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Aluminum-induced distortion in calcium signaling involving oxidative bursts and channel regulation in tobacco BY-2 cells☆

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Abstract

Trivalent cations such as those of Al, La, and Gd are phytotoxic. Our previous works showed that addition of $LaCl_3$ or $GdCl_3$ to tobacco cells triggers the generation of superoxide ($O_2^{\cdot-}$). Here, we show that $AlCl_3$ at normal physiological pH (5.8) induces much greater production of $O_2^{\cdot-}$ (detected with a specific chemiluminescence probe), indicating that these trivalent cations similarly induce the oxidative bursts. It was shown that NADPH oxidase is involved in the generation of $O_2^{\cdot-}$ and the yield of $O_2^{\cdot-}$ was dose-dependent (ca. 6 mM Al, optimal). Following the acute spike of $O_2^{\cdot-}$, a gradual increase in cytosolic-free Ca^{2+} concentration ($[Ca^{2+}]_c$) was detected with the luminescence of recombinant aequorin over-expressed in the cytosol. Interestingly, a $O_2^{\cdot-}$ scavenger and a Ca^{2+} chelator significantly lowered the level of $[Ca^{2+}]_c$ increase, indicating that the Al-induced $O_2^{\cdot-}$ stimulates the influx of Ca^{2+}. Compared to the induction of $O_2^{\cdot-}$ generation, the $[Ca^{2+}]_c$ elevation was shown to be maximal (340 nM) at relatively lower Al concentrations (ca. 1.25 mM). Thus, the Al concentration optimal for $O_2^{\cdot-}$ is too much (inhibitory) for $[Ca^{2+}]_c$. In addition, high concentrations of Al were shown to be inhibitory to the H_2O_2-induced Ca^{2+} influx. This explains the ineffectiveness of high Al concentration in the oxidative burst-mediated induction of $[Ca^{2+}]_c$ increase. It is likely that Al-induced $[Ca^{2+}]_c$ elevation is manifested from the finely geared balance between the $O_2^{\cdot-}$-mediated driving force and the channel inhibition-mediated brake. Furthermore, it is note-worthy that Al ($\leqslant$10 mM) showed no inhibitory effect on the hypo-osmolarity-induced Ca^{2+} influx, implying that Al may be a selective inhibitor of redox-responsive Ca^{2+} channels. Possible target channels of Al actions are discussed.

Keywords: Aequorin; Al; Ca; Metal toxicity; Oxidative burst; *Nicotiana tabacum*; Superoxide

Al ions are very toxic to plants, and number of studies documented the toxic impact 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].

In this study, we examine the impact of Al ions in tobacco (*Nicotiana tabacum*) BY-2 cells, since tobacco cell suspension culture has been frequently employed as a model system for the study of Al phytotoxicity [9–11].

Ions of lanthanides are also rhizotoxic and behave similarly to Al ions in binding to the negatively charged surface of plasma membrane in plant roots [12]. Recently, we have demonstrated that treatments of tobacco cell suspension culture with various salts of lanthanides (La and Gd), alkali earth metals (Mg and Ca) or alkali metals (Li, Na, and K) result in an immediate burst in production of reactive oxygen species (ROS), especially

☆ *Abbreviations:* ABA, abscisic acid; $[Ca^{2+}]_c$, cytosolic-free Ca^{2+} concentration; CLA, cypridina luciferin analog; DPI, diphenyleneiodonium chloride; EGTA, *O*,*O*′-bis(2-aminoethyl)ethyleneglycol-*N*,*N*,*N*′,*N*′-tetraacetic acid; HO·, hydroxyl radicals; MS, Murashige–Skoog (culture medium); $O_2^{\cdot-}$, superoxide anion; SOD, CuZn-superoxide dismutase; Tiron, 4,4-dehydroxy-1,3-benzene disulfonic acid disodium salt; rlu, relative luminescence units; ROS, reactive oxygen species.

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superoxide anion ($O_2^{\cdot-}$), via activation of NADPH oxidase [13–15]. Among the cations tested, La^{3+} and Gd^{3+} induced the greatest responses in $O_2^{\cdot-}$ production, while Ca^{2+} and Mg^{2+} showed moderate effects, and K^+ and Na^+ induced much lower responses, indicating that there is a tight relationship between the valence of cations and the level of $O_2^{\cdot-}$ production. In addition, activities of lanthanide trivalent cations on $O_2^{\cdot-}$ production were maximal at sub-mM to mM cations, while di- and monovalent ions require sub-M and M cations for maximal responses, respectively. Therefore, among the elements belonging to 1A, 2A, and 3B groups, cations with higher valency are extremely active in induction of $O_2^{\cdot-}$ production at minimal concentrations [13]. However, cations of other transition metals showed no detectable activity in induction of NADPH oxidase-dependent generation of $O_2^{\cdot-}$ in tobacco cells [16], although many of transition metal ions other than lanthanide ions, such as Cu^+ and Fe^{2+} are known to be strong prooxidants catalyzing the Fenton-type reactions yielding hydroxyl radicals ($HO^{\cdot}$), the most reactive ROS [17], thus inducing severe damages to plants [18]. It is likely that those metal cations behave differently in induction of ROS production. By analogy to lanthanide actions, it is tempting to test whether another rhizotoxic trivalent cation, Al^{3+}, has any impact on $O_2^{\cdot-}$ production in plants. Since Al^{3+} can be commonly found in plant-surrounding environments, especially in acidic soils, its phyto-toxic mechanism must be clarified. Here, we report the effect of $AlCl_3$ on the production of $O_2^{\cdot-}$ in tobacco cell suspension culture. In addition, impact of $AlCl_3$ treatment on the cytosolic-free calcium concentration ($[Ca^{2+}]_c$) in tobacco cells was also examined.

Experimental procedures

Cell culture. Tobacco (*Nicotiana tabacum* L. cv. Bright Yellow-2) suspension-cultured cells (cell line, BY-2) expressing apoaequporin exclusively in the cytosol [19] were propagated as previously described [20]. Briefly, the culture was maintained in Murashige–Skoog (MS) liquid medium (pH 5.8) containing 0.2 μg/ml of 2,4-dichlorophenoxyacetic acid at 28 °C with shaking on a gyratory shaker in darkness and subcultured once a week with a 4% (v/v) inoculum. The cells were harvested 3 days after subculturing, washed with, and resuspended in fresh MS medium supplemented with 20 mM K-phosphate buffer (pH 5.8) containing 10 μM CLA, and used for experiments after 2 h of resting incubation in dark. $AlCl_3$ or $LaCl_3$ was 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 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 $LaCl_3$.

Chemicals. A *Cypridina* luciferin analog (CLA) (2-methyl-6-phenyl-3,7-dihydroimidazol[1,2-a]pyrazin-3-one) and Tiron (4,5-dehydroxy-1,3-benzene disulfonic acid, disodium salt) were purchased from Tokyo Kasei Kogyo (Tokyo, Japan). A membrane-impermeable calcium chelator, *O*,*O*′-bis(2-aminoethyl)ethyleneglycol-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) was purchased from Wako Pure Chemical Industries (Osaka, Japan). Cu,Zn-superoxide dismutase (SOD) and all other reagents were from Sigma (St. Louis, MO). Chemically synthesized coelenterazine was a gift from Prof. M. Isobe (Nagoya University).

Detection of ROS by chemiluminescence. Generation of $O_2^{\cdot-}$ in cell suspension culture was monitored by chemiluminescence of CLA with a CHEM-GLOW Photometer (American Instrument, Maryland, USA) equipped with a pen recorder and expressed as relative luminescence units (rlu) as previously described [21]. CLA-chemiluminescence specifically indicates the generation of $O_2^{\cdot-}$ (and O_2^1 with a lesser extent) but not that of O_3, H_2O_2, or $HO^{\cdot}$ [22].

Monitoring of $[Ca^{2+}]_c$. The changes in $[Ca^{2+}]_c$ were monitored by the Ca^{2+}-dependent emission of blue light from the Ca^{2+}-sensitive luminescent protein, aequorin as described previously [21]. 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 the same equipment described for the measurement of CLA-chemiluminescence and expressed as "rlu." 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 plant use by Knight et al. [23] and applied to the tobacco BY-2 cells by Takahashi et al. [19].

Results and discussion

Induction of $O_2^{\cdot-}$ generation in tobacco cell suspension by Al treatment

To study the effect of Al on production of $O_2^{\cdot-}$, increases in the chemiluminescence of CLA specifically reflecting the generation of $O_2^{\cdot-}$ were measured after tobacco cell suspension culture was treated with $AlCl_3$. Addition of $AlCl_3$ dissolved in K-phosphate-buffered MS culture medium (0.2 ml, pH 5.8) to tobacco cell suspension culture (0.2 ml) resulted in transient production of $O_2^{\cdot-}$ that reaches to the maximal level immediately after Al treatment (Fig. 1A). The Al-induced generation of $O_2^{\cdot-}$ was shown to occur in a dose-dependent manner and the optimal $AlCl_3$ concentration was 6.25 mM (Fig. 1B). $AlCl_3$ concentrations higher than at 6.25 mM were less effective. Effects of La and Gd on generation of $O_2^{\cdot-}$ were also examined for comparison using the same batch of cell culture. The La- and Gd-induced production of $O_2^{\cdot-}$ was maximal at 1.25 mM, apparently lower than the optimal concentration of $AlCl_3$. Interestingly, the maximal production of $O_2^{\cdot-}$ induced by 6.25 mM $AlCl_3$ (optimal concentration) was ca. 60% greater than that induced by $LaCl_3$ (1.25 mM).

Effects of ROS scavengers and an inhibitor of NADPH oxidase

As it has been suggested that NADPH oxidase is involved in the $O_2^{\cdot-}$ production activated by divalent and

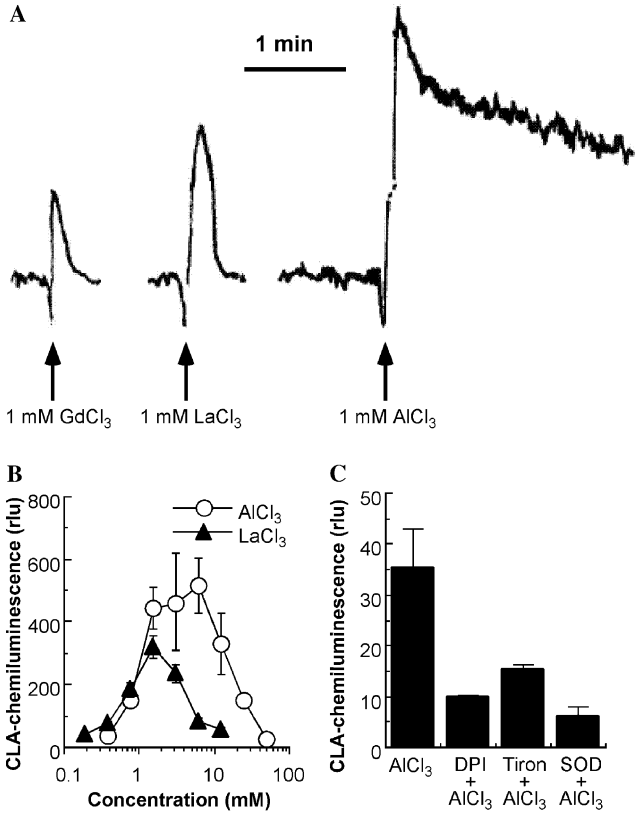

Fig. 1. Induction of $O_2^{\cdot-}$ generation in tobacco cell suspension culture by $AlCl_3$. (A) Typical traces of metal cation-induced increase in CLA-chemiluminescence reflecting the production of $O_2^{\cdot-}$ in tobacco cells. (B) Effect of increasing concentration of $AlCl_3$ and $LaCl_3$ on induction of $O_2^{\cdot-}$ generation. (C) Inhibition of $AlCl_3$-induced $O_2^{\cdot-}$ generation by ROS scavengers and an inhibitor of NADPH oxidase. Tobacco cells were pre-incubated with control media, 100 μM diphenyleneiodonium chloride (DPI), 330 U/ml SOD, or 5 mM Tiron for 5 min before addition of 6.25 mM $AlCl_3$. rlu stands for relative luminescence units.

trivalent cations [13], it is tempting to speculate that NADPH oxidase is also activated by Al ions. To clarify the nature of $O_2^{\cdot-}$-generating system activated by Al, the cell suspension culture was treated with an NADPH oxidase inhibitor, diphenyleneiodonium chloride (DPI, 100 μM), or two $O_2^{\cdot-}$ scavengers, SOD (330 U/cm^3) and Tiron (5 mM), 5 min prior to addition of 6.25 μM $AlCl_3$ (Fig. 1C). The Al-induced $O_2^{\cdot-}$ production was effectively inhibited by SOD, Tiron, and DPI, confirming that the observed chemiluminescence surely reflects the generation of $O_2^{\cdot-}$ (see the effects of Tiron and SOD) and that the $O_2^{\cdot-}$-generating mechanism involves NADPH oxidase (see the effect of DPI), similarly to $O_2^{\cdot-}$ production induced by other trivalent cations such as La^{3+} and Gd^{3+} [13]. Since SOD is a macro-molecule that does not readily penetrate across the plasma membrane, the observed effect of exogenously applied SOD strongly suggests that $O_2^{\cdot-}$ is released in the extracellular space. Similar phenomenon has been reported for human neutrophil NADPH oxidase in which binding of divalent cations such as Ca^{2+} or Mg^{2+} results in spontaneous activation of the $O_2^{\cdot-}$-releasing activity of the membrane bound enzyme [24,25]. In addition, it is also similar to plants that cations with higher valence are reportedly more active in enhancement of $O_2^{\cdot-}$-producing activity of NADPH oxidase from human neutrophils [24]. Taken together, it is likely that the cation-activated $O_2^{\cdot-}$-generating systems are widely conserved in both mammals and plants. Thus, the impact of Al ions on the $O_2^{\cdot-}$-generating activity in neutrophils must be examined.

Induction of a transient increase in $[Ca^{2+}]_c$

In addition to the Al-induced oxidative burst, we examined the Al impact on the $[Ca^{2+}]_c$ level. Treatment of tobacco cells with sub-mM and mM levels of $AlCl_3$ resulted in an increase in $[Ca^{2+}]_c$ (Fig. 2). Following the spike of $O_2^{\cdot-}$ generation, a gradual increase in $[Ca^{2+}]_c$) was observed. While the $O_2^{\cdot-}$ generation is an immediate event starting in a sub-second time scale and ceasing within initial 20 s, the changes in $[Ca^{2+}]_c$ are relatively slower events attaining the peak level spending ca. 1 min after the Al-impact.

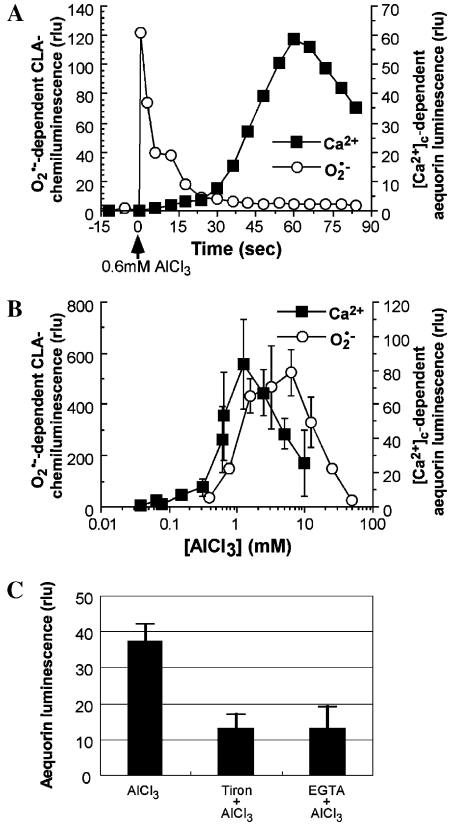

Fig. 2. $AlCl_3$-induced increase in $[Ca^{2+}]_c$ in tobacco cells. (A) Slower induction of the $[Ca^{2+}]_c$ increase following a rapid and transient $O_2^{\cdot-}$ generation. Typical data obtained after addition of 0.6 mM $AlCl_3$ are shown. (B) Effect of Al concentration on the induction of $[Ca^{2+}]_c$ increase. (C) Inhibition of $AlCl_3$-induced $[Ca^{2+}]_c$ increase by 5 mM Tiron and 10 mM EGTA, added 5 min prior to addition of 1.25 mM $AlCl_3$. rlu stands for the relative luminescence units.

The dose of Al required for $[Ca^{2+}]_c$ elevation was shown to be optimal at ca. 1.25 mM and the dose of Al higher than the optimal dose was shown to be less effective in induction of $[Ca^{2+}]_c$ elevation (Fig. 2B). Based on the equation proposed by Knight et al. [23], the maximal $[Ca^{2+}]_c$ manifested in the presence of 1.25 mM $AlCl_3$ was estimated to be 340 nM. Compared to the induction of $O_2^{\cdot-}$ generation, the $[Ca^{2+}]_c$ elevation required relatively lower concentrations of $AlCl_3$ for maximal $[Ca^{2+}]_c$ increase.

Evidence in support of $O_2^{\cdot-}$-dependent Ca^{2+} influx

The Al-treatment showed differential dose profiles for $O_2^{\cdot-}$ production and $[Ca^{2+}]_c$ elevation. Thus, it is tempting to examine if those two different events are regulated by different mechanisms or not. Addition of a strong $O_2^{\cdot-}$ scavenger, Tiron (5 mM), prior to Al addition resulted in significant inhibition of the Al-induced $[Ca^{2+}]_c$ elevation. The Al-induced $[Ca^{2+}]_c$ increase was also inhibited in the presence of a Ca^{2+} chelator, EGTA (10 mM), indicating that the source of Ca^{2+} required for Al-induced $[Ca^{2+}]_c$ elevation is the extracellular free calcium. Taken together, the data are indicative of the model that $O_2^{\cdot-}$ triggers the influx of Ca^{2+}.

We have previously reported similar models for salicylic acid [17,21] and aromatic monoamines [26,27], in which the generation of ROS ($O_2^{\cdot-}$ and H_2O_2 for both stimuli, $HO^{\cdot}$ for only aromatic amines) is rapidly induced and in turn, influx of Ca^{2+} is stimulated. Here, the working hypothesis assuming that Ca^{2+} influx and $O_2^{\cdot-}$ generation occur independently was finally rejected. Then, we had to seek for the alternative mechanism in explanation of differential dose requirements for the Al-dependent $O_2^{\cdot-}$ generation and $[Ca^{2+}]_c$ increment.

Lowered Ca^{2+} influx potency in the presence of Al

By analogy to the Ca^{2+} channel blocking actions of lanthanide ions, effect of Al on the influx of Ca^{2+} was examined (Fig. 3).

Addition of H_2O_2 (as a typical oxidative stress) induces a rapid and transient increase in $[Ca^{2+}]_c$ in tobacco BY-2 cells, as previously reported [28]. We found that Al-treatment effectively inhibits the H_2O_2-induced increase in $[Ca^{2+}]_c$ in a dose-dependent manner (Fig. 3A). The $[Ca^{2+}]_c$ elevation induced by 2 mM H_2O_2 was inhibited by 50% and 100%, in the presence of 0.3 and 10 mM $AlCl_3$, respectively. This explains the ineffectiveness of high Al concentrations for induction of $[Ca^{2+}]_c$ increase. It is likely that Al-induced $[Ca^{2+}]_c$ elevation is manifested from the finely geared balance between the $O_2^{\cdot-}$-mediated channel stimulation as the driving force and the channel inhibition-mediated lowering of Ca^{2+} influx potency as the brake.

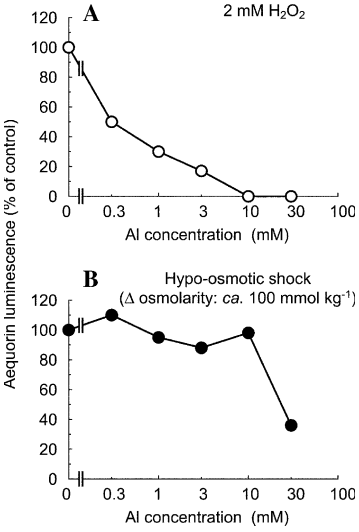

Fig. 3. Al-dependent inhibition of Ca^{2+} influx induced by H_2O_2 and hypo-osmotic shock in tobacco cells. (A) Effect of Al concentration on the H_2O_2-induced Ca^{2+} influx. Relative peak height in aequorin luminescence induced by 2 mM H_2O_2 in the absence of Al was expressed as 100%. (B) Effect of Al concentration on the hypo-osmotic shock-induced Ca^{2+} influx. Relative peak height in aequorin luminescence induced by addition of water to the culture media (ca. −100 mmol/kg) in the absence of Al was expressed as 100%.

Effect of Al on the Ca^{2+} influx induced by hypo-osmotic shock was also examined (Fig. 3B). It is noteworthy that Al ($\leqslant$10 mM) showed no inhibitory effect on the hypo-osmolarity (addition of equal volume of water to the cell suspension)-dependent Ca^{2+} influx. Hypo-osmotic shock is known to induce an immediate increase in $[Ca^{2+}]_c$ by stimulating the influx of Ca^{2+} through plasma membrane-localized Ca^{2+} channel(s) [19,29]. The present results imply that Al may be a selective inhibitor of ROS-responsive Ca^{2+} channels.

Recently, Furuichi et al. [30] have cloned the plant's first gene encoding a voltage-gated channel with high affinity for Ca^{2+} permeation, from *Arabidopsis*. The channel possesses extremely high homology with a recently cloned two pore channel (TPC1) from rats, thus designated as AtTPC1. Here, we hypothesize that the AtTPC1 homologs or equivalents in tobacco may be the possible targets of Al-stimulation via $O_2^{\cdot-}$ generation. In addition, we showed here that Al is inhibitory to the ROS-responsive Ca^{2+} influx, thus AtTPC1 equivalents in tobacco cells may be a target of Al-inhibition too.

Role of ROS in Al toxicity

ROS, including $O_2^{\cdot-}$, H_2O_2, and $HO^{\cdot}$, are known to be highly reactive causing oxidative damages to proteins, membrane lipids, and other cellular components [31]. Under both abiotic and biotic stresses, members of ROS including $O_2^{\cdot-}$ exacerbate damage and signal the activation of defense responses [32]. According to Ezaki et al.

[33], plants respond to sub-lethal Al stress by expressing the series of genes that contribute to development of plant tolerance to excessive environmental Al ions. Biological roles of those Al-inducible genes obtained from *Arabidopsis*, tobacco, wheat, and *Saccharomyces cerevisiae*, in tolerance development against Al stress and oxidative stress have been tested by over-expressing them in *Arabidopsis* plants. It was shown that Al-inducible genes, including an *Arabidopsis* blue-copper-binding protein gene (AtBCB), a tobacco glutathione *S*-transferase gene (parB), a tobacco peroxidase gene (NtPox), and a tobacco GDP-dissociation inhibitor gene (NtGDI1), confer a degree of resistance to both Al and oxidative stresses. Therefore, these genes introduced into the transgenic *Arabidopsis* plants are serving to protect against Al toxicity and oxidative stress. The above study indicates that Al toxicity is mediated via oxidative stress by production of ROS. As proposed in the present study, elevation of $[Ca^{2+}]_c$ is one of the notable impacts of Al-induced ROS.

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

Physiological roles for the $[Ca^{2+}]_c$ increase in Al toxicity is not well documented at present. It is generally believed that disturbance of $[Ca^{2+}]_c$ homeostasis is one of the primary triggers of Al toxicity [34–36]. Recent studies conducted by Rengel and his colleagues [37,38] have provided fluorescence probe-based evidence showing a correlation between the Al-induced increase in $[Ca^{2+}]_c$ and inhibition of root growth.

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 [39–41]. On the other hand, 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 was shown [42]. It is thus still controversial if Al disruption of Ca^{2+} transport in the root may play an important role in the mechanisms of Al toxicity in Al-sensitive plants, and that different Al tolerances may be associated with the ability of Ca-transport systems in cells of the root apex to resist disruption by potentially toxic level of Al in the soil.

Proposed mechanism

It has been implicated that Al stress is mediated with oxidative events, since toxic level of Al induces the oxidative stress-related gene expression in *Arabidopsis thaliana* [43]. In addition, those genes induced by Al-treatment were also shown to be induced by ozone, further confirming that Al signaling is mediated with oxidative events. Furthermore, Al-dependent oxidative damages to tobacco membranes were shown to occur in the presence of Fe ions [44]. The reported knowledge and the present results consistently support the view that Al stimulates the production of ROS (most likely, $O_2^{\cdot-}$) and the presence of transition metals such as Fe leads to generation of $HO^{\cdot}$ that finally damages the cellular components.

Model mechanisms of Al toxicity involving oxidative burst and Ca^{2+} signaling distortion are summarized in Fig. 4. First, Al stimulates the NADPH oxidase and induces the generation of $O_2^{\cdot-}$ that triggers the influx of Ca^{2+}. The resultant ROS and $[Ca^{2+}]_c$ elevation may lead to development of phytotoxicity (Fig. 4A).

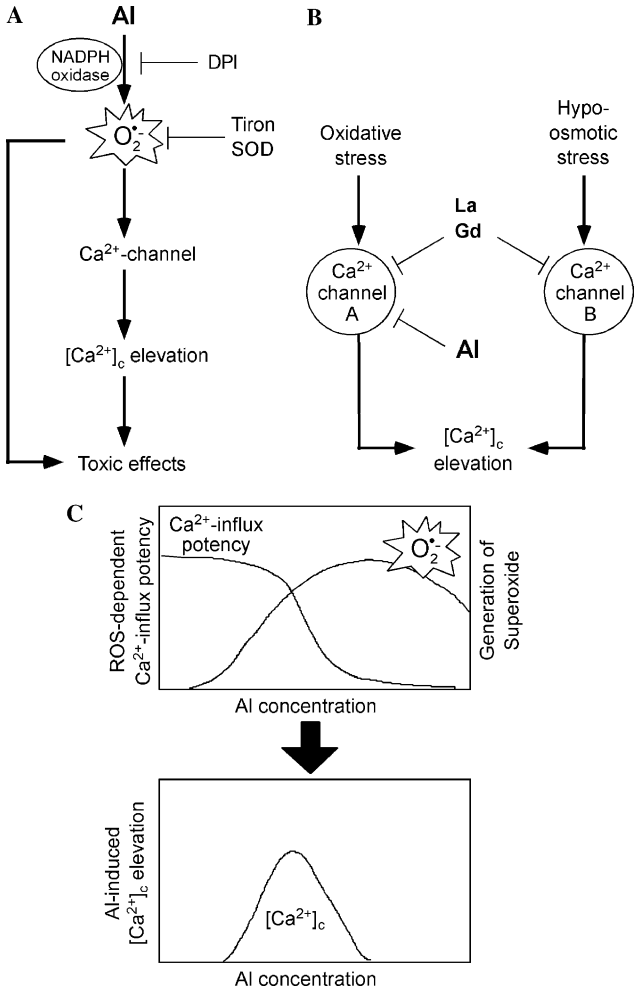

Fig. 4. Model mechanisms of Al toxicity involving oxidative burst and Ca^{2+} signaling distortion. (A) Al-induced $O_2^{\cdot-}$ triggers the influx of Ca^{2+}, and both oxidative stress and $[Ca^{2+}]_c$ elevation may result in phytotoxic phenomena. (B) Differential effects of known Ca^{2+} channel blockers and Al. Ions of La and Gd inhibit the Ca^{2+} influx induced by both hypo-osmotic shock and oxidative stress. In contrast, Al action is only against the oxidative stress-mediated mechanism. There may be at least two types of Ca^{2+} channels that differed in sensitivity to metal cations. (C) A model explaining the ineffectiveness of high concentration of Al in induction of Ca^{2+} influx. At low Al concentrations, the Ca^{2+} influx potency is high but the driving force is not sufficient. At high Al concentrations, there is much Ca^{2+} influx-inducing force but the Ca^{2+} channel's potency is low. Therefore, $[Ca^{2+}]_c$ elevation could be manifested only when the opposing effects compromise.

Jones et al. [43] proposed a view, based on the observation that Al treatment results in elevation of $[Ca^{2+}]_c$ in root hairs of *Arabidopsis*, that the phytotoxic action of Al in root hairs is not brought about through blockage of Ca^{2+} permeable channels required for Ca^{2+} influx into the cytoplasm. However, effects of Al on $[Ca^{2+}]_c$ homeostasis are not that simple. We showed the differential effects of Al and known channel blockers with no selectivity [45] on the Ca^{2+} influx. While the ions of La and Gd effectively inhibit the Ca^{2+} influx induced by both hypo-osmotic shock [19] and oxidative stress [21,28], the Al action is only against the ROS-mediated mechanism. Probably, two types of Ca^{2+} channels that differed in sensitivity to Al may be present in tobacco cells (Fig. 4B). Finally, we could propose a view clearly explaining the ineffectiveness of high concentration of Al in induction of Ca^{2+} influx. Al plays dual roles acting for and against Ca^{2+} influx at the same time, by releasing $O_2^{\cdot-}$ and by inhibiting the Ca^{2+} channel(s), respectively. At low Al concentrations, the ROS-responsive Ca^{2+} influx potency is high but the driving force (due to ROS) is not sufficient. At high Al concentrations, the Ca^{2+} influx-driving force is great but the Ca^{2+} channel's potency is low. Therefore, $[Ca^{2+}]_c$ elevation could be manifested only in the range of Al concentration in which the opposing effects compromise well (Fig. 4C).

Perspectives—similarity to abscisic acid signaling mechanism

Our present study may give a clue to the action of trivalent ions in plant cells. Our next concerns are impacts of trivalent ion-induced $O_2^{\cdot-}$ generation on gene expression that is regulated by salt-stress, draught stress, and ABA, since similarity between the stress hormone ABA and trivalent metal cations in induction of plant responses has been reported [46].

Trivalent ions have been shown to act as effectors of gene expression in plants [46,47] and animals [48]. It has been reported that trivalent lanthanides such as La^{3+} and Tb^{3+} stimulated the expression of ABA-responsive genes mimicking the action of ABA in rice [46,47], although mechanism of action has not been clarified.

Recently, some lines of studies have shown a relationship between the signal transduction pathways for ABA and the production of ROS [49]. Several studies revealed that ABA action on stomatal closure is mediated with the NADPH oxidase-catalyzed generation of ROS and elevation of cytosolic-free $[Ca^{2+}]_c$ [50,51]. In the proposed mechanisms, ABA elicits the production of H_2O_2 and, in turn, the resultant H_2O_2 stimulates the opening of Ca^{2+} channels, resulting in a rapid increase in $[Ca^{2+}]_c$. Actually, this is almost identical to the mechanism of Al action uncovered here.

In addition to Ca^{2+} disturbance, Al induces a distortion in K^+ homeostasis. Al enhances the efflux of K^+ out of the vacuole membrane vesicles from barley roots [52], and it inhibits the uptake of K^+ by wheat root hairs, by blocking the inward-rectifying K-channels on plasma membrane [53]. Effect of Al on K^+ homeostasis is also very similar to ABA action.

Earlier works of Kasai et al. [54] were also indicative of Al-ABA similar, pointing out the similarity effects of Al and ABA on increments in ATP-dependent and PPi-dependent H^+-pumping activities in tonoplast-based vesicles. They further proposed that ABA may be involved in the action of Al. However, this sounds unlikely that secondary induction of ABA mediates the rapid response to Al, such as immediate burst in ROS production. The above works and proposal should be re-examined.

Acknowledgments

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