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Aluminum Tolerance Acquired during Phosphate Starvation in Cultured Tobacco Cells[1]

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Al toxicity in cultured tobacco cells (*Nicotiana tabacum* L. cv Samsun; nonchlorophyllic cell line SL) has been investigated in nutrient medium. In this system, Al and Fe(II) (ferrous ion) in the medium synergistically result in the accumulation of both Al and Fe, the peroxidation of lipids, and eventually death in cells at the logarithmic phase of growth (+P cells). A lipophilic antioxidant, *N,N'*-diphenyl-*p*-phenylenediamine, protected +P cells from the peroxidation of lipids and from cell death, suggesting that a relationship exists between the two. Compared with +P cells, cells that had been starved of Pi (−P cells) were more tolerant to Al, accumulated 30 to 40% less Al and 70 to 90% less Fe, and did not show any evidence of the peroxidation of lipids during Al treatment. These results suggest that −P cells exhibit Al tolerance because their plasma membranes are protected from the peroxidation of lipids caused by the combination of Al and Fe(II). It seems likely that the exclusion of Fe from −P cells might suppress directly Fe-mediated peroxidation of lipids. Furthermore, since −P cells accumulated β-carotene, it is proposed that this carotenoid pigment might function as a radical-trapping antioxidant in the plasma membrane of cells starved of Pi.

Al is a major component of soils, and free Al ions solubilized at pH values of 5.0 and below are a major growth-limiting factor. Al inhibits root growth within 1 to 2 h of the start of exposure, and also interferes with the uptake of several elements and water from root apices. Although many different mechanisms of Al toxicity have been hypothesized, they have not yet been fully characterized (for reviews, see Foy, 1974; Foy et al., 1978; Haug, 1984; Taylor, 1988; Rengel, 1992; Kochian, 1995).

The primary site of Al accumulation and toxicity in the plant is reported to be the root meristem, which consists of actively dividing cells (Rincón and Gonzales, 1992; Ryan et al., 1993). At the cellular level, Al is mainly localized in the cell wall, although some is found in the nucleus. Although the ultimate target that mediates Al toxicity has not yet been identified, an increase in the rigidity of the actin network (Grabski and Schindler, 1995) and the inhibition of phospholipase C (Jones and Kochian, 1995) have been proposed as possible intracellular Al target sites that may be involved in root growth (for reviews, see Rengel, 1992; Delhaize and Ryan, 1995; Kochian, 1995).

We have previously investigated the responses of tobacco (*Nicotiana tabacum* L.) suspension culture cells (Yamamoto et al., 1994; Ono et al., 1995) to Al. The sensitivity of tobacco cells to Al changes depending on the phase of growth. Cells at the logarithmic phase are sensitive to Al, whereas cells at the stationary phase are resistant to it. Logarithmic-phase cells start to accumulate Al after about 10 h of exposure, and there is a good correlation between the extent of growth inhibition by Al and the amount of Al accumulated in the cells. However, since the stationary-phase cells do not take up Al, it appears that accumulation of Al, such as that which occurs in actively growing cells (Yamamoto et al., 1994), is a prerequisite for Al toxicity. Although we have not determined the cellular sites to which Al binds, the localization of Al in tobacco cells has been estimated by staining with hematoxylin, which indicated the presence of Al over the entire cell surface and in the nucleus. Because Al that has accumulated in cells is not released by chelators (EDTA and citrate), but is instead retained in cells after digestion of the cell walls by protoplasting enzymes, it seems likely that Al is either absorbed by the cell or is tightly bound to the plasma membrane or some minor part of the cell wall that remains after digestion (Yamamoto et al., 1994).

It has been reported that the peroxidation of lipids is caused by Al in the presence of Fe(II). In liposomes prepared with phospholipids from bovine brain, Gutteridge et al. (1985) observed that Al did not stimulate the peroxidation of lipids, but greatly accelerated the peroxidation induced by Fe(II) at low pH. Therefore, they suggested that Al ions cause an alteration in membrane structure that facilitates lipid peroxidation.

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Abbreviations: DPPD, *N,N'*diphenyl-*p*-phenylenediamine; FDA, fluorescein diacetate; ferrozine, 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-P,P'-disulfonic acid; Fe(II), ferrous ion; Fe(III), ferric ion; MDA, malondialdehyde; MS, Murashige and Skoog; PCV, pyrocatechol violet; TBA, thiobarbituric acid.

In root tips of soybean (*Glycine max*), it has been reported that peroxidation of lipids is induced after treatment with Al at concentrations that inhibit root elongation substantially (Cakmak and Horst, 1991: Horst et al., 1992). Soybean seedlings were treated with Al in nutrient solution, which enhanced the peroxidation of lipids caused by Fe(II). However, it has not been determined whether the Al-induced lipid peroxidation is a cause or an effect of cell death.

In cultured tobacco cells treated with Al in nutrient medium, we found that Al and Fe(II) act synergistically to reduce the viability of cells. Furthermore, cells treated with both Al and Fe(II) exhibit several responses indicative of the perturbation of the plasma membrane: a loss of potassium, a loss of plasmolyzing ability, a decrease in the extent of staining with FDA, and an increase in the extent of lipid peroxidation. Cells treated with either Al or Fe(II) alone do not exhibit any of these responses. On the basis of these results, we suggest that the peroxidation of lipids caused by Al in the presence of Fe alters the permeability of the plasma membrane and leads to cell death (Ono et al., 1995).

In this report we will show that the peroxidation of lipid induced by Al and Fe(II) is a cause of cell death in cultured tobacco cells, and that tobacco cells become tolerant to Al if they are deprived of Pi. The possible mechanism of Al tolerance exhibited by Pi-starved cells may involve a system that protects the plasma membrane from oxidative stress caused by the combination of Al and Fe.

MATERIALS AND METHODS

Strain, Media, and Culture Conditions

The nonchlorophyllic tobacco cell line SL (*Nicotiana tabacum* L. cv Samsun) (Nakamura et al., 1988; Nagai et al., 1989) was generously provided by Dr. C. Nakamura of Kobe University, Japan. Cells were grown in a modified version of MS medium (pH 5.8) that contained MS salts (macronutrients, 18.8 mM KNO_3, 20.6 mM NH_4NO_3, 1.25 mM KH_2PO_4, 3.0 mM $CaCl_2$, 1.5 mM $MgSO_4$; and micronutrients, 100 μM $FeSO_4$ with 100 μM Na_2-EDTA, 5 μM KI, 100 μM H_3BO_3, 92 μM $MnSO_4$, 30 μM $ZnSO_4$, 1 μM Na_2MoO_2, 0.1 μM $CuSO_4$, 0.1 μM $CoCl_2$), B5 vitamins (550 μM inositol, 30 μM thiamin-HCl, 5 μM pyridoxine-HCl, 6 μM nicotinic acid), Suc (3%), and 2,4-D (1.5 μM) on a rotary shaker operated at 100 rpm at 25°C in the darkness, as described previously (Yamamoto et al., 1994). Cells were serially subcultured at 7-d intervals at a dilution of 1:15 (7-d subculture). During the subculture, cells exhibited a logarithmic phase of growth from d 2 to 5 and reached the stationary phase on d 7. For Pi starvation, cells at the logarithmic phase on d 4 of the 7-d subculture were washed twice with Pi-free, modified MS medium (pH 5.8), resuspended in the same medium at a cell density of approximately 200 mg fresh weight/10 mL, and cultured for various numbers of days. Because both Pi and EDTA reduce the toxicity of Al (Yamamoto et al., 1995), Pi-EDTA-$FeSO_4$-free modified MS medium (medium A, pH 5.0) was used for washing cells before and after Al treatment, and Pi-EDTA-free modified MS medium (medium B, pH 4.0) was used for the Al treatment. For the preparation of medium B, a freshly prepared, filter-sterilized, 10 mM solution of $FeSO_4$ was added to medium A to give a final concentration of 100 μM $FeSO_4$ just before use.

Fe(II), one of the components of medium B present at 100 μM $FeSO_4$, acts synergistically with Al to cause the peroxidation of lipids and the loss of cell viability (Ono et al., 1995). Fe(III) (100 μM Fe[III]-EDTA) also stimulates the toxic effects of Al (Y. Yamamoto, A. Hachiya, and H. Matsumoto, unpublished data). However, Fe(III) needs a chelator such as EDTA to dissolve in water, and since EDTA also chelates Al, treatment with Al and Fe(III)-EDTA is not advisable. Therefore, we used Fe(II) instead of Fe(III) to investigate the interactive effect of Fe on Al cytotoxicity.

Quantitation of Monomeric Al in Solution

The amount of Al in the monomeric form in medium B was measured by the colorimetric method using PCV, as described by Menzies et al. (1992), with modifications to allow the reaction with Al (up to 200 μM) to occur in a linear range. A standard solution of Al (1000 parts per million, Ishizu Seiyaku, Osaka, Japan) was diluted in either medium B or 1.0 mM HCl (as a standard) to a final concentration of 0 to 200 μM. Then, 0.5 mL of each sample was added to 0.1 mL of the Fe interference reagent. After reaction for 1 min, 0.2 mL of the PCV reagent and 4 mL of 0.25 M imidazole buffer (pH 5.6) were added. After 20 min, the A_{578} was determined.

Treatment with Al

Cells were treated with Al and viability was determined as described previously (Yamamoto et al., 1994). For Al treatment, a solution of 10 mM $AlCl_3$ was prepared just before use and sterilized by passage through a 0.2-μm filter. Cells were washed three times with medium A and then treated with various concentrations of $AlCl_3$ in medium B at a cell density of 100 mg fresh weight/10 mL for various times on a rotary shaker operated at 100 rpm at 25°C. After the treatment, cells (from 10-mL aliquots of the culture, corresponding to 100 mg fresh weight at the start of the treatment) were harvested and washed twice with medium A. The cells were analyzed to determine viability, the amount of Al and Fe, or the ability to reduce Fe(III) to Fe(II), as described below. For the determination of viability, cells from 10-mL aliquots of the treatment cultures were cultivated in 30 mL of modified MS medium (pH 5.8) for 7 to 9 d (posttreatment culture). After the posttreatment culture, the cells were harvested by vacuum filtration on filter paper and their fresh weight was determined. Viability of Al-treated cells was estimated from their growth relative to that of untreated control cells as follows: viability = $(B/A) \times 100$ (A, fresh weight of untreated control cells; B, fresh weight of Al-treated cells). In another experiment, cells were treated with Al in the presence of DPPD, a lipophilic antioxidant, which was dissolved in DMSO and added to medium B at a concentration of 20 μM at the start of the treatment. The concentration of DMSO in the medium was 0.1% or less, which apparently had no effect on cell growth or on sensitivity to Al (data not shown).

Quantitation of Al, Fe, and Pi in Cells

Ten-milliliter aliquots of the cultures treated with or without Al were centrifuged at 500*g* for 5 min. The supernatant was collected for the direct determination of the amount of Al, and the pelleted cells were washed twice with medium A and then digested in the acid mixture described previously (Yamamoto et al., 1994). Concentrations of Al and Fe in the supernatant and the digested sample were determined by a simultaneous multi-element atomic absorption spectrophotometer with a graphite furnace atomizer (model Z-9000, Hitachi, Tokyo, Japan). For the quantitation of Al or Fe that had accumulated in cells during Al treatment, the amounts just before treatment were subtracted from the amounts in the cells after Al treatment. The level of P in 100 mg fresh weight of cells was determined as described elsewhere (Fiske and Subbarow, 1925) after digestion in the acid mixture described previously (Yamamoto et al., 1994).

Quantitation of Organic Acids in the Medium

The levels of malic and citric acid in the medium were determined by enzymic methods described previously (Dagley, 1974; Gutmann and Wahlefeld, 1974; Delhaize et al., 1993) but with minor modifications. The medium was evaporated at 50°C under a vacuum and the residue was resuspended in one-tenth volume of distilled water.

Assessment of Lipid Peroxidation

The peroxidation of lipids in cells was assessed by the TBA test, in which MDA is quantitated as an end product, as described previously (Ono et al., 1995). Cells from 10-mL aliquots of a culture that had been treated with or without Al, as described above, were washed twice with medium A without Suc and then suspended in 0.1% TCA (final concentration; final volume, 1 mL) and disrupted with a sonicator. Then, 0.5 mL of the sonicate was added to 3 mL of 1% H_3PO_4 and 1 mL of a 0.6% aqueous solution of TBA. The mixture was heated for 45 min, and then 4 mL of *n*-butanol was added with vigorous mixing. The A_{532} of the MDA-TBA complex in the butanol phase was determined. The value for nonspecific A_{520} was subtracted and the amount of MDA was calculated.

To determine if lipid peroxidation was a cause or an effect of cell death, we added DPPD, an effective inhibitor of the peroxidation of membrane lipids (Chen et al., 1990), to a cell suspension during treatment with Al, and examined the effect on cell viability.

Reduction of Fe(III) to Fe(II) by Cells

The reducing capacity of cells was determined as described by Horst et al. (1992) with a minor modification. Cells (200 mg fresh weight) were suspended in 10 mL of medium A that contained 100 μM $FeCl_3$-EDTA and 300 μM ferrozine. After aeration for 1 h, the formation of the Fe(II)-ferrozine complex was monitored at 562 nm (molar extinction coefficient = 27,900).

Analysis of Carotenoids

The carotenoids in cells were examined as described previously (Masamoto et al., 1993). The pigments were extracted from cells (500 mg fresh weight) twice with 90% acetone (5 mL) under dim light at room temperature. The pooled extracts were centrifuged for 3 min at 4000*g* and the resulting supernatant was filtered. The extracted pigments were separated by reverse-phase HPLC. The samples were loaded on a C_{18} column (LiChrosphere 100RP-18, 250 × 4 mm, Cica-MERK, Kanto Chemical Co., Tokyo, Japan) and the column eluted at a flow rate of 1.0 mL/min at 25°C. The mobile phase was 100% methanol for the first 5 min, and was then changed at a constant rate to 99.5% ethanol during the next 5 min. Finally, the column was eluted with 99.5% ethanol for 10 min. The eluate was monitored at 450 nm. Each peak after HPLC was identified by both its retention time and its absorption spectrum, and compounds were quantified by reference to authentic pigments and from comparisons with published values, as described previously (Masamoto et al., 1993). For the unidentified carotenoids eluted before and after β-carotene (see Fig. 6B, peaks 5 and 7), the calibration coefficients of violaxanthin and β-carotene, respectively, were used tentatively because of the similarity of their absorption spectra. The flavonoid content of cells was measured after extraction of the samples by a previously reported method (Yoshitama et al., 1992).

RESULTS

Estimation of the Viability of Al-Treated Tobacco Cells

The colorimetric determination with PCV indicated that more than 90% of the Al added to medium B (up to 200 μM) was present in monomeric form (Table I). During the Al treatment in medium B, the number of the cells in both control and Al-treated cultures did not change significantly (Yamamoto et al., 1994). After the Al treatment, when the cells were resuspended in nutrient solution and cultured, the growth of the treated cells was significantly retarded, depending on the Al concentration used for the treatment (Fig. 1) (Yamamoto et al., 1994). Loss of viability of Al-treated cells occurred before the posttreatment culture.

Table I. *Aluminum present in monomeric form in medium B (pH 4.0)*

Solutions of Al at various concentrations (up to 200 μM) were prepared in either medium B (pH 4.0) or 1.0 mM HCl (as a standard). The levels of soluble forms of Al in these solutions were then compared by the PCV colorimetric method; A_{578} of the complex formed between PCV and Al was determined as described in "Materials and Methods." Data show the means ± SE of results from three independent experiments.

Concentration of Al Added in Medium	Monomeric Al in Medium
μM	μM
50	49.0 ± 0.10
100	98.3 ± 0.01
150	138.9 ± 2.20
200	182.5 ± 1.00

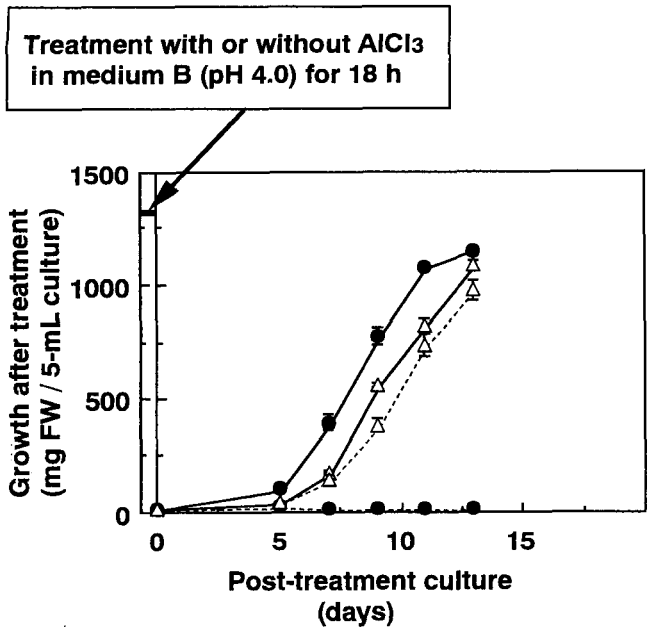

Figure 1. Growth of +P cells and −P cells after treatment with Al. +P cells and −P cells (6-d starvation) were treated without $AlCl_3$ in medium B (pH 4.0) as a control treatment or with $AlCl_3$ (120 μM) in medium B (pH 4.0) for 18 h at a cell density of 100 mg fresh weight/10 mL as described in "Materials and Methods." After the Al treatment, the cells (from 10-mL aliquots) were washed and then cultured in 30 mL of growth medium without Al for up to 13 d. At the indicated times during the posttreatment culture, fresh weights of cells in 5-mL aliquots were determined. All of the data show the means ± SE of results from four samples from two independent experiments. ● (solid line), control +P cells; ● (broken line), Al-treated +P cells; △ (solid line), control −P cells; △ (broken line), Al-treated −P cells.

This was confirmed by observing the reduction in vital staining with FDA and in the plasmolysis of Al-treated cells in 1 M mannitol (Ono et al., 1995). Cell mortality is often measured by a staining procedure using e.g. Evans blue (Levine et al., 1994); however, this procedure was not appropriate for a quantitative estimation of viability of the tobacco cell line used in this report, since even living cells were stained rather quickly and substantially (data not shown).

The Peroxidation of Lipids by Al and Fe(II) Is a Cause of Cell Death

Since the lipophilic antioxidant DPPD was very effective in preventing both the peroxidation of lipids and cell death (Table II), we concluded that the peroxidation of lipids is a cause of cell death.

Tolerance to Al Exhibited by −P Cells

During culture in the Pi-starved medium, the growth rate of the cells was repressed gradually (Fig. 2A) and the content of Pi in the cells decreased in parallel (Fig. 2B).

After the control treatment, the −P cells grew at the same rate as the +P cells in growth medium. However, the increase in the fresh weight of the −P cells was delayed by about 1 d compared with that of the +P cells, indicating that −P cells started to grow normally once sufficient Pi was supplied. After treatment with Al, the −P cells grew at a higher rate than the Al-treated +P cells. Viability estimated after posttreatment culture for 9 d was 69% for the −P cells and 3% for the +P cells (Fig. 1).

The viability of cells after treatment with Al (100 μM $AlCl_3$ for 18 h) increased almost linearly with increases in the duration of the Pi starvation, reaching a maximum value of 76% on d 4 (Fig. 2C). The Pi content of the −P cells that had been starved for 4 d was 56% of that of the +P cells (Fig. 2B). These results indicate that +P cells acquired maximum tolerance to Al when they had lost nearly half of the total cell content of Pi.

Figure 3 shows the viability of +P cells and −P cells (6-d starvation) after exposure to various concentrations of Al for 18 h. At 150 μM $AlCl_3$, +P cells almost completely lost their viability, whereas 43% of the −P cells remained viable. These results indicate that −P cells were more tolerant to Al than +P cells.

Decrease in the Accumulation of Both Al and Fe in −P Cells

+P cells accumulated 961 nmol Al/100 mg fresh weight, which corresponds to almost all of the Al added to the medium. Compared with the +P cells, all of the −P cells, which had been starved of Pi for 2 to 10 d, accumulated only 55 to 74% of the Al accumulated in the +P cells (Fig. 2D).

The endogenous levels of Fe did not change significantly during the starvation of Pi (data not shown). Figure 2D shows the amount of Fe accumulated during the treatment with Al. +P cells accumulated 152 nmol Fe/100 mg fresh weight, whereas the −P cells accumulated only 10 to 30% of that amount. The amount of Fe accumulated in the +P and −P cells was inversely correlated with the degree of tolerance to Al exhibited by the cells (Fig. 2, C and D).

Table II. *Effects of DPPD on the peroxidation of lipids and the loss of viability caused by Al and Fe(II) in tobacco cells*

+P cells were treated with or without 200 μM $AlCl_3$ in medium B (pH 4.0) in the presence or absence of 20 μM DPPD at a cell density of 100 mg fresh wt/10 mL for 18 h. The cells (from 10-mL aliquots) were harvested for determinations of the peroxidation product (MDA) and of viability, as described in "Materials and Methods." Data show the means ± SE of results from three samples from three independent experiments.

	−DPPD		+DPPD	
	−Al	+Al	−Al	+Al
Lipid peroxidation (nmol MDA 100 mg^{-1} fresh wt)	0.12 ± 0.08	3.36 ± 1.13	0.09 ± 0.06	0.03 ± 0.03
Viability (% of control)	100	3 ± 2	100	108 ± 3

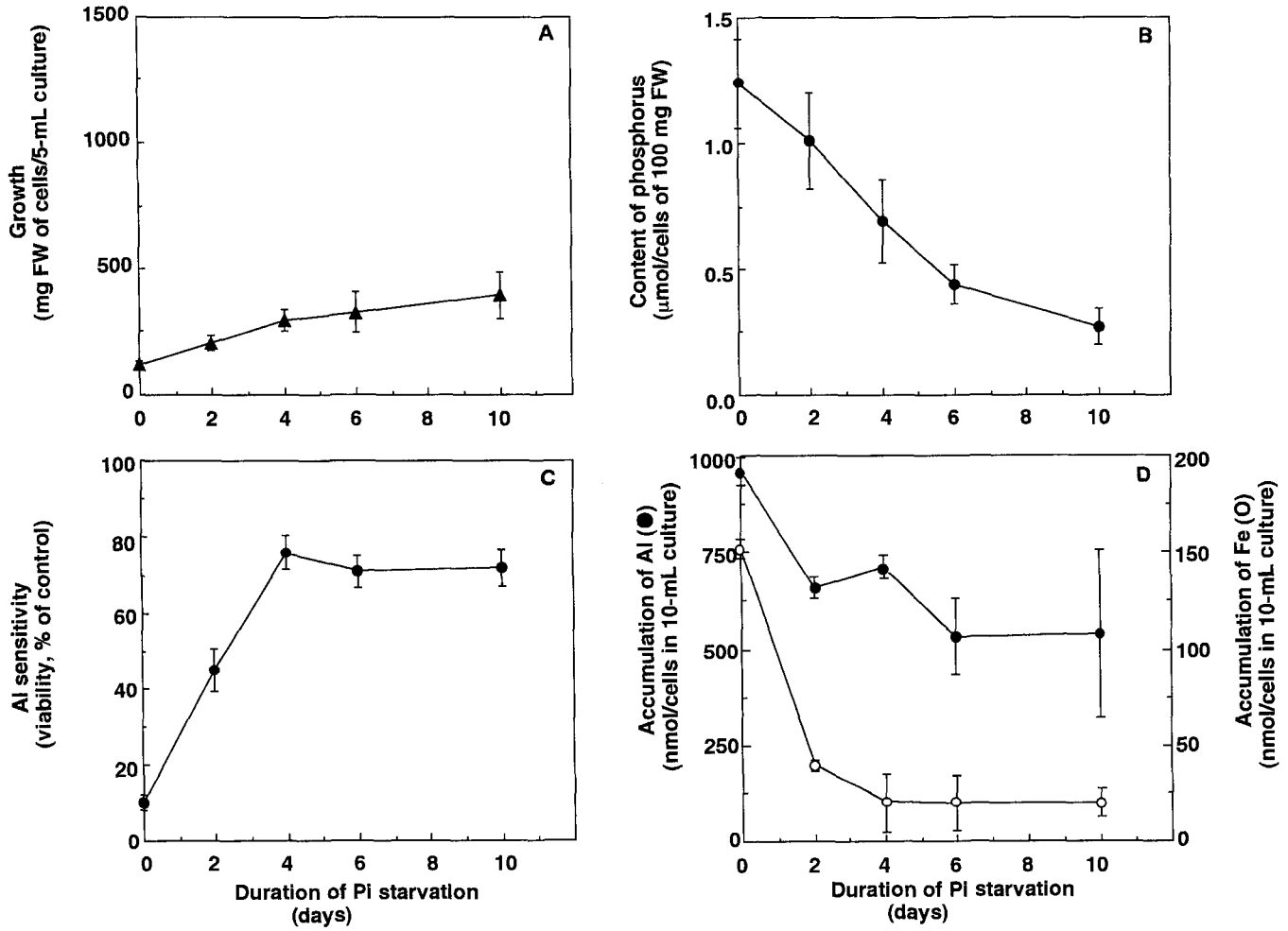

Figure 2. Changes in the amount of P and in the responses to Al (the sensitivity to Al, the accumulation of Al, and the accumulation of Fe) in cells during Pi starvation. +P cells were cultured in Pi-free modified MS medium for up to 10 d. At the indicated times, the growth (fresh weight of cells in 5 mL of culture) and the amount of P in the cells were monitored. The cells then were treated without (control) or with $AlCl_3$ (100 μM) in medium B (pH 4.0) for 18 h at a cell density of 100 mg fresh weight/10 mL. The cells (from 10-mL aliquots) were harvested for determinations of viability and the amounts of Al and Fe accumulated during the treatment with Al, as described in "Materials and Methods." All data show the means ± SE of the results from three independent experiments. A, Growth of cells in Pi-free medium; B, the amount of P in cells; C, Al sensitivity; and D, the accumulation of Al and Fe in cells during Al treatment.

Time Courses of the Accumulation of Al and Fe and the Loss of Cell Viability

We previously reported that there is a delay of about 10 h between the addition of Al to a suspension of cells and the start of the uptake of Al (Yamamoto et al., 1994). Figure 4 confirms this and indicates more clearly that most of the Al added to a suspension of +P cells remained in the medium for 8 h and then started to accumulate in the cells. Fe ions added to a cell suspension also remained in the medium before accumulating in the cells (data not shown).

In +P cells, Al began to accumulate 10 h after the start of exposure, reaching a maximum level of 804 nmol/cells in a 10-mL culture at 18 h; the level then decreased gradually until 44 h. The amount of Fe in the +P cells began to increase at 12 h, increasing gradually until it reached a maximum value of 722 nmol/cells in 10 mL of culture at 44 h. Thus, the +P cells had accumulated most of the Fe added to the medium during the 44-h exposure. The viability of the +P cells did not change until 10 h, but then it rapidly decreased at 12 h, reaching a minimum value of 24% at 18 h (Fig. 5C). When the +P cells were treated under the same conditions but without Al, Fe did not accumulate in the cells (Fig. 5A) and the viability of the cells was not affected by 100 μM $FeSO_4$ alone (Ono et al., 1995).

Al and Fe began to accumulate in −P cells at almost the same time as they did in +P cells, but increased more slowly and less extensively. At 24 h of exposure, the content of Al in the −P cells reached a maximum value of 399 nmol/cells in 10 mL of culture, 55% of that in the +P cells. The content of Fe in the −P cells reached a maximum value of 227 nmol/cells in 10 mL of culture at 44 h (Fig. 5B). Thus, the accumulation of both Al and Fe was repressed in the −P cells. The viability of the −P

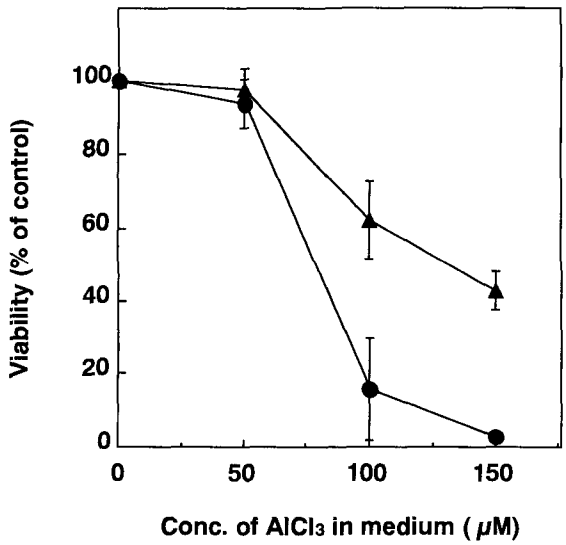

Figure 3. The viability of +P cells and −P cells after treatment with Al. +P cells and −P cells (6-d starvation) were treated with various concentrations of $AlCl_3$ in medium B (pH 4.0) for 18 h. After the 7-d posttreatment culture, the viability of the cells was determined as described in "Materials and Methods." All data show the means ± SE of results from four samples from two independent experiments. ●, +P cells; ▲, −P cells.

cells decreased at 18 h, and reached a minimum value of 69% at 24 h (Fig. 5C).

A rapid accumulation of Al in both +P and −P cells was the first event observed approximately 10 h after the addition of Al to a cell suspension. It was followed by the accumulation of Fe and a loss of cell viability that occurred almost simultaneously. We found that the kinetic pattern of loss of viability was negatively correlated with that of the

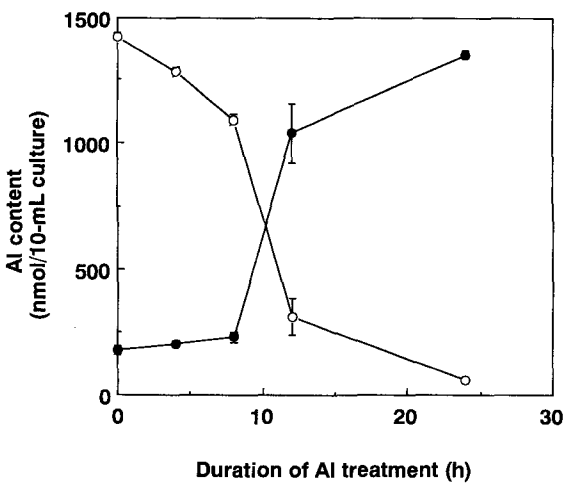

Figure 4. The amount of Al in the treatment medium and in cells during exposure of +P cells to Al. +P cells were treated with $AlCl_3$ (150 μM) in medium B (pH 4.0) at a cell density of 100 mg fresh weight/10 mL for 24 h. At the indicated times, 10-mL aliquots were withdrawn and centrifuged. The amount of Al in the supernatant (○) and in the cells (●) was determined as described in "Materials and Methods." All data show the means ± SE of results from four samples from two independent experiments.

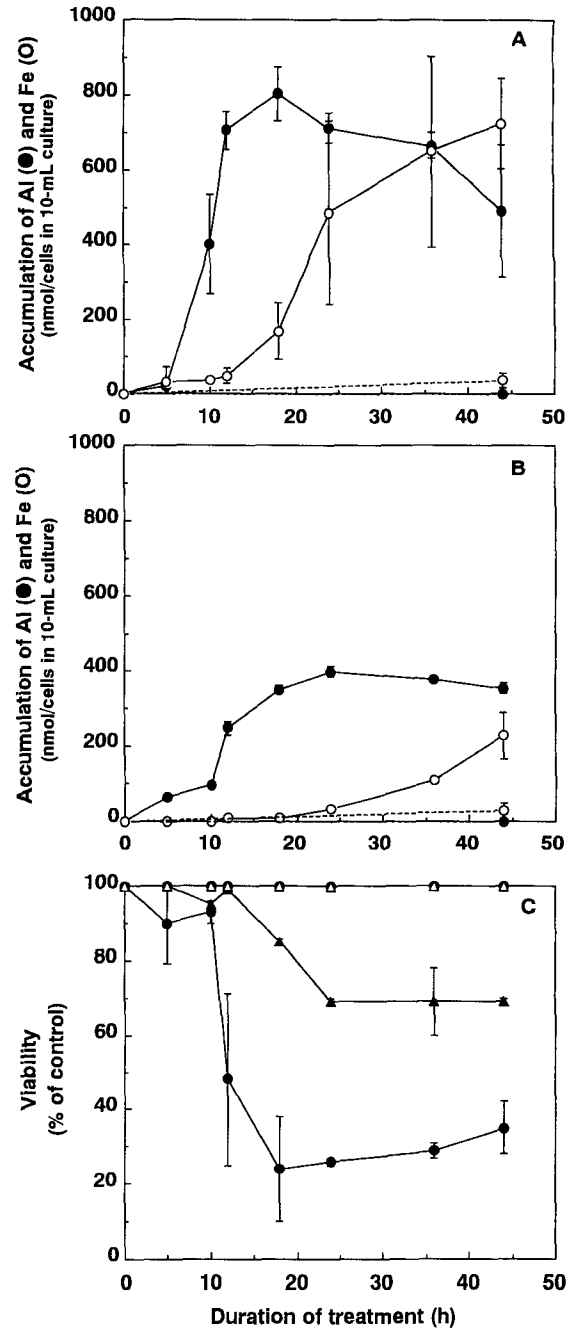

Figure 5. Time course of the responses to Al (the accumulation of Al, the accumulation of Fe, and the loss of viability) of +P cells and −P cells during treatment with Al. +P cells and −P cells (6-d starvation) were treated without (control) or with $AlCl_3$ (100 μM) in medium B (pH 4.0) at a cell density of 100 mg fresh weight/10 mL for 44 h. At the indicated times, the cells (from 10-mL aliquots) were harvested for determinations of the amounts of Al and Fe accumulated during the treatment, as well as for viability, as described in "Materials and Methods." All data show the means ± SE of results from three samples from two independent experiments. A, The accumulation of Al (●) and Fe (○) in +P cells during treatment with (solid line) or without (broken line) Al; B, the same as A, but in −P cells; C, the viability of +P cells (●) and −P cells (▲) after the treatment with Al, or without Al, which corresponds to 100% viability at each time, shown with open symbols.

accumulation of Al, but not with that of the accumulation of Fe in either +P or −P cells. This suggests that the amount of Al that accumulates in cells is the major determinant of the extent of cell death, whereas the accumulation of only a small amount of Fe is sufficient to cause cell death. The exclusion of both Al and Fe, particularly Fe, from −P cells might be responsible for the tolerance to Al demonstrated in this study.

−P Cells do not Excrete Malic and Citric Acids Significantly

The lower rate of Al accumulation in −P cells compared with +P cells might have been caused by the enhancement of organic acid (e.g. malic and citric acid) excretion, which could act to prevent the accumulation of Al in −P cells. However, both +P cells and −P cells (6-d starvation) excreted only a small amount of these organic acids at almost the same rates during treatment with or without Al (Table III).

Lipid Peroxidation Products Are Not Detected in −P Cells after Al Treatment

TBA-reactive products (MDA) in the cells were quantitated. The +P cells treated with Al generated 27-fold more MDA than did the same cells treated without Al, whereas the −P cells treated with Al did not generate MDA to any significant extent (Table IV), an indication that the peroxidation of lipids did not occur in −P cells during the treatment with Al.

−P Cells Retain Reducing Capacity for Conversion of Fe(III) to Fe(II) during Al Treatment

Treatment with 120 μM $AlCl_3$ for 18 h decreased the reducing capacity of the +P cells to 23% of that of the same cells treated without Al, whereas the −P cells retained 100% of the reducing capacity exhibited by the same cells treated without Al (Table IV). This was an indication that the plasma membrane of −P cells was still functional, whereas the membrane of +P cells was dysfunctional, likely due to the extensive peroxidation of lipids during Al treatment (as described above).

Table III. *Amounts of malic acid and citric acid released from +P cells and −P cells during treatment with Al*

+P cells and −P cells (6-d starvation) were treated with or without 120 μM $AlCl_3$ in medium B (pH 4.0) at a cell density of 100 mg fresh wt/10 mL for 18 h. Ten-milliliter aliquots were withdrawn and centrifuged to obtain the supernatants. The amounts of malic acid and citric acid in the supernatants were determined as described in "Materials and Methods." Data show the means ± SE of results from four samples from two independent experiments.

Organic Acid Released (nmol 100 mg^{-1} fresh wt)	+P cells		−P cells	
	−Al	+Al	−Al	+Al
Malic acid	263 ± 11	215 ± 24	205 ± 7	213 ± 17
Citric acid	113 ± 6	144 ± 19	144 ± 6	163 ± 6

Accumulation of Carotenoids during Pi Starvation

HPLC profiles of the carotenoid pigments extracted from +P cells revealed the presence of neoxanthin (peak 1), violaxanthin (peak 2), antheraxanthin (peak 3), zeaxanthin (peak 4), and β-carotene (peak 6) (Fig. 6A). −P cells (6-d starvation) exhibited increases in the amount of these components, particularly neoxanthin and β-carotene (Fig. 6B). Peaks 5 and 7, eluted before and after β-carotene, also increased in area after Pi starvation. They were assumed to represent β-carotene-epoxide and *cis*-β-carotene, respectively, in view of their retention times and absorption spectra (data not shown). Neither α-carotene nor lutein was detected in +P or −P cells.

Table V shows the amount of each carotenoid (pmol/mg fresh weight) in +P cells and in −P cells. The amount of β-carotene in the −P cells was 13-fold higher than that in the +P cells. Amounts of neoxanthin, violaxanthin, and the unidentified components (peaks 5 and 7 in Fig. 6) were also increased significantly by Pi starvation. Cells cultured with Pi for 6 d reached late logarithmic phase to stationary phase at a cell density of 983 ± 40 mg fresh weight/5-mL culture (mean ± SE, n = 3). From the late logarithmic phase to the stationary phase, the amounts of all of the carotenoid components in the cells were significantly lower than those in the +P cells. Compared with the +P cells, the amount of total carotenoid was 3.4-fold higher in the −P cells, and only 0.6 as much in the cells at stationary phase. These results indicate that the increase in carotenoid components, in particular β-carotene, in −P cells was caused specifically by the starvation of Pi and not by general starvation of nutrients.

Flavonoids were not detected at significant levels in either +P cells or in cells starved of Pi for up to 10 d (data not shown).

DISCUSSION

The sensitivity of cultured tobacco cells to Al changes depending on which solutions are used during Al treatment (e.g. nutrient medium, $CaCl_2$ solution) (see below). We studied Al toxicity in nutrient medium instead of $CaCl_2$ solution because the physiological condition of cells cultured in nutrient medium can change greatly in $CaCl_2$ solution. Furthermore, it is to be expected that plants will be exposed to Al in the presence of Fe and other mineral nutrients in acid soils, so it is interesting to examine the mechanism(s) of Al toxicity in nutrient medium.

Al and Fe(II) Synergistically Cause Oxygen Stress in Tobacco Cells in Nutrient Medium

In nutrient medium, Al or Fe(II) alone is not toxic to tobacco cells, but Al and Fe(II) synergistically cause a loss of cell viability (Ono et al., 1995). The combination of these two ions causes the accumulation of Al, the accumulation of Fe, the peroxidation of lipids, a decrease in the amount of potassium in cells, a decrease in the number of cells that can be plasmolyzed in 1 M mannitol, and a decrease in the number of cells that can be stained with FDA. These results strongly suggest that Al causes dysfunction of the plasma membrane in the presence of Fe(II) and that cell death

Table IV. *Effects of Al on the peroxidation of lipids and on the capacity to reduce from Fe(III) to Fe(II) in +P cells and −P cells*

+P cells and −P cells (6-d starvation) were treated with or without 120 μM $AlCl_3$ in medium B (pH 4.0) at a cell density of 100 mg fresh wt/10 mL for 18 h. They were then assessed for the peroxidation product (MDA) and the capacity to reduce Fe(III) to Fe(II), as described in "Materials and Methods." Data show the means ± SE of results from four samples from two independent experiments.

	+P cells		−P cells	
	−Al	+Al	−Al	+Al
Lipid peroxidation (nmol MDA 100 mg^{-1} fresh wt)	0.10 ± 0.05	2.67 ± 0.81[a]	0.05 ± 0.03	0.05 ± 0.03
Reducing capacity from Fe(III) to Fe(II) (μmol 200 mg^{-1} fresh wt)	0.135 ± 0.009	0.031 ± 0.005[b]	0.134 ± 0.027	0.136 ± 0.015

[a] 27-fold value of −Al. [b] 0.23-fold value of −Al.

occurs due to loss of membrane integrity (Ono et al., 1995). The autoxidation of lipids mediated by free radicals is usually (but not always) a deleterious process (for review, see Niki, 1987). In the cytotoxicity caused by the combination of Al and Fe in cultured tobacco cells, lipid peroxidation seems to be essential for cell death, because the antioxidant DPPD, an effective inhibitor of peroxidation of membrane lipids (Chen et al., 1990), also prevents the loss of viability (Table II).

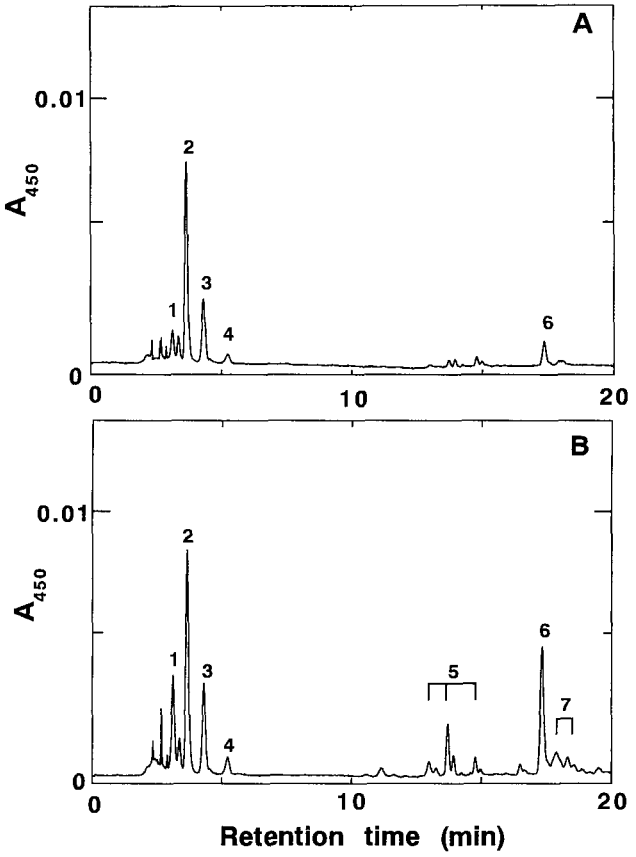

Figure 6. Profiles after HPLC of carotenoid pigments from +P cells and −P cells. Carotenoid pigments were extracted from +P cells and −P cells (6-d starvation) as described in "Materials and Methods" and analyzed by reverse-phase HPLC (LiChrosphere 100RP-18, 250 × 4 mm) at a flow rate of 1.0 mL/min. The mobile phase was 100% methanol for 5 min, which was then changed to 99.5% ethanol over the next 5 min, and then the column was eluted with 99.5% ethanol for 10 min. The contents of the numbered peaks in A, +P cells, and B, −P cells, were deduced as follows: 1, neoxanthin; 2, violaxanthin; 3, antheraxanthin; 4, zeaxanthin; 5, unidentified carotenoids; 6, β-carotene; 7, unidentified carotenoid.

The accumulation of Al in cells seems to be a prerequisite for cell death because it is strongly correlated with the extent of cell death (Yamamoto et al., 1994). Furthermore, the accumulation of Al is the first event observed after the addition of Al to cell suspensions, followed by the almost simultaneous accumulation of Fe and loss of viability (Fig. 5). The peroxidation of lipids is observed concurrently with the accumulation of Fe (Y. Yamamoto, A. Hachiya, and H. Matsumoto, unpublished data).

Kinetic patterns indicate that the accumulation rate of Al is much faster than that of Fe (Fig. 5), suggesting that the binding sites or mechanisms of these ions might be different. Cells that have lost viability continue to accumulate Fe (Fig. 5).

The accumulation of a large amount of Al is necessary for cell death, whereas only a small amount of Fe is sufficient (Fig. 5). Transition metals such as Fe(II), Fe(III), and copper play an important role in forming the hydroxyl radical, the alkoxy radical, and the lipid peroxyl radical at the initiation and propagation steps of a free-radical chain reaction during the oxidation of lipids (for review, see Niki, 1987). Thus, one possible explanation for the cell death caused by Al and Fe is that Al binds to the plasma membrane, altering its structure and rendering it permeable to Fe(II) or Fe(III), which enter the cell and catalyze the peroxidation of lipids, eventually causing cell death.

Stass and Horst (1995) reported short-term responses to Al in cultured soybean (*G. max*) cells treated with Al in a solution containing Suc (2%) and $CaCl_2$ (500 μM) (pH 4.3). In this system, Al did not initiate or enhance Fe(II)-promoted lipid peroxidation. This is in agreement with the response to Al in tobacco cells treated with Al and Fe(II) in a solution containing Suc (3%) and $CaCl_2$ (3 mM) (pH 5.0) (Y. Yamamoto, unpublished data). Furthermore, in the $CaCl_2$ solution, either Al alone or Fe(II) alone (at a concentration up to 50 μM) severely inhibits the growth of tobacco cells, whereas the synergistic inhibition of the growth by Al and Fe(II) is not observed (Y. Yamamoto, unpublished data). Thus, it seems that the enhancement of both the peroxidation of lipids and cell death by the combination of Al and Fe(II) occurs in nutrient medium, but not in a $CaCl_2$ solution. The mechanism of this difference is now under investigation in our laboratory.

Table V. *Amounts of various carotenoids in +P cells and −P cells*

+P cells were subcultured with or without Pi for 6 d. The cells subcultured with Pi for 6 d reached late log to stationary phase. The pigments were extracted from these cells and were analyzed by HPLC as shown in Figure 6. Data show the means ± SE of results from four independent experiments.

Pigment	Carotenoid Content		
	+P cells (log phase)	−P cells	+P cells (late log—stationary phase)
	pmol mg^{-1} fresh wt		
Neoxanthin	0.32 ± 0.18	1.31 ± 0.21 (4.1)[a]	0.21 ± 0.05 (0.7)[b]
Violaxanthin	0.96 ± 0.33	2.80 ± 0.53 (2.9)[a]	0.59 ± 0.03 (0.6)[b]
Antheraxanthin	0.55 ± 0.14	0.85 ± 0.11 (1.5)[a]	0.27 ± 0.08 (0.5)[b]
Zeaxanthin	0.11 ± 0.04	0.13 ± 0.02 (1.2)[a]	0.04 ± 0.03 (0.4)[b]
Unidentified carotenoids	0.18 ± 0.07	0.90 ± 0.18 (5.0)[a]	0.12 ± 0.02 (0.7)[b]
β-Carotene	0.09 ± 0.05	1.19 ± 0.58 (13.2)[a]	0.04 ± 0.01 (0.4)[b]
Total carotenoid	2.20 ± 0.74	7.42 ± 1.58 (3.4)[a]	1.28 ± 0.07 (0.6)[b]

[a] Parentheses indicate value of −P/+P. [b] Parentheses indicate value of +P (stationary)/+P (log).

Pi-Deficient Cells Are Tolerant to Al

After exposure to Al, the viability of −P cells is higher than that of +P cells (Figs. 1, 2, 3, and 5); −P cells starved of Pi for 4 d or more acquire maximum tolerance to Al. Cells become resistant to Al toxicity if they can exclude either Al or Fe or both during Al treatment; −P cells accumulate 30 to 40% less Al than do +P cells (Fig. 2D).

Organic acids such as malic acid or citric acid are supposed to chelate Al, thereby excluding it from root apices (Delhaize et al., 1993; for review, see Delhaize and Ryan, 1995). Furthermore, it has been reported that the exudation of these organic acids is enhanced in response to Pi starvation in whole-plant systems (Lipton et al., 1987; Hoffland et al., 1989). In carrot (*Daucus carota* L.) cell systems cultured in a medium that contains Al-phosphate as the sole source of Pi, cells excrete citric acid in response to Al-induced P deficiency. Since citric acid chelates Al in Al-phosphate, it was proposed that the solubilized Pi is utilized for cell growth (Ojima and Ohira, 1983; Koyama et al., 1990). However, in our system, the organic acids did not contribute to the exclusion of Al in −P cells because both +P cells and −P cells excreted the two organic acids at the same low rates, even during exposure to Al (Table III).

The exclusion of Fe from −P cells during Al treatment might be a more plausible cause of the cells' tolerance to Al than the exclusion of Al, since −P cells accumulate 70 to 90% less Fe than do +P cells and the amount of Fe accumulated in −P cells is negatively correlated with the degree of tolerance to Al (Fig. 2, C and D). Furthermore, in contrast to +P cells, −P cells do not show any evidence of the peroxidation of lipids and maintain a functional plasma membrane during Al treatment (Table IV). Thus, the tolerance of −P cells to the cytotoxicity caused by Al and Fe might be related to some unknown alterations in the structure and function of the plasma membrane that prevent the entry of Fe and inhibit the initiation step in the autoxidation of lipids. Conversely, antioxidants accumulated in −P cells (see below) might suppress the peroxidation of lipids that has been initiated by the combination of Al and small amounts of Fe, protecting the plasma membrane from further permeation of Fe into cells and, finally, from cell death. In fact, DPPD prevents not only the peroxidation of lipids, but also the accumulation of Fe in +P cells during Al treatment (Y. Yamamoto, A. Hachiya, and H. Matsumoto, unpublished data).

Protectants Against the Peroxidation of Lipids in −P Cells

Al tolerance and the prevention of lipid peroxidation are observed in −P cells and in +P cells treated with Al in the presence of DPPD, suggesting that −P cells might accumulate lipophilic antioxidants in the plasma membrane prior to Al treatment.

β-carotene is a quencher of singlet oxygen and can react directly with the peroxyl radicals involved in lipid peroxidation (for reviews, see Burton and Ingold, 1984; Krinsky, 1989: Pallett and Young, 1993). Krinsky and Deneke (1982) demonstrated that β-carotene is an effective inhibitor of Fe(II)-initiated lipid peroxidation in liposomes made of egg phosphatidylcholine. During Pi starvation of tobacco cells, the amount of β-carotene that accumulates in the −P cells (6-d starvation) is 13-fold higher than that in +P cells (Table V). Levels of other carotenoid pigments (neoxanthin and violaxanthin) also increase significantly during the starvation of Pi. Thus, β-carotene may be the antioxidant that protects cells from the lipid peroxidation caused by the combination of Al and Fe.

Flavonoids are also known to act as antioxidants (Takahama, 1985; Takahama, 1992); however, they are not detected at significant levels in either +P or −P cells. Vitamin E is a potent lipophilic antioxidant and its possible involvement in the Al tolerance of −P cells is currently under investigation. Glutathione-*S*-transferase is another candidate for the antioxidant enzyme that suppresses the peroxidation of lipids in −P cells. Glutathione-*S*-transferase and gluthathione have been shown to protect cells from the toxic products of oxidative stress (Mannervik et al., 1985) and limit lipid peroxidation (Prohaska, 1980; Price et al., 1990; Sharma and Davis, 1994). We previously isolated several cDNAs from −P cells that had been treated with Al in the presence of Fe(II) for 24 h (Ezaki et al., 1995). Among them, one cDNA clone (pAL142) had a sequence homolo-

gous to the *parB* gene of *N. tabacum*, the product of which has glutathione-*S*-transferase activity. The expression of pAL142 mRNA is enhanced by Pi starvation and by the treatment with Al in +P cells.

We were interested in determining why −P cells and stationary-phase cells, both growth-limiting cells, exhibit tolerance to Al. In our system, it seems that the peroxidation of lipids caused by both Al and Fe(II) is a cause of cell death; therefore, Al-tolerant cells should have some mechanism to protect them from either the direct interaction with Al and/or Fe or from the peroxidation of lipids caused by both Al and Fe. In the case of −P cells, it seems that the repression of Fe uptake and the accumulation of antioxidant molecules (as described above) might protect cells from the peroxidation of lipids caused by Al and Fe. In contrast to −P cells, the complete repression of Al uptake in stationary-phase cells during treatment with Al in nutrient medium might be a major cause of its tolerance to Al (Yamamoto et al., 1994). The details of the mechanism responsible for the absence of Al uptake have not yet been determined. The lower amount of carotenoids in stationary-phase cells suggests that carotenoids do not contribute to Al tolerance in stationary-phase cells or that the carotenoids do not contribute to Al tolerance in −P cells. That is, some other factor, common to growth-limiting cells, may account for Al tolerance in both cases.

In acid soils, there are two ways in which Al limits plant growth. One of them is the direct deleterious action of Al ions on root cells, and the other is the fixation of Pi in less readily available form in the soil, with resultant Pi starvation of plants (for review, see Foy et al., 1978). Fe is also a major component of soils, and the free Fe ions, Fe(III) and Fe(II), are solubilized in acid soils (for review, see Foy, 1978). Therefore, it is likely that Al and Fe ions attack root cells together in acid soils and cause the peroxidation of lipids. Under such conditions, if this lipid peroxidation could be prevented, as suggested in our study of Pi-starved tobacco cells, Pi-starved plants could avoid irreversible oxidative damage and grow again once Pi is supplied.

In summary, loss of viability of tobacco cells treated with Al in nutrient solution seems to be caused by oxidative stress produced by both Al and Fe(II). The tolerance to Al exhibited by −P cells seems to be based on the protection of the plasma membrane from the peroxidation of lipids, which might be achieved either by the exclusion of Al and Fe from cells or by antioxidants such as β-carotene that accumulate during the starvation of Pi.

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