Analyses of a Gravistimulation-Specific Ca$^{2+}$ Signature in Arabidopsis using Parabolic Flights$^1$[W][OPEN]

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Gravity is a critical environmental factor affecting the morphology and functions of organisms on the Earth. Plants sense changes in the gravity vector (gravistimulation) and regulate their growth direction accordingly. In Arabidopsis (Arabidopsis thaliana) seedlings, gravistimulation, achieved by rotating the specimens under the ambient 1g of the Earth, is known to induce a biphasic (transient and sustained) increase in cytoplasmic calcium concentration ([Ca$^{2+}$]). However, the [Ca$^{2+}$] increase genuinely caused by gravistimulation has not been identified because gravistimulation is generally accompanied by rotation of specimens on the ground (1g), adding an additional mechanical signal to the treatment. Here, we demonstrate a gravistimulation-specific Ca$^{2+}$ response in Arabidopsis seedlings by separating rotation from gravistimulation by using the microgravity (less than 10$^{-3}$g) conditions provided by parabolic flights. Gravistimulation without rotating the specimen caused a sustained [Ca$^{2+}$], increase, which corresponds closely to the second sustained [Ca$^{2+}$], increase observed in ground experiments. The [Ca$^{2+}$] increases were analyzed under a variety of gravity intensities (e.g. 0.5g, 1.5g, or 2g) combined with rapid switching between hypergravity and microgravity, demonstrating that Arabidopsis seedlings possess a very rapid gravity-sensing mechanism linearly transducing a wide range of gravitational changes (0.5g–2g) into Ca$^{2+}$ signals on a subsecond time scale.

Calcium ion (Ca$^{2+}$) functions as an intracellular second messenger in many signaling pathways in plants (White and Broadley, 2003; Hetherington and Brownlee, 2004; McInish and Pittman, 2009; Spalding and Harper, 2011). Endogenous and exogenous signals are spatio-temporally encoded by changing the free cytoplasmic concentration of Ca$^{2+}$ ([Ca$^{2+}$]), which in turn triggers [Ca$^{2+}$]-dependent downstream signaling (Sanders et al., 2002; Dodd et al., 2010). A variety of [Ca$^{2+}$] increases induced by diverse environmental and developmental stimuli are reported, such as phytohormones (Allen et al., 2000), temperature (Plieth et al., 1999; Dodd et al., 2006), and touch (Knight et al., 1991; Monshausen et al., 2009). The [Ca$^{2+}$], increase couples each stimulus and appropriate physiological responses. In the Ca$^{2+}$ signaling pathways, the stimulus-specific [Ca$^{2+}$] pattern (e.g. amplitude and oscillation) provide the critical information for cellular signaling (Scrase-Field and Knight, 2003; Dodd et al., 2010). Therefore, identification of the stimulus-specific [Ca$^{2+}$] signature is crucial for an understanding of the intracellular signaling pathways and physiological responses triggered by each stimulus, as shown in the case of cold acclimation (Knight et al., 1996; Knight and Knight, 2000).

Plants often exhibit biphasic [Ca$^{2+}$] increases in response to environmental stimuli. Thus, slow cooling causes a fast [Ca$^{2+}$] transient followed by a second, extended [Ca$^{2+}$], increase in Arabidopsis (Arabidopsis thaliana; Plieth et al., 1999; Knight and Knight, 2000). The Ca$^{2+}$ channel blocker lanthanum (La$^{3+}$) attenuated the fast transient but not the following increase (Knight and Knight, 2000), suggesting that these two [Ca$^{2+}$] peaks have different origins. Similarly, hypoosmotic shock caused a biphasic [Ca$^{2+}$] increase in tobacco (Nicotiana tabacum) suspension-culture cells (Takahashi et al., 1997; Cessna et al., 1998). The first [Ca$^{2+}$], peak was inhibited by gadolinium (Gd$^{3+}$), La$^{3+}$, and the Ca$^{2+}$ chelator EGTA (Takahashi et al., 1997; Cessna et al., 1998), whereas the second [Ca$^{2+}$], increase was inhibited.
by the intracellular Ca\(^{2+}\) store-depleting agent caffeine but not by EGTA (Cessna et al., 1998). The amplitude of the first \([Ca^{2+}]_c\) peak affected the amplitude of the second increase and vice versa (Cessna et al., 1998). These results suggest that even though the two \([Ca^{2+}]_c\) peaks originate from different Ca\(^{2+}\) fluxes (e.g. Ca\(^{2+}\) influx through the plasma membrane and Ca\(^{2+}\) release from subcellular stores, respectively), they are closely interrelated, showing the importance of the kinetic and pharmacological analyses of these \([Ca^{2+}]_c\) increases.

Changes in the gravity vector (gravistimulation) could work as crucial environmental stimuli in plants and are generally achieved by rotating the specimens (e.g. +180°) in ground experiments. Use of Arabidopsis seedlings expressing apoaequorin, a Ca\(^{2+}\)-reporting photoprotein (Plieth and Trewavas, 2002; Toyota et al., 2008a), has revealed that gravistimulation induces a biphasic \([Ca^{2+}]_c\) increase that may be involved in the sensory pathway for gravity perception/response (Pickard, 2007; Toyota and Gilroy, 2013) and the intracellular distribution of auxin during the rotation (Benjamins et al., 2003; Zhang et al., 2011). These results suggest that even though the two \([Ca^{2+}]_c\) changes have different characteristics. The first transient \([Ca^{2+}]_c\) increase depends on the rotational velocity but not angle, whereas the second sustained \([Ca^{2+}]_c\) increase depends on the rotational angle but not velocity. The first \([Ca^{2+}]_c\) transient was inhibited by Gd\(^{3+}\), La\(^{3+}\), and the Ca\(^{2+}\) chelator 1,2-bis(2-aminophenoxy)ethane-N,N',N''-tetraacetic acid but not by ruthenium red (RR), whereas the second sustained \([Ca^{2+}]_c\) increase was inhibited by all these chemicals. These results suggest that the first transient and second sustained \([Ca^{2+}]_c\) increases are related to the rotational stimulation and the gravistimulation, respectively, and are mediated by distinct molecular mechanisms (Toyota et al., 2008a). However, it has not been demonstrated directly that the second sustained \([Ca^{2+}]_c\) increase is induced solely by gravistimulation; it could be influenced by other factors, such as an interaction with the first transient \([Ca^{2+}]_c\) increase (Cessna et al., 1998), vibration, and/or deformation of plants during the rotation.

To elucidate the genuine Ca\(^{2+}\) signature in response to gravistimulation in plants, we separated rotation and gravistimulation under microgravity (\(\mu g\); less than 10\(^{-3}\)g) conditions provided by parabolic flight (PF). Using this approach, we were able to apply rotation and gravistimulation to plants separately (Fig. 1). When Arabidopsis seedlings were rotated +180° under \(\mu g\) conditions, the \([Ca^{2+}]_c\) response to the rotation was transient and almost totally attenuated in a few seconds. Gravistimulation (transition from \(\mu g\) to 1.5g) was then applied to these prerotated specimens at the terminating phase of the PF. This gravistimulation without simultaneous rotation induced a sustained \([Ca^{2+}]_c\) increase. The kinetic properties of this sustained \([Ca^{2+}]_c\) increase were examined under different gravity intensities (0.5g–2g) and sequences of gravity intensity changes (Fig. 2A). This analysis revealed that gravistimulation-specific Ca\(^{2+}\) response has an almost linear dependency on gravitational acceleration (0.5g–2g) and an extremely rapid responsiveness of less than 1 s.

**RESULTS**

\([Ca^{2+}]_c\) Increases Genuinely Induced by Gravistimulation

To help define how \([Ca^{2+}]_c\) changes observed throughout the various g phases of PF might be linked to rotation or gravistimulation, we first examined \([Ca^{2+}]_c\) increases induced by 180° rotation at 1g (i.e. during horizontal flight, the flight control; Fig. 3A). The kinetic parameters of the \([Ca^{2+}]_c\) increase induced under these conditions was almost the same as those seen in ground experiments (Supplemental Fig. S1): the delay between the onset of rotation and the peak of the second sustained \([Ca^{2+}]_c\) increase (t; 39.3 ± 1.2 s \([n = 14]\); Fig. 3F) and other parameters shown in Figure 3G were nearly the same (Table I). The signal amplitude of the flight control, however, was attenuated to approximately 30% of the ground control (Supplemental Fig. S1, C and D), probably due in part to the consumption of reconstituted aequorin induced by Ca\(^{2+}\) signals generated by the mechanical stresses experienced by the seedlings during takeoff of the aircraft (e.g. vibration).

PF created sequential changes in gravity intensity, ranging from \(\mu g\) for approximately 20 s to hypergravity of up to 2g before and after \(\mu g\) (Fig. 2A). Temperature, humidity, and pressure were constant throughout these changes in gravity (Fig. 2B). Such sequential gravitational changes during the PF did not affect the \([Ca^{2+}]_c\) in seedlings placed in the upright position (Fig. 3B). As \([Ca^{2+}]_c\) did change in response to rotation under these circumstances, this observation suggests that the plant may not generate active graviperception signals when growing at its preferred angle to gravity (gravitational set-point angle) despite alterations in g force but rather in response to displacements from this angle. Therefore, we next applied 180° rotation to plants during these
different phases of PF. Importantly, the tangential and centrifugal accelerations exerted on the seedlings by rotation were $3.6 \times 10^{-2} \, g$ and $5.6 \times 10^{-3} \, g$, respectively, both of which are too small to induce $[Ca^{2+}]_c$ increases by themselves (Toyota et al., 2007). Thus, any gravity-related $[Ca^{2+}]_c$ changes observed during these PF experiments should not be complicated by direct effects of acceleration associated with the act of rotation being superimposed on the effects of a background of $\mu g$. When seedlings were rotated under $\mu g$ (Fig. 1), a brief, transient $[Ca^{2+}]_c$ increase was elicited (Fig. 3, C and D). This change resembled the first transient $[Ca^{2+}]_c$ increase seen in response to 180° rotation in ground experiments. As gravitational acceleration was increased from $\mu g$ to 1.5g, $[Ca^{2+}]_c$ increased and peaked at $t_s = 63.6 \pm 3.6 \, s \,(n = 22)$ from the onset of the 180° rotation (Fig. 3C). This time to the peak of the sustained $[Ca^{2+}]_c$ increase ($t_s$ in Fig. 3G) was prolonged for approximately 20 s in comparison with the $1g$ controls (Fig. 3, A and F; Table I). This delay coincides with the time between the rotation and the end of $\mu g$, suggesting that the sustained $[Ca^{2+}]_c$ increase is induced by the 1.5g 180° gravistimulation after $\mu g$. To determine if this sustained phase was linked to the transition to greater than $\mu g$ specifically in rotated plants, we rotated seedlings 180°, followed by an immediate return back to 0°, during the period of $\mu g$. This treatment elicited the transient initial $[Ca^{2+}]_c$ increase but not the subsequent sustained $[Ca^{2+}]_c$, likely due to the plant experiencing the increase to 1.5g gravistimulation in an upright direction (Fig. 3E). As there were many sequential parabolas as part of the PF, the same seedlings were then rotated 180° during $\mu g$ (at 330 s), resulting in the sustained $[Ca^{2+}]_c$ increase by 1.5g (Fig. 3E). These data suggest that the transient $[Ca^{2+}]_c$ increase was induced by the rotation and would not affect the sustained $[Ca^{2+}]_c$ increase. The peak amplitude of the sustained $[Ca^{2+}]_c$ increase was almost the same irrespective of the $[Ca^{2+}]_c$ transient amplitude (Fig. 3, C and D), and there is no apparent correlation between the first transient and second sustained $[Ca^{2+}]_c$ increase induced by 180° rotation during $\mu g$ (Supplemental Fig. S2), showing that the amplitude of the transient $[Ca^{2+}]_c$ increase is not related to that of the sustained $[Ca^{2+}]_c$ increase ($n = 22; r = -0.09, P = 0.71$; Supplemental Fig. S2C).

The kinetic parameters of the sustained $[Ca^{2+}]_c$ increase (e.g. $r$ or $\tau$ in Fig. 3G) were almost the same as those of the flight control (Table I), supporting the idea that the sustained $[Ca^{2+}]_c$ increase is a $Ca^{2+}$ signature genuinely induced by gravistimulation, and it corresponds to the second $[Ca^{2+}]_c$ increase observed in plants gravistimulated by rotation on the ground. These PF experiments revealed that $[Ca^{2+}]_c$ increased with a delay after a transition from $\mu g$ to 1.5g (black bars in Fig. 3, C–E of 18.3 ± 0.6 s [n = 22]; $t_i$ in Fig. 3G). The aequorin signal during the latent period ($t_l$) was 10% ± 2% higher than the basal level ($P < 0.01; n = 22$), suggesting that gravistimulation induces a slight $[Ca^{2+}]_c$ increase prior to the sustained $[Ca^{2+}]_c$ increase. This increase was detected regardless of whether the seedlings

Figure 2. Acceleration, temperature, humidity, and pressure in an aircraft during flight experiments. A, Accelerations along $x$, $y$, and $z$ axes in the aircraft during PF. The direction of flight (FWD) and coordinates ($x$, $y$, and $z$) are indicated in the bottom graph. The inset shows an enlargement of the acceleration along the $z$ axis (gravitational acceleration) during $\mu g$ conditions lasting for approximately 20 s. B, Temperature, humidity, and pressure in the aircraft during PF. Shaded areas in graphs denote the $\mu g$ condition.
exhibited the transient initial change in \([Ca^{2+}]_c\) (Fig. 3, C and E) or not (Fig. 3D).

Hypergravity of 2g before entering the phase of \(\mu g\) is an inevitable element of PF. Therefore, we examined the effects of this component of hypergravity on the sustained \([Ca^{2+}]_c\) increase. The magnitude of the hypergravity was decreased to 1.2g from 2g (Supplemental Fig. S3A; for details, see “Materials and Methods”), which did not affect the kinetics and amplitude of the sustained \([Ca^{2+}]_c\) increase by 1.5g (Supplemental Fig. S4).

Gravity Dependency of the Sustained \([Ca^{2+}]_c\) Increase

Long-term (hours to days) morphological responses of plants under hypergravity or \(\mu g\) conditions have been described using plants grown in a centrifuge (Fitzelle and Kiss, 2001; Matsumoto et al., 2010) or aboard the space shuttle/station (Kiss et al., 1999, 2012; Paul et al., 2012; Roux, 2012), respectively. Such reports indicate that plants can respond with alterations in growth and development to a variety of gravitational accelerations. In contrast, short-term responses (seconds to minutes) of plants at different gravity conditions of 0.5g to 2g have rarely been examined. These short-term excursions to 0.5g to 2g were accessible using our PF regime. Therefore, we examined the \([Ca^{2+}]_c\) increase induced by gravistimulation at 0.5g, 1.5g, and 2g (Supplemental Fig. S3; for details, see “Materials and Methods”).

Gravistimulation by 180° rotation at 0.5g induced a biphasic \([Ca^{2+}]_c\) increase, but with smaller peaks than 1g (Fig. 4A), whereas gravistimulation at 1.5g and 2g induced larger biphasic \([Ca^{2+}]_c\) increases (Fig. 4, C and D) with almost the same time-dependent kinetics (Table I). The peak amplitude of the sustained \([Ca^{2+}]_c\) increase was almost linearly dependent on the magnitude of the gravitational acceleration (Fig. 4F), suggesting that Arabidopsis seedlings possess a gravity-sensing mechanism that can transduce 0.5g to 2g gravitational acceleration into Ca2+ signals whose amplitude depends on the magnitude of the gravitational acceleration but whose kinetic parameters remain constant.

Relationship between the Amplitude of the Sustained \([Ca^{2+}]_c\) Increase and the Duration of Gravistimulation

For experimentation on the Earth, the duration of gravistimulation cannot be changed easily without the use of rotation of the specimen for different periods of

between the groups \((P < 0.001, \text{one-way ANOVA followed by Tukey's multiple comparison test})\). G, Diagram of the \([Ca^{2+}]_c\) increase and kinetic parameters measured for statistical analysis. \(t_l\), Latent period, duration from the end of \(\mu g\) to the onset of the sustained \([Ca^{2+}]_c\) increase; \(r\), rate of rise of the sustained \([Ca^{2+}]_c\) increase; \(\tau\), decay time constant of the sustained \([Ca^{2+}]_c\) increase. Shaded areas in B to E and G denote the \(\mu g\) conditions. Black bars in C to E indicate a slight \([Ca^{2+}]_c\) increase during the \(t_l\) period.
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Table 1. Kinetic parameters of the sustained \([Ca^{2+}]_{i}\) increase induced by 180° rotation under different gravitational conditions

| Condition     | \(t_{1}\) (s) ± SE | \(r\) (\(\times 10^{-2}\) L\(_{\text{ratio}}\) s\(^{-1}\)) | \(\tau\) (s) ± SE | \(n\) | \(S_{\text{ratio}}\) (ng)
|---------------|---------------------|-------------------|------------------|-----|-------------
| 0.5g          | 40.0 ± 1.9a         | 1.5 ± 0.4a        | 56.0 ± 5.3a      | 6   | 1.5a 4.2   |
| 1g (ground)   | 41.3 ± 1.0a         | 7.8 ± 0.6b        | 57.2 ± 2.0a      | 20  | 0.5bd 54.5 |
| 1g (flight)   | 39.3 ± 1.2a         | 3.9 ± 0.4ac       | 55.3 ± 4.5a      | 14  | 0.2ac 57.6 |
| \(\mu\)g      | 63.6 ± 1.7b         | 3.6 ± 0.2ac       | 57.6 ± 3.6a      | 22  | 0.6b 57.2  |
| 1.5g          | 37.6 ± 0.8a         | 4.2 ± 0.4ad       | 52.0 ± 2.6a      | 5   | 0.4a 56.0  |
| 2g            | 36.9 ± 0.7a         | 6.0 ± 0.5bd       | 54.5 ± 1.9a      | 12  | 0.4a 56.0  |
| 2g (continuous)| 37.5 ± 1.0a         | 6.0 ± 0.4bcd      | 54.2 ± 3.8a      | 2   | 0.4bcd 54.2|

The presentation time estimated indirectly (less than 10 s in Lepidium sativum roots (Hejnowicz et al., 1998). Rapid transition between hypergravity and \(\mu\)g in the PF allowed us to apply a short gravistimulation of less than a few seconds without the need for rotation of the plant. Therefore, the presentation time for the sustained \([Ca^{2+}]_{i}\) increase was estimated by changing the duration of 2g exposure. The duration of 2g hypergravity exposure to the seedlings rotated through 180° was changed as shown with the horizontal bars below the aequorin signals in Figure 5A. The rotation of seedlings at 2g started 20 s before \(\mu\)g (“20-s exposure”) induced a large sustained \([Ca^{2+}]_{i}\) increase (black traces, Fig. 5A) with almost the same amplitude as observed under continuous 2g exposure (Fig. 4D). Because 5 s was required for 180° rotation, full gravistimulation at 2g was 15 s after rotation and induced a full \([Ca^{2+}]_{i}\) response (black traces, Fig. 5A). Gravistimulation with rotation started 6 s before \(\mu\)g (i.e. full gravistimulation for 1 s after rotation) induced an approximately half-maximal response (blue arrowhead in Fig. 5, A and C). A very small \([Ca^{2+}]_{i}\) response was detected when rotation was started 3 s before \(\mu\)g (red arrowhead in Fig. 5, A and C). In this case, 2g was applied to seedlings during rotation from 0° to 45°. Note that a “small” additional \([Ca^{2+}]_{i}\) increase was seen (blue and red double arrowheads in the inset of Fig. 5A) after the sustained \([Ca^{2+}]_{i}\) increases; the small \([Ca^{2+}]_{i}\) increase corresponds to the sustained \([Ca^{2+}]_{i}\) increase caused by 1.5g 180° gravistimulation after \(\mu\)g (Fig. 3, C and D). This small \([Ca^{2+}]_{i}\) increase was not detected in the seedlings rotated 20 s before \(\mu\)g, probably because the \([Ca^{2+}]_{i}\) response was fully activated. No apparent sustained \([Ca^{2+}]_{i}\) increase was detected in specimens not exposed to 180° rotation at 2g but exposed to upright (unrotated) 2g gravistimulation (measurements were made at 40 s after the rotation; Fig. 3C). We do not know the precise contribution of the gravity sensing during rotation (0°–45°) to the sustained \([Ca^{2+}]_{i}\) increase, but it is likely to be small because there is no statistical difference between responses to 0 and 3 s of 2g gravistimulation (Fig. 5C). Therefore, the presentation time for the sustained \([Ca^{2+}]_{i}\) increase is less than 6 s (i.e. 1 s of 2g full gravistimulation), which agrees with the presentation time estimated indirectly (less than 10 s; Hejnowicz et al., 1998).

Possible Involvement of Inositol 1,4,5-Trisphosphate Signaling in the Sustained \([Ca^{2+}]_{i}\) Increase

The PF experiments revealed that the sudden gravitational decrease from 2g to \(\mu\)g did not attenuate the sustained \([Ca^{2+}]_{i}\) increase induced by 2g 180° gravistimulation (Fig. 4D; Table I): that is, the rising phase of the response was not affected by the \(g\) decrease (Fig. 4D, inset). This finding indicates that once the gravity-sensing processes are activated, the \([Ca^{2+}]_{i}\) response proceeds less sensitively to absolute \(g\). This observation suggests a signal transduction pathway triggering \([Ca^{2+}]_{i}\) change subsequent to gravity perception via molecules that are not affected by the change in gravity. Our previous pharmacological study using RR (Toyota et al., 2008a) suggests the involvement of endomembrane \([Ca^{2+}]_{i}\)-permeable channels in the sustained \([Ca^{2+}]_{i}\) increase. Intracellular inositol 1,4,5-trisphosphate (InsP₃) level is known to increase within 15 s of gravistimulation in oat (Avena sativa) shoot pulvini (Perera et al., 2001), suggesting the involvement of phospholipase C (PLC)-dependent signaling in the response to gravistimulation (Perera et al., 2006; Smith et al., 2013). Thus, InsP₃-induced \([Ca^{2+}]_{i}\) release (Gilroy et al., 1990; Franklin-Tong et al., 1996; Knight et al., 1996) provides a potential mechanism for the sustained \([Ca^{2+}]_{i}\) increase that, once triggered, would be less sensitive to the gravity environment.
The effect of the PLC inhibitor U73122 on the sustained \([\text{Ca}^{2+}]_c\) increases was examined on the ground. U73122 attenuated the sustained \([\text{Ca}^{2+}]_c\) increase, but its inactive analog U73343 did not (Fig. 6), suggesting the involvement of PLC in the generation of the sustained \([\text{Ca}^{2+}]_c\) increase induced by the gravistimulation. Gravistimulation may trigger the PLC-dependent production of InsP3, which in turn induces InsP3-induced \(\text{Ca}^{2+}\) release via RR-sensitive endomembrane \(\text{Ca}^{2+}\)-permeable channels.

\([\text{Ca}^{2+}]_c\) Increases Induced by Rotatory Stimulation

Previously, we have suggested that the first transient \([\text{Ca}^{2+}]_c\) increase is related to rotational stimulation (Toyota et al., 2008a). Such a model is consistent with our observations here of the transient \([\text{Ca}^{2+}]_c\) increase induced by the rotation during \(\mu g\) (Fig. 3C). PF analysis allowed us to also determine that this transient \([\text{Ca}^{2+}]_c\) increase was dependent on the magnitude of the gravitational acceleration (Fig. 4E). Thus, the transient \([\text{Ca}^{2+}]_c\) increase induced by rotation under 2g at 20 s before \(\mu g\) was slightly larger than that induced by the same rotation at 6 s before \(\mu g\) (see the transients shown by black and blue arrows, respectively, in Fig. 5, A and B). The amplitude of the transient \([\text{Ca}^{2+}]_c\) increase decreased further when the seedlings were exposed to 2g for 3 s during the 5 s of rotation (red arrow in Fig. 5, A and B); the amplitude was almost the same as the amplitude of the transient \([\text{Ca}^{2+}]_c\) increase induced by the same rotation during \(\mu g\) (Figs. 3C and 5B), showing that the transient \([\text{Ca}^{2+}]_c\) increase was dependent on the duration of the gravistimulation (Fig. 5B) as well as the magnitude of the gravitational acceleration (Fig. 4E).

To gain further insight into the transient \([\text{Ca}^{2+}]_c\) increase, we designed a device to rotate the seedlings without changing their orientation with respect to the gravity vector (Fig. 7A) and analyzed \([\text{Ca}^{2+}]_c\) responses on the ground (Fig. 7, B and C). Horizontal 180° rotation induced a transient \([\text{Ca}^{2+}]_c\) increase without a sustained \([\text{Ca}^{2+}]_c\) increase (Fig. 7B). The amplitude of the transient \([\text{Ca}^{2+}]_c\) increase was angular acceleration dependent (Fig. 7B). Continuous rotation for 20 and 80 s induced the transient \([\text{Ca}^{2+}]_c\) increase at the onset and at the cessation of rotation but not during the rotation (Fig. 7C). These results suggest that Arabidopsis seedlings respond to angular acceleration (not to horizontal rotation itself). Taken together, the transient \([\text{Ca}^{2+}]_c\) increase could be a rotation-dependent \([\text{Ca}^{2+}]_c\) increase, which is presumably modulated by the angular and gravitational accelerations.

**DISCUSSION**

Gravistimulation of Arabidopsis seedlings at 1g caused a biphasic \([\text{Ca}^{2+}]_c\) increase consisting of first transient...
and second sustained $[\text{Ca}^{2+}]_c$ increases with different pharmacological and kinetic characters (Plieth and Trewavas, 2002; Toyota et al., 2008a, 2008b). In this study, 1.5g was applied to the specimen prerotated 180°, which induced a sustained $[\text{Ca}^{2+}]_c$ increase (Fig. 3, C and D). The kinetic parameters of this sustained $[\text{Ca}^{2+}]_c$ increase are almost the same as those of the sustained $[\text{Ca}^{2+}]_c$ increase in the ground experiments applying “gravistimulation” by rotation (Table I); the amplitude of the sustained $[\text{Ca}^{2+}]_c$ increase was rotational angle dependent (Plieth and Trewavas, 2002; Toyota et al., 2008a) but not rotational velocity dependent (Toyota et al., 2008a), and a vertical 360° rotation did not induce the sustained $[\text{Ca}^{2+}]_c$ increase (Plieth and Trewavas, 2002). These results strongly suggest that the sustained $[\text{Ca}^{2+}]_c$ increase, which corresponds to the second, sustained $[\text{Ca}^{2+}]_c$ increase induced by rotation at 1g, is the genuine response of the plant sensing a change in the gravity vector.

Gravistimulation by rotation under 0.5g to 2g induced biphasic $[\text{Ca}^{2+}]_c$ increases, and the amplitude of the response was almost linearly dependent on the magnitude of the gravitational acceleration (Fig. 4). Hypergravistimulation, achieved by reorienting the specimens 90° under hypergravity conditions (e.g. 5g), is known to enhance gravitropic curvature in Arabidopsis hypocotyls and roots (Fitzelle and Kiss, 2001), whereas gravistimulation less than 1g (e.g. 0.39g–0.93g), created by centrifugation in space, induced reduced gravitropic curvature in lentil (Lens culinaris) roots (Perbal et al., 2004). These observations are consistent with the gravity dependency of the sustained $[\text{Ca}^{2+}]_c$ increase we observed. Thus, plants that have evolved on Earth (1g) might be capable of transducing a wide range of gravitational changes into the gravitropic response via $\text{Ca}^{2+}$ signaling.

When the Arabidopsis seedlings were maintained in the upright configuration, the gravitational changes (e.g. 1g to 2g, 2g to $\mu$g, or $\mu$g to 1.5g) did not induce any detectable $[\text{Ca}^{2+}]_c$ increases (Fig. 3B), suggesting that these plants could only generate a gravitropic $[\text{Ca}^{2+}]_c$ when displaced from their vertical orientation by rotation. Yet, previous research has shown that Arabidopsis seedlings, geminated and grown in space, showed little gravitropic growth under continuous $\mu$g conditions (Kiss et al., 1999), whereas continuous hypergravity conditions, applied parallel to their growth axis, caused morphological changes (e.g. alterations in hypocotyl length and diameter; Matsumoto et al., 2010). These results suggest that plants can respond to changes in the intensity of gravity without reorientation. The disconnect between the $[\text{Ca}^{2+}]_c$ measurements in this study and the growth responses mentioned above could be due to the following two reasons. First, the gravitational changes (e.g. 2g to $\mu$g respectively. Data represent means ± se. Different letters above the bars denote significant differences between the groups ($P < 0.05$, one-way ANOVA followed by Tukey’s multiple comparison test). The shaded areas in A denote the $\mu$g condition.
or \( \mu g \) to 1.5g) in this study might induce a \([Ca^{2+}]_c\) change affecting gravitropic growth in space during the hours of observation, but if the signal is highly localized/undetectably small, we might not have observed it due to the detection limits of the aequorin measurement system. Second, the relatively short excursions to \( \mu g \) condition (20 s) might not have been long enough to trigger a detectable \([Ca^{2+}]_c\) change by the gravity change (\( \mu g \) to 1.5g). Indeed, our previous studies suggest that stronger hypergravity stimulation (i.e. 20g and 100g) is needed to trigger a \([Ca^{2+}]_c\) in- increase in upright-positioned seedlings (Toyota et al., 2007). Therefore, Arabidopsis seedlings grown vertically under 1g might be sensitive to reorientation but become...
adapted to 1g gravity in the top-to-bottom (upright) direction and so require a significant change from 1g to trigger a measurable response.

One other plant system where Ca\textsuperscript{2+} fluxes have been closely linked to gravity response is the polarization of the fern spore of *Ceratopteris richardii*. These spores exhibit a gravity-dependent Ca\textsuperscript{2+} current from the bottom to the top of the single cell, which appears important in determining its early polar development (Chatterjee et al., 2000). A recent study using electrophysiological analysis of Ca\textsuperscript{2+} currents around these spores during PF clarified that hypergravity or μg alters this transcellular Ca\textsuperscript{2+} current in the spore placed in the upright position (Salmi et al., 2011). Hypergravity (1.8g) increased the amplitude of the Ca\textsuperscript{2+} current, while μg decreased it 10 to 15 s after transition from hypergravity to μg. These observations suggest that this single-celled fern spore has a gravity-sensing mechanism that is highly sensitive to the direction and magnitude of gravity and rapidly converts changes in the gravity vector into alterations in Ca\textsuperscript{2+} current. These fern spore results suggest that flowering plants might also possess such a gravity-sensing mechanism modulating local Ca\textsuperscript{2+} currents. Indeed, gravistimulation causes a downward apoplastic Ca\textsuperscript{2+} movement across the horizontally oriented root tip of maize (*Zea mays*; Lee et al., 1983; Björkman and Cleland, 1991). Although our experimental setup could not detect hypergravity/μg-induced [Ca\textsuperscript{2+}] increase in upright plants (Fig. 3B), it is also possible that such coordinated influx and efflux mechanisms could lead to a gravity-modulated transcellular Ca\textsuperscript{2+} current, as seen in the fern spore, but with no net change in [Ca\textsuperscript{2+}].

The time course of the gravistimulation-induced sustained [Ca\textsuperscript{2+}] increase is analyzed here to our knowledge for the first time, which showed a latent period (approximately 18 s) from the start of 1.5g gravistimulation to the onset of the sustained [Ca\textsuperscript{2+}] increase (Fig. 3, C–E). Once the gravistimulus induced the [Ca\textsuperscript{2+}] increase, a sudden decrease from 2g to μg did not affect the time course of the sustained [Ca\textsuperscript{2+}] increase (Fig. 4D). These results suggest that gravity perception induces a biochemical cascade that takes approximately 18 s to trigger Ca\textsuperscript{2+} channel opening and Ca\textsuperscript{2+} increases that is independent of gravity. The amplitude of the sustained [Ca\textsuperscript{2+}] increases was dependent on the duration of the 2g gravistimulation; the 15 s of 2g gravistimulation induced the saturated response and 1 s of 2g gravistimulus induced the half-maximum response. These values basically agree with the values of “presentation time” estimated indirectly (Hejnolvicz et al., 1998; Plieth and Trewavas, 2002).

Kinetic parameters can be used to examine the mechanism proposed to account for the gravistimulation-induced [Ca\textsuperscript{2+}] increase (Perbal and Driss-Ecole, 2003). The most often referred to hypothesis of gravisensing is the starch-statolith hypothesis (Kiss, 2000; Morita, 2010); Perbal and Driss-Ecole (2003) proposed that sedimentation of the high-density plastids, amyloplasts, causes membrane stretch (or stress in the network of actin filaments), leading to the activation of mechanically sensitive channels in the plant plasma membrane. Analysis of shoot gravitropism (sgr) mutants (e.g., sgr2, zigzag [zig]/sgr4, or sgr9) has shown that their abnormal shoot gravitropism can be explained by the abnormalities in the amyloplast sedimentation (Kato et al., 2002; Morita et al., 2002; Yano et al., 2003; Nakamura et al., 2011). The amyloplast sedimentation after changing the gravity vector was directly observed with a vertical-stage fluorescence microscope (Saito et al., 2005; Nakamura et al., 2011), which showed that movement of the amyloplast sedimentation takes several minutes. This value fits a delay from the start of 1.5g gravistimulation to the peak of the sustained [Ca\textsuperscript{2+}] increase. However, the starch-statolith hypothesis cannot easily explain that (1) the t₀ was not changed by changing the magnitude of the gravity (Table I) and (2) a similar delay was observed when the [Ca\textsuperscript{2+}] increase was induced by the same gravistimulation in the endodermal-amyloplast-less1 (*eat1*) mutant that has no intact amyloplast in shoot endodermal cells (Fujihira et al., 2000; Morita et al., 2007; Toyota et al., 2008a). According to the starch-statolith hypothesis, the high gravity on amyloplasts will accelerate the sedimentation and shorten the delay, while conversely, the delay will be prolonged in *eat1*; thus, the experimental results from these PF experiments do not fit well with predictions of the starch-statolith hypothesis.

The sudden gravitational decrease from 2g to μg in the middle of the [Ca\textsuperscript{2+}] increase did not affect the profile of the sustained [Ca\textsuperscript{2+}] increase. This observation also does not fit easily to a starch-statolith hypothesis, where the amyloplasts are directly gating mechanically sensitive channels. Thus, the sedimentation of the amyloplasts should be nullified in μg, so their impact on sensors, such as inducing stress in the membrane or in the actin filaments, would be diminished. This reduction in stimulation should, in turn, deactivate the mechanically sensitive channels responsible for the Ca\textsuperscript{2+} increase and affect the time course of the [Ca\textsuperscript{2+}] increase, assuming that the deactivation of mechanosensitive cation channel is rapid (approximately 10 ms estimated in endothelial cells; Hayakawa et al., 2008). Therefore, it is likely that the sedimentation of amyloplasts will be involved in the early sensing process that will initiate “a gravityless-sensitive signal” that induces the sustained [Ca\textsuperscript{2+}] increase with a certain delay. A similar idea was proposed from the results of PF experiments using rhizoids of *Chara globularis* (Limbach et al., 2005): gravireceptor activation does not directly depend on the mechanical work that is provided by the gravity-induced statolith sedimentation process but, presumably, on the interaction of sedimented statoliths with the membrane-bound “receptor.”

Pharmacological experiments with U73122 and RR imply the involvement of PLC and endomembrane Ca\textsuperscript{2+}-permeable channels in the generation of the sustained [Ca\textsuperscript{2+}] increase; the intracellular InsP\textsubscript{3} level is known to be increased by gravistimulation in oat shoot pulvini and Arabidopsis (Perera et al., 2001, 2006), and inositol 1,4,5-triphosphate is a product of PLC action. The small
aequorin signal increase during the latent period (t1) may reflect a slight [Ca\(^{2+}\)] increase prior to the sustained [Ca\(^{2+}\)] increase. Taken together, Ca\(^{2+}\)-induced Ca\(^{2+}\) release and/or InsP\(_3\)-induced Ca\(^{2+}\) release are candidates for triggering the sustained [Ca\(^{2+}\)] increase and mediating the signal transduction of gravistimuli into the [Ca\(^{2+}\)] increase. Once triggered, Ca\(^{2+}\)-induced Ca\(^{2+}\) release and InsP\(_3\)-induced Ca\(^{2+}\) release would work in a gravityless-sensitive manner because the size and the mass of the main signaling molecules (Ca\(^{2+}\) and InsP\(_3\)) are too small to be directly affected by g force. These observations suggest a possibility that the slight [Ca\(^{2+}\)] increase is mediated by the activation of mechanosensitive channels (or by InsP\(_3\) signaling) and is followed by a large sustained [Ca\(^{2+}\)] increase via the Ca\(^{2+}\)-induced Ca\(^{2+}\) release and/or InsP\(_3\)-dependent Ca\(^{2+}\) release mechanisms. These responses might be followed by Ca\(^{2+}\)-related relocalization of PIN auxin transporter proteins and so modulation of auxin-dependent growth (Friml et al., 2002; Benjamins et al., 2003; Zhang et al., 2011).

In our previous ground-based study, the second sustained [Ca\(^{2+}\)] increase was observed in the hypocotyls and petals, but not cotyledons, of Arabidopsis seedlings upon rotation (Toyota et al., 2008a), implying that the [Ca\(^{2+}\)] increase during PF occurs at similar sites to those in the ground experiment. This finding is consistent with the above conclusion that the [Ca\(^{2+}\)] increase is involved in the early signal transduction for gravitropism (Pickard, 2007; Toyota and Gilroy, 2013). However, because luminescence from roots growing in an agar medium was not detected, most likely due to the poor penetration of coelenterazine into the agar during the reconstitution of aequorin (Toyota et al., 2008a), it is still unknown whether Arabidopsis roots show [Ca\(^{2+}\)] changes in response to gravistimulation (Legué et al., 1997). In the ground-based studies, the amplitude of the [Ca\(^{2+}\)] increase was almost the same in hypocotyls and petals (Toyota et al., 2008a), suggesting that the [Ca\(^{2+}\)] response occurs uniformly in these gravitropic organs. However, within these organs, we have yet to clarify the specific tissues/cells responding to gravistimulation with a change in [Ca\(^{2+}\)], due to the detection limits of aequorin luminescence. In these PF experiments, we monitored luminescence from entire seedlings, including organs that were most likely not responding to gravistimulation with a change in [Ca\(^{2+}\)] (e.g. cotyledons). Signal from these sites would increase the background luminescence and, in turn, reduce the signal-to-noise ratio of the data reported. Although aequorin is an essential tool that allows Ca\(^{2+}\) analysis during these PF experiments, high-resolution imaging using genetically encoded Ca\(^{2+}\) indicators such as Yellow Cameleon GFP-based sensors should be more appropriate to analyze the spatial nature of gravity-related [Ca\(^{2+}\)] signals (Choi et al., 2012).

The transient [Ca\(^{2+}\)] increase looks similar in its kinetics to the wind- or touch-induced [Ca\(^{2+}\)] spikes reported in seedlings of Nicotiana plumbaginifolia (Knight et al., 1991, 1992). These wind- and touch-induced transient [Ca\(^{2+}\)] spikes are thought to arise from intracellular Ca\(^{2+}\) release because they were inhibited by RR but not by Gd\(^{3+}\) or La\(^{3+}\) in N. plumbaginifolia seedlings (Knight et al., 1992) and Arabidopsis roots (Legué et al., 1997). The transient [Ca\(^{2+}\)] increase in this study was inhibited by Gd\(^{3+}\) and La\(^{3+}\) but not by RR (Toyota et al., 2008a), showing that the transient [Ca\(^{2+}\)] increase induced in response to rotation is pharmacologically distinct from the wind- and touch-induced [Ca\(^{2+}\)] spikes.

The peak amplitude of the transient [Ca\(^{2+}\)] increase was dependent on the rotational velocity but not on the angle, suggesting that the transient [Ca\(^{2+}\)] increase is induced by the rotational motion. The analysis of the transient [Ca\(^{2+}\)] increase in this study suggests that it is a genuine response to rotation because the first peak was induced by rotatory stimulation under μg and horizontal 180° rotation on the ground (1g) and its amplitude increases in an angular acceleration-dependent manner. However, the PF experiments have explored a new feature of the transient [Ca\(^{2+}\)] increase, that the amplitude of the transient [Ca\(^{2+}\)] increase was not only dependent on the angular acceleration but also on the gravitational acceleration (0.5g–2g), suggesting that it is mediated by a cellular sensing process sensitive to both angular and gravitational accelerations. Thus, the transient [Ca\(^{2+}\)] increase induced by the rotatory stimulation under μg could be genuinely angular acceleration dependent due to almost no gravitational acceleration being applied to the specimen. On the other hand, the transient [Ca\(^{2+}\)] increase under 0.5g to 2g conditions would be mediated via a complicated mechanism that is sensitive to both gravitational and rotatory accelerations, and its amplitude is larger than that induced under μg.

### MATERIALS AND METHODS

#### Plant Materials, Growth Conditions, and Reconstitution of Aequorin

Approximately 40 Arabidopsis (Arabidopsis thaliana; Columbia accession) seeds expressing cytoplasmically targeted aequorin were sterilized; sown on an agar plates containing a plant growth medium (1× Murashige and Skoog salts, 1% [w/v] Suc, 0.01% [w/v] myo-inositol, and 0.05% [w/v] MES, pH 5.8, adjusted with 1 m KOH) in a petri dish (diameter, 6 cm), as described previously (Toyoda et al., 2008a); and incubated at 22°C in a growth chamber under continuous white light for 5 to 7 d after stratification at 4°C in darkness for 2 d. These seedlings were incubated with the plant growth “liquid” medium (3 mL) containing 2.5 μM ω-fluro-dehydrocoelenterazine for approximately 6 h at 22°C in darkness to reconstitute aequorin. The liquid medium was then removed from the plate 2 h prior to the flight experiments.

[Ca\(^{2+}\)] Monitoring and a Device for Rotation

A plate of seedlings was mounted under a photomultiplier tube (model RP1942; Hamamatsu Photonics) with a 50-mm lens (f = 0.95; model YMV5095; Yakuco). [Ca\(^{2+}\)]-dependent aequorin luminescence was monitored and processed by a photon counter (model PHC3000-1; Scientex) at 0.5-s intervals. This configuration enables detection of the gravistimulation-induced aequorin signal from 40 seedlings, which is 10 times less than that reported previously as being needed (Plieth and Trewavas, 2002). Three sets of this configuration were mounted in a light-tight dark box, and the aequorin signal from each plate was simultaneously stored in a computer. This box was rotated 180° by a computer-controlled direct current motor system (model BX5120AM-100S; Oriental Motor) at an angular velocity of 6 rpm and an angular acceleration of 2.5 rad s\(^{-2}\). The plates were mounted 0.14 m from the rotation axis,
producing a centrifugal acceleration of $5.6 \times 10^{-3}$ g during uniform circular motion; the tangential acceleration at the start of rotation was $3.6 \times 10^{-3}$ g. These devices were mounted in a custom-made aluminum rack that was fixed on the floor of an aircraft via a shock absorber.

Data Analyses

The luminescence ratio (per $I_{flu}$) was calculated by dividing the aequorin luminescence intensity by the averaged intensity before 180° rotation ($I_{flu}$), as reported previously (Toyoda et al., 2007, 2008a). The aequorin luminescence was calibrated into [Ca$^{2+}$] by discharging the aequorin with 20% (v/v) ethanol and 2% CaCl$_2$ and estimating the amount of aequorin remaining at the end of each experiment, as described previously (Knight et al., 1996). In this paper, the luminescence ratio, rather than the calibrated [Ca$^{2+}$], (Supplemental Fig. S1B), was used for all analyses because it was not possible to discharge the remaining aequorin and monitor the signal for a further 5 min until values were within 1% of the highest discharge value in an aircraft. Data were analyzed by two-tailed Student’s t test or one-way ANOVA followed by Tukey’s multiple comparison test using GraphPad Prism (GraphPad Software). The number of observations (n) denotes the number of experiments made with independent samples/plates containing approximately 40 seedlings. All kinetic parameters (i.e. $t_s$ and $t_3$) and $30\%$ of the ground control (Supplemental Fig. S1, C and D). However, the other number of observations ($n$) was within 1% of the highest discharge value in an aircraft. Data were analyzed by two-tailed Student’s t test or one-way ANOVA followed by Tukey’s multiple comparison test using GraphPad Prism (GraphPad Software). The number of observations (n) denotes the number of experiments made with independent samples/plates containing approximately 40 seedlings. All experiments were repeated at least three times in independent PFs, and we analyzed all the data obtained.

The signal amplitude of the flight control was attenuated to approximately 30% of the ground control (Supplemental Fig. S1, C and D). However, the other kinetic parameters (i.e. $t_s$ and $t_3$) of the flight control were almost identical to those of the ground control (Table I), suggesting that mechanisms underlying the [Ca$^{2+}$] are not increased. As the frequency distribution of the transient and sustained [Ca$^{2+}$], increase appear to follow a normal/Gaussian distribution (Supplemental Fig. S2, A and B), we compared the results obtained from the PF with the flight control using parametric tests (e.g. Student’s t test or ANOVA) to assess statistical differences.

PFs and Circular Flights

PFs or circular flights were carried out with an aircraft (Mitsubishi MU-30; Diamond Air Service) over the Sea of Japan or the Pacific Ocean for 3 and 4 d in 2006 and 2007, respectively. After takeoff from Komaki/Nagoya Airport (Aichi, Japan), it took more than 30 min to enter airspace where we were permitted to perform the PF experiments. In a typical case, we started the experiments approximately 1 h after takeoff, which should allow the plants to acclimate to the flight conditions (e.g. mechanical stresses during the takeoff). Stress-induced rapid increases in superoxide are known to decline within 1 h after the PF experiments, suggesting that this period was suitable for acclimation to flight.

PFs create $\mu g$ (free-fall) conditions for approximately 20 s by minimizing the engine thrust (Fig. 1) and $0.5g$ for approximately 40 s by reducing the engine thrust (Supplemental Fig. S3B) after hypergravity ($2g$). The hypergravity condition before entering the phase of $\mu g$ was 2g for 20 s in a typical case (Fig. 1), but this could be changed to 1.2g or 1.5g for approximately 40 s by regulating the engine thrust and/or the pitch angle of the aircraft as it climbed (Supplemental Fig. S3A). PFs were repetitively performed more than 10 times with approximately 6-min intervals in one flight on each day. To create a continuous 2g condition for approximately 40 s, circular flight with a bank of approximately 45° was performed (Supplemental Fig. S3C). Gravitational acceleration in the aircraft was monitored with an accelerometer (model CXL04LP3; Crossbow Technology) and stored in a computer. Flight control experiments were made during steady horizontal flights at 1g before the PF experiments (Fig. 3A).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Ground and flight control [Ca$^{2+}$], increases.

Supplemental Figure S2. Correlation analysis of [Ca$^{2+}$], increases.

Supplemental Figure S3. Flight patterns to create 1.2g, 1.5g, 0.5g, and 2g.

Supplemental Figure S4. Effect of hypergravity before $\mu g$ on [Ca$^{2+}$], increases.
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