

Genetic Alterations of the Putrescine Metabolic Pathway in *Arabidopsis thaliana* Lead to Attenuated Morphological and Transcriptomic Responses to the Spaceflight Conditions of the International Space Station

Shih-Heng Su

ssu1@wisc.edu

University of Wisconsin–Madison

Patrick H. Masson

University of Wisconsin–Madison

Article

Keywords:

Posted Date: April 15th, 2026

DOI: <https://doi.org/10.21203/rs.3.rs-9349369/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Additional Declarations: No competing interests reported.

Abstract

This study investigates the impact of altering the polyamine (putrescine, Put) metabolic pathway on plant responses to spaceflight conditions in *Arabidopsis thaliana*. By engineering lines with modified Put synthesis and degradation pathways, we observed distinct transcriptional and phenotypic responses to spaceflight. Put-accumulating plants including an *ARGININE DECARBOXYLASE*-over-expressing (*OxPtADC*) line and *Copper-AMINE OXIDASE3* (*cuao3*) knockdown mutants, exhibited fewer differentially expressed genes (DEGs) than the wild type, suggesting that increased Put accumulation may mitigate some stress effects associated with spaceflight. Gene Ontology (GO) enrichment analysis revealed significant changes in biological processes related to spaceflight responses, particularly hypoxia, oxidative stress, and photosynthesis, with Put-modified genotypes showing simplified patterns of expression responses to spaceflight. Specifically, fewer hypoxia-related, and oxidative stress response genes, including those associated with ROS metabolism and cell wall modification, were responsive to spaceflight in the Put-accumulation lines than in the wild type. Similarly, fewer photosynthesis-associated genes were down-regulated under spaceflight conditions in Put-accumulation lines. Morphologically, ISS-grown seedlings exhibited an increase in petiole length, a phenotype previously associated with seedling exposure to hypoxia. This petiole-length response was notably reduced in the *OxPtADC* and *cuao3* genotypes. These findings suggest that manipulating the Put metabolic pathway may enhance plant adaptation to spaceflight conditions, facilitating their incorporation in bioregenerative life-support systems for space-exploration missions.

Introduction

Plants have been recognized as potential components of bioregenerative life support systems for space exploration, capable of recycling water, waste, and CO₂ to generate O₂ and biomass usable as food, feed, fiber and/or source of biopharmaceuticals, through photosynthesis. However, plants have evolved on Earth, constantly exposed to unidirectional gravity. Consequently, they have acquired the ability to use gravity as a key growth guide for their organs (gravitropism). They also developed an ability to counteract the mechanical load imposed by gravity on each organ by acquiring sturdy cell walls and developing specialized morphologies that effectively allow them to withstand load (gravi-resistance and gravi-morphogenesis) [1]. During spaceflight, a loss of significant unidirectional gravity prevents gravitropism, forcing plant organs to use alternative directional cues to guide their growth, such as light, gradients in water, ions, chemicals, oxygen and/or temperature [2–6]. Gravity-induced convection is also eliminated, thereby decreasing gas exchange at the surface of the plant, photosynthesis, respiration, and thermoregulation, and leading to hypoxia [1, 2, 5–8]. Additionally, elevated cosmic radiation during spaceflight can impact the morphology, biology, and genetic makeup of the plants [9]. Overall, the stressors associated with spaceflight trigger a range of biological responses that lead to phenotypes collectively named “space syndrome”, which include shorter organs, thinner leaves, roots tending to grow away from the light source (negative phototropism) while also skewing in some cases, abnormal root hairs, modified organelle size and shape, and altered metabolism and starch content, to cite only a few

[10–14]. These responses limit our ability to develop optimal plant-based bioregenerative systems capable of fully supporting space travel and exploration missions.

Recent research employing -omics and systems-biology approaches have identified several molecular pathways that are responsive to spaceflight conditions, including those involved in light responses, oxidative stress response, hypoxia, photosynthesis, ribosome genesis, cell wall remodeling, and defense responses [7]. Additionally, recent studies have suggested the involvement of epigenetic alterations, such as changes in DNA methylation, in plant responses to spaceflight conditions [8]. Unfortunately, variability in experimental outcomes has also been observed in these studies, likely due to differences in hardware, culture conditions, and plant genotypes [4, 6, 7, 9, 10]. Understanding the source of this variation will be essential to optimize plant growth conditions, develop optimized genotypes for cultivation during spaceflight, and improve plant resilience under stressful conditions.

Polyamines have been suggested as useful tools to mitigate the effects of environmental stressors on plant growth, development, and productivity (reviewed in [11–13]). These polycations are positively charged molecules under physiological pH that interact with negatively charged molecules such as proteins, nucleic acids, lipids and wall polymers, thereby contributing to expression regulation, modulation of enzymatic- or channel-activity, reactive oxygen species (ROS) scavenging, membrane protection against stress damage, crosstalk with hormonal (abscisic acid (ABA), salicylic acid (SA), ethylene and auxin) and calcium-ion (Ca^{2+}) pathways, wall-polymer cross-linking and modulation of cell-wall plasticity [14–19]. These complex activities allow polyamines to orchestrate a variety of biochemical and physiological adjustments that are crucial for plant survival under challenging environmental conditions [20–22].

The best-studied PAs in plants include putrescine (Put), spermidine (Spd), spermine (Spm) and thermospermine (tSpm). These PAs exist in free form within the plant as well as conjugated to small molecules such as hydroxycinnamic acid, wall polymers, and proteins. Two pathways lead to Put synthesis: one derives from ORNITHINE through a decarboxylation reaction catalyzed by ornithine decarboxylase (ODC, which does not exist in *Arabidopsis thaliana*), and the other derives from ARGinine through a decarboxylation reaction catalyzed by ARGinine DECARBOXYLASE (ADC). In the *Brassicaceae*, two genes have been shown to encode arginine decarboxylase: *ADC1* and *ADC2*. These genes are differentially regulated during development and in response to environmental stress, with *ADC2* being more highly expressed during vegetative growth under normal conditions and *ADC1* expression increasing dramatically upon exposure to stress [23]. This initial step of the pathway is rate-limiting, and *ADC* over-expression has been shown to significantly promote the accumulation of Put in the plant [24] while also conferring increased tolerance to stress [24].

In addition to directly contributing to the regulation of plant growth, development and environmental stress response, Put can be converted into Spd and Spm or tSpm by sequential addition of one or two aminopropyl groups generated by S-adenosylmethionine (SAM) decarboxylation (reviewed in [12, 25]). These reactions are catalyzed by SAM decarboxylases (SAMDCs), tightly regulated enzymes that specify

the amount of SAM diverted from other important pathways (such as ethylene synthesis and multiple methyl-transfer reactions) to the PA synthesis pathway. Spd is critical for mRNA translation and biotin synthesis whereas tSpm regulates vasculature development. Both Spd and Spm contribute to stress mitigation, either directly or indirectly through the signaling function of some of their degradation products (Reviewed in [26, 27]).

The catabolism of PAs is catalyzed by copper-dependent amine oxidases (CuAO) and flavin-containing polyamine oxidases (PAO). A back-conversion pathway leading to the conversion of tetraamines (like Spm or tSpm) to triamines (such as Spd), and/or triamines into diamines, is mainly catalyzed by PAOs [28–30] (Fig. 1A). On the other hand, CuAOs contribute to terminal catabolism, which leads to the production of H_2O_2 , GABA (for Put and Spd) and other catabolic products. These molecules also contribute to signal transduction and stress response by regulating ion homeostasis, modulating pH levels, and alleviating oxidative damage under stress conditions [14, 31–33].

Multiple environmental stimuli including hypoxia, heat, chilling, salinity, drought, ozone, UV and heavy metals, have been shown to promote the accumulation of Put and, sometimes, derived PAs (Spd and Spm) in plants [25, 34–36]. On the other hand, environmental stresses that induced the accumulation of Put in plant tissues did not automatically result in dramatic changes in Spd and Spm levels, probably because the levels of these compounds are highly regulated through feedback regulation of SAMDC activity as well as oxidation and back-conversion pathways (reviewed in [36]). In addition to responding to environmental stress by increased expression levels, key genes contributing to PA metabolism have been shown to improve plant tolerance to environmental stress when over-expressed. For instance, over-expression of *ODC* in tobacco led to increased salt tolerance [37], whereas over-expression of oat or *Datura stramonium ADCs* led to increased tolerance to drought in rice [38]. Similarly, over-expression of *ADC2* in *Arabidopsis thaliana* enhanced plant tolerance to drought [24]. Furthermore, over-expressing *Poncirus trifoliata ADC* (*PtADC*) in transgenic *adc1-1* *Arabidopsis* plants led to increased accumulation of Put, with only minor changes in Spd and Spm levels, a phenotype that was accompanied by enhanced resistance to multiple stressors, including high osmoticum, dehydration, long-term drought, and cold stress. This increased resilience to environmental stressors was accompanied by decreased accumulation of reactive oxygen species (ROS) under stress, probably due to the Put-dependent activation of ROS-scavenging enzymes and modulation of stress-related gene expression [39]. Many other examples of engineered tolerance to environmental stressors through modulation of the PA metabolic pathway have been published in the last few years, such as increased tolerance to environmental stressors conferred by SAMDC over-expression in tobacco and rice [40, 41], or increased tolerance to multiple stressors upon SPDS over-expression in transgenic pear [42].

Considering the stress imposed on plants by the spaceflight conditions, we reviewed previous transcriptomic analyses of plant responses to spaceflight, expecting increased expression of genes associated with Put metabolism. Surprisingly, no significant global expression changes of PA-metabolism genes were observed during spaceflight. Therefore, we hypothesized that a failure to activate the PA metabolic pathway might exacerbate plant responses to spaceflight conditions. We

genetically engineered *Arabidopsis thaliana* plants to accumulate either more or less Put in their tissues relative to wild type and tested these lines' abilities to respond morphologically and transcriptionally to the spaceflight conditions of the *International Space Station* (ISS). We show that seedlings engineered to accumulate more Put in their tissues relative to wild type display attenuated morphological and transcriptional responses to spaceflight relative to wild type or seedlings engineered to accumulate less Put in their tissues. Our results suggest engineering plants for increased Put accumulation may improve their tolerance to spaceflight conditions.

Results and Discussion

3.1 Spaceflight Does not Significantly Activate the Expression of Genes Involved in Put Metabolism

To investigate a possible effect of spaceflight conditions on the expression of Put-metabolism-associated genes, we performed a meta-analysis of expression responses to spaceflight using the results of 5 previous studies summarized in NASA's Open Science Data Repository database (<https://genelab.nasa.gov/>). **Supplemental Table S1** shows no evidence of global activation of Put-metabolism-related genes upon spaceflight in wild type plants despite widespread evidence of stress [9]. Therefore, we hypothesized that a lack of activation of expression of Put-metabolism-related genes under spaceflight conditions may contribute to strong, unmitigated stress-related responses under these conditions. We further postulated that engineering plants to accumulate more Put in their tissues might help mitigate the stress associated with exposure to spaceflight.

To investigate this possibility, we engineered *Arabidopsis thaliana* lines for altered Put accumulation in their tissues relative to wild type. A potential for increased Put accumulation was created by over-expressing *Poncirus trifoliata* ARGININE DECARBOXYLASE gene (*PtADC1*) in wild type *Col* plants under the control of the strong CaMV 35S promoter ([39]; Fig. 1), as well as using two *cuao3* knock-down/out mutants (*cuao3-1* and *cuao3-100^{cdr7}*). On the other hand, decreased Put accumulation was engineered by either over-expressing the *AtCUAO3* gene in a *cuao3-100^{cdr7}* background (*OxCuAO3*), or knocking out the *ADC1* gene (*adc1-1* mutant).

We then used a RT-qPCR strategy to verify the effect of these genetic alterations on the expression of the targeted genes. Results reported in Supplemental **Figure S1A** showed the *AtADC1* gene is expressed at wild type levels in wild type *Col*, *cuao3-1* and *cuao3-100^{cdr7}* knockout/down mutants and in *OxAtCuAO3* seedlings, and at very low levels in the *adc1-1* mutant seedlings, as expected. *AtADC1* transcript levels were also lower in *OxPtADC1* plants, suggesting over-expression of the *PtADC* transgene led to decreased expression of the native *AtADC1* gene. The latter plants expressed a high level of *PtADC1* transcripts, which was not found in other lines, as expected (**Supplemental Figure S1B**). Overall, we observed a 2.6-fold increase in *ADC* expression levels in *OxPtADC* relative to wild type. On the other hand, *AtCuAO3* transcripts were found at lower levels than wild type in *cuao3-1* mutant seedlings, and at

higher levels in *OxAtCuAO3* seedlings, as expected (**Supplemental Figure S1A, lower panel**). *cuao3-100^{cdr7}* mutant accumulated wild-type *CuAO3* transcript levels. However, this gene carries a R483K mutation that affects a region of the protein that may modulate substrate access to the nearby catalytic domain, suggesting altered enzymatic activity. [43]

We then quantified the levels of Put, Put precursors (Arginine and Agmatine: Fig. 1B), and GABA, a Putrescine catabolic product, in root and shoot tissues of wild type and genetically modified seedlings. Figure 1C shows increased Put accumulation in both shoots and roots of *OxPtADC*, *cuao3-1*, and *cuao3-100^{cdr7}*. Additionally, Put levels were not altered in the *adc1* mutant under these conditions, probably as a consequence of functional redundancy with *ADC2* [44]. We also observed an 80% reduction in arginine, the ADC substrate, in *OxPtADC* shoot tissues, and no changes in the roots, whereas agmatine, the ADC product, accumulated significantly (8-fold) in both root and shoot tissues of *OxPtADC* while slightly decreasing in *cuao3-1*. On the other hand, arginine accumulated 1.7-fold in *cuao3-100^{cdr7}* shoots, and only mildly in the roots, whereas agmatine decreased only mildly in *cuao3-100^{cdr7}* shoot tissues. Interestingly, we observed no significant alterations in GABA levels in any of the genotypes and/or tissues tested in this project (Fig. 1C), except for a mild decrease in *cuao3-100^{cdr7}* roots. This resiliency of GABA levels to alterations in the Put metabolic pathway under regular growth conditions may be a consequence of the tight control exerted by the GABA shunt on GABA levels under non-stressful conditions [45]. Similarly, spermidine and spermine levels remained relatively stable across all genotypes, suggesting stringent regulation of these polyamines in these backgrounds. (**Supplemental Figure S2**)

In conclusion, our genetic alterations of the Put metabolic pathway were successful at generating lines that accumulate either more (*OxPtADC* and *cuao3*) or less (*OxCuAO3*) Put than wild type in their tissues, enabling studies of potential mitigating effects of Put and/or Put-derived products on the space syndrome.

3.2 ISS-VEGGIE-Grown Seedling Shoots Are Larger than Ground Controls

To understand the effect of alterations in the Put metabolic pathway on plant responses to spaceflight conditions, we germinated and grew our wild type and genetically modified lines in Petri dishes within the VEGGIE growth chamber in the *International Space Station* (ISS) for nine days, alongside a ground-based control at the *Kennedy Space Center* that mimicked the growth conditions of ISS with a 48-h delay (GC). The 9-day-old seedlings were then photographed, harvested, fixed in *RNA/ater*, frozen at -80°C and returned to the laboratory for analysis.

The photographs taken before harvesting revealed the 9-day-old seedlings of all genotypes exhibited phenotypes previously seen for seedlings grown in Petri dishes under microgravity conditions in the ISS, with roots growing more randomly than the GC (**Supplemental Figure S3**). However, the shoot

phenotypes could not be evaluated on these images because the seedlings' density was too high, originally designed to generate enough biomass for RNA extraction and subsequent expression analysis.

To further evaluate the shoot phenotypes, we dissected the shoots from 16 *RNA/ater*-fixed seedlings per genotype. These shoots were manually flattened on a horizontal surface for morphometric analysis. We observed obvious differences between the ISS-grown and GC seedlings (Fig. 2A). The total shoot area confined within the borders outlined around each organ was generally larger for ISS-grown seedlings of all genotypes than those grown on Earth (Fig. 2A-B). The ratio between ISS-grown and GC seedling areas was not significantly different between genotypes (Fig. 2C).

A more careful evaluation of shoot morphology revealed a 1.5 to 2 times increase in petiole length between ISS-grown seedlings and the GC (Fig. 2A, D). Increased petiole length has previously been associated with hypoxia responses in Arabidopsis [46–48], suggesting a potential link to oxygen limitation during spaceflight. Interestingly, this ISS-induced increase in petiole length was significantly less pronounced in *OxPtADC* and *cuao3-100^{cdr7}* than the wild type and the other genotypes tested (Fig. 2E). Hence, two of the three genotypes engineered to accumulate more Put than wild type displayed an attenuated leaf petiole growth response to the spaceflight conditions relative to wild type and other genotypes.

3.3 Genotypes with Alteration in Put Metabolism Display Distinct Transcriptional Responses to the ISS Environment

To better understand the impact of genetic alterations in the Put metabolic pathway on the space syndrome, we compared the transcriptome profiles of dissected shoots and roots between ISS-grown and GC seedlings of our six genotypes. For Col, *adc1*, *OxPtADC*, and *OxCuAO3*, genes showing significant differential expression between ISS and GC using **both** DESeq ($p^{adj} < 0.05$) and EdgeR ($q < 0.05$) were considered representative DEGs. For *cuao3* mutant plants, only genes found to be significantly differentially expressed between ISS and GC in **both** independently isolated *cuao3-1* and *cuao3-100^{cdr7}* mutant lines were retained as DEGs. The complete list of DEGs for all genotypes is available in **Supplemental Table S2**.

The results from this expression analysis are summarized in Fig. 3. First, we observed dramatic differences in numbers of DEGs between genotypes, with wild type Col exhibiting the highest total number of DEGs (6,211 in shoots and 2775 in roots) and *OxPtADC* the lowest (2,260 in shoots and 506 in roots). Second, in all genotypes tested, the roots displayed fewer DEGs than the shoots. Third, the *OxPtADC* and *cuao3* genotypes showed a dramatic reduction (60–90%) in the total numbers of DEGs relative to the other genotypes. In fact, a *Principal Component Analysis (PCA)* grouped the *OxPtADC* and *cuao3* genotypes together, away from a second grouping made of the *adc1* and *OxCuAO3* genotypes, both separated from the wild-type Col genotype (Fig. 3C). Hierarchical Clustering of gene expression patterns led to similar genotype groupings (Fig. 3D). Interestingly, these groupings correlate with the

aforementioned PA quantification, which revealed significant increases in Put levels in the *OxPtADC* and *cuao3* lines relative to the other genotypes (Fig. 1C; **Supplemental Figure S2**).

While many genes were found to be differentially expressed between spaceflight and GC conditions in all genotypes tested, only a few were found to exhibit similar expression responses in all five genotypes and organs. Among them, only 55 DEGs were up-regulated and 24 were down-regulated in both roots and shoots of all genotypes (Fig. 3A and B; **Supplemental Table S2**). A Gene Ontology (GO) enrichment analysis of the common down-regulated DEGs revealed no significantly enriched GO groups, probably as a consequence of the small number of genes falling in this category. However, some interesting genes, such as *GUN5* (related to the gun retrograde signaling pathway) and *HY5* (related to biotic stress), were identified in this list. For the 55 up-regulated DEGs, GO groups related to hypoxia and the cell wall were enriched. These expression responses to spaceflight conditions are compatible with those previously reported for plants exposed to microgravity [49, 50]. In any case, the small list of common DEGs shared by shoots and roots of all genotypes despite dramatic tissue- and genotype-specific expression responses suggests that roots and shoots respond differently to the spaceflight conditions, as previously reported [49, 50].

3.4 GO Enrichment Analysis Suggests Genetic Alterations Leading to Increased Put Accumulation Result in Simplified Expression Responses to Spaceflight

To better understand the biological processes preferentially associated with root and shoot responses to spaceflight conditions, we performed a Gene Ontology (GO) enrichment analysis of the DEGs associated with each genotype. The results from this analysis are provided in **Supplemental Table S3**, with highlights summarized in Figs. 3 and 4. In wild type Col roots, the up-regulated DEGs are enriched for genes from GO groups related to ethylene response (GO:0009723), hormone signaling (GO:0009725), hydrogen peroxide (GO:0042542), cell communication (GO:0007154), and hypoxia (GO:0001666) (Fig. 4A). Conversely, the down-regulated DEGs are enriched for genes from GO groups related to the response to light (GO:0009314), temperature (GO:0009266), circadian rhythm (GO:0007623), and cell wall modification (GO:0042545) (Fig. 4B). In the shoots, the up-regulated DEGs are enriched for genes from GO groups associated with responses to hypoxia (GO:0001666), hormone-mediated signaling (GO:0009755), lipid response (GO:0033993), mRNA processing (GO:0006397), and auxin biosynthesis (GO:0009851) (Fig. 4C). The down-regulated DEGs are enriched for genes from GO groups associated with photosynthesis (GO:0015979), microtubule-based movement (GO:0007018), response to oxidative stress (GO:0006979), reactive oxygen species (ROS) (GO:0000302), and ribosome biogenesis (GO:0042254) (Fig. 4D). Overall, the GO enrichment analysis of Col DEGs aligns closely with findings from previous studies, underscoring the reliability of our data [6, 7, 49, 51, 52].

To evaluate the potential mitigating effect of Put on plant responses to the microgravity environment, we compared expression responses to spaceflight conditions between wild type Col and Put-accumulating lines: *cuao3* and *OxPtADC* (Figs. 3 and 4). A GO-enrichment analysis revealed a striking contrast between Put-accumulating lines and wild type Col, with *cuao3* and *OxPtADC* lines displaying a drastic and similar simplification of their transcriptomic responses to spaceflight, including a loss of significant enrichment for DEGs associated with cell wall modification (GO:0042545) amongst the down-regulated

root DEGs, ethylene response (GO:0009723), cell communication (GO:0007154) and ROS response (GO:0000302) amongst the upregulated root DEGs, photosynthesis (GO:0015979) and ribosome biogenesis (GO:0042254) amongst the down-regulated shoot DEGs, and auxin response (GO:0009851) and calcium signaling (GO:0019722) amongst the up-regulated shoot DEGs (Fig. 4). Additional GO groups were also found to be preferentially associated with spaceflight DEGs only in wild type Col organs, including some associated with ROS response (GO:0000302) (Fig. 4).

To better understand this simplification of expression responses to spaceflight conditions associated with increased Put accumulation, we performed a GO-enrichment analysis of genes found to be differentially expressed under microgravity in wild type Col roots and shoots, but **not** in the Put-accumulation lines. In the root, 2,162 genes were identified as differentially expressed in Col but unaltered in *OxPtADC* and *cuao3*. These genes were enriched in GO groups related to response to hypoxia (GO:0001666), photosynthesis (GO:0015979), and cell wall modification (GO:0042545) (**Supplemental Table S4, List 1-root**). In the shoot, 3,066 genes were differentially expressed under spaceflight conditions in Col, but not in *cuao3* or *OxPtADC*. GO groups related to photosynthesis (GO:0015979) and response to hypoxia (GO:0001666) were also significantly enriched in this list of Col shoot DEGs. Additionally, GO groups related to translation (GO:0006412), ribosome biogenesis (GO:0042254), and chloroplast RNA processing (GO:1901259) were enriched (**Supplemental Table S4, List 2-shoot**). Taken together, these exciting observations suggest that altering the Put metabolic pathway may alleviate some aspects of plant organ stress responses to spaceflight conditions. The next sections delve deeper into a comparative analysis of transcriptomic responses to spaceflight between genotypes, focusing more specifically on DEGs associated with defined GO groups previously implicated in plant responses to spaceflight such as hypoxia, oxidative stress, photosynthesis and cell wall metabolism.

3.5 Altering the Put Metabolic Pathway Significantly Affects Hypoxia-Related (GO: 0001666) Expression Responses to Spaceflight Conditions

Hypoxia is a well-known stressor for plants under microgravity ([5, 6, 53, 54]) and our study confirms a strong enrichment of up-regulated spaceflight DEGs for genes associated with GO-group *GO:0001666* in all tested genotypes (Fig. 4). Additionally, spaceflight-grown seedlings displayed longer petioles than GC (Fig. 2D **and E**), a phenotype previously associated with exposure to hypoxia [47, 48]. As a first step to better understand the *Hypoxia* component of plant responses to spaceflight, we used hierarchical clustering to compare hypoxia-related DEG expression responses to spaceflight between shoots and roots of wild type Col seedlings (Fig. 5A). Of the 265 genes included in GO-group *GO:0001666*, 164 were spaceflight DEGs in either roots or shoots of Col seedlings (Fig. 5A). However, only 35% of these genes showed similar expression responses to spaceflight (down or up) in both organs (Hypoxia groups I and II, respectively: I^h and II^h ; Fig. 5A). In addition to being enriched for biological processes associated with hypoxia and oxygen responses, the down-regulated group- I^h DEGs were also enriched for genes

associated with responses to water and temperature stimulus whereas the up-regulated group-*II^h* DEGs were significantly enriched for genes associated with anaerobic respiration, phospholipid catabolism, membrane metabolism and fatty acid metabolism (**Supplemental Table S5**).

The list of *II^h* up-regulated DEGs includes the *HUP29/PCO1* (*AT5G15120*) and *HUP43/PCO2* (*AT5G39890*) genes, which encode cysteine-oxidase enzymes functioning as primary oxygen sensors that target terminal cysteines in ERFVII-type transcription factors, thereby promoting their degradation by the N-end rule pathway under normoxic conditions [55]. One of their known targets in this pathway, *ERF73/HRE1* (*AT1G72360*), was also found amongst the *II^h* DEGs [56]. Importantly, 29 of the 32 *II^h* DEGs found in this project were reported to respond to ERFVII-type transcription factors in a previous study ([57]; Supplemental Table S5). Furthermore, 10 of them were found to co-immunoprecipitate with HRE2 in chromatin immunoprecipitation assays, supporting a role for this hypoxia-response pathway in their regulation [57] (**Supplemental Table S5**).

Additional putative transcription factor binding sites were also found in the promoter region of *I^h* - and *II^h* -cluster DEGs (Supplemental Figure S5). For instance, 7/26 *I^h* and 9/32 *II^h* DEGs are predicted targets of the bZIP16 (*AT2G35530*) transcription factor, which has been shown to integrate light and hormonal signaling pathways to modulate seed germination and seedling growth in *Arabidopsis thaliana* [58] (**Supplemental Table S5**; see section 3.8. below).

Groups *III^h* and *V^h* include root-specific DEGs. In group *III^h*, the DEGs are down-regulated under spaceflight conditions. This group is enriched for genes that are associated with programmed cell death and defense-response GO-groups, in addition to hypoxia responses. Several transcription factors are predicted to target multiple *III^h*-group genes, including PIF4, HY5 and bZIP16 (**Supplemental Table S5**). bZIP16 and HY5 are G-box binding proteins [58–60] that, along with *HY5-Homolog* (*HYH*), are down-regulated under microgravity conditions in both shoots and roots of all genotypes tested, except *cuao3-100^{cdt7}* for *HY5* (**Supplemental Table S2**).

Group *V^h*, on the other hand, includes a short list of root-specific DEGs that are up-regulated under spaceflight conditions. This *V^h* list is enriched for genes that belong to the phosphate-starvation and nutrient-response GO-groups. Groups *IV^h* and *VI^h* consist of shoot-specific DEGs that are either down- or up-regulated in response to spaceflight conditions, respectively. Group *VI^h* is enriched for genes that are associated with the ethylene-response and anaerobic-respiration GO groups (**Supplemental Table S5**). Two *VI^h*-group DEGs encode ERFVII-type transcription factors that were previously identified as key regulators of hypoxia responses: RAP2.12 (*AT1G53910*) and RAP2.2 (*AT3G14230*). Furthermore, 27 of the 33 *VI^h* DEGs were previously identified as ERFVII-responsive genes, with 12 of them carrying HRPE elements in their DNA (Supplemental Table S5; [57]). Together, these data support a role for hypoxia-related ERFVII-based expression regulation of *VI^h*-DEGs in *Arabidopsis* shoots under microgravity conditions.

A basic helix-loop-helix transcription factor (bHLH15) may also contribute to the regulation of V^h -DEG expression. Indeed, of the 33 DEGs associated with this group, 10 are predicted targets of bHLH15 (**Supplemental Table S5**). This transcription factor is known to interact with the PIF1 and PIL5 transcription factors, altering their DNA binding properties [61]. Together, these results suggest that the hypoxia response induced by microgravity may involve different regulatory mechanisms in roots and shoots, consistent with previous work [48].

To better understand the effects of alterations in the Put metabolic pathway on hypoxia-related expression responses to spaceflight, we used hierarchical clustering to compare the expression responses to spaceflight of *GO:0001666 (Response to Hypoxia)*-associated DEGs between genotypes (Fig. 5B). Although all genotypes showed significant enrichment for *hypoxia response*-related DEGs in both root and shoot (Fig. 5B), there were significant differences between genotypes within this GO group (Fig. 5B; **Supplemental Table S6**). Col exhibited the highest number of hypoxia-related DEGs between spaceflight and GC conditions (115 genes), with the other 4 genotypes showing significant decreases in DEG numbers. *OxPtADC* and *cuao3* showed the fewest hypoxia-related DEGs under spaceflight conditions (Fig. 5B).

In the root, very few hypoxia-related DEGs were common across all genotypes (clusters $r-a^h$ and $r-e^h$ for up-regulated and down-regulated DEGs, respectively). The 8 genes belonging to cluster $r-a^h$ in roots are also up-regulated in the shoot, and they are predicted targets for ERFVII-type transcription factors previously characterized as signal transducers of oxygen sensing during hypoxia in plants [57] (Fig. 5B; **supplemental figures S5 and S6**). They are not enriched for other defined biological processes. Cluster $r-c^h$, on the other hand, is made of hypoxia-related genes that are significantly up-regulated in wild type, *adc1* and *OxCuAO3* roots during spaceflight, and not in putrescine-accumulation mutants *cuao3* and *OxPtADC*. This cluster contains 11 DEGs that are enriched for genes that contribute to immune responses ($p = 4.45E-05$; **Supplemental Table S6**). Among the latter, *SPX-DOMAIN GENE 1 (SPX1: AT5G20150)* encodes an inositol polyphosphate-sensing SPX-domain containing protein that binds to transcription-factor PHR1 in the presence of inositol polyphosphate, affecting its ability to regulate the expression of phosphate starvation-induced genes [62]. Two other genes in this group, *AT1G73010 (Phosphate Starvation-Induced 2 = PS2)* and *AT3G02040 (SRG3)*, encode hydrolases that contribute to phosphate remobilization from organic molecules to maintain the cytosolic phosphate pool under phosphate starvation or other stressful conditions such as hypoxia [63, 64]. Interestingly, three $r-c^h$ genes (*AT1G03220*, *AT2G26560* and *AT3G07350*) also carry putative ERFVII-binding sites within their promoters.

The $r-f^h$ cluster includes a group of 42 hypoxia-related DEGs that are down-regulated in Col roots under spaceflight conditions, without being affected in the other genetic backgrounds. Twenty-five of these 42 $r-f^h$ genes are predicted targets for calmodulin-binding transcription activators (CAMTA), which contribute to Ca^{2+} signaling in response to abiotic and biotic stressors (**Supplemental Table S6**). Several $r-f^h$ DEGs encode proteins that also contribute to Ca^{2+} -signaling, including MYB30, a transcription factor

that inhibits the expression of Ca^{2+} signaling genes in response to oxidative and heat stress [65], a calcium-dependent lipid-binding protein (AT4G34150), Calmodulin-Like37 (CML37), Calmodulin-Binding Protein of 25 kDa (CAMBP25), Respiratory Burst Oxidase Homologue D (RBOHD; AT5G47910), Soybean Regulated by Cold 2 (SRC2; AT1G09070), and Xyloglucan Endotransglucosylase/hydrolase18 (XTH18; AT4G30280). Another seventeen *r-f^h* DEGs are predicted to encode defense response proteins (**Supplemental Table S6**). One of them, *AT4G11660*, encodes the HEAT Shock Transcription Factor B2B (HSFB2B), which was previously shown to mediate abiotic stress response of the circadian clock, fine-tune cold-induced vernalization and regulate the expression of pathogen resistance genes [66, 67].

DEGs in the *r-f^h* cluster (14 genes) showed downregulation in *Col* and *OxCuA03* roots under spaceflight conditions, without alterations in the other backgrounds, suggesting they might be responsive to GABA signaling. This cluster includes several transcription factor genes previously implicated in environmental stress response such as *DRE-Binding Protein 2A* (*DREB2A*; AT5G05410), *MULTIPROTEIN BRIDGING FACTOR 1C* (*MBF1C*; AT3G24500), *TOLL-Interleukin-Resistance (TIR) Domain-containing Protein* (AT1G72940) and *TRANSDUCIN/WD40 REPEAT-LIKE SUPERFAMILY PROTEIN*: AT1G78070). It also includes multiple signal-transducer genes such as *TCH4* (AT5G57560), *Auxin responsive family protein* (AT5G35735), *heat shock protein 101* (AT1G74310) and several additional transducers (Fig. 5; **Supplemental Table S6**).

It may be significant that most of the *r-f^h* and *r-c^h* genes are down-regulated in most genetically modified lines relative to *Col* under ground-control conditions, while displaying similar levels of expression between all lines under spaceflight conditions. This suggests alterations in the Put metabolic pathway triggered transcriptomic changes that are normally associated with wild type responses to hypoxia, better preparing the plants for exposure to hypoxic conditions. Similarly, many *r-c^h* genes are down-regulated in Put-modified lines relative to wild type under spaceflight conditions, suggesting an attenuation of plant responses to spaceflight (Fig. 5B).

In the shoot, a larger number of common hypoxia-related DEGs were found across all genotypes compared to the root (clusters *s-a^h* and *s-e^h*; Fig. 5B, **Shoot FL/GC panel**). Among them, shoot-specific *s-a^h*-cluster DEGs, which are similarly up-regulated in all genotypes under spaceflight conditions, include the ERFVII-type RAP2.2 transcription factor previously implicated in O_2 sensing [68], the Acyl-CoA-Binding Domain 3 (ACBP3) protein, known to sequester the RAP2 proteins at the membrane under normoxia conditions, ALANINE AMINOTRANSFERASE2, which converts pyruvate into alanine, contributing to nitrogen conservation under hypoxia, several genes that contribute to anaerobic respiration (*AT3G10020*, *AT1G33055*), and others that contribute to ethylene signaling (*ETR2*, *CTR1*, and *EIN3*) (Fig. 5B; **Supplemental Table S6**). A subgroup of *s-a^h* DEGs named *s-a^h'* include genes that are similarly up-regulated under spaceflight conditions in all genotypes tested, but with decreased level of upregulation in the *cuao3* loss-of-function mutant. This subcluster includes the *ALCOHOL DEHYDROGENASE1* (*ADH1*) gene involved in alcoholic fermentation, *HEMOGLOBIN1* (*HB1*), which encodes a nitrate reductase-associated protein that contributes to NO recycling into NO_3^- , and a few

other genes that encode proteins that function in ROS metabolism. Interestingly, under spaceflight conditions, most of these *s-a^h* cluster genes are downregulated in *cuao3* mutant shoots relative to wild type Col, but less in *OxPtADC*, suggesting a role for GABA in their regulation (Fig. 5B, **Shoot FL panel**).

Eight genes were upregulated in the shoots of most genotypes but *OxPtADC* and *atcuao3* (cluster *s-c^h*, Fig. 5B; **Supplemental Table S6**). Among them is the anoxia-responsive *HRE1* transcription factor gene (*AT1G72360* [68]), and several genes that contribute to phosphate homeostasis including key transcription-factor genes such as *HHO2* (*AT1G68670*, a myb-like transcription factor gene that functions downstream of *PHR1* to regulate phosphate homeostasis [69]) and *HYPERSENSITIVITY TO LOW PI-ELICITED PRIMARY ROOT SHORTENING 1* (*HRS1*, *AT1G13300*, a molecular logic gate that integrates P and N signals [70]). Additionally, a larger group of DEGs (14) were found to be upregulated in all genotypes but *OxPtADC* (*s-d^h*), including the *RAP2.12* transcription-factor gene (*AT1G53910*, also an O₂ sensor [71]), *WRKY22* (*AT4G01250*, which modulates ethylene biosynthesis [72]) and several signal transducers (Fig. 5; Supplemental Table S6). Comparing shoots and roots, only 4 genes overlapped within the *c^h* and/or *d^h* clusters (*AT5G41080*, *AT5G57220*, *AT1G03220* and *AT1G19530*), again indicating these organs respond very differently to the hypoxic stress associated with spaceflight conditions.

OxPtADC and *cuao3* showed a significant decrease in numbers of hypoxia-related DEGs relative to Col. *adc1* and *OxCuAO3* expression responses to spaceflight conditions were most similar to Col except for a cluster of 16 up-regulated DEGs that were not shared with Col (cluster *s-g^h*, Fig. 5B). Interestingly, a large proportion of cluster-*s-g^h* DEGs (10 out of 18) are predicted targets for calmodulin-binding transcription activator 1 (*CAMTA1*; **Supplemental Table S6**), a transcription factor that contributes to Ca²⁺/calmodulin signaling and plays a crucial role in regulating plant responses to auxin and both biotic and abiotic stresses [73]. Two CAMTA genes (*AT1G67310* and *AT2G22300*) showed increased expression under spaceflight conditions in the shoots of all but *OxPtADC* lines, and no changes in the roots (**Supplemental Table S2**). Importantly, other genes in the *s-g^h* cluster are annotated as also functioning in Ca²⁺ signaling, including *TCH3* (*AT2G41100*) and *TCH4* (*AT5G57560*), suggesting a role for Ca²⁺ signaling in shoot responses of Put under-producing lines. This is in sharp contrast with the decreased expression of potential CAMTA targets within the *r-f^h* cluster in Col roots, again suggesting a profound difference between root and shoot responses to the space environment (**Supplemental Table S2**; Fig. 5).

The *s-g^h* cluster also contains the *MAP Kinase Kinase 9* (*MKK9*) gene, which was previously shown to modulate plant tolerance to submergence via both membrane integrity and hypoxia signaling in a phosphatidic acid-dependent manner [74]. This cluster contains an *ALANINE AMINOTRANSFERASE1* (*A/AT1*) gene (*AT1G17290*) that contributes to Nitrogen conservation under hypoxia [68], and other genes previously reported to contribute to hypoxia stress response (Fig. 5; **Supplemental Table S6**). Differential expression of these genes under spaceflight conditions in *adc1* and *OxCuAO3* but not in wild type Col or Put-accumulation lines suggests a possible contribution of GABA in their regulation.

A small number of genes (6 out of 8) were downregulated in the shoot of all genotypes under spaceflight conditions (cluster *s-e^h*), including HSF-B2B [67] and a *Rubber Elongation Factor Protein* gene (*AT1G67360*). These genes were also downregulated in the roots of all tested genotypes. On the other hand, a larger group of 26 genes were downregulated in the shoots of only Col in response to spaceflight conditions (cluster *s-f^h*). 8 of them were also downregulated in Col roots, including several that encode Ca^{2+} signal transducers such as calcium-dependent lipid-binding protein (*AT4G34150*), Calmodulin-Like37 (*CML37*) and Respiratory Burst Oxidase Homologue D (*RBOHD*; *AT5G47910*). The *s-f^h* cluster also included the *PDC2* (*AT5G54960*) and *DIC2* (*AT4G24570*) genes, which encode a pyruvate decarboxylase-2 enzyme involved in anaerobic fermentation and a dicarboxylate carrier that may help maintain the balance between metabolic intermediates during anaerobiosis [75], the *WRKY46* and *WRKY70* genes, which encode transcription factors that contribute to defense response [76], and additional genes that encode proteins associated with the defense-response GO-group ($p = 3.29\text{E-}07$; **Supplemental Table S6**; Fig. 5). Every genetic alteration in the Put metabolic pathway tested in this project led to an elimination of spaceflight-induced upregulation of these *s-f^h* genes, suggesting homeostasis of Put and/or some of its metabolic products is/are critical for their regulation.

Overall, we conclude that altering the PA metabolic pathway leads to dramatic alterations in the patterns of anoxia-related expression responses to the spaceflight conditions of ISS, with simplification of response profiles in the genetically altered plants. Therefore, we wondered whether these alterations truly resulted from direct alterations in expression responses to the spaceflight conditions by our genetic manipulations of the Put metabolic pathway or instead reflected differential expression profiles between genotypes that mimicked some of the wild type expression responses to spaceflight conditions. To address these possibilities, we compared expression profiles between genetically altered plants and wild type Col under both spaceflight and ground-control conditions. The results are summarized in Figs. 5A and B, FL and GC panels respectively.

The expression profiles of anoxia-related genes under spaceflight conditions were remarkably similar between genotypes, with only a small fraction of the Col DEGs being differentially expressed between the genetically altered lines and Col under spaceflight. Those that were differentially expressed showed mostly a down-regulation in the genetically modified lines relative to Col, supporting an alleviation of the transcriptomic response to stress by alteration of the Put metabolic pathway. Furthermore, the DEG clusters discussed above displayed dramatic differences in the proportion of genes showing differential expression between modified lines and Col under spaceflight conditions, with *r-c^h* and *s-a^h* showing the largest proportion, and *r-f^h*, *r-h^h*, *s-b^h*, *s-g^h*, *s-c^h* and *s-d^h* showing the lowest.

The decreased expression responses to spaceflight conditions of *r-c^h* genes in *cuao3*, *OxPtADC* and *OxCuAO3* roots relative to Col under spaceflight conditions was dramatic, illustrating an attenuation of responses related to immunity and phosphate starvation in these lines (Fig. 5). Interestingly, under GC conditions, only one *r-c^h* DEG, *AT2G23270*, was upregulated in *OxPtADC* relative to wild type Col. The other DEGs were either unaffected or down-regulated in 1, 2 or 3 of the genetically modified backgrounds

relative to wild type (Fig. 5A, **Root GC panel**). Together, these data suggest the differential expression responses of *r-c^h* genes to spaceflight between genotypes is not a simple artefactual response to PA. As ROS signaling is an important component of these responses, and PAs have been shown to moderate ROS signaling in plants, the data are compatible with a potential mitigating effect of increased Put accumulation and/or altered Put metabolic product on stress response, possibly impacting stress tolerance in a positive way. A similar conclusion can be suggested for the attenuated response of shoot *s-a^h* genes in *cuao3* and *OxPtADC* lines (Fig. 5A, **Root GC panel**).

Similar analyses of *r-f^h* suggest another mechanism might be at play for this cluster. *R-f^h* genes expression decreased under spaceflight conditions in the wild type, without changing in any of the genetically modified lines (Fig. 5A **and B**). Under ground-control conditions, most genetically modified lines showed lower expression of *r-f^h*-cluster genes relative to wild type. Normally, under regular 1-g conditions, wild type plants exposed to hypoxia display a burst of ROS as a consequence of altered energy metabolism, leading to increased cytosolic Ca²⁺ levels, RBOHD activation, production of apoplastic ROS, RAP2.12 release from its ACP anchor in the plasma membrane and its translocation into the nucleus to promote the expression of glycolysis- and anaerobic metabolism-related genes, among others (reviewed in [68]). Glutamate decarboxylase is also activated, which catalyzes the conversion of glutamate into GABA. In turn, GABA contributes to ROS reduction and modulation of ethylene signaling (reviewed in [77]). The *RBOHD* gene is downregulated in wild type roots during the first 24h of hypoxia treatment, before returning to higher expression levels [78].

In APEX08, wild type Col plants exposed to the microgravity conditions for 9 days displayed decreased expression of all *r-f^h*-cluster genes, including *RBOHD* and other Ca²⁺-signaling genes described above. Hence, microgravity allows decreased *RBOHD* expression beyond the first 24h normally seen under anoxic conditions on the ground. Importantly, our genetic alterations of *CuAOX3*, whether knockout or over-expression, resulted in decreased *r-f^h* gene expression under GC conditions and abolished further inhibition by spaceflight, suggesting Put and/or its metabolic products such as GABA, contribute to this regulation (Fig. 5B). Together, our data suggest the alterations imposed upon the Put metabolic pathway by our genetic manipulations anticipated *r-f^h* gene expression responses to spaceflight conditions, possibly better preparing the plant to respond to the hypoxic stress associated with spaceflight exposure (Fig. 5).

3.6 Modifying the Put Metabolic Pathway Alters the Oxidative Stress Response Associated with Microgravity Exposure

Plants exposed to microgravity conditions are known to display strong oxidative-stress responses (reviewed in Veronica et al., 2023)[79]. To better understand the effects of alterations in the Put metabolic pathway on oxidative-stress responses to spaceflight conditions, we focused our investigations on four ROS-related Gene Ontology groups found to be significantly over-represented in

our lists of spaceflight-associated DEGs (*GO:0006979* ('*Response to Oxidative Stress*'), *GO:0000302* ('*Response to ROS*'), *GO:0072593* ('*ROS Metabolic Process*') and *GO:2000377* ('*Regulation of ROS Metabolic Process*')); **Supplemental Table S3**), noting that *GO:0006979* and *GO:0000302* belong to the same hierarchical branch, with the latter being a subgroup of the former Fig. 6, and **Supplemental Figure S4**).

We first compared the expression of DEGs associated with the '*Response to Oxidative Stress*' GO-group (*GO:0006979*) between root and shoot tissues in Col seedlings (Figs. 6A and **Supplemental Table S7**). As for the transcriptional responses to the hypoxic conditions associated with spaceflight conditions (see previous section), roots and shoots differed substantially from each other in their ROS-associated transcriptional responses to spaceflight (Fig. 6A; **Supplemental Table S7**). Several clusters of similarly expressed ROS-associated DEGs could be observed based on their relative expression responses in shoots and/or roots. In cluster *I^{OS}*, 35 DEGs were downregulated in both organs, with 21 of these genes predicted to encode plastid-localized proteins and 13 annotated as contributing to plant response to light stimuli. In addition to genes encoding enzymes involved in ROS scavenging, this group also includes *DRE-BINDING PROTEIN 2A* (*AT5G05410*), a gene that encodes a transcription factor of the AP2 family that regulates abiotic-stress inducible genes [80], and several abiotic-stress responsive genes such as *LOW TEMPERATURE INDUCIBLE78* (*LT178*) / *COLD RESPONSIVE 78* (*COR78*) / *DESSICATION-RESPONSIVE29A* (*RD29A*) (*AT5G52310*), *TOUCH2* (*TCH2*: *AT5G37770*), *CALMODULIN LIKE37* (*CML37*: *AT5G42380*), *SENESCENCE-ASSOCIATED FAMILY PROTEIN* (*AT1G66330*), *CHLOROPLAST DROUGHT-INDUCED STRESS PROTEIN of 32KD* (*CDSP32*: *AT1G76080*) and *MYB34* (*AT5G60890*, a transcription factor that modulates the synthesis of indolic glucosinolates in response to ABA and JA signaling [81]) (Fig. 6; **Supplemental Table S7**). Cluster *II^{OS}* contains 29 genes with increased expression in both shoot and root tissues under spaceflight conditions. These genes encode proteins predicted to be associated with a variety of cellular compartments, without obvious preferences for localization or molecular function. Seven and eight of these genes are predicted to carry bZIP16- and/or ABF2-binding motifs, respectively (**Supplemental Table S7**).

Clusters *III^{OS}* and *IV^{OS}* include DEGs that are down-regulated specifically in roots (*III^{OS}*) or shoots (*IV^{OS}*) whereas clusters *V^{OS}* and *VI^{OS}* include DEGs that are up-regulated in roots and shoots, respectively. Finally, the 5 DEGs represented in cluster *VII^{OS}* are down-regulated in roots and up-regulated in shoots. In fact, four of the 5 cluster-*VII^{OS}* genes are up-regulated in the shoots of all genotypes tested, but down-regulated in only Col or *cuao3* root tissues (Fig. 6A; **Supplemental Table S7**).

Next, we extended these studies to other genotypes. Spaceflight DEGs associated with *GO:0006979* (*Response to Oxidative Stress*) and *GO:0000302* (*Response to ROS*) were significantly over-represented in both root and shoot tissues of all genotypes, with the notable exception of *cuao3* roots for *GO:0000302* (*Response to ROS*) (Fig. 6A-B; **Supplemental Figure S4A**; **Supplemental Table S8**). Interestingly, the numbers of DEGs associated with these two GO groups were substantially reduced in the roots of all knockout and overexpressing lines compared to wild type Col, and in the shoots of *cuao3*

and *OxPtADC*. These data suggest a correlation between altered Put metabolism in engineered plant tissues and decreased ROS responses to spaceflight conditions (Fig. 6B and **Supplemental Figure S4A**).

Spaceflight DEGs associated with *GO:2000377* (*Regulation of ROS Metabolic Process*) were not significantly enriched in the root tissues of any of the genotypes included in this study. They showed only minor enrichment in Col, *adc1*, and *OxCuAO3* shoot tissues, and no enrichment in the *cuao3* and *OxPtADC* shoot tissues (**Supplemental Figure S4B**). Similarly, spaceflight DEGs associated with *GO:0072593* (*'ROS Metabolic Process'*) were only slightly enriched in *OxCuAO3* in shoot tissues (**Supplemental Figure S4C**). Taken together, these results indicate that Arabidopsis seedlings exhibit a robust ROS-related transcriptional response to spaceflight conditions, but this response is distinct from one involving ROS Metabolic Processes, at least at the transcriptional level.

Previous research has categorized Arabidopsis transcriptomic responses to disruptions in redox homeostasis into eight primary clusters, often called the "ROS wheel", based on the clustering of their expression profiles [82]. Therefore, we compared our lists of ROS-related DEGs from each tested genotype to the eight primary groups of the ROS wheel (*RW*) (Fig. 6C). For Col, 90% of the core genes associated with cluster-*I^{RW}* of the ROS wheel, mostly related to the *GENOME UNCOUPLED* (*GUN*) retrograde signaling pathway, were identified as being differentially expressed under spaceflight conditions in the shoot, but only 34% of them were identified as DEGs in the root. *GUN1* itself, which encodes a protein that modulates the import of nuclear encoded proteins into the plastids, relating information from at least three retrograde signaling pathways into the nucleus, is down-regulated in the shoots of all genotypes tested in this study. Several transcription-factor genes targeted by this pathway, including *GOLDEN2 LIKE 1* and *2* (*GLK1*, *GLK2*) [83], *ELONGATED HYPOCOTYL 5* (*HY5*) and *HY5-HOMOLOG* (*HYH*), are also globally downregulated in spaceflight exposed shoots (**Supplemental Table S2**), indicating a broad physiological and developmental adaptation to this stressful condition [84].

70% of Cluster- *I^{RW}* core genes, mostly related to high-light late responses, were identified as being differentially expressed in the shoot of spaceflight exposed plants, whereas 67% were identified as root DEGs. Less than 50% of ROS wheel core genes associated with other clusters were identified as spaceflight DEGs in Col seedlings. Similar observations were made in root (Fig. 6C). These results suggest that the ROS response caused by the microgravity environment of ISS in Col shoots and roots is similar to the ROS responses associated with alterations of the *GUN* retrograde signaling pathway and with high-light late responses.

For *cuao3* and *OxPtADC*, we observed a reduction in the numbers of differentially expressed core genes in most clusters of the ROS wheel (Fig. 6C). On the other hand, *OxCuAO3* shoot DEGs were strongly enriched in clusters *V^{RW}*, *VI^{RW}*, *VII^{RW}* and *VIII^{RW}* of the ROS wheel relative to the other genotypes. These clusters are associated with responses to chemical treatments to induce ROS, UV-B early responses, loss-of-function of RBOHF, and ROS acclimation [82]. Importantly, two *OxCuAO3*-specific clusters of DEGs are notable in Fig. 6B, one containing down-regulated DEGs that include genes encoding enzymes involved in auxin and ethylene biosynthesis (*YUCCA6* and *1-AMINOCYCLOPROPANE-1-CARBOXYLIC*

ACID SYNTHASE6 (*ACS6*), respectively), molecular chaperones (*HSP20*), and MAP-type protein kinases (*MPK8*), and another cluster that includes up-regulated DEGs such as the *WRKY53* transcription-factor gene, a key regulator of plant development and senescence that is targeted by multiple abiotic and biotic stress signaling pathways [85] including those mediated by MAP signaling cascades. Interestingly, the *ERK KINASE1* (*MEK1*) and *MAPKKK20* genes are also specifically up-regulated in *OXCUAO3* shoots under spaceflight conditions (Fig. 6; **Supplemental Table S6**). Furthermore, the *WRKY25* gene, which encodes a redox switch that drives up the expression of *WRKY53* during leaf senescence [86], is upregulated under microgravity conditions in all genotypes tested in this work whereas *WRKY18*, another modulator on *WRKY53* expression [86], is specifically upregulated in *OxCUAO3* and *adc1* shoots (**Supplemental Table S2**).

In roots, 4 DEGs are specifically downregulated in *OxCUAO3* under spaceflight conditions while not responding in the other backgrounds, including *HEAT SHOCK TRANSCRIPTION FACTOR A2* (*HSFA2*) [87], and *GRIM REAPER* (*GRI*), which encodes a peptide that contributes to the regulation of ROS-induced cell death in response to both abiotic and biotic stress conditions [88]. It also includes *ACONITASE2* (*ACO2*), which encodes a mitochondrion-associated isoform of an aconitase enzyme that converts citric acid into aconitic acid, then isocitrate in the Krebs cycle and serves as a central regulator of stress responses and signaling in plants, contributes to stress-induced organellar retrograde signaling, provides reducing equivalents and metabolic precursors for nitrogen metabolism and biosynthetic pathways, and maintains cell redox homeostasis under stress conditions [89]. This enzyme is an early target of ROS and Reactive Nitrogen Species (RNS) signals during stress responses due to the presence of an iron-sulfur cluster in the enzyme. These signaling molecules inhibit its activity, thereby resetting the metabolic and redox fluxes to better respond to the stress.

Six other genes are specifically up-regulated in *OxCUAO3* plants under spaceflight conditions, and not in the other backgrounds. One of them, *ATOZF1* (*AT2G19810*), encodes a CCCH-Type zinc-finger family plasma membrane-associated protein that was previously demonstrated to contribute to Arabidopsis plant tolerance to oxidative stress [90]. It also includes several genes that encode enzymes involved in ROS scavenging as well as a *GALACTINOL SYNTHASE2* (*GalS2*) gene, which encodes an enzyme involved in the biosynthesis of galactinol, an osmoprotective molecule.

Taken together, these important results suggest that increased *CuAO3* expression leads to ROS responses to spaceflight conditions that do not normally occur in the Col background. Whether these responses are associated with increased GABA production and/or other alterations in the Put metabolic pathway such as decreased Put accumulation, remains to be determined.

3.7 Down-regulation of Photosynthesis-associated Genes Under Spaceflight Conditions Is Abated in OxPtADC and *cuao3*

As mentioned in section 2.4., genes belonging to the '*Photosynthesis*' and '*Regulation of Photosynthesis*' GO groups (*GO:0015979* and *GO:0010109*, respectively) were significantly over-represented amongst the **shoot** down-regulated DEGs in space-flown wild type Col seedlings (Figs. 4 and 7). To investigate this further, we evaluated the expression under spaceflight and GC conditions of all genes belonging to these two photosynthesis-related GO groups for all the genotypes included in this study. For shoot, among the 196 genes belonging to *GO:0015979* (*Photosynthesis*), 143 were DEGs in at least one of the five genotypes tested. A majority of these genes were downregulated. Col exhibited the most significant enrichment in this GO group, with 120 out of 196 *GO:0015979*-associated genes being differentially expressed (115 down-regulated and 5 up-regulated; $p = 1.38E-277$). Sixteen, eleven and fifteen of these DEGs were found to carry bZIP16-, PIF4- and ABF2-binding motifs, respectively (**Supplemental Table S9**). While *PIF4* was not differentially expressed in any of the tested genotypes in response to spaceflight conditions, *ABF2* expression decreased in *adc1* and *OxCuAO3* backgrounds, whereas expression of its interacting partner, ARMADILLO BTB PROTEIN 1 (ABAP-1), decreased under spaceflight in all genotypes but *OxPtADC* and *cuao3* knockout plants. Similarly, while bZIP16 was not differentially expressed in any of the tested genotypes, its paralog GBF1 increased in expression in response to spaceflight in all genotypes tested whereas GBF2 increased in all genotypes but *OxPtADC* and *cuao3* backgrounds. Taken together, these results suggest a role for GBF1 and GBF2 in the regulation of this subgroup of photosynthesis-related genes in shoots.

The other genotypes included in this study differentially expressed fewer of these genes under spaceflight than wild type Col. Specifically, the *cuao3* and *OxPtADC* lines showed the most dramatic reduction in the number of down-regulated *GO:0015979*-associated genes under spaceflight conditions relative to GC, whereas *adc1* and *OxCuAO3* exhibited a milder reduction (Fig. 7A).

We also examined the expression of the 49 genes associated with *GO:0010109* ('*Regulation of Photosynthesis*'; Fig. 7B). 36 of these genes were found to be significantly differentially expressed between spaceflight and GC in at least one of the genotypes included in this study. Here again, significantly fewer of these genes were found to be differentially expressed in the *OxPtADC* and *cuao3* genotypes (Fig. 7B).

To better understand the effect of spaceflight conditions on photosynthesis-related expression responses, we mapped Col DEGs associated with GO groups *00159179* and *0010109* on three photosynthesis-related pathways: *ath00195* (*Light Reactions*), *ath00196* ('*Antenna Proteins*') and *ath00710* ('*Carbon Fixation*'), using the *Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway* database (Fig. 7C **and D**). A majority of the genes associated with *Light Harvesting* and *Photosynthetic Electron Transport* pathways were found to be significantly down-regulated under spaceflight conditions relative to GC in wild type Col shoots (11 out of 12 and 3 out of 4 genes, respectively; Fig. 7C). Furthermore, a majority of the genes involved in *Photosynthesis* (Fig. 7C) or in the *Dark Reaction* (Fig. 7D) were significantly down-regulated in wild type Col shoots, but not in *OxPtADC* (Fig. 7C **and D**). These findings suggest that the down-regulation of photosynthesis-related genes caused by spaceflight conditions can be rescued by over-expressing *ADC1*, hence increasing Put accumulation in plant tissues.

We conclude engineering the PA metabolic pathway to increase Put accumulation in plant cells can mitigate the negative effect of spaceflight conditions on the expression of photosynthesis-related genes in *Arabidopsis thaliana*. Future experiments will evaluate the potential impact of this transcriptional effect on the phototropic capability of these plants.

Only a limited number of genes associated with the *Photosynthesis* and *Regulation of Photosynthesis* GO groups were found to be up-regulated in the shoots under spaceflight conditions (Fig. 7A-D; **Supplemental Table S9**). The *PIF3* transcription factor and *FERRITIN1* genes were amongst the few generally up-regulated in all genotypes tested (**Supplemental Table S10**). This result is consistent with a previous study that suggested PIF3 may act as a repressor of chloroplast development [91].

We were also very surprised to observe an enrichment of '*Photosynthesis*' (*GO:0015979*)-related DEGs in Col root tissues. A total of 61 spaceflight DEGs associated with *GO:0015979* were identified from roots, including 48 that were shared with shoot tissues and 13 that are root-specific (**Supplemental Table S9**). Among the root-specific DEGs, 11 were down-regulated under spaceflight conditions and 2 were up-regulated. Ten of the thirteen DEGs were encoded from the plastid genome, in sharp contrast with the shoot DEGs (of which only 1 out of 71 was encoded by the plastid genome (**Supplemental Table S9**). The 2 root-specific, down-regulated nuclear DEGs encode Phytochrome B, previously shown to modulate root photomorphogenesis and growth regulation in *Arabidopsis* [92] and One-Helix Protein2, a photosystem-II (PSII) auxiliary protein that contributes to PSII assembly and is required for acclimation to high-light conditions [93]. The root-specific up-regulated nuclear DEG encodes a phosphofructokinase-like enzyme. The significance of these root-specific responses associated with *GO:0015979* remains unknown.

Our findings reveal that spaceflight conditions significantly affect photosynthesis-associated gene expression in *Arabidopsis*, with a pronounced down-regulation observed in the wild-type Col. This down-regulation primarily impacts genes involved in light reactions and carbon fixation, aligning with prior research indicating that microgravity impairs photosynthesis by altering gene expression patterns [7, 10, 94–96]. Notably, the *OxPtADC* and *cuao3* lines showed a significant reduction in the number of down-regulated photosynthesis genes, suggesting that increased Put levels may mitigate the adverse effects of microgravity on photosynthesis. This is consistent with studies highlighting the role of polyamines in stress response and photosynthetic efficiency. KEGG pathway analysis confirmed that spaceflight conditions disrupt genes related to light reactions and carbon fixation in Col, but the partial rescue of these down-regulations in *OxPtADC* and *cuao3* supports the hypothesis that polyamines enhance plant resilience to spaceflight stress by stabilizing photosynthesis-related gene expression. Additionally, up-regulated genes such as *PIF3* (from "regulation of photosynthesis" GO group) and *FERRITIN 1* (from "photosynthesis" GO group), which are involved in stress responses and chloroplast development [91, 97], further underscore the complex regulatory mechanisms at play. The differential responses of photosynthesis-related genes between root and shoot tissues also highlight the intricate nature of plant adaptation to microgravity.

3.8 Genetic Alterations of the Put Metabolic Pathway Leads to Decreased Transcriptional Responses to Spaceflight of Genes associated with Cell Wall Metabolism

Although APEX-08 was not specifically designed to examine the impact of microgravity on cell wall structure and composition, our transcriptomic data revealed a significant enrichment of root and shoot DEGs for genes associated with the *Cell Wall* Ontology Group (*GO:0005618*) in wild type Col seedlings (**Supplemental Table S11**), in agreement with previous studies [98]. To evaluate the effect of genetic alterations of the Put metabolic pathway on these responses, we analyzed the differential expression between spaceflight and GC of genes associated with *Cell Wall Modification* (*GO:0042545*; Fig. 8A), *Plant-Type Cell Wall Loosening* (*GO:0009828*; Fig. 8B), and *Cell Wall Biogenesis* (*GO:0042546*; Fig. 8C), using the five genotypes included in this project.

We observed a significant enrichment of spaceflight **root** DEGs for genes associated with *GO:0042545* (*Cell Wall Modification*) in wild-type Col, *adc1*, and *OxCuAO3* seedlings (Fig. 8A). Notably, most of these DEGs were not differentially expressed in the roots of *cuao3* and *OxPtADC* seedlings, suggesting a correlation between increased Put accumulation in these plants and reduced expression responses to microgravity conditions of *GO:0042545* (*Cell Wall Modification*)-associated genes (Fig. 8A). A similar enrichment for genes associated with *GO:0009828* (*Plant-Type Cell Wall Loosening*) was found in the microgravity-associated DEGs of wild-type Col, mutant *adc1*, and *OxCuAO3* roots, but not in *cuao3* and *OxPtADC* (Fig. 8B). Amongst them were genes that encode Expansin-like proteins as well as Endotransglucosylase/ Hydrolases (XTHs) (Fig. 8B and **Supplemental Table S12**), suggesting an impact on cell wall extensibility.

Genes associated with *Cell Wall Biogenesis* (*GO:0042546*) were not enriched amongst the microgravity-associated DEGs in any of the genotypes subjected to this analysis (Fig. 8C). However, we still found 32 and 57 DEGs associated with *GO:0042546* in roots and shoots of spaceflight seedlings relative to GC, respectively. Here again, many of the genes identified as DEGs in wild-type Col, mutant *adc1* and *OxCuAO3* seedlings were not differentially expressed in the *cuao3* and *OxPtADC* genotypes (Fig. 8C; **Supplemental Table 12**).

Taken together, our results are compatible with previous observations reporting on alterations in the expression of genes associated with cell wall modification, cell wall loosening and cell wall biogenesis under spaceflight conditions [49, 99–103]. They also suggest a role for putrescine accumulation in mitigating such expression changes. Future work should be aimed at directly investigating the effects of spaceflight conditions on cell-wall structure, composition and biophysical properties of wild type and the genetically altered plants.

Conclusion and Future Directions

During spaceflight, microgravity restricts convection, leading to diminished gas exchange at the surface of plant tissues and hypoxia. Additionally, exposure to space radiation and other stressors associated with the confined environment of ISS lead to adverse morphological and molecular responses including drastic changes in gene expression profiles relevant to hypoxia, oxidative stress responses, photosynthesis and cell-wall integrity and metabolism. On Earth, such stresses would lead to PA

accumulation which, in turn, would result in stress mitigation. Yet, previous expression studies of plants growing under microgravity conditions revealed no evidence of increased expression of Put-metabolism genes under microgravity. Therefore, we genetically engineered *Arabidopsis thaliana* to modify its ability to synthesize and/or catabolize Put and tested the ability of wild type and genetically altered seedlings to cope with the stresses associated with spaceflight on ISS. Our study revealed morphological and transcriptomic responses of wild type Col seedlings to spaceflight very similar to those encountered in previous studies, compatible with exposure to hypoxia, oxidative stress, altered photosynthesis and cell wall metabolism.

Our results also showed attenuated morphological and transcriptomic responses to spaceflight of Put-accumulating seedlings relative to wild type, including diminished petiole lengthening and a dramatic simplification of the transcriptomic response associated with hypoxia, photosynthesis, oxidative stress and cell wall integrity. These alterations are compatible with decreased stress response. Additionally, some of the altered transcriptional responses to spaceflight observed in Put-accumulation lines were associated with differential expression between the genetically modified lines and wild type already under ground control conditions, suggesting the alteration in Put metabolism preconditioned the plants for improved response to spaceflight. As discussed in the previous sections, these simplified responses to spaceflight are compatible with the demonstrated role of PAs and some of their catabolic products in radical scavenging, expression regulation, modulation of channel and enzyme activities, wall polymer cross-linking and membrane protection [14–19].

This study suggests an effective approach to mitigate the stress associated with spaceflight conditions, opening the road to the development of plant cultivars that are better adapted to the space environment and can be used in bioregenerative life support systems for long-term space exploration missions. The next step in these investigations will be to evaluate the effects of similar genetic alterations in the Put metabolic pathway on plant growth, development and stress response under microgravity conditions over the entire life cycle of the plant, from seed to seed, with careful quantification of physiological parameters associated with photosynthesis, respiration, gas exchange, nutrients uptake, PA accumulation, metabolic profiling, organs morphology and plant productivity. Furthermore, similar alterations in the PA metabolic pathway may be effective at mitigating some of the stresses associated with plant growth on lunar or Martian regolith, or exposure to cosmic radiations. Future work will address these possibilities.

Methods

5.1 Plant Material

In this study, we characterized *Arabidopsis thaliana* mutants with alterations in putrescine biosynthesis and metabolism, including *adc1* (SALK_085350C)[104], *cuao3-1* (SALK_095214C)[105], and the *cuao3-100^{cdr7}* (17693450C/T)[43] allelic mutants. Additionally, we developed transgenic plants overexpressing the *Poncirus trifoliata* ADC gene (*PtADC*) in *Arabidopsis* (hereafter referred to as *OxPtADC*). Golden gate

assembly strategy was used to generate this construct. Complete CDS of *PtADC* was synthesized (GenBank: HQ008237) and combined with the *CaMV35S*-promoter (pICH51277) and *Nos*-terminator (pICH41421) sequences, forming an intermediate cassette that was moved into the binary vector pAGM4673 to produce the final plasmid. This final construct was introduced into wild-type *Arabidopsis* (Col) using the *Agrobacterium*-mediated floral dip method. [106] For transgenic plants overexpressing the *AtCuAO3*, a similar construct fusing the *AtCuAO3* CDS to the *CaMV35S*-promoter (pICH51277) and *Nos*-terminator (pICH41421) sequences was generated and transformed into a *cuao3-100^{cdr7}* mutant. Transgenic plants were recovered and named *OXCuAO3* [43]. The expression level of all targeted genes in the mutants or overexpressing transgenic lines used in this study was confirmed by qRT-PCR, as described in section 4.3.

5.2 Spaceflight experimental set-up

At the time of the experiment, seeds were surface-sterilized with five successive 1-min washes with 95% (w/v) ethanol and then air-dried in a sterile hood for at least 20 mins. Fifty seeds of each genotype were plated on the surface of agar plates (1/2 MS, pH 5.7, 0.8% agar) and five biological repeats were prepared. Plates were sealed by micropore tape (3M). The seeded plates were immediately treated with far-red light in a cold room for 20 mins ($\sim 80\mu\text{mol}/\text{m}^2\text{S}$) in the dark, then wrapped in aluminum foil and stored at 4°C. The plates were handed over to the MEIT team at Kennedy Space Center for cold storage the following day. Upon transfer into the Dragon CRS-23 capsule, SpaceX-23 launched on August 29, 2021, and docked with the ISS approximately two days later. Once on the ISS, the plates were unwrapped and inserted into the VEGGIE growth unit by astronaut Shane Kimbrough on September 1, 2021. Before insertion, pictures of each plate were taken to ensure that seed germination had not occurred during launch and that the plates were not cracked. Germination was then triggered by a 24-hour red light treatment. After one day, both green and blue lights (on a low setting) were turned on, and the seedlings were allowed to grow for an additional eight days. Temperature, humidity, and CO₂ profiles were recorded using HOBO sensors located near the plates in VEGGIE, and this data was used to run an identical ground control (GC) experiment at KSC with a 48-hour delay. At the end of the growth period, all plates were photographed with a Nikon D5 camera, and the seedlings were harvested into RNeasy (Thermo Fisher Scientific, Waltham, MA, USA; Cat. No. AM7020) and placed in Kennedy Fixation Tubes (KFTs, <https://ntrs.nasa.gov/citations/20160005191>). After one day at room temperature, the KFTs were transferred to a -80°C freezer until returned to Earth on September 30, 2021.

5.3 RNA extraction and qRT-PCR

Frozen, RNeasy-fixed seedlings were thawed, rinsed with distilled water, and then dissected to separate the shoots and roots. Total RNA was extracted from both root and shoot tissues using the Direct-zol RNA Miniprep Plus extraction kit (Zymo Research, Irvine, CA, USA). DNase I treatment was performed on-column using the RNase-Free DNase I Set (Qiagen, Valencia, CA, USA). RNA quality was assessed using the Agilent 2100 Bioanalyzer with the Eukaryotic Total RNA NanoChip (Agilent Technologies, Santa Clara, CA, USA). For qPCR, 1 µg of total RNA per sample was reverse-transcribed into cDNA using the qScript cDNA SuperMix according to the manufacturer's protocol (QuantaBio, Beverly, MA). RT-qPCR was

performed using 250 ng RNA-equivalent cDNA per reaction, with 0.2 μ M primers and 1 $\times$ Bullseye EvaGreen qPCR Mastermix (MidSci, St. Louis, MO). Reactions were run on a LightCycler 480 II Instrument (Roche, Basel, Switzerland). Gene expression levels were calculated using the delta-Ct method, with data normalized to the *PP2A (AT2g13320)* reference gene. Student's t-test with 2-tailed distributions and equal variances was used to determine the statistical significance of observed differences between samples ($p < 0.05$).

5.4 Polyamine quantification

~ 200 Arabidopsis seeds of *Col*, *adc1*, *cuao3-1*, *cuao3-100^{cd7}*, *OXCuAO3*, and *OXPtADC* (described in section 4.1) were surface-sterilized with five successive 1-min washes with 95% (w/v) ethanol, air-dried in a sterile hood, and germinated on the surface of agar plates (1/2 MS, pH 5.7, 0.8% agar) at the density of 50 seeds per plate. All seedlings were grown side by side in the same growth chamber exposed to constant cool-white LED light (120–150 μ E.m⁻².s⁻¹), and 22°C, for nine days. Four plates per genotype (200 seeds) were combined as one bio-replicate, and three replicates were prepared for each genotype. At the end of this growth period, seedlings were harvested and dissected to separate shoot and root tissues. Around 1g of fresh tissue were frozen in liquid nitrogen and sent to *Creative Proteomics* (Creative Proteomics, NY) for polyamine extraction and quantification. This was done by grinding the tissue in a homogenization tube with MM 400 mill mixer. 100 mg of ground tissue was weighed, and 1 mL of 60% acetonitrile was added. The supernatant was collected for analysis. For absolute quantitation of free amines, the supernatant was mixed with isotope-labeled amines, dansyl chloride, and borate buffer, incubated at 40°C for 30 min, and analyzed using LC-MRM/MS on a Waters Acquity UPLC coupled to a Sciex QTRAP 6500 mass spectrometer. The concentrations of the analytes detected in the samples were calculated using internal standard calibration by interpolating their individual linear calibration curves (nmol/g). To avoid bias between different trials, the quantification of each trial was standardized to Col, and the standard deviation was calculated from three biological replicates with the relative fold change from mutants/transgenic lines compared to the wild-type (Col).

5.5 RNAseq analysis, Sequence Mapping and Transcription Profiling

For RNA-seq, approximately 1 μ g of total RNA from each sample was used to generate cDNA libraries with rRNA reduction using the TruSeq Stranded Total RNA Library Prep Plant Kit (Illumina, San Diego, CA). Paired-end sequencing (2 $\times$ 150 bp) was performed at the University of Wisconsin-Madison Biotechnology Center DNA Sequencing Facility on a NovaSeq 6000 (Illumina, USA). Libraries were multiplexed with a target of ~ 60 million reads per sample. The corresponding sequencing fastq files have been uploaded to the NASA's Open Science Data Repository (OSD971; DOI: 10.26030/j57a-7q73).

The Tuxedo pipeline was used to map RNA-seq reads to the *Arabidopsis thaliana* reference genome (Araport11; https://phytozome-next.jgi.doe.gov/info/Athaliana_Araport11). Briefly, paired-end 150-bp sequence reads were processed by filtering out low-quality bases with Phred scores below 30, after which they were joined. TopHat2 was then used to map splice junctions between exons, aligning the

reads to the *Arabidopsis thaliana* reference genome (Araport11/TAIR10) using Bowtie2 as the alignment engine. A maximum of 2-bp mismatches was allowed during this alignment step. HTSeq was used to count the number of reads assigned to each annotated transcript. Differential expression analysis was performed using DESeq2 and EdgeR (<https://www.r-project.org/>) to normalize the data and assess differences between microgravity-exposed and ground control samples. Three biological replicates per genotype were used to identify differentially expressed genes. P-values were adjusted for multiple testing using the Benjamini-Hochberg method (for DESeq2) or by setting the false discovery rate (FDR) at 0.05 (for EdgeR). The threshold for significance was set at $p(q) < 0.05$.

5.6 Bioinformatic Analysis

Differentially expressed genes (DEGs) were annotated using the information available in Phytozome for the *Arabidopsis thaliana* Col reference genome. Gene Ontology (GO) enrichment analysis was performed using the PANTHER platform (<https://geneontology.org/>). Gene identification numbers (At) associated with each annotated gene in Phytozome were directly used as input for all genotypes. Gene expression clustering analysis was performed using **Gene Cluster 3.0** software (<http://bonsai.hgc.jp/~mdehoon/software/cluster/software.htm>). Cluster data were visualized using **JAVA TreeView**. The correlation-uncentered method (Pearson correlation) was used for gene clustering based on expression levels. Multi-enrichment analysis for gene subgroups from clustering results was conducted using the ShinyGO platform (<https://bioinformatics.sdstate.edu/go/>). The databases used for these analyses included GO biological process, GO molecular function, GO cellular component, and transcription factor target data from AGRIS. The threshold for significant enrichment was set at an FDR of 0.05. For KEGG pathway enrichment analysis of photosynthesis-related DEGs, the Pathview platform (<https://pathview.uncc.edu/>) was used to generate plots for corresponding pathways.

5.7 Morphology Measurement

RNAlater-fixed seedlings were thawed, and 4 to 5 complete seedlings per genotype were randomly selected and rinsed with distilled water for morphological measurements. The seedlings were then flattened on microscope slides and scanned using an EPSON Perfection V33 scanner. Total shoot area, including leaves and petioles, was measured using ImageJ (<https://imagej.nih.gov/ij/>). Petiole length was determined by measuring the two longest petioles from each seedling and calculating the average to represent the petiole length for each seedling. Student's T-test with 2-tailed distributions and equal variances was used to determine the statistical significance of observed differences ($p < 0.05$).

Declarations

Author Contribution

Author Contributions: Conceptualization, P.H.M. and S.-H.S.; Methodology, S.-H.S. and P.H.M.; Data Analysis: S.-H.S. and P.H.M.; Investigation: S.-H.S.; Data Curation: S.-H.S.; Writing, review, and editing: S.-H.S. and P.H.M.; Figures: S.-H.S. and P.H.M.; Project administration: P.H.M. and S.-H.S.; Funding

Acquisition: P.H.M. and S.-H.S. All authors have read and agreed to the published version of the manuscript.

Acknowledgement

The authors thank Jeffrey T. Richards, the flight support team and the project managers at Kennedy Space Center, and the astronauts, particularly Shane Kimbrough, for all the help in making this project possible. This work was supported by grants from the National Aeronautics and Space Administration Space Biology Program (grant number 80NSSC19K1483), the National Science Foundation (grant number 1951182-IOS), a UW-Madison CALS Hatch award (grant number MSN233788), a Wisconsin Alumni Research Foundation Fall-Competition Award (grant number MSN282827), and Department of Genetics Bridge support to PHM.

Data Availability

The corresponding RNAsequencing fastq files have been uploaded to the NASA's Open Science Data Repository (OSD971; DOI: 10.26030/j57a-7q73).

References

1. Hoson, T., and Wakabayashi, K. (2015). Role of the plant cell wall in gravity resistance. *Phytochemistry* 112, 84–90.
2. Baba, A.I., Mir, M.Y., Riyazuddin, R., Cséplő, Á., Rigó, G., and Fehér, A. (2022). Plants in Microgravity: Molecular and Technological Perspectives. *Int J Mol Sci* 23.
3. Kiss, J., Millar, K., and Edelman, R. (2012). Phototropism of *Arabidopsis thaliana* in microgravity and fractional gravity on the International Space Station. *Planta* 236, 635–645.
4. Kiss, J.Z., Wolverton, C., Wyatt, S.E., Hasenstein, K.H., and van Loon, J.J.W.A. (2019). Comparison of Microgravity Analogs to Spaceflight in Studies of Plant Growth and Development. *Frontiers in Plant Science* 10, 1577–1577.
5. Porterfield, D. (2002). The biophysical limitations in physiological transport and exchange in plants grown in microgravity. *J Plant Growth Reg* 21, 177–190.
6. Choi, W., Barker, R., Kim, S., Swanson, S., and Gilroy, S. (2019). Variation in the transcriptome of different ecotypes of *Arabidopsis thaliana* reveals signatures of oxidative stress in plant responses to spaceflight. *Am J Bot* 106, 123–136.
7. Kruse, C.P.S., Meyers, A.D., Basu, P., Hutchinson, S., Luesse, D.R., and Wyatt, S.E. (2020). Spaceflight induces novel regulatory responses in *Arabidopsis* seedling as revealed by combined proteomic and transcriptomic analyses. *BMC Plant Biology* 20.

8. Zhou, M., Sng, N., LeFrois, C., Paul, A., and Ferl, R. (2019). Epigenomics in an extraterrestrial environment: organ-specific alteration of DNA methylation and gene expression elicited by spaceflight in *Arabidopsis thaliana*. *BMC Genomics* 20, 205.
9. Hughes, A.M., and Kiss, J.Z. (2022). -Omics studies of plant biology in spaceflight: A critical review of recent experiments. *Frontiers in Astronomy and Space Sciences* 9.
10. Su, S.-H., Levine, H.G., and Masson, P.H. (2023). *Brachypodium distachyon* Seedlings Display Accession-Specific Morphological and Transcriptomic Responses to the Microgravity Environment of the International Space Station. *Life* 13, 626.
11. Kusano, T., Berberich, T., Tateda, C., and Takahashi, Y. (2008). Polyamines: essential factors for growth and survival. *Planta* 228, 367–381.
12. Takahashi, T., and Kakehi, J. (2010). Polyamines: ubiquitous polycations with unique roles in growth and stress responses. *Ann Bot* 105, 1–6.
13. Alcázar, R., Bueno, M., and Tiburcio, A.F. (2020). Polyamines: Small Amines with Large Effects on Plant Abiotic Stress Tolerance. *Cells* 9.
14. Groppa, M., and Benavides, M. (2008). Polyamines and abiotic stress: recent advances. *Amino Acids* 34, 35–45.
15. Liu, K., Fu, H., Bei, Q., and Luan, S. (2000). Inward potassium channel in guard cells as a target for polyamine regulation of stomatal movements. *Plant Physiol* 124, 1315–1326.
16. Takahashi, Y., Berberich, T., Miyazaki, A., Seo, S., Ohashi, Y., and Kusano, T. (2003). Spermine signalling in tobacco: activation of mitogen-activated protein kinases by spermine is mediated through mitochondrial dysfunction. *Plant J* 36, 820–829.
17. Tang, W., and Newton, R. (2005). Polyamines reduce salt-induced oxidative damage by increasing the activities of antioxidant enzymes and decreasing lipid peroxidation in Virginia pine. *Plant Growth Reg* 46, 31–43.
18. Obata, T., and Fernie, A. (2012). The use of metabolomics to dissect plant responses to abiotic stresses. *Cell Mol Life Sci* 69, 3225–3243.
19. Shen, Y., Ruan, Q., Chai, H., Yuan, Y., Yang, W., Chen, J., Xin, Z., and Shi, H. (2016). The *Arabidopsis* polyamine transporter LHR1/PUT3 modulates heat responsive gene expression by enhancing mRNA stability. *Plant J* 88, 1006–1021.
20. Minocha, R., Majumdar, R., and Minocha, S.C. (2014). Polyamines and abiotic stress in plants: a complex relationship¹. *Frontiers in Plant Science* 5, 175.
21. Yamaguchi, K., Takahashi, Y., Berberich, T., Imai, A., Miyazaki, A., Takahashi, T., Michael, A., and Kusano, T. (2006). The polyamine spermine protects against high salt stress in *Arabidopsis thaliana*. *FEBS Letters* 580, 6783–6788.
22. Qiu, C.-W., Ma, Y., Wang, Q.-Q., Fu, M.-M., Li, C., Wang, Y., and Wu, F. (2023). Barley HOMOCYSTEINE METHYLTRANSFERASE 2 confers drought tolerance by improving polyamine metabolism. *Plant Physiology* 193, 389–409.

23. Hummel, I., Bourdais, G., Gouesbet, G., Couee, I., Malmberg, R., and Amrani, A. (2004). Differential gene expression of *ARGININE DECARBOXYLASE ADC1* and *ADC2* in *Arabidopsis thaliana*: characterization of transcriptional regulation during seed germination and seedling development. *New Phytol* 163, 519–531.
24. Alcázar, R., Planas, J., Saxena, T., Zarza, X., Bortolotti, C., Cuevas, J., Bitrián, M., Tiburcio, A., and Altabella, T. (2010). Putrescine accumulation confers drought tolerance in transgenic *Arabidopsis* plants over-expressing the homologous Arginine decarboxylase 2 gene. *Plant Physiol Biochem* 48, 547–552.
25. Jancewicz, A., Gibbs, N., and Masson, P. (2016). Cadaverine's functional role in plant development and environmental response. *Front Plant Sci* 7, 1–8.
26. Tiburcio, A., Altabella, T., Bitrián, M., and Alcázar, R. (2014). The roles of polyamines during the lifespan of plants: from development to stress. *Planta* 240, 1–18.
27. Noble, C.G., Hollinshead, T., Kende, A., Langford, M.P., Lim, P.P., Linney, E., Mattocks, J., Swindale, L.Y., and Green, K. (2025). The plant Diaminopelargonic acid aminotransferase uses spermidine as its amino donor. *The Plant Journal* 121, e70076.
28. Moschou, P., Sanmartin, M., Andriopoulou, A., Rojo, E., Sanchez-Serrano, J., and Roubelakis-Angelakis, K. (2008). Bridging the gap between plant and mammalian polyamine catabolism: a novel peroxisomal polyamine oxidase responsible for a full back-conversion pathway in *Arabidopsis*. *Plant Physiol* 147, 1845–1857.
29. Kamada-Nobusada, T., Hayashi, M., Fukazawa, M., Sakakibara, H., and Nishimura, M. (2008). A putative peroxisomal polyamine oxidase, AtPAO4, is involved in polyamine catabolism in *Arabidopsis thaliana*. *Plant Cell Physiol* 49, 1272–1282.
30. Fincato, P., Moschou, P., Spedaletti, V., Tavazza, R., Angelini, R., Federico, R., Roubelakis-Angelakis, K., and Tavladoraki, P. (2001). Functional diversity inside the *Arabidopsis* polyamine oxidase gene family. *J Exp Bot* 62, 1155–1168.
31. Wimalasekera, R., Tebartz, F., and Scherer, G. (2011). Polyamines, polyamine oxidases and nitric oxide in development, abiotic and biotic stresses. *Plant Sci* 181, 593–603.
32. Xu, M., Yang, Q., Bai, G., Li, P., and Yan, J. (2022). Polyamine pathways interconnect with GABA metabolic processes to mediate the low-temperature response in plants. *Front Plant Sci* 13, 1035414.
33. Planas-Portell, J., Gallart, M., Tiburcio, A.F., and Altabella, T. (2013). Copper-containing amine oxidases contribute to terminal polyamine oxidation in peroxisomes and apoplast of *Arabidopsis thaliana*. *BMC Plant Biology* 13, 109.
34. Gill, S., and Tuteja, N. (2010). Polyamines and abiotic stress tolerance in plants. *Plant Signal Behav* 5, 26–33.
35. Alcázar, R., Altabella, T., Marco, F., Bortolotti, C., Reymond, M., Koncz, C., Carrasco, P., and Tiburcio, A. (2010). Polyamines: molecules with regulatory functions in plant abiotic stress tolerance. *Planta* 231, 1237–1249.

36. Alcázar, R., Marco, F., Cuevas, J.C., Patron, M., Ferrando, A., Carrasco, P., Tiburcio, A.F., and Altabella, T. (2006). Involvement of polyamines in plant response to abiotic stress. *Biotechnology Letters* 28, 1867–1876.
37. Kumria, R., and Rajam, M. (2002). Alteration in polyamine titres during *Agrobacterium* mediated transformation of indica rice with ornithine decarboxylase gene affects plant regeneration potential. *Plant Sci* 162, 769–777.
38. Capell, T., Escobar, H., Liu, H., Burtin, D., Lepri, O., and Christou, P. (1998). Over-expression of the oat arginine decarboxylase cDNA in transgenic rice (*Oryza sativa* L.) affects normal development patterns *in vitro* and results in putrescine accumulation in transgenic plants. *Theor Appl Genet* 97, 246–254.
39. Wang, J., Sun, P., Chen, C., Wang, Y., Fu, X., and Liu, J. (2011). An arginine decarboxylase gene *PtADC* from *Poncirus trifoliata* confers abiotic stress tolerance and promotes primary root growth in *Arabidopsis*. *J Exp Bot* 62, 2899–2914.
40. Waie, B., and Rajam, M. (2003). Effect of increased polyamine biosynthesis on stress responses in transgenic tobacco by introduction of human S-adenosylmethionine decarboxylase gene. *Plant Sci* 164, 727–734.
41. Wi, S., Kim, W., and Park, K. (2006). Overexpression of carnation S-adenosylmethionine decarboxylase gene generates a broad-spectrum tolerance to abiotic stresses in transgenic tobacco plants. *Plant Cell Rep* 25, 1111–1121.
42. Wen, X., Pang, X., Matsuda, N., Kita, M., Inoue, H., Hao, Y., Honda, C., and Moriguchi, T. (2008). Over-expression of the apple spermidine synthase gene in pear confers multiple abiotic stress tolerance by altering polyamine titers. *Transgenic Res* 17, 251–263.
43. Jancewicz, A. (2017). A Genetic Study of Cadaverine Response Reveals Crosstalk Between Cadaverine and Putrescine Pathways in *Arabidopsis thaliana*. In *Cellular and Molecular Biology*, Volume PhD. (University of Wisconsin-Madison), p. 133.
44. Sánchez-Rangel, D., Chávez-Martínez, A.I., Rodríguez-Hernández, A.A., Maruri-López, I., Urano, K., Shinozaki, K., and Jiménez-Bremont, J.F. (2016). Simultaneous Silencing of Two Arginine Decarboxylase Genes Alters Development in *Arabidopsis*. *Frontiers in Plant Science* 7.
45. Bown, A.W., and Shelp, B.J. (2020). Does the GABA Shunt Regulate Cytosolic GABA? *Trends Plant Sci* 25, 422–424.
46. Millenaar, F.F., Cox, M.C., van Berkel, Y.E., Welschen, R.A., Pierik, R., Voosenek, L.A., and Peeters, A.J. (2005). Ethylene-induced differential growth of petioles in *Arabidopsis*. Analyzing natural variation, response kinetics, and regulation. *Plant Physiol* 137, 998–1008.
47. Loreti, E., and Perata, P. (2020). The Many Facets of Hypoxia in Plants. *Plants (Basel)* 9.
48. Ellis, M.H., Dennis, E.S., and Peacock, W.J. (1999). *Arabidopsis* roots and shoots have different mechanisms for hypoxic stress tolerance. *Plant Physiol* 119, 57–64.
49. Paul, A., Zupanska, A., Schultz, E., and Ferl, R. (2013). Organ-specific remodeling of the *Arabidopsis* transcriptome in response to spaceflight. *BMC Plant Biol* 13, 112.

50. Su, S.H., Levine, H.G., and Masson, P.H. (2023). *Brachypodium distachyon* Seedlings Display Accession-Specific Morphological and Transcriptomic Responses to the Microgravity Environment of the International Space Station. *Life (Basel)* **13**.
51. Sheppard, J., Land, E.S., Toennisson, T.A., Doherty, C.J., and Perera, I.Y. (2021). Uncovering Transcriptional Responses to Fractional Gravity in *Arabidopsis* Roots. *Life (Basel)* **11**.
52. Johnson, C., Subramanian, A., Pattathil, S., Correll, M., and Kiss, J. (2017). Comparative transcriptomics indicate changes in cell wall organization and stress response in seedlings during spaceflight. *Am J Bot* *doi: 10.3732/ajb.1700079*.
53. Paul, A., Daugherty, C., Bihn, E., Chapman, D., Norwood, K., and Ferl, R. (2001). Transgene expression patterns indicate that spaceflight affects stress signal perception and transduction in *arabidopsis*. *Plant Physiol* **126**, 613–621.
54. Liao, J., Liu, G., Monje, O., Stutte, G.W., and Porterfield, D.M. (2004). Induction of hypoxic root metabolism results from physical limitations in O₂ bioavailability in microgravity. *Adv Space Res* **34**, 1579–1584.
55. Weits, D.A., Giuntoli, B., Kosmacz, M., Parlanti, S., Hubberten, H.-M., Riegler, H., Hoefgen, R., Perata, P., van Dongen, J.T., and Licausi, F. (2014). Plant cysteine oxidases control the oxygen-dependent branch of the N-end-rule pathway. *Nature Communications* **5**, 3425.
56. Renziehausen, T., Dirr, A., Schmidt-Schippers, R., Flashman, E., and Schippers, J. (2025). Oxygen sensing and plant adaptation to flooding in a changing climate. *Philos Trans R Soc Lond B Biol Sci* **380**, 20240238.
57. Zubrycka, A., Dambire, C., Dalle Carbonare, L., Sharma, G., Boeckx, T., Swarup, K., Sturrock, C.J., Atkinson, B.S., Swarup, R., Corbineau, F., et al. (2023). ERFVII action and modulation through oxygen-sensing in *Arabidopsis thaliana*. *Nature Communications* **14**, 4665.
58. Hsieh, W.-P., Hsieh, H.-L., and Wu, S.-H. (2012). *Arabidopsis* bZIP16 Transcription Factor Integrates Light and Hormone Signaling Pathways to Regulate Early Seedling Development *The Plant Cell* **24**, 3997–4011.
59. Gao, Y., Li, J., Strickland, E., Hua, S., Zhao, H., Chen, Z., Qu, L., and Deng, X.W. (2004). An *Arabidopsis* Promoter Microarray and its Initial Usage in the Identification of HY5 Binding Targets in Vitro. *Plant Molecular Biology* **54**, 683–699.
60. Norén Lindbäck, L., Ji, Y., Cervela-Cardona, L., Jin, X., Pedmale, U.V., and Strand, Å. (2023). An interplay between bZIP16, bZIP68, and GBF1 regulates nuclear photosynthetic genes during photomorphogenesis in *Arabidopsis*. *New Phytologist* **240**, 1082–1096.
61. Gao, F., and Dubos, C. (2024). The *arabidopsis* bHLH transcription factor family. *Trends in Plant Science* **29**, 668–680.
62. Qi, W., Manfield, I.W., Muench, S.P., and Baker, A. (2017). AtSPX1 affects the AtPHR1-DNA-binding equilibrium by binding monomeric AtPHR1 in solution. *Biochem J* **474**, 3675–3687.
63. May, A., Berger, S., Hertel, T., and Köck, M. (2011). The *Arabidopsis thaliana* phosphate starvation responsive gene AtPPsPase1 encodes a novel type of inorganic pyrophosphatase. *Biochim Biophys*

Acta 1810, 178–185.

64. Cheng, Y., Zhou, W., El Sheery, N.I., Peters, C., Li, M., Wang, X., and Huang, J. (2011). Characterization of the Arabidopsis glycerophosphodiester phosphodiesterase (GDPD) family reveals a role of the plastid-localized AtGDPD1 in maintaining cellular phosphate homeostasis under phosphate starvation. *Plant J* 66, 781–795.
65. Liao, C., Zheng, Y., and Guo, Y. (2017). MYB30 transcription factor regulates oxidative and heat stress responses through ANNEXIN-mediated cytosolic calcium signaling in Arabidopsis. *New Phytologist* 216, 163–177.
66. Jeong, G., Jeon, M., Shin, J., and Lee, I. (2022). HEAT SHOCK TRANSCRIPTION FACTOR B2b acts as a transcriptional repressor of VIN3, a gene induced by long-term cold for flowering. *Sci Rep* 12, 10963.
67. Kumar, M., Busch, W., Birke, H., Kemmerling, B., Nürnberger, T., and Schöffl, F. (2009). Heat Shock Factors HsfB1 and HsfB2b Are Involved in the Regulation of Pdf1.2 Expression and Pathogen Resistance in Arabidopsis. *Molecular Plant* 2, 152–165.
68. Fukao, T., Barrera-Figueroa, B.E., Juntawong, P., and Peña-Castro, J.M. (2019). Submergence and Waterlogging Stress in Plants: A Review Highlighting Research Opportunities and Understudied Aspects. *Front Plant Sci* 10, 340.
69. Nagarajan, V.K., Satheesh, V., Poling, M.D., Raghothama, K.G., and Jain, A. (2016). Arabidopsis MYB-Related HHO2 Exerts a Regulatory Influence on a Subset of Root Traits and Genes Governing Phosphate Homeostasis. *Plant Cell Physiol* 57, 1142–1152.
70. Medici, A., Marshall-Colon, A., Ronzier, E., Szponarski, W., Wang, R., Gojon, A., Crawford, N.M., Ruffel, S., Coruzzi, G.M., and Krouk, G. (2015). AtNIGT1/HRS1 integrates nitrate and phosphate signals at the Arabidopsis root tip. *Nature Communications* 6, 6274.
71. Papdi, C., Pérez-Salamó, I., Prathiba Joseph, M., Giuntoli, B., Bögre, L., Koncz, C., and Szabados, L. (2015). The low oxygen, oxidative and osmotic stress responses synergistically act through the Ethylene Response Factor-VII genes RAP2.12, RAP2.2 and RAP2.3. *Plant J DOI: 10.1111/tpj.12848*.
72. Wang, Z., Wei, X., Cui, X., Wang, J., Wang, Y., Sun, M., Zhao, P., Yang, B., Wang, Q., and Jiang, Y.-Q. (2024). The transcription factor WRKY22 modulates ethylene biosynthesis and root development through transactivating the transcription of ACS5 and ACO5 in Arabidopsis. *Physiologia Plantarum* 176, e14371.
73. Dong, F., Yang, F., Liu, Y., Jia, W., He, X., Chai, J., Zhao, H., Lv, M., Zhao, L., and Zhou, S. (2021). Chapter 15 - Calmodulin-binding transcription activator (CAMTA)/ factors in plants. In *Calcium Transport Elements in Plants*, S.K. Upadhyay, ed. (Academic Press), pp. 249–266.
74. Zhou, Y., Zhou, D.M., Yu, W.W., Shi, L.L., Zhang, Y., Lai, Y.X., Huang, L.P., Qi, H., Chen, Q.F., Yao, N., et al. (2022). Phosphatidic acid modulates MPK3- and MPK6-mediated hypoxia signaling in Arabidopsis. *Plant Cell* 34, 889–909.
75. Lee, C.P., Elsässer, M., Fuchs, P., Fenske, R., Schwarzländer, M., and Millar, A.H. (2021). The versatility of plant organic acid metabolism in leaves is underpinned by mitochondrial malate-citrate

- exchange. *Plant Cell* **33**, 3700–3720.
76. Hu, Y., Dong, Q., and Yu, D. (2012). Arabidopsis WRKY46 coordinates with WRKY70 and WRKY53 in basal resistance against pathogen *Pseudomonas syringae*. *Plant Science* **185–186**, 288–297.
 77. Ansari, M.I., Jalil, S., Ansari, S., and Hasanuzzaman, M. (2021). GABA shunt: a key-player in mitigation of ROS during stress. *Plant Growth Regulation*.
 78. Wang, F., Chen, Z.H., Liu, X., Colmer, T.D., Shabala, L., Salih, A., Zhou, M., and Shabala, S. (2017). Revealing the roles of GORK channels and NADPH oxidase in acclimation to hypoxia in Arabidopsis. *J Exp Bot* **68**, 3191–3204.
 79. De Micco, V., Aronne, G., Caplin, N., Carnero-Diaz, E., Herranz, R., Horemans, N., Legué, V., Medina, F.J., Pereda-Loth, V., Schiefloe, M., et al. (2023). Perspectives for plant biology in space and analogue environments. *npj Microgravity* **9**, 67.
 80. Sakuma, Y., Maruyama, K., Osakabe, Y., Qin, F., Seki, M., Shinozaki, K., and Yamaguchi-Shinozaki, K. (2006). Functional Analysis of an Arabidopsis Transcription Factor, DREB2A, Involved in Drought-Responsive Gene Expression. *The Plant Cell* **18**, 1292–1309.
 81. Frerigmann, H., and Gigolashvili, T. (2014). MYB34, MYB51, and MYB122 distinctly regulate indolic glucosinolate biosynthesis in Arabidopsis thaliana. *Mol Plant* **7**, 814–828.
 82. Willems, P., Mhamdi, A., Stael, S., Storme, V., Kerchev, P., Noctor, G., Gevaert, K., and Van Breusegem, F. (2016). The ROS Wheel: Refining ROS transcriptional footprints. *Plant Physiol* **171**, 1720–1733.
 83. Waters, M.T., Wang, P., Korkaric, M., Capper, R.G., Saunders, N.J., and Langdale, J.A. (2009). GLK Transcription Factors Coordinate Expression of the Photosynthetic Apparatus in Arabidopsis The *Plant Cell* **21**, 1109–1128.
 84. Xiao, Y., Chu, L., Zhang, Y., Bian, Y., Xiao, J., and Xu, D. (2022). HY5: A Pivotal Regulator of Light-Dependent Development in Higher Plants. *Frontiers in Plant Science Volume 12–2021*.
 85. Zentgraf, U., and Doll, J. (2019). Arabidopsis WRKY53, a Node of Multi-Layer Regulation in the Network of Senescence. *Plants* **8**, 578.
 86. Andrade Galan, A.G., Doll, J., von Roepenack-Lahaye, E., Faiss, N., and Zentgraf, U. (2025). The transcription factor WRKY25 can act as redox switch to drive the expression of WRKY53 during leaf senescence in arabidopsis. *Scientific Reports* **15**, 27623.
 87. Schramm, F., Ganguli, A., Kiehlmann, E., Englich, G., Walch, D., and von Koskull-Döring, P. (2006). The Heat Stress Transcription Factor HsfA2 Serves as a Regulatory Amplifier of a Subset of Genes in the Heat Stress Response in Arabidopsis. *Plant Molecular Biology* **60**, 759–772.
 88. Wrzaczek, M., Brosché, M., and Kangasjärvi, J. (2009). Scorched earth strategy: Grim Reaper saves the plant. *Plant Signal Behav* **4**, 631–633.
 89. Rahikainen, M., Berkowitz, O., Whelan, J., Kangasjärvi, S., and Pascual, J. (2025). Role of aconitase in plant stress response and signaling. *Physiologia Plantarum* **177**, e70128.
 90. Huang, P., Chung, M.-S., Ju, H.-W., Na, H.-S., Lee, D.J., Cheong, H.-S., and Kim, C.S. (2011). Physiological characterization of the Arabidopsis thaliana Oxidation-related Zinc Finger 1, a plasma

- membrane protein involved in oxidative stress. *Journal of Plant Research* *124*, 699–705.
91. Stephenson, P.G., Fankhauser, C., and Terry, M.J. (2009). PIF3 is a repressor of chloroplast development. *Proceedings of the National Academy of Sciences* *106*, 7654–7659.
 92. Park, E., Kim, Y., and Choi, G. (2018). Phytochrome B Requires PIF Degradation and Sequestration to Induce Light Responses across a Wide Range of Light Conditions. *Plant Cell* *30*, 1277–1292.
 93. Maeda, H., Takahashi, K., Ueno, Y., Sakata, K., Yokoyama, A., Yarimizu, K., Myouga, F., Shinozaki, K., Ozawa, S.-I., Takahashi, Y., et al. (2022). Characterization of photosystem II assembly complexes containing ONE-HELIX PROTEIN1 in *Arabidopsis thaliana*. *Journal of plant research* *135*, 361–376.
 94. Chen, B., Zhang, A., Lu, Q., Kuang, T., Lu, C., and Wen, X. (2013). Characterization of photosystem I in rice (*Oryza sativa* L.) seedlings upon exposure to random positioning machine. *Photosynth Res* *116*, 93–105.
 95. Kitaya, Y., Kawai, M., Tsuruyama, J., Takahashi, H., Tani, A., Goto, E., Saito, T., and Kiyota, M. (2001). The effect of gravity on surface temperature and net photosynthetic rate of plant leaves. *Adv Space Res* *28*, 659–664.
 96. Vandenbrink, J.P., Herranz, R., Poehlman, W.L., Alex Feltus, F., Villacampa, A., Ciska, M., Javier Medina, F., and Kiss, J.Z. (2019). RNA-seq analyses of *Arabidopsis thaliana* seedlings after exposure to blue-light phototropic stimuli in microgravity. *American Journal of Botany* *106*, 1466–1476.
 97. Yang, Y., Zhang, J., Li, M., Ning, Y., Tao, Y., Shi, S., Dark, A., and Song, Z. (2023). Heterologous Expression of a Ferritin Homologue Gene PpFer1 from *Prunus persica* Enhances Plant Tolerance to Iron Toxicity and H₂O₂ Stress in *Arabidopsis thaliana*. *Plants* *12*, 4093.
 98. Jost, A.-I.K., Hoson, T., and Iversen, T.-H. (2015). The Utilization of Plant Facilities on the International Space Station—The Composition, Growth, and Development of Plant Cell Walls under Microgravity Conditions. *Plants* *4*, 44–62.
 99. Hoson, T., Soga, K., Mori, R., Asiki, M., Nakamura, Y., Wakabayashi, K., and Kamisaka, S. (2002). Stimulation of elongation growth and cell wall loosening in rice coleoptiles under microgravity conditions in space. *Plant Cell Physiol* *43*, 1067–1071.
 100. Hoson, T., Soga, K., Wakabayashi, K., Kamisaka, S., and Tanimoto, E. (2003). Growth and cell wall changes in rice roots during spaceflight. *Plant Soil* *255*, 19–26.
 101. Soga, K., Yano, S., Kamada, M., Matsumoto, S., and Hoson, T. (2022). Understanding the Mechanisms of Gravity Resistance in Plants. *Methods Mol Biol* *2368*, 267–279.
 102. Herranz, R., Vandenbrink, J., Villacampa, A., Manzano, A., Poehlman, W., Feltus, F., Kiss, J., and Medina, F. (2019). RNAseq analysis of the response of *Arabidopsis thaliana* to fractional gravity under blue-light stimulation during spaceflight. *Front Plant Sci* *10*, 1529.
 103. Barker, R., Kruse, C.P.S., Johnson, C., Saravia-Butler, A., Fogle, H., Chang, H.S., Trane, R.M., Kinscherf, N., Villacampa, A., Manzano, A., et al. (2023). Meta-analysis of the space flight and microgravity response of the *Arabidopsis* plant transcriptome. *NPJ Microgravity* *9*, 21.
 104. Cuevas, J.C., López-Cobollo, R., Alcázar, R.n., Zarza, X., Koncz, C., Altabella, T., Salinas, J., Tiburcio, A.F., and Ferrando, A. (2008). Putrescine Is Involved in *Arabidopsis* Freezing Tolerance and Cold

- Acclimation by Regulating Abscissic Acid Levels in Response to Low Temperature. *Plant Physiology* *148*, 1094–1105.
105. Liu, C., Atanasov, K.E., Arafaty, N., Murillo, E., Tiburcio, A.F., Zeier, J., and Alcázar, R. (2020). Putrescine elicits ROS-dependent activation of the salicylic acid pathway in. *Plant, Cell & Environment* *43*, 2755–2768.
106. Clough, S.J., and Bent, A.F. (1998). Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant J* *16*, 735–743.

Figures

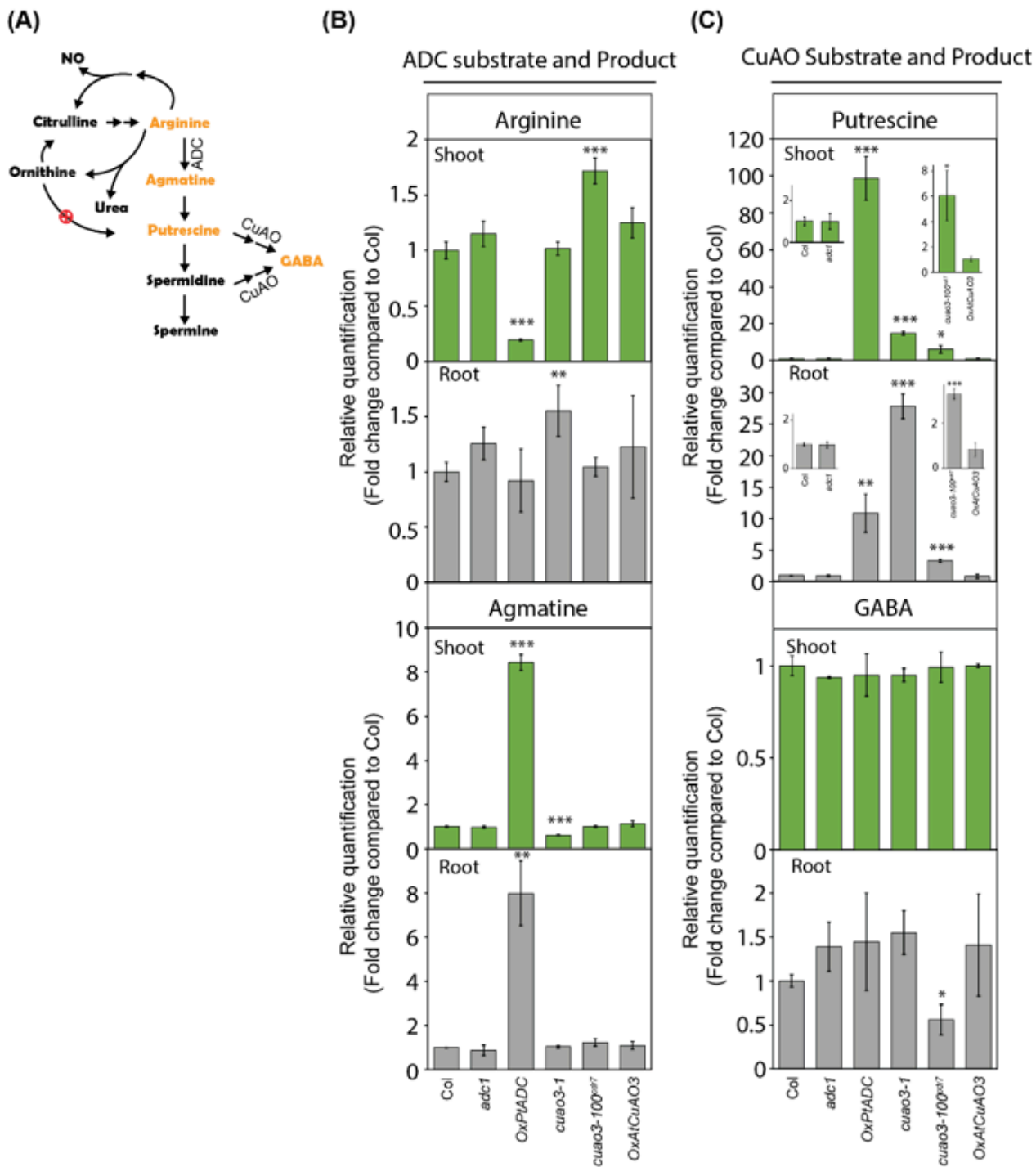


Figure 1

Quantification of putrescine precursors, putrescine, and its derivatives in wild-type Col and genetically modified lines with alterations in the putrescine metabolic pathway. (A) Diagram of the core putrescine pathway that we focus on in this study. (B) Relative quantification of ADC substrate and product. (C) Relative quantification of CuAO substrate and product. The quantified compounds are named at the top of each graph, with genotypes along the X-axis. Relative compound levels are represented by green bars for shoots (top) and grey bars for roots (bottom). Relative compound levels were standardized to wild-type Col levels (assigned an average value of 1). Error bars represent standard deviations. N=3.

Statistically significant differences from wild type are indicated by asterisks (t-tests; *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.005$).

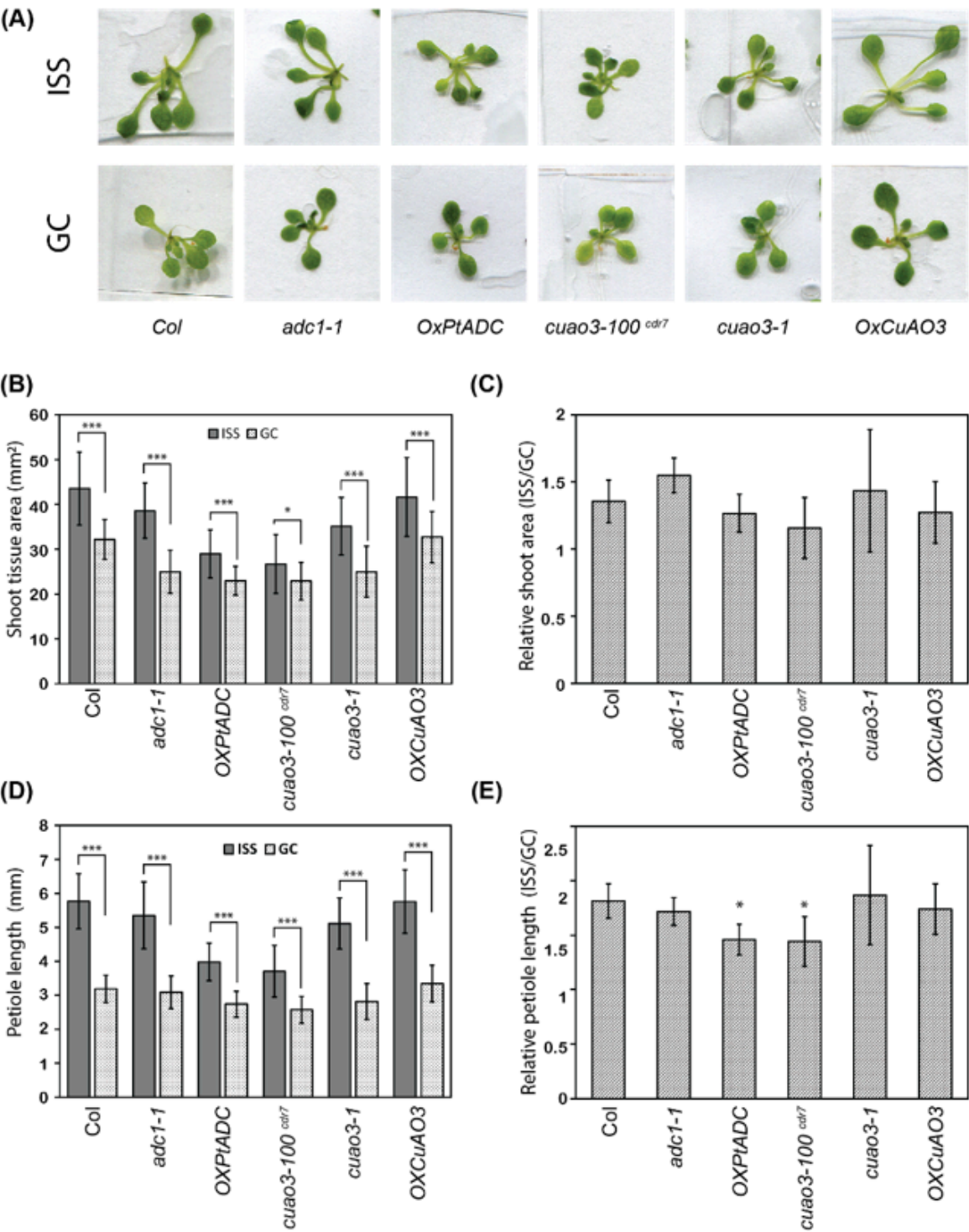


Figure 2

Arabidopsis seedlings display morphological responses to spaceflight conditions. (A) Representative shoot images from ISS-grown seedlings (upper panel) and ground control seedlings (lower panel) of each genotype. (B) Measurement of total shoot area and (C) relative shoot area (ISS/GC) for each

genotype compared to Col. (D) Petiole length for the first two true leaves and (E) relative petiole length (ISS/GC) for each genotype compared to Col. Quantification was performed on dissected fixed seedlings after careful flattening on a glass surface. Significant t-test p-values are indicated by asterisks (*: $p < 0.05$; ***: $p < 0.001$). $N \geq 4$ per genotype.

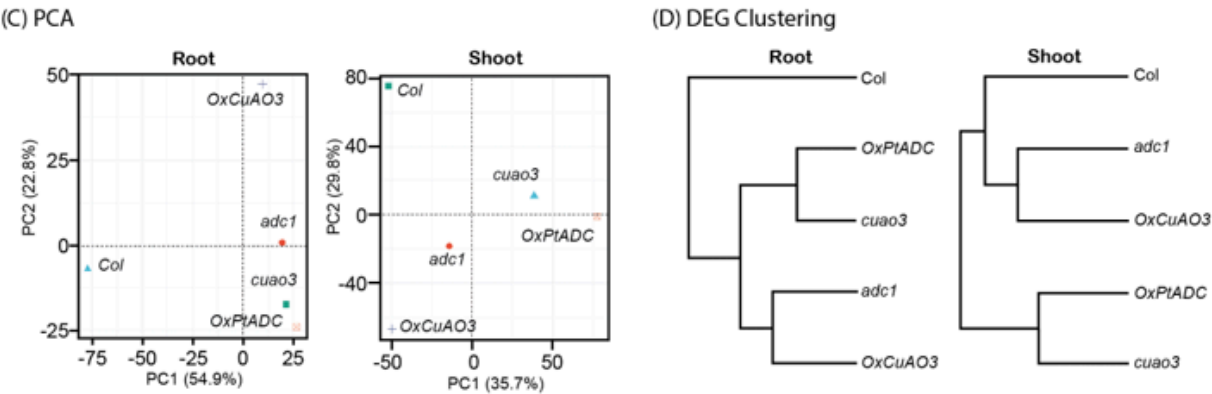
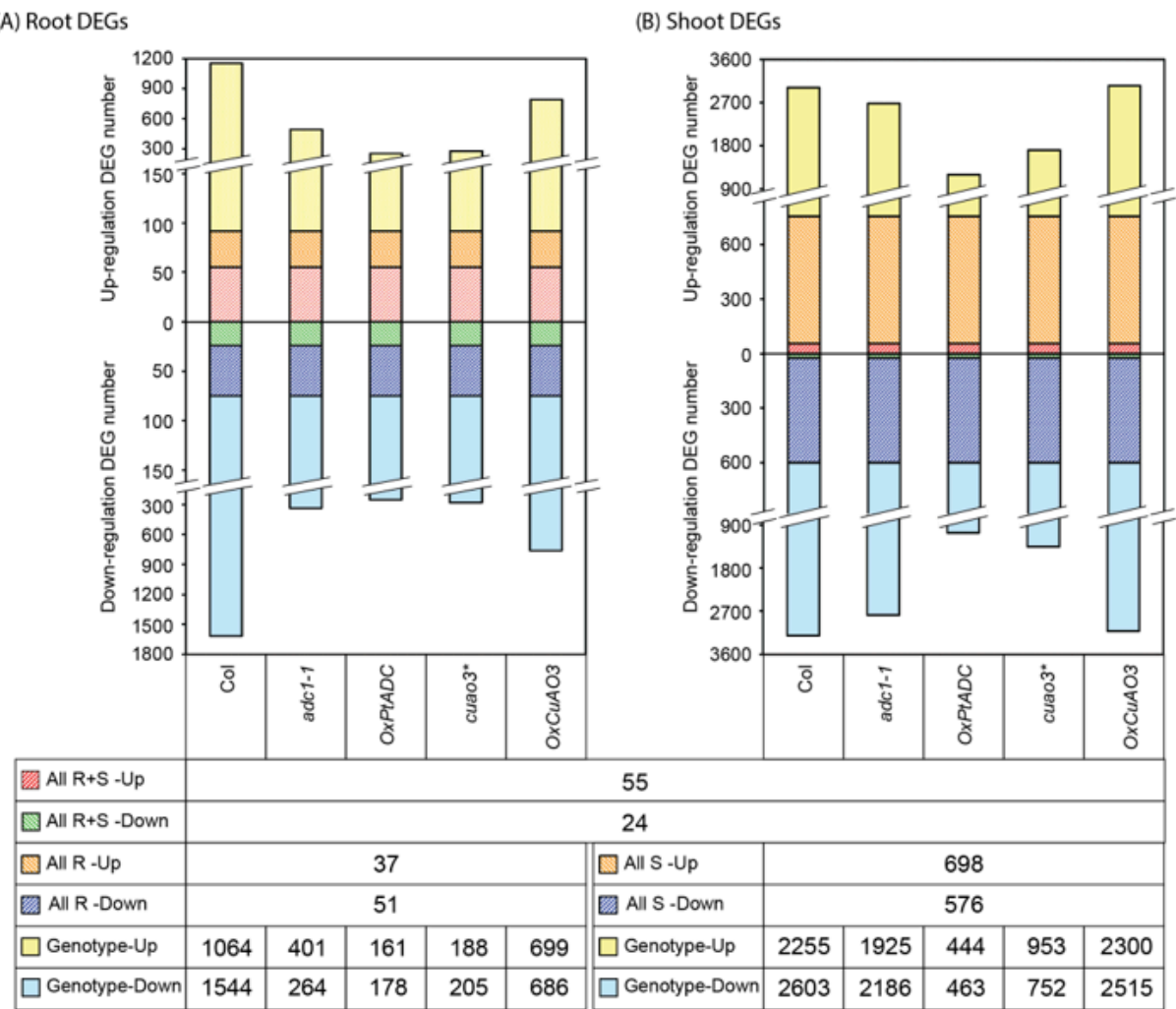


Figure 3

Genetic alterations in the putrescine metabolic pathway affect the number of differentially expressed genes (DEGs) under spaceflight conditions.Total number of DEGs in (A) root tissue and (B) shoot tissue for each genotype. The upper panel shows up-regulated DEGs, while the lower panel shows down-regulated DEGs. Based on DEG appearance and expression trends for each genotype, six categories are represented in an accumulated bar graph. 'R' represents root tissue, 'S' represents shoot tissue, and 'Genotype' refers to each corresponding genotype. The detailed numbers for each category are listed in the associated table. (C) Principal component analysis (two components) and (D) clustering analysis of DEGs expression level for five genotypes are shown for root and shoot, respectively.

(A) Up-regulated DEGs in root

Biological GO group	Col	adc1	OxCuAO3	cuao3	OxPtADC
response to hypoxia (GO:0001666)	7.10E-15	1.82E-25	2.50E-17	1.53E-07	1.21E-06
cellular response to chemical stimulus (GO:0070887)	5.62E-12	2.49E-18	4.06E-13	4.21E-05	1.71E-04
response to nutrient levels (GO:0031667)	2.62E-09	2.39E-09	4.63E-10	2.88E-02	1.21E-09
response to abiotic stimulus (GO:0009628)	4.46E-05	9.37E-09	1.86E-06	8.28E-03	6.08E-03
response to oxygen-containing compound (GO:1901700)	5.52E-06	9.21E-09	1.15E-06	5.80E-03	3.56E-04
response to ethylene (GO:0009723)	6.85E-05	1.91E-02	5.91E-03	N.S.	N.S.
cell communication (GO:0007154)	4.78E-03	1.92E-06	1.16E-02	N.S.	N.S.
indole glucosinolate metabolic process (GO:0042343)	1.09E-04	4.71E-06	1.76E-03	N.S.	N.S.
response to lipid (GO:0033993)	3.27E-02	2.10E-03	1.64E-03	N.S.	N.S.
response to hormone (GO:0009725)	3.31E-03	1.64E-03	5.94E-03	N.S.	N.S.
response to hydrogen peroxide (GO:0042542)	6.99E-03	N.S.	N.S.	N.S.	N.S.
tricyclic triterpenoid metabolic process (GO:0010683)	2.24E-02	N.S.	N.S.	N.S.	N.S.
metal ion transport (GO:0030001)	2.10E-02	N.S.	N.S.	N.S.	N.S.
hydrocarbon biosynthetic process (GO:0120251)	2.27E-02	N.S.	N.S.	N.S.	N.S.
response to reactive oxygen species (GO:0000302)	6.19E-03	N.S.	N.S.	N.S.	N.S.

(B) Down-regulated DEGs in root

Biological GO group	Col	adc1	OxCuAO3	cuao3	OxPtADC
response to radiation (GO:0009314)	8.36E-34	6.09E-11	3.36E-25	2.46E-13	1.28E-04
response to temperature stimulus (GO:0009266)	3.38E-33	3.83E-07	4.50E-16	6.98E-16	4.10E-03
response to cold (GO:0009409)	4.69E-21	2.35E-03	1.62E-07	6.26E-09	2.40E-02
response to oxygen-containing compound (GO:1901700)	7.91E-19	6.82E-04	1.80E-07	1.71E-05	8.70E-07
response to water deprivation (GO:0009414)	3.25E-15	6.21E-05	3.68E-08	6.86E-07	4.53E-05
circadian rhythm (GO:0007623)	3.51E-10	4.85E-02	1.98E-05	N.S.	N.S.
lipid biosynthetic process (GO:0008610)	3.13E-05	4.84E-02	3.32E-03	N.S.	N.S.
cell wall modification (GO:0042545)	3.39E-03	2.69E-04	2.82E-05	N.S.	N.S.
cell wall organization or biogenesis (GO:0071554)	1.63E-02	4.39E-04	1.09E-03	N.S.	N.S.
plant-type cell wall loosening (GO:0009828)	2.23E-02	5.93E-05	3.17E-04	N.S.	N.S.
RNA modification (GO:0009451)	6.89E-05	N.S.	N.S.	N.S.	N.S.
response to fatty acid (GO:0070542)	3.15E-02	N.S.	N.S.	N.S.	N.S.
hydrogen peroxide catabolic process (GO:0042744)	3.15E-02	N.S.	N.S.	N.S.	N.S.
regulation of photosynthesis, light reaction (GO:0042548)	1.20E-03	N.S.	N.S.	N.S.	N.S.
cell redox homeostasis (GO:0045454)	1.13E-02	N.S.	N.S.	N.S.	N.S.

(C) Up-regulated DEGs in shoot

Biological GO group	Col	adc1	OxCuAO3	cuao3	OxPtADC
protein modification process (GO:0036211)	3.49E-16	2.85E-15	4.35E-22	1.02E-12	8.33E-03
response to lipid (GO:0033993)	2.10E-14	3.46E-22	7.21E-22	9.65E-11	1.47E-11
post-translational protein modification (GO:0043687)	9.31E-13	6.56E-13	3.08E-12	2.52E-09	5.25E-04
response to hypoxia (GO:0001666)	5.13E-11	5.83E-28	1.71E-34	5.53E-15	2.66E-15
hormone-mediated signaling pathway (GO:0009755)	4.34E-08	1.24E-11	2.55E-10	3.86E-05	3.35E-04
cellular response to auxin stimulus (GO:0071365)	4.19E-06	7.54E-03	8.31E-03	N.S.	N.S.
mRNA splicing, via spliceosome (GO:0000398)	6.84E-06	4.06E-02	2.17E-02	N.S.	N.S.
regulation of hormone levels (GO:0010817)	1.97E-04	1.34E-02	1.52E-02	N.S.	N.S.
nitrogen compound transport (GO:0071705)	2.76E-04	3.61E-03	1.49E-03	N.S.	N.S.
calcium-mediated signaling (GO:0019722)	2.78E-02	9.65E-03	2.70E-02	N.S.	N.S.
mRNA processing (GO:0006397)	2.02E-06	N.S.	N.S.	N.S.	N.S.
protein transport (GO:0015031)	3.96E-04	N.S.	N.S.	N.S.	N.S.
epigenetic regulation of gene expression (GO:0040029)	7.90E-03	N.S.	N.S.	N.S.	N.S.
auxin biosynthetic process (GO:0009851)	2.94E-02	N.S.	N.S.	N.S.	N.S.
sugar mediated signaling pathway (GO:0010182)	3.83E-02	N.S.	N.S.	N.S.	N.S.

(D) Down-regulated DEGs in shoot

Biological GO group	Col	adc1	OxCuAO3	cuao3	OxPtADC
photosynthesis (GO:0015979)	3.33E-55	2.47E-49	3.89E-32	9.37E-17	4.29E-04
plastid organization (GO:0009657)	6.68E-33	4.03E-31	5.74E-34	1.02E-08	3.13E-07
response to temperature stimulus (GO:0009266)	1.98E-24	2.40E-15	2.77E-15	6.78E-10	1.20E-05
microtubule-based movement (GO:0007018)	5.00E-11	1.07E-10	3.05E-18	3.08E-05	7.28E-12
response to oxidative stress (GO:0006979)	4.91E-08	1.78E-02	3.32E-03	2.80E-05	7.60E-03
gene expression (GO:0010467)	1.06E-16	6.50E-13	2.99E-27	N.S.	N.S.
photosynthesis, light harvesting in photosystem I (GO:0009768)	4.88E-16	5.83E-11	7.07E-03	N.S.	N.S.
protein modification process (GO:0036211)	4.50E-07	1.07E-07	4.42E-05	N.S.	N.S.
plastid translation (GO:0032544)	5.03E-06	1.19E-05	2.83E-05	N.S.	N.S.
ribosome biogenesis (GO:0042254)	1.51E-03	3.84E-02	1.96E-04	N.S.	N.S.
starch catabolic process (GO:0005983)	1.11E-04	N.S.	N.S.	N.S.	N.S.
response to reactive oxygen species (GO:0000302)	1.27E-04	N.S.	N.S.	N.S.	N.S.
response to biotic stimulus (GO:0009607)	3.02E-03	N.S.	N.S.	N.S.	N.S.
spermine metabolic process (GO:0008215)	5.59E-03	N.S.	N.S.	N.S.	N.S.
reactive oxygen species metabolic process (GO:0072593)	1.10E-02	N.S.	N.S.	N.S.	N.S.

Figure 4

Representative lists of enriched GO groups within root and shoot DEGs under APEX-08. All DEGs are grouped based on the mode of expression change (up or down under spaceflight relative to ground-control conditions) and tissue type (root or shoot). Enrichment analysis was performed separately for each group. The number represents the p-value for the GO group in the enrichment analysis, and N.S. indicates non-significant results. FDR is set to 0.05.

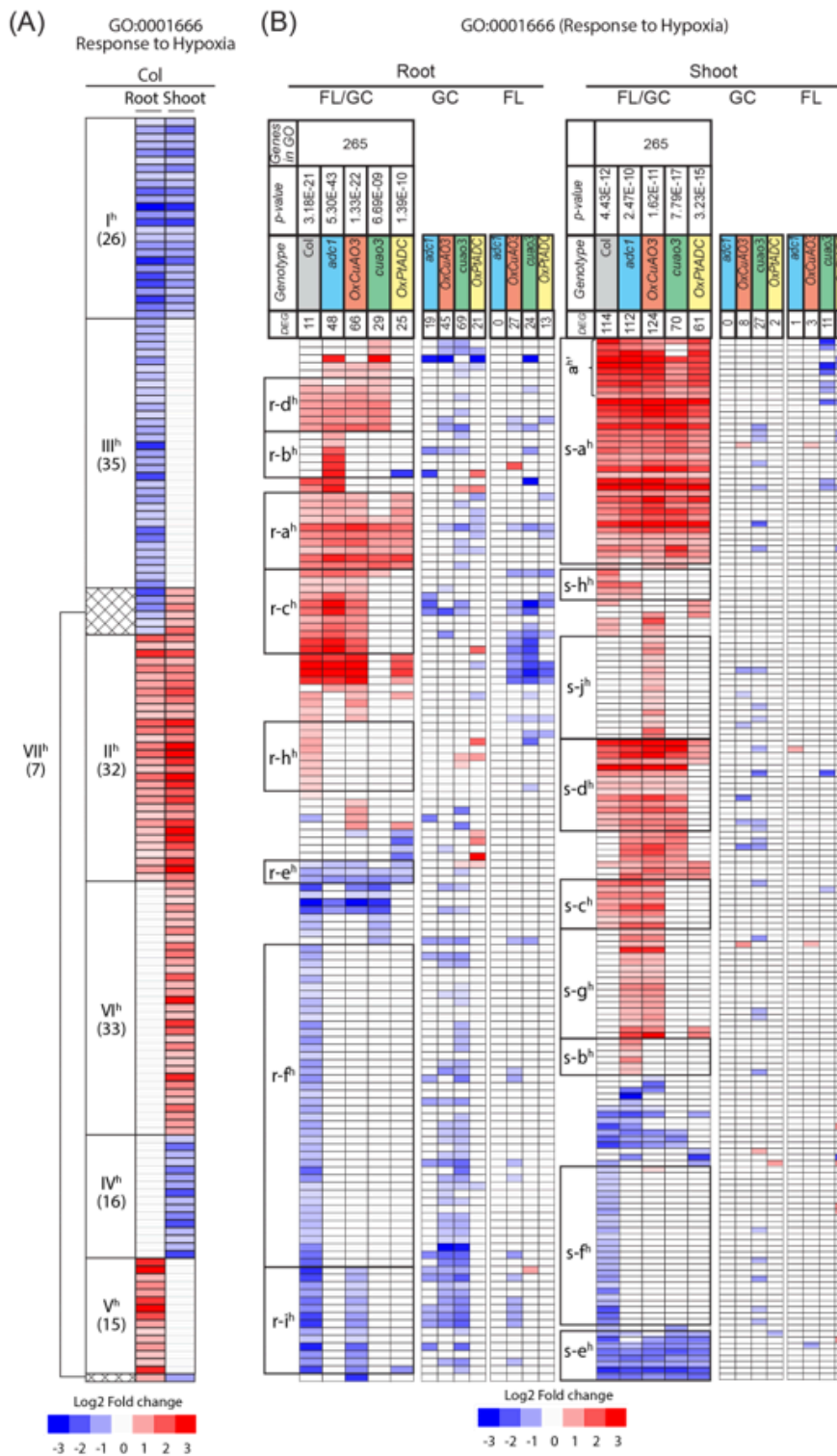


Figure 5

Hierarchical clustering of GO:000166-associated gene expression responses to spaceflight conditions in wild-type Col and other genotypes with alterations in the putrescine metabolic pathway.(A) Hierarchical clustering of expression responses to spaceflight conditions of GO:000166-associated DEGs in Col root and shoot tissues. Expression differences between ISS and GC conditions are shown in a heatmap, with red indicating up-regulation and blue indicating down-regulation, as indicated by the scale bar under the

graph. Each row represents a different gene. The full list of these DEGs, along with group information, is provided in supplemental table S5. (B) Hierarchical clustering of expression responses to spaceflight conditions (FL/GC) for the various accessions included in this study, based on similarities between expression profiles in root (left) and shoot (right). For each organ, expression responses to spaceflight conditions are shown on the left (FL/GC), whereas expression differences between each modified line and the wild type Col under ground-control (GC) and spaceflight conditions (FL) are displayed in the middle and right of the panel. The full list of these DEGs, along with group information, is provided in supplemental table S6.

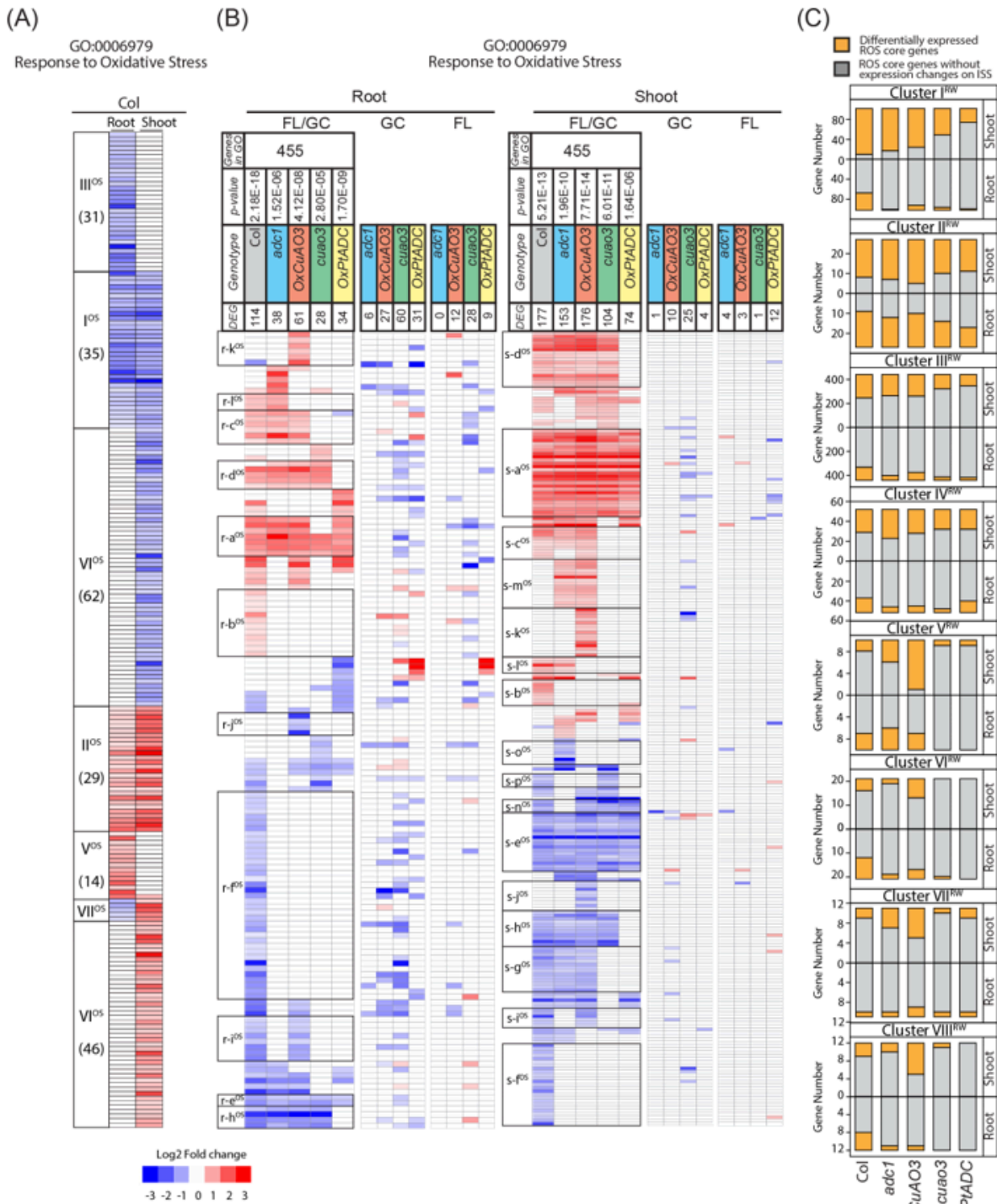


Figure 6

Hierarchical clustering of GO:0006979 (response to oxidative stress) associated gene expression responses to spaceflight conditions in wild-type Col root and shoot tissues (A) and other genotypes with alterations in the putrescine metabolic pathway (B). In A, expression differences between ISS and GC conditions in Col root and shoot tissues are shown in a heatmap, with red indicating up-regulation and blue indicating down-regulation, as indicated by the scale bar under the graph. Each row represents a

different gene. The full list of these DEGs, along with group information, is provided in supplemental table S7. (B) Hierarchical clustering of expression responses to spaceflight conditions (FL/GC) for the various accessions included in this study, based on similarities between expression profiles in root (left) and shoot (right). For each organ, expression responses to spaceflight conditions are shown on the left (FL/GC), whereas expression differences between each modified line and the wild type Col under ground-control (GC) and spaceflight conditions (FL) are displayed at the middle and right of the panel. The total number of genes belonging to this GO group and the probability of over-representation of this GO group for each genotype are shown in the table on the top of the chart. The full list of these DEGs, along with group information, is provided in supplemental table S8. (C) GO:0006979-group DEGs preferentially associate with specific transcriptional-response clusters from the Arabidopsis ROS wheel (Willems et al., 2016). Clusters I-VIII correspond to categories of ROS-related experiments used to establish the Arabidopsis ROS wheel. Each graph represents a different cluster from the ROS wheel. Genotypes are shown along the X-axis, while the Y-axis represents the number of genes in each genotype belonging to the cluster (core genes, in grey) and the number of those genes identified as DEGs in APEX-08 (yellow bars).

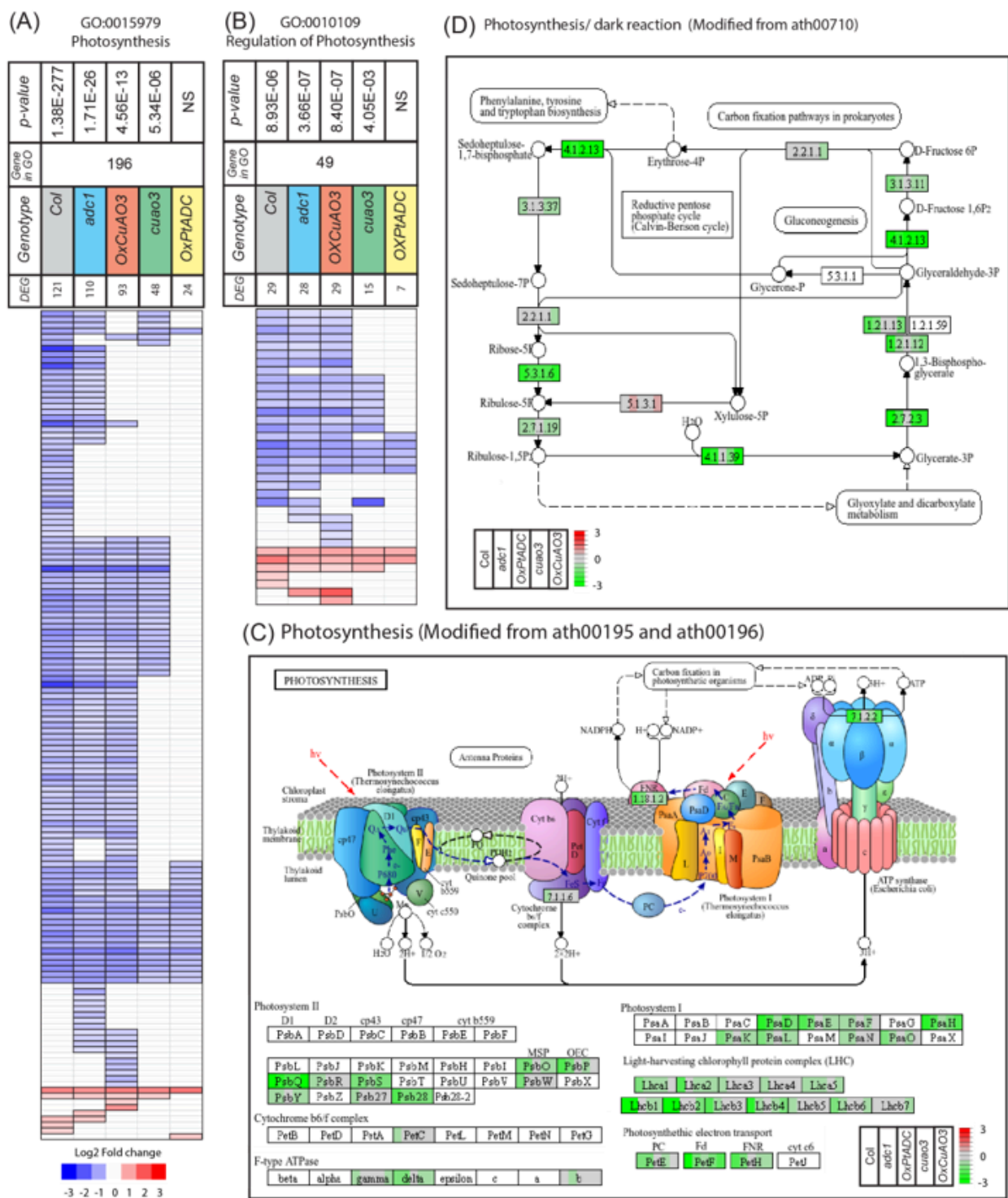


Figure 7

Decreased expression of photosynthesis-related genes under spaceflight conditions is attenuated in Put-accumulation lines. DEGs associated with (A)GO:0015979 and (B)GO:0010109 are shown here.

Hierarchical clustering methods were used to generate these data. The total number of genes belonging to this GO group and the probability of over-representation of this GO group for each genotype are shown in the table on the bottom of the chart. The color scale bar at the bottom of the graph indicates the Log2

of expression fold changes (C,D). Representation of expression fold changes between spaceflight conditions and ground control for genes involved in (C) photosynthesis and (D) the dark reaction. The KEGG pathways are illustrated in these panels, along with individual genes involved in multiple steps of the reactions. Differential expression of these genes between spaceflight and GC is illustrated by a color heat chart according to the scale bar shown at the bottom. The genotypes are represented by rows of colored boxes in the order provided at the bottom of each chart.

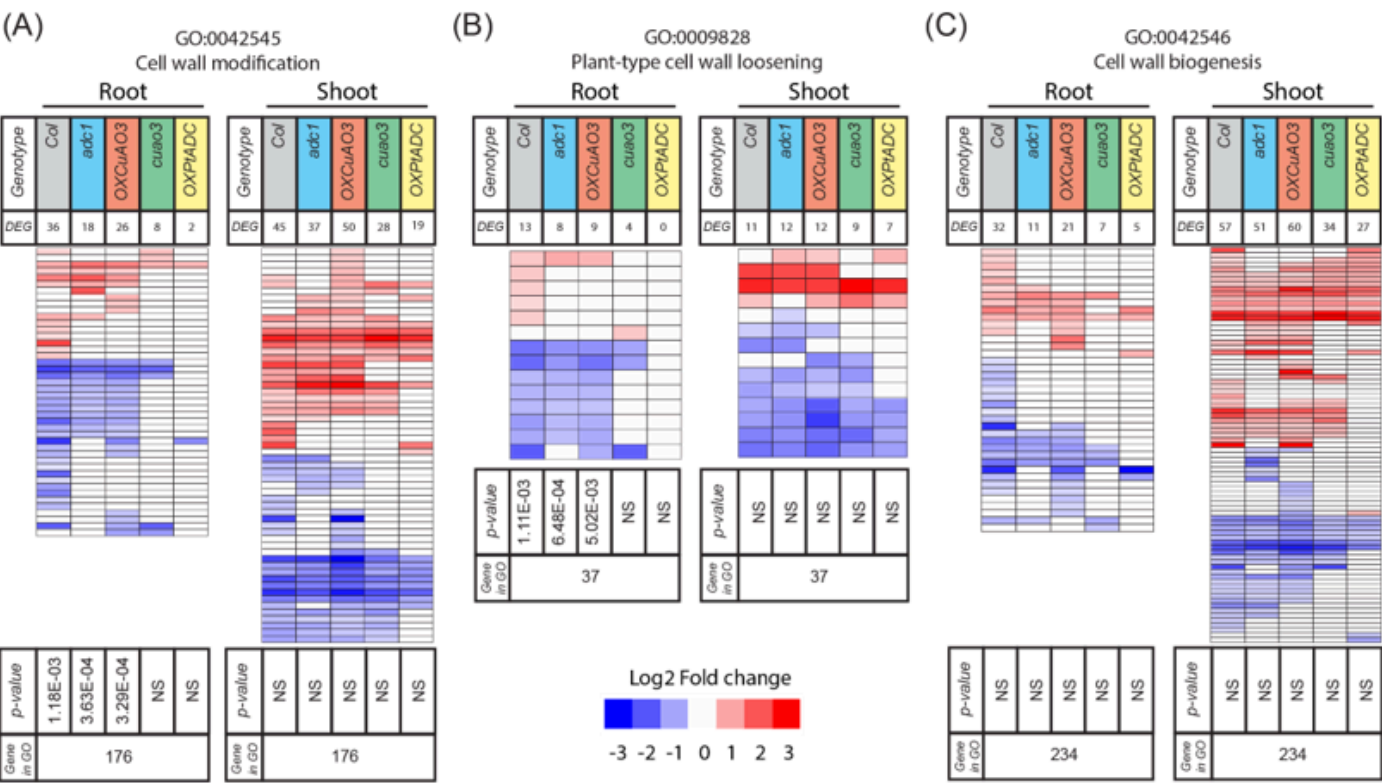


Figure 8

Over-expression of PtADC and *cuao3* knockout/down mitigate the effect of spaceflight conditions on the expression of genes associated with GO:0042545 (Cell Wall Modification; A), GO:0009828 (Plant-type Cell wall loosening; B), and G00042546 (Cell wll biogenesis; C). The heatmap colors represent the Log2 fold change in gene expression between ISS and GC for each gene, with the color scale shown at the bottom of the chart (red indicating up-regulation under microgravity and blue indicating down-regulation). Each column corresponds to a genotype, and each row represents a differentially expressed gene (DEG). Only genes that show significant differential expression in at least one genotype under microgravity conditions are included. At the bottom of each graph, the probability of over-representation of Gene Ontology (GO) groups within the DEGs is displayed, along with the total number of genes in each group. The total number of DEGs associated with each GO group and found to be differentially expressed in each genotype is listed above the chart, beneath the genotype name. The data were generated using hierarchical clustering methods.

Supplementary Files

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

- [SupplementalFigS1qRT.png](#)
- [SupplementalFigS2otherpolyaminequantification.png](#)
- [SupplementalFigS3Morphologycomparison.png](#)
- [SupplementalFigS4ROSGO.png](#)
- [SupplementalTableS1.xlsx](#)
- [SupplementalTableS2.xlsx](#)
- [SupplementalTableS3..xlsx](#)
- [SupplementalTableS4.xlsx](#)
- [SupplementalTableS5.xlsx](#)
- [SupplementalTableS6.xlsx](#)
- [SupplementalTableS7.xlsx](#)
- [SupplementalTableS8.xlsx](#)
- [SupplementalTableS9.xlsx](#)
- [SupplementalTableS10.xlsx](#)
- [SupplementalTableS11.xlsx](#)
- [SupplementalTableS12.xlsx](#)