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Selenium Volatiles as Proxy to the Metabolic Pathways of Selenium in Genetically Modified *Brassica juncea*

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In this study we demonstrate that the headspace selenium volatiles could be used as proxy to the metabolic pathways in the Se-accumulator plant *Brassica juncea*. The selenium metabolic pathways in wild type plants are compared to those of several genetically modified cultures. Complementary use of atomic and molecular mass spectrometric techniques also allowed for identification of yet unreported minor headspace Se-containing volatiles such as $CH_3SeSeSeCH_3$, $CH_3SeSSeCH_3$, and $CH_3SeCH_2CH_3$. By combining the information resulting from this research with the previously known information about selenium metabolism in *B. juncea*, it is possible that a more efficacious phytoremediation tool can be constructed.

Introduction

Selenate and selenite are major contaminants in soils especially in the Central Valley region of California where Se has been shown to be a factor in high rates of mortality and embryonic deformities of resident birds of the Kesterson Reservoir (*1*). The selenium originates from the erosion of natural cretaceous shale and travels via irrigation water which eventually evaporates, causing the concentration of Se to reach levels >12 mg Se kg^{-1} soil (*2*). To control the problem, some attempts have been made, such as pooling the drainage water into evaporation ponds, but via evapo-concentration, the Se levels ultimately increase and the cleanup of these polluted areas becomes a necessary objective.

The use of selenium accumulator plants to uptake and detoxify contaminants (phytoremediation) is an attractive solution for cleaning such sites. Certain plants extract selenium from the contaminated soil and ultimately convert it to virtually nontoxic volatile organic forms like dimethyl selenide. Selenium hyper-accumulator plants, such as *Astragalus bisulcatus*, have been examined for this purpose, but slow growth rate and small biomass limit their effectiveness for phytoremediation (*3*).

An attractive solution to this problem is the genetic engineering of fast-growing Se accumulator plants, such as Indian mustard (*Brassica juncea*) as the efficiency of Se phytoremediation, a naturally occurring process, can be dramatically enhanced this way (*4*−*7*). One strategy for genetically engineering plants for improved phytoremediation is to increase the levels of rate-limiting enzymes involved in the uptake and/or detoxification of selenium. An example of this strategy is the overexpression of the APS1 gene (encodes for ATP-sulfurylase (The ATP-sulfurylase overexpressing transgenic plants will be abbreviated as APS), EC 2.7.7.4) from *Arabidopsis thaliana* that overcomes the rate-limiting step of reducing selenate to selenite (*6*−*8*). A newer strategy for genetically engineering plants takes advantage of the increasing information about the Se-hyperaccumulation mechanism. An example of this approach involves the overexpression of a gene isolated from the Se-hyperaccumulator, *A. bisulcatus*, which encodes for selenocysteine methyltransferase (the selenocystein methyltransferase overexpressing transgenic plants will be abbreviated as SMT) (EC 2.1.1.9) (*5*). Overexpressing SMT increases levels of selenocysteine methyltransferase which methylates SeCys to form *Se*-(methyl)-selenocysteine (MeSeCys), a non-proteinogenic amino acid, thus diverting the Se flow away from SeCys and subsequent production of SeMet (see Figure 1), both of which can be mis-incorporated into proteins, altering their native structure and function.

Decreasing SeCys levels represent an important Se tolerance strategy. Recently, the two approaches were combined by developing *B. juncea* overexpressing both APS1 and SMT (*10*). The double transgenic plants (APS×SMT) appear to have the combined benefits of overexpressing both genes as they were able to accumulate significantly more Se than the single transgenics (APS and SMT) when grown on selenate-containing media.

In genetically modifying a plant, the transgene is introduced with the intent it will integrate into the genome. To determine whether this process occurred successfully, several approaches are practiced. Polymerase chain reaction (PCR) analysis is often the first step. In this process a DNA fragment specific for the used primers will be amplified from DNA extracted from genetically modified (GM) cultures, whereas no such DNA can be amplified from wild type (WT). Using gel electrophoresis to separate DNA fragments, a positive result can be visualized as an ethidium bromide stained band.

A second test is based on the fact that the overexpression of the gene results in increased production of the desired protein. Western or immuno-blotting is typically used to detect the protein of interest using an antibody that will selectively interact with the desired protein. In some cases, this can add practical complications since obtaining appropriate antibodies can be difficult and expensive. Also, there is the possibility that the antibody will hybridize with endogenous proteins (as is the case with the SMT antibody) thus leading to false positives. In most cases, however, this method (like PCR) is highly specific and presence of the modification can be detected as a stained protein band at the expected molecular weight.

The aim of this study is to investigate alternative elemental and molecular mass spectrometric methods for rapidly detecting molecular level changes produced by genetic modifications, thus differentiating wild type *B. juncea* from genetically modified *B. juncea* similar to the conventional electrophoresis methods.

Experimental Section

Chemicals and Standards. All reagents were of analytical grade and were used without any further purification. Dimethyl selenide (DMeSe), dimethyl sulfide (DMeS), and dimethyl disulfide (DMeDS) were purchased from Fluka

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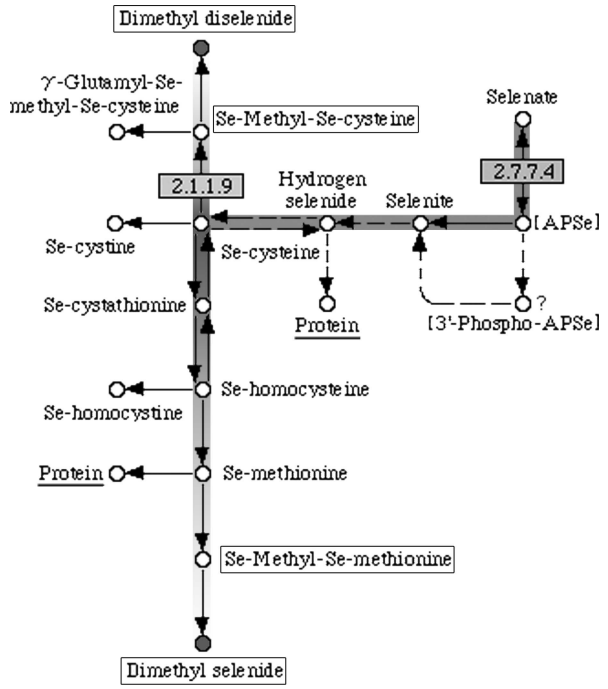

FIGURE 1. Outline of the selenium metabolic pathway in *B. juncea* shown as uncontrolled wild-type pathway (→dimethyl selenide) and genetically modified pathway (→ dimethyl diselenide) (*9*).

(Milwaukee, WI). Dimethyl diselenide (DMeDSe) and diethyl disulfide (DEtDS) were purchased from Sigma-Aldrich (Milwaukee, WI). Diethyl diselenide (DEtDSe) was purchased from Strem Chemicals (Newburyport, MA). The stock solutions of 1000 μg mL^{-1} were prepared by dilution of 2.5 μL of compound with 2500 μL of HPLC grade methanol (Fisher Scientific; Fair Lawn, NJ). CH_3SeSCH_3 was prepared via chalcogenide exchange reaction as described elsewhere (*19*). The liquid Ar used to run the ICP−MS as well as the Ar:N_2 optional gas (50:50) were obtained from Wright Brothers (Cincinnati, OH).

D,L-Selenomethionine (SeMet), selenocystine ($SeCys_2$), and sodium selenite (Na_2SeO_3) were purchased from Aldrich (Milwaukee, WI). *Se*-(methyl)-selenocysteine (MeSeCys) was purchased from Sigma (St Louis, MO). *Se*-(methyl)-selenomethionine (MeSeMet) was synthesized from SeMet and methyl iodide according to Fan et al. (*11*). All the solutions were prepared daily in 18 MΩ cm distilled deionized water (Sybron Barnstead, Boston, MA). Heptafluorobutyric acid (HFBA) was obtained from Sigma, and HPLC-grade methanol (Fisher Scientific, Pittsburgh, PA) was used to prepare mobile phases.

For the determination of total Se, solutions were prepared by appropriate dilutions of 1000 μg mL^{-1} Se(IV) standard solution (Spectrum Chemical, Gardena, CA). For digestion of samples, nitric acid (HNO_3, 68%, supra-pure) and hydrochloric acid (HCl, 36.5%, supra-pure) were purchased from Pharmco (Brookfield, CT).

Instrumentation. An Agilent Technologies (Agilent Technologies; Palo Alto, CA) model 6890 series gas chromatograph (GC) was used and was coupled to an Agilent 7500ce inductively coupled plasma mass spectrometer (ICP−MS) equipped with a reaction/collision cell (Agilent Technologies; Tokyo, Japan). The GC was interfaced to the ICP−MS through a heated transfer line (Agilent Technologies; Tokyo, Japan). A second Agilent 6890 GC was used with a quadrupole mass analyzer. For additional structural conformation, a third Agilent 6890 GC coupled to a Micromass (Manchester, UK) GCT orthogonal time-of-flight mass spectrometer (TOF−MS) was used with standard operating conditions (electron impact ionization at 180 °C, trap current 400 μA, 1 s scan time) using heptacosa(fluorotributylamine) as a calibration and as the lock mass compound (m/z 218.9856 ion). Average mass accuracy usually was less than 0.001 Da. Chromatographic settings are shown in Table 1.

The operating conditions for GC are listed in Table 1. ICP−MS was operated with the collision/reaction cell using the following standard conditions: 1150 W plasma power, 1.0 L Ar min^{-1} carrier gas, 2.0 mL H_2 min^{-1} collision cell gas, quadrupole bias −16 V, octopole bias −18 V, and 100 ms dwell time for each monitored isotope (^{32}S, ^{34}S, ^{77}Se, ^{78}Se, ^{80}Se, and ^{82}Se). A 50:50 Ar/N_2 mixture was used as an optional gas at 5% carrier gas flow.

For total Se analysis, the Agilent Integrated Autosampler was used for continuous flow sample introduction. ICP−MS was operated with the collision/reaction cell using the following standard conditions: 1500 W plasma power, 1.21 L Ar min^{-1} carrier gas, 3.5 mL H_2 min^{-1} collision cell gas, quadrupole bias −16 V, octopole bias −18 V, and 100 ms dwell time for each monitored isotope (^{77}Se, ^{78}Se, ^{80}Se, and ^{82}Se).

Volatiles were sampled from the headspace by using a 75 μm Carboxen/poly(dimethyl siloxane) solid-phase microextraction (SPME) fiber (Supelco; Bellefonte, PA). The fiber was cleaned every day by putting the fiber in the injection port for the entirety of the GC analysis.

Separation of selenium compounds extracted from plant material was carried out using an Agilent 1100 liquid chromatograph (LC) equipped with a binary HPLC pump, an autosampler, a vacuum degasser system, and temperature column compartment. The detector used was the Agilent 7500ce (conditions same as for total Se analysis). An Altech Alltima C_8 column was used for separation (250 mm × 4.6 mm, 5 μm). The mobile phase contained 0.1% heptafluorobutyric acid and 5% methanol (v/v) in water (pH ∼2.5). The flow rate was 1 mL min^{-1} and 50 μL of sample was injected.

Plant Growth. Indian mustard (*Brassica juncea*; Accession no. 173874) seeds were initially obtained from the North Central Regional Plant Introduction Station (Ames, IA) and propagated. Transgenic APS plants were obtained as described by Pilon-Smits et al. (*6*), by expression of the *Arabidopsis thaliana* APS1 gene (encoding ATP sulfurylase) and transgenic SMT plants were obtained as described by LeDuc et al. (*5*), by expression of the *Astragalus bisulcatus* SMT gene (encoding Se-cysteine methyltransferase). Double transgenic APS×SMT plants were obtained by crossing homozygous APS plants with homozygous SMT plants as described in LeDuc et al. (*10*).

GC/ICP−MS Experiments. Indian mustard seeds (WT, SMT, and APS×SMT) were sterilized and sown on media without adding any Se-compounds as described above. After 2 days of cold treatment (4 °C) and 3 days incubation in a growth chamber kept at 25 °C under continuous light, approximately 7−10 seedlings were transferred to each sterile glass bottle (Wheaton) containing 5 mL of liquid media, described above, without the phytagar, capped with aluminum foil, and returned to the 25 °C growth chamber for a 1 day incubation. Following that, some of the bottles were capped with a Teflon seal and crimped shut without addition of Se-compounds (controls). To other bottles, sodium selenate or sodium selenite was added to a final concentration of either 200 or 500 μM, and the bottles were capped with a Teflon seal and then crimped shut. The seedlings were returned to the 25 °C growth chamber for 7−10 days and frozen at −80 °C upon first indications of plant deterioration. The samples were frozen at −20 °C until analysis. Following GC/ICP−MS analysis, the plant material was retrieved from the bottles and dried in a 50 °C oven in order to determine plant dry mass.

TABLE 1. GC Conditions for Molecular and Atomic Mass Spectrometric Analysis

	GC/ICP−MS	GC/MS	GC/TOF−MS
column	DB−5; 30 m × 320 m i.d. × 0.25 m	HP−5; 60 m × 320 m i.d. × 1.0 m	HP−5; 30 m × 320 m i.d. × 0.25 m
purge time	0.5 min	0.5 min	0.5 min
purge flow	5 mL He min^{-1}	5 mL He min^{-1}	1 mL He min^{-1}
injection port temp	250 °C	250 °C	220 °C
injection type	pulsed/splitless	pulsed/splitless	splitless
pulse time	0.5 min	n/a	n/a
pulsed pressure	10 psi	n/a	n/a
temp program	40 °C for 4 min; 40−125 °C @ 15 °C min^{-1} hold 5 min; 125−300 °C @ 35 °C min^{-1} hold 1 min	40 °C for 4 min; 40−125 °C @ 15 °C min^{-1} hold 5 min; 125−300 °C @ 35 °C min^{-1} hold 1 min	50−220 °C @ 10 °C min^{-1}; hold 7.5 min
carrier gas	2.4 mL He min^{-1}	3.0 mL He min^{-1}	1.0 mL He min^{-1}
auxiliary 1 & 2	250 °C	250 °C	250 °C

TABLE 2. Summary of the Se-Containing Volatiles Identified from the Headspace of APS×SMT1 *B. Juncea* Supplemented with Selenite

structure[a]	retention time (min)[b]	relative abundance[c] Se	method of identification[d]
CH_3**Se**CH_3 (110 Da)	5.35	32.1%	RT, MS, EC, AS
CH_3**Se**CH_2CH_3 (124 Da)	7.42	2.1%	MS, EC, AS
CH_3**Se** $CH_2CH_2CH_3$ (138 Da)	10.63	1.4%	MS
CH_3CH_2**Se**$CH_2CH_2CH_3$ (152 Da)	11.83	0.7%	MS, AS
$CH_3CH_2CH_2$**Se**$CH_2CH_2CH_3$ (166 Da)	13.72	0.6%	MS, AS
CH_3**Se**SCH_3 (142 Da)	10.20	10.7%	RT, MS, EC
CH_3**Se**SCH_2CH_3 (156 Da)	12.10	0.7%	MS
CH_3**Se****Se**CH_3 (190 Da)	11.65	51.4%	RT, MS, EC
CH_3**Se**S**Se**CH_3 (222 Da)	19.4[e]	0.1%	RT, MS, EC
CH_3**Se****Se****Se**CH_3 (270 Da)	21.5[e]	<0.1%	MS, EC

[a] Monoisotopic molecular weight based on ^{80}Se. [b] Retention time based on GC/MS conditions from Table 1, unless otherwise noted [c] Based on the molecular ion peak intensity of all volatile Se species present. [d] RT: retention time matching with standards as performed on a 30 and 60 m DB-5 (0.25 μm and 1 μm film thickness respectively) capillary columns. AS: aliphatic series with retention time vs alkyl chain length. MS: electron impact mass spectra obtained from plants and compared to mass spectra of the matching standards. EC: elemental composition and isotope patterns verified with high-resolution GC/TOF−MS. [e] Calculated based on GC/TOF−MS conditions from Table 1.

HPLC−ICP−MS Experiments. Indian mustard seeds (WT, SMT, and APS×SMT) were sterilized by rinsing in 95% ethanol for 30 s, in 0.65% hypochlorite solution for 30 min, and in sterile deionized water for 5 × 10 min, on a rocking platform. Sterilized seeds (25) were sown in a grid pattern in Magenta boxes (Sigma) on half-strength MS medium with 10 g L^{-1} sucrose and 5 g L^{-1} phytagar (Sigma), with or without 100 μM selenite. After 2 days at 4 °C, the boxes were moved to a growth chamber kept at 25 °C under continuous light. On day 7, individual seedlings (both control and selenite-treated) were harvested, washed in running deionized water to remove any Se externally bound to the roots, roots and shoots separated, flash-frozen in liquid nitrogen, and ground to fine powder.

Sample Analysis and Optimization. *GC/ICP−MS.* The headspace of the standards and the plants was sampled by exposing the SPME fiber through the sample vial septum for 10 min and then injecting it into the GC using an injection port designed for SPME (0.75 mm i.d. injection liner). A mixture of Se and S volatile standards was used to optimize the GC separation. Optimized GC/ICP−MS conditions (from Tables 1 and 2) were employed, noting that pulsed/splitless injection was used, to minimize peak broadening of later eluting compounds. After optimization, standards of DMeSe and DMeDSe (ranging from 2.6 to 520 nmol) were used to create a calibration curve based on integrated peak area from the ^{78}Se signal of the ICP−MS. Previous data from Meija et al. (*12*) were also compiled and analyzed to determine the correlation between the concentration ratio of DMeSe to DMeDSe and genetic modification.

HPLC/ICP−MS. According to the proposed Se pathway in *B. juncea*, the precursors to the prevalent volatile compounds DMeSe and DMeDSe are MeSeMet and MeSeCys, respectively (*9*). These amino acids were extracted using a previous protocol by Montes-Bayón et al. (*13*). For the extraction, a ratio of 1 mL of 0.1 M HCl per 75 mg of sample material was used. The mixture was stirred for 24 h and centrifuged at 4000 rpm for 10 min. The supernatant was collected and diluted 1:1 with the mobile phase. The sample (50 μL) was injected and Se levels in eluates were monitored by the ICP−MS.

Results and Discussion

Evaluating Se Compounds in Relationship to Genetic Modification. In 1968 Evans et al. showed that the headspace of a Se-accumulator plant *A. bisulcatus* contained dimethyl diselenide and no traces of dimethyl selenide (*14*). In contrast to that, cabbage plants (Se non-accumulators) contained CH_3SeCH_3 as the only [major] Se-containing volatile (*15*). The marked differences in the observed Se-volatile profiles served as a tool to distinguish between the accumulator and non-accumulator plants even at the early stages of research in this area (*16*). In this study we expand on the use of the volatile Se profiles to follow the genetic modifications that increase Se accumulation thus making *B. juncea* more apt for Se phytoremediation. Since the genetic modifications of wild type *B. juncea* converts it from a Se-accumulator plant into a Se-hyper-accumulator plant, the resulting changes can be monitored from the headspace Se volatiles (see Figure 1).

Altering the expression of enzymes along the selenium metabolism pathway will, in most cases, lead to a detectable change in the relative amounts of different Se metabolites. As shown in Figure 1, the modified pathway versus the unmodified pathway can result in the following trends: (1) by modifying the pathway to increase SMT expression, more

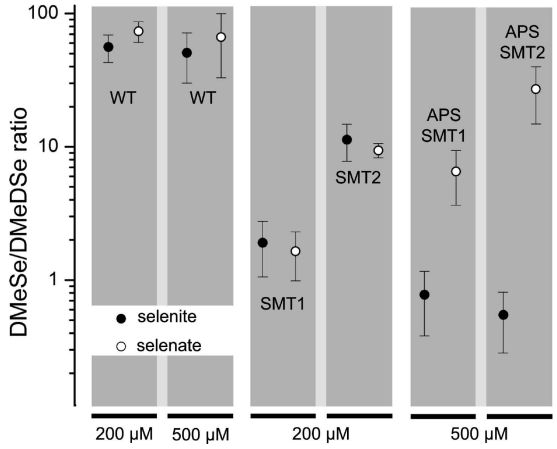

FIGURE 2. Average DMeSe/DMeDSe ratios for WT, SMT, and APS×SMT *B. juncea* seedling cultures supplemented with selenate or selenite.

Se is routed toward MeSeCys and then volatilized as dimethyl diselenide (DMeDSe), (2) if the *B. juncea* is left unmodified, the Se pathway should result in the formation of more MeSeMet that will ultimately be volatilized as dimethyl selenide (DMeSe). As such, Se-amino acids and Se-volatiles can be used as proxies to determine the overall effects of the genetic modification attempted. Accordingly, WT samples should have higher MeSeMet/MeSeCys and DMeSe/DMeDSe ratios whereas GM types will exhibit lower ratios, with the decrease in the ratio proportional to the extent of the enzyme expression. This also varies depending on the position in the genome—where the transgene integrates.

DMeSe and DMeDSe in Relationship to Genetic Modification. The DMeSe/DMeDSe ratios of the WT, SMT, and APS×SMT *B. juncea* samples were analyzed using GC/ICP–MS and the average results for each sample type are shown in Figure 2. For the WT cultures the DMeSe/DMeDSe ratio is independent of supplementation type; neither Se enrichment form nor concentration significantly altered the ratio

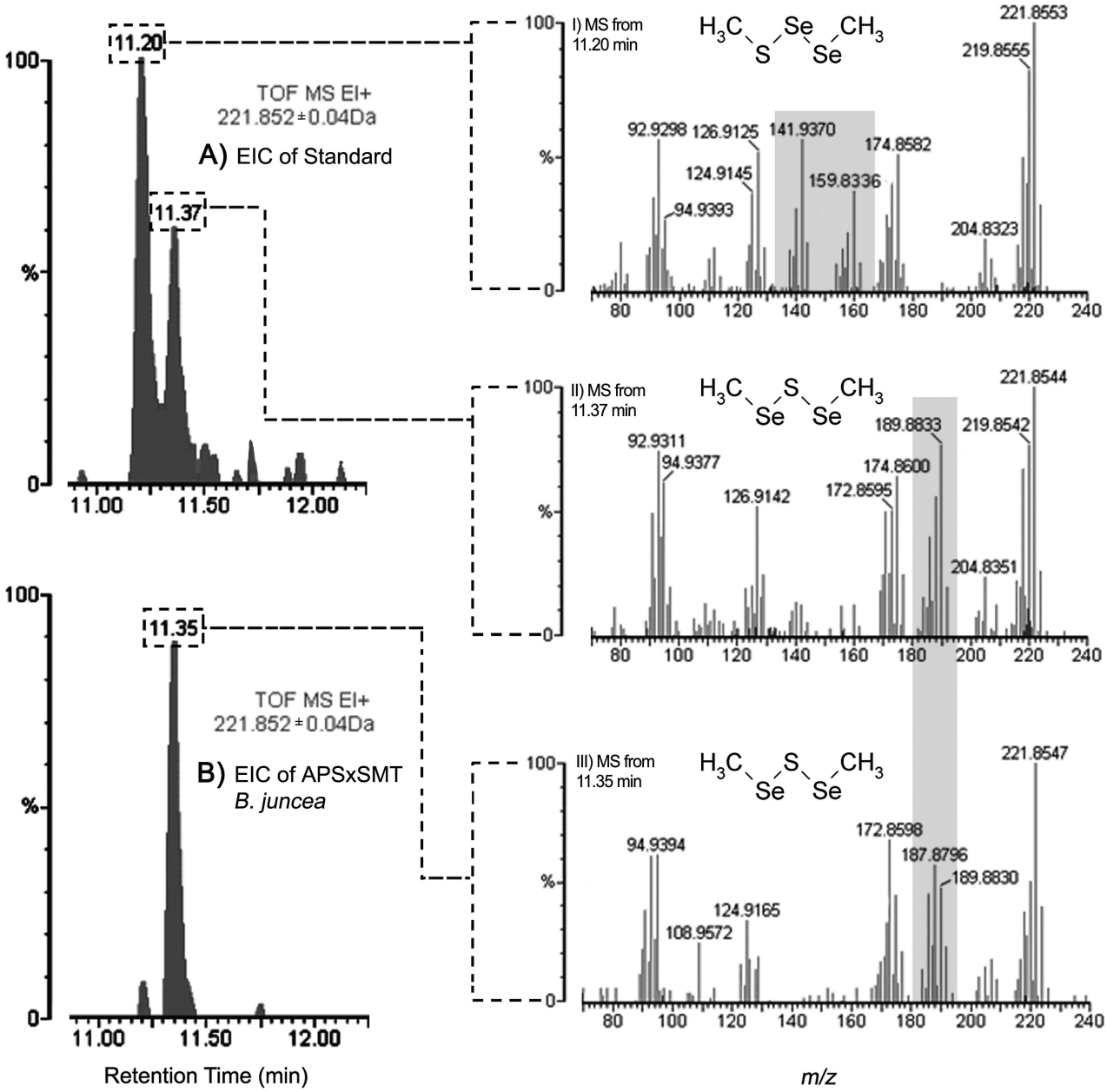

FIGURE 3. Extracted ion chromatogram from GC/TOF–MS of 221.852 of standard mixture including $CH_3SSeSeCH_3$ and $CH_3SeSSeCH_3$ (A) and APS×SMT1 *B. juncea* headspace sample (B). Right side shows the mass spectra of $CH_3SSeSeCH_3$ (11.20 min) and $CH_3SeSSeCH_3$ (11.37 min) standards and $CH_3SeSSeCH_3$ mass spectrum from *B. juncea* headspace (11.35 min).

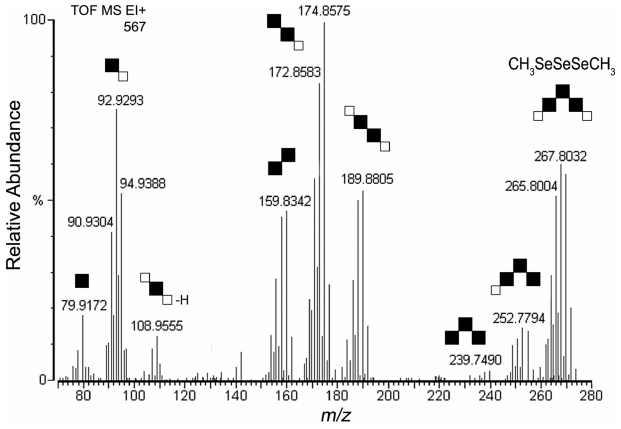

FIGURE 4. Mass spectrum of dimethyl triselenide, $CH_3SeSeSeCH_3$, from GC/TOF−MS analysis of APS×SMT1 *B. juncea* supplemented with selenite.

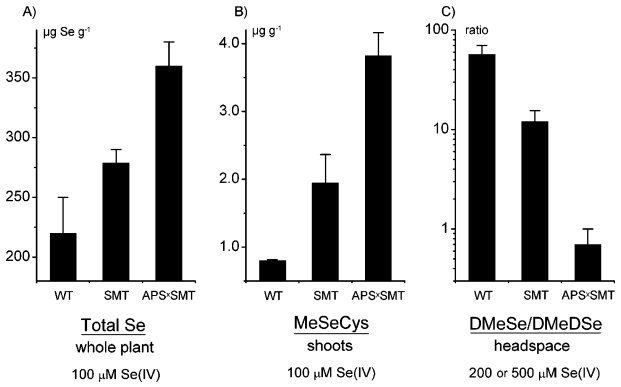

FIGURE 5. Overall summary of Se uptake and metabolism related to the various genetic modification (WT, SMT, APS×SMT) of *B. juncea* treated with selenite. The bar graphs are (A) total Se content (μg Se g^{-1} of sample (dry weight)) in each plant type, (B) total MeSeCys content (μg g^{-1} of sample) in plant extract from the shoot material for each plant type, and (C) DMeSe/DMeDSe ratio from the headspace of each plant type.

of DMeSe/DMeDSe. Though cultures SMT1 and SMT2 were both SMT modified, when grown under the same supplementation conditions, differences in DMeSe/DMeDSe ratio were exhibited. This is because the transgene, which is inserted randomly, could be in a more advantageous location in the SMT1 culture than the SMT2 culture, thus explaining the lower ratio for SMT1 when supplemented with selenite and selenate. This also agrees with earlier findings that SMT expression at the mRNA and protein level is lower in the SMT2 plants than in the SMT1 (*5*).

Another interesting point is that in the SMT1 and SMT2 cultures DMeSe/DMeDSe ratios are maintained whether supplemented with selenite or selenate. This is likely due to the fact that the SMT modification only affects the methylation of SeCys and does not directly affect the reduction of selenate to selenite, thus making its effects independent of the upstream selenium form added.

When the plant was doubly genetically modified (APS× SMT) and supplemented with selenite, the DMeSe/DMeDSe ratios were even lower than those of the SMT modified cultures. This trend is rather unexpected, since the APS modification should affect only the uptake of selenate, not selenite. However, since ATP sulfurylase is an early enzyme in the sulfur/selenium assimilation pathway this remains an open question as the exact mechanism of the APS modification is not fully understood. It is also possible that the SMT modification is simply in a more advantageous location in the both APS×SMT cultures than in the SMT cultures.

Note that only APS×SMT plant cultures show difference between the DMeSe/DMeDSe ratios when supplemented with selenate vs selenite. This is expected as overexpression with APS directly discriminates between the selenate and selenite: in our opinion, a rather elegant way of detecting the presence of the APS gene.

Though it was established that DMeSe/DMeDSe ratios for both WT and SMT overexpressing plants were independent of selenium source (selenate or selenite), the overall quantities of the DMeSe and DMeDSe produced were drastically different. Approximately 10 times more DMeSe and DMeDSe (normalized to sample weight) is produced in all plant types when the plants were supplemented with selenite than with selenate (*8*).

Minor Se and S Headspace Volatiles. It is well documented that there are two main selenium-containing volatiles emitted from Se-accumulating plants: dimethyl selenide, CH_3SeCH_3, and dimethyl diselenide, $CH_3SeSeCH_3$ (*17*, *18*). A third major Se volatile that is found in the headspace of *B. juncea* plants is dimethyl selenosulfenate, CH_3SeSCH_3 (*12*, *20*−*22*). In this study multiple steps were taken to identify as many of the minor Se compounds as possible. The first step toward this is the GC/ICP−MS retention time comparison with available alkyl selenide standards. In the second step, selected *B. juncea* samples were reanalyzed using GC/MS and high-resolution GC/TOF−MS to gather structural information and confirm the identities of those tentatively established by GC/ICP−MS. Analysis of selenium compounds is less selective and sensitive when using GC/MS, but the distinct Se isotope pattern makes interpreting the mass spectra easier.

A second basis for low-intensity peak identification using GC/MS is retention time when applied to an aliphatic series. When using a constant temperature ramp, the retention time of unbranched dialkyl selenides increases linearly with increasing alkyl length. When using these data in combination with molecular ion peaks derived from mass spectra, Se isotope pattern, and fragmentation patterns, relatively confident identifications of several dialkyl selenides could be made. Additional information is presented in Table 2 while the compound eluting at 10.63 min deserves more discussion. The molecular ion of this species is present at $m/z = 138$ (^{80}Se) and initially was thought to be EtSeEt; however, the commercial standard of EtSeEt elutes at 9.21 min under the same conditions. Therefore, this was tentatively identified as PrSeMe (further fragment ion analysis was not possible due to its low levels).

The third step of the analysis was to use the exact mass capability and the higher sensitivity of the GC/TOF−MS to further confirm the structures of the minor Se volatiles. The most intense of the minor Se-volatiles is ethylmethyl selenide, with the measured exact mass of 123.981_1 Da. Mass spectrum of this compound shows the presence of CH_3SeH^+ (measured at 95.9490 Da, ^{80}Se) and $CH_3CH_2Se^+$ (measured at 108.9565 Da, ^{80}Se). By searching the GC/TOF−MS for exact masses of various dimethyl polychalcogenides, two trichalcogenides were observed. The first compound was of the molecular formula $C_2H_6Se_2S$ (measured exact mass 221.853_1 Da, ^{80}Se). Such elemental composition corresponds to two isomers, the asymmetric MeSeSeSMe and the symmetric MeSeSSeMe. Since both synthesized isomers are available (*19*) the retention time and mass spectral comparison of these two compounds allowed us to establish the presence of MeSeSSeMe in the headspace of the genetically modified *B. juncea* plants (see Figure 3). Among the mass spectral differences of the two isomers, most notable is the loss of the central chalcogen atom (S for MeSeSSeMe and Se for MeSeSeSMe) leading to the abundant m/z 190 cluster for MeSeSSeMe and m/z 142 cluster for MeSeSeSMe.

A second trichalcogenide was discovered at the exact mass of 267.803$_2$ Da. This mass (along with the isotopic pattern) corresponds to $C_2H_6Se_3$. As shown previously, the most abundant m/z peak is not representative of $^{80}Se^{80}Se^{80}Se$, rather it is from $^{78}Se^{80}Se^{80}Se$ (*19*). The mass spectral fragmentation and the retention time comparisons agree with the dimethyl triselenide, MeSeSeSeMe (Figure 4). In recent work, other trichalcogenides such as MeSSSeMe, MeSSeSMe, and MeSeSeSMe were reported (*23*−*24*). To the best of our knowledge, this is the first report of MeSeSSeMe and MeSeSeSeMe in plants.

Se Amino Acids in Relationship to Genetic Modification. From all the Se-containing metabolites, to date only MeSeCys has been used as a proxy to *B. juncea* genetic modifications (*13*). While the MeSeCys levels in leaves remain unchanged with the extent of genetic modification (data not shown), the amount of MeSeCys in the shoot material exhibits strong correlation. In the wild type plants MeSeCys represents 23−41% of the total extracted Se while in the SMT and APS×SMT cultures MeSeCys represents 45−51% and 60−71% of the total Se extracted, respectively. The increase in MeSeCys shoot content from wild type to APS×SMT cultures is also in good agreement with other recent findings (*10*). As previously discussed in the Se volatile section, the increase in MeSeCys production from SMT to APS×SMT is unexpected and further supports the conclusion that the APS and SMT interaction is not as simple as that portrayed in Figure 1.

There have been reports of MeSeMet presence in WT *B. juncea* shoots when supplemented with selenite (*25*) or SeMet (*26*). When supplemented with selenite, MeSeMet comprises ∼10% of the total Se, while when supplemented with SeMet, it makes up ∼60% of the total Se. In our study the plants were supplemented with selenite, and MeSeMet (HPLC/ICP−MS analyses) represented less than 6% of the total extracted Se in the WT plants. Furthermore, there was no noticeable change in the MeSeMet content from WT to GM types. Therefore, it seems that MeSeMet levels are inadequate to follow the genetic modifications of selenium.

For comparison, the total selenium content of the various plants determined in previous experiments (*5*, *6*) is also assembled in Figure 5 along with total MeSeCys content and DMeSe/DMeDSe ratios.

In this study we have shown that the ratio of the two major headspace Se-volatiles, DMeSe and DMeDSe, seems a better proxy to the genetic modification than conventional total selenium content and plant tissue levels of Se-amino acids, such as MeSeMet and MeSeCys. Also, as seen in Figure 5, the DMeSe/DMeDSe ratios are more sensitive to change between modifications than total selenium and MeSeCys levels. Besides, the complementary use of GC/ICP−MS and GC/TOF−MS proved very useful in identification and confirmation of commonly neglected minor selenium volatile present in the headspace of *B. juncea*. As a result, previously unreported minor Se-containing volatiles, such as MeSeEt, MeSeSSeMe, and MeSeSeSeMe were discovered.

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