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Accumulation of Al in Root Mucilage of an Al-Resistant and an Al-Sensitive Cultivar of Wheat[1]

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To estimate rates of Al accumulation within the symplasm, all apoplastic pools of Al need to be eliminated or accounted for. We have developed a revised kinetic protocol that allows us to estimate the contribution of mucilage-bound Al to total, nonexchangeable Al, and to eliminate the mucilage as an apoplastic pool of Al. By comparing the Al content of excised root tips (2 cm) of wheat (*Triticum aestivum* L.) with and without the removal of the mucilage (using a 10-min wash in 1 M NH_4Cl), we found that Al bound to the mucilage accounted for approximately 25 to 35% of Al remaining after desorption in citric acid. The kinetics of Al uptake into mucilage were biphasic, with a rapid phase occurring in the first 30 min of uptake, followed by a linear phase occurring in the remainder of the experimental period (180 min). By adopting a step for removal of mucilage into our existing kinetic protocol, we have been able to isolate a linear phase of uptake with only a slight deviation from linearity in the first 5 min. Although we cannot unambiguously identify this phase of uptake as uptake into the symplasm, we believe this new protocol provides us with the most accurate quantitative estimate of symplastic Al yet available.

A number of recent studies have emphasized the importance of the root tip in the expression of Al toxicity and resistance in plants. This was perhaps most elegantly demonstrated by Ryan et al. (1993), who showed that Al must be supplied to the terminal 2 to 3 mm of the root apex of *Zea mays* for symptoms of Al toxicity to be expressed. This observation is consistent with an array of less direct evidence, which also supports the role of the root tip as the primary site of Al-related lesions. For example, in *Allium cepa* and *Vignia unguiculata*, decreased rates of mitosis have been associated with accumulation of Al in the root apex (Clarkson, 1965; Horst et al., 1982, 1983). Al has also been shown to bind to cell nuclei in root tips of *Z. mays* (Galsomies et al., 1992) and, more specifically, to DNA in roots of *Pisum sativum* and *A. cepa* (Matsumoto et al., 1976; Morimura et al., 1978). If the root tip is indeed the site where toxicity is most clearly expressed, we would expect potential resistance mechanisms to be most clearly expressed in this region as well. Although mechanisms of Al resistance are poorly understood, Delhaize et al. (1993b) demonstrated that the terminal 3 to 5 mm of root tips of an Al-resistant cultivar of *Triticum aestivum* L. were the primary source of Al-induced malic acid excretion. Similarly, Basu et al. (1994a) provided evidence that an Al-induced membrane protein was most abundant in the terminal 5 mm of roots of an Al-resistant cultivar of *T. aestivum*.

For Al to reach sensitive meristematic regions, it must first penetrate and cross the root mucilage. Because the root-tip region is the site of the most intense mucilage production (Paull and Jones, 1975), immobilization of Al in this layer could constitute an important mechanism that protects the meristem from Al injury (Horst et al., 1982) through exclusion of Al from the cell symplasm (Taylor, 1988, 1991). Chelate ligands present in the mucilage may bind Al and thereby present a physical or chemical barrier to the inward movement of Al (Henderson and Ownby, 1991). Enhanced exudation of malate (Delhaize et al., 1993b; Basu et al., 1994b; Ryan et al., 1995a, 1995b) and citric acid (Miyasaka et al., 1991; Pellet et al., 1995) have been reported in Al-resistant cultivars of *T. aestivum*, *Phaseolus vulgaris*, and *Z. mays*. Furthermore, Horst et al. (1982) showed that 50% of total Al in 5-mm root tips of *V. unguiculata* was bound to mucilage. Removal of the mucilage prior to treatment with Al facilitated the entry of Al into root tissue and rendered roots more sensitive to Al (Horst et al., 1982).

If mucilage plays a role in mediating exclusion of Al, it is expected that sensitive plants would accumulate Al in the symplasm more rapidly than resistant plants, and these differences would be more pronounced at the root tip. In *T. aestivum*, Rincon and Gonzales (1992) and Delhaize et al. (1993a) found that root tips of Al-sensitive plants absorbed more Al than those of Al-resistant plants. Unfortunately, their protocols did not allow them to differentiate between apoplastic and symplastic Al. Thus, these studies cannot provide direct evidence of exclusion mechanisms operating at the plasma membrane.

To determine if exclusion mechanisms play a role in resistance, Al uptake into the symplasm of root tips must be measured independently of apoplastic uptake. Progress in this regard has been hindered by the lack of a suitable radioisotope that can be purchased and detected at reasonable cost and the lack of analytical techniques capable of measuring minute quantities of Al internalized by plant cells. Nonetheless, several important obstacles have been overcome. Perhaps most important, we have shown that it is possible to virtually eliminate metabolism-dependent accumulation of Al in the apoplasm (Zhang and Taylor, 1990) by using low concentrations of Al in simple uptake solutions (50 μM $AlCl_3$ and 1.0 mM $CaCl_2$) with a subse-

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quent wash in 0.5 mM citric acid (Archambault et al., 1996). Under these conditions, concerns about contamination of the symplasm during fractionation are minimized, and binding of Al to membranes contributes only 4% of total, nonexchangeable uptake. However, identifying the remaining linear phase as uptake into a putative symplastic compartment remains speculative. The kinetics of Al uptake into mucilage remain to be studied and uptake into this apoplastic compartment could contribute to both the rapid, nonlinear phase and the linear phase of Al uptake.

In this study we have investigated the contribution of mucilage-bound Al to total uptake and the possibility of removing the mucilage to isolate the linear phase of uptake in roots of *T. aestivum*. Our studies demonstrate that the mucilage represents an important apoplastic pool for Al that can be removed with a 10-min wash in NH_4Cl. A revised kinetic protocol is proposed that may provide a more accurate estimate of symplastic levels of Al.

MATERIALS AND METHODS

Preparation of Plant Material

To prepare plants for experimentation, seeds of the Al-resistant cv PT 741 and the Al-sensitive cv Neepawa of *Triticum aestivum* L. were surface-sterilized in 1.2% sodium hypochlorite for 20 min and germinated for 24 h in a solution of Vitavax (0.005 g/L) to prevent fungal growth. Seedlings were grown on nylon mesh suspended in aquaria containing a full nutrient solution (Zhang and Taylor, 1989) for 4 to 7 d. In experiments requiring excised roots, 30 2-cm root tips were excised and placed into each of 50 to 55 replicate absorption tubes. The tubes were then placed in a full nutrient solution until excision was complete (<60 min). Following a 30-min equilibration period in 1.0 mM $CaCl_2$ (pH 4.5, 23°C), the tubes were transferred to uptake solutions.

Visualization of Mucilage

Roots of 5-d-old seedlings were observed and photographed at 100× magnification to reveal the presence of a droplet of substance at the root apex. To verify whether the droplet was mucilage, the roots were immersed in 25 mL of 100 μM Ruthenium red (Sigma-Aldrich Canada, Mississauga, Ontario), a stain for pectins, and rinsed with deionized, distilled water (>18 MΩ). Visual observations showed that the droplet stained an intense red and could not be removed by rinsing with water. Based on these observations we concluded that the droplets consisted of mucilage and we proceeded to test protocols that might allow us to remove this layer.

Kinetics of Al Removal from the Mucilage Using NH_4Cl

Excised roots of the Al-resistant cv PT 741 were prepared for experimentation as described above and transferred to uptake solutions containing 50 μM $AlCl_3$ in 1.0 mM $CaCl_2$ at pH 4.5 and 23°C. Following 2 h of exposure to Al, roots were desorbed in 0.5 mM citric acid at pH 4.5 and 0°C for 30 min and subsequently washed in 1 M NH_4Cl at pH 4.5 and 23°C for 0, 2, 4, 8, 10, 20, 40, and 60 min. Roots were then rinsed with deionized, distilled water, dried in an oven at 55°C, weighed, ashed in a muffle furnace at 500°C, solubilized in 200 μL of nitric acid, and the volume was adjusted using distilled, deionized water. Solutions were analyzed for Al using graphite furnace atomic absorption spectrophotometry as described by Zhang and Taylor (1989).

Testing for Cell Viability

Plants were subjected to either a negative control treatment (full nutrient solution, pH 4.5, 23°C), a positive control treatment (10 min in 70% ethanol), a 30-min wash in 0.5 mM citric acid (pH 4.5, 0°C), or a 10-min wash in 1 N NH_4Cl (pH 4.5, 23°C). Roots were then rinsed with distilled water, immersed in a 0.1% solution of Evans blue for 5 min, and subsequently rinsed in distilled water. Roots were examined and photographed under a dissecting microscope. Viable cells excluded the stain.

Removal of Mucilage

The potential role of mucilage as an apoplastic pool for Al was evaluated in a series of experiments in which mucilage was removed using a 10-min wash in 1 M NH_4Cl (Brams, 1969). To test the efficacy of this treatment, microscope studies were undertaken to visually observe the root-tip-mucilage region of the Al-resistant cv PT 741. We also compared this treatment with two other chloride salts, namely 1 M KCl and 1 M $CaCl_2$, as well as the sulfate salts of NH_4^+, K^+, and Ca^{2+}, to determine which part of the ion pair would be responsible for the observed effects. Plants were prepared for experimentation as described above. Roots of 5-d-old seedlings were left untreated (control) or washed for 10 min in 50 mL of a 1 M solution (pH 4.5, 23°C) of either chloride or sulfate salts of NH_4^+, K^+, or Ca^{2+}. After they were washed, roots were rinsed with deionized water and photographed under a dissecting microscope at 100× magnification.

Contribution of Mucilage-Bound Al to Uptake

The amount of Al tightly bound to mucilage was estimated by quantitative analysis of the Al content of excised roots from Al-resistant cv PT 741 and Al-sensitive cv Neepawa following a series of washing procedures. Excised roots were prepared as described above and loaded with Al in solutions containing 50 μM $AlCl_3$ in 1.0 mM $CaCl_2$ (pH 4.5, 23°C) for 2 h. Five replicate tubes containing roots of each genotype were given the following treatments: (a) harvested immediately for determination of total Al; (b) washed for 30 min in 0.5 mM citric acid (pH 4.5, 0°C); (c) washed for 30 min in 0.5 mM citric acid (pH 4.5, 0°C) and for 10 min with 1 M NH_4Cl (pH 4.5, 23°C); (d) washed for 30 min in 0.5 mM citric acid (pH 4.5, 0°C) and for 10 min with 1 M KCl (pH 4.5, 23°C); (e) washed for 30 min in 0.5 mM citric acid (pH 4.5, 0°C) and for 10 min with 1 M $CaCl_2$ (pH 4.5, 23°C); (f) washed for 10 min with 1 M NH_4Cl (pH 4.5, 23°C) and for 30 min in 0.5 mM citric acid (pH 4.5, 0°C);

or (g) washed for 30 min in 0.5 mM citric acid (pH 4.5, 0°C), for 10 min with 1 M NH_4Cl (pH 4.5, 23°C), and for 30 min in 0.5 mM citric acid (pH 4.5, 0°C). Roots were prepared for Al analysis as described above.

Patterns of Al Uptake in Mucilage

Measurements of total Al remaining after desorption in citric acid, NH_4Cl, and KCl suggested that the mucilage represents a significant apoplastic pool of Al. Thus, kinetic experiments were conducted to determine the time course of Al accumulation in this pool. These experiments allowed us to investigate the possibility that differences in the pattern of Al accumulation in mucilage might exist between Al-resistant and Al-sensitive cultivars. We looked at the kinetics of Al uptake into mucilage of cv PT 741 and cv Neepawa in both short-term (3 h) and long-term (6 h) exposure studies. For short-term studies, excised roots were prepared for experimentation as described above and transferred to uptake solutions containing 50 μM $AlCl_3$ in 1.0 mM $CaCl_2$ (pH 4.5, 23°C). After 0, 10, 30, 60, 120, and 180 min, five replicate tubes for each genotype were removed from uptake solutions and roots were desorbed for 30 min in 0.5 mM citric acid (pH 4.5, 0°C) and washed for 10 min with 1 M NH_4Cl (pH 4.5, 23°C) (Brams, 1969). Aliquots of the NH_4Cl wash were analyzed for Al using graphite furnace atomic absorption spectrophotometry without further preparation. Roots were then prepared for Al analysis as described above.

For long-term studies, whole plants were prepared for experimentation as described above. After 7 d of growth, plants growing on nylon mesh were removed from aquaria containing nutrient solution, rinsed with distilled water, and placed in aquaria containing 50 μM $AlCl_3$ and 1.0 mM $CaCl_2$ (pH 4.5 and 23°C) for 0, 2, 4, and 6 h. Following exposure to Al, roots were rinsed with distilled, deionized water, excised 2 cm from the root tip, and subjected to a desorption treatment and removal of the mucilage as described above. The NH_4Cl solution was then assayed directly to estimate the Al content of the root mucilage.

Kinetics of Al Uptake Using New Protocol

Experiments were performed to compare patterns of Al uptake using the protocol described previously by Archambault et al. (1996) and a new protocol that includes removal of mucilage. Excised roots of cv PT 741 were prepared as described above and transferred to uptake solutions containing 50 μM $AlCl_3$ in 1.0 mM $CaCl_2$ (pH 4.5 and 23°C). Following 0, 5, 10, 15, 20, 30, 60, 90, 120, 150, and 180 min of uptake, five replicate tubes were removed from uptake solutions and the roots were desorbed in 0.5 mM citric acid (pH 4.5, 0°C) for 30 min. Following desorption one-half of the roots from each tube were removed, rinsed with deionized, distilled water, and prepared for analysis. The remaining roots were placed in a 1 M solution of NH_4Cl (pH 4.5, 23°C) for 10 min to remove the mucilage, rinsed with deionized, distilled water, and prepared for Al analysis as described above.

RESULTS

Staining of excised roots with Ruthenium red, a stain for pectins, suggested that droplets of the substance at the root apex were mucilage (Fig. 1, A and B). Older portions of the roots stained less intensely (Fig. 1B), indicating that pectins were found along the entire length of the root, but at lower concentrations than at the root tip. Loss of small portions of the mucilage were commonly observed if roots were subjected to extensive manipulations. Careful preparation of roots for analysis completely overcame this problem.

The time course of Al removal using 1 M NH_4Cl from roots previously desorbed in citric acid was examined to determine the length of time required to completely remove nonexchangeable Al associated with the mucilage. Removal of Al was rapid in the first 8 min but slowed to nil in the remaining 52 min of exposure to NH_4Cl (Fig. 2), suggesting that the mucilage is rapidly removed and that root tissue was not damaged to the point of significant leakage of Al from the symplasm during the wash period. The viability of roots subjected to either the citric acid (30 min) or the NH_4Cl (10 min) wash did not differ from control roots exposed to full nutrient solution (negative control). Roots washed in ethanol (positive control), however, stained an intense blue with Evans blue, suggesting that viability was not sustained under this treatment (Fig. 3). Concurring results were also obtained using Neutral red as a vital stain (results not shown). Therefore, we conclude that the cells of roots exposed to wash treatments employed in this study remained viable throughout the steps required for removal of Al from the apoplasm. Leakage of Al from the symplasm would be minimal and should not significantly affect our estimates of intracellular Al levels.

To determine whether the cationic or anionic components of NH_4Cl dissociation were responsible for the removal of mucilage, microscopic observations were undertaken to compare the effects of various treatments on the removal of the mucilage. Microscopic observation of roots treated with NH_4Cl and KCl for 10 min showed that the mucilage was completely removed (Fig. 4, B and C). Although $CaCl_2$ had little effect (Fig. 4D) on the mucilage, the

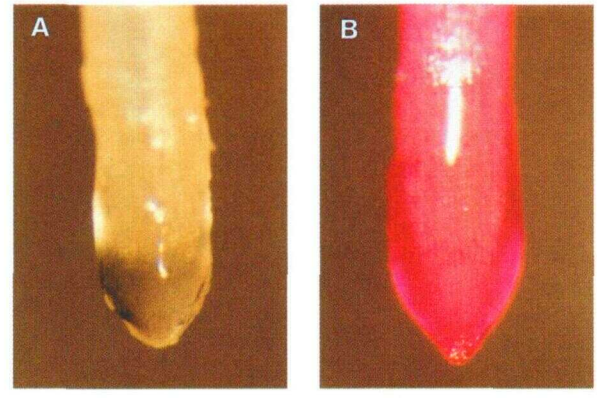

Figure 1. Roots of *T. aestivum* L. cv PT 741 that were untreated (A) or stained with Ruthenium red (B). Photographs were taken using a 35-mm camera mounted on a dissecting microscope using 100× magnification.

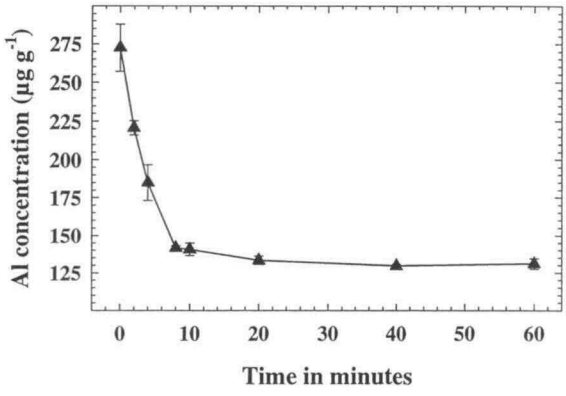

Figure 2. Kinetics of removal of Al from roots of Al-resistant cv PT 741 of *T. aestivum* L. exposed to 50 μM $AlCl_3$ in 1.0 mM $CaCl_2$ (pH 4.5, 23°C) for 2 h and subsequently desorbed in citric acid (pH 4.5, 0°C) for 30 min. Al was further removed from roots using 1 M NH_4Cl (pH 4.5, 23°C) for 0 to 60 min. Values represent means of five replicates ± SE.

layer seemed smaller than in the control. The same effects were observed when sulfate salts were used (data not shown). Our results indicate that high concentrations of monovalent cations (NH_4^+ and K^+) were responsible for the removal of the mucilage and that the Cl^- and SO_4^- anions had little or no effect.

To quantify Al tightly bound in the mucilage, we designed experiments to compare the effects of various desorption solutions on the Al content of root tips. We compared the amount of Al remaining in roots after a desorption in citric acid with that remaining after desorption in citric acid and removal of the mucilage using a 10-min wash in 1 M NH_4Cl. After desorption with citric acid, root tips of the Al-resistant cv PT 741 retained 301 ± 11 $\mu g\ g^{-1}$ of Al, whereas root tips of the Al-sensitive cv Neepawa retained 368 ± 5 $\mu g\ g^{-1}$ of Al. When mucilage was subsequently removed from root tips using NH_4Cl, the amount of Al remaining was approximately 25 to 35% lower than when the mucilage was left intact (Fig. 5, A and B). A similar effect was observed when NH_4Cl was substituted with KCl (Fig. 5). However, such was not the case when $CaCl_2$ was employed. Al levels were the same when the citric acid wash was followed by a $CaCl_2$ wash as when citric acid was used singly (Fig. 5). This observation is consistent with the results of our microscopic work, which suggested that Ca is not effective in removing the mucilage, and further demonstrates that Ca is incapable of removing Al remaining in the apoplasm after a citric acid wash. Although citric acid may be capable of desorbing a portion of the Al present in the mucilage, our results suggest that it is not completely effective in desorbing this apoplastic pool.

The effectiveness of NH_4Cl in displacing Al from root tips after a citric acid wash may be a direct result of the removal of the mucilage itself, removing a significant pool of tightly bound Al that cannot be desorbed using citric acid alone. Alternatively, intact mucilage might protect underlying apoplastic binding sites from desorption, and removal of the mucilage facilitates desorption of Al from these sites. It is also possible that NH_4^+ itself acts as a powerful desorption agent that is capable of removing Al not previously desorbed with citric acid. We cannot reject the latter hypothesis on the grounds that another monovalent cation, K^+, was also effective in removing a significant pool of Al. However, Ca^{2+} was an ineffective desorption agent. Inasmuch as divalent cations should be more effective than monovalent cations in desorbing Al, this argues against a direct role for these cations in direct desorption of Al. We have attempted to differentiate between the remaining alternative hypotheses by varying the order of the wash treatments (NH_4Cl followed by citric acid) and by including a second wash in citric acid (citric acid, followed by NH_4Cl, and a second wash in citric acid). If removal of the mucilage with NH_4Cl exposes underlying sites to the effect of a desorption agent, citric acid should be a more effective desorption agent when used after the mucilage has been removed. In both the Al-resistant cv PT 741 and the Al-sensitive cv Neepawa, changing the order of the NH_4^+ and citric acid washes or adding a second wash in citric acid following mucilage removal did not desorb additional Al from the roots (Fig. 5). Thus, we are inclined to believe that NH_4Cl effectively removes a significant apoplastic pool of Al that cannot be removed by citric acid alone.

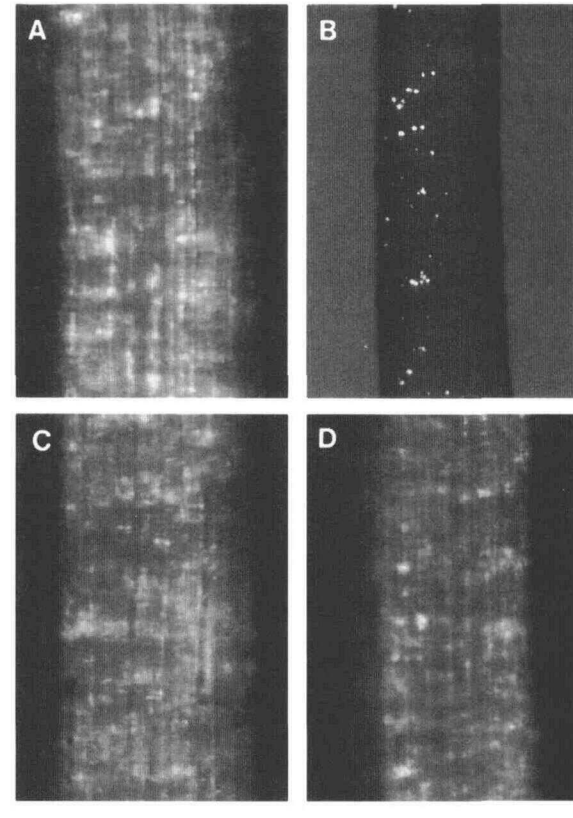

Figure 3. Roots of *T. aestivum* L. cv PT 741 that were tested for viability using Evans blue. Roots were either (A) soaked in full nutrient solution (negative control); (B) washed with 70% ethanol for 10 min (positive control); (C) washed with 0.5 mM citric acid for 30 min (pH 4.5, 0°C); or (D) washed with NH_4Cl for 10 min (pH 4.5, 23°C), stained with Evans blue for 5 min, rinsed in deionized, distilled water, and photographed using a 35-mm camera mounted on a dissecting microscope using 100× magnification.

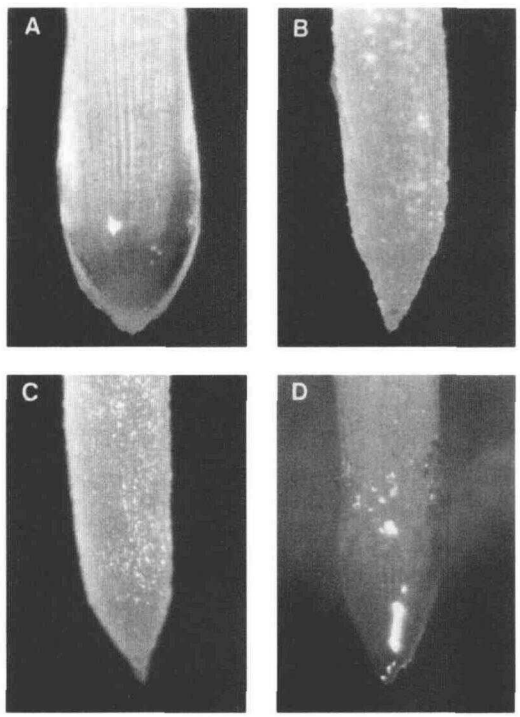

Figure 4. Roots of *T. aestivum* L. cv PT 741 that were (A) untreated; (B) washed in 1 M NH_4Cl (pH 4.5, 23°C); (C) washed in 1 M KCl (pH 4.5, 23°C); or (D) washed in 1 M $CaCl_2$ (pH 4.5, 23°C). Photographs were taken using a 35-mm camera mounted on a dissecting microscope using a 65× magnification. Experiments with the sulfate salts of NH_4^+, K^+, and Ca^{2+} gave similar results (data not shown).

Having ascertained that the mucilage represents a significant pool of apoplastic Al, we then focused our attention on the time course of Al accumulation in this pool. Is binding of Al to mucilage a rapid, saturable process, or can it contribute to the linear phase of uptake with time? Short-term (3 h) exposure experiments showed that patterns of Al uptake into root mucilage were biphasic for both cv PT 741 and cv Neepawa (Fig. 6, A and B). A rapid phase of Al uptake was observed in the first 30 min of exposure, followed by a linear phase of uptake occurring over the remainder of the 180-min experimental period. Despite the qualitative similarities, there were some quantitative differences. Extrapolation of the linear phase of Al accumulation back to time 0 indicated that rapid-phase accumulation was approximately 5 times greater in the Al-sensitive cv Neepawa (140 $\mu g\ g^{-1}$) than in the Al-resistant cv PT 741 (27 $\mu g\ g^{-1}$). Furthermore, although the linear phase of Al uptake was substantial in cv PT 741 (0.67 $\mu g\ g^{-1}\ min^{-1}$), it was weak in cv Neepawa (0.27 $\mu g\ g^{-1}\ min^{-1}$). Long-term studies (6 h) showed that in both cultivars the linear phase of Al uptake into the mucilage persisted throughout the experimental period with no sign of saturation (Table I). Thus, in both cultivars accumulation of Al into the mucilage has the potential to make a significant contribution to the rapid, saturable phase of uptake and to the linear phase of uptake that has been observed in excised roots (Zhang and Taylor, 1989; Archambault et al., 1996).

Given the importance of mucilage as a sink for apoplastic Al, we have incorporated a step for removal of mucilage into our kinetic protocol. This step provides a significant improvement in our ability to isolate the linear phase of Al uptake (putatively uptake into the symplasm). Comparison of the kinetics of Al uptake into roots subjected to a simple desorption in 0.5 mM citric acid (pH 4.5, 0°C for 30 min) to that of roots washed in citric acid followed by a 10-min wash in 1 M NH_4Cl (pH 4.5, 23°C) demonstrated that removal of the mucilage effectively eliminated most of the rapid phase of uptake and also reduced the magnitude of the linear phase. This left a linear phase of uptake that deviated only slightly from linearity during the first 5 to 10 min of uptake (Fig. 7).

DISCUSSION

The experiments reported here represent an ongoing effort to improve techniques that provide quantitative estimates of Al accumulation in the symplasm of plant roots.

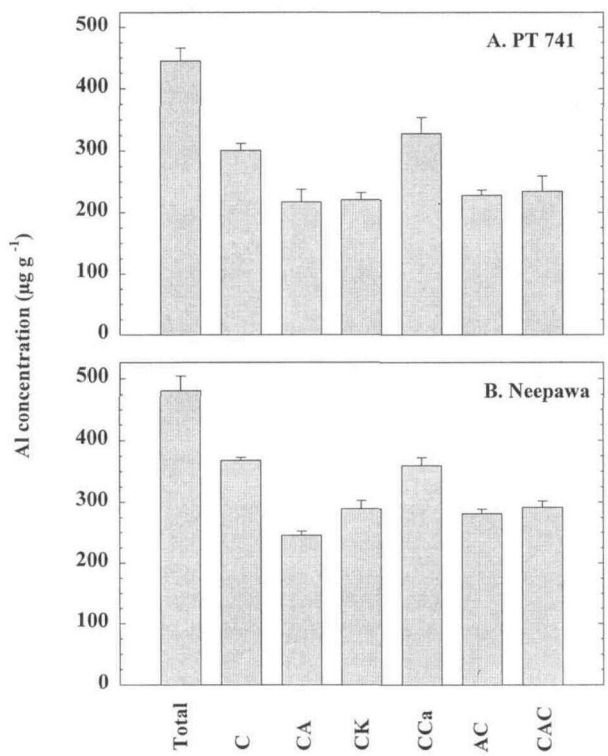

Figure 5. Al remaining in pretreated roots (2 cm) of *T. aestivum* L. cv PT 741 (A) and cv Neepawa (B) after various desorption protocols. Roots were exposed to 50 μM $AlCl_3$ in 1.0 mM $CaCl_2$ (pH 4.5, 23°C) for 2 h and analyzed directly for Al content (Total) or subjected to a 30-min desorption in 0.5 mM citric acid (pH 4.5, 0°C) (C); a 30-min desorption in 0.5 mM citric acid (pH 4.5, 0°C) followed by a 10-min wash in 1 M NH_4Cl (pH 4.5, 23°C) (CA); a 30-min desorption in 0.5 mM citric acid (pH 4.5, 0°C) followed by a 10-min wash in 1 M KCl (pH 4.5, 23°C) (CK); a 30-min desorption in 0.5 mM citric acid (pH 4.5, 0°C) followed by a 10-min wash in 1 M $CaCl_2$ (pH 4.5, 23°C) (CCa); a 10-min wash in 1 M NH_4Cl (pH 4.5, 23°C) followed by a 30-min desorption in 0.5 mM citric acid (pH 4.5, 0°C) (AC); or a 30-min desorption in 0.5 mM citric acid (pH 4.5, 0°C) followed by a 10-min wash in 1 M NH_4Cl (pH 4.5, 23°C) and a second citric acid treatment (CAC). Values represent means of five replicates ± SE.

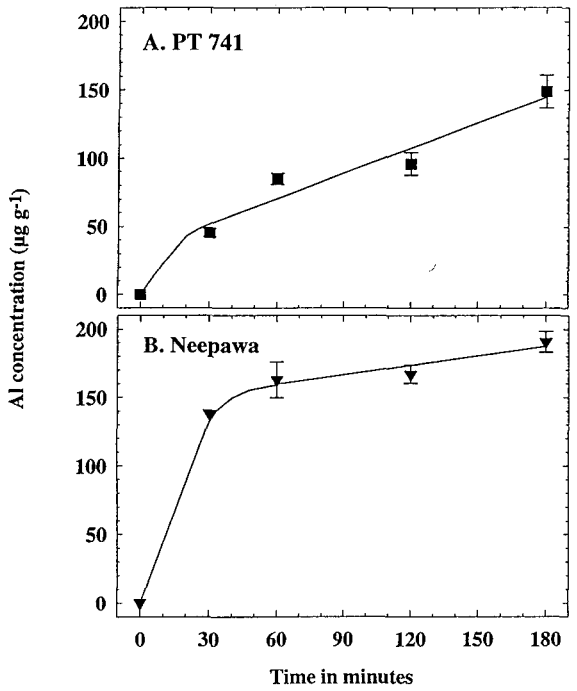

Figure 6. Kinetics of Al uptake into the mucilage of (A) Al-resistant cv PT 741 and (B) Al-sensitive cv Neepawa of *T. aestivum* L. Roots were exposed to 50 μM $AlCl_3$ in 1.0 mM $CaCl_2$ (pH 4.5, 23°C), desorbed for 30 min in 0.5 mM citric acid (pH 4.5, 0°C), and washed in 1 M NH_4Cl for 10 min (pH 4.5, 23°C). A sample of the NH_4Cl was taken and analyzed for Al content without further preparation. Values represent means of five replicates ± SE.

Given the lack of a suitable radioisotope for Al that can be purchased and detected at reasonable cost, direct, unambiguous measurement of the rate of Al accumulation within the symplasm has been problematic. Nonetheless, we still believe that a kinetic approach has the potential to provide an accurate estimate of the rate of symplastic uptake. The accuracy of this type of approach clearly depends on the validity of our operational definition of symplastic uptake. Two factors are particularly important in this regard. First, an efficient desorption protocol must be developed to effectively desorb Al that accumulates within the apoplasm. Ideally, this would allow experimental isolation of the linear phase of uptake, which might putatively be designated as uptake into the symplasm. If success in this endeavor is to be achieved, efforts will also be required to identify all possible apoplastic pools of Al that might contribute to the linear phase of uptake in vivo (and perhaps in vitro as a result of contamination arising from experimental perturbation).

In previous work considerable progress has been made toward achieving these goals. Zhang and Taylor (1989) demonstrated that the kinetics of Al uptake in excised roots were biphasic, with a rapid phase of uptake superimposed over a linear phase of uptake with time. Subsequently, they showed that the linear phase of Al uptake may include metabolism-dependent uptake into the cell wall (Zhang and Taylor, 1990). The potential contribution of metabolism-dependent uptake into the cell wall presents a barrier to measuring Al accumulation within the symplasm. However, we have since discovered that this metabolism-dependent binding can be virtually eliminated by the use of experimental conditions that are less conducive to the formation of solid-phase Al in the apoplasm (Tice et al., 1992; Archambault et al., 1996). Under these conditions, citric acid effectively desorbed Al in cell-wall material, and binding of Al to membrane components represented less than 4% of nonexchangeable Al (Archambault et al., 1996). Despite these advances, previous experiments cannot eliminate the possibility that other apoplastic pools contribute to the linear phase of uptake. In this paper we have tested the hypothesis that tight binding of Al to mucilage may prevent a complete desorption of Al from the apoplasm. If this is the case, elimination of mucilage-bound Al as a pool of apoplastic Al will be required to obtain accurate estimates of symplastic Al and rates of transmembrane transport of Al.

Brams (1969) used a 1-min wash in 1 M NH_4Cl to remove the mucilaginous layer surrounding the roots of citrus

Table I. *Long-term uptake of Al into the mucilage of Al-resistant cv PT 741 and Al-sensitive cv Neepawa of T. aestivum L.*

Roots were exposed to 50 μM $AlCl_3$ in 1.0 mM $CaCl_2$ (pH 4.5, 23°C) for 2, 4, and 6 h, desorbed for 30 min in citric acid (pH 4.5, 0°C), and washed in 1 M NH_4Cl for 10 min (pH 4.5, 23°C). A 2-mL sample of the NH_4Cl was taken and analyzed directly for Al content. Values represent means of three replicates ± SE.

Time	Al Concentration	
	PT 741	Neepawa
h	*μg g⁻¹*	
2	91 ± 19	84 ± 1
4	130 ± 12	100 ± 12
6	146 ± 19	118 ± 9
Rate of Al uptake (μg g^{-1} min^{-1})	0.23	0.14

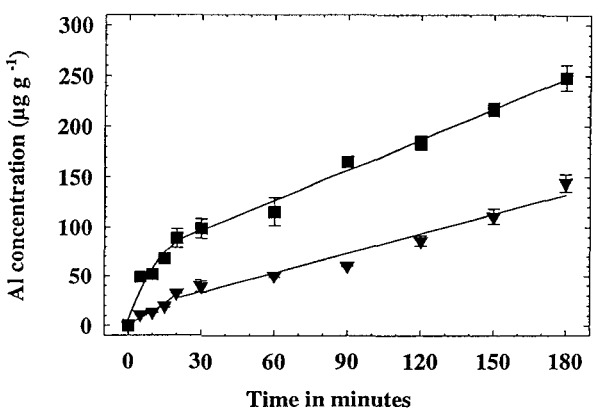

Figure 7. Kinetics of Al uptake in excised roots (2 cm) of the Al-resistant cv PT 741 of *T. aestivum* L. Roots were exposed to 50 μM $AlCl_3$ in 1.0 mM $CaCl_2$ (pH 4.5, 23°C) for 0, 5, 10, 15, 20, 30, 60, 90, 120, 150, and 180 min, followed by either a 30-min desorption in 0.5 mM citric acid at pH 4.5, 0°C (■) or a 30-min desorption in 0.5 mM citric acid at pH 4.5, 0°C and a 10-min wash in 1 M NH_4Cl at pH 4.5, 23°C (▲). Values represent means of five replicates ± SE.

plants. We decided to test the effectiveness of this method by removing the mucilage itself and the Al associated with the mucilage from roots of *T. aestivum*. The time course of Al removal using NH_4Cl was rapid for up to 8 min, with little removal occurring during the remaining 52 min of exposure (Fig. 2), suggesting that complete removal of the mucilage required a longer wash than that described by Brams (1969). Therefore, we adopted a 10-min wash in 1 M NH_4Cl as a step for the removal of mucilage in subsequent experiments. When roots were washed in 1 M NH_4Cl (pH 4.5, 23°C) for 10 min, visual and microscopic examination confirmed that the mucilage was removed (Fig. 4B). Although both the NH_4Cl and citric acid washes might be viewed as harsh treatments, staining with Evans blue (Fig. 3) and Neutral red (results not shown) confirmed that the cells remained viable throughout the experimental period.

Experiments with a variety of desorption agents demonstrated that desorption in citric acid alone was not sufficient to remove Al from the mucilage and that Al tightly bound to the mucilage can only be removed by removal of the mucilage itself. This would appear to be an important part of kinetic protocols, since Al in the mucilage accounted for up to 35% of nonexchangeable Al (Fig. 5). This value is consistent with that reported by Horst et al. (1982), who used physical removal of mucilage to estimate the amount of Al in this compartment. Examination of the pattern of Al uptake into the mucilage of Al-resistant and Al-sensitive plants demonstrated that accumulation of Al in the mucilage was rapid for the first 30 min and linear throughout the remainder of the experimental period (Fig. 6, A and B). Long-term experiments showed that the linear phase of Al uptake persisted for up to 6 h (Table I). This is also consistent with the results of Horst et al. (1982), who demonstrated that Al accumulation in *V. unguiculata* was time-dependent, with no sign that saturation occurs, even after 48 h of exposure.

Quantification of mucilage weight or volume was not possible using our technique; thus, results for the Al-resistant and Al-sensitive cultivars cannot be quantitatively compared on a mass of mucilage or volume of mucilage basis. Nonetheless, Al concentrations were calculated on a root dry weight basis, and interesting observations were made. The general (biphasic) pattern of Al uptake into the mucilage did not differ between cv PT 741 and cv Neepawa in short-exposure (3 h) studies (Fig. 6), although quantitatively, the relative importance of the linear phase was greater in cv PT 741. The reasons for quantitative differences in linear phase accumulation of Al in the mucilage are not clear. We must recognize, however, that production of mucilage itself has been shown to be inhibited by Al (Horst et al., 1982). If inhibition of mucilage production is more pronounced in sensitive plants, this might limit the extent of linear phase accumulation and the degree of protection afforded to underlying tissues of the root meristem. Saturation of binding sites within the mucilage would subsequently lead to a greater exposure to toxic Al ions. Higher Al sensitivity has been related to higher Al contents in root tips (Horst et al., 1982). These authors have also reported that root elongation can be considerably more inhibited when the mucilage is removed. In Al-resistant plants continued synthesis of mucilage in the face of Al stress could serve to maintain the binding capacity of the mucilage, providing ongoing protection for the growing region. Knowledge of such dynamic aspects of mucilage excretion and Al binding may be required for a complete understanding of the role of mucilage in mediating resistance.

The observation that citric acid is not capable of desorbing Al from the mucilage suggests that this layer binds Al tenaciously. Henderson and Ownby (1991) suggested that the mucilage layer by nature only allows for slow diffusion of substances, thus creating an area of high organic acid concentration. If organic acids such as malate (Delhaize et al., 1993b; Basu et al., 1994b) or citrate (Miyasaka et al., 1991; Pellet et al., 1995) are relatively immobile, they could decrease the activity of Al^{3+} in the apoplasm and hence the rate at which Al crosses the plasma membrane. This hypothesis is consistent with the results of McCormick and Borden (1974), who found localized accumulations of an Al-phosphate precipitate in mucilaginous material at the surface of root tips in *Hordeum vulgare*, and those of Horst et al. (1982), who showed that the mucilage of 5-mm root tips of *V. unguiculata* contained approximately 10 times more Al than the root tissue proper after a 6-h exposure to Al. Investigation of the dynamics of complex formation, however, has not yet been performed.

Mucilage represents a significant pool of apoplastic Al, accounting for as much as 35% of nonexchangeable Al. This substantial pool of Al in the apoplasm complicates the interpretation of previous kinetic work. Results from this study suggest that the mucilage must be removed to obtain an accurate estimate of symplastic Al levels. Having incorporated a step for removal of the mucilage into our kinetic protocol, we reviewed the kinetics of Al uptake using the Al-resistant cv PT 741. We found that it was possible to isolate the linear phase of uptake with deviation from linearity observed only during the first 5 min of uptake (Fig. 7). Although kinetic studies such as this cannot provide an unambiguous definition of symplastic Al, we believe that this linear phase provides the best available estimate of the rate of Al uptake into the symplasm (putative symplastic fraction).

The isolation of the linear phase of uptake provides a means of comparing the rate of Al uptake into the putative symplastic fraction of cultivars that exhibit differential Al resistance. Although this has not been the aim of the present study, Al-resistant and Al-sensitive cultivars did not differ appreciably in nonexchangeable Al levels in 2-cm root tips (Fig. 5). In subsequent experiments (D.J. Archambault, G. Zhang, G.J. Taylor, unpublished data), we have shown that substantial differences in the rate of accumulation of Al between cultivars exist only at the root tip (0–5 mm). Work is now underway to characterize these differences.

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