Ultrastructure of potato tubers formed in microgravity under controlled environmental conditions

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Abstract
Previous spaceflight reports attribute changes in plant ultrastructure to microgravity, but it was thought that the changes might result from growth in uncontrolled environments during spaceflight. To test this possibility, potato explants were examined (a leaf, axillary bud, and small stem segment) grown in the ASTROCULTURE™ plant growth unit, which provided a controlled environment. During the 16 d flight of space shuttle Columbia (STS-73), the axillary bud of each explant developed into a mature tuber. Upon return to Earth, tuber slices were examined by transmission electron microscopy. Results showed that the cell ultrastructure of flight-grown tubers could not be distinguished from that of tuber cells grown in the same growth unit on the ground. No differences were observed in cellular features such as protein crystals, plastids with starch grains, mitochondria, rough ER, or plasmodesmata. Cell wall structure, including underlying microtubules, was typical of ground-grown plants. Because cell walls of tubers formed in space were not required to provide support against the force due to gravity, it was hypothesized that these walls might exhibit differences in wall components as compared with walls formed in Earth-grown tubers. Wall components were immunolocalized at the TEM level using monoclonal antibodies JIM 5 and JIM 7, which recognize epitopes of pectins, molecules thought to contribute to wall rigidity and cell adhesion. No difference in presence, abundance or distribution of these pectin epitopes was seen between space- and Earth-grown tubers. This evidence indicates that for the parameters studied, microgravity does not affect the cellular structure of plants grown under controlled environmental conditions.

Key words: Cell wall, microgravity, pectin, plant, Solanum, spaceflight, tuber, ultrastructure.

Introduction
Plants on Earth develop under the influence of gravity. At the organismal and tissue levels, plants must support themselves against the force due to gravity; and at the cellular level, organelle movement takes place in the constant presence of gravity. Because it is impossible to produce a gravity-free environment on Earth, the effects of gravity on plants are poorly understood. Earth-based clinostat experiments, in which a plant is oriented horizontally and slowly rotated around its main axis, provide a means to expose the plant to gravity equally on all sides, but do not free the plant of gravitational effects. Spaceflight comes close to providing a gravity-free environment, although space craft orbiting Earth still experience a small fraction of Earth’s gravity. This small gravitational acceleration is caused by powering the space craft and its experiments, as well as by movement of crew members (see Kordyum, 1997, for a review).

A variety of effects have been reported in the experiments that have been conducted on plants in space (reviewed by Halstead and Dutcher, 1987; Claassen and Spooner, 1994; Kordyum, 1997), making it difficult to draw convincing conclusions. Plant features such as cell size and mitotic activity were affected in some experiments, but not in others. In some plants, cytology was affected by spaceflight, while at the same time morphology and growth were not (Halstead and Dutcher, 1987).
Variability in orbiters and flight hardware, in the length of spaceflights, in experimental designs, in plant genera, and in plant parts studied, makes comparison among space-based experiments difficult. Furthermore, insufficiently controlled and reported conditions in space make it impossible to prove that microgravity, rather than other environmental factors such as temperature, humidity, light or gas composition caused the diverse effects seen in plants that have undergone spaceflight (Halstead and Dutcher, 1987; Levine et al., 2001; Stankovic, 2001; Ferl et al., 2002).

In an attempt to determine which effects of spaceflight might, with confidence, be attributed to microgravity, potato (Solanum tuberosum L. cv. Norland) explants were examined that had flown on the space shuttle Columbia (STS-73) in the ASTROCULTURE™ plant growth unit, which is capable of controlling environmental conditions significant to plant growth: temperature, humidity, light, carbon dioxide, and ethylene levels (Morrow et al., 1995; Stankovic, 2001). Potato explants consisted of a small stem segment with attached leaf and axillary bud. Over a period of 14–16 d, when planted under appropriate conditions on Earth, the axillary buds develop into mature, fully differentiated sessile tubers approximately 1.5 cm in diameter (Wheeler, 1986). Tubers formed in both environments are similar in size, weight, shape, internal organization, and general cytology (Croxdale et al., 1997). Here space-grown tubers were examined at the ultrastructural level. It was hypothesized that the various ultrastructural changes reported in space-grown cells, previously attributed to the effects of microgravity, would not occur in cells grown in space under controlled environmental conditions. Since the need for support from cell walls would be diminished in microgravity compared to that on Earth, it was hypothesized that the cell walls might not contain the same wall components, or that the components might be present in differing amounts or in spatially different patterns. Of the many molecules that contribute to cell wall structure, pectins were chosen for examination because they are thought to contribute to wall rigidity and adhesion of adjoining cells (Willats et al., 2001), factors that it was thought might be affected by changes in the force due to gravity.

Materials and methods

Details of the potato explant system and the ASTROCULTURE™ plant growth unit have been reported previously (Duffie et al., 1995; Brown et al., 1996; Croxdale et al., 1997; see also http://wcesr.engr.wisc.edu/hardware.html and the photos of the plant tray which are supplementary online data associated with this article). The hardware design made it impossible for the crew to remove plant tissue from the unit during flight. Therefore, tuber development could not be followed over time, and tuber structure could only be examined at the end of the mission. Within 6 h of the space shuttle landing, the potato explants were removed from the growth medium in the ASTROCULTURE™ plant growth unit and prepared for microscopic examination. Control plants were grown in Madison in the ASTROCULTURE™ unit (see Croxdale et al., 1997, for a description).

Preparation for conventional ultrastructural examination

Tubers formed on all five potato explants in space. A disk of tissue 1–2 mm thick was taken by slicing vertically through the centre of each tuber with a Teflon-coated razor blade. Tuber slices were immersed in a solution of 4% v/v glutaraldehyde in 0.05 M sodium phosphate buffer (pH 7) for 4 h. Specimens were rinsed twice in buffer (10 min each rinse) and left in a third change of buffer in a refrigerator overnight. The following morning a fourth change of buffer was made, and the specimens were flown from Kennedy Space Center, FL to Madison, WI. Specimens were hand carried through airport security, and were not X-rayed. Tubers were then immersed in a 2% w/v aqueous solution of osmium tetroxide for 2 h, dehydrated in a graded acetone series, infiltrated in three steps in Spurr’s resin, embedded, and polymerized in a 70 °C oven overnight. Ground control specimens were treated the same way. Thin sections (silver-gold) were cut using a Sorvall MT2-B Ultra Microtome with a Diatome diamond knife, stained for 5–10 min with aqueous Reynolds’ lead citrate, and viewed using a JEOL JEM-1200 EX transmission electron microscope at either 60 or 80 kV. For each experimental and control tuber, cells of the periderm, the interior region just beneath the periderm, and the interior region at the centre of the tuber were examined.

Preparation for ultrastructural immunolocalization

Tuber slices were made in the same way as for conventional ultrastructural examination and immersed in a solution of 4% w/v paraformaldehyde with 0.5% v/v glutaraldehyde in 0.1 M sodium phosphate buffer (pH 8) for 4 h. Specimens were rinsed twice in buffer (10 min each rinse) and left in a third change of buffer in a refrigerator overnight. The following morning a fourth change of buffer was made, and the specimens were flown to Madison, WI. These specimens were then dehydrated in a graded ethanol series, infiltrated with LR White resin in three steps over a 24 h period, embedded, and polymerized in a 50 °C oven for 24 h. Ground control specimens were treated the same way. Thin sections (~90 nm, silver-gold) were cut using a Sorvall MT2-B Ultra Microtome with a Diatome diamond knife and collected on nickel grids.

Antibody staining protocol

John Innes Monoclonal (JIM) antibodies JIM 5 and JIM 7, which recognize pectin, were kindly provided by Maureen McCann of the John Innes Centre. Sections were blocked in 5% w/v non-fat dried milk for 1 h, exposed to JIM 5 (not diluted) or JIM 7 (diluted 1:80 with 2.5% w/v non-fat dried milk in sodium phosphate buffered saline with Tween (PBST)), rinsed three times (10 min each rinse) in PBST, exposed to secondary antibody (goat anti-rat complexed with 12 nm gold from Jackson Immuno Research Laboratories) for 1 h, rinsed three times (10 min each rinse) in PBST, rinsed with 30 drops of nanopure water from a syringe, and finally stained with 0.5% w/v uranyl acetate for 3–6 min. Sections treated with non-immune rat serum (diluted 1:80 with 2.5% w/v non-fat dried milk in PBST) were one control, and sections treated only with 2.5% w/v milk in PBST provided a second control. Sections were viewed using a Zeiss 10 transmission electron microscope at 60 kV.

Results and discussion

Plant evolution has taken place in the presence of an unchanging gravitational acceleration. Hence plant respon-
ses to gravity are likely to be highly conserved. Roots and shoots of plants on Earth are known to exhibit characteristic growth patterns (Sack, 1991; Kiss, 2000), and the orientation of plant cell division planes can be affected by force (Lintilhac and Vesecky, 1984). Because it is impossible to create an environment on Earth that does not include the force due to gravity, it is difficult to assess what the affects of gravity may be on plants. Since the area available for science experiments is at a premium on spacecraft, opportunities are limited for the study of plant growth in the absence of gravity.

While many ultrastructural studies have been conducted on roots in space, this study of potato tubers is one of relatively few space studies of stem ultrastructure (Halstead and Dutcher, 1987; Claassen and Spooner, 1994; Kordyum, 1997). In addition, this 16 d experiment is one of the longer plant experiments conducted in microgravity. The plants were grown in the ASTROCULTURE plant growth unit, which is designed to control for a number of environmental factors significant to plants: temperature, humidity, light, carbon dioxide, and ethylene levels. According to the findings for the parameters studied here, when these environmental conditions are controlled, the absence of gravity does not significantly affect the growth and structure of plant cells, tissues or organs.

**Crystals and amyloplasts**

Prominent features of the potato tuber cells are large crystals and amyloplasts containing starch grains. At the light microscopic (LM) level the crystals do not exhibit birefringence under polarized light, and have been shown to stain with Coumassie blue, indicating that they are proteinaceous (Croxdale et al., 1997). Crystal size is similar in Earth- and space-grown tubers (Croxdale et al., 1997), and it is shown here that crystals appear the same at the ultrastructural level as well (Fig. 1A, B, arrowheads). Depending on the plane of section, crystals may appear as triangles or other shapes in thin section.

Most amyloplasts in cells of these potato tubers contained one starch grain per plastid. A few plastids in both Earth- and space-grown tubers contained from two to four starch grains (not shown). Membranes separate multiple starch grains within a single plastid. The presence of multiple starch grains within a plastid did not appear to be more common in space-grown tubers than in Earth-grown tubers. By contrast, potato minitubers, grown from axillary buds of another cultivar, *Solanum tuberosum* L. cv. Zarero, for 8 d on the Russian space station Mir, were reported to contain more plastids with multiple starch grains than did control minitubers grown on Earth (Kordyum et al., 1997). In addition, the plastids of these tubers were more often reported to contain a large volume of electron-dense, lamellate stroma, than were plastids of Earth-grown minitubers (Kordyum et al., 1997). No differences were observed in stromal volume, density or lamellation between amyloplasts of space- and Earth-grown tubers (Fig. 1A, B, arrows). Electron-opaque inclusions found in both Earth- and space-grown amyloplasts in this study resemble an iron compound known as phytoferretin (Esau, 1977; Bowes, 1996, Fig. 2.40). The differences between this study and that of Kordyum et al. (1997) may be attributed to potato cultivar, flight length or environmental conditions.

**Other cytological changes**

A number of other cytological changes have been reported in previous studies of plants grown in space. Golgi bodies have been reported to display altered morphology in space-grown cells (Sytnik et al., 1983), but in this study’s space-grown tubers Golgi bodies exhibit normal morphology (Fig. 2B). Increased amounts of ER have been reported in columella cells of roots grown in space (Volkmann and Sievers, 1990), but the space-grown tubers in this study have normal amounts of ER (Fig. 2B). Plasmodesmata in

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**Fig. 1.** (A, B) Comparison of cellular organelles in an Earth-grown (A) and a space-grown (B) tuber. Mitochondria (M), amyloplasts with starch grains (arrows), and large crystals (arrowheads) appear similar in cells of both tubers. Bars=2 μm.
space-grown tubers are also typical of those in Earth-grown cells (Fig. 2B). Mitochondria with unusual electron-opaque matrices and well ordered cristae were reported in some studies of roots grown in space, while mitochondria with fewer and less ordered membranes were reported in other studies of space-grown roots (reviewed by Halstead and Dutcher, 1987; Claassen and Spooner, 1994; Kordyum, 1997). Mitochondria in cells of both Earth- and space-grown tubers from this study sometimes exhibit electron-opaque matrices. Furthermore, such mitochondria may be seen in numerous micrographs of Earth-grown tissue (see micrographs in Esau, 1977; Bowes, 1996). It was concluded that this condition is not an effect of microgravity in this study’s tubers.

In addition, no difference was observed in the relative numbers of mitochondria with electron-opaque matrices in space-grown as compared with Earth-grown tubers. By contrast, Kordyum et al. (1997) reported a greater percentage of mitochondria with electron-opaque matrices and more prominent cristae in space-grown minitubers than in Earth-grown controls. They believe that these differences indicate the presence of a greater number of functional mitochondria in space (Kordyum et al., 1997). However, changes in conformation and in electron opacity of osmium-fixed mitochondria have long been known to reflect different phases of respiration (Lehninger, 1964; Hackenbrock, 1968).

This cytological study is not quantitative, and neither were the other studies reporting differences between space- and Earth-grown tissue with which they were compared (Halstead and Dutcher, 1987; Claassen and Spooner, 1994; Kordyum, 1997). Such study at the TEM level would require extensive sampling, because the size of (for example) a mitochondrion as seen in thin section depends on whether one has made a section through the edge or the centre of the organelle. Quantitative studies would be more meaningful if conducted on tissue fixed in space and treated with an in-flight 1 g centrifuge control, methods incompatible with the hardware and hence not available to the authors.

Cell walls

Decreased amounts of cellulose and the enzymes involved in lignin production have been reported in walls of seedlings grown in space (Cowles et al., 1986; see Nedukha, 1997, for a review). In a more recent study, however, differences in cellulose and lignin in space-grown plants were shown to be due to poor water/nutrient delivery in space hardware, not to the effects of microgravity (Levine et al., 2001). While these wall polymers were not tested, walls of the space-grown tubers (including the underlying cortical microtubules thought to determine cellulose microfibril orientation; Giddings and Staehelin, 1991) appear at the ultrastructural level to be typical of those of ground-grown plants: lamellae were observed in periderm walls (not shown), and parallel microtubules were observed beneath the wall, just inside the plasma membrane (Fig. 2A, arrow). Thinner walls have previously been reported in tissue grown in spaceflight (Hoson et al., 2002; Claassen and Spooner, 1994; Nedukha, 1997), but a variety of wall thicknesses was observed in cells of tubers formed under controlled conditions during spaceflight and on Earth. No differences between wall thickness of space- and Earth-grown tubers were apparent. These findings at the ultrastructural level are consistent with results from semi-thin sections (1 μm

![Fig. 2. (A, B) Cellular organelles in space-grown tubers. (A) Glancing paradermal section through wall with straight, parallel microtubules (arrow) underneath, just inside plasma membrane. (B) Typical examples of Golgi bodies (G) in face and sectional views, mitochondria (M), rough ER (ER), and plasmodesmata (Pd). Bar=1 μm (A); bar=0.5 μm (B).](image-url)
thick; Fig. 3A, B) and with those of a previously reported light microscope study showing that cell dimensions and aspect ratios as well as cell patterns within tissue appear to be unaffected by microgravity (Croxdale et al. 1997). Given that no overall structural differences were apparent in cell walls grown on Earth and in space, it was thought that there might be differences in the molecular components of these structures. It was hypothesized that cell walls developed in space would not need to withstand the force due to gravity experienced on Earth and might lack or have reduced amounts of components that impart rigidity to the walls.

Among the most abundant of the cell wall components are pectins, a complex set of polysaccharides that are considered to be important structural molecules in the cell walls of plants grown on Earth (McCann et al., 2001; Ridley et al., 2001; Willats et al. 2001). Pectins participate in feedback loops that compensate for varying amounts of other wall components, and have been shown to vary in their localization patterns among different tissue types and developmental stages (Freshour et al., 1996; McCann et al., 2001; Ridley et al., 2001; Willats et al., 2001). Hence, it was thought they might be sensitive indicators of structural changes caused by microgravity. The best known of the pectins, homogalacturonan, is thought to contribute to wall stiffness and to adhesion of walls of adjoining cells (Willats et al., 2001). Homogalacturonans are esterified when synthesized in the Golgi, and can be modified (de-methyl esterified) by pectin methyl esterases in the wall (Cosgrove, 1997). Unesterified homogalacturonans can link together by means of Ca$^{2+}$, forming a gel that contributes to cell wall adhesion and stiffening (Willats et al., 2001). It was hypothesized that walls of tubers grown in the absence of gravity might need less of such a supporting gel and, therefore, have lower levels of the more highly unesterified pectins.

In this study, the presence, abundance, and spatial distribution of two epitopes of homogalacturonan were assessed to explore the possibility that walls formed in space differed from those formed on the ground with respect to these epitopes. Homogalacturonan epitopes were immunolocalized at the TEM level using John Innes Monoclonal (JIM) antibodies JIM 5 and JIM 7, commonly used antibodies which are known to bind to pectin with a range of esterification states (Willats et al., 2000, 2001). The epitope recognized by JIM 5 is thought to be more unesterified than that recognized by JIM 7 (Knox et al., 1990; Willats et al., 2000). These epitopes may be located on the same pectin molecule (Knox, 1997). The JIM 5 epitope is typically found in the middle lamella and outer sections of walls near intercellular spaces (Schindler et al., 1995; Knox, 1997), while the JIM 7 epitope is commonly distributed throughout the wall (Knox et al., 1990; Li et al., 1995; Schindler et al., 1995; Knox, 1997), although spatial distribution of these epitopes may vary with specimen or development, and may exclude certain tissues or cell types (Knox et al., 1990; Lynch and Staehelin, 1992; Knox, 1997; Willats et al., 2001).

In space-grown and control tubers JIM 5 bound to the middle lamella (Fig. 4A, B) and to portions of the wall adjacent to intercellular spaces, i.e. in a position equivalent to the middle lamella. In both space-grown and control tubers JIM 7 bound evenly throughout the wall (Figs 4C, D). No differences in abundance or distribution of the epitopes identified by JIM 5 and JIM 7 were found between flight- and ground-formed tubers.

Controls for the immunolocalization of the two pectin epitopes using rat pre-immune serum diluted 1:80 in Earth-grown and spaceflight tissue showed sparse labelling, and no label was present on sections treated with PBST and milk (controls not shown). This evidence indicates that microgravity does not affect the presence, abundance or spatial distribution of the pectin epitopes recognized by JIM 5 and JIM 7 antibodies in plant cell walls grown under controlled environmental conditions.

Because of the constraints of the hardware, it was not possible chemically to preserve the specimens in space. While it has been shown that some turnover of cell wall...
molecules may occur during plant growth and development (Fry, 1988; Labavitch, 1981; Gibeaut and Carpita, 1991), in many cells there is very little turnover of wall materials, even during cell expansion (Fry, 1988). Furthermore, most turnover of pectic substances occurs during cell division (Takeuchi and Komamine, 1980; Lozovaya et al., 1996). Therefore, it is not believed that a substantial turnover of pectins would have occurred in cells of the mature, fully differentiated tubers during the few hours between landing the shuttle and the fixation of the tuber tissue. Another hardware constraint was the lack of an in-flight 1 g centrifuge control for this experiment. Such a control, though rarely available, is much more accurate than a ground-based 1 g control because it takes into account vibrations and other forces experienced during flight (Stankovic, 2001). Because differences were not identified between tubers grown in space and those of control tubers grown on Earth, the lack of this control is less significant.

The hardware did allow controls for a number of variables important for plant growth, including gas levels. Kuang et al. (1996) and Musgrave et al. (1997) have found that a critical factor for successful plant reproduction in space is the regulation of environmental conditions, particularly, gas levels in the experiment chamber. Their results show that when space shuttle cabin air was circulated into the plant growth chamber, levels of carbon dioxide and ethylene were similar to those in ground controls, and plant reproduction was successful, although seed quality was compromised (Kuang et al., 2000; Musgrave et al., 2000). In a study of the root cell ultrastructure of the wild type and starch-compromised mutants, Guisinger and Kiss (1999) concluded that ethylene levels need to be carefully controlled during flight experiments. When not controlled, starch content in spaceflight samples is reduced. Ground controls exposed to elevated ethylene show similar declines. Similarly, ethylene levels, not microgravity were found to be
responsible for the male sterility of wheat plants grown on the Russian space station Mir (Campbell et al., 2001). Finally, an evaluation of Arabidopsis seedling growth in microgravity (Kiss et al., 2000) showed that there were ethylene effects in all of the four ecotypes flown.

Conclusion
A previous study of potato tubers grown in the ASTROCULTURE™ plant growth unit under controlled growing conditions (including controlled ethylene levels) showed no differences in size, weight, shape, tissue organization, or general cytology (Croxdale et al., 1997). In this study, no differences were observed between the cellular ultrastructure of flight-grown tubers and that of tubers grown in the same growth unit on Earth. Hence, despite numerous reports of ultrastructural changes of space-grown plants, these results for the parameters studied here indicate that microgravity did not affect the cellular ultrastructure or the pectin components that were studied in the walls of the potato stems grown under controlled environmental conditions.

These results point to the idea that growth in microgravity may not be as stressful as was previously thought. However, there could still be differences in parameters not studied. It is also possible that the internal forces of the spherical tuber itself may have been enough to maintain normal wall anatomy. These observations could best be tested on other plant organs without the same physical constraints, and using quantitative studies of plants grown with modern methods of space flight experimentation, including inflight fixation and an inflight 1 g centrifuge. This study indicates the need for further work to distinguish the effects of microgravity from among those of other environmental conditions found in space flight.

Supplementary data
Photographs of the plant tray of the ASTROCULTURE™ plant growth unit and a potato explant with attached tuber have been provided as supplementary online data associated with this article.

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