Simultaneous compartmentalization of lead and arsenic in co-hyperaccumulator Viola principis H. de Boiss.: An application of SRXRF microprobe

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A R T I C L E   I N F O

Article history:
Received 4 December 2007
Received in revised form 27 April 2008
Accepted 28 April 2008
Available online 20 June 2008

Keywords:
Heavy metal
Micro-distribution
Nutrient element
Phytoremediation
Synchrotron radiation X-ray fluorescence
Trichome

A B S T R A C T

The cellular distributions of Pb and As in the leaves of co-hyperaccumulator Viola principis H. de Boiss. were inspected by synchrotron X-ray fluorescence spectroscopy (SRXRF). The results revealed that Pb and As had similar compartmentalization patterns in the leaves. Both elements were enriched in the bundle sheath and the palisade mesophyll. In comparison with the sheath and the mesophyll, the vascular bundle and the epidermis contained lower levels of Pb and As. The palisade enrichment of Pb and As indicated that V. principis H. de Boiss. may have a special mechanism on detoxification of toxic metals within the mesophyll cells. Relative concentrations of both Pb and As in trichome bases were higher than those in trichome rays. The results of hierarchical cluster analysis and correlation analysis confirmed that the distribution of Pb was similar to that of As in the leaves, and their distribution patterns were different from the nutrient elements, such as K, Ca, Mn, Fe, Ni, Cu and Zn. In vivo cellular localization of Pb and As in the leaves provides insight into the physiological mechanisms of metal tolerance and hyperaccumulation in the hyperaccumulators.

1. Introduction

That plants accumulate extremely high concentrations of trace metals in their harvestable biomass may offer a sustainable method for phytoremediation of metal contaminated sites and an opportunity to metal-scavenging from the contaminated soils or mineral ores that cannot be used by conventional mining (Brooks et al., 1998; Chaney et al., 1999; Martinez et al., 2006; Quartacci et al., 2006; Tanhan et al., 2007). Some species of genus Viola have been reported to be endemic to metalliferous soils that containing cumulative Pb, As and Cd up to 2350, 1032, and 1201 mg kg⁻¹ dry mass in the shoot (Wei et al., 2004). Another Viola species, Viola baoshanensis grown in mining area hyperaccumulated Cd up to 2310 mg kg⁻¹ dry mass in the shoot (Wei et al., 2004). Another Viola species, Viola principis H. de Boiss. was also found to hyperaccumulate Pb, As and Cd up to 2350, 1032, and 1201 mg kg⁻¹ dry mass in the leaves, with transport factors (ratio of element concentration in shoot to that in root) of 3.27, 1.41 and 3.89, respectively. In the same time, the bioconcentration factor (ratio of element concentration in shoot to that in soil) of Cd was more than 1.31. In the same time, the bioconcentration factor of Cd was more than 1.31. So this species is believed to be a candidate for phytoremediation of contaminated soil with multi metals.

Hyperaccumulation of heavy metals requires that plants have a high capacity of detoxification (Baker and Reeves, 1989). Brooks (1998) stated that heavy metals might be stored where they could not interrupt the normal metabolic activities of the cell in the leaves of hyperaccumulators. There are evidences that the Zn/Cd hyperaccumulator Thlaspi caerulescens accumulated higher concentrations of the metals in the leaf epidermis than in the mesophyll (Küpper et al., 1999; Frey et al., 2000; Cosio et al., 2005). Accumulation of Ni in epidermal cells seemed to be a common feature in the leaves of Ni-hyperaccumulators, such as Thlaspi goresiense, Alysum species and Berkhaya coddi (Krämer et al., 1996; Küpper et al., 2000, 2001; Kerkeb and Krämer, 2003; Broadhurst et al., 2004a; McNear et al., 2005). Similar distribution patterns were reported as As in the pinna of Petriis vitatta (Lombi et al., 2002). However, the sequestration of toxic metals in leaf epidermis was not a universal detoxification mechanism in all hyperaccumulators. Essential metals like Zn, Mn and Ni, as well as toxic metals like Cd and As could be enriched in the mesophyll of hyperaccumulator leaves. Mesjasz-Przybylowicz et al. (2001) noticed that the enrichment of Ni occurred in the leaf mesophyll of B. coddi. Copper in Elsholtzia splendens (Shi et al., 2004) and Mn in Gossia bidwillii (Fernando et al., 2006) were more abundant in the mesophyll than in the epidermis of leaves. Besides compartmentalization in the mesophyll and the epidermis, the compartmentalization of Zn, Cd, As and Ni in leaf trichomes has been well documented (Blamey et al., 1986; Zhao et al., 2000; Broadhurst et al., 2004b; Li et al., 2006). The cellular distribution of Pb in leaf is less studied, with only one report on Pb distribution in the...
trichome of Nicotiana tabacum leaf (Martell, 1974). The cellular compartmentalization of Pb in hyperaccumulator leaves has not been reported.

To understand the mechanisms on hyperaccumulations of Pb and As in the co-hyperaccumulating plant, in vivo cellular localization of Pb and As in the leaves of V. principis H. de Boiss. was investigated by the synchrotron X-ray fluorescence spectroscopy (SRXRF), and their distribution patterns in the leaves were compared with those of nutrient elements.

2. Materials and methods

2.1. Sample collection and preparation for SRXRF analyses

Fresh green leaves were collected from healthy plants of V. principis H. de Boiss. growing at metalliferous soils of Baoshan in Southern China, where the soils are heavily contaminated with Zn, Pb, Cu, Fe and Zn. Each plant consisted of 2–4 shoots growing on the rhizome. The leaf samples were washed with tap water and then rinsed with deionized water to remove the possible dust deposited from air. Parts of leaves were used for SRXRF scanning, while the others were used for analyses of the element concentrations. The leaves used for SRXRF analyses were individually placed in an aluminum foil containers with deionized water inside and quickly frozen at −30 °C. The frozen leaf with ice around was then cut into sections with a thickness of 20 μm with a cryo-microtome (Figocut 2700; Reichert-Jung, Nussloch, Germany). The sections were attached with polyethylene film to the sample holders, and freeze-dried at −20 °C for 7 days (Ager et al., 2002).

The leaf samples for analyses of their elemental concentrations were dried at 65 °C for 24 h and then wet-digested in HNO3–HClO4 (7:3, v/v) for 12 h in a heating program with the highest temperature of 120 °C. Standard reference material GSV-2 and spiking standards were used. Concentrations of Pb, Cd, K, Ca, Mn, Ni, Cu and Zn in digests were analyzed with atomic absorption spectrometer (Vario 6, Jena, Germany), and As was analyzed with hydride generation coupled with atomic fluorescence spectrometer (APS-2202, Haigang, China). The mean values of Pb, As, Cd, K, Ca, Mn, Ni, Cu and Zn concentrations in the leaf samples were 1215, 708.9, 442.9, 255.6, 2046, 2977, 212.9, 105.1, and 34.61 μg g⁻¹ dry mass, respectively.

2.2. SRXRF analyses

X-ray fluorescence scanning was performed at an XRF station on Beamline 4W1A of the Beijing Synchrotron Radiation Facility (BSRF). The electron storage ring was operated at 2.2 GeV with the electron current ranging from 59 to 114 mA during the experiment. A special set of adjustable slits was used to confine the size of the exciting X-ray beam to 20 μm × 20 μm, which allowed us to get the elemental count of specific cells. The samples were fixed with a precision sample positioning stage, with 1 μm per translocation step in three-dimensions, driven by computer-controlled stepping motors. The sample profile was adjusted to 45° with respect to the beam direction and a fluorescent detector was located in the position of 30 mm away from the sample according to the signal intensity of the elements. The fluorescent radiation was detected using a PGT Si (Li) solid detector with a 7.5 μm thick beryllium window, and a resolution of 134 eV at 5.89 keV. The detector was located at 90° with respect to the beam direction, and the signals were connected to a multi-channel energy dispersive spectrometer. Scanning was performed in a spot-by-spot manner, and the exposure time was 100 s for each spot. Spectra resolutions were processed using the program AXIL (Chen et al., 2005) to integrate the excited peak area of Pb, As, K, Ca, Mn, Ni, Cu, Fe and Zn. Unfortunately, because the Kα line emission energy of Cd was excluded from the optimal energy range of SRXRF spectra, and Lx line was overlapped by the peaks of K and Ar, the distribution of Cd in leaf could not be analyzed by BSRF. Relative concentrations of each element were calculated by means of the normalization of the Compton scattering intensity after calibrating the peak area with electron current (Liu et al., 2000). The absorption edges of As Kγ line (11.85 keV) and Pb Lα line (12.60 keV) were used for calculating the relative concentration of Pb and As because the overlapping of As Kα line (10.51 keV) with Pb Lα line (10.53 keV) (Fig. 1).

For the leaf sections, SRXRF scanning points of the samples was selected by a microscope (Fig. 2A and D). To avoid the interference of trichome covering on the adaxial or abaxial surface of the leaf, all the point scanning was conducted on trichome-free leaf areas. The first group of scanning points was on the leaf surface where the midrib cross section, including the vascular tissue. The second group scanning points was on the leaf cross section with mesophyll cells adjacent to the midrib, and the third group of scanning points was on the leaf cross section between two longitudinal leaf veins near the leaf edge. The first group scanning was conducted for seven replicates, while the other two were conducted for six replicates.

SRXRF scanning was also conducted on the leaf surface where the trichomes were located. Two trichomes on the adaxial surface and two trichomes on the abaxial surface were randomly selected for scanning.

2.3. Statistical analyses

One way ANOVA was used to statistically test for significant differences. Bivariate correlations were analyzed with Pearson correlation coefficients after data transform for normal distribution. The method of between-group linkage was applied for the hierarchical cluster analysis. All of the statistical analyses were performed with software package SPSS 10.0 (SPSS, Chicago, IL).

3. Results

3.1. Pb and As micro-distribution in leaves

The SRXRF scanning results showed that the highest relative concentrations of both Pb and As in midrib cross sections were found in the vascular bundle sheath cells (Fig. 2B). The mean relative concentrations of Pb and As in sheath cells were 10.0 and 23.8 times higher than those in the vascular tissues (xylem and...
phloem), respectively. The lowest relative concentrations of both Pb and As were found in the collenchymas adjacent to adaxial epidermis. The relative concentrations of As found in vascular tissue, cortex, and epidermis showed no significant difference.

The palisade mesophyll cells adjacent to the bundle sheath sequestered significantly higher concentrations of Pb and As than the cells of other tissues did (p < 0.05, Fig. 2C). The relative concentrations of As found in vascular tissue, cortex, and epidermis showed no significant difference.

In the vascular-free leaf cross section near the leaf edge, again, both Pb and As showed the highest relative concentrations in the palisade mesophyll cells (Fig. 2E). Relative concentrations of Pb and As in the adaxial epidermis and the abaxial epidermis were lower than those of the palisade mesophyll cells. The relative concentrations of Pb or As in the abaxial epidermis were not significantly different from those in the adaxial epidermis and those in the cuticle (Fig. 2E).

3.2. Compartmentalization of Pb and As in trichome bases

The trichome on the leaf surface of V. principis is prickle, and is composed of ray and base part attaching to the epidermis (Fig. 3A). The SRXRF scanning results provided clear evidence that the relative concentrations of both Pb and As were higher in trichome bases than those in trichome rays (Fig. 3B). On the adaxial surface,
the relative concentrations of Pb and As in the trichome bases were 5.5 and 10.2 times of those in the trichome rays, respectively, while the concentration ratios of Pb and As were 2.6 and 3.6, respectively, on the abaxial surface.

3.3. Differences in micro-distribution between nutrient elements and Pb/As

Nine elements measured by SRXRF scanning were classified into two clusters according to the hierarchical cluster analysis of the relative concentrations in different types of cells of *V. principis* H. de Boiss. leaves. One cluster includes Pb and As, and the other includes the nutrient elements Fe, Cu, K, Zn, Mn, Ca and Ni (Fig. 4). The distributions of Pb and As were different from those of nutrient elements. In contrast with the enrichment of Pb and As in the bundle sheath, the relative concentrations of each nutrient element investigated did not show significant difference among the different types of cells in midrib part (Table 1). The relative concentrations of nutrient elements in the bundle sheath were similar to those in the vascular bundle. In contrast, the relative concentration of Pb or As in sheath cell was much higher than that in the bundle sheath, while the nutrient elements distributed evenly among bundle sheath and vascular bundle.

In the vascular-free leaf cross section near the leaf edge, the mesophyll enrichments of K, Mn, Fe, Ni, Cu and Zn were not observed (Table 1). The relative concentrations of nutrient elements in mesophyll were similar to those in the adjacent epidermis. Relative concentrations of each individual nutrient element did not show significant correlation with those of Pb or As in midrib cross section or the vascular-free leaf cross section near the leaf edge. However, the relative concentrations of Pb were significantly correlated to those of As (Table 2), indicating similar distribution patterns.

In the adaxial and abaxial trichome the relative concentrations of K, Ca, Mn and Zn were higher in trichome bases than those in trichome rays, but Fe, Ni and Cu did not show significant differences between the bases and the rays (Table 1). The relative concentrations of the nutrient elements of K, Ca, Mn, Fe and Zn were significantly correlated to those of Pb and As in the trichome (Table 2).

4. Discussion

The results from the SRXRF analyses of leaf sections (Fig. 2E) showed that Pb and As in *V. principis* H. de Boiss. were more concentrated in the mesophyll than in the epidermis. This result is different from most of the previous studies on metal cellular distribution, which supported the epidermal sequestration of Zn, Cd, As and Ni in hyperaccumulators (Krämer et al., 1996; Küpper et al., 1999,2001; Frey et al., 2000; Lombi et al., 2002; Robinson et al., 2003; Cosio et al., 2005; McNear et al., 2005). Similar to our results of As enrichment in the mesophyll of *V. principis* H. de Boiss., in the pinna of As hyperaccumulator *P. nervosa*, the highest concentration of As was found in palisade tissue (Chen et al., 2003). Although the Cd and Zn concentrations in the epidermal tissues of *T. caerulescens* (Ganges ecotype) were 2-fold higher than those of mesophyll tissues, 65–70% of total leaf Cd and Zn were distributed in the mesophyll tissues, suggesting that mesophyll was a major storage site of the two metals in the leaves (Ma et al., 2005). This study showed that Pb could be enriched in the palisade cells of *V. principis* H. de Boiss. In addition, this species could sequester As in mesophyll cells. Both Pb and As at high concentrations in photosynthetic tissue can exert a strong toxic effect on plants. Therefore, it is believed that *V. principis* H. de Boiss. may have a special mechanism on detoxification of toxic metals within the palisade cells.

After Pb enrichment in trichomes of N. tabacum leaf was found (Martell, 1974), the compartmentalization of heavy metals in the trichome has been reported for several elements, such as Cd.
in the leaf trichomes of Brassica juncea (Salt et al., 1995) and Arabidopsis thaliana (Ager et al., 2002), and Ni in the hyperaccumulator Alyssum lesbiacum (Krämer et al., 1997). Furthermore, in some hyperaccumulators, the trichome base compartmentalized higher concentrations of heavy metals than the trichome ray, such as As in P. vittata (Li et al., 2005), Cd and Zn in Arabidopsis halleri (Küpper et al., 2000; Zhao et al., 2000), Mn in Helianthus annuus (Blamey et al., 1986), Ni in Alyssum bertolonii (Broadhurst et al., 2004b), and Ni and Mn in Alyssum species (McNear et al., 2005). Our results showed that the trichome bases of V. principis H. de Boiss. also sequestered higher relative concentrations of Pb and As than the trichome rays (Fig. 3). The trichome (especially the trichome base) on the leaf surface of V. principis H. de Boiss. may be a sink for excessive Pb and As.

Unlike Pb and As, the nutrient elements were distributed evenly in epidermal cells and mesophyll cells (Table 1). In contrast to the enrichment of Pb and As in the mesophyll, the relative concentrations of nutrient elements in mesophyll were not higher than those in epidermis. A significant positive correlation was found between As and K in the leaves of the As hyperaccumulators, P. nervosa (Chen et al., 2003) and P. vittata (Lombi et al., 2002), which may be explained by the counterbalance of K anions to As anions. However, we did not observe a positive correlation between As and K in mesophyll and adjacent epidermis of V. principis H. de Boiss. (Table 2), possibly because As had the same distribution pattern as Pb, and they might form complexes to balance the charges. However, this should be considered with caution since in several studies As in hyperaccumulator plants such as P. vittata (Lombi et al., 2002; Webb et al., 2003) have shown that As was accumulated as arsinite, which, under physiological conditions, is uncharged and would not form complexes with Pb. Therefore, further studies are recommended to ascertain the speciation of As in this plant.

**Acknowledgements**

This research was funded by the National Science Fund for Distinguished Young Scholars (Grant No. 40325003). We wish to thank Mr. Wei-Xiang Chen and Dr. Xiao-Yong Liao for assistance in the plant sampling in field, Dr. Ying-Ru Liu for chemical analysis, and Dr. Yan Chen and Ms Xue-Ying Liu for comments on grammar of manuscript.

**References**

