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Expression of Mouse Dihydrofolate Reductase Gene Confers Methotrexate Resistance in Transgenic Petunia Plants

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Abstract—*Transgenic petunia plants containing an altered ($Leu_{22} \rightarrow Arg_{22}$) mouse dihydrofolate reductase gene fused to the cauliflower mosiac virus 35S (CaMV 35S) promoter and nopaline synthase (nos) polyadenylation site were obtained by transforming petunia leaf disks with an* Agrobacterium tumefaciens *strain carrying the chimeric gene. Transformants were directly selected for and rooted on medium containing 1 μM methotrexate (MTX). The chimeric gene was present in the regenerated plants at one to three copies and produced the expected 950-nucleotide-long transcript based on Southern and Northern hybridization analyses, respectively. Leaf pieces from the regenerated transgenic plants were able to form callus when cultured on medium containing 1 μM MTX and were able to incorporate ^{32}P into high-molecular-weight DNA in the presence of >100 μM MTX, thus demonstrating that the chimeric mouse* dhfr *gene was fully functional and useful as a selectable marker in plant transformation experiments. To date, this is the first report of successful expression of a vertebrate gene in transformed plant cells.*

INTRODUCTION

Dominant selectable markers are an essential and often the most critical feature of a transformation system. Numerous bacterial genes, primarily drug-resistance markers, have been used successfully as selectable markers in both plant and animal transformation systems (1–6). However, since all plant species are not equally sensitive to various drugs, there is no single drug-resistance gene that will function uniformly in all plants as a dominant selectable marker. Also, additional markers are needed for multiple transformations of individual plants. Thus, there is still a need for new selectable markers for plant transformation systems.

Many mammalian genes have been extensively characterized and several used successfully in animal cell transformation/selection systems (7–9). They make an excellent and available source of selectable markers for plant transformation experiments. We have focused our efforts on dihydrofolate reductase (*dhfr*) genes as selectable markers (10), since most plant species are sensitive to methotrexate (MTX) and it has already been demonstrated that chimeric constructs containing a bacterial *dhfr* gene can confer resistance to low levels of MTX in transformed tobacco and turnip cells (11, 12).

Simonsen and Levinson (10) isolated an altered mouse *dhfr* cDNA clone which encoded an enzyme that had a 270-fold lower

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affinity for MTX than the wild-type enzyme (13). They found that there was a single nucleotide change at position +68 in which a T to a G substitution produced a Leu to Arg amino acid change in the enzyme at position 22 (10). Because of the superior enzymatic properties associated with the mutant mouse *dhfr* gene (13), we had decided to focus our efforts on the mammalian rather than the bacterial *dhfr* gene used by others.

MATERIALS AND METHODS

Construction of CaMV 35S/dhfr/NOS Plant Transformation Vectors. The methotrexate-resistant mouse dihydrofolate reductase coding sequence was constructed as follows. A 1-kb Fnu4HI fragment was obtained by cleavage of the pDHFR-11 cDNA clone (14). Following treatment with Klenow polymerase, the fragment was inserted into the SmaI site of M13mp8 (15) so that the BamHI site of the multilinker was adjacent to the 5′ end of the coding sequence. The M13mp8-DHFR (M-23) clone was subjected to site-directed mutagenesis by the method of Zoller and Smith (16) using a synthetic primer containing the sequence 5′-AGAACGGAGACAGACCCTGGTCTC, where the underlined AG represents the two bases altered from the wild-type sequence CT to change the leucine codon to an arginine codon at position 22 (Fig. 1). The altered coding sequence was excised from the replicative form of the mutated mp8 DNA on a 660-bp BamHI-BglII fragment and inserted between the cauliflower mosaic virus major transcript promoter, CaMV 35S (17), and a 260-bp segment containing the 3′ untranslated sequence and polyadenylation site of the nopaline synthase (*NOS*) gene from the *Agrobacterium tumefaciens* tumor-inducing (Ti) plasmid pTiT37 (1). The segment of DNA carrying the CaMV 35S promoter extends from base 7146 to base 7464 of the CM4-184 sequence (18). The sequence at base 7464 had been

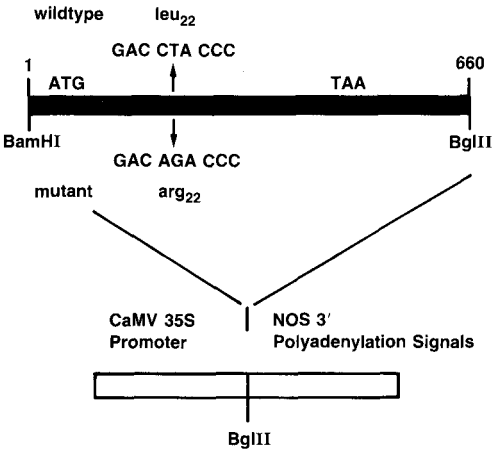

Fig. 1. Site-directed mutagenesis of mouse dihydrofolate reductase cDNA clone. The nucleotides CT at positions +67 to +68 were changed to AG in the mouse dhfr cDNA to produce a Leu-to-Arg amino acid change in the DHFR enzyme. The altered mouse dhfr sequence was then subcloned as a 660-bp BamHI-BglII fragment between the CaMV 35S promoter and NOS 3′ polyadenylation signals for expression in plant cells.

altered by insertion of a G to provide a BglII site (19).

Various components were assembled into an 11.4-kb binary plant transformation vector, pMON809 (Fig. 2), as follows. The RK2

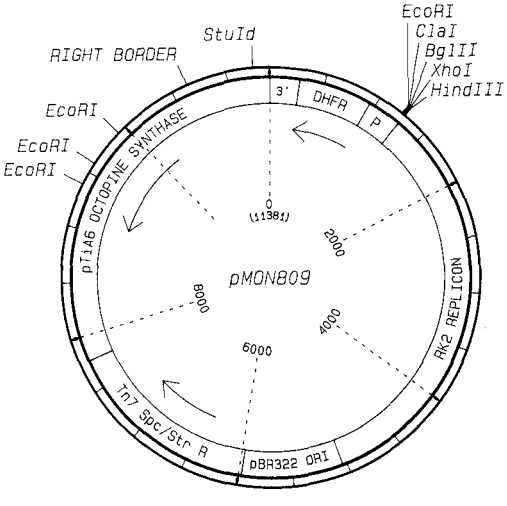

Fig. 2. Physical and genetic map of pMON809. P = CaMV 35S promoter and 3′ = nopaline synthase 3′ untranslated sequence and polyadenylation site.

broad host range plasmid replicon, carried on a 3.8-kb HindIII to SmaI fragment from pTJS75 (20), was fused at the HindIII site to the pMON200 synthetic multilinker (21). A 0.9-kb pBR322 DraI (n. 3232) to NdeI (n. 2297) fragment (22), which contained the ColE1 origin of replication, was fused to the RK2 fragment at the SmaI site. The spectinomycin/streptomycin gene from Tn7 (23) was carried on a 1.8-kb NdeI to StuI fragment and fused to the NdeI site of the pBR322 fragment. A 3.6-kb SmaI (n. 11,207) to StuI (n. 14,675) fragment, carrying the octopine synthase (*OCS*) gene and the adjacent right border sequence which delimits the left portion of the two-component octopine-type Ti plasmid transferred DNA (T_{left}-DNA) from the *Agrobacterium tumefaciens* pTiA6 plasmid (24), was fused to the Tn7 spectinomycin/streptomycin resistance gene at the StuI site. The nopaline synthase 3′ untranslated sequence was carried on a 0.4-kb StuI to BamHI fragment from pMON200 and was fused to the StuI site of the octopine synthase gene and right border fragment. The resulting StuI site was protected from cleavage due to *dcm* methylation in *dcm*$^+$ *E. coli*. A 0.9-kb BglII to EcoRI fragment carrying the chimeric CaMV 35S/mouse DHFR gene fusion was ligated to the BamHI site of the nopaline synthase 3′ untranslated sequence and to the EcoRI site of the multilinker.

Plant Transformation and MTX Selections. Petunia leaf disks were transformed as previously described (25) with *Agrobacterium tumefaciens* strain GV3111-SE containing the disarmed Ti plasmid pTiB6S3-SE and pMON505 or pMON809 which had been introduced by triparental matings (1). The binary plant transformation vector pMON505 (26) served as a negative control in transformation experiments. Plasmid pMON505 contained the CaMV 35S/NPTII/NOS Kan^R marker and nopaline synthase (*NOS*) gene in place of the pMON809 CaMV 35S/*dhfr*/NOS MTX^R marker and octopine synthase (*OCS*) gene. MTX^R calli were selected on MS104 medium, pH 5.7, containing MS salts (Gibco), B_5 vitamins, 3% sucrose, 0.1 mg/liter naphthalene acetic acid, 1.0 mg/liter benzyladenine, and 1 μM MTX. Shoots developed within three weeks following transformation and were excised and cultured on hormone-free medium containing 1 μM MTX for root regeneration. After four weeks, rooted shoots were potted in soil and grown in growth chambers. Leaf pieces from the mature transgenic plants were cultured on the above medium with 1 μM MTX for leaf callus assays to test for continued expression of the chimeric gene.

Southern and Northern Hybridization Analyses. For Southern hybridization analysis, DNA was extracted from each plant as follows: 4 g of mature leaves were frozen in liquid nitrogen, powdered in a mortar and pestle, then homogenized in 10 ml of 50 mM each Tris HCl, pH 8, EDTA, NaCl, 400 μg/ml ethidium bromide, and 2% Sarkosyl. Leaf debris was cleared by centrifugation at 10 K and CsCl was then added at 0.95 g/ml of supernatant. Following ultracentrifugation in a Beckman Ti 70.1 rotor at 45K and 20°C for 22 h, DNA bands were pulled from the CsCl gradients, ethidium bromide extracted with 20× SSC saturated isopropanol, and dialyzed against TE at 5°C. After ethanol precipitation, DNAs were digested with EcoRI, following the manufacturer's suggested conditions, electrophoresed on 0.7% agarose gels in 1× TBE, transferred to nitrocellulose as previously described (27), and hybridized with a nick-translated pMON809 probe for 40 h at 42°C in 50% formamide, 5× SSC, 5× Denhardt's, 0.2% SDS and 0.1 mg/ml salmon sperm DNA. Filters were washed 2× 30 min in 1× SSC and 0.5% SDS at 65°C and exposed to Kodak XAR-5 film at −80°C for 14 h with two screens.

For Northern hybridization analysis, RNA was extracted from each plant as follows: 8 g of mature leaves were frozen in

liquid nitrogen, powdered in a mortar and pestle and, using a polytron, extracted with 1% triisopropylnaphthalene sulfonic acid (Kodak), 6% *p*-amino salicylic acid, 100 mM Tris HCl, pH 7.6, 50 mM EGTA, 100 mM NaCl, 1% SDS, 50 mM β-mercaptoethanol, and an equal volume of a 1:1 TE saturated phenol–chloroform mixture. Organic and aqueous phases were separated by centrifugation and the aqueous phases were extracted a second time with equal volumes of the phenol–chloroform mixture, phases separated, and nucleic acids precipitated with 0.1 volumes of 3 M NaOAc, pH 6, and 2 vol of ethanol at −20°C. Nucleic acid pellets were resuspended in H_2O and total RNA was purified from the mixture by the addition of equal volumes of 4 M LiCl and precipitation on ice. After centrifugation, the pellets were resuspended in water and the RNA was reprecipitated with LiCl as above for a total of three precipitations followed by an ethanol precipitation. Forty-microgram aliquots of total RNA from each plant were denatured with glyoxal and electrophoresed on 1.5% agarose gels in 10 mM Na_2HPO_4 (28), transferred directly to nitrocellulose with 20× SCC, and hybridized exactly the same as above using a nick-translated probe containing the CaMV 35S/*dhfr*/NOS construct in pUC18. Filters were washed as described above and exposed for 27 h with two screens.

Methotrexate-Resistance Assay. Petunia plants transformed with pMON505 or pMON809 and a nontransformed control were assayed for MTX-resistant DHFR activity as previously described (12) with a few minor modifications. Approximately 0.2 g of the uppermost newly expanded leaves were collected from each plant, surface-sterilized for 15 min in a 10% Clorox® solution, and then rinsed three times in sterile water. Leaves were cut into 5-mm^2 segments and cultured in 5 ml of liquid medium, pH adjusted to 5.7, containing a Murashige and Skoog salt mixture without KPO_4 salts, B_5 vitamins, sucrose (30 g/liter), benzyladenine (1 mg/liter), naphthalene acetic acid (0.1 mg/liter), and MTX (Sigma) at 0 or 50 μg/ml. Cultures were incubated in the dark at 28°C for 24 h on a gyratory shaker. The medium was exchanged with 5 ml of fresh medium, and 35 μCi of ^{32}P as orthophosphoric acid in H_2O (New England Nuclear) was added to each culture. Following an additional 24-h incubation at 28°C in the dark on a gyratory shaker, the medium was removed and each culture washed three times in TE buffer. The cultures were then frozen in liquid nitrogen and total DNA was extracted from each in 0.5 ml of 50 mM NaCl, 10 mM EDTA, 1% Sarkosyl, and 10 mM Tris HCl, pH 7.5, followed by two phenol extractions, 1 vol each, and an isopropanol precipitation.

The pellets were resuspended in 50 μl of TE containing 20 μg/ml RNase A and incubated 1 h at 37°C. Then, 450 μl of a 200 μg/ml proteinase K–0.1% SDS solution was added followed by an additional 1-h incubation at 37°C. Each sample was then extracted with 1 vol of phenol, precipitated with isopropanol, and resuspended in 50 μl TE. Each sample was aliquoted such that all of the 0 μg/ml MTX treatments had equivalent amounts of radioactivity for equivalent exposures on autoradiographs. The 50 μg/ml MTX treatments were aliquoted such that the 0 and 50 μg/ml MTX treatments from each plant had equivalent amounts of DNA. Samples were electrophoresed on 1.2% agarose gels for 1 h at 100 V (~50 mA) in 1× TBE buffer containing 0.1 μg/ml ethidium bromide. The wet gels were then exposed to Kodak XAR-5 X-ray film at −80°C after excess moisture was removed by blotting gels overnight on Whatman 3 MM paper and paper towels.

RESULTS

Petunia Leaf Disk Transformation. The altered mouse *dhfr* coding sequence and nopaline synthase (NOS) polyadenylation site were placed downstream from the CaMV 35S

promoter to create pMON809 (Fig. 2), a binary plant transformation vector which also contained the octopine synthase (*OCS*) gene, the octopine T_{left}-DNA right border, Tn7 spectinomycin/streptomycin drug resistance marker, broad host range origins of replication and transfer, and the ColEl origin of replication. After triparental matings to introduce pMON809 into *Agrobacterium tumefaciens* strain GV3111-SE, the resulting strain was used to transform petunia leaf disks. Methotrexate-resistant calli were selected, shoots regenerated, and roots developed on 1 μM MTX (Fig. 3). When leaf pieces from mature transgenic plants were put back into culture on medium containing 1 μM MTX, 22 of 35 independent plants tested formed callus. This indicated that a majority of the transformed plants continued to express the chimeric gene. Petunia leaf disks transformed with the pMON505 control construct containing no *dhfr* gene did not produce callus on 1 μM MTX.

Southern and Northern Hybridization Analyses of Transgenic Petunias. DNA and RNA was extracted from the mature leaves of four MTX® transgenic plants (4041, 4113, 4033, 4067) and two MTS^s transformed (3615) and nontransformed (VR) control plants. Southern hybridization analysis was used to demonstrate the presence and determine the number of copies of the CaMV 35S/*dhfr*/NOS chimeric gene in the transgenic plants. DNAs were purified on CsCl gradients, digested with EcoRI, transferred to nitrocellulose filters, and hybridized with the nick-translated pMON809 plasmid (Fig. 4A). The EcoRI digests released an 8.45-kb internal fragment and two or more junction fragments per each plant. The internal EcoRI fragment contained the origins of replication and transfer functions. The junction fragments contained either the 3′ end of the *OCS* gene and the entire Tn7 *spc/str* resistance gene or the CaMV 35S/*dhfr*/NOS chimeric gene. Based on the copy number reconstructions (not shown) and the number of junction fragments, the Southern hybridization data show that the *dhfr* gene was present in the pMON809-transformed petunia plants at only one copy per haploid genome in plant 4067, two copies each in plants 4041 and 4113, and three copies in plant 4033. The pMON809 vector must have rearranged or undergone a deletion in plant 4067 because the 8.45-kb EcoRI internal fragment is not present, only two junction fragments. The pMON505-transformed plant, 3615, should have only had two bands hybridizing to the pMON809 probe, both junction fragments, since the pMON505 vector had a single EcoRI site. Since three bands

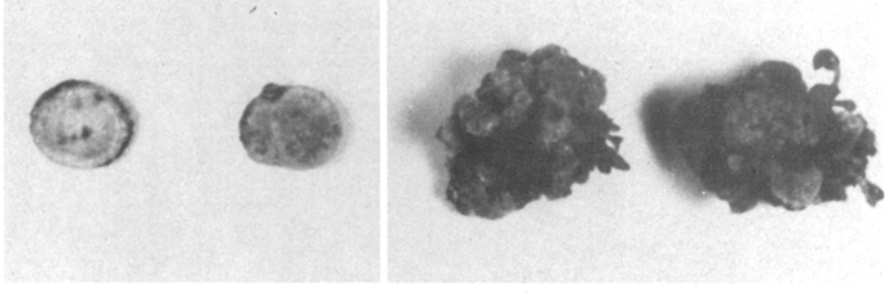

Fig. 3. Transformed petunia leaf disks on MTX selection medium. Petunia leaf disks had been transformed with *A. tumefaciens* containing pMON505 or pMON809 binary plant transformation vectors. They were then cultured on MS104 selection medium containing 1 μM MTX and photographed four weeks after culture.

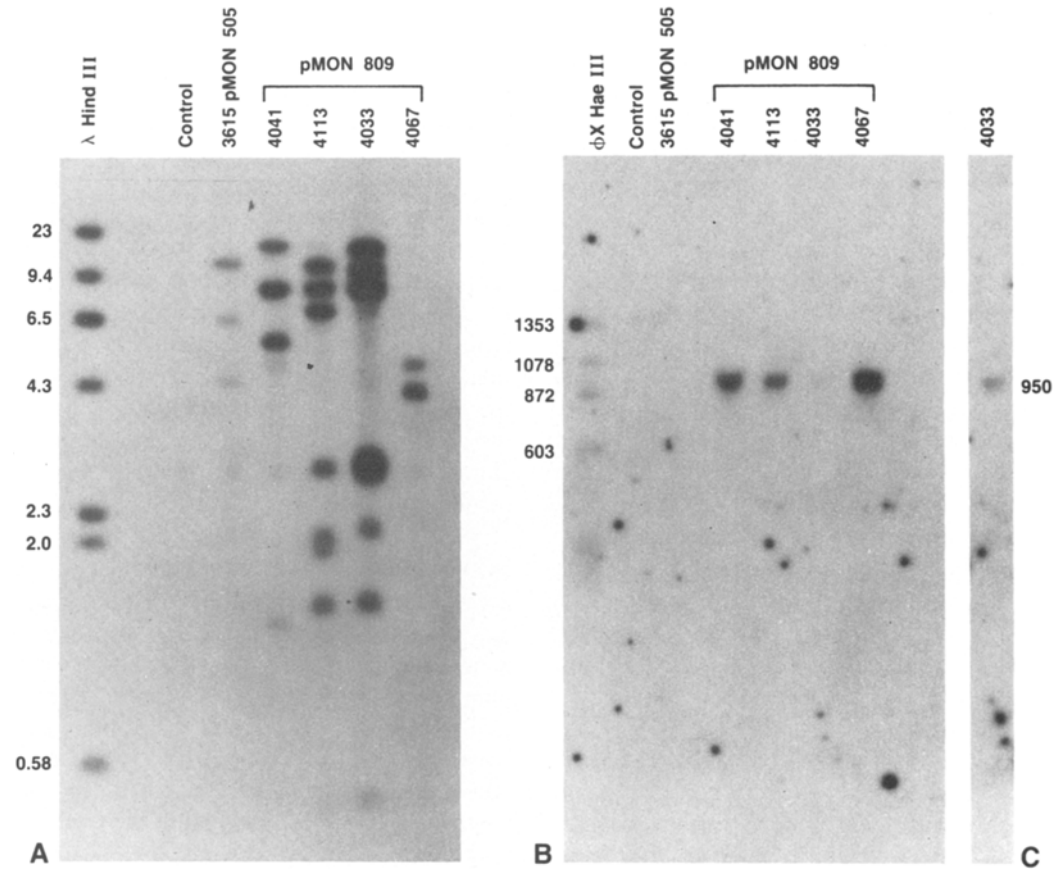

Fig. 4. Southern and Northern hybridization analyses of MTX^R transgenic and MTX^S transformed and nontransformed control petunia plants. (A) Southern hybridization analysis of leaf genomic DNAs using a nick-translated pMON809 probe. (B) Northern hybridization analysis of total leaf RNAs using a nick-translated probe containing the CaMV 35S/dhfr/NOS construct in pUC18. Filter exposed 27 h at −80°C. (C) Northern hybridization analysis of plant 4033 using the same nitrocellulose filter as in B, but with the plant 4033 lane exposed for 96 h at −80°C.

hybridized to the probe, it showed a hybridization pattern that would be indicative of either a rolling circle insertion mechanism, two independent insertion events, or tandem insertions. The nontransformed control VR showed no hybridization at all to pMON809, thus, the endogenous petunia *dhfr* gene did not cross-hybridize with the mouse *dhfr* gene under these conditions.

Northern hybridization analysis was used to demonstrate the presence of the altered mouse *dhfr* transcript in the transgenic petunia plants. Total and poly A+ RNA fractions from each plant were denatured with glyoxal and electrophoresed on 1.5% agarose gels in 10 mM Na_2HPO_4, transferred directly to nitrocellulose and hybridized with a nick-translated probe containing the CaMV 35S/*dhfr*/NOS construct in pUC18 (Fig. 4B, C). There was no hybridization to either of the methotrexate-sensitive control petunia plants, 3615 or the nontransformed wild-type VR. The nick-translated probe did hybridize to a single, abundant transcript 950 nucleotides in length in both the total and poly A+ RNA fractions from all of the pMON809-transformed MTX^R plants analyzed. Since the CaMV 35S promoter is a strong plant promoter (19, 29, P. Sanders, et al., in preparation) and produces an abundant

transcript, we were able to see the *dhfr* transcript in the total RNA fraction and show only that data. The Northern hybridization analysis of poly A+ RNA was identical. The 950-nucleotide-long transcript was the expected length and included 40 nucleotides of 5′ nontranslated sequence from the CaMV 35S CAP site to the *dhfr* ATG, 660 nucleotides of *dhfr* coding sequence, 180 nucleotides of NOS 3′ untranslated sequence and polyadenylation site, and roughly a 70-nucleotide-long poly A tail.

The level of expression of the chimeric gene in each of the transgenic plants was nearly identical, except in plant 4033 where the expression seemed to be reduced roughly 100-fold. That particular plant contained three copies of the chimeric *dhfr* gene based on Southern hybridization analysis. The reduced expression of the gene based on the lower transcript level had no effect on the level of MTX resistance observed at the concentration used in this study. The 4033 plant was as resistant as the other transgenic plants to MTX in direct selection, MTX leaf callus and [^{32}P]phosphorus incorporation assays.

Methotrexate Resistance Assay. Methotrexate, a folate antagonist, inhibits DHFR which catalyzes the reduction of dihydrofolate to tetrahydrofolate. That, in turn, blocks the synthesis of deoxythymidylate, resulting in a defect in DNA synthesis (30). Therefore, it was possible to assay MTX^{R} transgenic plants for DHFR activity by testing their ability to incorporate [^{32}P]phosphorus into high-molecular-weight DNA in the presence of methotrexate (12). Leaves from six MTX^{R} plants, a nontransformed MTX^{S} control plant (VR), and a pMON505-transformed MTX^{S} plant (3615) were cultured in the presence of 0 and 50 μg/ml (110 μM) MTX and [^{32}P]phosphorus. Total DNA was extracted from each

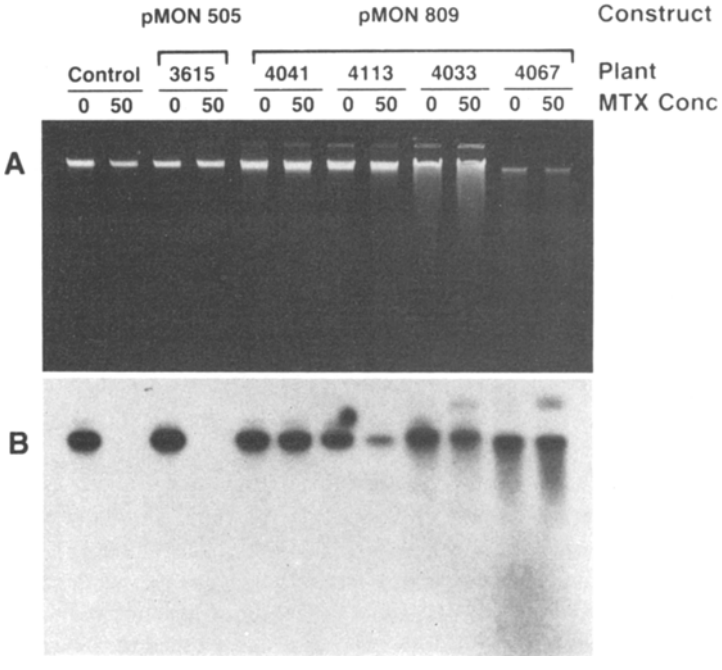

Fig. 5. Methotrexate-resistance assay. (A) Ethidium bromide-stained agarose gel containing total DNA extracted from pMON809 MTX^{R} and pMON505 and nontransformed VR control MTX^{S} petunia leaves. The lanes were loaded such that all of the 0 μg/ml MTX treatments had equivalent amounts of radioactivity. The 50 μg/ml MTX treatments were loaded such that 0 and 50 μg/ml MTX treatments from each plant had equivalent amounts of DNA. (B) Autoradiograph of gel in (A).

treatment, electrophoresed on agarose gels, and autoradiographed (Fig. 5).

All of the pMON809-transformed MTX^R petunia plants were able to incorporate [^{32}P]phosphorus into high-molecular-weight DNA in the presence of 50 μg/ml MTX, as shown for four of the six plants analyzed. Neither of the control plants incorporated [^{32}P]phosphorus into high-molecular-weight DNA at 50 μg/ml MTX concentrations. In fact, DNA synthesis was nearly completely inhibited in the control plants at 25 μg/ml MTX concentrations (data not shown).

DISCUSSION

Selectable markers are a key part of any successful transformation system. Our group and several others have previously reported success using the bacterial kanamycin-resistance gene, neomycin phosphotransferase II (NPTII) as a selectable marker in many plant transformation experiments (1–3). However, it does not function as a good selectable marker for many plant species. For that reason, more selectable markers are needed to aid in the development of transformation systems for many important agronomic crop species. Recently, resistance to hygromycin has been successfully engineered into plant cells (31, 32, Rogers et al., submitted).

The altered mouse *dhfr* gene has been an excellent selectable marker in mammalian cell transformations and now in plant cell transformations. There are several advantages to using it as a selectable marker. Plant cells are very sensitive to MTX at low concentrations (1 μM in petunia) and transformed cells can be directly selected for in its presence. Additionally, the selection can be maintained during shoot regeneration and root development. DeBlock et al. (11) had previously reported that they were unable to get MTX^R *Nicotiana* shoots, transformed with a chimeric bacterial *dhfr* gene fused to the nopaline synthase promoter and polyadenylation site (NOS/*dhfr*/NOS), to regenerate roots in the presence of MTX. The fact that we were able to get root regeneration from MTX^R shoots transformed with the chimeric altered mouse *dhfr* gene suggests that the CaMV 35S promoter is stronger than the NOS promoter and/or the altered mouse DHFR enzyme is more stable than the bacterial DHFR enzyme. Data from our laboratory show that the CaMV 35S promoter is significantly stronger than the NOS promoter. Based on enzymological data (13, 33, Kathryn H. Pattishall, personal communication), in the absence of MTX, the altered mouse DHFR enzyme is at least twofold more catalytically efficient than the bacterial R67 plasmid encoded DHFR enzyme. The R67 enzyme, with an ID_{50} of 1.1×10^{-3} M, is more resistant to MTX than the altered mouse enzyme which has an ID_{50} of only 6×10^{-7} M. However, at the cellular level, the altered mouse enzyme is very effective and has already been shown to be 270-fold less sensitive to MTX than the wild-type enzyme. The observed differences in resistance to MTX in the heterologous systems, i.e., the plant cell environment, could be due to enzyme stabilities. Thus, it seems that using either the CaMV 35S promoter and/or the altered mouse *dhfr* gene in chimeric constructs contributes to the greater resistance to MTX.

Another reason for having a number of selectable markers to choose from is for performing multiple transformation experiments. It may be necessary to introduce foreign genes sequentially for genetic mapping studies, pathogen infectivity studies, marker exchange and rescue experiments, or for obtaining the desired level of expression of a particular gene. We have recently retransformed petunia leaf disks from transgenic plants containing the Kan^R gene with our CaMV 35S/mouse *dhfr*/NOS construct and have obtained transgenic plants that are both KAN^R and MTX^R. This result shows that petunia cells can be doubly transformed, with each marker being stably maintained and selectable.

The results in this manuscript are the

first report of a vertebrate gene being accurately expressed and fully functional in a transformed plant cell. Previous work by other groups has shown that vertebrate regulatory DNA sequences examined to date do not function in plants (34–36). Several mammalian genes, including actin, β-globin, interferon, and chimeric genes containing the SV40 early promoter had been introduced into plant cells, but none of them were transcribed (34, 35). Koncz et al. (35) had reported that the chicken ovalbumin gene was transcribed in transformed tobacco tumor lines, producing transcripts of variable length which had either initiated or terminated prematurely and were not accurately processed. A similar result was observed with a mini human growth hormone (*hgh*) gene containing a single intron fused to the CaMV 35S promoter and transformed into tobacco cells. The *hgh* gene was transcribed but was not properly spliced or expressed (36). Recently, however, there has been success with plant DNA regulatory sequences functioning in animal cells. The maize AdhI plant promoter accurately expressed the *Ecogpt* and *neo* bacterial genes in monkey cells transfected with pSV2-derived vectors containing the chimeric constructs (37).

Our successful expression of the altered mouse *dhfr* chimeric gene in transgenic petunia plants was undoubtedly due to controlling expression with the CaMV 35S and NOS plant DNA regulatory sequences. We have also recently obtained similar success with fusing the CaMV 35S promoter and NOS polyadenylation site to the α-human chorionic gonadotropin gene (*αhcg*). Preliminary radioimmune assay and western hybridization data indicated that the chimeric gene was transcribed and translated rather abundantly, producing a protein that cross-reacted with the *α-hcg* antibodies.

Our data clearly show that the CaMV 35S/mouse dhfr/NOS chimeric gene was accurately expressed and produced a fully functional enzyme that was able to confer MTX resistance in transformed plant cells. The altered mouse DHFR enzyme is extremely resistant to MTX, even in the plant cell environment where little of the enzyme seemed to be required to confer high levels of MTX resistance. This observation was based on the results with plant 4033, where there was a significant reduction in the expression of the chimeric mouse *dhfr* gene.

Plant 4033 was the only transgenic plant, of those analyzed, which displayed any peculiarities in expression. There was roughly a 100-fold reduction in the expression of the chimeric gene when compared to the other transgenic plants, even though this plant contained three genomic copies of the chimeric gene. The transcripts were identical in size in all of the transgenic plants analyzed. Presumably, there were no qualitative differences in the transcripts since all of the transgenic plants were equally resistant to MTX. The only explanation we can give for the reduced transcription of the chimeric *dhfr* gene in plant 4033 is due to position effects: the differential expression of a foreign gene due to its chromosomal site of insertion. Since the genetic analysis of this plant has not been performed, we do not know if the sites of insertion are linked or are distributed throughout the genome. This same quantitative but not qualitative difference in expression of chimeric genes in transgenic plants has also been observed with the pea *rbcS-E9* gene in transgenic petunia and tobacco plants (38). Variable expression of the chimeric petunia chlorophyll a/b binding protein promoter/octopine synthase coding sequence fusion (Cab/ocs) in transgenic petunia and tobacco plants has also been attributed to position effects (39).

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