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Article

Selenium-Tolerant Diamondback Moth Disarms Hyperaccumulator Plant Defense

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Summary

Background: Some plants hyperaccumulate the toxic element selenium (Se) to extreme levels, up to 1% of dry weight. The function of this intriguing phenomenon is obscure.
Results: Here, we show that the Se in the hyperaccumulator prince's plume (*Stanleya pinnata*) protects it from caterpillar herbivory because of deterrence and toxicity. In its natural habitat, however, a newly discovered variety of the invasive diamondback moth (*Plutella xylostella*) has disarmed this elemental defense. It thrives on plants containing highly toxic Se levels and shows no oviposition or feeding deterrence, in contrast to related varieties. Interestingly, a Se-tolerant wasp (*Diadegma insulare*) was found to parasitize the tolerant moth. The insect's Se tolerance mechanism was revealed by X-ray absorption spectroscopy and liquid chromatography—mass spectroscopy, which showed that the Se-tolerant moth and its parasite both accumulate methylselenocysteine, the same form found in the hyperaccumulator plant, whereas related sensitive moths accumulate selenocysteine. The latter is toxic because of its nonspecific incorporation into proteins. Indeed, the Se-tolerant diamondback moth incorporated less Se into protein. Additionally, the tolerant variety sequestered Se in distinct abdominal areas, potentially involved in detoxification and larval defense to predators.
Conclusions: Although Se hyperaccumulation protects plants from herbivory by some invertebrates, it can give rise to the evolution of unique Se-tolerant herbivores and thus provide a portal for Se into the local ecosystem. In a broader context, this study provides insight into the possible ecological implications of using Se-enriched crops as a source of anti-carcinogenic selenocompounds and for the remediation of Se-polluted environments.

Introduction

Hyperaccumulation is an intriguing phenomenon in which some plant species accumulate unusually high levels of an element, typically two orders of magnitude higher than other species growing on the same site [1]. Selenium (Se)-hyperaccumulating plants such as *Stanleya pinnata* (Brassicaceae) accumulate more than 1000 mg Se · kg^{-1} DW (dry weight) from soils that contain 10 mg Se · kg^{-1} DW or less [2]. Although Se is an essential trace element for many organisms [3], it is toxic at high levels. Selenium is similar to sulfur (S), and plants readily assimilate selenate into selenocysteine (SeCys) via the sulfate assimilation pathway. In proteins, SeCys can nonspecifically replace cysteine, which is toxic [3]. Loss of livestock due to ingestion of Se hyperaccumulator plants costs more than $330 million each year in the U.S. [4, 5]. The hyperaccumulator plants themselves are tolerant to Se because of SeCys methylation, preventing its nonspecific incorporation in proteins [6]. Lately, there has been increasing interest in the ability of dietary methylselenocysteine (MeSeCys) to prevent cancer in humans and animals [7–9]. Private and government U.S. agencies are currently seeking to increase the concentration of MeSeCys in many agricultural food crops either naturally or by genetic modification by using genes from Se hyperaccumulator plants [10–13]. Certain hyperaccumulator plants may also be a useful source of dietary Se because they mainly accumulate Se as MeSeCys [14].

Several hypotheses have been put forward to explain the functional significance of elemental hyperaccumulation in plants [15, 16]. These include allelopathy, drought resistance, and defense against herbivory or pathogens. Most evidence to date supports the third, elemental-defense hypothesis. Hyperaccumulated Zn was shown to protect plants from invertebrate herbivory [17, 18], and Ni hyperaccumulation can protect plants from caterpillar herbivory as well as fungal and bacterial infection [19–23]. Selenium also protects plants from herbivory, both by vertebrates [24] and invertebrates. In choice studies, larvae of the Lepidoptera beet armyworm (*Spodoptera exigua*), cabbage white butterfly (*Pieris rapae*), and cabbage looper (*Trichoplusia ni*) all preferred to feed on diets without Se [25–27]. Furthermore, Se was shown to protect plants from green peach aphids (*Myzus persicae*) and fungal infections [27, 28].

If elemental hyperaccumulation serves as a defense mechanism in plants, some herbivores may have disarmed this defense and evolved tolerance. One herbivore that is known to rapidly disarm plant defense compounds and other organic toxins is the diamondback moth (*Plutella xylostella*), an important agricultural pest that incurs an estimated $1 billion in management costs annually worldwide [29]. *Plutella xylostella* can disarm glucosinolate-myrosinase and even preferentially oviposits on Brassicaceae containing isothiocyanates [30, 31]. It was also one of the first insects documented to be resistant to dichloro-diphenyl-trichloroethane (DDT) and has been shown on numerous occasions to develop tolerance to *Bacillus thuringiensis* (Bt) toxin [32, 33]. Some explanations for this ability of *P. xylostella* to quickly evolve resistance to toxins are its short generation time, large number of offspring, and large genetic variation in any given population [29, 34].

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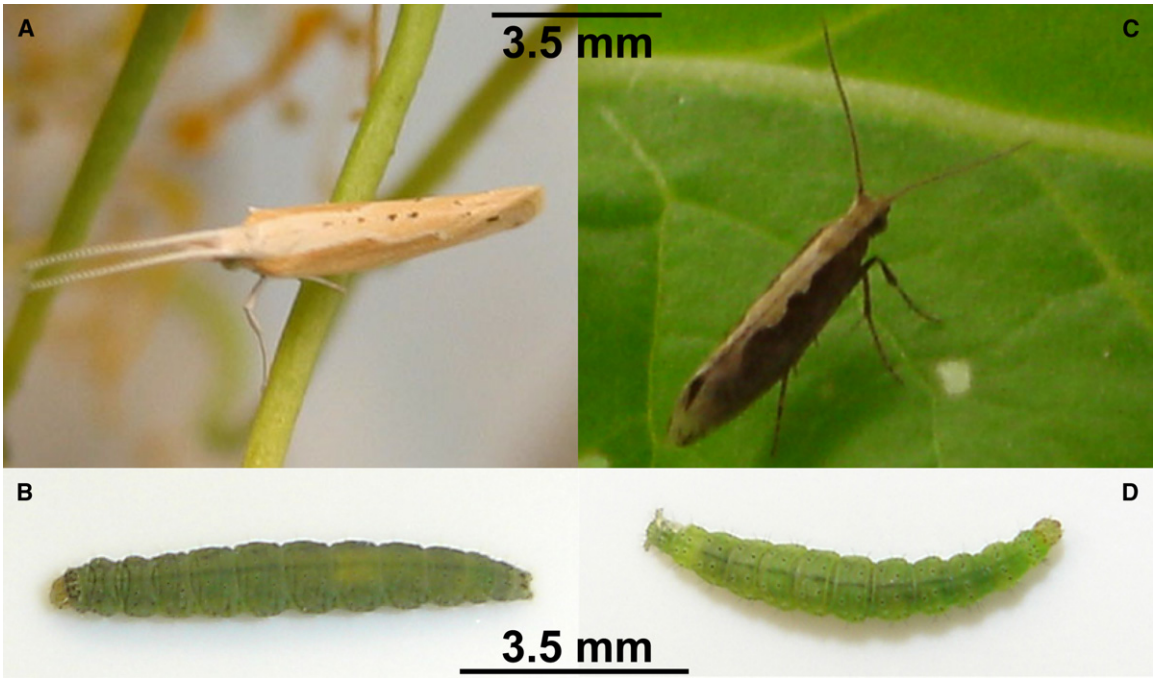

Figure 1. The Two Varieties of Diamondback Moth Used in This Study

(A and B) *Plutella xylostella* Stanleyi collected in Fort Collins, CO from the Se hyperaccumulator plant *Stanleya pinnata*.
(C and D) *P. xylostella* G88 laboratory population originally collected in Geneva, NY.

Elemental defenses are hypothesized to have the advantages of being energetically less costly to accumulate and more difficult to disarm because elements cannot be broken down [35]. So far, one example has been reported of an herbivore that has evolved tolerance to an elemental plant defense. *Melanotrichus boydi* accumulated nickel up to 770 mg · kg^{-1} DW from eating the hyperaccumulator *Streptanthus polygaloides,* and this Ni accumulation protected *M. boydi* from predation [36]. The mechanism of Ni tolerance in this herbivore remains uncharacterized.

The purpose of this study was to identify Se-tolerant herbivores of the Se hyperaccumulator *Stanleya pinnata* and to eludicate their tolerance mechanisms and trophic interactions.

Results

Discovery of *Plutella xylostella* Stanleyi on the Se Hyperaccumulator *Stanleya pinnata*

It has been hypothesized that Se hyperaccumulation by plants serves a defensive function [27, 28]. Various types of plant defenses have eventually been overcome as herbivores evolve tolerance. Therefore, it is possible that Se-tolerant herbivores that can feed on toxic hyperaccumulator plants have evolved. Indeed, we observed that *S. pinnata* plants growing in their natural habitat at our study site in Fort Collins, CO and containing Se levels around 0.2% of dry weight (2,000 μg Se · g^{-1} DW) showed significant leaf herbivory and were colonized by an unidentified Lepidopteran species. Incidentally, other plant species on the same site contained 10–20 μg Se · g^{-1} DW (M. Galeas, personal communication), illustrating the hyperaccumulator nature of these *S. pinnata* plants. Adults hatching from the caterpillars collected from *S. pinnata* (Figures 1A and 1B) showed resemblance to the diamondback moth, *P. xylostella* (Figures 1C and 1D), but were yellow rather than brown. The moths were dissected for identification and confirmed to be an uncharacterized variety of *P. xylostella.* The diamondback moth is an invasive species that has only been in the U.S. for about a century and is an important agricultural pest [30]. Further observations on the field site revealed that the yellow *P. xylostella* variety was abundant on *S. pinnata* plants but was not found on any other species in the area. We therefore suspect that this variety occurs predominantly on this Se hyperaccumulator species, and we will hereafter refer to it as *P. xylostella* Stanleyi.

When the *P. xylostella* Stanleyi larvae collected from the field were eclosed in the laboratory, a significant proportion of the larvae contained a parasitic microgastrine wasp, identified as *Diadegma insulare.* This wasp is a well-known North American parasite of *P. xylostella* [37]. Apparently, this moth and its parasite have evolved Se tolerance and may be specialists living on the Se hyperaccumulator *S. pinnata*.

P. xylostella Stanleyi Differs from Related Taxa in Se Tolerance and Se Avoidance Behavior

The finding that this newly discovered variety of diamondback moth is able to thrive on this hyperaccumulator that contains Se levels 1–2 orders of magnitude higher than those known to be toxic to other Lepidoptera larvae prompted us to further investigate its Se tolerance and feeding preference.

Selenium accumulation was measured in animals collected in the field, with Inductively Coupled Plasma

Table 1. Total Selenium Accumulation in Plants and Insects

Specimens	Tissue Se Concentration ($\mu g\ Se \cdot g^{-1}$ Dry Weight)	
Field Originating	Pooled Insects	*S. pinnata* Leaves (n = 10)
P. xylostella Stanleyi larvae	160	1246 ± 271
P. xylostella Stanleyi cocoons	47	1246 ± 271
P. xylostella Stanleyi adults	201	1246 ± 271
Adult parasitic wasps *D. insulare*	156	1246 ± 271
Lab	Pooled Larvae	*S. pinnata* Leaves (n = 3)
Pooled larvae after 24 hr of feeding on Se-enriched *S. pinnata* leaves		
P. xylostella G88 larvae (n = 2)	10	22 ± 10
P. xylostella Stanleyi larvae (n = 2)	34	22 ± 10
P. xylostella G88 larvae (n = 2)	35	284 ± 96
P. xylostella Stanleyi larvae (n = 3)	62 ± 10	284 ± 96
Pooled larvae after 48 hr of feeding on Se-enriched *S. pinnata* leaves		
P. xylostella G88 larvae (n = 2)	62	1860 ± 322
P. xylostella Stanleyi larvae (n = 3)	178 ± 26	1860 ± 322

Selenium concentration was determined with ICPAES. Data represent the mean total Se concentration ± SE. n = number of samples; each sample consisted of multiple individuals.

Atomic Emission Spectroscopy (ICP-AES). *P. xylostella* Stanleyi larvae and adults contained similar Se levels, around 200 $\mu g \cdot g^{-1}$ DW (Table 1). These tissue Se levels are 5-fold lower than those typically found in their host plant but an order of magnitude higher than those found to be lethal for the cabbage white butterfly, *Pieris rapae* [27]. These Se levels were also toxic to predators [38]. The silk cocoons of *P. xylostella* Stanleyi had 3- to 4-fold lower Se levels than the larvae and adults. The parasitic wasp *D. insulare* showed similar tissue Se levels as its host the *P. xylostella* Stanleyi larvae. Thus, based on their high tissue Se levels, it appears that both *P. xylostella* Stanleyi and its parasite *D. insulare* are remarkably Se tolerant, and their tolerance mechanisms appear to be based on metabolic detoxification rather than exclusion.

The Se tolerance of *P. xylostella* Stanleyi was analyzed more quantitatively in follow-up laboratory studies; in addition, two related taxa were analyzed for contrast. Our first species for comparison was a variety of *P. xylostella* (G88) originally collected in Geneva, NY. Furthermore, *Pieris rapae* was included because it was observed earlier to be sensitive to Se [27] and because it is an important agricultural pest on Brassicaceae [39]. Nonchoice experiments were performed in which larval Se tolerance was tested. After being reared on a diet of *S. pinnata* plants grown in the absence of Se, larvae of all three taxa were transferred to *S. pinnata* plants pretreated with or without Se, and animal survival and weight gain were monitored over the next 3 days. *P. xylostella* Stanleyi showed no significant difference in survival or weight gain between the ± Se treatments (Figures 2A and 2B). In contrast, *P. xylostella* G88 (Figures 2C and 2D) and *P. rapae* (Figures 2E and 2F) both showed a significant decrease in survival and weight gain when feeding on Se-enriched leaves as compared

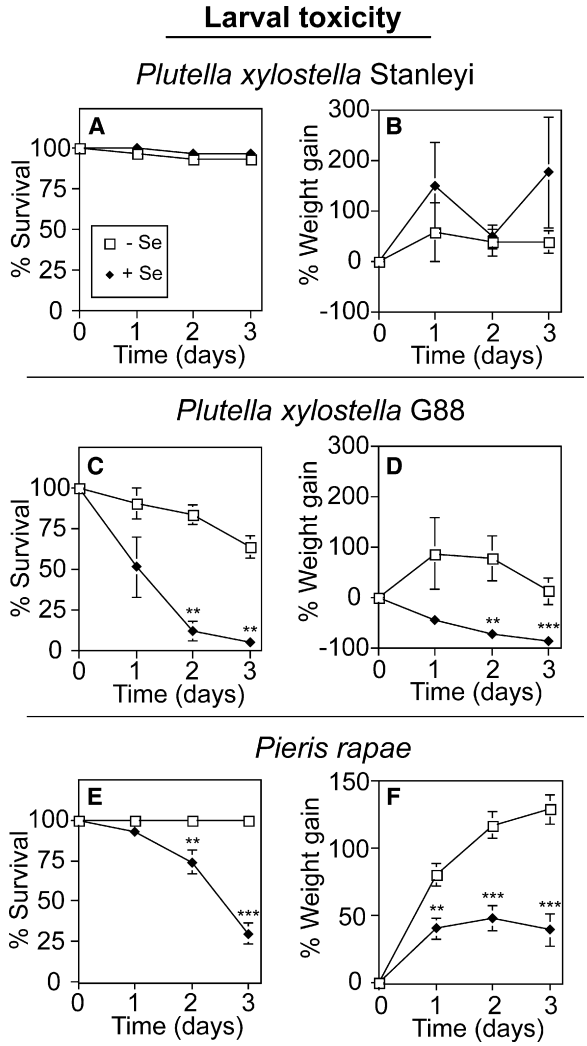

Figure 2. Nonchoice Se-Tolerance Comparisons of *P. xylostella* Stanleyi, *P. xylostella* G88, and *Pieris rapae*

Larvae were fed *S. pinnata* leaves with Se (black diamonds) or without Se (white squares); for leaf Se concentrations, see the Experimental Procedures. (A, C, and E) Larval survival and (B, D, and F) weight change were used as a measure of tolerance. Values represent the mean ± SE. (*p < 0.05; **p < 0.01; ***p < 0.001, t-test).

to leaves that were not Se enriched. These results confirmed that the newly discovered diamondback moth variety is remarkably Se tolerant.

For investigation of whether the difference in Se tolerance displayed by the two closely related varieties of diamondback moth is due to a difference in Se accumulation, similar nonchoice experiments using plant material with three different Se levels (Table 1) were performed. In each case, the Se-tolerant *P. xylostella* Stanleyi contained a 2- to 3-fold higher tissue Se concentration than the Se-sensitive *P. xylostella* G88. The lower Se accumulation in the Se-sensitive larvae was to some extent due to Se toxicity because they stopped eating within 24 hr and died between 24 and 48 hr, so they had less time to feed. In any case, the tolerance of the *P. xylostella* Stanleyi does not appear to be due to Se exclusion but rather to an enhanced Se-detoxification capacity.

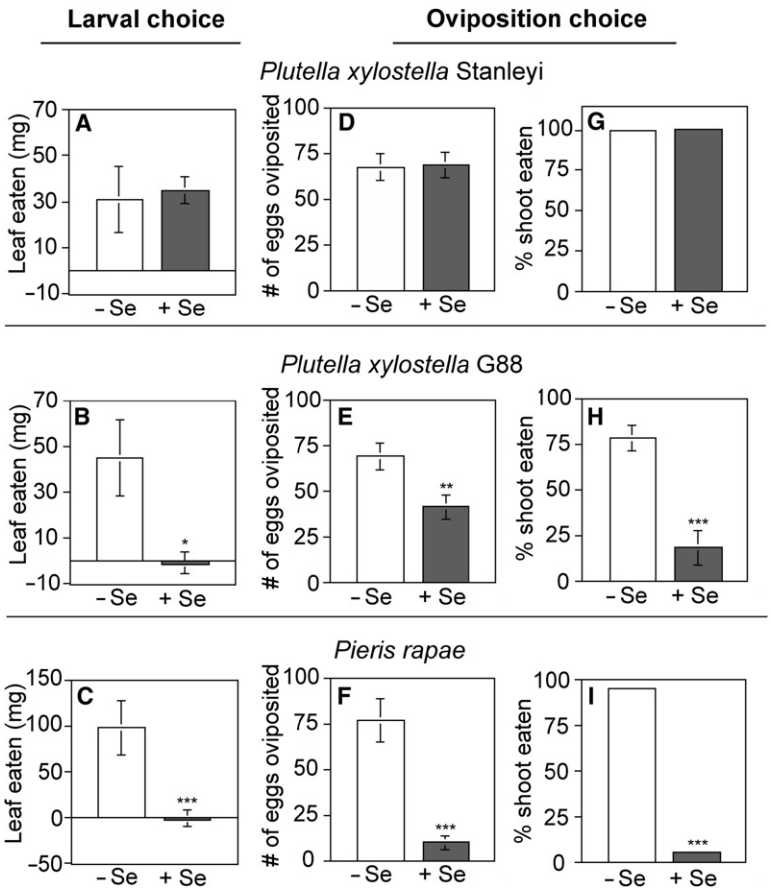

Figure 3. Choice Se Larval Feeding and Adult Oviposition Comparisons of *P. xylostella,* Stanleyi *P. xylostella* G88, and *Pieris rapae*

Larvae were given a choice between *S. pinnata* leaves with or without Se. For leaf Se concentrations, see the Experimental Procedures.

(A–C) Larval feeding choice measured by amounts eaten from leaves.

(D–F) Adult oviposition choice on *S. pinnata* plants with or without Se measured by the number of eggs laid.

(G–I) Larval herbivory damage to plants after 30 days. Values represent the mean ± SE. (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$, t-test) Note that in some cases, the SE was too small to be plotted.

Plant defense compounds are known to act as deterrents to generalist herbivores. Selenium in plants has indeed been shown to deter feeding by various invertebrate herbivores including *P. rapae* [27]. For investigation of whether the two diamondback-moth varieties showed any difference in Se avoidance, larval choice studies using leaves from *S. pinnata* plants treated with or without Se were performed. *Pieris rapae* was included as a control species. The Se-tolerant *P. xylostella* Stanleyi larvae showed no preference for leaves ± Se (Figure 3A). In contrast, the Se-sensitive *P. xylostella* G88 and *P. rapae* larvae showed a significant preference to feed on leaves with low Se concentration and avoided high-Se-concentration leaves (Figures 3B and 3C).

Another important host-plant choice is made in nature by the adult moths, with respect to where they lay their eggs. For investigation of whether the two diamondback-moth varieties differed in Se avoidance for oviposition, choice experiments using *S. pinnata* plants treated with or without Se were performed. *P. rapae* was again included for comparison. The Se-tolerant *P. xylostella* Stanleyi showed no oviposition preference for plants with or without Se (Figure 3D). In contrast, both Se-sensitive controls, *P. xylostella* G88 (Figure 3E) and *P. rapae* (Figure 3F), oviposited significantly less on *S. pinnata* plants containing high levels of Se than on plants with low Se levels.

Thirty days after oviposition, the *S. pinnata* plants were analyzed for herbivory damage from larvae hatched from the deposited eggs. The larvae of the Se-tolerant *P. xylostella* Stanleyi variety had completely eaten all *S. pinnata* plants regardless of their Se levels (Figure 3G). Larvae of *P. xylostella* G88 and *P. rapae*, however, had done significantly less damage to plants with high levels of Se than to those without Se (Figures 3H and 3I). Selenium treatment did not lead to any visible changes in the *S. pinnata* plants, so there was no indication that changes other than Se accumulation affected feeding or oviposition preference. It cannot be excluded that other, biochemical changes may have had effects. However, periodic randomized tests of total phenolic concentrations in leaves showed no differences when *S. pinnata* plants were grown + or −Se (data not shown).

Therefore, our results indicate that Se protected *S. pinnata* from herbivory by these two Lepidopteran pest species because of oviposition deterrence, larval feeding deterrence, and larval toxicity.

Se-Tolerant and -Sensitive *P. xylostella* Varieties Differ in Se Composition, Offering Insight into Se-Tolerance Mechanisms

The greater Se accumulation observed in the Se-tolerant *P. xylostella* Stanleyi compared to the Se-sensitive *P. xylostella* G88 suggests that the tolerant variety can more efficiently store or detoxify ingested Se. This led us to investigate the spatial distribution and chemical forms of Se accumulated in the two moth varieties, by using micro-scanning X-ray fluorescence (μ-SXRF) and micro-X-ray Absorption Near-Edge Structure (μXANES) at the Se K-edge.

μ-SXRF mapping was used to compare the spatial Se distribution in Se-tolerant *P. xylostella* Stanleyi

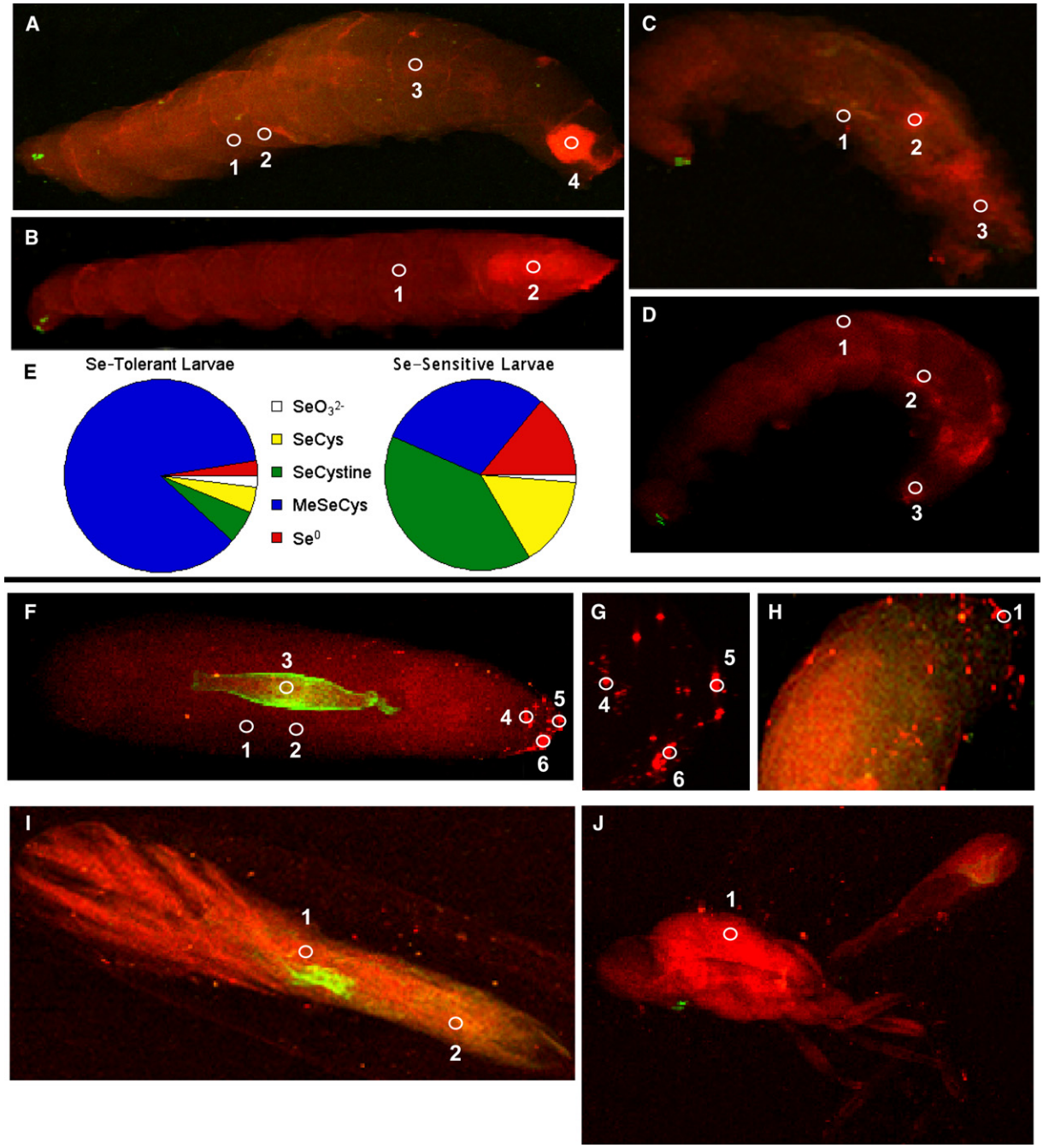

Figure 4. μ-SXRF Localization of Se and μXANES Speciation inside *P. xylostella* Stanleyi, *P. xylostella* G88, and *D. insulare*

Se and Zn channels are shown in red and green, respectively.

(A and B) μ-SXRF map of Se inside *P. xylostella* Stanleyi larvae fed Se-enriched *S. pinnata* leaves in the lab.

(C and D) μ-SXRF map of Se inside *P. xylostella* G88 larvae fed Se-enriched *S. pinnata* leaves in the lab. White numbered circles in (A)–(D) indicate locations of Se-K XANES scans presented in Table 2.

(E) Average composition of the Se in the Se-tolerant and Se-sensitive *P. xylostella*.

(F) μ-SXRF map of Se inside a field-collected *P. xylostella* Stanleyi pupa.

(G) Same as (F), except the abdomen is shown at a higher magnification.

(H) μ-SXRF map of Se inside the abdomen of a field-collected *P. xylostella* Stanleyi larva.

(I) μ-SXRF map of Se inside a field-originated *P. xylostella* Stanleyi adult.

(J) μ-SXRF mapping of Se inside a field-originated parasitic wasp *D. insulare*. White numbered circles in (F)–(J) indicate the locations of Se-K XANES scans presented in Table 4; all images are 20× except that (G) and (H) are 50×.

(Figures 4A and 4B) and Se-sensitive *P. xylostella* G88 larvae (Figures 4C and 4D) fed Se-containing *S. pinnata* leaves. The Se-tolerant larvae contained a higher total Se concentration than the Se-sensitive variety, as judged from signal intensities (shown in red); this is in agreement with the ICP-AES data (Table 1). The Se in

Table 2. Selenium Composition of Leaves and of the Larval Structures Shown in Figure 4 after 48 Hr of Feeding on Se-Enriched *S. pinnata* Leaves

	SeO_4^{2-}	SeO_3^{2-}	$SeGSH_2$	SeCys	SeCystine	MeSeCys	Gray Se^0	Red Se^0
Lab Specimens	%	%	%	%	%	%	%	%
S. pinnata								
Leaves (SNS; n = 14)	ND	ND	ND	8 ± 3	4 ± 2	87 ± 3	ND	ND
P. xylostella Stanleyi Larvae								
Thorax muscular tissue (A1, A2[a]; n = 4)	ND	2 ± 1	ND	ND	ND	98 ± 1	ND	ND
Abdomen between midgut and hindgut (A3, B1; n = 3)	ND	ND	ND	5 ± 5	ND	93 ± 4	ND	2 ± 2
Hindgut (A4, B2; n = 4)	ND	ND	ND	4 ± 4	6 ± 6	87 ± 5	ND	3 ± 3
P. xylostella G88 Larvae								
Thorax muscular tissue (C1, D1; n = 6)	ND	ND	ND	17 ± 17	38 ± 21	22 ± 13	6 ± 6	14 ± 10
Abdomen between midgut and hindgut (C2, D2; n = 6)	ND	2 ± 1	ND	ND	34 ± 20	52 ± 16	5 ± 4	7 ± 6
Hindgut (C3, D3; n = 6)	ND	1 ± 1	ND	ND	64 ± 20	26 ± 14	ND	8 ± 7

Speciation of Se was calculated from X-ray absorption near edge spectra. Data represent the average percentage of total selenium ± SE. n = total number of spectra. SNS = spot not shown. ND = not detected.
[a] Spot location as shown in Figure 3.

the Se-tolerant larvae was present throughout the animal but was highly concentrated in its posterior (on the right in Figures 4A and 4B), tentatively identified as the hindgut. Incidentally, the head is easily recognizable by the Zn in the mandibles of larvae (shown in green, Figures 4A–4D). Judged from Zn distribution, the mandibles appear to be digested in the pupa midgut during metamorphosis (Figure 4F). In addition to the hindgut, Se appeared to be concentrated to some extent at the segment junctions in the tolerant larvae (Figures 4A and 4B). μ-SXRF imaging of the Se-sensitive *P. xylostella* (G88) larvae suggested that their internal organs were losing their integrity because of Se toxicity (Figures 4C and 4D). Unlike the tolerant Stanleyi, *P. xylostella* (G88) did not exhibit obvious areas of Se accumulation.

We used XANES to investigate the Se composition of the two diamondback varieties at the spots shown in Figures 4A–4D. The forms of Se present in the plant were also determined. Table 2 shows the Se composition at various locations. Within individual animals, the different tissues tested showed a similar Se composition (Table 2). Based on XANES, the total Se in *S. pinnata* leaves was mainly methylselenocysteine (MeSeCys, 87% ± 3%), with small fractions of SeCys (8% ± 3%) and SeCystine (4% ± 2%). After having fed on this same plant material, the two diamondback moth varieties showed a striking difference in Se composition, as summarized in Figure 4E, which shows the average from all spots. The Se-tolerant moth larvae accumulated predominantly MeSeCys (~90%), whereas the Se in the sensitive variety consisted of more than 50% SeCystine and SeCys. Because the methylation of SeCys is known to be an effective way of preventing toxic nonspecific incorporation of SeCys into proteins, this striking difference in Se composition could explain the observed difference in Se tolerance between the two diamondback-moth varieties.

Because XANES cannot distinguish well among selenomethionine (SeMet), MeSeCys, the dipeptide γ-glutamyl-MeSeCys (γGMeSeCys), and selenocystathionine (SeCysth), we used Liquid Chromatography-Mass Spectroscopy (LC-MS) to compare the levels of these organic selenocompounds in larvae of the Se-tolerant *P. xylostella* Stanleyi and the Se-sensitive *P. xylostella* G88 that were fed leaves from Se-enriched *S. pinnata* plants. The leaves were also analyzed. The nonprotein organic Se compounds in the *S. pinnata* leaves consisted of 87% MeSeCys and 13% SeCysth (Table 3). After the Se-tolera/nt *P. xylostella* Stanleyi larvae had eaten these leaves, 92% of their total nonprotein organic Se was MeSeCys and 8% was γGMeSeCys. By contrast, in the Se-sensitive *P. xylostella* G88, MeSeCys made up only 15% of all nonprotein organic Se compounds, whereas 84% was SeCys and 1% SeCystine (Table 3). These results from LCMS support the XANES results and indicate that the most likely reason for the difference in tolerance between the two diamondback-moth varieties is their difference in SeCys methylation.

To investigate whether the Se-tolerant *P. xylostella* Stanleyi variety is indeed more efficient in preventing nonspecific replacement of Cys by SeCys in proteins than the Se-sensitive *P. xylostella* G88 variety, we

Table 3. Composition of Nonprotein Organic Selenocompounds in Plants and Insects

	SeCysth	SeMet	$SeGSH_2$	SeCys	SeCystine	MeSeCys	γGMeSeCys
Lab Specimens	%	%	%	%	%	%	%
S. pinnata leaves (n = 3)	13 ± 1	ND	ND	ND	ND	87 ± 1	ND
P. xylostella Stanleyi larvae (n = 3)	ND	ND	ND	ND	ND	92 ± 1	8 ± 1
P. xylostella G88 larvae (n = 6)	ND	ND	ND	84 ± 2	1 ± 0	15 ± 2	ND

Nonprotein organic Se compounds were measured with LCMS. Shown is the percentage of total nonprotein organic selenium ± SE. n = number of samples; each sample consisted of multiple individuals. ND = not detected.

Table 4. Selenium Composition of Field-Originating Specimens at Structures Shown in Figure 4

	SeO_4^{2-}	SeO_3^{2-}	$SeGSH_2$	SeCys	SeCystine	MeSeCys	Gray Se^0	Red Se^0
	%	%	%	%	%	%	%	%
P. xylostella Stanleyi Larva								
Thorax muscular tissue (SNS; n = 7)	ND	ND	5 ± 0	7 ± 1	ND	89 ± 1	ND	ND
Hindgut (SNS; n = 3)	ND	ND	3 ± 0	9 ± 1	ND	89 ± 1	ND	ND
Abdominal deposits (H1; n = 6)	ND	ND	ND	ND	ND	ND	100 ± 0	ND
Frass (SNS; n = 5)	ND	ND	ND	14 ± 8	6 ± 6	74 ± 4	ND	7 ± 4
P. xylostella Stanleyi Pupa								
Thorax muscular tissue (F1, F2; n = 3)	ND	ND	ND	ND	ND	100 ± 0	ND	ND
Midgut (F3; n = 3)	ND	ND	ND	7 ± 7	ND	83 ± 2	ND	10 ± 5
Abdominal deposits (F and G 4,5,6; n = 9)	ND	1 ± 1	ND	ND	6 ± 6	ND	93 ± 5	ND
P. xylostella Stanleyi Adult								
Thorax muscular tissue (I1; n = 3)	ND	ND	ND	ND	ND	100 ± 0	ND	ND
Abdomen (I2; n = 3)	ND	ND	ND	17 ± 0	ND	83 ± 0	ND	ND
Adult Parasitic Wasp *D. insulare*								
Thorax muscular tissue (J1; n = 5)	ND	8 ± 1	ND	ND	ND	93 ± 1	ND	ND

Speciation of Se was calculated from X-ray absorption near edge spectra. Data represent the average percentage of total selenium ± SE. n = total number of spectra. SNS = spot not shown. ND = not detected.

allowed larvae of both varieties to feed for 48 hr on *S. pinnata* leaves containing 2007 ± 295 μg Se · g^{-1} DW and then quantified the Se in the protein fraction by ICP-AES. A significantly smaller fraction of the ingested Se was incorporated into protein in the *P. xylostella* Stanleyi larvae compared to the G88 larvae ($p < 0.005$, Table 5). Sulfur was also significantly less incorporated into protein in the *P. xylostella* Stanleyi larvae compared to the G88 larvae ($p < 0.005$, Table 5).

Selenium Composition in Field-Collected Specimens Reveals a Similar Se-Tolerance Mechanism at Three Trophic Levels

In a subsequent study, field-collected *P. xylostella* Stanleyi larvae, pupae, and adults, as well as the parasitic wasp *D. insulare* were analyzed for Se composition and Se distribution. One purpose of this study was to test whether the results obtained with laboratory animals, which were fed Se for only several days, are relevant for animals that lived their entire life on high-Se *S. pinnata* plants in the field. The other purpose was to follow the fate of Se over three trophic levels and to compare the tolerance mechanisms at each of these levels.

μ-SXRF imaging was used so that the spatial Se distribution in the various field-collected animals could be analyzed. The pupae (Figures 4F and 4G) from the Se-tolerant *P. xylostella* Stanleyi showed a fairly homogeneous Se distribution throughout their tissues, with some Se accumulation in abdominal spots, which might be specific deposit sites. The larva (Figure 4H) concentrated Se in its hindgut but also in abdominal spots similar to the pupa. In *P. xylostella* Stanleyi adults (enclosed in the lab from pupae collected in the field), the Se appeared to be distributed homogeneously throughout the body (Figure 4I) without obvious hot spots. Judged from XANES, MeSeCys was the predominant form (~90%) of Se accumulated in these field-collected *P. xylostella* Stanleyi larvae, pupae, and adults (Table 4). Only the abdominal Se spots found in both larvae and pupae from the field (Figures 4F–4H) were approximately 95% elemental Se (Table 4). Thus, the predominant form of Se found in *P. xylostella* Stanleyi was the same in lab and field specimens, and likely, this accumulation as MeSeCys is key to its Se tolerance. The only difference between lab and field animals was that the field animals contained an abdominal hot spot consisting of elemental Se. This difference may be due to the longer exposure times to Se.

Because the parasitic wasp *D. insulare* derived all of its biomass from feeding upon the Se-enriched *P. xylostella* Stanleyi larvae without ill effects, it is interesting to investigate and compare its Se distribution and composition as well. When the adult wasp was analyzed by μ-SXRF (Figure 4J), it gave a strong Se signal indicative of substantial Se accumulation, in agreement with the results from ICP-AES (Table 1). Similar to the *P. xylostella* larvae, Zn (shown in green) was accumulated in the *D. insulare* mandibles, probably for the fortification of these chewing mouthparts. Se-K XANES scans revealed that 93% of the total Se in *D. insulare* was MeSeCys (Table 4). Thus, MeSeCys accumulated in these Se-tolerant organisms from all three trophic levels, *S. pinnata*, *P. xylostella* Stanleyi, and *D. insulare*. Accumulating Se as MeSeCys appears to be a unifying Se-tolerance mechanism in this ecosystem.

Table 5. Relative Incorporation Values of Selenium and Sulfur into Total Proteins Isolated from *P. xylostella* Stanleyi and *P. xylostella* G88 Larvae

	μg Se · g^{-1} Protein	μg S · g^{-1} Protein
P. xylostella Stanleyi larvae total protein (n = 4)	205 ± 2	12059 ± 166
P. xylostella G88 larvae total protein (n = 4)	252 ± 6	14910 ± 350

Discussion

The protective function of Se observed here in the hyperaccumulator species *S. pinnata* supports the

hypothesis that Se hyperaccumulation has evolved as an elemental defense mechanism. The Se in *S. pinnata* protected it from herbivory by a common variety of the diamondback moth (*P. xylostella* G88) as well as from the cabbage white butterfly (*Pieris rapae*). Both herbivores occur in the Fort Collins area where this plant grows naturally and thus these interactions are ecologically relevant. Both herbivores are important agricultural pests, and the finding that Se is effective in controlling them may have applications in agriculture. The Se in this hyperaccumulator *S. pinnata* is mainly present in the organic form MeSeCys, whereas in nonhyperaccumulators Se mainly accumulates as selenate [40, 41]. Earlier studies on Se as a defense compound have focused on nonhyperaccumulator plants [25–28], and this study is the first to show that this organic form of Se is effective as a defense compound against invertebrate herbivores, because of both deterrence and toxicity.

The finding of a Se-tolerant diamondback-moth variety that likely evolved on the hyperaccumulator and the elucidation of its tolerance mechanisms are significant because they represent the first discovery and characterization of an herbivore tolerance mechanism to Se or any other plant elemental defense. The characterization of a high Se-accumulating variety of the microgastrine-wasp *D. insulare*, apparently tolerant to the high Se concentrations ingested while actively parasitizing the Stanleyi diamondback moth, suggests that adaptation could also have occurred at the next trophic level. Se tolerance in this variety of *D. insulare* needs to be further confirmed through tests comparing it to a closely related Se-sensitive variety. The finding that Se accumulated in this parasitic wasp adds another dimension by showing that Se is capable of moving up food chains. Together, the three species likely form a multitrophic Se-tolerant, Se-accumulating system that could serve as a portal for Se into this natural ecosystem. It will be interesting for future studies to investigate the toxicological effects of the Se accumulated by this moth and parasitic wasp on other less tolerant ecological partners. It is very likely that their internal Se concentration protects them from generalist predators because of both deterrence and toxicity. Accumulation of Se in insects has clearly been shown to result in toxicity to some predators [38, 42]. The finding that the tolerant Stanleyi moth sequestered Se distinctly in its hindgut or in elemental deposits on the exterior of its abdomen suggests that these larvae could use the accumulated Se for their own defense against predation.

When feeding on *S. pinnata* plants containing mainly MeSeCys, the Se-tolerant Stanleyi diamondback moth variety accumulated MeSeCys, whereas the sensitive variety contained mainly SeCys. The ability to prevent the biotransformation or degradation of MeSeCys into other, more toxic forms is evidently responsible for the observed Se tolerance. The simplest explanation for this difference is that the Se-tolerant animals do not demethylate the ingested MeSeCys, whereas Se-sensitive animals do. Alternatively, it is possible that both insects demethylate the ingested MeSeCys and that only the Se-tolerant variety is able to remethylate it. In either case, the accumulation of SeCys in the Se-sensitive (G88) diamondback moth larvae led to higher incorporation rates of Se into protein compared to the Se-tolerant Stanleyi larvae. Sulfur was also significantly less incorporated into protein in the Se-tolerant larvae compared to the G88 larvae. This suggests that the Se-tolerant Stanleyi larvae are less able to incorporate sulfur from the ingested plant material into proteins. It is likely that a substantial fraction of the sulfur in these plants is present as S-methylcysteine (MeCys). Selenium hyperaccumulator plants accumulate large amounts of MeCys compared to nonhyperaccumulating relatives [14, 43, 44]. Many other Brassicaceae species including *B. juncea* also contain substantial concentrations of MeCys and MeCys sulfoxide, which can cause toxicity in animals when ingested [45, 46]. Because the diamondback moth is a Brassicaceae pest, the ability of this herbivore to demethylate MeCys and MeCys sulfoxide would be a great advantage, both to decrease the toxicity of the plant and to obtain the essential amino acid cysteine for its own nutrition. MeCys demethylase enzyme activity has been characterized in Chinese cabbage and in rats [47, 48], and it is likely that it is also present in the diamondback moth. If such Me(Se)Cys demethylase activity is lost or decreased in the Se-tolerant Stanleyi variety, different selection pressures may act on them in Se-rich habitats than in areas that lack Se. Whereas a lack of MeSeCys demethylation could protect the Stanleyi variety from the misincorporation of SeCys into proteins, the associated lack of MeCys demethylation may cause a decrease in its ability to assimilate sulfur from Brassicaceae that accumulate sulfur in the form of MeCys.

In addition to demethylating MeSeCys, the nontolerant (G88) diamondback moth may be able to further degrade SeCys into inorganic Se, as seen in Table 1. The predominantly accumulated SeCys may either represent an accumulating intermediate or have resulted from reassimilation from inorganic Se. In relation to the latter possibility, it was recently reported that another Se-sensitive Lepidopteran pest species, the beet armyworm, was able to absorb selenate from its diet and bioconvert it into organic Se, modeled as mainly SeMet [42]. The overall mechanisms by which MeSeCys is metabolized in the nontolerant (G88) diamondback moth remain to be discovered. Enzymes such as a MeCys demethylase, stomach acids, and gut microbial communities could all contribute to this process. Nonetheless, the ability to retain MeSeCys in its original form and thus prevent Se from misincorporation into proteins is apparently responsible for Se tolerance in the tolerant Stanleyi variety.

From these collective findings, we can envision the following model for the course of events in the evolution of Se tolerance in this diamondback moth: A Se hyperaccumulator habitat is invaded by a Se-sensitive moth that carries a recessive mutation in a gene encoding a Me(Se)Cys demethylase that renders the allele nonfunctional. If the moth is heterozygous at this locus, also carrying the dominant allele encoding the active enzyme, its ability to detoxify MeCys and mobilize Cys is not affected. The moth has a preference to oviposit on non-Se plants but lays some of its eggs on hyperaccumulator plants, allowing for the Se to act as selection pressure. The offspring that is homozygous for the recessive allele (25%) will be Se-tolerant and therefore will be the only ones surviving and reproducing on

hyperaccumulator plants. In parallel or subsequent to the evolution of Se tolerance, the moths lose their aversion to oviposit and feed on high-Se plants, for instance, because of the loss of an olfactory Se receptor. There will be selection for the loss of this aversion because Se-tolerant animals that oviposit more on high-Se plants have a higher reproductive success because of less competition and perhaps less predation. Other adaptations also evolve, such as the color change of the adult, which is pale yellow, whereas larva and pupa are yellow or green. These altered colorations may give both the Stanleyi larva and moth better camouflage on the pale yellow inflorescence of *S. pinnata*. Indeed, in the field, *P. xylostella* Stanleyi was observed to often graze, pupate, feed on nectar, and lay eggs in these inflorescences, in addition to leaves. This is all the more interesting considering that these inflorescences in the field contained very high concentrations of Se, often reaching 3,500 $\mu g\ Se \cdot g^{-1}$ DW. In addition, the *P. xylostella* Stanleyi may have evolved tolerance to other *S. pinnata* defense compounds, considering that even in the absence of Se, it showed slightly higher survival at day 3 compared to *P. xylostella* G88 (Figure 2C).

The biochemical characterization of these naturally occurring Se fluxes within and among trophic levels provides a framework for other natural and managed systems. Over the past few years, there has been an increasing interest in the use of plants for Se phytoremediation and as a dietary source of anticarcinogenic selenocompounds. This study provides insights into the possible ecological implications of the use of Se-enriched agricultural crops and of plants for the remediation of Se-polluted environments.

Experimental Procedures

Lepidopteran Populations

Two *Plutella xylostella* populations were used. *P. xylostella* G88 was obtained from Dr. Tony Shelton at Cornell University (Ithaca, NY) and was originally collected in Geneva, New York in 1988. *P. xylostella* Stanleyi was first collected in 2004 from the Se hyperaccumulator *S. pinnata* growing in the Pine Ridge Natural Area near Fort Collins, Colorado during an insect survey (M.L. Galeas, L. Bennett, E. Klamper, J.L.F., B. Kondratieff, and E.A.H.P.-S., unpublished data). *P. xylostella* Stanleyi was identified through the method of Moriuti [49], including dissection to confirm the characteristic peculiarly shaped aedeagus. The *Pieris rapae* population was obtained from Carolina Biological Supply Company (Burlington, North Carolina). These populations were maintained on *Brassica juncea* (accession number 173874, North Central Regional Plant Induction Station, Ames, Iowa) or *S. pinnata* plants. The plants were grown in Pro-Mix BX potting soil in a growth room (24°C/20°C, 12 hr/12 hr light/dark, 120 $\mu mol \cdot m^{-2} \cdot s^{-1}$ photosynthetic photon flux) and watered three times weekly. Adults of these populations oviposited on the plants for 6–9 days. The larvae that emerged fed on *B. juncea* or *S. pinnata* plants until pupation. Adults were fed artificial nectar, according to care instructions provided by Carolina Biological Supply, while they were allowed oviposition.

Plant Material

Stanleya pinnata leaves were used for testing Se tolerance in all three populations. *S. pinnata* was obtained from Western Native Seed in Coaldale, CO. *S. pinnata* plants were grown in Pro-Mix BX for 6–8 weeks in a growth room (24°C/20°C, 12 hr/12 hr light/dark, 120 $\mu mol \cdot m^{-2} \cdot s^{-1}$ photosynthetic photon flux). After 3 weeks of growth, half of these plants were watered with 80 $\mu M\ Na_2SeO_4$ for 4–6 weeks before use, whereas the other half were given water as a control. Directly prior to each experiment, Se levels in leaves were determined in the paired batches of ± Se grown plants, as described below. The *S. pinnata* ± Se leaf material used in each experiment was the same for both insect types. These leaf Se concentrations were as follows in Figures 2A–2D: 1860 $\mu g\ Se \cdot g^{-1}$ DW for +Se and 9 $\mu g\ Se \cdot g^{-1}$ DW for −Se. In Figures 2E–2F, the concentrations were as follows: 185 $\mu g\ Se \cdot g^{-1}$ DW for +Se and 5 $\mu g\ Se \cdot g^{-1}$ DW for −Se. The *S. pinnata* Se concentrations used in Figure 3 were: (A), 792 $\mu g\ Se \cdot g^{-1}$ DW for +Se and 47 $\mu g\ Se \cdot g^{-1}$ DW for −Se; (B), 914 $\mu g\ Se \cdot g^{-1}$ DW for +Se and 6 $\mu g\ Se \cdot g^{-1}$ DW for −Se ; (C), 202 $\mu g\ Se \cdot g^{-1}$ DW for +Se and 1 $\mu g\ Se \cdot g^{-1}$ DW for −Se; (D) and (G), 7715 $\mu g\ Se \cdot g^{-1}$ DW for +Se and 9 $\mu g\ Se \cdot g^{-1}$ DW for −Se; (E) and (H), 1326 $\mu g\ Se \cdot g^{-1}$ DW for +Se and 17 $\mu g\ Se \cdot g^{-1}$ DW for −Se; (F) and (I), 530 $\mu g\ Se \cdot g^{-1}$ DW for +Se and 11 $\mu g\ Se \cdot g^{-1}$ DW for −Se. All whole plants that were used were of the same size with equal leaf numbers, and no phenotypic differences were observed between ±Se plants. In addition to total Se concentration, the only noted difference was the presence or absence of the typical pungent dimethyldiselenide odor emanating from the +Se-treated plants.

Reagents

Na_2SeO_4 (S8295), Na_2SeO_3 (S1382), SeCystine (S1650), and SeMet (S3132) were obtained from Sigma-Aldrich, St. Louis, MO. MeSeCys, γGMeSeCys, SeCysth, and $SeGSH_2$ standards were obtained from PharmaSe, Lubbock, Texas. SeCys was obtained by reduction of SeCystine at 25°C overnight in 100 mM Na-Borohydride at a 1:1 molar ratio.

Selenium and Sulfur Concentrations in Biological Material

Biological samples were rinsed with distilled water and dried at 50°C for 48 hr. 20 mg (insect) or 100 mg (plant) replicate samples were acid-digested and analyzed for Se and S by Inductively Coupled Plasma Atomic Emission Spectrometry (ICP-AES) as described by Pilon-Smits, et al. [50].

Quantification of Larval Se Tolerance

Nonchoice feeding experiments used leaf tissue from *S. pinnata* pretreated with or without 80 $\mu M\ SeO_4^{2-}$. After 4–6 weeks of treatment, young leaves were excised from *S. pinnata* and their stems were inserted in a 1.5 ml tube filled with distilled water and sealed by a cotton plug so that desiccation could be prevented. Each tube containing a leaf was placed in a Petri dish with a larva from one of the three populations. Larval survival and larval weight gain were monitored over the course of the experiment (up to 5 days). Three experiments were performed for each of the Lepidopteran varieties, with 7–9 individuals per treatment.

Quantification of Larval Se Avoidance

Larvae were given a choice to feed on young *S. pinnata* leaves ± Se. These leaves were collected from plants that had been treated for 4–6 weeks with 0 or 80 $\mu M\ Na_2SeO_4$. For each replicate, one leaf from each treatment was placed with its petiole in a 1.5 ml tube filled with distilled water and sealed with a cotton plug so that desiccation could be prevented. The two tubes with leaves sticking out were placed inside a plastic container covered with mesh so that ventilation was allowed. A larva was placed in the middle and allowed to feed on its leaf of choice. Leaf weights were measured at the beginning and end of the experiment so that the amount of plant tissue consumed could be determined. The initial larval weight was also measured to ensure there were no differences between ± Se treatments. The replicate containers were placed in alternating orientation (with the +Se leaf on opposite sides) so that any influence from microclimate differences could be prevented. Every experiment was performed three times, with ten individuals for each of the two *P. xylostella* varieties and 18 for *P. rapae*.

Adult Oviposition Choice and Its Effect on Long-Term Plant Survival

In glass aquaria covered with mesh, adults from one of the three Lepidoptera populations were allowed to oviposit on *S. pinnata* plants pretreated for 4–6 weeks with either 0 or 80 $\mu M\ Na_2SeO_4$. All plants that were used were of the same size with equal leaf numbers, and no phenotypic differences were observed between ± Se plants. Three replicate 40 L aquaria were used for each of the three populations (*P. xylostella* Stanleyi, *P. xylostella* G88, and *P. rapae*).

For each aquarium, 4–6 plants were used per treatment (± Se) and 20 adults were allowed to oviposit. The number of eggs on each plant was recorded after 7 days. After oviposition was recorded, the adults were removed and the eggs on the plants were left to hatch. During oviposition and for 30 days afterward, the plants were treated with distilled water only. Larvae moved freely between *S. pinnata* plants pretreated with either 0 or 80 μM SeO_4^{2-}. Thirty days after oviposition, the herbivory suffered by each plant was recorded.

μ-XRF/μ-XANES

Samples collected from both the field and the lab were washed to remove any external Se, frozen in LN_2, and shipped on dry ice to the Advanced Light Source at the Lawrence Berkeley Laboratory for micro-spectroscopic analysis on Beamline 10.3.2 [51]. The total distribution of Se was mapped by scanning the samples in the focused X-ray beam at 13,000 eV. Samples were mounted onto a Peltier stage with a tiny amount of silicone grease and kept at −33°C so that potential radiation damage could be reduced. μ-SXRF mapping of Se was performed first on all samples. Insect maps were collected with a 7 × 7 μm beam at 13,000 eV sampled in 5 × 5 μm pixels or with a 5 × 5 μm beam at 13,000 eV sampled in 3 × 3 μm pixels. The Kα fluorescence line intensities of Se (and other elements of interest) were measured with a 7 element Ge solid state detector and normalized to the incident monochromatic beam intensity. Se K-edge XANES was used for probing the molecular speciation of the Se at specific points [52]. Aqueous solutions of the various selenocompounds were used as standard materials.

Extraction and Measurement of Nonprotein Organic Selenocompounds

S. pinnata leaves were ground with a micropestle in liquid N_2. Two hundred milligrams of this finely ground tissue was then suspended in 200 μL of 50 mM HCl. The solution was clarified by centrifugation for 5 min at 16,000 *g* at 4°C. This solution was then passed through a Sep Pak C18 syringe cartridge that had been charged with 100% acetonitrile and washed with distilled H_2O. The flow through was then stored at −80°C. *P. xylostella* Stanleyi and *P. xylostella* G88 were allowed feeding for 48 hr on Se-enriched *S. pinnata* leaves and then underwent 24 hr without food so that their intestines could be evacuated. The larvae were then frozen and ground with a micropestle in LN_2. One hundred milligrams of this finely ground tissue was suspended in 2 mL of 50 mM HCl. The solution was clarified by centrifugation for 5 min at 16,000 *g* at 4°C, and the supernatant was passed through a Sep Pak C18 syringe cartridge, which had been charged with 100% ACN and washed with distilled H_2O. The flow through was stored at −80°C until analysis.

The nonprotein organic selenocompounds in the *S. pinnata*, *P. xylostella* Stanleyi, and *P. xylostella* G88 extracts were analyzed by LC-MS with a Hewlett Packard/Agilent 1100 Series HPLC and a Finnigan LcQDuo thermo Quest MS system equipped with Xcalibur software. Through injecting 30 μL of these prepared solutions, the selenocompounds were separated at 15°C with a Phenomenex Hypersil 5 μm C18 (ODS) column (250 × 2.00 mm, 5 μm) at a flow rate of 0.36 mL · min^{-1}, with the following two eluents: (A) water + 0.1% formic acid and (B) acetonitrile + 0.1% formic acid. The following program was used: 0–2 min, 100% A; 2–10 min, gradient 0–40% B; 10–12 min, 40–0% B; 12–17 min, 100% A. The following pure aqueous selenocompounds were used for generation of 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, and this confirmed the exact characteristic Se isotopic signatures 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 so that the total abundance of the other less abundant Se isotopes could be calculated. 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 this small mass range.

Protein Selenium and Sulfur Content

With the method of Pilon, et al. [53], Se and S content in protein was determined in larvae from *P. xylostella* Stanleyi and *P. xylostella* G88 after 48 hr of feeding on leaves collected from a *S. pinnata* plant treated with 80 μm Na_2SeO_4. The insects were frozen and ground in liquid nitrogen. Aliquots of 0.1 g fresh weight were extracted in 0.5 mL · g^{-1} buffer containing 100 mM NaCl, 50 mM Tris-HCl (pH 7.5), 0.5% (v/v) Triton X-100, 1 mM DTT, and 0.1 mM phenylmethylsulfonyl fluoride. The homogenate was cleared by centrifugation (7,500 *g* for 10 min). A small sample was taken for protein determination, and the volume of the extract was measured. The proteins in the extract were precipitated by addition of trichloroacetic acid to a final concentration of 15% (w/v). The mixture was incubated on ice for 30 min and then centrifugated for 20 min at 7,000 *g* at 4°C in a glass tube. The pellet was washed with ice-cold acetone, dried, and dissolved in 1 mL of concentrated nitric acid. After acid digestion, the Se concentrations in these samples were determined by ICP-AES according to Pilon-Smits, et al. [50].

Data Analysis

Statistical analyses were performed with the software package JMP-IN from the SAS institute (Cary, North Carolina). Synchrotron data analysis was performed with a suite of LabVIEW programs (National Instruments) available at Beamline 10.3.2 and downloaded from http://xraysweb.lbl.gov/uxas/Beamline/Software/Software.htm.

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