Comprehensive report on the Auxin Transport space experiment: the analysis of gravity response and attitude control mechanisms of plants under microgravity conditions in space on the International Space Station

Junichi Ueda

Principal Investigator of the Auxin Transport space experiment
Professor Emeritus
Graduate School of Science, Osaka Prefecture University
1-1 Gakuen-cho, Naka-ku, Sakai, Osaka 599-8531
Japan

Abstract

The present paper is a comprehensive report on the Auxin Transport space experiment: the analysis of gravity response and attitude control mechanisms of plants under microgravity conditions in space on the International

Space Station. The Auxin Transport space experiment was conducted in 2016 and 2017 in the Japanese Experiment Module (JEM) on the International Space Station (ISS), with the principal objective being integrated analyses of the growth and development of etiolated pea (*Pisum sativum* L. cv Alaska) and maize (*Zea mays* L. cv Golden Cross Bantam) seedlings under true microgravity conditions in space relative to polar auxin transport. To clarify auxin dynamics at molecular levels, gene expression of *PsPIN1* and *ZmPIN1a* mRNA, and their products detected by immunohistochemistry were also investigated. In addition, the use of microarray data technology with *Medicago* (*Medicago truncatula*) microarrays to characterize global changes in the transcript abundance of etiolated Alaska pea seedlings grown under microgravity conditions in comparison with those under artificial 1 G conditions on the International Space Station was reported here. Comprehensive analyses of endogenous plant hormones in etiolated pea and maize seedlings grown under microgravity conditions in space as well as on 1 G conditions on Earth have already performed in the space experiment. ©2020 Jpn. Soc. Biol. Sci. Space; doi:10.2187/bss.34.12

Table of contents

1. Introduction
2. Research plan
 - 2.1 Research goals
 - 2.2 Organization
 - 2.3 Time lapse of the Auxin Transport space experiment until implementation
3. Materials, methods, and operation
 - 3.1 Plant materials
 - 3.2 Procedures for preparing plant material on orbit and procedures for returning samples
 - 3.3 Polar auxin transport experiment procedure on orbit
 - 3.4 Polar auxin transport analysis procedure on the ground after sample retrieval
 - 3.5 Control experiment under 1 G conditions
 - 3.6 Preparation of chemical fixative for immunohistochemistry
 - 3.6.1 Principle of chemical fixation
 - 3.6.2 Experimental limitation in space experiments
 - 3.6.3 Chemical fixative selection
 - 3.6.4 Improvement of chemical fixative composition
 - 3.6.5 Determination of chemical fixative
 - 3.6.6 Space experiment and post-flight analysis
 - 3.7 Extraction of total RNA
 - 3.8 Real-time quantitative PCR
 - 3.9 Extraction of membrane protein and Western blot analysis
 - 3.10 Immunohistochemical analysis
 - 3.10.1 Production of PsPIN1 and ZmPIN1a antibodies
 - 3.10.2 Immunohistochemistry of PsPIN1 and ZmPIN1a proteins
 - 3.11 Statistical analysis
4. Growth and development of etiolated pea and maize seedlings grown under μ G conditions in space
 - 4.1 Growth and development of etiolated pea seedlings grown under μ G conditions in space
 - 4.2. Growth and development of etiolated maize seedlings grown under μ G conditions in space
 - 4.3. Polar auxin transport of etiolated pea epicotyls and maize shoots grown under μ G conditions in space
 - 4.4 Effect of μ G conditions in space on *PsPIN1* mRNA and PsPIN1 protein accumulation
 - 4.5 Effect of μ G conditions in space on subcellular localization of PsPIN1 protein
 - 4.6 Effect of μ G conditions in space on *ZmPIN1a* mRNA and ZmPIN1a protein accumulation
 - 4.7 Effect of μ G conditions in space on subcellular localization of ZmPIN1 proteins in the coleoptiles and the mesocotyls
5. Microarray profile of gene expression in etiolated pea seedlings grown under μ G conditions in space
6. Comprehensive analyses of plant hormones in the etiolated pea and maize grown under μ G conditions in space

Received: April 24, 2020; Accepted: May 27, 2020

*To whom correspondence should be addressed:

Phone: +81-721-29-5306

E-mail: ueda@b.s.osakafu-u.ac.jp

1. Introduction

The growth and development of land plants are affected by such environmental factors as light, gravity, temperature, moisture, gas components, and contact. Each physiological process in the life cycle of a sessile plant is greatly affected by gravity, so plants have acquired various mechanisms to adapt or respond to terrestrial gravity stimuli. An important gravitational response is gravitational resistance. This allows plants to grow upward against gravity. Plants build a hardy structure by building rigid cell walls. Another characteristic gravitational response is gravitropism. This growth response is induced by the orientation of gravity vector, which spatially positions the plant's organs in a location appropriate for their functions. That is, the part aboveground grows upward (or negative with respect to the gravity vector) to capture light efficiently, and the underground part grows downward (in the direction of the gravity vector) to acquire water and nutrients and to keep the plant anchored firmly in the soil.

Plant growth and development is controlled by the dynamics of plant hormones and other plant growth substance. Auxin (indole-3-acetic acid: IAA) is a representative plant hormone that plays an important role in regulating multiple physiological processes such as cell elongation, apical dominance, rooting, and vascular patterning. Biosynthesized mainly at the shoot apex, auxin is transported to the root tip through the central cylinder in the aerial part of the plant, then to a specific root tip cell in a symmetrical manner, known as the auxin fountain model. Redirected towards the root elongation area, the unique transport of auxin in the polar direction and so is called polar auxin transport. It is a unique form of transport through carriers along the membrane diffusion and plant axially, thereby allowing auxin gradients to control growth and development as well as various plant movements (Teiz and Zeiger, 2002).

Polar auxin transport is believed to be controlled mainly by the AUX1 protein (Marchant *et al.*, 1999; Kerr and Bennett, 2007) as an auxin uptake carrier and a functional protein that is localized in the plasma membrane, such as the PIN protein as an efflux carrier (Okada *et al.*, 1991; Gählweiler *et al.*, 1998; Křeček, *et al.*, 2009; Luschnig and Vert, 2014). The PIN protein was discovered in a study of *Arabidopsis thaliana pin* mutants, which in fact showed reduced polar auxin transport in *pin* mutants (Okada *et al.*, 1991; Oka *et al.*, 1999). In *Arabidopsis*, AtPIN1 has been shown to be a fundamental protein for polar auxin transport. Since these PIN proteins are specifically localized at the base of the plasma membrane by secretory vesicles, PIN proteins are considered essential for polar auxin transport (Okada *et al.*, 1991; Marchant *et al.*, 1999).

In the dicotyledonous, pea plant (*Pisum sativum* L.), three *PIN* genes, (*PsPIN1*, *PsPIN2* and *PsPIN3*), have been isolated (Chawla and DeMason, 2004; Hoshino *et al.*, 2005; Ueda *et al.*, 2012). In the monocotyledonous, maize plant (*Zea mays* L.), four *ZmPIN1* subfamily genes, (*ZmPIN1a*, *b*, *c*, and *d*) have been identified (Carraro *et al.*, 2006; Forestan *et al.*, 2010; Forestan and Varotto, S.,

2010). Experiments using antibodies raised against AtPIN1 show that ZmPIN1 localized on the polar and nonpolar sides of the plasma membrane in some tissues and accumulated in the inner membrane of endosperm tissue cells (Forestan *et al.*, 2010; Forestan *et al.*, 2012). Using transgenic plants that express native endogenous ZmPIN1a protein, Gallavotti and colleagues showed in experiments that all maize branching events during vegetative and reproductive development respond to auxin through activation of ZmPIN1a protein (Gallavotti *et al.*, 2008). They also showed that the function of the ZmPIN1a protein is similar to that of AtPIN1 because it was able to rescue the *pin1-3* mutant of *Arabidopsis*.

In order to clarify how gravitational stimuli affect the growth and development of plants, the use of environments with different gravitational stimuli is the most important research tool; these include microgravity (μ G) of space and hypergravity environments. Also studied is research on various physiological responses, changes in plant hormone dynamics, and responses to the growth of plants grown under μ G conditions on the Space Shuttle (STS) and on the International Space Station (ISS, Halstead and Dutcher, 1987; Kamada *et al.*, 2000; Correll and Kiss, 2008; Hoson *et al.*, 2009; Paul *et al.*, 2013; Hoson *et al.*, 2014; Kiss, 2014; Wakabayashi *et al.*, 2015; Ferl *et al.*, 2016; Yamazaki *et al.*, 2016; Johnson *et al.*, 2017; Wakabayashi *et al.*, 2017; Soga *et al.*, 2018). In such a weightless environment, plants exhibit growth called automorphogenesis (see review of Stanković *et al.*, 1998). More recently, the term automorphogenesis has been used to describe the establishment of intracellular polarity and the determination of a growth direction in a weightless environment (Volkman *et al.*, 1986; Driss-Ecole *et al.*, 2008) and has also been used for morphogenesis of plants grown in a simulated μ G environment in a three-dimensional (3D) clinostat (Hoson *et al.*, 1992; Takahashi, 1994; Hoson *et al.*, 1995; Hoson, *et al.*, 1997; Miyamoto *et al.*, 2005). When 2,3,5-triiodobenzoic acid (TIBA, a polar auxin transport inhibitor), is given on a 3D clinostat, polar auxin transport is lower than under 1 G conditions on Earth. Changes in the growth direction of etiolated pea epicotyls (Hoshino *et al.*, 2006; Hoshino *et al.*, 2007) and those induced by the application of TIBA is deeply involved in the localization of PIN proteins to plasma membranes (Geldner *et al.*, 2001; Geldner *et al.*, 2003; Kojo *et al.*, 2014; Hille *et al.*, 2018). Given these facts, it was decided to study the effects of TIBA on auxin dynamics, including the localization of auxin carrier protein-related gene expression and its products, when TIBA regulates plant growth and development under μ G conditions in space.

To clarify the effects of gravity on plant growth and development from the perspective of plant hormone dynamics, our research team did an experiment (STS-95) on the space shuttle "Discovery" in 1998. The STS-95 experiment showed that polar auxin transport is affected by gravity in the etiolated Alaska pea epicotyls and the Golden Cross Bantam maize coleoptiles (Ueda *et al.*, 1999; Ueda *et al.*, 2000). This polar auxin transport in etiolated Alaska pea seedling epicotyls was shown to be

lower than that in seedlings grown under 1 G conditions on Earth (Ueda *et al.*, 1999; Ueda *et al.*, 2000). However, polar auxin transport in coleoptiles of etiolated Golden Cross Bantam maize seedlings was greater in μG conditions in space than on 1 G conditions on Earth (Ueda *et al.*, 1999; Ueda *et al.*, 2000). Comparable results were obtained in experiments in a simulated μG environment on a 3D clinostat (Ueda *et al.*, 1999; Miyamoto *et al.*, 2005). Recognized from STS-95 space experiments and ground experiments using such polar auxin transport inhibitors as TIBA, *N*-(1-naphthyl) phthalamic acid (NPA), and 9-hydroxyfluorene-9-carboxylic acid (HFCA) reduced polar auxin transport in automorphogenesis of etiolated Alaska pea seedlings and has been shown to be a cause, not a consequence, of automorphogenesis (Ueda *et al.*, 1999; Ueda *et al.*, 2014). However, this difference between etiolated pea and etiolated maize seedlings under μG conditions in space may be due to differences in plant species, organs and tissues, and the polar auxin transport system itself. The details are not clear yet.

Table 1. Organization of Auxin Transport space experiment in 2016 to 2019

Principal Investigator	
Junichi Ueda	Professor Emeritus, Osaka Prefecture University
Co-Investigators and support research workers	
Kensuke Miyamoto	Professor, Osaka Prefecture University
Eiji Uheda	Associate Professor, Osaka Prefecture University
Mariko Oka	Associate Professor, Tottori University
Motoshi Kamada	Ph D, AES, Co., Ltd
Chiaki Yamasaki	Ph D, JAXA/Japan Space Forum
Akira Higashibata	Ph D, JAXA
Toru Shimazu	Ph D, JAXA/Japan Space Forum
Haruo Kasahara	JAXA/JAMSS
Noriaki Ishioka	Professor, JAXA
Tomomi Suzuki	JAXA
Hiroshi Sano	JAMSS

Fig. 1. Time lapse of the Auxin Transport space experiment until implementation SDR; System Definition Review, CDR; Critical Design Review, PQR/PSR; Post Qualification Review/Pre Shipment Review

2. Research plan

2.1 Research goals

So far, the mechanism of gravity-controlled plant growth and development and its closely related polar auxin transport have been elucidated by the dynamics of the PsPIN and ZmPIN proteins at a molecular level in the Alaska pea (*Pisum sativum* L.) and Golden Cross Bantam maize (*Zea mays* L.) seedlings, respectively. Focusing on the expression of the genes encoding PsPIN1 and ZmPIN1a proteins, and their subcellular localization, the author planned a space experiment using the μG conditions in space on the ISS (according to NASA's operating nomenclature, Auxin Transport, Ueda, 2016). For this purpose, novel specific polyclonal antibodies against PsPIN1 and ZmPIN1a were generated (Kamada *et al.*, 2018a; Kamada *et al.*, 2018b). This paper reports the details of the growth and development of etiolated pea and etiolated maize seedlings grown under μG conditions in space and closely related polar auxin transport in the Auxin Transport space experiment. The present study also clarified the effects of μG conditions in space on the expression of *PsPIN1* mRNA in epicotyl cells of etiolated pea seedlings and *ZmPIN1a* mRNA in etiolated maize coleoptile and mesocotyl cells encoding PsPIN1 and ZmPIN1a proteins, respectively. The key role of the dynamics of PsPIN1 and ZmPIN1a proteins in polar auxin transport regulated by gravity is also reported. Comprehensive analyses of identification and quantification of endogenous plant hormones were done to investigate how endogenous plant hormone dynamics in etiolated pea and maize seedlings are affected by the influence of polar auxin transport under μG conditions in space. Based on these results, the final research goal is to clarify the mechanism of self-constructed plant attitude control by gravity.

2.2 Organization

Organization of the Auxin Transport space experiment carried out from 2016 to 2019 is shown in Table 1. The ISS crew, Mr. Timothy Peake and Mr. Thomas Pesquet, and their colleagues, also conducted important space experiments on the ISS in 2016 and 2017.

2.3 Time lapse of the Auxin Transport space experiment until implementation

Figure 1 shows the time lapsed the Auxin Transport space experiment until implementation. The plan for the Auxin Transport space experiment was approved in 2010 and the space experiments were implemented in 2016 and 2017.

3. Materials, methods, and operation

3.1 Plant materials

The seeds of pea (*Pisum sativum* L. cv. Alaska) and maize (*Zea mays* L. cv. Golden Cross Bantam) used in the Auxin Transport space experiment were purchased from Watanabe Seed Co., Ltd. (Miyagi Prefecture, Japan) and Kokaen (Hokkaido, Japan), respectively.

For etiolated pea seedlings, an acrylic pea box (95 mm wide x 50 mm deep x 63 mm high, Pea Chamber)

Fig. 2. Equipment and materials used in the Auxin Transport space experiment

Table 2. Time schedule of Auxin Transport space experiment on orbit

Run 2, Run 3 (all dates are in Japan time)
<ul style="list-style-type: none"> • Launch: "Space X No. 8" April 9, 2016 Start of Run2 culture: May 23, 2016 Run2 culture end: May 26, 2016 Start of Run3-1 culture: May 30, 2016 End of Run3-1 culture: June 2, 2016 Start of Run3-2 culture: June 7, 2016 Run3-2 culture end: June 11, 2016 • Return: Space X Unit 9 August 27, 2016
Run 1, Run 4 (all dates are in Japan time)
<ul style="list-style-type: none"> • Launch: Space X No. 10 February 19, 2017 Start of Run4 culture: March 7, 2017 Run4 culture completed: 11 March 11, 2017 Start of Run1 culture: March 12, 2017 Run1 culture end: 15 March 15, 2017 • Return: Space X No. 10 March 19, 2017

or an 80 mm wide, 65 mm deep, and 55 mm high pea box with an observation window was used (Fig. 2). In each case, there were four 1-cm holes in the top for ventilation and was covered with a hydrophobic fluoropore membrane (MilliSeal; Merck Millipore, Tokyo, Japan). Rock wool 16 mm thick (a culture mat from the Japan Rock Wool Co., Ltd.) was packed so as to fit the pea box exactly. On the ground, 12 dry pea seeds were placed in each pea box so that they were completely

buried in rock wool, with the hilum of the seed (ie, the line connecting the epicotyl and the radicle) toward the surface of the rock wool. It was firmly embedded in the rock wool horizontally. The pea boxes were loaded on the SpaceX-8 and the SpaceX-10. The SpaceX-8 and the SpaceX-10 was launched from the US Kennedy Space Center on April 9, 2016 and February 19, 2017, respectively, to the ISS.

For etiolated maize seedlings, an acrylic resin maize box (100 mm wide x 62 mm deep x 150 mm high, Maize Chamber) was used (Fig. 2). Each maize box had four 1-cm holes on the front and two on the sides for ventilation and was also covered with the hydrophobic fluoropore membrane. As a seed support, rock wool similar to that used in pea, and 20 dry seeds were embedded in each maize box accurately so that the embryo of the seed was perpendicular to the rock wool surface. These maize boxes were loaded on the SpaceX-10 and launched from the US Kennedy Space Center on February 19, 2017, bound for the ISS.

3.2 Procedures for preparing plant material on orbit and procedures for returning samples

Time schedules for the Auxin Transport space experiment on orbit are listed in Table 2. This space experiment consists of four Runs. The first experiments (Run 2 and Run 3-1 and Run 3-2) started at the SpaceX-8 mission on April 9, 2016 Japan Time. It was implemented at the ISS Increment-47 in May and June 2016. The SpaceX 10 mission experiments (Run 1 and

Run 4) on February 19, 2017 were performed at the ISS Increment-50 in March 2017. Each sample returned to Earth on August 27, 2016 and March 19, 2017 by the Cargo Dragon Capsule mounted on SpaceX-9 and SpaceX-10, respectively. The Run 2 and Run 3-1 and Run 3-2 experiments were performed earlier than the Run 1 experiments due to the constraint on ISS crew time. Figure 2 shows the equipment and materials used in the experiment.

Run 1: On orbit, an astronaut supplied 45 mL of water (Milli-Q water, autoclaved) or 30 μ M TIBA (Sigma-Aldrich, MO, USA) to the dry pea seeds of three pea boxes. The seed boxes were then placed in a compartment to create μ G conditions in space and artificial 1 G conditions in the Cell Biology Experiment Facility (CBEF). In this environment, etiolated pea seedlings were germinated and grown at 23.5°C in the dark for 3 days (68 hours and 10 minutes). After taking pictures at the end of the experiment, the etiolated pea seedlings were fixed with a fixative using a specially designed Chemical Fixation Bag (CFB) to store samples for later immunohistochemical analysis. Thereafter, the CFB was stored in a plastic box and stored in +2°C environment of the ISS cabin. The remaining seedlings were stored at -95°C in a laboratory freezer (Minus Eighty-degree Celsius Laboratory Freezer, MELFI).

Run 2: After water was supplied as described above, the etiolated pea seedlings were allowed to grow for 3 days (74 hours, 20 minutes) at 23.5°C in the dark under the μ G conditions in space and under artificial 1 G conditions in the ISS CBEF. Later, British astronaut Tim Peak conducted the polar auxin transport experiment using an “Auxin tube” as shown in Figs. 2 and 3A.

Run 3-1 and Run 3-2: The astronaut supplied 40 ml of water (Milli-Q water, autoclaved) to the dried pea

seeds in the pea observation chamber on orbit, and seedlings were allowed to grow at 23.5°C in the dark in the Video Measurement Experiment Unit (V-MEU) under μ G conditions in space and an artificial 1 G conditions in space. After 3 days (Run 3-1; 74 hours and 52 minutes) and 4 days (Run 3-2, 95 hours and 23 minutes), the astronauts removed each chamber and fixed each sample using the media sampling box. The samples were then transferred to MELFI for storage at -95°C. In Run 3-2, during the incubation, the plant growth and development were automatically recorded by the CCD camera in the CBEF V-MEU at 6 hour intervals from day 2 to day 4.

Run 4: On orbit, the astronaut supplied 120 ml of water (Milli-Q water, autoclaved) to each maize box, put it in a Ziplock bag, and allowed it to germinate under μ G conditions in CBEF. The etiolated maize seedlings were grown for 4 days (96 hours and 27 minutes) at 25°C in the dark under μ G conditions in space for later immunohistochemical analysis. After taking pictures, some etiolated maize seedlings were subjected to polar auxin transport experiments, and the remaining seedlings were fixed with CFB or frozen at -95°C in MELFI.

Preparation of Auxin tube including radiolabelled IAA (Run 2) and turn over (Run 1 and Run 4) in US Kennedy Space Center are shown in Fig. 3A.

A ground control experiment was performed to verify the results of the STS-95 space experiment using etiolated maize seedlings. Seedlings were fixed with a fixative in CFB to perform immunohistochemistry experiments and +2°C and -95°C in MELFI for analysis of gene expression and endogenous plant hormone levels, respectively. The Auxin tube with which the polar auxin transport experiment was performed on orbit was stored at -95°C in MELFI. These samples were returned to Earth by the Cargo Dragon Capsule carried by SpaceX-10, but were packed in dry ice during and after storage to maintain the storage temperature conditions, then sent to the laboratory. No thawing of the sample was observed during the return and transport of the sample.

3.3 Polar auxin transport experiment procedure on orbit

The on-orbit polar auxin transport experiment was performed with some modifications to the previously reported method (Ueda *et al.*, 1999; Ueda *et al.*, 2000). An aqueous lanolin solution (30%, w/w, 20 μ L) containing 14 C-IAA (indole-3-acetic acid, [1-^{14} C] IAA, 55 mCi/mmol, American Radiolabeled Chemicals Inc., St. Louis, MO, USA) diluted 2/100 (v/v) on the ground was added to the bottom of a 1.5 mL Eppendorf tube (Fig. 4).

The astronauts marked the epicotyl of the cotyledon side with a Sharpie pen (Newell Brands, Hoboken, USA) so that the cotyledon side and anticotyledon side of the epicotyl could be identified. Using a JAXA Stem Cutter, the first internode segment (20 mm long) of the epicotyl

Fig. 3. Activities in Kennedy Space Center
A: Preparation of Auxin tube including radiolabelled IAA (Run 2)
B: Turn over in Kennedy Space Center (Run 1 and Run 4)

Fig. 4. Schematic procedures for polar auxin transport in the segments of etiolated pea epicotyls (A) and maize shoots (B) using radiolabelled IAA

was cut from a three-day-old etiolated pea seedling. Since radioactive IAA did not substantially move from the basal side of the section (Ueda *et al.*, 1999; Ueda *et al.*, 2000), the apical side of the segment was inserted into lanolin containing ^{14}C -IAA at the bottom of a 1.5 mL Eppendorf tube. As a result, ^{14}C -IAA was taken in from the apical side of the segment, and a polarity shift was recognized. Next, after culturing the Eppendorf tube containing the segment at the cabin temperature for 19 hours, the tube was transferred to MELFI and fixed at -95°C .

It has been found that the aerial part of etiolated maize seedlings has significantly higher auxin transport activity in the coleoptile than in the mesocotyl (Ueda *et al.*, 2014b). Therefore, to examine the effect of μG conditions on polar auxin transport in both the coleoptile and the mesocotyl, a 20-mm segment consisting of the mesocotyl with the coleoptile and the node (the junction between the coleoptile and the mesocotyl) was examined. The coleoptile side of the segment prepared from the four-day-old etiolated maize seedling was inserted into an Eppendorf tube so that radioactive auxin was taken from the coleoptile side, and incubated at cabin temperature for 16 hours. At the end of the incubation, Eppendorf tubes were frozen at -95°C in

MELFI. After returning to Earth, the flight samples already frozen on the ISS were transferred to a freezing box with dry ice, and then transported to a laboratory in Japan. No thawing of the sample was observed during the return and transport of the sample.

3.4 Polar auxin transport analysis procedure on the ground after sample retrieval

The polar auxin transport in the segment having epicotyl of the etiolated pea seedling was carried out by partially modifying the previously reported method (Ueda *et al.*, 1999; Ueda *et al.*, 2000). Figure 4 shows the procedure for measuring polar auxin transport in segments of pea epicotyls and aerial parts of maize.

Frozen etiolated pea epicotyl segments were carefully divided in half vertically into the proximal side and the distal side and placed on dry ice to avoid thawing. After removing the portion immersed in lanolin containing radioactive IAA, each epicotyl segment was divided into five zones (3 mm long). A small piece of the divided epicotyl section was directly put into a vial containing a liquid scintillation cocktail (UniverSol ESTM, MP Biomedicals, LLC, OH, USA) and its radioactivity was measured with a scintillation counter (2200CA, Packard Instrument, CT, USA).

On the other hand, the 20 mm segment (consisting of an coleoptile, node, and mesocotyl) of frozen etiolated maize seedlings was divided into seven zones (about 3 mm long). In this case, the node was zone 4 and the end of the mesocotyl was zone 1. The estimated mean lengths of the coleoptile and the mesocotyl were 6.9 mm and 13.1 mm, respectively.

3.5 Control experiment under 1 G conditions

The ground control experiment was conducted at the JAXA Tsukuba Space Center (TKSC) and Osaka Prefecture University according to the same procedure and time schedule as the ISS on-orbit experiment of Runs 1 to 4.

3.6 Preparation of chemical fixative for immunohistochemistry

3.6.1 Principle of chemical fixation

In order to achieve the purpose of the space experiment, it is necessary to recover the biological sample obtained, while maintaining the morphology, gene expression and protein localization. For that purpose, chemical fixation is very important. It is necessary to select an optimal composition for the chemical fixative depending on the difference in biological species and organs (tissue hardness, water/lipid content, etc.) and the molecules (genes, proteins, etc.) to be analyzed. Representative types of chemical fixatives include aldehyde-based fixatives, organic solvent fixatives, chemical fixatives using metal ions, and combination of these. Chemical fixatives of aldehydes

and metal ions can prevent degradation and metabolism by crosslinking proteins in the sample. The organic solvent fixatives stabilize proteins by precipitation and dehydration, but also shrink tissue and cells as dehydration occurs. To prevent shrinkage, acetic acid is added because of its swelling effect.

3.6.2 Experimental limitation in space experiments

It is necessary to prepare a suitable and adequate chemical fixative for immunohistochemical analysis specialized in space experiments. If the chemical fixative is stored for a long period after preparation, it degrades due to decomposition or chemical change, and its original function is not sufficiently exhibited. Also, a chemically fixed sample also needs to be removed from the chemical fixative within several hours to at most two days later and to proceed to the next processing. If the sample remains in the chemical fixation too long, it becomes “over-fixed”, and it is difficult to obtain an acceptable scientific result due to destruction of the cell and steric structural changes (due to excessive crosslinking and precipitation of proteins in the sample).

3.6.3 Chemical fixative selection

Several types of chemical fixatives are shown in Table 3 and are used in immunohistochemical analysis of plant samples. In experiments using these chemical fixatives, the chemical fixation period is limited to two days. In an experiment in space, the sample remains immersed in the chemical fixative until it is recovered on the ground. The Space X Cargo Dragon Capsule used for launching and retrieving experiment samples from space has a mooring period of about one month and, considering the time needed to transport the sample to Japan after collection, the sample is chemically fixed for as much as one month. A chemical fixative is required that can analyze the localization of the target protein in the plasma membrane even when immersed in the solution.

After the etiolated pea seedlings were chemically fixed for one month with eight kinds of chemical fixatives, the localization of PsPIN1 was analyzed by immunohistochemistry. Compared to the obtained sample, it is possible to analyze the localization in a sample chemically fixed with an organic solvent. In particular, appropriate and adequate results were obtained with the chemical fixative AE50, and further investigations were repeated using AE50 as the basic solution.

3.6.4 Improvement of chemical fixative composition

Fixing chemically plant cells that contain high in lipids and performing immunohistochemical analysis of the cytoskeleton of surface microtubules, a small amount of a surfactant or dimethyl sulfoxide (DMSO) is used to enhance penetration of formaldehyde-based chemical fixatives into plant tissues. Therefore, a new solution has been devised, nAE50 in which 0.1% Nonidet P-40 was added as a surfactant and ndAE50 in which 0.1% Nonidet P-40 and 5% DMSO were added to AE50

Table 3. Candidates of chemical fixatives for immunohistochemistry

Fixatives	Chemical composition
AE50	50% (v/v) ethanol, 5% (v/v) acetic acid
Carnoy	60% (v/v) ethanol, 30% (v/v) chloroform, 10% (v/v) acetic acid
Zinc Fixative	0.05% (w/v) calcium acetate, 0.5% (w/v) zinc acetate, 0.5% (w/v) zinc chloride, 1M Tris-HCl pH 7.4
Zinc Formalin	3.7% (v/v) formaldehyde, 1.5% (w/v) zinc sulfate
PFA+GA	4% (w/v) paraformaldehyde, 0.25% (v/v) glutaraldehyde, 50 mM sodium phosphate pH 7.2
PFA	4% (w/v) paraformaldehyde, 50 mM PIPES pH 7.2, 5 mM MgSO ₄ , 5 mM EGTA
FNB	10% (v/v) formalin neutral buffer
FAA	3.7% (v/v) formaldehyde, 50% (v/v) ethanol, 5% (v/v) acetic acid
nAE50	50% (v/v) ethanol, 5% (v/v) acetic acid, 0.1% (w/v) Nonidet P-40
ndAE50	50% (v/v) ethanol, 5% (v/v) acetic acid, 0.1% (w/v) Nonidet P-40, 5% (v/v) DMSO
AM50	50% (v/v) methanol, 5% (v/v) acetic acid
nAM50	50% (v/v) methanol, 5% (v/v) acetic acid, 0.1% (w/v) Nonidet P-40
ndAM50	50% (v/v) methanol, 5% (v/v) acetic acid, 0.1% (w/v) Nonidet P-40, 5% (v/v) DMSO

Denoted from Kamada et al. (2018b)

Table 4. Schedule of the Auxin Transport space experiment for chemically fixed samples (all dates are Japan time)

Run 1 Schedule for etiolated pea seedlings		
Date	Description	Remarks
January 30, 2017	Preparation of nAE50 chemical fixative	
February 19, 2017	Launch of SpaceX-10	
March 12, 2017	Start cultivation of etiolated pea seedlings	
March 15, 2017	Etiolated pea seedlings primary chemical fixation	
Chemical fixative storage period before use: 44 days		
March 19, 2017	SpaceX-10 landing	
March 27, 2017	Chemically fixed sample arrived at TKSC	
March 28, 2017	Completion of primary chemical fixation, secondary chemical fixation with newly prepared nAE50 chemical fixative	
Primary chemical fixation period: 13 days		
March 29, 2017	Completion of secondary chemical fixation, dehydration	
March 31, 2017	Paraffin replacement	
April 3, 2017	Paraffin embedding	
Run 4 Schedule for etiolated maize seedlings		
Date	Description	Remarks
January 30, 2017	nAM50 chemical fixative preparation	
February 19, 2017	Launch of SpaceX-10	
March 7, 2017	Start cultivation of etiolated maize seedlings	
March 11, 2017	Etiolated maize seedlings primary chemical fixation	
Storage period of chemical fixative before use: 40 days		
March 19, 2017	SpaceX-10 landing	
March 27, 2017	Chemically fixed sample arrived at TKSC	
March 28, 2017	Primary chemical fixation completed, secondary chemical fixation with Carnoy chemical fixative solution	
Primary chemical fixation period: 17 days		
March 29, 2017	Completion of secondary chemical fixation, dehydration	
March 31, 2017	Paraffin replacement	
April 3, 2017	Paraffin embedding	

TKSC: Tsukuba Space Center in JAXA

(Table 3).

After remaining in the fixative for one month, the localization of PsPIN1 was analyzed by immunohistochemistry in the preliminary ground experiment. There was no difference in the localization of the target protein in the cross section of the stem, but the signal localized in the root-side plasma membrane in the longitudinal section was better in nAE50 and ndAE50 than in AE50. Therefore, we decided to narrow down to two candidates, nAE50 and ndAE50.

3.6.5 Determination of chemical fixative

In space experiments, the storage of chemical fixative before use is very important. Before launch, a chemical fixative may have to stay in storage for one or two months after preparation. To determine a suitable chemical fixative for immunohistochemistry of etiolated pea seedlings, nAE50 and ndAE50 were stored in a refrigerator for one to two months, and it was found that

there was no difference in the localization signals of PsPIN1 between nAE50 and ndAE50 stored for one month after preparation. After storage for two months after preparation, it was found that an excellent localization signal of PsPIN1 protein was obtained from nAE50, much better than from ndAE50. Therefore, it was decided that nAE50 was a suitable and an adequate chemical fixative for etiolated pea seedlings in the space experiments and would be used.

On the other hand, nAM50 was optimal for the secondary fixation of etiolated maize seedlings in ground preliminary experiments, but it overfixed in the space experiment samples. It was decided that Carnoy would be used to detect ZmPIN1a proteins in immunohistochemistry of the space experiment samples (Kamada *et al.*, 2018b).

3.6.6 Space experiment and post-flight analysis

Table 4 shows the schedule for chemical fixation of samples in the Auxin Transport space experiment. The storage periods of the chemical fixatives used in the space experiment before chemical fixation were 44 and 40 days. For the secondary fixation of etiolated maize seedlings, nAM50 was optimal in ground preliminary experiments, but it overfixed in actual space experiment samples. Because of this, Carnoy was mostly used for the samples of the space experiment. Immediately after chemical fixation, the process was shifted to immunohistochemical analysis, and the localization of PsPIN1 and ZmPIN1a proteins in μ G conditions in space was determined.

3.7 Extraction of total RNA

Frozen etiolated pea seedlings in the Run 1, Run 2, and Run 3-1 experiments, frozen etiolated maize seedlings in the Run 4 experiment, and frozen samples of pea and maize seedlings in a 1 G ground control experiment were used for RNA extraction.

In etiolated pea seedlings, the hook region (0-3 mm from the tip of the seedling) and the proximal and distal sides of the epicotyl (the region 3-8 mm below the hook) were cut from the frozen seedling. For etiolated maize seedlings, the tip of the coleoptile (0 to 3 mm), the remaining part of the coleoptile, the mesocotyl, the primary leaf, the tip of the root (10 mm long, from the root cap), and the remaining part of the root were cut out from the frozen seedling. These segments were subjected to grinding centrifugation at 5,500 rpm for 20 seconds using a Micro Smash MS-100 (Tomy Seiko, Tokyo, Japan) four times. Grinding samples were cooled on ice for 3 minutes between each round.

Total RNA was extracted using TRI reagent (Sigma-Aldrich, MO, USA) according to the product protocol. During total RNA extraction and purification, DNase I (Takara Bio, Shiga, Japan) treatment was performed to remove residual DNA. The total RNA was subjected to real-time quantitative PCR (qPCR) analysis. The experiment was performed with $n=3$.

3.8 Real-time quantitative PCR

Run 1, Run 2 and Run 3-1 RNAs were reverse

transcribed with the ExScript RT reagent kit (Takara Bio) and amplified using an iCycler thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA). The total amount of complementary DNA was measured using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad). Real-time PCR with fluorescence monitoring was performed using the Light Cycler 96 real-time PCR system (Roche Diagnostics, Switzerland) with primer pairs for PCR amplification. Primers specific to each gene were designed and real-time PCR was performed. For *PsPIN1* (accession number: AY222857), forward: 5'-TGTTTGGTGGACATGAATTTGG-3' and reverse: 5'-TCTCTATGACCGTCCACTTTTCC-3'; *PsDEAD-box* (accession number: AY167670) forward: 5'-TTGTGAACACCGACGGAAGT-3' and reverse: 5'-GGTCCATGTCTCCGTGTGTTG-3'. *PsPIN1* in qPCR was normalized based on the expression of the *PsDEAD-box* gene encoding DNA helicase (Bai and DeMason, 2006).

On the other hand, for Run 4 *ZmPIN1a* (accession number: DQ836239), specific primers were forward: 5'-TGTTAGGGACTTTTCATGCGC-3' and reverse: 5'-AAGAGCCCTAGCATCATTCCATCG-3', and *ZmAUX1* (accession number: AJ011794) forward: 5'-GTTCTCGCCATCATCTTCC-3' and reverse: 5'-TGATATAGACGGTGAAGCTGACG-3'. As a housekeeping gene for *ZmPIN1a*, the *ZmUPL* gene (accession number: AM749995), which is a ubiquitin-related gene that is not affected by different gravitational environments as judged from data of microarray analysis of a plurality of samples, was used. The relative expression ratio of the *ZmPIN1a* gene and the *ZmAUX1* gene was normalized with respect to the expression of the *ZmUPL* gene. The specific primers of the *ZmUPL* gene were forward: 5'-TGGGAGTTTCATCTCGTCCAT-3' and reverse: 5'-TCGTCAGCAGGCCCTTAGAC-3'. For each PCR, the temperature was increased from 65°C to 95°C at 0.2°C/sec. The melting curves of the products confirmed that each transcript was a homogeneous product. The analysis was performed three times on three independently extracted RNA samples.

3.9 Extraction of membrane protein and Western blot analysis

The three etiolated pea seedlings from Run 1, frozen apex hook, proximal and distal sides of epicotyl, and the three etiolated maize seedlings from Run 4, frozen the coleoptile tip (3 mm), the remaining part of the coleoptile, the mesocotyl, the primary leaf, the tip of the root (10 mm) and the remaining part of the root were homogenized with 0.2 ml of extraction buffer (8 M urea, 2% (w/v) 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulphonate (CHAPS), 50 mM dithiothreitol (DTT) containing proteinase inhibitor cocktail tablets (Roche, Germany). After adding the 5% (v/v) SDS solution to the homogenate, the homogenate was shaken for 1 hour at 4°C to extract the membrane protein, and centrifuged at 13,000 g for 5 minutes at 4°C. The protein in the membrane protein fraction was determined by a 2-D Quantitation according to the product description (GE Healthcare, Tokyo, Japan). Quantification was performed

using the Kit.

Western blot analyses of *PsPIN1* and *ZmPIN1a* proteins were performed according to the methods previously reported (Kamada *et al.*, 2018b). The total protein (8 µg) was subjected to SDS-PAGE using Ready Gel 5-20% (Wako Pure Chemicals Co., Osaka, Japan). After SDS-PAGE, proteins were transferred to polyvinylidene fluoride membrane (Bio-Rad Laboratories, Inc., Tokyo, Japan). The blotted membrane was washed with a washing buffer (20 mM Tris-HCl buffer containing 137 mM NaCl, pH 7.6), incubated with 5% (w/v) skim milk, and then the blotted membrane was incubated with an anti-*PsPIN1* antibody. After incubation with the primary antibody, the hybridization membrane was washed three times with a washing buffer. The hybridization membrane was incubated for two hours with 1:10,000 diluted anti-rabbit Ig, HRP binding hole Ab (GE Healthcare). After washing and incubation with ECL Select Western Blotting Detection Reagent (GE Healthcare), the chemiluminescent signal was detected using the VersaDoc system (Bio-Rad). As a loading control, a primary antibody of anti-β-actin (Clone C4, Santa Cruz Biotechnology, CA, USA) and a secondary antibody, HRP-conjugated Whole Ab (GE Healthcare) were used. After Western blotting, the signal strength of the *PsPIN1* and *ZmPIN1a* proteins was determined using ImageJ software (Wayne Rasband, National Institute of Health, USA) and normalized with actin used as an internal standard. Statistical analysis used intensity data obtained on three independent blotting membranes.

3.10 Immunohistochemical analysis

3.10.1 Production of *PsPIN1* and *ZmPIN1a* antibodies

PsPIN1 polyclonal antibody was produced by the following method (Kamada *et al.*, 2018a). Amino acids 387-400 of the hydrophilic region of *PsPIN1* (i.e., VDGHRETQEDYLEK) were synthesized according to a solid phase peptide synthesis method (Eurofins Genomics, Tokyo, Japan). The obtained peptide was conjugated to keyhole limpet hemocyanin for use as an antigen, and rabbits were immunized for 77 days. According to the antibody production protocol of the manufacturer, affinity purification showing specificity for the *PsPIN1* oligopeptide was performed to obtain a polyclonal anti-*PsPIN1* antiserum.

The production of *ZmPIN1a* antibody was performed by the same method (Kamada *et al.*, 2018b). Amino acids 282 to 297 of the hydrophilic region of *ZmPIN1a* (i.e., GATRPPSNYEEDPQ GK, accession number DQ836239) were synthesized according to a solid phase peptide synthesis method (Eurofins Genomics). After binding the obtained peptide to keyhole limpet hemocyanin, rabbits were immunized with 2 mg of the antigen mixed with adjuvant for 77 days, the obtained polyclonal anti-*ZmPIN1a* antiserum was purified according to the manufacturer's antibody production protocol. The peptide was subjected to affinity purification to obtain a polyclonal anti-*ZmPIN1a* antiserum.

3.10.2 Immunohistochemistry of PsPIN1 and ZmPIN1a proteins

In the Run 1 experiment, etiolated pea seedlings grown under μG conditions in space and 1 G conditions in space on the ISS for three days were chemically fixed. The samples were kept in the refrigerator at 2°C after and returned to ground, then sent to the JAXA Tsukuba Space Center. The etiolated pea seedlings in CFB were stored in nAE50 fixative for 13 days. After removing them from CFB, the epicotyl (proximal and distal sides) and apical hook region were stored overnight at 4°C with freshly prepared nAE50 fixative. After dehydration with an ethanol series and tert-butyl alcohol, the samples were embedded in Paraplast Plus (Sigma-Aldrich), 10 μm -thick slices were prepared with a microtome (model RM2135; Leica Biosystems, Germany) and coated with silicon and placed on a glass slide. Immunohistochemical staining and observation using a fluorescence microscope were done according to the method previously reported (Kamada *et al.*, 2018a). After immunohistochemical staining of five independent etiolated pea seedlings in Run 1, PsPIN1 accumulation and subcellular localization were detected using ImageJ software.

The immunohistochemistry of the ZmPIN1a protein was also carried out with the previously reported method (Kamada *et al.*, 2018b), partially modified. Other experimental steps were the same as for etiolated pea seedlings.

3.11 Statistical analysis

Statistical analysis was performed using an R software program (R ver.2.14.2; <http://www.r-project.org>;

R Development Core Team, R Foundation for Statistical Computing, Vienna, Austria). A two-factor (2w) or three-factor (3w) analysis of variance (ANOVA) was applied to the statistical analysis to confirm the differences between a gravity environment, presence/absence of TIBA, and difference in tissue. The differences were examined by a post-hoc Tukey multiple comparisons test for comparison of the means. $P < 0.05$ was considered statistically significant.

Table 5. Effect of μG conditions on pea and maize seed germination

Pea seed germination, %			
Water		TIBA	
Artificial 1 G conditions	Microgravity conditions	Artificial 1 G conditions	Microgravity conditions
95.8 $\pm$ 4.2	89.8 $\pm$ 8.8	97.2 $\pm$ 1.6	94.4 $\pm$ 0.8
Maize seed germination, %			
Water			
1 g conditions on Earth		Microgravity conditions	
67.5 $\pm$ 9.4		69.5 $\pm$ 9.6	

A: germination ratio of pea seeds was determined from the picture of pea seedlings grown for 3 days under artificial 1 G conditions and μG conditions in the CBEF on the ISS in the presence or absence of 30 μM TIBA.

B: germination ratio of maize seeds was determined from the picture of maize seedlings grown for 4 days on 1 G conditions on the ground and μG conditions in the CBEF on the ISS.

Results were expressed as the average with the standard error of the mean ($n=3$).

Fig. 5. Etiolated pea seedlings grown for 3 days under μG and artificial 1 G conditions in the CBEF on the ISS in the presence or absence of 30 μM TIBA.

Photographs are of Run 2 experiment. A: artificial 1 G in space - Water; B: microgravity in space - Water; C: artificial 1 G in space - TIBA; D: μG in space - TIBA. Denoted from Miyamoto *et al.*, 2019.

Fig. 6. Kinetics of elongation (A) and growth direction (B) of epicotyls of etiolated pea seedlings grown under μG conditions and artificial 1 G conditions in space. Pea seeds were allowed to germinate and grow under μG and artificial 1 G conditions in the CBEF on the ISS for 96 h (Run 3-2). Seedlings were photographed from 48 h to 96 h after watering at 6-h intervals. Results were expressed as the average with the standard error of the mean ($n=3$ pea boxes). Denoted from Miyamoto *et al.*, 2019 with modifications.

Fig. 7. Etiolated maize seedlings grown for 4 days under μG conditions in the CBEF on the ISS, and those grown under 1 G conditions on Earth. Photographs are of Run 4 experiment. Denoted from Miyamoto *et al.*, 2019.

4. Growth and development of etiolated pea and maize seedlings grown under μG conditions in space

4.1 Growth and development of etiolated pea seedlings grown under μG conditions in space

We compared the growth and development of etiolated pea seedlings under μG and artificial 1 G conditions in the CBEF on the ISS. The interaction between the μG conditions in space and a polar auxin transport inhibitor, TIBA was also investigated (Miyamoto *et al.*, 2019).

Seed germination and elongation growth of the etiolated pea seedlings was somewhat affected by μG conditions in space but the direction of the stem was strongly affected (Table 5 and Fig. 5). The epicotyls of

etiolated pea seedlings grown under artificial 1 G conditions in space with water for three days grew in a direction of about 90 degree away from the cotyledons (Figs. 5 and 6). Their epicotyls also bent near the cotyledonary node, with the epicotyls growing 40~50 degree away from the cotyledons under μG conditions in space. As a result, the etiolated pea seedlings grown under μG conditions in space showed automorphogenesis. Conversely, artificial 1 G generated by the centrifuge in space was completely same as 1 G conditions on Earth.

The application of TIBA had little effect on pea seed germination under both artificial 1 G and μG conditions in the CBEF on the ISS as shown in Table 5 and Fig. 5. The application of TIBA substantially induced automorphogenesis-like bending of epicotyls in etiolated

pea seedlings grown under artificial 1 G conditions in space (Figs. 5). Under μ G conditions in space, the application of TIBA reduced the bending of epicotyls. In the etiolated pea seedlings grown under μ G conditions with water, roots in the pea boxes grew into aerial space.

Growth and development of etiolated pea seedlings in the acrylic resin Observation Chamber were recorded automatically by CCD camera in the CBEF in Run 3-2 experiment. Kinetic analysis of etiolated pea seedling growth indicated that until three days after watering, μ G conditions in space somewhat affected elongation growth, but thereafter substantially inhibited it (Fig. 6). Kinetic studies also revealed that epicotyls bent at their basal region or near the cotyledonary node toward the direction far from the cotyledons with about 40 degree in both seedlings grown under both gravity conditions on the ISS until two days after watering (Fig. 6). Thereafter, epicotyls grew while maintaining this orientation under μ G conditions in space. However, the direction of growth of those grown under artificial 1 G conditions changed to an antigravity direction by negative gravitropic response. Automorphological epicotyl bending was even observed under artificial 1 G conditions. Thus, the early growth just after germination is apparently not under gravistimulation.

4.2. Growth and development of etiolated maize seedlings grown under μ G conditions in space

Growth and development of etiolated maize seedlings grown under μ G conditions in space was also investigated (Miyamoto *et al.*, 2019). Microgravity conditions on the ISS had a negligible little effect on

Fig. 8. Effect of μ G conditions in space and in the presence of TIBA on polar auxin transport in epicotyl segments of etiolated pea seedlings grown for 3 days (Run 2). Pea seeds were allowed to germinate and grow under microgravity and artificial 1 G conditions in the CBEF on the ISS for 72 h in the presence or absence of TIBA (Run 2). Results were expressed as the average with the standard error of the mean ($n=15$ segments). Denoted from Miyamoto *et al.*, 2019 with modifications.

maize seed germination based on the photographs of seedlings taken four days after watering, while the growth status of seedlings varied quite a bit compared with those grown under Earth-normal conditions. Judging from the picture taken four days after watering, the coleoptiles of maize seedlings grew straight, but the mesocotyls curved at random in the “maize boxes” under μ G conditions (Fig. 7). In contrast, the coleoptiles

Fig. 9. Positive correlation between epicotyl growth and polar auxin transport in epicotyls of etiolated pea seedlings. Contribution of the proximal side was greater than that of the distal side. Denoted from Miyamoto *et al.*, 2019.

Fig. 10. Effect of μ G conditions in space on polar auxin transport in shoot segments of etiolated maize seedlings. Maize seeds were grown under μ G conditions in the CBEF on the ISS and under 1 G conditions on Earth, and then the shoot segments excised from the seedlings were subjected to the determination of polar auxin transport using radiolabeled IAA. After removing the coleoptile portion dipped into the lanolin containing radiolabeled IAA, the segment was divided into seven zones, with the node (i.e. junction between coleoptile and mesocotyl) being in zone 4. The radioactivity of the side opposite to the donor side of the segments (zone 1) was used as the amount of IAA transported. Results were expressed as the average with the standard error of the mean ($n=14$ segments).

and mesocotyls of maize seedlings grown under 1 G conditions on Earth were almost straight, growing upward or in a direction against the gravity vector. The lengths of the coleoptiles and mesocotyls grown in space were substantially less than those in the 1 G ground experiment (Fig. 7).

4.3. Polar auxin transport of etiolated pea epicotyls and maize shoots grown under μ G conditions in space

Polar auxin transport of etiolated pea epicotyls in the samples of the Auxin Transport space experiment was investigated as follows (Miyamoto *et al.*, 2019).

Given our finding that polar auxin transport activity in the proximal side is significantly higher than that in the distal side of etiolated pea epicotyls (Hoshino *et al.*, 2006), polar auxin transport was determined in the proximal and distal segments of the epicotyls to the cotyledons. The frozen pea epicotyl segments were carefully divided into longitudinal proximal and distal halves on dry ice without thawing (Fig. 4). The radioactivity of the side opposite to the donor side (basal side) of the segments was taken as the amount of IAA transported. The polar auxin transport of seedlings grown under artificial 1 G conditions was almost same as that of seedlings grown under 1 G conditions on Earth (Fig. 8). Microgravity conditions substantially reduced the activity of polar auxin transport in both the proximal and distal sides of epicotyls. The application of TIBA substantially reduced polar auxin transport under both artificial 1 G and μ G conditions, and thus TIBA synergistically acts with μ G conditions to reduce polar auxin transport. The reduced polar auxin transport seems

to be closely related to the morphology of etiolated pea seedlings under μ G conditions as already suggested (Ueda *et al.*, 2000; Ueda *et al.*, 2014a). A close relationship was clearly found between epicotyl bending and polar auxin transport in epicotyls (Fig. 9).

Polar auxin transport of etiolated maize shoots grown under μ G conditions in space and on 1 G conditions on Earth was also investigated. After discarding the coleoptile portion dipped into the lanolin containing radiolabeled IAA, the segment was divided into seven zones, in which the node and the side opposite to the donor side were defined as zone 4 and zone 1, respectively (Fig. 4). Ground control experiments on polar auxin transport in maize seedlings were conducted using coleoptile-mesocotyl segments including the node comparable to that used in the space experiments.

The total amount of IAA from zones 7 to 5 gradually decreased, but a transient accumulation of transported IAA was observed in zone 4 (Fig. 10). Polar auxin transport in the segments of maize shoots grown under μ G conditions in space was significantly higher than in those grown on 1 G conditions on Earth. This observation supports our previous finding in STS-95 space experiments in 1998 and in simulated weightlessness on a 3-D clinostat (Ueda *et al.*, 2000; Ueda *et al.*, 2014a).

4.4 Effect of μ G conditions in space on *PsPIN1* mRNA and *PsPIN1* protein accumulation

As shown in Fig. 11, statistical differences in the accumulation of *PsPIN1* mRNA in the subapical region of etiolated pea seedlings were found in gravity conditions (G) and tissue differences (T). A significant difference was also found in the interactive effect of G x the presence or

Fig. 11. Effect of μ G conditions in the ISS on the accumulation of *PsPIN1* mRNA in the subapical (A) and apical hook region of epicotyls (B) in etiolated pea seedlings grown in the presence or absence of TIBA. The accumulation of *PsPIN1* mRNA was analyzed using the real-time quantitative PCR technique. The relative amount of *PsPIN1* mRNA was normalized by expression of *PsDEAD-box* mRNA. Data are shown as mean $\pm$ SE (n=3). Asterisks denote the significance of 2 w or 3 w ANOVA: **P<0.01, *P<0.05, ns: not significant. Abbreviations indicate each factor; G: gravity conditions, S: presence or absence of TIBA, T: tissue differences. Different letters denote significant differences among independent samples (P<0.05, Tukey test). Denoted from Kamada *et al.*, 2019.

Fig. 12. Effect of μ G conditions in the ISS on the amount of PsPIN1 in the subapical (A) and apical hook region of epicotyls (B) in etiolated pea seedlings grown in the presence or absence of TIBA. Eight μ G of total proteins were loaded into each lane and separated through SDS-PAGE. After SDS-PAGE and blotting of the gel onto the PVDF membrane, the signal of PsPIN1 was detected with affinity-purified anti-PsPIN1 antibodies. The relative amount of PsPIN1 protein was normalized by actin. Data are shown as mean $\pm$ SE ($n=3$). Asterisks denote the significance of 2 w or 3 w ANOVA: ** $P<0.01$, * $P<0.05$, ns: not significant. Abbreviations indicate each factor; G: gravity conditions, S: presence or absence of TIBA, T: tissue differences. Different letters denote significant differences among independent samples ($P<0.05$, Tukey test). Denoted from Kamada *et al.*, 2019.

Fig. 13. Effect of μ G conditions in the ISS on subcellular localization of PsPIN1 in endodermal tissues in the subapical region of epicotyls in etiolated pea seedlings grown in the presence or absence of TIBA. Etiolated pea seedlings were grown under 1 G conditions on Earth (a, d, e, j, m, n), artificial 1 G conditions in the ISS (b, f, g, k, o, p), and μ G conditions in the ISS (c, h, i, l, q, r). Etiolated pea seedlings were grown in the absence (a-i) or presence of TIBA (j-r). Signals for PsPIN1 by Alexa Fluor 488 and the nucleus by propidium iodide are green and red in color, respectively. White boxes of enlarged views of localization of PsPIN1 in the proximal and distal sides of epicotyls are shown in the lower panels, respectively. Bars are 200 μ m in a, b, c, j, k, l, g: direction of gravitational force. Denoted from Kamada *et al.*, 2019.

absence of TIBA (S) (Fig. 11A). While a post-hoc Tukey test revealed that the accumulation of *PsPIN1* mRNA in the proximal side of epicotyls of etiolated pea seedlings grown under different gravity conditions was not different compared with that of the distal side (Fig. 11A). In performing 2 w ANOVA, the accumulation of *PsPIN1*

mRNA in the apical hook region of the seedlings was affected by μ G conditions in the ISS (Fig. 11B). Regardless of gravity conditions, TIBA at a concentration of 30 μ M did not affect the accumulation of *PsPIN1* mRNA in the proximal and distal sides of epicotyls (Fig. 11A). In the apical hook region of the seedlings

grown under μG conditions in space in the presence of TIBA, however, the accumulation of *PsPIN1* mRNA was higher than that grown under artificial 1 G conditions in the ISS (Fig. 11B).

As shown in Fig. 12, a significant difference in the accumulation of PsPIN1 protein in the proximal side of epicotyls of etiolated pea seedlings in gravity conditions was found. Significant differences were also found in the interactive effects of “G” x “T” and “S” x “T” (Fig. 12A). A post-hoc Tukey test revealed that the accumulation of

PsPIN1 in the proximal side of epicotyls of etiolated pea seedlings grown under different gravity conditions was not different compared with that of the distal side (Fig. 12A). In performing 2 w ANOVA, the accumulation of PsPIN1 in the apical hook region of etiolated pea seedlings was not affected by gravity conditions in the presence or absence of TIBA (Fig. 12B). In etiolated pea seedlings grown under μG conditions on the ISS, the accumulation of PsPIN1 in the proximal side of epicotyls in the presence of TIBA was significantly lower than that in the distal side (Kamada *et al.*, 2018a).

4.5 Effect of μG conditions in space on subcellular localization of *PsPIN1* protein

Kamada *et al.* (2018a) showed the localization of PsPIN1 proteins in the plasma membrane in endodermal cells in the epicotyls was classified into four types: the basal side, the basal-lateral (vascular) side, the lateral (vascular) side, and others. PsPIN1 mainly localized in the basal side (rootward) of the plasma membrane in endodermal cells in the subapical region of etiolated pea seedlings grown under artificial 1 G conditions in the ISS and on 1 G conditions on Earth. Approximately 80 to 90 of PsPIN1 localized in the basal side of the plasma membrane in endodermal cells on the proximal side of the epicotyls grown under 1 G conditions on Earth (Figs. 13 and 14). The frequency of cells in which PsPIN1 localized in the basal side of the plasma membrane in the proximal side of epicotyls did not differ from that in the distal side (Fig. 14). PsPIN1 localization in the basal side of the plasma membrane was significantly reduced by the μG conditions in the ISS in the presence or absence of TIBA (Fig. 14).

Fig. 14. Effect of μG conditions in space on distribution of PsPIN 1 proteins in the proximal and the distal sides of epicotyls in etiolated pea seedlings grown in the presence or absence of 30 μM TIBA. PsPIN 1 proteins were detected by immunohistochemistry as shown in Fig. 13.

Fig. 15. Effect of μG conditions in the ISS on subcellular localization of PsPIN1 in endodermal tissues in the apical hook region of epicotyls in etiolated pea seedlings grown in the presence or absence of TIBA. Etiolated pea seedlings were grown under 1 G conditions on Earth (a, d, e, j, m, n), artificial 1 G conditions in the ISS (b, f, g, k, o, p), and μG conditions in the ISS (c, h, i, l, q, r). Etiolated pea seedlings were grown in the absence (a-i) or presence of TIBA (j-r). Signals for PsPIN1 by Alexa Fluor 488 and the nucleus by propidium iodide are green and red in color, respectively. White boxes of enlarged views of localization of PsPIN1 in the proximal and distal sides are shown in the lower panels, respectively. Bars are 200 μm in a, b, c, j, k, l, g: direction of gravitational force. Denoted from Kamada *et al.*, 2019.

Fig. 16. Effect of μ G conditions in space on distribution of PsPIN1 proteins in the proximal and the distal sides of subhook regions in etiolated pea seedlings grown in the presence or absence of 30 μ M TIBA. PsPIN1 proteins were detected by immunohistochemistry as shown in Fig. 15.

The localization of PsPIN1 proteins in the plasma membrane in endodermal cells in the apical hook was classified into three types: the basal side, the basal-lateral (vascular or epidermis) side, and others (Fig. 15). In ca. 70% of the endodermal cells in the proximal side of the apical hook, PsPIN1 localized in the basal side of the plasma membrane, and in ca. 70% of cells in the distal side of the apical hook, PsPIN1 localized in the basal-lateral side of the plasma membrane under 1 G conditions on Earth and under artificial 1 G conditions in the ISS (Figs. 15 and 16). In the presence of TIBA, however, μ G conditions in space substantially affected the localization pattern of PsPIN1. The frequency of PsPIN1 localized in the basal side of the plasma membrane in epicotyls was particularly reduced in the proximal side of the epicotyls (Figs. 15 and 16). This somehow affects polar transport in the epicotyls, as the interaction of TIBA and μ G conditions in space substantially disturbed polar auxin transport in etiolated pea seedlings.

Significant differences in the frequency of cells in which PsPIN1 localized in the basal side and basal-lateral side of the plasma membrane were found between the proximal and distal sides of the epicotyls (Fig. 16).

4.6 Effect of μ G conditions in space on *ZmPIN1a* mRNA and *ZmPIN1a* protein accumulation

Real-time PCR analyses revealed that the accumulation of *ZmPIN1a* mRNA in the tip and the subapical region of coleoptiles, and in the mesocotyls were almost the same as in etiolated maize seedlings grown on 1 G conditions on Earth.

In maize four *ZmPIN1* subfamily genes (*ZmPIN1a*, *b*, *c* and *d*) were identified in maize, and *ZmPIN1d* was specifically expressed in reproductive tissues (Forestan *et al.*, 2012). The accumulation of *ZmPIN1c* mRNA in the coleoptiles and the mesocotyls was not affected by μ G conditions in space (data not shown). The primer set for

Fig. 17. Distribution frequency of ZmPIN1 proteins in the plasma membrane of parenchymatous cells in the coleoptiles was shown as angles between epidermis and the line passing through the center of the arc of ZmPIN1 fluorescent signals detected by immunohistochemical staining. The x axis (0 degree) was parallel to the outer epidermis. The value is small when the direction of the signal of ZmPIN1 proteins is away from the vascular bundle. When the value is large, the direction is approaching the vascular bundle. Denoted from Oka *et al.*, 2020 with modifications.

Fig. 18. Effect of μ G conditions in space on the distribution frequency of subcellular localization of ZmPIN1 proteins in plasma membrane of parenchymatous cells of the coleoptiles in etiolated maize seedlings.

amplifying *ZmPIN1b* mRNA used in this experiment yielded three products, so it was somewhat difficult to amplify *ZmPIN1b* mRNA individually by RT-PCR, probably due to their high sequence similarity to *ZmPIN1a* and *ZmPIN1c*.

On the other hand, the accumulations of *ZmAUX1* mRNA, which encodes influx carrier proteins of auxin, in the tip and the subapical region of coleoptiles and in the mesocotyls, were almost the same as in etiolated maize seedlings grown under 1 G conditions on Earth. The

accumulation of *ZmAUX1* mRNA was not affected by μG conditions in space, regardless of the tissue or organ differences.

Western blotting analysis for the accumulation of ZmPIN1a proteins revealed that the accumulation level in the coleoptiles was almost identical to that in the mesocotyls of the etiolated maize seedlings grown under 1 G conditions. The accumulation of ZmPIN1a proteins in these organs of the seedlings grown in space tended to be relatively higher than that of the seedlings grown under 1 G conditions on Earth. The results described above have been reported in Oka *et al.*, 2020.

4.7 Effect of μG conditions in space on subcellular localization of ZmPIN1 proteins in the coleoptiles and the mesocotyls

Observing subcellular localization of ZmPIN1a proteins in the longitudinal sections revealed that ZmPIN1a proteins localized in the lateral side of the plasma membranes in parenchymatous cells of the etiolated coleoptiles regardless of gravity conditions, 1 G or μG conditions.

ZmPIN1 proteins in plasma membrane of parenchymatous cells in the coleoptiles was determined as angles between epidermis and the direction of ZmPIN1 fluorescent signals detected by immunohistochemical staining. The x axis (0 degree) was parallel to the outer epidermis. The value is small when the line passing through the center of the arc of the signal of ZmPIN1a protein is away from the vascular bundle. When the value is large, the direction is toward the vascular bundle (Fig. 17). Observing the transverse sections in parenchymatous cells of the coleoptiles of the etiolated seedlings grown under 1 G conditions

revealed that the majority of lateral localization of ZmPIN1a proteins was directed to the radial side, but not toward the vascular bundle side. However, in contrast, in the seedlings grown under μG conditions showed lateral localization of ZmPIN1a proteins, mostly toward the vascular bundles side (Fig. 18). This fact indicates that μG conditions in space substantially affect the subcellular localization of ZmPIN1a proteins in parenchymatous cells of the coleoptiles in etiolated maize seedlings and that it is possible to facilitate auxin loading into vascular tissues.

On the other hand, in the mesocotyls of etiolated maize seedlings grown under 1 G conditions, ZmPIN1a proteins were localized in the basal (rootward) side of the plasma membrane in endodermal cells along vascular tissues and in the outer epidermal cells. The localization pattern in the mesocotyls of etiolated maize seedlings grown under μG conditions was not different from that grown under 1 G conditions on Earth. These results have also been described in Oka *et al.*, 2020.

5. Microarray profile of gene expression in etiolated pea seedlings grown under μG conditions in space

Section 4 describes the reduction of polar auxin transport in μG conditions in space has been thought to be regulated by altered subcellular localization of PsPIN1 proteins in the plasma membrane, rather than by a reduced accumulation of *PsPIN1* mRNA or by a reduced total amount of PsPIN1 proteins (Kamada *et al.*, 2019; Miyamoto *et al.*, 2019).

It is worthwhile to survey novel genes that are responsive to altered gravity conditions. By controlling

Fig. 19. Volcano plot illustrating Fold Change (log base 2) compared with p-value ($-\log_{10}$) between μG and artificial 1 G conditions. Upregulated and Downregulated expression at least twofold with significant differences ($P < 0.05$) under μG conditions were indicated by red and blue plots, respectively. A: proximal side of epicotyls grown with water, B: distal side of epicotyls grown with water, C: proximal side of epicotyls grown with TIBA, D: distal side of epicotyls grown with TIBA. Denoted from Kamada *et al.*, 2020.

Fig. 20. Functional classification of genes upregulated and downregulated under μ G conditions in space regardless of the presence of TIBA and tissue differences. Denoted from Kamada *et al.*, 2020.

the expression of such genes, it is possible to clarify the regulatory mechanisms of plant growth and development in μ G conditions in space. It should be mentioned that comprehensive analyses using microarray methods to analyze gravity-dependent or μ G-dependent alterations of gene expression have been studied in a number of biological species (Kimbrough *et al.*, 2004; Lebsack *et al.*, 2010; Fengler *et al.*, 2015; Wakabayashi *et al.*, 2015; Higashibata *et al.*, 2016; Wakabayashi *et al.*, 2017).

The pea genome (about 4300 Mb) is five to ten times as large as that of the *Medicago* (*Medicago truncatula*), a closely-related species in the legume family (Kalo *et al.*, 2004), however, it is believed that more than half of the genome consists of repeated elements (Macas *et al.*, 2007). Several comprehensive analyses of gene expression in pea plants have already been made using the *Medicago* microarray platform (Fondevilla *et al.*, 2011; Hosseini *et al.*, 2015), but no pea microarray platform has been produced yet. To characterize global changes in mRNA abundance in etiolated Alaska pea (*Pisum sativum* L.) seedlings regulated by μ G conditions in space, we introduced microarray data of *Medicago* to etiolated pea seedlings grown under μ G and artificial 1 G conditions in the presence or absence of TIBA on the ISS.

Of the 44,000 genes of the *Medicago* microarray platform, more than 25,000 transcripts of pea seedlings were hybridized, suggesting that the microarray platform for *Medicago* could be useful in the study of gene expression of etiolated pea seedlings grown under μ G conditions in space. Gene expression data were analyzed according to stringent criteria that restricted the scored genes for specific hybridization values at least twofold. Expression of 1362 and 1558 genes in proximal side (the proximal side) and distal side of the epicotyl to the cotyledons (the distal side), respectively, were highly affected by μ G conditions in space. Of the genes analyzed, 407 of 1362 transcripts in the proximal side and 740 of 1558 transcripts in the distal side were expressed at ratios at least twofold. However, in the presence of the auxin transport inhibitor TIBA, 212 of 399 transcripts and 255 of 477 transcripts were expressed at

ratios at least twofold as high in the proximal and the distal sides of epicotyls in the seedlings grown under μ G conditions, respectively. Based on Venn diagram analysis, 31 transcripts and 24 transcripts were found to commonly increase and decrease, respectively, under μ G conditions in space. Volcano plot illustrating Fold Change (log base 2) compared with p-value (-log base 10) between μ G and artificial 1 G conditions was shown in Fig. 19. Functional classification of genes upregulated and downregulated under μ G conditions in space regardless of the presence of TIBA and tissue differences was also shown in Fig. 20. It should be mentioned that microarray profile of gene expression in etiolated pea seedlings grown under μ G conditions in space revealed six auxin-related genes and three water channel *AQUAPORIN* genes that were responsive to gravity. Among 6 auxin-related genes, the accumulation of transcripts of Auxin-induced protein 5NG4 and Indole-3-acetic acid-amido synthetase GH3.3 tended to increase, and that of Auxin-induced protein, Auxin response factor, SAUR-like auxin-responsive family protein and Auxin response factor tended to decrease under μ G conditions, whereas there were no statistic differences between under μ G and artificial 1 G conditions. Similarly there were no statistic differences between under μ G conditions and artificial 1 G, but the accumulation of NIP3-1 and Plasma membrane intrinsic protein11, and *AQUAPORIN1*/Tonoplast intrinsic protein tended to increase and decrease, respectively. Polar auxin transport-related gene products such as PIN proteins as well as auxin-related gene and water channel gene products have a possible significant role in regulating the growth and development of the etiolated pea seedlings grown under μ G conditions in space (Kamada *et al.*, 2020).

6. Comprehensive analyses of plant hormones in the etiolated pea and maize grown under μ G conditions in space

Other than Sections 4 and 5, comprehensive analyses of endogenous levels of plant hormones have already been performed in the etiolated pea and maize seedlings grown under μ G conditions in space as well as artificial 1 G conditions in space and 1 G conditions on Earth in the Auxin Transport space experiment. Auxins (IAA and IAA aspartate), gibberellins (GA₁ and GA₄), jasmonates (JA and JA isoleucine), abscisic acid and salicylic acid were successfully identified and quantified in the etiolated Alaska pea and Golden Cross Bantam maize seedlings grown under μ G conditions in space as well as those on artificial 1 G conditions in space and 1 G conditions on Earth. The total amounts of identified free cytokinins (*trans*-zeatin, *cis*-zeatin, dihydrozeatin, and isopentenyladenine) and their conjugates (riboside, glucoside and/or glucosyl ester) were also estimated. Possible cross-talk among these plant hormones on plant growth and development grown under different gravity conditions is now under progress. All results of the experiment and intensive discussion of the close

relationships between endogenous levels of plant hormones and the growth and development of plants grown under μG conditions in space will be reported elsewhere in the near future.

Finally the hypothesis that polar auxin transport affected by gravity is closely related to the posture control of plants is considered to be verified at a molecular level by the present ISS space experiment. In order to clarify the effects of gravity on plant hormone dynamics, we are conducting the ISS space experiment including comprehensive expression analyses of genes related to polar auxin transport and determination of subcellular localization of their products using immunohistochemistry as well as comprehensive analyses of endogenous plant hormones. The comprehensive report on the Auxin Transport space experiment substantially contributes to establish novel findings in gravitational plant physiology. In addition, it makes possible not only to control artificially plant growth and development in space, but also to establish cultivation and techniques for sufficient food production in long-term exploration of space.

Space radiation and μG conditions in space have a great influence on surviving organisms in space. Some important reviews have independently clarified characteristics and biological effects of space radiation and μG conditions in space. In the future and advance space experiments, it is desirable to establish a novel methodology for studying how the interaction between μG conditions and space radiation affects the plant growth and development.

Declaration of Interests

The author declares no competing interests.

References

- Bai, F. and DeMason, D.A. (2006) Hormone interactions and regulation of Unifoliata, PsPK2, PsPIN1 and LE gene expression in pea (*Pisum sativum*) shoot tips. *Plant Cell Physiol.*, **47**, 935-948. <https://doi.org/10.1093/pcp/pcj066>.
- Carraro, N., Forestan, C., Canova, S., Traas, J. and Varotto, S. (2006) *ZmPIN1a* and *ZmPIN1b* encode two novel putative candidates for polar auxin transport and plant architecture determination of maize. *Plant Physiol.*, **142**, 254-264. <https://doi.org/10.1104/pp.106.080119>.
- Chawla, R. and DeMason, D.A. (2004) Molecular expression of *PsPIN1*, a putative auxin efflux carrier gene from pea (*Pisum sativum* L.). *Plant Growth Regul.*, **44**, 1-14. <https://doi.org/10.1007/s10725-004-2139-9>.
- Correll, M.J. and Kiss, J. (2008) Space-based research on plant tropism. In: Gilroy, S., Masson, P. (Eds) *Plant Tropism*, Blackwell, Oxford, UK. pp. 161-182. <https://doi.org/10.1002/9780470388297.ch8>.
- Driss-Ecole, D., Legué, V., Carnero-Díaz, E. and Perbal, G. (2008) Gravisensitivity and automorphogenesis of lentil seedling roots grown on board the International Space Station. *Physiol. Plant.*, **134**, 191-201. <https://doi.org/10.1111/j.1399-3054.2008.01121.x>.
- Fengler, S., Spierer, I., Neef, M., Ecke, M., Nieselt, K. and Hampp, R. (2015) A Whole-genome microarray study of *Arabidopsis thaliana* semisolid callus cultures exposed to microgravity and nonmicrogravity related spaceflight conditions for 5 days on board of Shenzhou 8. *BioMed. Research International* 2015, e547495. <http://dx.doi.org/10.1155/2015/547495>.
- Ferl, R.J. and Paul, A.-L. (2016) The effect of spaceflight on the gravity-sensing auxin gradient of roots: GFP reported gene microscopy on orbit. *npj Microgravity*, **2**, 15023. <https://doi.org/10.1038/npjmgrav.2015.23>.
- Fondevilla, S., Küster, H., Krajinski, F., Cubero, J.I. and Rubiales, D. (2011) Identification of genes differentially expressed in a resistant reaction to *Mycosphaerella pinodes* in pea using microarray technology. *BMC Genomics*, **12**, e28. <https://doi.org/10.1186/1471-2164-12-28>.
- Forestan, C., Farinati, S. and Varotto, S. (2012) The maize *PIN* gene family of auxin transporters. *Front. Plant Sci.*, **3**, 16. <https://doi.org/10.3389/fpls.2012.00016>.
- Forestan, C., Meda, S. and Varotto, S. (2010) ZmPIN1-mediated auxin transport is related to cellular differentiation during maize embryogenesis and endosperm development. *Plant Physiol.*, **152**, 1373-1390. <https://doi.org/10.1104/pp.109.150193>.
- Forestan, C. and Varotto, S. (2010) PIN1 auxin efflux carriers localization studies in *Zea mays*. *Plant Signal Behav.*, **5**, 436-439. <https://doi.org/10.4161/psb.5.4.11339>.
- Gallavotti, A., Yang, Y., Schmidt, R.J. and Jackson, D. (2008) The Relationship between auxin transport and maize branching. *Plant Physiol.*, **147**, 1913-1923. <https://doi.org/10.1104/pp.108.121541>.
- Gälweiler, L., Guan, C., Müller, A., Wisman, E., Mendgen, K., Yephremov, A. and Palme, K. (1998) Regulation of polar auxin transport by AtPIN1 in *Arabidopsis* vascular tissue. *Science*, **282**, 2226-2230. <https://doi.org/10.1126/science.282.5397.2226>.
- Geldner, N., Anders, N., Wolters, H., Keicher, J., Kornberger, W., Müller, P., Delbarre, A., Ueda, T., Nakano, A. and Jürgens, G. (2003) The *Arabidopsis* GNOM ARF-GEF mediates endosomal recycling, auxin transport, and auxin-dependent plant growth. *Cell*, **112**, 219-230. [https://doi.org/10.1016/S0092-8674\(03\)00003-5](https://doi.org/10.1016/S0092-8674(03)00003-5).
- Geldner, N., Friml, J., Stierhof, Y.D., Jürgens, G. and Palme, K. (2001) Auxin transport inhibitors block PIN1 cycling and vesicle trafficking. *Nature*, **413**, 425-428. <https://doi.org/10.1038/35096571>.
- Halstead, T.W. and Dutcher, F.R. (1987) Plants in space. *Annu. Rev. Plant. Physiol.*, **38**, 317-345. <https://doi.org/10.1146/annurev.pp.38.060187.001533>.
- Higashibata, A., Hashizume, T., Nemoto, K., Higashitani, N., Etheridge, T., Mori, C., Harada, S., Sugimoto, T., Szewczyk, N.J., Baba, S.A., Mogami, Y., Fukui, K. and

- Higashitani, A. (2016) Microgravity elicits reproducible alterations in cytoskeletal and metabolic gene and protein expression in space-flown *Caenorhabditis elegans*. npj Microgravity, **2**, e15022. <https://doi.org/10.1038/npjmgrav.2015.22>.
- Hille, S., Akhmanova, M., Glanc, M., Johnson, A. and Friml, J. (2018) Relative contribution of PIN-containing secretory vesicles and plasma membrane PINs to the directed auxin transport: Theoretical estimation. Int. J. Mol. Sci., **19**, e3566. <https://doi.org/10.3390/ijms19113566>.
- Hoshino, T., Hitotsubashi, R., Miyamoto, K., Tanimoto, E. and Ueda, J. (2005) Isolation of *PsPIN2* and *PsAUX1* from etiolated pea epicotyls and their expression on a three-dimensional clinostat. Adv. Space Res., **36**, 1284-1294. <https://doi.org/10.1016/j.asr.2005.03.121>.
- Hoshino, T., Miyamoto, K. and Ueda, J. (2006) Requirement for the gravity-controlled transport of auxin for a negative gravitropic response of epicotyls in the early growth stage of etiolated pea seedlings. Plant Cell Physiol., **47**, 1496-1508. <https://doi.org/10.1093/pcp/pcl015>.
- Hoshino, T., Miyamoto, K. and Ueda, J. (2007) Gravity-controlled asymmetrical transport of auxin regulates a gravitropic response in the early growth stage of etiolated pea (*Pisum sativum*) epicotyls: studies using simulated microgravity conditions on a three-dimensional clinostat and using an agravitropic mutant, *ageotropum*. J. Plant Res., **120**, 619-628. <https://doi.org/10.1007/s10265-007-0103-2>.
- Hoson, T., Kamisaka, S., Masuda, Y. and Yamashita, M. (1992) Changes in plant growth processes under microgravity conditions simulated by a three-dimensional clinostat. Bot. Mag. Tokyo, **105**, 53-70. <https://doi.org/10.1007/BF02489403>.
- Hoson, T., Kamisaka, S., Masuda, Y., Yamashita, M. and Buchen, B. (1997) Evaluation of the three-dimensional clinostat as a simulator of weightlessness. Planta, **203**, S187-S197. <https://doi.org/10.1007/PL00008108>.
- Hoson, T., Kamisaka, S., Yamamoto, R., Yamashita, M. and Masuda, Y. (1995) Automorphosis of maize shoots under simulated microgravity don a three-dimensional clinostat. Physiol. Plant., **93**, 346-351. <https://doi.org/10.1111/j.1399-3054.1995.tb02238.x>.
- Hoson, T., Matsumoto, S., Soga, K., Wakabayashi, K., Hashimoto, T., Sonobe, S., Muranaka, T., Kamisaka, S., Kamada, M., Omori, K., Ishioka, N. and Shimazu, T. (2009) Growth and cell wall properties in hypocotyls of *Arabidopsis tua6* mutant under microgravity conditions in space. Biol. Sci. Space, **23**, 71-76. <https://doi.org/10.2187/bss.23.71>.
- Hoson, T., Soga, K., Wakabayashi, K., Hashimoto, T., Karahara, I., Yano, S., Tanigaki, F., Shimazu, T., Kasahara, H., Masuda, D. and Kamisaka, S. (2014) Growth stimulation in inflorescences of an *Arabidopsis* tubulin mutant under microgravity conditions in space. Plant Biol., **16**(S1), 91-96. <http://dx.doi.org/10.1111/plb.12099>.
- Hosseini, S., Elfstrand, M., Heyman, F., Jensen, D. F. and Karlsson, M. (2015) Deciphering common and specific transcriptional immune responses in pea towards the oomycete pathogens *Aphanomyces euteiches* and *Phytophthora pisi*. BMC Genomics, **16**, e627. <https://doi.org/10.1186/s12864-015-1829-1>.
- Johnson, C.M., Subramanian, A., Pattathil, S., Correll, M.J. and Kiss, J.Z. (2017) Comparative transcriptomics indicate changes in cell wall organization and stress response in seedlings during spaceflight. Amer. J. Bot., **104**, 1219-1231. <https://doi.org/10.3732/ajb.1700079>.
- Kalo, P., Seres, A. Taylor, S.A., Jakab, J., Kevei, Z., Kereszt, A., Endre, G., Ellis, T.H.N. and Kiss, G.B. (2004) Comparative mapping between Medicago sativa and Pisum sativum. Mol. Genet. Genomics, **272**, 235-246. <https://doi.org/10.1126/10.1007/s00438-004-1055-z>.
- Kamada, M., Fujii, N., Aizawa, S., Kamigaichi, S., Mukai, C., Shimazu, T. and Takahashi, H. (2000) Control of gravimorphogenesis by auxin: accumulation pattern of CS-IAA1 mRNA in cucumber seedlings grown in space and on the ground. Planta, **211**, 493-501. <https://doi.org/10.1007/s004250000321>.
- Kamada, M., Miyamoto, K., Oka, M., Ueda, J. and Higashibata, A. (2018a) Regulation of an asymmetric polar auxin transport by PsPIN1 in endodermal tissues of etiolated *Pisum sativum* epicotyls: Focus on immunohistochemical analyses. J. Plant Res., **131**, 681-692. <https://doi.org/10.1007/s10265-018-1031-z>.
- Kamada, M., Miyamoto, K., Oka, M., Uheda, E., Ueda, J. and Higashibata, A. (2018b) Procedures for chemical fixation in immunohistochemical analyses of PIN proteins regulating polar auxin transport: Relevance to spaceflight experiments. Life Sci. in Space Res., **18**, 42-51. <https://doi.org/10.1016/j.lssr.2018.05.005>.
- Kamada, M., Oka, M., Inoue R., Fujitaka, Y., Miyamoto, K., Uheda, E., Yamazaki, C., Shimazu, T., Kasahara, H., Sano, H., Suzuki T., Higashibata A. and Ueda, J. (2019) Gravity-regulated localization of PsPIN1 is important for polar auxin transport in etiolated pea seedlings: Relevance to the International Space Station experiment. Life Sci. Space Res., **22**, 29-37. <https://doi.org/10.1016/j.lssr.2019.07.001>.
- Kamada, M., Oka, M., Miyamoto, K., Uheda, E., Yamazaki, C., Shimazu, T., Kasahara, H., Sano, H., Kasahara, H., Suzuki T., Higashibata A. and Ueda, J. (2020) Microarray profile of gene expression in etiolated *Pisum sativum* seedlings grown under microgravity conditions in space: Relevance to the International Space Station experiment "Auxin Transport" Life Sci. Space Res., (in press).
- Kerr, I.D. and Bennett, M.J. (2007) New insight into the biochemical mechanisms regulating auxin transport in plants. Biochem. J., **401**, 613-622. <https://doi.org/10.1042/BJ20061411>.
- Kimbrough, J.M., Salinas-Mondragon, R., Boss, W.F., Brown, C.S. and Sederoff, H.K. (2004) The Fast and transient transcriptional network of gravity and mechanical stimulation in the Arabidopsis root

- apex. *Plant Physiol.*, **136**, 2790-2805. <https://doi.org/10.1104/pp.104.044594>.
- Kiss, J.Z. (2014) Plant biology in reduced gravity on the Moon and Mars. *Plant Biol.*, **16** (S1), 12-14. <https://doi.org/10.1111/plb.12031>.
- Kojo, K.H., Yasuhara, H. and Hasezawa, S. (2014) Time-sequential observation of spindle and phragmoplast orientation in BY-2 cells with altered cortical actin microfilament patterning. *Plant Signal Behav.*, **9**, e29579. <https://doi.org/10.4161/psb.29579>.
- Křeček, P., Skůpa, P., Libus, J., Naramoto, S., Tejos, R., Friml, J. and Zažímalová, E. (2009) The PIN-FORMED (PIN) protein family of auxin transporters. *Genome Biol.*, **10**, 249. <https://doi.org/10.1186/gb-2009-10-12-249>.
- Lebsack, T.W., Fa, V., Woods, C.C., Gruener, R., Manziello, A.M., Pecaut, M.J., Gridley, D.S., Stodieck, L.S., Ferguson, V.L., Deluca, D. (2010) Microarray analysis of spaceflown murine thymus tissue reveals changes in gene expression regulating stress and glucocorticoid receptors. *J. Cell Biochem.*, **110**, 372-381. <https://doi.org/10.1002/jcb.22547>.
- Luschnig, C. and Vert, G. (2014) The dynamics of plant plasma membrane proteins: PINs and beyond. *Development*, **141**, 2924-2938. <https://doi.org/10.1242/dev.103424>.
- Macas, J., Neumann, P. and Navratilova, A. (2007) Repetitive DNA in the pea (*Pisum sativum* L.) genome: comprehensive characterization using 454 sequencing and comparison to soybean and *Medicago truncatula*. *BMC Genomics*, **8**, e427. <https://doi.org/10.1186/1471-2164-8-427>.
- Marchant, A., Kargul, J., May, S.T., Muller, P., Delbarre, A., Perrot-Rechenmann, C. and Bennett, M.J. (1999) AUX1 regulates root gravitropism in *Arabidopsis* by facilitating auxin uptake within root apical tissues. *EMBO J.*, **18**, 2066-2073. <https://doi.org/10.1093/emboj/18.8.2066>.
- Miyamoto, K., Inui, A., Uheda, E., Oka, M., Kamada, M., Yamazaki, C., Shimazu, T., Kasahara, H., Sano, H., Suzuki T., Higashibata A. and Ueda, J. (2019) Polar auxin transport is essential to maintain growth and development of etiolated pea and maize seedlings grown under 1 g conditions: Relevance to the international space station experiment. *Life Sci. Space Res.*, **20**, 1-11. <https://doi.org/10.1016/j.lssr.2018.11.001>.
- Miyamoto, K., Hoshino, T., Yamashita, M. and Ueda, J. (2005) Automorphosis of etiolated pea seedlings in space is simulated by a three-dimensional clinostat and the application of inhibitors of auxin polar transport. *Physiol. Plant.*, **123**, 467-474. <https://doi.org/10.1111/j.1399-3054.2005.00472.x>.
- Oka M, Kamada M, Inoue R, Miyamoto K, Uheda E, Yamazaki C, Shimazu T, Sano H, Kasahara H, Suzuki T, Higashibata A, Ueda J. (2020) Altered localization of ZmPIN1a proteins plasma membranes responsible for enhanced-polar auxin transport in etiolated maize seedlings under microgravity conditions in space. *Func Plant Biol.* (*in press*)
- Oka, M., Miyamoto, K., Okada, K. and Ueda, J. (1999) Auxin polar transport and flower formation in *Arabidopsis thaliana* transformed with indoleacetamide hydrolase (*iaaH*) gene. *Plant Cell Physiol.*, **40**, 231-237. <https://doi.org/10.1093/oxfordjournals.pcp.a029532>.
- Okada, K., Ueda, J., Komaki, M.K., Bell, C.J. and Shimura, Y., 1991. Requirement of auxin polar transport system in early stage of *Arabidopsis* floral bud formation. *Plant Cell*, **3**, 677-684. <https://doi.org/10.1105/tpc.3.7.677>.
- Paul, A.-L., Wheeler, R.M., Levine, H.G. and Ferl, R.J. (2013) Fundamental plant biology enabled by the Space Shuttle. *Amer. J. Bot.*, **100**, 226-234. <https://doi.org/10.3732/ajb.1200338>.
- Soga, K., Yamasaki, C., Kamada, M., Tanigawa, N., Kasahara, H., Yano, S., Kojo, K.H., Kutsuna, N., Kato, T., Hashimoto, T., Kotake, T., Wakabayashi, K. and Hoson, T. (2018) Modification of growth anisotropy and cortical microtubule dynamics in *Arabidopsis* hypocotyls grown under microgravity conditions in space. *Physiol. Plant.*, **162**, 135-144. <https://doi.org/10.1111/ppl.12640>.
- Stanković, B., Volkmann, D. and Sack, F.D. (1998) Autotropism, automorphogenesis, and gravity. *Physiol. Plant.*, **102**, 328-335. <https://doi.org/10.1034/j.1399-3054.1998.1020222.x>.
- Takahashi, H. (1994) Hydrotropism and its interaction with gravitropism in roots. *Plant Soil*, **165**, 301-308. <https://doi.org/10.1007/BF00008073>.
- Teiz, L. and Zeiger, M. (2002) *Plant Physiology*, third ed. Sinauer Associates, Inc., Publishers, Sunderland, MA, USA.
- Ueda, J. (2016) https://www.nasa.gov/mission_pages/station/research/experiments/1991.html (accessed 27 November 2018, Principal Investigator: Prof. Junichi Ueda).
- Ueda, J., Miyamoto, K., Uheda, E., Oka, M., Yano, S., Higashibata, A. and Ishioka, N. (2014a) Close relationships between polar auxin transport and graviresponse in plants. *Plant Biol.*, **16**(S1), 43-49. <https://doi.org/10.1111/plb.12101>.
- Ueda, J., Miyamoto, K., Yuda, T., Hoshino, T., Fujii, S., Mukai, C., Kamigaichi, S., Aizawa, S., Yoshizaki, I., Shimazu, T. and Fukui, K. (1999) Growth and development, and auxin polar transport in higher plants under microgravity conditions in space: BRIC-AUX on STS-95 space experiment. *J. Plant Res.*, **112**, 487-492. <https://doi.org/10.1007/PL00013904>.
- Ueda, J., Miyamoto, K., Yuda, T., Hoshino, T., Sato, K., Fujii, S., Kamigaichi, S., Izumi, R., Ishioka, N., Aizawa, S., Yoshizaki, I., Shimazu, T., Fukui, K. (2000) STS-95 space experiment for growth and development, and auxin polar transport. *Biol. Sci. Space* **14**, 47-57. <https://doi.org/10.2187/bss.14.47>.
- Ueda, J., Sakamoto-Kanetake, M., Toda, Y., Miyamoto, K., Uheda, E. and Daimon, H. (2014b) Auxin polar

transport is essential for the early growth stage of etiolated maize (*Zea mays* L. cv. Honey Bantam) seedlings. *Plant Prod. Sci.*, **17**, 144-151. <https://doi.org/10.1626/pps.17.144>.

Ueda, J., Tada, T., Hoshino, T., Miyamoto, K., Uheda, E. and Oka, M. (2012) Isolation of *PsPINs* and *PsAUX1* cDNAs encoding putative auxin efflux and influx carriers and/or facilitators, respectively from etiolated epicotyls of an agravitropic pea (*Pisum sativum* L.) mutant, *ageotropum*. *Biol. Sci. Space*, **26**, 32-41. <https://doi.org/10.2187/bss.26.32>.

Volkman, D., Behrens, H.M. and Sievers, A. (1986) Development and gravity sensing of cress roots under microgravity. *Naturwissenschaften*, **73**, 438-441. <https://doi.org/10.1007/BF00367291>.

Wakabayashi, K., Soga, K., Hoson, T., Kotake, T., Kojima, M., Sakakibara, H., Yamazaki, C., Higashibata, A., Ishioka, N., Shimazu, T. and Kamada, M. (2017) Persistence of plant hormone levels in rice shoots grown under microgravity conditions in space: its relationship to maintenance of shoot growth. *Physiol. Plant.*, **161**, 285-293. <https://doi.org/10.1111/ppl.12591>.

Wakabayashi, K., Soga, K., Hoson, T., Kotake, T., Yamazaki, T., Higashibata, A., Ishioka, N., Shimazu, T., Fukui, K., Osada, I., Kasahara, H. and Kamada, M. (2015) Suppression of hydroxycinnamate network formation in cell walls of rice shoots grown under microgravity conditions in space. *PLoS One*, **10**, e0137992. <https://doi.org/10.1371/journal.pone.0137992>.

Yamazaki, C., Fujii, N., Miyazawa, Y., Kamada, M., Kasahara, H., Osada, I., Shimazu, T., Fusejima, Y., Higashibata, A., Yamazaki, T., Ishioka, N. and Takahashi, H. (2016) The gravity-induced re-localization of auxin efflux carrier CsPIN1 in cucumber seedlings: spaceflight experiments for immunohistochemical microscopy. *npj Microgravity*, **2**, 16030. <https://doi.org/10.1038/npjmggrav.2016.30>.

Publication list

Kamada M, Miyamoto K, Oka M, Ueda J, Higashibata A.

(2018). Regulation of asymmetric polar auxin transport by PsPIN1 in endodermal tissues of etiolated *Pisum sativum* epicotyls: focus on immunohistochemical analyses. *Journal of Plant Research* **131**, 681-692. <https://doi.org/10.1007/s10265-018-1031-z>

Kamada M, Miyamoto K, Oka M, Uheda E, Ueda J, Higashibata A. (2018). Procedures for chemical fixation in immunohistochemical analyses of PIN proteins regulating polar auxin transport: Relevance to spaceflight experiments. *Life Sciences in Space Research* **18**, 42-51. <https://doi.org/10.1016/j.lssr.2018.05.005>

Miyamoto K, Inui A, Uheda E, Oka M, Kamada M, Yamazaki C, Shimazu T, Kasahara H, Sano H, Suzuki T, Higashibata A, Ueda J. (2019). Polar auxin transport is essential to maintain growth and development of etiolated pea and maize seedlings grown under 1 g conditions: Relevance to the international space station experiment. *Life Sciences in Space Research* **20**, 1-11. <https://doi.org/10.1016/j.lssr.2018.11.001>

Kamada M, Oka M, Inoue R, Fujitaka Y, Miyamoto K, Uheda E, Yamazaki C, Shimazu T, Sano H, Kasahara H, Suzuki T, Higashibata A, Ueda J. (2019). Gravity-regulated localization of PsPIN1 is important for polar auxin transport in etiolated pea seedlings: Relevance to the International Space Station experiment. *Life Sciences in Space Research* **22**, 29-37. <https://doi.org/10.1016/j.lssr.2019.07.001>

Kamada M, Oka M, Miyamoto K, Uheda E, Yamazaki C, Shimazu T, Sano H, Kasahara H, Suzuki T, Higashibata A, Ueda J. (2020). Microarray profile of gene expression in etiolated *Pisum sativum* seedlings grown under microgravity conditions in space: Relevance to the International Space Station experiment "Auxin Transport". *Life Sciences in Space Research* (*in press*)

Oka M, Kamada M, Inoue R, Miyamoto K, Uheda E, Yamazaki C, Shimazu T, Sano H, Kasahara H, Suzuki T, Higashibata A, Ueda J. (2020). Altered localization of ZmPIN1a proteins in plasma membranes responsible for enhanced-polar auxin transport in etiolated maize seedlings under microgravity conditions in space. *Functional Plant Biology* (*in press*)