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Original Article

Germination and Growth Test in Four Strains of *Arabidopsis thaliana* in the Reference Model of European Modular Cultivation System

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Abstract

Japan Aerospace Exploration Agency (JAXA) has two plant physiological space experiments that utilize the European Modular Cultivation System (EMCS) facility of European Space Agency (ESA). The theme of the two experiments, namely, Cell Wall and Resist Wall (CWRW) are aimed at understanding the formation of plant cell wall and the mechanism of gravity resistance in plants. A ground-based study for monitoring the germination and growth of *Arabidopsis*, science test, was performed for 50 days in the EMCS experiment reference model (ERM), as a preparation of the onboard CWRW space experiment. Four strains of *Arabidopsis* seeds were germinated and the seedlings were cultivated in the dedicated plant cultivation chamber (PCC) in the EMCS ERM until the inflorescence stems grew to approximately 10-cm long. The objective of this science test was to successfully grow *Arabidopsis* seedlings in the EMCS ERM for determining the approximate duration of the onboard CWRW space experiment. As a result, the PCC could be used to grow three strains, i.e., wild type, *lefty* mutant, and gene modified *pCesA7::GUS*, *Arabidopsis* plants from seeds to 10-cm-long inflorescence stems within 50 days. This science test confirmed that the PCC is biocompatible and a good support system for these strains growth during the entire experiment. On the other hand, the *hmg* mutant seeds, which are more delicate and susceptible to outer environment than other strains, failed to germinate. Therefore, increase the number of sowing *hmg* mutant seeds into the PCC and the germination test using the PCC for selection the *hmg* mutant seeds with best germination rate were essential for the future onboard CWRW space experiment.

1. Introduction

The Cell Wall and Resist Wall (CWRW) experiment consists of two themes that have been selected by the 5th International Research Solicitation for Space Flight Experiments in the Fields of Life Science and Space Medicine (2004). The official titles of CWRW experiment are 'The Reverse Genetic Approach to Exploring Genes Responsible for Cell Wall Dynamics in Supporting Tissues of *Arabidopsis* under Microgravity Conditions' and 'Role of Microtubule-Membrane-Cell Wall Continuum in Gravity Resistance in Plants', respectively. The aim of the CWRW experiment is to explore the molecular mechanism by which cell wall formation in *Arabidopsis* is regulated by gravity. The goals and objectives of the CWRW experiment are as follows: (1) identification and characterization of gene sets that are responsible for gravity-dependent cell wall formation in *Arabidopsis* stem; (2) functional analysis of the genes identified as playing a central role in the gravity-dependent supporting-tissue formation; and (3) confirmation of the involvement of the 'microtubule-plasma membrane-cell wall continuum' in resistance of plants to gravity[1,2]. The CWRW experiment brings the following two results and findings. (1) The organization and activity of cell wall genes and the activity of signaling pathways leading to transcriptional regulation are essential for defining the cell wall components that will be incorporated in the cell wall during its elongation and those that will no longer grow and function as supporting structures of the shoot[1]. (2) It is generally expected that defects in the cell wall related mutants are rescued, and such plants can grow and develop almost normally under microgravity conditions in space, where the formation of a tough cell wall is not required[2]. In the present study, we will address the former issue by possibly identifying the missing link between gravitational

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response and morphology of plants, and thus, provide pioneering insights into the development of plants. If the expected results are obtained, the basic mechanism of gravity resistance in plants will be elucidated; this would remarkably enhance our knowledge of plant responses to gravity. That is, the spin-off applications are thought that the discovery of such a hidden mechanism would be of considerable importance. Moreover, the experiment will provide invaluable information that will directly contribute to our knowledge of how we can engineer plants with altered shapes and functions, thereby expanding the utilization of plants not only on earth but also in space and on other planets.

The seeds of four strains of *Arabidopsis thaliana*: wild type ecotype Columbia; the tubulin mutant *lefty*, which causes defects in the organization of cortical microtubules; the *hmg* mutant, which is defective in the synthesis of membrane sterols; and gene modified *pCesA7::GUS* (the promoter of cellulose synthase A7 gene, which is cell wall related gene that is preferentially expressed in the supporting tissue of *Arabidopsis* and responsible for gravitational responses, fused with β-glucuronidase gene construct) are used for the CWRW experiment[1,2]. We used these strains to study as follows: (1) *Arabidopsis* mutants defective in formation of cortical microtubules or the plasma membrane, i.e., *lefty* mutant or *hmg* mutant, are unable to form the tough cell wall, and therefore, they show disordered growth pattern on earth[2]. It is highly expected that the defects of such mutants are rescued and they can grow and develop more or less normally under microgravity in space, where formation of the tough cell wall is not required. (2) We generate gene-modified *Arabidopsis* plant, *pCesA7::GUS*, and characterize expression profile of the GUS reporter gene that is equivalent to gravitational responses responsible cell-wall related gene promoter activity, in stem at various developmental stages and under different mechanical stresses. In other words three *Arabidopsis* strains of *lefty*, *hmg*, and *pCesA7::GUS* are extremely important for study on gravity resistance and gravity-induced supporting tissues formation of plants.

The plant cultivation chamber (PCC) will be used in the onboard CWRW space experiment for cultivating of *Arabidopsis* plants. The PCC is a slightly modified version of the European Space Agency (ESA) multi-generation experiment-1 (MULTIGEN1) PCC equipment[3]. Briefly, the protocol for the CWRW experiment would be as follows: prior to launch, sterilized dry seeds of *Arabidopsis* will be sown in PCC, and then, on orbit, the PCC will be installed into the European Modular Cultivation System (EMCS) facility by the International Space Station (ISS) crew. *Arabidopsis* will be grown in an automatic sequence for 43 ± 10 days, until 10-cm-long inflorescence stems are obtained. The EMCS experiment reference model (ERM) is used for reference experiments for EMCS utilization (**Fig. 1**). Because the PCC is placed in a horizontal position in the EMCS facility on the ground, the growth direction from stem to root in the PCC design is different from the direction of gravitational force. However, the EMCS ERM is designed in such a way that the growth direction may be the same as that of the gravitational force on the ground. In addition, the conditions for plant growth, such as temperature, humidity, air, light, and ventilation, in the EMCS ERM can be controlled similar to those in the EMCS facility.

The main objective of the ground-based study for monitoring the germination and growth of *Arabidopsis*, also known as science test, was to grow *Arabidopsis* plants in the EMCS ERM to determine the approximate duration of the onboard CWRW space experiment. This test also functioned as an additional verification of the functionality, performance, and biocompatibility of the PCC during the process of germination to inflorescence stem growth of *Arabidopsis*.

2. Materials and Methods

2.1 *Arabidopsis* seeds

The seeds of four strains of *Arabidopsis thaliana*, i.e., wild type, *lefty* mutant, *hmg* mutant, and gene modified *pCesA7::GUS*, were used for the science test.

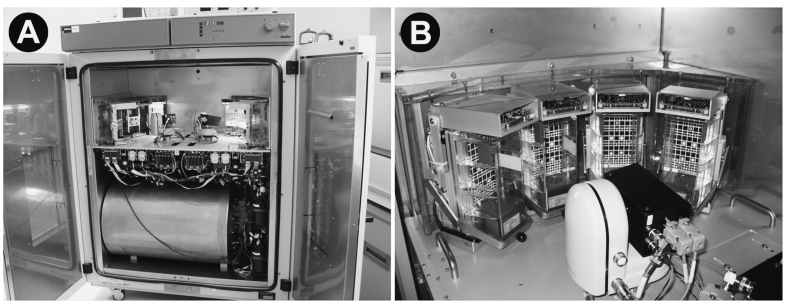

Fig. 1 The EMCS ERM facility system. The lower part comprises the atmosphere regulation system and water supply system (A). The upper part has a capacity of holding 8 PCC-ECs, and 4 PCC-ECs can be observed at the same time with 1 camera each fitted at the left and right side; photographs can be automatically taken. The photographs of 4 PCC-ECs are arranged in the EMCS ERM on the upper left side (B).

Before the science test, the three strain seeds except the *hmg* mutant, i.e., wild type, *lefty* mutant, and gene modified *pCesA7::GUS*, put on wet filter papers in the dishes for germination test. The germination test was performed at 23°C, and then we used the lot seeds of 90% or more germination rate on the science test. For the *hmg* mutant, we decided to use the same lot seeds which used to general experiment in the laboratory in Osaka City University with 90% or more germination rate[4]. These seeds were sterilized in 70% ethanol for 5 min and then soaked in 2.5% sodium hypochlorite and 0.5% Tween 20 for 5 min. Subsequently, the seeds were washed 3 times with sterilized water and dried on sterilized filter paper[5]. The sterilized dry seeds were screened on the basis of color and shape under a stereo microscope, and only healthy seeds were selected for the science test.

2.2 Experimental set-up for the science test

The test was conducted in the EMCS ERM at Norwegian-User Support Operation Center (N-USOC) in Trondheim, Norway. *Arabidopsis* was grown in the CWRW PCC mounted in the experiment container (EC) provided by Astrium GmbH (Friedrichshafen, Germany). The CWRW PCC was manufactured by CMR Prototech (Bergen, Norway). Two growth pots of the CWRW PCC (PCC FM03 mounted in EC FM062 and PCC FM07 mounted in EC FM063) were sterilized, and growth medium and seeds were added according to the PCC assembly procedure[3]. The growth medium, zeolite was purchased from ZeoponiX, Inc. (Boulder, CO., USA) and based on National Aeronautics and Space Administration (NASA) developed plant growth medium. The CWRW PCC has seven mini-lid holes for sowing seeds. These holes were numbered from #1 to #7 starting from the left rear to the right front end. In the mini-lid holes numbered from #1 to #7, 1 seed of wild type, 1 seed of *pCesA7::GUS*, 3 seeds of *lefty* mutant, 3 seeds of *hmg* mutant, 1 seed of *pCesA7::GUS*, 3 seeds of *lefty* mutant, and 3 seeds of *hmg* mutant, respectively, were sown (**Table 1**). The science test was performed for 50 days in the EMCS ERM from September 19 to November 7, 2007. The PCC-EC was periodically removed from the EMCS ERM in order to weigh water content and *Arabidopsis* plant. It was considered that most of the water was located inside the PCC pot and a maximum of 1 m*l* remained in the EC baseplate.

2.3 Environmental and growth conditions

The science test was conducted in accordance with the N-USOC test procedure. Initially, the environmental conditions such as temperature, photoperiodic cycle, humidity, and atmosphere were controlled. For initial watering, 12 m*l* water was injected in pulses inside the PCC-EC by using the 'EMCS ERM water pulse command schedule' such that the first 5 m*l* and the remainder were injected at 0.2 m*l* per min and per 5 min, respectively. The number of pulses required to reach 13 m*l* of total water content was calculated and the 'EMCS ERM water pulse command schedule' was accordingly adjusted so that the remaining water added up to 13 m*l* (**Fig. 2**). *Arabidopsis* plants were germinated and grown at 23°C under a 50 Wm^{-2} white light emitting diode (LED) with a photoperiodic cycle of 16 h light/8 h dark. The humidity inside the PCC-

Table 1 Seed configuration set-up and results of seed germination in the science test

PCC-EC	Mini-lid hole position	Strain	Number of seeds	Germination result*
PCC03-EC062	#1	wild type	1	G
	#2	gene modified *pCesA7::GUS*	1	G
	#3	*lefty* mutant	3	G2
	#4	*hmg* mutant	3	NG
	#5	gene modified *pCesA7::GUS*	1	G
	#6	*lefty* mutant	3	G2
	#7	*hmg* mutant	3	NG
PCC07-EC063	#1	wild type	1	G
	#2	gene modified *pCesA7::GUS*	1	NG
	#3	*lefty* mutant	3	G2
	#4	*hmg* mutant	3	NG
	#5	gene modified *pCesA7::GUS*	1	G
	#6	*lefty* mutant	3	G2
	#7	*hmg* mutant	3	NG

* G: germination, G2: germination of at least 2 seedlings (very difficult to verify the number of seedlings when 2 or more seeds have germinated), NG: no germination

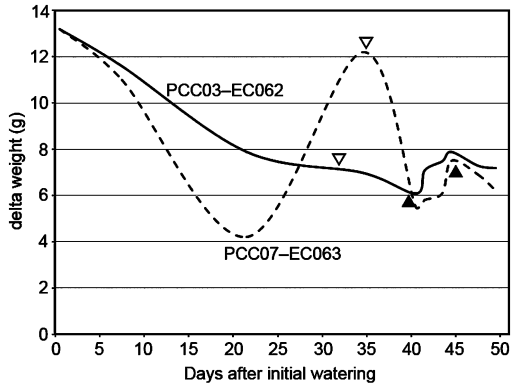

Fig. 2 The time course change of the PCC-EC delta weight. The delta weight is a defined as the difference between the weight of PCC-EC before it was set up to the EMCS ERM and the weight of PCC-EC per day after initial watering, including the water inside the PCC-EC and the *Arabidopsis* plant weight. The solid line and the broken line indicate the delta weights of PCC03-EC062 and PCC07-EC063, respectively. The open triangle and the closed triangle represent the day when the first inflorescence stem developed and when they grew up to 10-cm long, respectively.

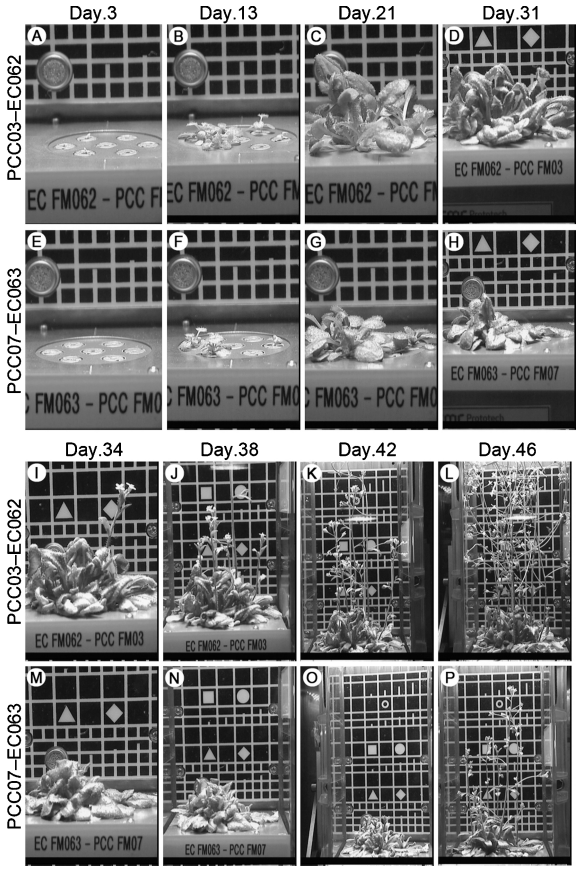

Fig. 3 The photographs of the developing *Arabidopsis* plants during the science test by using the PCC and the EMCS ERM facility. *Arabidopsis* grown in PCC03-EC062 and PCC07-EC063 are shown in A-D and I-L; and E-H and M-P, respectively. These photographs were taken at 3 (A, E); 13 (B, F); 21 (C, G); 31 (D, H); 34 (I, M); 38 (J, N); 42 (K, O); and 46 (L, P) days after initial watering. Each photographic plate has the dimensions of 10×10 mm.

EC was maintained at 50 to 95% by switching the air valves between the venting mode and the bypass mode. The functions of the venting mode and bypass mode have been described in a previous paper[5]. The atmospheric concentrations of O_2, N_2, and CO_2 were maintained at 20, 70, and $0.6 \pm 0.1\%$, respectively, using external gas bottles. After initial watering, water was automatically supplied, as required, by referring to the inner delta pressure of the PCC in the EMCS ERM program. Appropriate ventilation conditions were maintained by scheduling the venting mode for 3 min and the bypass mode for 15 min from the time of the initial water supply for 14 days. Later, the time intervals between venting mode and bypass mode were adjusted according to the development and transpiration of plants during the experiment. At 20 days after initial watering, the light intensity was switched from 50 to 75 Wm^{-2}. Water was supplied on the basis of the inner delta pressure of the PCC in the EMCS ERM program.

3. Results and Discussion

3.1 Germination

Germination of seeds started at 3 days after initial watering for the science test in the EMCS ERM and was observed in 5 of the 7 mini-lid holes in PCC03-EC062 and 4 of the 7 mini-lid holes in PCC07-EC063 (**Fig. 3**, Table 1). The gene modified *pCesA7::GUS* seeds that were sown in mini-lid hole #2 of PCC07-EC063 and all *hmg* mutant seeds that were sown in 2 mini-lid holes in both PCCs did not geminate. However, *lefty* mutant seeds sown 3 seeds per mini-lid hole germinated at least 2 seedlings from a mini-lid hole. An accurate number of seedlings that had germinated could not be determined since seed germination was judged from the images captured by the EMCS ERM camera.

3.2 Inflorescence stem development

The first inflorescence stem developed on day 32 in PCC03-EC062 and on day 35 in PCC07-EC063 (Figs. 2 and 3). The development of the inflorescence stem in PCC07-EC063 was delayed compared with that in PCC03-EC062 because the growth of *Arabidopsis* in PCC07-EC063 had temporarily stopped due to a hydration and water supply problem that occurred around day 20 (Fig. 2). After the problem was fixed by manual water injection using a syringe, the growth of *Arabidopsis* was restored. The inflorescence stems grew from small shoots up to 10-cm long in approximately 10 days, which is much better than the requirement of 10-cm-long stems within 50 days (Figs. 2 and 3). Since the 10-cm-long inflorescence stems had many rosette leaves, flowers, and siliques, analysis of these

parts is expected to be applicable for further research (Fig. 3).

3.3 Verification for PCC functions

The PCC is the EMCS experiment-specific hardware that is highly automated hardware to perform experiments autonomously on a running in microgravity and optimized for the cultivation of the model plant, *Arabidopsis*[3,5]. Therefore, the following four problems for the germination and growth of *Arabidopsis* in the PCC on the ground and/or under microgravity were assumed.

(1) Effect of gas accumulation on the growth of *Arabidopsis* by complete sealing up and closed system of the PCC: the ethylene and carbon dioxide gases were suggested to influence the growth of *Arabidopsis*.
(2) Effect of wetting characteristic on the germination of *Arabidopsis* on the polypropylene (PP)-felt membrane PCC pot: the hydrophilic and/or hydrophobic characters of PP-felt might influence the germination of *Arabidopsis*.
(3) Effect of particle size of the zeolite on the growth of *Arabidopsis*: the water-holding capacity of the zeolite is weak because the particle size of the zeolite, between 0.5 and 1.0 mm[3,5], is bigger than soil and rock wool fiber.
(4) Effect of gaseous exchange of the PCC pot on the root growth of *Arabidopsis* by permeability to the PCC pot dryness condition: the dryness stress would influence the root growth of *Arabidopsis*, if the inside of the PCC pot dried in excess because the PCC was structured to send air around the root in the PCC pot when water is supplied.

As a result of this science test, *Arabidopsis* inflorescence stems, leave and other organs had developed normally and healthy in the PCC. Moreover, it was observed that the root system had also developed normally when the PCC pot was disassembled after the science test. Therefore, we were able to confirm that the above-mentioned problems did not influence the germination and growth of *Arabidopsis* except the *hmg* mutant in the science test. On the other hand, the MULTIGEN1 was performed in the ISS on board from August to October, 2007[6]. The MULTIGEN1 which studied seed-to-seed and circumnutation of inflorescence stems used only wild type seeds of the *Arabidopsis*, but the humidity and ventilation controls inside the PCC were important for the early growth of the *Arabidopsis* under microgravity. In the MULTIGEN1, the ethylene and carbon dioxide gas accumulation in the PCC and particle size of the zeolite did not become the problem for the *Arabidopsis* growth. It was considerably useful that we were able to know this information before the future CWRW space experiment. Therefore, we decided on growth conditions for the CWRW space experiment on the basis of the matters which understood from the MULTIGEN1 and this science test.

3.4 Success criteria

We had set experiment success criteria for the germination of seeds and the development of inflorescence stems before the commencement of the science test **(Table 2)**, which were stated as follows: the seeds of the four strains of *Arabidopsis* would germinate and develop 10-cm-long inflorescence stems within 50 days. It is known that the *hmg* mutant plants show male sterility and its barely produced seeds are delicate and susceptible to outer environment[7]. Therefore, the germination rate for the *hmg* mutant was set as 50% (3 seeds of the total 6 seeds/PCC), whereas it was 80% (7 seeds of the total 9 seeds/PCC) for the other three strains, i.e., wild type, *lefty* mutant, and gene modified *pCesA7::GUS* (Table 2). On the other hand, the rate for inflorescence stem development for the *hmg* mutant from the mini-lid hole was set as 50% (1 stem of the 2 holes/PCC), and 60% (3 stems of the 5 holes/PCC) for the other three strains (Table 2). The results of the science test revealed that the seeds of the three strains, i.e., wild type, *lefty* mutant, and gene modified *pCesA7::GUS*, germinated and developed up to 10-cm-long inflorescence stems at a rate greater than that set in the success criteria (Table 2). Therefore, the

Table 2 Summary of the experiment success criteria for germination and inflorescence stem development

Criteria	Strain group	Requirements	Results
Germination rate	wild type, *lefty* mutant, and gene modified *pCesA7::GUS*	Average 80% of seeds (7 seeds of the total 9 seeds/PCC)	At least 7 seeds germinated in both PCCs, and this part of the test was therefore successful.
	hmg mutant	Average 50% of seeds (3 seeds of the total 6 seeds/PCC)	No seeds germinated at all, and this part of the test was therefore unsuccessful.
Inflorescence stem development rate	wild type, *lefty* mutant, and gene modified *pCesA7::GUS*	Average around 60% of the mini-lid holes (3 stems of the 5 holes/PCC)	Stems developed from all the seeds in both the PCCs and reached the length of 10 cm within 50 days. This part of the test was therefore successful.
	hmg mutant	Average around 50% of the mini-lid holes (1 stem of the 2 holes/PCC)	No germination and consequently no stem development, and this part of the test was therefore unsuccessful.

science test for these strains was successful. However, the *hmg* mutant seeds did not germinate at all; hence, we were unable to determine the success rate for their germination or inflorescence stem development (Tables 1 and 2). The same lot of the *hmg* mutant seeds germinated usually in the laboratory, and was able to be used for a wide variety of experiments[4]. Because the *hmg* mutant seeds are delicate and susceptible to outer environment, even if the *hmg* mutant seeds germinate normally on agar medium, germination rate tends low in the medium such as felt or rock wool that does not have a uniform water condition[4]. Therefore, because the germination rate of the *hmg* mutant seeds tends to be lower than wild type, we decided to increase the number of sowing seeds into the PCC. That is, three mutant seeds were sown each mini-lid hole of the PCC in the onboard CWRW space experiment. Additionally, the germination test using the PCC was essential to select the *hmg* mutant seeds suitable for the future onboard CWRW space experiment. The germination rate for another lot of the hmg mutant seeds, which were screened more carefully on the basis of color and shape under a stereo microscope, was approximately 80% in the PCC test conducted in December 2007.

4. Conclusion

The science test confirmed that the PCC can be used to grow *Arabidopsis* plants from seeds to 10-cm-long inflorescence stems within 50 days. The strains, i.e., wild type, *lefty* mutant, and gene modified *pCesA7::GUS*, seeds germinated successfully in both the PCCs excluding at one position (mini-lid hole ♯2 in PCC07-EC063). The science test also confirmed that the PCC is biocompatible and a good support system for *Arabidopsis* growth during the entire experiment. However, the *hmg* mutant seeds failed to germinate at any position in both PCCs, and hence, no stems developed; this was probably because they are more delicate and susceptible to outer environment than other strains.

Acknowledgments

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