

Roles of MAP65-1 and BPP1 in Gravity Resistance of *Arabidopsis* hypocotyls

Mana Murakami¹, Kouichi Soga^{1*}, Toshihisa Kotake², Takehide Kato³, Takashi Hashimoto³, Kazuyuki Wakabayashi¹, Takayuki Hoson¹

¹Graduate School of Science, Osaka City University, Sumiyoshi-ku, Osaka 558-8585, Japan

²Graduate School of Science and Engineering, Saitama University, Sakura-ku, Saitama 338-8570, Japan

³Graduate School of Biological Sciences, Nara Institute of Science and Technology, Ikoma, Nara 630-0192, Japan

Abstract

The present experiment aims to clarify the roles of 65 kDa microtubule-associated protein-1 (MAP65-1) and basic proline-rich protein1 (BPP1), which are involved in the maintenance of transverse microtubule orientation, in gravity resistance, using green fluorescent protein (GFP)-expressing *Arabidopsis* lines. Hypergravity at 300 G inhibited elongation growth and promoted lateral expansion of epidermal cells in the subapical region of hypocotyls in GFP-MAP65-1 line expressing by native promoter and BPP1-GFP line expressing by a constitutive cauliflower mosaic virus 35S promoter. In BPP1-GFP line, hypergravity showed smaller effects on modification of growth anisotropy than wild type. Also, hypergravity induced reorientation of cortical microtubules from transverse to longitudinal directions in both lines. However, in BPP1-GFP line, hypergravity showed smaller effects on reorientation of cortical microtubules. When the expression levels of MAP65-1 were determined by analyzing GFP fluorescence in hypocotyls of GFP-MAP65-1 line, hypergravity decreased the levels of MAP65-1 in the subapical region, where hypergravity modified growth anisotropy and orientation of cortical microtubules. These results indicate that the regulation of levels of MAP65-1 and BPP1 is involved in the hypergravity-induced reorientation of cortical microtubules, which may lead to modification of growth anisotropy, thereby developing a tough body against the gravitational force in *Arabidopsis* hypocotyls. ©2016 Jpn. Soc. Biol. Sci. Space; doi: 10.2187/bss.30.1

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*To whom correspondence should be addressed:

Tel./Fax: +81-(0)6-6605-3686;

E-mail: soga@sci.osaka-cu.ac.jp

Introduction

The development of a tough body to resist the gravitational force is a critical response for plants living on land. This phenomenon is termed 'gravity resistance' (Hoson and Soga, 2003) and hypergravity generated from centrifugation has been utilized for the assessment of the nature and mechanism of gravity resistance (Soga, 2010, 2013). Plants developed a short and thick body under hypergravity conditions (Hoson *et al.*, 1996; Soga *et al.*, 1999a, 1999b, 2001; Wakabayashi *et al.*, 2005; Nakano *et al.*, 2007). On the other hand, plant body became longer and thinner under microgravity conditions in space (Soga *et al.*, 2001, 2002, 2014; Hoson *et al.*, 2002). Therefore, modification of body shape in response to the magnitude of the gravitational force is considered as a key part of gravity resistance.

The body shape of a plant depends predominantly on the shape of individual cells and the direction of cell expansion. Cortical microtubules, a characteristic structure in interphase cells of plants, are assumed to be responsible for anisotropic expansion of plant cells by directing the orientation of cellulose microfibrils (Shibaoka, 1994; Baskin, 2001; Paredez *et al.*, 2006). Hypergravity induced reorientation of cortical microtubules from transverse to longitudinal directions in azuki bean epicotyls (Soga *et al.*, 2006) and *Arabidopsis* hypocotyls (Matsumoto *et al.*, 2010). Microtubule-associated proteins (MAPs) bind to microtubules and regulate their dynamics, stability, and organization (Gardiner, 2013; Hamada, 2014). It is reported that 65 kDa microtubule-associated proteins (MAP65s) and basic proline-rich protein1 (BPP1) are required for the maintenance of transverse microtubule orientation (Sawano *et al.*, 2000; Hamada *et al.*, 2013). We produced green fluorescent protein (GFP) lines in which GFP was fused with these MAPs in *Arabidopsis*. To clarify the roles of MAP65-1 and BPP1 in gravity resistance, the modifications in growth and microtubule orientation in the GFP lines under hypergravity conditions were analyzed. We also examined the effects of hypergravity on the expression levels of MAP65-1 by measuring strength of GFP fluorescence.

Materials and Methods

Plant materials and growth conditions

Two GFP-expressing lines of *Arabidopsis thaliana* Columbia [Col-0] ecotype were used in the present study: GFP-MAP65-1 expressing by native promoter and BPP1-GFP expressing by a constitutive cauliflower mosaic virus 35S promoter. As to GFP-MAP65-1, a genomic fragment of MAP65-1 gene (5 kbp) including 1.4 kbp of upstream sequence was amplified with a set of primers, gMAP65-1-F (5'-GAAAAGCTTC GACGAGGAGCGTGTGAAAG-3') and gMAP65-1-R (5'-CACGAATTCGGCTTTTGTGGAAGACAC-3'). The DNA fragment was digested with HindIII and EcoRI and subcloned into the binary vector pBI121. The restriction site of BamHI was introduced at the N-terminus, to which a fragment encoding for GFP was inserted to yield

the construct, GFP-MAP65-1/pBI121. The construct was introduced into Arabidopsis by an Agrobacterium (*Rhizobium radiobacter*, EHA105 strain)-mediated method. As to BPP1-GFP, the details were described previously (Hamada *et al.*, 2013).

The seeds were sterilized in 2% (v/v) sodium hypochlorite solution for 1 min, and then washed thoroughly with water. The sterilized seeds were planted on 1.5% (w/v) agar medium in a 50 ml centrifuge tube, kept at 4°C for 3 days, and exposed to white light (40 $\mu\text{mol s}^{-1}\text{m}^{-2}$ at seed level) for 6 h to induce germination, and then they were grown at 25°C in the dark for 42 h. To analyze the growth behavior, we exposed the seedlings to hypergravity at 300 G for 24 h at 25°C in the dark with a centrifuge (H-28-F; Kokusan, Tokyo, Japan). After the treatment, we measured the length of hypocotyls using a scale, and the diameter of hypocotyls with a digital multi-angle stereoscopic microscope system (VB-G25; Keyence, Osaka, Japan). Cell length was measured by a fluorescence microscope (Axio Imager. A1; Carl Zeiss, Göttingen, Germany) equipped with a cooled CCD camera (VB-7000, Keyence, Osaka, Japan). The data of hypocotyl length, cell length and hypocotyl diameter (Figs. 1-3) were analyzed by the Student's t-test.

Microscopy

For the observation of cortical microtubules, whole seedlings were fixed with a mixture of 1.5% (v/v) formaldehyde and 0.5% (v/v) glutaraldehyde in PEMT buffer (50 mM PIPES, 2 mM EGTA, 2 mM MgSO_4 , 0.05% (v/v) Triton X-100, pH 7.2) for 40 min and rinsed three times in PEMT buffer. They were subsequently treated with 0.05% (w/v) Pectolyase Y-23 (Kyowa Chemical Products, Osaka, Japan) and 0.05% (w/v) Cellulase Y-C (Kyowa Chemical Products, Osaka, Japan) in PEMT buffer with 0.4 M mannitol for 20 min at 30°C, and then rinsed three times in PEMT buffer. After incubating in 0.5% Triton X-100 for 1 h, seedlings were incubated with primary antibodies against α -tubulin (product T6199; Sigma, St. Louis, USA) diluted 1:1000 (v/v) in 1% BSA at 30°C for 16 h, and rinsed three times with PEMT buffer. Seedlings were then incubated with Cy3-conjugated anti-mouse IgG (product C2181, Sigma, St. Louis, USA) diluted 1:100 (v/v) in 1% BSA for 3 h at 37°C, and rinsed three times with PBS. They were mounted on a slide glass and covered with a solution containing 90% (v/v) glycerol. Fluorescence images were collected with a fluorescence microscope (Axio Imager. A1; Carl Zeiss, Göttingen, Germany) equipped with a cooled CCD camera (VB-7000, Keyence, Osaka, Japan), and processed with bundled image processing software (BZ-H1A, Keyence, Osaka, Japan).

The orientation of cortical microtubules adjacent to the outer tangential wall of each epidermal cell was determined. In most cells, the orientation of cortical microtubules was uniform, although it varied with the cell. Thus, we recorded the orientation of cortical microtubules in each cell. We determined the frequency of cells with cortical microtubules within a range of 90-70° (transverse), 70-20° (oblique), 20-0° (longitudinal) to the longitudinal

cell axis, and in a variety of directions (random).

For the observation of GFP-MAP65-1 and BPP1-GFP, seedlings were immediately mounted on a slide glass and covered with a solution containing 90% (v/v) glycerol after hypergravity treatment. Fluorescence images were collected and analyzed as described above. We also collected fluorescence images by a confocal microscope (SP8; Leica Microsystems GmbH, Wetzlar, Germany) for measuring intensity of GFP. The intensity of GFP was determined using ImageJ software (<http://rsbweb.nih.gov/ij/>, NIH). The data of GFP intensity (Fig. 5) were analyzed by the Student's t-test.

Results and Discussion

Figure 1 shows the length of hypocotyls of wild type, GFP-MAP65-1 line, and BPP1-GFP line grown at 1 G and 300 G. The length of wild type and both GFP line hypocotyls was significantly smaller in seedlings grown under hypergravity conditions than that of 1 G at 5% level. Hypocotyl length of wild type and GFP-MAP65-1 line under hypergravity conditions was ca. 80% of 1 G control. On the other hand, in BPP1-GFP line, hypocotyl length grown under hypergravity conditions

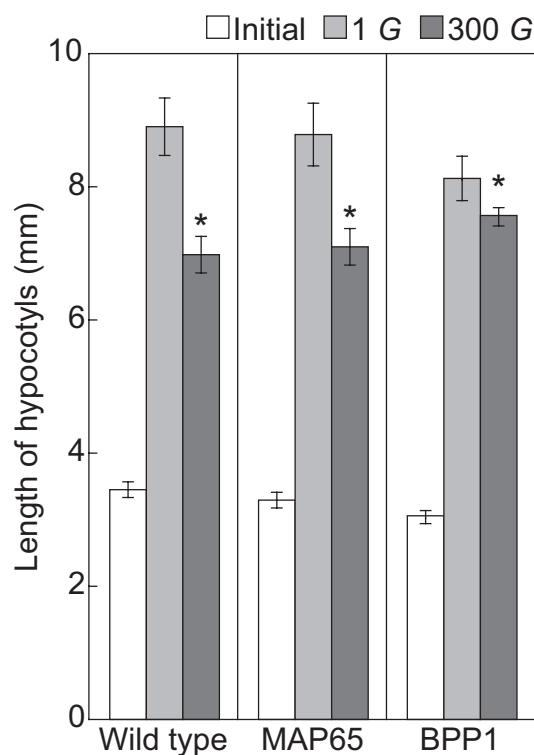


Fig. 1. Effects of hypergravity on hypocotyl length of Arabidopsis. Wild type and two GFP-expressing lines (GFP-MAP65-1 expressing by native promoter (MAP65) and BPP1-GFP expressing by a constitutive cauliflower mosaic virus 35S promoter (BPP1)) were grown on agar medium at 1 G for 48 h at 25°C. Seedlings were then transferred to 1 G or 300 G conditions, and grown for a further 24 h at 25°C. The length was measured using a scale. Values are means \pm SE ($n = 20$). *, Mean values with significant differences between 1 G and 300 G treatments ($P < 0.05$).

was ca. 90% of 1 G control. BPP1, which is required for the maintenance of transverse microtubule orientation (Hamada *et al.*, 2013), was constitutively overexpressed under the control of the cauliflower mosaic virus 35S promoter in BPP1-GFP line used in the present study. Thus, the overexpression of BPP1 may be responsible for the smaller effects of hypergravity on the growth of hypocotyls. In any case, both GFP-MAP65 and BPP1-GFP lines used in the present study responded to the gravitational force, indicating that we can utilize both lines for analysis of gravity resistance.

Gendreau *et al.* (1997) reported that elongation growth of epidermal cells mainly occurred in apical and subapical regions of etiolated *Arabidopsis* hypocotyls. To analyze the growth behavior in detail, we analyzed cell length in three regions of hypocotyls, apical (just below the hook), subapical (ca. 20% below the hook in hypocotyl length), and basal (ca. 85% below the hook in hypocotyl length) regions. During incubation, elongation

growth occurred at all regions in wild type and both GFP lines, irrespective of gravitational conditions, although the amount of growth in subapical region was remarkable (Fig. 2). Hypergravity suppressed elongation growth only in subapical region. Similarly, Matsumoto *et al.* (2010) showed that hypergravity-induced suppression of elongation growth mainly occurred at subapical region of *Arabidopsis* hypocotyls. In wild type and GFP-MAP65-1 line, hypergravity suppressed elongation growth by 65% in subapical region (Fig. 2). However, the degree of hypergravity-induced suppression of growth of BPP1-GFP line was almost a half of that in wild type and GFP-MAP65-1 line. The length of hypocotyls was correlated with amount of cell elongation in subapical region (Figs. 1 and 2). No cell division occurs in hypocotyls after germination (Gendreau *et al.*, 1997). Thus, hypergravity-induced decrease in the length of hypocotyls may be due to suppression of elongation growth in subapical region.

Figure 3 shows the changes in the diameter of

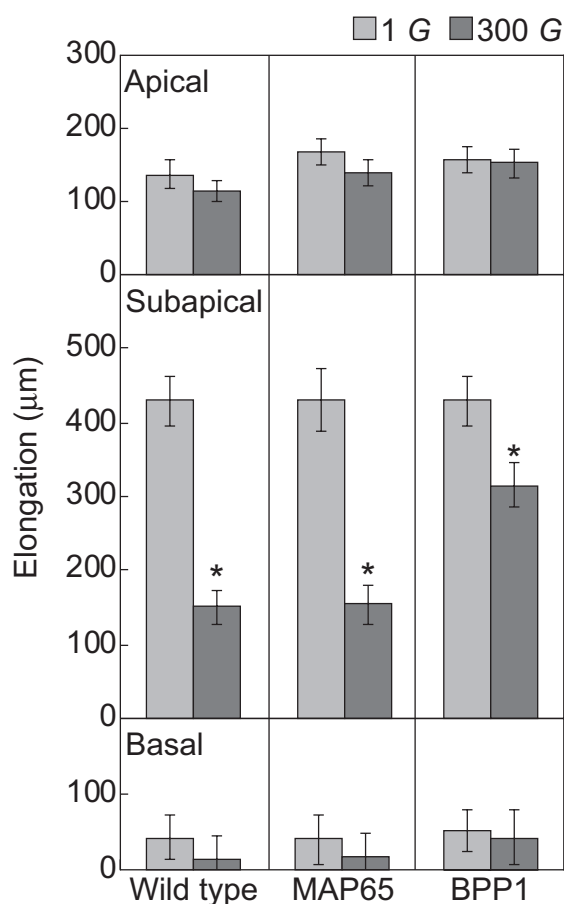


Fig. 2. Effects of hypergravity on cell elongation of epidermal cells in *Arabidopsis* hypocotyls. Wild type and GFP-expressing lines were grown as in Figure 1. The length of cells was measured with a fluorescence microscope and elongation for 24 h was calculated. Values are means \pm SE (n = 20). *, Mean values with significant differences between 1 G and 300 G treatments ($P < 0.05$).

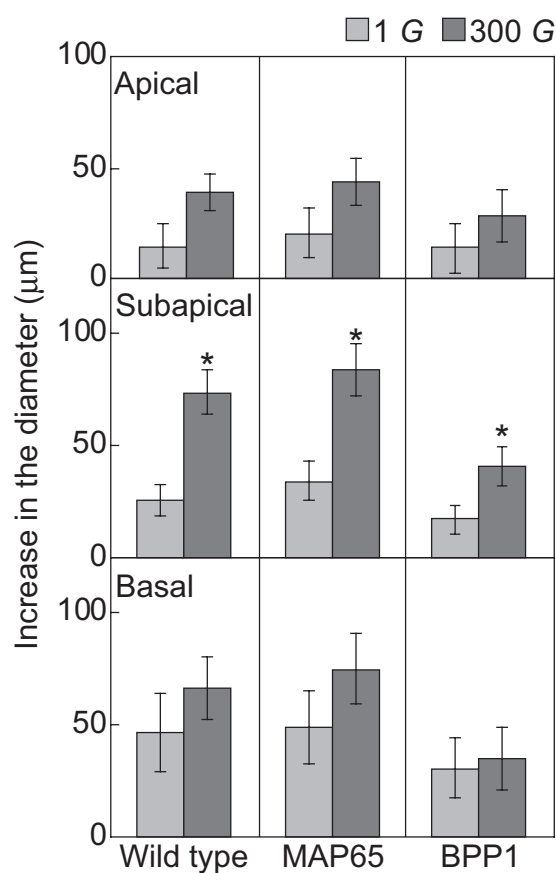


Fig. 3. Effects of hypergravity on lateral thickening in hypocotyls of *Arabidopsis*. Wild type and GFP-expressing lines were grown as in Figure 1. The diameter of hypocotyls was measured with a digital stereoscopic microscope and increase in the diameter for 24 h was calculated. Values are means \pm SE (n = 20). *, Mean values with significant differences between 1 G and 300 G treatments ($P < 0.05$).

hypocotyls grown at 1 G or 300 G conditions. During incubation, the diameter was increased at all regions in wild type and both GFP lines, irrespective of gravitational conditions. From the apical to toward the basal regions, the diameter of hypocotyls was increased. Hypergravity induced significant lateral expansion in subapical region at 5 % level (Fig. 3). In other regions, apical and basal regions, the diameter tended to be slightly increased by hypergravity. Hypergravity caused significant suppression of elongation growth only in subapical region at 5 % level

(Fig. 2). These results suggest that significant changes in growth anisotropy by hypergravity occurred only at subapical region of Arabidopsis hypocotyls. The degree of hypergravity-induced suppression of elongation growth and lateral expansion of BPP1-GFP line in subapical region was almost a half of those of wild type and GFP-MAP65-1 line (Figs. 2 and 3). Thus, regulation by gravity of BPP1 expression in an appropriate range may be required for the suitable modification of growth anisotropy. The orientation of cortical microtubules adjacent to

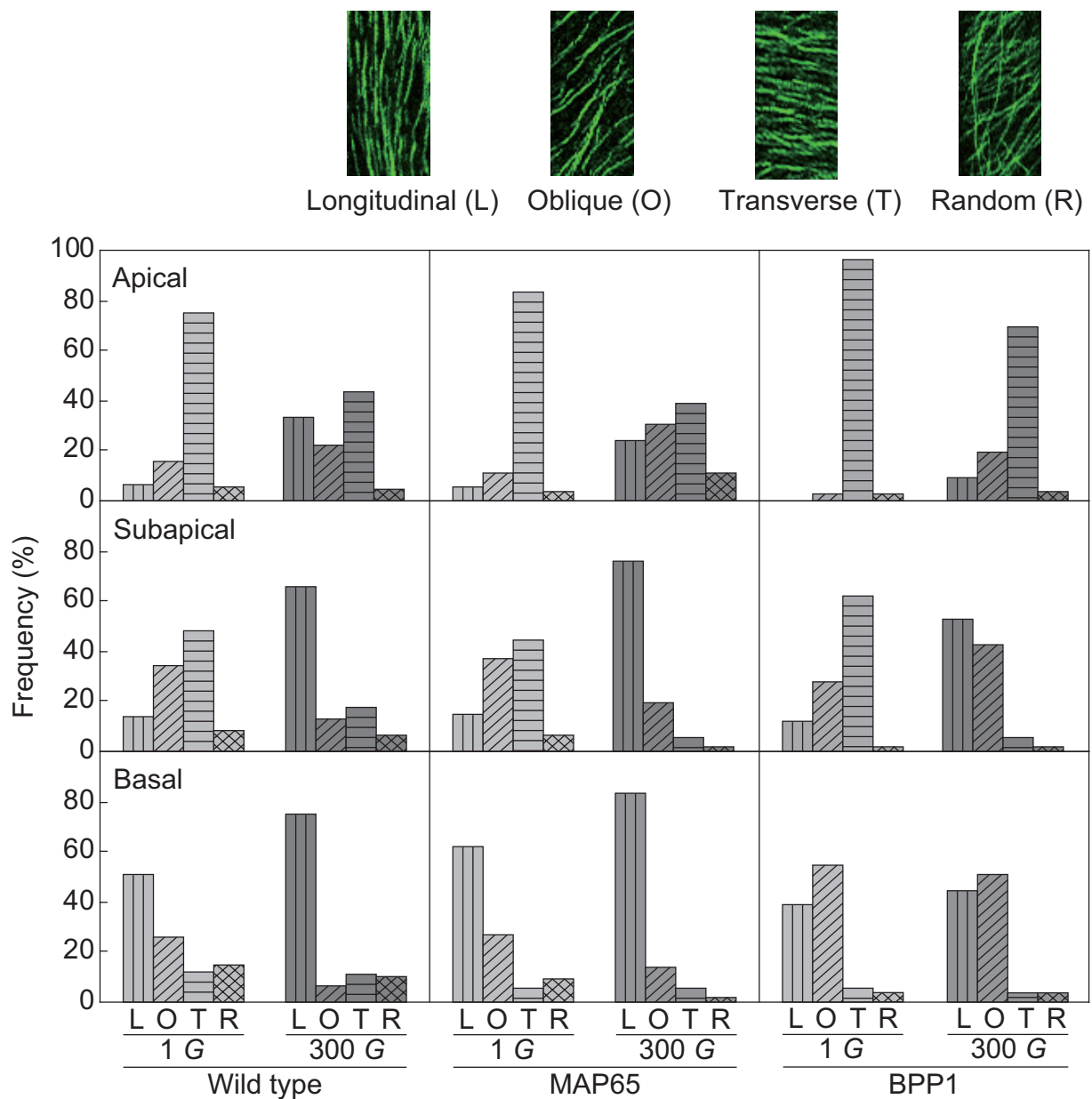


Fig. 4. Effects of hypergravity on the orientation of cortical microtubules in hypocotyls of Arabidopsis. Wild type and GFP-expressing lines were grown as in Figure 1. The orientation of cortical microtubules of adjacent to the outer tangential wall of epidermal cells was analyzed. The percentage of cells with microtubules within a range of 90-70° (transverse), 70-20° (oblique), 20-0° (longitudinal) to the longitudinal cell axis, and in various directions (random) was calculated for 200 cells.

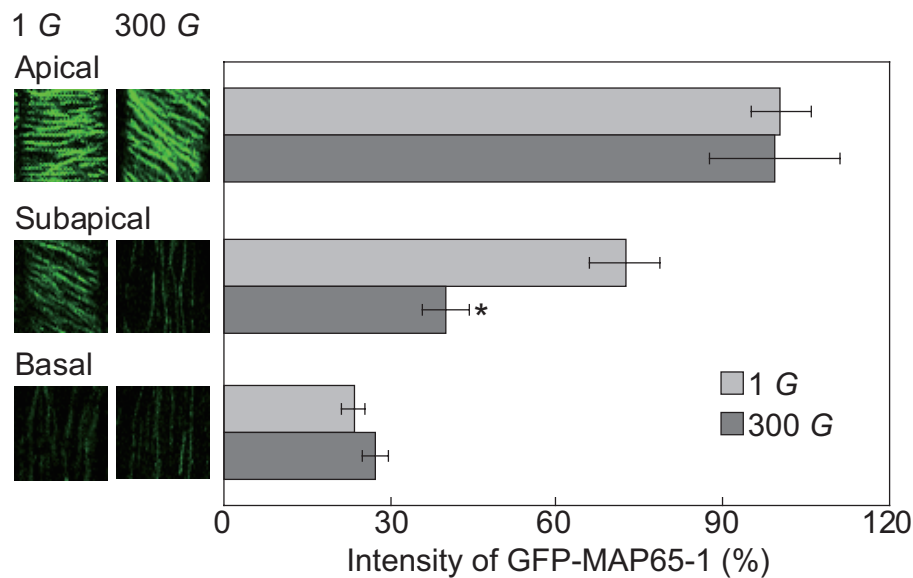


Fig. 5. Effects of hypergravity on the levels of MAP65-1 in epidermal cells of *Arabidopsis* hypocotyls. GFP-MAP65-1 line was grown as in Figure 1. Fluorescence images were collected by a confocal microscope and the intensity of GFP was determined using ImageJ software. *, Mean values with significant differences between 1 G and 300 G treatments ($P < 0.05$).

the outer tangential wall in epidermal cells of hypocotyls was analyzed by immunofluorescence microscopy. We also analyzed localization of GFP-MAP65-1 in epidermal cells of hypocotyls. The distribution of Cy3-tubulin and GFP-MAP65-1 was almost in the same pattern. These results suggest that GFP-MAP65-1 localized to cortical microtubules. Lucas *et al.* (2011) reported that GFP-MAP65-1 and cortical microtubules were colocalized. We previously reported that BPP1-GFP and cortical microtubules were colocalized (Hamada *et al.*, 2013). These results indicate that both GFP-MAP65-1 and BPP1-GFP lines can be utilized in the analysis of the orientation of cortical microtubules.

In wild type and GFP-MAP65-1 line, four types of cells with transverse, oblique, longitudinal or random microtubule orientation, were observed (Fig. 4). At 1 G, cells having transverse microtubules were predominant in the apical region of hypocotyls. The proportion of four types of cells varied along hypocotyls. The percentage of cells with transverse microtubules decreased from the apical to the basal regions, while that of cells with longitudinal microtubules increased (Fig. 4). In the apical region of BPP1-GFP line, cells having longitudinal microtubules were not observed, and the ratio of cells with transverse microtubules was higher than that of wild type or GFP-MAP65-1 line. However, the orientation of cortical microtubules changed along hypocotyls, as the same as that in wild type and GFP-MAP65-1 (Fig. 4). These results indicate that we can analyze the dynamics of cortical microtubules using both GFP-MAP65-1 and BPP1-GFP lines. Hypergravity decreased cells with transverse microtubules and increased cells with longitudinal microtubules in wild type and both GFP lines. Especially, in the subapical region, where

hypergravity clearly modified growth anisotropy (Figs. 2 and 3), hypergravity showed remarkable effects on reorientation of cortical microtubules (Fig. 4). It has been reported that hypergravity induced reorientation of cortical microtubules from transverse to longitudinal directions in azuki bean epicotyls (Soga *et al.*, 2006) and *Arabidopsis* hypocotyls (Matsumoto *et al.*, 2010). Taken together, the reorientation of cortical microtubules is involved in the regulation of growth anisotropy by gravity in plant cells.

As described above, hypergravity showed smaller effects on modification of growth anisotropy and reorientation of cortical microtubules in the BPP1 overexpressed line (Figs. 1-4). The BPP1 overexpressed lines are resistant to a microtubule depolymerizing drug, oryzalin, indicating that overexpression of BPP1 stabilizes cortical microtubules (Hamada *et al.*, 2013). In addition, the ratio of transverse microtubules was increased in the BPP1 overexpressed line (Hamada *et al.*, 2013). These results indicate BPP1 has roles for stabilization of microtubules and maintenance of transverse microtubule orientation. Thus, smaller effects on modification of growth anisotropy and reorientation of cortical microtubules in the BPP1 overexpressed line may be due to the excessive stabilization and maintenance of transverse microtubule orientation. These results suggest that regulation by gravity of BPP1 expression in an appropriate range may be required for constructing tough body against the gravitational force in *Arabidopsis* hypocotyls.

In the plant cells, microtubules including cortical microtubules in interphase are usually bundled by forming cross-bridges between adjacent microtubules (Sonobe *et al.*, 2001). MAP65 proteins are required for bundling of microtubules by forming cross-bridges between adjacent

microtubules (Jiang and Sonobe 1993). Sawano *et al.* (2000) reported that the protein levels of MAP65 in rapidly elongating regions of azuki bean epicotyls, which have predominantly transverse cortical microtubules, are higher than those in non-elongating regions which have predominantly longitudinal cortical microtubules. The transcript levels of MAP65-1 gene also decreased from the apical to the basal region in azuki bean epicotyls (Soga *et al.*, 2012). In the present study, we determined the protein levels of MAP65-1 by analyzing GFP fluorescence in hypocotyls of GFP-MAP65-1 line. At 1 *G*, the GFP fluorescence was decreased from the apical region, which had predominantly transverse cortical microtubules, to the basal regions, which had predominantly longitudinal cortical microtubules (Figs. 4 and 5). Hypergravity decreased the GFP fluorescence only in the subapical region where hypergravity modified growth anisotropy and orientation of cortical microtubules. In azuki bean epicotyls, hypergravity decreased the transcript levels of MAP65-1 gene during reorientation of cortical microtubules from transverse to longitudinal directions (Soga *et al.*, 2012). Similar changes in the orientation of cortical microtubules and transcript levels of MAP65-1 were observed in azuki bean epicotyls treated with 1-aminocyclopropane-1-carboxylic acid, the immediate precursor of ethylene (Soga *et al.*, 2012). Taken together, these results indicate that the decrease in the transcript and protein levels of MAP65-1 is accompanied by reorientation of cortical microtubules from transverse to longitudinal directions. Namely, changes in the protein levels of MAP65-1 via modification of expression of its gene may be one of the mechanisms for the regulation of orientation of cortical microtubules.

Plants generally had a shorter and thicker body under hypergravity conditions (Soga, 2010, 2013). On the other hand, plant developed a long and thin body under microgravity conditions in space (Hoson *et al.*, 2002; Soga *et al.*, 2002, 2014). The body shape of Arabidopsis varied in proportion to the logarithm of the magnitude of gravity in the range from microgravity to hypergravity (Soga *et al.*, 2001). The hypergravity-induced development of a short and thick body may be mediated by reorientation of cortical microtubules that is caused by the action of MAPs such as MAP65-1 and BPP1. Therefore, modification of dynamics of both cortical microtubules and MAPs may contribute to the formation of a long and thin body under microgravity conditions in space. We are carrying out space experiments, denoted as Resist Tubule and Aniso Tubule, to examine this possibility using GFP-expressing Arabidopsis lines.

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