Rhizosphere pH difference regulated by plasma membrane H^+-ATPase is related to differential Al-tolerance of two wheat cultivars

Y. Yang[1], Q.L. Wang[2], M.J. Geng[1], Z.H. Guo[1], Z. Zhao[1]

[1]*Key Laboratory of Subtropical Agriculture and Environment, Centre for Microelement Research of Huazhong Agricultural University, Ministry of Agriculture, Wuhan, P.R. China*

[2]*Wuhan Military Economic Academy, Wuhan, P.R. China*

ABSTRACT

Aluminum (Al)-tolerance of different cultivars shows considerable differences. Elevation of rhizosphere pH is an external Al-resistant mechanism of plants. To elucidate the correlation between Al tolerance and the capacity of plants to modify the rhizosphere pH at different Al-tolerant levels, a comparative study on the wheat *(Triticum aestivum* L.) cultivars ET8 (Al-tolerant) and ES8 (Al-sensitive) was performed. Rhizosphere pH of ET8 was much higher than that of ES8 under the same treatment, significant correlations were obtained among all the data of rhizosphere pH and relative root elongation (R^2 = 0.9209**), or Al content in root apex (R^2 = 0.9321**), which indicated that Al tolerance may be related to pH changes in the rhizosphere. The elevation of rhizosphere pH was inhibited by H^+-ATPase specific inhibitor DCCD (dicylcohexylcarbodiimide, 25 μmol). Relative PM (plasma membrane) H^+-ATPase activity of ET8 was significantly higher than that of ES8 under the same treatment. Significant correlation between all the data of relative PM H^+-ATPase activity and rhizosphere pH (R^2 = 0.8319**) were obtained. Taken together, these results suggest that PM H^+-ATPase was involved in regulating rhizosphere pH. Under Al stress, the Al-tolerant line showed a stronger capacity of up-regulating rhizosphere pH by PM H^+-ATPase than the Al-sensitive line, which may explain the observed differences in Al tolerance between the two wheat cultivars.

Keywords: acid soil; Al species; *Triticum aestivum* L.; root

In neutral or alkaline soil, Al mainly exists as oxide or silicate precipitates that are not toxic to plants. However, in acid soil, chemical Al speciation is known to be dominated by the soluble Al^{3+} form, which is believed to be toxic to plants (Kochian et al. 2005). Lime is widely used in acid soil (Puschenreiter et al. 2005). The application of lime can increase soil pH, reduce the content of soluble Al^{3+}, and alleviate the adverse effects of Al on root growth (Haling et al. 2010). However, liming may be ineffective in the subsoil, and in some cases heavy lime application may have a deleterious effect on soil structure (Rao et al. 1993). Therefore, selecting Al-resistant cultivars is of economic interest. The screening of Al-tolerant plants is based on the understanding of Al tolerance mechanisms. Elevation of rhizosphere pH is one of the effective external Al resistance mechanisms of plants. Increasing the rhizosphere pH can reduce Al solubility and its potential toxicity to achieve high resistance to Al. Foy et al. (1965) found that wheat can increase culture solution pH under Al stress. Later, scientists recognized that many plants (wheat, sorghum, and Arabidopsis mutant *alr-100*) can maintain relatively higher rhizosphere pH to tolerate Al toxicity. Variations in the Al resistance do exist among cultivars. Different rhizosphere pH changing capacity may relate to the differential Al tolerance of plants. Therefore, changes of rhizosphere pH of the wheat cultivars ET8 and ES8 known for their different Al tolerance were studied in this research.

PM (plasma membrane) H^+-ATPase regulates ion homeostasis and H^+ flux. It is a crucial enzyme for the survival of plants under various environmental stresses. PM H^+-ATPase was shown to participate

Supported by the Natural Science Foundation of China, Project No. 30571115.

in regulating rhizosphere pH (Ahn et al. 2002). However, most previous studies used NH_4^+-N, NO_3^--N, or combination of the two as nitrogen source in the growth medium to study the relationship between PM H^+-ATPase and rhizosphere pH. Yet, the N source provided in the growth medium has a significant impact on rhizosphere pH (Taylor 1991). The uptake of NO_3^--N leads to an alkalinization of rhizosphere, whereas NH_4^+-N can cause rhizosphere acidification (Galvez et al. 1991).

To exclude the effects of N sources on rhizosphere pH, N-free culture solution was applied in this research to investigate the effects of PM H^+-ATPase on rhizosphere pH. The aim of this research was to elucidate the relationship between pH changing capacity in the rhizosphere and the Al tolerance of two wheat cultivars.

MATERIALS AND METHODS

Plant materials and growth conditions. Seeds of wheat (*Triticum aestivum* L.) lines ET8 (Al-tolerant) and ES8 (Al-sensitive) were surface-sterilized by immersion in 1% (v/v) sodium hypochlorite for 15 min, 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 transferred onto a net made of cotton floating on 0.5 mmol $CaCl_2$ (pH 4.5) in a 2 L plastic container. 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, and a relative air humidity of 70%. After 4 days, seedlings of uniform length were selected for experiments. Prior to each experiment, the roots were rinsed by soaking in 0.5 mmol $CaCl_2$ solution pH 4.5 over night.

All treatments in this study contained 0.5 mmol $CaCl_2$ as background electrolyte, which was also used as the control (CK) treatment. Before treatment, all treatment solutions were adjusted to pH 4.5 with 0.1 mol/L HCl after other chemicals were added.

Aluminum treatment. In dose-response experiments, culture solutions were 200 ml 0.5 mmol $CaCl_2$ (pH 4.5), which contained 0, 25, 50 and 100 μmol $AlCl_3$, respectively. Culture solutions pH without (non-planted control)/with wheat seedlings were determined after 24 h treatment. Length of primary root was measured with a ruler before and after Al treatment. After 24 h Al treatment, roots were briefly rinsed with deionised water, and then root apexes (0–10 mm) were excised with a razor for the determination of Al content or PM H^+-ATPase activity. In time-course experiments, seedlings were exposed to 200 ml 0.5 mmol $CaCl_2$ (pH 4.5) with 0 or 50 μmol $AlCl_3$ for 3, 6, 9 and 12 h. In this experiment, solutions containing 0.5 mmol $CaCl_2$ (pH 4.5) without seedlings (non-planted control) were carried out simultaneously.

Na_2WO_4 treatment. Seedlings were exposed to 200 ml 0.5 mmol $CaCl_2$ (pH 4.5) with 0, 50 μmol $AlCl_3$, or 100 μmol Na_2WO_4 for 24 h.

DCCD (dicylcohexylcarbodiimide) treatment. Seedlings were exposed to 200 ml 0.5 mmol $CaCl_2$ (pH 4.5) with 0, 50 μmol $AlCl_3$, or 25 μmol DCCD for 24 h.

Determination of relative root elongation, rhizosphere pH and Al accumulation. Root elongation was estimated in 10 replicates, and Al tolerance was expressed as relative root elongation (RRE) [(root elongation with Al treatment)/(root elongation without Al treatment) × 100]. Rhizosphere pH was determined using a pH Meter (Mettler FE-20, Shanghai, China). Al contents in root apexes (0–10 mm) were determined by the method of Osawa and Matsumoto (2001). The excised root apexes were placed in a microcentrifuge tube (1.5 mL) containing 1.0 mL of 2 mol/L HCl. The tubes were mixed with an orbital shaker at 10 rpm for 24 h to release Al from the root apexes. The Al concentration in the HCl solution after dilution was determined by graphite furnace atomic absorption spectrophotometry (GFAAS Varian GTA 120, Mulgrave, Australia).

Preparation and determination of 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 P_i was measured with a spectrophotometer (Hitachi, U-1800, Tokyo, Japan) at 720 nm. Membrane protein contents were 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].

Statistical analysis. All experiments were run in three replicates. Data were pooled and subjected to one-way analysis of variance (ANOVA) followed by Tukey-Kramer test. $P \leq 0.05$ was set as the level of statistical significance. DPS v7.05 and OrigenPro7.5 software were used for computation, data analysis and graphic.

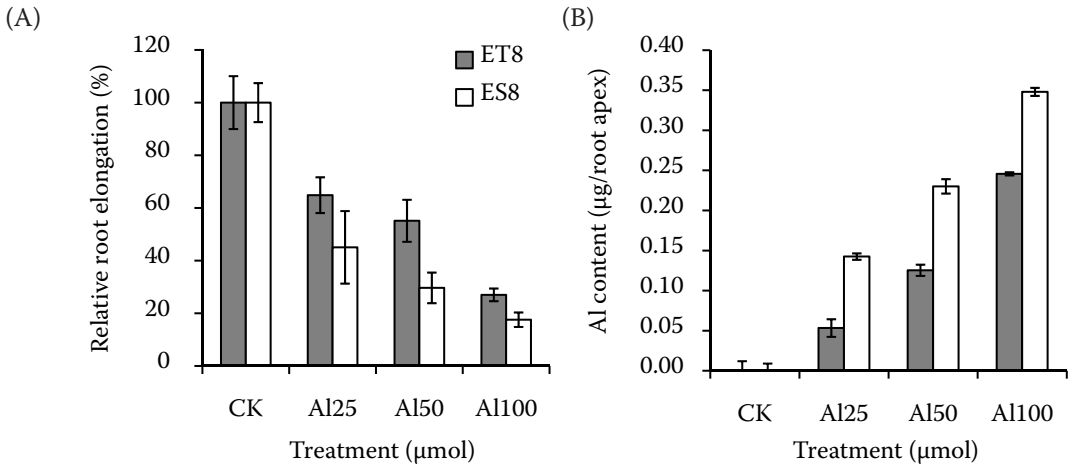

Figure 1. Effects of increasing $AlCl_3$ concentrations on relative root elongation (A) and Al content in root apexes (B) of ET8 and ES8. Seedlings were exposed to different concentrations $AlCl_3$. The values and standard errors (vertical bars) of three replicates are shown

RESULTS AND DISCUSSION

Zhao et al. (2003) studied 21 barley varieties, and confirmed that Al accumulated in the root apex, inhibited root elongation, and the correlation between root apex Al content and RRE was significant. In the present study, the RRE of ET8 and ES8 was decreased with increasing concentrations of $AlCl_3$ (25, 50 and 100 μmol, 24 h), and Al content in root apex (0–10 mm) was increased with the $AlCl_3$ concentrations (Figure 1). A highly significant (R^2 = 0.9079**) correlation between all the data of relative root elongation and Al content was obtained (Figure 2), revealing that the accumulation of Al in root apex inhibited root elongation.

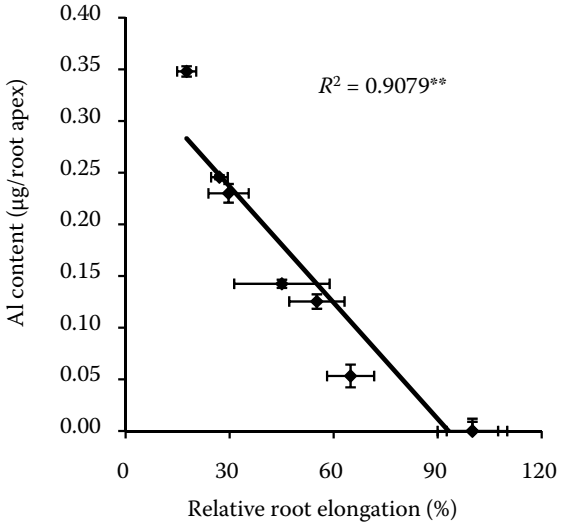

Figure 2. Correlation between all the data of relative root elongation and Al content in wheat root apex. Seedlings of ET8 and ES8 were exposed for 24 h to different concentrations $AlCl_3$. The values and standard errors (vertical bars) of three replicates are shown

Higher RRE and lower root apex Al content in the Al-tolerant as compared to the Al-sensitive line strongly suggests differential Al tolerance.

Rhizosphere pH determines the chemical speciation of Al. Stass et al. (2006) found that Al mainly existed as biologically toxic Al^{3+} at rhizosphere pH < 4.3. Contents of active Al^{3+} were decreased when the rhizosphere pH was increased from 4.0 to 5.0 (Zhu et al. 2005). In our dose-response experiments, pH of solutions without seedlings (non-planted control) was increased slightly. There were no differences among the solutions containing different concentrations Al (Table 1). It showed that Al^{3+} could not affect the pH of non-planted solution. The observed increases in rhizosphere pH compared to the control were significantly less pronounced at higher $AlCl_3$ concentrations (Figure 3A). In time-course experiments (Figure 3B), solutions pH of non-planted control was slightly increased by 0.06 pH units after 12 h. In the presence of seedlings, the pH increased in a time-dependent manner. After 12 h, the pH values of the Al-free control treatment of ET8 and ES8 (4.84 and 4.73) were significantly higher than that of the $AlCl_3$ treatment (4.71 and 4.66). Results above indicated that wheat could elevate rhizosphere pH under low pH, but Al could affect the elevation of rhizosphere pH by acting on wheat,

Table 1. Effects of $AlCl_3$ treatments (0, 25, 50 and 100 μmol, 24 h) on non-planted culture solution pH

CK	Al25	Al50	Al100
4.53 ± 0.004	4.54 ± 0.023	4.56 ± 0.012	4.53 ± 0.029

Results are expressed as mean ± SE (n = 3)

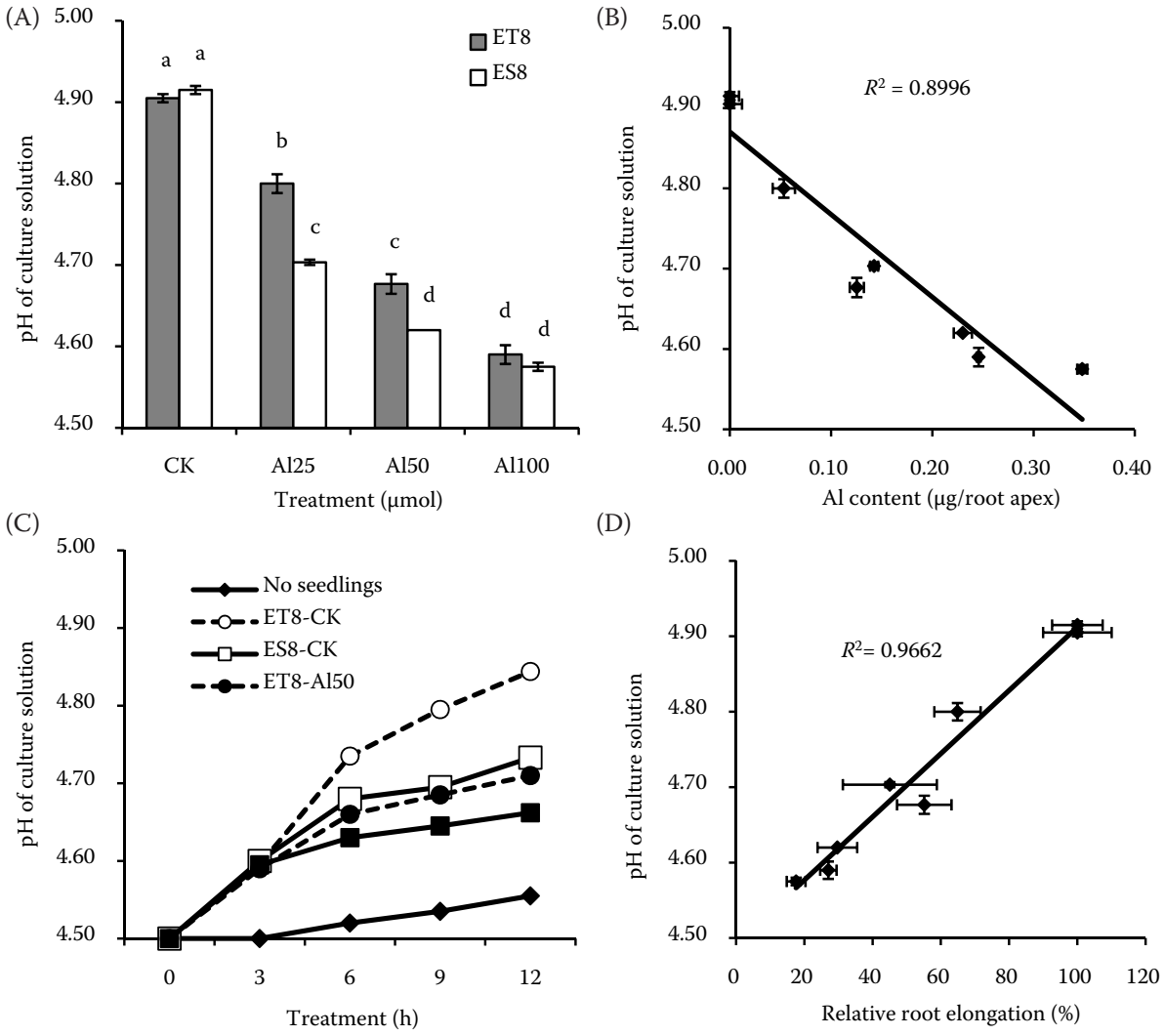

Figure 3. Dose-response of $AlCl_3$ treatments (0, 25, 50 and 100 μmol, 24 h) on rhizosphere pH of ET8 and ES8 (A). Correlations among all the data of culture solution pH and Al content in wheat root apex (B). Time-course (0, 3, 6, 9 and 12 h) of $AlCl_3$ treatments (0 or 50 μmol) on rhizosphere pH of ET8 and ES8 (C), relative root elongation (D). The values and standard errors (vertical bars) of three replicates are shown

and ET8 could maintain relatively higher rhizosphere pH than that of ES8 under same treatment.

Highly significant correlation among all the data of rhizosphere pH and Al content in root apexes (R^2 = 0.8996**, Figure 3B), or RRE (R^2 = 0.9662**, Figure 3D) were obtained, indicating that rhizosphere pH plays an important role in regulating the accumulation of Al in the root apex and root elongation. Compared with ES8, the higher rhizosphere pH of ET8 reflected the higher Al resistance.

Foy et al. (1982) reported that NO_3^--N could alleviate the toxicity effects of Al to plants. This can be due to OH^- ions produced and secreted into rhizosphere during NO_3^--N metabolism process catalyzed by nitrate reductase (Taylor and Foy 1985). However, nitrate reductase is induced by $N0_3^-$-N. There was no NO_3^--N in culture solution in present study, the activity of nitrate reductase (data not shown) or rhizosphere pH (Figure 4) was affected neither by Al nor by nitrate reductase inhibitor Na_2WO_4 treatments. These results indicated that nitrate reductase did not participate in the regulation of rhizosphere pH in the present study. It is likely that other mechanisms promoted the observed increases of rhizosphere pH.

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) found that *Arabidopsis thaliana* could increase rhizosphere pH by uptake of rhizosphere H^+ under low-pH stress (pH = 4.2), but this process could be abolished by Al exposure. In the present study we show that Al treatments markedly diminished the increase of rhizosphere pH of both cultivars, especially

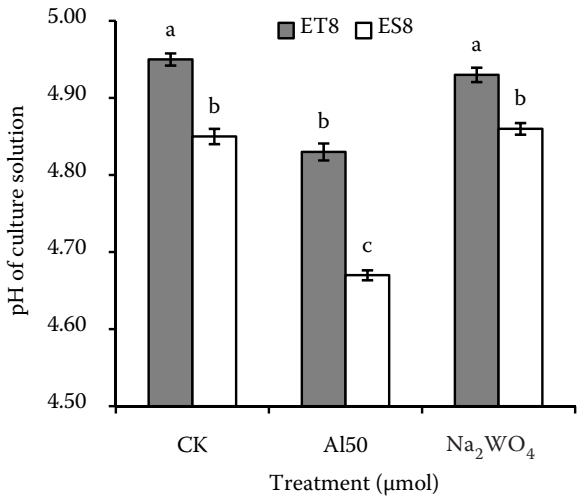

Figure 4. Effects of Na_2WO_4 on rhizosphere pH of ET8 and ES8. Seedlings were exposed for 24 h to 0.5 mmol $CaCl_2$ containing 0, 50 μmol $AlCl_3$, or 100 μmol Na_2WO_4. The values and standard errors (vertical bars) of three replicates are shown

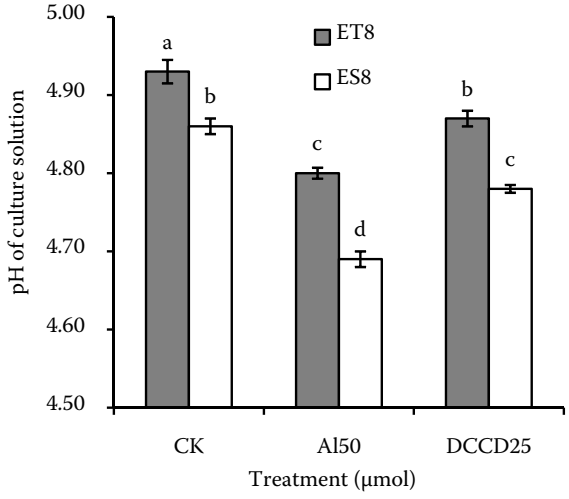

Figure 5. Effects of DCCD on rhizosphere pH of ET8 and ES8. Seedlings were exposed for 24 h to 0.5 mmol $CaCl_2$ containing 0, 50 μmol $AlCl_3$, or 25 μmol DCCD. The values and standard errors (vertical bars) of three replicates are shown

the more Al-sensitive line (Figure 3A and C). The PM H^+-ATPase activity can be inhibited by the specific inhibitor DCCD (Kisnierienë and Sakalauskas 2007). In this research, DCCD was used to identify the function of PM H^+-ATPase in regulating rhizosphere pH. Figure 5 shows that the amplitude of pH increases in the rhizospheres of ET8 and ES8 was smaller in the presence of 25 μmol DCCD. Compared with the control treatment, the rhizosphere pH of ET8 and ES8 were 0.06 and 0.08 pH unit lower, respectively, providing strong evidence that PM H^+-ATPase participated in regulating rhizosphere pH. In addition, we analyzed the changes of PM H^+-ATPase activity after Al treatment. The PM H^+-ATPase activities were inhibited by the treatments of $AlCl_3$ (Figure 6). As the Al concentrations in the root apex increased, the relative activities of PM H^+-ATPase of ET8 and ES8 decreased from 95.9% to 60.0%. The PM H^+-ATPase activity of ES8 was significantly lower than that of ET8 under the same treatment. The present observations indicate that the relatively higher PM H^+-ATPase activity in the Al-tolerant line reflected less destruction of PM function, associated with higher rhizosphere pH of the Al-tolerant line. In good agreement with our hypothesis, we obtained positive correlations among all the data of relative PM H^+-ATPase activity and rhizosphere pH (R^2 = 0.9106**), Al content in root apex (R^2 = 0.9079**), or relative root elongation (R^2 = 0.8820**) (Table 2), further confirming that root apex PM H^+-ATPase plays an important role in regulating rhizosphere pH, and the resistance against Al stress. Higher rhizosphere pH caused by PM H^+-ATPase would alleviate Al toxicity because of a shift in ionic speciation of Al to decrease the proportion of Al^{3+}. Thus, it resulted in relatively lower Al content in root apex and higher RRE. As under Al stress the Al-tolerant line ET8 possessed the stronger capacity of up-regulating rhizosphere pH than the Al-sensitive line, we suggest that the proposed mechanism may largely explain why cultivar ET8 exhibited significantly higher Al tolerance than ES8.

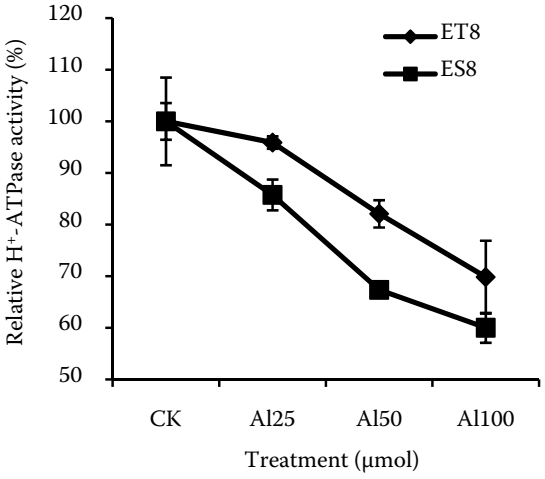

Figure 6. Effects of $AlCl_3$ on relative PM H^+-ATPase activity of ET8 and ES8. Seedlings were exposed for 24 h to different concentrations of $AlCl_3$. The values and standard errors (vertical bars) of three replicates are shown

Table 2. Correlation among all the data of relative PM H^+-ATPase activity, culture solution pH, Al content in root apex, or relative root elongation. Seedlings were exposed for 24 h to different concentrations of $AlCl_3$

	Culture solution pH	Al content in root apex	RRE
Relative PM H^+-ATPase activity	0.9106**	0.9079**	0.8820**

**$P < 99\%$

Acknowledgement

We thank Dr. Ma Jian Feng of the Research Institution for Bioresources of Okayama University for the ET8 and ES8 seeds.

REFERENCES

Ahn S.J., Sivaguru M., Chung G.C., Rengel Z., Matsumoto H. (2002): Aluminium-induced growth inhibition is associated with impaired efflux and influx of H^+ across the plasma membrane in root apices of squash (*Cucurbita pepo*). Journal of Experimental Botany, *53*: 1959–1966.

Bose J., Babourina O., Shabala S., Rengel Z. (2010): Aluminium-induced ion transport in Arabidopsis: the relationship between Al tolerance and root ion flux. Journal of Experimental Botany, *61*: 3163–3175.

Bradford M.M. (1976): Rapid and sensitive method for quantization of microgram quantities of protein utilizing principle of protein-dye binding. Analytical Biochemistry, *72*: 248–254.

Cui X.M., Zhang Y.K., Wu X.B., Liu C.S. (2010): The investigation of the alleviated effect of copper toxicity by exogenous nitric oxide in tomato plants. Plant, Soil and Environment, *56*: 274–281.

Foy C.D., Fleming A.L. (1982): Aluminum tolerance of two wheat genotypes related to nitrate reductase activities. Plant Nutrition, *5*: 1313–1333.

Foy C.D., Burns G.R., Brown J.C., Fleming A.L. (1965): Differential aluminum tolerance of two wheat varieties associated with plant-induced pH changes around their roots. Soil Science Society of America Journal, *29*: 64–67.

Galvez L., Clark R.B. (1991): Nitrate and ammonium uptake and solution pH changes for Al-tolerant and Al-sensitive sorghum (*Sorghum bicolor*) genotypes grown with and without aluminum. Plant and Soil, *134*: 179–188.

Haling R.E., Simpson R.J., Delhaize E., Hocking P.J., Richardson A.E. (2010): Effect of lime on root growth, morphology and the rhizosheath of cereal seedlings growing in an acid soil. Plant and Soil, *327*: 199–212.

Johansson F., Olbe M., Sommarin M., Larsson C. (1995): Brij 58, a polyoxyethylene acyl ether, creates membrane vesicles of uniform sidedness: a new tool to obtain inside-out (cytoplasmic side-out) plasma membrane vesicles. Plant Journal, *7*: 165–173.

Kisnierienë V., Sakalauskas V. (2007): The effect of aluminum on bioelectrical activity of the *Nitellopsis obtusa* cell membrane after H^+-ATPase inhibition. Central European Journal of Biology, *2*: 222–232.

Kochian L.V., Hoekenga O.A., Piñeros M.A. (2005): How do crop plants tolerate acid soils? Mechanisms of aluminum tolerance and phosphorous efficiency. Annual Review of Plant Biology, *55*: 459–493.

Palmgren M.G., Askerlund P., Fredrikson K., Widell S., Sommarin M., Larsson C. (1990): Sealed inside-out plasma membrane vesicles: optimal conditions for formation and separation. Plant Physiology, *92*: 871–880.

Osawa H., Matsumoto H. (2001): Possible involvement of protein phosphorylation in aluminum-responsive malate efflux from wheat root apex. Plant Physiology, *126*: 411–420.

Puschenreiter M., Horak O., Friesl W., Hartl W. (2005): Low-cost agricultural measures to reduce heavy metal transfer into the food chain – a review. Plant, Soil and Environment, *51*: 1–11.

Rao I.M., Zeigler R.S., Vera R., Sarkarung S. (1993): Selection and breeding for acid-soil tolerance in crops. Bioscience, *43*: 454–465.

Stass A., Wang Y., Eticha D., Horst W.J. (2006): Aluminium rhizotoxicity in maize grown in solutions with Al^{3+} or $Al(OH)_4^-$ as predominant solution Al species. Journal of Experimental Botany, *57*: 4033–4042.

Taylor G.J., Foy C.D. (1985): Mechanisms of aluminum tolerance in *Triticum aestivum* L. (wheat). II. Differential pH induced by spring cultivars in nutrient solutions. American Journal of Botany, *72*: 695–701.

Taylor G.K. (1991): Current views of the aluminum stress response the physiological basis of tolerance. Current Topics of Plant Biochemistry Physiology, *10*: 57–93.

Zhao Z., Ma J.F., Sato K., Takeda K. (2003): Differential Al resistance and citrate secretion in barley (*Hordeum vulgare* L.). Planta, *217*: 794–800.

Zhu X.Z., Huang Y., Zong L.G., Kong F.X. (2005): Effects of aluminum speciation on wheat root growth and its enzyme activity. Chinese Journal of Applied Ecology, *16*: 1043–1046.

Received on December 12, 2010

Corresponding author:

Prof. Zhuqing Zhao, Centre for Microelement Research of Huazhong Agricultural University, Key Laboratory of Subtropical Agriculture and Environment, Ministry of Agriculture, Wuhan 430070, P.R. China
phone: + 86 27 8727 8578, e-mail: zzq@mail.hzau.edu.cn