Dynamics of Actin Filaments in Epidermal Cells of Azuki Bean Epicotyls under Hypergravity Conditions

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Abstract

The effects of hypergravity on growth and dynamics of actin filaments were examined in azuki bean (Vigna angularis) epicotyls. Elongation growth occurred mainly in the apical region of epicotyls, which was inhibited by hypergravity at 300 G. The density of actin filaments in epidermal cells decreased from the apical to the basal regions of epicotyls, irrespective of the gravitational conditions, and in the apical region of epicotyls, hypergravity decreased the density. Actin filaments were arranged with longitudinal or radial direction in the epidermal cells of apical region of 1 G-grown epicotyls. On the other hand, actin filaments with transverse direction were observed in basal region of epicotyls grown at 1 G. Similar changes in the arrangement of actin filaments toward the basal region were observed even under hypergravity conditions. Hypergravity had no effects on the growth and reorientation of cortical microtubules, when actin filaments were disrupted by cytochalasin D treatment. These results suggest that modification of dynamics of actin filaments is responsible for reorientation of cortical microtubules, which leads to inhibition of elongation growth in azuki bean epicotyls under hypergravity conditions.

Introduction

Plants have developed a response, termed ‘gravity resistance’ (Hoson and Soga, 2003), to resist the gravitational force for survival during their evolution on the earth’s terrestrial habitats. It is effective to analyze the responses of plants when they are treated with different gravitational conditions for clarifying the mechanism of gravity resistance. Based on this point of view, the responses of various plant materials to hypergravity, the gravitational acceleration of more than 1 G, have been observed. Hypergravity treatments have been shown to inhibit elongation growth and promote lateral expansion of pea epicotyls (Waldron and Brett, 1990), radish and cucumber hypocotyls (Kasahara et al., 1995), garden cress hypocotyls (Hoson et al., 1996), azuki bean epicotyls (Soga et al., 1999a), maize coleoptiles and mesocotyls (Soga et al., 1999b), Arabidopsis hypocotyls (Soga et al., 2001) and inflorescence stalks (Nakabayashi et al., 2006), and wheat coleoptiles (Wakabayashi et al., 2005). Based on the results of hypergravity experiments, it was hypothesized that elongation growth would be promoted whereas lateral expansion would be inhibited in shoot organs under microgravity conditions in space. As hypothesized, microgravity was shown to promote elongation growth and inhibit lateral expansion of rice coleoptiles (Hoson et al., 2002), Arabidopsis hypocotyls (Soga et al., 2002, 2018a) and inflorescence stalks (Hoson et al., 2014), and azuki bean epicotyls (Soga et al., 2014). These results indicate that the development of a short and thick body is considered as a key response that enables plants to grow against the gravitational force.

In plant cells, two major types of cytoskeleton, microtubules and actin filaments, are involved in the cellular rearrangements. Both microtubules and actin filaments have many cellular functions, and have important roles in growth and development of plants. The shape of whole plant body is predominantly regulated by anisotropic growth of individual cells. Cortical microtubules, a characteristic cytoskeleton in interphase cells of plants, are likely involved in the modification of growth anisotropy of cells through the regulation of the orientation of cellulose microfibrils (Shibaoka, 1994; Baskin, 2001; Paredez et al., 2006). We have shown that the gravitational force affected the orientation of cortical microtubules during modification of anisotropic growth of stems. Hypergravity, which inhibited elongation growth and promoted lateral growth in shoots, increased cortical microtubules with longitudinal orientation, but decreased those with transverse orientation in azuki bean epicotyls (Soga et al., 2006) and Arabidopsis hypocotyls (Matsumoto et al., 2010; Murakami et al., 2016). On the other hand, microgravity, which promoted elongation growth and inhibited lateral growth in shoots, decreased cortical microtubules with longitudinal orientation, but increased those with transverse orientation in Arabidopsis hypocotyls (Soga et al., 2018a).

Actin filaments are also involved in the regulation of cell growth. Thimmann et al. (1992) reported that the disruption of actin filaments by cytochalasin D induce suppression of elongation growth, and the integrity of actin filaments is correlated with elongation growth in segments of oat coleoptiles. These results suggest that changes in the dynamics of actin filaments are involved in the regulation of cell growth of plants. In addition, it has been shown that actin filaments are required for normal gravitropism (Morita, 2010). However, roles of actin filaments in gravity resistance have not been examined yet. To clarify the roles of actin filaments in gravity resistance, the dynamics of actin filaments were examined in azuki bean epicotyls.
resistance, dynamics of actin filaments under hypergravity conditions were analyzed in azuki bean epicotyls. We also examined the effects of disruption of actin filaments by cytochalasin D on the hypergravity induced changes in growth and orientation of cortical microtubules.

Materials and Methods

Plant materials and growth conditions

Seeds of azuki bean (Vigna angularis (Willd.) Ohwi et Ohashi ‘Erimowase’) were soaked in running tap water for 1.5 days at 30°C and they were allowed to germinate on gauze spread on a plastic dish filled with water at 25°C in the dark. After 3 days, seedlings with an epicotyl about 45 mm long were selected, and then five 8-mm regions (A to E from the top) in epicotyls were marked with India ink. The marked seedlings were exposed to basipetal hypergravity at 300 G with a centrifuge (H-28-F, Kokusan Co., Japan) for 5 h in the dark. After grown at 1 G or 300 G conditions, the lengths of the marked regions were measured using a scale, and then the regions were excised. All manipulations were done under dim green light.

For the treatment of the actin disrupting reagent cytochalasin D, 15-mm long cuttings prepared from the seedlings were used, because actin filaments were not disrupted in epicotyls of intact seedlings that cytochalasin D was applied from roots. The cuttings were wrapped in a wiper cloth (Kimwiper S-200, Nippon Paper Crecia Co., Japan) soaked in 1 mM MES-KOH buffer solution (pH 6.0) and 1% (v/v) dimethyl sulfoxide with or without various concentration of cytochalasin D. Cytochalasin D at 3 μM caused fragmentation of actin filaments, and actin filaments were not observed in epidermal cells of 10 μM cytochalasin D-treated cuttings. Thus, we used 10 μM cytochalasin D for further analysis. After 10 μM cytochalasin D treatment for 2 h, a 8-mm subhook region corresponding to region A in an intact seedling was marked with India ink. The marked cuttings were exposed to basipetal hypergravity at 300 G in the presence or absence of 10 μM cytochalasin D. After the incubation, the length of the marked regions was measured using a scale, and then segments were excised from the regions. All manipulations were done under dim green light.

Microscopy

The segments excised from azuki bean epicotyls were immediately fixed with 4% (w/v) paraformaldehyde in PMEG buffer (50 mM PIPES, 1 mM MgSO4, 5 mM EGTA, 1% (v/v) glycerol, pH 6.8) at 25°C for 1 h after infiltration with a vacuum pump for 10 min. The air pressure was restored, and the samples were washed four times (15 min each) with phosphate-buffered saline (PBS) at 25°C. The epidermal strips were peeled from the segments. For the observation of actin filaments, the specimens were treated with Alexa Fluor 488 phalloidin (A12379, Thermo Fisher Scientific, USA) diluted 1:30 (v/v) in PBS at 25°C for 20 min, and washed with PBS. For the observation of cortical microtubules, the specimens were treated with a detergent solution (1% (v/v) Nonidet P-40 in PBS) for 30 min, and then washed four times (15 min each) with PBS. They were incubated in a blocking solution (1% (w/v) bovine serum albumin in PBS) at 37°C for 30 min and washed with PBS. These specimens were treated with FITC-conjugated monoclonal antibody against α-tubulin (F2168, Sigma, USA) diluted 1:100 (v/v) in PBS at 37°C for 20 min, and washed with PBS. Fluorescence images were collected with a fluorescence microscope (Axio Imager. A1; Carl Zeiss, Germany) equipped with a cooled CCD camera (VB-7000; Keyence, Japan) or a confocal microscope (SP8; Leica Microsystems, Germany).

The density of actin filaments adjacent to the outer tangential wall of each epidermal cell was analyzed by using ImageJ software (http://rsbweb.nih.gov/ij/, NIH). The density (pixel occupancy) of actin filaments was defined as the percentages of pixels above the brightness of background (cytoplasm) per cell. As to the orientation of cortical microtubules, we determined the frequency of cells with cortical microtubules within a range of 0-20° (longitudinal), 20-70° (oblique), 70-90° (transverse) to the longitudinal cell axis, and in a variety of directions (random).

Results and Discussion

The left panel of Fig. 1 shows elongation growth of azuki bean epicotyls grown at 1 G and 300 G. Elongation growth was large in the apical region (A), and that decreased from the apical to the basal regions. Hypergravity at 300 G significantly inhibited elongation growth only in the apical region. We reported previously that hypergravity clearly promoted lateral expansion in the apical region of azuki bean epicotyls (Nakano et al., 2007). Also, hypergravity-induced modification of the growth anisotropy mainly occurred in upper region of Arabidopsis hypocotyls (Murakami et al., 2016). These results indicate that the upper regions have larger capacity to modify the growth anisotropy in response to the changes in the magnitude of gravity.

The middle panel of Fig. 1 shows representative fluorescence micrographs of actin filaments stained with Alexa Fluor 488 phalloidin in epidermal cells of epicotyls grown at 1 G and 300 G. The density of actin filaments tended to be different between the apical and basal region of epicotyls. Thus, we analyzed the density per cell along epicotyls. Under 1 G conditions, the density of actin filaments in cells decreased from the apical to the basal regions of epicotyls (Right panel of Fig. 1). The density in basal region was ca. 20% of that in apical region. Under hypergravity conditions, the filament density in cells also decreased from the apical to the basal regions of epicotyls (Right panel of Fig. 1). Hypergravity significantly decreased the density of actin filaments in the apical region (A), whose growth anisotropy was modified clearly by hypergravity. On the other hand, in regions B to E, the density did not differ between epicotyls grown at 1 G and those at 300 G. The amounts of elongation growth were positively correlated with the density of actin filaments (Spearman’s correlation coefficient: r_s=0.936, P<0.05). Such relationship between the density of actin filaments
In addition to the density of actin filaments, the directional arrangement of actin filaments tended to be different between the apical and basal region of epicotyls. In the apical regions of epicotyls grown at 1 G, actin filaments with longitudinal or radial direction were mainly observed (Middle panel of Fig. 1). On the other hand, and plant growth has not been clarified yet. Recently, Li et al., (2018) reported that elongation growth of rice pollen tube was suppressed, when the density of actin filaments was decreased. Thus, regulation of the density of actin filaments may be involved in the modification of growth anisotropy by hypergravity in azuki bean epicotyls.
Actin Filament Dynamics in Azuki Bean under Hypergravity

Actin filaments with transverse direction were observed in basal region of epicotyls grown at 1 G. These results suggest that the directional arrangement of actin filaments changes with aging in azuki bean epicotyls. Seagull et al. (1987) showed that the arrangement of actin filaments varied from random to longitudinal direction with aging in alfalfa cell suspension culture. Even under hypergravity conditions, the changes in the directional arrangement of actin filaments toward the basal regions were similar to those in 1 G control (Middle panel of Fig. 1). Taken together, these results indicate that hypergravity did not affect the arrangement, although the arrangement of actin filaments varies with aging.

In order to further confirm the contribution of the changes in the density of actin filaments to the hypergravity-induced change in growth of epicotyl, we examined the effects of cytochalasin D, an actin-disrupting reagent, on elongation growth and reorientation of cortical microtubules of epicotyls grown at 300 G. Elongation growth of epicotyls grown at 1 G was inhibited by the treatment with cytochalasin D in dose-dependent manner. Cytochalasin D at 10 μM completely disrupted actin filaments and significantly inhibited elongation growth compared with that in the absence of cytochalasin D under 1 G conditions (Left panel of Fig. 2). Hypergravity did not affect elongation growth of epicotyls in the presence of 10 μM cytochalasin D, although it significantly inhibited elongation in the absence of cytochalasin D. These results suggest that cytochalasin D-treated epicotyls are hypersensitive to the gravitational force and the effects of gravity are saturated at 1 G. In the several models of graviperception in gravitropism, tension produced by actin filaments has been thought to be involved in the graviperception (Morita, 2010). We have shown that graviperception mechanism in gravity resistance may be different from that in gravitropism (Soga, 2010, 2013). These facts suggest that roles of actin filaments may be different between gravity resistance and gravitropism, even if actin filaments are involved in the graviperception of both gravity resistance and gravitropism. Anyway, these results support the hypothesis that actin filaments are involved in the modification of growth anisotropy by hypergravity in azuki bean epicotyls.

The effects of cytochalasin D on orientation of cortical microtubules of epicotyls were examined under 1 G or 300 G conditions (Right panel of Fig. 2). Four types of cells with longitudinal, oblique, transverse, or random microtubule orientation were observed, regardless of hypergravity or cytochalasin D treatment. In the absence of cytochalasin D, cells having transverse or longitudinal microtubules were predominant in epicotyls grown at 1 G.
or 300 G, respectively. The distribution pattern of four types of cells was significantly different between 1 G and 300 G conditions. On the other hand, in the presence of cytochalasin D, frequency of cells having longitudinal, oblique, or transverse was almost the same in epicotyls grown at 1 G. Furthermore, hypergravity did not induce reorientation of cortical microtubules in the presence of cytochalasin D. Thus, the distribution pattern of four types of cells was identical between 1 G and 300 G conditions. We have reported that the hypergravity-induced modification of growth anisotropy may be mediated by reorientation of cortical microtubules from transverse to longitudinal directions in azuki bean epicotyls (Soga et al., 2006) and Arabidopsis hypocotyls (Matsumoto et al., 2010; Murakami et al., 2016). Also, Sampathkumar et al. (2011) showed that the modification of dynamics of actin filaments may be associated with orientation of cortical microtubules in Arabidopsis hypocotyls. Taken together, these results suggest that modification of dynamics of actin filaments is responsible for reorientation of cortical microtubules, which leads to inhibition of elongation growth in azuki bean epicotyls under hypergravity conditions.

Hypergravity inhibited elongation growth and promoted lateral expansion in shoot organs (Soga, 2010, 2013). On the other hand, microgravity promoted elongation growth and inhibited lateral expansion in shoot organs (Soga et al., 2018b). The modification of growth anisotropy by both microgravity and hypergravity may be mediated by reorientation of cortical microtubules. Present results suggest that hypergravity-induced decrease in the density of actin filaments may induce reorientation of cortical microtubules. Therefore, the density of actin filaments may be increased under microgravity conditions in space, which may contribute to the modification of dynamics of cortical microtubules and growth anisotropy.

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References

Baskin, T. I. (2001) On the alignment of cellulose microfibrils by cortical microtubules: A review and a model. Protoplasma, 215, 150-171.

Hoson, T., Nishitani, K., Miyamoto, K., Ueda, J., Kamisaka, S., Yamamoto, R. and Masuda, Y. (1996) Effects of hypergravity on growth and cell wall properties of cress hypocotyls. J. Exp. Bot., 47, 513-517.

Hoson, T., Soga, K., Mori, R., Saiki, M., Nakamura, Y., Wakabayashi, K. and Kamisaka, S. (2002) Stimulation of elongation growth and cell wall loosening in rice coleoptiles under microgravity conditions in space. Plant Cell Physiol., 43, 1067-1071.

Hoson, T. and Soga, K. (2003) New aspects of gravity responses in plant cells. Int. Rev. Cytol., 229, 209-244.

Hoson, T., Soga, K., Wakabayashi, K., Hashimoto, T., Karahara, I., Yano, S., Tanigaki, F., Shimazu, T., Kasahara, H., Masuda, D. and Kamisaka, S. (2014) Growth stimulation in inflorescences of an Arabidopsis tubulin mutant under microgravity conditions in space. Plant Biol., 16(S1), 91-96.

Kasahara, H., Shiwa, M., Takeuchi, Y. and Yamada, M. (1995) Effects of hypergravity on elongation growth in radish and cucumber hypocotyls. J. Plant Res., 108, 59-64.

Li, G., Yang, X., Zhang, X., Song, Y., Liang, W. and Zhang, D. (2018) Rice morphology determinant-mediated actin filament organization contributes to pollen tube growth. Plant Physiol., 177, 255-270.

Matsumoto, S., Kumasaki, S., Soga, K., Wakabayashi, K., Hashimoto, T. and Hoson, T. (2010) Gravity-induced modifications to development in hypocotyls of Arabidopsis tubulin mutants. Plant Physiol., 152, 918-926.

Morita, M. T. (2010) Directional gravity sensing in gravitropism. Annu. Rev. Plant Biol., 61, 705-720.

Murakami, M., Soga, K., Kotake, T., Kato, T., Hashimoto, T., Wakabayashi, K. and Hoson, T. (2016) Roles of MAP65-1 and BPP1 in gravity resistance of Arabidopsis hypocotyls. Biol. Sci. Space, 30, 1-7.

Nakabayashi, I., Karahara, I., Tamaoki, D., Masuda, K., Wakasugi, T., Yamada, K., Soga, K., Hoson, T. and Kamisaka, S. (2006) Hypergravity stimulus enhances primary xylem development and decreases mechanical properties of secondary cell walls in inflorescence stems of Arabidopsis thaliana. Ann. Bot., 97, 1083-1090.

Nakano, S., Soga, K., Wakabayashi, K. and Hoson, T. (2007) Different cell wall polysaccharides are responsible for gravity resistance in the upper and the basal regions of azuki bean epicotyls. Biol. Sci. Space, 21, 113-116.

Paredes, A. R., Somerville, C. R. and Ehrhardt, D. W. (2006) Visualization of cellulose synthase demonstrates functional association with microtubules. Science, 312, 1491-1495.

Sampathkumar, A, Lindeboom, J. J., Debolt, S., Gutierrez, R., Ehrhardt, D. W., Ketelaar, T. and Persson, S. (2011) Live cell imaging reveals structural associations between the actin and microtubule cytoskeleton in Arabidopsis. Plant Cell, 23, 2302-2313.

Seagull, R. W., Falconer, M. M. and Weerdenburg, C. A. (1987) Microfilaments: dynamic arrays in higher plant cells. J. Cell Biol., 104, 995-1004.

Shibaoka, H. (1994) Plant hormone-induced changes in the orientation of cortical microtubules: Alterations in the cross-linking between microtubules and the plasma membrane. Ann. Rev. Plant Physiol. Plant Mol. Biol., 45, 527-544.

Soga, K., Wakabayashi, K., Hoson, T. and Kamisaka, S. (1999a) Hypergravity increases the molecular size of xyloglucans by decreasing xyloglucan-degrading activity in azuki bean epicotyls. Plant Cell Physiol., 40, 581-585.
Soga, K., Harada, K., Wakabayashi, K., Hoson, T. and Kamisaka, S. (1999b) Increased molecular mass of hemicellulosic polysaccharides is involved in growth inhibition of maize coleoptiles and mesocotyls under hypergravity conditions. J. Plant Res., 112, 273-278.

Soga, K., Wakabayashi, K., Hoson, T. and Kamisaka, S. (2001) Gravitational force regulates elongation growth of Arabidopsis hypocotyls by modifying xyloglucan metabolism. Adv. Space Res., 27, 1011-1016.

Soga, K., Wakabayashi, K., Kamisaka, S. and Hoson, T. (2002) Stimulation of elongation growth and xyloglucan breakdown in Arabidopsis hypocotyls under microgravity conditions in space. Planta, 215, 1040-1046.

Soga, K., Wakabayashi, K., Kamisaka, S. and Hoson, T. (2006) Hypergravity induces reorientation of cortical microtubules and modifies growth anisotropy in azuki bean epicotyls. Planta, 224, 1485-1494.

Soga, K. (2010) Gravity resistance in plants. Biol. Sci. Space, 24, 129-134.

Soga, K. (2013) Resistance of plants to gravitational force. J. Plant Res., 126, 589-596.

Soga, K., Biology Club, Kurita, A., Yano, S., Ichikawa, T., Kamada, M. and Takaoki, M. (2014) Growth and morphogenesis of azuki bean seedlings in space during SSAF2013 program. Biol. Sci. Space, 28, 6-11.

Soga, K., Yamazaki, C., Kamada, M., Tanigawa, N., Kasahara, H., Yano, S., Kojo, K. H., Kutsuna, N., Kato, T., Hashimoto, T., Kotake, T., Wakabayashi, K. and Hoson T. (2018a) Modification of growth anisotropy and cortical microtubule dynamics in Arabidopsis hypocotyls grown under microgravity conditions in space. Physiol. Plant., 162, 135-144.

Soga, K., Wakabayashi, K. and Hoson, T. (2018b) Growth and cortical microtubule dynamics in shoot organs under microgravity and hypergravity conditions. Plant Signal. Behav., 13, e1422468.

Thimann, K. V., Reese, K. and Nachmias, V. T. (1992) Actin and the elongation of plant cells. Protoplasma, 171, 153-166.

Wakabayashi, K., Soga, K., Kamisaka, S. and Hoson, T. (2005) Changes in levels of cell wall constituents in wheat seedlings grown under continuous hypergravity conditions. Adv. Space Res., 36, 1292-1297.

Waldron, K. W. and Brett, C. T. (1990) Effects of extreme acceleration on the germination, growth and cell wall composition of pea epicotyls. J. Exp. Bot., 41, 71-77.