J. Int. Environmental Application & Science, Vol. 3 (5): 336-345 (2008)

Phytoextraction of Cadmium from Contaminated Soil Using Transgenic Tobacco Plants[#]

Hatice Daghan[1,*], Andreas Schaeffer[2], Rainer Fischer[3, 4], Ulrich Commandeur[3]

[1]Mustafa Kemal University, Agriculture Faculty, Soil Science Department, 31040 Hatay, [2]RWTH-Aachen University, Institute for Environmental Research (Biology V), Environmental Biology and Chemodynamics, Worringerweg 1 52074 Aachen, [3]RWTH-Aachen University, Molecular Biotechnology Department, Biology VII, Worringerweg 1 52074 Aachen, [4]Fraunhofer Institute for Molecular Biology and Applied Ecology (IME) Forckenbeckstrasse 6, 52074 Aachen

Abstract: Phytoremediation, the use of plants to clean up toxic metals from contaminated soil or water, represents one of the most promising, effective and technically affordable solutions. Transgenic tobacco plants, constitutively expressing Metallothionein II (MTII) genes from Chinese hamster (Ch) and *Saccharomyces cerevisiae* (Sc), were designed for phytoextraction of cadmium (Cd) contaminated soil. The recombinant proteins were targeted to the vacuole, as confirmed by immunogold labelling. In this study, transgenic and nontransgenic (*Nicotiana tabacum, cv.* Petit Havana SR-1) plants were tested for their ability to extract and accumulate Cd from soil at three different concentrations (0, 10, 20 and 30 ppm). The production of dry matter of wild type and transgenic tobacco plants was slightly affected by the treatment with Cd. Among the transgenic plants, the cV-ChMTII-GFP cassette transformed plants showed the highest dry weight. The level of Cd in the wild type and transgenic tobacco plants did not significantly differ. The results showed that the over-expressions of metallothioneins (MTs) increased plant tolerance to Cd. However, feature researches are needed to confirm these findings under field conditions.

Key words: Phytoremediation, metallothionein, cadmium, vacuolar targeting, *Nicotiana tabacum*, transgenic tobacco.

Introduction

In the past there has been little concern about pollution because the population on earth was not too large and there was plenty of space to dispose of the wastes. However, the rapid population growth and the global changes that have occurred in the last century in our society have dramatically increased the production of wastes and new types of pollutants (Tan, 1994). Various physical, chemical and biological processes are already being used to remediate contaminated soil. Phytoremediation represents one of the most promising, effective and technically affordable solutions. Phytoremediation is the use of these plants to clean up the environment. This technology is an environmentally friendly, safe, cheap way to clean up contaminants. Phytoremediation can be classified into four sub-groups; phytoextraction- the use of metal-accumulating plants to remove toxic metals from soil, phytostabilization- the use of plants to eliminate the bioavailability of toxic metals in soil, phytorhizofiltration- the use of plant roots to remove toxic metals from polluted waters and phytovolatilisation- the use of plants to volatilise certain pollutants and remove them from air.

Certain plants called hyperaccumulators absorb unusually large amounts of metals in comparison to other plants (e.g. up to 0.1% chromium, cobalt, copper or nickel or 1% zinc, manganese in the aboveground shoots on a dry weight basis). Such hyperaccumulators are taxonomically widespread throughout the plant kingdom (Cunningham *et al.*, 1995) and are relevant to phytoremediation. These plants are often rare and grow in remote regions; in certain cases, their habitat is threatened by mining, development and other industrial activities. Hyperaccumulator plants

[*] *Corresponding: hdaghan@mku.edu.tr Tel: +90 326 245 58 45/1362; Fax: + 90 326 245 58 32*

[#] *This study has been presented at BIES'08 Symposium Giresun-Turkey*

have some limitations for phytoextraction of metal contaminated soils. These plants grow slowly and have small biomass. However the optimum plant for phytoextraction process should not only be able to tolerate and accumulate high levels of heavy metals in but also have rapid growth rate produce a high biomass in the field.

Biotechnology has already been successfully employed to manipulate metal uptake and tolerance properties in several species. For example, in tobacco (*Nicotiana tabacum*) increased metal tolerance has been obtained by expressing genes encoding mammalian metallothionein, metal-binding proteins (Lasat, 2000). Genetic engineering will make feasible to manipulate the capacity of plants to tolerate, accumulate, and metabolize pollutants and thus to create the ideal plant for containment of environmental pollution.

For human health and environmental restoration, the use of plants to clean up toxic metals from contaminated soil or water has been developed. Because a number of plant species are naturally capable of high levels of organic compound degradation or of heavy metal hyperaccumulation, plant biotechnology provides an opportunity to develop transgenic plants with increased metal binding or metal reducing capacity. Besides, phytoremediation is a low cost technology, and some extracted metals may be recycled. To balance the concentration of these toxic metals in cells, all organisms induce biosynthesis of low molecular weight, cysteine rich proteins called metallothionein (MT) (Liu *et al*., 2000). MTs can bind Zn, Cd, Ni, Pb, and Cu with affinities that depend on the concentration and bioavailability of the heavy metals.

The objective of the present study was to investigate the effect of MTII (ChMTII and ScMTII) gene expression on the growth of soil tobacco plants exposed to Cd, the ability of the plants to accumulate Cd, and the impact on non protein sulfhydryl (SH) group content.

Materials and Methods

Plasmids, Bacteria and Plants

E. coli strains DH5α and SCS 110 were used for cloning procedures. The following plasmids were used for cloning and gene transfer: pUC18 NdeI*GFP-S65C-VTS (kindly provided by Dr. Verena Hoppman, RWTH Aachen), p35SMG (Brandle 1992), pTRA-kc (kindly provided by Dr. Thomas Rademacher, RWTH Aachen) and *Agrobacterium tumefaciens* GV3101 (pMP90RK Gmr, Kmr, RifrR) (Koncz and Schell 1986). Tobacco (*Nicotiana tabacum*, cv. Petite Havana SR1) was used for agroinfiltration (Kapilla *et al.* 1996; Vaquero *et al*. 1999) and stable transformation (Horsch *et al*. 1985).

ChMTII Expression Construct with cv Targeting Signal

The *ChMTII* gene was amplified from p35SMG (Brandle 1992) using primers ChMTII N 5′ (5′-CAT GCC ATG GAC CCC AAC TGC TCC TGT GC-3′) and ChMTII N 3′ (5′-CAT GCC ATG GCG CAG CAG CTG CAC TTG TCC-3′). The PCR product was digested with *Nco*I and ligated into the *Nco*I site of pUC18-*Nde*I*GFP-S65C-VTS to generate the pUC18-cV-ChMTII-GFP fusion cassette. This comprised the 5′ untranslated region of the petunia chalcone synthase gene (CHS5′UTR), the vacuolar targeting signal from *Catharanthus roseus* strictosidine synthase (cV), and the ChMTII coding region joined in-frame to the mGFP4-S65C mutant of green fluorescent protein (GFP). The cV-ChMTII-GFP cassette was excised using *Eco*RI and *Xba*I, and was ligated into the plant expression vector pTRA-kc, linearized with the same enzymes, to generate p-cV-ChMTII-GFP (Fig. 1).

Since higher recombinant protein levels have been achieved using the potato sporamin A vacuolar targeting signal (Matsuoka *et al*. 1990; Koide *et al*. 1997) a new cassette was constructed containing the potato sporamin signal (S) rather than the *Catharanthus* sequence (cV). The sporamin A gene was amplified by three-step splice overlap extension PCR (SOE-PCR) (Horton *et al*. 1989) using primers P3 (5′-AAA AAA AAC AAT GCA TTC CAG GTT CAA TCC C-3′), P4 (5′-GGT TCG TGT GTG GTG GGG AGG CGG ATG GGA TTG AAC CTG GA-3′) and P5 (5′-CCA CCA CAC ACG AAC CCG CCA TGG ACC CCA ACT GCT CCT GTG CT-3′).

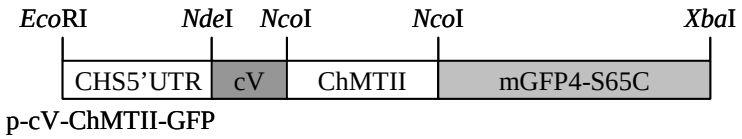

Figure 1. Schematic presentations of p-cV-ChMTIIGFP construct. CHS 5: 5′ untranslated region of *chalcone synthase*; cV: Vacuolar targeting signal from the *C. roseus* strictosidine synthase*;* ChMTII: Chinese Hamster Metallothionein II; GFP: green florescent protein (mGFP4-S65C).

In the first step, P5 and the appropriate MTII reverse primers (ChMTII 3′ S or ScMTII 3′ S; see above) were used to amplify the *ScMTII* and *ChMTII* sequences in order to incorporate a 5′ complementary region which would provide a template overlap in the final SOE-PCR step. In the second step, four primers with complementary regions of 11-21 bps were used to juxtaposition the CHS 5′ UTR and the S signal. These primers were P1 (5′-GAA TTC ACA ACA CAA ATC AGA TTT-3′), P2 (5′-TTG TTT TTT TTT TTT TTA TAA ATC TCT CTA TAA ATC TGA TTT GTG TTG TGA ATT-3′), P3 and P4 (see above). In the final step, 10 µl of reaction product from each of the first two steps were combined to assemble the complete CHS-S-MTII cassette, using the outer P1 primer and the MTII reverse primers (ChMTII 3′ S and ScMTII 3′ S; see above). The final SOE-PCR products (S-ChMTII and S-ScMTII) were digested with *Eco*RI and *Sal*I, and ligated into the linearized pUC18 plasmid upstream of the *c*-myc and His_6 tags.

To produce pS-ChMTII and pS-ScMTII plant expression constructs, the cassettes described above were excised with *Eco*RI and *Xba*I, and inserted downstream of the enhanced 35S promoter and upstream of the CaMV 35S polyadenylation signal (pA35SS) in the pTRA-kc plant expression vector. The S-ChMTII and S-ScMTII cassettes were verified by *Eco*RI/*Xba*I digestion and sequencing (Fig. 2).

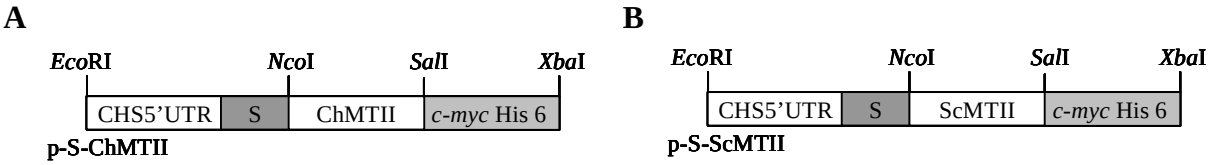

Figure 2. Schematic presentation of p-S-ChMTII (A) and p-S-ScMTII (B) constructs. CHS: 5' untranslated region of chalcon synthase; S: vacuolar targeting signal from potato Sporamin A*;* ChMTII: *Chinese hamster* Metallothionein, ScMTII: *Saccharomyces cerevisiae* Metallothionein II; *c-myc*: myc epitope for detection; His 6: His-6 tag for detection.

Plant Transformation, Regeneration and Screening

Transgenic tobacco plants were generated by the leaf disc transformation method (Fraley *et al*. 1983; Horsch *et al*. 1985) and 25 primary transformants from each line (p-cV-ChMTII-GFP, p-S-ChMTII and p-S-ScMTII) were regenerated and screened for the accumulation of recombinant protein. No abnormal morphological phenotypes were evident and all the plants set seeds normally following self-fertilization. Agroinfiltration was carried out according to Kapila *et al*. (1996)

Western Blotting Analysis

Fresh frozen leaves (0.5 g) were ground in liquid nitrogen to a fine powder with a mortar and pestle. Total soluble proteins (TPS) were extracted using a 1:2 (w/v) ratio of plant material and extraction buffer (200 mM Tris–HCl, pH 7.4, 5 mM EDTA, 5 mM DTT, 0.1% (v/v) Tween 20), boiled 3 min and centrifugated at 16000g for 30 min at 4°C. The supernatant was used for expression analyses by immunoblot. TSP extracted from leaves was resolved by 12 % SDS-polyacrylamide gel electrophoresis under reducing conditions and blotted onto PVDF membrane. After blotting the membrane was blocked with PBS buffer containing 3 % (w/v) skim milk powder (MPBS). As primary antibody both anti *cmyc*, anti His6 (Qiagen) were used in a dilution of 1:5000, anti GFP(Clontech,

Palo Alto, USA) and anti metallothionein (Santa Cruz Biotechnology) were used in a dilution of 1:1000 in 1xPBS. Attachment of the primary antibody was detected by addition of the secondary polyclonal antibody coupled to alkaline phosphatase (AP). Both, primary and secondary antibodies were diluted in blocking buffer. The target protein was finally revealed by addition of substrate BCIP/NBT (Bio-Rad).

Soil Characterisation

The soil used for the pot experiments was collected from Melaten, RWTH-Aachen (BioV) field from a depth of 0 to 30 cm. The soil was air-dried and sieved through a 2 mm sieve and characterised as follows; the sand, clay, and silt fractions of soil samples were determined by Bouyoucos hydrometer methods (Bouyoucus, 1952). Organic matter content was determined by Walkley, Black method (Nelson *et al.,* 1996). The soil pH measured by the $CaCl_2$-method (Lewandowski *et al.,* 1997).

The DTPA-method was used to determine the bioavailable Cd in the soil (Risser *et. al.* 1990). Cadmium analysis in the soil extract was performed by AAS. Cadmium standards were prepared by addition of 0.2-5 ppm $3CdSO_4.7H_2O$ (Merck) into the DTPA solution and analysed under identical conditions.

The total amount of Cd in the soil was determined by the aqua regia method (DIN 38414 Teil 7:1983-01. Cadmium analysis in the soil extract was performed by AAS (Perkin-Elmer 1100B). Cadmium standards were prepared by addition of 0.2-5 ppm $3CdSO_4$ $7H_2O$ (Merck) into the aqua regia solution (21 ml 32% HCl, 7 ml 63% HNO_3) and analysed under identical conditions. The soil properties are listed in Table 1.

Table 1. Properties of the soil used in the experiment.

Chemical Characters	Content
Organic matter (%)	4.300
pH ($CaCl_2$)	7.200
Salt (%)	0.073
$CaCO_3$ (%)	2.600
Total Cd (ppm)*	2.330
Bioavailable Cd (ppm)**	0.620

* Extracted with aqua regia

** Extracted with DTP

Soil texture Class was Loam (49.41%Sand, 8.49 % Clay and 42.10 % Silt).

Pot Experiments

One kg of soil was filled in 1.5 L plastic pots. To each pot the following amounts of fertilizer were applied: 200 ppm Ca $(NO_3)_2$ $4H_2O$, 100 ppm KH_2PO_4 and 2.5 ppm FeEDTA. Serial Cd concentrations ranging from 0 to 30 ppm were applied directly to the soil. Each treatment was replicated three times.

Nicotiana tobaccum Petit Havana SR-1 and transgenic tobacco plants used for the pot experiments were grown under controlled environmental conditions with a 16 h light period (light intensity of 320µmol/m^2/s), a 25/20^{o}C light/dark temperature regime, and 60 % relative humidity. Plants were harvested after 6 weeks of growth.

Harvesting and Analysis

Plant samples (shoots and roots) were rinsed briefly in deionised water and dried with tissue paper, then stored under liquid nitrogen (for later analysis of SH groups; see below) or processed for Cd quantitation by drying at 70^{o}C. After reducing particle size with a ball mill, samples were dry-ashed at 500^{o}C for 5 h in a muffle furnace and the ash was dissolved in 3.3% (v/v) HCl (Risser *et al.* 1990). The Cd in digested samples was measured using an atomic absorption spectrometer with auxiliary for nitrous oxide (AAS) (Perkin Elmer-1100B, California). The measurement of total non-protein SH-groups was carried out as described by Cakmak and Marschner (1992). After extracting 0.5 g and 1.0 g of fresh leaf and root samples, respectively, with 5 ml of 5% (v/v) meta-phosphoric acid, 2.5 ml 150 mM phosphate buffer (pH 7.4) was added and the mixtures incubated at room temperature prior to measuring absorbance at 412 nm using a Hitachi U-2000 spectrophotometer. Glutathione (0 to 100 µg ml^{-1}) was used as a standard. All measurements were randomized and carried

out in triplicate. Statistical analyses of the data were carried out using analysis of MSTAT-C package programme.

Results

No symptoms were observed on transgenic or wild type plants treated with the highest concentration (30 ppm) of Cd (data not shown). Morphologically, the only difference was observed for plants transformed with the p-cV-ChMTII-GFP cassette that flowered earlier than wild type plants.

Accumulation of the recombinant proteins in tobacco leaves was assayed by immunoblot analysis using crude extracts of the total soluble leaf proteins. The recombinant proteins migrated according to their predicted molecular weight of 37 kDa for ChMTII-GFP fusion protein (Figure 3A) and 17 kDa for ChMTII (Figure 3B/1-2) and ScMTII (Fig. 3B/3-4), respectively.

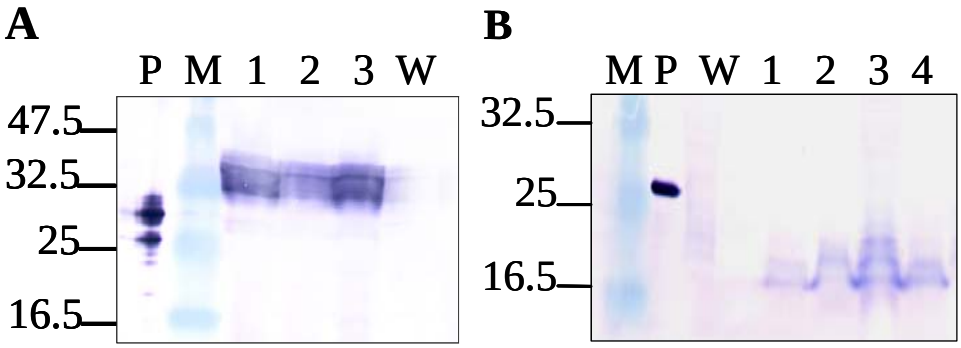

Figure 3. Screening of transgenic tobacco plants expressing ChMTII-GFP, ChMTII and ScMTII after Cd treatment. A: 1-3= 10 µl of TSP extracted from individual tobacco leaves expressing ChMTII-GFP fusion protein in the vacuole under the *Chatarantus roseus* vacuolar targeting signal; W: 10 µl of TSP from wild type fresh leaves; M: prestained protein marker. P: 200 ng GFP used as positive control. B: 10 µl of TSP extracted from individual tobacco leaves expressing ChMTII (Line: 1-2) and ScMTII (Line 3-4) in the vacuole under the Sporamin A vacuolar targeting signal; M: prestained protein marker; W: 10 µl of TSP from wild type fresh tobacco leaves; P: 200 ng of scFv4813 used as positive control.

Immunoblot analysis of crude protein extracts showed that plants transformed with the cV-ChMTII-GFP construct accumulated the highest level of recombinant metallothionein whereas the lowest accumulation was detected in plants transformed with the S-ScMTII construct (Figure 3A and B). No signal was detected in wild type plant (SR-1).

Analysis of the Plant Dry Weight

The production of dry matter of wild type and transgenic tobacco plants were only slightly affected by the treatment with Cd. Plants transformed with the cV-ChMTII-GFP cassette showed the highest dry weight compared to the other transgenic lines (Table 2).

Table 2. Effect of Cd supply on the dry weight (g/plant) of plants.

Cd dosage (ppm)	Plants variety								Average	
	SR-1		p-S-ScMTII		p-S-ChMTII		p-cV-ChMTII GFP			
0	11	c	10	de	12	b	13	a	*11*	*A*
10	9	d-f	10	de	10	de	13	a	*10*	*B*
20	8	g	9	d-f	9	d-f	11	c	*9*	*C*
30	8	g	9	d-f	8	g	10	de	*9*	*D*
Average	*9*	*C*	*9*	*BC*	*10*	*B*	*12*	*A*		

According to the results of cadmium effect on the plants dry weights, the plants dry weight values significantly decreased with the increasing Cd dosage. On the average, the highest dry weight value was found 12 g/plant in p-cV-ChMTII GFP plant and the lowest was found 9 g/plant in SR-1 and p-S-ScMTII. The average dry weights were change between 8-13 g/plant (Table 2).

Analysis of Cadmium Content

The level of cadmium in wild type and transgenic tobacco plants did not differ significantly (Table 3). The level of Cd in the plant tissues markedly increased with the dose of Cd in the soil.

Table 3. Effect of Cd supply on the cadmium content (µg/plant) of plants.

Cd dosage (ppm)	Plants variety								Average	
	SR-1		p-S-ScMTII		p-S-ChMTII		p-cV-ChMTII GFP			
0	74	i	80	i	81	i	114	i	*87*	*D*
10	276	gh	244	h	326	fg	402	c-f	*312*	*C*
20	406	c-f	371	ef	459	b-d	466	bc	*426*	*B*
30	441	c-e	382	d-f	535	b	790	a	*537*	*A*
Average	*299*	*C*	*269*	*C*	*350*	*B*	*443*	*A*		

According to general average, the cadmium content results of p-cV-ChMTII were significantly important for statistical aspects (Table 3). On the average, 30 ppm application of Cd effects on Cd content was important. The lowest Cd content (74 µg/plant) was obtained from 0 ppm Cd application in the pots grown SR-1 plants and the highest value (790 µg/plant) was obtained from 30ppm Cd application in the pots grown p-cV-ChMTII GFP plants.

Analysis of non protein Sulphydryl Groups

The concentration of non protein sulphydryl (SH) groups in leaves of transgenic as well as wild type plants decreased with inverse proportion to the concentration of Cd supplied in the soil (Table 4). Transgenic tobacco plants transformed with the cV-ChMTII-GFP cassette showed a higher concentration of non-protein SH groups than other transgenic and control plants. But only without Cd supplement S-ChMTII construct produced more non-protein SH than the other plants.

There were significant differences in SH group contents with the Cd application. The results of SR-1 and p-S-ChMTII SH content found significantly important for statistical aspects, while the results of p-S-ScMTII SH content found unimportant (Table 4). General average results showed that Cd application effects on plant SH contents were important. The SH content were decreased with the increasing of the Cd application dosage. The lowest free sulhydrl groups contents were obtained from p-S-ScMTII (3119 µg/leaf) and p-cV-ChMTII GFP plants (3599 µg/leaf) with the 30 ppm Cd application and the highest value was obtained from p-S-ChMTII plants (11886 µg SH/leaf) with the application of 0ppm Cd.

Table 4. Effect of Cd supply on the non protein sulphydryl (SH) groups content (µg/leaf) of plants.

Cd dosage (ppm)	Plants variety								Average	
	SR-1		p-S-ScMTII		p-S-ChMTII		p-cV-ChMTII GFP			
0	9791	b	8975	bc	11886	a	8501	b-d	*9788*	*A*
10	7634	c-e	7350	c-e	6800	de	9019	bc	*7701*	*B*
20	6912	de	6497	e	6163	e	7478	c-e	*6762*	*C*
30	6104	e	3119	f	6924	de	3599	f	*4936*	*D*
Average	*7610*	*A*	*6485*	*B*	*7943*	*A*	*7149*	*AB*		

Discussion

Phytoextraction is one aspect of phytoremediation and compared to other methods, it is cheaper, easier, and environmental-friendly. Phytoextraction has been used successfully for removing environmental pollution. However, much of the underlying biological processes that plants use for up taking and translocating the pollutants are still unknown.

The objectives of this study was to test whether these transgenic plants would be more efficient than wild type plants in the uptake of heavy metals, such us Cd, from polluted soils.

The rationale of the present study was to constitutively express recombinant MTIIs in tobacco plants under control of the strong CaMV 35SS promoter and to investigate metal uptake. At the sub-cellular level the distribution of heavy metals in plant cells is not homogenous. Cadmium, for instance, accumulates mainly in the cytosol whereas in organelles such as the nucleus, mitochondria, and microsomes a significant reduction of this metal has been described (Klassen *et al*., 1998). MTs are cytoplasmic proteins (Kägi *et al*, 1985; Klassen *et al*., 1999; Tapia *et al*., 2004). In mature plant cells the cytosol occupies a smaller volume than the vacuole and cannot accumulate high concentration of metals such as Cd, Zn, Cu, and Hg. On the contrary, vacuoles are large subcellular compartments with dual function of storage and/or degradation and may represent the ideal subcellular compartment for detoxifying heavy metals. Therefore, the expression cassettes for recombinant MTIIs were engineered with two different vacuolar targeting signals derived from the *Catharanthus roseus*'s *Stricosidine synthase* and from the potato *Sporamin* A.

The ability of these plants to uptake heavy metals was tested in soil experiments where Cd was used as the reference pollutant and was supplied at different concentrations to ascertain whether the transgenic plants had acquired tolerance to Cd toxicity.

Soil Experiments

The aim of these experiments was to quantify the genotypic variation in the uptake and distribution of Cd in transgenic and non-transgenic tobacco plants. No symptoms were observed on transgenic or wild type plants treated with the highest concentration (30 ppm) of Cd (data is not shown). Morphologically, the only difference was observed for plants transformed with the p-cV-ChMTII-GFP cassette that flowered earlier than wild type plants. de Borne *et al.*, (1998) introduced a chimeric gene encoding a human metallothionein (hMTII) into a different tobacco variety from the one of the present study. These authors observed that morphologically, the hMTII plants were normal, except that they flowered earlier than controls, a feature that has been reported also when other unrelated genes were introduced in tobacco. Our results are in accordance with the ones described by de Borne *et al*., (1998).

We observed a significant correlation between levels of the recombinant proteins, plant dry weight, and Cd accumulation in transgenic and wild type tobacco plants. Immunoblot analysis of crude protein extracts showed that plants transformed with the cV-ChMTII-GFP construct accumulated the highest level of recombinant metallothionein whereas the lowest accumulation was detected in plants transformed with the S-ScMTII construct (Figure 3A and B).

The dry weight production of wild type and transgenic plants was not affected by application of Cd in the soil to levels that simulated the polluting concentrations for Cd in agricultural soils (Table 2). Sauerbeck (1982) indicated that a critical content of 5-10 mg Cd per g shoot dry matter might affect plant growth. In our experiments, the Cd contents in the shoot dry matter were never above these critical levels. Depressions in dry matter production or toxicity symptoms were not observed. The production of dry matter of wild type and transgenic tobacco plants was only slightly affected by the treatment with Cd. Plants transformed with the p-cV-ChMTII-GFP cassette showed the highest dry weight compared to the other transgenic lines. This result could be related with high protein expression level of p-cV-ChMTII GFP plants.

The level of Cadmium in wild type and transgenic tobacco plants did not differ significantly (Table 3). Nevertheless, the level of Cd in the plant tissues markedly increased with the dose of Cd in the soil. According to Brandle *et al.,* (1992) the concentration of available Cd in the soil solution might induce plant uptake rates that exceed the binding capacity of the MT produced by the plants. Levels of MT accumulation were likely not sufficiently high to allow the MT to compete with other, more mobile, Cd-binding molecules. Alternatively, expression of the MT gene may have been not sufficiently high in those cell types involved in Cd uptake and translocation. It is also possible that the

greater mobility of Cd within the plants was a result of low stability of the Cd-MT complex despite the multiyear half-life of metallothionein-Cd complexes described for humans (Kägi and Schaeffer, 1988, Messerle, *et al*., 1990).

Suh *et al*., (1998), introduced the *N. glutinosa* MT cDNA into *N. tabacum* plants via *Agrobacterium*-mediated transformation. Overexpression of the MT gene conferred Cd tolerance to the transgenic plants. This was the first report suggesting the possibility of using MT genes of plant origin for the bioremediation of heavy metal contaminated soil. These authors reported reduced growth of T1 transgenic tobacco plants that was likely due to the exposure to Cd stress during root development. However, the flowering, seed development and germination processes of all transgenic tobacco plants showed increased Cd tolerance.

Large genotypic differences in Cd contents were found between plant species and even between cultivars and inbred lines of the same species (e.g. lettuce, wheat and barley, maize and tobacco). Uptake of Cd by plants differs between plant species and cultivars of the same species. In many of these cases however, it is not clear whether the observed differences are due to a different uptake or a different internal distribution of Cd between roots and shoots (Guo, 1995).

The concentration of non-protein SH groups in leaves of transgenic as well as wild type plants decreased with inverse proportion to the concentration of Cd supplied in the soil. Transgenic tobacco plants transformed with the cV-ChMTII-GFP cassette showed a higher concentration of non-protein SH groups than other transgenic and control plants. However only without Cd supplement the S-ChMTII transformed plants produced more non-protein SH than other plants. This indicated that non-protein SH groups are possibly involved in increased Cd tolerance of stable transgenic cV-ChMTII-GFP tobacco plants by increasing antioxidative defence mechanism.

Pan *et al.* (1994) and Zhu *et al.*, (1999) transformed mouse MT cDNA and *E. coli gsh*II encoding glutathione synthesise into tobacco and Indian mustard (*B. juncea*), respectively. The over expression of the MT gene or glutathione synthesise conferred up to 200mg/l kanamycin and 100µM Cd resistance to the transgenic plants.

Although transgenic plants overexpressing MT genes or PCs are more tolerant to acute Cd toxicity, it remains to be determined whether they are practically useful for phytoremediation. When the pea MT gene was introduced into *A. thaliana*, the expression of the PsMTA gene caused enhanced Cu accumulation and a reduction of Fe availability. No significant effect on the accumulation of either Zn or Cd was detected. When the progenies of transgenic plants overexpressing *N. glutinosa* MT were investigated for tolerance to Cu and Zn, T2 seedlings showed increased tolerance to Cu and Zn. Overexpression of *N. glutinosa* MT confers enhanced resistance for heavy metals such as Cd, Cu and Zn on transgenic plants, indicating that the MT gene in plant cells is probably involved in detoxification of excess metals. By analogy with the MTs of animals and microorganisms, the *N. glutinosa* MT may serve as an intracellular "sink" for excess metals (Liu *et al*., 2000). We did however not observe any differences of PCs concentration between transgenic cV-ChMTII-GFP plants and wild type SR-1 tobacco plants.

A wound and pathogen inducible MT cDNA was previously isolated from *N. glutinosa* while cloning plant disease resistance-response genes by subtractive hybridization (Choi *et al.*, 1996). Liu *et al*. (2000) introduced the same MT gene into tobacco plants via an *Agrobacterium* mediated transformation. In this study the overexpression of the MT gene conferred up to 200µM $CdSO_4$ tolerance to transgenic tobacco plants.

It is often difficult to predict the effects of the expression of transgenes at the level of the whole plant. However, an improved understanding of metal homeostasis in plants will be vital for the development of successful phytoremediation technologies. Future strategies are likely to involve the introduction of several transgenes in tandem, the use of tissue-specific and inducible promoters, the manipulation of regulatory processes in plant metal homeostasis and the generation of hybrids between metal hyperaccumulators and related high-biomass crop plants (Krämer *et al*., 2001).

Conclusion

The results of the present study showed that heterologous expression and subcellular targeting of MTIIs in tobacco plants was successfully achieved. Cd treatments on plants grown in soil proved the efficacy of our approach and confirmed findings reported by others using a similar strategy. However, *in vivo* function of vacuolar targeted MTIIs and the stabilizing effects that fusion partners, such as

GFP, exert on MTs represent novel findings not previously reported. Expression of MTs under the control of root or shoot specific promoters might help to tailor MT overexpression according to the requirements of specific applications (Krämer, *e. al.*, 2001).

The long term goal of the phytoextraction approach here described is the use of transgenic plants with improved extraction properties as a novel tool to remove metal pollutants from contaminated soils. The overexpressions of MTs can increase plant tolerance to specific metals, for example Cd or Cu. However, these findings should be confirmed under field conditions. Only in a few reports did MT overexpression result in slightly increased accumulation of metals in shoots. Thus, the use of MTs in phytoremediation shows still limitations but offers great potentials for improvement.

Acknowledgment: *We would like to thank Dr. Flora Schuster for her help in stable transformation of tobacco plants. Dr. Elsa Arcalis for help microscopic works. We thank Dr. Mehmet Aslan for critical reading of the manuscript. Special thanks to Dr. Kemal Dogan for statistical analysis of the data's and invaluable helps.*

References

Booyoucous GJ, (1952) A recalibration of hydrometer for making mechanical analysis of soils. *Agron. J.*, **43**, 434-438.

Brandle JE, Labbe H, Hattori J, Miki BL, (1992) Field performance and heavy metal concentrations of transgenic flue-cured tobacco expressing a mammalian metallothionein-beta-glucuronidase gene fusion. *Genome*, **36,** 255-60.

Cakmak I, Marschner H, (1992) Magnesium deficiency and high light intensity enhance activities of superoxide dismutase, ascorbate peroxidase and glutathione reductase in bean leaves. *Plant Physiol,* **98,** 1222-1227.

Choi D, Kim HM, Yun HK, Park JA, Kim WT, Bok SH, (1996) Molecular cloning of a metallothionein-like gene from Nicotiana glutinosa L. and its induction by wounding and tobacco mosaic virus infection. *Plant Physiol* **112**, 353-9.

Cunningham SD, Berti WR, Huang JW, (1995) Phytoremediation of contaminated soils. *Trends Biotechnol*, **13,**393-397.

de Borne FD, Elmayan T, de Roton C, de Hys L and Tepfer M, (1998) Cadmium partitioning in transgenic tobacco plants expressing a mammalian metallothionein gene. *Molecular Breeding*, **4,** 83-90.

DIN 38414 Teile 7, Deutsches Institut für Normung e.V. 1983-01.

Fraley RT, Rogers SG, Horsch RB, Sanders PR, Flick JS., Adams SP, Bittner ML, Brand LA, Flink CL, Fry JS, Galluppi GR, Goldberg SB, Hoffmann NL, Woo SC, (1983) Expression of bacterial genes in plant cells. *Proc Nat Acad USA* **80,** 4803-4807.

Guo Y, (1995) *Genotypic Differences in Uptake and Translocation of Cadmium and Nickel in Different Plant Species*, Ph.D. Thesis, In *Institute für Pflanzenernährung*. Stuttgart: Univ. Hohenheim.

Horsch R, Fry JE, Hoffman N, Eicholtz D, Rogers S, Fraley R, (1985) A simple and general method for transferring genes into plants. *Science*, **227,** 1229-1231.

Horton RM, Hunt HD, Ho SN, Pullen JK, Pease LR, (1989) Engineering hybrid genes without the use of restriction enzymes: gene splicing by overlap extension. *Gene* **77,** 61-68.

Kägi JHR, Kojima Y, (1985) *Metallothionein II. In: Second international meeting on metallothionein and other low molecular weight metal-binding proteins* (Ed by JHR Kägi and Y Kojima), Birkhäuser Verlag Basel, Zürich.

Kägi JHR and Schäfer A, (1988) Biochemistry of metallothionein. *Biochemistry* **27,** 8509–8515.

Kapila J, de Rycke R, van Montagu M, Agenon G, (1996) An agrobacterium mediated transient gene expression system for intact leaves. *Plant Sci*, **122,** 101-108.

Klaassen CD, Liu J, (1998) Induction of metallothionein as an adaptive mechanism affecting the magnitude and progression of toxicological injury. *Environ Health Perspect*, **106,** 297-300.

Klaassen CD, Liu J, Choudhuri S, (1999) Metallothionein: an intracellular protein to protect against cadmium toxicity. *Annu Rev Pharmacol Toxicol*, **39,** 267-94.

Krämer U, Chardonnens AN, (2001) The use of transgenic plants in the bioremediation of soils contaminated with trace elements. *Appl Microbiol Biotechnol*. **55,** 661-672.

Koide Y, Hirano H, Matsuoka K, Nakamura K, (1997) The N-terminal propeptide of the precursor to sporamin acts as a vacuole-targeting signal even at the C terminus of the mature part in tobacco cells. *Plant Physiol,* **114**, 863-70.

Koncz C, Schell J, (1986) The promoter of TL-DNA gene 5 controls the tissue-specific expression of chimaeric genes carried by a novel type of Agrobacterium binary vector. *Mol Gen Genet*, **204,** 382-396.

Lasat MM, (2000) The use of plants for the removal of toxic metals from contaminated soils: (Su Doc EP 1.2:2002011154): U.S. Environmental Protection Agency.

Lewandowski J, Leitschuh S and Koß V, (1997) Schadstoffe im Boden. Springer-Verlag, Heidelberg:

Liu JR, Suh MC, Choi D, (2000) Phytoremediation of cadmium contamination: Overexpression of metallothionein in transgenic tobacco plants. *Bundesgesundheitsbl-Gesundheitforsch-Gesundheitsschutz,* **43,** 126-130.

Matsuoka K, Matsumoto S, Hattori T, Machida Y, Nakamura K, (1990) Vacuolar targeting and posttranslational processing of the precursor to the sweet potato tuberous root storage protein in heterologous plant cells. *J Biol Chem*, **265,** 19750-7.

Messerle BA, Schäffer A, Vasak M, Kagi JH and Wuethrich K, (1990) Three-dimensional structure of human [113Cd7] metallothionein-2 in solution determined by nuclear magnetic resonance spectroscopy. *J Mol Biol*, **214**, 765-79.

Nelson DW and Sommers LE, (1996) Total carbon, organic carbon, and organic matter. In: *Methods of Soil Analysis* (Ed. by JM Bigham), pp. 961-1010. *Part 3. Chemical Methods*, Madison: Soil Science Society of America (SSSA) and American Society of Agronomy (ASA).

Risser JA and Baker DE, (1990) Testing soil for toxic metals. In: *Soil Testing and Plant Analysis*, pp. 275-298. Third edition, number 3, in the Soil Science Society of America Book Series Soil Science Society of America, Inc., Madison, Wisconsin, USA.

Pan A, Yang M, Tie F, Li L, Chen Z, Ru B, (1994) Expression of mouse metallothionein-I gene confers cadmium resistance in transgenic tobacco plants. *Plant Mol Biol*, **24,** 341-352.

Sauerbeck D, (1982) Welche Schwermetallgehalte in Pflanzen dürfen überschritten werden, um Wachstumsbeeinträchtigungen zu vermeiden? *Landwirtsch. Forsch., Sonderh*. **39,** 109-129.

Suh MC, Choi D, Liu JR, (1998) Cadmium resistance in transgenic tobacco plants expressing the Nicotiana glutinosa L. metallothionein-like gene. *Mol Cells*, **8,** 678-84.

Tan HK, (1994) *Environmental Soil Science*. New York: Marcel Dekker.

Tapia L, Gonzalez-Aguero M, Cisternas MF, Suazo M, Cambiazo V, Uauy R, Gonzalez M, (2004) Metallothionein is crucial for safe intracellular copper storage and cell survival at normal and supra-physiological exposure levels. *Biochem J,* **378,** 617-24.

Vaquero C, Sack M, Chandler J, Drossard J, Schuster F, Monecke M, Schillberg S, Fischer R, (1999) Transient expression of a tumor-specific single-chain fragment and a chimeric antibody in tobacco leaves. *Proc Natl Acad Sci USA,* **96,** 11128-11133.

Zhu Y, Pilon-Smits EAH, Jouanin L, Terry N, (1999) Overexpression of glutathione synthetase in Indian mustard enhances cadmium accumulation and tolerance. *Plant Physiol,* **119,** 73-79.