Journal of Biotechnology, 14 (1990) 333–344
Elsevier

BIOTEC 00521

The pFF plasmids: cassettes utilising CaMV sequences for expression of foreign genes in plants

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(Received 12 December 1989; accepted 20 December 1989)

Summary

A plant expression cassette was constructed using the cauliflower mosaic virus 35S 5′ regulatory region with the enhancer duplicated and the 35S polyadenylation signal. Insertion of a polylinker between the transcription initiation and polyadenylation sites allows for easy cloning of genes. To test the usefulness of the cassette chimeric bacterial genes were prepared. The constructs were introduced into *Nicotiana tabacum* suspension culture cells by the particle bombardment process. Expression of the β-glucuronidase reporter gene was verified by histochemical staining. Stable kanamycin and hygromycin resistant transgenic lines were obtained after introduction of chimeric genes encoding the enzymes neomycin phosphotransferase and hygromycin B phosphotransferase, respectively. The number of stable transformants was approximately 2% of the cells that transiently expressed the β-glucuronidase reporter gene.

Plasmid, pFF; CaMV sequence; Foreign gene; Plant

Introduction

Expression cassettes are modules of 5′ and 3′ regulatory regions for the construction of gene fusions with prokaryotic or eukaryotic genes. They are used to analyze

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specific regulatory sequences, to serve as internal controls (Kuhlemeier et al., 1987) or to express marker genes for the selection of drug-resistant cell lines. Chimeric gene constructions utilized regulatory sequences obtained from T-DNA opine synthase genes (Bevan et al., 1983; Fraley et al., 1983; Herrera-Estrella et al., 1983), from a variety of plant genes such as the small subunit of ribulose 1,5-biphosphate carboxylase (Herrera-Estrella et al., 1984; Broglie et al., 1984), or from cauliflower mosaic virus (CaMV). Because a high level of expression is often a critical factor, the relative strengths of some of these plant promoters have been evaluated. Quantitative measurements of transcript levels in transformed tobacco cells (Morelli et al., 1985; Harpster et al., 1988) or transgenic petunia plants (Sanders et al., 1987) showed that the CaMV 35S promoter is at least 30 times stronger than the widely used nopaline synthase (NOS) promoter.

The 35S promoter is a viral promoter isolated from cauliflower mosaic virus, a DNA virus that infects members of the *Cruciferae*. Information on the complete nucleotide sequence of CaMV (Gardner et al., 1981) and on the 35S transcriptional start site (Covey et al., 1981; Guilley et al., 1982) is available. Comparison of transcript levels of various deletion mutants of the 35S promoter identified the -343 to -46 upstream region as being responsible for the majority of the 35S promoter strength (Odell et al., 1985). Multiple *cis* regulatory elements were identified within this region not only regulating the level of transcription but also conferring tissue specificity and developmental control (Odell et al., 1987; Ow et al., 1987; Benfey et al., 1989; Fang et al., 1989). The -343 to -90 fragment was shown to be of interest because of its enhancer-like properties. When cloned 5′ of the NOS promoter, a 3-fold increase in transcription levels was measured (Odell et al., 1988). Duplication of the -343 to -90 sequences resulted in a 10-fold transcriptional enhancement (Kay et al., 1987).

Although construction of expression cassettes utilizing the 35S promoter has been reported before (Pietrzak et al., 1986; Topfer et al., 1987), these do not use the full potential of the 35S enhancer sequences. We have constructed cassettes consisting of the duplicated 35S enhancer, the promoter region and polyadenylation signal. The unique restriction sites outside the cassettes facilitate subcloning it into other vectors and make the 5′ and 3′ regulatory regions interchangeable with 5′ and 3′ sequences of other genes. A polylinker between the 5′ and 3′ *cis*-acting elements facilitates easy construction of chimeric genes.

The new expression cassette was tested by inserting the well characterized genes for the enzymes neomycin phosphotransferase type II (NPT II) (Bevan et al., 1983; Fraley et al., 1983; Herrera-Estrella et al., 1983), hygromycin B phosphotransferase (HPH) (Van den Elzen et al., 1985; Waldron et al., 1985) and β-glucuronidase (GUS) (Jefferson et al., 1986). We employed the particle bombardment process to introduce these chimeric gene constructs into suspension culture cells of *Nicotiana tabacum*. Histochemical staining for GUS activity was used to monitor transient expression one day after bombardment. Gene fusions with the NPT II and HPH coding regions were tested by selection of kanamycin and hygromycin stable transgenic lines.

Materials and Methods

Bacterial strains and plasmids

DNA constructions were carried out in *E. coli* strain JV30 (Vieira and Messing, 1987). Single-stranded DNA template for mutagenesis was prepared in *E. coli* strain BW313 (Kunkel, 1985). Plasmid pUC120 is identical to plasmid pUC118 except that it has a *Nco*I site 5′ to the *Eco*RI site (Vieira, 1988).

DNA manipulations

All DNA manipulations were performed as described previously by Maniatis et al. (1982) except for the preparation of single-stranded DNA which was prepared according to Vieira and Messing (1987). Oligonucleotide directed in vitro mutagenesis was carried out as described by Zoller and Smith (1984) and Kunkel (1985).

Introduction of DNA into cells by the particle gun

Suspension cultures of *Nicotiana tabacum* (line XD) were maintained in 250-ml Erlenmeyer flasks containing 50 ml of RMS medium (Klein et al., 1988a) and agitated under continuous light at 28° C. The cells were subcultured approximately every 4 days. Suspension culture cells (5 ml) were evenly distributed over a Whatman No. 4 filter paper (diameter 5.5 cm) using a Buchner funnel. The filter paper with cells was then placed on a RMS plate (0.7% agarose).

Tungsten particles (1 μm; 25 mg) were cleaned in 1 ml absolute ethanol by sonication at maximum power for 10 min. The particles were sedimented by centrifugation (2 min), washed twice with sterile distilled water, and resuspended in 0.5 ml of sterile distilled water. Tungsten was prepared for transformation by mixing 25 μl of the suspension with 2 μg DNA, dissolved in 5 μl $T_{10}E_1$-buffer (10 mM Tris, pH 8.0, 1 mM EDTA), 25 μl 1.0 M $CaCl_2$ and 10 μl 0.1 M spermidine. The particle/DNA mixture was incubated on ice for 2 min and centrifuged for 1 min in the Eppendorf centrifuge after which 30 μl of the supernatant was removed. The remaining solution was sonicated for 1 or 2 s. Two μl of the DNA-coated tungsten suspension was placed on the microprojectile for each bombardment which was performed as described by Klein et al. (1988b).

GUS assay

Cells were incubated on the filter paper on RMS medium for approximately 24 h at 28° C following bombardment. The filter paper was then transferred to a sterile Petri dish. GUS substrate mixture containing potassium ferricyanide (5 mM), potassium ferrocyanide (5 mM), sodium phosphate buffer (0.1 M, pH 7.0), Triton X-100 (0.06%, v/v) and the synthetic GUS substrate, 5-bromo-4-chloro-3-indoyl-β-D-glucuronic acid (0.3%, w/v) (Jefferson, 1987; Klein et al., 1988b), was pipetted dropwise onto the cells in order to cover all cells. After incubation overnight at 37°C, the cells expressing GUS were counted under a dissecting microscope.

Selection of kanamycin or hygromycin resistant calli

Following bombardment, the cells were incubated on the filter paper on RMS medium for 2 d at 28°C without drug selection. The filter paper was then placed in

a sterile flask with 30 ml RMS medium (0.5% agarose) containing 50 μg ml^{-1} kanamycin or 30 μg ml^{-1} hygromycin. The cells were resuspended from the filter and pipetted on three RMS plates (10 ml suspension per plate) containing the same antibiotic and at the same concentration as the top layer. Resistant calli were identified after 3–5 weeks of incubation at 28°C. Resistance of the selected calli was verified by their ability to repeatedly grow on the same selective medium.

Southern analysis of resistant calli

Genomic DNA was isolated from 10–15 g of calli. The tissue was ground in 20 ml extraction buffer (100 mM Tris, pH 8.5, 100 mM NaCl, 20 mM EDTA and 1% Sarcosyl) using a homogenizer. The tissue homogenate was extracted twice with phenol/chloroform and once with chloroform alone, then the DNA was alcohol-precipitated. The pellet was resuspended in $T_{10}E_1$-buffer (10 mM Tris, pH 8.0, 1 mM EDTA) and the DNA further purified on CsCl density gradients (Maniatis et al., 1982). DNA (5 μg) was digested with the appropriate restriction enzymes, electrophoresed through 1% agarose, and transferred onto nitrocellulose filter. Hybridisation was carried out with the coding region of the appropriate genes using nick-translated ^{32}P-labeled DNA. Digestions, electrophoresis, DNA transfer, nick translation and Southern blot hybridisation were performed as described by Maniatis et al. (1982).

Results

Construction of expression cassette pFF19

The 5′ and 3′ termini of the 35S transcript have been mapped with S1 nuclease and showed an overlapping sequence of 200 nucleotides (Covey et al., 1981; Guilley et al., 1982). The 35S 5′ region and polyadenylation signal could therefore be isolated on a single small DNA fragment. These regulatory sequences from the CaMV strain CM1841 (Gardner et al., 1981) were cloned as a *Hin*cII (7016)-*Bgl*II (7674) fragment into the *Hin*cII-*Bam*HI sites of pUC120 forming the clone pFF10 (Fig. 1). With the lacZ and 35S promoters being in opposite orientations, read through from the lacZ promoter was eliminated. To allow for easy cloning of the different genes in between the 35S 5′ and 3′ regions, it was necessary to introduce a multiple cloning site at that position. Oligonucleotide directed in vitro mutagenesis was used to introduce a unique *Xho*I site to separate the two regulatory regions. Subsequently, the remaining pUC120 polylinker sites were eliminated as described in the legend of Fig. 1, to enable the insertion of a unique polylinker in the *Xho*I site (see below). Duplication of the 35S enhancer sequence in pFF15 was achieved by cloning the 329 bp *Eco*RV-*Hin*dIII fragment from pFF14 into the *Hin*cII-*Hin*dIII sites from pFF12 (Fig. 1).

As a final step a multiple cloning site was introduced resulting in the vector pFF19 (Fig. 2). The polylinker of vector pFF19 derives from plasmid pIC20h (Marsh et al., 1984). The *Eco*RI, *Eco*RV and *Cla*I restriction sites were deleted from the pIC20h polylinker by digesting pIC20h with *Sst*I and ligation of the

plasmid. The sites were eliminated because the same sites are present at other positions in pFF19. The remaining polylinker was excised as a *Hin*dIII-mung bean nuclease-treated fragment and ligated into the *Xho*I site from pFF15 which was filled in with Klenow fragment. Removing the 5′ protruding ends generated by the *Hin*dIII restriction endonuclease rather than filling them in had two effects. It created a unique *Nsi*I recognition site adjacent to the *Sph*I site and more importantly, it prevented the reformation of the *Hin*dIII sites when ligated into the *Xho*I site filled in with Klenow fragment. Both the 35S 5′ region and the poly(A) addition signal therefore have unique flanking restriction sites allowing them to be easily substituted by 5′ and 3′ regulatory sequences of other genes.

Chimeric gene constructions

The GUS coding region was cloned as a *Pst*I fragment from pRAJ260 (Jefferson et al., 1986) into the *Pst*I site from pFF19 to produce pFF19G (Fig. 2).

Before cloning the Tn5 NPT II gene-coding region into our expression vector, we deleted the upstream ATG which is located 16 bp 5′ of the real translation initiation site. Deletion of this ATG ensures for initiation at the right start codon and therefore allows a higher level of drug selection (Rogers et al., 1985). The NPT II gene was digested with *Bcl*I-*Aha*II and ligated into *Bgl*II-*Cla*I digested pIC20h vector (Marsh et al., 1984) resulting in the plasmid pIC20h5K′. The 5′ end of the NPT II coding region was cloned as a 250 bp *Mbo*I-*Taq*I fragment into the *Bam*HI-*Cla*I sites of pIC20h forming pIC250neo. Since the *Mbo*I site is located in between the two ATGs, this 250 bp fragment only contains the right translational

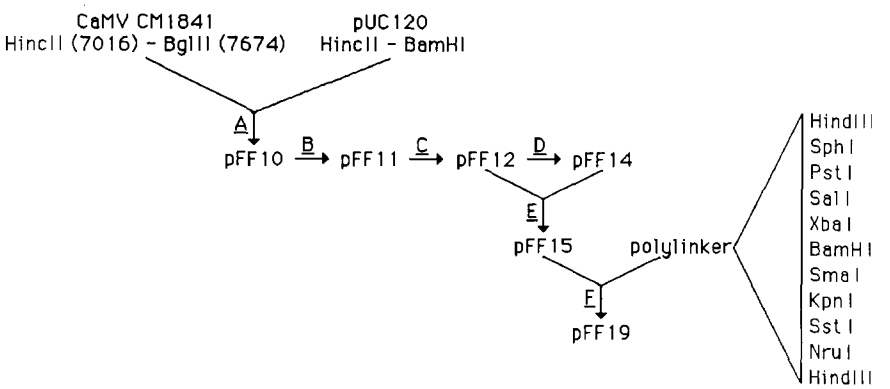

Fig. 1 Construction of plasmid pFF19. The 658 bp *Hin*cII-*Bgl*II fragment from the CaMV strain CM1841 was cloned into the *Hin*cII-*Bam*HI sites from pUC120 to yield pFF10 (A). Mutagenesis was used to change nucleotides 7477 and 7479 (CM1841 map positions) from Ts to Gs, creating a unique *Xho*I site (7474) yielding pFF11 (B). The flanking *Sst*I, *Kpn*I and *Sma*I restriction sites were deleted by digestion with *Sst*I and *Sma*I, treatment with the Klenow fragment and ligation of the blunt ends yielding pFF12 (C). Similarly, the *Hin*cII, *Pst*I and *Sph*I sites were deleted yielding pFF14 (D). The 35S enhancer was duplicated by inserting the *Eco*RV-*Hin*dIII fragment from pFF14 into the *Hin*cII-*Hin*dIII sites of pFF12 yielding pFF15 (E). The polylinker was inserted as a Mung Bean nuclease treated *Hin*dIII fragment into the *Xho*I site of pFF15 which was filled in with Klenow fragment yielding pFF19 (F).

start site. The unique *Pst*I site from the NPT II gene is also present on this fragment and was used to combine the 3′ end of the gene with this isolated 5′ end. To do so pIC20h5K′ was digested partially with *Pst*I and completely with *Xho*I. The 3.3 kb fragment was isolated and ligated to the 250 bp *Pst*I-*Sal*I fragment from pIC250neo. The resulting clone, pICNeo6, has the entire coding sequence from the Tn5 NPT II gene with the upstream ATG removed. It was digested with *Eco*RI, treated with Mung Bean nuclease and subsequently digested with *Sst*I. To construct the plasmid pFF19K, this fragment was cloned into the *Sst*I and *Sph*I (blunt-ended with Klenow fragment) sites of pFF19 (Fig. 2).

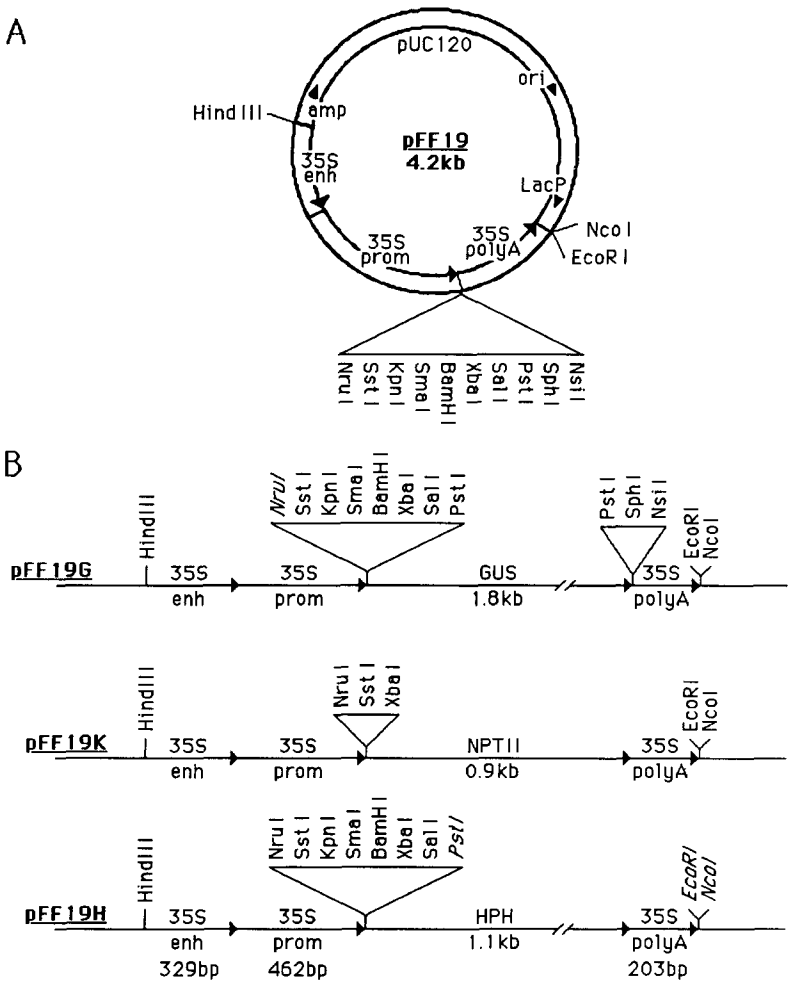

Fig. 2. The expression cassette pFF19 (A) and chimeric genes to express the GUS, NPT II and HPH enzymes (B). The direction of transcription of the regulatory and coding regions is indicated by the arrows. Restriction sites shown in italics are not unique because they are also present in the coding sequence of the reporter gene.

TABLE 1

The number of GUS-expressing cells and kanamycin or hygromycin resistant clones after bombardment with plasmids pFF19G, pFF19K and pFF19H, respectively

	pFF19G		pFF19K		pFF19H	
	Exp. I	Exp. II	Exp. I	Exp. II	Exp. I	Exp. II
	126	336	5	5	5	3
	153	268	2	3	2	4
	57	109	6	2	3	4
	138	418	1	5	2	3
			1	2	1	5
			2	3	2	7
			2	4	2	3
			1	3	4	4
			5	6	5	2
			–	5	3	3
Average	119	283	2.5	3.8	2.9	3.8

Plasmid pJD214Hy (Dougherty and Temin, 1986) was used to clone the HPH coding region into our expression cassette. This plasmid contains the HPH gene from pLG89 (Gritz and Davies, 1983) flanked at the 5′ end by a polylinker which includes a *Xba*I, *Sal*I and *Pst*I site and at the 3′ end by a *Cla*I site. The 1.1 kb *Cla*I-*Sal*I fragment from pJD214Hy was ligated into *Sph*I-*Sal*I digested pFF19 after treating the protruding ends of the *Cla*I and *Sph*I restriction sites with Klenow fragment (Fig. 2). In the resulting plasmid pFF19H, there are no unique sites flanking the 35S polyadenylation signal, because of the presence of both an *Eco*RI and a *Nco*I site within the hygromycin coding sequence.

Transient gene expression assay with pFF19G

The chimeric construct pFF19G was introduced into *N. tabacum* suspension culture cells by bombardment with the particle gun to analyze if the 35S regulatory regions of the pFF19 expression cassette would lead to transient expression of the chimeric gene. After a 1-d incubation, the bombarded cells were treated with the GUS histochemical substrate mixture. GUS expressing cells develop a distinctive blue color which can be easily detected microscopically. The number of blue cells in independent bombardments was determined and is given in Table 1. In two different experiments, an average of 119 (Experiment I) and 283 (Experiment II) cells expressed GUS, with a variation between 57 and 418 blue cells per bombardment.

Selection of kanamycin and hygromycin resistant clones

In order to test the efficiency of the chimeric NPT II and HPH genes for obtaining stable transformants, XD cells were bombarded with plasmids pFF19K and pFF19H. The bombarded cells were incubated for 2 d without drug selection to allow expression of the resistance gene. Stable transformants were then selected by

embedding the cells in medium containing 50 μg ml^{-1} kanamycin or 30 μg ml^{-1} hygromycin, respectively. The number of kanamycin and hygromycin resistant colonies that was obtained per bombardment is listed in Table 1. On average, about three resistant clones were recovered in each bombarded sample.

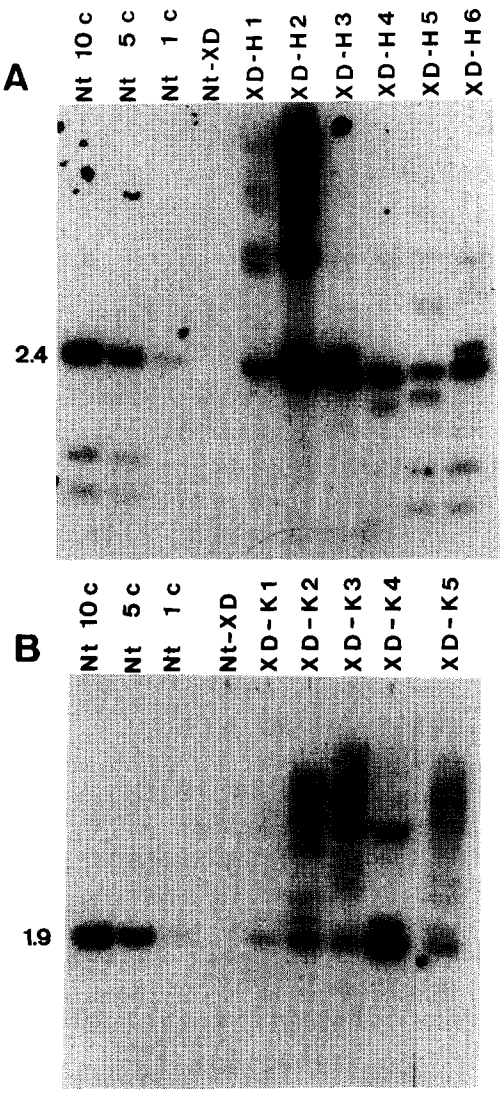

Fig. 3. Southern analysis of hygromycin (A) and kanamycin (B) resistant calli from transformed *N. tabacum* XD cells. Lane Nt-XD, DNA from nontransformed suspension culture cells. Lanes XD-H1 to XD-H6, DNA from hygromycin resistant call. Lanes XD-K1 to XD-K5, DNA from kanamycin resistant call. Lanes Nt 10c, Nt 5c and Nt 1c, genomic DNA mixed with pFF19H (A) or pFF19K (B) to reconstruct the integration of 10, 5 or 1 copy of the chimeric HPH (A) or NPT II (B) genes per diploid nucleus.

Southern analysis of stable transformants

DNA isolated from kanamycin and hygromycin resistant calli were probed for the presence of transforming DNA by the Southern procedure. DNA isolated from kanamycin resistant clones was digested with *Eco*RI-*Hin*dIII, and after transferring onto nitrocellulose filter probed with nick-translated *Eco*RI-*Hin*dIII insert from pFF19K. These two enzymes have unique recognition sites just outside the 35S regulatory regions producing a 1.9 kb fragment (Fig. 2). Whereas DNA from nontransformed tissue lacked hybridisation using this probe, all the kanamycin resistant calli showed a hybridizing band of the predicted size (Fig. 3). Comparison of the intensity of this band with reconstructed mix indicates that the transgenic clones carry 1 to 20 copies of the intact NPT II gene per diploid genome. DNA from hygromycin resistant colonies was analyzed in a similar manner. There are two *Pvu*II sites in the lacZ region, therefore digestion with the *Pvu*II restriction endonuclease excised the chimeric HPH gene from plasmid pFF19H as a 2.4 kb fragment. Nick-translated *Pvu*II insert from pFF19H was used as a probe resulting in a hybridizing band of 2.4 kb only in the lanes containing DNA from hygromycin resistant calli (Fig. 3). The number of intact HPH gene copies per diploid genome is around 3 to 30. Southern probing for both types of transgenic calli also revealed fragments with sizes deviating from the expected 1.9 or 2.4 kb. These are due to rearrangements of the plasmid DNA upon transformation, a phenomenon which has been observed before (Paszkowski et al., 1984; Klein et al., 1988a). The additional two bands seen in the first two lanes of Fig. 3A correspond to a size of approximately 1.0 and 1.4 kb and are most probably due to star activity of the restriction enzyme used.

Discussion

We described the construction of a new vector pFF19 utilizing the 35S promoter region and a duplicated enhancer as 5′ region to allow for high levels of transcription (Kay et al., 1987). The usefulness of the cassette was tested in a transient assay with the GUS reporter gene after introducing the plasmid pFF19G into tobacco suspension culture cells. Also stable transgenic lines were obtained using chimeric constructs carrying the selectable markers NPT II or HPH. Previous studies suggested that about 2–5% of the cells that transiently express a foreign gene stably integrate it (Klein et al., 1988a). Comparison of the number of GUS positive cells, as a measure of the success of DNA delivery, and of the number of kanamycin or hygromycin resistant clones, as the number of stable transformants, yielded a similar value. Efficiency of bombardment is variable (Table 1) and depends on a number of parameters (Klein et al., 1988b). Determining the number of GUS expressing cells is useful to compare efficiency of DNA delivery in independent experiments. In addition to the experiments described in this manuscript we tested expression of the GUS gene in pFF19G in suspension culture cells of *Avena sativa, Oryza sativa* and *Zea mays*. The number of GUS expressing cells was comparable to those obtained in *Nicotiana tabacum* (data not shown). This observation confirms

earlier findings which showed that the 35S upstream region is active both in transgenic tobacco and petunia plants (Odell et al., 1985; Jefferson et al., 1987; Sanders et al., 1987) as well as in a transient protoplast assay of several dicots and monocots (Fromm et al., 1985; On-Lee et al., 1986; Nagata et al., 1987).

The pFF19 cassette was designed to allow for maximum expression of a reporter gene based on the use of an efficient 5′ regulatory region. If desired, expression of genes in the cassette can be further improved. Alterations that may increase expression include exchange of sequences at the 3′ terminus (Ingelbrecht et al., 1989), insertion of an intron in between the transcription and translation initiation sites (Callis et al., 1987) and insertion of specific 5′ untranslated leader sequences at the same position (Gallie et al., 1987; Sleat et al., 1987; Jobling and Gehrke, 1987; Harpster et al., 1988). These sequences can be easily incorporated into our cassettes using the unique restriction sites present.

Acknowledgement

This research was supported by a grant from the New Jersey Commission on Science and Technology.

References

Benfey, P.N., Ren, L. and Chua, N.-H. (1989) The CaMV 35S enhancer contains at least two domains which can confer different developmental and tissue specific expression patterns. EMBO J. 8, 2195–2202.

Bevan, M.W., Flavell, R.B. and Chilton, M.-D. (1983) A chimeric antibiotic resistance gene as a selectable marker for plant cell transformation. Nature 304, 184–187.

Broglie, R., Coruzzi, G., Fraley, R.T., Rogers, S.G., Horsch, R.B., Niedermeyer, J.G., Fink, C.L., Flick, J.S. and Chua, N.-H. (1984) Light-regulated expression of a pea ribulose-1,5-biphosphate carboxylase small subunit gene in transformed plant cells. Science 224, 838–843.

Callis, J., Fromm, M. and Walbot, V. (1987) Introns increase gene expression in cultured maize cells. Genes Dev. 1, 1183–1200.

Covey, S.-N., Lomonossoff, G.P. and Hull, R. (1981) Characterization of cauliflower mosaic virus DNA sequences which encode major polyadenylated transcripts. Nucleic Acids Res. 9, 6735–6747.

Dougherty, J.P. and Temin, H.M. (1986) High mutation rate of spleen necrosis virus-based retrovirus vector. Mol. Cell. Biol. 168, 4387–4395.

Fang, R., Nagy, F., Sivasubramaniam, S. and Chua, N.-H. (1989) Multiple *cis* regulatory elements for maximal expression of the cauliflower mosaic virus 35S promoter in transgenic plants. Plant Cell 1, 141–150.

Fraley, R.T., Rogers, S.G., Horsch, R.B., Sanders, P.R., Flick, J.S., Adams, S.P., Bittner, M.L., Brand, L.A., Fink, C.L., Fry, J.S., Galluppi, G.R., Goldberg, S.B., Hoffmann, N.L. and Woo, S.C. (1983) Expression of bacterial genes in plant cells. Proc. Natl. Acad. Sci. USA 80, 4803–4807.

Fromm, M., Taylor, L.P. and Walbot, V. (1985) Expression of genes transferred into monocot and dicot plant cells by electroporation. Proc. Natl. Acad. Sci. USA 82, 5824–5828.

Gallie, D.R., Sleat, D.E., Watts, J.W., Turner, P.C. and Wilson, T.M.A. (1987) The 5′-leader sequence of tobacco mosaic virus RNA enhances the expression of foreign gene transcripts in vitro and in vivo. Nucleic Acids Res. 15, 3257–3273.

Gardner, R.C., Howarth, A.J., Hahn, P., Brown-Luedi, M., Shepherd, R.J. and Messing, J. (1981) The complete nucleotide sequence of an infectious clone of cauliflower mosaic virus by M13mp7 shotgun sequencing. Nucleic Acids Res. 9, 2871–2888.

Gritz, L. and Davies, J. (1983) Plasmid-encoded hygromycin B resistance: the sequence of hygromycin B phosphotransferase gene and its expression in *Escherichia coli* and *Saccharomyces cerevisiae*. Gene 25, 179–188.

Guilley, H., Dudley, R.K., Jonand, G., Balazs, E. and Richards, K.E. (1982) Transcription of cauliflower mosaic virus DNA detection of promoter sequences and characterisation of transcripts. Cell 30, 763–773.

Harpster, M.H., Townsend, J.A., Jones, J.D.G., Bedbrook, J. and Dunsmuir, P. (1988) Relative strengths of the 35S cauliflower mosaic virus, 1′, 2′ and nopaline synthase promoters in transformed tobacco, sugarbeet and oilseed rape callus tissue. Mol. Gen. Genet. 212, 182–190.

Herrera-Estrella, L., De Block, M., Messens, E., Hernalsteens, J.-P., Van Montagu, M.C. and Schell, J. (1983) Chimeric genes as dominant selectable markers in plant cells. EMBO J. 2, 987–995.

Herrera-Estrella, L., Van den Broeck, G., Maenhaut, R., Van Montagu, M.C., Schell, J., Timko, M. and Cashmore, A. (1984) Light-inducible and chloroplast-associated expression of a chimeric gene introduced into *Nicotiana tabacum* using a Ti plasmid vector. Nature 310, 115–120.

Ingelbrecht, I.L.W., Herman, L.M.F., Dekeyser, R.A., Van Montagu, M.C. and Depicker, A.G. (1989) Different 3′ end regions strongly influence the level of gene expression in plant cells. The Plant Cell 1, 671–680.

Jefferson, R.A., Burgess, S.M. and Hirsh, D. (1986) β-Glucuronidase from *Escherichia coli* as a gene fusion marker. Proc. Natl. Acad. Sci. USA 83, 8447–8451.

Jefferson, R.A., Kavanagh, T.A. and Bevan, M.W. (1987) GUS fusions: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J. 6, 3901–3907.

Jefferson, R.A. (1987) Assaying chimeric genes in plants: the GUS gene fusion system. Plant Mol. Biol. Rep. 5, 387–405.

Jobling, S.A. and Gehrke, L. (1987) Enhanced translation of chimeric messenger RNAs containing a plant viral untranslated leader sequence. Nature 325, 622–625.

Kay, R., Chan, A., Daiy, M. and McPherson, J. (1987) Duplication of CaMV 35S promoter sequences creates a strong enhancer for plant genes. Science 236, 1299–1302.

Klein, T.M., Harper, E.C., Svab, Z., Sanford, J.C., Fromm, M.E. and Maliga, P. (1988a) Stable genetic transformation of intact *Nicotiana* cells by the particle bombardment process. Proc. Natl. Acad. Sci. USA 85, 8502–8505.

Klein, T.M., Gradziel, T., Fromm, M.E. and Sanford, J.C. (1988b) Factors influencing gene delivery into *Zea mays* cells by high velocity microprojectiles. Bio/Technology 6, 559–563.

Kuhlemeier, C., Green, P. and Chua, N.-H. (1987) Regulation of gene expression in higher plants. Annu. Rev. Plant Physiol. 38, 221–257.

Kunkel, T.A. (1985) Rapid and efficient site-specific mutagenesis without phenotypic selection. Proc. Natl. Acad. Sci. USA 82, 488–492.

Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, New York.

Marsh, J.L., Erfle, M. and Wykes, E.J. (1984) The pIC plasmid and phage vectors with versatile cloning sites for recombinant selection by insertional inactivation. Gene 32, 481–485.

Morelli, G., Nagy, F., Fraley, R.T., Rogers, S.G. and Chua, N.-H. (1985) A short conserved sequence is involved in the light-inducibility of a gene encoding ribulose-1,5-biphosphate carboxylase small subunit of pea. Nature 315, 200–204.

Nagata, T., Okada, K., Kawazu, T. and Takebe, I. (1987) Cauliflower mosaic virus 35S promoter directs S phase specific expression in plant cells. Mol. Gen. Genet. 207, 242–244.

Odell, J.T., Nagy, F. and Chua, N.-H. (1985) Identification of DNA sequences required for activity of the cauliflower mosaic virus 35S promoter. Nature 313, 810–812.

Odell, J.T., Nagy, F. and Chua, N.-H. (1987) Variability in 35S promoter expression between independent transformants. In: McIntosh, L. and Key, J. (Eds.), Plant Gene Systems and their Biology, Alan R. Liss, New York, 62, pp. 329–331.

Odell, J.T., Knowlton, S., Lin, W. and Mauvais, C.J. (1988) Properties of an isolated transcription stimulating sequence derived from the cauliflower mosaic virus 35S promoter. Plant Mol. Biol. 10, 263–273.

On-Lee, T.M., Turgeon, R. and Wu, R. (1986) Expression of a foreign gene linked to either a plant-virus or a *Drosophila* promoter, after electroporation of protoplast of rice, wheat and sorghum. Proc. Natl. Acad. Sci. USA 83, 6815–6819.

Ow, D.W., Jacobs, J.D. and Howell, S.H. (1987) Functional regions of the cauliflower mosaic virus 35S RNA promoter determined by the firefly luciferase gene as a reporter of promoter activity. Proc. Natl. Acad. Sci. USA 84, 4870–4874.

Paszkowski, J., Shillito, R.D., Saul, M., Mandak, V., Hohn, T., Hohn, B. and Potrykus, I. (1984) Direct gene transfer to plants. EMBO J. 3, 2717–2722.

Pietrzak, M., Shillito, R.D., Hohn, T. and Potrykus, I. (1986) Expression in plants of two bacterial antibiotic resistance genes after protoplast transformation with a new plant expression vector. Nucleic Acids Res. 14, 5857–5868.

Rogers, S.G., Fraley, R.T., Horsch, R.B., Levine, A.D., Flick, J.S., Brand, L.A., Fink, C.L., Mozer, T., O'Connell, K. and Sanders, P.R. (1985) Evidence for ribosome scanning during translation initiation of mRNAs in transformed plant cells. Plant Mol. Biol. Rep. 3, 111–116.

Sanders, P.R., Winter, J.A., Barnason, A.R., Rogers, S.G. and Fraley, R.T. (1987) Comparison of cauliflower mosaic virus 35S and nopaline synthase promoters in transgenic plants. Nucleic Acids Res. 15, 1543–1558.

Sleat, D.E., Gallie, D.R., Jefferson, R.A., Bevan, M.W., Turner, P.C. and Wilson, T.M.A. (1987) Characterisation of the 5′-leader sequence of tobacco mosaic virus RNA as a general enhancer of translation in vitro. Gene 60, 217–225.

Topfer, R., Matzeit, V., Gronenborn, B., Schell, J. and Steinbiss, H. (1987) A set of plant expression vectors for transcriptional and translational fusions. Nucleic Acids Res. 15, 5890.

Van den Elzen, P.J.M., Townsend, J., Lee, K.Y. and Bedbrook, J.R. (1985) A chimeric hygromycin resistance gene as a selectable marker in plant cells. Plant Mol. Biol. 5, 299–302.

Vieira, J. and Messing, J. (1987) Production of single-stranded plasmid DNA. Methods Enzymol. 153, 3–11.

Vieira, J. (1988) Ph.D. thesis, University of Minnesota U.S.A.

Waldron, C., Murphy, E.B., Roberts, J.L., Gustafson, G.D., Armour, S.L. and Malcolm, S.K. (1985) Resistance to hygromycin B: a new marker for plant transformation. Plant Mol. Biol. 5, 103–108.

Zoller, M.J. and Smith, M. (1984) Oligonucleotide-directed mutagenesis: a simple method using two oligonucleotide primers and a single-stranded DNA template. DNA 3, 479–488.