View Article Online / Journal Homepage / Table of Contents for this issue

Micro X-ray fluorescence imaging and micro X-ray absorption spectroscopy of cadmium hyper-accumulating plant, *Arabidopsis halleri* ssp. *gemmifera*, using high-energy synchrotron radiation†

Naoki Fukuda,[a] Akiko Hokura,[a] Nobuyuki Kitajima,[ab] Yasuko Terada,[c] Hiroyuki Saito,[d] Tomoko Abe[d] and Izumi Nakai*[a]

Received 3rd March 2008, Accepted 4th June 2008
First published as an Advance Article on the web 9th July 2008
DOI: 10.1039/b803602k

Published on 09 July 2008. Downloaded by Pennsylvania State University on 16/09/2016 00:32:30.

Monochromatic high-energy X-rays (37 keV) focused into a microbeam (3.8 × 1.3 μm^2) with a Kirkpatrick-Baez mirror allows us for the first time to reveal the striking sub-cellular distribution and the chemical form of cadmium in the cadmium hyperaccumulating plant, *Arabidopsis halleri* ssp. *gemmifera*, by detecting the Cd Kα line. Micro X-ray fluorescence analysis (μ-XRF) and micro X-ray absorption near-edge structure analysis (μ-XANES) were used for the nondestructive analysis of the Cd distribution and chemical form of Cd at a cellular level in the plant in order to investigate the Cd accumulation mechanism. It was clearly observed by two-dimensional μ-XRF imaging that Cd was highly accumulated in a part of the trichomes, epidermal hairs existing on the surface of the leaves. The bases of the trichomes contained Cd, Zn and Mn at high concentrations and their distribution patterns were similar to each other. These elements were located near the surface of the trichome in the form of a ring. The μ-XANES analysis revealed that the majority of the Cd exists in the divalent state and bound to the O and/or N ligands. This study has demonstrated the potential of the Cd K-edge μ-XANES spectroscopy, which was applied here for the first time to plant samples.

1. Introduction

Arabidopsis halleri grows in Europe and is known to contain more than 10 000 mg kg^{-1} of cadmium and zinc in its shoot.[1] This trait of *A. halleri* has the potential to be exploited in phytoremediation, a soft method in which plants are used for the cleanup of heavy metal-polluted soils. A number of studies have been reported over the last decade.[1–12] Subspecies of this plant, *Arabis gemmifera*, a native species of Japan, is also known as a cadmium and zinc hyperaccumulator.[13] Since Cd is a highly toxic metal for plants,[14–16] it is interesting to reveal the mechanism involved in the Cd accumulation of this plant.

It was reported that Cd was highly accumulated in the trichomes of the leaves.[1,17] Trichomes are epidermal hairs approximately 30 μm in diameter, and present on the leaf surface. Leaf trichomes in *Arabidopsis thaliana*, a related variety of *A. halleri*, originate from a single epidermal cell that differentiates to form a characteristic three-branched structure.[18] Recent studies using a scanning electron microscope (SEM) with an energy-dispersive X-ray spectrometer (EDS) showed that the heavy metal elements, such as Zn and Cd, are distributed in the base of the trichome.[1,2] The concentration of Cd in this compartment, assuming that this vacuole contains 85% water, can be estimated to be higher than 1 mol L^{-1}, based on a quantitative analysis of Cd Lα obtained using the O Kα line as an internal standard with EDS.[1] For the EDS analysis of Cd, the L line is usually used because the excitation energy of the Cd K line (27 keV) is too high to be excited by an electron beam. However, in Cd Lα_1 (3.13 keV) and Cd Lβ_1 (3.32 keV), the most intense Cd lines overlap with the K Kα line (3.31 kev), which is a major component of plants, because energy resolution of the conventional Si-(Li) solid state detector (SSD) used in the EDS analysis is as low as ~140 eV (@5.9 keV).

Study of the chemical form of Cd in plant tissue is also important for elucidation of the accumulation mechanism. The number of studies applying synchrotron radiation (SR) to biological samples has increased in recent years because the analysis using SR X-rays enables chemical speciation with minimal sample preparation.[3,19–28] The high-energy X-ray based SR allows us to investigate the chemical form of heavy metals including cadmium in plant samples.[19–21] Cd K-edge X-ray absorption fine structure analysis (XAFS) has been applied to plant samples. Cd in the xylem sap of *Brassica juncea* L. has been found to be predominantly coordinated with O or N ligands, while the majority of the Cd in the root is coordinated with S ligands.[19,20] It has also been reported for *Thlaspi caerulescens* that ligands for Cd depended on the function and age of the plant tissue.[21] However, these previous analyses of the chemical form of Cd were carried out using freeze-dried ground samples with non-focused X-rays. Therefore, this method had no spatial resolution and the resultant data represent an average of the whole tissue.[22]

[a]*Department of Applied Chemistry, Tokyo University of Science, 1-3 Kagurazaka, Shinjuku-ku, Tokyo, 162-8601, Japan. E-mail: inakai@rs.kagu.tus.ac.jp; Fax: +81 3 5261 4631; Tel: +81 3 5228 8266*
[b]*Fujita Co., 2025-1 Ono, Atsugi, Kanagawa, 243-0125, Japan*
[c]*SPring-8, JASRI, 1-1-1 Kouto, Sayo-cho, Sayo-gun, Hyogo, 679-5198, Japan*
[d]*RIKEN, 2-1 Hirosawa, Wako, Saitama, 351-0198, Japan*

† Presented at the International Symposium on Metallomics 2007, Nagoya, Japan, November 28–December 1, 2007.

 This journal is © The Royal Society of Chemistry 2008

View Article Online

Published on 09 July 2008. Downloaded by Pennsylvania State University on 16/09/2016 00:32:30.

The key technology for the micro X-ray fluorescence analysis (μ-XRF) imaging of heavy elements is definitely the development of the focusing optics for high-energy X-rays. Various types of focusing optics for micron to nanometre-sized X-rays have been developed during the past decade at the third-generation synchrotron radiation facilities, such as SPring-8, ESRF, and APS. The recent advances in X-ray optics allow us to use the Fresnel Zone plate (FZP) or Kirkpatrick-Baez (K-B) mirror to produce focused X-ray microbeams of submicrometre sizes. The compartmentalization of Cs in a single plant cell was revealed using a focused beam 0.4 μm × 0.7 μm in size produced for 5.8 keV by FZP.[25] Mineral grains produced by tobacco trichomes were revealed to be Zn-substituted calcite by Zn K-edge micro extended X-ray absorption fine structure spectroscopy (μ-EXAFS).[26] On the other hand, the distribution and chemical form of cadmium in the trichome and root of *Arabidopsis thaliana* were investigated using μ-XRF imaging of Cd Lα line and Cd L_{III}-edge micro X-ray absorption near-edge structure spectroscopy (μ-XANES) with a beam size of 0.9 μm × 0.3 μm on the sample.[27] Furthermore, the microbeam produced using a pair of elliptically bent mirrors in the K-B configuration was used in the Zn K-edge μ-EXAFS analysis of the trichomes in the leaves of *A. halleri*.[3] However, these studies used K-B optics to produce a microbeam of relatively lower energy of less than 20 keV. The μ-XRF and μ-XAFS analyses of Cd in the plant samples using a Cd Kα line have never been reported.

Focusing optics for high-energy X-rays have recently been developed at SPring-8 using a FZP or K-B mirror.[29–31] A focused beam of 0.5 μm in size was obtained using an X-ray energy of 100 keV by FZP.[31] As such, for the first time, we measured the cellular distribution of cadmium on a cellular level using a Cd Kα line.[17] However, it was difficult for the FZP optics to change energy at the same focal length because of the chromatic aberration. Therefore, the optics has not allowed the XAFS analysis to be carried out. Therefore, a total reflection mirror with no chromatic aberration is required, and the development of the microbeam with the K-B mirror in the high-energy regions has advanced. In 2004, we succeeded in obtaining a focal spot size smaller than 1 μm with an X-ray energy as high as 100 keV.[32–34] The spot size was defined by the FWHM of the differential curve; the size at 37 keV was 1.3 μm in the horizontal direction and 1.5 μm in the vertical direction.[32,33]

The aim of the present study is to determine the distribution of Cd in the leaves of *A. gemmifera*, and also to determine the chemical form of Cd accumulated in the trichomes. The distribution of Cd in the leaves and trichomes was investigated by μ-XRF analysis with the K-B mirror in the high-energy regions. The chemical form of Cd was then studied by Cd K-edge μ-XANES analysis.

2. Experimental

2.1. Plant culture

Arabidopsis halleri ssp. *gemmifera* was used in the present study. This plant is a subspecies of *A. halleri* in Europe and one of the native species in Japan. The plants were germinated on a mixture of peat moss and vermiculite moistened with deionized water. After 2 months of germination, the seedlings were transferred to vessels containing a nutrient solution of the following chemical composition: KNO_3 (810 mg L^{-1}), $Ca(NO_3)_2 \cdot 4H_2O$ (950 mg L^{-1}), $MgSO_4 \cdot 7H_2O$ (500 mg L^{-1}), $NH_4H_2PO_4$ (155 mg L^{-1}), NaFe-ethylenediamine tetraacetate (20 mg L^{-1}), H_3BO_3 (3 mg L^{-1}), $MnCl_2 \cdot 4H_2O$ (1.81 mg L^{-1}), $CuSO_4 \cdot 5H_2O$ (0.05 mg L^{-1}), $Na_2MoO_4 \cdot 2H_2O$ (0.02 mg L^{-1}) and $ZnSO_4 \cdot 7H_2O$ (0.22 mg L^{-1}).

After 4 weeks, the plants were transferred to 100 mL plastic bottles containing a nutrient solution of 20 μM Cd. Analytical grade $Cd(NO_3)_2 \cdot 4H_2O$ was used. The nutrient solution was continuously aerated at 1.5 L min^{-1} and renewed every 3 days. Plants were grown in a room under the following conditions: 13 h day length, 22 °C /17 °C day/night temperature, and 55–85% relative humidity.

2.2. Sample preparation

For the XRF analysis, the healthy plants were carefully selected on the 7th day of the Cd treatment and their leaves were cut from the plants and then flattened to keep them smooth by placing them between two heavy books. A Mylar® film was stretched over a 4 × 4 cm^2 acrylic plate with a 2 cm diameter hole, on which the leaf was placed. The edge of the sample was fixed with Scotch® tape.

For the μ-XRF analysis, the sample should be prepared as a thin sample with a constant thickness to obtain a precise elemental distribution. Moreover, to reveal the chemical form of Cd in a living plant, the sample with minimal preparation was required for the μ-XANES analysis. Therefore, a thin section of the freeze-dried sample was prepared in order to maintain its tissue structure and chemical state. The cross-section of the leaf was cut to a thickness of 200 μm using a vertical slicer (Model HS-1, JASCO Co, Japan) and immediately put on dry ice. The section was freeze-dried by the dry ice overnight using a lyophilizer (Labconco Co. Lyph-Lock 6 Liter Freeze-Dry System Model 77530). The section was fixed with double-faced adhesive tape on the 4 × 4 cm^2 acrylic sample board. The samples were photographed using a digital microscope. They were also subjected to SEM observations after SR-XRF analysis using a scanning electron microscope (JEOL JSM-5600LV).

2.3. X-Ray analyses

SR-μ-XRF and Cd K-edge μ-XANES measurements of the plant leaves were performed using beam line 37XU at the SPring-8, operated at 8 GeV and *ca.* 100 mA. The X-rays from an undulator were monochromatized by a Si(111) double-crystal monochromator to 37 keV in order to excite the K-lines of cadmium and to minimize overlap of the K-line peak with the Compton scattering peak. The flux of the photons is *ca.* 10^8–10^9 photon s^{-1} at 37 keV.[32,33] Although the energy resolution of the Si(111) monochromator is not as high as the Si(311) double monochromator, a higher flux beam can be obtained by the former monochromator than the latter one. Therefore, in the present study, Si(111) was selected for the Cd XAFS measurement to obtain a high flux beam, which allows a measurement in order to be made over a shorter time. The beam was focused to a spot size of 3.8 μm (horizontal, *H*) × 1.3 μm (vertical, *V*) using

View Article Online

Published on 09 July 2008. Downloaded by Pennsylvania State University on 16/09/2016 00:32:30.

a pair of elliptically bent mirrors in the Kirkpatrick-Baez configuration.[32,33] The fluorescence X-rays were measured using a Si(Li)-SSD in air at room temperature. The sample on the acryl board was mounted on an x-y translation stage. The fluorescence X-ray intensity was normalized by the incident X-ray intensity, I_0, to produce a two-dimensional elemental map. Prior to the μ-XRF imaging of the freeze-dried section samples, XRF imaging using an X-ray beam of conventional size, $50 \times 50\ \mu m^2$, was carried out to determine the overall elemental distribution in the tissues.

The Cd K-edge μ-XANES spectra were measured at the accumulating points of Cd, which were revealed by the μ-XRF imaging, in the fluorescence mode with a Si(Li)-SSD. A monochromator stabilization (MOSTAB) system,[35] which was a feedback control to stabilize the X-ray beam of the synchrotron radiation, was used. This system applies a feedback voltage to a piezo electric transducer attached to a double-crystal monochromator to estimate any change in the beam position by the energy change. The XANES spectra were measured using an X-ray microbeam (3.8 μm (H) × 1.3 μm (V)) over the energy range of 26.672–26.772 keV with an energy step of 1 eV. The X-ray fluorescence intensities were measured for 1–4 s per point. The XANES spectra of the cadmium sulfate (CdS), cadmium oxide (CdO), cadmium acetate (AcCd), and hexakis(imidazole)cadmium(II) nitrate ($(Im)_6Cd(NO_3)_2$) reagents were measured as the reference spectra after dilution with boron nitride to the appropriate concentrations. That of phytochelatin-Cd (PC-Cd) and metallothionein (MT-Cd) was measured without dilution. $(Im)_6Cd(NO_3)_2$ was synthesized as previously described.[36] PC-Cd was a gift from Prof. Kazumasa Hirata (Graduate School of Pharmaceutical Science, Osaka University, Japan). Metallothionein from rabbit liver was purchased from Sigma (9038-94-2). CdS, CdO and AcCd were commercially available reagent grade chemicals. The $Cd(NO_3)_2$aq solution (1000 mg L^{-1}) for the atomic absorption analysis (Wako, Japan) was also measured as a reference sample. A grade 5C filter paper impregnated with the solution was enclosed with Mylar® film and used in the measurement. Energy calibration was carried out by measuring the XANES spectrum of a cadmium metal. The first energy inflection point of the metallic Cd was assumed to be 26.715 keV.

2.4. ICP-AES analysis

The plant was immediately harvested after SR-μ-XRF. The plant was divided into the roots and the shoots, and the shoots were then further divided into young leaves, which consisted of the ten newest leaves, and the old leaves. The leaves were quickly washed with distilled water and then oven-dried at 85 °C for 15 h. The dried samples were ground, and the subsample (approximately 10 mg) was weighed into a glass tube and digested with a mixed solution of HNO_3 and $HClO_4$.[2–4] A reagent blank and the standard reference material for the plant (NIES CRM No.1 'Pepper Bush', National Institute for Environmental Studies, Japan) were also analyzed to verify the accuracy and precision of the digestion procedure and subsequent analysis. The concentrations of Cd and Zn in the digested samples were determined using an inductively coupled plasma atomic emission spectrometer (ICP-AES), Hitachi P-4010.

3. Results and discussion

3.1. Plant growth and elemental concentrations of shoots

In the present study, the plant was cultivated and treated with 20 μM Cd for 2 weeks. The plants treated with 20 μM Cd for 7 days showed no phytotoxicity. After 2 weeks of cultivation, some shoots were found to contain more than 7000 mg kg^{-1} Cd on a dry-weight basis, but it showed a lethal toxicity. It has also been reported that subspecies of this plant accumulate Cd up to *ca.* 6000 mg kg^{-1} on a dry-weight basis in the shoots, and show a phytotoxicity.[1] Therefore, the healthy plant treated with Cd for 7 days was subjected to an XRF analysis. The biomass for each sample as dry-weight and the Cd and Zn concentrations in the shoots analyzed by ICP-AES are listed in Table 1. This plant accumulated approximately three times more Cd in the young leaves (1700 mg kg^{-1}) than in the old leaves (595 mg kg^{-1}), while the Zn concentrations for the young and old leaves were almost the same. The average concentrations of Cd and Zn in the entire shoots including the young and old leaves were calculated to be 870 mg Cd kg^{-1} and 128 mg Zn kg^{-1}, respectively. The young leaves were subjected to SR-μ-XRF imaging as their Cd levels were high.

3.2. XRF analysis of a whole leaf

Fig. 1 shows the distribution of Cd, Zn, Mn, and Ca of a leaf as elemental maps, in which the XRF intensity normalized by the incident X-rays are expressed by the color scale from red to blue, which corresponds to the XRF intensity from highest to lowest. A photograph of the sample is shown in Fig. 1(a). Fig. 1(f) shows an X-ray fluorescence spectrum measured in the leaf tissue at point A in Fig. 1(b). The Cd Kα peak can be clearly observed using a high-energy (37 keV) X-ray beam. As shown in Fig. 1(b), the Cd distribution of the leaves can also be clearly observed on the basis of the detection of the Cd Kα line. These images can be directly used to correlate the distribution of elements in relation to the plant tissue structure. In Fig. 1, some spots containing high levels of Zn, Cd, and Mn correspond to the trichomes of the leaf. It was found that Cd was preferentially distributed in the trichomes (Fig. 1(b)). Trichomes are epidermal hairs and present on the surface of the *A. gemmifera* leaves. There have been several reports concerning heavy metal accumulation in the trichomes.[1–3,17] Moreover, it has been found that both Zn and Mn accumulate in the trichomes (Figs. 1(c) and (e)). It seems that the Cd-accumulating trichomes (Fig. 1(b)) differ from those containing Mn (Fig. 1(e)), although the trichomes are present

Table 1 Concentration of cadmium and zinc in the leaves of *A. gemmifera* ($n = 1$)

		Concentration[b]/mg kg^{-1}	
Plant part	DW[a]/mg pot^{-1}	Cd	Zn
Young leaves	50.5	1700	156
Old leaves	152	595	119
Entire shoot[c]	202.5	870	128

[a] DW: dry-weight.[b] The concentration was estimated from the dry-weight.[c] A total of young and old leaves.

 This journal is © The Royal Society of Chemistry 2008

View Article Online

Published on 09 July 2008. Downloaded by Pennsylvania State University on 16/09/2016 00:32:30.

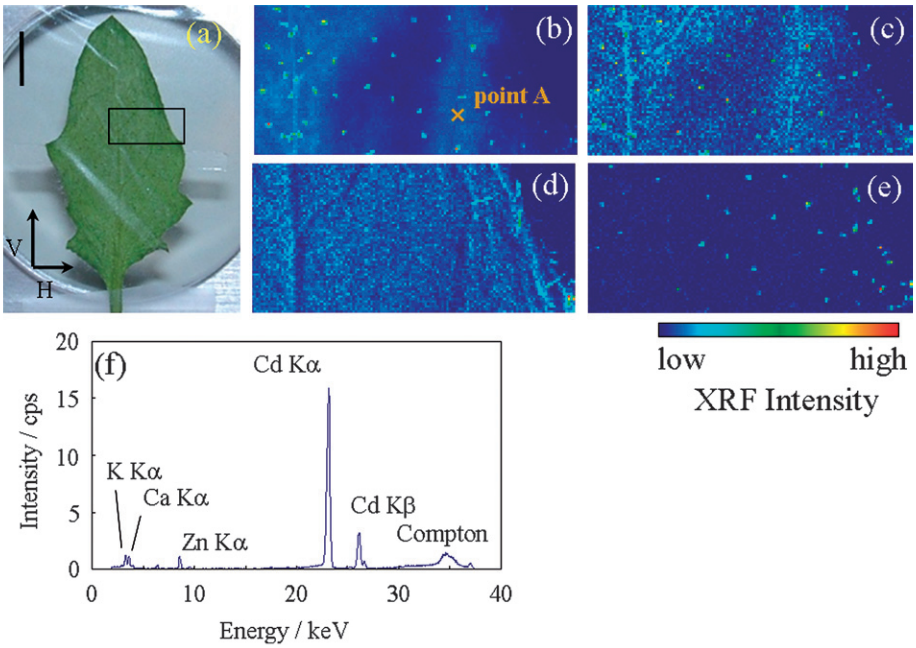

Fig. 1 μ-XRF imaging of a leaf of *A. gemmifera*. (a) Photograph of the leaf showing the measured imaging area, scale bar = 5 mm. The two-dimensional distribution of (b) Cd, (c) Zn, (d) Ca, and (e) Mn. (f) X-ray fluorescence spectra of the leaf tissue (point A in Fig.1(b)). Measurement time, 300 s. Measurement condition: imaging area, 6.5 mm (H) × 3 mm (V); X-ray energy, 37 keV; beam size, 50 μm × 50 μm, step size, 50 μm × 50 μm; step number, 131 point (H) × 61 point (V); dwell time, 0.5 s point^{-1}.

over the entire surface of the leaves. Cd was primarily distributed in the trichomes that are present in the surroundings of the main vein and the branch. In contrast, Mn was distributed in the trichomes that are present in the margin of the leaf. Mn accumulation was not clearly observed in the trichomes around the main vein and the branch. The distribution of Zn was observed in the trichomes in the entire leaf. Since Zn is an essential element, trichomes typically tend to accumulate high levels of zinc. However, it is noteworthy that there was almost no Mn at all distributed in the trichomes surrounding the vascular tissues. The accumulation of these elements in trichomes might be affected whether their location is in (or near) the surroundings of the main vein and the branch, when the exposure time is not long enough to reach the accumulation equilibrium.

Based on to the whole leaf, it was found that Cd and Zn were distributed around the main vein and its branch. However, the presence of Mn is not clear because it exists below the detection limit of the detection method. Ca was distributed throughout the whole leaf, and especially at the edge of the leaf, as seen in Fig. 1(d).

3.3. μ-XRF analysis of trichomes

The cellular distributions of Cd, Zn, Mn and Ca present in the trichomes were successfully revealed for the first time, as shown in Fig. 2, together with photographs taken by a digital microscope. The SEM image obtained using a low vacuum SEM with cool stage in the backscattering electron (BSE) mode under a chamber pressure of 20 Pa is also shown in Fig. 2(k). Two trichome samples taken from two individual leaves were subjected to the analysis to check the reproducibility (Fig. 2(a) and (f)). The trichomes were present in the area between the main vein and the margin of the leaf. The two-dimensional imaging clearly revealed the distributions of the various elements. High accumulations of Cd, Zn and Mn were found in the trichomes. Cd, Zn and Mn were accumulated in an *ca.* 20 μm area (Fig. 2(b), (c), (e), (g), (h) and (j)), which was 20–50 μm above the base of the trichome, especially located in a ring region of the trichome. In contrast, Ca was highly enriched in the upper part of the trichome (Fig. 2(d) and (i)), especially at the tip, while little was found in the base of the trichomes where Cd had highly accumulated. The high contrast portion of the SEM-BSE image corresponds well with the portion where these heavy metals had highly accumulated. Isaure *et al.* studied the trichome of *A. thaliana* using an SEM and named the border of the white and black portions as the strip.[27] It was found that Cd located in the trichomes exhibited the highest amounts in the strip of the trunk. Fig. 2(l) shows a close-up of the Cd distribution in the trichome visualized in Fig. 2(g). This clearly reveals that Cd locates at the cross-section of the trichome like a ring. In particular, Cd was found to be sequestered in a small compartment in a layer near the surface of the trichome. These findings are consistent with the prediction by Küpper *et al.*,[1] who revealed by an SEM-EDS analysis that Zn accumulated in a narrow ring in the trichome base and assumed that Cd and Zn may be compartmentalized in a similar way.

The present study provides new insights into the distribution of Cd and other elements in the trichomes. Such a finding could not be revealed by SEM-EDS, which may allow for the detection of the L line, but not the K line of Cd. The μ-XRF analysis of *A. thaliana* was conducted using an X-ray microbeam (0.9 μm × 0.3 μm) at the European Synchrotron Radiation Facility (ESRF, Grenoble).[27] The Cd distribution was measured using the Cd Lα line with an incident X-ray energy

View Article Online

Published on 09 July 2008. Downloaded by Pennsylvania State University on 16/09/2016 00:32:30.

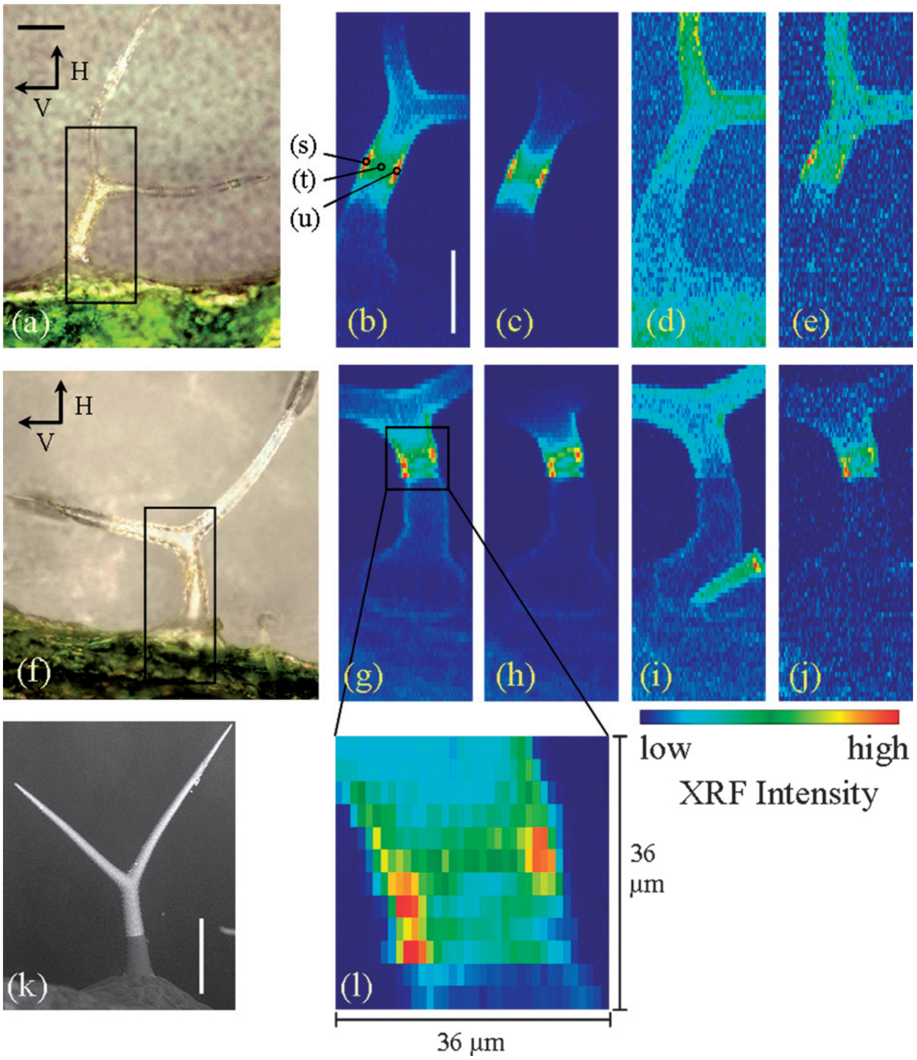

Fig. 2 μ-XRF imaging of trichome. (a) and (f) are photographs of the measured samples showing the imaging area, (b) Cd, (c) Zn, (d) Ca and (e) Mn of (a), (g) Cd, (h) Zn, (i) Ca, and (j) Mn of (f). For the maps, imaging area of 204 μm (*H*) × 81 μm (*V*), beam size of 3.8 μm (*H*) × 1.3 μm (*V*), step size of 3 μm (H) × 1 μm (V), step-number of 68 point (*H*) × 81 point (*V*), and dwell time of 0.3 s point^{-1} were used. (k) SEM image of trichome. (l) distribution of Cd in the area shown in the box in Fig. 2(g). (s)–(u) are measured points by μ-XANES of Cd. The scale bar is 50 μm except for (k), for which the scale bar is 100 μm.

below the absorption edge of K (3.608 keV). They showed that Cd is mostly located in the trichomes, with the highest amounts of metal encountered in the strip of the trunk, in the ends of the branches, and in the small bumps on the trichome surface.[27] The low-energy Lα line (3.134 keV) analysis should be surface-sensitive. Since the high-energy Cd Kα line is much more penetrating than the Cd Lα line, the ring-like shape of the three-dimensional distribution of Cd has been revealed for the first time by the present study.

3.4. Correlation of the distribution of elements

Fig. 3(a) and (b) show the correlations between the XRF intensities of Cd *vs*. Zn and Cd *vs*. Mn, respectively. The data shown in Fig. 1 are used in the plots. A significant positive correlation was observed between the XRF intensities of Cd and Zn in the leaf (correlation coefficient: $r^2 = 0.5021$, data number: $n = 7991$). In Fig. 3(a), the high-intensity area (Cd > 1000 cps, Zn > 40 cps) corresponds to the data from the trichomes. In this area, a positive correlation between Cd and Zn was found. In contrast, no correlation was observed between Cd and Mn (Fig. 3(b)).

Similarly, Fig. 3(c) and (d) show a correlation between the XRF intensities of Cd *vs*. Zn (c) and Cd *vs*. Mn (d) in the trichome sample using the data corresponding to the area shown in Fig. 2(l). Fig. 3(c) indicates a strong positive correlation between the distribution of Cd and that of Zn ($r^2 = 0.8674$, $n = 438$). Here, the correlation efficient was calculated using all data except for the zero intensity. It was found that this positive correlation in the trichomes is much stronger than that in the entire mesophyll tissue shown in Fig. 3(a) and (b). Mn was distributed in the bases of the trichomes together with Cd and Zn (Fig. 2). Fig. 3(d) shows that there was also a positive correlation between the distribution of Cd and that of Mn ($r^2 = 0.8009$, $n = 438$).

 This journal is © The Royal Society of Chemistry 2008

View Article Online

Published on 09 July 2008. Downloaded by Pennsylvania State University on 16/09/2016 00:32:30.

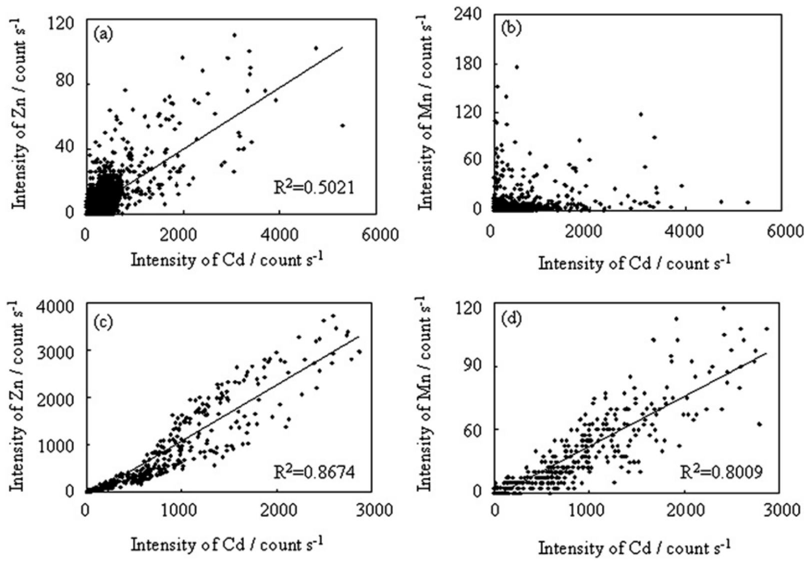

Fig. 3 Correlation between XRF intensities of (a) Cd *vs.* Zn and (b) Cd *vs.* Mn in the leaf of *A. gemmifera*, and (c) Cd *vs.* Zn, and (d) Cd *vs.* Mn correspond to the area given in Fig. 2(l). The XRF data shown in Figs. 1 and 2 were used in the plots.

The results showed that Cd and Zn behave in a similar way in the leaf and trichomes. It is known that the chemical behavior of Cd resembles that of Zn because both elements belong to Group 12 in the periodic table. As such, the accumulation of these elements may proceed *via* a similar transportation pathway in the plant. Bert *et al.* showed evidence of a generic correlation between the Cd and Zn accumulations in the aerial parts of *A. halleri*.[5] They suggested that the metals are taken up, at least in part, by the same transporter(s), or that their transporters, when different, are controlled by common regulators.[5] Zhao *et al.* also indicated that *A. halleri* can hyperaccumulate Cd, although the short-term uptake of Cd is at a lower rate than that of Zn.[6] Both the uptake and root-to-shoot translocation of Cd are inhibited by Zn, suggesting that Cd enters the *A. halleri* cells partly through the Zn transport pathway.[6] The present study clearly visualized the elemental distribution in the tissue with a cell level spatial resolution, which offers visual evidence supporting these important studies.

3.5. Chemical form of Cd in the trichomes

The Cd K edge μ-XANES spectrum of the plant was successfully measured for the first time by utilizing the K-B mirror and the MOSTAB system. The Cd K-edge μ-XANES spectra of the trichome and the reference materials are shown in Fig. 4. The spectra of CdS and CdO obtained using an X-ray microbeam are in good agreement with those obtained by a nonfocused beam.[23] The reference compounds more or less exhibited a similar absorption edge energy because the valence states of Cd are all divalent. However, the spectrum above the absorption edge, 26.72–26.76 keV, shows a characteristic difference in shape between Cd with the S ligand and that with the O ligand. Thus, the spectrum of Cd with the S ligand (CdS, PC-Cd, MT-Cd) can be distinguished from those of Cd with the O ligand (CdO, $Cd(NO_3)_2$aq, AcCd). The covalent nature is high in the Cd-S bond, while the ionic nature is high in the Cd-O bond. The intensity of the white line peak is related to both the covalent and ionic nature of the bond. The Cd K-edge XANES spectrum above the white line peak shows a very flat feature for the compounds containing Cd with the S ligand (Fig. 4(d)–(f)), which is consistent with the CdS and PC-Cd data reported by Pickering *et al.*[23] The Cd ion is hydrated in $Cd(NO_3)_2$ aqueous solutions and the Cd ion is coordinated by six O ligands. Fig. 4 shows that the spectrum of $Cd(NO_3)_2$aq is similar to that of AcCd. Salt *et al.* have also used an aqueous solution of $Cd(NO_3)_2$

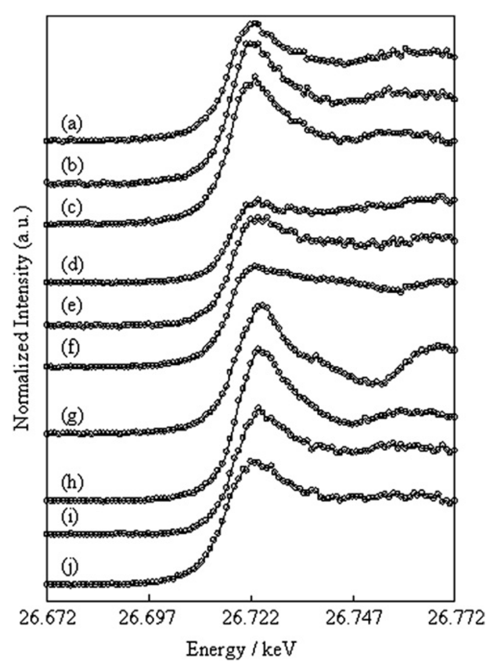

Fig. 4 Cadmium K-edge μ-XANES spectra for the trichome of *A. gemmifera* and the reference compounds. (a) Point s, (b) point t, (c) point u of the trichome sample in Fig. 2(b). (d) PC-Cd, (e) MT-Cd, (f) CdS, (g) CdO, (h) $Cd(NO_3)_2$ aq, (i) AcCd, (j) $(Im)_6Cd(NO_3)_2$. Beam size: 3.8 μm × 1.3μm, energy step: 1 eV, and dwell time of 1–4 s point^{-1}.

View Article Online

Published on 09 July 2008. Downloaded by Pennsylvania State University on 16/09/2016 00:32:30.

as a standard compound of Cd in the six-fold coordination of O, for their Cd K-edge XAFS analysis of *B. juncea* seedlings.[20] The Cd K-edge spectra of points (s), (t), and (u) (Fig. 2(b)) in the trichome were successfully acquired (Fig. 4(a)–(c)). Since Cd was highly accumulated in these hot spots, each spectrum could be measured by a counting time of 2 or 4 s point^{-1}. It was found that these spectra are similar in shape to those of the compounds containing Cd with the O ligand, especially to $Cd(NO_3)_2$aq and AcCd (Fig. 4(h)–(i)). Therefore, it was assumed that the Cd accumulated in the trichome was divalent Cd coordinated with O. It should be noted here that the XANES feature of $(Im)_6Cd(NO_3)$ (Fig. 4(j)) in which Cd is coordinated by N is similar to that of AcCd. Since we cannot distinguish the N ligand from the O ligand by the present qualitative XANES analysis, the possibility of the presence of the N coordinated Cd compound in the trichomes cannot yet be ruled out.

Sarret *et al.* carried out the chemical speciation of Zn on a micron scale by Zn K-edge μ-EXAFS in the base of the trichomes of *A. halleri*.[3] The spectrum of the trichomes was compared with a large number of Zn organic compounds, but no good match was obtained. The data suggest that in the base of the trichomes, Zn is four-fold coordinated and complexed to carboxyl and/or hydroxyl groups belonging to organic acids. Our results are consistent with their findings that ligands coordinated with metal elements in the trichome are oxygen. In view of the chemical similarly between Cd and Zn, both metals may coordinate with the same ligand. To the best of our knowledge, the chemical form of Cd in *A. halleri* has never been studied using X-ray absorption spectrometry, though there have been some reports regarding another plant.[19–21,27] Isaure *et al.* investigated the distribution and chemical form of cadmium in the trichomes and the root of *Arabidopsis thaliana* using Cd L_{III}-edge μ-XANES spectroscopy with a beam size of 0.9 μm × 0.3 μm.[27] Approximately 75% of the cadmium in the trichomes was bound to O or N atoms, likely belonging to the cell wall or cuticle of the trichome. Our results are consistent with theirs, although the plant species are different. Moreover, they have indicated that Cd in the root is unambiguously bound to S ligands and coordinated to the thiol groups of cysteine.[27] Salt *et al.* applied X-ray absorption spectrometry without focusing optics to *B. juncea* exposed to 0.6 μg mL^{-1} Cd for 7 days, and reported that Cd in the xylem sap is predominantly coordinated by O or N ligands, while the majority of the Cd in the root is coordinated by S ligands, probably as a Cd-S_4 complex.[19]

In the present study, a high-energy synchrotron radiation X-ray microbeam focused with a K-B mirror was utilized to study the chemical form of Cd on a cellular scale. The shape of the Cd μ-XANES spectrum suggested that O ligands are bound to Cd in the trichome. The XANES feature was more similar to $Cd(NO_3)_2$aq and AcCd than CdO, but the details of the chemical form of Cd in the trichomes remain unknown. Measurement of the X-ray absorption spectrum in the EXAFS region could be useful for future investigations. On the other hand, the distribution and chemical forms of Cd in the root were not studied in the present investigation because of the low Cd concentrations. In addition, the size of the root was too small (a diameter of about 100 μm) to prepare a good sample and await future improvements in both the sample preparation and measurement systems.

4. Conclusions

The combination of μ-XRF and μ-XANES with a submicron resolution have proved to be an indispensable tool for the study of Cd accumulation in biological samples on a cellular scale. A positive correlation between the XRF intensity and distribution of Cd and Zn in the leaf was found ($r^2 = 0.5021$, $n = 7991$). The Cd and Zn were highly accumulated in the trichomes (epidermal hairs) and a strong positive correlation was found between the XRF intensity of Cd and Zn ($r^2 = 0.8674$, $n = 438$) and between that of Cd and Mn ($r^2 = 0.8009$, $n = 438$). These elements were accumulated in a ring-shape at the border between the upper and lower parts of the trichome. The results indicated that a striking sub-cellular compartmentalization of Cd and Zn would occur in the vacuole of the trichomes, and these compartmentalizations play an important role in the hyperaccumulating process of this plant. In these specialized cells, the μ-XANES results showed that the majority of Cd was divalent and bound to O or N ligands, not S ligands. These data were obtained for the first time using a high-energy X-ray microbeam. In future research, we will investigate the change in distribution and the chemical form of Cd through the transportation process of Cd. It will include rate dependent uptake as a function of the medium in which the plant is grown. Similarly, competitive reaction between Zn and Cd in the process of uptake, transfer, and accumulation for them could also be demonstrated as a function of the concentration of either element in the plant environment.

Acknowledgements

The authors wish to express their appreciation to Professors Kazumasa Hirata (Graduate School of Pharmaceutical Science, Osaka University, Japan) and Hatsuo Maeda (School of Pharmacy, Hyogo University of Health Sciences) for their kind gift of the purified phytochelatin-Cd complex. This study was partly supported by a Grant-in-Aid for Scientific Research (No. 17350040) from the Ministry of Education, Culture, Sports, Science and Technology, Japan. This study was carried out under approval of the SPring-8 Program Advisory Committee (2005A0542-NXb-np, 2005B0743-NXb-np, 2006A1686-NXb-np).

References

1 H. Küpper, E. Lombi, F. J. Zhao and S. P. McGrath, *Planta*, 2000, **212**, 75.
2 F. J. Zhao, E. Lombi, T. Breedon and S. P. McGrath, *Plant, Cell Environ.*, 2000, **23**, 507.
3 G. Sarret, P. Saumitou-Laprade, V. Bert, O. Proux, J. L. Hazemann, A. Traverse, M. A. Marcus and A. Manceau, *Plant Physiol.*, 2002, **130**, 1815.
4 S. P. McGrath, E. Lombi, C. W. Gray, N. Caille, S. J. Dunham and F. J. Zhao, *Environ. Pollut.*, 2006, **141**, 115.
5 V. Bert, P. Meerts, P. Saumitou-Laprade, P. Sails, W. Gruber and N. Verbruggen, *Plant Soil*, 2003, **249**, 9.
6 F. J. Zhao, R. F. Jiang, S. J. Duham and S. P. McGrath, *New Phytol.*, 2006, **162**, 646.
7 M. Cho, A. N. Chardonnens and K. J. Dietz, *New Phytol.*, 2003, **158**, 287.
8 C. Cosio, E. Martinoia and C. Keller, *Plant Physiol.*, 2004, **134**, 716.
9 S. P. McGrath, E. Lombi, C. W. Gray, N. Caille, S. J. Dunham and F. J. Zhao, *Environ. Pollut.*, 2006, **141**, 115.
10 V. Bert, I. Bonnin, P. Saumitou-Laprade, Patrick de Lagueerie and D. Petit, *New Phytol.*, 2002, **155**, 47.

 This journal is © The Royal Society of Chemistry 2008

View Article Online

Published on 09 July 2008. Downloaded by Pennsylvania State University on 16/09/2016 00:32:30.

11 H. Dahmani-Muller, F. van Oort and M. Balabane, *Environ. Pollut.*, 2001, **114**, 77.
12 H. Dahmani-Muller, F. van Oort and L. Denaix, *Environ. Pollut.*, 2002, **117**, 487.
13 H. Kubota and C. Takenaka, *Int. J. Phytorem.*, 2003, **5**, 197.
14 L. S. Toppi and R. Gabbrielli, *Environ. Exp. Bot.*, 1999, **41**, 105.
15 M. P. Benavides, S. M. Gallego and M. L. Tomaro, *J. Plant Physiol.*, 2005, **17**, 21.
16 M. B. Kirkham, *Geoderma*, 2006, **137**, 19.
17 A. Hokura, R. Onuma, N. Kitajima, Y. Terada, H. Saito, T. Abe, Shigeo Yoshida and Izumi Nakai, *Chem. Lett.*, 2006, **35**, 1246.
18 G. Gutiérrez-Alcalá, G. Gotor, A. J. Meyer, M. Fricker, J. M. Vega and L. C. Romero, *Proc. Natl. Acad. Sci. U. S. A.*, 2000, **97**, 11108.
19 D. E. Salt, R. C. Prince, I. J. Pickering and I. Raskin, *Plant Physiol.*, 1995, **109**, 1427.
20 D. E. Salt, I. J. Pickering, R. C. Prince, D. Gleba, S. Dushenkov, R. D. Smith and I. Raskin, *Environ. Sci. Technol.*, 1997, **31**, 1636.
21 H. Küpper, A. Mijovilovich, W. Mever-Klaucke and P. M. H. Kroneck, *Plant Physiol.*, 2004, **134**, 748.
22 J. L. Freeman, L. H. Zhang, M. A. Marcus, S. Fakra, S. P. Mc Grath and E. A. H. P. -Smits, *Plant Physiol.*, 2006, **142**, 124.
23 I. J. Pickering, R. C. Prince, G. N. George, W. E. Rauser, W. A. Wickramasinghe, A. A. Watson, C. T. Dameron, I. G. Dance, D. P. Fairlie and David E. Salt, *Biochem. Biophys. Acta*, 1999, **1429**, 351.
24 G. de la Rosa, J. R. Peralta-Videa, M. Montes, J. G. Parsons, I. Cano-Aguilera and J. L. Gardea-Torresdey, *Chemosphere*, 2004, **55**, 1159.
25 P. Le Lay, M.-P. Isaure, J.-E. Sarry, L. Kuhn, B. Fayard, J.-L. Le Bail, O. Bastien, J. Garin and C. Roby, *J. Bourguignon, Biochim.*, 2006, 1.
26 G. Sarret, E. Harada, Y.-E. Choi, M.-P. Isaure, N. Geoffroy, S. Fakra, M. A. Marcus, M. Birschwilks, S. Clemens and A. Manceau, *Plant Physiol.*, 2006, **141**, 1021.
27 M. P. Isaure, B. Fayard, G. Sarret, S. Pairis and J. Bourguignon, *Spectrochim. Acta, Part B*, 2006, **61**, 1242.
28 A. Bernaus, X. Gaona, D. Van Ree and M. Valiente, *Anal. Chim. Acta*, 2006, **565**, 73.
29 S. Tamura, M. Yasumoto, N. Kamijo, Y. Suzuki, M. Awaji, A. Takeuchi, K. Uesugi, Y. Terada and H. Takano, *Vacuum*, 2006, **80**, 823.
30 M. Awaji, Y. Suzuki, A. Takeuchi, H. Takano, N. Kamijo, M. Yasumoto and Y. Terada, *Rev. Sci. Instrum.*, 2003, **74**, 4948.
31 N. Kamijo, Y. Suzuki, S. Tamura, M. Yasumoto, A. Takeuchi and M. Awaji, *Rev. Sci. Instrum.*, 2003, 74.
32 Y. Terada, A. Takeuchi, Y. Suzuki, R. Onuma, K. Shirai, N. Kitajima, A. Hokura and I. Nakai, *SPring-8 User Experiment Report 2004B*, p. 170.
33 A. Awaji, K. Uesugi, Y. Suzuki, A. Takeuchi and Y. Terada, *SPring-8 Annual Report*, 2004, 95.
34 A. Takeuchi, Y. Suzuki, Y. Terada and M. Awaji, *SPring-8 User Experiment Report 2004B*, p. 125.
35 T. P. Kudo, Y. Nishino, M. Suzuki, H. Tanida, Y. Furukawa, T. Hirono and T. Ishikawa, *J. Jpn. Soc. Synchrotron Radiat. Res.*, 2003, **16**, 39.
36 A. D. Mighell and A. Santoro, *Acta Crystallogr.*, 1971, **B27**, 2089.