Low-pH and Aluminum Resistance in Arabidopsis Correlates with High Cytosolic Magnesium Content and Increased Magnesium Uptake by Plant Roots

Jayakumar Bose1,2*, Olga Babourina1, Sergey Shabala2 and Zed Rengel1

1School of Earth and Environment, University of Western Australia, Crawley, WA 6009, Australia
2Tasmanian Institute of Agriculture and School of Agricultural Sciences, University of Tasmania, Hobart, TAS 7001, Australia

*Corresponding author: E-mail, Jay.Bose@utas.edu.au; Fax: +61-3-6226-2642.

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Introduction

The most severe symptom of aluminum (Al3+; hereafter Al) toxicity is inhibition of nutrient uptake and transport, affecting root growth (Kochian et al. 2005). Having a similar hydrated ionic radius, Al competes with Mg ions for apoplastic binding sites and plasma membrane Mg transporters (Bose et al. 2011). Indeed, Al-induced inhibition of Mg uptake occurs in diverse plant species (e.g. Grimme 1983, Keltjens 1988, Rengel and Robinson 1989, Rengel 1990). However, the magnitude of inhibition was not equal among the genotypes of a given plant species. Al-resistant genotypes showed higher Mg uptake and/or tissue Mg accumulation compared with Al-sensitive genotypes in a range of species, including maize (Mariano and Keltjens 2005, Giannakoula et al. 2008), wheat (Silva et al. 2010), sorghum (Baligar et al. 1993) and rice (Sivaguru and Paliwal 1993). The above positive correlation between the rate of Mg uptake and Al resistance may indicate a causal relationship between these two traits. However, given that most published reports consider relatively long-term Al exposure (hours to days), it remains unclear whether higher Mg uptake in Al-resistant genotypes is due to greater root development and/or greater resistance of uptake mechanisms. To test this hypothesis, a comparison of short-term (up to an hour) and long-term Mg uptake studies involving genotypes that differ in Al resistance in the presence of fixed Al activity is essential.

In many plant species, Al toxicity may be alleviated by adding relatively high (millimolar) concentrations of Mg (Bose et al. 2011). Increased ionic strength of the solutions (Noble and Summer 1988), reduction in Al saturation at the apoplastic binding sites (Grauer and Horst 1992) and decreased Al activity at the root cell plasma membrane surface (Kinraide 2003, Kinraide et al. 2004) have been proposed as mechanisms for Al toxicity alleviation by Mg. On the other hand, under constant Al activity, even micromolar concentrations of Mg in the rooting media enhanced root growth in soybean (Silva et al. 2001), rice bean (Yang et al. 2007) and...
rice (Watanabe and Okada 2005), suggesting that an ameliorative mechanism of Al toxicity by low concentrations of Mg is beyond the pure electrostatic effects. Further, overexpression of Mg transporter genes in yeast (MacDiarmid and Gardner 1998), *Nicotiana benthamiana* (Deng et al. 2006) and rice (Chen et al. 2012) conferred Al resistance, emphasizing the importance of enhanced Mg uptake in the presence of Al ions. However, there are no short-term Al exposure studies directly examining the immediate effects of Al on Mg uptake mechanisms by measuring Mg fluxes.

The root apex (Ryan et al. 1993), and particularly the distal elongation zone (also known as the transition zone), is the primary site of Al stress (Sivaguru and Horst 1998, Baluska et al. 2010). The distal elongation root zone also acts as a ‘plant command centre’ to integrate sensory inputs into adaptive mechanisms under diverse environmental stimuli (Baluska et al. 2004). In addition, Al-induced gene expression in the mature root zone may play a key role in Al resistance (Chandran et al. 2008). Therefore, Mg fluxes need to be compared between these two root zones (i.e. distal elongation and mature) to decipher primary mechanisms of Al toxicity and resistance in plants.

Intracellular Mg might play a pivotal role in maintenance of H\(^+\)-ATPase activity, acid phosphatase activity, organic acid synthesis and metabolism, cytosolic Ca\(^{2+}\) dynamics and reactive oxygen species homeostasis during Al stress (for references, see Bose et al. 2011). Surprisingly, little is known about the dynamics of intracellular Mg activity during Al stress. To address this knowledge gap, we have used, for the first time, an Mg-selective fluorescent dye (Magnesium Green \(^{TM}\)) to measure the intracellular Mg concentration in intact root cells of *Arabidopsis thaliana* during Al stress.

Using *A. thaliana* as a model system to study complex Al toxicity and resistance mechanisms has many advantages, such as availability of large collections of mutants differing in growth response to Al-toxic environments, rapid advancement in the knowledge of *Arabidopsis* (including a fully sequenced genome), and the relatively short time and small space needed to screen large numbers of genotypes. Thus, an Arabidopsis Al-resistant mutant, *alr104* (Degenhardt et al. 1998), and two Al-sensitive mutants, *als5* and *als3* (Larsen et al. 1996), along with the wild type (Col-0) were used in this study. The long-term (7 d) and short-term (0–60 min) effects of Al exposure on Mg uptake by these genotypes were compared to examine (i) whether these genotypes indeed differ in Mg uptake and (ii) how the changes in Mg uptake relate to Al resistance of these genotypes.

### Results

**Long-term effects of low-pH and combined low-pH/Al stresses on root Mg accumulation**

Growth characteristics of the wild type, *alr104*, *als5* and *als3* have been documented in our previous publication (Bose et al. 2010a). At pH 5.5, the wild type accumulated slightly more Mg in roots when compared with the mutants (Fig. 1A). The low-pH treatment decreased Mg accumulation in roots of the wild type and the *als3* mutant, but did not affect *alr104* and *als5* mutants. The combined low-pH/Al treatments inhibited Mg accumulation in all the genotypes, but the inhibition was most pronounced in the Al-sensitive genotypes (*als3* and *als5*), followed by the wild type, and was least in the Al-resistant mutant (*alr104*). The lowest Al concentration tested (10 \(\mu\)M) caused >55% inhibition of Mg accumulation in roots of the Al-sensitive mutants (*als3* and *als5*).

Regression of root dry weight and Mg accumulation during combined low-pH/Al stress revealed a positive relationship

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**Fig. 1** Long-term effects of Al stress on *A. thaliana* (A) root Mg accumulation and (B) the relationship between Mg accumulation and root dry weight. Means ± SE (\(n = 3\) replicates). In (A), points sharing common letters for each Al concentration are not significantly different by the LSD (least significant difference) test at \(P \leq 0.05\). For (B), only root dry weight and Mg accumulation values corresponding to 10–250 \(\mu\)M AlCl\(_3\) (pH 4.2) treatments were plotted. Arabidopsis seedlings were grown in diluted (1/10) Hoagland solution for 3 weeks followed by treatments imposed in buffered (0.5 mM HomoPIPES) Hoagland solution (1/10) for 7 d.
Moreover, root growth in the Al-resistant genotypes (Col-0 and alr104) and a lack of root growth in the Al-sensitive mutants (als3 and als5) can be explained by their good and poor capacity, respectively, to accumulate Mg during Al stress.

**Long-term effects of combined low-pH/Al exposure on root Al concentration**

The combined low-pH/Al treatment increased Al concentration in the roots of all four genotypes tested, but to a different extent (Fig. 2). The lowest Al concentration was observed in the Al-resistant alr104 mutant and the highest in the Al-sensitive als5 mutant. Interestingly, the Al-sensitive mutant als3 and the wild type had a similar Al concentration in roots.

**Mg$^{2+}$ fluxes along the different root zones of Arabidopsis mutants at pH 5.5**

Measurement of Mg$^{2+}$ fluxes along the longitudinal root axis (Fig. 3) revealed that net Mg$^{2+}$ influx was observed at the root cap, apical meristem, and distal and proximal elongation zones, but net efflux was observed at the mature zone. The highest Mg$^{2+}$ influx was observed in the distal elongation zone in all the genotypes. Among the genotypes, the alr104 mutant recorded a higher Mg$^{2+}$ influx (Fig. 3) than the other genotypes at the apical meristem and the distal elongation zone.

**Short-term effects of low-pH and combined low-pH/Al stresses on Mg$^{2+}$ and H$^+$ fluxes**

Under no stress (pH 5.5), small net Mg$^{2+}$ influx at the distal elongation zone and net Mg$^{2+}$ efflux at the mature zone was observed in all genotypes (Figs. 3–6). The low-pH treatment induced Mg$^{2+}$ influx from the distal elongation zone of all genotypes (Figs. 4–6, top panels). In the mature zone, the low-pH treatment did not cause any significant change in Mg$^{2+}$ fluxes that fluctuated around zero in all genotypes (Figs. 4–6, bottom panels).

Al dose dependency experiments with the wild type (Col-0) revealed that the Mg$^{2+}$ influx induced by low-pH/100 μM Al was smaller when compared with the low-pH/50 μM Al treatment, except during the first 10 min in the mature zone (Fig. 4). In addition, Mg$^{2+}$ influx induced by the combined low-pH/100 μM Al treatment was variable in the distal elongation zone and even turned into a small Mg$^{2+}$ efflux 26–32 min after the commencement of the treatment (Fig. 4A). The treatment with a high Al concentration (500 μM AlCl$_3$, pH 4.2) caused Mg$^{2+}$ efflux from the distal elongation zone within 5 min, and from the mature zone in 15 min, but then fluxes fluctuated around zero until the end of the measuring period (Fig. 4). Sequential introduction of low pH and Al revealed (Fig. 5) that Al concentrations from 25 to 75 μM increased the Mg$^{2+}$ influx in the distal elongation zone and from 10 to 75 μM in the mature zone when compared with the no Al treatment with pH 5.5 or 4.2. The maximum Mg$^{2+}$ influx was observed at 50 μM Al in both root zones.

In the distal elongation zone, the low-pH-induced Mg$^{2+}$ influx was higher in the als5 and alr104 mutants than in the wild type and als3 mutant (Fig. 6, top left panel). A similar difference was not noticed in the mature zone during the low-pH treatment, where Mg$^{2+}$ fluxes fluctuated around zero in all genotypes (Fig. 6, bottom left panel). The combined low-pH/Al treatment (50 μM AlCl$_3$) generally induced higher

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*(Fig. 2)* The Al concentration in roots of *A. thaliana* after 7 d with (+Al) or without (−Al) aluminium stress. The data for no Al treatments (−Al, pH 5.5 and 4.20) and Al treatments (+Al: 10, 25, 50, 75 and 100 μM AlCl$_3$, pH 4.20) were averaged. Means ± SE (n = 4–15 replicates). In each group, bars sharing common letters are not significantly different by LSD test at P ≤ 0.05. Arabidopsis seedlings were grown in diluted (1/10) Hoagland solution for 3 weeks followed by treatments imposed in buffered (0.5 mM HomoPIPES) Hoagland solution (1/10) for 7 d.

*(Fig. 3)* The Mg$^{2+}$ fluxes along the longitudinal root axis of 4- or 5-day-old *A. thaliana* seedlings in basal salt medium (0.1 mM CaCl$_2$ + 1 mM KCl + 0.2 mM MgCl$_2$, pH 5.5). Error bars indicate ± SE (n = 6–18 seedlings). For each distance from the root tip, points sharing a common letter are not significantly different by LSD test at P ≤ 0.05. NS, non-significant difference; DEZ, distal elongation zone; PEZ, proximal elongation zone.
Mg\(^{2+}\) influx than the low-pH alone treatment. This Al-induced Mg\(^{2+}\) influx was observed at both the distal elongation and mature zones of all genotypes (Fig. 6, right panel). Moreover, Al-induced Mg\(^{2+}\) influx was higher at the distal elongation zone than at the mature root zone. The wild type and Al-resistant alr104 mutant recorded higher Al-induced Mg\(^{2+}\) influx than the Al-sensitive mutants (als3 and als5).

The combined low-pH/50 \(\mu\)M AlCl\(_3\) treatment caused H\(^+\) efflux in the mature zone and H\(^+\) influx in the distal elongation zone in all genotypes (Fig. 7A). The AI-sensitive mutants (als3 and als5) exhibited greater efflux in the mature and smaller influx in the distal elongation zone when compared with the Al-resistant genotypes (Col-0 and alr104). To draw a functional relationship between H\(^+\) and Mg\(^{2+}\) fluxes, a regression fitting was done (Fig. 7B). A positive relationship was found between H\(^+\) and Mg\(^{2+}\) fluxes in both root zones \((R^2 = 0.93\) in the distal zone and \(R^2 = 0.79\) in the mature root zone). This observation makes it unlikely that Mg\(^{2+}\) uptake was driven by H\(^+\) efflux (H\(^+\)-ATPase) during the low-pH/50 \(\mu\)M AlCl\(_3\) treatment.

**Short-term effects of low-pH and combined low-pH/Al stresses on intracellular Mg\(^{2+}\) concentration**

The dynamics of intracellular Mg\(^{2+}\) concentrations were measured for the first time in planta using fluorescence lifetime imaging (FLIM) of Magnesium Green™ dye in the epidermal cells of the distal elongation zone of intact A. thaliana roots (Figs. 8–10). The intracellular Mg\(^{2+}\) concentration in the wild type (Col-0) did not differ between low-pH (4.2) and control (pH 5.5) treatments (Fig. 8). Among the Al concentrations tested, the combined low-pH/50 \(\mu\)M Al stress raised the intracellular Mg\(^{2+}\) concentration 2-fold, whereas no difference was observed between the low-pH/100 \(\mu\)M Al treatment and control. Among the treatments, the lowest intracellular Mg\(^{2+}\) concentration was observed at low-pH/500 \(\mu\)M Al (Fig. 8).
Fig. 6 Effect of low-pH and combined low-pH/50 μM AlCl₃ stresses on Mg²⁺ fluxes measured at the distal elongation zone (top panel) and the mature zone (bottom panel) of 4- or 5-day-old A. thaliana roots. The low-pH and the combined low-pH/Al³⁺ treatments were imposed at time = 0; the data recorded in the first 5 min before time = 0 represent Mg²⁺ fluxes at the respective root zones at pH 5.5. Negative Mg²⁺ flux values indicate Mg²⁺ efflux, and positive values indicate Mg²⁺ influx. Error bars represent ± SE (n = 6–15 seedlings). Arabidopsis seedlings were conditioned in basal salt medium (BSM; 0.1 mM CaCl₂ + 1 mM KCl + 0.2 mM MgCl₂, pH 5.5) for 20 min before treatments were imposed in unbuffered BSM.
Under no stress (pH 5.5), the intracellular Mg$^{2+}$ concentration varied among the genotypes. The Al-resistant mutant alr104 recorded the highest intracellular Mg$^{2+}$ concentration followed by als5/C24 wild type > als3. In all genotypes tested, the low-pH treatment did not cause any significant change in Mg$^{2+}$ concentration compared with the control (pH 5.5). In contrast, the combined low-pH/50 μM Al treatment raised the intracellular Mg$^{2+}$ concentration in all genotypes, but to a different extent (Fig. 9). The wild type and alr104 mutant recorded a higher intracellular Mg$^{2+}$ concentration than the Al-sensitive mutants (als3 and als5).

**Discussion**

Measurements of short-term Mg$^{2+}$ fluxes along the longitudinal root axis of *A. thaliana* genotypes demonstrated...
Fig. 10 Magnesium Green-AM™ fluorescent dye loading into *A. thaliana* roots and *in vivo* calibration using FLIM. Color coding of images A and B was based on the distribution of fluorescence molecules excited by 850 nm light. The mean lifetime ($\tau_m$) was calculated from a triple exponential decay. Red color lines in images represent regions of interest (epidermal root cells). (A) Effect of a 1 h incubation in basal salt medium (BSM; 0.1 mM CaCl$_2$ + 1 mM KCl + 0.2 mM MgCl$_2$, pH 5.5) containing 200 μM EGTA-AM and 2.0 mM EGTA on the lifetime distribution of fluorescence molecules in Arabidopsis root cells ($\tau_m$ 579 ps). (B) Effect of a 1 h perfusion with 2.0 mM Mg$^{2+}$ plus 200 μM A23187 ionophore on the lifetime distribution of fluorescence molecules in Arabidopsis root cells ($\tau_m$ 875 ps). (C) A representative image of the lifetime distribution of Magnesium Green fluorescence dye in root cells after 1 h of acid loading of 1.5 mM Magnesium Green-AM™ on ice and 12 h of recovery in basal salt medium. The $\tau_m$ after Magnesium Green-AM™ loading was 796 ps. The orange/yellow color indicates relatively lower, and blue color relatively higher, Mg$^{2+}$ concentration within cells. (D) Amplitudes calculated from the triple exponential decay of Magnesium Green-AM™ fluorescence dye for different Mg$^{2+}$ concentrations. (E) In vivo calibration curve of the $\tau_m$ used to calculate intracellular Mg$^{2+}$ concentrations.
variability between the root apex (influx) and the mature zone (efflux) at pH 5.5 (Fig. 3). This differential transport dynamics between the root apex and the mature zone might be due to the presence of specific transport systems. Indeed, in Arabidopsis, hyperpolarization-activated cation currents were observed only in the elongation zone (Kiegle et al. 2000), whereas non-selective cation channels predominate in the mature root zone (Demidchik et al. 2002). The Al-resistant alr104 mutant had a higher Mg2+ influx in the meristematic and the distal elongation zone (but not in the proximal elongation and the mature zones) (Fig. 3) and a higher intracellular Mg2+ concentration in the distal elongation zone (Fig. 9) compared with other genotypes at pH 5.5, suggesting differential uptake of Mg2+ along the root axis.

A study involving the 28Mg isotope demonstrated that the low-pH (pH 4.5) treatment could inhibit Mg uptake by rice seedlings through competitive interaction between Mg2+ and H+ (Kobayashi et al. 2012). Characterization of Arabidopsis mutants (alr104, als5 and als3) in the low-pH (pH 4.2) environment revealed that alr104 and als5 mutants were resistant to low-pH stress and had a superior capacity to reduce the H+ concentration in the rhizosphere by taking up H+ (influx) from the low-pH environment (Bose et al. 2010a). If H+ removal from the rhizosphere is bound to decrease H+ competition for Mg2+ uptake, the low-pH-resistant mutants should show enhanced Mg2+ influx. Indeed, the alr104 and als5 mutants recorded a higher Mg2+ influx than the wild type or the als3 mutant in the distal elongation zone (Fig. 6, top left panel). Such enhanced Mg2+ influx in the alr104 and als5 mutants further aids these mutants in resisting low-pH stress and provides independent support for an ameliorating effect of Mg2+ on H+ toxicity in Arabidopsis (Koyama et al. 2001).

Ten members of the CorA Mg transporter family were suggested to be involved in Mg uptake and transport in Arabidopsis (Li et al. 2001). Only three [AtMGT1 (Li et al. 2001), AtMGT7 (Gebert et al. 2009) and AtMGT9 (Chen et al. 2009)] are expressed in roots. Among these transporters, the plasma membrane high-affinity Mg2+ transporter AtMGT1 is a likely candidate for enhanced Mg2+ uptake in roots during Al stress because overexpression of the AtMGT1 gene in Nicotiana benthamiana resulted in increased Al resistance (Deng et al. 2006). Moreover, in rice, (i) knockout of OsMGT1 resulted in increased sensitivity to Al but not to La and Cd; and (ii) expression of OsMGT1 was rapidly enhanced following Al exposure and resulted in increased Mg2+ uptake and Mg concentration in the wild-type rice roots (Chen et al. 2012). However, it is highly unlikely that AtMGT1 could be responsible for enhanced Mg2+ influx from both the elongation and mature zones of the wild type and Al-resistant mutant (alr104) (Fig. 6) during moderate AI stress (50 μM AlCl3, pH 4.2). Indeed, all high-affinity Mg2+ transporters reported so far imply 2H+/Mg2+ antiporter operation (Shaoul et al. 1999, Shabala and Hariadi 2005), whereas our measurements showed a positive relationship between H+ and Mg2+ fluxes (Fig. 7). Thus, it appears that a low-affinity Mg2+ transport system (ion channels) may play a central role in Mg2+ uptake by the Al-resistant genotypes during moderate Al stress. This is further supported by thermodynamic considerations. Under our experimental conditions (0.2 mM external Mg2+ concentration; 0.9–1.3 mM intracellular Mg2+ concentration; Fig. 9), the calculated Nernst potential, E\text{M}_{\text{Mg}} was between −20 and −25 mV, whereas the measured membrane potential, E\text{m}, ranged between −50 and −100 mV (Bose et al. 2010a), making thermodynamically passive (channel-mediated) Mg2+ uptake plausible. Interestingly, low pH and Al stress depolarized the Em in the mature zone of the wild type and the Al-resistant mutant (alr104) to the same extent (Fig. 9 of Bose et al. 2010a), but Mg2+ uptake was enhanced in this zone by Al stress (Fig. 6, bottom right), suggesting that Al may activate Mg2+-permeable channels in the Al-resistant genotypes independently of its effect on the membrane potential.

Considering the importance of intracellular Mg2+ in plants (for references, see Bose et al. 2011), it is surprising to note that only Yazaki et al. (1988) measured the free Mg2+ concentration (0.4 mM) in the cytoplasm of mung bean root tips using in vivo 31P-NMR (nuclear magnetic resonance) spectroscopy. Although the intracellular Mg2+ concentration has been measured in animal cells using Magnesium-Green fluorescent dye (Gotoh et al. 1999, Sharikabad et al. 2001, Montezinho et al. 2002), to our knowledge the present study is the first intracellular Mg2+ measurement in plants using Mg-sensitive dyes (Fig. 10). Under no stress (pH 5.5), the intracellular Mg2+ ranged from 0.78 ± 0.06 to 1.24 ± 0.04 mM in the root epidermal cells of the distal elongation zone (Figs. 8, 9); this figure is higher than the 0.4 mM reported for mung bean root tips (Yazaki et al. 1988). The reason for this discrepancy is likely to be due to the fact that the measurements done by Yazaki et al. (1988) represented an average for the whole root tip tissues, whereas our measurements were on individual cells in the distal elongation zone (Fig. 10).

The intracellular Mg2+ concentration was elevated during 50 μM Al stress in all genotypes tested. However, this increase was more pronounced in the Al-resistant genotypes (wild type and alr104) than the Al-sensitive mutants (als3 and als5) (Fig. 9). A rise in intracellular Mg2+ concentration in the Al-resistant genotypes could be explained by (i) influx of Mg2+ ions into the root tissue from the external medium (Fig. 6) and/or (ii) intracellular ATP hydrolysis because ATP hydrolysis usually results in an increased intracellular Mg2+ concentration (Leysens et al. 1996, Gotoh et al. 1999). Indeed, Al concentrations below the phytotoxicity threshold enhanced ATP hydrolysis in maize roots (Facanha and Okorokova-Facanha 2002) and in the fungus Yarrowia lipolytica (Lobão et al. 2007). On the other hand, exposure of Arabidopsis wild-type roots to Al concentrations >50 μM (i.e. 100 and 500 μM AlCl3 treatments, pH 4.2) decreased the intracellular Mg2+ concentration in a dose-dependent manner (Fig. 8). This decline might have been due to decreased Mg2+ influx, or increased efflux, at 500 μM Al3+ (Fig. 4), caused by Al inhibition of plasma membrane cation channels because the driving force for Mg2+ transport within the first 10 min under these conditions would
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have been directed inward (see the above calculations for $E_{Mg}$).
Thus, it appears that the efficacy of phytotoxic Al to block Mg transport through cation channels is concentration and genotype dependent.

Efflux of $H^+$ by electrogenic proton-extruding ATPase is the main driving force for nutrient absorption by plants, especially under stress conditions (Palmgren and Nissen 2010). The $Mg^{2+}$ activity inside the cytoplasm is pivotal for the regulation of $H^+$-ATPase activity (Brooker and Slayman 1983, Costa and de Meis 1996). Moreover, increased free $Mg^{2+}$ ($K = 2.9 \text{mM}$) activated $H^+$-ATPase activity in maize roots at pH 6.0 (Costa and de Meis 1996). In contrast, Al stress inhibited $H^+$-ATPase by 37% in rice bean ($Vigna umbellata$) roots in the absence of $Mg^{2+}$ in a growth medium (Yang et al. 2007). However, addition of a micromolar concentration ($10 \mu M$) of $Mg^{2+}$ to the external medium restored the plasma membrane $H^+$-ATPase activity, even though Al activity was maintained constant (Yang et al. 2007). Given that the Al-resistant Arabidopsis genotypes recorded a larger $Mg^{2+}$ influx at the distal elongation and the mature zones (Fig. 6) as well as a higher intracellular $Mg^{2+}$ concentration (Fig. 9) and overall root $Mg^{2+}$ accumulation (Fig. 1) than the Al-sensitive genotypes, we suggest a relatively higher $H^+$-ATPase activity in the Al-resistant (als104 and wild type) vs. the Al-sensitive genotypes (als3 and als5).

Differential uptake of Al could account for differences in Al resistance among the genotypes (Delhaize et al. 1993). Indeed, the Al-resistant Arabidopsis mutant als104 had the lowest, and the Al-sensitive mutant als5 the highest, Al concentration in roots (Fig. 2). The lowest Al concentration in the als104 mutant was probably due to the superior Al exclusion mechanism via an Al-induced increase in the rhizosphere pH (Degenhardt et al. 1998, Bose et al. 2010a). In contrast, the highest Al concentration in the als5 mutant of all genotypes tested confirmed that this mutant is defective in the Al exclusion mechanism (Larsen et al. 1996). Interestingly, the root Al concentration in the wild type and Al-sensitive als3 was similar (Fig. 2), even though root growth inhibition by Al was more severe in the als3 mutant than in the wild type, potentially because of greater $Mg^{2+}$ uptake by the latter. Moreover, the als3 mutant in comparison with the wild type may lack the capacity to transport Al via an ABC transporter-like protein away from the sensitive sites in the root tissue (Larsen et al. 2005).

Exposure of Arabidopsis mutants to the combined low-pH/Al treatment for 7 d hampered Mg accumulation in all four genotypes (Fig. 1A). This was clearly an aggregate effect of prolonged Al exposure decreasing the nutrient-absorbing root surface area (Fig. 1B) (Clarkson 1985). However, the inhibition was lower in the Al-resistant als104 mutant and the wild type than in the Al-sensitive mutants (als3 and als5), suggesting the Al-resistant genotypes maintained relatively less-disturbed Mg uptake compared with the Al-sensitive genotypes.

In summary, the enhanced capacity of als3 and als104 mutants to take up $Mg^{2+}$ from the low-pH environment may help these mutants cope with low-pH stress. Relatively higher resistance to the combined low-pH/Al stresses in the wild type and the als104 mutant coincided with greater $Mg^{2+}$ influx and higher intracellular $Mg^{2+}$ concentration. In contrast, poor capacity to take up $Mg^{2+}$ from an Al-containing environment coincided with poor growth of the Al-sensitive genotypes (als3 and als5).

Materials and Methods

Long-term experiment

Seedlings of A. thaliana L. wild type (ecotype Col-0), als3, als5 and als104 were grown in diluted (1/10) Hoagland solution as described previously (Bose et al. 2010a, Bose et al. 2010b). Three-week-old seedlings were exposed to pH 5.5/no Al, or to a range of AlCl$_3$ concentrations (0, 10, 25, 50, 75, 100 or 250 $\mu$M) at pH 4.2. Treatments were replicated three times, and the experiment was repeated twice. At harvest, shoots and roots were separated, washed in 100 $\mu$L CaSO$_4$, rinsed with deionized water, dried in the oven at 70°C for 72 h and weighed. Oven-dried roots were digested with a HNO$_3$:HClO$_4$ (10:1) mixture. The Mg concentrations were analysed using inductively-coupled plasma-mass spectrometry (ICP-MS).

Short-term experiments

Arabidopsis thaliana seedlings were grown in 90 mm Petri dishes containing 0.8% (w/w) agar and basal salt medium (BSM) 0.1 mM CaCl$_2$ + 1 mM KCl + 0.2 mM MgCl$_2$, pH 5.5, as described elsewhere (Bose et al. 2010a, Bose et al. 2010b). Four- or five-day-old seedlings were used for all the short-term experiments.

Measurement of $Mg^{2+}$ and $H^+$ fluxes using MIFE®

The roots of an intact Arabidopsis seedling were secured horizontally in a measuring chamber with a Parafilm strip and a small glass cover slide. The seedling were supplied with BSM and conditioned for at least 20 min. Net fluxes of $Mg^{2+}$ were measured 40 $\mu$m away from the root surface using the non-invasive MIFE® system (University of Tasmania) as described by Newman (2001). The $Mg^{2+}$ flux was calculated according to the equation described earlier (Knowles and Shabala 2004). Briefly, microelectrodes were pulled from borosilicate glass capillaries (GC 150-10, SDR Clinical Technology), oven dried at 230°C for about 5 h, and silanized using tributylchlorosilane (Fluka catalog no. 90796). Electrodes were broken back to get an external tip diameter of 2–3 $\mu$m. The electrodes were back-filled with 0.5 mM MgCl$_2$ and front-filled with Mg-selective ionophore cocktail (Fluka catalog no. 63048). Prepared electrodes were calibrated in a set of Mg standards (0.1–2.0 $mM$) in the background of 0.1 mM Ca$_{2+}$ and 100 $\mu$L Al$_{2+}$. Electrodes with the slope responses of <25 mV per decade were discarded.

Inherent $Mg^{2+}$ flux characteristics of the wild type (ecotype Col-0) and als3, als5 and als104 mutants were ascertained by measuring $Mg^{2+}$ fluxes at the root cap (0–30 $\mu$m from the root tip), meristem (30–180 $\mu$m), the distal elongation zone (180–240 $\mu$m), the proximal elongation zone (240–480 $\mu$m)
and the mature zone (>480 μm). For Al dose dependency experiments, wild-type (Col-0) seedlings were exposed to either low-pH (pH 4.2; no Al) or Al treatments (50, 100 or 500 μM AlCl₃, pH 4.2). The time-course of Mg²⁺ uptake by the aforementioned genotypes during low-pH (pH 4.2) or combined low-pH/50 μM AlCl₃ treatment was assessed at the distal elongation zone (200 μm away from the root cap) and the mature zone (700 μm away from the root cap). To establish Al-specific effects on Mg²⁺ flux, wild-type (Col-0) seedlings were exposed to low pH (4.2), with the Al concentration increasing sequentially (0, 10, 25, 50, 75 and 100 μM AlCl₃), starting from the lowest and proceeding to a higher Al concentration every 15 min after the first 15 min of pH 5.5.

Net H⁺ fluxes were measured after 15 min of the combined low-pH/50 μM AlCl₃ treatment at the distal elongation and the mature zone as described elsewhere (Bose et al. 2010a).

**Intracellular Mg²⁺ measurements**

The Mg-sensitive fluorescent dye Magnesium Green™-AM (Molecular Probes) [glycine, N-[2-(carboxymethoxy)-4-[[2,7'-dichloro-3,6′-dihydroxy-3-oxo-spiro[isobenzofuran-1(3H),9′-[9H]xanthen]-5-yl]carbonyl][amino][phenyl]-N-carboxymethyl acetoxymethyl ester] was dissolved in dimethylsulfoxide (DMSO) (Sigma) and diluted with a loading solution (0.2 mM CaCl₂ and 50 mM mannitol, pH 4.2) to a final concentration of 1.5 μM. Preliminary experiments indicated that the 1.5 μM concentration was sufficient for measuring intracellular Mg²⁺ while being sufficiently low to avoid damage to root cells induced by laser scanning (data not shown). The final concentration of DMSO in the loading solution was 1% (v/v). Preliminary experiments with different recovery times and temperatures revealed that the minimum 10 h recovery period at 25°C was essential for Magnesium Green™-AM loading into the root tissue (data not shown). Therefore, the dye was loaded into the intact Arabidopsis roots for 1 h on ice (Guo et al. 2009), followed by recovery in BSM for 12 h at 25°C.

All the calibration and intracellular Mg²⁺ measurements were done using an inverted-stage confocal microscope (Leica TCS SP2 AOBs, Leica Microsystems GmbH). Light pulses were generated at a frequency of 80 MHz with a Mai Tai Laser (Spectra Physics). An emission wavelength of 850 nm was found in preliminary experiments to minimize the contribution from autofluorescence (data not shown). The mean lifetime (τₘ), assuming a triple exponential decay for Magnesium Green™ was used in calibrations (Szmacinski and Lakowicz 1996). Preliminary studies demonstrated a significant shift in τₘ values between in vitro and in vivo calibrations (data not shown); thus, in vivo calibration was performed for the concentration range 0.0–2.0 mM Mg using Magnesium Green™-AM (Fig. 10A, B, D, E). For zero Mg concentration, seedlings loaded with Magnesium Green™-AM were incubated for 1 h in BSM containing 200 μM EGTA-AM and 2.0 mM EGTA (Fig. 10A). For other Mg concentrations, seedlings loaded with Magnesium Green™-AM were perfused with the required Mg concentration plus 200 μM A23187 ionophore and allowed to equilibrate for 1 h (Fig. 10B). Linear regression analysis confirmed that amplitudes (a1 and a2, Fig. 10D) and τₘ (Fig. 10E) showed a linear response to the tested Mg²⁺ concentrations. Among these parameters, τₘ delineated autofluorescence effectively; thus, τₘ was used for calculating intracellular Mg²⁺ concentrations (Fig. 10E).

After dye loading and BSM recovery, seedlings were exposed to low-pH stress (pH 4.2) or Al stress (50, 100 or 500 μM AlCl₃, pH 4.2). After 10 min of treatment, FLIM images were collected for 500 s using photomultipliers, and the FLIM analysis was performed using electronics (SPC-730; Becker & Hickl) and software (SPC7.22; Becker & Hickl) for time-correlated single-photon counting (O’Connor and Phillips 1994). Intracellular Mg²⁺ was calculated for epidermal cells of the root distal elongation zone according to calibration curves (Fig. 10E) using data analysis software for FLIM microscopy systems (SPCImage Version 2.6, MP-FLIM and D-FLIM).

**Statistical analysis**

Significant differences among means were assessed by t-test and analysis of variance (ANOVA) using Genstat (10th edition) (VSN International Ltd.).

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**References**


