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Chimeric genes as dominant selectable markers in plant cells

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Opine synthases are enzymes produced in dicotyledonous plants as the result of a natural gene transfer phenomenon. Agrobacteria contain Ti plasmids that direct the transfer, stable integration and expression of a number of genes in plants, including the genes coding for octopine or nopaline synthase. This fact was used as the basis for the construction of a number of chimeric genes combining the 5′ upstream promoter sequences and most of the untranslated leader sequence of the nopaline synthase (*nos*) gene with the coding sequence of two bacterial genes: the aminoglycoside phosphotransferase (APH(3′)II) gene of Tn*5* and the methotrexate-insensitive dihydrofolate reductase (DHFR Mtx^R) of the R67 plasmid. The APH(3′)II enzyme inactivates a number of aminoglycoside antibiotics such as kanamycin, neomycin and G418. Kanamycin, G418 and methotrexate are very toxic to plants. The chimeric NOS-APH(3′)II gene, when transferred to tobacco cells using the Ti plasmid as a gene vector, was expressed and conferred resistance to kanamycin to the plant cells. Kanamycin-resistant tobacco cells were shown to contain a typical APH(3′)II phosphorylase activity. This chimeric gene can be used as a potent dominant selectable marker in plants. Similar results were also obtained with a NOS-DHFR Mtx^R gene. Our results demonstrate that foreign genes are not only transferred but are also functionally expressed when the appropriate constructions are made using promoters known to be active in plant cells.

Key words: selectable marker genes/Ti plasmid/plant-cell transformation/recombinant DNA/antibiotic resistance

Introduction

Selectable marker genes have been the key for the establishment of DNA-mediated transformation systems in bacteria, yeast, *Dictyostelium* and various animal systems. Such selectable marker genes can also be used to select for the introduction of other, unrelated DNA sequences. In such experiments the unrelated DNA sequences are either covalently linked to the marker gene, or both DNAs are introduced simultaneously by co-transformation (Wigler *et al.*, 1979; Mantei *et al.*, 1979).

Ideally, a selectable marker gene should be capable of expression in a variety of host cells; this expression should be easily distinguished from endogenous gene activities and should produce a selectable change in the phenotype of the normal host cells. Such genes have been called dominant-acting markers (Wigler *et al.*, 1980; Southern and Berg, 1982). With the exception of the tumor-controlling genes (T-region) of Ti plasmids (Martón *et al.*, 1979; Hernalsteens *et al.*, 1980), no good selectable marker genes have, as yet, been isolated or constructed for use with plant cells.

Bacteria contain a number of genes that could serve as dominant-acting selectable markers. For instance, the aminoglycoside phosphotransferases APH(3′)II and APH(3′)I carried by transposons Tn*5* and Tn*601* respectively (Davies and Smith, 1978) were shown to inactivate the related aminoglycoside antibiotics G418 (Jimenez and Davies, 1980), neomycin and kanamycin. Jimenez and Davies (1980) showed that the APH(3′)I gene of Tn*601* could be expressed in yeast, and was responsible for a selectable resistance to the G418 antibiotic. The APH(3′)II gene from Tn*5* was also shown to provide a selectable resistance in mammalian cells when expressed under control of the SV40 early promoter (Southern and Berg, 1982), and in *Dictyostelium* when expressed under control of the promoter of the *Dictyostelium* actin 8 gene (Hirth *et al.*, 1982).

Plant cells are sensitive to several aminoglycosides and the expression of an APH activity could, therefore, be expected to determine a resistant phenotype. Another example of a bacterial dominant selectable marker is the methotrexate-insensitive dihydrofolate reductase (DHFR Mtx^R) carried by transposon Tn*7* or by the R67 plasmid (Fling and Elwell, 1980). Methotrexate resistance, resulting from the expression of these genes under control of the early SV40 promoter, was used as a selectable marker in mammalian cells (O'Hare *et al.*, 1981). Plant cells are quite sensitive to low concentrations of methotrexate and the expression of the bacterial methotrexate-insensitive enzymes could be expected to provide a selectable resistance. Bacterial genes introduced into plant cells *via* simple insertion into Ti plasmid vectors were found to be expressed very poorly, or not at all (unpublished data). However, a vector for the expression of foreign genes in plants using a Ti-specific promoter has recently been constructed; chimeric genes consisting of the 5′ promoter sequences of the nopaline synthase gene (Depicker *et al.*, 1982), and the coding sequence of either octopine synthase (De Greve *et al.*, 1982) or the bacterial chloramphenicol acetyltransferase were shown to be expressed in plant cells (Herrera-Estrella *et al.*, 1983). Here, we report on the construction, transfer to plant cells, expression and use as selectable markers of chimeric genes coding for the Tn*5* aminoglycoside phosphotransferase and for the R67 methotrexate-insensitive dihydrofolate reductase (DHFR Mtx^R).

Results

Search for suitable selective markers

Plant cells are known to be sensitive to a number of antibiotics, such as kanamycin (Dix *et al.*, 1977; Maliga *et al.*, 1980), and to inhibitors of the activity of dihydrofolate reductase, such as methotrexate (Nielsen *et al.*, 1979). In order to test whether bacterial genes which confer resistance to these inhibitors can be used as selectable markers in *Nicotiana tabacum* we first determined the sensitivity of tobacco tumor cells to these drugs. The results of this experiment are sum-

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marized in Table I, and indicate that the growth of tobacco cells is completely inhibited in the presence of 16 mg/l of kanamycin or 1 mg/l of methotrexate; these antibiotics, therefore, can be used to test for the expression of chimeric genes containing the coding sequence of the aminoglycoside phosphotransferase (APH(3')II) and methotrexate-resistant dihydrofolate reductase (DHFR Mtx^R) bacterial genes.

Table I. Drug resistance of *N. tabacum* W38C58 tumor cells

Drug	Lowest concentration inducing partial inhibition (mg/l)	Concentration required for total inhibition (mg/l)
Kanamycin	2	16
Neomycin	30	250
G418	3	25
Methotrexate	0.01	0.5
Trimethoprim	8	16

Construction of chimeric genes consisting of the NOS promoter linked to the coding sequences of the Tn5 APH(3')II gene and to the R67 DHFR Mtx^R gene

A plasmid able to promote transcription of foreign genes in plant cells has been recently constructed. This vector (pLGV2381) contains the promoter and the 3' end of the nopaline synthase (*nos*) gene with a unique *Bam*HI site in the 5'-untranslated leader sequence (Figures 1 and 4).

We used this vector to determine whether the NOS promoter would allow the expression of the Tn*5* APH(3')II and of the R67 DHFR Mtx^R coding sequences, and whether the expression of these genes is sufficient to allow these chimeric

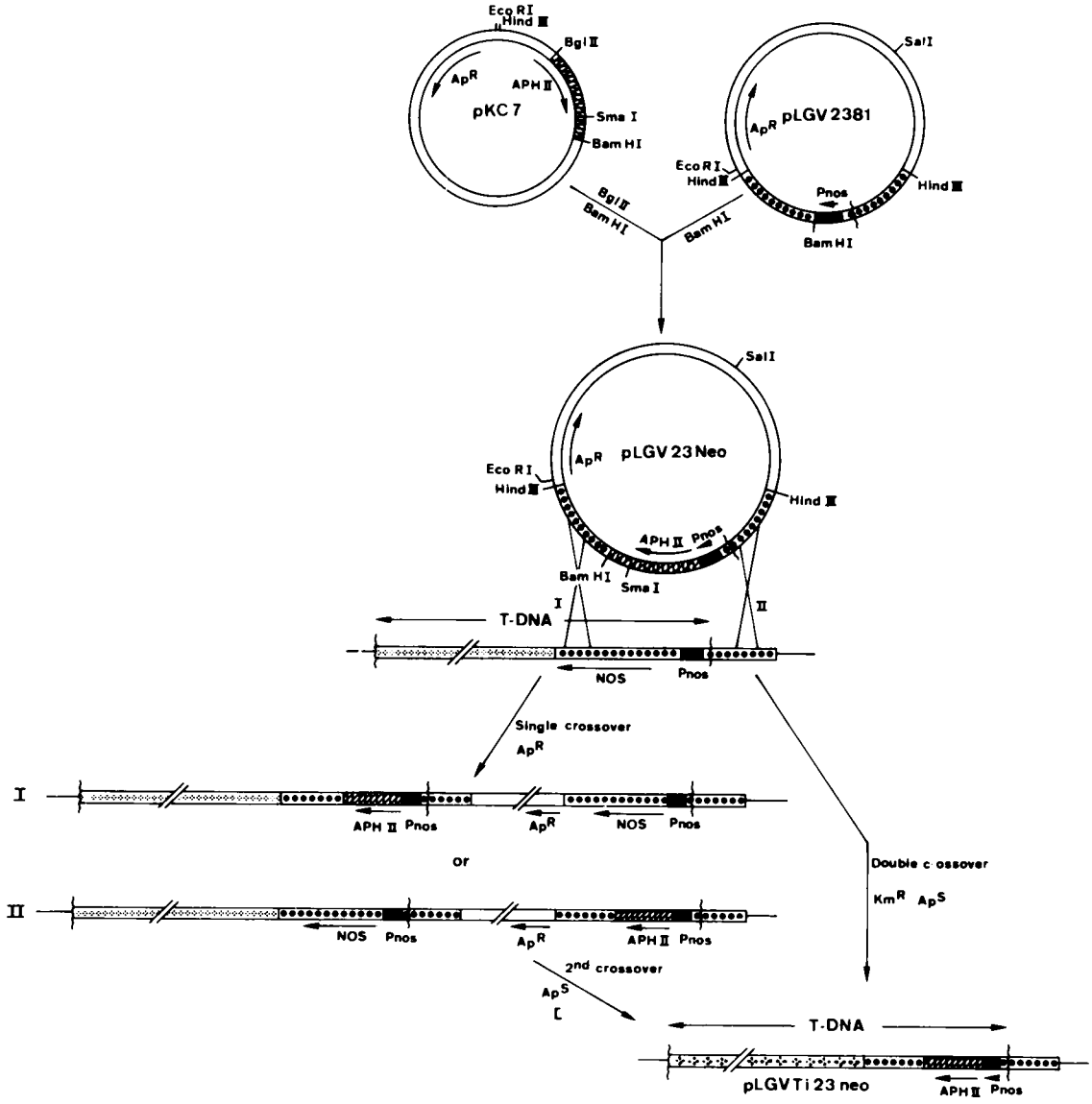

Fig. 1. Construction of the NOS-APH(3')II chimeric gene and its insertion in the T-region of pTiC58. The *Bam*HI-*Bgl*II fragment from pKC7 containing the coding sequence of the APH(3')II gene was cloned into the *Bam*HI site of pLGV2381. The resulting plasmid pLGV23neo was transferred to *Agrobacterium* and exconjugants containing co-integrates between pLGV23neo and pTiC58 were selected on ampicillin-containing medium. The two possibilities of co-integration depending on the region of homology where the cross-over occurred are shown in the Figure. Second recombinants were obtained by screening for the loss of the ampicillin marker. Second recombinants can also be directly obtained by searching for Km^R Ap^S exconjugants. This selection is possible because the NOS promoter allows a low expression of the APH(3')II gene in *Agrobacterium*. The following notations were used: (□) pBR322 sequences; (▨) pTiC58 fragment *Hind*III-23; (■) NOS promoter; (▩) T-region; and (⟨) border sequence.

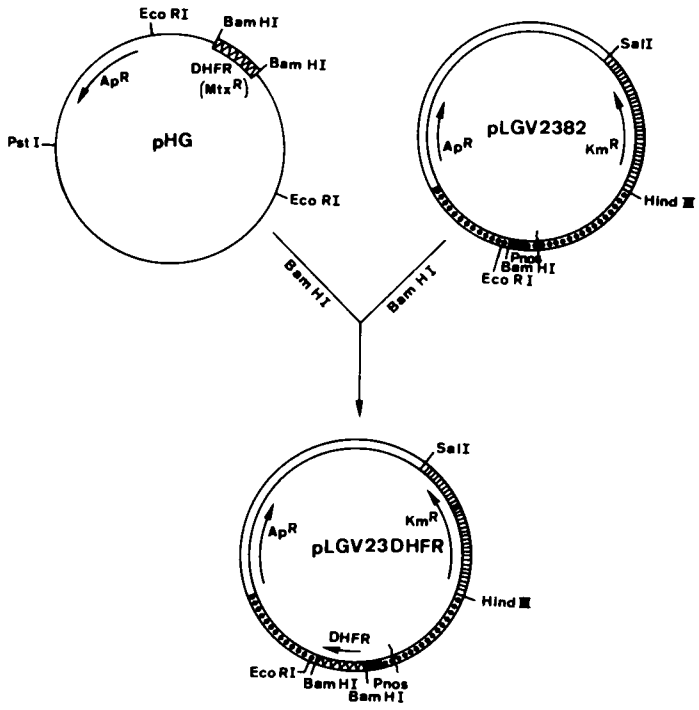

Fig. 2. Construction of a chimeric NOS-DHFR gene. A 370-bp *Bam*HI fragment from pHG (O'Hare *et al.*, 1981) containing the complete coding sequence of the DHFR from R67 was isolated from a 5% polyacrylamide gel and cloned into the *Bam*HI site of pLGV2382. pLGV2382 is a derivative of pLGV2381 (Herrera-Estrella *et al.*, 1983), the construction of which is explained in Figure 3.

genes to be used as dominant selectable marker genes in transformed plant cells growing in culture.

NOS-APH(3')II chimeric gene

The nucleotide sequence and position of the promoter of the Tn*5* APH(3')II gene has been determined (Rothstein *et al.*, 1980; Beck *et al.*, 1982). A *Bgl*II site is located in the 5'-untranslated leader sequence of the APH(3')II gene, 30 bp upstream of the putative initiation codon. This restriction site has been previously used in animal cells to express the APH(3')II gene under the control of the herpes simplex thymidine kinase promoter (Colbère-Garapin *et al.*, 1981). Thus, the 1.1-bp *Bgl*II-*Bam*HI fragment from Tn*5*, containing the complete coding sequence of the APH(3')II gene, was cloned into the *Bam*HI site of pLGV2381 (Figure 1). One of the plasmids resulting from this cloning (pLGV23neo) contained the *Bgl*II-*Bam*HI fragment inserted in the orientation that places the APH(3')II coding sequence under the control of the NOS promoter.

NOS-DHFR Mtx^R chimeric gene

A 370-bp *Bam*HI fragment from the vector pHG (O'Hare *et al.*, 1981) containing the complete coding sequence of the R67 DHFR gene was isolated and cloned into the *Bam*HI site of pLGV2382 (Figure 2). pLGV2382 is a derivative of pLGV2381, containing both an additional unique *Eco*RI cloning site adjacent to the NOS promoter and the APH(3')II gene as an additional marker, useful for genetic selection in *Agrobacterium*. The construction of pLGV2382 is described in Figure 3. One of the resulting plasmids pLGVDHFR (Figure 2) contained the DHFR gene properly oriented behind the NOS promoter.

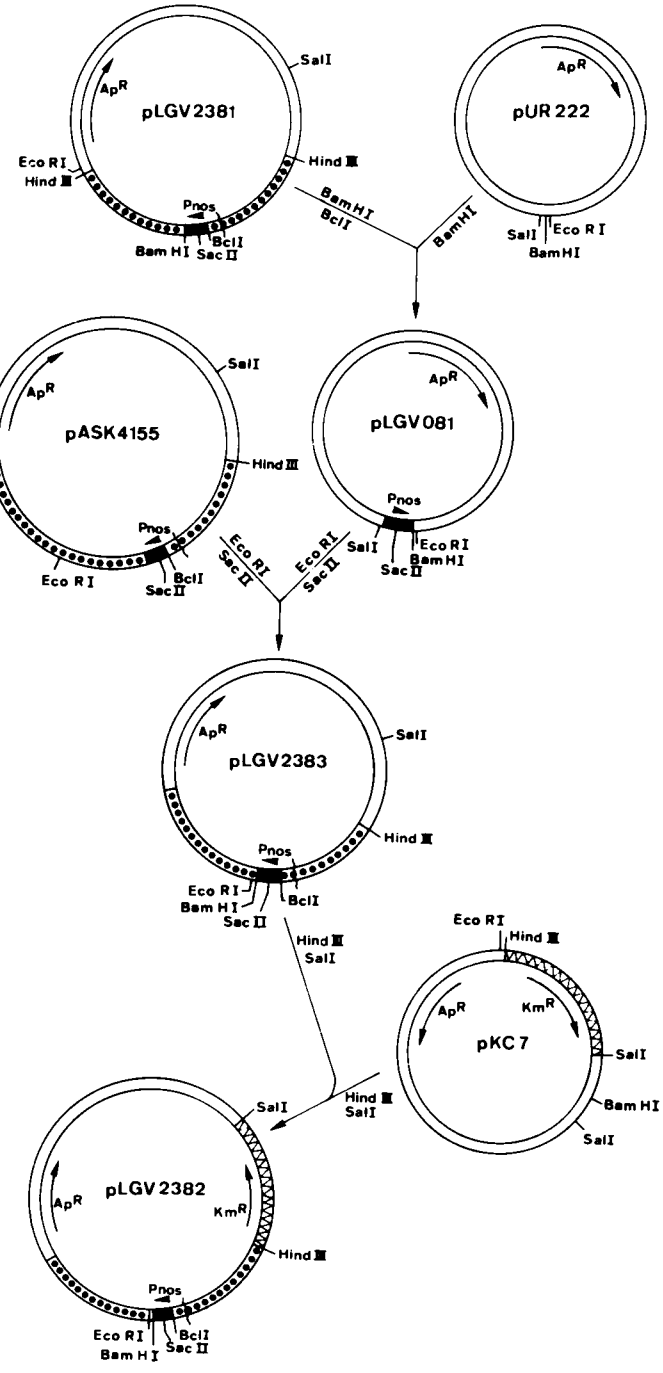

Fig. 3. Construction of pLGV2382. The *Bcl*I-*Bam*HI fragment of pLGV2381 containing the NOS promoter was subcloned in the *Bam*HI site of pUR222 (Ruther *et al.*, 1981). From the resulting plasmid (pLGV081) the small *Sac*II-*Eco*RI fragment was subcloned into pASK4155 (a derivative of pBR322 containing fragment *Hind*III-23 of the T-region of pTiC58, where the *Bam*HI site originally present in this fragment was exchanged for an *Eco*RI site; Shaw *et al.*, 1983) previously digested with the restriction endonucleases *Sac*II and *Eco*RI, resulting in pLGV2383. The *Hind*III-*Sal*I fragment carrying the APH(3')II gene from pKC7 was subcloned into pLGV2383 to produce pLGV2382. All notations are as in Figure 1.

Expression of the NOS-APH(3')II chimeric gene in Escherichia coli and A. tumefaciens

Expression of the nopaline synthase gene carried by Ti plasmids of *A. tumefaciens* has not been observed in its prokaryotic host. The structure of this gene (Depicker *et al.*,

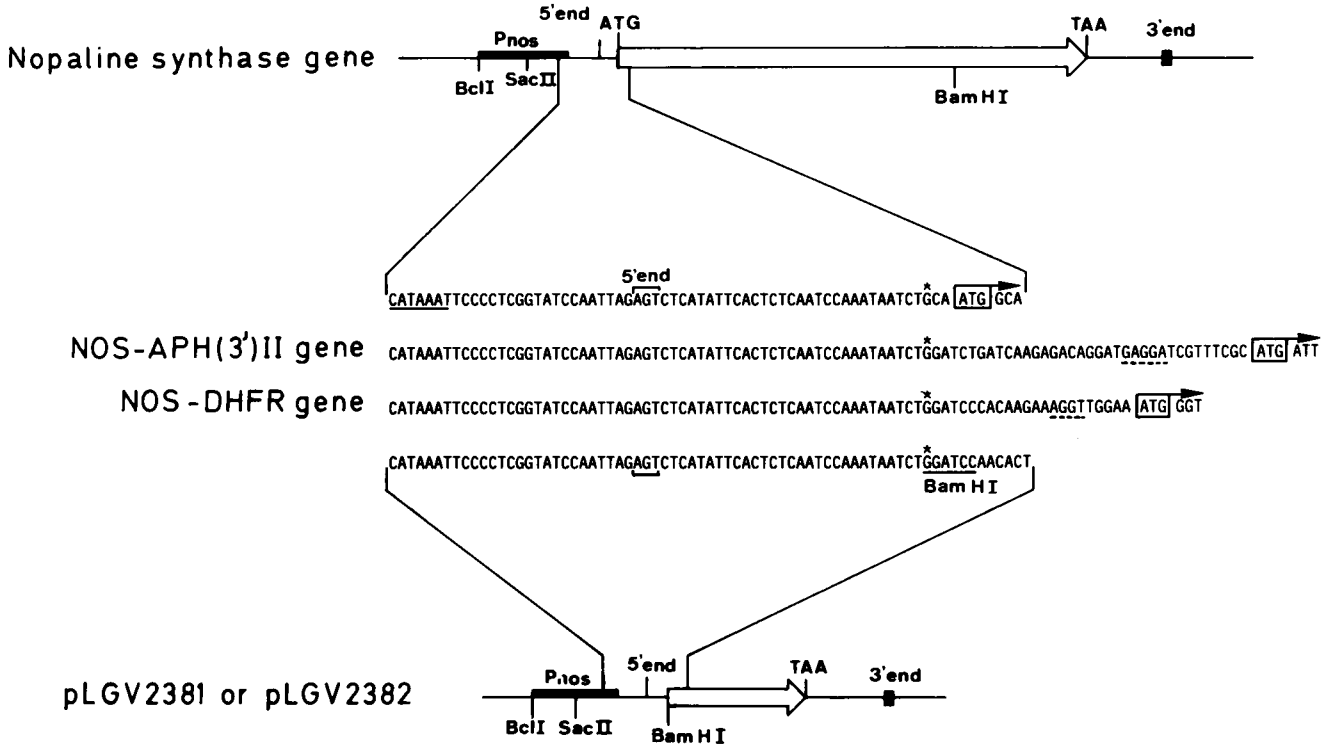

Fig. 4. Nucleotide sequence of the 5′ region at the fusion point of the NOS-APH(3′)II and NOS-DHFR chimeric genes. The sequences around the points of fusion between the NOS promoter and the APH(3′)II-DHFR Mtx^R coding sequences were deduced from published data (Depicker *et al.*, 1982; O'Hare *et al.*, 1981). The 5′-untranslated leader sequence of the *nos* gene and the endpoint of the deletion in the *nos* gene in pLGV2381 and pLGV2382 are also shown. The Shine-Dalgarno-like sequences present in the 5′-untranslated leader sequence of both the APH(3′)II and DHFR Mtx^R are underlined. The distance from the stop codon of the APH(3′)II gene to the *Bam*HI site of pLGV2381 is 737 bp, whereas the distance between this *Bam*HI site and the polyadenylation site in the 3′-untranslated region of the *nos* gene is 602 bp. Similarly, the distance between the stop codon of the DHFR Mtx^R gene and the *Bam*HI site in pLGV2382 is 179 bp. Therefore, the stop codon of APH(3′)II is located 1339 bp upstream of the 3′ end of the *nos* gene, and that of the DHFR Mtx^R 781 bp.

1982) allows two possible explanations for this observation: the 5′-upstream promoter sequence appears to be eukaryotic and may, therefore, not be functional in prokaryotes; secondly, it is conceivable that the promoter sequence is functional in prokaryotes but that the transcript cannot be translated because the untranslated leader sequence of the *nos* gene does not contain a Shine-Dalgarno-like sequence. As seen in Figure 4, the chimeric NOS-APH(3′)II gene contains the Shine-Dalgarno sequence of the APH(3′)II gene (Beck *et al.*, 1982). It was therefore possible to test whether the NOS promoter is functional in bacteria since it should produce a translatable mRNA from the chimeric NOS-APH(3′)II gene. *E. coli* with and without pLGV23neo was tested for kanamycin sensitivity. Whereas the strain without the chimeric NOS-APH(3′)II gene was completely inhibited by concentrations of 5 μg/ml, the pLGV23neo strain grew well at kanamycin concentrations of up to 15 μg/ml. Similarly, whereas the wild-type *A. tumefaciens* C58 strain was unable to grow in the presence of 5 μg/ml of kanamycin, the isogenic strain containing the NOS-APH(3′)II gene was able to grow well in the presence of up to 25 mg/ml of kanamycin. The NOS promoter is not as active as the Tn*5* promoter itself since *E. coli* or *Agrobacterium* carrying the complete Tn*5* are resistant to concentrations >50 μg/ml kanamycin (Rao and Rogers, 1979; Joos *et al.*, 1983). Nevertheless, these observations indicate that the NOS promoter can be functional in *E. coli* and *A. tumefaciens*. This conclusion was further supported by our observation that the chimeric NOS-DHFR Mtx^R gene in *E. coli* was able to confer resistance to up to 5 μg/ml of trimethoprim whereas *E. coli* without this gene was completely inhibited under these conditions. A putative Shine-Dalgarno-like sequence can also be seen in the untranslated leader sequence of this chimeric gene (Figure 4).

Transfer to Agrobacterium and recombination of the chimeric genes with the Ti plasmid

pLGV23neo and pLGV23DHFR were transferred to strain C58 containing the Ti plasmid pTiC58, by a method allowing the transfer of ColE1-like plasmids by direct mobilization (Van Haute *et al.*, 1983). As the origin of replication of these plasmids is not functional in *Agrobacterium*, all the exconjugants carrying the ampicillin-resistant (Ap^R) marker of pLGV23neo or the Ap^R and kanamycin-resistant (Km^R) markers of pLGV23DHFR, resulted from a co-integration event involving a cross-over through the homologous T-region DNA sequences in the acceptor Ti plasmid and in the pLGV2381-derived vectors. Recombinants between pLGV23neo and pTiC58 were isolated by selective plating on ampicillin-containing medium and were found at a frequency of 10^{-8}. Co-integrates between pLGV23DHFR and pTiC58 were isolated on kanamycin-containing plates and were found at a frequency of $10^{-6}-10^{-7}$. The lower frequency found for pLGV23neo::pTiC58 co-integrates is due to poor expression of the β-lactamase of pBR322 in *Agrobacterium*. This leads to a low recovery of exconjugants containing the Ap^R marker, since it is difficult to distinguish Ap^R exconjugants from the slow-growing background of *Agrobacterium* which express a low level of endogenous β-lactamase activity.

The structure of the two possible co-integrates between pLGV23neo and pTiC58 is shown in Figure 1. Second cross-over events leading to the exchange of the APH(3')II gene for most of the NOS-coding sequence, were obtained at a frequency of 10^{-4} by screening for the loss of the Ap^R marker. The structure of one of the resulting Ti plasmids (pLGV23neo) was confirmed by Southern blotting hybridization analysis (data not shown).

In view of the evidence that Ti plasmid co-integrates of type I (Figure 1) are able to both transfer and express chimeric genes in plant cells (Herrera-Estrella *et al.*, 1983), we decided to use a co-integrate of this type (pLGVTi23DHFR) to transfer the chimeric NOS-DHFR Mtx^R gene to tobacco protoplasts rather than searching for a double cross-over recombinant as we did in the case of the pLGV23neo gene. The demonstration that co-integrates derived from a single cross-over event can be used to transfer genes to plants, is a finding of practical importance. Second cross-over events are indeed difficult to isolate because of the low frequency with which they occur when the intermediate vector only has a small region of homology with the Ti plasmid (as was the case for pLGV2381 and pLGV2382).

Phenotypic expression of the chimeric genes

A modification of an *in vitro* cell transformation system (Martón *et al.*, 1979) based on the co-cultivation of regenerating plant cell protoplasts with *Agrobacterium*, was used to evaluate the usefulness of the chimeric drug resistance genes as dominant selectable markers. This system (see Materials and methods) has the advantage that hormone independence and drug resistance can be selected independently. This fact allowed us to test for the predicted linkage between the T-DNA-encoded phenotypes of hormone-independent growth and opine synthesis on the one hand, and the introduced drug resistance on the other hand.

In three independent experiments, using the oncogenic *Agrobacterium* strain C58C1Rif^R (pLGVTi23neo) harboring the chimeric APH(3')II gene in its T-DNA (Figure 1), a total of ~5000 tobacco calli grown in liquid medium supplemented with auxin and cytokinin, were screened for resistance to 75 mg/l of kanamycin. Four percent of these calli were resistant to kanamycin and were also shown to be able to grow autonomously in media without hormones (Table II). From the same three co-cultivation experiments, a total of ~1300 colonies were transferred to solid hormone-free growth medium, and 8% proved to be capable of hormone-independent growth. When subsequently tested for growth on solid media containing 100 mg/l of kanamycin, all hormone-independent calli were capable of further growth. To confirm that the kanamycin-resistant phenotype is linked to T-DNA-coded properties, eight randomly chosen kanamycin-resistant hormone-independent calli were tested for the presence of agrocinopine (Ellis and Murphy, 1981) as described in Materials and methods, and all were found to be positive. When the original co-cultivation procedure of Wullems *et al.* (1981) was used in a similar experiment, a lower frequency of kanamycin-resistant (0.5%) or hormone-independent (0.3%) clones was obtained. Notwithstanding these low transformation frequencies, the linkage between kanamycin resistance and hormone independence was again observed.

In two independent experiments, using the modified co-cultivation method with the *Agrobacterium* strain C58C1Rif^R (pLGVTiDHFR) harbouring the chimeric methotrexate-resistant dihydrofolate reductase gene in its T-DNA, a total of ~3000 calli grown in liquid hormone-containing medium were transferred to the same liquid medium, but containing 0.05 mg/l methotrexate. Two percent of these clones survived the methotrexate treatment, and 90% of these were shown to be capable of continued growth on 0.5 mg/l of methotrexate. All of these methotrexate-resistant clones were subsequently shown to be capable of growth on hormone-free medium. Twenty-five methotrexate-resistant hormone-independent calli were then screened for nopaline synthesis (Aerts *et al.*, 1979), 24 were found to be positive and one negative.

From the same co-cultivation experiment, another 1200 colonies were tested for hormone independence, and 7.8% such clones were obtained. All of these clones were subsequently shown to be capable of growth on hormone-free medium supplemented with 0.5 mg/l of methotrexate. Twenty-five hormone-independent and methotrexate-resistant clones were tested for nopaline synthesis (Aerts *et al.*, 1979), and 24 of these were positive.

The fact that two out of 50 hormone-independent, methotrexate-resistant calli were found not to synthesize nopaline was expected because, as seen in Figure 1, the co-integration of the vector containing the chimeric gene with the acceptor Ti plasmid produces a T-region with two right border sequences in tandem. Integration *via* the internal right border will produce cells not harbouring the *nos* gene.

Table II. Linkage of hormone independence and kanamycin resistance in calli transformed by *Agrobacterium* (pLGVTi23neo)

	No. colonies tested	No. of surviving colonies after 1st selection		No. of surviving colonies after 2nd selection		% of transformed calli	Linkage of hormone independence and Km^R
		Km^R	HI	Km^R	HI		
Exp. 1	2000	63	–	–	63	3.1%	100%
	500		29	29	–	5.8%	100%
Exp. 2	2000	91	–	–	91	4.5%	100%
	500	–	45	45	–	9.0%	100%
Exp. 3	1000	54	–	–	54	5.4%	100%
	300	–	30	30	–	10.0%	100%

Calli were tested independently for either kanamycin resistance or hormone independence 2–3 weeks after transformation (1st selection); a few weeks later, the colonies capable of growth in presence of the drug or in absence of hormones were tested for the presence of the unselected marker (2nd selection). Km^R, kanamycin resistance; HI, hormone independence.

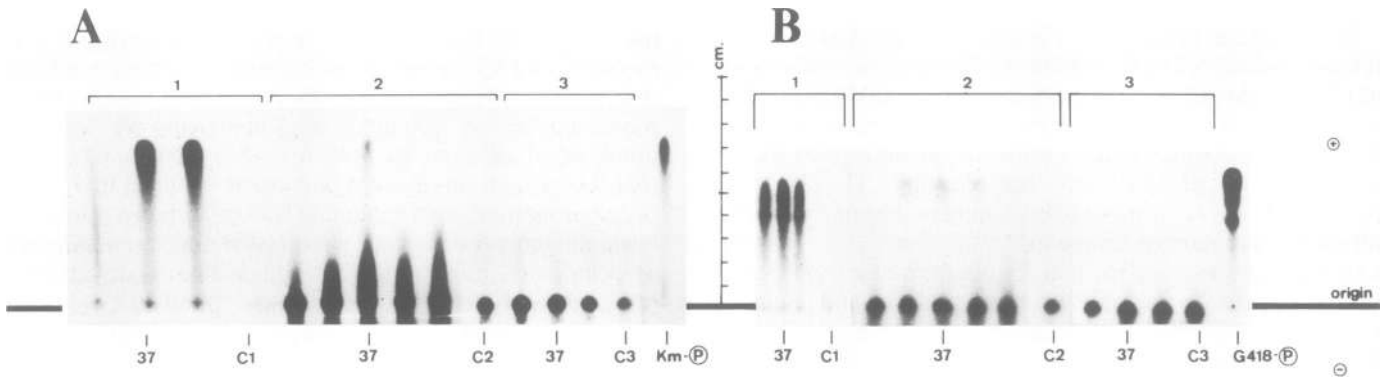

Fig. 5. APH(3′)II activity in Tn*5*- containing *E. coli* and crown gall tissue induced with *Agrobacterium* (pLGVTi23neo) after fractionation by gel filtration. The fractions obtained from high resolution gel filtration were assayed for APH(3′)II activity as described in Materials and methods. Fractions from extracts of (**1**) *E. coli* S605 (pGV0335::Tn*5*), (**2**) crown gall induced with *Agrobacterium* C58C1(pLGV23neo), or (**3**) crown gall induced with wild-type *Agrobacterium* C58C1(pTiC58) were incubated with either kanamycin (**panel A**) or G418 (**panel B**). The corresponding phosphorylated aminoglycosides were identified by paper chromatography. Lanes C1, C2, and C3 represent incubations of the fractions with maximum activity without the corresponding aminoglycoside. The retention time in minutes is indicated below the fraction containing the maximum APH(3′)II enzymatic activity. Km-P, purified kanamycin 3′-phosphate, and G418-P, purified G418 3′-phosphate.

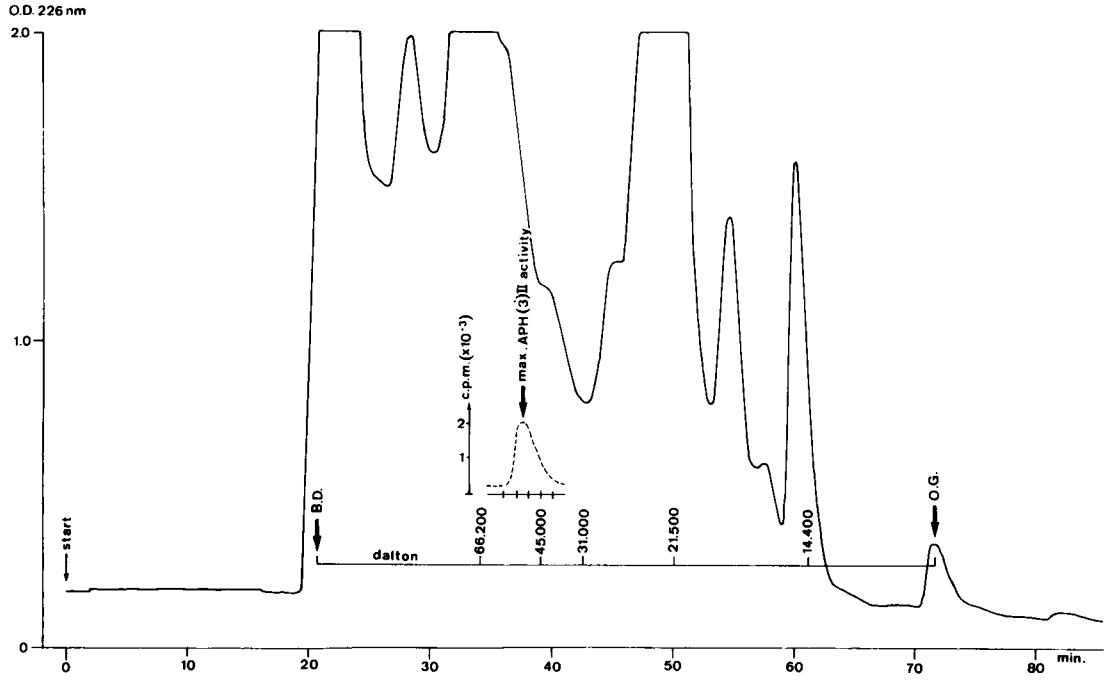

Fig. 6. Gel filtration pattern of the extract of crown gall tissue induced by pLGVTi23neo through a TSK-G3000 SW high resolution gel filtration column. 500 μl of the tumor extract was applied to the column and eluted with buffer B at a flow rate of 0.5 ml/min. Fractions of 0.5 ml were taken, and samples concentrated three times were assayed for APH(3′)II activity with an incubation mixture containing either kanamycin or G418 (at a final concentration of 100 μg/ml) (see Materials and methods). Peak fractions containing APH(3′)II activity eluted at the same retention time as APH(3′)II activity from *E. coli* S605 (pGV0335::Tn*5*) extracts prepared according to Neu and Heppel (1965). The fractions with a retention time of 37 min contained the highest activity. The column was calibrated with low mol. wt. protein standards from Bio-Rad (BSA 66 200; ovalbumin 45 000; carbonic anhydrase 31 000; soya bean trypsin inhibitor 21 500; lysozyme 14 400 daltons). (— — —) Relative c.p.m. ^{32}P incorporated into Km-P. Absorbance range: 2.0 OD at 226 nm.

Demonstration of the presence of the APH(3′)II enzymatic activities in transformed tobacco tissues

The APH(3′)II gene codes for an aminoglycoside phosphotransferase activity which phosphorylates, and thereby inactivates, antibiotics such as kanamycin and neomycin (Berg *et al.*, 1978), and also G418, which is a 2-deoxystreptamine aminoglycoside related to gentamicin (Jimenez and Davies, 1980). In order to test for the presence of APH(3′)II activity in tobacco crown gall tissue transformed by pLGVTi23neo, extracts from this tissue were prepared and the proteins were separated by high-resolution gel filtration on a LKB TSK-G3000 SW column; fractions were collected in a buffer allowing optimal measurement of APH(3′)II activity (Haas and Dowding, 1975). Enzyme activity was assayed by looking for phosphorylation of kanamycin (Figure 5A) or of G418 (Figure 5B).

As controls, *E. coli* extracts were prepared by osmotic shock as described by Neu and Heppel (1965) from a strain containing the Tn*5* transposon in a multicopy plasmid [*E. coli* S605 (pGV0354::Tn*5*)] and from a tobacco crown gall tissue induced by the wild-type *Agrobacterium* strain C58. Particular care was taken (see Materials and methods) to protect the APH(3′)II activity during extraction from endogenous plant proteases (Hershko and Ciechanover, 1982; Morejohn and Fosket, 1982).

Extracts of both the *E. coli* S605 (pGV0354::Tn*5*) and the tumor tissue induced with *Agrobacterium* containing pLGVTi23neo exhibited a kanamycin and G418 phosphotransferase activity, whereas no such activity was observed in similar fractions isolated from the control C58 tobacco crown gall tissues (Figure 5). The retention time in the gel filtration column, for the APH(3′)II activity from both the *E. coli* and tobacco extracts were found to be identical, corresponding to a protein mol. wt. of ~54 000 daltons (Figure 6). This mol. wt. is in agreement with data published by Colbère-Garapin *et al.* (1981) and Jimenez and Davies (1980); it may correspond to a dimer of the protein which has a real mol. wt. of 29 000 daltons as deduced from the nucleotide sequence (Beck *et al.*, 1982).

To confirm that the radioactive spots observed after incubation of kanamycin and G418 with [γ-^{32}P]ATP and the active fractions from the APH(3′)II-containing tobacco crown galls, were kanamycin 3′-phosphate (Km-P) and G418 3′-phosphate (G418-P), the corresponding spots were eluted and subjected to a second separation system on cation exchange thin-layer as described by Pauncz (1972) to differentiate them from other possible phosphorylated compounds. It was shown (data not presented) that these spots indeed correspond to Km-P and G418-P. The kanamycin-resistant tobacco crown galls containing the chimeric APH(3′)II gene have thus been shown to express this gene and to produce a functional enzyme.

Discussion

Dominant selectable markers have been an important key to the development of transformation methods in different types of organisms ranging from *E. coli* to animal cells. The Ti plasmid is a natural vector for the transfer of genes into plant cells. Dicotyledonous plants are transformed by *Agrobacterium* by the transfer, integration and expression of part of the Ti plasmid (T-DNA) in the plant genome. The hormone-independent growth of crown gall tumors as first described by Braun (1958) is a natural dominant selectable marker, and has been used successfully to develop transformation systems for plants (Martón *et al.*, 1979; Davey *et al.*, 1979; Krens *et al.*, 1982). This natural dominant selectable marker, however, interferes with plant morphogenesis and differentiation, and prevents the formation of whole plants from single cells or from callus tissue.

The T-DNA responsible for tumorous growth can be eliminated from Ti plasmids without interfering with the mechanism of T-DNA region transfer and integration (Leemans *et al.*, 1982; Joos *et al.*, 1983). Recently, it has been possible to construct Ti plasmid vectors lacking all T-DNA genes; such vectors have been shown to be efficiently transferred and the resulting transformed tobacco cells could be readily regenerated to form normal plants (Zambryski *et al.*, in preparation). The usefulness of such vectors could be enhanced if they were combined with dominant selectable marker genes.

The observation that chimeric genes, combining the 5′ promoter sequence of the nopaline synthase gene and the coding sequence of a bacterial chloramphenicol acetyltransferase, are expressed in tobacco and produce a functional enzyme (Herrera-Estrella *et al.*, 1983) prompted us to construct similar chimeric genes for the expression of the APH(3′)II gene from Tn*5* and of the methotrexate-insensitive DHFR gene from plasmid R67. These chimeric genes were inserted in the T-region of a wild-type Ti plasmid and introduced in independent tobacco clones by a modification of the method of Wullems *et al.* (1981) for the *in vitro* transformation of plant protoplasts by co-cultivation with *Agrobacterium* cultures. Using this method, it was possible to demonstrate that both chimeric genes are expressed in tobacco crown galls and that they can be used as dominant selectable marker genes. The chimeric NOS-APH(3′)II gene was found to confer the capacity to tobacco crown gall calli to grow on media containing 50 or 100 mg/l of kanamycin, whereas normal tobacco crown gall calli are completely inhibited by these concentrations of kanamycin.

Two independent lines of evidence support our conclusion that the kanamycin resistance phenotype is due to the expression of the chimeric NOS-APH(3′)II gene. (i) A very tight linkage was observed between the Km^R phenotype and the tumorous autonomous (hormone-independent) growth of the transformed calli on the one hand, and with the agrocinopine synthase activity on the other hand. These observations are as expected since the chimeric NOS-APH(3′)II gene was introduced in these calli *via* the T-region of a vector Ti plasmid which codes for tumorous growth and agrocinopine synthesis and they cannot be explained by assuming that the Km^R phenotype would be the consequence of a mutation. Mutations giving resistance to 50–100 mg/l of kanamycin were not observed in our control experiments. (ii) It was furthermore found that the kanamycin-resistant tobacco clones contained an aminoglycoside phosphotransferase activity able to phosphorylate both kanamycin and G418; this activity eluted from a high resolution gel filtration column with the same retention time as the one found for the enzyme in Tn*5*- containing *E. coli* strains. These results confirm that the kanamycin resistance observed is due to the functional expression of the NOS-APH(3′)II gene.

The origin of the genes encoded by the T-DNA of Ti plasmids and active in plant cells, is unknown. Recent evidence (Schröder *et al.*, 1983) suggests that at least some of these genes might also be expressed in *Agrobacterium*. However, expression of the nopaline synthase gene has not been observed in bacteria. The observation that both the chimeric NOS-APH(3′)II gene and the NOS-DHFR Mtx^R are expressed in *E. coli* or *A. tumefaciens*, suggests that the NOS promoter can be functional in prokaryotes.

The fact that no nopaline synthase activity has ever been observed in *Agrobacterium* is probably due to the fact that the 5′-untranslated leader sequence of this gene does not contain a Shine-Dalgarno sequence (Depicker *et al.*, 1982). These observations lend support to the idea that the T-DNA genes may be of prokaryotic origin but have evolved to be functional after transfer in plant cells.

These studies also provide information about the structure and nucleotide sequence requirements for the expression of foreign genes in plants. It is important to note that the absence of introns in chimeric genes combining the NOS promoter with coding sequences of various genes of bacterial origin (the chloramphenicol acetyltransferase of pBR325, the APH(3′)II of Tn*5* and the DHFR Mtx^R of R67) does not prevent the transcription and proper processing into functional mRNAs of these genes in plant cells. This is also the case for wild-type T-DNA genes such as octopine and nopaline synthase.

It is important to note that apparently one can generate functional 3′ ends in chimeric genes without having to make

very precise constructions. As indicated in the legend of Figure 4, the stop codons of the NOS-APH(3')II and of the NOS-DHFR Mtx^R genes are respectively located 1339 bp and 781 bp upstream of a 'natural' polyadenylation site. Notwithstanding this fact, both genes apparently produce functional mRNAs in plants. These results could be explained by the presence of alternative polyadenylation signals in these chimeric genes (Dhaese *et al.*, 1983).

The transfer of genes into various plants has been limited by the host range of oncogenic *Agrobacterium* strains. So far, there is no report of transfer of foreign DNA sequences to any monocotyledonous plant. It is expected that these, or similar, dominant selectable marker genes will open the way for the development of new transformation systems, and/or of gene vectors for both dicotyledonous and monocotyledonous plants, such as cereals.

Materials and methods

Bacterial strains

E. coli HB101 was used for all *in vitro* DNA transformation experiments. *Agrobacterium* C58C1Rif^R pTiC58 was used as a receptor for all bacterial conjugations.

Bacterial conjugation and transformation

Conjugations involving *E. coli* and *Agrobacterium* strains were done as described by Van Haute *et al.* (1983). Transformations of *E. coli* were as described by Maniatis *et al.* (1982).

Plasmids

pKC7 was described by Rao and Rogers (1979), pHG by O'Hare *et al.* (1981) and the detailed construction of pLGV2381 was previously reported (Herrera-Estrella *et al.*, 1983).

DNA manipulations

All restriction endonuclease digests were incubated in TA buffer (O'Farrell *et al.*, 1980). Plasmid vectors were dephosphorylated by incubation with 1 unit/μg calf intestine phosphatase in TA buffer during the restriction digest. Other methods were as described by Maniatis *et al.* (1982).

Plasmid DNA preparation

Plasmid DNA was isolated from overnight cultures grown in Luria broth (LB), using the rapid alkaline procedure of Birnboim and Doly (1979) for small scale preparation. For large scale DNA plasmid preparations the same method was used except that the proportions were modified as follows: 500 ml cultures were grown for 20 h in LB with vigorous agitation; cells were resuspended in 10 ml of solution I (without lysozyme), 20 ml of solution II were immediately added to lyse the cells and 17.5 ml of solution III were added to remove the cell debris and chromosomal DNA. After 30 min on ice, DNA was precipitated by adding two volumes of ethanol, and further purified by a CsCl-EtBr isopicnic gradient.

Nopaline and agrocinopine detection

The presence or synthesis of nopaline in crown gall tumors was detected as described (Aerts *et al.*, 1979).

The assay system for agrocinopine detection will be published later (Messens *et al.*, in preparation). Briefly, 30 mg of tumor tissue were incubated in Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) containing $H_3{}^{32}PO_4$ for 5 days. Tumor extracts were subjected to electrophoretic separation and radioactive agrocinopine was identified by autoradiography.

Suspension culture and plating of N. tabacum crown gall cells

A W38C58 tissue culture was obtained from a tumor induced by *A. tumefaciens* C58 on *N. tabacum* cv. Wisconsin 38 as described previously (Leemans *et al.*, 1981). Cell suspensions were cultured in hormone-free LS medium (Linsmaier and Skoog, 1965) in 250 ml Erlenmeyer flasks on a 90 r.p.m. gyrotary shaker, and diluted 5-fold each week. The mixture of single cells and small cell aggregates used for plating was obtained by filtration of a 7-day-old suspension through a sieve of ~1.5 mm. A sample (0.6 ml) of the resulting suspension was evenly spread on top of 10 ml of hormone-free LS medium containing the filter-sterilized drug, and solidified with 0.6% agar, in 5 cm Petri dishes. The plates were sealed with parafilm, and incubated for 1 month in the light (2000 lux) at 24°C. The lowest concentration of different drugs inducing partial and total inhibition of growth were determined by plating on MS solid media containing different concentrations of each drug (Table I).

Modifications of the method for transformation of tobacco cells by co-cultivation with Agrobacterium

N. tabacum cv. Petit Havana line SR1 (Maliga *et al.*, 1973) was used in all experiments. In one experiment using strain C58C1Rif^R (pLGVTi23neo), the co-cultivation procedure was as described by Wullems *et al.* (1981). In all other experiments, a modification of this procedure was used, which gives a higher transformation frequency or an enrichment in favour of the transformed cells. Cellulase Onozuka R10 and macerozyme Onozuka R10 were used at a concentration of 0.5% and 0.2%, respectively. The protoplasts were plated at 10^5/ml in K3 +H (Wullems *et al.*, 1981), and infected after 4 days at a multiplicity of 5 x 10^2 bacteria/protoplast, by adding 50 μl of a bacterial culture grown to saturation in minimal A medium (Miller, 1972) to 5 ml of protoplasts (10^4 protoplasts/ml). After 3 days, the protoplasts were diluted to 5 x 10^3/ml in K3 +H medium containing 500 mg/l cefotaxime. Two to three weeks later the hormonal concentration is lowered by 50% by addition of hormone-free K3 medium. After 2 weeks, the calli are pelleted (5 min at 80 g), and resuspended in hormone-free K3 medium. Four weeks later the hormone-independent calli can be transferred onto hormone-free LS medium (Linsmaier and Skoog, 1965). When drug resistance is selected, a similar procedure is used but the hormone level in the medium is not lowered.

Selection for drug-resistant cell clones

For the selection of kanamycin resistance in liquid medium, kanamycin is added to a final concentration of 75 mg/l 2 weeks after the addition of cefotaxime. In solid medium, 100 mg/l of kanamycin is used for selection. The difference between the sensitive calli which are completely inhibited in their growth and the resistant calli which grow well is clearly visible after 4 weeks.

For the selection of methotrexate resistance in liquid medium, 0.05 mg/l of methotrexate is added 3 weeks after the addition of cefotaxime. Ten days later, a clear difference is visible between the resistant calli and the sensitive cell clones which are killed by the drug. The resistant calli are transferred onto hormone-free LS medium without methotrexate because methotrexate inhibits the growth of the small resistant calli. When the cell clones reach a size of ~3–5 mm, they can be cultured on medium containing 0.5 mg/l of methotrexate. This concentration of methotrexate is routinely used for the selection of methotrexate resistance in solid medium.

APH(3')II activity determination

2 g of tumor tissue were ground with a pestle in the presence of 700 μl of extraction buffer [40 mM EDTA, 150 mM NaCl, 100 NH_4Cl, 0.13 mg/ml leupeptine (Sigma), 0.3 mg/ml soya bean trypsin inhibitor (Sigma), 15 mM dithiothreitol (DTT), 9 mg/ml bovine serum albumin (BSA), 12 mM Tris-HCl, pH 7.5] and 100 μl of a preincubated extract of nopaline C58 crown gall tissue. This latter extract was prepared by incubating 1 g of ground crown gall tissue for 30 min at 4°C in 350 μl of buffer B [30 mM NaCl, 15 mM NH_4Cl, 0.2 mM DTT, 3 mM Tris-HCl (pH 7.5), 3 mM $MgCl_2$, 5 μM EDTA]. All manipulations were done at 4°C. The total mix was centrifuged for 5 min in an Eppendorf centrifuge, the supernatant filtered through a 0.2 μm Millipore filter, and 500 μl of the filtrate was injected into a high-resolution gel filtration column (LKB TSK-G3000 SW: 7.5 mm ID, 600 mm long; particle size 10 ± 2 μm). The sample was eluted with buffer B. The elution pattern was determined by the absorbance of the different fractions at 226 nm. In each filtration Blue dextran and Orange G were added for the visual detection of the start and end points of the elution of the samples. A standard protein mixture was used for the calibration of the column (see Figure 6). Each fraction (0.5 ml) was concentrated three times, and the APH(3')II activity was determined by incubating 3 μl of each concentrated fraction with 3 μl of the two times concentrated incubation mix [100 μCi [γ-^{32}P]ATP (~3000 Ci/mmol; Amersham, Belgium), 0.24 mM ATP, 24 mM $MgCl_2$, 0.4 mM DTT, 40 mM Tris-HCl, pH 7.5, and 200 μg/ml of Km, or G418, as indicated] for 30 min at 37°C. The reaction was stopped by adding 6 μl of phenol. To identify the presence of phosphorylated kanamycin in the reaction mix, the aqueous phase was spotted on 3MM Whatman paper and subjected to electrophoresis in a buffer containing 0.05 M pyridine acetate (pH 6.5) for 40 min at 2000 volts. The phosphorylated derivatives of kanamycin or G418 were identified by a 24–36 h autoradiography.

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References

Aerts,M., Jacobs,M., Hernalsteens,J.P., Van Montagu,M. and Schell,J. (1979) *Plant Sci. Lett.*, **17**, 43-50.

Beck,E., Ludwing,G., Averswald,E.A., Reiss,B. and Schaller,H. (1982) *Gene*, **19**, 329-336.

Berg,D., Jorgenson,R. and Davies,J. (1978) in Schlessinger,D. (ed.), *Microbiology*, ASM Publications, Washington, pp. 13-15.

Birnboim,H.C. and Doly,J. (1979) *Nucleic Acids Res.*, **7**, 1513-1523.

Braun,A.C. (1958) *Proc. Natl. Acad. Sci. USA*, **44**, 344-349.

Colbère-Garapin,F., Horodniceanu,F., Kourilsky,P. and Garapin,A.-C. (1981) *J. Mol. Biol.*, **150**, 1-14.

Davey,M.R., Cocking,E.C., Freeman,J., Pearce,N., Tudor,I., Hernalsteens, J.P., De Beuckeleer,M., Van Montagu,M. and Schell,J. (1979) in Ferenczy,L. and Farkas,G.L. (eds.), *Advances in Protoplast Research*, Pergamon Press, Oxford, pp. 425-430.

Davies,J. and Smith,D.I. (1978) *Annu. Rev. Microbiol.*, **32**, 469-518.

De Greve,H., Dhaese,P., Seurinck,J., Lemmers,M., Van Montagu,M. and Schell,J. (1982) *J. Mol. Appl. Genet.*, **1**, 499-512.

Depicker,A., Stachel,S., Dhaese,P., Zambryski,P. and Goodman,H.M. (1982) *J. Mol. Appl. Genet.*, **1**, 561-574.

Dhaese,P., De Greve,H., Gielen,J., Seurinck,J., Van Montagu,M. and Schell,J. (1983) *EMBO J.*, **2**, 419-426.

Dix,P.J,. Joó,F. and Maliga,P. (1977) *Mol. Gen. Genet.*, **157**, 285-290.

Fling,M.E. and Elwell,J. (1980) *J. Bacteriol.*, **141**, 779-785.

Ellis,J.G. and Murphy,P.J. (1981) *Mol. Gen. Genet.*, **181**, 36-43.

Haas,M.J. and Dowding,J.E. (1975) *Methods Enzymol.*, **43**, 611-628.

Hernalsteens,J.P., Van Vliet,F., De Beuckeleer,M., Depicker,A., Engler,G., Lemmers,M., Holsters,M., Van Montagu,M. and Schell,J. (1980) *Nature*, **287**, 654-656.

Herrera-Estrella,L., Depicker,A., Van Montagu,M. and Schell,J. (1983) *Nature*, in press.

Hershko,A. and Ciechanover,A. (1982) *Annu. Rev. Biochem.*, **51**, 335-364.

Hirth,K.P., Edwards,C.A. and Firtel,R.A. (1982) *Proc. Natl. Acad. Sci. USA*, **79**, 7356-7360.

Jimenez,A. and Davies,J. (1980) *Nature*, **287**, 869-871.

Joos,H., Inzé,D., Caplan,A., Sormann,M., Van Montagu,M. and Schell,J. (1983) *Cell*, **32**, 1057-1067.

Krens,F.A., Molendijk,L., Wullems,G.J. and Schilperoort,R.A. (1982) *Nature*, **296**, 72-74.

Leemans,J., Shaw,C., Deblaere,R., De Greve,H., Hernalsteens,J.P., Maes, M., Van Montagu,M. and Schell,J. (1981) *J. Mol. Appl. Genet.*, **1**, 149-164.

Leemans,J., Deblaere,R., Willmitzer,L., De Greve,H., Hernalsteens,J.P. Van Montagu,M. and Schell,J. (1982) *EMBO J.*, **1**, 147-152.

Linsmaier,E.M. and Skoog,F. (1965) *Physiol. Plant.*, **18**, 100-127.

Maliga,P., Sz.-Breznovits,A. and Martón,L. (1973) *Nature New Biol.*, **244**, 29-30.

Maliga,P., Xuan,L.T., Dix,P.J. and Cséptö,A. (1980) in Sala,F., Parisi,B., Cella,R. and Ciferri,O. (eds.), *Plant Cell Cultures: Results and Perspectives*, Elsevier, Amsterdam, pp. 161-171.

Maniatis,T., Fritsch,E.F. and Sambrook,J. (1982) *Molecular Cloning*, published by Cold Spring Harbor Laboratory Press, NY.

Mantei,N., Boll,W. and Weissmann,C. (1979) *Nature*, **281**, 40-46.

Martón,L., Wullems,G.J., Molendijk,L. and Schilperoort,R.A. (1979) *Nature*, **277**, 129-130.

Miller,J.H. (1972) *Experiments in Molecular Genetics*, published by Cold Spring Harbor Laboratory Press, NY.

Morejohn,L.C. and Fosket,D.E. (1982) *Nature*, **297**, 426-428.

Murashige,T. and Skoog,F. (1962) *Physiol. Plant.*, **15**, 473-497.

Neu,H.C. and Heppel,L.A. (1965) *J. Biol. Chem.*, **240**, 3685-3695.

Nielsen,E., Rollo,F., Parisi,B., Cella,R. and Sala,F. (1979) *Plant Sci. Lett.*, **15**, 113-115.

O'Farrell,P.H., Kutter,E. and Nakanishi,M. (1980) *Mol. Gen. Genet.*, **179**, 421-435.

O'Hare,K., Benoist,C. and Breathnach,L. (1981) *Proc. Natl. Acad. Sci. USA*, **78**, 1527-1531.

Pauncz,J.K. (1972) *J. Antibiot.*, **15**, 677-678.

Rao,R.N. and Rogers,S.G. (1979) *Gene*, **7**, 79-82.

Rothstein,S.J., Jorgensen,R.A., Yin,J.C.-P., Yong-Di,Z., Johnson,R.C. and Reznikoff,W.S. (1980) *Cold Spring Harbor Symp. Quant. Biol.*, **45**, 99-106.

Ruther,U., Koenen,M., Otto,K. and Muller-Hill,B. (1981) *Nucleic Acids Res.*, **9**, 4087-4098.

Schröder,G., Klipp,W., Hillebrand,A., Ehring,R., Koncz,C. and Schröder,J. (1983) *EMBO J.*, **2**, 403-409.

Shaw,C.H., Leemans,J,. Shaw,C.H., Van Montagu,M. and Schell,J. (1983) *Gene*, in press.

Southern,P.J. and Berg,P. (1982) *J. Mol. Appl. Genet.*, **1**, 327-341.

Van Haute,E., Joos,H., Maes,M., Warren,G., Van Montagu,M. and Schell, J. (1983) *EMBO J.*, **2**, 411-418.

Wigler,M., Sweet,R., Sim,G.K., Wold,B., Pellicer,A., Lacy,E., Maniatis,T., Silverstein,S. and Axel,R. (1979) *Cell*, **16**, 777-785.

Wigler,M., Perucho,M., Kurtz,D., Dana,S., Pellicer,A., Axel,R. and Silverstein,S. (1980) *Proc. Natl. Acad. Sci. USA*, **77**, 3567-3570.

Wullems,G.J., Molendijk,L., Ooms,G. and Schilperoort,R.A. (1981) *Proc. Natl. Acad. Sci. USA*, **78**, 4344-4348.