Effects of Fifteen Rare-Earth Metals on Ca^{2+} Influx in Tobacco Cells

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Effects of naturally existing rare-earth metals (REMs; atomic numbers, 39, 57–60, 62–71; Y, La, Ce, Pr, Nd, Sm, Eu, Gd, Tb, Dy, Ho, Er, Tm, Yb and Lu), added as chloride salts, on Ca^{2+} influx induced by two different stimuli, namely hypoosmotic shock and hydrogen peroxide, were examined in a suspension-cultured transgenic cell line of BY-2 tobacco cells expressing aequorin, a Ca^{2+}-sensitive luminescent protein in cytosol. Most REM salts used here showed inhibitory effect against Ca^{2+} influx. Especially $NdCl_3$, $SmCl_3$, $EuCl_3$, $GdCl_3$ and $TbCl_3$ showed the most robust inhibitory action. In contrast, $LuCl_3$, $YbCl_3$, $ErCl_3$ and YCl_3 were shown to be poor inhibitors of Ca^{2+} influx. Since REMs tested here form a sequential range of ionic radii from 86.1 to 103.2 pm and the optimal range of ionic radii required for blocking the flux of Ca^{2+} was determined for each stimulus. The hydrogen peroxide-induced Ca^{2+} influx was optimally blocked by REMs with a broad range of ionic radii (93.8–101 pm) which is slightly smaller than or similar to that of Ca^{2+} (100 pm), while the hypoosmotically induced flux of Ca^{2+} was inhibited optimally by few REMs with a narrower range of relatively smaller ionic radii around that of Gd^{3+} (93.8 pm) a well known inhibitor of stretch-activated channels. Possible applications of such series of channel blockers in elucidation of plant signal transduction pathways are encouraged.

Key words: Calcium, Ion Channel, Ionic Radius, Rare-Earth Elements

Introductions

Calcium ions play a key role as a second messenger in the signal transduction in living cells including those of plants. As analogous to animal systems, it is likely that three different types of calcium channels, to be activated voltage-sensitively, mechano-sensitively, or ligand-sensitively, participate in the induced movement of extracellular Ca^{2+} into the plant cells (Hetherington and Brownlee, 2000). Previous studies with tobacco cell suspension culture have suggested that the actions of hydrogen peroxide (H_2O_2) or other reactive oxygen species (ROS) such as superoxide in induction of Ca^{2+} influx (Kawano *et al.,* 1998; Kawano and Muto, 2000) are likely mediated through activation of TPC1-type channels, the only voltage-dependently activated plant Ca^{2+} channels (Furuichi *et al.,* 2001; Kawano *et al.,* 2003, 2004). On the other hand, Ca^{2+} influx induced in response to hypoosmotic shock (Takahashi *et al.,* 1997) is likely mediated through activation of other types of Ca^{2+} channels distinct from TPC1-type channels (Kawano *et al.,* 2003, 2004). By analogy to other eukaryotic systems, hypoosmotical regulation of Ca^{2+} uptake may be mediated through opening and closing of mechano-sensitive (stretch-activated) cation channels functionally homologous to the yeast Mid1 channel (Kanzaki *et al.,* 1999), as such activity has been found in plant epidermal cells possibly serving to transduce the mechanical forces generated in the integrated cell wall-membrane-cytoskeleton system during turgor changes and cell expansion (Ding and Pickard, 1993). The aim of this study is to examine the effects of putative blockers of ion channels on two distinct types of Ca^{2+} influx-inducing stimuli namely hypoosmotic shock and H_2O_2 in tobacco cells.

The rare-earth metals (REMs) include scandium (Sc), yttrium (Y) and fourteen lanthanides such as lanthanum (La), cerium (Ce), praseodymium (Pr), neodymium (Nd), promethium (Pm), samarium (Sm), europium (Eu), gadolinium (Gd), terbium (Tb), dysprosium (Dy), holmium (Ho), erbium (Er), thulium (Tm), ytterbium (Yb) and lutetium (Lu). Among them Pm is a synthetic element not present in nature. In general, REMs are not present at high concentrations under normal

environmental conditions surrounding living organisms and thus biological roles for REMs had not been intensively tested, partly due to the classical belief, based on their membrane impermeability, that REMs are physiologically inert (Evans, 1990; Kawano, 2003). However, in fact, the ions of REMs, especially La^{3+} and Gd^{3+}, are not biologically inert at all.

Due to similarities of natures of multi-valent cations including that of aluminum and REMs to those of Ca^{2+} in terms of hydrated ionic radius, electron orbital configuration, or other chemical properties, multiple types of Ca^{2+} channels were shown to be interfered by such cations present in environments as contaminants (Atchison, 2003). Actually, La^{3+} and/or Gd^{3+} inhibit the current via Ca^{2+} channels in most animals (Evans, 1990), yeast (Kanzaki *et al.,* 1999), and plants (Muto, 1992), and thus La^{3+} and Gd^{3+} have been extensively employed as plasma membrane Ca^{2+} channel antagonists in plant researches (Muto, 1992), although non-specific inhibition of ion channels by treatment of *Arabidopsis* cells with La^{3+} has been reported (Lewis and Spalding, 1998).

Our previous works have documented the inhibitory effects of La^{3+} and Gd^{3+} against both the ROS-induced and hypoosmotic shock-induced influx of Ca^{2+} (Kawano *et al.,* 1998; Takahashi *et al.,* 1997), thus we observed no selectivity or specific tendency to be inhibited. To date, any test comparing the effects of all available REM salts as inhibitors of any specific Ca^{2+} channel has not been documented. Here, in order to obtain novel and more effective inhibitors of plant Ca^{2+} channels, we thoroughly analyzed the effects of all REMs available as chloride salts on Ca^{2+} influx induced by the hypoosmotic shock (ROS-independent stimuli inducing Ca^{2+} influx, Takahashi *et al.,* 1997) and H_2O_2 (typical ROS that induces Ca^{2+} influx; Price *et al.,* 1994; Takahashi *et al.,* 1998) using BY-2 tobacco cell suspension culture carrying the gene of cytosol-targeted aequorin, a Ca^{2+}-sensitive luminescent protein.

Materials and Methods

Plant material

A tobacco (*Nicotiana tabacum* L. cv. Bright Yellow-2) cell suspension culture (cell line BY-2) expressing apoaequorin exclusively in the cytosol (Takahashi *et al.,* 1997) was propagated as previously reported (Kamada and Muto, 1994). Briefly, the culture was maintained in Murashige-Skoog (MS) liquid medium (pH 5.8) containing 0.2 μg/ml of 2,4-dichlorophenoxy acetic acid at 23 °C with shaking on a gyratory shaker in the darkness and subcultured once a week with a 2% (v/v) inoculum. The cells harvested 5 d after subculturing were used for the experiments.

Chemicals

Chemically synthesized coelenterazine (Isobe *et al.,* 1994), a luminophore required for reconstitution of aequorin from apoaequorin, was a generous gift form Dr. M. Kuse and Prof. M. Isobe (Nagoya University). Other chemicals used here were of reagent level.

Treatments

REM salts were first dissolved in water. Onto the cell suspension in MS medium (200 μl), solutions of REMs (10 μl) were added 5 min prior to other treatments. Hypoosmotic shock was applied by addition of an equal volume of water to the culture media, which results in acute lowering of osmorality (−100 mOsmol). Oxidative stress was applied by adding 10 mM H_2O_2 (final fraction) to the culture.

Monitoring of $[Ca^{2+}]_c$

The changes in cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_c$) were monitored with the Ca^{2+}-dependent emission of blue light from aequorin as previously described (Kawano *et al.,* 1998). The active form of aequorin was reconstituted by addition of 1 μM coelenterazine to the suspension culture of apoaequorin-expressing tobacco cells, 8 h prior to the measurements of $[Ca^{2+}]_c$. The aequorin luminescence was measured using a CHEM-GLOW Photometer (American Instrument, Maryland, USA) equipped with a pen recorder and expressed as relative luminescence units (rlu).

Results

Inhibition of $[Ca^{2+}]_c$ increase induced by hypoosmotic shock

Addition of 15 REMs to the cell suspension resulted in inhibition of $[Ca^{2+}]_c$ increase induced by hypoosmotic shock (Fig. 1). Many of REMs showed inhibitory effect against the hypoosmolarity (−100 mOsmol)-dependent Ca^{2+} influx at 0.5 mM and 2.5 mM. It is noteworthy that the inhi-

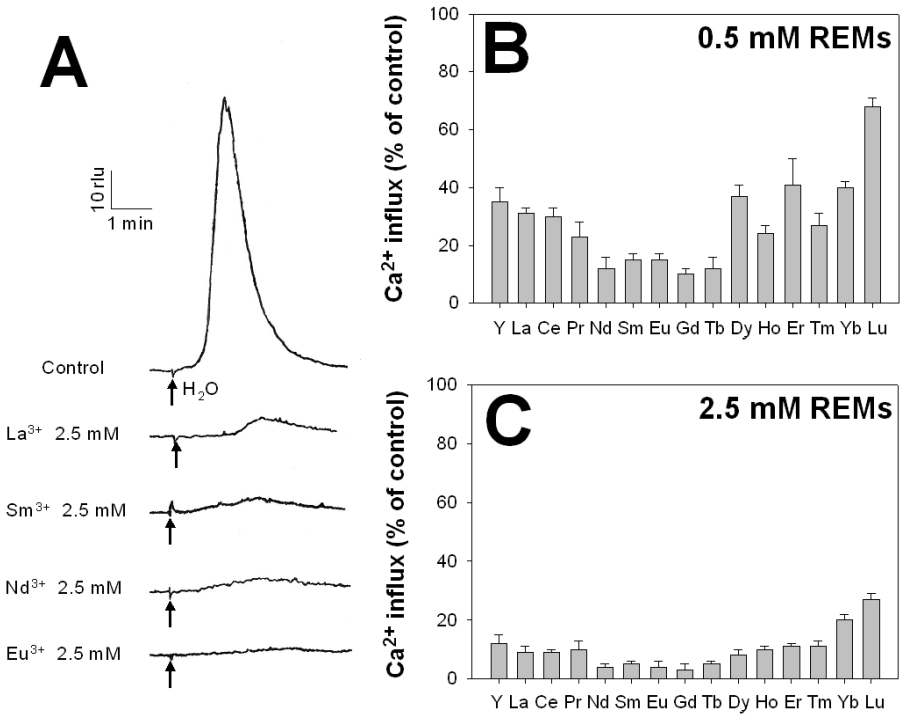

Fig. 1. Effects of REMs on Ca^{2+} influx induced by hypoosmotic shock. (A) Typical traces recorded for Ca^{2+} influx induced by hypoosmotic shock (−100 mOsmol). (B) Effect of 0.5 mM REMs. (C) Effect of 2.5 mM REMs. Bars, S.E. (n = 3); rlu, relative luminescence unit.

bition by Nd^{3+}, Sm^{3+}, Eu^{3+} and Tb^{3+} was much stronger than that by La^{3+}, the most well documented Ca^{2+} channel blocker. REMs with greater atomic numbers such as Lu^{3+}, Yb^{3+} and Er^{3+} were less active as Ca^{2+} channel blockers. For inhibition of Ca^{2+} influx by Lu^{3+}, much higher concentrations are required (data not shown). Since Lu is the most expensive metal among naturally existing elements on the earth, the use of such a precious element required in bulk is not economically appreciated.

Inhibition of $[Ca^{2+}]_c$ increase induced by H_2O_2

Effects of 15 REMs as Ca^{2+} channel blockers were also examined against the $[Ca^{2+}]_c$ increase induced by H_2O_2 (Fig. 2). Here, treatments with most REMs at 0.5 mM and 2.5 mM resulted in much greater inhibition compared to REM actions against hypoosmotic shock. This suggests the possibility that the channel stimulated by ROS (H_2O_2) is much more sensitive to REMs.

Ionic radii and channel inhibition

The relationship between ionic radii of REM ions and Ca^{2+}-channel inhibition was examined by plotting the REM ionic radii against the percentage of inhibition in Ca^{2+} influx. Plots were made by citing the data on ionic radii from Shannon (1976), assuming that both Ca^{2+} and trivalent REM ions are present as six-coordinated cations (Fig. 3). In case of H_2O_2-induced Ca^{2+} influx, Lu^{3+} (ionic radius 86.1 pm), Er^{3+} (ionic radius 89 pm), Yb^{3+} (ionic radius 86.8 pm), Y^{3+} (ionic radius 90 pm) and Tm^{3+} (ionic radius 88 pm) at 0.5 mM showed only weak inhibition of Ca^{2+} influx, while Gd^{3+}, Eu^{3+}, Sm^{3+}, Nd^{3+}, Pr^{3+}, and Ce^{3+} (ionic radii 93.8–101 pm) showed strong inhibition of Ca^{2+} influx (Fig. 3B). In general, the inhibitory effect of REMs increased as the ionic radii enlarged. It seems that REMs with the ionic radii closer to the ionic radius of Ca^{2+} (100 pm) are maximally active for performing the inhibitory effect. On the other hand, the optimal range of ionic radii for inhibition of hypoosmotically induced Ca^{2+} influx was between 92.3 and 98.3 pm (corresponding to Tb^{3+}, Gd^{3+}, Eu^{3+}, Sm^{3+}, Nd^{3+}), while REMs with much greater ionic radii (Pr^{3+}, Ce^{3+} and La^{3+}) were poorly effective (Fig. 3A).

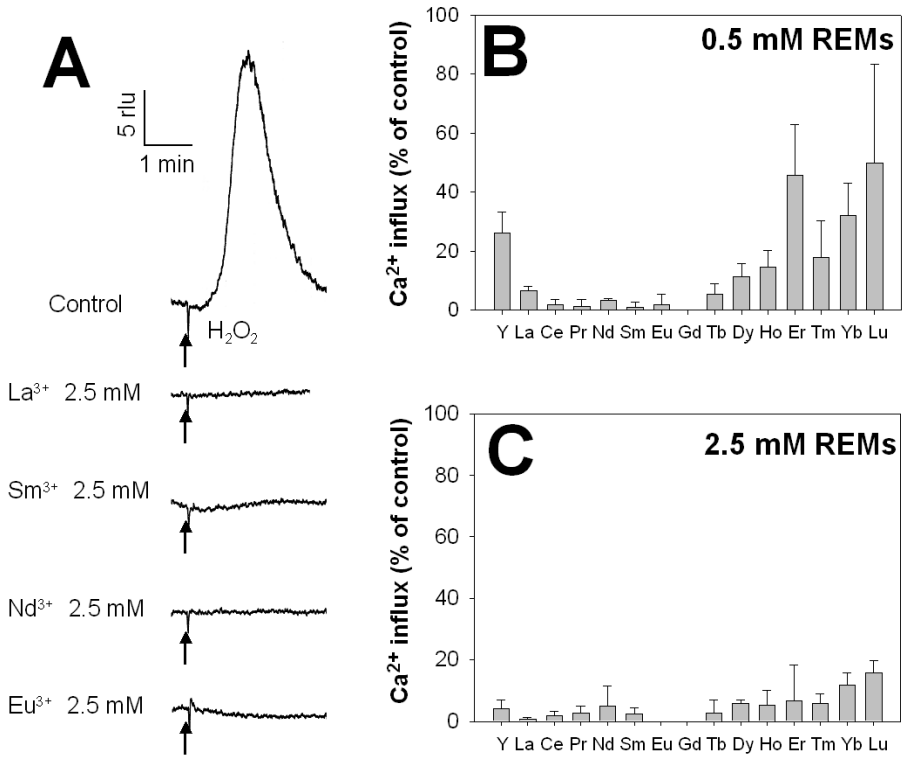

Fig. 2. Effects of REMs on Ca^{2+} influx induced by H_2O_2. (A) Typical traces recorded for Ca^{2+} influx induced by H_2O_2 (10 mM). (B) Effect of 0.5 mM REMs. (C) Effect of 2.5 mM REMs. Bars, S.E. ($n = 3$); rlu, relative luminescence unit

Discussion

In order to measure cytosolic Ca^{2+} levels non-invasively, the gene encoding apoaequorin, a protein found in the jellyfish *Aequorea victoria*, has been introduced into many plant species and cells (Knight, 2002). In the presence of coelenterazine, the functional protein aequorin is formed. When two of three highly specific Ca^{2+} binding domains of aequorin are filled, bound coelenterazine is oxidized to form coelenteramide and as a consequence blue light is emitted. This allowed us to measure the changes in $[Ca^{2+}]_c$ in tobacco cells and therefore the impacts of 15 REMs could be tested in the present study.

Previously, it has been shown that high concentration of Al^{3+} inhibits the influx of Ca^{2+} induced by ROS, but not by hypoosmotic shock, suggesting that Al^{3+} is a potent selective inhibitor of Ca^{2+} channels responsive to ROS (Kawano *et al.,* 2003). Moreover, our recent work demonstrated that TPC1-type plant Ca^{2+} channels are the only aluminum-sensitive Ca^{2+} channels responsible for such ROS-dependent influx of Ca^{2+} (Kawano *et al.,* 2004). However, the application of Al^{3+} for the study of Ca^{2+} signaling is limited since extremely high concentrations (5–10 mM) are required for inhibition of TPC1-type channels (Kawano *et al.,* 2004). In addition, effect on culture pH value due to application of $AlCl_3$ and the relatively lower efficiency of Al to be fully ionized at normal physiological pH must be considered. Therefore, the searches for other available tests are required.

This study investigated the effect of the 15 members of REMs as inhibitors of Ca^{2+} influx into the cytoplasm induced by H_2O_2 and hypoosmotic shock using tobacco cells. The results obtained from each element tested are still fragmental and not really informative, but as a whole, all REMs tested altogether tell us what is the desirable range of ionic radius for inhibition of the channels, and what is the possible mode of interaction between REMs and the channels. Since REMs tested here coordinately formed a sequential spectrum of ionic radii ranging from 86.1 to 103.2 pm, the optimal range of ionic radii required for blocking the flux of Ca^{2+} was determined for each stimulus. The H_2O_2-induced Ca^{2+} influx was optimally blocked by REMs with a broad spectrum of ionic radii

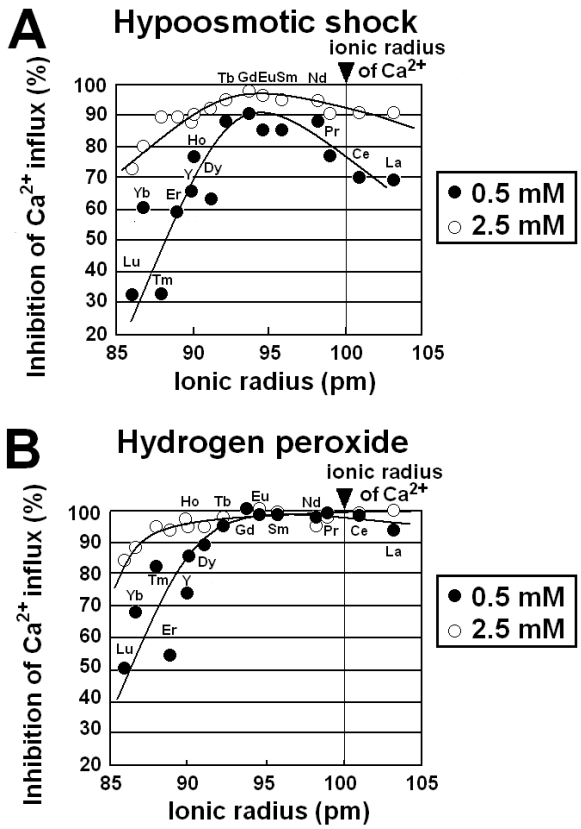

Fig. 3. Relationship between ionic radii of REMs and inhibition of Ca^{2+} influx. (A) Effect of REMs on hypoosmotic shock-induced Ca^{2+} influx. (B) Effect of REMs on H_2O_2-induced Ca^{2+} influx. Ionic radii shown here were of six-coordinated trivalent cations and Ca^{2+} (cited from Shannon, 1976). Each dot represents the corresponding ionic radius and the mean value of channel inhibition ($n = 3$).

(93.8–101 pm) which is similar to or slightly smaller than the ionic radius of Ca^{2+} (100 pm), while the hypoosmotically induced flux of Ca^{2+} was inhibited most effectively by the REMs with a narrower range of ionic radii around Gd^{3+} (93.8 pm). This implies the nature of Gd^{3+} well known as an inhibitor of stretch-activated (mechano-sensitive) calcium channels in mammals (Hamill and McBride, 1996) and as an inhibitor of hypotonic shock-gated channels in yeast cells (Batiza *et al.,* 1996; Kanzaki *et al.,* 1999). However, the selectivity of Gd^{3+} against stretch-activated channels is questioned due to its strong action against other types of channels (Lacampagne *et al.,* 1994; Caldwell *et al.,* 1998). Using the approach presented here, we can examine the ideal range of ionic radii for inhibition of the stretch-activated channels in various organisms.

Although one of the well recognized targets of REM action is the ion channels, the actual mode of REM action is not fully understood. The present data suggested that the modes of REM-dependent inhibition may differ between the two distinct channels responsible for two different stimuli. In case of H_2O_2-dependent Ca^{2+} influx, it is tempting to speculate that the similarity between the desired size of ionic radii and that of Ca^{2+} suggests the possible competition between Ca^{2+} and REM ions in interaction with or binding to the pore or some domains of the channels. In case of osmotic response, we hypothesize that the REM ions such as Gd^{3+}, with slightly smaller ionic radii compared to that of Ca^{2+}, may interfere with Ca^{2+} influx by reaching some specific sites on the channel pore deeper than Ca^{2+} can reach, or by binding to the membrane rather than or in addition to binding to the channel proteins.

To date, using patch-clamp techniques, some researchers have analyzed the blockade of Ca^{2+} channels by multiple REMs with regards to ionic radii of metals. Lansman (1990) studied the relationship between REM cationic radii and the mode of blockade of single calcium channels (measured with Ba^{2+} current) in cultured mouse skeletal muscle C2 cells, using six REM species (La, Ce, Nd, Gd, Dy and Yb). Accoding to above work, the kinetics of channel blockade by REMs seemed to follow a model in which the fluctuations of the single-channel current arose from the entry (blocking) and exit (unblocking) of occluding cations into/from the pore (Lansman, 1990). Blockade of Ca^{2+} current through T-type Ca^{2+} channels by eight REM ions was also performed in rat and human thyroid C cell lines (Mlinar and Enyeart, 1993). In addition, a recent report covered nine REMs and described their inhibitory effect on the Ba^{2+} current through α_{1G} T-type Ca^{2+} channels from human brain, which were cloned and transiently expressed in human embryonic kidney tsA-201 cells (Beedle *et al.,* 2002). These works on T-type Ca^{2+} channels have shown that the more increase in ionic radius, the less capability in blocking, with the extent of blockade as in the following orders: Y > Er > Gd > Ce > Ho > Yb > Nd > La (Beedle *et al.,* 2002); Ho > Y > Yb > Er > Gd > Nd > Ce > La (Mlinar and Enyeart, 1993). On the contrary, in L-type channels from mouse muscle

cells, the entry rates of blocking cations (the key step resulting in occlusion of the pore) decreased as cationic radius decreased (both entry rates and ionic radii, La > Ce > Nd > Gd > Dy > Yb), thus this is, in combination with other data, supporting the idea that the smaller the ions are, the slower they enter into the pore, presumably due to slow dehydration of such ions (Lansman, 1990). These studies suggest that the ionic species varying in ionic radii required for the maximal blockade may differ from channel to channel. Our present study with much higher resolution manifested with 15 REMs revealed that the results, especially the data on the blockade of H_2O_2-induced increase in $[Ca^{2+}]_c$, are similar to the data from animal L-type channels rather than T-type channels. Since the H_2O_2-responsive Ca^{2+} channels in tobacco cells may belong to the TPC1-type channels (Kawano *et al.,* 2004) which share structural similarity with animal L-type Ca^{2+} channels (Furuichi *et al.,* 2001), the observed similarity with animal L-type channels in preferred ionic size of REMs for channel blockade is likely due to structural similarity between the channels involved. In both cases (tobacco and mouse cells), the elements with ionic radius closest to that of competing cations, Ca^{2+} (100 pm) or Ba^{2+} (135 pm), were shown to be the most active blockers.

To date, interesting phenomena have been reported for the modes of lanthanide actions for and against transient receptor potential channels (TRPs). Mammalian members of the classical TRP subfamily (TRP1–7) are Ca^{2+}-permeable cation channels involved in receptor-mediated increase in intracellular Ca^{2+} (Jung *et al.,* 2003). Most TRP-related channels such as TRP3 (Kamouchi *et al.,* 1999; Zhu *et al.,* 1998), TRP6 (Inoue *et al.,* 2001), TRP7 (Okada *et al.,* 1999; Riccio *et al.,* 2002) are inhibited by La^{3+} and/or Gd^{3+}. However, according to Jung *et al.* (2003), the currents through TRP4 and TRP5 are largely potentiated by La^{3+} and Gd^{3+}. This may be the exceptional case of the Ca^{2+} channel-targeted actions of REMs. In addition, the report by Halaszovich *et al.* (2000) questioned the mode of lanthanide action against TRP3 since their data suggested that La^{3+} and Gd^{3+} can block the influx of Ca^{2+} from the cytosolic side of the plasma membrane in Chinese hamster ovary cells, suggesting that exogenously applied La^{3+} and Gd^{3+} enter the cells in concentrations relevant for block of TRP3 channels. Also in plants, similar evidence in support of La^{3+} action after uptake into the cells has been reported (Liu and Hasenstein, 2005). This may result in drastic changes in cytoskeletal organization and thus such changes possibly affect the plant response via stretch-activated channels likely connected to cytoskeletal components.

Although the modes of REM action need to be further investigated, the analysis with multiple REMs as performed here may be applicable for dissecting the involvement of different Ca^{2+} channels in different signaling mechanisms in plant cells.

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