Genetics and Molecular Biology, 37, 1, 81-92 (2014)

www.sbg.org.br

Research Article

Expression of stress-related genes in zebrawood (*Astronium fraxinifolium*, Anacardiaceae) seedlings following germination in microgravity

Peter W. Inglis[1], Ana Y. Ciampi[1], Antonieta N. Salomão[2], Tânia da S.A. Costa[3] and Vânia C.R. Azevedo[1]

[1]*Laboratorio de Genética Vegetal, Embrapa Recursos Genéticos e Biotecnologia, Parque Estação Biológica, Brasília, DF, Brazil.*
[2]*Laboratorio de Sementes, Embrapa Recursos Genéticos e Biotecnologia, Parque Estação Biológica, Brasília, DF, Brazil.*
[3]*Laboratório de Química de Produtos Naturais, Embrapa Recursos Genéticos e Biotecnologia, Parque Estação Biológica, Brasília, DF, Brazil.*

Abstract

Seeds of a tropical tree species from Brazil, *Astronium fraxinifolium*, or zebrawood, were germinated, for the first time in microgravity, aboard the International Space Station for nine days. Following three days of subsequent growth under normal terrestrial gravitational conditions, greater root length and numbers of secondary roots was observed in the microgravity-treated seedlings compared to terrestrially germinated controls. Suppression subtractive hybridization of cDNA and EST analysis were used to detect differential gene expression in the microgravity-treated seedlings in comparison to those initially grown in normal gravity (forward subtraction). Despite their return to, and growth in normal gravity, the subtracted library derived from microgravity-treated seedlings was enriched in known microgravity stress-related ESTs, corresponding to large and small heat shock proteins, 14-3-3-like protein, polyubiquitin, and proteins involved in glutathione metabolism. In contrast, the reverse-subtracted library contained a comparatively greater variety of general metabolism-related ESTs, but was also enriched for peroxidase, possibly indicating the suppression of this protein in the microgravity-treated seedlings. Following continued growth for 30 days, higher concentrations of total chlorophyll were detected in the microgravity-exposed seedlings.

Key words: microgravity, stress response, germination, suppression subtractive hybridization, zebrawood.

Received: July 13, 2013; Accepted: December 2, 2013.

Introduction

Plants have evolved under constant gravitational conditions and even transient exposure to microgravity is unnatural. Gravisensing is one of the most important factors in the regulation of plant growth and development, where plant shoots grow upward (negative gravitropism) and roots grow downward (positive gravitropism) (Morita and Tasaka, 2004). However, seed-to-seed growth experiments performed with plants such as *Brassica rapa, Arabidopsis thaliana* and peas have shown that microgravity may not be an impediment to development and completion of the life cycle (Musgrave *et* al. 2000; Laurinavicius *et al.*, 2001; Sychev *et al.*, 2007). Microgravity, experienced by plants during spaceflight, as distinct from simulated microgravity in clinostat or random positioning machine experiments, may cause physiological and ultrastructural changes that can provoke acceleration of growth and differentiation of cells and their aging as a result (Kordyum, 1994). Recent evidence from transcriptome profiling of seedlings and cultured cells confirms the fundamental hypothesis that survival in the spaceflight environment requires adaptive changes that are both governed and displayed by alterations in gene expression, primarily of heat shock-related and stress-related genes (Paul *et al.*, 2012).

Zebrawood (*Astronium fraxinifolium* Schott, Anacardiaceae) is a tropical tree species native to the Amazon Rainforest, Atlantic Forest, Caatinga, and Cerrado Biomes in Brazil (Santin PA, 1989, Masters Thesis - Instituto de Biologia, Universidade Estadual de Campinas, Campinas, Brazil). In conservation terms, the species is classified as being vulnerable by The Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis (Ibama) (http://www.arvoresbrasil.com.br), due to its exploitation for use as timber, as an ornamental tree in landscaping, for its medicinal uses, and due to the disappearance of its natural habitats. In a spacial autocorrelation study of two roadside populations of *A. fraxinifolium,* no genetic structure was detected between 1 and 78 km, where the morphologi-

Send correspondence to Vânia C.R. Azevedo. Laboratorio de Genética Vegetal, Embrapa Recursos Genéticos e Biotecnologia, Parque Estação Biológica, Caixa Postal 02372, 70770-900 Brasília, DF, Brazil. E-mail: vania.azevedo@embrapa.br.

cal traits analyzed appeared to be highly variable (Aguiar *et al.*, 2004). The adaptability of the species has attracted interest for its use in the reforestation of degraded soils in Brazil (Miranda *et al.*, 2011). The seeds are tolerant of osmotic (-10 Bars), and anoxic (1% O_2) stress, with germination that is both rapid and homogenous (A.N. Salomão, unpublished data). These characteristics made the germination of zebrawood seeds an attractive choice for one of eight experiments to be carried out by the first Brazilian astronaut, Lt. Col. Marcos Cesar Pontes, on the International Space Station (ISS), where the timeframe available to obtain seed germination results was strictly limited by the proposed six-day orbital flight.

On their return to Earth, the zebrawood seedlings were grown on for a further six days, along with parallel control batches of seedlings grown solely under terrestrial conditions. The germination process was both faster and more homogeneous in microgravity conditions, both for seeds maintained in the presence of light, and for those kept in the dark. A differential gene expression analysis was then conducted after the total 12-day growth period. To our knowledge, these are the first experiments of their kind to be carried out on a tropical tree species.

Materials and Methods

Germination

Experiment GSM formed part of the scientific research program performed by the Brazilian Astronaut, Marcos C. Pontes on the Russian Segment of the ISS in the framework of the CENTENARIO Brazilian Soyuz Mission Project. The zebrawood germination experiment was designed by researchers at Embrapa Genetic Resources and Biotechnology, who germinated identical seeds on the ground using the same materials as those used aboard the ISS. Four replicates of 10 *A. fraxinifolium* seeds were fixed to a blotting paper substrate by Kapton tape in sealed plastic bags. Two plastic bags were exposed to light and the other two were put into an aluminium foil coated bag. Activation of the germination process was performed by seed wetting using a water-filled syringe, incorporated in the germination kit, which was injected by the astronaut when on the ISS. To monitor the germination rate and phenotype, photography by an onboard digital camera was used, where the germination kits kept in the dark were periodically removed from their foil-coated bags to facilitate this. Following the nine day spaceflight, the germinated seeds were returned to earth. On arrival in the laboratory three days later, development was again monitored (12 days post-wetting) and samples of the microgravity-treated seedlings and terrestrial controls reserved, where roots and any emergent shoot tissue were dissected and frozen in liquid nitrogen for later RNA extraction.

The remaining seedlings germinated in both environments were also removed from their packs and transferred to a germinator, maintained at a temperature of 25 °C, with a photoperiod of 74.98 $\mu Mm^{-2}.s^{-1}$/12 h for about 30 days, for the development of leaflets. Chlorophylls and carotenoids were then extracted, separated, identified and quantified by scanning spectrophotometer at 180-600 nm, adapting methods of Gomes *et al.* (2003) and Rodriguez-Amaya (1999).

RNA methods and construction of subtractive cDNA libraries

Because of the extremely limited amounts of biological material available from the spaceflight samples, we opted to produce and sequence two cDNA libraries of expressed sequence tags (ESTs) from a subtractive hybridization experiment, to detect differential gene expression under microgravity and normal gravity, mixing light- and dark-grown seedlings together.

The roots and shoots from five dark-germinated, and five light-germinated seedlings from either microgravity or terrestrial samples were ground to a fine powder in liquid nitrogen with a mortar and pestle. Total RNA was then extracted using a mini-column purification kit (Invisorb spin plant RNA mini kit; STRATEC Molecular GmbH, Berlin, Germany). Messenger RNAs were purified using Oligo $(dT)_{25}$ magnetic beads (Dynabeads; Dynal - Life Technologies Corp., Carlsbad, USA) and quantified spectrophotometrically using a Nanodrop instrument (Thermo Scientific, Wilmington, USA).

Messenger RNA transcripts enriched either by microgravity or by terrestrial conditions were detected by PCR suppression subtractive hybridization (Diatchenko *et al.*, 1996; Gurskaya *et al.*, 1996), using the PCR-Select cDNA Subtraction Kit (Clontech Laboratories Inc., Mountain View, USA), following the manufacturer's protocols. A forward subtracted experiment was set up using cDNA from the seeds germinated in space as the tester and cDNA from the terrestrially germinated samples as driver, utilizing a 10-fold excess of driver cDNA over tester cDNA. A reverse subtracted experiment was also set up using the converse configuration of tester and driver cDNAs. For the primary subtraction the cDNAs were denatured at 98 °C for 1.5 min, and hybridized at 68 °C for eight hours. Samples were then secondarily subtracted for a further eight hours using an additional 10-fold excess of denatured driver cDNA. PCR amplified subtracted cDNAs were cloned in the pGEM T-easy vector (Promega, Madison, USA) and used to transform *E. coli* DH5α competent cells. Positive clones were unidirectionally sequenced using the Big Dye 3.1 kit and 3700 sequencer (Applied Biosystems - Life Technologies Corp., Carlsbad, USA). Sequences from the subtracted clones were stripped of vector and adapter sequence and of low-quality regions and assembled into contigs using the high sensitivity/medium setting of Geneious (v.5.4.3., Biomatters Ltd. Auckland, New Zealand).

EST analysis

EST analysis was carried out using the Blast2GO gene annotation and ontology assignment pipeline (Conesa and Götz, 2008). BLAST searches of the NCBI nucleotide database (nr) used the BlastX algorithm, with an ExpectValue of 1.0E-3 and HSP length cutoff of 33. The 20 most significant BlastX hits per sequence were saved, and the top hits then annotated with their gene ontology (GO)-terms, using the default annotation configuration and evidence code weights. Annotation augmentation (ANNEX) was applied, and the results of an InterProScan and GO-EnzymeCode mapping steps added. The gene ontologies were also simplified using the GO-Slim Plant ontology for comparative purposes. Overlap in GO-terms between the libraries was calculated and visualised using the BioVenn web application (Hulsen *et al.*, 2008).

Results

Germination

Radicle protrusion and growth of rootlets appeared both faster and more uniform in seeds sent to the ISS. Seven days after the start of the experiment, 60% of the seeds in the presence of light and 30% of those kept in darkness had germinated on the ISS. For seeds kept in the laboratory, germination values were 5% and 10% in the presence and absence of light, respectively, though these values did not reach statistical significance, due to the large variances obtained (Figure 1). On the day the experiment returned to Earth (nine days after the start of the experiment), the percentages of seeds germinated in microgravity were 65% (light) and 85% (dark) and the seeds germinated in the laboratory were 80% (light) and 60% (dark). At 12 days after the start of the experiment, the germination of the material from the ISS was 100% in both the presence and absence of light, with rootlets having an average length of 1.5 cm and starting to show positive geotropic curvature. The germination rate of the terrestrial control was also 100% by the 12th day, but the average length of the radicle was 0.5 cm. During later seedling development for 30 days in the germinator, no difference between the growth of the shoots derived from seeds exposed to microgravity and the control seedlings was observed (not shown). However, the microgravity-treated seedlings of seeds had greater root length and number of secondary roots.

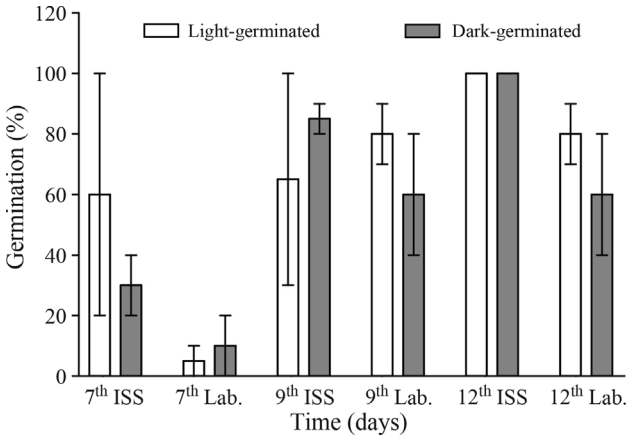

Figure 1 - Germination rates of seeds of *Astronium fraxinifolium* in microgravity (ISS) and terrestrially (Lab). There was no significant difference ($p > 0.05$) between the percentage germination in microgravity and terrestrial conditions, and in both light and dark, according to ANOVA with Bonferroni test). Error bars = SD.

Pigments content

The ratio of Chlorophyll a to b was 2.5 in leaflets of seedlings that started germination both in microgravity and in the laboratory (Figure 2). However, leaflets of plantlets that germinated in microgravity showed a significantly ($p < 0.001$) higher concentration of total chlorophyll (581.47 mg/100 g fresh weight) than those germinated in the laboratory (381.84 mg/100 g fresh weight), following 30 days of terrestrial growth. The concentration of α-carotene in leaflets of plantlets that initiated germination in microgravity was not significantly different ($p > 0.05$) from those germinated terrestrially, while the concentrations of both β-carotene ($p < 0.05$) and xanthophils ($p < 0.01$) were both significantly higher in the microgravity-treated samples (Figure 3).

Suppression subtractive hybridization library EST analysis

Of the microgravity SSH ESTs, 704 of 741 high quality sequence reads were assembled into 56 contigs, leaving 36 singletons. Filtering of ribosomal RNAs using *Aesculus pavia* rRNA 26S, 18S and 5.8S sequences found a massive 405 ESTs assembling to the 26S rRNA gene and a further 17 ESTs assembling to the 18S rRNA gene. Of the terrestrial SSH ESTs, 588 of 681 high quality reads were assem-

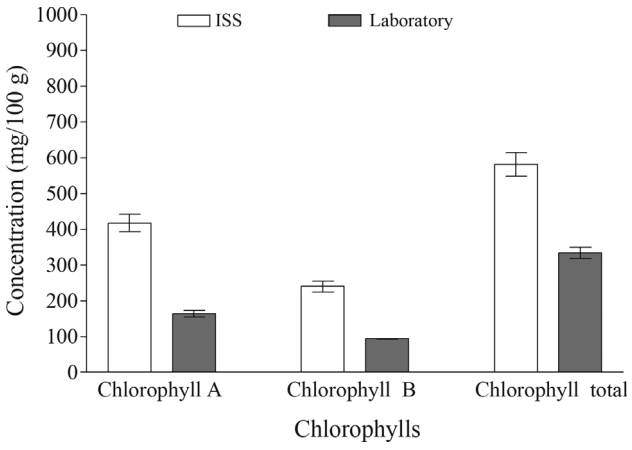

Figure 2 - Synthesis of chlorophylls in 30-day plantlets derived from seeds germinated in microgravity (ISS) and terrestrially (Laboratory). The chlorophyll content was measured relative to leaflet fresh weight, and differences are statistically significant ($p < 0.001$) according to ANOVA with Bonferroni test for all three pairs of measurements between the ISS and the terrestrial laboratory. Error bars = SD.

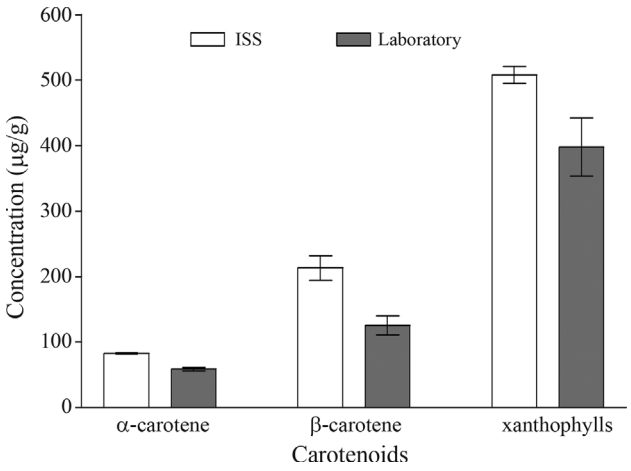

Figure 3 - Synthesis of carotenoids in 30-day plantlets derived from seeds germinated in microgravity (ISS) and terrestrially (Laboratory). The carotenoid content was measured relative to leaflet fresh weight, and pairs of measurements between the ISS and the terrestrial laboratory are significant at $p < 0.05$ for β carotene and $p < 0.01$ for xanthophylls, but were not significant at $p > 0.05$ for α carotene according to ANOVA with Bonferroni test. Error bars = SD.

bled into 101 contigs, leaving 93 singletons. Ribosomal RNA filtering found 136 ESTs matching 26S rRNA and 1 EST matching 18S rRNA. Cross-assembly between the two libraries, with the exception of the rRNA hits, found only three mixed-library contigs, demonstrating that the PCR suppression subtractive hybridization protocol had otherwise been highly effective.

The species distribution of the top BLAST hits for the rRNA filtered ESTs was similar for both microgravity and terrestrial SSH libraries, and the combined results are shown in Figure 4. The great majority of hits were with genes from other dicotyledonous plants, as might be expected. The top hits for both microgravity and terrestrial library contigs (excluding singletons) are given in Table 1 and Table 2 respectively.

The microgravity SSH library was notably rich in ESTs of stress-related genes, where seven hits with several different classes of heat shock proteins were noted (Table 1), as well as an additional six hits among the singleton reads (not shown). In contrast, there was just one hit for heat shock protein 70 in the terrestrial library (Table 2). Also related to a stress-response, there were three separate hits for glutathione transferase among the contigs in the *A. fraxinifolium* microgravity SSH library (Table 1) and an additional hit among the singleton reads (not shown), whereas no hit for this protein was found in the terrestrial library. Similar stress-related hits included polyubiquitin and 14-3-3-like protein. Notably enriched in the terrestrial SSH library was peroxidase (Table 2).

Despite the low frequency of common EST reads in the two libraries, there was a large overlap in all GO terms extracted from the microgravity and terrestrial SSH library top Blast hits (Figure 5). This was emphasised in the analysis of the GOSlims, where the great majority of GOs were common to both libraries. Unique plant GOSlims in the microgravity library were: GO:0009856 (fimbrin-like protein 2-like (Pollination)), from contig 29; GO:0030528 (btb and taz domain protein 4 (transcriptional regulator activity)), singleton; GO:0007049 (cell division cycle protein 48 homolog (cell-division cycle)), singleton; GO:0030234 (cystatin (enzyme modulator)), singleton. Because of the

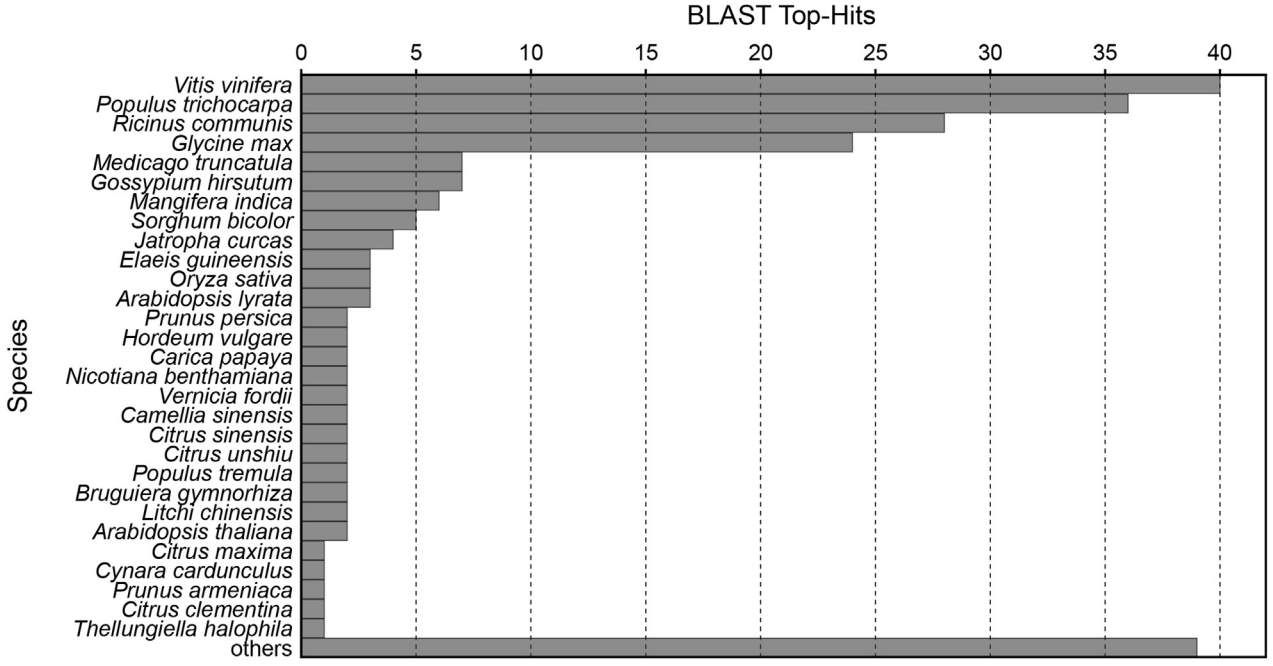

Figure 4 - Species distribution of the top BLAST hits from contiguous and singleton ESTs, obtained from both microgravity and terrestrial SSH libraries.

Table 1 - Top BLAST hits of microgravity SSH library assembled contigs containing 2 or more reads.

Contig	Top BLAST Hit	Species	GenBank No.	E-Value	Contig (bp)	No. reads
2	kinesin k39	*Nematostella vectensis*	XP001620916	1.0E-11	919	25
3	No hit	-	-	-	146	24
4	sec14 cytosolic factor	*Glycine max*	XP003541004	1.3E-39	212	23
6	pleiotropic drug resistance protein 1-like	*Vitis vinifera*	XP003632802	7.9E-42	261	11
7	Cysteine protease	*Populus trichocarpa*	ABK96252	1.5E-39	218	10
8	No hit	-	-	-	150	9
9	14-3-3-like protein	*Gossypium hirsutum*	ADK93081	9.8E-65	428	8
10	26s proteasome triple-a atpase subunit5a	*Oryza sativa*	EAZ35994	4.3E-96	488	7
11	Heat shock protein 70	*Triticum durum*	CBZ39500	6.5E-72	365	7
12	Nc domain-containing protein	*Populus trichocarpa*	XP002309251	3.0E-29	326	7
13	No hit	-	-	-	286	7
14	No hit	-	-	-	260	7
15	Splicing factor arginine serine-rich	*Ricinus communis*	XP002523584	1.8E-3	140	6
16	Polyubiquitin	*Oryza sativa*	ABR25718	3.1E-99	456	6
17	Glutathione peroxidase	*Ricinus communis*	XP002509790	9.1E-61	355	5
18	Pre-mrna-splicing factor clf1	*Vitis vinifera*	CBI34455	7.4E-103	570	5
19	5-enolpyruvylshikimate-3-phosphate synthase	*Lactuca sativa*	BAE20403	6.2E-104	511	5
20	2-alkenal reductase	*Populus trichocarpa*	XP002309720	2.4E-81	442	5
21	Probable l-type lectin-domain receptor kinase -like	*Vitis vinifera*	CBI37712	7.9E-23	430	5
22	Heat shock protein hsp20	*Mangifera indica*	ACD69682	6.0E-49	403	5
23	No hit	-	-	-	200	5
24	Vacuolar processing enzyme	*Populus trichocarpa*	XP002324151	3.1E-71	558	4
25	Multidrug pheromone mdr abc transporter family	*Thalictrum flavum*	AAX07468	1.1E-25	374	4
26	Kda class I heat shock protein	*Pisum sativum*	AAN74634	2.5E-33	280	4
27	Glutathione transferase	*Nicotiana benthamiana*	AAP04397	3.5E-17	149	4
28	No hit	-	-	-	563	3
29	fimbrin-like protein 2-like	*Populus trichocarpa*	XP002317323	2.81E-114	559	3
30	60s ribosomal protein l26	*Ricinus communis*	XP002525271	2.34E-36	535	3
31	protein in2-1 homolog b-like	*Jatropha curcas*	ADB85103	2.78E-56	520	3
32	glutathione s-transferase	*Glycine max*	AAG34804	8.06E-60	429	3
33	hairpin-inducing protein	*Casuarina glauca*	ABZ80409	4.80E-16	395	3
34	monoglyceride lipase-like	*Vitis vinifera*	CBI20835	5.40E-70	352	3
35	17.5 kDa small heat shock protein	*Carica papaya*	AAR25848	5.63E-38	341	3
36	alcohol dehydrogenase	*Gossypium hirsutum*	AAA98987	2.98E-24	144	3
37	cysteine proteinase	*Carica papaya*	P05993	4.61E-28	558	2
38	chalcone synthase	*Camellia grijsii*	AAO43487	1.16E-90	511	2
39	ubiquitin-protein ligase	*Ricinus communis*	XP002528983	3.28E-53	508	2
40	proline-rich 33 kda extensin-related	*Vitis vinifera*	CAN61377	0.075305	441	2
41	serine threonine-protein kinase	*Populus trichocarpa*	XP002330314	3.48E-07	438	2
42	s-adenosylmethionine decarboxylase	*Populus trichocarpa*	XP002314904	8.36E-20	437	2
43	NAC domain protein	*Gossypium hirsutum*	ACI15345	2.28E-09	418	2
44	No hit	-	-	-	391	2
45	heat shock protein 70 kDa	*Glycine max*	ACU17965	2.68E-63	314	2
46	heat shock protein 70	*Arabidopsis lyrata*	XP002873055	9.91E-36	274	2
47	glutathione-s-transferase omega	*Ricinus communis*	XP002525204	5.95E-31	257	2
48	programmed cell death 4	*Populus trichocarpa*	XP002318177	3.41E-25	245	2
49	protein	*Populus trichocarpa*	XP002322148	4.37E-31	221	2
50	transcription factor	*Lycoris longituba*	ADG57809	9.00E-25	213	2
51	No hit	-	-	-	210	2
52	methionine sulfoxide	*Vitis vinifera*	CBI26152	4.11E-22	193	2
53	protein	*Populus trichocarpa*	XP002300227	1.14E-25	170	2

Table 2 - Top BLAST hits of terrestrial SSH library assembled contigs containing 2 or more reads.

Contig	Top BLAST Hit	Species	GenBank No.	E-Value	Contig (bp)	No. reads
02	Peroxidase	*Bruguiera gymnorhiza*	ADD54644	6.37E-96	743	20
03	No hit	-	-	-	407	16
04	glyceraldehyde-3-phosphate dehydrogenase	*Glycine max*	AAC70010	7.31E-42	236	15
05	plasma membrane intrinsic protein	*Populus tremula*	CAH60718	2.09E-119	572	13
06	nucleotide binding	*Vitis vinifera*	CBI40569	1.71E-70	565	12
07	lipid-transfer protein seed storage 2s	*Ricinus communis*	XP002531954	2.34E-24	399	12
08	peroxidase	*Bruguiera gymnorhiza*	ADD54644	1.07E-59	315	12
09	14-3-3 protein	*Vitis vinifera*	CBI33672	6.49E-41	210	12
10	dihydroflavonol 4-reductase	*Citrus sinensis*	AAS00611	8.43E-83	431	8
11	lactoylglutathione lyase	*Vitis vinifera*	XP002273346	4.04E-30	365	8
12	NAD-dependent malic enzyme	*Cucurbita pepo*	AAG23798	2.52E-75	356	8
13	chalcone synthase	*Prunus persica*	BAC98340	5.99E-38	303	8
14	glycine-rich RNA-binding protein	*Citrus unshiu*	BAA92156	2.69E-43	411	7
15	alpha beta fold family protein	*Vitis vinifera*	XP002282804	6.11E-22	188	7
16	chaperonin	*Corchorus olitorius*	ABS72190	4.13E-91	555	6
17	translation factor sui1	*Ricinus communis*	XP002522857	6.24E-68	525	6
18	sigma factor sigb regulation protein rsbq	*Vitis vinifera*	XP002285308	1.05E-81	502	6
19	60S ribosomal protein L24	*Vitis vinifera*	XP002279048	1.59E-07	429	6
20	40s ribosomal protein s3-3-like	*Sonneratia alba*	ACS68715	5.80E-87	407	6
21	acyl- -binding protein	*Populus trichocarpa*	XP002326588	1.72E-42	405	6
22	60s ribosomal protein	*Paeonia suffruticosa*	ABQ65185	4.94E-19	118	6
23	glutamate-gated kainate-type ion channel receptor	*Ricinus communis*	XP002519690	5.68E-123	613	5
24	cytochrome p450	*Populus trichocarpa*	XP002318835	3.71E-60	535	5
25	ras-related protein RABC1	*Vitis vinifera*	XP002267387	1.12E-58	493	5
26	Translation elongation factor 1-	*Gossypium hirsutum*	ABA12221	1.10E-69	491	5
27	protein	*Populus trichocarpa*	XP002322246	2.32E-73	446	5
28	metallothionein	*Mangifera indica*	ADH04476	1.22E-18	425	5
29	hydrophobic protein lti6a	*Glycine max*	XP003554596	2.58E-29	318	5
30	enolase	*Prunus armeniaca*	AAY34909	1.95E-54	275	5
31	60s ribosomal protein l18a	*Populus trichocarpa*	ABK93213	1.58E-53	252	5
32	serine hydroxymethyltransferase	*Cucumis melo*	BAD93605	7.68E-41	232	5
33	chloroplast ferredoxin I	*Camellia sinensis*	AEI83424	3.09E-20	222	5
34	60s ribosomal protein	*Ricinus communis*	XP002518107	1.55E-30	182	5
35	peroxisomal targeting signal 1 receptor	*Ricinus communis*	XP002529211	3.35E-109	570	4
36	phosphoglycerate mutase	*Elaeis guineensis*	AEZ00838	3.14E-101	559	4
37	carrier protein mitochondrial-like	*Vitis vinifera*	CAN66307	3.74E-106	538	4
38	non-specific serine threonine protein kinase	*Ricinus communis*	XP002531832	1.50E-31	509	4
39	dcd (development and cell death) domain protein	*Vitis vinifera*	CBI21352	1.20E-63	488	4
40	serine carboxypeptidase-like 18	*Vitis vinifera*	XP002272116	1.52E-43	414	4
41	importin alpha	*Ricinus communis*	XP002512528	1.47E-54	388	4
42	calmodulin binding protein	*Ricinus communis*	XP002525175	5.69E-22	325	4
43	rna recognition motif-containing protein	*Glycine max*	XP003527209	2.00E-40	323	4
44	receptor kinase At1g27190-like	*Vitis vinifera*	XP002268171	1.89E-55	318	4
45	No hit	-	-	-	281	4
46	fructose-bisphosphate aldolase	*Plantago major*	CAL34034	7.77E-48	269	4
47	xyloglucan endotransglycosylase	*Arabidopsis lyrata*	XP002874875	2.32E-22	254	4
48	No hit	-	-	-	229	4
49	epoxide hydrolase	*Ricinus communis*	XP002516953	3.50E-11	194	4
50	GDSL esterase/lipase	*Glycine max*	XP003521784	3.48E-27	192	4
51	elongation factor 1-alpha	*Cynara cardunculus*	ACC99594	6.36E-33	169	4

Table 2 (cont.)

Contig	Top BLAST Hit	Species	GenBank No.	E-Value	Contig (bp)	No. reads
52	No hit	-	-	-	146	4
53	nucleoside-triphosphatase-like	*Vitis vinifera*	XP002269993	3.14E-68	575	3
54	Cu/Zn superoxide dismutase	*Tetradium ruticarpum*	AFF57842	3.43E-83	574	3
55	erd4 protein	*Davidia involucrata*	AAL47004	1.30E-53	533	3
56	atp binding	*Sorghum bicolor*	XP002441590	5.74E-21	505	3
57	sucrose synthase 1	*Citrus unshiu*	BAA89049	1.65E-81	405	3
58	transcription factor tcp14-like	*Gossypium barbadense*	ABL86669	6.18E-21	404	3
59	protein	*Glycine max*	NP001240231	3.33E-11	396	3
60	60s ribosomal protein l31	*Vernicia fordii*	ACJ02351	2.07E-46	391	3
61	polyketide synthase	*Acer maximowiczianum*	AEK80412	8.39E-74	372	3
62	p-type h+-atpase	*Phaseolus acutifolius*	AAQ19040	5.11E-74	365	3
63	alpha tubulin	*Arabidopsis thaliana*	BAD94893	2.66E-58	353	3
64	heat shock protein 70 kda	*Hordeum vulgare*	CAA10980	5.13E-61	291	3
65	leucine zipper and W2 domain-containing	*Medicago truncatula*	XP003624182	1.40E-33	268	3
66	dna-damage-repair toleration protein drt100	*Populus trichocarpa*	ABK94260	1.13E-26	257	3
67	p-loop containing nucleoside triphosphate hydrolase-like protein	*Glycine max*	XP003538031	3.48E-13	216	3
68	adenosylhomocysteinase s-adenosyl-l-homocysteine hydrolase	*Caragana jubata*	ABI22054	3.16E-38	214	3
69	No hit	-	-	-	182	3
70	No hit	-	-	-	161	3
71	at3g52930-like protein	*Glycine max*	ACU16628	3.01E-20	139	3
72	No hit				93	3
73	No hit				84	3
75	fasciclin-like arabinogalactan protein	*Vitis vinifera*	XP002270426	1.80E-49	544	2
76	FRIGIDA-like	*Vitis vinifera*	XP002282465	3.13E-83	527	2
77	Glycogen synthase kinase-3	*Medicago truncatula*	XP003592909	6.53E-121	518	2
78	translation factor sui1	*Ricinus communis*	XP002522857	1.42E-67	508	2
79	protein toc75	*Vitis vinifera*	CBI16091	9.31E-100	501	2
80	calnexin-like protein	*Populus trichocarpa*	XP002321768	3.49E-77	500	2
81	protein	*Glycine max*	ACU21243	1.26E-35	493	2
82	acyl-CoA thioesterase	*Ricinus communis*	XP002511811	3.95E-23	469	2
83	high mobility group b2 protein	*Gossypium hirsutum*	ADO34795	1.08E-24	468	2
84	pectinacetylesterase family protein	*Glycine max*	XP003536006	7.37E-55	458	2
85	gtp-binding protein	*Sorghum bicolor*	XP002445189	5.12E-64	438	2
86	mitochondrial respiratory chain complexes assembly protein	*Ricinus communis*	XP002530989	9.70E-78	429	2
87	proteasome subunit alpha type 3	*Oryza sativa*	ABR25575	1.80E-45	427	2
88	photolyase blue-light receptor 2	*Medicago truncatula*	ACJ85635	1.75E-62	405	2
89	beta-tubulin	*Populus tremula*	AEK64520	4.45E-90	397	2
90	catalase	*Jatropha curcas*	ADU56189	2.06E-45	359	2
91	protein notum homolog	*Litchi chinensis*	ACF05806	2.51E-37	340	2
92	NADPH cytochrome p450 reductase	*Citrus maxima*	ACP43317	9.76E-63	337	2
93	60s ribosomal protein L8-3	*Glycine max*	XP003537629	2.71E-49	332	2
94	hormone-sensitive lipase	*Ricinus communis*	XP002517206	3.50E-42	328	2
95	protein	*Populus trichocarpa*	XP002298558	8.31E-17	316	2
96	No hit	-	-	-	269	2
97	esterase lipase domain-containing protein	*Glycine max*	ACU23514	5.90E-39	261	2
98	seven transmembrane domain protein	*Populus trichocarpa*	XP002302451	2.49E-46	245	2
99	60s ribosomal protein L13A	*Vernicia fordii*	ACJ02350	2.26E-38	243	2
100	rna-binding csx1-like	*Vitis vinifera*	CBI26626	9.51E-18	212	2
101	beta-tubulin	*Vitis vinifera*	AAF25842	1.15E-28	157	2

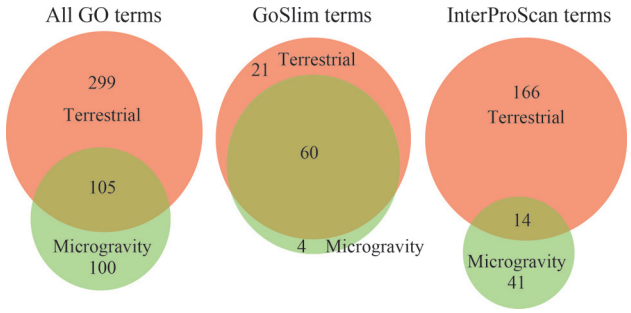

Figure 5 - Gene ontology Venn diagrams for overlap between microgravity and terrestrial SSH library top Blast hits of all contigs and singleton reads.

low differential resolution offered by the GOSlim terms, further comparative analysis was conducted on the full set of GO terms.

The elevated expression of stress-related genes in the spaceflight exposed seedlings was evident in the analysis of the GO terms for the top hits for all contigs and singleton reads. Figure 6 shows the result of a Fisher's Exact Test comparison (Bluthgen *et al.*, 2005) of the enrichment of all GO terms (full set) in the microgravity and terrestrial libraries, where 33% of the microgravity sequences (contigs and singletons) yielded the GO term "response to stress", compared to 13% of the terrestrial sequences. Similarly, the terms "glutathione metabolic process", "glutathione conjugation reaction" and "glutathione transferase activity" were exclusive to the microgravity library. Similar microgravity-exclusive, stress-related terms were "defence response to bacterium" and "response to fungus". Additionally, the term "response to stimulus" was almost doubled in representation in the microgravity library in comparison with the terrestrial sample, and the term "response to oxidative stress" was increased almost four-fold. Conversely, the terrestrial subtracted library was much richer in generalist growth-related terms such as "biological process", "structural molecule activity", and "cellular component biogenesis", among other structural GO terms, and, as stated earlier, possessed nearly double the number of distinct BLAST hits for contigs and an almost three-fold increase in diversity of singleton read hits.

Discussion

The high germination rate and rapid root growth seen in the *A. fraxinifolium* seeds exposed to spaceflight has been observed in other species, such as *Linum*

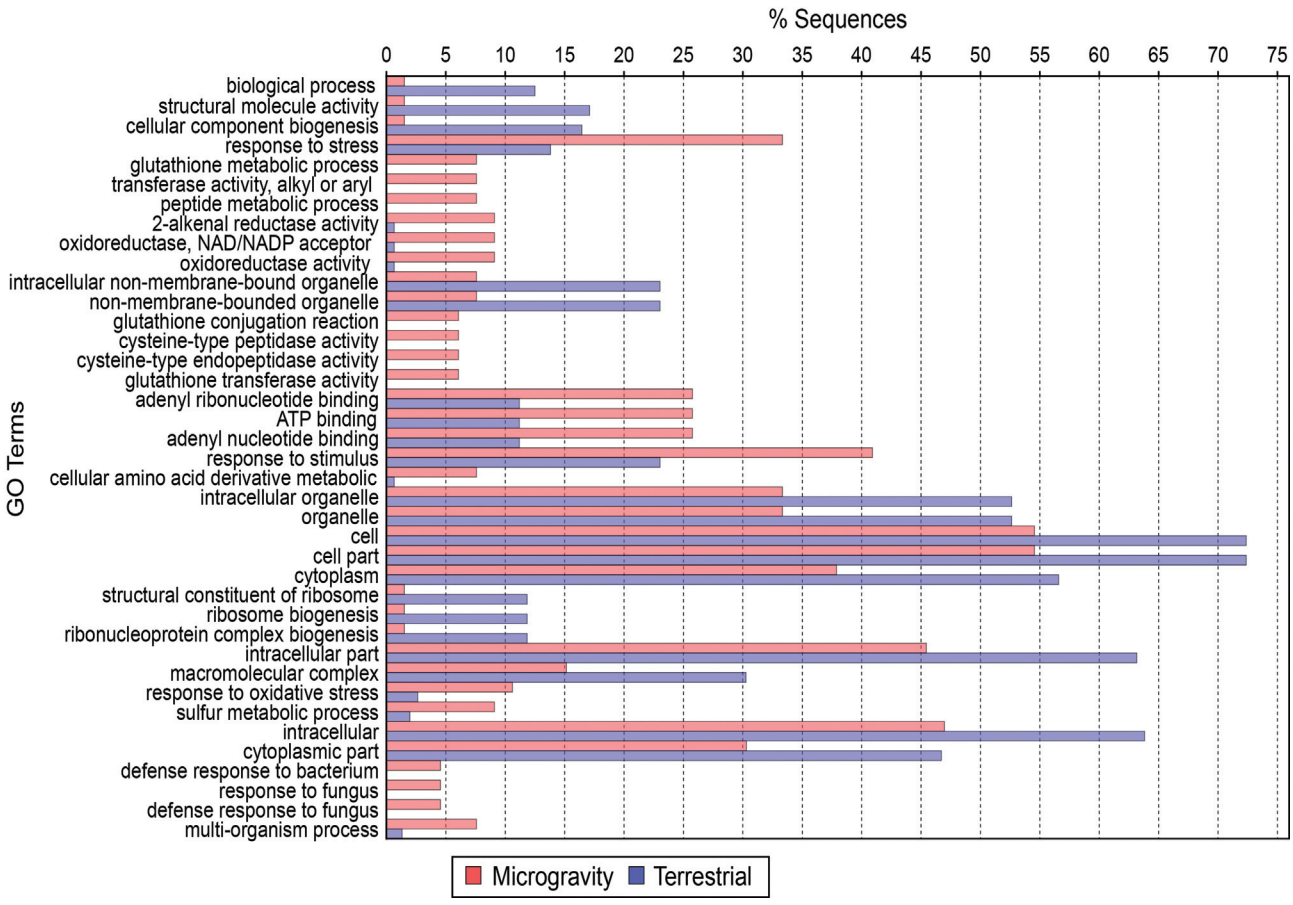

Figure 6 - Fisher's Exact Test (conducted in Blast2GO) comparison of the enrichment of GO terms in the microgravity and terrestrial libraries, with correction for multiple testing.

usitatissimum (Levine *et al.*, 2003), *Glycine max* (Levine *et al.*, 2000) and *Arabidopsis* (Millar *et al.*, 2011). The limited time available on the spaceflight precluded reliable observation of any difference in shoot length in the microgravity and terrestrially germinated seedlings, but early shoot growth stimulation has been observed in other, faster developing plants, such as rice seedlings subjected to simulated microgravity, such as clinorotation (Jagtap *et al.*, 2011), where chlorophyll content was also increased. After prolonged further development under normal gravity, shoot growth appeared not to be affected by microgravity treatment in *A. fraxinifolium*. However, an interesting persistent effect on chlorophyll content was observed, even after 30 days of subsequent terrestrial growth.

The abundance of reads representing rRNA genes in the cDNA libraries is surprising, since these molecules are thought not to be normally polyadenylated, and the library preparation method included both a mRNA purification step and oligo(dT)-primed first strand cDNA synthesis, to avoid spurious rRNA cloning. Possible explanations for the abundance of rRNA genes in our libraries include annealing of the oligo(dT) first strand cDNA synthesis primer to poly-A tracts in the *A. fraxinifolium* rRNA or self-priming by hairpin formation (Gonzalez and Sylvester, 1997). Although inspection of the unclipped rRNA sequences from our libraries showed no convincing evidence of polyadenylated tails, polyadenylation of rRNA has been periodically reported in the literature, where in plants, rRNA polyadenylation was first observed in *Nicotiana tabacum* stressed by exposed to cadmium (Lewandowska *et al.*, 2007).

The presence of multiple heat-shock protein reads in the microgravity-exposed *A. fraxinifolium* seedlings agrees with previous experimental findings in plants exposed to gravity perturbations. In this context, Kozeko and Kordyum (2006) showed that HSP70 and HSP90 levels were both significantly increased in pea seedlings grown under horizontal or vertical clinorotation, simulating microgravity. Similarly, levels of HSPs have been found to increase under hypergravity, where the HSPs could be involved in protein stabilization and quality control as well as signal transduction pathways under altered gravity (Kozeko and Kordyum, 2009). Using an agravitropic mutant of *Arabidopsis thaliana* grown under clinorotation or increased gravity (7 *g*), it was shown that HSP70 and glutathione s-transferase 6, among other proteins, are part of a generalized stress response to gravitational changes (Tan *et al.*, 2011). The glutathione antioxidant pathway has also been shown to be induced in *Xenopus laevis* embryos during simulated microgravity, and was suggested to play a protective role (Rizzo *et al.*, 2009). A further type of stress-related gene present among the microgravity ETSs was polyubiquitin (Table 1, 6 ESTs), where in budding yeast, the stress-inducible polyubiquitin gene, UBI4, has been shown to be upregulated in response to oxidative stress, and has been suggested to play an important role in increasing cellular ubiquitin levels to allow cells to survive under toxic stress conditions (Cheng *et al.*, 1994). Furthermore, polyubiquitin has been shown to be upregulated in rat muscle during spaceflight, and was associated with the stimulation of expression of ubiquitin-proteasome pathway genes and resultant muscle atrophy (Ikemoto *et al.*, 2001). Recent evidence suggests that protein ubiquitination also plays a critical role in regulating responses to abiotic stresses in plants (Lyzenga and Stone, 2012).

It has been shown that peroxidase activity can be depressed under weightlessness in pine seedlings (Cowles *et al.*, 1984), in *Brassica napus* protoplasts (Skagen and Iversen, 2000), and in germinating spores of the aquatic fern, *Ceratopteris richardii*, where three different genes likely to encode peroxidases were down regulated 1.5 to 2.5 fold during spaceflight (Salmi and Roux, 2008). Peroxidase repression in *A. fraxinifolium* during spaceflight may, therefore, have been indicated by its corresponding overrepresentation in the terrestrial SSH library, where peroxidase was the most abundant non-rRNA EST (Table 2; Contigs 02 and 08). In *Arabidopsis*, however, peroxidase expression was shown to be insensitive to six minutes exposure to microgravity on a sounding rocket (Martzivanou *et al.*, 2006).

14-3-3-like protein was also among the more abundant ESTs in the microgravity library (Table 1, contig 9, 8 ESTs), and has been associated with the defence response to abiotic and biotic stress. This protein has been shown to interact with ascorbate peroxidase and may play a regulatory role in the stress response on multiple levels. Important mechanisms of regulation by 14-3-3 include shuttling proteins between different cellular locations and acting as scaffolds for the assembly of larger signalling complexes (Roberts *et al.*, 2002). In *Drosophila* cells, expression of 14-3-3 protein has been shown to be heat shock-related and, in cooperation with Hsp70/Hsp40, was demonstrated to mediate the resolubilization and reactivation of heat-aggregated citrate synthase, where 14-3-3 protein or Hsp70/Hsp40 alone, lacked the activity (Yano *et al.*, 2006). In the context of an induction of a generalized stress response in the microgravity-treated seedlings, it would not be surprising, therefore, to detect the enrichment of 14-3-3-like protein transcripts along with several other stress-related ETSs. The enrichment of 14-3-3-like protein ESTs may also be correlated with the longer root length observed in the microgravity-treated seedlings, where these proteins have recently been demonstrated to play an important role in both root and chloroplast development in *Arabidopsis*. Here, 14-3-3 defective mutants have shorter roots than wild-type, but show increased root greening (Mayfield *et al.*, 2012). In the context of the observed higher chlorophyll content of the microgravity-treated seedlings, 14-3-3 proteins, together with the Hsp70 molecular chaperone, are thought to play a role in chloroplast development, guiding

phosphorylated chloroplast precursors towards their destinations (May and Soll, 2000).

It would appear that a major consequence for germination during spaceflight for the *A. fraxinifolium* seeds was an at least temporary switch to a "stress" mode of growth and a quantitative reduction in general metabolism, when compared to terrestrially germinated seeds. This was despite the phenotypic effects observed, where the microgravity sample germinated more rapidly and more homogenously. The latter observation may, in part, explain the increased variety of ESTs in the terrestrial sample, which, being less synchronized than the spaceflight-sample, may have possessed a greater variety of temporally-expressed mRNAs, quantitatively affecting the subtractive hybridization results.

The gene expression responses to microgravity-induced stresses are likely to be variable over time and difficult to standardize between different model- and experimental systems. An example of this was found in rat cells, where the activity of the intracellular antioxidant enzymes, superoxide dismutase, glutathione peroxidase, and catalase, was all significantly increased at 12 h after the microgravity onset, yet decreased at 96 h (Wang *et al.*, 2009). Preparation procedures for spaceflight and the non-ideal environmental conditions on board the ISS subject organisms to additional environmental stresses that demonstrably affect gene expression. The vibrational stresses incurred during space vehicle launch and re-entry and transient hypergravity during acceleration are likely to have a physiological impact on biological systems. The stress gene responses induced by vibration, however, may differ from purely microgravity-related responses, where, for example, mechanical stresses and vibration did not cause the up-regulation of mRNA for hsp70 and hsp27 in human lymphocytes (Cubano and Lewis, 2001). Importantly, the response of whole plants or seedlings may differ to that of cultured cells. In *Arabidopsis thaliana* seedlings, up-regulation of *TCH* (touch) gene expression and an increase in hypocotyl elongation was demonstrated in response to vibration at 50 Hz for 72 h, though the response was weaker than in touch-stimulated plants (Johnson *et al.*, 1998).

Logistical problems are a major complication for gene expression analysis in true spaceflight scenarios, where in the current study, three days of terrestrial development during transit to our laboratory in Brazil was added to the nine day spaceflight time, before samples could be stabilized. Despite this, however, the effects of microgravity on *A. fraxinifolium* seedlings appear to be persistent, at least for three days following their return to normal gravity, and longer when the increased chlorophyll content in the 30 day plantlets is considered. An example of the long-term effects of stress on plants is priming of defence in response to pathogen attack, which is a strategy employed by stressed plants to enhance resistance against future stress episodes with minimal associated costs on growth. Worrall *et al.* (2012) showed that tomato seeds treated with the signalling molecules, jasmonic acid or β-aminobutryric acid, displayed increased resistance for up to eight weeks to a range of pests and diseases. A growing body of evidence indicates that stress can induce persistent and substantial changes at the chromatin level in plants, with concomitant changes in gene expression. The long-term duration and heritability of these changes, however, is controversial (Pecinka and Scheid, 2012). Simulated microgravity experiments performed on the ground, under ideal conditions, using a random position machine or clinostat, may show much more subtle effects on gene expression (Herranz *et al.*, 2010).

Although localized hypoxia, caused by the design of the germination kits utilized in our experiment, may be a source of stress for the germinating seedlings, this aspect of the experiment could have been eliminated by the use of identical kits for the terrestrial controls. Differing convection currents and variations in heat and gas exchange during spaceflight and their effects on plants have been investigated (Kitaya *et al.*, 2006), but these effects are, however, difficult to reproduce accurately on the ground in the control kits. Also difficult to control and separate from the direct microgravity-induced effects is the increased exposure of living tissue to cosmic radiation as well as variation in magnetic flux during spaceflight. Despite the fact that reported gene expression effects are frequently similar in real and simulated microgravity experiments, the synergistic effects of the spaceflight environment on microgravity responses cannot be discounted (Beckingham, 2010; Herranz *et al.*, 2010).

Our results largely agree with many other experiments using more widely studied model plants, where a general theme of the induction of multiple stress genes in response to microgravity is usually seen. Since this is the first gene expression analysis of the germination during spaceflight of a tropical tree species, this study represents only the first glimpse of the response of these plants to this environment. Terrestrial clinostat experiments with accompanying EST analyses, involving *A. fraxinifolium* seedlings, would enable us to partially confirm some of the findings in the present study, where the timing and longevity of the response to gravitational stress would be interesting to investigate in detail, which would be greatly facilitated in purely ground-based experiments. More material reserved for molecular analysis would have allowed us to investigate the microgravity response in greater detail. In particular, it would be interesting to dissect the differential response of shoot and root, in both light and darkness, to microgravity. The logistical problems involved in future true microgravity experiments may be somewhat alleviated by sample fixation by rapid freezing, or more practically using room temperature chemical fixative reagents such as RNAlater (Qiagen), which can be applied by non-specialists during spaceflight. These measures will make future microgravity experiments much easier to interpret.

Acknowledgments

The authors thank the Brazilian astronaut, Lt. Col. Marcos Cesar Pontes, for his implementation of the GSM experiment aboard the International Space Station (ISS). Financial support was kindly provided by Empresa Brasileira de Pesquisa Agropecuária - Embrapa (Brazil) and Fundação Arthur Bernardes - Funarbe (Brazil).

References

Aguiar AV, Coelho ASG, Moura MF, Morais K, Pinheiro JB, Moraes MLT, Zucchi MI, Moura NF and Vencovsky R (2004) Autocorrelação espacial de caracteres morfológicos em populações naturais de gonçalo-alves (*Astronium fraxinifolium* Schott.) Biosci J (Uberlândia) 20:151-160.

Beckingham KM (2010) Synergy between stresses: An interaction between spaceflight-associated conditions and the microgravity response. Mol Ecol 19:4105-4107.

Blüthgen N, Brand K, Cajavec B, Swat M, Herzel H and Beule D (2005) Biological profling of gene groups utilizing gene ontology. Genome Inform 16:106-115.

Cheng L, Watt R and Piper PW (1994) Polyubiquitin gene expression contributes to oxidative stress resistance in respiratory yeast (*Saccharomyces cerevisiae*). Mol Gen Genet 243:358-362.

Conesa A and Götz S (2008) Blast2GO: A comprehensive suite for functional analysis in plant genomics. Int J Plant Genomics 2008:619832.

Cowles JR, Scheld HW, Lemay R and Peterson C (1984) Growth and lignification in seedlings exposed to eight days of microgravity. Ann Bot 54(suppl 3):33-48.

Cubano LA and Lewis ML (2001) Effect of vibrational stress and spaceflight on regulation of heat shock proteins hsp70 and hsp27 in human lymphocytes (Jurkat). J Leucoc Biol 69:755-761.

Diatchenko L, Lau YFC, Campbell AP, Chenchik A, Moqadam F, Huang B, Lukyanov S, Lukyanov K, Gurskaya N, Sverdlov ED, *et al.* (1996) Suppression subtractive hybridization: A method for generating differentially regulated or tissue-specific cDNA probes and libraries. Proc Natl Acad Sci USA 93:6025-6030.

Gomes MSO, Sinnecker P, Tanaka RT and Lanfer-Marquez UM (2003) Effect of harvesting and drying conditions on chlorophyll levels of soybean (*Glycine max* L. Merr). J Agric Food Chem 51:1634-1639.

Gonzalez IL and Sylvester JE (1997) Incognito rRNA and rDNA in databases and libraries. Genome Res 7:65-70.

Gurskaya NG, Diatchenko L, Chenchik A, Siebert PD, Khaspekov GL, Lukyanov KA, Vagner LL, Ermolaeva OD, Lukyanov SA and Sverdlov ED (1996) Equalizing cDNA subtraction based on selective suppression of polymerase chain reaction: Cloning of Jurkat cell transcripts induced by phytohemaglutinin and phorbol 12-myristate 13-acetate. Anal Biochem 240:90-97.

Herranz R, Benguría A, Laván DA, López-Vidriero I, Gasset G, Javier Medina F, van Loon JJ and Marco R (2010) Spaceflight-related suboptimal conditions can accentuate the altered gravity response of *Drosophila* transcriptome. Mol Ecol 19:4255-4264.

Hulsen T, de Vlieg J and Alkema W (2008) BioVenn - A web application for the comparison and visualization of biological lists using area-proportional Venn diagrams. BMC Genomics 9:e488.

Ikemoto M, Nikawa T, Takeda S, Watanabe C, Kitano T, Baldwin KM, Izumi R, Nonaka I, Towatari T, Teshima S, *et al.* (2001) Space shuttle flight (STS-90) enhances degradation of rat myosin heavy chain in association with activation of ubiquitin-proteasome pathway. FASEB J 15:1279-1281.

Jagtap SS, Awhad RB, Santosh B and Vidyasagar PB (2011) Effects of clinorotation on growth and chlorophyll contents of rice seeds. Microgravity Sci Technol 23:41-48.

Johnson JA, Sistrunk ML, Polisensky DH and Braam J (1998) *Arabidopsis thaliana* responses to mechanical stimulation do not require ETR1 or EIN2. Plant Physiol 116:643-649.

Kitaya Y, Kawai M, Takahashi H Tani A, Goto E, Saito T, Shibuya T and Kiyota M (2006) Heat and gas exchanges between plants and atmosphere under microgravity conditions. Ann N Y Acad Sci 1077:244-255.

Kordyum EL (1994) Effects of altered gravity on plant cell processes: Results of recent space and clinostatic experiments. Adv Space Res 14:77-85.

Kozeko L and Kordyum E (2006) The stress protein level under clinorotation in context of the seedling developmental program and the stress response. Microgravity Sci Tech 18:254-256.

Kozeko L and Kordyum E (2009) Effect of hypergravity on the level of heat shock proteins 70 and 90 in pea seedlings. Microgravity Sci Tech 21:175-178.

Laurinavicius R, Svegzdiene D, Rakleviciene D and Kenstaviciene P (2001) Ontogeny of plants under various gravity conditions. Adv Space Res 28:601-606.

Levine HG, Sharek JA, Johnson KM, Stryjewski EC, Prima VI, Martynenko OI and Piastuch WC (2000) Growth protocols for etiolated soybeans germinated within BRIC-60 canisters under spaceflight conditions. Adv Space Res 26:311-314.

Levine HG, Anderson K, Boody A, Cox D, Kuznetsov OA and Hasenstein KH (2003) Germination and elongation of flax in microgravity. Adv Space Res 31:2261-2268.

Lewandowska M, Borcz B, Kaminska J, Wawrzynski A and Sirko A (2007) Polyadenylation and decay of 26S rRNA as part of *Nicotiana tabacum* response to cadmium. Acta Biochim Pol 54:747-755.

Lyzenga WJ and Stone SL (2012) Abiotic stress tolerance mediated by protein ubiquitination. J Exp Bot 63:599-616.

Martzivanou M, Babbick M, Cogoli-Greuter M and Hampp R (2006) Microgravity-related changes in gene expression after short-term exposure of *Arabidopsis thaliana* cell cultures. Protoplasma 229:155-162.

May T and Soll J (2000) 14-3-3 Proteins form a guidance complex with chloroplast precursor proteins in plants. Plant Cell 12:53-63.

Mayfield JD, Paul AL and Ferl RJ (2012) The 14-3-3 proteins of *Arabidopsis* regulate root growth and chloroplast development as components of the photosensory system. J Exp Bot 63:3061-3070.

Millar KDL, Johnson CM, Edelman RE and Kiss JZ (2011) An endogenous growth pattern of roots is revealed in seedlings grown in microgravity. Astrobiology 11:787-797.

Miranda LPM, Tarsitano MAA, Alves MC and Rodrigues RAF (2011) Custo para implementação de *Astronium fraxini-*

folium Schott em área degredada utilizando-se adubos verdes e lodo de esgoto. Pesq Agropec Trop 41:475-480.

Morita MT and Tasaka M (2004) Gravity sensing and signaling. Curr Opin Plant Biol 7:712-718.

Musgrave ME, Kuang A, Xiao Y, Stout SC, Bingham GE, Briarty LG, Levenskikh MA, Sychev VN and Podolski IG (2000) Gravity independence of seed-to-seed cycling in *Brassica rapa*. Planta 210:400-406.

Paul AL, Zupanska AK, Ostrow DT, Zhang Y, Sun Y, Li JL, Shanker S, Farmerie WG, Amalfitano CE and Ferl RJ (2012) Spaceflight transcriptomes: Unique responses to a novel environment. Astrobiology 12:40-56.

Pecinka A and Scheid OM (2012) Stress-induced chromatin changes: A critical view on their heritability. Plant Cell Physiol 53:801-808.

Rizzo AM, Montorfano G, Negroni M, Corsetto P, Berselli P, Marciani P, Zava S and Berra B (2009) Simulated microgravity induce glutathione antioxidant pathway in *Xenopus laevis* embryos. Cell Biol Int 33:893-898.

Roberts MR, Salinas J and Collinge DB (2002) 14-3-3 Proteins and the response to abiotic and biotic stress. Plant Mol Biol 50:1031-1039.

Rodriguez-Amaya DB (1999) A Guide to Carotenoid Analysis in Food. ILSI Press, Washington, 65 pp.

Salmi ML and Roux SJ (2008) Gene expression changes induced by space flight in single-cells of the fern *Ceratopteris richardii.* Planta 229:151-159.

Skagen EB and Iversen TH (2000) Effect of simulated and real weightlessness on early regeneration stages of *Brassica napus* protoplasts. In Vitro Cell Dev Biol Plant 36:312-318.

Sychev VN, Levenskikh MA, Gostimsky SA, Bingham GE and Podolsky IG (2007) Spaceflight effects on consecutive generations of peas grown onboard the Russian segment of the International Space Station. Acta Astronaut 60:426-432.

Tan C, Wang H, Zhang Y, Qi B, Xu G and Zheng H (2011) A proteomic approach to analyzing responses of *Arabidopsis thaliana* root cells to different gravitational conditions using an agravitropic mutant, pin2 and its wild type. Proteome Sci 9:72.

Wang J, Zhang J, Bai S, Wang G, Mu L, Sun B, Wang D, Kong Q, Liu Y, Yao X, *et al.* (2009) Simulated microgravity promotes cellular senescence via oxidant stress in rat PC12 cells. Neurochem Int 55:710-716.

Worrall D, Holroy GH, Moore JP, Glowacz M, Croft P, Taylor JE, Paul ND and Roberts MR (2012) Treating seeds with activators of plant defense generates long-lasting priming of resistance to pests and pathogens. New Phytol 193:770-778.

Yano M, Nakamuta S, Wu X, Okumura Y and Kido H (2006) A novel function of 14-3-3 protein: 14-3-3 ζ is a heat-shock-related molecular chaperone that dissolves thermal-aggregated proteins. Mol Biol Cell 17:4769-4779.

Associate Editor: Adriana S. Hemerly