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RNA-seq analyses of *Arabidopsis thaliana* seedlings after exposure to blue-light phototropic stimuli in microgravity

Joshua P. Vandenbrink[1,2,5], Raul Herranz[3], William L. Poehlman[4], F. Alex Feltus[4], Alicia Villacampa[3], Malgorzata Ciska[3], F. Javier Medina[3], and John Z. Kiss[2]

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[1] School of Biological Sciences, Louisiana Tech University, Ruston, LA 71272, USA

[2] Department of Biology, University of North Carolina at Greensboro, Greensboro, NC 27402, USA

[3] Centro de Investigaciones Biológicas (CSIC), Madrid E28040, Spain

[4] Department of Genetics and Biochemistry, Clemson University, Clemson, SC 29634, USA

[5] Author for correspondence (e-mail: jpvdb@latech.edu)

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PREMISE: Plants synthesize information from multiple environmental stimuli when determining their direction of growth. Gravity, being ubiquitous on Earth, plays a major role in determining the direction of growth and overall architecture of the plant. Here, we utilized the microgravity environment on board the International Space Station (ISS) to identify genes involved influencing growth and development of phototropically stimulated seedlings of *Arabidopsis thaliana*.

METHODS: Seedlings were grown on the ISS, and RNA was extracted from 7 samples (pools of 10–15 plants) grown in microgravity (μ*g*) or Earth gravity conditions (1-*g*). Transcriptomic analyses via RNA sequencing (RNA-seq) of differential gene expression was performed using the HISAT2-Stringtie-DESeq2 RNASeq pipeline. Differentially expressed genes were further characterized by using Pathway Analysis and enrichment for Gene Ontology classifications.

RESULTS: For 296 genes that were found significantly differentially expressed between plants in microgravity compared to 1-*g* controls, Pathway Analysis identified eight molecular pathways that were significantly affected by reduced gravity conditions. Specifically, light-associated pathways (e.g., photosynthesis-antenna proteins, photosynthesis, porphyrin, and chlorophyll metabolism) were significantly downregulated in microgravity.

CONCLUSIONS: Gene expression in *A. thaliana* seedlings grown in microgravity was significantly altered compared to that of the 1-*g* control. Understanding how plants grow in conditions of microgravity not only aids in our understanding of how plants grow and respond to the environment but will also help to efficiently grow plants during long-range space missions.

KEY WORDS European Modular Cultivation System; phototropism; spaceflight; transcriptomics; tropisms.

Due to their sessile nature, plants need to be able to integrate multiple external stimuli to direct their growth and development. These growth-mediated movements (or "tropisms") were first scientifically characterized by Darwin and Darwin (1880), who theorized the movement was modified circumnutation. Multiple external stimuli affect plant growth and development such as water availability (Kiss, 2007), mechanical forces (Braam, 2005), gravity (Chen et al., 1999; Kiss, 2000), and light (Briggs, 2014; Liscum et al., 2014; Vandenbrink et al., 2014a; Kutschera and Briggs, 2016), among others. Taken together, these tropisms play a large role in determining the overall architecture of the plant (Correll and Kiss, 2002). In addition, sensing and responding to these external cues are important for the plant to be able to adapt to changing environmental conditions.

Plants take multiple environmental stimuli into account when determining growth strategy. Typically, plants orient their roots toward the gravity vector (positive gravitropic response), whereas aerial organs orient away from the gravity vector (negative gravitropic response) and toward a blue/white light source (positive phototropic response; Chen et al., 1999; Kiss et al., 2003; Briggs, 2014; Kutschera and Briggs, 2016).

In addition, plants utilize blue-light cues to influence lateral root growth and other root architectural characteristics (Moni et al., 2015).

Light irradiation also affects root length (Laxmi et al., 2008; Silva-Navas et al., 2015). The effect of light on roots is significant, as light has been shown to penetrate several millimeters into the soil (Woolley and Stoller, 1978; Tester and Morris, 1987), with red and far-red light penetrating to greater depths than the remaining spectrum (Mandoli and Briggs, 1984). This light exposure has led roots to evolve blue-light receptors in the upper portion of the roots and red-light receptors more distal in the root due to the more penetrative quality of red light (Mo et al., 2015).

Plant gravitropism is divided into three stages: perception, transduction, and response. During the perception phase, starch-filled statoliths interact with cytoplasmic objects in the specialized gravity-sensing columella cells (Sack, 1991; Salisbury, 1993; Kiss, 2000; Vandenbrink et al., 2014b). Once the gravity signal is perceived, a differential auxin gradient is sent along opposing sides of the root to the root elongation zone (transduction stage), where differential plant growth occurs and leads to reorientation of the root in the direction of the gravity vector (reviewed by Vandenbrink et al., 2014b).

Growth of plants in conditions of real or simulated microgravity have allowed for the study of phototropic response in the absence of significant gravitational influences (Kiss, 2015). Recently, studies have aimed to characterize the link between the two tropistic responses. For instance, our previous study found that light perception by the roots had an effect on shoot gravitropic response in *Arabidopsis thaliana* (Hopkins and Kiss, 2012). In addition, phototropic curvature of roots in response to blue light illumination was shown to be intimately tied to the magnitude of the gravity vector (Vandenbrink et al., 2016).

Our recent spaceflight study also identified an association between the red-light phototropic response in roots and the magnitude of the gravity vector (Vandenbrink et al., 2016). In terms of cell growth and cell proliferation, it has been shown that when seedlings are grown in darkness, there is a lack of balance between these key plant development functions in microgravity (Matía et al., 2010). Further evidence for this observation was provided by analyzing a dark-grown, synchronized cell culture grown in simulated microgravity (Kamal et al., 2018). Recent spaceflight results show that red light can compensate for this effect (Valbuena et al., 2018), particularly increasing cell growth (measured by means of ribosome biosynthesis in the nucleolus) that was depleted without light stimulation.

In the present study, we used RNA expression profiling to begin characterizing the molecular mechanisms affected by conditions of microgravity in phototropically stimulated seedlings. Thus, in the present set of space experiments, we use transcriptomic techniques to characterize gene expression related to seedling growth in conditions of microgravity. Our most significant results demonstrate that light-associated pathways show significant downregulation in microgravity.

MATERIALS AND METHODS

Spaceflight experiment

Experiments with seeds of *Arabidopsis thaliana* ecotype Landsberg erecta (Ler) were flown to the International Space Station (ISS) via the SpaceX Dragon. Spaceflight experiments were conducted utilizing the European Modular Cultivation System (EMCS) in the Columbus Module of the ISS. The EMCS facility provides two centrifuges for the production of gravity vectors and atmospheric, temperature, and hydration monitoring and control (Brinckmann and Schiller, 2002; Brinckmann, 2005; Kiss et al., 2014). In addition, the EMCS contains a video camera for image acquisition as well as visual monitoring of the experiment. The seedling growth series of experiments was conducted in two parts to accommodate the experiment's large sample size. The first set of experiments (termed SG1) was uploaded on to the ISS via SpaceX CRS-2 (March 2013) followed by return via CRS-3 (May 2014), and the second set of seedlings (termed SG2) was carried to the ISS on SpaceX CRS-4 (September 2014) and returned on CRS-5 (February 2015).

Spaceflight procedures

Experimental containers (ECs) were sent to the ISS and loaded into the EMCS. Experimental conditions were controlled remotely from the Norwegian User Support and Operations Centre (N-USOC; Trondheim, Norway). Temperature within the ECs was held between 21.5° and 24.3°C, and relative humidity was held at 95%. The experiments were initiated via hydration of the seeds (Fig. 1). Plants were grown in microgravity and in a 1.0-*g* control. Seedlings were illuminated under white light (30–40 μmol m^{-2} s^{-1}) for 96 h, followed by 48 h of unidirectional photostimulation with blue light (1–40 μmol m^{-2} s^{-1}) perpendicular to the white light and gravity vector. Light sources were LEDs as previously described (Kiss et al., 2014). After the conclusion of the experiments, seedlings were frozen and stored at −80°C in the General Laboratory Active Cryogenic ISS Experiment Refrigerator (GLACIER) freezer of the ISS. Upon return of frozen seedlings to Earth, samples were transported on dry ice, and promptly preserved with RNA*later* (1.5 mL; ThermoFisher Scientific, Waltham, MA, USA) for subsequent RNA-seq analysis.

RNA sequencing and differential gene expression analysis

RNA was extracted individually for each EC, which consisted of 10–14 seedlings. Pooled RNA from an individual EC denotes one replicate. ECs that shared the same gravity conditions were pooled to allow ample RNA for RNA-seq analysis. A plant specific RNA extraction NucleoSpin kit (Macherey-Nagel, Bethlehem, PA, USA) including a DNase treatment was used to isolate whole plant mRNA. The quantity and quality of the extracted RNA was determined by a Nanodrop 2000 (Thermo Scientific). RNA remained frozen at −80°C until delivery. Extracted RNA was shipped on dry ice to the David H. Murdoch Research Institute in Kannapolis, NC, USA. During sequencing, pooled RNA samples were used to generate sequencing libraries using the Illumina TruSeq RNA Library Preparation Kit (Illumina, San Diego, CA, USA). Samples were individually indexed. The samples were pooled, and the following groups were established: microgravity (± 0.00 × *g*, 4 EC replicates) and 1-*g* control (0.99 ± 0.06 × *g*, 3 EC replicates). A 125-bp paired-end sequencing run was performed on the Illumina HiSeq2500. Quality reports for the pooled RNA are available in Appendices S1–S7.

Paired-end 125-bp reads were aligned with the Arabidopsis TAIR10 genome using the PBS-GEM workflow (https://github.com/wpoehlm/PBS-GEM) on the Clemson University Palmetto Cluster (Kim et al., 2015). Fragments with a Phred score below 33 were filtered using Trimmomatic (Bolger et al., 2014). The program HISAT2 (v2.1.0; Kim et al., 2015) was used to align sequencing reads to the reference genome using a minimum intron length of 60 and a maximum intron length of 2000. Annotated reference gene abundances were quantified using StringTie (v1.3.4; Pertea et al., 2015). Annotated reference genes were identified using the TAIR10 genome GFF3

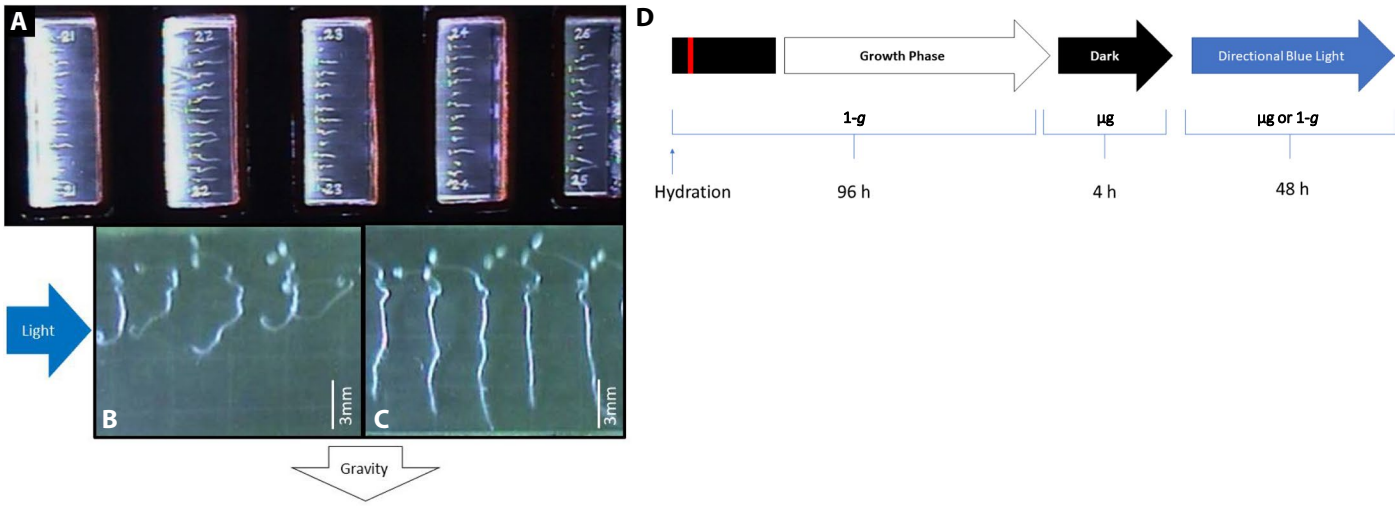

FIGURE 1. Images of seedlings of *Arabidopsis thaliana* growing on the International Space Station. (A) Five seedling cassettes positioned within the European Modular Cultivation System (EMCS). (B, C) Higher magnification view of seedlings grown in (B) conditions of microgravity and (C) 1-*g*. Blue light is introduced at 90° from the overhead white LEDs used during the growth phase. (D) Timeline of events for the seedling growth experiments. Seedlings are initially exposed to a 96-h, 1-*g* growth phase that begins with hydration of the seeds, followed by a short burst of red light to help initiate germination. Seedlings are initially grown at 1-*g* to ensure seedlings are properly oriented, then subjected to a 4-h microgravity dark period. Seedlings are then exposed to unidirectional blue light 90° from the initial white light present during the growth phase (either in µ*g* or 1-*g* control). Once the blue-light phase is complete, seedlings are frozen at −80°.

annotation file (https://www.arabidopsis.org/download_files/Genes/TAIR10_genome_release/TAIR10_gff3/TAIR10_GFF3_genes.gff).

Statistical analysis of differential gene expression was conducted with DESeq2 (v1.18.1; Anders and Huber, 2010). A multiple-test corrected *p*-value (*q*-value; Benjamini and Hochberg, 1995) of 0.05 was employed (Appendix S8). Principal component analysis (PCA) of the samples shows clustering of the microgravity and 1-*g* samples (Appendix S9). Genes identified as differentially expressed between the µ*g* and 1-*g* conditions were subsequently used for Generally Applicable Gene-set Enrichment (GAGE) for Pathway Analysis to identify genetic pathways enriched for differentially expressed genes (Luo et al., 2009). Last, gene ontology annotation of differentially expressed genes (µ*g* vs 1-*g*) was conducted utilizing Protein ANalysis THrough Evolutionary Relationships (PANTHER) Classification System version 13.1 (http://www.pantherdb.org; Thomas et al., 2003; Mi et al., 2013). The PANTHER statistical overrepresentation test was performed using the default settings. An outline of the RNA-seq and Pathway analysis is included (Appendix S10). The data were deposited into NASA's GeneLab (https://genelab.nasa.gov/; accession GLDS 251).

RESULTS

Identification of differentially expressed genes (DEGs)

We analyzed the transcriptome of *Arabidopsis thaliana* seedlings that were grown on the International Space Station (Fig. 1). Dry seeds were hydrated to initiate our spaceflight experiment as previously described (Vandenbrink and Kiss, 2019). Differential expression of genes between plants in µ*g* and Earth's gravity (1-*g*) was analyzed using DESeq2 (Anders and Huber, 2010) with genes from 1-*g* as the reference group. A *q*-value false discovery rate (Benjamini and Hochberg) of 0.05 was used to identify differentially expressed genes (DEGs). Comparison between genes from µ*g* and 1-*g* revealed 296 genes were significantly differentially expressed between microgravity and Earth's 1-*g* (Appendix S11).

Enrichment analysis of gene ontology caused by microgravity

After identification of DEGs, a Generally Applicable Gene-Set (GAGE)-Pathway Analysis was conducted between µ*g* and 1-*g* gravity conditions to determine whether any molecular pathways were significantly enriched with genes (identified as differentially expressed) to give a more holistic view of the effects of reduced gravity on plant molecular pathways (Luo et al., 2009). GAGE analysis revealed that eight pathways were significantly enriched (2 upregulated, 6 downregulated; FDR < 0.1) with genes identified as differentially expressed in the µ*g* vs 1-*g* group (Table 1). These pathways included photosynthesis (Fig. 2) and photosynthetic antenna proteins (Fig. 3) as being significantly downregulated. In addition, porphyrin and chlorophyll metabolism, starch and sucrose metabolism, carotenoid biosynthesis, and protein processing in the endoplasmic reticulum were all shown to be downregulated (Appendices S12–S15). Only two pathways, ribosome synthesis and oxidative phosphorylation were found to be upregulated (Appendices S16, S17).

In the Gene Ontology classifications of genes differentially expressed between µ*g* and 1-*g* using PANTHER (http://www.pantherdb.org; Thomas et al., 2003; Mi et al., 2013), the statistical overrepresentation test identified four molecular processes as overrepresented in the list of differentially expressed (DE) genes. In addition, analysis revealed six biological processes, five protein classes, and five cellular components showing statistical overrepresentation (Table 2). In regard to molecular function, catalytic activity accounted for 42% of genes differentially expressed, while binding and structural molecular activity accounted for 25% and 19%, respectively. Smaller contributing ontologies included receptor activity (2.0%), signal transducer activity (1.0%), and translation

TABLE 1. GAGE-Pathview overrepresentation test of genes identified as differentially expressed between μ*g* and 1-*g* gravity conditions.

Pathway	KEGG pathway identifier[a]	Gene set size	Geometric mean[b]	Stat mean[c]	*p*	FDR[d]
Unregulated PATHWAYS						
Ribosome	ath03010	307	2.34E-25	8.08	2.34E-15	2.62E-13
Oxidative phosphorylation	ath00190	120	1.73E-03	2.95	1.73E-03	9.80E-02
Downregulated pathways						
Photosynthesis – antenna proteins	ath00196	22	1.42E-05	–4.70	1.42E-05	1.61E-03
Photosynthesis	ath00195	45	2.08E-03	–2.98	2.08E-03	5.87E-02
Porphyrin and chlorophyll metabolism	ath00860	51	1.97E-03	–2.96	1.97E-03	5.87E-02
Protein processing in endoplasmic reticulum	ath04141	190	4.37E-03	–2.64	4.37E-03	8.23E-02
Starch and sucrose metabolism	ath00500	140	1.18E-03	–3.07	1.18E-03	5.87E-02
Carotenoid biosynthesis	ath00906	27	2.86E-03	–2.88	2.86E-03	6.46E-02

[a]Kyoto Encyclopedia of Genes and Genomes.
[b]Geometric mean of the individual *p*-values from multiple single array based gene set tests.
[c]Mean of the individual statistics from multiple single array based gene set tests.
[d]False discovery rate *q*-value adjustment of the global *p*-value using the Benjamini–Hochberg procedure.

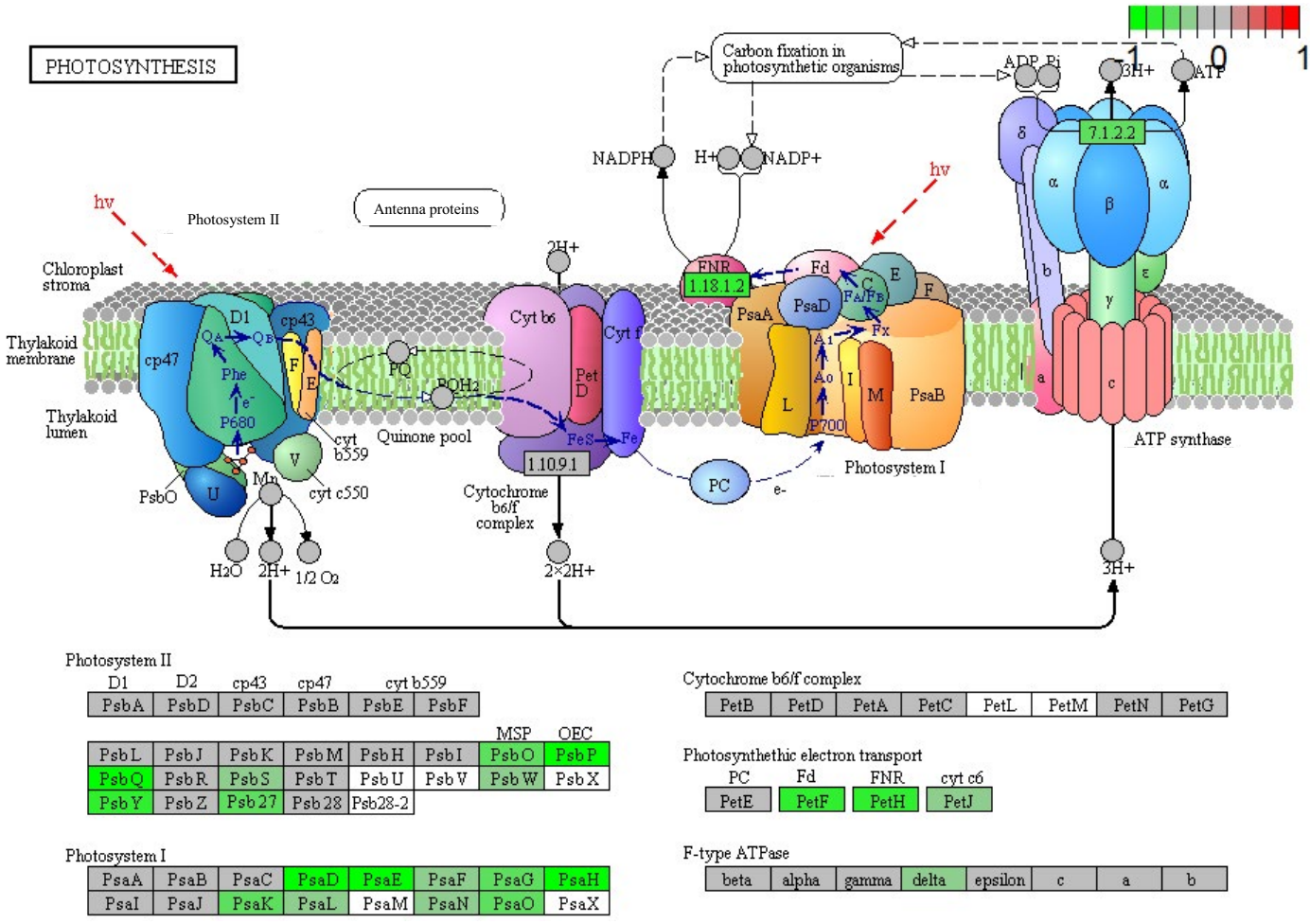

FIGURE 2. Photosynthesis pathview of differentially expressed genes identified in conditions of microgravity when compared to 1-*g* control. Differentially expressed genes were identified using the HISAT2-Stringtie-DESeq analysis pathway ($p = 5.87E-02$). Genes highlighted with green indicate reduced expression when compared to 1-*g* control.

regulation activity (1.0%). Further division into categories of these ontologies is provided in Fig. 4.

The ontological breakdown of biological processes affected by microgravity was also conducted. Metabolic processes and cellular processes each accounted for 37.8% of gene classifications, accounting for the majority of genes identified as differentially expressed (Fig. 5), followed by response to stimulus (9.2%), cellular component organization of biogenesis (7.2%), localization (4.1%), biological replication

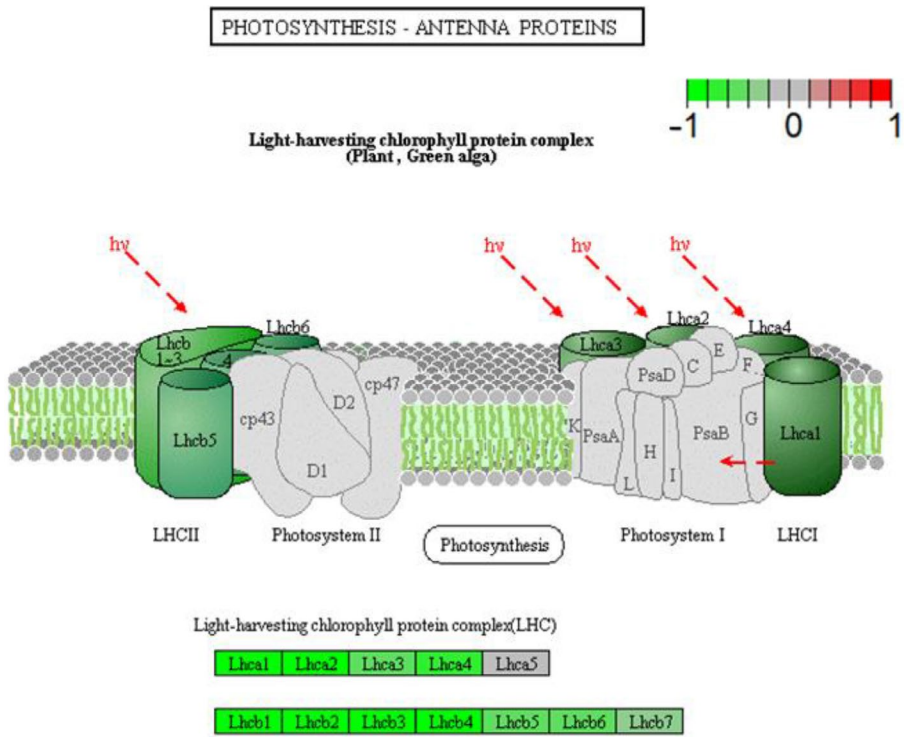

FIGURE 3. Photosynthesis – antenna proteins pathview of differentially expressed genes identified in conditions of microgravity when compared to 1-*g* control. Gene were identified using the HISAT2-Stringtie-DESeq analysis pathway (p = 1.61E-03). Genes highlighted with green indicate reduced expression when compared to 1-*g* control.

(2.0%), and single-multicellular organismal processes and death (1%). Further categorization of gene ontology classifications for cellular component and protein class are shown in Appendices S18 and S19.

DISCUSSION

Image analysis of seedlings grown during the seedling growth suite of experiments previously characterized a novel blue-light phototropic response in roots of *Arabidopsis thaliana* grown in conditions of microgravity (Vandenbrink et al., 2016). This relationship was shown to be linearly related to the magnitude of the gravity vector. To achieve a better understanding of how gravity affects these phototropically stimulated seedlings, we performed RNA-seq analyses to characterize changes in gene expression that may be associated with this novel phototropic response. Interestingly, GAGE-Pathview results indicated that genes constituting major pathways associated with light perception, photosynthesis, and biosynthesis of the photosynthetic complexes showed reduced expression in microgravity.

Photosynthesis and antenna proteins

The two pathways with the greatest statistical significance for downregulation in this study were involved with photosynthesis: antenna protein pathway and photosynthetic pathway (Figs. 2,3). Acclimation to changing light conditions is achieved through changes in expression to the antenna protein genes (Masuda and Fujita, 2008). Antenna proteins, which aid in plant acclimation to different light environments, had all but one light-harvesting chlorophyll protein complex (LHC) gene significantly downregulated (Appendix S12). In regard to the photosynthetic pathway, there was significant downregulation of genes associated with photosystems I and II (Fig. 3). This observation confirms a previous study that detailed an overall reduction in photosystem I (PSI) complexes, as well as a 30% reduction in photochemical activity on *Brassica rapa* plants grown aboard the space shuttle (Jiao et al., 2004). The reduction in photosystem I was accompanied by an overall reduction in biomass of the samples, correlating with a reduction in photosynthetic processes. In a simulated microgravity study, the observed reduction in photosystem I activity was supported by a study which grew *Oryza sativa* on a random positioning machine (RPM) and detailed a reduction in PSI activity, which was attributed to an overall reduction in the biosynthesis of PSI proteins (Chen et al., 2013).

In our present study, four genes associated with photosynthetic electron transport (petE: plastocyanin, petF: ferredoxin, petH: ferredoxin–NADP+ reductase, petJ: cytochrome c6) were significantly downregulated. This observation suggests that there is an interaction between gravity and the light harvesting machinery at a genetic level. This proposed interaction between gravity and light-related pathways may help explain the novel blue-light phototropic response in roots of *Arabidopsis* grown in microgravity (Vandenbrink et al., 2016).

Chlorophyll metabolism and chloroplast function

In addition to photosynthetic pathways, multiple pathways that are associated with light perception and photosynthesis were significantly downregulated. The porphyrin and chlorophyll metabolism pathway, which is responsible for the biosynthesis of chlorophyll pigment, also shows significant downregulation of genes (Appendix S12). Control of chlorophyll metabolism has been shown to be regulated by phytohormones, environmental signals, organ specificity, developmental stage, gene expression, proteolysis, among others (Masuda and Fujita, 2008). However, the relationship between gravity and chlorophyll biosynthesis is poorly understood.

When analyzing gene expression patterns, we found significant downregulation of genes responsible for heme biosynthesis (*HemA*, *HemL*, *HemB*, *HemC*, *HemD*, *HemE*, *HemF*, and *HemH*). These heme series of proteins are important for the biosynthesis of chlorophylls. In addition to the heme proteins, genes for multiple enzymes associated with the final processing of chlorophyll *a* and chlorophyll *b*, such as chlorophyll *b* reductase and chlorophyll synthase, were downregulated; Appendix S12). This observation suggests that reduction of gravity from 1-*g* to μ*g* potentially reduces the ability of the plant to synthesis mature chlorophyll. These latter results may have important implications for the use of plants in bioregenerative life support in space missions.

TABLE 2. PANTHER overrepresentation test of genes identified as differentially expressed (DE) between μ*g* and 1-*g* gravity conditions.

PANTHER Category	No. reference genes[a]	No. mapped genes[b]	Expected no. genes[c]	Fold enrichment[d]	+/–[e]	*p*-value[f]	FDR[g]
Biological process							
Generation of precursor metabolites and energy	263	21	2.89	7.27	+	6.61E-12	1.24E-09
Oxidative phosphorylation	58	10	0.64	15.7	+	3.28E-09	3.09E-07
Respiratory electron transport chain	153	13	1.68	7.74	+	3.70E-08	2.32E-06
Response to abiotic stimulus	175	13	1.92	6.76	+	1.60E-07	7.51E-06
Translation	308	14	3.38	4.14	+	1.32E-05	4.95E-04
Biosynthetic process	2090	41	22.95	1.79	+	4.26E-04	1.33E-02
Cation transport	67	5	0.74	6.8	+	1.13E-03	3.03E-02
Protein class							
Ribosomal protein	322	27	3.54	7.64	+	1.90E-15	3.35E-13
RNA binding protein	1115	33	12.24	2.7	+	4.03E-07	3.54E-05
Nucleic acid binding	1771	38	19.45	1.95	+	9.01E-05	3.96E-03
Ligand-gated ion channel	11	3	0.12	24.84	+	4.23E-04	1.49E-02
Winged helix/forkhead transcription factor	31	4	0.34	11.75	+	5.48E-04	1.61E-02
Anion channel	16	3	0.18	17.07	+	1.08E-03	2.38E-02
ATP synthase	47	4	0.52	7.75	+	2.28E-03	4.46E-02
Ion channel	57	3	0.63	4.79	+	2.76E-02	4.04E-01
Helix-turn-helix transcription factor	161	5	1.77	2.83	+	3.53E-02	4.78E-01
Cation transporter	140	4	1.54	2.6	+	7.24E-02	8.49E-01
Transporter	953	6	10.46	0.57	–	2.03E-01	1.70E+00
Receptor	74	1	0.81	1.23	+	5.60E-01	2.19E+00
Transcription factor	690	8	7.58	1.06	+	8.52E-01	2.42E+00
Cellular component							
Ribosome	325	23	3.57	6.44	+	6.44E-12	3.22E-10
Macromolecular complex	1919	52	21.07	2.47	+	2.46E-09	6.16E-08
Ribonucleoprotein complex	643	25	7.06	3.54	+	9.64E-08	1.61E-06
Mitochondrial inner membrane	128	11	1.41	7.83	+	3.77E-07	4.72E-06
Proton-transporting ATP synthase complex	25	5	0.27	18.21	+	1.67E-05	1.67E-04
Protein complex	1375	29	15.1	1.92	+	8.61E-04	7.17E-03
Cytoplasm	3618	60	39.73	1.51	+	1.12E-03	7.99E-03
Cytosol	776	18	8.52	2.11	+	4.35E-03	2.72E-02
Intracellular	5893	85	64.71	1.31	+	5.94E-03	3.30E-02
Membrane	2139	37	23.49	1.58	+	6.71E-03	3.36E-02
Cell part	6166	87	67.71	1.28	+	1.02E-02	4.25E-02
Thylakoid	46	2	0.51	3.96	+	9.58E-02	3.68E-01
Organelle	4563	59	50.11	1.18	+	1.86E-01	6.64E-01
Mitochondrion	417	3	4.58	0.66	–	6.35E-01	1.18E+00
Molecular function							
Structural constituent of ribosome	264	25	2.9	8.62	+	1.66E-15	2.60E-13
Structural molecule activity	530	28	5.82	4.81	+	2.28E-11	1.79E-09
Proton-transporting ATP synthase activity, rotational mechanism	25	5	0.27	18.21	+	1.67E-05	6.55E-04
Hydrogen ion transmembrane transporter activity	102	8	1.12	7.14	+	2.77E-05	8.71E-04
Ligand-gated ion channel activity	10	3	0.11	27.32	+	3.35E-04	8.76E-03
Anion channel activity	77	5	0.85	5.91	+	2.02E-03	4.52E-02
Ion channel activity	93	5	1.02	4.9	+	4.36E-03	8.57E-02
Transmembrane transporter activity	858	11	9.42	1.17	+	6.15E-01	1.97E+00
Transporter activity	996	11	10.94	1.01	+	8.78E-01	2.03E+00

[a]Number of genes in reference genome mapped to the annotation category.
[b]Number of identified DE genes mapped to annotation category.
[c]Number of DE genes predicted to be in annotation category.
[d]Fold enrichment of genes identified as DE (no. of DE genes/no. expected).
[e]Overrepresentation (+) or underrepresentation (–) when compared to expected.
[f]Determined by Fisher's exact test.
[g]False discovery rate determined by Benjamini-Hochberg procedure.

In addition to downregulation of genes associated with the production of chlorophyll, chloroplasts in plants grown in microgravity undergo significant changes, including more ovoid-shaped chloroplasts than plants grown in 1-*g*, and denser packing of thylakoid membranes of space-grown plants, with each of the membranes roughly 0.9 nm closer than those plants grown in 1-*g* (Stutte et al., 2006). In addition, a study of clinorotated *Arabidopsis* plants showed that the number of grana per chloroplast and overall size of the

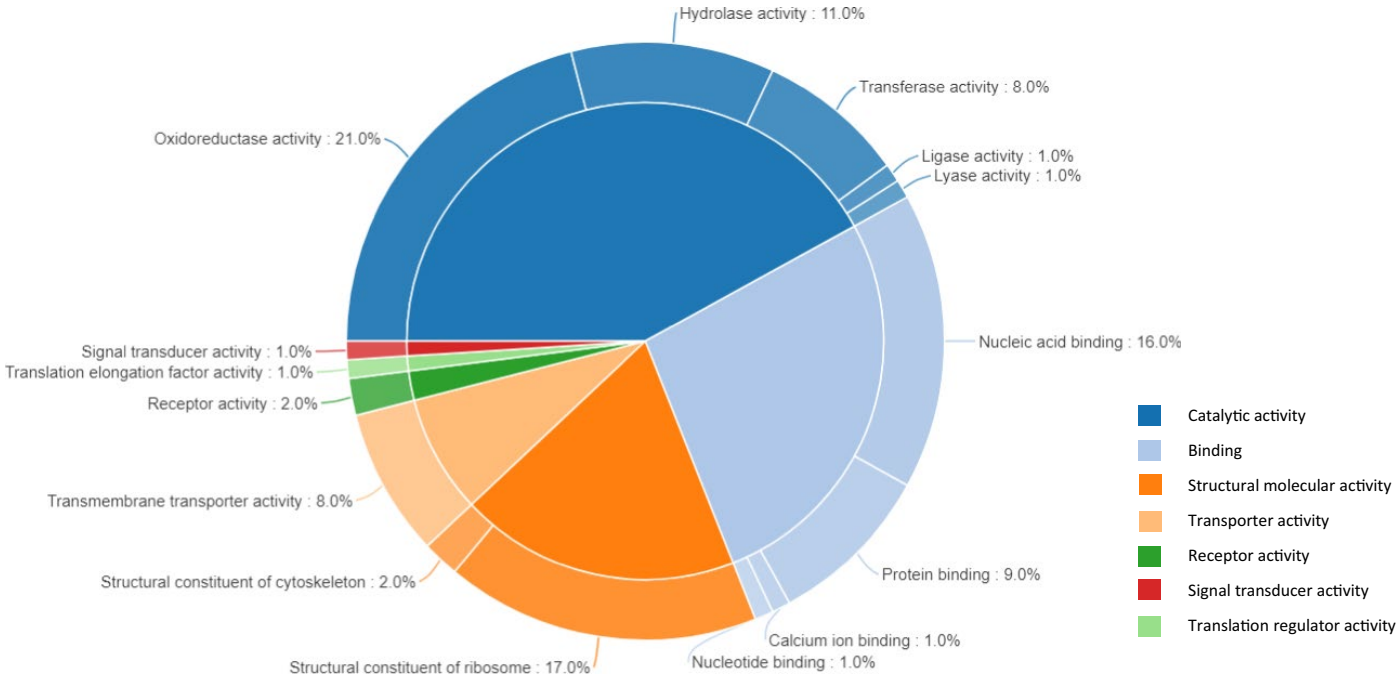

FIGURE 4. PANTHER molecular function of differentially expressed genes. Classification of the functions that proteins identified from differentially expressed genes perform on its direct molecular target (μ*g* vs. 1-*g*).

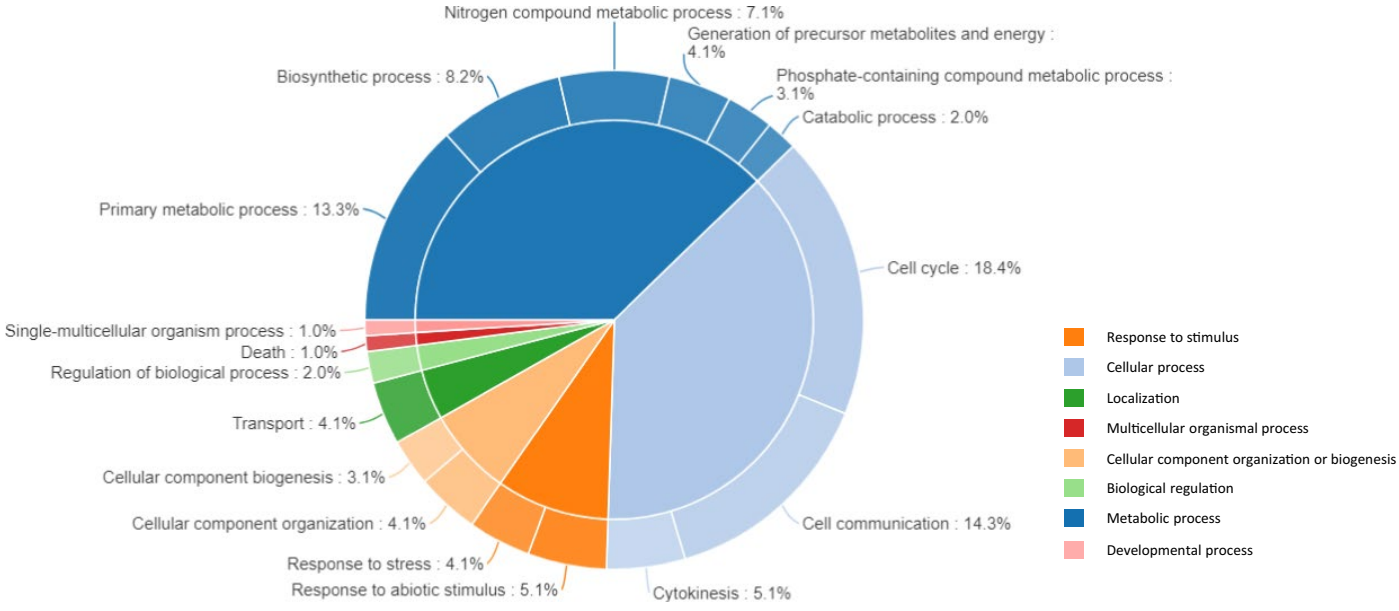

FIGURE 5. PANTHER biological process of differentially expressed genes. Classification of biological systems that identified differentially expressed genes belong to μ*g* vs. 1-*g*.

chloroplast were reduced (Adamchuk, 1998). An increase in proton permeability of the thylakoid membrane was also observed in seedlings grown on a 2-D clinostat (Mikhaylenko et al., 2001). Taken together, these observations suggest that the synthesis of chlorophyll and the formation of plant chloroplasts are significantly altered in conditions of microgravity.

Previous spaceflight research has considered the effects of microgravity environments on the biosynthesis of chlorophyll. Species of *Chlorella*, single-celled photosynthetic algae, showed alterations to their thylakoid membrane as well as a 35–50% decrease in chlorophyll content (Moleshko et al., 1991). Similarly, the chlorophyll content is reduced in wheat grown in microgravity for 19 days (Rumyantseva et al., 1990) and in pea plants grown in space (Laurinavichius et al., 1986). In addition to spaceflight studies, experiments utilizing simulated microgravity devices, such as clinostats and random positioning machines (RPMs), have also shown a decrease in chlorophyll content (Miyamoto et al., 2001). Also, Yamada et al. (1993) predicted that carbon metabolism was affected

by microgravity when starch granules in chloroplasts were found to be smaller in clinorotated plants.

Effects of experimental hardware on photosynthetic rates

There have been multiple studies of photosynthetic rates in plants exposed to microgravity, which have produced a wide range of results. Numerous reports (detailed above) have reported reductions in photosynthetic apparatuses, photosynthetic rates, chloroplast function and morphology, among others. However, in contrast, in other studies, no changes were found in chloroplast density (Stutte et al., 2006), chloroplast structure (Musgrave et al., 1998), or photosynthetic rate (Stutte et al., 2005), to name a few. When comparing results of these experiments, it is important to acknowledge the effects of the spaceflight hardware when interpreting the results. Multiple stresses and environmental stimuli are encountered when growing plants in microgravity (Kiss, 2015). These include (but are not limited to) lack of convection, reduced CO_2 levels, less than optimal temperature, elevated ethylene, spacecraft vibrations, increased radiation exposure, among others. Hardware exists to try to mitigate many of these environmental factors that are present on the International Space Station, but no perfect hardware exists for the growth of plants in space.

Stutte et al. (2006) grew plants in a facility (the Biomass Production System [BPS]) that aimed to limit the confounding hardware effects present in some spaceflight studies such as lack of convection, improper lighting, reduced CO_2 levels, temperature fluctuation, low humidity, and elevated ethylene. In the BPS, they found no apparent changes in photosynthetic rate and attributed previous findings of photosynthetic reduction in a microgravity environment to improper ventilation. However, here we used the European Modular Cultivation System, which contains an air scrubbing/filtration system designed to remove excess ethylene from the seedlings during the growth phase (Kiss et al., 2014; Kiss, 2015). Thus, even with proper ventilation of plants grown in space, expression of photosynthetic genes was reduced.

Interestingly, a few studies have shown an increase in chlorophyll content in space-grown pea plants (Abilov, 1986; Aliyev, 1987). This increase in chlorophyll content was also observed in clinorotated rice seedlings (Jagtap et al., 2011). However, these studies all observed this increased chlorophyll content in young seedlings. Jagtap et al. (2011) noted that chlorophyll content increases in clinorotated rice seedlings up to 5 days, then the trend reverses, and chlorophyll content starts to decrease. This observation suggests a temporal component to the effects of microgravity on chlorophyll production, with longer durations causing a decrease in chlorophyll biosynthesis.

Starch and sucrose metabolism

The relationship between starch and sucrose metabolism in microgravity has been explored in previous space flight and spaceflight analog studies. For example, soybean seedlings grown for 6 days under simulated microgravity conditions (clinorotation) were shown to have a decrease in starch concentration in cotyledons (Brown and Piastuch, 1994). In addition, in this study, the activity of ADP-glucose pyrophosphorylase was reduced. This observation corresponds with our own results, which show a downregulation of ADP-glucose pyrophosphorylase in the starch and sucrose metabolism pathway (Appendix S13). In addition, wheat leaves grown in conditions of altered gravity (clinorotation) were shown to have reduced sucrose and starch accumulation in leaves; however, ADP-glucose pyrophosphorylase activity did not decrease.

In contrast to the above reports, sweet potato stem cuttings flown in the Space Shuttle for 5 days showed substantially greater accumulation of soluble sugars, glucose, fructose, sucrose, and total starch. The space-flown sweet potatoes were exposed to ~50% higher concentrations of CO_2, which likely would account for the increase in sugar accumulation (Mortley et al., 2008). Similarly, soybean and potato plants grown for 5 days onboard the Space Shuttle observed compositional changes in starch granules; however, changes in starch content were attributed to ethylene effects (Kuznetsov et al., 2001). In addition, the reported ethylene effects were not observed in the sweet potato study (Mortley et al., 2008) nor in the present study, which both used methods of ethylene removal. Thus, reduction in starch and sucrose metabolism may be species or tissue specific, or alternatively, due to hardware effects.

Carotenoid biosynthesis

The carotenoid pathway also is associated with photosynthesis and light perception (Appendix S14). Carotenoids have been shown to be linked to chlorophyll production; carotenoid content increases in conjunction with chlorophyll biosynthesis (Howitt and Pogson, 2006). Xanthophylls are essential components of the plant photosynthetic apparatus (Lokstein et al., 2002) and important for the formation of stable pigment–protein complexes (Paulsen, 1995) and as ancillary light-harvesting pigments (Siefermann-Harms, 1987). In our present spaceflight experiment, the expression of xanthophyll biosynthetic genes was reduced. In addition, multiple genes associated with the lutein biosynthesis arm of the carotenoid pathway were downregulated. Lutein is the predominant carotenoid in photosynthetic tissue, playing a large role in the bulk antenna complex, or LHC II (Pogson et al., 1996). In addition, lutein optimizes antenna structure and organization to increase the efficiency of light harvesting (Lokstein et al., 2002).

Carotenoids, the second most-abundant group of pigments in nature, play an important protective role in light sensing. For instance, these photoreceptors act as photoprotective compounds that quench triplet chlorophyll and radical oxygen species derived from excess light absorption (Demmig-Adams et al., 1996). This nonphotochemical quenching also prevents damage to the thylakoid membrane, to which the chlorophyll and carotenoids are bound (Niyogi, 1999). In previous research on spaceflight-flown photosynthetic organisms, carotenoid content in organisms was reduced. For instance, a 50% reduction in carotenoid contents was found in the alga *Chlorella* (Moleshko et al., 1991) and a similar reduction in maize (Rumyantseva et al., 1990). These past studies further support our current findings of a reduction in gene expression associated with carotenoid biosynthesis in space-grown seedlings of *A. thaliana*.

Ribosome biogenesis and oxidative phosphorylation

In contrast with the downregulation of phototropism and related biosynthetic and metabolic pathways, we found two very clearly upregulated functions in microgravity-grown seedlings when compared to 1-*g* spaceflight samples: ribosome biosynthesis and oxidative phosphorylation (Appendices S16 and S17). In both true microgravity (Matía et al., 2010) and different simulated microgravity facilities using both seedlings and cell cultures (Manzano et al., 2013; Kamal et al., 2018), ribosome biogenesis was reduced.

Our recent spaceflight results showed that red light can compensate for this effect (Valbuena et al., 2018), particularly by increasing cell growth (measured by means of ribosome biosynthesis in the nucleolus) that was depleted without light stimulation. The results of our present space studies support that removing the gravitropic stimuli in combination with photostimulation can lead to higher levels of protein production and metabolism rates (oxidative phosphorylation).

CONCLUSIONS

The results of this study help to detail the influences of gravity on the development of phototropically stimulated seedlings. Analysis of the affected gene ontologies revealed that catalytic activity and binding activity were the most significantly affected molecular functions, while cellular processes and metabolic processes were the most significantly affected biological processes. Removal of the influence of gravity on blue-light-illuminated seedlings showed a reduction in gene expression in multiple pathways associated with photosynthesis, suggesting the gravity response influences development of seedlings exposed to directional blue light. In addition, pathways previously associated with light perception and response, such as carotenoid biosynthesis and starch metabolism, were also identified as downregulated, suggesting a possible interplay between plant growth in microgravity and plastid function. Taken together, these findings in concert suggest an intricate connection between gravity and growth of phototropically stimulated seedlings in *A. thaliana*.

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DATA AVAILABILITY

All sequences generated in this study are deposited in NASA GenLab (https://genelab.nasa.gov/) as data set GLDS 251.

SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

APPENDIX S1. FASTQC results for pooled sample #111: microgravity (A) forward and (B) reverse paired-end reads.

APPENDIX S2. FASTQC results for pooled sample #114: microgravity (A) forward and (B) reverse paired-end reads.

APPENDIX S3. FASTQC results for pooled sample #116: microgravity (A) forward and (B) reverse paired-end reads.

APPENDIX S4. FASTQC results for pooled sample #120: microgravity (A) forward and (B) reverse paired-end reads.

APPENDIX S5. FASTQC results for pooled sample #175: 1-*g* (A) forward and (B) reverse paired-end reads.

APPENDIX S6. FASTQC results for pooled sample # 179: 1-*g* (A) forward and (B) reverse paired-end reads.

APPENDIX S7. FASTQC results for pooled sample # 235-239: 1-*g* (A) forward and (B) reverse paired-end reads.

APPENDIX S8. MA plot showing the log2 fold-change of normalized counts vs. the mean of normalized counts for microgravity samples (1-*g* control).

APPENDIX S9. Principal component analysis of microgravity (pink) and 1-*g* (blue) data sets generated from the seedling Growth series of spaceflight experiments.

APPENDIX S10. Diagram detailing the steps and software used for pathway analysis of differentially expressed genes. Seedlings were pooled based on gravity exposure (μ*g* or 1-*g*).

APPENDIX S11. Table of results for differential gene expression analysis with DESeq2.

APPENDIX S12. Porphyrin and chlorophyll metabolism pathview of differentially expressed genes identified from HISAT2-Stringtie-DESeq. p = 5.87E-02

APPENDIX S13. Starch and sucrose metabolism pathview of differentially expressed genes identified from HISAT2-Stringtie-DESeq. p = 5.87E-02

APPENDIX S14. Carotenoid biosynthesis pathview of differentially expressed genes identified from HISAT2-Stringtie-DESeq. p = 4.99E-09

APPENDIX S15. Protein processing in the endoplasmic reticulum pathview of differentially expressed genes identified from HISAT2-Stringtie-DESeq. p = 8.23E-02

APPENDIX S16. Ribosome pathview of differentially expressed genes identified from HISAT2-Stringtie-DESeq. p = 2.62E-13

APPENDIX S17. Oxidative phosphorylation pathview of differentially expressed genes identified from HISAT2-Stringtie-DESeq. p = 9.80E-02

APPENDIX S18. PANTHER cellular location of differentially expressed genes. Cellular localization of the protein products derived from genes identified as differentially expressed in conditions of microgravity.

APPENDIX S19. PANTHER protein class of differentially expressed genes. Ontological classification of protein products derived from genes identified as differentially expressed in conditions of microgravity.

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