Spatial Imaging, Speciation, and Quantification of Selenium in the Hyperaccumulator Plants *Astragalus bisulcatus* and *Stanleya pinnata*[1]

John L. Freeman, Li Hong Zhang, Matthew A. Marcus, Sirine Fakra, Steve P. McGrath, and Elizabeth A.H. Pilon-Smits*

Biology Department, Colorado State University, Fort Collins, Colorado 80523 (J.L.F., L.H.Z., E.A.H.P.-S.); Advanced Light Source, Lawrence Berkeley Laboratory, Berkeley, California 94720 (M.A.M., S.F.); and Cross Institute Programme for Sustainable Soil Function, Rothamsted Research, Harpenden, Herts AL5 2JQ, United Kingdom (S.P.M.)

Astragalus bisulcatus and *Stanleya pinnata* hyperaccumulate selenium (Se) up to 1% of plant dry weight. In the field, Se was mostly present in the young leaves and reproductive tissues of both hyperaccumulators. Microfocused scanning x-ray fluorescence mapping revealed that Se was hyperaccumulated in trichomes in young leaves of *A. bisulcatus*. None of 10 other elements tested were accumulated in trichomes. Micro x-ray absorption spectroscopy and liquid chromatography-mass spectrometry showed that Se in trichomes was present in the organic forms methylselenocysteine (MeSeCys; 53%) and γ-glutamyl-MeSeCys (47%). In the young leaf itself, there was 30% inorganic Se (selenate and selenite) in addition to 70% MeSeCys. In young *S. pinnata* leaves, Se was highly concentrated near the leaf edge and surface in globular structures that were shown by energy-dispersive x-ray microanalysis to be mainly in epidermal cells. Liquid chromatography-mass spectrometry revealed both MeSeCys (88%) and selenocystathionine (12%) inside leaf edges. In contrast, both the Se accumulator *Brassica juncea* and the nonaccumulator *Arabidopsis thaliana* accumulated Se in their leaf vascular tissues and mesophyll cells. Se in hyperaccumulators appears to be mobile in both the xylem and phloem because Se-treated *S. pinnata* was found to be highly toxic to phloem-feeding aphids, and MeSeCys was present in the vascular tissues of a *S. pinnata* young leaf petiole as well as in guttation fluid. The compartmentation of organic selenocompounds in specific storage areas in the plant periphery appears to be a unique property of Se hyperaccumulators. The high concentration of Se in the plant periphery may contribute to Se tolerance and may also serve as an elemental plant defense mechanism.

In the 1930s, several plant species growing on selenium (Se)-enriched soil in the western United States were found to accumulate unusually high concentrations of Se (Beath et al., 1934; Knight and Beath, 1937; Trelease and Trelease, 1938, 1939). Species within the genera Astragalus and Stanleya, among others, are able to accumulate Se at concentrations of up to 10,000 μg g^{-1} Se dry weight in shoots while growing on naturally occurring soils containing 2 to 10 μg g^{-1} Se dry weight (Virupaksha and Shrift, 1965; Davis, 1972, 1986). Most plant species cannot tolerate high concentrations of Se in their tissues and contain less than 25 μg g^{-1} Se dry weight, even on high-Se soils (White et al., 2004). Therefore, these Astragalus and Stanleya species can be considered Se hyperaccumulators in analogy to plants that accumulate other elements (e.g. cadmium [Cd], nickel [Ni], or zinc [Zn]) to concentrations that are 2 orders of magnitude higher than other plants on the same site (Baker and Brooks, 1989). The distribution of Se hyperaccumulators in the United States and their Se hyperaccumulation abilities have been well documented (Cannon, 1960; Davis, 1972; Kubota and Allaway, 1972; Feist and Parker, 2001). Se hyperaccumulators are indicators of seleniferous soil, many of which can be found at the location of the prehistoric western interior seaway. This former inland sea resulted in a Se-enriched Cretaceous shale deposit known as the Morrison formation (Emmons et al., 1896).

Se is toxic at high concentrations, but it is also an essential element for many organisms. Ingestion of Se hyperaccumulator plants by animals can cause chronic or acute Se poisoning, called alkali disease and blind staggers (Draize and Beath, 1935; Cosgrove, 2001). Irrigated agriculture on Se-rich soil leads to Se leaching and concentration in shallow groundwater regions or wetlands. Accumulation of Se from agricultural drainage water can result in the death and deformity

[1] This work was supported by the National Science Foundation (grant no. IOB–0444471 to E.A.H.P.-S.). The operations of the Advanced Light Source at Lawrence Berkeley National Laboratory are supported by the Director, Office of Science, Office of Basic Energy Sciences, Materials Sciences Division of the U.S. Department of Energy (contract no. DE–AC02–05CH11231). Rothamsted Research receives grant-aided support from the U.K. Biotechnology and Biological Sciences Research Council.

* Corresponding author; e-mail epsmits@lamar.colostate.edu; fax 970–491–0649.

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www.plantphysiol.org/cgi/doi/10.1104/pp.106.081158

of birds and fish, as was found in the Kesterson reservoir in California (Ohlendorf et al., 1986). Se toxicity in the western United States is in stark contrast to some parts of the eastern United States, where Se deficiency causes white muscle disease in livestock. Se is an essential element for animals because selenocysteine (SeCys) is present in the active site of certain selenoproteins. Some selenoproteins play a role in free-radical scavenging, and, consequently, Se has been shown to have anticarcinogenic activity, especially when consumed as methylselenocysteine (MeSeCys; Ellis and Salt, 2003).

Se was originally thought to be essential for Se hyperaccumulator plants because they show decreased growth rates in its absence (Trelease and Trelease, 1938; Trelease and Beath, 1949). However, to date, there is no conclusive evidence that Se is essential for higher plants (Birringer et al., 2002; Ellis and Salt, 2003; Sors et al., 2005b). Certain green algae, however, do require Se (Fu et al., 2002). Hyperaccumulator plants seek out and rapidly assimilate selenate (SeO_4^{2-}) from the soil, preferentially over sulfate (SO_4^{2-}; Bell et al., 1992). The roots of Se hyperaccumulators were found to grow preferentially toward SeO_4^{2-}-enriched soils, suggesting that elemental chemotaxis may exist in roots (Goodson et al., 2003). The mechanisms and reasons for the increased preferential uptake and sequestration of Se by these specialized plant species remain largely unknown (Sors et al., 2005b).

In plants, Se and sulfur (S) follow the same pathways in their uptake and metabolism. SeO_4^{2-} is taken up through a SO_4^{2-} transporter such as Sultr1;2 in Arabidopsis (*Arabidopsis thaliana*), which has localized expression in the root tip, root cortex, and lateral roots (Shibagaki et al., 2002). After uptake into the roots, SeO_4^{2-}, like SO_4^{2-}, is thought to be transported to the shoot chloroplasts (Leustek, 2002; Ellis and Salt, 2003). Indistinguishable to most enzymes, Se analogs of S are assimilated and metabolized by S pathways, which results in Se being found in most S-containing biomolecules (Leustek, 2002). Whereas the biochemical forms of Se in plants support this, Se and S have atomic differences that cause a few biochemical processes involving Se to be excluded from those associated with S (Sors et al., 2005b). For instance, selenoglutathione has not been found in hyperaccumulator plants that synthesize glutathione (Shrift and Virupaksha, 1965). The Se atom is bigger than S with an ionic radius of 0.69 Å, compared to S at 0.37 Å. As a result, the bond between two Se atoms is weaker than the disulfide bond (Sors et al., 2005b). If Se is integrated into SeCys and selenomethionine (SeMet), these amino acids nonspecifically replace Cys and Met in proteins and result in Se toxicity (Brown and Shrift, 1981, 1982).

Diversion of Se away from proteins can provide Se tolerance and hyperaccumulation (Brown and Shrift, 1981). In hyperaccumulators, Se misincorporation is circumvented by the methylation of SeCys into Se- MeSeCys, which may be further converted to γ-glutamyl-Se-MeSeCys (γGMeSeCys; Dunnill and Fowden, 1967; Brown and Shrift, 1981; Burnell, 1981). The methylation of SeCys is mediated by a specialized enzyme SeCys methyltransferase (SMT; Neuhierl and Böck, 1996). The level of expression of this enzyme correlates with Se hyperaccumulation in Astragalus species (Sors et al., 2005a). Additionally, selenocystathionine (SeCysth) accumulates to high concentrations in some Se hyperaccumulator plants and this may also provide tolerance (Shrift and Virupaksha, 1965; Kotrebai et al., 2000).

Astragalus bisulcatus, the two-grooved milkvetch, a Fabaceae family member also nicknamed locoweed, accumulates high concentrations of Se up to 1% dry weight (Shrift and Virupaksha, 1965). *A. bisulcatus* accumulates a large amount of MeSeCys, mainly in young leaves, whereas the mature leaves have predominantly SeO_4^{2-} and 40- to 60-fold less MeSeCys (Pickering et al., 2000). The total Se amounts in young leaves were at least 10-fold higher than in old leaves (Pickering et al., 2000). The *SMT* gene is expressed equally in leaves of all ages, so changes in the expression of this enzyme did not account for the decrease in MeSeCys observed in older leaves (Pickering et al., 2003). This may suggest that the synthesis of MeSeCys in older leaves is blocked at an earlier metabolic step and that mature leaves cannot reduce SeO_4^{2-} to selenite (SeO_3^{2-}; Pickering et al., 2003). An alternative hypothesis is that the mature leaves of *A. bisulcatus* export MeSeCys to younger tissues (Pickering et al., 2003). MeSeCys can be converted to the conjugated form γGMeSeCys, a dipeptide that is a storage form of Se in *A. bisulcatus* seeds (Nigam and McConnell, 1969). MeSeCys is also an intermediate in the formation of dimethyl diselenide, the primary volatile form of Se from *A. bisulcatus*, which is responsible for its distinctive malodorous smell (Evens et al., 1968). If organic Se is indeed selectively transformed and translocated to different tissues in hyperaccumulator plants, such a cycle of Se localization and accumulation in specific tissues is likely to have an important function.

Another species of Se hyperaccumulators, the Brassicaceae family member *Stanleya pinnata* or Prince's plume, accumulates 0.1% Se of dry weight, which is severalfold less Se than found in *A. bisulcatus*. *S. pinnata* was also reported to accumulate Se in its leaves in the form MeSeCys (83% of total Se in leaves), with a minor fraction (up to 17%) of SeCysth (Shrift and Virupaksha, 1965).

Highly concentrated Se in hyperaccumulators may represent a toxic elemental defense. These plants hyperaccumulate Se from low external soil concentrations and are generally found only where Se is present in the soil, which suggests an environmental requirement for Se in the long-term survival of these plants. In 1957, Tadros and coworkers first noticed that some plant species were more sensitive to pathogen infection when grown in the absence of elements that they evolved to tolerate (Tadros, 1957). Metal hyperaccumulation in plants was then hypothesized to be a potent defense mechanism in plants because the excess metal was toxic to pathogens and herbivores

(Boyd and Martens, 1992). This intriguing idea has been termed the elemental defense hypothesis of metal hyperaccumulation (Martens and Boyd, 1994). Metal hyperaccumulation was found to be a potent form of defense against both herbivores and pathogens (Boyd and Martens, 1992; Pollard and Baker, 1997; Davis and Boyd, 2000; Ghaderian et al., 2000). Similarly, Se has been shown to protect plants from both herbivory and pathogen infection (Hurd-Karrer and Poos, 1936; Vickerman and Trumble, 1999; Vickerman et al., 2002; Hanson et al., 2003, 2004). So far, these Se studies were done using nonhyperaccumulator plant species. It is likely that the Se in hyperaccumulators protects them from herbivory as well, both due to Se toxicity and to the repellent odor of their volatile selenocompounds (Birringer et al., 2002).

Little is known about the sequestration processes in Se hyperaccumulators in terms of the cellular storage sites of Se. We hypothesize that Se would be concentrated in areas that would serve a specific function. Storage of Se in strategic areas could improve Se tolerance in planta and also result in a toxic Se-based plant defense mechanism. The distribution of Se in tissues may give a clue to the function of Se in the ecology of the plants and in the mechanisms underlying metal hyperaccumulation. In this study, we compare the forms and distribution of Se in two hyperaccumulator species, *S. pinnata* and *A. bisulcatus*, to the nonhyperaccumulator *Brassica juncea* and the nonaccumulator Arabidopsis. If Se indeed has a protective function in hyperaccumulator plant species, it would be expected to accumulate in structures and areas key to their survival and reproduction. To investigate this, we used micro x-ray absorption spectroscopy (μ-XAS), energy-dispersive x-ray microanalysis (EDXMA), and liquid chromatography (LC)-mass spectroscopy (MS) to examine the spatial distribution and speciation of Se in young, actively growing leaves. μ-XAS and EDXMA are extremely useful because Se can be imaged and speciated at a high resolution in intact frozen tissues. Inductively coupled plasma (ICP)-atomic emission spectrometry (AES) was also used to measure the Se content in different tissue types of these two hyperaccumulators, collected from their natural environment, to analyze Se distribution at the whole-plant level.

RESULTS

Imaging Se Concentration and Speciation in Se Hyperaccumulator Leaves

To investigate the spatial Se distribution in hyperaccumulator species, micro scanning x-ray fluorescence mapping (μ-SXRF) was performed on young leaves from *A. bisulcatus* and *S. pinnata*. μ-SXRF imaging in *A. bisulcatus* revealed an almost exclusive localization and accumulation of Se in the trichomes (leaf hairs; Fig. 1A). Judging from the intensity of the Se signal, the concentration of Se in the trichomes was around 10-fold higher than in the remainder of the leaf.

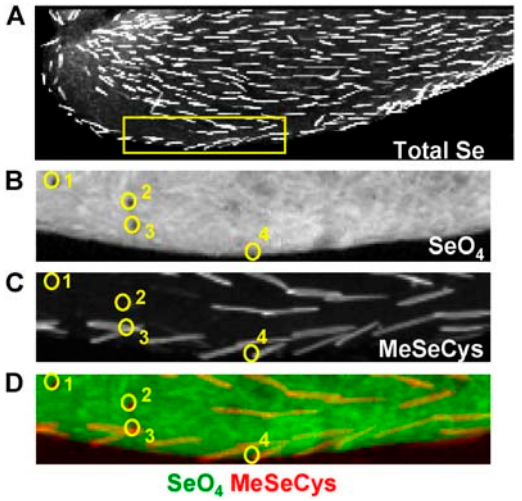

Figure 1. Localization and speciation of Se in *A. bisulcatus* using μ-XAS. Young leaf from a plant supplied with 40 μM Na_2SeO_4 is shown. A, Map showing spatial distribution of total Se (in white at normal gain). The yellow box indicates the area used for high-resolution chemical mapping of Se (magnified in B–D) and XANES (Fig. 4A). B, Chemical map showing spatial distribution of SeO_4 in the boxed area shown in A (in white at high gain). C, Chemical map showing spatial distribution of MeSeCys in the boxed area shown in A (in white at normal gain). D, Overlay of the chemical maps for SeO_4 (in green) and MeSeCys (in red). Yellow circles in B to D show locations of XANES speciation scans 1 and 2 off-trichome and 3 and 4 on-trichome (shown in Fig. 4A).

In *S. pinnata*, Se accumulation was observed mainly at the leaf margins and near the tips (Fig. 2, A and B). Multiple leaves from two *S. pinnata* ecotypes with and without trichomes were tested. Without exception, Se was most concentrated in globular structures situated within the leaf edges and tips, as shown in Figure 2, A and B. No Se accumulated in the trichomes of the *S. pinnata* variety that had trichomes (data not shown). Se was also observed in the vascular tissues of its petiole (Fig. 2C). A leaf cross-section image (Fig. 2D) reveals that the highly concentrated Se regions were located mainly in the leaf periphery and appeared concentrated in the epidermal region. Much lower concentrations of Se were found in the middle of this cross-section compared with the leaf edges. The Se concentration in the Se-enriched globular structures was 15 times greater than that of the leaf itself, as quantified from signal intensities. To investigate in more detail in which cell layers Se accumulates in *S. pinnata*, we performed EDXMA on intact freeze-fractured leaves. This higher-resolution map of the Se in a *S. pinnata* young leaf cross-section showed that the epidermal cell layer, particularly toward the leaf margins, had the highest concentration of Se inside its cells and represents the principal storage site of Se in the leaf (Fig. 2E).

Thus, both Se hyperaccumulator species appear to concentrate Se in the periphery of their leaves, either in the trichomes of *A. bisulcatus* or around the leaf edges and tips in the epidermis of *S. pinnata*. We next wanted

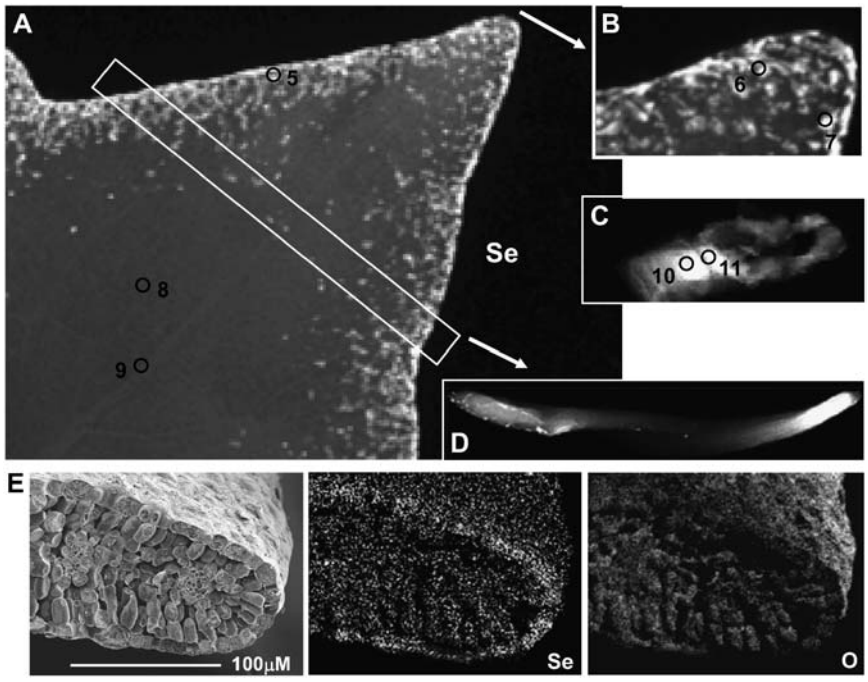

Figure 2. Localization and speciation of Se in *S. pinnata* using μ-XAS. Young leaf from a plant supplied with 40 μM Na_2SeO_4 is shown. A, Map showing spatial distribution of total Se (in white at normal gain). Black circles show locations of XANES speciation scans 5 (highest Se areas), 8 (leaf center), and 9 (midrib vascular tissues). The white box indicates the area used for cross-section imaging of total Se (enlarged in D). B, Magnification of leaf tip used for XANES speciation scans 6 and 7 of highest Se areas. C, Map showing spatial distribution of total Se (in white at normal gain) in petiole cross-section. Black circles show locations of XANES speciation scans 10 and 11 on Se in leaf petiole vascular tissues. D, Map showing spatial distribution of total Se (in white at normal gain) in leaf cross-section. E, Distribution of Se (central image, dot map) across different tissues of a frozen, hydrated *S. pinnata* leaf, as revealed by EDXMA. The dot map for oxygen is shown in the right image, as a reference, demonstrating only small areas of dropout of the signal for elements due to shading effects. The left image gives an overview of the leaf architecture.

to examine how this spatial distribution of Se in hyperaccumulators compares to nonhyperaccumulator species. To this end, we performed μ-SXRF mapping on *B. juncea*, which is related to *S. pinnata* but does not hyperaccumulate Se. In *B. juncea*, Se was found to be concentrated in the leaf vascular tissues (Fig. 3, A and B). Whereas this species has trichomes, it is clearly shown that these trichomes did not accumulate Se (Fig. 3, A and B, arrows). Interestingly, these trichomes did accumulate calcium (Ca) and manganese (Mn); the latter accumulated mainly at the base (Fig. 3B, inset).

The chemical forms of Se in the young *A. bisulcatus* and *S. pinnata* leaves were further investigated using microfocused x-ray absorption near-edge structure (XANES) at the Se-K absorption edge. Se-K XANES provides information about the oxidation state and, when compared to known Se standard compounds, information about its chemical form. XANES was performed at four different locations on the *A. bisulcatus* leaf, as indicated by circles in Figure 1, B to D. These XANES spectra are shown in Figure 4A. The leaf Se XANES spectra were then least-squares fitted to spectra obtained from a series of Se standards (Fig. 4, C and D), which led to an estimation of the Se composition in each spot, as listed in Table I. Different spectroscopic results were obtained in off-trichome regions (spots 1 and 2) compared to trichome regions (spots 3 and 4). Outside of the trichomes, 30% of the low-Se level found there was composed of the inorganic forms SeO_4^{2-} (20%) and SeO_3^{2-} (10%); the remaining 70% matched the organic form MeSeCys. In contrast, more than 98% of the highly concentrated Se inside the trichomes matched the organic form MeSeCys. To visualize the spatial distribution of the two Se species, SeO_4^{2-} and MeSeCys, microresolution chemical maps were obtained using relevant absorption energies specific for the two Se compounds. These maps confirm that SeO_4^{2-} was localized in the leaf itself and not in the trichomes (Fig. 1B), whereas MeSeCys was predominantly localized in trichomes (Fig. 1C). An overlay of these maps is shown in Figure 1D, where SeO_4^{2-} is shown in green and MeSeCys in red. The unmethylated organic forms SeCys and SeCystine were not detected in the *A. bisulcatus* leaf.

In *A. bisulcatus*, the leaf trichomes are mainly present on the abaxial (bottom) side of the leaves and, on this young leaf, numbered around 350. As mentioned above, the concentration of Se in the trichomes was 7 to 11 times greater than in the leaf itself, as quantified by XANES signal intensities using the postedge, main resonance peak. Quantification of total Se using the

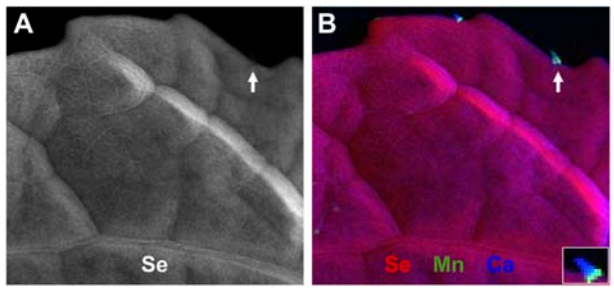

Figure 3. Localization and speciation of Se in *B. juncea* using μ-XAS. Young leaf from a plant supplied with 20 μM Na_2SeO_4 is shown. A, Map showing spatial distribution of total Se (in white at normal gain). The arrow points to the location of a trichome (leaf hair). Note that it is devoid of Se. B, Map showing overlay of spatial distribution of total Se (in red), Mn (in green), and Ca (in blue). Note that Ca is accumulated in the entire trichome, whereas Mn accumulates at the trichome base.

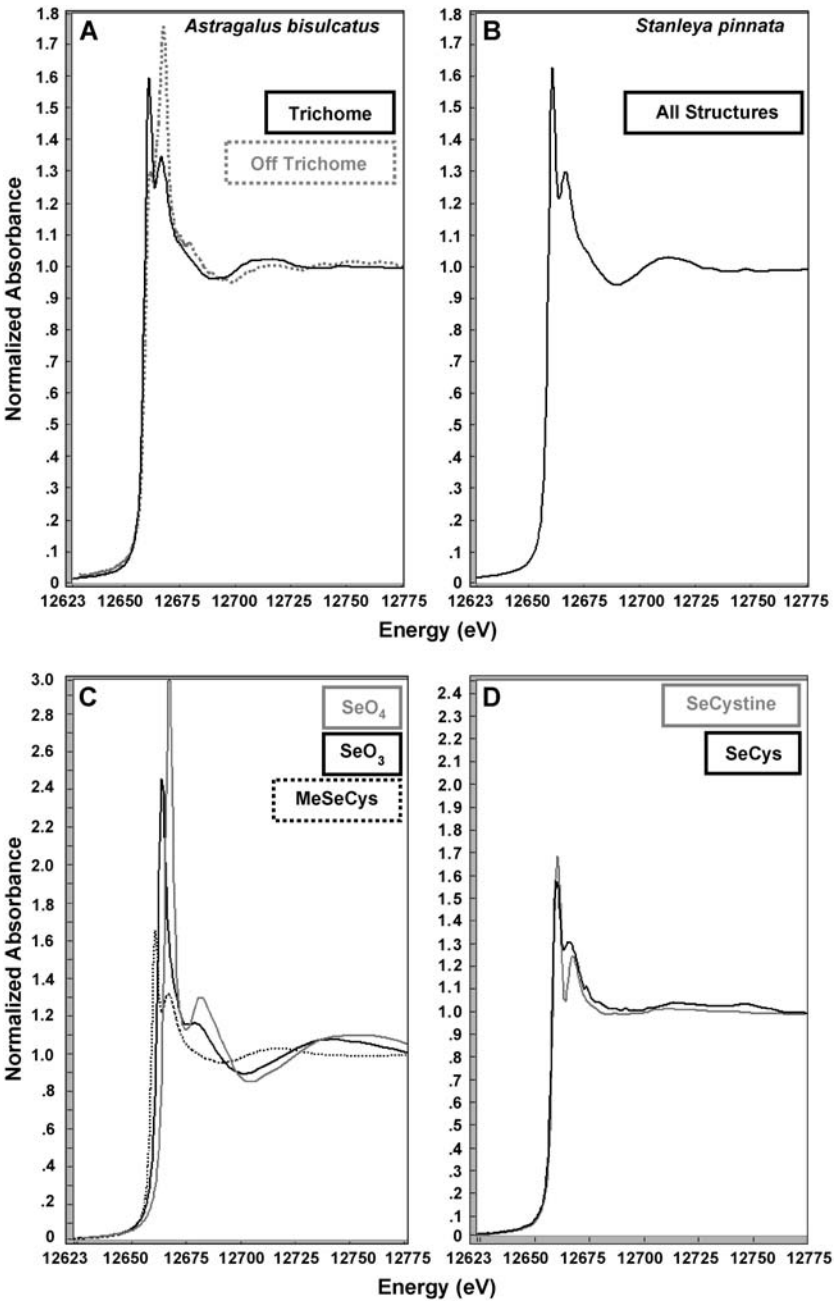

Figure 4. Se-K XANES in two different hyperaccumulator species and aqueous Se standards. The spectra were collected at the locations shown by circles in Figures 1 and 2. A, Young *A. bisulcatus* leaf. Dashed gray line represents the average (n = 3) of Se spectra at locations 1 and 2 off-trichome. Solid black line represents the average (n = 6) of Se spectra in leaf trichomes at locations 3 and 4. B, Young *S. pinnata* leaf. Solid black line represents the average (n = 20) of Se spectra collected at locations 5 to 11. Little or no difference in Se speciation was observed between any of these locations. C, Solid gray line, SeO_4; solid black line, SeO_3; dashed black line, MeSeCys. D, Solid gray line, SeCystine; solid black line, SeCys. All spectra have been normalized.

scan intensities from XANES and the binary areas acquired from the software program Image J revealed that 42% of the Se was localized specifically to the trichomes, which represent an area only 6% that of the total leaf. The remaining 58% of total Se was present at much lower concentrations, distributed homogeneously throughout the remaining 94% of the leaf.

XANES was also used to investigate the chemical forms of Se in young *S. pinnata* leaves. Because very little is known about the transport of Se into and out of the young leaves of *S. pinnata*, a variety of leaf structures were imaged and chemically speciated. Multiple XANES scans were collected at different locations both on the globular surface structures (Fig. 2A, spot 5; Fig. 2B, spots 6 and 7) and off these structures (Fig. 2A, spot 8), in addition to the leaf vascular tissues on the midrib near the tip (Fig. 2A, spot 9) and in the petiole cross-section (Fig. 2C, spots 10 and 11). All Se XANES spectra on this young *S. pinnata* leaf revealed that the major form of Se (86%–98%) matched MeSeCys (Fig. 4B; Table I). In the leaf-edge globular structures, the central leaf, and the leaf midrib, the remaining Se was composed of organic SeCys (3%–9%) and SeCystine (1%–7%). Only the petiole vascular tissues differed somewhat in Se composition. In addition to

Table I. *Se composition at leaf structures shown in Figures 1 and 2*

Speciation of Se was calculated from XANES (Fig. 4). Data represent the average percent of total Se ± SD. *n*, Total number of spectra; nd, not determined.

	SeO_4	SeO_3	SeCys	SeCystine	MeSeCys
	%	%	%	%	%
A. bisulcatus					
Leaf, off-trichome (spots 1 and 2[a]), *n* = 6	21.8 ± 2	8.4 ± 6.6	nd	nd	70.6 ± 6.7
Leaf trichome (spots 3 and 4), *n* = 3	0.5 ± 1	1.4 ± 2.4	nd	nd	98.2 ± 2.2
S. pinnata					
Leaf edge (spots 5–7), *n* = 8	nd	nd	8.9 ± 13.2	1.3 ± 3.6	87.7 ± 12.2
Leaf center (spot 8), *n* = 6	nd	nd	6.5 ± 10.2	6.9 ± 10.8	85.9 ± 10.5
Leaf midrib (spot 9), *n* = 3	nd	nd	3.6 ± 6.2	4.8 ± 8.4	91.9 ± 7.4
Petiole vein (spots 10 and 11), *n* = 3	0.4 ± 0.7	1.5 ± 2.7	nd	nd	98.5 ± 2.6

[a]Spot location as shown in Figure 1 or 2.

98% MeSeCys, it contained small portions of inorganic SeO_3^{2-} (2%) and SeO_4^{2-} (<1%), but no SeCys or SeCystine (Table I).

As mentioned above, Se concentrations in the *S. pinnata* globular structures were 10 to 15 times greater than in the remainder of the leaf, as quantified by XANES signal intensities. Quantification of total Se using the signal intensities from XANES and the areas acquired using Image J revealed that 60% of the Se was localized specifically to the globular structures, which represent only 9% of the total leaf surface area. The remaining 40% of total Se is present at a much lower concentration, distributed homogeneously throughout the remaining 91% of the leaf surface area.

None of the elements examined (Ca, cobalt [Co], copper [Cu], chromium [Cr], iron [Fe], potassium, Mn, Ni, and Zn) were accumulated in the *A. bisulcatus* trichomes (data not shown), although most of these elements were detected in the leaf itself. The spatial imaging of Se and S accumulation in *A. bisulcatus* also showed no colocalization of Se with S in trichomes (Fig. 5A). The elements tested were also not accumulated in the *S. pinnata* globular structures (data not shown), although most of the elements were detected in the leaf. In *S. pinnata*, a slight accumulation of S did appear in some of the globular structures, especially at the very edges of the leaf (Fig. 5B). However, the signal intensity for Se in the globular structures on the leaf edges was 110 times that of S. In the nonaccumulator *B. juncea*, Se and S are clearly colocalized throughout the leaf (Fig. 5C). The other elements tested were also detected in the *B. juncea* leaf itself.

Further Identification of Free Organic Selenocompounds in Se Hyperaccumulation Structures in *A. bisulcatus* and *S. pinnata* Using LCMS

The organic selenocompounds SeMet, MeSeCys, γ-GMeSeCys, and SeCysth have very similar Se-K XANES spectra, making it difficult to distinguish them using XAS. To further investigate the forms of Se accumulated in the apparently specialized Se hyperaccumulation structures in *A. bisulcatus* and *S. pinnata*, we performed LC-MS. In trichome extracts from young *A. bisulcatus* leaves, Se was found to be present in the two organic forms MeSeCys and γ-GMeSeCys at a ratio of 53% to 47%, respectively. LC-MS of extracts of young *S. pinnata* leaf edges and tips, where the globular structures are present, demonstrated that Se was present in the two organic forms MeSeCys and SeCysth at a ratio of 88% to 12%, respectively. To better understand how Se may be transported to these globular structures of *S. pinnata*, we induced guttation under high humidity conditions and collected guttation

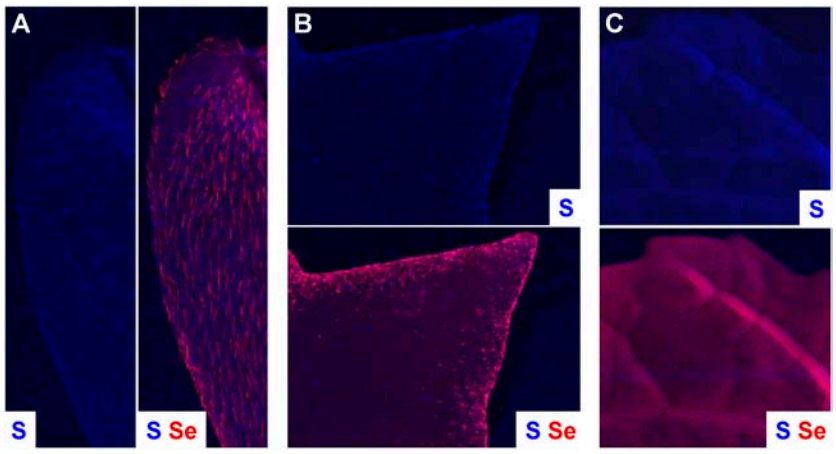

Figure 5. Localization of Se and S in young leaves of the hyperaccumulators *A. bisulcatus* and *S. pinnata* and the nonhyperaccumulator *B. juncea* using μ-XAS. The hyperaccumulator plants were supplied with 40 μM Na_2SeO_4 and the nonhyperaccumulator with 20 μM Na_2SeO_4. For each plant species, the spatial distribution of total S is shown in blue on the left, whereas the spatial distribution of total S imaged in blue is overlaid with total Se imaged in red on the right. A, *A. bisulcatus*. B, *S. pinnata*. C, *B. juncea*.

fluid from the young pinnate leaf tips. LC-MS of these droplets revealed that MeSeCys was present in *S. pinnata* guttation fluid at a substantial concentration (35 μM), indicating that Se is mobile in the xylem and may be transported in this organic form. In earlier studies, we observed that feeding SeO_4^{2-} to *S. pinnata* results in toxicity to aphids (Hanson et al., 2004), indicating that Se is also mobile in the phloem and can have a protective function against herbivory in hyperaccumulators.

Analysis of Whole-Plant Level Distribution of Se in Field-Grown *A. bisulcatus* and *S. pinnata* Using ICP-AES

To obtain more insight into potential long-distance Se flow patterns in hyperaccumulator plants, the Se distribution in *A. bisulcatus* and *S. pinnata* was analyzed at the whole-plant level in their natural habitat (Fort Collins, CO). *A. bisulcatus* showed approximately 3-fold higher total Se concentrations than *S. pinnata*, but both hyperaccumulator species demonstrated very similar Se distribution profiles (Fig. 6, A and B). Young leaves of both species had much higher Se concentrations than old leaves, and the reproductive organs had the highest concentrations overall. Total Se concentrations in young leaves of *A. bisulcatus* were 10- to 14-fold higher compared with old leaves. The flowers, fruit, and seeds had 18- to 20-fold more Se than old leaves and 1.5- to 1.7-fold higher Se concentrations than young leaves (Fig. 6A). Similarly, total Se concentrations in young *S. pinnata* leaves were 6- to 7-fold higher compared with old leaves, whereas the fruit and seeds had 18- to 20-fold more Se than old leaves and 3.2- to 3.5-fold more Se than young leaves (Fig. 6B). The flowers of *S. pinnata* had 12-fold more Se than old leaves and 1.7-fold more Se than young leaves. Thus, the highest Se concentrations in both plant species were found in newly developing leaves and reproductive tissues.

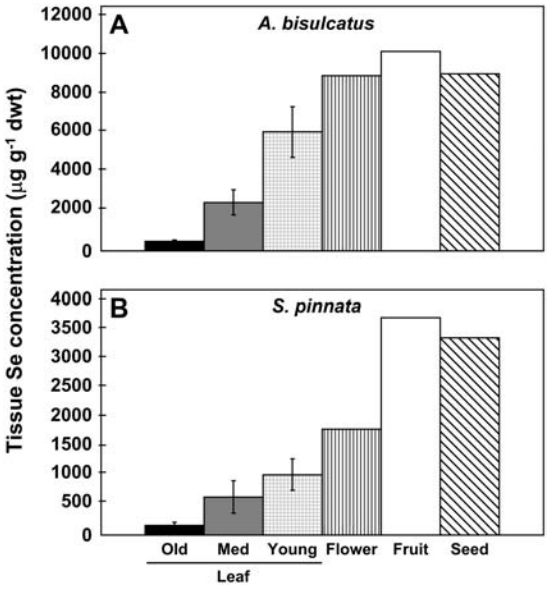

Figure 6. Total Se concentration (μg g^{-1} dry weight) in *A. bisulcatus* and *S. pinnata* tissues collected from their native habitat in Fort Collins, Colorado. A, *A. bisulcatus*. B, *S. pinnata*. For each species, we collected leaves at different developmental stages in addition to flowers, fruit, and seeds. Values shown represent the mean ± SE of 10 plants. Values shown for flowers, fruit, and seeds represent pooled samples from 10 plants each.

DISCUSSION

Both Se hyperaccumulator species accumulated Se near their leaf periphery. Young *A. bisulcatus* leaves hyperaccumulated Se predominantly in their trichomes. Young *S. pinnata* leaves hyperaccumulated Se in their epidermal cells, especially around the leaf edges and tips; no Se accumulation was observed in trichomes. This localization of Se in the leaf periphery of both plant species appears to be specific for Se because other elements, including S, did not show similar distribution patterns. Moreover, this Se sequestration pattern appears to be typical for hyperaccumulators because in the nonhyperaccumulators *B. juncea* and Arabidopsis Se appeared throughout the vascular tissues and mesophyll cells (Fig. 3; Van Hoewyk et al., 2005) even though they had trichomes. Therefore, specifically directed Se sequestration appears to be one of the major differences between Se hyperaccumulator and nonhyperaccumulator species.

In earlier studies, plants that hyperaccumulate metals showed similar metal localization patterns to the ones observed here for Se. In the Ni hyperaccumulators *Alyssum lesbiacum* and *Alyssum bertolonii*, the highest Ni concentrations were found inside the epidermal layers of leaf margins, inside epidermal cells, and also in leaf trichomes (Krämer et al., 1997; Küpper et al., 2001). Moreover, in the Zn and Cd hyperaccumulator *Arabidopsis halleri*, leaf trichomes had high concentrations of Zn or Cd (Küpper et al., 2000). These findings suggest that actively directed transport mechanisms for the hyperaccumulated elements are a unifying theme in hyperaccumulator species.

μ-XAS showed that the Se in trichomes of *A. bisulcatus* was in the organic form, with a XANES spectrum most similar to MeSeCys. LC-MS confirmed that Se concentrations were high in trichomes and revealed that the Se was in the organic forms MeSeCys and γ-GMeSeCys in equal concentration. These results are in agreement with the μ-XAS data because both forms of Se have identical XANES spectra (data not shown). Thus, both experimental approaches point to trichomes as the main site of Se storage in *A. bisulcatus* leaves. It is interesting to find so much γ-GMeSeCys in these trichomes because this compound has so far only been reported as a storage form of Se in *A. bisulcatus* seeds (Nigam and McConnell, 1969). The leaf area outside the trichomes exhibits 30% inorganic Se

(SeO_4^{2-}, 20%; SeO_3^{2-}, 10%) in addition to 70% MeSeCys. These ratios of inorganic Se are surprisingly high because, in earlier reports, young leaves were found to contain only MeSeCys (Pickering et al., 2003). The earlier studies were done by bulk XAS, however, which does not have the high spatial resolution of μ-XAS. Only now are we able to visualize these Se forms in the leaf without being obscured by the much higher trichome organic Se concentrations.

The presence of SeO_4^{2-}, SeO_3^{2-}, MeSeCys, and γ-GMeSeCys indicates that the young leaves are a site for SeO_4^{2-} reduction and for incorporation of Se into these organic forms. The accumulation of MeSeCys and γ-GMeSeCys in the trichomes may either reflect preferential sequestration of organic Se in trichomes or enhanced biosynthesis of organic selenocompounds in the trichomes themselves. In this context, it is interesting to note that trichomes in Arabidopsis have a 300-fold higher glutathione content than basement and epidermal cells (Gutierrez-Alcala et al., 2000) due to higher expression of S assimilation pathway enzymes and glutathione biosynthetic enzymes. Perhaps *A. bisulcatus* trichomes show similarly active S metabolism. Because Se and S are chemical analogs, this would likely lead to enhanced Se assimilation in these structures.

The much higher Se concentrations observed in *A. bisulcatus* young leaves and reproductive tissues suggest that Se is exported via the phloem to these sink tissues during leaf maturation. Because young leaves contain mainly organic Se and old leaves mainly SeO_4^{2-} (Pickering et al., 2003), it appears to be the organic Se fraction that is transported in the phloem. The transported form may be MeSeCys and the stored form may be both MeSeCys and γ-GMeSeCys. Other Fabaceae are known to produce and transport S analogs of these Se compounds, methylcysteine (MCys) and γ-glutamyl-MCys (GMCys; Baldi and Salamini, 1973; De Lourdes et al., 1998). It has been suggested that these genera use MCys as a transportable form of reduced S and GMCys as a storage form in their seeds.

In young *S. pinnata* leaves, Se was highly concentrated at the leaf edge and surface as globular structures. LC-MS revealed both MeSeCys (88%) and SeCysth (12%) in these leaf edges. Intriguingly, *S. pinnata* leaves have been reported to have a seleniferous wax, which is unique to this species (McColloch et al., 1963). However, judging from our EDXMA studies, the major storage site for Se was in the epidermal cells rather than the cuticle, and it is possible that the globular structures shown by μ-XAS are large epidermal cells (Fig. 2E). A similar finding was observed in the localization of Zn inside the hyperaccumulator *T. caerulescens*, where a linear relationship was observed between epidermal cell size and concentration of Zn (Küpper et al., 1999). Therefore, specialized cells may exist in some hyperaccumulator species for storing metals. It is currently unknown how metals or metalloids may be preferentially transported to specialized cells in a specific area.

In *S. pinnata*, MeSeCys was also present in the vascular tissues of a young leaf petiole, as well as concentrated in guttation fluid. Therefore, this organic form of Se is also mobile in the xylem. SeO_4^{2-}-treated *S. pinnata* plants were toxic to phloem-feeding aphids, which suggests that Se is also mobile in the phloem. The finding that total Se concentrations in *S. pinnata* plants growing in their natural habitat were highly elevated in young leaves and reproductive tissues also suggests that Se is actively exported via the phloem to these sink tissues during leaf maturation, likely in the organic form.

Although the predominant form of Se was MeSeCys in young leaves of both hyperaccumulator species, the composition of the remaining Se fraction was somewhat different. In addition to MeSeCys, *S. pinnata* also contained SeCysth, SeCys, and SeCystine, whereas, in addition to MeSeCys, *A. bisulcatus* also contained high concentrations of γ-GMeSeCys, as well as some SeO_4^{2-} and SeO_3^{2-}. Accumulation of the unmethylated metabolite SeCysth in some hyperaccumulator species could represent an ability to pool this form selectively, rather than convert it to SeMet. This could represent an uncharacterized Se tolerance mechanism in some species of Se-hyperaccumulating plants. Accumulation of some SeCysth, SeCys, and SeCystine in *S. pinnata* could also represent a decrease in the specificity or activity of the putative SMT enzyme for SeCys and may explain the somewhat lower level of both Se accumulation and tolerance when compared to *A. bisulcatus*, which had no detectable SeCysth, SeCys, or SeCystine.

Speciation of Se in leaves of SeO_4^{2-}-treated nonhyperaccumulator species like *B. juncea* and Arabidopsis have been published previously (de Souza et al., 1998; Van Hoewyk et al., 2005). In these species, the majority of total leaf Se is in the unreduced form of SeO_4^{2-}, as confirmed for *B. juncea* in this study (data not shown). The reduction of SeO_4^{2-} to SeO_3^{2-} therefore appears to be a limiting factor of Se assimilation in nonhyperaccumulator plants (de Souza et al., 1998). In addition, nonhyperaccumulators appear to have much lower rates of SeCys methylation mediated by the Se hyperaccumulator enzyme SMT (Neuhierl and Böck, 1996; Sors et al., 2005a).

Both hyperaccumulator species accumulated Se mostly in young leaves and reproductive tissues, with Se sequestered mostly in the leaf periphery. The question arises as to whether this distribution may have functional significance. One possible function of stored Se may be as a defense mechanism. The tissues with the highest Se concentrations, young leaves and reproductive tissues, are likely important for the plant to defend and, due to their nutritional value and tenderness, may also be the most prone to herbivore attack (McKey, 1979). The high concentrations of Se in seeds could function in the defense of both the developing embryos in their pods, the dormant seeds, and the newly germinated seedlings as they are becoming established. Similarly, allocation of large amounts of

organic Se to the developing young leaves may protect them from herbivory due to toxicity and deterrence. Within leaves, Se was sequestered along the edges and in the periphery, which may be most effective against herbivory or pathogen attack. The trichomes on *A. bisulcatus* leaves and the epidermal cells on the leaf edges and tip of *S. pinnata* likely are the first areas to come into contact with such attackers (McKey, 1979). Trichomes play a major role in plant defense mechanisms and are known to be important structural barriers protecting plants from insect herbivory (Fernandes, 1994; Traw and Dawson, 2002). Indeed, trichomes have been found to both accumulate and excrete many toxic chemicals known to inhibit herbivory, such as various alkaloids and other plant secondary compounds (Rodriguez et al., 1984; Cutler et al., 1986; Zador and Jones, 1986). Many plant species respond to insect damage by increasing the density and/or number of trichomes on new leaves (Myers and Bazely, 1991; Traw, 2002). Therefore, the finding that Se accumulates in trichomes and other epidermal cells supports the hypothesis that Se hyperaccumulation functions as an elemental plant defense mechanism.

An alternative hypothesis is that the observed Se distribution in these hyperaccumulators increases Se tolerance and hyperaccumulation ability because sequestration of Se in peripheral cells excludes toxic Se from other, more sensitive tissues like the parenchyma and allows for long-term storage of high concentrations of Se inside specialized cells. A similar hypothesis has been proposed for both Ni and Zn by Küpper et al. (2000, 2001). These hypotheses are not mutually exclusive because storage of Se in these cells could increase both Se tolerance and hyperaccumulation ability of these plants and may also serve as a toxic Se-based plant defense mechanism.

A better understanding of the molecular and biochemical basis of the Se hyperaccumulation processes in these two Se hyperaccumulators will offer insight into how this intriguing physiological phenomenon may have evolved. If the accumulation of Se in a certain compartment or area provides tolerance to Se and/or provides protection to herbivores or pathogens, this could provide a selective advantage, driving further evolution of this trait. A better understanding of Se hyperaccumulation in these two hyperaccumulator plant species may ultimately lead to the development of pathogen- and pest-resistant, anticarcinogenic, Se-fortified crops and to plants with superior properties for the phytoremediation of Se-polluted soil and water.

MATERIALS AND METHODS

Plant Growth

Astragalus bisulcatus and *Stanleya pinnata* seeds were obtained from the field near Fort Collins, Colorado. An additional *S. pinnata* ecotype, not depicted in the figures, was obtained from the field near Santa Fe, New Mexico. These plants were grown for 8 or 6 months, respectively, in Scotts Metro-Mix 350 and watered 3 times weekly with 40 μM SeO_4 for the entire time. *Brassica juncea* seeds (accession no. 173874), were obtained from the North Central Regional Plant Introduction Station, Ames, Iowa. They were grown for 1 month in Scotts Metro-Mix 350 and watered 3 times weekly with 20 μM SeO_4^{2-} for an additional month. All plants were cultivated in a growth room (24°C/20°C, 10 h/14 h light/dark, 120 μmol m^{-2} s^{-1} photosynthetic photon flux). Se concentrations used were all nontoxic to normal plant growth and reproduction.

Se Standards

Na_2SeO_4 (S8295), Na_2SeO_3 (S1382), SeCystine (S1650), and SeMet (S3132) were obtained from Sigma-Aldrich. MeSeCys, γGMeSeCys, SeCysth, and $SeGSH_2$ standards were obtained from PharmaSe. SeCys was obtained by reducing SeCystine at 25°C overnight in 100 mM sodium borohydride at a 1:1 molar ratio. Gray and red elemental Se were a gift from Amy Ryser and Dan Strawn.

μ-SXRF/μ-XAS

Young leaf samples from mature plants were washed to remove any external Se, frozen in liquid N_2, and shipped on dry ice to the Advanced Light Source at the Lawrence Berkeley Laboratory for microspectroscopic analysis on Beamline 10.3.2 (Marcus et al., 2004). The total distribution of Se was imaged by scanning the leaves in the focused beam at 13,085 eV. Intact young leaves were mounted, using a tiny amount of silicone grease, onto a Peltier stage and kept at −33°C to reduce radiation damage. μ-SXRF mapping of Se was performed first on one representative leaf for each plant type. A large *A. bisulcatus* map used a 16-μm (horizontal) × 6-μm (vertical) beam at 13,085 eV sampled in 5- × 5-μm pixels, followed by fine chemical mapping with a 5- × 5-μm beam at 12,660 eV for MeSeCys and 12,667 eV for SeO_4^{2-} in 3- × 3-μm pixels. This image was then decontaminated so that any interference was subtracted. This allowed us to differentially map the SeO_4 and MeSeCys signals. The K_α fluorescence line intensities of Se (and other elements of interest) were measured with a seven-element Ge solid-state detector and normalized to the incident monochromatic beam intensity. Both *S. pinnata* and *B. juncea* maps used a 7- × 7-μm beam at 13,000 eV sampled in 5- × 5-μm pixels. Se-K XANES was used to provide molecular speciation of the Se at specific points (Pickering et al., 2000). Aqueous solutions of the various selenocompounds were used as standard materials.

EDXMA

Young leaves from mature *S. pinnata* plants were washed to remove any external Se before their petioles were placed into water-filled test tubes and shipped overnight to Rothamsted Research for EDXMA. EDXMA was then performed on flash-frozen, freeze-fractured, gold-coated leaves as described by Küpper et al. (1999). We used a JEOL LV6360 scanning electron microscope (JEOL UK) with a GATAN Alto 2100 Cryo preparation system (Gatan UK) and an OXFORD Inca 2000 microanalysis system (Oxford Instruments) and the *L* line for Se (to avoid interference on the K_α line) and an acceleration voltage of 5,000 eV.

Extraction and Measurement of Nonprotein Organic Selenocompounds

Using a new clean scalpel, approximately 6,000 *A. bisulcatus* trichomes were carefully shaved off 20 young leaves and placed in 10 mL of absolute ethanol in a glass test tube. This solution was dried completely in a vacuum concentrator before the resulting crystals were resuspended in 100 mL of 50 mM HCl and stored at −80°C for 2 d. Using new clean scissors, the leaf edges and tips from 10 young *S. pinnata* leaves were excised and ground using a micropestle in liquid N_2. Two hundred milligrams of this finely ground tissue were then suspended in 200 μL of 50 mM HCl. This solution was then passed through a Sep Pak C18 syringe cartridge, which had been charged with 100% acetonitrile and washed with distilled, deionized water. The flow-through was then stored at −80°C for 2 d.

Using a high-humidity guttation box, three *S. pinnata* plants were exposed to a normal light period and intensity before being placed into the dark overnight. Approximately 1 mL of the resulting fluid droplets, which gathered mainly on the tips of young pinnate leaves, was collected and stored at −80°C for 2 d.

The nonprotein organic selenocompounds in the *A. bisulcatus* and *S. pinnata* extracts were analyzed by LC-MS using a Hewlett-Packard Agilent 1100 series HPLC and a Finnigan LcQDuo thermoquest MS system equipped with Xcalibur software. Through injecting 30 μL of these prepared solutions, the selenocompounds were separated at 15°C using a Phenomenex Hypersil 5-μm C18 (ODS) column (250 $\times$ 2 mm, 5 μm) at a flow rate of 0.36 mL min^{-1}, using two eluents: (A) water + 0.1% formic acid; and (B) acetonitrile + 0.1% formic acid. The following program was used: 0 to 2 min, 100% A; 2 to 10 min, gradient 0% to 40% B; 10 to 12 min, 40% to 0% B; 12 to 17 min, 100% A. The following pure aqueous selenocompounds were used for generating standard curves: SeCystine, SeCys, MeSeCys, γ-GMeSeCys, SeCysth, and SeMet. Through MS, the different nonprotein selenocompounds were measured at the appropriate masses observed for each of the standards, while confirming the exact characteristic Se isotopic signature and retention times. Peak values were integrated for both the complete Se isotopic mass range in addition to the major Se-containing isotope. The abundance of the major isotope was used to calculate the total abundance of the other less abundant Se isotopes. This calculation is based on the ratio of isotopic abundance for Se. Both of these methods resulted in the same values, indicating that other compounds were not present in the small mass range.

Se Concentrations in Plant Tissues

Plant tissue samples (leaves, flowers, fruits, and seeds) were collected from *A. bisulcatus* and *S. pinnata* plants (n = 10) in July 2005 at Pine Ridge Natural Area, Fort Collins, Colorado. The plant material was rinsed with distilled water and dried at 50°C for 48 h. One-hundred-milligram samples were acid digested and analyzed for Se by ICP-AES as described by Pilon-Smits et al. (1999).

Data Analyses

Statistical analyses were performed using the software package JMP-IN from the SAS Institute (Cary, NC). X-ray data analysis was performed using a suite of LabView programs (National Instruments) available at beamline 10.3.2 and freely available at http://xraysweb.lbl.gov/uxas/Beamline/Software/Software.htm in addition to the ImageJ program (National Institutes of Health, Bethesda, MD; http://rsb.info.nih.gov/ij).

ACKNOWLEDGMENTS

We thank Donald Dick for his help in developing the LC-MS protocols and data analysis, and Jean Devonshire at the Rothamsted Centre for Bioimaging for help with EDXMA. We are grateful to Colin Quinn for his help with plant growth and to Miriam Loeffler for collecting seeds from the field.

Received March 29, 2006; accepted July 20, 2006; published August 18, 2006.

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