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Original Paper

The Transcript Level of Katanin Gene is Increased Transiently in Response to Changes in Gravitational Conditions in Azuki Bean Epicotyls

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Abstract

Development of a short and thick body by reorientation of cortical microtubules is required for the resistance of plants to the gravitational force. Katanin has microtubule-severing activity and is involved in the reorientation of cortical microtubules. Here, we investigated the effects of hypergravity produced by centrifugation on the expression of *VaKTN1* gene encoding katanin. Hypergravity at 300 *G* increased the transcript level within 15 min, and the level reached maximum at 45 min. Then, the level was decreased and returned to the control range at 2 h. Also, the expression of *VaKTN1* gene was increased transiently by removal of hypergravity stimulus. Changes in the microtubule-severing activity as a result of the modification of *VaKTN1* expression in response to changes in gravitational conditions may be involved in the regulation of the orientation of cortical microtubules, leading to changes in the shape of plant body. Lanthanum and gadolinium ions, potential blockers of mechanosensitive calcium ion-permeable channels (mechanoreceptors), nullified the up-regulation of *VaKTN1* gene, suggesting that mechanoreceptors are responsible for regulation by gravity of *VaKTN1* expression.

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Introduction

Development of a tough body to resist the gravitational force is a principal graviresponce in organisms living on land. The mechanisms of gravity resistance in plants have been analyzed using hypergravity conditions produced by centrifugation and microgravity conditions in space experiments (Hoson and Soga 2003). Hypergravity increased the cell wall rigidity in shoots of various plants (Hoson *et al*., 1996; Soga *et al*., 1999a, 1999b, 2001). Under microgravity conditions in space, the cell wall rigidity was decreased oppositely (Hoson *et al*., 2002; Soga *et al*., 2001, 2002). These results indicate that the regulation of cell wall rigidity is involved in the resistance of plants to the gravitational force (Hoson and Soga 2003). Hypergravity treatment has been shown to inhibit elongation growth and promote lateral expansion in shoot organs (Waldron and Brett, 1990; Kasahara *et al*., 1995; Hoson *et al*., 1996; Soga *et al*., 1999a, 1999b, 2001; Wakabayashi *et al*., 2005; Nakano *et al*., 2007). Namely, plant body becomes shorter and thicker under hypergravity conditions. On the other hand, plant shape becomes longer and thinner under microgravity conditions in space (Hoson *et al*., 2002; Soga *et al*., 2001, 2002). Thus, development of a short and thick body may be regarded as a part of the response that enables plants to grow against the gravitational force.

The shape of plant body depends generally on the shape of its individual cells, and the shape of plant cells is in turn primarily controlled by the orientation of cellulose microfibrils. The orientation of cortical microtubules has been considered to determine the orientation of cellulose microfibrils (Giddings and Staehelin, 1991; Shibaoka, 1994). The orientation of cortical microtubules is under the control of environmental signals, such as gravity. For example, hypergravity promoted reorientation of microtubules into parallel arrays in protoplasts from *Nicotiana tabacum* (Wymer *et al*., 1996) and *Brassica napus* (Skagen and Iversen, 1999). Also, hypergravity induced reorientation of cortical microtubules from transverse to longitudinal directions in azuki bean epicotyls (Soga *et al*., 2006). Taken together, reorientation of cortical microtubules may be involved in the development by gravity of a short and thick body.

Murata *et al*. (2005) have shown that plant ϒ-tubulin complex binds onto pre-existing cortical microtubules and nucleates microtubules as branch. Recently, we reported that the transcript level of genes encoding ϒ-tubulin complex (*VaTUG* and *VaGCP3*) was transiently increased during reorientation of cortical microtubules by gravity in azuki bean epicotyls (Soga *et al*., 2008). These facts suggest that a transient increase in the levels of ϒ-tubulin complex via up-regulation of the expression of *VaTUG* and *VaGCP3* by gravity induces branching of microtubules, which leads to reorientation of cortical microtubules. When cortical microtubules are reoriented, the original microtubules should be severed from the newly synthesized microtubules, and then depolymerized. Katanin has microtubule-severing activity and is involved

in the reorientation of cortical microtubules (Burk *et al*., 2001; Bouquin *et al*., 2003). Thus, the expression of katanin gene may also be modified in response to the changes in gravitational conditions. Table 1 summarizes the events during hypergravity-induced changes in the growth direction (construction of short and thick body) of azuki bean epicotyls. To confirm a working model for microtubule reorientation (Fig. 1), we examined the changes in the expression of katanin (*VaKTN1*) gene in azuki bean epicotyls grown under hypergravity conditions produced by centrifugation. We also examined the effects of lanthanum or gadolinium ions, potential blockers of mechanosensitive calcium ion-permeable channels (mechanoreceptors), on hypergravity-induced changes in expression of *VaKTN1*, because hypergravity-induced reorientation of cortical microtubules as well as up-regulation of expression of *VaTUG* and *VaGCP3* were nullified in the presence of lanthanum and gadolinium ions (Soga *et al*., 2006, 2008).

Materials and methods

Plant material and hypergravity experiments

The growth experiments were carried out essentially as described previously (Soga *et al*., 1999a). Seeds of azuki bean (*Vigna angularis* Ohwi et Ohashi cv. Erimowase) were soaked in running tap water for 1 day 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 5 days, seedlings with an epicotyl 30 to 35 mm long were selected. In some experiments, the seedlings were treated with lanthanum chloride ($LaCl_3$) or gadolinium chloride ($GdCl_3$). The seedlings were transplanted into a plastic dish filled with 1 mM MES-KOH buffer (pH 6.0) with or without 0.1 mM lanthanum or gadolinium ions. After pretreatment for 2 h, a 10 mm subhook region (3-13 mm below the hook) was marked with India ink. The marked seedlings were transplanted into test tubes (16 mm in diameter, 100 mm in length) containing 1.5 mL of 1 mM MES-KOH buffer (pH 6.0) with or without 0.1 mM lanthanum or gadolinium ions. The marked seedlings were then exposed to basipetal hypergravity with a centrifuge (H-28-F; Kokusan Co., Tokyo, Japan) at 25˚C in the dark. The magnitude of acceleration was regulated by changing the speed of rotation. The seedlings for 1 *G* control were placed on top of spinning centrifuge during incubation. All manipulations were done under dim green light (ca. 0.09 μmol m^{-2} s^{-1} at handling level).

cDNA cloning

Total RNA was extracted from 5-day-old azuki bean epicotyls using RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA). Single strand cDNA was synthesized from 2 μg of total RNA using a random hexamer primer. For cloning of the partial cDNA encoding katanin p60 (KTN1), degenerate primers (forward, 5'-T(AG)GCTG(AC)(ACT)ATGCT(GT)GAAAGGG-3'; reverse, 5'-TC(AG)AA(AG)TCACACAT(AGT)GC(ACG)AC(AT)GG-3') were designed based on Arabidopsis At1g80350 and rice Os01g0683100. The PCR was performed with the degenerate primers using the single strand cDNA as a template under the following conditions: 0.5 min denaturing at 95˚C, 0.5 min annealing at 55˚C and 1.5 min amplification at 72˚C, 35 cycles. The amplified cDNA fragments were subcloned into a pGEM T-Easy vector (Promega, Madison WI, USA) and the nucleotide sequence of 36 clones was determined. The cloned cDNA was designated *VaKTN1* (AB453280).

Quantitative real-time RT-PCR

The marked regions excised from epicotyls were immediately frozen with liquid nitrogen and kept at -80˚C until use. The frozen segments (ca. 100 mg FW) were homogenized in a mortar with a pestle. Total RNA was prepared using RNeasy Plant Mini Kit (Qiagen), including a DNA elimination step (RNase-Free DNase

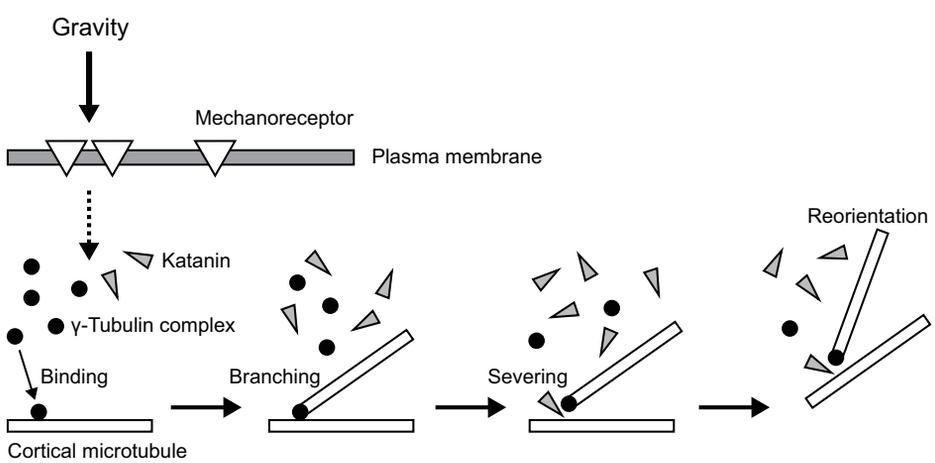

Fig. 1. A working model for reorientation of cortical microtubules by gravity. The gravity signal perceived by mechanoreceptors on the plasma membrane transiently increases the levels of ϒ-tubulin complex and katanin. ϒ-Tubulin complex binds onto pre-existing cortical microtubules and nucleates microtubules as branch. Then, katanin severs the newly synthesized microtubule branch. Repeat of these processes may induce the reorientation of cortical microtubules.

Set, Qiagen). Single strand cDNA was synthesized from 2 μg of total RNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) with a random hexamer primer. One μL of the reaction mixture (total volume: 20 μL) was used subsequently for PCR. Real time RT-PCR was performed with the Applied Biosystems 7500 Real Time PCR System with Power SYBR Green PCR Master Mix (Applied Biosystems) according to the manufacturer's instructions. All data were normalized with respect to 18S rRNA, which was measured as an internal standard. Primers were designed by the Primer Express program (Applied Biosystems) and had the following sequences: for *VaKTN1* (forward, 5'-TCCCTTGTGGATGCCTGAATA-3'; reverse, 5'-AAACATGAGAACGCCTTTCCAT-3'): and 18S rRNA for normalization (forward, 5'-AGTCATCAGCTCGCGTTGAC-3'; reverse, 5'-TCAATCGGTAGGAGCGACG-3'). Concentrations of primers were 50 nM. Double-strand cDNA was generated by initial steps (50˚C for 2 min, 95˚C for 10 min) and by PCR (95˚C for 15 sec, 60˚C for 1 min) for 40 cycles. We determined nucleotide sequence of the RT-PCR products, and confirmed the specificity of the primers.

Results and Discussion

Hypergravity-induced inhibition of elongation growth and promotion of lateral growth mainly occurred in the upper region of azuki bean epicotyls (Nakano *et al*., 2007). Therefore, in this experiment, we marked 10 mm of the upper region (3-13 mm below the hook) of epicotyls and examined the expression profiles of *VaKTN1* gene in this region under hypergravity conditions. Figure 2 shows the time course changes in the expression level of *VaKTN1* in epicotyls grown under 1 or 300 *G* conditions. The expression level of *VaKTN1* of control epicotyls was almost constant during incubation (Fig. 2). Meanwhile, hypergravity significantly increased the level within 15 min after the transfer (5% level by the Student's t-test). The maximum level was achieved at 45 min after transfer to hypergravity conditions (Fig. 2). Then, the level was decreased and returned to control range at 2 h. Also, the expression of level of *VaKTN1* was increased transiently by removal of hypergravity stimulus (the level at 15-60 min after the transfer was significantly different from the control value at 5% level). These results indicate that the transcript level of *VaKTN1* is increased transiently in response to the changes in gravitational conditions (Table 1).

Katanin, the only microtubule-severing protein in plants, couples ATP hydrolysis to disassemble microtubules into tubulin subunits. It has been assumed that katanin severs microtubules from the nucleation site (Lloyd and Chan, 2004; Stoppin-Mellet *et al*., 2006). Cortical microtubules are nucleated as branches on pre-existing microtubules via recruitment of ϒ-tubulin complex (Murata *et al*., 2005). It has been shown that both katanin and ϒ-tubulin are required for proper organization of cortical microtubules (Burk *et al*., 2001; Bouquin *et al*., 2003; Pastuglia *et al*., 2006). Recently, we reported that the gene expression of components of ϒ-tubulin complex, *VaTUG* and *VaGCP3*, was transiently increased during the changes in the orientation of cortical microtubules by gravity in azuki bean epicotyls (Soga *et al*., 2008). Our present results revealed that the expression level of *VaKTN1*, catalytic subunit p60 of katanin, was also transiently increased in response to changes in gravitational conditions (Fig. 2). When expression profiles of *VaKTN1* were compared with those of *VaTUG* and *VaGCP3*, the peak of *VaKTN1* expression was delayed by 30 to 45 min, although hypergravity increased the expression levels of all genes within 15 min (Soga *et al*., 2008; Fig. 2). The expression levels of *VaTUG*, *VaGCP3* and *VaKTN1* returned to control range after 2 h. Also, the changes in the orientation of cortical microtubules were detected within 30 min and settled within 2 h after hypergravity or removal treatment (Soga *et al*., 2006). These lines of results suggest that the synthesis of ϒ-tubulin complex via up-regulation of the expression of *VaTUG* and *VaGCP3* may induce the branching of microtubules (Fig. 1). Then, microtubule-severing activities may be increased by up-regulation of *VaKTN1* gene expression, resulting in the separation of the newly synthesized microtubule branch. Repeat of these processes may induce the reorientation of cortical microtubules, which contributes to development of a tough body to resist the gravitational force.

We have previously revealed that gravity is perceived by mechanoreceptors on plasma membrane in gravity resistance in plants (Soga *et al*., 2004, 2005a, 2005b). In the present study, we also examined the effects of lanthanum and gadolinium ions, potential blockers of mechanoreceptors, on the expression of *VaKTN1*. After pretreatment with 0.1 mM lanthanum or gadolinium ions for 2 h, the seedlings were grown 1 *G* or 300 *G* conditions

Table 1 The summary of events during hypergravity-induced changes in the growth direction of azuki bean epicotyls.

Event	1 *G* to 300 *G*	300 *G* to 1 *G*	References
Growth direction	Lateral	Longitudinal	Soga *et al.* (2006)
Microtubule orientation	Longitudinal	Transverse	Soga *et al.* (2006)
Transcript levels			
VaTUG	Transient increase	Transient increase	Soga *et al.* (2008)
VaGCP3	Transient increase	Transient increase	Soga *et al.* (2008)
VaKTN1	Transient increase	Transient increase	Present study

VaTUG, *VaGCP3*, and *VaKTN1* encode ϒ-tubulin, ϒ-tubulin complex protein 3 (GCP3), and catalytic subunit of katanin in azuki bean (*Vigna angularis*), respectively. ϒ-Tubulin and GCP3 are required for microtubule nucleation, and katanin has microtubule-severing activity from their nucleation sites.

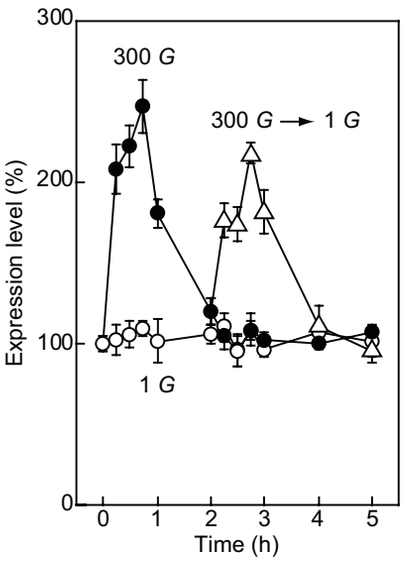

Fig. 2. Changes in the expression of *VaKTN1* gene in azuki bean epicotyls under hypergravity conditions. Azuki bean seedlings with marked epicotyls were kept under basipetal hypergravity at 300 *G* for 2 h, and then a half of the hypergravity-treated seedlings were transferred to 1 *G* conditions for an additional 3 h. The expression of *VaKTN1* in the marked region was determined by a real time RT-PCR. The values were compensated with levels of 18S rRNA as an internal standard, and expressed as percentage of initial level. Values are means ± SE (n=3).

for 5 h, and the transcript level of *VaKTN1* was measured at several time points. Under 1 *G* conditions, lanthanum and gadolinium ions had no effects on expression of *VaKTN1*. The expression of *VaKTN1* was significantly increased by hypergravity at 300 *G* in the absence of lanthanum or gadolinium ions (Fig. 3). On the other hand, the up-regulation of *VaKTN1* expression was not observed in the lanthanum or gadolinium-treated epicotyls. Figure 3 shows the data of 45 min-treatment, when the maximum expression by hypergravity was obtained in the absence of blockers. In the presence of both blockers, expression level of *VaKTN1* was almost the same between 1 *G* control and hypergravity treatment during the whole 5 h-incubation period. Hypergravity transiently increased the concentration of cytoplasmic calcium ions in Arabidopsis, and the increase was cancelled in the presence of lanthanum or gadolinium ions (Toyota *et al*., 2007). Taken together, azuki bean cells may utilize the changes in calcium ion levels via modifications of the activity of mechanoreceptors to regulate the expression of *VaKTN1* (Fig. 1).

The increase in level of *VaKTN1* gene was transient, and the level returned to the control range during continuous hypergravity treatment (Fig. 2). In addition, transient increase in the *VaKTN1* level was induced by removal of hypergravity (Fig. 2). Similar modifications of expression levels were observed in *VaTUG* and *VaGCP3* (Soga *et al*., 2008). Hypergravity-induced changes in growth anisotropy in azuki bean epicotyls were nullified immediately after transfer of azuki bean seedlings kept for several hours at 300 *G* conditions to 1 *G* conditions (Soga *et al*., 2003, 2006). Also, hypergravity-induced modifications of cell wall properties, such as cell wall rigidity, metabolisms of cell wall polysaccharides and apoplastic pH (Soga *et al*., 2000a, 2000b, 2007a), were cancelled immediately after transfer to 1 *G* conditions (Soga *et al*., 2003, 2007a). In addition, the synthesis of cell wall polysaccharides was similar between the 300 and 1 *G* treatment (Soga *et al*., 2007b). These lines of evidence strongly indicate that hypergravity at 300 *G* is not an extraordinary stimulus for plants and that plant response to this magnitude of gravity can be recognized as normal physiological responses.

The expression level of *VaKTN1* gene was transiently increased, when azuki bean seedlings were transferred from 1 *G* to 300 *G* and from 300 *G* to 1 *G* (Fig. 2). Namely, the expression of *VaKTN1* was changed in response to the increase and decrease in the gravitational force in the range between 1 *G* and 300 *G*. Our present results suggest that changes in the expression of *VaKTN1* are involved in the regulation of the shape of plant body. We previously reported that plants developed a long and thin body under microgravity conditions in space (Hoson *et al*., 2002; Soga *et al*., 2001, 2002). Thus, the expression of *VaKTN1* may also be modified in response to the changes in gravitational conditions in the range between 0 *G* and 1 *G*. The analysis of expression profiles of *VaKTN1* under microgravity conditions in space may further clarify the function of katanin in gravity resistance in plants.

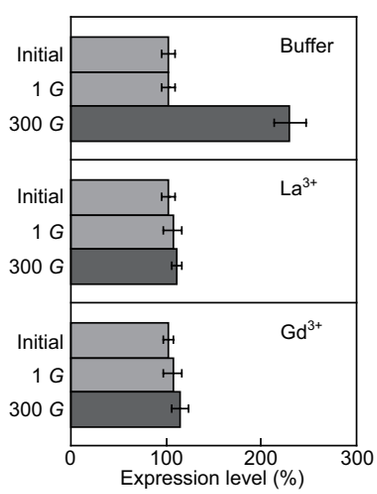

Fig. 3. Effects of lanthanum and gadolinium ions on the expression of *VaKTN1* gene in azuki bean epicotyls. Azuki bean seedlings were grown at 1 *G* in the presence or absence of 0.1 mM lanthanum or gadolinium ions for 2 h. Then, a 10 mm subhook region was marked with India ink, and the seedlings were kept at 1 *G* or 300 *G* in the presence or absence of 0.1 mM lanthanum or gadolinium ions for 45 min. The expression of *VaKTN1* was determined as indicated in Fig. 2. Values are means ± SE (n=3).

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