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

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*To whom correspondence should be addressed:
Phone: +81-721-29-5306
E-mail: ueda@b.s.osakafu-u.ac.jp

Review
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; Gälweiler et al., 1998; Křeček, et al., 2009; Luschning 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 (μG) 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 μG 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; Feri 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 (Volkmann et al., 1986; Driss-Ecole et al., 2008) and has also been used for morphogenesis of plants grown in a simulated μG 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 μG 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
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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          | Co-Investigators and support research workers     |
|---------------------------------|---------------------------------------------------|
| Junichi Ueda, Professor Emeritus, Osaka Prefecture University | Kensuke Miyamoto, Professor, Osaka Prefecture University |
| Eiji Uheda, Associate Professor, Osaka Prefecture University | Eiji Uheda, Ph D, AES Co., Ltd. |
| Mariko Oka, Associate Professor, Tottori University | Chiaki Yamasaki, Ph D, JAXA/Japan Space Forum |
| Motoshi Kamada, Ph D, JAXA | Akira Higashibata, Ph D, JAXA |
| Toshi Shimazu, Ph D, JAXA/Japan Space Forum | Noriaki Ishioka, Professor, JAXA |
| Tomomi Suzuki, JAXA | Hiromi Sano, JAMSS |

### 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 by 50 mm deep by 63 mm high, Pea Chamber)
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.

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

| Run 2, Run 3 (all dates are in Japan time) |
|------------------------------------------|
| • 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) |
|-------------------------------------------|
| • 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    |

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
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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 μM 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 μG 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 μG 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 μG 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 μG conditions in CBEF. The etiolated maize seedlings were grown for 4 days (96 hours and 27 minutes) at 25°C in the dark under μG 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 μL) containing 14C-IAA (indole-3-acetic acid, [1-14C] 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)
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 because of its swelling effect.

### 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 MgSO4, 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 form Kamada et al. (2018b)
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 μg 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'-TGGTTTGGTGGACATGAAATTGG-3' and reverse: 5'-TCTCTATGACGCTCCACTTTTTCC-3'; *PsDEAD-box* (accession number: AY167670) forward: 5'-TGTTAGGGACTTTTCATGCGC-3' and reverse: 5'-TGTGGAACACCGACGGAAAGT-3'. As a housekeeping gene, the *ZmUPL* gene 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'-TGGAGGACTTTCATCGCC-3' and reverse: 5'-AAGAGCCCTAGCATCCATCG-3', and *ZmAUX1* (accession number: AY011794) forward: 5'-GTTCCTGCCATCATCTTCC-3' and reverse: 5'-GGTCATGTGCTCGGTGTTG-3'. *PsPIN1* in qPCR was normalized based on the expression of the *PsDEAD-box* gene encoding DNA helicase (Bai and DeMason, 2006).

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., VDHGRETQEDYLEK) 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., GATRPPSNYEEDPGK, 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, % | Maize seed germination, % |
|----------------|-------------------------|---------------------------|
|                | Artificial 1 G conditions | Microgravity conditions | Artificial 1 G conditions | Microgravity conditions |
| Water          | 95.8±4.2                | 89.8±8.8                  | 97.2±1.6                  | 94.4±0.8                 |
| TIBA at 30 μM  |                         |                           |                           |                           |

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.
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 cotyledary 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

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.
pea seedlings grown under artificial 1G conditions in space (Figs. 5). Under μG conditions in space, the application of TIBA reduced the bending of epicotyls. In the etiolated pea seedlings grown under μG 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, μG 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 μG conditions in space. However, the direction of growth of those grown under artificial 1G conditions changed to an antigravity direction by negative gravitropic response. Automorphological epicotyl bending was even observed under artificial 1G conditions. Thus, the early growth just after germination is apparently not under gravistimulation.

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

Growth and development of etiolated maize seedlings grown under μG conditions in space was also investigated (Miyamoto et al., 2019). Microgravity conditions on the ISS had a negligible little effect on 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 μG conditions (Fig. 7). In contrast, the coleoptiles...
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 μG 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 μG conditions, and thus TIBA synergistically acts with μG conditions to reduce polar auxin transport. The reduced polar auxin transport seems to be closely related to the morphology of etiolated pea seedlings under μG 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 μG 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 μG 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 μG 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 (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 means±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.
absence of TIBA (S) (Fig. 11A). While a post-hoc Tukey test revealed that the accumulation of \textit{PsPIN1} mRNA in the proximal side of epicotyls 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 \textit{PsPIN1} mRNA in the apical hook region of the seedlings was affected by \( \mu \text{G} \) conditions in the ISS (Fig. 11B). Regardless of gravity conditions, TIBA at a concentration of 30 \( \mu \text{M} \) did not affect the accumulation of \textit{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 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).

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 grew 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 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, l, q, r). Etiolated pea seedlings 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.
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, μG 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 μG 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 μG 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 μG conditions in space (data not shown). The primer set for 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...
the expression of such genes, it is possible to clarify the regulatory mechanisms of plant growth and development in μG conditions in space. It should be mentioned that comprehensive analyses using microarray methods to analyze gravity-dependent or μG-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 μG conditions in space, we introduced microarray data of Medicago to etiolated pea seedlings grown under μG 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 μG 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 μG 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 μG conditions, respectively. Based on Venn diagram analysis, 31 transcripts and 24 transcripts were found to commonly increase and decrease, respectively, under μG conditions in space. Volcano plot illustrating Fold Change (log base 2) compared with p-value (-log base 10) between μG and artificial 1 G conditions was shown in Fig. 19. Functional classification of genes upregulated and downregulated under μG 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 μG 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 μG conditions. whereas there were no statistic differences between under μG and artificial 1 G conditions. Similarly there were no statistic differences between under μG 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 μG conditions in space (Kamada et al., 2020).

6. Comprehensive analyses of plant hormones in the etiolated pea and maize grown under μG 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 μG 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 asparatate), gibberellins (GA, 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 μG 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.

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