JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

ARTICLE

pubs.acs.org/JAFC

Biofortification of Tomato (*Solanum lycopersicum*) Fruit with the Anticancer Compound Methylselenocysteine Using a Selenocysteine Methyltransferase from a Selenium Hyperaccumulator

David A. Brummell,[*,†] Lyn M. Watson,[†] Ranjith Pathirana,[†] Nigel I. Joyce,[‡] Phillip J. West,[†] Donald A. Hunter,[†] and Marian J. McKenzie[†]

[†]The New Zealand Institute for Plant & Food Research Limited, Food Industry Science Centre, Private Bag 11600, Palmerston North 4442, New Zealand

[‡]The New Zealand Institute for Plant & Food Research Limited, Canterbury Agriculture and Science Centre, Private Bag 4704, Christchurch 8140, New Zealand

ABSTRACT: Methylselenocysteine (MeSeCys) is an amino acid derivative that possesses potent anticancer activity in animals. Plants that can tolerate growth on soils with high Se content, known as Se hyperaccumulators, do so by converting inorganic Se to MeSeCys by the enzyme selenocysteine methyltransferase (SMT). A cDNA encoding the SMT from a Se hyperaccumulator was overexpressed in tomato (*Solanum lycopersicum*). Transgenic plants were provided with selenite or selenate to the roots during fruit development, and liquid chromatography−mass spectrometry was used to show that MeSeCys accumulated in the fruit but not in the leaves. Depending on the transgenic line and Se treatment, up to 16% of the total Se in the fruit was present as MeSeCys. MeSeCys was produced more effectively from selenite on a percentage conversion basis, but greater accumulation of MeSeCys could be achieved from selenate due to its better translocation from the roots. MeSeCys was heat stable and survived processing of the fruit to tomato juice.

KEYWORDS: Methylselenocysteine, selenocysteine methyltransferase, tomato, *Solanum lycopersicum*, anticancer compounds, transgene expression

INTRODUCTION

Selenium (Se) is a micronutrient essential for human health due to the presence of 25 selenoproteins in the human proteome.[1] For example, Se is an essential component of glutathione peroxidase, an enzyme that protects cells from oxidative damage.[2] Se is ingested in food, but in many countries (including New Zealand, Scandinavia, and parts of China, Russia, Eastern Europe, Africa, and North America), large proportions of the population may be consuming inadequate Se due to the low concentration of Se in the soil in which crops are grown.[3,4] Supplementation of soils using high-Se fertilizers has been used to increase the Se content of crops and pastures in several countries.[4−6]

Symptoms of severe Se deficiency in humans include a compromised immune system, cardiovascular disease, hypothyroidism, and bone and joint diseases in children.[3,4,7] Symptoms of Se deficiency are rare in the western world, even though many people do not meet the recommended daily intake (RDI) for Se of 55 μg. However, an increased consumption of Se is beneficial, since supranutritional intake of Se above the RDI has been shown to have anticarcinogenic effects. In human trials, intakes of Se of ∼200 μg day^{-1} reduced cancers of the stomach, esophagus, liver, and colon-rectum and reduced total deaths due to cancer.[8,9] Although both inorganic and organic forms of Se appear to possess anticancer activity, particular organic Se compounds are more effective at suppressing the development of precancerous lesions. Se compounds from garlic and broccoli had greater cancer preventative effects than the Se content alone would predict.[10−12] Species of the genera *Allium* and *Brassica*, such as garlic and broccoli, manufacture the methylated amino acid derivative methylselenocysteine (MeSeCys) from inorganic Se, and it appears that MeSeCys possesses potent antitumorigenic activity.[13−16]

MeSeCys is produced naturally by various plant species when grown on seleniferous soils. Some plants, known as Se hyperaccumulators, tolerate the uptake of large amounts of Se from Se-rich soil by converting the majority of the toxic inorganic Se into MeSeCys, which is not incorporated into proteins and can be safely accumulated.[17] The most bioavailable form of Se in soils is selenate, which is taken up by plants using enzymes of the sulfur assimilation pathway since most of the transporters and enzymes involved in uptake, translocation, and assimilation cannot discriminate between the S and the Se forms of their substrates.[18] The first step of assimilation of selenate into the organic pathway is carried out by ATP sulfurylase (ATPS), which may be a rate-limiting step in some species.[19] Selenate is eventually reduced to selenite and converted to selenocysteine (SeCys).[18] In Se hyperaccumulators, alliums and brassicas SeCys is methylated to MeSeCys by the action of selenocysteine methyltransferase (SMT).[17]

The *SMT* gene has been cloned from the Se hyperaccumulator *Astragalus bisulcatus*,[20] and introduced into *Arabidopsis thaliana* and *Brassica juncea* (Indian mustard) for the purpose of phytoremediation of Se-contaminated soils.[21] However, studies aimed at nutritional improvement by accumulation of MeSeCys in food

Received: June 29, 2011
Accepted: September 9, 2011
Revised: August 28, 2011
Published: September 26, 2011

ACS Publications © 2011 American Chemical Society

crops have not so far been attempted. We have shown that the *A. bisulcatus AbSMT* gene will also function in the model Solanaceous species *Nicotiana tabacum* (tobacco),[22] suggesting that the gene can be used to engineer the production of MeSeCys in related crop species. Here, we show that expression of *AbSMT* in transgenic tomato enables production of MeSeCys from selenate or selenite and that biofortification of a food crop with the anticancer compound MeSeCys is a possibility.

MATERIALS AND METHODS

Generation of *SMT*-Overexpressing Plants. The plasmids used for plant transformation were the same as those previously described.[22] Plasmid 1007 (*35S:AbSMT:nos*) contained a transgene derived from the SMT gene *SMT1* of the selenium hyperaccumulator *A. bisulcatus*, under the transcriptional control of the constitutive cauliflower mosaic virus 35S promoter. Plasmid 1008 (*35S:AbSMT:nos; UBQ10:BoATPS1:nos*) contained two transgenes, the *35S:AbSMT:nos* transgene described above, plus a transgene consisting of the *Brassica oleracea* ATP sulphurylase gene *BoATPS1* under the transcriptional control of the constitutive *UBQ10* promoter from *A. thaliana*. These constructs in the binary vector pART27,[23] which confers resistance to kanamycin, were introduced into *Agrobacterium tumefaciens* strain LBA-4404 by electroporation.

Cotyledons from aseptically grown 8 day old seedlings of tomato (*Solanum lycopersicum* L.) cultivar 'Moneymaker' were submerged for 5 min in cultures of *Agrobacterium* harboring the constructs above, then cocultivated on solid basal medium (BM) consisting of modified Murashige and Skoog salts and B5 vitamins as described,[24] supplemented with 3% (w/v) sucrose, 0.05 mg L^{-1} indole-3-acetic acid, 1 mg L^{-1} zeatin, and 100 μM acetosyringone. Explants were maintained under light (30 μmol m^{-2} s^{-1}, 16 h photoperiod) at 24 °C for 48 h and transferred to termination regeneration medium [solid BM supplemented with 3% (w/v) sucrose, 0.05 mg L^{-1} indole-3-acetic acid, 0.5 mg L^{-1} zeatin, and 500 mg L^{-1} cefotaxime] for 48 h. The explants were then transferred to termination regeneration medium supplemented with 50 mg L^{-1} kanamycin and maintained under light with transfer to fresh media every 3 weeks until fresh shoots were formed from callus ends. A single healthy shoot per explant was transferred to rooting media (BM with 500 mg L^{-1} cefotaxime and 50 mg L^{-1} kanamycin). After hardening off, plants in 16 L size plastic bags containing potting mix were grown to maturity in a greenhouse during spring and summer under ambient lighting, with a maximum daytime temperature of 27 °C and a minimum nighttime temperature of 16 °C.

Characterization of SMT-Overexpressing Plants. Plants transformed with constructs 1007, 1008, and empty vector pART27, together with wild-type untransformed plants that had been through similar tissue culture procedures (T_1 generation), were allowed to self-fertilize, and red ripe fruits were collected.

Seeds from the primary transformants (96 per plant) were germinated in potting mix in 2.5 cm × 2.5 cm cells of a potting tray, and when the seedlings reached the two-leaf stage, they were sprayed twice with 25 mg mL^{-1} kanamycin. Two weeks later, the ratio of survivor to dying plants was determined, and lines with a 3:1 ratio of alive:dead were selected. This segregation ratio indicated insertion of functional T-DNA at a single locus. Several survivors of the kanamycin spraying (T_2 generation) were transferred to plastic bags of potting mix and grown to maturity. To separate hemizygous plants from those that were homozygous for the T-DNA, 48 seedlings from each of the T_2 generation plants (i.e., T_3 generation) were grown, together with 12 wild-type per line, and treated with kanamycin as above. Lines of T_3 seedlings where all of the control wild-type seedlings had died yet 100% of the transgenic seedlings survived showed that the T_2 parent was homozygous.

One homozygous line transformed with plasmid 1007 (line 1007-11) and two homozygous lines transformed with plasmid 1008 (lines 1008-3 and 1008-8) were selected, and two or four plants of each were grown in a greenhouse together with wild-type controls.

RNA was prepared using the hot borate method,[25] separated by electrophoresis in 1.2% (w/v) agarose and formaldehyde denaturing gels, then blotted to Amersham Hybond-XL nylon membrane (GE Healthcare, Little Chalfont, United Kingdom) overnight, and immobilized by ultraviolet irradiation. cDNA probes were synthesized from templates consisting of the entire open reading frames of *AbSMT* and *BoATPS1* using [α-^{32}P]dATP and the Klenow fragment of DNA polymerase I. The labeled probes were hybridized with the RNA gel blots in Church and Gilbert[26] buffer at 65 °C overnight. Gel blots were washed four times for 30 min in 0.1 X SSC/0.1% SDS at 65 °C and then exposed to MS X-ray film (Kodak, Rochester, NY) or to phosphorimager plates that were scanned with an FLA-5100 fluorescent scanner (Fujifilm, Tokyo, Japan). ATPS activity assays in chloroplast preparations from freshly harvested young leaves and SMT activity assays in total protein extracts or chloroplast preparations from freshly harvested young leaves were carried out as described.[22]

Selenium Treatments and Plant Material. Selenium treatments were begun when small fruit had set on multiple trusses of mature plants. Aliquots (20 mL) of stock solutions of sodium selenite or sodium selenate at the concentrations indicated in the tables were added to the soil twice per week for 10 weeks (20 applications in total). Plants were watered twice per day for 1 min using an automatic watering system, with the selenium application being made shortly after the first watering. Fully expanded leaves and three red ripe fruit per replicate were harvested 3 days after the final selenium application. Leaves were frozen in liquid nitrogen then freeze-dried. Fruit were separated into pericarp, which was pooled, frozen in liquid nitrogen, and freeze-dried, and locules (discarded) and seeds (retained for later use). Leaf and pericarp weights were recorded before and after freeze-drying to allow data obtained on a dry weight (DW) basis to be calculated on a fresh weight (FW) basis. Freeze-dried leaf and fruit pericarp samples were powdered using a coffee grinder and then analyzed for selenium content and speciation as below.

Preparation of Tomato Juice. Red ripe fruits were harvested from plants that had been watered with solutions of Se as above. Fruits free from surface blemishes (12−15 fruit, totaling 1.6−1.9 kg) were washed, dried, and then diced into cubes of ~1 cm. For each line, all of the tissue and juice was collected into a preweighed dish, covered with plastic film, and subjected to a hot break in a commercial microwave (650 W output), heating at full power for 5 min, stirring the contents, and then heating for another 3 min. The dish and contents were cooled on ice and were then made back to the original weight with distilled water. The cooked tissue was sieved to remove seeds and skins, which were discarded, and the pulp was homogenized in a Waring blender at low power using two bursts of 5 s each. A sample of the homogenate was degassed under vacuum, and aliquots of 8 g were dispensed into tubes, freeze-dried, and weighed. The dried powder was assayed for contents of total Se and MeSeCys.

Determination of Se and MeSeCys. The total Se concentration in dried samples (on a mg kg^{-1} DW basis) was determined by a commercial company (Gribbles Veterinary, Hamilton, New Zealand), using the method of Watkinson.[27] For determination of MeSeCys concentration, freeze-dried powder (~200 mg) was shaken with 5 mL of chloroform:methanol (3:7, v/v), and then, 5 mL of 10 mM HCl was added. Extraction into the aqueous phase was assisted by using vortex/ultrasound agitation for 5 min and end-over-end shaking for 2 h. After centrifugation at 2931*g* for 10 min, the aqueous phase was transferred to a 20 mL vial, and the extraction step was repeated with 2 mL of 10 mM HCl. Extracts were combined and concentrated under vacuum to approximately 1 mL and transferred into a preweighed 1.7 mL microvial, and the volume was recorded by mass (g). After centrifugation at

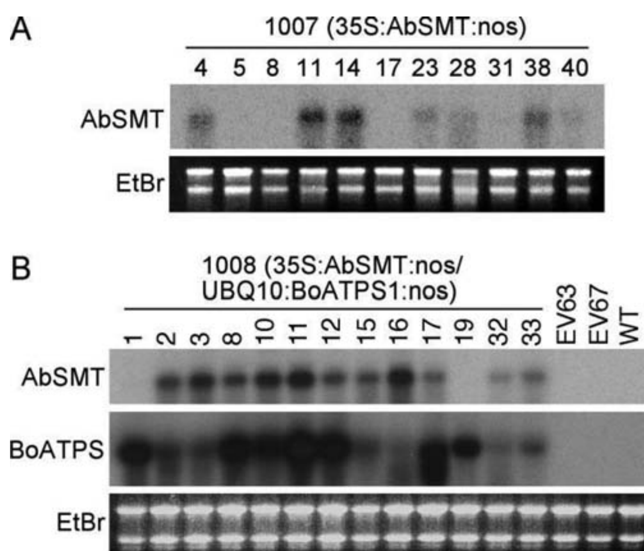

Figure 1. Screening of primary transformant (T_1) 1007 and 1008 populations for expression of transgenes. RNA gel blots show (A) mRNA accumulation of an *AbSMT* transgene in 1007 lines (20 μg total RNA per lane) and (B) mRNA accumulation of both *AbSMT* and *BoATPS1* transgenes in 1008 lines (5 μg total RNA per lane). EV, empty vector control transformants; WT, wild-type; and EtBr, ethidium bromide staining of RNA.

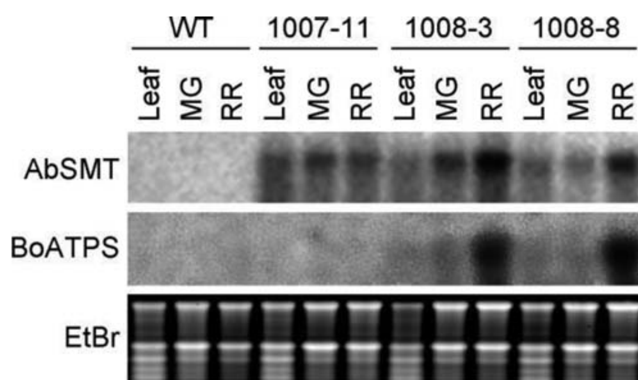

Figure 2. Transgene mRNA abundance in different tissues of homozygous (T_2) transgenic lines. Primary transformants were allowed to self-fertilize, and in the first segregating population, plants that were homozygous for the T-DNA were identified by segregation analysis of their progeny. The RNA gel blot (5 μg total RNA per lane) was hybridized with an *AbSMT* probe, stripped, and then rehybridized with a *BoATPS* probe. MG, mature green fruit; RR, red ripe fruit; and EtBr, ethidium bromide staining of RNA.

20800*g* for 10 min, an aliquot was transferred to a glass vial for liquid chromatography−mass spectrometry (LCMS) analysis.

The LC system consisted of a Thermo Electron Corp. (San Jose, CA) Finnigan Surveyor MS pump, Finnigan MicroAS autosampler, and a ThermaSphere TS-130 column heater (Phenomenex, Torrance, CA). A 2 μL aliquot of each prepared extract was separated with a mobile phase flowing at 100 μL min^{-1} consisting of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B) by reverse phase chromatography (Aqua guard cartridge 4 mm × 2 mm, 10 μ, and Synergi-HydroRP C18, 4 μ, 80 Å, 250 mm × 2.1 mm, Phenomenex) maintained at 30 °C. A gradient was applied from 100% A, held for 6 min, to 10% B at 10 min, 70% B at 20 min, then equilibrated for 10 min at the start conditions. The eluent was analyzed by atmospheric pressure ionization mass spectrometry (API-MS) (LTQ, 2D linear ion-trap, Thermo-Finnigan, San Jose, CA) with electrospray ionization in the positive mode. Data were acquired by selective reaction monitoring by selecting the parent mass $[M + H]^+$, m/z 184, and daughter ion 167 for MeSeCys with an applied collision energy of 35 units. Quantification was based on integration of the area under the curve produced by plotting selected daughter ions with the aid of external standards (0.1−10 μg mL^{-1}). LCMS data were processed with the aid of Xcalibar 2.0.5 software (Thermo Electron Corp., Waltham, MA). Reference standard Se-methylselenocysteine was purchased from Sigma (St. Louis, MO).

RESULTS

Molecular and Biochemical Analysis of Transformants. Two populations of transgenic tomato plants were created. The first population, plants with the prefix 1007, constitutively overexpressed the *AbSMT* gene from *A. bisulcatus*. Eleven plants were confirmed by polymerase chain reaction (PCR) to possess both the transgene and the selectable marker gene (data not shown), and ripe fruits from these plants were analyzed for *AbSMT* mRNA abundance (Figure 1A). Plants 1007-11 and 1007-14 exhibited high mRNA accumulation of the *AbSMT* transgene, and five other plants showed moderate accumulation. No transcripts cross-hybridizing with the *AbSMT* probe were detected in control wild-type or empty vector transformants (Figure 1B). The second population, plants with prefix 1008, overexpressed both the *AbSMT* gene and a *BoATPS1* gene from broccoli as independent transgenes within the same T-DNA. For the 1008 population, fruits from 13 plants confirmed by PCR to possess both transgenes and the selectable marker gene (data not shown) were analyzed for *AbSMT* and *BoATPS1* mRNA abundance (Figure 1B). No correlation in the accumulation of mRNA from the two transgenes was observed, with plants exhibiting high mRNA abundance of one transgene but not the other (1008-1, 1008-16), and either low abundance (1008-32) or high abundance (1008-10) of both transgenes. Some of the most promising lines (1008-2, 1008-10, and 1008-11) did not produce seeds.

Several plants showing high overexpression of the transgenes were selected for further study. Segregation analysis (data not shown) was used to determine that lines 1007-11, 1008-3, and 1008-8 possessed a single expressing locus of the selectable marker gene, as demonstrated by resistance to kanamycin.

For the three above-mentioned lines, plants in the succeeding T_2 generation were identified as homozygous for the selectable marker gene using segregation analysis of the T_3 generation. Leaf, green fruit, and red fruit from homozygous T_2 plants of these lines were analyzed for the abundance of transgene mRNA (Figure 2). *AbSMT* mRNA accumulation, which was under the control of the constitutive 35S promoter, was strong in all of the tissues of the transgenic lines examined and was particularly high in red ripe fruit of the two 1008 lines. However, the accumulation of mRNA from the *BoATPS1* transgene was less constitutive, even though it was under the control of the nominally constitutive *Arabidopsis UBQ10* promoter. In the two 1008 lines, *BoATPS1* mRNA was present at high abundance in red ripe fruit and at detectable but low abundance in leaf and mature green fruit. As would be expected, *BoATPS1* mRNA was not present in tissues from line 1007, and neither probe showed any cross-hybridization with mRNAs from wild-type tissues.

Plastids were prepared from young leaves of mature plants and examined for ATPS activity (Figure 3). ATPS activity in

wild-type tomato was substantial and was increased by almost 2-fold in both 1008-3 and 1008-8 transgenic lines. Line 1007-11, which did not possess the *BoATPS1* transgene, had an ATPS activity similar to wild type. Repeated attempts to assay SMT activity in leaves and fruit in vitro using a thin-layer chromatography method were unsuccessful, despite the constructs being the same as those that produced SMT activity in vitro when overexpressed in tobacco,[22] the *AbSMT* transgene being clearly expressed (Figures 1 and 2) and the reaction product MeSeCys being present (see below). This suggests that some endogenous inhibitor(s) may be present in tomato that interferes with activity in tissue homogenates.

Accumulation of Se and MeSeCys in Fruit and Leaves. Mature plants that were beginning to set fruit were watered with aliquots of solutions of sodium selenite or sodium selenate twice per week for 10 weeks, during which time fruit developed and ripened on numerous trusses. A comparison of total Se accumulated in fruit from the first, second, and sixth trusses found differences of up to 2-fold between trusses (data not shown), but differences were generally relatively low within a truss (see standard deviations in Table 1). To reduce variability, at the end of the 10 week treatment period, replicate fruit batches were collected only from the sixth truss, plus samples of expanded leaves.

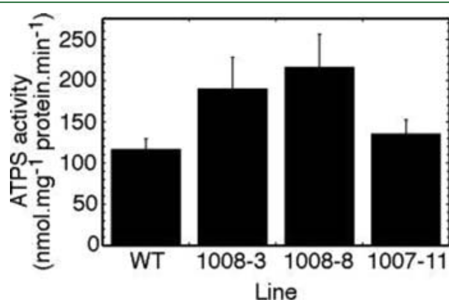

Figure 3. ATPS activity of transformed lines. Plastids were prepared from leaves of lines overexpressing *BoATPS1* (1008 lines) or controls (WT, 1007-11) and ATPS activity determined as nmol of adenosine 5′-phosphosulfate produced mg^{-1} protein min^{-1}. Error bars denote SD between replicate determinations ($n = 2$).

Se provided as selenite may more easily enter the organic assimilation pathway than does selenate,[18] but in wild-type plants, selenite provided to the roots was inefficiently transported to leaves or fruit (Table 1). Selenate was much more effective than selenite as a source of Se, with greater concentrations accumulating in both leaves and fruit on a DW basis. The expression of an *AbSMT* transgene in line 1007-11 increased the accumulation of Se in both fruit and leaves, particularly in the latter, with the fold increase varying with tissue and with the form and concentration of Se applied. MeSeCys accumulated in fruit but not in leaves (Table 1), despite the expression of the *AbSMT* transgene in both tissues (Figure 2). Whether this is due to a lack of MeSeCys production in the leaf or to transport of MeSeCys out of the leaves and into sinks such as fruit is not clear. Low concentrations of MeSeCys were present in fruit from wild-type plants provided with the higher concentration of selenite or with either concentration of selenate. The accumulation of MeSeCys in fruit from overexpressing line 1007-11 was substantial when plants had been watered with aliquots of 5 mM selenite or selenate. Although larger concentrations of MeSeCys resulted in fruit after watering plants with selenate rather than with selenite, the greater accumulation of total Se meant that the efficiency of conversion of inorganic Se to MeSeCys was less from selenate (10.9%) than from selenite (16.4%).

Table 1. Accumulation of Se and MeSeCys in the Fruit and Leaves of Plants Overexpressing an *A. bisulcatus SMT* Gene[a]

tissue	line	selenium salt	concn applied (mM)	Se concn (mg kg^{-1} DW)	MeSeCys concn (mg kg^{-1} DW)[b]	conversion (%)[c]
fruit	WT		0	0.3 ± 0.3	ND	0
		selenite	0.5	0.5 ± 0.2	ND	0
			5	5.0 ± 0.1	0.9 ± 0.5	7.4
		selenate	0.5	9.5 ± 1.9	0.8 ± 0.5	3.8
			5	60.5 ± 7.4	2.1 ± 0.4	1.5
	1007-11		0	0.7 ± 0.4	ND	0
		selenite	0.5	7.3 ± 1.0	1.5 ± 0.1	9.0
			5	54.0 ± 20.7	20.4 ± 8.5	16.4
		selenate	0.5	16.2 ± 1.9	2.9 ± 0.7	7.7
			5	137.5 ± 18.0	34.7 ± 3.5	10.9
leaf	WT		0	0.3 ± 0.1	ND	0
		selenite	0.5	0.8 ± 0.2	ND	0
			5	6.9 ± 0.4	ND	0
		selenate	0.5	96.5 ± 12.2	ND	0
			5	595.5 ± 48.8	ND	0
	1007-11		0	0.3 ± 0	ND	0
		selenite	0.5	52.9 ± 15.0	ND	0
			5	134.0 ± 0.3	ND	0
		selenate	0.5	293.3 ± 7.3	ND	0
			5	783.2 ± 60.5	ND	0

[a] Sodium selenite or sodium selenate (20 mL per application) at the indicated concentration was added to the soil twice per week for 10 weeks, while fruit were developing. Data are expressed on a DW basis and are means ± SDs (n = 4−6 for fruit, 2 for leaf). Spiking experiments indicated that MeSeCys recovery was 42−56%. A correcting factor has not been applied. [b] ND, not detected (at detection limit of ∼0.3 mg kg^{-1} DW). [c] Calculated using only the Se component (MW = 79) of MeSeCys (MW = 182).

Table 2. Accumulation of Se and MeSeCys in the Fruit and Leaves of Plants Overexpressing an *A. bisulcatus SMT* Gene and a Broccoli *ATPS1* Gene[a]

tissue	line	selenium salt	concn applied (mM)	Se concn (mg kg^{-1} DW)	MeSeCys concn (mg kg^{-1} DW)[c]	conversion (%)[d]
fruit	WT	selenite	5	1 ± 0	ND	0
		selenate	5	68 ± 13	6.0 ± 4.3	3.8
			10	99 ± 16	9.1 ± 4.8	4.0
			25	332 ± 10	32.0 ± 4.0	4.2
	1008-3	selenite	5	29 ± 12	7.5 ± 3.0	11.3
		selenate	5	130 ± 6	24.4 ± 6.4	8.1
			10	172 ± 2	20.7 ± 6.3	5.2
			25[b]	NC	NC	
	1008-8	selenite	5	13 ± 2	4.1 ± 2.0	13.4
		selenate	5	144 ± 7	28.7 ± 7.2	8.7
			10	216 ± 11	53.3 ± 11.7	10.7
			25	349 ± 11	105.0 ± 27.2	13.1
leaf	WT	selenite	5	6 ± 0	ND	0
		selenate	5	561 ± 72	ND	0
			10	1102 ± 80	ND	0
			25	1264 ± 122	ND	0
	1008-3	selenite	5	32 ± 3	ND	0
		selenate	5	1021 ± 50	1.6 ± 1.6	0.1
			10	1772 ± 327	1.6 ± 1.6	0
			25	2581 ± 330	3.1 ± 1.5	0.1
	1008-8	selenite	5	9 ± 0	ND	0
		selenate	5	882 ± 134	2.1 ± 1.6	0.1
			10	1341 ± 70	2.6 ± 1.7	0.1
			25	1702 ± 43	2.9 ± 1.9	0.1

[a] Sodium selenite or sodium selenate (20 mL per application) at the indicated concentration was added to the soil twice per week for 10 weeks, while fruit were developing. Data are expressed on a DW basis and are means ± SDs ($n = 2$). Spiking experiments indicated that MeSeCys recovery was 42−56%. A correcting factor has not been applied. [b] NC, not collected (fruit either did not set or did not develop). [c] ND, not detected (at detection limit of ∼0.3 mg kg^{-1} DW). [d] Calculated using only the Se component of MeSeCys.

The presence of enhanced ATPS activity in plants of line 1008 would be expected to improve the flow of inorganic Se from selenate into organic selenium compounds. In a separate experiment to that described above, plants of line 1008 and wild-type controls were provided with selenite and with three concentrations of selenate (Table 2). The highest concentration of selenate used, 25 mM, resulted in considerable stress to the plants, with reduced leaf development, browning, and death of the youngest leaves, and in the case of line 1008-3, the normal development of fruit did not occur. Se accumulated to extraordinary concentrations in leaves of 1008-3, exceeding 2500 mg kg^{-1} DW. In line 1008-8, provision of 25 mM selenate resulted in the accumulation of MeSeCys to substantial concentrations in fruit from the sixth truss, exceeding 100 mg kg^{-1} DW. MeSeCys was also present in wild-type fruit, confirming the results of the previous experiment (Table 1), with a conversion efficiency of inorganic Se to MeSeCys of ∼4%. In line 1008-8, a conversion efficiency of inorganic Se to MeSeCys of ∼13% was observed in fruit from plants treated with either 5 mM selenite or 25 mM selenate, although the accumulation of both Se and MeSeCys was ∼25-fold greater in the latter. Low concentrations of MeSeCys were detected in leaves of both lines 1008-3 and 1008-8 after watering the plants with selenate.

The above data show that MeSeCys can be produced in fruit of tomato and that this can be greatly increased in transgenic lines overexpressing an *AbSMT* transgene. The presence of an additional transgene for overexpression of *BoATPS1* appeared to have little effect on the efficiency of conversion of inorganic Se to MeSeCys. However, the presence of substantial amounts of inorganic Se in the fruit is a problem, since although small amounts of Se are beneficial, in greater amounts Se is toxic. Considerable variability between fruit on different trusses was observed in the amounts of Se accumulated. From a food safety point of view, it would be difficult to guarantee the quantity of Se in any one particular fruit to be within certain strictly defined limits. If MeSeCys is stable to heat and the effects of processing, an alternative way to provide dietary Se and MeSeCys with more controlled parameters is as a processed tomato juice.

Se and MeSeCys Contents of Tomato Juice. Tomato juice was produced using a lab-based method from the lines and treatments described in Table 2, collecting fruit from all trusses. A microwave hot break was used to cook and sterilize the fruit, followed by passing the cooked tissue through a sieve and blending of the pulp into juice. The juice needed to be freeze-dried to concentrate the samples sufficiently for quantification of MeSeCys, but data have been back-calculated (DW was 7.0−10.2% of FW) to be expressed on a FW basis (Table 3). The Se concentration was ∼4-fold greater in the transgenic lines than in wild-type, but MeSeCys concentration was 30−50-fold greater. The efficiency of conversion of inorganic Se to MeSeCys was 7.7−9.7% in the transgenic lines, suggesting that losses of MeSeCys

Table 3. Se and MeSeCys Concentrations in Tomato Juice Prepared from Fruit of Plants Overexpressing an *AbSMT* Gene and a *BoATPS1* Gene[a]

line	concn applied (mM)	Se concn (mg kg^{-1} FW)	MeSeCys concn (mg kg^{-1} FW)	conversion (%)[b]
WT	5	3.73 ± 0.01	0.07 ± 0.05	0.8
1008-3	5	12.71 ± 0.14	2.26 ± 0.19	7.7
1008-8	5	15.48 ± 0.30	3.41 ± 0.64	9.6
	10	15.63 ± 0.32	3.49 ± 0.46	9.7

[a] Plants were treated with sodium selenate as in Table 2. Chopped red ripe fruit was subjected to a microwave hot break, juiced, freeze dried, and then assayed for concentration of total Se (in both inorganic and organic forms) and MeSeCys. Data have been back-calculated to be expressed on a FW basis and are means ± SDs (n = 2). Spiking experiments indicated that MeSeCys recovery was 42−56%. A correcting factor has not been applied. [b] Calculated using only the Se component of MeSeCys.

during processing were low. This shows that the bulk of MeSeCys present in the fruit was heat-stable and survived in vitro processing.

DISCUSSION

In tomato, expression of an *AbSMT* transgene was found to increase the assimilation of Se into leaves and fruit. Relative to wild-type, Se accumulation from soil-supplied selenate was increased by ∼1.5−3-fold in both leaves and fruit (Tables 1 and 2). There was some variability between leaves and fruit on the same plant (data not shown), which is not surprising considering that trusses develop at different rates and that some leaves were already mature when Se watering began and others developed during the treatment period. Expression of an *AbSMT* transgene in *A. thaliana*, Indian mustard, and tobacco also increased total Se accumulation as well as the accumulation of MeSeCys.[22,28,29] As in Indian mustard tissues,[29] wild-type tomato fruit were able to produce some MeSeCys from inorganic Se due to the activity of endogenous methyltransferases,[20] but this capacity was greatly increased by the expression of the *AbSMT* transgene. In transgenic tomato, a watering regime including aliquots of 5 mM selenate (20 applications of 20 mL each = 2 mmol selenate in total provided to the soil) produced MeSeCys concentrations of ∼30 mg kg^{-1} DW in the fruit (Tables 1 and 2), which could be increased to >100 mg kg^{-1} DW at applied selenate concentrations that were severely detrimental to plant growth (Table 2).

Experiments with Indian mustard found that accumulation of organic Se compounds occurred when Se was provided as selenite but not as selenate.[30] Overexpression of *ATPS*, the first enzyme of the Se assimilation pathway that begins by reducing selenate to selenite, allowed the production of organic Se compounds from selenate,[19] and overexpression of *ATPS* and *SMT* together allowed the production of MeSeCys.[31] In transgenic tobacco, the enhancement of ATPS activity increased the proportion of Se becoming incorporated into MeSeCys, relative to that caused by enhancement of SMT activity alone.[22] In tomato, enhanced ATPS activity (by ∼2-fold in leaves) had little apparent effect on the proportion of total Se that accumulated as MeSeCys in fruit, although the comparison was between different independent transgenic lines in different experiments (Tables 1 and 2). Expression of the *BoATPS1* transgene was much higher in red fruit than in leaves (Figure 2), suggesting that ATPS activity may have been more greatly enhanced in this tissue. However, it was not possible to assay ATPS activity in ripe fruit since the plastids were too labile to survive purification on a density gradient.

Watering plants with selenite rather than with selenate resulted in much lower incorporation of Se into aerial organs (Tables 1 and 2). Although selenite enters the assimilation pathway at a higher point and is therefore more easily incorporated into organic Se compounds, selenite is poorly translocated out of the roots.[30,32] Nevertheless, substantial accumulation of MeSeCys occurred in tomato fruit when selenite was provided as the source of Se, and the proportional conversion of inorganic Se to MeSeCys was slightly greater than that when selenate was the source of Se (Tables 1 and 2). This is consistent with data from Indian mustard, where a greater proportion of Se was converted to MeSeCys from selenite than from selenate.[29]

Although increased dietary consumption of Se is correlated with reduced susceptibility to cancer, the window for safe Se consumption between sufficiency and toxicity is quite narrow and varies between individuals. An RDI of 55 μg can be beneficially increased by at least 200 μg or to the reference dose (a measure of the maximal safe intake) of 400 μg day^{-1} without ill effects, but sustained consumption of >2000 μg day^{-1} produces adverse symptoms of selenosis, and intakes of >6000 μg day^{-1} can be toxic.[3,7] Toxicity may not be avoided by presenting the Se as the organic forms common in dietary supplements derived from yeast (mainly selenomethionine),[33] but MeSeCys appears to lack toxicity since it can be safely broken down and excreted in breath or urine.[3,34] Combined with the anticancer effects of MeSeCys demonstrated in vitro,[13−16] this suggests that dietary presentation of Se predominantly as MeSeCys could have a considerable benefit. Currently what might be an effective anticancer dose of MeSeCys in humans in vivo is not known. In transgenic tomato fruit, MeSeCys concentrations of >100 mg kg^{-1} DW could be achieved, although this required applied selenate concentrations that were severely detrimental to plant growth. Spiking experiments indicated that these concentrations of MeSeCys were an underestimate based on a recovery of ∼50%, suggesting that the actual MeSeCys concentrations could have been twice these values.

However, from a human nutrition point of view, the presence of substantial amounts of inorganic Se in the fruit is a concern. A small amount of inorganic Se as a nutritional supplement may be beneficial, but the variability in Se content observed between batches of fruit and the narrow window for safe Se consumption argue against the development of a reliably MeSeCys-supplemented fresh market tomato product. MeSeCys appears to be heat stable, since no marked loss occurred during a lab-based processing procedure converting fresh whole fruit to tomato juice (cooked and homogenized pulp). This implies that a processed MeSeCys-supplemented tomato juice could be produced in which juice from fruit of Se-treated, *SMT*-overexpressing plants is blended with juice from fruit of wild-type plants to carefully control the final concentration of Se.

Future work will need to circumvent the problems associated with Se application to plants (poor translocation of selenite out of the roots, low conversion of selenate to organic forms), contamination of soils with Se (perhaps by using recirculating hydroponic systems), and the current relatively low conversion of inorganic Se to MeSeCys. The latter may require increased expression of the *AbSMT* transgene or engineering of the recombinant enzyme to have higher activity or manipulation of the

expression of other enzymes in the assimilation pathway. If these issues can be resolved and MeSeCys accumulation increased to the concentrations seen in Se hyperaccumulators without excess inorganic Se, the technology will enable the enrichment of crops with a powerful anticancer agent.

■ AUTHOR INFORMATION

Corresponding Author

*Tel: +64-6-355-6121. Fax: +64-6-351-7050. E-mail: david.brummell@plantandfood.co.nz.

Funding Sources

This work was funded by the New Zealand Foundation for Research, Science and Technology (FRST) under contract number C02X0701 and by Vital Vegetables, a Trans-Tasman research project jointly funded and supported by Horticulture Australia Limited, The New Zealand Institute for Plant & Food Research Limited, FRST, the Victorian Department of Primary Industries, Horticulture New Zealand, and the Australian Vegetable and Potato Growers Federation Inc.

■ ACKNOWLEDGMENT

We thank Jun Zhou for technical assistance, Bernhard Neuhierl for the *Astragalus SMT* cDNA, Adam Matich and Daryl Rowan for helpful contributions, Simon Deroles and Kevin Davies for comments on the manuscript, and Ian King for care of the transgenic plants.

■ ABBREVIATIONS USED

RDI, recommended daily intake; MeSeCys, methylselenocysteine; SeCys, selenocysteine; ATPS, ATP sulfurylase; SMT, selenocysteine methyltransferase; BM, basal medium; DW, dry weight; FW, fresh weight; LCMS, liquid chromatography−mass spectrometry; API-MS, atmospheric pressure ionization mass spectrometry

■ REFERENCES

(1) Kryukov, G. V.; Castellano, S.; Novoselev, S. V.; Lobanov, A. V.; Zehtab, O.; Guigo, R.; Gladyshev, V. N. Characterization of mammalian selenoproteomes. *Science* **2003**, *300*, 1439–1443.

(2) Rotruck, J. T.; Pope, A. L.; Ganther, H. E.; Swanson, A. B.; Hafeman, D. G.; Hoekstra, W. G. Selenium: Biochemical role as a component of glutathione peroxidase. *Science* **1973**, *179*, 588–590.

(3) Combs, G. F. Selenium in global food systems. *Br. J. Nutr.* **2001**, *85*, 517–547.

(4) Gupta, U. C.; Gupta, S. C. Quality of animal and human life as affected by selenium management of soils and crops. *Commun. Soil Sci. Plant Anal.* **2002**, *33*, 2537–2555.

(5) Broadley, M. R.; White, P. J.; Bryson, R. J.; Meacham, M. C.; Bowen, H. C.; Johnson, S. E.; Hawkesford, M. J.; McGrath, S. P.; Zhao, F. J.; Breward, N.; Harriman, M.; Tucker, M. Biofortification of UK food crops with selenium. *Proc. Nutr. Soc.* **2006**, *65*, 169–181.

(6) White, P. J.; Broadley, M. R. Biofortification of crops with seven mineral elements often lacking in human diets—Iron, zinc, copper, calcium, magnesium, selenium and iodine. *New Phytol.* **2009**, *182*, 49–84.

(7) Rayman, M. P. The argument for increasing selenium uptake. *Proc. Nutr. Soc.* **2002**, *61*, 203–215.

(8) Combs, G. F. Current evidence and research needs to support a health claim for selenium and cancer prevention. *J. Nutr.* **2005**, *135*, 343–347.

(9) Whanger, P. D. Selenium and its relationship to cancer: An update. *Br. J. Nutr.* **2004**, *91*, 11–28.

(10) Ip, C.; Birringer, M.; Block, E.; Kotrebai, M.; Tyson, J. F.; Uden, P. C.; Lisk, D. J. Chemical speciation influences comparative activity of selenium-enriched garlic and yeast in mammary cancer prevention. *J. Agric. Food Chem.* **2000**, *48*, 2062–2070.

(11) Finley, J. W.; Davis, C. D.; Feng, Y. Selenium from high selenium broccoli protects rats from colon cancer. *J. Nutr.* **2000**, *130*, 2384–2389.

(12) Finley, J. W.; Davis, C. D. Selenium (Se) from high-selenium broccoli is utilized differently than selenite, selenate and selenomethionine, but is more effective in inhibiting colon carcinogenesis. *BioFactors* **2001**, *14*, 191–196.

(13) Sinha, R.; Kiley, S. C.; Lu, J. X.; Thompson, H. J.; Moraes, R.; Jaken, S.; Medina, D. Effects of methylselenocysteine on PKC activity, cdk2 phosphorylation and *gadd* gene expression in synchronized mouse mammary epithelial tumor cells. *Cancer Lett.* **1999**, *146*, 135–145.

(14) Kim, T.; Jung, U.; Cho, D. Y.; Chung, A. S. Se-methylselenocysteine induces apoptosis through caspase activation in HL-60 cells. *Carcinogenesis* **2001**, *22*, 559–565.

(15) Unni, E.; Koul, D.; Yung, W. K. A.; Sinha, R. Se-methylselenocysteine inhibits phosphatidylinositol 3-kinase activity of mouse mammary epithelial tumor cells *in vitro*. *Breast Cancer Res.* **2005**, *7*, R699–R707.

(16) Hurst, R.; Elliott, R. M.; Goldson, A. J.; Fairweather-Tait, S. J. Se-methylselenocysteine alters collagen gene and protein expression in human prostate cells. *Cancer Lett.* **2008**, *269*, 117–126.

(17) Neuhierl, B.; Böck, A. On the mechanism of selenium tolerance in selenium-accumulating plants—Purification and characterization of a specific selenocysteine methyltransferase from cultured cells of *Astragalus bisculatus*. *Eur. J. Biochem.* **1996**, *239*, 235–238.

(18) Sors, T. G.; Ellis, D. R.; Salt, D. E. Selenium uptake, translocation, assimilation and metabolic fate in plants. *Photosynth. Res.* **2005**, *86*, 373–389.

(19) Pilon-Smits, E. A. H.; Hwang, S. B.; Lytle, C. M.; Zhu, Y. L.; Tai, J. C.; Bravo, R. C.; Chen, Y. C.; Leustek, T.; Terry, N. Overexpression of ATP sulfurylase in Indian mustard leads to increased selenate uptake, reduction, and tolerance. *Plant Physiol.* **1999**, *119*, 123–132.

(20) Neuhierl, B.; Thanbichler, M.; Lottspeich, F.; Böck, A. A family of *S*-methylmethionine-dependent thiol/selenol methyltransferases—Role in selenium tolerance and evolutionary relation. *J. Biol. Chem.* **1999**, *274*, 5407–5414.

(21) Pilon-Smits, E. A. H.; LeDuc, D. Phytoremediation of selenium using transgenic plants. *Curr. Opin. Biotechnol.* **2009**, *20*, 207–212.

(22) McKenzie, M. J.; Hunter, D. A.; Pathirana, R.; Watson, L. M.; Joyce, N. I.; Matich, A. J.; Rowan, D. D.; Brummell, D. A. Accumulation of an organic anticancer selenium compound in a transgenic Solanaceous species shows wider applicability of the selenocysteine methyltransferase transgene from selenium hyperaccumulators. *Transgenic Res.* **2009**, *18*, 407–424.

(23) Gleave, A. P. A versatile binary vector system with a T-DNA organizational structure conducive to efficient integration of cloned DNA into the plant genome. *Plant Mol. Biol.* **1992**, *20*, 1203–1207.

(24) Pathirana, R.; Harris, J. C.; McKenzie, M. J. A comparison of microtubers and field-grown tubers of potato (*Solanum tuberosum* L.) for hexoses, sucrose and their ratios following postharvest cold storage. *Postharvest Biol. Technol.* **2008**, *49*, 180–184.

(25) Wan, C.; Wilkins, T. A. A modified hot borate method significantly enhances the yield of high-quality RNA from cotton (*Gossypium hirsutum* L.). *Anal. Biochem.* **1994**, *223*, 7–12.

(26) Church, G. M.; Gilbert, W. Genomic sequencing. *Proc. Natl. Acad. Sci. U.S.A.* **1984**, *81*, 1991–1995.

(27) Watkinson, J. H. Semi-automated fluorimetric determination of nanogram quantities of selenium in biological materials. *Anal. Chim. Acta* **1979**, *105*, 319–325.

(28) Ellis, D. R.; Sors, T. G.; Brunk, D. G.; Albrecht, C.; Orser, C.; Lahner, B.; Wood, K. V.; Harris, H. H.; Pickering, I. J.; Salt, D. E. Production of Se-methylselenocysteine in transgenic plants expressing selenocysteine methyltransferase. *BMC Plant Biol.* **2004**, *4*, 1.

(29) LeDuc, D. L.; Tarun, A. S.; Montes-Bayon, M.; Meija, J.; Malit, M. F.; Wu, C. P.; AbdelSamie, M.; Chiang, C. Y.; Tagmount, A.;

DeSouza, M.; Neuhierl, B.; Bock, A.; Caruso, J.; Terry, N. Overexpression of selenocysteine methyltransferase in *Arabidopsis* and Indian mustard increases selenium tolerance and accumulation. *Plant Physiol.* **2004**, *135*, 377–383.

(30) de Souza, M. P.; Pilon-Smits, E. A. H.; Lytle, C. M.; Hwang, S.; Tai, J.; Honma, T. S. U.; Yeh, L.; Terry, N. Rate-limiting steps in selenium assimilation and volatilization by Indian mustard. *Plant Physiol.* **1998**, *117*, 1487–1494.

(31) LeDuc, D. L.; AbdelSamie, M.; Móntes-Bayon, M.; Wu, C. P.; Reisinger, S. J.; Terry, N. Overexpressing both ATP sulfurylase and selenocysteine methyltransferase enhances selenium phytoremediation traits in Indian mustard. *Environ. Pollut.* **2006**, *144*, 70–76.

(32) Arvy, M. P. Selenate and selenite uptake and translocation in bean plants (*Phaseolus vulgaris*). *J. Exp. Bot.* **1993**, *44*, 1083–1087.

(33) Rayman, M. P.; Infante, H. G.; Sargent, M. Food-chain selenium and human health: Spotlight on speciation. *Br. J. Nutr.* **2008**, *100*, 238–253.

(34) Finley, J. W. Increased intakes of selenium-enriched foods may benefit human health. *J. Sci. Food Agric.* **2007**, *87*, 1620–1629.