The decrease of extracted apoplast protein in soybean root tip by aluminium treatment

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Abstract

Aluminium effect on the mobility of apoplast protein in root tips was studied. Two-day seedlings of soybean (*Glycine max.* (L.) Merr. cv. Tsurunoko) were treated with 50 µM $AlCl_3$ for 2 h. Using infiltration method, the apoplast protein in root tips was extracted with 20 or 100 mM $MgCl_2$. When 20 mM $MgCl_2$ was used to collect weakly bound protein to apoplast, the amount of protein extracted was reduced to be about 20 % compared with that of control and the band of 97 kDa disappeared in SDS-PAGE gel. However, the 97 kDa protein could be extracted by 100 mM $MgCl_2$, which were used for extraction of more tightly bound protein to apoplast, and the amount was estimated to be the same as that of control. When the protein was further developed in two-dimensional electrophoresis, three spots were found between pI 6.4 and 6.5. This is the first report of an Al effect on the mobility of apoplast protein.

Additional key words: Glycine max, protein mobility, SDS-PAGE.

Introduction

Aluminium ion is generally toxic to plants. In acid soils Al ions has been reported as one of the main factors inhibiting plant growth and crop production (Wright 1989). As the soil amelioration techniques are frequently restricted, breeding for Al tolerance is considered a useful way to improve crop production on acid soils. It is now generally accepted that the root tip is a major portion where Al is accumulated and the toxicity is initially induced. The root response to Al in the soil solution varies with the plant species or genotypes. In some Al tolerant plants, it was reported that the ability to release organic anions (Ma 2000, Ma *et al.* 2001, Ryan *et al.* 2001) from the root tips was one of the mechanism to keep the content of Al lower than that of Al sensitive ones.

In contrast to Al tolerance, the mechanism of Al toxicity mechanism is still not clear. The symptoms are similar to nutrient deficiencies (Bennet *et al.* 1986,

Taylor 1988), but these symptoms appear to be the consequence of inhibition of root development. Effects of Al on various cell constituents (cell wall, chromatin, Golgi body, plasma membrane, vacuole) have been reported (Matsumoto and Morimura 1980, Johnson and Bennet 1990, Ikeda and Tadano 1993, Van *et al.* 1994, Zhang *et al.* 1996), but the most critical sites of Al toxicity are not still understood. One of serious problems to study mechanism of Al toxicity is lacking sensitive method to detect trace amount of Al in plant tissues. Therefore, in many cases, plants with long-term Al treatments have been used, in spite of the importance to know what triggered the root growth inhibition in an early stage of Al treatment.

It is well known that the callose formation (Zhang *et al.* 1994) and a reduction of K^+ net-efflux (Horst *et al.* 1992) are induced by short time Al treatment. Horst (1995) suggested the sensitive binding sites to Al in

apoplast and the competition for these binding sites might be responsible for root growth inhibition in short term Al treatment. However, the knowledge of Al toxicity to the plasma membrane or cell wall is very limited. Moreover, in case of the other components of apoplast, there is very little information about an Al effect. In the present report, we focused on the mobility and the distribution of apoplast protein in root tip for the first time.

Materials and methods

Two-day seedlings of soybean [*Glycine max* (L.) Merr. cv. Tsurunoko], which root is approximately 5 cm in length, were treated with 0.2 mM $CaCl_2$ (pH 4.4) with or without 50 µM $AlCl_3$. To begin with the experiments, an effect of the Al concentration on the root tissues was determined by measuring the root elongation and the viability after 24 h treatment. To know the viability of the plasma membrane, the root tip was doubly stained with 1 mg m^{-3} FDA + 15 mg m^{-3} PI for 5 min at room temperature. When the permeability of plasma membrane is normal, FDA absorbed into cytoplasm reacts with esterase and green fluorescence is emitted, which is excited by UV light. In the case when the activity of the plasma membrane is lowered, PI is incorporated into cytoplasm and reacts with nucleotide and emits red fluorescence.

For experiments of protein extraction from apoplast in root tips, seedlings treated for 2 h were used. After the treatment, all the seedlings were washed with distilled water (pH adjusted to 4.4) to remove Al weakly bound to the root surface and then the root tips were excised into two parts. The first part (position 1) was between 0 and 2 mm from root apex including meristem area as well as root cap. The second one was between 2 and 5 mm from the root apex, corresponding to an elongation zone, *i.e.*, without root cap. Each part collected from about 100 seedlings, was washed with distilled water and then the segment was put side by side in an ultrafree microtube with a filter which pore size was 0.22 µm (*Millipore*, Bedford, USA). Approximately 0.12 cm^{-3} of 20 mM $MgCl_2$ was added into the microtubes as extraction solution for extraction of weakly bound protein to apoplast and the microtubes of the packed tissues were placed in vacuum for 5 min so that the extraction solution infiltrated into apoplast space. Then the microtubes were centrifuged for 5 min at 2 000 *g* to collect the apoplast protein. The vacuum infiltration and the centrifugation were repeated once. The collected infiltrate was immediately mixed with 0.02 cm^{-3} of protein stabilization solution, 0.3 M acetate buffer (pH 5.0) containing 5 mM NaEDTA, 10 mM PMSF and 0.5 M DTT, and then, ultrafiltrated through molecular membrane (*Millipore*, Mr 30 000).

When a 100 mM $MgCl_2$ solution was applied as extraction solution to obtain protein more tightly bound to apoplast, the root tips between 0 and 5 mm from root apex were used. The root tips were cut further, approximately every 200 µm in thickness to collect the tightly bound protein to apoplast at infiltration more easily. The following procedures were the same as the case of 20 mM $MgCl_2$.

A part of the extracted solution was used for protein determination by *Bio-Rad* protein assay kit (Richard, USA). Aliquot was dried under vacuum and was dissolved with solution consisted of 10 % (m/v) glycerol, 5 % (v/v) 2-mercaptoethanol, 2.3 % (m/v) SDS and 62 mM Tris-HCl (pH 6.8). Then the sample was applied to an electrophoresis, *Phast System* (*Pharmacia*, Tokyo, Japan), with a 7.5 % SDS-polyacrylamide gel and the gel was treated with silver staining.

Two-dimensional electrophoresis was performed according to O'Farell (1975). Extracted protein was loaded at the end of focusing gel. Isoelectric focusing was conducted for 15 h at 400 V, followed by 1 h at 800 V. After extrusion, the gels were loaded onto a second resolving gel, 7.5 % polyacrylamide. The electrophoresis was run for 3 h at 30 mA per gel, then removed, fixed and stained with silver staining kit DAIICHI (*Daiichi Pure Chemicals*, Tokyo, Japan).

Results

After 24 h treatment of 50 µM $AlCl_3$, the root elongation was inhibited to be about 40 % of that in control. However, root growth inhibition was not induced within 2 h, nor the light green fluorescence showing viability observed in root tips was different from that of control. The results suggest the decrease of viability was not induced and the membrane function was maintained normal within 2 h (data not shown).

In control, the amount of apoplast protein extracted from position 1 was 3 times higher than that of the elongation zone. Though there was no influence of Al treatment on amount of extracted protein in the elongation zone, remarkable decrease (approximately 20 % of control) was shown in position 1 by Al treatment (Table 1).

Table 1. Protein amount [μg g^{-1}(d.m.)] extracted with 20 mM MgCl$_2$ from root tip (position 1, 0 - 2 mm) and elongation zone (2 - 5 mm) of control soybean root or root treated with 50 μM AlCl$_3$ for 2 h. Means ± SE, n = 4.

Control root tip	elong. zone	Al-treated root tip	elong. zone
95.0 ± 12.8	29.1 ± 2.6	20.2 ± 5.7	25.8 ± 1.8

Table 2. Protein amount [μg g^{-1}(d.m.)] extracted with 100 mM MgCl$_2$ from soybean root tips treated with or without 50 μM AlCl$_3$ for 2 h. Means ± SE, n = 4.

Control (0 - 5 mm)	Al (0 - 5 mm)
1.95 ± 0.06	1.99 ± 0.06

When the *lane* 2 and 4 were compared, the pattern of protein distribution was similar with each other, but the amount of high molecular mass protein of *lane* 2 was found to be higher than that of *lane* 4. In the case of Al treated samples, the high molecular mass proteins disappeared in both position 1 (*lane* 1) and position 2 (*lane* 3) (Fig. 1A).

When 100 mM MgCl$_2$ was applied to obtain apoplast protein including more tightly bound protein, the amount of extracted apoplast was increased drastically (Table 2) compared with that of 20 mM (Table 1). In contrast to the case of 20 mM, there was no difference in amount of extracted apoplast protein between control and Al treatment, and the distribution of the protein pattern in the root treated with Al was also similar to that of the control, when extracted with 100 mM MgCl$_2$. Furthermore, the high molecular mass protein, approximately 97 kDa, which disappeared at Al treatment with extraction using
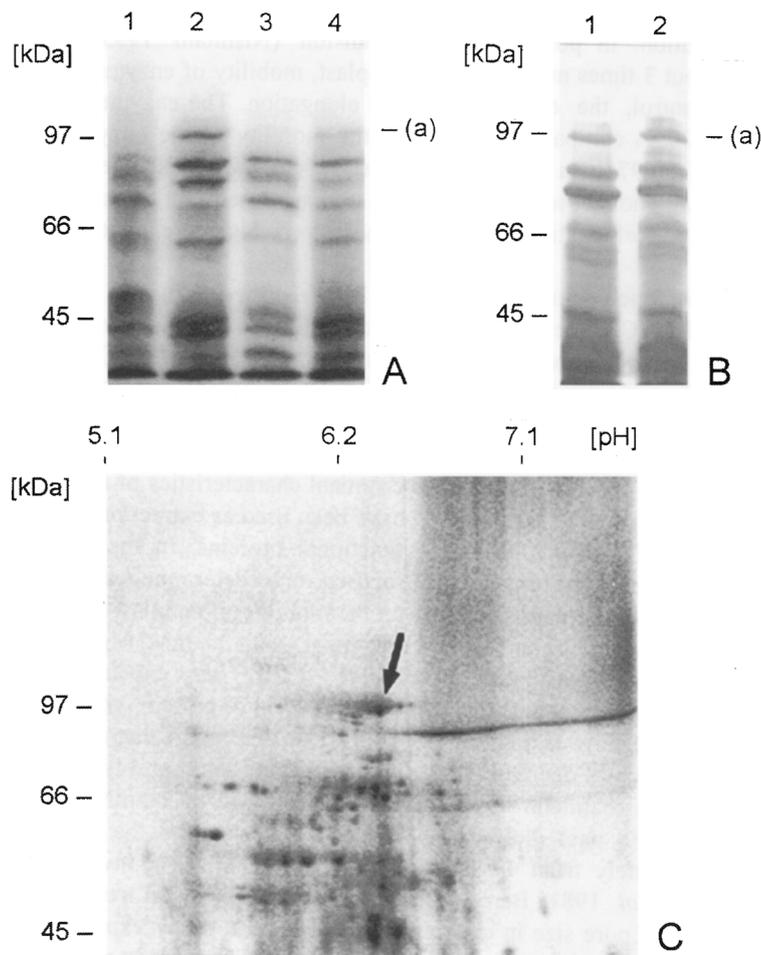

Fig. 1. *A* - SDS-PAGE of apoplast protein extracted with 20 mM MgCl$_2$ from position 1 (*lanes 1, 2*) and elongation zone (*lanes 3, 4*) of soybean root treated with (*lanes 1, 3*) or without (*lanes 2, 4*) 50 μM AlCl$_3$ for 2 h. The gel was stained with silver. *B* - SDS-PAGE of apoplast protein extracted with 100 mM MgCl$_2$ from root tips within 5 mm from root apex treated with (*lane 1*) or without (*lane 2*) 50 μM AlCl$_3$ for 2 h. The gel was stained with silver. *C* - Two-dimensional protein profile extracted with 100 mM MgCl$_2$ from apoplast in control root tips within 5 mm from root apex. The gel was stained with silver.

20 mM MgCl₂ (Fig. 1*A*) was shown in both control and Al-treated samples and the amount of the protein was estimated to be the same with each other (Fig. 1*B*).

The 97 kDa protein in one-dimensional gels was separated to three spots after applying to the two-dimensional electrophoresis (Fig 1*C*). The positions of the three spots were found between pI 6.4 and pI 6.5.

Discussion

When soybean seedlings were treated with 50 μM AlCl₃ (pH 4.4) for 2 h, neither root growth inihibition nor the viability decrease was observed, suggesting that the permeability of plasma membrane was kept normal throughout the treatment (data not shown).

The protein studies about an Al effect on root have been limited to the induction of stress-protein of root (Aniol 1984). The present study is the first report focusing on the mobility of the apoplast protein in root tips. Enormous difference in amount of the extracted protein between position 1 and the elongation zone after 2 h treatment was observed (Table 1), when 20 mM MgCl₂ was used as extraction solution. In position 1, where amount of the protein was about 3 times more than that of the elongation zone in control, the extracted protein was decreased to be about 20 % compared with that of control by Al treatment. The 97 kDa protein, shown by SDS-PAGE analysis, was not observed in Al-treated root both in position 1 and elongation zone (Fig 1*A*).

When the concentration of the extraction solution was increased from 20 mM to 100 mM MgCl₂, the 97 kDa band was found in Al-treated root tips as well as in control, and the amount of the 97 kDa protein was almost same between the root tips with and without Al treatment (Fig. 1*B*). The total amount of the extracted protein from root tips was also quite similar to that of control (Table 2).

Although neither the function nor the sequence of the protein has been identified yet, because of the limiting amount of the apoplast protein extracted, there was not any difference in the amount of 97 kDa protein and the total apoplast protein between control and Al treatment in case of 100 mM MgCl₂ extraction, which amount was drastically decreased by 20 mM MgCl₂ extraction (Table 1, Fig. 1*A*). These results suggested the decrease in the mobility of the apoplast protein by Al treatment.

The pore size of cell wall enables to pass globular protein with molecular size, approximately from 17 to 70 kDa (Carpita *et al.* 1979, Tepfer *et al.* 1981, Baron-Epel *et al.* 1998). When the decrease of pore size in cell wall was induced by the binding of Al to cell wall, the mobility of protein, especially with high molecular mass, may be interrupted. However, the cell wall is not the only site to bind Al in apoplast. Putterill and Gardner (1988) reported that acid polypeptide such as poly-L-asparagine acid and poly-L-glutamic acid combines with Al rapidly even at low concentration of Al.

The discussion about the apoplast protein had to be based on the knowledge in leaf and shoot tissues, because the research about apoplast protein in root was very limited. The mechanism of cell expansion in detail is not still clear, but it is known, for example, that the reaction of cleavage and reconnection of cross-link network of xyloglucan and cellulose is crucial in cell wall at cell expansion (Nishitani 1995). Among the proteins in apoplast, mobility of enzyme protein is very important in cell elongation. The enzyme activity has to be depended on the mobility of the enzyme in apoplast, because the substrate can not be expected to move or diffuse. In root tip, it is supposed that the activity of enzyme in apoplast may be very high to expand the cells smoothly after the cell division.

It is reported that one of the major enzyme of the reaction, endoxyloglucantransferase, is able to be extracted with 50 mM MgCl₂ (Nishitani and Tomionaga 1991). In most studies on apoplast protein, not only much higher concentration than 50 mM MgCl₂ to cut stronger ionic bond, but also DMSO, CDTA or enzyme to change chemical characteristics of cell wall or cut covalent bond have been used as extraction solution for discovering new functional proteins. In the present study, however, we focused on to determine whether Al treatment influenced on the mobility of apoplast protein in an early stage of Al treatment, and 20 mM MgCl₂ was used for extraction process which was expected to extract weakly ionic bound protein or protein with higher mobility in apoplast. This is the first report that relatively short time treatment of Al inhibited the mobility of protein bound to apoplast before root growth inhibition or serious damage was induced and suggests the decrease of the mobility in apoplast protein may be involved in induction of the root growth inhibition by Al treatment. The mechanism of Al toxicity in root tip is expected to be clarified further, focusing more on apoplast protein.

References

Aniol, A.: Induction of aluminum tolerance in wheat seedlings by low doses of aluminum in nutrient solution. - Plant Physiol. 76: 551-555, 1984.

Baron-Epel, O., Gharyal, P.K.: Schindler, M.: Pectins as mediators of wall porosity in soybean cells. - Planta 17: 389-395, 1998.

Bennet, R.J., Breen, C.M., Fey, M.V.: Aluminum toxicity and induced nutrient disorders involving the uptake and transport of P, K, Ca and Mg in *Zea mays* L. - S. Afr. J. Plant Soil 3: 11-17, 1986.

Carpita, N., Sabularse, D., Montezions, D., Delmer, D.P.: Determination of the pore size of cell walls of living plant cells. - Science 205: 1144-1147, 1979.

Horst, W.J.: The role of the apoplast in aluminium toxicity and resistance of higher plants: a review. - Z. Pflanzenernähr. Bodenk. 158: 419-428, 1995.

Horst, W.J., Asher, C.J., Cakmak, I., Szulkiewicz, P., Wissemeier, A.H.: Short-term responses of soybean roots to aluminium. - J. Plant Physiol. 140: 174-178, 1992.

Ikeda, H., Tadano, T.: Ultrastructural changes of the root tip cells in barley induced by comparatively low concentration of aluminum. - Soil Sci. Plant Nutr. 39: 109-117, 1993.

Johnson, P.A., Bennet, R.J.: Aluminum tolerance of root cap cells. - J. Plant Physiol. 137: 760-762, 1990.

Ma, J.F.: Role of organic acids in detoxification of aluminum in higher plants. - Plant Cell Physiol. 41: 383-390, 2000.

Ma, J.F., Ryan, P.R., Delhaize, E.: Aluminium tolerance in plants and complexing role of organic acids. - Trends Plant Sci. 6: 273-278, 2001.

Matsumoto, H., Morimura, S.: Repressed template activity of chromatin of pea roots treated by aluminum - Plant Cell Physiol. 21: 951-959, 1980.

Nishitani, K.: Endo-xyloglucan transferase, a new class of transferase involved in cell wall construction - J. Plant Res.

108: 137-148, 1995.

Nishitani, K., Tominaga, R.: *In vitro* molecular weight increase in xyloglucans by an apoplastic enzyme preparation from epicotyls of *Vigna angularis*. - Physiol. Plant. 82: 490-497, 1991.

O'Farell, P.H.: High resolution two-dimensional electrophoresis of proteins, L. - J. biol. Chem. 262: 10035-10038, 1975.

Putterill, J.J., Gardner, R.C.: Proteins with the potential to protect plants from aluminum toxicity. - Biochem. biophys. Acta 964: 137-145, 1988.

Ryan, P.R., Delhaize, E., Jones, D.L.: Function and mechanism of organic anion exudation from plant roots. - Annu. Rev. Plant Physiol. Plant mol. Biol. 52: 527-560, 2001.

Taylor, G.J.: The physiology of aluminum phytotoxicity. - In: Sigel, H. (ed.): Metal Biological Systems. Aluminum and its Role in Biology. Vol. 24. Pp. 123-163. Marcel Dekker, New York 1988.

Tepfer, M., Taylor, I.E.P.: The permeability of plant cell walls as measured by gel filtration chromatography. - Science 213: 761-763, 1981.

Van, H.L., Kuraishi, S., Sakurai, N.: Aluminum-induced rapid root inhibition and changes in cell-wall components of squash seedlings. - Plant Physiol. 106: 971-976, 1994.

Wright, R.L.: Soil aluminum toxicity and plant growth. Commun. Soil Sci. Plant Anal. 20: 1479-1497, 1989.

Zhang, G., Hoddinott, J., Taylor, G.J.: Characterization of 1,3-β-D-glucan (callose) synthesis in roots of *Triticum aestivum* in response to aluminum toxicity. - J. Plant Physiol. 144: 229-234, 1994.

Zhang, G., Slaski, J.J., Archambault, D.J., Taylor, G.J.: Aluminum-induced alterations in lipid composition of microsomal membranes from an aluminum-resistant and an aluminum-sensitive cultivar of *Triticum aestivum*. - Physiol. Plant. 96: 683-691 1996.