Aluminium-induced ion transport in Arabidopsis: the relationship between Al tolerance and root ion flux

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Abstract

Aluminium (Al) rhizotoxicity coincides with low pH; however, it is unclear whether plant tolerance to these two factors is controlled by the same mechanism. To address this question, the Al-resistant alr104 mutant, two Al-sensitive mutants (als3 and als5), and wild-type Arabidopsis thaliana were compared in long-term exposure (solution culture) and in short-term exposure experiments (H+ and K+ fluxes, rhizosphere pH, and plasma membrane potential, EM). Based on biomass accumulation, als5 and alr104 showed tolerance to low pH, whereas alr104 was tolerant to the combined low-pH/Al treatment. The sensitivity of the als5 and als3 mutants to the Al stress was similar. The Al-induced decrease in H+ influx at the distal elongation zone (DEZ) and Al-induced H+ efflux at the mature zone (MZ) were higher in the Al-sensitive mutants (als3 and als5) than in the wild type and the alr104 mutant. Under combined low-pH/Al treatment, alr104 and the wild type had depolarized plasma membranes for the entire 30 min measurement period, whereas in the Al-sensitive mutants (als3 and als5), initial depolarization to around –60 mV became hyperpolarization at –110 mV after 20 min. At the DEZ, the EM changes corresponded to the changes in K+ flux: K+ efflux was higher in alr104 and the wild type than in the als3 and als5 mutants. In conclusion, Al tolerance in the alr104 mutant correlated with EM depolarization, higher K+ efflux, and higher H+ influx, which led to a more alkaline rhizosphere under the combined low-pH/Al stress. Low-pH tolerance (als5) was linked to higher H+ uptake under low-pH stress, which was abolished by Al exposure.

Key words: Aluminium toxicity, distal root elongation zone, H+ flux, K+ flux, low pH, mature root zone, plasma membrane potential.

Introduction

Aluminium (Al) affects root growth in acidic soils. A number of mechanisms responsible for Al tolerance have been characterized in plants, such as (i) release of organic acid anions (e.g. Ma et al., 1997; Kochian et al., 2004; Hoekenga et al., 2006; Liu et al., 2009; Ryan et al., 2009); (ii) release of phenolic compounds (Kidd et al., 2001); (iii) rhizosphere alkalization (Degenhardt et al., 1998); (iv) internal detoxification of Al by complexation with organic acid anions (Ma et al., 2001; Shen et al., 2002); and (v) redistribution of accumulated Al away from sensitive root tissues (Larsen et al., 2005). However, the low pH itself can affect root growth in various plant species (Arnon and Johnson, 1942; Llugany et al., 1995; Lazof and Holland, 1999; Kidd and Proctor, 2001; Koyama et al., 2001; Kinraide, 2003; Rangel et al., 2005; Iuchi et al., 2007; Sawaki et al., 2009). Nevertheless, our knowledge of proton toxicity and the molecular mechanisms underlying low-pH tolerance is rather limited when compared with Al tolerance.

Low-pH tolerance and K+ nutrition appear to be interlinked in some plant species because the addition of K+ to the external medium alleviated H+ toxicity in maize (Yan et al., 1992), common bean (Rangel et al., 2005), and sugar beet (Lindberg and Yahya, 1994). Also, down-regulation of CIPK23, which encodes the regulatory kinase of a major K+ transporter AKT1, may be responsible for the higher sensitivity of the Arabidopsis stop1 mutant to low pH (Iuchi et al., 2007).
et al., 2007; Sawaki et al., 2009). Although the detailed mechanism underlying this phenomenon is unclear, the increased internal K\(^+\) concentration could be due to changes in K\(^+\) transport at the root-soil interface, via either increased K\(^+\) uptake or decreased K\(^+\) efflux. Lower K\(^+\) efflux under Al exposure in comparison with low pH exposure has been reported for soybean cells grown in suspension culture (Stass and Horst, 1995), which might indicate Al-induced changes in the plasma membrane potential (\(E_m\)) because K\(^+\) transport and accumulation in roots is highly dependent on \(E_m\). Hence, \(E_m\) and K\(^+\) flux need to be assessed simultaneously to elucidate the role of K\(^+\) nutrition in low-pH tolerance.

In Arabidopsis, low pH (H\(^+\) toxicity) causes irreversible damage to primary and lateral roots, with the pattern of damage being different from the one caused by Al rhizotoxicity (Koyama et al., 1995). Furthermore, an Arabidopsis quantitative trait locus (QTL) analysis revealed that Al tolerance and H\(^+\) tolerance are controlled by different genetic factors (Ikka et al., 2007). In contrast, the proton-hypersensitive Arabidopsis stop1 (sensitive to proton rhizotoxicity 1) mutant is also hypersensitive to Al (Iuchi et al., 2007; Sawaki et al., 2009). Therefore, it appears that H\(^+\) and Al\(^{3+}\) toxicities and tolerances are controlled by some separate and some common mechanisms, which would need to be elucidated.

Based on this hypothesis, low pH tolerance of an Al-resistant mutant, \(alr104\), which has higher rhizosphere alkalinization capacity than the wild-type (Degenhardt et al., 1998; Larsen et al., 1998), and two Al-sensitive mutants, \(als3\) [defective in an ABC transporter-like protein (Larsen et al., 2005)] and \(als5\) [defective in Al exclusion (Larsen et al., 1996)], were studied. The Al stress is inevitably studied in combination with the low-pH stress; as a result, genotypes more sensitive to one stress were occasionally classified as being tolerant to the other stress (for references, see Lazof and Holland, 1999). Hence, the effects of low pH were separated from combined low-pH/Al effects and Al susceptibility or tolerance of these Arabidopsis mutants was re-examined by measuring rhizosphere alkalinization capacity, internal K\(^+\) concentration, changes in the plasma membrane potential (\(E_m\)), and the H\(^+\) and K\(^+\) net fluxes. It was found that the \(als5\) mutant was tolerant to low pH but sensitive to Al, whereas \(alr104\) was tolerant and \(als3\) was sensitive to both low pH and Al.

Materials and methods

Long-term exposure experiments: hydroponic culture

Arabidopsis thaliana L. seeds were surface sterilized with 1% (w/v) calcium hypochlorite for 10 min. Seeds were then sown on rockwool strips (1-2 mm thick and 5-6 cm long) that were placed into 250 ml plastic containers containing 1/10 Hoagland solution. The containers were kept at 4°C for 2 d to achieve synchronized germination. Seedlings were then moved to a growth cabinet with 16 h light (150 \(\mu\)mol m\(^{-2}\) s\(^{-1}\)) and 8 h dark at 20±1°C. Three-week-old seedlings were used to conduct two sets of long-term exposure experiments. During the first set of experiments, \(A.\) thaliana L. wild-type ecotype Col-0 and Al-sensitive mutant (\(als3\) and \(als5\)) seedlings were exposed to either pH 5.5 or 4.2 with or without 0.5 mM homo-PIPES buffer for 10 d in quadruplicate. Nutrient solutions were changed daily. During the treatment period, the bulk solution pH was measured on the second day at 12 h and 24 h after the nutrient solution was changed.

During the second set of experiments, 3-week-old seedlings [the wild type (ecotype Col-0), \(als3\), \(als5\), and \(alr104\)] were exposed to pH 5.5 and a range of Al concentrations (0, 10, 25, 50, 75, 100, or 250 \(\mu\)M \(AlCl_3\); pH 4.2) in 0.5 mM homo-PIPES buffer for 7 d. The treatments were performed in triplicate, and the treatment solutions were changed daily.

At the end of the experiments, shoots and roots were separately harvested, washed with 100 \(\mu\)M CaSO\(_4\), rinsed with deionized water, dried in an oven at 70°C for 72 h, and weighed. Dried shoots were digested with a HNO\(_3\)/HClO\(_4\) (10:1) mixture. The K\(^+\) concentration was analysed using inductively coupled plasma–mass spectrometry (ICP-MS).

Short-term exposure experiments

Arabidopsis thaliana L. wild-type (ecotype Col-0), \(als3\), \(als5\), and \(alr104\) seeds were surface sterilized with 1% (w/v) calcium hypochlorite and seedlings were grown in 90 mm Petri dishes under constant fluorescent light (150 \(\mu\)mol m\(^{-2}\) s\(^{-1}\)) and temperature (23–25°C) in 0.8% (w/v) agar medium containing basal salt medium (BSM) with 0.1 mM CaCl\(_2\), 1 mM KCl, and 0.2 mM MgCl\(_2\), pH 5.5. The Petri dishes were oriented upright, so the roots grew down along the agar surface without penetrating it. However, roots were anchored in the agar by root hairs.

Four- to five-day-old seedlings of the wild type (ecotype Col-0) and \(als3\), \(als5\), and \(alr104\) mutants were conditioned in BSM at pH 5.5 for 20 min followed by either low pH (pH 4.2) or combined low-pH/50 \(\mu\)M \(AlCl_3\) treatment. All measurements were made at the root distal elongation zone (DEZ; 200 \(\mu\)m away from the root cap) and the mature zone (MZ; 700 \(\mu\)m from the root cap).

Net fluxes of H\(^+\) and K\(^+\) and rhizosphere pH were measured 40 \(\mu\)m away from the root surface using the non-invasive MIFE\(^®\) system (University of Tasmania, Hobart, Australia) as described by Newman (2001). Microelectrodes were pulled from borosilicate glass capillaries (GC 150-10, SDR Clinical Technology, Middle Cove, Australia), oven dried at 230°C for ~5 h, and silanized using tributylchlorosilane (Fluka catalogue no. 90796). Electrodes with an external tip diameter of 2–3 \(\mu\)m were used. The electrodes were back-filled with the appropriate solution (0.15 mM NaCl and 0.4 mM KH\(_2\)PO\(_4\) adjusted to pH 6.0 using NaOH for the H\(^+\) electrode and 0.5 M KCl for the K\(^+\) electrode). The electrode tips were then front-filled with ionophore cocktails (Fluka catalogue no. 95297 for H\(^+\) and no. 60031 for K\(^+\)). The prepared electrodes were calibrated with a set of standards (pH from 3.2 to 6.5; K\(^+\) from 0.5 mM to 10 mM). Electrodes with slopes of <50 mV per 10 units were discarded.

The roots of an intact 4- to 5-day-old Arabidopsis seedling were gently secured horizontally in a measuring chamber with a Parafilm strip and small plastic blocks. The seedling was then placed in the measuring chamber containing BSM and conditioned for at least 20 min. The \(E_m\) measurements were conducted according to the procedure outlined in Cuin and Shabala (2005). The borosilicate glass microelectrodes (Shabala and Lew, 2002) (Clarke Electrochemical Instruments, Reading, UK) were filled with 1 M KCl, connected to an IE-251 electrometer (Warner Instruments, Hampden, CT, USA) via the Ag–AgCl half-cell, and inserted into the root tissue (DEZ or MZ) with a manually operated micromanipulator (MMT-5, Narishige, Tokyo, Japan). The \(E_m\) was monitored continually using the CHART software (for details, see Newman, 2001). Once a stable measurement of \(E_m\) was obtained for 1 min, treatments with either low pH (pH 4.2) or combined low-pH/50 \(\mu\)M \(AlCl_3\) were initiated, and the \(E_m\) was measured for ~30 min. Eight to 12 plants were measured for every treatment, and the data were averaged.
Results

Long-term exposure experiments: low pH and low-pH/Al stresses in buffered and non-buffered media

Alkalinization capacity and growth of Arabidopsis mutants under low pH: Measurements of the bulk solution pH were made after 12 h and 24 h (Fig. 1). The results revealed that 0.5 mM homo-PIPES buffer was sufficient to keep the pH at ~4.2 or 5.5 (depending on the treatment) for at least 24 h. In non-buffered medium, within 12 h the plants increased the media pH from 4.2 to 5.1–5.6 depending on the genotype (Fig. 1). The als5 mutant was the most and the als3 mutant was the least effective of the three genotypes tested in increasing the media pH.

After 10 d in non-buffered medium, low-pH treatment decreased the biomass of the wild type and the als3 mutant, whereas low-pH stress enhanced growth of the als5 mutant (Fig. 2); there were no differences in growth of als5 at the two pHs in the buffered medium. For the wild type and als3 mutant, biomass was similar in the buffered and non-buffered media.

Effect of low pH and Al on root biomass and shoot K+ concentration: In the homo-PIPES-buffered medium, the low-pH treatment retarded root biomass of the wild type and als3 mutant, whereas root biomass of the alr104 and als5 mutants was unaffected. The als3 mutant showed a greater reduction in root biomass under low pH treatment compared with the wild type (Fig. 3A). The addition of Al severely inhibited root biomass of the Al-sensitive mutants (als3 and als5), even at a low concentration (10 μM), whereas root biomass reduction in the wild type was observed at Al concentrations ≥25 μM. Root biomass of the Al-resistant mutant alr104 was inhibited only at ≥75 μM Al (Fig. 3A). Al-related root biomass reduction was greater in the sensitive mutants (als3 and als5) compared with the wild type and was the lowest in the resistant mutant (alr104).

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Fig. 1. Bulk solution pH of Arabidopsis thaliana genotypes with and without 0.5 mM homo-PIPES buffer measured on the second day at 12 h and 24 h after the nutrient solution change. Mean ± SE (n=4 replicates). Within each buffer×pH treatment, different letters represent significant difference by LSD test at P <0.001. Arabidopsis seedlings were grown in diluted (1/10) Hoagland solution for 3 weeks; treatments were then imposed for 10 d.

Fig. 2. Biomass of Arabidopsis thaliana genotypes at pH 4.2 with and without 0.5 mM homo-PIPES buffer. Mean ± SE (n=4 replicates). In each graph, different letters within each genotype represent significant difference between pH 5.5 and pH 4.2 by t-test at P ≤0.001.
Both root zones of all genotypes tested. However, the Al-sensitive mutants had lower rhizosphere pH compared with the wild type and alr104 in both root zones (Fig. 4).

Fig. 3. Effect of low pH and Al stresses on Arabidopsis thaliana root biomass [A] and shoot K+ concentration [B]. Means ±SE (n=3 replicates). In each AlCl3 concentration, genotypes sharing a common letter are not significantly different by LSD test at \( P \leq 0.05 \). Arabidopsis seedlings were grown in diluted (1/10) Hoagland solution for 3 weeks, and treatments were then imposed in buffered medium (0.5 mM homo-PIPES) for 7 d.

The shoot K+ concentration did not differ among the genotypes in the pH 5.5 treatment (Fig. 3B). Under low pH treatment, the Al-sensitive mutants (als5 and especially als3) had higher shoot K+ concentrations compared with the wild type. Interestingly, the shoot K+ concentration of the Al-resistant mutant alr104 did not differ from that of the wild type under any treatment. In all Al treatments, the Al-sensitive mutant als3 had higher shoot K+ concentrations than any of the other genotypes.

Short-term exposure experiments: microelectrode measurements during low pH and low-pH/Al stresses

**Rhizosphere pH:** Under the low-pH treatment, the alr104 and als5 mutants had higher rhizosphere pH than the wild type and als3 mutant in both the DEZ and MZ. No significant rhizosphere pH change was observed between the wild type and als3 mutant in both root zones, except for the first 12 min in the DEZ when the als3 mutant demonstrated a slightly lower pH than the wild type (Fig. 4).

The combined low-pH/50 \( \mu M \) Al treatment resulted in a lower rhizosphere pH than the low-pH treatment alone in both root zones of all genotypes tested. However, the Al-resistant alr104 mutant had a rhizosphere pH that was 0.1±0.01 units higher than that of the wild type in the DEZ. In the MZ, the alr104 mutant demonstrated a higher pH than the wild type for the first 4 min, but there was no significant difference afterwards. Both of the Al-sensitive mutants had lower rhizosphere pH compared with the wild type and alr104 in both root zones (Fig. 4).

**H+ flux:** At pH 5.5, a small net H+ influx in the DEZ and a small net H+ efflux in the MZ were observed in all the genotypes tested (Fig. 5). The low-pH treatment induced a net H+ influx in both root zones of all genotypes, but the magnitude was greater in the DEZ than in the MZ. The alr104 and als5 mutants exhibited higher H+ influxes than the wild type and als3 at both root zones. Under the low-pH treatment, als3 and the wild type had similar H+ influxes in both root zones, except for the first 12 min in the DEZ, where a lower level of H+ influx was observed in the als3 mutant compared with the wild type.

In the low-pH/Al treatment (50 \( \mu M \)), the Al-resistant alr104 mutant maintained the highest H+ influx in the DEZ, followed by the wild type; H+ influx was the lowest in the Al-sensitive mutants (als3 and als5; Fig. 5). In the MZ, the 50 \( \mu M \) Al treatment induced H+ efflux in all genotypes tested for the first 45 min. The highest levels of H+ efflux were observed in the als3 and als5 mutants. The highest H+ efflux was observed 17 min after 50 \( \mu M \) AI exposure in the Al-sensitive mutants (als3 and als5) and after 22 min in the wild type and alr104 mutant (Fig. 5).

**K+ efflux:** The low-pH treatment induced K+ efflux from the DEZ, which decreased gradually over time in all genotypes (Fig. 6). Among the mutants, als3 and als5 had lower levels of K+ efflux than the wild type and alr104. During the first 30 min of the low-pH treatment, K+ efflux was higher in the alr104 mutant than in the wild type, but the opposite occurred between 30 min and 60 min after the start of the treatment.

The combined low-pH/50 \( \mu M \) Al treatment generally induced lower levels of K+ efflux in the DEZ than the low-pH treatment alone after ~10–15 min in all genotypes, except for the alr104 mutant which showed little difference between K+ flux in the low-pH and low-pH/Al treatments (Fig. 6). Interestingly, under the combined low-pH/50 \( \mu M \) Al treatment, the K+ flux changed from efflux to influx in the als3 and als5 mutants after 12 and 20 min, respectively, whereas the wild type and alr104 mutant maintained K+ influx for the entire 60 min period.

In the MZ, no distinct differences in K+ flux were observed between the low-pH and combined low-pH/50 \( \mu M \) Al treatments for all genotypes tested (Fig. 7). There was a tendency toward K+ efflux for the wild type and alr104, whereas the net fluxes oscillated around zero for the two Al-sensitive mutants.

**Plasma membrane potential (\( E_{pm} \):** The resting \( E_{pm} \) (at pH 5.5) of the alr104 mutant was more negative than the \( E_{pm} \) of the wild type and the Al-sensitive mutants (als3 and als5) in both root zones (Figs 8, 9). There was no difference in the resting potential observed between the wild type and Al-sensitive mutants (als3 and als5) in the DEZ (Fig. 8).
However, in the MZ, the wild type had a more negative resting potential than the sensitive mutants (als3 and als5; Fig. 9).

The low-pH treatment depolarized the plasma membrane in both root zones and in all genotypes, but to different extents. Low pH induced more depolarization in the als3 and als5 mutants than in the wild type and alr104 mutant (Figs 8, 9).

In the DEZ of the Al-sensitive mutants (Fig. 8), the initial depolarization of the plasma membrane was less in the combined low-pH/50 μM Al treatment than in the low-pH treatment. $E_m$ depolarization in the DEZ lasted for 30 min in the wild type and in alr104. In the Al-sensitive mutants (als3 and als5), $E_m$ depolarization was maintained for the 60 min measuring period in the low-pH treatment, whereas the low-pH/50 μM Al treatment hyperpolarized the plasma membrane after 20 min in both Al-sensitive mutants. After 60 min of low-pH/Al treatment, $E_m$ was still depolarized in alr104, but became hyperpolarized in the other three genotypes.

In the MZ of wild type and alr104 mutant roots, the low-pH/Al treatment did not induce a significant difference in the depolarization pattern when compared with the low-pH treatment for up to 60 min (Fig. 9). In contrast, in the Al-sensitive mutants, the low-pH/Al treatment depolarized the plasma membrane to a lesser extent than the low-pH treatment. After 60 min of low-pH/Al treatment, plasma membrane hyperpolarization was observed in the Al-sensitive mutants, whereas the wild type and alr104 maintained depolarized states.

**Discussion**

*Arabidopsis* mutants differed in their responses to low-pH and Al stresses: als5 grew better under the low-pH
treatment (Fig. 2) and poorly in the Al treatment (Fig. 3A), whereas als3 was sensitive and alr104 was tolerant to both stresses (Figs 2, 3A). These results agree with those of Ikka et al. (2007), who classified 260 A. thaliana strains for Al and low-pH tolerance based on the results of QTL analysis.

Several mechanisms have been proposed for the increased plant tolerance to Al toxicity (Matsumoto, 2000; Kochian et al., 2004), some of which could also increase tolerance to low-pH stress, such as increased rhizosphere alkalinization. It was found that the als5 and alr104 mutants had higher rhizosphere pH than the wild type and the als3 mutant, reflecting the low-pH tolerances of the former mutants (Fig. 4). In line with the impaired rhizosphere alkalinization mechanism under the combined low-pH/Al treatment, the Al-sensitive mutants (als3 and als5) exhibited lower rhizosphere pHs in both root zones during short-term exposure experiments if compared with the Al-tolerant genotypes (wild type and alr104; Fig. 4). Interestingly, a suppressor mutant of als3 (alt1-1) has enhanced capability for pH adjustment of the rhizosphere (Gabrielson et al., 2006). Therefore, rhizosphere alkalinization appears to be a regulatory mechanism of plant tolerance to low-pH and Al stresses.

Although rhizosphere pH changes are the net result of the dynamics of cation/anion uptake and release (including H+, OH−, and organic acids), the MIFE® technique does not allow separate ion flux measurements; hence, it is difficult to establish which ion fluxes are responsible for a specific pattern of pH changes. However, a close correlation was found between rhizosphere pH changes and changes in H+ flux ($r \geq 0.93$). Thus, H+ flux across the root tissue is likely

Fig. 5. Effect of low pH and combined low pH/50 μM Al on H+ fluxes measured at the distal elongation zone (top panel) and the mature zone (bottom panel) of 4- to 5-day-old Arabidopsis thaliana roots. The low-pH and combined low-pH/Al treatments were imposed at time=0; the data recorded in the first 5 min before time=0 represent H+ fluxes at pH 5.5. Negative H+ flux values indicate efflux, and positive values influx. Error bars are ±SE (n=10–12 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.

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to be an important contributor to pH changes in the rhizosphere under low-pH and combined low-pH/Al stresses.

The low-pH treatment induced an increase in H+ influx in both root zones for all the genotypes tested (Fig. 5). This H+ influx could be the result of (i) passive entry of H+ from the external media into the root tissue because acidification of the external pH by one unit can increase the H+ electrochemical gradient across the plasma membrane by 60 mV (Babourina et al., 2001; Yamashita et al., 2003) and/or (ii) decreased activity of the H+-ATPase (Kasamo, 1986; Zhao et al., 2008). However, a decrease in H+-ATPase activity under low pH conditions was not supported by Yan et al. (1998, 1992); instead, they reported that re-entry of H+ ions into the root cells was enhanced at low pH. Increased H+ influx into the root tissue would cause intracellular acidification (Gerendas et al., 1990; JB, OB, and ZR, unpublished results), thereby disturbing the cytoplasmic pH. Earlier reports showed that cytoplasmic pH regulatory genes were down-regulated in the low-pH-hypersensitive Arabidopsis stop1 mutant (Iuchi et al., 2007; Sawaki et al., 2009). In the present experiments, higher H+ influx (and greater biomass growth) was observed in the low pH-tolerant mutants (als5 and alr104) compared with the low-pH-sensitive wild type and als3 mutant (Figs 2, 3A). Hence, it is proposed that the low-pH-tolerant mutants (als5 and alr104) have a better cytoplasmic pH regulatory mechanism than the wild type and als3 mutant.

The combined low-pH/Al treatment decreased net H+ influx in the DEZ and increased net H+ efflux in the MZ for all the genotypes tested (Fig. 5). This could result from Al ions either inhibiting H+ influx or inducing H+ efflux, which is consistent with earlier studies on squash roots where Al-treated root apices were not able to alkalinize media to the same extent as in control low-pH media (Ahn et al., 2002). The MIFE technique used in the present study estimates the net H+ flux across the plasma membrane. Hence, it can only be speculated that Al ions, because of their strong affinity for the plasma membrane surface, might have shifted the plasma membrane surface potential towards relatively positive values (Ahn et al., 2001, 2004a). A positively charged plasma membrane surface would impede the uptake of cations, including H+ ions. This might explain the observed inhibition of H+ influx in the DEZ. The H+ influx inhibition by Al ions would also result in measured enhancement of
the net H⁺ efflux in the MZ. Furthermore, Ahn et al. (2004a) reported that Al ions shifted the plasma membrane surface potential towards positive values in an Al-sensitive (ES8) but not in an Al-tolerant wheat genotype (ET8). Similarly, plasma membrane surface potential differences between Arabidopsis genotypes exposed to low-pH/Al treatment in the present study could have been linked to greater H⁺ influx inhibition and enhanced H⁺ efflux in the Al-sensitive mutants (als3 and als5) compared with the wild type and the Al-tolerant alr104 mutant.

Similarly to Al-sensitive maize (Calba and Jaillard, 1997) and wheat roots (Kinraide, 1988), enhanced net H⁺ release from Al-sensitive mutants (als3 and als5) under Al stress would decrease the net H⁺ influx from the DEZ and increase the net H⁺ efflux from the MZ (Fig. 5). Protein kinases can regulate H⁺-ATPase activity across the plasma membrane (Trofimova et al., 1997). Though up-regulation of protein kinases was reported for Al-tolerant genotypes of some plant species (Osawa and Matsumoto, 2001; Shen et al., 2005), protein kinase inhibition by Al ions was observed for the Al-sensitive Arabidopsis stop1 mutant (Sawaki et al., 2009). It is tempting to hypothesize that Al can specifically inhibit the Arabidopsis protein kinase PKS5, a negative regulator of the membrane H⁺-ATPase, thereby inducing H⁺ efflux and acidification of the external medium (cf. Fuglsang et al., 2007).

The low-pH treatment depolarized the $E_m$ in all the genotypes tested (Figs 8, 9). This could be the result of a transient increase in H⁺ influx and/or a decrease in H⁺-ATPase activity. Under the combined low-pH/Al treatment, $E_m$ depolarization was higher in the Al-tolerant genotypes (wild type and alr104) than in the Al-sensitive mutants (als3 and als5) in both root zones (Figs 8, 9). Similar results were observed in wheat (Papernik and Kochian, 1997; Wherrett et al., 2005), indicating that Arabidopsis and wheat might employ similar mechanisms to combat combined low-pH/Al stress. This Al-induced $E_m$ depolarization in the Al-tolerant genotypes could be a result of (i) currents caused by the H⁺ flux across the plasma membrane (see Raven, 1991, and references therein) because higher H⁺ influx in the DEZ or lower H⁺ efflux in the MZ of the Al-tolerant genotypes (alr104 and wild type) would cause the $E_m$ to depolarize.

Fig. 7. Effect of low pH and combined low pH plus 50 μM Al³⁺ on K⁺ fluxes measured at the mature zone of 4- to 5-day-old Arabidopsis thaliana roots. The low-pH and combined low-pH/Al³⁺ treatments were imposed at time=0; the data recorded in the first 5 min before time=0 represent K⁺ fluxes at pH 5.5. Negative K⁺ flux values indicate efflux and positive values indicate influx. Error bars are ±SE ($n=10–12$ 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.
more than in the Al-sensitive mutants (als3 and als5); or (ii) release of organic anions from the Arabidopsis roots upon Al exposure, which would depolarize the $E_m$ (Olivetti et al., 1995; Papernik and Kochian, 1997; Kollmeier et al., 2001). Under Al stress, Arabidopsis has been reported to release malate (Hoekenga et al., 2006), citrate (Liu et al., 2009), pyruvate, and succinate (Larsen et al., 1998). Characterization of the AtALMT transporter revealed that Arabidopsis falls into the pattern II category (Ma et al., 2001), requiring 4 h induction to achieve maximum malate release (Kobayashi et al., 2007). For this reason, malate efflux could not have caused the observed $E_m$ depolarization in the tolerant genotypes as measured in the present study because the experimental period was only 60 min.

Larsen et al. (1998) reported that the air104 mutant and the wild type release similar amounts of citrate upon Al exposure. Tricarboxylate citrate$^3$– has a 6- to 8-fold greater ability to chelate Al than bicarboxylate malate$^{2–}$ (Ryan et al., 2001). Citrate efflux occurs through MATE transporters in Arabidopsis (Liu et al., 2009), wheat (Ryan et al., 2009), barley (Furukawa et al., 2007), and sorghum (Magalhaes et al., 2007). The MATE transporters are present in the plasma membrane of epidermal cells along the root apex as well as the MZ (Furukawa et al., 2007; Magalhaes et al., 2007; Ryan et al., 2009) and rapidly (within 20 min) release citrate following Al exposure (Zhao et al., 2003). The release of large amounts of citrate$^3$– upon Al exposure from the Al-tolerant genotypes (air104 and the wild type) would decrease the intracellular negatively charged citrate thereby maintaining $E_m$ depolarization, whereas release of smaller amounts of citrate from Al-sensitive genotypes would diminish $E_m$ depolarization (Fig. 8).

Given that K$^+$ transport in plants usually occurs near the electrochemical equilibrium, theoretically any change in $E_m$ would affect K$^+$ flux. The low-pH treatment caused immediate depolarization in all the genotypes tested (Fig. 8). This $E_m$ depolarization should lead to increased K$^+$ efflux from the roots through K$^+$ channels as shown previously under low-pH conditions (Babourina et al., 2001; Shabala et al., 2006), which was indeed observed in the DEZ of all genotypes in this study (Fig. 6). The combined low-pH/Al treatment caused a smaller initial depolarization in Al-sensitive mutants (both zones) compared with the low-pH treatment (Figs 8, 9). This shift in $E_m$ towards less
depolarization or even hyperpolarization should decrease K⁺ efflux or even induce K⁺ influx, which was observed in the DEZ of all the genotypes tested (Fig. 6). Similar results were reported in soybean suspension cells (Stass and Horst, 1995) and wheat, wherein inhibition of K⁺ efflux by Al ions was more pronounced in Al-sensitive Scout than Al-tolerant Atlas wheat (Sasaki et al., 1995). Interestingly, Al treatment hyperpolarised the $E_m$ in the DEZ of Al-sensitive Arabidopsis mutants ($als3$ and $als5$) after 20 min (Fig. 8). K⁺ influx occurred together with this $E_m$ hyperpolarization in the Al-sensitive mutants (Fig. 6), which might have been the result of hyperpolarization-activated K⁺ inward-rectifying channels (KIRCs) (Maathuis and Sanders, 1995; Lebaudy et al., 2007).

The DEZ-type regulation of K⁺ flux by $E_m$ was not found in the MZ, with no specific pattern of K⁺ flux changes observed under either low-pH or combined low-pH/Al stress (Fig. 7). Indeed, the MZ has a larger number of K⁺ transport systems than the root apex (Hanson and Kahn, 1957; Ahn et al., 2004b; Vallejo et al., 2005), but not all K⁺ transport systems are voltage gated in the MZ of Arabidopsis roots (Lebaudy et al., 2007).

Compared with other genotypes, the highest shoot K⁺ concentration (Fig. 3B) and lowest K⁺ efflux (Fig. 6) observed for $als3$ under low-pH stress independently supports the observation of Koyama et al. (2001) that K⁺ transport is altered at low pH values in Arabidopsis. The higher shoot K⁺ concentration in the Al-sensitive mutants ($als3$ and $als5$ mutants) could be linked to decreased K⁺ efflux or enhanced K⁺ influx in the DEZ (Fig. 6), indicating a disturbance in K⁺ homeostasis. This shift in K⁺ flux towards influx might be due to a direct or indirect effect of $ALS3$ and $ALS$ mutations. $ALS3$ is a plasma membrane-localized ABC transporter-like protein (Larsen et al., 2005). Although it shares high similarity with other plant ABC transporters, it lacks the ATP-binding cassette. It has been proposed that $ALS3$ is involved in translocation of Al from Al-sensitive tissues (Larsen et al., 2005). This conclusion is based on its high expression in the phloem. However, 24 h exposure to Al had no effect on $ALS3$ expression in the phloem, but shifted $ALS3$ expression from external to internal cells (Larsen et al., 2005). In the current study, an immediate difference between the wild type and $als3$ mutants in ion fluxes and $E_m$ was observed after exposure.
to Al. It indicates that ALS3 functioning may be linked to maintenance of $E_m$ depolarization, K$^+$ efflux, H$^+$ influx, and, in the longer term, to K$^+$ homeostasis. These findings are consistent with physiological studies on plants with alr1-1 mutation (a suppressor of als3 mutation), which demonstrated that alr1-1 mutation increased Al resistance by pH adjustment rather than Al exclusion (Gabrielson et al., 2006).

In summary, the enhanced ability of the als5 and alr104 mutants to alkalinize the rhizosphere and take up H$^+$ from a low-pH environment is responsible for the low-pH tolerance in these mutants. Higher tolerance to combined low-pH/Al stress in the wild type and alr104 mutant coincided with a higher resting $E_m$ and continuous $E_m$ depolarization, higher K$^+$ efflux, and higher H$^+$ influx, which are linked to the plant’s ability to make the rhizosphere less acidic. Low-pH tolerance (als5 mutant) was associated with higher H$^+$ uptake under low-pH stress; however, this ability was abolished by exposure to Al. Therefore, the mechanisms that underlie plant tolerance to acidic and Al stresses appear to be different.

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