Comparison of cauliflower mosaic virus 35S and nopaline synthase promoters in transgenic plants

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ABSTRACT

We have compared the level of expression of the Cauliflower Mosaic Virus 35S promoter and the nopaline synthase promoter when fused to a common reporter gene. A cassette containing the neomycin phosphotransferase (type II) coding sequence followed by the nopaline synthase 3' nontranslated region was used for transcriptional and translational evaluation of the two different promoters. These chimeric genes were introduced into petunia plants and the copy number of the gene, the steady state level of NPTII transcript and the levels of NPTII enzyme activity were determined. In this paper, we report that the NPT II transcript levels are on the average 30 fold higher in plants containing CaMV 35S promoter and leader sequences than in plants containing the same reporter gene but nopaline synthase promoter and leader sequences. Similarly, plants containing the CaMV 35S promoter had an average of 110 fold higher levels of NPTII enzyme activity than those containing the nopaline synthase promoter. The significance of these results for expression of foreign genes in plants is discussed. In addition, we describe the construction of a convenient plant expression cassette vector (pMON316) which utilizes the CaMV 35S promoter.

INTRODUCTION

One of the most significant recent advances in the area of plant molecular biology has been the development of the *Agrobacterium tumefaciens* Ti plasmid as a vector system for the transformation of plants (1). The Ti plasmid transformation vectors provide a facile means to introduce homologous and heterologous genes into plants to study the stability of the gene product, the timing and quantity of gene product expression as well as the tissue specificity of that expression. With this tool, the promoter regions responsible for light regulated expression of certain genes (2, 3, 4, 5) and the portions of transit peptides responsible for proper compartmentalization of proteins within the plant cell are being elucidated (6, 7). The tissue specific and developmentally regulated expression of several reintroduced genes have also been demonstrated (8, 9, 10). On the applied end of plant science, the Ti transformation system has been adapted for the study of virus host range, symptom production and replication (11, 12) as well as host cell

recombination processes. Applications of the Ti transformation system aimed at crop improvement include: the introduction of modified 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase genes into plants, rendering these plants resistant to the nonselective herbicide Roundup® (13, 14); the introduction of the *Bacillus thuringiensis* crystal toxin gene into plants to obtain tobacco hornworm resistance (15) and the introduction of viral coat protein genes to obtain virus resistant transgenic plants (16).

The above examples have utilized a variety of Ti plasmid based expression vector systems to facilitate transcription of desired coding sequences. Many early chimeric gene constructions utilized 5' regulatory regions obtained from T-DNA opine synthase genes (17, 18, 19, 20, 21). More recently, promoter fragments derived from cauliflower mosaic virus (14, 16, 22, 23) and from a variety of plant genes such as the small subunit of ribulose 1,5 biphosphate carboxylase (RuBP) (3, 24, 25) have been utilized in expression cassettes. While these various promoters have been used successfully to obtain foreign gene expression, very little is known about the relative efficiencies of the various promoters. Since promoter strength is a critical factor in allowing convenient, high level transcription of selected coding sequences, we felt it important to evaluate relative strengths of available plant promoters. In this paper we compare two widely used promoters, the cauliflower mosaic virus (CaMV 35S) transcript and nopaline synthase (NOS) promoters, in transgenic plants. In addition, we describe the construction of a convenient plant expression cassette vector which utilizes the highly expressed, constitutive CaMV 35S promoter.

MATERIALS AND METHODS

Plasmids

Plasmid pMON273 is a derivative of pMON200 (26) in which the nopaline synthase (NOS) promoter of the chimeric NOS-NPTII-NOS gene has been replaced with the CaMV full length (35S) transcript promoter.

The CaMV 35S promoter fragment was isolated from plasmid pOS-1, a derivative of pBR322 carrying the entire genome of CM4-184 as a *Sa*I insert (27). This plasmid was kindly provided by R. J. Shepherd. The CM4-184 strain is a naturally occurring deletion mutant of strain CM1841. The nucleotide sequences of the CM1841 (28) and Cabb-S (29) strains of CaMV have been published as well as some partial sequence for a different CM4-184 clone (30). The nucleotide sequences of the 35S promoter regions of these three isolates are essentially identical. In Fig. 1 and in the following the description, nucleotide numbers reflect the sequence of Gardner et al. (28). The 35S

Figure 1. CONSTRUCTION OF pMON200 AND pMON273

- 1a. The structure of the pMON273 vector, containing the CaMV promoter, the NPTII coding region and the NOS 3' polyadenylation signal (NPT'), cloned into a pMON120 intermediate vector (17). Plasmid pMON273 contains the following segments of DNA: 1. a segment of pBR322 DNA for replication in *E. coli*; 2. a segment from pTiT37 that contains a functional nopaline synthase gene (NOS) for identification of transformants; 3. a segment of Tn7 carrying the spectinomycin/streptomycin resistance determinant for selection in *A. tumefaciens*; 4. the LIH fragment of the pTiA6 plasmid which is homologous with a resident octopine-type Ti plasmid in *A. tumefaciens* for recombination to occur. Nucleotide sequence of the CaMV35S promoter: the overlined sequences are the nucleotide at the 5' end of the 35s promoter and engineered BglIII site. The overlined extreme 5' sequences including an EcoRI site are from linkers added to facilitate promoter manipulations. The arrow indicates the CAP site (33). The underlined ATG is the start of NPTII translation.
- 1b. Map of pMON200 which is identical to pMON273 except that the CaMV35S promoter has been replaced with the NOS promoter fragment. Nucleotide sequence of the NOS promoter (18). The overlined sequences are the 5' EcoRI site and 5' nucleotide of the NOS promoter sequence. The intervening nucleotides are from synthetic linkers added during promoter manipulations. The arrow indicates the CAP site.

promoter was isolated as an *AluI* (n 7143)-*EcoRI** (n 7517) fragment which was inserted first into pBR322 cleaved with *Bam*HI, treated with the Klenow fragment of DNA polymerase I and then cleaved with *EcoRI*. The promoter fragment was then excised from pBR322 with *Bam*HI and *EcoRI*, treated with Klenow polymerase and inserted into the *SmaI* site of M13 mp8 so that the *EcoRI* site of the mp8 multi-linker was at the 5' end of the promoter fragment. Site directed mutagenesis (31) was then used to introduce a G at nucleotide 7464 to create a *Bgl*III site. The 35S promoter fragment was then excised from the M13 as a 330 bp *EcoRI*-*Bgl*III fragment which contains the 35S promoter and 30 nucleotides of the 5' nontranslated leader, but does not contain any of the CaMV translational initiators nor the 35S transcript polyadenylation signal that is located 180 nucleotides downstream from the start of transcription (32, 33). The 35S promoter fragment was joined to a 1.3 kb *Bgl*III-*EcoRI* fragment containing the Tn5 neomycin phosphotransferase II coding sequence modified so that the translational initiator signal in the bacterial leader sequence had been removed (34) and the NOS 3' nontranslated region and inserted into pMON120 to give pMON273. The chimeric 35S-NPTII'-NOS gene is oriented in the same direction in the pMON273 vector as is the construction of pMON316 NOS-NPTII'-NOS gene in pMON200.

These plasmids were transferred into *E. coli* strain JM101 and then mated into *Agrobacterium tumefaciens* strain GV3111 carrying the disarmed pTiB6S3-SE plasmid as described by Fraley *et al.* (17).

Construction of pMON316

The 330 bp *EcoRI* to *Bgl*III 35S promoter fragment was also inserted into pMON200 at the *EcoRI* and *Bgl*III sites. The resulting plasmid was cleaved with *EcoRI*, treated with the Klenow fragment of DNA polymerase I and ligase. This creates a new *Xmn*I site between the NOS and 35S promoter fragments. Next a synthetic multilinker with the sequence:

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      BglIII  ClaI           KpnI   EcoRI
      |      |             |      |
5'- AGATCTATCGATTCCCGGGTACCTCGAGAATT

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and the 260 bp *Mbo*I fragment carrying the NOS 3' polyadenylation signals which had an *EcoRI* linker at the 5' end of the fragment and *Bam*HI, *Sal*I and *Hind*III sites at the 3' end were inserted into the *Bgl*III and *Hind*III sites. The resultant plasmid, pMON316, contains unique cleavage sites for *Bgl*III, *Cla*I, *Kpn*I and *EcoRI* located between the 5' leader and the NOS polyadenylation signals. The pMON316 plasmid retains all of the properties of pMON200.

Plant Transformation

Cocultivation of *Petunia* protoplasts with *A. tumefaciens*, selection of kanamycin resistant transformed callus and regeneration of transgenic plants was carried out as described previously (35).

Preparation of DNAs

Plant DNA was extracted by grinding the frozen tissue in extraction buffer (50mM TRIS-HCl pH 8.0, 50mM EDTA, 50mM NaCl, 400µg/ml ethidium bromide, 2% sarcosyl). Following low speed centrifugation, cesium chloride (CsCl) was added to the supernatant (0.85gm/ml). The CsCl gradients were centrifuged at 150,000x g for 48 hours. The ethidium bromide was extracted with isopropanol, the DNA was dialyzed, and ethanol precipitated.

Southern Hybridization Analysis

10µg of each plant DNA was digested with *Bam*HI for pMON200 plant DNAs and *Eco*RI for pMON273 plant DNAs. The fragments were separated by electrophoresis on a 0.8% agarose gel and transferred to nitrocellulose (36). The blots were hybridized (50% formamide, 3xSSC, 5X Denhardt's, 0.1% SDS and 20µg/ml tRNA) with nick-translated pMON273 plasmid DNA for 48-60 hrs. at 42°C.

RNA Isolation and Northern Hybridization Analysis

Petunia leaf RNA was extracted (37) and poly (A) + RNA isolated by passing total RNA over an Oligo d(T) cellulose Type III (Collaborative Research) column. Quantitation of the poly (A) + RNA involved annealing an aliquot of the RNA to radiolabeled poly U [(uridylylate 5,6-3H)-polyuridylic acid] (New England Nuclear), followed by RNase A treatment (10 µg per ml for 30 minutes at 37°C). The reaction mix was spotted on DE-81 filter paper, washed 4X with 0.5M sodium phosphate (pH 7.5) and counted (38). Globin poly (A) + RNA (BRL) was used as a standard.

5µg of poly (A) + RNA from each plant source was treated with glyoxal and dimethylsulfoxide (39). The glyoxylated RNAs were electroblotted (25mM NaH₂PO₄/NaHPO₄, pH 6.5) for 16 hrs. at 125 amps from the gel to GeneScreen® (New England Nuclear). The filters were hybridized as per manufacturer's instructions for 48-60 hrs. at 42°C with constant shaking. The nick-translated DNAs used as probes were the 1.3kb *Bgl*II/*Eco*RI NPTII fragment purified from the pMON273 plasmid (Fig 1) for detecting the NPTII transcript, and the *petunia* small subunit gene (25) as an internal standard for comparing the amount of RNA per lane.

Neomycin Phosphotransferase Assay

The gel overlay assay was used to determine the steady state level of NPTII enzyme activity in each plant (40). Several parameters were investigated

for optimizing the sensitivity of the assay for plant tissue. Early observations showed that the level of NPTII activity varied between leaves from different positions on the same plant. This variability was minimized when the plant extract was made from pooled tissue. A paper hole punch was used to collect 15 disks from both young and old leaves. Grinding the plant tissue in the presence of microbeads (Ferro Corp) rather than glass beads increased the plant protein yield 4-fold.

To optimize detection of low levels of NPTII activity a titration curve was done with 10-85 $\mu\text{g}/\text{lane}$ of plant protein. For the pMON200 (NOS) plants, NPTII activity was not detectable at less than 50 $\mu\text{g}/\text{lane}$ of total protein (2 hr. exposure) while activity was detectable at 10 $\mu\text{g}/\text{lane}$ for the pMON273 plants. There was a nonlinear increase in NPTII activity between 40 and 50 μg of protein per lane for pMON200 NOS plants. This suggested that the total amount of protein may affect the stability of the NPTII enzyme. Supplementing plant cell extracts with 30-45 μg per lane of bovine serum albumin (BSA), resulted in a linear response; NPTII activity increased proportionately as plant protein levels increased. The addition of BSA appears to stabilize the enzyme, resulting in a 10-fold increase in the sensitivity of the assay. Experiments indicate that 25 $\mu\text{g}/\text{lane}$ of pMON273 plant protein and 70 $\mu\text{g}/\text{lane}$ of pMON200 plant protein was within the linear range of the assay in the presence of BSA.

Leaf disks were pooled from each plant for the assay. The tissue was homogenized with a glass rod in a microfuge tube with 150-200 μl of extraction buffer (20% glycerol, 10% B-mercaptoethanol, 125mM Tris-HCl pH 6.8, 100 $\mu\text{g}/\text{ml}$ bromophenol blue and 0.2% SDS). Following centrifugation in a microfuge for 10 minutes, total protein was determined using the Bradford assay. 25 μg of pMON273/3111SE plant protein or 70 μg of pMON200/3111SE plant protein, both supplemented with BSA, was loaded on a native polyacrylamide gel as previously described (40). The polyacrylamide gel was equilibrated for 30 minutes in water and then 30 minutes in reaction buffer (67 mM TRIS-maleate pH 7.1, 42mM MgCl₂, 400mM NH₄Cl), transferred onto a glass plate, and overlaid with a 1.5% agarose gel. The overlay gel contained the neomycin phosphotransferase substrates: 450 μCi [γ -³²P] ATP and 27 $\mu\text{g}/\text{ml}$ neomycin sulfate (Sigma). After 1 hr at room temperature a sheet of Whatman P81 paper, two sheets of Whatman 3MM paper, a stack of paper towels and a weight were put on top of the agarose gel. The phosphorylated neomycin is positively charged and binds to the P81 phosphocellulose ion exchange paper. After blotting overnight, the P81 paper was washed 3X in 80°C water, followed by 7 room temperature washes. The paper

was air dried and exposed to XAR-5 film. Activity was quantitated by counting the ^{32}P -radioactivity in the NPTII spot.

RESULTS

In order to permit a direct comparison of the relative strengths of the CaMV 35S and NOS promoters, each promoter fragment was fused in a similar fashion to an NPTII reporter gene containing a NOS 3' untranslated tail (Fig. 1). The resulting chimeric gene constructs were introduced into pMON120 to create pMON200 (NOS-NPTII-NOS) and pMON273 (CaMV 35S-NPTII-NOS). Transgenic plants containing pMON200 and pMON273 were produced as described previously (41) and were analyzed for insert copy number, NPT transcript and enzyme levels as described below.

DNA Hybridization Analysis of pMON200 and pMON273 Transformed Plants

The T-DNA copy number for each transgenic plant was determined by DNA blot hybridization. *Eco*RI digestion of the pMON273 plasmid generates a 1.6kb fragment containing the CaMV35S-NPTII-NOS3' gene (Fig. 1a). Following hybridization to the pMON273 probe, the intensity of the 1.6kb band in each plant was compared to the reconstruction copy controls, and gene copy number was determined for each plant (Fig. 2a). All of the pMON273 plants contain 1 copy of the T-DNA with the exception of #3271 which appears to contain 2 copies.

*Bam*HI digestion of the pMON200 plasmid generates a 3.2kb fragment containing the NOS-NPTII-NOS3' gene (Figure 1b). The intensity of the 3.2kb *Bam*HI fragment in each transformed plant DNA was determined relative to the T-DNA copy controls (Fig. 2b). Of the 7 pMON200 transformed plant DNAs analyzed, 4 appear to have a single copy of the gene (Figure 2b, plants 2782, 2813, 3612 and 2823) while 3 plants appear to have 2 copies (plant 2505, 2822, and 2818).

RNA Hybridization Analysis of pMON200 and pMON273 Transformed Plants

To determine NPTII transcript levels, Northern blots of poly (A) + RNA isolated from each transformed plant were hybridized with the 1.3kb NPTII fragment. As shown in Fig. 3a, the steady state transcript levels in pMON273 plants are significantly greater than that of the pMON200 transformants (Fig. 3b).

To verify that this difference was due to increased transcript levels and not an artifact of the amount of RNA loaded per lane, the blots were hybridized with a petunia small subunit probe and reexposed for 5 minutes. To quantitate the difference in NPTII transcript levels between the pMON273 and pMON200 plants, the silver grains in each band were eluted in 1 M NaOH and light scattering monitored at 500 nm (42). The values for each of the NPTII

bands was normalized for the variance observed in the petunia small subunit band. According to this analysis, the 5 pMON273 CaMV35S-NPTII plants have 30 times the level of NPTII transcripts observed in the 7 different pMON200 NOS-NPTII plants (Table 1).

There is obvious variability in transcript levels within each set of plants which is likely to be due to insert position effects. These differences do not alter the conclusion that pMON273 NPTII genes are more highly expressed than pMON200 genes, since the most highly expressed NPTII gene in a pMON200 plant is still less than the NPTII expression in the pMON273 plants.

Neomycin Phosphotransferase Activity

A confirmation of the higher level of expression observed in the pMON273 transformants was obtained from the results of the NPTII assay shown in Figure 4. A comparison of equivalent exposures (10 minutes) of the phosphorylated neomycin indicates a significantly greater level of NPTII activity in the pMON273 plant extracts (Figure 4a) than from the pMON200 plant extracts (Figure 4b). The pMON273 plant #3216 on the pMON200 gel allows direct visualization of the enhanced NPTII activity level.

Monitoring of radioactivity migrating with ^{32}P -neomycin phosphate allowed relative quantitation of the variance in NPTII activity. After adjustment of the values to compensate for the different levels of protein loaded on the gels, the average phosphorylated neomycin signal from the pMON273 extracts was found to be 110 times more intense than the signal from the pMON200 extracts (Table 1).

DISCUSSION

We have compared the NPTII transcript levels and enzyme activities in two sets of transgenic petunia plants. In one set of plants (pMON273) the NPTII coding sequence is preceded by the CaMV 35S promoter and leader sequences, in the other set of plants (pMON200) the NPTII coding region is preceded by the nopaline synthase promoter and leader sequences. We estimate that the pMON273

Figure 2. DNA BLOT HYBRIDIZATION ANALYSIS OF TRANSFORMED PLANTS.

- Each lane contains 10 μg of DNA from an individual plant. The molecular weight of λ -HindIII markers are indicated. The control lanes contain nontransformed Mitchell diploid petunia DNA. The numbers above each lane identify individual transformed plants.
- 2a. DNA blot hybridization analysis of 5 pMON273 transformed plants. All plant DNAs have been digested with *Eco*RI enzyme. 10c, 5c and 1c are copy reconstruction controls.
 - 2b. DNA blot hybridization of 7 pMON200 transformed plants. All plant DNAs have been digested with *Bam*HI enzyme. The first two lanes are pMON200 5 and 1 copy reconstruction controls.

Figure 3. NORTHERN BLOT ANALYSIS OF TRANSFORMANTS.

Northern blot analysis of poly (A) + RNA (5 μ g/lane) from transformed plants. The filters were hybridized with the 1.3kb NPTII fragment (6.2 $\times$ 10⁸ cpm/ μ g) and exposed to X-ray film for 2 hours. The filters were subsequently hybridized with a petunia small subunit gene (25) (1.0 $\times$ 10⁹ cpm/ μ g) and exposed to X-ray film for 5 minutes. The first lane is non-transformed petunia plant RNA. The numbers above each lane identify individual transformed plants.

3a. Blot of poly (A) + RNA isolated from pMON273 transformed plants.

3b. Blot of poly (A) + RNA isolated from pMON200 transformed plants.

plants contain a 30 fold greater level of NPTII transcript than the pMON200 plants. Consistent with this observation is the finding that the pMON273 leaf extracts have 110 fold higher NPTII enzyme activity than the pMON200 leaf extracts. In several of the transgenic plants, there is a substantial variation in both RNA and enzyme levels which cannot be accounted for by the slight difference in gene copy number. The observed differences may be due to the chromosomal location of the T-DNA insertion. Similar variability in expression of inserted genes in plants has been previously observed; investigators

TABLE 1
QUANTITATION OF NPTII TRANSCRIPT LEVELS AND NPTII ENZYME ACTIVITY IN
pMON273 AND pMON200 PLANTS

Plant number	Relative ^a NPTII transcript levels	Relative ^b NPTII activity (cpm X 10 ⁻³)
<u>pMON 273</u>		
3272	545.8	113.5
3271	415.8	1148.1
3349	432.0	447.3
3350	307.0	650.0
3343	501.7	1539.3
Average	440.4	779.6
<u>pMON 200</u>		
2782	0.0	0.2
2505	0.0	5.8
2822	0.0	0.0
2813	27.3	19.0
2818	0.3	1.0
3612	17.7	0.4
2823	62.6	23.1
Average	15.4	7.1

- ^a The transcriptional activities of different transgenic plants were determined for each of the RNA samples in Figure 3 by comparing the ratio of NPTII to petunia ssu transcripts. Quantitation of silver grains eluted from bands on x-ray films was carried out as described in Materials and Methods. The average NPTII transcript level determined for pMON273 plants was 28.6-fold higher than that determined for pMON200 plants.
- ^b The NPTII enzyme activity levels of different transgenic plants was determined for each of the protein extracts in Figure 4. Quantitation was carried out by direct monitoring by radioactivity migrating with ³²P-neomycin. The values shown in the table have been corrected for protein load. The average NPTII enzyme levels determined for pMON273 plants was 109.8-fold higher than that determined for pMON200 plants.

have reported >200 fold variation in the expression of transgenic plants containing a chlorophyll a/b binding protein gene promoter fused to the octopine synthase coding sequence (43). Such "position effects" have been reported in transgenic mice (44, 45, 46) and fruit flies (47, 48) and have not yet been adequately explained at the molecular level.

Although there is not a clear correlation between insert copy number and

Figure 4. DETERMINATION OF NEOMYCIN PHOSPHOTRANSFERASE ACTIVITY.

Each autoradiogram is a 10 minute exposure of phosphorylated neomycin bound to PE-81 paper. The control lane is leaf tissue extract from a nontransformed Mitchell diploid plant.

- 4a. NPT assay on the pMON273 plant extracts, 25µg of plant protein loaded on the gel per lane.
- 4b. NPT assay on the pMON200 transformed plants, 70µg of plant protein loaded per lane. The last lane was loaded with 25µg of pMON 273 plant protein.

level of chimeric gene expression, the fact that 3 of the 7 pMON200 transgenic plants contain 2 copies of the NOS-NPTII-NOS gene would suggest that the differential expression of the CaMV 35S promoter may actually be slightly underestimated in our studies. In addition, all plants were selected to be kanamycin resistant prior to analysis of gene expression such that the pMON200 plants tested may be biased or enriched for a higher level of expression. In contrast, because of the stronger CaMV 35S promoter, the pMON273 plants are likely to represent an unbiased population. This selection bias would also underestimate the CaMV35S promoter strength. This enhanced transcription of the CaMV 35S promoter and leader sequences has also been observed in tobacco and tomato transgenic plants.

Figure 5. Construction of pMON316 Expression Vector.

pMON316 is a derivative of pMON200 (26) in which the CaMV 35S promoter (P) and NOS 3' polyadenylation signal (3') have been inserted into the *Bgl*III and *Hind*III sites as described under Materials and Methods. A synthetic multilinker containing unique *Bgl*III, *Cla*I, *Kpn*I and *Eco*RI sites is located between the CaMV 35S promoter and the NOS polyadenylation signals. The pMON316 plasmid retains all of the properties of pMON200.

Previous analyses of CaMV 35S and NOS 5' sequences have indicated that the CaMVs promoter is only slightly stronger than the NOS promoter. For example, it has been reported that the transcript level from a chimeric CaMV 35S-human growth hormone mini-gene was ~3-fold greater than that from a chimeric NOS-NPTII-NOS gene (49). Similarly, a 3-5 fold difference in transcript levels was found when a CaMV-TMV coat protein cDNA-NOS gene was compared to the NOS-NPTII-NOS gene in regenerated tobacco plants (50). It is likely that the use of different coding sequences in these studies have led to an underestimate of the efficiency of the CaMV 35S promoter. Using electroporation to transfect carrot, tobacco and maize protoplasts, the pCaMVCAT construct gave much higher levels of chloramphenicol acetyltransferase activity than the pNOSCAT plasmid (51).

The constructs described in this paper have identical coding regions and 3' nontranslated tails, allowing us to attribute the differences in the steady state transcript levels of our chimeric genes directly to the 5' sequences.

We are currently doing experiments to determine whether these observed differences are entirely due to differences in promoter strengths, or whether the 5' nontranslated leader may also contribute to transcript stability.

Based on the above results, the CaMV 35S promoter and 5' nontranslated region has been incorporated into an expression cassette vector, pMON316 (Fig. 5) which has been used successfully to express a variety of coding sequences in transgenic plants. A mouse dihydrofolate reductase coding sequence (52) and a hygromycin phosphatase coding sequence (53, 54) inserted into pMON316 have been shown to function as extremely efficient selectable marker genes in plant transformation experiments. Similarly, a cDNA clone encoding a petunia EPSP synthase engineered in pMON316 is expressed 20-40 fold over its normal level; this overproduction results in enhanced tolerance to Roundup® herbicide in petunia (14). Finally insertion of a cDNA of TMV coat protein gene in pMON316 results in TMV coat protein being produced at levels of ~0.1% of the total leaf protein in transgenic tobacco (16). Such plants show significant resistance to infection by TMV virus.

It will be of interest in future studies to carefully compare the level of expression of the CaMV 35S promoter with other potentially highly expressed promoters derived from the TR-DNA 1' and 2' transcript genes (21, 55) as well as from plant genes such as the RuBP ssu carboxylase (3, 56, 57) *chl a/b* binding protein (5, 43) and the cell wall hydroxy proline-rich glycoprotein (58).

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