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Adaptation to real 1/6 g moon gravity contributes to plant development and expeditious acclimation to super-freezing

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ABSTRACT

Understanding how terrestrial life responds to planet microgravity is essential for humankind's ambitious solar system exploration. Using the life-regeneration ecosystem carried by Chang'e 4 probe landed on the Moon, for the first time in human history, we followed the life trajectory of an earth cotton seed germination, development, and final fate after long term exposure to super cold temperature. We compared this life trajectory on the moon to that on earth in a controlled environment with matching parameters, except that the gravity is different. We found that 1/6 g Moon gravity causes no significant interruption to seed germination speed, but slows down seedling growth and contributes to an apparently shortened hypocotyl and thinner cotyledon. Most surprisingly, Moon seedling showed expeditious acclimation to super-freezing under 1/6 g microgravity, remaining erect and green after exposure to long term super cold temperature during the lunar night. We propose plausible mechanisms for the cold resilience based on moon-microgravity induced cellular and molecular responses. These unique findings will extend our understanding of plant adaptive responses to space suboptimal environments.

Statement of experimental research and field studies on plants

The authors used seeds in this manuscripts complying with the IUCN Policy Statement on Research Involving Species at Risk of Extinction and the Convention on the Trade in Endangered Species of Wild Fauna and Flora.

Introduction

The past half century has witnessed a great advance in human exploration of space and solar system. Three bold spacecrafts launched this summer, the United State of America NASA's Perseverance mission, China's Tianwen-1 mission, and United Arab Emirates' Hope mission, declared a new stage of human exploration to Mars. As humankind is preparing for the ambitious goal of constructing bases on the moon and on Mars, a fundamental question is how terrestrial life responds to real space environment. This question remains largely unanswered due to a series of technological and financial challenges.

Organisms in earth exhibit strong adaptability to numerous environmental variables, such as temperature, humidity, nutrition, CO₂ concentration, etc. However, there is very little information on how terrestrial life responds to different gravitation in the space environment. This is important to explore, as gravity is a critical factor involved in the development of terrestrial life, and plays a key role in evolution¹.

Over the past 20 years, our understanding of the impact of microgravity on organism physiology has increased. Plant-based life regeneration ecosystems have helped scientists to study plant growth in a ground-controlled environment by simulating short duration of altered-gravity, and the improved design of plant growth chambers in the International Space Station (ISS) has allowed researchers to study the mechanisms of life adaptation to approximate zero-gravity condition²⁻⁶. Because plants could be an essential integral part of long-term bioregenerative life support system², plant culture in space remains a high priority strategy for all space exploration plans. Life-support systems have been continuously developed to give optimal growth condition for plants in space^{7,8}, i.e., stably controlled light, temperature, air humidity, CO₂ concentration, and so on. A number of plants have been reported to be able to germinate and grow in ISS, including *Arabidopsis thaliana*⁴, *Brassica rapa*⁹, *Cucumber*¹⁰, *Rice*³, *Triticum aestivum*¹¹, and *pisum sativum*¹². However, no previous experiment was carried out in low-gravity planets (e.g. Moon or Mars) - the potential destinations of planetary migration, due to the extreme difficulty in conducting such experiments. How plants or other earth organisms respond to real microgravity and extreme environments in these planets remains unknown.

To address the above questions, we recently performed humankind's first experiment to grow plants on the 1/6 g gravity Moon, which was reported in the news by *Science* and *Nature* in January 15, 2019. Using the ecosystem carried by Chang'e 4 probe landed on the Moon, we followed the life trajectory of an cotton seed. Compared with the earth control, 1/6 g Moon gravity causes no significant interruption to seed germination speed, but slows down seedling

growth and contributes to an shortened hypocotyl and thinner cotyledon. Most surprisingly, Moon seedling showed expeditious acclimation to super-freezing under 1/6 g microgravity, remaining erect and green after exposure to long term super cold temperature during the lunar night.

5 **Methods**

Equipment design: the experiment was carried out by Chang'e 4 lander in a sealed, one atmospheric pressure, and climate-controlled ecosystem equipment which forms a mini biosphere under the condition of low gravity and wide temperature variation (Fig. 1A-D). In this small container, a biological warehouse fixed on the bottom contains rapeseeds, cotton, potatoes and Arabidopsis seeds, as well as yeast and fruit-fly eggs (Fig. 1B-D). Two radiators and multilayer heat insulation are designed to accurately control the container internal temperature. All biological samples were covered by water soluble PVA fiber cotton, and a pipe can load water from storage tank to the biological warehouse. A light pipe on the container's top surface was designed to import natural light for plant photosynthesis. Other interfaces for telemetry and power, as well as CMOD are mounted for accurate control. The biological growth can be recorded in real time by two cameras above the warehouse.

Biological experiments: Figure1E illustrates the biological experiments performing on the Moon. The power of the ecosystem was switched on exactly 12.87 hours after the craft landing at 10:26 on Jan 3, 2019. Half an hour later, water pipe successfully loaded water to the biological warehouse, the container temperature was temporarily adjusted to 54°C, and the water pipe sealing paraffin plug was melted in a few minutes. The cameras and sensors recorded and transmitted back to earth real time data in the period that we named "the first lunar day after landing", until the power was switched off at 20:03 on Jan 12, 2019. After that the Moon entered a so-called "the first lunar night after landing", which lasted for about 18 earth-days, and was dark and cold. The temperature of the container in such environment cooled down quickly to about -52°C in 23 hours after the power was switched off, according to the heat balance test performed on earth by China Academy of Space Technology (Test report number: KPZH-DLPG-05). Finally, the power re-switched on at 01:33 on Jan 31, 2019, marking the beginning of "the second lunar day after landing". We performed the same experiments in parallel in earth in a growth container, with all the parameters and growth conditions accurately matched to that on the moon.

Results and Discussions

We real-time recorded the life trajectory of plant germination, development, and final fate after the lethal strike from the exposure to the 18 days of super-frozen lander night. For life-regeneration ecosystems carried by Chang'e 4 probe or the parallel control on the earth, all physical parameters were identical except the space radiation and gravity (Fig. 2A), the latter being $1/6\ g$ on the Moon and $1g$ on earth. As recorded by the cameras, both the Moon and the earth ecosystem grew one seedling. The moon seedling appeared ~ 22 hours after water injection and was slight, while the earth seedling grew up ~ 82 hours after water injection and was fleshy. This result indicates that $1/6\ g$ gravity has no significant interruption to seed germination. We speculate that the quicker germination of Moon seed is caused by the strong vibration that moved the seed closer to the surface during the launch of Chang'e 4 probe. Meanwhile, compared with the $1\ g$ condition plant growth, Moon seedling grew slower, and developed an obviously shorter hypocotyl and thinner cotyledon (Fig. 2B-C).

Generally, the absence of gravity is a stress condition for plants, and specifically for the functions of meristematic cells¹³. Plants are supposed to sense the direction of gravity and re-orientate themselves according to the gravitational pressure model or starch-statolith hypothesis^{1,14-16}. For plants grown on ISS, it was reported that microgravity can modify the plant body shape, increase the number of cells with transverse microtubules, and suppress lateral expansion in shoot organs including hypocotyls and epicotyls¹⁷. Three days exposure of the Alaska pea seedlings to ISS microgravity resulted in polar auxin transport greatly disarrayed in a PsPIN1 membrane localization dependent manner¹⁸. Furthermore, RNA-seq analysis of *Arabidopsis thaliana* seedling grown on ISS revealed that light-associated pathways (e.g., photosynthesis-antenna proteins and chlorophyll metabolism, which were involved in plants grown) were significantly downregulated in microgravity¹⁹. In addition, it was reported that *Arabidopsis thaliana* seedlings grew for 4 days in darkness in the ISS resulted in an increase in the rate of cell proliferation and a decrease in the cell growth rate²⁰. Meanwhile, mRNA sequencing data from *A. thaliana* seedling on spaceflight showed that the expression of the genes associated with the increase of cell volume was strongly suppressed in the hypocotyl²¹. The above experiments suggest that, the cotton seedling of moon should adapt to $1/6\ g$ gravity by mechano-transduction of cellular substance like microtubules, amyloplasts, or hormone auxins, which together with microgravity induced gene expression changes, contribute to shape up the short height and thin leaf features. It is also worth noting that, probably influenced by the temperature higher than 30°C (Fig 2A), other model plants including rapeseeds, potatoes

and arabidopsis seeds, whose appropriate germination temperatures are lower than 30°C, didn't germinate in either the Moon or the earth container.

A major challenge for life during space exploration is the extreme environment. Understanding how plants respond to the extreme conditions under different gravity is not only important for constructing the life support system for the future space base, but also important for understanding the survival of living systems in general. To gain some insight, we followed the survival trajectory of the cotton seedling which experienced extreme cold temperature in an extended period of time during the lunar night under 1/6 *g* gravity. The seedling first grew for 190 hours after gemination, then was exposed to dark and ultra-low temperature (as low as -52°C in the containers; according to a heat-balance-experiment performed in a simulated environment on earth) of lunar night for ~18 earth days. During the lunar night, the power for the life-regeneration ecosystems was switched off to conserve energy. Surprisingly, on Jan 31, 2019, when the container power was re-switched on after being kept in frozen condition for ~18 days, and rewarmed to room temperature, the moon seedling was still green and erect (Fig. 3A). In comparison, the seedling on earth had already died, showing a fragmental appearance and black-yellow color (Fig. 3A). During the following 8 days of the second lunar day, the moon seedling kept standing with green leaves, and finally crinkled (Fig. 3A). Although it seemed that the slim seedling didn't continue to grow in the second lunar day, we suspect that this was due to oxygen exhaustion in the container (Fig. 2A). The above observations suggest an extraordinary phenomenon: a slim seedling probably can survive after the attack from sustained super-cold under moon microgravity!

Studying the mechanism of this unusual phenomenon is difficult, as the experiment cannot be repeated on earth. Here we suggest some potential mechanisms by considering possible answers to the following questions. First, what are the mechanisms for cold resistance in plants? Secondly, how does microgravity influence those cold adaptation mechanisms?

Previously, it was found that earth boreal plants possess very high freezing resistance. Under the seasonal programed control of photoreceptors and circadian clock system, plants acclimate severe subzero temperatures in winter²². The mechanisms of adaptation to subfreezing temperatures differs in different tissues. Xylem parenchyma cells avoid intracellular freezing by deep supercooling, while extracellular freezing is the most common mechanism of adaptation to freezing for many plant cells, e.g., cambium and leaf cells²²⁻²⁴. By extracellular freezing, plant cells can even tolerate liquid nitrogen temperature²⁵.

Overwintering vegetative buds of trees have been reported to survive freezing by different survival strategies like supercooling or extra-organ freezing^{26,27}.

Although the mechanisms involved in the above freezing resistance strategies are complicated and remain unclear, several cellular physiologic processes are thought to be important for cold protection: 1) Membrane stability is a key factor in cold tolerance, which is probably achieved both by changes in membrane lipid composition and by accumulation of solutes in the surrounding cytosol, including soluble sugars, carbohydrates, proline and betaines²⁸⁻³¹; 2) Plant auxins are also involved in cold tolerance, among them, abscisic acid is a protective factor³¹⁻³³, while ethylene is a negative regulator^{34,35}; 3) Transient influx of calcium into the cytosol should be a key step in the response to freezing^{36,37}; 4) A number of cold-responsive genes are activated during cold-acclimation, including heat shock, antioxidant, and metabolism associated genes involved in lipid or carbohydrate metabolism^{23,36,38,39}; 5) The probable involvement of mitochondria mediated energy metabolism⁴⁰; and, 6) Cell cycle arrest or dormancy.

We speculate that the moon seedling's resistance to the super-cold temperature may involve some of the aforementioned mechanisms. It was suggested that plants on earth develop freezing resistance through adaptation to long term seasonal cold²². How could the moon seedling develop expeditious acclimation to super-freezing in such a short time? The most likely possibility is that moon microgravity contributes to this process.

An interesting review published in 2017 discussed the effect of real and simulated microgravity on certain cell components. Briefly, the properties and functions of cytoplasmic membrane can be viewed as the most sensitive indicators of the effect of microgravity on the cell⁴¹. Under microgravity condition, the cytoplasmic membrane of chlorella cells was found to form higher levels of sinuosity and complicated folding configuration, which was associated with enhanced metabolism and active transport⁴². Membrane isolated from low-gravity grown pea seedling roots showed increased phosphatidylcholines and phosphatidylethanolamines, as well as enhanced unsaturated fatty acids and decreased saturated fatty acids content⁴³. Membrane from pea seedlings under clinostatic conditions had significantly increased sterines, an important component of "lipid rafts", supposed to be involved in microgravity induced alteration of membrane permeability and protection from stress^{41,44}. An *A. thaliana* seedling transcriptome and proteome study reported microgravity induced membrane-bound proteins participating in the metabolism and cell defense⁶. These observations prompt us to suggest one possible mechanism for the fast cold acclimation by the moon seedling: 1/6 g gravity alters the

components of the cell membrane, which in turn promotes the folding of cytoplasmic membrane, and alters membrane permeability, ion transport, distribution of protective membrane proteins, and consequently contributes to freezing resistance.

- 5 How is change in gravitational force translated into an initial cell membrane biochemical signaling cascade when plants respond to long-term microgravity exposure? As reviewed by Masatsugu and Simon⁴⁵, plants gravitropic response exhibits both rapid phasic and extended tonic components. Numerous available data on plants rapid signaling events to mechanical/gravitropic stimulation
- 10 indicate that, cells sense and carry out signal transduction by sedimentation of amyloplasts, as well as the cytoplasmic increase of Ca^{2+} through mechanosensitive ion channels, reflecting auxin-triggered signaling⁴⁶⁻⁴⁸. While the duration of mechanosensitive ion channel activity is too short to exhibit a long-term gravitropism response, Masatsugu and Simon suggested that the
- 15 cytoskeleton should be a candidate in this long-term perception system. Plant's microtubule cytoskeleton plays an important role in the regulation of cell expansion, and gravistimulation results in changes in the orientation of cortical microtubules directions in plants hypocotyls⁴⁹. Cortical actin cytoskeleton underlying the cell membrane also appears to plays a role in initial
- 20 graviperception. As an important component of biochemical mechanosensory network⁵⁰, actin is involved in clathrin-mediated endocytosis which is influenced by cell membrane tension⁵¹. In vitro experiment also showed that in reconstituted actin-and-membrane systems, actin shells can control buckling and wrinkling of biomembranes⁵². Most interestingly, Michele and his
- 25 colleagues found that mechanical stretch can deform the nucleus, leading to changed chromatin rheology and architecture⁵³; the latter is generally believed to influence genes expression. These studies may provide one model for how plant cytoskeleton proteins operate to transduce the mechanical signal from long-term microgravity to membrane biochemical activity.
- 30 In addition to gravity-associated membrane alteration, we think several microgravity induced physiological changes may also be beneficial to frozen protection: 1) Concentrations of soluble sugars, glucose, sucrose and total starch were substantially increased in Space shuttle flight sweetpotato stem⁵⁴, and soluble sugars enhanced in tobacco callus cells under rotation simulated
- 35 microgravity⁵⁵; 2) In space, microgravity environment had no obvious effect to rice shoots auxins, but reduced the expression of ACC synthase, the key enzyme of ethylene biosynthesis³, and ethylene is a negative regulator of cold resistance; 3) Numerous experiments indicated that calcium in cells increased under real or simulated conditions^{5,56}; 4) Genes expression changed under real

microgravity in space and simulated ground experiments. Functions of “major space genes” include Ca^{2+} and lipid signaling, cell membrane biosynthesis, total metabolism, carbohydrates and lipid response to stress, auxin-signaling, and heat shock protein (HSP) thought to support the organization of cytoskeleton in adaptive response to microgravity⁵⁷⁻⁶⁰. In food-borne pathogen *Listeria*, low-shear modeled microgravity elevated cold resistance in a cold-stress-regulated gene dependent manner⁶¹.

Conclusion

In conclusion, for the first time, we found that earth plant seed can sprout under real microgravity planet, and moon’s 1/6 *g* gravity contributes to the decreased growing speed and shortened hypocotyl and thinner cotyledon appearance. Most importantly, it seemed that moon seedling developed expeditious acclimation to super-freezing, keeping green and erect for several days after the exposure to the long term super cold lunar night. We propose a hypothetic model for the fast acclimation of the moon seedling to super-freezing stress. As summarized in figure3B, through moon-microgravity-induced adaption, plants can fold cells cytoplasmic membrane, alter membrane components and permeability, enhance cytoplasmic soluble sugars as well as other carbohydrates, reduce ethylene production, provide an increased flow of calcium ions into cells, and alter “major space genes” expression, thus contribute to cold resistance.

This first experiment on plant growth on the moon explores how plant response to real low-gravity, and extends our knowledge on life survival under extreme space conditions. It will be exciting to test in future space explorations whether plants in general better survive super cold temperature under microgravity conditions. It will also be important to see whether such phenomenon generalizes to other multicellular organisms such as animals. Deeper exploration of microgravity associated physiology should be helpful for solving some critical problems for humans, such as deep freezing of human body as a way to preserve life, which may be important for long time space travel, and can be utilized for patients with currently untreatable diseases.

Figure 1

Figure1 Ecosystem equipment and workflow of the moon biological experiment carried out by Chang'e 4 lander.

A: ecosystem equipment exterior. It is a sealed, atmospheric pressure and climate well-controlled container which can form a mini biosphere under the moon environment. B-C: Internal layout of the container. A biological warehouse is fixed on the bottom and covered by water soluble PVA fiber cotton. Two radiators and multilayer heat insulation are equipped to accurately control the container internal temperature. A pipe can load water from storage tank to the biological warehouse. A light pipe on the container top surface is to import natural light for plant photosynthesis. And two cameras above the warehouse are designed for realtime recording. D: Biological warehouse appearance. It contains rapeseeds, cotton, potatoes and arabidopsis seeds, as well as yeast and fruit-fly eggs. E: biological experiment procedures performing on the Moon. The ecosystem power was switched on exactly 12.87 hours after the craft landing. Half an hour later, water pipe successfully loaded water to the biological warehouse by temporally adjusting container temperature to 54°C to melting the water pipe sealing paraffin plug. The ecosystem kept working for several days we defined as “the first lunar day after landing”. And the power was switched off at 20:03 Jan 12, 2019, means the Moon entered “the first lunar night after landing”, which lasted for about 18 earth-days, and was dark and frozen. Finally, the power re-switched on at 01:33 Jan 31, 2009, and it was the beginning of “the second lunar day after landing”.

Figure 2

Figure2 Cotton seed germination and growth diversity between lunar and earth parallel control.

A: Left panel, temperature was well controlled in both moon and earth containers; Right panel, the atmospheric pressure and oxygen content dynamics of the earth container

- 5 during the whole experiment. The double slash indicates the period of “the first lunar night after landing”, which lasted for about 18 earth-days. B: Seedling growth of the lunar during the first lunar day after landing. Upper panel, pictures realtime captured by cameras; Middle panel, magnified images of the local part in red boxes of the upper panel. Red arrowhead points to the seedling; Bottom panel, 3-D simulation images of lunar seeding during the first lunar day. C: Seedling growth of the earth during the first lunar day after landing. Upper panel, pictures realtime captured by cameras; Bottom panel, magnified images of the local part in yellow boxes of the upper panel. Yellow arrowhead points to the seedling.

Figure 3

Figure3 Lunar seedling seems developing expeditious acclimation of super-freezing under real moon 1/6 g microgravity.

A: The variation between images of the second lunar day and last frame of the first lunar day, for both seedlings on the lunar and the earth. Red arrowhead points to the seedling. After being kept in frozen condition for about 18 days and rewarmed to room temperature, the moon seedling still be green and erect for several days, while the earth control had already died and shew a fragmental appearance and black-yellow color. B: A hypothetic model of fast acclimation of moon seedling to super-freezing stress under microgravity environment.

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15 and wrote the manuscript.

Figures

Figure 1

Figure 1

Ecosystem equipment and workflow of the moon biological experiment carried out by Chang'e 4 lander. A: ecosystem equipment exterior. It is a sealed, atmospheric pressure and climate well-controlled container which can form a mini biosphere under the moon environment. B-C: Internal layout of the container. A biological warehouse is fixed on the bottom and covered by water soluble PVA fiber cotton. Two radiators and multilayer heat insulation are equipped to accurately control the container internal temperature. A pipe can load water from storage tank to the biological warehouse. A light pipe on the container top surface is to import natural light for plant photosynthesis. And two cameras above the warehouse are designed for realtime recording. D: Biological warehouse appearance. It contains

rapeseeds, cotton, potatoes and arabidopsis seeds, as well as yeast and fruit-fly eggs. E: biological experiment procedures performing on the Moon. The ecosystem power was switched on exactly 12.87 hours after the craft landing. Half an hour later, water pipe successfully loaded water to the biological warehouse by temporally adjusting container temperature to 54° to melting the water pipe sealing paraffin plug. The ecosystem kept working for several days we defined as “the first lunar day after landing”. And the power was switched off at 20:03 Jan 12, 2019, means the Moon entered “the first lunar night after landing”, which lasted for about 18 earth-days, and was dark and frozen. Finally, the power re-switched on at 01:33 Jan 31, 2009, and it was the beginning of “the second lunar day after landing”.

Figure 2

Cotton seed germination and growth diversity between lunar and earth parallel control. A: Left panel, temperature was well controlled in both moon and earth containers; Right panel, the atmospheric pressure and oxygen content dynamics of the earth container during the whole experiment. The double slash indicates the period of “the first lunar night after landing”, which lasted for about 18 earth-days. B: Seedling growth of the lunar during the first lunar day after landing. Upper panel, pictures realtime

captured by cameras; Middle panel, magnified images of the local part in red boxes of the upper panel. Read arrowhead points to the seedling; Bottom panel, 3-D simulation images of lunar seeding during the first lunar day. C: Seedling growth of the earth during the first lunar day after landing. Upper panel, pictures realtime captured by cameras; Bottom panel, magnified images of the local part in yellow boxes of the upper panel. Yellow arrowhead points to the seedling.

Figure 3

Lunar seedling seems developing expeditious acclimation of super-freezing under real moon $1/6 g$ microgravity. A: The variation between images of the second lunar day and last frame of the first lunar day, for both seedlings on the lunar and the earth. Red arrowhead points to the seedling. After being kept in frozen condition for about 18 days and rewarmed to room temperature, the moon seedling still be green and erect for several days, while the earth control had already died and shew a fragmental appearance and black-yellow color. B: A hypothetic model of fast acclimation of moon seedling to super-freezing stress under microgravity environment.