Response of Rice (*Oryza sativa*) with Root Surface Iron Plaque Under Aluminium Stress

RONG FU CHEN[1,2], REN FANG SHEN[1,*], PEI GU[1,2], XIAO YING DONG[1,2],
CHANG WEN DU[1] and JIAN FENG MA[3]

[1]*State Key Laboratory of Soil and Sustainable Agriculture (Institute of Soil Science, Chinese Academy of Sciences), Nanjing 210008, China,* [2]*Graduate School of the Chinese Academy of Sciences, Beijing 100039, China and* [3]*Research Institute for Bioresources, Okayama University, 2-20-1, Chuo, Kurashiki, 710-0046 Japan*

● *Background and Aims* Rice (*Oryza sativa*) is an aquatic plant with a characteristic of forming iron plaque on its root surfaces. It is considered to be the most Al-tolerant species among the cereal crops. The objective of this study was to determine the effects of root surface iron plaque on Al translocation, accumulation and the change of physiological responses under Al stress in rice in the presence of iron plaque.
● *Methods* The japonica variety rice, Koshihikari, was used in this study and was grown hydroponically in a growth chamber. Iron plaque was induced by exposing the rice roots to 30 mg L^{-1} ferrous iron either as Fe(II)-EDTA in nutrient solution (6 d, Method I) or as $FeSO_4$ in water solution (12 h, Method II). Organic acid in root exudates was retained in the anion-exchange resin and eluted with 2 M HCl, then analysed by high-performance liquid chromatography (HPLC) after proper pre-treatment. Fe and Al in iron plaque were extracted with DCB (dithionite–citrate–bicarbonate) solution.
● *Key Results and Conclusions* Both methods (I and II) could induce the formation of iron plaque on rice root surfaces. The amounts of DCB-extractable Fe and Al on root surfaces were much higher in the presence of iron plaque than in the absence of iron plaque. Al contents in root tips were significantly decreased with iron plaque; translocation of Al from roots to shoots was significantly reduced with iron plaque. Al-induced secretion of citrate was observed and iron plaque could greatly depress this citrate secretion. These results suggested that iron plaque on rice root surfaces can be a sink to sequester Al onto the root surfaces and Fe ions can pre-saturate Al-binding sites in root tips, which protects the rice root tips from suffering Al stress to a certain extent.

Key words: Rice (*Oryza sativa*), formation of iron plaque, root surface, Fe(II)-EDTA, aluminium (Al) stress, citrate secretion, DCB (dithionite–citrate–bicarbonate), root tip, Al translocation.

INTRODUCTION

Approximately 40–50 % of the world's potentially arable soils are acidic, and aluminium (Al) toxicity has been recognized as one of the most important limiting factors of plant productivity on acidic soils (Kochian *et al.*, 2004). Al toxicity was also deemed to be one of the major concerns of low rice productivity in acid upland and lowland acid sulfate soils (IRRI, 1978; Fageria and Carvalho, 1982; Vasconcelos *et al.*, 2002).

Rice (*Oryza sativa*) is an aquatic plant with the characteristic of forming iron plaque on its root surfaces due to its capability to release oxygen to the rhizosphere, and that results in the oxidation of ferrous to ferric iron and the precipitation of iron oxide/hydroxide on the root surface. Rice cultivars, growth stage and soil type were significantly related to the amount of iron plaques on field-grown rice roots (Chen *et al.*, 1980*a*). In wetland fields, iron (Fe) forms precipitates on root surfaces, which is composed mainly of goethite for *Juncus bulbosus* (Chabbi, 1999) or predominantly of ferrihydrite (approx. 63 %) for *Phalaris arundinacea* (Hansel *et al.*, 2001) depending on the species and growing conditions. In field and laboratory conditions, iron plaque was present as a continuous precipitate (Hansel *et al.*, 2001) or an amorphous coating on roots with an uneven distribution (Batty *et al.*, 2000, 2002).

It has been proposed that the presence of iron plaque may act as a barrier, buffer or something else to reduce the uptake of potentially phytotoxic metals and metalloids into plant tissues (Greipsson, 1994, 1995; Batty *et al.*, 2000; Liu *et al.*, 2004*a*). Rice is generally considered to be the most Al-tolerant species among the cereal crops (Foy, 1988; Wagatsuma *et al.*, 1988). There have been several studies to explore the Al tolerance mechanisms on genetic bases (Wu *et al.*, 2000; Nguyen *et al.*, 2001, 2002; Ma *et al.*, 2002). Iron plaque on aquatic plant roots is ubiquitous (Hansel *et al.*, 2001); however, the response of rice with root surface iron plaque under Al stress remains unknown. Thus the objective of this study was to determine the effects of root surface iron plaque on Al translocation, accumulation and the change of physiological responses under Al stress in rice in the presence of iron plaque.

MATERIALS AND METHODS

Plant materials and seedling growth

The *Oryza sativa* japonica variety rice, 'Koshihikari', was used in this study. Seeds were soaked in deionized water

* For correspondence. E-mail rfshen@issas.ac.cn

for 24 h and then germinated on nets that were floated on 0·5 mM CaCl₂ (pH 4·5) solution. After growth at 30 °C for 5 d, uniform seedlings were selected and transplanted to 1 L pots (15 seedlings per pot) containing half-strength Kimura B nutrient solution (modified from Kimura B macronutrients and Arnon micronutrients). The nutrient solution contained the macronutrients (mM): $(NH_4)_2SO_4$ (0·18), $MgSO_4.7H_2O$ (0·27), KNO_3 (0·09), $CaNO_3.4H_2O$ (0·18) and KH_2PO_4 (0·09), and the micronutrients (μM): Na_2EDTA-Fe(II) (20), $MnCl_2.4H_2O$ (9), H_3BO_3 (46), $Na_2MoO_4.4H_2O$ (9), $ZnSO_4.7H_2O$ (0·7) and $CuSO_4.5H_2O$ (0·3). The pH of this solution was adjusted to 4·5 using 0·1 M HCl; the solution's volume was restored daily with the same nutrient solution and renewed every 3 d. The plants were grown in a controlled environment growth chamber (Microclim-1000, Snijders Scientific, Tilburg, The Netherlands) with a 14 h/30 °C day and 10 h/20 °C night regime, light intensity of 375 μmol m⁻² s⁻¹ and 65 % relative humidity.

Experimental treatments

Previous studies showed that there are a number of methods for inducing the formation of iron plaques, and different culture methods might result in different results (Greipsson, 1994; Ye *et al.*, 1997; Zhang *et al.*, 1999; Batty *et al.*, 2000, 2002; Liu *et al.*, 2004*a*, *b*; Chen *et al.*, 2005). Thus two methods for inducing iron plaque were used to find the most suitable one. After 2 or 3 weeks, iron plaque was induced on the rice roots as follows.

Method I. Two-week-old seedlings grown in half-strength Kimura B nutrient solution were used to induce the formation of iron plaque by 30·0 mg L⁻¹ Fe(II)-EDTA (+Fe) [the control (–Fe) was 1·12 mg L⁻¹ Fe(II)-EDTA] in half-strength Kimura B nutrient solution (pH 4·5) in a 1 L pot for 6 d, during which the solutions were changed twice and the same nutrient solutions were supplemented every day to keep the culture solution at the same volume.

Method II. Three-week-old seedlings grown in half-strength Kimura B nutrient solution were chosen as the materials. Prior to induction of iron plaque, all seedlings were placed in 0·5 mM CaCl₂ solution (pH 4·5) for 2 h to minimize any interference from other elements with Fe. They were then transferred to a freshly prepared 1 L solution containing 30·0 mg L⁻¹ ferrous iron as $FeSO_4.7H_2O$ (+Fe) or without ferrous iron (–Fe) for 12 h. The solution pH was adjusted to 4·5 or 5·0 with 0·1 M HCl or 0·1 M NaOH to determine a suitable pH value of the solutions for healthy root growth.

After iron plaque was induced, seedlings were placed in 0·5 mM CaCl₂ solution (pH 4·5) overnight and then exposed to 0·5 mM CaCl₂ solution (pH 4·5) with 50 μM AlCl₃ (+Al) or without Al (–Al) as described by Ma *et al.* (2002). After 24 h of Al treatment, rice plant materials and root exudates were harvested as described below. Each experiment was repeated at least twice with three replicates each.

Scanning electron microscopy (SEM) and energy dispersive X-ray spectrometric microanalysis (EDAX)

Roots pre-treated with Fe(II)-EDTA or $FeSO_4$ were harvested after Al treatment and were quick-frozen and freeze-dried. After coating with gold, root segments were investigated by scanning electron microscopy (SEM; Sirion 200, FEI, The Netherlands) and energy dispersive X-ray spectroscopy (EDAX; Genesis, EDAX Inc., USA).

Collection and analysis of root exudates

After 24 h exposure to 0·5 mM CaCl₂ solution (pH 4·5) with 50 μM AlCl₃ (+Al) or without Al (–Al), the rice was harvested and the solutions were collected and passed through a column (20 mL, Bio-RAD) filled with 5 mL of cation-exchange resin Amberlite IR-120 (H-form, 16–45 mesh), followed by a column filled with 2 g of anion-exchange resin AG 1-X8 (formate form, 100–200 mesh) for collection of root exudates. The organic acids retained in the anion-exchange resin were eluted with 2 M HCl, and the eluate was concentrated to dryness by a rotary evaporator at 40 °C. Then the residue was dissolved in 1 mL of ultra-pure water and detected by HPLC (LC-10AT *VP*, Shimadzu, Tokyo, Japan) equipped with a Shim-pack SCR-102H column (8·0 mm i.d. × 30 cm) according to Ma *et al.* (2002).

Extraction and determination of Fe and Al on the root surface

At harvest, the plant material was split into roots and shoots and rinsed thoroughly in distilled water. Then the iron plaque deposited on the root surface was extracted using the dithionite–citrate–carbonate (DCB) method of Taylor and Crowder (1983) and McLaughlin *et al.* (1985): The fresh roots were incubated for 3 h at 25 °C in 45 mL of a solution (pH 6·5) containing 0·27 M sodium citrate ($Na_3C_6H_5O_7.2H_2O$) and 0·11 M sodium bicarbonate ($NaHCO_3$), with the addition of 3·0 g of sodium dithionite ($Na_2S_2O_4$). $Na_2S_2O_4$ is a strong reducer in $NaHCO_3$ solution, which reduces Fe^{3+} to Fe^{2+}; while citrate in $Na_3C_6H_5O_7.2H_2O$ can form a complex with Al^{3+} and Fe^{2+}. Thus they remove iron plaques on root surfaces. After filtration, the extraction solution was then analysed for Fe and Al as described below, and roots and shoots were oven dried at 75 °C for 3 d and weighed before digestion.

Analysis of tissue elements

At harvest (before DCB extraction), ten roots tips (0–1 cm) in each pot were randomly selected and excised with a razor and then placed in a plastic tube (1·5 mL) containing 1 mL of 2 M HCl for extraction of total Al and Fe in root tips (Ma *et al.*, 2002). The tubes stood for at least 24 h with occasional shaking before determination of Al and Fe contents.

Dried plant materials were ground to a fine powder and digested with concentrated HNO_3 (heavy-metal grade). The
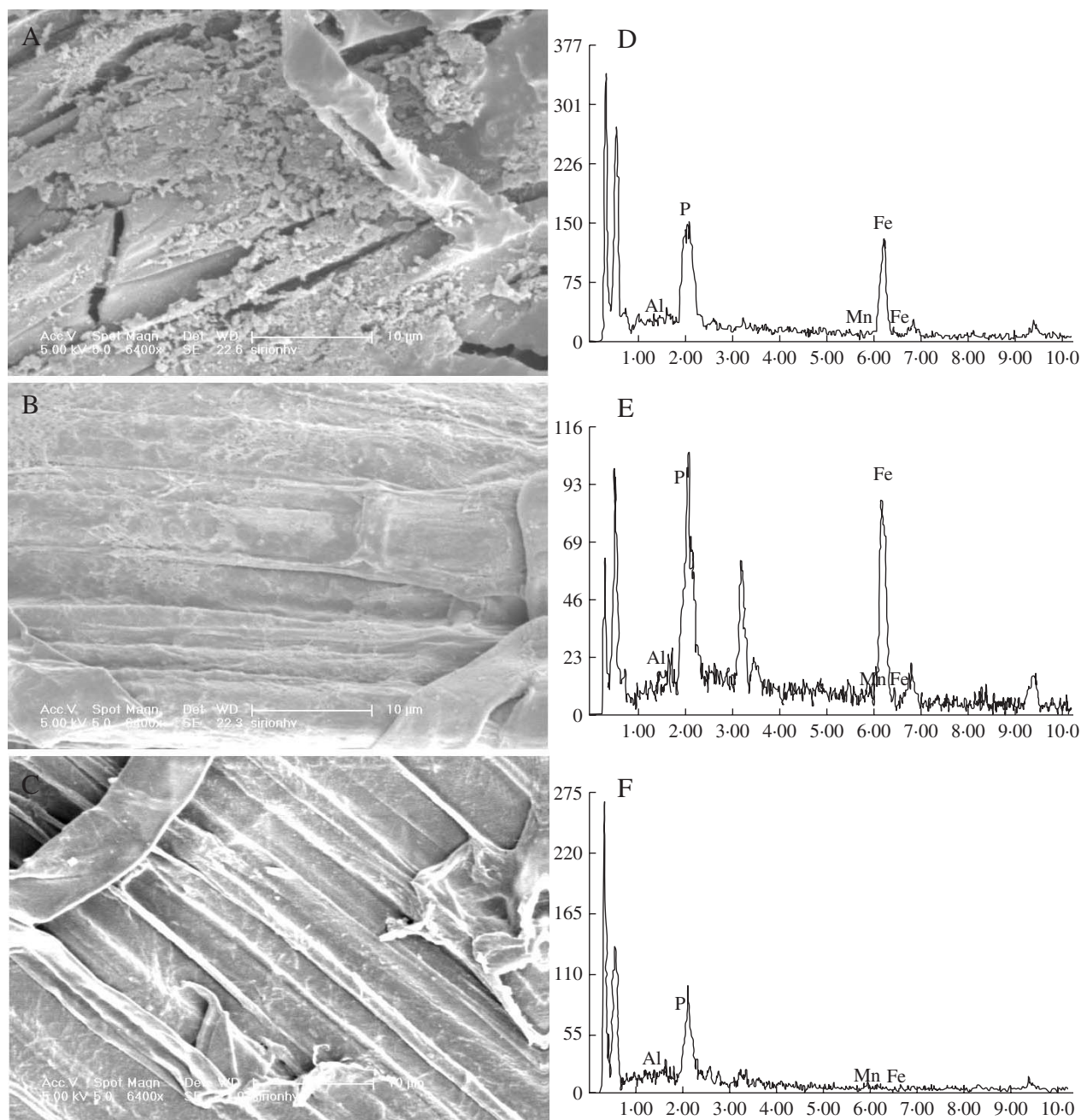
FIG. 1. Scanning electron micrographs (A, B and C) and energy-dispersive X-ray analysis (EDAX) (D, E and F) of precipitates formed on rice (Koshihikari) roots in hydroponic culture supplemented with iron or aluminium. (A) Rice was grown in Kimura B nutrient solution (pH 4·5) containing 30 mg L^{-1} Fe(II)-EDTA for 6 d (Method I). (B) Rice was pre-treated with a solution (pH 4·5) containing 30·0 mg L^{-1} FeSO$_4$ for 12 h (Method II). (C) Rice was not pre-treated with iron but was exposed for 24 h to 0·5 mM CaCl$_2$ solution (pH 4·5) containing 50 μM AlCl$_3$. (D), (E) and (F) correspond to (A), (B) and (C), respectively. These micrographs are representative of the general morphology and image.

concentrations of Al in digested solutions, concentrations of Al and Fe in the DCB extracts and root tip extractions were determined by ICP-AES (IRIS-Advantage, Thermo Elemental, MA, USA), after an appropriate dilution.

Statistical analysis

Statistical analyses were carried out on plant biomass, concentrations of metals and organic acid data using a one-way analysis of variance (ANOVA) in the SPSS statistical package.

RESULTS

Formation of iron plaque on rice root surfaces

Morphology of iron plaque on rice roots. After a 6 d pre-treatment with Fe(II)-EDTA in nutrient solution

(Method I), rice roots showed normal growth with good root development. Thin yellowish coatings appeared, indicating the formation of iron plaque, and SEM-EDAX studies also demonstrated that Fe(II)-EDTA induced the uneven distribution of iron plaque with a porous structure on the root surfaces (Fig. 1A, D). For Method II, after 12 h of pre-treatment with $FeSO_4$ in the water solution, plaque deposits were clearly visible as a dense and massive reddish coating on the root surfaces (Fig. 1B, E). There were visible differences between micrographs of precipitates formed in $FeSO_4$ solution and in Fe(II)-EDTA (Fig. 1A, B), but data from EDAX showed no significant differences for the key elements in iron plaque such as Fe and P (Fig. 1D, E). The roots with –Fe treatment remained white, indicating the absence of iron plaque (Fig. 1C, F). However, unlike the treatment with Fe(II)-EDTA, the root tips treated with $FeSO_4$ appeared bulgy and approx. 0–5 mm from the tip turned a brown colour, which showed that the root tips were injured.

Fe and Al contents in iron plaques of rice roots. The amounts of Fe on the root surfaces extracted by DCB under both methods increased remarkably after the induction of iron plaque. There was more Fe in Method I than in Method II (Fig. 2A). The Al concentrations in DCB extracts from both methods showed that there was significant enrichment of Al after Fe pre-treatment and there was more Al in Method I than in Method II (Fig. 2B).

Effect of pH on the formation of iron plaque and Al adsorption

The pH of the environment was regarded as one of the important factors in the formation of iron plaque and controlling uptake of metals (Batty *et al.*, 2000). In order to find out the reason for the root damage, a different pH regime of the treatment solution was set. The results showed that there were no visible differences in the degree of damage between pH 4·5 and 5·0 of the pre-treatment solution, i.e. the pH value of the solution should not be mainly responsible for the damage of root tips. The pH also had no significant effect on the formation of iron plaque and the adsorption of Al (data not shown).

Based on the information obtained thus far, Method I was chosen in this study and the following results were all based on the results using Method I.

Effect of iron plaque on the growth of rice seedlings

Throughout the experiment of Method I, rice seedlings showed normal growth, and no visible difference during growth was observed between +Fe and –Fe treatments. The dry weights of shoots and roots of rice seedlings were not significantly different between the presence and absence of iron plaques (Table 1), indicating that iron plaques had no significant effects on rice growth.

Effects of iron plaque on Al accumulation in roots and translocation from roots to shoots

Rice accumulated less Al in root tips in the presence of iron plaque than in the absence of iron plaque (Fig. 3). Al
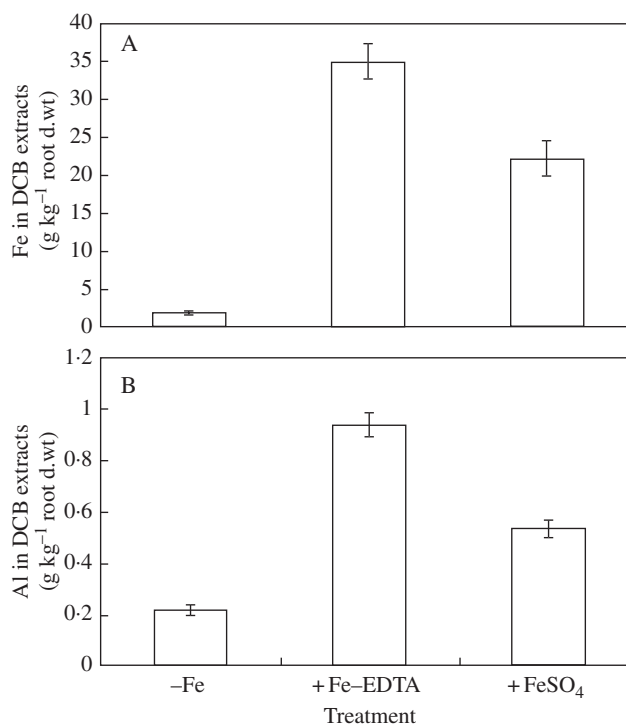

FIG. 2. Fe (A) and Al (B) concentrations in dithionite–citrate–bicarbonate (DCB) extracts. Two-week-old seedlings were pre-treated on the root surfaces with 30 mg L^{-1} Fe(II)-EDTA [the control (–Fe) was 1·12 mg L^{-1} Fe(II)-EDTA] in Kimura B nutrient solution (pH 4·5) for 6 d (Method I) or 3-week-old seedlings were pre-treated with water solution (pH 4·5) containing 30·0 mg L^{-1} $FeSO_4$ for 12 h (Method II). Error bars represent ± s.e. ($n = 3$).

TABLE 1. *Biomass (dry weight) of shoots and roots of 3-week-old rice (Oryza sativa) seedlings*

Treatment	Shoot (g)	Root (g)
–Fe	1·68 ± 0·07[a]	0·31 ± 0·02[a]
+Fe	1·48 ± 0·08[a]	0·27 ± 0·01[a]

Seedlings (5 d old) were transplanted to a 1 L pot containing half-strength Kimura B nutrient solution. After 2 weeks, the half-strength Kimura B nutrient solution containing 30·0 mg L^{-1} Fe(II)-EDTA was used to induce the formation of iron plaque for 6 d. Data are means (per 15 plants values) ± s.e. of three replicates. Means with the same superscript within columns do not differ significantly at $P = 0.05$ using one-way ANOVA.

contents in roots (the remaining Al after DCB extraction) showed no significant difference between treatments with and without iron plaque, while Al in shoots was lower with iron plaque although Al contents were much higher in roots than in shoots (Fig. 4).

Citrate exudation from rice roots and Fe contents in rice root tips

Citrate secretion was not detected unless the rice roots were exposed to Al stress in treatments both with and without iron plaques; however, it was remarkably depressed when there were iron plaques on the root surface (Fig. 5).
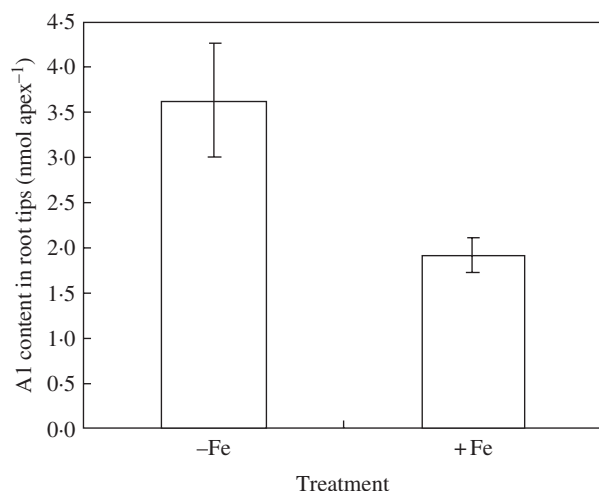
FIG. 3. Aluminium (Al) content in the root apex of rice (Koshihikari). When the seedlings [pre-treated with Fe(II)-EDTA] were harvested, root apices (1 cm) were excised and extracted with 2 M HCl. The Al concentration was detected by ICP-AES. Error bars represent ± s.e. ($n = 3$).

FIG. 5. Effect of iron plaque on the release of citrate from rice (Koshihikari) under aluminium (Al) stress. Fe(II)-EDTA (30·0 mg L^{-1}) was used to pre-treat the root surfaces for 6 d, and then the seedlings were exposed for 24 h to 0·5 mM CaCl$_2$ solution (pH 4·5) containing 50 μM AlCl$_3$, and root exudate was collected. Error bars represent ± s.e. ($n = 3$).
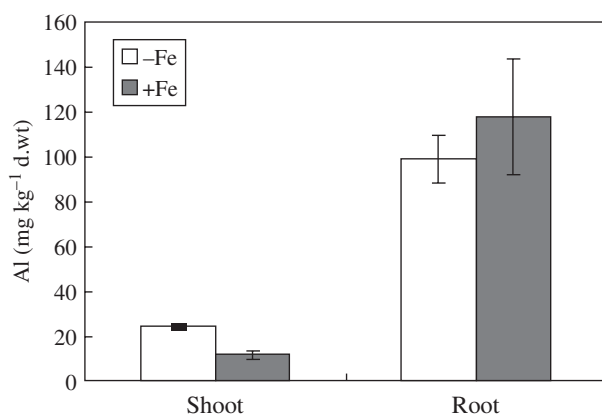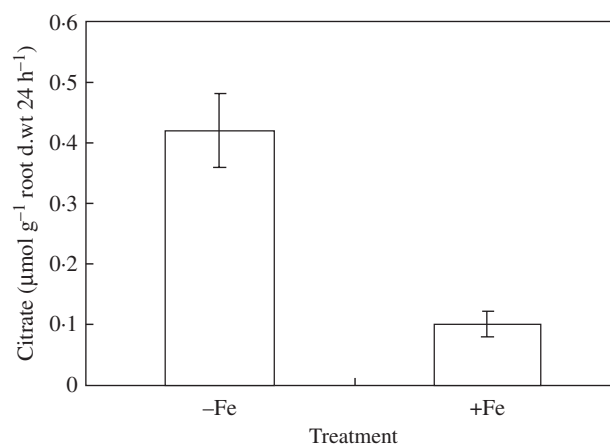

FIG. 4. Effect of iron plaque on the distribution of aluminium (Al) in whole roots and shoots. After extraction with DCB, the whole root and shoot were dried and digested with concentrated HNO$_3$. Error bars represent ± s.e. ($n = 3$).
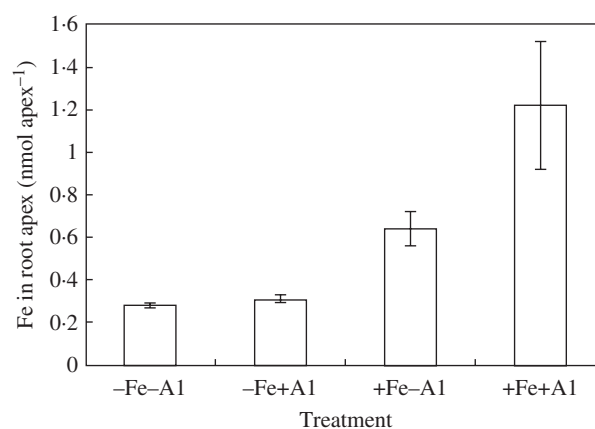

FIG. 6. Iron (Fe) content in the root apex of rice (Koshihikari). When the seedlings were harvested, root apices (1 cm) were excised and extracted with 2 M HCl. The iron concentration was detected by ICP-AES. Error bars represent ± s.e. ($n = 3$).

Fe contents in rice root tips extracted by 2 M HCl were significantly higher in the +Fe treatment. Al treatment had no significant effect on Fe content in both –Fe and +Fe treatments (Fig. 6).

DISCUSSION

Formation of iron plaque

The iron plaque on root surfaces is, in principle, facilitated by the release of oxygen and oxidants into the rhizosphere by aquatic plants; it could be induced in a number of ways in the field and laboratory depending on the purposes of the experiments. For example, in the laboratory, it can be induced by ferrous iron (FeSO$_4$) solution (Greipsson, 1994, 1995; Liu *et al.*, 2004*a*), by nutrient solution containing Fe(OH)$_3$ or FeSO$_4$ (Ye *et al.*, 1997; Zhang *et al.*, 1999) or by nutrient solution containing (NH$_4$)$_2$Fe(SO$_4$)$_2$, FeSO$_4$ or Fe(II)-EDTA, but not containing phosphate (Batty *et al.*, 2000, 2002; Liu *et al.*, 2004*b*). Some were induced under field conditions such as rice grown in flooded soil (Chen *et al.*, 1980*a*, *b*), *J. bulbosus*, the pioneer species of acid lakes (Chabbi, 1999), and *Phragmites australis* collected from a field contaminated by coal mine drainage (Batty *et al.*, 2000). In the present study, 30·0 mg L^{-1} Fe(II)-EDTA in half-strength Kimura B nutrient solution (pH 4·5) containing 0·09 mM phosphate was employed to pre-treat the rice root for 6 d; Fe concentrations in DCB extracts were enhanced greatly (Fig. 2A), and results from SEM-EDAX (Fig. 1) also showed the formation of iron plaque on the root surface in rice. Thus, Fe(II)-EDTA in nutrient solution could also induce the formation of iron plaque compared with the common method using FeSO$_4$, and the effects of the two methods of induction on the formation and Al

sequestration were analogous, although fewer iron plaques formed on the root surfaces in Method II than that in Method I and less Al was deposited on the root surfaces (Fig. 2). Since the targeting area for Al^{3+} toxicity is the root tip (Ryan *et al.*, 1993), it is crucial to keep the root tip healthy before it is subjected to Al stress; thus Method I was chosen to evaluate the effect of iron plaque on Al stress in rice in the present study.

A sink for Al in iron plaque

Iron plaque was regarded as a 'buffer' for arsenic (As) in the rhizosphere (Liu *et al.*, 2004*a*) and it might sequestrate As and consequently reduce the translocation of As from roots to shoots (Liu *et al.*, 2004*b*). In contrast, investigations into the growth of *Typha latifolia* demonstrated that roots with plaque adsorbed less nickel (Ni) (Ye *et al.*, 1997), and less cadmium (Cd) (Ye *et al.*, 1998) than those without. These discrepancies may be partly attributed to the differences in the culture methods, the metal/metalloid characters and the plant species. In our experiment, more Al was found on root surfaces with iron plaque than those without (Fig. 2B), indicating that Al might be sequestrated onto the root surfaces as a sink.

The chemical signature of coatings formed in the absence of Fe was dominated by Al and P (Fig. 1F) and, in the presence of Fe, the coatings comprised a combination of Fe, Al and P (Fig. 1D, E), suggesting that Fe, Al and P were co-precipitated. Batty *et al.* (2002) also found that Al was not adsorbed onto the surface of plaque but formed a separate phosphate deposit resembling the iron plaque in *P. australis*.

Al contents in rice root tips

The prime site of Al toxicity is the root tip (Ryan *et al.*, 1993). So what is the response of the root tips in the presence of iron plaques under Al stress? Chen *et al.* (1980*b*) reported that Fe plaques tended to form on older root parts rather than the more active root tips and fresh root hairs of rice. Batty *et al.* (2002) observed using SEM that iron plaques started approx. 1 cm behind the *P. australis* root tip and gradually thinned with distance from this region to the tip. Our results regarding iron plaque determined by EDAX also showed that less Fe was present on root tips than on other regions (data not shown). However, the Fe contents in root tips extracted with 2 M HCl did increase in +Fe treatment compared with –Fe treatment (Fig. 6); this might lead to the pre-saturation of Al-binding sites in root tips with Fe ions, and consequently contribute to depress the accumulation of Al in the root tips (Fig. 3). This reduction of Al contents in root tips could protect the rice root tips from suffering Al stress to a certain extent.

Effects of iron plaque on Al translocation in rice

Rice is not an Al accumulator; much less Al was accumulated in shoots (Chen and Shen, Institute of Soil Science, CAS, China, unpubl. res.) compared with shoots of buckwheat (Shen and Ma, 2001). DCB-extractable Al on root surfaces was increased significantly with iron plaque (Fig. 2B), but Al in the roots (the remaining Al after DCB extraction) was not influenced by iron plaque (Fig. 4), which suggested that iron plaque could not prevent the Al from entering the roots. However, Al in the shoots was reduced with iron plaque (Fig. 4). Thus one of the key effects of iron plaques might be to hold back Al translocation from roots to shoots, though the underlying mechanisms remains to be elucidated. This effect is consistent with the results of Liu *et al.* (2004*b*) who studied the role of iron plaque on As uptake in rice.

Depression of citrate exudation

Secretion of organic acid from plant roots when subjected to Al stress is considered as an important mechanism for plants to achieve high tolerance to Al (Ma, 2000). Anion channels, a kind of membrane-bound transport proteins existing on the plasma membrane, mediate the secretion of organic acid anion, and Al activates these anion channels by interacting directly with the channel protein or with a specific receptor on the membrane (Ma *et al.*, 2001). Less citrate was exuded from roots coated by iron plaque (Fig. 5). This might be explained partially by the high Fe contents in the root tip with iron plaque (Fig. 6), because competition between Al and Fe on the membrane of root tip cells may depress the ability for Al activation.

In conclusion, Fe(II)-EDTA in nutrient solution could also induce the formation of iron plaque on root surfaces of rice. Iron plaque on rice root surfaces can act as a sink for Al and sequester Al onto the root surfaces and reduce the translocation of Al from roots to shoots. Increasing Fe contents in root tips with Fe pre-treatment may pre-saturate the Al-binding sites and consequently depress the accumulation of Al in root tips. The Al-induced secretion of citrate could be greatly depressed with iron plaque. Further investigation is needed to see whether and how iron plaque on root surface contributes to Al tolerance in rice.

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