Volume 12 Number 22 1984 Nucleic Acids Research

Binary *Agrobacterium* vectors for plant transformation

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Received 24 July 1984; Revised and Accepted 25 October 1984

ABSTRACT

A vector molecule for the efficient transformation of higher plants has been constructed with several features that make it efficient to use. It utilizes the trans acting functions of the vir region of a co-resident Ti plasmid in Agrobacterium tumefaciens to transfer sequences bordered by left and right T-DNA border sequences into the nuclear genome of plants. The T-region contains a dominant selectable marker gene that confers high levels of resistance to kanamycin, and a lac alpha-complementing region from M13mp19 that contains several unique restriction sites for the positive selection of inserted DNA.

INTRODUCTION

Agrobacterium tumefaciens cells that harbour large Ti plasmids are able to cause tumourous growths, or crown galls, on a wide range of dicotyledonous plants and gymnosperms.[1] This transformation is brought about by the transfer to the nuclear genome of a susceptible plant of a large segment of the Ti plasmid called T-DNA.[2] T-DNA contains genes for growth regulator autonomy[3,4] and the synthesis of a wide variety of opines[5], which are novel metabolites able to be catabolised by the inciting Agrobacterium.

T-DNA is delineated by imperfect 25 bp terminal repeats[6,7]; that is, any sequences between these elements are able to be integrated into plant nuclear DNA. A large region of the Ti plasmid called the vir locus is responsible for this transfer of T-DNA[8], although the vir locus has not been detected in transformed cells. The vir locus contains several genes which act in trans to T-DNA[9,10]; presumably T-DNA terminal repeat sequences are recognised by one or more of the products of the vir region.

This natural gene transfer system requires several modifications before it can be used as an efficient gene-vector system for producing transformed plants. First, the genes for growth regulator autonomy have to be deleted, as the expression of these genes interferes with the regeneration of normal

fertile plants.[11] Second, because growth regulator autonomy can no longer be used to select transformed cells, a marker gene is needed for selection. Recently, chimaeric genes composed of the regulatory sequences of an opine (nopaline) synthase gene and the coding sequence of a bacterial neomycin phosphotransferase gene have been shown to confer on plant cells resistance to the toxic antibiotics kanamycin and G 418,[12,13,14] and can be used as dominant selectable markers.

Finally, a means has to be found to introduce into the Ti plasmid sequences to be transferred to plants. Until now, single or double homologous recombinations into resident Ti plasmid T-DNA sequences have been used.[12,13,14,15] While the recombination steps are easy, they are limited by possible recombination out of repetitive sequences (commonly found in plant DNA)during or after recombination into the Ti plasmid replicon in Agrobacterium.

Here I describe the construction of an efficient Agrobacterium vector. It utilises the trans acting functions of the vir region on a Ti plasmid to transfer a modified T-DNA region to plant cells. The modified T-DNA, on a separate small replicon, contains only border repeats, and a chimaeric nopaline synthase - neomycin phosphotransferase gene. Sequences for transfer can be conveniently inserted into a multiple cloning site in T DNA and positively selected.

MATERIALS AND METHODS

The wide host-range plasmid pRK252 (derived from RK2) was obtained from Wayne Barnes (Washington University, St. Louis, Mo.), pAT21,[16] a pBR322 plasmid containing a type III APH 3' phosphotransferase from Streptococcus faecalis was obtained from Patrick Trien-Cuot (Institut Pasteur, Paris), and Agrobacterium tumefaciens strain LBA4404[9] was obtained from Mary-Dell Chilton (Ciba-Geigy Biotechnology, Raleigh, N.C.).

Recombinant vectors were mobilised from E. coli HB101 to LBA4404 in a triparental mating with HB101 harboring pRK2013.[17] Transconjugants were selected on minimal medium containing 50 µg/ml kanamycin. DNA manipulations and plasmid isolations were conducted using standard procedures.[18] Nopaline synthase was assayed using ^{14}C arginine as described elsewhere.[14]

Plant tissues were infected with A. tumefaciens by applying 5 µl of a rapidly growing culture of bacteria to the basal end of 5 mm lengths of stems of 8 week-old Nicotiana plumbaginafolia plants embedded apical end down in solidified MS medium supplemented with 0.5 mg/l NAA and 0.2 mg/l kinetin.

After approximately 5 days the stem segments were washed in liquid MS medium containing 500 µg/ml carbenicillin for 24 hrs to remove and kill the bacteria, and then plated, basal end up, in MS medium containing 0.5 mg/l NAA, 0.2 mg/l kinetin, and 500 µg/ml carbenicillin. After 2 weeks, callus from the basal end was removed and placed on the same medium containing 100 µg/ml kanamycin to select transformants.

RESULTS

1. Construction of vector molecules

Chimaeric nopaline synthase-neomycin phosphotransferase genes constructed to date[11,12,13] have all utilised a restriction fragment from Tn5 containing the coding region of neomycin phosphotransferase that has an additional, out-of-frame, AUG codon 5' to the correct AUG.[19] Unpublished experiments (R. Fraley, pers. comm.) indicate that, as expected, the removal of the spurious AUG greatly increases the level of kanamycin resistance obtained using the nopaline synthase promoter, presumably by making an mRNA more efficient in the translation of neomycin phosphotransferase. Therefore I decided to construct a more efficient selectable marker by deleting the spurious AUG. These steps are described in Figure 1 and resulted in the creation of pUC9-nopneoΔ.

This molecule provided a convenient skeleton upon which left and right borders of T-DNA from pTiT37,[6] could be assembled (see Fig. 2). The Hind III restriction fragment containing the right border also contained an intact nopaline synthase gene,[20,21] which is a useful screenable marker as nopaline is not found in untransformed plant tissues. The T-DNA array of left and right borders and selectable marker was ligated into a derivative of the wide host range plasmid pRK252 that contained a type III aminoglycoside phosphotransferase for selection in Agrobacterium. The prototype binary vector Bin 6 (15 kb) was obtained, which contains restriction sites within T-DNA for Sal 1 and EcoRI, an efficient selectable marker gene and a screenable gene for identifying putative transformants. However, in practice this vector was too large for routine blunt end ligations. The prototype was modified by the deletion of unwanted sequences as described in Figure 2, steps 3 and 6. The nopaline synthase gene was removed by a partial SStII digestion and religation, and excess Ti plasmid sequences (approximately 1.5 kb) flanking the left and right border repeats were removed by partial Bal 31 exonuclease treatment. The truncated T region thus obtained was cloned into a pRK252 derivative that had suffered a 2.5 kb deletion during the insertion of

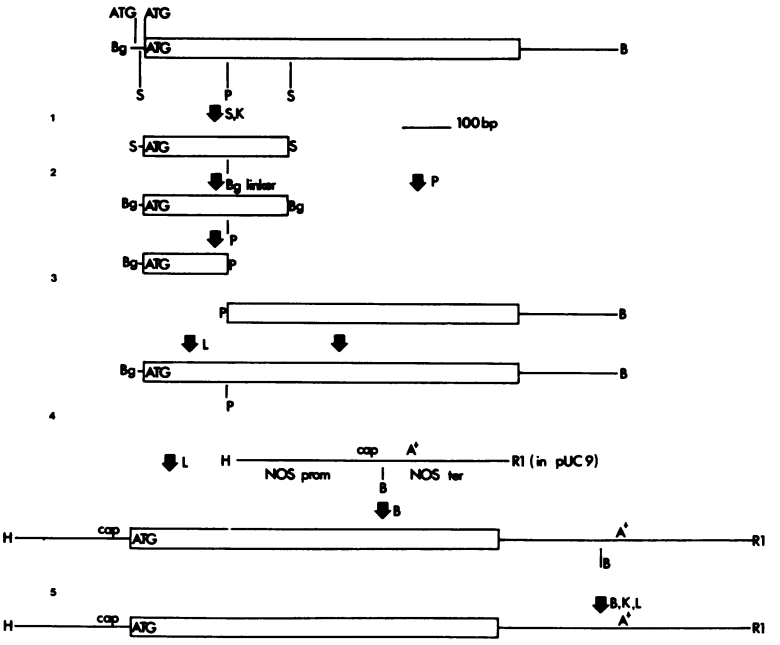

Fig. 1 Construction of selectable marker gene.

The coding region of aminoglycoside phosphotransferase type II, represented as the open box, was excised from a fragment of Tn5 cloned in pBR325[10] using Bam Hl and Bgl II. A 310 bp Sau3a fragment containing the NH_2-terminus of the gene was rendered flush with Klenov polymerase (step 1), ligated to Bgl II linkers (step 2) and replaced in the gene via a Pst 1 site (step 3). These steps removed the spurious ATG codon indicated. The modified phosphotransferase gene was ligated (step 4) between a nopaline synthase promoter fragment of 310 bp[18], and a 260 bp Sau3a fragment containing the nopaline synthase polyadenylation site[20,21]. Finally, the Bam HI site separating the phosphotransferase gene and the polyadenylation site was removed by filling-in (step 5). Abbreviations: A^+, polyadenylation site; B, Bam Hl; Bg, Bgl II; H, Hind III; L, T_4 DNA ligase; P, Pst 1; S, Sau3a; K, Klenov fragment of E. coli DNA polymerase; R_1, EcoRI; b.p., base-pairs.

the kanamycin resistance gene from Streptococcus. The deletion did not affect the mobilisation frequency to or stability of the plasmid in A. tumefaciens (see below). Finally, to aid insertion of sequences into the T-region of the vector, a 440 bp HaeII fragment from ml3mpl9[22,23] was inserted in place of a 1.6 kb EcoRI fragment 80 bp from the left border of T-DNA to provide unique sites for EcoRI, Bam Hl, Hind III, SStI, Kpnl, Sma 1, Xba 1, and Sal 1 within T-DNA.

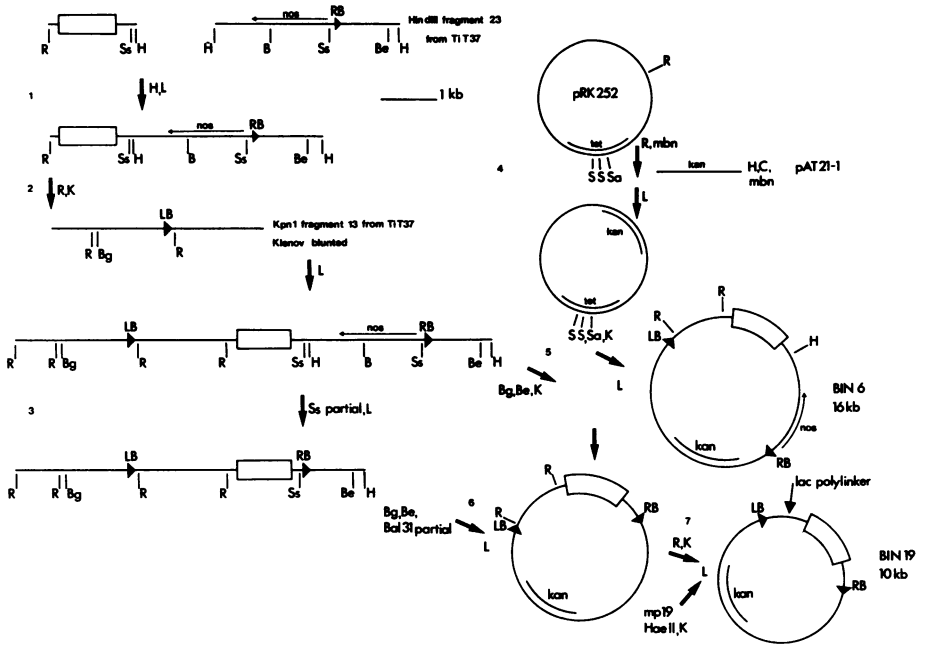

Fig. 2 Construction of Binary vector.

The right T-DNA border of pTiT37 and an intact nopaline synthase gene, found on a 3.2 kb Hind III fragment[6,20] was ligated (step 1) into the Hind III site of the dominant marker gene described in Fig. 1. The left T-DNA border of pTiT7, on a 3.8 kb Kpn 1 fragment[7], was made flush-ended with Klenov polymerase and ligated into the flush-ended R_1 sites. This artificial T-DNA was truncated by removing the nopaline synthase gene by a partial Sst II digestion (step 3) and by a partial Bal31 nuclease treatment of a 5.5 kb BstE 11 - Bgl II fragment which removed approximately 0,8 kb from each end of the molecule, but spared both T-DNA border elements (step 6).
Both T-DNA arrays were ligated (steps 5 and 6) into a wide host-range plasmid, pRK252, that had been modified by the insertion of a kanamycin resistance gene from Streptoccocus[16] (step 4), and by a fortuitous 4 kb deletion which occurred during this ligation. The vector molecule Binary 19 was constructed by replacing a 1,6 kb EcoRI T-DNA fragment with a 440 bp Hae II fragment from M13 mp19[22] that contains the alpha-complementary region of β-galactosidase and an array of restriction enzyme sites (step 7). Abbreviations: B, Bam Hl; Be, Bste II; Bg, Bgl II; H, Hind III; K, Klenov fragment of E. coli DNA polmerase; C, Cla 1; mbn, mung bean nuclease; R, EcoRI; L, T4 DNA ligase; S, Sma 1, Sa, Sal 1; Ss, Sst II; LB, RB, left and right T-DNA border repeats; nos, nopaline synthase gene.

Plasmids harboring inserts in this sequence can conveniently be identified on plates containing kanamycin, IPTG, and X-gal. This binary vector, called Bin 19, is approximately 10 kb in length, and is efficiently

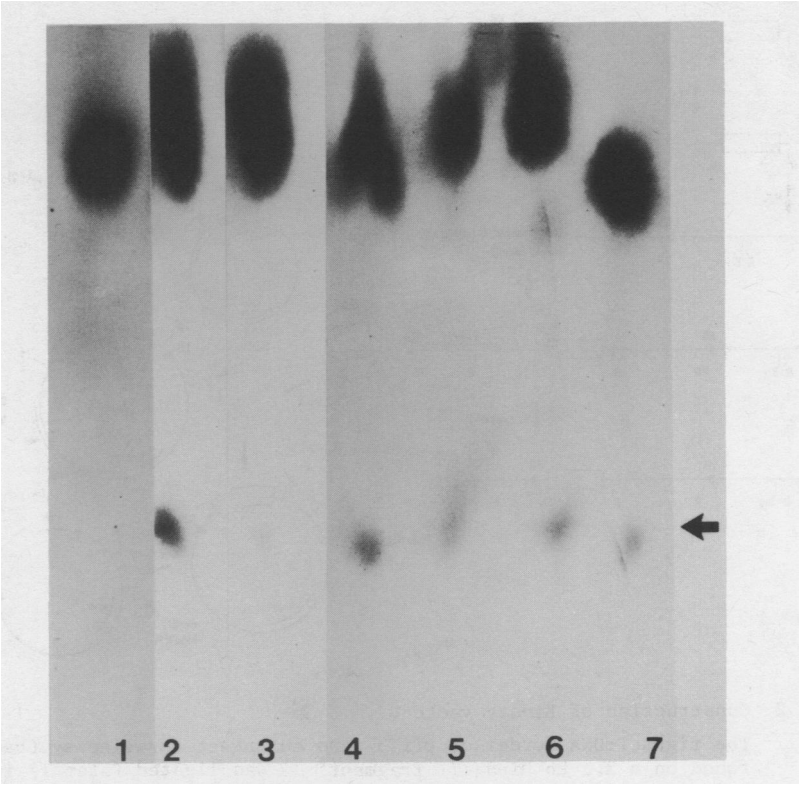

Fig. 3 Nopaline synthase activity in putative transformants.

10 mg of tissue was extracted into 25 µl buffer, of which 2.5 µl was assayed.

Lane 1	tissue treated with Agrobacterium tumefaciens LBA 4404 devoid of Bin 6 vector.
Lanes 2, 4	standard nopaline producing teratoma HT37
Lane 3	tissue transformed with Bin 6 vector
Lanes 5, 6	tissue transformed with Bin 6 vector and selected on kanamycin-containing medium.
Lane 7	leaf tissue from a plantlet regenerated from a kanamycin-resistant callus.

The arrow indicates the position of radioactive nopaline on the paper electrophoretogram. The large radioactive spot with a high Rf present in each lane is the substrate, radioactive arginine.

mobilised by the helper plasmid pRK2013 into A. tumefaciens LBA4404, which contains Ti plasmid pAL4404[9]. After a typical overnight plate mating, approximately 1-2% of the Agrobacterium cells contain the Binary vectors, when HB101/pRK2013 is used as a mobilising strain.

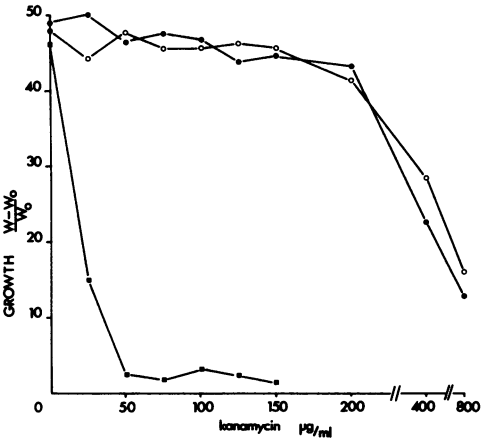

Fig. 4 Growth rates of transformed tissues on kanamycin containing medium. Ten pieces of callus, original weight (W_0) ca. 50 mg, were analysed for each point on the graph. The final weight (W) was measured after 30 days, and the growth was calculated.

■ untransformed callus.

○ callus transformed with Bin 6 and selected on 100 µg/ml kanamycin for 1 month before testing.

● callus transformed with Bin 19 and selected on 100 µg/ml kanamycin for 1 month before testing.

An important consideration in the use of binary vectors is the stability of the component plasmids. Both Bin 6 and Bin 19 are rapidly lost from Agrobacterium tumefaciens LBA4404 in the absence of selection. Consequently, strains for transformation of plants must be grown under kanamycin selection, and then removed by centrifugation and resuspended in plant growth medium. The short time of exposure to plants, usually 24-48 hrs, and the unsuitability of the medium for bacterial growth, means that plasmid loss is probably unimportant at this stage.

2. Transformation of plant tissue

Bin 6, the prototype, was used to transform stem explants of N. plumbaginafolia as described above. The inciting bacteria were killed efficiently by carbenicillin treatment, and abundant callus formed on the infected stem after two weeks. When this tissue was assayed for nopaline production, low levels were detected (Fig. 3 compare lane 2 and 3), while none was detected in callus obtained from stems infected with the helper strain LBA 4404 that did not contain Bin 6 plasmids (Fig. 3 lane 1). Callus pieces were then transferred to the same medium containing 100 µg/ml kanamycin to select

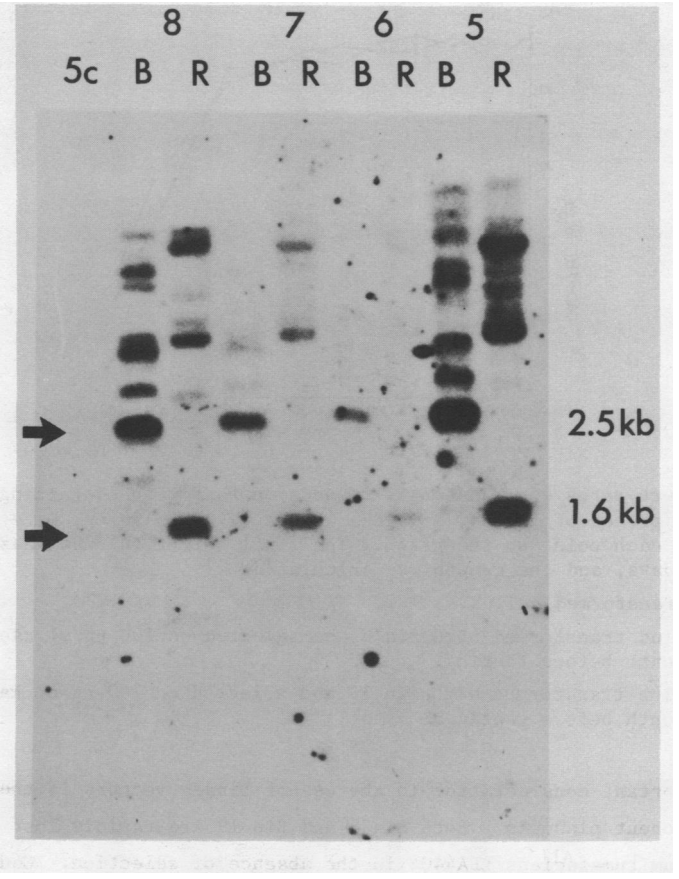

Fig. 5 Southern blot analysis of kanamycin resistant callus lines.

The figure shows an autoradiograph of a Southern blot of total callus DNA digested with Bam HI (B) or EcoRI (R). Samples 5, 6, 7, 8 were from different transformation experiments. 5c is a 5 copy EcoRI reconstruction of the Bin 6 vector (37.5 pg). The radioactive probe was a nick-translated 8 kb Bgl II - Bste II fragment containing the entire T-region of the Binary vector. The arrow indicates the expected 1.6 kb internal EcoRI fragment. The position of the expected 2.5 kb Bam HI internal fragment is also shown.

transformed cells. After 2-3 weeks, callus infected by LBA4404 that did not contain Bin 6 were dead, while callus infected by LBA4404 containing Bin 6 produced excrescences of vigorously growing white callus on its surface.

The rate of appearance of kanamycin-resistant callus varied according to the age of the plants from which the explants were taken, and the position on the plants from which the explants were taken. In general however, kanamycin resistant callus appeared more rapidly than when explants were transformed

with A208 neo^{+13}, which contained a kanamycin resistance gene in T-DNA *cis* to the virulence locus. I presume the more rapid appearance of callus on kanamycin containing medium is due to higher levels of resistance conferred by the gene construction used in these experiments. When this callus was assayed for nopaline synthase activity it gave an abundant nopaline spot (Fig. 3 lanes 5,6) on the electrophoretogram, and upon transfer to fresh kanamycin-containing medium it continued to grow.

The growth rates of calli transformed with Bin 6, Bin 19, and untransformed callus, were compared on MS medium supplemented with 0.5 mg/l NAA, 0.2 mg/l kinetin, and various concentrations of kanamycin. Figure 4 shows that the growth of callus transformed by both Bin 6 and Bin 19 is unaffected by up to 200 µg/ml kanamycin, and growth continues at a steadily reduced rate from 400-800 µg/ml kanamycin. This is in accord with the unpublished observations of Fraley *et al.*, and contrasts with the relatively low levels of resistance conferred by earlier "two AUG" constructions.[12,13,14] In practice, kanamycin can be used at concentrations between 75-200 µg/ml for selection. Untransformed tobacco cells are killed by concentrations of kanamycin exceeding 30 µg/ml.

Callus from four separate transformation experiments using Bin 6 was grown on kanamycin medium for a further four weeks, when samples were taken for DNA analysis.[23] Figure 5 shows an autoradiograph of a Southern blot of EcoRI and Bam HI digested total DNA probed with a nick-translated Bgl II-BstE II fragment that spans the predicted T-region of the binary vector (Fig. 2, step 5). All four samples from separate transformation experiments contained T-DNA with a predicted EcoRI internal fragment of 1.6 kb and an internal Bam HI fragment of 2.5 kb (both arrowed). The intensity of hybridisation of these internal fragments and the number and size of other fragments indicated that tandem or even multimeric insertions in plant DNA were common, as they are in other T-DNA structures.[6] A comparison of the intensity of hybridisation of reconstruction mixtures and genomic DNA indicated that the copy number of internal T-DNA fragments varied from 5-20 copies per diploid genome. This estimate is supported by the large number of faintly hybridising presumptive fusion fragments of T-DNA and plant DNA. Transformation experiments using Bin 19 have shown the production of tobacco callus resistant to high levels of kanamycin as shown in Fig. 5 that do not produce nopaline, as expected.

3. Regeneration of plants from transformed cells

After approximately 6-8 weeks growth on selective medium containing 0.5

mg/l NAA, 0.2 mg/l kinetin, and 100 μg/ml kanamycin, callus was transferred to MS medium supplemented with only 0.5 mg/l kinetin. This medium promoted the formation of morphologically normal shoots which produced roots upon transfer to MS medium that did not contain sucrose, or growth regulators.[24] Leaf tissue from these plants produced nopaline (Fig. 3 lane 7). This showed that, as expected, T-DNA transferred to plant cells does not affect the totipotency of those cells.

CONCLUSIONS

This work describes the construction and use of a high utility Agrobacterium vector for plant transformation. It has several novel advantages: firstly, it contains a large number of restriction sites conveniently located in lac DNA for the positive identification of foreign DNA inserted in the T-region, and these sites are assymetrically arranged so that all the benefits of construction afforded by the pUC plasmids[25] can be enjoyed in the binary vector. Secondly, the relatively small size of the vector molecule allows for efficient ligation of passenger DNA into the multiple cloning site. In practice it is the large size of wide host range plasmids that places a lower limit on the size of binary vectors and therefore makes them less efficient vectors than, for example, pUC plasmids. An increase in knowledge of the functions on wide host range plasmids may lead to further reductions in the size of binary vectors. Finally, binary vectors require no recombinational steps for integration into the Ti plasmid. This feature is useful in situations where intrinsically unstable sequences, such as those containing repeated sequences, are to be transformed.

The efficiency of DNA transfer mediated by a trans configuration of T-DNA and virulence functions, compared to the normal cis configuration of these functions will be an interesting question to answer. The experiments reported here have shown there is no qualitative difference in the ease of transformation using explanted leaf tissues with Binary vectors compared with "cis" vectors, as transformed tissues are obtainable within two weeks of culture on kanamycin.

ACKNOWLEDGEMENTS

The author gratefully acknowledges the skilled technical assistance of Sue Mason. This research was funded in part by the British Technology Group.

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