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Plasma Membrane Permeability of Root-Tip Cells Following Temporary Exposure to Al Ions Is a Rapid Measure of Al Tolerance among Plant Species

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No correlations were recognized between Al tolerance among four plant species, rice (*Oryza sativa* L.), maize (*Zea mays* L.), pea (*Pisum sativum* L.), and barley (*Hordeum vulgare* L.), in rank order of Al tolerance, and cation exchange capacities of root-tip (0–1 cm) cells or of their cell walls. The plasma membrane of root-tip of Al sensitive plant species (pea and barley) was considerably permeabilized with elongation of root in Al-free solution following 0.5 h pretreatment with Al. K^+ release from and Al permeation into the protoplasts isolated from the root-tip of Al-sensitive plant species were more significant than those for Al-tolerant plant species (rice and maize) on 10 or 30 min treatment with Al. The permeability of the plasma membrane for protoplasts isolated from Al sensitive plant species was considerably increased by treatment with hypotonic Al-free control solution following 10 min pretreatment with Al. To our knowlege, these are the most rapid responses to Al ions reported to date, i.e., within 0.5 h in whole plant and within 10 min in protoplast. These results suggest that a temporary contact with Al ions irreversibly alters the plasma membrane of root-tip cells of Al-sensitive plant species: the cells become more leaky and rigid due to binding of Al ions to the plasma membrane.

Key words: Aluminum (Al) tolerance — CEC of cell wall — PM permeability — Protoplast.

Aluminum (Al) toxicity is known to be a major factor limiting plant growth in acid soils. Micromolar concentrations of Al can inhibit root elongation of Al-sensitive wheat cultivar within 1 h (Ownby and Popham 1989). Al-tolerant plant species (Wagatsuma et al. 1995a) and Al-tolerant wheat lines (Delhaize et al. 1993a) accumulate less Al in the root-tip than Al-sensitive species. Several mechanisms for this reduced accumulation have been proposed, including metabolism-dependent exclusion of Al (Wagatsuma 1983, Zhang and Taylor 1989), and detoxification of Al by the secretion of organic acids (Delhaize et al. 1993b, de la Fuente et al. 1997, Ma et al 1997).

The binding of Al ions to carboxyl groups in pectic substances of the cell wall inhibits apoplastic movement of water and nutrients (Blamey and Dowling 1995) and the metabolism of cell wall polysaccharides (Le Van et al. 1994). In addition, Rengel (1992) speculated that Al ions repress the cell elongation induced by acid-mediated wall loosening. However, there is no consensus on the correlation between CEC of roots and Al tolerance among plant species or cultivars (Munn and McCollum 1976, Mugwira and Elgawhary 1979, Wagatsuma 1983, Rengel and Robinson 1989b, Allan et al. 1990, Masion and Bertsch 1997).

Al ions immediately inhibit not only the influx of K^+ (Miyasaka et al. 1989), Ca^{2+} (Rengel and Elliott 1992), Mg^{2+} (Rengel and Robinson 1989a), or NO_3^- (Durieux et al. 1995) by the binding of Al ions to the PM protein, but also accelerate the efflux of K^+ (Wagatsuma et al. 1995a), or phosphate ions (Ownby 1993) across the PM lipid. Chen et al. (1991) observed a decrease in water and increase in nonelectrolyte (for example, urea) permeation across cell membranes in intact *Quercus rubra* L. root cortex cells on Al treatment, and suggested the alteration is induced by the binding of Al^{3+} to negatively charged phospholipid head groups in cell membranes. Vierstra and Haug (1978) using electron paramagnetic resonance spectroscopy showed that Al^{3+} decresed membrane fluidity in isolated and intact cell membranes of *Thermoplasma acidophilum*. Yamamoto et al. (1996) suggested that Al significantly induced the peroxidation of the PM lipid of cultured tobacco (*Nicotiana tabacum* L.) cells at logarithmic phase in the presence of Fe with cell death resulting from the increase in permeability of the PM. Zhang et al. (1997) showed several genotype-specific changes in the lipid composition of the PM from roots of wheat cultivars that differed in tolerance to Al on 3-d treatment with Al. Jones and Kochian (1995) firstly reported in plants that Al^{3+} could inhibit the phosphoinositide signal-transduction pathway. The significance of PM of root-tip cells in Al tolerance is still unclear.

In the present study we suggest that the PM of the root-tip for Al-tolerant plant species does not easily leak and is less susceptible in the short-term (within 1 h) to Al.

Abbreviations: CEC, cation exchange capacity; FDA, fluorescein diacetate; IPP, the increase in PM permeability; PI, propidium iodide; PM, plasma membrane.

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Materials and Methods

Plant materials and growth conditions—Seeds of rice (*Oryza sativa* L. cv. Sasanishiki; an Al-tolerant plant species), maize (*Zea mays* L. cv. Pioneer 3352; a plant species that is moderately tolerant to Al), and pea (*Pisum sativum* L. cv. Kinusaya; an Al-sensitive plant species) were soaked in tap water composed of 8 mg liter^{-1} Ca^{2+}, 3 mg liter^{-1} Mg^{2+}, and 1.5 mg liter^{-1} K^+ under aeration for 1 d. Seeds of barley (*Hordeum vulgare* L. cv. Manriki; a plant species that is very sensitive to Al) were not soaked, but germinated on a nylon screen that was put on a polypropylene container filled with 8 liters of tap water under aeration at 27°C in a growth room under fluorescent white light (18 μE m^{-2} s^{-1}) with a 14-h photoperiod. To maintain high humidity, each container was covered with a polyvinyl sheet and the seeds sprayed with deionized water. Three or four-d-old seedlings with roots approximately 5 cm in length were used in the experiments.

Value of Al tolerance—The length of the longest root was measured for ten seedlings with a ruler prior to the treatments with control or Al. These seedlings were then transferred to the 8-liters of 0.2 mM $CaCl_2$ solution with (Al) or without (control) 5 μM $AlCl_3$ and treated for 3 d under aeration at 27°C. The solutions were renewed every day, and pH was maintained at 5.0 by using diluted HCl and NaOH every 6 to 8 h. After 3 d, the root lengths of the same ten seedlings were measured again. Al tolerance was calculated based on the ratio of the net root elongation in Al treatment to that in control.

Cell wall isolation and determination of CEC—Cell wall was isolated basically by the method of Whitman and Travis (1985), as described below. Twenty g of apical 1-cm segments of fresh root was homogenized with 100 ml of a homogenizing medium consisting of 50 mM Tris-HEPES (pH 7.8), 5 mM EGTA, 5 mM EDTA, 10 mM NaF, 2.5 mM DTT, 100 μg ml^{-1} butylated hydroxytoluene, and 250 mM sucrose with a homogenizer (HG 300; Hitachi, Tokyo, Japan) at 15,000 rpm for 30 s three times. The homogenates were filtered through a nylon cloth of pore diameter 30 μm, and fully washed with deionized water. This procedure was repeated twice. The crude cell wall was soaked in the homogenizing medium for 24 h at 5°C, filtered with a nylon cloth, homogenized twice in a mixture of chloroform and methanol (1 : 2, v/v) for 30 s each time, and filtered. The homogenates were soaked in the mixture for 24 h at 5°C, filtered and finally washed with deionized water. The final material on the nylon cloth was designated as purified cell wall. The purity of this material was judged by its phosphorus (P) concentration. P was determined colorimetrically by the molybdeum blue method after digestion with a mixture of concentrated HNO_3 and 60% $HClO_4$ (5 : 3, v/v).

The CECs of apical 1 cm root segments and purified cell wall material were determined according to the method of Wagatsuma (1983). Briefly, dry powder (0.2 g) of root and cell wall was incubated first in 40 ml of 0.5 M HCl for 3 h, then in 80 ml of 0.1 M $Ca(CH_3COO)_2$ containing 0.2 mM $CaCl_2$ (pH 5.0) for 3 h and washed with deionized water until Cl^- free. The adsorbed Ca was desorbed by incubating with 50 ml of 0.05 M HCl, and the Ca concentration in the filtrates was determined by inductively coupled plasma atomic emission spectroscopy (ICP; Liberty 220, Varian Australia Pty., Ltd., Victoria, Australia).

Permeability of the PM and length of re-elongation of root—Seedlings of four plant species were treated with a 0.2 mM $CaCl_2$ solution containing 0 (control), 5, 20, or 100 μM $AlCl_3$ (Al) for 0.5 (rice, barley and maize) or 1 h (pea). The pH of each Al solution and its control solution were equalized with the corresponding values when 10 mM $AlCl_3$ stock solution was diluted in each Al concentration with deionized water; 5.0 for 5 μM Al, 4.9 for 20 μM Al, and 4.6 for 100 μM Al. Permeability of roots was observed by staining with fluorescein diacetate-propidium iodide (FDA-PI) according to the method of Ishikawa et al. (1996). Briefly, a stock solution of FDA (Aldrich Chemical Company, Inc., Milwaukee, WI, U.S.A.) or PI (Sigma, St. Louis, MO, U.S.A.) was prepared by dissolving 5 mg ml^{-1} in acetone or 40 μg ml^{-1} in deionized water, respectively. Roots were stained for 10 min with a mixture of FDA (12.5 μg ml^{-1})-PI (5 μg ml^{-1}) solutions, both diluted with deionized water. The concentration of PI was 15 μg ml^{-1} for pea roots. After extra dye was removed by washing with deionized water for 1 min, the root-tip was observed under a stereoscopic microscope (SMZ-10; Nikon, Tokyo, Japan) equipped with a macro-fluorescence apparatus (MX-100F; Nikon) (excitation filter, 450–490 nm; barrier filter, 520 nm), and photographed using a photographic apparatus (AFX-II; Nikon) and a film of ASA 400. Experiments were replicated more than three times.

Roots which had been pretreated with the 0.2 mM $CaCl_2$ solution containing 0, 5, 20, or 100 μM $AlCl_3$ for 0.5 or 1 h were washed with deionized water for a few seconds, and thereafter treated with a solution of 0.2 mM $CaCl_2$ with or without 2 mM citrate (pH 4.5) for 0.5 h at 5°C. The roots were washed with deionized water, and re-elongated in 0.2 mM $CaCl_2$ (pH 4.9) for 5 (rice, barley and maize) or 8 h (pea) at 27°C. The permeability of the re-elongated roots was observed by staining with FDA-PI as described above. Pea roots of each of the 10 seedlings were pretreated with 0.2 mM $CaCl_2$ solution containing 0, 5, 20, or 100 μM $AlCl_3$ for 1 h, then treated with or without 2 mM citrate (pH 4.5) for 0.5 h at 5°C, and finally re-elongated in 0.2 mM $CaCl_2$ (pH 4.9) for 24 h. The net lengths of re-elongation were compared.

Permeability of protoplasts isolated from root-tip—The isolation and purification of protoplasts from root-tip were carried out as described by Wagatsuma et al. (1995b). Briefly, apical 1 cm segments were cut into 2 mm pieces and segments weighing 3 g were digested by shaking at 30 cycles min^{-1} for 3.5 h at 27°C with 20 ml of a medium composed of 0.6 M mannitol, 2% (w/v) Cellulase Onozuka RS (Yakult Pharmaceutical Industry Co., Ltd., Tokyo, Japan), 0.1% (w/v) Pectolyase Y-23 (Seishin Pharmaceutical Industry Co., Ltd., Tokyo, Japan), 0.05% (w/v) BSA (fraction V; Sigma), 1 mM $CaCl_2$, and 0.5 mM DTT (pH 5.6). Protoplasts were separated from the digested tissues by passage through a nylon cloth with a pore diameter of 95 μm. The residue on the nylon cloth was gently agitated in suspension medium, which consisted of 0.7 M mannitol, 0.1 mM $CaCl_2$, 0.5 mM DTT, and 5 mM Tris-MES (pH 6.5), and then filtered through the nylon cloth. Both flitrates were centrifuged at 200 × g for 6 min and the pellet of crude protoplasts was gently suspended in 30% (w/v) Ficoll (Ficoll 400; Pharmacia Biotech, Uppsala, Sweden) in 0.7 M mannitol and 2 mM Tris-MES (pH 6.5). A discontinuous gradient was formed by successive layering of solutions of 8, 5, and 0% Ficoll in 0.7 M mannitol and 2 mM Tris-MES (pH 6.5). The fraction at the interface between the layers of 0% and 5% Ficoll was collected after centrifugation at 380 × g and the Ficoll was removed by washing twice with 0.7 M mannitol solution. Protoplasts, in which the cell wall was hardly discernible by staining with fluorescent brightener 28 (Sigma), could be isolated by this procedure (data not shown).

Four ml of 0.2 mM $CaCl_2$ in 0.7 M mannitol (pH 4.5, isotonic control solution) was added to the purified protoplasts which were then incubated for 10 min at room temperature. This suspension was centrifuged at 130 × g for 5 min and the supernatant was discarded. Next, 4 ml of isotonic control solution was

added and the protoplast pellet resuspended. The suspension was divided into 4 aliquots (of approximately 1 ml). Three ml of the isotonic control solution was added to two of these 1-ml aliquots (pH 4.5, isotonic control pretreatment), and 1 ml of isotonic control solution and 2 ml of 0.2 mM $CaCl_2$ containing 200 μM $AlCl_3$ in 0.7 M mannitol were added to the other two aliquots (pH 4.5, isotonic Al pretreatment); the Al concentration in the latter solution was 100 μM. These suspensions were incubated for 10 min at 27°C, then centrifuged at 130 × *g* for 5 min, and the supernatants were discarded. Two ml each of 0.2 mM $CaCl_2$ in 0.5 M mannitol (pH 4.5, hypotonic treatment) or 0.2 mM $CaCl_2$ in 0.7 M mannitol (pH 4.5, isotonic treatment) was added to each pellet, and the mixtures were incubated for 5 min at 27°C. After centrifugation at 130 × *g* for 5 min, each supernatant was reduced to 0.5 ml by pipetting out. A few drops of the solution of FDA-PI (FDA, 25 μg ml^{-1}; PI, 8 μg ml^{-1}) were added to the residues. The preparations were observed under a fluorescence microscope equipped with B2 filter (excitation filter, 450–490 nm; barrier filter, 520 nm) (EFDA-2; Nikon), and photographed as described above. The increase in PM permeability (IPP) was estimated from the ratio of the number of protoplasts exhibiting red fluorescence to the total number of protoplasts. Experiments were replicated more than three times.

K^+ release from protoplasts—Protoplasts isolated from root-tip were incubated with isotonic control solution for 10 min, then centrifuged at 200 × *g* for 5 min. Four ml of isotonic control solution was added to the pellet of protoplasts and mixed. The suspension was divided into two portions and the protoplasts were enumerated with a hemacytometer (Erma Inc., Tokyo, Japan). Two ml of isotonic control (0.2 mM Ca) or isotonic Al (0.2 mM Ca containing 200 μM Al) solution was added to each portion and the mixtures were treated for 0.5 h at 27°C. The protoplast suspensions were centrifuged at 300 × *g* for 5 min and the K concentration of the supernatants was determined by atomic absorption spectrophotometry (170-50 A; Hitachi).

Al concentration of protoplasts and of protoplast ghosts—Two ml of each solution (pH 4.5), i.e., isotonic control solution and 0.2 mM Ca without mannitol, was added to each protoplast pellet of which the number of protoplasts had been counted, and incubated for 10 min. Two ml of isotonic 0.2 mM $CaCl_2$ plus 200 μM $AlCl_3$ solution was added to the former and 2 ml of 0.2 mM $CaCl_2$ plus 200 μM $AlCl_3$ solution without mannitol to the latter. These mixtures were treated for 10 min at 27°C. Mixtures were filtered through a cellulose acetate membrane filter (pore size, 0.1 μm). The protoplasts on the membrane filters were washed several times with deionized water. The membrane filter materials were soaked in 10 ml of 0.1 M HCl for 24 h to extract Al. Al was determined by ICP.

Results

Relationships between Al tolerance and CEC of roots or cell wall—Fig. 1 shows the correlations between Al tolerance among the four plant species and CECs of the root-tips (0–1 cm) or of purified cell wall isolated from root-tips. When treated with 5 μM Al for 3 d, the plants ranked as follows in terms of Al tolerance; rice (79.9%) > maize (69.2%) > pea (59.6%) > barley (4.4%). The CEC of root-tip of rice, maize, and barley, which are monocotyledons, was approximately 10 $cmol_c$ (kg dry weight)$^{-1}$ of root, whereas that of pea, a dicotyledon, was approximately 2 times that value. The CEC of the purified cell wall of monocotyledons was approximately 10 $cmol_c$ (kg dry weight)$^{-1}$ of cell wall and that of pea approximately 6 times higher. The P concentration of the purified cell wall as a percentage of that of the root-tip for each plant species was 3 to 5% (data not shown); more than 95% of the protoplasm was removed from the cell wall materials. The correlation coefficients between Al tolerance and CEC of root-tip or of cell wall from root-tip among the four species was 0.011 and 0.109, respectively, and each correlation was non-significant.

Changes in permeability of the PM of root-tip cells and in root elongation following temporary exposure to Al ions—When stained with FDA-PI, cells with normal permeablity can exclude PI from their PMs; in such cells, FDA passes through PM and is hydrolyzed by intracellular esterases to produce fluorescein, and exhibits green fluorescence when excited by UV light. On the other hand, the permeabilized cells exhibit a bright red fluorescence by the passage of PI through their PMs and intercalation with DNA and RNA. When the seedlings were treated with control solution above pH 4.5, the PM permeability of root-tip cells was normal in all plant species (data not shown). Just after the roots of rice and barley were treated with 100 μM Al for 0.5 h, normal permeability of the PM was apparently retained in the root-tip cells (Fig. 2A, E). Normal permeability of the PM was also demonstrated in maize (da-

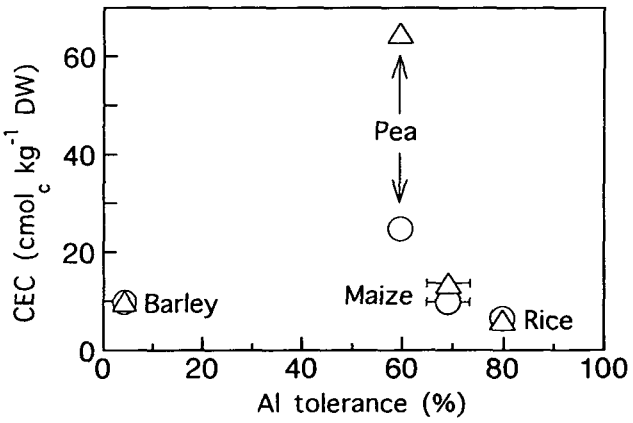

Fig. 1 Correlation between Al tolerance and CEC of root-tip (0–1 cm) or cell wall isolated from root-tip among four plant species. Al tolerance (%) was calculated based on the ratio of net root elongation of the longest root of plant treated with 5 μM Al for 3 d to that of control. The purity of cell wall isolated by homogenizing with homogenizing medium and subsequently chloroform-methanol was approximately 95% in every plant species based on the comparison of P concentrations between root-tip and cell wall. CEC was determined as the amount of Ca saturation to cation exchange sites at pH 5. The correlation coefficients between Al tolerance and CEC of root-tip (○) and cell wall (△) were 0.011 and 0.109, respectively (not significant). The experiment was duplicated and values are represented with SE.

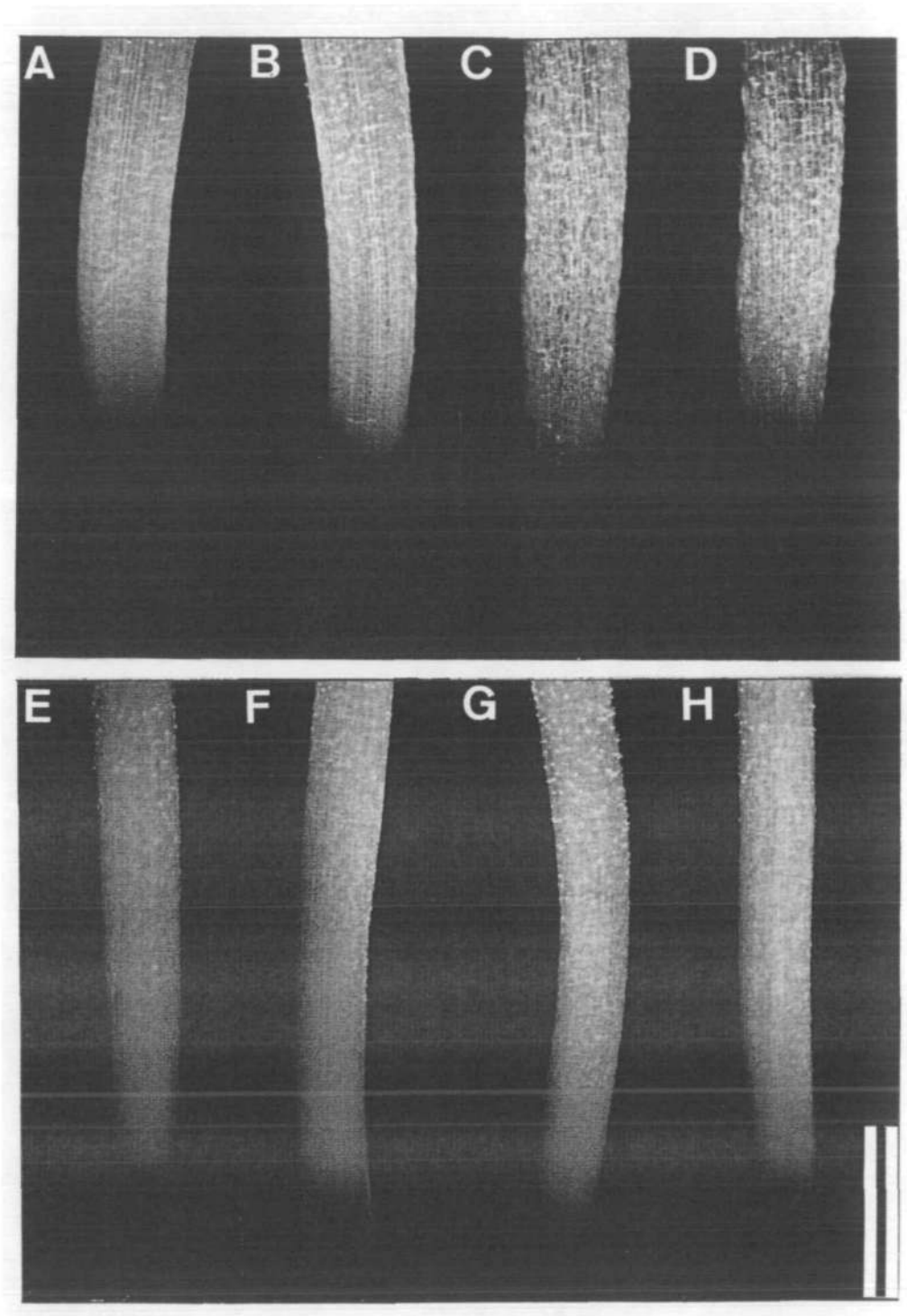

Fig. 2 Effect of a short Al treatment and subsequent root re-elongation on permeability of the PM in root-tip. Seedlings were pretreated with 5, 20, or 100 μM $AlCl_3$ for 0.5 h, and the roots were re-elongated in Al-free 0.2 mM $CaCl_2$ solution for 5 h. The re-elongated roots were stained with FDA-PI. Green fluorescence and red fluorescence exhibit normality or increase of permeability of the PM, respectively. A-D and E-H, root-tip portions of barley and rice, respectively; A and E, just after the treatment with 100 μM $AlCl_3$ for 0.5 h; B and F, C and G, D and H, after re-elongation of roots pretreated with 5, 20, or 100 μM Al, respectively. Scale bar indicates 1 mm.

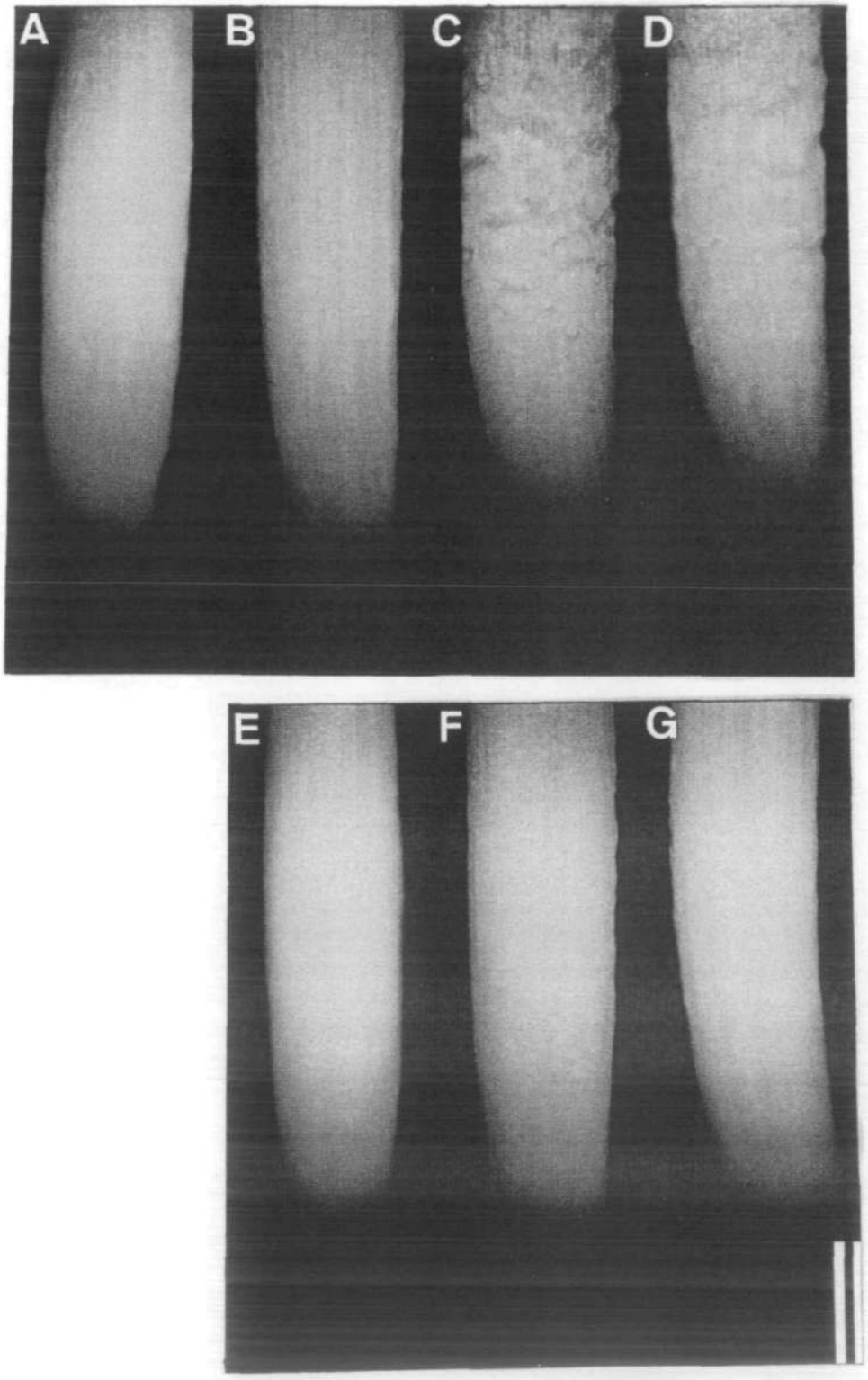

Fig. 3 Effect of the treatment of citrate on permeability of the PM in root-tip when re-elongated following a short pretreatment with Al. Pea seedlings were pretreated with 5, 20, and 100 μM Al for 1 h, and the Al-pretreated roots were treated with or without 2 mM citrate (pH 4.5) for 0.5 h, and subsequently, re-elongated in Al-free solution for 8 h. A, just after the treatment with 100 μM Al; B–G, after the re-elongation of roots following the pretreatment with 5 (B and E), 20 (C and F), or 100 (D and G) μM Al and the treatment with (E–G) or without (B–D) 2 mM citrate. Scale bar indicates 1 mm.

ta not shown) and pea (Fig. 3A). When the roots of Al-pretreated plants were re-elongated for 5 to 8 h in Al-free 0.2 mM $CaCl_2$ solution, the permeability of root-tip cells, especially in the epidermis and the outer cortex cells in the elongation zone of barley (Fig. 2B–D) and pea (Fig. 3B–D), increased significantly with increasing concentrations of Al pretreatment. Permeability of root-tip cells of maize (data not shown) and rice (Fig. 2F–H) re-elongated in the same way was normally retained irrespective of the concentrations used in Al pretreatment.

In pea roots which had been pretreated with 5, 20, or 100 μM $AlCl_3$ for 1 h and treated with 2 mM citrate (pH 4.5) for 0.5 h and thereafter re-elongated in Al-free 0.2 mM $CaCl_2$ solution for 8 h, the permeability of the PM of root-tip cells was retained irrespective of the concentrations in Al pretreatment (Fig. 3E–G). Similar results were observed for other plant species (data not shown). The permeability of control roots without Al pretreatment was normal at all pH and temperature settings in the present experimental conditions (data not shown).

The elongation of pea root in Al-free 0.2 mM $CaCl_2$ solution for 24 h following 1 h-pretreatment with different concentrations of Al was inhibited proportionally to the concentrations in Al treatment, i.e., approximately 10 and 20% inhibitions at 20 and 100 μM $AlCl_3$, respectively (Fig. 4). Treatment with citrate following 1 h-pretreatment with Al abrogated the inhibition of root elongation.

Effect of temporary exposure to Al ions on permeability of the PM of, K^+ release from, and Al uptake by the protoplasts isolated from root-tip—The permeability of the PM for pea protoplasts treated with isotonic control solution for 10 min was almost normally retained as these protoplasts exhibited green fluorescence (16% of IPP, Fig. 5A). By the 10 min-treatment with isotonic 100 μM $AlCl_3$ solution, pea protoplasts exhibited slightly more red fluorescence, which means a slight increase in PM permeability (30% of IPP, Fig. 5B). After the 5 min-treatment with hypotonic control solution, the permeability of the PM was almost normally retained in pea protoplasts pretreated with the isotonic control solution (20% of IPP,

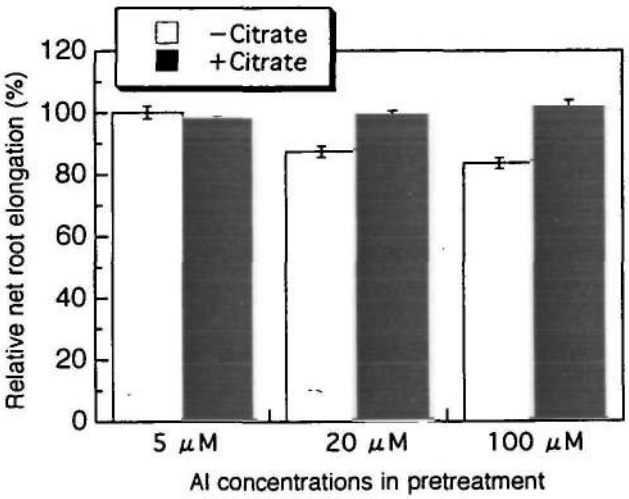

Fig. 4 Effect of the treatment of citrate on the re-elongation of pea roots pretreated temporarily with Al ions. Pretreatment with Al ions and treatment with citrate were carried out as described for Fig. 3. Roots were re-elongated in 0.2 mM $CaCl_2$ solution without Al for 24 h. Relative net root re-elongation was calculated based on the ratio of the re-elongation of roots pretreated with Al to that of roots pretreated without Al. The columns and the vertical bars in the figure are the mean and the SE in ten seedlings.

Fig. 5C), whereas it was considerably increased in pea protoplasts pretreated with the isotonic 100 μM $AlCl_3$ solution (66% of IPP, Fig. 5D). Considerably less permeabilization was observed in rice protoplasts on the same treatment (<25% of IPP, Fig. 5E–H).

Based on the relative K^+ release from protoplasts treated with isotonic 100 μM $AlCl_3$ solution for 0.5 h to that from protoplasts treated with isotonic control solution, the plants ranked in the order; barley, pea>>maize>rice (Table 1).

For Al content of protoplasts treated with 100 μM $AlCl_3$ for 10 min, the plants ranked as follows: barley>maize>pea>rice (Table 2). Little amount of the cell wall material was presumed to be the surface of protoplasts be-

Table 1 Effect of Al ions on K^+ release from protoplasts

	Rice	Maize	Pea	Barley
Control	0.36±0.07	0.95±0.02	0.27±0.03	0.64±0.08
Al	0.34±0.03	1.02±0.03	0.51±0.07	1.21±0.03
Al/Control	0.94	1.07	1.89	1.89

Protoplasts were treated with isotonic control (Control) or 100 μM Al (Al) solutions for 0.5 h. After centrifugation, the supernatants were collected and the K^+ concentration in the supernatants was determined using atomic absorption spectrophotometry. The values of Control and Al are means±SE in duplicate and represented as [pmol K^+ (protoplast)$^{-1}$]. The value of Al/Control indicates the ratio of K^+ release in both treatments.

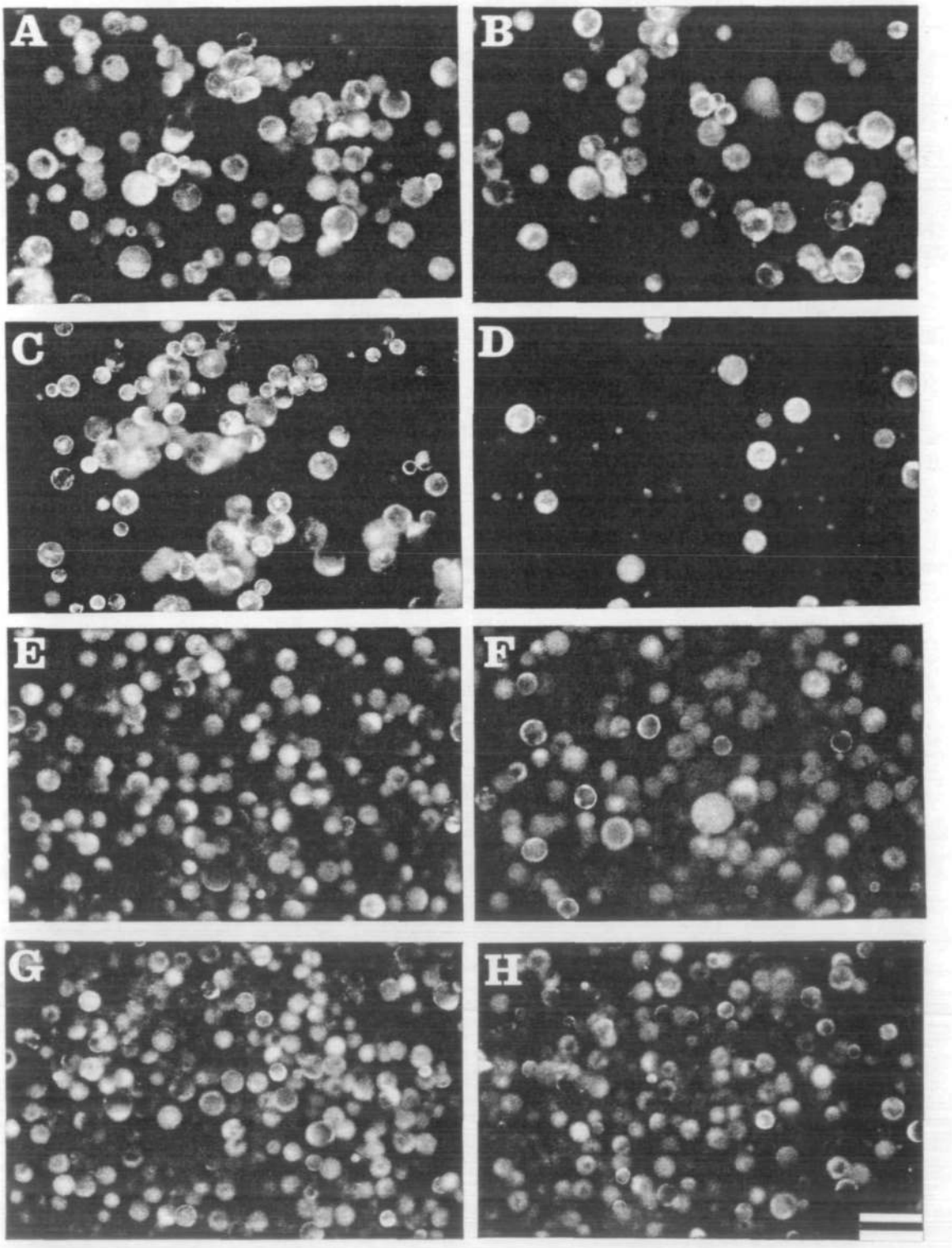

Fig. 5 Effect of Al ions and hypotonic conditions on permeability of the PM of protoplasts. Protoplasts isolated from root-tip of pea (A–D) and rice (E–H) were pretreated with isotonic control (A and E) or 100 μM Al (B and F) solution for 10 min, and subsequently treated with hypotonic Al-free solution for 5 min. Protoplasts were treated with hypotonic treatment following pretreatment with isotonic control (C and G) and Al solution (D and H). Protoplasts were observed under a fluorescence microscope following staining with FDA-PI. Scale bar indicates 100 μm.

Table 2 Al contents of protoplasts and protoplast ghosts

	Rice	Maize	Pea	Barley
Protoplasts (A)	20.9±4.3	58.7±2.0	48.6±5.9	62.3±3.2
Protoplast ghosts (B)	59.9±4.2	104.3±9.2	57.9±1.7	74.4±7.8
(B)/(A)	2.87	1.78	1.19	1.19

(A), protoplasts isolated from root tip (0–1 cm) were treated with isotonic 100 μM Al solution for 10 min; (B), protoplast ghosts were prepared by the treatment of protoplasts with control solution in the absence of 0.7 M mannitol, and subsequently with 100 μM Al solution in the absence of 0.7 M mannitol for 10 min. The values of (A) and (B) are means (n=3) ±SE of Al contents of protoplasts [fmol Al (protoplast)$^{-1}$] and protoplast ghosts [fmol Al (protoplast ghost)$^{-1}$], respectively. The value of (B)/(A) indicates the ratio of Al content of protoplast ghosts to that of protoplasts.

cause of the short period of the treatment (within 30 min) and the simple composition of the nutrient solution (Mock et al. 1990). Protoplast ghosts took up more Al in all plant species, but on the ratio of the Al content of protoplasts to that of protoplast ghosts the plants ranked as follows: rice > maize > pea, barley (Table 2).

Discussion

Most investigations on mechanisms of Al tolerance have treated cultivars or near-isogenic lines differing in Al tolerance within the same species. In the present study, we focused on the effect of Al ions on the permeability of PM and the negativities of cell wall and PM among several plant species that differ widely in Al tolerance.

Munn and McCollum (1976) and Wagatsuma (1983) reported no correlation between root CEC and Al tolerance, whereas Mugwira and Elgawhary (1979) and Rengel and Robinson (1989b) suggested a negative correlation between the two. In the present experiment, no correlation was recognized between root CEC and Al tolerance among the four plant species (r=0.011, Fig. 1). We also found no correlation between the two among ten plant species and cultivars within the same species that differ in Al tolerance (data not shown). The CEC of the purified cell wall from pea was approximately 6 times greater than those from the other species and was approximately 2.6 times greater than the root CEC of pea (Fig. 1). The CECs of cell walls from the other species were similar to those of roots. The high value of the CEC of cell wall from pea may be attributable to a considerable removal of cellular components with little negative charge in the preparation of cell wall materials. A strong positive correlation was recognized between root CEC and the CEC of cell wall among the four plant species (r=0.994, data not shown). Although EDTA and EGTA used in cell wall isolation may partly remove water soluble and loosely bound pectins in cell wall, the difference in the CECs of cell walls among the species was consistent with the difference in pectin contents among them (Sakurai et al. 1991). No correlation was recognized between cell wall CEC and Al tolerance among the species (r=0.109, Fig. 1). Cosgrove and co-workers found two proteins (expansins) involved in wall extension from the cell wall of growing hypocotyls, and reported that Al^{3+} inhibited both their activities (McQueen-Mason et al. 1992, Cosgrove 1996). However, rapid response to a temporary contact with Al ions (within 1 h) is assumed to be characteristic of the PM of root-tip cells (Fig. 2, 3, 4). Short-term contact with Al ions (10 min) revealed a similar effect on the PM of cell-wall removed cells, i.e., protoplast (Fig. 5). These results suggest a reduced significance of cell wall materials in Al tolerance. The role of expansins in Al tolerance remains to be elucidated.

Ishikawa et al. (1996) suggested an association of permeability of the PM with Al tolerance on treatment with 20 μM $AlCl_3$ for 2 h. In the present experiment, temporary treatment with Al ions (less than 1 h) did not induce any change in permeability of the PM and no relation between permeability and Al tolerance was observed (Fig. 2A, E, 3A). However, when roots pretreated with Al ions for a short period (less than 1 h) were re-elongated in Al-free solution, the PM of root-tip cells was clearly permeabilized depending on the Al concentrations in treatment and on Al tolerance (Fig. 2B–D, F–H, 3B–D). These results suggest that the PM of Al-sensitive plant species becomes rigid and less extensible on short-term contact with Al ions. The PM of protoplasts isolated from Al-sensitive plant species, which had been pretreated with isotonic Al solution for 10 min, was permeabilized considerably after the 5 min-treatment with hypotonic Al-free control solution (66% of IPP, Fig. 5D). These results can be interpreted as follows: in Al-sensitive plant species, contact with Al ions induces a rigid PM, reduces the extension of it, permeabilizes it during the re-elongating period or under hypotonic conditions, and inhibits root elongation.

When Al-pretreated roots were treated with citrate for 0.5 h, and subsequently re-elongated in Al-free solution for several hours, the permeability of the PM in root-tip cells

was retained in every plant species irrespective of the Al concentrations in pretreatment (Fig. 3E–G). The 0.5 h of treatment with 2 mM citrate at pH 4.5 results in the desorption of the apoplastic Al bound to the cell wall and outer leaflet of PM (Ownby and Popham 1989, Zhang and Taylor 1989), though some Al ions enter the symplast (Lazof et al. 1994). In addition to this, the permeation of citric acid and aluminum citrate through the lipid bilayer is limited by hydrogen bonding between hydrated water molecules and carboxyl groups of citrate, and the hydrated water shell of Al ions (Akeson and Munns 1989). The recovery of normal permeability of the PM by treatment of citrate is ascribable to the desorption of Al from the PM.

Al associated with protoplast is composed of two fractions: Al bound to the PM and permeated Al. Microtubule, nucleus and cytoplasmic proteins may be retained in the protoplast ghost (Sonobe and Takahashi 1994), and therefore the permeated Al ions can easily bind to symplastic contents. A greater Al content in protoplast ghost relative to protoplast indicates difficulty in permeation of protoplast by Al ions. Table 2 suggests that the PM of root-tip for Al-tolerant plant species is less permeable to Al ions. The smaller release of K^+ from protoplast (Table 1) and root-tip portion (Wagatsuma et al. 1995a) of Al-tolerant plant species supports this. That there were no differences in K^+ release from (Table 1) and Al permeation into (Table 2) the protoplasts between pea and barley in spite of the significant difference in Al tolerance between them (Fig. 1) may be due to the high concentration of Al in the medium, i.e., 100 μM Al.

Ishikawa et al. (1996) investigated the comparative toxicity of Al^{3+}, Yb^{3+}, and La^{3+} to root-tip cells and suggested that Al^{3+} with the largest ionic potential among the three metal ions most preferentially binds covalently to the negative sites of PM. When hydrated Al^{3+}, i.e., $[Al(OH_2)_6]^{3+}$, binds to the negatively charged phospholipid, seven to eight hydrated water molecules of the phosphate group (Cevc 1982) and six hydrated water molecules of Al^{3+} may be dehydrated together by the Eigen mechanism. Dispersed phospholipid molecules in the normal PM form a partial packing area by binding Al ions; membrane in a liquid crystal state becomes rigid and gel-like as a result of dehydration (Hauser and Phillips 1979, Chen et al. 1991). This dehydration may make the packing area more hydrophobic. Membrane proteins can also join this packing area with their carboxyl groups and Al ions. The boundary between the packed area (e.g., phospholipids and proteins) and non-packed area (e.g., sterols) may be enlarged by re-elongation or under hypotonic conditions; permeabilization of the PM may be the result of the notch effect of stress concentration in a mechanical sense.

We speculated that the negativity of the PM of root-tip cells determines the extent to which Al ions bind to the PM of root-tip cells and finally the Al tolerance of several plant species (Wagatsuma and Akiba 1989, Wagatsuma et al. 1995a, b). Takabatake and Shimmen (1997) and Jones and Kochian (1997) reported phospholipids or lipids of PM as the primary site for Al toxicity. Yermiyahu et al. (1997) suggested that PM surface negativity and Al sorptive capacity probably account for some of the sensitivity to Al^{3+}. On the other hand, Zhang et al. (1997) reported that the ratio of phospholipids to total lipid in the PM from whole roots was smaller in Al-sensitive wheat cultivar. Further investigations on the characteristics of PM in association with Al tolerance should be carried out. Al tolerance among plant species is rapidly revealed in the intactness of the PM permeability of root-tip cells after temporary exposure to Al ions. Similar results have been observed between cultivars of several plant species in other experiments, and this will be reported elsewhere. To the authors' knowledge, no investigations have been reported on this close association following short-term Al treatment; less than 0.5 h in whole plant and 10 min in protoplast.

Whether or not the amount of organic acids exuded from root-tip is enough to detoxify Al ions in medium is still unclear, though there is a higher concentration of exuded organic acids near the surface and in the cell wall of root. We have explained one of the mechanisms of Al tolerance even in protoplasts free from such apoplastic space.

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