

# Aluminum Toxicity Is Associated with Mitochondrial Dysfunction and the Production of Reactive Oxygen Species in Plant Cells<sup>1</sup>

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Potential mechanisms of Al toxicity measured as Al-induced inhibition of growth in cultured tobacco cells (*Nicotiana tabacum*, nonchlorophyllous cell line SL) and pea (*Pisum sativum*) roots were investigated. Compared with the control treatment without Al, the accumulation of Al in tobacco cells caused instantaneously the repression of mitochondrial activities [monitored by the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide and the uptake of Rhodamine 123] and, after a lag of about 12 h, triggered reactive oxygen species (ROS) production, respiration inhibition, ATP depletion, and the loss of growth capability almost simultaneously. The presence of an antioxidant, butylated hydroxyanisole, during Al treatment of SL cells prevented not only ROS production but also ATP depletion and the loss of growth capability, suggesting that the Al-triggered ROS production seems to be a cause of ATP depletion and the loss of growth capability. Furthermore, these three late events were similarly repressed in an Al-tolerant cell line (ALT301) isolated from SL cells, suggesting that the acquisition of antioxidant functions mimicking butylated hydroxyanisole can be a mechanism of Al tolerance. In the pea root, Al also triggered ROS production, respiration inhibition, and ATP depletion, which were all correlated with inhibition of root elongation. Taken together, we conclude that Al affects mitochondrial functions, which leads to ROS production, probably the key critical event in Al inhibition of cell growth.

Al is a most abundant metal in the earth's crust and is solubilized as the free Al<sup>3+</sup> ion under acidic conditions. A wide range of toxic effects of Al ions has been demonstrated in plants and animals, although the mechanisms of Al toxicity have not been elucidated. In animals, Al is a potent neurotoxin, whereas in plants, Al is a major factor reducing crop production in acid soils (for review, see Horst, 1995; Kochian, 1995; Rengel, 1996; Flaten et al., 1997; Kochian and Jones, 1997; Matsumoto, 2000). In plant roots, Al accumulates predominantly in the cells located within the apical elongation zone and inhibits cell elongation rapidly. However, the primary causal event for Al inhibition of cell elongation has not yet been elucidated.

The involvement of oxidative stress in Al toxicity has been suggested, although Al itself is not a transition metal and cannot catalyze redox reactions. Instead, Al ions have a strong affinity for biomembranes and can cause the rigidification of membranes

(Deleers et al., 1986), which seems to facilitate the radical chain reactions mediated by Fe ions enhancing the peroxidation of lipids in phospholipid liposomes (Oteiza, 1994), soybean (*Glycine max*) roots (Cakmak and Horst, 1991), and cultured tobacco (*Nicotiana tabacum*) cells (Ono et al., 1995; Yamamoto et al., 1997). The Al-enhanced Fe-mediated peroxidation of lipids leads to the loss of plasma membrane integrity and eventually cell death in cultured tobacco cells (Yamamoto et al., 1997; Yamaguchi et al., 1999). On the contrary, in pea (*Pisum sativum*) roots, the peroxidation of lipids was enhanced by Al but without an external supply of Fe (Yamamoto et al., 2001). Furthermore, the peroxidation of lipids is an early symptom and appears to cause, in part, callose production, but not the inhibition of root elongation. Genetically biased studies in plants also suggest that Al enhances oxidative stress because Al induces the expression of several genes encoding antioxidant enzymes such as glutathione S-transferase, peroxidase, and superoxide dismutase (Richards et al., 1998; Ezaki et al., 2000).

Cultured plant cells have been used as a model system for investigation of cellular mechanisms of Al toxicity in roots. Roots are usually treated with Al in a simple Ca solution. Thus, we have recently developed a new Al treatment condition for cultured tobacco cells using a simple Ca solution containing 3 mM CaCl<sub>2</sub> and 3% (w/v) Suc (Ca medium; Ikegawa et al., 2000). In this medium, the response to Al of

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cultured tobacco cells at the logarithmic phase of growth was comparable with that of plant cells in the root meristematic region growing in Ca solution. In Ca medium, Al accumulated immediately in tobacco cells and induced callose production, but not the loss of plasma membrane integrity. In addition, Al alone did not cause the peroxidation of lipids (Ikegawa et al., 2000), but reduced growth capability (Devi et al., 2001). In this paper, to elucidate mechanisms of Al-induced inhibition of cell growth and of Al-induced root elongation inhibition in a simple Ca solution, we investigated the effect of Al on the ability of mitochondria to produce ATP and reactive oxygen species (ROS). Evidence for a new mechanism of Al toxicity involving ROS evolution probably via mitochondrial dysfunction is presented.

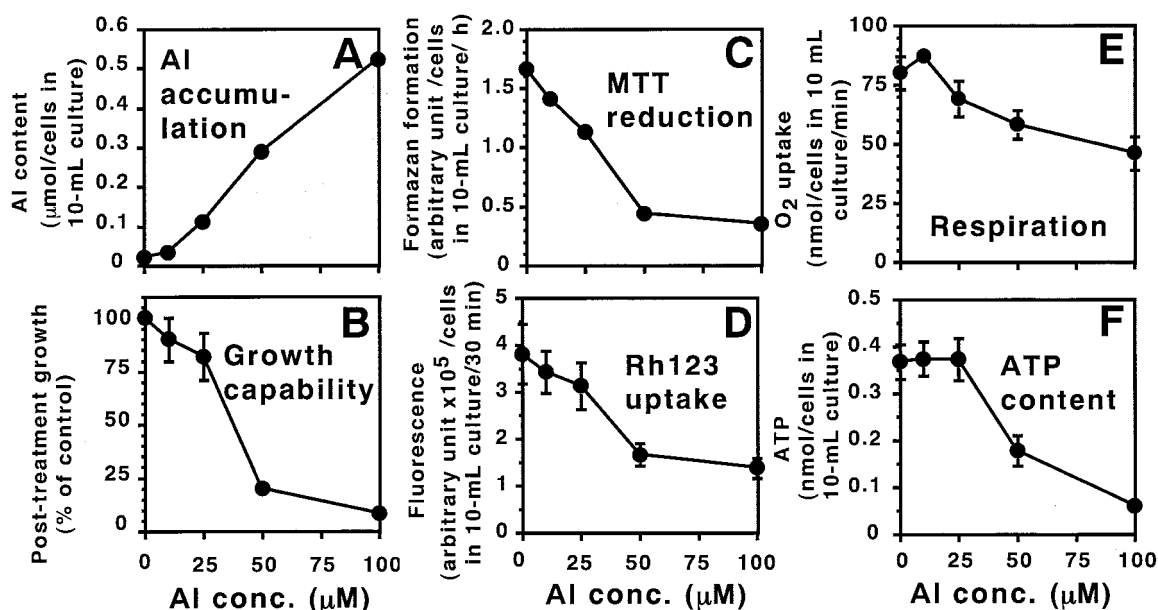
## RESULTS

### Al Decreases Mitochondrial Activities, Respiration, ATP Content, and Growth Capability in Tobacco Cells

In Ca medium, tobacco cells (a nonchlorophyllous cell line SL) did not grow significantly, but they maintained growth capability for at least 24 h. The growth capability of the cells was judged from the degree of growth after transferring the cells into growth medium. SL cells were exposed to various concentrations of AlCl<sub>3</sub> in Ca medium for 24 h. With an increase in Al concentration, the amount of Al accumulated in cells increased (Fig. 1A), and with an increase in the level of Al accumulation, Al-

accumulated cells lost their growth capability (Fig. 1B). After treatment with 50  $\mu\text{M}$  AlCl<sub>3</sub> or more for 24 h, the growth capability of the cells decreased to less than 25% of that of control cells. Under these conditions, however, it seems that Al-accumulated cells maintain the integrity of the plasma membrane because after treatment with 50  $\mu\text{M}$  AlCl<sub>3</sub> for 24 h the cells did not incorporate nonpermeating dyes such as Evans blue (Ikegawa et al., 2000) or propidium iodide (Y. Yamamoto, unpublished data), but they did incorporate and maintain a permeating dye such as fluorescein diacetate (Ikegawa et al., 2000).

To elucidate the mechanism of the Al-induced loss of growth capability, we investigated mitochondrial activities during Al treatment. Mitochondrial activities in living cells were monitored by either the formation of formazan from MTT or the uptake of Rh123 into mitochondria. MTT can freely enter into cells and is supposed to be cleaved to formazan via coupling reducing reaction based on the electron transport chain located in the inner mitochondrial membrane (Slater et al., 1963). Rh123 is recognized as a specific fluorescent probe to monitor active mitochondria. Its uptake into the mitochondrial matrix depends on the transmembrane electrochemical potential of the mitochondrial inner membrane ( $\Delta\Psi\text{m}$ ; for review, see Kroemer et al., 1998; Scheffler, 1999). SL cells that accumulated Al exhibited decreases in MTT reduction and Rh123 uptake (Fig. 1, C and D), as well as growth capability with similar Al dose dependency (Fig. 1B). Under the fluorescence micros-



**Figure 1.** Al causes the reduction of mitochondrial activities, respiration inhibition, ATP depletion, and the loss of growth capability in cultured tobacco cells (SL). SL cells were treated with various concentrations of Al for 24 h in Ca medium, pH 4.5. After Al treatment, Al accumulation (A), growth capability (B), mitochondrial activities [monitored by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction (C) and Rhodamine 123 (Rh123) uptake (D)], respiration (E), and ATP content (F) were determined as described in "Materials and Methods." All values are the means and SE of nine replicates, except for Rh123 uptake with 15 replicates and for respiration with six replicates.

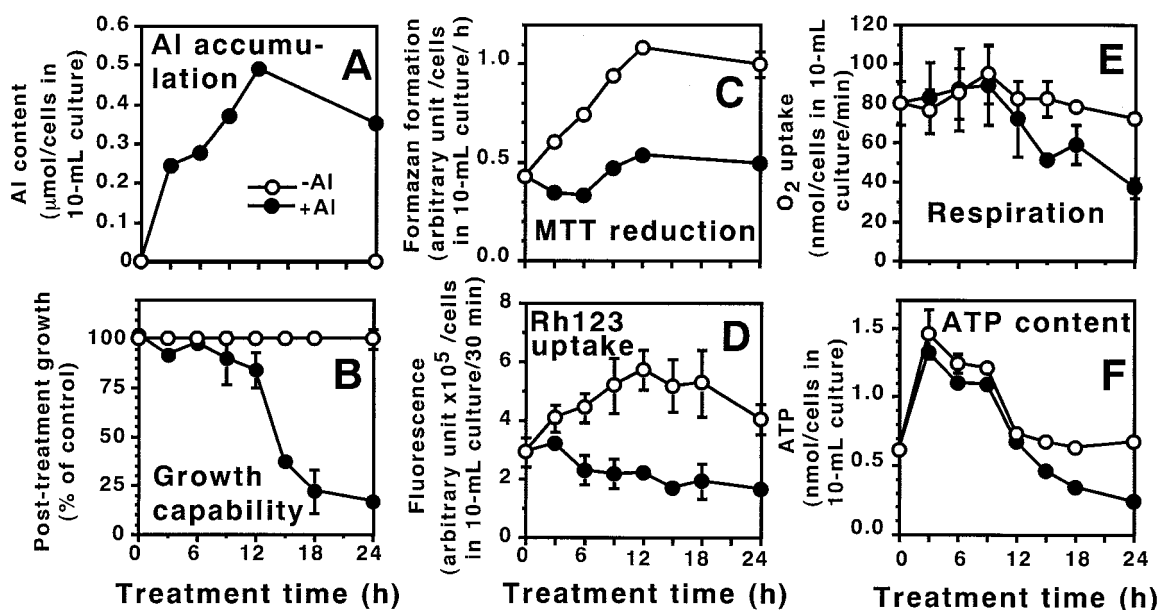
copy, all the control cells were stained extensively with Rh123, whereas the Al-treated cells (100  $\mu\text{M}$  Al) were stained at lower intensity or were not stained (Fig. 3A). At a higher magnification, Rh123 fluorescence in control cells was observed in numerous small particles (Fig. 3B) which indicated mitochondria.

Time course experiments with 100  $\mu\text{M}$  Al indicated that the accumulation of Al and the Al-induced decreases in the mitochondrial activities started simultaneously after the start of Al exposure (Fig. 2, A, C, and D). In control cells treated without Al, MTT reduction and Rh123 uptake increased from a start of the treatment until 12 h, then decreased slightly (MTT reduction) or near to the initial level (Rh123 uptake) at 24 h. The increases in these mitochondrial activities may be induced by a change of culture medium from growth medium (a modified Murashige and Skoog medium) to Ca medium. In Al-treated cells, however, such increases in the mitochondrial activities were not observed from the start of a treatment (Fig. 2, C and D), suggesting that the repression of the mitochondrial activities is an early response to Al. Before accessing to mitochondria, Rh123 must pass the plasma membrane, which also depends on the plasma membrane potential ( $\Delta\Psi_p$ ; for review, see Scheffler, 1999). Thus, as a possibility, a decrease of  $\Delta\Psi_p$  might affect mitochondrial accumulation of the probe. However, Rh123 uptake and MTT reduction were repressed by Al in the same dose-dependent and time-dependent manners (Figs. 1 and 2). Thus, we concluded that the repression of mitochondrial activities, namely the electron flow

(detected by MTT reduction) and  $\Delta\Psi_m$  in the inner membrane (detected by Rh123 uptake), is a relatively early symptom induced by the accumulation of Al in tobacco cells.

We also found that the respiration level detected by oxygen uptake (Fig. 1E) and ATP content (Fig. 1F) decreased with a decrease in growth capability in the same Al dose-dependent manner. During the treatment period, control cells maintained almost the same level of oxygen uptake, whereas Al-treated cells exhibited a decrease in oxygen uptake after 12 h (Fig. 2E). Just after a start of the Al treatment, the content of ATP in control and Al-treated cells temporarily increased and returned to the initial level at 12 h (Fig. 2F). Then, from 12 to 24 h, control cells maintained the ATP level exhibited at 12 h, whereas Al-treated cells lost ATP gradually and had only 36% of the control level at 24 h (Fig. 2F). The growth capability started to decrease from 12 h and reached a minimum level at 24 h (Fig. 2B). Thus, the loss of growth capability and the decreases in oxygen uptake and ATP content were late symptoms and occurred almost simultaneously after a 12-h exposure to Al in tobacco cells.

Taken together, it seems that Al causes the repression of the mitochondrial activities (the impediment of an electron flow and the dissipation of  $\Delta\Psi_m$ ) from a start of the exposure time, which is not toxic, but after 12 h, may lead to the critical late events such as respiration inhibition, ATP depletion, and the loss of growth capability.



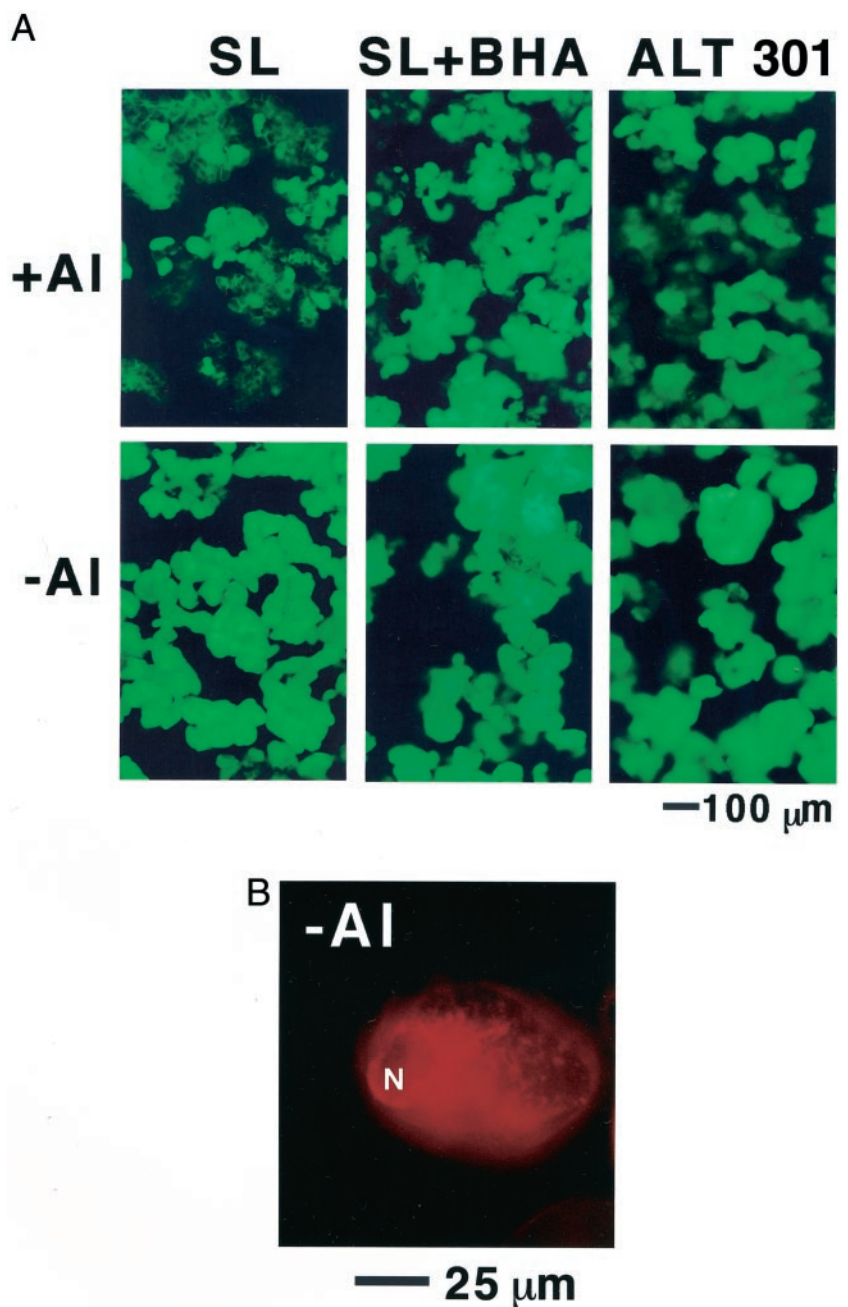
**Figure 2.** Time course of the events triggered by Al (the reduction of mitochondrial activities, respiration inhibition, ATP depletion, and the loss of growth capability) in cultured tobacco cells (SL). SL cells were treated with 100  $\mu\text{M}$  Al for various duration in Ca medium, pH 4.5. At times, Al accumulation (A), growth capability (B), mitochondrial activities [monitored by MTT reduction (C) and Rh123 uptake (D)], respiration (E), and ATP content (F) were determined as described in "Materials and Methods." All values are the means and SE of nine replicates, except for Rh123 uptake with 15 replicates and for respiration with six replicates.

### Al Triggers ROS Production in Tobacco Cells

The Al-induced repression of mitochondrial activities may induce hypergeneration of ROS. Thus, the possible production of ROS by Al inside cells was investigated by staining SL cells with ROS-specific indicators, the superoxide anion ( $O_2^-$ )-specific indicator, dihydroethidium (DHE), or the peroxide-specific indicator, 2', 7'-dichlorofluorescein diacetate (Costa-Pereira and Cotter, 1999). The presence of  $O_2^-$  oxidizes DHE to ethidium, and ethidium is free to in-

tercalate with DNA, whereupon it emits fluorescence (Costa-Pereira and Cotter, 1999). When compared with control SL cells, a part of the Al-treated SL cells had significant DHE-derived fluorescence by 12 h, and greater than 50% of Al-treated SL cells fluoresced by 24 h (Fig. 4A). The DHE-derived ethidium fluorescence in Al-treated cells (Fig. 4B) was observed in nuclei, and in particles that correspond in number and morphology to Rh123-stained mitochondria in control cells (Fig. 3B). These observations clearly in-

**Figure 3.** Rh123 uptake in Al-sensitive tobacco cells (SL) with or without the antioxidant butylated hydroxyanisole (BHA) and in Al-tolerant tobacco cells (ALT301) after treatment with Al. SL cells were treated with Al (0 and 100  $\mu M$ ) for 24 h in the absence or presence of BHA (100  $\mu M$ ) as described in "Materials and Methods." ALT301 cells were also treated with Al (0 and 100  $\mu M$ ) for 24 h. After treatment, cells were stained with Rh123 and the uptake of Rh123 into cells was observed under fluorescence microscope with a filter set No. 10 (A; for higher intensity; Carl Zeiss, Oberkochen, Germany). In addition, the uptake of Rh123 into mitochondria in a control SL cell treated without Al for 24 h was observed with a filter set No. 15 (B; for higher resolution; Carl Zeiss). B, A nucleus is indicated with N, and small numerous particles are mitochondria.

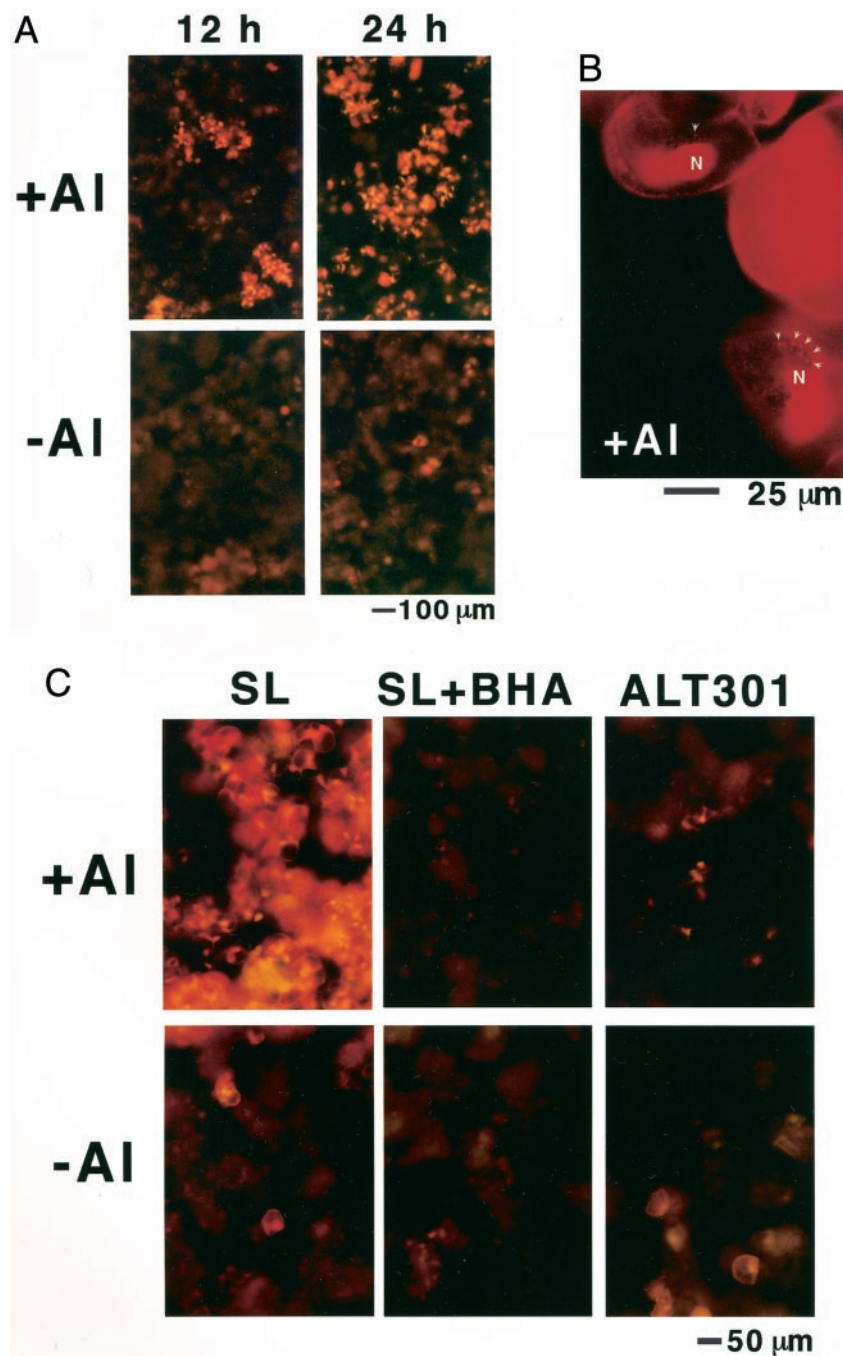


dicates that Al enhances  $O_2^-$  production inside cells, which is strongly correlated to the loss of growth capability.

Judging from the dichlorofluorescein stain under fluorescence microscope, the intracellular peroxide level seemed to be enhanced by Al, but only temporarily during Al treatment (data not shown).

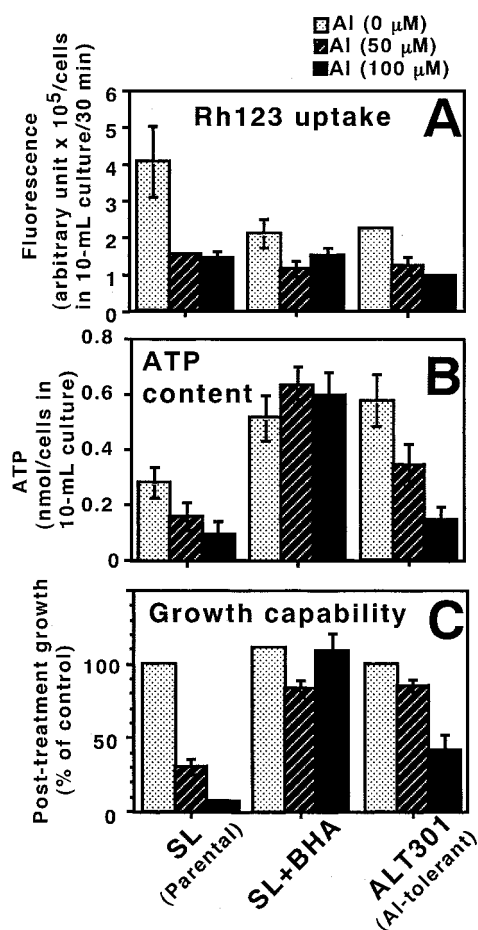
#### Protective Effects of BHA on Al-Triggered ROS Production, ATP Depletion, and Loss of Growth Capability in Tobacco Cells

BHA, a lipophilic antioxidant, has been reported to prevent ROS formation induced by the impediment of electron flow by antimycin A in mitochondria in



**Figure 4.** Al triggers ROS production in Al-sensitive tobacco cells (SL), but not in the presence of BHA and in Al-tolerant tobacco cells (ALT301). SL cells were treated with Al (0 and 100  $\mu\text{M}$ ) for 12 h or 24 h in Ca medium (pH 4.5) as described in "Materials and Methods" (A and B). SL cells in the absence or presence of BHA (100  $\mu\text{M}$ ) and ALT301 cells were also treated with Al (0, 100  $\mu\text{M}$ ) for 24 h (C). After treatment, ROS production (presumably  $O_2^-$ ) in the cells was observed by staining with DHE as described in "Materials and Methods." Fluorescence images were obtained with a filter set No. 9 (A and C; for higher intensity; Carl Zeiss) and No. 15 (B; for higher resolution; Carl Zeiss). B, the localization of ethidium fluorescence derived from  $O_2^-$ -oxidized DHE in Al-treated SL cells for 24 h was observed. Nuclei are indicated with N, and small numerous particles indicated with arrow heads are most likely mitochondria (for details, see text).

cultured tobacco cells (Maxwell et al., 1999). In this study, BHA also effectively protected SL cells from the Al-induced ROS production (Fig. 4C). In control SL cells, compared with the absence of BHA, the presence of BHA reduced the uptake level of Rh123 to one-half of that without BHA (Fig. 5A). However, all the cells in the presence of BHA were stained with Rh123 less intensively but more homogeneously than the cells without BHA (Fig. 3A). Time-course experiments revealed that control SL cells in the presence of BHA maintained the initial Rh123 uptake level at a start of the culture until 24 h in Ca medium (data not shown) without the temporal increase in Rh123 uptake observed in the absence of BHA (Fig. 2D). Furthermore, in the presence of BHA, the inhibition rate of Rh123 uptake by Al in SL cells (28%–46%) was



**Figure 5.** Al-sensitive tobacco cells (SL) in the presence of the antioxidant BHA and Al-tolerant tobacco cells (ALT301) similarly overcome Al toxicity (the repression of mitochondrial activity, ATP depletion, and the loss of growth capability). SL cells were treated with Al (0, 50, and 100  $\mu\text{M}$ ) for 24 h in the absence or presence of BHA (100  $\mu\text{M}$ ). ALT301 cells were also treated with Al for 24 h. After treatment, mitochondrial activity (A; detected by Rh123 uptake), ATP content (B), and growth capability (C) were determined as described in “Materials and Methods.” Values shown are the means and SE of 15 replicates (Rh123 uptake), nine replicates (ATP content), and three replicates (growth capability).

less than that in SL cells without BHA (63%–75%; Fig. 5A). These results suggest that BHA stabilizes  $\Delta\Psi\text{m}$  in SL cells during culture in Ca medium with or without Al. In the presence of BHA, ATP content in control SL cells increased 1.8 times that without BHA, and the high ATP content was maintained even after Al treatment (Fig. 5B). Furthermore, in the presence of BHA, Al-treated SL cells maintained growth capability as much as control cells (Fig. 5C). Thus, the content of ATP was well correlated with the degree of growth capability in Al-treated SL cells (Fig. 5, B and C). In addition, the presence of BHA did not affect the accumulation level of Al in SL cells significantly (105% and 78% of the Al contents in SL cells treated in the absence of BHA for 24 h with 50 and 100  $\mu\text{M}$   $\text{AlCl}_3$ , respectively). The presence of BHA did not affect the respiration level in control and Al-treated cells (data not shown).

Taken together, it seems that BHA causes the stabilization of mitochondrial activities, the prevention of ROS production, and the maintenance of high ATP level, which leads to the acquisition of Al tolerance.

#### An Al-Tolerant Tobacco Cell Line Prevents Al Toxicity in a Similar Manner as BHA

An Al-tolerant tobacco cell line (ALT301) and its parental cell line SL accumulate Al to the same extent (Devi et al., 2001). However, ALT301 cells exhibited lower ROS production than SL cells after Al treatment (Fig. 4C). Compared with control SL cells treated without Al, the level of Rh123 uptake in control ALT301 cells was about one-half (Fig. 5A), and the inhibition rate of Rh123 uptake by Al in ALT301 cells (46%–58%) was less than that in SL cells (63%–75%; Fig. 5A). In addition, the ATP content in control ALT301 cells was two times higher than that in control SL cells, and ALT301 cells maintained higher levels of ATP even after Al treatment than SL cells (Fig. 5B). Furthermore, the higher ATP levels in ALT301 cells were well correlated with higher growth capability of ALT301 cells (Fig. 5, B and C). On the other hand, the respiration inhibition by Al was observed in both cell lines to the same extent (data not shown). Thus, the responses to Al similarly demonstrated by ALT301 cells and by the BHA-protected SL cells (see above) suggest that the acquisition of the functions mimicking that of BHA leads to Al tolerance in ALT301 cells.

#### Al Triggers ROS Production, Respiration Inhibition, and ATP Depletion in Pea Roots

Finally, we investigated the possible involvement of ROS production, respiration inhibition, and ATP depletion in Al-induced inhibition of root elongation in pea seedlings. After a 24-h Al treatment, root elongation (Fig. 6A) and ATP content (Fig. 6B) decreased in the same Al dose-dependent manner. In a similar

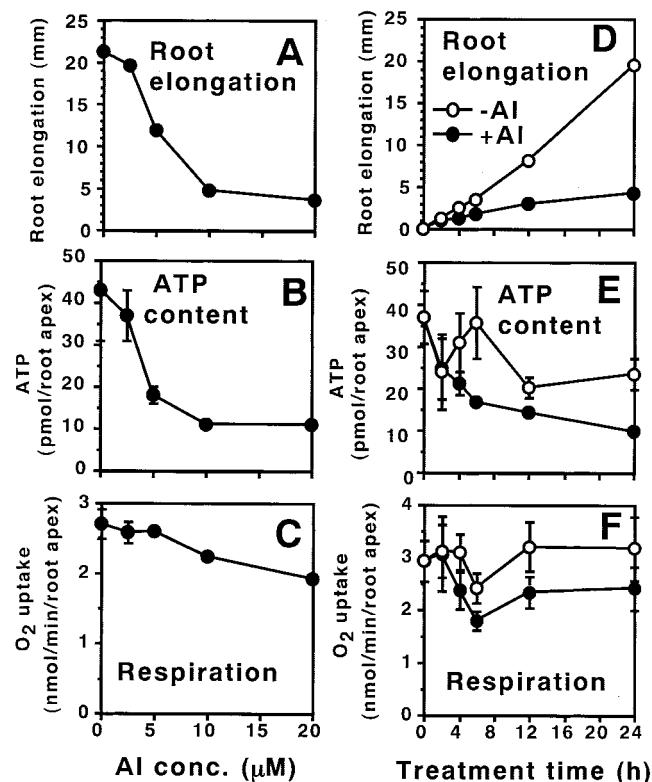
manner, the respiration level decreased with an increase in Al concentration (Fig. 6C). Time-course experiments with 10  $\mu\text{M}$  Al for up to 24 h indicate that the Al-dependent decreases in the root elongation (Fig. 6D), ATP content (Fig. 6E), and respiration (Fig. 6F) started simultaneously from 4 h, whereas the ATP content in control roots fluctuated during the treatment. Compared with control roots, the apex of the roots (specifically at elongation zone) treated with 10  $\mu\text{M}$  Al for 12 h was stained significantly stronger with DHE (Fig. 7), indicating the Al-induced ROS production at elongation zone. The ROS production was detected by a 2-h exposure to Al (data not shown), and increased with an increase in the exposure time. These chronological relationships suggest that the Al-enhanced ROS production leads to respiration inhibition, ATP depletion, and the inhibition of root elongation.

## DISCUSSION

The data presented in this study indicate that Al triggers ROS production (presumably  $\text{O}_2^-$ ) inside cells, respiration inhibition, and ATP depletion, which are well correlated with the inhibition of cell growth (tobacco cells) and the inhibition of root elongation (pea roots; Figs. 1–4, 6, and 7). Thus, ROS production, respiration inhibition, and ATP depletion seem to be critical events of Al toxicity in cultured plant cells and whole roots. In plant cells,  $\text{O}_2^-$  is mainly evolved in mitochondria (via respiration) and in chloroplasts (via photosynthesis; Scandalios, 1993). Since the tobacco cell lines used in this study and pea roots do not have chlorophyll, and Al treatments were carried out in the dark, it is likely that the mitochondrion is a primary organelle for the Al-induced  $\text{O}_2^-$  production. It is interesting that Hamilton et al. (2001) have recently reported the enhancement of activities of vacuolar  $\text{H}^+$ -ATPase and mitochondrial ATP synthase by Al specifically in an Al-resistant cultivar (cv PT741) of wheat (*Triticum aestivum*), suggesting that the vacuolar ATPase responds specifically to Al stress with the ATP supplied by mitochondrial ATP synthase.

The chronological analyses in tobacco cells suggest that the accumulation of Al in cells initially affects the activities of mitochondrial inner membranes by the impediment of an electron flow (detected by MTT reduction) and the dissipation of  $\Delta\Psi\text{m}$  (detected by Rh123 uptake), which seems to lead later (after a 12-h exposure to Al) to the death process, including the evolution of ROS, respiration inhibition, ATP depletion, and the loss of growth capability, ultimately (Figs. 1–4). It has been well documented in animal cells that mitochondria play a major role in the regulation of physiological cell death (apoptosis) and accidental cell death (necrosis; for review, see Kroemer et al., 1998; Scheffler, 1999). Animal cells undergoing cell death (either apoptosis or necrosis) exhibit

a reduction of the cellular uptake of  $\Delta\Psi\text{m}$ -sensitive fluorochromes (e.g. Rh123 and JC-1), and the dissipation of the  $\Delta\Psi\text{m}$  constitutes an early event during cell death processes. The disruption of  $\Delta\Psi\text{m}$  suggests that the proton-moving force and/or the inner membrane permeability has been affected, and the permeability transition (PT) has been postulated to be a central, rate-limiting event in cell death processes. A number of toxic metabolites such as ROS and supra-physiological  $\text{Ca}^{2+}$  concentrations can trigger PT. The PT level beyond a threshold is believed to lock the cell into an irreversible death program, which leads to apoptosis or necrosis, accompanying major changes in cellular redox potentials (e.g. hyperproduction of  $\text{O}_2^-$  and depletion of nonoxidized glutathione), energy metabolism [e.g. depletion of ATP and NAD(P)H], and ion compartmentalization (e.g. outflow of  $\text{Ca}^{2+}$  from the mitochondrial matrix). This working hypothesis would be compatible with the Al symptoms observed in tobacco cells: Al causes the impediment of an electron flow and the dissipation of  $\Delta\Psi\text{m}$  as early events, which may lead to a threshold level of massive PT after a 12-h exposure to Al, then the cell death process is initiated accompanying redox catastrophe (respiration inhibition and the hy-



**Figure 6.** Al triggers root elongation inhibition, ATP depletion, and respiration inhibition simultaneously in pea roots. Pea seedlings were treated with various concentrations of Al for 24 h (A–C) or with 10  $\mu\text{M}$  Al for various duration (D–F). After treatment, root elongation (A and D), ATP content (B and E), and respiration (C and F) in root apices (5 mm) were determined. Values shown are the means and SE of three replicates.



**Figure 7.** Al triggers ROS production in pea roots. Pea seedlings were treated with or without  $10 \mu\text{M}$  Al for 12 h. After treatment, the production of ROS was observed by staining with DHE as described in “Materials and Methods.” Note that DHE stains exclusively the elongation zone of Al-treated pea roots, but not in untreated roots. Bar indicates 1 mm.

perproduction of  $\text{O}_2^-$ ) and the bioenergetic catastrophe (ATP depletion; Figs. 2 and 3). Judging from the major site of Al localization of plant cells (Rengel, 1996; Kochian and Jones, 1997), we tentatively suggest that Al accumulation on the outer plasma membrane affects mitochondrial functions by an unknown signal transduction pathway. Another possibility is that a small amount of Al transported across the plasma membrane (Taylor et al., 2000) may

directly interfere with mitochondrial functions. In this regard, an *in vitro* study with isolated mitochondria from rat liver reported Al as an inducer of the mitochondrial PT (Toninello et al., 2000). The initial process of Al to cause mitochondrial dysfunctions remains to be elucidated.

An antioxidant (BHA) seems to partly preserve mitochondrial functions in tobacco cells under Al stress. In the presence of BHA, the stabilization of  $\Delta\Psi\text{m}$  and an increase of ATP content were observed in control SL cells, and ROS production, ATP depletion, and the loss of growth capability were prevented in Al-treated SL cells (Figs. 3–5). The BHA effects seem to be due to scavenging  $\text{O}_2^-$  by hydrogen donation or supposedly by the induction of antioxidant genes such as NAD(P) H-quinone reductase and glutathione *S*-transferase as reported in animal cells (Pinkus et al., 1996; for review, see Halliwell and Gutteridge, 1999). Our results suggest that the Al-triggered impediment of an electron flow (suggested by the inhibition of MTT reduction; Figs. 1 and 2) leads to the inhibition of normal oxygen consumption by cytochrome oxidase (respiration inhibition), but to the enhancement of  $\text{O}_2^-$  production by the leakage of electrons directly to oxygen. Since BHA prevented  $\text{O}_2^-$  production and the loss of growth capability but not the respiration inhibition, it is likely that  $\text{O}_2^-$  production is a key critical event leading to the loss of growth capability.

Judging from the resemblance of the phenotypes exhibited by BHA-protected SL cells and by Al-tolerant ALT301 cells, ALT301 cells seem to acquire the functions mimicking that of BHA. A search for a putative gene(s) that gives rise to the Al tolerance in ALT301 cells is now under investigation.

## MATERIALS AND METHODS

### Plant Materials and Al Treatment

A tobacco (*Nicotiana tabacum* L. cv Samsun) cell line SL (nonchlorophyllous; Nakamura et al., 1988) and an Al-tolerant cell line ALT301 derived from SL (Devi et al., 2001) were used. Cells were grown in a modified version of Murashige and Skoog medium (growth medium) and were subcultured at 7-d intervals (Yamamoto et al., 1994). Al treatment was performed as described previously (Ikegawa et al., 2000). In brief, exponentially growing cells 4 d after subculture were treated with  $\text{AlCl}_3$  in Ca medium (3 mM  $\text{CaCl}_2$  and 3% [w/v] Suc), pH 4.5, at 10 mg fresh weight cells  $\text{mL}^{-1}$  for up to 24 h on a rotary shaker at 100  $\text{rev min}^{-1}$  at 25°C in darkness. Then, 10-mL aliquots of the treated cells, corresponding to 100 mg fresh weight at the start of treatment, were harvested, washed, and used for quantitation and detection of various events as described below. The growth capability of Al-treated cells was determined from the extent of growth of Al-treated cells relative to that of untreated control cells after subsequent culture in Al-free growth medium for 7 d (Yamamoto et al., 1994). For BHA treatment, cells were precultured in Ca medium (pH



4.5) containing 100  $\mu\text{M}$  BHA (Sigma, St. Louis) at 10 mg fresh weight cells  $\text{mL}^{-1}$  for 2 h, and they were then harvested, resuspended in Ca medium (pH 4.5), and treated with Al as described above in the presence of 100  $\mu\text{M}$  BHA. Pea (*Pisum sativum* L. cv Alaska) seedlings (4 d after germination) were treated with  $\text{AlCl}_3$  in an aerated 100  $\mu\text{M}$   $\text{CaCl}_2$  solution, pH 4.75, for up to 24 h at 25°C under a 12-h photoperiod. Root elongation during Al treatment was determined as described (Yamamoto et al., 2001).

### Quantitation of Al

Cells were digested with acids, and the concentration of Al was determined using an atomic absorption spectrophotometer with graphite furnace atomizer (model Z-9000; Hitachi, Tokyo) as described previously (Yamamoto et al., 1994, 2001).

### Quantitation of Mitochondrial Activities in Tobacco Cells

#### Reduction of MTT

The assay was based on the procedure modified for tobacco cells (Ikegawa et al., 1998). After Al treatment, cells (10-mL aliquots) were suspended in 10 mL of Ca medium, pH 5.0, containing MTT (250  $\mu\text{g mL}^{-1}$ ; Dojindo Laboratories, Kumamoto, Japan), and the suspension was shaken gently at 25°C for 1 h in darkness. Cells were then harvested, resuspended in 5 mL of isopropanol containing 0.04 M HCl, and they were mixed vigorously to dissolve the formazan produced by the cleavage of MTT. Cells were removed by centrifugation, and the absorbance of formazan was determined at 590 nm spectrophotometrically.

#### Uptake of Rh123

The assay (Darzynkiewicz et al., 1999) was modified for tobacco cells. After Al treatment, cells (1.5-mL aliquots) were suspended in 1 mL of Ca medium, pH 5.0, containing Rh123 (2  $\mu\text{g mL}^{-1}$ ; Molecular Probes, Eugene, OR), and the suspension was shaken gently at 25°C for 30 min in darkness. Cells were then harvested, washed, and resuspended homogeneously in a Percoll solution (12.6% [v/v] Percoll [Amersham Pharmacia Biotech, Uppsala] and 10 mM MES [2-(*N*-morpholino)-ethanesulfonic acid]-1,3-bis[Tris-(hydroxymethyl)methylamino]propane, pH 5.0, in Ca medium). The fluorescence of Rh123 was measured using a fluorescence spectrophotometer (excitation 488 nm, emission 526 nm, slit 10 nm; model F4500; Hitachi). Uptake of Rh123 into cells and into mitochondria was observed under a fluorescence microscope (model Axiotron; Carl Zeiss) with a filter set No. 10 (excitation 450–490 nm, emission 515–565 nm, for higher intensity) and a filter set No. 15 (excitation 546/12 nm, emission 590 nm or more, for higher resolution), respectively.

### Quantitation of ATP

After Al treatment, tobacco cells (10-mL aliquots) or four pea root apices (5-mm section from root tip) were incu-

bated at 100°C for 15 min in 1 mL (tobacco cells) or 0.5 mL (roots) of  $\text{dH}_2\text{O}$ , and they were then disrupted at 4°C by ultrasonication (tobacco cells) or by mechanical homogenization (roots). After centrifugation at 9,000g for 15 min, ATP content in the supernatants was determined in an assay mixture containing 25 mM Glycyl-Gly-NaOH, pH 7.8, 15 mM  $\text{MgSO}_4$ , and luciferase-luciferin (Wako Pure Chemicals, Osaka) using a luminometer (model LB9506; EG&G Berthold, Germany) as described previously (Strehler, 1963).

### Quantitation of Respiration

Oxygen uptake by tobacco cells and pea roots was determined by use of a Clark-type oxygen electrode apparatus (Hansatech, King's Lynn, UK). After Al treatment, tobacco cells (2-mL aliquots) were withdrawn, put into the reaction chamber directly without wash, and the consumption of oxygen was determined immediately under continuous mixing condition at 25°C. The presence of  $\text{AlCl}_3$  (100  $\mu\text{M}$ ) in solution did not interfere in the oxygen detection by the electrode (Y. Yamamoto, unpublished data). In a similar manner, after Al treatment, pea roots were washed briefly, and then root apices (5-mm section from root tip) were cut and put into the reaction chamber containing 1 mL of 100  $\mu\text{M}$   $\text{CaCl}_2$ , pH 4.75, and oxygen consumption was determined immediately as described above.

### Detection of ROS

The assay (Costa-Pereira and Cotter, 1999) was modified for tobacco cells and pea roots. Al-treated tobacco cells (2-mL aliquots) were suspended in 2 mL of Ca medium, pH 5.0, containing 10  $\mu\text{M}$  DHE (Molecular Probes), and they were then shaken gently at 25°C for 30 min. Cells were then harvested, washed, and resuspended in 2 mL of Ca medium, pH 5.0. Fluorescence of ethidium derived from DHE oxidation by ROS (presumably  $\text{O}_2^-$ ) was observed under a fluorescence microscope (model LSM510 with a filter set no. 9 [excitation 450–490 nm, emission 520 nm or more] for higher intensity; and model Axiotron with a filter set no. 15 [see above] for higher resolution, both Carl Zeiss). The production of ROS in pea roots was observed by staining roots with 10  $\mu\text{M}$  DHE in 100  $\mu\text{M}$   $\text{CaCl}_2$ , pH 4.75, for 30 min. After washing, the roots were observed under a fluorescence microscope (LSM510 with a filter set no. 9; Carl Zeiss).

### Statistical Analysis

Each experiment was repeated at least three times with similar results. All values are shown as the means  $\pm$  SE of at least three replicates obtained from three independent experiments (see figure legends).

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