PHYTOREMEDIATION STUDIES USING TRANSGENIC TOBACCO ACCUMULATING Cd

T. Macek[1,2], M. Macková[1,2], D. Pavlíková[3], J. Száková[3], P. Kotrba[1], M. Surá[1,2]

[1,2]Department of Biochemistry and Microbiology, Institute of Chemical Technology, CZ-166 28 Prague, [2]Department of Natural Products, Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic, CZ-166 10 Prague, [3]Department of Agrochemistry and Plant Nutrition, Czech University of Agriculture in Prague, CZ-165 21 Prague, Czech Republic

Abstract

Tobacco, *Nicotiana tabacum* L., var. Wisconsin 38 as the control (WSC), and genetically modified line of the same variety, bearing the transgene coding for the polyhistidine cluster, combined with yeast metallothionen (HisCUP) was tested for cadmium accumulation and tolerance. The screening test was conducted on sand nutrient medium with addition of Knop's nutrient solution modified by addition of various cadmium doses. Significantly increasing Cd accumulation in above ground biomass of HisCUP tobacco growing in Cd concentration 0.2 and 0.6 mg $Cd.l^{-1}$ of nutrient solution was determined comparing to the control. Dose of 5.4 mg $Cd.l^{-1}$ induced cadmium toxicity symptoms of control tobacco. The highest dose of Cd was lethal for this treatment. HisCUP showed increasing plant resistance against stress response induced by cadmium. The experiment continued in model pot experiment with two various polluted soils. The Cd content in the above ground biomass of transgenic tobacco was increased by 45-75 % compare to control.

Introduction

Heavy metal concentrations in soils are locally quite high and are still increasing due to many human activities, resulting in risk for man's health and environment. The total metal load on agricultural soils is the sum of metal input from atmospheric deposition, and input from the addition of fertilizers (mainly phosphate fertilizers), metal-containing pesticides, biosolids (for example sewage sludge, industrial wastes) and wastewater. Cd is of great environmental concern because it is highly toxic to plants and animals and it is highly mobile in the terrestrial environment. Atmospheric input and the use of phosphate fertilizers are the major sources of Cd for total plant concentrations of agricultural crops (1). Use of specific plant species known as hyperaccumulators and microorganisms for remediation of soils contaminated with metals is a relatively new field of technology. Phytoremediation depends on high accumulation of heavy metals by plants, especially by above ground portions of plant, and production of a relatively large amount of biomass. These two features determine the efficiency of the process. Baker and Brooks (2) have reported about 400 metal-accumulating wild plants that accumulate high concentrations of heavy metals in their shoots. Natural hyperaccumulator plants often grow slowly and have low biomass yield. *Thlaspi caerulescens* was reported as a hyperaccumulator of cadmium and zinc. It can accumulate over 3 % of zinc and at the same time over 0.1 % of cadmium per dry biomass. The practical use of this plant for phytoremediation is restricted by its small biomass yield (3). Metal hyperaccumulators are highly attractive model organisms, because they have overcome major physiological bottlenecks limiting metal accumulation in biomass and metal tolerance. Improvement of plants by genetic engineering opens up new possibilities for phytore-mediation. Genetic engineering is a technique that might be applied advantageously to the search for better phytoremediation plants combining high metal accumulating capacity and high above ground biomass yield (4). The introduction of an additional metal binding domain to the implemented protein should further enhance the metal binding capacity (5,6).

The main objective of this investigation was focused on the evaluation of Cd accumulation ability of transgenic tobacco with a yeast metallothionein combined with a polyhistidine tail.

Materials and Methods

Tobacco, *Nicotiana tabacum* L., var. Wisconsin 38 as the control (WSC), and genetically modified line of the same variety, bearing the transgene coding for the polyhistidine cluster, combined with yeast metallothionen (HisCUP) was tested in sand media and pot experiment. The plants were genetically transformed as described by Macek et al. (7). Based on the previous experiments the best Cd accumulating line HisCUP-X was chosen from HisCUP transgenic tobacco lines for this test (7).

Screening test

The screening test was conducted on sand nutrient medium with addition of Knop's nutrient solution modified by addition of various cadmium doses (0.2 – 0.6 – 1.8 – 5.4 – 16.2 mg Cd .l^{-1} of nutrient solution as $Cd(NO_3)_2.4H_2O$. The plants were inserted into medium, planted during the elongation period and harvested after six weeks of growth.

Pot experiment

The model pot experiment was set up using two soils with different Cd content – unpolluted Chernozem and Cambisol with contamination caused by the atmospheric emissions. In experiment 5 kg of soil were thoroughly mixed with 2 g N applied as NH_4NO_3. P and K were added as K_2HPO_4 at a rate of 0.44 g P and 1.1 g K per pot at the same time. The pots were irrigated by deionised water and humidity of the soil was kept on 60% of maximal water capacity for duration of whole experiment. The plants were cultivated for three months. Both experiments consisted of four replicates of each treatment. The plants were of the same size at the start of the experiments.

Chemical analyses

For total Cd content, the soil samples were decomposed by dry ashing procedure and ash was dissolved in diluted Aqua Regia. Before and after the pot experiment the plant-available content of Cd was determined by 0.01 mol.l^{-1} $CaCl_2$ (16). For determination of Cd, atomic absorption spectrometry (Varian SpectrAA-300) was used equipped by flame atomizer. Quality of analyses of Cd soil content was controlled by reference material RM 7001 Light sandy soil. Certified value of Cd in reference material was 0.32±0.05 mg Cd.kg^{-1} and obtained value 0.37±0.03 mg Cd.kg^{-1}.

After harvest an above ground biomass and roots were gently washed with deionised water, dried, ground and analyzed for total Cd content. Plant material was decomposed by dry ashing procedure and ash was dissolved in diluted Aqua Regia. Cd concentration in plants and roots was determined by atomic absorption spectrometry (Varian SpectrAA-300). The accuracy of analyses was estimated by comparison with reference material RM 12-02-03 Lucerne with a certified content of 0.136±0.003 mg Cd.kg^{-1} dry mass for which contents of 0.167±0.022 mg Cd.kg^{-1} dry mass was obtained.

Results and Discussion

Screening test in sand medium

The HisCUP construct proved to have a positive effect on Cd accumulation. The Cd content in above ground biomass of tobacco growing in Cd concentration 0.2 mg Cd.l^{-1} was increased by 80 % compare to the non-transformed control (Tab. 1). This result confirmed our previous experiments that accumulation of cadmium significantly increased much in plants bearing the transgene coding for the polyhistidine cluster, combined with yeast metallothionein (7).

Table 1: Cd content in tobacco plants growing in sand medium

Cd dose (mg Cd.l^{-1})	Cd content (mg . kg^{-1})			
	WSC-38		HisCUP	
	Roots	Above-ground biomass	Roots	Above-ground biomass
0.2	13.6 ± 2.9	16.6 ± 5.9	11.6 ± 6.2	30.1 ± 3.5
0.6	34.4 ± 4.17	46.8 ± 4.6	36.8 ± 2.9	74.7 ± 3.3
1.8	142.5 ± 0.7	125.5 ± 2.1	150.0 ± 6.4	144.6 ± 1.3
5.4	520.0 ± 15.5	259.0 ± 4.5	514.0 ± 23.6	336.0 ± 5.9
16.2	1908.5 ± 51.2	647.5 ± 111.6	1886.8 ± 100.5	736.5 ± 269.2

Significantly increasing accumulation was also determined in HisCUP tobacco growing in Cd concentration 0.6 mg Cd.l^{-1}. Cadmium content in roots of control tobacco did not differ from transgenic plants and it was lower compare to above ground biomass. Next three doses 1.8, 5.4 and 16.2 mg $Cd.l^{-1}$ caused lower differences of Cd contents in plant above ground biomass and roots between control and HisCUP. Dose of 5.4 mg $Cd.l^{-1}$ induced cadmium toxicity symptoms in leaves of control tobacco resembled Fe chlorosis. Adriano (1) described these toxicity symptoms. In addition to chlorosis, control plants exhibited necrosis, red coloration of young leaves and mainly reduction in growth. According Sappin-Didier et al. (8) interveinal chlorosis and necrosis were observed on the leaves of tobacco grown on contaminated soil. In radish, Cd toxicity was associated with reduction of Zn levels in plant tissues (9). Using nutrient culture, the following Cd concentrations in solution were found to be associated with 50 % reduction of biomass: beet, bean and turnip 0.2 ppm; corn and lettuce 10 ppm; tomato and barley 5.0 ppm; cabbage 9 ppm (1). The highest dose of Cd (16.2 mg $Cd.l^{-1}$) in sand medium depressed root and plant growth and was lethal for this control treatment. HisCUP tobacco showed increasing plant resistance against stress response induced by cadmium. Both doses were not toxic for transgenic plants. Studies of many different authors (4,10,11) suggest that metallothionein gene may be useful in improving metal tolerance of plants. It seems to have significant effect on Cd tolerance of HisCUP tobacco in our experiment.

Model pot experiment

The experiment continued in model pot experiment with two various polluted soils, where risk element bioavailability was substantially lower compared to sand media (Tab. 2). The bioavailability of Cd to plants can be more accurately predicted determining available portion of Cd than the soil total content (1). Before experiment plant-available Cd formed 8 % from total Cd content on polluted Cambisol. Our results did not show changes of available Cd content after harvest of tobacco plants on this soil. Unpolluted Chernozem contained 0.56 % available Cd from total Cd content. Significant decrease of Cd was observed only on this soil after harvest of transgenic tobacco.

Table 2 Total and available content of Cd in soils used in pot experiments

Soil		Chernozem	Cambisol
Total content of Cd ($mg.kg^{-1}$)		0.42±0.07	4.73±1.18
Plant available content of Cd ($mg.kg^{-1}$)			
Before experiment		0.0025±0.0004	0.382±0.124
After experiment	WSC	0.0021±0.0005	0.408±0.123
	HisCUP	0.0012±0.0004	0.388±0.131

After harvest of tobacco plants the yields of above ground biomass and roots were determined. Both yield and content of dry matter of transgenic plants were not significantly different compared to control. Numerous studies lend support to plants' tendencies to accumulate Cd. Radish shoots can accumulate 5 ppm of Cd when grown on soil containing 0.6 ppm Cd. Leafy plants accumulated 175 to 354 ppm Cd when grown on soils treated with sewage sludge (1). Mainly Cd content in soils influenced the Cd content in tobacco plants cultivated in this experiment. Content of Cd in tobacco growing on polluted Cambisol with high portion of available Cd was 14-17 fold higher compared to Chernozem. The content of Cd in the above ground biomass of transgenic tobacco cultivating on non-polluted soil increased by 75 % compare to control and by 45 % on polluted soil (Tab. 3).

Table 3: Cd content in tobacco plants growing in pot experiment

Soil	Treatment	Cd content ($mg.kg^{-1}$) Roots	Cd content ($mg.kg^{-1}$) Above ground biomass
Chernozem	WSC	0.24 ± 0.12	1.25 ± 0.60
	HisCUP	0.13 ± 0.03	2.19 ± 0.95
Cambisol	WSC	3.61 ± 0.65	21.33 ± 4.84
	HisCUP	2.50 ± 0.41	31.43 ± 5.65

Very important fact for phytoremediation is, that most of the accumulated cadmium was stored in the leaves, while the metal content in roots was lower comparing to the control plants. According King and Hajjar (12) in tobacco, Cd concentrations declined markedly with higher leaf position on the stalk. This general decline with increasing leaf height in the tobacco plant suggests that Cd has limited mobility within the plant and its concentration is related to the age of the leaf.

For evaluation of uptake of heavy metals by plants growing on various soils transfer factors values are used (13). Transfer factor is calculated as quotient of metal total content in plant biomass and in soil. According Kloke et al. (14) Cd transfer factor is 1-10. Higher values are showed for leafy plants. Tlustoš et al. (15) calculated transfer factor values 1.50 - 3.59 for spinach leaves and 1.65 – 3.32 for spinach roots. Our results showed high transfer factor for tobacco (Tab. 4), mainly for above ground biomass of transgenic plants 5.26 – 6.65.

Table 4: Values of Cd transfer factor for tobacco plants

Soil	Treatment	Cd transfer factor	
		Roots	Above ground biomass
Chernozem	WSC	0.54	3.00
	HisCUP	0.27	5.26
Cambisol	WSC	0.76	4.51
	HisCUP	0.52	6.65

4. Conclusions

The results showed high Cd accumulation ability of HisCUP construct and confirmed a good chance to prepare improved transgenic plants for phytoremediation purposes. The Cd content in above ground biomass of transgenic tobacco was increased by 80 % compare to the non-transformed control. HisCUP tobacco also showed increasing plant resistance against stress response induced by cadmium. Toxicity symptoms were not observed even at concentrations, which were detrimental to the control non-transgenic plants. Content of Cd in the above ground biomass of transgenic tobacco cultivating on soils was increased by 45 - 75 % compare to control. Very important fact for phytoremediation is, that most of the accumulated cadmium was stored in the leaves, while the metal content in roots was lower comparing to the control plants. High values of Cd transfer factor (5.26 – 6.65) were calculated for above ground biomass of transgenic plants.

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