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Aluminum as a specific inhibitor of plant *TPC1* Ca^{2+} channels☆

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Abstract

In plant cells, Al ion plays dual roles as an inducer and an inhibitor of Ca^{2+} influx depending on the concentration. Here, the effects of Al on Ca^{2+} signaling were assessed in tobacco BY-2 cells expressing aequorin and a putative plant Ca^{2+} channel from *Arabidopsis thaliana*, *AtTPC1* (two-pore channel 1). In wild-type cells (expressing only aequorin), Al treatment induced the generation of superoxide, and Ca^{2+} influx was secondarily induced by superoxide. Higher Al concentrations inhibited the Al-stimulated and superoxide-mediated Ca^{2+} influx, indicating that Ca^{2+} channels responsive to reactive oxygen species (ROS) are blocked by high concentration of Al. H_2O_2-induced Ca^{2+} influx was also inhibited by Al. Thus, inhibitory action of Al against ROS-induced Ca^{2+} influx was confirmed. Similarly, known Ca^{2+} channel blockers such as ions of La and Gd inhibited the H_2O_2-induced Ca^{2+} influx. While La also inhibited the hypoosmotically induced Ca^{2+} influx, Al showed no inhibitory effect against the hypoosmotic Ca^{2+} influx. The effects of Al and La on Ca^{2+} influx were also tested in the cell line overexpressing *AtTPC1* and the cell line *AtTPC1*-dependently cosuppressing the endogenous *TPC1* equivalents. Notably, responsiveness to H_2O_2 was lost in the cosuppression cell line, thus *TPC1* channels are required for ROS-responsive Ca^{2+} influx. Data also suggested that hypoosmotic shock induces *TPC1*-independent Ca^{2+} influx and Al shows no inhibitory action against the *TPC1*-independent event. In addition, *AtTPC1* overexpression resulted in a marked increase in Al-sensitive Ca^{2+} influx, indicating that *TPC1* channels participate in osmotic Ca^{2+} influx only when overexpressed. We concluded that members of *TPC1* channel family are the only ROS-responsive Ca^{2+} channels and are the possible targets of Al-dependent inhibition.

Keywords: Aequorin; Al; *AtTPC1*; Calcium signaling; Metal toxicity; *Nicotiana tabacum*

A number of studies have documented the toxic impacts of Al ions on roots [1–4], hypocotyls [5], and germinating pollens [6,7]. It has been proposed that early effects of Al toxicity at the root apex, such as those on cell division, cell extension or nutrient transport, involve the direct intervention of Al on cell function [8]. Cell suspension culture of tobacco (*Nicotiana tabacum*) has been frequently employed as a model system for the study of Al phytotoxicity [9–11]. Using tobacco BY-2 cells, we

☆ *Abbreviations:* $[Ca^{2+}]_c$, cytosolic free Ca^{2+} concentration; DAPI, 4,6-diamino-2-phenylindole; MS, Murashige–Skoog (culture medium); $O_2^{\bullet-}$, superoxide anion; rlu, relative luminescence units; ROS, reactive oxygen species.

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have previously examined the impacts of trivalent cations such as those of Al, La, and Gd [12–14]. We found that $LaCl_3$ and $GdCl_3$ added to tobacco cells trigger the generation of cytotoxic superoxide ($O_2^{\bullet-}$) [12,13]. Interestingly, $AlCl_3$ at normal physiological pH (5.8) triggers much greater production of $O_2^{\bullet-}$ in a dose-dependent manner (ca. 6 mM Al, optimal) [14]. Pharmacological experiments suggested that NADPH oxidase is involved in the cation-induced generation of $O_2^{\bullet-}$ [12–14].

Jones et al. [15] proposed a view that the phytotoxic action of Al in root hairs is not due to blockage of Ca^{2+} channels required for Ca^{2+} influx into the cytoplasm, based on the observation that Al treatment results in elevation of the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$) in root hairs of *Arabidopsis thaliana*. However, effects of Al on $[Ca^{2+}]_c$ homeostasis are not that simple.

In our previous work, we have examined the Al impact on $[Ca^{2+}]_c$ with the luminescence of recombinant aequorin overexpressed in the cytosol, and found that treatment of tobacco cells with sub-mM to mM levels of $AlCl_3$ results in an increase in $[Ca^{2+}]_c$ [14]. Following the spike of Al-induced $O_2^{\bullet-}$ generation, a gradual increase in $[Ca^{2+}]_c$ was shown. Compared to the $O_2^{\bullet-}$ spike, the secondarily induced change in $[Ca^{2+}]_c$ is a relatively slower event attaining the peak level spending ca. 1 min after the Al-induced $O_2^{\bullet-}$ generation. We have also showed that both $O_2^{\bullet-}$ scavengers and Ca^{2+} chelators significantly lower the level of Al-induced $[Ca^{2+}]_c$ elevation, confirming that the Al-induced $O_2^{\bullet-}$ in turn stimulates the influx of Ca^{2+} across the plasma membrane [14]. Since the $[Ca^{2+}]_c$ response is saturated at relatively lower Al concentrations compared to the induction of $O_2^{\bullet-}$ generation, we have hypothesized that the Al concentration optimal for $O_2^{\bullet-}$ is too much (inhibitory) for $[Ca^{2+}]_c$ response, suggesting that Al at high concentration behaves as an inhibitor of Ca^{2+} influx [14], despite the view of Jones et al. [15]. Supporting our view, inhibitory action of high Al concentration against Ca^{2+} influx was confirmed also in the H_2O_2-treated tobacco cells [14].

Hypoosmotic shock is known to induce an immediate increase in $[Ca^{2+}]_c$ in tobacco cells by stimulating the influx of Ca^{2+} through plasma membrane-localized Ca^{2+} channel(s) [16,17]. While non-selective channel blockers such as La^{3+} and Gd^{3+} [18] effectively inhibit the Ca^{2+} influx induced by both hypoosmotic shock [16] and oxidative stress [19,20], Al (up to 10 mM) showed no inhibitory effect on the hypoosmolarity-induced Ca^{2+} influx [14], suggesting that Al is a novel type of selective inhibitor acting specifically against the redox-responsive Ca^{2+} channel, assuming that two types of Ca^{2+} channels differed in sensitivity to Al may be present in tobacco cells [14].

Recently, Furuichi et al. [21] have cloned the plant's first gene encoding a voltage-gated channel with high affinity for Ca^{2+} permeation, from *A. thaliana*. The channel possesses extremely high homology with a recently cloned *TPC1* (two pore channel 1) from rats [22], thus designated as *AtTPC1*.

It has been shown that *AtTPC1* is expressed in various tissues in *Arabidopsis*. Sense-antisense experiments in *Arabidopsis* and complementation tests in a Ca^{2+} uptake-defective yeast mutant have confirmed that *AtTPC1* functions as a Ca^{2+} channel [21]. According to Kadota et al. [23], tobacco BY-2 cells possess two orthologs of *AtTPC1* highly identical to *AtTPC1* which are designated as *NtTPC1A* and *B*, most likely behaving as elicitor-responsive Ca^{2+} channels.

In this study, we conducted a series of overexpression and cosuppression experiments using transgenic cell lines transformed with the *AtTPC1*-encoding vector construct. Here, we report that the plant's first voltage-dependent Ca^{2+} channel, *AtTPC1*, is the Al-sensitive ROS-responsive Ca^{2+} channel in plants.

Experimental procedures

Cell culture. Tobacco (*Nicotiana tabacum* L. cv. Bright Yellow-2) suspension-cultured cells (cell line, BY-2) expressing apoaequoporin exclusively in the cytosol [16] were further exposed to *Agrobacterium*-based transformation [24] using binary vectors encoding the green fluorescence protein (GFP)-tagged *AtTPC1* gene [21]. Following transformation, the cell lines with *AtTPC1*-overexpression and *AtTPC1*-dependent cosuppression (of tobacco *TPC1* equivalents) were screened by monitoring the level of *AtTPC1* expression with immunoblotting using *AtTPC1A*-specific antibody as described [23]. The cell lines derived from the aequorin expressing BY-2 cells, used in this study, were termed as "wild-type" (expressing only aequorin), "overexpression" (expressing both aequorin and *AtTPC1*), and "cosuppression" (expressing aequorin but endogenous *TPC1* expression cosuppressed). These cell lines were propagated as usual. Briefly, the cultures were maintained in Murashige–Skoog (MS) liquid medium (pH 5.8) containing 0.2 μg/ml of 2,4-dichlorophenoxyacetic acid at 23 °C with shaking on a gyratory shaker in darkness and subcultured once a week with a 4% (v/v) inoculum. The cells harvested 3 days after subculturing were used for experiments.

Chemicals. Coelenterazine used for reconstitution of aequorin was chemically synthesized as reported previously [25]. Other chemicals used in this study were of reagent grade.

Treatments. $AlCl_3$ or $LaCl_3$ were first dissolved in water and diluted with the same volume of 2× MS medium supplemented with 40 mM K-phosphate buffer (pH 5.8). To compare the effect of metal salts at the physiologically normal pH, the tobacco cells were suspended in the MS medium supplemented with K-phosphate buffer (pH 5.8) and incubated for at least for half an hour prior to addition of metal salts. Then the cell suspension (0.2 ml) was added with solutions (0.2 ml) of $AlCl_3$ or $LaCl_3$. Hypoosmotic shock (ca. 100 milli-osmol difference) was applied by adding 0.2 ml of water to 0.2 ml of culture. Oxidative stress was applied by adding 1 mM H_2O_2 (final conc.) to the culture.

Monitoring of $[Ca^{2+}]_c$. The changes in $[Ca^{2+}]_c$ were monitored by the Ca^{2+}-dependent emission of blue light from aequorin as previously described [20]. 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, MD, USA) equipped with a pen recorder, and expressed as relative luminescence units (rlu) as previously described [20].

After each measurement, all remaining aequorin was discharged with 1 M $CaCl_2$ and 10% ethanol, and the resultant luminescence was measured to estimate the amount of remaining aequorin. $[Ca^{2+}]_c$ was calculated using the equation: $pCa = 0.332558(-\log k) + 5.5593$, where k is a rate constant equal to luminescence counts per second divided by total counts. The equation was proposed for the plant use by Knight et al. [26] and applied to the tobacco BY-2 cells by Takahashi et al. [16].

Detection of ROS by chemiluminescence. Generation of $O_2^{\bullet-}$ in cell suspension culture was monitored by chemiluminescence of *Cypridina* luciferin analog as described previously [19]. The $O_2^{\bullet-}$-specific chemiluminescence was measured using the same equipment described for measurement of aequorin luminescence and expressed as "rlu."

Fluorescence microscopy. For detection of GFP-tagged *AtTPC1* protein expressed in tobacco cells, the green fluorescence was determined with fluorescence microscopy with an *Optiphoto* microscope (Nicon, Tokyo, Japan) equipped with a CCD camera (C4742-95-12R, Hamamatsu Photonics, Hamamatsu, Japan). For comparison, localization of nuclei in the cells was also examined by staining the cells with a fluorescent reagent, 4,6-diamino-2-phenylindole (DAPI), as described [27].

Results

ROS-responsive Ca^{2+} channel may be a possible target of Al-dependent inhibition

As demonstrated previously [14], Al treatment of tobacco BY-2 cells resulted in the rapid generation of superoxide (Fig. 1A). Then Ca^{2+} influx was secondarily induced (Fig. 1B). This is due to superoxide-dependent stimulation as previously reported [14]. Based on the equation proposed by Knight et al. [26], the maximal $[Ca^{2+}]_c$ manifested in the presence of optimal concentration of $AlCl_3$ (1.25 mM) was estimated to be 340 nM. Al concentrations higher than 5 mM inhibited the Al-stimulated (superoxide-mediated) Ca^{2+} influx (Fig. 1B). H_2O_2-induced Ca^{2+} influx was also inhibited by Al, indicating that Ca^{2+} channels responsive to reactive oxygen species (ROS) are blocked (Fig. 1C). Similarly, La^{3+}, a known Ca^{2+} channel blocker, inhibited the H_2O_2-induced Ca^{2+} influx (Fig. 1C). La^{3+} also inhibited the hypoosmotically induced Ca^{2+} influx, but treatment with Al resulted in no significant decrease in the hypoosmotically induced Ca^{2+} influx (Fig. 1D). Only effect resulted from Al addition observed here was slight delay of the peak, but influx of Ca^{2+} was not inhibited at all. Therefore, unlike many of nonselective channel blocking lanthanides such as La and Gd, Al may be a novel channel blocker selectively inhibiting the ROS-responsive Ca^{2+} channels as illustrated in Fig. 1E.

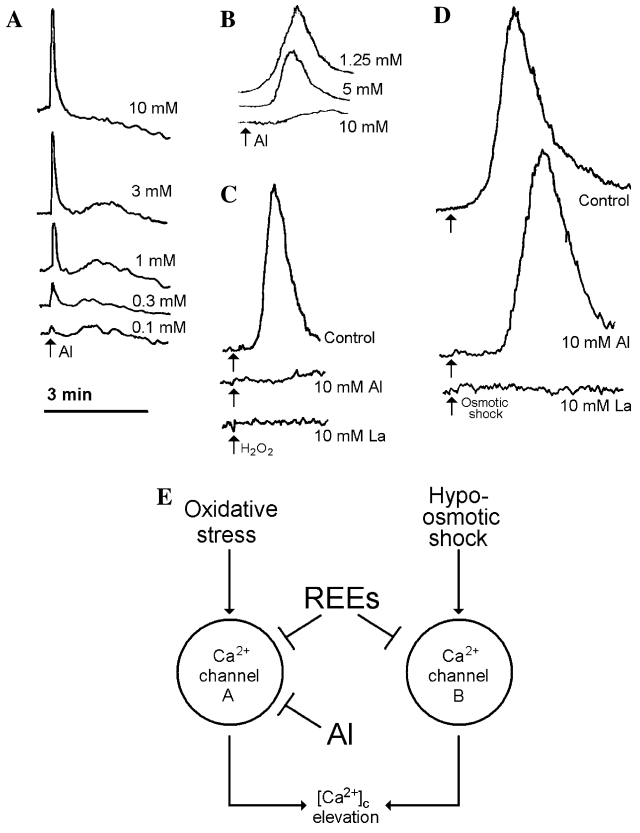

Fig. 1. $AlCl_3$-induced oxidative burst and Ca^{2+} influx in tobacco BY-2 wild-type cells. (A) Induction of $O_2^{\bullet-}$ generation by addition of Al to the culture. Note that there is a good dose-dependency. (B) Al-induced increase in $[Ca^{2+}]_c$. Note that higher Al concentration is inhibitory. (C) Inhibition of H_2O_2-induced increase in $[Ca^{2+}]_c$ by Al and La. (D) Different effects of Al and La against hypoosmotically induced increase in $[Ca^{2+}]_c$. (E) A model for explaining the selective action of Al. There may be two distinct groups of Ca^{2+} channels that differed in sensitivity to Al. The Al-sensitive group may correspond to the ROS-responsive channels. The Al-insensitive group may participate in the hypoosmotic response. Production of $O_2^{\bullet-}$ was monitored with $O_2^{\bullet-}$-specific chemiluminescence of *Cypridina* luciferin analog (A). The increase in $[Ca^{2+}]_c$ was monitored with aequorin luminescence (B–D). Arrows (A–D) indicate the time points of indicated treatments. Al or La (10 mM, each) was added to the culture 5 min prior to addition of H_2O_2 (C) or hypoosmotic treatment (D). Horizontal bar, 3 min. REEs, rare earth elements.

Localization of AtTPC1 overexpressed in BY-2 cells

Before examining the effect of *AtTPC1* overexpression on the Ca^{2+} influx, actual expression and localization of *AtTPC1*-GFP fusion protein in *AtTPC1* overexpressing cells were confirmed by visualizing the plasma membrane-localized fluorescence of GFP (Fig. 2). In the wild-type and cosuppression cell lines, the GFP-dependent fluorescence was not detected. Overexpression of *AtTPC1* and its cosuppression events were previously examined by immuno-blotting analysis using GFP-targeted antibody [23].

Hypoosmotic shock induces TPC1-independent Ca^{2+} influx

The extents of $[Ca^{2+}]_c$ increases in Ca^{2+} channel-modified cell lines (overexpression and cosuppression), induced by hypoosmotic shock and H_2O_2 in the presence and absence of $AlCl_3$ and $LaCl_3$, were compared with that in wild-type cell line (Fig. 3). For enabling

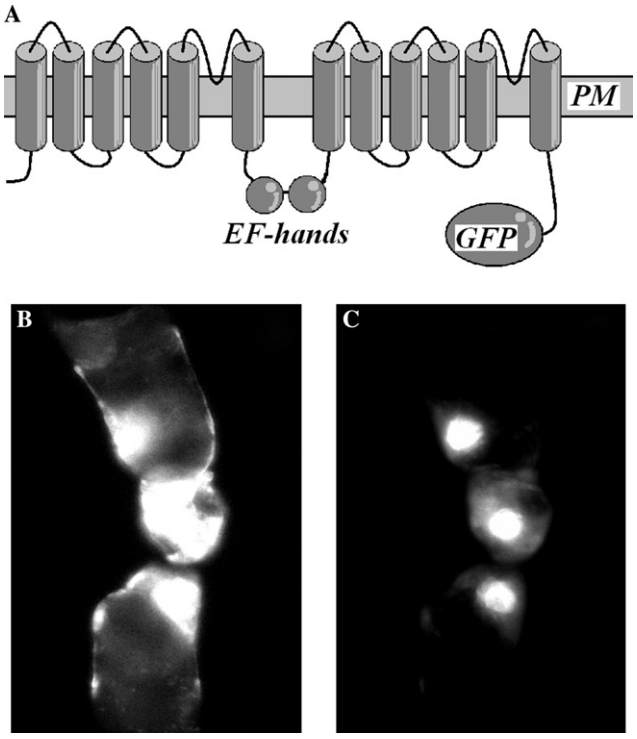

Fig. 2. Expression and localization of GFP-fused *AtTPC1* in BY-2 cells. (A) Likely structure of GFP-fused *AtTPC1* protein embedded in plasma membrane. (B) Localization of GFP fluorescence at cell peripheral. (C) Staining of nuclei with DAPI. PM, plasma membrane.

the comparison of Ca^{2+} influx potency in different cell lines, the Al-insensitive increase in $[Ca^{2+}]_c$ inducible by hypoosmotic shock was expressed as 100% rlu, since Al (up to10 mM) [14] is almost inert to the hypoosmotically induced Ca^{2+} influx in the wild-type cells.

The osmotically induced Ca^{2+} influx in the absence of Al was only slightly greater than that in Al-pretreated cells in wild-type cell line, thus no significant inhibition was observed while La showed strong inhibition (ca. 97% inhibited). Inhibition of H_2O_2-induced influx of Ca^{2+} by 10 mM Al and La was ca. 94% and 98%, respectively (Fig. 3A, right). This suggests that ROS-induced response is sensitive to both Al and La ions.

In the *TPC1*-cosuppressed cell line, hypoosmotic $[Ca^{2+}]_c$ increase was equally induced both in the presence and absence of 10 mM $AlCl_3$ (Fig. 3B, left-1, 2), clearly indicating that *TPC1* channels are not required for mechanical induction of Ca^{2+} influx by hypoosmotic treatment, and that Al has nothing to do with this *TPC1* channel-independent event. In contrast, La effectively inhibited the *TPC1*-independently occurring hypoosmotic induction of Ca^{2+} influx by 96% (Fig. 3B, left-3).

TPC1 channel cosuppression lowers H_2O_2-responsive Ca^{2+} influx

On the other hand, we observed that *TPC1* cosuppression resulted in drastically lowered responsiveness

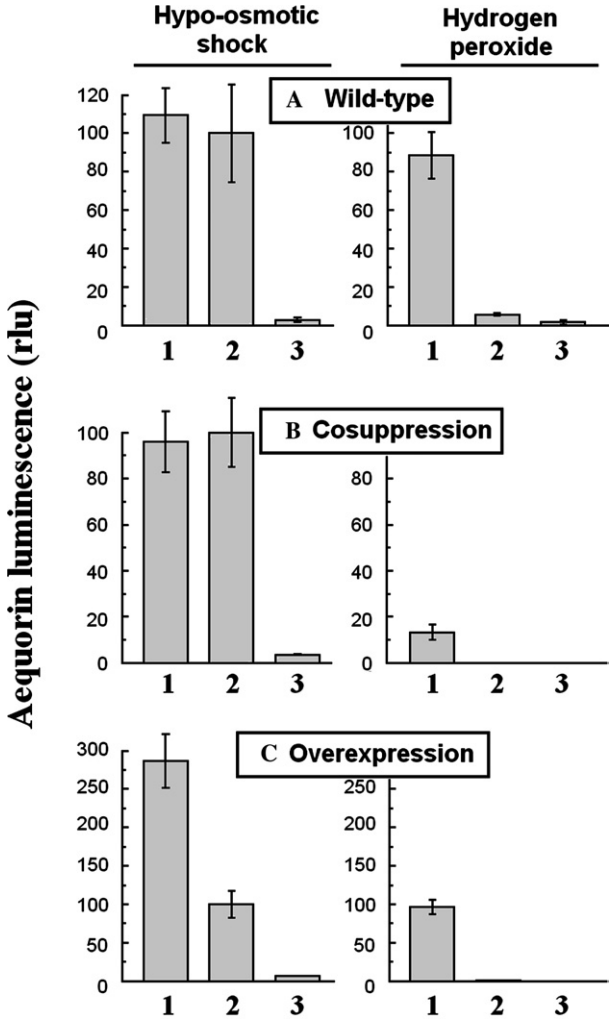

Fig. 3. Inhibitory actions of Al and La against Ca^{2+} influx in three cell lines of tobacco BY-2 cells. Tobacco cells were exposed to hypoosmotic shock (−100 milli-osmol) (left) or 1 mM hydrogen peroxide (right). (A) Wild-type. (B) *AtTPC1*-dependently cosuppressed cell line. (C) *AtTPC1*-overexpressing cell line. (1) Control. (2) 10 mM $AlCl_3$. (3) 10 mM $LaCl_3$. rlu, relative luminescence unit. Comparison was made by expressing Al-insensitive aequorin luminescence peak height manifested after hypoosmotic treatment as 100 rlu.

to H_2O_2 attaining only 13% of Al-independently inducible increase in aequorin luminescence (Fig. 3B, right-1) while wild-type cells were able to induce much greater level of Ca^{2+} influx which was almost 90% of Al-insensitive osmotic $[Ca^{2+}]_c$ increase (Fig. 3A, right-1). It is conclusive that members of *TPC1* channels are the only major Ca^{2+}-permeable channels responsive to oxidative stress, since major portion of Ca^{2+} influx potency was lost by cosuppression of *TPC1* channels. In the presence of either Al or La, the H_2O_2-dependent increase in $[Ca^{2+}]_c$ was no longer observed.

AtTPC1 overexpression increases Al-sensitive Ca^{2+} influx

Although Al-sensitive Ca^{2+}-permeable channel does not participate in the hypoosmotically induced $[Ca^{2+}]_c$

increase in the wild-type cell line, the overexpression of *AtTPC1* in BY-2 cells resulted in a marked enhancement in induction of Ca^{2+} influx after hypoosmotic shock (Fig. 3C, left). Interestingly, such enhancement of Ca^{2+} influx by *AtTPC1* overexpression was shown to be highly sensitive to Al, further confirming that *TPC1* channels are the Al-sensitive Ca^{2+}-permeable ion channels. In the overexpression cell line, H_2O_2-induced Ca^{2+} influx observed was as great (ca. 97%) as the Al-insensitive osmotically induced Ca^{2+} influx, and was again, highly sensitive to Al (99% inhibition) (Fig. 3C, right-1, 2). La inhibited both the hypoosmotic and oxidative induction of Ca^{2+} influx by 94% and 100%, respectively, as expected (Fig. 3C, left-3 and right-3).

Discussion

Kawano et al. [14] have reported that the status of Al-induced activation and inhibition of Ca^{2+} fluxes into tobacco cells are highly dependent on the concentration of treated Al. It has been shown that wide range of Al stimulates the production of $O_2^{\bullet-}$ in tobacco cells and the radical species are likely stimulating the opening of Ca^{2+} channels. However, in the presence of high Al, the $O_2^{\bullet-}$-responsive Ca^{2+} uptake was completely inhibited. Other ROS, such as H_2O_2 also stimulated the Ca^{2+} influx and the H_2O_2-induced Ca^{2+} influx was also inhibited in the presence of high concentration of Al. The inhibitory action of Al against Ca^{2+} influx was limited only to the responses to ROS. The Ca^{2+} influx induced by physical stimuli such as hypoosmotic shock rather than ROS was insensitive to Al treatment.

It has been shown that hyperpolarization-activated Ca^{2+} channels at the tip of *Arabidopsis* root hairs [28], and those found in the cells in the elongating zones in *Arabidopsis* roots [29], are susceptible to both La^{3+} and Al^{3+}. Very and Sentenac [30] have predicted in their review article that the Al-sensitive Ca^{2+} channels may be either of channels belonging to *CNGC*, *GLR* or *TPC1* family. The evidences presented here are strongly indicative that Ca^{2+} channels belonging to *TPC1* channel family are the ROS-responsive Ca^{2+} channels and are the possible targets of Al-dependent inhibition.

$[Ca^{2+}]_c$ distortion and Al toxicity

Physiological roles for the $[Ca^{2+}]_c$ increase and its inhibition in Al toxicity have not been well documented at present. It is generally believed that disturbance of $[Ca^{2+}]_c$ homeostasis is one of the primary triggers of Al toxicity [31–33]. Recent studies conducted by Rengel and his colleagues [34,35] have provided fluorescence probe-based evidence showing a correlation between the Al-induced increase in $[Ca^{2+}]_c$ and inhibition of root growth. Our previous work also revealed that Al induces a transient increase in $[Ca^{2+}]_c$ in tobacco BY-2 cells [14]. Controversially, it has been reported that Al-treatment inhibits the Ca^{2+} absorption by root apices of Al-sensitive, but not of Al-resistant, wheat seedlings [36–38]. Further puzzlingly, it has been reported that the $[Ca^{2+}]_c$ elevation induced by Al is observable in the root hairs of Al-sensitive but not of Al-resistant *Arabidopsis* mutants, and no tight correlation between $[Ca^{2+}]_c$ level and root hair growth inhibition has been obtained [39]. It is thus still controversial if Al disruption of Ca^{2+} transport may play an important role in the mechanisms of Al rhizo-toxicity in Al-sensitive plants. Since now it is obvious that the Al-sensitive Ca^{2+} channels in plants are *TPC1* channels and their roles in ROS-response is indicated, we have to examine the roles of *TPC1* channels in Al toxicity in growing plants, and the relationship between ROS stress and Ca^{2+} signaling during Al toxicity development must be reexamined.

Lastly, we propose the use of Al, in addition to lanthanides, as a strong tool to study the role of Ca^{2+} channels, since Al may be a novel channel blocker specific for ROS-responsive Ca^{2+} channels and such a selective channel blocker has not been reported to date.

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