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Phytoremediation of cadmium contamination: Overexpression of metallotionein in transgenic tobacco plants

Summary

The contamination of heavy metals by mining and combustion of fossil fuel has brought about significant deleterious consequences not only to environment but also to human health. Cadmium is a potentially toxic metal which causes renal dysfunction, pulmonary emphysema, and possibly bone demineralization. To explore the potential of plants to tolerate this metal, we engineered a model plant, Nicotiana tabacum, to express a metallothionein under the control of CaMV 35S promoter. Transgenic plants expressing metallothionein were able to grow in concentrations of up to 200 µM $CdSO_4$. Non-transgenic plants promptly underwent leaf chlorosis and their growth and development were inhibited on the medium containing 50 µM $CdSO_4$. PCR analysis for $CdSO_4$ from sensitive and resistant T_2 seedlings confirmed a high correlation between increased Cd-tolerance and the transgenic genotype, indicating that cadmiun resistance is stably inherited in the next generation. This result suggests one possible strategy for phytoremediation of heavy metals by expression of plant MT.

Key words

Agrobacterium mediated transformation · Cadmium · Metallothionein · Nicotiana tabacum · Phytoremediation

Metal contamination of soil and water is caused by coal mining, fossil fuel combustion, application of fertilizers, pesticides and sewage sludge. Because heavy metals, unlike organic pollutants, cannot be chemically degraded or biodegraded by microorganisms, their content has steadily increased in soil and subsequently accumulated in plants and animals. Needless to say, such chronic exposure to toxic metals can cause significant deleterious consequences in the environment and for human health. Cadmium is a potentially toxic metal that can accumulate in the human body with a half-life exceeding ten years. Chronic exposure to Cd is associated with renal dysfunction, pulmonary emphysema, and possibly bone demineralization [1, 2, 3]. For human health and environmental restoration, the use of plants to clean up toxic metals from contaminated soil or water has been developed [4, 5]. Because a number of plant species are naturally capable of high levels of organic compound degradation [6] or of heavy metal hyperaccumulation [7], plant biotechnology provides an opportunity to develop transgenic plants with increased metal binding or metal reducing capacity (Table 1). Besides, phytoremediation is a low cost technology, and some extracted metals may be recycled [8].

Da Schwermetalle im Boden und Wasser nicht durch Mikroorganismen abgebaut werden können, wurde die Verwendung von Pflanzen zur Reinigung schwermetallverseuchten Bodens oder Wassers weiterentwickelt.

Heavy metals, such as copper and zinc, are essential micronutrients in cellular metabolism, and serve as structural and catalytic components of proteins and enzymes. However, these micronutrients and heavy metals such as cadmium, lead, mercury and nickel are extremely toxic to cells in excessive concentrations. To balance the concentration of these toxic metals in cells, all organisms induce biosynthesis of low molecular weight, cysteine-rich proteins called metallothionein (MT) [16, 17]. MTs are present in various eukaryotic organisms including fungi, invertebrates, insects, mammals and plants. The cysteine residues in MT probably play an important role in metal chelation considering sequence conservation across species. The function of animal MTs has been reported as a „storehouse" for zinc, a free-radical scavenger, or a protector against Cd [18]. Recently, plant MT genes have been characterized from several plants [19, 20, 21]. They are also differentially induced by various environmental stresses such as heavy metals, heat-shock, plant hormones, wounding, senescence and virus infection. Although little is known about the biochemical mechanisms involved in such induction responses, plant MTs are thought to play a role in metal metabolism and detoxification due to their metal-binding activity and inducibility by heavy-metal ions [22].

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Phytoremediation von Kadmiumbelasteten Böden: Überexpression von Metallothionein in transgenen Tabakpflanzen

Zusammenfassung

Die Verunreinigung mit Schwermetallen aus Bergbaubetrieben und durch Verbrennung von fossilen Brennstoffen hat zu deutlichen schädlichen Folgen nicht nur für die menschliche Gesundheit, sondern auch für die Umwelt geführt. Cadmium ist ein potentiell toxisches Metall, das Nierenerkrankungen, Lungenemphyseme und möglicherweise Entmineralisierung der Knochen bewirken kann. Um die Fähigkeit von Pflanzen zur Aufnahme dieses Metalls aus dem Boden zu untersuchen, haben wir Nicotiana tabacum als Modellpflanze benutzt und sie durch Einbringen eines Konstruktes aus einem Gen für ein Metallothionein unter der Kontrolle des CAMV 35S Promoters gentechnisch verändert. Transgene Tabakpflanzen, die das Metallothionein-Gen exprimierten, konnten in Gegenwart von $CdSO_4$-Konzentrationen bis zu 200 µM wachsen. Nichttransgene Pflanzen bekamen sofort chlorotische Blätter und das Wachstum der Pflanzen wurde bei einer $CdSO_4$-Konzentration von 50 µM gehemmt. Die PCR-Analyse der Cd-sensitiven und der Cd-resistenten Nachkommen aus der T_2-Generation bestätigte die hohe Korrelation zwischen der phänotypischen Expression des Metallothionein-Gens und dem transgenen Genotyp; damit war gezeigt, dass die Cadmium-Resistenz gentechnisch stabil auf die Folgegeneration vererbt worden war. Dies Ergebnis zeigt eine mögliche Strategie zur Phytoremediation von Schwermetallen durch Expression des pflanzlichen Methallothioneins auf.

Schlüsselwörter

Schwermetall · Cadmium · Metallothionein · Phytoremediation · Gentechnisch veränderte Pflanze

CaMV, Cauliflower Mosaic Virus;
MT, metallothionein;
nptII, neomycin phosphotransferase II;
PC, phytochelatin
TMV, Tobacco Mosaic Virus

Das Ergebnis zeigt, dass durch Nutzung von Pflanzen-MT-Genen die Phytoremediation von schwermetallverseuchtem Erdreich oder Wasser möglich ist!

We were interested in understanding the role of plant MT genes in plants during exposure to high concentrations of heavy metals. A wound- and pathogen-inducible MT cDNA was previously isolated from *Nicotiana glutinosa* [23]. We introduced MT cDNA into tobacco plants via an *Agrobacterium* mediated transformation system. Overexpression of the MT gene conferred cadmium tolerance in transgenic tobacco plants. This result provides the possibility of phytoremediation of heavy metal contaminated soil or water using plant MT genes.

Results

Transformation of the chimeric gene encoding MT into tobacco plants

The MT gene was previously isolated from a subtractive cDNA library constructed with mRNA from *N. glutinosa* plant tissues showing a systemic HR to TMV [23]. The recombinant plasmid carrying the MT gene was isolated from KC9-10 clone and digested with *Bam*HI and *Nsi*I. Approximately 450 bp DNA fragments including an 18 bp 5' UTR, an ORF encoding MT, and 163 bp 3' UTR were eluted and ligated in the *Bam*HI and *Pst*I sites of pBluscript SK(+) vector. The pMBP1 was chosen as a binary vector used for plant transformation. The purified DNA fragments from a recombinant pBluscript SK(-) vector carrying a full-length MT gene were cloned in the *Bam*HI and *Kpn*I sites of pMBP1 for overexpression of the MT gene. The resulting structure was called pMBP1-MTS. The construction of the resultant plasmids is described in Fig. 1A. The recombinant binary vector, pMBP1-MTS, was introduced into *A. tumefaciens* strain LBA4404 harboring the disarmed plasmid pAL4404. Tobacco leaf discs were cocultivated with *Agrobacterium* carrying the MT gene. Transformed shoots were directly formed on the cutting edges of tobacco leaf discs on medium supplemented with 0.1 mg/L NAA, 1 mg/L AP, 100 g/L kanamycin and 300 g/L carbenicillin and regenerated in vitro. More than 90% of the regenerated tobacco plants with the *npt*II gene were normal in morphology and growth rate.

The MT gene was stably integrated into the nuclear genomes

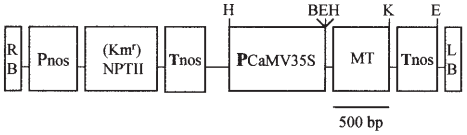
Genomic DNA was isolated from fully grown leaves of a nontransgenic plant and a transgenic plant. Transgenic plant line #+1 showed PCR bands for the *npt*II gene and cadmium resistance. From Southern analysis of the genomic DNA, major bands of approximately 3.2 kb were detected from *Eco*RI and *Eco*RI/*Hin*dIII digested genomic DNA of both transgenic and nontransgenic tobacco plants. This result suggests that the nascent tobacco MT gene which has a high nucleotide sequence homology with the introduced MT gene. Each of the bands, approximately was present in the genome as multicopies in repeated sequences 1.0 b for *Eco*RI and 0.9 kb for *Eco*RI/*Hin*dIII, was detected only in the transgenic plant and represented a foreign MT gene which was introduced *via Agrobacterium* mediated transformation. This result confirmed the stable integration of the foreign MT gene into the nuclear genomes of the transformed tobacco plants (Fig. 1B).

The MT transcript was highly expressed in the transgenic tobacco plants

Total RNA was isolated from nontransgenic and transgenic tobacco plants at the same developmental stage, and equally loaded onto an agarose gel. Northern hybridization showed specific bands for the MT transcripts at approximately 600 bp (Fig. 2). Although the transcriptional levels of the MT gene from individual tobacco plants varied, the expression level of the MT transcripts from transgenic plant lines, such as #+1 (lane 3), #+5 (lane5), and #+6 (lane 6), was higher than those of nontransgenic plant lines. This indicates the active transcription of the introduced gene in these transgenic tobacco plants (Fig. 2).

Resistance of transgenic tobacco plants to cadmium sulfate

The nontransgenic tobacco plants were previously tested for cadmium resistance on a medium containing various concentrations of cadmium sulfate. Con-



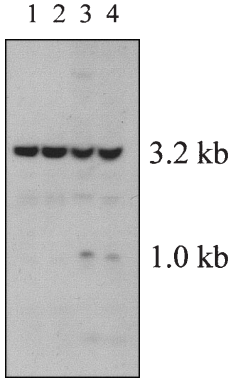


Fig. 1 ◀ **A restriction enzyme map of a binary vector carrying the MT gene, pMBP1-MTS (A) and a genomic Southern blot analysis for the metallothionein gene from T_1 transgenic tobacco plants (B). (A) The thick line below the MT box, labelled „500 bp" is for the MT gene coding sequence. B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; and K, *Kpn*I; LB, T-DNA left border; RB, T-DNA right border; *npt*II, neomycin phosphotransferase gene II; Pnos, nopaline synthase promoter; Tnos, nopaline synthase terminator; P_{35S}, CaMV 35S promoter. (B) Chromosomal DNA was digested with *Eco*RI and *Eco*RI/*Hin*dIII, run on a 0.7% agarose gel, blotted onto a Nytran membrane and hybridized with ^{32}P-labeled *Bam*HI/*Kpn*I DNA fragment of the metallothionein gene. *Eco*RI (*lane 1*) and *Eco*RI/*Hin*dIII (*lane 2*) digested DNA from untransformed plant tissues. *Eco*RI (*lane 3*) and *Eco*RI/*Hin*dIII (*lane 4*) digested DNA from MT transgenic tobacco line #+1**

trol plants suffered leaf chlorosis on a medium containing only 10 µM $CdSO_4$ and their growth was severely retarded at a concentration of 100 µM $CdSO_4$. At concentrations higher than 200 µM $CdSO_4$, nontransgenic plants promptly turned white and eventually died (data not shown). Ninety individually regenerated shoots having the *npt*II gene were directly rooted in MS media supplemented with both 100 mg/L kanamycin and 50, 100, or 200 µM $CdSO_4$. Approximately 60% of the transgenic plants rooted in the medium containing 50 µM $CdSO_4$ developed normally. Only two plants were found to be severely growth-inhibited. In the medium containing 100 µM $CdSO_4$, the number of unaffected transgenic plants decreased to approximately 20% and the number of severely growth-retarded transgenic plants increased to approximately 20%. At a concentration of 200 µM $CdSO_4$, approximately 30% of the transgenic tobacco plants were not affected, and growth was normal in all plants (Fig. 3A and 3B). After acclimatization, approximately 70 individual transgenic plants showing either cadmium tolerance or kanamycin resistance were moved to soil in a green house. The growth of T_1 transgenic tobacco plants may have been delayed because they were exposed to cadmium stress during their root development. However, the flowering, seed development and germination processes of all transgenic plants showed increased cadmium tolerance.

Inheritance of the MT gene into progenies

To investigate whether *npt*II and MT genes were stably inherited by the next generation, self-pollinated seeds from T_1 transgenic lines #+1, #+5, and #+6 were germinated on a MS medium containing 300 mg/L of kanamycin or 100 µM $CdSO_4$. The germination of all seeds was normal on MS media supplemented with either 300 mg/L of kanamycin or 100 µM $CdSO_4$, but kanamycin-sensitive seedlings promptly turned white and cadmium-sensitive seedlings were severely inhibited in their leaf development and root growth. The root length of sensitive seedlings was markedly shorter with severe leaf chlorosis when compared to resistant seedlings (Fig. 4A). From the T_1 transgenic line #+1, 127 kanamycin-resistant seedlings and 40 kanamycin-sensitive seedlings were counted on the media containing 300 mg/L kanamycin, whereas 115 cadmium-resistant seedlings and 39 cadmium-sensitive seedlings were enumerated on the media supplemented with 100 µM $CdSO_4$. The 3:1 Medelian pattern of segregation for kanamycin and cadmium resistance (data not shown), indicates that the *npt*II and MT genes were stably inherited by progeny. The correlation between the phenotypic and genotypic patterns of T_2 seedlings of the T_1 transgenic line #+1 was investigated by isolating DNA from the samples and then performing PCR. Primers specific for the transgenic MT gene were designed to avoid amplification of the endogenous MT gene. All six of the sensitive seedlings were negative for the transgenic MT gene. Among the resistant seedlings, 15 out of 18 (87%) were positive for the gene. There is, therefore, a high correlation between the phenotypic expression and the transgenic genotype (Fig. 4B).

Discussion

Recent studies have demonstrated that transgenic plants with an increased capacity to metabolize or chelate heavy metals could be used for remediation of metal-contaminated soil and water (Table 1). Cadmium is a favorable target metal for phytoremediation because it is readily transported and accumulated in the shoots of several species [24]. To explore the potential of transgenic plants to sequester this metal, a wound- and pathogen-inducible MT cDNA was integrated into tobacco plants via an *Agrobacterium* mediated transformation system. The transgenic tobacco plants overexpressing MT had an increased Cd tolerance on culture medium containing up to 200 µM $CdSO_4$ (Fig. 3). Whereas control plants suffered from leaf chlorosis on medium containing 10 µM $CdSO_4$ (data not shown), their growth was severely retarded at a concentration of 100 µM $CdSO_4$ (Fig. 3 and Fig. 4).

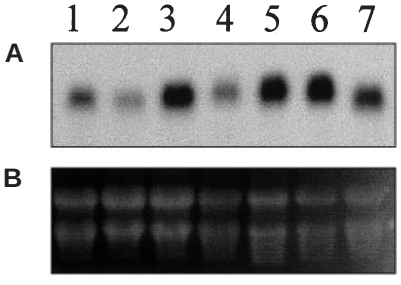


Fig. 2 ▲ **Northern blot analysis for the MT gene from T_1 transgenic tobacco plants. Total RNA (20 µg/lane) of nontransgenic (1-2) and transgenic (3-7) tobacco plants was subjected to electrophoresis on a 1.2% denaturing formaldehyde gel. The RNA was blotted and probed with ^{32}P-labelled 0.45 kb *Bam*HI/*Pst*I DNA fragments of the MT gene (A), and ribosomal DNA fragments from petunias as a control (B). Northern bands indicate the position for the nascent and introduced MT gene transcripts at 0.6 kb**

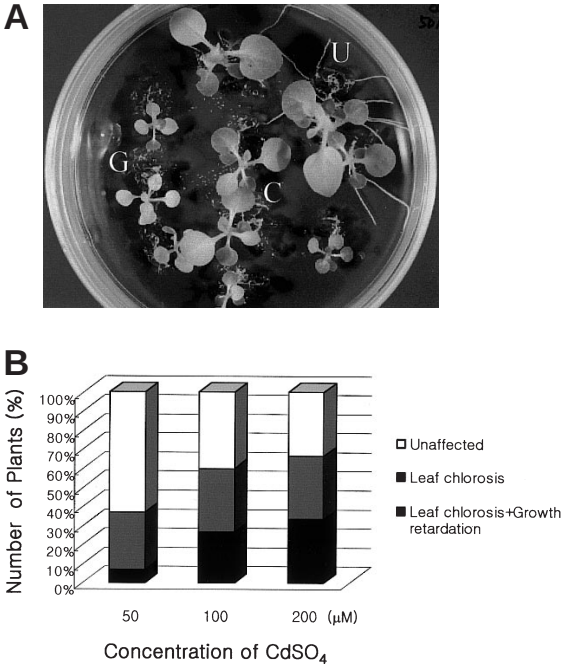


Fig. 3 ▲ **Cadmium resistance of kanamycin resistant tobacco shoots after transformation.** (A) **and** (B)**: Ninety individual regenerated shoots having *npt*II and *N. glutinosa* MT genes were directly rooted on a MS medium containing both 100 mg/L of kanamycin and 50, 100, and 200 μM $CdSO_4$, respectively. After approximately one month, the number of individual plants tested were divided into three groups – unaffected (U), leaf chlorosis (C), and leaf chlorosis and growth retardation (G) by their developmental phenotypes, and counted.**

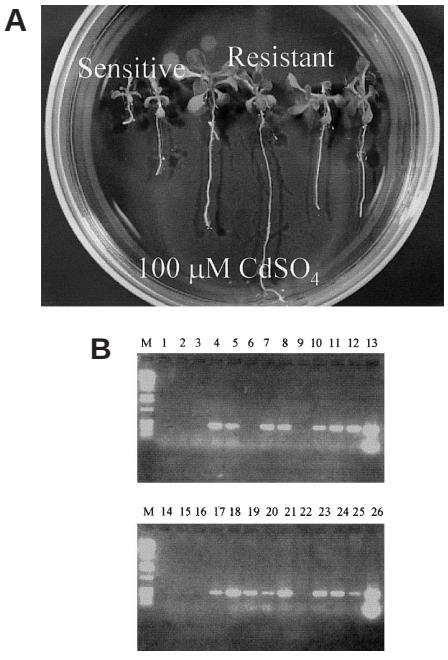


Fig. 4 ▲ **Inheritance of the transgenic MT gene into progenies.** (A) **The self-pollinated seeds were aseptically germinated on a MS medium containing 100 μM $CdSO_4$, and then resistant and sensitive seedlings for cadmium sulfate were found.** (B) **PCR analysis of the cadmium sensitive and resistant seedlings for the transgenic MT gene. M, BRL's 1 kb ladder; 1-3 and 14-16, cadmium sensitive seedlings; 4-12 and 17-25, cadmium resistant seedlings, 13 and 26, the recombinant plasmid, pMBP1-MTs as a positive control**

When the self-pollinated seeds of transgenic tobacco line #+1 were germinated on medium supplemented with 100 μM $CdSO_4$, cadmium sensitive and resistant seedlings segregate at a 3:1 ratio, indicating that the cadmium resistant character is stably inherited into progenies. PCR analysis of the cadmium sensitive and resistant seedlings for the transgenic MT gene showed that there was approximately 87% correlation between the increased tolerance and the transgenic genotype. However, three phenotypically resistant seedlings showed negative results. The degree of correlation between the increased tolerance and the transgenic genotype may be increased if seedlings having approximately ten leaves are used, or if seedlings are grown on medium supplemented with $CdSO_4$ at a concentration higher than 100 μM.

Kadmium ist ein geeignetes Zielmetall zur Phytoremediation, da es leicht transportiert wird und in den Schößlingen verschiedener Spezies angesammelt wird.

According to Pan et al. [9] and Zhu et al. [14], mouse MT cDNA and *E. coli gsh*II encoding glutathione synthetase were transformed into tobacco and Indian mustard (*B. juncea*), respectively. The overexpression of the MT gene or glutathione synthetase confers cadmium resistance on transgenic plants. Our results are in accordance with theirs. Phytochelatins (PCs) including MT contain a high percentage of Cys sulfhydryl residues, which are able to bind and sequester heavy metal ions in stable complexes. PCs were induced by heavy metals such as Cd in all plants tested [25]. Recent studies using yeast and *Arabidopsis* mutants with abolished PC production suggest that the role of PCs is correlated with metal tolerance [26, 27, 28]. Therefore, PCs including *N. glutinosa* MT might be useful proteins in the production of heavy metal tolerant plants. However, although transgenic plants overexpressing MT genes or PCs are more

Table 1
Transgenic plants showing hypertolerance and/or hyperaccumulation of heavy metals

Metals tested	Transgenic plants	Gene engineered	Source of gene	
Cd	Nicotiana tabacum	Metallothionein	Mus musculus	[9]
Hg	Arabidopsis thaliana	Mercury reductase (merA)	Escherichia coli	[10]
Al	N. tabacum	Citrate synthase	Pseudomonas aeruginosa	[11]
Hg	Liriodendron tulipifera	Modified merA	E. coli	[12]
Cd	N. tabacum	Metallothionein	N. glutinosa	[13]
Cd	Brassica juncea	Glutathione synthetase	E. coli	[14]
Hg	A. thaliana	merB	E. coli	[15]

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 [29]. 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 of Cu and Zn, T_2 seedlings showed increased tolerance for Cu and Zn (data not shown). 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 to the MTs of animals and microorganisms, the *N. glutinosa* MT may serve as an intracellular „sink" for excess metals.

Die Verwendung des von einer Pflanze stammenden Gens könnte gegenüber der Verwendung eines von einem Tier oder Mikroorganismus stammenden Gens zusätzliche Vorteile liefern.

Often, animal and microorganism derived genes have been used in creation of transgenic plants. Use of a plant derived gene, however, may provide many additional advantages over the use of an animal or microoganism derived gene. For instance, the gene regulatory mechanisms and translational system such as codon usage are very different when genes of animals or microorganisms are utilized in heterologous plant cells [12, 29]. In addition, to elevate the efficiency of foreign gene expression in transgenic plants, tissue-specific promoters which could be switched on in only root, leaf or seed tissues have been investigated. High biomass plants with the ability to concentrate Cd to high levels within their shoots or weeds having a fast growth rate could be subjected to genetic transformation for phytoremediation of heavy metal contaminated areas.

Outlook

Phytoremediation using transgenic plants for removing heavy metals from contaminated soil or water is a potentially more cost-effective technology than soil replacement, solidification, and washing methods which are currently used. Recently transgenic tobacco plants with enhanced capacities to chelate or metabolize toxic metals have been tested in the field [30, 31]. By transforming fast growing trees such as poplar phytoremediation may be commercially used in near future.

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