

Exogenous indole-3-acetic acid could reduce the accumulation of aluminum in root apex of wheat (*Triticum aestivum* L.) under Al stress

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Abstract

Indole-3-acetic acid (IAA) is hormones in higher plants and participates in plant growth regulation and stress resistance including Al stress. The reduction of root apex aluminum (Al) content by exogenous IAA treatment was hypothesized to be effective in alleviating the adverse effect of high Al concentration in wheat root growth. To investigate the role of IAA in lowering root apex Al content, Al-tolerant wheat (ET8) was studied in Al solution (50 μ M), co-treated with IAA (25 μ M) and anion channel inhibitors (5 μ M NIF or A9C) or IAA transport inhibitors (5 μ M TIBA or NPA) under acidic condition for 24 h. Treatments were as follows: control (0.5 μ M CaCl₂), Al (50 μ M), Al (50 μ M) + IAA (25 μ M), Al (50 μ M) + IAA (25 μ M) + NIF (or A9C, 5 mM), Al (50 μ M) + NPA (or TIBA, 5 μ M). Al content in root apex, rhizosphere pH and PM H⁺-ATPase activity were studied. The results showed that co-treatment with IAA reduced root apex Al content by 42% compared to Al treatment. Anion channel inhibitors enhanced the accumulation of Al in root apex by 27% (or 32%) (NIF or A9C), while addition of IAA neutralized its promoting effect. The co-treatment with IAA increased rhizosphere pH by alleviating the decrease of plasma membrane H⁺-ATPase activity, while IAA transport inhibitors (NPA or TIBA) suppressed the elevation of rhizosphere pH. The current results suggested that IAA could be effective in alleviating Al toxicity through reducing Al accumulation in wheat root apex.

Keywords: Aluminum toxicity, indole-3-acetic acid, malic acid, rhizosphere pH, PM H⁺-ATPase

Abbreviations: A9C, Anthracene-9-carboxylic acid; NIF, Niflumic acid; NPA, N-1-naphthyl-phtalamic acid; TIBA, 2,3,5-triiodobenzoic acid.

1. Introduction

Indole-3-acetic acid is one of the most important signal hormones in higher plants, which not only functions as plant growth regulator but also as an essential stress resistant substance. It could relieve the inhibitory effect of HgCl_2 on internodes diameter of sponge loofah *Luffa cylindrica* L. (*Cucurbitaceae*) (Khan and Chaudhry, 2006). It also has the potential of reducing the toxic effect of Cu^{2+} in sunflower (*Helianthus annuus* L.) roots and improving the stability of the light-harvesting complex photosystem 2 reaction centers (Ouzounidou and Ilias, 2005). IAA has been reported to regulate the activity of plant cell PM H^+ -ATPase. Six hours after imposing Fe deprivation, IAA concentration increased in shoots and roots, which in turn upregulates the activity of PM H^+ -ATPase (rhizosphere pH) in root apex of cucumber (Bacacoa *et al.*, 2011). Exogenous application of IAA stimulates the activity of PM H^+ -ATPase and P uptake (Shen *et al.*, 2006).

Al mainly exists as oxide or silicate precipitates in the crust of the Earth and is usually not toxic to plants. However, as the soil pH drops below 5, the octahedral hexahydrate, $\text{Al}(\text{H}_2\text{O})_6^{3+}$, more commonly referred to as Al^{3+} , is solubilized into the soil solution. This form of Al is the most important rhizotoxic Al species to plant (Kinraide, 1991). On the other hand, there is a wide genetic variation of both intra and inter-species in Al tolerance, suggesting that Al-resistant species or cultivars possess several mechanisms for detoxifying Al. Two mechanisms are suggested for the detoxification of Al, one is the exclusion of Al from the root apex (exclusion mechanism), and the other is the tolerance of Al that enters plant (internal tolerance mechanism) (Ma and Furukawa, 2003). With regard to possible exclusion mechanisms, these could include the secretion of Al-chelating organic acid from roots (Ma and Furukawa, 2003), Al-binding by mucilage secreted from roots (Cai *et al.*, 2011) and the formation of a rhizosphere pH barrier (Wang *et al.*, 2006; Yang *et al.*, 2011a; Vidal-Bardán and Villa-Bermejo, 2012). Internal tolerant mechanisms could include Al fixation

in the cell wall (Arroyave *et al.*, 2011), complexation via organic ligands, and sequestration in the vacuole (Shen *et al.*, 2002).

The uptake of Al by plant may cause both physiological disturbance as well as the structural damage (Claudio *et al.*, 2011; Qin *et al.*, 2010). However, a simple and effective way for detoxification is to reduce Al accumulation in root apex. Our earlier results indicated that IAA could increase the efflux of malic acid under Al stress (Yang *et al.*, 2011b), but effects of IAA in root apex Al content were still waiting to be explored. As is known that exogenous IAA could enhance the efflux of malic acid and the increase of rhizosphere pH, we therefore hypothesized that exogenous IAA treatment could be effective in reducing root apex aluminum (Al) content. The present study focused on the activity of root PM H^+ -ATPase, rhizosphere pH and Al content in root apex to elucidate the possible involvement of IAA in Al-tolerance of wheat roots exposed to Al stress.

2. Materials and Methods

2.1. Plant materials and growth conditions

The seeds line ET8 (Al-resistant) of wheat (*Triticum aestivum* L.) were surface-sterilised by immersing them in 1% (v/v) sodium hypochlorite for 15 min, rinsed several times, and soaked for 12 h with deionized water, before they were subjected to germination on a layer of moistened filter-paper at 25 °C for 24 h in darkness. The germinated seeds were transferred onto a cotton net floating on 0.5 mM CaCl_2 , pH 4.5 in a 2 L plastic container for 4 days. The uniform seedlings were selected for experiments which were carried out in a growth room with a day/night temperature at 25/22 °C and 14/10 h duration, the light intensity of 150 $\mu\text{mol photon m}^{-2} \text{s}^{-1}$ at the plant-canopy level, and a relative air humidity of 70%.

2.2. Treatments

Five types of treatments were performed, which all contained 0.5 mM CaCl₂. Four of the treatments were performed with Al (50 μM), Al (50 μM) + IAA (25 μM), Al (50 μM) + IAA (25 μM) + NIF (or A9C, 5 μM), and Al (50 μM) + NPA (or TIBA, 5 μM) respectively, and treatment with 0.5 mM CaCl₂ as the control. All treatments started by exposing the seedlings for 24 h to 0.5 mM CaCl₂ (pH 4.5) containing other chemicals.

Al treatments

Treatment 1 contains: CK; 25; 50 or 100 μM Al (AlCl₃·6H₂O, Alfa Aesar, Lancaster). At sampling time, roots were briefly rinsed with distilled water and then three of the longest root apices (0-10 mm) of each seedling were excised with a razor for the determination of Al content.

IAA treatments

Treatment 2 contains: CK; 50 μM Al; 25 μM IAA (Sigma, USA) and 50 μM Al. At sampling time, rhizosphere pH was determined, then roots were briefly rinsed with distilled water and then three of the longest root apices (0-10 mm) of each seedling were excised with a razor for the determination of Al content or PM H⁺-ATPase activity.

A9C and NIF treatments

Treatment 3 (or 4) contains: CK; 50 μM Al; 50 μM Al and 5 μM A9C (or NIF) (Sigma, USA); 50 μM Al, 5 μM A9C (or NIF) and 25 μM IAA, respectively. At sampling time, roots were briefly rinsed with distilled water and then three of the longest root apices (0-10 mm) of each seedling were excised with a razor for the determination of Al content.

NPA and TIBA treatments

Treatment 5 contains: CK; 50 μM Al; 5 μM NPA (Sigma, USA) and 50 μM Al; 5 μM TIBA and 50 μM

Al (Sigma, USA). At sampling time, rhizosphere pH was determined.

2.3. Determination of rhizosphere pH and root apex Al content

Rhizosphere pH was determined by pH Meter (Mettler FE-20, Shanghai). Al contents in root apices (0-10 mm) were determined by graphite furnace atomic absorption spectrometry (GFAAS Varian GTA 120, USA) (Osawa and Matsumoto, 2001).

2.4. Preparation and determination of PM H⁺-ATPase activity

PM vesicles were prepared at 4 °C by the method of Palmgren *et al.* (1990). PM H⁺-ATPase activity was measured by the method of Johansson *et al.* (1995). The liberated Pi was measured with a spectrophotometer (Hitachi, U-1800, Japan) at 720 nm. Membrane protein content was determined by the protein-dye binding method of Bradford (1976), using bovine serum albumin as the standard. PM H⁺-ATPase activity was expressed as relative PM H⁺-ATPase activity [(PM H⁺-ATPase activity with Al treatment)/(PM H⁺-ATPase activity without Al treatment) × 100].

2.5. Statistical analysis

The experiments were done in triplicate, and data were pooled and subjected to one way analysis of variance (ANOVA) followed by Tukey-Kramer test. *P* ≤ 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.

3. Results

3.1. Effect of Al on root apex Al content

Root apex Al contents were 0.05, 0.13 and 0.25 μg root apex⁻¹ respectively, under the treatments of 25, 50 and 100 μM Al. In the present study, root apex Al

content significantly increased with the increase of Al concentrations (Figure 1). Meanwhile, severe visible Al-induced damage could be observed in the roots at the concentration above 50 μM Al, therefore, 50 μM Al was used for all further experiments.

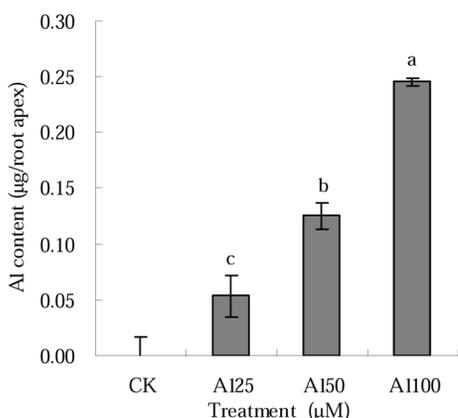


Figure 1. Al content in root apices of wheat 24 h after exposure to three different concentrations (25, 150 and 100 μM) of Al. The values are mean \pm SE (n=3).

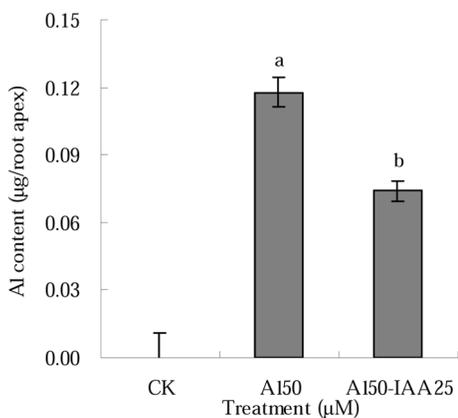


Figure 2. Effect of IAA on root apices Al content in wheat. The values are mean \pm SE (n=3).

3.2. Effect of IAA on root apex Al content

The contents of Al in root apex were 0.12 and 0.07 mg root apex⁻¹ under the treatments of 50 μM Al and 50 μM Al +25 mM IAA respectively (Figure 2). Al content in the group merely treated by Al was significantly higher than the groups co-treated by IAA with Al. It indicated that exogenous IAA could alleviate the accumulation of Al in root apex.

3.3. Effect of anion channel inhibitors on root apex Al content

In order to further confirm our speculation, anion channel inhibitor was applied in the following experiments. Al content in the root apex was 1.27 (or 1.32) times higher than the group treated merely by 50 μM Al after being treated with 5 μM A9C (or NIF) + 50 μM Al (Figure 3). The changing trend was consistent with malic acid efflux characters in Figure S3. Root apex Al content of the group treated by 5 μM A9C (or NIF) + 50 μM Al + 25 μM IAA was higher than that merely treated by 50 μM Al, but was 10% (or 14%) lower than the co-treated ones by 5 mM A9C (or NIF) + 50 M Al. This changing trend also consistent with the corresponding malic acid efflux changing trend (Figure S3).

The results described above further suggested that exogenous IAA could alleviate the Al accumulation in root apex.

3.4. Effect of IAA on rhizosphere pH

Effects of exogenous IAA on rhizosphere pH were examined to investigate the role of IAA in Al resistant comprehensively. As shown in Figure 4, although the initial rhizosphere pH of all the treatments (CK, A150, A150 + IAA25) were 4.5, obvious difference among these three treatments after 24 h could be observed. The rhizosphere pH of CK was 4.87, 4.73 of 50 μM Al and 4.96 of 25 μM IAA + 50 μM Al co-treatment. These results indicated that exogenous IAA could promote the elevation of rhizosphere pH of wheat under Al stress.

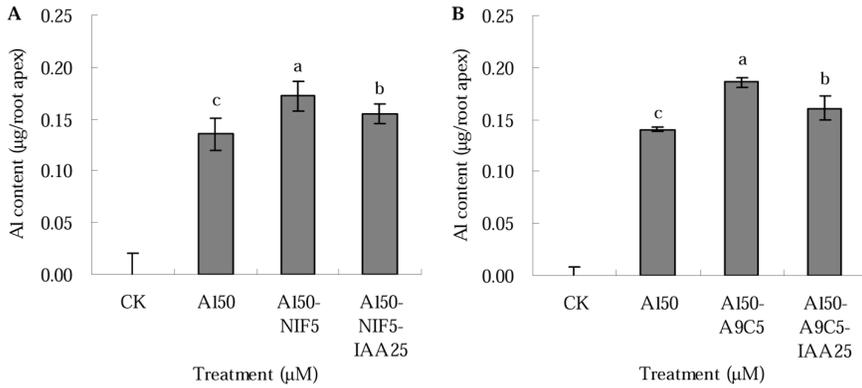


Figure 3. Effect of anion channel inhibitor (A9C, A or NIF, B) or IAA on root apices Al content of wheat. The values are mean ± SE (n=3).

In order to further confirm that IAA participated in regulating rhizosphere pH, IAA transport inhibitors NPA and TIBA were applied. The rhizosphere pH of 50 µM Al and 5 µM TIBA (or NPA) co-treated groups was significantly lower than CK or 50 µM Al treated alone (Figure 5), which showed that NAP or TIBA inhibited the elevation of rhizosphere pH, and this provided evidence that IAA was involved in promoting rhizosphere pH.

3.5. Effect of IAA on PM H⁺-ATPase activity

PM H⁺-ATPase is known to regulate the charge balance and H⁺ movement at plasma membrane surfaces. In order to investigate mechanisms of IAA regulated rhizosphere pH, effects of IAA on PM H⁺-ATPase activity were studied (Figure 6). The relative PM H⁺-ATPase activity was 45.27% under the treatment of 50 µM Al, which was significantly lower than the co-treatment of 50 µM Al and 25 µM IAA (59.84%). The involvement of IAA in regulating PM H⁺-ATPase activity was confirmed.

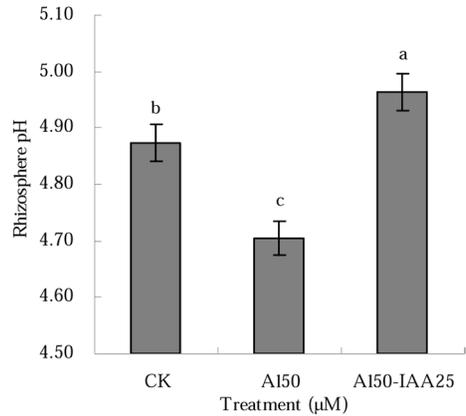


Figure 4. Effects of IAA on rhizosphere pH of wheat. The values are mean ± SE (n=3).

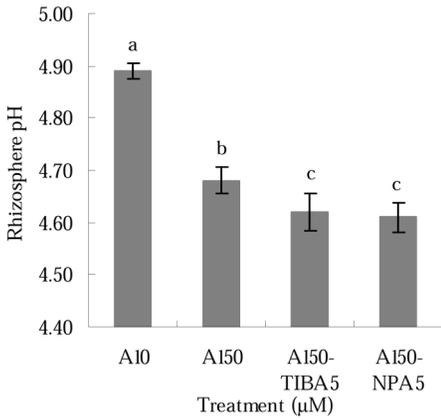


Figure 5. Effects of TIBA (or NPA) on rhizosphere pH of wheat. The values are mean \pm SE (n=3).

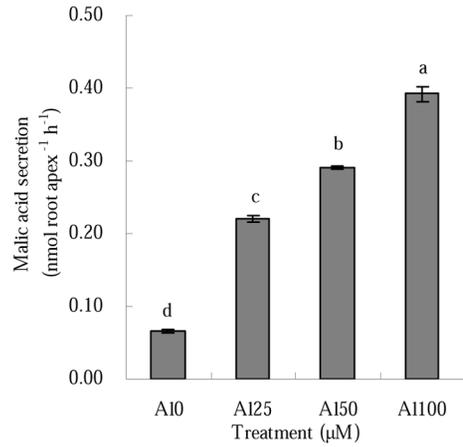


Figure S1. Dose-response of Al-induced malic acid efflux from wheat root after 24 h exposure to different concentrations Al. The values are mean (n=3).

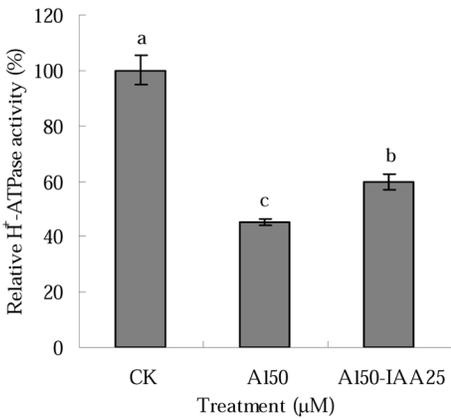


Figure 6. Effects of IAA on PM H⁺-ATPase activity of wheat root apex (0-10 mm). The values are mean \pm SE (n=3).

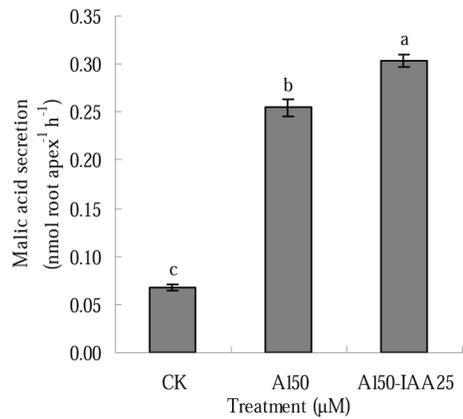


Figure S2. Effect of 50 μM Al and 25 μM IAA co-treatment for 24 h on malic acid efflux from wheat. The values are mean \pm SE (n=3).

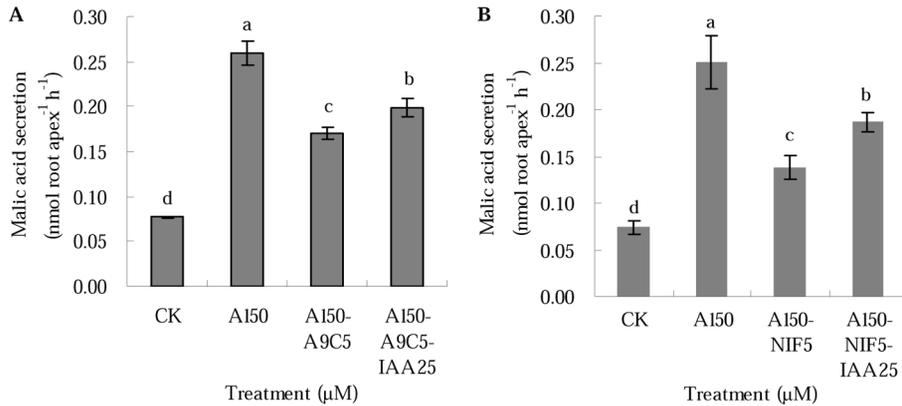


Figure S3. Effect of anion channel inhibitor (A9C, A or NIF, B) or IAA on malic acid efflux from wheat. The values are mean (n=3).

4. Discussion

IAA is an important signal substance in plants that extensively participates in environmental stresses. Salt-tolerance of wheat can be improved significantly by soaking seeds in IAA (Iqbal and Ashraf, 2007). When *Medicago truncatula* is nodulated by an IAA-overproducing *Sinorhizobium meliloti* strain, it increases tolerance to high (55°C) or low (4 °C) temperature, UV-irradiation, NaCl stress (0.5 M) and low pH (3) (Bianco and Defez, 2009).

Al mainly accumulates in plant root apex within 0-10 mm and is toxic to cells (Tahara *et al.*, 2008; Yang *et al.*, 2011c). Organic acid secreted by plants roots possesses higher binding capacity with rhizosphere active Al, which could reduce the binding of Al to root cells (Ma and Furukawa, 2003). Reducing the root apex Al content could be an effective way to improve Al resistance (Reyes-Díaz *et al.*, 2011). To promote the efflux of malic acid is a prominent way to resist Al toxicity of wheat. In the present study, Al contents in the root apex increased with the elevation of Al content in culture solution significantly (Figure 1), and malic

acid was induced by Al in a dose-dependent manner (Figure S1), which was in consistency with the study of Zhao *et al.* (2003).

Our earlier results indicated that IAA could increase the efflux of malic acid under Al stress (Yang *et al.*, 2011b), which was reproduced in this study (Figure S2). Exogenous IAA could cause difference of malic acid efflux between treatments of Al and IAA + Al respectively. The effects of IAA on Al content of root apex (0-10 mm) of wheat were studied to investigate the role of IAA in Al-resistance. Figure 2 showed that IAA could reduce the accumulation of Al in root apex. We speculated that IAA could enhance efflux of Al-chelating malic acid, which led to the decrease of Al content in root apex. In order to further confirm our speculation, anion channel inhibitor was applied in our study. IAA relieved the inhibiting effect of A9C (or NIF) on Al induced malic acid efflux (Figure S3), which consisted with our previous results (Yang *et al.*, 2011b). In accordance with these changing characters, IAA abolished the promoting effects of anion channel inhibitor on Al accumulation in root apex (Figure 3).

The reduction of Al content in root apex could further confirm that IAA participated in Al-resistance.

As mentioned previously, efflux of organic acid is a main but not the only mechanism of plant to resist Al toxicity, the elevation of rhizosphere pH is also a barrier to defect Al stress (Wang *et al.*, 2006). Yang *et al.* (2011a) reports that at low rhizosphere pH condition (pH<5.00), root apex Al content decreased with the increase of rhizosphere pH. There was a significant negative correlation between all the date of rhizosphere pH and Al content in root apex. In present study, exogenous IAA could promote the elevation of rhizosphere pH of wheat under Al stress (Figure 4), which indicated that IAA participated in Al resistance not only by inducing malic acid efflux but also by increasing rhizosphere pH.

In order to further confirm that IAA participated in regulating rhizosphere pH, NPA and TIBA were applied in this study. NPA and TIBA are IAA transport inhibitors, which can cause the accumulation of IAA in the merismatic zone and insufficient accumulation in the elongation zone (Kollmeier *et al.*, 2000). Our results showed that NAP or TIBA treatment inhibited the elevation of rhizosphere pH (Figure 5), which provided further evidence that IAA was involved in the promotion of rhizosphere pH under Al stress.

PM H⁺-ATPase is known to regulate the charge balance and H⁺ movement at plasma membrane surfaces. It is of great significance for plant survival under a variety of external stresses (Cui *et al.*, 2010). Bose *et al.* (2010) find that *Arabidopsis thaliana* could increase rhizosphere pH by the uptake of rhizosphere H⁺ under low-pH stress (pH = 4.2), but this process could be abolished by Al exposure. That IAA participates in the regulation of plant cell PM H⁺-ATPase activity has been proved. Bacaicoa *et al.* (2011) report that Fe-starved plants show an increase in IAA concentration in shoots and roots after 6 and 24 h from the beginning of the treatments, which in turn upregulates the activity of PM H⁺-ATPase (rhizosphere pH) in root apex of cucumber. Shen *et al.* (2006) have studied PM H⁺-

ATPase activity of soybean root under Al stress, and confirmed that exogenous IAA could stimulate the activity of PM H⁺-ATPase and P uptake, while NPA blocked IAA effects.

Consistent with the study of Yang *et al.* (2011a), Al treatment markedly inhibited the activity of PM H⁺-ATPase in the present study (Figure 6). But this inhibiting effect could be abolished by exposing to exogenous IAA. The relative PM H⁺-ATPase activity of the IAA and Al co-treatment was significantly higher than the mere Al treatment. So IAA participated in regulating rhizosphere pH by acting on PM H⁺-ATPase directly or indirectly was speculated. Previous studies of Yang *et al.* (2011a) find that there is a significant positive correlation between PM H⁺-ATPase activity and rhizosphere pH. Rhizosphere pH of the IAA and Al co-treated group was shown to be significantly higher than the control group in this research (Figure 5), while the PM H⁺-ATPase activity was significantly lower than the control group (Figure 6), which was not fully consistent with the previous results of Yang *et al.* (2011a). So we could deduce that IAA up-regulated rhizosphere pH not only by regulating PM H⁺-ATPase, but also other mechanisms.

5. Conclusions

The current results suggested that IAA could be effective in alleviating Al toxicity through reducing Al accumulation in wheat root apex. Inducing efflux of malic acid by IAA and increasing rhizosphere pH could be considered the two effective ways to improve the resistance of plants to Al. Our study was to confirm that exogenous indole-3-acetic acid could reduce the accumulation of aluminum in root apex of wheat under Al stress. Further research is under way in our laboratory to explore the main mechanism and the contribution rate.

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