Cytoplasmic Calcium Increases in Response to Changes in the Gravity Vector in Hypocotyls and Petioles of Arabidopsis Seedlings[1]

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Plants respond to a large variety of environmental signals, including changes in the gravity vector (gravistimulation). In Arabidopsis (*Arabidopsis thaliana*) seedlings, gravistimulation is known to increase the cytoplasmic free calcium concentration ($[Ca^{2+}]_c$). However, organs responsible for the $[Ca^{2+}]_c$ increase and the underlying cellular/molecular mechanisms remain to be solved. In this study, using Arabidopsis seedlings expressing apoaequorin, a Ca^{2+}-sensitive luminescent protein in combination with an ultrasensitive photon counting camera, we clarified the organs where $[Ca^{2+}]_c$ increases in response to gravistimulation and characterized the physiological and pharmacological properties of the $[Ca^{2+}]_c$ increase. When the seedlings were gravistimulated by turning 180°, they showed a transient biphasic $[Ca^{2+}]_c$ increase in their hypocotyls and petioles. The second peak of the $[Ca^{2+}]_c$ increase depended on the angle but not the speed of rotation, whereas the initial peak showed diametrically opposite characters. This suggests that the second $[Ca^{2+}]_c$ increase is specific for changes in the gravity vector. The potential mechanosensitive Ca^{2+}-permeable channel (MSCC) inhibitors Gd^{3+} and La^{3+}, the Ca^{2+} chelator 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA), and the endomembrane Ca^{2+}-permeable channel inhibitor ruthenium red suppressed the second $[Ca^{2+}]_c$ 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. Moreover, the second $[Ca^{2+}]_c$ increase was attenuated by actin-disrupting drugs cytochalasin B and latrunculin B but not by microtubule-disrupting drugs oryzalin and nocodazole, implying that actin filaments are partially involved in the hypothetical activation of Ca^{2+}-permeable channels. These results suggest that the second $[Ca^{2+}]_c$ increase via MSCCs is a gravity response in the hypocotyl and petiole of Arabidopsis seedlings.

Gravity is a ubiquitous force on the earth and affects the growth and morphogenesis in plants. Many higher plants sense gravity and orient their growth direction with respect to the gravity vector, a phenomenon known as gravitropism. Changes in the gravity vector (gravistimulation) are supposed to be transduced into certain intracellular signals in the early process of gravitropic response (Chen et al., 1999; Blancaflor and Masson, 2003; Morita and Tasaka, 2004). However, the signaling molecules and the underlying transduction mechanisms remain largely obscure.

It is widely accepted that Ca^{2+} plays a crucial role in the growth and development of plants (Trewavas and Malho, 1998; White and Broadley, 2003; Hepler, 2005). The relationship between Ca^{2+} and gravitropism has been closely investigated in the past decades. Gravitropism in maize (*Zea mays*) roots and oat (*Avena sativa*) coleoptiles was nearly completely eliminated by soaking them in Ca^{2+} chelators (e.g. EGTA) or distilled water (Lee et al., 1983a; Daye et al., 1984; Millet and Pickard, 1988), indicating the necessity of extracellular Ca^{2+} for gravitropic responses. An asymmetrical distribution of cytoplasmic, vacuolar, and apoplastic Ca^{2+} across oat coleoptiles was observed after gravistimulation, which was spatially correlated with the bending region (Slocum and Roux, 1983). Furthermore, gravistimulation caused a downward redistribution of apoplastic Ca^{2+} across the horizontally placed root tip in maize (Lee et al., 1983b; Björkman and Cleland, 1991). The gravistimulation-induced redistribution of Ca^{2+} took place in decapitated maize roots that regenerated gravitropic sensitivity but not in decapitated roots that lost it (Björkman and Cleland, 1991). These results suggest that Ca^{2+} plays a key role in gravitropism

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and is involved in the process of gravity sensing and/or following signal transduction.

Because cytoplasmic Ca^{2+} is considered as a ubiquitous intracellular second messenger, a possible involvement of changes in cytoplasmic free calcium concentration ($[Ca^{2+}]_c$) in gravity response has repeatedly been pointed out (Sinclair and Trewavas, 1997; Fasano et al., 2002; Blancaflor and Masson, 2003). Increases in $[Ca^{2+}]_c$ induced by gravistimulation were observed in maize coleoptiles using the $[Ca^{2+}]_c$ indicator, fluo-3 (Gehring et al., 1990). Several minutes after gravistimulation, $[Ca^{2+}]_c$ increased in the lower side of the horizontally placed maize coleoptiles. This result raised the possibility that gravistimulation is transduced into increases in $[Ca^{2+}]_c$, although the underlying molecular mechanisms are still unclear. Recently, refined technologies of $[Ca^{2+}]_c$ imaging revealed changes in the $[Ca^{2+}]_c$ induced by gravistimulation in Arabidopsis (*Arabidopsis thaliana*) seedlings expressing the luminous Ca^{2+}-reporting protein, apoaequorin (Plieth and Trewavas, 2002). Hundreds of Arabidopsis seedlings mounted on a wheel in front of a photomultiplier tube (PMT) showed a biphasic $[Ca^{2+}]_c$ transient lasting for over 10 min in response to gravistimulation. Continuous gravistimulation using a clinorotation caused a sustained increase in $[Ca^{2+}]_c$ for more than 30 min. On the other hand, when gravitational acceleration increased to 100*g* from 1*g* using a centrifuge, a monophasic $[Ca^{2+}]_c$ transient lasting for several minutes was detected in Arabidopsis seedlings expressing apoaequorin (Toyota et al., 2007). The peak amplitude of the monophasic $[Ca^{2+}]_c$ transient was dependent on the magnitude of the gravitational acceleration. These findings imply an involvement of $[Ca^{2+}]_c$ increases in gravity response and/or signal transduction in Arabidopsis seedlings. However, the organs where $[Ca^{2+}]_c$ increased in response to changes in the gravity vector (including direction and magnitude of gravitational acceleration) and the underlying cellular and molecular mechanisms remain to be solved.

We developed an imaging system to provide a spatial resolution of aequorin luminescence during gravistimulation, which clarified the organs that responded to gravistimulation in Arabidopsis seedlings. Furthermore, the properties of the biphasic $[Ca^{2+}]_c$ increase were investigated in more detail by fine control of gravistimulation in combination with pharmacology. Our results provide a deeper insight into the cellular and molecular mechanisms of the $[Ca^{2+}]_c$ increase, leading to a conclusion that the second $[Ca^{2+}]_c$ increase is specific for changes in the gravity vector, while the initial one is related to rotational motion in the shoots of Arabidopsis seedlings.

RESULTS

$[Ca^{2+}]_c$ Increases in Hypocotyls and Petioles in Response to Gravistimulation

A plate of seedlings of Arabidopsis expressing apoaequorin was mounted under an ultrasensitive photon-counting camera (PCC) in a light-tight dark box (Fig. 1A) and subjected to gravistimulation. Aequorin luminescence from individual seedlings was integrated for 7 min before and 20 s after a rotation (Fig. 2A). When the seedlings were turned 180° at the speed of 6 rpm, increases in luminescence intensity were observed in a hypocotyl and its petioles (Fig. 2A). By subtracting the control image as background luminescence (Fig. 2A, a) from the image after the 180° rotation (Fig. 2A, b), gravistimulation-responsive organs were visualized more clearly (Fig. 2A, c and d). Quantitative analyses of changes in the integrated luminescence intensity showed that aequorin luminescence increased by 40% to 45% in hypocotyls and petioles but not in cotyledons to the gravistimulation (Fig. 2, B and C). Hypocotyls and petioles in general show gravitropic responses (Fukaki et al., 1998; Mano et al., 2006), which is spatially related to the $[Ca^{2+}]_c$ increase. Thus, our observations raise the possibility that the $[Ca^{2+}]_c$ increase is a gravity response in shoots of Arabidopsis seedlings. We did not detect aequorin luminescence from roots, probably due to an incomplete reconstitution of aequorin (see "Materials and

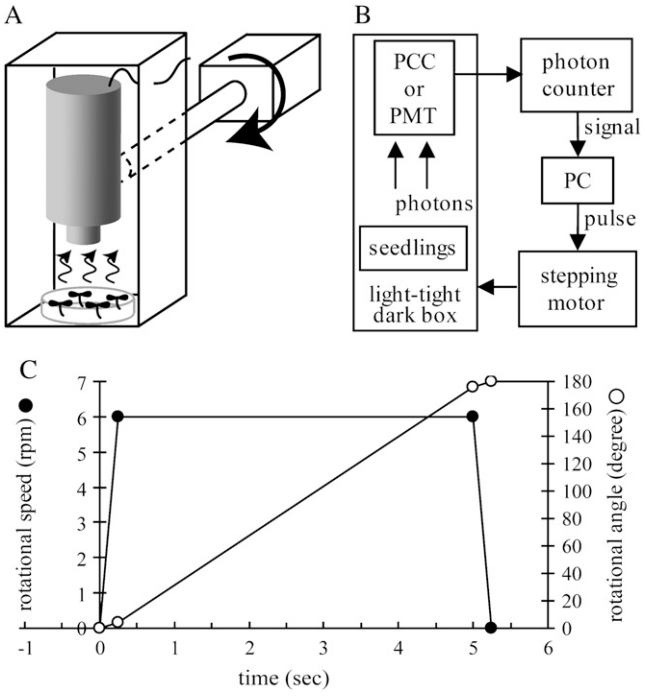

Figure 1. Schematic diagrams of a device for gravistimulation and a photon-counting system. A, A plate of Arabidopsis seedlings was mounted under an ultrasensitive PCC or a PMT in a light-tight dark box connected to a stepping motor, enabling gravistimulation while monitoring the intensity of aequorin luminescence. B, The luminescence intensity was monitored with a photon counter and stored in a personal computer. The rotational speed and angle of the stepping motor was controlled by the same personal computer. C, Time course of changes in the rotational speed (black circle) and angle (white circle) while turning 180° at the speed of 6 rpm.

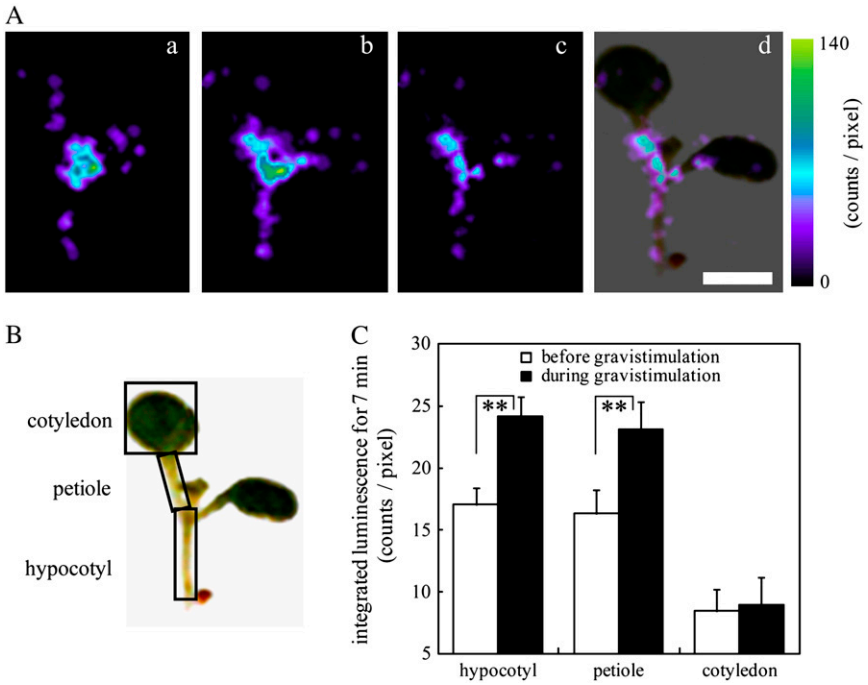

Figure 2. Increases in $[Ca^{2+}]_c$ induced by gravistimulation in hypocotyls and petioles. A, $[Ca^{2+}]_c$-dependent aequorin luminescence from a whole seedling was integrated for 7 min before and during gravistimulation (180° rotation) using an ultrasensitive PCC system. Typical images with pseudocolor before (a) and during (b) gravistimulation, the subtracted (c; b − a), and the overlaid image with a bright-field image (d) are shown. The color scale indicates photon counts per pixel. B and C, Quantitative analysis with averaging the integrated luminescence intensities at each pixel in three regions (hypocotyls, petioles, and cotyledons) before (white bar) and during (black bar) gravistimulation. Data represent means ± SES (n = 40). **, Statistically significant differences ($P < 0.05$, the two-tailed Student's t test). Scale bar = 2 mm.

Methods''). The maximum luminescence intensity (L_{max}) induced by adding ethanol plus $CaCl_2$ was detected in hypocotyls, petioles, and cotyledons but was extremely small in roots (data not shown), suggesting that aequorin luminescence from seedlings is mainly originated from shoots in our system. Therefore, changes in $[Ca^{2+}]_c$ in roots were not examined.

Properties of Biphasic $[Ca^{2+}]_c$ Transient Induced by Gravistimulation

Aequorin luminescence was monitored with a PMT to examine the time course of the $[Ca^{2+}]_c$ increase induced by gravistimulation. When a plate of seedlings was subjected to gravistimulation by turning 180° at the speed of 6 rpm, a biphasic $[Ca^{2+}]_c$ transient was observed (Fig. 3A, white and black arrowheads), which was consistent with the previous report (Plieth and Trewavas, 2002). The initial $[Ca^{2+}]_c$ transient peaked within 4 s when the gravistimulation was applied and decayed exponentially with a time constant of approximately 3 s (Fig. 3A, white arrowhead). The second $[Ca^{2+}]_c$ transient peaked at around 40 s from the start of the turning and decayed exponentially with a time constant of approximately 60 s (Fig. 3A, black arrowhead). When the seedlings were turned back 180° at the same speed, the peak amplitude of the second $[Ca^{2+}]_c$ transient was strongly attenuated (Fig. 3A, asterisk), whereas that of the initial $[Ca^{2+}]_c$ transient remained almost at the same level. Subsequently, the same seedlings were subjected to the same series of gravistimuli (±180°) within 15 min after the cessation of the second gravistimulation. The initial $[Ca^{2+}]_c$ transient was almost unchanged to the third and fourth gravistimuli, whereas the second $[Ca^{2+}]_c$ transient was strongly attenuated (data not shown), indicating that the second $[Ca^{2+}]_c$ transient showed a desensitization to repetitive gravistimulation. These results suggest that the initial and second $[Ca^{2+}]_c$ transients arise from different cellular and/or molecular mechanisms.

To characterize the biphasic $[Ca^{2+}]_c$ transient in more detail, we examined the effect of rotational angle and speed on the peak amplitudes of the initial and second $[Ca^{2+}]_c$ transients. The normalized amplitudes of the initial peak induced by turning through different angles showed no significant difference (Fig. 3B), indicating that the amplitude of the initial $[Ca^{2+}]_c$ transient is independent of the angle of rotation. On the other hand, the peak amplitudes of the second $[Ca^{2+}]_c$ transients were dependent on the angle of rotation (Fig. 3C). The maximum peak of the second $[Ca^{2+}]_c$ transient was detected when the seedlings were turned 135°, which was consistent with the previous report (Plieth and Trewavas, 2002).

Next, the effect of rotational speed on the initial and second $[Ca^{2+}]_c$ transients was examined. As the rotational speed was step-wisely decreased to 0.6 rpm, the initial peak was attenuated in a rotational speed-dependent manner (Fig. 4, A and B). In contrast, the second peak was not significantly affected by slower rotational speed than 6 rpm (Fig. 4, A and C), whereas the time to peak of the second $[Ca^{2+}]_c$ transient was slightly delayed (Fig. 4A), probably due to a delay in reaching 180°. Taken together, the amplitude of the second $[Ca^{2+}]_c$ transient was dependent on the angle of gravistimulation (Fig. 3C) but not on its speed (Fig. 4C), suggesting that the second $[Ca^{2+}]_c$ transient is induced by changes in gravity vector. Therefore, we

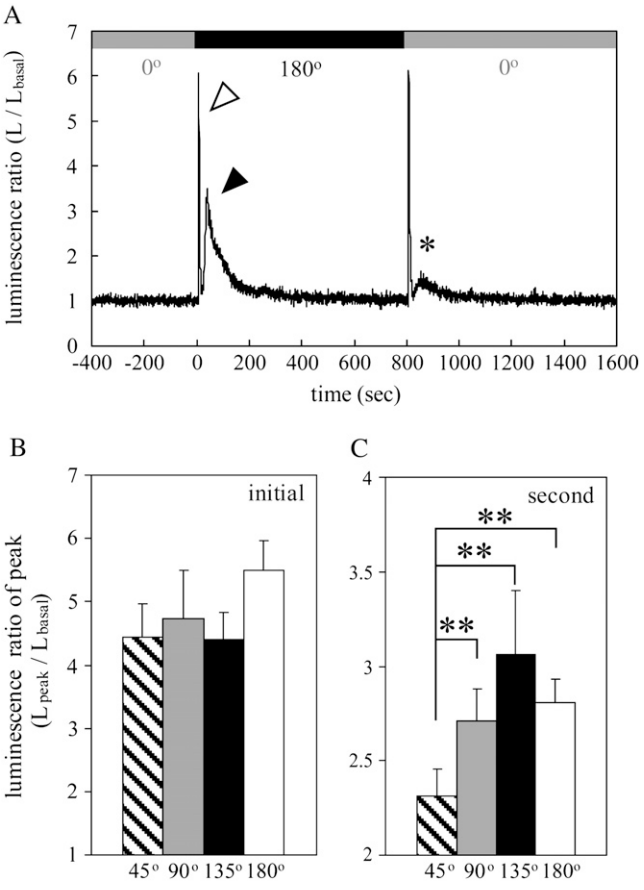

Figure 3. Biphasic $[Ca^{2+}]_c$ transient induced by gravistimulation in Arabidopsis seedlings. A, Typical time course of changes in luminescence ratio induced by gravistimulation (±180° rotation). Approximately 40 Arabidopsis seedlings grown in a plate were turned 180° at the speed of 6 rpm at 0 s on the abscissa and retained at 180° for 800 s, which induced a biphasic $[Ca^{2+}]_c$ transient with an initial peak and a second one (white and black arrowheads, respectively). Subsequently, seedlings were turned back −180° at the same speed, which also induced the biphasic $[Ca^{2+}]_c$ transient with a small second peak (asterisk). The shaded and black bars above the data represent the rotational angles (0° and 180°, respectively). B and C, The effect of rotational angles on the initial and second $[Ca^{2+}]_c$ peaks. Plates of seedlings were turned through different angles at the speed of 6 rpm. The peak amplitudes of the initial (B) and second $[Ca^{2+}]_c$ transients (C) in response to 45°, 90°, 135°, and 180° gravistimulation are presented as hatched ($n = 18$), shaded ($n = 22$), black ($n = 16$), and white bars ($n = 32$), respectively. Data represent means ± SES. **, $P < 0.05$, the two-tailed Student's t test.

named the second $[Ca^{2+}]_c$ transient the graviinduced $[Ca^{2+}]_c$ transient and focused mainly on this response in this study. On the other hand, the amplitude of the initial $[Ca^{2+}]_c$ transient was dependent on the speed of gravistimulation (Fig. 4B) but not on its angle (Fig. 3B), suggesting that the initial $[Ca^{2+}]_c$ transient is induced by forces related to the rotational speed. No correlation of the peak amplitude between the initial and second $[Ca^{2+}]_c$ transients was observed (Figs. 3, B and C, and 4, B and C), further supporting the idea that these $[Ca^{2+}]_c$ transients have different origins.

Inhibitor Analyses on the Biphasic $[Ca^{2+}]_c$ Transient

Gravistimulation is often regarded as a sort of mechanical stimulation (Trewavas and Knight, 1994). In general, mechanical stimulus is thought to be transduced into a certain intracellular signal thorough mechanosensitive (MS) channels (Hamill and Martinac, 2001). We examined a possible involvement of MS Ca^{2+}-permeable

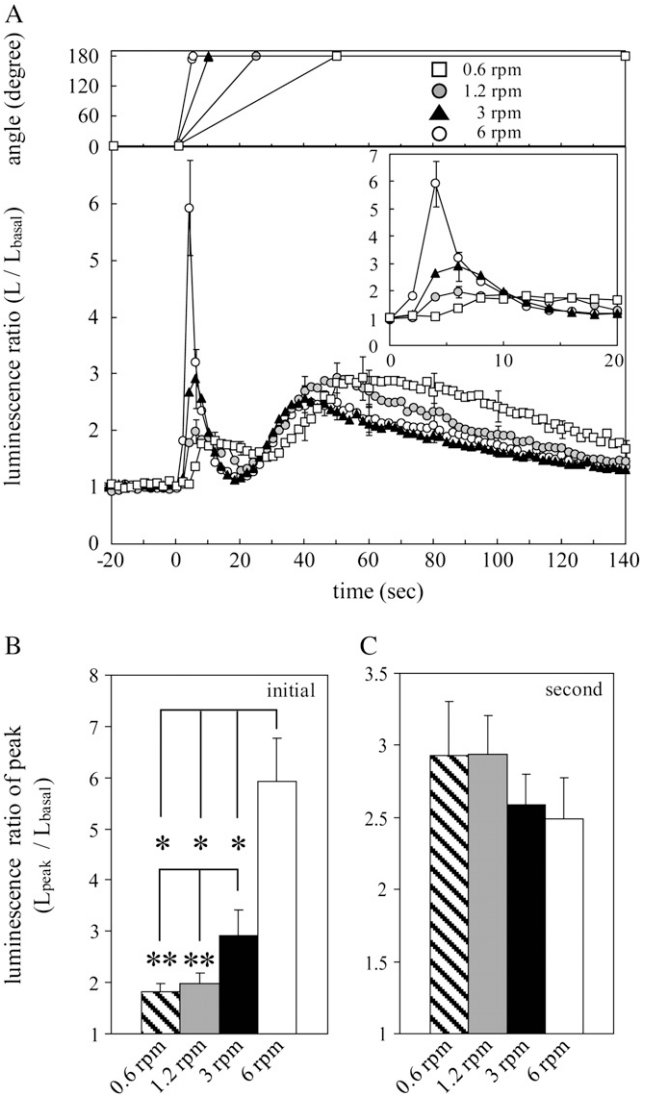

Figure 4. The effect of rotational speed on the initial and second $[Ca^{2+}]_c$ transients. A, The averaged trace of changes in luminescence ratio in response to gravistimulation at the speeds of 0.6, 1.2, 3, and 6 rpm (white square, shaded circle, black triangle, and white circle, respectively). Plates of seedlings were turned 180° at the above speeds at 0 s on the abscissa and retained at 180°. Time course of changes in the rotational angle at each speed is shown in the top graph. The inset shows an enlargement of the initial $[Ca^{2+}]_c$ transients. B and C, The peak amplitudes of the initial (B) and second $[Ca^{2+}]_c$ transients (C) induced by 0.6, 1.2, 3, and 6 rpm gravistimulation are presented as hatched ($n = 8$), shaded ($n = 13$), black ($n = 6$), and white bars ($n = 18$), respectively. Data represent means ± SES. *, $P < 0.01$; **, $P < 0.05$, the two-tailed Student's t test.

channels (MSCCs) in the biphasic $[Ca^{2+}]_c$ transient. Potential MSCC inhibitors, Gd^{3+} and La^{3+}, suppressed the graviinduced $[Ca^{2+}]_c$ transient in a concentration-dependent manner (Fig. 5, A, B, and F). The Ca^{2+} chelator, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA), also nearly completely inhibited the graviinduced $[Ca^{2+}]_c$ transient (Fig. 5, C and F). After washing out these chemicals with growth medium, nearly the same amplitude of the graviinduced $[Ca^{2+}]_c$ transient without drug treatments was recovered (data not shown). These results suggest that the graviinduced $[Ca^{2+}]_c$ transient arises from Ca^{2+} influx via putative MSCCs in the plasma membrane. On the other hand, the potential endomembrane Ca^{2+}-permeable channel inhibitor, ruthenium red (RR), also suppressed the graviinduced $[Ca^{2+}]_c$ transient in a concentration-dependent manner (Fig. 5, D and F), suggesting the involvement of Ca^{2+} release from intracellular Ca^{2+} stores in the graviinduced $[Ca^{2+}]_c$ transient. The slow vacuolar channels are hypothesized to play an important role in Ca^{2+}-induced Ca^{2+} release in *Beta vulgaris* (Ward and Schroeder, 1994; Bewell et al., 1999). Because it is known that RR inhibits the Ca^{2+}-perameable channels in the endoplasmic reticulum (Bauer et al., 1998) and the slow vacuolar channels in the vacuole (Pottosin et al., 1999), it is plausible to expect that gravistimulation causes the Ca^{2+} influx via the MSCCs followed by the Ca^{2+}-induced Ca^{2+} release. In contrast, the initial $[Ca^{2+}]_c$ transient was inhibited by Gd^{3+}, La^{3+}, and BAPTA but not by RR (Fig. 5, A–E). This result suggests that the initial $[Ca^{2+}]_c$ transient originates mainly from Ca^{2+} influx through putative MSCCs in the plasma membrane and is mechanistically distinct from the graviinduced $[Ca^{2+}]_c$ transient.

Previous studies indicate that the cytoskeleton plays a certain role in the mechanosensitivity of MSCCs (Hamill and Martinac, 2001). Recent evidence showed that activation of MSCC by stretch force imposed in the plasma membrane is modulated by actin filaments in human leukemia cells (Staruschenko et al., 2005) and guard cells of *Vicia faba* (Zhang et al., 2007), suggesting that MSCCs are coupled with the cortical actin filaments functionally and/or structurally. Internal or external forces resulting from mechanical stimuli (e.g. gravistimulation) are hypothesized to be effectively transmitted to MSCCs thorough the cytoskeleton (Fasano et al., 2002; Perbal and Driss-Ecole, 2003). The involvement of cytoskeleton, especially actin filaments and microtubules, in the biphasic $[Ca^{2+}]_c$ transient induced by gravistimulation was examined in this study. The graviinduced $[Ca^{2+}]_c$ transient was attenuated by actin-disrupting drugs, latrunculin B, and cytochalasin B but not by the microtubule-disrupting drugs oryzalin and nocodazole (Fig. 6, A, B, and D), suggesting that actin filaments are partially involved in the hypothesized activation of MSCCs, leading to the graviinduced $[Ca^{2+}]_c$ transient. The initial $[Ca^{2+}]_c$ transient was also slightly attenuated by latrunculin B and cytochalasin B but not by oryzalin and nocodazole (Fig. 6, A–C), suggesting an actin filament-dependent machinery for the initial $[Ca^{2+}]_c$ transient. Forces generated by the rotational motion may also be effectively transduced into MSCCs in the plasma membrane via actin filaments.

DISCUSSION

Changes in $[Ca^{2+}]_c$ induced by various endo- and exogenous signals have been extensively investigated in plants (Gilroy et al., 1993; Sanders et al., 2002; Scrase-Field and Knight, 2003). Recent work demonstrated that changes in the gravity vector induced a $[Ca^{2+}]_c$ transient in hundreds of Arabidopsis seedlings expressing apoaequorin (Plieth and Trewavas, 2002). On the other hand, no $[Ca^{2+}]_c$ change to gravistimulation was detected in Arabidopsis roots, in which indo-1 was used to probe $[Ca^{2+}]_c$ levels (Legue et al., 1997). It has been controversial whether gravistimulation induces changes in $[Ca^{2+}]_c$ in Arabidopsis seedlings, because the organs where the intensity of aequorin luminescence increased have remained to be determined. In this study, we confirmed the previous observation (Plieth and Trewavas, 2002) and gain new insights into the debated issue by utilizing advanced optical techniques.

We clarified that gravistimulation induced a $[Ca^{2+}]_c$ increase in hypocotyls and petioles (Fig. 2), which is not contradictory to the previous results (Legue et al., 1997). In our setup, we did not detect aequorin luminescence from roots due to the incomplete reconstitution of aequorin in roots growing inside the agar. We tried to detect $[Ca^{2+}]_c$ changes in response to gravistimulation in roots by reconstituting aequorin in another way (see "Materials and Methods"). However, we still did not detect it in the roots during gravistimulation using PCC (data not shown). In general, gravistimulation may cause an increase in $[Ca^{2+}]_c$ at a detectable level in shoots, as reported previously (Gehring et al., 1990), but not in roots. Recently, transient increases in cytoplasmic pH induced by gravistimulation were observed in columella cells of Arabidopsis roots (Fasano et al., 2001; Hou et al., 2004). Cytoplasmic pH rather than $[Ca^{2+}]_c$ may play an important role in gravity response of roots.

Leaf petioles as well as hypocotyls generally show gravitropic responses (Hangarter, 1997; Fukaki et al., 1998). The conventional gravitropic mutants in hypocotyls, *phosphoglucomutase* and *shoot gravitropism2*, also exhibit reduced gravitropism in petioles, suggesting that petioles and hypocotyls share the same mechanism of gravity response (Mano et al., 2006). The mechanism of the $[Ca^{2+}]_c$ increase may also be shared in hypocotyls and petioles, because we observed $[Ca^{2+}]_c$ increases with comparable amplitude in both the organs (Fig. 2C). There are a large number of endodermal cells that are believed to be a gravity perceptive cell in hypocotyls and petioles (Fukaki et al., 1998; Mano et al., 2006). Genetic studies using gravitropic

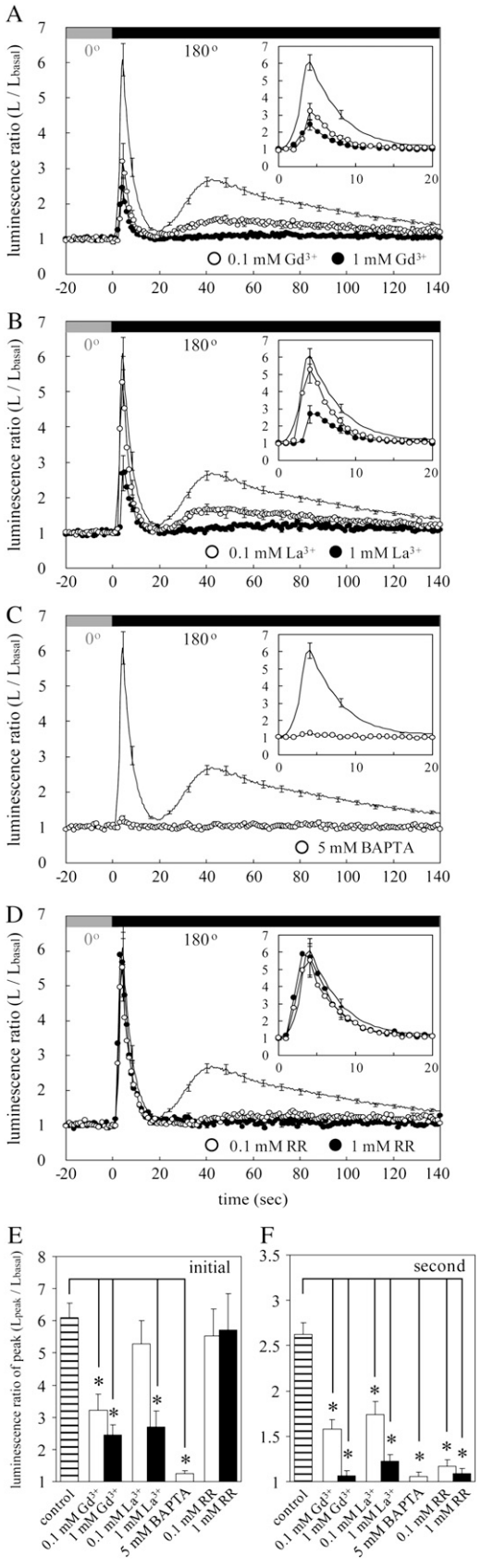

Figure 5. The effect of chemical agents on the initial and second $[Ca^{2+}]_c$ transients. A to D, Potential MSCC inhibitors (Gd^{3+} and La^{3+}), a Ca^{2+} chelator (BAPTA), and an endomembrane Ca^{2+}-permeable channel inhibitor (RR) were extracellularly applied to Arabidopsis seedlings for 1 h. Each averaged trace shows changes in luminescence ratio induced by gravistimulation at the speed of 6 rpm in control (solid line) and chemical agent-treated seedlings (white or black circles). The inset in each figure shows an enlargement of the initial $[Ca^{2+}]_c$ transients. E and F, The peak amplitudes of the initial (E) and second (graviinduced) $[Ca^{2+}]_c$ transients (F) in control (hatched bar) and chemical agent-treated seedlings (white or black bars) are shown. Symbols and concentrations of the chemicals are presented in each figure (A–D) and below the bar graphs (E and F). Data represent means ± SES, $n = 19$, 8, 12, 10, 8, 7, 8, and 9 from the left bar in E and F, respectively. *, $P < 0.01$, the two-tailed Student's t test.

mutants in Arabidopsis suggest that sedimentation of highly dense, starch-filled plastids (amyloplasts) in shoot endodermal cells is involved in gravity sensing (Sack, 1997; Tasaka et al., 1999; Kiss, 2000). According to this hypothesis, $[Ca^{2+}]_c$ would increase in the shoot endodermal cells in response to sedimentation of amyloplasts, as discussed previously (Perbal and Driss-Ecole, 2003; Morita and Tasaka, 2004). However, we could not resolve the responsive cells because of the limitation of optical resolution with our setup. To uncover the relationship between the $[Ca^{2+}]_c$ increase and amyloplasts, we examined $[Ca^{2+}]_c$ changes in response to gravistimulation in the *endodermal-amyloplast-less1* (*eal1*) mutant that has no intact amyloplast in shoot endodermal cells (Fujihira et al., 2000; Morita et al., 2007). Our preliminary experiments showed that both the amplitude and decay time constant of the $[Ca^{2+}]_c$ increase in *eal1* were almost the same as those in wild-type seedlings, implying that intact amyloplasts in endodermal cells of hypocotyls were not necessary for the $[Ca^{2+}]_c$ increase observed in this study. During gravistimulation, both the side and end cell walls are supposed to be exposed to mechanical stress. The $[Ca^{2+}]_c$ increase may be initiated by interaction between the cell wall and the plasma membrane via adhesion molecules (Pickard, 2007) rather than by sedimentable organelles.

Gravistimulation caused a biphasic $[Ca^{2+}]_c$ increase consisting of the initial and second $[Ca^{2+}]_c$ transients with different characters (Fig. 3A). The initial $[Ca^{2+}]_c$ transient looks similar in its kinetics to the wind- or touch-induced $[Ca^{2+}]_c$ spikes in seedlings of *Nicotiana plumbaginifolia* (Knight et al., 1991, 1992). The wind- and touch-induced $[Ca^{2+}]_c$ spikes are supposed to arise from intracellular Ca^{2+} release, because they were inhibited by RR but not by Gd^{3+} or La^{3+} in *Nicotiana* seedlings (Knight et al., 1992) and Arabidopsis roots (Legue et al., 1997). However, the initial $[Ca^{2+}]_c$ transient in this study was inhibited by Gd^{3+} and La^{3+} but not by RR (Fig. 5, A–E), which is diametrically opposite to the above-mentioned results. Collectively, it is suggested that the initial $[Ca^{2+}]_c$ transient is pharmacologically distinct from the wind- and touch-induced $[Ca^{2+}]_c$ spikes. Certainly, the effect of wind during rotation

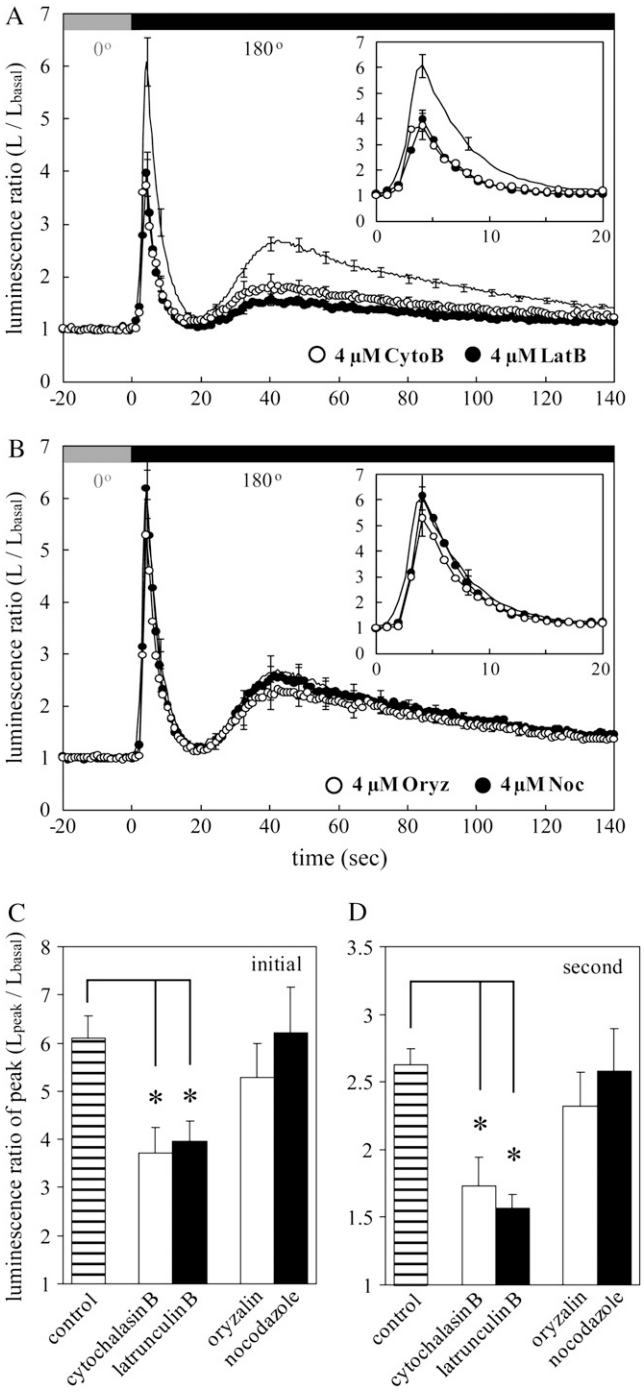

Figure 6. The effect of cytoskeleton-disrupting drugs on the initial and second $[Ca^{2+}]_c$ transients. A and B, Actin-disrupting drugs (cytochalasin B and latrunculin B) and microtubule-disrupting drugs (oryzalin and nocodazole) were extracellularly applied to Arabidopsis seedlings at the final concentration of 4 μM. Each averaged trace shows changes in luminescence ratio induced by gravistimulation at the speed of 6 rpm in control (solid line) and drug-treated seedlings (white and black circles). The inset in each figure shows an enlargement of the initial $[Ca^{2+}]_c$ transients. C and D, The peak amplitudes of the initial (C) and second (graviinduced) $[Ca^{2+}]_c$ transients (D) in control (hatched bar) and drug-treated seedlings (white and black bars) are shown. Symbols and concentrations of the chemicals are presented in each figure (A and B) or below the bar graphs (C and D). Data represent means ± SES, $n = 19$, 11, 18, 19, and 11 from the left bar in A and B, respectively. *, $P < 0.01$, the two-tailed Student's t test.

must be extremely small in this study, because the seedlings are grown in a capped petri dish.

The peak amplitude of the initial $[Ca^{2+}]_c$ transient was dependent on the rotational speed (Fig. 4B) but not on the angle (Fig. 3B), suggesting that the initial $[Ca^{2+}]_c$ transient is induced by forces related to the rotational speed. Centripetal acceleration during rotation depends on the rotational speed and exerts a centrifugal force on the seedlings. However, the centripetal acceleration during rotation at the speed of 6 rpm was $5.6 \times 10^{-3}g$ in our setup (Fig. 1A), which was much smaller than the gravitational acceleration ($1g$). To exclude the effect of prospected centrifugal force, the seedlings were turned at the speed of 6 rpm on the rotation axis, which did not affect the initial $[Ca^{2+}]_c$ transient at all (data not shown). This suggests that the initial $[Ca^{2+}]_c$ transient is not induced by the centrifugal force during rotation. All gravistimuli here were performed at the rotational acceleration of $3.6 \times 10^{-2}g$ (Fig. 1C). To examine the effect of the rotational acceleration on the initial $[Ca^{2+}]_c$ transient, the seedlings were turned at the rotational acceleration of $7.2 \times 10^{-1}g$, which did not affect the initial $[Ca^{2+}]_c$ transient (data not shown). This suggests that the initial $[Ca^{2+}]_c$ transient is not dependent on the rotational acceleration. The acceleration time to reach the speed of 6 rpm at $7.2 \times 10^{-1}g$ was 0.01 s, which might not be long enough to induce a response in the seedlings. Rotation causes a shift in weight bearing of the seedlings, leading to a slight deformation in hypocotyls and petioles, because they are supported on one end. The mechanical stress resulting from the deformation might induce the initial $[Ca^{2+}]_c$ transient.

The second $[Ca^{2+}]_c$ transient showed a rotational angle dependency (Fig. 3C), whereas the rotational speed had no significant effect on its peak amplitude (Fig. 4C). A single rotation around 360° did not induce the second $[Ca^{2+}]_c$ transient, whereas the initial one was observed (Plieth and Trewavas, 2002). These results suggest that the second $[Ca^{2+}]_c$ transient is specific for changes in the gravity vector but not for the rotation. As the rotational speed was decreased, the peak amplitude of the second $[Ca^{2+}]_c$ transient was slightly increased and the time to the peak was delayed (Fig. 4, A and C). When the seedlings were turned 135° and returned back to the vertical position (0°) within 5 s, a small but distinguishable second $[Ca^{2+}]_c$ transient was observed (Plieth and Trewavas, 2002), suggesting that the presentation time, the time of gravistimulation to elicit the second $[Ca^{2+}]_c$ transient, is less than 5 s. It requires approximately 50 s to reach 180° at the speed of 0.6 rpm (Fig. 4A). Because the presentation time is much shorter than the above rotational time, the seedlings might be gravistimulated

at each angle during the slow rotation, resulting in an apparent increase in the second $[Ca^{2+}]_c$ transient (Fig. 4A). The delayed time to the peak is probably due to the delay to reach 180°, because the second $[Ca^{2+}]_c$ transient is induced by changes in the gravity vector, as discussed above. Recently, Toyota et al. (2007) demonstrated that increases in the gravitational acceleration caused a monophasic $[Ca^{2+}]_c$ transient in Arabidopsis seedlings, which resembles the second $[Ca^{2+}]_c$ transient in terms of time course. The monophasic $[Ca^{2+}]_c$ was inhibited by La^{3+} or Gd^{3+} and was also attenuated by repetitive hypergravity stimuli. $[Ca^{2+}]_c$ spikes like the initial $[Ca^{2+}]_c$ transient were not evoked by hypergravity stimuli, implying that gravity-related stimuli (e.g. changes in the gravity vector or increases in the gravitational acceleration) cause a long lasting $[Ca^{2+}]_c$ increase. Based on this perspective, our observations further support the idea that the second $[Ca^{2+}]_c$ transient was induced by changes in the gravity vector.

The second (graviinduced) $[Ca^{2+}]_c$ transient was inhibited by La^{3+}, Gd^{3+}, RR, and BAPTA (Fig. 5), suggesting that it arises from Ca^{2+} influx via putative MSCCs in the plasma membrane and Ca^{2+} release from intracellular Ca^{2+} stores. Plant MS channels have been characterized by the patch clamp technique in *V. faba* guard cells (Cosgrove and Hedrich, 1991), epidermal cells of *Allium cepa* (Ding and Pickard, 1993), and Arabidopsis mesophyll cells (Qi et al., 2004). Recently, several genes of putative MS channels were identified in Arabidopsis. Nakagawa et al. (2007) isolated *MID1-COMPLEMENTING ACTIVITY1* encoding a plasma membrane protein that is responsible for Ca^{2+} influx in response to mechanical stress such as hypoosmotic shock. Haswell and Meyerowitz (2006) characterized two MscS-like (MSL) proteins, MSL2 and MSL3, which are homologous to the well-known bacterial MS channel MscS and are localized to the plastids such as chloroplasts. A homolog of MscS expressing in chloroplasts (MSC1) was also cloned in *Chlamydomonas* and its functional reconstitution was succeeded in *Escherichia coli* (Nakayama et al., 2007). These putative MS channels may be involved not only in mechanosensing in the plasma and endomembrane but also in the $[Ca^{2+}]_c$ transient in Arabidopsis seedlings, which is induced by changes in the gravity vector, as proposed here.

Intracellular levels of inositol 1,4,5-trisphosphate ($InsP_3$) increased within 15 s of gravistimulation and peaked at around 60 s in the lower pulvinus of the horizontally placed oat shoot (Perera et al., 2001). Arabidopsis inflorescence stems also showed increases in the $InsP_3$ level within 5 min of gravistimulation (Perera et al., 2006). The graviinduced $[Ca^{2+}]_c$ transient could be observed at approximately 20 s after the onset of rotation and lasted for more than 5 min, which resembles the $InsP_3$ increase in terms of time course. Thus, the graviinduced $[Ca^{2+}]_c$ transient may be related to the increased level of $InsP_3$ that leads to an $InsP_3$-induced Ca^{2+} release (Bush, 1995). Plieth and Trewavas (2002) demonstrated that both the initial and second $[Ca^{2+}]_c$ transients were attenuated by auxin transport inhibitors (naphthylphthalamic acid, NPA; and 2,3,5-triiodobenzoic acid, TIBA) and raised the possibility that $[Ca^{2+}]_c$ increases while auxin is redistributed asymmetrically. We do not exclude this possibility, because we also confirmed that NPA and TIBA affected the $[Ca^{2+}]_c$ transient with our setup (data not shown). The biphasic $[Ca^{2+}]_c$ transient may be related to auxin via its multiple signaling pathways (Woodward and Bartel, 2005).

Recently, an intriguing model for gravity sensing was proposed based on a careful analysis of gravitropism in maize roots (LaMotte and Pickard, 2004). In this model, maize roots require at least two gravity sensing steps, termed gravifacilitation and vectorial graviinduction steps. Pickard (2007) speculates that the biphasic $[Ca^{2+}]_c$ transient is involved in the gravifacilitation step that does not induce gravitropism directly but operates the second vectorial graviinduction step. The pharmacological properties of the biphasic $[Ca^{2+}]_c$ transient in this study are almost consistent with the speculation. The roles of the biphasic $[Ca^{2+}]_c$ transient in gravitropism remain to be solved.

MATERIALS AND METHODS

Plant Materials and Growth Condition

We used the transgenic Arabidopsis (*Arabidopsis thaliana*) ecotype Columbia-0 (Col-0) expressing cytoplasmic apoaequorin under the control of the 35S promoter of *Cauliflower mosaic virus* (Knight et al., 1996) for all experiments. Approximately 40 surface-sterilized seeds of homozygous luminous plants were sown on a 0.3% [w/v] gelrite (Wako Pure Chemicals) plate containing plant growth medium (Murashige and Skoog salts, 1% [w/v] Suc, 0.01% [w/v] myoinositol, and 0.05% [w/v] MES, pH 5.8, adjusted with 1 M KOH) in petri dishes (diameter, 6 cm). The plates were incubated at 4°C in the dark for 2 d and subsequently cultivated at 22°C in a growth chamber under continuous light at approximately 80 μmol m^{-2} s^{-1} with daylight fluorescent lamps (model FL32S NA-G, Matsushita Electric Industrial). All plates of seedlings light grown on the agar were used for experiments 5 to 7 d after cultivation.

Reconstitution of Aequorin

Chemically synthesized *o*-fluoro-dehydrocoelenterazine (F-DCT; Isobe et al., 2002), a kind gift from Dr. M. Kuse and Prof. M. Isobe, Nagoya University, was prepared in ethanol to give a 1-mM stock solution and then added into the plant growth medium to make a 2.5-μM solution. To reconstitute aequorin, Arabidopsis seedlings grown on the agar were filled with 3 mL of the plant growth medium containing 2.5 μM F-DCT for approximately 8 h at 22°C in the dark. After incubation, the plant growth medium was removed from the dish 2 h before experiments.

Recently, a coelenterazine was considered as a chemiluminescent indicator for reactive oxygen species (ROS) in addition to $[Ca^{2+}]_c$ (Plieth, 2005). We examined whether the transient increase in intensity of aequorin luminescence during gravistimulation is induced by an increase in ROS. Untransformed Col-0 seedlings treated with F-DCT showed no changes in luminescence intensity during gravistimulation (data not shown), indicating that the increase in luminescence intensity is induced by $[Ca^{2+}]_c$ increases but not by ROS.

Treatments with Chemical Agents

La^{3+} or Gd^{3+}, potential MSCC inhibitors; RR, a potential endomembrane Ca^{2+}-permeable channel inhibitor; and BAPTA, a Ca^{2+} chelator were prepared in distilled water to give a 10-mM stock solution. Cytochalasin B or latrunculin B, actin-disrupting drugs, and oryzalin or nocodazole, microtubule-disrupting drugs, were prepared in dimethyl sulfoxide to give a 10-mM stock solution. Each drug was added into the plant growth medium containing F-DCT in a

petri dish 1 h prior to removal of the medium. The plant growth medium containing F-DCT and drugs was removed from the dish 2 h before the experiments. The final dimethyl sulfoxide concentration did not exceed 0.1%, which had no effect on the $[Ca^{2+}]_c$ increases induced by gravistimulation (data not shown).

Gravistimulation and $[Ca^{2+}]_c$ Monitoring

Approximately 40 Arabidopsis seedlings cultivated on the agar in a petri dish were mounted under an ultrasensitive PCC (a CCD camera equipped with an image intensifier, model C2741-35A, or a Peltier cooled image intensifier, model C8600-04, Hamamatsu Photonics) and a PMT (model RP1942, Hamamatsu Photonics) in a light-tight dark box (Fig. 1A). The box was set on the folder and turned by a computer-controlled stepping motor system (model RK569BA, Oriental Motor) to change the gravity vector against the seedlings, termed gravistimulation (Fig. 1B). The radius of rotation, the length from the rotation axis to seedlings, was approximately 0.14 m. All gravistimuli were performed at the rotational acceleration of 0.35 m s^{-2} (Fig. 1C, black circle) and at the rotational speed of 6 rpm except for one experiment (Fig. 4). In this system, the relative position between Arabidopsis seedlings and the detector was not shifted during gravistimulation, which made it possible to monitor aequorin luminescence with a spatial resolution.

To visualize the organ that increases $[Ca^{2+}]_c$, photons of aequorin luminescence from individual plants attached on the agar surface were integrated for 7 min using PCC with a 25-mm lens ($F = 0.95$; model CM 120, Schneider Kreuznach) before and during gravistimulation as described previously (Furuichi et al., 2001). The integrated images were processed with the image analysis software, MetaMorph (Universal Imaging) or HPD-LIS (Hamamatsu Photonics) and presented through low-pass filtering (Fig. 2A). Aequorin luminescence was integrated from 20 s after the start of rotation to exclude the luminescence of the initial $[Ca^{2+}]_c$ transient. Bright-field images of Arabidopsis seedlings were taken with a digital camera (model D70s, Nikon) under approximately 80 μmol m^{-2} s^{-1} white light at the end of each experiment.

The intensity of aequorin luminescence from a group of Arabidopsis seedlings in a petri dish were monitored with PMT with a 50-mm lens ($F = 0.95$; model YMV5095, Yakumo). The signals from the seedlings were processed by a photon counter (model PHC3000-1, Scientex) at 0.5-s intervals and stored in a computer (Fig. 1B).

Data Analysis

For the statistical analysis of the data obtained from different gravistimuli, the luminescence ratio (L_{peak}/L_{basal}) was calculated by dividing the initial and second peak intensities of aequorin luminescence (L_{peak}) with the steady luminescence intensity before gravistimulation (L_{basal}). The relative luminescence (L_{peak}/L_{max}) was obtained by dividing L_{peak} by the L_{max} induced by the addition of 20% (v/v) ethanol plus 2 M $CaCl_2$ at the end of each experiment. Almost the same statistical significances were obtained using either L_{peak}/L_{basal} or L_{peak}/L_{max} (data not shown) in this study. All experiments were repeated more than three times independently with similar results. All data represent means ± SE.

The levels of aequorin reconstitution in hypocotyls, petioles, cotyledons, and roots were estimated by the measurements of the relative steady luminescence (L_{basal}/L_{max}). The L_{basal}/L_{max} was monitored with the PCC and almost the same level was observed in hypocotyls, petioles, and cotyledons but was quite small in roots (data not shown). When the reconstitution of aequorin was performed by floating seedlings in the growth medium containing F-DCT, as described previously (Knight et al., 1991), we could detect almost the same levels of the L_{basal}/L_{max} in all organs (data not shown). These results suggest that the reconstitution of aequorin is not completed in roots in the agar, probably due to poor penetration of F-DCT into the agar. Thus, aequorin luminescence from seedlings was mainly originated from shoots in our system.

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