Arabidopsis Mutants with Increased Sensitivity to Aluminum

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Al-sensitive (als) mutants of Arabidopsis were isolated and characterized with the aim of defining mechanisms of Al toxicity and resistance. Most als mutants selected on the basis of root growth sensitivity to Al were recessive, and together the mutants constituted eight complementation groups. Also, in most als mutants, Al sensitivity appeared to be specific for Al relative to La (another trivalent cation), except als2, which was more sensitive to La than wild type. The tendency of roots on mutant seedlings to accumulate Al was examined by staining with morin and hematoxylin, dyes used to indicate the presence of Al. A significant increase in morin staining was observed in als5, consistent with its increased sensitivity to Al. Unexpectedly, als7 and als4 showed less morin staining, suggesting that the roots on these mutants accumulate less Al than wild type after exposure to Al-containing solutions. Roots of wild-type seedlings produce callose in response to AlCl3 concentrations that inhibit root growth. Only als5 accumulated more callose than wild type in response to low levels (25 μM) of AlCl3. However, als4 and als7 did not accumulate callose at this AlCl3 concentration even though root growth was significantly inhibited. The lack of callose accumulation in als4 and als7 suggests that there is not an obligatory relationship between callose deposition and Al-induced inhibition of root growth.

Al is the most abundant metal in the earth's crust and is found in soils primarily in the form of insoluble aluminosilicates or oxides. When solubilized in acid soils, Al (primarily in the form of Al3+) is toxic to many crop plants (Foy et al., 1978; Rao et al., 1993; Kochian, 1995). Acid soils are particularly abundant in the developing world, and the use of Al-resistant plants may form part of a crop management strategy for agricultural production on acid soils.

In plants, Al produces general toxic symptoms that are similar to nutrient deficiencies (Bennet et al., 1986; Taylor, 1988). However, these general symptoms appear to be the consequence of inhibition of root development caused by targeted action of Al at root tips (Ryan et al., 1993). Visible symptoms of Al toxicity include inhibition of root growth (Delhaize and Ryan, 1995), swelling of the root tip, and/or sloughing off of the epidermis. Other responses include plasma membrane depolarization (Olivetti et al., 1995), alteration of Ca2+ fluxes at the root tip (Huang et al., 1992a, 1992b), stimulation of callose deposition (Wissemeier et al., 1987; Schreiner et al., 1994; Zhang et al., 1994), and induction of rigor in the actin cytoskeleton (Grabski and Schindler, 1995). Resistance to Al occurs naturally in cultivars of different crop plants (Foy et al., 1978; Taylor, 1988). Two general forms of resistance are recognized: Al exclusion, in which Al is prevented from entering the root tip, and Al tolerance, in which Al is not excluded but is tolerated in the symptoms (Kochian, 1995). The production of phytochelatins confers heavy metal tolerance in plants (Grill et al., 1985; Rauser, 1990; Howden et al., 1995a, 1995b); however, phytochelatins do not contribute to Al tolerance, most likely because they do not bind Al effectively.

The genetics of Al resistance has been extensively studied in cereal crops. Al resistance in some wheat cultivars is multigenic (Aniol and Gustafson, 1984; Aniol, 1991) but is conditioned by a single dominant gene in other cultivars (Kerridge and Kronstad, 1968; Aniol and Gustafson, 1984; Fisher and Scott, 1987; Larkin, 1987). For example, Al resistance is conditioned by the Al1 gene in the wheat cv Carazinho (Delhaize et al., 1993a). In other Al-resistant cultivars, such as Atlas 66, Al resistance is conditioned by two or more major genes (Berzonsky, 1992). Resistance in certain wheat and maize genotypes has been correlated with the ability to release organic acids, such as malic and citric acid, in response to Al (Delhaize et al., 1993b; Pellet et al., 1995; Ryan et al., 1995a). Released organic acids are thought to complex with Al3+ and prevent its uptake. Al1 in wheat has been shown to condition the Al-inducible release of malic acid (Delhaize et al., 1993a, 1993b). Various wheat genotypes show a range of Al sensitivities (Polle et al., 1978), and Ryan et al. (1995b) found that the major difference among genotypes is their capability to release malate. In screening 36 wheat lines, they found that Al resistance generally correlated with the release of malate from roots in the presence of Al. Wheat genotypes rank ordered with respect to Al resistance generally follow the same rank order with respect to malate release. This suggests that malate release is an important mechanism by which wheat genotypes differ in their capability of resisting the growth-inhibiting effects of Al. More recently, D.M. Pellet, L.A. Papernik, and L.V. Kochian (unpublished re-

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Abbreviations: als, aluminum sensitive; Col-0, Columbia; EMS, ethyl methylsulfonate; I50, 50% inhibitory concentration; Ls-0, Landsberg; Ws-0, Wassilewskija.
sults) found that Al resistance in the Al-resistant line Atlas 66 is associated with constitutive phosphate release in addition to Al-induced malate release from the root apex.

In snapbean (Phaseolus vulgaris) Al tolerance can be induced in cv. Dade but not in cv. Romano (Cumming et al., 1992). Olivetti et al. (1995) recently reported that in response to Al membrane potentials depolarize more in the root-cap cells of cv. Dade than in cv. Romano. They argued that Al reduces K⁺ efflux, presumably via outward-rectifying K⁺ channels, in cv. Dade more than in cv. Romano. This response could be part of the signaling pathway that mediates Al-induced citrate release in cv. Dade.

We have taken a genetic approach to the problem of Al toxicity by identifying Arabidopsis mutants that have increased sensitivity to Al. Arabidopsis shows classic signs of Al toxicity, including severe inhibition of root growth and, at high Al concentrations, root death. Through the analysis of sensitive mutants, we hope to identify genes that encode targets of Al toxicity and genes involved in Al-resistance mechanisms.

MATERIALS AND METHODS

Isolation of Mutants

Mutants were generated in the Arabidopsis thaliana ecotype Col-0 by treating seeds with EMS. M₂ seedlings were screened for mutants with increased sensitivity to Al by using a two-layer, solid-medium system in which the lower layer contained subtoxic levels of AlCl₃.

The two-layer medium was set up by pouring a lower layer consisting of 40 mL of nutrient medium (pH 4.2) plus 0.125% gellan gum (Gel-Gro, ICN Biomedicals) in GA7 Magenta jars (Magenta Corp., Chicago, IL). Nutrient medium consisted of 2 mM KNO₃, 0.2 mM KH₂PO₄, 2 mM MgSO₄, 0.25 mM (NH₄)₂SO₄, 1 mM Ca(NO₃)₂, 1 mM CaSO₄, 1 mM K₂CO₃, 1 mM MnSO₄, 5 μM H₃BO₃, 0.05 mM CuSO₄, 0.2 mM ZnSO₄, 0.02 mM NaMoO₄, 0.1 mM CaCl₂, 0.001 mM CoCl₂, and 1% Suc. Al was introduced by overlaying the solidified lower layer with 20 mL of “soak solution,” consisting of the nutrient solution medium described above but with the following changes: 0.1 mM KH₂PO₄, 1.1 mM K₂SO₄ plus 0.75 mM AlCl₃. To adjust the pH of the soak solution, the amount of base (0.1 N KOH) to be added was determined empirically by adjusting the pH on an aliquot of the soak solution containing 0.75 mM AlCl₃. The amount of base determined from the trial pH adjustment was added to the actual soak solution prior to adding AlCl₃. This was done to prevent the formation of the very toxic polymeric Al species Al₂O₂Al₂(OH)₂₄(H₂O)₁₂⁺, called Al₁₃⁺ (Bertsch et al., 1986; Bertsch, 1987; Parker et al., 1989), at high pH. The soak solution was allowed to equilibrate with the lower layer for 2 d and was then poured off. The lower layer was rinsed with deionized water, and an upper layer consisting of 20 mL of nutrient medium (pH 4.2) plus 0.125% gellan gum was poured onto the lower layer and allowed to gel.

Mutagenized seeds were sterilized and cold stratified (4°C) for 2 d in the dark to synchronize germination. Two hundred M₃ seeds suspended in 0.15% agarose were planted around the periphery of the upper layer in each Magenta jar. The jars were incubated in a growth chamber at 20°C with a day/night cycle of 16 h/8 h. After 6 d, seedlings with roots that grew through the upper layer but did not penetrate the lower layer were marked. Marked seedlings with roots that did not grow significantly during the next 24 h were identified and rescued on plant nutrient medium plus Suc (Lincoln et al., 1990). After 2 weeks of growth, the putative mutants were transferred to soil and grown for seed production in a light room at 20°C, 40% RH, and 50 μE m⁻² s⁻¹ light. The putative mutants were selfed, and M₄ progeny were rescreened using the same two-layer media procedure.

Genetic Analysis

Analysis of inheritance was performed by crossing each als mutant (male parent) to wild-type (Col-0) plants (female parent) bearing the glabrous-1 mutation (used as a crossing marker). Inheritance tests and complementation crosses were analyzed using a single-layer gel medium. Single-layer gels consisted of 85 mL of the nutrient medium (pH 4.2) plus 0.125% gellan gum in 100-× 25-mm Petri dishes. The gel was equilibrated for 2 d with an overlaying soak solution containing 1.0 mM AlCl₃. The soak solution was poured off, and seeds were planted around the periphery of the plate and incubated for 7 d under the previously described growth conditions.

For mapping purposes, als mutants (male parent) were crossed to the Ws-0 ecotype (female parent). Chromosome location was determined by identification of microsatellite markers that co-segregate with the Al-sensitive trait in F₂ progeny (Bell and Ecker, 1994). Map distances were determined using the Mapmaker II program (Lander et al., 1987).

Solution Culture

Arabidopsis seedlings were grown in solution culture in modified GA7 Magenta jars. Polycarbonate blocks glued to the floor of each Magenta jar were used to support a 6-× 6-cm, 250-μm mesh, polypropylene screen (Small Parts, Inc., Miami Lakes, FL). Both screens and Magenta jars were sterilized by autoclaving, and the jars were filled with 55 mL of nutrient medium (pH 4.2). Cold-stratified seeds were sown directly onto this screen in 0.15% agarose. The jars were incubated without aeration under the previously described growth conditions, and after 4 d, one-half of the seedlings were removed for root measurement. At this time, the solution was replaced with nutrient solution containing varying concentrations of AlCl₃ or LaCl₃. For transfer into LaCl₃-containing medium, the seedling roots were rinsed with deionized water and placed in modified nutrient medium (pH 4.2) containing LaCl₃, but without KH₂PO₄ to prevent the precipitation of La. The seedlings were grown for an additional 2 d, after which time the roots of the remaining seedlings were measured.

Morin and Hematoxylin Staining

Morin (2',3',4',5'-pentahydroxyflavanone) and hematoxylin are histochemical indicators used to indicate the presence of Al. For morin staining, roots of seedlings grown in solution culture were exposed for 1 h to 25 μM Morin.
AlCl$_3$ in nutrient medium. The seedlings were washed in Mes buffer (pH 5.5) for 10 min and stained with 100 μM morin in the same buffer for 1 h. Morin fluorescence was visualized using a Zeiss microscope with epifluorescence attachments. Samples were photographed using Kodak Ektachrome Elite 100 slide film. For hematoxylin staining, roots were exposed for 60 min to 75 μM AlCl$_3$ in nutrient medium (pH 4.2), rinsed in deionized water (30 min), and placed for 30 min in hematoxylin stain solution (0.2 g of hematoxylin and 0.02 g of KI$_3$ in 100 mL of deionized water). Roots were then washed again in deionized water (30 min) and photographed.

**Callose Production**

Five-day-old seedlings were exposed to various concentrations of AlCl$_3$ for 24 h, after which they were transferred to fixative containing 10% formaldehyde, 5% glacial acetic acid, and 45% ethanol and vacuum infiltrated for 4 h. Fixed seedlings were stored in 0.1% aniline blue (pH 9.0, 0.1 M K$_3$PO$_4$). Callose production was visualized under conditions described for morin staining.

### RESULTS

**Isolation of Mutants with Increased Al Sensitivity**

als mutants were identified by screening M$_2$ populations of EMS-mutagenized Arabidopsis for seedlings with roots capable of growing normally in the absence of Al but inhibited by the presence of subtoxic levels of Al. A two-layer, gel-medium system was developed for screening. The top gel layer consisted of normal nutrient medium with no added Al, and the bottom gel layer contained nutrient medium equilibrated with a subtoxic level of AlCl$_3$ (Fig. 1). Gellan gum, instead of agar or agarose, was used as a gel matrix because gellan gum solidifies at low pH (pH 4.2) and does not seem to compromise the toxicity of AlCl$_3$-containing medium as much as other gelling agents. Al was introduced into the lower gel layer by soaking the gels with an AlCl$_3$-containing soak solution rather than by adding Al to the medium prior to gelation, because Al toxicity was variable when AlCl$_3$-containing medium was autoclaved with the gellan gum. It also appeared that Al affected the strength of the gel when it formed in the presence of different concentrations of AlCl$_3$. This raised concerns that different gel strengths might influence the rate of root growth independently of the phytotoxic effects of Al.

Another concern in setting up conditions to screen for Al-sensitive mutants related to the control of rhizosphere pH. When these experiments were initiated, pH buffers with pKs of approximately 4 to 4.5 that do not chelate Al were not available. Without adequate buffers, maintaining an acidic pH in the rhizosphere was difficult because the standard Arabidopsis growth medium contains high levels of nitrate as a nitrogen source (Lincoln et al., 1990). Nitrate uptake results in an alkalization of the rhizosphere, and in unbuffered medium, the pH increased from 4.2 to approximately 6.0 during 7 d of growth. The problem was resolved by including ammonium in the growth medium, because ammonium uptake stimulates acidification of the rhizosphere. It was found that a nutrient medium in which the NO$_3^-\cdot$NH$_4^+$ was 8:1 effectively maintained the pH of the medium during the 7 d of growth used for screenings.

To determine the appropriate concentration of AlCl$_3$ to use in the soak solution for mutant screening, AlCl$_3$ dose responses were determined for root growth in wild-type seedlings of different ecotypes, Col-0, La-0, andWs-0 (Fig. 2A). Total AlCl$_3$ concentrations in the soak solution ranged from 0 to 1.5 mM. (These concentrations do not represent the final AlCl$_3$ concentration in the gel but only the total AlCl$_3$ concentration in the solution used to equilibrate with the gel. The total AlCl$_3$ concentration in the gel is not known, but the Al$^{3+}$ activity, as judged by Al toxicity, appears to be much lower in gels than in solution culture. Al$^{3+}$ activity can be more accurately calculated for the medium used in solution culture and is presented in a later section.) After 7 d, root growth in gels equilibrated with 0.50 mM or less AlCl$_3$ was not significantly inhibited, whereas above that concentration, root growth was progressively inhibited. Sensitivity to Al varied somewhat among Arabidopsis ecotypes (Fig. 2A). Ecotype La-0 was more sensitive to Al than were Col-0 andWs-0. For example, in gels equilibrated with 1.0 mM AlCl$_3$, root growth was inhibited by approximately 36% in Col-0 andWs-0, whereas root growth in La-0 was inhibited 64%. (Ecotype variation in Al sensitivity was particularly relevant to the choice of parents used in mapping crosses in a later section.) From the Al dose-response curve for the Col-0 andWs-0 ecotypes, it was concluded that a soak solution containing 0.75 mM AlCl$_3$ would provide a subtoxic level of AlCl$_3$ in a gel layer to be used in screening for Al-sensitive mutants.

To screen for sensitive mutants, mutagenized Arabidopsis seeds were planted on the upper layer of the two-layer system. After 5 d, seedlings with roots that grew...
Tablesensitive. An AlCl₃ dose-response curve for one of the 60 putative mutants from a total of approximately 10⁵ seedlings. Rescreening of M₁ seedlings confirmed that 11 mutants derived from different pools were truly Al-containing medium.

Figure 2. Growth of Arabidopsis roots in gel medium containing Al. Root growth in a one-layer gel equilibrated with growth medium containing various concentrations of AlCl₃. Relative root growth (expressed as a percentage of root growth in the absence of added AlCl₃) was compared among wild-type seedlings in three Arabidopsis ecotypes, Col-0, La-0, and Ws-0 (A), and between wild type (Col-0) and als1-1 (B). Error bars represent SE values (n = 50).

unimpeded through the upper layer but did not penetrate the bottom, Al-containing layer were isolated (Fig. 1). Mutant screening performed for approximately 2500 M₁ seeds from each of 40 mutagenized pools resulted in the isolation of 60 putative mutants from a total of approximately 1 × 10⁵ seedlings. Rescreening of M₂ seedlings confirmed that 11 mutants derived from different pools were truly Al-sensitive. An AlCl₃ dose-response curve for one of the Al-sensitive mutants, als1-1, illustrates its Al-sensitivity compared to Col-0 wild type (Fig. 2B). Concentrations of AlCl₃ that were noninhibitory for wild type caused severe root growth inhibition in als1-1. This was evident in medium equilibrated with 0.50 mM AlCl₃, in which Col-0 wild type showed only a 6% reduction in root length, whereas als1-1 was inhibited by approximately 55%.

Several of the als mutants (als1-1, als3, and als5) appeared to be normal when grown on growth medium or soil with no added Al. Roots on other mutants (als2, als4, als6, als7, and als8) grew more slowly than on wild type. Only als3 showed a pronounced delay in growth when recovered from Al-containing medium.

Genetic Analysis of als Mutants

Inheritance characteristics of the mutants were determined by crossing each mutant (male) to Col-0 glabrous-1 (female). The seeds of the F₁ and the F₂ progeny were analyzed on single-layer plates equilibrated with a 1.0 mM AlCl₃ solution. The F₂ progeny of most mutants segregated 3:1 (wild type:sensitive), as expected for a single recessive mutation, except for als5 (Table I). The mutation in als5 was inherited in a pattern consistent with it being a semidominant mutation (data not shown).

To determine the complexity of the genes that condition the Al-sensitive phenotype in Arabidopsis, nonreciprocal complementation crosses were conducted among nine mutants. F₁ and F₂ progeny were analyzed on single-layer plates equilibrated with a 1.0 mM AlCl₃. It was found that als1-1 and als1-2 were noncomplementing, because, when crossed, the F₂ progeny were more sensitive to Al than wild type (Fig. 3B). These two mutants constitute the als1 complementation group (Table I). All of the other mutants complemented each other to some extent. An example of complementation is shown in the F₂ progeny from the cross of als1-1 × als7. If the mutants complement each other, then the expected ratio of wild type to sensitive progeny in this cross should be 9:7. The actual distribution of root lengths in the progeny supports that prediction even though the distribution of root lengths in the resistant progeny appears to be broad (Fig. 3C).

Complementation analysis for the group of nine mutants showed that they belonged to eight different

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<th>Table 1. Characteristics of als mutants</th>
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<sup>a</sup> Inhibition of root growth during 2 d of exposure to 35 μM AlCl₃ in solution culture.  
<sup>b</sup> Inhibition of root growth during 2 d of exposure to 5 μM LaCl₃ in solution culture.  
<sup>c</sup> Not applicable.  
<sup>d</sup> n = 50.  
<sup>e</sup> Experiment 1 is not in parentheses; experiment 2 is in parentheses.

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Figure 3. Complementation analysis of als mutants. Segregation of AI-sensitive phenotype in F2 progeny from crosses used for complementation analysis. All seedlings were grown in a one-layer medium equilibrated with 1.0 mM AlCl3. Distribution of progeny according to root lengths in wild type (wt, Col-0) and als1-1 parent seedlings (A), in F2 progeny from a cross of als1-1 x als1-2 compared to the als1-2 parent (B), and in F2 progeny from a cross of als1-1 x als7 compared to the als7 parent (C). n = 50 for P1; n = 200 for F2.

complementation groups, als1 to als8, with two mutants in the als1 complementation group (Table I). Thus, the als mutants are a genetically complex group of mutants. More mutations will be needed to saturate the loci that condition this trait.

Growth of als Mutants in Solution Cultures Containing AI

As stated previously, Al3+ appears to bind to or interact with the matrix in agar or gellan gum gels, effectively lowering the phytotoxicity and the apparent Al3+ activity in solid-gel medium. Therefore, als mutants were grown hydroponically in solution culture, in which Al3+ activity can be more accurately determined. Seedlings were germinated and grown for 4 d in the full nutrient solution in the absence of added AlCl3, after which one-half of the seedlings were removed from culture and their roots were measured. The remaining one-half were grown an additional 2 d in varying concentrations of AlCl3 (0-100 μM) in nutrient solution. Root growth (relative increment of root length) during 2 d in response to Al dose was determined for three different mutants and compared to wild type (Fig. 4). For wild-type (Col-0) seedlings, root growth steadily declined with increasing concentrations of AlCl3 above 10 μM AlCl3. Between 5 and 10 μM AlCl3, growth of wild-type roots was stimulated. At 50 μM AlCl3, root growth in wild-type seedlings was inhibited by 50% (L50 = 50 μM), and at 100 μM root growth was inhibited by 92%. The Al3+ activity in medium containing 50 μM AlCl3 calculated using GEOCHEM-PC (Parker et al., 1987) was 3.9 μM, indicating that the Al sensitivity in Arabidopsis is comparable to that in crop plants (Kochian, 1995).

As expected, the als mutants als1-1, als2, and als4 were significantly more sensitive to Al (L50 = 15-30 μM) than wild type (Fig. 4). At 50 μM AlCl3, root growth in Col-0 wild type was one-half maximally inhibited; however, root growth in each tested als mutant was completely inhibited. It is interesting that the stimulation in root growth seen in Col-0 wild type at low AlCl3 concentrations was also observed in both als2 and als4 but not in als1-1. At 10 μM AlCl3, root growth in als1-1 was slightly inhibited, whereas root growth in als2 and als4 seedlings was stimulated 25% more than at 0 μM AlCl3. For the other als mutants, root growth inhibition was measured in response to 35 μM AlCl3 (Table I). This survey demonstrated that in solution

Figure 4. Root growth response of als mutants to Al in solution culture. Growth of roots of wild type (wt, Col-0), als1-1, als2, and als4 seedlings with their roots in liquid nutrient medium. Seedlings were grown for 4 d in solution culture in the absence of added AlCl3, and then for an additional 2 d (6 d) in varying concentrations of AlCl3. Root growth (increments of root length) in the presence of Al was expressed as (RL d 6 - RL d 4)/RL d 4, where RL is root length. Data are expressed as relative increment of root length (RIRL), where the increment of root length at zero AlCl3 concentration has been normalized to 100. Error bars represent ses (n = 50).
culture all of the als mutants were more sensitive to Al than wild type.

**Growth of als Mutants in Solution Cultures Containing La**

To determine whether the sensitivity to Al in the als mutants was Al specific, the mutants were grown in solution culture in the presence of another trivalent cation, La$^{3+}$ (as LaCl$_3$). La causes symptoms similar to Al toxicity (Kinraide et al., 1992). In wheat, most Al-tolerant lines are not tolerant to La, indicating that mechanisms that exclude Al in tolerant wheat lines do not protect from La toxicity (Kinraide et al., 1992; Ryan et al., 1995a).

Root growth in wild-type Arabidopsis seedlings was quite sensitive to LaCl$_3$ ($I_{50} = 7-8 \mu M$) (not shown). Most of the als mutants were similar to wild type with respect to their sensitivity to 5 $\mu M$ LaCl$_3$ (0.9 $\mu M$ activity), an La concentration in which root growth in wild type was inhibited by about 25% (Table I). Root growth in als2 was more sensitive to La than wild type and was inhibited by about 60% in 5 $\mu M$ LaCl$_3$. Therefore, Al sensitivity in als2 does not appear to be Al specific. Surprisingly, root growth in als5 was more resistant to La than wild type (Table I).

**Al Uptake Assessed by Al Indicator Stains**

Morin fluorescence has been used as a means to detect the presence of Al in plant tissue after short-term exposure to Al (Tice et al., 1992). Morin is a fluorescent dye with a high sensitivity for Al. In Arabidopsis, morin staining was barely visible in root tips not exposed to Al but quite vivid in roots exposed to 25 $\mu M$ AlCl$_3$ for 1 h (Fig. 5). Exposure to 25 $\mu M$ AlCl$_3$ was used to examine Al uptake in other als mutants, because this Al concentration was well below saturation for morin staining (data not shown). Surprisingly, root tips on most of the sensitive mutants did not show enhanced morin staining (Table I). Only one mutant, als5, stained more intensely than wild type (Fig. 5), consistent with the expectation for an als mutant that might be unable to exclude Al. Quite unexpectedly, two of the als mutants, als4 and als7, showed less morin staining in the root tip compared to wild type. We attempted to determine whether the differences between wild type and the mutants could be amplified by desorbing Al from the root cell walls with 0.5 mM sodium citrate after Al exposure. However, we found that desorption of roots with citrate had no effect on the intensity or pattern of morin fluorescence (data not shown).

Hematoxylin staining has also been used as an indicator of Al accumulation (Polle et al., 1978; Delhaize et al., 1993a). An Al dose-response analysis was carried out on wild-type seedlings, and it was found that following 1 h of exposure to 75 $\mu M$ AlCl$_3$ hematoxylin staining was easily visible and yet the staining was not saturating. The pattern and intensity of staining for most of the als mutants was similar to wild type (data not shown). Consistent with the morin staining, als5 stained more intensely with hematoxylin than wild type, and als7 did not (data not shown). The only inconsistency between hematoxylin and morin staining was in Als7, which showed intensely with hematoxylin than wild type, and als7 stained more intensely with hematoxylin than wild type. We did not observe any enhanced staining of Als7 mutant seedlings with Al but intense staining with hematoxylin. Therefore, Al sensitivity in als7 does not appear to be Al specific. Surprisingly, root growth in als5 was more resistant to La than wild type (Table I).

**Callose Accumulation**

Callose is accumulated in response to damage caused by Al in the roots of other plants (Wissemeier et al., 1987; Schreiner et al., 1994; Zhang et al., 1994). We investigated whether callose accumulated in the roots of wild-type Arabidopsis and various als mutants in response to treatment with AlCl$_3$ in solution culture. Five-day-old seedlings grown in the absence of added AlCl$_3$ were transferred to nutrient medium containing various concentrations of AlCl$_3$ and grown for an additional 24 h. Seedlings were fixed, stained with aniline blue, and examined by fluorescence microscopy for callose accumulation. Increasing callose fluorescence in response to AlCl$_3$ was observed in roots of Col-0 wild-type seedlings incubated in nutrient medium containing AlCl$_3$ at concentrations equal to or greater than 25 $\mu M$ (not shown). The presence of als mutant seedlings...
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Figure 6. Callose accumulation in root tips of Al-treated mutant seedlings. Five-day-old seedlings in solution culture were grown for an additional 24 h in nutrient medium containing 25 μM AlCl₃, except in the first panel where no Al was added (−Al). Seedlings were fixed and stained with 0.1% aniline blue (pH 9.0). Wild type (wt) is of the Col-0 ecotype.

was examined at 25 μM AlCl₃, a concentration that induced moderate callose accumulation in wild type (Fig. 6) and yet inhibited root elongation in the als mutants (Fig. 4). In response to 25 μM AlCl₃, callose accumulated much more in als5 than wild type, much less in als4 and als7, and somewhat less in als2 and als8 (Fig. 6). Although callose staining was much lighter generally in als7 root tips, there was significant staining localized to the presumed promeristem region of the root. Therefore, in only one of the als mutants was greater Al sensitivity in root growth correlated with greater Al induction of callose accumulation (Table I).

Chromosome Mapping of als1 and als4

Two mutants from different complementation groups (als1 and als4) were chosen for further study and mapped on the Arabidopsis genome. For mapping purposes, mutants in the Col-0 background (male) were crossed to wild type (female) of the Ws-0 ecotype. This ecotype was chosen for a mapping partner because roots on Ws-0 seedlings have Al sensitivities similar to Col-0 roots (Fig. 2A). Since most of the als mutants were recessive, homozygous Al-sensitive F₂ progeny from each cross were obtained by selecting for short roots on single-layer plates equilibrated with 1.0 mM AlCl₃. The genotypes of the F₂ progeny were confirmed by analyzing F₃ families under similar selection conditions.

In mapping crosses, als1-1 co-segregated with markers toward the bottom of chromosome 5, near the microsatellite marker nga129 (8–9 centimorgans), whereas als4 co-segregated with markers toward the middle of chromosome 5 and mapped near the microsatellite marker nga151 (2–3 centimorgans) (Bell and Ecker, 1994). The separate chromosome locations are consistent with the observation that the two mutants belong to separate complementation groups (Table I).

DISCUSSION

Al Sensitivity in Arabidopsis

To our knowledge, this study is the first report in plants of mutants selected for altered responses to Al. Prior investigations of other plants focused on Al tolerance in natural cultivars or varieties. In this study we selected for Al sensitivity rather than Al resistance, with the goal of finding mutants either with defects in resistance mechanisms or with alterations in the targets of Al toxicity. Al sensitivity has not been sought as a trait, most likely because Al resistance and not Al sensitivity has agronomic value. However, it is our contention that mutations that confer Al sensitivity might occur in genes that condition Al resistance in their normal state. Therefore, mutations in genes that confer Al sensitivity might be as important in understanding Al resistance as are mutations that endow Al resistance.

An assumption made in undertaking this study was that wild-type forms of the standard Col-0 ecotype have mechanisms that confer some degree of Al resistance and that loss-of-function mutations can be found that render seedlings more sensitive to Al. The recovery of recessive Al-sensitive mutations is consistent with the assumption that loss-of-function mutations can be found that render the standard ecotype more sensitive to Al. The finding that mutants analyzed so far constitute eight different complementation groups indicates that the group of Al-sensitive mutants are genetically complex and that many more mutants will be needed to saturate the group. However, two alleles were recovered in one complementation group, als1. Given the apparent complexity of the trait, it was surprising that mutants were not found at a higher frequency in mutagenized populations. It is possible that only severe alleles can be recognized.

We have also selected for Al-resistant Arabidopsis mutants (P.B. Larsen, unpublished results). Unlike most of the sensitive mutants, all of the resistant mutants were found to be semidominant. Furthermore, it appears that Al resistance may be less complex genetically, because fewer different mutants have been recovered from that screen. As
yet, we have not found any resistance mutations that map to loci conferring sensitivity.

**Al Accumulation and Toxicity Responses**

Following short-term exposure to Al, some of the Arabidopsis *als* mutants characterized in this study show significant differences from wild type in morin and hematoxylin staining (Table I). Both stains have been used to indicate the presence of Al (Tice et al., 1992; Delhaize et al., 1993b). Morin is a fluorochrome with sensitivity to Al, and hematoxylin is a stain for which Al serves as a mordant for binding. One of the *als* mutants in this study stains in a manner that might be expected for mutants that are less capable of excluding Al. Roots on *als5* stain more intensely with morin and hematoxylin and, therefore, appear to take up Al more readily than wild type. This suggests that *als5* may have a defect in a process that excludes Al from the root tip. Many of the *als* mutants exhibited hematoxylin and morin staining that was similar to wild type. It is possible that the defects in these mutants condition greater sensitivity to cellular targets of Al toxicity rather than affecting mechanisms of Al exclusion. Contrary to expectations, morin and hematoxylin staining in *als7* was less intense than wild type, suggesting that this mutant accumulated less Al compared to wild type. A possible way to reconcile these observations with the Al sensitivity in *als7* is that the cell wall might be the site of Al toxicity, and the cell walls in *als7* might bind Al more avidly than wild type. Enhanced binding in the cell-wall fraction could reduce the availability of Al for uptake into the symplasm during short-term Al exposure. This would result in reduced morin fluorescence, since it appears that morin interacts with Al in the symplasm (Tice et al., 1992). These findings point out the need to quantify Al accumulation in roots by appropriately sensitive analytical methods, such as inducibly coupled plasma MS. Nonetheless, the staining behavior of the roots does indicate that Al sensitivity cannot be assessed in all cases purely by hematoxylin- or morin-staining behavior.

Al causes visible injury in roots accompanied by the deposition of callose. Callose is a particularly sensitive indicator of Al toxicity because callose accumulation in roots can be readily observed by fluorescence microscopy, as it has been in soybean, wheat, and maize (Wissemeier et al., 1987; Lugany et al., 1994; Schreiner et al., 1994; Zhang et al., 1994). We have also observed callose accumulation in wild-type Arabidopsis seedlings in response to Al. Increasing callose deposition in roots was observed with increasing Al concentrations over a range of 0 to 100 μM AlCl₃, consistent with a correlation between the degree of Al-induced root growth inhibition and callose accumulation. Along these lines, *als5* accumulates more callose than wild type at AlCl₃ concentrations that are only slightly inhibitory to wild type. However, this correlation does not hold for the other *als* mutants, particularly for *als4* and *als7*, in which root growth is blocked by concentrations of AlCl₃ (25 μM) that do not induce significant callose deposition. This suggests that mechanisms other than callose deposition are responsible for the blockage of root growth by Al.

**Al Specificity of *als* Mutants**

The metal specificity of most of the *als* mutants in this study is specific for Al, relative to La. La³⁺ is another trivalent cation that produces toxicity symptoms in plants similar to Al³⁺. Only *als2* showed greater sensitivity to La than wild type, suggesting that the sensitivity defect in *als2* may not be Al specific. Unexpectedly, *als5* was more resistant to La than wild type. As described above, *als5* appears to be less capable of excluding Al. That property may be related to an ability to exclude La; however, we have not measured La accumulation in these mutants and do not understand the basis for this increased resistance. The specificity of the sensitivity to Al indicates that specific targets of Al toxicity or mechanisms of Al resistance have been altered in these mutants. Efforts are underway to discover the nature of these targets or mechanisms.

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