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ORIGINAL PAPER

Genotype-dependent effects of phosphorus supply on physiological and biochemical responses to Al-stress in cultivated and Tibetan wild barley

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Abstract Aluminium (Al) toxicity and phosphorus (P) deficiency often co-exist in acidic soils and limit plant growth and crop production. To investigate the alleviating effects of different levels of phosphorus on Al stress, greenhouse hydroponic experiments were conducted using two contrasting Tibetan wild barley genotypes XZ16 and XZ61 of Al tolerant and sensitive, respectively, and Al tolerant *cv*. Dayton. The results showed that Al stress induced reduction in P accumulation in plants; and stem and leaf P concentrations of the three genotypes, except of XZ16 under HP + Al (100 μM Al with high level of 360 μM P) which was close to the control level. XZ16 recorded significantly higher P accumulation in plants, compared with XZ61 and Dayton, and P concentrations in leaves under Al stress, and in stems under NP + Al (100 μM Al with normal level of 180 μM P) and HP + Al. Meanwhile, H^+-, $Ca^{2+}Mg^{2+}$-, and Total- ATPase activities in XZ16 and Dayton under Al stress were markedly higher than in XZ61. Normal or high level of P under Al stress could relieve Al stress as enhanced plant biomass, with increased photosystem II photochemistry (Fv/Fm) and P content, relative to the low level of 90 μM P. Compared with XZ61, addition of high P concentration for XZ16 significantly increased the values of Gs and Tr, with higher root GPX and H^+-ATPase activities, and such nutrient elements as P, Mg and Ca in stems and leaves, and induced more malate secretion, but less MDA accumulation.

Keywords Wild barley (*H. vulgare* ssp. *Spontaneum*) · Al stress · Phosphorus · Alleviation

Introduction

Aluminum (Al) concentration in soil solution is the most important factor restricting plant growth in acidic soils. Plant growth can be influenced by a variety of chemical factors and interactions between these factors on acid soils (Marschner 1991). Aluminum toxicity and phosphorus deficiency were considered to be the most important factor limiting the crop yield in acid soil (Kochian et al. 2004; Fukuda et al. 2007). It is estimated that 30–40% of arable land and up to 50% of potentially arable land in the world are acidic and may be prone to Al toxicity (Vance et al. 2003). Moreover, Al ions dissociated from alumina or other minerals and combined with phosphorus under low pH, further aggravating the symptoms of phosphorus deficiency (Kochian et al. 2004). Phosphate fertilizers are often applied in acid soils to decrease Al activity and increase crop production. Although an improved crop production in acidic soils is possible with lime application, such application is neither economical for all farmers nor a thorough method for correcting subsoil acidity (Kochian et al. 2005). It is therefore important to identify genetic resources that have high Al tolerance and to understand the mechanisms of Al–P interactions in plants.

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Barley (*Hordeum vulgare* L.), the fourth important cereal crop of the world, is one of the most Al-sensitive species among small-grain cereals (Zhao et al. 2003). Tibetan Wild barley is a unique resource in China, as it contains a variety of specific genes for stress resistance and provides a rich gene pool for the genetic improvement of crops. In view of narrow genetic diversity in cultivated barley, wild barley has been paid more attention in order to identify the elite germplasm for use in barley breeding (Ellis et al. 2000). The utilization rate to phosphorus in plant has large genetic diversity in the nature resource (Ramaekers et al. 2010). McCormick and Borden (1972) found that the interaction between Al and P was related to the epidermal and cortical cells and the plasma membrane of barley cell wall using molybdenum blue staining. Tan and Keltjens (1990) proposed the reason that Al stress in plants relieved after increased P content was likely to be achieved through phosphorus to promote the growth and development of roots and the absorption of mineral nutrients.

Studies have found that both Al addition and P deficiency impaired the whole electron transport chain from photosystem II (PSII) donor side up to the photosystem I (PSI), led to photosynthetic electron transport capacity declining, thus decreasing the rate of CO_2 assimilation, and finally affected photosynthesis (Jiang et al. 2008; Lin et al. 2009). Jiang et al. (2009) indicated that P addition increased P level in roots and shoots, prevented Al-induced impairment of the whole photosynthetic electron transport chain and alleviated Al-induced inhibition of growth. In addition, with the increase of P content in roots, the toxicity of Al to plant growth gradually decreased, moreover, P content in roots was higher and the transport capacity of P was stronger in resistant cultivars than sensitive cultivars (Gaume et al. 2001). Zheng et al. (2005) found that the resistance to Al was related to the amount of oxalic acid secretion in root tips and the chelation between P and Al. It was reported by Liao et al. (2006) that the soybean cultivars with high P efficiency could improve the secretion of organic acid in roots through Al–P interaction to increase the resistance to Al. In this scenario, the interaction between Al and P is very important for the plant's resistance to Al. There is no similar report on whether Al–P interaction is related to the Al tolerance in Tibetan wild barley at present. Therefore, a complete understanding of the Al–P interaction of plants to these stress and the effects on plant growth is of considerable, practical and ecological significance for the improvement of abiotic stress tolerance.

Our recent studies have demonstrated that Tibetan wild barley genotypes XZ16 and XZ61 of Al tolerant and sensitive, respectively, compared with Al tolerant *cv*. Dayton (Dai et al. 2011). However, so far there is no study on genotypic difference in the effect of exogenous phosphorus on the changes in morpho-physiolocal of barley plants under Al stress. The present study reports genotypic difference in Al-induced changes in growth, photosynthesis, mineral concentrations and antioxidative metabolism, and the role of exogenous phosphorus in Al tolerance using two Tibetan wild barley genotypes varying in Al tolerance and accumulation. These results would be useful to understand the mechanisms of Al tolerance in Tibetan wild barley genotypes, and open novel prospective for improving Al tolerance of plants in acid soil.

Materials and methods

Plant materials and experimental designs

Hydroponic experiments were carried out in the greenhouse of Huajiachi Campus of Zhejiang University, Hangzhou, China. Seeds of the Tibetan wild barley genotypes XZ16 and XZ61 (*H. vulgare* L. ssp. *spontaneum*) and *cv*. Dayton were disinfected with 3% H_2O_2 for 30 min and rinsed with distilled water; then, they were soaked for 4 h at room temperature. Seeds were transferred onto moist filter papers in germination boxes in a growth chamber (22/18 °C, day/night) in dark for 3 days and incubated for another 4 days with light. Seven-day-old healthy and uniform plants were selected and transplanted into 4.5 L containers filled with 4.3 L basal nutrient solution (BNS) and fixed by sponge. The composition of BNS was according to Chen et al. (2010).

Twenty days after transplanting, P (as KH_2PO_4) and Al (as $AlCl_3$) were added to the containers to form the following 5 treatments: NP 6.0 (control, BNS, containing normal P of 180 μM KH_2PO_4) at pH 6.0; NP 4.3 (BNS, containing normal P of 180 μM KH_2PO_4) at pH 4.3; LP + Al (BNS, containing 90 μM P + 100 μM Al) at pH 4.3; NP + Al (BNS, 180 μM P + 100 μM Al) at pH 4.3 and HP + Al (BNS, 360 μM P + 100 μM Al) at pH 4.3. KCl concentration was adjusted according to different P concentrations to keep the same nutrient solution composition, while other nutrient element content remained the same. The solution pH in each container was adjusted once a day with 1 M HCl or NaOH as required. The solution was continuously aerated with an air pump and renewed every 3 days. The experiment was laid in a split-plot design with treatment as the main plot and genotype as sub-plot, and there were seven replicates for each treatment.

Measurement of chlorophyll fluorescence, photosynthesis parameters

After 20 days Al treatment, chlorophyll fluorescence and photosynthetic parameters were measured on the second uppermost fully expanded leaf with 5 replicates. LI-6400

portable photosynthesis system (LI-COR, Lincoln, NE) was used to measure net photosynthetic rate (Pn), stomatal conductance (Gs), transpiration rate (Tr) and intracellular CO_2 concentration (Ci) of the 2nd fully expanded leaves. The photochemical efficiency (Fv/Fm) of leaf photosystemII (PSII) was measured by IMAGING-PAM modulated chlorophyll fluorescence imaging system (Walz, Eveltrich, Germany).

Measurements of dry weight and Al concentration

Plant samples were harvested 20 days after Al treatment, rinsed thoroughly with deionized water, and divided into roots, stems and leaves, then dried at 80 °C and weighed. Dried roots and shoots were ashed and weighed, nitrified by10 ml HNO_3/$HClO_4$ mixture (4:1, v/v) for 6 h, constant volume with the appropriate amount of deionized water. Filtered by filter paper, the contents of P, K, Ca, Mg, Fe, Mn, Cu, Zn and Al in the filtrate were determinate using the plasma reflection spectroscopy (ICP/AES) (IRIS/AP optical emission spectrometer, Thermo Jarrell Ash, San Jose, CA, USA). And root tips were used for fluorescence imaging of Al. Specific method for details were referenced to Cao et al. (2014).

Measurements of antioxidant enzyme activity, ATPase activity and MDA content

The fresh roots and the second fully opened leaves were sampled, immediately frozen in liquid nitrogen and stored at −80 °C for further analysis or directly used for the determination of antioxidative enzyme activities and MDA content. Superoxide dismutase (SOD), glutathione reductase (GR), glutathione peroxidase (GPX), catalase (CAT), peroxidase (POD), and ascorbate peroxidase (APX) activities were determined according to Ahmed et al. (2013a). The content of MDA was determined according to Wu et al. (2003). ATPase activities were determined according to Ahmed et al. (2013b) with an enzyme assay kit according to the manufacturers' protocol (Jiancheng Bio Co., Nanjing, China).

Collection and determination of root organic-acid (OA) secretion

Root organic acids (citric acid and malic acid) were determined according to the method of Zheng et al. (2005) with some modifications. Root exudates were collected by the cation exchange column (16 mm × 14 mm, USA Bio-Rad company) with 5 g Amberlite IR-120B type cation exchange resin (H^+ type, American Alrich-Sigma), and then through an anion exchange column (16 mm × 14 mm, USA Bio-Rad company) with 2 g Dowex × 8 anion exchange resin (formate type, 100–200 meshes), in order to wipe off cations and make anions adsorbed on the anion resin. 1 M HCl was used to wipe off the material retain on the anion resin then. The vacuum concentration of the desorption solution was concentrated to dry at 40 °C by rotary evaporators and dissolved in deionized water, then measured organic acids using a high performance liquid chromatography (HPLC) (Waters 2695, Waters Corp, MA, USA) with a symmetrical C18 cation exchange column(4.6 mm i.d × 200 mm, 5 μm). With 10 mM $(NH_4)_2HPO_4$ (pH 2.5) as mobile phase, the flow rate was 0.8 mL min^{-1}, the column temperature was 25 °C, and injection volume was 20 μL. The detection wavelength was set at 214 nm.

Statistical analysis

All data were analyzed by Data Processing System (DPS) statistical software for variance analysis and multiple comparisons (Tang and Feng 1997) using ANOVA followed by the Duncan's Multiple Range Test to evaluate treatment effects ($P < 0.05$). Origin Pro version 8.5 (Origin lab corporation, Wellesley Hills, Wellesley, MA, USA) was used to prepare graphs.

Results

Effect of P on biomass and P concentration in three barley genotypes under Al stress

Barley plants exposed to 100 μM Al for 20 days showed a significant decrease in dry weight, with the largest reduction in the low P level (LP + Al) (Table S1) and sensitive genotype XZ61, i.e. root, stem and leaf dry weight decreased by 20.0, 19.8 and 14.9% in XZ16, 58.3, 29.0 and 27.0% in XZ61, and 24.4, 23.7 and 17.6% in Dayton respectively, over the NP(4.3) case (Al absent basic nutrition solution but containing normal P of 180 μM KH_2PO_4 at pH 4.3). With the increase of P level of 180 and 360 μM P (NP + Al, HP + Al), the root, stem and leaf dry weights were significantly relieved; and the highest increase was recorded in XZ16.

Leaf and stem P concentrations were clearly decreased in the plants of the three genotypes when exposed to Al, especially in XZ61, which may induce shoot P-deficiency. Furthermore, significant genotypic difference in the effect of Al on P concentration was detected under NP + Al or HP + Al conditions. The highest leaf/stem P concentration and accumulation were observed in XZ16 when exposed to Al (Fig. 1, S1) in the three genotypes. e.g. leaf/stem P concentrations in XZ16 were 25.7/6.8% and 38.5/6.0% higher than that in XZ61 under NP + Al and HP + Al. Plant P accumulation in XZ16 was 25.1, 36.0 and 39.4% higher

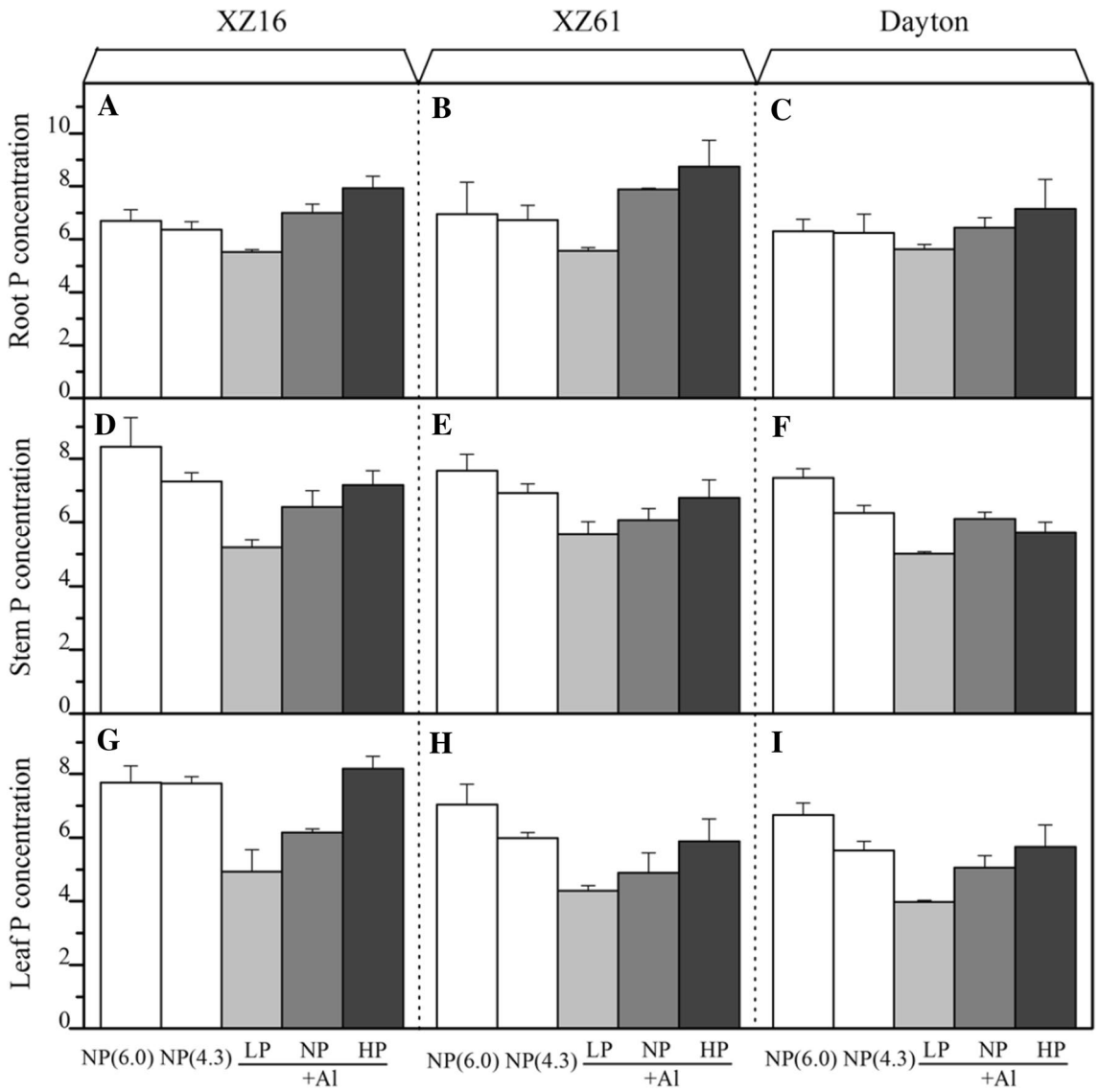

Fig. 1 P concentrations (mg g^{-1} DW) in roots (**a**–**c**), stems (**d**–**f**) and leaves (**g**–**i**) of XZ16 (*left* panel), XZ61 (*middle*) and Dayton (*right*) exposed to 100 μM Al. Data are means ± SD (n = 3). NP(6.0), NP(4.3); and LP + Al, NP + Al, HP + Al correspond to basic nutrition solution (BNS, containing normal P of 180 μM KH_2PO_4) at pH 6.0, BNS at pH 4.3; and at pH 4.3 under BNS (containing 90 μM P) + 100 μM Al, BNS (180 μM P) + 100 μM Al, BNS (360 μM P) + 100 μM Al

than XZ61 under LP + Al, NP + Al and HP + Al, respectively, while 26.1, 37.3 and 49.1% higher than Dayton.

Effect of P on fluorescence parameter and photosynthesis in three barley genotypes under Al stress

The changes of fluorescence parameters in barley leaves were examined and shown in Table S2, which reflected toxicity degree under Al stress. The photosystem II photochemistry (Fv/Fm) value of Al tolerant genotype XZ16 and Dayton were not changed under different P concentration, while Al sensitive cultivar decreased by 16.9% under low P concentration (LP + Al) and were not restored to the control level (pH 4.3) under high P concentration (HP + Al), however, XZ16 and Dayton were restored to control level.

The changes of photosynthetic parameters in barley leaves are shown in Fig S2. Of the three genotypes, photosynthetic rate (Pn) in XZ61 and Dayton significantly decreased under LP + Al and were not restored to the control level (pH 4.3) under high P concentration (HP + Al), while this rate was not significantly changed in Al tolerant genotype XZ16 (Fig. S2). Moreover, the Pn rate of Al tolerant genotypes Dayton and XZ16 was improved with increasing of P level, while the sensitive genotypes XZ61 had no changed. The stomatal conductance (Gs) was

significantly decreased under LP + Al accompanied with decrease in intercellular CO_2 concentration (Ci) and transpiration rate (Tr) in all genotypes, while increasing the P level caused raise in Gs and Tr compared to LP + Al in XZ16 and Dayton genotypes except in XZ61.

Effect of P on antioxidant system and MDA content in three barley genotypes under Al stress

The antioxidant responses in the Tibetan wild and cultivated barley genotypes to Al stress are presented in Fig. 2 and S3. After 20 d Al treatment, different antioxidants showed distinct variations among the genotypes and P level. The SOD activity in the roots significantly ($P < 0.05$) increased in XZ61 and Dayton while exposed to Al stress and decreased with the increasing in P level; however, increased in XZ16 (Fig. 2a–c). The POD activity in roots was significantly increased in all genotypes while exposed to Al stress (Fig. 2d–f) while the activity of GR in XZ16 was not significantly changed, but XZ61 and Dayton increased respectively by 118.5 and 37.7% in average compared with the control (pH 4.3) (Fig. 2g–i). Moreover, the POD and GR activity decreased with the increasing in P level except in Dayton. The GPX activity of XZ16 increased by 105.7% under control (pH 4.3) compared to pH 6.0, while decreased in LP + Al and restored to the control level (pH 4.3) under high P concentration (HP + Al). On the other hand, the activity of GPX in XZ61 and Dayton were down regulated by 83.7 and 15.5% (Fig. 2j–l). The CAT activity increased in all genotypes while exposed to Al stress and decreased with the increase level of P, but no significant effect difference was seen in XZ16 under LP + Al (Fig. 2m–o). The APX activity of XZ16 was decreased significantly under Al stress, and the activity of APX slightly increased under HP + Al in Dayton and XZ16, which were not significantly different from the control (Fig. 2p–r). The results showed that the POD, GR and APX activities of XZ16 were more likely to be related to Al tolerant under the interaction of P and Al stress.

The activity of SOD in the leaves significantly ($P < 0.05$) decreased in XZ16 and XZ61 under Al stress and increased with the increasing in P level; however, no significant effect of P level was observed in Dayton (Fig. S3a–c). It is interesting that under low P concentration, the POD activity in leaves of XZ16 decreased by 17.9% compared with the control, while XZ61 and Dayton were increased by 181.1 and 46.9%, respectively (Fig. S3d–f). Compared with control the GR activity of three barley genotypes decreased with the increasing in P level (Fig. S3g–i). The GPX activity in XZ16 and Dayton leaves were increased by 33.7 and 24.1% in average under Al stress, while XZ61 decreased by 57.0% (Fig. S3j–l). The activity of CAT in XZ16 leaves was increased with the increase of the P level and it was 34.4% higher under high P level than that of control, while no significant difference was observed in XZ61 leaves when compared with control, but decreased in Dayton (Fig. S3m–o). The APX activity in all genotypes was significantly decreased with the increasing of P level (Fig. S3p–r). Moreover, the effects of different pH conditions on the antioxidant system in leaves were small which was different from roots (Fig. S3).

After 20 d of Al treatment, on average of three P level the MDA contents of XZ16, XZ61 and Dayton were increased by 25.9, 62.9 and 38.1%, respectively. Our results showed that P nutrition increased in the solution significantly alleviated oxidation of root membrane lipids in Dayton and XZ61 (Fig. 3a–c). Importantly, MDA content in HP + Al treated leaves of XZ16 remained similar with the control. However, MDA content of sensitive genotype XZ61 was higher than that of tolerant genotype XZ16 and Dayton (Fig. 3d–f).

Effect of P on ATPase activity in three barley genotypes under Al stress

ATPase activity in both roots and leaves of XZ16 and Dayton increased gradually with the increase of P level, while the ATPase activity of XZ61 was not changed enough (Fig. 4, S4). Compared with pH 6.0, the activity of ATPase in roots and leaves were significantly higher in pH 4.3. With the increase of P level, the average percent raised in the activities of H^+-, $Ca^{++}Mg^{++}$- and total-ATPases between the three genotypes in descending order were as follows: Dayton > XZ16 > XZ61 (Fig. 4a–i, S4a–i).

Effect of P on Al accumulation and Al fluorescence localization in root tips in three barley genotypes under Al stress

Al concentration and accumulation in roots, stems and leaves of three barley genotypes were significantly improved after Al treatment (Table 1, Table S1). The Al concentration in barley roots, stems and leaves was not significantly affected by P level. Under Al stress, the Al concentration in the roots, stems and leaves of sensitive cultivar XZ61 was higher than that of XZ16. Exposure of plants to pH 6.0 and pH 4.3 did not result in a distinct fluorescence in the root epidermis in Fig. 5. However, there was an obvious fluorescence among root tips exposed to 100 μM Al, which showed that Al accumulated in the root tips of the all barley genotypes, yet the whole cross section in XZ16 exhibited an overall minor fluorescence. On the whole, the fluorescence intensity of XZ61 was higher than that of XZ16 and Dayton, which was consistent with the trend of Al content in relevant roots.

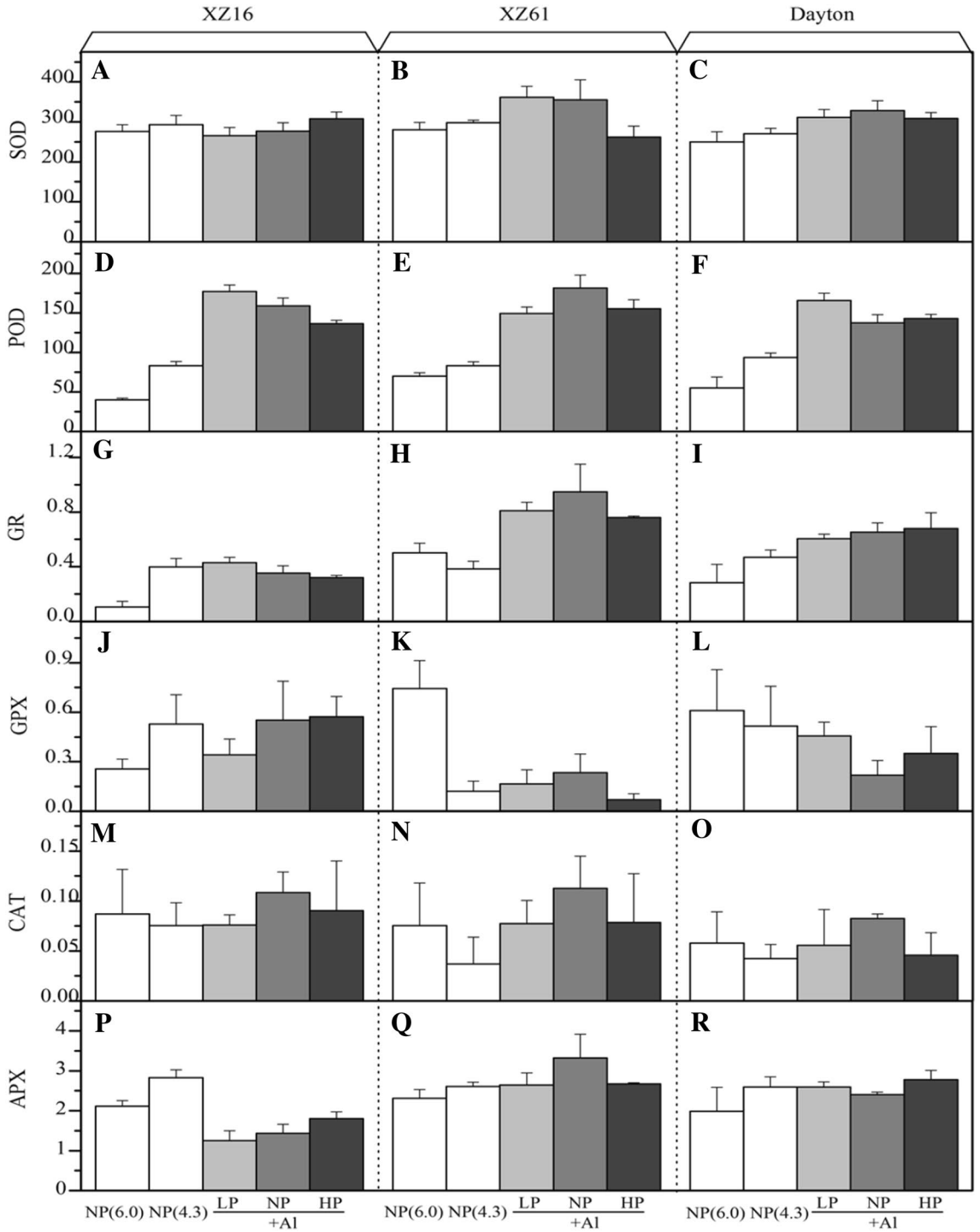

Fig. 2 Activities (U g^{-1} FW) of SOD (**a**–**c**), POD (**d**–**f**), GR (**g**–**i**), GPX (**j**–**l**), CAT (**m**–**o**), APX (**p**–**r**) in roots of XZ16 (*left* panel), XZ61(*middle*) and Dayton (*right*)exposed to 100 μM Al. Data are means ± SD (n = 3).NP(6.0), NP(4.3); and LP + Al, NP + Al, HP + Al correspond to basic nutrition solution (BNS, containing normal P of 180 μM KH_2PO_4) at pH 6.0, BNS at pH 4.3; and at pH 4.3 under BNS (containing 90 μM P) + 100 μM Al, BNS (180 μM P) + 100 μM Al, BNS (360 μM P) + 100 μM Al

Effect of P on organic acid secretion in three barley genotypes under Al stress

After 20 d of Al treatment, with the increase of P level in Al stress the secretion of citric acid in Al tolerant genotypes XZ16 and Dayton was about 2–3 times more to Al sensitive genotype XZ61, and there was no significant difference between Dayton and XZ16 (Fig. 6). However, the secretion

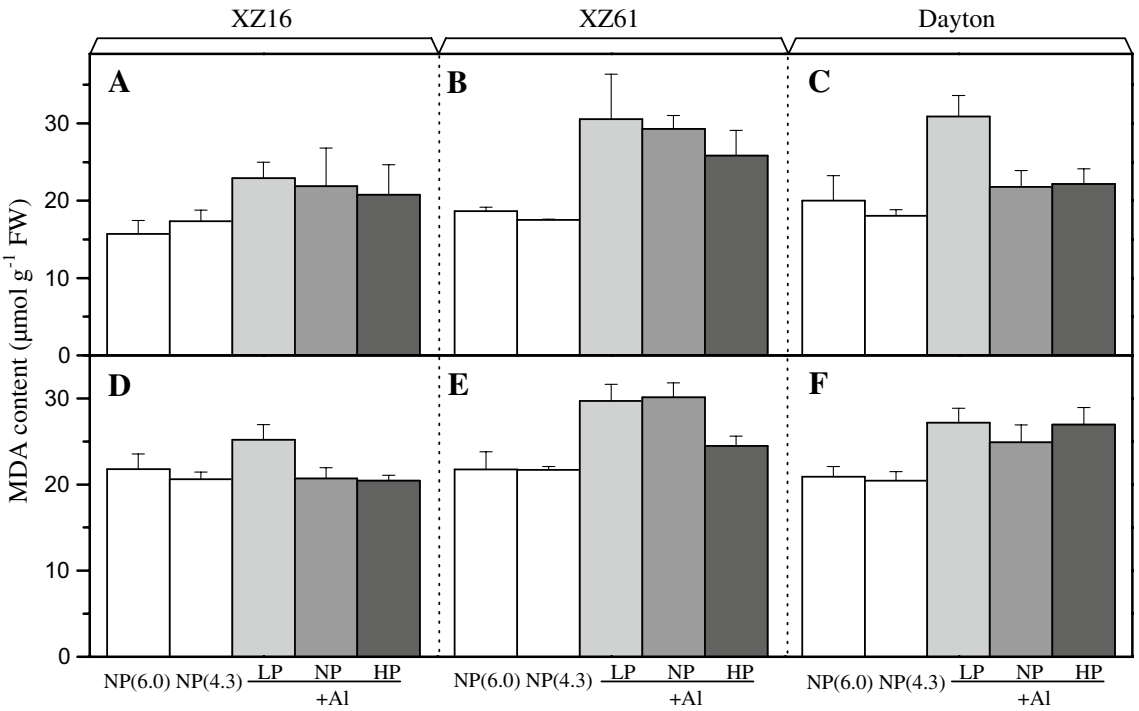

Fig. 3 MDA content in barley roots (*upper*) and leaves (*below*) of XZ16 (*left* panel), XZ61(*middle*) and Dayton (*right*) exposed to 100 μM Al. Data are means ± SD (n = 3). NP(6.0), NP(4.3); and LP + Al, NP + Al, HP + Al correspond to basic nutrition solution (BNS, containing normal P of 180 μM KH_2PO_4) at pH 6.0, BNS at pH 4.3; and at pH 4.3 under BNS (containing 90 μM P) + 100 μM Al, BNS (180 μM P) + 100 μM Al, BNS (360 μM P) + 100 μM Al

of citric acid in XZ16 was increased in HP + Al, while in Dayton and XZ61 was not changed with the increase of P level in Al stress. In addition, the secretion of malic acid in XZ16 was significantly higher than that of Dayton and XZ61 under Al stress, but it did not show significant change with the increase of P level in 3 genotypes.

Effect of P on the content of mineral elements in three barley genotypes under Al stress

Al stress significantly affected the absorption and distribution to macro- and micro-nutrients in barley, and there were significant differences among the genotypes (Table S3; Table S4 and Table S5). With the increase of P level, the contents of K and Mo in barley roots were significantly increased, and that of Mn and Cu were significantly decreased in XZ16, while those in Dayton and XZ61 were not significantly different from control (Table S3). Meanwhile, the contents of Mo and S were significantly increased and the contents of Cu, Mg, Zn, Mn and Ca were significantly decreased at pH 4.3 rather than pH 6.0, which indicated that low pH conditions for a long time could be unfavorable for the growth of barley.

The contents of Mo, Mn, Mg and Ca in the barley stems were decreased under Al treatment and the contents of Fe and Cu in XZ16 were decreased by 33.5 and 41.1% at low P concentration, but there was no significant change in XZ61 and Dayton (Table S4). With the increase of P level in Al treatment, the contents of P, Mg, Mn, S, Fe, Zn and other elements in XZ16 increased continually, while in Dayton and XZ61, only the content of Ca, Mg and P increased significantly.

The contents of Mn, Mg and Ca in barley leaves were significantly decreased under Al stress; however, K in XZ61 and Dayton increased significantly, while K content had no significant change in XZ16. On the other hand, the content of Zn in XZ61 and XZ16 decreased significantly, but the change in Dayton was not obvious; Al stress decreased the Cu content in XZ61 and Dayton, but had no effect on XZ16 (Table S5). Among three different P level in Al stress, macroelements except K and microelements in XZ16 showed an increasing trend to the control level, while only contents of P, Mg and Ca showed an increasing trend in XZ61.The contents of P, Mg and Fe increased significantly in Dayton, which may indicated that content of P, Mg, Ca, Fe in barley leaves was positively correlated with barley tolerance to Al.

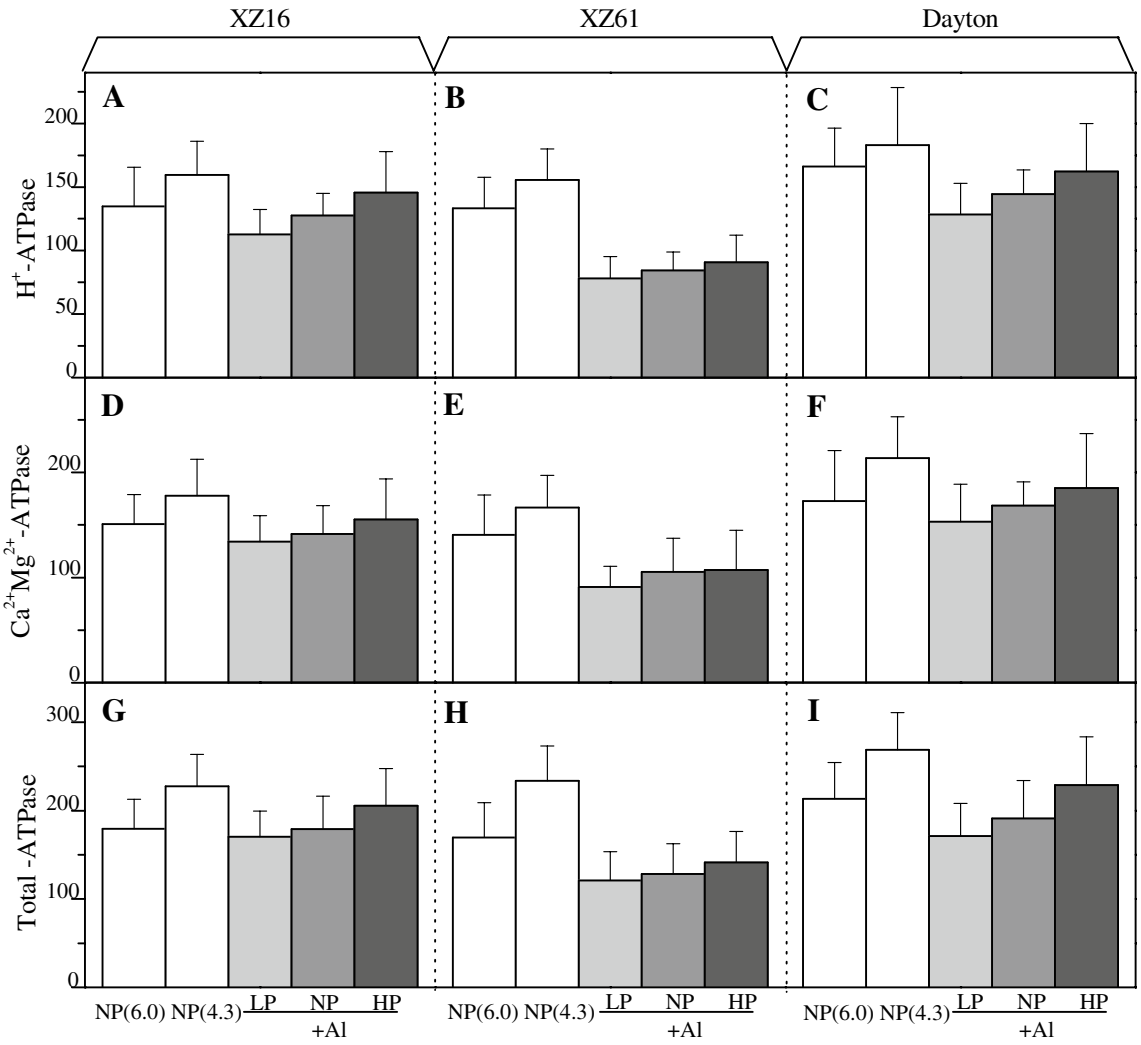

Fig. 4 H^+-(**a–c**), $Ca^{2+}Mg^{2+}$-(**d–f**), and Total-ATPase activities (**g–i**) (μmol pi g^{-1} FW h^{-1}) in roots of XZ16 (*left* panel), XZ61 (*middle*) and Dayton (*right*) exposed to 100 μM Al. Data are means ± SD (n = 3). NP(6.0), NP(4.3); and LP + Al, NP + Al, HP + Al correspond to basic nutrition solution (BNS, containing normal P of 180 μM KH_2PO_4) at pH 6.0, BNS at pH 4.3; and at pH 4.3 under BNS (containing 90 μM P) + 100 μM Al, BNS (180 μM P) + 100 μM Al, BNS (360 μM P) + 100 μM Al

Discussions

Aluminum inhibits the growth of crops in acid soil, as well as deficiencies of phosphorus (P) being the major limiting nutrient due to toxicity of aluminum (Hairiah et al. 1995). Many studies showed that exogenous P can relieve the stress level of plants suffered from Al stress (Tan and Keltjens 1990; Gaume et al. 2001; Nakagawa et al. 2003; Liao et al. 2006; Zheng et al. 2005). In the present study, barley plants exposed to 100 μM Al for 20 days showed a significant decrease in dry weight, with the largest reduction in the low P level (LP + Al) (Table S1) and sensitive genotype XZ61. With the increase of P level of 180 and 360 μM P (NP + Al, HP + Al), the root, stem and leaf dry weights were significantly relieved; and the highest increase was recorded in XZ16. Our result was consistent with the previous findings that P can alleviate the growth of barley under Al stress and Al tolerance was superior in resistant cultivar than sensitive cultivar. Compared with the Al-sensitive XZ61, XZ16 recorded significantly higher P accumulation in plants, and P concentrations in leaves under Al stress, and in stems under NP + Al and HP + Al. (Fig. 1, S1). Indicating that Al tolerance of XZ16 is associated with its higher leaf/stem P concentrations, which was more closely related to the alleviating effect of P on Al toxicity. i.e. maintaining shoot P concentration is important concerning the alleviative effect of P on Al toxicity.

Compared with the Al-sensitive XZ61, XZ16 recorded significantly higher P accumulation in plants, and P concentrations in leaves under Al stress, and in stems under

Table 1 Effect of P supply on Al concentration in roots, stems and leaves of three barley genotypes exposed to 100 μM Al for 20 days

Genotype	Treatment	Al concentration (mg kg^{-1}DW)					
		Root		Stem		Leaf	
XZ16	NP(6.0)	174.5	c	110.2	b	31.1	b
	NP(4.3)	164.6	c	96.5	b	29.5	b
	LP ± Al	1305.1	b	238.2	a	57.1	a
	NP ± Al	1492.1	a	237.9	a	71.7	a
	HP ± Al	1503.9	a	236.0	a	62.0	a
XZ61	NP(6.0)	175.2	b	99.6	b	43.4	b
	NP(4.3)	155.4	b	75.8	b	39.8	b
	LP ± Al	1490.7	a	227.3	a	76.9	a
	NP ± Al	1470.6	a	282.1	a	67.8	ab
	HP ± Al	1478.4	a	318.7	a	66.2	ab
Dayton	NP(6.0)	142.9	b	83.4	c	27.6	b
	NP(4.3)	129.7	b	77.7	c	33.6	b
	LP ± Al	1330.3	a	228.0	a	62.5	a
	NP ± Al	1453.6	a	189.9	ab	87.4	a
	HP ± Al	1399.7	a	162.2	b	72.2	a

NP(6.0), NP(4.3); and LP + Al, NP + Al, HP + Al correspond to basic nutrition solution (BNS, containing normal P of 180 μM KH_2PO_4) at pH 6.0, BNS at pH 4.3; and at pH 4.3 under BNS (containing 90 μM P) + 100 μM Al, BNS (180 μM P) + 100 μM Al, BNS (360 μM P) + 100 μM Al. Means with the same letter are not significantly different at $P = 0.05$ among the 5 treatments at each of the 3 genotypes

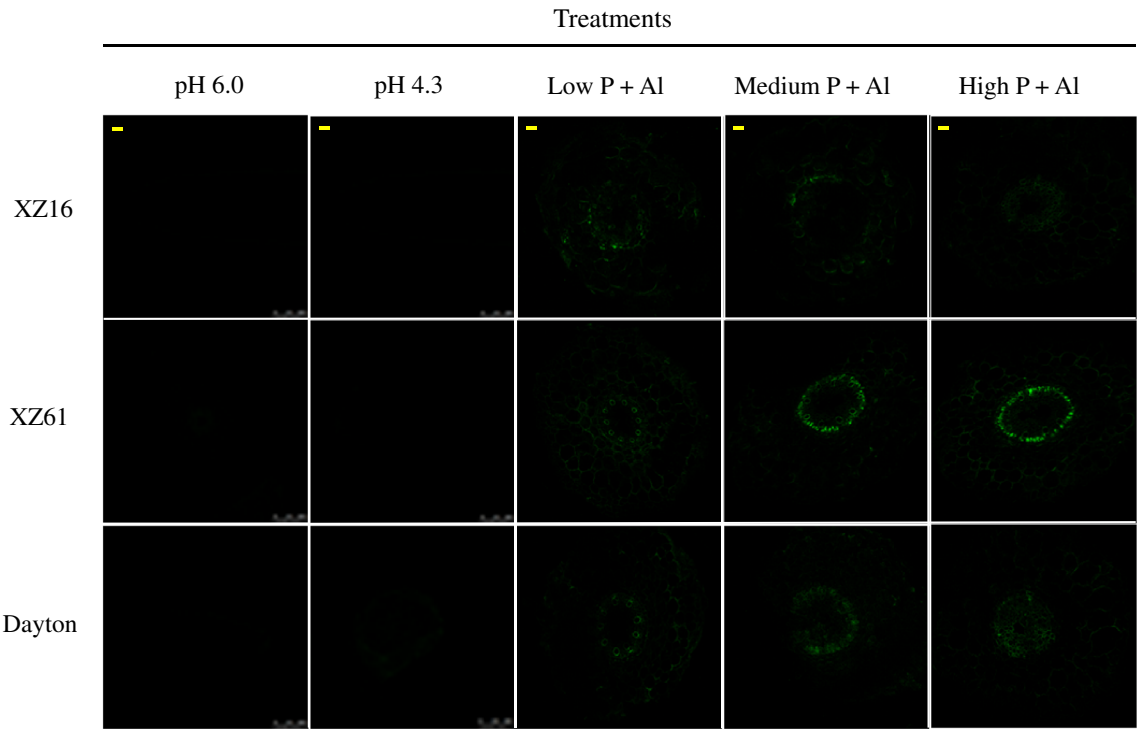

Fig. 5 Al localization in barley roots exposed to 100 μM Al, monitored by morin fluorescence using confocal laser scanning microscopy. NP(6.0), NP(4.3); and LP + Al, NP + Al, HP + Al correspond to basic nutrition solution (BNS, containing normal P of 180 μM KH_2PO_4) at pH 6.0, BNS at pH 4.3; and at pH 4.3 under BNS (containing 90 μM P) + 100 μM Al, BNS (180 μM P) + 100 μM Al, BNS (360 μM P) + 100 μM Al

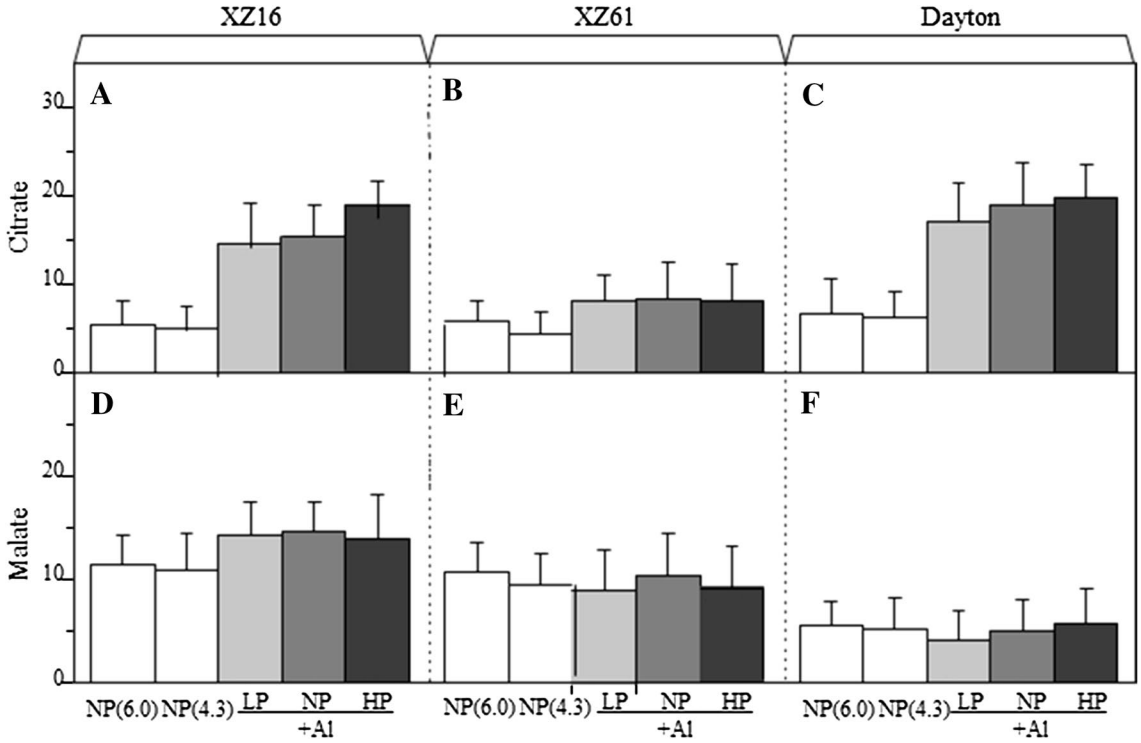

Fig. 6 Citrate (**a**–**c**) and malate (**d**–**f**) secretions (nmol g^{-1} FW) from roots of XZ16 (*left* panel), XZ61 (*middle*) and Dayton (*right*) exposed to 100 μM Al. Data are means ± SD (n = 3). NP(6.0), NP(4.3); and LP + Al, NP + Al, HP + Al correspond to basic nutrition solution (BNS, containing normal P of 180 μM KH_2PO_4) at pH 6.0, BNS at pH 4.3; and at pH 4.3 under BNS (containing 90 μM P) + 100 μM Al, BNS (180 μM P) + 100 μM Al, BNS (360 μM P) + 100 μM Al

NP + Al and HP + Al. (Fig. 1, S1), which was also consistent with the report that Al resistant rice (Sivaguru and Paliwal 1993) or wheat (Ramirez and Lopez 2000) cultivars were more efficient in P than the corresponding Al sensitive species. Indicating that Al tolerance of XZ16 is associated with its higher leaf/stem P concentrations, which was more closely related to the alleviating effect of P on Al toxicity. i.e. maintaining shoot P concentration is important concerning the alleviative effect of P on Al toxicity. The increasing Al accumulation in roots and P accumulation in plants was also consistent with the result of Jiang et al. 2009 which supported the hypothesis that P alleviated impairment of the whole photosynthetic electron transport chain from PSII donor side up to the reduction of end acceptors of PSI, preventing the decrease of CO_2 assimilation and thus relieved Al-induced inhibition of growth.

Additionally, Al resistant genotype XZ16 had a smaller decreasing amplitude on P, Ca, Mg, Fe than XZ61 under Al treatment, and increased as the contents of P increased(Table S3, S4, S5). The research showed that Ca played an important role in plant growth and development (Hossain et al. 2005). And Ca can alleviate Al toxicity by maintaining the stability of the biological membrane and the activity of H^+-ATP, Ca^{2+}-ATP and Mg^{2+}-ATP (Zhang et al. 2000). Mg is an important component of the chloroplast in leaf cells, plays a key role in the process of photosynthesis, and it is also a cofactor of ATP in many enzymatic reactions. Studies showed that Mg plays a very important role in alleviating inhibition of roots caused by Al in wheat (Ryan and Kochian 1993), soybean (Silva et al. 2001), sorghum (Keltjens 1988) and rice (Watanabe and Okada 2005). Fe mainly exists in plants in the form of Fe-P protein, which is called plant ferritin, while the 75% Fe in the cell combined with the chloroplast and plays a key role in the process of photosynthesis. Therefore, under Al stress, the addition of P in Al tolerant genotype XZ16 increased the uptake of other mineral elements such as Ca, Mg, Fe, and maintained the activity of H^+-, $Ca^{2+}Mg^{2+}$- and total -ATPases in roots and shoots at a high level (Fig. 4).

A series of changes also happened in the antioxidant enzyme activity of roots and shoots in barley while exposed to Al stress (Fig. 2), including that POD activity was significantly increased among three barley genotypes exposed to Al stress, which was found to be consistent with the findings of Simonovicova et al. (2004). Meanwhile, Al stress induced the production of CAT, SOD, APX, GPX, GR and other antioxidant enzymes in different plants (Pereira et al. 2010; Guo et al. 2007; Jeong and Kim 2004; Darko et al. 2004), but Al stress on different plants or different time in the same plant caused the different changes of antioxidant

enzymes, indicating the complexity of antioxidant system on Al toxicity alleviation. This study found that with the increase of P application, APX and GR activity changes in XZ16 were different from XZ61 and Dayton, which may be related to the specific tolerance to Al, but more research is needed. Besides, MDA content decreased with the increasing of P level, which indicated that the P addition may be in favor of alleviating membrane oxidative damage.

The main mechanism of many Al resistant plants is to secrete more organic acids such as citric acid, oxalic acid and malic acid to alleviate the toxicity of Al to the root system (Ma et al. 2001). Studies have indicated that the deficiency of P can also induce the secretion of malic acid and citric acid (Ryan et al. 2001). Ligaba et al. (2004) found that the lack of P prompted the purple lupin root to secrete more citric acid, rather than Al stress. Dong et al. (2004) reported that the interaction between P and Al in root system had an effect on the secretion of organic acids; P deficiency resulted in the secretion of oxalic acid and malic acid while Al stress stimulated the secretion of citric acid. Through the research of two high P efficient soybean cultivars, Liao et al. (2006) found taproot secreted more malic acid, application of P promoted the secretion of organic acid under Al stress, meanwhile, Al induced secretion of citric acid and low P induced oxalate secretion, while both would promote the secretion of malic acid. However, in *Lespedeza bicolor* pre-cultured by P, Sun et al. (2008) found that after the addition of P, citrate and malate secretion induced by Al was less than control plants without P, indicating that Al tolerance improving in the *Lespedeza bicolor* after the addition of P was unrelated to the secretion of organic acids in root. In this study, Al stress induced similar citric acid secretion between XZ16 and Dayton, but both are significantly higher than XZ61 (Fig. 6), which indicated that citric acid many play an important role in the process of alleviating Al toxicity. In addition, the malate acid secretion increased in XZ16 under Al stress, being markedly higher than in Dayton, while there was no such change in Dayton and XZ61. That might be one of the mechanisms that XZ16 chelated more Al cations by organic acids around rhizosphere and reduced the accumulation of Al in stems and leaves (Table 1).

Taken together, exogenous P directly promotes P accumulation in plants under Al stress, maintains the activity of H^+-, $Ca^{2+}Mg^{2+}$- and total -ATPases in roots and shoots at a high level, and increased the uptake of such other mineral elements as Ca, Mg and Fe, alleviates oxidative stress and membrane oxidative damage, and ultimately relieves Al stress. In addition, the Al tolerant mechanism of XZ16 may complete through higher P accumulation in plants and P concentration in leaves and stems, improved much more in activities of ATPases and the secretion of malate acid, and transferred more mineral elements such as P, Ca, Mg and Fe to the upper part, ensuring the normal operation of leaf photosynthesis and a series of ways.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

References

Ahmed IM, Dai H, Zheng W, Cao F, Zhang GP, Sun D, Wu FB (2013a) Genotypic differences in physiological characteristics in the tolerance to drought and salinity combined stress between Tibetan wild and cultivated barley. Plant Physiol Biochem 63:49–60

Ahmed IM, Cao FB, Zhang M, Chen XH, Zhang GP, Wu FB (2013b) Difference in yield and physiological features in response to drought and salinity combined stress during anthesis in tibetan wild and cultivated barleys. PLoS ONE 8(11):e77869

Cao FB, Chen F, Sun HY, Zhang GP, Chen ZH, Wu FB (2014) Genome-wide transcriptome and functional analysis of two contrasting genotypes reveals key genes for cadmium tolerance in barley. BMC Genom 15:611–611

Chen F, Wang F, Wu FB, Mao WH, Zhang GP, Zhou MX (2010) Modulation of exogenous glutathione in antioxidant defense system against Cd stress in the two barley genotypes differing in Cd tolerance. Plant Physiol Biochem 45:663–672

Dai HX, Shan WN, Zhao J, Zhang GP, Li C, Wu FB (2011) Difference in response to aluminum stress among Tibetan wild barley genotypes. J Plant Nutr Soil Sci 174:952–960

Darko E, Ambrus H, Stefanovits-Banyai E, Fodor J, Bakos F, Barnaba B (2004) Aluminium toxicity, Al tolerance and oxidative stress in an Al-sensitive wheat genotype and in Al-tolerant lines developed by in vitro microspore selection. Plant Sci 166:583–591

Dong D, Peng X, Yan X (2004) Organic acid exudation induced by phosphorus deficiency and/or aluminium toxicity in two contrasting soybean genotypes. Physiol Plant 122:190–199

Ellis RP, Forster BP, Robinson D, Handley LL, Russell JRG, Powell W (2000) Wild barley, a source of genes for crop improvement in the 21st century? J Exp Bot 51:9–17

Fukuda T, Saito A, Wasaki J, Shinano T, Osaki M (2007) Metabolic alterations proposed by proteome in rice roots grown under low P and high Al concentration under low pH. Plant Sci 172:1157–1165

Gaume A, Machler F, Frossard E (2001) Aluminum resistance in two cultivars of *Zea mays* L.: Root exudation of organic acids and influence of phosphorus nutrition. Plant Soil 234:73–81

Guo TR, Zhang GP, Zhou MX, Wu FB, Chen JX (2007) Influence of aluminum and cadmium stresses on mineral nutrition and root exudates in two barley cultivars. Pedosphere 17:505–512

Hairiah K, Vannoordwijk M, Setijono S (1995) Tolerance and avoidance of Al toxicity by *Mucuna pruriens* var. *utilis* at different levels of P supply. Plant Soil 171:77–81

Hossain AKMZ, Ohno T, Koyama H, Hara T (2005) Effect of enhanced calcium supply on aluminum toxicity in relation to cell

wall properties in the root apex of two wheat cultivars differing in aluminum resistance. Plant Soil 276:193–204

Jeong MS, Kim SH (2004) Aluminum stress in the roots of naked barley. J Plant Biol 47:65–74

Jiang HX, Chen LS, Zheng JG, Han S, Tang N, Smith BR (2008) Aluminum-induced effects on Photosystem II photochemistry in citrus leaves assessed by the chlorophyll a fluorescence transient. Tree Physiol 28:1863–1871

Jiang HX, Tang N, Zheng JG, Li Y, Chen LS (2009) Phosphorus alleviates aluminum-induced inhibition of growth and photosynthesis in *Citrus grandis* seedlings. Physiol Plant 137(3): 298–311.

Keltjens WG (1988) Short-term effects of Al on nutrient-uptake, H^+ efflux, root respiration and nitrate reductase-activity of 2 sorghum genotypes differing in Al-susceptibility. Commun Soil Sci Plant Anal 19: 1155–1163

Kochian LV, Hoekenga OA, Pineros MA (2004) How do crop plants tolerate acid soils?—Mechanisms of aluminum tolerance and phosphorous efficiency. Annu Rev Plant Biol 55:459–493

Kochian LV, Pineros MA, Hoekenga OA (2005) The physiology, genetics and molecular biology of plant aluminum resistance and toxicity. Plant Soil 46:237–260

Liao H, Wan HY, Shaff J, Wang XR, Yan XL, Kochian LV (2006) Phosphorus and aluminum interactions in soybean in relation to aluminum tolerance, exudation of specific organic acids from different regions of the intact root system. Plant Physiol 141:674–684

Ligaba A, Yamaguchi M, Shen H, Sasaki T, Yamamoto Y, Matsumoto H (2004) Phosphorus deficiency enhances plasma membrane H^+-ATPase activity and citrate exudation in greater purple lupin (*Lupinus pilosus*). Funct Plant Biol 31:1075–1083

Lin ZH, Chen LS, Chen RB, Zhang FZ, Jiang HX, Tang N (2009) CO_2 assimilation, ribulose-1, 5-bisphosphate carboxylase/oxygenase, carbohydrates and photosynthetic electron transport probed by the JIP-test, of tea leaves in response to phosphorus supply. BMC Plant Biol 9(1):43

Ma JF, Ryan PR, Delhaize E (2001) Aluminium tolerance in plants and the complexing role of organic acids. Trends Plant Sci 6:273–278

Marschner H (1991) Mechanisms of adaptation of plants to acid soils. Plant Soil 134:1–20

McCormick L, Borden F (1972) Phosphate fixation by aluminium in plant roots. Soil Sci Soc Am J 36:779–802

Nakagawa T, Mori S, Yoshimura E (2003) Amelioration of aluminum toxicity by pretreatment with phosphate in aluminum-tolerant rice cultivar. J Plant Nutr 26:619–628

Pereira LB, Mazzanti CMD, Goncalves JF, Cargnelutti D, Tabaldi LA, Becker AG, Calgaroto NS, Farias JG, Battisti V, Bohrer D, Nicoloso FT, Morsch VM, Schetinger MRC (2010) Aluminum-induced oxidative stress in cucumber. Plant Physiol Biochem 48:683–689

Ramaekers L, Remans R, Rao IM, Blair MW, Vanderleyden J (2010) Strategies for improving phosphorus acquisition efficiency of crop plants. Field Crops Res 117:169–176

Ramirez R, Lopez M (2000) Agronomic effectiveness of phosphate rock and superphosphate for aluminum-tolerant and non-tolerant sorghum cultivars. Commun Soil Sci Plant 31:1169–1178

Ryan PR, Kochian LV (1993) Interaction between aluminum toxicity and calcium uptake at the root apex in near isogenic lines of wheat (*Triticum aestivum* L.) differing in aluminum tolerance. Plant Physiol 102:975–982

Ryan P, Delhaize E, Jones D (2001) Function and mechanisms of organic anion exudation from plant roots. Annu Rev Plant Physiol Plant Mol Biol 52:527–560

Silva IR, Smyth TJ, Israel DW, Raper CD, Rufty TW (2001) Magnesium is more efficient than calcium in alleviating aluminum rhizotoxicity in soybean and its ameliorative effect is not explained by the Gouy-Chapman-Stern model. Plant Cell Physiol 42:538–545

Simonovicova M, Huttova J, Mistrik I, Siroka B, Tamas L (2004) Root growth inhibition by aluminum is probably caused by cell death due to peroxidase-mediated hydrogen peroxide production. Protoplasma 224:91–98

Sivaguru M, Paliwal K (1993) Differential aluminum tolerance in some tropical rice cultivars.2. Mechanism of auminum tolerance. J Plant Nutr 16:1717–1732

Sun QB, Shen RF, Zhao XQ, Chen RF, Dong XY (2008) Phosphorus enhances Al-resistance in Al resistant *Lespedeza bicolor* but not in Al-sensitive *L. cuneata* under relatively high Al stress. Ann Bot 102:795–804

Tan KZ, Keltjens WG (1990) Interaction between aluminium and phosphorus in sorghum plants 0.1. Studies with the aluminum sensitive sorghum genotype Tam428. Plant Soil 124:15–23

Tang QY, Feng MG (1997) Practical statistics and its DPS statistical software package. Agriculture Press, Beijing

Vance CP, Uhde SC, Allan DL (2003) Phosphorus acquisition and use: critical adaptations by plants for securing a nonrenewable resource. New Phytol 157:423–447

Watanabe T, Okada K (2005) Interactive Effects of Al, Ca and other cations on root elongation of rice cultivars under low pH. Ann Bot 95:379–385

Wu FB, Zhang G, Dominy P (2003) Four barley genotypes respond differently to cadmium: lipid peroxidation and activities of antioxidant capacity. Environ Exp Bot 50:67–78

Zhang F, Li X, Wang C, Shen Z (2000) Effect of cadmium on autoxidation rate of tissue and inducing accumulation of free proline in seedlings of mung bean. J Plant Nutr 23:357–368

Zhao Z, Ma JF, Sato K, Takeda K (2003) Differential Al resistance and citrate secretion in barley (*Hordeum vulgare* L.). Planta 217(5):794–800

Zheng SJ, Yang JL, He YF, Yu XH, Zhang L, You JF, Shen RF, Matsumoto H (2005) Immobilization of aluminum with phosphorus in roots is associated with high aluminum resistance in buckwheat. Plant Physiol 138:297–303