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

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ABSTRACT

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 can not 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 high concentrations of Zn, Pb and Cd (Bizoux et al., 2004). *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. 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 meosphyll (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 goesingense*, *Alyssum* species and *Berkheya coddii* (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 *Pteris vittata* (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. coddii*. 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

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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 Pb, Zn, Cd and As because of the long mining history of Zn and Pb. 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 HNO_3–$HClO_4$ (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 (AFS-2202, Haiguang, China). The mean values of Pb, As, Cd, K, Ca, Mn, Ni, Cu and Zn concentrations in the leaf samples were 1215, 708.9, 255.6, 2046, 2977, 212.9, 105.1, 34.61 and 389.5 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 in a high 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 Lα line was overlapped by the peaks of K and Ar, the distribution of Cd in leaf could not be analyzed with 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 Kβ line (11.85 KeV) and Pb Lβ 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).

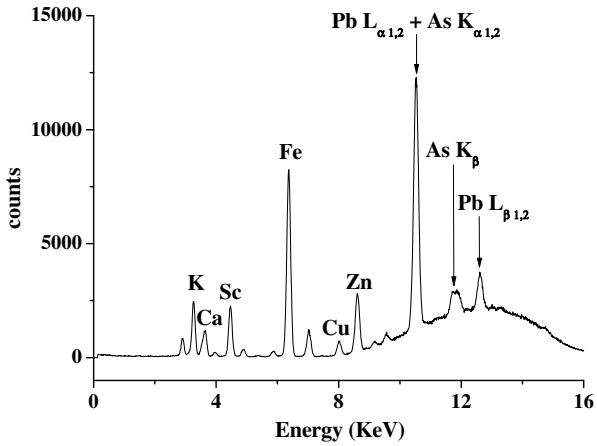

Fig. 1. Typical SRXRF spectra in leaf cell of *V. principis* H. de Boiss.

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 along the vertical middle axis of 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

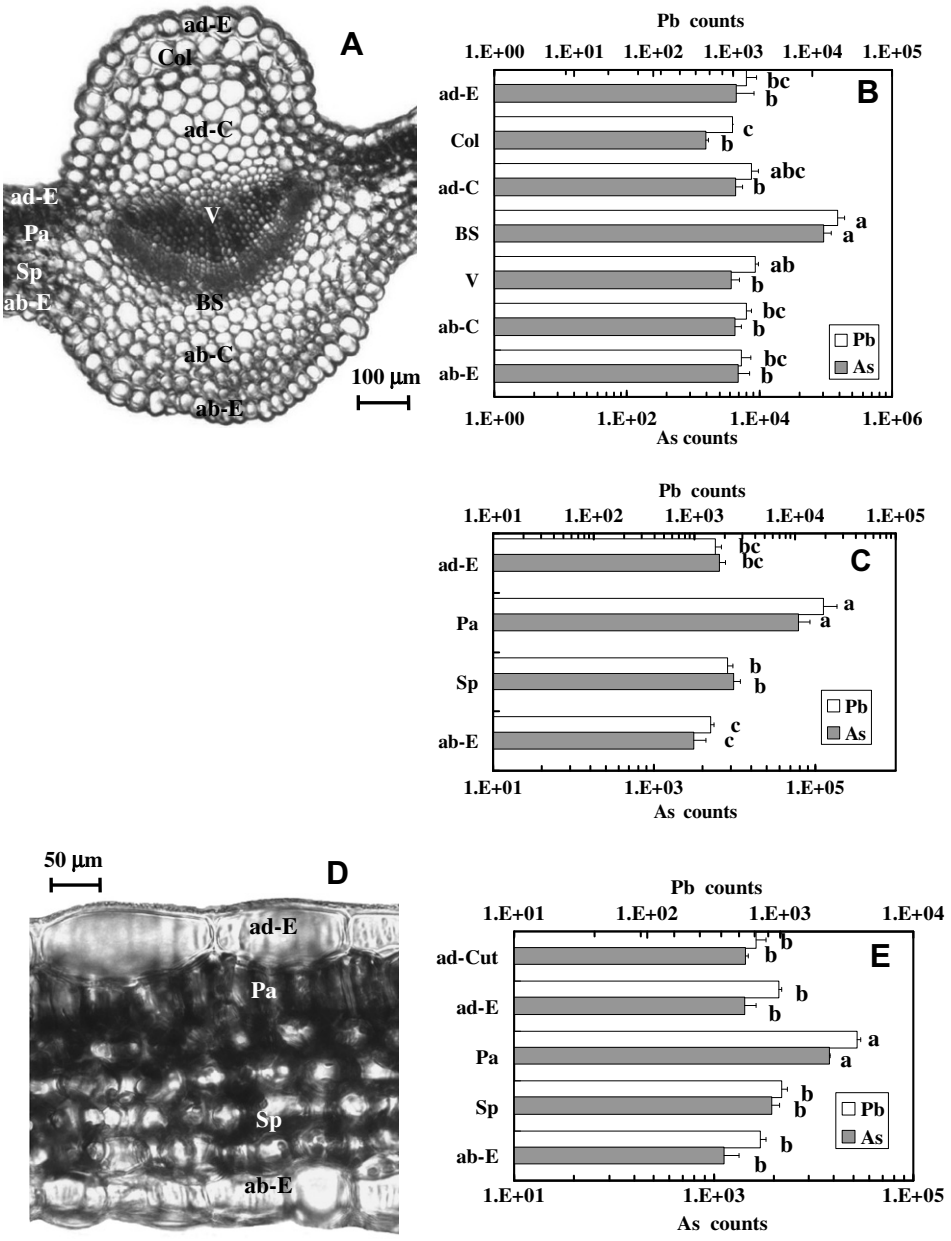

Fig. 2. SRXRF micro-analyses of Pb and As distribution in the leaf cross section of *V. principis* H. de Boiss. grown in metalliferous soils of Baoshan in Southern China. (A) The anatomical structure of midrib; (B) counts along the vertical axis with vascular tissue; (C) counts in the leaf cross section adjacent to midrib; (D) the anatomical structure of leaf cross section near leaf edge; (E) counts in the leaf cross section near leaf edge. Value is mean ± SE of 3–7 replicates for (B), 3–4 replicates for (C), and 2–6 replicates for (E). ad-E, adaxial epidermis; Col, collenchymas; ad-C, adaxial cortex; BS, bundle sheath; V, vascular bundle; ab-C, abaxial cortex; ab-E, abaxial epidermis; Pa, palisade; Sp, spongy; ad-Cut, adaxial cuticle. Different letters of a, b and c in each figure denote significant difference at the 0.05 level among different tissue of each element.

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 Pb and As in spongy mesophyll cells were less than those in palisade mesophyll cells, but were greater than those in neighboring abaxial epidermis cells. There was no significant difference for the relative concentrations of Pb or As between the abaxial and the adaxial epidermis cells, respectively (Fig. 2C).

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,

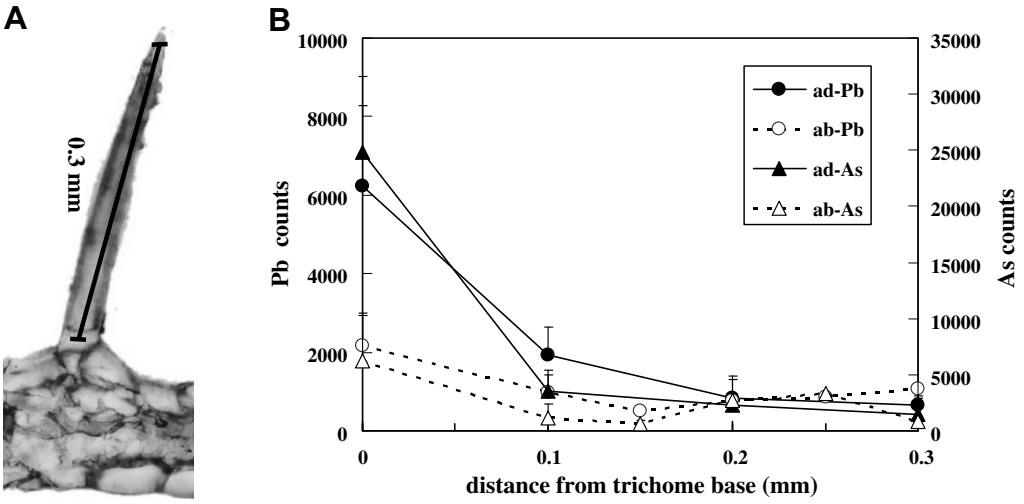

Fig. 3. SRXRF micro-analyses of Pb and As distribution in the trichome on the leaf surface of *V. principis* H. de Boiss. (A) Trichome on leaf surface; (B) counts of Pb and As in the trichome. Values are means ± SE of two replicates.

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 comparison, the relative concentration of Pb or As in sheath cell was much higher than that in the vascular bundle, reaching 11.0 and 24.8 times, respectively. So for *V. principis* H. de Boiss., Pb and As were enriched in the bundle sheath of leaf midrib, 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).

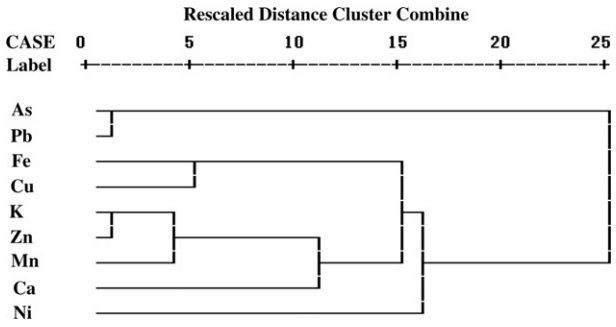

Fig. 4. Hierarchical cluster analyses of element micro-distribution in the leaf cross section of *V. principis* H. de Boiss. without trichome ($n = 53$, dendrogram using average linkage between groups).

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

Table 1
Nutrient elemental levels determined by SRXRF in different leaf tissues of *V. principis* H. de Boiss. grown in metalliferous soils in South China

Part	Cell	K	Ca	Mn	Fe	Ni	Cu	Zn
		(Counts)						
Midrib	Adaxial epidermis	38 259 ± 19257a	15 831 ± 8853a	845 ± 383a	13 713 ± 5323a	328 ± 87a	626 ± 151a	28 146 ± 14246a
	Collenchymas	17 222 ± 2271a	3583 ± 1727a	339 ± 208a	13 024 ± 10700a	164 ± 19a	588 ± 532a	10 927 ± 6093a
	Adaxial cortex	26 784 ± 4507a	3323 ± 533a	507 ± 74a	9154 ± 1180a	289 ± 51a	544 ± 90a	14 433 ± 2579a
	Bundle sheath	34 712 ± 25634a	3474 ± 2185a	635 ± 400a	11 441 ± 1576a	297 ± 45a	440 ± 199a	21 768 ± 17370a
	Vascular bundle	29 003 ± 9426a	3350 ± 972a	501 ± 124a	7487 ± 1212a	214 ± 27a	400 ± 136a	15 969 ± 4989a
	Abaxial cortex	18 157 ± 4543a	4860 ± 1706a	524 ± 133a	10 896 ± 3280a	241 ± 29a	306 ± 66a	11 889 ± 3216a
	Abaxial epidermis	16 845 ± 9662a	3324 ± 1729a	561 ± 396a	19 562 ± 19967a	180 ± 99a	609 ± 615a	14 484 ± 8101a
Cross section with mesophyll	Adaxial cuticle	11 408 ± 169a	936 ± 496a	478 ± 34a	7398 ± 1302a	108 ± 31a	442 ± 206a	12 168 ± 2499a
	Adaxial epidermis	8356 ± 3421a	1434 ± 566a	354 ± 189a	7686 ± 3866a	116 ± 46a	293 ± 186a	10 620 ± 6366a
	Palisade	9456 ± 3152a	3123 ± 1836a	380 ± 124a	4552 ± 1269a	94 ± 58a	273 ± 166a	10 177 ± 4148a
	Spongy	6756 ± 1214a	1773 ± 523a	237 ± 46a	3792 ± 789a	61 ± 10a	123 ± 23a	6398 ± 1320a
	Abaxial epidermis	9866 ± 2521a	825 ± 203a	509 ± 239a	10 245 ± 9206a	90 ± 5a	310 ± 208a	9286 ± 2419a
Adaxial trichome	Base	19 326 ± 25955a	6452 ± 8722a	579 ± 711a	20 905 ± 18621a	580 ± 775a	652 ± 695a	13 483 ± 18155a
	Ray	1531 ± 497b	156 ± 22b	64 ± 23b	5423 ± 907a	97 ± 11a	130 ± 32a	599 ± 240b
Abaxial trichome	Base	5055 ± 3419a	2669 ± 3177a	214 ± 154a	5494 ± 2430a	56 ± 4a	199 ± 140a	3879 ± 2832a
	Ray	1278 ± 257b	220 ± 50b	83 ± 19b	4268 ± 825a	62 ± 11a	331 ± 227a	1209 ± 374b

Values are means ± SE of 3–7 replicates for midrib, 2–6 replicates for cross section with mesophyll, 2–7 replicates for trichome. For each part analyzed, different letters of a and b denote significant difference at 0.05 level.

Table 2
Correlation coefficient of metal concentrations between Pb or As and nutrient elements in the leaves of *V. principis* H. de Boiss. based on the data from Table 1 and Figs. 2 and 3

	Correlation coefficient							
	K	Ca	Mn	Fe	Ni	Cu	Zn	As
Midrib (n = 33)								
Pb	0.06	−0.08	0.15	0.20	0.29	0.12	0.03	0.87**
As	−0.01	−0.06	0.08	0.14	0.23	0.04	−0.03	
Leaf (n = 20)								
Pb	−0.13	0.25	−0.12	−0.19	−0.27	−0.20	−0.11	0.85**
As	0.05	0.36	−0.03	−0.40	−0.29	−0.20	0.07	
Trichome (n = 20)								
Pb	0.67**	0.69**	0.61**	0.67**	0.36	0.38	0.60**	0.90**
As	0.61**	0.59**	0.47*	0.51*	0.36	0.25	0.48*	

* $P < 0.05$.
** $P < 0.01$.

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 arsenite, 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.

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