Inhibition of cadmium ion uptake in rice (*Oryza sativa*) cells by a wall-bound form of silicon

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Summary

- The stresses acting on plants that are alleviated by silicon (Si) range from biotic to abiotic stresses, such as heavy metal toxicity. However, the mechanism of stress alleviation by Si at the single-cell level is poorly understood.
- We cultivated suspended rice (*Oryza sativa*) cells and protoplasts and investigated them using a combination of plant nutritional and physical techniques including inductively coupled plasma mass spectrometry (ICP-MS), the scanning ion-selective electrode technique (SIET) and X-ray photoelectron spectroscopy (XPS).
- We found that most Si accumulated in the cell walls in a wall-bound organosilicon compound. Total cadmium (Cd) concentrations in protoplasts from Si-accumulating (+Si) cells were significantly reduced at moderate concentrations of Cd in the culture medium compared with those from Si-limiting (−Si) cells. *In situ* measurement of cellular fluxes of the cadmium ion (Cd^{2+}) in suspension cells and root cells of rice exposed to Cd^{2+} and/or Si treatments showed that +Si cells significantly inhibited the net Cd^{2+} influx, compared with that in −Si cells. Furthermore, a net negative charge (charge density) within the +Si cell walls could be neutralized by an increase in the Cd^{2+} concentration in the measuring solution.
- A mechanism of co-deposition of Si and Cd in the cell walls via a [Si-wall matrix]Cd co-complexation may explain the inhibition of Cd ion uptake, and may offer a plausible explanation for the *in vivo* detoxification of Cd in rice.

Introduction

Among the well-known phytotoxic heavy metals in the environment, cadmium (Cd) is of considerable importance because of its high water solubility, mobility, persistence, and toxicity even in minute amounts (Wagner, 1993; Sanita di Toppi & Gabrielli, 1999). Cd in rice (*Oryza sativa*) and other grains poses a potential health problem for human safety, and increased dietary intake of Cd has been correlated with an increased consumption of rice (Egan *et al.*, 2007). The source of Cd in rice grains is soil; paddy rice is able to accumulate high concentrations of Cd, and Cd is absorbed by rice roots and transported to the grains, resulting in considerable Cd accumulation even when plants are grown on slightly to moderately Cd-polluted soils (Uraguchi *et al.*, 2009). A major transporter of Cd, *Nramp*5, is responsible for the transport of Cd from the external solution to root cells (Sasaki *et al.*, 2012). Subsequently, Cd is transported into grains by another transporter, *OsLCT*1 (Uraguchi *et al.*, 2011).

Silicon (Si) is the second most abundant element in soils, but its availability to plants as silicic acid may be limiting, and hence silicate fertilizers in rice production are applied (Ma & Takahashi, 2002a). Rice is the most effective known Si-accumulating plant (Epstein, 2009), taking up > 10% of its dry weight (DW) (Ma & Takahashi, 2002b). Moreover, Cd toxicity in rice plants (Wang *et al.*, 2000; Zhang *et al.*, 2008) has been shown to be alleviated by the presence of Si in the cell walls. Liang *et al.* (2005) and Vaculik *et al.* (2009) described this phenomenon in maize (*Zea mays*) plants. Differences in Cd uptake of roots and shoots are probably related to the development of the apoplastic fraction (Vaculik *et al.*, 2012). Recently, Nwugo & Huerta (2011) used a proteomic approach to investigate the effect of Si on Cd tolerance in rice plants, suggesting a more active involvement of Si in plant physiological processes than previously proposed.

Despite evidence of the role of Si in the amelioration of heavy metal Cd toxicity in plants at the whole-plant level, our understanding of the mechanisms involved in Si-induced Cd tolerance at the single-cell level remains very limited. Recently, a study was designed to investigate aluminium (Al)–Si interactions at the cellular level using suspension cultures of Norway spruce (*Picea abies*) (Prabagar *et al.*, 2011). Notably, the presence of Si reduced the concentration of free Al in the cell wall, and formation of aluminosilicate complexes in the wall was proposed (Prabagar *et al.*,

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2011). Suspension culture avoids the complicated influences of the whole plant, tissue or organs, and provides an opportunity to explore heavy metal uptake and understand the cellular and chemical mechanisms of Si-induced alleviation of Cd toxicity. In this study, we employed a noninvasive ion flux technique to measure the cellular fluxes of Cd^{2+} in suspension cells and root cells of rice exposed to Cd and/or Si treatments. The aim of this study was to investigate the Si-induced alterations of Cd^{2+} ion fluxes in rice cells and to identify the cellular response to Cd toxicity and its chemical mechanism that can be used to re-evaluate the results of whole-plant studies.

Materials and Methods

Cell culture

Suspension-cultured cell lines of rice (*Oryza sativa* L. cv Zhonghua 11) were established following the processes of Chu *et al.* (1975) and Thomas *et al.* (1989): mature seeds were dehusked, sterilized with 75% ethanol for 1 min and 0.1% mercury chloride for 10 min and washed with sterilized water five times. Sterilized seeds were incubated at 28°C in the dark for 1 month in a modified N6 medium and subcultured three times in subculture medium for callus formation. The calli (50–100) were transferred to 125-ml plastic Erlenmeyer flasks containing 40 ml of liquid AA medium in the presence of 2,4-D at 1 mg l^{-1} and cultivated on a rotary shaker (110 rpm) at 28°C in the dark. The suspension cells were subcultured at 5-d intervals for 2–3 months by replacing old liquid medium with newly prepared nutrient solution every 5 d to supplement nutrients and Si. To apply different culture conditions, rice cells were cultivated in the absence (−Si) or presence (+Si) of 1.0 mM silicic acid at various concentrations of Cd (5, 30 and 60 μM) in the liquid medium (pH 5.6). Specifically, freshly made silicate was added as a basic Na_2SiO_3 solution (Casey *et al.*, 2003) for each subculture use. The solutions were then acidified to pH 5.6 with 1.0 M HCl, generating the corresponding concentration of NaCl which was supplemented in the control solutions. Only then were the other constituents added to dilute culture solutions to the final Si concentration. In order to maintain a constant concentration of Si during the 5 d of culture, only a certain number of cells were retained in the plastic flask to minimize depletion of the solution (< 2% Si was depleted after the 5-d period) according to an established method (Chu *et al.*, 1975). The Si content of the liquid medium in the plastic flasks was determined to be 0–2 mg kg^{-1} using an inductively coupled plasma mass spectrometer (ICP-MS; Elan DRC-e; PerkinElmer, Concord, Ontario, Canada). Cell samples were kept sealed during most manipulations to avoid Si contamination from dust. All solutions were prepared using ultra-high-purity water, 18 MΩ-cm, from a two-step purification treatment including double distillation (YaR; SZ-93, Shanghai, China) and deionization (Milli-Q, Billerica, MA, USA). The total Si content of the ultra-high-purity water was determined to be close to zero using ICP-MS. All chemicals were high purity (Aldrich-Sigma, Shanghai, China, unless otherwise indicated). To visualize cells and determine their viability (Supporting Information Methods S1),

we used a digital CCD camera (DS-Fi1; Nikon, Tokyo, Japan) coupled to a light microscope (BX51; Olympus, Tokyo, Japan), and measured cell sizes using the software NIS-ELEMENTS F 3.0 (Nikon).

Protoplast culture

The protoplasts were generated from rice suspension cells cultivated for 2–3 months (Yamada *et al.*, 1986). Briefly, suspension cells (*c.* 0.5 g FW) were added to 10 ml of enzyme solution that consisted of 2.5% cellulase from *Trichoderma viride*, 1% macerozyme R-10, 0.4 M mannitol, 80 mM $CaCl_2$, 0.125 mM $MgCl_2$ and 0.5 mM MES. The pH of the solution was adjusted to 5.6. After 8 h of incubation at 25°C in the darkness without shaking, the released protoplasts were separated from the undigested suspension cells by filtration using a 40-μm nylon filter, and collected by centrifugation at 68 *g* for 5 min. The protoplasts were washed three times with protoplast suspension medium (0.4 M mannitol, 80 mM $CaCl_2$, 0.125 mM $MgCl_2$ and 0.5 mM MES at pH 5.6). The protoplasts were suspended in the above medium and lyophilized for the Si and Cd determinations by ICP-MS.

Plant growth

Hydroponic culture of rice plants was carried out, as described previously (Zhang *et al.*, 2008). Briefly, seeds of rice were surface-sterilized with 0.3% H_2O_2 solution for 20 min and then rinsed with double-distilled water. All sterilized seeds were placed in quartz sand to germinate. After 5 d, the seedlings were transplanted to 1-l pots, at a density of 20 seedlings per pot, which contained nutrient solution, with 1.0 mM silicic acid, consisting of (mM) K_2SO_4: 0.75, $MgSO_4$: 0.65, $Ca(NO_3)_2$: 2, KCl: 0.1, NH_4Cl: 1, KH_2PO_4: 0.25, FeEDTA: 0.08, H_3BO_3: 0.01, $MnSO_4$: 0.001, $ZnSO_4$: 0.001, $CuSO_4$: 1×10^{-4}, and $(NH_4)_6Mo_7O_{24}$: 1×10^{-6}. The solution pH was maintained at 5.0–6.0. Silicic acid (1.0 mM) was added to the solution first, followed by adjustment of the pH to 5.5 with 1 M HCl once a week. The rice plants were grown in growth chambers under controlled environmental conditions for 1 month.

Measurement of Cd^{2+} flux

The net Cd^{2+} flux was measured noninvasively using a scanning ion-selective electrode technique (SIET) (SIET system BIO-001A; Younger USA Sci. & Tech. Corp., Amherst, MA, USA; Applicable Electronics Inc., Sandwich, MA, USA; and Science Wares Inc., Falmouth, MA, USA) at Xu-Yue Sci. & Tech. Co. Ltd (Beijing, China; http://www.xuyue.net). Ion-selective microelectrodes with an external tip diameter of *c.* 3 μm were manufactured and silanized with tributylchlorosilane and the tips backfilled with a commercially available ion-selective cocktail (Cadmium Ionophore I, 20909; Fluka, Buchs, Switzerland). The microelectrodes were calibrated in 50 and 500 μM Cd^{2+} before the Cd^{2+} flux measurement according to the standard procedure (Xu-Yue Sci. & Tech. Co. Ltd). Only electrodes with Nernstian slopes > 25 mV per decade were used.

Rice suspension cells (200 µl) were added to glass coverslips pretreated with a poly-L-lysine (Sigma-Aldrich) solution and transferred to the measuring chamber containing 3 ml of measuring solution (0.03 mM $CdCl_2$, 0.1 mM KCl, 0.05 mM $CaCl_2$, 0.05 mM $MgCl_2$, 0.5 mM NaCl, 0.1 mM Na_2SO_4, 0.3 mM 2-(N-morpholino) ethanesulfonic acid (MES), and 0.1% sucrose, pH 5.7). A measuring chamber was mounted on the micromanipulator, and the electrodes were positioned close to the surfaces of cells and root samples, and left to equilibrate. Gradients of Cd^{2+} adjacent to the cells were measured by moving the Cd^{2+}-selective microelectrode between two positions in a pre-set excursion of 20 µm, completing a whole cycle in 5.45 s. The Cd^{2+} flux data were recorded for a period of 10–20 min. The flux data were obtained with the ASET software, which is part of the SIET system. They were analysed with an Excel spread sheet to convert data from the background potential (mV) estimation of concentration and the microvolt difference estimation of the local gradient into specific ion flux ($pmol\,cm^{-2}\,s^{-1}$) using MAGEFLUX, developed by Xu-Yue Sci. & Tech. Co. Ltd (http://xuyue.net/mageflux). All experiments were repeated at least three times and their mean values ± SD are presented. Statistical differences in mean values at each point were determined with a Student's t-test.

The cell surface potential difference was measured *in situ* using the SIET by moving the Ca^{2+}-selective microelectrode between two positions in a pre-set excursion of *c.* 20 µm. V_a is the background potential of the aqueous medium, and V_s is the surface potential of suspension cells (mV).

Total Si and Cd concentration measurement

Three millilitres of concentrated HNO_3 and 2 ml of H_2O_2 were added to 0.05 g of the samples (lyophilized cells or isolated cell walls or protoplasts) in a teflon bottle and left overnight. The teflon bottle was put into a high-temperature digestion tank to prevent nitric acid volatilization. The digestion tank was then placed in an oven at a temperature of 80°C for 3 h followed by 160°C for 5 h. The digestion tank was heated on a hot plate at a temperature of 150°C, and evaporated to dryness. The dried material was made up to a volume of 10 ml with 2% HNO_3. An ICP-MS was used for Si and Cd concentration measurements. All experiments were repeated three times, and their mean values ± SD are presented.

X-ray photoelectron spectroscopy (XPS) studies

Before spectroscopy measurement, all samples were dried under vacuum for at least 8 h. Lyophilized cells and isolated cell walls were ground with a teflon mortar. A powder sample was placed on an Al platform and homogenized with a spatula in order to obtain a relatively smooth surface. Roots of rice were cut into small fragments and placed on the Al platform for XPS measurements (VG multilab 2000 equipment; ThermoVG Scientific, East Grinstead, UK) using the Al Kα X-ray line of 1486.6 eV excitation energy at 300 W. To correct for sample charging, high-resolution spectra were used as a reference by setting the C

1s hydrocarbon peak to 284.6 eV. The background was linearly subtracted. Data analysis was performed using the THERMAL ADVANTAGE software (http://www.tainstruments.com). The ratios of atomic concentrations were calculated using the peak areas normalized on the basis of acquisition parameters and sensitivity factors proposed by the manufacturer. XPS experiments were repeated at least five times to ensure reproducibility of the results.

Results

Suspension rice cells were treated with 30 µM Cd^{2+} and then the Cd^{2+} flux was measured using SIET (Fig. 1). A net Cd^{2+} influx with mean values of 1.39 and 0.76 $pmol\,cm^{-2}\,s^{-1}$ for cells cultivated in solution for 2 months in the absence and presence, respectively, of 1 mM silicic acid in the medium was measured immediately following exposure to 30 µM $CdCl_2$. Meanwhile, the transient Cd^{2+} flux was measured from different regions (both the root apex and root hair zone) along the root axis (at *c.* 0.2 mm increments) after 30–60 min of exposure to 30 µM Cd^{2+} (Fig. 2a,b). Responses in the root apex (0–800 µm from the root tip) in the absence of Si (−Si treatments) were much greater than in the root hair zone (at 2000 µm from the root tip), with a Cd^{2+} influx of *c.* 70 $pmol\,m^{-2}\,s^{-1}$ measured *in situ* (Fig. 2c). This is about seven-fold greater than the average Cd^{2+} influx in the root hair zone (Fig. 2c). By contrast, no such obvious Cd^{2+} influx was observed in the presence of Si (+Si treatments) (Fig. 2), although
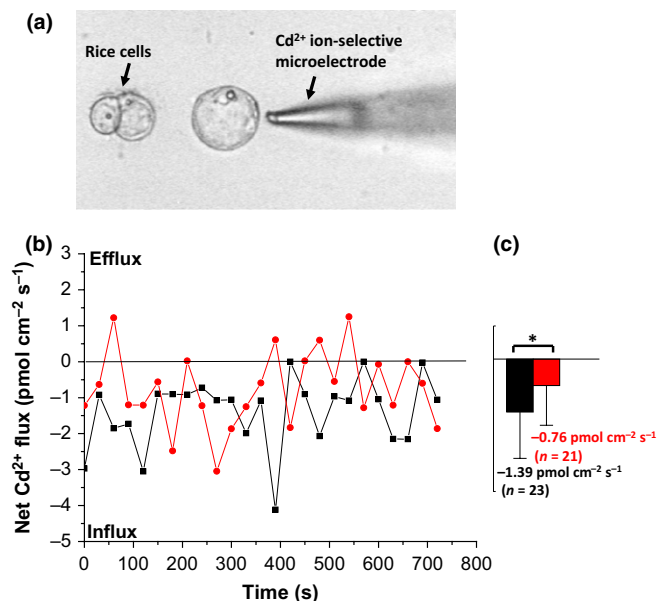

Fig. 1 The Cd^{2+} flux of rice suspension cells. (a) Rice (*Oryza sativa*) suspension cells cultured in the absence and presence of 1.0 mM silicic acid for 2 months after transplanting to solution medium from solid culture medium. (b) Kinetics of cadmium (Cd^{2+}) fluxes in a suspension-cultured cell in the presence of 30 µM Cd^{2+} in the measuring medium determined using the scanning ion-selective electrode technique (SIET). Black, −Si +Cd; red, +Si +Cd. (c) The mean rate of Cd^{2+} fluxes in suspension cells, using the mean value of three independent measurements (mean ± SD; $n = 3$). Every independent measurement contains > 20 replicates. The asterisk in (c) indicates a significant difference at $P < 0.05$.
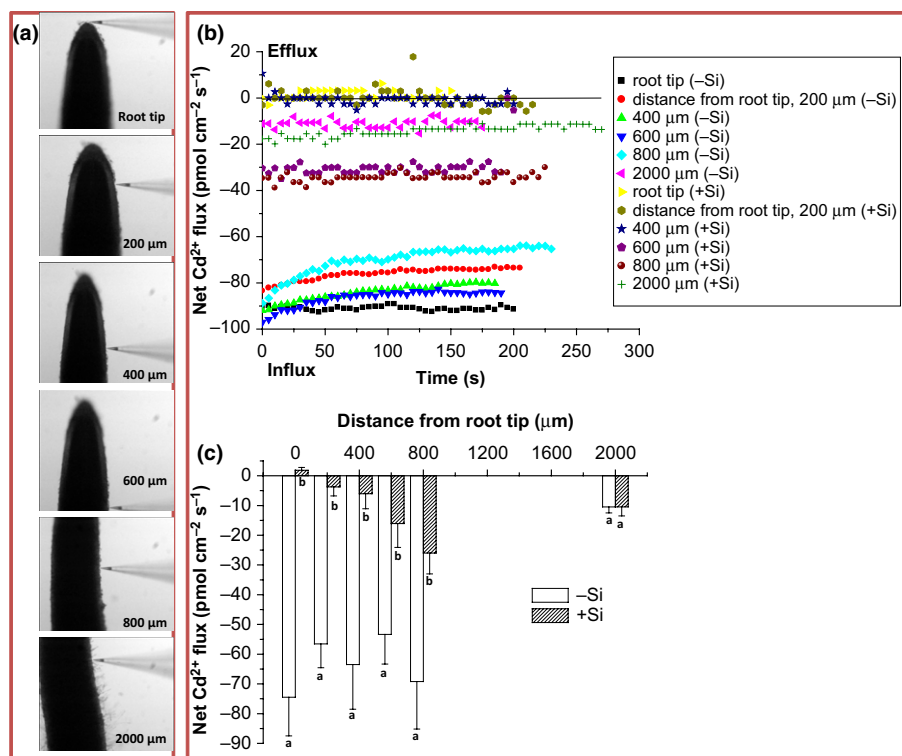
Fig. 2 The Cd^{2+} flux in root cells. (a, b) Net cadmium (Cd^{2+}) fluxes in root cells of rice (*Oryza sativa*) from different distances from the root tip grown in the absence and presence of 1.0 mM silicic acid for 1 month in a hydroponic culture of whole plants. Net Cd^{2+} fluxes were measured after 30 min of exposure to 30 µM $CdCl_2$, starting from the root tip. At each position, an average Cd^{2+} flux was measured for 5 min before the electrode was repositioned. (c) The mean Cd^{2+} fluxes in root cells after exposure to 30 µM Cd^{2+}. Means ± SD ($n = 3$). Different lowercase letters in (c) indicate a significant difference at $P < 0.01$.

slightly higher Cd^{2+} uptake (*c.* 20 pmol m^{-2} s^{-1}) was observed in the region of 600–800 µm from the root tip.

We further investigated whether there existed a difference in Ca^{2+} ion fluxes in the absence and presence of Cd^{2+} ions in the medium. The results showed that, before and after addition of 30 µM Cd^{2+}, the suspension cells and roots cultivated in the absence and presence of Si exhibited a stable and constant Ca^{2+} efflux with a rate of *c.* 7 and 14 pmol cm^{-2} s^{-1}, respectively (Figs S1, S2), suggesting that either the presence of Si in cells/roots or the short duration (10 min) of exposure to Cd^{2+} stress did not significantly influence Ca^{2+} fluxes.

In order to investigate the subcellular distributions of Si and Cd following their uptake, we cultivated protoplasts from suspension cells, and determined total Si and Cd concentrations in cells and protoplasts, respectively. Si concentration in whole cells, which were suspension-cultivated for 2 months in the presence of 1.0 mM silicic acid, was 108.0 ± 24.0 mg kg^{-1} ($n = 3$) of cell dry weight (Fig. 3a), whereas in its absence, cells contained Si at 11.0 ± 5.0 mg kg^{-1} ($n = 3$). In comparison, in protoplasts formed from suspension cells cultivated in the presence and absence of 1.0 mM Si, the average Si concentration was 1.8 ± 1.0 ($n = 3$) and 0.7 ± 0.4 ($n = 3$) mg kg^{-1}, respectively. This suggests that most Si in suspended cells accumulated in the cell walls. Moreover, total Cd concentration in whole cells, which were suspension-cultivated for 2 months in the absence and presence of 1.0 mM silicic acid, increased with the Cd concentration in the medium, ranging from 5 to 60 µM (Fig. 3b). Total Cd concentration in cells cultivated without Si and with 5, 30, or 60 µM Cd in the medium increased to 11.4 ± 0.2 ($n = 3$), 46.9 ± 5.5 ($n = 3$), and 101.0 ± 8.0 ($n = 3$) µg g^{-1} DW, respectively. By contrast, the presence of Si in the medium did not affect the Cd

uptake of cells; the total Cd concentrations in cells (10.2, 46.0 or 108.6 µg g^{-1}) were not significantly changed (Fig. 3b). Surprisingly, the total Cd concentrations in protoplasts, which were formed by adding both cellulase and macerozyme to remove the cell walls of suspension cells cultivated for 2 months in the presence of 1.0 mM Si, significantly decreased to 10 ± 2.1 ($n = 3$) µg g^{-1} DW at a Cd concentration of 30 µM in the medium as compared with 24.8 ± 3.7 ($n = 3$) µg g^{-1} DW in the absence of Si in the medium (Fig. 3b). Therefore, we can reasonably reckon that the Cd concentration in the cell wall in the absence and presence of Si was 22.1 and 36.9 µg g^{-1} DW, respectively, that is, *c.* 32% ((36.9–22.1)/46.0) and 47% (22.1/46.9) inhibition of Cd uptake by the wall modified by Si and the wall unmodified by Si, respectively.

When the Cd concentration in the medium was low (5.0 µM), the unmodified cell wall was able to inhibit the uptake of most Cd ions into the cells (Fig. 3b). However, when the Cd concentration in the medium was elevated to 60 µM, no significant differences in total Cd concentrations in protoplasts/cells were found to exist regardless of the absence or presence of Si in the cell walls (Fig. 3b). This may suggest that the Cd binding and absorption sites in the cell walls with and without Si reached complete saturation, beyond the capability of the cell walls to exert an influence on the Cd uptake of cells. Moreover, the total Cd concentration in +Si cells cultured at 60 µM Cd in the medium was a little bit higher than that of −Si cells (Fig. 3b), suggesting that the −Si cells were much more likely to be dead at the elevated Cd concentration than +Si cells, thus decreasing Cd uptake into the cells, or that the cell wall in the absence of Si may not prevent leaking of cytosol containing relatively high Cd, or both. The above analyses can be partly substantiated based on the
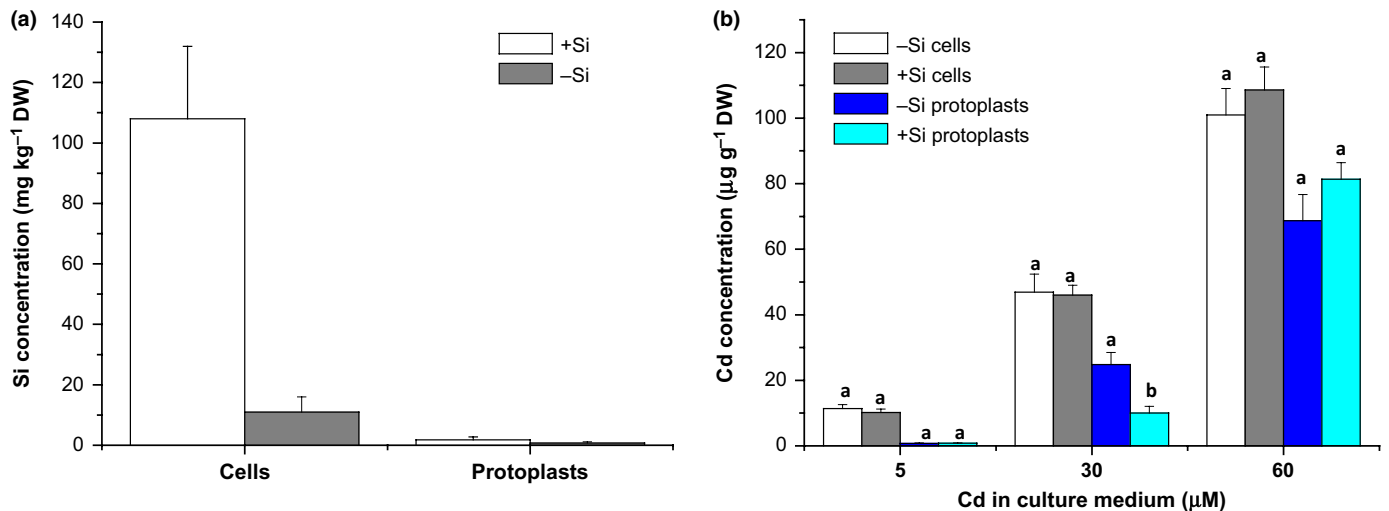
Fig. 3 Silicon and cadmium concentrations in cells and in protoplasts of rice. (a) Total silicon (Si) concentration in whole cells and in protoplasts of rice (*Oryza sativa*), cultivated for 2 months in the presence or absence of 1.0 mM silicic acid. (b) Total cadmium (Cd) concentration in whole cells and in protoplasts of rice cultivated at various Cd concentrations (5, 30, or 60 µM) in the absence and presence of 1.0 mM silicic acid for 2 months after transplanting to solution medium from solid culture medium. Values are mean ± SD of three independent sets of experiments. Different lowercase letters in (b) indicate a significant difference at $P < 0.05$.

result of the viability of the cells induced by heavy metal Cd stress (Notes S1, Fig. S3). Accordingly, most +Si cells, and especially their organelles, remained nearly intact and were less affected by high Cd treatments (Fig. S4). All −Si cells experienced dramatic damage and severe structural changes in organelle integrity (Fig. S4). These observations clearly show the role of Si in both the Cd tolerance of individual cells and in maintenance of the integrity of the cells and their walls.

To understand how Si in the walls helps the cells to withstand heavy metal stress, we looked at the Si-containing components and structures formed in cell walls using XPS. The Si_{2p} core-level XPS spectra of isolated walls and suspension cells that were cultivated in the presence of 1.0 mM silicic acid showed an obvious peak at 101.3 ± 0.1 eV ($n = 5$) (Fig. 4a,b). However, for hydroponically cultured rice plants containing *c.* 225–389 mg kg^{-1} DW Si in roots, the Si_{2p} peak of roots was decomposed into two components, at *c.* 101.3 eV (37.0 in at%, *c.* 83–144 mg kg^{-1}) and 102.1 eV (63.0 in at%), respectively (Fig. 4c). The concentration of this constituent at *c.* 101.3 eV was close to that in suspension cells (Fig. 3a). For the species at 102.1 eV, it may belong to monomeric silicic acid or (oligo/poly)silicic acid (Tesson *et al.*, 2009), and it may not be completely translocated to shoots from roots following uptake.

In an attempt to understand how the wall-bound form of Si is involved in the detoxification of Cd, we investigated the changes of the cell surface potential in a measuring solution similar to the cell culture medium in the presence of various Cd concentrations (5, 10 or 30 µM). We observed that a potential difference of −0.39 mV existed between the −Si and +Si wall surfaces of suspension-cultured cells (Fig. 5a,b), resulting in a net negative charge (charge density) within the +Si cell wall. However, the potential difference ($V_a − V_s$) was significantly decreased for +Si cell wall surfaces with the increase of Cd concentration in the measuring solution, compared with that for −Si cell wall surfaces

(Fig. 5c), whereas the presence of Ca^{2+} ions in the measuring medium did not obviously affect the potential difference ($V_a − V_s$), regardless of the presence or absence of Si in the cell walls (Fig. 5d). This result was consistent with the measurements of Cd^{2+} and Ca^{2+} fluxes in suspension-cultured cells (Figs 1, S1), underlining that the Si-modified wall matrix may interact/chelate with Cd^{2+}, not Ca^{2+}, thus preventing further Cd^{2+} uptake into the cells.

Discussion

The flux of a free metal ion species such as Cd^{2+} towards relevant sites at the plasma membrane after passing through the cell wall is governed (Town *et al.*, 2012) by: its mass transport properties connected to concentration gradients in the diffusion layer and coupled to chemical reaction rates in the reaction layer; interactions with various electric fields; and biological affinity (such as Cd^{2+} transporters) (Uraguchi *et al.*, 2011; Sasaki *et al.*, 2012). The cell walls typically carry a number of negatively charged groups such as carboxyl and hydroxy that generate a net electrostatic field which may have a significant impact on local equilibrium as well as kinetic and transport parameters pertaining to metal species (Duval & van Leeuwen, 2012). The structural and immobile charge gives rise to a Donnan potential in the cell wall, which influences the partitioning of Cd^{2+} ions (Ohshima & Kondo, 1990; Kalis *et al.*, 2009a).

Rice suspension cells have a primary cell wall (PCW) (Selvendran & O'Neill, 1987), the wall layer that is formed first, which is composed of polysaccharides (pectins, hemicelluloses and cellulose) and smaller proportions of glycoproteins (Fry, 2004). The ratio and exact composition of the various wall polysaccharides and glycoproteins differ temporally during the development of a given cell (Josè & Puigdomènech, 1993; Brett & Waldron, 1996). Polysaccharides in the intact wall are cross-linked to
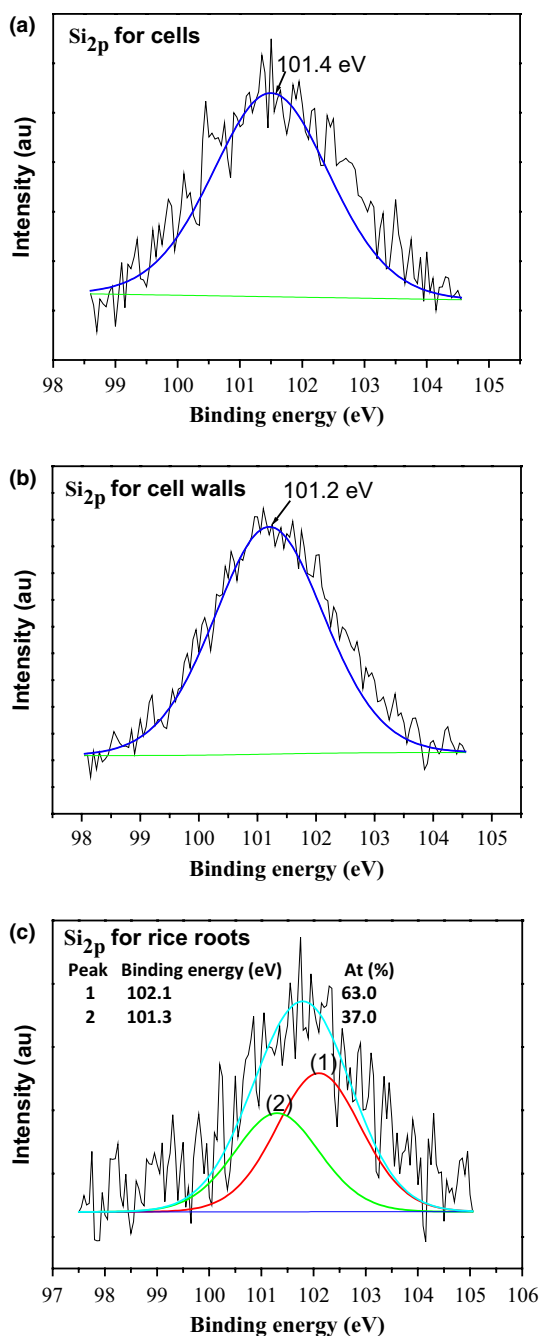
Fig. 4 X-ray photoelectron spectroscopy (XPS) characterization of the chemical composition for Si_{2p} of (a) the suspension cells, (b) isolated cell walls and (c) roots of rice (*Oryza sativa*). Suspension cells were cultivated at 1 mM silicic acid for 2 months and rice plants were grown in a hydroponic culture for 1 month. Si_{2p} intensity for cells and isolated walls in the absence of 1.0 mM silicic acid was not measurable or was below the detection limit for the experimental conditions used. au, arbitrary units.

form a 'fabric' (Fry, 1986) by the interaction of noncovalent and covalent links (O'Neill *et al.*, 2001). Rather high Donnan potentials have been measured in the cell walls of plants, ranging from *c.* −30 to −60 mV, as a result of the presence of various functional groups (Shomer *et al.*, 2003). Therefore, the cell walls possess substantial metal-binding capacity (Fig. 3b) through

complexing sites in polysaccharides with negatively charged functional groups (Slaveykova & Wilkinson, 2002) that are able to selectively bind divalent cations such as Ca^{2+} and Cd^{2+} ions (Davis *et al.*, 2003). This is confirmed by our finding that the unmodified cell wall can inhibit the influx of most Cd ions into cells at a low concentration of Cd (5 μM) in the medium (Fig. 3b).

At physiological pH, Si exists mainly as undissociated silicic acid ($pK_{a1} = 9.84$) in the medium. Considering the relatively high permeability of the wall for a neutral molecule, the passive diffusion of silicic acid into the walls may be allowed. The present (Fig. 4) and previous XPS determinations of individual cells and their walls demonstrated that most Si in the walls of suspension cells (Fig. 3a) was bound, not soluble (He *et al.*, 2013), suggesting that Si in some form may cross-link the plant cell wall matrix through forming a Si–wall complexation (He *et al.*, 2013). Kinrade *et al.* (1999) have shown that Si binds to aliphatic polyols (simple sugar-like molecules) in aqueous solution to form stable hypervalent silicon complexes via the formation of Si-O-C bonds under favourable conditions. Such hypervalent complexes may play a vital role in the biochemistry of Si; the presence of Si-O-C bonds is likely in the biosphere, or even in cell walls in which their components are closely related analogues of polyols (Kinrade *et al.*, 1999). According to the simple chemical reactions of coordination/complexation of Si and polyols, the Si-containing complexes are negative charged ([Si (polyol)]$^{q-}$; $q = 1–4$) (Kinrade *et al.*, 1999). This may account for the observation that +Si cell surfaces were more negative than −Si cell surfaces (Fig. 5a,b), and the potential difference ($V_a − V_s$) was significantly decreased for +Si cell surfaces with the increase of Cd concentration in the measuring solution, compared with that for −Si cell surfaces, through the electrostatic interaction/complexation between the Si–wall matrix complexes and Cd cations (Fig. 5c), thus inhibiting Cd ion uptake (Figs 1, 2).

Cd is present in the form of both free Cd^{2+} and wall-bound complexes (deposition) (Kacurakova *et al.*, 2000; Davis *et al.*, 2008). The chemically heterogeneous walls produced by Si modification could provide more binding sites for the Cd ions compared with cells without Si within the walls (Fig. 5c). For a given cell wall, the actual proportion of specifically and electrostatically bound Cd will depend on the nature of the functional groups in the cell walls. For multidentate ligands of carbohydrates and glycoproteins in cell walls that carry the Si–wall matrix anions and, as primary binding sites (PBSs), carboxylates or amino residues, the interactions between divalent metal ions (M) and the Si-modified walls must be reflected in stability enhancement. (Sigel & Sigel, 2007; Al-Sogair *et al.*, 2011; Knobloch *et al.*, 2011). The coordination sites of the metal ion to the walls should increase for the formation of an [Si-PBS]M complex (the charge of PBSs remaining undefined). The total electrostatic force/strength should increase from [PBS]M to [Si-PBS]M (Al-Sogair *et al.*, 2011; Knobloch *et al.*, 2011).

Furthermore, we noted that the presence of Ca^{2+} ions in the measuring medium did not obviously affect the potential difference ($V_a − V_s$) (Fig. 5d). In fact, Ca^{2+} ions are present at
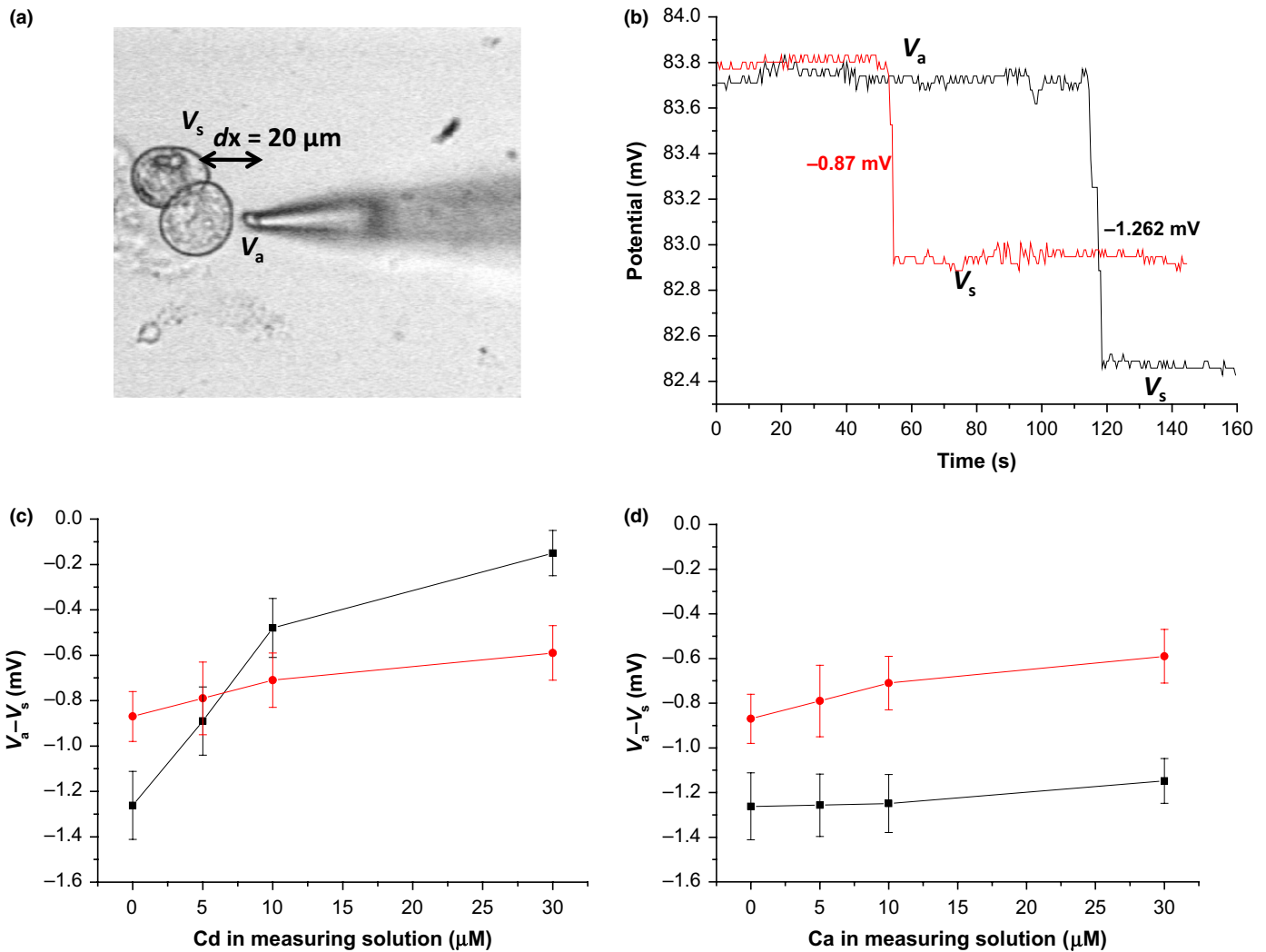
Fig. 5 The cell surface potential difference. (a) A schematic illustration of the rice (*Oryza sativa*) cell surface potential difference *in situ* measured by moving the Ca^{2+}-selective microelectrode between two positions in a pre-set excursion of *c*. 20 μm. V_a, the background potential of the aqueous medium; V_s, the surface potential of suspension cells (mV). (b) Representative plots of potential as a function of time for suspension cells cultivated for 3 months in the absence (−Si) and presence (+Si) of Si. (c, d) Plots of potential differences ($V_a - V_s$) against free (c) Cd^{2+} or (d) Ca^{2+} concentrations in the bulk measuring solutions. Black, +Si; red, −Si cells. Values are mean ± SD; +Si cells, $n = 30$; −Si cells, $n = 32$ for both measurements in (c) and (d).

appreciable concentrations in the pectins of plant cell walls (Ishii *et al.*, 1999; Cosgrove, 2005). Ca^{2+} binding and absorption sites in cell walls with and without Si may reach complete saturation after cultivation of cells in solution in the presence of Ca ions in the medium, or there may exist specific Ca transporters to take up Ca ions into the cells (Evans *et al.*, 1991). In a simple alginate gel system representing cell wall matrices, the Ca^{2+} cation is required to stabilize the alginate gel network under a wide range of ionic strengths (Kalis *et al.*, 2009a). Below an ionic strength of 9 mM the gel swelling is rather small and remains constant. This implies that up to this ionic strength the gels retain structural stability, that is, the level of Ca^{2+} cross-linking remains approximately constant (Kalis *et al.*, 2009a). In our SIET measuring system for Cd^{2+} ion flux at a constant Ca : Na ratio, the ionic strength is *c*. 1.35 mM (including 0.05 mM $CaCl_2$) that is below an ionic strength of 9 mM, therefore, the wall integrity of suspension cells is maintained. This suggests that immobilized Cd and Ca in

the walls do not contribute to the steady-state Cd and Ca fluxes, respectively (Figs 1, 2, S1, S2). Kalis *et al.* (2009b) also confirmed that the bound Cd in the alginate gel would not be released with time.

During homeostatic regulation of metal ions in many cell types, any perturbations resulting from changes in extracellular conditions, such as excess Cd^{2+} ions in the medium, rapidly activate various cell response mechanisms. Phytochelatins (Zenk, 1996), chitinase and heat-shock protein (Romero-Puertas *et al.*, 2002; Metwally *et al.*, 2003), calcium (Rodriguez-Serrano *et al.*, 2009) and intracellular Ca-binding proteins (Rivetta *et al.*, 1997) are all involved in the cell response to the heavy metal Cd. However, the first responses involve changes in ion flux across the plasma membrane, which can be detected in animal, plant and fungal cells within 1–10 min (Zonia & Munnik, 2007; Li *et al.*, 2012). Later, the above-mentioned secondary responses are mediated by signalling cascades on the cell surface and protein kinase

cascades (Zonia & Munnik, 2007). In the present study, significant changes in Ca ion flux in the presence of Cd in the medium were not detected (Figs S1, S2). Therefore, we reasoned that the Si-modified cell walls play a crucial role in amelioration of Cd toxicity in rice suspension cells via a chemical mechanism of co-deposition through the complexation interaction rather than a cellular response.

Previous work on Si/Al showed that Si treatment leads to the formation of hydroxyaluminiumsilicate (HAS) complexes in the apoplast of the root apex of maize, thus detoxifying Al (Wang et al., 2004). There is an in planta component to the amelioration phenomenon. Microanalytical investigations have revealed co-deposition of Al and Si in root cell walls, which are the main internal sites of aluminosilicate and/or HAS formation and of Al detoxification (Cocker et al., 1998). Our findings may explain these previous Al/Si results and broaden the application of a co-complexation mechanism.

Conclusions

In the present study, we found that, when suspended rice cells were exposed to Cd alone, they accumulated Cd in the cell wall in a wall-bound constituent of Si with net negative charges, which may be a natural ligand for Cd ions, binding them firmly to the wall matrix. These findings suggest that a co-complexation mechanism may exist that results in in vivo detoxification of Cd in rice.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Effect of Cd^{2+} on the uptake of Ca^{2+} in suspension cells of rice.

Fig. S2 Effect of Cd^{2+} on the uptake of Ca^{2+} in root cells of rice.

Fig. S3 Viability of suspension cells.

Fig. S4 TEM images of the ultra microstructure of rice suspension cells cultivated in the absence and presence of 1 mM silicic acid and/or 30–60 μM Cd^{2+}.

Methods S1 Details of viability measurements of suspension cells and transmission electron microscopy.

Notes S1 Details of viability of cells.

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