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RESEARCH PAPER

# 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, $E_m$). 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 $E_m$ 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 $E_m$ 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 alkalinization (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 plants 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; 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 alkalinizing 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% (v/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 μmol $m^{-2}$ $s^{-1}$) 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 μM $AlCl_3$; 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 μM $CaSO_4$, 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 μmol $m^{-2}$ $s^{-1}$) and temperature (23–25 °C) in 0.8% (w/w) 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 μM $AlCl_3$ treatment. All measurements were made at the root distal elongation zone (DEZ; 200 μm away from the root cap) and the mature zone (MZ; 700 μm from the root cap).

Net fluxes of $H^+$ and $K^+$ and rhizosphere pH were measured 40 μm 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 μm were used. The electrodes were back-filled with the appropriate solution (0.15 mM NaCl and 0.4 mM $KH_2PO_4$ 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 μM $AlCl_3$ 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*).

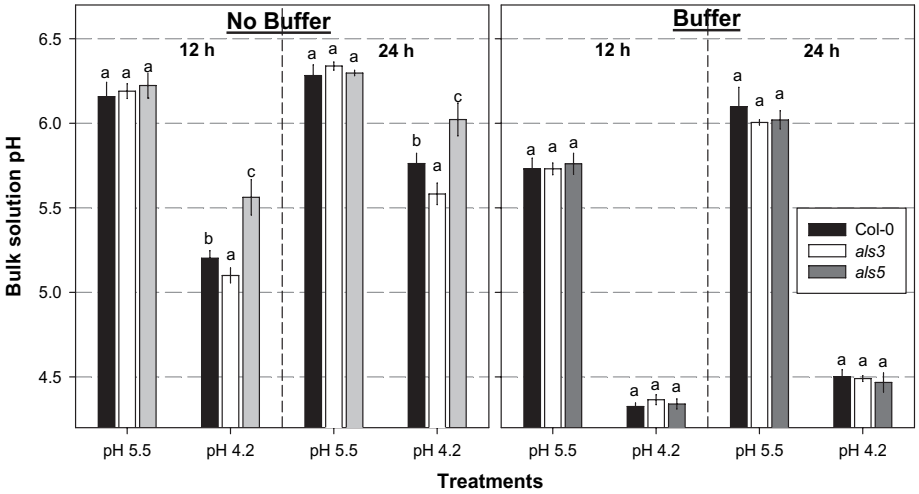


**Fig. 1.** Bulk solution pH of *Arabidopsis thaliana* genotypes with and without 0.5 mM homo-PIPES buffer measured on the second day at 12 h and 24 h after the nutrient solution change. Mean ±SE ($n$=4 replicates). Within each buffer×pH treatment, different letters represent significant difference by 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.

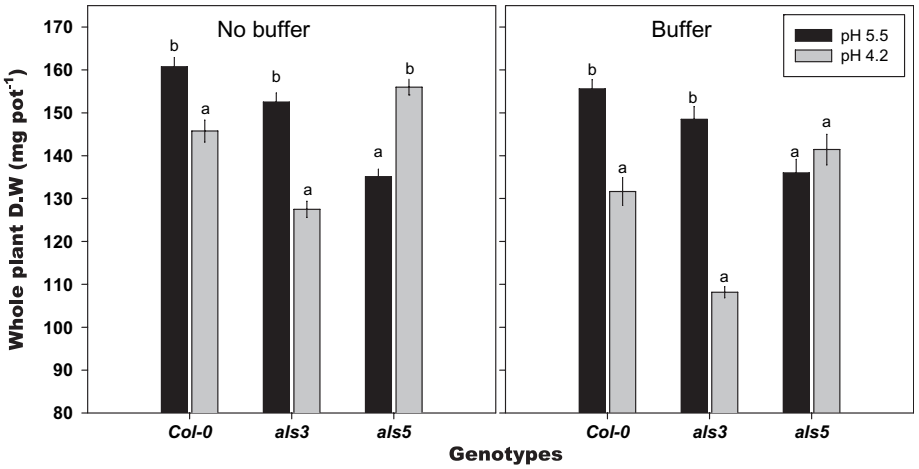


**Fig. 2.** Biomass of *Arabidopsis thaliana* genotypes at pH 4.2 with and without 0.5 mM homo-PIPES buffer. Mean ±SE ($n$=4 replicates). In each graph, different letters within each genotype represent significant difference between pH 5.5 and pH 4.2 by $t$-test at $P$ ⩽0.001.

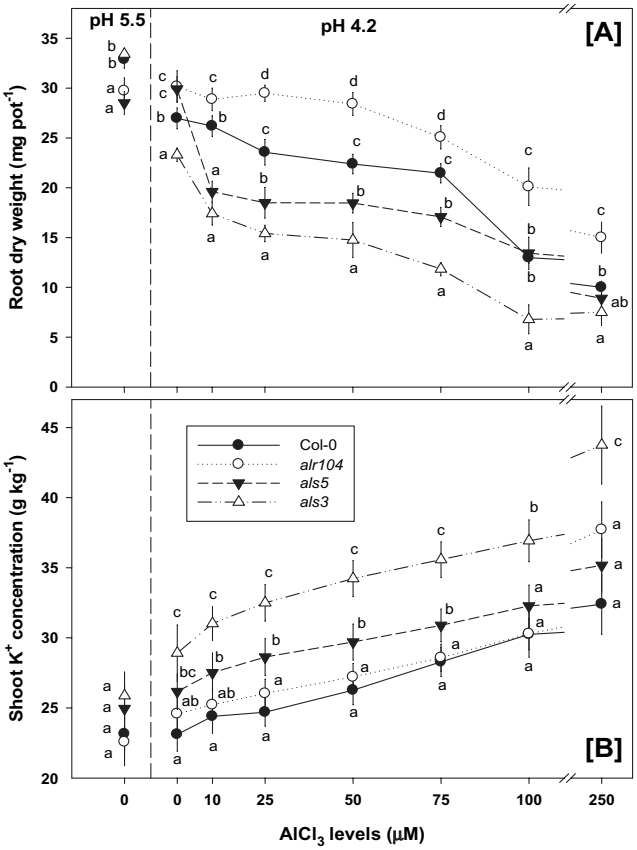


**Fig. 3.** Effect of low pH and Al stresses on *Arabidopsis thaliana* root biomass [A] and shoot $K^+$ concentration [B]. Means ±SE ($n$=3 replicates). In each $AlCl_3$ concentration, genotypes sharing a common letter are not significantly different by LSD test at $P \leqslant 0.05$. *Arabidopsis* seedlings 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 μM 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 μM), 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 μM 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 μM Al exposure in the Al-sensitive mutants (*als3* and *als5*) and after 22 min in the wild type and *alr104* mutant (Fig. 5).

*$K^+$ flux:* 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 μM 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 μM 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 $K^+$ efflux 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 μM 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 ($E_m$):* The resting $E_m$ (at pH 5.5) of the *alr104* mutant was more negative than the $E_m$ 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).

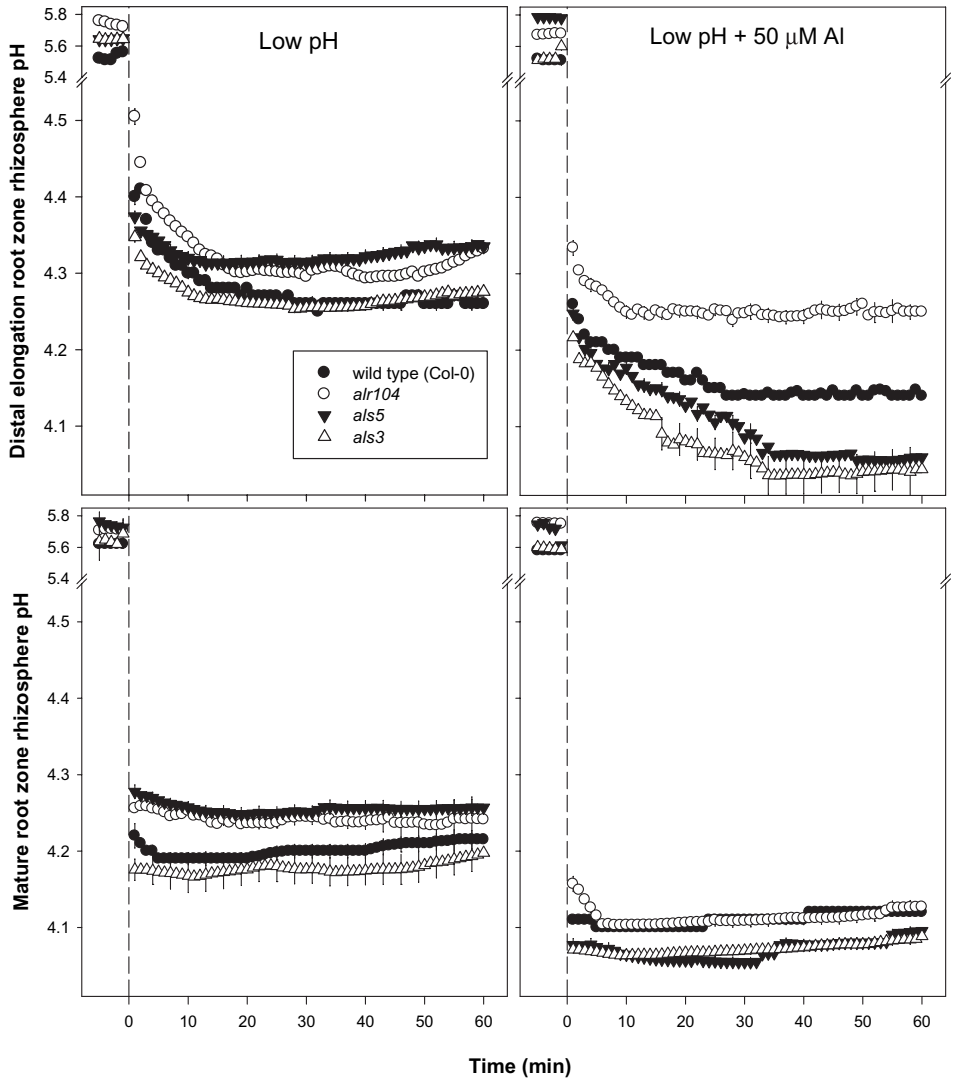


**Fig. 4.** Effect of low-pH and combined low-pH/Al treatments on rhizosphere pH at the distal elongation zone (top panel) and mature zone (bottom panel) of 4- to 5-day-old *Arabidopsis thaliana* roots. The low-pH and combined low-pH/Al treatments were imposed at time=0; the data recorded in the first 5 min before time=0 represent rhizosphere pH at pH 5.5. Error bars indicate ±SE ($n$=10–12 seedlings). *Arabidopsis* seedlings were conditioned in basal salt medium (BSM; 0.1 mM $CaCl_2$+1 mM KCl+0.2 mM $MgCl_2$, pH 5.5) for 20 min before treatments were imposed in unbuffered BSM.

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

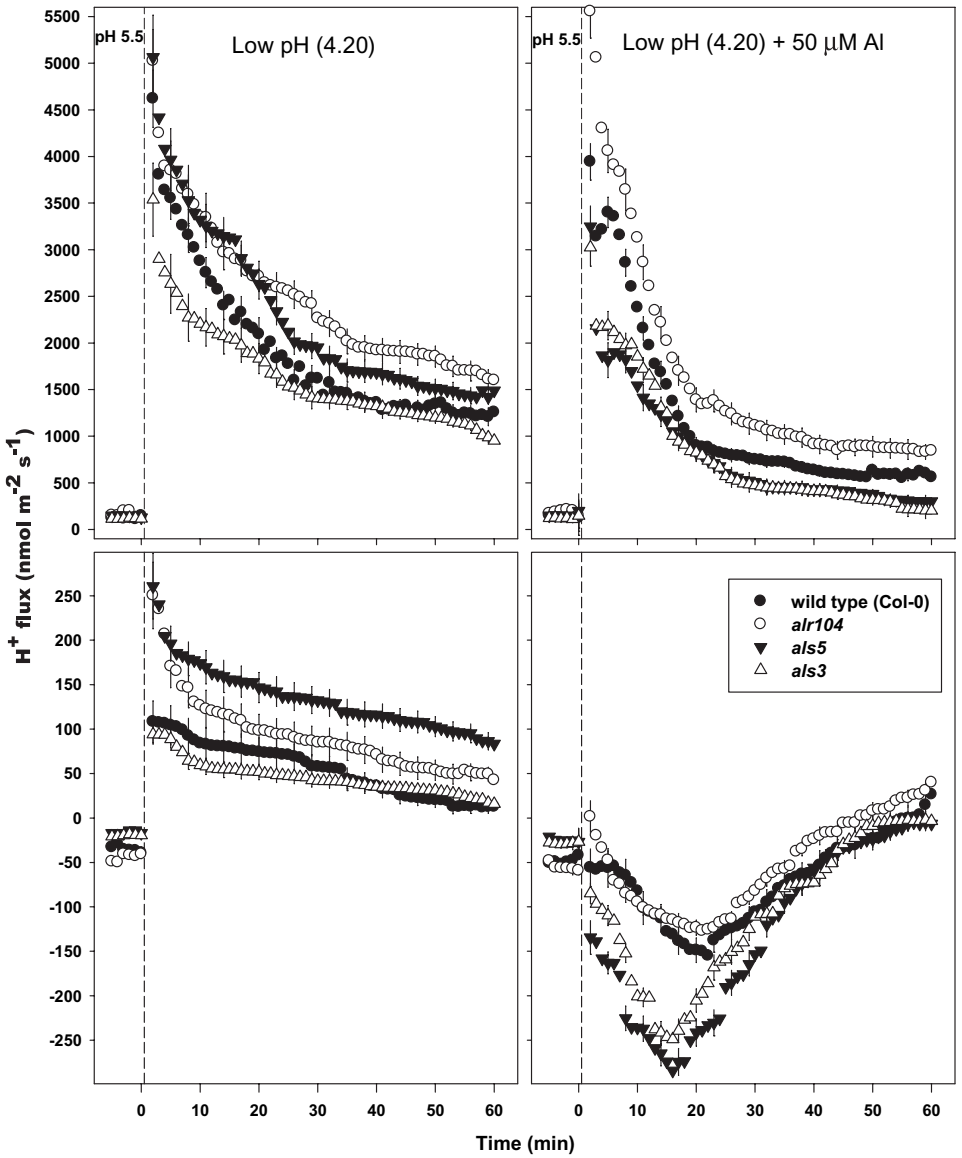


**Fig. 5.** Effect of low pH and combined low pH/50 μM Al on $H^+$ fluxes measured at the distal elongation zone (top panel) and the mature zone (bottom panel) of 4- to 5-day-old *Arabidopsis thaliana* roots. The low-pH and combined low-pH/Al treatments were imposed at time=0; the data recorded in the 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 $CaCl_2$+1 mM KCl+0.2 mM $MgCl_2$, pH 5.5) for 20 min before treatments were imposed in unbuffered BSM.

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 ($r \geqslant 0.93$). Thus, $H^+$ flux across the root tissue is likely

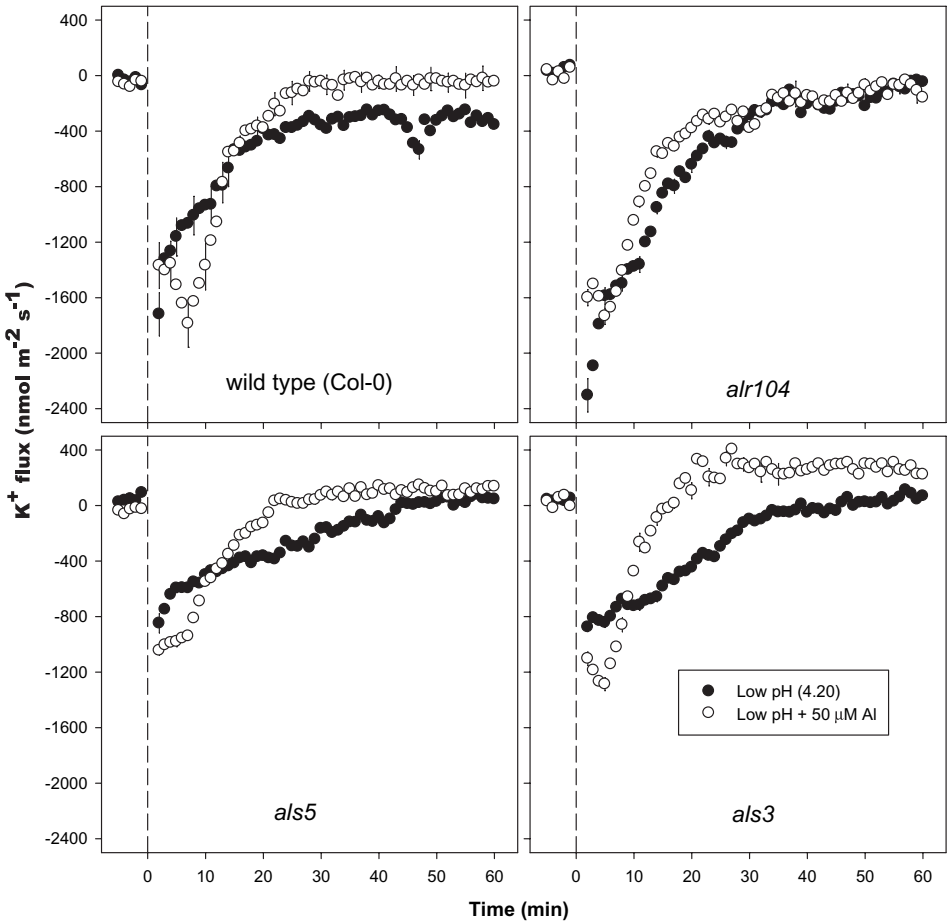


**Fig. 6.** Effect of low pH and combined low pH plus 50 μM $Al^{3+}$ on $K^+$ fluxes measured at the distal elongation zone of 4- to 5-day-old *Arabidopsis thaliana* roots. The low-pH and combined low-pH/$Al^{3+}$ treatments were imposed at time=0; the data recorded in the first 5 min before time=0 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 $CaCl_2$+1 mM KCl+0.2 mM $MgCl_2$, pH 5.5) for 20 min before treatments were imposed in unbuffered BSM.

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, 2004*a*). 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

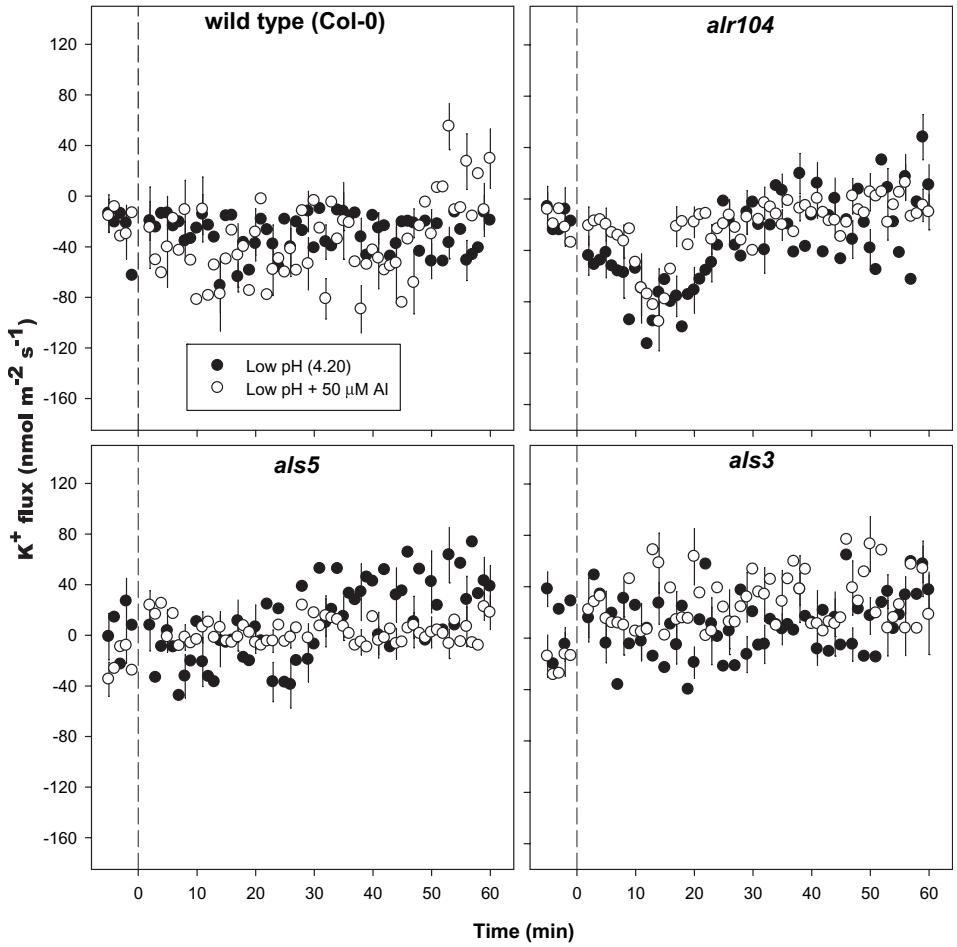


**Fig. 7.** Effect of low pH and combined low pH plus 50 μM $Al^{3+}$ on $K^+$ fluxes measured at the mature zone of 4- to 5-day-old *Arabidopsis thaliana* roots. The low-pH and combined low-pH/$Al^{3+}$ treatments were imposed at time=0; the data recorded in the first 5 min before time=0 represent $K^+$ 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 $CaCl_2$+1 mM KCl+0.2 mM $MgCl_2$, pH 5.5) for 20 min before treatments were imposed in unbuffered BSM.

the net $H^+$ efflux in the MZ. Furthermore, Ahn *et al.* (2004*a*) 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

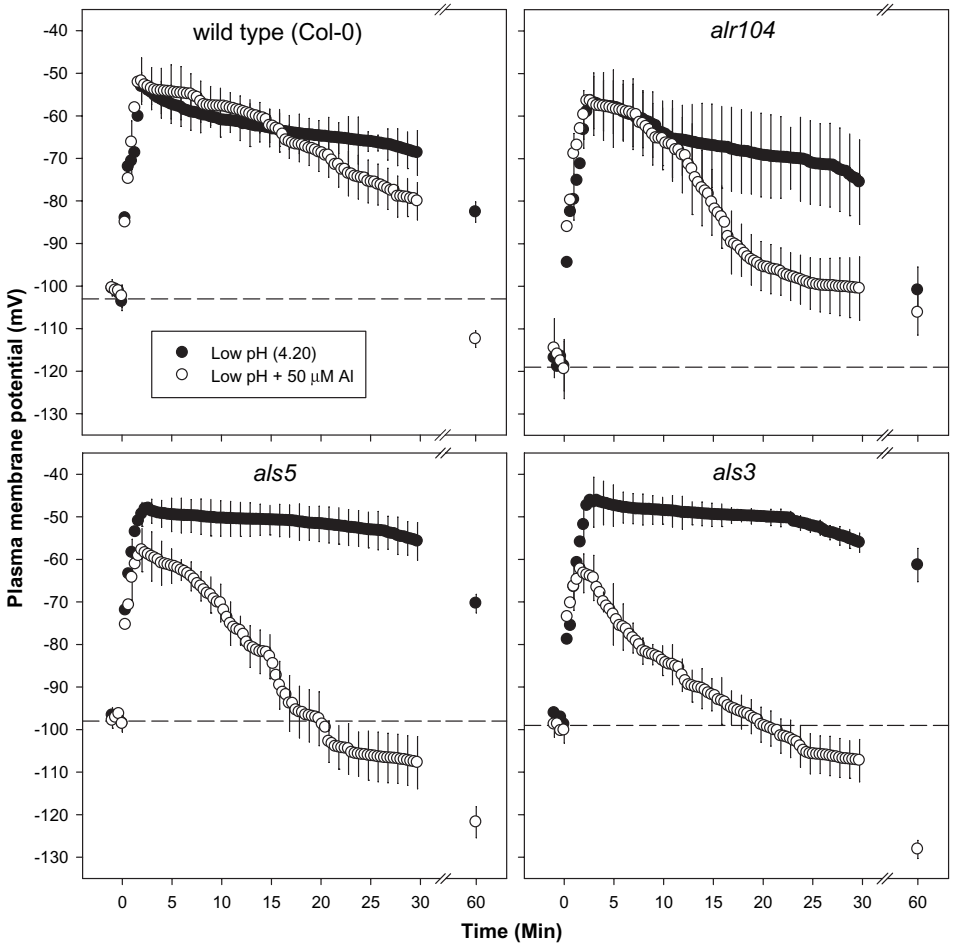


**Fig. 8.** Effect of low pH and combined low pH/Al treatments imposed at time=0 on the plasma membrane potential in the distal elongation zone of 4- to 5-day-old *Arabidopsis thaliana* roots. Error bars indicate ±SE ($n$=8–10 seedlings). The horizontal dotted lines represent the resting plasma membrane potential of the respective genotypes at pH 5.5. *Arabidopsis* seedlings were conditioned in basal salt medium (BSM; 0.1 mM $CaCl_2$+1 mM KCl+0.2 mM $MgCl_2$, 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 *alr104* 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 (*alr104* 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

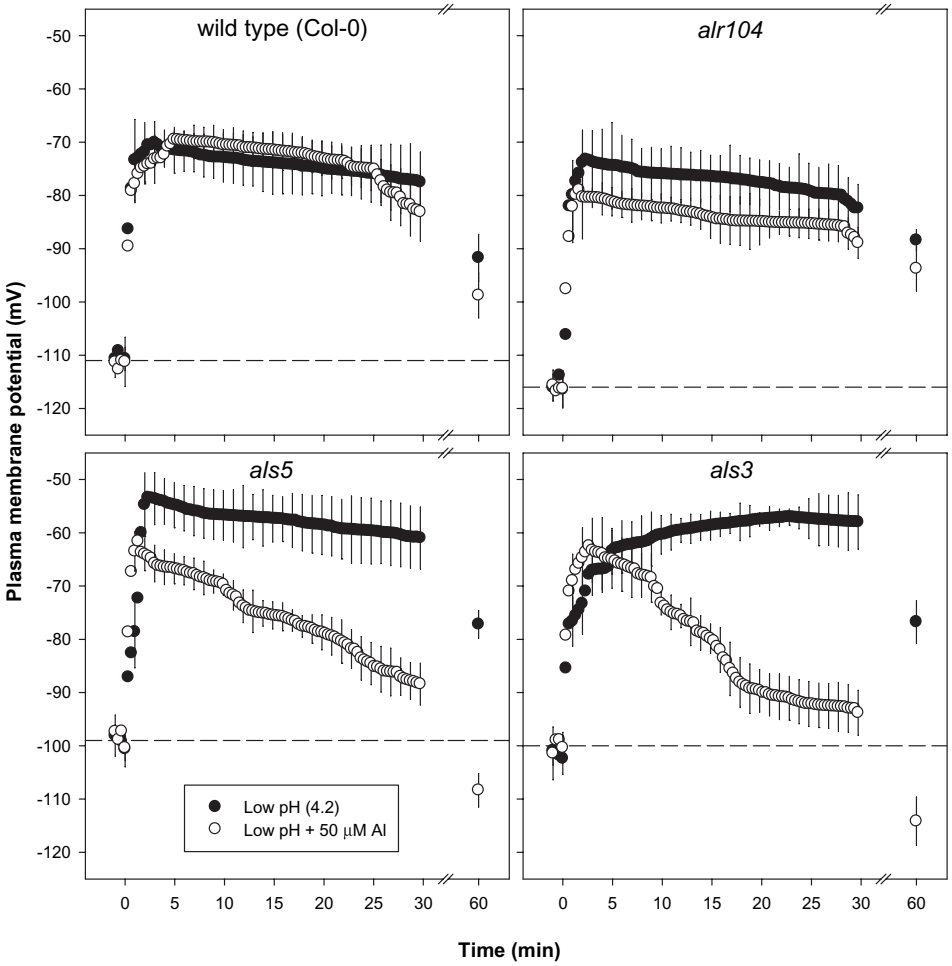


**Fig. 9.** Effect of low pH and combined low pH/Al treatments imposed at time=0 on the plasma membrane potential in the mature zone of 4- to 5-day-old *Arabidopsis thaliana* roots. Error bars indicate ±SE ($n$=8–10 seedlings). The horizontal dotted lines represent the resting plasma membrane potential of the respective genotypes at pH 5.5. *Arabidopsis* seedlings were conditioned in basal salt medium (BSM; 0.1 mM $CaCl_2$+1 mM KCl+0.2 mM $MgCl_2$, pH 5.5) for 20 min before treatments were imposed in unbuffered BSM.

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.*, 2004*b*; 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 *alt1-1* mutation (a suppressor of *als3* mutation), which demonstrated that *alt1-1* 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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