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Aluminium reduces sugar uptake in tobacco cell cultures: a potential cause of inhibited elongation but not of toxicity

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Abstract

Aluminium is well known to inhibit plant elongation, but the role in this inhibition played by water relations remains unclear. To investigate this, tobacco (Nicotiana tabacum L.) suspension-cultured cells (line SL) was used, treating them with aluminium (50 μM) in a medium containing calcium, sucrose, and MES (pH 5.0). Over an 18 h treatment period, aluminium inhibited the increase in fresh weight almost completely and decreased cellular osmolality and internal soluble sugar content substantially; however, aluminium did not affect the concentrations of major inorganic ions. In aluminium-treated cultures, fresh weight, soluble sugar content, and osmolality decreased over the first 6 h and remained constant thereafter, contrasting with their continued increases in the untreated cultures. The rate of sucrose uptake, measured by radio-tracer, was reduced by approximately 60% within 3 h of treatment. Aluminium also inhibited glucose uptake. In an aluminium-tolerant cell line (ALT301) isogenic to SL, all of the above-mentioned changes in water relations occurred and tolerance emerged only after 6 h and appeared to involve the suppression of reactive oxygen species. Further separating the effects of aluminium on elongation and cell survival, sucrose starvation for 18 h inhibited elongation and caused similar changes in cellular osmolality but stimulated the production of neither reactive oxygen species nor callose and did not cause cell death. We propose that the inhibition of sucrose uptake is a mechanism whereby aluminium inhibits elongation, but does not account for the induction of cell death.

Key words: Aluminium toxicity, cell death, elongation, osmotic potential, reactive oxygen species, sugar uptake, water uptake.

Introduction

Aluminium is the most abundant metal element in the Earth’s crust, and free aluminium ions are the main factor responsible for inhibiting root growth in acidic soils (Horst, 1995; Matsumoto, 2000; Kochian et al., 2004; Ma, 2007). The primary site of aluminium accumulation and toxicity is the growing root tip, where aluminium inhibits elongation after a short-term exposure, and impairs root development and causes necrosis after longer exposure. Because aluminium mainly accumulates within the cell wall and outer surface of the plasma membrane, with only a limited amount of the element entering the cell (Reid et al., 1996; Taylor et al., 2000), primary targets of aluminium are considered to be within the cell wall and at the plasma membrane. However, other than that, the targets whereby
aluminium inhibits elongation have not been elucidated definitively.

Essentially, elongation depends on three events: synthesis of cell wall constituents, loosening of the cell wall, and water uptake. In principle, aluminium might inhibit any or all of these; however, aluminium’s effect on water relations has been little studied but is likely to be important based on previous studies of cell walls.

In root tips of wheat (Tabuchi and Matsumoto, 2001) and squash (Le Van et al., 1994), aluminium has no effect on the quantity of cellulose or pectin and notably increases the abundance of hemicellulose, findings that are inconsistent with an inhibition of cell wall synthesis. Although aluminium has been reported to inhibit the proton pump (Ahn et al., 2002), an action that could be expected to inhibit elongation according to the acid-growth theory, aluminium toxicity occurs in media with a pH of 5.0 or less, making a shortage of protons seem unlikely. Cell wall extensibility, however, might depend on factors other than protons. Indeed, several groups have reported that cell walls of aluminium-treated roots are less extensible than those of untreated controls (Günsé et al., 1997; Tabuchi and Matsumoto, 2001; Ma et al., 2004). Therefore, aluminium might exert a direct effect on the wall-loosening reactions within the cell wall.

Nevertheless, the investigations on wall extensibility are not definitive. Günsé et al. (1997) examined mature rather than growing regions of the root. Ma et al. (2004) found that cell wall extensibility decreased progressively with time in aluminium for 6 h and then tended to increase whereas the elongation rate was constant over the entire period (albeit slower than untreated roots). Furthermore, Ma et al. (2004) showed that treating killed wheat roots with aluminium caused no change in extensibility, implying a role for the living cell rather than a direct interaction between aluminium and the apoplast. Along with decreased cell wall extensibility and root elongation in an aluminium-sensitive wheat line (Scout 66), Tabuchi and Matsumoto (2001) found that aluminium also decreased the extensibility of an aluminium-tolerant-line (Atlas), despite the fact that aluminium had no effect on the elongation rate of this line.

Subsequently, Tabuchi et al. (2004) found for the tolerant line that the concentration of soluble sugars was increased in root elongation zone cells, thereby presumably allowing the cells to compensate for their stiffer walls and to maintain their elongation rate. Concomitantly, cells in the sensitive cultivar had greatly decreased levels of soluble sugars. These findings suggest that decreased water influx as a result of lower solute concentrations might be at least as important as stiffened walls for the ability of aluminium to inhibit elongation.

Although water relations in short-term aluminium responses have been little explored, that they are central is suggested by a small but growing body of research. Aluminium has been reported to alter hydraulic conductivity of the root cortex both in the mature zone of maize (Zea mays) after a 24 h treatment (Günsé et al., 1997) and in the apex of Northern red oak (Quercus rubra) after a 1 h treatment (Zhao et al., 1987). Furthermore, among genes in rye (Secale cereale) that respond to aluminium treatment within 6 h are the tonoplast aquaporins, which were down-regulated (Milla et al., 2002). Decreased levels of tonoplast aquaporins would be expected to decrease turgor pressure, and hence to reduce elongation. Offering recent, albeit indirect, evidence for reduced water uptake under aluminium treatment, Tamás et al. (2006) reported that exposure of barley (Hordeum vulgare) roots to aluminium for 3 h induced the expression of a dehydrin, a well-known marker for water deficit. Therefore, it appears warranted to investigate the role of water relations in the inhibition of elongation caused by aluminium.

This investigation was undertaken in tissue culture cells. Characterizing water relations in roots is hindered by their various cell types and tissues, complex geometry, and multiple pathways for water movement. By contrast, tissue culture cells are relatively homogenous and each cell is in contact with the medium. Furthermore, roots are active sensors of their environment and, for example, have been reported to respond to aluminium by the rapid synthesis of cytokinin (Massot et al., 2002) and a decrease in auxin transport (Kollmeier et al., 2000; Sun et al., 2009). By contrast, suspension culture cells arguably lack such whole-plant level sensory systems in consequence of their more or less unspecialized differentiation.

A system for studying aluminium responses has been developed using a pair of isogenic tobacco cell lines. The parental line (SL) presents toxicity symptoms similar to those of roots (Yamamoto et al., 2002, 2003); whereas, a derived cell line, ALT301, is aluminium tolerant and, although accumulating aluminium and synthesizing callose to the same extent as the parental line, ALT301 produces less reactive oxygen and undergoes less cell death (Devi et al., 2001, 2003; Yamamoto et al., 2002). Suppression of reactive oxygen species in ALT301 is associated with aluminium tolerance, as indicated, for example, by ALT301 also tolerating hydrogen peroxide. The isogenic tolerant and sensitive lines help distinguish between processes that cause toxic symptoms and those that accompany them.

The mechanism of elongation inhibition by aluminium was investigated here with a focus on water relations. It is reported that, along with blocked elongation, aluminium-treated cells fail to accumulate soluble sugars and have a low cellular osmolality. Aluminium reduces sucrose uptake substantially within 3 h, well before reactive oxygen species increase. It is suggested that the inhibition of sucrose uptake by aluminium is a primary event, responsible for lowered osmolality and hence lowered water uptake and the inhibition of elongation, but not for cell death.

Materials and methods

Tobacco cells, media, aluminium treatment, and sugar starvation

Two non-chlorophyllous tobacco cell lines were used: a wild-type line (SL) derived from Nicotiana tabacum L. cv. Samsun (Nakamura et al., 1988), and an aluminium-tolerant line (ALT301) derived from the SL after chemical mutagenesis (Devi et al., 2001). Cells were maintained by subculturing, at 7 d intervals, 2 ml of cell suspension into 30 ml of fresh medium in a 100 ml flask. The cells
were cultured on a rotary shaker operated at 100 rpm at 25 °C in the dark. The nutrient medium employed for cell growth was a modified version of Murashige-Skoog medium (pH 5.0, after autoclaving; Yamamoto et al., 1994). Under these conditions, the doubling time of the lines was approximately 30 h.

Tobacco cells at the logarithmic phase of growth were washed in medium containing 3 mM CaCl₂ and 3% (88 mM) sucrose, pH 5.0, adjusted with HCl (washing medium), and then suspended in medium containing 0.5 mM CaCl₂, 88 mM sucrose, and 20 mM MES, pH 5.0 adjusted with bis-tris propane (treatment medium) and various concentrations of AlCl₃ at a cell density of 10 mg fresh weight per ml and cultured for up to 18 h on a rotary shaker operated at 100 rpm at 25 °C in the dark. When cells were treated with aluminium in medium containing glucose (or fructose), instead of sucrose, the washing and treatment media contained 88 mM glucose (or fructose), unless otherwise indicated. For fresh weight measurement, cells in 10 ml aliquots were harvested on filter paper by vacuum filtration and the fresh weight was determined as described previously (Yamamoto et al., 1994). For dry weight measurement, 30 ml aliquots were harvested on filter paper, and dried together with the filter paper at 80 °C for 24 h or more until the dry weight had been stabilized. Filter paper without cells was processed similarly, weighed, and subtracted to find the dry weight of the harvested cells.

Aluminium-treated cells tended to adhere to the glassware; therefore, glassware was coated with Sigmacoat (Sigma-Aldrich, St Louis, MO, USA).

Aluminium sensitivity of cell lines was determined as post-treatment growth capacity in the absence of aluminium, as described previously (Yamamoto et al., 2002). In brief, cells for assay were harvested (in 10 ml aliquots), washed with the washing medium, suspended in 30 ml of nutrient medium, and cultured for 7 d. The increase in fresh weight over that week was called post-treatment growth and expressed as a percentage of the control.

For sugar starvation, cells were treated under the same conditions as for aluminium treatment, except the washing medium and the treatment medium contained 88 mM mannitol instead of sucrose.

**Assessment of the loss of membrane integrity of the plasma membrane**

Cell death was evaluated by the loss of membrane integrity based on an impermeant dye (Evans Blue) retained by cells, as described previously, with minor modifications (Ikegawa et al., 2000). Briefly, cells in 10 ml aliquots were suspended in 2 ml of the washing medium containing 0.05% Evans Blue and gently shaken for 15 min at room temperature. Then the cells were washed with the washing medium, and observed through a microscope (Axion; Carl Zeiss, Oberkochen, Germany).

**Protoplast preparation and determination of cell number**

Protoplasts were prepared as described previously (Potrykus and Shillito, 1988; Yamamoto et al., 1994) with minor modifications. Briefly, cells in 10 ml aliquots were incubated with 2% cellulase ‘Onozuka’ R10 (Yakult Pharma, Tokyo, Japan) and 0.5% pectolyase Y23 (Seishin Pharma, Tokyo, Japan) in a solution containing 0.4 M mannitol, 25 mM CaCl₂, and 20 mM MES (pH 5.6, adjusted with bis-tris propane) at 25 °C with rotation at 30 rpm for 1 h. The protoplasts were sedimented by centrifugation and resuspended in a solution containing 0.29 M mannitol, 125 mM CaCl₂, and 20 mM MES (pH 5.6). The number of protoplasts was determined with a haemocytometer. Protoplasts were also used for the determination of water flux rates as described below.

**Assessment of osmotic water permeability and cellular osmolality**

Protoplasts were prepared as described above in a solution containing 0.4 M mannitol, 25 mM CaCl₂, and 20 mM MES (pH 5.6) and resuspended in the same solution. The protoplasts were photographed for an assessment of protoplast volume at the beginning of the experiment. Then to assess the osmotic water permeability, protoplast aliquots were collected and resuspended in solutions consisting of 25 mM CaCl₂, 20 mM MES (pH 5.6), and various mannitol concentrations (osmolality of the solutions was determined with a freezing point osmometer; Model 210, Fiske Associates, Norwood, MA, USA). Protoplasts were immediately mounted on a haemocytometer and photographed at 1 min intervals from 1 min after the suspension in each mannitol solution until 5 min and then at 5 min intervals until 16 min. The volume of protoplasts was assessed by measurement of the diameter of each protoplast. For each experiment, all the spherical protoplasts of various sizes in the fields randomly selected (total 50 protoplasts) were monitored.

To assess the osmolality of the intact cells by the degree of plasmolysis, cells were suspended in a solution containing various concentrations of mannitol and then observed by light microscopy for 2 h.

**Measurements of osmolality and inorganic ion concentrations of cell sap**

Measurements of osmolality and ion content were performed according to Mimura et al. (2003), with minor modifications. Cells in 50 ml aliquots were harvested on filter paper by vacuum filtration and immediately transferred to liquid nitrogen in a mortar and homogenized with a pestle. The homogenate was collected in 1.5 ml tubes, centrifuged for 10 min at 12,000 rpm at 4 °C twice, and the supernatant was harvested as cell sap. The osmolality of cell sap was measured with the freezing-point osmometer. A part of the cell sap was diluted and incubated at 97 °C for 7 min, and then centrifuged to remove debris. Ion concentrations in each sample were determined by the Tosoh Analysis and Research Center (Syunan-city, Yamaguchi, Japan) using ion chromatography systems. Anion concentrations were determined using an ion chromatography system (IC-2001, Tosoh, Tokyo, Japan) equipped with a suppresser, conductivity detector together with an UV detector (for the detection of nitrate ion) and an anion-exchange column (TSK gel Super IC-AP, Tosoh). Cation concentrations were determined using the conductivity detector (CM-8010, Tosoh), pump (CCPM, Tosoh), oven (CO-8010, Tosoh), autosampler (AS-8010, Tosoh) and a cation-exchange column (TSK gel IC-Cation I/II HR, Tosoh).

**Measurements of starch and soluble sugar contents, sugar composition, and sucrose uptake**

The measurement of starch and soluble sugar contents was performed by the anthrone reaction as described previously (Jensen, 1962) with minor modifications. This assay quantifies principally free hexose and the hexose moiety derived from oligosaccharides during heat treatment with anthrone-sulphuric acid reagent. Briefly, cells in 5 ml aliquots were harvested and washed twice with 3 mM CaCl₂ and then soluble sugars were extracted twice with 5 ml of 80% ethanol at room temperature. The cells were further washed three times with 5 ml of 80% ethanol and twice with 5 ml distilled H₂O. Starch was then extracted with perchloric acid (29% v/v) at 0 °C. Sugar content of the samples (soluble sugar, starch) was determined spectrophotometrically (at 630 nm) after the reaction with anthrone-sulphuric acid reagent (at 100 °C, for 7.5 min) using glucose as a standard. Soluble sugar content was expressed as glucose equivalents, and starch content was calculated by multiplying the equivalents by 0.90.

For determining the composition of the soluble sugar fraction described above, the ethanol-extract was dried and trimethylsilylated, and the derivatized sugars were analysed with gas chromatography (GC-16A, detected with FID, Shimadzu Co. Ltd., Kyoto, Japan) in a stainless steel column (3 mm i.d.×2 m) packed.
with 2% silicon OV-17 on Chromosorb W AW/DMCS (80–100 mesh, GL Science Inc., Tokyo, Japan) at 160 °C (2 min) then at 160–320 °C (7.5 °C min⁻¹), as described previously (Choen et al., 2001).

The rate of sucrose uptake in tobacco cells was determined in two different treatment media: (i) medium containing 0.5 mM CaCl₂, 88 mM sucrose, and 20 mM MES, pH 5.0 as described above, and (ii) the same medium but containing various concentrations of sucrose (0 to 75 mM) and mannitol (250 to 175 mM) where the combined concentration of sucrose and mannitol equaled 250 mM, to make the osmolality of the treatment media constant as described previously (Delrot and Bonnemain, 1981). To determine the rate of sucrose uptake, [U-¹⁴C] sucrose (either 18.4 GBq mmol⁻¹, 3.7 MBq ml⁻¹ from Moravek Biochemicals, Inc., Brea, California, USA; or 21.8 GBq mmol⁻¹, 37 MBq ml⁻¹ from MP Biomedicals, Inc., Irvine, USA) was added to the culture at 3.7×10⁵ Bq ml⁻¹ and incubated for 1 h, unless otherwise indicated. Then 5 ml aliquots of the culture were immediately harvested on a glass membrane filter by vacuum filtration and washed three times with 5 ml of the treatment medium. The cells on the filter were dried at 80 °C for 30 min, the dried filter was incubated in scintillation cocktail (Clear-sol 1, Nacalai Tesque, Inc., Kyoto, Japan), and the radioactivity on the filter was determined with a scintillation counter (Wallac 1410; Pharmacia, Wallac Oy, Turku, Finland). Non-specific binding was estimated by incubating cells in ¹⁴C-sucrose under the same conditions but on ice for 1 h, and processing as above. Uptake at 0 °C was less than 10% of the uptake value at 25 °C, and was subtracted from all of the data reported here.

To compare the effects of aluminium and some inhibitors [carbonyl cyanide m-chlorophenylhydrazone (CCCP), 4-chloromercuri] benzene sulfonic acid (PCMBs), orthovanadate (VA)] on sucrose uptake, cells were treated with these inhibitors for 1 h prior to the addition of ¹⁴C-sucrose, unless otherwise indicated, and then the uptake of ¹⁴C-sucrose was determined after incubation for 1 h, as described above. CCCP and vandadate were purchased from Wako Pure Chemical Industries, Osaka, Japan, and PCMBs was purchased from Tronto Research Chemicals Inc., North York, Ontario, Canada.

To determine the rate of glucose uptake, tobacco cells treated with or without aluminium for 9 h in the treatment medium containing 88 mM sucrose were washed with 3 mM CaCl₂, and then treated in the presence or absence of aluminium in a medium containing 0.5 mM CaCl₂, 20 mM MES (pH 5.0), 3 mM glucose, and [¹-H (NJ) D-glucose, (740 GBq mmol⁻¹, 37 MBq ml⁻¹, Moravek Biochemicals, Inc.) at 9.25 KBq ml⁻¹. At various times, cell aliquots were harvested and the radioactivity of the cells was determined as described above.

The contents of sucrose and glucose in medium were determined enzymatically by use of a sucrose assay kit (Sigma-Aldrich) and glucose assay kit (HK; Sigma-Aldrich), respectively.

Detection of reactive oxygen species
Cell suspensions were treated with 10 μM dihydroethidium (DHE; Invitrogen, Carlsbad, CA, USA) for 30 min, and observed with fluorescence microscopy (Axiotron, Carl Zeiss, with filter set no. 9) as described previously, with minor modifications (Yamamoto et al., 2002). In the presence of superoxide, DHE is oxidized to ethidium, which is free to intercalate with DNA, whereupon it fluoresces (Costa-Pereira and Cotter, 1999).

Measurement of callose content
Callose content in the cells was determined as described previously (Chang et al., 1999). Briefly, callose was extracted from the cells with 1 M NaOH, and the alkaline-extracted callose was quantified with aniline blue (water soluble; Wako) by fluorometry, using curdlan (Wako) as a standard.

Results

Aluminium reduces fresh and dry weights of cultured cells
In previous experiments with the tobacco cultured cell lines used here, the treatment medium contained 3 mM CaCl₂ and 3% (88 mM) sucrose, pH 4.5 (Ikegawa et al., 2000; Devi et al., 2001; Yamamoto et al., 2002). Here, the CaCl₂ concentration was reduced to 0.5 mM (the lowest level maintaining viability of the cells, data not shown) to minimize the potential for interaction between calcium and aluminium. In addition, the medium was buffered at pH 5.0 with 20 mM MES, which is the lowest concentration able to maintain a constant pH in the presence of up to 100 μM AlCl₃ (data not shown). Therefore, the toxicity of 20 mM MES and the aluminium-sensitivity of the sensitive line (SL) cells was evaluated in the modified medium. Cells at the logarithmic phase of growth were treated with or without aluminium for 18 h, and sensitivity was evaluated by the amount of growth during a subsequent 7 d culture in an aluminium-free nutrient medium. In the absence of aluminium, cells treated in the modified medium grew during the post-treatment culture as vigorously as in the original medium (data not shown), indicating that 20 mM MES is not toxic. In the presence of aluminium, cells showed an aluminium sensitivity similar to the original reports (Devi et al., 2001; Yamamoto et al., 2002). Specifically, aluminium reduced post-treatment growth in a concentration-dependent manner, with 50 μM causing about 70% reduction and 100 μM nearly eliminating growth (Fig. 1A). Subsequent experiments, 50 μM AlCl₃ was selected as a standard treatment, because it provoked a strong but not completely toxic response.

To assess the growth processes affected by aluminium, fresh and dry weights of cells were measured before and after the 18 h treatment, as well as cell number. Because the cell line grows in clumps, to quantify cell number, protoplasts were prepared. Protoplasts derived from controls and cells treated with 50 μM AlCl₃ remained intact apparently to the same extent (Fig. 1B, C). Over the 18 h treatment, control cells increased in fresh and dry weight, as well as in number (Table 1). By contrast, aluminium treatment markedly reduced gains in fresh and dry weight but, interestingly, did not diminish the gain in cell number. Based on the cell number data, fresh and dry weight could be expressed on a per cell basis. The contents of both water and dry matter per cell increased modestly in control cells but decreased substantially in aluminium-treated cells. The decreased contents of water and dry mass reflect the inhibition of elongation by aluminium and the continued occurrence of cell division. Over the 18 h treatment, these data point to the selectivity of aluminium in this cell line for influencing elongation compared to division.

Aluminium decreases cell osmolality and soluble sugars
As an initial assessment of water relations, the consequences of the 18 h treatment with 50 μM AlCl₃ on membrane
integrity were examined by means of Evans Blue exclusion (Fig. 1D–G). Immediately following the treatment, few cells had stained nuclei (Fig. 1E), whereas after a 24 h post-treatment culture in nutrient medium, aluminium-treated cultures contained many cells with Evans Blue-positive nuclei (Fig. 1G), indicating a breached membrane. Evidently, during aluminium treatment, cells maintained a plasma-membrane integrity sufficient to exclude Evans Blue.

To examine water relations quantitatively, hydraulic conductivity was assayed. Protoplasts of control and aluminium-treated cells were prepared in a 0.4 M mannitol solution (pH 5.6; 550 mOsm kg\(^{-1}\)), and then resuspended in mannitol solutions of various osmolality, and the diameter of individual protoplasts was recorded every minute with light microscopy. In a low osmolality solution (0.05 M mannitol), protoplasts from both treatments swelled (Fig. 2A); at moderate osmolality (0.2 M mannitol), protoplasts from controls enlarged while those from aluminium-treated cells shrank (Fig. 2B); and at high osmolality (0.6 M mannitol), both types of protoplasts shrank (Fig. 2C). In all cases, there was an initial period of rapid flux followed by the attainment of a steady-state diameter. The initial as well as the final volume of control cells was always larger than that of the aluminium-treated cells, consistent with the smaller cell volume of aluminium-treated cells reported in Table 1.

The initial rate of water flux was obtained by linear regression, and was plotted versus medium osmolality (Fig. 2D). Within the limits of this method, the curves for both treatments have the same shape, suggesting that aluminium has little effect on plasma membrane permeability to water. However, the curves differ substantially in the place where they cross the x-axis, at which there is zero movement of water across the plasma membrane, corresponding theoretically to the point where the cell and the medium are isotonic. The x-intercepts were approximately 400 mOsm kg\(^{-1}\) for controls and 160 mOsm kg\(^{-1}\) for aluminium-treated cells. Although these cells did not behave as ideal osmometers, nevertheless the large difference in x-intercept implies that aluminium caused a marked decrease in cellular osmolality.

To confirm that aluminium decreased cellular osmolality, the susceptibility of intact cells to plasmolysis was examined. After the 18 h treatment, with incubation in osmotica for 30 min, control cells were slightly plasmolysed in 0.3 M mannitol (425 mOsm kg\(^{-1}\)) and distinctly plasmolysed in 0.35 M mannitol (490 mOsm kg\(^{-1}\)), implying that the osmolality of control cells was a little less than 425 mOsm kg\(^{-1}\), consistent with Fig. 2D. By contrast, aluminium-treated cells

Table 1. Growth parameters of the tobacco cell line SL as affected by aluminium

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Fresh weight (mg)</th>
<th>Dry weight (mg)</th>
<th>Cell number (x 10^5)</th>
<th>Cell water content(^a) (ng cell(^{-1}))</th>
<th>Cell dry mass (ng cell(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial</td>
<td>10(^b)</td>
<td>0.71±0.07</td>
<td>1.16±0.08</td>
<td>80</td>
<td>6.1</td>
</tr>
<tr>
<td>Control</td>
<td>15.8±2.0</td>
<td>1.04±0.09</td>
<td>1.50±0.22</td>
<td>99</td>
<td>6.9</td>
</tr>
<tr>
<td>Al (50 (\mu)M)</td>
<td>11.2±1.4</td>
<td>0.68±0.07</td>
<td>1.61±0.19</td>
<td>65</td>
<td>4.2</td>
</tr>
</tbody>
</table>

\(^a\) Estimated from the ratio of the water content (fresh weight– dry weight) and cell number.

\(^b\) Adjusted at a start of treatment subculture to ensure equal cell densities in the treated cultures.
were extensively plasmolysed in 0.3 M mannitol but not plasmolysed at all in 0.25 M mannitol (370 mOsm kg\(^{-1}\)), suggesting that the osmolality of aluminium-treated cells was a little greater than 370 mOsm kg\(^{-1}\) and thus approximately 50 mOsm kg\(^{-1}\) less than that of the control. This supports the finding that aluminium decreases cellular osmolality, but by a smaller amount than inferred from changes in protoplast volume.

To resolve this discrepancy, cell sap was extracted from control and aluminium-treated cells and osmolality was measured directly (Table 2). Osmolality of control cells was the same before and after treatment (~260 mOsm kg\(^{-1}\)) whereas that of aluminium-treated was decreased by about 50 mOsm kg\(^{-1}\). Although the absolute value of osmolality determined by sampling cell sap was lower than determined by incipient plasmolysis, the methods agree that aluminium treatment substantially lowered cellular osmolality.

To account for the difference in osmolality between control and aluminium-treated cells, inorganic ions were investigated first because they often contribute to changes in cellular osmolality. Over the 18 h culture period, certain ions decreased, nitrate substantially so, while others increased, notably chloride; however, these changes occurred irrespective of the presence of aluminium (Fig. 3A, B). An

Fig. 2. Effects of aluminium on hydraulic conductivity in SL cells. After treatment without or with 50 \(\mu\)M AlCl\(_3\) for 18 h, protoplasts were prepared in a 0.4 M mannitol solution, and resuspended in the solutions containing mannitol at 0.05 M (A), 0.2 M (B), or 0.6 M (C), and protoplast diameters were measured through the light microscope. The experiment was repeated three times with similar results; data from a representative experiment are shown. Each point represents the mean ±SE of 50 protoplasts. In (D), the initial rates of water flux shown by the protoplasts are plotted versus the osmolality of the mannitol solution.

Table 2. Osmolality and carbohydrate content of SL cells as affected by aluminium

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Osmolality (mOsm kg(^{-1}))</th>
<th>Starch (pg cell(^{-1}))</th>
<th>Soluble sugar (ng cell(^{-1}))</th>
<th>Glucose (%)</th>
<th>Fructose (%)</th>
<th>Sucrose (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial</td>
<td>262±17</td>
<td>75±2</td>
<td>1.22±0.07</td>
<td>32±3</td>
<td>35±3</td>
<td>12±2</td>
</tr>
<tr>
<td>Control</td>
<td>260±4</td>
<td>88±3</td>
<td>2.34±0.05</td>
<td>32±2</td>
<td>36±2</td>
<td>16±3</td>
</tr>
<tr>
<td>Al (50 (\mu)M)</td>
<td>205±3</td>
<td>65±3</td>
<td>0.40±0.02</td>
<td>34±1</td>
<td>38±1</td>
<td>8±1</td>
</tr>
</tbody>
</table>
exception was calcium, which increased to a greater extent in aluminium-treated cells compared with controls. Nevertheless, the calcium concentration was too small to influence osmolality appreciably and, what is more, a similar change in concentration occurred in the aluminium-tolerant cell line (ALT301; Fig. 3C, D). These measurements of ion levels support our conclusion that aluminium did not cause appreciable leakiness of the plasma membrane over 18 h of treatment, and show that inorganic ion levels do not play a major role in the osmotic changes reported above.

Next the contribution of carbohydrate to the difference in osmolality between control and aluminium-treated cells was investigated. After the 18 h treatment, control cells increased starch levels slightly and soluble sugar content by 2-fold, whereas aluminium-treated cells decreased starch slightly and soluble sugar by one-third (Table 2). Therefore, after 18 h, soluble sugar levels in the aluminium-treated cells were almost 6-fold less than that of the control. Soluble sugars comprised mainly glucose, fructose, and sucrose (Table 2). The percentage of glucose and fructose remained nearly constant with or without aluminium, while that of sucrose increased in control cells and decreased in aluminium-treated cells. The amount of soluble sugars per cell, taken as glucose and referring to the estimated cell volumes (Table 1), represents 131 mM in control cells and 34 mM in aluminium-treated cells (assuming no partitioning between cytoplasm and vacuole), a 100 mM decrease that plausibly accounts for the decreased osmolality.

Aluminium inhibits sucrose uptake in the short term

To clarify the relationship between water uptake, soluble sugar content, and osmolality, a time-course experiment was performed (Fig. 4). During the first 3 h of treatment, the fresh weight of both cultures decreased slightly; subsequently, the fresh weight of control cultures increased steadily whereas that of aluminium-treated cultures remained almost constant, implying that aluminium inhibited elongation almost completely after 3 h (Fig. 4A). Soluble sugar content in control cells increased gradually over the first 6 h and after that more steeply, whereas, in aluminium-treated cells, soluble sugar content decreased over the first 6 h, reaching about 70% of the initial value at 6 h, and remaining at that level thereafter (Fig. 4B). Similarly osmolality of control cells increased gradually after 3 h, whereas in aluminium-treated cells, the osmolality decreased over the first 6 h, and remained constant thereafter (Fig. 4C).

Fig. 3. Effects of aluminium on inorganic ion contents in SL (wild-type) and ALT301 (aluminium-tolerant) cells. Cell sap was prepared from logarithmic phase cells (‘Initial’) and from cells treated with or without aluminium (50 μM) for 18 h. Insets in (A) and (C) show data for calcium and magnesium at a smaller scale. Each bar represents the mean ± SE of four measurements from two replicates.

Fig. 4. Time-course of the effects of aluminium on fresh weight, soluble sugar content, and osmolality. Cells were treated with or without 50 μM AlCl₃. At the times indicated, the fresh weight of the cells (A), soluble sugar content of the cells (B), and osmotic concentration of cell sap (C) were determined. Each point represents the mean ± SE of four replicates from three independent experiments [note that in (C), the SE’s are smaller than the symbols].
To examine the basis for the difference in soluble sugar levels between control and aluminium-treated cells, the effect of aluminium on sucrose uptake was investigated.

When cells were labelled continuously with \textsuperscript{14}C-sucrose, uptake in control cells was linear for at least 9 h, with the uptake rate approximately 6.5 nmol sucrose mg\textsuperscript{-1} fresh wt h\textsuperscript{-1} (Fig. 5A). In aluminium-treated cells, the uptake rate was unaffected for 1 h, and was then reduced, to approximately 50\% of the control. The rate of sucrose uptake was next determined by pulse-labelling cells. At various times, cultures were spiked with \textsuperscript{14}C-sucrose and uptake determined after 1 h (Fig. 5B). Again, in control cells, the uptake rate was nearly constant for 6 h and tended to increase by 15 h, whereas in aluminium-treated cells the rate was reduced by about 60\% at 3 h and by 75\% at 15 h. Note that aluminium inhibited sucrose uptake after 1 h of treatment, which precedes the fresh weight increase in controls (Fig. 4A), consistent with inhibited sucrose uptake accounting for the reduced soluble sugar levels in aluminium-treated cells.

The sucrose uptake rate was pH sensitive, decreasing significantly from pH 4.5 to pH 6.0 (Fig. 5C), behaviour that is consistent with an uptake method based on proton-coupled symport. To test this further, incubation in aluminium was compared to incubation in three inhibitors known to affect sucrose transporters, namely: CCCP (a protonophore), PCMBs (an inhibitor of certain sucrose carriers; M'Batchi and Delrot, 1984), and vanadate (an inhibitor of the plasma-membrane proton ATPase). Over 1 h incubation all of these inhibitors reduced sucrose uptake to about the same extent as did aluminium (Fig. 5D). In another experiment, at 3 h after of treatment, some cultures were supplemented with CCCP for an additional hour, and then labelled sucrose added to measure the uptake rate. As expected, CCCP inhibited sucrose uptake substantially; however, inhibitory effects of CCCP and aluminium were not additive (Fig. 5E), suggesting that aluminium inhibits sucrose uptake via a CCCP-sensitive pathway.

Next, the concentration dependence of sucrose uptake was determined (Fig. 6A). To keep the osmolality of the medium constant, mannitol was added so that the total sugar (mannitol plus sucrose) concentration was 250 mM (370 mOsm kg\textsuperscript{-1}). Under this condition, two regimes appeared: a high affinity uptake, which neared saturation around 10 mM sucrose and a low affinity regime, which started to be saturated at 75 mM. This concentration dependency resembled that reported for \textit{Vicia faba} leaves (Delrot and Bonnemain, 1981). Uptake in both regimes was inhibited effectively by CCCP (Fig. 6A). Aluminium was strongly inhibitory for both low affinity (Fig. 6B) and high affinity uptake (Fig. 6C). Importantly, inhibition appeared to be established from the earliest time measured (30 min), demonstrating the rapidity of the aluminium effect.

\textbf{On the role of invertase}

Sucrose could be taken up either directly in the form of sucrose, or as glucose and fructose after conversion by a cell-wall associated invertase (Roitsch and González, 2004). Thus, it was examined whether SL cells could utilize glucose or fructose, and the effects of aluminium on growth and soluble sugar content when cells were grown on these carbon sources. On all three carbon sources, aluminium similarly inhibited fresh weight gain (Fig. 7A), and decreased soluble sugar content (Fig. 7B). These results tend to exclude a specific effect of aluminium on a sucrose
transporter but cannot distinguish between parallel effects on sucrose and hexose transporters or only on the latter in the presence of an apoplastic invertase.

To help distinguish between these possibilities, the uptake and production of glucose were examined. Glucose uptake rate was measured directly following a 9 h aluminum treatment, which establishes a clear effect on uptake (Fig. 5A), and using 3 mM glucose in the uptake assay which is within the putative high affinity range (Fig. 6A). Aluminum decreased glucose uptake rate (Fig. 7C). The inhibition was concentration dependent and at 50 μM AlCl₃, glucose uptake rate was reduced by 50%. Glucose uptake rate was also reduced substantially by the protonophore (CCCP). The sensitivity to the protonophore and the magnitude of inhibition by aluminum were similar for glucose and sucrose uptake (Figs 5B, 7C), consistent with an important role for apoplastic invertase in carbon uptake from sucrose.

To assess the role of invertase further, glucose concentration was measured in the medium of cells grown on sucrose. In control cells, glucose level in the medium reached a steady level after about 3 h of culture; whereas, in the presence of aluminum, the glucose concentration increased almost linearly over the entire treatment interval (Fig. 7D). The amount of glucose accumulating in the medium under aluminum treatment (~0.6 mM, namely ~100 μg glucose ml⁻¹ of treatment culture) was commensurate with the amount of dry mass accumulating in control cells (~330 μg ml⁻¹; Table 1). This suggests that a substantial amount of carbon from sucrose enters these cells in the form of hexose. Taken together, our results imply that the inhibited expansion and lowered cellular osmolality of aluminum-treated tobacco cells occur because the element inhibits hexose uptake.

The effect of aluminium on sugar uptake is separate from the induction of cell death

To examine the relationship between the inhibition of sucrose uptake, elongation, and aluminium tolerance, it was determined to what extent aluminium inhibited sugar uptake in the tolerant, ALT301 cell line. Surprisingly, over the 18 h treatment, aluminium prevented the increase in fresh weight, and decreased osmolality and soluble sugar levels indistinguishably in both lines (see Supplementary
Fig. S1 at JXB online). Likewise, aluminium reduced the uptake rate of radiolabelled sucrose similarly in both lines (data not shown). To examine the relation further, a time-course experiment was done where culture aliquots were removed after various times in 50 μM AlCl₃ and tested for growth capability over a standard 7 d culture without aluminium (Fig. 8A). Interestingly, during the first 6 h of treatment, the growth capability decreased similarly in both lines, reaching about one-half of the initial level. For longer treatments, growth capability in the sensitive line continued to decline whereas it stabilized in the tolerant line.

Tolerance in the ALT301 line has been suggested previously to be based on limiting the production of reactive oxygen species (Yamamoto et al., 2002; Devi et al., 2003). Consistently, 6–9 h of treatment were required before the SL cells, but not the ALT301 cells, became brightly stained with dihydroethidium (DHE), a reporter for superoxide and probably other reactive species (Costa-Pereira and Cotter, 1999) (Fig. 8B). Insofar as aluminium-treated cells had stopped elongation by 6 h (Fig. 4), these results imply that the inhibition of elongation and the production of reactive oxygen species are sequential and separate responses.

As an additional way to examine the relation between decreased sugar uptake and toxicity, it was determined to what extent aluminium treatment could be mimicked by sugar starvation. Cells were starved for sugar by replacing the sucrose in the treatment medium with an equivalent concentration of mannitol. Sucrose starvation for 18 h prevented the increase in fresh weight to about the same extent as did aluminium treatment (Fig. 9A) and was even more effective at decreasing soluble sugar content (Fig. 9B). Also, sucrose starvation decreased cellular osmolality to 86±0.7% (mean±SE, n=3) of the control level, which is similar to the decrease caused by aluminium (Fig. 4C; see Supplementary Fig. S1B at JXB online).

By contrast, aluminium treatment, but not sugar starvation, induced callose production (Fig. 9C), which is a hallmark of aluminium toxicity (Zheng et al., 1994). Sugar-starved cells were stained a little more with DHE than were control cells, but much less than were aluminium-treated cells (Fig. 9D). After a 24 h post-treatment culture in nutrient medium, while aluminium-treated cultures contained many Evans Blue-positive cells (indicating cell death), the sugar-starved cultures had few such cells (Fig. 9E), and after a 7 d post-treatment culture period, sucrose-starved cells grew to a much greater extent than aluminium-treated cells (Fig. 9F). These results are consistent with the inhibition of elongation by aluminium being caused by sugar deficit; and, moreover, that inhibited elongation and decreased sugar levels are, in themselves, insufficient to elicit symptoms associated with aluminium toxicity, including callose production, excessive reactive oxygen species, and cell death.

**Discussion**

**Aluminium and sucrose uptake**

The inhibition of water uptake has previously been suggested as a primary effect caused by aluminium (Zhao et al., 1987; Chen et al., 1991; Tabuchi et al., 2004; Tamás et al., 2006). Here, using cultured tobacco cells, it was found that aluminium decreases cellular osmolality, soluble sugar content, and uptake rates of sucrose and glucose (Figs 4–7), all of which indicate a reduced driving force for water uptake, whereas it seems that aluminium does not affect water flux directly (Fig. 2D). It is hypothesized that depressed sugar uptake is pivotal in the ability of aluminium to inhibit cellular elongation.

In contrast to our hypothesis, some authors have suggested that aluminium inhibits water uptake directly through its effects on membrane fluidity (Zhao et al., 1987; Chen et al., 1991). In the cortex of Northern red oak roots, effects on water flux were suggested to result from aluminium reducing membrane lipid fluidity and increasing the packing density and abundance of straight-chain lipids. This suggestion was based on the observed rates of plasmolysis, calculations of the energy of activation for water transport, and the changes in the lipid partiality of the cell membrane; however, aluminium was added during plasmolysis and not before, which might not be physiologically relevant. Here, following an 18 h exposure to 50 μM aluminium, tobacco SL cells excluded Evans Blue (Fig. 1E) and had unaltered levels of inorganic ions (Fig. 3), both
indicating relatively undisturbed membranes; furthermore, water moved across the membrane of protoplasts according to the difference in water potential for both control and aluminium-treated cultures (Fig. 2D), which indicates that any changes in membrane structure are modest. That aluminium inhibits elongation by reducing the force for water uptake was hypothesized by Tabuchi et al. (2004), who found that aluminium treatment decreases soluble sugar content (glucose, fructose, and sucrose) in the root apex of aluminium-sensitive wheat cultivars (Scout, ES8). However, the mechanism responsible for decreasing the sugar levels remained unclear. In tobacco cells, it was found that total soluble-sugar content decreased by 6 h of exposure and then stayed constant (Fig. 4B), and, moreover, that after the first 3 h exposure to aluminium, the rate of sucrose uptake in tobacco cells was inhibited by 60% (Fig. 5B). Although the concentration of free sugar in the cytoplasm reflects metabolism as well as uptake, and aluminium treatment could, in theory, stimulate glycolysis or other catabolic pathways, the large reduction in sugar uptake-rate is plausibly responsible for most, if not all, of the reduction of cytoplasmic sugar levels.

The tobacco cells used here are heterotrophic and depend on the uptake of sugar from the medium, and in this way they resemble root cells. Although phloem unloading of sucrose into root tissues may be mainly through plasmodesmata, it has been calculated that symplastic continuity is insufficient to satisfy the demand of the elongating and dividing cells and that a transcellular pathway is also required (Bret-Harte and Silk, 1994). Huck (1972) reported previously that aluminium impaired sucrose utilization in the roots of cotton seedlings (Gossypium hirsutum). After a pulse of 14CO2 to shoots, labelled sucrose was translocated to the roots and assimilated into the cell wall; however, aluminium treatment led to an accumulation of the label as soluble sucrose (ethanol-soluble fraction), along with fragile root tissues. Huck assumed that free sucrose accumulated at the root apex because aluminium inhibited cell wall synthesis; it is proposed instead, given the undiminished, or even increased, cell wall synthesis in the

![Figure 9](image.png)

**Fig. 9.** Comparison of the responses to aluminium and sucrose starvation in SL cells. Cells were treated with or without 50 μM AlCl3 or sucrose-starved for 18 h. For sucrose starvation, mannitol replaced sucrose in the culture medium. (A) Fresh weight. (B) Soluble sugar content (glucose equivalent). (C) Callose content (curdian equivalent). (D) DHE staining. Paired fluorescence and phase-contrast images are shown. Bar, 100 μm. (E) Evans Blue uptake (bright-field) following a 24 h post-treatment culture in nutrient medium. Bar, 100 μm. (F) Post-treatment growth capacity.
presence of aluminium (Le Van et al., 1994; Tabuchi and Matsumoto, 2001), that the inhibition occurs directly at the level of sucrose uptake.

In principle, the uptake of sucrose takes place through different routes: (i) sucrose–proton symport; (ii) cleavage of sucrose by an apoplastic invertase and subsequent import of hexoses by transporters; and (iii) endocytosis (Kühn et al., 1999; Roitsch and González, 2004; Etxeberria et al., 2005). In the tobacco cells used here, the contribution of each route to sucrose utilization is not known. Our results are most consistent with sucrose being converted in the apoplast to glucose and fructose and subsequent uptake by means of proton-coupled symport. First, aluminium inhibits the utilization of sucrose, glucose, and fructose, (Fig. 7A, B), as well as the uptake rates of sucrose and glucose (Figs 5B, 7C) essentially to the same extent which is consistent with a specific effect on a hexose transporter. Although for cells grown on sucrose, aluminium reduced the intracellular sucrose level more than that of either glucose or fructose (Table 2), the intracellular levels of all three sugars were greatly reduced and the specific levels attained reflect metabolism as well as uptake. Second, although PCMB inhibited sucrose uptake, this compound exerts pleiotropic effects on membranes and is not specific for sucrose transporters (Naccache and Sha’afi, 2005). Third, the tobacco cells have cell wall invertase activity, which is not inhibited by aluminium (Qi Ge Qi, T Sasaki, Y Yamamoto, unpublished results). Finally, for cells grown on sucrose, the amount of glucose accumulating in the medium in the presence of aluminium was comparable to the amount of dry weight gained by untreated cells (Fig. 7D; Table 1; see Results).

Regardless of the route used for carbon uptake, aluminium inhibited this process. Although parallel inhibitory effects on sucrose and hexose transporters, and even on endocytosis, cannot be ruled out, it is more parsimonious to invoke aluminium acting on a hexose transporter. Alternatively, aluminium might decrease the proton gradient, and hence proton-coupled symport. Results with CCCP (Figs 5D, 6A, 7C) are consistent with this idea, and aluminium is known to inhibit the plasma-membrane proton-ATPase (Ahn et al., 2002). However, nearly 75% of the proton pumping activity was maintained in the plasma-membrane fraction isolated from SL cells after an 18 h treatment with even 1 mM AlCl$_3$ (Y Yamaguchi, T Sasaki, Y Yamamoto, unpublished results). Thus, the inhibition of the proton pump by aluminium is probably insufficient to account for the reduced sugar uptake.

**Elongation and cell death**

Taken together, comparative studies of SL and its isogenic aluminium-tolerant derivative, ALT301, reveal that aluminium affects these cells in two distinct regimes (Fig. 10). The early phase involves the inhibition of elongation, which is well advanced if not complete by 3 h of exposure and which appears to be mediated, at least in part, by low sugar uptake. The late phase, which is apparent after 6 h of aluminium exposure, involves the production of reactive oxygen species as well as of callose (Yamamoto et al., 2003; Sivaguru et al., 2005), and culminates eventually in cell death. Interestingly, these late-phase symptoms are not due to decreased soluble sugar levels per se, because these symptoms are induced to a much lesser extent, if at all, by sugar starvation. Furthermore, in the case of ALT301, the production of reactive oxygen species is reduced (Fig. 8B), while the production of callose is induced as much as in SL cells (Devi et al., 2001), indicating that these late symptoms are separable. The modest decreases in post-treatment growth capacity observed in sugar-starved cells and ALT301 are possibly due to the delay of regrowth related to the lowered soluble sugar concentration but not due to cell death, whereas additional effects of aluminium beyond low sugar levels are required to cause plentiful production of reactive oxygen species, callose, and widespread cell death.

However, while a reduced level of soluble sugar might not be sufficient to cause the late-phase symptoms, it might be necessary. Soluble sugars are implicated in regulating responses to stress in various metabolic reactions, such as...
respiration and photosynthesis (Couée et al., 2006). Oxida-
tive stress typically occurs when mitochondrial function is
compromised and, notably, aluminium induces symptoms of
mitochondrial dysfunction (Yamamoto et al., 2002; Devi
et al., 2003). Prolonged aluminium treatment inhibits
respiration and decreases ATP content; but even during
early exposure, inner-membrane electrochemical potential
dissipates and electron transport rate slows, events that
occur over the same time frame as soluble sugar levels
decrease. Therefore, during aluminium exposure, impaired
mitochondrial function might interact synergistically with
reduced sugar levels to induce a massive burst of reactive
oxogen species and, eventually, cell death. Discovering
whether the lowered sugar levels of the early phase and the
burst of reactive oxygen species of the late phase are linked
mechanistically becomes an important challenge for un-
derstanding how the plant survives or dies when coping
with environmental aluminium.

Supplementary data

Supplementary data are available at JXB online.

Supplementary Fig. S1. Effects of aluminium treatment
(50 μM AlCl₃ for 18 h) on fresh weight, osmolality, soluble
sugar content, and post-treatment growth capacity were
compared between SL (wild-type) and ALT301 (aluminium-
tolerant) cells.

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