

The role of root anatomy, organic acids and iron plaque on mercury accumulation in rice

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Abstract

Background and aims Rice contaminated by mercury [Hg, especially methylmercury (MeHg)] has given rise to great concern in recent years. This study investigated variations in ecophysiological features (anatomy, organic acid secretions, Fe plaque formation) of rice roots and their effects on the uptake and accumulation of total mercury (THg) and MeHg by rice plants.

Methods The development of apoplastic barriers in roots of four rice cultivars was observed by a hydroponic experiment while the concentrations of five organic acids, Fe and THg in Fe plaque were determined using a rhizobag trial with different Hg treatments.

Results Cultivars with low Hg accumulation tended to develop strong apoplastic barriers in endodermis, secrete less organic acids and form more Fe plaque on root surfaces and in rhizosphere. Fe concentrations were positively correlated with THg concentrations in rhizosphere's Fe plaque ($R^2=0.60$, $P<0.01$), whereas the latter was negatively correlated with bioavailable Hg concentrations in rhizosphere ($R^2=0.40$, $P<0.01$).

Conclusions Organic acids and Fe plaque formation of rice roots play important roles in Hg uptake and accumulation. The development of apoplastic barriers in root restricts Hg uptake but the significance of suberin deposition on Hg uptake needs further investigations.

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Introduction

Mercury (Hg) is recognized as an extremely toxic global contaminant which has received considerable attention because of its accumulative and persistent nature (Jiang et al. 2006). Mercury can be methylated to the extremely toxic methylmercury (MeHg) under certain environmental conditions (Ullrich et al. 2001). The production of MeHg is of greater concern due to its higher toxicity to humans and its ability to be more readily biomagnified in trophic levels along food chains (Mergler et al. 2007). Recent studies have shown that rice (*Oryza sativa* L.), a staple food in Asia, is the

primary source of MeHg for people living in Hg-mining areas and also in certain inland areas in southwestern China (Feng et al. 2008; Zhang et al. 2010). There is therefore an urgency to develop effective management practices to reduce total Hg (THg) and MeHg in rice production, especially in Hg-contaminated regions.

Previous studies have reported that there are significant differences in the uptake and accumulation of THg and MeHg between rice cultivars (Peng et al. 2012; Rothenberg et al. 2012; Li et al. 2013). This implies that appropriate cultivar selection can be a possible way to reduce THg and MeHg levels in rice grains, but how to 'select' appropriate cultivars (or based on what features or characters) is still a question. From the limited studies in this area, the abilities to translocate Hg from straw to brown rice (Li et al. 2013), and from the caryopsis to the endosperm (Rothenberg et al. 2012) appeared to be the important factors affecting the levels of THg and MeHg in brown rice. However, the reasons for variations in THg and MeHg accumulation between rice cultivars are not fully understood.

Roots play a dominant role in the accumulation of toxic elements in plants as they have direct contact with these elements in soil and affect many uptake processes, particularly the uptake from soil and translocation to shoots or leaves through the xylem tissues (Mendoza-Cozatl et al. 2011; DalCorso et al. 2013). The rhizodermis, exodermis and endodermis of roots act as barriers to the entry of toxic elements such as zinc (Zn) and cadmium (Cd) into the xylem (Cheng et al. 2010; Lux et al. 2011). However, toxic elements can induce changes in root anatomy (Martinka and Lux 2004; Vaculik et al. 2009), and the responses of root anatomy to toxic elements differ between species (or genotypes) (Lux et al. 2004; Cheng et al. 2010). Cheng et al. (2010) reported that Zn induces the development of apoplastic barriers in roots of mangrove plants, and the species with thicker outer cell layers and more lignification in the epidermis possess a higher Zn tolerance. Lux et al. (2004) compared the development of endodermal Casparian bands (CBs) in different *Salix* clones, and observed that CBs in clones with high accumulation of Cd occurred more distant from the root tip than in clones with low accumulation. However, the role of root anatomy in Hg uptake and accumulation in the rice plant is still unclear, and how the responses of root anatomy to Hg differ between rice cultivars has never been reported.

Plants can alter the conditions in the rhizosphere through the secretory activities of their roots in order to

adapt to the local environment (Bais et al. 2006). Recent studies have shown that toxic elements such as aluminum (Al), Zn and Cd can induce changes in the amount and composition of organic acids secreted by roots (Guo et al. 2007; Xu et al. 2007; Zhu et al. 2011). The secreted organic acids can influence the mobility of toxic elements in the rhizosphere and then the uptake by plants (Cieslinski et al. 1998; Sasaki et al. 2004; Shen et al. 2005; Zeng et al. 2008). On one hand, these organic acids can help to 'exclude' toxic elements such as Al and lead (Pb) from roots through chelation or precipitation (Yang et al. 2000, 2006; Sasaki et al. 2004). On the other hand, they may form complexes with toxic elements and enhance their mobility and bioavailability, leading to higher uptake by plants (Liu et al. 2007; Zeng et al. 2008). The effects of organic acids on the uptake and accumulation of toxic elements seem to vary with both the plant species and the toxic element (Ma et al. 1997; Zeng et al. 2008; Zhu et al. 2011). The effects of organic acids secreted by roots on the uptake and accumulation of Hg by rice plants have never been reported although the amount and composition of organic acids secreted by rice roots are known to differ between cultivars and growth stages (Aulakh et al. 2001).

It has been widely observed that iron (Fe) plaque can be formed on root surfaces and in the rhizosphere of wetland plants including rice (Taylor et al. 1984; Chen et al. 2005; Yang et al. 2014). Fe plaque is able to sequester metal (loid)s by adsorption and/or co-precipitation, thus affecting the bioavailability of these elements in the rhizosphere, which can influence the metal tolerance and uptake by wetland plants (Ye et al. 1997; Heikens et al. 2007). Cheng et al. (2014) reported that Fe plaque can promote Pb and Cd deposition onto root surfaces, limit their transfer from root to above-ground tissues and subsequently reduce their distribution in rice grains. The amounts of Zn in Fe plaque in the rhizosphere are much larger than those on root surfaces, suggesting that Fe plaque in the rhizosphere may have a more important role than that on root surfaces (Yang et al. 2014). However, our recent study (Wang et al. 2014a) showed that it is the root tissue rather than Fe plaque on root surfaces that plays a dominant role in reducing Hg transfer from roots to above-ground tissues although this study did not investigate the effects of Fe plaque in the rhizosphere on the accumulation and translocation of Hg in rice plants.

Fe plaque formation on root surfaces and in the rhizosphere of wetland plants can be affected by Fe (III)-reducing bacteria (FeRB) (Weiss et al. 2003),

which have been established to control the Fe (III)-reducing process and are involved in the Fe cycle in non-sulphidogenic sedimentary environments, including paddy fields (Weber et al. 2006). FeRB have also been shown to be the dominant members of the rhizosphere microbial community (Weiss et al. 2003; Chen et al. 2008). FeRB can also be related to the methylation of Hg (Fleming et al. 2006; Kerin et al. 2006). This implies that the activities of FeRB may directly or indirectly affect the mobility and transformation of Hg in the rhizosphere, thus controlling the uptake and accumulation of Hg in rice plants.

Based on the above findings and observations, we hypothesize that low Hg-accumulating rice cultivars tend to develop stronger apoplastic barriers, secrete less organic acids and form more Fe plaque on root surfaces and in the rhizosphere, thus lowering the bioavailability of Hg in the rhizosphere and restricting the uptake of Hg by root tissues. In order to test this hypothesis, a hydroponic experiment was set up to observe the development of apoplastic barriers in roots of four rice cultivars possessing different abilities in Hg accumulation. A rhizobag trial was also conducted for determining the amounts of five organic acids and concentrations of Fe and THg in the Fe plaque of these cultivars. The present study investigated the variations in ecophysiological features of rice roots in different cultivars and evaluated their potential association with the uptake and accumulation of THg and MeHg. The results of this study will be important for the selection of appropriate rice cultivars for Hg-contaminated paddy fields.

Materials and methods

Pre-culture of rice seedlings

Four rice cultivars, Zixiang (ZX), Zhongdao 097 (ZD), Nanfeng (NF) and Wufengyou 128 (WFY) were selected based on their differences in Hg accumulation properties as shown in our previous study (Li et al. 2013). The first two cultivars, ZX ($28.5 \pm 2.2 \text{ ng g}^{-1}$ THg, $20.5 \pm 1.7 \text{ ng g}^{-1}$ MeHg) and ZD ($34.4 \pm 3.2 \text{ ng g}^{-1}$ THg, $12.7 \pm 3.1 \text{ ng g}^{-1}$ MeHg), have a higher Hg accumulation in brown rice than those in the latter two cultivars, NF ($18.4 \pm 0.7 \text{ ng g}^{-1}$ THg, $6.7 \pm 1.3 \text{ ng g}^{-1}$ MeHg) and WFY ($20.3 \pm 2.8 \text{ ng g}^{-1}$ THg, $6.8 \pm 1.7 \text{ ng g}^{-1}$ MeHg). Seeds were surface sterilized with 30 % v/v hydrogen peroxide (H_2O_2) for 30 min, washed thoroughly with deionized

water and germinated in acid-washed quartz sand for 7 days. Seedlings were subsequently transferred to plastic containers (12 L) and grown in 1/4-strength Hoagland's solution for 21 days with the following nutrient composition ($\mu\text{mol L}^{-1}$): NH_4NO_3 500, K_2SO_4 200, CaCl_2 400, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 1500, KH_2PO_4 1000, Fe-EDTA 50, H_3BO_3 10, $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ 1.0, $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ 1.0, $\text{MnSO}_4 \cdot 5\text{H}_2\text{O}$ 5.0, $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ 0.5 and $\text{CoSO}_4 \cdot 7\text{H}_2\text{O}$ 0.25. The nutrient solution was adjusted to pH 6.0 with NaOH or HCl, without any forced aeration, and changed every 5 days. The seedlings were grown in a growth cabinet with the following conditions: day/night temperatures 25/20 °C, relative humidity 60/80 % and 16 h of light with $>350 \mu\text{mol m}^{-2} \text{ s}^{-1}$ photon flux density.

Plant cultivation under hydroponic conditions

At the end of 28 days growth in the hydroponic conditions, seedlings of each of the four rice cultivars were transplanted into 1/4-strength Hoagland's solution (the same as mentioned above) containing four Hg treatments (added as HgCl_2): CK (no Hg added), 0.5, 1.0 and 2.5 mg L^{-1} . The nutrient solution was renewed and supplemented with Hg every 3 days. There were 4 replicates with 16 seedlings per treatment and the growth conditions were the same as described above. At the end of 2-weeks cultivation, the seedlings were harvested and divided into two batches. The first batch was used for the observation of apoplastic barriers in roots and the other batch was divided into roots and shoots, freeze-dried at $-50 \text{ }^\circ\text{C}$, weighed, ground to powder, and stored at $4 \text{ }^\circ\text{C}$ prior to further analysis. The concentration of Hg in each plant component was measured, with four replicates per treatment.

Histochemical studies to detect apoplastic barriers in the roots

Healthy adventitious roots were cross-sectioned at a distance of 5.0 cm from the root tip for the observations on the development of apoplastic barriers in the endodermis. This was based on the development of root endodermis and results as reported in previous studies. The development of the endodermis in plant roots can be distinguished in three consecutive stages. The primary developmental stage is characterized by the impregnation of lipophilic and aromatic substances (Casparian bands) into radial and transverse endodermal cell walls. In the secondary developmental stage, a thin, lipophilic suberin

lamella can be observed to deposit on the inner surface of radial and tangential walls of endodermal cells. According to the previous studies by Schreiber et al. (1999, 2005), the region about 5.0 cm from the root apex is a suitable site for the observations on the primary and secondary developmental stage of the endodermis. Our previous work (Wang et al. 2014b) also showed that the occurrence of Casparian bands could only be detected in the root apex but not the deposition of suberin lamella. Briefly, healthy adventitious roots were cross-sectioned at a distance of 5.0 cm from the root tip. The sections were stained with 0.1 % (*w/v*) berberine hemisulphate for 1 h and with 0.5 % (*w/v*) aniline blue for another hour (Kotula et al. 2009), then viewed under a fluorescence microscope (Zeiss, Germany) to detect the development of Casparian bands in the root endodermis.

Plant cultivation under soil conditions

Two soils, an Hg-contaminated soil (from Wanshan in Guizhou Province, southwest China) and a non-contaminated soil (from the campus of South China Agricultural University) were collected from the plough layer (0–20 cm) of paddy fields. The Hg-contaminated soil has a pH of 7.0 (measured in the water extract following the method of ISO 10390 (2005)), contained 4.8 mg g⁻¹ total C (Total Organic Carbon Analyzer, Shimadzu, Japan), 1.4 mg g⁻¹ total N, 1.4 mg g⁻¹ total P (Automated Discrete Analyzer, Smart Chem 200, Alliance, France), 46 µg g⁻¹ total Hg (THg) and 3.7 ng g⁻¹ MeHg (following method of USEPA 1630: 2001). The non-contaminated soil has a pH of 6.5, contained 8.4 mg g⁻¹ total C, 1.3 mg g⁻¹ total N, 1.0 mg g⁻¹ total P, and the THg concentration was below the National Guidance Limit for soils (0.5 µg g⁻¹) (Li et al. 2013). After air-drying, soils were homogenized, sieved to <10 mesh and supplemented with basal fertilizers at a rate of 125 mg N kg⁻¹ soil as (NH₂)₂CO, 80 mg P kg⁻¹ and 125 mg K kg⁻¹ soil as KH₂PO₄ and K₂SO₄, mixed thoroughly and equilibrated for a month.

Cylindrical nylon rhizobags were designed to separate rhizosphere from non-rhizosphere. Each was constructed of 30 µm nylon mesh, 12 cm in diameter and 15 cm in height, and separated into two halves by a thin plastic card in the centre. Each rhizobag was filled with 350 g dry sand (the same acid-washed quartz sand as used for seed germination), and was placed in the centre of a PVC pot (20 cm diameter and 18 cm high), filled with 1.3 kg paddy soils prepared as above. The system

was inundated with deionized water to a depth of around 2 cm above the soil surface for 2 weeks before planting. The seedlings of each of the four rice cultivars were carefully transplanted into the rhizobags, with one seedling in each half. A total of 80 pots were prepared (10 pots for each cultivar grown in each soil type). The pots were kept submerged using deionized water as described above and the growth conditions were the same as for the hydroponic culture.

Harvest and sampling

Rice plants of similar size were harvested at the ear emergence (about 70 days after transplanting) and maturation stages (about 100 days after transplanting), with four replicates of each cultivar at each stage in each treatment. Since the exudation rate of organic acids by rice roots was shown to be the highest at the ear emergence stage (Aulakh et al. 2001) and Fe plaque formation reached its peak at this stage and remained stable (Wang et al. 2014a), root exudates and Fe plaque were only collected and extracted at this stage in the present study. The two plants from a pot were separated and washed thoroughly with deionized water. One plant was used for the measurement of organic acids secreted by roots and the other was separated into roots, straw and panicle. The separated roots were used for dithionite-citrate-bicarbonate (DCB) extraction of Fe plaque and the determination of FeRB on root surfaces. After the DCB extraction, the roots, straw and panicle were freeze-dried at -50 °C, weighed for dry mass, ground to a fine powder and stored at 4 °C prior to analysis of THg and MeHg. The sand of the two halves in each pot were mixed, homogenized and then separated into two portions, one for DCB extraction of Fe plaque and the determination of bioavailable Hg after being freeze-dried at -50 °C, crushed to pass a 150-mesh sieve and stored at 4 °C. The second portion was used for determining the FeRB in the rhizosphere after freeze-drying and stored at -80 °C. At the maturation stage, the plants were separated into roots, straw, husk and brown rice, all of which were freeze-dried, weighed for dry mass, ground and stored at 4 °C prior to further analysis of THg and MeHg.

Collection and measurement of organic acids

The collection of organic acids was based on the methods described by Aulakh et al. (2001) and Zeng et al. (2008). In brief, an individual plant was placed in a

PVC pot containing 500 ml of 0.5 mM CaCl₂ solution (pH 5.10) in such a position that the complete root system was submerged in the solution. After 4 h, the solution containing the root exudates was collected from each pot. Each sample of exudate solution was successively filtered through a 0.45 µm membrane filter to remove root detritus and microbial cell debris, and then concentrated to a volume of 10 ml by freeze-drying at –50 °C, prior to the analysis of organic acids, including malic, lactic, oxalic, citric and succinic acid. The quantitative determination of these organic acids was carried out using a High-Performance Liquid Chromatography Mass Spectrometer (HPLC-MS, LCQ Deca XP, Thermo Fisher Scientific Inc, USA).

DCB extraction of Fe plaque on root surfaces and in the rhizosphere

Fe plaque on fresh root surfaces or on sand surfaces (the rhizosphere material) was extracted using the DCB method (Otte et al. 1989). In brief, roots or sand were incubated for 60 min in 30 ml DCB solution (0.03 M sodium citrate and 0.125 M sodium bicarbonate with the addition of 0.6 g sodium dithionite) at room temperature. After incubation, roots or sand were rinsed three times with deionized water and the rinsing was added to the DCB extract. Concentrations of THg and Fe in DCB-extracted solutions were determined by Atomic Absorption Spectroscopy (AAS, Z-5000, Hitachi, Japan) (for THg) and Inductively-Coupled Plasma Optical Emission Spectrometry (ICP-OES, Optima 2000 DV, Perkin Elmer, USA) (for Fe), respectively.

Determination of the abundance of FeRB by qPCR

Microbial community DNA was extracted from approximately 10 g of rhizosphere sand or 5 g root samples (with plaque) using a Power Max DNA extraction kit (MOBIO Laboratory, USA), with four replicates for each treatment. Quantitative-PCR (qPCR) assays targeting FeRB were performed, using the group specific primer sets and qPCR conditions as presented in Supplementary Data. The FeRB was determined by targeting the family Geobacteraceae and the genus *Shewanella* (Somenahally et al. 2011a, b). PCR reactions with 40 amplification cycles were conducted at the temperatures listed in the Supplementary Data. Melting curve analyses of the PCR products were performed after each assay to confirm the quality of PCR-

amplification. The qPCR was performed using a Lightcycler480 thermocycler (Roche, USA).

Chemical analysis

For THg analysis, plant samples were microwave-digested in concentrated HNO₃ (16 mol L⁻¹) and measured by atomic fluorescence spectrometry (AFS, Beijing Titan Instrument Co., Ltd.). For MeHg analysis, soil and plant samples were extracted by KBr-CuSO₄/solvent and KOH-methanol/solvent, respectively (Liang et al. 1996), and determined with a MERX Automatic Methylmercury System (Brooks Rand Laboratories, Seattle, WA), following method 1630 (USEPA, 2001). For the analysis of Hg bioavailability, a sequential extraction technique for soil and sediment was employed, and only the water-soluble fraction representing the ‘bioavailable inorganic Hg’ (Bloom et al. 2003) was considered. Blanks, tea standard material (GBW-08303) (China Standard Materials Research Center, Beijing, P.R. China) and lobster standard material (TORT-2) (National Research Council of Canada) were used for quality control. Recoveries of THg, Fe and MeHg from the standard materials and matrix spikes ranged from 95–102 %, from 97–104 %, and from 86–104 %, respectively.

Statistical analysis

Data were analyzed using the statistical software package SPSS 17.0 and summarized as means±standard errors (SE). Comparisons of Fe, THg and MeHg concentrations, organic acids amounts and FeRB numbers between rice cultivars were performed using one-way analysis of variance (ANOVA) followed by least significant difference (LSD) tests at the 5 % level. Coefficients of determination (R²) and significance probabilities (*P*) were computed for linear regression fits between THg/MeHg concentration and Fe concentration in Fe plaque, bioavailable Hg concentration and FeRB numbers.

Results

Growth and accumulations of THg and MeHg in rice plants

There were significant differences (*P*<0.05) between cultivars in the biomass of each part of the rice plants grown under both soil conditions (Table S1) and

hydroponic conditions (Table S2). Hg-contaminated soil significantly reduced the biomass of the above-ground parts of plants ($P < 0.05$), with 40–86 % decreases in the biomass of brown rice of cvs. ZX, ZD and WFY grown in the Hg-contaminated than those grown in the non-contaminated soil (Table S1).

Significant differences ($P < 0.05$) in both THg and MeHg concentrations in brown rice (Fig. 1) and other tissues (roots, straw, ears and husks) (Table S3) were detected between rice cultivars grown in Hg-contaminated soil, with higher levels of THg and MeHg in cvs. ZX and ZD than in cvs. NF and WFY. However, there were no significant differences between rice cultivars grown in non-contaminated soil. A similar phenomenon was also detected in the concentrations of THg in roots and shoots of rice plants grown under hydroponic conditions (Fig. S1), following the trend of cvs. ZD > ZX > WFY ~ NF.

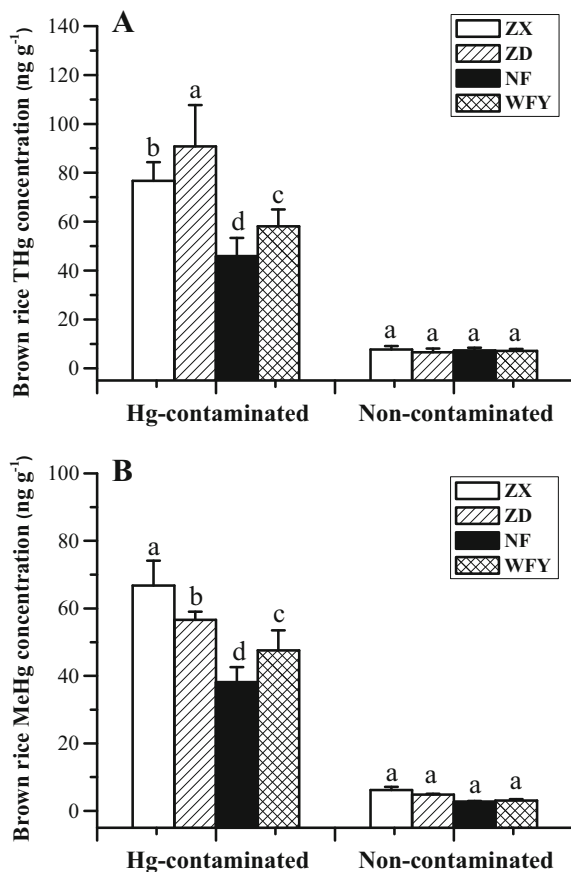


Fig. 1 THg (a) and MeHg (b) concentrations in brown rice of four rice cultivars (cvs. ZX, ZD, NF and WFY) grown in Hg-contaminated and non-contaminated soils (ng g⁻¹, mean ± SE, $n = 4$). Different letters within the same soil indicate significant differences between cultivars at the level of $P < 0.05$

Root anatomy: development of casparian bands (CBs)

As the root anatomy of the four rice cultivars grown in the 2.5 mg Hg L⁻¹ treatment was severely destroyed due to the toxicity of added Hg, it was difficult to observe the development of CBs under this condition. Thus, only the observations in the CK, 0.5 and 1.0 mg Hg L⁻¹ treatments are shown in Fig. 2. The dot-like and ‘U’-shaped green-yellow fluorescence indicated by white arrows refers to incomplete CBs, which are formed by the impregnation of lipophilic and aromatic substances into radial and/or transverse endodermal cell walls. The ring-like green-yellow fluorescence indicated by red arrows can be regarded as well-developed CBs, which are due to the deposition of a thin, lipophilic suberin lamella on the inner surface of radial and tangential walls of endodermal cells. For cvs. ZX and ZD, incomplete CBs were found in all treatments. However, for cvs. NF and WFY, the appearance of CB varied between treatments and cultivars. There were no obvious CBs in the CK treatment of both cultivars. In the 0.5 mg Hg L⁻¹ treatment, incomplete CBs occurred in the endodermis

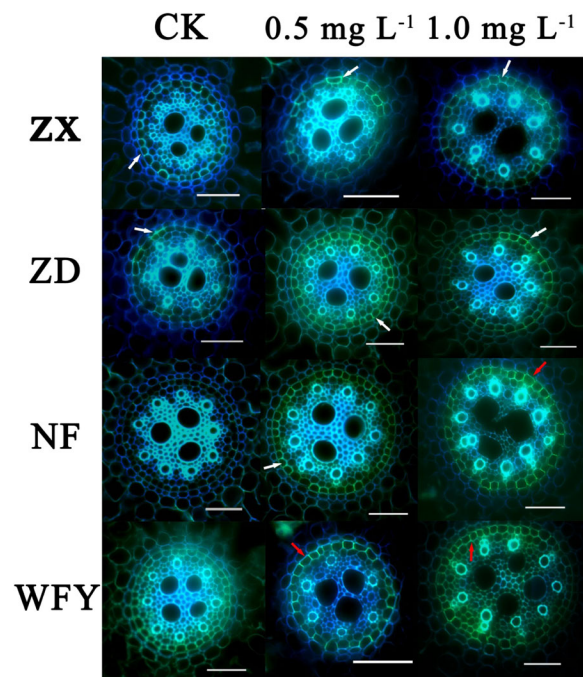


Fig. 2 Appearance of Casparian bands in the endodermis of roots of four rice cultivars (cvs. ZX, ZD, NF and WFY) in different Hg treatments (CK, 0.5, 1.0 mg Hg L⁻¹) under hydroponic culture. The presence of incomplete Casparian bands was indicated by white arrows, with well-developed Casparian bands were indicated by red arrows. Bars = 50 μm

of cv. NF but well-developed CBs were observed for cv. WFY. In the 1.0 mg Hg L⁻¹ treatment, well-developed CBs were detected in both cultivars.

Organic acids secreted by rice roots

The concentrations of the five organic acids declined in the order of malic>lactic>oxalic >>citric~succinic acids (Fig. 3 and Table S4). The concentrations of malic, lactic and oxalic acids secreted by rice roots grown in the Hg-contaminated soil, with mean values of 9.5, 0.45 and 0.14 $\mu\text{mol g}^{-1}$ d.w. root, respectively, were significantly higher ($P<0.05$) than those in the non-contaminated soil, with respective mean values of 3.9, 0.32 and 0.10 $\mu\text{mol g}^{-1}$ d.w. root. Significant differences in the concentrations of organic acids were observed between rice cultivars, with higher concentrations of malic, lactic and oxalic acids secreted by cvs. ZX and ZD than by cvs. NF and WFY grown in both Hg-contaminated soil and non-contaminated soils (Fig. 3). However, no obvious trend could be observed in the concentrations of citric and succinic acids between cultivars in both of the soils (Table S4).

Concentrations of Fe and THg in Fe plaque and abundance of FeRB

Fe concentrations in Fe plaque on root surfaces, ranging from 18 to 33 mg g⁻¹, were significantly higher ($P<0.05$) than those in the rhizosphere, ranging from 4.1 to 12 mg g⁻¹ (Table S5). Significant differences were detected in Fe concentrations in Fe plaque on root surfaces and in the rhizosphere between rice cultivars, following the trend of cvs. NF>WFY>ZD>ZX.

The concentrations of THg in Fe plaque on root surfaces and in the rhizosphere of rice plants grown under non-contaminated soil conditions were extremely low and did not show any significant differences between cultivars (data not shown). On the other hand, plants accumulated significant amounts of THg when grown in Hg-contaminated soil, with higher THg concentrations on root surfaces than in the rhizosphere (Fig. 4a). The THg concentrations in Fe plaque of cvs. NF and WFY were higher than those of cvs. ZD and ZX. Positive correlations were found between Fe concentrations and THg concentrations in Fe plaque on root surfaces ($R^2=0.59$, $P<0.01$) (Fig. 4b) and in the rhizosphere ($R^2=0.60$, $P<0.01$) (Fig. 4c). The THg content in each component (the product of the THg

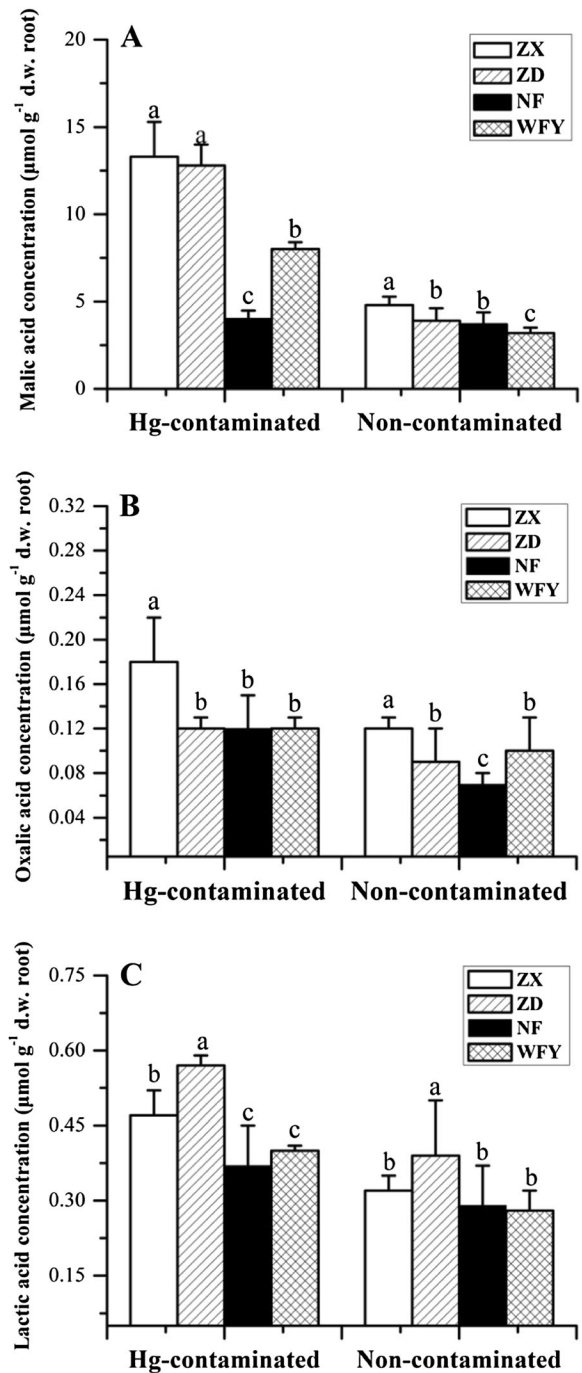


Fig. 3 Concentrations of malic acid (a), oxalic acid (b) and lactic acid (c) secreted by roots of four rice cultivars (cvs. ZX, ZD, NF and WFY) grown in Hg-contaminated and non-contaminated soils ($\mu\text{mol g}^{-1}$ d.w. root, mean \pm SE, $n=4$). Different letters within the same soil indicate significant differences between cultivars at the level of $P<0.05$

concentration and the dry weight of the component) expressed as a proportion of the total content of THg

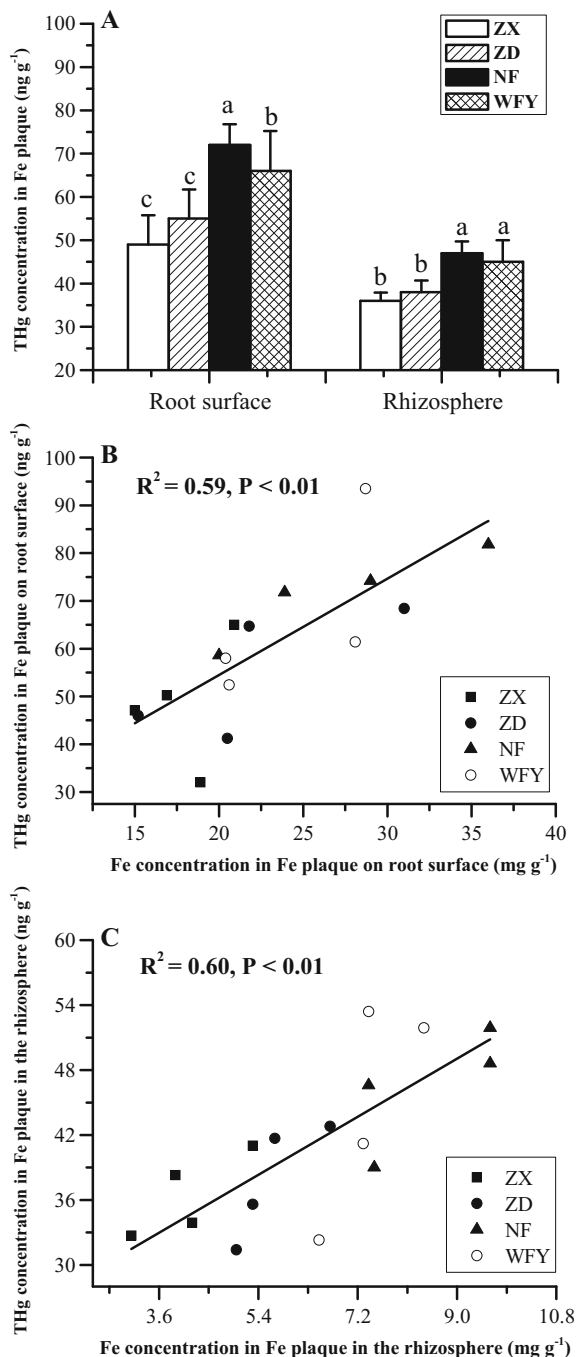


Fig. 4 Concentrations of THg in Fe plaque (a) (ng g⁻¹, mean±SE, n=4) and relationships between Fe concentrations and THg concentrations in Fe plaque on root surfaces (b) and in the rhizosphere (c) of four rice cultivars (cvs. ZX, ZD, NF and WFY) grown in Hg-contaminated soil. Different letters within the same item (root surface or rhizosphere) in Fig. 4a indicate significant differences between cultivars at the level of $P<0.05$

accumulated in whole plant, followed the trend of Fe plaque in the rhizosphere>root tissues>straw >>ear> Fe plaque on root surfaces (Fig. 5).

The abundance of FeRB on root surfaces and in the rhizosphere was represented as the numbers of the family Geobacteraceae and the genus *Shewanella* (Table 1). The numbers of Geobacteraceae were much higher (~1000 fold) than those of *Shewanella*. Significantly higher numbers of Geobacteraceae were observed in the rhizosphere (mean value: 9.6×10^5 copies g⁻¹ dry soil) than those on root surfaces (mean value: 2.1×10^5 copies g⁻¹ dry soil), whereas there was no significant difference in the numbers of *Shewanella* between the rhizosphere and root surfaces. Significant differences ($P<0.05$) in the numbers of Geobacteraceae were detected between rice cultivars, irrespective to whether in the rhizosphere or on root surfaces. The log number of *Geo* gene copies was negatively correlated with the Fe concentrations in Fe plaque in the rhizosphere ($R^2=0.63$, $P<0.01$) (Fig. 6).

Bioavailability of Hg in rhizosphere soil

The concentrations of bioavailable Hg in rhizosphere soil ranged from 163 to 267 ng g⁻¹ (Fig. 7a), accounting for 0.35–0.58 % of the THg content in the soil. Significant differences ($P<0.05$)

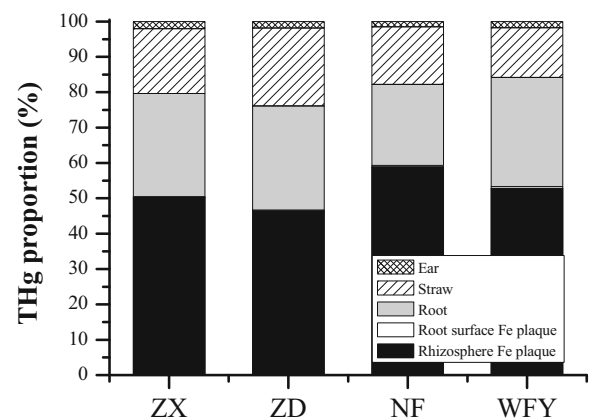


Fig. 5 Distribution of THg in different parts (ears, straw, roots and Fe plaque on root surfaces and in the rhizosphere) of four rice cultivars (cvs. ZX, ZD, NF and WFY) grown in Hg-contaminated soil at ear emergence stage (%)

Table 1 Numbers of FeRB on root surfaces and in the rhizosphere of four rice cultivars (cvs. ZX, ZD, NF and WFY) grown in Hg-contaminated and non-contaminated soils (mean±SE, n=4)

Soil	Cultivar	Numbers of FeRB (copies g ⁻¹ dry soil)			
		Geobacteraceae		<i>Shewanella</i>	
		Root surface	Rhizosphere	Root surface	Rhizosphere
Hg- contaminated	ZX	2.7×10 ⁵ ±9.0×10 ⁴ b	1.2×10 ⁶ ±2.0×10 ⁵ a	5.4×10 ³ ±6.2×10 ² b**	6.0×10 ³ ±1.1×10 ³ b**
	ZD	3.7×10 ⁵ ±1.0×10 ⁵ a**	1.4×10 ⁶ ±4.1×10 ⁵ a	4.2×10 ³ ±6.4×10 ² b**	3.9×10 ³ ±4.0×10 ² c**
	NF	2.1×10 ⁵ ±3.0×10 ⁴ c**	5.3×10 ⁵ ±2.3×10 ⁵ c**	1.0×10 ⁴ ±4.8×10 ³ a*	6.7×10 ³ ±1.8×10 ³ b**
	WFY	1.9×10 ⁵ ±2.7×10 ⁴ c	9.5×10 ⁵ ±2.8×10 ⁵ b*	9.2×10 ³ ±3.1×10 ³ a	1.0×10 ⁴ ±2.7×10 ³ a
Non- contaminated	ZX	2.3×10 ⁵ ±2.5×10 ⁴ a	1.4×10 ⁶ ±5.0×10 ⁵ a	3.4×10 ³ ±1.9×10 ² c	3.9×10 ³ ±6.4×10 ² c
	ZD	1.7×10 ⁵ ±3.1×10 ⁴ b	1.3×10 ⁶ ±8.9×10 ⁵ a	6.0×10 ³ ±3.5×10 ² b	6.2×10 ³ ±1.6×10 ³ c
	NF	6.6×10 ⁴ ±9.3×10 ³ c	1.5×10 ⁵ ±3.9×10 ⁴ c	1.4×10 ⁴ ±2.3×10 ³ a	2.7×10 ⁴ ±1.9×10 ⁴ a
	WFY	1.7×10 ⁵ ±3.2×10 ⁴ b	7.8×10 ⁵ ±2.1×10 ⁵ b	8.8×10 ³ ±1.9×10 ³ b	1.3×10 ⁴ ±6.0×10 ³ b

Different letters within the same column under the same soil conditions indicate significant difference between cultivars at the level of $P<0.05$. For each cultivar, * and ** indicate that the differences between the Hg-contaminated and non-contaminated soils were significant at $P<0.05$ and $P<0.01$, respectively

were observed in bioavailable Hg concentrations between rice cultivars, following the trend of cvs. ZD>ZX~WFY>NF. The THg concentrations in Fe plaque in the rhizosphere were negatively correlated with bioavailable Hg concentrations ($R^2=0.40$, $P<0.01$) (Fig. 7b), but were positively correlated with THg concentrations in brown rice ($R^2=0.69$, $P<0.01$) (Fig. 7c).

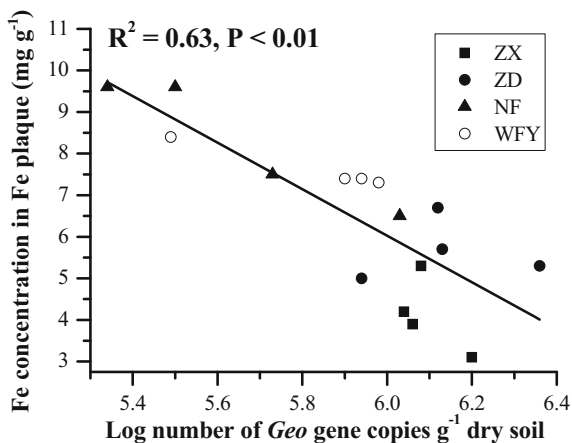


Fig. 6 Relationship between log number of *Geo* gene copies and Fe concentrations in Fe plaque in the rhizosphere of four rice cultivars (cvs. ZX, ZD, NF and WFY) grown in Hg-contaminated soil

Discussion

Response of root anatomy and influences on Hg accumulation

It has been proposed that apoplastic barriers play important roles in restricting the entry of toxic elements into the xylem and subsequent translocation (Lux et al. 2011; Wang et al. 2014b). In the present study, the responses of root anatomy to the increased Hg exposure differed between the four rice cultivars (Fig. 2). Cultivars such as NF and WFY tended to develop stronger apoplastic barriers, whereas the root anatomy of cvs. ZX and ZD showed no obvious changes as the concentration of Hg increased. Recent studies have shown that Hg was observed to be associated with cell walls, accompanied by their structural changes in *Medicago sativa* roots (Carrasco-Gil et al. 2013). Most of the Hg was co-localized with sulphur in forms similar to β -HgS and Hg-cysteine in root tissues of *Brassica juncea* (Wang et al. 2012) and *M. sativa* (Carrasco-Gil et al. 2011), respectively. This suggests that the better-developed apoplastic barriers in the endodermis at 5 cm from the root apex of cultivars such as cvs. NF and WFY may inhibit the uptake and transfer of Hg to the above-ground parts. However, the

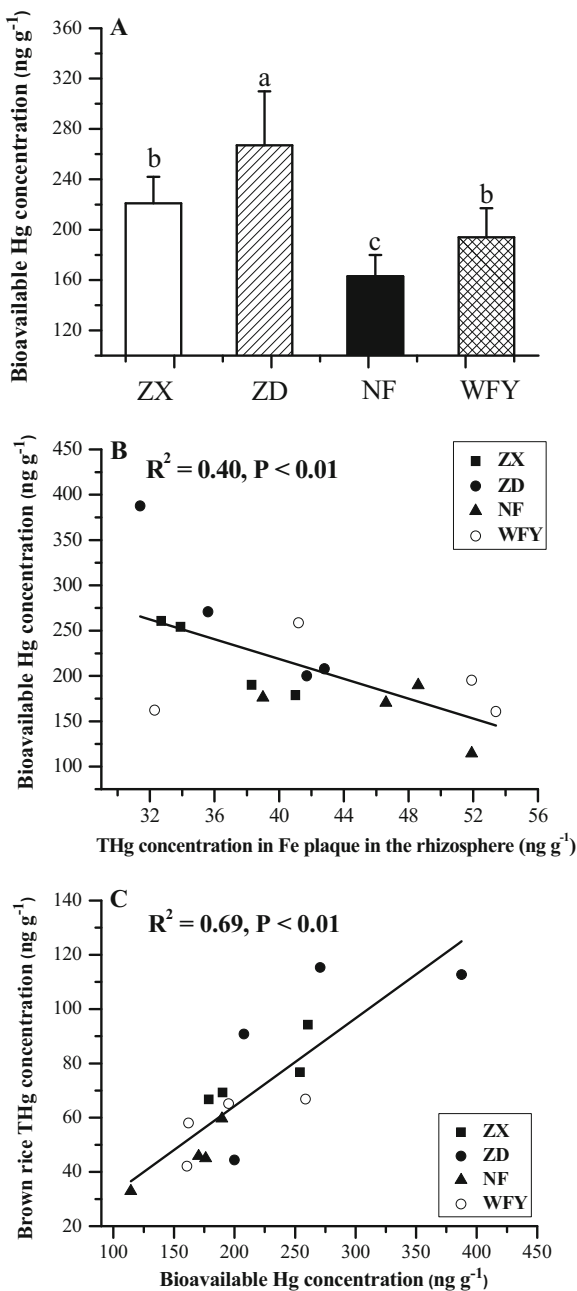


Fig. 7 Bioavailable Hg concentrations in the rhizosphere (a) and its relationships with THg concentrations in Fe plaque in the rhizosphere (b) and THg concentrations in brown rice (c) of four rice cultivars (cvs. ZX, ZD, NF and WFY) grown in Hg-contaminated soil. Different letters in Fig. 7a indicate significant differences between cultivars at the level of $P < 0.05$

significant of suberin deposit at 5 cm on Hg uptake still needs more in-depth studies, as Carrasco-Gil et al. (2013) has shown that Hg mainly enters the root at 0–5 mm from the apex. Future work should

focus on the relationship between Hg uptake and apoplastic barriers including suberin deposition at different distances from the root tip, particularly at 0–5 mm.

Variations in the organic acid secretions and influences on Hg accumulation

Hg exposure stimulated the exudation of malic, lactic and oxalic acids by roots (Fig. 3 and Table S4). The concentration of malic acid was the greatest among the five organic acids assayed. Aulakh et al. (2001) also found that malic acid secreted by ten rice cultivars grown under soil conditions had the highest concentration followed by tartaric, succinic, citric and lactic acids. These findings suggest that malic acid secreted by roots may play an important role in adapting to the local environment. However, acetic and formic acids were found to be the major organic acids secreted by rice roots treated with Cd (Liu et al. 2007), whereas oxalic, citric and malic acids were dominant when exposed to chromium (Cr) (Zeng et al. 2008). To the best of our knowledge, the amounts and compositions of organic acids secreted by rice roots under Hg exposure have never been reported. The contribution and significance of different organic acids needs to be further verified. In the present study, the compositional profiles of organic acids secreted by roots followed the same trend for all cultivars, with the concentrations of malic acid 20- to 100-fold higher than those of oxalic and lactic acids, whereas the concentrations of citric and succinic acids were approximately 1000-fold lower. However, the amounts of these organic acids varied between cultivars. Cultivars such as ZX and ZD secreted greater amounts of organic acids (especially malic acid) under Hg exposure and appeared to accumulate more THg and MeHg in the plants (Figs. 1 and 3), suggesting that the secretion of organic acids may enhance the uptake and accumulation of Hg by rice plants. It is possible that the variations in the amounts of organic acids (especially malic acid) between cultivars may affect the metabolism of FeRB in the rhizosphere, since organic acids in root exudates have been found to exert both stimulatory and inhibitory influences on rhizosphere microbial community structure and composition (Bais et al. 2006; Hartmann et al. 2009). The greater amounts of organic acids secreted by roots of these two cultivars may increase the abundance of FeRB, which can inhibit the formation of Fe

plaque in the rhizosphere (Fig. 6), thus enhancing the bioavailability of Hg in the rhizosphere (Fig. 8a). However, further research is required to confirm how organic acids affect the mobility, bioavailability, uptake and accumulation of Hg in rice plants.

Variations in Fe plaque formation, FeRB abundance and their relationships with Hg accumulation

The rice cultivars with higher degrees of Fe plaque formation tended to sequester more Hg on root surfaces and in the rhizosphere (Figs. 4b, c), which may help lower the bioavailability of Hg in the rhizosphere (Fig. 7b), leading to less THg accumulated in the rice plants (Fig. 7c). The abilities of Fe plaque to sequester other metal (loid) s such as Cu, Pb, Zn and As by

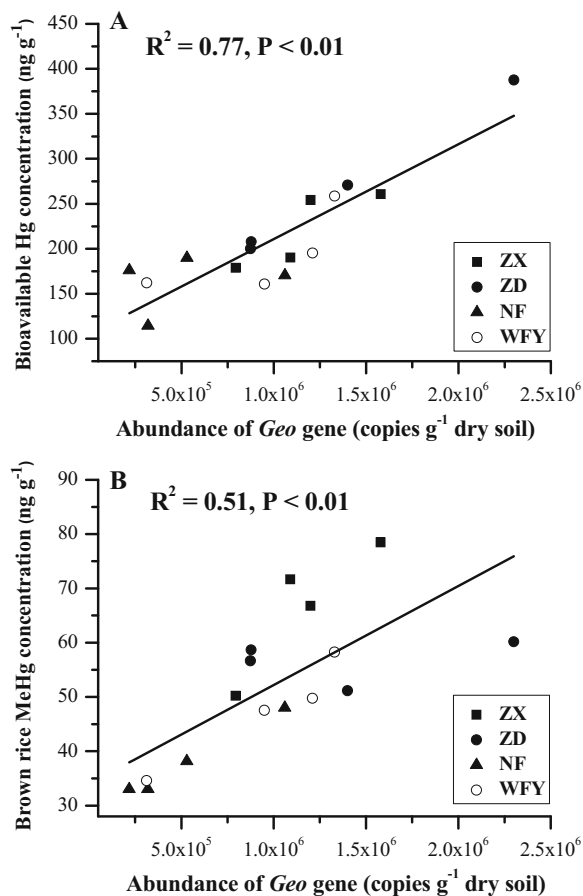


Fig. 8 Relationships between *Geo* gene number in the rhizosphere and bioavailable Hg concentrations in the rhizosphere (a) and MeHg concentrations in brown rice (b) of four rice cultivars (cvs. ZX, ZD, NF and WFY) grown in Hg-contaminated soil

adsorption and/or co-precipitation, so affecting their bioavailability in the rhizosphere, the uptake and metal tolerance of wetland plants have been reported by several workers (Ye et al. 1997; Hansel et al. 2001; Heikens et al. 2007; Cheng et al. 2014). The relative importance of Fe plaque formed on the root surface and in the rhizosphere in binding metals has been debatable. Wang et al. (2014a) found that the proportion of THg in Fe plaque on root surfaces was much lower compared to that accumulated in root tissues, but the work ignored the effects of Fe plaque in the rhizosphere. Conversely, the role of Fe plaque in the rhizosphere in regulating metal absorption was found to be more important than that on root surfaces (Yang et al. 2012, 2014). In the present study, the largest proportion of THg was accumulated in Fe plaque in the rhizosphere, followed by that accumulated in root tissues, whereas the lowest amount was absorbed onto root surfaces (Fig. 5). This indicates that Fe plaque in the rhizosphere played the most dominant role in controlling the uptake and accumulation of THg in rice plants, and the degrees of Fe plaque formation (especially in the rhizosphere) can be a meaningful criterion for cultivar selection.

FeRB have been established to be an important microbial group involved in the Fe (III)-reducing process and Fe cycle in paddy fields (Weber et al. 2006). In this study, results on the abundance of FeRB suggest that the family Geobacteraceae (rather than the genus *Shewanella*) played the dominant role in inhibiting the formation of Fe plaque on root surfaces and in the rhizosphere (Table 1, Fig. 6). FeRB have also been shown to take part in the methylation process of Hg (Fleming et al. 2006; Kerin et al. 2006). The positive correlation between *Geo* gene numbers in the rhizosphere and MeHg concentrations in brown rice ($R^2=0.51$, $P<0.01$) (Fig. 8b) suggests that the rice cultivars with higher abundance of FeRB in the rhizosphere tended to accumulate more MeHg. However, the genus *Shewanella* in the present study did not show any obvious difference between cultivars, suggesting the complex interactions between rice plant and FeRB. The activities of FeRB may also be affected by radial oxygen loss (ROL), a feature in roots closely linked with Fe plaque formation, since FeRB are anaerobic microorganisms and sensitive to O₂ (Weber et al. 2006). More in-depth studies on the role of root exudates and ROL on FeRB in the rhizosphere are needed.

Conclusions

The present study has revealed that the cultivars with lower Hg accumulation (cvs. NF and WFY) tended to develop stronger apoplastic barriers in the endodermis, secrete lower amounts of organic acids and form more Fe plaque on root surfaces and in the rhizosphere, compared to the cultivars with higher Hg accumulation (cvs. ZX and ZD). This suggests that the formation of Fe plaque can help restrict the uptake and accumulation of Hg by rice plants, whilst the secretion of organic acids presents an opposite effect. The development of apoplastic barriers in the endodermis at 5 cm from the root apex may also inhibit the uptake and upward transportation of Hg but this needs to be further investigated as Hg mainly enters the root at 0–5 mm from the apex. The formation of Fe plaque in the rhizosphere (rather than that on root surfaces) plays an important role in sequestering Hg and reducing the bioavailability of Hg in the rhizosphere, thus inhibiting the accumulation of THg in rice plants. The results presented here demonstrate that ecophysiological features (anatomy, organic acid secretions and Fe plaque formation) of rice roots can be useful criteria for selecting appropriate cultivars for Hg-contaminated paddy fields.

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