Aluminum Targets Elongating Cells by Reducing Cell Wall Extensibility in Wheat Roots

Jian Feng Ma 1,4, Renfang Shen 2, Sakiko Nagao 1 and Eiichi Tanimoto 3

1 Faculty of Agriculture, Kagawa University, Ikenobe 2393, Miki-cho, Kita-gun, Kagawa, 761-0795 Japan
2 State Key Laboratory of Soil and Sustainable Agriculture, Institute of Soil Science, Chinese Academy of Science, Nanjing, 210008 China
3 Graduate School of Natural Sciences, Nagoya City University, Mizuho-ku, Nagoya, 467-8501 Japan

Phytotoxicity of aluminum is characterized by a rapid inhibition of root elongation at micromolar concentrations, however, the mechanisms primarily responsible for this response are not well understood. We investigated the effect of Al on the viscosity and elasticity parameters of root cell wall by a creep-extension analysis in two cultivars of wheat (Triticum aestivum L.) differing in Al resistance. The root elongation and both viscous and elastic extensibility of cell wall of the root apices were hardly affected by the exposure to 10 μM Al in an Al-resistant cultivar, Atlas 66. However, similar exposure rapidly inhibited root elongation in an Al-sensitive cultivar, Scout 66 and this was associated with a time-dependent accumulation of Al in the root tissues with more than 77% residing in the cell wall. Al caused a significant decrease in both the viscous and elastic extensibility of cell wall of the root apices of Scout 66. The “break load” of the root apex of Scout 66 was also decreased by Al. However, neither the viscosity nor elasticity of the cell wall was affected by in vitro Al treatment. Furthermore, pre-treatment of seedlings with Al in conditions where root elongation was slow (i.e. low temperature) did not affect the subsequent elongation of roots in a 0 Al treatment at room temperature. These results suggest that the Al-dependent changes in the cell wall viscosity and elasticity are involved in the inhibition of root growth. Furthermore, for Al to reduce cell wall extensibility it must interact with the cell walls of actively elongating cells.

Keywords: Aluminum toxicity — Cell wall — Extensibility — Root elongation — Triticum aestivum L.

Introduction

Phytotoxicity of aluminum ions (Al3+) is characterized by a rapid inhibition of root elongation at micromolar concentrations (Kochian 1995). However, the primary mechanisms involved in Al toxicity are still poorly understood. Numerous studies have been carried out intensively on Al phytotoxicity and a number of events caused by Al were observed. For example, Al was reported to block Ca2+ channels on the root-cell plasma membrane in wheat roots (Huang et al. 1992). Aluminum caused membrane damage and peroxidation of membrane lipids (Cakmak and Horst 1991, Wagatsuma et al. 1995). Aluminum affected signal transduction pathways such as the phosphoinositide pathway in the plasma membrane, which would result in concomitant disruptions of cytoplasmic Ca2+ homeostasis, the phospholipids bilayer of the plasma membrane, and components of the cytoskeleton (Jones and Kochian 1995). Recent studies showed that Al blocked symplastic transport and communication by inducing callose deposition at plasmodesmata in wheat roots (Sivaguru et al. 2000) and affected mitochondrial functions by triggering reactive oxygen species in pea roots (Yamamoto et al. 2002). It seems that Al interacts with multiple sites of the root cells including cell wall, plasma membrane and symplasm. However, it is unclear whether these events caused by Al are the result or cause of Al-induced inhibition of root elongation.

Root elongation consists of cell division and cell elongation. Since Al-induced inhibition of root elongation occurs within hours (e.g. Ownby and Popham 1989, Ryan et al. 1993), the contribution of cell division to the elongation in the short-term would be small. This is supported by the evidence that only exposure of the elongation zone, rather than other apical regions to Al resulted in inhibition of root elongation (Ryan et al. 1993, Sivaguru and Horst 1998). Therefore, initial Al-induced inhibition of root elongation is likely to be caused by the inhibition of cell elongation. Cell elongation is regulated by turgor pressure and physical properties of the cell wall. Several studies have shown consistently that most of the Al is bound to the cell wall. Clarkson (1967) reported that 85–90% of the total Al accumulated by barley roots was tightly bound to cell walls. In giant algal cells of Chara corallina, up to 99.9% of the total cellular Al accumulated in the cell wall (Rengel and Reid 1997). Chang et al. (1999) reported that 89% of total Al was associated with the cell wall in cultured tobacco cells. Using hypocotyls of okra as an experimental model, it was found that 95% of the total Al was associated with the cell wall of epidermis (Ma et al. 1999). However, the effect of Al bound on the cell wall on its properties is less investigated. Tabuchi and Matsumoto (2001) reported that exposure to Al for 6 h decreased the extensibility of root cell walls in an Al-sensitive cultivar of wheat, Scout 66 by using tensile test method. How-

4 Corresponding author: Email, maj@ag.kagawa-u.ac.jp; Fax, +81-87-891-3137.
However, the time-sequence of mechanical changes in the cell walls during Al-induced inhibition of root elongation has not been examined. Here, we report a detailed study on the effect of Al on the viscosity and elasticity of root cell wall in two cultivars of wheat (Triticum aestivum L.) differing in Al resistance. We found that Al caused a rapid decrease in both viscosity and elasticity of the cell wall of the root apices.

**Results**

These experiments examined the changes that occur in the viscosity and elasticity of root cell walls during Al treatments that reduce root elongation. Exposure to 10 µM Al hardly inhibited the root elongation in an Al-resistant cultivar, Atlas 66 (Fig. 1a), while significantly inhibited (about 40% inhibition) the root elongation in an Al-sensitive cultivar, Scout 66 (Fig. 1c). The Al content of root apices (0–1 cm) in Scout 66 increased with time and the Al in the cell wall accounted for 77–102% of total Al (Fig. 1d). The Al content of root apices in Atlas 66 was much less than that in Scout 66 (Fig. 1b, d). Microscopic observation showed that Al was accumulated mainly on the epidermis and outer cortex, rather than inner cortex and stele in Scout 66, after the roots were stained with 0.1% Eriochrome Cyanine R solution (Fig. 2).
When the roots were exposed to Al and then fixed with methanol (in vivo treatment), the elasticity parameters ($E_0$, $E_1$, and $E_2$) and the viscosity parameters ($\eta_0$, $\eta_1$, $\eta_2$) were hardly affected in Atlas 66 (Fig. 3a, b), while in Scout 66 these parameters were increased by 6–22% after 3 h and more thereafter as compared to controls (Fig. 3d, e). Among these parameters, $E_0$ and $\eta_0$ were most affected by Al. These parameters describe the physical properties of the cell wall such that smaller values reflect a greater extensibility (Tanimoto et al. 2000). Therefore the increases in both sets of parameters measured in this study indicate that Al decreases the elastic and viscous extensibilities of the root cells in Scout 66 (Fig. 3f). The total extensibility was decreased by 20–30% by Al in Scout 66 at 3 and 6 h and there was no significant difference in the extensibility between 6 and 9 h. The break load of the root cell wall in this cultivar was also decreased by Al (Fig. 4), suggesting that Al makes the cell wall brittle.

However, when the root apices of Scout 66 were exposed to Al after fixing in methanol (in vitro treatment), neither the elastic nor the viscosity extensibility was affected by Al (Fig. 5), although the root apex contained 2.33 nmol apex$^{-1}$, which is comparable to the alive root apex (Fig. 1d). Since the cell extensibility of dead roots was not affected by in vitro Al treatments, living and actively growing roots was suggested to be a target for Al.

To examine the targeting site of Al, pretreatments of seedlings were performed in conditions where root elongation was slow and normal at 7°C and 26°C, respectively. At 7°C, the
metabolic activity such as cell wall synthesis etc. will be more significantly suppressed than the physicochemical reactions such as Al-diffusion into apoplast and Al-binding to cell wall components. The roots of Scout 66 were subjected to a solution at 7°C or 26°C in the presence and absence of Al for 6 h and subsequently to a solution without Al at 26°C for a further 6 h. At 7°C, the roots hardly elongated both in the presence or absence of Al, while the root elongation was 0.46 and 0.19 cm per 6 h at 26°C in the absence and presence of Al, respectively, during pretreatment (Table 1). Exposure of the roots to 10 µM Al at 7°C for 6 h did not affect the root elongation during the next 6 h at 26°C in the absence of Al although Al was bound to the root apices (1.10 nmol root apex⁻¹) (Table 1). By contrast, the root elongation did not recover (40% of the control) after exposure to 10 µM Al at 26°C during the 0–6 h period and then no Al for the next 6 h. More Al was bound to the root apices (2.30 nmol root apex⁻¹) by this treatment. At both 7 and 26°C, more than 75% of total Al was in the cell wall of the root apices (Table 1). Pre-treatment with low temperature in –Al solution did not affect the root elongation thereafter in the absence of Al (0.47 cm vs. 0.46 cm) (Table 1).

**Discussion**

It is still under debate whether the primary target of Al toxicity is in the apoplast or symplast (Horst 1995, Kochian 1995). Furthermore it is possible that the mechanisms of Al toxicity vary with the treatment conditions such as Al concentrations, period of exposure, pH etc. (Ma 2000). For example, in the roots exposed to a low concentration of Al for a short period, only the apoplasm (cell wall) of the roots may be influenced by Al. However, in the roots exposed to a high concentration of Al for a long period, the plasma membrane, DNA, and enzymes may also be influenced. However, it should be noted that the Al-induced inhibition of root elongation occurs within several hours at very low concentrations (micromolar). Therefore, to understand the primary mechanism of Al toxicity, an experiment with short exposure to low Al concentration is required. In the present study, the wheat roots were exposed...
an analysis was carried out with root apices between 1 and 4 mm, viscoelastic extensibility of the cell wall, a creep-extension modulus. To understand the effect of Al binding on the two physical parameters, i.e. viscosity coefficient and elasticity: a viscosity or plastic component and an elastic component. (Sakurai 1991). There are two components of wall extensibility, however, the Al content was too low to be detected. We attempted to identify the Al form in the cell wall of cultured tobacco cells, most of the Al was bound to the pectin fraction (Chang et al. 1999). In cultured tobacco cells, most of the Al was bound to the pectin fraction (Chang et al. 1999). Recently, evidence shows that there is a close positive correlation between pectin content and Al-induced loss of cell viability in maize suspension cells (Horst 1995, Chang et al. 1999, Blamey 2001). In cultured tobacco cells, most of the Al was bound to the pectin fraction (Chang et al. 1999). Furthermore, most of the Al was localized on epidermis and outer cortex (Fig. 2).

The components which bind Al to the cell wall have not been identified, but pectin has been suggested as a candidate to bind Al (Horst 1995, Chang et al. 1999, Blamey 2001). In cultured tobacco cells, most of the Al was bound to the pectin fraction (Chang et al. 1999). Recently, evidence shows that there is a close positive correlation between pectin content and Al-induced loss of cell viability in maize suspension cells (Schmohl and Horst 2000), suggesting that binding of Al to pectin matrix is an important step in the expression of Al toxicity. We attempted to identify the Al form in the cell wall of wheat root apices (cv. Scout 66) by applying solid 27Al-NMR, however, the Al content was too low to be detected.

Extensibility of the cell wall is regarded as an important factor in the regulation of cell elongation in plant tissues (Sakurai 1991). There are two components of wall extensibility: a viscosity or plastic component and an elastic component. The viscosity component is the ability of the wall to be deformed irreversibly in a time-dependent manner; the elastic component is instant deformation and reversible after the deforming force is removed. The cell elongation is accompanied by an increase in cell wall extensibility that is regulated by the two physical parameters, i.e. viscosity coefficient and elasticity modulus. To understand the effect of Al binding on the viscoelastic extensibility of the cell wall, a creep-extension analysis was carried out with root apices between 1 and 4 mm, which represents the elongation zone of wheat roots. The physical parameters of three elastic (E₀, E₁, E₂) and three viscous (η₀, η₁, η₂) parameters were measured and analyzed by using a Kelvin-Voigt-Burgers’ model. This method was developed for plant roots by Tanimoto et al. (2000). Although the physiological functions of the viscoelastic parameters (E₀, E₁, E₂, η₀, η₁, η₂) in creep-extension analysis are not fully understood, some characteristics of these parameters have been elucidated. For example, low-pH treatment in vitro predominantly decreased the viscosity coefficients rather than the elastic moduli (Tanimoto et al. 2000). Changes in these parameters were also reported to correlate with the elongation growth of sorghum roots that had been treated with or without silicon in vivo (Hattori et al. 2003). Thus the changes of these parameters are possibly regulating factors of elongation zone of roots both in vitro and in vivo.

Among the parameters, E₀ and η₀ are the most significant parameters to represent the whole extensibility of the roots. Time-course experiments showed that Al rapidly increased both elastic moduli (E₀, E₁, E₂) and viscosity coefficients (η₀, η₁, η₂) of the cell wall of root apices (Fig. 3d, e), therefore decreased both elastic and viscous extensibility and the whole extensibility (Fig. 3f). This decrease is consistent with the inhibition of root elongation occurring in intact roots during the same treatment (Fig. 1c). A similar conclusion was reached by Tabuchi and Matsumoto (2001) who used an alternative tensile test method to show that Al decreased the extensibility of root cell walls. However, our results of time-course changes in viscoelastic parameters of root cell walls gave further insights into the sequence of mechanical changes in cell walls during Al-induced inhibition of root elongation. Furthermore, we found that the cell wall was more easily broken by Al treatment in Scout 66 (Fig. 4), suggesting that Al makes root cell walls brittle. The breaking load of control roots decreased with incubation time (Fig. 4). Since the rigidity of cell walls is maintained by proper supply of cell wall components, insufficient supply of new cell wall materials in rapidly elongating zone of the root may cause such decline of breaking load and Al may interfere this proper assembly of new cell wall components.

### Table 1  Recovery of root elongation after pre-treatments with low temperature and Al

<table>
<thead>
<tr>
<th>Pretreatment (0–6 h)</th>
<th>Root elongation during pretreatment (0–6 h) (cm)</th>
<th>Recovery of root elongation (6–12 h) (cm) a</th>
<th>Al content at 6 h (nmol apex⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>—Al, 7°C</td>
<td>0.06±0.02</td>
<td>0.47±0.02</td>
<td>nd</td>
</tr>
<tr>
<td>+Al, 7°C</td>
<td>0.04±0.02</td>
<td>0.41±0.03</td>
<td>1.10±0.09</td>
</tr>
<tr>
<td>—Al, 26°C</td>
<td>0.46±0.02</td>
<td>0.46±0.02</td>
<td>nd</td>
</tr>
<tr>
<td>+Al, 26°C</td>
<td>0.19±0.02</td>
<td>0.20±0</td>
<td>2.30±0.12</td>
</tr>
</tbody>
</table>

The roots were exposed to a 0.5 mM CaCl₂ solution (pH 4.5) with or without 10 µM Al at 7 or 26°C for 6 h, and then transferred to the same solution without Al at 26°C and grown for a further 6 h. Data are means ± SE (n = 10 for root elongation and 3 for Al content). nd, not detected.

a The root elongation during 6–12 h in the absence of Al at 26°C after different pretreatments.
Interestingly, in vitro exposure of cell walls to Al did not affect elasticity or viscosity (Fig. 5). These results suggest that Al may interact differently with living elongating cells and non-elongating dead cells although similar amount of Al was bound to these cells (Fig. 1d). To test these possibilities temperature treatments were used to expose living roots to Al while they were elongating normally (26°C) or elongating very slowly (7°C). The root elongation occurring after these treatments was then compared at 26°C without Al. The results indicated that exposing the roots to Al at low temperature did not affect the subsequent elongation rate even though a large amount of Al still accumulated in the cell wall (Table 1). These results suggest that Al must bind with elongating cells to ultimately inhibit root growth and that Al bound to the cells during non-elongation process does not affect the elongation and the cell wall extensibility.

The mechanisms responsible for the Al-decreased extensibility of the cell wall are unknown. Evidence has shown that Al causes accumulation of cell-wall polysaccharides, especially hemicellulosic polysaccharides in the growing region (Le Van et al. 1994, Tabuchi and Matsumoto 2001), resulting in the thickening of cell wall. However, this increase may be the result of the inhibition of cell elongation. As shown in time course effects of Al on viscoelastic parameters (Fig. 2), Al-induced increase in viscoelastic parameters was found within a 3-h treatment. Changes in wall extensibility could come about either as a result of the new cell wall synthesis or as a result of the enzymatic breaking of bonds and/or enzymatic deformation of polysaccharides within the wall framework (wall loosening). Al possibly affects the process of synthesis and/or loosening of cell wall by interfering these processes. However, considering that over twofold more Al accumulated in the cells of actively elongating roots than in the other root cells (Table 1), it is likely that more binding site of Al is generated during the loosening process of root cell wall. Wall loosening and continued deposition of new material into the wall must be tightly integrated events. We therefore suggest that tight binding of Al to the newly generated site of the cell wall hampers the binding of newly synthesized cell wall materials to newly generated sites of the cell wall, which is necessary for reorganization of the cell wall during cell elongation. Such an interruption of cell wall deformation may result in the decrease in both elastic and plastic extensibility and break load of the cell wall.

The elongation growth of plant cells are also regulated by osmotic concentration of cells in addition to the extensibility of cell walls. Recently Tabuchi and Matsumoto (2004) reported the decrease in osmotic potential of Scout roots and the increase in Atlas roots. Further, as Al has a strong binding affinity for the oxygen donor compounds, the binding of Al to various cellular components may result in multiple damage in the structure and function of the roots. However, as the cell wall is the first and major site of Al accumulation, our results indicate that the loss of viscoelastic extensibility in the cell wall of elongating cell is at least one of the important mechanisms of Al-induced inhibition of root elongation.

Materials and Methods

Sample preparation and root elongation measurement

Seeds of wheat (T. aestivum L. cvs. Scout 66 (Al-sensitive) and Atlas 66 (Al-resistant)) were soaked in distilled water for 2 h and then placed on a net floated on a 0.5 mM CaCl₂ (pH 4.5) solution in a 1.5-liter plastic container. The container was covered with aluminum foil for 3 d and then removed. The solution was renewed daily. On day 4, selected seedlings with similar size were exposed to a 0.5 mM CaCl₂ (pH 4.5) solution containing 0 or 10 µM AlCl₃. The root elongation was measured with a ruler at 0, 3, 6, and 9 h. At each sampling time, the roots were briefly rinsed with distilled water and then the root apices (0–1 cm) were excised with a razor. For measurement of cell wall viscoelasticity, the excised roots were immediately fixed in boiling methanol for 5 min, followed by washing three times with fresh methanol. The samples were stored at 4°C until the measurement. For in vitro treatment, the roots were first fixed in boiling methanol for 5 min and then exposed to a 0.5 mM CaCl₂ (pH 4.5) solution containing 0 or 10 µM AlCl₃ for 15 h before creep extension analysis. For determination of Al content, the excised roots were stored in a freezer at −80°C until use as described below. The plants were grown in a growth cabinet with a day/night temperature regime of 25°C (14 h)/20°C (10 h). Ten replicates for root elongation and three replicates for Al content were made. Replicates for measurements of cell wall properties were indicated in each legend to figure.

Cell wall extraction and Al determination

The content of Al bound to the cell walls of the root cells was estimated by homogenizing the frozen root apices (20 for each sample) with 0.5 ml ice-cold distilled water in an Eppendorf tube using a plastic grinder (Ma et al. 1999). The homogenate was centrifuged at 13,000 g for 10 min, and the precipitate was resuspended in ice-cold water and centrifuged again. The precipitate was then washed three times with 10 volumes of 80% ethanol and one with 10 volumes of methanol : chloroform mixture (1 : 1), followed by 10 volumes of acetone. After drying, the precipitate was resuspended in 1 ml of 2 M HCl at room temperature for at least 2 d. For total Al determination of the root apices, the excised root apices (10 per sample) were placed in a 1.5-ml Eppendorf tube containing 1 ml of 2 M HCl. The tubes were stood for at least 24 h with occasional shaking. The Al concentrations in the solutions were determined after appropriate dilution by graphite furnace atomic absorption spectrophotometer (model Z-5000, Hitachi, Tokyo, Japan).

Measurement of cell wall viscosity and elasticity

A creep-extension analysis was carried out to investigate the effect of Al on viscoelastic properties of root cell wall as detailed by Tanimoto et al. (2000). Briefly, root segments prepared as described above, were rehydrated with 10 mM MES (2-(Nmorpholino)-ethanesulfate) buffer, pH 6.0, at 0°C for 15 min. The diameter of root segment was measured under a microscope at 1 mm and 4 mm behind the apex and the mean cross area of each segment was calculated to obtain the amount of load for each root. The root was secured between two clamps of a Rheoner creep meter (Yamaden RE-33005, Tokyo), one at a position 1 mm from the apex and the other at 4 mm, leaving 3 mm for extension (1–4 mm behind the apex). The creep extension was carried out in the MES buffer at room temperature. Depending on the cross area of each root, a constant load, 15 g mm⁻², was applied to the root by driving the lower clamp down in the maximum speed at
0.5 mm s⁻¹. The extension process was recorded by a computer at 0.5-s intervals for 10 min and then the load was released to record the shrinkage of the root for 5 min. The residual extension after the shrinkage was designated as plastic extension and the difference between total extension and plastic extension was designated as elastic extension. The data was analyzed by the computer program installed in the Rheon creep meter and the least square methods using Burgers’ viscoelastic model to calculate three elastic (Eᵦ, E₁, E₂) and three plastic (ηᵦ, ηᵥ, ηₚ) parameters.

For break load measurement, the apical 1–4 mm portion of roots were extended for 10 min at the speed of 0.5 mm s⁻¹ by stepwise increasing the load at 10 g mm⁻² intervals.

**Low temperature treatment experiment**

Seedlings (4 days old) prepared as described above, were exposed to a 0.5 mM CaCl₂ (pH 4.5) solution containing 0 or 10 or viscosity (ηᵦ, ηᵥ, ηₚ) parameters.


(Received September 8, 2003; Accepted February 23, 2004)