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Short communication

Aluminum-Induced Ethylene Production is Associated with Inhibition of Root Elongation in *Lotus japonicus* L.

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Inhibition of root elongation by toxic aluminum (Al^{3+}) occurs rapidly and is one of the most distinct and earliest symptoms of Al toxicity. To elucidate mechanism underlying Al^{3+}-induced inhibition of root elongation, we investigated the involvement of ethylene in Al^{3+}-induced inhibition of root elongation using the legume model plants *Lotus japonicus* and *Medicago truncatula*. Root elongation of *L. japonicus* and *M. truncatula* was rapidly inhibited by exposure to $AlCl_3$. A similar rapid inhibition of root elongation by the ethylene-releasing substance, ethephon, and the ethylene precursor, 1-aminocyclopropane-1-carboxylic acid (ACC), was also observed. The Al^{3+}-induced inhibition of root elongation was substantially ameliorated in the presence of antagonists of ethylene biosynthesis [Co^{2+} and aminoethoxyvinylglycine (AVG)]. Al^{3+} increased the activity of ACC oxidase (ACO), and induced a rapid evolution of ethylene from root apices and expression of genes of ACC synthase (ACS) and ACO. These findings suggest that induction of ethylene evolution resulting from up-regulation of ACS and ACO plays a critical role in Al^{3+}-induced inhibition of root elongation.

Keywords: Aluminum toxicity — Ethylene production — *Lotus japonicus* — *Medicago truncatula* — Root elongation.

Abbreviations: ACC, 1-aminocyclopropane-1-carboxylic acid; ACO, ACC oxidase; ACS, ACC synthase; AVG, aminoethoxyvinylglycine; PVPP, polyvinyl polypyrrolidone; RT–PCR, reverse transcription–PCR.

Aluminum (Al) is the most abundant mineral in the soil and it becomes phytotoxic to plants when it is solubilized to phytotoxic Al^{3+} species under acidic conditions. Al toxicity is one of the most important factors limiting crop production on acid soils (Kochian 1995). Inhibition of root elongation is one of the most distinct and earliest symptoms of Al toxicity, which occurs within hours or even minutes of exposure to Al^{3+} (Zhang and Rengel 1999). Inhibition of root elongation by Al^{3+} requires the root apex +to be directly exposed to Al (Ryan et al. 1993, Sivaguru and Horst 1998), suggesting that root apex is a critical site of perception and expression of Al toxicity and resistance. Although numerous physiological parameters are altered when plants are exposed to toxic Al^{3+}, including inhibition of auxin transport into root apices (Kollmeier et al. 2000) and disruption of cytosolic Ca^{2+} homeostasis (Zhang and Rengel 1999), the primary mechanism underlying the Al^{3+}-induced inhibition of root growth remains to be deciphered (Matsumoto 2000, Barcelo and Poschenrieder 2002, Rengel and Zhang 2003).

In addition to inhibition of root elongation, plants suffering from Al toxicity also display symptoms such as formation of barrel-shaped cells (Gunsé et al. 1997) and swelling of the root apex (Vázquez et al. 1999). The rapid inhibition of root elongation and alterations of root morphology by Al^{3+} resemble those of plants resulting from increased ethylene production (Lynch and Brown 1997, Morgan and Drew 1997). Ethylene, a well-known phytohormone, is closely associated with numerous physiological processes in plants, ranging from seed germination to senescence (Abeles et al. 1992). Ethylene is synthesized from methionine through *S*-adenosyl-L-methionine and 1-aminocyclopropane-1-carboxylic acid (ACC) (Kende 1993). The rate-limiting step in ethylene biosynthesis lies in the production of ACC by ACC synthase (ACS), which is followed by the conversion of ACC to ethylene by ACC oxidase (ACO) (Kende 1993). The ACS and ACO are encoded by multigene families, and are regulated by developmental and environmental factors (Barry et al. 1996, Bouquin et al. 1997). Evolution of ethylene in response to biotic (van Loon et al. 2006) and abiotic stress (Morgan and Drew 1997) resulting from up-regulation of ACS and ACO is a common phenomenon. The burst of ethylene evolution induced by stress, referred as to stress ethylene (Abeles et al. 1992), may act as an

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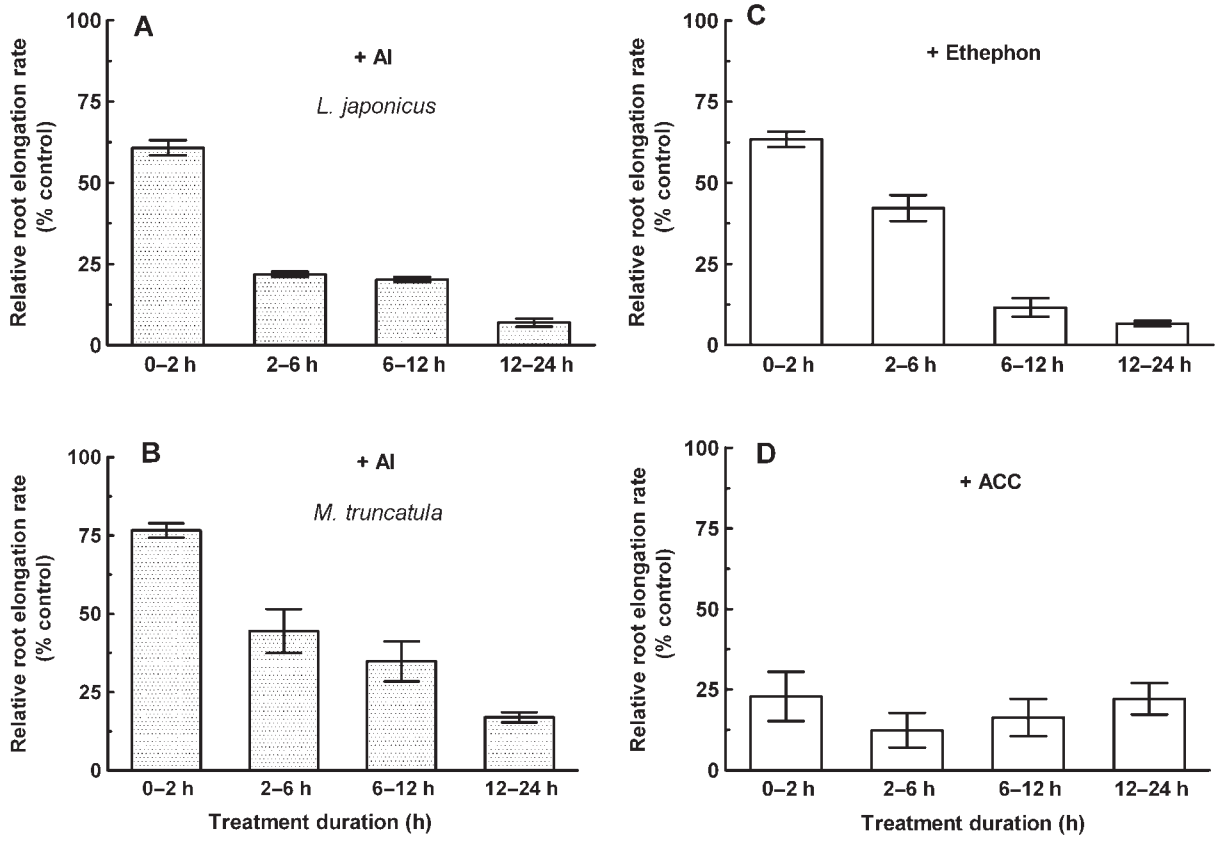

Fig. 1 Root elongation of *Lotus japonicus* (A) and *Medicago truncatula* (B) following exposure to 10 μM $AlCl_3$ (pH 4.5) and 10 μM of the ethylene-releasing substance ethephon (C) and ethylene biosynthesis precursor ACC (D) for varying times. The control solution contained 0.5 mM $CaCl_2$, pH 4.5. Root length was measured prior to treatments and then the roots were exposed to $AlCl_3$ (ethephon or ACC), and root length was measured again under the stereomicroscope after exposure for 2, 6, 12 and 24 h. Data are expressed as root elongation relative to controls, and are presented as the mean ± SE of >8 roots.

important signal to elicit biochemical and physiological changes in response to environmental stress. The involvement of ethylene in Al^{3+}-induced root elongation has been reported in the literature (Gunsé et al. 2000, Massot et al. 2002). For instance, in maize, Al^{3+} inhibits root elongation, but it does not affect ethylene evolution (Gunsé et al. 2000). In contrast, the same group reported that Al^{3+} rapidly stimulates ethylene synthesis, induces a decrease in cytokinin and inhibits root elongation in *Phaseolus vulgaris* (Massot et al. 2002). These results are indicative of the possible involvement of ethylene-dependent changes in cytokinin in Al^{3+}-induced arrest of root elongation. However, the above study only provides a casual relationship between Al^{3+}-induced inhibition of root elongation and ethylene production, and there has been no direct evidence showing the involvement of ethylene production in Al toxicity.

To characterize the role of ethylene in Al phytoxicity to plants, we investigated the effects of Al^{3+} on root elongation and ethylene evolution in the legume model plants *Lotus japonicus* and *Medicago truncatula*. We also compared the effects of Al^{3+} on root elongation with those of an ethylene biosynthesis inhibitor, exogenous ethylene and an ethylene biosynthesis precursor (ACC). Finally, we studied effects of Al^{3+} on ACO activity and expression of genes for ASC and ACO in *M. truncatula*.

Exposure of *L. japonicus* and *M. truncatula* to 10 μM $AlCl_3$ (pH 4.5) led to a rapid inhibition of root elongation (Fig. 1A, B). Root elongation was inhibited by 81 and 72% for *L. japonicus* and *M. truncatula*, respectively, after 24 h incubation in 10 μM $AlCl_3$ solution. When *L. japonicus* was treated with 10 μM ethephon, an ethylene-releasing substance, a similar rapid inhibition of root elongation was also observed (Fig. 1C). To confirm whether the ethephon-induced inhibition of root elongation is associated with ethylene, the effect of the ethylene biosynthesis precursor ACC was investigated. Fig. 1D shows that treatment of *L. japonicus* with 10 μM ACC also markedly inhibited root elongation. For example, root elongation in *L. japonicus* was inhibited by 82 and 77%

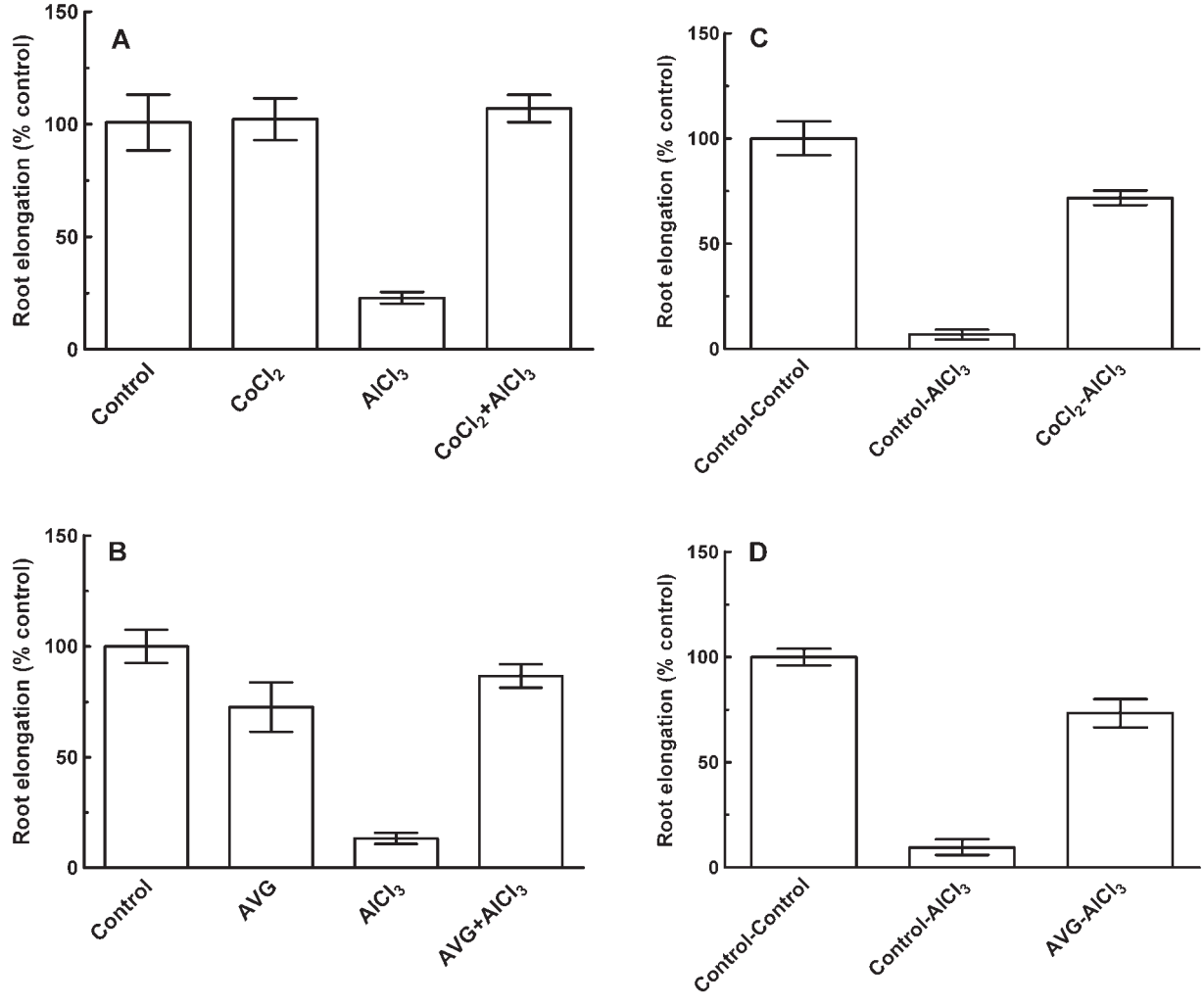

Fig. 2 Effects of 10 μM $CoCl_2$, 10 μM AVG and 10 μM $AlCl_3$ alone and their combinations on root elongation of *L. japonicus* (A, B). Effect of Co^{2+} and AVG on Al-induced inhibition of root elongation by incubating the roots in solutions supplemented with 10 μM $AlCl_3$ for 12 h followed by another 12 h incubation in 10 μM $CoCl_2$ or 10 μM AVG (C, D). Root elongation was expressed as relative to root elongation in the control solution of 0.5 mM $CaCl_2$, pH 4.5. Data are the mean ± SE of >10 roots.

after treatment with 10 μM ethephon and ACC, respectively.

The similarities between responses of root elongation to Al^{3+} and external ethylene prompted us to propose that the effect of Al^{3+} on root elongation could be related to alterations of ethylene production. To test this hypothesis, we studied the effect of Al^{3+} on root elongation in the presence of antagonists of ethylene biosynthesis. Co^{2+}, an inhibitor of ethylene synthesis, at 10 μM had no effect on root elongation (Fig. 2A), while the Al^{3+}-induced inhibition of root elongation was substantially ameliorated by Co^{2+} (Fig. 2A). Root elongation was inhibited by aminoethoxyvinylglycine (AVG), another commonly used inhibitor of ethylene (Fig. 2B). However, when exposed to 10 μM ACl_3 and AVG together, the Al^{3+}-induced inhibition of root elongation was also recovered (Fig. 2B). The ameliorating effects of Co^{2+} and AVG on the Al^{3+}-induced inhibition of root elongation may result from lowering Al^{3+} activity in the apoplasm by Co^{2+} due to a decrease in the negative potential of the membrane surface by charge screening and chelating Al^{3+} with AVG, thus rendering Al^{3+} non-toxic. Similar alleviating effects of Co^{2+} and AVG on Al^{3+}-induced inhibition of root elongation were observed when roots were treated with Co^{2+} (or AVG) and Al^{3+} separately (Fig. 2C, D). Thus these results discount the possibility that the ameliorating effects of AVG and Co^{2+} on the Al^{3+}-dependent inhibition of root elongation are due to decreases in effective Al^{3+} activity by AVG and Co^{2+}.

To test further whether the Al^{3+}-induced inhibition of root elongation is related to induction of ethylene

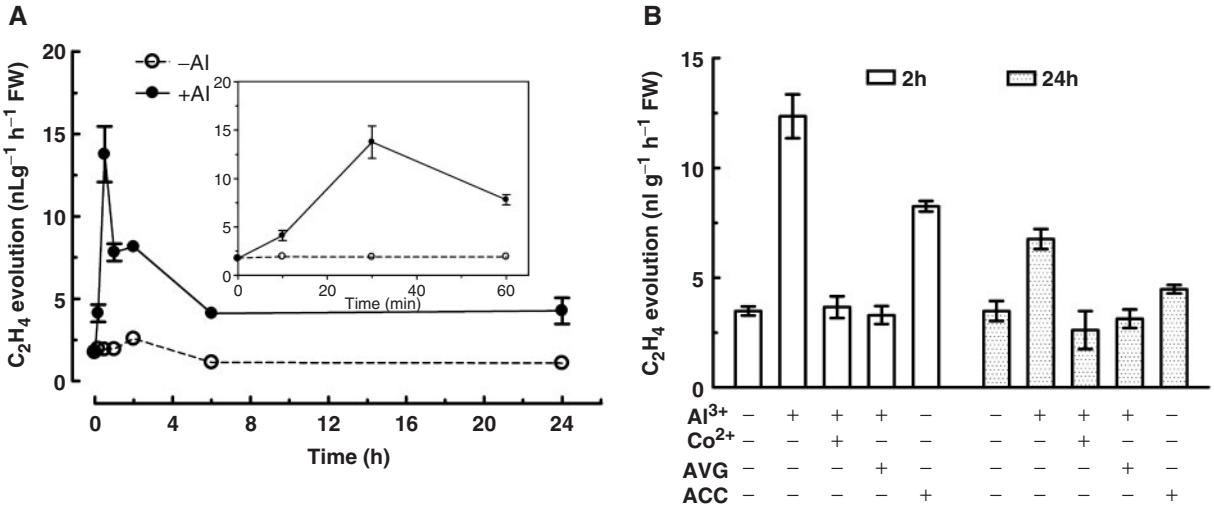

Fig. 3 Time-course of ethylene evolution from root apices of *L. japonicus* in response to exposure to 50 μM $AlCl_3$ (A). Effect of 10 μM $AlCl_3$ and 10 μM ACC alone, and 10 μM $AlCl_3$ together with 10 μM Co^{2+} and AVG for 2 and 24 h on ethylene evolution (B). Values are the mean ± SE of four replicates.

production, we studied the effect of Al^{3+} on ethylene evolution from roots of *L. japonicus*. As shown in Fig. 3A, a rapid burst of ethylene evolution from root apices of *L. japonicus* was observed upon exposure of *L. japonicus* to 50 μM $AlCl_3$. The Al^{3+}-induced ethylene evolution reached a maximum after 30 min exposure to Al^{3+}, and thereafter the evolution was reduced to a relatively constant level after exposure to Al^{3+} for 6 h. A similar transient increase in ethylene evolution from root apices was also observed when *L. japonicus* was exposed to 10 μM $AlCl_3$ (Fig. 3B). Furthermore, the Al^{3+}-induced ethylene evolution was abolished by ethylene biosynthesis inhibitors Co^{2+} and AVG (Fig. 3B), suggesting that the ameliorating effect of Co^{2+} and AVG on Al^{3+}-induced inhibition of root elongation is associated with Al^{3+}-dependent ethylene biosynthesis. Fig. 3B also shows that 10 μM ACC caused a similar ethylene evolution to that induced by 10 μM $AlCl_3$, confirming that ACC's effect is associated with ethylene. To test whether an increase in activity of ACO underpins the observed Al^{3+}-induced ethylene evolution, the effect of Al^{3+} on ACO was also investigated. As shown in Fig. 4, there was a 2-fold increase in activity of ACO when exposed to 10 and 50 μM Al^{3+} for 2 h. The Al^{3+}-induced increase in activity of ACO was slightly greater after 2 h exposure to Al^{3+} than after 24 h exposure to Al^{3+} (Fig. 4).

There are no available gene sequence data for ACS and ACO in *L. japonicus*. Therefore, *M. truncatula* was chosen to test whether the observed induction of ethylene production by Al^{3+} is due to regulation of expression of the two genes, *MtACS* and *MtACO*, using a semi-quantitative reverse transcription–PCR (RT–PCR) method. Both the

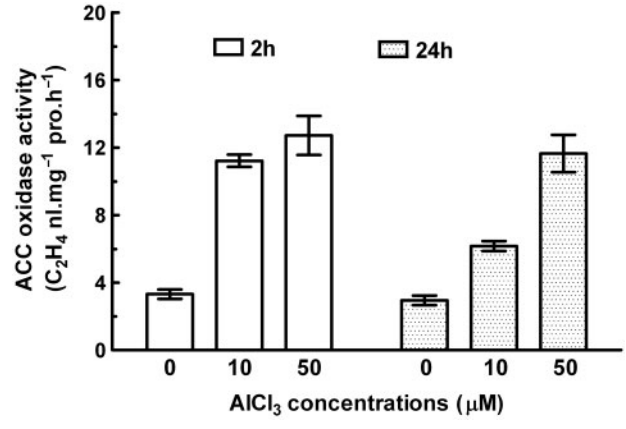

Fig. 4 Effect of $AlCl_3$ on the activity of ACC oxidase of *L. japonicus* root apices. The roots were exposed to 10 and 50 μM $AlCl_3$ for 2 and 24 h, and the ACC oxidase activity was determined by measuring ethylene evolution by gas chromatography. Data are the mean ± SE of four replicates.

MtACS2 and *MtACO* genes were expressed weakly in the absence of Al^{3+} (Fig. 5). Expression of the *MtACS2* and *MtACO* genes increased dramatically after exposure to 10 μM $AlCl_3$, and the expression levels were higher in root apices treated with Al^{3+} for 2 h than in those treated for 24 h (Fig. 5).

In the present study, we found that the two model legumes, *L. japonicus* and *M. truncatula*, were sensitive to Al^{3+} as shown by the rapid inhibition of root elongation upon exposure to 10 μM $AlCl_3$ (Fig. 1). A similar effect of an ethylene-releasing substance (ethephon) and an ethylene

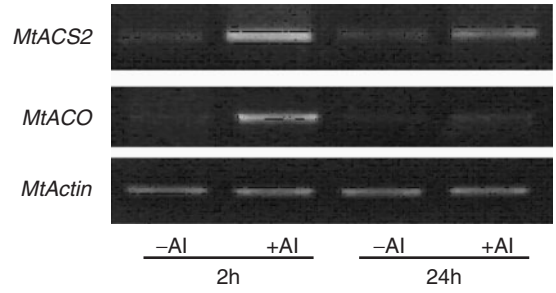

Fig. 5 Changes in gene expression of *MtACS* and *MtACO* upon exposure to 10 μM $AlCl_3$ monitored by RT–PCR. RNA was extracted from *M. truncatula* root apices grown under control (–Al) conditions and 2 h and 24 h of exposure to 10 μM $AlCl_3$.

precursor (ACC) on root elongation was observed (Fig. 1). These findings led us to speculate on whether the induction of ethylene production by Al^{3+} is involved in Al^{3+}-dependent inhibition of root elongation. The following observations are in line with the proposition that induction of ethylene production resulting from expression of ACS and ACO by Al^{3+} is an important event in triggering Al^{3+}-induced inhibition of root elongation: (i) the antagonists of ethylene biosynthesis alleviated the Al^{3+}-induced inhibition of root elongation (Fig. 2); (ii) Al^{3+} elicited a rapid burst of ethylene evolution from the root apices and the Al^{3+}-induced ethylene evolution was abolished by the antagonists of ethylene biosynthesis (Fig. 3); and (iii) Al^{3+} stimulated ACO activity (Fig. 4) and markedly induced expression of *MtACS* and *MtACO* in the root apices.

The rapid production of ethylene from root tips of *L. japonicus* upon exposure to Al^{3+} is comparable with that reported in roots of *P. vulgaris* (Massot et al. 2002). In contrast, Gunsé et al. (2000) reported that treatments of maize roots with Al^{3+} for 4 and 24 h do not elicit an enhanced ethylene evolution from maize roots. As the observed increase in ethylene evolution occurred rapidly after exposure to Al^{3+} (an ~8-fold increase in ethylene evolution after 30 min exposure of roots to Al^{3+}) (Fig. 4), it is likely that the enhanced evolution of ethylene is a trigger for rather than a consequence of Al^{3+}-induced inhibition of root elongation. The time-course of Al^{3+}-induced ethylene evolution displayed a similar pattern to that of the Al^{3+}-induced increase in ACO activity and expression of the two genes underlying ethylene biosynthesis (*MtACS2* and *MtACO*), i.e. the Al^{3+}-induced ethylene production and expression of *MtACS2* and *MtACO* occurred rapidly and then was attenuated (cf. Figs. 3, 5). The increased expression of the genes underlying ethylene biosynthesis is consistent with the Al^{3+}-induced enhancement of ACO activity. These results reveal that stimulation of ACS and ACO expression and enhanced ACO activity is likely to account for the observed Al^{3+}-induced ethylene evolution. Several metals have been shown to elicit ethylene production, including Cd^{2+} (Fuhrer, 1982), Cu^{2+} and Zn^{2+} (Gora and Clijsters 1989). The enhanced ethylene production by the metals can result from stimulation of activities of ACS and/or ACO, or enhancement of lipid peroxidation (Abeles 1992). It has been shown that Al^{3+}-induced lipid peroxidation is an early symptom rather than the cause of Al^{3+}-induced inhibition of root elongation in pea (Yamamoto et al. 2001). Our findings that Al^{3+} induced rapid evolution of ethylene and enhanced ACO activity discount the possibility that the Al^{3+}-induced lipid peroxidation is a trigger of ethylene production. In this context, it has been shown that induction of ethylene production by Cd^{2+} from carrot suspension cells is not associated with Cd^{2+}-induced lipid peroxidation (di Toppi et al. 1998). Therefore, it is conceivable that Al^{3+}-induced ethylene evolution is due to up-regulation of ACS and ACO. The increased levels of ethylene then elicit changes in physiological processes, thus leading to the observed inhibition of root elongation by Al^{3+}. The close relationship between the Al-induced inhibition of root elongation and stimulation of ethylene production implies that plant species or genotypes with different tolerance to Al may vary in their capacity to synthesize ethylene in response to Al. This hypothesis can be evaluated by examining the effect of Al on ethylene evolution from root apices of *L. japonicus* genotypes displaying contrasting tolerance to Al.

Previous studies have revealed that Al^{3+} inhibits auxin transport from shoot to root (Kolleimer et al. 2000), reduces endogenous nitric oxide (NO) levels in root apical cells (Tian et al. 2007), increases cytokinin levels in roots (Massot et al. 2002) and disrupts cytosolic Ca^{2+} homeostasis (Zhang and Rengel 1999). There is compelling evidence that there is cross-talk among these molecules in modulating physiological processes in plants (Neill et al. 2003, Guo and Ecker 2004, Etheridge et al. 2006). As the Al^{3+}-induced ethylene production occurred very rapidly upon exposure to Al^{3+} (Fig. 3; also see Massot et al. 2002), it is likely that induction of ethylene production is an early event among the Al^{3+}-dependent changes in the messenger molecules. Future studies using Arabidopsis mutants that have a defect in sensing and/or producing ethylene will unravel the signaling cascades involved in Al-induced inhibition of root elongation.

Materials and Methods

Seeds of *L. japonicus* (Gifu-129) and *M. truncatula* (Gaertn.) seeds, line A17 of cv Jemalong, were sterilized in 5% (v/v) sodium hypochlorite solution for 5 min, and then rinsed three times with de-ionized water. Seeds germinated on filter paper were grown in aerated Hornum solution containing (mg l^{-1}) (400 NH_4NO_3,

300 KNO_3, 300 $MgSO_4$, 100 NaH_2PO_4, 20 Fe-EDTA, 1.2 $MnSO_4$, 1.2 H_3BO_3, 0.4 $CuSO_4$, 0.4 $ZnSO_4$ and 0.08 $NaMoO_4$) in a greenhouse under conditions of a 14 h light/10 h dark cycle and a temperature of 23°C. Seedlings of 3-week-old plants were incubated in solutions containing different chemicals with a basal composition of 0.5 mM $CaCl_2$, pH 4.5. During the pre-treatment culture, the nutrient solution was changed every 3 d. Three seedlings were transferred to 250 ml glass chambers containing 0.5 mM $CaCl_2$ (pH 4.5), and $AlCl_3$ or ethephon (an ethylene donor) was added to the chosen concentrations. Root length before and after 24 h incubation in the presence of various chemicals was measured with a ruler. Root elongation was calculated from the difference in root length between the two measurements.

To study the short-term effect of $AlCl_3$ and ethephon on root elongation, *L. japonicus* seedlings were incubated in Hornum solution for 3 weeks and then transferred into Petri dishes with solutions containing either $AlCl_3$ or ethephon for varying time periods. Root length was measured before exposure to $AlCl_3$ or ethephon and it was measured again, following the different time periods (2, 6, 12, 24 h) after exposure to $AlCl_3$ or ethephon, under an Olympus steromicroscope. The difference in root length in the absence and presence of Al for varying time periods was used to compute root elongation and was expressed as relative root elongation, i.e. (Root elongated in different treatment/root elongated in control solution) × 100. Values were given as the mean ± SE of at least eight independent measurements.

After exposure of *L. japonicus* to $AlCl_3$ (10 and 50 μM) for varying time periods, root tips (about 1.5 cm in length) of approximately 0.2 g were excised and put into 5 ml gas-tight vials. One milliliter of the headspace was taken from the vials, and then injected into a gas chromatograph equipped with an alumina column (GDX502) and a flame ionization detector (GC-7AG; Shimadzu Japan) for measuring the ethylene concentration.

To determine the activity of ACO, root tips (~1.5 cm) were cut and frozen in liquid nitrogen and ground with a mortar and pestle in 2 ml g^{-1} tissue of extraction medium containing 0.1 M Tris (pH 7.2), 10% (w/v) glycerol, 30 mM sodium ascorbate and 5% (w/v) polyvinyl polypyrrolidone (PVPP). The slurry was centrifuged at 15,000×*g* for 20 min. The supernatant was used for enzyme assays. The activity of ACO was assayed immediately by mixing 0.2 ml of crude extract with a 2 ml reaction mixture containing 1.7 ml of extraction buffer (without PVPP), 50 μM FeSO4, 2 mM ACC, and incubated at 30°C. Ethylene produced in the head space of 5 ml capped tubes after 1 h incubation was determined as described above.

RT–PCR was used to study the effect of Al on the expression pattern of *ACO* and *ACS2* genes in *M. truncatula*. Total RNAs were extracted from *M. truncatula* root apices (~1.5 cm in length) with Trizol reagent (Invitrogen) and treated with RNase-free DNase I (Progma). The total RNAs were reverse-transcribed into first-strand cDNA with SuperScript™ II reverse transcriptase (Invitrogen), and the cDNAs obtained were used as templates for PCR amplification with specific primers. Gene-specific primers for *MtACO* were 5′-AAA TCA AGG ATG CTT GTG AAA ACT GGG-3′ and 5′-TGG TTC CTT GGC CTG AAA CTT TAA CC-3′, for *MtACS2* 5′-TAA TAA TGG GAC TTG TGA GC-3′ and 5′-TAT GTG AAC GAG GTT ACG GT-3′, and for *Actin* in *M. truncatula* 5′-ACG AGC GTT TCA GAT G-3′ and 5′-ACC TCC GAT CCA GAC A-3′. The same amplification reaction was conducted with an *M. truncatula Actin* gene and used as template RNA loading control. RT–PCRs were repeated three times.

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