Transgenic Res (2009) 18:407–424
DOI 10.1007/s11248-008-9233-0

ORIGINAL PAPER

Accumulation of an organic anticancer selenium compound in a transgenic Solanaceous species shows wider applicability of the selenocysteine methyltransferase transgene from selenium hyperaccumulators

Marian J. McKenzie · Donald A. Hunter · Ranjith Pathirana · Lyn M. Watson · Nigel I. Joyce · Adam J. Matich · Daryl D. Rowan · David A. Brummell

Received: 27 January 2008 / Accepted: 17 November 2008 / Published online: 3 December 2008
© Springer Science+Business Media B.V. 2008

Abstract Tolerance to high selenium (Se) soils in Se-hyperaccumulating plant species is correlated with the ability to biosynthesise methylselenocysteine (MeSeCys), due to the activity of selenocysteine methyltransferase (SMT). In mammals, inclusion of MeSeCys in the diet reduces the incidence of certain cancers, so increasing the range of crop plants that can produce this compound is an attractive biotechnology target. However, in the non-Se accumulator *Arabidopsis*, overexpression of *SMT* does not result in biosynthesis of MeSeCys from selenate because the rate at which selenate is reduced to selenite by ATP sulfurylase (ATPS) is low. This limitation is less problematic in other species of the Brassicaceae that can produce MeSeCys naturally. We investigated the potential for biosynthesis of MeSeCys in other plant families using *Nicotiana tabacum* L., a member of the Solanaceae. When plants were watered with 200 μM selenate, overexpression of a *SMT* transgene caused a 2- to 4-fold increase in Se accumulation (resulting in increased numbers of leaf lesions and areas of necrosis), production of MeSeCys (up to 20% of total Se) and generation of volatile dimethyl diselenide derived directly from MeSeCys. Despite the greatly increased accumulation of total Se, this did not result in increased Se toxicity effects on growth. Overexpression of *ATPS* did not increase Se accumulation from selenate. Accordingly, lines overexpressing both *ATPS* and *SMT* did not show a further increase in total Se accumulation or in leaf toxicity symptoms relative to overexpression of *SMT* alone, but directed a greater proportion of Se into MeSeCys. This work demonstrates that the production of the cancer-preventing compound MeSeCys in plants outside the Brassicaceae is possible. We conclude that while the *SMT* gene from Se hyperaccumulators can probably be utilised universally to increase the metabolism of Se into MeSeCys, the effects of enhancing ATPS activity will vary depending on the species involved.

Keywords ATP sulfurylase · Dimethyl diselenide · Methylselenocysteine · Selenium accumulation · Selenocysteine methyltransferase · Tobacco

M. J. McKenzie (✉) · D. A. Hunter · R. Pathirana · L. M. Watson · D. A. Brummell
New Zealand Institute for Plant & Food Research Ltd., Food Industry Science Centre, Private Bag 11600, Palmerston North 4442, New Zealand
e-mail: mckenziem@crop.cri.nz

N. I. Joyce
New Zealand Institute for Plant & Food Research Ltd., Canterbury Agriculture and Science Centre, Private Bag 4704, Christchurch 8140, New Zealand

A. J. Matich · D. D. Rowan
New Zealand Institute for Plant & Food Research Ltd., John Lyttleton Building, Private Bag 11030, Palmerston North 4442, New Zealand

Introduction

Plants can be divided into three groups according to their ability to take up and metabolise selenium (Se)

(Terry et al. 2000; Ellis and Salt 2003). The majority, termed Se 'non-accumulators', accumulate only low levels of Se from the soil (no more than 100 mg Se kg^{-1} DW, and usually much less). These plants take up Se non-specifically via the sulfur (S) assimilation pathway (White et al. 2007; see Fig. 1), ultimately forming seleno-Cys (SeCys) and seleno-Met (SeMet), which are mis-incorporated into proteins and can result in Se toxicity symptoms such as stunting, necrotic lesions on the leaves and reduced root growth. In contrast, Se hyperaccumulators, such as *Astragalus bisulcatus* and *Stanleya pinnata*, when grown in Se-rich soils can take up and accumulate large quantities of Se (up to several thousand mg Se kg^{-1} DW) without apparent ill-effects (Pickering et al. 2003; Freeman et al. 2006). Between these extremes are the secondary Se accumulators, which can grow on moderate Se soils and accumulate moderate quantities of Se (up to 1000 mg Se kg^{-1} DW) without showing signs of toxicity. Certain *Brassica* spp., such as Indian mustard (*B. juncea*), broccoli (*B. oleracea* var. *italica*) and canola (*B. napus*) are in this group, together with some species of the *Alliaceae* including garlic (*Allium sativum*).

Se uptake by plants occurs via the S assimilation pathway since most of the enzymes involved in uptake, translocation and assimilation cannot discriminate between the Se and S forms of their substrates (Sors et al. 2005b). The production of organic Se compounds is thus largely dependent on the enzymes of S metabolic pathways. Se is generally taken up from the soil as selenate, which must be reduced to selenite by plastidic ATP sulfurylase (ATPS) and adenosine 5′-phosphosulfate (APS) reductase before proceeding along the S assimilation pathway to SeCys, the first organic Se compound (Fig. 1). The major mechanism for accumulation of, and tolerance to, large quantities of Se in Se-hyperaccumulating species such as *A. bisulcatus* is the activity of selenocysteine methyltransferase (SMT) (Neuhierl and Böck 1996; Neuhierl et al. 1999). This enzyme is found only in Se accumulators and hyperaccumulators, and methylates SeCys to form methylseleno-Cys (MeSeCys), an amino acid derivative that is not incorporated into proteins and that can be tolerated at high concentrations by the plant, where it is stored in cells on the periphery of young leaves (Pickering et al. 2000; Freeman et al. 2006). MeSeCys can also be volatalized as dimethyl diselenide (Me_2Se_2), or transformed into the dipeptide γ-glutamyl-MeSeCys (GluMeSeCys) (Fig. 1). SMT is relatively specific for SeCys, whose concentrations are rapidly reduced without depleting Cys (Neuhierl and Böck 1996). In this way, Se accumulators remove SeCys, and the subsequently-produced SeMet, from the pool of substrates available for protein synthesis, preventing their mis-incorporation into proteins and consequent deleterious effects on protein native structure and function (Brown and Shrift 1981).

In addition, this plant detoxification mechanism has important implications for human health. It is well established that supplementation of the diet with Se helps reduce the incidence of certain cancers (Clark et al. 1996; Finley 2007) and boosts the immune system (Lyons et al. 2004), but further benefits may result from the form in which the Se is ingested. Rat cancer models fed with Se as high-Se broccoli or garlic had lower rates of cancer than control rats fed equivalent amounts of inorganic Se and regular broccoli or garlic (Finley et al. 2000, 2001; Ip et al. 2000a, b; Finley and Davis 2001). This is thought to be due to the ability of broccoli and garlic, both secondary Se accumulators, to convert high levels of Se from the soil into MeSeCys. Supporting this, MeSeCys has been shown to have potent anti-carcinogenic effects when applied to animal cancer cell lines (Sinha et al. 1999; Kim et al. 2001), and was the most effective anti-carcinogenic Se-containing compound in animal mammary cancer trials (Whanger 2004). Therefore, the development of food crops intended to increase dietary Se intake (Lyons et al. 2004; Broadley et al. 2006) would be even more beneficial if a proportion of this supplementary Se were present as MeSeCys.

The availability of the gene encoding SMT from *A. bisulcatus* (Neuhierl et al. 1999) allows the possibility of moving the trait of Se accumulation, and therefore MeSeCys production, into a wider variety of plants. Previous work, however, has focused primarily on the development of rapidly-growing Se-accumulating transgenic plants for the phytoremediation of selenate-contaminated soils (Ellis et al. 2004; LeDuc et al. 2004, 2006), where MeSeCys production is part of the mechanism for Se accumulation and volatalization. We are interested in using the *SMT* gene to develop crop plants that when used for human dietary Se supplementation contain

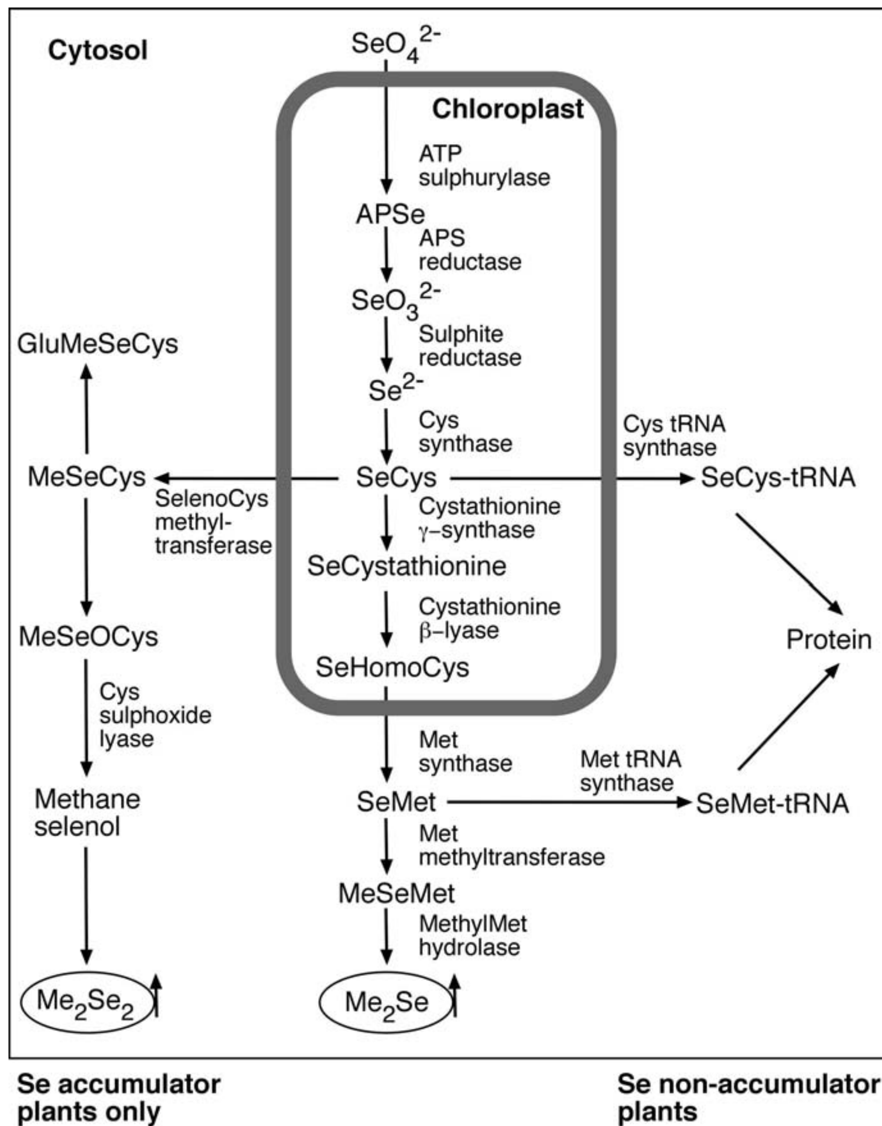

Fig. 1 Simplified scheme showing assimilation of Se by the S pathway of plants. Selenate is first accumulated into the cell by sulfate transporters on the plasma membrane (not shown), then activated and reduced in the chloroplast. SeCys is converted into SeMet, both of which can be mis-incorporated into proteins (with toxic effects) in Se non-accumulating plants. Some SeMet is methylated to form MeSeMet, which is hydrolyzed to the volatile Me_2Se. In Se-accumulating plants, selenoCys methyltransferase converts SeCys to MeSeCys, which can be accumulated non-toxically or transformed to the dipeptide GluMeSeCys or the volatile Me_2Se_2. Possible alternative pathways of conversion of APSe to SeCys and MeSeMet to Me_2Se have been omitted for clarity. The enzyme activities converting MeSeCys to GluMeSeCys and Me_2Se_2 are not completely characterised in Se accumulators. Abbreviations: APS, adenosine 5′-phosphosulfate; APSe, adenosine 5′-phosphoselenate; Cys, cysteine; GluMeSeCys, γ-glutamyl-methylselenocysteine; MeSeCys, methylselenocysteine; MeSeOCys, methylselenocysteine selenooxide; MeSeMet, methylselenomethionine; Met, methionine; Me_2Se, dimethyl selenide; Me_2Se_2, dimethyl diselenide

MeSeCys, a compound possessing powerful anti-cancer properties. An example of an economically significant group of plants is the Solanaceae, which contains many important crops with edible organs including potato, pepper and eggplant and for which tobacco is a common model system. Transformation of model phytoremediation plants with a constitutively-expressed *SMT* transgene has resulted in different results in different species. In *Arabidopsis thaliana*, MeSeCys production and consequent

increases in Se uptake and Se tolerance were achieved, but only when the transgenic lines were watered with selenite, and not with selenate (Ellis et al. 2004). This is thought to be because the reduction of selenate to selenite by ATPS may be a limiting step in many species (Leustek 1996; Leustek and Saito 1999; Pilon-Smits et al. 1999). In Indian mustard, a secondary accumulator, the natural ability to produce MeSeCys from selenate was increased in lines overexpressing *SMT* (LeDuc et al. 2004). Transformation of Indian mustard with both *ATPS* and *SMT* showed that in this species the simultaneous overexpression of these genes increased the accumulation of Se and production of MeSeCys, compared with either gene alone (LeDuc et al. 2006).

However, ATPS activity does not appear to limit Se accumulation in all species. Both Indian mustard and *Arabidopsis* are from the Brassicaceae family, many species of which possess significant secondary S metabolic pathways (Halkier and Gershenzon 2006). In cultured cells of tobacco (*Nicotiana tabacum* L.), a Se non-accumulator from the Solanaceae, overexpression of ATPS activity did not alter sulfate uptake or cell growth in the presence of selenate (Hatzfeld et al. 1998). The aim of the present work was: firstly, to examine if ATPS activity limits Se accumulation from selenate in whole tobacco plants, and therefore limits the amounts of Se able to enter organic pools such as MeSeCys; and secondly, to determine if the *SMT* gene from *A. bisulcatus* would allow this non-accumulating plant species that is not related to the Brassicaceae to become a Se accumulator capable of producing MeSeCys. This would indicate the universality of this gene's function and its potential for human health applications outside the Brassicaceae, and presents the possibility for the accumulation of anti-cancer Se compounds in a wide variety of vegetable species.

Materials and methods

Construction of plasmids

Three populations of tobacco plants were created: one constitutively overexpressing an *Arabidopsis thaliana ATPS1* transgene (*AtATPS1*), the second constitutively overexpressing an *Astragalus bisulcatus SMT* transgene (*AbSMT*), and the third constitutively overexpressing both a *Brassica oleracea ATPS1* (*BoATPS1*) transgene and an *AbSMT* transgene. The *ATPS1* gene from *Arabidopsis thaliana* (*AtATPS1*, GenBank accession number U05218) was a generous gift from Dr T. Leustek, and was provided as a *35S:AtATPS1:nos* cassette in binary vector pBI101. The *SMT-A* gene from *Astragalus bisulcatus* (*AbSMTA*, GenBank accession number AJ13143) was a generous gift from Dr B. Neuhierl. The ORF of the *AbSMT* gene was PCR-amplified and ligated into a shuttle vector, between the CaMV 35S promoter and the *nos* transcription termination sequence from *Agrobacterium tumefaciens*, to create a *35S:AbSMT:nos* expression cassette.

For preparation of a construct to constitutively express both *AbSMT* and a gene encoding ATPS, a 700-bp region of the promoter of the *Arabidopsis thaliana UBQ10* gene (Norris et al. 1993) was amplified from genomic DNA by PCR, omitting the intron immediately upstream of the translation start. A probe for isolation of a broccoli cDNA encoding ATPS was prepared by random prime labelling of the *AtATPS1* cDNA (Leustek et al. 1994), and used to screen a broccoli cDNA library according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). The longest clone resulting from this screen was sequenced, and named *BoATPS1* (GenBank accession number EU346738). *BoATPS1* was highly homologous to *AtATPS1*, being 90% identical and 95% similar at the amino acid level, and like *AtATPS1* also possessed a predicted transit peptide for chloroplast entry. The ORF of *BoATPS1* was amplified by PCR and a *UBQ10:BoATPS1:nos* expression cassette was assembled. The completed cassette was ligated into the *35S:AbSMT:nos* expression vector described above to create a *35S:AbSMT:nos/UBQ10:BoATPS1:nos* construct. All constructs were verified by DNA sequencing.

The expression cassette *35S:AbSMT:nos* was transferred into the binary vector pHZbar2 (a derivative of pMLBART) conferring resistance to Basta, and the *35S:AbSMT:nos/UBQ10:BoATPS1:nos* cassette was transferred into the binary vector pART27 (Gleave 1992) conferring resistance to kanamycin. Binary vectors and empty binary vectors (as controls) were transformed into *A. tumefaciens* strain LBA4404 by electroporation.

Plant transformation

Tobacco (*Nicotiana tabacum L.* cv. Samsun) was transformed using the method of Horsch et al. (1985). Leaf discs were inoculated with overnight cultures of *A. tumefaciens* harbouring the binary plasmids described above and co-cultivated on MS medium (Murashige and Skoog, 1962) supplemented with 0.1 μg ml^{-1} α-naphthaleneacetic acid, 1 μg ml^{-1} 6-benzylaminopurine (both Sigma Life Science, St Louis, MO, USA) and 3% sucrose at 25°C for two days in the dark followed by selection on MS medium containing 200 μg ml^{-1} kanamycin (Sigma) or 20 μg ml^{-1} Basta (Hoescht, Melbourne, Australia) as appropriate. Green shoots from independent leaf discs were rooted on 0.5× MS selection medium under a 16 h light, 8 h dark photoperiod at a light intensity of 30 μmol m^{-2} s^{-1}. In addition to control plants transformed with pBI101, pART27 and pHZbar2 empty vectors, non-transformed control (wild-type) plants were also regenerated.

Molecular analyses

Putative transgenic seedlings were screened by PCR for the presence of the transgene(s) and the selectable marker gene, and plants positive for both were screened for transgene mRNA accumulation by RNA gel blot analysis. RNA was prepared from young leaves of independent lines using the Trizol reagent (Invitrogen), and 20 μg total mRNA per line was separated by electrophoresis in 1.2% agarose and 5% formaldehyde denaturing gels. RNA was blotted to Hybond-XL membrane (Amersham, Little Chalfont, UK) according to the manufacturer's instructions, and cross-linked by UV irradiation. RNA gel blots were hybridised with probes prepared from the cDNAs of *AbSMT*, *AtATPS1* or *BoATPS1* using random hexamers, α-[^{32}P]-dATP and the Klenow fragment of DNA polymerase I. Hybridisation was in Church and Gilbert (1984) buffer at 65°C overnight, then blots were washed with 0.1× SSC and 0.1% SDS at 65°C three times and exposed to phosphorimager plates.

Extracted protein was separated by electrophoresis in denaturing polyacrylamide gels (Laemmli 1970) and electroblotted to PVDF membrane (Bio-Rad, Hercules, CA, USA). Membranes were blocked with 3% BSA in PBST, hybridised with the primary antibody (raised against onion ATPS, a generous gift of Prof M. McManus) at a dilution of 1:200, washed, hybridized with goat anti-rabbit antibody coupled to alkaline phosphatase (Bio-Rad) and re-washed. Bound antibodies were detected using 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium as substrate.

Plant growth and selenium watering

Transgenic lines showing high mRNA accumulation of the transgene(s) were identified as above, and multiple clonal copies of each independent line were produced using axilliary bud culture. Eight to twelve clonal plants of each line were ex-flasked into pots of medium grade vermiculite (Nuplex, Auckland, New Zealand) and acclimatized for 1–2 weeks in a mist tent before moving to the glasshouse, where they were placed in trays and watered from the bottom with liquid 0.5x Hoagland's solution (Hoagland and Arnon 1950). After a further two weeks, when seedlings were at the 5–6 leaf stage, plants were split into groups of 4–6 and sodium selenate (BDH, VWR International, Poole, UK) was added to the watering liquid to a final concentration of 0, 50, 200 or 300 μM. Solutions were changed every 2–3 days. Watering with sodium selenite was not conducted since, unlike selenate, selenite is not accumulated actively and is transported poorly out of the roots (Ulrich and Shrift 1968; Arvy 1993; de Souza et al. 1998; Zayed et al. 1998). Also, selenate is the form of Se generally present in the soil and available to plants (Broadley et al. 2006).

At the conclusion of the experiment, shoot weight and leaf number were recorded for each plant, and toxicity symptoms recorded for each leaf based on the severity of visual toxicity symptoms: 0 for no discernible symptoms; 1 for mild symptoms (a few black lesions or small necroses covering less than 10% of the leaf blade); 2 for moderate symptoms (numerous black lesions or brown necroses covering from 10 to 50% of the leaf blade); and 3 for severe symptoms (numerous black lesions or brown necroses covering greater than 50% of the leaf blade). For each plant the leaf ratings were added together and divided by total leaf number to give an overall toxicity symptom value.

As an approach for reducing plant-to-plant variability, leaves from 4–6 plants of the same genotype

and treatment were pooled and snap frozen in liquid nitrogen then stored at −80°C. Replicate samples were taken from this pooled tissue for analysis as below.

Measurement and speciation of selenium compounds

Frozen leaves were freeze dried then powdered. Total selenium concentration was determined by a commercial company (Gribbles, Hamilton, New Zealand) using the method of Watkinson (1979). Se content was expressed as mg total Se per kg dry weight.

Organic selenium compounds were extracted by a modification of the method of Lyi et al. (2005). Briefly, duplicate aliquots (100 mg) of freeze dried powdered leaf material were extracted with constant agitation in 10 ml of 50 mM HCl at 4°C for 3 h in the dark, and supernatants were clarified by centrifugation. Aliquots (1 µl) of prepared extracts were injected into a LC-MS system consisting of a Finnigan Surveyor MS pump (Thermo Electron Corporation, San Jose, CA, USA), Finnigan Micro-AS auto-sampler and a ThermaSphere TS-130 column heater (Phenomenex, Torrance, CA, USA). Separation was achieved by reverse phase chromatography (Aqua guard cartridge 4 × 2 mm, 10 μ and Synergi-HydroRP C18, 4 μ, 80 Å, 250 × 2.1 mm, Phenomenex, Torrance, CA, USA) at 30°C, with a mobile phase consisting of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B) flowing at 100 µl min^{-1}. 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 analysed by a Finnigan API-MS (LTQ, 2D linear ion-trap) with electrospray ionisation in the positive mode. Data were acquired by selective reaction monitoring by selecting each compound's parent mass $[M + H]^+$ and then applying a collision energy to produce daughter ions (MS^2). Quantification was based on integration of the area under the curve produced by plotting selected daughter ions with the aid of external standards (0.1–20 µg ml^{-1}). Reference materials S-methyl-L-cysteine and Se-methyl-selenocysteine (Sigma) were used for calibration. γ-Glutamyl-methyl-selenocysteine was estimated based on the response curve for MeSeCys, and its daughter ions were identified based on data from Larsen et al. (2006).

Analysis of volatile S and Se compounds

Preliminary experiments indicated that when plants were grown in 200 µM selenate the production of volatiles was low, so a separate experiment with increased selenate was carried out. After 16 days of watering with 300 µM selenate, individual tobacco plants were enclosed in oven bags (Glad brand, Clorox New Zealand Ltd., Auckland) and sealed about the stem just above vermiculite level. The headspace was allowed to equilibrate for 60 min prior to sampling with a 75 µm Carboxen™-PDMS Solid Phase Microextraction (SPME) fibre (Supelco, Bellefonte, PA, USA) inserted through the wall of the oven bag for 17 h.

GC-MS analyses were carried out using an Agilent 6890 N GC coupled to a Waters GCT time of flight (ToF) mass spectrometer. Separations used a 20 m × 0.18 mm i.d., 0.18 µm film thickness DB-Wax (Agilent) column at a helium flow of 1 ml min^{-1}. SPME samples were analysed by splitless injection (60 s) into a standard split/splitless injection port at 220°C. The oven temperature ramp was 30°C for 1 min, 3°C min^{-1} to 62°C, 10°C min^{-1} to 240°C. Liquid samples were analyzed by splitless injection (30 s) into a HP6890 PTV cryogenic injection port at −40°C (liquid CO_2). This temperature was maintained for 3 s, then increased by 700°C min^{-1} to 260°C, held for 5 min, reduced by 5°C min^{-1} to 220°C and held for 30 min. The oven temperature ramp was 30°C for 2 min, 2°C min^{-1} to 50°C, 5°C min^{-1} to 90°C, 10°C min^{-1} to 180°C, and held for 5 min. Organoselenium compounds were identified by comparison with authentic standards of dimethyl selenide (Lancaster Synthesis, Morecambe, UK) and dimethyl diselenide (Acros, Geel, Belgium), analysis of the high resolution mass spectra and comparison of mass spectra with those in the literature and in commercial mass spectral libraries (NIST, John Wiley & Sons, Hoboken, NJ, USA).

Enzyme activity assays

For ATPS activity, ~10 g of fresh young leaves was homogenized using the Polytron method

described by Kunst (1998), except that centrifugation on a 40%/80% Percoll discontinuous gradient was used. Intact chloroplasts were collected from the interface and washed as described, then resuspended and frozen in aliquots for storage at −80°C. Chloroplast suspensions were thawed and homogenized with a plastic pestle and a small amount of acid-washed sand to disrupt them, then centrifuged prior to use. Aliquots of ruptured chloroplasts were assayed similar to the method of Murillo and Leustek (1995). Reaction volumes of 200 µl contained 50 mM Tris (pH 8.0), 5 mM $MgCl_2$, 5 mM glucose, 0.3 mM NAD, 0.1 mM adenosine 5′-phosphosulfate, 5 U hexokinase and 5 U glucose-6-phosphosulfate dehydrogenase. Reactions were started by the addition of tetrasodium pyrophosphate to 1 mM, and absorbance at 340 nm was recorded using a platereader in kinetic mode for calculation of V_{max}.

For SMT activity, protein was extracted according to the procedure of James et al. (1995). The slurry was filtered through 2 layers of Miracloth (Calbiochem, San Diego, CA, USA) into a chilled microfuge tube and clarified by centrifugation at 4°C. The supernatant was desalted through a Sephadex G-25 column. SMT activity was assayed using the quantitative method described by Neuhierl and Böck (2002), using reduced seleno-DL-cystine (Sigma) and *S*-adenosyl-L-[*methyl*-^{14}C] methionine (2.05 GBq/mmol^{-1}, Amersham Biosciences UK Ltd.) as substrates. After 30 min, reactions were stopped by pipetting into an equal volume of glacial acetic acid. Products were resolved on Polygram SIL G/UV254 TLC plates (Alltech Associates, Deerfield, IL, USA) using butanol:acetic acid:water (4:1:1), and radioactive spots were quantified via a Fujifilm FLA-5100 using Fujifilm Science Lab 2001 Image Gauge Version 4. 0 software (Fujifilm Corp., Tokyo, Japan). One unit of radioactivity (in arbitrary phosphorimager units) was defined as the radiation dose minus background 1 mm^{-2}.

Protein concentrations of extracts were determined colorimetrically with a Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA, USA) based on the Bradford assay, using bovine serum albumin as a standard.

Results

Molecular and biochemical analysis of transformants

Three populations of tobacco plants were created: constitutively overexpressing an *Arabidopsis thaliana ATPS1* transgene (*AtATPS1*), or an *Astragalus bisulcatus SMT* transgene (*AbSMT*), or both a *Brassica oleracea ATPS1* (*BoATPS1*) transgene and an *AbSMT* transgene, respectively. For the first population, out of eight transformants confirmed as positive for the presence of the *AtATPS1* transgene using PCR, four exhibited strong expression of the transgene (Fig. 2a). Cross-reactivity of the recombinant protein with onion ATPS antiserum showed that accumulation of the recombinant protein paralleled the accumulation of transgene mRNA (Fig. 2a). Endogenous tobacco ATPS did not hybridise with the *AtATPS1* probe and reacted only weakly with the ATPS antiserum. For the second population, out of 21 transformants confirmed as positive for the presence of the *AbSMT* transgene using PCR, 14 exhibited strong expression of the transgene (Fig. 2b). For the third population, 13 transformants confirmed by PCR as positive for the presence of both the *AbSMT* and *BoATPS* transgenes were assessed by RNA gel blot analysis (Fig. 2c). Several lines had strong expression of one of the transgenes, and several had moderate expression of both.

Leaves of selected transgenic seedling plants were assayed for activity of ATPS and SMT relative to wild-type. ATPS activity was assayed in purified chloroplasts of lines ATPS20, 111 and 117 (line ATPS116 had poor root development and could not be used). ATPS activity ranged from 40- to 55-fold greater than wild-type in the three ATPS transgenic lines (Fig. 3a). SMT activity was assayed in lines SMT2, 4 and 37 (lines SMT33 and 36 had poor root or leaf development and could not be used). All three lines had substantial SMT activity compared with the wild type control, in which activity was undetectable (Fig. 3b). Line SMT4 had the highest activity, while line SMT37 had less than half the activity of lines SMT4 or 2. In lines ATPS/SMT24 and 47, with the double transgene construct in which expression of *BoATPS1* was controlled by the weaker *UBQ10* promoter, ATPS activity in purified chloroplasts was

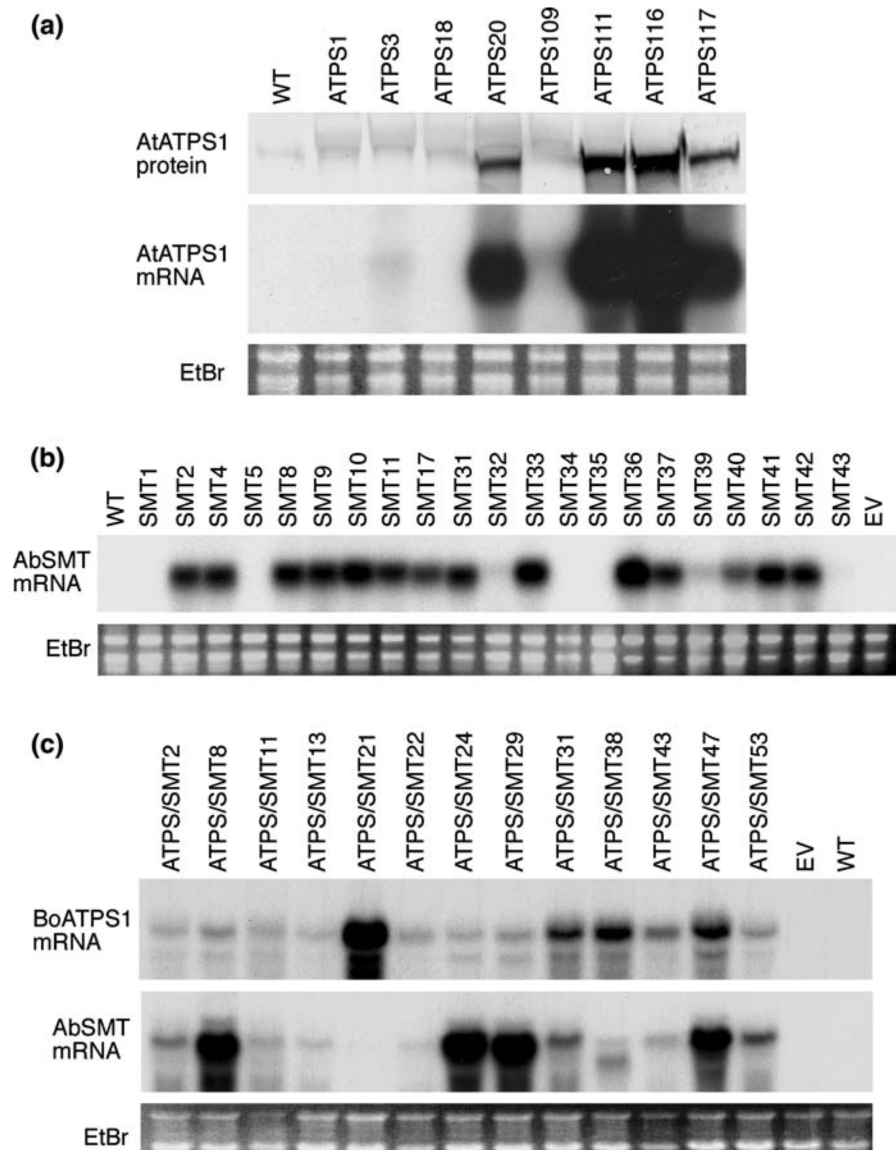

Fig. 2 Screening of tobacco primary transformants. **a** *ATPS* overexpression in plants transformed with a *35S:AtATPS1:nos* construct. RNA gel blot showing transgene mRNA abundance (lower panels), and protein gel blot showing recombinant ATPS protein cross-reacting with antiserum to onion ATPS (upper panel). **b** *SMT* overexpression in plants transformed with a *35S:AbSMT:nos* construct. RNA gel blot showing transgene mRNA abundance. **c** *ATPS* and *SMT* overexpression in plants transformed with a *35S:AbSMT:nos/UBQ10:BoATPS1:nos* construct. mRNA abundance of transgene *BoATPS1* (upper panel) and *AbSMT* (lower panel). WT, wild-type; EV, empty vector transformants

approximately 10-fold greater than in wild-type (Fig. 3c upper panel), and SMT activity was present (Fig. 3c lower panel) at levels similar to that in lines overexpressing *AbSMT* alone.

Effect of selenate on *ATPS* transformants

Plants of three independent transgenic lines with enhanced ATPS activity were watered with sodium selenate at 0, 50, 200 or 300 μM in 0.5× Hoagland's solution, and monitored over the subsequent 14 days. Leaf toxicity symptoms were recorded relative to a standardised scale (Fig. 4) and, when expressed as an average value per leaf per plant, transgenic lines exhibited no more signs of toxicity than did wild-type or empty vector lines (Fig. 5a). Treatment with 50 μM selenate had little effect on growth, but the higher selenate concentrations reduced shoot growth with the Se dose effect being similar for wild-type, empty vector and the transgenic lines (Fig. 5b). Plants accumulated similar amounts of foliar Se when watered with 50, 200 or 300 μM selenate, and transgenic plants with enhanced ATPS activity did not accumulate greater amounts of Se than wild-type or empty vector lines (Fig. 5c).

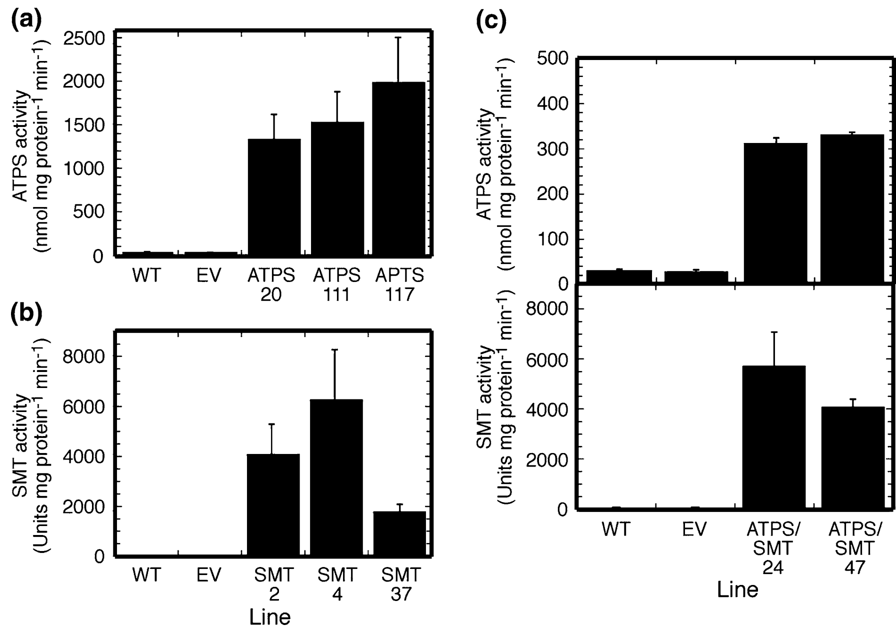

Fig. 3 Enzyme activities of transformed lines. **a** Lines overexpressing *ATPS*. **b** Lines overexpressing *SMT*. **c** Lines overexpressing both genes, *ATPS* (upper panel) and *SMT* (lower panel). ATPS activities are nmol of APS produced mg^{-1} protein min^{-1}. SMT activities are expressed as arbitrary phosphorimager units detected in [^{14}C]MeSeCys after thin layer chromatography of reaction products, as mg^{-1} protein min^{-1}. Error bars denote SE between replicate determinations ($n = 3$). WT, wild-type; EV, empty vector transformants

Effect of selenate on *SMT* transformants

Plants of three transgenic lines with enhanced SMT activity were watered with selenate at rates of 0, 50, 200 and 300 µM. Both plant growth and appearance were strongly affected by exposure to selenate at high concentrations. Plants watered with 200 and 300 µM selenate displayed significant symptoms of Se toxicity compared with control plants (Fig. 6a), including black spots, regions of necrosis and death of entire leaves. *SMT*-overexpressing lines exhibited leaf toxicity symptoms 1.5- to 3-fold higher than wild-type or empty vector lines.

Overall growth of the plants was also affected by high selenate concentrations. The shoot weight of all plants was substantially reduced by selenate treatment above 50 µM, with 300 µM selenate generally having a greater effect than 200 µM (Fig. 6b). The Se dose effect was similar for all lines. At 300 µM treatment, the reduced shoot weight was related both to a substantial decrease in shoot height and a reduction in overall leaf number, of on average one per plant (data not shown).

At the termination of selenate watering, total foliar Se content was determined (Fig. 6c). After watering with 200 or 300 µM selenate, all *SMT*-overexpressing lines recorded a large increase in Se content compared with controls, at 200 µM selenate ranging from 2-fold in line SMT4, to 4-fold in lines SMT2 and 37. However, when the shoot weight of plants watered with 200 µM selenate was expressed as a percentage of the shoot weight of plants from the same line watered without selenate, the reduction in growth of SMT lines 2, 4 and 37 (55, 54 and 41%, respectively) was no worse than that of wild-type and empty vector controls (54 and 63%, respectively). This was despite the much larger amounts of Se accumulated in the *SMT*-overexpressing lines than in wild-type or empty vector lines.

Effect of selenate on *ATPS/SMT* transformants

Plants of two *ATPS/SMT* transgenic lines were watered with selenate at rates of 0, 50 and 200 µM. Due to the severe effects of 300 µM selenate on plant development (see above), this treatment was omitted. *ATPS/SMT* transformant plants watered with 200 µM selenate displayed leaf toxicity symptoms at least 2-fold higher than wild-type or empty vector lines (Fig. 7a), similar to that observed with the *SMT*-overexpressing lines (Fig. 6a).

The shoot weight achieved after 14 days of selenate treatment was not affected by 50 µM selenate, but was reduced for all plants exposed to 200 µM selenate (Fig. 7b), as seen with the *SMT*-overexpressing lines (Fig. 6b). When the shoot

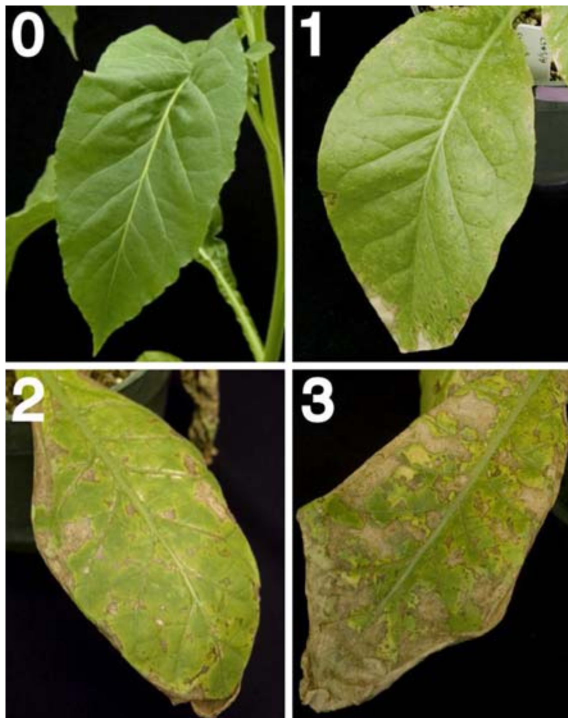

Fig. 4 Typical Se toxicity symptoms on tobacco leaves. Leaves were assessed relative to this standardized scale, and assigned the score shown: 0 for no visible symptoms; 1 for a few black lesions or small necroses covering less than 10% of the leaf blade; 2 for numerous black lesions or brown necroses covering 10 to 50% of the leaf blade; 3 for numerous black lesions or brown necroses covering greater than 50% of the leaf blade

weight of plants watered with 200 μM selenate was expressed as a percentage of the shoot weight of plants from the same line watered without selenate, the reduction in growth of the ATPS/SMT24 and 47 was not significantly different from the reduction observed in wild-type and empty vector lines.

Following watering with 200 μM selenate, both *ATPS/SMT*-overexpressing lines had a substantial increase in leaf Se content compared with wild-type and empty vector lines, ranging from a 2-fold increase in line ATPS/SMT47, to a 3-fold increase in line ATPS/SMT24 (Fig. 7c).

Conversion of inorganic selenate to MeSeCys

The *AbSMT* transgene encodes the SMT enzyme, which is responsible for the generation of MeSeCys from SeCys (Fig. 1). To assess the functionality of the recombinant enzyme in tobacco plants, LC-MS

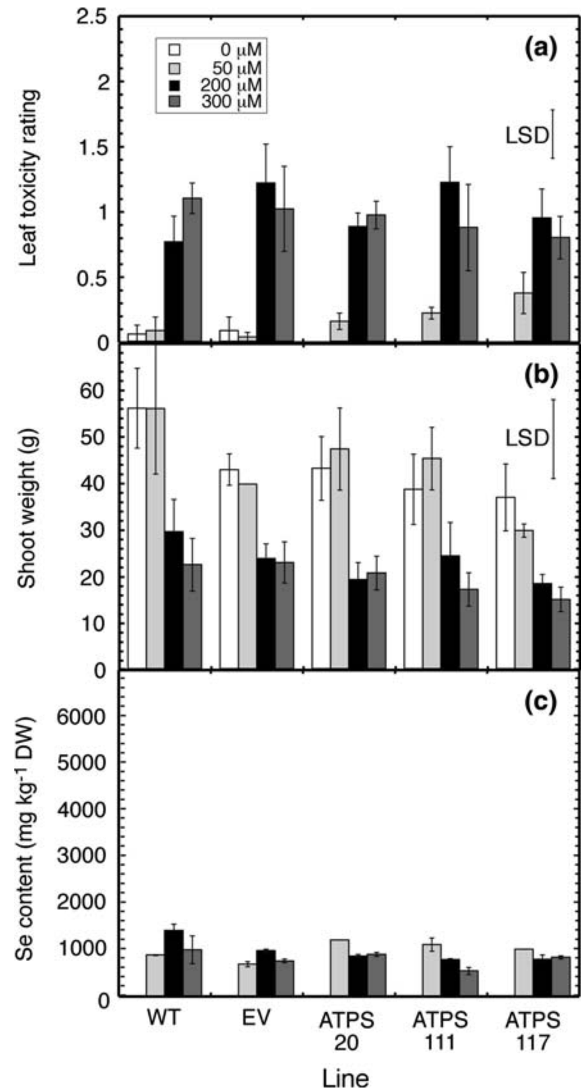

Fig. 5 Effect of selenate on *ATPS*-overexpressing lines. After 14 d of treatment with 0, 50, 200 or 300 μM selenate, leaf toxicity symptoms (**a**) and fresh weight of leaves (**b**) were recorded. Total Se accumulated by the leaves was determined on a mg kg^{-1} dry weight basis (**c**). Error bars in (**a**) and (**b**) denote SE between replicate plants of the same genotype ($n = 4$–6) and the least significant difference (LSD) at $P < 0.05$ is indicated. In (**c**) error bars denote replicate samples of pooled tissue from 4–6 plants ($n = 3$); the coefficient of variability between replicates was 10%. A representative experiment (of two) is shown. WT, wild-type; EV, empty vector transformants

analysis was used to detect MeSeCys in leaf material from the *SMT*-transformed lines after watering with 50, 200 and 300 μM selenate (Table 1). In wild-type, neither MeSeCys nor MeCys was present above the detection limit at any watering rate. In contrast, all

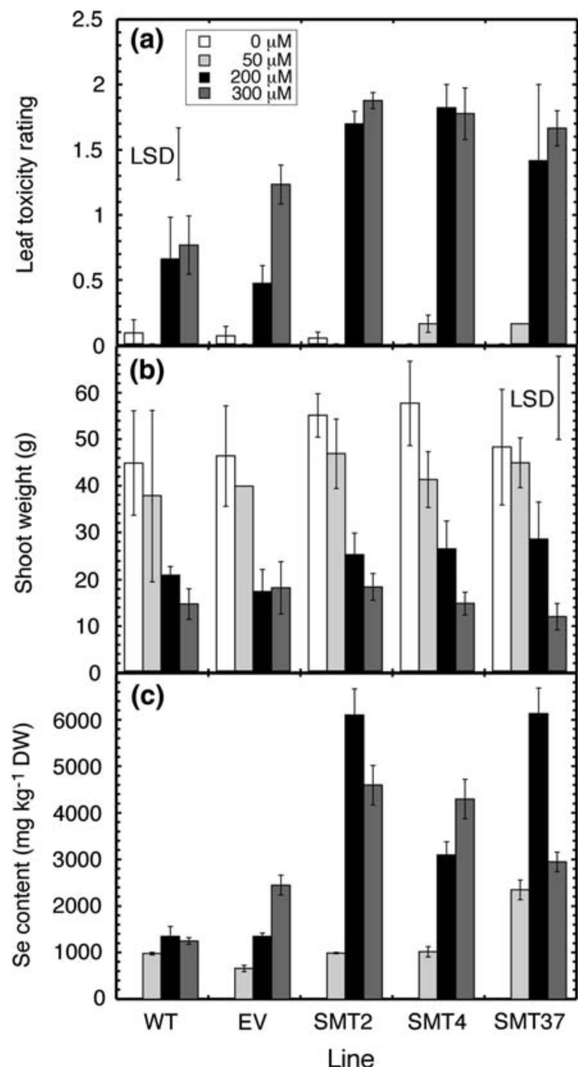

Fig. 6 Effect of selenate on *SMT*-overexpressing lines. After 14 d of treatment with 0, 50, 200 or 300 μM selenate, leaf toxicity symptoms (**a**) and fresh weight of leaves (**b**) were recorded. Total Se accumulated by the leaves was determined on a mg kg^{-1} dry weight basis (**c**). Error bars in (**a**) and (**b**) denote SE between replicate plants of the same genotype ($n = 6$) and LSD at $P < 0.05$ is indicated. In (**c**) error bars denote replicate samples of pooled tissue from 4–6 plants ($n = 3$); the coefficient of variability between replicates was 10%. A representative experiment (of two) is shown. WT, wild-type; EV, empty vector transformants

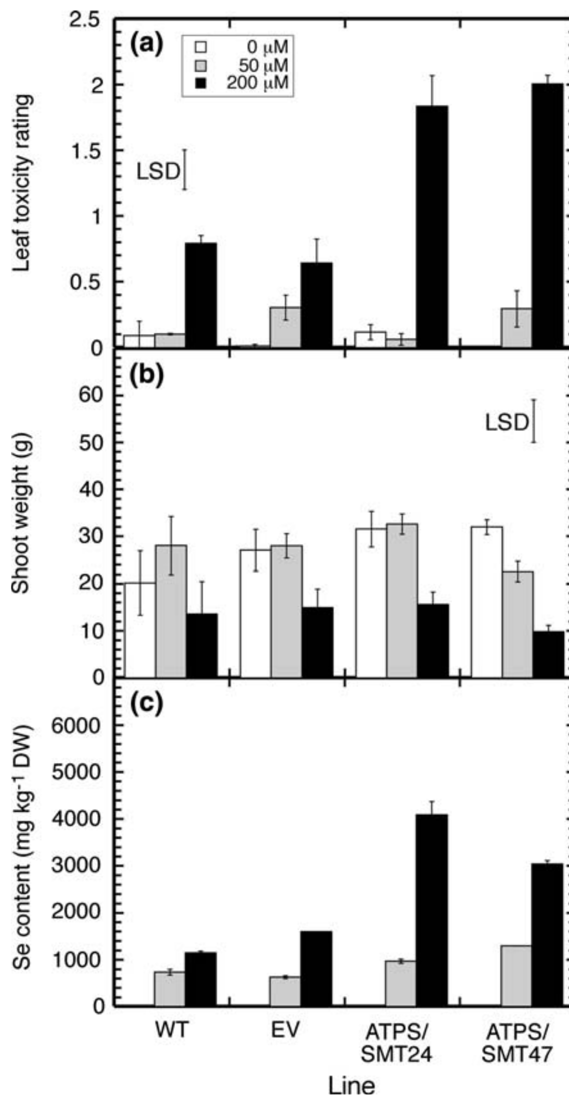

Fig. 7 Effect of selenate on *ATPS/SMT*-overexpressing lines. After 14 d of treatment with 0, 50 or 200 μM selenate, leaf toxicity symptoms (**a**) and fresh weight of leaves (**b**) were recorded. Total Se accumulated by the leaves was determined on a mg kg^{-1} dry weight basis (**c**). Error bars in (**a**) and (**b**) denote SE between replicate plants of the same genotype ($n = 5$) and LSD at $P < 0.05$ is indicated. In (**c**) error bars denote replicate samples of pooled tissue from 4–6 plants ($n = 3$); the coefficient of variability between replicates was 9%. A representative experiment (of two) is shown. WT, wild-type; EV, empty vector transformants

transgenic lines overexpressing *SMT* accumulated MeSeCys, relatively low amounts at a watering rate of 50 μM selenate, but substantial amounts of MeSeCys, ranging from 532 to 1443 mg kg^{-1} DW, at higher watering rates. The percentage conversion of inorganic Se into the Se component of MeSeCys was low at a watering rate of 50 μM selenate (1.1–2.2%), but substantially greater at higher watering rates where up to 20% of the total Se accumulated by the plants was present in the MeSeCys form. MeCys was also detected in all the transformants,

although at much lower concentrations than MeSeCys (Table 1), consistent with other studies (Ellis et al. 2004; Lyi et al. 2005).

After watering with selenate, MeSeCys also accumulated in plants transformed with both the *ATPS* and *SMT* transgenes (Table 2). As with the *SMT* alone transformants, when the Se content of MeSeCys in these plants was expressed as a percentage of total Se accumulated, the percentage conversion of inorganic Se into MeSeCys was higher at a watering rate of 200 μM selenate than at 50 μM. At a watering rate of 200 μM selenate, up to 10% of the plant's total Se was present in MeSeCys in line ATPS/SMT47 (Table 2). However, in this experiment the amounts of total Se accumulated (Fig. 7c) and the amounts of MeSeCys present (Table 2) were both less than in the experiment with the *SMT*-overexpressing transgenic plants (cf. Fig. 6c, Table 1).

To further examine the function of the *ATPS* transgene, Se accumulation by *SMT* and *ATPS/SMT* transformants was examined when plants of the same age had been watered with 300 μM selenate under identical conditions (Fig. 8). Despite the lack of direct comparability inevitable when comparing plants that are the result of different, independent transgenic events, Se accumulation was similar in all four transgenic lines, indicating that the presence of the *ATPS* transgene had little effect on total Se accumulation (Fig. 8). As found previously, the *SMT* transgene increased Se accumulation in the leaves by several fold relative to wild-type. The Se content of roots was much lower than leaves, and was not increased by the presence of the transgenes. However, as shown in Table 3, plants with enhanced ATPS activity (lines ATPS/SMT24 and 47) had somewhat greater accumulation of MeSeCys and GluMeSeCys than plants possessing wild-type levels of ATPS activity (lines SMT2 and 37).

Analysis of volatile S/Se compounds produced by *SMT* and *ATPS/SMT* transformants

As a further confirmation of the *in planta* function of the SMT enzyme, two transformed lines from each *SMT*-overexpressing population were chosen for analysis of volatile Se-containing compounds produced after watering with 300 μM selenate. No Se volatiles were detected in any of the transformed lines in the absence of selenate watering (data not shown), but after selenate watering three related molecular species, dimethyl selenide (Me_2Se), dimethyl diselenide (Me_2Se_2) and dimethyl selenosulfenate (Me_2SeS), were detected amongst head space volatiles (Table 4). All four transformants produced substantial amounts of dimethyl diselenide, which is formed from MeSeCys (Fig. 1), thus indirectly confirming the presence of MeSeCys-forming SMT activity in these plants. Substantial amounts of dimethyl selenide were also produced by all transgenic lines, but not by wild-type controls. Dimethyl selenide is produced from SeCys via SeMet, and does not require the activity of SMT (Fig. 1). The low levels of dimethyl selenide produced by selenate-watered wild-type plants was initially surprising, but

Table 1 Quantity of MeSeCys (mg kg^{-1} DW) and % of total Se in MeSeCys in tobacco lines overexpressing a *SMT* transgene, following watering with 50, 200 or 300 μM selenate for 14 days

Line	Se treatment (μM)	MeCys (mg kg^{-1} DW)	MeSeCys (mg kg^{-1} DW)	Se in MeSeCys (% total Se)[a]
Wild-type	50	nd[b]	nd	–
	200	nd	nd	–
	300	nd	nd	–
SMT2	50	77	50	2.2
	200	91	975	6.9
	300	158	866	8.2
SMT4	50	74	44	1.9
	200	164	1443	20.2
	300	192	1180	11.9
SMT37	50	211	59	1.1
	200	129	1047	4.0
	300	74	532	7.8

[a] Calculated using only the Se component of MeSeCys

[b] nd, not detected at detection limit of 40 mg kg^{-1}

Table 2 Quantity of MeSeCys (mg kg^{-1} DW) and % of total Se in MeSeCys in tobacco lines overexpressing *ATPS* and *SMT* transgenes, following watering with 50 or 200 μM selenate for 14 days

Line	Se treatment (μM)	MeCys (mg kg^{-1} DW)	MeSeCys (mg kg^{-1} DW)	MeSeCys (% total Se)[a]
Wild-type	50	nd[b]	nd	–
	200	nd	nd	–
ATPS/SMT24	50	170	101	4.5
	200	nd	659	7.0
ATPS/SMT47	50	60	113	3.8
	200	125	686	9.8

[a] Calculated using only the Se component of MeSeCys

[b] nd, not detected at detection limit of 40 mg kg^{-1}

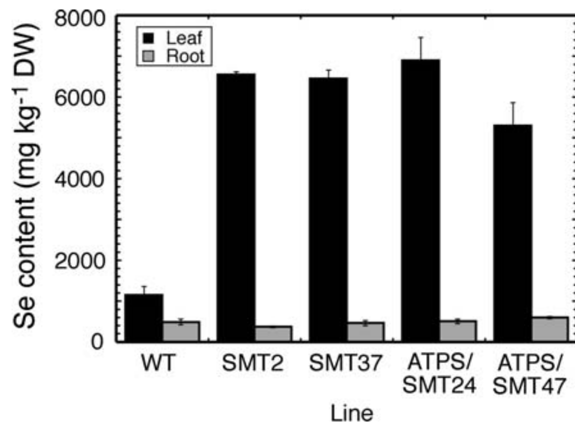

Fig. 8 Total Se accumulated by transgenic lines overexpressing *SMT* or *ATPS/SMT*. After 17 d of treatment with 300 μM selenate, total Se accumulated by the leaves and roots was determined on a mg kg^{-1} dry weight basis. Error bars denote SE between replicate plants of the same genotype ($n = 2$). The coefficient of variability between replicates was 11%. WT, wild-type

these plants accumulated much less Se than plants overexpressing an *SMT* transgene (Figs. 6c, 7c). Low amounts of dimethyl selenosulfenate were also

Table 4 Relative amounts (peak areas sample^{-1}) of volatile Se compounds detected in head-space of tobacco lines overexpressing a *SMT* transgene or *ATPS* and *SMT* transgenes, following watering with 300 μM selenate for 14–17 days. Data are from a representative experiment (of two)

Line	Me_2Se	Me_2SeS	Me_2Se_2
Wild-type	6	nd[a]	nd
SMT2	374	8	241
SMT37	47	nd	132
ATPS/SMT24	1077	54	1370
ATPS/SMT47	208	8	115

[a] nd, not detected at detection limit of <1

detected in the head space in three of the four transformed lines.

Discussion

To examine the role of ATPS in the uptake of Se by whole tobacco plants, wild-type controls and transgenic lines with at least a 10-fold increase in

Table 3 Quantity of MeSeCys and its γ-glutamyl derivative (mg kg^{-1} DW) and % of total Se in MeSeCys or its derivative in tobacco lines overexpressing a *SMT* transgene or *ATPS* and *SMT* transgenes, following watering with 300 μM selenate for 17 days (± SD)

Line	MeCys (mg kg^{-1} DW)	MeSeCys (mg kg^{-1} DW)	GluMeSeCys (mg kg^{-1} DW)	MeSeCys (% total Se)[a]
Wild-type	nd[b]	12 ± 1	nd	–
SMT2	90 ± 2	723 ± 3	11 ± 1	4.8
SMT37	66 ± 3	799 ± 10	10 ± 0	5.4
ATPS/SMT24	125 ± 15	1467 ± 122	32 ± 2	9.3
ATPS/SMT47	80 ± 14	894 ± 41	25 ± 2	7.4

[a] Calculated using only the Se component of MeSeCys

[b] nd, not detected at detection limit of 10 mg kg^{-1}

chloroplast ATPS specific activity were exposed to selenate under a continuous watering regime. Total Se accumulation by the *ATPS*-overexpressing plants was not increased relative to controls and no evidence of increased Se toxicity or growth inhibition was observed (Fig. 5). In some species, ATPS has been proposed as the rate-limiting activity controlling the pathway of S assimilation (Leustek 1996). Indeed, in Indian mustard a 2-fold overexpression of ATPS activity increased selenate reduction, plant tolerance to Se and the accumulation of both total Se and Se in the form of organic compounds (Pilon-Smits et al. 1999). In contrast, in *Arabidopsis* a 10-fold enhancement of ATPS activity resulted in an increased rate of selenate reduction, but reduced total Se accumulation and selenate tolerance (Sors et al. 2005a), and in cultured tobacco cells an 8-fold overexpression of ATPS activity did not affect growth in the presence of selenate or appear to be limiting for cell metabolism (Hatzfeld et al. 1998). Our data are thus consistent with the findings of Hatzfeld et al. (1998) who studied cultured cells of the same species used in our study, and suggest that ATPS activity is not always limiting to S/Se assimilation. Indeed, in maize the activity of the second enzyme necessary for selenate reduction, APS reductase, appears to be a major control point for sulfate reduction (Martin et al. 2005), and studies of various species of *Astragalus* with differing Se accumulation ability have found that ATPS activity does not correlate with Se hyperaccumulation (Sors et al. 2005a).

Tobacco is a member of the Solanaceae, and lacks detectable SMT activity (Fig. 3) or the ability to produce more than negligible amounts of MeSeCys (Table 3). In the Se hyperaccumulator *A. bisulcatus*, Se tolerance apparently results from the constitutive expression of *SMT* in all tissues (Pickering et al. 2003). Expression of this gene allows the majority of the Se accumulated in active young leaves to be stored in the form of MeSeCys (or GluMeSeCys), or subsequently volatalized, with much lower levels accumulating as toxic inorganic selenate or selenite (Pickering et al. 2000; Freeman et al. 2006). In broccoli, a secondary Se accumulator, expression of the orthologous gene is low or undetectable unless plants are watered with selenate, when mRNA abundance dramatically increases (Lyi et al. 2005). Transgenic tobacco lines overexpressing *SMT* had greatly increased Se content (Fig. 6c), consistent with observations in transgenic *Arabidopsis* and Indian mustard (Ellis et al. 2004; LeDuc et al. 2004). Despite this increase, which presumably explains the severe Se toxicity symptoms observed (Fig. 6a), growth inhibition in plants watered with 200 μM selenate was no worse than that in controls (Fig. 6b). Since increasing Se accumulation is increasingly inhibitory to growth (White et al. 2004), this suggests that in the transgenic lines the ability to sequester a proportion of the Se entering the organic pathway as MeSeCys partially protected them from Se damage. Indeed, plants overexpressing *SMT* produced up to 1400 mg kg^{-1} DW MeSeCys in the leaf tissue (up to 20% of the total Se), compared with wild-type plants in which no MeSeCys could be detected following watering with selenate (Table 1). As a percentage of total Se accumulated, this ability to convert inorganic Se into organic forms increased with exposure to increasing concentrations of selenate (Tables 1, 2). Presumably this protects enzymatic metabolism by directing organic Se away from misincorporation into proteins (Fig. 1).

Overexpression of *SMT* in *Arabidopsis* and Indian mustard has produced varying results, despite both species being in the Brassicaceae. In transgenic *Arabidopsis* watered with 100 μM selenite, Se tolerance was achieved, and correlated with the ability to sequester Se as MeSeCys (Ellis et al. 2004). However, in contrast to *SMT*-overexpressing tobacco, MeSeCys was not produced if selenate was the sole source of Se and, since the bulk of the Se remained as selenate, this suggests that reduction of selenate to selenite is probably the limiting step in *Arabidopsis* (Ellis et al. 2004). In Indian mustard, *SMT* overexpression improved tolerance to both selenate and selenite (LeDuc et al. 2004). MeSeCys accumulation was increased relative to wild-type after watering with either compound, although a proportionally greater conversion occurred after watering with selenite. Tobacco is thus more similar to Indian mustard than *Arabidopsis*, although it should be noted that Indian mustard is a secondary Se accumulator and even wild-type plants have the ability to biosynthesise MeSeCys (LeDuc et al. 2004), and that some *Arabidopsis* accessions have a selenite-inducible putative *SMT* gene (Zhang et al. 2006).

A large amount of the selenate taken up by plants and translocated to the shoot remains as selenate, with only a proportion being reduced to selenite by ATPS and ultimately being converted into organic Se

(Asher et al. 1967; de Souza et al. 1998; Zayed et al. 1998). This unconverted selenate also has toxic effects on metabolism, additional to those mediated through the altered properties of Se-containing proteins (Terry et al. 2000). Tobacco plants overexpressing both *ATPS* and *SMT* did not accumulate greater amounts of total Se than transgenic plants overexpressing *SMT* alone (Fig. 8), confirming the results of experiments overexpressing *ATPS* alone where no restriction of total Se accumulation from selenate due to limiting ATPS activity was observed (Fig. 5). Tobacco thus differs substantially from Indian mustard (already a Se accumulator), lines of which overexpressing both *SMT* and *ATPS* accumulated greater amounts of Se from selenate than those overexpressing *SMT* alone (LeDuc et al. 2006). However, in tobacco enhanced ATPS activity (in the presence of SMT activity) caused a greater proportion of the total accumulated Se to be channelled into organic forms (Table 3), suggesting that upregulation of ATPS may be beneficial in attempts to increase MeSeCys content, even in Solanaceae.

The type of Se volatiles produced by different species of plants is characteristic of their Se accumulation status. In enzyme extracts of cabbage, the only major Se-containing volatile produced was dimethyl selenide (Lewis et al. 1971), whereas in the Se hyperaccumulator *A. bisulcatus* dimethyl diselenide was the only major volatile detected (Evans et al. 1968). Only in Se accumulators, where Se flow is diverted into MeSeCys, can dimethyl diselenide be produced (Fig. 1), and this ability gives such plants an additional capacity to discharge a proportion of the accumulated Se as volatiles. Wild-type tobacco plants watered with 300 μM selenate produced small amounts of dimethyl selenide but dimethyl diselenide was not detected, as would be expected of a non-accumulator (Table 4). On the other hand, tobacco plants transformed with a *SMT* transgene produced both dimethyl selenide and dimethyl diselenide. In Indian mustard plants watered with selenate or selenite, the ratio of dimethyl selenide to dimethyl diselenide produced has been successfully used to discriminate between wild-type, and *SMT*- and *ATPS/SMT*-overexpressing plants (Kubachka et al. 2007). Such plants also produced smaller amounts of a third major Se-containing volatile, dimethyl selenosulfenate (Meija et al. 2002; Kubachka et al. 2007), which was similarly detected at low amounts in transgenic tobacco (Table 4).

In this study we have demonstrated that it is possible to convert a Solanaceous species, which unlike the Brassicaceae lacks the S-based secondary metabolism responsible for producing compounds such as glucosinolates and S-containing phytoalexins (Halkier and Gershenzon 2006; Macías et al. 2007), from a Se non-accumulator into an accumulator of the cancer-preventing compound MeSeCys. This extends the observed functionality of the *SMT* gene beyond the Brassicaceae and a small number of other species. Expanding the range of crops that can produce the cancer-preventing compound MeSeCys is useful, since garlic is generally eaten in small quantities and the number of Brassicaceous food crops is limited. In investigating the putative rate limiting step of Se uptake we found that, unlike in *Arabidopsis* (Ellis et al. 2004), wild-type levels of ATPS activity are apparently sufficient to allow MeSeCys accumulation from supplied selenate in tobacco, and that additional ATPS activity did not increase total Se assimilation, as was the case in Indian mustard (LeDuc et al. 2004). This indicates that ATPS is not rate limiting for total Se uptake in tobacco, and perhaps for the Solanaceae in general. However, a proportionally greater incorporation of inorganic Se into MeSeCys was observed when ATPS activity was enhanced. We conclude that while the *SMT* gene can presumably be widely utilized for moving the trait of MeSeCys accumulation into a variety of crop plants, the effects of ATPS on Se assimilation may be specific to the plant family. The data also suggest that, on a percentage basis, the conversion of inorganic Se into MeSeCys increases with increasing concentration of selenate supplied, and therefore that substantial accumulation of MeSeCys may require exposure of the plants to high levels of selenate, even if ATPS is upregulated. If significant production of MeSeCys can be achieved in crop plants without excess inorganic Se accumulation, the *SMT* gene of *A. bisulcatus* could be a route to the enrichment of crops with a compound possessing powerful anticancer properties.

Acknowledgements We thank Dr Thomas Leustek (Rutgers University) for the *Arabidopsis ATPS* vector; Dr Bernhard Neuhierl (Institute for Genetics and Microbiology, Munich) for the *Astragalus SMT* cDNA; Prof Michael McManus (Massey University, Palmerston North) for the onion ATPS antiserum;

Ian King for care of the transgenic plants; Dr John Finley (AM Todd Inc., Montgomeryville, PA) for many useful discussions; and Duncan Hedderley for statistical analysis. This work was funded by the New Zealand Foundation for Research, Science and Technology (FRST), and by Vital Vegetables, a Trans-Tasman research project jointly funded and supported by Horticulture Australia Ltd., the New Zealand Institute for Plant & Food Research Ltd., FRST, the Victorian Department of Primary Industries, Horticulture New Zealand and the Australian Vegetable and Potato Growers Federation Inc.

References

Arvy MP (1993) Selenate and selenite uptake and translocation in bean plants (*Phaseolus vulgaris*). J Exp Bot 44:1083–1087. doi:10.1093/jxb/44.6.1083

Asher CJ, Evans CS, Johnson CM (1967) Collection and partial characterization of volatile selenium compounds from *Medicago sativa*. Aust J Biol Sci 20:737–748

Broadley MR, White PJ, Bryson RJ, Meacham MC, Bowen HC, Johnson SE, Hawkesford MJ, McGrath SP, Zhao FJ, Breward N, Harriman M, Tucker M (2006) Biofortification of UK food crops with selenium. Proc Nutr Soc 65:169–181. doi:10.1079/PNS2006490

Brown TA, Shrift A (1981) Exclusion of selenium from proteins of selenium-tolerant *Astragalus* species. Plant Physiol 67:1051–1053

Church GM, Gilbert W (1984) Genomic sequencing. Proc Natl Acad Sci USA 81:1991–1995. doi:10.1073/pnas.81.7.1991

Clark LC, Combs GF, Turnbull BW, Slate EH, Chalker DK, Chow J, Davis LS, Glover RA, Graham GF, Gross EG, Krongrad A, Lesher JL, Park HK, Sanders BB, Smith CL, Taylor JR (1996) Effects of selenium supplementation for cancer prevention in patients with carcinoma of the skin. JAMA 276:1957–1963. doi:10.1001/jama.276.24.1957

de Souza MP, Pilon-Smits EAH, Lytle CM, Hwang S, Tai J, Honma TSU, Yeh L, Terry N (1998) Rate-limiting steps in selenium assimilation and volatilization by Indian mustard. Plant Physiol 117:1487–1494. doi:10.1104/pp.117.4.1487

Ellis DR, Salt DE (2003) Plants, selenium and human health. Curr Opin Plant Biol 6:273–279. doi:10.1016/S1369-5266(03)00030-X

Ellis DR, Sors TG, Brunk DG, Albrecht C, Orser C, Lahner B, Wood KV, Harris HH, Pickering IJ, Salt DE (2004) Production of Se-methylselenocysteine in transgenic plants expressing selenocysteine methyltransferase. BMC Plant Biol 4:1. doi:10.1186/1471-2229-4-1

Evans CS, Asher CJ, Johnson CM (1968) Isolation of dimethyl diselenide and other volatile selenium compounds from *Astragalus recemosus*. Aust J Biol Sci 21:13–20

Finley JW (2007) Increased intakes of selenium-enriched foods may benefit human health. J Sci Food Agric 87:1620–1629. doi:10.1002/jsfa.2943

Finley JW, Davis CD (2001) Selenium (Se) from high-selenium broccoli is utilized differently than selenite, selenate and selenomethionine, but is more effective in inhibiting colon carcinogenesis. Biofactors 14:191–196

Finley JW, Davis CD, Feng Y (2000) Selenium from high selenium broccoli protects rats from colon cancer. J Nutr 130:2384–2389

Finley JW, Ip C, Lisk DJ, Davis CD, Hintze KJ, Whanger PD (2001) Cancer-protective properties of high-selenium broccoli. J Agric Food Chem 49:2679–2683. doi:10.1021/jf0014821

Freeman JL, Zhang LH, Marcus MA, Fakra S, McGrath SP, Pilon-Smits EAH (2006) Spatial imaging, speciation, and quantification of selenium in the hyperaccumulator plants *Astragalus bisulcatus* and *Stanleya pinnata*. Plant Physiol 142:124–134. doi:10.1104/pp.106.081158

Gleave AP (1992) 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 20:1203–1207. doi:10.1007/BF00028910

Halkier BA, Gershenzon J (2006) Biology and biochemistry of glucosinolates. Annu Rev Plant Biol 57:303–333. doi:10.1146/annurev.arplant.57.032905.105228

Hatzfeld Y, Cathala N, Grignon C, Davidian JC (1998) Effect of ATP sulfurylase overexpression in Bright Yellow 2 tobacco cells—regulation of ATP sulfurylase and SO_4^{2-} transport activities. Plant Physiol 116:1307–1313. doi:10.1104/pp.116.4.1307

Hoagland DR, Arnon DI (1950) The water-culture method for growing plants without soil. Calif Agric Exp Sta Circ 347, Berkeley, CA

Horsch RB, Fry JE, Hoffmann NL, Eichholtz D, Rogers SG, Fraley RT (1985) A simple and general method for transferring genes into plants. Science 227:1229–1231. doi:10.1126/science.227.4691.1229

Ip C, Birringer M, Block E, Kotrebai M, Tyson JF, Uden PC, Lisk DJ (2000a) Chemical speciation influences comparative activity of selenium-enriched garlic and yeast in mammary cancer prevention. J Agric Food Chem 48:2062–2070. doi:10.1021/jf000051f

Ip C, Thompson HJ, Zhu ZJ, Ganther HE (2000b) In vitro and in vivo studies of methylseleninic acid: evidence that a monomethylated selenium metabolite is critical for cancer chemoprevention. Cancer Res 60:2882–2886

James F, Nolte KD, Hanson AD (1995) Purification and properties of *S*-adenosyl-L-methionine:L-methionine *S*-methyltransferase from *Wollastonia biflora* leaves. J Biol Chem 270:22344–22350. doi:10.1074/jbc.270.43.25827

Kim T, Jung U, Cho DY, Chung AS (2001) Se-methylselenocysteine induces apoptosis through caspase activation in HL-60 cells. Carcinogenesis 22:559–565. doi:10.1093/carcin/22.4.559

Kubachka KM, Meija J, Leduc DL, Terry N, Caruso JA (2007) Selenium volatiles as proxy to the metabolic pathways of selenium in genetically modified *Brassica juncea*. Environ Sci Technol 41:1863–1869. doi:10.1021/es0613714

Kunst L (1998) Preparation of physiologically active chloroplasts from *Arabidopsis*. In: Martínez-Zapater J, Salinas J (eds) Methods in molecular biology, vol 82 *Arabidopsis* protocols. Humana Press, Totowa, NJ

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685. doi:10.1038/227680a0

Larsen E, Lobinski R, Burger-Meÿer K, Hansen M, Ruzik R, Mazurowska L, Rasmussen P, Sloth J, Scholten O, Kik C

(2006) Uptake and speciation of selenium in garlic cultivated in soil amended with symbiotic fungi (mycorrhiza) and selenate. Anal Bioanal Chem 385:1098–1108. doi:10.1007/s00216-006-0535-x

LeDuc DL, Tarun AS, Montes-Bayon M, Meija J, Malit MF, Wu CP, AbdelSamie M, Chiang CY, Tagmount A, DeSouza M, Neuhierl B, Bock A, Caruso J, Terry N (2004) Overexpression of selenocysteine methyltransferase in *Arabidopsis* and Indian mustard increases selenium tolerance and accumulation. Plant Physiol 135:377–383. doi:10.1104/pp.103.026989

LeDuc DL, AbdelSamie M, Montes-Bayon M, Wu CP, Reisinger SJ, Terry N (2006) Overexpressing both ATP sulfurylase and selenocysteine methyltransferase enhances selenium phytoremediation traits in Indian mustard. Environ Pollut 144:70–76. doi:10.1016/j.envpol.2006.01.008

Leustek T (1996) Molecular genetics of sulfate assimilation in plants. Physiol Plant 97:411–419. doi:10.1034/j.1399-3054.1996.970228.x

Leustek T, Saito K (1999) Sulfate transport and assimilation in plants. Plant Physiol 120:637–643. doi:10.1104/pp.120.3.637

Leustek T, Murillo M, Cervantes M (1994) Cloning of a cDNA encoding ATP sulfurylase from *Arabidopsis thaliana* by functional expression in *Saccharomyces cerevisiae*. Plant Physiol 105:897–902. doi:10.1104/pp.105.3.897

Lewis BG, Johnson CM, Broyer TC (1971) Cleavage of Se-methylselenomethionine selenonium salt by a cabbage leaf enzyme fraction. Biochim Biophys Acta 237:603–605

Lyi SM, Heller LI, Rutzke M, Welch RM, Kochian LV, Li L (2005) Molecular and biochemical characterization of the selenocysteine *Se*-methyltransferase gene and *Se*-methylselenocysteine synthesis in broccoli. Plant Physiol 138:409–420. doi:10.1104/pp.104.056549

Lyons GH, Stangoulis JCR, Graham RD (2004) Exploiting micronutrient interaction to optimize biofortification programs: the case for inclusion of selenium and iodine in the *HarvestPlus* program. Nutr Rev 62:247–252. doi:10.1301/nr2004.jun247-252

Macías FA, Galindo JLG, Galindo JCG (2007) Evolution and current status of ecological phytochemistry. Phytochemistry 68:2917–2936. doi:10.1016/j.phytochem.2007.10.010

Martin MN, Tarczynski MC, Shen B, Leustek T (2005) The role of 5′-adenylylsulfate reductase in controlling sulfate reduction in plants. Photosynth Res 86:309–323. doi:10.1007/s11120-005-9006-z

Meija J, Montes-Bayon M, LeDuc DL, Terry N, Caruso JA (2002) Simultaneous monitoring of volatile selenium and sulfur species from Se accumulating plants (wild type and genetically modified) by GC/MS and GC/ICPMS using solid-phase microextraction for sample introduction. Anal Chem 74:5837–5844. doi:10.1021/ac020285t

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473–497. doi:10.1111/j.1399-3054.1962.tb08052.x

Murillo M, Leustek T (1995) Adenosine-5′-triphosphate-sulfurylase from *Arabidopsis thaliana* and *Escherichia coli* are functionally equivalent but structurally and kinetically divergent: nucleotide sequence of two adenosine-5′-triphosphate-sulfurylase cDNAs from *Arabidopsis thaliana* and analysis of a recombinant enzyme. Arch Biochem Biophys 323:195–204. doi:10.1006/abbi.1995.0026

Neuhierl B, Böck A (1996) 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 239:235–238. doi:10.1111/j.1432-1033.1996.0235u.x

Neuhierl B, Böck A (2002) Selenocysteine methyltransferase. Methods Enzymol 347:203–207. doi:10.1016/S0076-6879(02)47019-X

Neuhierl B, Thanbichler M, Lottspeich F, Böck A (1999) A family of *S*-methylmethionine-dependent thiol/selenol methyltransferases—role in selenium tolerance and evolutionary relation. J Biol Chem 274:5407–5414. doi:10.1074/jbc.274.9.5407

Norris SR, Meyer SE, Callis J (1993) The intron of *Arabidopsis thaliana* polyubiquitin genes is conserved in location and is a quantitative determinant of chimeric gene expression. Plant Mol Biol 21:895–906. doi:10.1007/BF00027120

Pickering IJ, Prince RC, Salt DE, George GN (2000) Quantitative, chemically specific imaging of selenium transformation in plants. Proc Natl Acad Sci USA 97:10717–10722. doi:10.1073/pnas.200244597

Pickering IJ, Wright C, Bubner B, Ellis D, Persans MW, Yu EY, George GN, Prince RC, Salt DE (2003) Chemical form and distribution of selenium and sulfur in the selenium hyperaccumulator *Astragalus bisulcatus*. Plant Physiol 131:1460–1467. doi:10.1104/pp.014787

Pilon-Smits EAH, Hwang SB, Lytle CM, Zhu YL, Tai JC, Bravo RC, Chen YC, Leustek T, Terry N (1999) Overexpression of ATP sulfurylase in Indian mustard leads to increased selenate uptake, reduction, and tolerance. Plant Physiol 119:123–132. doi:10.1104/pp.119.1.123

Sinha R, Kiley SC, Lu JX, Thompson HJ, Moraes R, Jaken S, Medina D (1999) Effects of methylselenocysteine on PKC activity, cdk2 phosphorylation and *gadd* gene expression in synchronized mouse mammary epithelial tumor cells. Cancer Lett 146:135–145. doi:10.1016/S0304-3835(99)00250-5

Sors TG, Ellis DR, Na GN, Lahner B, Lee S, Leustek T, Pickering IJ, Salt DE (2005a) Analysis of sulfur and selenium assimilation in *Astragalus* plants with varying capacities to accumulate selenium. Plant J 42:785–797. doi:10.1111/j.1365-313X.2005.02413.x

Sors TG, Ellis DR, Salt DE (2005b) Selenium uptake, translocation, assimilation and metabolic fate in plants. Photosynth Res 86:373–389. doi:10.1007/s11120-005-5222-9

Terry N, Zayed AM, de Souza MP, Tarun AS (2000) Selenium in higher plants. Annu Rev Plant Physiol Plant Mol Biol 51:401–432. doi:10.1146/annurev.arplant.51.1.401

Ulrich JM, Shrift A (1968) Selenium absorption by excised *Astragalus* roots. Plant Physiol 43:14–20

Watkinson JH (1979) Semi-automated fluorimetric determination of nanogram quantities of selenium in biological materials. Anal Chim Acta 105:319–325. doi:10.1016/S0003-2670(01)83763-6

Whanger PD (2004) Selenium and its relationship to cancer: an update. Br J Nutr 91:11–28. doi:10.1079/BJN20031015

White PJ, Bowen HC, Parmaguru P, Fritz M, Spracklen WP, Spiby RE, Meacham MC, Mead A, Harriman M, Trueman LJ, Smith BM, Thomas B, Broadley MR (2004) Interactions between selenium and sulphur nutrition in *Arabidopsis thaliana*. J Exp Bot 55:1927–1937. doi:10.1093/jxb/erh192

White PJ, Bowen HC, Marshall B, Broadley MR (2007) Extraordinarily high leaf selenium to sulfur ratios define 'Se-accumulator' plants. Ann Bot (Lond) 100:111–118. doi:10.1093/aob/mcm084

Zayed A, Lytle CM, Terry N (1998) Accumulation and volatilization of different chemical species of selenium by plants. Planta 206:284–292. doi:10.1007/s004250050402

Zhang LH, Abdel-Ghany SE, Freeman JL, Ackley AR, Schiavon M, Pilon-Smits EAH (2006) Investigation of selenium tolerance mechanisms in *Arabidopsis thaliana*. Physiol Plant 128:212–223. doi:10.1111/j.1399-3054.2006.00739.x