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The Outline and Significance of the Resist Wall Experiment: Role of Microtubule-Membrane-Cell Wall Continuum in Gravity Resistance in Plants

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Abstract Resistance to the gravitational force is one of two major graviresponses in plants. However, only limited information has been obtained for its mechanism. The Resist Wall experiment aims to examine the role of the cortical microtubule-plasma membrane-cell wall continuum in gravity resistance, thereby clarifying its mechanism. For this purpose, we will cultivate *Arabidopsis* mutants defective in organization of cortical microtubules (*tua6*) or synthesis of membrane sterols (*hmg1*) as well as the wild type Columbia under microgravity and 1*G* conditions in the European Modular Cultivation System on the International Space Station up to reproductive stage, and compare phenotypes on growth and development using video images. These mutants are unable to form the normal cell wall and show disordered growth pattern on Earth. However, it is 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. We will also analyze changes in expression of genes involved in formation of the continuum and properties of related cellular components under microgravity conditions. The results of the Resist Wall experiment will clarify the molecular mechanism of gravity resistance and benefit efficient plant production not only in space but on Earth.

Introduction

Plants have utilized gravity, which is present in a constant direction and magnitude on Earth, as the most reliable signal for morphogenesis. Gravitropism is the principal gravity response of plants and enables them to orient their leaves to sunlight and roots to water and minerals. The mechanism of gravitropism has been well studied. Plants also show the other gravity response that is to resist the gravitational force by constructing a tough body. The presence of this second response has not been properly recognized for long, and there was even no suitable name for it. We have termed this reaction "gravity resistance" and examined its nature and mechanism using conditions of water submergence, with hypergravity conditions produced by centrifugation, and by space experiments during the Space Shuttle STS-95 mission (Hoson and Soga, 2003; Hoson *et al.*, 2005). As a result, we have clarified the outline of the sequence of events leading to the final response in gravity resistance. One important hypothesis raised by the study is that the structural or physiological continuum of cortical microtubule-plasma membrane-cell wall may play an important role in gravity resistance. We have considered and proposed a space experiment to confirm this hypothesis on the occasion of the 5th International Research Solicitation for Space Flight Experiments in the Fields of Life Science and Space Medicine. The proposal entitled "Role of microtubule-membrane-cell wall continuum in gravity resistance in plants" was selected, nicknamed the Resist Wall experiment, and recently approved as the experiment to be conducted in the European Modular Cultivation System (EMCS) on the International Space Station (ISS). In the present article, we describe the outline of the Resist Wall experiment and its scientific significance.

Gravity Resistance in Plants

Gravity resistance consists of the sequence of events, signal perception, transformation and transduction of the perceived signal, and response to the transduced signal,

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Table 1 The sequence of events leading to gravitropism and gravity resistance.

Events	Gravitropism	Gravity resistance
Perception	Sedimentation of amyloplasts in statocytes	Sensing of whole protoplast mass by mechanoreceptors in almost all cells
Signal transformation and transduction	Intercellular polar transport of auxin	Intracellular signal transduction
Response	Differential growth due to differential cell wall modifications	Increase in the cell wall rigidity in each cell

as other environmental responses. For graviperception of gravitropism, the starch-statolith hypothesis has been proposed. The *pgm* (*phosphoglucomutase*) and *sgr1* (*shoot gravitropism 1*) mutants of *Arabidopsis* show reduced or no gravitropic responses in inflorescence stems and hypocotyls, because of lack of sedimentable amyloplasts (Tasaka *et al.*, 2001). If graviperception mechanism is common in gravity resistance and gravitropism, both *pgm* and *sgr1* mutants are expected not to show or only weakly show gravity resistance response. However, hypocotyls of these mutants showed normal gravity resistance responses similar to those of wild types (Soga *et al.*, 2004). In addition, the removal of root cap did not influence gravity resistance in azuki bean roots, although the gravitropic curvature was completely inhibited (Soga *et al.*, 2005). These results suggest that the graviperception mechanism in gravity resistance is independent of that in gravitropism (Table 1). On the other hand, mechanoreceptors (mechanosensitive ion channels) are present in various organisms, including plants, on the plasma membrane (Kanzaki *et al.*, 1999; Nakagawa *et al.*, 2007). In epicotyls and roots of azuki bean and *Arabidopsis* hypocotyls, lanthanum and gadolinium ions, which are blockers of mechanosensitive ion channels, strongly diminished gravity resistance responses, without affecting gravitropism (Soga *et al.*, 2004, 2005). The results suggest the involvement of mechanoreceptors in the perception of gravity signal in gravity resistance, but not in gravitropism (Table 1). The results that horizontal- and acropetal-hypergravity induced gravity resistance responses as did basipetal-hypergravity support the hypothesis.

Plant cells are enclosed with the cell wall which provides the cells with structural rigidity. We have obtained evidence supporting the view that the cell wall is responsible for the final resistance response of plants to the gravitational force, as do the bones and muscles in an animal body, by extensive analyses of the changes in the mechanical and chemical properties of the cell wall in plant seedlings grown under different gravity conditions (Hoson and Soga, 2003; Hoson *et al.*, 2005). An increase in cell wall rigidity under hypergravity conditions was detected in various plant materials, suggesting that plants in general resist the gravitational force by constructing tough cell walls (Table 1). The mechanical properties of the cell wall are determined by the chemical nature of cell wall constituents and interactions among them. Hypergravity has been shown to increase cell wall thickness in shoots and roots of a number of plants. Hypergravity also caused a polymerization of certain matrix polysaccharides, the types of which were different between dicotyledonous plants and monocotyledonous Gramineae plants. In dicotyledons, hypergravity increased the molecular mass of xyloglucans, whereas hypergravity increased that of 1,3,1,4-β-glucans in Gramineae plants. In addition, hypergravity decreased xyloglucan-degrading activity in azuki bean epicotyls and *Arabidopsis* hypocotyls and 1,3,1,4-β-glucanase activity in coleoptiles and mesocotyls of maize. Thus, xyloglucans and 1,3,1,4-β-glucans may act as anti-gravitational cell wall polysaccharides, in cooperation with cellulose microfibrils. The hypothesis was supported by space experiments during the Space Shuttle STS-95 mission. The cell wall rigidity of both *Arabidopsis* hypocotyls and rice coleoptiles was lower in space-grown seedlings than in the controls (Hoson *et al.*, 2002; Soga *et al.*, 2002). Also, the space-grown *Arabidopsis* hypocotyls and rice coleoptiles had a decreased cell wall thickness and a lower molecular mass xyloglucans and 1,3,1,4-β-glucans, resulting from the increases in xyloglucan-degrading activity and 1,3,1,4-β-glucanase activity. These results clearly support the principal role of the cell wall, in particular of xyloglucans and 1,3,1,4-β-glucans, in gravity resistance in plants.

Transformation and transduction of a perceived gravity signal is the step connecting signal perception and response to the signal. In gravity resistance, this step appears to occur within each cell (Table 1). Because the plasma membrane is the site of location of mechanoreceptors and it also plays an important role in sustaining the metabolism of cell wall constituents, the plasma membrane may be responsible for signal transformation and transduction processes in gravity resistance. Actually, the involvement of H^+-ATPase and cellulose synthase located on the plasma membrane in gravity resistance has been suggested (Hoson and Soga, 2003; Hoson *et al.*, 2005). Also, membrane sterols may play a role in gravity resistance. Under hypergravity

conditions, the expression of a gene encoding 3-hydroxy-3-methylglutaryl-Coenzyme A reductase (HMGR), which catalyzes a reaction producing mevalonic acid, a key precursor of terpenoids, was greatly up-regulated (Yoshioka *et al.*, 2003). Out of various membrane constituents of azuki bean epicotyls, the level of sterols was specifically increased under hypergravity conditions (Koizumi *et al.*, 2007). In addition, lovastatin, an inhibitor of HMGR, made the epicotyls hypersensitive to the gravitational force. These results suggest that membrane sterols are involved in maintenance of normal growth capacity of plant organs against the gravitational force. It has been shown that sterols consist of microdomains called rafts. Sterols may act in gravity resistance in plants as constituents of membrane rafts (Hoson *et al.*, 2005) (Fig. 1).

Cortical microtubules, in cooperation with the cell wall and other cytoskeletal components, give the cytoplasm structural stability and mechanical strength, and thus may also play a role in gravity resistance in plants. Actually, the analyses of the changes in gene expression under hypergravity conditions have shown that most α- and β-tubulin genes are up-regulated in *Arabidopsis* hypocotyls (Matsumoto *et al.*, 2007). Moreover, microtubule-disrupting agents made the hypocotyls hypersensitive to the gravitational force. Under hypergravity conditions, not only the levels but the orientation of cortical microtubules was modified. In the epidermis of azuki bean epicotyls grown at 1*G*, cells with transverse cortical microtubules were predominant. With increasing the gravitational force, the percentage of cells with transverse microtubules was decreased, whereas that with longitudinal microtubules was increased (Soga *et al.*, 2006). Lanthanum and gadolinium ions suppressed the reorientation of cortical microtubules, suggesting their involvement in gravity resistance. The direction of plant cell growth is primarily determined by the pattern of deposition of cellulose microfibrils, which, in turn, is thought to be regulated by cortical microtubules. The co-alignment hypothesis states that the movement of cellulose synthase complexes in the plasma membrane is constrained by interactions with cortical microtubules (Baskin, 2001). Cellulose microfibrils and cortical microtubules are thus mutually dependent in their functions. Take together, these results suggest that the structural or physiological continuum of

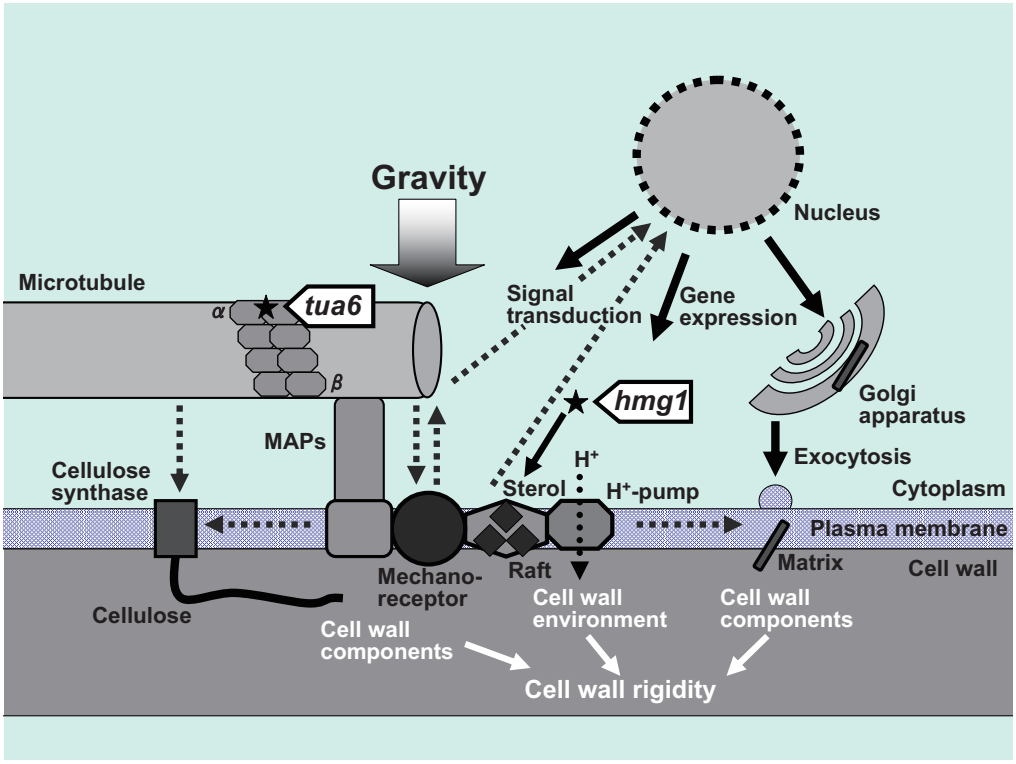

Fig. 1. Mechanism of gravity resistance and *Arabidopsis* mutants used for the Resist Wall Experiment. The gravity signal is perceived by the mechanoreceptors located on the plasma membrane, and then transformed and transduced through the plasma membrane and into the cells via the cortical microtubule-plasma membrane-cell wall continuum. The transduced signal induces the expression of diverse genes and influences the structure and function of various cellular components. The modifications of the cell wall environment as well as the metabolism of cell wall components determine the cell wall rigidity, leading to gravity resistance response. *Arabidopsis* mutants defective in organization of cortical microtubules (*tua6*) and in synthesis of membrane sterols (*hmg1*) will be used for the Resist Wall Experiment.

cortical microtubule-plasma membrane-cell wall plays a principal role in gravity resistance in plants (Fig. 1).

Strategy and Objective

The objective of the Resist Wall experiment is to prove the essential role of the continuum of cortical microtubule-plasma membrane-cell wall in resistance of plants to the gravitational force, thereby clarifying the mechanism of gravity resistance. In general, resources such as crew time, electrical power, and refrigeration/freezing for space experiments are limited. In particular, given the constraints and priority of ISS assembly requirements, resources available for the Resist Wall experiment will be extremely limited. Also, in the 5th International Research Solicitation for Space Flight Experiments, usable plant species was limited to *Arabidopsis thaliana*. In consideration of these conditions, we have proposed the space experiment using *Arabidopsis* mutants altered in formation of the cortical microtubule-plasma membrane-cell wall continuum.

A number of amino acid substitution mutants in α- or β-tubulins have been isolated in *Arabidopsis* (Hashimoto, 2002). In hypocotyls of these tubulin mutants, the length was shorter, the thickness was larger, and the cell-wall extensibility was smaller than those in wild-type hypocotyls under 1*G* conditions (Matsumoto *et al.*, 2006). Hypergravity suppressed elongation growth, stimulated lateral thickening, and decreased the cell-wall extensibility of wild-type hypocotyls. The degree of such changes was smaller in tubulin mutants than in wild-type, suggesting that tubulin mutants are hypersensitive to the gravitational force. Moreover, tubulin mutants showed either left-handed or right-handed helical growth, derived from disordered organization of cortical microtubules, in hypocotyls even under 1*G* conditions, and such a phenotype was intensified under hypergravity conditions. Similar results were obtained for mutants of some microtubule-associated proteins (MAPs) such as *mor1*, which are essential for normal organization of cortical microtubules. These results support the hypothesis that cortical microtubules play an important role in maintenance of normal growth capacity of plant organs against the gravitational force (Fig. 1).

T-DNA insertion mutants for two HMGR genes have been isolated in *Arabidopsis* (Suzuki *et al.*, 2004). These loss of function mutants showed a decreased sterol level and pleiotropic phenotypes such as dwarfism, early senescence, and sterility. Hypergravity suppressed elongation growth and stimulated lateral expansion of wild-type hypocotyls. In *hmg* mutants, such changes in growth parameters occurred even under 1*G* conditions and hypergravity did not further modify the parameters, suggesting that *hmg* mutants are hypersensitive to the gravitational force (Koizumi *et al.*, 2006). Hypergravity also decreased the cell wall extensibility in wild-type hypocotyls. In *hmg* mutants, the cell wall extensibility was lower than that in wild-type even under 1*G* conditions, and hypergravity did not further decrease it. Thus, membrane sterols may also be involved in maintenance of normal growth capacity of plant organs against the gravitational force, probably via sustaining capacity of cell wall expansion (Fig. 1).

The above-mentioned results obtained with mutants suggest that functions of cortical microtubules, the plasma membrane, and the cell wall in gravity resistance are mutually and deeply dependent, and without normal organization of the continuum of cortical microtubule-plasma membrane-cell wall, plants cannot resist the gravitational force or survive on Earth. To prove this hypothesis, we have originally proposed the space experiment using five related mutant strains. Because of severe limitation of available resources, we have finally decided to select only *tua6* and *hmg1* mutants for the Resist Wall experiment (Fig. 1).

Experimental Design

In the Resist Wall experiment, we will cultivate *tua6* and *hmg1* mutants as well as the wild type Columbia under microgravity and 1*G* conditions in EMCS on ISS up to reproductive stage and compare phenotypes on growth and development using video images. Plants are supplied with nutrients and water automatically during cultivation. Once the experiment is complete, plant materials are chemically fixed or frozen on orbit and collected to Earth. Using collected materials, we will analyze the changes in expression of genes involved in formation of the cortical microtubule-plasma membrane-cell wall continuum, and properties of related cellular components under microgravity conditions.

Pre-flight procedures

Dry seeds (seven each) of Columiba, *tua6*, and *hmg1*, which have been surface-sterilized and selected, will be sown on a polypropylene felt in the Plant Cultivation Chamber (PCC) , developed for MULTIGEN-1 experiment by Dr. T.-H. Iversen. Culture media used is zeolite enriched with MS nutrients. Two sets of PCCs are prepared: one is for microgravity and the other for flight 1*G* control. These PCCs will be stowed and launched at ambient temperature.

On-orbit procedures

On orbit, PCCs will be set into two rotors on EMCS by crew operation. One rotor is kept static (microgravity) and the other is rotated to produce acceleration at 1*G*. Plants are germinated and grown at 23°C in a photoperiodic cycle of 16 h light/8 h dark at 75 Wm^{-2} of white LED. Cultivation starts by water supply and plants are supplied with nutrients and water automatically thereafter. Humidity will be kept at 60 ± 10%. Plants are regularly observed with a video camera by automated system equipped in EMCS. Video images are down-linked to Earth in real time or within a minimum delay. When flower stalks will fully develop (after cultivation for 43 ± 10 days), the ECCs will be taken out of the

EMCS. Plant stems in each ECC will then be cut with scissors by crew operation. A part of plants will be put into the Kennedy Fixation Tube (KFT) and fixed with RNAlater solution. KFTs will be kept at 4˚C for several days and then frozen in a freezer at −20˚C. The remaining plants will be put into Ziploc bags, and frozen at −80˚C. The samples will be kept frozen on orbit and during recovery to earth at −20˚C in the freezer. An identical experiment in terms of materials, hardware, and procedure will be carried out in the laboratory of PI as the ground control.

Post-flight analyses

Two types of data will be obtained in the Resist Wall experiment: video images and plant materials collected. From video images down-linked, we will quantify parameters on growth and development, such as the length and the shape of hypocotyls, roots, flower stalks and siliques, and the size and the number of rosette leaves, cauline leaves and flowers. Based on measured data, we will compare differences in these phenotypes among strains (Columiba, *tua6*, and *hmg1*), cultivation period, and gravity conditions (microgravity, flight control, and ground control). Also, we will extract total RNA from buds, leaves, and young flower stalks of collected plant materials, that had been fixed with RNAlater solution in KFTs on orbit and collected to Earth, and synthesize the first-strand cDNA using commercial preparation kits. The expressions of genes encoding α- and β-tubulins, MAPs (MAP-65, SPRs, MOR1, and katanin), HMGRs, and xyloglucan endotransglucosylase/hydrolases (XTHs) are analyzed with the real-time PCR method. In addition, from the collected frozen plant materials, we will excise flower stalks and prepare materials for determination of the cell wall properties and levels of cellular components. The mechanical properties of the cell walls of thawed flower stalks will be measured by the strain/stress and the stress-relaxation methods with a tensile tester attached to a computer. After measurement, the plant materials will be immediately homogenized and separated by centrifugation. The cell wall constituents are collected by low speed centrifugation and hemicellulosic polysaccharides are extracted with 4 M KOH solution. The levels and the molecular size of xyloglucans are measured colorimetrically and with a gel filtration column on HPLC, respectively. If enough amounts of plant materials will be available, we intend to determine also the levels of tubulins and MAPs by western blotting and those of membrane sterols with GLC and HPLC. In the Resist Wall experiment, the number of plants per PCC is reduced from 16 to 7 due to various limitations. The amounts of plant materials obtained will determine how many parameters can be determined in the experiment.

Expected Results and Significance

As mentioned above, *Arabidopsis* mutants defective in formation of cortical microtubules or the plasma membrane are unable to form the normal cell wall, and therefore, they show disordered growth pattern, such as dwarfism and helical growth, on Earth. The unfavorable phenotypes are intensified and the mutants show a low viability under hypergravity conditions. However, it is 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. In the Resist Wall experiment, we intend to prove this hypothesis using *Arabidopsis tua6* and *hmg1* mutants. As the background of changes in growth and development of the mutants in space, the levels of expression of genes encoding tubulins, MAPs, HMGRs, and XTHs, as well as the levels and biochemical properties of related cellular components, which are greatly increased or modified under hypergravity conditions, should be kept at the same level as the control or changed oppositely under microgravity conditions in space.

We have analyzed the mechanism of gravity resistance mainly using conditions of centrifugal hypergravity. Such ground-based experiments have brought us much important information, as mentioned above. However, hypergravity, after all, is artificial conditions for plants, and we are uncertain whether the results obtained are applicable to normal gravity resistance of plants to 1*G* gravity on Earth. Therefore, we need to clarify the changes induced under microgravity condition in space, which is close to the environment, in terms of gravity, where plant ancestors had been born and stayed until they first went ashore more than 450 million years ago. Moreover, because effects of gravity on growth and development vary in proportion to the logarithm of the magnitude of gravity (Hoson and Soga, 2003), the changes brought about in space (at about $10^{-5}G$) are almost twice as those induced by 300*G* hypergravity. Thus, space experiments are very effective to understand the mechanism of gravity resistance.

Gravity resistance is the major graviresponse in plants, comparable to gravitropism. However, its precise mechanism remains to be clarified. The Resist Wall experiment, which aims to prove the essential role of the continuum of cortical microtubule-plasma membrane-cell wall in gravity resistance, will greatly deepen our knowledge of plant responses to gravity signal. Because the mechanism by which plants respond to other environmental signals such as mechanical forces is often common to that of gravity resistance, the present study will also advance our understanding of the general mechanisms of environmental responses in plants. Also, the role of the cortical microtubule-plasma membrane-cell wall continuum in other physiological functions of plants will also be clarified by the present experiment.

On Earth, plants are forced to use more than a half of energy, which they have fixed from the sun light by photosynthesis, for constructing the touch cell wall to resist the gravitational force. Under microgravity conditions, the accumulation of a significant part of the cell wall materials should not be necessary, and plants would be able to use the corresponding energy for other purposes such as formation of storage products. Thus, information brought about by the present study will enable efficient plant production indispensable for human life not only in space but also on Earth.

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