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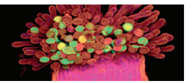

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RESEARCH PAPER

Growth stimulation in inflorescences of an *Arabidopsis* tubulin mutant under microgravity conditions in space

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ABSTRACT

Cortical microtubules are involved in plant resistance to hypergravity, but their roles in resistance to 1 *g* gravity are still uncertain. To clarify this point, we cultivated an *Arabidopsis* α-tubulin 6 mutant (*tua6*) in the Cell Biology Experiment Facility on the Kibo Module of the International Space Station, and analyzed growth and cell wall mechanical properties of inflorescences. Growth of inflorescence stems was stimulated under microgravity conditions, as compared with ground and on-orbit 1 *g* conditions. The stems were 10–45% longer and their growth rate 15–55% higher under microgravity conditions than those under both 1 *g* conditions. The degree of growth stimulation tended to be higher in the *tua6* mutant than the wild-type Columbia. Under microgravity conditions, the cell wall extensibility in elongating regions of inflorescences was significantly higher than the controls, suggesting that growth stimulation was caused by cell wall modifications. No clear differences were detected in any growth or cell wall property between ground and on-orbit 1 *g* controls. These results support the hypothesis that cortical microtubules generally play an important role in plant resistance to the gravitational force.

INTRODUCTION

Gravity is unique, of various environmental signals on earth, in that it is always present in a constant direction and magnitude. Plants have utilized gravity as the most reliable signal for morphogenesis. Gravitropism is a typical mechanism of gravimorphogenesis that enables plants to develop an appropriate body form for efficient life processes. At the same time, plants have had to develop a tough body to resist the gravitational force. This was an especially critical process for plant ancestors to survive at a terrestrial environment, when they moved from the sea to the land about 450 million years ago (Hoson 2003, 2006; Volkmann & Baluska 2006). Mechanical resistance to the gravitational force is thus a principal graviresponse in plants, comparable to gravitropism. We have termed this response 'gravity resistance' and examined its mechanism mainly using hypergravity conditions, produced by centrifugation (Hoson & 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. In gravity resistance, the gravity signal may be perceived by mechanoreceptors (mechanosensitive ion channels) on the plasma membrane of not only statocytes but also other types of cells (Soga *et al.* 2004, 2005). The perceived signal may be then transformed and transduced intracellularly within each cell, which may involve modulations of the expression of diverse genes and of the formation and functions of various cellular components. As the final step in gravity resistance, plants increase the rigidity of their cell walls via modifications to the cell wall metabolism as well as to the cell wall (apoplastic) environment.

The gravitational signal greatly modifies the expression of a wide range of genes (Martzivanou *et al.* 2006; Paul *et al.* 2012). Genes related to organization and functions of microtubules are included in those whose expression is altered under different gravity conditions. The expression of most α- and β-tubulin genes in *Arabidopsis* hypocotyls was up-regulated under hypergravity conditions (Yoshioka *et al.* 2003; Matsumoto *et al.* 2007). Hypergravity also modified the orientation of cortical microtubules. In the epidermis of azuki bean epicotyls grown at 1 *g*, cells with transverse cortical microtubules were predominant. The percentage of cells with transverse microtubules was decreased, whereas that with longitudinal microtubules was increased under hypergravity conditions (Soga *et al.* 2006). Furthermore, hypergravity increased transiently the expression of γ-tubulin and katanin genes (Soga *et al.* 2008, 2009), which are assumed to be responsible for reorientation of cortical microtubules (Murata *et al.* 2005). These results suggest that cortical microtubules are involved in gravity resistance, probably in signal transduction and transduction processes, leading to the cell wall changes.

The important role of cortical microtubules in plant resistance to hypergravity has been suggested, as mentioned above. However, it is uncertain whether the hypothesis is applicable to gravity resistance of plants to 1 *g* gravity, as to the resistance to hypergravity. To clarify this point, we conducted the space experiment using an *Arabidopsis* α-tubulin 6 mutant (*tua6*) in the Kibo Module on the International Space Station, as a part of the experiment termed Space Seed (PI, S. Kamisaka). The tubulin mutants showed disordered growth pattern, such as dwarfism and helical growth, at 1 *g*, and hypergravity further intensified such phenotypes (Matsumoto *et al.* 2010). It is expected that the mutants can grow and develop more or less normally under microgravity in space, where gravity resistance is not required and the defects of mutants are not disadvantageous for their growth. In the present paper, we report the modifications to growth and cell wall properties in inflorescences of the *tua6* mutant under microgravity conditions in space.

MATERIALS AND METHODS

Plant materials and space experiment

An amino acid substitution mutant of α-tubulin 6 (A281T, *tua6*) in *Arabidopsis thaliana* (L.) Heynh. (Ishida *et al.* 2007) was used in the present experiment. Healthy seeds of the mutant were selected under a stereo-microscope based on the outward appearance, and surface-sterilized with 70% ethanol. The seeds were then glued with 1% (w/v) gum arabic to the seedbed made of rock wool in the plant growth chamber of the Plant Experiment Unit (PEU). The PEUs containing the mutant seeds were launched on Space Shuttle STS-128 (17A) on August 28, 2009 (Yano *et al.* 2013). In orbit, the PEUs were unstowed and installed into the Cell Biology Experiment Facility of the Kibo Module. One PEU was placed in microgravity compartment, and the other was rotated to produce acceleration at 1 *g*. Initial watering of seeds was started on September 10, and germinated plants were grown at 23.5 ± 0.1 °C under continuous light of blue/red LEDs at 110 $\mu mol \cdot m^{-2} \cdot s^{-1}$ (Yano *et al.* 2013). After the initial watering, an infra-red moisture analyzer was operated every 10 min and the water pump delivered suitable amounts of water when water was below the stipulated level. Watering was carried out 6–14 times a day and relative humidity was kept between 70 and 80%. Daily image acquisition was carried out with an automated acquisition system in PEU. The length of each inflorescence stem was measured with down-linked daily images using a ruler. After 33 days, *Arabidopsis* plants were taken out of the plant growth chamber and then fixed with RNAlater® solution in a Kennedy Space Center Fixation Tube (KFT). The frozen KFTs containing fixed plant materials were frozen and recovered to earth on Space Shuttle STS-131 (19A) on April 19, 2010, and then transported to the laboratory of Osaka City Univ. for analyses. Ground control experiments were carried out at the laboratory of PI at Univ. of Toyama, by reproducing materials, hardware, and procedures during flight experiment, as much as possible.

Measurement of the mechanical properties of the cell wall

The frozen plant materials were thawed and the inflorescence stems were immediately cut into 10 mm segments from the apex to the base. The mechanical properties of the cell wall of each region were measured by a sequential analysis with the stress-strain and the stress-relaxation methods with a tensile tester (Tensilon RTM-25, Toyo Baldwin, Tokyo, Japan) (Hoson *et al.* 2009). The middle parts of each region of inflorescences were fixed between two clamps (1 mm apart) and stretched by lowering the bottom clamp at a speed of 10 $mm \cdot min^{-1}$ until a stress of 1 *g* was produced. The stress as a function of time was recorded at 1 ms intervals. The extensibility was calculated based on the slope of the stress-strain curve immediately before the stress of 1 *g* was produced. The minimum stress-relaxation time was also calculated from the stress-relaxation curves.

Microarray analysis

Inflorescence stem segments were immediately frozen with liquid nitrogen, after measurement of the mechanical properties of the cell wall. The frozen segments were homogenized with a mortar and a pestle. Total RNA was prepared using RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA), including a DNA elimination step (RNase-Free DNase Set, Qiagen). cDNA was then synthesized from the RNA samples, and the cRNA was subsequently labeled with Cyanin3 using the Quick-Amp Labeling Kit (Agilent Technologies, Santa Clara, CA. USA). Microarray analysis was performed by custom service of DNA Chip Research Inc. (Yokohama, Japan) with *Arabidopsis* Oligo DNA microarray ver. 4.0 (Agilent Technologies).

Statistical analysis

For each measurement, the means and the standard errors of the means (SE) were calculated. The significance of differences between microgravity and ground or on-orbit 1 *g* controls was analyzed by the Student's paired *t*-test.

RESULTS

Germination and development

Germination of the *tua6* mutant was first observed 3 days after the initial watering, and germination ratio reached 97% by the end of day 10 under ground 1 *g*, on-orbit (space) 1 *g*, and microgravity conditions. After germination, the mutant plants produced, in succession, rosette leaves, inflorescences, and flowers (Fig. 1). There were no apparent differences in development of the mutant between microgravity and ground or on-orbit 1 *g* controls, except for the color of rosette leaves. The rosette leaves of the mutant remained dark green for longer under microgravity conditions as compared with both controls, as observed in the wild-type Columbia (Yano *et al.* 2013).

Growth of inflorescences

The bolting of the *tua6* mutant was first observed 23 or 24 days after the initial watering. The average date of bolting tended to be 1–2 days early under microgravity conditions than under ground and on-orbit 1 *g* conditions, although the difference was not statistically significant (Table 1).

Figure 2 shows the time course of longitudinal growth in inflorescence stems of the mutant. When the length of the stem

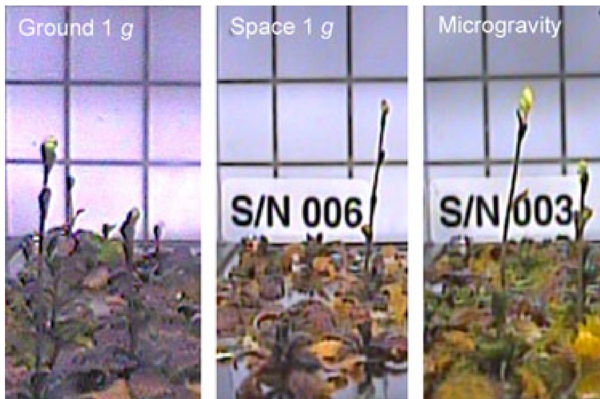

Fig. 1. The *tua6* mutant grown on the ground or in space. The plants were cultivated under ground 1 *g*, on-orbit 1 *g*, and microgravity conditions for 26 days, as mentioned in Materials and Methods. Images were taken with an automated acquisition system in the PEUs.

Table 1. The time of bolting of the *tua6* mutant on the ground or in space.

	the time of bolting (days)		
	ground 1 *g*	space 1 *g*	microgravity
first bolting	24	23	23
average	27.1 ± 0.9 (n = 9)	27.8 ± 1.4 (n = 4)	26.1 ± 0.7 (n = 8)

The mutant plants were cultivated under ground 1 *g*, on-orbit (space) 1 *g*, and microgravity conditions, as mentioned in Materials and Methods. The day when the first bolting was observed and the average date (±SE) of bolting after the initial watering are shown.

grown under microgravity conditions was compared with those of ground and on-orbit controls at each day after initial watering, it was shown that the stems were as a whole longer under microgravity conditions than at both controls. Because the time of bolting is variable, the differences in the length between microgravity and 1 *g* conditions were not statistically significant, except for some time points in the late growth phase. So, we aligned the timing of emergence and calculated the average length of inflorescence stems each day after that (Fig. 3). The stems were 10–45% longer, depending on the day, under microgravity conditions than those at ground and on-orbit controls, and the differences were significant over wide range of growth phase. No clear differences were observed in the length between ground and on-orbit 1 *g* controls.

The daily growth rate of inflorescence stems increased up to 4 days after emergence, and then decreased, irrespective of gravity conditions (Fig. 4). The height of the plant growth chamber of the PEU effective for plant growth was about 50 mm (Yano *et al.* 2013), which may limit longitudinal growth of inflorescences. The growth rate was 15–55% higher under microgravity conditions than that at both controls, and the differences were statistically significant in the early growth phase. No differences were detected in the growth rate between ground and on-orbit controls.

The mechanical properties of the cell wall

The mechanical extensibility of the cell wall was high in the apical elongating region of inflorescences and decreased sharply toward the basal region. The cell wall extensibility in apical and subapical regions was significantly higher under microgravity conditions than those at ground and on-orbit controls (Fig. 5).

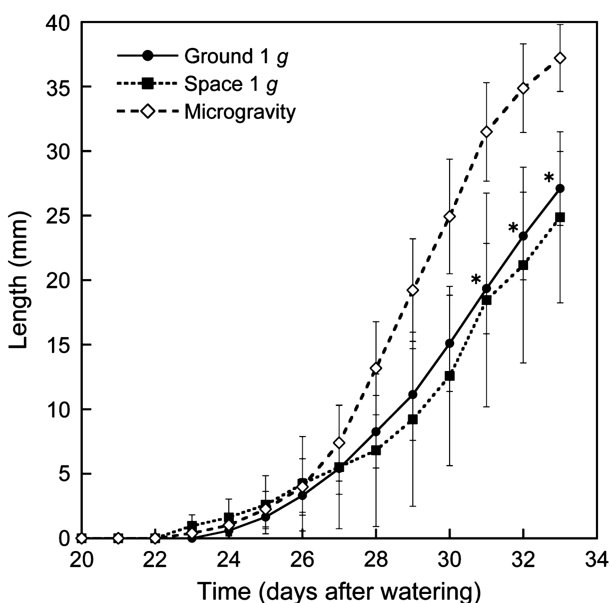

Fig. 2. The time course of longitudinal growth in inflorescence stems of the *tua6* mutant. The plants were cultivated as in Materials and Methods, and the length of each inflorescence stem was measured with down-linked daily images using a ruler. Means ± SE (n = 4–9). *Mean values significantly different between ground 1 *g* and microgravity conditions ($P < 0.05$).

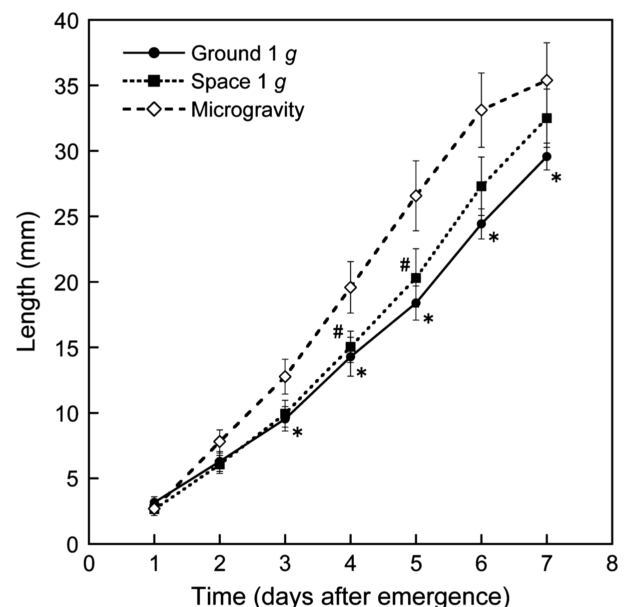

Fig. 3. The changes in inflorescence length of the *tua6* mutant after emergence. The plants were cultivated and the length of inflorescence stems was measured as in Fig. 2, and then the average length of the stems after emergence was calculated. Means ± SE (n = 4–9). * and #Mean values significantly different between ground 1 *g* and microgravity, and between on-orbit 1 *g* and microgravity conditions, respectively ($P < 0.05$).

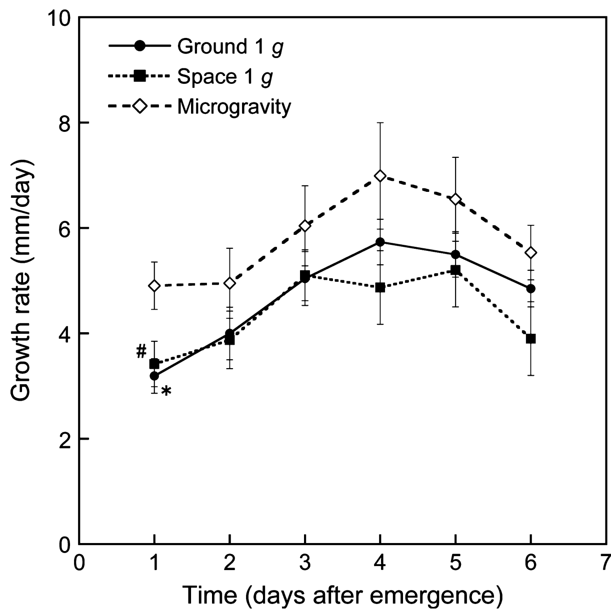

Fig. 4. The changes in the growth rate of the *tua6* mutant after emergence. The plants were cultivated and the length of inflorescence stems was measured as in Fig. 2, and then the daily growth rate of the stems after emergence was calculated. Means ± SE (n = 4–9). * and #Mean values significantly different between ground 1 *g* and microgravity, and between on-orbit 1 *g* and microgravity conditions, respectively ($P < 0.05$).

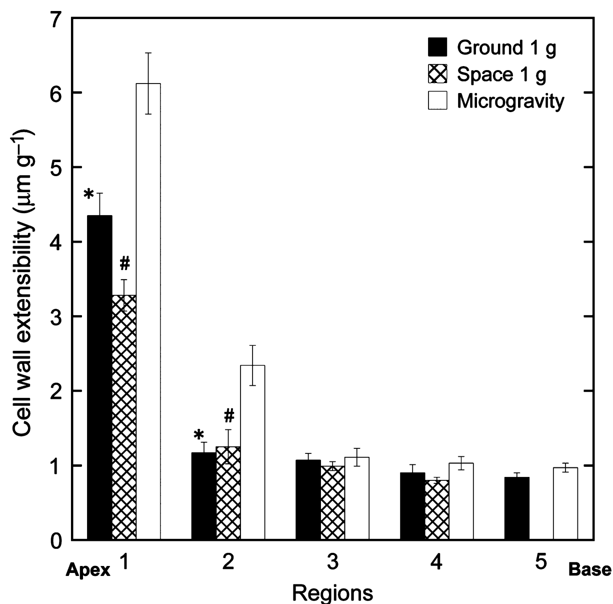

Fig. 5. The cell wall extensibility along inflorescence regions of the *tua6* mutant. The plants were cultivated under ground, on-orbit 1 *g*, and microgravity conditions for 33 days. The recovered inflorescences were cut into 10 mm segments from the apex (region 1) to the base (region 5), and the cell wall extensibility of each region were measured, as mentioned in Materials and Methods. Means ± SE (n = 4–9). * and #Mean values significantly different between ground 1 *g* and microgravity, and between on-orbit 1 *g* and microgravity conditions, respectively ($P < 0.05$).

On the other hand, the minimum-stress relaxation time of the cell wall slightly increased from the apical to the basal regions and tended to be lower under microgravity conditions, although the difference was not statistically significant (data not shown). No clear differences were detected in the cell wall mechanical properties between both controls.

Changes in gene expression

Microarray analyses were carried out, to examine the change in transcriptional profiles under microgravity conditions. Because the amount of materials obtained from on-orbit control was limited, statistical analysis was performed between microgravity and ground 1 *g* conditions. Under microgravity conditions, the amounts of 752 transcripts, which are classified into 92 gene ontology (GO) categories, were more than twice, whereas those of 725 transcripts of 26 GO categories were less than 50% of those of the ground control ($P < 0.05$). The expression of five microtubule-related genes was up-regulated, but that of 17 microtubule-related genes, including β-tubulin 1 and MAP-65 genes, was down-regulated by microgravity. Tables S1 and S2 show the list of major genes whose expression was modified under microgravity conditions.

DISCUSSION

The *tua6* mutant developed normal inflorescences under microgravity conditions, as well as under ground and on-orbit 1 *g* conditions (Fig. 1). When longitudinal growth of inflorescence stems was compared between microgravity and 1 *g* conditions, it was shown that the stems were longer and their growth rate was higher under microgravity conditions than those at both controls (Figs 2–4). Because no clear differences were detected in the length or the growth rate between ground and on-orbit 1 *g* controls, stimulation of inflorescence growth may be caused by microgravity, not by space flight. Growth stimulation under microgravity conditions has been reported in etiolated seedlings of some cultivars in rice (Hoson *et al.* 2002) and *Arabidopsis* (Soga *et al.* 2002). In the present space experiment, full analysis of growth or cell wall properties has not been carried out for the wild-type Columbia, because of operational limitations. However, the comparison of the daily growth rate after emergence showed that the degree of growth stimulation by microgravity tended to be higher in the *tua6* mutant than in Columbia, in particular in the late growth phase (Figure S1). These results support the hypothesis that cortical microtubules play an important role in plant resistance to the gravitational force.

The cell wall extensibility in apical elongating regions of inflorescences from the mutant was significantly higher under microgravity conditions than ground and on-orbit controls (Fig. 5). No clear differences were detected in the cell wall mechanical properties between both controls. These results suggest that stimulation by microgravity of inflorescence growth was caused by cell wall modifications. We have expected that growth of stem organs in tubulin mutants may be more or less restored under microgravity conditions, even if cell wall properties are kept constant, because the gravity resistance is not required and the defects of mutants are not disadvantageous for their growth (Hoson *et al.* 2009). Although this view was not directly examined in the present study because of cell wall modifications under microgravity conditions, the results as a whole are compatible with the above-mentioned hypothesis.

In tubulin mutants, organs such as hypocotyls, flower petals, rosette leaves, and roots, show either left-handed or right-handed helical growth at 1 *g*, which may be caused by inclination of microtubule arrays (Hashimoto 2002). Helical growth of hypocotyls in tubulin mutants was further intensified by hypergravity (Hoson *et al.* 2010; Matsumoto *et al.* 2010). On the other hand, the helical growth phenotype was not clear in inflorescence stems, as compared with the above organs, which was not strongly influenced by microgravity (data not shown). It was also shown that the calculated decrease in longitudinal length of hypocotyls based on the pitch angle of inclined cell files was too small for the measured decrease (Matsumoto *et al.* 2010). Thus, helical growth may not be the direct cause of dwarfism in tubulin mutants. The effects of microgravity on formation and orientation of cortical microtubule arrays in tubulin mutants are important issues, but not examined in the present study. Further space experiments are needed for this topic.

Under hypergravity conditions, the expression of most α- and β-tubulin genes in *Arabidopsis* hypocotyls was up-regulated (Yoshioka *et al.* 2003; Matsumoto *et al.* 2007). Hypergravity also increased the expression of genes encoding some of microtubule-associated proteins (MAPs), such as γ-tubulin and katanin (Soga *et al.* 2008, 2009). In the present study, we examined by microarray analyses whether microgravity induced opposite changes in expressions of microtubule-related genes. The number of the transcripts of such genes, whose expression was down-regulated under microgravity conditions, was more than three times that of up-regulated (data not shown), supporting the hypothesis. However, the magnitude of observed changes in gene expression was not always so large. Because the up-regulation of expression of microtubule-related genes by hypergravity was mostly transient, a kind of adaptation may also occur under constant microgravity conditions in space. The time-course analysis of gene expression, which involves a transfer of plant materials from on-orbit 1 *g* to microgravity conditions, may be effective to clarify this point. Also, we need to keep in mind that the present results are biased by the mutation in α-tubulin 6 gene. In the present study, wild-type materials were not available for gene expression analysis.

Microarray analyses also showed the modifications of expression of other genes under microgravity conditions (Tables S1 and S2). The expression of genes encoding cell wall proteins, such as xyloglucan endotransglucosylase/hydrolases, expansins, and pectinesterases, was down-regulated at microgravity, which may be responsible for the increase in cell wall extensibility. The modifications of expression of respiratory burst oxidase and superoxide dismutase genes might be related to modifications of metabolism of cell wall phenolics. Also, up- or down-regulation of expression of genes responsible for metabolism of plant hormones, such as auxin, ethylene, brassinosteroids, jasmonate, and abscisic acid, might be involved in growth stimulated under microgravity conditions.

The role of cortical microtubules in plant resistance to the gravitational force was supported with only one tubulin mutant in the present study, and it is important to quantify their contributions. We have shown that there is a good correlation between the degree of defects of various tubulin mutants on formation of cortical microtubules and growth properties, and the capacity of gravity resistance under hypergravity conditions (Hoson *et al.* 2010; Matsumoto *et al.* 2010). It appears that tubulin mutants may become hypersensitive to the gravitational force, dependent on the degree of defects. Also, we have shown that parameters on gravity resistance may vary in proportion to the logarithm of the magnitude of gravity over microgravity and hypergravity range (Hoson & Soga 2003). We intend to confirm such a dose-response relationship by the next space experiment, termed Resist Tubule, on the Kibo Module of the International Space Station.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Increase in the growth rate under microgravity conditions of the wild-type Columbia and the *tua6* mutant after emergence.

Table S1. Transcripts whose expression levels increased more than five-fold under microgravity conditions as compared with ground 1 *g*.

Table S2. Transcripts whose expression levels decreased by more than 80% under microgravity conditions as compared with ground 1 *g*.

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