Inheritance and Expression of the Mouse Metallothionein Gene in Tobacco

Impact on Cd Tolerance and Tissue Cd Distribution in Seedlings

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ABSTRACT

Genetically engineered seedlings obtained from self-fertilized transgenic tobacco (Nicotiana tabacum) contained and expressed the mouse metallothionein and kanamycin resistance marker genes and were more tolerant to cadmium stress than untransformed controls. Cadmium accumulation in leaves of transgenic seedlings exposed to a low, field-like Cd concentration (0.02 micromolar) was about 20% lower than that in untransformed controls. Genetic analysis of R1 and R2 progeny showed inheritance of the marker gene to be as a dominant Mendelian trait. These results suggest the possibility of developing transgenic plants with modified tolerance to heavy metal stress and food crops having lower Cd content.

Remarkable recent progress has been made in efforts to transfer useful, foreign genes to plants (4, 20). Principal targets have been increased resistance to disease and insect herbivory and increased tolerance to herbicide damage. Transformation and subsequent whole plant regeneration and characterization of field crop species including soybean, alfalfa, cotton, flax, oil seed rape, and tomato has now been demonstrated (6, 13 and references therein). We are interested in modifying plants to reduce the content of undesirable heavy metals in crops. To this end, we recently introduced the mouse gene encoding for the heavy metal chelating protein metallothionein (MT) into the model system tobacco (10). Our intent is to use this foreign chelator to effect desirable tissue partitioning of heavy metals in a seed-transmissible manner and also to probe endogenous mechanisms of heavy metal accommodation in plants. Present studies focus on influencing responses of plants to the potentially toxic, nonessential metal Cd, principally because vegetable components of the human diet are thought to contribute ≥70% of Cd intake in man (23). Cadmium has been implicated in several human pathological conditions and occurrence of Cd in agricultural soils is thought to be increasing as a result of the use of Cd-containing phosphate rock and sewage sludge as fertilizers (18).

Metallothionein is a metal (Cd, Hg, Zn, Cu, Ag, etc.) binding protein which occurs in animals (5, 8). Animal genes encoding MT are well characterized and are highly conserved (3, 5, 12). It is thought that MT functions in toxic heavy metal sequestration and, at least for multicellular animals, in Zn and Cu homeostasis (5). While higher plants and certain yeasts apparently do not form MT, they do form cysteine-rich, Cd-binding peptides (also called cadysteins, phytochelatins, etc.) when exposed to high levels of Cd (21). Plant peptides apparently have a lower affinity for Cd (Kd, about 10^19) than does Cd, Zn thionein (Kd, about 10^25) (17). Therefore, it is anticipated that the occurrence of the high affinity chelator MT in a transformed plant may modify responses of the plant upon exposure to Cd. The present paper demonstrates stable integration and expression of the MT gene in tobacco. In addition, a Cd-tolerance response and a lowered leaf Cd concentration in transformed seedlings is shown.

MATERIALS AND METHODS

Plant Transformation, Regeneration, and Growth

Transformation of Nicotiana tabacum cv Ky 14 was achieved using the binary vector pKYLX-7 (19) and an engineered Agrobacterium tumefaciens stain as described earlier (10). Primary transformants were regenerated from kanamycin resistant calli and propagated as described earlier (19).

Segregation Analysis

Seeds from self-pollinated transgenic plants (R1 and R2 progeny) were surface-sterilized with 70% ethanol for 2 min, then 0.5% sodium hypochlorite for 10 min, washed thoroughly with sterile water, then germinated on rooting medium (15) containing kanamycin (300 μg/mL). Sensitivity to kanamycin was scored after 3 weeks and the χ² test was used to evaluate results.

Analysis of DNA, RNA, and Protein

Genomic DNA was isolated from leaves of transformed R1 progeny and untransformed tobacco plants, and Southern analysis was as previously described (10). Total DNA (10 μg) was digested with ScaI and HindIII, separated on 1% agarose gel, transferred to nitrocellulose and hybridized with a nick translated BglII to PstI fragment of pM169 (10) containing the MT coding sequence. This same fragment was used for determination of gene copy number.

Total RNA was extracted from leaves of transformed R1
progeny and untransformed control plants for Northern blot analysis as described elsewhere (10). Total RNA (30 μg) was denatured in glyoxal at 50°C, fractionated on a 1.5% agarose gel, and transferred to nitrocellulose. Northern blots were hybridized with a nick translated pUCM901 probe containing mouse MT cDNA. Hybridization conditions and autoradiography were as described earlier (10).

Protein was extracted from mature, fully expanded leaves. Leaves were pulverized in liquid N2, homogenized in 25 mM K-phosphate (pH 7.2), 10 mM β-mercaptoethanol, and the homogenate was centrifuged at 100,000g for 30 min, 4°C. To label MT, 109Cd2+ was added prior to protein separation on Sephadex G-50 (fine). The Sephadex G-50 gel filtration column (90 x 15 cm) was standardized using blue dextran, Cyt c, insulins β chain, and rat liver metallothionein, and protein was eluted with 25 mM K-phosphate (pH 7.2), 10 mM β-mercaptoethanol. MT was detected by 109Cd-binding to the peak eluting at ν/ν0 of 1.6 which corresponded to standard rodent metallothionein.

Cd-Tolerance and Cd-Accumulation Analysis

Surface-sterilized seeds were germinated on Whatman No.1 filter paper wet with 3 mL of rooting medium (15) containing CdCl2 (0–1000 μM). Closed Petri dishes were placed under constant cool-white fluorescent lighting (300 μL/cm2) at 22 to 26°C. After 10 d, fresh weight and Chl content (11) of combined seedlings were determined. Values were normalized for 100 germinated seedlings and expressed as percentage of control (not exposed to CdCl2).

For Cd-accumulation analysis, Kan-resistance Ky 14M707 and untransformed Ky 14 seedlings were grown for 15 d in nutrient solution containing 0.02 μM Cd2+ (as 106CdCl2 2.5 μg/g RPI) employing a bare root culture method described earlier (22). Cd content was determined by atomic absorption spectrometry after wet oxidation (22) of individual seedling roots and shoots and was expressed as μg Cd/g or dry weight of tissue. Statistical analysis utilized the F test and significance is expressed as LSD0.05.

RESULTS AND DISCUSSION

We have previously (10) described the introduction of a cauliflower mosaic virus 35 promoter-MT gene into tobacco. This chimeric gene was carried on a Ti plasmid-associated binary vector (pKYX7; 19) and was designated pKYXL M707. Primary transformants obtained with pKYXL M707 were designated Ky 14 M707. Inheritance analysis of R1 progeny of 12 independent Ky 14 M707 lines is presented in Table I.

Selfed seeds from eight primary transformants of Ky 14 M707 segregated with an expected ratio of 3:1 for the kanamycin-resistant versus kanamycin-sensitive phenotype (Table I). Four others produced a higher proportion of resistant seedlings, suggesting insertion of T-DNA at more than one chromosomal locus. The low probabilities of significance in three out of four plants tested for a 15:1 ratio may reflect insertion of more than two copies of the Kan’ gene or that the population was too small to obtain an accurate representation. Similar results have been reported from inheritance analysis of foreign genes in petunia (7), tobacco (2, 16), and tomato (14).

Southern analysis (Fig. 1A) of leaf DNA from transgenic R1 plants indicated the presence of the MT gene in the plant genome. The gene identified as a 0.4 kb band from HindIII-Scal cleaved DNA of transformed plants (lanes 1–3 and 5) was absent from control, untransformed plants (lane 6) or plants transformed with the CAT-gene (IDK-8) (lane 7) lacking the MT sequence. The number of DNA copies was estimated in these plants by densitometry and was shown to be equivalent to two to six copies per tobacco genome (Fig. 1A). Northern analysis of these R1 plants showed the presence of expected transcripts (Fig. 1B). The presence of MT in primary transgenic plants was demonstrated earlier by Western analysis (10).

Leaves of four separate R1 plants were assayed and shown to contain a macromolecule that binds 109Cd and has chromatographic behavior on Sephadex G50 that is characteristic of rodent MT (8). Untransformed tobacco and transformed lines that do not carry the MT gene did not possess this macromolecule (Fig. 1C). We have previously shown that transformed plants synthesize a polypeptide that is immunologically related to MT (10). The results shown in Figure 1C demonstrate that transgenic plants that carry the MT gene of pM707 (10) possess a macromolecule capable of binding 109Cd2+. Thus, the MT-related polypeptide described earlier is probably authentic, functional MT capable of forming complexes with Cd2+. In addition to the MT-like Cd-binding activity, transgenic plants also contain a unique Cd2+-binding component of MW 4300 (Fig. 1C). This component is not seen in untransformed plants. The nature of this component is not clear at this time, but its chromatographic properties are clearly distinct from MT. We are currently characterizing this component in more detail.
When seeds from MT-containing and noncontaining lines were germinated in the presence of different concentrations (0–1000 μM CdCl₂) of Cd²⁺, clear differences in the initial growth of very young seedlings were seen (Table II). At the highest Cd²⁺ level tested (1000 μM), MT-containing transgenic seedlings retained a significant level of Chl and were marginally impaired in overall growth. MT-lacking seedlings were severely retarded in growth and had virtually no Chl at this Cd²⁺ level. Prolonged exposure to Cd²⁺ led to decline of all seedlings, but more tolerant seedlings could be rescued by transfer to Cd-free media (not shown). This behavior was seen with different independent MT transgenic lines (data not shown) and never with untransformed plants (Table II) or transgenic lines that carry the kan' gene but not the chimeric MT gene of pKYLX M707 (data not shown). Thus, the observed Cd²⁺ tolerance is a consequence of the presence of MT in the transgenic seedlings.

To test the possibility that transformed seedlings exclude Cd and thereby avoid toxicity, about 40 seeds each of transformed, untransformed, and a transgenic line which carries the kan' gene but not the chimeric MT gene were germinated and grown in the presence of 500 μM Cd²⁺ for 10 d. The very young seedlings were then pooled, washed thoroughly with 5 mM CaCl₂ to remove adsorbed metal, and their Cd²⁺ content was determined. Values for transformed Ky 14 M707, untransformed Ky 14, and the transgenic line lacking MT were 421, 429, and 406 μg Cd/gm dry weight, respectively. Thus, no evidence was found for exclusion or differential Cd uptake in seedlings of these three lines.

Lefebvre et al. (9) have shown that expression of MT in turnip leaves using a cauliflower mosaic virus (CaMV) vector confers tolerance to 10 mM Cd²⁺ in leaf segments after virus infection of plants. Our approach, unlike that of Lefebvre et al., has resulted in seed-transmissible expression of MT. These authors suggested that chelation of excess free Cd was the mechanism of tolerance in the in vitro system. Our results corroborate these studies, and extend them to intact plants.

Table II. Comparative Cd Tolerance of Transformed and Untransformed Seedlings

<table>
<thead>
<tr>
<th>[CdCl₂]</th>
<th>Ky 14</th>
<th>Ky 14 M707</th>
<th>Homozygote No. 1</th>
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<tbody>
<tr>
<td>μM</td>
<td>untransformed</td>
<td>MT-containing</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>100</td>
<td>100</td>
<td>a</td>
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<tr>
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<td>31</td>
<td>72</td>
</tr>
<tr>
<td>750</td>
<td>54</td>
<td>16</td>
<td>65</td>
</tr>
<tr>
<td>1000</td>
<td>33</td>
<td>2</td>
<td>63</td>
</tr>
</tbody>
</table>

* Fresh weight of seedlings 10 d after planting Ky 14 seedlings (100) had a combined fresh weight of 190 mg and 100 Ky 14 M707 homozygous seedlings had a combined fresh weight of 260 mg. In the absence of Cd, germination rates were 89 and 99%, respectively, for untransformed and transformed seedlings. Corresponding values in the presence of 1000 μM Cd were 70 and 91%, respectively. b Chl content of seedlings 10 d after germination. The Chl level in the Ky 14 seedlings was about 57 mg/100 seedlings and that in MT-containing seedlings was about 67 mg/100 seedlings.

Figure 1. A. Southern blot analysis of transformants containing the MT-DNA sequence. DNA from leaf of Ky 14 M707 plants 1, 2, 3, 4, and 5 (lanes 1–5, respectively). Loss of sample of plant 4 resulted in poor detection in this case. However, the sequence was detectable to the eye on the negative. KY 14 nontransformed leaf sample (lane 6) and KY 14 IDK-8 sample (lane 7) which does not have MT-DNA sequence. A reconstruction representing two copies (lane 9) and four copies (lane 8) per genome equivalent of pKYLX M707. B. Northern blot analysis of total RNA isolated from leaves of Ky 14 M707 plants. RNA from leaves of transformed KY 14 IDK-8 (lane 1), leaves of individual Ky 14 M707 plants 1 to 5 (lanes 2–6), respectively), and liver RNA from Cd-injected mouse (lane 7). Northern blots were hybridized with a nick translated pUCM1901 containing mouse MT cDNA. Markers at left of autoradiogram indicate position and size in kb of HindIII digested λ DNA. C, Sephadex G-50 gel filtration analysis of MT. The profile shows the presence of metallothionein (MT) in an extract from Ky 14 M707 plant 3, but not in a control Ky 14 leaf extract or a leaf extract from Ky 14 IDK-8 (10) transformed with CAT gene.
Table III. Cadmium Accumulation in Seedlings of N. tabacum, Ky 14 M707 versus Control

<table>
<thead>
<tr>
<th>Experiment</th>
<th>No. of Plants</th>
<th>X Leaf 707</th>
<th>X Root 707</th>
<th>707 LSD from</th>
<th>707 Root so from 14 Leafa</th>
<th>707 Root so from 14 Leafb</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8 25</td>
<td>0.68</td>
<td>0.79</td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>2</td>
<td>23 23</td>
<td>0.72</td>
<td>1.18</td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>3</td>
<td>16 22</td>
<td>0.84</td>
<td>1.24</td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>4</td>
<td>14 15</td>
<td>0.64</td>
<td>1.01</td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>5</td>
<td>20 20</td>
<td>0.97</td>
<td>0.92</td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>6</td>
<td>10 7</td>
<td>0.73</td>
<td>1.18</td>
<td>*</td>
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</tr>
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</table>

\[\bar{x} = 0.76 \pm 0.12 \quad \bar{x} = 1.05 \pm 0.17\]

* Supplied 0.02 μM Cd as 106Cd for 15 d.  
* Mean top/root ratios were 1.81 and 2.32 for 707 and control, respectively.  
* LSD = 0.05.

In addition, we find that Cd tolerance in very young seedlings can be affected by levels of MT 5- to 10-fold lower than that attained by Lefebvre et al. using the CaMV vector (we have previously reported a range of MT levels of 0.06-0.1% of soluble leaf protein [10], compared with levels of 0.5% reported by Lefebvre et al. [9]).

We have examined the potential of MT to alter accumulation of Cd in transgenic plants exposed to 0.02 μM Cd, a level predicted to occur in a mildly polluted soil solution (1). Six-week-old Ky 14 M707 and Ky 14 plants were grown for 15 d in nutrient solution containing 0.02 μM Cd (as 106CdCl2, 2.5 Ci/g, RPI) employing a bare-root culture method (22). Results of six independent experiments (about 18 seedlings each of transformed and untransformed per experiment, 0.5 and 0.15 g fresh weight top and root, respectively) indicated that mean leaf Cd (dpm/g dry weight) of transformed plants was 76% that of untransformed plants and mean root Cd was 105% that of untransformed plants. In five of the six experiments (Table III), transformed plant leaf Cd was significantly lower (P = 0.05) than that in untransformed controls. These results suggest that MT affects the potential of tissues to accumulate Cd. We are currently studying Cd accumulation and tissue (root versus top) Cd partitioning in other transformed tobacco species and are determining the extent of MT expression in various tissues of transformed plants. Efforts are underway to increase expression of MT, and to test the root-specific expression case.

The finding that MT reduces Cd accumulation even in tissues that contain MT is unexpected and presently not understood. MT may affect the steady state levels of Cd in leaves of transgenic plants by altering the rate of uptake or discharge of Cd (or Cd-macromolecule complexes) in leaves; reduced uptake (perhaps due to an altered intracellular partitioning of Cd) or increased 'excretion' (perhaps of foreign MT-Cd complexes) would alter Cd levels and lead to a modestly lowered Cd accumulation in leaves. Alternatively, MT may reduce the translocation of Cd from roots to upper parts of the plants, once again leading to an overall decrease in Cd in leaves. A greater understanding of the processes that underlie metal ion homeostasis in plants is needed to distinguish between these possibilities.

In summary, we have found that seed-transmissible expression of a mouse metallothionein gene in transgenic tobacco can confer increased Cd tolerance to very young tobacco seedlings and results in reduced leaf Cd after low-level Cd exposure of older seedlings. The ability of this foreign gene product to alter the response of plants to Cd provides a tool with which to study various aspects of the interaction of toxic heavy metals with the metabolic apparatus of plants. Since MT also binds the nutrient ions Zn2+ and Cu2+ and has been shown to donate metals to Zn2+ and Cu2+—requiring enzymes in vitro, tobacco expressing MT may also be useful for studying nutrient metal homeostasis in plants. Finally, since the bulk of Cd intake in man is derived from agricultural crops, MT may be generally useful in decreasing chronic, low-level Cd exposure in humans.

LITERATURE CITED