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RESEARCH ARTICLE

Variation in the transcriptome of different ecotypes of *Arabidopsis thaliana* reveals signatures of oxidative stress in plant responses to spaceflight

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PREMISE OF THE STUDY: Spaceflight provides a unique environment in which to dissect plant stress response behaviors and to reveal potentially novel pathways triggered in space. We therefore analyzed the transcriptomes of *Arabidopsis thaliana* plants grown on board the International Space Station to find the molecular fingerprints of these space-related response networks.

METHODS: Four ecotypes (Col-0, Ws-2, Ler-0 and Cvi-0) were grown on orbit and then their patterns of transcript abundance compared to ground-based controls using RNA sequencing.

KEY RESULTS: Transcripts from heat-shock proteins were upregulated in all ecotypes in spaceflight, whereas peroxidase transcripts were downregulated. Among the shared and ecotype-specific changes, gene classes related to oxidative stress and hypoxia were detected. These spaceflight transcriptional response signatures could be partly mimicked on Earth by a low oxygen environment and more fully by oxidative stress (H_2O_2) treatments.

CONCLUSIONS: These results suggest that the spaceflight environment is associated with oxidative stress potentially triggered, in part, by hypoxic response. Further, a shared spaceflight response may be through the induction of molecular chaperones (such as heat shock proteins) that help protect cellular machinery from the effects of oxidative damage. In addition, this research emphasizes the importance of considering the effects of natural variation when designing and interpreting changes associated with spaceflight experiments.

KEY WORDS *Arabidopsis thaliana*; BRIC; ecotype; heat shock protein; hypoxia; peroxidase; reactive oxygen species; spaceflight.

In spaceflight, a complex suite of interacting stimuli operating against the unique background of reduced gravity yield an, as yet poorly understood, range of biological stress responses that have been collectively termed "space syndrome" (National Academies, 2011). For example, the transcriptomic data from *Arabidopsis thaliana* grown in a variety of hardware on board the International Space Station (ISS) shows transcriptional profiles implying a broad range of stress responses in both seedlings and cell cultures (e.g., Paul et al., 2012a, b, 2013, 2017; Correll et al., 2013; Kwon et al., 2015). The likely widespread nature of such space-related stress responses in plants is suggested by the observation that stress-associated transcripts are induced by spaceflight not only in *Arabidopsis* but also in species ranging from the fern *Ceratopteris richardii* (Salmi and Roux, 2008) to *Brassica rapa* (Sugimoto et al., 2014) and rice (Jin et al., 2015).

Two of the most obvious features of the spaceflight environment that are likely to alter plant growth and development are the loss of the directional cue that gravity provides on Earth and the lack of mechanical loading normally derived from the plant's own weight. However, other factors such as fluid and gas flow are altered in the spaceflight environment relative to the Earth's surface, and these other features also have important effects on biological systems that have evolved against a constant background of 1× gravity. For example, there is a loss of buoyancy-driven convection in microgravity

(Porterfield, 2002), and the associated lack of convective gas movement can impact on a range of responses from thermoregulation to respiration, e.g., imposing hypoxic stress (Porterfield, 2002; Liao et al., 2004). Similarly, spaceflight exposes biology to elevated levels of solar and galactic cosmic radiation (Chancellor et al., 2014). Thus, in addition to alterations in plant physiology and development from reduced gravitropism and gravimorphogenesis, an array of effects linked to these other properties of spaceflight are expected from plants in this environment. However, precisely which response networks are being triggered remains poorly defined.

Transcriptional profiling provides one approach to begin to assess the spectrum of the biological changes being elicited by spaceflight. Indeed, a suite of such studies of *Arabidopsis* plants have revealed potential roles for processes ranging from defense signaling to cell wall remodeling in plant reactions to the spaceflight environment (e.g., Paul et al., 2012a, b, 2013, 2017; Correll et al., 2013; Zupanska et al., 2013; Kwon et al., 2015). Variability in the transcriptional profiles found between such studies likely resides in part in the different hardware, growth conditions, and mission profiles between experiments. However, differences in the geographical origins (ecotypes) of the *Arabidopsis* lines being analyzed may also be playing an important role because *Arabidopsis* shows distinct ecotypic variations on Earth to a host of environmental factors (e.g., Lefebvre et al., 2009). Thus, when comparing responses between different spaceflight experiments, ecotypic effects may be superimposed on those of any core reaction to this environment. For example, the degree of root skewing is a trait known to be influenced by spaceflight, but also to show distinctly different ecotypic responsiveness (Millar et al., 2011; Vaughn and Masson, 2011; Paul et al., 2012a; Nakashima et al., 2014). Initial studies have indeed suggested potential differences in growth during spaceflight between the Col, Ws, Cvi and C24 *Arabidopsis* ecotypes and in patterns of gene expression in the root tips of Col-0 and Ws in space (Kiss et al., 2000; Paul et al., 2017).

Many spaceflight experiments aimed at profiling transcriptome-level changes have used the Col-0 ecotype as their reference wild type for both seedling (e.g., Paul et al., 2012a, b, 2017; Nakashima et al., 2014; Kwon et al., 2015; Li et al., 2017) and cell culture experiments (e.g., Zupanska et al., 2013; Fengler et al., 2015). In addition, the transcriptional responses of the Ws-2 ecotype have been studied using equipment such as the Advanced Biological Research System (ABRS; Paul et al., 2012a, b) and in the CARA experiment (Paul et al., 2017). Similarly, the Ler-0 ecotype has been monitored in both the Space Shuttle era (e.g., Millar et al., 2011) and on board the ISS (e.g., Correll et al., 2013; Johnson et al., 2017). Comparisons between these experiments become complex when the effect of the ecotypic background on reactions to spaceflight is not known (reviewed by Vandenbrink and Kiss, 2016).

In this research, we present a comparison of transcriptome-level responses between four Arabidopsis ecotypes (Col-0, Ws-2, Cvi-0, and Ler-0) grown under identical conditions on board the ISS as part of the BRIC19 flight (NASA, 2015) and its associated GeneLab experiment (GeneLab, 2015; NASA, 2015). We report that the four ecotypes tested show a core set of shared transcriptional responses to spaceflight characterized by induction of transcripts related to heat shock, oxidative stress, and hypoxia, coupled with repression of peroxidases. In addition, each ecotype had unique changes in transcript levels that highlight the need to carefully account for ecotypic background when designing spaceflight experiments or comparing spaceflight transcriptome data.

MATERIALS AND METHODS

Plant material

Seeds of *Arabidopsis thaliana* ecotypes Col-0, Ler-0, Ws-2, and Cvi-0 were obtained from Lehle Seeds (Round Rock, TX, USA) and were surface-sterilized in 70% (w/v) ethanol for 2 min, dried on sterile Whatman 3M filter paper (Sigma-Aldrich, St. Louis, MO, USA), and 64 seeds per 60 mm Petri dish of each were planted on 15 mL of sterile 1/2 strength Epstein's medium (Wymer et al., 1997) supplemented with 1% (w/v) Phytagel (Sigma-Aldrich) and 10 mM sucrose, pH 5.7. The seeded dishes were then irradiated for 12 h with 70 μmol m^{-2} s^{-1} white light from a fluorescent fixture (F20T12/CW; Osram Sylvania, Wilmington, MA, USA) at 22°C to synchronize subsequent germination once transferred to the ISS. The irradiated dishes were then stored at 4°C in the dark for ~8 h until integration into the flight hardware.

Flight hardware

Each Petri dish was assembled into a single stage Petri Dish Fixation Unit cassette (PDFU; Wells et al., 2001) under sterile conditions. The PDFU liquid injection reservoir was filled with 13 mL RNAlater solution (Life Technologies, Grand Island, NY, USA) and five PDFUs plus a HOBO data-logger (Onset Computer Corp., Bourne, MA, USA) were integrated into the Biological Research in Canister (BRIC) hardware (Wells et al., 2001), which was stored at 4°C before launch to inhibit seed germination. To reduce the potential for effects of any variation between BRICs, we distributed samples of each ecotype across more than one BRIC with a pattern mirrored between flight and ground samples. This hardware was then transferred in a Double Cold Bag (Hutchinson and Campana, 2009), held at 4°C, to the Dragon capsule of SpaceX mission CRS-4 where it was stowed 48 h before launch. Four BRICs were used for the analysis, allowing for an experimental design with four plates each of Col-0 and Ws-2 and three plates each of Ler-0 and Cvi-0.

Mission profile

SpaceX CRS-4 launched on 21 September 2014 and docked with the ISS ~2 d later. After a further ~2 d on orbit, the BRICs were removed from the cold bag and allowed to warm to ISS ambient temperature over 3 h (~24°C; Appendix S1). This timeline meant that the seeds were stored at 4°C for 150 h from the time of integration into the BRIC hardware to removal from the cold bag on orbit (Appendix S1). Plates were then left at ISS ambient for the seeds to germinate and seedlings to grow for 8 d. RNAlater was then injected onto the sample plates by actuating the PDFUs. Samples were allowed to fix for 12 h, then the BRICs were loaded into the Minus Eighty Laboratory Freezer on the ISS (MELFI) for sample storage. For return to Earth, the BRICs were removed from the MELFI, packed into a freezer bag (that maintained −32°C throughout the return process) and loaded into the Dragon capsule. The payload splashed down on 25 October 2014 and was shipped to Kennedy Space Center, FL, USA, where the BRIC canisters were transferred to a −80°C laboratory freezer. The hardware was then thawed at 4°C to allow disassembly of the BRIC and the Petri dishes were de-integrated from their PDFUs. A further 10 mL of RNAlater was added to each dish, and the

dishes were then frozen at −20°C, packed on dry ice and shipped to Madison, WI, USA where they were stored in a −80°C freezer for subsequent analysis.

Ground controls

A duplicate set of BRICs was used for the ground control experiments. These samples were treated identically to the flight materials but were transferred to the ISS Environmental Simulator (ISSES) for the period that the flight samples were grown on board the ISS. To facilitate programming the environmental conditions of the ISSES to mimic those on the ISS, the ground controls were run with a 48 h delay to the flight experiment. After 8 d of growth in the ISSES, RNAlater was injected, with the timing and order of RNAlater actuation for each PDFU closely mirroring that of the flight protocol. The samples were left for 12 h, then the BRICs containing the sample plates were transferred to a −80°C laboratory freezer. These samples were de-integrated from the hardware alongside the flight materials as described above. From this point onward, all other manipulations of the samples were identical to the flight material.

RNA isolation and sequencing

Samples were removed from the −80°C freezer and thawed at 4°C. Twenty-five seedlings per sample from each plate were then collected using forceps, blotted on sterile filter paper, then added to 500 µL of sterile extraction buffer (100 mM Tris-HCl, 50 mM EDTA, 150 mM LiCl, 1% (w/v) SDS and 5% (v/v) β-mercaptoethanol, pH 8.0). Samples were pulverized using a GenoGrinder (Spex SamplePrep, Metuchen, NJ, USA) and total RNA isolated using the RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Residual genomic DNA was removed by RNase-free DNase I treatment using the TURBO DNase kit (Ambion, Waltham, MA, USA) also according to the manufacturer's instructions.

Purified total RNA was subjected to rRNA reduction (Ribo-Zero plant leaf kit, Illumina, San Diego, CA, USA), and a library was created using the Illumina TruSeq RNA Sample Preparation Kit according to the manufacturer's directions. Paired-end, 1×100 bp next-generation sequencing was performed at the University of Wisconsin-Madison Sequencing Facility using a HiSeq2000 (Illumina, USA) and CASAVA 1.8.2 as the base caller software. Libraries were multiplexed, with four libraries sequenced in each lane, producing ~80 million total reads per sample.

RNA sequence analyses

The paired-end, 100-bp fragments were aligned to the Arabidopsis TAIR10 genome using the Tuxedo pipeline resources (Trapnell et al., 2013) at CyVerse (CyVerse, 2015). Briefly, fragments were joined and then aligned using the CyVerse HT-Process (CyVerse, 2015), filtering out any low-quality base reads with Phred scores below 30, as shown in Appendix S2A. TopHat version 2.0.13 was then used as the spliced junction mapper and Bowtie 2-2.2.4 as the alignment engine (see Appendix S3 for Trimmomatic and TopHat settings). The TAIR10 Arabidopsis genome FASTA file was used as a scaffold to align the RNA fragments guided by the TAIR10 genome GTF annotation file. This analysis led to between 70–80% alignment of reads. Count dispersion plots between samples were similar (Appendix S2B), suggesting subsequent comparisons between ecotypes and treatments were likely to be robust.

Subsequent normalization and statistical analysis of differential transcript abundance used either DESeq (Anders and Huber, 2010) or edgeR (Robinson, McCarthy, and Smyth, 2010) with a p-value cutoff of 0.05 and the Benjamini and Hochberg (1995) method for multiple testing correction ($q = 0.05$). In addition, Cuffdiff 2.1.1 was used to also identify significantly differentially abundant transcripts with a q-value cutoff of 0.05 and using upper quartile normalization and multi-hit correction with a false discovery rate of 0.05 (Trapnell et al., 2013). The number of biological and technical replicates per ecotype sequenced and used in the subsequent analysis of differential gene expression varied between 6 and 8: i.e., two independent sample isolations from each of 3 (Cvi-0, Ler-0) or 4 (Ws-2, Col-0) separate sample plates. The custom R scripts used for subsequent Gene Ontology (GO) analysis have been deposited at GitHub: https://github.com/dr-richard-barker. The FASTQ files of the transcriptomic data sets used for the ecotypic analysis can be obtained from the GeneLab data repository (http://genelab.nasa.gov/data) through accession number GLDS-37.

When p-value cutoffs were lowered to 0.1, essentially identical classes of gene ontology enrichments were detected, suggesting that use of the more stringent 95% confidence level was not biasing the results toward a subset of transcripts where differences between ecotypes were at the borderline of $p < 0.05$ significance levels. It is also important to note that because we have taken advantage of the well-annotated TAIR10 *Arabidopsis* Col-0 genome to align reads for all ecotypes, there is the potential to have introduced bias against diverged sequences in genes in the other ecotypes studied. To address this issue, we allowed 3 mismatches when aligning reads to gene sequence (increasing to 4 mismatches or higher did not increase the degree of alignment). In addition, the proportion of sequence alignments from the total number of reads was similar between all ecotypes (Col-0: 80%, Ler-0: 81%, Cvi-0: 82%, Ws-2: 81%), suggesting that we had not introduced a large degree of mismatch in Ler-0, Cvi-0, and Ws-2 by using Col-0 as the reference genome.

Quantitative PCR analysis

Total RNA was isolated from ~10 seedlings using the RNeasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions. Genomic DNA was eliminated using RNase-free DNase I treatment (TURBO DNase kit, Ambion) and 1–2 µg of the RNA was reverse transcribed using SuperScript III (Invitrogen, Waltham, MA, USA). The qPCR analysis was performed using an Applied Biosystem 7500 qPCR machine (Applied Biosystems, Waltham, MA, USA) and results analyzed using the MxPro qPCR software (Stratagene, Santa Clara, CA, USA). The *UBQ10* gene was used as the internal reference (Czechowski et al., 2004) and qPCR performed essentially as by Choi et al., (2014). Each reaction contained cDNA proportional to 10 ng of starting total RNA, 200 nM of primer, 7.5 µL of 2X EvaGreen qPCR master mix with ROX passive reference dye (Biotium, Hayward, CA, USA) made to a final volume of 15 µL with water. Quantitative PCR analysis was performed in 96-well optical PCR plates (ABgene, Epsom, UK) with one cycle of 15 min at 95°C; 40 cycles of 20 s at 95°C, 15 s at 58°C, and 15 s at 65°C; and one cycle of 58 to 95°C at 0.5° increments. Expression was calculated using the comparative threshold cycle (Ct) method (Choi and Roberts, 2007). The qPCR primers used are described in Appendix S4.

H_2O_2 and hypoxic treatment

Hypoxia was administered using custom-built hypoxic chambers (Appendix S5). Seeds were planted as for the flight experiment, the Petri dishes were then placed in the hypoxic chambers and nitrogen gas introduced until O_2 levels reached either 0, 3, 6, 10, or 21% (v/v). The plants were then grown for 8 d in the dark at 22°C. The chamber O_2 levels were reset when the levels had drifted more than 0.5% (v/v) from the set point. RNA was harvested at the end of the growth period and qPCR performed as described above. Seeds maintained under continuous anoxia (0% O_2) failed to germinate, so no seedling data were collected from these samples.

For analysis of H_2O_2 response, repeated treatment with H_2O_2 was used to mimic the likely long-term oxidative stress experienced in spaceflight. Seeds were planted as for the flight experiment in 60 mm Petri dishes and grown vertically in the dark at 22°C for 4 d. Thereafter, at 2-d intervals, plates were opened in a sterile hood under a green safelight, 5 mL H_2O_2 solution (0, 1, 10, 100, or 1000 μM) added and the seedlings incubated with the plates horizontal for 20 min. The solution was then poured off and the plates returned to vertical growth in the dark. RNA was extracted on day 8 and qPCR performed as described above.

RESULTS

Transcriptional responses to spaceflight common to all ecotypes

To robustly define up- and downregulated transcripts in the spaceflight data relative to ground controls, we used three separate methods to define significantly different transcript levels: DESeq, edgeR, and Cuffdiff 2 (Anders and Huber, 2010; Robinson et al., 2010; Trapnell et al., 2013). We used *q*-value cutoffs and false discovery rates of 0.05 to maintain stringent selection criteria. These packages use different normalization and statistical approaches to identify differentially expressed genes and so often do not precisely agree on which genes are showing alterations in transcript abundance (Seyednasrollah et al., 2015). We chose DESeq and edgeR because they share the same underlying statistical model, and Cuffdiff 2 because it is characterized as an analytical approach that generates results most different from these other software packages (Seyednasrollah et al., 2015). Combining results from these packages allowed us to further maximize confidence in the identification of spaceflight-responsive transcripts by looking for agreement of these approaches that a transcript was differentially accumulated during growth in space (Appendix S6). Fig. 1A shows that such analysis revealed changes in abundance of a range of transcripts. There were between 29 and 485 genes upregulated depending on the ecotype analyzed. The number of downregulated genes was lower, with Ler-0 showing the smallest number of 37 and Cvi-0 the largest at 149. A list of all significantly altered transcripts agreed upon by the three approaches to defining differentially expressed genes in spaceflight vs. ground for all four ecotypes is shown in Appendix S7.

Multidimensional scaling comparisons of the data from each ecotype between all the ground samples and separately between all the flight samples is shown in Appendix S8. This analysis indicates that gene expression patterns in Cvi-0 and Ler-0 are most similar to each other when comparing between samples only grown on the ground as well as when comparing between the flight sample data. However, Fig. 1 suggests responses to spaceflight (i.e., differentially expressed genes comparing flight to ground samples within each ecotype) are most different (at least in terms of absolute number of differentially expressed genes) between these two ecotypes. This result implies that the differences between ecotypes seen when comparing flight responsive transcripts do not simply mirror the intrinsic degree of differential gene expression seen between ecotypes under any growth conditions and so most likely reflect specific ecotype-related changes induced by spaceflight.

We therefore next applied Gene Ontology (GO) analysis using a consensus between DAVID 6.7 (Huang da et al., 2009), AgriGO (Du et al., 2010), and our own custom R script-based analysis of the GO database (Gene Ontology, 2015) to the spaceflight responsive transcriptomes to ask what classes of genes appear to be responding to the spaceflight environment. This analysis revealed that some processes were commonly regulated in spaceflight in multiple ecotypes, but relatively few were shared across all four ecotypes studied. For example, Appendix S9 shows that, among a range of enriched GO terms, Cvi-0 and Ws-2 exhibited a common enhancement for plastid- and photosynthesis- related terms, but these were not detected as being over-represented in Col-0 and Ler-0. Similarly, Col-0 and Cvi-0 shared enrichment in defense-related processes, and Col-0 and Ws-2 also showed enrichment in processes related to protein folding (likely reflecting an abundance of transcripts related to protein chaperone activity; see discussion of heat shock proteins below). However, all ecotypes showed enrichment in terms related to the apoplast and cell wall and to oxidative stress and response to reactive oxygen species (ROS; Appendix S9, S10). Other classes of

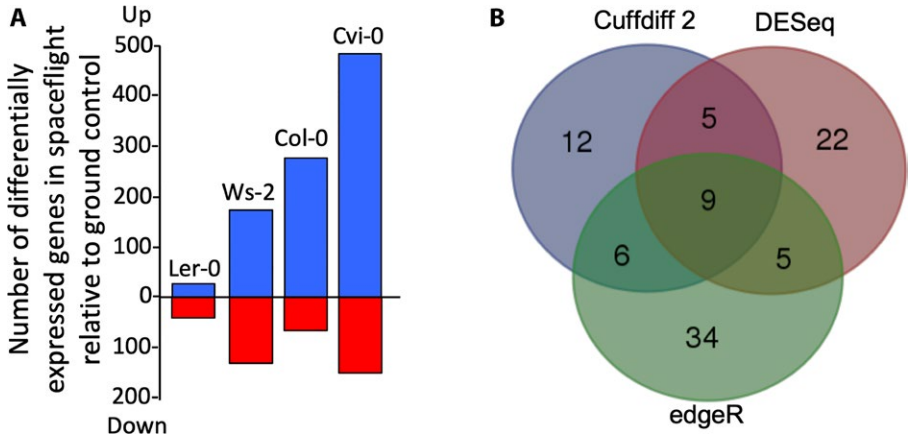

FIGURE 1. Changes in transcript abundance accompany spaceflight. (A) Significantly up- and downregulated transcripts across ecotypes of *Arabidopsis thaliana* when comparing spaceflight to ground control RNAseq data from a consensus between three packages for analyzing differential gene expression: Cuffdiff 2, DESeq and edgeR. (B) Venn diagram comparing the three packages for the significantly differentially expressed transcripts (up and down) identified across the four genotypes. The three packages identified the same nine transcripts and a consensus of two of the three approaches identified 25 (see also Table 1).

biological process that were enriched across ecotypes included response to light and temperature.

Individual ecotypes also exhibited enrichment in transcripts related to GO term categories not seen in the other ecotypes. Thus, e.g., Col-0 showed unique enrichment of hormone (abscisic acid) and water and osmotic response-related transcripts (Appendix S9A) that may reflect a suite of spaceflight-triggered changes in this particular ecotype that are normally linked to water stress on Earth.

Commonly up- and downregulated genes across ecotypes show response to oxidative stress

By using the consensus analysis between DESeq, edgeR, and Cuffdiff 2, five transcripts were identified as commonly upregulated and four downregulated by spaceflight in comparison to ground controls in all the ecotypes (Fig. 1B, Table 1). All five upregulated transcripts were from the "heat shock" superfamily. This pattern of enrichment was retained when the stringency of selection was lowered to requiring only two of the three approaches to agree (Table 1). Under these conditions, 13 transcripts were up- and 12 downregulated, with genes encoding proteins related to heat shock still over-represented in the upregulated set, forming 75% of this class. Table 2 further summarizes the transcripts annotated as heat shock protein (HSP) or heat shock factor (HSF) that change in some, but not all, of the ecotypes in response to spaceflight. All show induction, further emphasizing the link between this class of genes and response to the spaceflight environment across ecotypes.

In addition to heat shock-related proteins, the group of 13 commonly upregulated transcripts (from the analysis using consensus of two of the packages defining differential expression) contained the genes *BAG6* (*Bcl-2 associated athanogene homolog 6*) and *PDX1.2* (*PYRIDOXINE BIOSYNTHESIS 1.2*) that have also been linked to heat shock/oxidative stress response (see discussion section for more detailed description). The last two genes in this commonly upregulated group are *WTF1* (*WHAT'S THIS FACTOR 1*), a plastid RNA binding protein involved in intron splicing (Kroeger et al., 2009) and an uncharacterized member of the alpha/beta-hydrolases superfamily (AT1G73480), where links to abiotic stress and HSP-related events are less obvious.

In the commonly downregulated group of transcripts from this analysis, genes encoding peroxidases were over-represented (33% of total, Table 1). Table 3 also shows peroxidases that exhibited differential transcript levels in spaceflight in some but not all ecotypes. All these other peroxidase transcripts were also downregulated, reinforcing the idea that the levels of RNA of this family of enzymes are suppressed by spaceflight. In addition to peroxidases, the commonly downregulated grouping (Table 1) included two membrane transporters (amino acid permease 2, a transcript also downregulated in the *Arabidopsis* spaceflight data of Kwon et al. (2015), and a nodulin MtN21/EamA-like transporter family protein), a cysteine protease (*CP2*) and a number of genes encoding proteins of unknown function. The GO analysis of these commonly up- and downregulated transcripts shown in Fig. 2 revealed significant enrichment in cellular responses linked to ROS, temperature, and light stress. Despite the possibility that lack of buoyancy-driven

TABLE 1. Transcripts commonly up- and downregulated across ecotypes in spaceflight versus parallel ground controls.

		Fold-change				Standard error between methods			
Transcript	**Transcript ID**	**Col-0**	**Ler-0**	**Cvi-0**	**Ws-2**	**Col-0**	**Ler-0**	**Cvi-0**	**Ws-2**
HSP101	**AT1G74310.1**	**12.83**	**14.08**	**5.51**	**5.08**	**1.56**	**4.29**	**1.38**	**1.12**
HSP17.4	AT1G54050.1	6.78	37.72	158.36*	39.91	0.36	21.50	103.28*	21.78
HSP20 like	AT1G07400.1	11.82	9.87	16.62	4.60	3.20	2.30	7.40	0.65
HSP70	**AT3G12580.1**	**6.40**	**5.20**	**2.65**	**3.58**	**0.45**	**1.13**	**0.38**	**0.64**
HSP17.6A	AT5G12030.1	9.18	14.35	16.60	3.19	1.52	2.88	6.85	0.60
HSP23.5	**AT5G51440.1**	**4.48**	**4.83**	**4.10**	**2.88**	**0.46**	**0.80**	**0.84**	**0.47**
WTF	AT4G01037.1	3.33	2.37	2.34	3.50	0.16	0.27	0.26	0.70
—	AT1G73480.1	3.84	9.46	2.92	4.82	0.20	2.32	0.45	1.13
PDX1.2	AT3G16050.1	4.50	5.01	2.46	3.84	0.59	0.98	0.34	0.55
BAG6	AT2G46240.1	2.81	5.02	4.42	2.39	0.16	0.85	0.88	0.27
HSP81.1	**AT5G52640.1**	**3.32**	**8.21**	**2.44**	**2.59**	**0.17**	**2.08**	**0.29**	**0.34**
HSP70T-2	AT2G32120.1	1.60	15.26	2.36	2.27	0.73	8.94	1.24	1.19
HSP70T-2	**AT2G32120.2**	**2.23**	**5.71**	**3.19**	**3.13**	**0.12**	**1.21**	**0.54**	**0.51**
AtPrx39	AT4G11290.1	0.48	0.38	0.40	0.47	0.13	0.05	0.04	0.04
AtPrx32	AT3G32980.1	0.58	0.35	0.43	0.41	0.02	0.04	0.04	0.04
AAP2	AT5G09220.1	0.48	0.31	0.50	0.51	0.03	0.03	0.04	0.04
UMAMIT17	AT4G08300.1	0.32	0.22	0.30	0.43	0.09	0.04	0.04	0.04
DUF599	AT5G10580.1	0.38	0.47	0.33	0.34	0.04	0.04	0.04	0.04
—	AT1G61590.1	0.46	0.48	0.40	0.46	0.01	0.04	0.04	0.04
—	AT1G14160.1	0.33	0.19	0.27	0.36	0.04	0.02	0.04	0.05
CP2	AT4G11320.1	0.46	0.14	0.19	0.26	0.04	0.03	0.04	0.04
—	**AT5G44417.1**	**0.28**	**0.14**	**0.49**	**0.10**	**0.05**	**0.03**	**0.04**	**0.02**
RCI3	**AT1G05260.1**	**0.29**	**0.30**	**0.38**	**0.32**	**0.03**	**0.04**	**0.04**	**0.04**
—	**AT1G64370.1**	**0.17**	**0.27**	**0.22**	**0.41**	**0.02**	**0.04**	**0.04**	**0.04**
AtPrx22	**AT2G38380.1**	**0.20**	**0.23**	**0.28**	**0.17**	**0.01**	**0.04**	**0.04**	**0.02**

Notes: Transcripts in the list were agreed upon as being significantly differentially up- or downregulated in spaceflight vs ground controls by at least two of the three approaches used (Cuffdiff 2, DESeq, and edgeR); transcripts for which all three techniques agreed are in bold. Values represent the mean of the fold-changes of flight versus ground samples from the three methods. Standard error is between all three methods. At2G32120.1 and At2G32120.2 represent transcript variants of the same gene.

*Large fold-change and SE arise largely from the edgeR results and the almost undetectable levels of expression of this gene in the ground controls. Enrichment in both *HSP* and *PDX* genes is significant $P < 0.05$, χ^2, df = 1.

TABLE 2. Heat shock protein or heat shock factor transcripts showing differential level in spaceflight vs. ground controls in some but not all four of the ecotypes tested.

		Fold-change spaceflight/ground			
Transcript	Transcript ID	Col-0	Ler-0	Cvi-0	Ws-2
HSP17.4B	AT1G54050.1	—	37.72	—	—
HSP17.6A	AT5G12030.1	9.18	—	16.60	—
HSP17.6B	AT2G29500.1	—	11.62	—	—
HSP17.8	AT1G07400.1	11.82	—	16.62	—
HSP18.5	AT2G19310.1	2.39	—	3.62	—
HSP22.0	AT4G10250.1	—	38.72	—	—
HSP70	AT3G09350.1	4.22	—	—	—
HSP70b	AT1G16030.1	4.44	5.91	6.36	—
HSP70-2	AT5G02490.1	2.50	—	—	—
HSP70-3	AT3G09440.2	2.99	—	—	2.98
HSP81-2	AT5G56030.1	2.04	—	—	—
HSP81-3	AT5G56010.1	1.66	—	—	—
HSA32	AT4G21320.1	-	3.16	—	—
HOP3	AT4G12400.1	6.00	5.35	—	—
MTHSC70-2	AT5G09590.1	1.77	—	—	—
CPHSC70-2	AT5G49910.1	—	—	2.22	—
ATHSFA1E	AT3G02990.1	2.61			
ATHSFA2	AT2G26150.1	—	—	—	4.41
HSFB2A	AT5G62020.1	1.91	—	—	—
HSFB2B	AT4G11660.1	2.42	—	—	—
HSFA6B	AT3G22830.1	2.97	—	—	—

Notes: Consensus of all three techniques used to define differentially expressed genes (Cuffdiff 2, DESeq, and edgeR). Values represent the mean of the fold-changes from the three methods.

TABLE 3. Differential regulation of peroxidase transcripts in spaceflight across ecotypes.

		Fold-change spaceflight/ground			
Transcript	Transcript ID	Col-0	Ler-0	Cvi-0	Ws-2
PRX7	AT1G30870.1	—	—	—	0.21
PRX10	AT1G49570.1	—	—	0.44	—
PRX16	AT2G18980.1	—	0.30	—	0.17
PRX27	AT3G01190.1	—	—	0.22	0.28
PRX29	AT3G17070.1	0.40	—	—	—
PRX30	AT3G21770.1	—	0.30	—	0.36
PRX39	AT4G11290.1	—	0.39	0.40	0.47
PRX44	AT4G26010.1	—	—	—	0.19
PRX47	AT4G33420.1	—	—	0.47	—
PRX57	AT5G17820.1	—	0.35	-	0.25
PRX59	AT5G19890.1	—	—	0.30	0.34
PRX69	AT5G64100.1	—	—	—	0.47
PRX72	AT5G66390.1	0.29	—	—	—
PRX73	AT5G67400.1	—	—	—	0.20

Notes: Class III peroxidase transcripts showing significant repression in spaceflight in some but not all ecotypes agreed upon by Cuffdiff 2, edgeR, and DESeq. Values represent the mean of the fold-changes from these three methods.

convection in spaceflight could lead to hypoxia around respiring organisms, as discussed in the introduction, such GO analysis did not identify genes related to hypoxic response as being enriched in the spaceflight responsive transcriptome. To further probe potential links to ROS, we analyzed these up- and downregulated genes using the "ROS wheel" (Willems et al., 2016). The ROS wheel is a meta-analysis of the publicly available gene expression data from experiments that tested ROS treatments or response systems. The technique uses a radial mapping approach in which experimental classes yielding co-regulation of transcripts reside in close proximity to each other as adjacent sectors in the circular tree, or "ROS wheel". Comparison of transcripts showing differential expression to ROS wheel groupings therefore allows inference of whether they also share patterns of response with one of the major ROS-related experiment clusters. Consistent with the GO analyses outlined in Fig. 2, cross-referencing our spaceflight data to the ROS wheel shown in Fig. 3 revealed that the most obvious overlap of the commonly upregulated transcripts in spaceflight across ecotypes is to the ROS wheel's Cluster III, the "high light early" grouping of responses, suggesting the high light stress response and ROS-related events detected in the GO analysis in Fig. 2 may be part of a linked ROS-related response system. The commonly downregulated genes showed no equivalent enrichment in this ROS-related analysis. It is also important to note here that although hypoxia and ROS responses have been closely linked in the literature (see discussion for a fuller description), the ROS wheel does not explicitly define a hypoxia-related cluster in its analysis.

To ensure the robustness of the inferences made from these RNA-sequencing (RNAseq) data, we validated the changes in transcript levels in our spaceflight and ground control samples using the independent technique of qPCR. We chose *HSP101* and *HSP70* as genes that were upregulated in all genotypes in our RNAseq analysis (Table 1), *PRX22* as a gene downregulated across ecotypes in this analysis and *WRKY33* as a gene with a small induction in spaceflight in Col-0 and no change in the other ecotypes in the spaceflight RNAseq data. Appendix S11 shows that qPCR broadly agreed with the RNAseq data, with upregulation of *HSP70* transcript levels and downregulation of *PRX22* across ecotypes as predicted by the RNAseq analysis. For *HSP101*, both Col-0 and Ws-2 had the expected increase in transcript abundance; however, Cvi-0 had no statistically significant change, and the 1.6-fold increase in transcript levels in Ler-0 was not significant at the 95% confidence level. Transcript levels of *WRKY33*, a gene showing no statistically significant (Ler-0, Cvi-0, Ws-2) or minor induction (1.9-fold, Col-0) due to spaceflight in the RNAseq data, showed no significant changes in the qPCR data except for an ~2-fold reduction in the Cvi-0 background. Unfortunately, the limited amount of RNA from the spaceflight materials precluded us repeating this analysis to test whether the disparity between the RNAseq and qPCR results for a few samples reflected qPCR artifacts. However, in general, the qPCR indicated the same trends in transcript accumulation or reduction in levels seen in the RNAseq analysis.

Uniquely up- and downregulated genes across ecotypes

In the previous results section, we identified genes that were commonly up- or downregulated across all ecotypes. We next looked for genes that changed in transcript abundance in only one ecotype. Figure 4 shows a consensus analysis of the RNAseq data using DESeq, edgeR, and Cuffdiff 2 to find such genes. Ler-0 stood out from this analysis as having the fewest unique changes, with only seven uniquely regulated genes (3 up, 4 down). In comparison, Cvi-0 had the most uniquely responding genes with 216 (160 up and 56 down). Figure 4C also shows that this result likely in part reflects the larger total number of spaceflight responsive genes in Cvi-0 vs Ler-0, i.e., as the pool of genes being drawn from enlarges, the number of unique genes increases.

The GO analysis of these "spaceflight ecotype-unique" transcripts in Appendix S12 indicates Col-0 showed enriched in transcripts

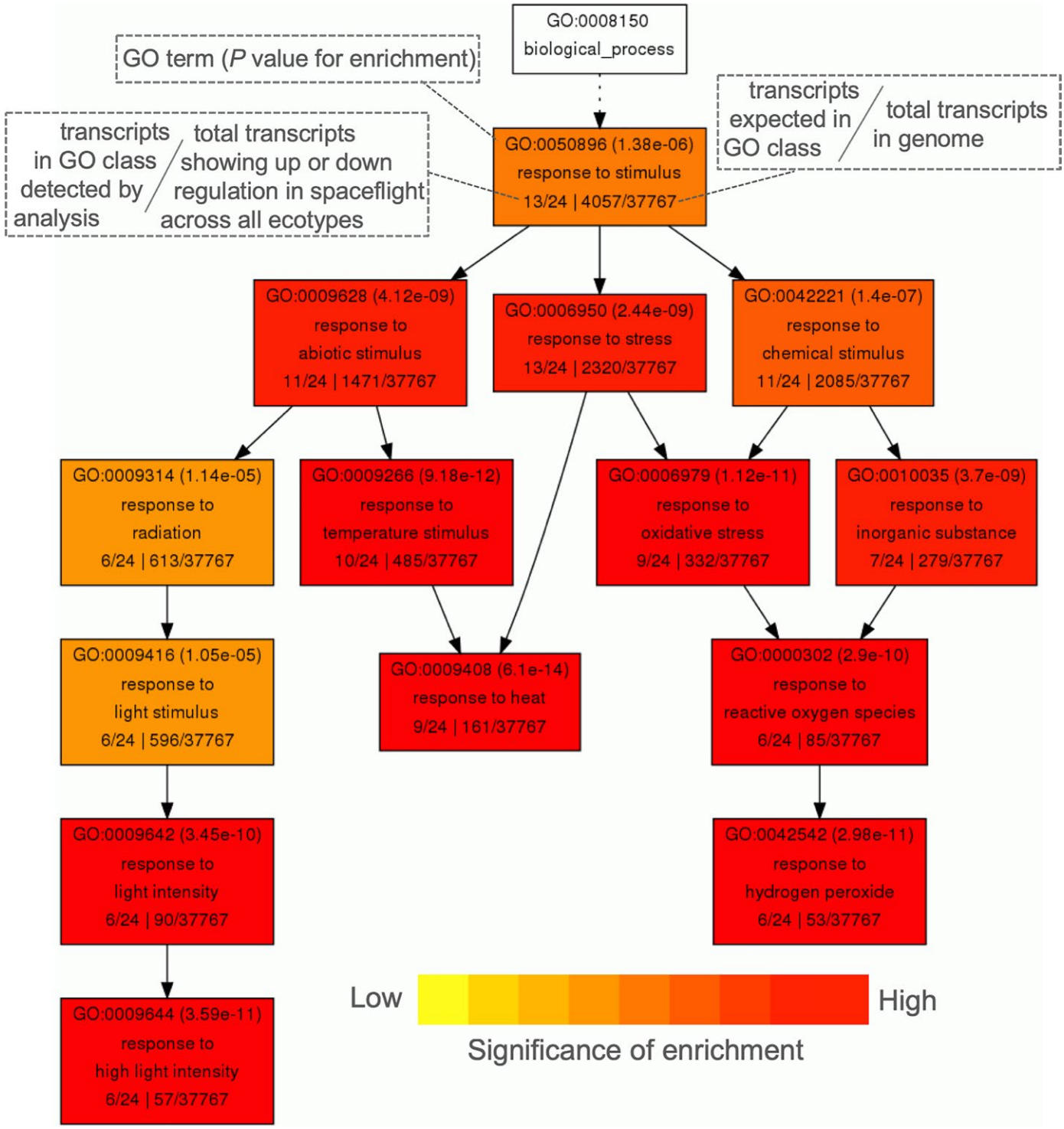

FIGURE 2. Gene Ontology enrichment analysis of transcripts showing significant differential expression in spaceflight versus ground controls agreed upon by DESeq, edgeR and Cuffdiff 2 and that are shared by Col-0, Ler-0, Cvi-0 and Ws-2. Enrichment diagram generated by AgriGo using singular enrichment analysis and using the Bonferroni multiple test correction (Du et al., 2010).

related to cold and sugar responses, whereas Cvi-0 was enriched in transcripts related to light stress, kinase signaling, and cell wall modifications. Ws-2 showed no consensus in transcript enrichment between the tools used although David 6.7 did detect enrichment in responses related to oxidative stress/response. Ler-0 also showed no significantly over-represented processes in any of the analysis tools used, which we attribute to the small number of genes changing in this ecotype reducing the sensitivity of this transcript enrichment analysis approach.

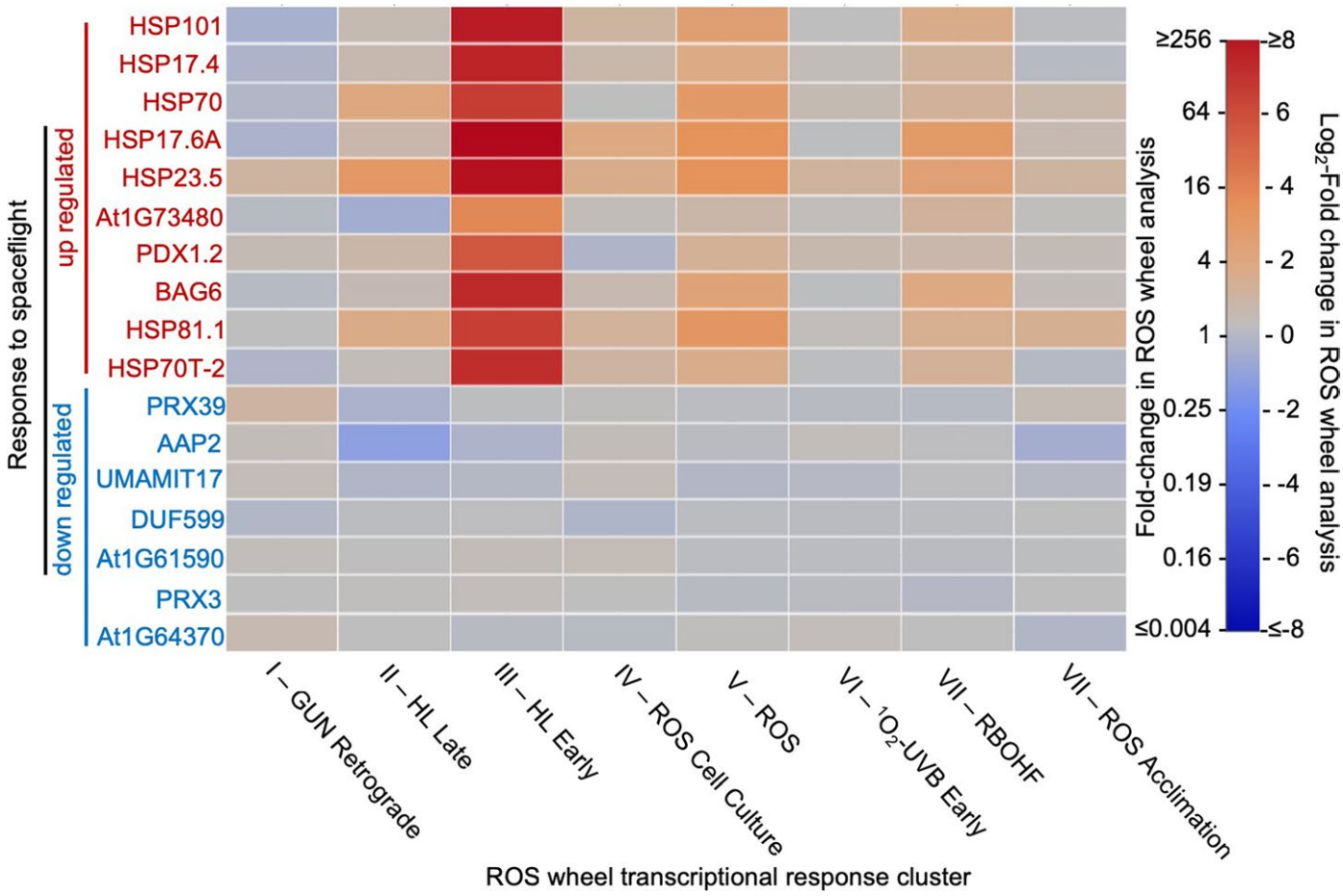

FIGURE 3. Spaceflight up- and downregulated transcripts common to Col-0, Ler-0, Cvi-0, and Ws-2 (from Table 1) compared to transcriptional response clusters from the ROS wheel meta-analysis of ROS responses by Willems et al. (2016). Only 17 of the commonly regulated genes in Table 1 are found in the microarrays used to construct the ROS wheel clusters. I–VII reflect categories of ROS-related experiments and treatments used for clustering transcriptional responses in the ROS wheel analysis: GUN, genome uncoupled mutants (known plastid retrograde signaling components); HL Late, 3 to 8 h of high light exposure; HL Early, 30 min to 2 h high light exposure; ROS cell culture, mitochondrial electron transport/ATP synthase inhibitor or H_2O_2 treatment of cell cultures; ROS, direct application or indirect generation of ROS in plants; $^1O_2$, exposure to singlet oxygen; UVB Early, 15 min to 1 h of UV-B exposure, RBOHF, responses in *rbohF* mutant background; ROS Acclimation, redox mutants leading to long-term (12 h to many days) ROS stress. For a fuller description of clusters I–VII, see Willems et al. (2016).

Ground-based analysis of ecotypic variability in hypoxic and ROS-stress response

The literature on spaceflight responses outlined in the introduction suggests that spaceflight may be imposing hypoxic and/or oxidative-stress. Similarly, our GO analyses highlight oxidative stress as an enriched class in the spaceflight responsive transcripts. Therefore, we assessed phenotypic responses of the four ecotypes in space to hypoxia and ROS-related stress in those on the ground. To mimic potential spaceflight-induced hypoxia, we grew plants in conditions mimicking the spaceflight experimental protocols (growth on ½ strength Epstein's medium with sucrose, 60 mm Petri dish for 8 d in the dark) in custom-built hypoxic chambers (Appendix S5) where we could set the O_2 levels from ambient (~21%) to 0% O_2 by flushing with N_2 gas. Hypoxia has been reported to elicit transcriptional responses in plants at around 5–8% O_2 (e.g., van Dongen et al., 2009), so we spanned this level by setting the hypoxic chambers at 3, 6, 10%, and ambient O_2. Fig. 5 shows qPCR analysis of the effects of such treatments on the expression of *HSP101*, *HSP70* (representing our common spaceflight upregulated transcripts), *PRX22* (commonly downregulated across ecotypes in space) and on two genes (*YUP18H12.4* and *WRKY33*) that are known to be induced in response to hypoxia on Earth and increase in transcript level in spaceflight but only significantly in Col-0 (Appendix S7). qPCR analysis of samples from the Col-0 ecotype showed that O_2 levels of between 3 and 10% led to the induction of the genes upregulated in spaceflight (*HSP101* and *HSP70*) and also modulated *PRX22* (Fig. 5). We compared these transcript profiles to the Col-0 spaceflight samples to pinpoint 6% O_2 as the closest likely match to the potential O_2 level triggering spaceflight responses. We then compared expression of the marker genes described above across all four ecotypes at this level of hypoxic challenge. Only *HSP70* showed the patterns of increased transcript in nearly all ecotypes expected from the spaceflight data (Appendix S13). Thus, although hypoxia

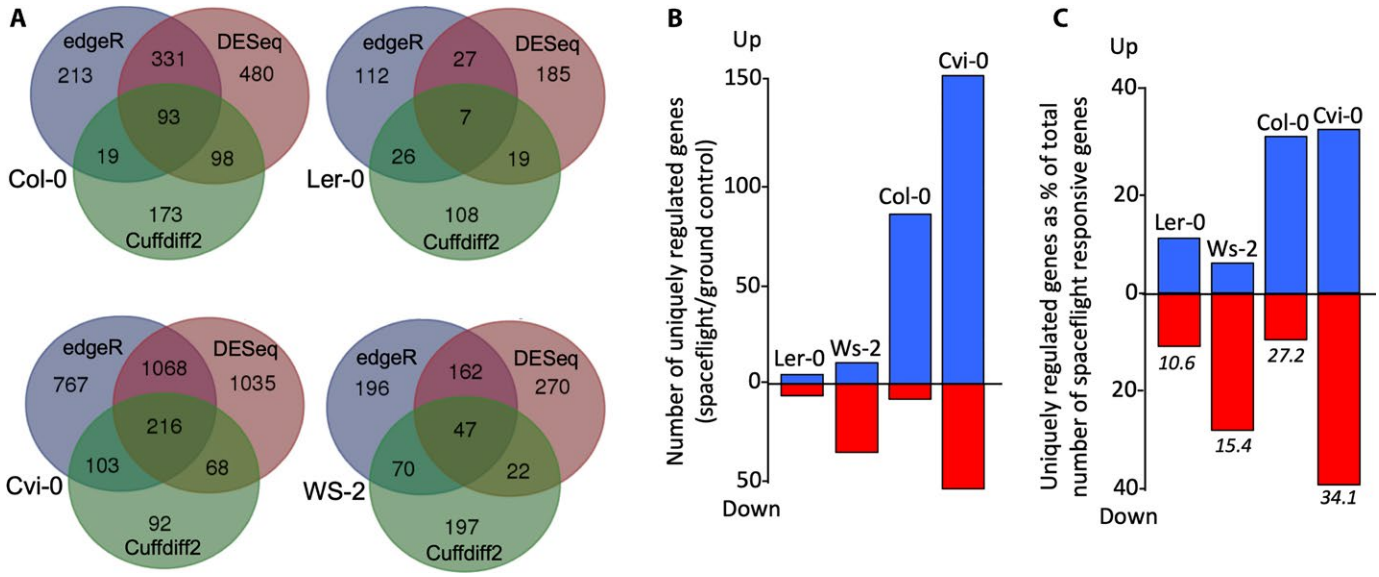

FIGURE 4. Genes uniquely regulated in each ecotype comparing flight to ground. (A) Venn diagrams comparing the DESeq, edgeR, and Cuffdiff 2 analyses of differential gene expression showing consensus for differentially regulated genes (up and down). (B) Comparison of total number of genes identified by all three analyses grouped by up- and downregulated transcripts. (C) Number of genes uniquely up- or downregulated in each genotype as a percentage of the total number of genes with significant up- or downregulation induced by spaceflight. Italics, total unique up- plus downregulated genes as a percentage of the total number of genes per ecotype with significant up- or downregulation induced by spaceflight.

could mimic some aspects of spaceflight on transcript abundance, the spaceflight pattern was not simply mimicked by applying low O_2 stress.

To assess the possible contribution of ROS-response in spaceflight, seedlings were treated with 0 to 1 mM H_2O_2 for 20 min every 2 d to trigger transcriptional cascades to a relatively long-term, sustained ROS signaling/stress. Although ROS covers a wide range of potential oxidative species (Swanson and Gilroy, 2010), H_2O_2 was chosen as a relevant ROS to apply because our RNAseq analysis of spaceflight data highlighted response to H_2O_2 as one of the most widely enriched GO terms across ecotypes in the spaceflight transcriptome (Appendix S9). Under these assay conditions, treatments above 100 µM H_2O_2 led to plant growth that was significantly attenuated (Appendix S14), so we focused on assaying a dose response between 0 and 100 µM. After H_2O_2 treatment, RNA was isolated and qPCR performed for *HSP101*, *HSP70*, *PRX22*, *WRKY33*, and *YUP8H12.4*. These analyses showed that in Col-0, this oxidative stress treatment led to the induction of a suite of genes seen in spaceflight (*HSP101*, *HSP70*, *YUP8H12.4*), whereas *PRX22* and *WRKY33* showed no significant change in transcript level upon H_2O_2 treatment (Fig. 6). Comparison of this H_2O_2 response across ecotypes (Appendix S15) showed that *HSP101* and *HSP70* showed a trend for induction by increasing H_2O_2 levels and *YUP8H12.4* showed no significant difference in transcript levels in the other ecotypes (with a significant decrease at 1 µM H_2O_2 treatment in Cvi-0), broadly in agreement with the predictions of the spaceflight RNAseq data. However, *WRKY33* showed significant induction at 100 µM H_2O_2 across Cvi-0, Ler-0, and Ws-2, whereas the RNAseq found no significant change in *WRKY33* transcript levels induced by spaceflight.

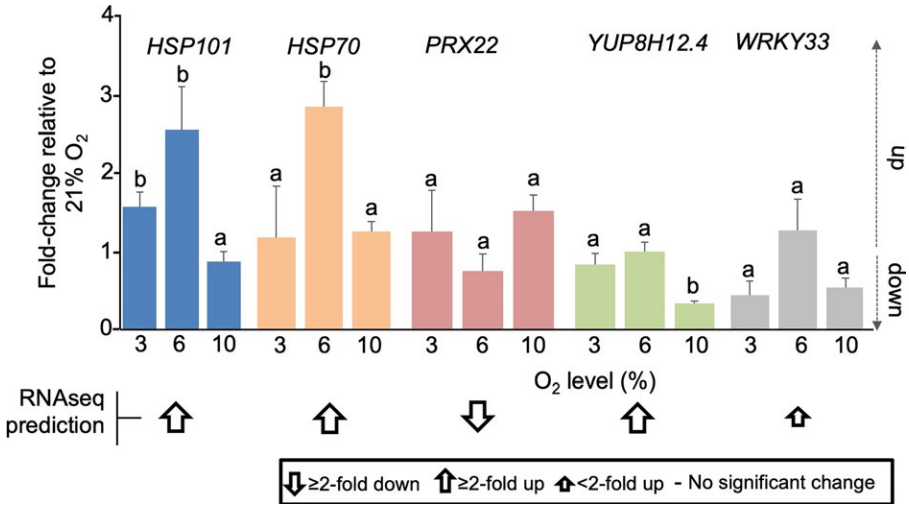

FIGURE 5. Effect of hypoxia on gene expression in Col-0. RNA was extracted from seedlings treated at 3, 6, 10, or 21% O_2 and subjected to qPCR analysis for genes with transcripts predicted to be up- (*HSP*101, *HSP*70, *YUP*8H12.4, *WRKY*33) or downregulated (*PRX*22) by spaceflight from RNAseq analysis (arrows). Results represent mean ± SEM, n = 6–12 from 3 to 4 biological replicates with fold-change calculated to the 21% ambient control. Letter "a" indicates no significant difference from the 21% O_2 control, $P > 0.05$, t-test. Bars with different letters differed significantly, $P < 0.05$, t-test (3% O_2, *HSP*101: $t = 2.34$, $P = 0.03$, df = 14; 6% O_2 *HSP*101: $t = 2.92$, $P = 0.01$, df = 14; *HSP*70: $t = 5.00$, $P = 4 \times 10^{-4}$, df = 11; 10% O_2 *YUP*8H12.4: $t = -5.7$, $P = 9.3 \times 10^{-5}$, df = 12). Significance comparisons (i.e., letters) were only made between values within a particular gene.

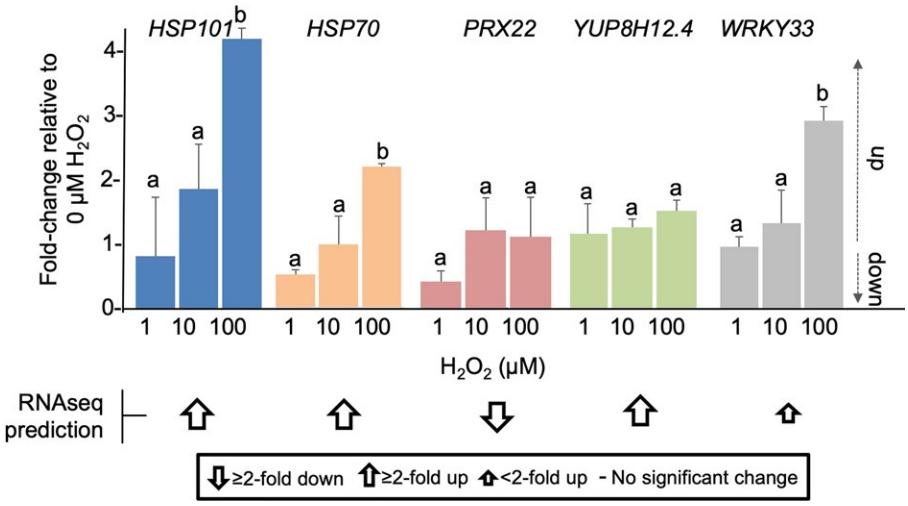

FIGURE 6. Effect of H_2O_2 on gene expression in Col-0. RNA was extracted from seedlings treated with 0 (water, control), 1, 10 and 100 µM H_2O_2 and subjected to qPCR analysis for genes with transcripts predicted to be up- (*HSP*101, *HSP*70, *YUP*8H12.4, *WRKY*33) or downregulated (*PRX*22) by spaceflight from RNAseq analysis (arrows). Results represent mean ± SEM, $n = 6–12$ from 3 to 4 biological replicates with fold-change calculated against the 0 µM control. Letter "a" indicates no significant difference from 0 µM control, $P > 0.05$, *t*-test. Bars with different letters differed significantly, $P < 0.05$, *t*-test (100 µM H_2O_2 treatment versus control, *t*-test for *HSP*101: $t = 2.27$, $P = 0.03$, df = 6; *HSP*70: $t = 2.35$, $P = 0.03$, df = 6; *WRKY*33: $t = 2.21$, $P = 0.04$, df = 6). Significance comparisons (i.e., letters) were only made between values within a particular gene.

DISCUSSION

Common transcriptional response to spaceflight among ecotypes

Using a comparison between different *Arabidopsis* ecotypes grown under identical spaceflight conditions, we are now able to better define ecotype-specific changes and a set of commonly up- and downregulated transcripts that may help to understand core spaceflight responses (Table 1). Of the 12 commonly upregulated genes across all ecotypes identified by at least two approaches to analyzing the RNAseq data, HSPs were prominently over-represented, comprising 75% of the total. Similarly, a range of other HSP and HSF transcripts appear to be induced in some of the ecotypes (Table 2). Heat shock protein and HSF upregulation in spaceflight has been previously reported in both seedlings and in cultured cells (Paul et al., 2005, 2012a, b; Correll et al., 2013; Zupanska et al., 2013; Kwon et al., 2015; Johnson et al., 2017; Li et al., 2017) with *HSP101*, *HSP70*, and *HSP17.6A* shared among various members of these previous studies and our list of upregulated transcripts common across ecotypes. Although these previous studies were performed mostly using the BRIC hardware, they also included experiments with the European Modular Cultivation System and SIMBOX, suggesting that modulation of the heat shock-related transcripts is not limited to a single experimental platform.

Heat shock proteins are a ubiquitous, conserved set of proteins that are associated with response to a wide range of abiotic and biotic stresses (Swindell et al., 2007). The HSPs are thought to protect cellular components from damage, alleviate protein unfolding and aggregation and reduce the generation of cytotoxic cellular products (Feder and Hofmann, 1999; Basha et al., 2004; Doyle et al., 2013). However, temperature recordings from the data loggers that sat within the spaceflight hardware give no indication of temperature excursions away from the ISS ambient of 22–24°C during germination or plant growth (Appendix S1). Similarly, comparison to the suite of heat shock-activated transcripts in *Arabidopsis* found in the literature (e.g., Zhang et al., 2015a) indicates the spaceflight environment only upregulated a small subset of these in each ecotype studied. Thus, HSP gene induction was most likely linked to the role of these proteins in response to some other biological stress rather than reflecting a high temperature in the experimental hardware.

Heat shock proteins are known to be involved in responses to ROS (Swindell et al., 2007), and spaceflight transcriptomes also bear other hallmarks of altered oxidative stress (Figs. 2, 3; Appendix S9; see also Correll et al., 2013; Paul et al., 2013; Sugimoto et al., 2014; Kwon et al., 2015), a pattern even seen in plants experiencing short periods of microgravity during parabolic flight (Aubry-Hivet et al., 2014; Hausmann et al., 2014). Consistent with these ideas, two of the non-HSP spaceflight upregulated transcripts seen across all four ecotypes in this study, *BAG6* and *PDX1.2* also have links to molecular chaperone activity and oxidative stress (Kabbage and Dickman, 2008; Leuendorf et al., 2014; Moccand et al., 2014). Induction of stress response genes such as *HSP101* is seen in spaceflight in undifferentiated cell cultures (Zupanska et al., 2013) and fern spores (Salmi and Roux, 2008) as well as in intact plants. Therefore, this response is likely due to an alteration in the physical environment of the cell rather than disruption of the normal gravitropic sensing and response machinery in whole plants.

A lack of convective gas mixing in microgravity is thought to potentially limit oxygen supply to respiring tissues (Liao et al., 2004), an idea tentatively supported by alterations in transcripts from hypoxia-related genes and/or markers seen in some, but not all, spaceflight experiments (e.g., Paul et al., 2001, 2005; Stout et al., 2001; Liao et al., 2004; Correll et al., 2013; Kwon et al., 2015; Johnson et al., 2017). *Arabidopsis* has also been reported to develop enhanced root skewing in space (Millar et al., 2011; Paul et al., 2012a, b; Nakashima et al., 2014), and hypoxia enhances this root trait on Earth (Eysholdt-Derzso and Sauter, 2017). However, our GO analyses did not highlight transcripts related to hypoxic response as being enriched in our ecotype spaceflight-responsive transcriptomes. We therefore manually inspected available hypoxia-linked transcriptional responses in the literature to ask whether the sensitivity of traditional GO analysis might be insufficient to detect a hypoxic signature in these data. Appendix S16 shows that many of the ecotype-common spaceflight upregulated transcripts in Table 1 (*HSP17.6A*, *HSP20*, *HSP81.1*), along with other spaceflight upregulated transcripts such as the heat shock associated scaffold *HOP3* or the stress-related transcriptional regulator *WRKY33*, are also upregulated in the polysomal RNA samples from hypoxically challenged *Arabidopsis* described in Branco-Price et al. (2005). Similarly, of the 49 core hypoxia markers used by Mustroph et al. (2009) and Gasch et al. (2016), between ~20 to 50% are significantly up- (more than

1.5-fold) or downregulated (to less than 0.5-fold) in our spaceflight data, depending on ecotype (Appendix S16). Taken together, these results suggest that hypoxia could be one part of the spaceflight environment of the plant. However, although our analysis of qPCR markers in response to lowered O_2 levels is consistent with some spaceflight responses, such as induction of *HSP101* and *HSP70*, being linked to hypoxia (Fig. 4; Appendix S13), hypoxic challenge alone does not recapitulate the spectrum of spaceflight changes such as suppression of peroxidase transcripts (*PRX22*; Appendix S13). Thus, it is likely that other factors are interacting with a possibly hypoxic environment to elicit the full suite of changes observed in spaceflight.

Hypoxia and oxidative stress are closely linked, with hypoxic challenge leading to ROS production and response (e.g., Pucciariello et al., 2012). *Brassica rapa* (mizuna) grown in space has been reported to show transcriptional fingerprints of oxidative stress (Sugimoto et al., 2014), and *Arabidopsis* spaceflight transcriptomes often show evidence of ROS response and oxidative stress (e.g., Correll et al., 2013; Kwon et al., 2015). Cross-referencing the transcriptome from the mizuna study to our own reveals many commonly upregulated genes associated with oxidative stress in Col-0 and Cvi-0 (Appendix S17). Indeed, of the common 12 genes that show upregulation across all four ecotypes in this present study, 50% (*HSP70T-2*, *HSP17.4*, *BAG6*, *HSP23.5*, *HSP101*, *HSP17.6A*) were also identified by Pucciariello et al. (2012) as markers of both low oxygen and ROS effects in their meta-analysis of hypoxia and ROS-related microarrays. Consistent with these ideas, our experiments imposing oxidative stress using H_2O_2 mimicked changes in transcript levels seen in spaceflight, especially the induction of HSPs (Fig. 5; Appendix S15). Induction of chaperones such as HSPs under hypoxic challenge and oxidative stress appears to be a response conserved across kingdoms (Vandenbroucke et al., 2008; Mustroph et al., 2010), and HSFs have even been proposed as ROS sensors for plants (Miller and Mittler, 2006). Further, comparison to the ROS wheel meta-analysis of ROS responses (Willems et al., 2016) shows our commonly altered genes in spaceflight map to ROS responsive transcripts of the "high light early" class (Fig. 3). This result suggests a possible upregulation of the high light ROS-related stress response pathway in space, even in the absence of a high light stress (the BRIC flight hardware used maintains plants in the dark). Indeed, GO analysis of all significantly up- and downregulated transcripts across ecotypes (Appendix S9) reinforces a high light response pathway as likely a common response in all the seedlings in our spaceflight samples. Defining whether these alterations in the spectrum of ROS-related transcripts represent the triggering of an inappropriate light-linked response cassette by the unique spaceflight environment, or whether the high light pathway provides some adaptive advantage under these circumstances will be an important goal for future work in this area.

The major class of downregulated genes seen in our spaceflight-responsive transcriptomes across all ecotypes were peroxidases (4 of the 12 commonly repressed genes, Table 1), with other peroxidases showing downregulation but not in all ecotypes (Table 3). Kwon et al. (2015) reported suppression in cell wall peroxidase transcript levels in their BRIC 16 flight experiment. Peroxidase protein levels were also seen to be reduced in Col-0 cell cultures flown as part of the SZ-8 mission (Zhang et al., 2015b) and in the spaceflight response transcriptome of Ler-0 (Correll et al., 2013), implying this response may be to a common cellular-level stress of the spaceflight environment. Interestingly, unlike the commonly upregulated transcripts described above, these commonly downregulated transcripts did not consistently map to a cluster within the ROS wheel meta-analysis (Fig. 3). Further, transcript levels of *PRX22* were not suppressed in our H_2O_2 treatments (Fig. 5; Appendix S15), although they were in the spaceflight data, suggesting H_2O_2-imposed oxidative stress at least does not readily mimic this aspect of the spaceflight-responsive transcriptional response.

Peroxidases likely play a role in cell wall remodeling (Kwon et al., 2015), and a possible role for alterations in cell wall structure and function has also been noted in several other spaceflight transcriptome and proteome data sets (e.g., Paul et al., 2012a, b, 2013; Correll et al., 2013; Johnson et al., 2017; Li et al., 2017). These changes are consistent with reported alterations in wall structure in rice and *Arabidopsis* plants under spaceflight conditions (Hoson et al., 2003; Johnson et al., 2017). Indeed, our GO analyses consistently found genes related to "extracellular" and "cell wall" annotations to be over-represented in our spaceflight transcriptomes across ecotypes (Appendix S10). Taken together, these results support that a further potentially conserved component of the *Arabidopsis* spaceflight response is an alteration in cell wall remodeling capacities. We speculate that altered wall characteristics may be triggered by the altered mechanical environment of the plant growing in microgravity, but further analyses are required to test these ideas.

Spaceflight experiments are inherently linked to the available hardware certified for use during flight, and it has been reported that in comparison to specimens grown under more standard laboratory growth conditions (Petri dish in growth chamber), plants grown in the BRIC can show alterations in growth and molecular responses that suggest the BRIC may impose intrinsic stresses on the plants (Johnson et al., 2015; Basu et al., 2017). In addition, at a practical level, relatively high seedling densities are needed to ensure adequate tissue for RNA extraction from etiolated seedlings in the limited growth space within the BRIC PDFU. These observations raise the important possibility that the BRIC may sensitize some seedling stress response pathways and cause some spaceflight-responsive genes to be missed in analyses of transcriptome responses (Basu et al., 2017). The sealed nature of the BRIC, coupled with the relatively high seedling planting density also holds the possibility for the buildup of ethylene within the sample chamber. Indeed, effects of elevated ambient levels of ethylene in the spacecraft cabin has been linked to some plant growth responses seen in flight samples where cabin air was exchanged with the sample chamber (e.g., Kiss et al., 1999). However, such crowding and stress effects related to the architecture of the BRIC are likely to be similar between ground controls and the flight samples, so we have endeavored to make the ground controls as faithful to the flight samples in treatment, timing, and environmental conditions as possible. In addition, GO analyses of our RNAseq data did not reveal enrichment of ethylene response markers in spaceflight, suggesting that the flight samples were not exposed to higher ethylene levels on the ISS than the ground controls were.

It is also important to note here that the samples analyzed in these studies were returned from spaceflight as RNAlater-fixed, frozen specimens. This approach is required as standard sample preservation approaches used on Earth, such as snap-freezing in liquid nitrogen, are not available on board the ISS. However, Park and Hasenstein (2016) raised the important concern that in the microgravity environment, the RNAlater fixative may reach the seedlings at a different rate to the ground controls leading

to fixation-rate-based stress gene induction. The transcript-level changes we observed in spaceflight do not directly mirror the changes reported by Park and Hasenstein (2016) when they compared slow vs. fast RNAlater fixation but with the important caveat that Park and Hasenstein (2016) studied *Brassica rapa*. This concern about fixation effects is mitigated somewhat in our study because the volume of RNAlater injected per PDFU exceeded the entire free air volume of the Petri dishes above the growth medium and seedlings by >1 mL. Actuation of the PDFUs by the astronaut to inject fixative into the plates also took only a few seconds per plate. Together, the speed of injection and excess volume of fixative suggest that the seedlings growing on the media surface are likely to have been rapidly and completely covered with the fixative.

Even with uniform fixation, the RNAlater fixation process itself does have the potential to affect transcript profiles, potentially superimposing patterns of RNAlater responsive transcription on any spaceflight-related changes (Kruse et al., 2017). However, we think such potential RNAlater artifacts are less likely to play a significant role in the conclusions presented herein on two counts. First, the comparisons are between samples grown on the ISS and samples treated and fixed identically on the ground, so any RNAlater-related effects should be similar in both and accounted for in the analysis of spaceflight responsive genes. Second, the spectrum of transcript changes seen in the flight-based analyses reported here do not correlate well with RNAlater-induced alterations in transcript levels reported for *Arabidopsis* by Kruse et al. (2017). For example, only 183 of the 2785 protein-coding transcripts showing >2-fold increase in abundance in response to RNAlater (Kruse et al., 2017) were significantly altered in abundance by spaceflight in the RNAseq analyses of this study.

In summary, our results highlight significant differences between ecotype behavior outside the core sets of commonly shared spaceflight-related genes. For example, Ler-0 showed a relatively spaceflight-resistant transcriptome, whereas Col-0, and especially Cvi-0, were much more responsive to the same conditions. Similarly, Cvi-0 and Ws-2 show hallmarks of plastid-related responses that were also suggested by Kwon et al. (2015) from the BRIC16 study, but these features are not as prominent in our Col-0 and Ler-0 spaceflight data. In a comparison of the root-tip spaceflight-responsive transcriptome, Paul et al. (2017) also observed that, similar to this study, Ws and Col-0 had roughly the same number of differentially regulated transcripts induced by spaceflight. However, this result was dependent on the growth conditions on orbit, with the Ws ecotype showing a significant increase in transcripts exhibiting changes if the roots were exposed to light during their growth on the ISS. Such results highlight the need for careful consideration of both ecotypic background and environmental variables when both comparing spaceflight data sets and in the design of future spaceflight experiments.

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AUTHOR CONTRIBUTIONS

W.C., S.G., R.B., and S.S. designed experiments; W.C., R.B., S.K., S.S., and S.G. performed the research; W.C., R.B., S.K., and S.G. analyzed data; W.C., S.G., R.B., and S.S. wrote the manuscript.

DATA ACCESSIBILITY

RNAseq data from this study are available at NASA's GeneLab data repository (http://genelab.nasa.gov/data; accession GLDS-37).

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

APPENDIX S1. Temperature profile of BRIC canisters throughout the spaceflight.

APPENDIX S2. RNAseq quality control.

APPENDIX S3. RNAseq analytical pipeline.

APPENDIX S4. qPCR primers used in this study.

APPENDIX S5. Hypoxic chamber design and operation.

APPENDIX S6. Total number of differentially expressed genes in flight samples compared to ground controls by ecotype as detected by edgeR, DESseq, and Cuffdiff2.

APPENDIX S7. Significantly differentially accumulated transcripts in spaceflight compared with ground control for four Arabidopsis ecotypes.

APPENDIX S8. Multidimensional scaling plot of RNAseq data by ecotype from ground or flight samples.

APPENDIX S9. Gene Ontology enrichment for biological process.

APPENDIX S10. Gene Ontology enrichment for cellular component.

APPENDIX S11. qPCR validation of RNAseq data.

APPENDIX S12. Gene Ontology enrichment for biological process.

APPENDIX S13. Effect of hypoxia on gene expression across ecotypes.

APPENDIX S14. Shoot length in seedlings of the four ecotypes used in this study treated with the indicated H_2O_2 levels.

APPENDIX S15. Effect of H_2O_2 on gene expression in the Cvi-0, Ler-0, and Ws-2 ecotypes.

APPENDIX S16. Significantly up- and downregulated transcripts in spaceflight versus ground controls by ecotype also found in data from hypoxic treatments in the literature.

APPENDIX S17. Significantly up- and downregulated transcripts in spaceflight versus ground controls by ecotype also found in oxidative stress/ROS microarray analysis of space flown mizuna from Sugimoto et al. (2014).

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