Identification of dominant mutations that confer increased aluminium tolerance through mutagenesis of the Al-sensitive Arabidopsis mutant, als3-1

Kelly M. Gabrielson, Jesse D. Cancel, Luis F. Morua and Paul B. Larsen*

Department of Biochemistry, University of California, Riverside, CA 92521, USA

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Abstract

Aluminium (Al) toxicity is a global agricultural problem that occurs in acid soil environments and severely limits root growth and crop productivity. The isolation and characterization of a gene, ALS3, which is absolutely required by Arabidopsis seedlings for growth in an Al-toxic environment was reported previously. Since the als3-1 loss-of-function mutant has extreme root growth inhibition even in the presence of very low levels of Al, it was an excellent candidate for using a mutagenesis approach to identify suppressor mutations that would increase either Al resistance or tolerance in Arabidopsis roots. EMS-mutagenized als3-1 seedlings were screened for mutants that could sustain root growth in an Al-containing environment that is highly toxic to als3-1 but not Col-0 wt. This approach resulted in identification of 12 strong suppressor mutants that reversed the als3-1 phenotype and grew as well or better than Col-0 wt in the presence of high levels of Al. Subsequent analysis of three representative suppressor mutants revealed that the phenotype of each probably arises from dominant gain-of-function mutations at the same locus. Detailed analysis of one of these, alt1-1 (Al tolerant), suggests that this mutation positively impacts Al resistance in a manner dependent on pH adjustment rather than enhanced Al exclusion. Identification of these suppressor mutations, should not only further elucidate the biochemical and molecular mechanisms underlying Al toxicity and tolerance but also will develop a collection of mutations that may be useful for engineering crop plants that can grow and thrive in Al-toxic environments.

Key words: ALS3, alt, aluminium, Arabidopsis, root.

Introduction

Aluminium toxicity in acid soils is a worldwide problem that severely limits agricultural productivity primarily through the inhibition of root growth. Greater than 30% of the world’s agriculturally available land is considered to be acidic (von Uexkull and Mutert, 1995), which leads to speciation of Al to the phytotoxic Al^{3+} cation (Kochian, 1995). The consequences of the Al^{3+} ion are severe for root growth, especially at the root tip, which is thought to be one of the most important sites of Al toxicity (Kochian, 1995). These consequences include inhibition of cell division and cell elongation coupled with severe morphological damage after long-term exposure. At the cellular level, targets of the Al^{3+} ion are both apoplastic and symplastic, including cell wall components (Tabuchi and Matsumoto, 2001), members of signalling pathways (Jones and Kochian, 1995), the cytoskeleton (Grabiski and Schindler, 1995; Blancaflor et al., 1998), metabolic enzymes, and membrane constituents (Jones and Kochian, 1997).

Although much is known about the biochemical targets of Al^{3+}, current understanding of how plants cope with Al in their environment remains limited. A significant advance in this understanding came with the discovery that Al-resistant roots of wheat and maize release Al-chelating organic acids, specifically malate or citrate, into the rhizosphere following Al exposure and that these organic acids serve to protect growing roots from Al-dependent damage through Al exclusion (Delhaize et al., 1993; Pellet et al., 1995; Ryan et al., 1995a, b). Subsequently, a gene that encodes a malate transporter, ALMT1, was identified from wheat and it was found that overexpression of this in barley conferred a substantial increase in malate exudation and, consequently, Al resistance for barley roots (Delhaize et al., 2004; Sasaki et al., 2004), indicating that
this approach may be useful for genetic engineering of Al-resistant crop plants.

Besides the release of Al-chelating organic acids, it is also speculated that plants may resist toxic levels of Al in their environment by raising the pH of the rhizosphere. Since Al toxicity is dependent on speciation in an acidic environment, it is likely that plants that are capable of increasing the pH of the environment would force solubilized Al to speciate to less toxic forms, thus reducing the overall Al toxicity of the environment. Although never described as a mechanism utilized by plants outside of the laboratory, a mutagenesis approach using Arabidopsis to identify mutations that could confer increases in Al resistance did identify one mutant, alr-104, that could grow better in an Al-toxic environment due to an increased capability to alkalinize the rhizosphere (Degenhardt et al., 1998; Larsen et al., 1998).

A third strategy for coping with environmental Al involves true tolerance of internalized Al. Several plant species have been found to accumulate very high levels of Al in their leaves. This Al tolerance is dependent on uptake and redistribution of chelated Al to tissues that are less sensitive to Al toxicity. Physiological studies have revealed that Al can exist as either an oxalate or citrate complex during transport and storage, suggesting that complex formation significantly reduces the toxic effects of internalized Al (Ma et al., 1997, 1998; Zheng et al., 1998; Ma, 2000).

Although progress has been made toward describing the biochemical basis for Al-resistance mechanisms, as shown by ALMT1, understanding of how these mechanisms function remains incomplete, especially with regard to the constituents of these mechanisms. Because of this, Arabidopsis thaliana is being used as a model system to identify genes that encode factors that are required for root growth in an Al-toxic environment. This approach has relied on the identification of als (Al-sensitive) mutants, which have roots that are incapable of growth in the presence of concentrations of AlCl3 that have little to no effect on wild-type (wt) root growth (Larsen et al., 1996, 1997, 2005). The als3-1 mutation has been cloned (Larsen et al., 2005), with this negatively impacting a gene that encodes a putative ABC transporter that is required for Al tolerance in Arabidopsis and possibly in plants in general. ALS3 has been extensively characterized and was found to localize primarily to the plasma membrane of phloem sieve tube elements throughout the plant along with the root epidermis (Larsen et al., 2005). Based on the als3 loss-of-function phenotype and its localization, ALS3 is thought to mediate the redistribution of Al from the root to less Al-sensitive tissues, including the hydathodes, possibly for exudation of Al.

The als3-1 mutant has given a unique opportunity to identify mutations that confer increased Al resistance and/or tolerance. Although Arabidopsis was previously used to identify Al-resistant mutants, this work was eventually abandoned due to the extreme difficulty regarding fine scale mapping of the dominant als mutation (Degenhardt et al., 1998; Larsen et al., 1998). This difficulty primarily arose from an inability consistently to follow the Al-resistance phenotype in mapping populations, which made it impossible to isolate the dominant mutations of interest. Since the als3-1 mutant is extremely Al hypersensitive compared with wt, the als3-1 mutant was mutagenized in order to overcome this issue, with the expectation being that it will be possible to generate large genuine mapping populations which will allow rapid cloning of the mutations of interest. By taking the approach of identifying suppressors of als3-1, it is expected that mutations will be identified that will be useful not only for isolating factors previously not known to be involved in Al resistance and/or tolerance mechanisms, but also for giving new strategies to engineer crop plants that are capable of strong root growth in an Al-toxic environment.

Materials and methods

EMS mutagenesis of als3-1

Approximately 50 000 als3-1 seeds, which were also homozygous for the glabrous loss-of-function mutation (Oppenheimer et al., 1991), were mutagenized overnight in the dark using 0.3% EMS (Sigma Aldrich, St Louis, MO) for 15 h. Mutagenized seeds were subsequently washed several times with deionized water and planted in 118 M1 pools consisting of approximately 500 seeds each. M2 seeds were collected from this population and approximately 2000 seeds from each pool were screened for capability to grow on nutrient gel plates soaked with 0.75 mM AlCl3 (pH 4.2). From the approximately 250 000 M2 seeds that were screened, putative suppressor mutants were identified, allowed to self-fertilize, and M3 seeds were re-screened on nutrient gel plates soaked with either 0.75 mM or 1.50 mM AlCl3 (pH 4.2).

Plants and growth conditions

For all seedling growth experiments, Col-0 wt, als3-1, and suppressor mutant seeds were surface-sterilized and cold stratified at 4 °C for 4 d in the dark to synchronize germination. Unless otherwise indicated, all experiments with alt1-1 were performed using alt1-1 in the als3-1 mutant background. Seeds were then suspended in 0.15% (w/v) agarose and sown on either soaked gel plates or hydroponic plates. For soaked gel plates, nutrient medium (pH 4.2) consisted of 40 ml of 1 mM KNO3, 0.2 mM KH2PO4, 2 mM MgSO4, 0.25 mM (NH4)2SO4, 1 mM Ca(NO3)2, 1 mM CaSO4, 1 mM K2SO4, 1 mM MnSO4.5 H2O, 0.05 μM CuSO4, 0.2 μM ZnSO4, 0.02 μM NaMoO4, 0.1 μM CaCl2, 0.001 μM CoCl2, 1% sucrose, and 0.125% Gellan gum (Gel-Gro, ICN). For Al experiments, the solidified nutrient medium was equilibrated for 2 d with 25 ml of the nutrient medium, without agar, with or without AlCl3 (pH 4.2), after which the soak solution was removed and seeds were planted and allowed to grow for 7 d. For dose–response analysis in a hydroponic environment, seedlings were grown on 250 μm mesh polypropylene screens in 100×15 mm Falcon X-plate dishes with 40 ml of nutrient medium (pH 4.2) containing 5 μM LaCl3 or varying concentrations of AlCl3. For dose–response analysis in the presence of a buffer, plants were grown hydroponically as described either in the presence or absence of 10 mM Homo-PIPES (Fluka Chemical). All dose–response analyses...
were performed in a Percival 136LLVL plant growth chamber under a 24 h light cycle at 20 °C. All adult plants were grown in soil under a 24 h light cycle at 20 °C in a plant growth room supplemented with Sylvania Gro-Lite fluorescent bulbs.

Northern analysis
For determination of Al-dependent gene expression in mutant and Col-0 wt roots, plants were grown hydroponically (pH 4.2) for 7 d. Following this, plants were transferred to nutrient media containing either no or 25 µM AlCl₃ (pH 4.2) for 24 h, after which tissue was collected and frozen for RNA extraction. Total RNA was extracted from plant tissue using the RNeasy Plant Mini Kit (Qiagen, Valencia, CA). For all experiments, 10 µg of total RNA were separated by electrophoresis in a 1% (w/v) denaturing agarose gel and subsequently transferred to Zeta-Probe GT Blotting Membrane (Bio-Rad, Hercules, CA). 32P-labelled probes were generated using the Prime-a-Gene labeling system (Promega, Madison, WI). Prehybridization and hybridization were both carried out at 42 °C and washes were done at 42 °C and 65 °C following the manufacturer’s instructions. Results were visualized by autoradiography.

Visualization of callose accumulation
Seedlings of Col-0 wt, als3-1, and alt1-1 were grown in hydroponic media (pH 4.2) for 7 d and subsequently transferred to hydroponic media that contained 25 µM AlCl₃ (pH 4.2) for 24 h. Following treatment, seedlings were transferred to fixative containing 10% formaldehyde, 5% glacial acetic acid, and 45% ethanol and vacuum infiltrated for 4 h. Fixed seedlings were stained with 0.1% aniline blue (pH 9.0, 0.1 M K₃PO₄). Levels of callose were qualitatively determined as described by Larsen et al. (1996).

ICP-OES analysis
For quantification of total Al, roots of 5-d-old wt and als3-1 grown hydroponically were exposed to 25 µM AlCl₃ (pH 4.2) for 2 d, after which they were washed with water. Following this, the distal 25% (approximately 3–4 mm) of sample roots was harvested, dried, weighed using a microbalance, and ashed in pure nitric acid. Samples were resuspended in 5 ml of 1% nitric acid and analysed using a Perkin-Elmer Optima 3000 DV ICP-OES.

Determination of alt1-1 map position
A mapping population was generated from a cross of alt1-1 (male; Col-0 background) to als3-1 (female; introgressed into the La-0 background). F₂ seedlings were grown on nutrient medium soaked with 0.75 mM AlCl₃ (pH 4.2) for 7 d and those that displayed the als3-1 phenotype, consisting of severely inhibited root and shoot growth, were isolated and planted in soil for subsequent collection of leaf tissue and isolation of genomic DNA. Genomic DNA was prepared as described (Larsen and Cancel, 2003) and used as a template for PCR-based mapping. Linkage was determined based on rate of appearance of La-0 polymorphisms for each polymorphic marker.

Results
Identification of als3-1 suppressor mutants
Seeds of the Arabidopsis als3-1 mutant, which has roots with extreme sensitivity to low concentrations of AlCl₃, were treated with the chemical mutagen ethyl-methane sulphonate (EMS) to induce nucleotide substitutions. M₂ seedlings were screened (~250 000 total M₂ seeds) for suppressor mutants that had the capability to grow in a soaked gel environment containing 0.75 mM AlCl₃ (pH 4.2), which is a concentration that almost completely inhibits als3-1 root growth, but has little effect on Col-0 wt growth (Larsen et al., 1997). From this suppressor screen, 161 putative Al tolerant (alt) mutants with significantly increased root growth compared with als3-1 were identified.

Putative alt mutants were subsequently rescreened by growing these for 7 d in a soaked gel environment (pH 4.2) that contained no, 0.75 mM, or 1.50 mM AlCl₃, the latter of which is highly toxic to both als3-1 and Col-0 wt (Larsen et al., 1996). From this analysis, it was found that 50 putative mutants no longer displayed the suppressor phenotype, suggesting that the previously observed increase in root growth for these resulted from environmental variability or conditioning due to the high density of seedlings in the primary screen. A second group of 99 putative alt mutants displayed root growth that was greater than that of als3-1 at 0.75 mM AlCl₃ but little to no growth at 1.50 mM AlCl₃, indicating that these were weak suppressor mutants that will not be initially pursued. A third group of 12 putative mutants displayed strong root growth at both 0.75 mM and 1.50 mM AlCl₃, indicating that these suppressor mutants represent molecular changes that enhance Al resistance and/or tolerance beyond the level observed for Col-0 wt. Interestingly, all but one of the 12 strong alt mutants segregated for the als3-1 suppressor phenotype in the M3 generation, indicating that these represent dominant mutations.

Genetic analysis of suppressors of als3-1
Of the group of 12 strong suppressor mutants, three were initially chosen for characterization as the first step in cloning the respective mutations. Each of these (male) was crossed to the als3-1 mutant (female) and the F₁ and F₂ progeny from the crosses were analysed by growth in a soaked gel environment supplemented with 0.75 mM AlCl₃ (pH 4.2). For the three suppressor mutants, F₁ progeny grew similar to or better than Col-0 wt, with none of the progeny demonstrating an als3-1 phenotype, indicating that each mutation is dominant in nature. Analysis of the F₂ progeny from each cross resulted in segregation ratios of approximately 3:1 (suppressor mutant phenotype:als3-1 loss-of-function phenotype) further demonstrating the dominance of each mutation (Table 1).

Table 1. Dominance analysis for three als3-1 suppressor mutants

<table>
<thead>
<tr>
<th>Cross</th>
<th>Number of progeny</th>
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<tr>
<td></td>
<td>Suppressor phenotype</td>
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<tr>
<td>line 1-6 x als3-1</td>
<td>111</td>
</tr>
<tr>
<td>line 23-4 x als3-1</td>
<td>109</td>
</tr>
<tr>
<td>line 63-2 x als3-1</td>
<td>123</td>
</tr>
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Chi-square analysis indicated that there was no significant difference between the predicted and observed ratios for this $F_2$ population.

For complementation analysis, the initial three suppressor mutants examined were crossed to each other and $F_2$ progeny were tested for appearance of the $als3-1$ root phenotype in the presence of levels of Al that are toxic only to $als3-1$ roots. From this analysis of each cross, no $F_2$ progeny presented the severe Al-dependent root growth inhibition associated with $als3-1$, suggesting that these three initial suppressor mutations are probably allelic (Table 2), although the dominant nature of these makes it almost impossible to determine by genetic analysis if these are indeed allelic or are closely linked mutations at independent loci. Because of the likelihood that these three suppressor mutants are allelic based on complementation analysis and map position, line 23-4, which displayed the best growth in the presence of Al and will be referred to as $alt1-1$ for the remainder of this report, was chosen for further intensive examination to determine the biochemical basis for the observed increase in Al resistance associated with it.

**Al-responsive root growth of $alt1-1$**

Col-0 wt, $als3-1$, and the suppressor mutant $alt1-1$ were grown in both soaked gel and hydroponic environments supplemented with a range of Al concentrations to demonstrate not only that the $alt1-1$ mutation reverses the Al hypersensitivity associated with $als3-1$ but also confers greater than wt root growth at very high Al concentrations. For soaked gel analysis, seedlings of Col-0 wt, $als3-1$, and $alt1-1$ were grown for 7 d in medium (pH 4.2) that was equilibrated with either no Al or a range of Al concentrations up to 2.0 mM, after which root length was measured. For analysis of growth in a hydroponic environment, seedlings were grown similarly except that the Al concentration range used was from 0–50 $\mu$M since there was no gel medium in this experimental setup to bind to Al. For both experiments, it was found that the $alt1-1$ mutation effectively reverses the hypersensitivity of $als3-1$ roots and gives greater than wild-type root growth at concentrations of Al that are highly inhibitory to Col-0 wt (Fig. 1) as shown by the large difference in root growth compared with Col-0 wt at 35 $\mu$M AlCl$_3$ (pH 4.2) in a hydroponic environment. It should be noted that for these particular

| Table 2. Complementation analysis for three $als3-1$ suppressor mutants |
|---------------------------------|-----------------|-----------------|
| Cross                           | Number of progeny | Suppress phenotype | $als3-1$ phenotype |
| line 1-6 × line 23-4            | 118              | 0                |
| line 1-6 × line 63-2            | 117              | 0                |
| line 23-4 × line 63-2           | 129              | 0                |
analyses, the als3-1 mutation was still present in a homozygous state in alt1-1 and after prolonged exposure to high levels of Al, there was at least partial manifestation of the als3-1 loss-of-function phenotype with regard to Al-dependent inhibition of leaf expansion.

In order to determine if the alt1-1 phenotype is Al specific, its capability for growth in the presence of La, which is another toxic trivalent cation, was tested. Seedlings of Col-0 wt, als3-1, and alt1-1 were grown hydroponically in the presence or absence of 5 μM LaCl3 (pH 4.2), which is a level of La that is highly inhibitory to Arabidopsis root growth. After 7 d, root length was measured for all samples (Fig. 2). It was determined from this that alt1-1 roots were as sensitive to La toxicity as Col-0 wt and als3-1, with each of these exhibiting similar levels of inhibition when grown in the presence of La.

It was also of interest to determine if the recessive als3-1 mutation had any impact on the Al-tolerance phenotype conferred by the dominant alt1-1 mutation. To assess this, alt1-1 was crossed to Col-0 wt and als3-1. F1 seeds were collected and their capability to grow in the presence of 1.5 mM AlCl3 in a soaked gel environment (pH 4.2) was determined. For this experiment, F1 seeds from the cross of alt1-1 to Col-0 wt were heterozygous for both alt1-1 and als3-1 whereas F1 seeds from the cross of alt1-1 to als3-1 were heterozygous for alt1-1 and homozygous for als3-1. It was found that when als3-1 was in a heterozygous state, there was a significant increase in the effect of the alt1-1 mutation on root growth in the presence of Al (Fig. 3) indicating that having the als3-1 mutation in a homozygous state in the alt1-1 mutant has a measurable deleterious effect. This suggests that the effect of the alt1-1 mutation on growth in the presence of high levels of Al is larger than what was observed when the als3-1 mutation is present.

Callose accumulation as an assessment of Al-dependent damage

In order to determine if the basis for the alt1-1 phenotype was dependent on altered Al uptake, several physiological

Fig. 1. Growth of alt1-1 in an Al toxic environment. (A) Col-0 wt, als3-1, and the als3-1 suppressor mutant alt1-1 were grown for 7 d on nutrient media (pH 4.2) that was infiltrated with either no or increasing concentrations of AlCl3 ranging from 0.25 mM to 2.0 mM. Following growth, root lengths were measured for all treatments. Mean ±SE values were determined from 30 roots for each treatment. T-tests were performed by comparing either als3-1 or alt1-1 to the value of the relevant Col-0 wt sample from the same treatment: An asterisk indicates that a significant difference was found with P < 0.01.

Fig. 2. Growth of alt1-1 roots in the presence of La3+. Col-0 wt, als3-1, and alt1-1 were grown for 7 d either in the presence or absence of 5 μM LaCl3 (pH 4.2), after which root growth was measured for all samples. Mean ±SE values were determined from 30 roots for each treatment. T-tests were performed by comparing either als3-1 or alt1-1 to the value of the relevant Col-0 wt sample from the same treatment: An asterisk indicates that a significant difference was found with P < 0.01.

Fig. 3. Effects of the als3-1 mutation on alt1-1 root growth in the presence of Al. alt1-1 in the als3-1 background was crossed to either Col-0 wt or als3-1. F1 seeds were collected and grown in the presence of 1.5 mM AlCl3 in a soaked gel environment (pH 4.2) for 7 d after which root growth was measured. Mean ±SE values were determined from 20 roots for each treatment. T-tests were performed by comparing either als3-1 or alt1-1 to the value of the relevant Col-0 wt sample from the same treatment: An asterisk indicates that a significant difference was found with P < 0.01.
aspects that are accepted indirect measures of Al exclusion were analysed. Callose accumulation has been described as an excellent qualitative indicator of Al-dependent damage and reduced callose production is correlated with an increased capacity for Al exclusion in maize, wheat, and Arabidopsis. Al-treated roots of Col-0 wt, als3-1, and alt1-1 were qualitatively examined to determine whether they produce callose following Al exposure (Fig. 4). It was found that all three were indistinguishable with regard to callose production, with each accumulating significant levels of callose at an AlCl$_3$ concentration that results in profound differences in root growth when comparing alt1-1 with both als3-1 and Col-0 wt. This suggests that the mechanism impacted by the alt1-1 mutation is not related to a positive change in Al exclusion since Al-dependent damage occurs in alt1-1 roots.

Assessment of Al-dependent gene induction

In Arabidopsis, expression of specific genes, including a homologue of the malate transporter ALMT1 and the Al tolerance gene ALS3, increases following Al treatment. Northern analysis was performed to determine whether Al-treated roots of alt1-1 display normal induction of these Al-responsive genes as a means of understanding whether Al exclusion plays a role in the alt1-1 phenotype. Seedlings of Col-0 wt, als3-1, and alt1-1 were grown for a period of 7 d in hydroponic media (pH 4.2) after which they were transferred to media supplemented with either no or 25 μM AlCl$_3$ (pH 4.2) for 24 h. Root samples were subsequently harvested and total RNA was extracted from these. For northern analysis, 10 μg of total RNA from each sample were electrophoretically separated and blotted. Both an Arabidopsis ALMT1 homologue (At1g08440), which was found to be Al-inducible, and ALS3 were used as probes to assess levels of Al-dependent stress response in the wt and mutants (Fig. 5). It was found that, for ALMT1 and ALS3, each gene was induced similarly in wt and the mutants, indicating that each was experiencing equally this particular aspect of Al-dependent stress, which is not consistent with the increased Al-related growth seen for alt1-1 being dependent on a positive change in an Al-exclusion mechanism.

**AI accumulation is not altered in alt1-1 roots**

In order to answer directly whether Al exclusion was positively impacted by the alt1-1 mutation, total Al content in root samples from 5-d-old seedlings of Col-0 wt, als3-1, and alt1-1 exposed to 25 μM AlCl$_3$ for 24 h was determined by ICP-OES analysis. From this analysis it was found that there was no significant difference in overall AI accumulation when comparing wt, als3-1, and alt1-1 (Fig. 6), which further argues that the alt1-1 mutation does not impact on an Al-exclusion mechanism.

**The alt1-1 phenotype is dependent on capability to modify environmental pH**

Since Al toxicity in soils is thought to occur only when the Al$^{3+}$ ion forms in an acidic environment, it was of interest to determine if the alt1-1 phenotype was related to an increased capability to alkalinize its growth environment. In order to test this, seedlings of Col-0 wt, als3-1, and alt1-1 were grown either in the presence or absence of 25 μM AlCl$_3$ in media that was either supplemented or not supplemented with 10 mM Homo-PIPES, which is an effective buffer at or near pH 4.2. Following a 7 d growth period, root lengths were measured. From this analysis, it was found that addition of Homo-PIPES, which effectively prevents growing roots from raising the pH of their

![Fig. 4](image-url). Callose accumulation in roots following Al treatment. Col-0 wt, als3-1, and alt1-1 were grown hydroponically for 7 d after which they were transferred to nutrient media supplemented with 25 μM AlCl$_3$ (pH 4.2) for 24 h. Seedlings were subsequently fixed, stained with aniline blue, and callose was visualized using an epifluorescent dissecting microscope.

![Fig. 5](image-url). Al-dependent induction of ALMT1 and ALS3 in roots following Al treatment. Col-0 wt, als3-1, and alt1-1 were grown hydroponically for 7 d after which they were transferred to nutrient media supplemented with either no or 25 μM AlCl$_3$ (pH 4.2) for 24 h. Root tissue was harvested, total RNA was extracted, and 10 μg of total RNA from each sample was electrophoretically separated and blotted. Northern analysis was performed using the Al-inducible genes ALMT1 and ALS3 as probes. Tomato 18S rDNA was used for comparison of loading.
growth environment, had little or no effect on the degree of Al toxicity found for either Col-0 wt or als3-1 (Fig. 7).

By contrast, whereas alt1-1 roots in an unbuffered environment without Homo-PIPES showed the characteristic strong root growth, even in the presence of high levels of Al, the addition of Homo-PIPES to the growth media resulted in a profound decrease in the capability of alt1-1 roots to grow in the presence of high levels of Al, with very little effect in the absence of Al.

Map position of alt1-1

A map-based cloning approach is currently being used to isolate the alt1-1 mutation. For this approach, the als3-1 mutation was introgressed into an ecotype other than Col-0 wt in order to build a mapping population. La-0 was chosen for this since roots of this ecotype have a similar Al sensitivity compared with wt (Larsen et al., 1996). Four backcrosses were made between La-0 and successive generations of als3-1 (Col-0 as the starting background). After each backcross, an F2 seedling that displayed the als3-1 phenotype in the presence of 0.75 mM AlCl3 in a soaked gel environment (pH 4.2) was selected and subsequently crossed to La-0 wt. After the fourth backcross, alt1-1 was crossed to an F2 plant from this backcross that displayed the als3-1 phenotype. F2 progeny from this cross were then screened on 0.75 mM AlCl3 in a soaked gel environment (pH 4.2) for those that displayed the als3-1 phenotype (approximately 25%). Genomic DNA was isolated from these progeny and a PCR-based survey to determine the genomic location of the alt1-1 mutation was conducted. From this analysis, it was found that the alt1-1 mutation along with the alleles represented by lines 1-6 and 63-2 lie at approximately the same position between the microsatellite markers nga76 and CIW9 on the top half of chromosome 5.

Discussion

Al toxicity in acid soils is a global problem that has garnered significant interest both in terms of developing an understanding of its physiological effects and identifying the biochemical mechanisms underlying Al resistance and tolerance. The latter effort has resulted in a clear relationship between Al resistance and release of Al-chelating organic acids such as malate or citrate into the rhizosphere (Delhaize et al., 1993; Pellet et al., 1995). Al tolerance is also dependent on chelation of internalized Al by organic acids (Ma, 2000), although the mechanisms underlying Al uptake and redistribution following chelation remain to be elucidated. Based on this knowledge, advances have been made toward identifying the biochemical components of these Al-resistance and tolerance mechanisms through varied approaches. This includes the identification of the wheat malate transporter, ALMT1, which when over-expressed in barley significantly enhances Al resistance (Delhaize et al., 2004; Sasaki et al., 2004). Progress has
also been made towards the identification of factors that mediate Al tolerance in plants, primarily through isolation and characterization of Arabidopsis mutants that have Al hypersensitivity (Larsen et al., 1996). This effort has resulted in the cloning of ALS3, which encodes an ABC transporter-like protein that is predicted to mediate the redistribution of internalized Al as part of a mechanism of basal tolerance to Al in Arabidopsis (Larsen et al., 2005). Even with these advances, the identification of the molecular components of Al resistance and tolerance mechanisms remains an arduous process.

In order to facilitate the isolation of these components, an approach of mutagenizing the Al-sensitive Arabidopsis mutant als3-1 has been taken to identify suppressor mutants that can grow as well as or greater than even Col-0 wt. By using this approach, gain-of-function mutations will be isolated, with these not only being useful for identifying novel factors required for Al resistance and/or tolerance but will also give new approaches for engineering Al resistance and/or tolerance in agriculturally important plants. This unique approach of mutagenizing als3-1 was used because of the severe Al sensitivity presented by this mutant, which will make map-based cloning of the alt (Al-tolerant) mutations of interest achievable. A similar approach was previously utilized whereby Col-0 wt was mutagenized and two mutants, alr-128 and alr-104, with significant increases in Al resistance were identified (Degenhardt et al., 1998; Larsen et al., 1998). Unfortunately, because of inherent variability in root growth and the dominant nature of these mutations, it was not possible easily to follow the mutant phenotypes in crosses, making map-based cloning of these mutations extremely difficult. By having the alt mutations in a mutant background that results in extreme Al sensitivity, map-based cloning of the alt mutations has become a routine exercise and should result in their rapid isolation.

From the initial screen for suppressor mutants, two mutant groups were identified including those that represented those with either marginal or moderate growth in Al-containing medium that was toxic to als3-1 but not Col-0 wt and those that had strong growth in Al-containing medium that was highly inhibitory to both als3-1 and Col-0 wt. The latter group is represented by 12 suppressor mutations from which three were initially chosen for further characterization. Further analysis of these three revealed that all of these are likely dominant gain-of-function alleles of the alt1 locus based on complementation analysis, map position, and phenotype characterization. Because of this, alt1-1 was chosen for further analysis in order to determine the biochemical basis of the increase in Al tolerance and to begin work necessary for cloning the mutation of interest.

alt1-1 represents an als3-1 suppressor mutant with a profound increase in root growth even at Al concentrations that are almost completely inhibitory to Col-0 wt root growth. This is particularly impressive when one considers that the alt1-1 mutant for the majority of this study still is homozygous for the als3-1 mutation. Crosses to remove the als3-1 mutation further enhanced the effects of alt1-1 on root growth in the presence of inhibitory levels of Al and this suggests that this mutation may have great utility for genetic engineering of Al tolerance in crop plants of interest.

From the analyses, the alt1-1 mutant phenotype is not dependent on enhanced Al exclusion. Indirect evidence against an enhancement in Al exclusion in alt1-1 includes the observation that callose accumulates to similar levels in alt1-1 roots compared with Col-0 wt following Al treatment, which is inconsistent with enhanced Al exclusion and indicates that alt1-1 roots perceive the Al challenge similarly to Col-0 wt. Callose accumulation has been argued to be an excellent qualitative indicator of Al resistance since resistant roots tend to produce reduced callose compared with sensitive roots, but exceptions to this have been reported, including the Al-resistant Arabidopsis mutant alr-104, which accumulates levels of callose similar to Col-0 wt following Al exposure (Larsen et al., 1998), and the Al-sensitive mutants als4 and als7, which do not accumulate callose following Al treatment even though they are hypersensitive to low levels of AlCl3 (Larsen et al., 1996). It was also found that following Al treatment, two Al-responsive Arabidopsis genes were induced similarly in both Col-0 wt and alt1-1, which is also inconsistent with enhanced Al exclusion. For this study, an Arabidopsis homologue of wheat ALMT1 was used to assess Al-dependent gene expression since its expression is Al responsive, which is unique compared to what was reported for ALMT1 expression in wheat (Sasaki et al., 2004). Further work is necessary to determine if this ALMT1 homologue encodes a protein functionally equivalent to wheat ALMT1, but for this study, the function of this reporter was not relevant since the focus of this experiment was the determination of whether alt1-1 roots demonstrate Al-dependent gene expression comparable with Col-0 wt following Al treatment.

In support of this indirect evidence, quantification of Al accumulation by alt1-1 roots following Al treatment revealed no measurable difference compared with Col-0 wt, although this is a gross measurement of Al accumulation that can only determine overall Al concentrations rather than tissue- or cell-specific levels of Al. It appears that the alt1-1 phenotype is dependent on the capability to adjust the pH of the rhizosphere, since addition of a buffer appropriate for a moderately acidic pH virtually eliminates the increased Al tolerance observed for alt1-1 roots with little or no effect on the growth of Col-0 wt and als3-1. The phenotype that results from the alt1-1 mutation is similar to what was reported for the alr-104 mutation, which was also found to result in an increased capability for rhizosphere alkalization with little to no effect on Al uptake or callose production, yet alt1-1 and alr-104 are localized to distinctly different areas of the Arabidopsis genome (Degenhardt et al., 1998). It is not clear if the alt1-1 Al tolerance
mechanism is directly dependent on pH adjustment or if it represents an alteration in transport of an unidentified substrate that mediates the observed Al tolerance, with this transport mechanism being dependent on capability for proton flux. Identification of the alt1-1 mutation should help elucidate this.

Currently, work is underway to isolate the alt1-1 mutation using a map-based cloning approach and subsequently to introduce this, either singly or in combination with other alt1 alleles, into an agriculturally important crop species such as maize or barley to determine whether the observed increase in Al tolerance in *Arabidopsis* can be replicated in a different model system. Work will also continue to analyse the large group of suppressor mutants in an attempt to identify other affected loci as part of this programme to isolate mutations that could be useful for genetic engineering of Al tolerance. By taking this approach, it is hoped that a better understanding of this critical global problem will be gained and new biotechnological approaches to address it will be generated.

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