Seed-to-Seed-to-Seed Growth and Development of *Arabidopsis* in Microgravity

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Abstract

Arabidopsis thaliana was grown from seed to seed wholly in microgravity on the International Space Station. *Arabidopsis* plants were germinated, grown, and maintained inside a growth chamber prior to returning to Earth. Some of these seeds were used in a subsequent experiment to successfully produce a second (back-to-back) generation of microgravity-grown *Arabidopsis*. In general, plant growth and development in microgravity proceeded similarly to those of the ground controls, which were grown in an identical chamber. Morphologically, the most striking feature of space-grown *Arabidopsis* was that the secondary inflorescence branches and siliques formed nearly perpendicular angles to the inflorescence stems. The branches grew out perpendicularly to the main inflorescence stem, indicating that gravity was the key determinant of branch and silique angle and that light had either no role or a secondary role in *Arabidopsis* branch and silique orientation. Seed protein bodies were 55% smaller in space seed than in controls, but protein assays showed only a 9% reduction in seed protein content. Germination rates for space-produced seed were 92%, indicating that the seeds developed in microgravity were healthy and viable. Gravity is not necessary for seed-to-seed growth of plants, though it plays a direct role in plant form and may influence seed reserves. **Key Words:** *Arabidopsis*—Branch—Inflorescence—Microgravity—Morphology—Seed—Space. *Astrobiology* 14, 866–875.

1. Introduction

GRAVITY AND PLANT FORM have been systematically studied for more than 200 years. Plant space biology has been closely associated with human space exploration in that plants are key parts of biologically based life support. Learning to grow plants in space is an essential goal for long-duration space missions since crop growth in space will aid with air regeneration, food production, and water recycling (Sager and Drysdale, 1996; Stankovic, 2001).

Plants have been used in space experiments from the early days of the space program. Periodic literature updates on plant space biology have reviewed the documented influence of gravity on both plant growth and cellular and molecular responses, including cell cycle, embryogenesis and seed development, photosynthesis and gas exchange, gravitropic sensing and response, phototropism, cell wall development, and gene expression changes (Wolverton and Kiss, 2009). Plant science experiments during the space shuttle era produced key science insights on biological adaptation to spaceflight and especially plant growth and tropisms, which were thoroughly reviewed by Paul *et al.* (2013a).

Many plant space biology experiments have shown abnormalities such as chromosomal breakage (Krikorian and O'Connor, 1984), failure to produce seed (Mashinsky *et al.*, 1994; Campbell *et al.*, 2001), altered or nonviable embryos (Merkys and Laurinavicius, 1983), alterations in the cell wall composition and properties (Hoson *et al.*, 2003), increased breakdown of xyloglucans (Soga *et al.*, 2002), changes in polar auxin transport (Ueda *et al.*, 2000), or other morphological abnormalities (Link and Cosgrove, 2000). Most plant space experiments last less than 18 days. Prior to the present study, plants had only been successfully grown from seed to seed during the course of two experiments, each of which showed developmental alterations.

The first successful seed-to-seed experiment in microgravity was reported by Merkys and Laurinavicius (1983), who used *Arabidopsis thaliana*. They observed some viable seed, but most seed had nonviable embryos. The second successful experiment was performed with *Brassica rapa* and was reported by Musgrave *et al.* (2000) and Kuang *et al.* (2000). Though the *Brassica* seed was healthy and viable, the seed was observed to have less protein, fewer cotyledon cells, and aberrant deposition of starch grains. In both

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experiments, the results were more likely due to the rigors of the microgravity environment than to the lack of gravity itself. For example, altered starch content has been reported by numerous investigators for different species of space-grown plants: pepper plants (Johnson and Tibbitts, 1968), *Lepidium* root (Volkmann *et al.*, 1986), *Arabidopsis* (Laurinavichius *et al.*, 1986; Brown *et al.*, 1996; Kuang *et al.*, 1996; Musgrave *et al.*, 1998), and maize root columella (Moore *et al.*, 1987). However, improving plant ventilation during spaceflight was found to eliminate carbohydrate differences (Musgrave *et al.*, 1997, 1998). In addition, ethylene, a plant stress hormone, is a common problem in microgravity experiments. Plant ethylene production increases in clinostat studies (Hilaire *et al.*, 1996) and in space (Klymchuk *et al.*, 2003). Elevated ethylene levels (1100–1600 ppb on a shuttle) caused anomalous seedling growth of *Arabidopsis* in spaceflight studies, although they had no effect on relative graviresponsiveness (Kiss *et al.*, 1999). Furthermore, ethylene levels on the Mir space station were very high (800–1200 ppb) during a *Brassica* study reported by Kuang *et al.* (2000). It is notable that *Brassica* plants produced seed at this ethylene level, since the same environment stopped a wheat crop from producing seed on board Mir (Campbell *et al.*, 2001).

At the University of Wisconsin-Madison, the Wisconsin Center for Space Automation and Robotics (WCSAR) developed the ADVanced ASTroCulture (ADVASC) plant growth unit to improve the plant growth environment on long-duration missions to the International Space Station (ISS). ADVASC was designed as a state-of-the-art growth chamber capable of providing nutrients to the plants and controlling soil moisture, light, air temperature, humidity, ethylene, and CO₂ levels (Zhou *et al.*, 2002). In a partnership agreement, WCSAR and Space Explorers, Inc., an educational company in Green Bay, Wisconsin, grew *Arabidopsis thaliana* in the ADVASC unit on the ISS. ADVASC represented a substantial advance in plant growth facilities in that it was fully automated, required very little care from astronauts, and was remotely controlled from the ground (Zhou *et al.*, 2002; Link *et al.*, 2003). The first flight of ADVASC provided an opportunity to study the patterns of plant growth and development as well as seed and plant morphology in microgravity (first seed-to-seed *Arabidopsis* experiment on the ISS). The subsequent flight of ADVASC was used to obtain a second generation of microgravity-grown *Arabidopsis* plants (second seed-to-seed *Arabidopsis* experiment on the ISS) and to obtain fresh plant tissue for subsequent DNA microarray analysis. Since previous investigators found abnormalities in seed produced on long-duration missions, we wanted to discern whether ADVASC's improvements in remote plant care had translated into improved seed quality. Taking advantage of growing two generations, that is, seed to seed to seed, of *Arabidopsis thaliana* on the ISS, we were also interested in learning whether microgravity would alter plant form and cause biochemical, cellular, and molecular changes.

2. Materials and Methods

2.1. Plant growth

Plants were grown in the ADVASC unit, whose specifications and performance in microgravity were thoroughly

described by Zhou *et al.* (2002). ADVASC is configured as two single-Middeck-Locker inserts that can be installed in an EXPRESS Rack on the ISS. One insert contains the plant growth chamber; the other contains the control unit and the support systems. ADVASC consists of six major subsystems: environmental chamber, temperature and humidity control unit, light module, fluid and nutrient delivery system, atmospheric composition control unit, and a computer control and data management system (Zhou *et al.*, 2002). ADVASC thus provides a completely enclosed, environmentally controlled plant growth chamber capable of supporting plant growth for up to several months in a reduced gravity environment. Detailed information about the ADVASC growth chamber and its performance on the ISS is also available in numerous publicly available NASA documents (see NASA, 2001, and the links therein).

The inner dimensions of the root tray were 20.3 × 19.7 × 3.1 cm (L × W × D), and the interior of the chamber was 34.3 cm high. Prior to launch, the root tray was filled with a baked particulate calcined-clay mixture (arcillite) with irregular particles ranging from 0.5 to 4 mm. The arcillite was held in place by a fine stainless steel screen with 6.5 mm holes spaced 14 mm apart (center to center). The holes allowed the plants to emerge and were arranged in rows spaced 25 mm apart. Two planting techniques were used. In the first, a double layer of cheesecloth (40 weight) was placed between the arcillite and the screen to cover the holes and prevent the arcillite from floating away in microgravity. The cheesecloth also provided a germinating layer for the seeds. The second technique used "cartridges" made by loosely stuffing rolls of germination paper with cotton and securing them in a hole under the edges of the screen. Prior to launch, 91 *Arabidopsis thaliana* seeds of the Col-0 ecotype were attached to the cheesecloth or the cartridge with 1% (w/w) gum guar. Seeds were purchased from Lehle Seed Co. (Round Rock, Texas). The nutrient reservoir was charged with 550 mL of half-strength Hoagland's solution (Hoagland and Arnon, 1950).

The payload for the first seed-to-seed experiment in microgravity was turned over to NASA for delivery to the ISS as a science payload on the STS-100 mission (ISS assembly flight 6A in April 2001) and returned on the STS-104 mission (ISS assembly flight 7A in July 2001). For the second seed-to-seed experiment, the growth chamber included seeds that were developed in microgravity (during the first experiment). In the second experiment, the growth chamber was delivered to the ISS on the STS-108 mission in December 2001 and was returned to Earth on the STS-111 mission in June 2002 (both were ISS supply/crew rotation missions).

The experimental protocol was essentially similar in both spaceflight experiments. Once the astronauts installed the growth chamber in an EXPRESS Rack facility on the ISS, the experiment was telemetrically controlled from WCSAR's laboratory at the University of Wisconsin-Madison. The experiment was automated as much as possible. Water and nutrients were pumped into the arcillite matrix from the nutrient reservoir via four porous tubes buried in the arcillite. The nutrient reservoir was continuously recharged with water condensed from the evapotranspiration stream. At four time points during the mission, 300 mL of liquid was removed from the reservoir and replaced with half-strength

Hoagland's solution. The growth phase of the experiment lasted 46 days, after which the water supply to the root tray was terminated so that the chamber containing the plants could be desiccated prior to shutdown, transferred to a space shuttle, and returned to Earth (a 2-week process). During the growth phase, the temperature in the chamber was maintained at 22°C at 70% humidity with a 16/8 h day/night cycle. The photosynthetically active radiation came from a custom-made array of red and blue LEDs. The photosynthetically active radiation at the root tray was $230/25 \mu\text{mol m}^{-2} \text{s}^{-1}$ red/blue. The CO_2 level exceeded ADVASC's maximum sensor limit of 3000 ppm for the first 16 days of the experiment. The minimum CO_2 concentration was 500 ppm. Ethylene was photocatalytically removed from the air to levels below 100 ppb by using proprietary air-purifying technology based on ultraviolet light-activated modified titanium dioxide ($\text{TiO}_2/\text{ZrO}_2$) thin film as a photocatalyst. Exposed to ultraviolet light, this photocatalyst functions as an ethylene scrubber, oxidizing unsaturated hydrocarbons into CO_2 and H_2O (Zhou *et al.*, 2002).

Ground control experiments were in parallel conducted at the University of Wisconsin in an engineering reproduction of the spaceflight hardware. All dimensions, light, temperature, humidity, and control set points were the same as for the spaceflight experiment but several days behind the spaceflight experiment.

2.2. Light microscopy

Seeds were fixed 4 h in 5% (v/v) glutaraldehyde in 0.05 M sodium cacodylate buffer, pH 7.0 with a change of fixative after 2 h. Following fixation, a longitudinal incision was made with a 28-gauge needle between the hypocotyl-root axis and cotyledons of the ungerminated seeds. All tissues were then rinsed in buffer, dehydrated with ethanol, embedded in LR White Resin (London Resin Company), and polymerized at 50°C. Two-micrometer-thick sections were cut with a Sorvall Porter-Blum MT-2 ultramicrotome, attached to glass slides with heat, and stained with periodic acid/Schiff's reaction (PAS) with 2,4-dinitrophenyl-hydrazine as an aldehyde block (O'Brien and McCully, 1981). Sections were counterstained with aniline blue-black.

2.3. Transmission electron microscopy

Initial fixation, seed-coat incising, and buffer washes were performed as described for light microscopy. Seeds were postfixed with 2% (w/v) osmium tetroxide in 0.05 M sodium cacodylate buffer, followed by a buffer rinse and dehydration through a graded acetone series. Seeds were then transferred to propylene oxide before being embedded in Spurr's resin and polymerized at 70°C (Spurr, 1969). Gold sections were obtained and mounted on 0.5% pioloform-coated 75-mesh or uncoated 300-mesh copper grids and stained with 3% (w/v) uranyl acetate in 30% (v/v) ethanol and poststained in Reynold's lead citrate. Sections were viewed at 60 kV with a JEOL JEM-1200EX transmission electron microscope (TEM) and photographed with 4489 ESTAR Kodak electron microscope film.

2.4. Protein content

Two techniques were used to determine the seed protein content. The first technique measured the percent of cell

cross-sectional area covered by protein bodies seen in TEM images. The areas were measured by tracing the protein bodies and cells with Adobe Photoshop and counting the pixels within the boundaries. The second technique directly measured SDS soluble protein levels. First, the average seed weight was determined. Seeds were counted in lots of 500 and weighed so that the average mass of an individual seed could be determined. At least three lots were weighed for each batch of seed. No significant difference was found between space seed ($15.18 \pm 0.55 \mu\text{g}$) and ground control seed ($14.82 \pm 0.71 \mu\text{g}$), so protein mass per seed or per unit mass was considered to be equivalent. Protein levels were measured by incubating 12 seeds in 100 μL of 5% (w/v) SDS at 50°C for 1 h, grinding them, incubating them for an additional hour at 60°C, and repeating the grinding. BioRad DC Protein Assay Kit with BSA standard (part number 500-0112) was then used to estimate the protein concentration of 33 μL of extract. Triplicates were done for each treatment (space, ground control, or stock seed). All experiments were repeated two to four times. The technique was tested by varying the number of seeds in an extraction and plotting the results. A linear relationship was found between the final measured protein concentration and the number of seeds that were ground.

2.5. Microarray analysis

The second experiment on the ISS provided the opportunity to harvest fresh plant tissue for subsequent gene expression analysis with the use of DNA microarrays. Pertinent Supplementary Data are available online at www.liebertonline.com/ast.

3. Results

3.1. Seed ultrastructure

Seeds were examined by using light microscopy and transmission electron microscopy to check for alterations in embryo development, deposition of starch grains, reduction in cell numbers, or reduced seed protein content, since these phenotypes were reported for seed produced on previous long-duration spaceflights (Merkys and Laurinavicius, 1983; Kuang *et al.*, 2000). We also looked for cell wall thickening, since this was seen in *Arabidopsis* leaves grown under reduced oxygen atmospheres and may be indicative of hypoxia (Ramonell *et al.*, 2001).

Figure 1 shows light micrographs of the seed. The structural organization of the seeds was the same for both the space-grown and ground control material and was no different from normal seed (Busse and Evert, 1999). All seed contained mature embryos with well-formed cotyledons, root-shoot axis, and root and shoot meristems. A single layer of endosperm cells was found interior to the seed coat, as expected. The dimensions of the cells and the number of cells in an organ were the same (no statistical difference) between the space- and ground-produced seed. No starch grains were seen in any sections. Embryo and seed development were almost entirely normal in space.

The only difference between the two treatments at the light microscope level was the poor definition of the protein bodies in space-developed seed. Figure 2 shows the TEM images that were used to resolve fine structural details. The

FIG. 1. Light micrographs of mature seed sectioned transversely through the cotyledons and stained with PAS to reveal carbohydrates and aniline blue-black to reveal protein. Arrows indicate protein bodies that are more easily recognized in ground control seed (a) than in space-developed seed (b). Bar = 50 μ m. (Color images available online at www.liebertonline.com/ast)

FIG. 2. TEM images of sectioned seed. Frames (a) and (c) show ground control seed, while (b) and (d) are space-developed seed. Protein bodies (P) and lipid bodies (L) are indicated. Arrows show where globoid crystals existed within protein bodies. Bar = 2 μ m.

cells of both space and ground control seed were packed with protein and lipid reserves. TEM imaging revealed that protein bodies and osmophilic lipid droplets filled every cell, as expected, and that the protein bodies contained globoid crystals—presumably a phytate salt important for storing minerals during seed development (Otegui *et al.*, 2002). Many crystals fell out of the sections during preparation; however, some nearly electron-opaque crystals were found. The key difference between the two treatments was the reduction, in terms of area, of the protein bodies in space-developed seed. Protein bodies were found to occupy $35.89\% \pm 3.98\%$ of ground control seed and $16.25\% \pm 1.01\%$ of the cells for space seed. This represents a 54.73% decrease for space-developed seed. The reduction in the area of the protein bodies explains their poor definition in light micrographs. However, measuring areas did not take into account differences in the density of protein bodies or how well filled they were. To account for this we also measured SDS extractable protein levels.

The seed protein content is shown in Fig. 3. Both the ground control and space-developed seed had lower protein levels compared to the starting seed purchased from Lehle Seed Co. Ground control seed contained $90.93\% \pm 2.3\%$ of protein found in commercial seed, while the space seed had $81.96\% \pm 2.0\%$. The large discrepancy between this estimate of seed protein content and the area estimate from TEM images may be partially due to the reliance of BioRad's DC assay on reactions with a few amino acids to estimate the total protein content (Goossens *et al.*, 1999). However, protein levels were always expressed as a percentage compared to Colombia seed stock from the original batch from Lehle seed as an internal reference for each experiment to reduce or eliminate the effect of amino acid bias.

Seed were also tested for germination competency on germination paper soaked in half-strength Hoagland's solution. The space seed germinated better (92.5% , $n=40$) than the ground control seed (82% , $n=40$) despite having lower protein content.

3.2. Branch angles

The inflorescence branches of space-grown plants were unusual. Most branched off nearly perpendicular to the in-

FIG. 3. Protein content. Protein was extracted from seed (space, ground control, or original stock seed) with 5% (w/v) SDS and measured photometrically. The results were compared to the stock seed on a percentage basis to reduce inherent amino acid bias and as an internal control for each experiment. The error bars show one standard error ($n=6$).

FIG. 4. Comparison of the branch angles from spaceflight and ground control plants. The angles were measured between the branch and inflorescence at the base of the branch. Zero degrees is straight down parallel to the gravity vector (on Earth), 90° is perpendicular to the stem, and 180° is straight up toward the light source. The error bars show one standard error ($n=58$).

florescence, with the branches often growing away from the light source, toward the root tray. Some branches were observed to grow all the way across the surface of the root tray (similar to diagravitropism). Figure 4 shows a comparison of the initial branch angles. Inflorescence branch angles can be highly variable, but branches were never observed to grow downward in ground-based tests of *Arabidopsis*.

The siliques of space-grown plants also emerged nearly perpendicular to the stem. Figure 5 shows a comparison between the silique angles for the space plants and ground control plants. Both the branch and silique phenotypes are also apparent in Fig. 6, which shows silhouettes of typical space- and ground-grown plants. The image in Fig. 6a depicts an Earth-grown *Arabidopsis* control plant. The images in Fig. 6b, 6c illustrate plants from the first seed-to-seed *Arabidopsis* spaceflight experiment. The images in Fig. 6d, 6e are from the second seed-to-seed *Arabidopsis* spaceflight experiment. Since the plants were dried in space prior to their return to Earth, it was conceivable that the branches

FIG. 5. Comparison of the silique angles from spaceflight and ground control plants, with respect to the inflorescence stem. The measured angle was between the inflorescence and the long axis of the silique. On Earth, 0° is straight down parallel to the gravity vector, 90° is perpendicular to the stem, and 180° is straight up toward the light source. The error bars show one standard error ($n=49$ for space and 40 for the ground controls).

FIG. 6. Images of *Arabidopsis thaliana* grown in space. (a) Silhouette of a dried *Arabidopsis* plant grown on Earth in the ground control experiment with typical upward-oriented branches. (b and c) Silhouettes of typical dried plants grown in space. (d) A living plant photographed in space during a sampling opportunity. (e) Silhouette of the plant shown in (d) at the end of the experiment, demonstrating that the main inflorescence grew in an upward spiral while the branches grew primarily away from the light source. (Color images available online at www.liebertonline.com/ast)

and siliques “wilted” during the drying process. This was highly unlikely since there was no gravity to pull the branches downward, and furthermore branches are lignified. Figure 6d also shows a living plant photographed in space during the second seed-to-seed experiment. In this image, it is clear that the branches generally grew horizontally or even slightly toward the root tray. The “downward” orientation of the main inflorescence in Fig. 6d was not typical and was the result of “spring back” from resisting the airflow. The second experiment on the ISS had to be temporarily paused, and the growth chamber had to be turned off, which eliminated air circulation, to photograph the plants from the side (Fig. 6d). The final form of this plant’s inflorescence main stem, seen at landing, was an upward spiral to the light cap shown in Fig. 6e. The branches remained “down.”

4. Discussion

The absence of natural convection in space makes it easy for plants to become oxygen starved (Porterfield, 2002).

Hypoxia symptoms in seed include reduction in size of the protein bodies, failure of the protein bodies to fill, free-floating lipid droplets in the cytoplasm, abnormally vacuolated cells, and degeneration of portions of the embryo (Kuang *et al.*, 1998). Other symptoms may include aberrant deposition of starch grains and thickened cell walls (Ramonell *et al.*, 2001). In a full life-cycle experiment with *Brassica*, Kuang *et al.* (2000) found protein bodies that were 44% smaller (cross-sectional area). There was an accompanying 80% reduction in the cotyledon cell number, and starch grains were aberrantly deposited in the seed. This study concluded that alterations in the oxygen and ethylene concentrations within developing siliques were problematic in the experiment (Kuang *et al.*, 2000; Musgrave *et al.*, 2000). While the Svet greenhouses used to grow *Brassica* on Mir used a fan to circulate air, it is possible the circulation rate was insufficient (below 0.5 m/s) to prevent hypoxia (Porterfield, 2002).

We observed a 55% reduction in protein body size; however, since the protein bodies in space-developed seed were filled, and we did not observe any other signs of hypoxia such as degeneration of the embryos, deposition of starch grains, or alterations in cell structures or cell numbers, we conclude that the aerial portions of the plant were not starved for oxygen. The high forced airflow rates (2–3 m/s) and accompanying ethylene removal provided by ADVASC improved growing conditions for the aerial part of the plants when compared to the previous studies by Kuang *et al.* (2000) and Merkys and Laurinavicius (1983).

Root zone hypoxia could explain the reduced seed protein content. ADVASC uses passive airflow to move air through the root tray. Previous investigators found that root zone hypoxia was prevalent in spaceflight experiments (Stout *et al.*, 2001; Porterfield *et al.*, 1997). In this experiment, we used a porous arcillite matrix that is one of the better rooting systems for space (Porterfield *et al.*, 2000). Arcillite reduces root zone hypoxia by allowing air to penetrate between the arcillite grains. Nonetheless, air movement through arcillite is restricted, especially if the spaces between arcillite grains are filled with roots, water, or both. If passive airflow through the arcillite is cut off, then oxygen can only reach the roots by diffusion from the air above the soil or by the arrival of oxygenated water. Diffusion rates are negligible when the diffusion distances are more than a few millimeters (Porterfield, 2002).

When we opened the ADVASC root tray upon return to Earth, we visually estimated that 80% or more of the roots formed a dense mat in the top 13 mm of arcillite, while the roots of the ground control plants penetrated deeply throughout the root tray. Evapotranspiration data show that the porous tubes delivered an average of 110 mL/day of aerated water during the major growth portion of the experiment. There was not enough oxygen in this amount of water to meet the physiological needs of the roots (Porterfield, 2002). An anoxic root zone in space resembles an environment similar to flooded soil on Earth. Anoxia reduces nitrogen uptake by the roots; therefore, seed protein content is reduced. On Earth, applying fertilizer to flooded plants improves seed protein content (Bacanamwo and Purcell, 1999). Because ADVASC used an artificial soil with no native nutrient value, the plants were fertilized four times during the experiment. This may explain how the

plants achieved 82% of the normal, SDS-extractable, protein content in the seed (versus 91% for control seed).

The reduced branch angles and perpendicular growth of the siliques in space appear to be true microgravity phenotypes. The branching pattern seen in the first experiment was replicated during the second microgravity experiment (Fig. 6), indicating that this phenotype is persistent in *Arabidopsis* development on long-duration spaceflights. Light plays a principle role in the “upright” or light-seeking growth habit of the primary axis of many plants and is responsible for houseplants curving toward the nearest window. On Earth, this response interacts with negative gravitropism in the shoot and requires that shoot gravitropism experiments be conducted in the dark (Hangarter, 1997; Weise and Kiss, 1999; Correll and Kiss, 2002). In this experiment, the primary axis of *Arabidopsis* always grew toward the light source, supporting a central role for light in the primary axis.

The effect of light on axillary organs, however, is variable. Darwin (1884) observed that the tendrils of climbing plants are negatively phototropic. Numerous investigators found that runners, stolons, and/or prostrate stems of many plants grew upright when the plants were shaded or placed in darkness (Langham, 1941; Palmer, 1956). However, the change to upright growth is not directly related to light. Willmoes *et al.* (1988) demonstrated that feeding sucrose to cut *Paspalum vaginatum* stolons promoted diageotropic growth even when the plant was in total darkness. Since fructose and glucose did not show this effect, they concluded that light alters plant form indirectly via photosynthesis. Studies by Digby and Firm (2002) with *Tradescantia fluminensis* and the *lazy-2* mutant of tomato reached similar conclusions.

There are no direct studies on *Arabidopsis* inflorescence branches relating either to light or gravity, though Fukaki *et al.* (1996) noted that the branches of the shoot gravitropism mutants *sgr1*, *sgr2*, and *sgr3* grew horizontally. One caveat of working with mutants, however, is that mutations generally affect more than a single pathway. For example, the *sgr1* mutant is allelic to *scarecrow* (*scr*) and reduces the gravity response by eliminating the endodermal cell layer in hypocotyls and in the inflorescence where gravity is perceived (Di Laurenzio *et al.*, 1996; Fukaki and Tasaka, 1999; Morita *et al.*, 2002). Elimination of an entire tissue layer is a major alteration of plant architecture. Other gravitropic mutants, such as *endodermal-amyloplast less 1* (*eal1*), have branches that curve upward (Fujihira *et al.*, 2000), but this is believed to be due to a failure to completely eliminate gravisensing. Determining which mutants truly reproduce the plant's form in the absence of gravity can only be done by growing plants in space.

In this study, the reduced branch angles and tendency of the branches to ignore or curve away from the light source in space show that gravity plays the key role in signaling branches to curve upward on Earth for *Arabidopsis*. Secondly, the reduced angles that the siliques made with the stems also show that gravity has a direct role in determining the silique angles. Thirdly, since *Arabidopsis* branches do not naturally curve toward the light in microgravity, light plays either a negative or a secondary role in the branch form of *Arabidopsis*. Spaceflight appears to initiate cellular remodeling throughout the plant, yet specific strategies of the response are distinct among specific organs of the plant.

In the absence of gravity, plants rely on other environmental cues to initiate the morphological responses essential to successful growth and development through differential expression of genes in an organ-specific manner (Paul *et al.*, 2013b). Finally, since our plants phenocopied the *sgr2* and *sgr3* mutants, we conclude that these mutants are good candidates for continuing to study gravity and inflorescence branching on Earth.

We believe that this is the first report of altered branch and silique angles for space-grown plants. This is due primarily to the fact that there have been very few opportunities for long-term plant growth experiments in space. Most investigators have had to make do with shuttle flights that rarely last more than 16 days. Space experiments have also been plagued by high ethylene levels. In this experiment, ethylene levels were kept below 100 ppb by using a photocatalytic converter to remove ethylene from the growth chamber (Zhou *et al.*, 2002; Link *et al.*, 2003).

Though we report here the first attempt of transcriptional profiling of plants fully grown in microgravity, our results are presented only as Supplementary Data for a number of reasons. We caution with respect to deriving conclusions from this gene expression profiling study and advise that additional experimentation is needed, because the observed expression patterns may be at least in part induced by other interacting suboptimal environmental conditions, for example, an anoxic root zone in space. During the second seed-to-seed experiment on the ISS (that provided plants used for transcriptional profiling), technical issues interfered with the priming of the growth chamber and its transition into steady state. Most likely contributing factors to the technical issues in the second experiment were as follows:

- (i) possible air leakage through the porous cups and subsequent entry into the condensate recovery/nutrient delivery system;
- (ii) frequent repriming of the condensate recovery/nutrient delivery system and the heat sink caused extensive flooding of the root tray and interfered with the desired operation of ADVASC at 22°C and 70% humidity;
- (iii) difficulty in correlating the root tray pressure set point to moisture level in the root tray;
- (iv) the air vent redesign may have changed airflow in an undesirable way;
- (v) different root trays showed different soil moistures for the same pressure set points; thus the same root tray may have performed differently when it was in a different growth chamber, making each growth chamber/root tray/sensor combination unique and hence requiring testing with that specific combination;
- (vi) final adjustment of one of the root tray pressure sensors, conducted just before turnover;
- (vii) insufficient image quality of the available daily pictures, preventing accurate assessment of the dynamics of plant germination.

The above factors may have thus contributed to the observed gene expression patterns. This set of transcriptional profiling data also suffers from insufficient experiment information, and the usual statistical measures for array data are not available (see Supplementary Data).

The ADVASC growth chamber had to be repeatedly reprimed over several days in the initial stage of the experiment, and for that reason the root tray remained largely flooded with nutrient solution. Repriming of the condensation nubs and the required cooler heat sink activity delayed establishment of the desired set points of 22°C temperature and 70% humidity. We surmise that the accompanying moisture fluctuations (*i.e.*, hypoxia) in the rooting medium resulted in poor germination rate. The majority of these plants, which underwent germination stress and delayed sprouting, were then harvested for gene expression analysis at approximately 4 weeks of age. It is therefore difficult to compare the results of our study with the recently conducted well-controlled transcriptional analysis of 12-day-old *Arabidopsis* plants grown on phytigel plates within the Advanced Biological Research System on a space shuttle (Paul *et al.*, 2012, 2013b).

We conclude that, while *Arabidopsis* plants grown in microgravity may have shown some signs of root zone hypoxia, the ADVASC growth chamber in general provided a very good environment for growing plants on the ISS and successfully eliminated most of the problems seen in previous plant spaceflight experiments, allowing us to discover alterations in plant form and architecture and to confirm that branching phenotypes seen in the *sgr2* and *sgr3* mutants represent the form of wild-type *Arabidopsis* grown in microgravity. We were thus able to successfully grow two consecutive generations of *Arabidopsis thaliana* in space, that is, seed to seed to seed. Future experiments should be conducted to discern whether these alterations can be generalized across different species of plants. As well, future designs of space growth chambers should improve the root zone aeration to determine whether the reduction in seed protein content is due to root zone hypoxia or some other aspect of the microgravity environment.

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Author Disclosure Statement

No competing financial interests exist.

Abbreviations

ADVASC, Advanced Astroculture; ISS, International Space Station; TEM, transmission electron microscope; WCSAR, Wisconsin Center for Space Automation and Robotics.

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