Growth and development, and auxin polar transport of transgenic *Arabidopsis* under simulated microgravity conditions on a three-dimensional clinostat

Toru Shimazu[§], Kensuke Miyamoto and Junichi Ueda

College of Integrated Arts and Sciences, Osaka Prefecture University, 1-1 Gakuen-cho, Sakai, Osaka 599-8531, Japan

Abstract Growth and development, and auxin polar transport in *Arabidopsis thaliana* transformed with *iaaH* gene were studied under simulated microgravity conditions on a three-dimensional (3-D) clinostat. Simulated microgravity conditions on a 3-D clinostat did not affect the number of rosette leaves but promoted the growth and development (fresh weight of plant and the elongation of flower stalk) of transformants. Final growth of transformants under simulated microgravity conditions on a 3-D clinostat was almost equivalent to that grown on 1 g conditions in the presence of 1 μ M IAM (indole-3-acetamide). The activities of auxin polar transport in the segments of flower stalk (inflorescence axis) of transformants grown on 1 g conditions were significantly promoted by the addition of IAM. Interestingly, simulated microgravity conditions on a 3-D clinostat also promoted the activities of auxin polar transport of transformants grown on the medium with or without IAM. Based on the results in this study, transgenic plants may not have an efficient homeostatic mechanism for the control of growth and development, and auxin polar transport activity in microgravity conditions in space.

Introduction

Plant hormone, auxin (indole-3-acetic acid) has been recognized to play an important role in plant growth and development through the mode of actions of its polar transport on the earth. We have studied plant growth and development, and auxin polar transport under simulated microgravity conditions on a 3-dimensional (3-D) clinostat. A series of our studies using a 3-D clinostat showed that auxin polar transport in flower stalk of *Arabidopsis thaliana* Columbia ecotype was substantially affected by a long duration under simulated microgravity conditions on a 3-D clinostat, but not affected by a short duration (Oka *et al.* 1995, Ueda *et al.* 1996, 1997, 1999).

Higher plants have been considered to mainly biosynthesize indole-acetic acid (IAA) from tryptophan via indole-3-pyruvic acid and indole-3-acetaldehyde. Those infected with *Pseudomonas* (Kosuge *et al.* 1966, Morris 1986), *Agrobacterium* (Thomashow *et al.* 1984, Van Onckelen *et al.* 1985, Kemper *et al.* 1985) or *Bradyrhizobium* (Sekine *et al.* 1988), however, have been found to biosynthesize IAA via indole-3-acetamide (IAM) of the product catalyzed by tryptophan monooxygenase (Fig. 1). Although IAM has substantially been detected in higher plants (Kawaguchi *et al.* 1993), biosynthetic pathway of IAA via IAM in higher plants has not been

confirmed yet. It seems, therefore, that the biosynthetic pathway of IAA via IAM is independent from the native biosynthetic pathway of IAA in higher plants. The endogenous level of free IAA in tobacco callus which was transformed using Ti plasmid with *iaaM* gene and indoleacetamide hydrolase (*iaaH*) gene was double of non-transformed one (Sitbon *et al.* 1991).

In order to know the mechanism of gravity on the regulation of plant growth and development in relation to auxin polar transport, the introduction of transgenic plants was intended. *Arabidopsis thaliana* Columbia ecotype transformed with *iaaH* gene was used, in which the endogenous auxin level will be expected to be high by biosynthesizing from indole-3-acetamide as a substrate. The purpose of the present study is to know the effects of a simulated microgravity conditions on a 3-D clinostat on the growth and development, and auxin polar transport in transgenic *Arabidopsis*. A possibility introducing transgenic plants as cultivating plants in microgravity conditions in space will also be discussed.

Materials and Methods

Plant materials

Transgenic *Arabidopsis* with *iaaH* gene referred to as R12 was constructed by the same method reported previously (Oka *et al.* 1999). Wild type of the transgenic plants was Columbia ecotype. Seeds of wild type and transgenic one were sterilized by immersing in a 70% (v/v) ethanol for 1 minute and a solution of 10% (v/v) liquid bleach (Haitar, Kao Co., Ltd., Japan) for 10 minutes, followed by extensive washing with sterilized water. Seeds were sowed on 2% agar, then kept at 4 °C for 4 days. After that, they were germinated at 25 °C in the light. The seedlings were transplanted to pots (TECHNOPOT, Sumitomo Bakelite Co., Ltd., Japan) with the *Arabidopsis* basic medium (5 mM KNO₃, 2 mM MgSO₄, 2 mM

Original paper
Received October 8, 2003
Accepted December 1, 2003

Address for correspondence: Junichi Ueda
College of Integrated Arts and Sciences,
Osaka Prefecture University, 1-1 Gakuen-cho, Sakai,
Osaka 599-8531, Japan
E-mail: ueda@el.cias.osakafu-u.ac.jp

[§]Present address:
Japan Space Forum, 1-29-6 Hamamatsu-cho,
Minato-ku, Tokyo 105-0013, Japan

Ca(NO₃)₂, 2.5 mM KH₂PO₄, 50 µM Fe-EDTA, 70 µM H₃BO₃, 14 µM MnCl₂, 0.5 µM CuSO₄, 1 µM ZnSO₄, 0.2 µM NaMoO₄, 10 µM NaCl and 0.01 µM CoCl₂) with 1% agar in the presence or absence of appropriate concentrations of IAM. Plants were grown under continuous white fluorescent light (8 W/m²) at 25 °C under 1 g conditions or under simulated microgravity conditions on a 3-D clinostat for 30 days.

Extraction of DNA

Ten mg leaves of plants which have Kanamycin resistant were minced in 100 µl of DNA extraction buffer (0.14 M sorbitol, 0.22 M Tris-HCl (pH 8.0), 0.022 M EDTA, 0.8 M NaCl, 0.8% (w/v) hexadecyltrimethylammonium bromide, 1% (w/v) N-lauroylsarcosine) with sea sand. A hundred µl of chloroform was added to this solution, mixing strongly using a mechanical vibrator. Then they were kept for 30 minutes at 65 °C. They were centrifuged at 12,000 rpm for 5 minutes and their supernatant was transferred to a new tube. A hundred µl of isopropanol was added to the solution, then kept for 20 minutes at 4 °C. They were centrifuged at 15,000 rpm for 10 minutes, then the pellet obtained was washed with 70% (v/v) ethanol and dried under reduced pressure conditions.

PCR amplification

A 50 ng of DNA extracted from the plants was used as a template for PCR. We used a PCR solution of 2 µM primer including mixture of 10mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 800 µM dATP • dCTP • dGTP • dTTP. We used a primer from a sequence of *iaaHF1* gene (5'-GTTTGAAGTAGTTGCGATAG-3') and of *iaaHR1* gene (5'-GCATGAGTTATCGTTTGGAA-3'). PCR reactions included a denaturing step at 94 °C for 10 minutes followed by 30 cycles at 94 °C for 1 minute, at 55 °C for 1 minute and at 72 °C for 2 minutes with a final extension cycle at 72 °C for 7 minutes. We used a MiniCyclerTM (PTC-150, MJ Research, Inc., USA) for the PCR amplification.

Analysis of auxin polar transport

Activities of auxin polar transport in the segment of flower stalk of *Arabidopsis* R12 transformants were determined using the method previously reported (Oka *et al.* 1995). Fifty µl of 1% [¹⁴C] indole-3-acetic acid (American Radiolabeled Chemical Inc., USA) at the concentration of 1 mCi/ml were applied in a 1.5 ml Eppendorf tube. Segments (30 mm in length) were prepared from flower stalks of the seedlings to be applied the radioactive auxin from the upper or the basal side of the segments. These tubes were incubated in the dark for 22 hours at 25°C. After the incubation, 5-mm pieces of the opposite side from the donor side were excised, and their radioactivities were directly measured by a liquid scintillation counter to determine auxin polar transport.

Simulated microgravity conditions

Simulated microgravity conditions were made by a 3-D clinostat with 2 axes which was designed by Professor Masamichi Yamashita, Japan Aerospace Exploration Agency (former Institute of Space and Astronautical Science). The 3-D clinostat (Model CS-2, Nihonikakikai

Fig. 1. Biosynthetic pathway of IAA via IAM catalyzed by indoleacetamide hydrolase.

Co., Ltd., Osaka, Japan) was constructed according to the report (Hoson *et al.* 1992). The 3-D clinostat was controlled by clinostat control system (CL-CS1, Minamide System Engineering Co., Ltd., Osaka, Japan).

Statistical analyses

The significance of differences in each data was determined by Student's t-test for equal variance.

Results and Discussion

Growth and development of R12 transformed with *iaaH* gene under simulated microgravity conditions on a 3-D clinostat

The presence of *iaaH* gene in genomic DNA of R12 transformants was confirmed by PCR amplification of an internal fragment of *iaaH* gene. PCR products were verified to have an *iaaH* gene sequence by Southern hybridization probed *iaaH* gene (Fig. 2).

Exogenously applied IAM substantially inhibited root growth and extensively promoted the formation of lateral roots in R12 although IAM did not inhibit the root growth of Columbia ecotype (Oka *et al.* 1999). These results indicate that *iaaH* gene in R12 is always expressing and indoleacetamide hydrolase is produced during the growth of R12, resulting in the overproduction of IAA when R12 is grown on the medium containing IAM. The results of previous reports support our considerations. Cauliflower mosaic virus 35S promoter driving *iaaH* gene has been well known as a strong promoter that shows no tissue-specificity of gene expression (Odell *et al.* 1985). It induces high gene expression in all organs of *Arabidopsis* (Atanassova *et al.* 1992), in the apical meristem of tobacco (Lepetit *et al.* 1992) and in floral organs of *Arabidopsis* (Tsukaya *et al.* 1993). In addition, IAA applied exogenously has been shown to inhibit root growth and to promote the initiation of lateral root primordia (Torrey 1976, Wightman

Fig. 2. PCR amplification of *iaaH* gene in transgenic *Arabidopsis* (A) and Southern hybridization using *iaaH* gene probe (B). Lanes 1 to 4: transgenic *Arabidopsis*, 5: Columbia ecotype, 6: DNA size marker (λ DNA digested by HindIII).

et al. 1980, Blakesley *et al.* 1991).

Number of rosette leaves was not affected with *iaaH* gene in the presence or absence of IAM on 1 g conditions (data not shown). On the contrary, fresh weight of plants (aerial part) was slightly promoted with *iaaH* gene in the presence of low concentrations (0.1 to 1.0 μ M) of IAM (Fig. 3), but not in the presence of relatively high concentration (10 μ M). The growth promotion of plants in the presence of low concentrations of IAM depended on the significant promotion of flower stalk growth (Fig. 4). Although it is difficult to explain the growth promotion of plants, it might be due to the concentration of endogenous free IAA derived from IAM in the presence of *iaaH* gene. Relatively high concentration of endogenous IAA in R12 transformants in the presence of IAM has been already reported (Oka *et al.* 1999).

Simulated microgravity conditions on a 3-D clinostat substantially affected growth and development of *Arabidopsis* R12 transformants in the presence of IAM except the number of rosette leaves (data not shown). Fresh weight of R12 transformants in the presence of low concentration (0.1 μ M) of IAM was significantly promoted by simulated microgravity conditions on a 3-D clinostat (Fig. 3), but slightly inhibited in the presence of relative high concentration (10 μ M). However, growth of flower stalk of transformants in the presence of IAM (0.1 to 10 μ M) was not affected by simulated microgravity conditions on a 3-D clinostat (Fig. 4). As described above, growth promotion of flower stalk of non-transformants (Columbia ecotype) observed in the presence of 1 μ M IAM was almost equal to that on simulated microgravity conditions on a 3-D clinostat. These results suggest that growth promotion of flower stalk of transformants on simulated microgravity conditions on a 3-D clinostat due to relatively high levels of endogenous IAA concentrations and/or promotion of IAA polar transport activities in the presence or absence of IAM (Fig. 5) describing below. There is a possible explanation that endogenous levels of IAA in R12

Fig. 3. Effect of simulated microgravity conditions on a 3-D clinostat on the growth (fresh weight of aerial part) of transgenic *Arabidopsis* in the presence or absence of IAM. Results are expressed as the mean with standard error (n=7 to 21).

transformants in the presence or absence of IAM are enhanced by simulated microgravity conditions on a 3-D clinostat, although weight and hormone content of the microgravity maize seedlings were, with minor exceptions, not statistically different from seedlings grown in normal gravity (Schulze *et al.* 1992).

Auxin polar transport of *iaaH* transformants under simulated microgravity conditions on a 3-D clinostat

Activities of auxin polar transport in the segment of flower stalk of R12 transformants were measured using [14 C] labeled IAA. Auxin polar transport of R12 transformants on 1 g conditions was significantly promoted in the presence of IAM at concentrations of 1 to 10 μ M due to relatively high levels of endogenous IAA converted from IAM with *iaaH* gene product (Fig. 5). On the other hand, simulated microgravity conditions on a 3-D clinostat enhanced activities of auxin polar transport in R12 transformants in the presence or absence of IAM, they being almost equivalent to those of R12 in the presence of IAM

Fig. 4. Effect of simulated microgravity conditions on a 3-D clinostat on the growth of flower stalk of transgenic *Arabidopsis* in the presence or absence of IAM. Results are expressed as the mean with standard error (n=7 to 21).

Fig. 5. Effect of simulated microgravity conditions on a 3-D clinostat on IAA polar transport in flower stalk of transgenic *Arabidopsis* in the presence or absence of IAM. Results are expressed as the mean with standard error (n=7 to 21).

on 1 g conditions (Fig. 5). The reason why simulated microgravity conditions promoted auxin polar transport is not clear yet as well as that of growth promotion of flower stalk. It might depend on relatively high levels of endogenous IAA. Extremely high levels of IAA due to *iaaH* gene product in R12 transformants were reduced by metabolizing into its conjugate form (Oka *et al.* 1999).

Based on the results in this study, transgenic plants may not have an efficient homeostatic mechanism for the control of growth and development, and auxin polar transport activity in microgravity conditions in space.

Acknowledgement

The authors thank Professor Kiyotaka Okada of Kyoto University and Dr. Robert Masterson of Max-Planck-Institute for supplying seeds of transformants, and also thank Professor Masamichi Yamashita of Japan Aerospace Exploration Agency (former Institute of Space and Astronautical Science, Japan) for supporting to build the 3-D clinostat system. This work was partially supported by the fund of the Institute of Space and Astronautical Science, Ministry of Education, Science, Sports and Culture of Japan, for Basic Experiments Oriented to Space Station Utilization, and by the fund for Ground Research for Space Utilization promoted by former NASDA and Japan Space Forum.

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