

THE CADMIUM PHYTOEXTRACTION EFFICIENCY OF SCMTII GENE BEARING TRANSGENIC TOBACCO PLANT

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ABSTRACT

Cadmium pollution is a serious world-wide problem affecting the human health and the environmental quality. Phytoremediation, the use of green plants to remove, sequester or detoxify pollutants offers an environmentally-friendly alternative to engineering-based methods for remediation. The T2 generations of the ScMTII gene bearing transgenic and non-transgenic tobacco plants were grown hydroponically in Hoagland nutrition solution containing 0, 5 and 10 mg/L Cd in controlled growth room to determine their ability to uptake and accumulate Cd within the shoots and roots. There were no significant shoot and root dry weight differences between transgenic and non-transgenic tobacco plants. The ScMTII gene bearing transgenic tobacco plant accumulated 19.8% higher Cd than the non-transgenic tobacco plant in the above ground parts of the plant during the two weeks exposure period in hydroponic culture. In non-transgenic plant, however, Cd is accumulated mainly in the roots. The results of current study indicate that the use of the ScMTII gene bearing transgenic tobacco plant for Cd phytoremediation is limited. Further studies are needed to test the effectiveness of the ScMTII gene for phytoextraction of other heavy metal ions.

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Introduction

Any metallic element that has a relatively high density (above 5.0 g/cm³) and is toxic or poisonous even at low concentration is considered as heavy metal (30). Small amounts of heavy metals are common in our environment and diets and may be necessary for good health, but large amounts of any of them threats human health and environment. There are hundreds of sources of heavy metal pollution, including mining, smelting and dumping of municipal sewage wastes, natural gas, paper, and chlor-alkali industries.

Metal accumulating plant species can uptake heavy metals like Cd, Zn, Co, Mn, Ni, Pb up to 100 or 1000 times those taken up by non-accumulator (excluder) plants. The phytotechnology using plants for cleaning of contaminated sites, soil or water is known as phytoremediation. Phytoremediation technologies, depending on the type and speciation of the pollutant, are based on the applicable characteristics of the plant species (38). Advantages of phytoremediation technologies include low cost, speed of deployment, preservation of natural soil properties, and reliance on solar energy (48). The success of phytoremediation technology depends on the level of the heavy metal contamination in the soil, bioavailability of the metal, and the ability of the plant to absorb and accumulate the metal in shoots.

Metallothioneins (MTs), a family of low molecular weight cysteine-rich proteins, play a major role in the processes of

plant metal homeostasis and detoxification (8, 10, 11, 37, 45). Plants can be genetically engineered for phytoremediation that combines a high accumulative capacity for metals with a high yield of aerial biomass (26, 27). The introduction of extra metal-binding domains to the implemented protein should further improve the metal-binding capacity.

Cadmium (specific density exceeding 5 g/cm³ and relative atomic mass above 40) (41) is considered one of the most toxic heavy metal pollutants for the environment and its toxic effects on plants and animals are well known (1, 4, 13, 21). Uncontaminated soils contain Cd in the range of 0.04-0.32 µM, while moderately polluted soils contain 0.32-1.00 µM Cd (46). Cadmium tolerant or hyper-accumulate plants are capable of surviving in soils that contain more than 35 µM Cd in the soil solution (6).

Cadmium is a permanent metal ion, which cannot be degraded by any organisms; hence, it can be removed from the environment by heavy metal accumulators such as metal-accumulating plant species. Increased mining and industrial activities, chemical fertilizers, pesticide, and urban and industrial wastes have led to input of large amount of Cd in the environment (2, 46).

Higher plants are highly sensitive to Cd stress. Elevated concentration of Cd in the soil inhibits seed germination and exerts adverse effects on the growth, development and metabolism of plants (3, 19, 33, 34). Toxic amount of cadmium interferes with chlorophyll biosynthesis, inhibits enzymes of the photosynthetic Calvin cycle (44), alters electron transport in both chloroplasts and mitochondria (36, 42, 47). Unlike most of the other heavy metals, Cd is a non-redox metal

unable to perform single electron transfer reactions and does not produce active oxygen species (AOS) such as superoxide radicals (O_2^-), single oxygen (1O_2), hydroxyl radical ($OH^\cdot$) and hydrogen peroxide (H_2O_2) but generates oxidative stress by interfering with the antioxidant defense system (3, 18). The exposure of plants to Cd affects various cellular processes mostly concerned with the functioning of membrane systems. Cadmium tolerant plants show some protective mechanisms to eliminate or alleviate these damages, such as enhancement of lipid peroxidation and alteration of antioxidant enzyme activities and antioxidant levels (36, 42). In addition, Cd has been reported to inhibit the activities of several other enzymes involved in nitrogen metabolism (5, 28), glycolysis, and the pentose phosphate pathway (9) and sulfate assimilation (29).

Cadmium is a highly mobile element in the soil-plant system that can easily enter into food chain and can create risk for human and environmental health (17). Therefore, phytoremediation of the Cd contaminated environments have great importance. The present study was undertaken to determine the Cd uptake efficiency of ScMTII gene bearing transgenic plant and to determine its suitability for phytoextracting of Cd contaminated environments.

Materials and Methods

The tested plant was tobacco (*Nicotiana tabacum* L., cv. Petite Havana SR1). It was used as the control and the genetically modified lines of the same variety were investigated. They were bearing the ScMTII gene which was under a constitutive promoter that enabled a follow up of the heavy metal tolerance and uptake changes as a function of the present transgene.

The ScMTII cDNA clone (kindly provided by Dr. Martin Zimmermann, RWTH Aachen), was amplified by PCR using primers ScMTII N 5' (5'-CAT GCC ATG GTC AGC GAA TTA ATT AAC TTC C-3') and ScMTII S 3' (5'-CTA GTC GAC TTT CCC AGA GCA GCA TGA C-3'). The products were inserted into the pUC18 (Verena Hoppmann, RWTH-Aachen, Biologie VII) (23) vector downstream of the *potato Sporamin A vacuolar targeting signal* (S) and upstream of *c-myc* and His₆ tags to generate the CHS-S- ScMTII cassette (Fig. 1). To produce pS-ScMTII plant expression construct, the above described cassettes were excised with *EcoRI* and *XbaI*, and inserted downstream of the enhanced 35S promoter and upstream of the CaMV 35SS polyadenylation signal (pA35S) in the pTRA-kc (p) plant expression vector. The S-ScMTII cassette was verified by *EcoRI/XbaI* digestion and sequencing (Fig. 1).

pTRA (p) plant expression vector (Thomas Rademacher, RWTH-Aachen, Biologie VII) was used for transformation of *Agrobacterium tumefaciens* and expression of metallothionein II into the plant cell. This vector was used as an optimized plant expression vector containing the 35S promoter and the pA35S untranslated region from CaMV. The plants were genetically transformed as described in (12) by using *A. tumefaciens*. Transgenic tobacco plants were generated by the leaf disc transformation method (15, 24) and 25 primary BIOTECHNOL. & BIOTECHNOL. EQ. 24/2010/3

transformants from 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. (25).

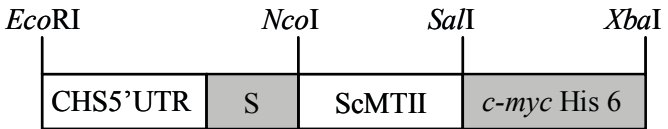


Fig. 1. Schematic presentation of the p-S-ChMTII construct. CHS: 5' untranslated region of chalcon synthase; S: vacuolar targeting signal from potato Sporamin A; ScMTII: *Saccharomyces cerevisiae* Metallothionein II; *c-myc*: myc epitope for detection; His 6: His-6 tag for detection

Non-transgenic tobacco seeds were germinated in a mixture of peat and sand. Transgenic tobacco seeds were germinated on Murashige and Skoog minimal organics medium (Sigma, Deisenhofen, Germany) supplemented with 2% (w/v) sucrose, 0.4µg/mL thiamin, 0.4% (w/v) glycine, 0.1% (w/v) nicotinic acid, 0.1% (w/v) pyridoxine, 200µg/mL claforan (cefotaxime) and 200µg/mL kanamycin as a selectable marker. After 4 weeks, the seedlings were transferred into a nutrient solution consisting of macronutrients (1mM KH_2PO_4 , 3mM KNO_3 , 0.25mM $MgSO_4 \cdot 7H_2O$, 2 mM $Ca(NO_3)_2 \cdot 4H_2O$, 0.1 mM Fe-EDTA, 1×10^{-3} mM $MnSO_4 \cdot H_2O$, 1×10^{-3} mM $ZnSO_4 \cdot H_2O$, 0.25×10^{-3} mM $CuSO_4 \cdot H_2O$, 0.25×10^{-3} mM $(NH_4)_6Mo_7O_{24}$, 2.5×10^{-2} mM KCl, 1.25×10^{-2} mM H_3BO_3) (43). Four weeks later the seedlings were transferred to plastic vessels containing aerated nutrient solution, that was changed every 2-3 days. Plants were grown in a growth chamber under controlled environmental conditions (16-h photoperiod, 25/20°C day/night temperature, relative humidity 60% and light intensity 10 klux or 120 µmol m⁻² s⁻¹). After 6 days in nutrient solution, plants were supplied with increasing concentrations of Cd, administered as $3CdSO_4 \cdot 7H_2O$ and increasing from 0 to 50 to 100 mg/L for a week. After 15 days, the plants were assessed for the severity of Cd toxicity in leaves and roots and then harvested.

Plant samples were rinsed briefly in deionized water and dried with tissue paper, then stored under liquid nitrogen (for later analysis of SH groups; see below) or processed for Cd quantification by drying at 70°C. After reducing particles size with an agate mill, samples were subjected to wet combustion with HNO_3 and H_2O_2 in microwave oven (MarsXpress). Total metal (Cd, Fe, Cu, and Zn) concentrations of digested samples were measured using an ICP-AES (Inductively Coupled Plasma-Atomic Emission Spectrometry; Varian Series-II).

The measurement of total non-protein SH-groups was carried out as described by Cakmak and Marschner (7). After extracting 0.5 g and 1.0 g of fresh leaf and root samples, respectively, with 5 ml of 5% 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 Schimatzu 1240 UV/VIS spectrophotometer.

Glutathione (0 to 100 $\mu\text{g ml}^{-1}$) was used as a standard. All measurements were randomized and carried out in triplicate.

The experiment was conducted in a completely randomized design with four replications. Analysis of variance was performed using a general linear model procedure (40). Comparisons between means were carried out using the Least Significant Difference (LSD) test at the significance level of $p < 0.05$.

Results and Discussion

The applied Cd had no visible Cd morphological toxicity symptoms such as dry tip, leaf chlorosis and root browning. Dry shoot weights of plant grown in applied nutrient solution of 5 mg/L Cd were 2.52 and 3.07 g/plant for ScMTII gene bearing transgenic and nontransgenic tobacco cultivar SR-1, respectively (**Table 1**). Transgenic and non-transgenic tobacco cultivars had 0.18 and 0.21 g/plant root dry weight, respectively. However, the dry shoot and root weight differences between transgenic and non-transgenic tobacco cultivars were not significant. The insignificant difference between transgenic and non-transgenic plants most probably resulted from the lower range of the applied Cd doses (5 and 10 mg/L).

Comparison of transgenic and non-transgenic plants in hydroponic culture showed that transgenic tobacco plant accumulated 730.44 mg Cd/kg dry mass in the above-ground parts and 2965.00 mg Cd/kg dry mass in the roots while non-transgenic plant accumulated 612.44 mg Cd/kg dry mass in above-ground parts and 4144.00 mg Cd/kg dry mass in the roots (**Table 1**). Genetically modified tobacco plant accumulated 19.8% higher Cd than the non-transgenic one in above ground parts (**Table 1**). However, roots of transgenic tobacco plant accumulated 28% less Cd than the roots of non-transgenic

tobacco plant. Higher amount of Cd in the transgenic tobacco was an evidence for higher Cd translocation and accumulation in the above ground parts of the transgenic tobacco plant. It was suggested that the metallothionein gene may be useful in improving metal tolerance of plants (14, 20, 26). The growth conditions and development stages, such as concentration of Cd and exposure duration, the balance between Cd and any other metal in the growth medium and age of plants may affect the metal concentrations in plants (16). In the present study, the Cd accumulation difference between transgenic and non-transgenic plant was not significant. *Our findings on Cd uptake were different from those reported* previously by Macek et al. (31) and Pavlikova et al (35) that accumulation of Cd significantly increased in transgenic plants.

Like Cd content, Cu, Fe and Zn content of shoot and roots of transgenic and nontransgenic tobacco plants were not statistically significant. Most of the metal ions were accumulated in the roots of both transgenic and non-transgenic tobacco plants. There were no significant free non-protein SH-groups differences in the crude extracts of shoots and roots between transgenic and non-transgenic tobacco plants (**Table 1**).

The Cd concentrations ranged from 0.0 to 1181 mg/kg in shoots, and from 8.0 to 6814 mg/kg in roots among the Cd application doses. At the 5 and 10 mg/L Cd concentrations, shoot and root dry weights significantly decreased. The mean dry shoot and root values for plants grown on 10 mg/L Cd in the Hoagland nutrient solution were lower than those for plants grown at 0 and 5 mg/L Cd concentrations (**Table 2**). Plants exposed to 10 mg/L Cd showed a significant decrease in both shoot and root dry weight ($p \leq 0.05$). The Cd content in plant tissues increased linearly with the increase in Cd concentration of the nutrient solution (**Table 2**). No visual symptoms of Cd

TABLE 1

Comparison of shoot and root dry weight, Cd, Cu, Fe, Zn, and SH accumulation by tobacco plants bearing the ScMTII transgene and non-transgene in hydroponic culture

Cultivar	Dry weight (g/plant)		Cd (mg/kg)		Cu (mg/kg)		Fe (mg/kg)		Zn (mg/kg)		SH ($\mu\text{g/mL}$)	
	Shoot	Root	Shoot	Root	Shoot	Root	Shoot	Root	Shoot	Root	Shoot	Root
p-S-ScMTII	2.52	0.18	730.44	2965	14.88	52.63	108	4407	29.35	157	463	610
SR-1	3.07	0.21	612.44	4144	15.16	47.93	101	4487	27.61	114	453	513
LSD 0.05	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.

TABLE 2

Response of transgenic p-S-ScMTII and nontransgenic SR-1 tobacco cultivars on shoot and root dry weight, Cd, Cu, Fe, Zn, Cd content and SH in hydroponic culture

Dose (mg/L)	Dry weight (g/plant)		Cd (mg/kg)		Cu (mg/kg)		Fe (mg/kg)		Zn (mg/kg)		SH ($\mu\text{g/mL}$)	
	Shoot	Root	Shoot	Root	Shoot	Root	Shoot	Root	Shoot	Root	Shoot	Root
0	3.96	0.29	0.0	8.0	20.53	29.72	153.0	3718	35.21	123	255	175
5	2.46	0.20	833	3852	11.88	50.37	75.61	3006	24.60	129	487	699
10	1.95	0.10	1181	6804	12.63	70.74	84.82	6613	25.63	155	633	810
LSD 0.05	0.76	0.06	96.44	1715	1.85	6.79	10.04	779	4.58	N.S.	210	222

toxicity were observed in above ground parts of the seedlings grown on Cd concentrations ranging from 0 to 10 mg/L (Table 2). Compared with the control, 5 mg/L Cd application significantly decreased Cu, Fe, Zn and Cd content in both shoot and roots. However, Cu, Fe, Zn and Cd content tended to increase at 10 mg/L Cd application compared with 5 mg/L Cd. The SH content in plants exposed to 5 and 10 mg/L Cd was significantly higher than that of the control treatment. This may have resulted from the competitions between Cd and the other heavy metals. In the present study, Cu, Zn and Fe content in shoots and roots decreased under 10 mg/L Cd, but were still within the optimal range for plant growth (32). At 5 mg/L Cd application, the content of Cu, Fe, and Zn decreased in both shoots and roots (Table 2) which might resulted from impaired uptake of these ions, their leakage from cells due to impaired membrane structure and function (22) or redistribution of these ions within the plant tissue. Since these elements are constituents of Cu, Zn superoxide dismutases, they have a role to participate in protection against oxidative stress in leaf (39). There were no significant differences between transgenic and non-transgenic tobacco plants in terms of free non-protein SH-groups in the crude extracts of shoots and roots (Table 2).

Conclusions

The current study showed that the introduction of ScMTII gene into tobacco plant did not significantly enhance the ability of the plant to accumulate higher amount of Cd in plant grown in hydroponic culture. Therefore, ScMTII gene bearing transgenic tobacco plant is unsatisfactory for phytoextraction of Cd, but the ScMTII gene could be more satisfactory for uptake and accumulation of other heavy metal ions rather than Cd. Further studies are needed to test the effectiveness of the ScMTII gene on uptake of other heavy metal ions.

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