

# Calcium mobilizations in response to changes in the gravity vector in *Arabidopsis* seedlings

## Possible cellular mechanisms

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**Abbreviations:**  $[Ca^{2+}]_c$ , cytoplasmic free calcium ion concentration; PF, Parabolic flights; MSCC, mechanosensitive  $Ca^{2+}$ -permeable channel; PLC phospholipase C; RR, ruthenium red; IICR,  $InsP_3$ -induced  $Ca^{2+}$ -release;  $InsP_3$ , inositol 1,4,5-trisphosphate; HGMF, High-gradient magnetic field; HUVECs, human umbilical vein endothelial cells; FN, fibronectin; MscS, mechanosensitive channel of small conductance; MS, mechano-sensitive

Gravity influences the growth direction of higher plants. Changes in the gravity vector (gravistimulation) immediately promote the increase in the cytoplasmic free calcium ion concentration ( $[Ca^{2+}]_c$ ) in *Arabidopsis* (*Arabidopsis thaliana*) seedlings. When the seedlings are gravistimulated by reorientation at 180°, a transient two peaked (biphasic)  $[Ca^{2+}]_c$ -increase arises in their hypocotyl and petioles. Parabolic flights (PFs) can generate a variety of gravity-stimuli, and enable us to measure gravity-induced  $[Ca^{2+}]_c$ -increases without specimen rotation, which demonstrate that *Arabidopsis* seedlings possess a rapid gravity-sensing mechanism linearly transducing a wide range of gravitational changes into  $Ca^{2+}$  signals on a sub-second timescale. Hypergravity by centrifugation (20 g or 300 g) also induces similar transient  $[Ca^{2+}]_c$ -increases. In this review, we propose models for possible cellular processes of the gravistimulus-induced  $[Ca^{2+}]_c$ -increase, and evaluate those by examining whether the model fits well with the kinetic parameters derived from the  $[Ca^{2+}]_c$ -increases obtained by applying gravistimulus with different amplitudes and time sequences.

### Introduction

Plants respond to changes in the gravity vector (gravistimulation); plants sense gravity and orient their growth direction with respect to the gravity vector, known as gravitropism. Changes in the gravity vector are supposed to be transduced into certain intracellular signals such as

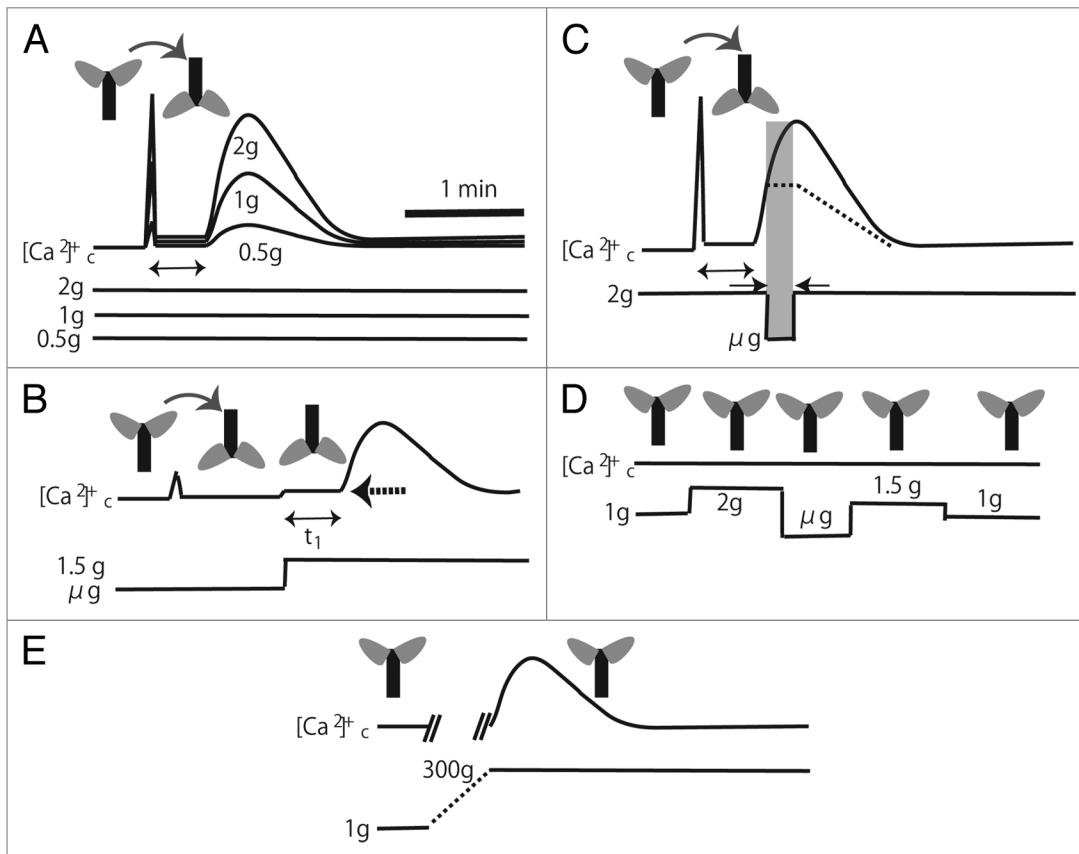
reactive oxygen species,<sup>1</sup> ionic gradient,  $pH^{3,4}$  and cytoplasmic free calcium concentration ( $[Ca^{2+}]_c$ )<sup>2-5</sup> in an early process of gravitropic response.<sup>6,7</sup>

Changes in the  $[Ca^{2+}]_c$  in gravity responses have repeatedly been reported in the field of plant biology; e.g.,  $[Ca^{2+}]_c$ -increases in maize coleoptiles were observed by the  $[Ca^{2+}]_c$  indicator, fluo-3.<sup>8</sup> Changes in the  $[Ca^{2+}]_c$  induced by gravistimulation have been studied in more detail in *Arabidopsis* (*Arabidopsis thaliana*) seedlings expressing the luminous  $Ca^{2+}$ -reporting protein, apoaequorin.<sup>9-11</sup> When the seedlings were gravistimulated by turning 180°, they show a transient biphasic  $[Ca^{2+}]_c$ -increase in their hypocotyls and petioles;<sup>9</sup> a fast transient and a following slow  $[Ca^{2+}]_c$ -increases. These two  $Ca^{2+}$  changes have different characteristics. The first transient  $[Ca^{2+}]_c$ -increase (at 1g) depends on the rotational velocity but not the rotational angle, whereas the second slow  $[Ca^{2+}]_c$ -increase depends on the rotational angle but not the velocity, suggesting that the first transient and second slow  $[Ca^{2+}]_c$ -increases are related to the rotational stimulation and the gravistimulation, respectively, and are mediated by distinct molecular mechanisms.<sup>9</sup> Thus, the response to rotation may overlap the pure gravistimulus-induced response. In this review gravistimulation denotes the changes in the direction and magnitude of the gravity vector (some of them are shown in **Figure 1**).

Recently, a gravistimulation-specific  $Ca^{2+}$  response in *Arabidopsis* seedlings has been demonstrated by separating gravity-vector-changes during rotation from gravistimulation by using the  $\mu g$  conditions  $<10^{-4}g$  provided by parabolic flights (PF).<sup>12</sup> When *Arabidopsis* seedlings were rotated +180° under  $\mu g$  conditions, only the fast transient was observed and almost totally attenuated in a few seconds. Gravistimulation (transition from  $\mu g$  to 1.5g, see more detail in **Figure 1**) was then applied to pre-rotated specimens at the terminating phase of the PF. This gravistimulation without simultaneous rotation induced a slow  $[Ca^{2+}]_c$ -increase, which corresponds to the slow  $[Ca^{2+}]_c$ -increase observed in the ground experiments,<sup>9,10</sup> and the amplitude of the

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**Figure 1.** Schematic illustrations of the typical time course of gravistimulus-induced  $[Ca^{2+}]_c$ -increases. (A), Transient biphasic  $[Ca^{2+}]_c$ -increases by 180° rotation at 0.5, 1, and 2g conditions. (B), A slow  $[Ca^{2+}]_c$ -increase by transition from  $\mu g$  to 1.5g. Plants were rotated at  $\mu g$  before the gravity change, and a small transient  $[Ca^{2+}]_c$ -increase was detected. The aequorin signal during the delay (shown by double headed arrow,  $t_1$ ) is slightly higher (pointed by a dotted arrow) than the basal level. (C), A sudden gravitational decrease from 2g to  $\mu g$  does not attenuate the slow  $[Ca^{2+}]_c$ -increase induced by 180°-gravistimulation at 2g. The dotted line denotes a hypothetical  $[Ca^{2+}]_c$  change according to the model in panel (E) in **Figure 2**. (D), No apparent changes in  $[Ca^{2+}]_c$  is detected in plants in an upright position by the 1g-2g- $\mu g$ -1.5g-1g change. (E), 300g-hypergravity stimulation induces a slow  $[Ca^{2+}]_c$ -increase with a similar time course. The gravitational acceleration increased from 1g to 300g gradually (dotted line). The accurate  $[Ca^{2+}]_c$  was not monitored during the gravitational acceleration increase due to the experimental setup<sup>13</sup>(not illustrated).

slow  $[Ca^{2+}]_c$ -increase is not influenced by that of the fast transient. Interestingly, hypergravity stimulation (20g or 300g) by centrifugation induces a similar slow transient  $[Ca^{2+}]_c$ -increase.<sup>13</sup>

These accumulations of experimental data in a variety of conditions allow us to estimate the kinetic parameters of the slow  $[Ca^{2+}]_c$ -response, and to evaluate a variety of cellular mechanisms proposed to account for the gravistimulation-induced slow  $[Ca^{2+}]_c$ -increases. Here, a potential molecular process of the gravistimulus-induced  $[Ca^{2+}]_c$ -increase in hypocotyls and petioles of *Arabidopsis* seedlings is proposed and discussed with an updated knowledge of biophysics of cellular and molecular mechanism of cell mechano-sensing.<sup>14-16</sup>

### Time Courses and Kinetic Parameters of $[Ca^{2+}]_c$ -Increases to Various Gravistimuli

Typical time courses and kinetics parameters of the gravistimulation-induced  $[Ca^{2+}]_c$ -increases are schematically illustrated in **Figure 1**. Gravistimulation of seedlings by turning

180° induces a transient biphasic  $[Ca^{2+}]_c$ -increase (**Fig. 1A**). A wide variety of stimulation scenarios is possible with parabolic flights (PFs), e.g., sequential changes in the gravity intensity ranging from  $\mu g$  to 2g. Gravistimulation by 180° rotation at 0.5g induces a biphasic  $[Ca^{2+}]_c$ -increase but with smaller peaks than at 1g (**Fig. 1A**), while gravistimulation at 1.5g or 2g induces larger biphasic  $[Ca^{2+}]_c$ -increases with almost the same kinetics.<sup>12</sup> The peak amplitude of the slow  $[Ca^{2+}]_c$ -increase is nearly linearly dependent on the magnitude of gravitational acceleration, suggesting that *Arabidopsis* seedlings possess a gravity-sensing mechanism that can transduce 0.5g to 2g gravitational acceleration into the amplitude of the slow  $[Ca^{2+}]_c$ -increase, while their kinetic parameters remain unchanged. This study<sup>12</sup> revealed that the amplitude of the first peak is also gravity dependent as well as speed of rotation (for details, see “Discussion” in the original paper<sup>12</sup>).

When seedlings were rotated under  $\mu g$ , a small transient  $[Ca^{2+}]_c$ -increase was detected. Gravitational acceleration from  $\mu g$  to 1.5g induced  $[Ca^{2+}]_c$ -increases with a time course similar to that of the slow  $[Ca^{2+}]_c$ -increase (**Fig. 1B**), demonstrating that

the gravistimulation without rotation of specimen causes only the slow  $[Ca^{2+}]_c$ -increase. It also demonstrated that the  $[Ca^{2+}]_c$  increased with a delay between the transition ( $\mu g$  to  $1.5g$ ) and the onset of the slow  $[Ca^{2+}]_c$ -increase, named  $t_1$  ( $t_1 \sim 20$  s) (Fig. 1B). The aequorin signal during the delay ( $t_d$ ) was 10% higher than the basal level. This denotes that gravistimulation induces a slight  $[Ca^{2+}]_c$ -increase without delay prior to the initiation of a slow  $[Ca^{2+}]_c$ -transient (dotted arrow in Figure 1B).

The parabolic flight experiment also revealed that a sudden gravitational decrease from  $2g$  to  $\mu g$  did not attenuate the slow  $[Ca^{2+}]_c$ -increase induced by  $180^\circ$ -gravistimulation at  $2g$  (Fig. 1C). The rising phase of the response was not affected by the sudden  $g$  decrease (the shaded period in Figure 1C). This indicates that once the gravity sensing process is triggered, the  $[Ca^{2+}]_c$ -response proceeds irrespective of gravity changes.

The gravitational changes (e.g.,  $1g$ - $2g$ - $\mu g$ - $1.5g$ ) during the PF did not affect the  $[Ca^{2+}]_c$  in seedlings placed in the upright position (Fig. 1D). The plant may not generate active graviperception signals when growing at its preferred angle to gravity despite alterations in  $g$  force but rather in response to displacements from this angle, suggesting the plants adapt to the gravity in the preferred angle (e.g., upright position).

The  $[Ca^{2+}]_c$  transiently increases and decays exponentially during  $20g$  to  $300g$ -hypergravity stimulation (Fig. 1E), the time course of which resembles that shown in Figure 1B. The  $[Ca^{2+}]_c$ -response showed a strong desensitization. For example,  $[Ca^{2+}]_c$ -response was not elicited even 45 min after the first stimulation.<sup>13</sup> Similar but less substantial desensitization to the repeated  $180^\circ$ -gravistimulation was also seen in  $1g$  ground experiments.<sup>9</sup>

The potential mechanosensitive  $Ca^{2+}$ -permeable channel (MSCC) inhibitors  $Gd^{3+}$  and  $La^{3+}$ , the endomembrane  $Ca^{2+}$ -permeable channel inhibitor ruthenium red (RR), and the phospholipase C (PLC) inhibitor U73122 profoundly attenuated the slow transient  $[Ca^{2+}]_c$ -increase, suggesting that it arises from  $Ca^{2+}$  influx via putative MSCCs in the plasma membrane and  $Ca^{2+}$  release from intracellular  $Ca^{2+}$  stores. The slow transient  $[Ca^{2+}]_c$ -increase was attenuated by actin-disrupting drugs cytochalasin B and latrunculin B, implying that actin filaments are involved in the activation of the MSCCs.<sup>9</sup> The slow transient  $[Ca^{2+}]_c$ -increase is presumably induced in the following sequence based on the pharmacological experiments: 1) activation of plasma membrane MSCCs and PLC, 2) production of  $InsP_3$ , and 3)  $InsP_3$ -induced  $Ca^{2+}$ -release (IICR) from ER (or vacuole).

### Possible Cellular Mechanisms to Transduce Forces Generated by Gravity Vector Changes

“Starch-statolith” and “non-starch-statolith” hypotheses have been proposed to explain the gravity sensing.<sup>3,17,18</sup> We briefly introduce these hypotheses and a reinforced “starch-statolith” hypothesis with knowledge of the mechano-sensing mechanism explored in mammalian endothelial cells with biophysical techniques.

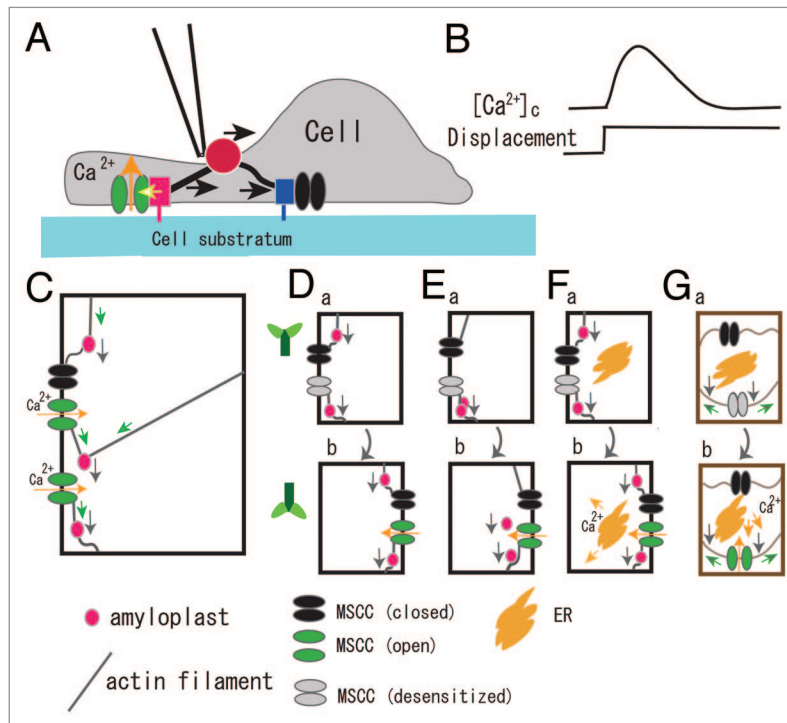
“Starch-statolith” and “non-starch-statolith” hypotheses are schematically illustrated in Figure 2. The starch-statolith mechanism typically includes sedimentation of the high-density plastids, amyloplasts, which may cause 1) stress increase in the actin network, resulting in activation of MSCCs, which is followed by  $[Ca^{2+}]_c$ -increases or 2) membrane deformation and stress increase in the membrane, which may also activate MSCCs.<sup>19</sup> The non-starch-statolith mechanism includes protoplast pressure model;<sup>20-22</sup> cells are assumed to be a water-filled balloon inside a cardboard box, and cells determine the membrane to be in the “downward direction,” when pressure to the plasma membrane increases and stretches the membrane (Fig. 2G). MSCCs sense the tension increase in the membrane, and will be activated. Although the non-starch-statolith model is still controversial in higher plants, it could be supported by observations that a certain class of starchless mutants responds to gravity essentially in the same way as the wild type (ca. 70% of the wild type)<sup>23</sup> and the elongation zone rather than the root cap containing amyloplasts contributes to gravity sensing in maize roots.<sup>24</sup> Here, it should also be noted that the starchless plastids may also function as statolith.<sup>25</sup>

“Starch-statolith” hypothesis is often referred to account for gravity-induced responses, and recent studies reinforced the “starch-statolith” hypothesis significantly. High-gradient magnetic field (HGMF) effectively mimics a gravitational field. Amyloplast displacement is induced under the HGMF, which in turn results in the organ curvature like the gravitropic response, and HGMF exploits differences in the diamagnetic susceptibilities between starch and cytoplasm.<sup>26,27</sup> HGMF does not affect root gravitropism of starchless mutants, which indicates that HGMF is likely to act on amyloplasts.

The hypergravity condition has been used to examine the mechanism of gravitropism in roots and hypocotyls,<sup>28</sup> and recently the hypergravity condition generated on a centrifuge microscope reveals that sedimentary movements of amyloplasts under hypergravity conditions are strongly correlated with gravitropic curvature in *Arabidopsis* stems even in the mutant which did not show amyloplast sedimentation at  $1g$ .<sup>29</sup> These studies<sup>28,29</sup> strongly support and reinforce the “starch-statolith” hypothesis.

*Arabidopsis* seedlings may use MSCCs and actin fibers to detect changes in the direction of the gravity vector as mentioned above section. Thus, the gravistimulus-induced  $[Ca^{2+}]_c$ -increase may be accounted for by the “starch-statolith” hypothesis with MSCCs and actin fibers. To test the “starch-statolith” hypothesis it is indispensable to directly manipulate the actin fiber and examine the MSCCs activation. However, no useful experimental methodology have been available in plants.

By manipulating a bead attached on a dorsal surface of cultured human umbilical vein endothelial cells (HUVECs) with a micropipette, it has become possible to apply forces directly to actin fibers and to detect activation of MSCCs, which demonstrates that the actin stress fiber works as a force-transmitting molecular device to activate MSCCs with an extremely small magnitude of force<sup>16</sup> in the range of the driving force of amyloplast sedimentation.<sup>18</sup>



**Figure 2.** Schematic illustrations of the possible cellular process of sensing the tensile stress increase and the gravistimulus-induced slow  $[Ca^{2+}]_c$ -increase. (A), Displacement of the FN bead (ca. 1  $\mu\text{m}$  for 10 s) generates tensile stress in the left-side actin stress fiber, activates MSCCs (a red box denotes an FA, and a yellow arrow designates stress that activates the MSCC colored green), while the compressive stress in the right side stress fiber is loaded to an FA (designated by a blue box), but not activate the MSCC colored black. The black arrow above a glass bead (red) indicates the direction of displacement. (B), A transient increase in  $[Ca^{2+}]_c$  (upper trace) induced by a displacement of the FN bead (lower trace). (C), Activation of MSCCs by the tensile increase in actin filaments. The gravitational force acting on amyloplasts (gray arrows) generates tensile stress (green arrows) and compressive stress designated by buckled lines of actin filaments. Open MSCCs are colored green and closed ones black. See text for more descriptions. (D), A "starch-statolith" model, which consist of amyloplasts, actin filaments, and MSCCs. A MSCC in upper panel (a) is desensitized (colored gray) to continuously applied tensile stress. The MSCC is activated (colored green) by a tensile tension increase when the plant is rotated 180° in panel (b). (E), A conventional "starch-statolith" model, which includes sedimentation of amyloplasts when the specimen rotated 180°. (F), A "starch-statolith" model with a signal transduction pathway that triggers  $Ca^{2+}$  release. (G), A "non-statolith-statolith" model. The cell wall (brown) and the plasma membrane (dark brown) are illustrated separately. Pressing the membrane (gray arrows) causes the stress increase in the membrane (green arrows).  $Ca^{2+}$  flux is shown by yellow arrows. The direction of plants is illustrated in the side of panel D.

In the newly developed method, glass beads conjugated with fibronectin (FN) were plated on cultured HUVECs. The beads adhered to the apical cell surface, leading to the formation of focal adhesions (FAs) underneath the beads. These FAs were connected to the pre-existing FAs at the basal cell surface via actin stress fibers (Fig. 2A). With this bead–apical–FA–stress-fiber–basal–FA linkage, it is possible to apply localized mechanical forces to the basal FAs by displacing the bead with a piezoelectric-driven glass pipette. Immediately after a displacement of the FN bead, the  $[Ca^{2+}]_c$  increased transiently (Fig. 2B). High speed imaging of the locus of the  $[Ca^{2+}]_c$  increase showed that MSCCs in the vicinity of the basal FAs were specifically activated by the tensile stress in the actin stress fibers, but MSCCs were not activated by compressive stress as shown in Figure 2A.<sup>16</sup> This enables cells to sense the direction of force applied externally.

The time course of the  $[Ca^{2+}]_c$ -increase reflects the accumulated  $Ca^{2+}$  via the MSCCs activated by the mechanical force, and of the decline of the  $[Ca^{2+}]_c$  denotes the cessation of MSCCs activation and sequestration of  $Ca^{2+}$  to intracellular store sites and the extrusion of  $Ca^{2+}$  to extracellular space by  $Ca^{2+}$ -pumps. When

the bead was displaced and hold at the same position, the  $[Ca^{2+}]_c$  increased transiently, and returned to a resting  $[Ca^{2+}]_c$  level in a few minutes (Fig. 2B). Generally the rate of sequestration and extrusion of  $Ca^{2+}$  is low comparing with the rate of  $Ca^{2+}$  influx via  $Ca^{2+}$  channels.<sup>30</sup> The  $Ca^{2+}$  influx via MSCCs would be nearly terminated at around the peak of  $[Ca^{2+}]_c$ -increase. This implies the inactivation of MSCCs whereas the bead was displaced continuously. This desensitization (i.e., inactivation of MSCCs to bead displacement) was confirmed by an observation that the mechano-stimuli induced  $[Ca^{2+}]_c$ -increase was attenuated by repeated stimulations. The mechanism of the desensitization is not identified at present, but decreases in the tension increase in the stress fiber during the repeated bead-displacement is known,<sup>15</sup> which would result in less activation of MSCCs in HUVECs.

The high-speed imaging of the  $[Ca^{2+}]_c$ -increase shows tensile stress-increase in actin stress fibers is transmitted to FAs and activates the MSCCs in less than a few ms,<sup>16</sup> suggesting the direct activation of MSCCs by the tension increase.

Recent time-lapse video analysis of the dynamic movement of the amyloplasts in an endodermal cell after gravistimulation by

reorientation (90 degrees) revealed that some amyloplasts moved along a newly applied gravity direction and others did not.<sup>31</sup> The amyloplasts that did not move are presumably tightly adhered to the actin filaments,<sup>32</sup> and those moved in the direction of gravity will interact with actin filaments after sedimentation.

These ideas are included in the “starch-statolith” hypothesis to reinforce it in the following section. We use this “reinforced starch-statolith hypothesis” in **Figure 2** (panels C to F) to explain the kinetics of the gravistimulus induced  $[Ca^{2+}]_c$ -increase under different scenarios of gravistimulation as follows.

### Possible Cellular Mechanisms That Explain the Cytoplasmic Calcium Increases by Gravistimuli in Hypocotyls and Petioles of *Arabidopsis* Seedlings

Among a wide variety of “starch-statolith” models of gravisensing,<sup>18,33,34</sup> we choose models that include actin fibers as a force transmitting device, since the gravistimulus-induced slow  $[Ca^{2+}]_c$ -increase in hypocotyls and petioles is attenuated by actin-disrupting drugs.

The model in Panel C in **Figure 2** shows activation of MSCSSs by tension increases in actin filaments generated by gravitational force acting on the amyloplasts tightly adhered to the filaments. MSCCs connected to actin filaments with different angle are illustrated. The tensile stress increases activate two MSCCs (green channels), whereas the compressive stress does not (black channel) as illustrated in the case of HUVECs (panel A). This model can generate some simplified versions as depicted in panels D to G. Only two MSCCs are illustrated in these panels; one is activated and the other is not.

Three types of “starch-statolith” models are shown in panels D to F in **Figure 2**. The model in panel D shows amyloplasts tightly adhered to actin filaments. The model in panel E allows sedimentation of amyloplasts, which will introduce a delay between the onset of gravistimulation and the activation of MSCCs.<sup>7,18</sup> The model in panel F includes intracellular organs (e.g., endoplasmic reticulum, ER, and vacuole in endodermal cells) that release  $Ca^{2+}$  in response to the second messengers, e.g.,  $Ca^{2+}$  and inositol 1,4,5-trisphosphate ( $InsP_3$ ). ER serves as a major  $Ca^{2+}$  storage component, and is confined to the bottom of columella cells. A vacuole locates in the center of cells as well as occupies a major portion of the cell volume in endodermal cells.<sup>35</sup>

The model in panel E is often employed to account for the graviresponse in plants.<sup>34</sup> The delay in **Figure 1** ( $t_1$ ) is around 20 s, which is within the time range of amyloplasts sedimentation. This delay cannot be afforded by the model in panel D, because the force generated by the gravity will activate MSCCs immediately according to the biophysical analysis of force dependent MSCC activation,<sup>16</sup> which will increase the  $[Ca^{2+}]_c$  within a few milliseconds.

Desensitization to the gravistimulation (**Fig. 1D**) can be accounted for by the desensitization of MSCCs (gray closed channels) to the continuously applied tensile stress generated by the gravitational force (**Fig. 2D–F**). The idea of desensitization to the mechanical stimulation is borrowed from the HUVECs

studies (**Fig. 2A and B**). When plants are rotated 180°, newly generated tensile stress in the actin filament activates the MSCCs transiently (green MSCCs). The model in panel E also accounts for the hypergravity (> 20 g)-induced  $[Ca^{2+}]_c$ -increase. A large tensile stress will activate MSCCs and may induce a  $[Ca^{2+}]_c$ -increase with a similar time course.

The model in panel E, however, hardly accounts for the delay of the graviresponse in different gravitropic acceleration conditions (0.5g–2g, or 200g), because the delay owing to the sedimentation of amyloplasts must be strongly affected by the degree of gravity vector alteration; the delay should be shortened at higher gravitropic acceleration conditions and elongated at lower g conditions. According to the model in panel E, the sudden decrease in the gravity must affect the time course of the  $[Ca^{2+}]_c$ -increase as shown by the dotted line in **Figure 1C** (the  $[Ca^{2+}]_c$  will no longer elevate under  $\mu g$ , and it should decline due to the  $Ca^{2+}$  sequestration), because the sedimentation of amyloplasts must be terminated immediately due to that the small Reynolds number of the amyloplasts in the cytoplasm,  $< 10^{-6}$ <sup>36</sup> if they sediment passively by the gravity.

We prefer the model in panel F, which includes a signal transduction pathway for triggering  $Ca^{2+}$  release subsequent to gravity perception via signals (e.g.,  $InsP_3$  and  $Ca^{2+}$ ) that are presumably not affected by changes in gravity.<sup>12</sup> This model is supported by a pharmacological study<sup>9</sup> that shows the involvement of phospholipase C (PLC) and endomembrane  $Ca^{2+}$ -permeable channels.

The delay ( $t_1 \sim 20$  s) in the slow  $[Ca^{2+}]_c$ -increase can be explained by a slow production of  $InsP_3$  according to the following observations (1 and 2). (1) The minimum duration of gravistimulation needed to induce a detectable response is in a sub-second range.<sup>12</sup> Within this time plants sense the change in gravity vector and trigger a signaling process (e.g., PLC). (2) The quantitative time-resolved measurements of cytosolic  $Ca^{2+}$  release by photolysis of caged  $InsP_3$  shows that the  $[Ca^{2+}]_c$ -increases less than a second by  $InsP_3$  increases,<sup>37</sup> and pharmacological results<sup>9</sup> suggest the major component of gravistimulus-induced  $[Ca^{2+}]_c$ -increase is PLC dependent. According to these (1 and 2), the delay in the gravistimulus-induced slow  $[Ca^{2+}]_c$ -increase is likely due to a slow onset of PLC dependent  $InsP_3$  production (ca. 20 s). The idea of such slow production of  $InsP_3$  (ca. 20 s) is not unusual because slow production of  $InsP_3$  is reported in muscle cells,<sup>38</sup> and in human endothelial cells by shear stress.<sup>39</sup>

The hypothesis in panel F is summarized as follows; gravity changes (e.g., rotation) loaded onto the amyloplasts tightly adhered to the actin filaments generate tension increase immediately in the actin filament, which activate MSCCs, and PLC. PLC produces  $InsP_3$  with a considerable delay, and  $InsP_3$  causes  $Ca^{2+}$ -release (IICR) from ER (or vacuole) in cell periphery.<sup>40,41</sup> The  $Ca^{2+}$  influx through MSCCs increases the  $[Ca^{2+}]_c$  slightly without a delay, and  $InsP_3$  increases the  $[Ca^{2+}]_c$  profoundly with a delay ( $t_1$ ). This model fits well with that the gravistimulation-induced slight  $[Ca^{2+}]_c$ -increases prior to the slow large  $[Ca^{2+}]_c$ -increase (**Fig. 1B**). The slight  $[Ca^{2+}]_c$ -increases may also trigger the  $Ca^{2+}$ -induced  $Ca^{2+}$ -release.

This model gives a good fit to the results in **Figure 1A**, assuming that the delay  $\tau_1$  is not dependent on the magnitude of gravity change. Gravity stimulus at 0.5g will activate the above signaling process with less magnitude, and the same stimulus at 2g will activate it with greater magnitude. The  $[Ca^{2+}]_c$ -increases in proportion to the magnitude of gravity changes, and can be explained by the model with nearly the same kinetic parameters. The model also fits well **Figure 1D**, since MSCCs are desensitized to continuous stress increases as shown in Figure 2Fa. The MSCCs will be activated by large stress increase by the hypergravity stimulation as shown in **Figure 1E**.

The importance of this model is in giving a satisfactory fit to various aspects of the time course of the gravistimulus-induced slow  $[Ca^{2+}]_c$ -increase as mentioned above, while requiring only two additional components “desensitization of MSCCs” and “InsP<sub>3</sub> production with delay.”

Note that all observations mentioned above do not exclude the “non-starch-statolith” model of graviresponse. One of the possible models is shown in **Figure 2G**; pressure increases to the cell membrane induced by the gravistimulus activate the MSCCs, PLC-dependent production of InsP<sub>3</sub>, and IICR. It might also work in parallel with the statolith sedimentation model.

## Future Perspective

The key components of our proposed “starch-statolith model” are mechano-sensitive (MS) channels and actin filaments.<sup>9</sup> However, the molecular entities of plasma membrane MS channels responsible for the gravisensing have not been identified yet. Recent studies identified four types of plant MS channels; plant homologs of bacterial mechanosensitive channel of small conductance (MscS), *MSL*,<sup>42,43</sup> Ca<sup>2+</sup>-permeable plasma membrane protein, *MCA*,<sup>44,45</sup> two pore potassium channel, *TPK*,<sup>46</sup> and newly identified MS channel, *Piezo*.<sup>47</sup> In *Arabidopsis* genome, 10 of *MSL* (*MSL1* – 10), 2 of *MCA* (*MCA1* and 2), 5 of *TPK* (*AtTPK1* – 5), and 1 of *Piezo* (*At2g48060*) are present. Some of these MS channels in *Arabidopsis* are functionally characterized; *MSL2* and 3 play a role in chloroplast division; *MSL9* and 10 are plasma membrane MS anion channel; *MCA1* and *AtTPK4* are plasma membrane MS cation channels.<sup>42,43,48-50</sup> Ca<sup>2+</sup> uptake was altered in *Arabidopsis* seedling and yeast cells overexpressing *MCA1*-GFP proteins in the plasma membrane of *Arabidopsis* root cells. Seedlings of knockout line (*mca1*) lack the ability to penetrate their roots into a harder agar.<sup>44</sup> These suggest that *MCA* may encode MS Ca<sup>2+</sup>-permeable channel. Among *Arabidopsis* *TPKs*, only *AtTPK4* localizes in the plasma membrane but not permeate Ca<sup>2+</sup>. The function of the *MSLs* and *Piezo* remains to be demonstrated. The plasma membrane MS channels responsible for the gravistimulation-induced  $[Ca^{2+}]_c$ -increase, and the molecular process of activation and desensitization of MSCCs by tension is remained to be elucidated.

Recent studies using actin filaments disrupting drug latrunculin B have provided controversial results:<sup>51,52</sup> root and shoot curvatures

were enhanced in *Arabidopsis* by the treatment with the drug. Amyoplast sedimentation in *Arabidopsis* endodermal cells appears to be enhanced by latrunculin B treatment.<sup>31</sup> However, it should be noted that the drug treatment affects the entire plant, and the effect of latrunculin B cannot be attributed to the effect of the drug to statocytes alone as pointed out,<sup>33</sup> we should also keep in mind that actin filaments play multiple roles in cells such as endomembrane dynamics or trafficking.

A recent in vitro study reveals that the actin filament itself functions as a mechanosensor in human endothelial cells.<sup>14</sup> A tiny force as small as 2 pN can be sensed by actin filaments, which affords an intriguing new hypothesis of gravity sensing by actin filaments; i.e., the sedimentation of amyoplasts increases the stress in the actin filaments,<sup>18</sup> which may modulate the interaction between actin filaments and actin-binding proteins<sup>53</sup> and lead to gravistimulus-induced responses.

The detail of signaling processes from tension increase in the actin fibers to the activation of MSCCs has not been explored, and the signaling molecules responsible for PLC activation are not identified at present. The processes from the  $[Ca^{2+}]_c$ -increase to the gravitropic response, which are highly likely auxin dependent,<sup>54</sup> are also not identified yet, and left for future studies.

The durations of gravistimuli (especially those of parabolic flight experiments) reviewed here are mostly short (e.g., less than 20 s). Thus the analysis and interpretation of the effect of gravistimuli to the  $[Ca^{2+}]_c$  is limited. Effects of gravistimuli with longer duration and different range to the  $[Ca^{2+}]_c$  will be required for understanding the pathway from gravi-sensing toward gravitropism. Mechanisms underlying the starch-statolith model have been examined in space flight experiments,<sup>55</sup> which enables observation of gravitropism only made in an environment where plants are exposed to different magnitude of gravity for days or months.

The cellular and molecular mechanism of the gravity sensing is still enigmatic; “starch-statolith” and “non-starch-statolith” mechanisms are relevant for explaining the graviresponse. The gravisensing mechanism of shoots and roots might be considerably different. Therefore, the molecular characterization of gravisensing-deficient mutants<sup>33</sup> and direct imaging of molecules crucial for the gravisensing in shoots and roots will accelerate the understanding of gravisensing machineries in plants.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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