The evaluation of cadmium, zinc and nickel accumulation ability of transgenic tobacco bearing different transgenes

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ABSTRACT

Tobacco, *Nicotiana tabacum* L., var. Wisconsin 38 as the control (WSC), and four genetically modified lines of the same variety, were tested for Cd, Zn and Ni accumulation. Genetically modified lines of the same variety, bearing the transgene *CUP1* (gene coding a yeast metallothionein), GUS (reporter gene for β-glucuronidase), HisCUP (CUP combined with a polyhistidine tail), and HisGUS (reporter gene for β-glucuronidase, combined with a polyhistidine tail) under a constitutive promoter, enabling it to follow the heavy metal tolerance and uptake changes as a function of the transgene present. Control and transgenic lines were tested for accumulation of risk elements on sand nutrient medium with the addition of cadmium, zinc and nickel. The results showed high Cd accumulation ability of HisCUP line. The Cd content in aboveground biomass was increased by 90% compared to the non-transformed control and Cd content in roots was decreased by 49%. Determination of Zn content in aboveground biomass did not confirm higher uptake by transgenic plants significant for phytoremediation. The Ni content was significantly increased in aboveground biomass of HisGUS construct. GUS construct introduced the ability to accumulate all investigated metals; the others accumulated only one in extended amount.

Keywords: transgenic tobacco; metallothionein; polyhistidine; β-glucuronidase; cadmium; zinc; nickel; accumulation

The concept of phytoremediation – using plants to remove or inactivate pollutants from soils has received increasing attention in recent years (Cunningham et al. 1995, Baker and Brooks 1998, Krämer and Chardonnens 2001, Němeček et al. 2001, Pilon-Smits and Pilon 2002, Vysloužilová et al. 2003). Compared to existing physical and chemical methods of soil remediation the use of plants is cost-effective and less disruptive to the environment. Very important is the rhizospheric cooperation of plants and micrrorganisms, both in remediation of soils contaminated by inorganic or organic pollutants and xenobiotics (Macek et al. 2000). In addition to its advantages, phytoremediation does suffer from a number of limitations (Cunningham et al. 1995). Hyperaccumulators often accumulate only one specific element. Many of these plants grow slowly and have low biomass yield. Little is known about their breeding potential and physiology. Baker and Brooks (1998) found that some native plant species were able to accumulate unusually high concentrations of potentially phytotoxic elements such as Cd, Cu, Pb, Ni and Zn from metalliferous soils. *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. *Alyssum* sp. can contain a very high concentration of up to 2% Ni. Metal hyperaccumulators are highly attractive model organisms, because they have overcome major physiological bottlenecks limiting metal accumulation in shoots and metal tolerance.

Genetic engineering is a technique that might be applied advantageously to the search for more suitable phytoremediation plants combining high metal accumulating capacity and high aboveground biomass yield (Kärenlampi et al. 2000). The introduction of an additional metal binding domain to the implemented protein should further enhance the metal binding capacity (Macek et al. 1996, Kotrba et al. 1999). This hypothesis was tested using transgenic plants with different constructs. These results are encouraging because it suggests that metal accumulation results obtained from the nutrient media are a valuable indication of

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transgenics' metal accumulation potential from environmental soils containing metal mixture. The obtained results from nutrient media showed transgenic plants as valuable indicators of elevated metal accumulation.

The main objective of this investigation was focused on the evaluation of Cd, Zn and Ni accumulation ability of transgenic tobacco bearing four different transgenes.

MATERIAL AND METHODS

The plants were genetically transformed as described by Macek et al. (2002b), by using *Agrobacterium tumefaciens*. The vector was plasmid pBI121, bearing a CaMV 35S promoter from cauliflower mosaic virus, the reporter gene for β-glucuronidase (GUS) and all regions necessary for a successful transformation using *Agrobacterium*, purchased from CLONTECH (BD Biosciences). The tested plants involve tobacco, *Nicotiana tabacum* L., var. Wisconsin 38, as the control (WSC-38), and genetically modified lines of the same variety, bearing the transgenes CUP (gene coding a yeast metallothionein), GUS (reporter gene for β-glucuronidase), HisCUP (CUP combined with a polyhistidine tail), and HisGUS (reporter gene for β-glucuronidase, combined with a polyhistidine tail) under a constitutive promoter, thus enabling us to follow the heavy metal tolerance and uptake changes as function of the transgene present.

Non-transformed control – WSC-38 and four transgenic tobacco lines (CUP, HisCUP, GUS and HisGUS) were tested for accumulation of Cd, Zn and Ni in aboveground biomass and roots. Based on the previous experiments (Macek et al. 2002b) the best Cd accumulating line HisCUP-X was chosen from HisCUP transgenic tobacco lines for this test. The presence of the introduced transgene was proven at the DNA level by PCR, using the commercial DNeasy Plant Mini Kit (Qiagen) for extraction and isolation, and primers to 35S CaMV promoter and NOS termination gene (Vollenhofer et al. 1999). The formation of corresponding RNA was confirmed after RNA isolation and RT-PCT with the appropriate specific primers designed for HisCUP gene construct.

All plant material was vegetatively multiplied, aseptically under *in vitro* conditions and transferred to pots after 6 weeks of growth at the mean size of about 10 cm. The plants were hardened to survive under greenhouse conditions by keeping them in open vessels for a three-day period.

The screening test was conducted on a sand nutrient media with the addition of Knop's nutrient solution modified by the addition of cadmium [0.2 mg Cd/l of nutrient solution as $Cd(NO_3)_2.4\ H_2O$), zinc (0.5 mg Zn/l of nutrient solution as $Zn(NO_3)_2.6\ H_2O$] and nickel [0.2 mg Ni/l of nutrient solution as $Ni(NO_3)_2.6\ H_2O$]. The plants were harvested after 6 weeks of growth at the mean size of about 30 cm. The results were obtained on an average of four independent experiments consisting of three to six replicates of each plant. The plants were of the same size at the start of the experiments, and there were no significant differences in the size of the different lines at the harvest.

After harvest the aboveground biomass and roots were gently washed with deionised water, dried, ground and analysed for total Cd, Zn and Ni content. The plant material was decomposed by using the dry ashing procedure and Cd, Zn and Ni concentrations in plants and roots were determined by using atomic absorption spectrometry (Varian SpectrAA-300). The accuracy of analyses was estimated by a comparison with reference material RM 12-02-03 Lucerne with a certified content of 0.136 ± 0.003 mg Cd/kg, 33.2 ± 0.5 mg Zn/kg, 2.54 ± 0.08 mg Ni/kg dry mass for which contents of 0.137 ± 0.022 mg Cd/kg, 34.5 ± 1.4 mg Zn/kg, 2.25 ± 0.41 mg Ni/kg dry mass were obtained.

Table 1. Yield of dry aboveground biomass and Cd, Zn and Ni content in tobacco plants

Tested tobacco plant lines	Yield of dry aboveground biomass (g per pot)	Content of risk elements in aboveground biomass (mg/kg dry mass)		
		Cd	Zn	Ni
WSC-38	2.63 ± 0.69	15.8 ± 2.9	93.5 ± 13.5	3.7 ± 1.1
CUP	2.55 ± 0.63	16.9 ± 6.6	113.7 ± 3.1	3.4 ± 0.2
HisCUP	2.48 ± 0.25	33.2 ± 2.7	104.3 ± 9.4	3.9 ± 0.3
GUS	2.38 ± 0.33	24.4 ± 4.8	123.1 ± 20.3	5.4 ± 0.8
HisGUS	2.32 ± 0.64	16.6 ± 6.1	115.6 ± 11.6	6.5 ± 2.4
$D_{min\ (\alpha = 0.05)}$	n.s.	8.73	n.s.	2.50

n.s. = non significant at $\alpha = 0.05$

Table 2. Yield of dry root biomass and Cd, Zn and Ni content in tobacco roots

Tested tobacco plant lines	Yield of dry roots (g per pot)	Content of risk elements in roots (mg/kg dry mass)		
		Cd	Zn	Ni
WSC-38	0.63 ± 0.14	19.9 ± 2.1	380.0 ± 35.3	27.2 ± 4.7
CUP	0.64 ± 0.21	14.3 ± 5.1	418.0 ± 28.0	27.7 ± 1.9
HisCUP	0.73 ± 0.21	10.8 ± 0.3	326.8 ± 28.4	43.7 ± 6.0
GUS	0.53 ± 0.13	15.7 ± 1.4	475.0 ± 30.0	64.6 ± 10.1
HisGUS	0.59 ± 0.13	15.2 ± 2.7	376.0 ± 33.9	44.2 ± 6.2
$D_{\min(\alpha = 0.05)}$	n.s.	5.10	n.s.	11.53

n.s. = non significant at $\alpha = 0.05$

The obtained data was statistically processed by means of a one-way analysis of variance and linear regression model using Statgraphics Plus 5.0.

RESULTS AND DISCUSSION

After harvest of the tobacco plants the yields of the aboveground biomass and roots were determined. Both yield and content of dry matter of transgenic plants were not significantly different compared to the controlled ones (Tables 1 and 2). For phytoremediation purposes it is important that the distribution of the accumulated metals within the plant, especially for the rate of the translocation to the harvestable parts (Macek et al. 2002a).

The results of cadmium analysis in tobacco plants showed that the control WSC-38 whole plants had accumulated on an average 15.8 mg Cd/kg in leaves and stems and 19.9 mg Cd/kg in the roots (Tables 1 and 2). The presence of the *CUP1* yeast metallothionein gene alone did not significantly increase the accumulation compare to the average of the controlled treatment. Studies of many other authors (Ow 1996, Dorlhac de Borne et al. 1998, Kärenlampi et al. 2000) suggest that the metallothionein gene may be useful in improving metal tolerance of plants. The most pronounced effect of metallothionein overexpression was observed by Hasegawa et al. (1997), who over-expressed the yeast metallothionein gene *CUP1* in cauliflower, leading to a 16-fold higher Cd accumulation. Our results are not in agreement with their observation. Although according to our determinations the effect of gene *CUP1* on the uptake of metals seems to be insignificant.

The GUS line exhibited an increased Cd-binding activity. Cadmium content in the aboveground biomass had increased by 54% compared to the control plants (Table 1). Due to a higher variation among replications the increase was not statistically significant. Elmayan and Tepfer (1994) found different results – Cd concentration in seedling shoots of tobacco bearing MT-GUS transgene by 60–70% were lower than in control. According to Brandle et al. (1993) and Hattori et al. (1994) Cd contents in the leaf tissues of transformed lines were not significantly different from the untransformed control.

Cd concentration in HisGUS constructs did not differ from the control treatment. The HisCUP construct proved to have a positive effect on Cd accumulation. The Cd content in aboveground biomass had increased by 90% comparing to the non-transformed control and the Cd content in roots had decreased by 49%. These results confirm our previous observation of a significantly increased cadmium accumulation much in plants bearing

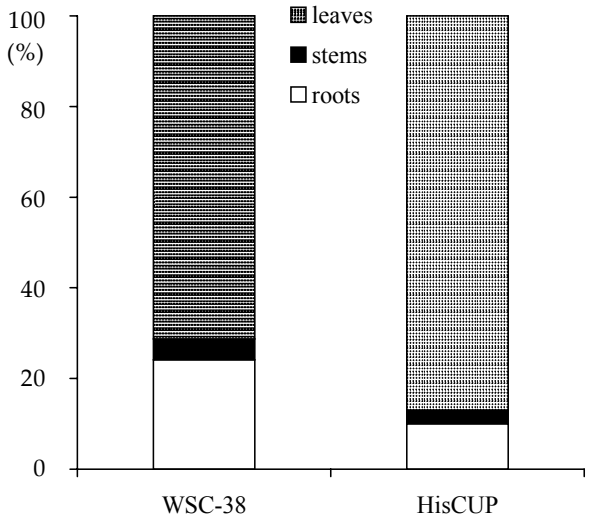

Figure 1. Distribution of Cd uptake among main parts of tobacco plants (100% WSC-38 = 80 μg Cd and 100% HisCUP = 87 μg Cd)

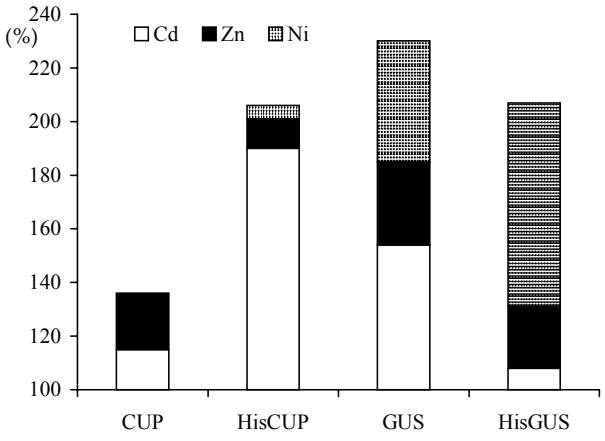

Figure 2. Relative growth of risk element contents in aboveground biomass of transgenic tobacco constructs compare to control treatment (control treatment = 100%)

the transgene coding for the polyhistidine cluster, combined with yeast metallothionein, marked HisCUP (Macek et al. 2002b).

Since the shoots are the most easily harvestable part of the plant, an effective phytoextraction technology requires the accumulation of a high concentration of risk elements in aboveground biomass. For this reason cadmium distribution in the control treatment and HisCUP tobacco plants were tested. Distribution of this element among the leaves, stems and roots showed a majority of Cd in leaves of both treatments (87% of the total uptake by HisCUP treatment and 71% by control one) (Figure 1). The uptake by stems was the lowest and was equal to 5% from the total uptake in the control plants and 3% in HisCUP plants.

The plants produced a range of ligands for cadmium, nickel and zinc. In the xylem, sap moving from roots to leaves is histidine one of the principal ligands for Zn and Ni (Rauser 1999). The Zn content in roots of plants expressing HisCUP and HisGUS was decreased and the content in aboveground biomass had increased but not significantly different from the non-transformed control ones (Tables 1 and 2). The highest Zn content in aboveground biomass and also in roots was determined in GUS plants (Tables 1 and 2).

Free histidine has been found as a metal chelator in xylem exudates in plants that accumulate Ni and the amount of free histidine increases after Ni exposure (Krämer et al. 1996). According to Kärenlampi et al. (2000) the modification of histidine metabolism, it might be possible to increase the Ni-accumulating capacity of plants. Our results showed that nickel accumulation in tobacco was higher in the roots compared to the aboveground biomass (Tables 1 and 2). The highest content was determined in the roots of GUS (64.6 mg Ni/kg) and HisGUS transgenic plants (44.1 mg Ni/kg). The Ni content of HisGUS treatment was significantly higher in the aboveground biomass in contrast to the control plants, but Ni concentration of all transgenic plants as well as control plants was very low (2.9–6.5 mg Ni/kg).

Evaluation of accumulation potential of transgenic tobacco from medium containing metal mixture showed the highest relative change of combined metal uptake in the aboveground biomass of GUS treatment (Figure 2). The uptake of each tested element was increased by 31–54% compare to the control treatment. The GUS construct introduced the ability to remove a wider range of metals but HisCUP and HisGUS constructs confirmed the high accumulation ability only by one element – Cd or Ni and they are suitable for remediation of soils contaminated only by individual elements. The increase of the elements uptake by CUP construct was not significant compare to the control treatment.

The experiments continue on soil substrates, where risk elements bioavailability is substantially lower. Based on these results it will be possible to evaluate the chances to improve transgenic tobacco for phytoremediation in particular and perspectively other plants including energy or technology crops, too.

REFERENCES

Baker A.J.M., Brooks R.R. (1998): Terrestrial higher plants which accumulate metals elements: A review of their distribution, ecology and phytochemistry. Biorecovery, *1*: 81–126.

Brandle J.E., Labbe H., Hattori J., Miki B.L. (1993): Field performance and heavy metal concentrations of transgenic flue-cured tobacco expressing a mammalian metallothionein-beta-glucuronidase gene fusion. Genome, *36*: 255–260.

Cunnigham S.D., Berti W.R., Huang J.W. (1995): Phytoremediation of contaminated soils. Trends Biotechnol., *13*: 393–397.

Dorlhac de Borne F., Elmayan T., De Roton Ch., De Hys L., Tepfer M. (1998): Cadmium partitioning in transgenic tobacco plants expressing a mammalian metallothionein gene. Mol. Breed., *4*: 83–90.

Elmayan T., Tepfer M. (1994): Synthesis of a bifunctional metallothionein beta-glucuronidase fusion protein in transgenic tobacco plants as a means of reducing leaf cadmium levels. Plant J., *6*: 433–440.

Hasegawa I., Terada E., Sunairi M., Wakita H., Schimmachi F., Noguchi A., Nakajima M., Yazaki J. (1997): Genetic improvement of heavy metal tolerance in plants by transfer of the yeast metallothionein gene (*CUP1*). Plant Soil, *196*: 277–281.

Hattori J., Labbe H., Miki B.L. (1994): Construction and expression of a metallothionein beta-glucuronidase gene fusion. Genome, *37*: 508–512.

Kärenlampi S., Schat H., Vangronsveld J., Verkleij J.A.C., van der Lelie D., Mergeay M., Tervahauta A.I. (2000): Genetic engineering in the improvement of plants for phytoremediation of metal polluted soils. Environ. Pollut., *107***:** 225–231.

Kotrba P., Macek T., Ruml T. (1999): Heavy metal-binding peptides and proteins in plants. Collect. Czech Chem. Commun., *64*: 1057–1086.

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

Krämer U., Cotter-Howells J.D., Charnock J.M., Baker A.J.M., Smith J.A.C. (1996): Free histidine as a metal chelator in plants that accumulate nickel. Nature, *379*: 635–638.

Macek T., Macková M., Kás J. (2000): Exploitation of plants for the removal of organics in environmental remediation. Biotechnol. Adv., *18*: 23–35.

Macek T., Macková M., Kučerová P., Chromá L., Burkhard J., Demnerová K. (2002a): Phytoremediation. In: Agathos S., Reineke W. (eds.): Biotechnology for the environment: Soil remediation. Kluwer Acad. Publ., Brussels, Belgium: 115–137.

Macek T., Macková M., Pavlíková D., Száková J., Truksa M., Singh-Cundy A., Kotrba P., Yancey N., Scouten W.H. (2002b): Accumulation of cadmium by transgenic tobacco. Acta Biotechnol., *22*: 101–106.

Macek T., Macková M., Truksa M., Singh-Cundy A., Kotrba P., Yancey N., Scouten W.H. (1996): Preparation of transgenic tobacco with a yeast metallothionein combined with a polyhistidine tail. Chem. Listy, *90*: 690.

Němeček J., Podlešáková E., Vácha R. (2001): Prediction of the transfer of trace elements from soils into plants. Rostl. Výr., *47*: 425–432.

Ow D.W. (1996): Heavy metal tolerance genes: prospective tools for bioremediation. Resour. Conserv. Recycl., *18*: 135–149.

Pilon-Smits E., Pilon M. (2002): Phytoremediation of metals using transgenic plants. Crit. Rev. Plant Sci., *21*: 439–456.

Rauser W.E. (1999): Structure and function of metal chelators produced by plants – The case for organic acids, amino acids, phytin, and metallothioneins. Cell Biochem. Biophys., *31*: 19–48.

Vollenhofer S., Burg K., Schmidt J., Kroath H. (1999): Genetically modified organisms in food-screening and specific detection by PCR. J. Agr. Food. Chem., *47*: 5038–5043.

Vysloužilová M., Tlustoš P., Száková J., Pavlíková D. (2003): As, Cd, Pb and Zn uptake by different *Salix* spp. grown at soils enriched by high loads of these elements. Plant Soil Environ., *49*: 191–196.

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ABSTRAKT

Příjem kadmia, zinku a niklu geneticky modifikovanými liniemi tabáku

Tabák, *Nicotiana tabacum* L., var. Wisconsin 38 jako kontrola (WSC), a jeho čtyři transgenní linie byly testovány na akumulaci Cd, Zn a Ni. Geneticky modifikované linie byly připraveny vnesením genu *CUP1* pro kvasniční metalothionein (CUP), genu pro β-glukuronidázu (GUS), genů *CUP1* a pro histidinovou kotvu (HisCUP) a genů pro β-glukuronidázu a pro histidinovou kotvu (HisGUS) s cílem zvýšit akumulaci rizikových prvků. Kontrolní a transgenní rostliny byly pěstovány jako písková kultura s přídavkem Cd, Zn a Ni. Nejvyšší obsah Cd v nadzemní biomase opakovaně vykazovaly rostliny HisCUP. V porovnání s kontrolními rostlinami byl zjištěn nárůst obsahu Cd o 90 %. Ve všech transgenních rostlinách byl stanoven nižší obsah Cd v kořenech v porovnání s kontrolními rostlinami. V kořenech varianty HisCUP bylo zjištěno nejnižší množství Cd – o 49 % méně v porovnání s kontrolou. Významné zvýšení obsahu Zn nebylo potvrzeno v nadzemní biomase žádné z testovaných transgenních linií. Schopnost významně zvýšené akumulace Ni byla stanovena pouze u rostlin HisGUS. Rostliny transgenního tabáku GUS kumulovaly ve zvýšené míře všechny sledované prvky, ostatní testované linie přijímaly ve zvýšené míře pouze jeden prvek.

Klíčová slova: transgenní tabák; metalothionein; polyhistidin; β-glukuronidáza; kadmium; zinek; nikl; akumulace

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