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Selection-expression plasmid vectors for use in genetic transformation of higher plants

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ABSTRACT

Plasmid vectors containing both a selectable marker for plant transformation (kanamycin resistance) and a second, directly adjacent, divergent promoter for the transcription of inserted DNA fragments have been constucted. These vectors make use of a small (479 bp) dual-promoter DNA fragment, originally isolated from the T-DNA of Agrobacterium tumefaciens, fused to the neomycin phosphotransferase gene of Tn5. Several unique restriction enzyme cleavage sites, as well as a polyadenylation signal sequence, have been introduced downstream of the open promoter, allowing simple insertional cloning of DNA fragments to be expressed in plants. To test the vectors, the coding region for the chloramphenicol acetyltransferase gene (CAT) from Tn9 was inserted, and the resulting plasmids introduced into tobacco cells. Transformed calli, selected only for Km resistance, contained, in every case tested, both NPTII and CAT activities.

INTRODUCTION

The development and analysis of genetic transformation systems for plants has, until recently, been hindered by the lack of any simple and effective dominant genetic markers which allow the selection of transformed cells without disrupting their normal development. Recently, chimaeric genes, fusing transcriptional control signals from either the T-DNA of the Ti plasmid (1,2,3,4,5,6) or viral genes (7) to the coding region of the neomycin phosphotransferase II (NPTII) gene from Tn5, have been constructed. The chimaeric NPTII genes have been shown to provide high levels of resistance to the aminoglycoside antibiotics, kanamycin (Km) and G418 when introduced into cells from several different plant species. Use of these and similar hybrid genes has greatly facilitated both the genetic analysis of transformed plants (8,9) and the development of alternative

systems for the introduction of foreign DNA into plant cells (7,10,11).

A further, and potentially very useful, application of the chimaeric dominant selectable genetic markers is their direct linkage to other, non-selectable, genes within a single vector and the subsequent use of the resistance phenotype to select for the introduction of the unselected genes into the target plant genome. Two chimaeric NPTII genes, recently constructed using a 479 bp dual plant-active transcriptional promoter fragment isolated from the T-DNA of an octopine type (pTiAch5) Ti plasmid (4) are excellent candidates for the construction of such direct selection plasmid vectors for use in plants. The dual promoter constructs, each containing two adjacent, bidirectional, transcriptional control regions, have the advantage, over other plant transformation vectors (5,6) of providing a second, unused, promoter for the transcription of inserted DNA. In a selection-expression vector system derived from the bidirectional T-DNA promoter fragment, the second promoter, as well as any inserted segment of DNA to be transcribed, would be genetically linked, as closely as possible, to the selectable marker gene (consisting of the NPTII gene fused to the opposite T-DNA promoter). Such an arrangement should increase the probability of simultaneous transfer, integration into the plant chromosome, and expression of both the selectable marker and inserted gene of interest.

In this report we describe the construction and testing of a family of selection-expression plasmid cloning vectors based on the dual T-DNA promoter fragment. These vectors are designed to be used for the introduction and transcription of foreign DNA in plants, using either Ti plasmid-*A. tumefaciens* derived systems (12), direct DNA transformation of plant protoplasts (7,10,11) or by electroporetic uptake of plasmid DNA by isolated protoplasts (personal communication; from the laboratory of A. Szalay and from H. Kohn).

MATERIALS AND METHODS

Bacterial strains and plant lines

Escherichia coli strains HB101, DH1 and JM83 (13) were used

for all DNA transformations and plasmid preparations. Conjugation between E. coli and A. tumefaciens used the intermediate E. coli strain, GJ23 (14). A. tumefaciens strain, C58C1rif (15), containing the pGV3850 Ti plasmid recombined with the indicated selection-expression plasmid, was used to transform Nicotiana tabacum cv. Petit Havana SR1 (16).

DNA preparation and manipulation

Techniques used for the preparation of plasmid DNA, small amounts for analysis (17) and large scale preparation (13), were as described previously. Enzymatic reactions conditions (including: restriction endonuclease digestions, T4 DNA ligase mediated reactions, and filling-in of restriction enyzme generated DNA ends using Klenow fragment of E. coli DNA polymerase I) were those described by the commercial source (BRL). Electrophoresis and purification of separated DNA fragments (18) and hybridization analysis of total A. tumefaciens DNA (19) have been previously described. Radioactive hybridiation probes were prepared using a nick-translation kit from Bethesda Research Laboratories.

Growth media and culture conditions

The bacterial culture media used was Lauria broth (20). Antibiotic concentrations used for selection were: (E. coli::A. tumefaciens) a) ampicillin (100 µg/ml::--), b) carbenicillin (--::100 µg/ml), c) kanamycin (25 µg/ml::25 µg/ml), d) streptomycin (25 µg/ml::150 µg/ml), e) spectinomycin (10 µg/ml::50 µg/ml), and f) rifampicin (--::100 µg/ml).

Screening for Beta-galactosidase activity was on LB agar containing 40 µg/ml X-gal and 160 µg/ml IPTG (21).

Plant media used were K3 (22) for liquid and MS for solid (23). Callus culture was maintained at 24° C at low light (aproximately 500 lux) on MS agar with the following hormone concentrations: 0.5 mg/l 2,4-Dichlorophenoxyacetic acid (Sigma), 2 mg/l Napthyl-1-acetic acid (Merck), 0.1 mg/l 6-Benzylaminopurine riboside (Serva) and 0.1 mg/l Kinetin (Serva). Bacteria were removed from protoplast cocultivations by the addition of Cefotaxim (Hoechst) to 500 µg/ml. Km resistant plant tissue was selected in K3 media containing 100 µg/ml Kanamycin sulfate (Sigma).

Transfer of chimaeric genes to plants

Plasmid constructions were transfered to tobacco protoplasts using the Agrobacterium-Ti plasmid system described previously (15) (see Figure 4). A modified cocultivation system (4) was used to transform isolated tobacco mesophyll protoplasts, which were then selected for Km resistance prior to analysis.

Enzymatic assays

NPTII. The in situ gel assay used for determining NPTII activity had been developed previously for use in bacterial systems (24). Crude extracts from transformed plant tissues were separated electrophoretically in a non-denaturing polyacrylamide gel system. Reaction substrates (Km Sulfate and gamma-^{32}P labeled ATP) were then introduced by diffusion and the resulting phosphorylated Km was bound to phosphocellulose paper, washed and autoradiographed. The phosphorylated Km spot coresponds to the location of the NPTII enzyme within the gel.

CAT. Enzymatic assays for CAT activity were done essentially as reported earlier (25). The concentrations of acetylCoA and leupeptine (Sigma) were doubled (to 1.0 mM each) and the reaction time reduced to 10 minutes at 37° C. The ^{14}C radiolabeled chloramphenicol (Cm), and its reaction products, 1-acetyl, 3-acetyl, and 1,3-acetyl Cm were then concentrated, separated by thin layer chromotography and an autoradiograph made. Tobacco tissue containing no chimaeric CAT gene was found to have a very weak backround activity converting Cm into 3-acetyl Cm. This activity was, however, less than 1% of the activity asociated with the active chimaeric CAT gene.

NOS. Detection of NOS activity within transformed plant tissue was as described previously (26). Crude extract from the individual calli were applied to Whatman 3MM filter paper, electrophoretically separated and stained. Tissues containing NOS enzyme were detected by the presence of a distinctive nopaline spot under long-wave ultraviolet light (366 nm).

RESULTS

Construction of the Selection-Expression Plasmids.

A 479 bp DNA fragment, previously isolated from the T-DNA of the Ti plasmid, had been shown to contain two, bidirectional,

transcriptional promoters active within transformed plant cells (4). The dual promoter fragment, originally part of the 1′ and 2′ genes from the T-DNA of pTiAch5 were used as the basis for the construction of the selection-expression plasmid vectors (see 27,28, and 29 for a description of the location and function of the 1′and 2′ T-DNA promoter regions contained within the dual promoter fragment).

Plasmid vectors containing unique cloning sites downstream of the open T-DNA promoter. Of the original dual promoter plasmids, only pAK1003, fusing the 1′ T-DNA promoter to the NPTII gene, contained a convenient unique restriction endonuclease cleavage site (EcoRI) downstream from the open, 2′, promoter (Figure 2). During the construction of a second plasmid, pAK2004, a DNA segment containing several convenient cloning sites was created adjacent to the 1′ promoter region (4). However, cut sites for most of these restriction enzymes are also present elsewhere within the pAK2004 plasmid, precluding simple insertional cloning of specific DNA segments downstream of the unused promoter. The interfering restriction sites were all contained within a single SalI fragment containing the bacterial kanamycin (Km) resistance marker (originally a 1.2 kb AvaII fragment of Tn903, (30)) required for selection of recombinant plasmids within A. tumefaciens (see Figure 4 for a brief description of the procedure used for the in vivo introduction of foreign DNA into the T-DNA of the Ti plasmid vector, pGV3850).

In order to remove the duplicate restriction sites within pAK2004, the 1.2 kb SalI fragment was replaced by a 2.4 kb BamHI-HindIII fragment (originally isolated from the bacterial transposon, Tn1831 (31)) containing a bacterial selectable genetic marker for Streptomycin and Spectinomycin resistance (St/Sp). For ease of manipulation, the actual replacement of the Tn903 fragment was done using the plasmid pLGV1103 (Herrera-Estrella et al., in preparation) and resulted in the St/Sp resistant construction, pNS1171 (for details see Figure 1). The 2′ promoter-NPTII chimaeric gene of pAK2004 was then reconstructed by replacing the nopaline synthase promoter of pNS1171 with the same bidirectional T-DNA promoter fragment originally used in the construction of pAK2004 (isolated from the

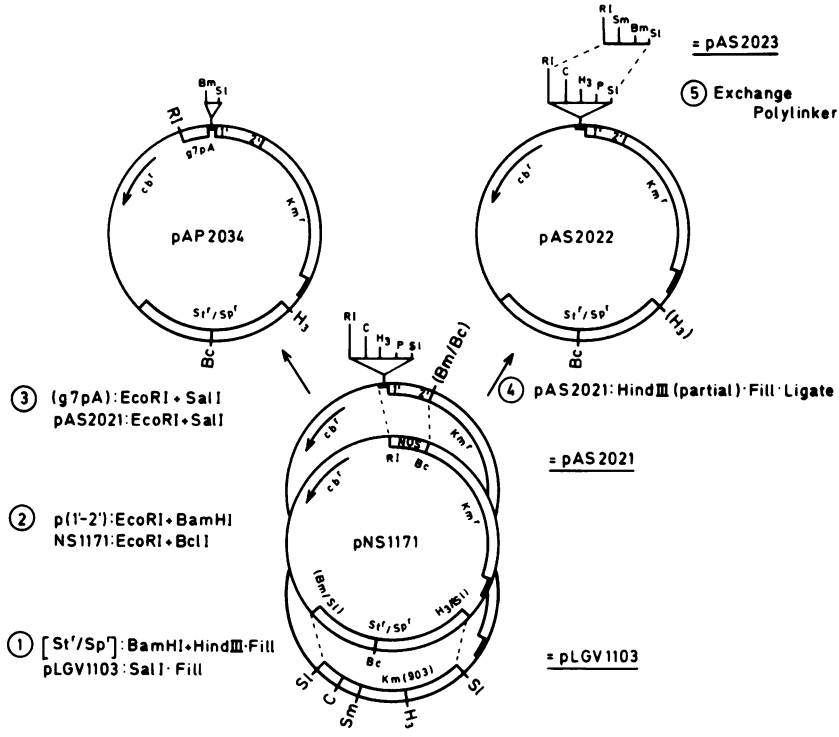
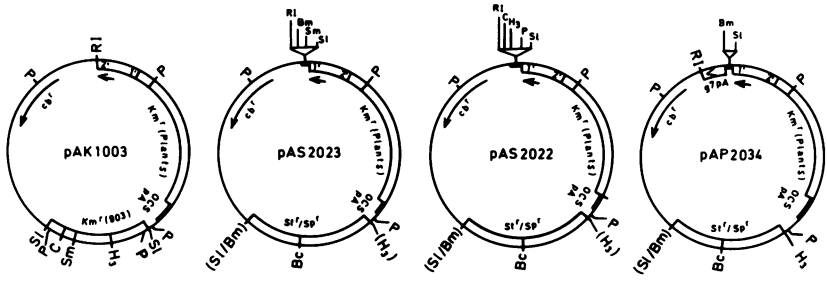


Figure 1. The various plasmid manipulations involved in the construction of the selection-expression vectors are outlined, proceding from bottom to top. STEP 1: The Tn903 derived Km resistance gene, contained within the indicated, 1.2 kb, SalI fragment, was replaced by a BamHI-HindIII 2.4 kb, St/Sp resistance fragment from Tn1831. The original SalI sites were destroyed during blunt-end ligation. STEP 2: The NOS promoter fragment of the resulting plasmid, pAS1171, was then replaced by the dual promoter EcoRI-BamHI fragment (from pOP44392, (4)). The resulting plasmid, pAS2021, was further modified in two ways; First (STEP 3), a polyadenylation start site from the 3' end of g7 was added downstream from the open 1' promoter, creating the plasmid pAP2034. Second (STEP 4), an extraneous HindIII cleavage site created during the insertion of the St/Sp fragment was removed by partial digestion with HindIII, followed by filling-in and blunt-end ligation. Finally (STEP 5), the polylinker segment (EcoRI-SalI) of the resulting plasmid, pAS2022, was replaced by a small DNA fragment containing cloning sites for BamHI and SmaI, creating the plasmid pAS2023. Restriction endonuclease cleavage sites are indicated by letter codes (A=ApaI, Bc=BclI, Bm=BamHI, C=ClaI, RI=EcoRI, H3=HindIII, P=PstI, Sl=SalI and Sm=SmaI). Sites destroyed during ligations are shown within parentheses. The selectable genetic markers are: Cb (carbenicillin resistance), Km (Km resistance for plant selection), Km(903) (Km resistance for bacterial selection) and St/Sp (streptomycin, spectinomycin resistance).



OPEN Promoter	2'	1'	1'	1'
Unique Cloning Sites	EcoRI	EcoRI, BamHI, SmaI, SalI	(EcoRI), ClaI, HindIII, SalI	BamHI, SalI
Polyadenylation	(-)	(-)	(-)	(+)
Plant Selectable marker	Km^r	Km^r	Km^r	Km^r
Bacterial Selectable marker	Km^r	St^r/Sp^r	St^r/Sp^r	St^r/Sp^r

Figure 2. Plasmid maps (not to scale) of the four selection-expression vectors are given, as well as a list of the pertinent characteristics of each individual plasmid. The direction of transcription of the open (unused) promoter is shown by a small arrow below the bidirectional promoter region. Unique cloning sites downstream of the open promoter are listed below and indicated on each plasmid map. The EcoRI site of pAS2022 is listed in parentheses due to the presence of a potential start codon (ATG) just upstream of the EcoRI site (see Figure 3). The structural components of the plasmids have been derived from several sources. The carbenicillin resistance and replication origin (counterclockwise from the top, EcoRI to SalI) are from pBR322. The Km(903) (SalI to SalI) or St/Sp (BamHI to HindIII) resistance markers are from Tn903 or Tn1831, respectively. The plant selectable marker (Km(Plants)) consists of a chimaeric gene containing a T-DNA promoter (4), the coding region from the NPTII gene of Tn5 (from pKm4 (42)) and the polyadenylation signal sequence (OCS pA) from the octopine synthase gene (a PvuII fragment, (43)). Finally, in the pAP2034 plasmid, the second polyadenylation signal sequence (the polarity of which is shown by the arrowhead) is from gene 7.

plasmid pOP44392 (4)). The resulting plasmid, pAS2021, is identical to pAK2004 except for the substitution of the St/Sp marker for the Tn903 Km resistance fragment and the production of an additional HindIII cleavage site at the junction of the St/Sp fragment and the 3' end of the NPTII chimaeric gene (Figure 1). This second HindIII site was subsequently destroyed by partial digestion of the pAS2021 plasmid with HindIII, followed by filling-in of the staggered ends and blunt end ligation.

The resulting plasmid, pAS2022 (see Figures 2 and 3),

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pAK1003
  TR 2'  --> | EcoRI
CGATACACCAAATCGAATTCTTGAAGACG

pAS2022
  TR 1' --> |          SalI  PstI   HindIII ClaI                    EcoRI
CTTCCACACGTGGATCGATCCGTCGACCTGCAGCCAAGCTTATCGATGATAAGCTGTCAAACatgAGAATTC

pAS2023
  TR 1' --> |          SalI BamHI SmaI EcoRI
CTTCCACACGTGGATCGATCCGTCGACGGATCCCCGGGAATTC

pAS2034
  TR 1' --> |          SalI  BamHI      | -->      g7 poly A signal sequence
CTTCCACACGTGGATCGATCCGTCGACGGATCCCCCGATGAGCTAAGCTAGCTATATCATCAATTTATGTATTACACAT

AATATCGCACTCAGTCTTTCATCTACGGCAATGTACCAGCTGATATAATCAGTTATTGAAATATTTCTGAATTTAAACT
                                  >> (polyadenylation)
TTGCATCaataaaTTATGTTTTTGCTTGGACTATAATACCTGACTTATTTTATCaataaaTATTTAAACTATATTTCTT
  --> |   EcoRI
TCAAGATGGGAATTC
```

Figure3. The predicted DNA sequence at the cloning sites and polyadenylation region of the various selection-expression vectors is given. In each case, the polarity of transcription from the T-DNA promoter is from left to right with the junction between the T-DNA, polylinker and polyadenylation sequence blocks indicated by vertical lines above the sequence. The EcoRI site marks the beginning of the pBR322 sequences within the vectors. The location of the proposed polyadenylation signal sequences (AATAAA) within pAP2034 and the potential start codon in pAS2022 are indicated by lower case letters. The start of polyadenylation within the g7 message is shown by two arrowheads above the sequence.

contains four unique cloning sites downstream of the open (1') promoter: SalI, HindIII (from pUC8 (21)), ClaI and EcoRI (from pBR322 (32)). A second selection-expression vector, pAS2023, was generated from pAS2022 by replacement of the DNA sequence between the EcoRI and SalI sites with a short DNA segment (from pUC8) containing cleavage sites for BamHI and SmaI (Figure 2 and 3). Of the two vectors, pAS2023 would be the vector of choice for cloning and expressing EcoRI fragments due to the presence, within pAS2022, of a potential start codon just upstream of the EcoRI site. Since, in eukaryotes, the initiatiation of translation appears to begin, predominantly, at the first AUG codon encountered downstream from the 5' end of a messenger RNA (33), the extra start codon in pAS2022 could serve to initiate translation prior to, and potentially out-of-frame from, the genuine start codon. Conversely, if a start codon were required

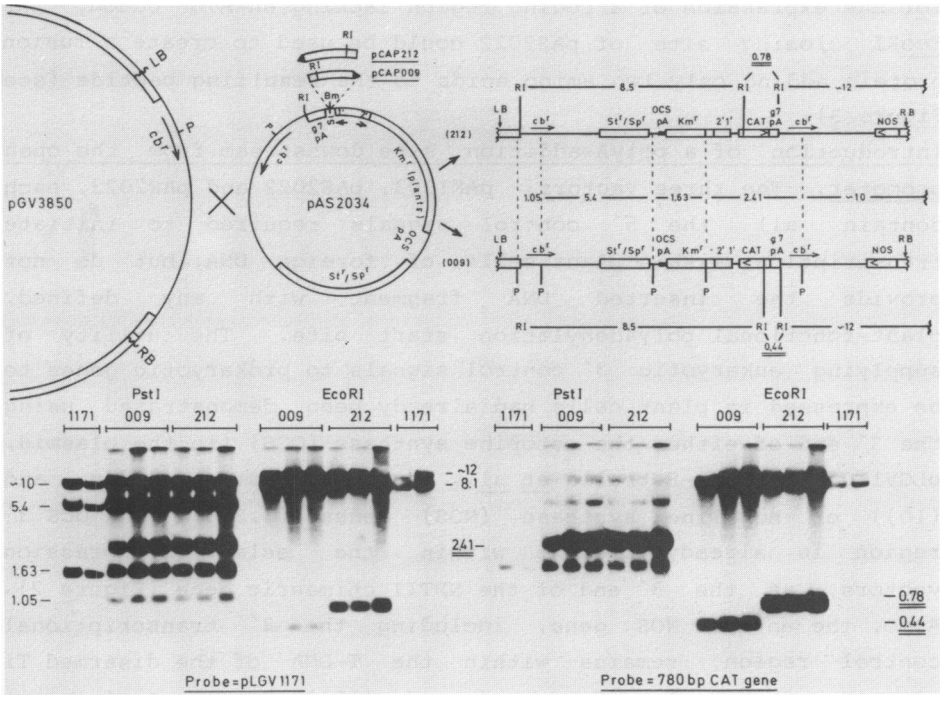

Figure 4. The two plasmids, pCAP009 and pCAP212, contain the coding region of the CAT gene inserted, either correctly (pCAP212) or incorectly (pCAP009) oriented, within the BamHI site of pAP2034. The linear restriction maps show the predicted structure of cointegrate plasmids formed by homologous recombination between pBR322 sequences, present both in the Ti plasmid vector, pGV3850, and in the CAP plasmids. The recombinant T-DNA contains: two copies of pBR322, the St/Sp selectable marker, both NPTII and CAT chimaeric genes, and the NOS gene. The structure of the cointegrates was confirmed by Southern hybridization analysis. For each construction (the pNS1171 cointegrate was included as size marker), total Agrobacterium DNA from three independent colonies was digested with either PstI or EcoRI, and electrophoretically separated. The resulting filters were then hybridized; first with labeled CAT coding region (autoradiograph, right) and subsequently with labeled total pCAP212 plasmid DNA (autoradiograph, left). The sizes (in kb) of the observed T-DNA fragments are indicated on the cointegrate map and beside the appropriate autoradiograph (the CAT 780 bp probe was slightly contaminated with part of the NPTII gene). The entire recombinant T-DNA, flanked by the left (LB) and right (RB) border regions, is eventially integrated into plant genome after transfer, via A. tumefaciens, to the plant cell.

for the expression of a coding region lacking an ATG codon, the EcoRI cloning site of pAS2022 could be used to create a fusion protein adding only two amino acids to the resulting peptide (see Figure 3).

Introduction of a polyA-addition site downstream from the open promoter. The three vectors; pAK1003, pAS2022 and pAS2023, each contain all the 5′ control signals required to initiate transcription, within plant cells, of foreign DNA but do not provide the inserted DNA fragment with any defined, plant-functional polyadenylation start site. The utility of supplying eukaryotic 3′ control signals to prokaryotic genes to be expressed in plant cells had already been demonstrated using the 3′ end of either the octopine synthase (OCS) (in the plasmid, pLGV1103, Herrera-Estrella et al., personal communication, and (10)) or nopaline synthase (NOS) genes (3,2,6). The OCS 3′ region is already present within the selection-expression vectors, at the 3′ end of the NPTII chimaeric gene (Figure 2). Also, the entire NOS gene, including the 3′ transcriptional control region, remains within the T-DNA of the disarmed Ti plasmid, pGV3850, (see Figure 4 and (15)) a potential plant transformation vector for introducing the selection-expression plasmids into plant cells. Thus, in order to avoid duplication of DNA sequences within the vector or the introduction of an additional segment of homology for potential recombination with the Ti-plasmid vector, pGV3850, a different polyadenylation start site was isolated for use in the selection-expression vectors.

A 212 bp, EcoRV to ClaI, DNA fragment (34) containing the 3′ untranslated region of gene 7 (g7) was isolated and introduced into the SmaI site of pUC8. The plasmid clone containing the correct orientation of the g7 3′ fragment, pUA110, was cut at the flanking EcoRI and SalI sites and inserted into the plasmid pAS2021 (Figure 1). The resulting vector, pAP2034, contains unique SalI and BamHI restriction sites between the open 1′ promoter and the g7 3′ untranslated region (Figure 3). The entire plant-functional segment of the pAP2034 vector; comprising the g7 3′ region, 1′-2′ promoters, NPTII coding sequence and OCS polyadenylation signal, is contained within a 2.5 kb EcoRI to HindIII fragment (Figure 2).

In vivo testing of the Selection-Expression Vector System.

The two promoters (1′ and 2′) of the isolated dual promoter fragment had been shown previously to be independently functional in transformed tobacco cells (4). Although both promoters would appear to function simultaneously within plant tissue transformed with wild-type T-DNA (27,28,29), no direct experimental evidence had yet proven that concurrent expression of two genes was possible using the isolated 1′-2′ promoter fragment. In order to test the function of the selection-expression vectors, as well as the utility of the g7 3′ control sequence, a DNA fragment, containing the coding region of the Tn9 gene for chloramphenicol resistance (chloramphenicol acetyltransferase, CAT) was inserted between the 1′ promoter and the g7 polyadenylation signal of the vector pAP2034 (Figure 4). The inserted fragment was derived from the pSV2-cats plasmid (35) and had been slightly modified to provide convenient restriction endonuclease cleavage sites within 20 bp of the 5′ end of the CAT coding region (L. Herrera-Estrella, personal communication and unpublished work from this laboratory).

The resulting plasmids, pCAP212 (oriented correctly, 5′-promoter and 3′-polyadenylation) and pCAP009 (opposite orientation), were recombined into the T-DNA of the Ti plasmid vector, pGV3850 (15), and the structure of the resulting cointegrate plasmids tested by hybridization analysis of Southern blots from total Agrobacterium DNA (Figure 4). Three independent transconjugant colonies from each construction were used to transfer the CAT constructions into tobacco protoplasts by a modified cocultivation technique (4).

Transformed tobacco protoplasts containing either orientation of the CAT gene were found to be resistant to 100 µg/ml kanamycin and were subsequently tested for both NPTII (Km resistance) and CAT activities (Figure 5). Pooled extracts (containing between 25 and 50 individual small calli) from tobacco cells transformed with either pCAP212 or pCAP009 showed high levels of NPTII activity consistent with their Km resistant phenotype. Assays for the CAT enzyme, made using similar pooled extracts from pCAP212 and pCAP009 transformed tissues, showed high levels of CAT activity within the 212 tissue but only

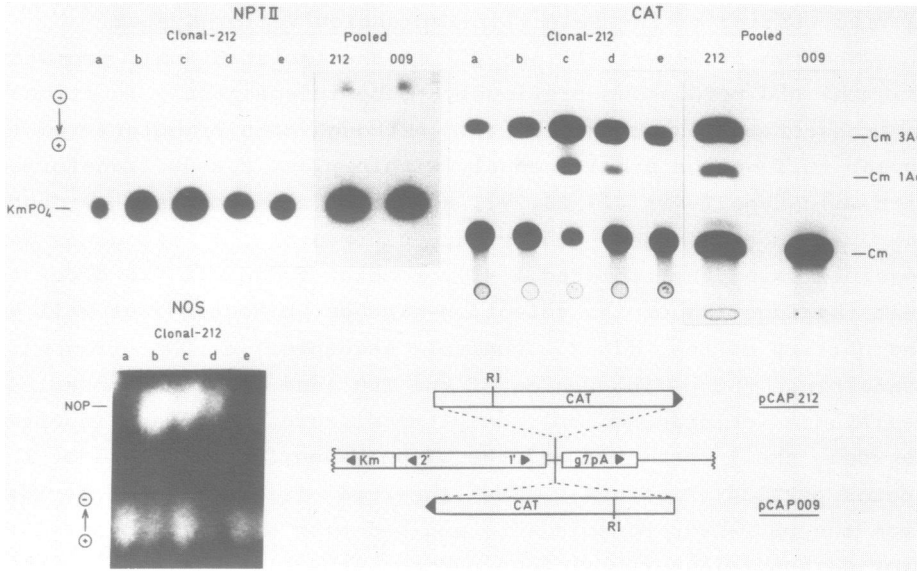

Figure 5. The orientation of the inserted CAT coding region in pCAP212 and pCAP009, is shown at the bottom right. Enzymatic assays for both chimaeric genes, NPTII and CAT, as well as the wildtype NOS gene (from pGV3850) were performed on either pooled or individual (a-e) tobacco calli. The pooled samples represent 25-50 separate calli. The spots on the NPTII autoradiograph (upper left) represent phosphorylated Km produced by the electrophoreticly separated NPTII enzyme. The location of the various acetylation products of Cm (at the 1 or 3 position), produced by the catalytic action of CAT, are indicated next to the CAT autoradiograph (upper right). The location of the nopaline spot and the direction of electrophoresis for the opine assay are indicated.

backround activity within the 009 derived callus (Figure 5).

Individual Km resistant calli from the pCAP212 cocultivation were separately propagated and tested for both NPTII and CAT activity, as well as for the presense of nopaline (resulting from expression of the NOS gene present within the pGV3850 T-DNA). These calli clones were assumed to represent independent transformation events since the observed frequency of transformation (5-10% of surviving protoplasts) suggests a low probability of multiple transformation. Of five calli tested, all contained high levels of both the NPTII and CAT enzymes. However, two of the five callus lines (a and e, Figure 5) showed

no measurable nopaline, indicating little, if any, NOS activity within these tissues. Subsequent testing of five additiional pCAP212 transformed calli gave similar results, with all five being positive for both NPTII and CAT activities but only four containing detectable quantities of nopaline (data not shown).

DISCUSSION

The 1′-2′ dual promoter region, recently isolated from the T-DNA of an octopine type Ti plasmid (4), has been used as the basis for the construction of a series of selection-expression plasmid vectors for the genetic transformation of plant cells. These vectors are unique in that they combine a dominant selectable marker for plants (Km resistance), with a second unused plant promoter available for the transcription of any DNA sequences inserted downstream from that promoter. The compactness and bidirectional arrangement of the two T-DNA promoters used within the vectors offers several advantages for the transcription of foreign DNA in plants. First, the foreign DNA sequences to be transcribed and the Km resistance selectable marker gene are directly linked, at their 5′ ends, within the vector plasmid (separated only by the 479 bp fragment containing the two T-DNA promoters). This arrangement should increase the probability of simultaneous transfer and integration of both DNA segments into the plant genome, irrespective of which genetic transformation system is used. Second, since both of the T-DNA promoters are contained within less than 479 bp, factors influencing the the general transcriptional state of the host genome at the site of insertion (including: DNA methylation, chromatin structure, histone content) would be likely to affect both transcriptional control regions similarly. Thus, expression of the resistance marker by the 2′ promoter, indicated by the Km resistance phenotype of the transformed plant tissue, would suggest a high probability of the concurrent transcription of the inserted DNA, initiated by the directly adjacent 1′ promoter region.

The results of the enzymatic analysis of tobacco cells transformed with the NPTII-CAT construction, pCAP212, support the idea of an enhanced genetic linkage of the Km resistance

phenotype with gene expression initiated by the second of the bidirectional T-DNA promoters. Testing of individual pCAP212 derived calli for NPTII, CAT, and NOS (from the NOS gene present within the pGV3850 Ti plasmid vector) activities, showed the expected high correlation (100%, from ten calli tested) of CAT enzyme activity (unselected) with NPTII activity (Km resistance) (Figure 5 and data not shown). Several of the calli tested, however, showed no NOS activity despite the relatively close physical linkage (promoter regions separated by 6-7 kb) of the NPTII and NOS genes, both of which are within the T-DNA borders of the Ti plasmid vector (Figure 4). It is currently not clear if the observed lack of NOS activity results from a failure to transfer, integrate or maintain the NOS gene or from some other factor(s) influencing the expression of the introduced genes. A more detailed examination of the relative expression levels and T-DNA structure of the inserted DNA is currently underway and will hopefully help to discern the basis of the observed lack of NOS activity as well as a noted variablity in the level of expression of the introduced foreign genes. In any case, the extremely close physical linkage, within the selection-expression plasmid, of the Km resistance marker and the DNA segment transcribed by the second of the dual T-DNA promoters would appear to increase their frequency of co-transfer and expression.

The presence of both NPTII and CAT activities within all pCAP212 derived calli tested also strongly suggests that both the 1′ and 2′ promoters of the selection-expression vectors function simultaneously within transformed tobacco cells. Both promoters have been shown to be independently active within tobacco cells (4) and it seems likely that promoter regions from the first two genes of a three step biosynthetic pathway would be able to function concurrently within the host cell (27, 36). An alternative explanation to simultaneous expression of both the CAT and NPTII genes within the transformed plant tissue, would be that each of several newly introduced T-DNA copies (each containing both the NPTII and CAT gene fusions) is expressing either one or the other of the two chimaeric genes. This possibility would appear unlikely, but is extremely difficult to conclusively disprove.

The plasmid vectors described in this report have been designed to provide unique cloning sites for many of the commonly used restriction endonucleases, directly downstream of the open T-DNA promoter (Figure 2). Additionally, in the case of the plasmid pAP2034, a DNA fragment containing the 3′ polyadenylation signal sequence from gene 7 of the octopine type T-DNA has been introduced 3′ from the promoter. Any DNA fragments inserted between the open promoter and the g7 3′ region would be provided with both transcriptional initiation and polyadenylation control signals. The vectors presented are especially suited for expressing prokaryotic genes and cloned cDNAs which would be expected to lack plant transcriptional control regions. A second, potentially exciting application of the vectors would be the transcription of either artificial or natural DNA fragments representing specific segments of particular genes or virus genomes. Such fragments could be oriented so as to produce RNA complementary to the normal messenger RNA and potentially interfer with normal gene expression or viral replication in plants. The practicality of such experiments has already been demonstrated in bacteria (37,38) and in mammalian cells (39).

In our experiments, the NPTII-CAT plasmids were introduced into plants using A. tumefaciens and the disarmed Ti plasmid vector, pGV3850 (15). To facilitate their use in this and similar recombinational plant transformation systems, a bacterial selectable marker, St/Sp resistance, suitable for use within A. tumefaciens, was included within the selection-expression vectors. Preliminary experiments (data not shown) indicate that it is also possible to transform the selection-expression plasmids (and their derivatives) directly into plant protoplasts using calcium-phosphate coprecipitation (7,10). A third plant genetic transformation technique, electroporetic introduction of DNA into plant protoplasts, should also be applicable (A. Szalay and H. Kohn, personal communication). Finally, as it appears that the T-DNA promoters function within at least some species of monocotyledonous plants (40,41,11,), it is possible that the utility of the expression-selection vectors will extend beyond the normal host range of Agrobacterium.

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