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REGULAR ARTICLE

Effect of indole-3-acetic acid on aluminum-induced efflux of malic acid from wheat (*Triticum aestivum* L.)

Ye Yang · Qiao Lan Wang · Ming Jian Geng · Zai Hua Guo · Zhuqing Zhao

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Abstract Indole-3-acetic acid (IAA) has been found to be involved in plant resistance to various types of environmental stress. Aluminum (Al) toxicity, as one of the most important environmental stress in acid soils, is coped by most plants through the efflux of organic acids via anion channel. This study aims to evaluate the effect of IAA on efflux of malic acid from wheat (*Triticum* aestivum L.) under Al stress. Hydroponic experiments were performed by wheat ET8 (Al-tolerant). The efflux of malic acid was investigated under different treatments. Results showed that Al treatments increased the accumulation of endogenous IAA, but decreased the activity of IAA oxidase in a dose-dependent manner. A good correlation between all the data of malic acid efflux rate and endogenous IAA content was obtained (R^2= 0.9859**). IAA treatment alone had no effect on the efflux of malic acid. But compared to Al (50 μM) treatment, the efflux of malic acid increased significantly under the co-treatment of IAA (50 μM) and Al (50 μM). In split-root experiments, the root with half of it being treated with Al (CK/Al), the other part (CK) showed significantly higher malic acid efflux rate and endogenous IAA content in root apexes, compared with the root without such treatment (CK/CK). The Al-induced malic acid efflux decreased under the treatments of IAA transport inhibitor *N*-1-napthyl-phtalamic acid (NPA) (or 2,3,5-triiodobenzoic acid, TIBA). These above results suggested the possible involvement of IAA in the stimulation of malic acid efflux under Al stress. In addition, anion channel inhibitor treatment experiment showed that IAA (50 μM) relieved the inhibiting effect of 5 μM anthracene-9-carboxylic acid (A9C) (or niflumic acid, NIF) on malic acid efflux induced by Al (50 μM), compared to the co-treatment of Al (50 μM) and 5 μM anion channel inhibitor A9C (or NIF) it is thus speculated that the anion channel might have been activated when IAA was involved in malic acid efflux. This study showed that IAA was involved in aluminum-induced efflux of malic acid from wheat.

Keywords Aluminum-toxicity · Anion channel · Indole-3-acetic acid · Malic acid · *ALMT1* gene · Wheat

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Abbreviations

A9C	Anthracene-9-carboxylic acid
DCP	Dichlorophenol
DIDS	4,4′-diisothiocyano stilbene-2,2′-disulfonic acid
IAA	Indole-3-acetic acid
IAA-94	R(+)-methylindazone; indanyloxyacetic acid-94
MAPK	Mitogen-activated protein kinases
NIF	Niflumic acid
NPA	*N*-1-napthyl-phtalamic acid
TaALMT1	*Triticum aestivum* Aluminum-activated malate transporter
TIBA	2,3,5-triiodobenzoic acid

Introduction

Aluminum (Al) is abundant in the crust of the Earth and Al toxicity is believed to be one of the major factors limiting the growth of roots and the overall development of plants in acid soils due to dissolution of Al-containing species. In response to Al stress, many plant species and cultivars secrete organic acids capable of chelating Al into non-rhizotoxic complexes, which increase their Al resistance (Ma and Furukawa 2003). However, the organic acid species and secretion patterns differ among plant species. There are two patterns of organic acid release with respect to timing of the secretion (Ma 2000). In pattern 1, no delay is observed between the addition of Al and the onset of secretion. In pattern 2, the efflux of organic acid anions is delayed for several hours after exposure to Al. The rapidity of pattern 1 response suggests that Al activates a pre-existing anion channel on the plasma membrane without the induction of genes. By contrast, the delay observed in pattern 2 type secretion might indicate that gene induction is required. Genes that encode the anion channel, which are responsible for Al-induced malic acid release have been identified in various plants. For example, in wheat cultivars *TaALMT1* was identified as the first Al tolerance gene, encoding a protein that facilitates the release of malic acid from roots (Sasaki et al. 2004). Heterogeneous expression of the *TaALMT1* gene in Xenopus oocytes revealed the kinetic properties of malic acid transport (Sasaki et al. 2004; Piñeros et al. 2008). When expressed in barley (Delhaize et al. 2004) and tobacco suspension cells (Sasaki et al. 2004; Zhang et al. 2008) *TaALMT1* conferred an Al^{3+}-activated efflux of malic acid which was associated with improved resistance to Al^{3+} stress. Heterologous expression of *TaALMT1* in barley plants showed a 2-fold increase in grain production on acid soil compared with the untransformed controls (Delhaize et al. 2009). In recent years, homologs of *TaALMT1* have been characterized in many species e.g., *AtALMT1* (Hoekenga et al. 2006; Kobayashi et al. 2007), *AtALMT9* (Kovermann et al. 2007) and *AtALMT12* (Sasaki et al. 2010) from *Arabidopsis thaliana*, *BnALMT1*and *BnALMT2* from *B. napus*, *ZmALMT1* from *Zea mays* (Ligaba et al. 2006), *HvALMT1* from *Hordeum vulgare* (Gruber et al. 2010). Some of these genes (*AtALMT1*, *BnALMT1*and *BnALMT2*) confer Al^{3+} resistant in a similar manner to *TaALMT1*, whereas others have different functions (*AtALMT9*, *AtALMT12*, *ZmALMT1* and *HvALMT1*). The former three encode the Al-activated root malic acid efflux transporter associated with Al tolerance. The latter four encode transporters that are implicated in the transport of anions, but do not confer the major Al^{3+} resistance. Although the functions of these genes had been studied intensively, the details of how Al^{3+} activates *ALMT1* protein are still not very clear.

It has been speculated that Al activated the opening of anion channel in three ways (Delhaize and Ryan 1995). (1) A1 binds to a membrane-bound receptor and activates the protein indirectly via a series of secondary signals. (2) Al binds to the membrane surface which is required for the simulation of malic acid efflux. The binding site of Al might be attached to an anion-channel protein, which could affect its configuration and alter its gating behavior in the Al-tolerant seedlings. (3) Al enters the cytoplasm and alters channel activity either directly by binding with the channel or indirectly through a signal transduction pathway.

As a signal hormone in higher plants, IAA regulates the growth and development of plants, and has a crucial function in stress resistance. For example, when it was applied together with $HgCl_2$, it caused less inhibition of the internodes diameter than sponge loofah *Luffa cylindrica* L. (*Cucurbitaceace*) treated with $HgCl_2$ alone (Khan and Chaudhry 2006). Besides, Ouzounidou and Ilias (2005) reported that IAA could reduce the toxic effect of Cu^{2+} in sunflower (*Helianthus annuus* L.) roots, leading to greater root length and root hair formation. It also has been reported that IAA regulates the activity of plant cell anion channel.

Marten et al. (1991) demonstrated that IAA could interact directly with the extracellular face of the anion channel in guard cells to elicit stomata opening. IAA could induce a time- and concentration-dependent negative shift of the activation potential of the whole-cell anion current of tobacco protoplasts from cell-suspension cultures (Sabine et al. 1994).

However, the effect of IAA on malic acid efflux from wheat to whether it is realized via the anion channel are rather under researched. This study was designed to determine the role of IAA in malic acid efflux under Al stress.

Materials and methods

Plant materials and culture conditions

Seeds of wheat (*Triticum aestivum* L.) lines ET8 (Al-tolerant) were provided by Faculty of Agriculture, Kagawa University. The seeds were immersed in 1% (v/v) sodium hypochlorite for 15 min for the purpose of surface-sterilization, rinsed several times with deionized water, and then soaked for about 12 h before germination on a layer of moistened filter-paper at 25°C for 24 h in darkness. The germinated seeds were then transferred onto a net made of cotton floating on 0.5 mM $CaCl_2$, pH 4.5 in a 2 L plastic container; the solution was renewed daily. After 4 days, some of the seedlings of uniform length were selected for experiments, while others were trans-planted into plastic pots (nine seedlings per pot) containing aerated one-fifth-strength Hoagland solution. This nutrient solution contained macronutrients (mM) including: KNO_3 (1.0), $Ca(NO_3)_2$ (1.0), $MgSO_4$ (0.4) and $(NH_4)H_2PO_4$ (0.2), and micro-nutrients (μM) including: Fe-EDTA (20), H_3BO_3 (3), $MnCl_2$ (0.5), $CuSO_4$ (0.2), $ZnSO_4$ (0.4) and $(NH_4)_6Mo_7O_{24}$ (1). The solution was adjusted to pH 4.5 with 0.2 M HCl and renewed every other day. After 19 days, seedlings of uniform length were selected and exposed to 0.5 mM $CaCl_2$, pH 4.5 over night. These seedlings were used for experiments on the 20th day. All experiments were done in an environmentally controlled growth room with a 24 h cycle of 14 h at 25°C in light/10 h at 22°C in darkness, a photon flux density of 150 μmol photon m^{-2} s^{-1} (photosynthetic active radiation) at the plant-canopy level, and a relative air humidity of 70%.

Treatments

This study has adopted 12 types of treatments, which all contained 0.5 mM $CaCl_2$ treatment as the control (CK) treatment. All treatments started by exposing the seedlings for 24 h to 0.5 mM $CaCl_2$, pH 4.5 containing other chemicals, which differed among treatments. All the solution was collected for the measurement of malic acid. Ten 4-day-old and three 20-day-old seedlings were selected for the treatment. Each experiment was conducted three times.

Al treatments

Treatment 1 contains: CK, 25, 50 or 100 μM Al ($AlCl_3 \cdot 6H_2O$, *Alfa Aesar*, Lancaster). At sampling time, roots were briefly rinsed with deionized water and then three of the longest root apexes (0–10 mm) of each seedling were excised with a razor. The excised roots were stored in a freezer at −80°C for the determination of endogenous IAA content and IAA oxidase activity in wheat. (If there were no particular emphasis, all the seedlings used in these experiments were 4 days old.)

IAA treatments

Treatment 2 contains: CK; 50 μM IAA (Sigma, USA); 50 μM Al.

Treatment 3 contains: CK; 50 μM IAA; 50 μM Al (20-day-old seedlings).

Treatment 4: seedlings were treated with 50 μM Al for 3 h, and then the roots were washed with 0.5 mM $CaCl_2$ for three times; then treatment contains: CK; 50 μM IAA; 50 μM Al.

Treatment 5: seedlings were treated with CK or 50 μM IAA for 3 h, respectively. Then the roots were washed with

0.5 mM $CaCl_2$ for three times; then the roots were all treated with 50 μM Al.

Treatment 6 contains: CK; 50 μM IAA and 50 μM Al; 50 μM Al. At sampling time, roots were briefly rinsed with deionized water and then three of the longest root apexes (0–20 mm) of each seedling were excised with a razor. The excised roots were stored in a freezer at −80°C for the determination of *TaALMT1* expression level of wheat.

Split-root experiments

Roots were split into two equal parts (part A and part B) by the root manipulation box described as Yang et al. (2001).

Treatment 7 contains: group 1, part A (CK) and part B (CK); group 2, part A (50 μM Al) and part B (50 μM Al); group 3, part A (CK) and part B (50 μM Al). Root apexes (0–10 mm) of the root group of the two parts were excised, frozen in liquid nitrogen immediately, and stored at −80°C for endogenous IAA determination.

Treatment 8 contains: group 1, part A (CK) and part B (CK); group 2, part A (CK) and part B (50 μM Al); group 3, part A (CK) and part B (50 μM IAA); group 4, part A (50 μM IAA) and part B (50 μM IAA); group 5, part A (CK) and part B (50 μM Al and 50 μM IAA).

NPA and TIBA treatments

Treatment 9 contains: CK; 50 μM IAA; 50 μM IAA and 50 μM Al; 50 μM IAA and 5 μM or 10 μM NPA (Sigma, USA) respectively; 5 or 10 μM NPA and 50 μM Al respectively; 50 μM Al.

Treatment 10 contains: CK; 50 μM IAA; 50 μM IAA and 50 μM Al; 50 μM IAA and 5 μM or 10 μM TIBA (Sigma, USA) respectively; 5 or 10 μM TIBA and 50 μM Al respectively; 50 μM Al.

A9C and NIF treatments

Treatment 11 contains: CK; 50 μM Al and 5 μM A9C (Sigma, USA); 50 μM Al, 5 μM A9C and 50 μM IAA; 50 μM Al and 50 μM IAA; 50 μM Al.

Treatment 12 contains: CK; 50 μM Al and 5 μM NIF (Sigma, USA); 50 μM Al, 5 μM NIF and 50 μM IAA; 50 μM Al and 50 μM IAA; 50 μM Al.

Determination of malic acid

For the measurement of malic acid, the solution was first passed through a cation-exchange column (16 mm×14 cm) filled with 5 g of Amberlite IR-120B (H^+ form) resin (Muromachi Chemical, Tokyo), and then through an anion-exchange column filled with 2 g of Dowex 1×8 resin (100–200 mesh, formate form) in a cold room. Malic acid retained on the anion-exchange resin was eluted with 2 M HCl and the eluent was concentrated in a rotary evaporator at 40°C. The residue was dissolved in deionized water and measured with enzymic methods as described Delhaize et al. (1993). 1.35 mL of sample was incubated with 1.5 mL of buffer (0.5 M Gly, 0.4 M hydrazine, pH 9.0), 0.1 mL of 40 mM NAD (Ames Co., USA). The reaction mixture was incubated for 30–60 min to obtain a stable A_{340} reading before the addition of 5 μL of malate dehydrogenase (Sigma, USA). The increase in A_{340} due to the production of NADH was measured with a spectrophotometer (Hitachi, *U-1800*, Japan) and is directly proportional to the amount of malic acid in the sample.

Determination of IAA oxidase activity

The assay for the IAA oxidase activity was carried out in weak light laboratory at 35°C following Kevers et al. (1981) with slight modifications. Root apexes were ground in 2 ml of chilled phosphate buffer (0.06 M, pH 6.0), and then centrifuged for 20 min at 4 000 g at 4°C. The supernatants were collected and IAA oxidase activity was assayed. The reaction mixtures contained 2 ml phosphate buffer (0.06 M, pH 6.0), 0.25 ml gel fraction, 0.15 ml $MnCl_2$ (1 mM), 0.3 ml DCP (1 mM), 0.3 ml IAA (4 mM). The IAA oxidase activity was estimated as ΔA_{254} nm compared with the control without enzymic fraction (Hitachi, *U-1800*, Japan). IAA oxidase activity was expressed as relative IAA oxidase activity[(IAA oxidase activity with Al treatment)/(IAA oxidase activity without Al treatment) × 100].

Determination of endogenous IAA content in wheat

Tissue samples were weighed, homogenized at 4°C, and extracted with homogenization buffer [80% (v/v) aqueous methanol +10 mg L^{-1} butylated hydroxytoluene]. Then after the methanol solution was dried under reduced pressure (Labconco Freezone, U.S.A.), its residue was dissolved in 0.5 ml of TBS-buffer for indirect enzyme-linked immunosorbent assay (ELISA) as described by Mertens et al. (1983) with monoclonal antibodies. The procedure followed manufacturers' recommendations of a commercial kit (Chinese Agriculture University, Beijing, China). Finally endogenous IAA is determined by an automatic enzyme-linked immunosorbent assay systems (Freedom EVOlyzer 150–8, Switz).

Real-time quantitative RT-PCR

Root apexes were frozen in liquid nitrogen and homogenized with a pestle and microcentrifuge tube. The *TaALMT1* expression analysis was based on the procedure previously described by Sasaki et al. (2004). Total RNA was isolated using TRIzol reagent (Invitrogen, CA, USA) to extract the RNA from 100 mg of root tissue. First-strand cDNA was synthesized from 2 μg of total RNA using the M-MLV Reverse Transcriptase (Promega, WI, USA) according to the manufacturer's instructions. The reaction mixture was incubated at 42°C for 50 min and the reaction was stopped by heating at 70°C for 15 min. The final reaction volume was diluted to 40 μl and aliquots of 2 μl were subsequently added to 20 μl of PCR solution. Quantitative PCR was performed on the CFX96™ Real-Time PCR Detection System (Bio-Rad, CA, USA), with the SYBR Green Realtime PCR Master Mix kit (TOYOBO, Japan) for amplification of the *TaALMT1* or Actin according to the manufacturer's protocol. The primers 5′-AAGAGCGTCCDTTAATTCG-3′ and 5′-CCTTA CATGATAGCTCAGGG-3′ were used for amplification of *TaALMT1* transcript (Sasaki et al. 2004); 5′-GAATCCATGAGACCACCTAC-3′ and 5′-AATC CAGACACTGTACTTCC-3′ were used for amplification of Actin gene in wheat (Paolacci et al. 2009). Reaction condition for thermal cycling were: 10 min at 95°C, followed by 40 cycles of 10 s at 95°C, 10 s at 55°C, and 10 s at 72°C. For each gene, a standard curve was prepared using a serial dilution of the reverse-transcribed cDNA sample. To account for differences in total RNA present in each sample, the amount of *TaALMT1* mRNA was normalized to the amount of Actin detected in the same sample.

Statistical analysis

Data were pooled and subjected to one-way analysis of variance (ANOVA) followed by Turkey tests. $P \leq 0.05$ was set as the level of statistical significance. DPS v7.05 and OrigenPro7.5 software were used for computation, data analysis and graphics.

Results

Effect of Al on endogenous IAA content and IAA oxidase activity in wheat roots

Aluminum treatment stimulated the efflux of malic acid from the root of ET8 in a dose-depend manner (Supplemental Fig. S1). The roots showed severe visible Al-induced damage at concentrations above 50 μM Al, and so in all further experiments 50 μM Al was used. The efflux of malic acid was stimulated by 50 μM Al in each treatment, which was significantly higher than that of the control treatment.

Endogenous IAA content was examined after Al treatment. The endogenous IAA content increased with the increasing of Al concentrations (25, 50, 100 μM),

which were 23.8, 28.5, 31.0 and 34.4 ng g^{-1} FW, respectively (Fig. 1A, treatment 1). A positive (R^2= 0.9859**) correlation was found between all the data of malic acid efflux rate and endogenous IAA content (Fig. 1B), which indicated that IAA might be involved in the Al-induced efflux of malic acid.

With the increase of Al concentrations, relative activity of IAA oxidase decreased significantly (Fig. 2, treatment 1) from 79.5% to 53.8%, consistent with the increase of endogenous IAA content (Fig. 1A). Results indicated that Al-induced decreasing of IAA oxidase activity reduced the catabolic metabolism of IAA.

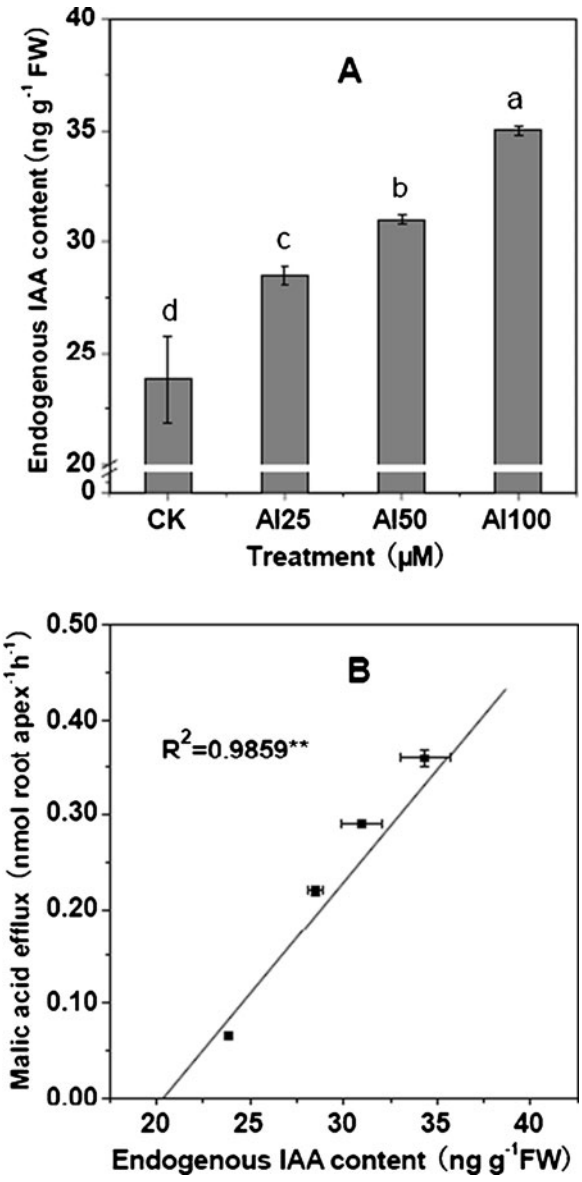

Fig. 1 Effect of Al treatments on endogenous IAA content (**A**) and the correlation between all the data of malic acid efflux and endogenous IAA content in wheat roots (**B**). Four-day-old wheat seedlings were exposed to 0.5 mM $CaCl_2$ solution (pH 4.5) containing 0, 25, 50, or 50 μM $AlCl_3$ for 24 h. Root apexes were excised (0–10 mm) after treatment for the determination of endogenous IAA content. Data are means ± SD (n=3). *Small letter* differences in the same figure mean significant difference at P<0.05

Effect of IAA on malic acid efflux

The above section showed the efflux of malic acid increased with the content of endogenous IAA under Al treatment, but it remained unknown whether this applied to exogenous IAA as well. So the possible effect of exogenous IAA on malic acid efflux was also examined. After being treated with IAA (Fig. 3A, treatment 2) the malic acid efflux of 4-day-old seedlings did not reveal significant difference compared with the control treatment, which might result from the limited absorption capacity of IAA of the 4-day-old seedlings. Therefore, the malic acid efflux rate of 20-day-old seedlings was examined. It was found that the rate was nearly the same with the control treatment after being treated with 50 μM IAA (Fig. 3B, treatment 3). So neither for the 4-day-old seedlings nor for the 20-day-old seedlings, the treatment of IAA alone could secrete

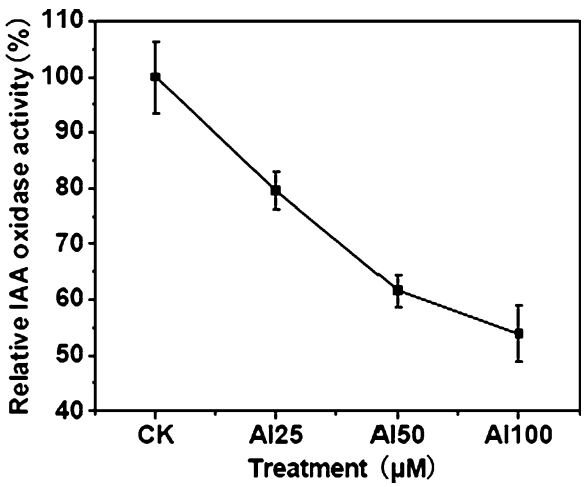

Fig. 2 Effect of Al treatments on the activity changes of IAA oxidase. Four-day-old wheat seedlings were exposed to 0.5 mM $CaCl_2$ solution (pH 4.5) containing 0, 25, 50, or 50 μM $AlCl_3$ for 24 h. Root apexes were excised (0–10 mm) after treatment for the determination of IAA oxidase activity. Data are means ± SD (n=3)

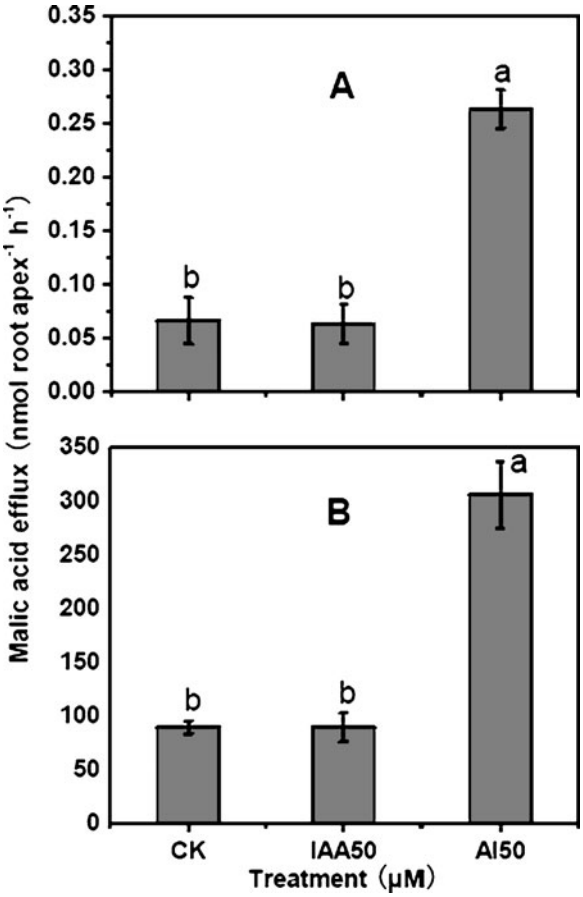

Fig. 3 Effect of IAA treatments on malic acid efflux from 4-day-old wheat seedlings (**A**) or 20-day-old wheat seedlings (**B**). Seedlings were exposed to 0.5 mM $CaCl_2$ solution (pH 4.5) containing 0, 50 μM $AlCl_3$ or 50 μM IAA for 24 h. Data are means ± SD (n=3). *Small letter* differences in the same figure mean significant difference at $P<0.05$

malic acid. It also showed that IAA treatment at current concentration could keep the integrity of plasma membrane without causing the leakage of malic acid.

In treatment 4, seedlings were pretreated with 50 μM Al for 3 h, and then were treated with 50 μM IAA under Al-free conditions. The seedlings under such treatment also did not show a significant malic acid efflux difference from the control treatment (Fig. 4A, treatment 4).

In treatment 5, Seedlings were pretreated with 50 μM IAA for 3 h, then exposed to 50 μM Al for 24 h. It was found that the malic acid efflux rate of the seedlings under IAA pretreatment was 1.23 times higher than that of the seedlings without the IAA pretreatment but with exposure to Al (Fig. 4B, treatment 5).

In treatment 6, co-treatments of Al and IAA were carried out. Compared with 50 μM Al treatment alone (0.27 nmol root $apex^{-1}$ h^{-1}), the efflux of malic acid was stimulated significantly higher under the treatment of 50 μM Al and 50 IAA (0.39 nmol root $apex^{-1}$ h^{-1}) together (Fig. 5, treatment 6), which indicated that IAA was involved in enhancing malic acid efflux under Al stress.

In addition, the effects of ABA and GA on the efflux of malic acid were also investigated. It was found that ABA or GA had no significant effect on the malic acid secretion in the presence or absence of Al (supplemental Fig. S2, S3, S4).

Split-root experiments

Split-root experiments were carried out in order to find out whether IAA was involved in the Al-induced malic acid efflux, in which the roots of single wheat seedling were divided into part A and part B for different treatment.

Malic acid secreted from both part A to part B was detected (Fig. 6A, treatment 7). When part A and part B were both treated with control (group 1, CK/CK), the malic acid efflux rate of part A and part B were 0.06 and 0.05 nmol root $apex^{-1}$ h^{-1}, respectively, which were consistent with those in whole root control treatment (Supplemental Fig. S1). When part A and part B were both treated with Al (group 2, Al/Al), the malic acid efflux rate of part A and part B were 0.26 and 0.25 nmol root $apex^{-1}$ h^{-1}, respectively, which were consistent with those in whole root Al treatment (Supplemental Fig. S1). When part A and part B were treated with control and Al (group 3, CK/Al), the malic acid efflux rate of part A and part B were 0.15 and 0.26 nmol root $apex^{-1}$ h^{-1}, respectively. The malic acid efflux rate of part A was significantly lower than that of part B, but was significantly higher than that of group 1 (CK/CK). Malic acid efflux rate in part B was consistent with group 2. Results showed that the Al treatment of one half of the root (part B) increased the efflux of malic acid of other half (part A) though it was not treated with Al, which suggested that malic acid efflux regulating signal (s) might be transferred from part B, to involved in the efflux of malic acid in part A.

Then endogenous IAA content was detected (Fig. 6B, treatment 7). When part A and part B were both treated with control (group 1, CK/CK), endog-

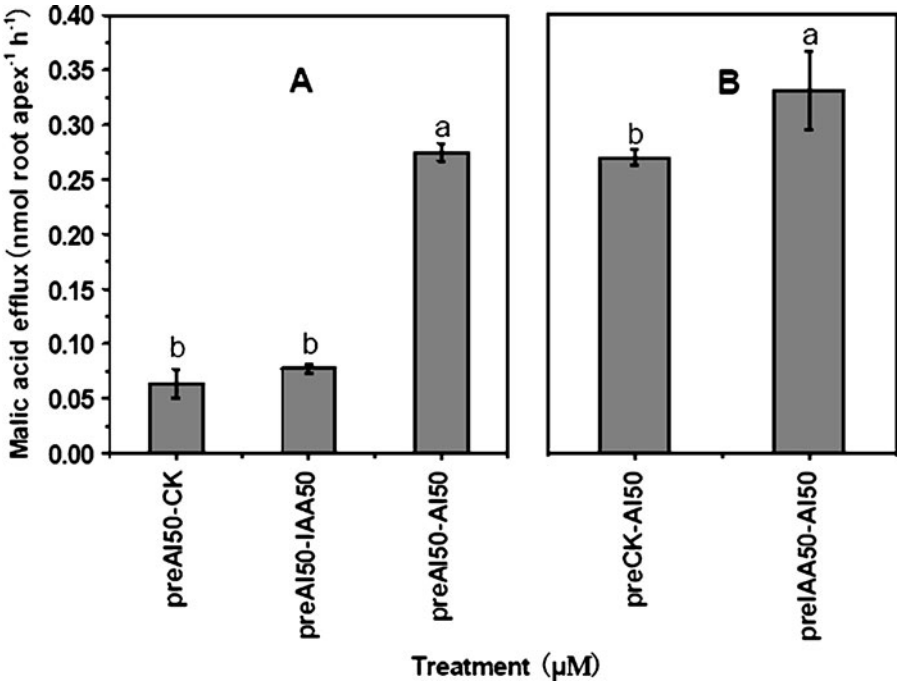

Fig. 4 Effect of IAA treatments on malic acid efflux. Four-day-old wheat seedlings were exposed to 0.5 mM $CaCl_2$ solution (pH 4.5) containing 50 μM $AlCl_3$ for 3 h, and then the roots were washed with 0.5 mM $CaCl_2$ for three times. Then seedlings were exposed to 0.5 mM $CaCl_2$ solution (pH 4.5) containing 0, 50 μM $AlCl_3$, or 50 μM IAA for 24 h (**A**). Four-day-old wheat seedlings were exposed to 0.5 mM $CaCl_2$ solution (pH 4.5) containing 0 or 50 μM IAA for 3 h, and then the roots were washed with 0.5 mM $CaCl_2$ for three times. Then seedlings were exposed to 0.5 mM $CaCl_2$ solution (pH 4.5) containing 50 μM $AlCl_3$ for 24 h (**B**). Data are means ± SD (n=3). *Small letter* differences in the same figure mean significant difference at P<0.05

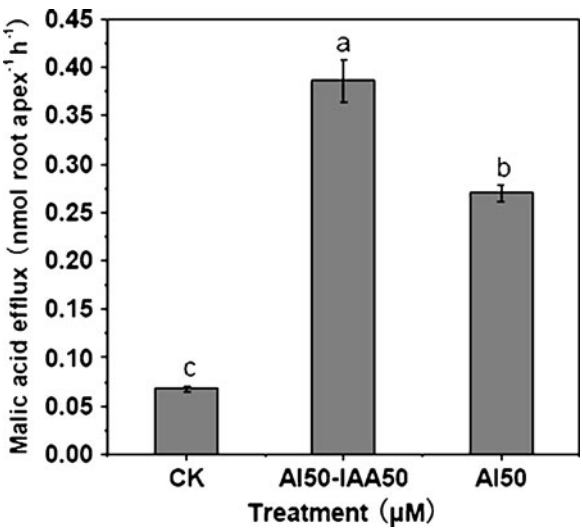

Fig. 5 Effect of Al and IAA co-treatments on malic acid efflux. Four-day-old wheat seedlings were exposed to 0.5 mM $CaCl_2$ solution (pH 4.5) containing 0, 50 μM $AlCl_3$, or 50 μM $AlCl_3$ and 50 μM IAA for 24 h. *Small letter* differences in the same figure mean significant difference at P<0.05

enous IAA content in part A and part B were 23.3 and 23.7 ng·g^{-1} FW, respectively, which were consistent with those in whole root control treatment (Fig. 1A). When part A and part B were both treated with Al (group 2, Al/Al), endogenous IAA content in part A and part B were 31.9 and 31.1 ng·g^{-1} FW, respectively, which were consistent with those in whole root Al treatment (Fig. 1A). When part A and part B were treated with control and Al (group 3, CK/Al), endogenous IAA content in part A and part B were 26.3 and 31.8 ng·g^{-1} FW, respectively. Endogenous IAA content in part A was significantly lower than that of part B, but was significantly higher than that of group 1 (CK/CK). Endogenous IAA content in part B was consistent with group 2 (Al/Al). Results in the above split-root experiments showed that the Al treatment of one half of the root (part B) increased the endogenous IAA content of other half (part A) though it was not treated with Al, which suggested IAA signal might involve in the Al-induced efflux of malic acid.

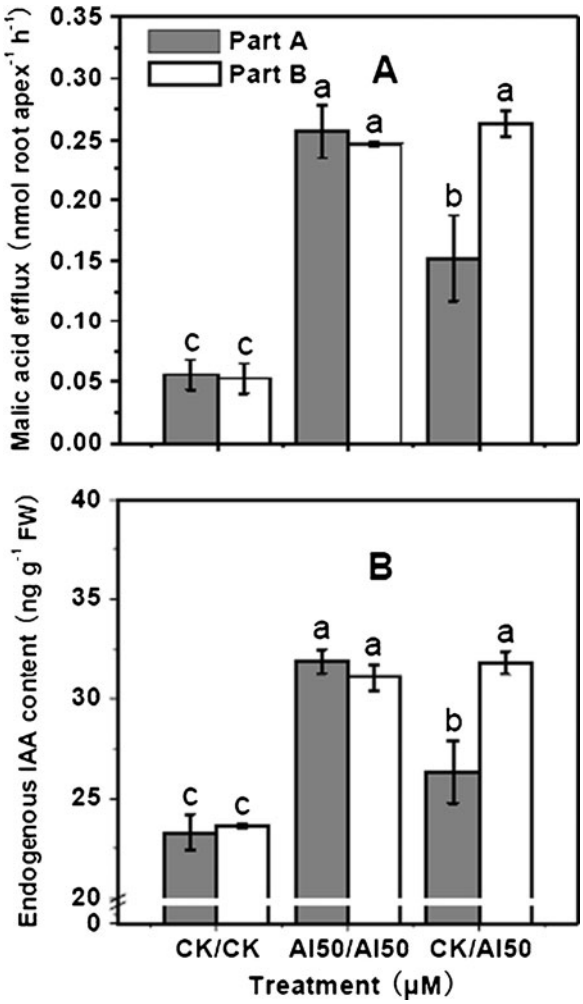

Fig. 6 Effect of Al treatments on malic acid efflux (**A**) and endogenous IAA content (**B**) from 4-day-old wheat seedlings in split-root experiment. Roots were split into two equal parts (part A and part B) using the *root manipulation box*. The split roots were exposed to 0.5 mM $CaCl_2$ solution (pH 4.5) containing other chemicals: group 1, part A (CK) and part B (CK); group 2, part A (50 μM $AlCl_3$) and part B (50 μM $AlCl_3$); group 3, part A (CK) and part B (50 μM $AlCl_3$) for 24 h. Root apexes (0–10 mm) were excised after treatment for endogenous IAA content determination. Data are means ± SD (n=3). *Small letter* differences in the same figure mean significant difference at P<0.05

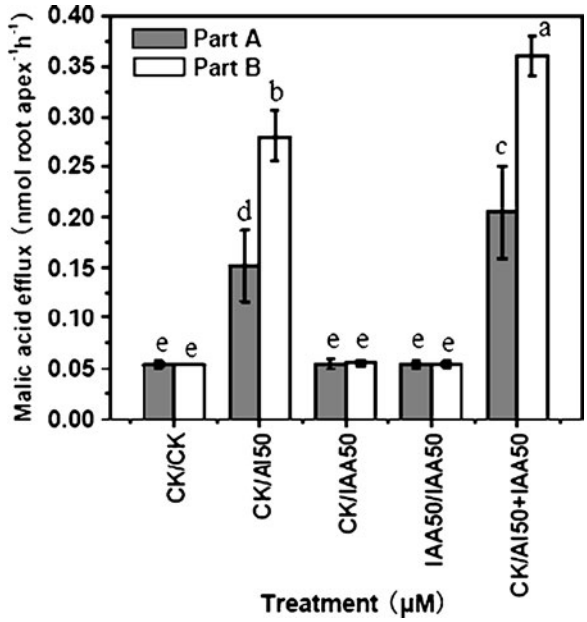

Fig. 7 Effect of IAA treatments on malic acid efflux from 4-day-old wheat seedlings in split-root experiment. Roots were split into two equal parts (part A and part B) using the *root manipulation box*. The split roots were exposed to 0.5 mM $CaCl_2$ solution (pH 4.5) containing other chemicals: group 1, part A (CK) and part B (CK); group 2, part A (CK) and part B (50 μM $AlCl_3$); group 3, part A (CK) and part B (50 μM IAA); group 4, part A (50 μM IAA) and part B (50 μM IAA); group 5, part A (CK) and part B (50 μM $AlCl_3$ and 50 μM IAA) for 24 h. Data are means ± SD (n=3). *Small letter* differences in the same figure mean significant difference at P<0.05

For the purpose of further investigating the effects of IAA on malic acid efflux, another split-root experiment was conducted (Fig. 7, treatment 8). When part A and part B were treated with control and IAA, and IAA and IAA (group 3, CK/IAA; group 4, IAA/IAA), respectively, the malic acid efflux rate of part A and part B in both groups were the same, which were consistent with the rate of part A and part B under treatment of control and control (group 1, CK/CK). When part A and part B were treated with control and Al + IAA (group 5, CK/Al + IAA), respectively, the malic acid secretion in part A was 1.35 times higher than the part A when its part B was treated with Al (CK/Al). The malic acid efflux in part B was consistent with those in whole root experiments (Fig. 5). Results above indicated that in split-root experiment (CK/Al + IAA), exogenous IAA treatment under Al stress in part B of the root system enhanced the efflux of malic acid in part A, which confirmed that IAA signal was involved in the Al-induced malic acid efflux.

Effect of NPA or TIBA on Al-induced malic acid efflux

The transport of IAA can be inhibited by treatment of IAA transport inhibitor NPA or TIBA. The efflux of

malic acid under the treatment with 5 μM or 10 μM NPA (or TIBA) and 50 μM IAA was not different from the control treatment, which indicated that the co-treatment of NPA (or TIBA) and IAA could not induce the efflux of malic acid. Compared with 50 μM Al treatment alone, the malic acid efflux rate of 5 μM (or 10 μM) NPA and 50 μM Al treatment decreased by 33.6% (or 45.0%) (Fig. 8A, treatment 9). The malic acid efflux rate decreased by 43.6% (or 53.2%) after treatment with 5 μM (or 10 μM) TIBA and 50 μM Al (Fig. 8B, treatment 10). These results indicated that the efflux of malic acid induced by Al was inhibited significantly with increasing concentrations of NPA or TIBA. The involvement of IAA in the Al-induced efflux of malic acid was further confirmed.

Effect of anion channel inhibitors (A9C or NIF) on IAA participation of Al-induced malic acid efflux

The effect of anion channel inhibitors A9C and NIF on IAA participation of Al-induced malic acid efflux was shown in Fig. 9A and B. The malic acid efflux rate was 0.17 nmol root apex^{-1} h^{-1} under the co-treatment of 5 μM A9C and 50 μM Al, which was significantly lower than that of 50 μM Al treatment alone (0.26 nmol root apex^{-1} h^{-1}, Fig. 9A, treatment 11). Compared with the co-treatment of Al and A9C, the application of IAA (50 μM Al, 5 μM A9C and 50 μM IAA) significantly induced the malic acid efflux. The malic acid efflux rate was 0.23 nmol root apex^{-1} h^{-1}, which was 1.37 times higher than that of the co-treatment of A9C and Al.

The malic acid efflux rate was 0.14 nmol root apex^{-1} h^{-1} under the co-treatment of 5 μM NIF and 50 μM Al, which was significantly lower than that of 50 μM Al treatment alone (0.25 nmol root apex^{-1} h^{-1}, Fig. 9B, treatment 12). Compared with the co-treatment of Al and NIF, the application of IAA (50 μM Al, 5 μM NIF and 50 μM IAA) significantly induced the efflux of malic acid. The malic acid efflux rate was 0.22 nmol root apex^{-1} h^{-1}, which was 1.58 times higher than that of the co-treatment of NIF and Al.

These results suggested that by acting on anion channel IAA signal was involved in the Al-induced efflux of malic acid.

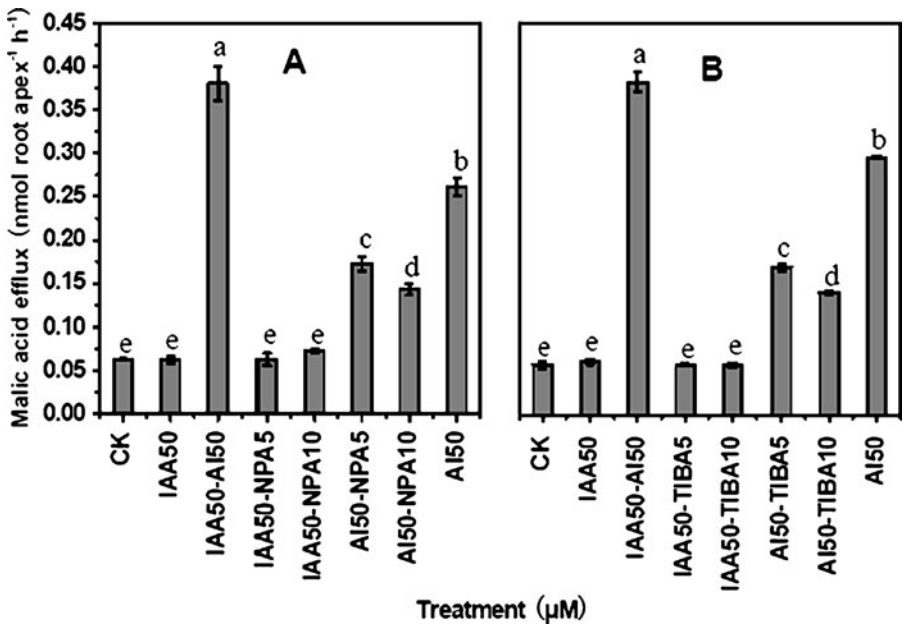

Fig. 8 Effect of IAA transport inhibitors on malic acid efflux from 4-day-old wheat seedlings. Seedlings were exposed to 0.5 mM $CaCl_2$ solution (pH 4.5) containing other chemicals: 0 μM $AlCl_3$, 50 μM IAA, 50 μM IAA and 50 μM $AlCl_3$, 50 μM IAA and 5 (10) μM NPA, 5 (10) μM NPA and 50 μM $AlCl_3$, or 50 μM $AlCl_3$ for 24 h (**A**); 0 μM $AlCl_3$, 50 μM IAA, 50 μM IAA and 50 μM $AlCl_3$, 50 μM IAA and 5 (10) μM TIBA, 5 (10) μM TIBA and 50 μM $AlCl_3$, 50 μM $AlCl_3$ for 24 h (**B**). Data are means ± SD (n=3). *Small letter* differences in the same figure mean significant difference at P<0.05

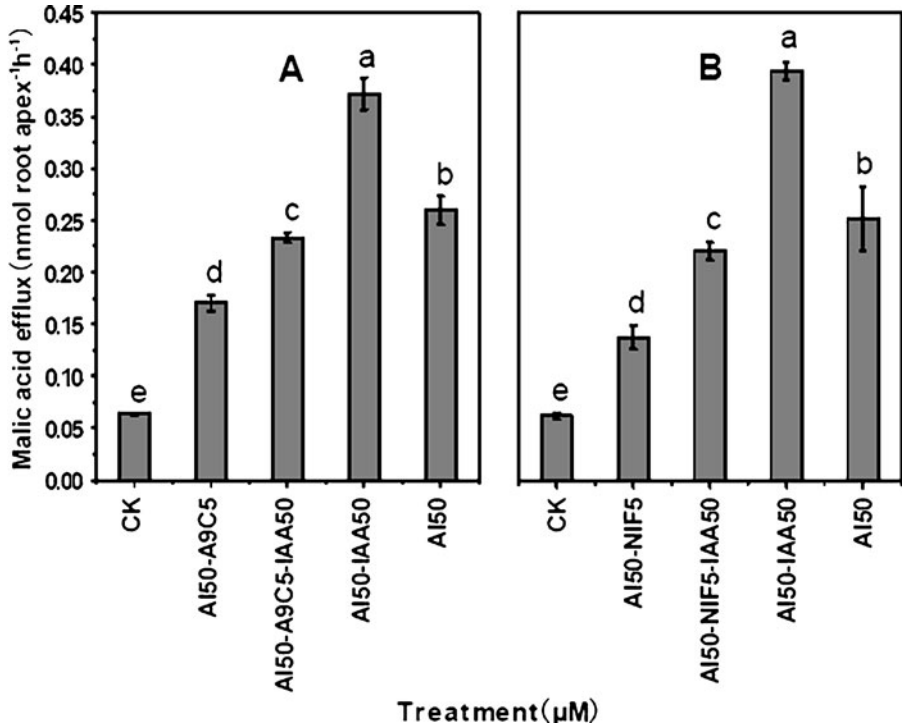

Fig. 9 Effect of anion channel inhibitors A9C (**A**) and NIF (**B**) on IAA stimulation of Al-induced malic acid efflux from 4-day-old wheat seedlings. Seedlings were exposed to 0.5 mM $CaCl_2$ solution (pH 4.5) containing other chemicals: 0 μM $AlCl_3$, 50 μM $AlCl_3$ and 5 μM A9C, 50 μM $AlCl_3$ and 5 μM A9C and 50 μM IAA, 50 μM $AlCl_3$ and 50 μM IAA, 50 μM $AlCl_3$ for 24 h; 0 μM $AlCl_3$, 50 μM $AlCl_3$ and 5 μM NIF, 50 μM $AlCl_3$ and 5 μM NIF and 50 μM IAA, 50 μM $AlCl_3$ and 50 μM IAA, 50 μM $AlCl_3$ for 24 h. Data are means ± SD (n= 3). *Small letter* differences in the same figure mean significant difference at $P<0.05$

Expression of the *TaALMT1* gene

Analysis of root apex by real-time quantitative RT-PCR verified *TaALMT1* expression in ET8. There were no significant differences of *TaALMT1* expression level between control treatment and Al treatment, but the expression level in the co-treatment of IAA and Al was much higher than that of Al or control treatment (Fig. 10, treatment 6). Result indicated that application of exogenous IAA under Al stress could improve the expression of *TaALMT1*.

Discussion

Extensive studies have found that many Al-resistant species and cultivars of plants respond to Al stress by expressing specific organic acid anions (citrate, oxalate and malic acid) from the roots which can bind Al strongly, thereby limiting the toxicity (Ma and Furukawa 2003). In this study, malic acid was induced and increased with the increasing concentrations of Al (Supplemental Fig. S1), which was consistent with Osawa and Matsumoto (2002).

Phytohormones such as ABA, GA and IAA are important signal substances that participate extensively in plant resistance to environmental stresses. Exogenous application of ABA could enhance Al-induced citrate secretion in soybean (Shen et al. 2004), oxalate secretion from buckwheat in Al free condition (Ma et al. 2001), and ameliorate Al-induced root elongation inhibition in soybean (Shen et al. 2004). By seed soaking of GA, mercury-induced hypocotyls growth inhibition and decline of fresh weight of *Phaseolus vulgaris* could be alleviated (Mor et al. 2002). However, this study found that ABA or GA just slightly influenced or had no effect on the malic acid efflux in the presence or absence of Al (supplemental Fig. S2, S3, S4), which were consistent with the results of Ryan et al. (2003) and Shen et al. (2004).

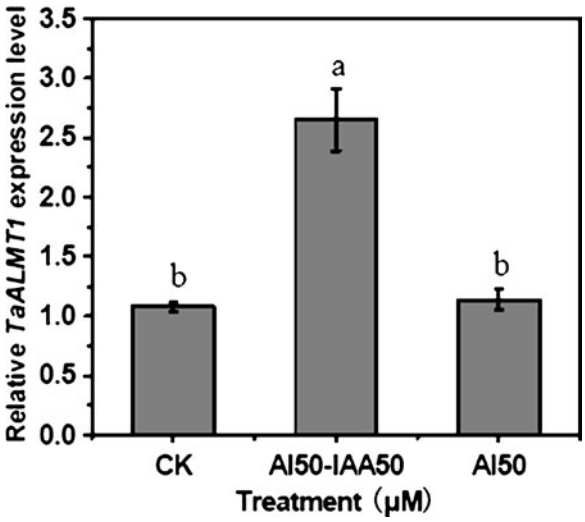

Fig. 10 Expression of *TaALMT1* in wheat. Real time quantitative RT-PCR was used to assess *TaALMT1* expression in root apexes (0–20 mm) of wheat lines ET8. Four-day-old wheat seedlings were exposed to 0.5 mM $CaCl_2$ solution (pH 4.5) containing 0, 50 μM $AlCl_3$, or 50 μM $AlCl_3$ and 50 μM IAA for 24 h. Data are means ± SD (n=3). *Small letter* differences in the same figure mean significant difference at $P<0.05$

It had been suggested that the salt-tolerance of wheat can be improved significantly by soaking seeds in IAA (Iqbal and Ashraf 2007). When $HgCl_2$ was applied with IAA, there was less inhibition of the diameter of internodes in *Luffa cylindrica* L. (Cucurbitaceace), compared with those treated with $HgCl_2$ alone, revealing the dominant role of IAA (Khan and Chaudhry 2006). Ouzounidou and Ilias (2005) found that treatment with 100 μM IAA lessened the toxic effects of 80 μM Cu in roots, as reflected in greater root length and root hair formation of sunflower, and improved stability of the light-harvesting complex photosystem 2 reaction centers. *Medicago truncatula* showed an increased production of IAA and an increased tolerance to several stress conditions (55°C, 4°C, UV-irradiation, 0.5 M NaCl, and pH 3) when nodulated by an indole-3-acetic acid-overproducing *Sinorhizobium meliloti* strain (Bianco and Defez 2009). Our study found that increased endogenous IAA content (Fig. 1A) and malic acid efflux (Supplemental Fig. S1) were promoted by Al treatment, and there was a good correlation between malic acid efflux rate and endogenous IAA content (R^2=0.9859**) (Fig. 1B). It suggested that IAA signal might be involved in the Al-induced efflux of malic acid signal transduction path way of wheat roots.

At cellular level, IAA oxidase participates in IAA catabolism by oxidative decarboxylation controlling the endogenous IAA content. The degradation of IAA could be reduced by inhibiting the activity of IAA oxidase (Xu et al. 2010). In this study, we found that Al treatment reduced the activity of IAA oxidase (Fig. 2), which helped the improvement of IAA level in wheat roots (Fig. 1A).

Since the efflux of malic acid increased with the endogenous IAA content under Al treatment (Fig. 1B), possible effect of exogenous IAA on malic acid efflux was identified. A significant increase of malic acid efflux induced by Al after the pretreatment of IAA was observed (Fig. 4B), and an obvious increase of malic acid efflux was also detected under the co-treatment of IAA and Al (Fig. 5). These suggested that Al-induced efflux of malic acid signal was amplified by the pre- or co-treatment of exogenous IAA, and thus intensify the physiological response, which further confirmed that IAA was involved in the Al-induced efflux of malic acid.

Long-distance communication by the transmission of chemical signals between roots and shoots are very common in plants. It was well known that nitrogen status signal generated in shoot can lead to the alterations of root physiology, activation of nitrate uptake (Forde 2002). Higher amounts of IAA in *M. xiaojinensis* were transported from the stem apex into roots under Fe deficiency stress, which resulted in a great enhancement of the root IAA content, being 4–5 times higher at Fe deficiency than at a normal Fe level (Han et al. 2005). Al caused a transport of ABA from the half of a root system in Al free condition to the half of the root system with Al treatment of soybean to adapt to Al stress (Hou et al. 2010). In present experiment (treatment 7, group 3, CK/Al), significant efflux of malic acid was detected in the exudates (Fig. 6A) and endogenous IAA content (Fig. 6B) increased obviously in the root apexes of the half root system treated with control when the other half of the root system was exposed to Al. We could exclude the possibility that the increased malic acid efflux and endogenous IAA content in part A (CK) was induced by Al, which transferred from part B (Al), for our results showed that Al could not be transported from Al treatment portion (part B) to the control treatment portion (part A) (Supplemental Fig. S5). So the polar

transportation of IAA to part A was enhanced probably by Al signal from part B, to IAA signal transport involved in the process of Al-induced efflux of malic acid was reflected by the enhanced release of malic acid in part A. In treatment 8, malic acid efflux rate of part A in group 2 (CK/Al) was significantly lower than the corresponding part A in group 5 (CK/Al + IAA) (Fig. 7), which was caused by the additional application of IAA in part B of group 5. This can be another evidence that IAA involved in the stimulation of Al-induced efflux of malic acid.

IAA is transported from auxin-synthesizing shoot tissues via the phloem toward the root apical meristems, where it is thought to be unloaded from the central stele into cortical to epidermal cells, and then translocated basipetally to the elongation zone (Estelle 1998; Hasenstein and Evans 1988). NPA and TIBA can cause the accumulation of IAA in the merismatic zone and insufficient accumulation in the elongation zone (Kollmeier et al. 2000). Our experiments showed that NAP or TIBA treatment inhibited the efflux of malic acid (Fig. 8), which provided evidence that IAA was involved in the stimulation of malic acid efflux under Al stress.

However, although IAA signal was found to be involved in Al-induced malic acid efflux signal path way, exogenous IAA treatment alone (Figs. 3, 4A and 7 groups 3 and 4) could not simulate the efflux of malic acid. In other words, the presence of Al was a prerequisite of exogenous IAA for enhancing the efflux of malic acid from wheat roots. Studies of Ryan et al. (1995) and Kobayashi et al. (2007) also discovered that malic acid efflux from wheat or Arabidopsis decreased rapidly after Al was removed from the external solution, which suggests that the efflux of malic acid needs the continuous activation of Al in roots. So IAA signal might not be able to activate the malic acid efflux path way in Al-free condition, but could involve in the stimulation of this path way when Al was added simultaneously. A possible explanation is that an unknown signal (s) prior/simultaneously with IAA signal might be stimulated by Al. Then the efflux of malic acid was achieved through the coordinate regulation of this unknown signal (s) and IAA signal. It is possible that this unknown signal (s) also could be transported in plant. In split-root experiment (Fig. 6A, treatment 7, group 3, CK/Al), the unknown signal (s) simulated by Al in part B was transported to part A, then induced the efflux of malic acid with IAA signal in part A. In Al free whole (Fig. 3, treatment 2 and 3; Fig. 4A, treatment 4)/split root experiment (Fig. 7, treatment 8, group 3 and 4) due to the absence of this unknown Al activated signal (s), the efflux of malic acid could not be induced even exogenous IAA could amplify the IAA signal.

In recent years, studies with patch-clamp and anion channel inhibitors have given some insight into the role of anion channel in organic acid secretion under Al stress. Al could trigger the opening of organic acid channel in the plasma membrane of plant roots, which facilitates the efflux of organic acid into the rizosphere (Ma and Furukawa 2003). Application of the patch-clamp technique to protoplasts isolated from maize apical root cortex in the distal part of the transition zone (DTZ) with incubation of intact roots with 90 μM Al for 1 h induced a citrate and malate-permeable, large conductance anion channel in 80% of the protoplasts from Al resistant cultivar, but only 30% from sensitive cultivar (Kollmeier et al. 2001). It was reported that the Al-induced secretion of citrate in wheat was inhibited significantly by the anion channel inhibitors NIF or A9C (Zhao et al. 2003). Our results were consistent with those reported in the literature (Fig. 9).

Delhaize and Ryan (1995) speculated that A1 may activate the channel indirectly via a series of secondary signals. Just like other phytohormones, IAA was involved in the regulation of anion channel activities, as it could interact directly with the extracellular face of the channel in guard cells to elicit stomata opening (Marten et al. 1991). Sabine et al. (1994) demonstrated that IAA induced a time- and concentration-dependent negative shift of the activation potential of the whole-cell anion current of tobacco protoplasts from cell-suspension cultures, which is in accordance with the findings for guard cell protoplasts in terms of time dependence, range of active IAA concentrations and amplitude of the shift in current peak potential (Marten et al. 1991). Anion channel inhibitors such as A9C, DIDS and IAA-94, which produced little or no stimulation of *Arabidopsis thaliana* hypocotyl elongation by themselves, were found able to counteract the inhibition and the disintegration induced by IAA specifically with various efficiencies (Thomine et al. 1997). They put forward the hypothesis that molecules such as A9C and DIDS was involved in early IAA signal trans-

duction by acting on an anion channel. IAA activated anion channels in the stomata guard cells and the hypocotyl might show similar activity in the roots. Our study indicated that Al-induced IAA signal might be involved in the efflux of malic acid by acting on anion channel, since the IAA participation of Al-induced efflux of malic acid was inhibited significantly by the application of A9C or NIF (Fig. 9).

The efflux rate of malic acid to rhizosphere depended on the number and activity of anion channel. The numbers of anion channel were probably reflected on *TaALMT1* gene expression level. The expression of *TaALMT1* gene in ET8 was constitutive and was not induced by Al (Fig. 10) (Sasaki et al. 2004). Al resistance in wheat was correlated with the Al-activated efflux of malic acid from the root apexes but poorly correlated with *TaALMT1* expression, which indicated that other factor (s) in addition to the level of *TaALMT1* expression were involved in the control of malic acid efflux (Sasaki et al. 2006). So, we deduced that the increased endogenous IAA content induced by Al improved the activities of anion channel, which then caused the increased efflux of malic acid. It seemed that the application of exogenous IAA increased the activities of anion channel, which promoted the efflux of malic acid. But, it was surprising to note that the expression level of *TaALMT1* was increased simultaneously under the co-treatment of IAA and Al (Fig. 10), which meant that the exogenous IAA participation of Al-induced efflux of malic acid might be caused by the co-action of increased activities and numbers of anion channel.

Based on the data obtained in our experiments and the results of other researches, the mechanism of IAA signal involved efflux of malic acid via anion channel under Al stress was suggested. In recent years, some researchers indicated that the involvement of protein reversible phosphorylation was implicated in the activation of *ALMT* protein. For example, Martínez-Estévez et al. (2001) reported that in vivo treatment of *Coffea* cells with $AlCl_3$ affected the activity of some protein kinases. The transient activation of a 48 kDa protein kinase and irreversible repression of a 42 kDa protein kinase were observed preceding the initiation of the malic acid efflux, and these changes were canceled by the inhibitor of protein kinases K-252a, which effectively blocked the Al-induced malic acid efflux (Osawa and Matsumoto 2001). Kobayashi et al. (2007) also showed that Al-inducible malic acid release was significantly reduced in plants pretreated with staurosporine (protein kinase inhibitors) or calyculin A (protein phosphatase inhibitors), and blocked all changes in *AtALMT1* gene expression. Their research revealed the important role of reversible phosphorylation in the transcriptional and post-translational regulation of *AtALMT1*, and it also proved the involvement of protein kinase in the activation/deactivation of the *AtALMT1* transporter. More recently, Ligaba et al. (2009) provided evidence that *TaALMT1* activity was regulated by protein kinase C-mediated phosphorylation. They also established that *TaALMT1* activity was disrupted when the serine residue at position 384 was replaced with an alanine, and concluded that the serine residue needed to be phosphorylated before Al^{3+} can activate *TaALMT1*.

The mitogen-activated protein kinase (MAPK) cascade is one of the protein kinase. MAPK-based signal transduction pathways regulate a large number of physiological processes, including environmental stimulators, such as phytohormones (Seo et al. 1999), heavy metals (Ding et al. 2009), drought (Mizoguchi et al. 1996), and Al (Arroyo-Serralta et al. 2005). Arroyo-Serralta et al. (2005) showed that addition of a toxic concentration of Al to cell suspension cultures of *Coffea arabica* L. induced the rapid and transient activation of a 58 kDa protein kinase that is a member of the MAP kinase family. Several other studies have implied MAPK pathway components in auxin signaling. Mockaitis and Howell (2000) reported that IAA treatment led to an increase in the activity of unidentified MAPKs in Arabidopsis, while extracts from auxin-treated tobacco cells also displayed enhanced MAPK activity (Mizoguchi et al. 1994). It has been suggested that a MAPK cascade was activated during the cucumber explants adventitious rooting process induced by IAA (Pagnussat et al. 2004). Pharmacological evidences showed that MAPK pathway (s) may function downstream of auxin-induced ROS in gravitropism of maize primary roots (Liu et al. 2009). Recently, Lee et al. (2009) identified an arabidopsis MAPK (MPK12) as a negative regulator of auxin signaling. They also showed that IAA activated MPK12 in vivo, and that suppression of MPK12 in transgenic plants led to the upregulation of auxin-responsive genes. These researches proved that MAPK acted downstream of IAA. So, we inferred that IAA might be involved in

the efflux of malic acid by modulation of the activity of MAPK, which is related to the activation of anion channel in wheat root cells. Research is underway in our laboratory to test the above hypothesis.

In conclusion, IAA might be involved in the stimulation of malic acid efflux from wheat by acting on anion channel under Al-stress.

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