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Spaceflight Transcriptomes: Unique Responses to a Novel Environment

Anna-Lisa Paul,[1] Agata K. Zupanska,[2] Dejerianne T. Ostrow,[2] Yanping Zhang,[2] Yijun Sun,[2] Jian-Liang Li,[2] Savita Shanker,[2] William G. Farmerie,[2] Claire E. Amalfitano,[2] and Robert J. Ferl[3]

Abstract

The spaceflight environment presents unique challenges to terrestrial biology, including but not limited to the direct effects of gravity. As we near the end of the Space Shuttle era, there remain fundamental questions about the response and adaptation of plants to orbital spaceflight conditions. We address a key baseline question of whether gene expression changes are induced by the orbital environment, and then we ask whether undifferentiated cells, cells presumably lacking the typical gravity response mechanisms, perceive spaceflight. Arabidopsis seedlings and undifferentiated cultured Arabidopsis cells were launched in April, 2010, as part of the BRIC-16 flight experiment on STS-131. Biologically replicated DNA microarray and averaged RNA digital transcript profiling revealed several hundred genes in seedlings and cell cultures that were significantly affected by launch and spaceflight. The response was moderate in seedlings; only a few genes were induced by more than 7-fold, and the overall intrinsic expression level for most differentially expressed genes was low. In contrast, cell cultures displayed a more dramatic response, with dozens of genes showing this level of differential expression, a list comprised primarily of heat shock–related and stress-related genes. This baseline transcriptome profiling of seedlings and cultured cells confirms the fundamental hypothesis that survival of the spaceflight environment requires adaptive changes that are both governed and displayed by alterations in gene expression. The comparison of intact plants with cultures of undifferentiated cells confirms a second hypothesis: undifferentiated cells can detect spaceflight in the absence of specialized tissue or organized developmental structures known to detect gravity. Key Words: Tissue culture—Microgravity—Low Earth orbit—Space Shuttle—Microarray. Astrobiology 12, 40–56.

1. Introduction

One of the accepted and fundamental premises of spaceflight biology, and one which carries over into astrobiology, is that an understanding of adaptive processes and environmental limits of terrestrial biology in spaceflight is key to successful space exploration strategies (Des Marais *et al.*, 2008). During spaceflight and exposure to other extraterrestrial environments, organisms experience growth and development conditions that are distinctly unlike the terrestrial environment that has directed the evolution of gene expression patterns that typically guide growth and ensure survival. Biology in space must therefore interpret novel stimuli, integrate new solutions, and express potentially unusual suites of genes in an attempt to cope with spaceflight environments. Some of these responses might be directly interpretable as appropriate responses that clearly inform science of the stresses induced by spaceflight. Other responses might be confused or inappropriate due to the novel signals or biological processes stimulated by those spaceflight conditions. Defining both kinds of responses is critical to understanding response and adaptation of any biology to all kinds of extraterrestrial environments—from vehicles in orbit to lunar habitats.

The fundamental scientific questions are: Do seedlings exhibit gene expression changes that characterize the adaptation to spaceflight, and do undifferentiated cells perceive and similarly adapt to spaceflight? Past approaches to similar questions have been hampered by spaceflight manifest opportunities and have been particularly limited with regard to biological replication. Current approaches seek to address the replication issue, and the present experiment is dedicated

[1]Horticultural Sciences and Genetics Institute, University of Florida, Gainesville, Florida.
[2]University of Florida, Gainesville, Florida.
[3]Interdisciplinary Center for Biotechnology and Research, Horticultural Sciences and Plant Molecular and Cellular Biology, University of Florida, Gainesville, Florida.

to robust, parallel replicated samples to answer these questions to a refined state of statistical significance while employing current gene expression profiling technologies.

Historical data indicate that plants mount stress responses in the spaceflight environment (Ferl *et al.,* 2002). Some of the responses appear directly and are appropriately correlated to certain environmental parameters in the vehicles and support hardware, or are directly attributable to gravity effects, including the absence of convective mixing in microgravity (*e.g.,* Porterfield *et al.,* 1997; Levinskikh *et al.,* 2000; Liao *et al.,* 2004; Johnsson *et al.,* 2009). However, it is also clear that aspects of spaceflight affect the ability of plants to process biological signals from one tissue type, or organ, to another (*e.g.,* Paul *et al.,* 2001; Roux *et al.,* 2003). Such processing errors can subvert the engagement of adaptive metabolisms, and the perception of certain signals may be inappropriately processed into a stress response when true stress conditions do not exist. It is not known how novel environmental signals are perceived and interpreted by plants or what genes play a role in signaling events during spaceflight.

In the present experiment, the use of both whole seedlings and undifferentiated Arabidopsis tissue culture enables an evaluation of the fundamental mechanisms associated with plant responses to spaceflight. Specific plant organs and tissues are often associated with the detection and transduction of signals from the environment, including gravity. For instance, the columellar cells of roots play a central role in gravity perception, and movement of air through stomata plays a critical role in a plant's perception of drought and engagement of ABA pathways. But what if organs such as these were absent? Would gravity still exert an effect on plant cells, and would the effect be the same? What are some of the underlying mechanisms of environmental sensing? To what extent can individual cells, absent typically organized tissues or organs, perceive the spaceflight environment?

Plants have evolved to deal with environmental challenge through changes in metabolism guided by the activation and repression of response genes. There are numerous reviews of the many environmental response genes and pathways that have been examined in Arabidopsis (*e.g.,* Chen *et al.,* 2002; Kreps *et al.,* 2002; Rossel *et al.,* 2002; Seki *et al.,* 2002; Kimura *et al.,* 2003; Martzivanou and Hampp, 2003; Kimbrough *et al.,* 2004; Kittang *et al.,* 2004; Branco-Price *et al.,* 2005; Liu *et al.,* 2005; Benedict *et al.,* 2006; Lim *et al.,* 2006; Jiang *et al.,* 2007), and several large databases have been populated with gene responses to a variety of environmental challenges (*e.g.,* Kilian *et al.,* 2007; Kant *et al.,* 2008). Spaceflight is unique in that it is a novel environment for plants; thus any response is one that can provide insight into how eukaryotes cope with abiotic signals that lie completely outside their evolutionary experience.

Plants offer valuable potential for exploring the eukaryotic response to spaceflight and other extraterrestrial environments. This potential arises from the confluence of the life-support and astrobiology agendas and has kept plant biology firmly within the spaceflight experiment community. Numerous plant experiments have flown in the Space Shuttle and International Space Station payload programs in the last 20 years, and the following citations are only a sampling of this research: Saunders (1968), Bucker (1974), Krikorian *et al.* (1981, 1992), Kordyum *et al.* (1983), Guikema *et al.* (1994), Kuang *et al.* (1996, 2000), Levine and Krikorian (1996), Brown *et al.* (1997), Musgrave *et al.* (1997), Porterfield *et al.* (1997), Adamchuk *et al.* (1999), Kiss and Edelmann (1999), Nedukha *et al.* (1999), Sato *et al.* (1999), Gao *et al.* (2000), Levinskikh *et al.* (2000), Levine *et al.* (2001), Kern and Sack (2001), Paul *et al.* (2001, 2005), Hoson *et al.* (2003), Klymchuk *et al.* (2003), Stutte *et al.* (2006), Salmi and Roux (2008), Johnsson *et al.* (2009), Kiss *et al.* (2009), Ou *et al.* (2009), Solheim *et al.* (2009), Visscher *et al.* (2009). Conclusions from plant biology experiments have highlighted biological responses to spaceflight environments and have also illuminated engineering and operational advancements necessary for conducting sound biological experiments in space (reviewed in Halstead and Dutcher, 1987; Dutcher *et al.,* 1994; Ferl *et al.,* 2002; Clement and Slenzka, 2006; Brinckmann, 2007; Gilroy and Masson, 2007).

Plants grown in low Earth orbital environments often display an altered physiology compared to plants in ground-based controls. At the cellular level, spaceflight has been associated with disruptions of microtubular self-organization (Papaseit *et al.,* 2000), changes in amyloplast distribution (Legue *et al.,* 1997; Kiss and Edelmann, 1999; Driss-Ecole *et al.,* 2000), and energy metabolism (Hampp *et al.,* 1997). At the organismal level, plants have responded to spaceflight with variations in basic physiology such as electron transport rates in photosynthetic processes (Tripathy *et al.,* 1996) and stress metabolism responses related to hypoxia (Porterfield *et al.,* 1997), and disruptions of calcium localization and calcium-mediated signaling (Merkys and Darginaviciene, 1997; Klymchuk *et al.,* 2001; Nedukha *et al.,* 2001; Paul *et al.,* 2001; Roux *et al.,* 2003; Salmi and Roux, 2008). However, there are also examples where remarkably little disruption of basic processes occurs (Stutte *et al.,* 2006), and it is possible that the better we get at designing microgravity habitats, the fewer stress responses we will see in our biology.

Almost all experiments that have evaluated the effects of spaceflight on plants have been conducted with intact whole plants, and the focus has often been related to gravisensing and the roles of specific cells and organs that act as sensors and contribute to gravity signal transduction. Spaceflight experiments in which tissue explants of roots and tissue culture–derived plantlets have been used have flown as well (Merkys *et al.,* 1989; Levine and Krikorian, 1992, 1996) but with no available molecular analyses. A notable exception is the fern single-cell model system of *Ceratopteris richardii* spores, which does respond to spaceflight at the molecular level and appears to be especially sensitive to changes in the gravity vector and calcium signaling (Salmi and Roux, 2008).

A powerful approach to evaluating spaceflight adaptation is to assess the patterns of gene expression as they change in response to the spaceflight environment. This experimental approach has been limited to a small number of experiments with few replicates. Gene expression studies that have been conducted to date are neither highly replicated nor technically robust enough to assay gene expression alterations approaching the 2-fold level of change with statistical significance (Paul *et al.,* 2005; Stutte *et al.,* 2006; Salmi and Roux, 2008). The results presented here are from highly replicated biological samples of developmentally distinct forms of Arabidopsis: etiolated seedlings and undifferentiated cell cultures. Exploration of their respective transcriptomes in response to the spaceflight environment provides new

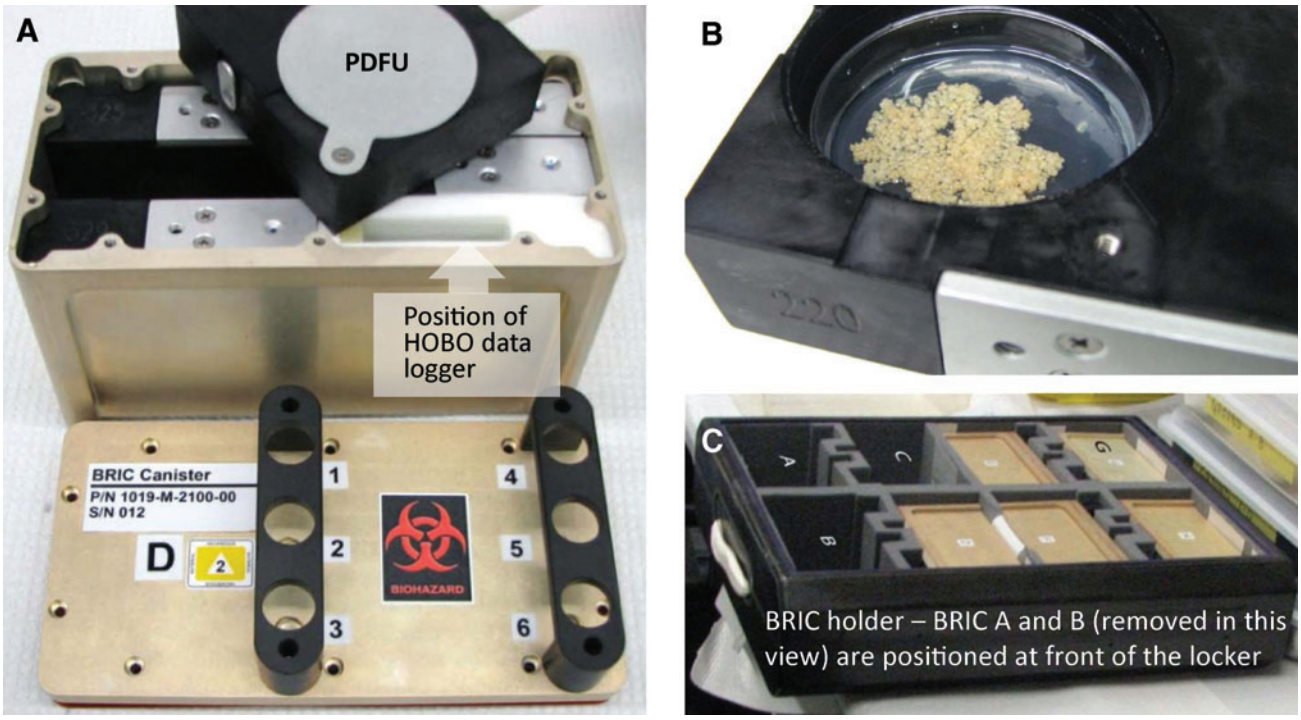

FIG. 1. BRIC spaceflight hardware. Panel (**A**) shows the opened BRIC with one of the PDFUs removed from its slot in the unit and the position of the HOBO data logger indicated with a white arrow. (**B**) An open PDFU containing a plate of cultured cells. (**C**) The distribution of the BRICs in the middeck locker. Photo 1A courtesy of Kim Slater, NASA, KSC. Color images available online at www.liebertonline.com/ast

insight in the perception and adaptation of terrestrial organisms to an extraterrestrial environment.

2. Materials and Methods

2.1. BRIC spaceflight hardware

The hardware for this experiment was the NASA Biological Research in a Canister–Light-Emitting Diode (BRIC-LED). The LED component of the hardware was unavailable due to mission resource constraints, and all biology was held in the dark for the duration of the experiment. The BRIC-LED canisters hold six 60 mm diameter Petri plates in small subcompartments called Petri Dish Fixation Units (PDFUs). The PDFUs allow an externally activated fixation step without manipulating the samples. We were allocated 13 PDFUs: five in BRIC A, five in BRIC B, and three in BRIC G. The distribution of the BRICs in the middeck locker is shown in Fig. 1C.

2.2. General biology operations

Wild-type (non-transgenic) Arabidopsis plants and Arabidopsis tissue culture cells were planted on 60 mm round, nutrient agar Petri plates cultured in a horizontal position until turnover and installation into the BRIC hardware. All the subculturing on the plates was conducted in the laboratory of the principal investigators and the University of Florida. Some planting of the seeded plates was conducted at Kennedy Space Center (KSC).

Power analysis calculations were used to determine optimal number of replicates (individual Petri plates) among the two treatment groups: (Spaceflight) and a control group (Ground Control) (Tables 1 and 2). Plates were planted in a laminar flow hood and stored in sterile BioTransport Carrier (BTC, Nalgene). Plates with both seeds and cells were kept in the dark, and the seeds vernalized at 4°C.

The biological material for both Flight and Ground Control were prepared and treated exactly the same, with the exception that the Ground Control material was delayed 24 h in all phases. This delay was to facilitate the programming of the Ground Control Orbiter Environmental Simulator (OES) with near–real-time download of middeck environmental data from the orbiter in order to present the Ground Control with as similar an environmental profile as operationally possible (see below).

2.3. Preparation of specialized plates and biology

2.3.1. Petri plates. Because of their configuration within the BRIC flight hardware, sterility was maintained on all surfaces (interior and exterior) of the Petri plates. The media in the plates for BRIC had to be 5 mm deep or less to

Table 1. Sample Size Required to Detect a 2-Fold Change in Arrays

Power	*Estimated sample size per group*
95%	6
90%	5
85%	4
80%	4
75%	3
70%	3

Table 2. The Effect of Sample Size on the Power to Detect a 2-Fold Change

Sample size per group	*Estimated power*
3	0.773
4	0.887
5	0.947
6	0.977
7	0.990
8	0.996

accommodate the biology and the PDFU cover hardware. For a 60 mm dish, this calculates out to 6.7 mL. After pouring, the plates were allowed to solidify and were then transferred to a sterile BTC (to maintain sterility of external surface) until planting.

2.3.2. Preparation of seeded plates. Arabidopsis seed was surface sterilized (after Paul *et al.,* 2001) in a laminar flow hood. Seeds were planted on the surface of the Petri plates at a density of about 75 seeds per plate. The cover was replaced, but not taped, and the Petri plates were transferred to a sterile BTC. The BTC was wrapped in black cloth and stored for four days at 4°C until turnover, thereby ensuring that the seeds remained dormant prior to launch. Further, plates were inspected before loading into the PDFU to verify all seeds remained dormant.

2.3.3. Preparation of cell culture plates. Murashige Skoog (MS) culture media was dispensed onto 60 mm Petri plates as described above. The liquid media from a sterile cell suspension was decanted, and the cells were washed once with fresh liquid media and then decanted again. A sterile scoop was used to place about 1 g of cells on the surface on the plate and disperse it evenly across the surface. The cover was replaced, but not taped; the Petri plates were transferred to a sterile BTC. The BTC was wrapped in black cloth and stored at room temperature for 6 days until turnover. This Arabidopsis callus line (which is far more slow-growing than a liquid suspension culture) is typically subcultured once a month; thus preparation along this time line provided in-flight research material in the middle of the typical growth cycle.

2.4. RNA extractions

Total RNA was extracted with Qiashredder and RNeasy kits from Qiagen according to the manufacturer's instructions. Residual DNA was removed by performing an on-column digestion with use of an RNase Free DNase (Qiagen). Integrity of the RNA was evaluated with the Agilent 2100 BioAnalyzer (Ferl *et al.*, 2011).

2.5. Quantitative RT-PCR

For quantitative reverse transcription–polymerase chain reaction (RT-qPCR), 150 ng of RNA were reverse transcribed into cDNA by using High Capacity RNA to cDNA Master Mix (Applied Biosystems). One-eighth or one-sixth of total cDNA for seedlings or tissue, respectively, was used as a template for a single RT-qPCR run. RT-qPCR was carried out with TaqMan technology (Applied Biosystems) on the ABI 7500 Fast instrument (Applied Biosystems) (Bustin, 2000). The TaqMan Fast Advanced Master Mix reagent was used for the duplex RT-qPCR reaction with 6FAM and VIC-dye labeled, TAMRA-quenched probes. In all reactions, the UBQ11 (At4g05050) served as an internal control. Each duplex PCR mixture contained 900 n*M* target gene–specific forward and reverse primers each, 150 n*M* UBQ11 forward and reverse primers each, 250 n*M* 6FAM labeled target gene–specific probe and 250 n*M* VIC-labeled UBQ11 probe. Primers and probes were designed with Primer Express software and supplied by Applied Biosystems. The complete list of RT-qPCR probes and primer sets is shown in Table 3.

The thermal cycling program consisted of 20 s at 95°C, followed by 40 cycles of 3 s at 95°C, and 30 s at 59°C. Reactions were quantified by selecting the amplification cycle when the PCR product was first detected (threshold cycle, Ct). Three randomly selected primer and probe sets were chosen for validation experiments to test the efficiency of the target and reference amplifications. The Ct values for six biological replicas of each experimental group (seedlings Flight, seedlings Ground Control, tissue Spaceflight, tissue Ground Control) were analyzed with 7500 Software v2.0.5 along with Microsoft Excel and the comparative CT(ΔΔCT) method. The ΔCt was calculated as the difference between the threshold cycle value of a target gene and that of UBQ11 (endogenous control) in the same sample, while ΔΔCt as the difference between the ΔCt value of a Spaceflight sample and that of the Ground Control (calibrator). The fold difference of the target gene expression in Spaceflight samples relative to Ground Control samples (calibrator) was calculated as $2^{(-\Delta\Delta Ct)}$ and then log2-transformed.

2.6. Microarray preparation and analyses

Extracted total RNA (60 ng) was amplified with the Ovation Pico WTA System (NuGEN Technologies Inc.) in accordance with manufacturer's protocol. Fragmentation and

Table 3. Quantitative PCR Primer and Probe Sets

Gene	*qPCR Primer/Probe sets*
MLP423	**F** 5′ AACCATCACCGTTATCCCTAAGG 3′
At1g24020	**R** 5′ GGAAGCCTCGTACTGAGAGTTCA 3′
	P 5′ 6FAMCCTCGGTGTTGCTCCGGC TAMRA 3′
MAPKKK13	**F** 5′ GCCAAAGCTCCATGGTCAAG 3′
At1g07150	**R** 5′ GCCCTCGGTGTGTGGATTC 3′
	P 5′ 6FAMTAGCCGATTTTGGGTCGGCGT TCTAMRA 3′
AGP12	**F** 5′ TCTCCGCCGTAGGAAACGT 3′
At3g13520	**R** 5′ AGCATCGGAAGTAGGACTTGGA 3′
	P 5′ 6FAMCTGCGCAGACAGAGGCTCCG GTAMRA 3′
HSP17.6A	**F** 5′ CAAAGGCAATGGCTGCTACA 3′
At5g12030	**R** 5′ TGTCCACGGCGAAAACGTA 3′
	P 5′ 6FAMCTGACGTTATCGAGCACCCGG ATAMRA 3′
UBQ11	**F** 5′ AACTTGAGGACGGCAGAACTTT 3′
At4g05050	**R** 5′ GTGATGGTCTTTCCGGTCAAA 3′
	P 5′ 6VICCAGAAGGAGTCTACGCTTCAT TTGGTCTTGCTAMRA 3′

The gene symbol and Atg number are shown in the left-hand column, and the sequences for the forward (F), reverse (R) and primer (P) are shown in the right-hand column.

biotinylation occurred with the Encore Biotin Module (NuGEN Technologies, Inc.) per the manufacturer's protocol. Amplified and labeled cDNA (5 μg/sample) was fragmented and hybridized with rotation onto Affymetrix GeneChip Arabidopsis ATH1 Genome Arrays for 16 h at 45°C. Arrays were washed on a Fluidics Station 450 (Affymetrix) with the Hybridization Wash and Stain Kit (Affymetrix) and the Washing Procedure FS450_0004. Fluorescent signals were measured with an Affymetrix GeneChip Scanner 3000 7G.

2.7. SAGE preparation and analyses

SAGE SOLiD barcoded libraries were constructed at Life Technologies facilities located in Carlsbad, California, with assistance of University of Florida, ICBR Gene Expression Core personnel. All SAGE SOLiD barcoded libraries are prepared by using SOLiD SAGE with Barcode Module (catalog#: 4452811) according to manufacturer's protocols. Briefly, 1 μg of total RNA was bound to Dynabeads to capture the polyA-containing RNA. Double-stranded cDNA was synthesized on the Dynabeads by using SuperScript III Reverse Transcriptase and *E. coli* DNA polymerase. The bound double-stranded cDNA was cleaved with Nla III to generate DNA fragments that had an average length of approximately 250 bp fragments. SOLiD SAGE barcode Adaptor A was ligated to the Dynabead-bound cDNA fragments, and the Adaptor A–linked cDNA was digested with EcoP15I, which binds to Adaptor A adjoining to the NlaIII CATG cleavage recognition site and cleaves the cDNA ~27 bp downstream from the adaptor. As a result, a tag with a 2 bp overhang containing the Adaptor A sequence and 27 bp of unique sequence from a single transcript was released. After the ethanol precipitation, SOLiD SAGE Adaptor B was ligated to the 5′ end of each tag; then each library was amplified with a different barcoded SOLiD 3′ primer from the SOLiD RNA Barcoding Kit (Cat#: 4427046) and a SOLiD 5′ primer, primer, producing a clean 130 bp product. Finally, equal molar amounts of the barcoded libraries were pooled together and purified for SOLiD sequencing. Amplified libraries were ligated to beads by using the reagents and protocol following Applied Biosystems SOLiD 4 Templated Beads Preparation Guide (Cat #: 4448378). High-throughput sequencing of the 35 bp was carried out on SOLiD Version 4 sequencer. Sequencing was conducted on one slide of a SOLiD 4 Analyzer (Life Technologies at Carlsbad, CA), and tags were mapped to the Arabidopsis reference.

3. Results

3.1. Experiment design

To determine the experimental setup, power analysis was performed based on a completely randomized treatment-control experimental design. In the design summarized in Tables 1 and 2, two groups of biological samples were considered: a treatment group (Spaceflight) and a control group (Ground Control). Type I error (two-tailed) was held at 0.05. The standard deviation for the analysis summarized in both Tables 1 and 2 was estimated by using our previous Arabidopsis expression dataset with similar design (Paul *et al.*, 2005). Given the programmatic and physical constraints of the BRIC payload distributions, balance between experiment design and payload opportunity was key to deciding the distribution of sample type and size. With a sample size of four for each group of samples, the design was expected to have a power near 89% to detect 2-fold difference between the control (Ground Control) and treatment (Spaceflight) groups (Table 1). With a sample size of six, the power rises to almost 98% (0.977, Table 2). Based on our previous spaceflight data and Arabidopsis seedling array data from KC-135 parabolic flights (Paul *et al.*, 2011), we sought to maximize the opportunity to confidently detect gene expression differences at the 2-fold sensitivity such that the array analyses should reveal significant changes in gene expression caused by spaceflight. Therefore, the experiment was designed to have 6-fold replication of biological samples in the Spaceflight and Ground Control hardware to facilitate confidence in changes in gene expression at the 2-fold level. Thus, the availability of 13 PDFUs within the payload allowed for two sample types: seedlings and cultured cells.

3.2. Experiment hardware

The hardware for this experiment was the NASA BRIC-LED (Kern *et al.*, 1999; NASA.gov, 2011). The BRIC-LED was flown as a nonpowered middeck payload by program design, so the LED component of the hardware was not available to the researchers; all biology was held in the dark for the duration of the experiment. The BRIC-LED canisters hold six 60 mm diameter Petri plates in small subcompartments called PDFUs. The PDFUs allow an externally activated fixation step without manipulating the samples. Figure 1A shows an open BRIC container with a view of the slots for five PDFUs, with one PDFU extracted for comparison. The six replicate plates of seedlings and seven replicate plates of cultured cells (13 plates total) were distributed among three BRIC canisters: BRIC A (all seedlings), BRIC B (all cultured cells), and BRIC G (two PDFUs of seedlings, three of cultured cells). One of the PDFU slots in each BRIC was used to hold a HOBO data logger to record temperature. Figure 1B shows an open PDFU containing a plate of cultured Arabidopsis cells. The BRICs were contained within a foam-lined tray (Fig. 1C) for launch and stowage in a Shuttle middeck locker for the flight.

An additional set of Petri plates within BRIC hardware, identical to that launched for Spaceflight, was prepared for the Ground Control. The Ground Control was housed in the OES chamber in the Spaceflight Life Sciences Laboratory (SLSL) at KSC. The Ground Control was initiated with a precise 24 h delay to enable the OES environment to be programed with the environmental profile taken from telemetry of the Space Shuttle. Thus, 24 h later, the OES assumed an identical environmental profile as the Shuttle cabin that contained the BRIC hardware and biology on orbit, and the Ground Control BRIC hardware and samples experienced the same external environmental profile as the hardware and samples on the Space Shuttle. Environmental parameters that were controlled on the OES and made identical to flight were cabin temperature, humidity, and CO_2 concentration. Environmental telemetry from within the interior of the BRIC units could not be collected and transmitted in real time; however, the inclusion of HOBO data loggers enabled the collection of those data for postflight reference (details below).

3.3. Flight profile and environmental conditions

The BRIC-16 experiment was turned over to payload engineers in the SLSL at KSC 24 h before the scheduled launch time. The payload launched on STS-131 on April 5, 2010, at 06:25, and achieved orbit approximately 8 min later. The biology was in orbit 12 days and 21 h (04/05/10 06:25:28 to 04/18/10 03:37:28) before being fixed on orbit with RNAlater (Ambion, Austin, TX). The orbiter landed on April 20 (04/20/10 07:37:28), and the biology was returned to the principal investigators at SLSL approximately 7 h later. Launch, Fixation, and Landing points are marked on the thermal profiles shown in Fig. 2, in terms of hours after turnover.

The temperature profiles collected by each BRIC data logger are also shown in Fig. 2. Figure 2 presents each BRIC unit individually, with the Spaceflight and Ground Control temperatures for each BRIC displayed on the same graph as solid and dotted lines, respectively. The HOBO temperature data collection began immediately after the biology was sealed in the BRICs, 24 h before launch. After a 30 h period of adjustment from a laboratory loading temperature of 21°C, all BRICs (A, B, G, Spaceflight, and Ground Control) maintained a temperature between 21.7°C and 24.8°C until they were fixed on orbit on day 12 (Fig. 2).

3.4. Sample recovery and processing

Figure 3 shows representative pictures of the plant materials removed from their respective PDFUs after flight. In each set of images, there is a picture that shows the entire Petri plate and a closer view to its right. Spaceflight samples are shown in the top row and Ground Controls on the bottom. Other than the Ground Control samples showing growth oriented along the gravity vector, there are no visible gross differences in morphology between the Spaceflight and Ground Control treatments.

3.5. Seedlings and cell cultures have unique and different responses

Array analysis of RNAs from seedlings and cultured cells fixed in RNALater on orbit relative to Ground Controls revealed a range of genes differentially expressed in Spaceflight. At $p<0.01$, there were approximately 300 genes identified as over- or under-expressed in Spaceflight in either seedlings or cell cultures (see Fig. 4).

Figure 4 shows the distribution of all genes represented in the microarrays with respect to p value and fold change with respect to Ground Controls. Figure 4 (left panel) displays the gene expression data for etiolated seedlings, and Fig. 4 (right panel) shows the data for cultured cells. Genes displaying statistically significant expression are indicated in red and are found above the horizontal red line that shows the $p<0.01$ demarcation. The position on the y axis indicates $-$log10 of p value, and position on the x axis indicates fold change ($+$ or $-$) in terms of log2.

Table 4 shows all genes that were differentially expressed by more than 5-fold in seedlings and provides the average fold change for the six replicate samples. Nearly half of the genes upregulated by 5-fold or more in seedlings are associated with pathogen response and wounding. The remaining genes are quite typical of generalized stress-response genes, including those associated with drought and cold stress. Several genes of specific, well-defined developmental function are represented as well, such as ALF4, which plays a role in auxin-mediated lateral root development (Dubrovsky *et al.*, 2008), and SHM1, an essential regulator of

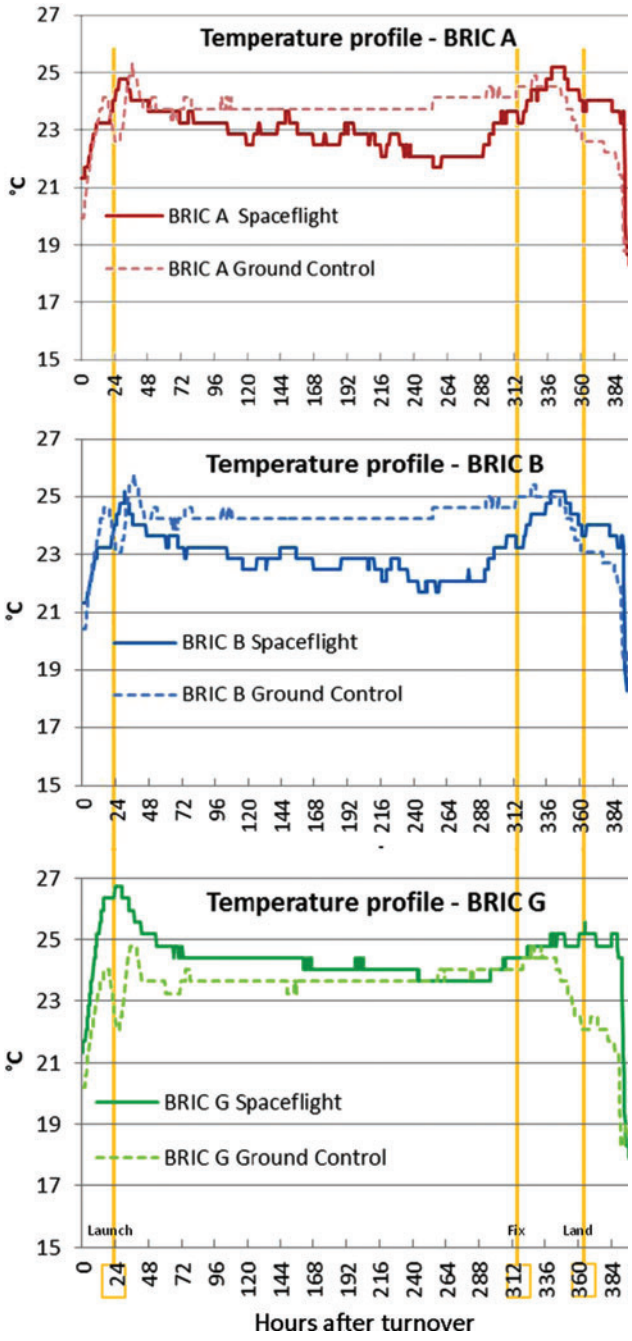

FIG. 2. Spaceflight and Ground Control temperature profiles. The panels provide graphs of the temperature profiles of each BRIC as recorded by the HOBO data loggers. Ground Controls are displayed as dotted lines and Spaceflight as solid lines. The individual BRICs are color-coded: BRIC A, red; BRIC B, blue; BRIC G, green. The y axis shows temperature in degrees Celsius, and the x axis shows time points as hours after turnover. The time points of Launch, Fixation, and Landing are indicated with yellow vertical lines. Color images available online at www.liebertonline.com/ast

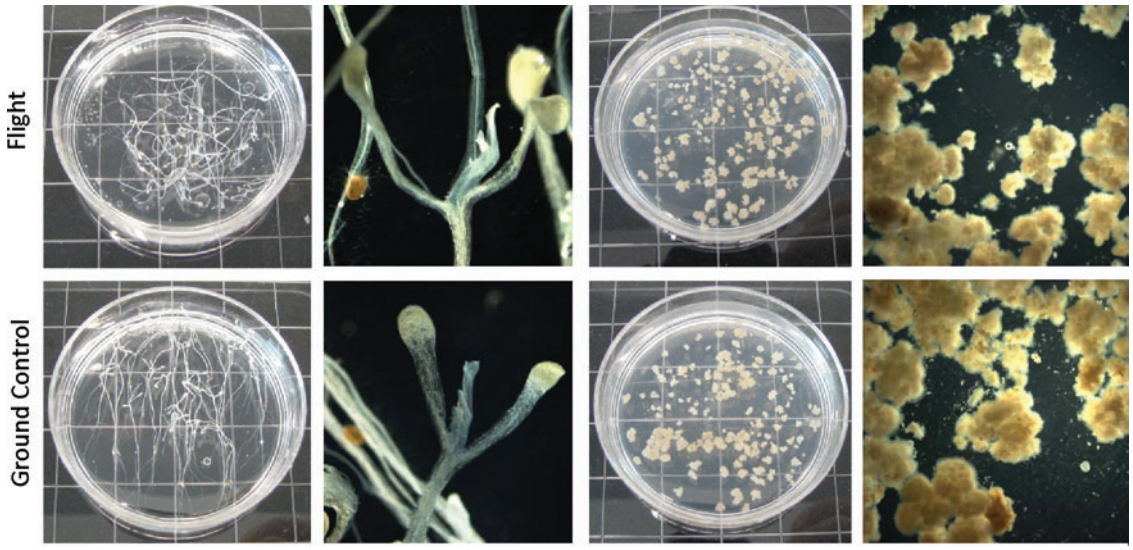

FIG. 3. Spaceflight and Ground Control biology after landing. Representative pictures of the plant materials removed from their respective PDFUs. The top row shows plant material from the Spaceflight and the bottom row from the Ground Controls. In each set of images, there is a picture showing the entire Petri plate and a closer view to its right. The grids subtending the entire Petri plate measure 13 mm square. Color images available online at www.liebertonline.com/ast

photorespiration (McClung *et al.*, 2000). The group of genes downregulated by 5-fold or more appears more mixed in functional categorization. Genes encoding transcription factors appear to be more highly represented in the group of downregulated genes, as are genes associated with cell wall metabolism and cell elongation. Also present are examples of genes encoding proteins that function in calcium-mediated signaling and play a role in gravitropism.

Table 5 shows all the genes that were differentially expressed by more than 5-fold in cultured cells in response to spaceflight and provides the average fold change for the six replicate samples. In cultured cells, the largest category, which comprises nearly half the genes differentially expressed by 5-fold or more, is that of genes associated with a heat shock response. This category expands in representation as higher fold-induction, with 75% of the genes showing greater than 7-fold induction being heat shock–related. All six genes that were induced greater than 10-fold are heat shock–related. The remaining genes induced at 5-fold or greater represent a wide diversity of function and categories, including recognizable stress responses, such as those involved with salt, drought, metals, wounding, phosphate, and ethylene. There are also some single representatives in metabolisms associated with senescence, terpenoids, seed

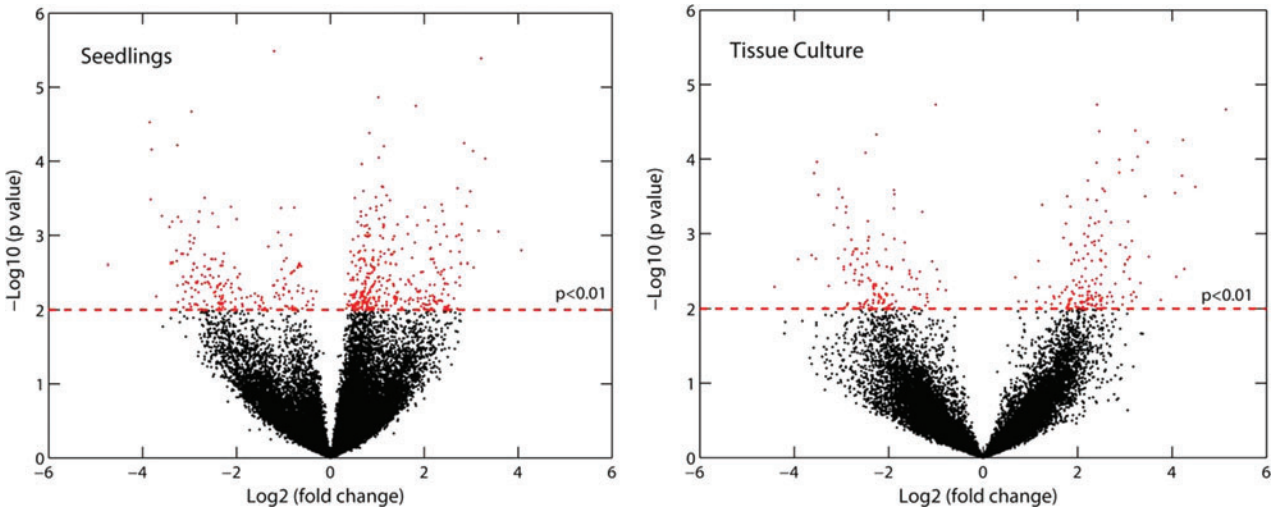

FIG. 4. Volcano plots of microarray data. Each graph shows the distribution of all genes represented in the microarrays with respect to p value and fold change with respect to Ground Controls; seedling data are shown on the left, and cultured cell data are shown on the right. Genes displaying statistically significant expression are indicated in red and are found above the horizontal red line showing the $p < 0.01$ demarcation. The position on the y axis indicates $-\log10$ of p value, and position on the x axis indicates fold change (+or−) in terms of log2. Color images available online at www.liebertonline.com/ast

Table 4. Seedlings, $p < 0.01$, 5× Differential Expression

FC	*Atg number*	*Gene*	*Associated metabolism/function*
16.80	At3g08770	LTP6; lipid binding	drought stress response
9.85	At1g29860	WRKY71; transcription factor	pathogen response
8.28	At5g08280	HEMC; hydroxymethylbilane synthase	pathogen response
6.96	At1g12880	atnudt12; hydrolase	pathogen and oxidative stress
6.96	At3g55740	PROT2 ; L-proline transmembrane transporter	cold and drought stress
6.87	At5g13630	GUN5; magnesium chelatase	ABA signaling
6.77	At1g27460	NPGR1; calmodulin binding	calcium-mediated signaling
6.50	At2g42800	AtRLP29; protein binding	pathogen response
6.36	At1g67840	CSK; ATP binding/kinase	photosynthesis
6.19	At3g18690	MKS1, MAP kinase substrate 1; protein binding	pathogen response
5.94	At1g04110	SDD1; serine-type endopeptidase	drought stress response
5.74	At4g16265	NRPB9B; DNA binding/transcription regulator	transcription factor
5.70	At1g26560	BGLU40, BETA GLUCOSIDASE 40	pathogen response
5.66	At2g28950	ATEXPA6, ARABIDOPSIS THALIANA EXPANSIN A6	pathogen response
5.58	At1g72670	iqd8; calmodulin binding	calcium-mediated signaling
5.43	At5g11030	ALF4, ABERRANT LATERAL ROOT FORMATION 4	root development
5.35	At1g55870	AHG2, ABA-HYPERSENSITIVE GERMINATION 2	ABA signaling
5.35	At5g54570	BGLU41, BETA GLUCOSIDASE 41	pathogen response
5.31	At4g13770	CYP83A1, CYTOCHROME P450 83A1; oxidoreductase	pathogen response
5.24	At5g23320	ATSTE14A; carboxyl-O-methyltransferase	ABA signaling
5.21	At1g26820	RNS3, RIBONUCLEASE 3	RNA catabolism
5.21	At4g37930	SHM1, SERINE TRANSHYDROXYMETHYLTRANSFERASE 1	photorespiration
5.17	At2g28355	LCR5, Low-molecular-weight cysteine-rich 5	light and carbon metabolism
5.17	At1g04710	PKT4; acetyl-CoA C-acyltransferase	pathogen response
5.10	At2g38960	AERO2; FAD binding	ER oxidoreductin
0.20	At3g13784	AtcwINV5, *Arabidopsis thaliana* cell wall invertase 5	drought stress response
0.20	At3g09530	ATEXO70H3, exocyst subunit EXO70 family protein	transport
0.20	At4g21440	ATMYB102; transcription factor	transcription factor
0.20	At3g09790	UBQ8; protein binding	proteosome
0.20	At2g30770	CYP71A13, cytochrome P450	pathogen response
0.19	At3g62740	BGLU7, BETA GLUCOSIDASE 7	seed maturation
0.19	At5g64530	XND1; Xylem NAC domain transcription factor	transcription factor/senescence
0.19	At5g43610	ATSUC6, Sucrose-proton symporter 6	phloem loading
0.17	At3g26120	TEL1, TERMINAL EAR1-LIKE 1	development
0.14	At3g21500	1-deoxy-D-xylulose-5-phosphate synthase	phosphate stress
0.13	At3g50980	XERO1, DEHYDRIN XERO 1	drought and cold
0.13	At1g73280	SCPL3; serine-type carboxypeptidase	development/pathogen response
0.13	At1g34650	HDG10, HOMEODOMAIN GLABROUS 10	transcription factor/development
0.12	At5g61980	AGD1, ARF-GAP domain 1; ARF GTPase activator	root orientation
0.11	At1g69930	ATGSTU11; glutathione transferase	pathogen response
0.11	At2g30790	PSBP-2, photosystem II subunit P-2; Ca^{2+} binding	photosynthesis
0.11	At1g17870	S2P-like putative metalloprotease	stress response
0.10	At4g21200	GA2OX8; gibberellin 2-beta-dioxygenase	gibberellin, lignification
0.10	At2g34980	SETH1; phosphatidylinositol N-acetylglucosaminyltransferase	pollen tube growth
0.09	At3g58160	XI-J myosin	pollen tube growth

Differentially expressed genes in seedlings. The table shows all genes that were differentially expressed by more than 5-fold in seedlings. The average fold change for the six replicate samples is provided in the left-hand column, the name of the gene in the middle column, and the pathway or metabolic with which that gene is associated is shown in the right-hand column.

development, cell walls, photosynthesis, and auxin. The list of the most highly downregulated genes shows more diversity than the upregulated genes and includes several representatives each of kinases, transcription factors, and genes encoding wounding and cell wall–associated proteins.

Examining the Gene Ontology categories for all Spaceflight upregulated genes with p values < 0.01 between seedlings and cell cultures provides a broader look at the differences between the seedling and cell culture data sets. Although distributions of differentially expressed genes are similar for most categories, there are four categories that display striking differences between the two sample types. Whereas in seedlings there are more than twice as many genes upregulated in the category of cell organization and biogenesis than in cell cultures, cultured cells display about two and a half times as many stress and abiotic stimulus response genes as seedlings. Cell cultures also show induction of almost three times as many genes in the category of transcription as seen in seedlings. In a comparison of all genes with p values of less than 0.01, and displaying a 2-fold differential expression or greater, only one gene (MT3—At3g15353) is common to both the seedling and cell culture data sets, and it is upregulated in seedlings while being downregulated in cell cultures (Table 6).

Table 5. Cultured Cell, $p < 0.01$, 5× Differential Expression

FC	Atg number	Gene	Associated metabolism/function
35.26	At2g26150	ATHSFA2; DNA binding/transcription factor	heat shock
22.47	At3g12580	HSP70 (heat shock protein 70); ATP binding	heat shock
19.16	At1g74310	ATHSP101 (ARABIDOPSIS THALIANA HEAT SHOCK PROTEIN 101); ATP binding	heat shock
18.77	At5g52640	ATHSP90.1 (HEAT SHOCK PROTEIN 90.1); ATP binding	heat shock
18.51	At1g16030	Hsp70b (heat shock protein 70B); ATP binding	heat shock
16.68	At3g46230	ATHSP17.4	heat shock
10.06	At5g46350	WRKY8; transcription factor	wounding
9.65	At4g00950	MEE47 (maternal effect embryo arrest 47); transcription factor	seed development
9.45	At3g51910	AT-HSFA7A; DNA binding/transcription factor	heat shock
9.32	At4g23980	ARF9 (AUXIN RESPONSE FACTOR 9); transcription factor	auxin
8.88	At5g48570	peptidyl-prolyl *cis-trans* isomerase, putative/FK506-binding protein	Hsp90 co-chaperone
8.57	At5g12030	AT-HSP17.6A (HEAT SHOCK PROTEIN 17.6A); unfolded protein binding	heat shock
8.57	At3g25230	ROF1 (ROTAMASE FKBP 1); FK506 binding/calmodulin binding	heat shock
7.78	At5g47220	ERF2 (ETHYLENE RESPONSIVE ELEMENT BINDING FACTOR 2); transcription	ethylene and pathogen
7.73	At2g46240	BAG6 (BCL-2-ASSOCIATED ATHANOGENE 6); calmodulin binding	heat shock
7.36	At4g21810	DER2.1 (DERLIN-2.1)	heat shock
6.59	At4g02500	XT2 (UDP-XYLOSYLTRANSFERASE 2); UDP-xylosyltransferase	cell wall
6.06	At5g10030	TGA4 (TGACG MOTIF-BINDING FACTOR 4); calmodulin binding/transcription	calcium mediated
5.70	At5g61410	RPE; catalytic/ribulose-phosphate 3-epimerase	photosynthesis?
5.66	At5g23260	TT16 (TRANSPARENT TESTA16); transcription factor	seed development
5.58	At5g48010	THAS1 (THALIANOL SYNTHASE 1); catalytic/thalianol synthase	terpinoid pathway
5.54	At2g29460	ATGSTU4; glutathione transferase	stress response
5.31	At4g25460	ATNAP8; ATPase, coupled to transmembrane movement of substances	plant senescence
5.28	At3g16050	A37; protein heterodimerization	stress response
5.28	At2g39450	MTP11; cation transmembrane transporter/manganese ion	stress response
5.21	At2g33770	PHO2 (PHOSPHATE 2); ubiquitin-protein ligase	stress response
5.17	At5g37420	AGL105; transcription factor	MADS box
5.13	At4g16320	LDL3 (LSD1-LIKE3); amine oxidase/electron carrier/ oxidoreductase	histone demethylase paralog
5.13	At1g70290	ATTPS8; alpha,alpha-trehalose-phosphate synthase (UDP-forming)	wounding
5.03	At5g05410	DREB2A; DNA binding/transcription activator/transcription factor	drought cold
0.20	At3g05710	SYP43 (SYNTAXIN OF PLANTS 43); SNAP receptor	pathogen response
0.20	At1g05760	RTM1 (restricted tev movement 1); sugar binding	pathogen response
0.20	At3g20290	ATEHD1 (EPS15 HOMOLOGY DOMAIN 1); GTPase/calcium ion binding	calcium mediated
0.19	At2g31960	ATGSL03 (GLUCAN SYNTHASE-LIKE 3); 1,3-beta-glucan synthase/transferase	glucan synthase—wounding
0.19	At1g26830	ATCUL3 (CULLIN 3); protein binding/ubiquitin-protein ligase	embryogenesis
0.19	At1g74660	MIF1 (MINI ZINC FINGER 1); DNA binding/transcription factor	development
0.19	At3g28890	AtRLP43 (Receptor Like Protein 43); kinase/protein binding	kinase
0.18	At4g03550	ATGSL05 (GLUCAN SYNTHASE-LIKE 5); 1,3-beta-glucan synthase/transferase	glucan synthase—wounding
0.18	At5g22570	WRKY38; transcription factor	pathogen response
0.18	At1g53570	MAP3KA; ATP binding/kinase/protein serine/threonine kinase	kinase
0.18	At2g39660	BIK1 (BOTRYTIS-INDUCED KINASE1); kinase	pathogen response
0.18	At3g15353	MT3 (METALLOTHIONEIN 3); copper ion binding	stress response
0.18	At2g32920	ATPDIL2-3 (PDI-LIKE 2-3); protein disulfide isomerase	stress response
0.17	At2g23030	SNRK2.9 (SNF1-RELATED PROTEIN KINASE 2.9); serine/ threonine kinase	kinase
0.17	At4g16890	SNC1 (SUPPRESSOR OF NPR1-1, CONSTITUTIVE 1); nucleotide binding	pathogen response
0.16	At5g59320	LTP3 (LIPID TRANSFER PROTEIN 3); lipid binding	cell wall

(*continued*)

Table 5. (Continued)

FC	Atg number	Gene	Associated metabolism/function
0.15	At3g15030	TCP4 (TCP family transcription factor 4); transcription factor	transcription factor
0.15	At3g57860	UVI4-LIKE (UV-B-INSENSITIVE 4-LIKE)	meiosis and UV stress
0.15	At3g10640	VPS60.1—associated with endosomal sorting for transport (ESCRT)	transport
0.15	At3g46590	TRFL1 (TRF-LIKE 1); DNA bending/DNA binding/telomeric DNA binding	telomere homeostasis
0.15	At3g09880	ATB' BETA; protein phosphatase 2A beta	stress response
0.14	At2g36880	MAT3 (methionine adenosyltransferase 3); copper ion binding	stress response
0.14	At3g23010	AtRLP36 (Receptor Like Protein 36); protein binding	pathogen response (RLPs)
0.12	At1g47230	cyclin, putative	cell division, cell cycle
0.12	At5g40890	ATCLC-A (CHLORIDE CHANNEL A); anion channel/nitrate transmembrane	stress response
0.10	At5g56650	ILL1; IAA-amino acid conjugate hydrolase/metallopeptidase	auxin and seed development
0.05	At4g31120	SKB1 (SHK1 BINDING PROTEIN 1); protein methyltransferase	vernalization

Differentially expressed genes in cultured cells. The table shows all genes that were differentially expressed by more than 5-fold in seedlings. The average fold change for the six replicate samples is provided in the left-hand column, the name of the gene in the middle column, and the pathway or metabolic with which that gene is associated is shown in the right-hand column.

The differentially expressed genes of cultured cells are generally genes of higher intrinsic expression level than the differentially expressed genes of seedlings. Figure 5 illustrates a measure of the quantitative difference in the responses observed for seedlings and cultured cells in a scatter plot of all genes with p values < 0.01 between Spaceflight and Ground Control. Each point reflects the differential expression between Spaceflight and Ground Control; the farther above or below the central axis, the greater the differential expression. The relative x, y coordinate position indicates transcript abundance; the farther from (1, 1), the higher the absolute expression level of the transcript. The expression levels of spaceflight-affected genes in seedlings are indicated with blue squares, and those from cell cultures with red circles. The differentially expressed seedling genes are generally of lower absolute expression level, with the highest

Table 6. Identical Gene Sets in Seedlings and Cell Cultures

ProbID	Atg number	Gene	Fold change	Fold change
Genes show significant ($p < 0.01$) *change in* ***seedlings*** *but not cell cultures*			*Seedlings*	*Cell cultures*
258675_at	At3g08770	LTP6	**16.80**	1.62†
255999_at	At1g29860	WRKY71	**9.85**	0.67†
246033_at	At5g08280	HEMC	**8.28**	0.85†
261212_at	At1g12880	atnudt12	**6.96**	0.81†
257751_at	At3g18690	MKS1	**6.19**	0.50†
252137_at	At3g50980	XERO1	**0.13**	1.74†
267569_at	At2g30790	PSBP-2	**0.11**	0.68†
254459_at	At4g21200	GA2OX8	**0.10**	1.27†
267416_at	At2g34980	SETH1	**0.10**	0.39†
251564_at	At3g58160	XI-J	**0.09**	0.51†
Genes show significant ($p < 0.01$) *change in* ***cell cultures*** *but not seedlings*			*Seedlings*	*Cell cultures*
266841_at	At2g26150	HSFA2	0.37†	**35.26**
256245_at	At3g12580	HSP70	0.91†	**22.47**
254194_at	At4g23980	ARF9	1.42†	**9.32**
250351_at	At5g12030	HSP17.6A	0.30†	**8.57**
257822_at	At3g25230	ROF1	1.13†	**8.57**
257054_at	At3g15353	MT3	2.57	**0.18**
267254_at	At2g23030	SNRK2.9	0.83†	**0.17**
263838_at	At2g36880	MAT3	2.13†	**0.13**
260505_at	At1g47230	cyclin	1.40†	**0.12**
249327_at	At5g40890	ATCLC-A	1.00†	**0.12**

A comparison between identical gene sets in seedlings and cell cultures. The top panel shows a selection of genes that are statistically significant ($p < 0.01$) and were differentially expressed (fold change) by greater than 5-fold in seedlings. The column to the right shows the fold change values of the same gene identified in arrays from cell cultures; none of these corresponding genes show statistically significant changes in gene expression (indicated by a dagger superscript). The bottom panel shows the same comparison conducted with genes that showed significant ($p < 0.01$) changes in cell cultures but not seedlings.

†Not significant—$p < 0.01$.

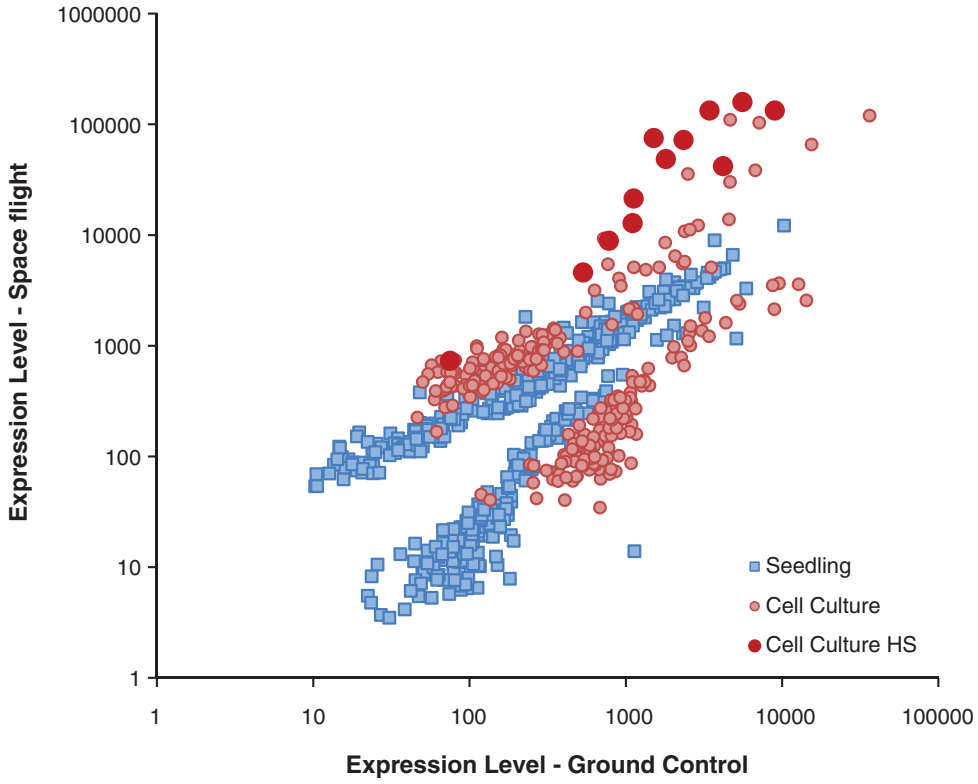

FIG. 5. Expression levels of differentially expressed genes in seedlings and cultured cells. The scatter plot illustrates the quantitative differences in the responses observed for seedlings and cultured cells for all genes with p values <0.01 between Spaceflight and Ground Control. The expression levels of spaceflight-affected genes in seedlings are indicated with blue squares and those from cell cultures with red circles. A number of the highly expressed heat shock–associated genes in cultured cells are highlighted with bold red circles. Color images available online at www.liebertonline.com/ast

fold changes occurring in the genes with the lowest expression levels. The differentially expressed genes in cell cultures are, however, generally of moderate to high expression levels, with the heat shock–associated genes showing the highest expression levels in addition to the highest fold-change values (large red circles).

3.6. Quantitative and SAGE analyses confirm differential expression

Both quantitative real-time PCR (qRT-PCR) and Serial Analysis of Gene Expression (SAGE) were employed to confirm expression changes in selected genes. Representative up- and downregulated genes from each comparison were chosen for quantitative PCR (ABI—Taqman) analyses: MLP423 (up in seedlings), MAPKKK13 (down in seedlings), HSP17.6A (up in tissue culture), and AGP12 (down in tissue culture). The qRT-PCR was performed independently on each of the six biological replicates for each sample. For SAGE analysis, the six replicate RNAs from each experiment set were normalized and pooled before processing. The SAGE analysis therefore yielded a single, averaged expression value for each gene, while the qRT-PCR analyses produced six independent values for each gene and treatment that were then averaged.

Figure 6 shows relative transcript abundance of the four above representative genes from cell culture and seedling Spaceflight and Ground Control samples' qRT-PCR and SAGE data, compared to data derived from the microarrays. The y axis represents the fold change of gene expression in Spaceflight samples relative to Ground Control samples calculated as a log2 function. The qRT-PCR error bars represent the standard error of the mean of the log2 fold change. The microarray data do not show error bars, as all presented genes exhibited p values <0.01. As stated, the SAGE data are obtained from pooled samples and therefore do not support error bar calculation. The complementary methods of SAGE and qRT-PCR confirmed the trends in differential expression on the microarrays for these representative genes.

SAGE data were used to expand the confirmation of Spaceflight activation of a variety of heat shock genes in cultured cells. Figure 7 presents fold-change values derived from SAGE (purple bars) compared to fold-change values derived from the microarrays (green bars). These heat shock–associated genes are also highlighted as bold red circles in the scatter plot of Fig. 5. For all the heat shock–related genes examined, SAGE data support the microarray data and confirm the activation of heat shock genes in the Spaceflight cultured cells.

4. Discussion

Arabidopsis seedlings and cell cultures clearly responded to the spaceflight environment with changes in gene expression. Microarray, SAGE, and quantitative PCR data fully support the conclusion that Arabidopsis, whether as

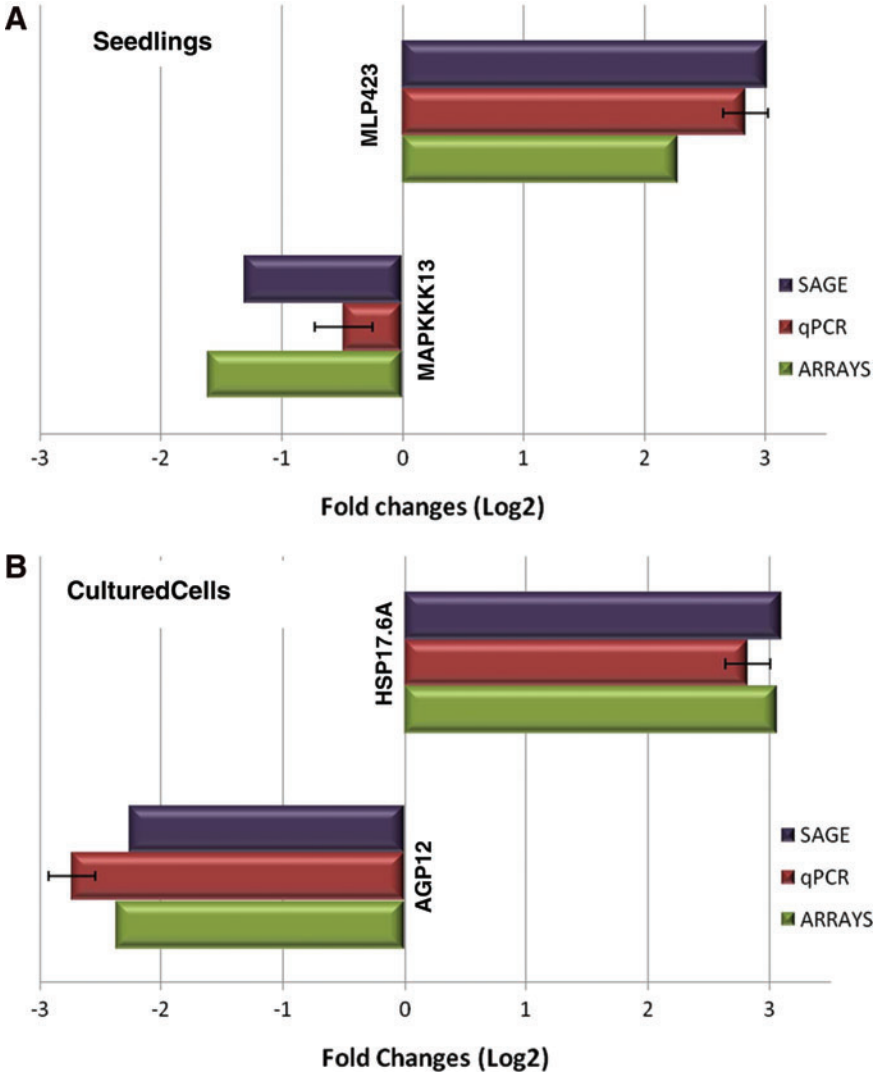

FIG. 6. Quantitative analyses. The transcript abundance for four representative genes is shown for seedlings (**A**) and cultured cells (**B**). Both panels show relative transcript abundance from seedling and cell culture Spaceflight and Ground Control samples' qRT-PCR (red) and SAGE (purple) data, compared to data derived from the microarrays (green). The *y* axis represents the fold change of gene expression in Spaceflight samples relative to Ground Control samples calculated as a log2 function. The qRT-PCR error bars represent the standard error of the mean of the log2 fold change. The microarray data are $p < 0.01$, and SAGE data are obtained from pooled samples and do not support error bar calculation. Color images available online at www.liebertonline.com/ast

etiolated seedlings or undifferentiated cells, senses that it is in a modified environment and mounts changes in gene expression as a response to spaceflight. The 6-fold replication of the experiment design allowed an examination of differences in gene expression down to 2-fold while retaining p values <0.01, which allowed a confident examination of fairly small quantitative changes in gene expression.

At a minimum, any genes affected by gravity or orientation on Earth might be expected to be changed in spaceflight. However, the range of genes affected by spaceflight appears to span beyond those genes whose expression might be easily explained by changes in gravity, and include a number of familiar genes with roles in the response to a wide variety of environmental factors. In addition, the results of these experiments reflect relatively long-term adaptive responses as the plants and cells were on orbit for 12 days before they were fixed. Thus, the patterns of gene expression reflect adaptive strategies of these two forms of the plant, rather than a rapid initial response to a new stress or environment. Time course analyses of any number of stress-response surveys repeatedly showed that initial responses are often diminished within minutes of the application of the stress, and patterns of gene expression change as longer-term adaptive strategies are engaged and maintained. Thus, these spaceflight-induced changes in gene expression likely reflect changes toward longer-term survival in the spaceflight environment. Future experiments should include the initial responses to spaceflight.

In etiolated seedlings, the genes most highly induced by spaceflight (by 5-fold or more) are almost equally divided between those associated with pathogen defense and those that are associated with environmental stress responses (Table 4). Differentially regulated genes reflect categories of genes that are broadly associated with drought stress, wounding, and calcium- and auxin-mediated signaling. The seedlings grew on sterile media, and none experienced drought or wounding stress. The temperature was well controlled, and any excursions from the Ground Control environmental profiles were minimal. The media compositions and atmospheric conditions were identical between Spaceflight and Ground Control. Therefore, a direct and obvious relationship between the affected genes and a defined stress is missing.

A possible basis for many of these responses may be traced to a translation of changes in forces associated with the cytoskeleton and cell wall. Pathogen and wounding

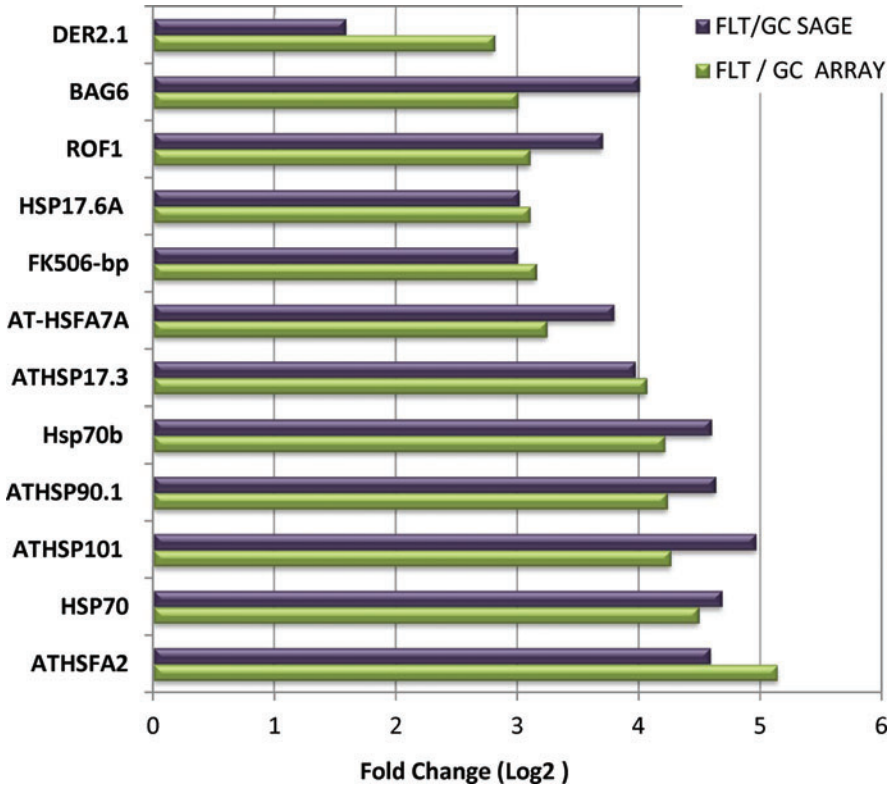

FIG. 7. Microarray versus SAGE values for differential expression. The fold-change values are shown for a selection of heat shock–associated genes from cultured cells. SAGE data (purple bars) are compared to fold-change values derived from the microarrays (green bars) in terms of log2 (*x* axis). Color images available online at www.liebertonline.com/ast

responses are very tightly connected, as both can involve a breach of the cell wall. In the Tensegrity-based model of the gravisensing, an actin-based cytoskeletal network throughout the cytoplasm is coupled to stretch-sensitive receptors in the plasma membrane (Yoder *et al.,* 2001). Cytoskeletal actin filaments attached to the plasma membrane, specialized proteins that contain an actin binding domain at their carboxyl terminus, and protein phosphorylation or phosphoinositide binding (Tsukita and Yonemura, 1997) have been postulated to participate in gravity-related signaling (Yoder *et al.,* 2001). It is possible that any of these signaling mechanisms by which a cell detects a breach are balanced by the force of gravity, and the absence of gravity may confound the signaling system, engaging pathogen signal transduction pathways as a consequence. The presence of other stress-response genes may have similar explanations; they appear inappropriate only because the foundations of the stimulus are unclear.

About 20% of the upregulated genes in seedlings do not fall obviously into either pathogen or environmental stresses. It is from among these examples that it may be possible to identify some "appropriate," or at least adaptively meaningful, responses to the spaceflight environment. Several candidates in this category include ALF4 (Aberrant Lateral Root Formation 4), ARR7 (Response Regulator 7), and PIN3 (Pin-Formed 3), all of which are either influenced by, or play a role in, auxin or cytokinin signaling and may be impacted by changes in the gravity vector (Lomax, 1997; Friml *et al.,* 2002; DiDonato *et al.,* 2004; Perrin *et al.,* 2005; Aloni *et al.,* 2006; Harrison and Masson, 2008).

The cells in culture responded in a dramatically different fashion to spaceflight than did the seedlings. While similar numbers of genes were differentially affected by spaceflight, there is almost no similarity among the identities of the genes affected. In addition, the cell cultures exhibited a greater number of highly (greater than 8-fold) differentially expressed genes (see Tables 4 and 5). In seedlings, most of the genes differentially expressed by 2-fold or more had low intrinsic signal values, while nearly all the differentially expressed genes in cultured cells had moderate to high signal values. About one-third of the differentially expressed cell culture genes had expression levels of greater than 2000, while only four of the differentially expressed genes from seedlings were as abundant (Fig. 5). These data suggest that cell cultures mount a more dramatic response to spaceflight and affect more genes with higher expression levels and with greater fold change.

None of the genes significantly impacted by spaceflight in cultured cells were similar to those in seedlings. Even the Gene Ontology categories of genes are substantially different. Cell cultures also exhibit less diversity in the types of genes that are highly induced. Pathogen and wounding, general stress response, and transcription factor categories are present, as in the seedling data sets, but the genes that populate those categories differ from those induced in seedlings. The cell culture samples illustrate repression of more kinases, and fewer proteases than seedlings, but transcription factors, transporters, and cell wall–associated gene categories are about equally affected—however, with different individual genes in each case.

By far the most dramatic molecular response to spaceflight in the cultured cells was a heat shock response, which was not manifested in seedlings. Genes encoding heat shock proteins, heat shock–related proteins, or heat shock transcription factors comprise the majority of the genes induced greater than 8-fold, and over 40% of those induced more than

5-fold. Clearly, no temperature differentials capable of classic heat shock were present between the Spaceflight samples and the Ground Control samples. Heat shock proteins are, however, generally related to responses to unfolded proteins and can be part of a variety of signal transduction pathways. It has been shown in both plants and animals that heat shock–related proteins are widely distributed in response to an assortment of abiotic stresses. In addition to their roles as molecular chaperones that manage post-translational processes, heat shock proteins are thought to participate in the cross talk among stress-response pathways, especially where an organism is exposed to multiple, concomitant stresses. Over-expression of heat shock proteins may contribute to a generalized tolerance for multiple environmental stresses (Swindell *et al.,* 2007). In a situation involving a novel environment such as spaceflight, it is possible that a plant cell responds as if it were being exposed to multiple stressors, and the induction of heat shock proteins reflects this perception.

There are few previous studies to provide ground for direct comparison with the current data, yet interesting correlations emerge. Three genome-scale plant molecular space studies have been published to date (Paul *et al.,* 2005; Stutte *et al.,* 2006; Salmi and Roux, 2008). One positive correlation is that evidence of spaceflight heat shock was seen in Arabidopsis (Paul *et al.,* 2005), albeit in light-grown seedlings quite distinct from those of the current study. Another positive, but less direct, correlation is the effect of spaceflight on the expression of genes involved in calcium signaling during fern spore development (Salmi and Roux, 2008). However, there were virtually no statistically supportable changes in the patterns of gene expression between wheat for comparison (Stutte *et al.,* 2006). Genome-wide studies of the impact of spaceflight on animal systems are slightly more common, particularly for cell lines from bone-derived cultures, mice, and drosophila models. While a full review of that literature is beyond the scope of this study, recent studies do demonstrate clear transcriptome differences during spaceflight, including studies of murine muscle (Allen *et al.,* 2009) and thymus tissue (Lebsack *et al.,* 2010). Drosophila and *Caenorhabditis elegans* show six differentially expressed genes common to these two diverse species (Leandro *et al.,* 2007; Adenle *et al.,* 2009). And while the divergence of plants and animals largely prevents specific gene comparisons in spaceflight responses, certain classes of genes seem similarly affected by spaceflight. Calcium-mediated signal transduction is impacted by spaceflight in both plants and animals (Hughes-Fulford *et al.,* 1998; Hashemi *et al.,* 1999; Paul *et al.,* 2001; Salmi and Roux, 2008), and genes affected by calcium signaling are differentially expressed. Heat shock proteins are highly conserved in both kingdoms, and heat shock genes are upregulated in some spaceflight studies. At least one of the subclass of heat shock genes highly induced in this study (Hsp90) is also induced in mouse thymus after exposure to spaceflight (Lebsack *et al.,* 2010). Stress also exacerbates sensitivity to pathogen attack in both plants and animals, and there are common mechanisms for triggering an immune response, such as nitric oxide and mitogen-activated protein kinase cascades (Nurnberger *et al.,* 2004). A number of genes associated with Drosophila immune pathways were downregulated in the spaceflight environment, and larvae were in fact more susceptible to pathogen infection (Marcu *et al.,* 2011). Although none of the Drosophila genes in that study were orthologous to the Arabidopsis genes presented here, many of genes in the present seedling data are nonetheless associated with pathogen defense. While many more studies will be required to support informed deductions regarding commonalities in spaceflight response (especially considering the dissimilarity of biology, hardware, flight profiles, and even replicates), current data suggest that such commonalities may exist.

That plants and plant cells under BRIC experimental conditions respond to spaceflight with significant changes in gene expression indicates that changes in metabolism are engaged to cope with spaceflight. Spaceflight demands an adaptive response. Many of these changes make some sense with regard to the primary effects of gravity, but others suggest stresses that are not simply explained by the lack of gravity. That cultured cells respond dramatically to spaceflight in a markedly different way than seedlings suggests that either undifferentiated cells have more biological difficulty with spaceflight or that there are technical reasons affecting the results. One possibility is that this difference between cultured cells and seedlings is due to the fact that seedlings have organs with which to sense and sample their environment, and that undifferentiated cell cultures, in the absence of such organs, propagate inappropriate stress responses. In support of this possibility, seedlings displayed a greater abundance of differentially expressed genes associated with plant hormone signaling and gravity sensing, while cultured cells did not. Another possible explanation for the differences is simply that all the cells in the culture were responding in an identical manner, enhancing the technical observation of the response, while in seedlings different tissues were responding differently, diluting the apparent effect as induction in one tissue balances repression in another. The analysis of spaceflight effects on seedlings would benefit from recovering enough material to look at individual organs or cell types. Regardless, it remains clear that there is not an intense and simple response to spaceflight that is shared by all cell types within the differentiated cells of an intact plant.

5. Conclusions

Seedlings and cell cultures both engage gene expression changes to alter metabolic responses to the spaceflight environment, but they engage dramatically different responses. This response differential between seedlings and cell cultures could be as simple as a consequence of examining a very uniformly responding system in undifferentiated cells compared to a complex organism composed of differentiated organs and tissues. Or it could be as complex as the presence of an organ-specific feedback sense and response system that allows seedlings, but not undifferentiated cells, to more properly sense the environment and integrate a response that makes sense based on input from organ systems. Regardless, it is clear that even undifferentiated plant cells sense spaceflight as an environment that demands molecular responses. Many of those responses tap familiar pathways—auxin, calcium signaling, stress—and there is a tendency to place those responses into familiar Earth-based context. But given the truly novel nature of spaceflight, responses may propagate in ways that are distinct from well-defined terrestrial analogues.

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Abbreviations

BRIC, Biological Research In a Canister; BTC, BioTransport Carrier; KSC, Kennedy Space Center; LED, light-emitting diode; OES, Orbiter Environmental Simulator; PDFUs, Petri Dish Fixation Units; qRT-PCR, quantitative real-time PCR; RT-qPCR, quantitative reverse transcription–polymerase chain reaction; SAGE, Serial Analysis of Gene Expression; SLSL, Spaceflight Life Sciences Laboratory.

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Address correspondence to:
Robert J. Ferl
Interdisciplinary Center for Biotechnology Research
Horticultural Sciences and Plant Molecular and Cellular Biology
University of Florida
Gainesville, FL 32611-0580

E-mail: robferl@ufl.edu

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