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Operationally Defined Apoplastic and Symplastic Aluminum Fractions in Root Tips of Aluminum-Intoxicated Wheat

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ABSTRACT

Knowledge of the mechanistic basis of differential aluminum (Al) tolerance depends, in part, on an improved ability to quantify Al located in the apoplastic and symplastic compartments of the root apex. Using root tips excised from seedlings of an Al-tolerant wheat cultivar (*Triticum aestivum* L. cv Yecora Rojo) grown in Al solutions for 2 d, we established an operationally defined apoplastic Al fraction determined with six sequential 30-min washes using 5 mM $CaCl_2$ (pH 4.3). Soluble symplastic Al was eluted by freezing root tips to rupture cell membranes and performing four additional 30-min $CaCl_2$ washes, and a residual fraction was determined via digestion of root tips with HNO_3. The three fractions were then determined in Yecora Rojo and a sensitive wheat cultivar (Tyler) grown at 18, 55, or 140 μM total solution Al (Al_T). When grown at equal Al_T, Tyler contained more Al than Yecora Rojo in all fractions, but both total Al and fractional distribution were similar in the two cultivars grown at Al_T levels effecting a 50% reduction in root growth. Residual Al was consistently 50 to 70% of the total, and its location was elucidated by staining root tips with the fluorophore morin and examining them using fluorescence and confocal laser scanning microscopy. Wall-associated Al was only observed in tips prior to any washing, and the residual fraction was manifested as distinct staining of the cytoplasm and nucleus but not of the apoplastic space. Accordingly, the residual fraction was allocated to the symplastic compartment for both cultivars, and recalculated apoplastic Al was consistently approximately 30 to 40% of the total. Distributions of Al in the two cultivars did not support a symplastic detoxification hypothesis, but the role of cytoplasmic exclusion remains unsettled.

Root lesions caused by Al toxicity can cause disruption of membrane structure and function, disruption of DNA synthesis and mitosis, cell wall rigidification and reductions in cell elongation, and/or disturbance in mineral assimilation and metabolism, and these postulated toxicity mechanisms have recently been reviewed by Taylor (29). Despite vast quantities of published research, however, the principal physiological mechanism(s) of Al rhizotoxicity remains unresolved, and it remains unclear which are primary dysfunctions and which should more properly be considered secondary effects. There are broad, genetically determined differences in Al tolerance between plant species and genotypes (8). Intraspecific differences in responses to Al may provide clues to mechanisms of toxicity and aid in plant breeding for superior Al tolerance (8).

In general, theories concerning mechanisms of differential tolerance may be divided into three categories (3, 30): (a) the primary lesion of Al rhizotoxicity is cytoplasmic, and differential tolerance is the result of variation in the ability of a plant to detoxify or tolerate Al within the symplast; (b) the primary lesion is cytoplasmic, and differential tolerance is a result of genotypic variation in ability to exclude Al from the symplast; and (c) the primary lesion is apoplastic, and differential tolerance is achieved via modification of the apoplastic environment to effectively protect cell wall and/or membrane structure and function. Symplastic tolerance mechanisms could, for example, involve Al chelation in the cytosol, vacuolar compartmentation, complexation by Al-binding proteins, or Al-tolerant enzymes (30). Cytoplasmic exclusion could be accomplished via selective permeability of the plasma membrane, formation of a plant-induced pH barrier in the rhizosphere, immobilization of Al on the cell wall, or exudation of chelating ligands (30). The latter three mechanisms could, presumably, also impart resistance to apoplastic injuries.

To evaluate postulated tolerance mechanisms, it is important to measure Al entry into the cytoplasm and to differentiate quantitatively between apoplastic and symplastic Al (34, 35). To date, such quantification has been limited, and reports are conflicting. Root uptake data suggest that >50% of Al in the root is apoplastic (6, 12, 35). In contrast, x-ray microanalysis indicated that Al is primarily localized in the cytoplasm and nuclei of meristematic cells, not in cell walls, although Al was also found in the root cap cell wall (21).

Horst et al. (11) observed that about 50% of Al in the root tips of cowpea (*Vignia ungiculata* [L.] Walp.) was located in the mucilage surrounding the apical 2 mm of the root tip. Conflicting results in Al quantification may be the result of different experimental approaches, some of which may have been inadvertently flawed. Because Al can exist in solution as a number of chemical species, it is important to ultimately relate root responses to those that seemingly dictate rhizotoxicity, and this is best accomplished by keeping test solutions chemically simple (15). For example, Al speciation can be difficult to predict when Al is added to solutions (including complete nutrient solutions) that contain ligands such as phosphate or sulfate. In such instances, much of the Al added may exist as polymers, precipitates, or complexes that may have little influence on the expression of toxicity (15). Reports suggesting that the majority of Al accumulates on root surfaces and does not penetrate the cortex (e.g. see ref. 26) may actually reflect surface precipitation of Al with phosphate present in the test solution. Moreover, as noted by Zhang

and Taylor (34), test solutions have often contained high, physiologically unrealistic levels of Al_T[1] that were not selected to correspond with specific degrees of rhizotoxicity (e.g. see refs. 12, 21, and 26).

Previous attempts to allocate root Al into apoplastic and symplastic components have largely been indirect, and have often been based on extrapolation of short-term, biphasic uptake kinetics (12, 34, 35). Absorption of Al demonstrates an initial rapid, nonlinear phase followed by a slower, linear phase. According to common interpretation, the initial, rapid phase represents apoplastic binding, and the linear phase corresponds to transport across the plasma membrane (34). These assumptions have not been directly substantiated, and the interpretation of biphasic uptake has recently been questioned (35). Other experimental approaches have included microscopy, wherein thin sections of tissue are subjected to severe chemical fixation and dehydration processes. It is unknown what artifacts such procedures may generate with respect to Al localization within the root, because loosely bound Al may be removed by chemical fixation and dehydration (10, 21). EDAX analyses of root Al localization were also hampered by freeze-drying; tissue dehydration rendered the cell wall and cytoplasm regions virtually indistinguishable (13). Procedures utilized to isolate cell wall material are likewise harsh (1, 2, 6), and redistribution of Al or alteration of binding properties is possible (35). Thus, Al binding on homogenized wall material may not accurately reflect binding on the heterogeneous, highly organized cell wall in vivo, nor is the complex interrelationship between wall, plasma membrane, and cytoskeleton (28) retained during such procedures.

In this study, we sought to improve on previous methods of differentiating between apoplastic and symplastic Al. Our objectives were to (a) develop a method whereby Al in the root tips of wheat (*Triticum aestivum*) seedlings could be fractionated into operationally defined apoplastic and symplastic components, with minimal risk of redistribution or other artifactual results; (b) validate the method and localize Al with fluorescence and confocal laser scanning microscopy; and (c) evaluate possible mechanistic bases for differential Al tolerance. The method was employed using two wheat cultivars differing in Al tolerance and evaluated at prescribed levels of solution Al_T selected to achieve specific reductions in root elongation.

MATERIALS AND METHODS

Root Growth Responses to Al

General procedures for root growth experiments were as described previously by Kinraide and Parker (16). Wheat seeds (*Triticum aestivum* cv Yecora Rojo or Tyler) were germinated for 2 d in the dark on filter paper moistened with double-deionized water. Five seedlings were selected for uniform primary root lengths of 15 ± 2 mm, placed in polyethylene foam floats, and transferred to a beaker containing 500 mL of test solution. Beakers were loosely covered to minimize evaporation and the solutions were aerated. Seedlings were grown at 25°C for 2 d in the dark. At termination, lengths of the two longest roots of each seedling were recorded and the mean of the 10 values for each beaker used as a measure of Al rhizotoxicity.

All reagents used were either of analytical grade or higher purity. Double-deionized water was used and all glassware was acid washed. A stock solution of 4 mM $AlCl_3$ in 2 mM HCl, prepared from $AlCl_3 \cdot 6H_2O$, was used to prepare all test solutions. Test solutions ranged from 0 to 216 μM Al_T and contained 1.5 mM $CaCl_2$, with two replications for each cultivar at each Al_T. Solutions were adjusted to pH 4.3 by addition of 5 mM HCl as needed, and were checked and readjusted approximately every 12 h. Base was never added to adjust pH, thus avoiding the formation of the acutely rhizotoxic polynuclear species "Al_{13}" (15). Using the GEOCHEM-PC program (25) and hydrolysis constants from Nordstrom and May (23), Al^{3+} activity was computed to be 81% of the sum of the activities of mononuclear Al species in all solutions. RRG was computed according to the following equation:

$$RRG = \frac{RL - RL_{sat}}{RL_C - RL_{sat}} \times 100,$$

where RL = mean root length at a given Al_T, RL_{sat} = mean root length at maximal toxic levels of Al_T, and RL_C = mean root length of control seedlings (15, 17).

Fractionation of Al in Root Tips

Seedlings were reared as described above, except that 2-L solutions with 25 seedlings per container were used. Growth solutions contained 0, 18, 55, or 140 μM Al_T and 1.5 mM $CaCl_2$, were prepared and readjusted at regular intervals to pH 4.3, and there were three replications at each Al_T. At termination, seedlings were removed from the flotation devices and rinsed in 1.5 mM $CaCl_2$ (pH 4.3) for several seconds. Roots were blotted, and 70 tips, 5 mm in length, were excised from the roots of each container and placed in small polyethylene cups. The wash cups were then weighed (fresh weight of root tips was determined by difference), covered, and refrigerated (4°C) until initiation of the fractionation procedure. Delay between excision and fractionation totaled ≤1.5 h. The fractionation of Al was performed as follows: (Step 1) Root tips were sequentially washed six times for 30 min each with 20-mL aliquots of a desorbing solution; solutions were gently agitated with a 10 × 3-mm stir-bar. (Step 2) Cell membranes were ruptured by freezing or sonication. (Step 3) Root tips were sequentially washed four more times, 30 min per wash, using the same method and desorbing solution as in Step 1. (Step 4) Root tips were placed in 6 × 3.5-cm Teflon containers and digested overnight in 1 mL of concentrated trace-metal-grade HNO_3 at 80°C. Solutions were diluted to 25 mL and adjusted to pH 2.0 with trace-metal-grade 12 N NH_4OH.

Polyethylene wash cups and Teflon digest vessels were prewashed in 2 N HCl and 0.01 M Na_4EDTA. All washes were decanted and retained for analysis of Al. Wash and digest solutions were analyzed spectrofluorometrically using

[1] Abbreviations: Al_T, total solution aluminum; morin, 2,3,4,5,7-pentahydroxyflavone; EDAX, energy-dispersive x-ray analysis; NH_4OAc, ammonium acetate; Al_{13}, $AlO_4Al_{12}(OH)_{24}(H_2O)_{12}^{7+}$; CLSM, confocal laser scanning microscopy; CEC, cation exchange capacity; RRG, relative root growth.

8-hydroxyquinoline with butyl acetate extraction (4). A Perkin-Elmer LS-5 fluorescence spectrophotometer was employed with excitation and emission slits = 10, and excitation and emission wavelengths of 391 and 513 nm, respectively. Al standards (pH 3.3) ranging from 0 to 30 $\mu g\ L^{-1}$ were prepared from an atomic absorption reference standard (1000 mg L^{-1} in 1% HCl).

Three desorbing solutions were evaluated using Yecora Rojo grown at 55 μM Al_T: 5 mM $CaCl_2$, 5 mM $CaCl_2$ plus 100 μM EDTA, and 5 mM $CaCl_2$ plus 100 μM citrate. The EDTA solution was prepared from the tetrasodium salt, and the citrate solution was prepared from the trisodium salt. Citrate solutions were prepared fresh daily to avoid microbial degradation. All desorbents were adjusted to pH 4.3. Sonication and freezing were evaluated as techniques to rupture cell membranes. Sonication was conducted with a Bronwill Biosonik III sonicator with a 3-mm diameter head for 1 min at high power. Freezing duration was approximately 18 h at −15°C.

Morin Staining and Fluorescence Microscopy

Morin (338.3 mol wt; ICN Biochemicals Corp., Cincinnati, OH) was used to stain Al in wheat root tips. Morin has been used previously to quantify aqueous Al (5) and to a limited extent for staining Al in plant tissue (7) and may offer advantages over other fluorescent stains. In the acidic pH range, morin shows high specificity for Al (5). The Al-morin complex fluoresces green, with an excitation wavelength of 420 nm, and an emission wavelength of 510 nm (5). Root tips of seedlings, reared as for the fractionation experiments, were stained immediately after excision, after fractionation Step 1 or after fractionation Step 3. The tips were (a) washed in 5 mM NH_4OAc buffer (pH 5.0) for 10 min; (b) stained in 100 μM morin plus 5 mM NH_4OAc (pH 5.0) for 1 h; and (c) washed in 5 mM NH_4OAc buffer (pH 5.0) for 10 min.

Squashes of root tips were examined microscopically and photographed using a Zeiss standard epi-fluorescence microscope fitted with a Nikon Microflex UFX-II camera and filter set consisting of an excitation filter (BP 400–440 nm) and a barrier filter (LP 470). Micrographs were taken with Fujichrome ASA 1600 color slide film. Whole root tips were observed and photographed on a Bio-Rad MRC-600 confocal laser scanning microscope (Cambridge, MA) with a fluorescein filter cube. The excitation source was an argon laser (wavelength = 488 nm). Micrographs were taken on a Polaroid Freeze Frame Video Recorder with T-Max 100 film and processed for black and white negatives.

RESULTS AND DISCUSSION

Root Growth Responses to Al

RRG is a standard measure of plant sensitivity to Al (15, 29). Tyler showed a much greater sensitivity to Al than Yecora Rojo, as evidenced by the marked decrease in root elongation with increasing Al_T (Fig. 1). A Weibull-type equation was employed to describe the sigmoidal relationship between RRG and solution Al_T. Using a general purpose nonlinear regression program, the RRG data were fit to equation 3 from Kinraide and Parker (17). The calculated coefficients were

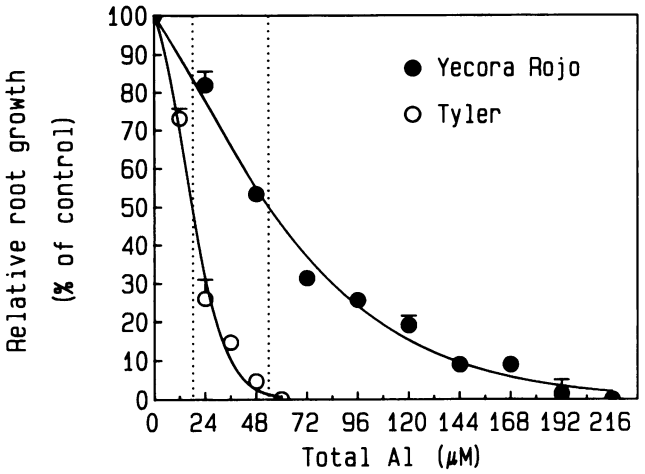

Figure 1. RRG of Yecora Rojo and Tyler wheat as a function of total Al in the test solution. Each point is the mean of duplicate determinations and error bars represent 1 SE where it exceeds symbol size.

used to determine Al_T levels required to achieve 50% reductions in root elongation, and these are indicated by the dashed lines in Figure 1: 18 μM and 55 μM Al_T for Tyler and Yecora Rojo, respectively. This allowed comparison of cultivars both at equal solution Al_T and at equal degrees of growth inhibition. Root growth of Yecora Rojo was 84 and 10% of control at 18 and 140 μM solution Al_T, respectively, whereas root growth of Tyler was 1% of control at 55 μM Al_T (Fig. 1).

Evaluation of Fractionation Methods

Al recovery followed a similar pattern regardless of choice of desorbent, and Figure 2 depicts typical results using 5 mM $CaCl_2$. We operationally define apoplastic Al as the total recovered in washes 1 through 6, after which the cell membranes were ruptured. Soluble symplastic Al corresponds to washes 7 through 10, and residual Al was determined in the final digests. Forty and 70% of apoplastic Al was recovered in the first wash for Yecora Rojo and Tyler, respectively, and net recovery quickly dropped to near zero in the next five washes. A small spike occurred after freezing, which is attributed to soluble Al that was eluted from the symplast following membrane rupture, but a large residual Al fraction remained (Fig. 2). High Al concentrations in the residual fraction of control tips was common, and it was ascertained that this was primarily due to Al contamination from the digestion vessels, which could not be fully removed despite prewashing in 2 N HCl and 0.01 M Na_4EDTA. At 18 μM solution Al_T, Al concentrations in many of the wash solutions approached detection limits of spectrofluorometric analysis, and thus even small shifts in background Al resulted in noticeable variations in recovery across replicate determinations.

We tested several desorption protocols based on the aforementioned definitions of apoplastic and symplastic compartments, and percent distributions were very similar (Table I). We initially chose $CaCl_2$ as a desorbent because Ca is an

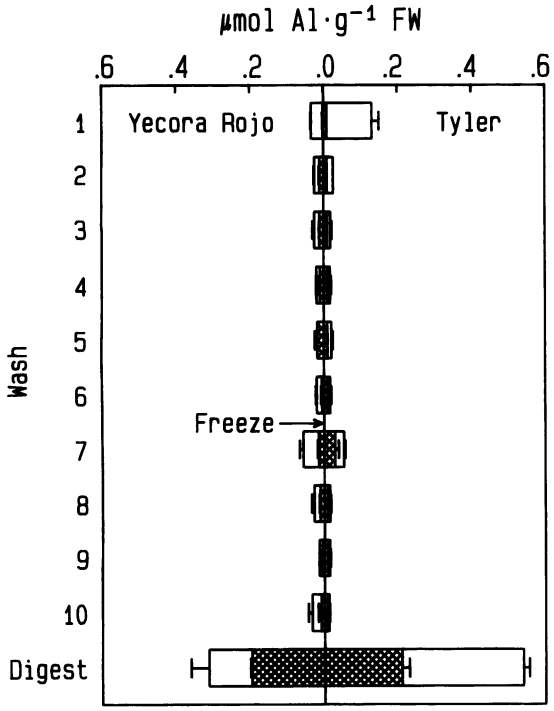

Figure 2. Al recovery in root tips of Yecora Rojo and Tyler wheat grown in 18 μM Al_T using 5 mM $CaCl_2$ as desorbing agent (pH 4.3). Each bar is the mean of three replications, and error bars represent 1 SE. Crosshatch area represents Al recovered in control root tips (0 Al), and open area represents net Al recovered.

effective cation exchanger and should be effective in displacing apoplastic Al by mass action while helping to maintain cell membrane integrity. $CaCl_2$ was, therefore, used alone or in conjunction with EDTA or citrate, both strong ligands for Al. Readily exchangeable apoplastic Al recovery was similar for each desorbent tested (Table I), totaling about 40%. Zhang and Taylor (34) reported similar Al recoveries following 180-min desorption of excised 2-cm root tips using several different desorbing agents. Increase in the percentage of Al assigned to the symplastic fraction with citrate, and to a lesser extent with EDTA (Table I), probably reflects enhanced elution of Al from nonlabile forms in the cytoplasm.

Analytical problems were encountered with desorbing agents containing Al-complexing ligands and were especially pronounced with citrate. Low recovery of standard solutions when either EDTA or citrate was present was overcome with rapid preadjustment to pH 10, where the aluminate anion predominates, and 100% recovery was achieved. When citrate was used, wash solutions had to be analyzed immediately following this pH adjustment. Otherwise, pH reductions to near neutral occurred due to microbial metabolism, causing precipitation of Al and low recoveries. Because there was no a priori criterion for choosing any one desorbent and because of the greater simplicity in analysis, 5 mM $CaCl_2$ alone was selected as the desorbing agent for the remainder of the study. Experimental results obtained subsequently justified this selection (see below).

Sonication yielded greater soluble symplastic Al than freezing, with a concomitant decrease in residual Al (Table I). Apoplastic recovery, unsurprisingly, was identical, because the procedures up to membrane rupture were the same. Although sonication yielded greater recovery in the soluble symplastic fraction, it is not known whether this increase was due to greater mechanical disruption of the cytoplasmic contents or to more thorough membrane rupture. For this reason and for simplicity, we used freezing as the membrane-rupturing method for the remainder of the study.

Fractionation of Al in Root Tips

Both cultivars were grown at 18 and 55 μM Al_T, and Yecora Rojo was also grown at 140 μM Al_T. Each wash solution and the residual digests were analyzed for Al, and apoplastic, symplastic, and residual Al were calculated for each treatment as described above. When compared at equal Al_T, the sensitive cultivar, Tyler, accumulated more Al in each fraction than Yecora Rojo, and this was especially pronounced at 55 μM Al_T (Fig. 3). At 140 μM Al_T, Yecora Rojo still accumulated less Al than Tyler at 55 μM Al_T, despite severe growth inhibition in both cultivars (Fig. 1). At 55 μM Al_T, root growth of Tyler was only 1% of the control, and Al concentrated in cells that had ceased elongating or dividing. At the same Al_T, root growth of Yecora Rojo was 50% of the control, and Al accumulation in root tips may have been partially offset by cell division and elongation, such that lower accumulation in Yecora Rojo may be partly ascribable to a dilution effect. At levels of Al_T that resulted in equal inhibition of root growth (18 μM for Tyler and 55 μM for Yecora Rojo), Al recoveries in each fraction were similar for both cultivars (Fig. 3). The location of the consistently large residual fraction (50–70% of total Al recovered) remained a quandary, and fluorescence microscopy was employed to disclose its location.

Table I. *Percentage Al Distribution in 5-mm Root Tips of Yecora Rojo Wheat Grown at 55 μM Al_T Using Different Desorbents and Membrane-Rupturing Methods*

Desorbent	Membrane-Rupturing Technique	Apoplast	Symplast	Residual
5 mM $CaCl_2$	Freeze	44.4 ± 6.1[a]	1.6 ± 0.3	54.0 ± 6.2
5 mM $CaCl_2$ + 100 μM EDTA	Freeze	38.2 ± 6.9	5.5 ± 2	65.3 ± 4.9
5 mM $CaCl_2$ + 100 μM citrate	Freeze	44.3 ± 1.2	14.8 ± 0.8	40.9 ± 0.6
5 mM $CaCl_2$	Sonicate	41.5 ± 6.6	25.6 ± 0.4	32.9 ± 6.2

[a] Values are mean net Al recovered (see Fig. 2) ± 1 SE for three replications.

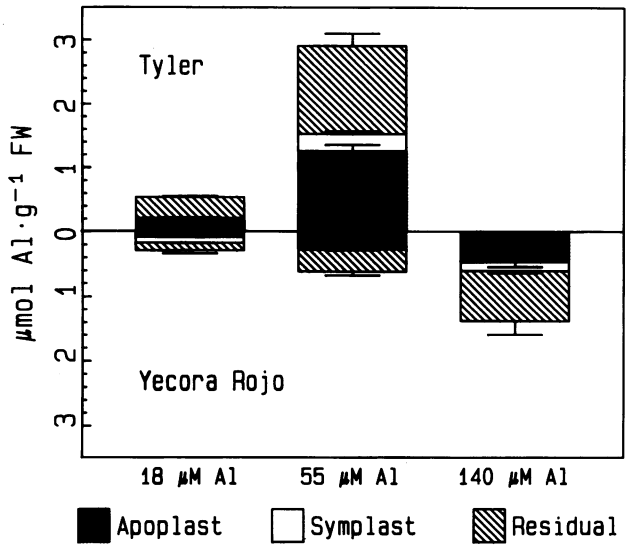

Figure 3. Al distribution in 5-mm root tips of Yecora Rojo and Tyler wheat grown in test solutions containing 18, 55, and 140 μM Al_T. Each bar represents the mean net Al recovered (see Fig. 2) for three replications, and error bars represent 1 SE.

Fluorescence Microscopy

Squashes of stained root tips of Yecora Rojo and Tyler grown both at 55 and 18 μM Al_T were examined, and representative root tips of the two cultivars grown at equal inhibition levels of Al_T were photographed at identical camera exposure settings (Fig. 4, a–f). This allowed comparison of fluorescence intensities for both cultivars at selected steps in the fractionation procedure. Because control root tips (0 Al) photographed virtually black (not shown), competitive staining and/or autofluorescence were not interfering. Fluorescence intensities of both cultivars prior to washing were similar (Fig. 4, a and b). Intensities decreased following fractionation Step 1, suggesting effective elution of apoplastic Al (Fig. 4, c and d). After fractionation Step 3, fluorescence intensities for both cultivars markedly increased (Fig. 4, e and f), probably due to enhanced morin permeation into the cells after membrane rupturing. The similar fluorescence intensities observed in both cultivars at each step of the fractionation procedure qualitatively agreed with the results of the fractionation procedure (Fig. 3). Overall, morin seemed to represent a substantial improvement in specificity and sensitivity compared to other staining methods for detecting Al (e.g. see refs. 10 and 32).

Minor cultivar differences were observed in the pattern of staining in the apical region. The apical portion of the Tyler root tip stained preferentially, even after six $CaCl_2$ washes (Fig. 4, a and c). To determine whether Tyler accumulated more Al in this region, 5-mm root tips of Tyler and Yecora Rojo grown at their respective 50% inhibition levels of Al_T were excised and washed six times with 5 mM $CaCl_2$. Then, 1 ± 0.5-mm apical segments were excised from the 5-mm tips, and both the apical and basal portions were digested to estimate the longitudinal distribution of Al. In both cultivars, Al in the apical 1-mm segments was 40 to 50% of the total present in 5-mm tips (data not shown). Greater fluorescence intensity in the apical region of Tyler, therefore, was not due to greater accumulation of Al, but was perhaps the result of equal Al accumulation in smaller, more tightly packed cells, or of greater morin permeation into these cells.

We observed what appeared to be apoplastic Al in tips stained prior to fractionation Step 1 (no washes), although this tended to be apparent only in the basal half of the excised root tips (Fig. 4g). Despite close scrutiny, we could not identify any wall-associated Al in any region of the tips that had been stained after fractionation Step 1 (six $CaCl_2$ washes) (Fig. 4h), suggesting that our fractionation procedure effectively removes the majority of apoplastic Al. Due to the limited spatial resolution of the fluorescence microscope, however, we sought to verify this observation with confocal microscopy.

CLSM

Root tips of Yecora Rojo and Tyler grown at 18 and 55 μM Al_T were examined using CLSM. Sites of Al localization were similar in the two cultivars, and representative micrographs of root tips stained at selected steps in the fractionation procedure are presented in Figure 5. In cells near the apex of Tyler grown at 18 μM Al_T, staining occurred primarily in the cytoplasm and nuclei prior to washing, and no Al was observed in vacuoles or in the cell wall (Fig. 5a). In more basal regions, Al in the cell wall area was observed prior to washing (Fig. 5b), in agreement with the results of fluorescence microscopy. After fractionation Step 1, virtually no Al was observed in the cell wall area in the more basal area of the root tips of Yecora Rojo grown in 55 μM Al_T, but Al staining in cytoplasm and nuclei was still apparent (Fig. 5c). Very little, if any, Al was ever observed in the cell wall area in any region of root tips of either cultivar following fractionation Step 1 (six $CaCl_2$ washes), but Al staining remained pronounced in the cytoplasm and nuclei.

Observable cell wall Al seemed less than expected, given that about 40% of the Al recovered was assigned to the apoplast in the fractionation procedure (Table I, Fig. 3), and we tested the hypothesis that some Al was removed during the staining process. Yecora Rojo was grown in 55 μM Al_T, and excised root tips were stained. The three staining solutions were decanted, adjusted such that each contained 100 μM morin, and analyzed for Al spectrofluorometrically using the parameters described by Browne et al. (5). Al equivalent to about 20% of the apoplastic component determined by fractionation (Fig. 3) was removed in the staining process, with most being removed in the initial NH_4OAc buffer wash (data not shown), and may partially account for the absence of more obvious wall Al. Moreover, the excitation wavelength of the confocal microscope (488 nm) is not optimum for the Al-morin complex (420 nm), and diminished fluorescence intensity may also have been a contributing factor.

Tyler grown at 18 μM Al_T and stained after fractionation Step 3 was photographed with the focal plane near the center of the root axis (Fig. 5d), and the dark region to the left illustrates the absence of Al staining in the central regions of the root. This lack of staining could be ascribable to limited morin permeation into inner regions of the root cylinder, but

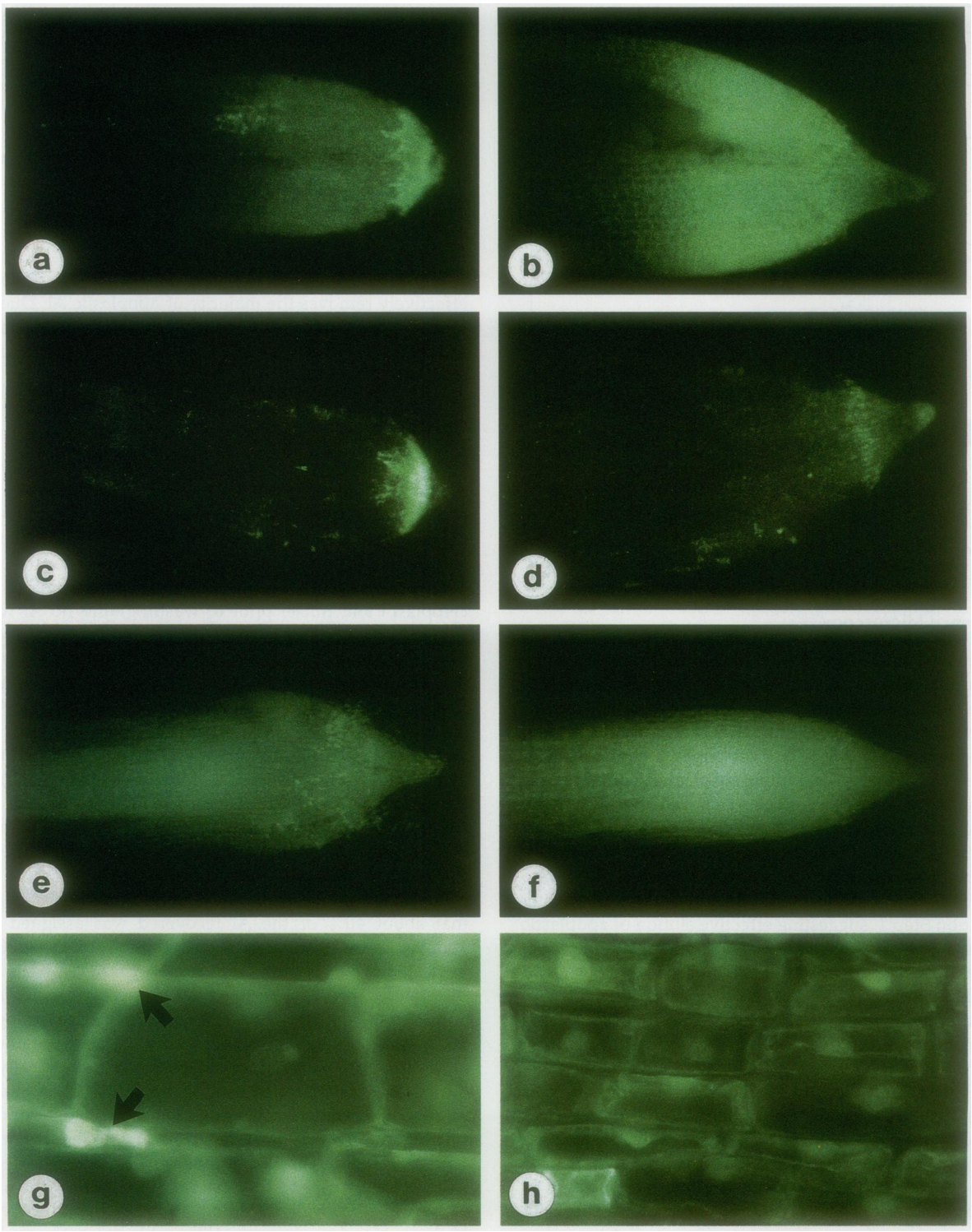

Figure 4. Al staining in root tips of Tyler and Yecora Rojo wheat grown at equal inhibition levels of Al_T (18 and 55 μM, respectively) at progressive steps in the fractionation procedure. Stained control root tips photographed virtually black and are not shown. Tyler root tips are shown on the left, and Yecora Rojo is to the right. a and b, Prior to fractionation Step 1 (no washes). c and d, Following fractionation Step 1 (six $CaCl_2$ washes). e and f, Following fractionation Step 3. g, Al staining prior to washing in Tyler, approximately 2 mm from root tip. Arrows denote apparent apoplastic Al. h, Al staining after fractionation Step 1 in Yecora Rojo, approximately 1 mm from root tip. Apoplastic space appears relatively free of Al. Magnifications: a–f are 16× actual; g is 250× actual; h is 160× actual size.

may represent the actual localization pattern, because previous research has shown that, in wheat grown at sublethal Al concentrations, entry was restricted primarily to meristematic, epidermal, and outer cortical cells (10). It was also noted that, after freezing, cells appeared disrupted and staining was more uniform throughout the cytoplasm, with a faint outline of the vacuolar region still discernible (Fig. 5d). Cytoplasmic Al was still pronounced, but even under high magnification apoplastic Al was not observed (not shown).

Allocation of Al into Apoplastic and Symplastic Compartments

Because apoplastic Al was almost never observed in stained root tips after Step 1 of the fractionation procedure, whereas staining continued to be pronounced in the cytoplasm, it is proposed that the residual Al fraction for both Yecora Rojo and Tyler (Fig. 3) be allocated to the symplast. Accordingly, the Al recoveries depicted in Figure 3 were recalculated and expressed as percentages assignable to the symplastic and apoplastic compartments (Table II). For both cultivars, the operationally defined apoplastic Al is consistently about 30 to 40% of the total recovered, regardless of solution Al_T (Table II). The remainder is symplastic, and microscopic evidence suggests it is primarily associated with the cytoplasm and nuclei, but not with vacuoles or other organelles.

Table II. *Percentages of Operationally Defined Apoplastic and Symplastic Al in Yecora Rojo and Tyler Wheat Grown at Different Levels of Solution Al_T*

Values are recalculated from results depicted in Figure 3, and represent means of three replications.

Al_T in Test Solution	Cultivar	Apoplast	Symplast	SE
μM				
18	Yecora Rojo	30.5	69.5	6.3
	Tyler	32.1	68.9	2.5
55	Yecora Rojo	44.4	55.6	6.1
	Tyler	43.6	56.4	1.4
140	Yecora Rojo	35.3	64.7	3.2

Conflicting reports of Al distribution between apoplastic and symplastic compartments have been given. Seventy-five

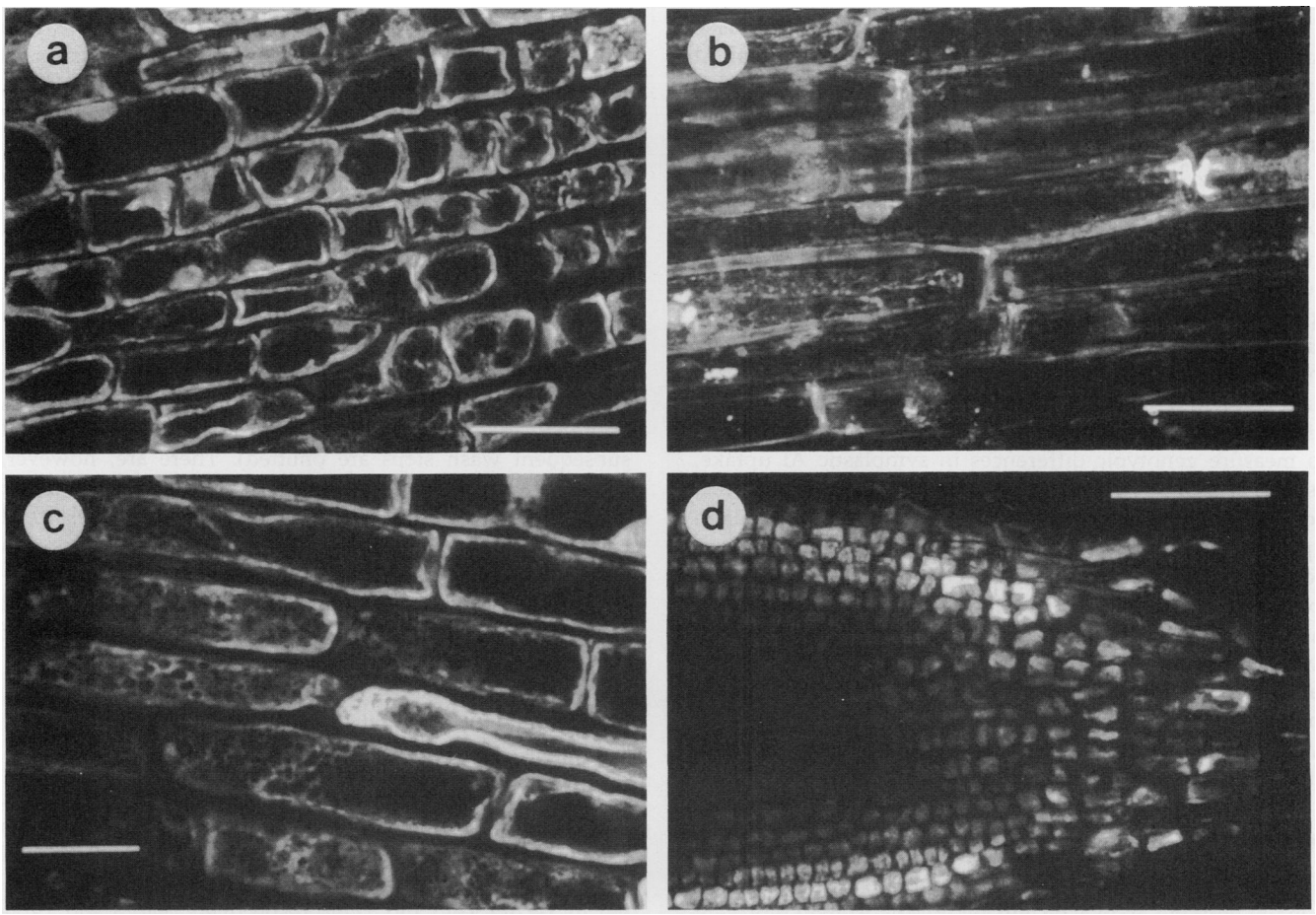

Figure 5. Confocal micrographs of stained root tips of Tyler and Yecora Rojo wheat grown in 18 and 55 μM Al_T, respectively. a, Al staining near root apex in Tyler prior to washing. Multi-vacuolated, meristematic region is to the right. Scale bar = 50 μm. b, Al staining prior to washing in Tyler, approximately 3.5 mm from tip. Scale bar = 50 μm. c, Al staining in Yecora Rojo following fractionation Step 1 (six $CaCl_2$ washes), approximately 2 mm from tip. Scale bar = 25 μm. d, Tyler root tip stained following fractionation Step 3. Scale bar = 100 μm.

to 95% of Al taken up by roots was reported to be associated with the cell wall by Huett and Menary (12) and Clarkson (6). These reports were based on extrapolative interpretation of biphasic patterns of uptake kinetics, and employed different experimental conditions: Huett and Menary (12) measured uptake by excised root tips using 1 mM $Al_2(SO_4)_3$, pH 4.2 and 4.0, for 3 h; Clarkson (6) measured uptake by whole roots using 100 μM $Al_2(SO_4)_3$ in a complete nutrient solution for 24 h, and by isolated cell wall material using 1 mM $Al_2(SO_4)_3$ (pH not specified). Solution Al_T levels employed were not correlated with decreases in root growth in the genotypes tested, and in some cases were substantially greater than those typically required to achieve 100% inhibition (34). It is possible that, in some instances, experimental conditions favored Al precipitation (15) and, hence, resulted in overestimations of Al uptake into the putative apoplastic compartment.

Zhang and Taylor (34) measured Al uptake in excised 2-cm wheat root tips using 75 μM $AlK(SO_4)_2$, at pH 4.5. Based on kinetics data, they estimated that apoplastic Al was <50% of total uptake over a 3-h period, in agreement with our results. They suggested, however, that the overall apoplastic fraction would be underestimated if the slow, linear uptake phase represented apoplastic as well as symplastic uptake. In a subsequent study (35), it was reported that the apoplastic compartment actually amounted to 45 to 75% of total Al uptake, but precipitation reactions may have confounded these estimates. Using stability constants from Nordstrom and May (23), we computed the speciation of Zhang and Taylor's (34, 35) solutions. Formation of polynuclear or precipitated hydroxo-Al species was likely (17), and the solutions were also oversaturated with respect to the basic Al-sulfate minerals alunite and jurbanite (22). Moreover, less than one-quarter of the total Al in solution was present as Al^{3+}, the species reportedly responsible for rhizotoxicity in wheat (17). Little difference in uptake was seen in four cultivars differing in Al sensitivity (34), and Al precipitation, if it occurred, could have masked differences. Moreover, the short duration (3 h) of these excised-root experiments may have been insufficient to measure genotypic differences in symplastic Al uptake, especially if these depend on plant responses to Al stress (i.e. acclimation). Our data suggest that the majority of Al taken up from undersaturated Al solutions over longer durations accumulates in the symplast of the two wheat cultivars we examined. Symplastic Al, however, may be overestimated if Al exists in tightly bound, nonexchangeable forms on the cell wall or membrane surface, which would limit complexation (and thus observable staining) by morin. Additional experiments may be needed to test the ability of morin to stain such nonlabile forms of Al and could help verify the existence of a purported nonexchangeable apoplastic fraction.

Method Assessment and Possible Improvements

For several reasons, the method reported here for operationally defining apoplastic and symplastic Al may represent an improvement over previous approaches. The only component of the test solutions other than Al is 1.5 mM $CaCl_2$, and Al is supplied as the chloride salt. In these solutions, the computed ion activity ratio (IAR = $\{Al^{3+}\}/\{H^+\}^3$) is $10^{7.78}$ and $10^{8.26}$ at Al_T = 18 and 55 μM, respectively. Both are below the value of $10^{8.8}$, which appears to be the threshold for onset of polynucleation and/or precipitation reactions (17), although pH in the free space may be higher than in the bulk solution, and polynucleation in the apoplast could conceivably occur (15). At pH 4.3, over 80% of total mononuclear Al in solution is present as Al^{3+}, which has been identified as the most toxic Al species toward Tyler wheat (17). This assures that both growth inhibition and Al distribution are primarily a function of toxic forms of Al, thus enhancing our ability to correctly deduce the mechanisms underlying differential tolerance.

The primary assumptions of our method are that apoplastic Al is relatively easy to exchange and that cytoplasmic Al is nonlabile. The first assumption may be reasonable if the majority of apoplastic Al is bound to pectin residues in the cell wall (32). The Ca/Al molar ratios in the first wash of the fractionation procedure were consistently $>10^4$, and even higher in subsequent washes, providing a large "driving force" for Al displacement. Apoplastic binding of Al may, however, involve other, metabolically driven processes that render some Al nonexchangeable (27, 35). Evidence for a nonexchangeable apoplastic fraction, however, is derived in part from experiments conducted using purified cell wall material (35), which may or may not reflect Al binding on the cell wall in vivo (28). The second assumption is also reasonable, given the abundance of potential ligands for Al in the cytoplasm, many of which may be structural (e.g. cytoskeletal) (18) or macromolecular (2), and thus effectively retain Al against efforts to desorb it. Moreover, the near-neutral cytoplasmic pH would favor rapid precipitation of Al as Al-hydroxides (30).

The proposed method is relatively straightforward and suitable for routine use. The detection limit of the spectrofluorometric analysis has been reported as about 10^{-8} M (4), thus allowing quantification of Al in roots of seedlings grown in low, physiologically relevant concentrations. The method can be simplified by combining the first six washes and then analyzing for apoplastic Al, followed by digestion in HNO_3 and analysis for symplastic Al (the membrane rupture and subsequent wash steps are omitted). There are, however, some drawbacks to the method as well. The submicromolar levels of Al present in samples make even trace levels of Al contamination problematic, although selection of digestion vessels that do not yield the high background levels encountered in this study may help. Our experience is that good reproducibility can be obtained but that a certain amount of practice is required.

Implications for Mechanistic Bases of Differential Tolerance

Tyler accumulated considerably more Al in both apoplastic and symplastic compartments compared to Yecora Rojo when grown at equal Al_T (Fig. 3). At 55 and 18 μM Al_T, both total accumulation and compartmental distribution were very similar in Yecora Rojo and Tyler, respectively (Fig. 3 and Table II). Accordingly, our results do not explicitly suggest a symplastic tolerance mechanism in wheat. The most compelling argument for such a mechanism would arise if, at equal degrees of inhibition, the tolerant cultivar accumulated more

Al in the symplasm (with a concomitant increase in the percentage of symplastic Al). Such a result would clearly suggest that the tolerant cultivar has an ability to detoxify some portion of the symplastic Al. In fact, we observed equal symplastic Al, and equal Al distributions, in the cultivars when compared at equal inhibition levels. Similarly, Al was never observed in vacuoles (Fig. 5), suggesting the absence of a vacuolar compartmentation mechanism.

A tolerance mechanism predicated on cytoplasmic exclusion could be most readily inferred if the percentage of symplastic Al in Yecora Rojo was less than that of Tyler when root tips the two cultivars contained equal amounts of total Al. This was not the case (Fig. 3, Table II), yet our results do not entirely rule out an operative exclusion mechanism. In absolute terms, Tyler did accumulate more symplastic Al when the cultivars were grown at equal Al_T (Fig. 3, Table II), and this could be viewed as evidence for an exclusion mechanism in Yecora Rojo. If exclusion is responsible for superior tolerance in Yecora Rojo, cell wall CEC may play a role.

Wagatsuma (32) observed greater levels of Al absorbed by roots of plant species whose dry root powder had greater estimated CEC values. Greater root CEC has been reported for sensitive cultivars of ryegrass (*Lolium* spp.) (27, 31) and wheat and barley (*Hordeum vulgare* L.) (9). Wagatsuma and Akiba (33) related interspecific differences in Al sensitivity in the order rice (*Oryza sativa* L.) < oat (*Avena sativa* L.) < maize (*Zea mays* L.) < pea (*Pisum sativa* L.) < barley to corresponding increases (more negative) in surface charge of the plasmalemma of isolated protoplasts. In contrast, however, others have observed no significant differences in root CEC between cultivars of cotton (*Gossypium hirsutum* L.) (14) and triticale (*X Triticale*) (20), and isolated cell walls of Al-sensitive wheat cultivars have been shown to have lower CEC values than those from tolerant cultivars (1). Thus, although conflicting evidence exists, greater apoplastic CEC in Tyler could exert indirect control over symplastic uptake, and a lower CEC in Yecora Rojo could lead to less accumulation in both apoplast and symplast and, thus, greater Al tolerance.

Our results also do not rule out the possibility that Al accumulation in the apoplast is responsible for the primary lesion, since apoplastic Al accumulation was also greater in Tyler when the cultivars were grown at equal Al_T (Fig. 3). Ownby and Popham (24) observed root growth inhibition in wheat exposed to a 5-h pulse of 40 μM Al, which may have principally reflected uptake into the apoplastic compartment. Because Al desorption by 2 mM citrate resulted in resumption of root growth to 75% of control rates, these authors proposed that Al toxicity may be at least partially due to apoplastic Al. The exact nature of apoplastic injury is unclear, but previous research has furnished theories that suggest membrane, cell wall, or signaling dysfunction (3, 17, 19, 28).

Further research is required to elucidate the location of the primary Al lesion(s), and our proposed method may prove useful in this regard. We examined Al accumulation in only two cultivars differing in Al tolerance. The method could be used to examine differences in Al accumulation in the root tips of several cultivars exhibiting a range of Al tolerance, or could be extended to the study of other species, thus facilitating an improved understanding of differential tolerance mechanisms. In addition, fluorescence microscopy using morin may provide additional insights into Al localization in root apices.

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