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Al Binding in the Epidermis Cell Wall Inhibits Cell Elongation of Okra Hypocotyl

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Al inhibits root elongation at micromolar concentrations, but the mechanisms leading to this process are unknown. In these studies, Al-induced inhibition of cell elongation was examined using hypocotyl of okra (*Abelmoschus esculentus* Moench cv. Clemson Spineless) as an experimental model. One-h exposure to Al (0.5 mM $AlCl_3$) in the presence of 10 μM auxin in 0.5 mM $CaCl_2$, pH 4.0 significantly inhibited auxin-induced cell elongation of okra hypocotyl segments. Elongation was further suppressed with increasing Al concentrations up to 1 mM. Treatment of the hypocotyl with 1 mM citrate for 10 minutes after 2-h exposure to Al resulted in significant recovery of elongation. The amount of Al in the cell wall relative to the total in the tissue was 96.0, 96.2, and 85.4%, respectively, following 1-, 2-, and 3-h exposure to the Al solution. The total and cell wall Al content was decreased by half after the citrate desorption treatment. Furthermore, 95% of Al was found in the epidermis, and 95% of the Al in the epidermis was associated with the cell wall. Experiments using split hypocotyl segments showed that Al exposure increased the outward bending of hypocotyl segments, suggesting that the epidermis elongation was specifically inhibited by Al. Al inhibited the autolysis of epidermis by about 20%, but had little effect on the autolysis of core tissue. Taken together, these results suggest that Al binding in the epidermal cell wall inhibits critical components in cell wall loosening mechanism, resulting in inhibition of cell elongation.

Key words: Al toxicity — Autolysis — Elongation inhibition — Epidermal cell wall — Hypocotyl — Okra.

Aluminum ion (Al^{3+}) is toxic to plants at micromolar concentrations. The initial and most dramatic visual symptom of Al toxicity is inhibition of root elongation (for a review, see Kochian 1995). Al-induced inhibition of root elongation has been reported in many plant species such as onion (e.g. Clarkson 1995), cotton (e.g. Lance and Pearson 1969), corn (Ryan et al. 1993, Sivaguru and Horst 1998), wheat (Wallace and Anderson 1984, Ownby and Popham 1989, Ryan et al. 1992), and so on. The inhibitory effect of Al on root elongation occurs very rapidly. For instance, a 1-h exposure to Al caused significant inhibition of root elongation in cotton (Lance and Pearson 1969), wheat (Ownby and Popham 1989, Ryan et al. 1992), and corn (Ryan et al. 1993, Sivaguru and Horst 1998) although plant species, cultivars, Al concentrations, and experimental conditions differed among experiments.

The root apex has been identified as the primary site of Al toxicity. Ryan et al. (1993) found that only exposure of the terminal 10 to 15 mm of the maize root to Al resulted in inhibition of growth, while application Al to all but this apical region of the root had little or no effect on growth over 24 h interval. They further indicated that the terminal 2.0 to 3.0 mm of root (root cap and meristem) must be exposed to Al to inhibit root elongation. Sivaguru and Horst (1998) developed a polyvinyl chloride-block technique to supply Al to individual 1-mm root zones of intact seedlings of maize and found that applying Al only to the three apical 1-mm root zones inhibited root elongation after 1 h. They concluded that the distal part of the transition zone of the root apex, where the cells are undergoing a preparatory phase for rapid elongation, is the primary target of Al toxicity (Sivagura and Horst 1998). However, the mechanism of Al toxicity leading to the rapid inhibition of the elongation of root apex is still unknown (Kochian 1995).

Many different mechanisms of Al toxicity have been proposed (for reviews, see Taylor 1988, Delhaize and Ryan 1995, Kochian 1995). As Al has a high binding affinity to extracellular and intracellular substances, Al binding by these substances may result in structural and functional damage to the roots. It has been hypothesized that Al binding with cell wall affects cell wall extensibility (see Rengel 1992). Huang et al. (1992) found that Al immediately blocked Ca^{2+} transport across the root cell plasma membrane in an Al-sensitive cultivar, Scout 66. Based on the rapid inhibition and reversibility of the effects of Al^{3+} on Ca^{2+} influx, they suggested that the Ca^{2+} channels in the root-cell plasma membrane was blocked by Al^{3+} and

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that this blockage could be involved in Al toxicity. Al-induced blockage of Ca^{2+} influx was also found in *Amaranthus tricolor* protoplasts (Rengel and Elliott 1992). However, there is no direct evidence showing that Al-induced blockage of Ca^{2+} channels would cause a rapid inhibition of root elongation. For instance, Ryan et al. (1994) designed concentrations of Al^{3+} and Ca^{2+} which severely inhibited root elongation of wheat, but did not affect Ca^{2+} uptake. They argued against the hypothesis that Al inhibits root elongation by reducing Ca^{2+} uptake. Al was also reported to inhibit K^+ uptake (e.g. Gassmann and Schroeder 1994), however, Al blocked the inward-rectifying K^+ channel in the mature region of roots, and it is unlikely that Al-induced K^+ uptake is involved in rapid inhibition of growth in the immature region of roots (Gassmann and Schroeder 1994). In addition, the interaction of Al with a range of molecules that include calmodulin, cytoskeletal elements, DNA, inositol 1,4,5-trisphosphate-mediated molecules, etc, have also been proposed to be involved in Al toxicity (Kochian 1995). To date, however, there is no strong evidence directly supporting any one hypothesis.

Root elongation consists of cell division and cell elongation. In view of the observation, Al can inhibit root elongation rapidly (1 h), the contribution of cell division to the elongation in the short-term would be small. Therefore, initial Al-induced inhibition of root elongation is likely to be caused by the inhibition of cell elongation. In the present study, the mechanism of Al-induced inhibition of cell elongation was investigated using hypocotyl of okra (*Abelmoschus esculentus* Moench cv. Clemson Spineless) as an experimental model. Direct evidence was shown that Al binding in the cell wall of epidermal cells causes rapid inhibition of cell elongation.

Materials and Methods

Plant materials—Seeds of okra (*Abelmoschus esculentus* Moench cv. Clemson Spineless) were subjected to concentrated sulfuric acid for 5 h, and then soaked in running tap water at 25°C overnight for germination. Germinated seeds were placed on a gauze spread on a plastic tray filled with distilled water and kept under continuous fluorescent light (15 W m^{-2}) at 25°C. At day 5 after germination, segments (longer than 1 cm) were excised from the upper region of hypocotyls with a razor blade and abraded with Carborundum powder between the thumb and the index finger to breach the cuticle. The segments were incubated in a petri dish with distilled water for 3 h and then cut into 1 cm segments with a double bladed cutter and used in following experiments. All experiments were repeated at least three times independently.

Al treatment—Twenty segments were incubated in a Petri dish (diameter 4 cm) with 4 ml treatment solution at 25°C with slow agitation. The treatment solution consisted of 0.5 mM $CaCl_2$ with or without $AlCl_3$ and in the presence and absence of 10 μM auxin (IAA). The Al concentration used was 0.5 mM except in dose-response experiments, in which Al concentrations ranging from 0.25 to 1 mM were used, and the pH of all treatment solutions was adjusted to 4.0 using 0.1 M HCl. No pH change was found during the treatment. A desorption experiment with sodium citrate (1 mM, pH 4.0) was conducted with the segments treated with or without Al for 2 h. The segments were placed in 4 ml citrate solution which was replaced once during a 10 min desorption process. After washing the segments with distilled water several times, they were incubated in the solution without Al for an additional 2 h. Elongation of the segments was measured with a binocular microscope equipped with an ocular micrometer (7× magnification) before and after the Al treatment. Auxin-induced elongation is presented as elongation over control (EOC) according to the formula $EOC(\%) = L_A(\%) - L_B(\%)$, in which L_A is the segment elongation in the presence of auxin, L_B is the segment elongation in the absence of auxin. After treatment, the segments were washed with distilled water at least 3 times and subjected to Al analysis and cell wall extraction. Some segments were stained in 0.1% Eriochrome Cyanine R solution for 10 min after the Al treatment, and a hand cross section was prepared using a razor blade. Photomicrographs were taken on Kodak film (Ektachrome 160T) using a microscope (Nikon Eclipse E600) at a magnification of 40. At the end of some experiments, the 0.5 mm ends of the hypocotyl surface were removed with a razor blade and the epidermis and core tissue of the trimmed segments were then carefully separated by tweezers.

Cell wall extraction—Whole segments (5 for each sample) or the peeled epidermis were homogenized with 2 ml ice-cold distilled water using a glass grinder. The homogenate was centrifuged at 1,000× *g* for 10 min, and the precipitate was resuspended in ice-cold water and centrifuged again (Hu and Brown 1994). The precipitate was then washed three times with 10 volumes of 80% ethanol and one with 10 volumes of methanol : chloroform mixture (1 : 1, v/v), followed by 10 volumes of acetone. In every step of washing, the supernatant was collected by centrifugation. Finally, the precipitate was transferred into porcelain crucible and air-dried.

Al determination—The hypocotyl segments or the cell wall were placed in porcelain crucibles and dried at 60°C and then dry ashed at 500°C for 5 h. Ashed samples were suspended in 2 ml of 2 M HNO_3 and heated at 80°C for 1 h. After the sample was filtered, the Al concentration was analyzed by inductively coupled plasma-mass spectrometry (Perkin Elmer-Sciex, Elan 500).

Split experiment—The hypocotyl segments prepared as described above were split in the middle (0.5 cm) with a razor blade and then exposed to 0.5 mM $CaCl_2$ solution (pH 4.0) with or without 10 μM auxin and with or without 0.5 mM Al. At 1 and 3 h, the distance between two cuts were measured with the aid of a binocular microscope equipped with an ocular micrometer (9× magnification). After 3 h, the segments were stained with 0.1% Eriochrome Cyanine R solution for 10 min and then photographed.

Autolysis assay—Segments exposed to 0.5 mM $CaCl_2$ solution with or without auxin (10 μM) and with or without Al (0.5 mM), pH 4.0 for 3 h, were carefully separated into epidermis and core tissue using tweezers on ice. Both the epidermis and core tissue were homogenized with 4 ml of cold Na-acetate buffer (50 mM, pH 4.5) with a glass grinder at 0°C and the insoluble wall materials were transferred to a compact chromatography column (8 × 40 mm, Bio-Rad) according to Inouhe and Nevins (1997). The walls retained within the column were washed extensively with ice-cold 50 mM Na-acetate buffer (pH 4.5), cold acetone (−20°C), and again with 50 mM Na-acetate buffer to remove membrane-bound cytoplasmic components. The column was equilibrated with 50 mM Na-acetate buffer, pH 4.5 at 0°C. After

equilibration, the remaining buffer was removed under reduced pressure. Autolysis was allowed to proceed with shaking of the column containing 1.5 ml of 50 mM Na-acetate buffer (pH 4.5) and 0.01% NaN_3 at 34°C. After 22 h, the reaction solution was filtered by passing the column and the total sugar content in the solution was determined by phenol-sulfuric acid method (Dubois et al. 1956).

Results

A one-h exposure to Al significantly inhibited the auxin-induced cell elongation of okra hypocotyl segments (Fig. 1). Elongation was suppressed by 65% during a 3-h treatment. A dose-response experiment indicated that the elongation decreased with increasing Al concentrations (Fig. 2). At 1 mM Al, the hypocotyl elongation was completely suppressed. Segments exposed to Al for 2 h, followed by exposure to a solution without Al (+Al−Al) did not significantly recover when compared to the segments without Al exposure (−Al−Al) (Fig. 3). However, when the segments exposed to Al for 2 h were desorbed with 1 mM citrate (pH 4.0) for 10 min, the elongation (+Al+C −Al) was significantly restored during the following 2 h growth in −Al solution. Hypocotyl elongation with the treatment (+Al+C−Al) during the second 2 h was comparable to that of segments without Al treatment (−Al −Al or −Al+C−Al). Citrate desorption treatment had no affect on the elongation of segments not subjected to Al

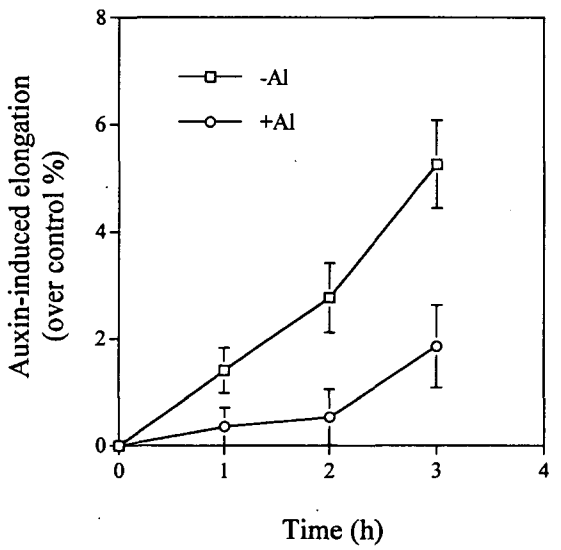

Fig. 1 Time course of Al-induced inhibition of elongation of okra hypocotyl segments. The segments (1 cm length) were exposed to 0.5 mM $CaCl_2$ solution with or without auxin (10 μM) and with or without Al (0.5 mM), pH 4.0, for 1, 2, and 3 h. The length of segments was measured with the aid of a microscope (magnification ×7) before and after treatment. Elongation over control (%)=(elongation (%) with auxin)−(elongation (%) without auxin). Bars represent standard error (n=20).

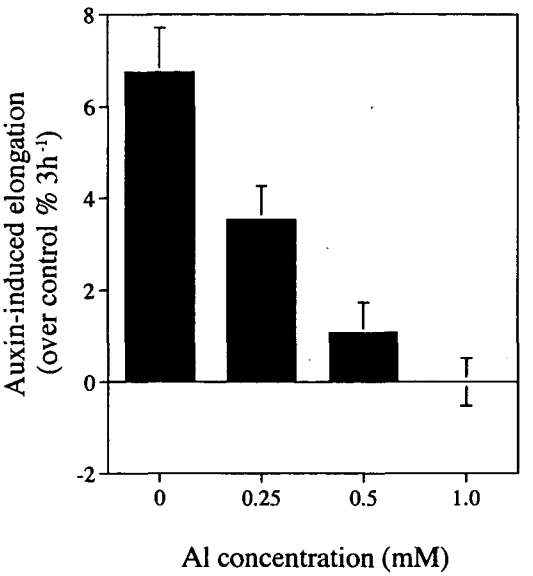

Fig. 2 Effect of Al concentration on the elongation of okra hypocotyl segments. Okra segments (1 cm length) were exposed to 0.5 mM $CaCl_2$ solution with or without auxin (10 μM) and with 0, 0.25, 0.5, or 1.0 mM Al, pH 4.0, for 3 h. The length of segments was measured under a microscope (magnification ×7) before and after treatment. Elongation over control (%)=(elongation (%) with auxin)−(elongation (%) without auxin). Bars represent standard error (n=20).

treatments (−Al−Al and −Al+C−Al) (Fig. 3). To observe cellular localization of Al, the segments were stained with a dye, Eriochrome Cyanine R, which complexes with Al. The epidermis was heavily stained (Fig. 4B), while the core tissue was not, suggesting specific accumulation of Al in the epidermis. High magnification further indicated that only 2 to 3 cell layers of the epidermis were stained (data not shown). When the epidermis was peeled, no staining was observed in the core tissue (Fig. 4C). Citrate desorption treatment significantly decreased the staining of the epidermis (Fig. 4D).

Al content in the okra hypocotyl segments increased with increasing exposure time to 0.5 mM Al (Table 1). The percentage of Al in the cell wall relative to the total in the tissue was 96.0, 96.2, and 85.4%, respectively, following 1-, 2-, and 3-h exposure to the Al solution. The total Al content of the tissue increased as a function of Al concentrations in the treatment solution after 3-h exposure; from 45.2 nmol Al hypocotyl^{-1} at 0.25 mM Al to 82.9 nmol Al hypocotyl^{-1} at 1.0 mM Al. When the segment was separated into epidermis and core tissue following Al treatment, 94.2% of Al was found in the epidermis and 95.4% of the Al in the epidermis was in the cell wall (Table 2). Both the total and cell wall Al content were decreased by about half by citrate desorption treatment (Table 3).

Experiments using the split hypocotyl segments showed

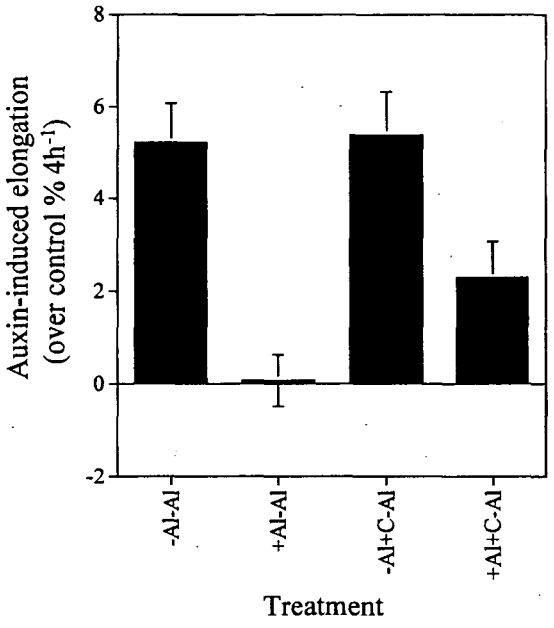

Fig. 3 Effect of citrate desorption on Al-induced inhibition of elongation of okra hypocotyl segments. The segments (1 cm length) were exposed to 0.5 mM $CaCl_2$ solution with or without auxin (10 μM) and with or without Al (0.5 mM), pH 4.0, for 2 h, followed by desorption with 1 mM sodium citrate (pH 4.0) for 10 min. The segments were then exposed to 0.5 mM $CaCl_2$ solution without Al and in the presence and absence of 10 μM auxin for a further 2 h. The length of segments was measured under microscope (magnification ×7) at 0 and 4 h. Elongation over control (%)=(elongation (%) with auxin)−(elongation (%) without auxin). C, citrate desorption treatment. Bars represent standard error (n=20).

that Al exposure increased the outward bending of hypocotyl segments as early as 1 h after the treatment. This occurred both in the presence and absence of auxin (Table 4, Fig. 5). Elongation of hypocotyl is regulated by epidermis (Masuda 1990). When the segments were split, the core tissues elongated faster than the epidermis because the cell are not constrained by the intact epidermal cylinder, resulting in outward bending of the split segments either with or without Al at 1 h (Table 4). As the elongation of the epidermis rather than core tissue responds to auxin (Kutschera and Briggs 1987), the epidermis of the segments with auxin elongated gradually in the absence of Al, and achieved the same length as the core tissue at 3 h, resulting in reduced bending (Fig. 5). However, the segments with auxin could not reverse the bending at 3 h in the presence of Al, suggesting that the epidermis elongation was specifically inhibited by Al. The segments exposed to Al also bent more than those without Al in the absence of auxin (Table 4, Fig. 5). This might be attributed to the acid-induced elongation because the pH of the incubation solution was 4.0. The cut surface of core tissue was also stained (Fig. 5), but no inhibitory effect of the Al on the elongation of the core tissue was observed.

Cell wall autolysis in vitro is a useful and standardized technique applied to the study of changes in cell wall polysaccharides mediated by multi-enzyme system activity (Gallego 1996). Cell wall autolysis is related to cell wall loosening, which is an integral step for cell elongation. Sugar content released into incubation medium during the autolysis process was compared between epidermis and core tissue treated with or without Al. Al inhibited the autolysis of epidermal cell walls by about 20% in segments either with or without auxin, but had little effect on the autolysis of core tissue (Fig. 6).

Discussion

The hypocotyl is a useful experimental system for the study of cell elongation. In the present study, we used the hypocotyl as an experimental model to study Al toxicity for two reasons. First, because only cell elongation contributes to the elongation of the hypocotyl, making it possible to discriminate Al effects on cell elongation from Al effects on cell division. Second, much information on the mechanisms of cell elongation in hypocotyls has been accumulated while little is known about the mechanisms of root cell elongation (for a review, see Masuda 1990). Therefore,

Table 1 Al content in okra hypocotyl segments and the cell wall with different treatment time of Al

	Treatment time (h)		
	1	2	3
Total Al (nmol Al hypocotyl^{-1})	55.0±3.8	58.6±4.8	73.8±1.8
Cell wall Al (nmol Al hypocotyl^{-1})	52.8±1.4	56.4±2.0	63.0±1.9
% of Al in cell wall	96.0	96.2	85.4

Okra segments (1 cm length) were exposed to 0.5 mM $CaCl_2$ solution containing 10 μM auxin and 0.5 mM Al, pH 4.0 for 1, 2, and 3 h. The cell wall fraction was then isolated and subjected to Al determination using ICP-MS after ashing and digestion. Shown are means±SE of three replicates.

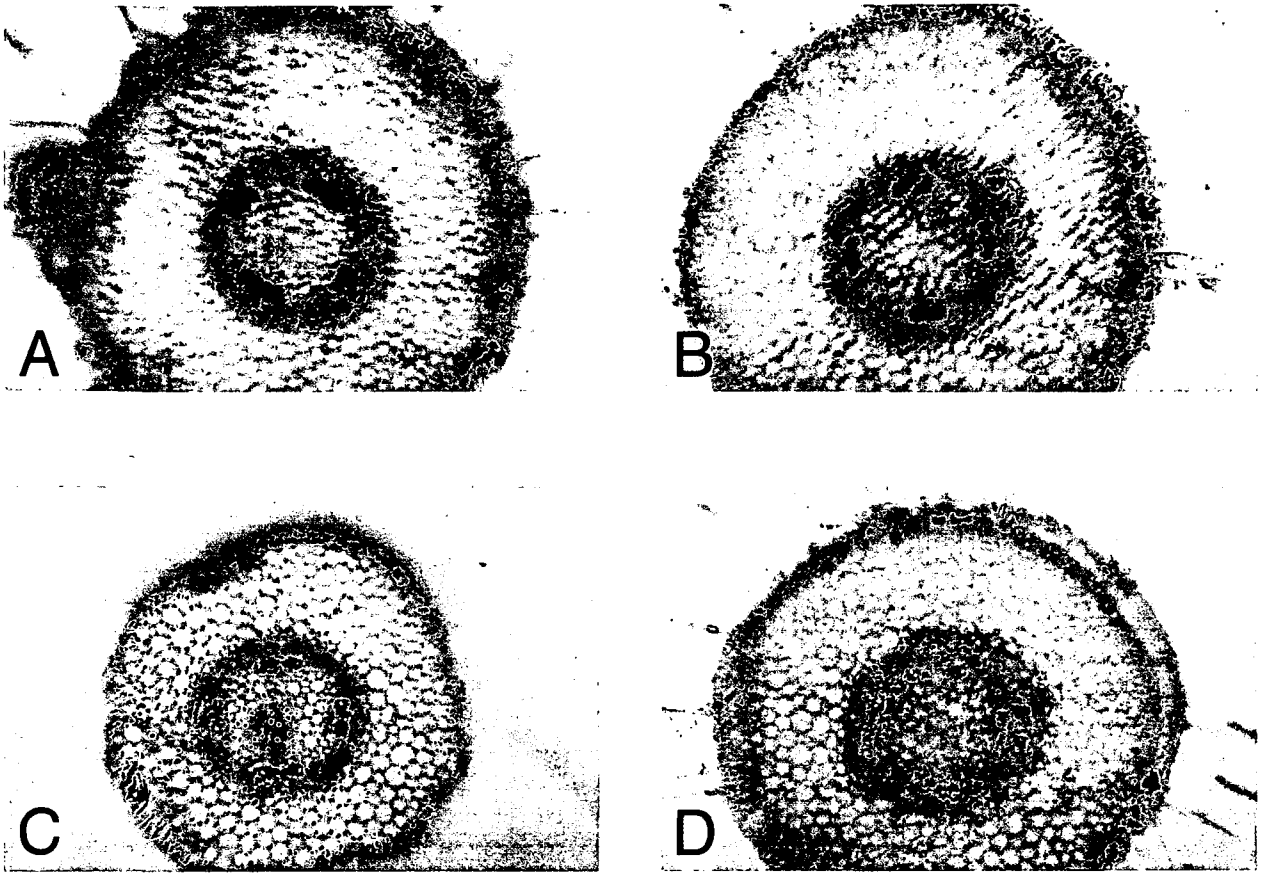

Fig. 4 Aluminum staining of cross section of okra hypocotyl segment. The segments were exposed to 0.5 mM $CaCl_2$ solution (pH 4.0) containing 10 μM auxin and 0 (A) or 0.5 mM Al (B) for 2 h. The core tissue (C) was obtained by removing the epidermis using tweezers after the Al treatment and then stained. Al staining after 10 min desorption treatment with 1 mM citrate (pH 4.0) is shown in D. The segments were stained by 0.1% Eriochrome Cyanine R for 10 min. Cross sections were prepared using a razor blade by hand. Photomicrographs were taken at a magnification of 40.

by studying the effects of Al on the cell elongation of hypocotyls, this may provide clues for better understanding Al toxicity mechanisms in the root. Al inhibited the auxin-induced elongation of okra hypocotyl as early as 1 h after application (Fig. 1). This result is consistent with those found in Al-induced inhibition of root elongation (Ownby and Popham 1989, Sivaguru and Horst 1998). The Al concentration for the inhibition of hypocotyl elongation is higher than that for root elongation (Fig. 2). This is attributed largely to the presence of cuticle in the hypocotyl,

Table 2 Al distribution in epidermis and core tissue of okra hypocotyl segment

Total Al (nmol Al hypocotyl^{-1})	Core tissue Total (nmol Al hypocotyl^{-1})	Epidermis	
		Total (nmol Al hypocotyl^{-1})	Cell wall (nmol Al hypocotyl^{-1})
41.2±1.3	4.0±0.6	38.8±1.1	37.0±1.9

The segments (1 cm length) were exposed to 0.5 mM $CaCl_2$ solution containing 0.5 mM Al and 10 μM auxin, pH 4.0, for 3 h. After treatment, 0.5 mm of the hypocotyl surface was removed and the remaining epidermis and core tissue were then carefully separated by tweezers. Cell wall fractions in the epidermis were then isolated and subjected to Al determination using ICP-MS after ashing and digestion. Shown are means±SE of three replicates.

also identified a gene, *phi-1*, which was rapidly induced by the addition of phosphate to phosphate-starved cells. These results are discussed in terms of resolving the relationship between phosphate availability and plant cell division.

Materials and Methods

Plant materials and culture conditions—Tobacco BY-2 cells (*Nicotiana tabacum* L. cv. Bright Yellow 2) were maintained by weekly subculture in modified Linsmaier and Skoog medium (1965), as described (Nagata et al. 1992).

Phosphate-starvation was attained by incubating 10 ml aliquots of 8 d-old cell suspension in phosphate-free medium for 3 d. Subsequently, concentrated KH_2PO_4 (100 mg ml^{-1}, pH 5.8) was added to the culture medium to give a final concentration of 370 mg $liter^{-1}$, while control cells were continually cultured in the phosphate-free medium.

Auxin starvation and re-addition was carried out as described by Ishida et al. (1993). In brief, 10 ml of 8 d-old cell suspension was washed with 10-volumes of culture medium without 2,4-D and incubated in medium without 2,4-D for 3 d. Subsequently, 2,4-D was re-added to the culture medium to give a final concentration of 0.2 mg $liter^{-1}$.

Synchronization of tobacco BY-2 cells was carried out as described by Nagata et al. (1992). In brief, 7 d-old cell suspension was transferred to fresh medium containing 5 μg ml^{-1} of aphidicolin (Sigma Chemical Co., St. Louis, U.S.A.) and incubated for 24 h. After washed with fresh medium, cell synchrony starting from S phase was obtained.

Determining DNA synthesis and cell division of the BY-2 cells—Cell division percentages were determined by counting MI after staining the cells with 4′,6-diamidino-2-phenylindole (DAPI) and viewing under a fluorescence microscope (BH-2, Olympus Optical Co., Tokyo, Japan), as described in Yasuda et al. (1988).

DNA synthesis was detected after the incorporating of 5-bromo-2′-deoxyuridine (BrdU) into cells for 30 min using a Cell Proliferation Kit, RPN20 (Amersham, Buckinghamshire, U.K.), by staining the cells with an antibody against BrdU (Amersham) under fluorescence microscopy (Miyake et al. 1997). After incorporating BrdU, cells were fixed with 3.7% formaldehyde in PMEG buffer (50 mM PIPES, 2 mM $MgSO_4$, 5 mM EGTA and 2% glycerol, pH 6.8) for overnight at 4°C. After fixation, cells were digested in an enzyme solution [1% Cellulase Onozuka RS (Yakult Honsha Co., Tokyo, Japan), 0.1% Pectolyase Y-23 (Seishin Co., Tokyo, Japan) and 0.4 M mannitol in PMEG buffer] for 5 min and then incubated with 1% Nonidet P-40 (Sigma) in phosphate-buffered saline (PBS) (20 mM Na-phosphate and 150 mM NaCl, pH 7.0)] for 15 min. After treatment with a blocking solution (0.1 mM glycine and 1% bovine serum albumin in PBS) for 10 min, cells were incubated with an anti-BrdU monoclonal antibody (Amersham) for 1 h, according to the manufacture's protocol. After washing with PBS, cells were incubated with a fluorescein isothiocyanate (FITC)-conjugated sheep anti-mouse IgG antibody (Sigma) for 30 min. The cells in which all the cell nucleus was stained were scored as S phase cells.

Isolation of RNA—Cells were harvested by centrifugation (1,000 × *g* for 1 min) and frozen in liquid nitrogen. Cell pellets were ground to a fine powder with a pestle and a mortar, which was suspended in a denaturing solution (4 M guanidium thiocyanate, 100 mM Tris, 0.5% Sarcosyl and 140 mM 2-mercaptoethanol, pH 7.0) and mixed. Total RNAs were extracted with phenol-chloroform-isoamylalchol (25 : 24 : 1) and ethanol precipitated. Poly(A)$^+$ RNAs were prepared using Oligo(dT) Cellulose columns according to the manufacture's protocol (Life Technologies, Gaitherburg, U.S.A.).

Differential plaque hybridization—Double stranded cDNAs were synthesized from poly(A)$^+$ RNA isolated from cells cultured for 2 h after the re-addition of phosphate using a cDNA synthesis kit according to the manufacture's protocol (Amersham). A cDNA library was then constructed in the λgt10 vector (Amersham). Differential screening of this cDNA library was carried out on Nylon membranes (Biodyne B, Pall BioSupport, East Hills, U.S.A.), which were hybridized with ^{32}P-labeled cDNA probes prepared from poly(A)$^+$ RNA isolated from cells cultured for 2 h in the presence or absence of phosphate. Putative clones were subcloned into pUC18 vector and sequenced with the Thermo Sequenase fluorescent labeled primer cycle sequencing kit with 7-deaza-dGTP according to the manufacture's protocol (Amersham).

RNA gel blot analysis—Total RNAs were separated on 0.8% formaldehyde-agarose gels, blotted on to Biodyne B membranes and hybridized to ^{32}P-labeled cDNA probes in 6 × SSC and 0.1% SDS at 65°C for 16 h. Membranes were washed for 30 min three times with 2 × SSC and 0.1% SDS at 65°C and exposed to an X-ray film (X-OMAT, Kodak, Rochester, U.S.A.).

Preparation of polyclonal antibodies—The *phi-1* cDNA was subcloned into the pET30 vector (Novagen Inc., Madison, U.S.A.). To do this, an *Eco*RI site was created at position of 388 by PCR using the primer: 5′-GGGAATTCCTTTCACTCTATTTGGGC-3′ and a *Sal*I site was created at position 1074 using the primer:5′-GGGTCGACATTCACTACACTAGATTA-3′. The resulting 700 bp PCR product was ligated into the pET30 vector and the protein was expressed in *E. coli* strain BL21 (DE3). The protein was purified with an Ni-NTA-agarose column (Qiagen, Hilden, Germany) and injected into a mouse to produce anti-phi-1 antiserum.

Affinity-purification of antiserum was carried out according to the method described in Olmsted et al. (1981) with some modifications. The purified phi-1 protein described above was separated on 10% polyacrylamide gel and it was transferred to a PVDF membrane. The transferred portion of the protein was cut out and incubated with anti-phi-1 antiserum for 30 min at 30°C. After washed with PBS containing 0.5% Tween-20 for 5 min five times, the antibody was eluted by incubating with 0.2 M glycine buffer (pH 2.6) for 2 min. The purified antibody was used after neutralization with 2 M Tris-HCl (pH 8.0).

Immunoblotting and immunolocalization—Cells were harvested by centrifugation, frozen by liquid nitrogen and homogenized to a fine powder with a pestle and a mortar. This powder was suspended in 50 mM Tris-HCl buffer (pH 7.0) containing 10% glycerol and 2% SDS, mixed and centrifuged for 10 min at 10,000 × *g*. The resulting supernatant was considered to be the whole protein extract. The whole protein extract was separated on 10% polyacrylamide gel and transferred to a PVDF membrane (Pall BioSupport). The blotted membrane was blocked with 5% skim milk and washed with Tris-bufferd-saline. After incubated with the affinity-purified anti-phi-1 antibody for overnight followed by alkaline phosphatase-conjugated anti-mouse antibody (Tago Inc., Burlingame, U.S.A.) for 1 h, phi-1 protein was detected using an alkaline phosphatase substrate kit (Vector Laboratories Inc., Burlingame, U.S.A.).

To examine the localization of the phi-1 protein, cells were pretreated with 250 μM *m*-maleimidobenzoyl-*N*-hydroxysuccimide ester (MBS) (Pierce Chemical Co., Rockford, U.S.A.) in

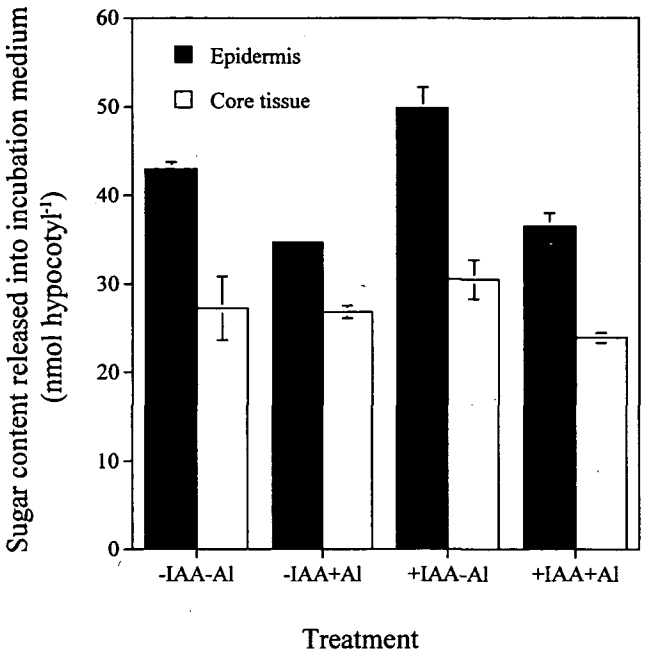

Fig. 6 Effect of Al on autolysis of epidermis and core tissue. Okra segments (1 cm length) were exposed to 0.5 mM $CaCl_2$ solution with or without auxin (10 μM) and with or without Al (0.5 mM), pH 4.0. After 3-h treatment, the epidermis and core tissue were separated using tweezers, and the cell walls were isolated using 50 mM Na-acetate buffer (pH 4.5). Autolysis was allowed to proceed in a column containing 50 mM Na-acetate buffer (pH 4.5) and 0.01% NaN_3 at 34°C. After 22 h, sugar content in the autolysis product was analyzed by phenol-sulfuric acid method. Bars represent standard error (n=4).

elongation of the epidermis, but not that of core tissue, although the core tissue was also exposed to Al in the split test (Fig. 5).

Desorption experiments with citrate also provided evidence that Al binding in the epidermal cell wall is directly responsible for the Al-induced inhibition of hypocotyl elongation. Citrate is a strong chelator of Al ion, and its secretion from the root and formation of the Al-citrate complex have been demonstrated to be the external and internal detoxification mechanisms of Al in some plant species (Miyasaka et al. 1991, Pellet et al. 1995, Ma et al. 1997a, b). When the hypocotyl was exposed to Al for 2 h and then desorbed with citrate for 10 min, the Al content in both whole tissue and cell wall was decreased by half (Table 3), and the elongation recovered over the following 2 h incubation in Al-free solution (Fig. 3). This result suggests that Al bound in the cell wall of the epidermis was desorbed by citrate, resulting in recovery of hypocotyl elongation. A similar finding was also reported by Ownby and Popham (1989) in wheat roots. They found that a short desorption with citrate (2.0 mM, 30 min) enabled wheat roots to resume growth following a 5 h pulse with Al, and suggested that most of the absorbed Al was in the apoplastic compartment of the root and some component of growth inhibition resulted from apoplastic Al.

Results of autolysis experiments further suggest that Al in the epidermis cell wall was responsible for the inhibition of the hypocotyl elongation (Fig. 6). Autolysis can be used to assess what potentially happens in vivo with respect to changes in the cell wall. Autolysis represents the reaction of endogenous enzymes with constitutive wall components, in a preparation which is free from the cytoplasmic or membrane associated fraction (Inouhe and Nevins 1997). Upon exposure to Al, the autolysis of the epidermis was inhibited by 20% (Fig. 6), but the autolysis of core tissue was hardly affected by Al. This result suggests that the putative cell wall loosening process of epidermis was affected by Al, resulting in inhibition of the hypocotyl elongation.

Auxin-induced cell elongation includes both loosening process of the outer epidermis wall and cell wall synthesis (Masuda 1990). A rapid inhibition (1 h) of the hypocotyl elongation by Al suggests that the loosening process of cell wall rather than the cell wall synthesis process was affected (Fig. 1). During loosening process of cell wall, the matrix polysaccharides, but not cellulose, undergo biochemical modifications; breakdown of xyloglucans in dicotyledons and (1 → 3),(1 → 4)-β-glucans in Poaceae (Hoson 1993). However, it is not clear which step is affected by Al. There are two possibilities; one is that Al binding in the epidermis cell wall cause deformation of matrix polysaccharides, resulting in inhibiting breakdown of the polysaccharides by enzymes. Pectins which have substantial negatively charged carboxyl groups have been suggested to be candidates for Al binding (Blamey and Dowling 1995). However, it remains to be elucidated what structural units and how Al is bound to the pectins and other substances as well. Another possibility is that the loosening enzymes are inhibited by Al. Many enzymes have been suggested to be involved in loosening process of cell wall. McQueen-Mason et al. (1992) isolated two endogenous proteins, expansins, that induce cell wall extension from cucumber. They found that 1 mM Al inhibited the extension activity mediated by the isolated proteins. As the protein are not responsible for glycanase activity, which is a key enzymes during cell wall loosening, further work is needed to examine the effect of Al on other enzymes related with loosening processes such as glucanase and xyloglucanase.

Although sites of Al phytotoxicity may be multiple, the results obtained in this study provided first evidence that Al binding in the epidermis cell wall directly causes rapid inhibition of hypocotyl elongation in okra.

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