Environ. Sci. Technol. **2005**, *39,* 1771−1777

Field Trial of Transgenic Indian Mustard Plants Shows Enhanced Phytoremediation of Selenium-Contaminated Sediment

GARY BAÑUELOS,[†] NORMAN TERRY,*[,‡] DANIKA L. LEDUC,[‡] ELIZABETH A. H. PILON-SMITS,[§] AND BRUCE MACKEY[||]

ARS-USDA, 9611 South Riverbend Avenue, Parlier, California 93648, Department of Plant and Microbial Biology, 111 Koshland Hall, University of California, Berkeley, California 94720-3102, Biology Department, Colorado State University, Fort Collins, Colorado 80523, and ARS-USDA, 800 Buchanan, Albany, California 94710

Three transgenic Indian mustard [*Brassica juncea* (L.) Czern.] lines were tested under field conditions for their ability to remove selenium (Se) from Se- and boron-contaminated saline sediment. The transgenic lines overexpressed genes encoding the enzymes adenosine triphosphate sulfurylase (APS), γ-glutamyl-cysteine synthetase (ECS), and glutathione synthetase (GS), respectively. The APS, ECS, and GS transgenic plants accumulated 4.3, 2.8, and 2.3-fold more Se in their leaves than wild type, respectively ($P < 0.05$). GS plants significantly tolerated the contaminated soil better than wild type, attaining an aboveground biomass/area almost 80% of that of GS plants grown on clean soil, compared to 50% for wild type plants. This is the first report showing that plants genetically engineered for phytoremediation can perform successfully under field conditions.

Introduction

Selenium (Se) contamination of soil and irrigation drainage water represents one of the most serious problems confronting agriculture in the western United States and other parts of the world with similar environmental and geological conditions (*1*). Selenium is a naturally occurring metalloid primarily found in sedimentary rock formations in drier areas of the world. Soils in the Western United States are derived from shale rock containing high concentrations of Se. As these soils are irrigated, Se is leached into the subsurface drainage water, which is eventually channeled into evaporation ponds. With evaporation and continual loading of these ponds, the amount of bioavailable Se builds up to very high concentrations that can cause substantial damage to birds and wildlife. This was strikingly illustrated by the environmental disaster at the Kesterson Reservoir in the San Joaquin Valley, California (*2*, *3*). The safe disposal of huge quantities of Se-contaminated drainage water is a major concern that has yet to be resolved.

* Corresponding author telephone/fax: (510)642-3510; e-mail: nterry@nature.berkeley.edu.
[†] ARS-USDA, Parlier.
[‡] University of California, Berkeley.
[§] Colorado State University.
[||] ARS-USDA, Albany.

In the 1980s an attempt was made to manage the problem of drainage water in the San Joaquin Valley, CA, by channeling it through the San Luis Drain (SLD), small sloughs, and the San Joaquin River toward the San Francisco Bay Delta (*4*). After objections from environmentalists and others, this approach was abandoned, leaving large quantities of Se-, salt-, and boron-contaminated sediment remaining in the drain. This sediment should be dredged and cleaned out (*5*). However, this has not been done because of the lack of a suitable method of managing the highly toxic sediment. Removing and transporting the sediment is prohibitively expensive − possibly as much as \$11−34 per m^3 soil (*6*). Since it is estimated that there are approximately 100 000 m^3 of contaminated sediment in the SLD, this approach would cost millions of dollars. Other methods of handling contaminated sediments such as soil washing, excavation, and reburial of the contaminated sediment are also cost-intensive (*7*). An alternative and much less expensive approach for remediating Se-contaminated sediments is phytoremediation (*8*−*10*). Banuelos et al. (unpublished, 2004) investigated the possibility of using phytoremediation to clean up the sediments by evaluating Se tolerance and accumulation of different plant species grown in mixtures (3:0, 2:1, 1:2) of Se-laden drainage sediment and good quality soil; they observed that *Brassica* species accumulated the greatest amount of Se among the tested species grown in various sediment-soil mixtures.

The use of phytoremediation as a technology for the cleanup of Se and other toxic trace elements is currently limited by the slow rate of the biological processes involved. There is a need to develop plants that can remove toxic trace elements from soil at much faster rates than are presently available. Genetic engineering offers an innovative and promising approach for increasing the efficiency of phytoremediation. Specifically, the goal is to generate high-biomass plants with enhanced abilities to tolerate and remove heavy metals or metalloids from contaminated soil. To date most of the research on genetic engineering has centered on enhancing the accumulation of heavy metals and Se by plants in laboratory and greenhouse experiments (*11*−*18*); to our knowledge, no studies have yet been reported where the efficacy of genetically altered plants for phytoremediation has been tested under actual field conditions.

In our previous research (UC Berkeley), three different types of Indian mustard [*Brassica juncea* (L.) Czern.] transgenics were developed that each exhibited an increased ability to take up and remove Se and/or heavy metals from soil in laboratory and greenhouse experiments (*19*−*21*). These three Indian mustard transgenic lines overexpressed genes encoding the enzyme adenosine triphosphate sulfurylase (APS transgenic line), γ-glutamyl-cysteine synthetase (ECS line), and glutathione synthetase (GS line), respectively. The APS enzyme mediates sulfate and selenate reduction, while ECS and GS are involved in glutathione synthesis. Recently, these transgenics showed promising results when they were compared with wild type plants for their capacity to accumulate Se or metals from environmental soils in greenhouse pot experiments. The GS and ECS plants accumulated significantly more Cd and Zn from mine tailing sediment (*14*), and the APS plants showed 3-fold higher Se accumulation from Se-rich shale rock sediment (*22*). The goal of the present research was to compare the ability of the three types of transgenic Indian mustard plants with wild type Indian mustard (WT) in terms of their ability to remove Se from highly Se-contaminated sediment under actual field conditions. The plants were grown in Se-sediments taken from

10.1021/es049035f CCC: \$30.25
Published on Web 02/01/2005

the SLD and placed in field plots located at the USDA-ARS Research Facilities at Parlier, CA.

Experimental Section

Field Characteristics. Drainage sediment was collected (August 30, 1999) at 0−25 cm depth from the San Luis Drain, Mendota, CA, and spread to a depth of 25 cm in a previously excavated field plot at USDA-ARS research facility in Parlier, CA. Ten cm of good quality soil (sandy loam; pH 7.2, EC 0.9 dS/m) was applied as a topsoil to enhance plant survival and to encourage biological activity. The topsoil and sediment were mixed using a tractor to construct twenty 33 m long and 1 m wide beds on the field sediment plot. Similarly, a nonsediment field plot (control) was constructed to the same dimensions from the sandy loam soil. A surface drip irrigation system was installed consisting of in-line turbulent flow emitters with a 4 L/h emitter discharge rate and an emitter spacing of 0.45 m, with one line per bed.

Plant Genotypes. Indian mustard (*Brassica juncea*; Accession No.173874) seeds were obtained from the North Central Regional Plant Introduction Station, Ames, IA, and propagated. The three transgenic lines were obtained by transformation of this genotype with different DNA constructs. Transgenic APS8 plants were obtained by overexpression of the mouse-ear cress [*Arabidopsis thaliana* (L.) Heynh.] APS1 cDNA (encoding the enzyme ATP sulfurylase) including its own chloroplast transit sequence, under the control of the CaMV 35S promoter, as described by Pilon-Smits et al. (*19*).

Transgenic cytECS3 plants were obtained by transforming *B. juncea* plants with the construct described by Noctor et al. (*23*), containing the *Escherichia coli* gshI gene (encoding the enzyme γ-glutamylcysteine synthetase) driven by the cauliflower mosaic virus CaMV 35S promoter with a double enhancer sequence. The cytECS3 plants were obtained essentially as the cpECS plants described by Zhu et al. (*21*), except that no chloroplast transit sequence was used, so that the ECS enzyme was expressed in the cytosol. The cytECS plants demonstrated tolerance to and accumulation of a variety of metals (*24*). To summarize briefly, cytECS3 seedlings had significantly greater root lengths and fresh weights when grown on 10 mg/L chromium (K_2CrO_4), 4 mg/L arsenic ($NaAsO_2$), or a combination of metals (7.5 mg/L cadmium ($CdCl_2$), 1. 5 mg/L As, and either 25 mg/L lead ($Pb(NO_3)_2$) or 12 mg/L zinc ($ZnCl_2$)). CytECS3 seedlings also accumulated more As and molybdenum (85 mg/L Mo as Na_2MoO_4) than wild type. The mature cytECS plants exhibited greater tolerance than wild type when grown on liquid media containing either 2.5 mg/L As, 7.5 mg/L Cd, or 60 mg/L Mo. This improved ability to tolerate and accumulate heavy metals was correlated with increased concentrations of glutathione and phytochelatins.

Transgenic cytGS7 plants were obtained as described by Zhu et al. (*20*), by expression of the *E. coli* gshII gene (encoding the enzyme glutathione synthetase), driven by the double-enhanced 35S CaMV promoter. For this field study, one representative line was chosen for each type of transgenic, as well as untransformed *B. juncea*. For all transgenic lines, only seeds homozygous for the introduced gene were used.

Plant Growth Conditions. In view of the small quantity of seed available for the transgenic plants, the seeds were individually planted in germination trays under controlled greenhouse conditions at 24 ± 2 °C with an average photosynthetic flux of approximately 450 μmol m^{-2} s^{-1}. Seven days after emergence, plantlets were lightly fertilized with a non-sulfur containing ammonium nitrate fertilizer (15−15−15). Eighteen days after emergence, plantlets were hardened by increasing the temperature to 32 ± 2 °C and by reducing the amount of irrigation water. At day 30, the plants were transplanted to the sediment and control field plots in April 2003. Two beds were randomly selected from the sediment and control plot, respectively. Each bed was divided into two blocks. Within each block, five 3.3 m × 1 m plots were randomly designated for the three transgenic lines, one untransformed wild type Indian mustard, and for one bare plot. Each planted bed in sediment and control field sites was surrounded by a 1 m high chicken wire fence to prevent entry by rabbits, birds, and other animals. Initial plant density was 55−60 plants per plot, planted in double rows. Prior to transplanting, a low rate of non-sulfur nitrate fertilizer was applied (50 kg N ha^{-1}). Soil samples were taken only at preplant in duplicate from each plot at depths of 0−25 cm and from 25 to 50 cm. Due to the short growing season, we did not expect to observe measurable differences in Se concentrations in the soil; hence, soil samples were not collected at harvest. Safer Insecticide Soap (containing 49% potassium salt of fatty acids as the active ingredient) was sprayed on all plants and bare plots every 2−3 days to reduce insect infestation of aphids and spider mites. All plants were surface-drip irrigated based upon weather data collected from a CIMIS weather station located at UC Kearney, Parlier, CA, and from estimated crop water use data collected from plants grown on control plots.

Collection of Volatile Se. During the growing season, measurements of volatile Se production were periodically taken from each genotype on selected plots. Multiple Se collection chambers (6.6 mm thick Plexiglas with dimensions of 0.71 m long, 0.71 m wide, and 0.76 m high), as described in detail in Lin et al. (*25*), were placed over plants, and volatile Se was trapped for 24 h in an alkaline peroxide trapping solution [(6% H_2O_2 and 0.05 M NaOH), as described by Lin et al. (*25*)] and measured as described below. As there were no significant differences between plant lines, individual data points are not included. Volatilization rates ranged from 3.9 to 13.4 μg Se m^{-2} day.

Plant Harvesting. As mandated by the USDA-Animal and Plant Health Inspection Service, all plants were harvested when 25% of any transgenic line flowered—in this case 45 days after transplanting. At harvest, plant population density was counted in two 1-m^2 sections randomly selected from each plot. Eighteen plants per plant line were harvested by removing the shoots 2 cm above the soil surface. On harvesting the roots, we visually observed the root distribution in the contaminated soil from both 0−25 and 25−50 cm depths. Shoots (leaves) were separated from harvested plant material, washed with deionized water, oven-dried at 50 °C for 7 days, weighed, and ground in a stainless steel Wiley Mill equipped with a 1-mm screen. A low drying temperature of 50 °C was selected to reduce any potential loss of Se through volatilization during sample dehydration.

Elemental Analysis. Pre-plant sediment samples were combined from within each plot, dried at 50 °C for 7 days, and ground to pass an 850 μm sieve. Water-soluble fractions of soil Se, B, S, Na, and electrical conductivity (EC) were determined from a sediment/soil water extract of approximately 1:1. The water extractable form of Se is presumed to be predominately selenate and immediately available for plant uptake. Total sediment Se (or the total reservoir of all forms of Se) was determined in a 500 mg ground sediment sample after wet acid digestion with nitric acid, hydrogen peroxide, and hydrochloric acid (*26*). Other forms of Se (e.g., selenite, organic Se, or elemental Se) were not specifically identified; however, they are presumably included as part of the total Se identified. Plant samples were acid digested with nitric acid, hydrogen peroxide, and hydrochloric acid as described by Banuelos and Akohoue (*27*). Selenium in plant and soil, including trapped volatile Se, were analyzed by an atomic absorption spectrometer (Thermo Jarrell Ash, Smith Hieftje 1000, Franklin, MA) with an automatic vapor accessory (AVA 880); B, S, and Na were analyzed using an inductively

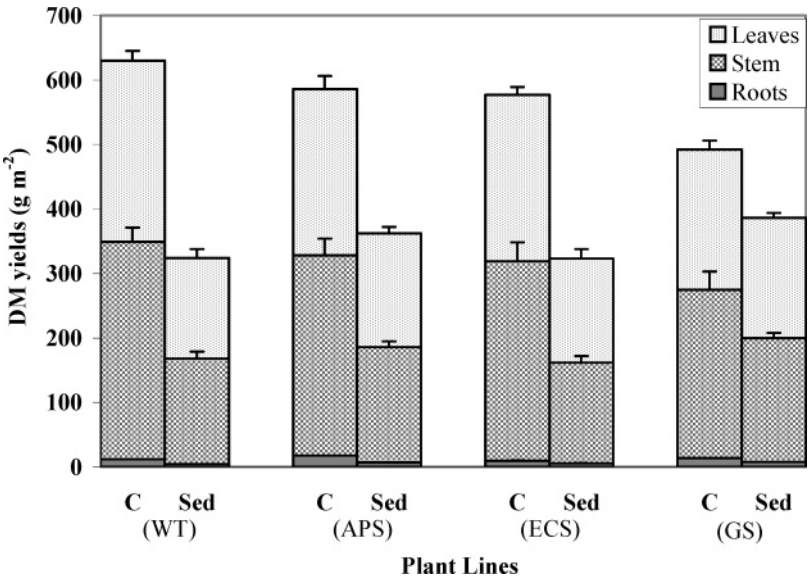

FIGURE 1. Plant dry weight. Dry weights of leaves, stems, and roots of plants grown on clean soil (first column, designated C for control) and on contaminated sediment−soil (second column, designated Sed). Values represent the means and standard errors from four blocks. Each block consisted of a composite sample composed of a minimum of 30 plant samples. There was significant decrease in dry yields at the $P < 0.05$ level for each plant line grown in sediment (Sed) compared to control (C).

TABLE 1. Mean Values for Total and Extractable Se and Other Chemical Parameters from 0 to 50 cm in Sediment and Control Plots prior to Planting[a]

treatment	total Se (μg g^{-1})	water-extractable concentrations (μg mL^{-1})					EC (mS cm^{-1})	pH
		Se	S	Na	B	Cl		
				0−25 cm				
control soil	0.15 ± 0.0^{b§}	0.02 ± 0.0^{b}	285 ± 35^{c}	100 ± 18^{d}	1 ± 0^{d}	98 ± 12^{d}	0.8 ± 0.0^{c}	7.3 ± 0.01^{b}
bare soil	4.02 ± 0.7^{a}	0.62 ± 0.2^{a}	1134 ± 298^{ab}	871 ± 101^{bc}	7 ± 2^{bc}	424 ± 52^{c}	5.2 ± 0.2^{b}	8.1 ± 0.01^{a}
WT	4.53 ± 1.0^{a}	1.09 ± 0.6^{a}	1019 ± 126^{ab}	1039 ± 135^{b}	7 ± 2^{bc}	517 ± 56^{b}	6.4 ± 0.3^{ab}	8.0 ± 0.01^{a}
APS	5.13 ± 1.0^{a}	1.02 ± 0.3^{a}	1358 ± 406^{a}	1640 ± 142^{a}	10 ± 2^{a}	630 ± 58^{a}	7.2 ± 0.2^{a}	8.0 ± 0.01^{a}
ECS	4.58 ± 0.8^{a}	0.79 ± 0.1^{a}	1094 ± 135^{ab}	687 ± 83^{c}	8 ± 2^{ab}	395 ± 41^{c}	5.5 ± 0.2^{b}	7.9 ± 0.01^{a}
GS	3.79 ± 1.0^{a}	1.08 ± 0.6^{a}	901 ± 107^{b}	759 ± 85^{c}	5 ± 1^{c}	422 ± 46^{c}	5.2 ± 0.2^{b}	7.9 ± 0.01^{a}
				25−50 cm				
controlsoil	0.24 ± 0.0^{d}	0.04 ± 0.0^{c}	365 ± 41^{c}	140 ± 22^{c}	1 ± 0^{c}	175 ± 18^{c}	0.9 ± 0.0^{c}	7.3 ± 0.01^{b}
bare soil	7.52 ± 2.0^{bc}	3.36 ± 1^{b}	1629 ± 126^{b}	2132 ± 193^{b}	12 ± 3^{ab}	1093 ± 125^{b}	10.4 ± 0.8^{b}	8.2 ± 0.01^{a}
WT	11.28 ± 2.0^{a}	3.17 ± 1^{b}	1947 ± 148^{a}	2957 ± 221^{a}	13 ± 2^{ab}	1804 ± 132^{a}	13.5 ± 2^{a}	8.1 ± 0.01^{a}
APS	5.41 ± 0.8^{c}	3.22 ± 0.7^{b}	1684 ± 137^{b}	1961 ± 180^{b}	10 ± 2^{b}	1192 ± 130^{b}	10.1 ± 1^{b}	8.0 ± 0.01^{a}
ECS	13.39 ± 2.0^{a}	4.29 ± 1^{ab}	2173 ± 222^{a}	3371 ± 282^{a}	14 ± 2^{a}	1846 ± 141^{a}	14.1 ± 2^{a}	8.1 ± 0.01^{a}
GS	8.48 ± 3.0^{b}	4.58 ± 1^{a}	1597 ± 271^{b}	2136 ± 183^{b}	10 ± 1^{b}	1257 ± 133^{b}	10.2 ± 1^{b}	8.0 ± 0.01^{a}

[a] WT, wild type Indian mustard; APS, adenosine triphosphate sulfurylase-overexpressing Indian mustard; GS, glutathione synthetase-overexpressing Indian mustard; ECS, γ-glutamylcysteine synthetase-overexpressing Indian mustard; bare soil (unplanted). Values represent the means and standard errors from four blocks. Means followed by the same letter are not significantly different at the $P < 0.05$ level between plant lines, bare and control plots for soil parameters at each respective depth.

coupled plasma spectrometer (Perkin-Elmer Plasma 2000 Emission spectrometer, Norwalk, CT). External quality control standards for soils, soil extract, and plant tissue samples were obtained from The National Institute of Standards and Technology (NIST). The standards used were wheat flour (SRM 1567; Se content of 1.1 ± 0.2 μg g^{-1} DM, 94% recovery) and internal soil standards (sediment collected from Kesterson Reservoir, CA) with a total Se content of 7.5 and 25 mg kg^{-1}, 94% recovery, respectively. Soil and plant samples were also acidified with nitric acid and extracted with 2% acetic acid, respectively, and analyzed for chloride by potentiometric titration with silver nitrate. The model 160 conductivity/salinity meter measured soil EC in the soil extract. Analysis of variance (*28*) was used to analyze the data and least mean comparisons between the wild type Indian mustard and transgenic lines were made with Dunnett's tests.

Results

Tolerance to Sediment. All plants grew at substantially lower rates on the Se-, boron-, and salt-contaminated sediment-soil when compared to their growth on clean soil (Figure 1). Table 1 shows concentrations of Se and other soil characteristics from 0 to 50 cm that all plant roots were exposed to during the short growing season. There was normal field variability in total Se and water-extractable concentrations of the selected elements from 0 to 25 cm and 25−50 cm depths. At the time of planting in 2003, the concentrations of total and extractable Se, EC values, and extractable B were generally greater at the 25−50 cm depth compared to 0−25 cm (likely due to their movement caused by natural weather conditions; e.g., rainfall, prior to the experiment). Most of the root biomass was observed to be between 0 and 25 cm, although secondary and tertiary root fibers were found between 25 and 50 cm. No plants exhibited any visible symptoms of ion or salt toxicity. There were also no effects of sediment on final plant density (55 plants m^{-2}) for any plant lines growing in the sediment or control plots. Wild type, APS, and ECS plants grew equally well on clean soil; when grown on contaminated sediment−soil, they also grew equally well but at lower rates (Figure 1). Plants of the GS

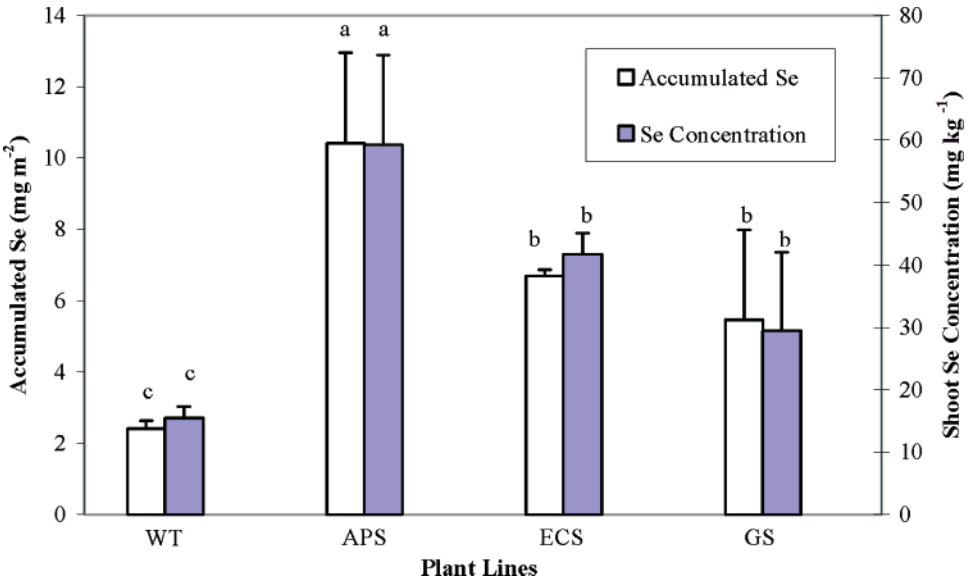

FIGURE 2. Amount of Se accumulated in shoot biomass and shoot Se concentrations for WT, APS, ECS, and GS plants grown on contaminated sediment−soil. Values represent the means and standard errors from four blocks. Each block consisted of a composite sample composed of a minimum of 30 plant samples. Means followed by the same letter are not significantly different at the $P < 0.05$ level between plant lines for accumulated Se and Se concentrations, respectively.

line, however, grew less well on clean soil than plants of the other genotypes but grew better than plants of the other genotypes when grown on sediment-soil. As a result, plants of the GS line grown on sediment-soil attained a total aboveground dry weight (shoot and stems) per unit ground area that was 79% of that of GS plants grown on clean nonsediment soil (control), while wild type plants attained an aboveground dry weight of only 51% on contaminated sediment-soil versus clean soil (Figure 1).

Selenium Accumulation. The three transgenic lines accumulated substantially greater amounts of Se in their shoots than wild type (Figure 2). The APS transgenic line accumulated the most Se: it contained 4.3-fold more Se in its shoots than wild type, while ECS and GS lines accumulated 2.8-fold and 2.3-fold more Se than wild type, respectively (Figure 2). The increases in Se accumulation in transgenic plants were mostly due to increases in Se concentration; Se concentrations in APS, ECS, and GS shoots were 3.8, 2.7, and 1.9-fold higher, respectively, than those of wild type (Figure 2). In terms of bioaccumulation, the APS transgenic plants accumulated Se in shoots to concentrations 12 times higher than the mean total Se concentration in soil (0−25 cm depth; where most of the root mass was located); ECS and GS plants accumulated Se at 9 and 8 times those in soil at preplant, respectively, while wild type plants accumulated Se in shoots to only 3 times those in soil at preplant. When the shoot bioaccumulation ratio was determined based on extractable rather than total soil Se concentration (from 0 to 25 cm), the values were 58, 53, 27, and 14 for APS, ECS, GS, and wild type, respectively. With respect to the concentrations of other elements, APS plants attained the highest concentrations of total sulfur and sodium, followed by ECS, GS, and wild type plants in descending order (Table 2). The boron concentration was significantly lower in APS and ECS plants than in WT and also nonsignificantly lower in GS plants; chloride concentrations did not differ significantly among the four genotypes (Table 2).

TABLE 2. Concentrations of Total S, Na, B, and Chloride (Cl^-) in Shoot Tissue of Wild Type, APS, ECS, and GS Indian Mustard Plants Grown in Contaminated Sediment−Soil[a]

plant lines	shoot tissue concentration			
	Na (mg/kg)	B (mg/kg)	S (g/kg)	Cl (g/kg)
APS	$8042 \pm 305^{a§}$	185 ± 6^{c}	40 ± 0.56^{a}	29 ± 0.82^{a}
ECS	7749 ± 229^{a}	198 ± 6^{bc}	33 ± 0.71^{b}	26 ± 0.67^{a}
GS	5800 ± 327^{b}	231 ± 15^{ab}	25 ± 0.34^{c}	27 ± 0.76^{a}
WT	5246 ± 60^{b}	256 ± 3^{a}	16 ± 0.23^{d}	26 ± 0.58^{a}

[a] WT, wild type Indian mustard; APS, adenosine triphosphate sulfurylase-overexpressing Indian mustard; GS, glutathione synthetase-overexpressing Indian mustard; ECS, γ-glutamylcysteine synthetase-overexpressing Indian mustard. Values represent the means and standard errors from four blocks. Sample size consisted of a composite sample from 18 plants for each respective block. Means followed by the same letter are not significantly different at the $P < 0.05$ level within each column.

Discussion

The results of the present work show that all three transgenic plant lines were able to accumulate significantly more Se in their shoots than wild type plants. These results are of significance since, to our knowledge, they are the first report of transgenic plants showing enhanced phytoremediation potential in the field. The APS transgenics were the most efficient at Se phytoaccumulation: they accumulated 4.3-fold more Se in leaves than the wild type plants. This was more due to the 3.8-fold increase in Se concentration in leaves (Figure 2) than to an increase in biomass. These results are especially meaningful because the plants were allowed to accumulate Se for the relatively short growth period of only 6 weeks (as mandated by the regulatory agency, USDA-APHIS). One might reasonably expect an even greater Se accumulation if the plants had been allowed to grow to maturity and had been able to tap into higher concentrations of water extractable Se between 25 and 50 cm depth.

The APS transgenics used in the present study were tested previously in pot experiments carried out under greenhouse conditions (*22*). Although this study showed that the APS plants accumulated 3-fold higher shoot Se concentrations than wild type, the data are of limited value because of the unusual root/soil conditions associated with pot experiments. For example, the growth of roots is confined by the size of the pot so that roots can occupy an unusually large proportion of the root-soil volume compared to the unrestricted growth of roots under field conditions. Thus, pot-bound roots could potentially absorb much higher concentrations of Se than under field conditions. Because of these limitations, it is

essential that genetically engineered plants be tested under field conditions. We are therefore encouraged by the fact that the APS plants of the present field study exhibited a 4.3-fold increase in Se accumulation compared to wild type. The ECS and GS plants were also previously tested in a greenhouse pot experiment, but using metal-polluted soil. In that experiment they accumulated more Cd and Zn than wild type plants (*13*).

Possible explanations for the enhanced ability of APS Indian mustard to accumulate Se may be the following. First, APS plants overexpress ATP sulfurylase, a rate-limiting step in the conversion of selenate to selenite (*29*). This enables the APS plants treated with selenate to rapidly convert selenate via selenite to organic Se forms, while wild type plants accumulate mostly selenate (*19*). Second, APS plants may accumulate Se to high concentrations in shoot tissues by accumulating more Se in nontoxic organic Se forms such as the nonprotein amino acid methylselenocysteine (MetSeCys). The evidence in support of this latter view is that (i) Indian mustard has been shown to produce MetSeCys, even in wild type plants (*18*) and (ii) APS plants produce approximately 50% more MetSeCys than wild type (LeDuc et al., unpublished). Thus, we propose that APS plants efficiently reduce selenate to selenite, and then to selenocysteine of which some is methylated to form MetSeCys. This methylation may be carried out by a methyltransferase similar to that which has been cloned from the Se hyperaccumulator, *Astragalus bisulcatus* (*30*). The enhanced rate of sulfate/selenate reduction in APS plants appears to stimulate the uptake of sulfate and selenate, leading to higher tissue concentrations of S and Se (Table 2). This same phenomenon was observed earlier in lab and greenhouse studies (*19*, *22*).

The ECS and GS lines accumulated 2.8- and 2.3-fold more Se in their shoot than the wild type, respectively. In the case of ECS transgenic plants, the accumulation was almost entirely due to a 2.7-fold increase in shoot Se concentration and not to an increase in shoot biomass. In the GS plants, part of the increase in Se accumulation compared to wild type was due to a 19% increase in dry shoot biomass, although most was due to a 1.8-fold increase (compared to wild type) in shoot Se concentration. Part of the increase in shoot Se concentration by ECS and GS lines may have been due to upregulation of the early steps in the sulfate uptake and assimilation pathway. As with the APS plants, which overexpress a critical initial step in the S assimilation pathway, ECS and GS plants also accumulated much higher levels of S (as well as Se) than wild type (ECS > GS > WT, Table 2) suggesting that overexpression of ECS and GS may also have enhanced the uptake and assimilation of sulfate and selenate through an effect on the S assimilation pathway. In earlier hydroponic studies, the ECS and GS plants also exhibited enhanced shoot S and thiol levels (*20*, *21*). These two sets of results may be related to the fact that upregulation of ECS and GS (e.g., under conditions of stress) has been shown to lead to an upregulation of sulfate permease and ATP sulfurylase activity (*31*−*33*). It is possible therefore that the ECS and GS plants, which can be thought of as having permanently upregulated ECS and GS activity, would also increase S and Se uptake and assimilation through increased activity of sulfate permease and ATP sulfurylase.

In view of the fact that the plants were in the ground for such a short period (∼6 weeks), it seemed unlikely that we would be able to detect significant decreases in soil Se. We therefore did not attempt to measure post harvest changes in soil Se. As a consequence, an exact mass balance on the fate of Se was not determined. However, based on theoretical considerations, we estimate that the total Se removed by APS plants was ∼4.4% of the extractable Se available in 1 m^2 sediment (to a depth of 25 cm).

These Se accumulation results are comparable with other phytoremediation studies. For example, Van Mantgem et al. (34) reported values of Se accumulation ranging from 0.01 to 5.19 mg/m^2 for a variety of plant species grown at the Kesterson Natural Wildlife Refuge, CA. In this work, we found that wild type *B. juncea* accumulated 2 mg/m^2 Se and that the transgenic *B. juncea* lines accumulated 6−10 mg/m^2. Importantly, in only six weeks the transgenic *B. juncea* were able to achieve higher rates of Se accumulation than observed in the nine-month trial in the Van Mantgem et al. study (*33*) on sediment of similar water-extractable Se and sulfate concentrations.

In the present study, shoot Se concentrations ranged from 10 to 60 mg Se/kg. These values are lower than the shoot Se concentrations (e.g., 407−1017 mg Se/kg) obtained in an earlier greenhouse study (*9*). However, we do not believe the studies are comparable because of a number of differences between the two studies. The plants of the greenhouse study were grown for 60 days in low sulfate, water and soil cultures enriched with Se added as sodium selenate, a highly bioavailable form of Se. In our field trial, the plants were grown for 45 days in a soil containing Se in several different forms (some less bioavailable than others) as well as high levels of sulfate, which is known to strongly inhibit the uptake of Se.

The fact that that the *B. juncea* plants were able to survive on such poor quality sediment-soil, which contained not only high Se levels but also high B and high salinity in general, supports the view that these genetically engineered Indian mustards are particularly useful plants for Se phytoremediation. The mean levels of soil salinity (5.2−7.2 dS m^{-1} at 0−25 cm and 10.1−14.1 dS m^{-1} at 25−50 cm) and extractable B (5-10 mg L^{-1} at 0−25 cm and 10 to 16 mg L^{-1} at 25−50 cm) are considered to be detrimental for normal plant growth (*35*), especially under the hot and arid climatic conditions commonly present in this part of California. The transgenics were at least equally tolerant to these soil and field conditions compared to wild type, despite their higher Se accumulation. The GS transgenics also grew somewhat better than wild type: they grew to almost 80% of their own (clean soil) control, while wild type plants attained 51% on contaminated versus clean soil. The better growth of the GS transgenic line may have been due to a greater tolerance to high levels of soil Se and Na^+, as well as greater tolerance to other adverse growth conditions, such as heat or drought. Since the production of GSH is known to be part of the plant's protective response to a variety of stresses including salinity stress (*33*), chilling, heat shock, pathogen attack, active oxygen species, air pollution, and heavy metals (*36*−*38*), the better growth of the GS line on contaminated soil could well have been due to an elevated glutathione concentration (*20*, *21*).

When using genetically engineered organisms to remediate contaminated environments, it is essential to reduce the risk to wildlife in the local ecosystem by optimizing phytoremediation. Such work is already being actively pursued. For example, enhancing the plants' ability to volatilize Se, will increase the amount of Se removed entirely from the local ecosystem in addition to the amount of Se accumulated in plants (*39*). Volatilization should decrease the overall ecotoxic risk to wildlife by diminishing the amount of Se moving through the food chain. In the present work, the transgenic lines did not volatilize significantly more Se than wild type, despite the fact that they accumulated Se much better than wild type. This result was not entirely unexpected. In laboratory and greenhouse experiments, we have found that although APS plants accumulate more Se and reduce selenate to organic forms, downstream rate-limiting steps in the S/Se assimilation pathway greatly limit the production of volatile Se (*17*, *40*). Based on these findings, we have recently developed other transgenic Indian mustard

lines that show enhanced rates of Se volatilization, either through overexpression of cystathionine-γ-synthase (*17*) or selenocysteine methyltransferase (*18*). The combined overexpression of either of these enzymes with APS/GS/ECS in double-transgenic plants may further enhance Se phytoremediation potential in *B. juncea* while minimizing environmental risk.

The promising results of this study strongly support the need for further field-testing of genetically altered plants for phytoremediation. It is only by conducting such field tests that we can fully evaluate the ability of genetically altered plants to perform under real-life conditions.

Acknowledgments

We gratefully acknowledge the continuous support of the Electric Power Research Institute in providing funds (to University of California, Bekeley) to facilitate the research that enabled the development of the transgenic plant lines tested in this field trial as well as the financial support provided by the Agriculture Research Initiative, California State University (Fresno, CA). This work was also supported by Environmental Protection Agency Research Grant G8A11586 awarded to E.A.H.P.S. and N.T. and by U.S. National Science Foundation Grant MCB-9982432 awarded to E.A.H.P.S. Both G.B. and N.T. contributed equally to this work.

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Received for review June 24, 2004. Revised manuscript received November 19, 2004. Accepted December 14, 2004.

ES049035F