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RESEARCH PAPER

Aluminium-induced inhibition of root elongation in *Arabidopsis* is mediated by ethylene and auxin

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Abstract

Aluminium (Al) is phytotoxic when solubilized into Al^{3+} in acidic soils. One of the earliest and distinct symptoms of Al^{3+} toxicity is inhibition of root elongation. To decipher the mechanism by which Al^{3+} inhibits root elongation, the role of ethylene and auxin in Al^{3+}-induced inhibition of root elongation in *Arabidopsis thaliana* was investigated using the wild type and mutants defective in ethylene signalling (*etr1-3* and *ein2-1*) and auxin polar transport (*aux1-7* and *pin2*). Exposure of wild-type *Arabidopsis* to $AlCl_3$ led to a marked inhibition of root elongation, and elicited a rapid ethylene evolution and enhanced activity of the ethylene reporter *EBS:GUS* in root apices. Root elongation in *etr1-3* and *ein2-1* mutants was less inhibited by Al^{3+} than that in wild-type plants. Ethylene synthesis inhibitors, Co^{2+} and aminoethoxyvinylglycine (AVG), and an antagonist of ethylene perception (Ag^{+}) abolished the Al^{3+}-induced inhibition of root elongation. There was less inhibition of root elongation by Al^{3+} in *aux1-7* and *pin2* mutants than in the wild type. The auxin polar transport inhibitor, naphthylphthalamic acid (NPA), substantially alleviated the Al^{3+}-induced inhibition of root elongation. The Al^{3+} and ethylene synthesis precursor aminocyclopropane carboxylic acid (ACC) increased auxin reporter *DR5:GUS* activity in roots. The Al^{3+}-induced increase in *DR5:GUS* activity was reduced by AVG, while the Al^{3+}-induced increase in *EBS:GUS* activity was not altered by NPA. Al^{3+} and ACC increased transcripts of *AUX1* and *PIN2*, and this effect was no longer observed in the presence of AVG and Co^{2+}. These findings indicate that Al^{3+}-induced ethylene production is likely to act as a signal to alter auxin distribution in roots by disrupting AUX1- and PIN2-mediated auxin polar transport, leading to arrest of root elongation.

Key words: Aluminium toxicity, *Arabidopsis thaliana*, auxin polar transport, ethylene, root elongation.

Introduction

Aluminium (Al) is the most abundant metal in the Earth's crust. Most Al occurs in soil as aluminosilicate, which is usually non-toxic to living organisms (May and Nordstrom, 1991). However, Al^{3+} is hydrolysed into Al^{3+} cations in acidic environments, and becomes a major factor limiting crop production and yield in many acid soils throughout the world (Foy, 1988). Inhibition of root elongation is one of the earliest and most distinct symptoms exhibited by plants exposed to micromolar concentrations of Al^{3+} in solution cultures (Zhang and Rengel, 1999; Doncheva *et al.*, 2005). Although recent studies suggested that Al^{3+} can induce a rapid change in the position of cell division activity in maize (Doncheva *et al.*, 2005), it is generally believed that the rapid inhibition of root growth induced by Al^{3+} is primarily caused by inhibition of cell elongation (Horst, 1995; Matsumoto, 2000). Furthermore, several studies have demonstrated that the root apex, particularly the root distal transition zone, is a critical site of perception and expression of Al toxicity (Ryan *et al*, 1993; Sivaguru and Horst, 1998). Although extensive research has demonstrated that Al^{3+} alters numerous physiological processes, including disruption of cytosolic Ca^{2+} homeostasis, alterations of

cytoskeleton dynamics (see reviews by Matsumoto, 2000; Barcelo and Poschenrieder, 2002; Rengel and Zhang, 2003), and disturbance of endogenous nitric oxide in root tips (Illes *et al.*, 2006; Tian *et al.*, 2007), the primary mechanisms underlying Al toxicity in plants remain largely unknown.

Phytohormones, particularly auxin and ethylene, play critical roles in modulating root growth. For instance, it has been shown that ethylene affects root growth by inhibiting the rapid expansion of cells leaving the root meristem (Le *et al.*, 2001; Swarup *et al.*, 2007). This feature resembles the widely observed rapid inhibition of root elongation by Al^{3+}. In higher plants, ethylene is produced from methionine through *S*-adenosyl-L-methionine and 1-aminocyclopropane-1-carboxylic acid (ACC), catalysed by ACC synthase (ACS) and ACC oxidase (ACO), respectively (Kende, 1993). ACS and ACO are encoded by multigene families and regulated by many biotic and abiotic factors (Wang *et al.*, 2002). Our previous work has established that ethylene plays a critical role in Al-induced inhibition of root elongation in *Lotus japonicus* such that inhibition of Al^{3+}-induced ethylene production from root apices by ethylene synthesis antagonists markedly alleviates the Al-induced inhibition of root elongation (Sun *et al.*, 2007). In addition to ethylene, root growth and development are also closely related to auxin synthesis, distribution, and transport (Blilou *et al.*, 2005; Tanaka *et al.*, 2006). Several studies have demonstrated that Al^{3+} may interact with auxin signalling pathways by possibly targeting auxin polar transport systems, leading to alterations of auxin accumulation and distribution in roots (Kollmeier *et al.*, 2000; Doncheva *et al.*, 2005; Shen *et al.*, 2008). Auxin, which is transported to roots by polar transport systems through the specific subcellular localization of auxin efflux and auxin influx machineries, modulates root growth and development (Benjamins *et al.*, 2005). It has been identified that the PIN FORMED (PIN) proteins function to mediate auxin efflux (Blilou *et al.*, 2005; Paponov *et al.*, 2005; Teale *et al.*, 2006). Among the PIN proteins, PIN2 is involved in the transport of auxin from the root tip into the elongation zone and back again via the cortex toward the root tip (Blilou *et al.*, 2005). AUXIN RESISTANT 1 (AUX1) is an auxin influx carrier (Bennett *et al.*, 1996) which facilitates polar auxin delivery to the root apex (Swarup *et al.*, 2005). A recent study indicated that Al^{3+} reduces the auxin concentration in the transition zone of *Arabidopsis* roots by inhibiting the transport of PIN2 vesicles from plasma membranes to endosomes (Shen *et al.*, 2008). However, there has been no detailed study to investigate the role of PIN2 and AUX1 in Al^{3+}-induced inhibition of root elongation.

Synergistic effects of auxin and ethylene on root growth have been extensively studied using an array of *Arabidopsis* mutants defective in signalling of ethylene and auxin (Stepanova *et al.*, 2005, 2007; Růžička *et al.*, 2007; Swarup *et al.*, 2007). For example, Růžička *et al.* (2007) demonstrated that ethylene stimulates auxin biosynthesis and basipetal auxin transport toward the elongation zone, leading to the inhibition of root cell elongation. Because ethylene (Massot *et al.*, 2002; Sun *et al.*, 2007) and auxin (Kollmeier *et al.*, 2000; Doncheva *et al.*, 2005; Shen *et al.*, 2008) have been implicated in Al^{3+}-dependent inhibition of root growth, it is conceivable that cross-talk between the two hormones may exist in Al^{3+}-dependent inhibition of root growth in plants. In the present study, this issue was addressed by using pharmacological agents and several *Arabidopsis* mutants with impaired auxin and ethylene signalling (*pin2*, *aux1-7*, *etr1-3*, and *ein2-1*). The effect of Al^{3+} on auxin and ethylene production and distribution was also studied by monitoring activities of auxin (*DR5:GUS*) and ethylene (*EBS:GUS*) reporter genes.

Materials and methods

Plant materials and growth conditions

Arabidopsis thaliana ecotype Columbia (Col), ethylene-insensitive mutants *etr1-3* and *ein2-1*, and auxin polar transport mutants *aux1-7* and *pin2* were obtained from the Arabidopsis Biological Resource Centre, Columbus, OH, USA. The EBS–GUS reporter line, in which the *GUS* reporter gene is driven by a synthetic EIN3-responsive promoter, was generously provided by Dr J Alonso, and was originally generated by Dr Anna Stepanova (Stepanova *et al.*, 2005). *DR5* is a synthetic auxin-responsive promoter which has been widely used to monitor auxin responses *in planta*. The DR5–GUS report line used in the present study is described by Ulmasov *et al.* (1997) and is a kind gift of Professor Tom Guilfoyle. All seeds were surface-sterilized by incubation for 1 min in 75% ethanol, and rinsed thoroughly with sterile distilled water followed by exposure to 10% (v/v) sodium hypochlorite for 15 min, and then washed with sterile water. The sterilized seeds were sown on 1/2 MS agar plates [0.6% agar (w/v), pH 5.8]. Wild-type, *etr1-3*, *ein2-1*, *aux1-7*, and *pin2*, and DR5–GUS and EBS–GUS reporter seedlings (5 d old) grown on 1/2 MS agar plates were transferred to agar medium containing 1/2 MS nutrients, 0.8% sucrose, and 0.7% (w/v) agar, with pH adjusted to 5.8 for another 7 d. All seedlings were grown in 9 cm diameter glass dishes, oriented vertically, in a controlled environment with a temperature of 20/23 °C, 14/10 h light cycle, and photosynthetic photon flux density of 100–120 μmol m^{-2} s^{-1}.

Root elongation assays

To study the inhibitory effect of $AlCl_3$ on root elongation, *Arabidopsis* seedlings were incubated in 1/2 MS agar plates for 7 d and then transferred into Petri dishes with solutions containing 0.5 mM $CaCl_2$ with and without 50 μM $AlCl_3$ (pH 4.5) for 24 h or with agar (0.7%) containing $AlCl_3$ (0, 50, 100, and 200 μM, pH 4.5) for 4 d. Elongation of the primary root was measured after treating the roots for varying periods under a microscope. To study the effect of $AlCl_3$ on root elongation, seedlings of Col-0, *etr1-3*, *ein2-1*, *aux1-7*, and *pin2* were exposed to 50 μM $AlCl_3$ and root elongation was measured after exposure of seedlings to $AlCl_3$ for 24 h. To study the effect of aminoethoxyvinylglycine (AVG), Co^{2+}, $AgNO_3$, and naphthylphthalamic acid (NPA) on root elongation in the absence and presence of 50 μM $AlCl_3$, seedlings of *Arabidopsis* wild type (Col-0) were first exposed to 10 μM AVG, 10 μM $CoCl_2$, or 10 μM NPA for 2 h and then incubated in 50 μM $AlCl_3$ for another 24 h. For treatment with Ag^+, seedlings were first incubated in 10 μM $AgNO_3$ as control and then exposed to 50 μM $Al(NO_3)_3$ for 24 h to determine the effect of Ag^+ on root elongation of *Arabidopsis* wild-type (Col-0). Values are given as the mean±SE of at least 10 independent measurements. All experiments were repeated at least three times.

Determination of ethylene production

After exposure of *Arabidopsis* seedlings to 50 μM $AlCl_3$ for varying durations, root tips (~1 cm in length) of ~0.2 g were

excised and put into 5 ml gas-tight vials containing 1 ml of agar medium (0.7% agar). A 1 ml volume of the headspace was taken from the vials, and then injected into a gas chromatograph (GC) equipped with an alumina column (GDX502) and a flame ionization detector (GC-7AG; Shimadzu Japan) for measurement of the ethylene concentration.

GUS staining

GUS staining was carried as described in the literature (Jefferson *et al.*, 1987; Malamy and Benfey, 1997; Stepanova *et al.*, 2005). Briefly, 7-d-old seedlings were pulled out of agar and exposed to control solution and to solutions supplemented with 50 μM $AlCl_3$ (pH 4.5), 10 μM ACC, or 10 μM NPA for 2 h, fixed in an ice-cold 90% acetone, washed once with the rinse buffer, which is composed of 100 mM $NaPO_4$ buffer (pH 7.0), 1 mM $K_3Fe(CN)_6$, and 1 mM $K_4Fe(CN)_6$, and stained for 4 h in the dark at 37 °C. Staining buffer comprises 100 mM $NaPO_4$, pH 7.0, 1 mM $K_3Fe(CN)_6$, 1 mM $K_4Fe(CN)_6$, 10 mM Na_2EDTA, 0.1% (v/v) Triton X-100, 20% (v/v) methanol and 0.5 mg ml^{-1} X-Gluc. For observation of whole mounts, stained seedlings were transferred to small Petri dishes containing 0.24 N HCl in 20% methanol and incubated on a 57 °C heat block for 15 min. This solution was replaced with another solution containing 7% NaOH, 7% hydroxylamine-HCl in 60% ethanol for 15 min at room temperature. Roots were then rehydrated for 5 min in 40, 20, and 10% ethanol, respectively, and infiltrated for 15 min in 5% ethanol, 25% glycerol. Roots were mounted in 50% glycerol on glass microscope slides and individual seedlings were photographed as described by Malamy and Benfey (1997).

Gene expression analysis

Real-time RT-PCR was used to study the expression patterns of *ACS2*, *ACS6*, *ACS8*, *ACO1*, *ACO2*, *AUX1*, *PIN1*, and *PIN2* genes in *Arabidopsis* in response to different treatments including $AlCl_3$, ethylene precursors, and ethylene synthesis inhibitors. Total RNAs were extracted from *Arabidopsis* roots with Trizol reagent (Invitrogen) and treated with RNase-free DNase I (Promega). The total RNAs were reverse-transcribed into first-strand cDNA in a 20 μl volume with M-MLV reverse transcriptase (Promega). The samples were diluted to 100 μl with water, and 5 μl of each sample (~8 ng RNA equivalent) were PCR amplified using SYBR GreenER™ qPCR SuperMix Universal (Invitrogen) in a 25 μl reaction, containing 5 μl of diluted cDNA, 12.5 μl of SYBR GreenER™ qPCR SuperMix Universal, 0.5 μl of Rox Reference Dye, 1 μl of 10 μM forward primer, 1 μl of 10 μM reverse primer, and 5 μl of water. The Mx3000P machine was used to run quantitative RT-PCR with the following eight primer pair combinations: *AtACS2*, 5′-TCATGGGAAAAGCTAGAGGTGGAAG-3′ and 5′-TCAACGGTTAATTTGAAATTGTCGG-3′; *AtACS6*, 5′-AAACCGATGGCTGCAACAACTATGAT-3′ and 5′-TAAGTCTGTGCACGGACTAGCGGAG-3′; *AtACS8*, 5′-TGGGGTGATTTACTCCAACGATGATT-3′ and 5′-GACACTCGATGCCTGCAGCCTCTAG-3′; *AtACO1*, 5′-CCGTGTAATGACAGTGAAGCATGGAAG-3′ and 5′-TCTCAAGTCTGGGGCCTTTGTCTCC-3′; *AtACO2*, 5′-GGATGTCGGTTGCATCGTTTTA-3′ and 5′-TACGGCTGCTGTAGGATTCAGTTC-3′; *AtAUX1*, 5′-AGACGCACTTCTCGACCACTCCA-3′ and 5′-GCATCCCAATCACTTTCTCCCACA-3′; *AtPIN2*, 5′-CGCTCTTTTCACTATCAACACTGCCTAA-3′ and 5′-GTCTCCTATTCCGCATCGGTCTG-3′. In addition, a housekeeping gene, *AtActin11*, was employed as a control: 5′-CCACATGCTATTCTGCGTTTGGACC-3′ and 5′-CATCCCTTACGATTTCACGCTCTGC-3′

Primers were designed across exon–exon junctions of cDNA to avoid potential problems due to contamination of genomic DNA. The amplification efficiency for each primer pair was calculated using serial cDNA dilutions. After correcting the cycle threshold values according to the amplification efficiency, the expression values of the eight genes were normalized to the corresponding controls.

Results

Al^{3+} inhibited root elongation and evoked ethylene production

To examine the sensitivity of primary root elongation to Al^{3+}, *Arabidopsis* seedlings were exposed to hydroponic solutions with varying concentrations of $AlCl_3$ (0, 20, 50, and 100 μM, pH 4.5) for 24 h. As shown in Fig. 1A, root elongation was rapidly inhibited by exposure to Al^{3+}, and the inhibition of root elongation was positively dependent on $AlCl_3$ concentrations. For instance, root elongation was inhibited by 32, 71, and 97% after 24 h exposure to 20, 50, and 100 μM $AlCl_3$, respectively. A previous study has revealed that Al^{3+} evokes a rapid ethylene burst from root tips of *Lotus japonicas* (Sun *et al.*, 2007). To test whether a similar mechanism is operative in *Arabidopsis*, the effect of Al^{3+} on ethylene evolution from root tips of *Arabidopsis* was investigated. A rapid burst of ethylene evolution was

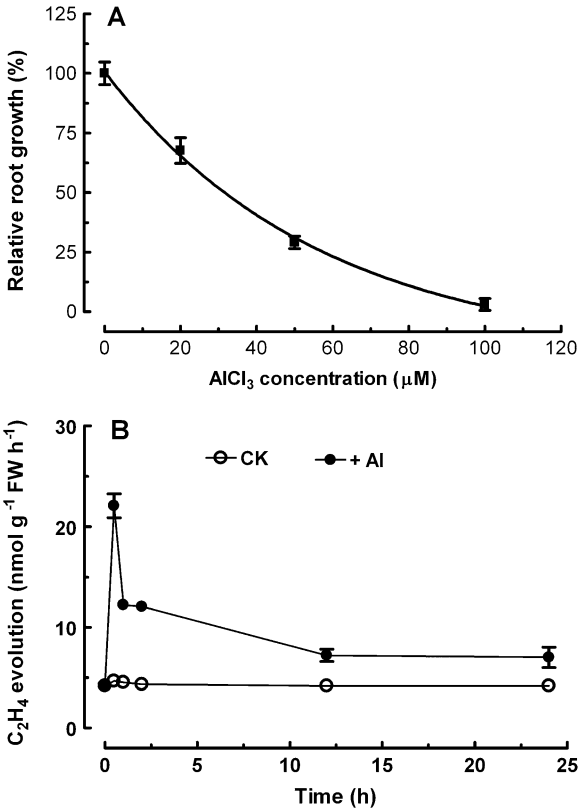

Fig. 1. (A) Response of root elongation in *Arabidopsis* wild-type (Col-0) seedlings to varying concentrations of $AlCl_3$ (A). The lengths of primary roots were measured after the seedlings were exposed to solutions containing 0, 20, 50, and 100 μM $AlCl_3$ in addition to 0.5 mM $CaCl_2$ pH 4.5 for 24 h. Data are presented as relative root elongation compared with control values. All values are means ±SE of >8 roots. (B) Time course of ethylene evolution from root tips of wild-type *Arabidopsis* upon exposure to 50 μM $AlCl_3$. Values are means ±SE of five replicates.

observed upon exposure of *Arabidopsis* roots to $AlCl_3$ (Fig. 1B). The ethylene production reached a maximum after 30 min of exposure to Al^{3+}, and thereafter the evolution rapidly declined to a relatively steady level after exposure to Al^{3+} for 12 h.

To evaluate the role of ethylene in Al^{3+}-induced inhibition of root elongation, a genetic approach was employed by using the ethylene-insensitive mutants *etr1-3* and *ein2-1*. Exposure of *etr1-3* and *ein2-1* mutants to 50 μM $AlCl_3$ led to less inhibition of root elongation than that of the wild type (Col-0), i.e. root elongation was reduced by 71, 23, and 21% for wild-type, *etr1-3* and *ein2-1* plants, respectively, upon 24 h exposure to 50 μM $AlCl_3$. Note that root elongation of *etr1-3* and *ein2-1* was ~20% greater than that of wild-type plants in the absence of $AlCl_3$ (Fig. 2A). A similar less inhibitory effect of Al^{3+} on root elongation in the two ethylene-insensitive mutants than in wild-type plants was also observed when these plants were grown on agar containing varying concentrations of $AlCl_3$ (0, 50, 100, and 200 μM) for 4 d (Fig. 2B). The lower inhibitory effect of $AlCl_3$ on root elongation when grown in agar could be ascribed to reduced Al^{3+} activity due to its complexing with agar.

Al^{3+} had less effect on root elongation in auxin-insensitive mutants

In addition to ethylene, the role of auxin in Al-induced inhibition of root elongation was also examined using the auxin polar transport mutants *aux1-7* and *pin2*. In contrast to wild-type plants, root elongation in both *aux1-7* and *pin2* was relatively insensitive to Al^{3+} when treated with 50 μM Al^{3+} hydroponically for 24 h (Fig. 3A). When both wild-type and mutant seedlings were grown in agar containing

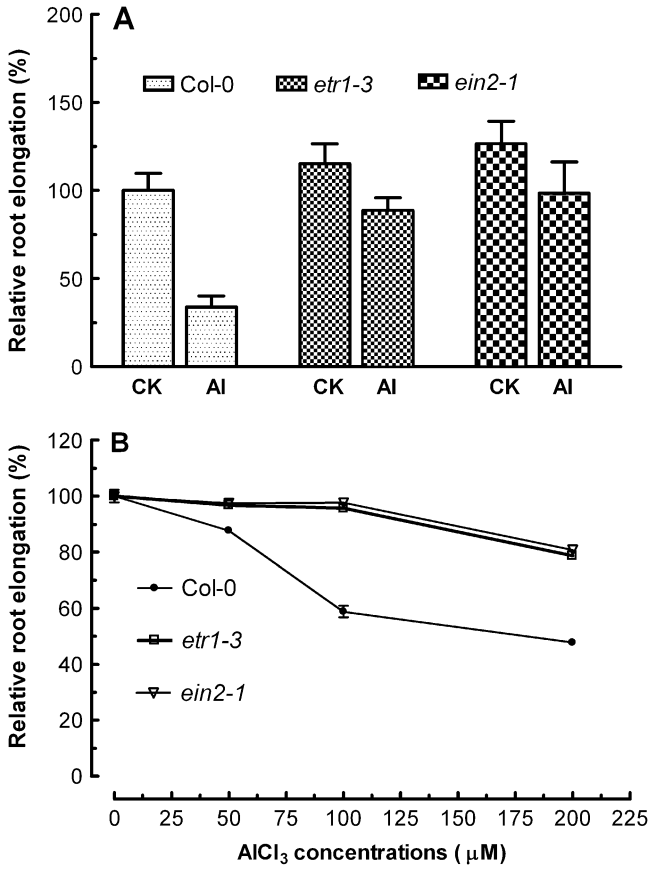

Fig. 2. Effect of $AlCl_3$ on root elongation of *Arabidopsis* wild-type (Col-0) and ethylene-insensitive mutants, *etr1-3* and *ein2-1*. (A) Seedlings of Col-0, *etr1-3*, and *ein2-1* were exposed to 50 μM $AlCl_3$ and root elongation was measured under a stereomicroscope after exposure of seedlings for 24 h. (B) Effect of varying concentrations of $AlCl_3$ on root growth of seedlings grown on agar. Primary root length was measured before and after transferring wild-type seedlings to agar medium with varying concentrations of $AlCl_3$ (0, 50, 100, and 200 μM, pH 4.5) for 4 d. Data are expressed as root elongation relative to controls, and given as means ±SE of >8 roots. The root elongation rate in the absence of $AlCl_3$ for Col-0, *etr1-3*, and *ein2-1* was 5.41±0.13 mm d^{-1} (n=17), 6.06±0.59 mm d^{-1} (n=6), and 6.66±0.66 mm d^{-1} (n=6), respectively.

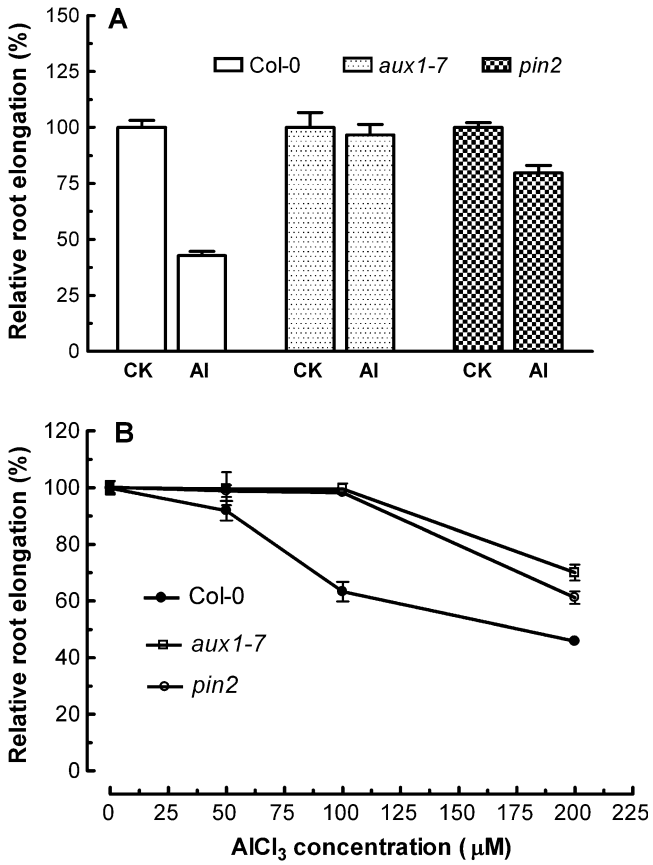

Fig. 3. Effect of $AlCl_3$ on root elongation of *Arabidopsis* wild-type (Col-0) and auxin polar transport mutants, *aux1-7* and *pin2*. (A) Seedlings of Col-0, *aux1-7*, and *pin2* were exposed to 50 μM $AlCl_3$ (pH 4.5) and root length was measured under a stereomicroscope after 24 h. (B) Effect of $AlCl_3$ on root elongation of seedlings grown on agar medium containing varying concentrations of $AlCl_3$ (pH 4.5) for 4 d. Data are expressed as relative root elongation relative to controls, and are presented as means ±SE of >8 roots. The root elongation rate in the absence of $AlCl_3$ for Col-0, *aux1-7*, and *pin2* was 5.41±0.13 mm d^{-1} (n=17), 7.14±0.47 mm d^{-1} (n=13), and 5.26±0.11 mm d^{-1} (n=10), respectively.

varying concentrations of $AlCl_3$ for 4 d, root elongation of *aux1-7* and *pin2* was also less inhibited than that of the wild type (Fig. 3B). For instance, root elongation in wild-type plants was reduced by 38% when grown in agar containing 100 μM $AlCl_3$ (pH 4.5), while root elongation in *aux1-7* and *pin2* was not affected when grown under the identical $AlCl_3$ conditions (Fig. 3B). These results are indicative that AUX1 and PIN2 are involved in Al-induced inhibition of root elongation in *Arabidopsis*.

AVG, Co^{2+}, and NPA alleviated Al-induced inhibition of root elongation

The less inhibitory effect of Al^{3+} on root elongation in the *Arabidopsis* mutants insensitive to ethylene and auxin than that in wild-type plants suggests that both ethylene and auxin may be involved in Al-induced inhibition of root elongation. To verify this hypothesis, the effects of Al^{3+} on root elongation in the presence of antagonists of ethylene biosynthesis (AVG and Co^{2+}) and ethylene perception (Ag^{+}) were examined. The Al^{3+}-induced inhibition of root elongation was markedly recovered when *Arabidopsis* seedlings were exposed to 10 μM AVG and $CoCl_2$ for 12 h prior to treatment with Al^{3+} (Fig. 4A, B). In the absence of Al^{3+}, AVG reduced root elongation by ~35% (Fig. 4A), while $CoCl_2$ had no effect on root elongation in the absence of Al^{3+} (Fig. 4B). A similar ameliorative effect on the inhibition of root elongation caused by Al^{3+} was also observed by treatment with the ethylene perception inhibitor, $AgNO_3$ (Fig. 4C). As root elongation in *aux1-7* and *pin2* mutants was also less sensitive to Al^{3+} than in wild-type plants (Fig. 3), the effect of an antagonist of auxin polar transport, NPA, on root elongation in the absence and presence of Al^{3+} was studied. As shown in Fig. 4D, NPA marginally inhibited root growth in the absence of Al^{3+}, while NPA substantially alleviated the Al-induced inhibition of root elongation.

Al^{3+} stimulated the activity of EBS:GUS *and* DR5:GUS

To investigate the mechanism by which Al^{3+} affects the synthesis and distribution of ethylene and auxin, the expression levels of the ethylene reporter construct, *EBS:GUS*, in which the *GUS* reporter gene is driven by a synthetic EIN3-responsive promoter, were first tested. In the absence of Al^{3+} or ACC, no visible expression of *EBS:GUS* in root tips was observed (Fig. 5). Upon exposure to Al^{3+}, there was a marked increase in the activity of *EBS:GUS* in the root apices (Fig. 5). A comparable increase in *EBS:GUS* activity was also observed when roots were treated with the ethylene synthesis precursor ACC (Fig. 5). In addition to *EBS:GUS* activity, the response of the auxin reporter *DR5:GUS* to Al^{3+} and ACC was also investigated. In control roots, *DR5:GUS* was mainly expressed in the quiescent zones and surrounding columella cells in root apices (Fig. 5). Treatment with Al^{3+} and ACC enhanced the levels of *DR5:GUS* in these areas as well as in the transition zone (Fig. 5). The similarity in response of *DR5:GUS* expression to Al^{3+} and ACC highlights cross-talk between ethylene and auxin in the Al-induced arrest of root elongation.

To unravel the relationship between the Al-induced increases in the activities of *EBS:GUS* and *DR5:GUS*, the effect of an ethylene synthesis inhibitor (AVG) and an auxin polar transporter inhibitor (NPA) on Al-dependent *DR5:GUS* and *EBS:GUS* expression, respectively, was investigated. As shown in Fig. 6, the Al-induced increase in activity of *DR5:GUS* was reduced by AVG. In contrast, NPA appeared to have a limited effect on the Al-induced increase in *EBS:GUS* activity (Fig. 6). These results are indicative that disruption of auxin distribution may result from elevated ethylene production evoked by Al^{3+}. In addition, AVG may also alter auxin distribution in the absence of Al^{3+}, as evidenced by AVG reducing *DR5:GUS* activity in roots without exposure to Al^{3+} (Fig. 6).

To test whether Al-induced disruption of auxin distribution in root apices occurs through Al-elicited ethylene production, the effect of Al^{3+} on ethylene production in *aux1-7* and *pin2* was studied. Ethylene evolution from the two auxin-insensitive mutants was lower than that in their wild-type counterpart in the absence Al^{3+} (Fig. 7A). Despite the lower basal levels of ethylene in the two mutants, both mutants exhibited increases in ethylene production when exposed to Al^{3+} (Fig. 7A). For instance, ethylene evolution was increased by 221, 290, and 262% in response to a 2 h exposure to Al^{3+} in the wild-type, *aux1-7* and *pin2* mutants, respectively. Moreover, it was found that NPA had no effect on Al-dependent ethylene evolution from root tips of wild-type seedlings (Fig. 7B).

Al^{3+} up-regulated expression of ASC, ACO, AUX1, PIN1, *and* PIN2

It was previously demonstrated that Al-induced ethylene evolution in *L. japonicas* roots is due to up-regulation of genes encoding ACS and ACO (Sun *et al.*, 2007). To confirm whether a similar mechanism accounts for ethylene evolution in *Arabidopsis* roots in response to Al^{3+}, the effect of Al^{3+} on expression of *AtACS and AtACO* was investigated by quantitative RT-PCR. In *Arabidopsis*, there are 12 *ACS* genes that encode eight functional ACS proteins (Tsuchiasaka and Theologis, 2004). It has been shown that *AtACS2, AtACS6*, and *AtACS8* are highly expressed in roots and responsive to environmental stress and auxin (Tsuchiasaka and Theologis, 2004). Similar to *AtACS*, expression of both *AtACO1* and *AtACO2* was rapidly up-regulated in response to Al^{3+} treatment (Fig. 8A). The Al-dependent up-regulation of these genes exhibited transient characteristics such that the expression peaked after exposure to Al^{3+} for 30 min and thereafter declined with exposure time (Fig. 8A). In addition to *ACS* and *ACO*, the effect of Al^{3+} on expression patterns of *AtAUX1* and *AtPIN2* was also studied. Unlike *ACS* and *ACO*, expression of *AtAUX1* and *AtPIN2* was increased marginally after exposure to Al^{3+} for 30 min, and the expression of these genes reached a maximum after 2 h exposure to Al^{3+}

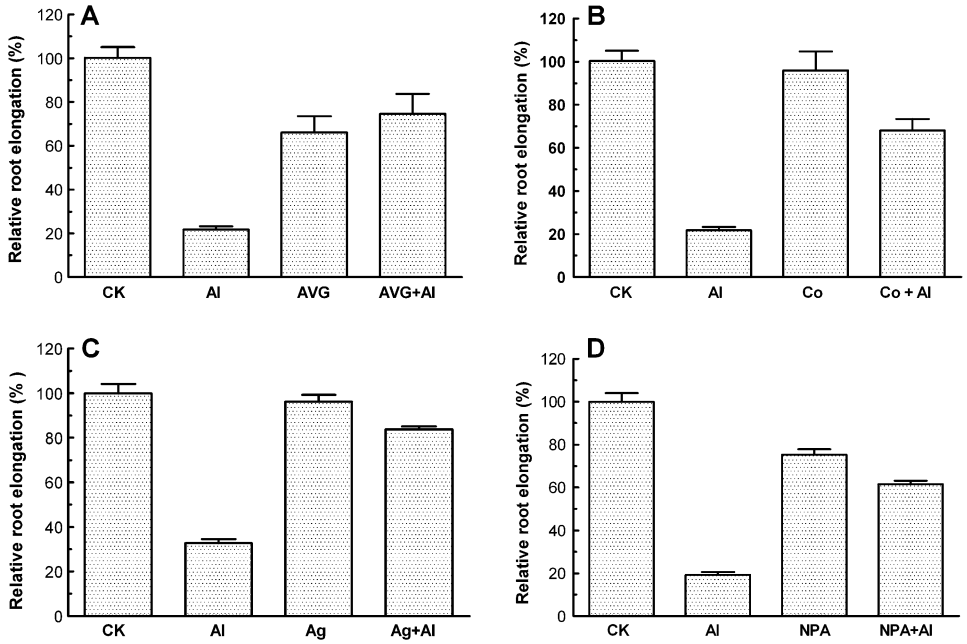

Fig. 4. Effect of (A) AVG, (B) Co^{2+}, (C) $AgNO_3$, and (D) NPA on root elongation in the absence and presence of 50 μM $AlCl_3$ (pH 4.5). To minimize the effect of these chemical agents on Al^{3+} activity, seedlings were first exposed to 10 μM AVG (A), 10 μM $CoCl_2$ (B), or 10 μM NPA (D) for 2 h followed by incubation in 50 μM $AlCl_3$ for another 24 h. For treatment with Ag^+, seedlings were first incubated in 10 μM $AgNO_3$ and then exposed to 50 μM $Al(NO_3)_3$ for 24 h to determine the effect of Ag^+ on root elongation of *Arabidopsis* wild-type (Col-0) (C). Root elongation was expressed relative to root elongation in the control solution (0.5 mM $CaCl_2$, pH 4.5). Data are means ±SE of >8 roots.

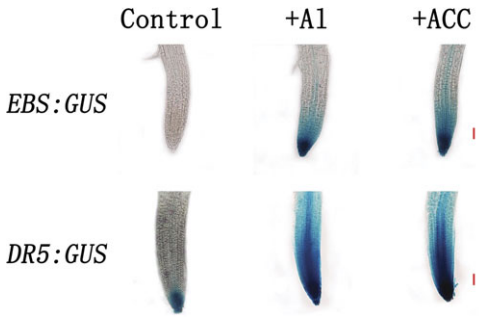

Fig. 5. Effect of Al^{3+} and ACC on expression of the ethylene reporter *EBS:GUS* and the auxin reporter *DR5:GUS* after 2 h incubation in the incubating solutions with and without 50 μM $AlCl_3$ (pH 4.5) and 10 μM ACC. The bar is 50 μm. The images are representatives of at least three independent experiments with >8 seedlings examined for each experiment.

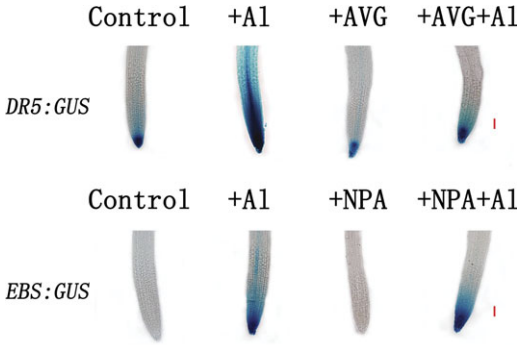

Fig. 6. Effect of AVG and NPA on Al-induced activity of *DR5:GUS* and *EBS:GUS*. *Arabidopsis* seedlings were incubated in solution containing 10 μM AVG or 10 μM NPA for 12 h and thereafter the seedlings were incubated in 50 μM $AlCl_3$ solution (pH 4.5) for 2 h. The bar is 50 μm. The images are representatives of at least three independent experiments with >8 seedlings examined for each experiment.

and was reduced markedly after 12 h exposure to Al^{3+} (Fig. 8A). A comparable up-regulation of *AtAUX1* and *AtPIN2* was found in response to the ethylene synthesis precursor ACC (Fig. 8B), implying that Al-induced expression of *AtAUX1*and *AtPIN2* may result from Al-induced ethylene evolution. To test this possibility, the responses of expression of *AtAUX1* and *AtPIN2* to AVG and Co^{2+} were further investigated. The transcriptional levels of *AtAUX1* and *AtPIN2* were not affected by either AVG or Co^{2+} in the absence of Al^{3+} (Fig. 8C, D). In contrast, the Al-induced increases in expression of *AtAUX1*and *AtPIN2* genes were abolished by AVG and Co^{2+} (Fig. 8C, D).

Discussion

In a previous study, it was found that exposure of *L. japonicus* to Al^{3+} led to a rapid inhibition of root elongation and that the inhibition of root elongation was closely associated with the ethylene burst (Sun *et al.*, 2007). In the present study, it was confirmed that a similar mechanism exists in *Arabidopsis* as evidenced by the following observations. Al^{3+} enhanced the expression of *AtACS2*, *AtACS6*, *AtACS8*, *AtACO1*, and *AtACO2* genes (Fig. 8A). The

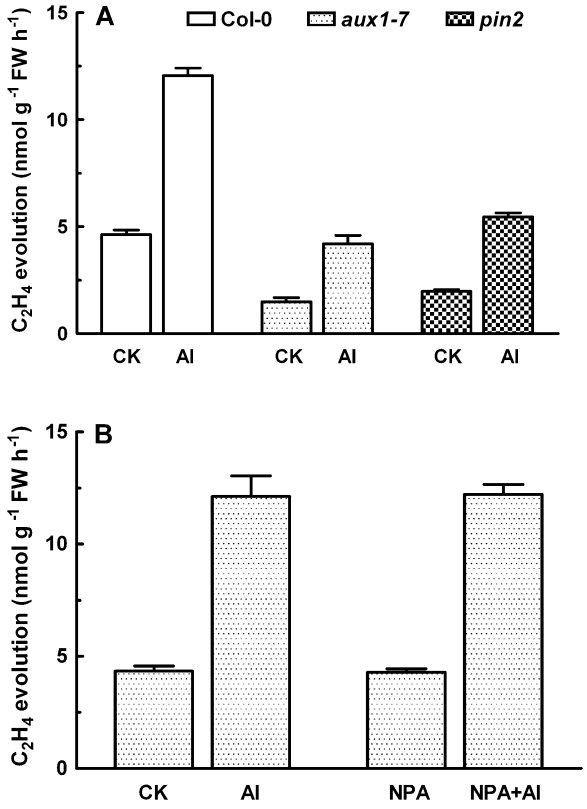

Fig. 7. Effect of Al^{3+} on ethylene evolution from root tips of *Arabidopsis* wild-type (Col-0) and auxin polar transport mutants, *aux1-7* and *pin2*. (A) Both wild-type and mutants seedlings were treated with 50 μM $AlCl_3$ (pH 4.5) for 2 h and the ethylene evolution from excised root tips (~5 mm) was determined. (B) Effect of 10 μM NPA on Al-induced increase of ethylene evolution from root tips ofthe *Arabidopsis* wild type (Col-0). Seedlings of Col-0 were treated with 10 μM NPA solution for 12 h and then incubated in 50 μM $AlCl_3$ (pH 4.5) for another 2 h. Values are means ±SE of five replicates.

enhanced expression of these genes would account for the observed Al-induced rapid ethylene production from root tips (Fig. 1B) and stimulation of *EBS:GUS* activity in the root apex (Fig. 5). In addition, the Al^{3+}-induced inhibition of root elongation was remarkably alleviated in the presence of inhibitors of ethylene biosynthesis (AVG and Co^{2+}) and an antagonist of ethylene perception (Ag^{+}) (Fig. 4A–C). The responses of ethylene-insensitive mutants to Al^{3+} were further analysed and it was found that root elongation was less inhibited by Al^{3+} in the ethylene-insensitive mutants (*etr1-3* and *ein2-1*) than in the wild-type plants (Fig. 3). The *etr1-3* mutant has a reduced ethylene response due to the dominant-negative versions of the membrane ethylene receptor (O'Malley *et al.*, 2005). The *ein2-1* mutant is also insensitive to ethylene, but the biochemical function of EIN2 remains to be characterized (Alonso and Stepanova, 2004). The lower sensitivity of *etr1-3* and *ein2-1* to Al^{3+} than the wild type could be explained by the Al^{3+}-induced ethylene signal in these plants being unable to activate downstream targets that underpin root elongation. Thus these findings highlight the important role of ETR1 and EIN2 in Al-induced inhibition of root elongation in *Arabidopsis*. Taken together, these findings corroborate that induction of ethylene production is a critical event in Al-induced inhibition of root elongation in *Arabidopsis*.

In addition to ethylene, the results revealed that Al-induced inhibition of root elongation may also be associated with disruption of auxin distribution and/or signalling. For instance, it was found that Al^{3+} up-regulated expression of *AtAUX1* and *AtPIN2* (Fig. 8A). PIN2, which is localized predominantly in cortical cells, is a key component for mediating basipetal auxin transport, and plays a pivotal role in control of cell division and growth (Blilou *et al.*, 2005). Shen *et al.* (2008) recently reported that Al^{3+} up-regulated *PIN2* expression and inhibited transport of PIN2 vesicles from plasma membranes to endosomes in *Arabidopsis*, leading to reductions in auxin concentration in root apical cells. The *AtAUX1* gene encodes a transmembrane protein and was believed to be associated with the influx of auxin across the plasma membrane (Swarup *et al.*, 2001; Tanaka *et al.*, 2006). Like PIN2, the transcriptional levels of *AtAUX1* were also enhanced when exposed to Al^{3+} (Fig. 8A). This result indicates that, in addition to PIN2 (Shen *et al.*, 2008), Al^{3+} may also target the AUX1-mediated auxin transport system, leading to disruption of auxin distribution in roots. Because PIN2 and AUX1 play critical roles in mobilizing auxin [indoleacetic acid (IAA)] between root apical cells and cells in the elongation zone (Swarup *et al.*, 2007), the enhanced expression of *AtPIN2* and *AtAUX1* by Al^{3+} may account for the changes in *DR5:GUS* activity in both root apical and elongation zones (cf. Fig. 6). The changes in auxin distribution would in turn contribute to the observed Al-induced inhibition of root elongation. Future studies focusing on the spatial and temporal changes in auxin distribution in response to Al^{3+} will shed light on the role of auxin in Al phytotoxicity.

The *aux1-7* mutant that has a single lesion in the auxin influx carrier *AUX1* gene is insensitive to auxin and ethylene in terms of root growth (Pickett *et al.*, 1990). *PIN2* encodes an auxin efflux carrier protein, exhibiting asymmetric PIN2 distribution in the *pin2* mutant and more protein degraded at the upper side of the gravistimulated root (Roman *et al.*, 1995). The observation that root elongation in *aux1-7* and *pin2* mutants was less sensitive to Al^{3+} in terms of inhibition of root elongation than in wild-type plants (Figs 3, 4) is consistent with the involvement of AUX1 and PIN2 in Al-induced inhibition of root elongation. It has been shown that root elongation in *aux1* and *pin2* mutants is less sensitive to exogenous ACC than that of wild-type plants (Růžička *et al.*, 2007). Moreover, both Al^{3+} and ACC induced a similar increase in expression of *AtAUX1* and *AtPIN2* (Fig. 8) and of *DR5:GUS* activity in roots (Fig. 6). These findings imply that the same mechanism may underlie the inhibitory effect of Al^{3+} and ethylene on root elongation. In maize, it has been shown that accumulation of IAA in the root elongation zone is reduced by Al^{3+}, while the IAA content in root apical cells is enhanced by Al^{3+} (Kollmeier *et al.*, 2000). The present finding that expression of *AtAUX1* and *AtPIN2* was enhanced by Al^{3+} may

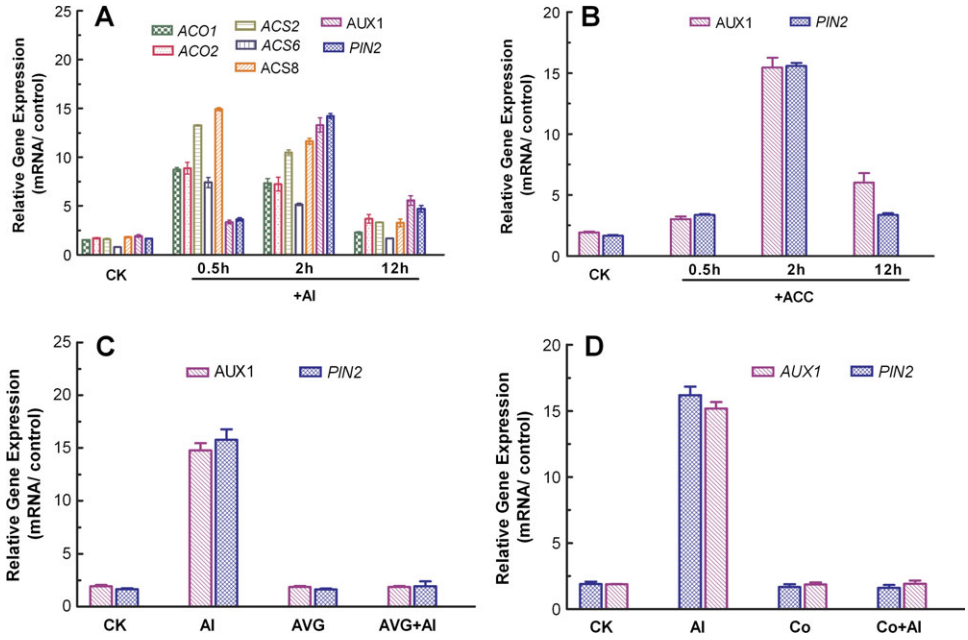

Fig. 8. Effect of Al^{3+} on expression of *ACS*, *ACO*, *AUX1*, and *PIN2* of wild-type (Col-0) *Arabidopsis* seedlings (A). Expression of *AtACS2*, *AtACS6*, *AtACS8*, *AtACO1*, *AtACO2*, *AtAUX1*, and *AtPIN2* was determined after exposure of Col-0 seedlings to 50 μM $AlCl_3$ for varying durations (0.5, 2, and 12 h). Effect of ACC on expression of *AtAUX1* and *AtPIN2* of wild-type (Col-0) seedlings by exposing the seedlings to 10 μM ACC solutions for 0.5, 2, and 12 h (B). Effect of AVG and Co^{2+} on Al-induced up-regulation of *AtAUX1* and *AtPIN2* from roots of *Arabidopsis* wild-type (Col-0) by incubating the roots in solutions supplemented with 50 μM $AlCl_3$ for 2 h followed by another 12 h incubation in 10 μM AVG or 10 μM $CoCl_2$ (C, D). The relative mRNA level was normalized based on the mRNA in roots grown in 0.5 mM $CaCl_2$ solutions. Data are means ±SE of three replicates.

account for the Al-induced changes in IAA distribution in maize roots because these two proteins play important roles in mobilizing auxin distribution between root apical and elongating cells (Tanaka *et al.*, 2006; Růžička *et al.*, 2007). In the present study, it was observed that Al^{3+} enhanced *DR5:GUS* activity in both root apical and elongating cells (Fig. 5), suggesting that Al^{3+} alters patterns of auxin accumulation and distribution in roots. This may in turn contribute to the observed inhibition of root elongation. The auxin polar transport inhibitor, NPA, inhibited root elongation (Fig. 4D), whereas NPA was also effective in alleviating the Al-induced inhibition of root elongation (Fig. 4D). These findings may suggest that Al^{3+} and NPA have opposite effects on auxin distribution such that Al^{3+} stimulates auxin polar transport by up-regulating *AtAUX1* and *AtPIN2* expression. In this context, Doncheva *et al.* (2005) reported that treatment with Al^{3+} and NPA led to a rapid inhibition of cell division in maize roots probably by changing cell patterning. Unfortunately, the authors did not investigate the interactive effects of Al^{3+} and NPA on cell division and root elongation.

There is ample evidence demonstrating the synergistic effects of auxin and ethylene on root growth and development (Stepanova *et al.*, 2005, 2007; Růžička *et al.*, 2007; Swarup *et al.*, 2007). In the present study, it was found that both ethylene and auxin were involved in Al-induced inhibition of root elongation. Attempts were therefore made to unravel the network associated with interactive effects of auxin and ethylene on root growth in the presence of toxic Al^{3+} using mutants defective in ethylene signalling and auxin polar transport, and inhibitors of ethylene synthesis and perception and of auxin polar transport. It was found that treatments with the ethylene synthesis precursor ACC and Al^{3+} induced comparable increases in *DR5:GUS* activity in *Arabidopsis* roots (Fig. 5), while the Al-dependent increases in *DR5:GUS* activity were substantially reduced by the ethylene synthesis inhibitor AVG (Fig. 6). On the other hand, the auxin polar transport inhibitor NPA had a marginal effect on Al-induced increases in *EBS:GUS* activity (Fig. 6) and Al-induced ethylene production (Fig. 7B). These results prompted the hypothesis that Al-induced ethylene production may act as a trigger to evoke changes in auxin distribution by affecting auxin polar transport systems such as AUX1 and PIN2. In the present study, it was found that the Al-induced up-regulation of ethylene synthesis genes (*ACS* and *ACO*) preceded the Al-induced up-regulation of the *AUX1* and *PIN2* genes (Fig. 8). In addition, both ACC and Al^{3+} induced comparable expression of AUX1 and PIN2 (Fig. 8A, B). The inhibitor of ethylene synthesis AVG abolished the Al^{3+}-evoked up-regulation of AUX1 and PIN2 (Fig, 8D). Taken together, these observations indicate that disruption of auxin distribution by Al^{3+} is a downstream event of Al-induced ethylene production. This claim is also in line with the consensus that auxin synthesis, transport, and signalling are required for the ethylene-induced inhibition of root elongation in *Arabidopsis* (Růžička *et al.*, 2007; Stepanova *et al.*, 2007; Swarup *et al.*, 2007).

In the present study, the effect of relatively low concentrations of Al^{3+} on root elongation was investigated using the wild type and mutants defective in ethylene signalling and auxin polar transport. It has been well documented that Al^{3+} can alter a myriad of biochemical and physiological processes (Matsumoto, 2000; Rengel and Zhang, 2003). Therefore, the inhibitory effect of Al^{3+} on root elongation, especially at relatively high Al^{3+} concentrations, may not be exclusively accounted for by the interaction of Al^{3+} with ethylene and auxin signalling cascades. The overall findings indicate that Al^{3+}-induced ethylene may act as a trigger to inhibit root elongation by disrupting auxin distribution in roots. However, the possibility cannot be ruled out that Al^{3+} may interact with auxin in an ethylene-independent manner. The disruption of auxin may in turn affect ethylene-dependent root growth (Stepanova *et al.*, 2007). This possibility may also account for the observation that Al^{3+} elicited less ethylene evolution from root apices of *aux1-7* and *pin2* than wild-type plants (Fig. 7).

In summary, we demonstrated that Al^{3+}-induced inhibition of root elongation was positively correlated with ethylene production in *Arabidopsis* root tips, and that *etr1-3* and *ein2-1* mutants were insensitive to Al^{3+} when compared with wild-type plants. These results highlight the critical roles played by ETR1 and EIN2, two key proteins in ethylene signalling (Alonso and Stepanova, 2004), in Al toxicity. In addition to ethylene, it was found, by monitoring changes in *DR5:GUS* activity in *Arabidopsis* roots, that Al^{3+} disrupted auxin distribution in roots. The up-regulation of *AtAUX1* and *AtPIN2* expression by Al^{3+} and the greater tolerance of *aux1-7* and *pin2* mutants to Al^{3+} than wild-type plants suggest that AUX1 and PIN2 proteins are likely to be involved in Al^{3+}-induced inhibition of root elongation. More importantly, we found that Al^{3+}-induced ethylene evolution occurred very rapidly and that Al^{3+}-induced up-regulation of *ACS* and *ACO* preceded Al^{3+}-induced expression of *AUX1* and *PIN2*. The up-regulation of *AtAUX1* and *AtPIN2* expression by Al^{3+} was mimicked and abolished by ACC and AVG, respectively. These findings indicate that Al-induced ethylene evolution may serve as a signal to elicit downstream changes in auxin distribution in roots by interacting with AUX1 and PIN2 proteins, leading to inhibition of root elongation in the presence of toxic Al^{3+}.

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References

Alonso JM, Stepanova AN. 2004. The ethylene signaling pathway. *Science* **306,** 1513–1515.

Barcelo J, Poschenrieder C. 2002. Fast root growth responses, root exudates, and internal detoxification as clues to the mechanisms of aluminium toxicity and resistance: a review. *Envrionmental and Experimental Botany* **48,** 75–92.

Benjamins R, Malenica N, Luschnig C. 2005. Regulating the regulator: the control of auxin transport. *BioEssays* **27,** 1246–1255.

Bennett MJ, Marchant A, Gren HG, May ST, Ward SP, Milner PA, Walker AR, Schultz B, Feldmann KA. 1996. Arabidopsis AUX1 gene: a permease-like regulator of root gravitropism. *Science* **273,** 948–950.

Blilou I, Xu J, Wildwater M, Willemsen V, Paponov I, Friml J, Heidstra R, Aid M, Palme K, Scheres B. 2005. The PIN auxin efflux facilitator network controls growth and patterning in *Arabidopsis* roots. *Nature* **433,** 39–44.

Doncheva S, Amenos M, Poschenrieder C, Barcelo J. 2005. Root cell patterning: a primary target for aluminium toxicity in maize. *Journal of Experimental Botany* **56,** 1213–1220.

Foy CD. 1988. Plant adaptation to acid, aluminum-toxic soils. *Communications in Soil Science and Plant Analysis* **19,** 959–987.

Horst WJ. 1995. The role of the apoplast in aluminium toxicity and resistance of higher plants: a review. *Zeitschrift für Pflanzenernährung und Bodenkunde* **158,** 419–428.

Illes P, Schlicht M, Pavlovkin J, Lichtscheidl I, Baluska F, Ovecka M. 2006. Aluminium toxicity in plants: internalization of aluminum into cells of the transition zone in Arabidopsis root apices relates to changes in plasma membrane potential, endosomal behaviour, and nitric oxide production. *Journal of Experimental Botany* **57,** 4201–4213.

Jefferson RA, Kavanagh TA, Bevan MW. 1987. GUS fusions: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *EMBO Journal* **6,** 3901–3907.

Kende H. 1993. Ethylene biosynthesis. *Annual Review of Plant Physiology and Plant Molecular Biology* **44,** 283–307.

Kollmeier M, Felle HH, Horst WJ. 2000. Genotypical differences in aluminum resistance of maize are expressed in the distal part of the transition zone. Is reduced basipetal auxin flow involved in inhibition of root elongation by aluminum? *Plant Physiology* **122,** 945–956.

Le J, Vandenbussche F, Van Der Straeten D, Verbelen J-P. 2001. In the early response of Arabidopsis roots to ethylene, cell elongation is up- and down regulated and uncoupled from differentiation. *Plant Physiology* **125,** 519–522.

Malamy JE, Benfey PN. 1997. Organization and cell differentiation in lateral roots of Arabidopsis thaliana. *Development* **124,** 33–44.

Massot N, Nicander B, Barcelo J, Poschenrieder CH, Tillberg E. 2002. A rapid increase in cytokinin levels and enhanced ethylene evolution precede Al^{3+}-induced inhibition of root growth in bean seedlings (*Phaseolus vulgaris* L.). *Plant Growth Regulation* **37,** 105–112.

Matsumoto H. 2000. Cell biology of aluminum toxicity and tolerance in higher plants. *International Review of Cytology* **200,** 1–46.

May HM, Nordstrom DK. 1991. Assessing the solubilities and reaction kinetics of aluminous minerals in soils. In: Ulrich B, Sumner ME, eds. *Soil acidity*. Berlin: Springer Verlag, 125–148.

O'Malley RC, Rodriguez FL, Esch JJ, Binder BM, O'Donnell P. 2005. Ethylene-binding activity, gene expression levels, and receptor system output for ethylene receptor family members from Arabidopsis and tomato. *The Plant Journal* **41,** 651–659.

Paponov IA, Teale WD, Trebar M, Blilou I, Palme K. 2005. The PIN auxin efflux facilitators: evolutionary and functional perspectives. *Trends in Plant Science* **10,** 170–177.

Pickett FB, Wilson AK, Estelle M. 1990. The AUX1 mutation of Arabidopsis confers both auxin and ethylene resistance. *Plant Physiology* **94,** 1462–1466.

Rengel Z, Zhang WH. 2003. Role of dynamics of intracellular calcium in aluminum toxicity syndrome. *New Phytologist* **159,** 295–314.

Roman G, Lubarsky B, Kieber JJ, Rothenberg M, Ecker JR. 1995. Genetic analysis of ethylene signal transduction in Arabidopsis thaliana: five novel mutant loci integrated into a stress response pathway *Genetics*. **139,** 1393–1409.

Růžička K, Ljung K, Vanneste S, Podhorska R, Beeckman T, Friml J, Benkova E. 2007. Ethylene regulates root growth through effects on auxin biosynthesis and transport-dependent auxin distribution. *The Plant Cell* **19,** 2197–212.

Ryan PR, DiTomaso JM, Kochian LV. 1993. Aluminium toxicity in roots: an investigation of spatial sensitivity and the role of the root cap. *Journal of Experimental Botany* **44,** 437–446.

Shen H, Hou NY, Schlicht M, Wan YL, Baluska F. 2008. Aluminum toxicity targets PIN2 in Arabidopsis root apices: effects on PIN2 endocytosis vesicular recycling, and polar auxin transport. *Chinese Science Bulletin* **53,** 2480–2487.

Sivaguru M, Horst WJ. 1998. The distal part of the transition zone is the most aluminum-sensitive apical root zone of maize. *Plant Physiology* **116,** 155–163.

Stepanova AN, Hoyt JM, Hamilton AA, Alonso JM. 2005. A link between ethylene and auxin uncovered by the characterization of two root-specific ethylene-insensitive mutants in *Arabidopsis*. *The Plant Cell* **17,** 2230–2242.

Stepanova AN, Yun J, Likhacheva AV, Alonso JM. 2007. Multilevel interactions between ethylene and auxin in Arabidopsis root. *The Plant Cell* **19,** 2169–2185.

Sun P, Tian QY, Zhao MG, Dai XY, Huang JH, Li LH, Zhang WH. 2007. Aluminum-induced ethylene production is associated with inhibition of root elongation in *Lotus japonicus* L. *Plant and Cell Physiology* **48,** 1229–1335.

Swarup R, Friml J, Marchant A, Ljung K, Sandberg G, Palme K, Bennett M. 2001. Localization of the auxin permease AUX1 suggests two functionally distinct hormone transport pathways operate in the *Arabidopsis* root apex. *Genes and Development* **15,** 2648–2653.

Swarup R, Kramer EM, Perry P, Knox K, Leyser HMO, Haseloff J, Beemster GTS, Bhalerao R, Bennett MJ. 2005. Root gravitropism requires lateral root cap and epidermal cells for transport and response to a mobile auxin signal. *Nature Cell Biology* **7,** 1057–1065.

Swarup R, Perry P, Hagenbeek D, Van Der Straeten D, Beemster GTS, Sandberg G, Bhalerao R, Ljung K, Bennett MJ. 2007. Ethylene regulates auxin biosynthesis in Arabidopsis seedlings to enhance inhibition of root cell elongation. *The Plant Cell* **19,** 2186–2196.

Tanaka H, Dhonukshe P, Brewer PB, Friml J. 2006. Spatiotemporal asymmetric auxin distribution: a means to coordinate plant development. *Cellular and Molecular Life Sciences* **63,** 2738–2754.

Teale WD, Paponov IA, Palme K. 2006. Auxin in action: signalling, transport and the control of plant growth and development. *Nature Reviews Molecular Cell Biology* **7,** 847–859.

Tian QY, Sun DH, Zhao MG, Zhang WH. 2007. Inhibition of nitric oxide synthase (NOS) underlies aluminium-induced inhibition of root elongation in *Hibiscus moscheutos* L. *New Phytologist* **174,** 322–331.

Tsuchisaka A, Theologis A. 2004. Unique and overlapping expression pattern among the Arabidopsis 1-amino-cyclopropane-1-carboxylate synthase gene family members. *Plant Physiology* **136,** 2982–3000.

Ulmasov T, Murfett J, Hagen G, Guilfoyle TJ. 1997. Aux/IAA proteins repress expression of reporter genes containing natural and highly active synthetic auxin response elements. *The Plant Cell* **9,** 1963–1971.

Wang KLC, Li H, Ecker JR. 2002. Ethylene biosynthesis and signaling networks. *The Plant Cell* **14,** S131–S151.

Zhang WH, Rengel Z. 1999. Aluminum induces an increase in cytoplasmic calcium in intact wheat root apical cells. *Australian Journal of Plant Physiology* **26,** 401–409.