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Expression of mouse metallothionein-I gene confers cadmium resistance in transgenic tobacco plants

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Abstract

Transgenic tobacco plants containing a mouse metallothionein-I (MT-I) gene fused to the cauliflower mosaic virus 35S (CaMV 35S) promoter and nopaline synthase (nos) polyadenylation site were obtained by transforming tobacco leaf discs with an *Agrobacterium tumefaciens* strain carrying the chimaeric gene. Transformants were directly selected and rooted on medium containing cadmium and kanamycin. A total of 49 individual transgenic tobacco plants were regenerated. Among them 20% showed a very high expression level and their growth was unaffected by up to 200 μM cadmium, whereas the growth of control plants was severely affected leaf chlorosis occurred on medium containing only 10 μM cadmium. The concentration of MT-I in leaves of control and transgenic tobacco was determined with Cd/haemoglobin saturation assay, a polarographic method and western blotting. In addition, seeds from self-fertilized transgenic plants were germinated on medium containing toxic levels of cadmium and scored for tolerance/susceptibility to this heavy metal. The ratio of tolerant to susceptible plants was 3:1 indicating that the metallothionein gene is inherited as a single locus.

Introduction

Metallothioneins (MT) are an ubiquitous class of low-molecular-weight proteins consisting of a single polypeptide chain of 61 amino acid residues, of which 20 are cysteines that chelate seven bivalent cations, leaving neither free thiol groups nor a disulphide bridge. They are widely distributed among both animal and plant species, in which their synthesis is induced by the metal ions to which they bind, *i.e.* Cd, Zn, Hg, Cu, Ag and Pb [11]. Taking into account structural relationships, MTs have been subdivided into three classes. Class I includes mammalian MTs and polypeptides from other phyla with a related primary structure [10]. Class II comprises MTs displaying no or only very distant correspondence to the mammalian forms, such as MTs from sea urchin, wheat, yeast, and certain prokaryotes [26]. Class III subsumes typical polypeptides containing γ-glutamylcysteinyl units [18]. All class I and II MTs characterized thus far are single-chain proteins. Mammalian forms contain 61 to 62 amino acid residues; chicken MT and sea urchin MT contain 63 and 64 residues, respectively. Shorter chains are found in invertebrates and in certain fungi, the shortest one with 25 residues in *Neurospora crassa*. Class III MTs

are often oligomeric structures made up of two or more polypeptide chains of variable length. The most conspicuous feature of all forms is the abundance of Cys totalling up to one-third of all residues, the frequent occurrence of Cys-X-Cys tripeptide sequences, where X is an amino acid residue other than Cys [12].

Over the past years, modern agricultural practices such as the excessive use of phosphate fertilizer and sewage sludge have resulted in contamination of agricultural soils with heavy metals. The passive uptake of metals such as cadmium (Cd) brings them into the food chain, and consumption of such contaminated food and tobacco results in chronic exposure, which poses a serious threat to human health [22]. In addition, industrial activities such as mining and smelting operations have produced large areas with copper (Cu)- and zinc (Zn)-contaminated soils [1], where climatic factors are otherwise favourable for crop production. The increasing levels of toxic metals in the soils necessitate the production and use of plant varieties capable of (1) heavy-metal tolerance and (2) sequestration of toxic metals in unconsumed plant parts. Despite the presence of phytochelatins (small peptides, which are not gene products), plants are generally susceptible to enhanced levels of Cd [17]. In this paper, we have successfully transferred the gene coding mouse liver metallothionein into tobacco plant using Ti-plasmid vectors. Our results show that expression of the gene confers cadmium resistance in transgenic tobacco plants.

Materials and methods

DNA preparation

Plasmids were purified in quantity by cesium chloride/ethidium bromide gradient centrifugation [13]. Small amount of plasmids for screening purposes were prepared by the method of Maniatis *et al.* [14]. DNA fragments (< 500 bp) were eluted from acrylamide gels by cutting out ethidium bromide-stained bands, crushing the gel slice and mixing the fragments overnight in 0.5 M ammonium acetate at 37 °C. Large fragments of DNA (> 500 bp) were eluted from agarose gels by cutting out ethidium bromide-stained bands, crushing the gel slice and extracting with phenol.

Enzymes

All restriction endonucleases and T4 DNA ligase were obtained from New England Biolabs and were used according to the instructions of the supplier.

Transformation of Escherichia coli *cells*

Plasmid DNAs were introduced into *E. coli* cells by using $RuCl_2$-treated cells strain DHV α [21]. Cells carrying recombinant plasmids were selected or grown on Luria broth medium plates at 37 °C containing appropriate antibiotics (ampicillin 100 μg/ml, spectinomycin 50 μg/ml, kanamycin 50 μg/ml).

Construction of chimaeric gene

For cloning of the mouse metallothionein I gene into the intermediary transformation vector, a 335 bp fragment containing the entire MT coding sequence was excised from the pBX-mMT-I plasmid [6] by restriction digestion with *Bam* HI. This digest, fragment of lengths about 335 bp containing the translation initiation and termination sites was isolated after preparative polyacrylamide gel electrophoresis. The purified DNA fragment was cloned directly into the *Bgl* II site of pCo24 which is expression cassette vector containing 35S promoter [20]. The positive colonies were screened by *in situ* hybridization using (γ-^{32}P) ATP labelled mMT-I DNA as probe, followed by rapid plasmid digestion of the recombinant DNA. The structure of the resultant plasmid with sense orientation of the gene was confirmed by analysis of rapid plasmid digests of pCO24-mMT-I, using different restriction endonucleases.

The recombinant plasmids with sense orienta-

tion of gene were prepared from positive colonies and cut with *Bam* HI. The fragment of length about 2.2 kb, containing CaMV 35S promoter mMT-I gene and a nos terminater, was isolated and cloned into the *Bam* HI site of binary vector pBin 19 [2]. The entire construction of the resultant plasmid is described in Fig. 1.

Introduction of pBin 19 derivatives into A. tumefaciens

The resultant plasmid (pBin 19-mMT-I), carrying the chimaeric MT gene (CaMV 35S:mMT-I:nos 3), in addition to the chimaeric neomycin phosphotransferase II (Nos:NPT:NOS) gene for selection of transformed tissue of kanamycin, was introduced into *A. tumefaciens* strain LBA 4404 harbouring the disarmed plasmid pAL 4404 [15]. The transformation method was described by Hofegen and Willmitzer [8]. Briefly, competent agrobacteria (LBA 4404) were mixed with 0.5–1.0 μg chimaeric DNA. The cells were incubated on ice for 5 min, in liquid nitrogen for 5 min and at 37 °C for 5 min. After dilution in 1 ml YEB medium, the cells were shaken 2–4 h at 28 °C. Aliquots of 200 μl were plated on YEB plates containing 25 μg/ml rifampicin and 50 μg/ml kanamycin and incubated for 2 days at 28 °C.

Transformation, selection and regeneration

Transformation and regeneration of tobacco was essentially as described by Horsch *et al.* [9]. Leaf discs of *Nicotiana tabacum* cv. NC89 were punched from surface-sterilized leaves with a paper punch and submerged in a culture of *A. tumefaciens* grown overnight in Luria broth at 28 °C which contained the chimaeric CaMV 35S:mMT-I/nos gene. After gentle shaking to ensure that all edges were infected, the discs were blotted dried and cultured on carrot feeder plates containing Murashige and Skoog's basal medium (3% sucrose, 1 mg/l BA and 0.1 mg/l NAA pH 5.8; SR medium for cocultivation. After 2 to 3 days, the discs were transferred to plates containing the SR medium but without feeder cells and filter papers and containing carbenicillin (500 mg/l) to inhibit further growth of bacteria and 100 mg/l kanamycin or 10 μM Cd for selection. At 2–3 weeks, the explants were subcultured on the SR medium containing 30 μM of cadmium. Developing shoots were removed and replated onto MS medium lacking growth regulators but containing 200 mg/l kanamycin and 100 μM cadmium to root. 5–6 weeks after inducing root formation, mature transformants were transferred to a greenhouse.

Southern blotting analysis

Plant DNA was isolated from leaves of control and transformed plants according to the method described by Doyle *et al.* [5]. DNA digested with *Bam* HI was subjected to electrophoresis in 0.7% agarose gel and transferred to nitrocellulose. Prehybridization and hybridization conditions were same with Maniatis *et al.* [14]. Briefly, prehybridization was done for 2–6 h at 60–65 °C in buffer consisting of 6× SSC, 0.5% SDS, 5× Denhardt's solution and 100 μg/ml denatured salmon sperm DNA. Filters were hybridized to α-^{32}P-labelled denatured probe DNA (CaMV 35S/mMT-I/nos fragment).

Western blot

Metallothionein was induced by administering 22 subcutaneous injections of $CdCl_2$ (2 mg Cd per kg body weight) to the rabbits on alternate days. The purification of the protein from livers excised 1 to 3 days after the last injection was a modification of a procedure previously described [19]. The purification protocol involved homogenization, centrifugation, extraction of chloroform/ethanol, Sephadex G-50 gel filtration and DEAE Sephadex A-50 ion exchange chromatography. Monoclonal antibodies against metallothioneins were prepared by combining intraperitoneal injection with intraplenic injection [23] with a mixture of the two forms of MT (MT-1 and MT-2).

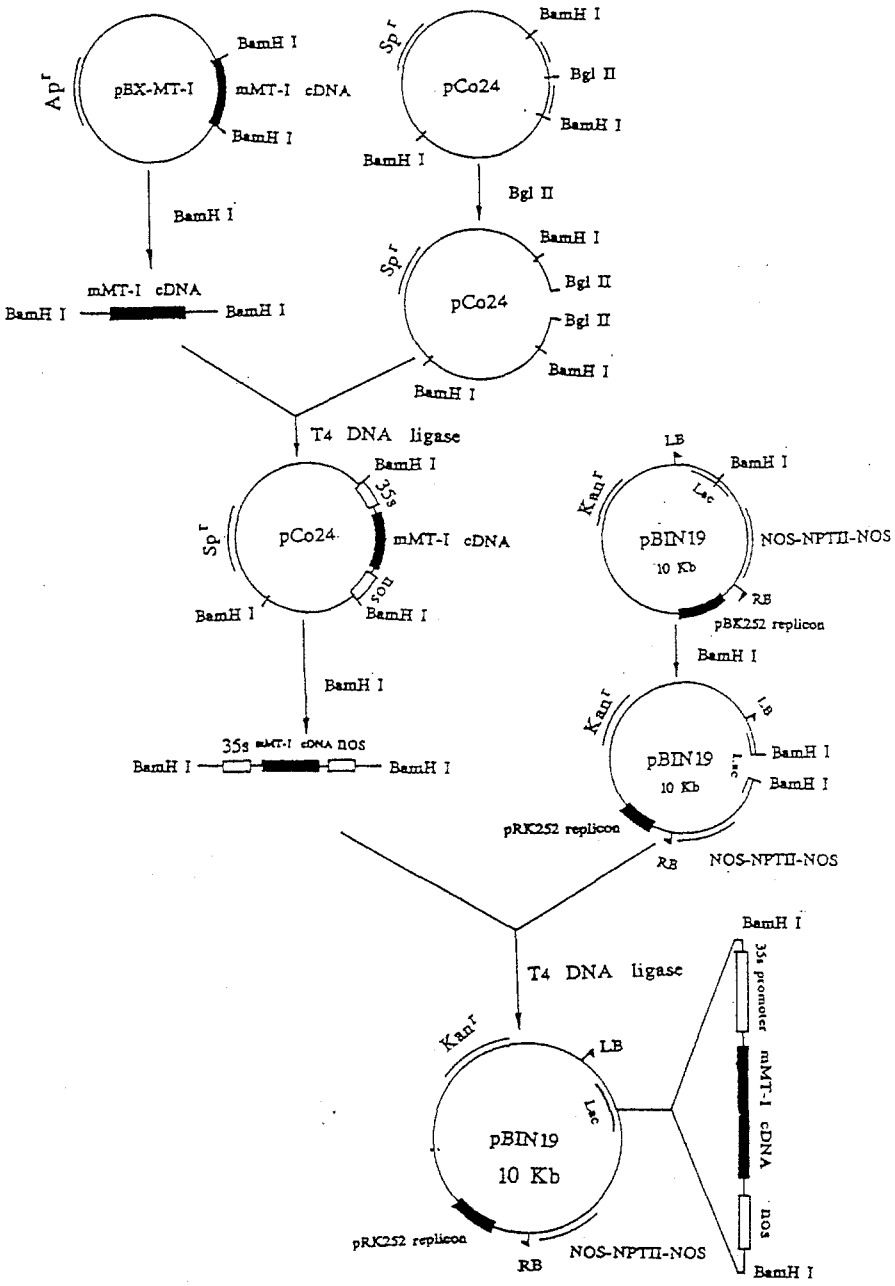

Fig. 1. Plant transformation vector containing chimaeric mouse metallothionein I gene. The promoter is derived from the cauliflower mosaic virus 35S gene. Polyadenylation signals are derived from nopaline synthase gene. Important functional regions of the vector are also shown: NOS-NPT-NOS which confers kanamycin resistance in plant cells; KM^R, a bacterial selectable maker; LB and RB, left and right borders for T-DNA transfer: pRK252 replicon, for mobilization and maintenance without integration into the resident Ti plasmid.

It was shown by western blot and ELISA that this monoclonal antibody had a unique cross-reactivity pattern when tested against MTs from the livers of several mammals.

Leaves of transgenic tobacco and control plants were homogenized in 0.02 M Tris-HCl buffer pH 8.0 (1 ml/g wet tissue) using a glass homogenizer. The homogenate was then centri-

fuged at 4 °C, 10000 × *g* for 10 min. The supernatant was bathed in boiling water for 3 min, then cooled and centrifuged as above. The supernatant can be used for the western blots, the Cd/haemoglobin saturation assay and the polarographic method. The western blot was done according to the procedure described by Burnette [4]. Initially, polyacrylamide gel electrophoresis (PAGE) was performed according to the procedure of Zhang *et al.* [5]. The separated sample was then transferred to nitrocellulose paper in a BioRad TransBlot apparatus in 0.025 M Tris, 0.192 M glycine buffer at pH 8.3 with 20% methanol. The nitrocellulose paper was blocked with 5% bovine serum albumin in 0.01 M Tris, 0.15 M NaCl pH 7.4 for 1 h at 37 °C. The strips were washed in 0.01 M Tris, 0.15 M saline buffer at pH 7.4 with (then without) 0.05% Tween 20. A conjugate of goat anti-mouse IgG/alkaline phosphatase (Sigma) diluted 1:500 in 5% bovine serum albumin was then applied. After similar washing, the strip was placed in an alkaline phosphatase substrate for 10 to 20 min and finally was washed with water.

Cd/haemoglobin saturation assay

An 100–200 μl of the prepared supernatant or purified MT was placed in an Eppendorf tube. Then 160 μl of 40 mg/l Cd was added to the tubes and mixed, resulting in a final Cd concentration of 8 mg/l in a total volume of 800 μl. The samples were allowed to incubate at room temperature for 10–15 min, followed by adding 100 μl of the 2% haemoglobin solution (prepared daily). The tubes were then heated in a boiling water bath for 2 min. After cooling in ice water, the tubes were centrifuged at 10000 × *g*, 4 °C for 5 min to remove precipitated proteins. The addition of haemoglobin, heating, cooling and centrifugation were then repeated twice. This procedure resulted in a tightly packed pellet and a clear supernatant from which a 50 to 200 μl aliquot was transferred to a 5 ml solution of 0.02 M HCl and 0.02 M KI for polarographic detection of cadmium. The solution was electrolysed at −0.40 V for 5 s, resulting in preconcentration of cadmium at the mercury electrode, followed by a cathodic sweep to −0.90 V at a scanning rate of 250 m V/s. The second-order derivative of peak current at −0.68 V (vs. SCE) was recorded. The MT content was calculated assuming 7 g Cd atoms bound per mole of MT with a molecular weight of 6050 [3].

Polarographic method

Linear sweep polarographic using the Brdicka procedure was carried out with a Model MP-1 polarographic analyser (Shan Tong 7th Electronic Equipment Factory, China) coupled to a Laser pp40X-Y printer plotter. A 50–100 μl of the prepared samples or purified MT were added into the polarographic cell in which contains 2 ml Brdicka solution (0.1 M NH_4Cl, 0.1 M $NH_3 \cdot H_2O$, 0.2 mM $CoCl_2$). Analysis was performed on 2 ml of supporting electrolyte scanning from −0.05 V to −0.18 V for 2 s using a dropping mercury electrode. The first order derivative of peak current at −1.45 V (vs. SCE) was recorded.

Seedling test

Seeds from self-fertilized transgenic plants were surface-sterilized with 2% sodium hypochloride for 20 min. After rinsing in distilled water, the seeds were aseptically germinated on MS agar medium containing 100 μM Cd. The ratio of tolerant to susceptible plants was determined after 3 weeks.

Results

Construction of a chimaeric gene encoding the MT protein

A metallothionein-processed gene (mMT-I) was supplied by Palmiter (1981) and its complete sequence was determined (data not shown). The strategy for constructing the chimaeric gene (pBin

19-mMT-I) containing mouse metallothionein-I gene is shown in Fig. 1. For insertion of the MT gene into the intermediary transformation vector, a 335 bp fragment was excised from the pBX-mMT-I plasmid (Fig. 1) by restriction digestion with *Bam* HI. This fragment was cloned at the *Bgl* II site of the expression cassette vector pCo24 by sticky ends. The positive colonies were screened by *in situ* hybridization (data not shown) followed by rapid plasmid digests of the recombinant DNA. Four out of six colonies were recombinant. The insert and its proper orientation with respect to the CaMV 35S promoter was confirmed by restriction endonuclease digestion of pCo24-mMT-I. The purified *Bam* HI-cut DNA fragments containing CaMV 35S promoter, mMT-I gene and a nos termination were cloned into the *Bam* HI site of binary vector pBin 19.

Transformation, selection and regeneration of tobacco

Leaf discs of tobacco were inoculated with *A. tumefaciens* strain LBA 4404 containing the binary vector pBin 19 mMT-I. The transformed cells of tobacco were selected and regenerated on SR media containing 100 mg/l kanamycin or 10 μM $CdCl_2$. Shoots developed within four weeks (Fig. 2B) following transformation, whereas the uninfected leaf discs did not produce callus on the same selection medium as above (Fig. 2A). Difference between transformation efficiencies using different selection agent (kanamycin 100 mg/l or Cd 10 μM) was slight. About half of infected leaf discs formed calli and shoots on selection medium. For shoot elongation, the explants were subcultured on SR medium

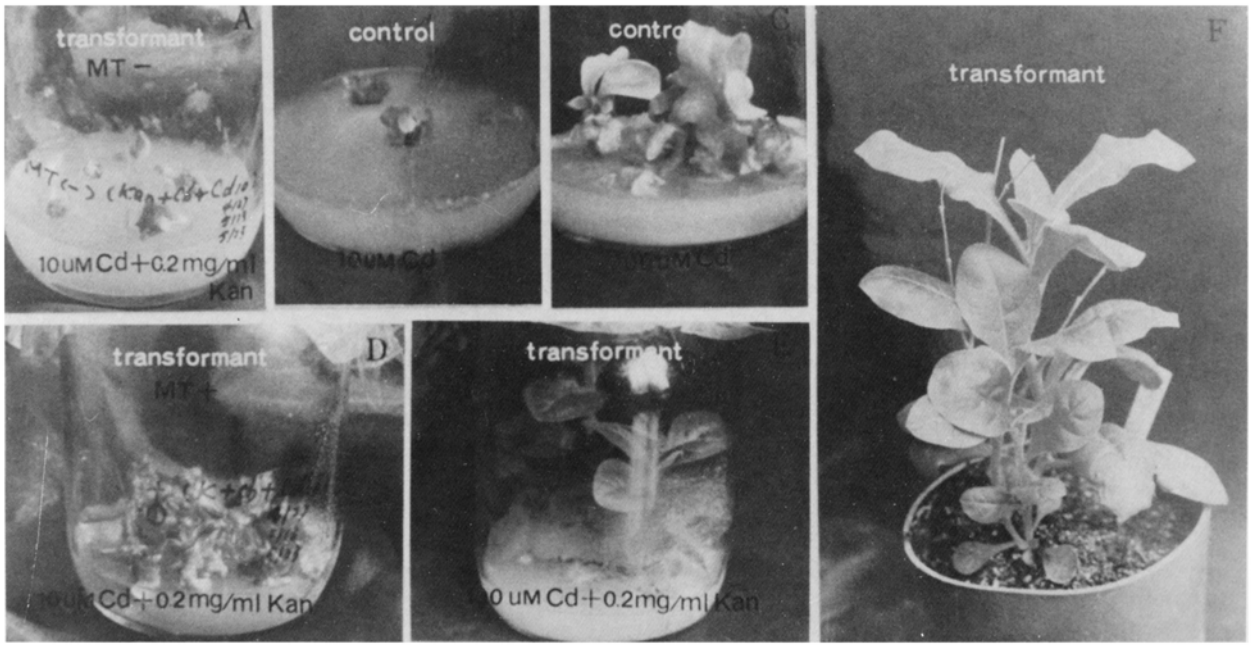

Fig. 2. Leaf discs transformation and selection of antibiotic-resistant and cadmium-resistant cells. Leaf discs were punched from a surface-sterilized of tobacco, inoculated with *Agrobacterium tumefaciens* strain (LBA 4404), cultured on feeder plates, and transferred 3 days later to a medium containing 100 μg of kanamycin and 300 μg/ml carbenicillin. The cultures were photographed 24 days after inoculation, and transferred to medium containing 10 μM cadmium. A week after inoculation, shoots transformed with pBin 19-mMT-I with respect or unrespect to orientation and control shoots are shown in A, B and D, respectively. Picture E shows root formation on select MS medium containing 100 μM of cadmium and 200 μg/ml kanamycin. Picture C shows the control plants on an MS medium containing 100 μM of cadmium. Picture F was taken from mature transformants, which are vigorous, and phenotypically.

containing 30 μM cadmium. Finally, regenerated shoots were rooted on MS medium containing 0.2 mg/ml kanamycin and 100 μM Cd. A total of 49 individual transgenic tobacco plants were regenerated. Among them 20% plants showed very high expression and their growth was unaffected by up to 200 μM Cd (Fig. 2D), whereas the control plants showed severe inhibition of root and shoot growth and chlorosis of leaves when growing on rooting medium containing 100 μM Cd (Fig. 2C). The three transgenic plants rooted on MS medium containing 100 μM Cd were chosen for Southern blot, western blot and other analysis. They were grown normally (Fig. 2F) and set seeds after transfer to the greenhouse.

Analysis of gene organization and expression in plant

Southern blot

To identify the mMT-I gene in chromosomal digests of transgenic plants, genomic DNA was extracted from mature leaves of three resistant transgenic plants and untransformed control plant. For Southern blot analysis, *Bam* HI-digested DNA was fractionated on a 0.7% agarose gel, transferred to nitrocellulose and hybridized to a P-labelled probe containing CaMV 35S promoter, mMT-I and the nos termination region. As expected, the probe hybridized to an internal *Bam* HI fragment of about 2.2 kb in the leaf DNA from tobacco transformant. No hybridization was observed in control tobacco leaf DNA (Fig. 3A).

Western blot

The No. 3 transformant showed in Fig. 3A was analysed for the expression of MT protein. As expected, the transgenic plant produced a 6.3 kDa protein that cross-reacted MT with antibodies and comigrated with the MT protein standard (Fig. 3B). In addition, the other protein found in transgenic plants (respected to multiple bands in the positive land) also showed cross-hybridization to MT antibodies, these protein may be the polymers of metallothionein since MT protein is

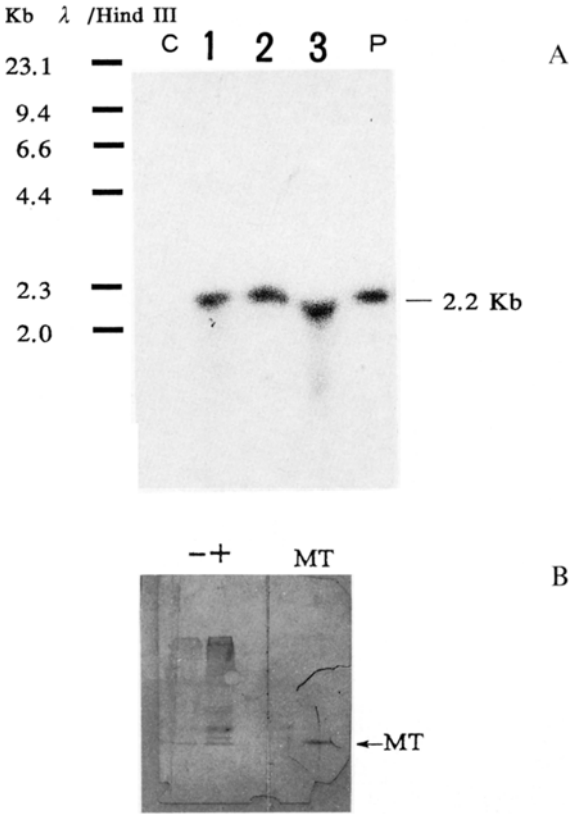

Fig. 3. Confirmation of chromosomal integration and expression of the chimaeric mMT-I gene in transgenic plants. A. Southern blot: lane C, *Bam* HI-cut DNA from control plant; lanes 1, 2, 3, *Bam* HI-cut DNA from transformants 1, 2, 3; lane P, probe (35S/mMT-I/nos) with a length of about 2.2 kb. B. Western blot: right lane, standard MT from mouse liver; middle lane, sample from transformant; left lane, control plant.

not stable and forms polymers in normal condition [10].

Cd/haemoglobin saturation assay

It was known that, per mole MT protein 7 g Cd atoms are bound, which could be dissociated in 0.02 M HCl solution and coordinated with iodide. Figure 4D shows single sweep polarograms of the complex of cadmium iodide and a linear relationship between cadmium and MT protein. The concentration of MT protein expressed in three transgenic tobacco plants that showed tolerance to 100 μM Cd was 10.5, 27.3 and 30 μg per gram leaf, respectively, whereas MT in control tobacco cannot be detected.

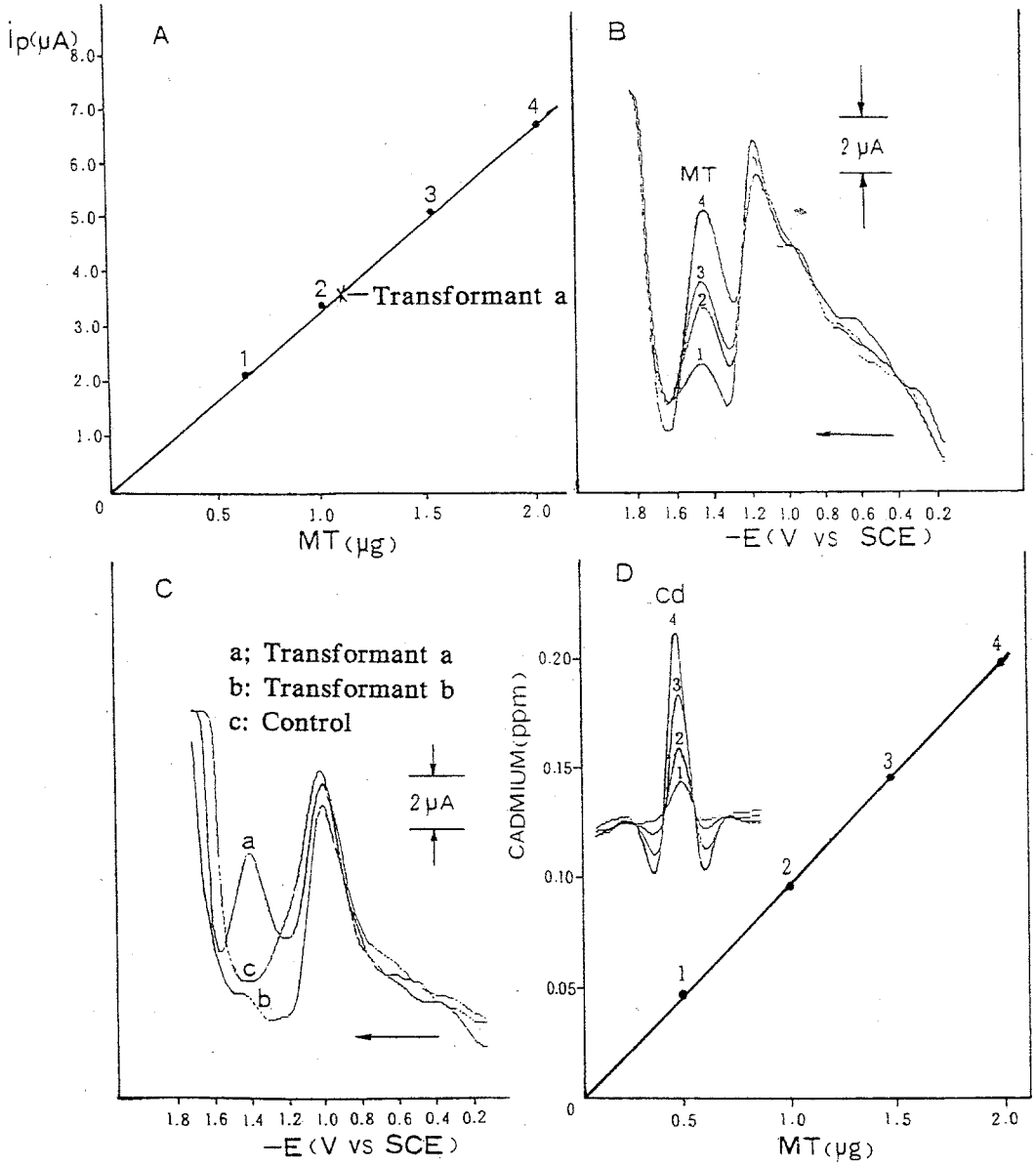

Fig. 4. Determinations of mouse metallothionein-I expressed in transgenic plants. About 0.4–0.5 g of tobacco leaves were homogenized in an equal volume of 0.01 M Tris-HCl, pH 8.6, buffer. The homogenate was heated in boiling water for 3 min and then cooled to 4 °C. The supernatant was directly added in ammoniacal solution of Co(II) salt to record linear sweep polarogram. The content of cysteine-rich MT can be obtained according to the peak height at −1.46 V (vs. SCE) (shown in A, B and C). D shows a linear relationship between cadmium and MT determined by Cd/haemoglobin saturation assay/single-sweep polarography. A different amount of purified MT or heat-denatured tobacco leaf supernatant were saturated with Cd. The unbound Cd in the solution was precipitated by adding bovine haemoglobin and heating in boiling water. After centrifugation, the supernatants were electrolysed at −0.40 V for 5 s followed by a cathodic sweep to −0.90 V at a scanning rate of 250 m V/S. The 2nd order derivative of peak current at −0.68 V (vs. SCE) was recorded. The MT contents were calculated, assuming 7 g Cd atoms bound per mole of MT with a molecular weight of 6050.

Polarographic method

The concentration of MT in transgenic plants were estimated by the polarographic method. Figure 4A and B give the calibration curve for MT protein. The leaves of transgenic tobacco and control plants were homogenized, heated and centrifuged. The 100 μl of prepared supernatant was directly added in ammoniacal solution of Co(II) salt for recording linear sweep polarogram. The content of MT which a rich cysteine content could be obtained according to the peak height at −1.46 V (vs. SCE) (Fig. 4C). The concentration of MT expressed in three transgenic tobacco that showed tolerance to 100 μM Cd was 14.3, 35.5 and 50.8 μg per gram leaf, respectively.

Inheritance of the cadmium-tolerant phenotype

Seed progeny from three self-pollinated transformants (R_1 generation) and control (untransformed) plants were germinated on MS medium containing 100 μM $CdCl_2$. The seedlings were then scored for root length for 3 weeks after germination. When control tobacco seeds were germinated on medium containing 100 μM $CdCl_2$, sensitivity to Cd was clearly indicated by inhibition of root growth. In contrast, the progeny of the selfed transformants segregated for root growth. As shown in Fig. 5, the seed progeny segregated into two distinct populations. The smaller population of seedlings had small stumpy roots with an average root length of 0.5 cm. In the large population, the seedling growth appeared to be unaffected on medium containing 100 μM Cd, the average root length of seedlings was 4.0 cm. A χ^2 analysis was conducted on data from the three transformants and it demonstrated that the ratio of tolerant to susceptible plants was 3:1 (Table 1). This ratio indicates that the MT gene was inherited as a single locus.

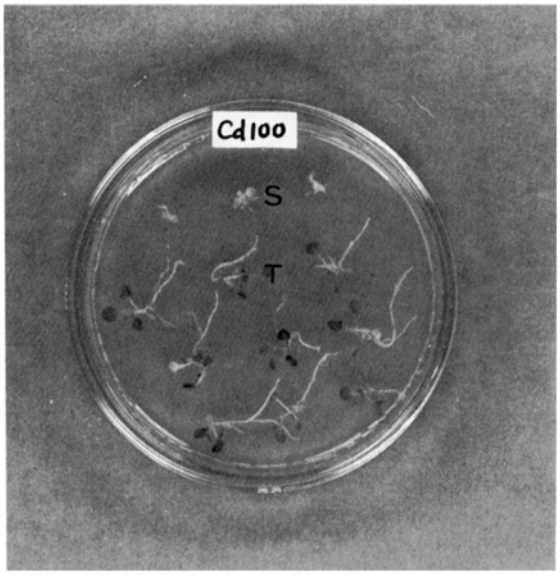

Fig. 5. Segregation of Cd-tolerant and susceptible phenotype in a R_1 population of transgenic tobacco seed sample No. 3. Seeds were germinated on MS medium with 100 μM $CdCl_2$ and scored for segregation 3 weeks after germination. T, tolerant; S, susceptible.

Discussion

We here describe the introduction of a chimaeric gene containing a cloned mouse metallothionein-I (mMT-I) cDNA into tobacco cells. The results in this paper reported that a mouse MT-I cDNA is integrated and expressed in transgenic tobacco plants. Several heavy-metal-tolerant transgenic plants were obtained. The growth of root and shoots of these transformed plants was unaffected by up to 200 μM Cd, whereas control plants showed severe inhibition of root and shoot growth and chlorosis of leaves cultured of the medium containing only 20 μM Cd.

Our approach of conferring heavy metal tolerance by a stable integration and expression of a single gene coding for heavy-metal binding and/or sequestering protein clearly demonstrated that plants can be genetically engineered for heavy-metal tolerance. In addition, the concentrations of metallothionein expressed in transgenic plants were determined by the Cd/haemoglobin saturation assay and the polarographic method. The values obtained by the polarographic method were slightly higher than those by the Cd/haemoglobin saturation assay. These results were in agreement with those described by Onosaka and Cherian [16].

Our results show that *Nicotiana tabacum* is sensitive to cadmium. The growth of uninfected tobacco leaf discs was almost completely inhibited at a cadmium concentration of 10 μM. Whereas the leaf discs cocultivated with *A. tumefaciens* LBA4404 (pBin 19 MT-I) were able to form healthy-looking shoots in shoot-inducing medium containing 10 μM cadmium at a frequency up to 50% (Fig. 2B). The cadmium resistant shoots continuously selected on rooting medium appeared to be cadmium-resistant up to 200 μM cadmium. Similar results were obtained for petunia. These results indicated that cadmium resistance provides an additional mode of selection and more flexibility in genetic manipulation of plant cells.

The Ti-plasmid-mediated genetic transformation of MT gene in plants provides a valuable method of generating metal-tolerant varieties, which could be useful for reclamation of wastelands and mine spoils. Also, this approach has a potential of regulating MT synthesis in a tissue-specific manner, thereby partitioning toxic metals in unconsumed parts of the plant. Analysis of plants grown on agricultural soils contaminated with sewage sludge and phosphate-fertilizers, which may contain high levels of Cd and other heavy metals, has shown that the highest concentration of these metals accumulate in leaf tissue. It is not surprising, therefore, to find high levels of cadmium in leafy vegetables. Expression of MT in root tissue specifically may overcome this problem to some extent. Efforts are now underway to express MT in roots of tobacco and petunia and examine its effect in partitioning of Cd between various plant parts.

Acknowledgements

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