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The Ca^{2+} pump inhibitor, thapsigargin, inhibits root gravitropism in *Arabidopsis thaliana*

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ABSTRACT

Thapsigargin, a specific inhibitor of most animal intracellular SERCA-type Ca^{2+} pumps present in the sarcoplasmic/endoplasmic reticulum, was originally isolated from the roots of the Mediterranean plant *Thapsia gargancia L.* Here, we demonstrate that this root-derived compound is capable of altering root gravitropism in *Arabidopsis thaliana*. Thapsigargin concentrations as low as 0.1 μM alter root gravitropism whereas under similar conditions cyclopiazonic acid does not. Furthermore, a fluorescently conjugated thapsigargin (BODIPY FL thapsigargin) suggests that target sites for thapsigargin are located in intracellular organelles in the root distal elongation zone and the root cap, regions known to regulate root gravitropism.

Key terms: *Arabidopsis thaliana*, Ca^{2+}-ATPases, calcium, gravitropism, thapsigargin, root.

INTRODUCTION

The ability of a plant to change developmental and metabolic processes in response to environmental stimuli is essential for survival. Response to gravitational stimuli enables crop plants to survive strong weather conditions, such as wind and rainstorms, by directing their growth and development. This response ensures that the root will grow downward in the soil, thereby optimizing water, mineral and nutrient uptake that are all vital for growth and development. Shortly after germination, the root senses gravitational forces and grows towards this gravity vector (gravitropism). Sensing of gravitational stimuli and subsequent response to these stimuli, by altering growth and developmental processes, is a change from a physical force to a physiological signal, which in turn leads to a physiological response (Blancaflor and Masson, 2003).

It has been demonstrated that dense amyloplasts (statoliths) in specialized root columella (cap) cells (statocytes) sediment in response to gravitational forces (physical force). This sedimentation of amyloplasts is then translated into a physiological signal by the plant, so that the root can grow and elongate in the direction of the new gravitational vector (physiological response) (Moore and Evans, 1986; Sack, 1991; Sack, 1997; Chen et al., 1999; Boonsirichai et al., 2002; Morita and Tasaka, 2004). The transduction of amyloplasts sedimentation to a physiological signal is poorly understood. It has been proposed that the pulling action generated by amyloplast sedimentation could stretch specific plasma membranes and/or the endoplasmic reticulum to activate mechanosensitive channels. The opening of these channels leads to a local transient increase in cytosolic-free Ca^{2+} levels, which in turn triggers a signal transduction cascade resulting in a physiological response (Sievers et al., 1991; Belyavskaya, 1996;

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Chen et al., 1999; Boonsirichai et al., 2002; Kordyum, 2003). Following the physiological signal (signal transduction cascade), the plant cells must return to a basal level, such that they may respond to future stimuli. It has been proposed that Ca^{2+} pumps (Ca^{2+}-ATPases) participate in this process by returning cytosolic Ca^{2+} levels to the basal level (Sze et al., 2000).

The Arabidopsis Calcium ATPase, ACA3 (ECA1), has been demonstrated to be inhibited by cyclopiazonic acid but not by thapsigargin (Liang and Sze, 1998). Cyclopiazonic acid and thapsigargin are both inhibitors of SERCA Ca^{2+}-ATPases in mammalian and yeast systems (Sorin et al., 1997; Treiman et al., 1998). The differential sensitivity to cyclopiazonic acid and thapsigargin by plant Ca^{2+}-ATPases has been suggested to evolve because thapsigargin is a plant-derived compound and the plant Ca^{2+}-ATPases, therefore, may have developed insensitivity to thapsigargin (Liang and Sze, 1998). However, the Ca^{2+}-pump TcSCA from *Trypanosoma cruzi* also is sensitive to cyclopiazonic acid and insensitive to thapsigargin (Furuya et al., 2001), indicating that not only plant-derived Ca^{2+}-ATPases are differentially sensitive to these inhibitors.

Thapsigargin-sensitive Ca^{2+}-pump activity has been identified in pea and cauliflower Golgi-derived vesicles (Ordenes et al., 2002). Therefore, unidentified thapsigargin-sensitive Ca^{2+}-pumps are present in plants. However, their physiological role is poorly understood.

In an effort to determine the physiological role of thapsigargin-sensitive Ca^{2+}-pumps, we studied the physiological effects that exogenous thapsigargin treatment had on *Arabidopsis thaliana* root growth and curvature in response to gravitropic stimuli. We provide evidence that thapsigargin inhibits Arabidopsis root gravitropism, whereas under similar conditions, cyclopiazonic acid does not. Furthermore, our results suggest that thapsigargin is able to target intracellular organelles of cells within the distal elongation zone and the root cap, regions of the root that play a role in root gravitropism.

MATERIALS AND METHODS

Plant material and growth conditions

Seven-day-old *Arabidopsis thaliana* seedlings, ecotype Columbia (Col), were grown vertically under sterile conditions on plates containing 1/2X MS (pH 5.7), 1% sucrose, 0.9% agar. Plants were grown vertically under 61.6 $\mu mol/m^{-2}s^{-1}$ cooled white fluorescent light (Philips TLD 30W/33), 16-h light cycle, 25° C, unless otherwise indicated.

Inhibitor treatment

Thapsigargin (Molecular Probes) and cyclopiazonic acid (Sigma) were dissolved in dimethyl sulfoxide. Sodium orthovanadate (Sigma) was dissolved in deionized water. The maximum concentration of dimethyl sulfoxide in any inhibitor solution used in these studies did not exceed 0.5% (minimum concentration in which root growth was unaffected, data not shown). Control plants for thapsigargin and cyclopiazonic acid studies were treated with DMSO at the same concentration used in the highest inhibitor concentration, respectively. Control plants for sodium orthovanadate studies were treated with H_2O.

Seedlings were infiltrated for 15 min in the dark with Ca^{2+}-ATPase/P-Type ATPase inhibitors at varying concentrations. Thapsigargin treatments were performed at 0, 0.1, 0.5, 1.0, and 5.0 μM. Cyclopiazonic acid treatments were performed at 0, 0.5, 1.0, 5.0, and 20 μM. Sodium orthovanadate treatments were performed at 0, 1.0, 5.0, 10, and 50 μM. Post treatment, plants were aligned linearly on 1/2X MS (pH 5.7), 1% sucrose, 0.9% agar containing plates, rotated 90° relative to the gravity vector and placed in the dark to avoid changes due to phototropism. Six hours post treatment, the angle of root curvature and the length of root growth were measured under a stereomicroscope. The angle between the plant axis and the tip of the root were measured as shown in figure 1D.

Control plants were aligned on the same plates as the treated plants, thereby serving as controls for solvent treatment as well as

the direction of gravitational forces. All assays were performed in triplicate, with a minimum of 10 treated and 10 untreated plants in each assay. All results are presented as the mean ± standard error. Statistical significance of the results was determined by performing the Student's t-test on the mean ± standard error of each treatment, with a certainty of 95% and degree of freedom of n-1.

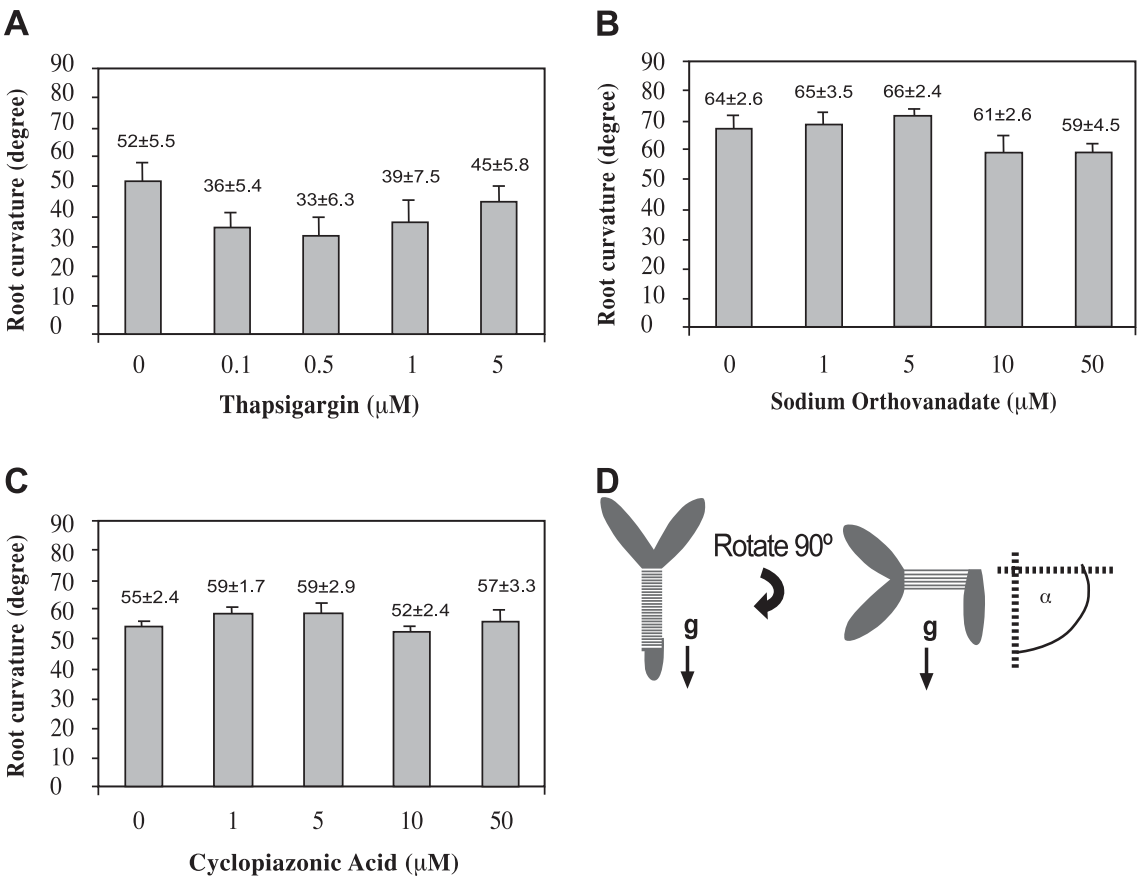

Figure 1: Gravitropic root curvature response in the presence of different P-type ATPase inhibitors. A-C, the angle of root curvature in response to gravitropic stimuli was measured 6 h post treatment with different concentrations of P-type ATPase inhibitors. A, thapsigargin, a specific inhibitor of most animal intracellular SERCA-type Ca^{2+}-pumps but not plant type IIA Ca^{2+}-ATPases (0 indicates control plants that have been treated with DMSO at the same concentration used for the thapsigargin-treated plants); B, sodium orthovanadate, a general inhibitor of P-type ATPases, (0 indicates control plants that have been treated with water); C, cyclopiazonic acid, a specific inhibitor of animal SERCA type and plant type IIA Ca^{2+}-ATPases, (0 indicates control plants that have been treated with DMSO at the same concentration used for the cyclopiazonic acid-treated plants). Bars indicate the mean ± the standard error. Experiments were performed in triplicate with at least ten plants for each experiment. The most representative experiment is shown. D, diagram demonstrating the angle used to measure root curvature in response to gravitropic stimuli in Arabidopsis. Vertically grown Arabidopsis seedlings were treated with different inhibitors, followed by a 90° change in the direction of gravity. Six hours post-treatment, the angle of the root curvature, and the length of root growth were measured. α is the angle measured in these experiments.

Fluorescent analysis

To visualize the cellular and intracellular targets of thapsigargin, 7-day-old seedlings were incubated with 1 μM BODIPY FL thapsigargin (Molecular Probes) in 0.1 mM phosphate buffer (pH 7.0) for 2 h in the dark. Samples were subsequently washed three times with 0.1 mM potassium phosphate buffer and visualized on an Olympus IX70 epifluorescence microscope with a Sony DXC-390 3CCD color camera. Differential Interference Contrast imaging was performed using this equipment and an UPLAN F1 objective. Fluorescence imaging was performed using a U-N3001 FITC filter (excitation D480/30X, dichroic 505 DCLP, emission D535/40m). Images were processed using Adobe Photoshop.

RESULTS

Calcium ATPase Inhibitors alter Arabidopsis root gravitropism

Treatment of Arabidopsis seedlings with low concentrations of thapsigargin (a specific inhibitor of most animal intracellular SERCA-type Ca^{2+} pumps but not plant type IIA Ca^{2+}-ATPases) showed a significant inhibition of root curvature in response to gravitropic stimuli (Figure 1A). 0.1 μM thapsigargin inhibited root curvature by 31%. The greatest inhibition of root curvature was seen with 0.5 μM thapsigargin. This concentration inhibited root curvature by 37%. However, higher concentrations of thapsigargin did not significantly inhibit root curvature in response to gravitropic stimuli (Figure 1A). To determine if the change in root curvature post-thapsigargin treatment was due to a change in the gravitropic response or a general inhibition of growth, root growth post treatment was analyzed (Table 1). Under the conditions utilized in these studies, no significant changes were observed in the root growth of thapsigargin-treated seedlings.

Arabidopsis seedlings treated with the P-type ATPase inhibitor sodium orthovanadate showed significant inhibition of root curvature when 50 μM sodium orthovanadate was used (Figure 1B). Additionally, root growth increased significantly when seedlings were treated with this concentration of sodium orthovanadate (Table 1). Lower concentrations of sodium orthovanadate did not significantly alter root curvature or alter root growth (Figure 1B, Table 1).

TABLE 1

Effects of Thapsigargin, Cyclopiazonic Acid and Sodium Orthovanadate on Arabidopsis Root Growth

	Root Growth (mm)*
Thapsigargin (μM)	
0	1.8 ± 0.6
0.1	1.8 ± 0.6
0.5	1.7 ± 0.6
1	2.4 ± 0.8
5	2.1 ± 0.5
Sodium Orthovanadate (μM)	
0	1.8 ± 0.1
1	1.7 ± 0.1
5	1.6 ± 0.1
10	1.7 ± 0.2
50	2.0 ± 0.0
Cyclopiazonic Acid (μM)	
0	1.6 ± 0.1
0.5	1.4 ± 0.1
1	1.7 ± 0.2
5	1.8 ± 0.1
20	1.7 ± 0.1

* Values represent the average root growth ± SE (n=10), 6 hours post treatment. Experiments were performed in triplicate. The most representative experiment is shown here.

Surprisingly, Arabidopsis seedlings treated with cyclopiazonic acid (a specific inhibitor of animal SERCA type and plant type IIA Ca^{2+}-ATPases) were not significantly altered in their ability to curve in response to gravitropic stimuli (Figure 1C). No alterations in root curvature and root growth in response to gravitropic stimuli were detected when plants were treated with concentrations as high as 20 μM cyclopiazonic acid (Figure 1C, Table 1).

The cellular and intracellular targets of thapsigargin

In order to determine whether thapsigargin targets cells in regions known to play a role in root gravitropism and to determine the intracellular target of thapsigargin, Arabidopsis seedlings were treated with a membrane-permeable green fluorescent derivative of thapsigargin, BODIPY FL thapsigargin. This green fluorescent derivative of thapsigargin has been utilized in various systems to determine the target of thapsigargin (Csordas and Hajnoczky, 2001; Lee and Aarhus, 2000; Abrenica and Gilchrist, 2000).

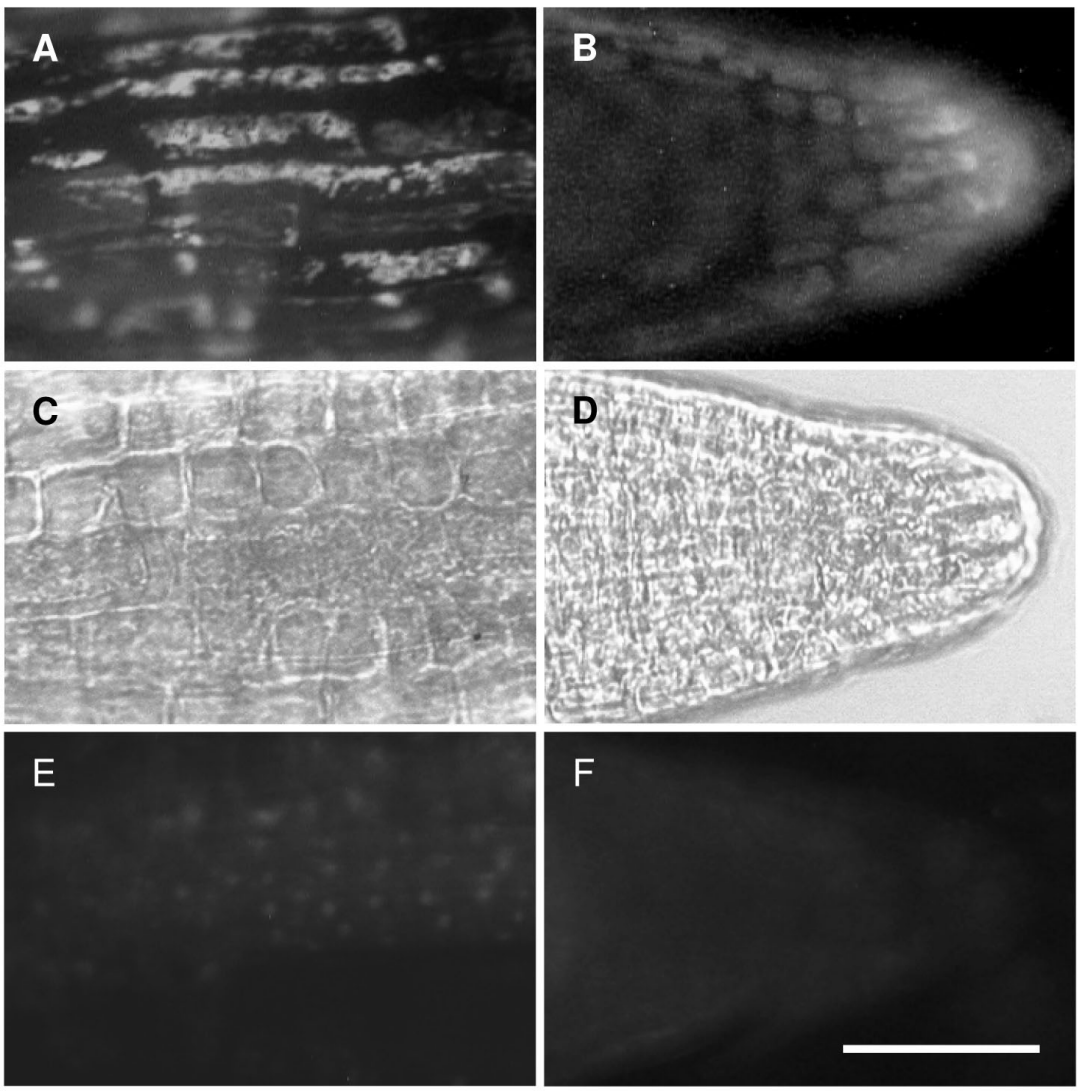

Figure 2: Thapsigargin targets regions of the root that are responsible for the root gravitropic response. To determine the intracellular target for thapsigargin, 7-day-old Arabidopsis seedlings were treated with BODIPY FL thapsigargin. Different regions of the root were analyzed for localization of the BODIPY FL fluorescence (A and B). Regions of the root analyzed include: A, C, and E, distal elongation zone of the root; B, D, and F, root tip (including root cap, quiescent center and root meristem); C and D, roots visualized by DIC microscopy; E and F, auto-fluorescence of untreated roots. Bar represents 50 μm.

Fluorescence from the BODIPY FL thapsigargin was detected in the distal elongation zone of the root (Figure 2A) as well as the root cap (Figure 2B). Interestingly, reduced fluorescence was detected in the quiescent center and meristematic zone of the root (Figure 2B). The BODIPY FL thapsigargin targeted intracellular reticulate structures in the distal elongation zone of the root (Figure 2A) and the root cap (Figure 2B). There is no notable co-localization between BODIPY FL thapsigargin and the plasma membrane in all tissues analyzed. The intracellular localization of thapsigargin also has been analyzed in Arabidopsis protoplasts, onion epidermal cells, and tobacco epidermal cells. All tissues showed similar patterns with numerous intracellular organelles co-localizing with the BODIPY FL thapsigargin (data not shown).

DISCUSSION

In this study, we have demonstrated that the Ca^{2+}-ATPase inhibitor, thapsigargin, inhibits root gravitropism in *Arabidopsis thaliana*. Concentrations of thapsigargin as low as 0.1 μM, inhibited root gravitropism in Arabidopsis. This concentration is within the range utilized to characterize thapsigargin-sensitive Ca^{2+}-ATPase activities in several animal studies as well as in the recently identified Ca^{2+}-ATPase activity in Golgi vesicles of etiolated pea seedlings (Simpson and Russell, 1997; Thastrup et al., 1990; Zhong and Inesi, 1998; Ordenes et al., 2002). Furthermore, 0.1 μM thapsigargin is lower than the concentration that has been shown to inhibit L-type Ca^{2+} channels (5 μM) (Nelson et al., 1994; Treiman et al., 1998). When Arabidopsis seedlings were infiltrated with 5 μM thapsigargin, no significant difference was detected in the gravitropic response between treated and non-treated plants. This lack of inhibition of root gravitropism with 5 μM thapsigargin may be due to the simultaneous inhibition of L-type Ca^{2+} channels and thapsigargin-sensitive Ca^{2+}-ATPases. This is further evidence that the inhibition of root gravitropism by thapsigargin at lower concentrations is specifically due to an inhibition of thapsigargin-sensitive Ca^{2+}-ATPases and not due to an inhibition of L-type Ca^{2+} channels.

Thapsigargin is the most widely used SERCA inhibitor (Treiman et al., 1998). It is a naturally occurring sesquiterpene lactone, found in several species belonging to the genus Thapsia. Thapsigargin was isolated originally from the roots of the Mediterranean plant *Thapsia garganica* L. (Linnaeus) (Treiman et al., 1998). Our findings suggest that under normal physiological conditions *in planta*, thapsigargin may play a role in signaling and regulating root gravitropism.

Sievers and Busch (1992) have demonstrated that cyclopiazonic acid sensitive Ca^{2+}-ATPases are involved in the transduction of gravitational stimuli in *Lepidum sativum* L. Cyclopiazonic acid is an indole tetramic acid that has been reported to be a specific inhibitor of SER-type Ca^{2+}-ATPases (SERCA) (Seidler et al., 1989). The study by Sievers and Busch (1992) demonstrated that *Lepidium sativum* L. seedlings treated with 20 μM cyclopiazonic acid inhibited root curvature in response to a change in the gravity vector. However, we have demonstrated that 20 μM cyclopiazonic acid is unable to inhibit root gravitropism in Arabidopsis after a 15 min treatment. Sievers and Busch (1992) treated *Lepidum sativum* L. with 20 μM cyclopiazonic acid for 2 h. Longer treatment with cyclopiazonic acid may alter root gravitropism in Arabidopsis roots. However, such a long treatment also may cause alterations in other physiological processes that may result in changes in root curvature. For example, a change in root growth may give rise to changes in root curvature.

To date, a thapsigargin-sensitive Ca^{2+}-ATPase has not been identified in Arabidopsis. Rather, two distinct families of Ca^{2+}-ATPases have been proposed based on sequence identities (Geisler et al., 2000). These are type IIA (or ECA for ER- type Ca^{2+}-ATPase) and type IIB (ACA for autoinhibited Ca^{2+}-ATPase). The type IIA Ca^{2+}-ATPases are insensitive to calmodulin

and sensitive to cyclopiazonic acid. While the type IIB Ca^{2+}-ATPases are stimulated by calmodulin. Neither the Ca^{2+}-ATPases, type IIA nor the type IIB have been demonstrated to be sensitive to thapsigargin (Sze et al., 2000). However, Ordenes et al. (2002) recently have identified a thapsigargin-inhibited Ca^{2+}-ATPase activity in Golgi vesicles isolated from pea epicotyls. Similarly, J Watkins, AK Campbell, MR Knight and AJ Trewavas recently have detected changes in endoplasmic reticulum and nuclear calcium levels due to thapsigargin treatment (unpublished data). This suggests that a thapsigargin sensitive Ca^{2+}-ATPase activity exists in plants. Our results suggest that these as yet unidentified thapsigargin-sensitive Ca^{2+}-ATPases are involved in physiological processes such as root gravitropism. BODIPY FL thapsigargin labels numerous cells, including cells in the distal elongation zone as well as the root cap. This suggests that these thapsigargin-sensitive Ca^{2+}-ATPases are localized in cells that are known to play an important role in the root gravitropic response.

We cannot, however, rule out the possibility that thapsigargin-sensitive targets, other than Ca^{2+}-ATPases, may exist in Arabidopsis and may be playing a role in root gravitropism. However, our results do demonstrate that a thapsigargin-sensitive factor is involved in Arabidopsis root gravitropism. Furthermore, the target for thapsigargin is localized in reticulate organelles. This thapsigargin-sensitive factor may be a Ca^{2+}-ATPase activity localized in organelles, such as the Golgi apparatus or the ER, but other molecular targets for thapsigargin also may exist. Further molecular genetic analyses in Arabidopsis to identify the factor(s) that are affected by thapsigargin, the subcellular localization of these factors, and their role in root gravitropism will help to unveil these possibilities.

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