

G. W. Stutte · O. Monje · R. D. Hatfield · A. -L. Paul
R. J. Ferl · C. G. Simone

Microgravity effects on leaf morphology, cell structure, carbon metabolism and mRNA expression of dwarf wheat

Received: 3 March 2006 / Accepted: 3 April 2006 / Published online: 10 May 2006
© Springer-Verlag 2006

Abstract The use of higher plants as the basis for a biological life support system that regenerates the atmosphere, purifies water, and produces food has been proposed for long duration space missions. The objective of these experiments was to determine what effects microgravity (μg) had on chloroplast development, carbohydrate metabolism and gene expression in developing leaves of *Triticum aestivum* L. cv. USU Apogee. Gravity naive wheat plants were sampled from a series of seven 21-day experiments conducted during Increment IV of the International Space Station. These samples were fixed in either 3% glutaraldehyde or RNAlater™ or frozen at -25°C for subsequent analysis. In addition, leaf samples were collected from 24- and 14-day-old plants during the mission that were returned to Earth for analysis. Plants grown under identical light, temperature, relative humidity, photoperiod, CO_2 , and planting density were used as ground controls. At the morphological level, there was little difference in the development of cells of wheat under μg conditions. Leaves developed in μg have thinner cross-sectional area than the

1 g grown plants. Ultrastructurally, the chloroplasts of μg grown plants were more ovoid than those developed at 1 g, and the thylakoid membranes had a trend to greater packing density. No differences were observed in the starch, soluble sugar, or lignin content of the leaves grown in μg or 1 g conditions. Furthermore, no differences in gene expression were detected leaf samples collected at μg from 24-day-old leaves, suggesting that the spaceflight environment had minimal impact on wheat metabolism.

Keywords Bioregeneration · Bioregenerative life support · Lignin · Carbohydrate metabolism · Microarray · *Triticum aestivum* L.

Abbreviations BLSS: Bioregenerative life support system · BPS: Biomass production system · BRIC: Biological Research in Canisters · CDS: Communication and data system · DAI: Days after imbibition · ISS: International Space Station · KSC: Kennedy Space Center · LN2: Liquid nitrogen · NASA: National Aeronautics and Space Association · OES: Orbiter environment simulator · P_{net} : Net photosynthesis rate · PAR: Photosynthetically active radiation · PESTO: Photosynthesis experiment system testing and operation · PGC: Plant growth chamber · PPF: Photosynthetic photon flux · PSI: Photosystem I · PSII: Photosystem II · QY: Quantum yield · STS: Space transport system · WCE: Whole chain electron transport

Electronic Supplementary Material Supplementary material is available for this article at <http://dx.doi.org/10.1007/s00425-006-0290-4> and is accessible for authorized users.

G. W. Stutte (✉) · O. Monje
Space Life Sciences Laboratory, Dynamac Corporation,
Mail Code Dyn-3, Kennedy Space Center,
Kennedy, FL 32899, USA
E-mail: stuttgw@kscems.ksc.nasa.gov
Tel.: +1-321-8613493
Fax: +1-321-8612925

R. D. Hatfield
US Dairy Forage Research Center,
USDA-Agricultural Research Service,
Madison, WI, USA

A. -L. Paul · R. J. Ferl
Department of Horticultural Sciences,
University of Florida, Gainesville, FL, USA

C. G. Simone
Department of Biology, University of South Florida,
Tampa, FL, USA

Introduction

The use of higher plants as the basis for a biological life support system (BLSS) that regenerates the atmosphere, purifies water and produces food during long-duration space missions has been researched by NASA for over 40 years (Myers 1954; Eley and Myers 1964; Miller and Ward 1966; Halstead and Dutcher 1987; Wheeler et al. 2001). The photosynthetic rate of higher plants is the critical component of plant-based atmospheric regeneration systems being proposed for long duration space

missions (> 3 years) since it is the fundamental biological process driving atmospheric regeneration (e.g. CO₂ removal and O₂ production) as well as food production (Brown et al. 1996; Wheeler et al. 2001). Because of that criticality, it is essential to determine the impacts of the microgravity (μ g) environment on the development of the photosynthetic apparatus and its functioning in space.

Although NASA's interest in plant biology is over 40 years old, and extensive research has been conducted during several spaceflight experiments to understand the basic physiological responses of plants in space, there is a paucity of information on photosynthesis per se. In addition, long-term experiments to determine biological adaptations of higher plants are not possible with drop towers (1–2 s μ g), parabolic flights (20–35 s μ g) or space shuttle (5–16 day μ g) experiments.

Canopy level photosynthetic rates from wheat launched and subsequently grown in space onboard the International Space Station during Increment IV was not different from photosynthetic rates of gravity naïve plants grown entirely in μ g (Stutte et al. 2005). These measurements suggest that the spaceflight environment (hereafter referred to as μ g) does not adversely affect the development of the photosynthetic apparatus or the efficiency of photosynthesis of developing tissue or canopy photosynthesis during vegetative growth (Monje et al. 2005; Stutte et al. 2005). These observations are consistent with reports of Aliyev et al. (1987) who reported no significant changes in the nuclei, mitochondria, rhizomes or chloroplasts of *Pisum sativum* leaves grown in space for 29 day on Salyut 7. However, Aliyev et al. (1987) observed vascularization in the chloroplast stroma, some separation within the grana, and reported a reduction in ribosome density within the stroma.

A change in grana structure was also reported by Tairbekov et al. (1981) in *Zea mays* grown in space for 19 day on Cosmos-1129 and by Kordyum et al. (1981) on *Funaria hydrometrica* that had been in space for 96 day onboard Salyut 6. In contrast, Musgrave et al. (1998) found no changes in the ultrastructure of chloroplast or mitochondrial membranes of *Arabidopsis thaliana* in a series of spaceflight experiments ranging from 6 to 11 day in space (Chromex-03; Chromex-04; Chromex-05). However, they found that reproductive development did not proceed normally on orbit if the physical environment around the plant under spaceflight conditions did not meet the metabolic demands of the plant. Cook and Croxdale (2003) examined the ultrastructure of space grown potato tubers that were grown under well controlled environmental conditions and could not distinguish μ g grown tissue from 1 g controls.

Musgrave et al. (1998) reported a strong relationship between ventilation and chlorophyll concentration and plastid development. These data suggested that previous observations of lower starch and chlorophyll concentrations in μ g grown tissue than 1 g controls (Brown et al. 1996), could be partially explained by the quality of the environmental conditions in the growth chamber.

Early space-flight experiments reported a reduction in certain components of the cell wall, such as cellulose and lignin (Cowles et al. 1984; Nedukha 1996). These changes were generally consistent with decreases in lignin content observed in clinostat studies (data reviewed in Halstead and Dutcher 1987; Brown and Piastuch 1994), but changes in cell wall composition have not been consistently observed when adequate environmental control is provided (Levine et al. 2001; Soga et al. 2002; Kraft et al. 2000).

Levine et al. (2001) did not observe any differences in the cell wall organization, root orientation, protoxylem or metaxylem thickening patterns or lignin content of wheat leaves that had been germinated at 1 g and exposed to 10 day of μ g during STS-51. Similarly, Kraft et al. (2000) did not detect any differences in either cell size or plastid area of columella cells of *A. thaliana* that were exposed to spaceflight, random positioning machine (RPM), or clinostat.

Soga et al. (2002) reported that the level of cell wall polysaccharides per unit length of hypocotyls decreases in space and there was no difference in the total concentration of cell wall polysaccharides on either a fresh or dry mass basis. The authors suggest that the decline in the concentration of cell wall polysaccharides with time is associated with an increase in cell wall extensibility, and that the apparent composition of the cell wall constituents did not change. Similarly, Hoson et al. (2002, 2003) reported that the concentration of the cell wall polysaccharides, cellulose, hemicellulose, and pectin decreased per unit length of rice roots in spaceflight. There was no difference in total lignin per root between the flight samples and the controls. They also did not see any difference in the relative proportion of cell wall polysaccharides between the flight and ground samples. The difference in concentration per unit length was associated with an increase in root length of spaceflight grown rice.

During a recent experiment onboard the International Space Station (ISS), the development of leaves from wheat plants grown for 14 and 21 days in space was studied. The Photosynthesis Experiment System Testing and Operation (PESTO) experiment provided the opportunity to obtain a tissue from replicated studies and allowed sampling of leaf tissues that had developed its photosynthetic apparatus entirely in μ g. The PESTO study was conducted with wheat cv. USU Apogee, an F1 progeny line of the wheat cv. Super Dwarf cultivar used in the study of Levine et al. (2001). A primary objective of these analyses was to determine what effect μ g had on the leaf development, cell structure, carbohydrate metabolism and gene expression patterns of semi-dwarf wheat harvested during PESTO.

The plants were grown in flight hardware that was optimized to minimized the most glaring of the "space-flight" factors, such as lack of convective currents, inadequate CO₂, temperature control, relative humidity, soil moisture and ethylene control (Morrow and Crabb 2000; Iverson et al. 2003) that confounded interpretation of early spaceflight experiments (Monje et al. 2003).

Additional steps were taken to control for the spacecraft environment effects included analyzing samples that were gravity naïve, conducting 1 g controls in identical flight hardware used in μ g under identical environmental conditions, and pairing post-flight statistical analysis between the 1 g and μ g samples.

Materials and methods

Spaceflight hardware

The Biomass Production System (BPS; Orbitec, Madison, WI) is a space shuttle double mid-deck locker-sized plant growth unit. It provides four plant growth chambers (PGCs) composed of a light bank, chamber walls and a root module. Each PGC permits independent control of air temperature, relative humidity, light level, CO₂ concentration, and root zone matrix potential. The plant chambers are removable for on-orbit access to plants for sampling and manipulation such as harvest or pollination. Light in each PGC is provided by cool white fluorescent lamps, and ethylene is removed by a photo-catalytic TiO₂ scrubber. Each PGC has 0.0264 m² of plant growth area with 13 cm tall chamber walls and a 3 cm deep root tray. The root tray is separated from the aerial portion of the chamber by a foam cover and a manifold to circulate air. Water is supplied to the substrate via three porous tubes by a metered peristaltic pump in order to maintain a constant root zone matrix potential. The PESTO experiment was conducted in PGCs 1–3 of the BPS. The fourth PGC was planted with *Brassica rapa* as part of a separate hardware validation test (Morrow and Crabb 2000; Iverson et al. 2003).

Spaceflight activities

The BPS was loaded with imbibed root modules at 3 days before launch. The root modules contained plants that were 8 (PGC2), 2 (PGC3) or 0 (PGC1) days after imbibition (DAI). One day before launch the BPS was transferred to the middeck-locker of Shuttle Endeavour. BPS was launched from the Kennedy Space Center (KSC) during the STS-110/8A mission on April 8, 2002. The BPS remained on Endeavour until it was transferred to the ISS on April 12, 2002. The BPS was installed onto EXPRESS Rack 4 of the Destiny module of ISS (Fig. 1a). The PESTO experiment was maintained by the payload engineer, who was responsible for on-orbit operations including tissue fixation, plant harvests and imbibing pre-planted root modules. The BPS returned to Earth on June 19, 2002 on STS-111/UF-2 (Edwards Air Force Base landing). The total on-orbit duration for the BPS was 73 days.

The PESTO experiment had a total of nine different planting/harvest cycles that resulted in wheat samples comprising: germinated and developed leaf structures at 1 g and were harvested at μ g (Harvest #1,2); germinated at 1 g, developed leaf structures and were harvested at μ g (Harvest #3); germinated, developed and harvested entirely in μ g (Harvest #4,5,6,7) and germinated and developed in μ g and harvested at 1 g (Harvest # 8,9). The conditions and chamber associated with each of these treatments, and the source of samples used in the following analysis, is shown in Table 1.

Plants were harvested in orbit by removing the appropriate PGC from the BPS chamber, taking photographs, and then selecting two representative plants for preservation in the Kennedy Space Center fixation tubes (KFTs). The KFT hardware has three levels of containment provided by a series of nested tubes and valves. The main

Fig. 1 Schematic of procedures conduct a dye-swap microarray experiment using RNA extracted from *T. aestivum* cv. USU Apogee leaves of developed μ g (FLT) and 1 g ground controls (GC). Each sample was contained internal controls. Following reverse transcription of the total RNA and internal controls, the samples were split and coupled with either Alexa 647 or Alexa 555 fluorescent dyes. Slide 26 was hybridized with cDNA from flight coupled to Alexa 647 and ground controls to Alexa 555. The dye pattern was reversed for Slide 27. Microgels were run to confirm the quality of the dye coupling prior to hybridization and analysis (Figure courtesy of D. Laudencia-Chinguanco, USDA, Albany, CA, USA)

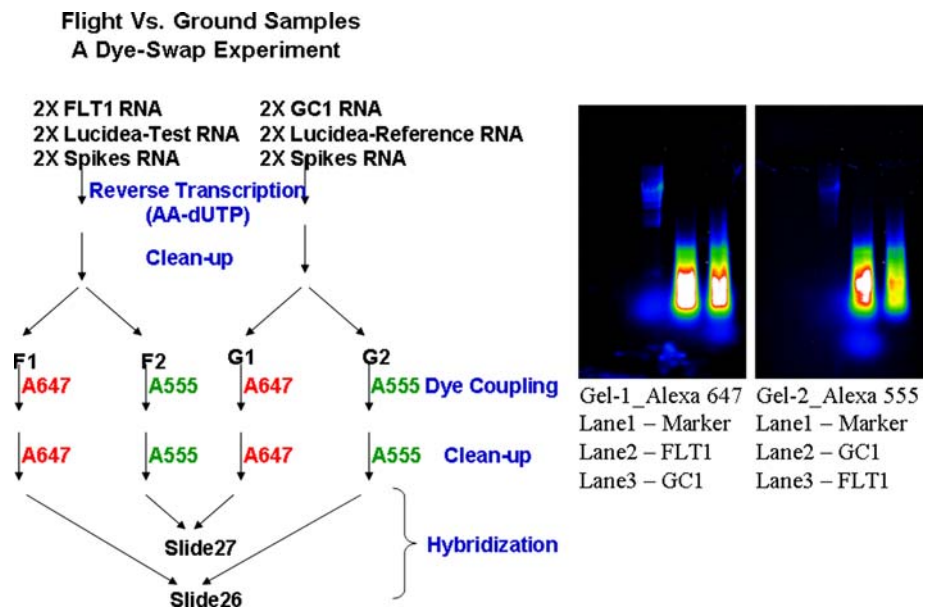


Table 1 Microgravity exposure of the multiple harvest of *T. aestivum* cv. USU Apogee obtained during the PESTO ISS experiment

Harvest		g environment				Notes ^a	
No.	PGC	Imbibe	Harvest	Age	µg days	Sampling ^b	Comments
1	PGC2	1 g	µg	21	11		Shoots developed
2	PGC3	1 g	µg	20	16		Shoot emergence
3	PGC1	1 g	µg	20	18	TEM	Gravity naïve shoots
4	PGC2	µg	µg	23	23	No samples used due to temperature anomaly.	
5	PGC1	µg	µg	21	21	LM, TEM, CHO, CW	Gravity naïve
6	PGC3	µg	µg	21	21	LM, TEM, CHO, CW, mRNA ^c	Gravity naïve
7	PGC4	µg	µg	14	14	LM, CHO	Gravity naïve
8	PGC1	µg	1 g	24	24	LM, CHO	9 h after landing
9	PGC3	µg	1 g	14	14	LM, CHO	9 h after landing

Leaf tissue from harvest 3, 5, 6 and 7 were considered to be gravity naïve since the tissue developed and was either fixed or frozen onboard ISS. All plant tissue collected during harvest 8 and 9 developed under µg conditions and was either fixed or frozen within 9 h of landing. Data obtained from harvests 1, 2 and 4 are not reported in this study

^a Growth, gas exchange, and photosynthetic responses of these tissues have been previously reported in Monje et al. (2005) and Stutte et al. (2005)

^b The tissues used for the light microscopy (LM), transmission electron microscopy (TEM), carbohydrate (CHO), cell wall composition (CW), and gene expression (mRNA) analysis reported from PESTO experiment

^c Two wheat plants fixed in RNALater™ and stored onboard ISS as described in Paul et al. (2004) until landing

sample tube can contain up to 35 ml of fixative. A description of the KFT operation has been fully described by Wells (1999). Briefly, the excised tissue is placed into the sample tube and an actuating plunger assembly is inserted into the top of the KFT. Rotation of the actuating plunger assembly releases the fixative from the storage tube and allows it to flow over the sample. The entire KFT was then re-stowed.

Two plants were preserved from each harvest (except for harvest #4, Table 1) for structural analysis using 3% glutaraldehyde solution in phosphate buffer as the reagent in the KFT as described by Stryjewski et al. (2000). The KFT's containing the samples were then stored under ambient ISS middeck conditions until return to Earth, where they were transferred to fresh 3% glutaraldehyde solution and stored 4°C until analysis.

Two plants were collected for RNA analysis during harvest #6 (Table 1) and placed a KFT containing RNALater™ (Ambion, Austin, TX) plus 0.1% Tween 80 as the fixative (Paul et al. 2005a, b). This operation was completed within 2 min of excision, and stowed at the -25°C in ARTIC freezer onboard ISS. On 14 June, the KFT's containing tissue fixed in RNALater™ were transferred to the mid-deck locker of the Space Shuttle Endeavor (STS-112) and stored under ambient conditions until landing on 19 June. Upon landing, the samples were transferred to -80°C cryogenic conditions where they were stored until analysis. The resulting RNA was of good quality upon extraction (Paul et al. 2005a, b).

The plants remaining in the PGC were then excised, wrapped in aluminum foil and stored at -25°C in the ARTIC freezer onboard the ISS. At 10 days before landing, the foil sample packets were placed in Biological Research in Canisters (BRICs) and transferred to the KSC gaseous N₂ (GN2) freezer at -176°C. The GN2 freezer was transferred to the mid-deck of the Space Shuttle Endeavor 5 days prior to landing. The samples were then transferred to -80°C cryogenic conditions

where they were stored until analysis. Half of the plants from each harvest were lyophilized prior to performing carbohydrate, cell wall, and lignin analysis.

Horticultural conditions

Triticum aestivum L., cv. USU Apogee was used for this experiment. USU Apogee is a semi-dwarf, spring wheat variety that has been selected for growth in controlled environments expected for spaceflight (Bugbee and Koerner 1997). It was selected for the experiment because vernalization is not required for germination, it grows rapidly following germination, and gas exchange rates at high CO₂ concentrations have been characterized (Monje and Bugbee 1998). Each PESTO root module was packed with ~500 g of Turface™ (calcined montmorillonite clay sifted to 1–2 mm) mixed with Osmocote™ (7 g l⁻¹ or 0.012 g g⁻¹ of substrate) slow release fertilizer. The root modules contained 32 wheat plants seeded in four rows at a planting density of 1,200 plants m⁻² (Stutte et al. 2000). This density corresponds to planting densities used during large scale (20 m² growing area) BLSS testing of wheat (Wheeler et al. 2003). The root zone matrix potential (suction) was -0.30 kPa, which equals a hydrostatic head of -3.0 cm measured at the center the root module. The root modules were removed from stowage, imbibed until saturated using a syringe, and placed in the BPS by the payload engineer.

The plants germinated and grew at a light level of ~280 µmol m⁻² s⁻¹ (measured at the top of the chamber), and a photoperiod of 20 h light/4 h dark. During the each planting cycle, the relative humidity and air temperature setpoints were 75% and 24°C, respectively. Ethylene concentration was maintained at < 50 nmol mol⁻¹ by a photocatalytic scrubber. CO₂ concentration during the light cycle was not allowed to fall below 1500 ± 50 µmol mol⁻¹. CO₂ was not controlled during the dark cycle.

Ground control experiment

The ground control experiment was started on Earth 2 weeks after the launch in a BPS chamber identical to the flight unit. The environmental parameters (air temperature, relative humidity, and CO₂ concentration) of the ISS cabin observed during the flight were reproduced in the Orbital Environmental Simulator (OES) chamber at the Kennedy Space Center (KSC). There were no statistical differences between the flight and ground treatments for PPF (260 ± 23 vs. $270 \pm 24 \mu\text{mol m}^{-2} \text{s}^{-1}$), air temperature (24.1 ± 0.2 vs. $24.1 \pm 0.2^\circ\text{C}$), relative humidity (79.4 ± 6 vs. $76.5 \pm 1\%$), or CO₂ concentration (2538 ± 870 vs. $2600 \pm 1100 \mu\text{mol mol}^{-1}$). It should be noted that CO₂ concentration was controlled to the $1,500 \mu\text{mol mol}^{-1}$ setpoint only during the 20 h light cycle, and not during the 4 h dark cycle when CO₂ increased in the chambers due to canopy respiration. All the on-orbit operations performed during spaceflight were mimicked during the ground control experiment.

Light microscopy preparation

Small, (~2 mm wide) sections of mid-leaf tissue were excised from flag leaves fixed in 3% glutaraldehyde (Harvest #3,5,6,7,8,9; Table 1). The mid-leaf sections were dehydrated through an ethanol series and embedded in paraffin. The resulting blocks were sectioned (12 μm), affixed to glass slides and stained with safranin and fast green. The samples were examined using an Olympus BH-2 microscope equipped with a Sony 390 CCD video camera (Sony Corp., Tokyo, Japan). Video images were captured using a Scion Corporation CG-7 frame grabber card (Scion Corp., Frederick, MD) and saved for subsequent analysis. Leaf thickness, number of palisade and spongy mesophyll cells, and number of chloroplasts per cell were determined using image analysis software (NIH Image).

Transmission electron microscopy preparation

Upon return to Earth, mid-leaf samples of two fully expanded leaves from gravity naïve, 21-day-old plants (harvest #3,5,6; Table 1) were transferred to fresh 3% glutaraldehyde in phosphate buffer saline solution (PBS), pH 7.2, and washed several times in PBS. The leaf samples were post-fixed in 1% buffered osmium tetroxide, washed in distilled water and dehydrated in a graded ethanol series. Then the samples were placed in two 15-min washes of acetone followed by a graded increase in embedding resin that was diluted in acetone (Spurr 1969). At 100% resin the samples were positioned in molds using a dissecting microscope, then allowed to cure in a 60°C oven before mounting. Thin 70 nm sections were made with a Reichert Ultracut E using a diamond knife, placed on copper grids and post stained in uranyl acetate followed by lead citrate. The grids were then observed in a Hitachi S-7000 transmission electron microscope (Hitachi Instruments, Inc. Danbury CT) and

pictures were digitally taken using Soft Imaging Systems (Soft Imaging Systems, Lakewood CO). Digital image analysis was used to determine the length and width of chloroplasts from flight and ground samples and to quantify the grana density within the thylakoid.

Carbohydrate analysis

Starch was analyzed in all samples using a modification of the amyloglucosidase assay described by Stutte et al. (1996). Briefly, ~15 mg of finely ground, lyophilized leaf tissue is placed in conical centrifuge tube and extracted three times with 80% MeOH at 80°C. The insoluble starch in the pellet was then gelatinized by adding 2 ml of 0.2 M KOH and incubating in a boiling water bath for 30 min. The sample was cooled, and pH adjusted to pH 3 by the addition of acetic acid. Approximately 90 units of amyloglucosidase (EC 3.2.1.3, A-7255, Sigma, St Louis, MO) in citrate buffer (30 units ml⁻¹) were added to the tube and the sample incubated at 55°C for 1 h. The sample was then centrifuged, supernatant decanted, pellet washed 2× with DI H₂O, and sample brought to 10 ml with DI H₂O. Glucose was determined following procedures of R-Biopharm D-glucose kit (0716251, Roehringer Mannheim/R-Biopharm, Darmstadt, Germany), and absorbance at 340 nm was measured with a spectrophotometer (Beckman Coulter DU 800, Fullerton, CA).

Cell wall preparation and analysis

Lyophilized leaf tissue from gravity naïve (harvest #5, 6; Table 1) and the corresponding 1 g ground controls were placed in a 55°C oven for 2 days before weighing 40–50 mg into dry pre-weighed 2 ml screw cap microfuge tubes containing two glass beads. Tightly capped tubes were placed in liquid nitrogen for 5 min, removed and ground in a bead beater mill for 30 s before being returned to the liquid nitrogen. This cycle was repeated 3× until the plant tissue was a fine powder (< 0.5 mm average). Ground samples were hydrated in 1.5 ml of TRIS-acetate buffer (50 mM, pH 6.0). The supernatant was removed after centrifugation (12,000g) and added to a 15 ml centrifuge tube. The insoluble residue was suspended in 250 μl of TRIS-acetate buffer, capped and placed in a boiling waterbath for 2 h. After 2 h the tubes were transferred to a 55°C bath and 1 unit each of amylase and amyloglucosidase was added and incubated for another 3 h in the 55°C bath. Ethanol was added to each tube (1.5 ml) to precipitate any cell wall polysaccharides that might have been solubilized during the starch removal step. After mixing the insoluble residue was pelleted by centrifugation (12,000g) and the supernatant removed and saved. The insoluble residue was washed 4× with 80% ethanol and 3× with acetone using centrifugation the pellet the insoluble material after each wash step. All supernatants were saved. After the final acetone wash the pelleted residue (alcohol insoluble residue, AIR) was left uncapped in the hood for drying. Tubes were placed in the 55°C oven overnight before

determining the weight of recovered material and sub samples removed for cell wall analyses. Leaf samples from each row of an individual PGC were collected and pooled. Two rows per PGC analyzed from harvest five and six, for a total of four individual samples. Each sample was analyzed 2×.

Lignin determination

Dry alcohol insoluble residue (AIR) was weighed (8–10 mg) into 2.5 ml reaction vials, 0.5 ml of 25% acetyl bromide in acetic acid added, and the vials placed in a 50°C heating block for 2 h. Vials were gently mixed periodically throughout the heating period. After heating the samples were cooled in an ice bath before quantitatively transferring the vial contents to a 10 ml volumetric flask containing 0.4 ml of 2 M NaOH, 0.48 ml of glacial acetic acid and 70 µl of 0.5 M hydroxylamine. Transfer was aided with glacial acetic acid and the volume was made up to 10 ml with glacial acetic acid. Samples were thoroughly mixed before a 1 ml subsample was removed placed in a microfuge tube (1.7 ml) and centrifuged for 2 min at 12,000g. Samples were scanned from 250 to 350 nm using Beckman 7400 spectrophotometer. Absorbance values at 280 nm were determined from digital recordings of the scans. Calculations of total lignin were determined using the extinction coefficient for wheat determined by Fukushima and Hatfield (2004).

Neutral sugar determinations of AIR

Sub-samples (4–6 mg) of AIR were weighed into 2 ml reaction vials and 100 µl of cold 12 M H₂SO₄ added. Samples and acid were mixed with a small stirring rod to disperse the AIR into the acid and allowed to stand at room temperature for 2 h with occasional stirring. At the end of 2 h distilled water was added (0.8 ml) to mix and rinse off the stirring rods; vials were capped and placed in a heat block at 100°C for 3 h. At the end of the heating samples were cooled, transferred to 50 ml centrifuge tube with the aid of distilled water, and internal standard (inositol 0.5 mg/tube) added. The dilute acid solution was neutralized with BaCO₃ and the insoluble BaSO₄ separated from the supernatant by centrifugation (2,500g, 20 min). Subsamples (4 ml) were transferred to 8 ml reaction vials evaporated to dryness under a stream of filter air. Monosaccharides from the cell wall hydrolysis were converted to alditol acetates (Blakeney et al. 1983) analyzed by GC-FID using SP-225 capillary column (Supelco 30 m × 0.25 mm) using a temperature program of 215°C initial for 2 min, 4 C/min to 230°C and hold for 11.25 min.

Lignin composition nitrobenzene oxidation

AIR samples (4–5 mg) were weighed into Teflon vials (8 ml) and 0.75 ml of 2 M NaOH along with 50 µl nitrobenzene added before capping tightly and placing into a larger Teflon reaction vessel (5 vials/tube) with 2 ml of

water and sealing tightly. Reaction vessels were heated in a forced air oven for 2 h at 170°C. After heating vessels were removed and cooled under running cold tap water. Individual reaction vials were opened and contents quantitatively transferred to culture tubes (25 ml with Teflon lined caps) using distilled water (total volume 5 ml) and internal standard isovanillin added (25 µg). Samples were initially extracted with 5 ml of chloroform before adjusting the pH to < 2 with 12 M HCl and extracting 2× with dichloromethane and 1× with ether (5 ml each extract). Ether and dichloromethane extracts were combined, washed once with acidified water, and the organic phase evaporated to dryness in a reaction vial (5 ml). Dried samples were derivatized with BSTFA and analyzed by GC-FID as described in Fukushima and Hatfield (2001).

Gene expression

Two gravity naive plants were collected during the PESTO harvest #6 (Table 1) in order to assess mRNA expression using microarray analysis. Due to the limited number of plants, the leaves of the gravity naive plants fixed in RNALater™ were pooled and extracted using RNeasy™ kits from Qiagen (Valencia, CA) using the protocols of Paul et al. (2004).

A dye-swap experiment was conducted by coupling Alexa 647 or 555 dyes with reversed transcribed RNA using protocols of The Institute of Genomic Research (TIGR) as described by Hegde et al. (2000) and modified by Laundencia-Chingcuanco (personal communication). Briefly, the total RNA extracted from each sample was reverse transcribed using oligo (dT)₂₃ (Sigma O-4387, St Louis, MO) and SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA) and amplified with PCR for 1.5 h at 42°C. The Superscript activity was inactivated by incubating at 70°C for 15 min. Random primers (3 µg/µl Gibco-BRL, Invitrogen, Carlsbad, CA), spiking controls from the AFGC microarray control set (2.5 ng/µl; SP1-SP10), and Lucidea ScoreCard mRNA test/reference mix (Amersham Biosciences, Piscataway, NJ) were added to the transcription reaction (Fig. 1).

Transcribed RNA was hydrolyzed by adding 250 mM EDTA (pH 8) and 1 N NaOH, and incubating at 65°C for 20 min. The solution was neutralized with 1 N HCL and the aa-cDNA purified from the unincorporated aa-dUTP and free amines with Microcon 30 filtration (Millipore, Bedford, MA).

Either Alexa 647 or Alexa 555 fluorescent dyes (Molecular Devices, Inc, Sunnyvale, CA) were coupled to the purified first strand cDNA in order to perform a dye-swap experiment. The dye-coupled cDNA was purified following manufacture instructions (Invitrogen, Carlsbad, FL, Cat #L1014-05) and microgels were run to confirm the quality of the dye coupling to the transcribed RNA (Fig. 1)

Each labeled sample was suspended in hybridization solution (50% formamide, 10× SSC, and 0.2% SDS) with Poly(A)-DNA (4 µg/µl), salmon sperm DNA (10 µg/µl),

heated for 90°C for 5 min and centrifuged for 1 min. The hybridization mix was pipetted onto the center of the HybriSlip™ hybridization cover, and allowed to hybridize at 42°C for 16–20 h. Following hybridization, the microarray slides were washed with solutions of increasing stringency, and then air dried (Fig. 1).

The arrays were scanned with GenePix 4000B (Molecular Devices Inc, Sunnyvale, Ca) at 635 and 532 nm wavelengths with PMT of 600, and 400 V and laser power at 3.8 and 3.9 V, respectively. The resulting signals were analyzed using GenePix Pro 5.1.0.0 (Molecular Devices Inc, Sunnyvale, CA).

Each microarray had a total of 21,500 features, 7,821 of which were genes. The arrays had 48 grids per array with a matrix of 21 × 22 spots per array with a nominal 200 μm center spacing and 150 μm spot diameter. Each sample was spotted at least twice. Controls (4 position markers, 2 buffer, 12 positive, 26 spiking, and 2 negative) were included on the first two rows of each grid. Lucidea score card controls (Amersham BioSciences, Piscataway, NJ) were included in blocks 7, 18, 31 and 42.

Results

Leaf morphology

There is no difference in the leaf transverse thickness between the μg grown and 1 g ground controls at 14 DAI (Table 2). There was no change in leaf thickness in the 1 g ground control plants with increasing age. In contrast, there was a linear decrease in leaf thickness μg environment (thickness, mm = -0.0075 (DAP) + 0.2103; $r^2 = 0.98$) with increasing age. This resulted in the leaves from μg grown wheat being thinner than comparable aged 1 g ground control tissue at 20 and 24 DAI.

Cell structure

Cell density from 21-day-old, gravity naïve plants was 13% higher in μg compared to 1 g ground controls (Table 3). There were no significant differences in cell cross-sectional area of cells from the μg and 1 g samples (Table 2). The cell structure (cell wall, nucleus, and mitochondria) also appeared to be normal in both the μg and 1 g samples.

Table 2 Cross-sectional thickness (mm) of *T. aestivum* leaves developed in μg and comparable aged 1 g ground controls

	μg	1 g	<i>P</i> > 0.05
14 DAI	0.203	0.202	NS
21 DAI	0.195	0.205	**
24 DAI	0.188	0.202	***

Sections from the leaf samples were obtained from leaves that developed in μg and were fixed in 3% glutaraldehyde either onboard International Space Station or within 9 h of landing. Mean separation analyzed using paired *t* test

DAI Days after imbibition, NS non-significant

P* > 0.01, *P* > 0.001

Chloroplast development

There was no difference in chloroplast density between μg and 1 g, and chloroplasts developed in μg conditions were slightly rounder than those developed in 1 g ground controls. However, differences in the development of the thylakoid membranes were observed between the μg and 1 g samples. While there was no statistical difference in the number of thylakoids per grana stack, each of the membranes were stacked approximately 0.9 nm closer in the tissue developed under μg conditions, relative to the 1 g ground controls (Table 3). While some degradation of the thylakoid membranes developed in μg was observed the disruption was isolated and sporadic.

Starch concentration

There were no differences in insoluble starch content between the μg grown and 1 g ground control leaf tissue of 21-day-old, gravity naïve plants harvested on orbit, or the 14-day-old μg grown plants harvested in 1 g after landing. The insoluble starch concentration was 13% lower in 24-day old μg grown plants that were harvested in 1 g after landing (Table 4). This decrease in starch is likely due to an environmental anomaly experienced with this PGC following transfer from ISS to the Space Shuttle prior to return to Earth when real-time environmental data was not available from space. This lack of data resulted in the air temperature in the ground control chamber being ~3°C cooler than the flight chamber for the last 5 day of development of this chamber. This would be expected to correspond to a higher respiration rate which would deplete carbohydrate reserves at a faster rate.

Cell wall composition

There was no difference in the lignin content, composition of insoluble lignin components, coniferyl, pHB, or sinapyl or in the proportion of alcohol insoluble sugars of the cell walls between the 21-day-old, gravity naïve plants and the corresponding 1 g ground controls (Table 5).

Table 3 Cell morphology and chloroplast development characteristics of 21-day-old *T. aestivum* leaves developed in μg and comparable 1 g ground controls

Measurement	μg	1 g	<i>P</i> > 0.05
Cell density (#/mm ²)	1,786	1,581	***
Cell area (μm ²)	72.8	79.8	NS
Chloroplast density (#/cell)	10.4	9.8	NS
Chloroplast area (μm ²)	0.534	0.522	NS
Chloroplast L/W	2.06	2.23	**
Thylakoid density (#/grana)	12.23	11.4	NS
Thylakoid spacing (nm)	18.4	19.3	**

Sections from the leaf samples were obtained from leaves that developed in μg and were fixed in 3% glutaraldehyde onboard International Space Station. Mean separation analyzed using paired *t* test

NS Non-significant, L length, W width

P* > 0.01, *P* > 0.001

Table 4 Starch concentration (mg gDM^{-1}) of *T. aestivum* leaves developed in μg and either onboard International Space Station or within 9 h of landing

	μg	1 g	$P > 0.05$
Harvested on orbit			
21 DAP	11.2	10.9	NS
Harvested after landing			
14 DAP	10.9	11.8	NS
24 DAP	10.6	12.2	*

Mean separation analyzed using paired *t* test

NS Non-significant

* $P > 0.05$

Gene expression

A total of 820 genes represented on the arrays were found to be expressed in the leaves of both the μg and 1 g ground control tissue. However, there was no evidence of differential expression in these genes between the μg and 1 g ground treatment. Any variation that was seen was below the twofold cutoff (Fig. 2).

The microarray chips were developed from cDNA sequence derived from field wheat lines and did not represent the full gene complement of the wheat genome (Morris, personal communication). None-the-less, the arrays do contain genes representing most of the primary biochemical pathways and represent the most complete arrays available at the time. The full list of genes expressed in the μg grown and 1 g ground control tissue, and the intensity of expression, is included as supplementary material.

Discussion

These experiments represent the first direct, replicated comparison of cell structure, carbohydrate and gene expression of wheat in a well controlled spaceflight

Table 5 Concentration of lignin (g gCW^{-1}), alcohol insoluble components of lignin ($\mu\text{mol g lignin}^{-1} \text{gCW}^{-1}$), and alcohol extractible components of cell walls ($\mu\text{g gCW}^{-1}$) from gravity naive leaves of *T. aestivum* cv. USU Apogee compared to 1 g ground controls

Component	Flight	Ground	$P > 0.05$
Lignin	0.69	0.062	NS
Alcohol insoluble components			
Coniferyl	1.68	1.65	NS
pHB	0.52	0.57	NS
Sinapyl	0.61	0.58	NS
Alcohol extractable components			
Rhamnose	2.17	2.10	NS
Fucose	0.94	0.50	NS
Arabinose	8.98	10.40	NS
Xylose	100.20	117.50	NS
Mannose	1.14	1.29	NS
Galactose	8.08	8.81	NS
Glucose	320.10	344.90	NS

Mean separation analyzed using paired *t* test

pHB *p*-hydroxybenzaldehyde

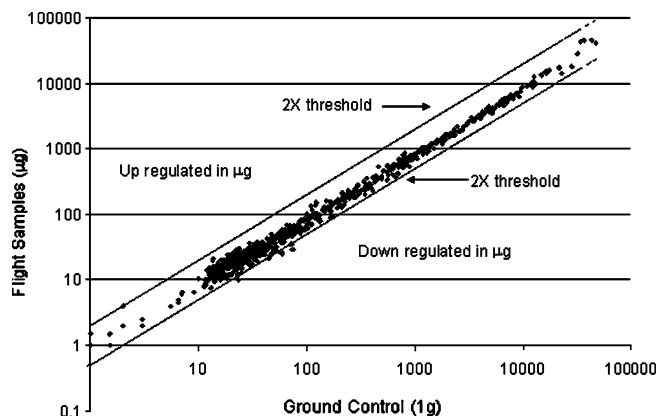


Fig. 2 Assessment of differential gene expression in between *T. aestivum* cv. USU Apogee leaves of developed μg and 1 g ground controls. Alaxa labeled total RNA was extracted from leaves that were grown in μg environment and fixed in RNALater™ onboard the International Space Station and hybridized to wheat arrays (courtesy of M. Giroux, Montana State University). Flip dyes were used for replicate hybridizations. The *solid lines* represent a difference of a factor of 2

environment with a controlled ground environment. These data suggest that spaceflight conditions alter leaf development, cell structure, and chloroplast morphology, but that these morphological changes do not compromise their physiological function. This result is consistent with the results of Stutte et al. (2005) and Monje et al. (2005), who showed that canopy level gas exchange and dry mass accumulation of these plants were not negatively impacted by the μg environment experienced during Increment IV onboard ISS.

At 14 DAI, there were no differences in the cross sectional area of leaves between the μg grown and the 1 g grown ground control tissue. There was also no difference in the thickness of the leaves between 14 and 24 days in the ground controls. In contrast, the cross-sectional thickness of the flight grown tissue decreased linearly over the same 10-day period, resulting in significantly thinner leaves between the μg and 1 g tissue at 20 and 24 DAI. At 21 DAI, the cell density was $\sim 12\%$ greater in the μg grown plants than in the 1 g ground control tissue. Although more densely packed, there was no difference in the cross sectional area of the individual cells between μg and 1 g. There was a tendency for the cells developed in μg to be more ovoid than those developed at 1 g but this difference could not be statistically resolved. Similarly, there was no statistical difference in the specific leaf mass between the μg (0.212 mg mm^{-2}) and 1 g (0.204 mg mm^{-2}) grown leaves. However, the trend towards slightly higher specific leaf mass is consistent with the increased cell density and reduced thickness of the tissue developed in μg . These structural changes are consistent with morphological observations of the plants previously reported by Monje et al. (2005) and Stutte et al. (2005).

At the chloroplast level, there were no difference in the number of chloroplast, size of chloroplast, or thylakoid density of cells developed in either μg or 1 g. How-

ever, the chloroplasts were somewhat more ovoid in the cells that developed in μg than in cells developed in the 1 g ground controls. Similarly, the thylakoid spacing in chloroplasts developed in μg was slightly less than in comparable 1 g ground controls. While statistically significant, this change in chloroplast structure did not impact the Fv/Fm, PSI, PSII or WCE activity at a light intensity of $280 \mu\text{mol m}^{-2} \text{s}^{-1}$ (Stutte et al. 2005). This suggests that the changes in leaf and cell morphology associated with the μg environment were not sufficient to induce physiological changes, or if they did, they may off-set each other. Alternatively, it is possible that the physiological functions had acclimated to the μg conditions, and the changes could not be detected in the tissues sampled.

The latter hypothesis is not supported by the chemical analysis of the tissue, however. No differences in the leaf starch concentration of between leaves developed in μg and 1 g environments were observed. There was a slight reduction in starch concentration in the 24 DAI plants that were grown in μg and harvested at 1 g, but it is likely that this was associated with lower light penetration into the canopy of μg plants because they were slight taller than the 1 g controls (Monje et al. 2001, 2005). The increase in cell density of the μg grown plants was not associated with a change in either the total amount of lignin in the cell wall, or the composition of the cell wall.

While the lack of changes in cell structure of leaves developed in μg contrasts with the observations of Cowles et al. (1984) and Nedukha (1996) they are consistent with many later observations showing no difference in cell structure of wheat (Levine et al. 2001), *A. thaliana* (Musgrave et al. 1998; Kraft et al. 2000), *Brassica rapa* (R.C. Morrow, personal communication), potato (Cook and Croxdale 2003) and rice (Soga et al. 2002). The differences in cell structure from early spaceflight experiments were likely related to secondary effects of the μg environment, caused by inadequate ventilation and limited environmental control of the plant chambers during spaceflight (Brown and Piastuch 1994; Musgrave et al. 1998; Monje et al. 2003). Similarly, the finding that cell wall lignin content or its composition is unchanged in cells developed under μg conditions contrasts with results reported from the early spaceflight experiments (Cowles et al. 1984; Nedukha 1996), but is consistent with results reported for plants grown in well-ventilated systems with good environmental control in μg (Levine et al. 2001; Kraft et al. 2000).

Although changes in leaf morphology of the leaves developed in μg were observed, there is only limited data to suggest any negative physiological impacts on photosynthetic function. The total insoluble starch concentration was the same in leaves developed in μg and 1 g and no differences in the soluble sugar content were observed (data not shown). It should be noted that the starch concentrations are relatively low compared to field grown plants, but likely represents the attenuation of light in the canopy (Monje et al. 2005) since the concentrations are low in both environments.

The lignin concentrations are also much lower than those commonly reported in field-grown wheat straw (Hatfield and Fukushima 2005). This is likely due to the young age of the wheat and rapid growth rate (Stutte et al. 2003) under relatively low light conditions. Although the PPF level of $280 \mu\text{mol m}^{-2} \text{s}^{-1}$ PAR is near the limit available in existing spaceflight certified plant growth chambers, it is only 10% of what is commonly observed under ambient field conditions. Although the concentration of lignin is lower than typically observed from the field, the composition is similar to field grown tissue, suggesting that the observed result is a function of the plant growth environment.

There were no examples of differential gene expression unique to either μg or 1 g ground control samples, suggesting that environmental and physiological stresses were below threshold levels to induce major changes in gene expression, and ultimately, physiological function. There have been two *A. thaliana* flight experiments that examined genome-wide patterns of gene expression in response to the spaceflight environment. Transgenic *A. thaliana* plants were launched for a 5-day mission on orbiter Columbia (STS-93) in 1999. Reporter gene analyses indicated that at the minimum, there was differential expression of alcohol dehydrogenase in root and shoot tissues compared to the ground control plants, and that the spaceflight environment impacted calcium mediated signal transduction (Paul et al. 2001). Follow-on experiments evaluated genome-wide patterns of native gene expression utilizing the Agilent Technologies DNA array of 21,000 *A. thaliana* genes (Paul et al. 2005a, b). Probing the arrays with RNAs made from flight and ground control *A. thaliana* shoot tissue revealed 182 genes that were differentially expressed in excess of fourfold, although only 50 genes from that set were expressed at levels high enough to be considered further by the authors. Among the 50 highly differentially expressed genes were those related to heat shock. However, the pattern of expression was not that of a typical heat shock response, nor were there any excursions into high temperatures recorded for either flight or ground controls. Chlorophyll binding proteins were down regulated in the flight compared to the control samples. Although some of the examples of differential expression were significant, the data indicate that the environmental stress imposed by spaceflight is minimal when the orbital growth systems are optimized for aeration, hydration and confined volatiles.

The limited stress responses demonstrated by these wheat arrays and the STS-93 *A. thaliana* arrays is in marked contrast to results reported by Link et al. (personal communication) in *A. thaliana* grown on ISS during Increment IV. They reported that several hundred genes were differentially expressed in the space grown tissue. An unknown percentage of the genes that were detected to be either up or down regulated in the μg samples may be associated with environmental factors affecting differences in growth conditions between the μg and 1 g plants. The plants grown in μg during that experiment experienced water logging, which delayed

germination and development, resulting in stunted growth on ISS. The water logged conditions were not replicated in the ground control plants used to probe for differential gene expression (B. Stankovic, personal communication).

Differential expression of 141 genes within 30 min of *g*-stimulation and mechanical stimulation has been reported in *A. thaliana* by Moseyko et al. (2002) in 3-week old seedlings using the Affymetrix (Santa Clara, CA) 8 K Gene Chips. There was a significant overlap in the genes activated by mechanostimulation and *g*-stimulation suggesting that common pathways are induced by the two stimuli. Moseyko et al. (2002) suggested that gene expression in *A. thaliana* is very sensitive to both stresses and that caution must be used in interpreting results, because of the limited number of genes, and the use of whole plants.

Kimbrough et al. (2004) conducted a more detailed study of gravity perception in root tips of *A. thaliana* by probing for changes in transcript abundance using the microarrays containing the entire genome of 22,744 genes. They reported transient responses within 2 min of the stimulation. As with Moseyko et al. (2002), the vast majority (96%) of the differentially expressed genes responded to both stimuli. Differential expression of genes in the root tips, the site of *g* perception would not be detected in the PESTO samples since only leaf tissues were used.

The ground controls used to evaluate the differential expression of wheat in PESTO were grown in flight hardware under environmental conditions identical to those of the gravity naïve plants grown on ISS. While the high fidelity environmental control suggests that no genes unique to the μg environment were induced in the leaves, it is possible that the arrays used (courtesy of Montana State University) did not include probes for genes which had been differentially expressed in the μg grown leaves. The wheat arrays utilized for these assays were developed using cDNA from field wheat (which is not semi-dwarf), and may therefore exhibit a different gene profile than the Apogee wheat used in this experiment (R.J. Ferl and A-L. Paul, personal communication). This suggests the need to develop gene arrays specific for the wheat cultivars used in spaceflight experiments in order to effectively assess the entire range of genomic responses to the spaceflight environment.

The lack of physiological changes in starch concentration, lignin content, lignin composition, and gene expression for tissue grown in μg compared to 1 *g* is consistent with the lack of response of canopy level photosynthesis and biomass accumulation rates to μg that has been previously reported for these plants. Stutte et al. (2005) reported that the P_{net} of plants initially germinated less than 1 *g* conditions and developed in μg were identical to P_{net} from gravity naïve plants. In addition, no differences in canopy level photosynthetic rates of wheat grown in μg for up to 24 days under elevated CO₂ and moderate (280 $\mu mol m^{-2} s^{-1}$) PPF conditions. Stutte et al. (2005) also reported no difference in the chlorophyll content of wheat leaves developed in μg or 1 *g* for 14 or 24 days. The Fv/Fm of the leaves was > 0.77 for all samples.

Monje et al. (2005) reported that the individual leaf blade developed μg was ~25% less than comparable 1 *g* controls and that the plant height was ~2 cm taller in the μg (10%) of these same plants. The decrease in leaf thickness is consistent with an increase in plant height, without a corresponding decrease in total plant DM or canopy level P_{net} (Stutte et al. 2005). The change in density in thylakoid stacking density may partially explain the 12–16% reduction in PSII, PSI, and WCE activity of isolated chloroplasts in the 24-day old μg grown plants over the 1 *g* ground controls reported by Stutte et al. (2005) in *T. aestivum* cv. USU Apogee and Tripathy et al. (1996) for *T. aestivum* cv. Super Dwarf. The changes in photosynthetic efficiency associated with thylakoid processes are typically manifested at high PPF levels (> 850 $\mu mol m^{-2} s^{-1}$ PAR), and are not expected to be observed at the PPF used in this experiment.

These results suggest that morphological changes occur in developing wheat leaves grown under well-controlled environmental conditions in μg environment. We found no evidence of developmental, physiological, or genomic changes associated with the indirect effects of μg associated with lack of convective currents or poor environmental control of air temperature, relative humidity, or CO₂ concentration (Porterfield 2002; Monje et al. 2003). Especially significant is the presence of adequate environmental control to overcome the deleterious impact of secondary effects related to poor ventilation, low light levels, and insufficient CO₂ concentrations present in the chambers used in earlier spaceflight plant growth experiments. The effectiveness of this control is evidenced not only by the environmental monitoring data, but from the lack of expression of hypoxia or water stress induced genes in leaf tissues and no changes in canopy dry mass (Monje et al. 2005; Stutte et al. 2005). Although the expression of hypoxia or water stress induced genes could not be analyzed in root tissues, any changes that may have been induced were not significant enough to altered biomass accumulation rates (Stutte et al. 2005) The high fidelity environmental control of the BPS chambers, and the replication of the growth cycles in microgravity, provides support to the conclusion that physiological function can be maintained in photosynthetic tissue of μg grown wheat at the light levels used in this experiment.

Acknowledgments This research was funded in whole or in part by a grant from the Office of Biological and Physical Research of the National Aeronautics and Space Administration. The authors gratefully acknowledge support of Sylvia Anderson for data collection and summarization, F. Bennett, Electron Microscopy Core Facility, University of Florida, Gainesville for preparation of electron micrographs, M. Giroux, Montana State University, Bozeman MT for wheat microarrays, D. Laudencia-Chinguanco, USDA, Albany, CA for hybridization of microarray chips, M. Popp, Molecular Biology Core Facility, University of Florida, Gainesville, for analysis of gene expression data, N. Chatterdon, USDA-ARS, Logan, UT for soluble sugar analysis. The authors acknowledge the support of personnel at Orbitec (Madison, WI), Ames Research Center, Moffett Field, CA, Kennedy Space Center, FL, Johnson Space Center, TX, and Marshall Space Flight Center, Huntsville, AL. Finally, the authors wish to express their gratitude to ISS Increment IV Flight Engineer Dan Bursch for his commitment to microgravity research.

References

- Adamchuk NI, Mikaylenko NF, Zolotareva EK, Hilaire E, Guikema JA (1999) Spaceflight effects on structural and some biochemical parameters of *Brassica rapa* photosynthetic apparatus. *J Gravitat Physiol* 6:95–96
- Aliyev AA, Abilov ZK, Mahinsky AL, Ganiyeva RA, Ragimova GK (1987) The ultrastructure and physiological characteristics of the photosynthesis system of shoots of garden pea grown for 29 days on the “Salyut-7” space station. *USSR Space Life Sci Digest* 10:15–16
- Blakeney AB, Harris PJ, Henery RJ, Stone BA (1983) A simple and rapid preparation of alditol acetates for monosaccharide analysis. *Carbohydr Res* 113:291–299
- Brown CS, Tibbitts TW, Croxdale JG, Wheeler RM (1997) Potato tuber formation in the spaceflight environment. *Life Support Biosph Sci* 4:71–76
- Brown CS, Tripathy BC, Stutte GW (1996) Photosynthesis and carbohydrate metabolism in microgravity. In: Suge H (ed) *Plants in space biology*. Institute of Ecology, pp 127–134
- Brown CS, Piastuch WC (1994) Starch metabolism in germinating soybean cotyledons is sensitive to clinorotation and centrifugation. *Plant Cell Environ* 17:341–344
- Bugbee B, Koerner G (1997) Yield comparisons and unique characteristics of the dwarf wheat cultivar “USU-Apogee”. *Adv Space Res* 20:1891–1894
- Cook ME, Croxdale JM (2003) Ultrastructure of potato tubers formed in microgravity under controlled environmental conditions. *J Exp Bot* 54:2157–2164
- Cowles JR, Scheld HW, LeMay R, Peterson C (1984) Experiments on plants grown in space: growth and lignification in seedlings exposed to eight days of microgravity. *Ann bot [Suppl 3]* 54:33–48
- Croxdale JM, Cook ME, Tibbitts TW, Brown CS, Wheeler RM (1997) Structure of potato tubers formed during spaceflight. *J Exp Bot* 48:2037–2043
- Eley JH, Myers J (1964) Study of a photosynthetic gas exchanger: a quantitative repetition of the Priestley experiment. *Tex J Sci* 16:296–333
- Fukushima RS, Hatfield RD (2001) Extraction and isolation of lignin for utilization as a standard to determine lignin concentration using the acetyl bromide spectrophotometric method. *J Agric Food Chem* 49:3133–3139
- Fukushima RS, Hatfield RD (2004) Comparison of the acetyl bromide spectrophotometric method with other lignin methods for determining lignin concentration in forage samples. *J Agric Food Chem* 52:3713–3720
- Halstead TW, Dutcher FR (1987) Plants in space. *Ann Rev Plant Phys* 38:317–345
- Hatfield R, Fukushima RS (2005) Can lignin be accurately measured? *Crop Sci* 45:832–839
- Hegde P, Rong Q, Abernathy K, Gay C, Dharap S, Gaspard R, Earle-Huges J, Snesrud E, Lee N, Quackenbush J (2000) A concise guide to cDNA microarray analysis-II. *Biotechniques* 29:548–562
- Hoson T, Soga K, Mori R, Asiki M, Nakamura Y, Wakabayashi K, Kamisaka S (2002) Stimulation of elongation growth and cell wall loosening in rice coleoptiles under microgravity conditions in space. *Plant Cell Physiol* 43:1067–1071
- Hoson T, Soga K, Wakabayashi K, Kamisaka S, Tanimoto E (2003) Growth and cell wall changes in rice roots during spaceflight. *Plant Soil* 255:19–26
- Iverson JT, Crabb TM, Morrow RC, Lee MC (2003) Biomass production system hardware performance. *SAE Technical Paper* 2003-01-2484
- Jiao S, Hilaire E, Paulsen AQ, Guikema JA (1999) Ultrastructural observation of chloroplast morphology in space-grown *Brassica rapa* cotyledons. *J Grav Physiol* 6:93–94
- Kimbrough JM, Salinas-Mondragon R, Boss WF, Brown CS, Sederoff HW (2004) The fast and transient transcriptional network of gravity and mechanical stimulation in the *Arabidopsis* root apex. *Plant Physiol* 136:2790–2805
- Kordyum EL, Nedukha EM, Sytnik KM, Mashinsky AL (1981) Optical and electron-microscopic studies of the *Funaria hygrometrica* protonema after cultivation for 96 days in space. *Adv Space Res* 1:159–162
- Kraft TFB, van Loon JJWA, Kiss JZ (2000) Plastid position in *Arabidopsis columella* cells is similar in microgravity and on a random-positioning machine. *Planta* 211:415–422
- Kuang A, Xiao Y, Musgrave ME (1996) Cytochemical localization of reserves during seed development in *Arabidopsis thaliana* under spaceflight conditions. *Ann Bot* 78:343–351
- Levine LL, Heyenga AG, Levine HG, Choi J-W, Davin LB, Krikorian AD, Lewis NG (2001) Cell-wall architecture and lignin composition of wheat developed in a micro gravity environment. *Phytochemistry* 57:835–846
- Liu F, VanToai T, Moy LP, Bock G, Linfor LD, Quackenbush J (2005) Global transcription profiling reveals comprehensive insights into hypoxic response in *Arabidopsis*. *Plant Physiol* 137:1115–1129
- Miller RL, Ward CH (1966) Algal bioregenerative systems. In: Kammermeyer E (ed) *Atmosphere in space cabins and closed environments*. Appleton-Century-Croft Pub, New York
- Moseyko N, Zhu T, Chang H-S, Wang X, Feldman LJ (2002) Transcription profiling of the early gravitropic response in *Arabidopsis* using high-density oligonucleotide probe microarrays. *Plant Physiol* 130:720–728
- Merkys AJ, Laurinavičius RS, Jarosius AV, Rupainiene OJ (1987) Growth, development, anatomy and morphological structure of *Arabidopsis thaliana* (L.) Heynh. under spaceflight conditions. Institute of Botany, Academy of Science of the Lithuanian SSR, Vilnius, pp 105–116
- Monje O, Bugbee B (1998) Adaptation to high CO₂ concentration in an optimal environment: radiation capture, canopy quantum yield and carbon use efficiency. *Plant Cell Environ* 21:315–324
- Monje O, Stutte GW, Goins GD, Porterfield DM, Bingham GE (2003) Farming in space: environmental and biophysical concerns. *Adv Space Res* 31:151–167
- Monje O, Stutte GW, Chapman D (2005) Microgravity does not alter plant stand gas exchange of wheat at moderate light levels and saturating CO₂ concentration. *Planta* 222:336–345
- Monje O, Wang HT, Kelly C, Stutte GW (2001) Nutrient delivery system water pressures affect growth rate by changes in leaf area, not single leaf photosynthesis. *SAE Technical Paper* 2001-02-2207
- Moore R, Fondren WM, McClelen CE, Wang C-L (1987) Influence of microgravity on cellular differentiation in root caps of *Zea mays*. *Am J Bot* 74:1006–1007
- Morrow RC, Crabb TM (2000) Biomass production system (BPS) plant growth unit. *Adv Space Res* 26:289–298
- Musgrave ME, Kuang A, Matthews SW (1997) Plant reproduction during spaceflight: importance of the gaseous environment. *Planta* 203:S177–184
- Musgrave ME, Kuang A, Brown CS, Matthews S (1998) Changes in *Arabidopsis* leaf ultrastructure, chlorophyll content and carbohydrate content during spaceflight depend on ventilation. *Ann Bot* 81:503–512
- Myers J (1954) Basic remarks on the use of plants as biological gas exchanges in a closed system. *J Aviat Med* 35:507–411
- Nechitailo GS, Mashinsky AL (1993) *Space biology: studies at orbital stations*. Mir Publishers, Moscow
- Nedukha EM (1996) Possible mechanisms of plant cell wall changes at microgravity. *Adv Space Res* 1:109–111
- Nedukha OM (1997) Effects of weightlessness on photosynthesizing cells structure of plants. *J Gravit Physiol* 4:79–80
- Paul A-L, Daugherty CJ, Bihn EA, Chapman DK, Norwood KLL, Ferl RJ (2001) Transgene expression patterns indicates that spaceflight affects stress signal perception and transduction in *Arabidopsis*. *Plant Physiol* 126:613–621
- Paul A-L, Levine HG, McLamb W, Norwood KL, Reed D, Stutte GW, Wells HW, Ferl RJ (2005a) Plant molecular biology in the space station era: utilization of KSC fixation tubes with RNA-Later. *Acta Astronaut* 56:623–628

- Paul A-L, Popp MP, Gurley WB, Guy C, Norwood KL, Ferl RJ (2005b) *Arabidopsis* gene expression patterns are altered during spaceflight. *Adv Space Res* 36:1175–1181
- Paul A-L, Schuerger AC, Popp M, Richards JT, Manak M, Ferl RJ (2004) *Arabidopsis* gene expression at low atmospheric pressure—hypobarica does not equal hypoxia. *Plant Physiol* 134:215–223
- Porterfield DM (2002) The biophysical limitations in physiological transport and exchange in plants grown in microgravity. *J Plant Growth Regul* 21:177–190
- Soga K, Wakabayashi K, Kamisaka S, Hoson T (2002) Stimulation of elongation growth and xyloglucan breakdown in *Arabidopsis hypocotyls* under microgravity conditions in space. *Planta* 215:1040–1046
- Spurr AR (1969) A low viscosity epoxy resin embedding medium for electron microscopy. *J Ultrastruct Res* 26:31–43
- Stryjewski E, Peterson BV, Stutte GW (2000) Long term storage of what plants for light microscopy. SAE technical paper 200-01-2231
- Stutte GW, Yorio NC, Wheeler GW (1996) Interacting effects of photoperiod and photosynthetic photon flux on net carbon assimilation and starch accumulation in potato leaves. *J Am Soc Hortic Sci* 121:264–268
- Stutte GW, Monje O, Goins GD, Chapman DK (2000) Measurement of gas exchange characteristics of developing wheat in the biomass production chamber. SAE technical paper 2001-01-424
- Stutte GW, Monje O, Goins G, Ruffe L (2001) Evapotranspiration and photosynthesis characteristics of two wheat cultivars grown in the biomass production system. SAE technical paper 2001-01-2180
- Stutte GW, Monje O, Anderson S (2003) Wheat growth on board the international space station: germination and early development. *Proc Plant Growth Regul Soc Am* 30:66–71
- Stutte GW, Monje O, Goins GD, Tripathy BC (2005) Microgravity effects on thylakoid, single leaf, and whole canopy photosynthesis of dwarf wheat 2005. *Planta* 223:46–56
- Sytnik KM, Popova AF, Nichitailo GS, Machinsky AL (1992) Peculiarities of the submicroscopic organization of *Chlorella* cells cultivated on a solid medium in microgravity. *Adv Space Res* 12:103–106
- Tairbekov MG, Parfyonov GP, Platonova RW, Aramova VM, Golov VK, Rostopshina AV, Lyubchenko VYU, Chuchkin VG (1981) Biological Investigations aboard the biosatellite Cosmos-1129. *Adv Space Res* 1:89–94
- Tripathy BC, Brown CS, Levine HG, Krikorian AD (1996) Growth and photosynthetic responses of wheat plants grown in space. *Plant Physiol* 110:801–806
- Ward CH, King JM (1978) Effects of simulated hypogravity on respiration and photosynthesis of higher plants. In: Holmquist R (eds) *Life sciences and space research*. Pergamon, Oxford, pp 291–296
- Ward CH, Wilks SS, Craft HL (1970) Effects of prolonged near weightlessness on growth and gas exchange of photosynthetic plants. *Dev Ind Microbiol* 11:276–295
- Wells HW (1999) Portable device for chemical fixation of a biological sample. *NasaTech Briefs*, July; KSC-11993
- Wheeler RM, Mackowiak CL, Yorio NC, Sager JC (1999) Effects of CO₂ on stomatal conductance: do stomata open at very high CO₂ concentrations. *Ann Bot* 83:243–251
- Wheeler RM, Stutte GW, Subbarao GV, Yorio NC (2001) Plant growth and human life support for space travel. In: Passarakli M (eds) *Handbook of plant and crop physiology*, 2nd edn. Marcel Dekker Inc, New York, pp 925–941
- Wheeler RM, Sager JC, Prince RP, Knott WM, Mackowiak CL, Yorio NC, Ruffe LM, Peterson BV, Goins GD, Hinkle CR, Berry WL (2003) Crop production for life support systems. *NASA TM-20032-11184*