Auxin enhances aluminum-induced citrate exudation through upregulation of

*GmMATE* and activation of the plasma membrane H$^{+}$-ATPase in soybean roots

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**Running title:** Mechanism of auxin enhancing aluminum-induced citrate exudation
ABSTRACT

**Background and Aims** Aluminum (Al) toxicity is a limiting factor for plant growth and crop production in acidic soils. Citrate exudation and activation of the plasma membrane H⁺-ATPase are involved in soybean responses to Al stress. Auxin has a crucial function in plant growth as well as stress responses. However, little is known about possible interactions between auxin and citrate exudation under Al stress. In this study, we elucidated the regulatory roles of IAA on Al-induced citrate exudation in soybean roots.

**Methods** We measured IAA content, Al concentration, citrate exudation, plasma membrane H⁺-ATPase activity, expression of the relevant genes, and phosphorylation of the plasma membrane H⁺-ATPase by integrating physiological characterization and molecular analysis using hydroponically-grown soybean.

**Key Results** The concentration of IAA was increased by 25 and 50 μM Al, but decreased to the control level at 200 μM Al. External addition of 50 μM IAA to the root medium containing 25, 50 or 200 μM Al decreased root Al concentration and stimulated Al-induced citrate exudation and the plasma membrane H⁺-ATPase activity. The RT-PCR analysis showed that exogenous IAA enhanced the expression of citrate exudation transporter (*GmMATE*) but not the plasma membrane H⁺-ATPase gene. The Western blot results suggested that IAA enhanced phosphorylation of the plasma membrane H⁺-ATPase under Al stress.

**Conclusions** Auxin enhanced Al-induced citrate exudation through upregulation of *GmMATE* and an increase in phosphorylation of the plasma membrane H⁺-ATPase in
soybean roots.

Key words: soybean; auxin; aluminum; citrate exudation; *GmMATE*; plasma membrane H\(^+\)-ATPase
Aluminium (Al) is the most abundant metal in the earth’s crust (Tesfaye et al., 2001). In acidic soils (pH_{water}<5.5), Al is released into the soil solution, becoming toxic to plants and limiting to crop production (Rengel and Zhang 2003). The Al-activated release of organic acid anions from roots has been characterized as one of the most important Al-resistance mechanisms in plants (Ma 2000), including the efflux of malate from wheat roots (Ryan et al. 1995), oxalate from buckwheat (Zheng et al., 1998), and citrate from soybean (Silva et al., 2001), rice bean (Yang et al., 2007) and broad bean (Chen et al., 2012). Exudation of organic acid anions decreases Al uptake by chelating Al to form stable and nontoxic complexes in the rhizosphere. Exudation of malate and citrate anions is mediated by membrane-localized anion transporters belonging to two families, ALMT1 (Al-activated malate transporter) and MATE (multidrug and toxic compound extrusion) (Eticha et al., 2010; Liu et al., 2011; Magalhaes et al., 2007; Sasaki et al., 2004; Wu et al., 2012). Several genes encoding ALMT1 and MATE protein families have been identified in roots of Arabidopsis (Liu et al., 2009), sorghum (Magalhaes et al., 2007), rice (Yokosho et al., 2011) and soybean (Wu et al., 2012).

The plasma membrane H⁺-ATPase, as the most abundant plasma membrane protein, belongs to a large superfamily of pumps termed P-type ATPases (Rengel et al., 2015). Using the chemical energy of ATP, the H⁺-ATPase extrudes protons from cells to create an electrochemical gradient across the plasma membrane that is necessary to activate secondary transporters and plays an important role in physiological functions.
such as nutrient uptake (Palmgren 2001), stomatal opening (Kinoshita and Shimazaki 1999), and polar auxin transport and cell growth (Rober-Kleber et al., 2003). Under P deficiency or Al toxicity, activation of the plasma membrane H\textsuperscript{+}-ATPase is also involved in citrate exudation since the release of citric acid by plant roots is attributed to citrate anion export through citrate transporter (MATE) and proton export through the plasma membrane H\textsuperscript{+}-ATPase (Yan et al., 2002; Yu et al., 2016). For example, citrate exudation from cluster roots of white lupin (*Lupinus albus* L.) under stress of P deficiency was dependent on the plasma membrane H\textsuperscript{+}-ATPase (Tomasi et al., 2009; Yan et al., 2002). In soybean and broad bean roots, an increase in citrate exudation accompanied by an elevation of plasma membrane H\textsuperscript{+}-ATPase activity was observed in the Al-resistant but not Al-sensitive cultivars (Chen et al., 2013; Guo et al., 2013; Shen et al., 2005). Furthermore, the upregulation of plasma membrane H\textsuperscript{+}-ATPase and interaction with the 14-3-3 protein are involved in regulating Al-induced citrate exudation in Al-resistant soybean and broad bean roots (Chen et al., 2013; Chen et al., 2015; Guo et al., 2013; Shen et al., 2005). However, it is still poorly understood how plants modulate the activity of the plasma membrane H\textsuperscript{+}-ATPase in response to Al stress.

Auxin, an important growth regulator in plants, modulates not only plant growth and development, but also has a crucial function in the stress responses. The auxin concentration and distribution have been shown to be altered upon exposure to abiotic stresses such as Cd (Xu et al., 2010), salt (Albacete et al., 2008) and low P (Shen et al., 2006). External application of IAA can alleviate heavy metal toxicity in *Arabidopsis*,
wheat and eggplant (Zhu et al., 2013b; Yang et al., 2011; Agami and Mohamed, 2013; Singh and Prasad, 2015). For example, treatment with 100 μM IAA alleviated the toxic effects of 80 μM Cu in roots, as reflected in greater root length and root hair formation in sunflower (Ouzounidou and Ilias, 2005). Exogenous auxin application alleviated Cd toxicity by decreasing Cd-induced chlorosis and Cd accumulation in Arabidopsis roots and shoots (Zhu et al. 2013b). Auxin is also considered to be involved in mediating the P-starved effect because external application of auxin in P-sufficient plants resulted in the proteoid roots formation in white lupin (Lupinus albus) (Gilbert et al., 2000). Furthermore, P starvation induced an increase in endogenous IAA concentration and exogenous application of IAA stimulated both activity of plasma membrane H⁺-ATPase and P uptake in soybean roots (Shen et al., 2006).

Under Al stress, the Al-induced auxin accumulation in the root-apex transition zone was crucial for Al-induced root growth inhibition (Yang et al., 2014; Zhu et al., 2013a). Primary root elongation was less inhibited by Al in the Arabidopsis auxin-polar-transport mutants PIN2 (PIN-FORMED) and AUX1 (AUXIN RESISTANT 1) than wild-type plants (Sun et al., 2010). Consistently, Ruiz-Herrera (2013) found that auxin signaling is involved in Al induced primary root growth inhibition, but promoted lateral root formation and maturation. In addition, transgenic rice overexpressing OsPIN2 showed greater Al resistance than wild type plants (Wu et al., 2014). Also, Al-induced endogenous IAA accumulation correlated significantly with malate exudation, and the exogenous treatment with 10 μM IAA or 30 μM NPA
(its efflux transport inhibitor) enhanced or decreased malate efflux and Al concentration in wheat roots (Yang et al., 2011), thus demonstrating a possible role of IAA in alleviating Al toxicity. So far, there is no direct evidence regarding the mechanisms by which IAA enhances Al-activated organic acid anion exudation from roots.

In this study, we provided evidence regarding whether and how IAA regulates Al-activated citrate exudation from soybean roots. The results showed that the concentration of IAA was increased by Al, and that external application of IAA decreased Al concentration in roots. Furthermore, exogenous IAA increased Al-induced citrate exudation through up-regulation of GmMATE and activation of the plasma membrane H^+-ATPase.

MATERIALS AND METHODS

Plant culture and growth conditions

Seeds of soybean (Glycine max Merr. “Tamba kuro”), considered an Al-resistant cultivar (Guo et al., 2013; Wu et al., 2013), were soaked in deionized water for 12 h in the dark at 25 °C followed by surface-sterilization with 1% (v/v) sodium hypochlorite. Then, the seeds were placed on a filter paper moistened with half-strength Hoagland solution for germination in the dark at 25 °C. Seedlings with roots approximately 1-2 cm long were transferred onto a floating mesh in a polypropylene pot with half-strength Hoagland solution (5 L) and grown under glasshouse conditions at 30/25°C day/night temperatures with 12 h of light. The
nutrient solution was renewed every other day.

**Measurement of endogenous IAA**

Two-week-old seedlings were pre-treated with 0.5 mM CaCl$_2$ (pH 4.2) at 25°C under constant light (100 μmol m$^{-2}$ s$^{-1}$) in a tissue culture room for 12 hours. The seedlings were then transferred into 0.5 mM CaCl$_2$ solution containing AlCl$_3$ at 0, 25, 50 or 200 μM (pH 4.2) for the 24-h treatment. After treatment, root apices (0-2 cm) were excised, weighed and immediately frozen in liquid nitrogen. Free IAA concentration in roots was measured by HPLC according to Sun et al. (2014).

**Effect of IAA on plant growth under Al stress**

To analyze the effects of IAA on plant growth, uniform five-day-old seedlings (without lateral roots) with the taproot about 1 cm long were transferred onto a mesh floating in a polypropylene pot with half-strength Hoagland solution (1 L) with 50 μM AlCl$_3$ plus 0, 0.5, 1, 25, 50 μM IAA or 50 μM IAA+10 μM TIBA for 8 days. Seedlings grown in half-strength Hoagland solution with 0 or 50 μM IAA or 50 μM IAA plus 50 μM AlCl$_3$ were used as controls. The pH of the treatment solutions was adjusted to 4.2 by the addition of 1 M HCl and the nutrient solution was renewed every other day. After 8-day treatments, the lateral root number and fresh weigh were determined.

**Measurement of Al concentration in roots and citrate concentration in root exudates**
Two-week-old seedlings were pre-treated with 0.5 mM CaCl$_2$ solution (pH 4.2) at 25°C overnight under constant light (as described above). The roots were thoroughly rinsed with deionized water and gently blotted; the seedlings were transferred into 0.5 mM CaCl$_2$ solution with the following treatments: control (CK, 0.5 mM CaCl$_2$), Al$_{25}$ (25 μM AlCl$_3$), Al$_{25}$IAA$_{50}$ (25 μM AlCl$_3$ + 50 μM IAA), Al$_{50}$ (50 μM AlCl$_3$), Al$_{50}$IAA$_{50}$ (50 μM AlCl$_3$ + 50 μM IAA), Al$_{200}$ (200 μM AlCl$_3$), Al$_{200}$IAA$_{50}$ (200 μM AlCl$_3$ + 50 μM IAA), or Al$_{50}$IAA$_{50}$TIBA$_{10}$ (50 μM AlCl$_3$ + 50 μM IAA + 10 μM TIBA). The pH was adjusted to 4.2 with 1 M HCl. After 24-h treatments, the root apices (0-2 cm) were excised, weighed, immediately frozen in liquid nitrogen and stored at -80°C for later RNA and plasma membrane extraction. The root exudates were collected and concentrated for citrate analysis by HPLC (Chen et al., 2012).

For measurement of Al concentration in soybean roots, the excised roots were thoroughly rinsed with deionized water, gently blotted, weighed, transferred to borosilicate tubes, and ashed at 550°C for 12 h. The resulting ash was dissolved overnight in 1 mL concentrated HNO$_3$ and then diluted to 50 mL with deionized water. Al was analyzed by Inductively-Coupled Plasma-Atomic Emission Spectroscopy (ICP-AES, model PS-1000, Leeman Labs., Lowell, MA, USA).

Isolation of the plasma membrane proteins and measurement of plasma membrane $H^+\text{-ATPase}$ activity

Two-week-old seedlings were treated as described above. After treatments, the root apices (0-2 cm) were excised, weighed, immediately frozen in liquid nitrogen and...
stored at -80°C for isolation of the plasma membrane proteins. The plasma membrane proteins were isolated and the activity of the plasma membrane H⁺-ATPase was assessed as we described elsewhere (Chen et al., 2015).

**Membrane potential**

The membrane potential was detected by a fluorescent probe, Bis-(1,3-dibutylbarbituric acid) trimethine oxonol (DiBAC₄, a voltage-sensitive fluorescent dye, Sigma-Aldrich) as previously described (Sun et al. 2012). After treatments, a stock solution of DiBAC₄ (2 mM in DMSO) was added to excised root tips (0-2 cm) for 10 min at 25°C. The final concentration of DiBAC₄ was 2 μM. Subsequently, the root tips were thoroughly rinsed with deionized water three times, mounted on glass slides and examined by a Nikon A1 confocal fluorescence system. The membrane potential dye was excited at 488 nm with an argon laser; fluorescence emission was filtered at 530 nm and fluorescence intensity was captured through a 20× objective. DiBAC₄ enters depolarized cells and exhibits fluorescence upon binding to endomembranes or proteins, whereas a return of plasma membrane to the resting potential or hyperpolarization causes extrusion of the dye and a decrease in fluorescence (Sivaguru et al., 2005). The experiments were conducted three times, and one representative set of results was presented.

**Real-time RT-PCR analysis**

The excised 0-2 cm root tips were used to isolate total RNA by Trizol reagent, and the
first strand cDNA was synthesized as we described previously (Chen et al., 2011).

Gene expression analyses were performed by adding 1 μL of tenfold-diluted cDNA in an ABI Plus (Applied Biosystems StepOne, Applied Biosystems) 96-well reverse transcription-PCR system with ABI SybrGreen PCR Master Mix following the manufacturer’s instructions. The sequences of the gene-specific primers pairs for *GmMATE* (GLYMA13G27300) and the plasma membrane H⁺-ATPase gene (AF091303) were designed as follows: 5′-AGTAAGCGTAGCCACAGAA-3′ (sense primer)/5′-CTGAGATAGAGCCAAGGTC-3′ (antisense primer) and 5′-TGAGTGGGAAGGCTTGGGATAA-3′ (sense primer)/5′-AAGGTTGGTTTTCAGGTGTC-3′ (antisense primer), respectively. The 18s rRNA gene (M16859) was used as a reference gene with 5′-CCCGTCTCAGATTGGTGTCATT-3′ (sense primer)/5′-ATAGCGAGCAAGTCGGTGGATT-3′ (antisense primer). PCR was performed for 40 cycles, and triplicates were performed on three independent experiments.

**Western blot analysis**

After treatments, plasma membrane proteins were extracted, and a aliquot of 60 μg plasma membrane proteins was separated by SDS-PAGE (10 %, w/w) for western analysis. The separated proteins were transferred to polyvinylidene difluoride (PVDF) membranes with a semi-dry transfer system. The membranes were first treated with the rabbit antibodies raised against the C-terminal domain of the *Arabidopsis* plasma membrane H⁺-ATPase (AHA3) or with the rabbit antibodies raised against the
specifically phosphorylated Thr-951 in the *Vicia faba* plasma membrane H\(^+\)-ATPase VHA2. The phosphorylation-specific antibody for VHA2, which has been shown to also detect the phosphorylation levels of the plasma membrane H\(^+\)-ATPase in soybean, was produced against a 20-amino-acid peptide (N’-ESVVKLGIDTIQQHYT (p)V-C’) with a phosphorylated modification according to the amino acidic sequence of *Vicia faba* VHA2 (Chen *et al.*, 2013).

**Statistical analysis**

Experiments contained from three to twelve replicates, and the data are expressed as means and SE. SPSS 12.0 for Windows (SPSS, Chicago, IL, USA) software packages were used to conduct the LSD test to determine statistical significance at \( p \leq 0.05 \).

**RESULTS**

*Al exposure induces auxin accumulation in soybean roots*

Because endogenous auxin accumulation is involved in plant response to Al stress, we first analyzed the changes in auxin concentration in soybean roots. Compared with the control treatment, the concentration of IAA was increased by 14 and 28\% after the roots were treated by 25 and 50 \( \mu \)M Al, respectively, but decreased to the control level at 200 \( \mu \)M Al (Fig. 1).

*External application of IAA increased plant growth and root development under Al stress*
Having ascertained that auxin accumulation in soybean root tips was induced by Al, we then asked the role of IAA on soybean growth under Al stress. As shown in Fig. 2A, lateral root number decreased significantly after treatment with 50 µM Al for 8 days. In contrast, adding 50 µM IAA to the control (0 µM Al) solution, or increasing additions of IAA (from 0.5 to 50 µM) to the solution with 50 µM Al, resulted in an increase in lateral root number (Fig. 2A). The 50 µM Al treatment decreased the root and shoot fresh weight and root/shoot ratio, but these parameters were significantly restored by the application of up to 50 µM IAA in the Al-treatment solution (Fig. 2 B, C and D). These results suggested that external application of IAA increased plant growth and root development under Al stress.

*IAA decreased Al accumulation in soybean root tips*

Increasing Al concentration in the treatment solution from 25 to 200 µM resulted in a significant increase in Al concentration in root tips (from 3.7 to 6.9 mg/g DW) (Fig. 2). Inclusion of 50 µM IAA in the treatment solutions containing 25, 50 or 200 µM Al decreased Al concentration in root tips by, respectively, 18, 29 and 31% (Fig. 3).

*Activation of the plasma membrane H+-ATPase activity by IAA was involved in the regulation of Al-induced citrate exudation*

Aluminum-induced organic acid exudation from plant root tips is an Al-exclusion mechanism that can reduce Al accumulation in plant roots. We investigated the involvement of IAA in Al-induced citrate exudation under Al stress in soybean roots.
No citrate exudation was detected in the control (0 μM Al) treatment (Fig. 4A). With increasing Al concentration to 25 or 50 μM, citrate exudation increased progressively and significantly, but was lower at 200 than 25 or 50 μM Al (Fig. 4A). Compared with the Al-only treatments, citrate exudation was significantly increased after 50 μM IAA was applied to 25, 50 and 200 μM Al treatment solutions (by 2.5-, 1.7- and 6.7-fold, respectively) (Fig. 4A).

It has been shown that citrate exudation from roots is accompanied by activation of the plasma membrane H⁺-ATPase in soybean under Al stress (Guo et al. 2013; Shen et al. 2005); therefore, the activity of this enzyme was also determined. The IAA-only treatment significantly increased the plasma membrane H⁺-ATPase activity in soybean root tips compared to the control (0 μM IAA added) (Fig. 4B). Similarly, exposing soybean plants to 25 and 50 μM Al increased (up to 2-fold) the plasma membrane H⁺-ATPase activity in the root tips, whereas at 200 μM Al, the activity of this enzyme decreased to the control level (Fig. 4B). Adding exogenous IAA at 50 μM to the Al-containing treatment solutions resulted in a significant increase in the plasma membrane H⁺-ATPase activity in case of 50 μM Al (74% increase) and 200 μM Al (45% increase) compared with the Al-only treatment (Fig. 4B). Adding TIBA (an IAA polar transport inhibitor) or vanadate (VA, a plasma membrane H⁺-ATPase inhibitor) to the treatment solution containing 50 μM Al plus 50 μM IAA resulted in a significant decrease in the plasma membrane H⁺-ATPase activity (Fig. 4B) as well as citrate exudation (Fig. 4A). These results indicated that the plasma membrane H⁺-ATPase was involved in IAA-dependent enhancement of Al-induced citrate
DiBAC4(3), a kind of fluorescent probe, is usually used for monitoring the changes of membrane potential. When membrane becomes hyperpolarized, the dye across the cell membrane and fluorescence decreases. Therefore, the changes of membrane potential were monitored to investigate the action of Al and IAA using DiBAC4(3) in this study. Soybean roots exhibited relatively weaker fluorescence after 24 h of exposure to Al compared with the control treatment (Fig. 4C). After application of IAA into the Al treatment solution, the relative fluorescence intensity was significantly decreased (about 68% relative to the control and 38% relative to the Al-only treatment) (Fig. 4D). However, TIBA supplied to the Al- and IAA-containing treatment solution increased the relative fluorescence intensity to the control level. These results suggested that both Al and IAA promoted hyperpolarization (Fig. 4C and D) by enhancing the plasma membrane H\(^+\)-ATPase (Fig. 4B) under Al stress.

**IAA enhanced GmMATE, but not the plasma membrane H\(^+\)-ATPase gene expression under Al stress**

To understand how IAA modulates the Al-induced citrate efflux and the plasma membrane H\(^+\)-ATPase activity, we explored the mRNA abundance of *GmMATE* and the plasma membrane H\(^+\)-ATPase gene. A 24-h exposure of soybean to 25 or 200 \(\mu\)M Al resulted in a similar increase in the mRNA abundance of *GmMATE* in the root tips, and exposure to 50 \(\mu\)M Al increased it even further (Fig. 5A). In case of all three Al treatments, adding 50 \(\mu\)M IAA increased the expression of *GmMATE* significantly.
with the Al+IAA treatment, application of TIBA reduced the expression of *GmMATE* by 62% (Fig. 5A).

The expression of the plasma membrane H^+^-ATPase gene was not influenced by an addition of 50 μM IAA, but was increased by 76, 95 or 59% after roots were treated with 25, 50 or 200 μM Al, respectively (Fig. 5B). However, neither IAA nor IAA+TIBA addition to the Al-containing treatment solutions changed the expression of the plasma membrane H^+^-ATPase gene. These results indicated that IAA enhanced Al-induced citrate exudation through increasing the expression of *MATE* but not the plasma membrane H^+^-ATPase gene in the soybean root tips.

**IAA enhanced phosphorylation of the plasma membrane H^+^-ATPase under Al stress**

To assess whether translational and/or post-translational regulation was involved in IAA-dependent enhancement of the plasma membrane H^+^-ATPase activity, we analyzed protein biosynthesis and phosphorylation. The total amount of the plasma membrane H^+^-ATPase protein in the root tips was increased by 35, 26 and 13% after soybean plants were treated by Al, Al+IAA and Al+IAA+TIBA, respectively (Fig. 6B). Phosphorylation of the plasma membrane H^+^-ATPase increased by 10% after treatment by 50 μM Al and 34% after Al+IAA. However, addition of TIBA to the Al+IAA treatment decreased phosphorylation to the control level (Fig. 6A).

**DISCUSSION**
As one of the most widely accepted mechanisms of plant responses to Al, organic acid anion exudation has been validated by cloning of the relevant transporter genes such as ALMT1 and MATE (Eticha et al., 2010; Liu et al., 2011; Magalhaes et al., 2007; Sasaki et al., 2004). In the present study, we found significantly increased abundance of GmMATE transcripts after a 24-h exposure to 25, 50 or 200 μM Al (Fig. 5A). Moreover, the addition of IAA into the Al-containing treatment solutions significantly enhanced the mRNA abundance of GmMATE compared with Al-treated roots. There results suggested that upregulation of GmMATE is involved in IAA-enhanced citrate release under Al toxicity.

Acidification of the cell wall through activation of the plasma membrane H⁺-ATPase is an important part of the growth-promoting effect of auxin (Frias et al., 1996; Hager et al., 1991), although the exact mechanisms mediating the effect of auxin on the plasma membrane H⁺-ATPase are unclear (Fuglsang et al., 2007). The auxin polar transport and auxin gradient formation were demonstrated to be dependent on the activity of the plasma membrane H⁺-ATPase (Hohm et al., 2014; Yang et al., 2006). In Arabidopsis, the pin2 mutant exhibited much reduced plasma membrane H⁺-ATPase activity, auxin transport, proton secretion and root elongation under alkaline stress (Xu et al., 2012). In soybean roots, an increase in endogenous IAA was observed under low-P, and an exogenous application of 10 μM IAA stimulated the activity of plasma membrane H⁺-ATPase and enhanced P uptake (Shen et al., 2006). The plasma membrane H⁺-ATPase is the pivotal enzyme for creating an electrochemical H⁺ gradient and generating the proton motive force across the plasma
membrane that is necessary to activate citrate transporters under Al stress and low-P conditions (Rengel et al., 2015; Yu et al., 2016). For example, the plasma membrane H⁺-ATPase is involved in citrate exudation from cluster roots of Lupinus albus under P deficiency (e.g., in cluster roots of Lupinus albus, Yan et al., 2002) and from soybean (Shen et al., 2005) and Vicia faba roots (Chen et al., 2012) of the Al-resistant but not the Al-sensitive cultivars. In the present study, 50 μM IAA added to the Al-containing solution enhanced both Al-induced citrate exudation and Al-mediated increase in the plasma membrane H⁺-ATPase activity (Fig. 4B and C); this enhancement was repressed by the addition of plasma membrane H⁺-ATPase inhibitor vanadate or the auxin-polar-transport inhibitor TIBA (Fig. 4). The results from using DiBAC₄ dye also suggested that IAA strengthened hyperpolarization of the plasma membrane caused by Al, such a hyperpolarization effect (Fig. 4C, D) of Al or IAA was underpinned by increased activity of H⁺-ATPase caused by Al or IAA (Fig. 4B).

In many experimental systems Al caused depolarization of the plasma membrane, suggesting decreased H⁺-ATPase activity, including wheat, barley, maize, and squash (for references see Rengel and Zhang, 2003), tobacco (Sivaguru et al., 2005), and Arabidopsis (Illes et al., 2006), but both depolarization and hyperpolarization of the plasma membrane due to Al exposure may occur in Arabidopsis depending on the root part and a degree of Al sensitivity (Bose et al., 2010a; b). Similarly, in wheat root tips, Al exposure caused depolarization in the Al-sensitive and hyperpolarization in the Al-resistant genotypes (Ahn et al., 2004). In the apoplastic space (pH~5.5), IAA is protonated as IAAH (pKa 4.85); whereas, higher cytosolic pH (7-7.5) promoted the
IAAH dissociates into deprotonated IAA (IAA⁻) and H⁺ (Florent Villiers and June M. Kwak, 2012). This indicates that the efflux of IAAH across the membrane must be attributed to at least two structurally separated plasma membrane transport processes: PIN mediated IAA (IAA⁻) efflux and plasma membrane H⁺-ATPase mediated H⁺ efflux. In the present study, hyperpolarization of the root-cell plasma membrane was caused by Al, and that effect was further enhanced by the exposure to Al+IAA (Fig. 4C and D); these results were consistent with Al and IAA individually and in combination enhancing the plasma membrane H⁺-ATPase activity (Fig. 4B).

Therefore, it is speculated that hyperpolarized of the membrane through activation of the plasma membrane H⁺-ATPase is necessary to activate auxin and citrate transport across the membrane. Whereas, whether PIN mediated IAA efflux is involved in the regulation of citrate exudation under Al stress need further determination.

The activity of the plasma membrane H⁺-ATPase can be modulated at transcriptional, translational and post-translational levels (Portillo 2000). For example, external IAA treatment enhanced the mRNA abundance and protein biosynthesis of the plasma membrane H⁺-ATPase in maize (Frias et al., 1996; Hager et al., 1991). Aluminum toxicity stress induced the gene expression and protein biosynthesis of the plasma membrane H⁺-ATPase in the Al-resistant cultivars of soybean and broad bean (Chen et al., 2013; Shen et al. 2005). Phosphorylation at the penultimate Thr residue of the plasma membrane H⁺-ATPase is a common example of post-translational modification altering the activity of this enzyme. In Arabidopsis, IAA activates the plasma membrane H⁺-ATPase by enhancing phosphorylation and the interaction with
14-3-3 protein during hypocotyl elongation under P-deficiency conditions (Takahashi et al., 2012). The penultimate Thr-directed phosphorylation of the plasma membrane 

H⁺-ATPase is also involved in citrate exudation from soybean (Shen et al., 2005) and *Vicia faba* roots (Chen et al., 2013) under Al toxicity. Furthermore, the 

post-translational modification of the plasma membrane H⁺-ATPase, rather than 

transcriptional and translational modification, was involved in Mg enhancement of 

Al-induced citrate exudation in *Vicia faba* roots (Chen et al., 2015). The results 

presented here showed that IAA did not induce changes in transcription (Fig. 5A) and 

translation (Fig. 6B) of the plasma membrane H⁺-ATPase under Al stress, whereas 

phosphorylation of the penultimate Thr-oriented residue in the plasma membrane 

H⁺-ATPase was increased in the presence of IAA under Al stress (Fig. 6B).

**CONCLUSIONS**

The results of this study provide evidence that Al induced IAA accumulation in 

soybean root tips, and that external application of IAA reduced Al uptake in the 

soybean root tips. An IAA-dependent upregulation of *GmMATE* expression together 

with phosphorylation of the plasma membrane H⁺-ATPase were involved in 

enhancing activity of the plasma membrane H⁺-ATPase, resulting in increased citrate 

exudation from soybean roots under Al stress.

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and induction by auxin in coleoptiles. Plant Cell, 8: 1533-44.


Figure legends

Fig. 1 Effect of Al on IAA accumulation in soybean root tips. Two-week-old seedlings were treated with 0, 25, 50 or 200 μM Al in 0.5 mM CaCl₂ for 24 h. Values are means ± S.E. (n=6). Means with different letters are significantly different (P≤0.05).

Fig. 2 Effect of external application of IAA on lateral root number (A), root (B) and (C) shoot fresh weigh, root/shoot ratio (D) of soybean under Al stress. Five-day-old seedlings were grown in half-strength Hoagland solution (1 L, pH 4.2) with 50 μM AlCl₃ plus 0, 0.5, 1, 25 or 50 μM IAA for 8 days. Seedlings grown in half-strength Hoagland solution with 0 or 50 μM IAA were used as controls. The treatment solution was renewed every other day. Values are means ± S.E. (n=12).

Fig. 3 Effect of IAA on Al concentration in soybean root tips (0-2 cm). The two-week-old seedlings were treated with 0, 25, 50 or 200 μM Al with 0 or 50 μM IAA and with or without 10 μM TIBA in 0.5 mM CaCl₂ for 24 h. Values are means ± S.E. (n=6). Means with different letters are significantly different (P≤0.05).

Fig. 4 Effect of IAA on citrate exudation (A), the plasma membrane H⁺-ATPase activity (B) and membrane potential (C and D) in soybean root tips (0-2 cm) under Al stress. Two-week-old seedlings were treated with 0, 25, 50 or 200 μM Al with 0 or 50 μM IAA and with or without 10 μM TIBA or 50 μM vanadate (VA) in 0.5 mM CaCl₂ for 24 h. For A, B and D, values are means ± S.E. (n=6). For C and D, membrane
potential was measured using fluorescence dye DiBAC$_4$. Decreased fluorescence indicates hyperpolarization of the plasma membrane. Quantification of the fluorescence intensity was done using ImageJ (http://rsb.info.nih.gov/ij/). Means with different letters are significantly different (P≤0.05).

Fig. 5 Real-time RT-PCR analysis of the transcription (mRNA abundance) of GmMATE (A) and the plasma membrane H$^+$-ATPase gene (B) in soybean root tips (0-2 cm). The two-week-old seedlings were treated with 25, 50 or 200 µM Al with 0 or 50 µM IAA and with or without 10 µM TIBA in 0.5 mM CaCl$_2$ for 24 h. Values are means ± S.E. (n=6). Means with different letters are significantly different (P≤0.05).

Fig. 6 Translation and phosphorylation of the plasma membrane H$^+$-ATPase isolated from soybean root tips (0-2 cm). A, Western blot analysis for detection of the protein biosynthesis (bottom) and phosphorylation (top) of the plasma membrane H$^+$-ATPase. B, Coomassie Brilliant Blue staining of the membrane proteins. Two-week-old seedlings were treated with 0 (control, CK), 50 µM Al (A$_{50}$), 50 µM Al+50 µM IAA (A$_{50}$I$_{50}$) or 50 µM Al+50 µM IAA+10 µM TIBA (A$_{50}$I$_{50}$T$_{10}$) in 0.5 mM CaCl$_2$ for 24 h. Relative-intensity band quantification of western blots was performed using ImageJ (http://rsb.info.nih.gov/ij/). The experiment was conducted three times, and one representative set of results is shown.
Figures

Fig. 1

![Bar chart showing IAA concentration (µg g⁻¹ FW) across different Al treatments (µM). The chart includes bars labeled with letters indicating significant differences.]
Fig. 2

(A) Lateral root number

(B) Root fresh weight (g plant⁻¹)

(C) Shoot fresh weight (g plant⁻¹)

(D) Root shoot ratio
Fig. 3

![Bar chart showing root Al concentration (mg g⁻¹ DW)]
Fig. 4

A

B

C

D

Relative fluorescent intensity

Activity of 5'ATase

(Fig. 5)
Fig. 5

A

Relative expression (relative to ACTIN)

B

Relative expression (relative to ACTIN)

Legend:

- OK
- IAA
- Al
- Al + IAA
- Al + IAA + TIBA
Fig. 6

A

<table>
<thead>
<tr>
<th>CK</th>
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<th>A50/150 T10</th>
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Phospho PM H^+ -ATPase

B

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<th>A50/250 T10</th>
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Total PM H^+ -ATPase

Markers:

- 116 kD
- 66 kD
- 45 kD
- 35 kD