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Aluminum Tolerance in Wheat (*Triticum aestivum* L.)

I. Uptake and Distribution of Aluminum in Root Apices

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We investigated the uptake and distribution of Al in root apices of near-isogenic wheat (*Triticum aestivum* L.) lines differing in Al tolerance at a single locus (*Alt1*: aluminum tolerance). Seedlings were grown in nutrient solution that contained 100 μM Al, and the roots were subsequently stained with hematoxylin, a compound that binds Al in vitro to form a colored complex. Root apices of Al-sensitive genotypes stained after short exposures to Al (10 min and 1 h), whereas apices of Al-tolerant seedlings showed less intense staining after equivalent exposures. Differential staining preceded differences observed in either root elongation or total Al concentrations of root apices (terminal 2–3 mm of root). After 4 h of exposure to 100 μM Al in nutrient solution, Al-sensitive genotypes accumulated more total Al in root apices than Al-tolerant genotypes, and the differences became more marked with time. Analysis of freeze-dried root apices by x-ray microanalysis showed that Al entered root apices of Al-sensitive plants and accumulated in the epidermal layer and in the cortical layer immediately below the epidermis. Long-term exposure of sensitive apices to Al (24 h) resulted in a distribution of Al coinciding with the absence of K. Quantitation of Al in the cortical layer showed that sensitive apices accumulated 5- to 10-fold more Al than tolerant apices exposed to Al solutions for equivalent times. These data are consistent with the hypothesis that *Alt1* encodes a mechanism that excludes Al from root apices.

Al toxicity is one of the major factors that limit plant growth in many acid soils (Wright, 1989). The primary effect of Al is to inhibit root growth in Al-sensitive genotypes with subsequent effects on nutrient and water uptake (Foy, 1983). Root elongation is affected within hours of Al exposure (Wallace et al., 1982), and, as in many plant species, the primary site of Al toxicity in wheat (*Triticum aestivum* L.) appears to be the root apex (Bennet and Breen, 1991). Ryan et al. (1993) have shown that in wheat and maize, root elongation is inhibited only when apices are exposed to Al, whereas selectively exposing the remainder of the root does not inhibit elongation. Hematoxylin, a stain for Al, stains root apices of Al-sensitive wheat genotypes more intensely than root apices of Al-tolerant genotypes, but the remainder of the root shows the same degree of staining in different genotypes (Polle et al., 1978; Wallace et al., 1982), indicating that tolerance might be a property of the root apex.

Differential uptake of Al into roots could account for differences in tolerance between genotypes, but conflicting results have been reported regarding differences in Al uptake in roots of different wheat genotypes. Some of these conflicting results appear to be due to the size of the root portion analyzed and the time of exposure to Al. Recently Rincón and Gonzales (1992) showed that an Al-sensitive wheat cultivar accumulated more Al in its root apices (2 mm terminus of root) than an Al-tolerant cultivar, which is consistent with the above discussion regarding the site of Al toxicity. Reports showing little or no difference in Al uptake between genotypes (Wallace et al., 1982; Zhang and Taylor, 1988, 1989) may have resulted from the use of longer sections of roots, which could mask differences occurring at the root apex.

Analysis of root apices for total Al, although preferable to analysis of whole roots, does not identify differences in Al distribution within root apices, which may be important in understanding the basis of Al tolerance. Methods have been developed to define apoplastic versus symplastic Al using kinetic analysis of Al uptake (Zhang and Taylor, 1989) or selective fractionation of Al in roots (Tice et al., 1992). These methods do not provide information regarding the distribution of Al within specific regions of the root apex. The use of x-ray microanalysis can complement such studies and help define the physiological basis of Al tolerance. Previous studies using elemental microanalysis have shown that Al is taken up by plant roots, but these studies were primarily concerned with defining sites of Al accumulation in roots rather than comparing genotypes differing in Al tolerance (Rasmussen, 1968; Matsumoto et al., 1976; Naidoo et al., 1978; Huett and Menary, 1980; Jentschke et al., 1991). Furthermore, the Al concentrations used in some of these microanalytical studies were in the millimolar range, and roots were exposed to Al for times ranging from 4 d (Rasmussen, 1968) to 17 weeks (Jentschke et al., 1991). The effects of Al on root physiology

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Abbreviations: *Alt1*, aluminum tolerance locus; XRFS, x-ray fluorescence spectroscopy; ZAF, atomic mass, fluorescence, and absorption.

are apparent within hours, and it is important that experiments aimed at identifying the physiological basis of Al tolerance should encompass time points prior to the visible onset of Al stress. Experiments in which parameters are measured after extended exposure to Al will provide limited information regarding either the primary effects of Al toxicity or Al tolerance mechanisms because root apices of sensitive seedlings will be dead or severely inhibited and almost any parameter measured is likely to show a difference between genotypes.

Al tolerance in some wheat cultivars is inherited in a simple manner consistent with the presence of a major dominant gene conferring Al tolerance (Kerridge and Kronstad, 1968; Larkin, 1987). Other cultivars show a more complex inheritance, indicating the presence of several additive genes (Aniol, 1991). It is preferable when comparing the physiology and biochemistry of genotypes differing in Al tolerance that the genetic backgrounds be similar so as to eliminate differences that are unrelated to Al tolerance. It is also advantageous to use lines differing by a single gene rather than systems where the Al tolerance is due to several mechanisms encoded by different genes. In this work we have used near-isogenic wheat lines differing in Al tolerance at a single locus. As part of our studies aimed at understanding the physiological basis of Al tolerance we describe the Al uptake and the elemental distribution in root apices of these lines.

MATERIALS AND METHODS

Plant Material

The wheat (*Triticum aestivum* L.) lines used were derived from crosses between Carazinho, an Al-tolerant cultivar, and Egret, an Al-sensitive cultivar. Briefly, F_1 progeny resulting from a Carazinho × Egret cross were backcrossed three times to Egret or derivatives of Egret. In the third backcross population, an F_2 group segregating for Al tolerance was identified and a pair of sibling lines, one homozygous Al-tolerant and the other homozygous Al-sensitive, was selected (Fisher and Scott, 1987). The lines were designated ET3 (homozygous Al-tolerant) and ES3 (homozygous Al-sensitive). The ES3 line was crossed to ET3 and the resulting F_1 progeny was backcrossed to the ES3 parent an additional six times. Pairs of lines consisting of a homozygous Al-tolerant and a homozygous Al-sensitive line were developed at the various backcrosses in the way described above. The lines were designated as ETX and ESX, where X denotes the total number of crosses including the three initial backcrosses. For experiments requiring large amounts of seed, such as for total Al analysis of root apices, lines ES3 and ET3 were used. For genetic analysis and experiments requiring few seed, we used the more advanced near-isogenic lines (ETX/ESX; where X = 6, 7, or 8).

Growth of Seedlings

Seed was germinated and seedlings were grown in solution culture using previously described methods (Delhaize et al., 1991). The basal nutrient had a pH of 4.1 and consisted of 500 μM KNO_3, 500 μM $Ca(NO_3)_2$, 250 μM NH_4NO_3, 125 μM $MgSO_4$, 2 μM KH_2PO_4, 2 μM $FeCl_3$, 11 μM H_3BO_3, 2 μM $MnCl_2$, 0.35 μM $ZnCl_2$, and 0.2 μM $CuCl_2$. Seedlings were grown at 22°C with a 16-h light/8-h dark regime. For the preparation of nutrient solutions that contained Al, the pH of the basal solution was adjusted to 4.5 prior to the addition of stock $AlK(SO_4)_2$. After addition of Al the pH was readjusted to 4.10 with 0.05 N HCl. The low pH, low ionic strength, and low Pi concentration ensured that Al remained phytotoxic (Blamey et al., 1983). The use of alkali was avoided when adjusting the pH of nutrient solutions that contained Al to prevent the formation of toxic polymeric Al species (Kinraide, 1991).

After 3 d of growth without Al, the seedlings were exposed to nutrient solution that contained 100 μM $AlK(SO_4)_2$. The seedlings were exposed to Al solutions for times ranging from 1 to 16 h; they were then washed for 30 min in aerated water and the root apices (2–3 mm) were collected for assay of total Al. In parallel experiments, root elongation was measured over a similar time scale. Seedlings were fixed to Petri plates with tape and incubated in 30 mL of the Al solution. Root growth was determined with an Olympus microscope using an eyepiece reticule (40× magnification) to measure the distance of the root apex from a reference line marked on the Petri plate.

Populations of F_2 seedlings segregating for Al tolerance were analyzed by growing the seedlings fully immersed in aerated nutrient solution that contained 20 μM Al. After 5 d of growth with daily changes of nutrient solution, Al tolerance was assessed on the basis of root growth; sensitive seedlings failed to develop roots beyond 5 mm, whereas the length of the primary root in tolerant seedlings was in excess of 20 mm.

Hematoxylin Staining of Roots

Roots of 3-d-old seedlings were stained using modifications of the method described by Polle et al. (1978). Roots were exposed to Al solutions for various times, then washed for 30 min in distilled water with several changes of water before being stained with hematoxylin for 30 min, washed for a further 30 min in distilled water, and then photographed. The stain was prepared the day before the experiment by placing 0.2 g of hematoxylin (BDH Chemicals) and 0.02 g of KIO_3 in 100 mL of water and stirring the solution overnight to dissolve the hematoxylin.

Al Assay

A modification of the method described by Zhang and Taylor (1989) was used to analyze total Al in root apices. Apices (terminal 2–3 mm of root) from 60 to 120 seedlings were collected, placed into preweighed, disposable borosilicate tubes (10 mm × 75 mm), and dried overnight at 70°C. The tubes were reweighed to determine the dry weight, then the samples were ashed at 550°C for 16 h. The ash was dissolved in a mixture consisting of 100 μL of concentrated HNO_3 and 100 μL of 30% (w/w) H_2O_2. The ash solution was heated for 2 h at 70°C, then transferred with distilled water to 16-mL glass test tubes and made up to 10 mL final volume. Samples were diluted prior to analysis depending on their Al concentration. Samples were analyzed by atomic absorption

spectroscopy on a Varian spectrophotometer equipped with a graphite furnace. Sample (10 μL) was combined with 15 μL of water and injected into a pyrolytically coated graphite tube. Samples were dried at 95°C for 40 s and 120°C for 10 s, heated to 1000°C for 8 s, then atomized at 2500°C for 4.8 s. All glassware and plasticware used for Al determinations was washed in 20% (v/v) nitric acid and thoroughly rinsed with distilled water. Using this method, recovery from 5 to 7 mg of ground pine needles spiked with 3 μg of Al (Al concentration, 430 μg g^{-1}) was 105 ± 6% (± SD).

Preparation of Freeze-Dried Samples for X-Ray Microanalysis

Roots from 3-d-old seedlings exposed to Al nutrient solutions for various times were washed in aerated distilled water for 30 min then snap frozen in liquid nitrogen. Root apices were fractured 2 mm from the tip with a razor blade cooled by liquid nitrogen. The fractured apices were freeze dried and mounted onto Tempfix-coated (Neubauer Chemikalien) stubs using methods described by the manufacturer. The mounted samples were carbon coated in a Balzer's sputter coater using carbon thread, and the samples were stored in a desiccator until analyzed.

X-Ray Microanalysis

Samples were analyzed in a JEOL JSM-6400 scanning electron microscope equipped with an energy-dispersive x-ray detector (Link eXL x-ray analyzer and associated software). The accelerating voltage was 15 kV with an emission current of 70 μA. The incident angle was 90° and the take-off angle was 50° at a working distance of 39 mm. Samples and standards were measured at an instrument magnification of 3000 using a Be window for x-ray analysis. A nominal probe current of 0.6 nA produced about 300 counts per second with a dead time of 25%. Spectra were collected for 100 s live time and analyzed with the Link ZAF-PB software for peak identification and quantitation (version eXL-ZPB.70). This software uses a process of peak stripping and background modeling to calculate peak-to-background ratios with subsequent estimation of elemental concentrations by ZAF-type calculations and use of correction factors. Standards for most of the elements were prepared by soaking cellulose powder (Whatman CF-11) in solutions containing a varying range of concentrations of $AlK(SO_4)_2$, $MgSO_4$, or NaCl. The powder was dried, pulverized into a finer powder for 10 min in a puck mill, then pressed into a pellet. For Ca and P, crystalline $CaHPO_4$, because of its low solubility in water, was combined directly with cellulose powder, then ground and pelleted. The concentrations of elements in these cellulose-based standards were determined with a Phillips PW 1404 x-ray spectrometer using conventional methods (Norrish and Hutton, 1977). These standard samples were homogeneous and approximated the matrix and mineral content of freeze-dried plant material. The standards were analyzed in the electron microscope to obtain correction factors that are used in calculations by the ZAF-PB software. The ZAF-PB method was compared with conventional XRFS over a range of concentrations for those elements analyzed in root apices.

Elemental maps of root samples were produced by the Link x-ray map and image acquisition software (version eXL-MAP.01) with a resolution of 256 × 256 with a dwell time of 10 ms and 10 cumulative scans of the sample. Microscope conditions were as described above. Raw data were processed with the Link imaging software (version eXL-SIP.01), which involved subtracting a background window from the elemental window then dividing the result by the background. This procedure corrected the data to minimize effects due to sample topography and matrix effects. After this procedure the data were converted, with appropriate threshold values, to binary data.

RESULTS

Genetics of Al Tolerance

Al tolerance assessed on the basis of root elongation segregated as a single dominant locus in F_2 populations from various backcrosses in the derivation of the near-isogenic lines (Fig. 1, Table I). A clear difference in root length was observed between Al-tolerant and Al-sensitive seedlings at 20 μM Al (Fig. 1), and there was about a 10-fold difference in Al tolerance based on the concentration causing 50% inhibition of root growth (Fig. 2). This locus (*Alt1*; aluminum tolerance) showed complete dominance, with heterozygous *Alt1* seedlings showing the same Al tolerance as homozygous *Alt1* seedlings (Fig. 2). The Al tolerance of ET8 at 50 and 100 μM Al was less than that of Carazinho, the original donor of *Alt1*, indicating that additional genes present in Carazinho were lost in the derivation of the near-isogenic lines (Fig. 3). A two-factor analysis of variance was used to analyze the data for the two wheat lines. The factors were (a) genotype and (b) Al concentrations, with 15 replicate roots per treatment (Fig. 3). There was a significant interaction of genotype with Al concentration ($P < 0.001$). Further analysis of the data at 50 and 100 μM Al showed that ET8 was less Al-

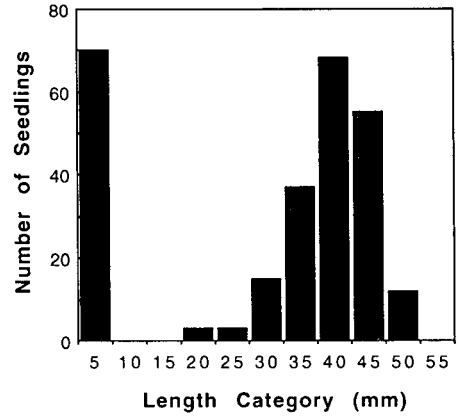

Figure 1. Root lengths of 5-d-old seedlings segregating for Al tolerance. The seedlings are an F_2 population derived from the sixth backcross and were grown in nutrient solution containing 20 μM Al. Root lengths were measured to the nearest mm and are denoted as categories 5 (0–5 mm), 10 (6–10 mm), etc. Al tolerance segregated as a single locus (192 tolerant and 70 sensitive seedlings were observed; P = 0.5–0.7 for a 3:1 ratio).

Table I. *Analysis of the Al tolerance of F_2 populations segregating for Alt1*

The Al tolerance of seedlings was assessed by hematoxylin staining and root elongation. BC, Backcross.

Genotype and Method	Tolerant	Sensitive	P (3:1 ratio)
	Number of seedlings		
F_2, BC3			
Hematoxylin[a]	585	202	0.5–0.7
Root length[b]	588	199	0.5–0.7
F_2, BC9			
Root length[c]	117	34	0.3–0.5

[a] Seedlings were grown for 3 d without Al then exposed to 100 μM Al in nutrient solution for 1 h and stained with hematoxylin. [b] After hematoxylin staining, seedlings were grown fully immersed in nutrient solution containing 20 μM Al for a further 5 d, and Al tolerance was assessed on the basis of root length. [c] Seeds were germinated directly in nutrient solution containing 20 μM Al, and seedlings were grown immersed in the nutrient solution for 5 d before being assessed for Al tolerance on the basis of root length.

tolerant than Carazinho at both of these concentrations ($P < 0.001$), although at 20 μM Al root growth in the ET8 line was stimulated above the control and was greater than root growth of Carazinho ($P < 0.001$).

Hematoxylin Stain

Seedlings differing in Al tolerance could be differentiated by staining with hematoxylin after short exposures to nutrient solutions that contained 100 μM Al (Fig. 4). Al-sensitive seedlings exposed to 100 μM Al for 1 h and stained with hematoxylin stained at the root apices, whereas Al-tolerant seedlings treated in the same way showed little staining.

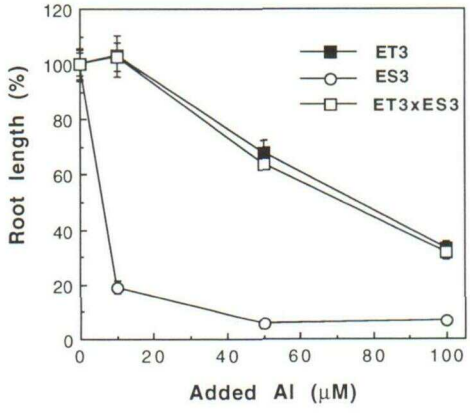

Figure 2. Root elongation of the homozygous Al-sensitive line ES3 (○), the homozygous Al-tolerant line ET3 (■), and F_1 seedlings (heterozygous for *Alt1*) of an ES3 × ET3 cross (□) in a range of Al solutions. Seedlings were grown for 5 d in the nutrient solutions, and root elongation is expressed as a percentage of the 0 Al controls (mean ± SE, $n = 5$). The absence of error bars indicates that the size of the error does not exceed the size of the symbol).

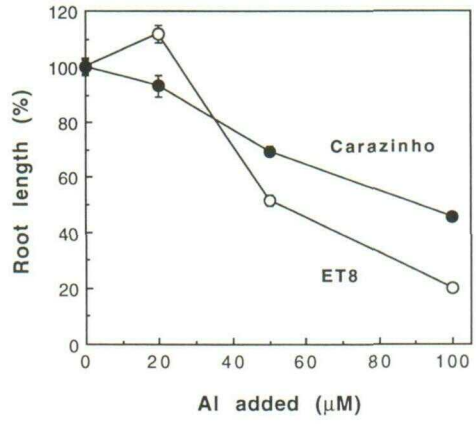

Figure 3. Root lengths of seedlings grown at various Al concentrations comparing Carazinho (●), the original donor of *Alt1*, to line ET8 (○). Seedlings were grown for 5 d in nutrient solutions, and root length is expressed as a percentage of the 0 Al controls (mean ± SE, $n = 15$). The absence of error bars indicates that the size of the error does not exceed the size of the symbol.

Differences in staining were also apparent after 10 min of exposure to 100 μM Al (Fig. 4).

Al-tolerant and Al-sensitive genotypes could be identified by hematoxylin staining before differences in root elongation were measurable (Fig. 5) and before gross effects of Al toxicity became evident in root apices of sensitive lines. Because hematoxylin is nondestructive to root apices, seedlings could be first stained and then grown on and assessed for root growth in nutrient solution containing Al. Roots of Al-sensitive seedlings exposed to 100 μM Al for 1 h and stained with hematoxylin continued to grow after transfer to solutions without Al, indicating that the seedlings were not adversely affected by either the exposure to Al or the hematoxylin staining (data not shown). The presence of stain in root apices after short exposures to Al could then be correlated with the *Alt1* phenotype based on root growth in Al solutions.

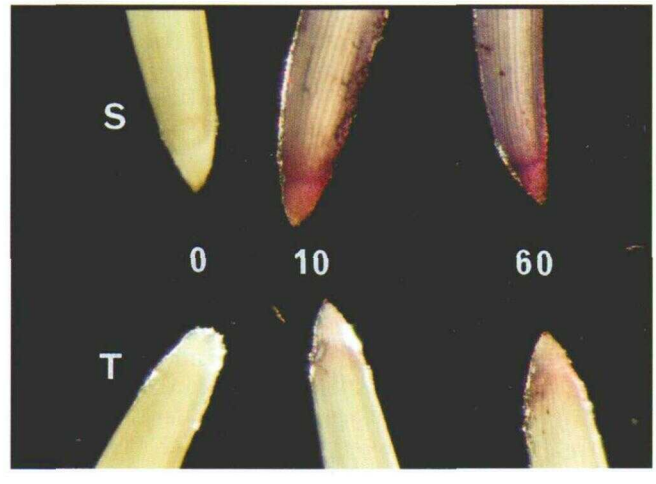

Figure 4. Hematoxylin staining of root apices of Al-sensitive (S) and Al-tolerant (T) seedlings exposed to 100 μM Al for 0, 10, and 60 min.

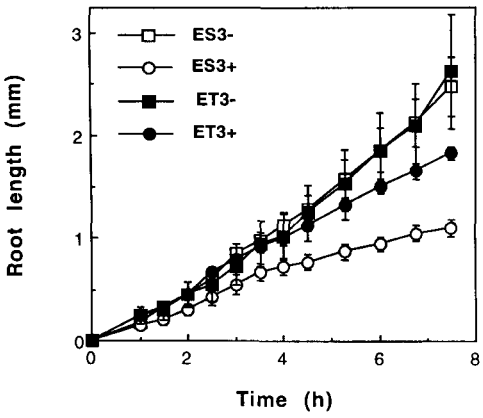

Figure 5. Time course of root elongation of ET3 and ES3 wheat lines in nutrient solution without Al (■, ET3−; □, ES3−) and in nutrient solution containing 100 μM Al (●, ET3+; ○, ES3+). The mean ± SE is shown ($n = 6$).

An F_2 population segregating for Al tolerance was assessed in this way. Seedlings were separated according to their hematoxylin staining pattern after a 1-h exposure to 100 μM Al and then grown in 20 μM Al for 5 d. These Al concentrations were chosen because they allowed the genotype of seedlings to be clearly identified by each of the methods. There was good agreement in identifying the Al genotype by the two methods (Table I), and both were consistent with a single locus conferring Al tolerance. Eight seedlings that stained in an anomalous way, i.e. were sensitive by stain and tolerant by root length or vice versa, were grown in soil and the progeny were tested. In all cases the progeny showed staining consistent with the Al tolerance genotype as determined by root growth in Al solutions. This indicated that the anomalous staining of the parental seedlings was due to poor staining or insufficient washing of those individuals in the particular experiment and was not genetically based. These results indicate that the differential hematoxylin staining is due to a locus tightly linked to *Alt1*, or due to *Alt1* itself, and reflects a difference in uptake or binding of Al by root apices of the different genotypes.

Al Uptake in Root Apices

The differential staining between Al tolerance genotypes suggested an apparent difference of Al uptake or binding by root apices. Treatment of Al-exposed root apices with citrate or Ca^{2+} solutions has been used in other studies to desorb apoplastic Al (Zhang and Taylor, 1989; Tice et al., 1992). Treatment of Al-exposed roots with citrate solutions prior to hematoxylin attenuated the staining intensity of sensitive seedlings (data not shown), indicating that apoplastic Al is likely to have been at least partly responsible for the differential staining observed. Since hematoxylin showed a clear difference in staining after roots were washed in water for 30 min, roots were not pretreated with citrate or Ca^{2+} solutions to desorb apoplastic Al before analysis of total Al. We had no reason to presuppose that apoplastic Al was not important in differential Al tolerance. We used intact seedlings for Al exposures because exposure of excised root apices to Al may have resulted in accumulation or uptake of Al at the cut surface, making the data difficult to interpret.

Figure 6 shows a time course of total Al accumulated in root apices of Al-tolerant and Al-sensitive genotypes exposed to 100 μM Al. Al concentrations in root apices of both genotypes increased with time of exposure, and the most marked increase was found in the sensitive genotype. Al-sensitive apices had accumulated more Al than the Al-tolerant apices after 4 h of exposure to Al at about the same time that the first measurable differences in root elongation could be detected (Fig. 5). Although hematoxylin clearly differentiated the genotypes after a 1-h exposure to 100 μM Al (Fig. 4), no statistically significant difference in total Al concentration of root apices was found between the genotypes after 1 h of exposure to Al (ES3, 542 ± 85 μg g^{-1} dry weight Al; ET3, 566 ± 65 μg g^{-1} dry weight Al [mean ± SE, $n = 3$]). Roots not exposed to Al did not stain with hematoxylin, yet both genotypes had about 300 μg g^{-1} dry weight Al. This apparently high concentration of Al was confirmed using a pyrocatechol violet assay (data not shown) and was of the same order as reported by Rincón and Gonzales (1992) for wheat root apices 2 mm long and not exposed to Al. Hematoxylin was clearly more sensitive than analysis of total Al in root apices for differentiating the genotypes but did not react with the large fraction of Al present in seedlings not exposed to Al.

X-Ray Microanalysis

X-ray microanalysis does not rely on the chemical form of Al for detection and can provide quantitative data for Al and other elements present in root apices. Before x-ray microanalysis of root apices was undertaken, the performance of the Link analytical system to detect and quantitate elements in freeze-dried root apices was assessed. Cellulose powder dosed with varying concentrations of elements provided a

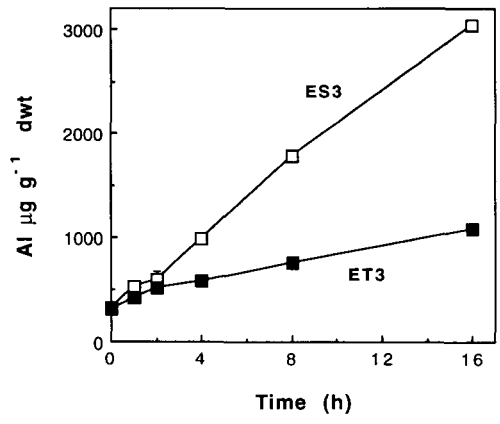

Figure 6. Time course of total Al accumulated in root apices (terminal 2–3 mm of root) of Al-tolerant (ET3, ■) and Al-sensitive (ES3, □) genotypes after exposure to 100 μM Al in nutrient solution. The mean (±range) of duplicate experiments is shown and the absence of error bars indicates that the range does not exceed the size of the symbol. dwt, Dry weight.

matrix comparable to freeze-dried root material and could be analyzed by conventional XRFS to determine the elemental composition. Good agreement was obtained in Al quantitation between conventional XRFS and x-ray microanalysis using the ZAF-PB software provided by Link. A linear relationship was obtained between the two methods ($y = 0.957x$; $r^2 = 0.99$) for standard samples containing from 200 to 800 $\mu g\ g^{-1}$ dry weight Al. Similar linear relationships were obtained for the other elements analyzed in root apices. Analysis was restricted to Mg, Al, P, S, Cl, and K, the elements readily detectable in root apices (Fig. 7). Ca was not quantitated because K was present in large concentrations and the $K_{K\beta}$ x-ray energy peak overlaps the $Ca_{K\alpha}$ peak (Lazof and Läuchli, 1991). Although peak deconvolution is possible, Figure 7 shows that the concentration of Ca was likely to be very low on the basis of the size of the combined $K_{K\beta}$ and $Ca_{K\alpha}$ peaks.

Analysis of the elemental distribution in sensitive root apices exposed to Al for 24 h showed that Al was consistently concentrated in the epidermal cells and subepidermal cortical cells (Fig. 8B). Accumulation of Al in cell wall regions was apparent in Al-sensitive apices exposed to Al for 2 h (Fig. 9), but this effect was not always reproducible and in many instances the Al could not be detected above the background. The presence of Al coincided with the absence of K in sensitive apices exposed to Al for 24 h, whereas the distribution of P was unaffected by Al (Fig. 8). By contrast, Al-tolerant apices accumulated less Al, and the Al, P, and K distributions (Fig. 8, F, G, and H) were similar to control apices not exposed to Al (data not shown).

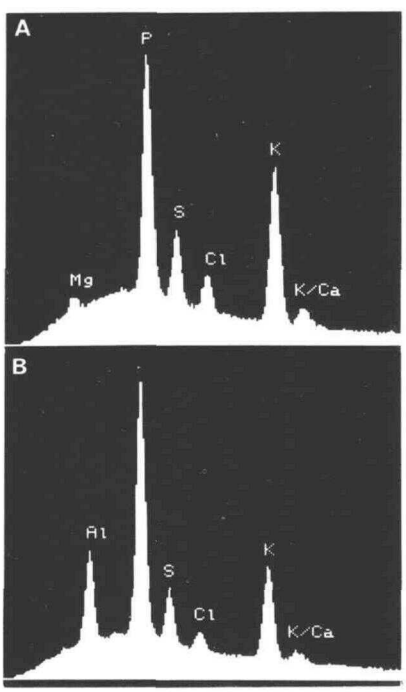

Figure 7. Typical spectra of transverse sections of freeze-dried root apices analyzed for their elemental content by x-ray microanalysis. The spectra were obtained from root apices of Al-sensitive seedlings grown in nutrient solution without Al (A) or with 100 μM Al for 24 h (B). The approximate region analyzed is shown in Figure 9. The K/Ca peak denotes the combined peak for the $K_{K\beta}$ and $Ca_{K\alpha}$ overlapping peaks.

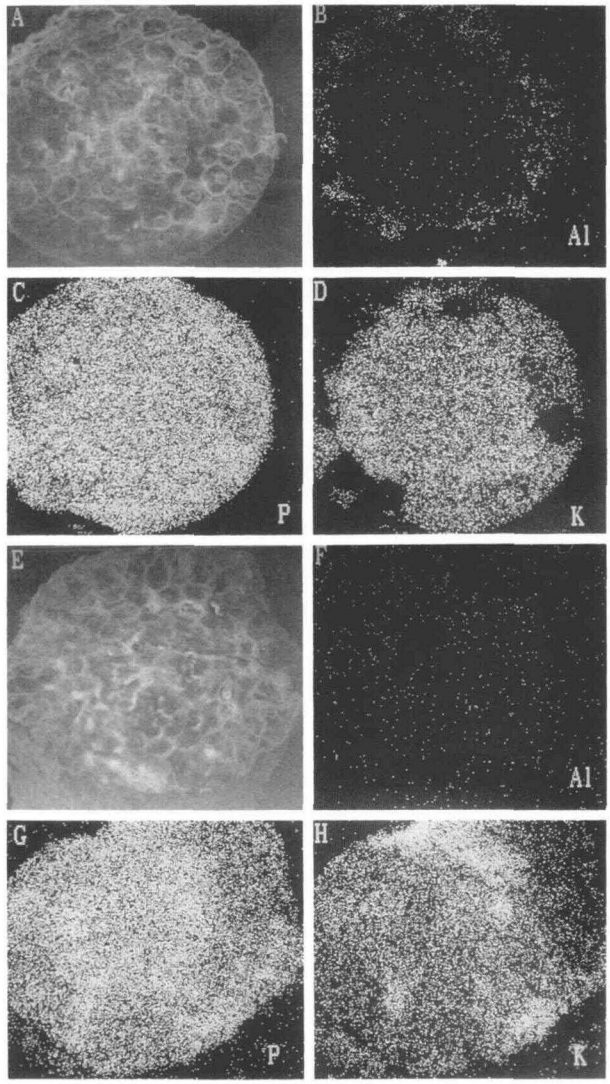

Figure 8. *Distribution maps of Al, K, and P in transverse sections* of freeze-dried root apices (2 mm from tip) from seedlings exposed to 100 μM Al for 24 h. A-D, Al-sensitive root apex; E-H, Al-tolerant root apex. A and E, Digitized electron image of section analyzed; B and F, Al distribution; C and G, P distribution; D and H, K distribution. Samples were analyzed at 160× magnification, and data for elements were corrected for surface topography and matrix effects before they were mapped.

Elemental quantitation was confined to the region identified in sensitive apices where Al accumulated (Fig. 9). Al had accumulated in sensitive seedlings in the cortex after 8 h of exposure of the roots to 100 μM Al, and high concentrations were evident after 24 h of exposure (Fig. 10). By contrast, Al-tolerant seedlings showed much less Al accumulation in equivalent regions after 8- and 24-h exposures to Al. Of the other elements quantitated, the most marked effects were a decline in Cl concentrations for both genotypes, whereas Mg and K decreased specifically in sensitive apices after 24 h of

Al exposure. The decreased concentration of K reflects its distribution observed in Al-sensitive apices exposed for 24 h to Al (Fig. 8D). The relatively large errors associated with the K concentrations indicate the variability in K distribution, with some regions showing near normal levels and other regions being almost devoid of K.

DISCUSSION

Al tolerance in the near-isogenic lines used in this study segregated as a single locus based on both hematoxylin staining and root length measurements. These near-isogenic lines provide a useful system for the study of the physiology and biochemistry of Al tolerance in plants because they avoid the common problem of comparisons between genetically unrelated or poorly defined cultivars or species. The original parental donor of *Alt1*, Carazinho, has additional genes that appear to be additive and confer a higher degree of Al tolerance. These genes are minor in comparison with *Alt1*, since the Al tolerance of the near-isogenic tolerant line was only marginally less than that of Carazinho (Fig. 3).

Hematoxylin staining, analysis of total Al in root apices, and x-ray microanalysis all provided evidence that *Alt1* could encode a mechanism that excludes Al from root apices. The methods we used for detecting Al in root apices were complementary and present a picture of Al uptake and distribution in root apices. Although analysis of total Al in root apices showed an increase in both genotypes over the initial 2 h of Al exposure, it was possible to differentiate only the genotypes with longer exposures. Some of the difference observed in Al uptake can be explained by differences in root growth, since Al-tolerant roots continued to grow in the presence of Al and would have effectively diluted the Al in apices. Root growth for both genotypes was approximately linear over the initial 4 h of Al exposure (Fig. 5; Al-tolerant, 0.27 mm h^{-1}; Al-sensitive, 0.20 mm h^{-1}), whereas Al accumulation was linear over 2 to 16 h (Fig. 6; Al-tolerant, 36 $\mu g\ g^{-1}$ dry weight h^{-1}; Al-sensitive, 167 $\mu g\ g^{-1}$ dry weight h^{-1}). The rate of Al accumulation over the 2- to 4-h period was about 4.6-fold greater in Al-sensitive apices than in Al-tolerant apices, whereas the root elongation was only about 1.3-fold greater for Al-tolerant roots than for Al-sensitive roots. If dilution were the only factor contributing to the differences in Al content of apices, then we would have expected Al-tolerant roots to be growing 4.6-fold faster than Al-sensitive roots.

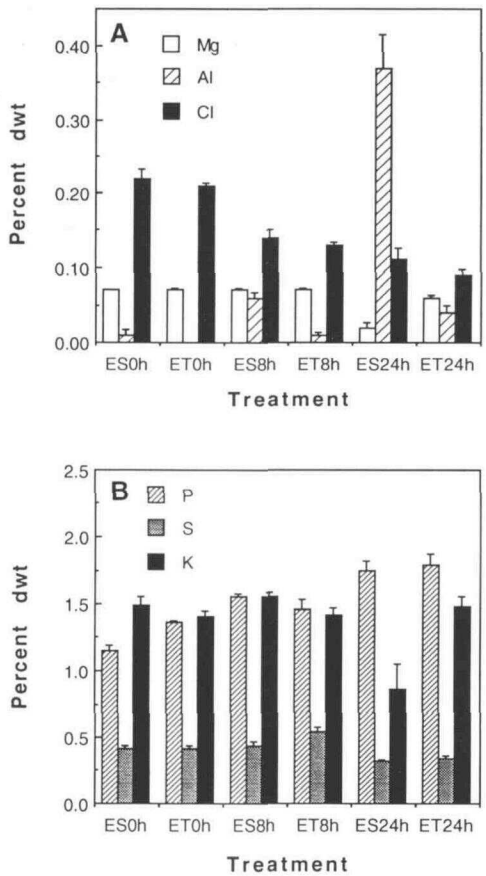

Figure 10. Concentrations of Mg, Al, and Cl (A), and P, K, and S (B) in freeze-dried root apices of Al-sensitive (ES) and Al-tolerant (ET) seedlings exposed to 100 μM Al for 0, 8, and 24 h. The approximate region analyzed is shown in Figure 9, and the signals were quantitated using the Link ZAF-PB software. The mean ± SE are shown (n = 9) from the analysis of three different regions from each of three root apices. dwt, Dry weight.

After 4 h, growth rates declined for both Al-tolerant (Fig. 5; 0.25 mm h^{-1}) and Al-sensitive roots (Fig. 5; 0.11 mm h^{-1}). This resulted in Al-tolerant roots having a 2.2-fold greater rate, which is still too low to explain the differences in Al accumulation of root apices on the basis of dilution only.

Rincón and Gonzales (1992) also showed that Al appears

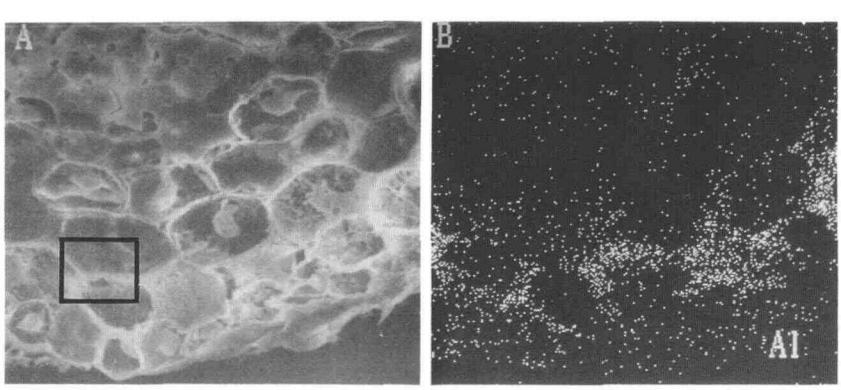

Figure 9. Digitized electron image (A) and distribution of Al (B) in a region of a transverse section of an Al-sensitive root apex exposed to Al for 2 h. The sample was analyzed at 600×, magnification and Al data were corrected for surface topography and matrix effects before they were mapped. The area enclosed by the rectangle approximates the region analyzed at a magnification of 3000× by ZAF-PB quantitation.

to be excluded from root apices of Al-tolerant wheat. However, in Atlas, the Al-tolerant wheat cultivar they used, hematoxylin staining and kinetics of Al accumulation showed a different pattern than in our Al-tolerant lines. Atlas showed an initial staining of root apices with hematoxylin after a 1-h exposure to Al. The staining intensity declined as time of Al exposure progressed, and this was accompanied by decreases in Al concentrations of root apices after an initial maximum. By contrast, in the ET lines, hematoxylin staining, although much less intense than in the ES lines, increased with increasing Al exposure, and this was accompanied by a gradual accumulation of Al in root apices (Fig. 6). These differences may indicate that the tolerance mechanism in Atlas differs fundamentally from that encoded by *Alt1* or that additional genes, encoding different Al tolerance mechanisms, are present in Atlas. In support of the latter case, at least two major genes controlling Al tolerance have been reported for Atlas (Rajaram et al., 1991). Similar results showing differences in Al concentrations of root apices between the cultivars Yecora Rojo (Al-tolerant) and Tyler (Al-sensitive) over a 2-d exposure to Al have been reported (Tice et al., 1992). Fractionation of the Al into symplastic and apoplastic compartments for these cultivars indicated that Al appeared to be excluded from both symplasm and apoplasm in Al-tolerant apices over 2 d of exposure to Al.

X-ray microanalysis was useful in determining the distribution and quantity of Al in apices and showed that Al entered Al-sensitive root apices and accumulated in cortical regions to concentrations 5- to 10-fold greater than those observed in Al-tolerant apices. Although cellular integrity and elemental distributions at a subcellular level are altered by freeze-drying tissue, the method can provide useful information regarding the distribution of elements across plant organs (Lazof and Läuchli, 1991). The detection limits for elements of low atomic mass are at least 10-fold and up to 50-fold lower for freeze-dried tissue compared with frozen-hydrated tissue (Lazof and Läuchli, 1991). In the current work we estimate that the detection limit for Al was about 100 to 200 $\mu g\ g^{-1}$ dry weight. However, even in freeze-dried apices this was too insensitive to consistently detect Al in sensitive roots exposed to Al for 2 h. Although the distribution of Al into apoplastic and symplastic compartments is not definitive in freeze-dried tissues, most of the Al in sensitive apices appeared to be associated with cell walls of cortical tissue (Fig. 9) and, with prolonged exposure, could be detected within cells. The effects of prolonged Al exposure on K distribution are consistent with damage to the plasma membrane and leakage of soluble contents, such as K, out of cells. Alternatively, Al may promote net efflux of K from cells without damaging the plasma membrane, since it has been shown that even in severely Al-intoxicated roots plasma membranes remain intact (Kinraide, 1988).

The ability to detect genotypic differences with hematoxylin after exposing seedlings to Al for 10 min indicates that *Alt1* is likely to be constitutively expressed. Although activation of genes can occur rapidly, as in the case of elicitor-treated plants, where gene transcription is detected as early as 10 min after treatment, the products of these genes may take several hours to accumulate and function (Dixon and Harrison, 1990). Huang et al. (1992) have shown that effects of Al on Ca fluxes around root apices occur within minutes in wheat and that genotypic differences in Al tolerance can also be demonstrated over this time. This differential response of Ca fluxes after short exposures to Al was also apparent in the ET6 and ES6 near-isogenic lines used here, providing additional evidence that Al tolerance encoded by *Alt1* is likely to be constitutively expressed (Ryan and Kochian, 1993).

Possible Al tolerance mechanisms based on exclusion of Al from root apices have been extensively reviewed (Taylor, 1988, 1991). Some of these mechanisms include (a) active exclusion at the level of the plasma membrane, (b) differential Al-binding properties by cell walls, and (c) excretion of organic acids that chelate and detoxify Al external to the symplasm. In the current study Al-tolerant seedlings appeared to exclude Al from whole apices, which is inconsistent with exclusion occurring solely at the plasma membrane. In an accompanying paper (pp. 695–702), we provide evidence to support the hypothesis that *Alt1* encodes a mechanism that responds to Al by excretion of malic acid, which in turn chelates and excludes Al from root apices.

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