

New Insights in Plant Biology Gained from Research in Space

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ABSTRACT

Recent spaceflight experiments have provided many new insights into the role of gravity in plant growth and development. Scientists have been taking seeds and plants into space for decades in an effort to understand how the stressful environment of space affects them. The resultant data have yielded significant advances in the development of advanced life-support systems for long-duration spaceflight and a better understanding of the fundamental role of gravity in directing the growth and development of plants. Experiments have improved as new spaceflight hardware and technology paved the way for progressively more insightful and rigorous plant research in space. The International Space Station (ISS) has provided an opportunity for scientists to both monitor and control their experiments in real-time. Experiments on the ISS have provided valuable insights into endogenous growth responses, light responses, and transcriptomic and proteomic changes that occur in the microgravity environment. In recent years most studies of

plants in space have used *Arabidopsis thaliana*, but the single-celled, *Ceratopteris richardii* spore is also a valuable model system that has been used to understand plant gravity response. Experiments using these fern spores have revealed a dynamic and gravity-responsive trans-cell Ca^{2+} current that directs polarization of these spores and a possible role of extracellular nucleotides in establishing or contributing to this current. As technology continues to improve, spaceflight experiments will provide many new insights into the role and effects of gravity on plant growth and development.

INTRODUCTION

Studies on the role of gravity in plant growth and development, especially tropisms, have been ongoing for well over a century. With the advent of space travel, the microgravity environment has provided a unique experimental condition where scientists can investigate how altering the gravity stimulus affects signaling and the subsequent response. Plants have always been a topic of interest in relation to long-term human space travel because of their ability to provide food and to clean used water and air — essential components of a life support system. In recent years, plant biology experiments in space have provided many new insights about gravity-related signaling and how the microgravity allows for novel or altered responses to environmental stimuli.

One major goal of plant space research is the development of a self-sustained life support system (Galston, 1992; Paul et al., 2013b). Plants

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have the unique ability to purify the air using photosynthesis — a process by which water and carbon dioxide are converted into carbohydrates and oxygen. Plants also have the ability to purify water through the process of transpiration — a process where water is filtered before being transported through the plant until it eventually evaporates out of pores in the leaves. In an enclosed life-support system, the condensation formed from evaporated water would be potable. Additionally, if crops are the plants chosen to grow in this life-support system, they will provide food for the crew. All of these resources would be valuable on long-duration missions where carrying large amounts of supplies or going through re-supply missions is not feasible.

The National Aeronautics and Space Administration (NASA) has begun developing portions of the advanced life-support system on the International Space Station (ISS). A current study that could provide a crop-based addition to the life-support system is the development of the Vegetable Production System (VEGGIE). VEGGIE was developed in an effort to provide the crew not only with salad-type vegetables, but also the relaxation that comes from observing green things growing (Retrieved from http://www.nasa.gov/mission_pages/station/research/experiments/863.html; Accessed 10/7/15). This system will provide an additional resource for plant space research because the tissue from crops grown inside can be preserved and used to evaluate how plants sense and respond to gravity. Although understanding the role of gravity in plant growth and development through space research is valuable, increasing our understanding will also help improve our ability to grow plants on Earth. The two missions VEG-01 and VEG-03 have provided useful information about the VEGGIE hardware, microbial load, and growth media (Retrieved from http://www.nasa.gov/mission_pages/station/research/experiments/863.html, accessed 10/7/15 and http://www.nasa.gov/mission_pages/station/research/experiments/1294.html, accessed 10/7/15).

In addition to developing plants for a self-sustained, advanced life-support system, space travel has provided a venue for fundamental plant research. Since the beginning of the space program, scientists have been sending plants to space and analyzing its effects by assessing

changes in plant growth and development, genetic material, tropisms, and endogenous movements (Paul et al., 2013b). The first plant experiments in space were focused on understanding how the microgravity and cosmic radiation of the space environment affect biological systems. Dormant seeds of many different plant species were flown on Discoverer 17 in 1960 and Sputnik 4 in 1961 (Halstead and Dutcher, 1984). After multiple unmanned, orbiter missions, the first plant growth experiments began to take place on the manned Skylab spacecraft and on the Russian Salyut space station in the early 1970s. The experiments conducted on the Russian spacecraft flights and aboard the Salyut space station showed how microgravity, long-term space exposure, and flight conditions caused genetic changes that were deleterious to seeds and seedlings (Dubinin et al., 1973; Vaulina et al., 1981; Kordyum et al., 1983; Kostina et al., 1984).

Experiments conducted on the first space station developed by the United States — Skylab — showed the effects of the space environment on plant growth, phototropism, and cytoplasmic streaming (Summerlin, 1977). During the Skylab experiments, germination was delayed during spaceflight but growth progressed normally after the initial interruption. However, the direction of plant growth was random and stems were not phototropic (Summerlin, 1977). Cytoplasmic streaming was initially observed by astronauts but stopped by the second observation because the plants died, most likely because of their inability to photosynthesize due to a lack of access to CO₂. Although there were many complications associated with these experiments, they provided valuable insight into the growth and development of plants in space and the need for further study (Summerlin, 1977).

The construction of the space shuttle allowed for larger projects, advanced hardware, and repeated experiments due to the shuttle's capacity and the crew's ability to work on experiments (Paul et al., 2013b). During the experiments flown on the shuttle, plant scientists initially encountered difficulties when they tried to grow plants from seed-to-seed during spaceflight. In the unfamiliar condition of space, plants experienced delayed development. Through a series of experiments over the course of multiple missions, plant growth chambers were modified to include

an air exchange system and supplemental carbon dioxide (Kuang et al., 1996b; Musgrave et al., 1997). These changes allowed reproductive development to proceed normally and for pollen transfer and fertilization, leading to seed production. These experiments, along with many others (Morrow et al., 1994; Kuang et al., 1996b; Kuang et al., 1996a; Musgrave et al., 1997; Musgrave et al., 1998; Porterfield et al., 2000), allowed plant scientists to optimize plant growth conditions in order to achieve results that could be attributed confidently to true spaceflight conditions, not the poor growth conditions of the hardware.

As the shuttle program progressed and continued to succeed, the United States took up the task of developing a bigger and better space station for scientific experiments. The components were built, launched, and assembled beginning in the late 1990s. The launch and assembly of the ISS paved the way for the development of more equipment designed for plant space research. The European Space Agency has provided a plant research platform with the European Modular Cultivation System (EMCS) and Biolab (Brinckmann, 2005). Both of these plant growth facilities contain incubators equipped with a centrifuge that can provide acceleration from 0.001 *g* to 2.0 *g*. They also have illumination and video options that allow experiments to be tailored to collect plant growth images and videos. The installations of Biolab and the EMCS have also improved investigators' ability to monitor and adjust environmental parameters. Both of these plant growth chambers are hooked up to the Life Support System, creating a controlled atmosphere where O₂, CO₂, and humidity levels can be monitored and optimized. This Life Support System also enables scientists to reduce CO₂ and ethylene levels during experiments (Brinckmann, 2005). Novel experiments investigating the threshold of gravity needed to induce gravitropism (Driss-Ecole et al., 2008), circumnutation (Johnsson et al., 2009), rosette leaf movements (Solheim et al., 2009) in space, and the positive phototropic reaction of *Arabidopsis* to red-light in microgravity (Millar et al., 2010), have been completed in the EMCS since it was installed (Brinckmann, 2005; Kittang et al., 2014).

NASA designed and installed a similar system on the ISS called the Advanced Biological

Research System (ABRS). The ABRS was designed to support small biology experiments with plants, microorganisms, and small arthropods (Camacho, 2015; Paul and Ferl, 2015). The ABRS is useful for plant biology experiments because it can be equipped with a hydrated foam base and a Green Fluorescent Protein (GFP) imaging system — two tools that have been used by plant biologists to conduct experiments in space (Paul and Ferl, 2015). Similar to the EMCS and Biolab, the environmental and light conditions can be managed and recorded throughout experiments (Camacho, 2015; Paul and Ferl, 2015).

Additionally, two units designed for vegetable growth have recently been installed on the ISS. The Lada-Vegetable Production Unit was launched by the Russian Space Agency in 2002 and was installed in the Russian segment of the ISS — the Zvezda module. This unit contains two independent greenhouse modules that are not temperature-regulated and are open to the cabin for air exchange. This unit was designed in an effort to provide a tool for investigating food production and safety (Paul and Ferl, 2015). NASA also designed a similar chamber for vegetable growth called Veggie-Vegetable Production System (Veggie-VPS). Engineers installed LED lights in this unit that provide optimal light for plant growth. Like the Lada-Vegetable Production Unit, Veggie-VPS is not temperature-controlled and is open to the ISS for gas exchange (Paul and Ferl, 2015).

In the near future, NASA will be installing a new growth habitat on the ISS called the Advanced Plant Habitat. The Advanced Plant Habitat will be a large volume chamber designed for multigenerational studies. The atmosphere and light in this chamber will be tightly monitored and regulated during experiments. The Advanced Plant Habitat is being designed as a tool that will contribute to the Bioregenerative Life Support System, an essential component of long-duration space travel (Paul and Ferl, 2015). Space agencies worldwide have designed and installed plant growth facilities on the ISS that provide the tools necessary for plant biologists to continue conducting spaceflight experiments that contribute a better understanding of the role of gravity in plant growth and development. These tools also further improve the Bioregenerative Life Support system necessary for long-term space travel.

Another major advance for space research in recent decades has been optimizing the process of in-flight preservation of samples for cellular or molecular analysis after they return to Earth. Initially, samples needed to be chemically fixed to maintain the integrity of cell structure or frozen to maintain high-quality nucleic acids and proteins. Due to issues with flash freezing and maintaining ultra-cold temperature during their return to Earth, scientists began to use a chemical fixative — RNAlater™ (Ambion) — to preserve the molecular integrity of samples. The study of this fixative under multiple conditions (Paul et al., 2005) — along with the development of the Kennedy Space Center Fixation Tube (KFT) — has allowed molecular biologists to conduct transcriptomic (Paul et al., 2013a; Kwon et al., 2015) and proteomic (Ferl et al., 2015) studies on tissue samples preserved in space and returned to Earth. RNAlater has also been shown to be an effective tool for preserving samples in space for morphological studies using scanning electron microscopy (Schultz et al., 2013). More recently, Nakashima et al. (2014b) optimized a glutaraldehyde fixation method using KFTs by increasing the concentration of glutaraldehyde, storing the fixative at 4°C, and bubbling nitrogen (N₂) gas over the fixative prior to loading the KFTs. All of these changes were made based on previous studies where seedlings had not been adequately preserved for morphological studies. These new methods of tissue preservation, and others like them, have greatly improved the way scientists investigate morphological changes associated with the extraterrestrial environment.

Since the beginning of plant experiments in space, scientists and engineers have constantly worked to improve the condition of plant growth facilities, ensuring future experiments would be better than the last. As we have now transitioned from the space shuttle to a new era of commercial space travel, the technology available for plant biology experiments in space has improved dramatically. These improvements have led to many new insights about plant space biology. Paul and Ferl (2015) and Kiss (2015) recently published thorough reviews that describe the lessons learned from decades of experiments in space. Both reviews covered the approach and constraints associated with spaceflight experiments. Kiss (2015) also described the

process of applying for support to fund plant spaceflight experiments. These reviews outlined the considerations that have to be taken into account when planning an experiment, and they provided information on data-collection strategies to gain the most valuable information from a plant spaceflight experiment (Kiss, 2015; Paul and Ferl, 2015). Collectively, these articles provided valuable information that will help future space plant biologists design and fly experiments. As the multiple commercial companies continue to improve their space travel vehicles and the international cooperation among space agencies progresses, this new age of plant space biology should generate even more exciting and credible discoveries.

INSIGHTS ON THE EFFECTS OF GRAVITY ON PLANT GROWTH GAINED FROM RECENT SPACEFLIGHT EXPERIMENTS

Since the beginning of the space program, astronauts and scientists have collaboratively explored how gravity affects plant growth using the space environment. In recent years, scientists have begun to focus on understanding the role of gravity in plant rhythmic movements. Specifically, the question, “is the force of gravity needed to initiate and sustain ultradian leaf movements?” has been explored. Ultradian leaf movements follow a recurrent cycle repeated in periods ranging from minutes to hours, and a recent report by Solheim et al. (2009) has convincingly demonstrated that they can be affected or initiated by the force of gravity. These authors carried out their novel studies by growing *Arabidopsis* in the EMCS. In the microgravity space environment, rhythmic leaf movements still occurred and their periods varied based on the presence of light. After years of studies by several labs on these ultradian leaf movements in the 1 *g* environment on Earth, there was still debate on the role of gravity in initiating them. However, Solheim et al. (2009) showed that even when the force of gravity is minimal, leaves have rhythmic movements. In addition, they also observed when leaves are grown in microgravity they have a more pronounced gravitropic response when they are transitioned to a 1 *g* environment. Both of these findings provide novel insights on gravity-

independent leaf movements and beg the question of what mechanisms cause them.

The mechanism behind the ultradian leaf movements may be explained by an additional experiment using the data collected during the MULTIGEN-1 (MULTIple GENerations 1) experiment use by Solheim et al. (2009). In this experiment, the MULTIGEN-1 data were also used by Fisahn et al. (2015) to analyze the effects of lunar gravity on leaf movement. The original hypothesis, made by Dr. Gunter Klein, states that the movement of bean leaves grown in constant environmental conditions with no entraining stimuli are a result of the lunar tidal force (Barlow et al., 2008). Barlow et al. (2008) demonstrated this hypothesis by establishing a correlation between the two by monitoring the downward movement of leaves and comparing this rhythm to changes in the tidal force. In order to find a direct connection between the tidal force and leaf movements, Fisahn et al. (2015) designed an experiment where the lunisolar gravitational force would be altered by monitoring plants grown on the ISS. The ISS provides a unique environment because the gravitational pull from Earth is reduced and the location of the Moon changes constantly during orbit. In this experiment, the periodicity and phase of leaf ultradian rhythms were compared to changes in the lunisolar gravitational force. Fisahn et al. (2015) showed lunisolar gravitational profiles had a periodicity of 45 minutes in orbit and that *Arabidopsis* leaf movements had a similar periodicity and synchronous phase. This discovery corroborates the data collected by Klein and analyzed and summarized by Barlow et al. (2008) on Earth. All of these findings provide unique insights into the behavior of leaf movements when the interference of Earth's gravity is minimal. However, the answer to the question of the necessity of gravity in initiating and sustaining leaf movements is still not clear.

In addition to leaf movements, scientists have long been fascinated by plant rotational growth patterns associated with growth oscillations called circumnutations. Much like the leaf movements mentioned above, circumnutation could be an endogenous plant action that can be altered by environmental factors — like gravity — or it may be caused by environmental stimuli. In order to investigate this question, *Arabidopsis* plants were

grown on rotors aboard the ISS and images of inflorescence stems were taken over time to document the effect of microgravity on circumnutation (Johnsson et al., 2009). Johnsson et al. (2009) showed that circumnutations in microgravity, although present, have a much lower magnitude. Using controlled centrifuge pulses on the ISS, circumnutations at 0.8 *g* were monitored. When stems were exposed to a higher *g*-force, circumnutations were amplified by a factor of five to ten (Johnsson et al., 2009). Data from monitoring leaves and stems of plants grown on the ISS showed that although there are gravity-independent endogenous growth movements, the force of gravity has an effect on their magnitude or amplitude, showing these responses are endogenous but can be affected by environmental factors like the force of gravity.

Other plant movements associated with growth are root skewing and waving. These growth movements, which were first observed in *Arabidopsis* roots (Okada and Shimura, 1990; Mullen et al., 1998; Thompson and Holbrook, 2004; Oliva and Dunand, 2007), were initially hypothesized to be an integrated response to gravity, mechanical, and touch stimulation as the tip of the root grows along the surface of a growth medium. However, experiments performed in microgravity showed unexpectedly that root waving and skewing patterns do not require a gravity stimulus. In fact, three spaceflight experiments showed significant differences in growth patterns between space and ground-grown *Arabidopsis* roots (Millar et al., 2011; Paul et al., 2012a; Nakashima et al., 2014a). Millar et al. (2011) found *Arabidopsis* (ecotype Landsberg) roots exhibited increased skewing to the left and suggested that this was due to an endogenous response. *Arabidopsis* (ecotypes Wassilewskija and Columbia) roots also showed exaggerated skewing in microgravity conditions (Paul et al., 2012a). Another spaceflight study found that the actin cytoskeleton actively suppresses this endogenous skewing response in *Arabidopsis* roots (Nakashima et al., 2014a). Thus it seems more likely that root waving and skewing are directed mainly by mechanical stimuli, rather than by gravity (Roux, 2012).

In addition to modulating root waving and skewing, mechanical stimuli also induce the release of adenosine triphosphate (ATP) into the

extracellular matrix (ECM) of *Arabidopsis* roots (Weerasinghe et al., 2009). Extracellular ATP (eATP) can modulate the growth of plant cells (Clark et al., 2014), and the addition of ATP inhibits root gravitropism and induces root curling (Tang et al., 2003). Exogenous ATP was also shown to increase root skewing in a pH-dependent manner (Haruta and Sussman, 2012). The concentration of eATP is regulated partially by enzymes called ecto-apyrases (ecto-nucleoside triphosphate diphosphohydrolases). In plants, apyrases play a pivotal role in controlling growth and development (Clark et al., 2014). Recently, we found apyrases also play an important role in regulating the root skewing response in *Arabidopsis* (Yang et al., 2015). In combination with the results from spaceflight experiments, these new insights indicate that endogenous actin-dependent signals and mechanical stimuli mediated by eATP and ecto-apyrases help regulate the *Arabidopsis* root skewing response. Recently, a lectin kinase receptor — DORN1 — was identified as the first plant eATP receptor and found to play a role in defense responses (Choi et al., 2014). Thus, it will be important to determine if DORN1 mediates apyrase and eATP effects on root skewing.

Also recently, a plasma membrane receptor-like kinase — FERONIA — was demonstrated to play an important role in *Arabidopsis* root skewing responses (Shih et al., 2014). FERONIA is a receptor-like kinase in the CrRLK1L subfamily that has previously been implicated in regulation of many aspects of plant growth and development (Escobar-Restrepo et al., 2007; Deslauriers and Larsen, 2010; Kessler et al., 2010; Duan et al., 2011). The study by Shih et al. (2014) found calcium signals induced by mechanical stimulation were lacking or altered in *feronia* (*fer*) mutants. In addition to an impaired skewing response, *fer* mutant roots showed disrupted ability to penetrate agar surfaces and altered growth response to impenetrable objects. The *fer* mutant also showed decreased levels of expression of certain touch-inducible genes in response to hypoosmotic stress compared to wild type (WT).

The space environment provides a unique opportunity to study environmental effects without the interference of gravity. For example, phototropism — the directed growth of a plant

due to unidirectional light — is an essential aspect of plant survival, but studies of this phenomenon on Earth always have to consider the force of gravity as a contributing factor. Recently, Millar et al. (2010) conducted a study of red-light and blue-light phototropic responses using the EMCS on the ISS in order to avoid the complications of the 1 *g* environment. One advantage of the EMCS is the ability to use the centrifuge for a 1 *g* space environment control. Using this growth chamber, Millar et al. (2010) observed a novel red-light phototropic response in *Arabidopsis* hypocotyls grown from seed in microgravity. This response was not seen in Earth-grown controls or in the 1 *g* space environment controls. Millar et al. (2010) also showed a more robust blue-light response compared to the 1 *g* control.

In addition to providing an environment where the interference of gravity is minimal, spaceflight experiments provide an opportunity for organisms to experience an extraterrestrial environment where conditions are distinctly different from Earth. This allows scientists to evaluate changes in gene expression caused by the multiple environmental changes of the space environment. Microarray analysis of RNA from seedlings and culture cells grown in space revealed a statistically significant difference in the expression of about 300 genes when compared to ground controls (Paul et al., 2012b). Although both seedlings and cell cultures showed changes in gene expression due to spaceflight, the genes that were differentially expressed in each system were not the same. Paul et al. (2012b) suggest this could be due to the uniformity of the cell culture compared to the complexity of the multi-cellular seedling, or that it could be the presence of a coordinated, organ-specific response in seedlings compared to a generic response in undifferentiated cells. This pioneering study showed how complex the spaceflight response is in both undifferentiated cells and multi-cellular seedlings. The gene expression changes could not easily be explained by changes in gravity or other environmental factors we associate with the terrestrial environment.

Another gene-expression study, conducted by Kwon et al. (2015), examined how the space environment alters gene expression by comparing the transcripts of plants grown in space for two weeks to plants grown on Earth. The evaluation of

the two transcriptomes showed genes associated with oxidative stress, cell wall remodeling, and the endomembrane system are repressed in space-grown *Arabidopsis* plants. Additionally, after further analysis, Kwon et al. (2015) discovered many of the transcripts down-regulated in space are enriched in root hairs. Data showing the average root hair length of space-grown seedlings was significantly less than ground controls support this observation. Kwon et al. (2015) independently confirmed the transcriptome changes seen in space-grown seedlings caused altered root hair growth by assessing root hair phenotypes of homozygous mutants of the genes down-regulated in microgravity. This mutant screen led to the identification of two peroxidase mutants with defective root hairs. These root hair changes could affect water and nutrient uptake in space — an essential component of plant survival and growth. This novel observation provides valuable insight into the effects of long-term spaceflight on plant development.

To complement the studies of transcriptome changes done by Paul et al. (2012b), Ferl et al. (2015) recently analyzed the proteome of space-grown *Arabidopsis* seedlings. This study pioneered the use of RNA later for the preservation of samples for protein analysis. This method worked well enough to provide protein for the identification of 1570 proteins and the quantitation of 1432 proteins using the iTRAQ method, with biological replicates allowing for a statistical analysis of quantitative differences. This analysis was the first proteomic study in space-grown seedlings. Moreover, the lot of seedlings used came from a very similar group of plants grown on the ISS for transcriptomic studies completed by Paul et al. (2013a), so the transcriptome changes could be compared to proteomic changes. As technology improves and the ability to do more experiments on the ISS increases, scientists will have the opportunity to identify both cell-specific and generic responses to the space environment (Ferl et al., 2015).

In addition to using the ISS to evaluate gene-expression changes associated with spaceflight, scientists have now developed methods to use parabolic flights for molecular biology experiments. *Arabidopsis* seedlings that went through parabolic flights on different days with different logistical aspects, showed gene

expression changes similar enough to be compared using a relatively stringent statistical analyses (Paul et al., 2011). The gene expression changes due to parabolic flight were related to changes in gravity and stress (Paul et al., 2011). Aubry-Hivet et al. (2014) showed roots exposed to transient microgravity on parabolic flights have distinct transcriptomic changes that are dependent upon auxin signaling by comparing the transcripts from WT, *pin2*, and *pin3* *Arabidopsis* seedlings. Hausmann et al. (2014) used transgenic *Arabidopsis* callus to monitor H₂O₂ and Ca²⁺ during parabolic flight. The same cells were used to analyze transcripts after microgravity exposure; many of the genes up-regulated were Ca²⁺ and ROS related (Hausmann et al., 2014).

Collectively, hardware improvements along with knowledge gained from decades of spaceflight experiments have paved the way for scientists to design and conduct experiments that provide valuable insight into the effects of gravity on plant growth. Recent experiments have explored the role of gravity in plant movements, revealed a novel red-light phototropic response, and provided valuable information about plant gene expression changes in space. This new information will help engineers and scientists design a Bioregenerative Life Support System for long-duration missions using plants — a major goal of international space agencies — and provide information that can be used to optimize plant growth on Earth, which is a key contribution as agricultural land availability decreases and our population increases.

ASSAYING GRAVITY EFFECTS IN SINGLE CELLS USING *CERATOPTERIS RICHARDII* SPORES AS A MODEL SYSTEM

In recent years, most studies of plants in space have focused on the model plant, *Arabidopsis thaliana*. However, another valuable model system — the single-celled *Ceratopteris richardii* fern spore — has also been used to understand the effects of gravity on plant cells. This single-celled model is valuable because it provides a simplified system to monitor how plant cells sense gravity and direct subsequent development based on this mechanical force. *Ceratopteris richardii* is a homosporous fern with large, 100-150 µm diameter, spores. The fern life cycle, like all

pteridophytes, consists of alternation of free-living haploid and diploid generations. *Ceratopteris* spores are produced on diploid fronds through meiosis. *Ceratopteris* spore germination is a red-light induced process mediated by the photoreceptor phytochrome. Germination results in the development of haploid, photosynthetic, monoecious, or male gametophytes. *Ceratopteris* spores are extremely resilient to environment change; spores germinate after exposure to 4°C or 37°C for up to 30 days. Spores remain dormant for many years if stored dry, and hydrated spores will remain dormant if kept in the dark for up to 90 days.

Ceratopteris spores germinate readily in standard laboratory culture conditions (Hickok et al., 1987). The basic progression of spore germination events is depicted in Figure 1. While it is still a single cell, the spore can sense and respond to gravity. Developmental polarity is established in response to the vector of gravity, as demonstrated by Edwards and Roux (1994), and it results in the downward migration of the cell nucleus, followed 48 hours later by the emergence of the primary rhizoid from the spore coat and its growth in the direction established in the first 30 hours of germination.

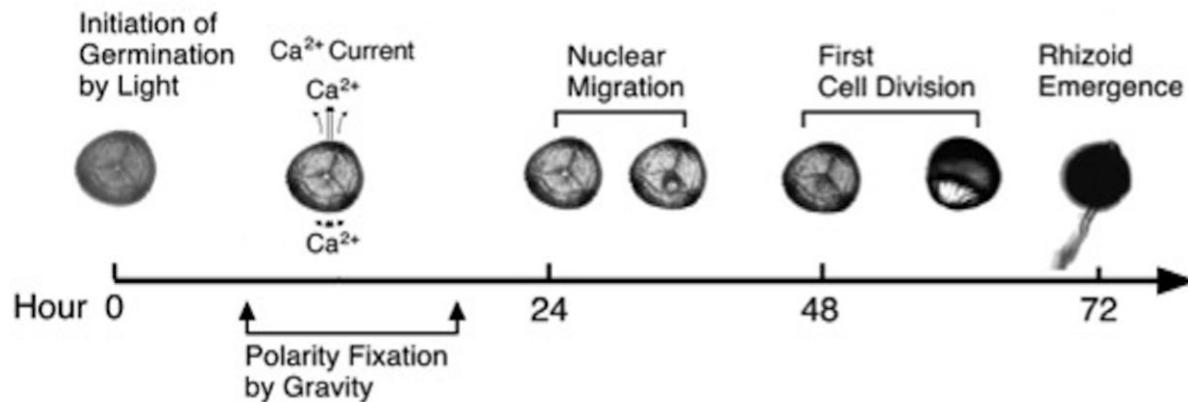


Figure 1. Early growth and developmental time-line of *Ceratopteris richardii* spores. Spore germination is initiated by water and red light. Within hours of initiation, calcium enters through channels at the bottom of spores and there is an efflux of calcium out of the top. The calcium efflux peaks between 7 and 12 hours after light-initiated germination begins. Polarity of development is set by gravity after 24-30 hours of growth and it results in the downward migration of the cell nucleus and a subsequent asymmetrical cell division. A visual representation of the direction of polarity set by gravity is the emergence of a downward-growing rhizoid 72 hours after germination begins.

The continuous microgravity conditions on the space shuttle flight STS-93 resulted in random orientation of nuclear migration and rhizoid emergence in germinating *Ceratopteris* spores, as demonstrated by Roux et al. (2003). In the same space shuttle mission, spore samples were exposed to light and allowed to develop for various time points in microgravity before being frozen and returned to Earth for analysis of gene expression changes (Salmi and Roux, 2008). All of the samples included in this study were frozen prior to the first division of the spore cell, allowing gene expression analysis of differences in transcript abundance within the same cell over

time and induced by the absence of the polarity directing force of gravity. Around 5% of the unique transcripts analyzed in this study exhibited a difference induced by microgravity conditions in at least one of the developmental time points analyzed. The stress of the space environment did not result in a general trend toward gene suppression, as an approximately equal number of down- and up-regulated changes were induced under the conditions of this experiment. Transferase- and hydrolase-type enzymes were the molecular functions most abundantly represented in the differentially regulated transcripts. Enzymes with these molecular

functions modify other proteins, suggesting the post-translational modifications involved gravity perception and might be altered in microgravity conditions.

Within the developmental stage when spores are responsive to gravity reorientation (e.g., the first 30 hours of development), there is a differential calcium ion flux around spores (Chatterjee and Roux, 2000; Salmi et al., 2011). Efflux is strongest at the top of the spore relative to gravity, and influx occurs primarily at the bottom of the spore. This calcium transport differential reorients coincident with spore rotation (Salmi et al., 2011), indicating the ion channels and pumps responsible for calcium flux are regulated almost instantaneously by gravity stimulation.

This rapid regulation of calcium flux was also observed in the changing *g*-force environment of parabolic flight. These flight experiments, in which the periods of hyper-*g* (hypergravity) and hypo-*g* (hypogravity) were of short-duration, demonstrated that the magnitude of the calcium differential is correlated with the level of gravity applied to spores (Salmi et al., 2011). They revealed how rapid the responses to gravity fluctuations are in germinating spores, suggesting changes in the magnitude and direction of the *g*-force can rapidly alter the transport activity of calcium channels and pumps.

Control of the gravity-directed calcium ion distribution in spores is the subject of continued investigation. Calcium signaling is important in diverse physiological processes of bacteria, fungi, plants, and animals. Cells generally keep their cytoplasmic calcium concentrations low — typically below micromolar — so temporal and location-specific increases in calcium concentration can serve as a specific signal (Gilroy et al., 1993). This intracellular low calcium concentration means that the efflux of calcium observed at the top of germinating *Ceratopteris* spores would be the result of an energy-requiring calcium pump, and the influx of calcium observed at the bottom of the spore would be mediated by a calcium channel. There has been recent progress in identifying the transport proteins that facilitate these processes.

A candidate for calcium efflux activity has been identified in a plasma membrane-type Ca^{2+} -ATPase that is expressed coincident with the

developmental period when spores are responsive to gravity (Salmi et al., 2011). Heterologous expression in yeast demonstrated this enzyme had functional pump activity (Bushart et al., 2013). Spore plasma membrane specific Ca^{2+} -ATPase activity was inhibited by treatment with 2',4',5',7'-tetrabromofluorescein (Eosin Yellow), and this inhibition of calcium efflux was verified using the CEL-C calcium sensor. Even though the extracellular calcium differential was eliminated by treatment with Eosin Yellow, when this treatment was limited to the period of gravity perception it did not alter the gravity-directed polar growth of the primary rhizoid. However, continuous treatment with Eosin Yellow does completely inhibit rhizoid development, indicating this calcium pumping activity is necessary for polar tip growth of the rhizoid. These data are consistent with the important role of Ca^{2+} -ATPase in maintaining a low cytoplasmic calcium concentration and facilitating tip growth. The results are also consistent with the model of spore gravity perception that implicates a localized region of high cytoplasmic calcium at the bottom of a germinating spore as the key signal for the direction of gravity-directed polarization. Consistent with this interpretation, the calcium channel antagonist, nifedipine, blocks the gravity-directed downward polarization of rhizoid growth (Chatterjee and Roux, 2000). The predicted gravity-induced calcium entry specifically at the bottom of a spore may be mediated by a mechanosensitive channel.

To date, three classes of MechanoSensitive (MS) channels have been identified in plants based on their sequence similarity to channels in other cell types: MscS-like, Mid1 complementing activity, and two-pore potassium channels (Hamilton et al., 2015). MscS channels are well-characterized MS channels of small conductance. In bacteria (i.e., *E. coli*) the MscS proteins form homoheptomers which have an open pore size of just under 13Å (Wang et al., 2008). Recently, point mutational analysis has demonstrated the MscS channel is kept in a closed state due to intrinsic membrane pressure, and it is the application of tension to the membrane that causes the pore to open as a relief valve, like a jack-in-the-box (Malcolm et al., 2015). In the open conformation, the bacterial MscS channel allows

ions into cells to relieve the osmotic stress that exerts tension on the plasma membrane.

In eukaryotic cells — including plants — MS channels have long been proposed as a gravity sensing mechanism. In this model, some settling mass (e.g., statolith or protoplasm) exerts tension on the bottom of the cell that results in opening of MS ion channels (Toyota and Gilroy, 2013). This results in local areas of high ion concentration. This model of gravity perception is consistent with the data obtained from *Ceratopteris* thus far. We have recently identified three members of the MscS family of MS channel in *Ceratopteris* based on sequence similarity (Salmi and Roux, unpublished results). It will be important to demonstrate MS activity of the proteins encoded by these genes and, if they prove to be functional, characterize their role in *Ceratopteris* spore development.

The current model of calcium directed gravity perception in spores also involves candidates for gravity-induced downstream signaling steps including the Ca^{2+} -binding proteins, calmodulin, Ca^{2+} -dependent protein kinase (CDPK), and annexin. All of these proteins would be the likely signal transducers that help mediate cell polarization events guided by gravity. Annexins are a multigene family of multifunctional calcium-dependent membrane proteins found in animal and plant cells (Clark et al., 2012). In plants, the number of annexin genes in any particular species varies; e.g., the model plant *Arabidopsis* has eight distinct annexin genes and rice has ten (Clark et al., 2012). An early study revealed annexins are likely targets of calcium action during gravity responses in plants because gravity induced a redistribution of annexin immunostain in pea plumules (Clark et al., 2000). The role of annexins in gravity responses was hypothesized based on their participation in the Golgi-mediated directional secretion of materials to the plasma membrane and ECM (Konopka-Postupolska et al., 2011). Localized secretion is a key component required for polarity establishment and maintenance in plant cells (Belanger and Quatrano, 2000). Control of secretion is likely to be important in the response phase of gravitropism, because this phase requires the asymmetrically directed transport of wall polysaccharides and newly synthesized plasma membrane to the expanding cell periphery.

In the model plant *Arabidopsis*, annexin 1 (AnnAt1) is the best characterized of the eight annexins and is a strong candidate for participating in the mediation of gravity responses. There is abundant evidence that AnnAt1 facilitates calcium transport, either directly or indirectly (Laohavisit and Davies, 2009), and also plays an important role in stress responses (Clark et al., 2012). Recently, experiments on *Arabidopsis* cell cultures during parabolic flight showed the onset of microgravity conditions induced an increase in calcium and H_2O_2 (Hausmann et al., 2014). This same study found phosphorylation of AnnAt1 was rapidly induced in *Arabidopsis* callus culture cells by hypergravity condition.. This kind of post-translational change would be expected to decrease the peroxidase activity of AnnAt1 (Gorecka et al., 2005). Because changes in gravitational fields induce changes in reactive oxygen species (ROS) levels (Barjaktarović et al., 2009; Hausmann et al., 2014), and peroxidase activity can reduce ROS levels, changes in the expression and phosphorylation status of AnnAt1 might play an important role in regulating the changes in ROS that occur in microgravity.

AnnAt2 is closely related to AnnAt1 and hypergravity conditions, as well as horizontal clinorotation (simulated weightless conditions), induced an increase in AnnAt2 protein levels in root apices (Tan et al., 2011). This study also found AnnAt2 was differentially expressed in the root-cap columella cells of wild-type and *pin2* mutants. This and other findings on gravity effects on annexins raise the question, “Do *Ceratopteris* spores express any annexins during the period when gravity is fixing the polarity of these cells?”

Two full-length *Ceratopteris* annexins — AnnCr1 and AnnCr2 — have been identified and both of these annexins are expressed at the 10 hour time point after induction of spore germination during the peak of the trans-cell Ca^{2+} current. Transient suppression of AnnCr1 expression by RNAi resulted in polarity disruption in *Ceratopteris* spores (Stout et al., 2003). So these annexins could play an important role in responding to the calcium current and/or maintaining this current in germinating spores. A key question that needs to be addressed is whether the distribution of AnnCr1 and/or AnnCr2 in spores becomes polarized as gravity fixes the

polarity of the cells between 9 and 18 hours after they are induced to germinate, and, if so, does this polarity change when the spores are turned upside down?

HYPOTHETICAL MODEL OF THE INITIAL GRAVITY RESPONSE IN *CERATOPTERIS* SPORES

The data collected from experiments investigating the mechanism of gravity perception and response in *Ceratopteris* have led to the development of a hypothetical model of the cellular events that contribute to the gravity response in *Ceratopteris richardii* spores. This model is based largely on the pharmacological observation that blocking calcium channels with nifedipine randomized the direction of rhizoid emergence (Chatterjee and Roux, 2000). This observation along with data showing that within hours of germination initiation, gravity induces a calcium current that rapidly changes in parallel with changes in the *g*-force (ul Haque et al., 2007; Salmi et al., 2011), outlines the importance of the calcium current to the gravity response.

In addition to the calcium differential between the top and bottom of spores, there is now evidence for another gravity-induced chemical gradient in spores that could help to regulate their polarization (i.e., an eATP gradient). A role for eATP in gravity-directed polarization would be consistent with studies showing calcium channels in plant cells can be regulated by extracellular nucleotides and that the concentration of these nucleotides is regulated by ecto-apyrases (Clark and Roux, 2011) — enzymes necessary for the polarized growth of pollen tubes and root hairs. The documented expression of an apyrase-like enzyme in *Ceratopteris* during the period of polarity fixation led us to hypothesize extracellular nucleotides and apyrases might play a role in gravity-directed early growth and development (Bushart et al., 2013).

Data in Bushart et al. (2013) show spores release ATP as they germinate and grow, and applied nucleotides and a purinoceptor antagonist suppress gravity-directed polarization. Collectively, these observations are consistent with the hypothesis that extracellular nucleotides could influence calcium transport in *Ceratopteris* spores as they do in *Arabidopsis* (Demidchik et

al., 2009; Demidchik et al., 2011). The hypothetical model shown in Figure 2 postulates how applied ATP and PPADS (Pyridoxal-phosphate-6-azophenyl-2', 4'-disulfonate) could suppress the gravity response. In this model, gravity induces the opening of stretch-activated channels preferentially along the bottom of the spore as predicted by the data in Salmi et al. (2011), and these channels would release ATP, as they are known to do from animal and *Arabidopsis* cells (Weerasinghe et al., 2009). If ATP is released primarily from the bottom of the spore, this asymmetric distribution could induce the opening of calcium channels (Demidchik et al., 2009) primarily along the bottom of spore. The bottom-focused gradient of ATP would be disrupted when extracellular nucleotides or ATP receptor antagonists are applied, thus altering the asymmetrical entry of calcium.

Many of the aspects of this hypothetical model have been verified. A key test of the model will be to measure the eATP in order to determine if a gradient between the top and bottom of germinating spores exists. A self-referencing electrochemical biosensor was developed by Vanegas et al. (2015) to directly measure eATP in living cells. Using this tool, a gradient of eATP was measured in germinating *Ceratopteris* spores within 0.5 hours of light exposure and between 16-22 hours after light-initiated germination. This gradient persisted throughout the critical polarization period. Support that this gradient could play a role in mediating gravity-induced cell polarization is that disrupting it by applying extracellular nucleotides uniformly around the spores, or blocking its activity by a purinoceptor antagonist, inhibits the gravity response in *Ceratopteris* spores when they are applied during the polarization period.

Additional data are needed to verify the model. Among the model's predictions, one of the most important that remains to be demonstrated is that extracellular ATP can actually establish or help contribute to the calcium current. To help address this question, *Ceratopteris* spores harvested from plants expressing the Yellow Cameleon 3.60 FRET-based Ca^{2+} sensor will be used. ATP will be applied both uniformly and unilaterally in order to determine if there is a link between extracellular nucleotides and calcium uptake in *Ceratopteris* spores. Using the newly

developed *Ceratopteris richardii* transformation methods (Plackett et al., 2014; Bui et al., 2015; Plackett., 2015), scientists can begin investigating

if knocking-out specific genes related to calcium and eATP signaling can block spore gravity response.

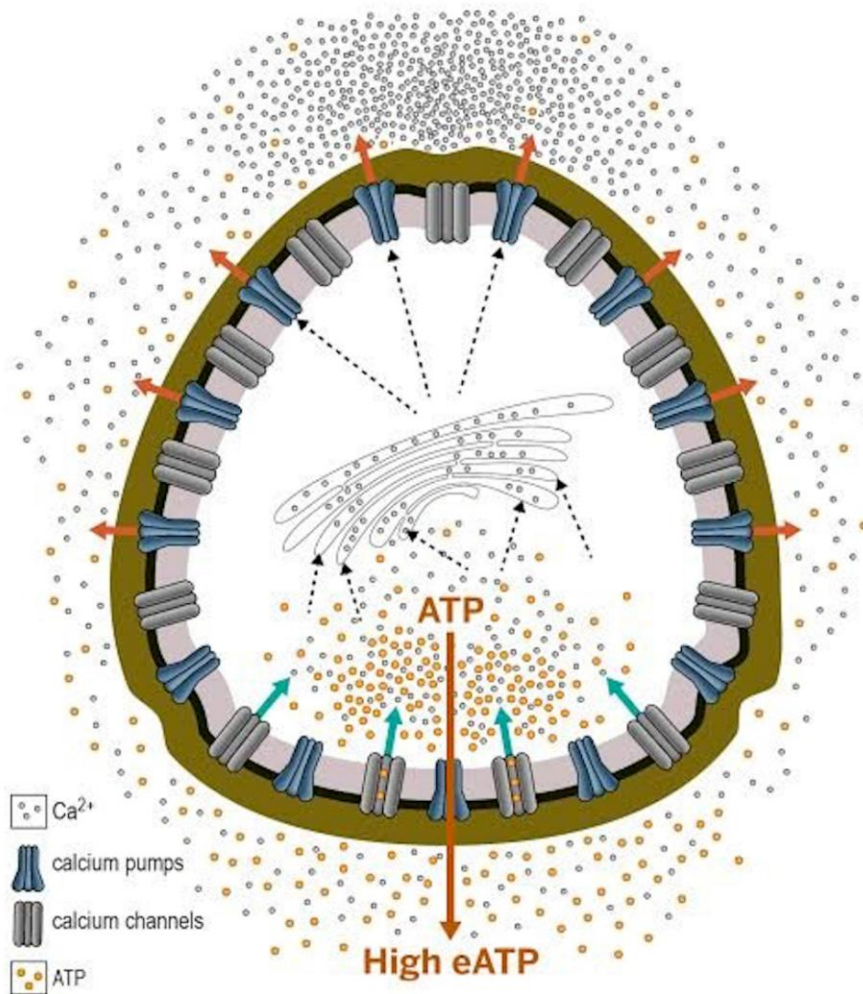


Figure 2. Hypothetical model showing the role of eATP and calcium in polarization of *Ceratopteris richardii* spores. In response to gravity, an undefined mass would settle, selectively activating mechano-sensitive channels primarily along the bottom of the spore. These mechano-sensitive channels could release ATP. The ATP released accumulates asymmetrically, resulting in a gradient where the eATP on the bottom of the spore is higher than the top. The eATP could activate calcium channels directly or indirectly, establishing or contributing to the initiation of the trans-cell Ca²⁺ current that is essential for gravity-directed polarization.

Ground-based experiments, along with results from spores grown in space, have provided valuable insight into the molecular mechanisms of gravity perception and response in single plant cells. These results are unique because they can be used to explain how many different eukaryotic cells are affected by changes in mechanical forces, such as gravity. As these experiments progress,

the results could assist both plant and animal scientists in understanding how cells sense and respond to gravity, allowing them to improve conditions for astronauts and plants in space.

CONCLUSION

During the last several decades, the unique environment of space has provided valuable

insights into the role of gravity in plant growth and development. As science and technology progresses, the experimental conditions for carrying out research in space have greatly improved. These improvements included, prominently, providing “smarter” and more versatile growth chambers, opportunities for longer-term experiments, better environmental monitoring and control, and advanced data telemetry of real-time operations. Additionally, scientists and engineers have also developed better preservation methods, ensuring genomic and morphological studies will have high-quality results. Using all of these new tools, scientists have gained and will continue to gain many new insights about the role of gravity in plant growth and development of multicellular and single-cell plant systems.

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