Proteomic Signature of Arabidopsis Cell Cultures Exposed to Magnetically Induced Hyper- and Microgravity Environments

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Abstract

Earth-based microgravity simulation techniques are required due to space research constraints. Using diamagnetic levitation, we exposed *Arabidopsis thaliana in vitro* callus cultures to environments with different levels of effective gravity and magnetic field strengths (B) simultaneously. The environments included simulated $0g^*$ at $B=10.1$ T, an internal $1g^*$ control ($B=16.5$ T), and hypergravity ($2g^*$ at $B=10.1$ T). Furthermore, samples were also exposed to altered gravity environments that were created with mechanical devices, such as the Random Positioning Machine (simulated μg) and the Large Diameter Centrifuge ($2g$). We have determined the proteomic signature of cell cultures exposed to these altered-gravity environments by means of the difference gel electrophoresis (DiGE) technique, and we have compared the results with microarray-based transcriptomes from the same samples. The magnetic field itself produced a low number of proteomic alterations, but the combination of gravitational alteration and magnetic field exposure produced synergistic effects on the proteome of plants (the number of significant changes is 3–7 times greater). Tandem mass spectrometry identification of 19 overlapping spots in the different conditions corroborates a major role of abiotic stress and secondary metabolism proteins in the molecular adaptation of plants to unusual environments, including microgravity. Key Words: DiGE—Microgravity simulation—Magnetic levitation—Proteome/transcriptome comparison—Callus cell cultures. *Astrobiology* 13, 217–224.

TO STUDY THE EFFECT of an environment with suppressed gravitational forces, we have to place samples in orbit by means of spaceflights or sounding rockets, or use simulation facilities on the ground. Mechanical facilities for microgravity, such as 2-D clinostats or random positioning machines, and centrifuges for hypergravity, like the Large Diameter Centrifuge (LDC) (Hoson *et al.*, 1992; Kraft *et al.*, 2000; van Loon *et al.*, 2004; van Loon, 2007), are used as experimental approaches to the study of the effects of altered gravity, including gravitation-dependent proteomic analyses (Wang *et al.*, 2006; Barjaktarovic *et al.*, 2007, 2009), and help avoid the costs and constraints of space experimentation. Nevertheless, it is not clear whether the use of such machines creates either a stimulus-free environment with respect to gravity (simulated weightlessness) or an omnilateral gravistimulation with strong mechanical disturbances. An alternative approach to

study the response of organisms to changes in gravity is the use of diamagnetic levitation (Beaugnon and Tournier 1991a, 1991b; Berry and Geim 1997; Valles *et al.*, 1997). Since diamagnetic material is repelled by magnetic fields, when a diamagnetic object is positioned in a magnetic field gradient it experiences a magnetic force away from regions of high field. The magnitude of the force is proportional to the product of the field strength (B) by the field gradient (the spatial derivative of the field, B'). When $[B \times B']$ is strong enough, this magnetic force can be used to counterbalance the gravitational force, leading to the levitation of a large variety of materials such as water and other fluids. Since the bulk of living organisms is composed of diamagnetic material, mostly water, organisms can be magnetically levitated, provided $[B \times B']$ is about $1400 \text{ T}^2/\text{m}$. Stable magnetic levitation is only possible in a dozen magnets around the world, at a field

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FIG. 1. Altered-gravity ground-based facilities and experimental approach. (A) Magnetic levitator picture including a detail of the sample container and location of the samples in the magnet bore. (B) Mechanical ground-based facilities used to produce altered gravity on ground (RPM as a microgravity simulator and LDC, a 2g centrifuge). (C) Example of the three-step experimental approach performed to locate, quantify, and identify one protein affected by hypergravity treatments (GAPC1). (Color images available online at www.liebertonline.com/ast)

strength of at least 16 T, so this technology was made available to the space biologist community only some years ago (Valles *et al.*, 2005; Guevorkian and Valles 2006; Beuls *et al.*, 2009; Hammer *et al.*, 2009; Dijkstra *et al.*, 2011; Herranz *et al.*, 2012; Hill *et al.*, 2012; Manzano *et al.*, 2012).

We have studied the effect of an exposure of 200 min to an environment of altered gravitational and magnetic forces on the overall proteomic profile of *Arabidopsis thaliana* semisolid cell cultures (callus). The present study is the first systematic multifacility, high-throughput, environmentally controlled collection of experiments that has been performed with the same setup, almost simultaneously in two mechanical facilities (RPM for simulated μg and LDC for 2g) and in a magnet-based facility, with the use of three different effective gravity (g^*) conditions [2g*, 1g*, and 0g*, namely, the calculated effective gravity for pure water in the center of each culture chamber, assuming that variations due to differential magnetic susceptibility of intracellular material are below biological sensitive (Schenck 1992; Valles *et al.*, 1997); see also Herranz *et al.* (2013) for a discussion about terminology]. This made possible inter-experiment comparisons of the results and a pooled analysis with multiple inner controls at similar magnetic fields. Apart from testing what kind of facility would be most suitable and reliable as an altered gravity simulator, a major achievement of this study was the comparison of the proteomic profiles with the transcriptomic results obtained from the same samples [Agilent two-color 44k whole genome microarray data sets, GEO ID: GSE29787 (Manzano *et al.*, 2012)].

A detailed description of the experimental setup, the levitation magnet (Fig. 1A), and the mechanical simulators (Fig. 1B) was provided in a parallel transcriptomic paper (Manzano *et al.*, 2012). In short, we exposed samples to three different conditions (g^* and B fields) within a high field magnet (HFML, Radboud University Nijmegen, the Netherlands) (Perenboom *et al.*, 2004; Wiegers *et al.*, 2010). The magnitude g^* denotes the value of the effective gravity (the asterisk reflects the presence of the background magnetic field) in each of the three positions within the magnet (Table 1). In the center of the magnet bore (1g* position), the magnetic field strength is maximal (16.5 T), but the magnetic field gradient is zero. At a distance of 81.6 mm above this position, at $B=10.1$ T, the diamagnetic force on water counterbalances the force of gravity, which leads to stable levitation; this is the 0g* position

(zero gravity is only reached in a single point). At a distance of 81.6 mm below the field center, also at $B=10.1$ T, the sum of the levitation and gravitational forces produces a 2g* effective force. In both cases, we consider that the effective gravity applied on *Arabidopsis* culture cells (free of starch statoliths) is similar to the one of water. We base this assumption on the fact that cells' main component is water, and most organic compounds within the cell have similar diamagnetic properties as those of water (Schenck 1992; Valles *et al.*, 1997). Additionally, we exposed samples to a mechanical simulator of microgravity, the Random Positioning Machine (μg -RPM, real random mode), and to the Large Diameter Centrifuge (2g-LDC) for mechanical generation of hypergravity, both facilities located in the European Space Research and Technology Centre [ESA-ESTEC, Noordwijk, the Netherlands, Fig. 1B (van Loon, 2007; van Loon *et al.*, 2008)]. In each facility, samples were exposed for 200 min, at $22^\circ\text{C} \pm 0.1^\circ\text{C}$, in darkness. External controls were kept at room temperature ($22^\circ\text{C} \pm 0.1^\circ\text{C}$, 1g ground gravity), away from the magnet, LDC, or RPM. As a model system, we used callus semisolid cultures of *Arabidopsis thaliana* prepared from MM2d suspension cultures (Menges and Murray, 2006). For all devices and conditions, the suspension cultures were strewn on the agar surface 1 week before the start of the experiment and grown at 22°C to allow the callus to reach maximum density in a 1–2 mm thick layer to minimize variations in the magnetic field and of the effective g -force. The samples were preserved immediately after treatment (less than 2 min) by quick freezing in liquid nitrogen and subsequent storage, first in dry ice for transport and then under deep freezing.

Samples were analyzed as to their proteomic profile as summarized in the example in Fig. 1C by the two-dimensional difference gel electrophoresis (2-D-DiGE) technique. Protein was extracted from three biological replicates as described by Barjaktarovic *et al.* (2007) and purified by methanol/chloroform precipitation. An amount of 40 μg of control or experimental protein samples was stained with 400 pmol of Cy3 or Cy5 (CyDryes manufacturer, GE Healthcare). Standard control for normalization between gels consisted of a pool of the samples at the same concentration stained with Cy2. DiGE first dimension of electrophoresis was performed in an IPG-phor IEF System with a linear 3–11 pH (GE Healthcare). DiGE second dimension was performed on an Ettan Dalt Six by using 12.5% SDS-PAGE at 20°C . Gels were scanned at 100 μm resolution in a Typhoon 9400 scanner (GE Healthcare). Data analysis was performed by using two or three replicates with DeCyder software v7.0, Differential In-Gel Analysis (DIA) and Biological Variation Analysis (BVA) for which, as filter, a fold change ≥ 1.2 or ≤ -1.2 was used with a Student t test to extract significant results between replicates ($p < 0.05$).

The results show that a 200 min treatment in mechanical facilities did not produce any significant effect at the proteomic scale (Table 1). In fact, we were able to detect one spot significantly altered in the RPM and two in the LDC only when removing the most variable replicate, which suggests that we were only detecting highly significant variations. The effect of a high magnetic field is moderate, producing more decreased than increased spots; a clearly stronger effect is observed in the 0g* and 2g* positions, especially detected in the 2g* position. These effects are quite similar to the synergic effect found at the transcriptomic level with the same samples (Manzano *et al.*, 2012), reinforcing the idea of an

TABLE 1. NUMBER OF SPOTS SHOWING ALTERED CONCENTRATIONS ($p < 0.05$) UNDER DIFFERENT EFFECTIVE GRAVITY (g^*) AND MAGNETIC/MECHANICAL CONDITIONS

Effective force (g)	0g*	1g*	2g*	μg RPM	2g LDC
Magnetic field (B)	10.1 T	16.5 T	10.1 T	—	—
Mechanical forces	—	—	—	YES	YES
Spots ≥ 1.2 -fold	22 (13)	4 (15)	56 (34)	1 (0)	1 (0)
Spots ≤ -1.2 -fold	38 (17)	14 (20)	61 (55)	0 (0)	2 (0)
TOTAL	60 (30)	18 (35)	117 (89)	1 (0)	3 (0)

— indicated that magnetic/mechanical perturbations are minimal and similar to the control for those samples. Number of significantly altered spots was determined by using a Student t test ($p < 0.05$) with two replicates. The numbers obtained, including a more variable third replicate, are shown between brackets for comparison.

TABLE 2. LOCALIZATION, FOLD CHANGE, AND DATABASE IDENTIFICATION OF THE 19 SPOTS AFFECTED IN SEVERAL ALTERED GRAVITY/MAGNETIC FIELD CONDITIONS

Experiment sample	Master sample number	Spot number	t test (p < 0.05)	Average MASCOT ratio	Candidate protein gi and description
RPM	Control RPM	1945	1854	0.021	1.26 <72 Not significant results in the database
LDC	Control LDC	907	996	0.046	1.44 519# gi15227981, fructose-bisphosphate aldolase
					308# gi15229231, GAPC1 (GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE C SUBUNIT 1)
					227 gi15239741, cinnamyl-alcohol dehydrogenase (CAD)
					226 gi15238762, GDH1 (GLUTAMATE DEHYDROGENASE 1)
	Control LDC	1193	1291	0.034	-1.39 204 gi15217446, ATCEL3 (CELLULOSE 3)
					128 gi297849086, T6D22.2 (<i>Arabidopsis lyrata</i>)
	Control LDC	1683	1747	0.031	-1.21 622 gi15221116, ATGLX1 (GLYOXALASE I Homolog)
1g*	Control 1g	1899	1859	0.046	-2.52 250 gi15225374, ATPHB6 (PROHIBITIN 6)
		2375	2316	0.0069	-2.55 197 gi13899069, <i>A. thaliana</i> unknown protein
0g*	Control 1g	928	1058	0.0064	-1.28 <72 Not significant results in the database
	Control 1g*	1157		0.011	-1.36
	Control 1g	2180	2365	0.016	-2.24 <72 Not significant results in the database
	Control 1g*	2364		0.040	-1.54
	Control 1g	717	844	0.00043	-2.25 270# gi15222981, Sks5 (SKU5 Similar 5)
	Control 1g	846	975	0.039	2.07 650 gi227202752, AT1G56340 ATCRT1A (calreticulina)
0g* and 2g*	Control 1g	663	788 (0g*)	0.020	-1.66 687 gi4467097, heat shock protein 70 like protein
			664 (2g*)	0.015	-2.69 208 gi15219234, VHA-A (VACUOLAR ATP SYNTHASE SUBUNIT A)
		749	877 (0g*)	0.0022	-2.08 620# gi15222981, Sks5 (SKU5 Similar 5)
			776 (2g*)	0.016	-1.46

(continued)

TABLE 2. (CONTINUED)

Experiment sample	Master sample number	Spot number	t test (p < 0.05)	Average ratio	MASCOT score	Candidate protein gi and description
2g*						
Control 1g	939	1062	0.014	4.91	141	gi15229595, chaperonin, putative
Control 1g*	1076		0.035	4.86	117#	gi15224879, BGLU15 (BETA GLUCOSIDASE 15)
Control 1g	960	1083	0.0070	6.79	194#	gi15224879, BGLU15 (BETA GLUCOSIDASE 15)
Control 1g*	1082		0.0047	9.78		
Control 1g	1186	1318	0.022	-1.84	302	gi14594802, translation initiation factor eIF-4A1
					254	gi15229033, MTO3 (Methionine over-accumulator3)
Control 1g*	1266		0.021	-1.71	123	gi119698385, putative cytosolic glutamine synthetase [<i>Populus tremula</i> × <i>Populus alba</i>]
Control 1g	1400	1543	0.0013	-1.87	354#	gi15229231, GAPC1 (GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE C SUBUNIT 1)
					241	gi42572317, <i>A. thaliana</i> unknown protein
Control 1g*	1527		0.0046	-1.55	185	gi15224592, ASP1 (Aspartate aminotransferase1)
					115#	gi15227981, fructose-bisphosphate aldolase
Control 1g	1876	2043	0.0013	-1.49	142	gi297808075, hypothetical protein ARALYDRAFT_910048 [<i>Arabidopsis lyrata</i>]
Control 1g*	1972		0.028	-1.42		
Control 1g	2100	2285	0.015	-1.90	326	gi15228276, TCTP (TRANSLATIONALLY CONTROLLED TUMOR PROTEIN)
Control 1g*	2193		0.023	-1.71		
Control 1g	2256	2462	0.00081	-2.57	301	gi30697298, ADF3 (ACTIN DEPOLYMERIZING FACTOR 3)
Control 1g*	2318		0.024	-2.00		

Spots appearing in more than one gravitational condition (comparison based on master and spot numbers) are shown, including statistical significance (*t* test) and average fold change values (in Average ratio, in italics when reduced, in bold when increased in concentration). Mascot values are also shown for the significant candidates only (it is indicated by dashed lines when more than one candidate is found for the same spot). # highlights candidates identified in another spot. See Supplementary Material for additional method details.

enhanced impact of altered gravity on biological systems under suboptimal environments (Herranz *et al.*, 2010, 2012).

To analyze more deeply the proteomic response, we decided to identify the more representative altered spots in several pair comparisons. We chose 19 spots (Table 2), namely, (a) those altered in the RPM/LDC samples, (b) two spots especially easy to isolate with high fold changes in the 0g* position, (c) those spots appearing simultaneously altered in any g* level after comparing its proteomic profile with both the internal (1g*) and the external (1g) controls, (d) two common spots of the comparisons of 0g* and 2g* with 1g, and (e) two outstanding spots revealed by the comparison of 0g* with the external 1g control. Once the spots of interest were identified and located, trypsin-digested spots were analyzed by a 4800 MALDI-TOF-TOF facility (MacCarrone *et al.*, 2010). MASCOT searches with nrNCBI protein database (viridiplantae only) were used to identify the candidate proteins that better fit with the mass spectrum obtained for each spot tryptic peptides, up to eight fragmentation peptides analyzed per spectrum (score higher than 72 to have a $p < 0.05$).

Candidate proteins that appeared during the analysis were easily identified as involved in cellular response to stress conditions, like chaperonin (4.91-fold increase in 2g*), β -glucosidase-15 (almost 10-fold increase in 2g*), and Hsp70 (slightly but significantly decreased in both 0g* and 2g*), together with ATP synthase VHA-A and oxidoreductase Sks5, which appeared in two different spots. Primary and secondary metabolism enzymes usually appear to be affected by environmental stress conditions (Liu *et al.*, 2011), and some of them were also found to be modified in our study (Table 2). Previously performed proteomic analyses with the use of 2-D electrophoresis cross comparisons identified similar types of proteins that were affected by mechanically altered gravity conditions, among which fructose-bisphosphate aldolase in particular was mentioned (Wang *et al.*, 2006; Barjaktarovic *et al.*, 2007, 2009). In our work, in which spots were quantified in the same gel (DiGE), only a few variations were found to be significant in the mechanical facilities, whereas the synergic action of two stressing conditions (magnetic field and altered gravity) revealed proteomic changes similar to those reported in the literature.

Finally, we compared these specific proteins with the genes we previously found to be affected with the use of an Agilent array on the same samples. Most of the identified

matches affected similarly both hypergravity environments (2g* in the magnet and LDC) (Table 3). It must be kept in mind that the decrease of one spot could mean that the affected protein has been postrationally modified (transformed into a new spot), becoming even more active; this would explain apparent inconsistencies between proteomic and transcriptomic data sets. In fact, we found additional partial matches [e.g., protein GAPC-1, used as example in Fig. 1C, match gene is not affected; but a similar gene, GAPC-2 (AT1G12900), is] that would cross-validate both proteomic and transcriptomic data sets.

Summarizing, we conclude that a 200 min exposure to mechanically altered gravity produces very few alterations in our environmentally controlled system in comparison with other studies in which *Arabidopsis* callus was exposed to clinorotation or centrifugation with other designs (Wang *et al.*, 2006; Barjaktarovic *et al.*, 2007, 2009). This result can be explained by the different sensitivities of other proteomic approaches (DiGE technology produces more statistically reliable results than independent 2-D gels but provides less number of spots detected) and also by the different duration of the treatment and source of the biological material (for instance, when using seedlings, cellular plus tissular proteomic responses will be observed). Nevertheless, our results are consistent with those of previous studies, which reinforces the idea that the alteration of gravity, together with other physical forces, promotes an abiotic stress response that provides the cells some adaptation benefits in the context of a new environmental situation at genomic and proteomic levels (Barjaktarovic *et al.*, 2007, 2009; Manzano *et al.*, 2012). In fact, several plant species have the capacity to cope with extreme environmental conditions by modifying secondary metabolism, stress, and repair pathways in a concerted way (Van Cutsem *et al.*, 2011; Forster *et al.*, 2012; Payyavula *et al.*, 2012).

Using magnetic forces seems to increase the system susceptibility to altered gravity and affects similar proteins as those that have been described in other studies in which mechanical simulators were used. The same gene ontology functions have been detected by transcriptomic analyses (Manzano *et al.*, 2012). Similar enhanced effects of microgravity on the transcriptomic profile have been observed before when suboptimal environmental conditions were added to the altered gravity stimulus (Herranz *et al.*, 2010, 2012; Manzano *et al.*, 2012). It is possible that the observed

TABLE 3. COINCIDENCES BETWEEN PROTEOMIC AND TRANSCRIPTOMIC IDENTIFIED PROTEIN/GENE

Protein description	Proteome changes		Transcriptome changes	
	Affected positions	Array ID	Affected positions	
gi15227981, fructose-bisphosphate aldolase	LDC and 2g*	AT2G36460	LDC and 2g*	
gi15229231, GAPC1 (Glyceraldehyde-3-phosphate dehydrogenase c subunit 1)	LDC and 2g*	AT3G04120	LDC and 2g*	
gi15239741, CAD (Cinnamyl-alcohol dehydrogenase)	<i>LDC</i>	AT3G19450	<i>LDC</i>	
gi15238762, GDH1 (Glutamate dehydrogenase 1)	LDC	AT5G18170	LDC and 0g*	
gi14594802, translation initiation factor eIF-4A1	<i>2g*</i>	AT1G51380	2g* and LDC	
gi15224592, ASP1 (Aspartate aminotransferase 1)	<i>2g*</i>	AT2G30970	2g*	
gi15228276, TCTP (Translationally controlled tumor protein)	<i>2g*</i>	AT3G16640	<i>2g* and LDC</i>	

Changes in gene expression or protein levels are indicated in bold (increase) or italics (decrease).

effects at the 0g* and 2g* positions could be related to forces generated by differences in magnetic susceptibility between different components of the cell. Further experiments are required to assess this possibility.

Investigation of “pure” microgravity effects should be performed in space, but mechanical and magnetic simulators could be used to study similar phenomena if we are able to distinguish the mechanical/magnetic effects from the gravitational effects in our systems. In addition, magnetic levitation can be an alternative to other ground-based methodologies and allow us to test the biological effects of altered gravitational forces in an unusual environment.

Supplementary Material

All raw and identification data is available as Supplementary Material (available online at www.liebertonline.com/ast and deposited in the PRIDE database (<http://www.ebi.ac.uk/pride>) following CNB computational proteomics unit/proteomics facility guidelines (Kenyani *et al.*, 2011) (PRIDE ID: 22049, 22050).

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Author Disclosure Statement

The authors have declared no conflict of interest.

Abbreviations

BVA, Biological Variation Analysis; DIA, Differential In-Gel Analysis; DiGE, difference gel electrophoresis; LDC, Large Diameter Centrifuge; RPM, Random Positioning Machine.

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