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Differential Exudation of Polypeptides by Roots of Aluminum-Resistant and Aluminum-Sensitive Cultivars of *Triticum aestivum* L. in Response to Aluminum Stress[1]

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Cultivars of *Triticum aestivum* differing in resistance to Al were grown under aseptic conditions in the presence and absence of Al and polypeptides present in root exudates were collected, concentrated, and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Upon exposure to 100 and 200 μM Al, root elongation in Al-sensitive cultivars was reduced by 30 and 65%, respectively, whereas root elongation in resistant cultivars was reduced by only 15 and 30%. Accumulation of polypeptides in the growth medium increased with time for 96 to 120 h, with little additional accumulation thereafter. This pattern of exudation was virtually unaffected by exposure to 100 μM Al in the Al-resistant cultivars Atlas 66 and Maringa, whereas total accumulation was reduced in sensitive cultivars. Changes in exudation were consistent with alterations in root elongation. Al-induced or Al-enhanced polypeptide bands were detected in Atlas 66 and Maringa after 72 h of exposure to Al. Increased accumulation of 12-, 22-, and 33-kD bands was observed at 75 μM Al in Atlas 66 and 12-, 23-, and 43.5-kD bands started to appear at 50 μM Al in Maringa. In the Al-sensitive cultivars Roblin and Katepwa, no significant effect on polypeptide profiles was observed at values up to 100 μM Al. When root exudates were separated by ultrafiltration and the Al content was measured in both high molecular mass (HMM; >10 kD) and ultrafiltrate (<10 kD) fractions, approximately 2 times more Al was detected in HMM fractions from Al-resistant cultivars than from Al-sensitive cultivars. Dialysis of HMM fractions against water did not release this bound Al; digestion with protease released between 62 and 73% of total Al, with twice as much released from exudates of Al-resistant than of Al-sensitive cultivars. When plants were grown in the presence of 0 to 200 μM Al, saturation of the Al-binding capacity of HMM exudates occurred at 50 μM Al in Al-sensitive cultivars. Saturation was not achieved in resistant cultivars. Differences in exudation of total polypeptides in response to Al stress, enhanced accumulation of specific polypeptides, and the greater association of Al with HMM fractions from Al-resistant cultivars suggest that root exudate polypeptides may play a role in plant response to Al.

The presence of Al in acid soils has long been recognized as a serious agricultural problem (Foy et al., 1978), although plant species and ecotypes vary in their capacity to withstand Al stress. A possible mechanism of Al resistance is exudation of chelator ligands into the apoplasm or rhizosphere. Chelators may form stable complexes with Al, thereby reducing the activity of monomeric Al in the apoplasm and inhibiting its absorption across the plasma membrane (Taylor, 1991). Several studies have attempted to identify potential chelator ligands and assess their role in Al resistance. Increased exudation of organic acids has been observed under conditions of Al stress in Al-resistant cultivars of *Phaseolus vulgaris* (Miyasaka et al., 1991) and *Triticum aestivum* (Delhaize et al., 1993; Basu et al., 1994), but not in *Lotus* (Blamey et al., 1990). In *T. aestivum*, malate is the dominant organic acid exuded. Increased exudation of malate is consistently expressed by Al-resistant plants in a population segregating for Al resistance (Delhaize et al., 1993) and can be attributed to increased synthesis and export (Basu et al., 1994).

Other chelator ligands may also play a role in reducing the activity of Al in the apoplasm. Tremaine and Miller (1983) found that an Al-resistant strain of *Rhizobium* produced significantly more EPS than sensitive strains. Furthermore, this EPS complexed 10 to 100 times more Al than EPS from other strains. In other studies with *Rhizobium*, acid tolerance was positively correlated with production of EPS but not with the Al-binding capacity of the exuded polysaccharide (Cunningham and Munns, 1984). Acidic polypeptides could also be involved in mediating resistance. Putterill and Gardner (1988) found that certain naturally occurring and synthetic acidic polypeptides, such as poly-L-Asp and poly-L-Glu, can bind Al at low concentrations. Although they did not provide evidence that these polypeptides were exuded by plant roots, they found that the preferential binding of acidic polypeptides to Al ameliorated the toxic effect of Al on various calmodulin-dependent processes. Similar studies by Konishi et al. (1988) showed that poly-L-Asp and poly-L-Glu have a protective effect on the growth of pollen tubes exposed to Al stress.

Roots are also known to exude a variety of macromolecules under certain conditions. Roots of *Medicago sativa* L. exuded three isoflavanoids in the presence of symbiotic *Rhizobium meliloti* that were not found in exudates of uninoculated plants (Dakora et al., 1993). Grayling and Hanke (1992) analyzed leaf and root exudates from *Perilla frutescens* for

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Abbreviations: EPS, extracellular polysaccharide; GFAAS, graphite furnace atomic absorption spectrophotometry; HMM, high molecular mass; REP, root exudate polypeptides.

cytokinins and found that the principal activity in root exudates corresponded to zeatin riboside. Polypeptides have also been reported to be exuded by roots (Vancura, 1964; Vancura and Hovadik, 1965). Bozarth et al. (1987) extracted a 28-kD polypeptide from cell walls of water-stressed soybean seedlings, and Iraki et al. (1989) reported that NaCl-adapted cells of *Nicotiana tabacum* exuded unique 40-, 29-, and 11-kD polypeptides under conditions of salt stress. In addition, phosphate starvation increased the secretion of at least six polypeptides by cell suspensions of *Lycopersicon esculentum* (Goldstein et al., 1989). Progress has been made on elucidating the pathway for secretion of polypeptides from plant cells (Akazawa and Hara-Nishimura, 1985), but the possible function of these exudates is not well understood.

In the present work we have analyzed polypeptides exuded from roots of Al-resistant and Al-sensitive cultivars of *T. aestivum* grown with and without Al under aseptic conditions. We have observed the induction or overaccumulation of several polypeptides in Al-resistant cultivars along with down regulation of some polypeptides under conditions of Al stress. We have also determined that the amount of Al associated with polypeptides in root exudates from Al-resistant cultivars is higher than that from sensitive cultivars, suggesting a possible role of these polypeptides as chelators in Al resistance.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Seeds of four cultivars of *Triticum aestivum*, Atlas 66, Maringa (Al resistant), Roblin, and Katepwa (Al sensitive), were selected. Atlas 66 is derived from Frondosa/3/Redhart 3/2/(Noll 28, Hussar/Forward) and Maringa is derived from Frontana//Kenya 58/Ponta Grossa 1. The relatedness of these two cultivars arises from Frontana and Frondosa, both of which are siblings from a cross between Fronteira and Mentana. Both Roblin and Katepwa are hard, red, spring wheats. Roblin is derived from crosses between RL 4302/RL 4356/RL 4359/RL 4353 (RL 4302 is obtained from the cross Manitou/Tobari 66; RL 4356 from CT615/Neepawa; RL 4359 from CT 615/Neepawa, and RL 4353 from CT 934/Neepawa/Era/Park). Katepwa is obtained from Neepawa *6/RL 2938/3/Neepawa *6//C18154/2 *Frocor.

Seeds were sterilized in 1% sodium hypochlorite for 20 min, allowed to imbibe in sterile deionized water for 4 to 6 h, and grown in Petri dishes containing Murashige and Skoog medium solidified with 8 g L^{-1} agar (Murashige and Skoog, 1962). After 48 h of growth, healthy, sterile seedlings were selected and transferred to nylon mesh supported by polypropylene rafts with 8-mm legs. Rafts (each supporting five seedlings) were then placed inside Magenta vessels (Sigma) containing 60 mL of liquid nutrient medium (pH 4.5). The nutrient medium contained (in μM): 1000 Ca, 300 Mg, 800 K, 2900 NO_3, 300 NH_4, 100 PO_4, 101 SO_4, 34 Cl, 20.2 Na, 10 Fe, 6 B, 2 Mn, 0.5 Zn, 0.15 Cu, 0.1 Mo, and 10 EDTA.

Experimental Design

Vessels containing plants and growth solutions were placed on a gyratory shaker (60 rpm) in a controlled environment with 16 h of light (20°C, 68% RH) and 8 h of darkness (16°C, 85% RH). The photosynthetic photon flux was 335 ± 12 μmol m^{-2} s^{-1} at plant base level. After 24 h, plants were transferred to fresh nutrient solutions with and without Al (100 μM) and grown for another 120 h under the same conditions. For time-course experiments, seedlings were grown in nutrient solution containing 0 and 100 μM Al and root exudates were collected after 24, 48, 72, 96, 120, and 144 h. For dose-response experiments, plants were grown for 120 h in medium containing 0, 25, 50, 75, 100, 150, and 200 μM Al. At least three independent vessels containing five plants each were raised for each treatment, and all experiments were repeated at least twice to verify results. To maintain solution pH below 4.5, aliquots of ammonium chloride were added after 3 d of growth in treatment solutions to give a final concentration of 400 μM in each Magenta vessel. At the termination of Al exposure, plants were removed and root lengths were measured. Aliquots (300 μL) of growth solution were plated on Petri dishes containing Murashige and Skoog medium to ensure that aseptic conditions had been maintained throughout the experiment. Our preliminary experiments demonstrated that filtration of root exudates through Millex-HV 0.45-μm filters to remove sloughed-off cells and particulate material had no effect on polypeptide profiles. Thus, in the experiments reported here, root exudates were stored at −20°C without filtration. Prior to analysis, exudates were thawed and concentrated 30-fold using an Amicon pressure ultrafiltration system with Amicon YM 10 membranes (cutoff 10 kD) at 4°C. This step separated high molecular mass components (the HMM fraction) from the low molecular mass components (the ultrafiltrate fraction).

Separation of Polypeptides by SDS-PAGE

For SDS-PAGE analysis, sample volumes were further reduced by 10-fold in a Sorvall Speed Vac concentrator and brought to a final volume of 200 μL. Total protein in the root exudate samples was estimated (Lowry et al., 1951) using BSA as a standard. Polypeptides were separated on 12.5% separating and 4% stacking gels by the method of Laemmli (1970). Samples were heated at 80°C for 3 min in sample buffer (0.125 M Tris-Cl, 4% [w/v] SDS, 20% [v/v] glycerol) before loading onto the gel. Gels were electrophoresed at 10 mA in the stacking gel followed by 20 mA in the separating gel. Polypeptides were stained with silver nitrate following a procedure modified from Morrissey (1981). Gels were incubated for 30 min in a solution of 45% (v/v) methanol and 12% (v/v) acetic acid, 3× 10 min in a solution of 10% (v/v) ethanol and 5% (v/v) acetic acid, 30 min in 2.5% (v/v) glutaraldehyde, 3× 30 s and 3× 10 min in water, 30 min in DTT (5 μg mL^{-1}), and 30 min in 0.012 M $AgNO_3$. For protein coloration, the gels were washed three more times with water and then soaked in a solution of 0.28 M Na_2CO_3/2 mM formaldehyde until bands appeared. The bands were fixed by placing the gels in 1% (v/v) glacial acetic acid for 5 to 10 min. The molecular mass of polypeptides was calculated based on the mobilities of the protein standards β-galactosidase (116 kD), phosphorylase *b* (97 kD), BSA (66 kD), fumarase (48.5 kD), carbonic anhydrase (29 kD), β-lactoglobulin (18.4 kD), and α-lactalbumin (14.2 kD) (Sigma).

Determination of Al in Exudate Fractions

For measurement of Al associated with polypeptides, root exudate samples were concentrated by ultrafiltration, which provided two fractions for further analysis: a concentrated HMM fraction containing polypeptides and other macromolecules (2 mL), and the remaining ultrafiltrate (approximately 58 mL) containing low molecular mass compounds. After determination of total protein in the HMM fractions, 100 μg of protein was diluted to 3 mL with deionized water for estimation of Al. Ultrafiltrates were concentrated to 2 mL using a Speed Vac concentrator and 200 μL were diluted to 3 mL for analysis of Al. To determine if dialysis could remove Al from HMM fractions, samples were placed in a dialysis tube (10-kD cutoff) and dialyzed against water for 24 h at 4°C. Dialyzed samples were measured directly for Al content. To determine if Al in HMM fractions was specifically bound to polypeptides, 2 mg mL^{-1} protein was taken up in a digestion buffer (8 M urea, 50 mM Tris-HCl bicarbonate, 1 mM $CaCl_2$, pH 8.0). Protease (type I: crude from bovine pancreas, Sigma) was added in a ratio of 1:5 (w/w) and incubated overnight at room temperature. Al content was measured after dialysis against water as above. For in vitro analysis of Al interaction with exudates, plants were grown in the absence of Al and root exudates were collected, concentrated by ultrafiltration, and dialyzed against 100 μM Al solution for 24 h at 4°C. Samples were further dialyzed against several changes of deionized water (4°C) and analyzed directly for Al content.

Concentrations of Al in HMM and ultrafiltrate fractions were determined using a Perkin-Elmer 3030 atomic absorption spectrophotometer with a HGA 500 graphite furnace attachment (GFAAS). Twenty microliters of each sample were mixed with 20 μL of $Mg(NO_3)_2$ as a matrix modifier, dried for 60 s at 150°C, pretreated for 45 s at 1700°C, and atomized for 5 s at 2500°C on a L'Vov platform in a pyrolytically coated graphite tube. Concentrations were calculated based on the integration of peak area and expressed as ng Al μg^{-1} protein and ng Al mL^{-1} filtrate. All plasticware, microfuge tubes, sample cups, and pipette tips were soaked in dilute HNO_3 for 3 to 4 d and then rinsed thoroughly with deionized water (>18 MΩ cm^{-1}) to reduce background contamination of Al.

RESULTS

Seedlings of Atlas 66, Maringa (Al resistant), Roblin, and Katepwa (Al sensitive) were grown in nutrient solution under aseptic conditions with Al concentrations ranging from 0 to 200 μM. Treatment with Al reduced root elongation in all four cultivars, but the extent of inhibition was greater in the Al-sensitive cvs Roblin and Katepwa (Fig. 1). Root elongation in Al-sensitive cultivars was inhibited by approximately 30% at 75 μM Al and by approximately 65% at 200 μM. In Al-resistant cultivars, root elongation was inhibited by only 30% at 200 μM Al (Fig. 1). Treatment with Al also affected exudation of polypeptides from roots. Again, the effect was more prominent in Al-sensitive cultivars (Fig. 2). In Katepwa, REPs declined steadily with increasing concentrations of Al and 69% inhibition was observed at 100 μM Al. In contrast, exudation of REPs in Atlas 66 was inhibited by 25% at 100 μM (Fig. 2A). In Roblin, REPs declined by 55% at 100 μM Al and 200 μM Al nearly abolished exudation. In comparison, exudation of REPs in Maringa was unaffected by Al below 100 μM (Fig. 2B).

Analysis of REPs by SDS-PAGE revealed marked differences among cultivars. In the Al-resistant cv Atlas 66, accumulation of three polypeptide bands representing 12, 22, and 33 kD was observed (Fig. 3). Growth in media containing 75 μM Al or higher was accompanied by increased accumulation of these polypeptides, and their level was maintained up to 200 μM Al (Fig. 3). Reductions in the levels of 25- and 29-kD polypeptides were also observed with Al exposure (Fig. 3). In the Al-resistant cv Maringa, polypeptide bands corresponding to 12, 23, and 43.5 kD were induced under Al stress (Fig. 4). The 43.5-kD band started to appear at 50 μM Al and the 12- and 23-kD bands started to appear at 75 μM Al. These polypeptides were near their highest level at 100 μM Al (Fig. 4). Lesser amounts of the 24- and 48-kD polypeptides in Maringa were also observed with exposure to Al (Fig. 4). In the Al-sensitive cv Roblin, accumulation of a 45-kD band was reduced by 150 μM Al and bands at 80 and 58 kD were accumulated with Al exposure (Fig. 5A). In Katepwa, 100 μM and higher Al concentrations resulted in a marked reduction of a 31-kD polypeptide (Fig. 5B).

In all cultivars, the total amount of REPs accumulating in the growth media increased approximately 2-fold after 48 to 72 h of growth and continued to increase until at least 96 h. In both Al-resistant cultivars, Atlas 66 and Maringa, accumulation of REPs was similar in the presence and absence of Al. However, in both sensitive cultivars, accumulation of REPs was reduced after 96 to 120 h of Al treatment (Fig. 6). These changes were consistent with the time course of root growth, which indicated almost no effect in Atlas 66 and Maringa, whereas root elongation was inhibited in Roblin and Katepwa after 72 h of exposure to Al (Fig. 7). Patterns of root elongation showed some unusual behavior. All culti-

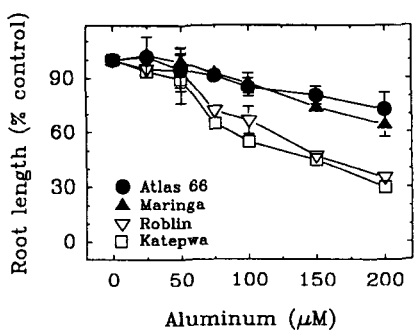

Figure 1. The effect of varying concentrations of Al on root elongation in *T. aestivum*. Seedlings of four cultivars, Atlas 66, Maringa (Al-resistant), Roblin, and Katepwa (Al-sensitive), were grown for 48 h on agar plates, 24 h in nutrient solution without added Al, and another 120 h in nutrient solutions containing 0 to 200 μM Al (pH 4.5). Values represent the mean (±SE) of triplicate samples. Root length in the absence of added Al was 11.3 ± 1.7, 11.9 ± 0.6, 9.2 ± 1.4, and 9.1 ± 1.0 cm for Atlas 66, Maringa, Roblin, and Katepwa, respectively.

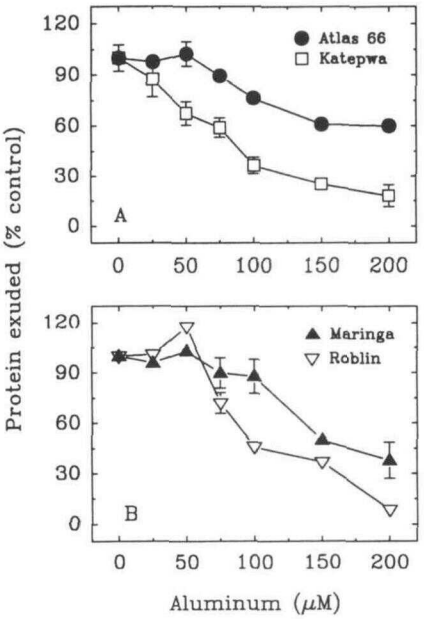

Figure 2. The effect of Al on exudation of polypeptides from roots of Atlas 66 and Katepwa (A) and Maringa and Roblin (B). Plants were exposed to concentrations of Al ranging from 0 to 200 μM for 120 h. Results presented in A and B represent data from two independent experiments. Values represent the mean (±SE) of triplicate samples. Protein exuded in the absence of added Al was 23 ± 0.9, 14.7 ± 0.26, 15.2 ± 0.1, and 26 ± 2 μg mL^{-1} for Atlas 66, Maringa, Roblin, and Katepwa, respectively.

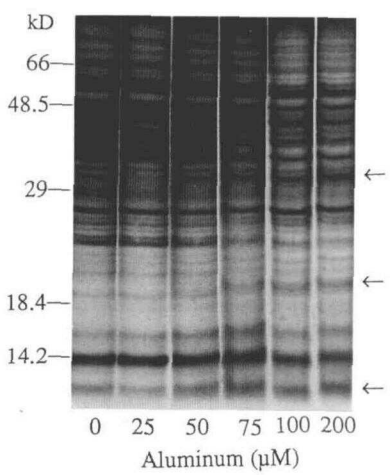

Figure 3. Polypeptide profiles showing the effect of varying concentrations of Al on induction of 12-, 22-, and 33-kD polypeptides in Atlas 66. Plants were grown under aseptic conditions for 120 h in nutrient solution containing 0 to 200 μM Al. Root exudates were collected, and 10 μg of protein was loaded onto each lane. Gels were silver stained. Results presented here are representative of three replicate samples, which were prepared and analyzed independently. Arrows indicate Al-induced polypeptides.

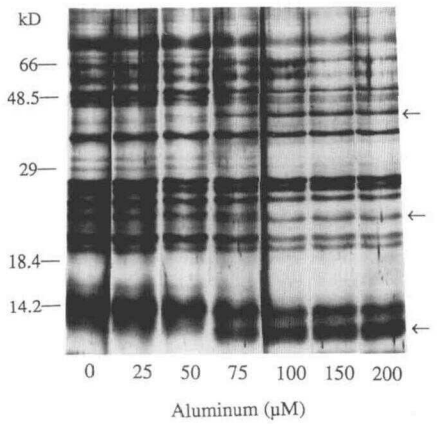

Figure 4. Polypeptide profiles showing the effect of varying concentrations of Al on induction of 12-, 23-, and 43.5-kD polypeptides in Maringa. Plants were grown under aseptic conditions for 120 h in nutrient solution containing 0 to 200 μM Al. Root exudates were collected, and 10 μg of protein was loaded onto each lane. Gels were silver stained. Results presented here are representative of three replicate samples, which were prepared and analyzed independently. Arrows indicate Al-induced polypeptides.

vars showed a small but perceptible lag in growth after plants were placed in culture vessels, and growth leveled off in all cultivars after 96 h. An Al-induced inhibition in root growth of Al-sensitive cultivars was apparent after 72 h. We believe that this unusual behavior reflects pretreatment procedures required to ensure sterility and a carbon limitation in sealed culture vessels. When plants were grown under nonsterile conditions in Magenta vessels with a small opening to the atmosphere, Al-induced inhibition in growth was observed in less than 24 h and the initial lag and subsequent leveling off of growth were not observed (data not shown). It is worth emphasizing that the Al-induced polypeptides observed in Atlas 66 (Fig. 8) and Maringa (Fig. 9) were almost at their highest level by 72 h; thus, changes in polypeptide profiles were observed prior to the Al-induced inhibition and the subsequent leveling of growth.

To determine if REPs were interacting with Al, root exudates from plants treated with Al were separated into two major fractions by ultrafiltration. The Al content of both fractions was analyzed by GFAAS. HMM fractions from the Al-resistant cvs Atlas 66 and Maringa showed 1.7- to 2.3-fold higher concentrations of Al than the same fractions from Al-sensitive cultivars (Table I). Dialysis of the HMM fractions released little or no additional Al (Table I). Much of this Al appeared to be specifically associated with REPs. Treatment with protease released 73 and 69% of the Al associated with the HMM fractions from Atlas 66 and Maringa, and 63 and 62% of the Al in Roblin and Katepwa (Table I). As a plant-free control, nutrient solutions containing 100 μM Al and 20 μg mL^{-1} BSA (a concentration based on polypeptide levels found in root exudates) were incubated for 24 h and concentrated by ultrafiltration. Only small amounts of Al were associated with BSA, and protease was less effective in releasing this bound Al. Furthermore, the concentration of Al in the ultrafiltrates isolated from solutions spiked with BSA

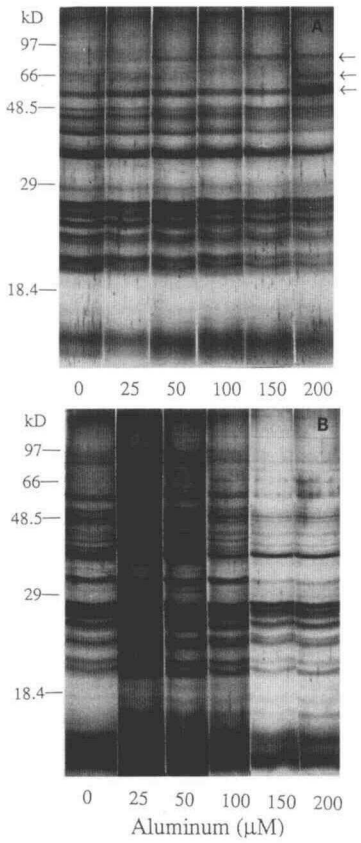

Figure 5. Polypeptide profiles showing the effect of varying concentrations of Al on polypeptide profiles in Roblin (A) and Katepwa (B). Plants were grown under aseptic conditions for 120 h in nutrient solution containing 0 to 200 μM Al. Root exudates were collected, and 10 μg of protein was loaded onto each lane. Gels were silver stained. Results presented here are representative of three replicate samples, which were prepared and analyzed independently. Arrows indicate Al-induced polypeptides.

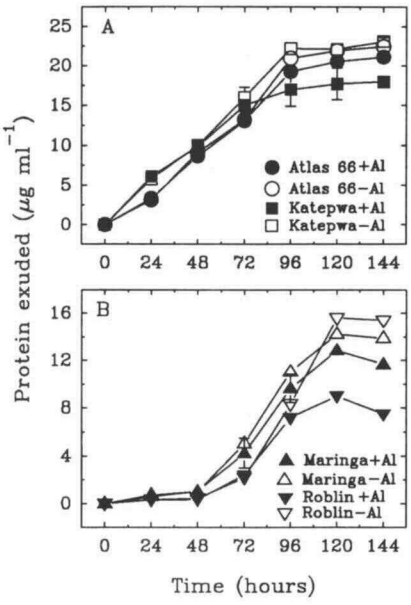

Figure 6. The time course of exudation of polypeptides from roots of Atlas 66 and Katepwa (A) and Maringa and Roblin (B). Plants were grown in the presence (100 μM) and absence of Al, and root exudates were collected after 0, 24, 48, 72, 96, 120, and 144 h. Results presented in A and B represent data from two independent experiments. Values represent the mean (±SE) of triplicate samples.

was more than 1 order of magnitude higher than the concentration of Al in ultrafiltrates of root exudates (Table I).

When HMM fractions from root exudates of plants grown in 0 to 200 μM Al were analyzed for the presence of Al, saturation of Al binding was observed at 50 μM Al in Katepwa and at 100 μM Al in Roblin. Saturation did not occur in the Al-resistant cvs Atlas 66 and Maringa, even at 200 μM Al (Fig. 10A). Aluminum measured in the ultrafiltrate fractions of all cultivars increased with increasing Al supplied to the plants (Fig. 10B).

The potent Al-binding capacity of exudate materials observed in this study could reflect either Al-induced polypeptides or the normal suite of polypeptides that are exuded under control conditions. To determine the interaction of Al with constitutive polypeptides, plants were grown in the absence of Al, root exudates were collected, and HMM fractions were dialyzed against 100 μM Al for 24 h at 4°C. They were further dialyzed against water for 24 h to remove any unassociated Al. The amount of Al associated with the HMM fractions was again greater in the Al-resistant cvs Atlas 66 (0.26 ± 0.08 ng Al μg^{-1} protein) and Maringa (0.19 ± 0.04

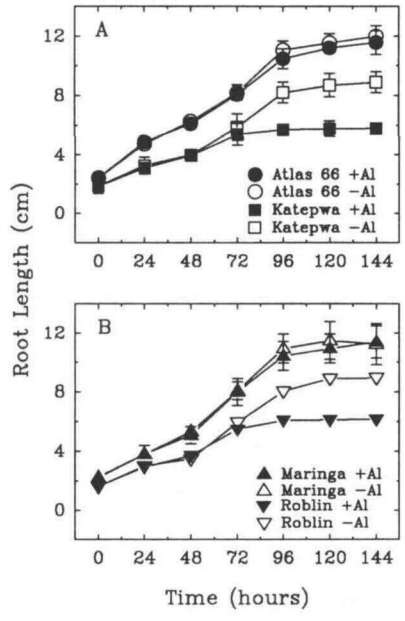

Figure 7. Increase in root length with time in Atlas 66 and Katepwa (A) and Maringa and Roblin (B). Plants were grown under aseptic conditions in the presence (100 μM) and absence of Al, and root lengths were measured after 0, 24, 48, 72, 96, 120, and 144 h. Results presented in A and B represent data from two independent experiments. Values represent the mean (±SE) of triplicate samples.

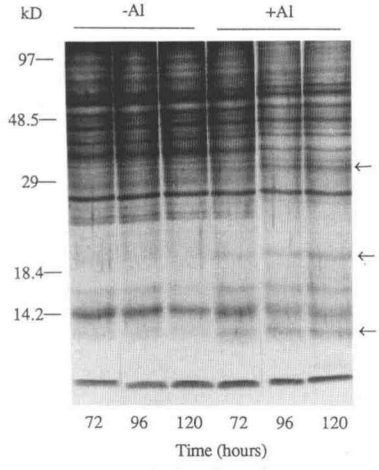

Figure 8. The time course of Al-induced appearance of polypeptides in root exudates of Atlas 66. Plants were grown under aseptic conditions in the presence (100 μM) and absence of Al, and root exudates were collected after 0, 24, 48, 72, 96, 120, and 144 h. Root exudates were concentrated, and 10 μg of protein was loaded onto each lane. The amount of protein collected from plants after 24 and 48 h of growth in Al was insufficient for resolution by SDS-PAGE. Results presented here are representative of three replicate samples, which were prepared and analyzed independently. Arrows indicate Al-induced polypeptides.

ng Al μg^{-1} protein) than in the Al-sensitive cvs Roblin (0.13 $\pm$ 0.05 ng Al μg^{-1} protein) and Katepwa (0.10 $\pm$ 0.03 ng Al μg^{-1} protein). The capacity for binding Al, however, was less than half of that of plants grown in the presence of Al (0.77 $\pm$ 0.09, 0.58 $\pm$ 0.07, 0.35 $\pm$ 0.02, and 0.34 $\pm$ 0.04 ng Al μg^{-1} protein in Atlas 66, Maringa, Roblin, and Katepwa, respectively).

DISCUSSION

Of the different mechanisms suggested to explain Al resistance in plants, an important first line of defense could be chelation of Al in the rhizosphere (Taylor, 1988, 1991). If this mechanism is to be effective, plants must be capable of synthesizing chelator ligands and exporting them to the apoplasm, where they must be able to form stable complexes with Al under physiological conditions. We have explored this possibility by collecting and analyzing root exudates from Al-resistant and Al-sensitive cultivars of *T. aestivum*. To date, the effect of Al on exudation of polypeptides into the apoplasm/rhizosphere and the possible role that REPs might play in resistance to Al have not been studied. Synthesis of specific Al-binding polypeptides in response to Al stress has been suggested (Aniol, 1984; Ownby and Hruschka, 1991), but it has been postulated that these Al-induced polypeptides would function in the cytosol in a manner analogous to other low molecular mass metal-binding peptides such as the phytochelatins. None of the phytochelatins have been shown to be induced by Al (Steffens, 1990); thus, unique Al-binding polypeptides would have to be postulated. REPs might complement such putative cytosolic Al-binding polypeptides by chelating soluble Al around the roots, limiting its entry into the symplasm, and thereby improving plant growth on Al-toxic soils.

The results reported here suggest that exudation of REPs in Al-resistant and Al-sensitive cultivars is affected by Al stress. Furthermore, it appears possible that REPs may play a role in plant response to Al. This conclusion is based on four observations. (a) Aluminum inhibited exudation of REPs in sensitive cultivars, but no effect was observed in resistant cultivars up to 100 μM Al. Inhibition of exudation of REPs was paralleled by reductions in root elongation in Al-sensitive cultivars. (b) Although Al had no effect on the total amount of REPs exuded by Al-resistant cultivars, SDS-PAGE analysis revealed a dose- and time-dependent accumulation of specific polypeptides upon Al exposure, including 12-, 22-, and 33-kD polypeptides in Atlas 66 and 12-, 23-, and 43.5-kD polypeptides in Maringa. (c) Measurement of Al in HMM fractions obtained after ultrafiltration of root exudates showed more Al associated with HMM exudates from Atlas 66 and Maringa than from the Al-sensitive cultivars Roblin and Katepwa. HMM exudates released from roots of the Al-resistant cultivars under control conditions (without Al) also showed a greater Al-binding capacity than exudates from Al-sensitive cultivars. (d) Digestion with protease released approximately 70% of the Al from the HMM fractions of the Al-resistant cvs Atlas 66 and Maringa, and 62% from HMM fractions of the Al-sensitive cvs Roblin and Katepwa.

There is evidence that formation of Al complexes with acidic polypeptides may result in detoxification of Al. Putterill and Gardner (1988) found that acidic polypeptides, such as poly-L-Asp and poly-L-Glu, could bind Al at very low concentrations (micromolar), resulting in an amelioration of the toxic effects of Al on calmodulin-mediated processes. These polypeptides also had a protective effect on the growth of

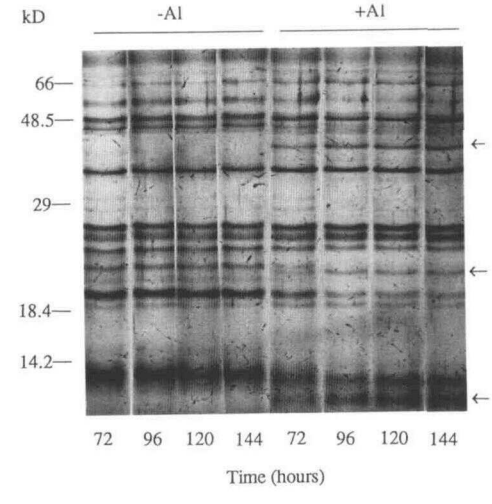

Figure 9. The time course of Al-induced appearance of polypeptides in root exudates of Maringa. Plants were grown under aseptic conditions in the presence (100 μM) and absence of Al, and root exudates were collected after 0, 24, 48, 72, 96, 120, and 144 h. Root exudates were collected and concentrated, and 10 μg of protein was loaded onto each lane. The amount of protein collected from plants after 24 and 48 h of growth in Al was insufficient for resolution by SDS-PAGE. Results presented here are representative of three replicate samples, which were prepared and analyzed independently. Arrows indicate Al-induced polypeptides.

pollen tubes in the presence of Al (Konishi et al., 1988). From our study it appeared that Al was associated both with a protease-sensitive (constitutive and induced proteins) fraction and a protease-resistant fraction. The total amount of Al released from Al-resistant cultivars after protease digestion was approximately twice the amount of Al released from Al-sensitive cultivars, indicating that differential binding was resulting largely from polypeptides in the exudates. This capacity to bind Al is not a property of polypeptides in general. Only a small amount of Al was associated with BSA, and treatment with protease released about 30% of this bound Al. Cultivars also differed in the amounts of Al that remained associated with the HMM fractions after protease treatment. This strongly bound Al could reflect incomplete digestion of polypeptides or the interaction of Al with other HMM exudates such as carbohydrates and lipids. It is interesting to note that Al associated with the HMM fraction (both the residual Al and that released by proteolysis) was not removed by repeated dialysis against water.

HMM fractions from exudates of plants grown in the absence of Al also showed the capacity to interact with Al in vitro. Fractions from the Al-resistant cvs Atlas 66 and Maringa bound more Al than fractions from the Al-sensitive cvs Roblin and Katepwa. This indicates a possible involvement of the constitutive polypeptides in Al chelation as well. It is important to note, however, that in vitro association of Al with the HMM fraction from plants grown in the absence of Al was less than half that of in vivo accumulation by plants grown in the presence of Al.

In looking at the possibility that exudation of polypeptides plays a role in resistance, several concerns arise. First, if differences in resistance to Al are normally observed within hours, we would expect to see increases in specific REPs over the same time interval. In this study, growth of plants in sealed culture vessels affected patterns of root growth such that an Al-induced reduction in root growth was observed 72 h after Al exposure. We were able to detect Al-induced or Al-enhanced REPs in Al-resistant cultivars after 72 h of exposure to Al. Changes in synthesis and exudation of polypeptides may have occurred even more rapidly; however, our technique of collecting exudates from a bulk nutrient solution may not be sufficiently sensitive to detect such short-term responses.

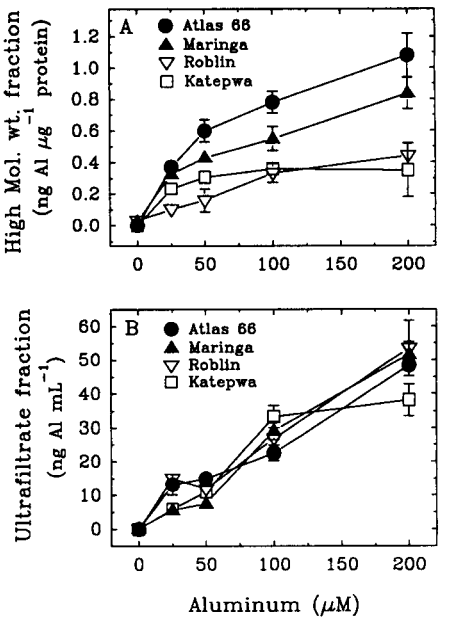

Figure 10. The effect of varying concentrations of Al in the growth medium on the association of Al with root exudates in Al-resistant (Atlas 66 and Maringa) and Al-sensitive (Roblin and Katepwa) cultivars. Plants were grown in the presence of varying concentrations of Al for 120 h. Root exudates were collected and separated by ultrafiltration. Al was estimated in both the HMM (A) and ultrafiltrate (B) fractions by GFAAS. Values represent the mean (±SE) of triplicate samples.

We might also predict that accumulation of REPs in bulk nutrient solution may not accurately reflect the high concentrations in the root apoplasm. A second concern about the possible role of REPs in mediating resistance to Al is the potential energetic cost involved in providing enough ligand to effectively reduce the activity of Al within the apoplasm

Table I. *Al associated with HMM and ultrafiltrate fractions of root exudates*

Root exudates from plants grown under aseptic conditions in the presence and absence of Al were separated by ultrafiltration. For dialysis treatments, HMM fractions were dialyzed for 24 h against several changes of water at 4°C. For protease treatments, 2 mg mL^{-1} protein was taken up in a digestion buffer, protease was added at a ratio of 1:5, and samples were incubated overnight at room temperature before dialysis as above. As a plant-free control, BSA at 20 μg mL^{-1} was added to the nutrient solution containing 100 μM Al, incubated for 24 h, and separated by ultrafiltration. Values represent the means (±SE) of triplicate samples.

Sample	HMM Fraction	HMM Fraction after Dialysis	HMM Fraction after Protease Treatment and Dialysis	Ultrafiltrate Fraction
		ng Al μg^{-1} protein		*ng Al mL^{-1}*
Atlas 66	0.77 ± 0.09	0.70 ± 0.10	0.21 ± 0.04	23.6 ± 3.7
Maringa	0.58 ± 0.07	0.58 ± 0.10	0.18 ± 0.02	27.7 ± 4.1
Roblin	0.35 ± 0.02	0.34 ± 0.06	0.13 ± 0.03	24.6 ± 3.2
Katepwa	0.34 ± 0.04	0.34 ± 0.07	0.13 ± 0.01	24.9 ± 1.4
BSA	0.07 ± 0.002	0.08 ± 0.005	0.05 ± 0.001	541 ± 40

and/or rhizosphere. This issue has been raised with respect to exudation of chelator ligands in general (Taylor, 1988). However, if Al resistance is viewed in a dynamic context, with root exudates localized primarily within the apoplasm, protecting the sensitive root tip (Ryan et al., 1993) for a short period of time before maturation occurs, the energetic cost of exudation need not be prohibitive. This view of resistance also allows us to reconcile the relatively small quantity of polypeptides that are exuded from roots of resistant plants. In this study the quantity of polypeptides released into the growth medium (polypeptides present within the apoplasm were not measured directly) and the Al-binding capacity of exuded polypeptides were sufficient to bind approximately 0.6% of total Al. If polypeptides are exuded primarily at the root tip, localized within the apoplasm, and required only to protect cells during early stages of maturation, such levels may be physiologically relevant.

Another concern arises from the possibility that specific Al-induced polypeptides could arise as a result of proteolytic degradation or polypeptides leaching out from cells due to an Al-induced loss of membrane integrity as a result of Al poisoning rather than de novo synthesis and export. Zhao et al. (1987) suggested that Al may induce leakiness by affecting either membrane lipids or membrane carriers. However, if this were the case, treatment with Al would be expected to affect REP profiles in Al-sensitive cultivars more dramatically than in Al-resistant cultivars. Because changes in REP profiles were observed in Al-resistant cultivars at concentrations of Al that were not toxic to Al-resistant plants, we are inclined to believe that these polypeptides reflect specific changes in synthesis or degradation of polypeptides. To determine if our Al-induced REPs are newly synthesized in response to Al stress, pulse-labeling experiments using [^{35}S]Met are currently underway. We are also looking at these polypeptides in segregating populations arising from crosses between the Al-resistant cv Maringa and the Al-sensitive cv Katepwa. Although our data suggest that REPs may have a strong Al-binding capacity, further studies showing specific binding of Al to purified polypeptides will be needed to conclusively demonstrate the role of REPs in Al chelation and to demonstrate a specific physiological function.

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