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Aluminum-induced deposition of (1, 3)-β-glucans (callose) in *Triticum aestivum* L.

Kymberly A. Schreiner[1], John Hoddinott and Gregory J. Taylor
Department of Botany, University of Alberta, Edmonton, Alberta, T6G 2E9, Canada. [1]*Present address: Department of Natural Resources, Northern Forestry Centre, Edmonton, Alberta, Canada*

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Abstract

Aluminum (Al)-induced damage to leaves and roots of two Al-resistant (cv. Atlas 66, experimental line PT741) and two Al-sensitive (cv. Scout 66, cv. Katepwa) lines of *Triticum aestivum* L. was estimated using the deposition of (1, 3)-β-glucans (callose) as a marker for injury. Two-day-old seedlings were grown for forty hours in nutrient solutions with or without added Al, and callose deposition was quantified by spectrofluorometry (0–1000 μ*M* Al) and localized by fluorescence microscopy (0 and 400 μ*M* Al). Results suggested that Al caused little damage to leaves. No callose was observed in leaves with up to 400 μ*M* Al treatment. In contrast, root callose concentration increased with Al treatment, especially in the Al-sensitive lines. At 400 μ*M* Al, root callose concentration of Al-sensitive Scout 66 was nearly four-fold that of Al-resistant Atlas 66. After Al treatment, large callose deposits were observed in the root cap, epidermis and outer cortex of root tips of Scout 66, but not Atlas 66. The identity of callose was confirmed by a reduced fluorescence in Al-treated roots: firstly, after adding an inhibitor of callose synthesis (2-deoxy-D-glucose) to the nutrient solution, and secondly, after incubating root sections with the callose-degrading enzyme β-D-glucoside glucohydrolase [EC 3.2.1.21]. Root callose deposition may be a good marker for Al-induced injury due to its early detection by spectrofluorometry and its close association with stress perception.

Abbreviations: DDG-2-deoxy-D-glucose, PAS – periodic acid - Schiffs reagent, PE – pachyman equivalents

Introduction

Soil acidity is a major growth-limiting factor in crop production worldwide, and yield losses are frequently attributed to aluminum (Al) toxicity (Foy, 1983). Such yield losses would be reduced if Al-resistant crops were developed. Selection for resistance to Al is facilitated by screening plants using an early stress response such as callose deposition as a physiological marker for Al injury (Wissemeier et al., 1987). Callose consists primarily of (1, 3) -β-glucans that are deposited extracellularly after a stress causes the localized influx of calcium ions (Kauss, 1989) and the release of vacuolar β-furfuryl-β-glucoside, which both induce the membrane-bound callose synthase (Ohana et al., 1992). Due to the sensitivity of callose synthase to disturbances at the plasma membrane, callose has been studied in response to a variety of biological, physical, and chemical stresses (see Schreiner, 1992; Stone, 1984). Of particular relevance to the present study, callose deposition has been correlated with Al treatment in the roots of *Glycine max* (Wissemeier et al., 1987) and *Picea abies* (Jorns et al., 1991), and with Al-resistance in the mesophyll protoplasts of *Avena sativa* and *Triticum aestivum*, but not *Hordeum vulgare* (Schaeffer and Walton, 1990).

The purpose of this study was to determine whether wheat plants of different genotypes respond similarly to Al in different parts of the plant. To address this question, we examined the concentration and location of Al-induced callose deposits in the leaves and roots of Al-resistant and Al-sensitive wheat seedlings. We found that the roots were the primary site of Al toxicity symptoms, with increased callose deposition occur-

ring with Al treatment, especially in the Al-sensitive cultivars. These results suggest that root callose concentration may be useful in screening Al resistance in wheat.

Materials and methods

Seeds of *Triticum aestivum* L. were surface-sterilized in 1.2% sodium hypochlorite for 20 minutes and germinated overnight in an aerated solution of 0.005 g L^{-1} Vitavax fungicide (Uniroyal Chemical). Seeds were grown on polyethylene mesh held at the surface of 10 L of an aerated nutrient solution containing (m*M*): NO_3^--N (3.30), NH_4^+-N (0.30), P(0.10), K(0.800), Ca(1.00), Mg(0.30), S(0.10), and (μM) Cl(34.0), Na(20.2), Fe(10.0), EDTA(10.0), B(6.0), Mn(2.0), Cu(34.0), Zn(0.5), and Mo(0.1) at pH 4.50. Plants were grown in a controlled environment chamber at 24°C and 16 hours of light at an irradiance of 315 μmol m^{-2} s^{-1} photosynthetically active radiation at plant base level. After 1 day of growth, 9 uniform seedlings were transferred to 600-mL glass beakers and grown for 40 hours on discs of polyethylene mesh buoyed by strips of Styrofoam on aerated nutrient solution. The nutrient solution was treated with various levels of Al ($AlK(SO_4)_2$ $12H_2O$) and the callose synthesis inhibitor, 2-deoxy-D-glucose (DDG, adjusted to pH 4.5), as described in the following experiments.

Experimental design and harvest procedures

For each of the following experiments, uniform seedlings (5–8 per beaker; three beakers per treatment) were selected for observations on mean leaf length (seed emergence to the tip of the longest blade), mean maximum root length (seed emergence to the tip of the longest seminal root), quantification of leaf and/or root callose deposits, and localization of leaf and/or root callose deposits. Each experiment was replicated 3 times, with complete randomization of treatments. Replicates of Experiment 2 (genotype response to Al) were staggered by a day to ensure that harvests occurred within 24 hours and that growth conditions were similar for the duration of the experiment.

All data were analyzed by the General Linear Model and Analysis of Variance procedures available on SAS version 6.06 (SAS 1989). Primary growth data were analyzed without transformation, while callose concentration data were log transformed to achieve homogeneity of variance. Statistical significance was defined at the 0.05 probability level.

Experiment 1: Callose concentration of leaves and roots after Al treatment

Seedlings of Al-resistant Atlas 66 and Al-sensitive Scout 66 were treated with 3 levels of Al (0, 50, and 400 μM) for 40 hours. Plants from a total of 18 beakers were used to determine plant growth responses to Al and to quantify callose deposits in the leaves and roots.

Experiment 2: Root callose concentration of four genotypes after Al treatment

Seedlings of Al-resistant cultivar Atlas 66, Al-resistant experimental line PT741, and Al-sensitive cultivars Scout 66 and Katepwa were treated with 17 levels of Al (0, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 150, 200, 300, 400, 700 and 1000 μM) for 40 hours. Plants from a total of 204 beakers were used to determine plant growth responses to Al and to quantify root callose deposits.

Experiment 3: Inhibition of callose deposition

Seedlings of Al-sensitive Scout 66 were treated with 2 levels of Al (0 and 400 μM) and 4 levels of DDG (0, 10, 50 and 100 μM) for 40 hours. Plants from a total of 24 beakers were used to determine plant growth responses to Al and to quantify root callose deposits.

Experiment 4: Localization of leaf and root callose deposits after Al treatment

Seedlings of Al-resistant Atlas 66 and Al-sensitive Scout 66 were treated with 2 levels of Al (0 and 400 μM), and 2 levels of DDG (0 and 50 μM) for 40 hours. Leaves and roots of plants from a total of 24 beakers were prepared for observation under fluorescence microscopy.

Experiment 5: Localization of root callose deposits after enzyme digestion

Seedlings of Al-resistant Atlas 66 and Al-sensitive Scout 66 were treated with 2 levels of Al (0 and 400 μM) for 40 hours. The roots of plants from a total of 6 beakers were prepared for observation under fluorescence microscopy. Prior to staining with periodic acid - Schiff's reagent (PAS), sections were incubated for

10 hours at 35°C with either 14 unit·mg^{-1} of the callose degrading enzyme β-D-glucoside glucohydrolase ([EC 3.2.1.21]; Sigma Chemical Co.) in acetate buffer (20 m*M*, pH 4.8–5.0), or with buffer alone, and rinsed for 5 minutes in distilled water.

Callose quantification by spectrofluorometry

Approximately 110 mg [fresh-weight) of leaf tissue or 85 mg of root tissue were excised and soaked for 60 minutes in 2 mL of 95% ethanol, containing 100 μM of the callose synthesis inhibitor, 2-deoxy-D-glucose (DDG), to remove soluble autofluorescent material (Kauss, 1989) and reduce callose deposition during the soak (Jaffe and Leopold, 1984). Tissues were homogenized and the callose was extracted in hot 1 *M* NaOH, as described by Bonhoff and Grisebach (1987). Using twice the volume of sample and reagents as described by Kauss (1989), callose was measured fluorometrically with aniline blue (water soluble, C.I. 42755, lot # 60894; PolySciences) using a Perkin-Elmer spectrofluorometer (excitation 398 nm, emission 495 nm) against a calibration curve of a freshly-prepared solution of the (1,3)-β-glucan pachyman from *Poria coccus* (lot # 902569, Calbiochem) in 1 *M* NaOH. Callose concentration was expressed as pachyman equivalents (PE) per tissue fresh weight (mg PE·g$^{-1}_{fw}$).

Callose localization by fluorescence microscopy

The mid 10-mm leaf segment and the distal 5-mm root tip of each plant were excised and fixed by freeze substitution. Tissues were immersed in 12% methylcyclohexane in 2-methyl butane cooled by liquid nitrogen, placed in an anhydrous solution of 1.3% acrolein in acetone over molecular sieves (type 13X, Sigma Chemical Co.), and cooled over dry ice for 13 days, as described by Hughes and Gunning (1980). Tissues were embedded in glycol methacrylate (GMA) in size 00 gelatin capsules and polymerized by irradiation from a long wavelength (> 315 nm) ultraviolet light source (Osram Ultra Vitalux lamp 300 W, 10-cm distance) for 15–20 hours at 10°C in the dark, as described by Brander and Wattendorff (1989). Tissues were sectioned on a Reichert-Jung Ultracut microtome at 1 μm with glass knives on a 10% acetone-water bath, and mounted on gelatin-coated slides.

Mounted sections were soaked in 2,4-dinitrophenylhydrazine (Calbiochem) in 15% (v/v) acetic acid in distilled water for 10 minutes, and rinsed in distilled water for 10 minutes, as described by Feder and O'Brien (1968). Sections were pre-stained with PAS and stained for 30 minutes with 0.003% (w/v) Sirofluor (sodium 4,4'[carbonylbis(benzene-4,1-diyl)bis(imino)]bisbenzene sulfonate; Biosupplies, Parkville, Victoria, Australia) in 0.067 *M* potassium phosphate buffer (pH 8. 5). Callose deposits were viewed by UV epifluorescence using a Zeiss fluorescence photomicroscope with a mercury lamp (G365 excitation filter and no barrier filters).

Results

Symptoms of Al toxicity were not observed in the leaves of seedlings. Leaf length measurements of Al-treated plants were variable, with no clear treatment response detected. In contrast, the roots of Al-treated seedlings exhibited typical symptoms of Al toxicity including stunting, brittleness, and browning of the root tips. Symptoms, especially reduced root length, were more prominent in the Al-sensitive cultivars Scout 66 and Katepwa than in the Al-resistant cultivar and line (Fig. 1A). Root length was significantly reduced by Al treatments as low as 30 μM in Scout 66, (23% less than control), 100 μM in Katepwa (26%), 700 μM in Atlas 66 (30%), and 1000 μM in in PT741 (25%). There was a significant interaction effect between Al and cultivar. Maximum reduction of root length occurred at 1000 μM Al with a loss of 60% in Scout 66, 60% in Katepwa, 40% in Atlas 66, and 25% in PT741.

Callose quantification

Plant tissues did not interfere with fluorescence, as a mixture of root tissue homogenate and pachyman standard had a 105% (± 5% standard error) recovery of fluorescence. Leaf callose was at the detection limit of the spectrofluorometer; callose deposits were not detected in the leaves of Scout 66 or Atlas 66, with or without Al treatment (Table 1). In contrast, root callose concentration increased after Al treatment in all cultivars tested (Tables 1 and 2; Fig. 1B), especially in the Al-sensitive cultivars Scout 66 and Katepwa. In Experiment 1, root callose concentration increased by nearly 140% in Atlas 66, and 730% in Scout 66 after 400 μM (Table 1). No interaction was detected between Al and wheat cultivar for callose concentration (Table 1), although a significant interaction effect was detected in Experiment 2 when more cultivars and a wider range of Al treatments were examined (Fig. 1B). In

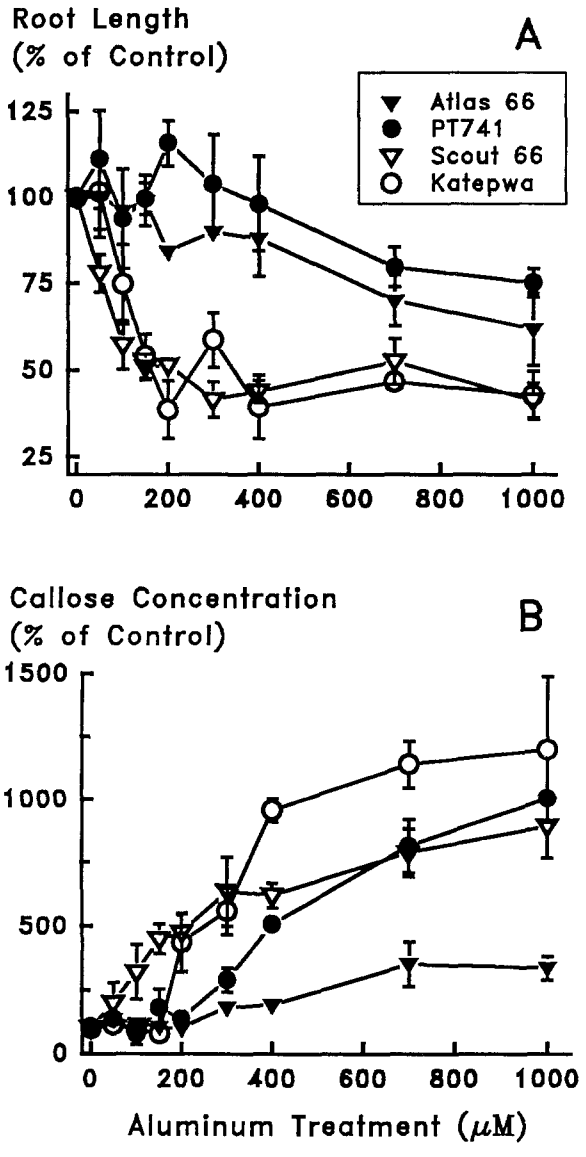

Fig. 1. Dose responses of root length (**A**) and root callose concentration (**B**) of four wheat genotypes to Al treatment. Two-day-old plants were transferred to nutrient solutions containing Al (0–1000 μM) for 40 hours. Roots were homogenized and callose deposits quantified by spectrofluorometer with a pachyman equivalent (PE) standard curve. Data are averages of 3 replicates, pooling of 5–8 plants per replicate, ± the standard error of the mean.

Experiment 2, roots of Scout 66 and Katepwa were more sensitive to Al treatment than the Al-resistant cultivars (Fig. 1B). Root callose concentration was significantly increased by Al treatments as low as 70 μM in Scout 66 (160% increase over control), 200 μM in Katepwa (340%), 300 μM in PT741 (190%) and 400 μM in Atlas 66 (90%). This ranking of plants by Al sensitivity was similar to that of the root length study, although on average, nearly three times more Al was required for the Al-sensitive cultivars to induce a significant change in root callose concentration than in root length. The Al-resistant line PT741 was more sensitive in the callose deposition study. Maximum root callose concentration occurred at 1000 μM Al with an increase of 1100% in Katepwa, 900% in Scout 66, 900% in PT741, and 240% in Atlas 66.

Table 2 summarizes the results of Experiment 3, which examined the effect of an inhibitor of callose synthesis on root callose concentration. In the absence of Al, high concentrations of DDG damaged seedlings: leaves were slightly chlorotic, roots were stunted, brittle, and had dark-brown tips. Increasing concentrations of DDG generally caused a corresponding decrease in leaf length, root length, and root callose concentration. Treatment with 10 μM DDG had no significant effect on leaf length and root length, whereas root callose concentration declined by 63% in the absence of Al, and by 46% in the presence of Al. Maximum inhibition of callose deposition (80%) was observed in roots treated with 100 μM DDG and Al, however this DDG treatment also reduced leaf length by 34% while not significantly affecting root length. A significant interaction effect was detected between Al and DDG for root length, leaf length, and root callose concentration.

Callose localization

Autofluorescence by the cell walls and embedding medium was low, and not noticeably affected by prestaining with PAS. In Experiment 4, callose was not observed in the leaves of either Atlas 66 or Scout 66, with or without Al treatment. Furthermore, few deposits were observed in the roots of either cultivar without Al treatment, or of Atlas 66 with Al treatment. Micrographs of tissues without callose deposits were dark and were therefore not included. In contrast, large deposits of callose were observed at the root tips of Al-sensitive Scout 66 after treatment with 400 μM Al (Fig. 2A); fewer deposits were observed in Scout 66 roots after treatment with Al and the callose inhibitor, DDG (Fig. 2B), and almost no deposits were observed in Experiment 5 after embedded sections of Al-treated roots were incubated with callose hydrolase (Fig. 2C). Incubating root sections with a buffer lacking callose hydrolase did not affect callose fluorescence. In general, callose was localized at the root cap, outer cortex (Fig. 2D), and at the pit fields, where it was particular-

Table 1. Effect of Al treatment on leaf and root callose concentration in Al-resistant Atlas 66 and Al-sensitive Scout 66

Al level (μM)	Callose concentration (mg PE·g_{gw}^{-1})			
	Leaf		Root	
	Atlas 66	Scout 66	Atlas 66	Scout 66
0	0.01 ± 0.006 a	0.01 ± 0.001 a	0.16 ± 0.017 b	0.18 ± 0.003 c
50	0.02 ± 0.002 a	0.01 ± 0.002 a	0.15 ± 0.030 b	0.28 ± 0.011 b
400	0.02 ± 0.002 a	0.01 ± 0.002 a	0.38 ± 0.029 a	1.48 ± 0.012 a

Values are means of 3 replicates, pooling 5-8 plants per replicate, ± the standard error of the mean. Values in a column followed by the same letter are not significantly different at the 5% level according to Duncan's multiple range test performed on log transformed data.

Table 2. Effects of aluminum and the callose inhibitor, 2-deoxy-D-glucose, on leaf length, root length, and root callose concentration in Al-sensitive Scout 66

DDG (μM)	Leaf length (mm)	Root length (mm)	Root callose (mg PE·g_{fw}^{-1})
Aluminum treatment - 0 μM			
0	42 ± 1.4 a	30 ± 1.4 a	0.16 ± 0.010 c
10	39 ± 1.9 a	27 ± 0.5 a	0.06 ± 0.016 d
50	26 ± 0.3 bc	13 ± 0.7 b	0.07 ± 0.006 d
100	22 ± 0.7 cd	11 ± 0.4 bc	0.07 ± 0.003 d
Aluminum treatment - 400 μM			
0	29 ± 1.8 b	13 ± 0.6 b	1.19 ± 0.064 a
10	27 ± 1.9 bc	13 ± 0.8 bc	0.64 ± 0.010 b
50	24 ± 2.7 cd	11 ± 0.2 c	0.43 ± 0.008 b
100	19 ± 1.7 d	11 ± 0.5 bc	0.24 ± 0.067 c

Values are means of 3 measurements, pooling of 5–8 plants per replicate, ± the standard error of the mean. Values in a column followed by the same letter are not significantly different at the 5% level according to Duncan's multiple range test performed on untransformed leaf length and root length, and log transformed root callose concentration.

ily concentrated (arrows, Fig. 2E). Few deposits were observed at the root meristem.

Discussion

Few callose deposits were detected or observed in the leaves of Al-resistant and Al- sensitive wheat seedlings after Al treatment. Nevertheless, leaf cells appear to be capable of synthesizing callose as deposits were observed in wheat mesophyll protoplasts after Al treatment (Schaeffer and Walton, 1990). This suggests that little direct injury occurs in leaves as a result of exposure of whole plants to Al, possibly because toxic species of Al do not reach leaves in sufficient quantity to induce injury. In contrast to leaves, roots were the primary site of Al injury. Aluminum treatment was associated with decreased root length and increased root callose deposition, especially in the Al-sensitive cultivars. Both parameters ranked cultivars for Al resistance similarly to published reports (Briggs et al., 1989), although root length was a more sensitive indicator than root callose concentration. Compared to Al-free controls, root length in the two Al-sensitive cultivars decreased by 23% after treatment with only 50 μM Al, whereas root callose concentration increased

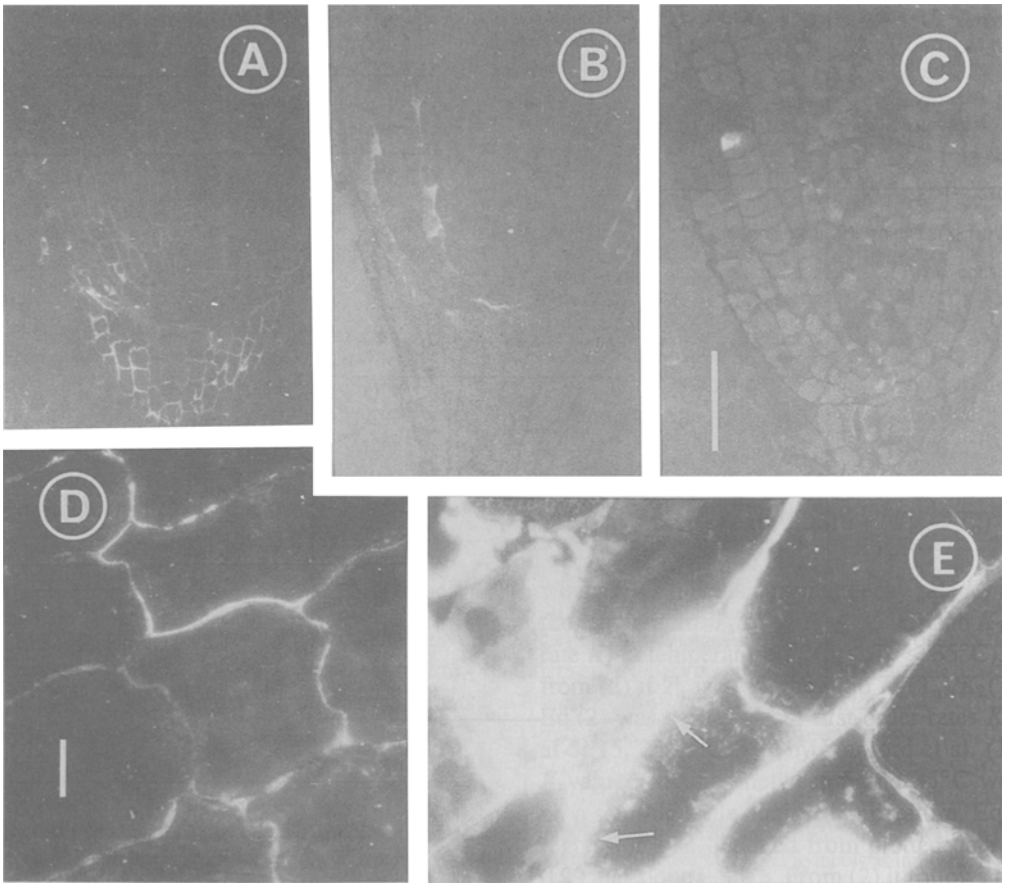

Fig. 2. Epifluorescence micrographs of longitudinal sections of root-tips of the Al-sensitive wheat cultivar, Scout 66. Two-day-old plants were transferred to nutrient solutions containing 400 μM Al and two levels of the callose synthesis inhibitor, 2-deoxy-D-glucose (DDG, 0 and 50 μM) for 40 hours. Roots were freeze-substituted, embedded, and sectioned at 1-μM. Some sections were incubated with β-D-glucoside glucohydrolase ([EC 3.2.1.21], 14 unit·mg^{-1}) for 10 hours at 35 °C, before staining with Sirofluor. Fluorescing callose deposits at root periphery and cortical cells (**A**); Reduced fluorescence after adding DDG to nutrient solution (**B**); No detectable fluorescence after incubating root sections with the callose-degrading enzyme (**C**); Callose deposits along cortical cell walls (**D**); Callose deposits at pit fields (arrows, **E**). *Bar* = 100 μm (A–C) or 10 μm (D–E).

by 250% after treatment with 150 μM Al. Differences in the rank and Al sensitivity of the four genotypes between the root length and root callose deposition studies may be due to the presence of several species of Al. Similar results could occur if plants were grown in a nutrient solution containing a rhizotoxic Al species of high activity and a callose-inducing Al species of low activity. It is also possible that significant increases in callose deposition could be observed at lower concentrations of Al and/or with shorter exposure times if root tips rather than entire roots were harvested for callose quantification. Since callose concentration was determined from entire roots, it likely underestimated callose deposits at the root tip - where Al entry is most rapid (Polle et al., 1978; Rincon and Gonzales, 1992) and Al sensitivity is greatest (Ryan et al., 1993). Callose has been observed in *Picea abies* root tips after 3 h treatment with 170 μM Al (Jorns et al., 1991).

Callose deposition is closely associated with membrane injury (Kauss, 1989), and Al is known to damage membranes (Wagatsuma et al., 1987). Hematoxylin staining of the Al-sensitive wheat cultivar Brevor indicated that Al is localized mostly at the outer layers of root cells, and not near the root tip, unless exposed to very high Al levels (Polle et al., 1978). Rincon and Gonzales (1992) found that the Al-resistant cultivar Atlas 66 accumulated less Al in the root meristem than the Al-sensitive cultivar Tam 105. This distribution of Al in roots is similar to the pattern of root callose deposition observed in the present study, and others (Jorns et

al., 1991; Wissemeier et al., 1987). These observations could be explained if toxic species of Al penetrated the Al-sensitive lines but were prevented from entering Al-resistant cultivars, except under conditions of severe Al stress. Additional study of callose localization in root caps separated from the root tip meristem may provide clues on the uptake of Al in plants. The plasma membrane is an important barrier to the passive movement of Al (Wagatsuma, 1983), perhaps reflecting the operation of a metabolism-dependent exclusion mechanism (Rincon and Gonzales, 1992; Zhang and Taylor, 1991). Alternatively, it is possible that the mechanism responsible for Al resistance also prevented membrane injury and/or callose deposition.

Although callose has similar staining and fluorescing properties as other compounds, several experiments indirectly confirmed its identity in this study. In both the callose quantification and localization experiments, adding a callose synthesis inhibitor, DDG, to the nutrient solution of Scout 66 roots reduced fluorescence. Although the action of this compound is not well-known, DDG may be metabolized into UDP-deoxyglucose and GDP-deoxyglucose, which inhibit the biosynthesis of lipid-linked oligosaccharides, and then inhibit protein glycosylation (Datema et al., 1983). Such a general action might explain the observed toxic effects of DDG on wheat, including reduced root and leaf lengths and severe root damage. It should be emphasized, however, that these more general effects were observed at higher concentrations of DDG than required to reduce callose deposition. Fewer toxic effects might have occurred if wheat seedlings were treated with lower levels of DDG and/or for shorter periods of time. Interestingly, Jaffe and Leopold (1984) did not observe any toxic effects of DDG after 1,000 to 10,000 μM DDG was added to the roots of *Plsum sativum* and *Zea mays.*

In the localization experiments, interference from other fluorescing compounds was reduced by prestaining tissues with periodic acid-Schiff's reagent and staining with Sirofluor, a concentrated preparation of the active ingredient in the fluorochrome aniline blue. Incubating sections of the Al-treated roots with the callose degrading enzyme β-D-glucoside glucohydrolase (EC 3.2.1.21) also reduced fluorescence. Although this enzyme is not specific for (1,3)-β linkages, it did not noticeably affect the fluorescence of cellulose, which might interfere with callose fluorescence, after staining with calcofluor white M2R (Sigma). As a whole, these results suggest that callose was indeed observed in our experiments.

In summary, resistance to Al was negatively associated with root length and root callose deposition (quantification and localization). These results supported previous studies that found root responses are more sensitive to Al than are leaf responses (Briggs et al., 1987). Attempts to detect callose deposition in the leaves provided little evidence of Al injury, perhaps indicating that toxic species of Al were not transported into the leaves in sufficient quantities to induce injury. Thus, resistance to Al appears to occur at the root level. Fluorescence microscopy suggests that toxic species of Al may have penetrated further into the roots of Al-sensitive cultivars than Al-resistant cultivars, perhaps due to some resistance mechanism operating in the outer region of the root. Although root length was more sensitive to Al treatment, use of root tips for quantifying callose deposition may improve the sensitivity and accuracy of callose deposition as a marker for Al-induced injury. We believe that callose quantification may be useful in short-term physiological studies as a biological marker of Al-induced injury, or for measuring the passage of toxic Al species. Further study is needed to identify where callose deposition is most prevalent and to determine the rapidity and dose required for a response.

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