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A simple method to transfer, integrate and study expression of foreign genes, such as chicken ovalbumin and α-actin in plant tumors

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A simple method for inserting foreign genes into the T-region of *Agrobacterium* Ti-plasmids is described. A modified cosmid (pHC 79) was introduced into a predetermined site of the T-region of pTi C58. An *Agrobacterium* strain harboring this modified Ti-plasmid was used as an acceptor strain into which genes, cloned in pBR322, can be introduced by mobilization from *Escherichia coli*. pBR322-derived plasmids cannot replicate in *Agrobacterium*, but can be maintained by integration into the T-region of the modified Ti-plasmids by homologous recombination. This method was used to introduce the genes for ovalbumin and α-actin from chicken into tobacco tumors. Southern blotting and re-isolation of the inserted genes by reverse cloning showed that the animal DNA was transferred and integrated into the plant genome without rearrangements. The α-actin gene is not transcribed in plant tumors, whereas transcription of the ovalbumin gene was observed, however the initiation point of transcription was different from the one used in the chicken oviduct. The RNA transcribed from the chicken ovalbumin gene is polyadenylated and ranges in size between 2 and 7 kb.
Key words: Ti-plasmid/integration/foreign genes/plant cell transformation/actin/ovalbumin/chicken genes/transcription/animal gene in plants

Introduction

Crown gall tumors on wounded dicotyledonous plants are the result of a natural DNA transfer in which a specific region (T-region) of the *Agrobacterium tumefaciens* Ti-plasmid is integrated into the nuclear genome of the host plant. Foreign DNA sequences inserted in the T-region are co-transfected to plants and integrated into the plant genome (Schell *et al.*, 1979; Hernalsteens *et al.*, 1980; Leemans *et al.*, 1981; Matzke and Chilton, 1981; Nester and Kosuge, 1981).

Two main strategies have been developed to introduce foreign DNA sequences into the T-region of Ti-plasmids. The first is a site-specific mutagenesis technique. A foreign gene is fused with a selectable antibiotic resistance marker and inserted into a subfragment of the T-DNA. This fragment is subsequently cloned into plasmid pRK290 and mobilized to an *Agrobacterium* acceptor strain, in which the plasmid can replicate. Double exchange recombinants betwen the Ti-plasmid of the acceptor strain and the modified T-DNA fragment of pRK290 can be selected for by transforming the bacteria with a plasmid incompatible with pRK290 (pR751, pR751-pMG2, pPH1J1) (Matzke and Chilton, 1981; Garfinkel *et al.*, 1981).

An alternative method was described by Leemans *et al.* (1981, 1982). A foreign gene is introduced into a subfragment of the T-DNA and cloned into a plasmid containing the origin of replication of the W-type plasmid Sa. This plasmid can replicate in both *Escherichia coli* and *Agrobacterium*. Co-integrates formed between such a recombinant intermediate vector and the resident Ti-plasmid are selected. The Ti-plasmid of the recipient *Agrobacterium* carries an antibiotic resistance gene in its T-DNA which is exchanged for the foreign gene by a double cross-over. Thus, the foreign DNA is stably integrated in the T-region of the resident Ti-plasmid. In the method described here, the cosmid pHC79 containing a Gm^R gene is integrated into the T-region of the Ti-plasmid. A foreign gene, cloned in pBR322 is then mobilized from *E.coli* to *A.tumefaciens* and maintained in the host by homologous recombination with the cosmid pHC79. Stable integration of the foreign DNA can be achieved by double recombination between pBR322 and the cosmid. A single conjugation between *E.coli* and *A.tumefaciens* allows screening for double recombination events. The method has been used to transfer and integrate the chicken genes ovalbumin and α-actin into the genome of tobacco tumor cells. Transcription in plants from the ovalbumin, but not from the α-actin gene, was detected.

Results

Cloning of the cosmid pHC79 into the nopaline synthase gene of pTiC58

To construct a suitable acceptor Ti-plasmid for homologous recombination with pBR322 derivatives, we cloned the cosmid pHC79 into the T-region of pTiC58, a nopaline-type plasmid. This was carried out by the *in vitro* mutagenesis technique described by Leemans *et al.* (1981). A derivative of the W-type plasmid Sa carrying *Hind*III fragment 23 was cleaved with *Bam*HI and pHC79 was partially digested with *Bgl*II. The two plasmids were ligated and recombinants were isolated that contained the cos site of phage λ (plasmid pCHC2379 Figure 1a) *E.coli* AB1157, containing the plasmids R64drd11 and pGJ28, was transformed with plasmid pCHC2379. These plasmids are necessary to mobilize the plasmid pCHC2379 to *Agrobacterium* strain GV3101 containing the Ti-plasmid pGV3105 (Van Haute *et al.*, 1983). pCHC2379 can be mobilized to *Agrobacterium* with frequencies of 1.5×10^{-3}. Co-integrates between Ti-plasmid and intermediate vector were obtained with a frequency of 1.9×10^{-5} by Ti-plasmid conjugation and selection for Km^R Cb^R occ^+ transconjugants.

Ti-plasmid recombinants resulting from a double cross-over, eliminating the pCH23 vector and the duplicated *Hind*III (23) fragment, were obtained by a further Ti-plasmid conjugation and screening for Km^S Cb^R occ^+ transconjugants (Table I). One of eight recombinants selected was pMP61. DNA/DNA hybridization, using the plasmids pCH23 and pHC79 as probes, showed that the cosmid pHC79 was integrated in *Hind*III fragment (23) of the Ti-

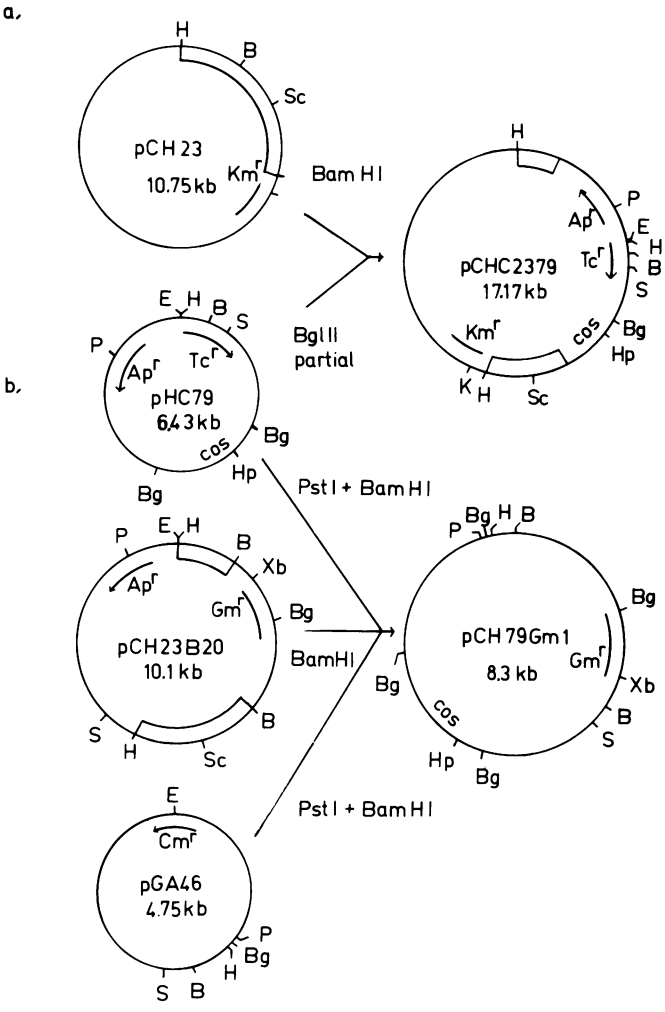

Fig. 1. Construction of intermediate vectors pCHC2379 and pHC78Gm1. **(a)** pCHC2379 cosmid was obtained by ligation of *Bam*HI digested plasmid pCH23 DNA to cosmid pHC79 digested partially with *Bgl*II followed by transformation to *E.coli* HB101. Recombinants were selected on plates containing kanamycin, tetracycline and ampicillin and screened for the presence of a *Bgl*II-*Hind*III fragment, carrying the *cos* sequence of phage λ DNA. **(b)** Plasmid pCH23B20 contains the gentamycin resistance gene of plasmid pJR88. pCH23B20 was constructed by subcloning the *Bam*HI fragment of plasmid p45-2 (S.Kagan, Ph.D. Thesis, Univ. Wisconsin) carrying the gentamycin resistance gene of pJR88 into vector pGVO601 (Leemans *et al.*, 1981). Intermediate vector pHC79Gm1 was constructed by ligation of an isolated *Bam*HI fragment of pCH23B20 to an isolated *Pst*I-*Bam*HI fragment of pHC79 and a *Pst*I-*Bam*HI digested plasmid pGA46 (An and Friesen, 1979). *E.coli* HB101 was transformed with the ligated DNA and colonies were selected that were Gm^R Ap^S Tc^S Cm^S.
Abbreviations: H = *Hind*III; B = *Bam*HI; Sc = *Sac*II; K = *Kpn*I; E = *Eco*RI; S = *Sal*I; P = *Pst*I; Bg = *Bgl*II; Hp = *Hpa*I; Xb = *Xba*I; Ap = ampicillin; TC = tetracycline; Km = kanamycin; Gm = gentamycin; Cm = chloramphenicol.

plasmid pGV3105. To characterize further the integration of pHC79 into the *Hind*III fragment (23) of pTiC58, the entire recombinant T-region of pMP61 was re-isolated by reverse cloning. The Ti-plasmid containing the cosmid was partially digested with *Kpn*I, re-ligated and *E.coli* cells were transformed. Clones obtained by this procedure overlapped Ti-plasmid regions located to the right and to the left of the pHC79 insert. pT616, one of 12 clones analysed, contained the full length T-region of nopaline Ti-plasmid and continued further to the left of the T-region (Figures 2a,3e and 4). pHC79 carries ampicillin and tetracycline resistance genes. The *tet* gene is expressed at a very low level in *Agrobacterium* and therefore cannot be used as a resistance marker. The β-lactamase (ampicillin gene) also inactivates carbenicillin which can be used as an antibiotic for *Agrobacteria*. In most cases a foreign gene is cloned into the *tet*-region of pBR322 (donor molecule) leaving only the ampicillin resistance gene intact for screening of integration into the Ti-plasmid (acceptor molecule). If the acceptor and donor molecules contain the same resistance marker no screening for integration or double cross-overs (loss of resistance marker from pHC79) would be possible. Therefore we decided to delete the *Bam*HI-*Pst*I fragment of pHC79 and to replace it by a gentamycin resistance gene. An intermediate vector pHC79Gm1 was constructed in which the *Pst*I-*Bam*HI fragment of pHC79 was replaced by the gentamycin resistance gene of plasmid pJR88 (Figure 1b). pHC79Gm1 was introduced into *E.coli* AB1157 (containing the plasmids R64drd11 and pGJ28 for mobilization; Van Haute *et al.*, 1983) and transferred to an *Agrobacterium* strain harboring the Ti-plasmid pMP61. The exchange of Gm^R for Cb^R marker by double crossing-over was analysed by replica plating. Colonies containing the co-integrate are Gm^R and Cb^R resistant. After double crossing-over the carbenicillin marker is lost. Double recombinants were identified with a frequency of 10^{-8} (Table I) and analysed further by DNA/DNA hybridization using pCH23B20 as a probe (Figure 3b).

Figure 4 shows the structure of the T-region of the pMPGR1 Ti-plasmid containing the modified cosmid pHC79Gm1.

Cloning of isolated genes into the T-region of pMPGR1

Foreign genes, cloned into the tetracycline-resistance gene of pBR322 can be integrated into the Ti-plasmid pMPGR1 by the following method. The derivative of pBR322 is transformed to donor *E.coli* AB1157 and mobilized to *Agrobacterium* containing the plasmid pMPGR1 as described above. Stabilization of the mobilized plasmid occurs by single crossing-over. Co-integrates can be selected for by their phenotype (Cb^R; Gm^R; occ^+). Loss of gentamycin resistance indicates the exchange of the pHC79Gm1 sequence of the Ti-plasmid for the incoming pBR322 derivative by double crossing-over.

Double recombinants can be identified in a single step by replica plating of Cb^R occ^+ colonies on plates containing gentamycin (Gm^S). This method has been used to insert two plasmids; pOV12 and pαA6.2, into the T-region of pMPGR1. These plasmids contain the ovalbumin (pOV12) and the α-actin (pαA6.2) genes from chicken. pOV12 is a chimeric plasmid containing the entire chicken ovalbumin gene (12 kb) in the *Hind*III site of pBR322 (Lai *et al.*, 1980). pOV12 is cleaved by *Hind*III into three fragments of the following size: 4.8, 4.0, 3.2 kb. *Bam*HI cuts the plasmid at only one position (Figure 2b). pOV12 has been transferred to *E.coli* strain AB1157 (R64drd11, pGJ28) and mobilized to *Agrobacterium* as described above. Co-integrates were selected with a frequency of 10^{-6} after plating on octopine indicator plates containing carbenicillin (100 μg/ml). Double recombinants were obtained with a frequency of $10^{-2}-10^{-3}$ by replica plating the colonies (Cb^R; occ^+) immediately on gentamycin plates (Table I). Gm^S, Cb^R, occ^+ colonies were tested for the presence of pOV12 by Southern blotting (Figures 3c and 4).

The α-actin gene (pαA6.2) from chicken is located on a 6.2-kb fragment and cloned into the *Hind*III site of pBR322

Table I. Construction of recombinant Ti-plasmids

Bacterial crosses			Selection	Frequency of exconjugants relative to number of recipients
(a)				
E.coli AB1157	(R64drd11) (pGJ28) (pCHC2379)	x *A.t.* GV3101 (pGV3105)	Rif^R Cb^R Km^R occ^+	1.5×10^{-3}
A.tumefaciens GV3101	(pGV3105) (pCHC2379)	x *A.t.* GV3103	Sm^R Sp^R Cb^R Km^R occ^+	1.3×10^{-5}
A.tumefaciens GV3103	(pGV3105::pCHC2379)	x *A.t.* GV3101	Rif^R Cb^R Km^S occ^+	1.0×10^{-3}
(b)				
E.coli AB1157	(R64drd11) (pGJ28) (pHC79Gm1)	x *A.t.* GV3101 (pMP61)	Rif^R Gm^R Cb^S occ^+	1.2×10^{-8}
(c)				
E.coli AB1157	(R64drd11) (pGJ28) (pOV12)	x *A.t.* GV3101 (pMP61)	Rif^R Gm^S Cb^R occ^+	0.9×10^{-8}
(d)				
E.coli AB1157	(R64drd11) (pGJ28) (pαA6.2)	x *A.t.* GV3101 (pMP61)	Rif^R Gm^S Cb^R occ^+	2.1×10^{-8}

(e)

Intermediate vector	Recombinant Ti-plasmid	Phenotype of incited tumor	
pCHC2379 Cb^R Km^R	pMP61	IMP61	Nos^-
pHC79Gm1 Gm^R	pMPGR1	IMPGR1	Nos^-
pOV12 Amp^R Cb^R	pMPOV12	IMPOV12	Nos^-
pαA6.2 Amp^R Cb^R	pMPαA6.2	IMPαA6.2	Nos^-

[a]Frequency of exconjugants in the second Ti-plasmid conjugation has been corrected for the value of co-integrate dissociation. The value shown corresponds to the frequency of recombinants formed by double cross-over.
[b,c,d]Donor strains for mobilization of pBR322 recombinants were constructed by transformation of *E.coli* strain AB1157 (R64drd11, pGJ28) with DNA of isolated plasmids.
[e]Intermediate vectors used for construction of recombinant Ti-plasmids and phenotype of tumors incited on *N.tabacum* SR1 plants by *Agrobacterium* strains carrying recombinant Ti-plasmids.

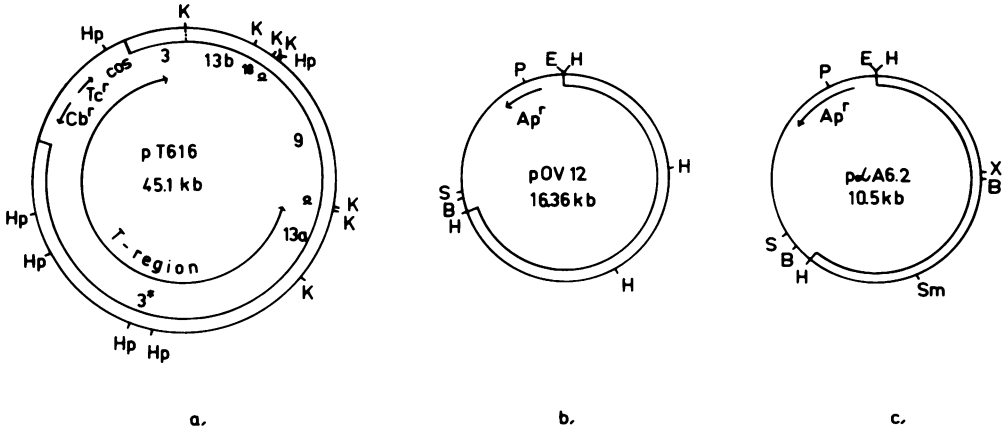

Fig. 2. Physical map of plasmids pT616, pOV12 and pαA6.2. Arrows show the extension of the T-region in plasmid pT616. Numbers inside the pT616 physical map correspond to *Kpn*I fragments of pTiC58 nopaline Ti-plasmid (Depicker *et al.*, 1980). Abbreviations of restriction endonuclease cleavage sites in plasmid figures correspond to those used in Figure 1. Sm = *Sma*I; X = *Xho*I..

(Figure 2c). The gene has single *Bam*HI, *Sma*I and *Xho*I sites. Although the α-actin fragment has only half the size of the ovalbumin insert, double recombinants were isolated with the same frequency ($10^{-8} - 10^{-9}$; Table I). Southern hybridization showed the exchange of pHC79Gm1 for pαA6.2. The resulting Ti-plasmid is pMPαA6.2 (Figures 3d and 4).

Transfer of cloned genes to plants

Sterile *Nicotiana tabacum* SR1 plants were inoculated with *Agrobacterium* strains containing the plasmids pMP61, pMPGR1, pMPOV12 and pMPαA6.2. Primary tumors were cultured on hormone-free medium as described by Leemans *et al.* (1981). The bacterium-free tumor culture grew without additional phytohormones as well as wild-type tumor control lines which contained the T-DNA of the pGV3105 Ti-plasmid. The tumors IMP 61, IMPGR 1, IMPOV 12 and IMPαA6.2 do not produce nopaline, indicating the insertion of the foreign DNA into the nopaline synthase gene. To support these findings the tumor lines IMPOV 12 and IMPαA6.2 were analysed further. Total plant DNA was isolated, cut with various restriction enzymes, run on agarose gels, blotted on nitrocellulose filters and hybridized with isolated fragments of pOV12 (*Hind*III fragments 4.0, 3.2, 4.8 kb) and pαA6.2 (*Hind*III fragment).

The hybridization patterns were identical to those obtained from Southern blot hybridization of the Ti-plasmids pMPOV12 and pMPαA6.2 carrying the ovalbumin and α-actin genes, respectively.

DNA from the tumor lines IMPOV 12 and IMPαA6.2 were cut with the restriction enzyme *Hind*III, run on agarose gels, blotted on nitrocellulose filters and hybridized with the *Kpn*I fragments 3 and 13 from the T-region of pTiC58 (Figure 4). The autoradiogram in Figure 6 shows several bands belonging to the T-region of the Ti-plasmid and in both cases (pOV12, pαA6.2) four additional bands. These fragments presumably contain the border sequences of the T-DNA integrated into the plant genome. The copy number

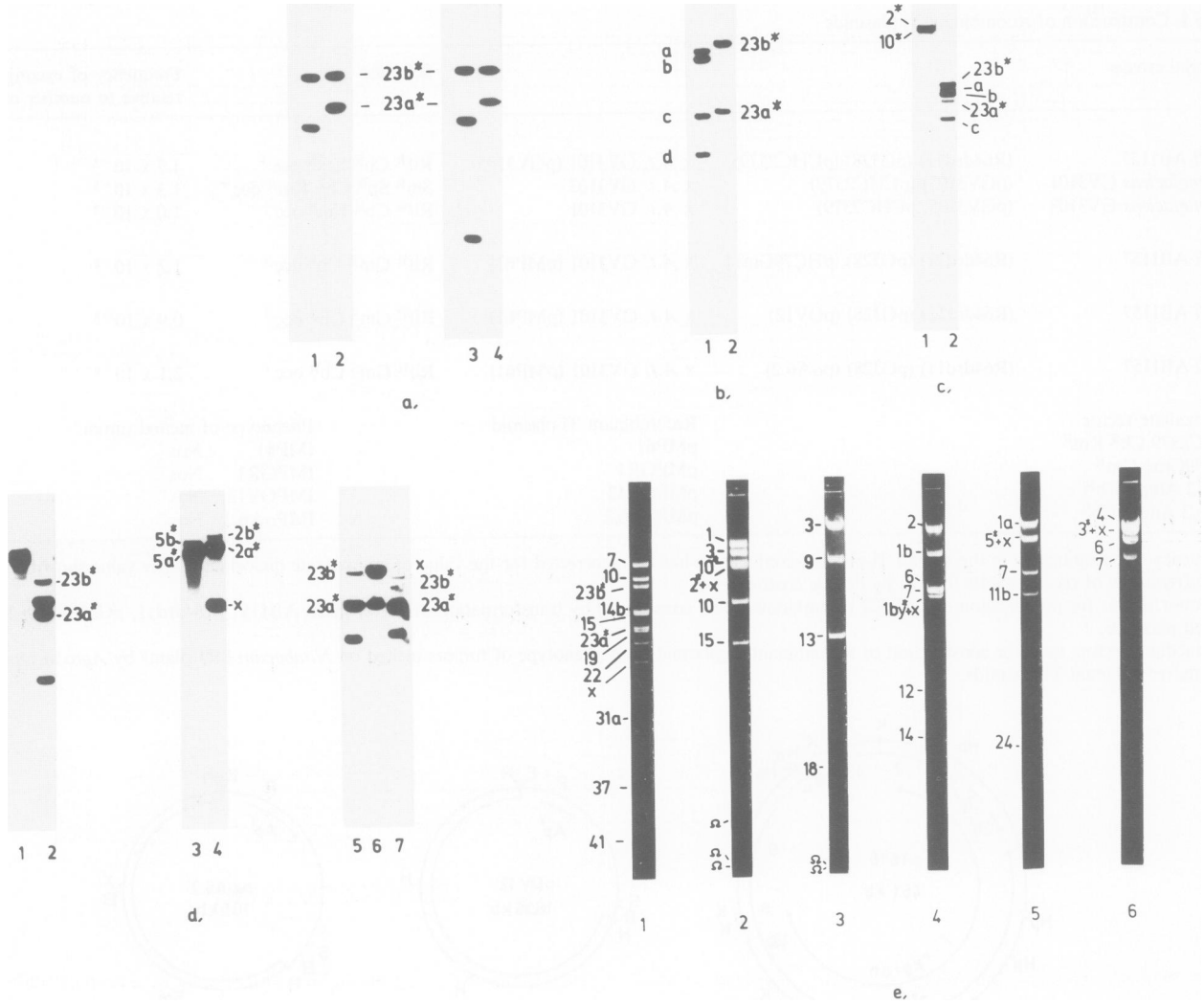

Fig. 3. DNA-DNA hybridization analysis of recombinant Ti-plasmid pMP61, pMPGR1, pMPOV12 and pMPαA6.2. Total DNA was prepared from *Agrobacterium* strains carrying recombinant Ti-plasmids, digested with large excess of restriction endonuclease, subjected to agarose gel electrophoresis, blotted on nitrocellulose filters (Southern, 1975) and hybridized to nick-translated probes (Rigby *et al.*, 1977) of appropriate plasmids. **(a)** Hybridization of pCH23 **(1,2)** and pHC79 **(3,4)** DNA probes to *Hind*III + *Pst*I **(1,3)** and *Hind*III **(2,4)** digested total DNA of *Agrobacterium* strain GV3101 (pMP61). **(b)** Hybridization of pCH23B20 pobe to *Bgl*II **(1)** and *Hind*III **(2)** digested total DNA of *Agrobacterium* strain GV3101 (pMPGR1). **(c)** Blots of *Bam*HI **(1)** and *Hind*III **(2)** digested total DNA of *Agrobacterium* GV3101 (pMPOV12) after hybridization to plasmid pOV12 DNA probe. **(d)** Hybridization of *Xho*I **(1)**, *Hind*III + *Sma*I **(2)**, *Sma*I **(3)**, *Bam*HI **(4)**, *Hind*III + *Xho*I **(5)**, *Hind*III **(6)**, and *Hind*III + *Bam*HI **(7)** digested total DNA of *Agrobacterium* GV3101 (pMPαA6.2) to labeled probe of plasmid pαA6.2 DNA. **(e)** Restriction endonuclease analysis of plasmid pT616 DNA with *Hind*III **(1)**, *Bam*HI **(2)**, *Kpn*I **(3)**, *Hpa*I **(4)**, *Sma*I **(5)**, and *Xba*I **(6)** endonucleases. Restriction endonuclease fragments hybridized to DNA probes and shown in fingerprints of plasmid pT616 are labeled according to physical maps of T-regions shown in Figure 4. The fragment nomenclature has been described earlier (Depicker *et al.*, 1980). Fragments labeled with +x in **e** are fusion fragments generated by ligation of *Kpn*I fragments 13b and 3 in plasmid pT616. Fragments marked with a star correspond to T-DNA fragments changed in size by insertions in the nopaline synthase gene of pTiC58.

of the T-DNA per tumor cell was estimated according to Barton *et al.* (1983) and the results showed that only one or two copies were integrated per plant genome (data not shown).

To see whether the structure of the T-DNA was rearranged during integration into the plant genome and to characterize the plant DNA next to the T-DNA border, a DNA fragment from the plant tumors was isolated containing the α-actin gene, part of the T-DNA and a 1.8-kb fragment of plant DNA. This fragment was isolated by reverse cloning (see Materials and methods). Mapping of this large plasmid (Figure 6) with restriction enzymes showed that no rearrangements seemed to occur during integration into the host genome. The plant DNA next to the point of integration was used as a probe in 'Southern' hybridizations with DNA from normal tobacco cells and shown to hybridize to highly repetitive DNA sequences (data not shown).

Transcription of animal genes in plant cells

RNA from tumor lines IMPOV 12 and IMPαA6.2 was isolated, poly(A)$^+$ and poly (A)$^-$ RNA were separated on oligo d(T)-cellulose and electrophoresed on agarose gels in formaldehyde. After blotting the RNA on nitrocellulose, the filters containing ovalbumin (RNAov, A$^+$; A$^-$) and α-actin RNA (RNAαA, A$^+$; A$^-$) were hybridized with ovalbumin and α-actin genomic DNA, respectively. The results failed to indicate any transcription of the α-actin gene in plant tumors. Northern blots with RNA from the ovalbumin tumor line IMPOV 12 showed hybridization with the isolated genomic DNA from pOV12 (Figure 5b). The size of the RNA transcribed from the ovalbumin gene ranges between 2 and 7 kb. To ensure that this hybridization is not an artefact, ^{32}P-

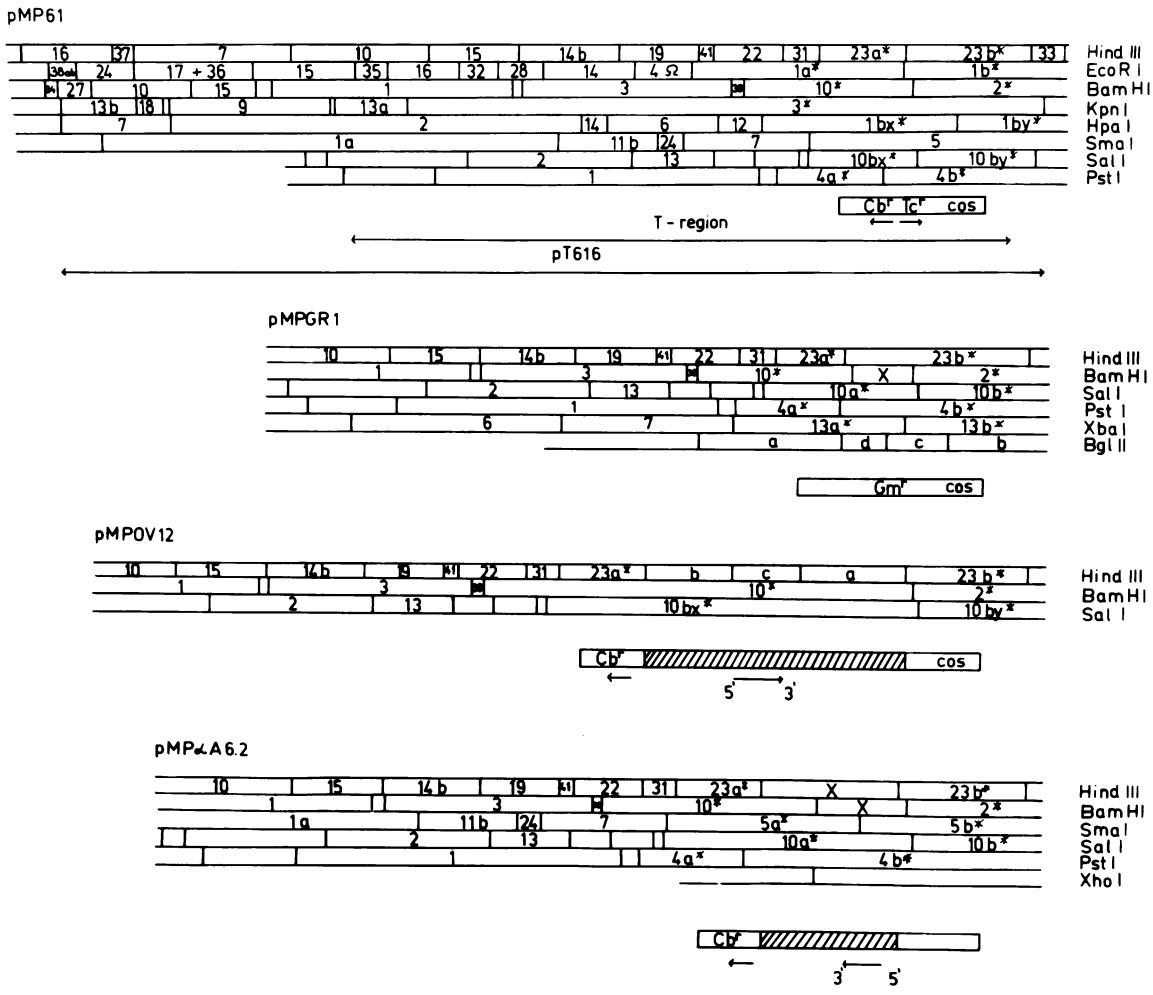

Fig. 4. Physical map of the T-region of recombinant Ti-plasmids pMP61, pMPGR1, pMPOV12 and pMPαA6.2. *Pst*I sites in the α-actin gene are not shown.

labeled cDNA was made with polyadenylated RNA from the tumor lines IMPOV 12 and IMPαA6.2 and hybridized to filter-bound restriction fragments from pOV12 and pαA6.2, respectively, (Figures 5c,6b). The results show that genomic DNA from α-actin does not hybridize to cDNA from IMPαA6.2 whereas Southern blots from pOV12 show hybridization to cDNA prepared from the IMPOV 12 tumor line. As an internal hybridization control, the DNA from three other plasmids (pGV342, pGV354, pGV415) (Depicker *et al.*, 1980) containing various fragments from the T-region of Ti-plasmids, were cut with *Hind*III, blotted on nitrocellulose filters and hybridized to the cDNAs from IMPOV 12 and IMPαA6.2 tumor lines. Figures 5c and 6d show the expected hybridization of cDNA from IMPOV 12 and IMPαA6.2 to T-DNA fragments of pGV354, pGV415 and pGV342. To analyse the startpoint of transcription of the ovalbumin gene in plants the DNA was cut with *Hind*III (yielding fragments: 4.0, 4.8, 3.2 kb in length). The three fragments were separated on agarose and isolated from the gel. The DNA fragments were cut further with some other restriction enzymes, electrophoresed on agarose gels and blotted on nitrocellulose. These filters were then hybridized with ^{32}P-labeled cDNA made from polyadenylated RNA (RNA OV A$^+$) by reverse transcriptase. Figure 5d shows that two areas of the ovalbumin gene hybridize intensively to the cDNA. The first area is located within the 4.0-kb fragment (Figure 5d) which means upstream from the '*in vivo*' initiation point of transcription. The second area of hybridization is located in the 3.2-kb piece. Between both areas a region of ~1.5 kb does not seem to hybridize to the cDNA. The 4.8-kb fragment does not hybridize to the ^{32}P-labeled cDNA.

Discussion

An important recent development in plant molecular biology was the construction of different Ti-plasmids which can be used as vectors for genetic engineering in plants. It has been shown that the T-region of the Ti-plasmid of *A.tumefaciens* is integrated into the genome of plant cells after infection and therefore can be used as a vehicle for incorporation of foreign genes into plants (Hernalsteens *et al.*, 1980). One way to use the Ti-plasmid as an experimental gene vector is to insert the foreign DNA sequences in the T-DNA region of an acceptor Ti-plasmid. The foreign genes are subsequently transferred to the nucleus of plant cells by infection with *Agrobacteria* carrying the vector Ti-plasmid. Here we report a fast and simple method by which foreign genes can be stably integrated into the T-region of a Ti-plasmid. The procedure involves two recombination steps. It should be emphasized that both recombination steps can be detected after a single conjugation of *E.coli* containing the foreign gene inserted into pBR322 (donor) with *A.tumefaciens* containing the recipient Ti-plasmid. Co-integrates formed by a single crossing-over are usually fairly stable in *Agrobacteria* under selective

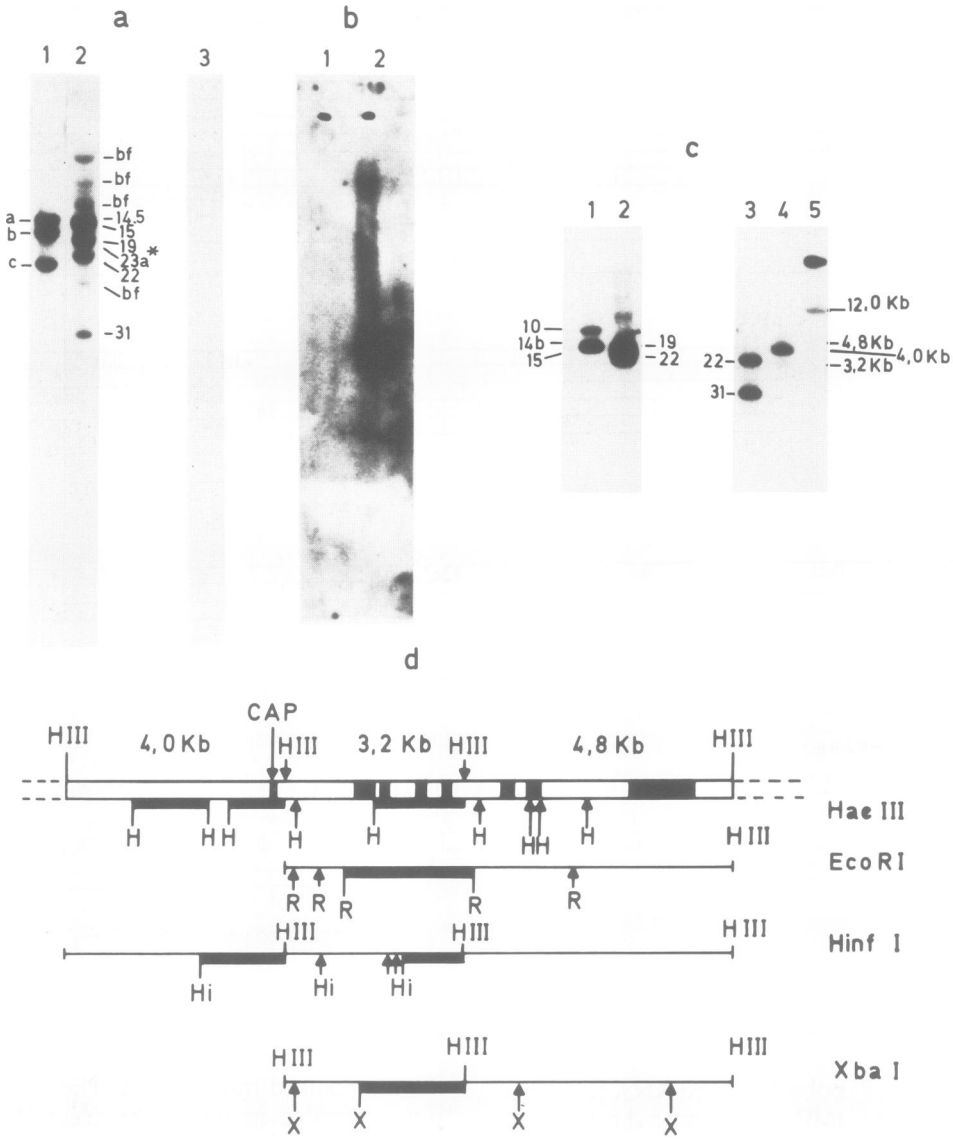

Fig. 5. a, (1 and 2) Southern blot of DNA from SRI tumor line IMPOV 12 containing the chicken ovalbumin gene. The DNA was cut with *Hind*III. **1;** hybridization with the *Hha*I fragment of pOV12 (chicken DNA). Fragments a,b,c see Figure 4. **2;** hybridization with the *Kpn*I fragments 3,13 (see Figure 4). Fragments 13a and 3 contain the left and right border of the T-DNA, respectively. Fragments 14b,15,19,23a*,22,31 see Figure 4. The four fragments named bf contain the border fragments of the T-DNA and the neighbouring plant DNA. **(3)** Southern blot W 38 tobacco DNA (no tumor). This filter was hybridized with the *Hha*I fragment (chicken DNA) of pOV12. **b, 1,2;** Northern blot of RNA [**lane 1** = poly(A)$^-$; **lane 2** = poly(A)$^+$] from tumor line IMPOV 12. The filter was hybridized with the *Hha*I fragment of pOV12 (chicken DNA). The polyadenylated RNA hybridizes with the ^{32}P-labeled DNA. **c;** Southern blots of the following plasmids: **1;** pGV342 (cut *Hind*III). **2;** pGV354 (cut *Hind*III). **3;** pGV415 (cut *Hind*III) (Depicker *et al.*, 1980). **4;** pOV12 (cut *Hind*III). **5;** pOV12 (cut *Hha*I). The filter was hybridized with ^{32}P-labeled cDNA homologous to polyadenylated RNA isolated from the tumor line IMPOV 12. The fragments (10), 14b, 15, 19, 22, 31 hybridize to cDNA homologous to mRNA transcribed from the T-DNA genes. The ovalbumin gene is cut into three pieces by *Hind*III; (4.0; 3.2; 4.8 kb). Only the 4.0-kb piece (5′ end of the gene) hybridizes strongly to the cDNA. **Lane 4.** The hybridization of the 3.2-kb piece is much weaker. **Lane 5** shows the hybridization of the 12-kb long *Hha*I fragment of pOV12 to the cDNA from plant tumors. **d;** The plasmid pOV12 was cut with *Hind*III, the three fragments of chicken DNA (4.8; 4.0; 3.2 kb) were isolated, recut with *Hae*III, *Eco*RI, *Hin*f, *Xba*I and blotted on nitrocellulose filters after electrophoresis on agarose gels. The filters were then hybridized with ^{32}P-labeled cDNA from polyadenylated RNA from IMPOV 12. The picture shows the ovalbumin gene with eight exons (black boxes) and seven introns (white boxes). The black bars show the areas of the ovalbumin gene which hybridize to the ^{32}P-labeled cDNA.

pressure. However, without continuous selection on carbenicillin and gentamycin, the resistant markers and the integrated foreign genes are lost with a frequency of $10^{-2}-10^{-3}$. If the T-region of a co-integrate pBR322 Ti-plasmid is used to transform plants, the foreign genes might also be lost in the plant, when they are not under selective pressure. Therefore selection of double recombinants prior to plant transformation might be necessary.

The ovalbumin and α-actin genes from chicken have been integrated into the tobacco genome. Both genes are present in the corresponding tumor lines IMPαA6.2 and IMPOV 12 (Figures 5a and 6a). To analyse further the integrated vector DNA and the plant sequences at the integration site, we re-isolated a large (19.4 kb) fragment from the tumor line IMPαA6.2 containing the ampicillin resistance marker, the α-actin gene, part of the T-DNA and a 1.8-kb piece of plant DNA (Figure 6c). This was carried out by the reverse cloning technique described in Materials and methods. This simple and fast method allows re-isolation of the vector DNA integrated into the host genome and part of the plant DNA pro-

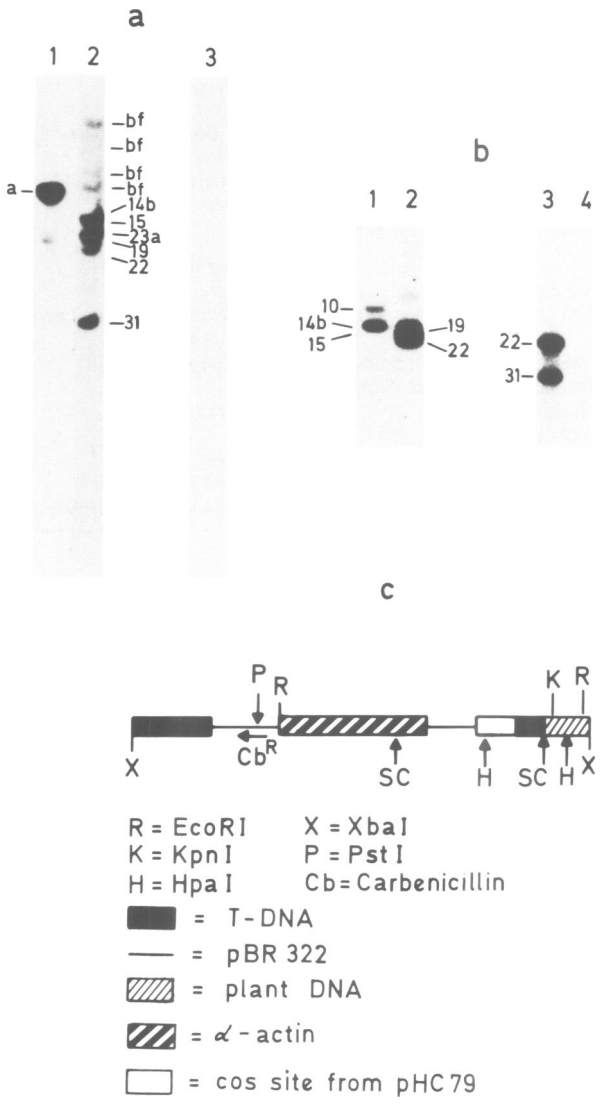

Fig. 6. a, (1 and 2) Southern blot of DNA from the SRI tumor line IMPαA6.2 containing the α-actin gene from chicken. The DNA was cut with *Hind*III. **1;** hybridization with the isolated *Hind*III fragment (chicken DNA) of plasmid pαA6.2. Fragment a; see Figure 4. **2;** hybridization with the *Kpn*I fragments 3, 13 (see Figure 4). Fragments 13a and 3 contain the left and right border of the T-DNA, respectively. Fragments 14b, 15, 19, 23a*, 22, 31 (see Figure 4). The four fragments named bf contain the border fragments of the T-DNA and the neighbouring plant DNA. **a, (3)** Southern blot of W 38 tobacco DNA. This filter was hybridized with the isolated *Hind*III fragment (chicken DNA) of plasmid pαA6.2. The plant α-actin gene does not hybridize to the chicken probe under the stringency used in this hybridization. **b;** Southern blots of the plasmids (cut with *Hind*III). **1;** pGV342, **2;** pGV354, **3;** pGV415 (Depicker *et al.*, 1980), **4;** pαA6.2. The filter was hybridized with ^{32}P-labeled cDNA synthesized from oligo d(T)-primed poly(A)$^+$ RNA isolated from the tumor line IMPαA6.2. The fragments (10), 14b, 15, 19, 22, 31 hybridize to cDNA homologous to mRNA transcribed from the T-DNA genes. **Lane 4** shows no hybridization of the α-actin gene from chicken to cDNA from IMPαA6.2 mRNA poly(A)$^+$. **c;** Structure of the plasmid pTPαA17. DNA from the tumor line IMPαA6.2 has been cut with *Xba*I and ligated with T4 DNA ligase. Transformed bacteria have been selected on ampicillin plates (reverse cloning).

vided the vector contains a resistant marker gene expressed in the bacterial cloning host. Restriction mapping demonstrated that the T-DNA was integrated into the plant DNA without detectable rearrangement. The plant DNA located next to the integration site was shown to be highly repetitive.

Northern blots with isolated genomic DNA containing the α-actin or the ovalbumin gene as probes, indicated that the ovalbumin gene was transcribed in plant tumors, whereas no RNA was synthesized from the α-actin gene. Figure 5b shows that the transcribed RNA is polyadenylated and ranges in size between 2 and 7 kb. The heterogenous size of the RNA transcribed from the ovalbumin gene in plant tumor cells could be the result of unspecific initiation or termination of transcription or of unspecific splicing of a large precursor. If transcription terminates at a different position in the plant cells compared with the oviduct tissue, a new polyadenylation site has to be used as signal for polyadenylating the transcribed RNA.

The ovalbumin structural gene consists of seven large introns and eight small exons which all together are transcribed to a precursor of ~7.8 kb (Dugaiczyk *et al.*, 1979; Tsai *et al.*, 1980; Gompton *et al.*, 1983). This precursor is processed by splicing to the mature mRNAov of ~1.8 kb.

The ovalbumin gene (pOV12) can be cut by the restriction enzyme *Hind*III into three fragments, 4.0, 4.3, 4.8 kb in size (Lai *et al.*, 1980).

The promoter of the gene and the initiation point of transcription are located on the 4.0-kb fragment. The start point of transcription is located 226 bp, and the region necessary for specific regulation of transcription ~360–470 bp, upstream from the *Hind*III site (Figure 5d). Two areas of the ovalbumin gene hybridized to cDNA from polyadenylated RNA from the IMPOV 12 plant tumor line. The first area is located within the 4.0-kb fragment (Figure 5d) and the second in the 3.2-kb piece. Possibly the RNA polymerase from plants starts initiation of transcription ~2 kb upstream from the cap site within the 4-kb *Hind*III fragment of the ovalbumin gene. Transcription of the polymerase preferentially terminates at two positions within the gene. One termination signal for the plant polymerase is located close to the cap site in the first intron and the second in the fifth intron. The synthesis of the first strand of the cDNA hybridization probe starts at the 3′ end of the mRNA and the average size of the cDNA normally lies between 400 and 800 bp. With these short cDNA molecules as probes only those DNA fragments close to the termination point can hybridize. Our data do not indicate whether any splicing or translation of the transcribed mRNA occurs in plant cells.

The data in this paper confirm that foreign genes (e.g., from animals) can be transported into plant cells by the Ti-plasmid and integrate into the genome. The technique used for this integration is simple and allows the transport of large foreign DNA pieces into the cell. Animal genes integrated into plants are either not transcribed (α-actin gene) or expressed in an abnormal way (ovalbumin gene). It might contribute to our understanding of the mechanisms of initiation of transcription by plant RNA polymerase to identify the sequences in the ovalbumin gene that play a role in its transcription in plant cells.

Materials and methods

Bacterial strains and plasmids are listed in Table II.

Cloning techniques

For large and small scale preparation of pBR322-related plasmid DNA the procedure of Birnboim and Doly (1979) was used. Ti-plasmid DNA was prepared according to Casse *et al.* (1979). Total bacterial DNA was isolated as described by Dhaese *et al.* (1979). Conditions of restriction endonuclease digestion, ligation, transformation of *E.coli*, agarose gel electrophoresis, nick-translation, blotting of agarose gels and DNA/DNA-hybridization were described previously (Holsters *et al.*, 1982; Dhaese *et al.*, 1979; Willmitzer *et*

Table II. Bacterial strains and plasmids

Bacterial strains	Relevant markers	Specification	Reference
E.coli HB101	pro leu his lacY Sm^R r_K^- m_K^-		H.W.Boyer; D.Roulland-Dussoix
E.coli AB1157	arg his leu pro thr lae gal ara Sm^R		D.J.Sherratt
A.tumefaciens GV3101	C58C1 Rif^R		Van Larebeke *et al.* (1974)
A.tumefaciens GV3103	C58C1 Sm^R Sp^R		Holsters *et al.*, 1980
Plasmids			
pBR322	Tc^R, Ap^R/Cb^R		Bolivar *et al.*, 1977
R64drd11	Tc^R Sm^R	I-type plasmid transfer derepressed	N.Datta
pGJ28	Km^R/Nm^R	Cda^-/da^- ColD replicon carrying ColE1 mob and bom	G.J.Warren; Finnegan and Sherratt (1982)
pGV3105	Tra^c noc^c occ^+	pTiC58 nopaline Ti-plasmid derepressed for autotransfer, nopaline catabolism constitutive, catabolizes octopine	Holsters *et al.* (1980)
pOV12	Ap^R	pBR322 carrying 12.2-kb chicken DNA	Lai *et al.* (1980)
pαA6.2	Ap^R	pBR322 carrying 6.2-kb chicken DNA	
p45-2	Ap^R Gm^R	pBR322 carrying *Bam*HI fragment of pJR88	J.Davies, S.Kagan
pGA46	Cm^R		An and Friesen (1979)
pHC79	Ap^R Tc^R		Hohn and Collins (1980)

al., 1982; Van Haute *et al.*, 1983; Leemans *et al.*, 1981).

In vitro mutagenesis techniques

The *'in vitro'* mutagenesis technique used for the construction of the recombinant Ti-plasmid pMP61 was described previously (Leemans *et al.*, 1981; Van Haute *et al.*, 1983). Intermediate vector pCHC2379 has been constructed as described in the text. Transformation of *E.coli* strain AB1157 (R64drd11, pGJ28) and mobilization of the plasmid to *Agrobacterium* recipient strain GV3101 (pGV3105) was as described by Van Haute *et al.* (1983). Transconjugants were purified and crossed with *Agrobacterium* recipient GV3103. GV3103 exconjugants carrying pGV3105:pCHC2379 co-integrates were selected on LB-agar media containing streptomycin (200 μg/ml), spectinomycin (100 μg/ml) and kanamycin (25 μg/ml) as described (Leemans *et al.*, 1981). Double recombinants were selected by a second Ti-plasmid conjugation by mating GV3103 (pGV3105: pCHC2379) x GV3101. Exconjugants were enriched on octopine minimal media (Leemans *et al.*, 1981) containing rifampicin (100 μg/ml) and carbenicillin (100 μg/ml) and replica plated on octopine indicator agar media (Hooykaas, 1979) and LB-agar containing carbenicillin (100 μg/ml) and kanamycin (25 μg/ml), respectively. Rif^R Cb^R Km^S occ^+ exconjugants were purified three times and subjected to DNA/DNA hybridization analysis.

pBR322 exchange cloning

pBR322 recombinants, pOV12 and pαA6.2 were transformed to *E.coli* strain AB1157 (R64drd11, pGJ28). Transformants were selected on LB-plates containing streptomycin (20 μg/ml), tetracycline (20 μg/ml), kanamycin (25 μg/ml) and carbenicillin (100 μg/ml). pBR322 recombinants were transmitted to *Agrobacterium* recipient GV3101 (pMPGR1) by mobilization as described (Van Haute *et al.*, 1983).

Exconjugants were selected on minimal indicator (BTB) plates containing 2% sucrose instead of glucose and 100 μg/ml carbenicillin and replica plated on LB-agar plates containing 25 μg/ml gentamycin. Rif^R, Cb^R, Gm^S, occ^+ colonies were purified three times.

Analysis of plant tumors

Tumor induction, cultivation of tobacco crown gall tumor tissue and detection of nopaline were described previously (Leemans *et al.*, 1981; Otten and Schilperoort, 1978).

Plant DNA and RNA from tobacco tumors were isolated and purified according to Murray and Thompson (1980). Poly(A)$^+$ and poly(A)$^-$ RNA were separated on an oligo d(T) column, electrophoresed and blotted on nitrocellulose filters according to Kreuzaler *et al.* (1983). cDNA from the poly(A)$^+$ RNA fraction was synthesized according to Willmitzer *et al.* (1982).

Reverse cloning of DNA integrated in the plant genome

300 μg of plant DNA were cut with 500 units of *Xba*I, phenol extracted, precipitated and self-ligated in a volume of 6 ml with 400 units of T4 DNA ligase (Boehringer, Mannheim). *E. coli* JF 1754 (McNeil and Friesen, 1981) was transformed with the ligated DNA and the bacteria were plated on ampicillin plates (100 μg/ml). The plasmid DNA from those clones growing on the antibiotic were analysed by restriction mapping.

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