Gene, 40 (1985) 343–348
Elsevier

GENE 1482

Chimeric vector construction for higher-plant transformation

(Recombinant DNA; plant protoplasts; callus; kanamycin resistance; cauliflower mosaic virus promoter, Tn*5*; transposon)

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(Received May 25th, 1985)
(Revision received July 30th, 1985)
(Accepted September 1st, 1985)

SUMMARY

A chimeric vector pKR612B1 was developed containing the neomycin phosphotransferase (APH) gene from the Tn*5* transposon under the control of the gene VI promoter of cauliflower mosaic virus (CaMV), and was used to transform higher plant protoplasts. Plasmid pDOB612, the parental vector of pKR612B1, has two unique restriction sites, *Sma*I and *Bam*HI, positioned just downstream of the CaMV gene VI promoter sequence. These unique cloning sites can be used for any kind of gene insertion into this vector. Using the polyethylene glycol transformation procedure, a large number of turnip and tobacco protoplasts were transformed and proved to be resistant to kanamycin (Km). From tobacco protoplasts whole Km-resistant plants were regenerated and shown to contain the integrated foreign gene. APH activity was detected in both transformed calli and in regenerated plants. DNA from transformed clones was analysed by Southern blot hybridization, showing the presence of the Tn*5*-derived gene.

INTRODUCTION

Within the last few years plant viruses have drawn the attention of molecular biologists because of their potential use as vectors for gene transfer in higher plants. It has been shown by Brisson et al. (1984), that CaMV can be employed as such a vector by replacing the nonessential open reading frame II with the dihydrofolate reductase gene. The principal drawbacks of such an approach stem from (i) the restricted host range of the virus, (ii) the limited space available for foreign DNA due to packaging constraints, and (iii) the need to precisely insert the foreign DNA, eliminating essentially all 5′- and 3′-noncoding sequences. An approach circumventing these drawbacks is to integrate the foreign gene into the plant genome using CaMV DNA only as a source of transcription initiation and termination signals.

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Abbreviations: Ap, ampicillin; APH, [APH(3)II] neomycin phosphotransferase; CaMV, cauliflower mosaic virus; Δ, deletion; EtdBr, ethidium bromide; kb, kilobases or 1000 bp; Km, kanamycin; nt, nucleotide(s); ORF, open reading frame; PEG, polyethylene glycol; PolIk, Klenow (large) fragment of *E. coli* DNA polymerase I; ^{R}, resistant.

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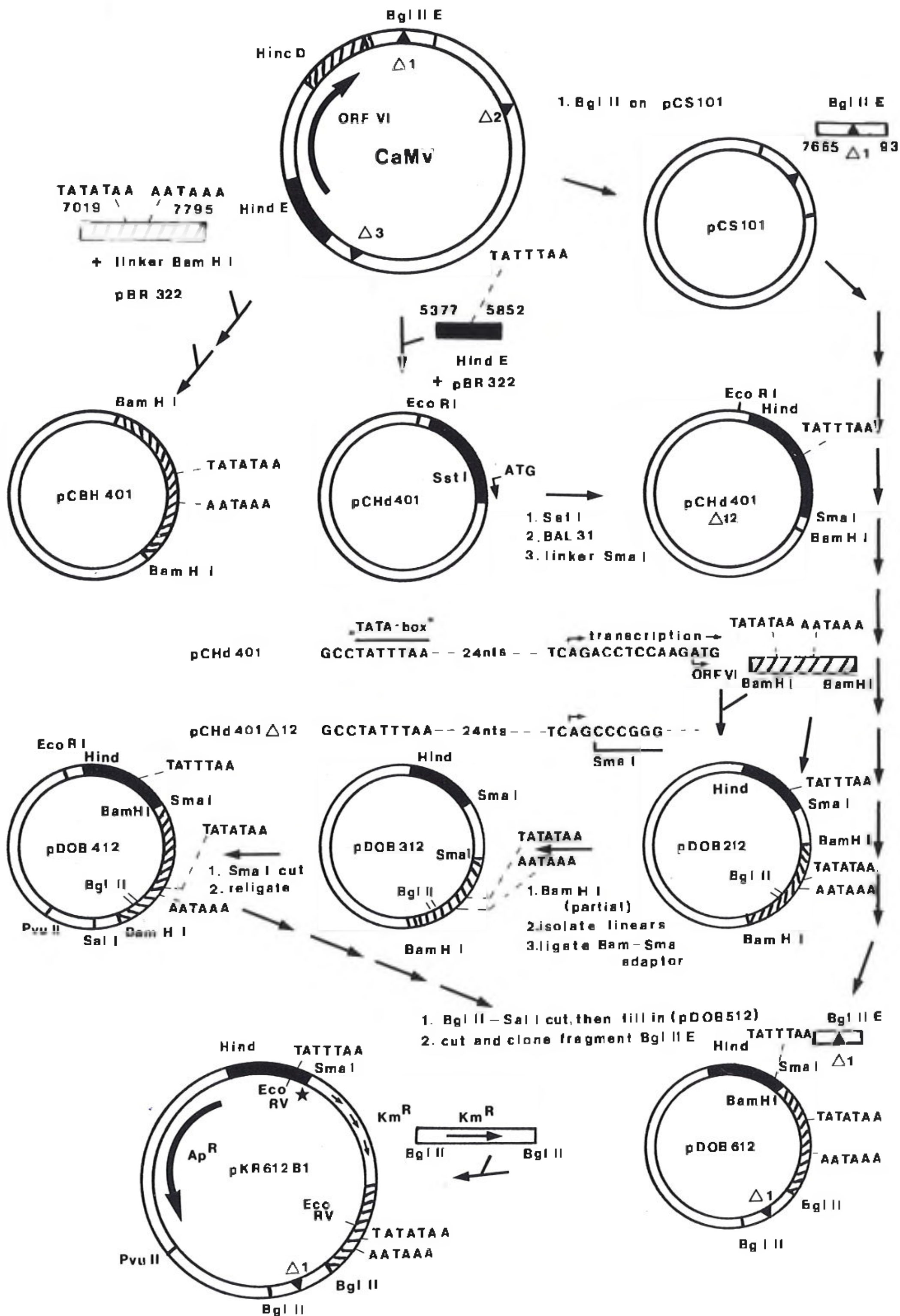

Fig. 1. Construction scheme for pKR612B1. Restriction enzymes were purchased from Amersham, Boehringer Mannheim, Genofit and Bethesda Research Laboratories. DNA polymerase, T4 DNA ligase and calf intestinal phosphatase were from Boehringer Mannheim, polynucleotide kinase from Amersham and BAL 31 nuclease from BRL. The *Bam*HI linker (dCGGATCCG), *Bgl*II linker (CAGATCTG) and *Bam*HI-*Sma*I adaptor (dGATCCCCCGGG) were purchased from New England Biolabs. To provide convenient starting material, three pieces of CaMV DNA were subcloned into pBR322. The *Hin*dIII-E fragment (nt 5376–5851; black boxes; all numbering refers

Recently, several groups have reported the successful use of this approach either by Ti-plasmid-mediated transfer of the foreign gene plus a CaMV promoter (Koziel et al., 1984; Odell et al., 1985) or by direct transformation of protoplasts with a vector containing a selectable marker under the control of CaMV transcription signals (Paszkowski et al., 1984; Potrykus et al., 1985b). We describe here a plasmid pKR612B1 containing the Tn*5* APH gene under the control of the CaMV gene VI promoter. The construct is similar to but more versatile than the vector pABD1 described earlier by Paszkowski et al. (1984). The plasmid has been successfully used to transform plant protoplasts. Regenerated plants have been shown to contain and express the foreign gene.

EXPERIMENTAL AND DISCUSSION

(a) Transformation of protoplasts

In preliminary experiments protoplasts of turnip (a host of CaMV) were transformed with pKR612B1, either in circular or linearized form using the PEG transformation procedure. We were able to regenerate microcalli from turnip protoplasts resistant to 40 μg G418/ml, a concentration lethal to calli regenerated from mock-inoculated protoplasts (not shown). Due to the slow rate of growth of turnip calli and the difficulty of regenerating this material into plants we decided to use pKR612B1 to transform tobacco protoplasts from which whole plants can be easily regenerated. Furthermore, there is now evidence (Paszkowski et al., 1984; Odell et al., 1985), that CaMV promoters function efficiently in the non-host tobacco. Tobacco mesophyll protoplasts of *Nicotiana tabacum* L.cv. Petit Havanna line SR1 (Maliga et al., 1973) were isolated from sterile shoot culture obtained from Dr. I. Potrykus by the method of Nagy and Maliga (1976). Protoplasts were transformed as described by Krens et al. (1982) with *Nru*I-digested pKR612B1 and microcalli resistant to 200 μg Km/ml were selected as described by Paszkowski et al. (1984). Cultures were grown in agarose beads as described by Shillito et al. (1983). With this procedure 1–3 colonies per 10^6 treated protoplasts were Km-resistant. Southern blot analy-

to the Cabb S sequence; Franck et al., 1980) containing the gene VI promoter and the beginning of the gene VI coding region was cloned into the *Hin*dIII site of pBR322 to give pCHd401. The *Hin*cII-D fragment (nt 7018–7794; hatched boxes) which contains the AATAAA polyadenylation signal, was ligated to *Bam*HI linkers and then cloned into the *Bam*HI site of pBR322 to give pCBH401. pCHd401 was digested with *Sst*I which cuts in the gene VI coding region and, after limited BAL 31 digestion, the plasmid was recircularized by ligation in the presence of *Sma*I linkers. After transformation, recombinant clones were screened for those containing a *Sma*I site and the size of the BAL 31 deletion in such plasmids was measured by appropriate restriction enzyme digestion and sequence analysis (Maxam and Gilbert, 1977). Clone pCHd401Δ12 in which the BAL 31 digestion has eliminated all but the first 2 nt of the gene VI transcription unit, was chosen for further use. pCHd401Δ12 was cut with *Bam*HI and the CaMV *Hin*cII-D fragment with *Bam*HI linkers at its extremities was inserted to give pDOB212. That the inserted fragment was in the desired orientation in this and other steps of the construction was verified by digestion of candidate plasmids with appropriate restriction enzymes. pDOB212 was subjected to partial digestion with *Bam*HI in the presence of EtBr and full-length linear molecules purified by agarose gel electrophoresis, were recircularized by ligation in the presence of a *Bam*HI-*Sma*I adaptor. After selection of a clone in which the new *Sma*I site was introduced next to the first *Bam*HI site (pDOB312) this plasmid was digested with *Sma*I and recircularized to give pDOB412. Plasmid pDOB412 was cut with *Bgl*II + *Sal*I, 5′ overhangs were filled-in with PolIk and the plasmid was recircularized to give pDOB512. This step eliminates the sequence between the first of the two closely spaced *Bgl*II sites in the cloned CaMV sequence (nt 7644 of CaMV) and the *Sal*I site of pBR322 while regenerating a *Bgl*II site. The *Bgl*II-E fragment of CaMV (nt 7664–92; boxed black triangle) was purified from a *Bgl*II digest of pCS101, a plasmid carrying the entire CaMV DNA sequence, and cloned into the *Bgl*II site of pDOB512 to give pDOB612. pDOB612 contains the gene VI promoter region (CaMV nt 5376–5765) positioned just upstream of unique *Sma*I site and *Bam*HI cloning sites. The cloning sites are followed by CaMV sequence (nt 7018–92) which contains the viral polyadenylation signal and presumably elements governing transcription termination. Note that the sequence between the two closely spaced *Bgl*II sites of CaMV (nt 7644–7664) has been eliminated in the construction. The Tn*5* APH gene (Km^R) carried on the plasmid pNEO (Pharmacia-PL Biochemicals) was inserted as a selectable marker behind the gene VI promoter in pDOB612. The DNA fragment encompassing the Km^R gene extended from the *Bgl*II site at nt −35 (Beck et al., 1982; numbering from the start of the coding region to the unique *Sma*I site at nt 969). For purposes of cloning the *Sma*I site was transformed into a *Bgl*II site by linearizing pNEO with *Sma*I and religating in the presence of *Bgl*II linkers. The resulting *Bgl*II fragment containing the Km^R sequence was purified by agarose gel electrophoresis and cloned into the *Bam*HI site of pDOB612. The final construction pKR612B1 with the Km^R gene in the same orientation as the CaMV promoter sequence was used in the protoplast transformation experiments. Asterisk indicates the position of the sequence shown in Fig. 2.

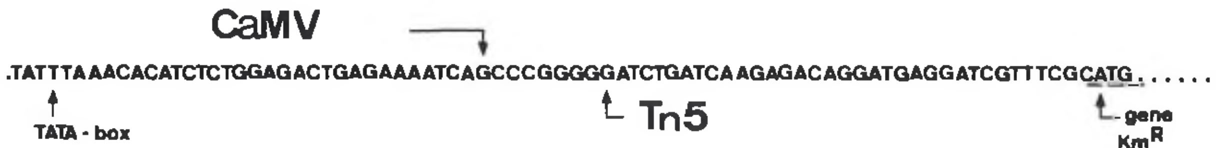

Fig. 2. Sequence between the TATA-box region of CaMV and the 5′ extremities of Tn*5* Km^R gene (see asterisk in plasmid pKR612B1 in Fig. 1).

sis of transformed callus DNA digested with *Eco*RV, which cuts in the CaMV DNA sequences of pKR612B1 near the extremities of the APH gene (Fig. 1), revealed the presence of one prominent and two minor bands for three separate clones (Fig. 3 A, B, C). The bands were absent from the DNA of nontransformed tobacco calli (Fig. 3 D).

One of the minor bands hybridizing with the pNEO probe was the length of the *Eco*RV fragment spanning the APH gene in pKR612B1 while a second minor band had slightly higher mobility (Fig. 3). Interestingly enough, however, the most prominent hybridizing *Eco*RV band in the transformed cell lines was about 300 bp longer than the corresponding fragment in pKR612B1. Thus it appears likely that the transforming DNA has undergone similar rearrangements or deletions in all three examples presented. The significance of these modifications remains unknown but it is noteworthy that similar effects were observed by Paszkowski et al. (1984) in transformation experiments with the CaMV-promoter-based vector pABD1. Perhaps the rearrangements facilitate expression of the inserted APH sequence or eliminate viral sequences which are toxic to the transformed plants.

(b) Test for APH activity

From several resistant microcalli whole tobacco plants were regenerated. The plantlets were resistant to 500 μg Km/ml (higher concentrations not tested) and were phenotypically normal. To demonstrate that the Km resistance of the transformed tobacco tissue was accompanied by expression of the APH gene, we assayed APH activity in a transformed tissue extract by the method of Reiss et al. (1984) as modified by Paszkowski et al. (1984). The results (Fig. 4) show that expression of the Tn*5* gene was obtained in transformed callus (lanes 5, 7) and leaf tissue derived from transformed plants (lanes 10, 11) but not expressed in nontransformed tobacco tissues (lanes 4, 6). Note that the activity band from leaf or callus tissue transformed with pKR612B1 has a mobility similar to that of APH from bacteria carrying the Tn*5* transposon (lane 1), whereas, as noted earlier (Paszkowski et al., 1984), the APH activity present in callus transformed by pABDI migrates as a slower-moving doublet of bands (lane 3).

The foregoing results confirm and extend the observations of Paszkowski et al. (1984) that a vector containing CaMV promoter and transcription termination signals and a selectable marker can be used to transform protoplasts from higher plants. Plasmid pABD1 contains a hybrid selectable-marker gene, the predicted product of which is a fusion protein containing 27 N-terminal amino acid residues coded for by the 5′-terminal part of gene VI of CaMV. This construct is useful for transformation of

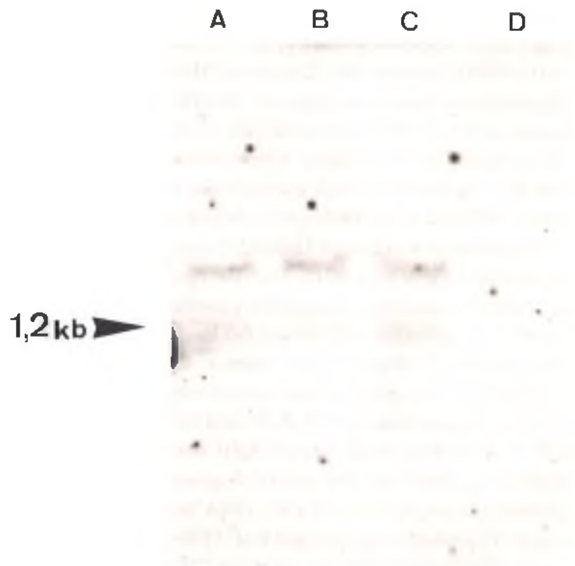

Fig. 3. Southern blot of DNA extracted from callus tissue regenerated from transformed tobacco protoplasts. Lanes A, B, C, samples from independent transformed cell lines. Lane D mock-inoculated cell line. Arrow shows the position of the *Eco*RV fragment of pKR612B1, containing the APH gene. DNA was extracted from 1 g of callus by a procedure developed by T. Huyuh as described and modified by Paszkowski et al. (1984). Probe was nick-translated [α-^{32}P]pNEO.

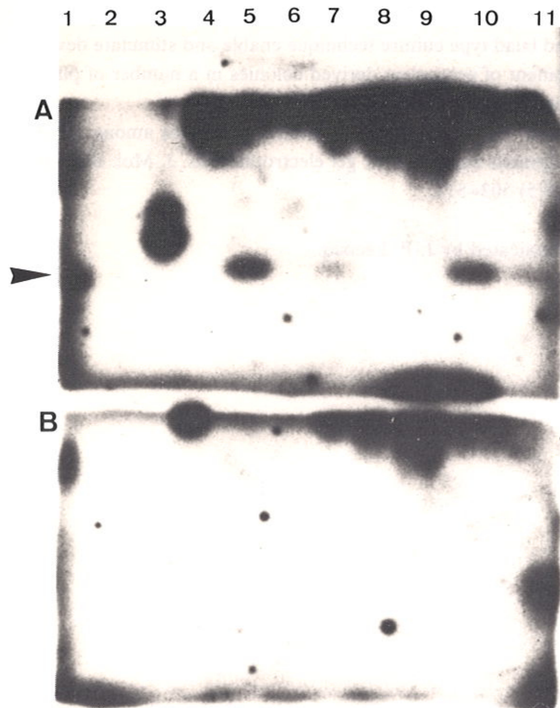

Fig. 4. APH enzyme activity test in Km^R callus and plants derived from them. Gels were allowed to react with [γ-^{32}P]ATP in the presence of 30 μg Km/ml (A) or in its absence (B). The test was performed according to Reiss et al. (1984). Arrowhead indicates the position of the APH activity from bacterial enzyme and that produced in plant tissues. Upper part of gels shows non-specific phosphorylation. Lanes: (1) bacterial APH enzyme from osmotic shock extract of *E. coli* carrying Km^R gene; (2) empty; (3) extract from Km-resistant plant transformed with pABDI (kind gift of Dr. Shillito); (4) and (6) extracts from wild-type tobacco calli; (5) and (7) extracts from calli derived from transformed protoplasts; (8) wild-type tobacco plant extract; (9) empty; (10) and (11) extracts from plants derived from transformed protoplasts.

protoplasts of several plant species (Potrykus et al., 1985a) but cannot be easily adapted for expression of other genes.

pDOB612 represents a considerable improvement over pABDI (Paszkowski et al., 1984) in that its transcript should direct synthesis of a polypeptide with an authentic N terminus rather than a fusion protein. Thus pDOB612 should prove useful for expression of other polypeptides in such transformation experiments.

ACKNOWLEDGEMENTS

We thank Daniele Beck for her excellent technical assistance, and we are grateful to Dr. L. Hirth for his continuous support. The help in the Km test of Dr. R. Shillito is highly appreciated. The senior author wishes to express his gratitude to Dr. T. Hohn for his support to finish part of these experiments in his laboratory.

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Communicated by J.-P. Lecocq.