Ethylene negatively regulates aluminium-induced malate efflux from wheat roots and tobacco cells transformed with TaALMT1

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Abstract

An important mechanism for Al3+ tolerance in wheat is exudation of malate anions from the root apex through activation of malate-permeable TaALMT1 channels. Here, the effect of ethylene on Al3+-activated efflux of malate was investigated using Al3+-tolerant wheat genotype ET8, which has high expression of TaALMT1. Exposure of ET8 plants to Al3+ enhanced ethylene evolution in root apices. Treatment with the ethylene synthesis precursor 1-aminocyclopropane-1-carboxylic acid (ACC) and ethylene gas suppressed Al3+-induced malate efflux from root apices, whereas the intracellular malate concentrations in roots were not affected. Malate efflux from root apices was enhanced in the presence of Al3+ by two antagonists of ethylene biosynthesis, aminoethoxyvinylglycine (AVG) and 2-aminoisobutyric acid (AIB). An increase in Al accumulation in root apices was observed when treated with ACC, whereas AVG and AIB suppressed Al accumulation in root apices. Al3+-induced inhibition of root elongation was ameliorated by pretreatment with AIB. In addition, ethylene donor (Ethrel) also inhibited Al3+-induced malate efflux from tobacco cells transformed with TaALMT1. ACC and the anion-channel blocker niflumate had a similar and non-additive effect on Al-induced malate efflux from root apices. Treatment of ET8 plants with ACC enhanced expression of TaALMT1, suggesting that the inhibitory effect of ethylene on Al-induced malate efflux is unlikely to occur at the transcriptional level. These findings indicate that ethylene may behave as a negative regulator of Al3+-induced malate efflux by targeting TaALMT1-mediated malate efflux by an unknown mechanism.

Key words: Ethylene, aluminium tolerance, malate efflux, ALMT1, wheat (Triticum aestivum L.).

Introduction

Aluminium (Al) is the most abundant metal in the Earth’s crust. Fortunately, the majority of Al occurs in the non-toxic form of aluminosilicate. However, Al is hydrolysed into phytotoxic Al3+ cations in acidic environments, and becomes a major constraint for crop growth and yield in acid soils (Kochian, 1995). Inhibition of root elongation is one of the earliest and most distinct symptoms of Al3+ toxicity that can be easily observed in solution culture (Zhang and Rengel, 1999). Although Al3+ can induce a rapid change in cell division in maize (Doncheva et al., 2005), the rapid suppression of...
root elongation by Al\(^{3+}\) within 1 h of exposure to Al\(^{3+}\) (Zhang and Rengel, 1999) suggests that Al\(^{3+}\)-induced inhibition of root growth probably results from arrest of cell elongation (Horst, 1995; Matsumoto, 2000). It has been established that the root apex, particularly the distal transition zone, is a critical site for perception of Al\(^{3+}\) and in determining whether a plant exhibits tolerance to Al\(^{3+}\) (Ryan et al., 1993; Šivágru and Horst, 1998). Extensive studies have demonstrated that numerous molecular and physiological processes are targeted by Al\(^{3+}\), such as Ca\(^{2+}\)-dependent signalling cascades, cytoskeleton dynamics (see reviews of Matsumoto, 2000; Rengel and Zhang, 2003), phytohormones (auxin, Kollmeier et al., 2000; Illes et al., 2006; Shen et al., 2008; ethylene, Sun et al., 2010), and nitric oxide (Wang and Yang 2005; Illes et al., 2006; Tian et al., 2007). However, the primary mechanisms underlying Al\(^{3+}\) toxicity in plants remain largely controversial and elusive.

In contrast to Al\(^{3+}\) toxicity, substantial progress has been made in our understanding of Al\(^{3+}\) tolerance (see reviews of Ryan et al., 2011; Delhaize et al., 2012). An important tolerance mechanism is the exudation of carboxylic anions (malate, citrate, oxalate) that can complex extracellular Al\(^{3+}\) (Ryan et al., 2001; Ryan et al., 2011; Ma et al., 2001). In wheat, malate is exuded from the root apex upon exposure to Al\(^{3+}\) (Delhaize et al., 1993b; Ryan et al., 1995). Further studies have revealed that Al\(^{3+}\)-induced exudation of malate is mediated by anion channels permeable to malate (Ryan et al., 1997; Zhang et al., 2001, 2008). Sasaki et al. (2004) identified that a membrane protein ALMT1 underpins the Al\(^{3+}\)-induced malate exudation from root apices in wheat. Heterologous expression of the TaALMT1 gene in Xenopus oocytes and in tobacco BY2 cells revealed the kinetic properties of malate transport (Sasaki et al., 2004; Zhang et al., 2008 Piñeros et al., 2008). When expressed in barley (Delhaize et al., 2004) and tobacco BY2 cells (Sasaki et al., 2004; Zhang et al., 2008), TaALMT1 conferred an Al\(^{3+}\)-activated efflux of malate that improved resistance to Al\(^{3+}\). Transporters homologous to ALMT1 have been identified to mediate Al\(^{3+}\)-induced malate efflux in species such as Arabidopsis thaliana (Hoekenga et al., 2006), rye (Collins et al., 2008), barley (Gruber et al., 2011), Brassica napus (Ligaba et al., 2006), and soybean (Liang et al., 2013). The function and regulation of TaALMT1 have been characterized at both transcriptional and post-transcriptional levels. For instance, the promoter characteristics (Sasaki et al., 2006; Ryan et al., 2010), membrane topology (Motoda et al., 2007), N-terminal and C-terminal domains (Ligaba et al., 2013; Furuichi et al., 2010), and putative protein phosphorylation sites have been shown to be involved in regulating the function of TaALMT1 (Oosawa and Matsumoto, 2001; Ligaba et al., 2009). Although numerous studies have investigated the mechanisms of Al\(^{3+}\)-induced malate efflux mediated by ALMT1, it is unclear how Al\(^{3+}\) activates the ALMT1 channels (Furuichi et al., 2010; Ryan et al., 2011; Ligaba et al., 2009, 2013).

Our previous studies revealed that Al\(^{3+}\) evokes ethylene evolution from root apices of Lotus japonicas (Sun et al., 2007) and Arabidopsis (Sun et al., 2010). In vascular plants, ethylene is produced from methionine through S-adenosylmethionine and 1-aminocyclopropane-1-carboxylic acid (ACC), catalysed by ACC synthase (ACS) and ACC oxidase (ACO), respectively (Kende, 1993). We demonstrated that the Al\(^{3+}\)-induced suppression of root elongation is negatively correlated with Al\(^{3+}\)-elicited ethylene production, such that inhibition of ethylene biosynthesis with antagonists markedly alleviates the inhibitory effect of Al\(^{3+}\) on root growth (Sun et al., 2007). In Arabidopsis, we further demonstrated that the Al\(^{3+}\)-induced ethylene may act as a signal to alter auxin distribution by targeting PIN2 and AUX1, leading to suppression of root growth (Sun et al., 2010). There is emerging evidence indicating that ethylene is involved in regulation of several membrane transporters at the transcriptional level, including both high- and low-affinity nitrate transporters in Arabidopsis (Tian et al., 2009) and oilseed rape (Leblanc et al., 2008), a high-affinity potassium transporter in Arabidopsis (Jung et al., 2009), high-affinity phosphate transporters in Arabidopsis (Lei et al., 2011) and Medicago falcata (Li et al., 2011), and an iron transporter in Arabidopsis (Garcia et al., 2010). In addition to regulation of nutrient transporters at the transcriptional level, ethylene can activate Ca\(^{2+}\)-permeable cation channels, leading to an increase in cytosolic Ca\(^{2+}\) activity in tobacco BY2 cells (Zhao et al., 2007). Given that Al\(^{3+}\) triggers ethylene production in roots of some plants and ethylene can regulate some ion channels, we explored the possibility that ethylene may be involved in regulation of Al tolerance by targeting ALMT1-mediated malate efflux. Our results showed that Al\(^{3+}\)-induced malate efflux from root apices and TaALMT1-expressing tobacco BY2 cells was correlated with ethylene production, suggesting the regulatory role of ethylene in TaALMT1-dependent tolerance to Al\(^{3+}\).

Materials and methods

Plant materials and growth conditions

Seeds of ET8, the Al-tolerant genotype of wheat Triticum aestivum L. (Delhaize et al., 1993b), were surface-sterilized by incubation for 1 min in 75% ethanol, rinsed with sterile distilled water followed by exposure to 10% (v/v) sodium hypochlorite for 20 min, and then washed with sterile water. The seeds were transferred to 100 ml flasks (10 seeds/flask) containing 40 ml medium (0.7% agar) for 1 h, and then the vials were sealed with a gas-tight stopper. The excised roots were kept moist during the 1-h collection time and injected into a gas chromatograph (GC) equipped with an alumina column (GDX502) and a flame ionization detector (GC-7AG; Shimadzu Japan) for determination of the ethylene concentration.

Determination of ethylene production

Roots of five-day-old seedlings were exposed to solutions containing 0, 50, and 200 µM AlCl\(_3\) (pH 4.5) with basal composition of 0.2 mM CaCl\(_2\) for 2 h, before root apices (about 2 cm long) of about 0.3 g were excised. To minimize the wounding effect, the excised roots were placed into 5 ml gas-tight vials containing 0.5 ml of agar medium (0.7% agar) for 1 h, and then the vials were sealed with a gas-tight stopper. The excised roots were kept moist during the 1-h period. One millilitre of headspace gas was taken from the vials after 1 h collection time and injected into a gas chromatograph (GC) equipped with an alumina column (GDX502) and a flame ionization detector (GC-7AG; Shimadzu Japan) for determination of the ethylene concentration.
Staining Al by haematoxylin and determination of Al in root apices

Al distribution in root apices was visualized using lumogallion following protocols described by Delhaize et al. (1993a). Briefly, root apices were first exposed to 0 μM and 10 μM 1-aminocyclopropane-1-carboxylic acid (ACC) for varying durations, and then incubated in 20 μM AlCl₃ (pH 4.5) for 30 min. After rinsing thoroughly with deionized water, they were transferred to 100 ml solutions containing 0.2 g haematoxylin and 2 mg KIO₃ for 30 min. The roots were photographed after being washed with deionized water.

To examine the effect of ethylene on Al accumulation, five-day-old wheat seedlings were first exposed to either 10 μM aminoethoxyvinylglycine (AVG) or 50 μM 2-aminoisobutyric acid (AIB) for 6 h and then incubated in 20 μM AlCl₃ for 30 min. Control roots that were not treated with AVG and AIB were also exposed to an identical Al solution. Al contents in root apices were determined following the protocols used by Rangel et al. (2007). Briefly, about 20 root apices that were thoroughly rinsed with 0.2 mM CaCl₂ (pH 4.5) were transferred into 2 ml Eppendorf reaction vials and digested in 500 μl ultra-pure HNO₃ (65%) on a rotary shaker for 24 h. The digestion was completed by heating the samples in a water bath at 80 °C for 20 min. Samples were diluted by addition of 1.5 ml distilled water after cooling. All samples were passed through a 0.45 μm filter (Millipore, USA). Al concentration in the extract solution was measured by Inductively Coupled Plasma Emission Spectrometer (ICP-OES, Thermo Electron Corporation, USA)

Determination of malate efflux and intracellular malate contents

Malate exudation from root apices was determined according to the method of Ryan et al. (1995) with minor modifications. Root apices (1 cm) were excised with a razor blade from plants incubated in control solution (0.2 mM CaCl₂, pH 4.5). Thirty root-apices for each measurement were transferred into 5 ml vials and washed three times with control solution to remove malate released from the cut surface. Excised root apices were exposed to control solution and to solutions supplemented with 10 μM ACC for 2 h, and then incubated in 1 ml solution containing 20 μM AlCl₃ for another 2 h. During the treatment, the vials were placed on a reciprocating shaker (100 rpm). After 2 h, the solution was collected for malate analysis. To determine the effect of ethylene gas on malate efflux, the excised root apices were transferred into 5 ml vials containing 0.15 ml control solution. The solubility of ethylene in solution is very low, thus a minimum volume of solution was used to maximize effective ethylene concentration. The vials were sealed and 1 ml ethylene gas (500 ppm) or air was injected into the vials. After treatment for 2 h, root apices were exposed to 0 or 200 μM AlCl₃ for another 2 h. To study the effect of ethylene synthesis inhibitors AVG and AIB on malate efflux, roots were incubated in solutions containing 10 μM AVG and 50 μM AIB for 6 h. Root apices were excised and placed into vials containing 1 ml 200 μM AlCl₃ to collect malate for 1 h. To determine the additive effect of ACC and the anion channel blocker niflumic acid (NA), thirty root apices were treated with 1 ml solutions containing various concentrations of NA (0, 2, 5, 10, 20 μM) or ACC (0, 5, 10, 15, 20, 30, 50 μM) and 200 μM AlCl₃ for 1 h.

Malate concentrations of the exudation solution were determined following protocols used by Delhaize et al. (1993b). One ml sample solution was incubated with 1 ml buffer (0.5 M Glycer; 0.4 M hydroxyamine, pH 9.0) and 0.1 ml NAD. After 5 min, the reaction solutions were used to determine the absorption at 340 nm (the first Aₙₔ). The reaction mixture was then incubated for 40 min after the addition of 5 μL malate dehydrogenase (MDH). The production of NADH leads to the increase in Aₕₜ. The change of Aₕₜ before and after addition of MDH was used to calculate malate content.

Malate efflux from tobacco BY2 cells was measured as described by Zhang et al. (2008). BY2 cells (Nicotiana tabacum L. cv. Samsun, a cell line SL) transformed with the TaALMT1 gene from wheat, or an empty vector (Sasaki et al., 2004) were grown in MS media. The transgenic BY2 cells were grown in MS media solution on a rotary shaker until the logarithmic phase of growth. Aliquots of suspension containing approximately 1 g of cells were centrifuged and the cells were gently resuspended in 15 ml of 3 mM CaCl₂ and 3 mM sucrose (pH 4.5). Aliquots were then collected and cells resuspended in the above solution treated with or without added treatments at approximately 0.15 g FW per 10 ml. Treatments included 10 μM Ethrel, 100 μM AlCl₃ (pH 4.5) or 10 μM Ethrel plus 100 μM AlCl₃ (pH 4.5) for 60 min. After the treatment, the suspensions were centrifuged and malate concentrations in the supernatant were assayed as described above.

To measure malate concentrations in root apices, thirty root apices were homogenized in liquid N₂ and extracted using a pestle in 1 ml of ice-cold 0.6 N perchloric acid after washing thoroughly with control solutions. The extract was centrifuged at 15000 × g for 5 min and 0.9 ml of supernatant solution was collected and neutralized with 80 μl of K₂CO₃ (69 g/100 ml⁻³). The solution was centrifuged at 15000 × g for 5 min. The contents of malate were assayed as described above after mixing 0.5 ml of the supernatant with 0.5 ml distilled water.

Measurements of root elongation

Roots of 5-day-old seedlings were exposed to solutions containing different concentrations of AlCl₃ (0, 10, 20, 50, 100 μM, pH 4.5) or ACC (0, 0.01, 0.1, 1, 10 μM, pH 4.5) for 24 h. Root elongation was determined by a ruler (± 0.5 mm) before and after treatments. To study the short-term effect of AlCl₃ and ACC on root elongation, ET₅ₐ seedlings were incubated in control solution and solutions containing 10 μM ACC or 20 μM AlCl₃ (pH 4.5) for 1 h, and root length was measured under a microscope (SZX12, OLYMPUS, Japan) before and after treatments. To measure the effect of ethylene synthesis inhibitors (AVG, AIB) on root elongation, roots of 5-day-old seedlings were pretreated with control solution or solutions containing 10 μM AVG or 50 μM AIB for 6 h, and then exposed to 0 μM or 20 μM AlCl₃ (pH 4.5) for another 2 h. Root length was measured under microscope before and after exposure to AlCl₃.

Analysis of TaALMT1 gene expression

Real-time PCR was used to study the expression patterns of TaALMT1 in ET₅ₐ in response to AlCl₃ and ACC. After exposure of ET₅ₐ to AlCl₃ (20 μM) or ACC (10 μM) for varying time periods (0, 2, 6, 24 h), approximately 50 root apices were excised and frozen in liquid N₂. RNA was isolated using RNAiso Plus reagent (TaKaRa). The RNA was reverse-transcribed into first-strand cDNA with PrimeScript RT reagent Kit (TaKaRa): 0.5 μg of total RNA, 1 μL of 5×DNA Eraser Buffer, 0.5 μL of gDNA Eraser, and DEPC-H₂O to 5 μL. The solution was incubated at 42 °C for 2 min to remove any contaminating genomic DNA. Each reaction was adjusted to 10 μL by adding 2.0 μL of 5×Primer Script buffer, 0.5 μL Primer Script RT Enzyme Mix 1, 0.5 μL RT-Primer Mix, and DEPC-H₂O. The reverse-transcription was performed for 30 min at 37 °C and terminated at 85 °C for 5 s. Three endogenous genes TaActin, TaTubulin, and TaGAPDH were used as control genes. TaALMT1 expression relative to the control genes was determined by real-time quantitative RT-PCR (qRT-PCR) on an ABI StepOne Plus instrument. Each reaction contained 5.0 μl 2×UltraSYBR Mixture (With ROX) reagent (Cwbio), 1.5 μL cDNA samples, and 1.2 μl of 10 mM gene-specific primers in a final volume of 10 μL. Thermocycling conditions were 95 °C for 10 min followed by 40 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s. The primers used for real-time PCR of TaALMT1 were those used by Sasaki et al. (2006): 5′-AAGAGCGTCCTTAATTCG-3′ and 5′-CTTACATGATAGCTACTAGG-3′. Three endogenous genes TaActin, TaTubulin, and TaGAPDH were amplified with the following primers: TaActin-F (5′-CTATCCTTGTTGACCTT-3′), TaActin-R (5′-AGGAGCTTCTCCTATTTG-3′), TaTubulin-F (5′-TCCATGTCGGACTGGTGC-3′), TaTubulin-R (5′-TCCAGGCGTCTCCTCCACAGG-3′), TaGAPDH-F (5′-GTGGA
GGGTTTGTGACCCAG-3'), TaGADPH-R (5'-TCGGACCTTCCTTGATAGC-3'). Sequencing of PCR products was used to confirm whether the primers amplified the target genes. Three biological and three technological repeats were performed in RT-PCR. The relative expression level was analysed by the comparative C_{T} method.

Results

Al-stimulated ethylene evolution from root apices

To establish a link between ethylene and Al^{3+}-induced malate efflux from root apices, the effect of Al^{3+} on ethylene evolution from excised root apices of ET8 plants was determined. Similar to L. japonicas (Sun et al., 2007) and A. thaliana (Sun et al., 2010), exposure of 5-day-old ET8 seedlings to solutions containing 50 and 200 μM AlCl_{3} for 2 h (pH 4.5) led to an increase in ethylene evolution above control levels (Fig. 1). The magnitude of ethylene evolution from root apices was positively dependent on Al^{3+} concentrations, such that an increase in ethylene evolution was increased by 33.6% and 65.0% after treatment with 50 and 200 μM AlCl_{3}, respectively.

Ethylene inhibited Al^{3+}-induced malate efflux from root apices

Previous studies revealed that Al^{3+} can induce a rapid malate efflux from ET8 root apices (Ryan et al., 1995). The observation that Al^{3+} also increased ethylene production in root apices of ET8 seedlings prompted us to examine whether the Al^{3+}-induced ethylene is involved in regulation of malate efflux from root apices. To evaluate the role of ethylene in malate efflux from root apices, we first examined the effect of ethylene biosynthesis precursor ACC on malate efflux. As shown in Fig. 2A, ACC abolished the basal level of malate efflux from root apices. A marked increase in malate efflux from root apices was observed upon exposure to Al^{3+}, and the Al^{3+}-induced malate efflux was significantly suppressed by ACC (Fig. 2A). To validate that the inhibitory effect of ACC on Al^{3+}-induced malate efflux is related to ethylene, the effect of ethylene gas on Al^{3+}-induced malate efflux from ET8 root apices was further studied by exposing the roots to ethylene gas before treatment of roots with Al^{3+}. Figure 2B. ACS and ACO are two key enzymes catalysing ethylene production in vascular plants. In contrast to ACC and ethylene gas, AVG and AIB (ACS and ACO inhibitors, respectively), stimulated Al^{3+}-induced malate efflux from root apices (Fig. 2C), whereas AVG and AIB had no effect on malate efflux from root apices in the absence of Al^{3+} (Fig. 2C). These results suggest that ethylene may negatively regulate Al^{3+}-induced malate efflux from wheat roots.

Ethylene and niflumic acid had similar effect on Al^{3+}-induced malate efflux

It has been shown that Al^{3+}-induced malate efflux is mediated by anion channels (Ryan et al., 1997; Zhang et al., 2001, 2008). The anion channel blocker niflumic acid (NA) inhibits Al^{3+}-induced malate efflux from root apices (Ryan et al., 1995) and blocks malate-permeable channels (Zhang et al., 2001, 2008). The effect of NA and ACC on Al^{3+}-induced malate efflux from ET8 root apices was compared by analysing their dose-response curves. Our results show that both NA and ACC inhibited Al^{3+}-induced malate efflux, and the IC_{50} (concentration of inhibitor producing 50% inhibition) values for NA and ACC were not significantly different (Fig. 3A, B). However the extent of inhibition was larger for NA (72.1%) compared with ACC (31.0%). Moreover, there was no additive effect of ACC and NA on Al^{3+}-induced malate efflux, as treatment with NA had an identical effect on malate efflux to treatment with NA and ACC together (Fig. 3C). These results suggest the inhibition of Al^{3+}-activated malate efflux by ethylene may result from blockade of malate-permeable anion channels.

Intracellular malate concentrations in roots were not affected by ethylene

In addition to malate efflux, we also determined the effect of ACC and ethylene synthesis inhibitors (AVG, AIB) on intracellular malate concentrations of ET8 root apices. Our results showed no effect of ACC, AVG, and AIB on malate concentrations in ET8 root apices (Fig. 4). These results reveal that ethylene negatively regulates Al^{3+}-induced malate efflux from ET8 root apices.

Ethylene inhibited Al^{3+}-induced malate efflux from transgenic tobacco suspension cells

Previous studies showed that expression of TaALMT1 in tobacco suspension cells resulted in Al^{3+}-induced malate efflux (Zhang et al., 2008). To further evaluate the role of
Fig. 2. Effect of ethylene biosynthesis precursor (ACC), ethylene gas ($C_2H_4$), ethylene synthesis inhibitors (AVG, AIB) and Al on malate efflux from the root apex. (A) Thirty root apices (1 cm in length) of five-day-old seedlings were exposed to 200 μM AlCl$_3$ for 2 h after first being incubated for 2 h in and/or 10 μM ACC. (B) Thirty root apices were transferred to 5 ml gas-tight vials containing 100 nl ml$^{-1}$ ethylene gas for 2 h and then incubated to 200 μM AlCl$_3$ for 2 h. (C) Root apices were exposed to 200 μM AlCl$_3$ for 1 h, after seedlings were incubated in 10 μM AVG and 50 μM AIB for 6 h. The malate in solution was measured by enzyme method. Data are the means ± SE of four replicates. The different letters indicate significant difference at $P<0.05$ tested with SAS Software.
ethylene in regulation of Al$^{3+}$-dependent malate efflux, malate efflux from tobacco suspension cells exposed to Al and ethylene was determined. Malate efflux from the transgenic tobacco cells was significantly enhanced by exposure to 100 μM AlCl$_3$ (pH 4.5), and the Al$^{3+}$-induced malate efflux was suppressed by 94% when the cells were pretreated with 10 μM Ethrel (Fig. 5). A similar inhibitory effect of Ethrel on Al$^{3+}$-induced malate efflux from the tobacco suspension cells was also found when the suspension cells were treated with Al$^{3+}$ and 10 μM Ethrel simultaneously. The same concentration of Ethrel had no effect on malate efflux from the tobacco cells expressing either empty vector or TaALMT1 (Fig. 5). These results indicate that the inhibition of Al$^{3+}$-induced malate efflux from ET8 root apices by ethylene is likely to result from the suppression of TaALMT1-mediated malate efflux.
Ethylene and Al-induced malate efflux

Ethylene enhanced Al accumulation in root apices

Malate released from root apices acts as a ligand to complex external Al\(^{3+}\), thus minimizing the toxic effect of Al\(^{3+}\) on root growth by preventing accumulation of Al\(^{3+}\) in root apices (Delhaize et al., 1993b). The inhibition of malate efflux by ethylene should lead to greater accumulation of Al in the root apices. To test this hypothesis, Al content in root apices was measured by staining roots with the Al-sensitive probe haematoxylin, as well as quantitatively determined Al contents in root apices by inductively coupled plasma optical emission spectrometry (ICP-OES). Figure 6 shows that Al was mainly accumulated in the quiescent zones in the absence of ACC, and that exposure of ET8 seedlings to ACC led to an enhanced accumulation of Al in these areas as well as in the differentiation zone. A similar increase in Al content in ET8 root apices after treatment with ACC was observed (Fig. 6A). Moreover, the increase in Al content in root apices by ACC pretreatment increased with increasing pretreatment time (Fig. 6B). For example, Al content in the root apices was increased by 32%, 95%, and 216% after exposure to ACC for 2, 4, and 6 h before application of Al, respectively, whereas Al content in root apices exposed to solution without ACC showed relatively lower Al content (Fig. 6B). In contrast to treatment with ACC, Al contents in root apices were significantly reduced by AVG and AIB (Fig. 6C). The involvement of ethylene in Al accumulation in root apices was further evaluated by comparing the effect of exogenous application of malate on Al content in root apices with that of ACC. Exogenous application of malate significantly reduced Al content in root apices, whereas ACC increased the Al content (Fig. 6D). The increase in Al content by ACC was markedly suppressed by malate (Fig. 6D).

Ethylene inhibited root elongation similar to Al\(^{3+}\)

The most distinct symptom of Al\(^{3+}\) toxicity is inhibition of root elongation. Efflux of organic anions alleviates the Al\(^{3+}\)-induced inhibition of root growth by complexing toxic Al\(^{3+}\) in the rhizosphere. Ethylene gas and ethylene synthesis precursor ACC suppressed Al\(^{3+}\)-induced malate efflux from ET8 root apices and enhanced Al accumulation in root tips (Figs 2 and 6), suggesting that ethylene may be involved in the Al\(^{3+}\)-induced inhibition of root elongation. To test this hypothesis, we compared the effect of ACC and AlCl\(_3\) on root elongation. As shown in Fig. 7, treatment with ACC and AlCl\(_3\) for 24 h markedly suppressed root elongation. The IC\(_{50}\) values for inhibition of root elongation by Al\(^{3+}\) and ACC were 12.4 \(\mu\)M and 0.04 \(\mu\)M, respectively (Fig. 7A, B), suggesting that root elongation is more sensitive to ACC than Al\(^{3+}\). A similar rapid

Fig. 6. Effect of ethylene biosynthesis precursor (ACC), ethylene synthesis inhibitors (AVG, AIB) and malate on Al accumulation in root apices. (A) ET8 seedlings were first exposed to 0 \(\mu\)M and 10 \(\mu\)M ACC for 6 h followed by 0 \(\mu\)M and 20 \(\mu\)M AlCl\(_3\) for 30 min, then stained by haematoxylin. (B) Al contents in root apices were measured after being pretreated with 10 \(\mu\)M ACC for varying time periods (0, 2, 4, 6 h). (C) The roots of ET8 seedlings were treated for 6 h with 10 \(\mu\)M AVG and 50 \(\mu\)M AIB, followed by 20 \(\mu\)M AlCl\(_3\) for 30 min. (D) Thirty root apexes were pretreated with 10 \(\mu\)M ACC and 50 \(\mu\)M malate for 6 h, and exposed to 20 \(\mu\)M AlCl\(_3\) for 30 min. The root tips were washed for 30 min in control solution after Al treatments and then the Al concentrations were determined by ICP-OES. Data are the mean ± SE of four replicates and bars with different letters indicate significant difference at \(P<0.05\) tested with SAS Software.
inhibition of root elongation by ACC also occurred. For instance, root elongation was inhibited by 54% and 62% after exposure to 10 μM ACC and 20 μM ACC (pH 4.5), respectively, for 1 h (Fig. 7C). Root elongation was inhibited by AVG and Al when treated alone, whereas AIB had no effect on root elongation in the absence of Al (Fig. 7D). However, pretreatment of wheat roots with AIB reversed Al-induced inhibition of root elongation, leading to greater root elongation than control roots that were exposed to control solution (Fig. 7D). In contrast, pretreatment with AVG potentiated Al-induced suppression of root elongation (Fig. 7D).

Ethylene up-regulated TaALMT1 expression

Previous studies demonstrated that TaALMT1 was expressed constitutively in wheat roots (Sasaki et al., 2004). To test whether the suppression of Al-induced malate efflux by ethylene is related to TaALMT1 at the transcriptional level, the effect of ACC and AlCl₃ on TaALMT1 expression was investigated. As shown in Fig. 8, regardless of the reference genes used in qRT-PCR, the expression of TaALMT1 was enhanced after exposure to ACC, whereas expression of TaALMT1 in ET8 root apices was not responsive to Al (Fig. 8). These results suggest that regulation of ALMT1-mediated malate efflux from wheat root apices is unlikely to occur at the transcriptional level.

Discussion

There have been numerous studies reporting the involvement of ethylene in morphological responses of plants to nutrient deficiency and metal toxicity (Jung et al., 2009; Tian et al., 2009; Sun et al., 2007, 2010). In addition to modulation of root morphology, emerging evidence indicates ethylene may also play a regulatory role in physiological processes in response to mineral stress (Nagarajan and Smith, 2012; Iqbal et al., 2013). Our previous studies showed that Al evoked a rapid and marked ethylene evolution in L. japonicus (Sun et al., 2007) and A. thaliana (Sun et al., 2010). In the present

Fig. 7. Effect of aluminium (Al), ethylene biosynthesis precursor (ACC) and ethylene synthesis inhibitors (AVG, AIB) on root elongation of ET8. (A) Root elongation in response to various concentrations of AlCl₃. Roots of 5-day-old seedlings were incubated in solutions containing different concentrations of AlCl₃ (0, 10, 20, 50, 100 μM) and elongation was measured by ruler after 24 h. (B) Effect of varying concentrations of ACC on root elongation. Root elongation was determined after roots were treated with different concentrations of ACC (0, 0.01, 0.1, 1, 10 μM) for 24 h. (C) Short-term effect of ACC and Al on root elongation. Five-day-old seedlings were fixed in 10-cm Petri dishes and roots were incubated for 1 h in control solution (0.2 mM CaCl₂, pH 4.5) or treatment solutions containing 10 μM ACC or 20 μM AlCl₃. Root elongation was measured by microscope. (D). Root elongation in response to ethylene synthesis inhibitors (AVG, AIB). Roots of five-day-old seedlings were pretreated with control solution or the solutions containing 10 μM AVG or 50 μM AIB for 6 h, and then exposed to 0 μM or 20 μM AlCl₃ for another 2 h. Root elongation were measured after exposing to AlCl₃ for 2 h by microscope. Values are given as means ± SE of at least eight independent measurements. The different letters indicate significant difference at P<0.05 tested with SAS.
study, we found that Al\(^{3+}\) also evoked an evolution of ethylene from root apices of an Al-tolerant ET8 wheat genotype (Fig. 1). We evaluated the role of Al-induced ethylene production in the overall tolerance of ET8 to Al by experimentally manipulating the endogenous ethylene level using ethylene gas, ethylene donors, ethylene biosynthesis precursor, and ethylene synthesis inhibitors. Our results reveal that ethylene negatively regulates Al\(^{3+}\)-induced malate efflux from root apices of Al-tolerant wheat plants and from tobacco BY2 cells expressing TaALMT1 (Figs 2 and 5). We further demonstrate that ethylene may act on the TaALMT1 protein as shown by a similar, non-additive effect of ethylene and anion channel blocker niflumic acid on malate efflux from wheat root apices (Fig. 3). These findings, together with the observations that treatment with ethylene synthesis precursor ACC and ethylene synthesis inhibitors (AVG, AIB) enhanced and reduced accumulation of Al in the root apex, respectively (Fig. 6), provide evidence in support of the involvement of ethylene in Al tolerance in wheat by regulating ALMT1-mediated malate efflux. The enhanced accumulation of Al in root apices treated with ACC owing to suppression of malate efflux can also account for the results that inhibition of ethylene production by antagonists of ethylene synthesis (AIB) alleviated Al-induced arrest of root elongation. Antagonist of ethylene biosynthesis AVG enhanced Al-induced malate efflux and reduced Al accumulation in root apices (Figs 2 and 6), but Al-induced suppression of root elongation was potentiated, rather than alleviated, by treatment with AVG (Fig. 7D). This observation suggests that AVG may have other effects on root elongation in addition to the inhibition of ethylene biosynthesis. A recent study shows that AVG can inhibit root growth by affecting nitrogen metabolism (Lemaire et al., 2013). A similar explanation may also account for our observation in the present study. Therefore, results obtained from effects of AVG on plant growth cannot be conclusively attributed to an ethylene effect.

In a recent study, Yang et al. (2011) reported that Al\(^{3+}\)-induced malate efflux from root apices of ET8 wheat plants was stimulated by exogenous application of IAA, and that endogenous IAA content was enhanced owing to inhibition of IAA oxidase activity by Al\(^{3+}\). Similar to our results, the authors found that the Al\(^{3+}\)-induced malate efflux is inhibited by antagonists of auxin polar transport (TIBA; 2,3,5-triiodobenzoic acid, and NPA; naphthylphthalamic acid), and anion channel blockers (niflumate and A-9-C) (Yang et al., 2011). A close crosstalk between ethylene and auxin in regulation of root growth and development has been reported in the literature (see review of Stepanova and Alonso, 2009). Our previous results showed that ethylene evoked by Al\(^{3+}\) via up-regulating ACS and ACO at the transcriptional level may act as an up-stream signal to alter auxin transport and distribution in roots, leading to the arrest of root elongation (Sun et al., 2010). Whether a similar interaction between ethylene and auxin in regulation of Al\(^{3+}\)-induced malate efflux operates warrants further investigation by experimentally manipulating ethylene and/or auxin production and distribution with antagonists of ethylene synthesis and perception, auxin polar transport and exogenous application of auxin and ethylene.

The inhibitory effect of ethylene on Al\(^{3+}\)-induced malate efflux is unlikely to occur at the transcriptional level as ACC did not suppress expression of TaALMT1, rather an up-regulation of TaALMT1 in response to ACC was observed (Fig. 8). Anion channel antagonist NA that blocks Al\(^{3+}\)-activated ALMT1 channels (Zhang et al., 2008) and Al\(^{3+}\)-induced malate efflux (Ryan et al., 1995) exhibited similar IC\(_{50}\) value to ACC in their effect on malate efflux (Fig. 3). Moreover, we found that ethylene and NA had non-additive effect on Al\(^{3+}\)-induced malate efflux (Fig. 3). These results suggest that ethylene may act as a channel blocker to depress TaALMT1-mediated malate efflux. Alternatively, ethylene may regulate ALMT1 by preventing its activation by Al\(^{3+}\). Our observation that ethylene inhibited Al\(^{3+}\)-induced malate efflux from tobacco BY2 cells expressing TaALMT1 seems to be in line with these hypotheses. However, to elucidate the mechanisms responsible for suppression of Al\(^{3+}\)-induced malate efflux,
additional experiments will be needed such as probing the interaction between Al$^{3+}$ and ethylene on ALMT1-mediated currents with electrophysiological techniques.

There are many reports showing that ethylene can regulate expression of genes encoding membrane transporters such as phosphate, nitrate, and iron (Li et al., 2011; Tian et al., 2009; Garcia et al., 2010), but few studies have focused on the effect of ethylene on transport of ions at protein and cellular levels. Léblanc et al. (2008) showed that treatment of oilseed rape seedlings with ACC and AVG reduced and enhanced nitrate uptake, respectively, by monitoring $^{15}$N uptake. They suggested that a posttranscriptional regulation of nitrate transporters may be involved in the regulation of nitrate transport by ethylene (Léblanc et al., 2008). Our previous study showed that ethylene can increase the concentrations of cytosolic Ca$^{2+}$ by activating Ca$^{2+}$-permeable cation channel in tobacco cells (Zhao et al., 2007). Although there has been no report showing the involvement of cytosolic Ca$^{2+}$ activity in regulation of Al$^{3+}$-activated ALMT1-mediated malate efflux in the literature so far, we cannot rule out the possibility that inhibition of ALMT1-mediated malate efflux by ethylene may occur through changes in cytosolic Ca$^{2+}$ activity.

Recent studies shed some lights on the mechanisms by which Al$^{3+}$ activates anion channels. For instance, in Arabidopsis, upstream transcription factors AtSTOP1 and AtWRKY64 were reported to be involved in Al$^{3+}$-induced expression of TaALMT1 (Iuchi et al., 2007; Sawaki et al., 2009; Ding et al., 2013). In wheat, Al$^{3+}$-induced malate efflux is mainly controlled by ALMT1 protein, the expression of which is constitutive and not induced by Al (Sasaki et al., 2004). A post-transcriptional regulation of TaALMT1 by Al$^{3+}$ seems to be an important mechanism (Furuichi et al., 2010; Ryan and Delhaize, 2010). Although the extracellular C-terminal domain is proposed to be a key site interacting directly with external Al$^{3+}$ and the structural integrity of TaALMT1 is considered to be involved in Al$^{3+}$-sensing of TaALMT1, the mechanism underlyinig the activation of ALMT1 by Al$^{3+}$ remains unknown (Furuichi et al., 2010; Ligaba et al., 2013). It is unclear whether Al$^{3+}$ activates the ALMT1 channel directly or through signalling molecules. There is emerging evidence suggesting that reversible phosphorylation may also be involved in Al$^{3+}$-induced malate efflux from wheat roots (Osawa and Matsumoto, 2001) and Arabidopsis roots (Kobayashi et al., 2007) as shown by the inhibition of Al-activated malate efflux by protein kinase inhibitors (K252a and staurosporine). Ligaba et al. (2009) demonstrated that malate current in Xenopus laevis oocytes expressing TaALMT1 is regulated by protein kinase C-mediated phosphorylation. Moreover, Al can induce a 48kDa protein kinase in wheat roots and coffee (Coffeea arabica) suspension cells (Osawa and Matsumoto, 2001; Martinez-Estevez et al., 2001). It is conceivable that ethylene may regulate phosphorylation of TaALMT1 by targeting a protein kinase, because protein kinases and phosphorylation play important roles in ethylene signalling cascades (Ju et al. 2012). For example, five ethylene receptors identified in Arabidopsis possess kinase activity (Gamble et al., 1998). The receptors interact with a Raf-like protein kinase CTR1, a negative regulator of the ethylene signalling pathway (Kieber et al., 1993), leading to inactivation of downstream signalling components EIN2 and EIN3 (Alonso et al., 1999). Ethylene binding results in the inactivation of the receptor–CTR1 complex and the accumulation of EIN3 and EIN3-like transcription factors EILs in the nucleus (Guo and Ecker, 2003), which in turn activate and repress hundreds of genes by initiating a transcriptional cascade (Alonso et al., 1999). The negative regulation of Al-dependent malate efflux by ethylene suggests that some components of ethylene signalling cascades may interact with ALMT1 directly or indirectly. Alternatively, ethylene elicited by Al$^{3+}$ may target ALMT1 by interacting with other unknown signalling molecules, leading to the observed suppression of Al-induced malate efflux. Future work using mutants of ethylene biosynthesis and signalling and Xenopus oocytes expressing TaALMT1 may unravel the molecular mechanism underlying the interaction between Al and ethylene in modulation of malate efflux.

In summary, we show that pretreatment of wheat roots with ethylene gas and ACC suppressed Al$^{3+}$-induced malate efflux. The suppression of Al-induced malate efflux by ethylene is likely to result from inhibition of ALMT1-mediated malate efflux as shown by a similar effect of ethylene on Al-induced malate efflux from tobacco cells expressing TaALMT1. The suppression of ALMT1-mediated malate efflux by ethylene may occur through post-transcriptional regulation of ALMT1 because ethylene enhanced rather than inhibited expression of TaALMT1. Although the mechanism by which ethylene inhibits Al-dependent malate efflux remains to be elucidated, our findings demonstrate that ethylene may be an important component in the regulation of ALMT1-dependent malate efflux. Finally, our results show that the effect of ethylene on ALMT1-dependent malate efflux occurs at the post-transcriptional level. Therefore, future research to decipher the molecular mechanisms underlying the regulation of ALMT1-dependent malate efflux by Al and ethylene at protein level is warranted.

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References


