination also generates a stress that might be additive to other stresses, such as osmotic and salt stress or nutrient deprivation (Silva-Navas et al., 2015; Yokawa et al., 2014). In effect, cell proliferation is decreased in a mechanism dependent on flavonols (Silva-Navas et al., 2016). This is in agreement with our results (Figure 6), which show that root length was reduced in seedlings fully exposed to light in comparison to the seedlings grown with the root protected from light exposure. Furthermore, in the latter light condition, negative root phototropism partially restores the longitudinal axis of root growth in microgravity. In addition, exogenous sucrose supplementation, as in our conditions with 1% (w/v) sucrose in the medium, can influence root growth and directional root growth adaptation in different illumination conditions (García-González, Lacek, & Retzer, 2021; García-González, Lacek, Weckwerth, et al., 2021; García-González et al., 2022; Miotto et al., 2021). This could influence the root growth and elongation in the different light conditions, especially in etiolated seedlings where roots are expected to be more reduced in the absence of sucrose supplementation (García-González, Lacek, & Retzer, 2021).

Our results show that etiolated seedlings present altered morphology and the lack of light has a strong impact on seedling development. Low proliferation activity, as shown by reduced meristem size, cyclin B1 expression and superoxide levels, was observed in control (1 *g*) and simulated microgravity-grown etiolated seedlings. Seedlings grown in total darkness present a specific growth pattern, called skotomorphogenesis, that is a developmental program executed in the absence of light. It is characterized by increased hypocotyl elongation and reduced cotyledon and root growth (reviewed by Josse & Halliday, 2008).

In natural conditions, when seeds germinate in soil, skotomorphogenesis is activated early in the plant development and assures the protection of the shoot apical meristem and cotyledons or primary leaves by the apical hook formation before they reach the light source and undergo photomorphogenesis (Alabadí et al., 2004; Lee et al., 2017). Recently, it has been reported that early gravitropic root response regulates this process through auxin gradient formation that exceeds the root/shoot barrier and extends into hypocotyl (Zhu et al., 2019). During this early developmental stage, the limited resources in the seed are dedicated to hypocotyl growth to reach the light and initiate photosynthesis, whereas the growth of the root is inhibited. When photosynthesis is initiated, the generated sucrose mediates and fuels root growth to source water and nutrients from the soil (Kircher & Schopfer, 2012). Whereas the growth of the seedlings in darkness or fully exposed to light respectively promotes skotomorphogenesis or photomorphogenesis, the growth of the seedlings in the D-ROOT device enables the fulfillment of the two subsequent developmental stages. This is in agreement with the phenotypes observed in different light conditions under normal gravity conditions.

Gravitropism and the subsequent formation of the auxin gradient early in the development have an essential role in determining the direction of growth of both the shoot and the root. Therefore, it is not surprising that, in microgravity, the seedling, without any other cue to

guide organ growth, is not able to establish the proper longitudinal axis. The development in the minimal gravity condition (real or simulated) is called automorphogenesis or automorphosis (Driss-Ecole et al., 2008; Hoson et al., 1995; Stanković et al., 1998). It consists of spontaneous curvature and/or altered growth direction of roots and shoots, possibly due to differences in cell wall properties between two sides of the organ (Hoson & Soga, 2003). Our results suggest that providing a diminishing light gradient during seed germination and seedling growth can be enough to stimulate a proper root growth direction through photomorphogenesis. Previous results suggest that providing unilateral light source above the seedlings in microgravity was enough to restore the direction of hypocotyl growth towards the light (Manzano et al., 2021). Nevertheless, prolonged exposure of the root to light has a negative impact on root growth, as shown by ours and previous results (Silva-Navas et al., 2016) and, therefore, a system where the root is protected from light exposure should be applied to grow plants in minimal gravity environment.

Surprisingly, a growth pattern similar to skotomorphogenesis, with increased hypocotyl length and decreased root length, was observed, not only in etiolated seedlings but also in both light and D-ROOT-grown seedlings exposed to microgravity (Figure 1F). This phenotype was also observed previously in both real (Soga et al., 2002, 2018) and simulated (Lionheart et al., 2018; Polinski et al., 2017) microgravity. It was accompanied by a reduction in chlorophyll biosynthesis measured by gene expression levels or chlorophyll content (Colla et al., 2007; Laurinavichius et al., 1986; Rumyantseva et al., 1990; Vandenbrink et al., 2019; Villacampa, Ciska, et al., 2021; Villacampa, Sora, et al., 2021). It is possible that this phenotype is related to the induction of hypoxia-related pathways in microgravity, which has been observed in multiple studies (Basu et al., 2017; Choi et al., 2019; Johnson et al., 2017; Kruse et al., 2020; Kwon et al., 2015). Hypoxia is known to induce skotomorphogenesis (Abbas et al., 2015). It is possible that the mechanisms activated in response to microgravity overlap with mechanisms induced during hypoxia. Moreover, the plastid genome overexpression found in multiple transcriptomic studies of *A. thaliana* seedlings grown in space (Angelos et al., 2021; Kruse et al., 2020; Vandenbrink et al., 2019; Villacampa, Ciska, et al., 2021; Villacampa, Sora, et al., 2021), suggests an affected retrograde signaling, which is a critical step in chloroplast development and maintenance. Impairment of chloroplast development in microgravity could also lead to skotomorphogenesis-like growth pattern (Vinti et al., 2000).

ROS play an important role in the regulation of proliferation, differentiation (Liu et al., 2015; Tsukagoshi et al., 2010), response to biotic and abiotic stresses (Cruz De Carvalho, 2008; Kwak et al., 2003; Sharma & Dietz, 2009) and multiple tropisms, such as gravi-, photo- and hydrotropism (Joo et al., 2001; Krieger et al., 2016; París et al., 2018; Peer et al., 2013). Nevertheless, apart from being signaling molecules, ROS are also strong oxidizers that react with biological molecules and may cause severe damage to plant tissues (Petrov & Van Breusegem, 2012). Flavonoids (particularly flavonols) act as ROS scavengers that neutralize the excess of ROS. The balance between both factors is needed for proper developmental and physiological responses (Cruz De Carvalho, 2008). When roots are illuminated, a burst of ROS (Yokawa et al., 2011) followed by accumulation of flavonols on the illuminated side takes place (Silva-Navas et al., 2016). Silva-Navas et al. (2016) demonstrated that flavonols play a role in the root light avoidance mechanism and that the root angle in mutants deficient in flavonoids (*tt4*) or accumulating these components (*constitutive photomorphogenesis 1–4*; *cop1-4*), exposed to unilateral light, was reduced, probably due to the inability to form a flavonol gradient. Flavonols, in particular quercetin, are natural inhibitors of auxin transport (Gayomba et al., 2016; Peer et al., 2013) and, through this mechanism, they regulate cell growth and differentiation (Brunetti et al., 2018; Gayomba et al., 2016). In *tt4* mutant, polar auxin transport is enhanced, and in *tt3* repressed (Peer et al., 2004). The disrupted auxin transport could cause the reduction in shoot and root growth in *tt* mutants in D-ROOT-grown seedlings and the ones fully exposed to light. Moreover, flavonols modulate gravi-, photo- and thigmotropism through their function as an auxin transport inhibitors. Flavonoid-deficient *tt4* mutant exhibits delayed gravitropic bending (Buer & Muday, 2004; Peer et al., 2004; Peer & Murphy, 2007) and also light-regime-dependent root coiling (looping) when grown on slanted hard-agar surface (Buer & Djordjevic, 2009). We have shown that *tt3* mutant, with higher flavonoid levels (*tt3-5* allele, see Kuhn et al., 2011 for a description of its specific features), presents enhanced root growth towards the darker side of the plate in the D-ROOT-grown seedlings, which strongly suggests that the light avoidance mechanism regulated by flavonols (Silva-Navas et al., 2016) is involved in this growth pattern correction.

On the other hand, flavonoids do not seem to regulate mechanisms involved in the appearance of skotomophogenesis-like phenotype in microgravity, as the increase in the shoot length in microgravity is observed independently to flavonoid levels (in *tt3*, flavonoid-accumulating, and *tt4*, flavonoid-deficient mutants). Nevertheless, flavonoids could modulate the root growth in response to microgravity when the root is exposed to light, since we did not observe a significant reduction in the root length in either *tt* mutant fully exposed to light.

Our results show that, when the plant is unable to perceive the gravity vector and a diminishing light gradient is applied, it directs the root growth according to light avoidance tropism and is able to restore the direction of the root growth. This response is regulated by flavonols. Our findings show the necessity to protect the roots from light when grown in microgravity conditions, for example, in the International Space Station, to assure the establishment of the longitudinal axis of the seedling and the efficient water and nutrient uptake from the soil or medium. The application of this cultivation system in "space farming" could provide an optimal seedling development and growth in the space environment, to be used for life support in human space exploration enterprises.

AUTHOR CONTRIBUTIONS

Conceptualization: Malgorzata Ciska and F. Javier Medina; methodology: Alicia Villacampa, Malgorzata Ciska; validation: Alicia Villacampa, Iris Fañanás-Pueyo, and Malgorzata Ciska; formal analysis: Alicia

Villacampa and Iris Fañanás-Pueyo; investigation: Malgorzata Ciska and F. Javier Medina; data curation: Alicia Villacampa; writing—original draft preparation: Malgorzata Ciska and Alicia Villacampa; writing—review and editing: Malgorzata Ciska, F. Javier Medina, Alicia Villacampa, and Iris Fañanás-Pueyo; visualization: Alicia Villacampa, Iris Fañanás-Pueyo, and Malgorzata Ciska; supervision: Malgorzata Ciska and F. Javier Medina; project administration: F. Javier Medina; funding acquisition: F. Javier Medina. All authors have read and agreed to the published version of the manuscript.

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DATA AVAILABILITY STATEMENT

The data supporting the findings of this study are available from the corresponding author, (F. Javier Medina), upon request.

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