

A reduced aquatic angiosperm lacking canonical gravitropism genes retains robust gravity-responsive transcriptional programs

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Abstract

Gravitropism is the ability of plants to sense and respond to gravity, a fundamental trait ensuring proper orientation of roots and shoots. While well-characterised in terrestrial plants, how aquatic species respond to altered gravity remains unknown. *Wolffia australiana*, one of the smallest known flowering plants, is morphologically reduced, rootless, and fast-growing, making it an ideal model for studying plant adaptation under extreme space-like conditions. We examined *W. australiana*'s growth, morphology, and transcriptomic responses under different gravity levels (μ g, 0.16g, 2g and 4g) using random positioning machines and a large-diameter centrifuge. Lunar gravity increased growth rate, while hypergravity suppressed it. Morphological changes in mother fronds were observed only under hypergravity. Transcriptomic profiling revealed that ~ 25% of genes were differentially expressed under low gravity, compared to ~ 7% under 2g and ~ 11% under 4g. Despite lacking canonical gravity-sensing genes, *W. australiana* exhibited distinct and coordinated transcriptional responses across treatments. Notably, circadian clock genes and those with time-of-day (TOD)-dependent expression were significantly overrepresented among gravity-responsive genes; many of these were enriched in the epidermis. These findings highlight *W. australiana*'s unique transcriptional and morphological plasticity, suggesting alternative gravitropic mechanisms with direct implications for space agriculture.

Summary

- *Wolffia* growth rate is increased by low gravity (simulated Moon gravity) while decreased by high gravity (hypergravity).
- *Wolffia* mother fronds morphology is responsive to hypergravity but not simulated low gravities.
- ~25% of genes are differentially expressed under low gravity while high gravity results in ~7% for 2g and ~11% for 4g.
- Low gravity (simulated gravity) resulted in coordinated transcriptional changes with the upregulation of growth and biosynthetic genes and downregulation of stress response, homeostasis, and light response genes.
- High gravity (hypergravity) resulted in coordinated downregulation of certain surface protection and barrier biosynthesis genes and uncoordinated upregulation of stress response genes at 2g versus assembly and secondary metabolite biosynthesis genes at 4g.
- Circadian clock genes and genes that have TOD expression are overrepresented in the gravity related DEGs.
- Genes responsive to gravity in *Wolffia* are more often found in the epidermis.

Introduction

The study of gravitropism, the process by which plants orient their growth in response to gravity, has intrigued scientists since the time of Darwin, who explored this fundamental plant behavior in his seminal work “The Power of Movement in Plants” (Darwin & Darwin, 2022). Over the years, research has

steadily advanced our understanding of how plants perceive and respond to gravitational cues, primarily through experiments conducted under Earth's conditions. The unique conditions of space environment offer an unparalleled platform to deepen our understanding of gravitropism. Moreover, the rapid progress in space exploration has opened a new window of opportunity to extend this research beyond our planet. Research aimed at understanding the effects of space-related factors on plant biology is fundamental to the success of long-term space missions, such as Artemis III, which seek to establish a sustainable human presence on the Moon and, ultimately, Mars (Romano & Aronne, 2021). In this context, conducting complementary Earth-based studies prior to spaceflight experiments is essential for reducing costs, accelerating discovery, and refining experimental approaches before deployment in space. Understanding the mechanisms of gravitropism under simulated microgravity and partial gravity conditions on Earth is necessary for the successful integration of plants into bioregenerative life support systems (BLSS), where they will play a critical role not only as a food source but also in the recycling of air and water within closed-loop ecosystems (Izzo et al., 2023; Gòdia et al., 2002; Santomartino et al., 2023; Romano et al., 2024).

In recent decades, growing interest in plant adaptation to the space environment has brought renewed scientific attention to how plants respond to gravity and to microgravity conditions in its absence. Gravitropism is a complex plant mechanism referring to the orientation of growth toward gravity, involving an intricate process starting from gravity perception, signal transduction, to growth responses (Baldwin et al., 2013; Hashiguchi et al., 2013). In most higher plants, this is accounted for by gravity perception through sedimentation of amyloplasts in the specialized cells, such as the columella cells in roots and endodermal cells in shoots (Morita, 2010). Such sedimentation provokes a signaling cascade, from actin cytoskeleton rearrangement and mechanosensitive ion channels activation to changes in secondary messengers like inositol-1,4,5-triphosphate (InP3), calcium ions (Ca²⁺), and pH (Fasano et al., 2001; Plieth & Trewavas, 2002; Toyota & Gilroy, 2013). Especially important in this context is the redistribution of the phytohormone auxin, mediated by auxin transport proteins such as PIN-FORMED (PIN) efflux carriers and the AUXIN RESISTANT 1 (AUX1) influx transporter. This redistribution sets up asymmetries in growth rates on either side of the plant organ, which allow the plant to redirect itself (Marchant et al., 1999; Band et al., 2012). Genes encoding auxin-responsive factors (ARFs) and other related transcriptional factors are responsible for this gravitropic response (Kimbrough et al., 2004; Zhang et al., 2019). This highly streamlined genetic network, therefore, enables the plant to detect and respond to changes within its gravitational environment.

Interactions between plant gravitropism and altered gravity conditions have been studied extensively, primarily using the model species *Arabidopsis thaliana* (Fitzelle & Kiss, 2001; Kiss, 2010), but also in a range of crop species in anticipation of their incorporation into BLSS and their use as food sources for space crews (Aronne et al., 2020; Izzo et al., 2022). More recently, members of the family Lemnaceae, including *Wolffia arrhiza* and *Wolffia globosa*, which have been consumed in Southeast Asia for centuries under names such as Khai Nam, have emerged as promising candidates for sustainable “superfood” production on both Earth and in space due to their rapid growth rates, high protein content, and minimal cultivation requirements (Yuan & Xu, 2017; Romano & Aronne, 2021; Pietryka et al., 2022; Romano et al.,

2024). *Wolffia* sp. are recorded as the smallest flowering plants, and their adaptability is widespread across different environments of the world (Pietryka et al., 2022). Nutritional value combined with the small size makes this species most suitable for BLSS, which will be able to meet the astronauts' dietary needs at ease and contribute most effectively in resource recycling efficiencies (Yuan & Xu, 2017; Romano & Aronne, 2021). Moreover, these *Wolffia* have evolved into a simplified plant system, lacking common organs such as leaves, stems and roots. Therefore, to fully realize their potential in such complex systems, it is important to understand how these plants respond under the unusual conditions of space. Within the *Wolffia* genus, *Wolffia australiana* was chosen for these studies due to the growing number of resources emerging for this species, as well as its small genome (~ 350 Mb) and streamlined transcriptome (> 20k genes) (Reinard et al., 2020; Lam & Michael, 2022). Additionally, it is noteworthy considering that recent genomic studies on *W. australiana* revealed gene losses of gravity-sensing proteins, pointing out that this species might possibly utilize alternative or simplified pathways for gravitropic response (Michael et al., 2020). Plants of *W. australiana* exhibit complex transcriptional responses to altered gravity environments, such as microgravity and partial gravity, which are critical for understanding plant adaptation in space (Sathasivam et al., 2021; Land et al., 2023). All this, together with its small structural complexity, makes *W. australiana* a very compelling model for studying plant gravitropic reaction.

Here, we bridge the gap of knowledge using the genetic responses of *W. australiana* to understand gravitropic response under varied gravity conditions, including simulated Moon-like partial gravity, simulated microgravity, and hypergravity. These environments can simulate the numerous gravitational forces experienced in the course of a space mission, ranging from the microgravity of orbit to the hypergravity experienced when spacecraft take off and land (Jamšek et al., 2021; Hosamani et al., 2022). Exploring a broader gravity spectrum also addresses the gravity continuum paradigm (van Loon, 2016). The core mechanisms and genetic components underlying plant gravitropism are well established, and more recent studies have extended these analyses to plants exposed to altered gravity environments, including microgravity and hypergravity (Su et al., 2017; Nie et al., 2025). We leveraged this body of work to examine how gravity-related genes behave in *Wolffia* under both micro- and hypergravity conditions. Specifically, we report on how different conditions in gravity affect the growth, morphology, and genetic expression of *W. australiana* using random positioning machines and a large-diameter centrifuge. We reasoned that understanding the responses of *W. australiana* to altered gravity conditions would not only provide insight into the evolutionary adaptability of this species during the transition from terrestrial ancestors back to aquatic environments, but also help inform the development of sustainable plant-based agricultural systems for future space missions.

Results

RGR and DT for Control vs Simulated gravities (sim- μ g, sim-Moon)

The relative growth rate (RGR) of *Wolffia australiana* clone 8730 (wa8730) was studied under different conditions: simulated microgravity (sim- μ g), Moon partial gravity (sim-Moon), and tested against the control (LowGravityControl) group (Fig. 1A). The results from the one-way ANOVA revealed significant differences in both RGR ($F = 5.526$, $p = 0.016$) and doubling time (DT) ($F = 5.289$, $p = 0.018$). Plants grown under sim-Moon conditions had a significantly higher mean RGR (0.25 ± 0.021) compared to the control group (0.22 ± 0.011) and the sim- μ g group (0.21 ± 0.022). Similarly, DT, which is derived from RGR, showed the same trend. The sim-Moon group exhibited the average shortest DT (2.76 ± 0.22 days), while the sim- μ g group had the longest DT (3.19 ± 0.31 days). Further analysis using Student-Newman-Keuls *post hoc* tests identified two distinct groups: the first containing the LowGravityControl and sim- μ g ($p = 0.797$ for RGR) and the second for the sim-Moon group, which was statistically distinct from the others.

RGR and DT for Control vs. Hypergravity (2g and 4g)

The RGR and DT of wa8730 were examined under hypergravity conditions (2g and 4g) and compared against their control group (HighGravityControl). One-way ANOVA revealed significant differences in both RGR ($F = 6.409$, $p = 0.010$) and DT ($F = 5.742$, $p = 0.014$). *W. australiana* plants grown under the hypergravity condition showed a notable decrease in RGR. More specifically, those grown under 2g conditions had a mean RGR of 0.25 ± 0.029 , significantly lower than the control group's RGR of 0.30 ± 0.013 . Under 4g conditions, the RGR was 0.27 ± 0.026 , which, while still significantly lower than the control. Similarly, DT followed the same pattern. The control group had the shortest DT at 2.33 ± 0.10 days. In contrast, DT increased under hypergravity, with the 2g condition resulting in the longest DT at 2.82 ± 0.34 days and the 4g condition showing a DT of 2.57 ± 0.24 days. *Post hoc* analysis using the Student-Newman-Keuls *post hoc* tests in both ANOVA analyses identified two homogeneous subsets. For RGR, one subset included the 2g and 4g treatments ($p = 0.114$), while the second included the 4g and control group ($p = 0.077$), indicating that the 4g condition is intermediate. (Fig. 1B).

Morphological comparison of Long and Short Axis Lengths and Ratios among Simulated gravities (sim- μ g, sim-Moon) and Hyper-gravities (2g and 4g)

Morphological parameters of the plants grown under different gravity levels were also measured. Specifically, the morphological changes in the long and short axes of the mother fronds of wa8730 plants were compared between the 1g control group (LowGravityControl) and two simulated gravity conditions (sim- μ g, sim-Moon). Results showed that the differences in morphological parameters (long and short axis) between the different simulated gravity treatments (sim- μ g, sim-Moon) compared against their control were not statistically significant (Table 1). The measured parameters for the control group for long and short axis lengths were 0.95 mm and 0.59 mm, respectively. In the sim- μ g condition, these lengths were slightly reduced to 0.93 mm and 0.58 mm, respectively. Under sim-Moon conditions, the long and short axis lengths were 0.97 mm and 0.60 mm, respectively. Comparisons of the ratios in which the two measured sides of the mother fronds are in relation were calculated. Results of the ANOVA showed that the long side ratio did not show significant differences across treatments for the Long side ratio ($F = 0.063$, $p = 0.939$) and for the Short side ratio ($F = 0.042$, $p = 0.959$). These findings

suggest that simulated gravity conditions do not significantly alter the proportional dimensions of the fronds.

The morphological data for wa8730 show differences in the long and short axes of the mother fronds when grown under hypergravity conditions (2g and 4g) compared to the control group (HighGravityControl). Results of the one-way ANOVA highlighted the morphological differences between plants affected by the hypergravity treatments and the control group HighGravityControl (1g). More specifically, the measured parameters for the mean long axis of the HighGravityControl were 0.95 mm and the mean short axis length of 0.59 mm. Under 2g hypergravity, the long axis length increased to 1.03 mm; under 4g hypergravity, it further increased to 1.06 mm. The short axis lengths also increased slightly, reaching 0.60 mm under 2g and 0.62 mm under 4g. Unlike for the simulated gravities, the morphological shift in the measured parameters for the Hypergravity conditions manifested significant differences for both the Long Side ratio ($F = 3.578$, $p = 0.033$) and the Short Side ratio ($F = 3.643$, $p = 0.031$). For the Long Side ratio, the control group had a mean ratio of 1.62, which increased to 1.73 under both 2g and 4g hypergravity conditions. This indicates that hypergravity caused an elongation of the long axis. The Student-Newman-Keuls *post hoc* test confirmed significant differences between the control and hypergravity treatments. The short side ratio of the Hypergravity treatment showed that the control group had a mean ratio of 0.62, which decreased to 0.58 under both 2g and 4g treatments. Confirming the relative elongation of the long axis under increased gravity, with the *post hoc* test again indicating significant differences between the control and hypergravity treatments (Table 1).

Table 1
Wolffia australiana growth measurements under different gravities.

Treatment	Mean Long Axis Length (mm)	Std. Dev.	Mean Short Axis Length (mm)	Std. Dev.	Long Side Ratio	Std. Dev.	Short Side Ratio	Std. Dev.
LowGravityControl	0.95	0.07	0.59	0.06	1.62	0.14	0.62	0.05
Simulated Microgravity	0.93	0.09	0.58	0.06	1.62	0.14	0.62	0.05
Simulated Moon	0.97	0.10	0.60	0.06	1.63	0.14	0.62	0.06
HighGravityControl	0.95	0.07	0.59	0.06	1.62	0.14	0.62	0.05
Hypergravity 2g	1.03	0.11	0.60	0.08	1.73	0.16	0.58	0.06
Hypergravity 4g	1.06	0.10	0.62	0.07	1.73	0.20	0.58	0.06

Wolffia australiana expression responses under different gravities

We compared transcriptional responses of wa8730 plants grown under our four gravity conditions (sim- μ g, sim-Moon, 2g and 4g) to determine if plants of *W. australiana* exhibit similar or distinct transcriptional responses to altered gravity environments. Both the sim- μ g and sim-Moon shared a LowGravityControl, while 2g and 4g shared a HighGravityControl and controls were used to drop out outlier samples (Supplemental Fig. 1, 2; Supplemental Table 1). The sim- μ g and sim-Moon conditions resulted in ~ 25% and ~ 29% differentially expressed genes (DEGs), respectively, while the 2g and 4g hypergravity samples were lower with ~ 7% and ~ 11% DEGs, respectively. Within each condition, down- and up-regulated genes demonstrated a consistent DEG percentage, with the max difference being ~ 1.3% (Table 2; Fig. 2; Supplemental Tables S2-6).

Table 2				
<i>Wolffia australiana</i> RNA-seq differentially regulated genes.				
Simulated microgravity, sim- μ g	UP (#)	UP (%)	DOWN (#)	DOWN (%)
	1,575	12%	1,630	13%
Moon partial gravity, sim-Moon	1,950	15%	1,872	14%
Hypergravity, 2g	389	3%	469	3.6%
Hypergravity, 4g	630	4.9%	802	6.2%

Next, we looked at the gene ontology (GO) terms overrepresented in the eight gene lists (Supplemental Tables S7-18). The two low gravity conditions demonstrated coordinated responses to their respective gravity treatments. Low gravity upregulated genes resulted in overrepresented GO terms relating to nucleic acid metabolic process, RNA binding, nucleolus, cell wall, ribosome biogenesis, and other translation-related processes (Fig. 3; Supplementary Table S8,S11). This enrichment would be characteristic of rapid development, growth, and an anabolic response to the altered gravitational environment. In contrast, low gravity downregulated genes resulted in overrepresented GO terms relating to response to oxidative stress, cellular oxidant detoxification, antioxidant activity, cellular response to high light intensity, and response to UV-A. These terms describe a coordinated downregulation of stress response and homeostasis restoration methods (Fig. 3; Supplementary Table S7,S10).

Wolffia australiana exposed to high gravity had a slightly smaller transcriptional response as compared to low gravity (Table 2), and the response was less coordinated across the upregulated GO terms of 2g and 4g. Conversely, downregulated terms of the high gravity samples were coordinated, reflecting the low gravity samples. The enriched GO terms for 2g upregulated genes highlight gene functions involved in response to metal and cadmium ions, response to heat and temperature stimulus, and *de novo* protein folding, while the 4g upregulated genes show enrichment for several ribosome related terms related to structure, biogenesis, and assembly, as well as peptide, pigment, and flavonoid biosynthetic processes (Fig. 3; Supplementary Table S14, S17). The downregulated genes under 2g and 4g were enriched for genes involved in an array of biosynthetic processes including alkane, cuticle hydrocarbon, hydrocarbons, and wax (Fig. 3; Supplementary Table S13,S16).

When all of the DEGs (up and down regulated genes) for low gravity were analyzed together they focused on cytosolic ribosome, ribosome biogenesis, cell wall, and brassinosteroid homeostasis, which are typical of actively dividing or differentiating cells, or tissues undergoing stress adaptation or metabolic remodeling, such as in developmental transitions, environmental response, or tissue repair (Fig. 3; Supplementary Table S9,S12). When considering all DEGs, including both up- and down-regulated genes under high-gravity conditions, we also identified enrichment for ribosome-related functions, circadian rhythm and rhythmic processes, as well as several stress-response pathways (Fig. 3; Supplementary Table S15,S18). Such a pattern is consistent with stress-adapted tissues, such as plants under combined heat and oxidative stress, pathogen attack, or even simulated space conditions, where both environmental stimuli and internal regulatory responses must be tightly coordinated. There were four GO terms shared across all four DEG sets (GO:0005840, GO:0022626, GO:0005618, GO:0030312). Together, these GO terms indicate involvement in ribosome function, cytosolic ribosome, cell wall, and external encapsulating structure. This set would be typical in rapidly growing tissues, responses to changing metabolic demands, or cell wall specific stress recovery where ribosome recycling is important. Taken together we observe four distinct gravity responses in *W. australiana*.

We next examined the overlap between genes responsive to low- and high-gravity conditions, as well as genes that responded consistently across all treatments. Both hypergravity and microgravity induced substantial and overlapping transcriptional responses (Fig. 4A-B). In the hypergravity dataset, 524 genes were shared across conditions and GO enrichment analysis revealed predominant stress- and stimulus-responsive processes, including abiotic and biotic stress responses, hormone signaling, and extensive regulation of ribosome function, protein folding, and chloroplast-associated translation. Similarly, the microgravity dataset exhibited a broad overlap of differentially expressed genes with GO enrichment revealed a strong and specific signature of ribosome biogenesis, rRNA processing, and cytosolic and plastid translation, indicating coordinated regulation of protein synthesis capacity. Analysis of the complete set of differentially expressed genes across all four gravity conditions identified 147 shared genes with a strikingly restricted set of enriched GO terms that mirrored microgravity, including polysomal ribosome, polysome, cytosolic ribosome, and ribosomal subunit. These results indicate that altered gravity conditions exert a convergent effect on protein synthesis capacity in *W. australiana*. Notably, among these 147 genes were five conserved circadian-associated genes, including the core clock components *LHY*, *PRR7*, *FKF1* and *RVE*, as well as the central light-signaling regulator *SPA1*, suggesting that gravity-induced perturbations to translational machinery intersect with circadian and light-regulated pathways.

Responses of known gravitropism and gravity-related genes

Gravitropism can be broadly organized into four stages: gravity perception (statolith sedimentation in statocytes), signal transduction (including the LAZY pathway, Ca^{2+} /ROS signaling, and PIN relocalization), auxin redistribution (establishment of lateral auxin asymmetry), and differential growth and angle maintenance (execution and stabilization of organ orientation). Based on the literature, we

compiled a set of 69 *Arabidopsis* genes representing these four stages and identified their putative *W. australiana* orthologs using a gene-family-based approach (Orthogroups; Supplementary Table S19). This analysis revealed that nine genes lack identifiable *W. australiana* orthologs. Consistent with previous observations, the entire *LAZY* gene family is absent from *W. australiana* (Michael et al., 2020); the remaining missing genes include *SGR9*, *DOA*, and *TAC1*. Notably, while *W. australiana* contains a single *PIN1* ortholog, it harbors nine tandemly duplicated *PIN4* orthologs clustered on chr05. Of these, only four are full-length and detectably expressed, whereas the remaining five represent partial gene models, likely reflecting ongoing degeneration of the tandem duplicates (Supplementary Table S19). Of the 69 gravity-related *W. australiana* orthologs, 24 were identified as DEGs in at least one condition; however, this overlap is not significantly different from random expectation based on a hypergeometric (Fisher's exact) test (Table 3). Three of the 24 DEGs correspond to the tandemly duplicated *PIN4* genes, and only two genes, *MAPK3* and *EXP14*, were consistently differentially expressed across all four gravity conditions tested. Notably, although gravity-related genes were not globally enriched among differentially expressed genes, the 24 responsive genes represent a functionally coherent subset spanning multiple stages of the gravitropic response, including auxin transport and signaling (*PIN4*, *LAX3*, *IAA17*, *IAA19*), hormone crosstalk (*BRI1*, *CKX1*), signal transduction and mechanosensing (*MPK3*, *MKK5*, *CNGC14*, *GLR3.3*), vesicle trafficking and organelle dynamics (*SYP22*, *VTI11*, *TOC120*), cytoskeletal organization (*ACT2*), and growth execution (*EXP14*), as well as regulators of stress and metabolic state (*GRV2/KAM2*, *RSH1*, *ADK1*). The selective responsiveness of these genes suggests that altered gravity in *W. australiana* primarily impacts downstream signaling, trafficking, and growth execution modules. Notably, the simultaneous regulation of *TOC120*, *ACT2*, and *MAPK3* hints at a mechanotransduction pathway linking plastid dynamics to cellular stress responses, rather than broadly activating the canonical gravitropism pathway.

Table 3
Gravity-related differentially expressed genes (DEGs)

#	<i>W. australiana</i> Gene ID	Gene name	Condition
1	Wa8730c001g006520_T001	<i>SGR8</i>	2g ALL $\cap$ 4g ALL $\cap$ gravity genes
2	Wa8730c005g011450_T001	<i>PIN4</i>	4g ALL $\cap$ gravity genes
3	Wa8730c004g008000_T001	<i>BRI1</i>	4g ALL $\cap$ gravity genes
4	Wa8730c011g009350_T001	<i>CNGC14</i>	4g ALL $\cap$ gravity genes
5	Wa8730c017g004850_T001	<i>ACT2</i>	4g ALL $\cap$ gravity genes
6	Wa8730c010g001380_T001	<i>MKK5</i>	4g ALL $\cap$ gravity genes
7	Wa8730c005g011340_T001	<i>PIN4</i>	sim-Moon ALL $\cap$ gravity genes
8	Wa8730c005g011420_T002	<i>PIN4</i>	sim-Moon ALL $\cap$ gravity genes
9	Wa8730c009g002860_T001	<i>IAA19</i>	sim-Moon ALL $\cap$ gravity genes
10	Wa8730c018g001060_T003	<i>TOC120</i>	sim-Moon ALL $\cap$ gravity genes
11	Wa8730c019g001360_T001	<i>CKX1</i>	sim-ug ALL $\cap$ 4g ALL $\cap$ gravity genes
12	Wa8730c011g004400_T001	<i>SGR3</i>	sim-ug ALL $\cap$ gravity genes
13	Wa8730c018g002240_T001	<i>5PTASE13</i>	sim-ug ALL $\cap$ gravity genes
14	Wa8730c007g010550_T001	<i>GLR3.3</i>	sim-ug ALL $\cap$ gravity genes
15	Wa8730c019g004450_T001	<i>MPK3</i>	sim-ug ALL $\cap$ sim-Moon ALL $\cap$ 2g ALL $\cap$ 4g ALL $\cap$ gravity genes
16	Wa8730c009g009450_T001	<i>EXP14</i>	sim-ug ALL $\cap$ sim-Moon ALL $\cap$ 2g ALL $\cap$ 4g ALL $\cap$ gravity genes
17	Wa8730c004g005860_T002	<i>HY5</i>	sim-ug ALL $\cap$ sim-Moon ALL $\cap$ gravity genes
18	Wa8730c001g000350_T001	<i>SGR4</i>	sim-ug ALL $\cap$ sim-Moon ALL $\cap$ gravity genes
19	Wa8730c011g000640_T002	<i>IAA17</i>	sim-ug ALL $\cap$ sim-Moon ALL $\cap$ gravity genes
20	Wa8730c014g004220_T001	<i>ADK1</i>	sim-ug ALL $\cap$ sim-Moon ALL $\cap$ gravity genes
21	Wa8730c009g008850_T001	<i>EXP14</i>	sim-ug ALL $\cap$ sim-Moon ALL $\cap$ gravity genes
22	Wa8730c001g002610_T001	<i>RLD3</i>	sim-ug ALL $\cap$ sim-Moon ALL $\cap$ gravity genes
23	Wa8730c002g007210_T001	<i>LAX3</i>	sim-ug ALL $\cap$ sim-Moon ALL $\cap$ gravity genes
24	Wa8730c015g001350_T001	<i>PLT2</i>	sim-ug ALL $\cap$ sim-Moon ALL $\cap$ gravity genes

Temporal and spatial analysis of the high and low gravity DEGs

Given *W. australiana*'s highly reduced body plan, we hypothesized that its gravity response may exhibit unique temporal and spatial characteristics. Among the DEGs identified across gravity treatments, several are known components of the circadian clock (Supplementary Table S20), a regulatory system previously implicated in gravity responses (Tolsma et al., 2021). The circadian clock functions as an internal timing mechanism that, together with daily light and temperature cues, orchestrates the rhythmic expression of up to 90% of plant genes at the appropriate time of day (Michael et al., 2008). Notably, several core circadian clock genes were among the DEGs under different gravity conditions (Supplementary Table S21), with *LHY*, *FKF1*, and *GI* consistently differentially expressed across all four treatments. *LHY* functions within the morning loop of the circadian oscillator, while *FKF1* and *GI* are key components of the evening loop. These results suggest that gravity-induced transcriptional changes may intersect with circadian regulation in *W. australiana*, potentially contributing to its adaptation under altered gravitational environments.

In a separate study, we examined the diel transcriptional behavior of *W. australiana* under light–dark cycles and found that only ~ 10% of genes exhibited TOD-specific cycling, in contrast to most plant species, where more than 50% of genes display rhythmic expression patterns (Michael et al., 2020). Here, we asked whether DEGs identified across the four gravity conditions were enriched for TOD-regulated genes. We found that a greater proportion of DEGs exhibited cycling behavior under all four conditions (15%, 18%, 37%, and 27% for sim- μ g, sim-Moon, 2g, and 4g, respectively) compared to the genomic background rate of 8.8%. Another important aspect of TOD regulation is that specific biological pathways are often coordinately expressed at particular times of day; for example, phytohormone-associated pathways frequently exhibit phased transcriptional regulation (Michael et al., 2008). We therefore tested whether gravity-responsive DEGs showed coordinated TOD expression patterns by assigning the peak expression TOD to the gravity DEGs. However, the temporal distribution of DEGs closely mirrored that of all expressed genes, indicating no evidence for coordinated or phased circadian expression among gravity-responsive genes (Supplemental Fig. 3).

In a separate study, we characterized *W. australiana* using single-cell RNA sequencing (scRNA-seq) and identified four predominant cell types: above-water epidermis and parenchyma, and below-water epidermis and parenchyma (Denyer et al., 2024). Although not all genes could be assigned to specific cell types due to the limitations of 10X scRNA-seq in capturing low-abundance transcripts, we investigated the cell-type distribution of gravity-responsive DEGs wherever annotations were available. Overall, DEGs were more frequently associated with epidermal cell types than with parenchyma. These findings suggest that the epidermis may play a central role in gravity perception and response in *W. australiana*, consistent with our observation that genes down-regulated under high-gravity conditions were enriched for cuticle biosynthesis functions (Supplemental Table S22).

Discussion

Our investigation of *Wolffia australiana* responses across a gravity continuum revealed distinct morphological and transcriptional adaptations that highlight the utility of this species as a model system for space plant biology. Simulated lunar gravity promoted growth, whereas hypergravity inhibited growth and altered mother frond morphology, effects that were not observed under low-gravity conditions. These findings emphasize the value of examining plant responses across multiple gravity regimes rather than focusing solely on microgravity. At the transcriptional level, low-gravity conditions elicited substantially broader responses than hypergravity, with approximately 25% and 29% of genes differentially expressed under simulated microgravity (sim- μ g) and simulated lunar gravity (sim-Moon), respectively, compared to ~ 7% and ~ 11% under 2g and 4g conditions. This suggests that reduced gravity requires more extensive physiological and developmental reprogramming in *W. australiana*.

Under simulated microgravity, genes associated with growth and biosynthetic activity, including nucleic acid metabolism, RNA binding, ribosome biogenesis, and nucleobase-containing compound metabolism, were coordinately upregulated, while pathways related to oxidative stress responses, ion homeostasis, and UV-A responses were generally downregulated. In contrast, hypergravity induced transcriptional signatures associated with heat and stress responses while repressing pathways involved in lipid and wax biosynthesis, secondary metabolism, and cuticle development. Across all gravity treatments, genes associated with circadian regulation and time-of-day (TOD) expression were significantly enriched among differentially expressed genes, suggesting an intersection between gravity sensing and temporal regulatory networks. Furthermore, gravity-responsive genes were disproportionately associated with epidermal cell types, indicating that the epidermis may play a central role in perceiving and responding to altered gravitational forces in *W. australiana*.

Wolffia australiana has previously been shown to lack several canonical gravitropism-associated genes, most notably members of the *LAZY* gene family and *SHOOT GRAVITROPISM 9* (*SGR9*), which in other land plants are key components of the starch–statolith gravity-sensing pathway (Michael et al., 2020; Ernst et al., 2025). Their absence suggests that *W. australiana* may perceive and respond to gravity through mechanisms distinct from those characterized in model systems such as *Arabidopsis thaliana*. In *Arabidopsis*, *SGR9* regulates the detachment and sedimentation of amyloplasts within specialized gravity-sensing cells; however, in *W. australiana*, chloroplasts appear to remain more tightly associated with the cytoskeletal network. Previous studies have shown that *Wolffia* species accumulate substantial starch reserves within chloroplasts of palisade-like parenchyma and mesophyll cells (Yang et al., 2021), raising the possibility that these starch-filled chloroplasts may nevertheless provide sufficient mass to function as gravity-responsive mechanosensory structures. Further investigation will be required to test this hypothesis directly.

An alternative explanation is provided by tensegrity-based models of gravity perception, in which gravitational forces are sensed not through organelle sedimentation but through mechanically induced tension within the cytoskeleton (Staehein et al., 2000). Consistent with this model, we identified differential expression of the chloroplast-associated protein import factor *TRANSLOCON AT THE OUTER ENVELOPE MEMBRANE OF CHLOROPLASTS 120* (*TOC120*) and the cytoskeletal component *ACTIN2*

(ACT2) under altered gravity conditions. In addition, the consistent upregulation of *MITOGEN-ACTIVATED PROTEIN KINASE 3* (MAPK3) across all gravity treatments suggests that altered gravity may primarily be perceived as a mechanical stress signal, activating generalized stress and growth-response pathways, including induction of *EXPANSIN 14* (EXP14), rather than a canonical directional gravitropic program. Together, these observations support a model in which *W. australiana* relies predominantly on mechanotransductive, cytoskeleton-associated gravity sensing rather than classical statolith sedimentation, potentially explaining its atypical responses to altered gravity environments.

Structurally, *W. australiana* possesses an extremely reduced body plan consisting of a spheroid-like frond composed primarily of two major tissue types epidermal and parenchymal cells with distinct spatial differentiation between aerial and submerged regions (Denyer et al., 2024). Under simulated lunar gravity, *W. australiana* displayed an increased relative growth rate (RGR) accompanied by the upregulation of genes associated with genome maintenance, including pathways involved in DNA replication, DNA repair, and cell-cycle regulation, while genes related to broader biosynthetic and metabolic functions were generally downregulated. Similar activation of genome integrity and stress-associated pathways has been reported in *Arabidopsis* exposed to altered gravity conditions (Land et al., 2023); however, *Arabidopsis* typically exhibits delayed or inhibited growth under microgravity. The contrasting response observed in *W. australiana* suggests that reduced gravity may be interpreted as a state of decreased mechanical or spatial constraint, promoting a reallocation of cellular resources toward proliferation and nucleic acid metabolism at the expense of secondary biosynthetic processes. In duckweeds, growth and morphology are strongly influenced by population density and crowding, with dense colonies often exhibiting reduced frond expansion, altered nutrient allocation, and changes in developmental dynamics. Because *W. australiana* naturally grows as tightly packed floating mats at the air-water interface, altered gravity may partially relieve physical compression, buoyancy-related contact forces, or frond-to-frond mechanical interactions that normally constrain growth. This “uncrowding-like” response could therefore represent a growth-priming strategy under reduced mechanical load, potentially explaining the enhanced proliferation observed under simulated lunar gravity.

In contrast to the growth-promoting effects observed under simulated lunar gravity, hypergravity conditions did not enhance growth but did induce significant morphological changes, particularly in the long and short axes of the mother fronds (Table 1). Both 2g and 4g treatments elicited a consistent and coordinated upregulation of heat stress response genes, suggesting that *W. australiana* may interpret increased gravitational force as a physiological stress analogous to elevated temperature. Downregulated genes, while less consistently shared between the two hypergravity levels, were largely enriched for pathways involved in cuticle hydrocarbon biosynthesis and cellular alkane metabolic processes, functions closely tied to membrane structure and surface properties. Given that gravity-responsive DEGs were disproportionately expressed in epidermal tissues, it is plausible that *W. australiana* adapts to hypergravity by modifying the composition or mechanical properties of its outer cell layers. Such changes may facilitate the observed alterations in frond morphology, possibly through adjustments in turgor pressure, membrane elasticity, or cell wall dynamics that contribute to a more flattened or structurally altered frond profile under increased gravitational load. These results underscore

a gravity-dependent remodeling of epidermal architecture, providing insight into how *W. australiana* modulates its form and surface physiology in response to mechanical stimuli.

Circadian clock components and TOD-regulated genes are known to be sensitive to gravitational changes, with numerous studies showing that microgravity disrupts or reprograms circadian networks in *Arabidopsis*, including altered expression of core oscillator genes such as *CIRCADIAN CLOCK ASSOCIATED 1 (CCA1)*, *LATE ELONGATED HYPOCOTYL (LHY)*, and *TIMING OF CAB EXPRESSION 1 (TOC1)* (Paul et al., 2013; Califar et al., 2021; Tolsma et al., 2021, 2022). Consistent with these findings, we observed a significant overrepresentation of circadian clock genes among the gravity-responsive DEGs in *W. australiana*. Notably, *LHY*, *GIGANTEA (GI)*, and *FLAVIN-BINDING, KELCH REPEAT, F-BOX 1 (FKF1)* were differentially expressed across all four gravity conditions, alongside numerous additional genes associated with circadian regulation of growth, flowering time, and hormone signaling (Table S21). Gene ontology (GO) enrichment analysis revealed that DEGs specific to hypergravity were significantly enriched for terms related to circadian rhythmicity and rhythmic biological processes (Supplementary Tables S3-18). These results suggest that gravity acts as a modulator of circadian regulation in *W. australiana*, and that gravitational changes may either entrain or disrupt internal timing mechanisms, potentially influencing broader physiological outputs including growth rate, cell cycle progression, and hormone dynamics. The consistent response of core clock components across divergent gravitational regimes further supports the idea that circadian systems may serve as integrators of environmental signals, including gravitational force, in both simplified and complex plant systems. However, *W. australiana* has a reduced set of genes controlled by the circadian clock and TOD (Michael et al., 2020), which could make it ideal for growth under different gravity regimes.

Together, our findings position *W. australiana* as a uniquely valuable model for space agriculture and plant biology under altered gravity conditions. Its simplified morphology, rapid growth, and ability to mount robust transcriptional responses to both simulated micro- and partial g as well as hypergravity make it especially well-suited for bioregenerative life support systems (BLSS) in space. Unlike typical land plants such as *Arabidopsis*, *W. australiana* exhibited increased growth under simulated lunar gravity and displayed gravity-responsive gene expression without relying on canonical gravitropism pathways. Its gravity-associated transcriptome included coordinated activation of genome integrity and stress response pathways, as well as significant involvement of circadian clock and TOD regulated genes. These responses, particularly the enrichment of differentially expressed genes in epidermal tissue and its reduced dependence on complex tissue organization, suggest that *W. australiana* could thrive in the constrained and dynamic environments of space habitats. As space agencies plan for longer-duration missions and off-Earth cultivation, *W. australiana*'s minimal gene set, compact architecture, and adaptive transcriptional flexibility in addition to rapid growth rate and high nutritional value, offer a promising foundation for optimizing plant-based systems in extraterrestrial environments. Ultimately, this unique evolutionary trajectory toward reduction appears to have favored a more essential design, that rather than maintaining specialized *ad hoc* plastids, has evolved a simplified gravity perception mechanism that leverages starch-filled chloroplasts to function as efficient, dual-purpose sensors.

Methods

Data Collection and Analysis

This study utilized the same imaging protocol and imaging techniques as described (Romano et al., 2024). More specifically, data were collected by imaging multiwell plates at the start (t0) and after 168 hours (t168). A Sony Alpha II camera with an 18–70 mm lens captured top-down images on a uniformly lit lighting table. These images were used to calculate the relative growth rate (RGR) by comparing the plant area in pixels at t0 and t168. The RGR was then used to determine the doubling time (DT). A Leica® MZ8 Stereo zoom Microscope captured detailed images of *Wolffia australiana*. The long and short axes of the mother fronds were measured using Fiji® software to assess frond roundness. Image analysis focused on growth parameters (RGR and DT) and morphological differences. RGR was calculated by frond counting at t0 and t168 (N = 12). DT was determined based on RGR (Naumann et al., 2007). For morphological analysis, 50 mother fronds with attached daughter fronds were randomly selected and measured for their long and short axes. Frond's measurements were performed with Fiji® software (N = 100).

Data Analysis

Image analysis data were processed and analysed using SPSS Statistics ver. 27 (IBM Corp.). Differences in relative growth rates (RGR), doubling time (DT) and morphological traits of *W. australiana* among gravity treatments were assessed by one-way ANOVA ($p < 0.05$). In all cases, a split experimental approach has been adopted so that Simulated Microgravity and Partial Gravity (Moon) were always compared against their control (LowGravityControl), and 2g and 4g were always compared with their control (HighGravityControl). Pairwise comparisons were performed using Student-Newman-Keuls's post-hoc test ($P < 0.05$) to identify differences among individual treatments against their controls.

Plant growth conditions and experimental hardware

Plants of *W. australiana* clone 8730 (wa8730) have been kindly provided by Dr. Laura Morello from the Institute of Agricultural Biology and Biotechnology (IBBA) National Research Council (CNR) Milan, Italy. Upon receipt, plant material was surface sterilised with 0.2% bleach/water solution for 5 min. After 14 days from disinfection, plants were subcultured for 15 days in N-medium under axenic conditions. Therefore, plant material has been transferred under a laminar flow hood in sets of 6-well plates, each filled with 5 ml of N-medium solution and sealed with micropore® tape. Before the experimental run, plants were acclimated for 48 h at 30°C. After acclimatisation, an average of 31 ± 7 fronds were transferred in each well of a 6-multiwell plate previously filled with 5 ml of N-medium and 0.8% of Agar to achieve a semi-solid substrate. The experiment was conducted at $30 \pm 0.5^\circ\text{C}$ average temperature with a photoperiod of 16/8 h light/dark and a total Photon Flux Density (PFD) of $72.37 \pm 10.30 \mu\text{mol m}^{-2} \text{s}^{-1}$ for 168 h2. The experimental procedures were conducted in custom-developed experimental hardware, as Romano et.al 2024 described.

Gravity treatments

The study included four different gravity treatments: simulated microgravity (sim- μ g), simulated Moon partial gravity (sim-Moon), and hypergravity conditions (2g and 4g) (Table S1). Two control groups under Earth gravity conditions were also included for comparison. Each treatment was implemented with two 6-well plate setups (Romano et al., 2024). Simulated microgravity (sim- μ g) was achieved using an RPM (Random Positioning Machine, original Dutch Space Leiden, NL; currently Yuri Space, Meckenbeuren, DE) that allowed the plants to be positioned within 10 cm from the centre of rotation. The RPM was set to a maximum random speed of 60 °/s, minimising residual acceleration. RPM was also set to random interval and random direction. Simulated Partial gravity (sim-Moon) was simulated using a similar RPM, which was running a different program that does not provide a complete random rotation during the experiment but generates biased vector orientations and values such that the averaged resulting gravity vector simulates the Moon (Manzano et al., 2018). This proprietary software-generated motion can examine plant responses to partial gravity ranging from nearly zero to 0.9 g levels. Hypergravity conditions were obtained using the large-diameter centrifuge (LDC) (van Loon et al., 2008). Two hypergravity levels were tested: 2g and 4g, corresponding to twice and four times the gravitational force on Earth, respectively. These conditions allowed investigation of how *W. australiana* responded to increased gravitational forces. Two control groups were included, in which plants were grown under normal gravity conditions (1g) in a static gondola and in the incubator containing the RPMs for sim- μ g and sim-Moon. These control groups served as a baseline for comparing the growth and morphology of *Wolffia australiana* under different gravity treatments.

RNA extraction and sequencing

Collection of Plant Material for Genetic Analysis

The collection of plant material for RNA sequencing (RNA-seq) began with the sequential stopping of the experimental setups, followed by the collection of fresh plant material from the multiwell plates. This procedure was carried out 168 hours after the start of the experiment at approximately mid-day and took approximately twenty minutes to complete. Each experimental treatment was paused individually, and plant material was collected as quickly as possible using a spatula. About 300 mg of plant material was taken from each multiwell plate and divided into three separate Eppendorf tubes ($n = 3$). The 2 ml Eppendorf tubes were pre-filled with 1 ml of RNA-Later solution. Once the plant material was placed into the tubes, they were immediately immersed in liquid nitrogen to preserve them. RNA extraction was performed using the Tiangen RNAprep Pure Plant Plus kit, which is designed for samples rich in polysaccharides and polyphenolics.

RNA sequencing (RNA-seq) analysis

Library Construction, Quality Control and Sequencing

RNA-seq was performed by NOVOgene. Messenger RNA was purified from total RNA using poly-T oligo-attached magnetic beads. After fragmentation, the first strand cDNA was synthesized using random hexamer primers, followed by the second strand cDNA synthesis using either dUTP for directional library

or dTTP for non-directional library. The library was checked with Qubit and real-time PCR for quantification and bioanalyzer for size distribution detection. Quantified libraries will be pooled and sequenced on Illumina platforms, according to effective library concentration and data amount. The resulting reads were filtered and aligned to the indexed *Wolffia australiana* clone 8730 (wa8730) (Ernst et al., 2025) primary transcripts using Salmon (v1.6.0) to generate gene counts for each set of reads. These Salmon quant files were then merged into a counts table for all samples. The Salmon NumReads counts table along with sample metadata were then imported into R for analysis with DESeq2 (v1.44.0). Preliminary RNASeq counts plots, PCA plots, and heatmaps were generated in order to identify any outliers and perform quality control. One of the Simulated Gravity Control samples was subsequently removed due to strong similarity to the experimental groups. Comparisons in DESeq2 were computed between single experimental groups and their associated control samples. The percent of up- and down-regulated genes were calculated and a list of the most significant differentially expressed genes was compiled for each experimental group. Gene ontology (GO) was carried out using goatools (Klopfenstein et al., 2018).

Declarations

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Author contributions

L.E.R. conceived the study. L.E.R., J.J.W.A.v.L., and G.A. carried out experiments. L.E.R., N.A. and T.P.M. analyzed data and wrote the manuscript, J.J.W.A.v.L. and G.A. reviewed the manuscript.

Conflict of Interest

The authors declare no conflict of interest.

References

1. **Aronne G, Romano LE, Izzo LG. 2020.** Subsequent inclusion/exclusion criteria to select the best species for an experiment performed on the ISS in a refurbished hardware. *Life Sciences in Space Research*, 27: 19–26.
2. **Baldwin KL, Strohm AK, Masson PH. 2013.** Gravity sensing and signal transduction in vascular plant primary roots. *American journal of botany* **100**: 126–142.

3. **Band LR, Wells DM, Larrieu A, Sun J, Middleton AM, French AP, Brunoud G, Sato EM, Wilson MH, Péret B, et al. 2012.** Root gravitropism is regulated by a transient lateral auxin gradient controlled by a tipping-point mechanism. *Proceedings of the National Academy of Sciences of the United States of America* **109**: 4668–4673.
4. **Califar B, Zupanska A, Callaham JA, Bamsey MT, Graham T, Paul A-L, Ferl RJ. 2021.** Shared metabolic remodeling processes characterize the transcriptome of *Arabidopsis thaliana* within various suborbital flight environments. *Gravitational and Space Research: Publication of the American Society for Gravitational and Space Research* **9**: 13–29.
5. **Darwin F, Darwin C. 2022.** *The power of movement in plants*. Legare Street Press.
6. **Denyer T, Wu P-J, Colt K, Abramson BW, Pang Z, Solansky P, Mamerto A, Nobori T, Ecker JR, Lam E, et al. 2024.** Streamlined spatial and environmental expression signatures characterize the minimalist duckweed *Wolffia australiana*. *Genome research* **34**: 1106–1120.
7. **Ernst E, Abramson B, Acosta K, Hoang PTN, Mateo-Elizalde C, Schubert V, Pasaribu B, Albert PS, Hartwick N, Colt K, et al. 2025.** Duckweed genomes and epigenomes underlie triploid hybridization and clonal reproduction. *Current Biology*, **35**(8):1828-1847.
8. **Fasano JM, Swanson SJ, Blancaflor EB, Dowd PE, Kao TH, Gilroy S. 2001.** Changes in root cap pH are required for the gravity response of the *Arabidopsis* root. *The plant cell* **13**: 907–921.
9. **Fitzelle KJ, Kiss JZ. 2001.** Restoration of gravitropic sensitivity in starch-deficient mutants of *Arabidopsis* by hypergravity. *Journal of Experimental Botany*, **52**(355): 265–275.
10. **Gòdia F, Albiol J, Montesinos JL, Pérez J, Creus N, Cabello F, Mengual X, Montras A, Lasseur C. 2002.** MELISSA: a loop of interconnected bioreactors to develop life support in space. *Journal of biotechnology* **99**: 319–330.
11. **Hashiguchi Y, Tasaka M, Morita MT. 2013.** Mechanism of higher plant gravity sensing. *American journal of botany* **100**: 91–100.
12. **Hosamani R, Swamy BK, Dsouza A, Sathasivam M. 2022.** Plant responses to hypergravity: a comprehensive review. *Planta* **257**: 17.
13. **Izzo LG, Romano LE, Muthert LWF, Iovane M, Capozzi F, Manzano A, Ciska M, Herranz R, Medina FJ, Kiss JZ, van Loon JJW.A, Aronne G. 2022.** Interaction of gravitropism and phototropism in roots of *Brassica oleracea*. *Environmental and Experimental Botany*, **193**: 104700.
14. **Izzo LG, El Nakhel C, Rouphael Y, Proietti S, Paglialunga G, Moscatello S, Battistelli A, Iovane M, Romano LE, De Pascale S, Aronne G. 2023.** Applying productivity and phytonutrient profile criteria in modelling species selection of microgreens as Space crops for astronaut consumption. *Frontiers in Plant Science*, **14**: 1210566.
15. **Jamšek M, Kunavar T, Blohm G, Nozaki D, Papaxanthis C, White O, Babič J. 2021.** Effects of simulated microgravity and hypergravity conditions on arm movements in normogravity. *Frontiers in neural circuits* **15**: 750176.
16. **Kimbrough JM, Salinas-Mondragon R, Boss WF, Brown CS, Sederoff HW. 2004.** The fast and transient transcriptional network of gravity and mechanical stimulation in the *Arabidopsis* root apex.

Plant physiology **136**: 2790–2805.

17. **Kiss JZ. 2000.** Mechanisms of the Early Phases of Plant Gravitropism. *Critical Review Plant Science*. **19(6)**:551-73.
18. **Klopfenstein DV, Zhang L, Pedersen BS, Ramírez F, Warwick Vesztrocy A, Naldi A, Mungall CJ, Yunes JM, Botvinnik O, Weigel M, et al. 2018.** GOATOOLS: A Python library for Gene Ontology analyses. *Scientific Reports* **8**: 10872.
19. **Lam E, Michael TP. 2022.** Wolffia, a minimalist plant and synthetic biology chassis. *Trends in plant science* **27**: 430–439.
20. **Land ES, Sheppard J, Doherty CJ, Perera IY. 2023.** Conserved plant transcriptional responses to microgravity from two consecutive spaceflight experiments. *Frontiers in plant science* **14**: 1308713.
21. **Manzano A, Herranz R, Den Toom LA, Te Slaa S, Borst G, Visser M, Medina FJ, van Loon JJW A. 2018.** Novel, Moon and Mars, partial gravity simulation paradigms and their effects on the balance between cell growth and cell proliferation during early plant development. *NPJ Microgravity*. **4**: 4:9.
22. **Marchant A, Kargul J, May ST, Muller P, Delbarre A, Perrot-Rechenmann C, Bennett MJ. 1999.** AUX1 regulates root gravitropism in Arabidopsis by facilitating auxin uptake within root apical tissues. *The EMBO journal* **18**: 2066–2073.
23. **Michael TP, Ernst E, Hartwick N, Chu P, Bryant D, Gilbert S, Ortleb S, Baggs EL, Sree KS, Appenroth KJ, et al. 2020.** Genome and time-of-day transcriptome of *Wolffia australiana* link morphological minimization with gene loss and less growth control. *Genome research*.
24. **Michael TP, Mockler TC, Breton G, McEntee C, Byer A, Trout JD, Hazen SP, Shen R, Priest HD, Sullivan CM, et al. 2008.** Network Discovery Pipeline Elucidates Conserved Time-of-Day–Specific cis-Regulatory Modules. *PLoS genetics* **4**: e14.
25. **Morita MT. 2010.** Directional gravity sensing in gravitropism. *Annual review of plant biology* **61**: 705–720.
26. **Naumann B, Eberius M, Appenroth K-J. 2007.** Growth rate based dose-response relationships and EC-values of ten heavy metals using the duckweed growth inhibition test (ISO 20079) with *Lemna minor* L. clone St. *Journal of plant physiology* **164**: 1656–1664.
27. **Nie H, Zhou W, Zheng Z, Deng Y, Zhang W, Zhang M, Jiang Z, Zheng H, Yuan L, Yang J, et al. 2025.** Exploring plant responses to altered gravity for advancing space agriculture. *Plant Communications* **6**: 101370.
28. **Paul A-L, Zupanska AK, Schultz ER, Ferl RJ. 2013.** Organ-specific remodeling of the Arabidopsis transcriptome in response to spaceflight. *BMC plant biology* **13**: 112.
29. **Pietryka M, Richter D, Podlaska M. 2022.** Distribution and ecology of *Wolffia arrhiza* (L.) Horkel ex Wimm. In the lowland part of Lower Silesia (Poland). *Biologia*.
30. **Plieth C, Trewavas AJ. 2002.** Reorientation of seedlings in the earth’s gravitational field induces cytosolic calcium transients. *Plant physiology* **129**: 786–796.

31. **Reinard T, Londenberg A, Brychcy M, Lühmann K, Behrendt G, Wichmann M. 2020.** Editing the Genome of *Wolffia australiana*. In: Cao XH, Fourounjian P, Wang W, eds. *The Duckweed Genomes*. Cham: Springer International Publishing, 165–177.
32. **Romano LE, Aronne G. 2021.** The world smallest plants (*Wolffia* Sp.) as potential species for bioregenerative life support systems in space. *Plants* **10**: 1896.
33. **Romano LE, van Loon JJWA, Vincent-Bonnieu S, Aronne G. 2024.** *Wolffia globosa*, a novel crop species for protein production in space agriculture. *Scientific reports* **14**: 27979.
34. **Santomartino R, Aversch NJH, Bhuiyan M, Cockell CS, Colangelo J, Gumulya Y, Lehner B, Lopez-Ayala I, McMahon S, Mohanty A, et al. 2023.** Toward sustainable space exploration: a roadmap for harnessing the power of microorganisms. *Nature communications* **14**: 1391.
35. **Sathasivam M, Hosamani R, K Swamy B, Kumaran G S. 2021.** Plant responses to real and simulated microgravity. *Life Sciences in Space Research* **28**: 74–86.
36. **Stahelin LA, Zheng HQ, Yoder TL, Smith JD, Todd P. 2000.** Columella cells revisited: novel structures, novel properties, and a novel gravisensing model. *Gravitational and Space Biology Bulletin: Publication of the American Society for Gravitational and Space Biology* **13**: 95–100.
37. **Su S-H, Gibbs NM, Jancewicz AL, Masson PH. 2017.** Molecular mechanisms of root gravitropism. *Current Biology* **27**: R964–R972.
38. **Tolsma JS, Ryan KT, Torres JJ, Richards JT, Richardson Z, Land ES, Perera IY, Doherty CJ. 2021.** The circadian-clock regulates the *Arabidopsis* gravitropic response. *Gravitational and space research: publication of the American Society for Gravitational and Space Research* **9**: 171–186.
39. **Tolsma JS, Torres JJ, Richards JT, Perera IY, Doherty CJ. 2022.** Evaluating the Effects of the Circadian Clock and Time of Day on Plant Gravitropic Responses. *Methods in molecular biology* **2368**: 301–319.
40. **Toyota M, Gilroy S. 2013.** Gravitropism and mechanical signaling in plants. *American journal of botany* **100**: 111–125.
41. **van Loon JJWA, Krausse J, Cunha H, Goncalves J, Almeida H, Schiller P. 2008.** The Large Diameter Centrifuge, LDC, for Life and Physical Sciences and Technology. *Life in Space for Life on Earth* **553**: 92.
42. **van Loon, JJWA. 2016.** Centrifuges for Microgravity Simulation. *The Reduced Gravity Paradigm*. *Frontiers in Astronomy and Space Sciences* **3**: 202594.
43. **Yang J, Zhao X, Li G, Hu S, Hou H. 2021.** Frond architecture of the rootless duckweed *Wolffia globosa*. *BMC Plant Biology* **21**: 387.
44. **Yuan J, Xu K. 2017.** Effects of simulated microgravity on the performance of the duckweeds *Lemna aequinoctialis* and *Wolffia globosa*. *Aquatic botany* **137**: 65–71.
45. **Zhang Y, Xiao G, Wang X, Zhang X, Friml J. 2019.** Evolution of fast root gravitropism in seed plants. *Nature Communications* **10**: 3480

Figures

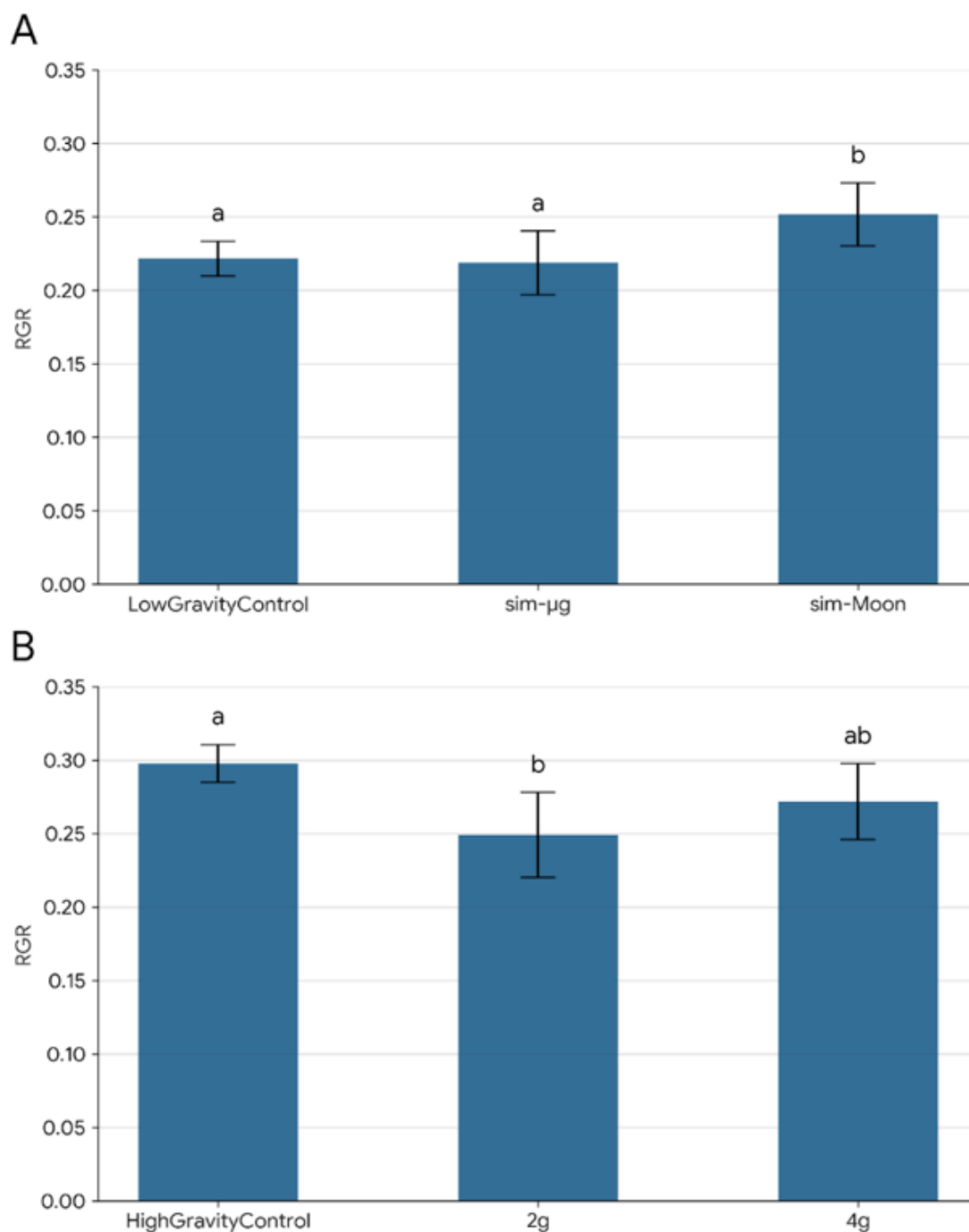


Figure 1

***Wolffia australiana* growth rate under different gravities.** A) Observed Mean RGR of *W. australiana* under simulated microgravity (sim- μ g), simulated partial gravity (sim-Moon), and control (LowGravityControl). The control group (LowGravityControl) has a median RGR with low variability. Simulated microgravity (sim- μ g) shows the lowest mean RGR with similar low variability. Simulated partial gravity (sim-Moon) displays the highest median RGR with higher variability. Different letters (a, b) represent statistically

significant differences between groups as determined by the Student-Newman-Keuls *post hoc* test. B) RGR of *W. australiana* under hypergravity conditions (2g and 4g) and the control group (HighGravityControl). The control group shows the highest median RGR with low variability. The 2g condition exhibits the lowest median RGR with higher variability, while the 4g condition has an intermediate median RGR with moderate variability. Different letters (a, b, ab) represent statistically significant differences between groups as determined by the Student-Newman-Keuls *post hoc* test.

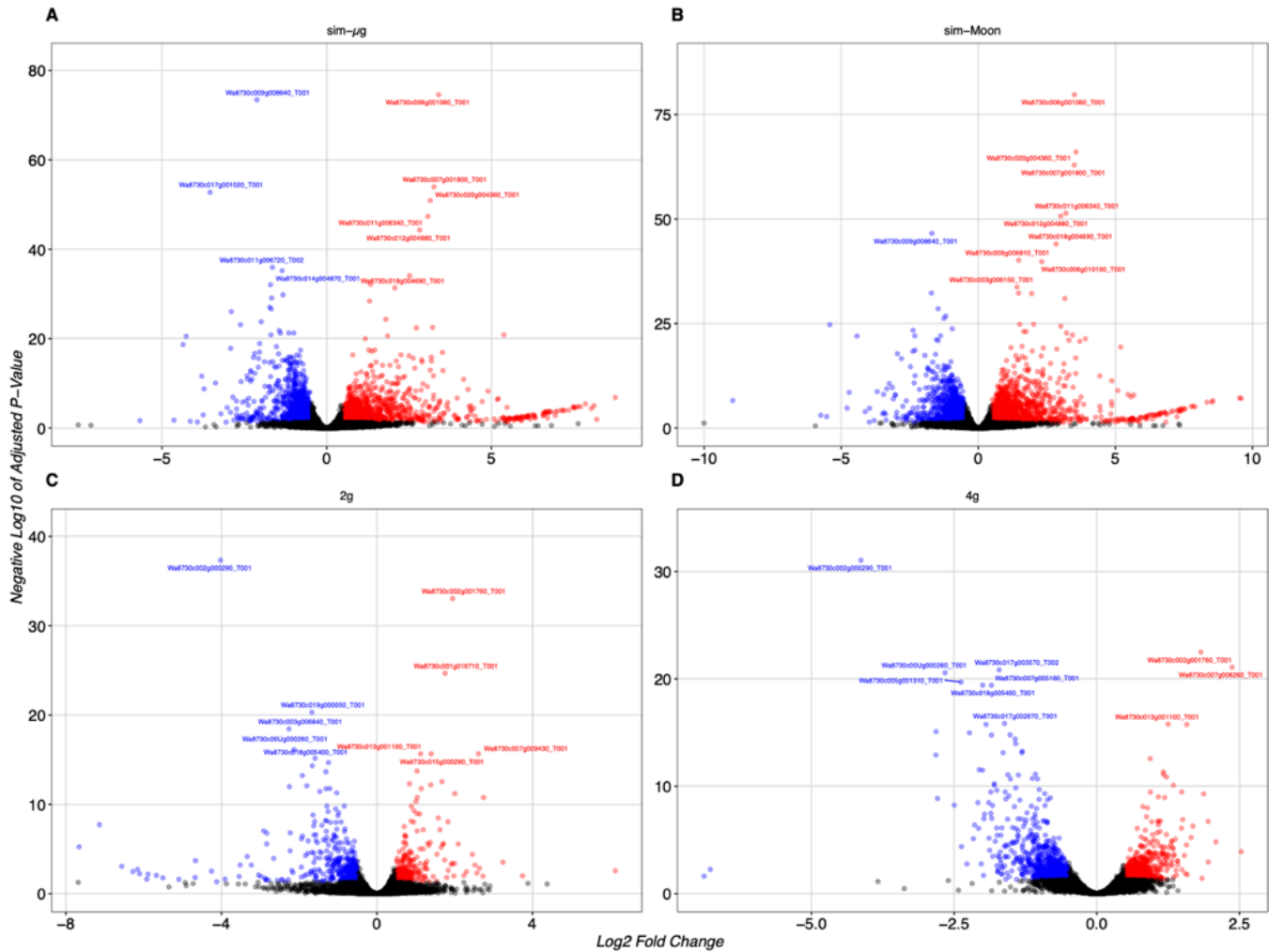


Figure 2

Differential gene expression under altered gravity conditions. Volcano plots show differential gene expression in *W. australiana* exposed to simulated microgravity (sim-μg) (A), moon partial gravity (sim-Moon) (B), hypergravity (2g) (C), and hypergravity (4g) (D), relative to Earth-gravity controls. Each point represents a gene, plotted by log₂ fold change (x-axis) and -log₁₀ adjusted p-value (y-axis). Genes meeting significance thresholds are highlighted in red or blue, while non-significant genes are shown in black. Labeled points indicate genes with the strongest differential expression. Both reduced and

elevated gravity conditions induce distinct transcriptional responses, with greater divergence from Earth gravity associated with a higher percentage of differentially expressed genes.

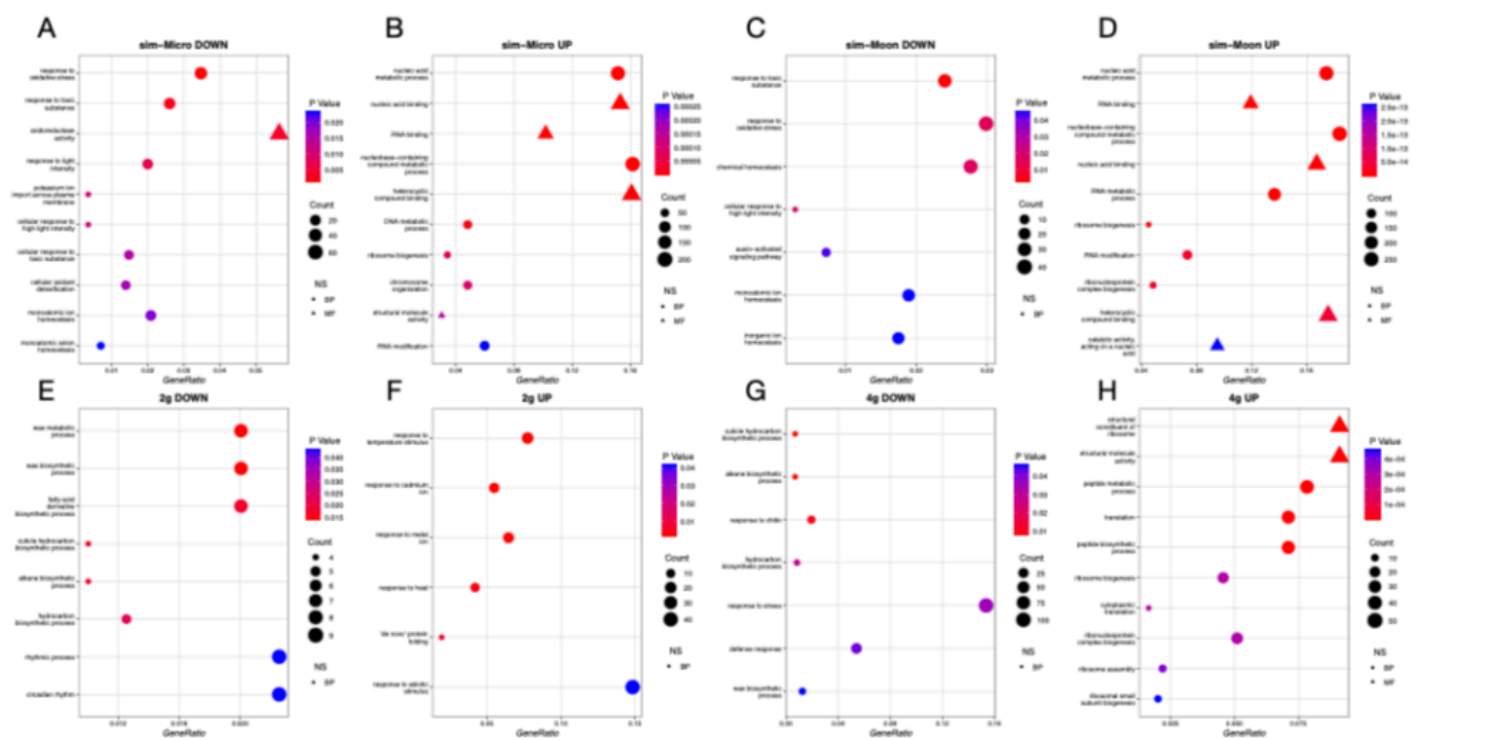


Figure 3

Gene Ontology (GO) enrichment analysis of differentially expressed genes under altered gravity conditions. Bubble plots show the top enriched GO Biological Process and GO Molecular Function terms among genes that are up-regulated (B, D, F, H) or down-regulated (A, C, E, G) under simulated microgravity (sim- μ g), moon partial gravity (sim-Moon), hypergravity (2g) and hypergravity (4g), respectively. The x-axis indicates the percentage of genes in the study associated with each GO term, while the y-axis lists the GO term names. Dot size reflects the number of genes contributing to each term, and color intensity corresponds to the Benjamini-Hochberg p-value of enrichment (scale shown at right), with red indicating higher significance. Distinct gravity conditions elicit partially overlapping but condition-specific functional responses, including processes related to nucleic acid metabolism and cell cycle regulation under reduced gravity, and stress responses, secondary metabolism, and cuticle and wax biosynthesis under increased gravity.

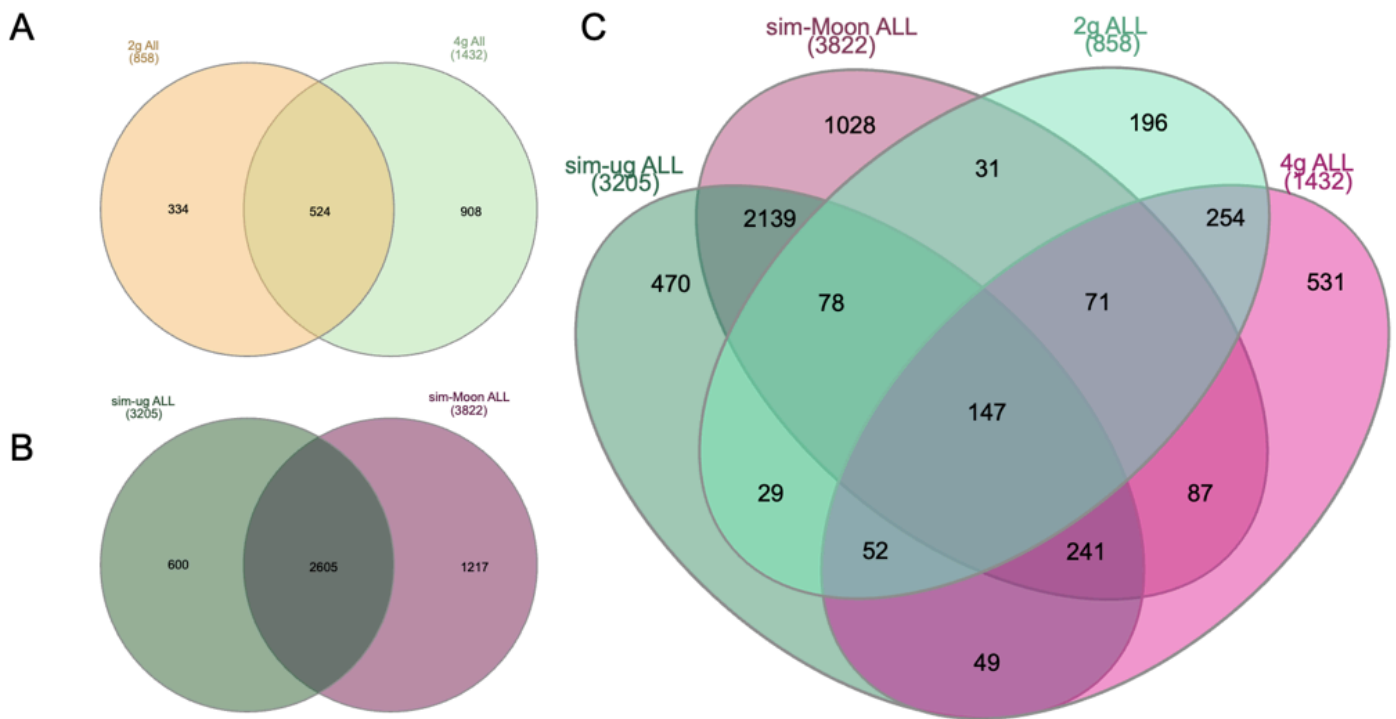


Figure 4

Overlap of differentially expressed genes across altered gravity conditions.

Venn diagrams summarize the overlap of differentially expressed genes (DEGs) identified under simulated gravity conditions. A) Overlap between DEGs identified hypergravity (2g) and hypergravity (4g), showing both shared and condition-specific gene sets. B) Overlap between DEGs identified under simulated microgravity (sim-μg) and simulated Moon gravity (sim-Moon), highlighting a substantial shared transcriptional response between reduced-gravity conditions. C) Four-way Venn diagram showing the overlap of DEGs across sim-μg, sim-Moon, 2g, and 4g conditions. Numbers within each region indicate the count of genes unique to or shared among the indicated gravity treatments. Together, these comparisons reveal both a core set of gravity-responsive genes shared across conditions and distinct gene subsets that respond specifically to reduced versus increased gravity environments.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Wolffiagravitytables051726.xlsx](#)
- [SupplementalFigures.docx](#)