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

Raul Herranz,1,* Ana I. Manzano,1,* Jack J.W.A. van Loon,2 Peter C.M. Christianen,3 and F. Javier Medina1

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 \(\mu g\)) 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.

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 \(T^2/m\). 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).

Table 1. Number of Spots Showing Altered Concentrations (p < 0.05) under Different Effective Gravity (g*) and Magnetic/Mechanical Conditions

<table>
<thead>
<tr>
<th>Effective Force (g)</th>
<th>0g*</th>
<th>1g*</th>
<th>2g*</th>
<th>μg</th>
<th>RPM</th>
<th>LDC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Magnetic field (B)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10.1 T</td>
<td>16.5 T</td>
<td>10.1 T</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>Mechanical forces</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>YES</td>
<td>YES</td>
<td></td>
</tr>
<tr>
<td>Spots ≥ 1.2-fold</td>
<td>22 (13)</td>
<td>4 (15)</td>
<td>56 (34)</td>
<td>1 (0)</td>
<td>1 (0)</td>
<td></td>
</tr>
<tr>
<td>Spots ≤ –1.2-fold</td>
<td>58 (17)</td>
<td>14 (20)</td>
<td>61 (55)</td>
<td>0 (0)</td>
<td>2 (0)</td>
<td></td>
</tr>
<tr>
<td>TOTAL</td>
<td>60 (30)</td>
<td>18 (35)</td>
<td>117 (89)</td>
<td>1 (0)</td>
<td>3 (0)</td>
<td></td>
</tr>
</tbody>
</table>

— 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.

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>
<thead>
<tr>
<th>Experiment sample</th>
<th>Master number</th>
<th>Spot number</th>
<th>t test value</th>
<th>Average ratio</th>
<th>MASCOT score</th>
<th>Candidate protein gi and description</th>
</tr>
</thead>
<tbody>
<tr>
<td>RPM Control RPM</td>
<td>1945</td>
<td>1854</td>
<td>0.021</td>
<td>1.26</td>
<td>&lt;72</td>
<td>Not significant results in the database</td>
</tr>
<tr>
<td>LDC Control LDC</td>
<td>907</td>
<td>996</td>
<td>0.046</td>
<td>1.44</td>
<td>519*</td>
<td>gi15227981, fructose-bisphosphate aldolase</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>308*</td>
<td>gi1529231, GAPC1 (GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE C SUBUNIT 1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>227</td>
<td>gi15239741, cinnamyl-alcohol dehydrogenase (CAD)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>226</td>
<td>gi15238762, GDH1 (GLUTAMATE DEHYDROGENASE 1)</td>
</tr>
<tr>
<td>Control LDC</td>
<td>1193</td>
<td>1291</td>
<td>0.034</td>
<td>-1.39</td>
<td>204</td>
<td>gi15217446, ATCEL3 (CELLULASE 3)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>128</td>
<td>gi297849086, T6D222.2 (Arabidopsis lyrata)</td>
</tr>
<tr>
<td>1g* Control 1g</td>
<td>1899</td>
<td>1859</td>
<td>0.046</td>
<td>-2.52</td>
<td>250</td>
<td>gi15225374, ATPHB6 (PROHIBITIN 6)</td>
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<tr>
<td></td>
<td>2375</td>
<td>2316</td>
<td>0.0069</td>
<td>-2.55</td>
<td>197</td>
<td>gi13899069, A. thaliana unknown protein</td>
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<tr>
<td>0g* Control 1g</td>
<td>928</td>
<td>1058</td>
<td>0.0064</td>
<td>-1.28</td>
<td>&lt;72</td>
<td>Not significant results in the database</td>
</tr>
<tr>
<td>Control 1g*</td>
<td>1157</td>
<td></td>
<td>0.011</td>
<td>-1.36</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control 1g</td>
<td>2180</td>
<td>2365</td>
<td>0.016</td>
<td>-2.24</td>
<td>&lt;72</td>
<td>Not significant results in the database</td>
</tr>
<tr>
<td>Control 1g*</td>
<td>2364</td>
<td></td>
<td>0.040</td>
<td>-1.54</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control 1g</td>
<td>717</td>
<td>844</td>
<td>0.00043</td>
<td>-2.25</td>
<td>270*</td>
<td>gi15222981, Sks5 (SKU5 Similar 5)</td>
</tr>
<tr>
<td>Control 1g</td>
<td>846</td>
<td>975</td>
<td>0.039</td>
<td>2.07</td>
<td>650</td>
<td>gi227202752, AT1G56340 ATCRT1A (calreticulina)</td>
</tr>
<tr>
<td>0g* and 2g* Control 1g</td>
<td>663 788 (0g*)</td>
<td>0.020</td>
<td>-1.66</td>
<td>687</td>
<td>4467097, heat shock protein 70 like protein</td>
<td></td>
</tr>
<tr>
<td></td>
<td>664 (2g*)</td>
<td>0.015</td>
<td>-2.69</td>
<td>208</td>
<td>gi15219234, VHA-A (VACUOLAR ATP SYNTHASE SUBUNIT A)</td>
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<tr>
<td></td>
<td>749 877 (0g*)</td>
<td>0.0022</td>
<td>-2.08</td>
<td>620*</td>
<td>gi15222981, Sks5 (SKU5 Similar 5)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>776 (2g*)</td>
<td>0.016</td>
<td>-1.46</td>
<td></td>
<td></td>
<td></td>
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</table>

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

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, tryptic-digested spots were analyzed by a 4800 MALDI-TOF-TOF facility (Mac carrone 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). 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 reinforce 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>
<thead>
<tr>
<th>Protein description</th>
<th>Proteome changes</th>
<th>Transcriptome changes</th>
</tr>
</thead>
<tbody>
<tr>
<td>gi15227981, fructose-bisphosphate aldolase</td>
<td>LDC and 2g*</td>
<td>LDC and 2g*</td>
</tr>
<tr>
<td>gi15229231, GAPC1 (Glyceraldehyde-3-phosphate dehydrogenase c subunit 1)</td>
<td>LDC and 2g*</td>
<td>AT2G36460</td>
</tr>
<tr>
<td>gi15239741, CAD (Cinnamyl-alcohol dehydrogenase)</td>
<td>AT2G04120</td>
<td>LDC and 2g*</td>
</tr>
<tr>
<td>gi15238762, GDFH1 (Glutamate dehydrogenase 1)</td>
<td>LDC</td>
<td>AT3G19450</td>
</tr>
<tr>
<td>gi14594802, translation initiation factor elf4-A1</td>
<td>LDC</td>
<td>AT5G18170</td>
</tr>
<tr>
<td>gi155224592, ASPI (Aspartate aminotransferase 1)</td>
<td>2g*, 2g*</td>
<td>2g* and LDC</td>
</tr>
<tr>
<td>gi15528276, TCTP (Translationally controlled tumor protein)</td>
<td>2g*</td>
<td>AT3G16640</td>
</tr>
</tbody>
</table>

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 (Kenyan 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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