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The promoter of T_L-DNA gene *5* controls the tissue-specific expression of chimaeric genes carried by a novel type of *Agrobacterium* binary vector

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Summary. A "plant gene vector cassette" to be used in combination with various *Escherichia coli* gene-cloning vectors was constructed. This cassette contains a replication and mobilization unit which allows it to be maintained and to be transferred back and forth between *E. coli* and *Agrobacterium tumefaciens* hosts provided these hosts contain plasmid RK2 replication and mobilization helper functions. The cassette also harbors a transferable DNA unit with plant selectable marker genes and cloning sites which can be combined with different bacterial replicons, thus facilitating the reisolation of transferred DNA from transformed plants in *E. coli*. The vector cassette contains two different promoters derived from the T-DNA-encoded genes *5* and nopaline synthase (*NOS*). By comparing the levels of expression of the marker enzymes linked to each of these promoter sequences, it was found that the gene *5* promoter is active in a tissue-specific fashion whereas this is not the case for the *NOS* promoter. This observation provides the first documented instance of a gene derived from a procaryotic host the expression of which is apparently regulated by plant growth factors.

Key words: *Agrobacterium* binary vector system – Plant transformation – Tissue specificity – T-DNA-encoded genes

Introduction

The *Agrobacterium* Ti-plasmids are extensively used for the introduction of foreign genes and for the analysis of their expression in higher plants (Murai et al. 1983; Odell et al. 1985; De Block et al. 1984; Facciotti et al. 1985; Simpson et al. 1985; Lamppa et al. 1985; Spena et al. 1985; Kaulen et al. 1986). The use of *Agrobacterium tumefaciens* Ti-plasmids as gene-transfer vectors was based on the recognition that during interaction of agrobacteria with wounded plant tissues a well-defined segment of their Ti-plasmid, called T-DNA, is transferred to plant cells, integrated into the plant nuclear DNA and transcribed by the host RNA polymerase II. (For reviews see: Kahl and Schell 1982; Caplan et al. 1983; Schell et al. 1984; Verma and Hohn 1984.)

The position and polarity of flanking 25 bp direct repeats, bordering the T-DNA sequences on the Ti-plasmid, appear to be essential for the transfer and/or integration of T-DNA into the plant genome (Wang et al. 1984). Transfer and integration of T-DNA is not dependent on the expression of T-DNA encoded genes, but rather governed by genes unlinked to the T-DNA, in the virulence (*vir*) region of the Ti-plasmid (Joos et al. 1983; Hoekema et al. 1983; Klee et al. 1983; Hille et al. 1984). These genes are known to be expressed in bacteria on induction by a small molecular weight metabolite produced in wounded plant tissues (Stachel et al. 1985a, b). Preceding the transfer, the products of the induced *vir* loci initiate formation of a T-DNA intermediate which carries a single border, presumable originating from the recombination of the right- and left-hand 25 bp border repeats (Koulikova-Nicola et al. 1985.) T-DNA inserted into Ti-independent plasmid replicons (or in the *Agrobacterium* chromosome) is transferred to plant cells, with the help of the *vir* function provided in *trans*, as efficiently as T-DNA physically linked to the *vir* loci in *cis* (Hoekema et al. 1983, 1984; De Framond et al. 1983).

New Ti-plasmid-derived vectors, referred to as *Agrobacterium* binary cloning systems, have two elements: a broad host-range cloning vector carrying functional T-DNA border sequences and a Ti-derived helper plasmid providing virulence functions in *trans* (De Framond et al. 1983; Hoekema et al. 1983; Bevan 1984). In this report we describe the characterization of a novel binary vector system which was used to study the promoter of the Ti-plasmid T_L-DNA encoded gene *5* in transformed plants.

A new feature of this system, compared to others published recently (Bevan 1984; An et al. 1985; Klee et al. 1985; Van den Elzen et al. 1985) is that the different elements needed in plant cloning vectors (such as selectable and screenable marker genes flanked by T-DNA borders, cloning sites, appropriate bacterial genes to facilitate identification of transconjugants, broad host-range replication and mobilization functions) are all combined in a single unit, referred to as a "plant vector cassette". This cassette can easily be combined with a number of different

Abbreviations: OCS, octopine synthase (gene); NOS, nopaline synthase (gene); NPT-II, neomycin phosphotransferase (gene) of transposon Tn*5*; *vir*, Ti-plasmid region encoding virulence functions; Cb, carbenicillin; Gm, gentamycin; Km, kanamycin; Cm, chloramphenicol; Sm, streptomycin; Sp, spectinomycin; Rif, rifampicin; Ery, erythromycin; *bom*, basis of mobilization; ori_T, origin of conjugational plasmid transfer; Tra, Mob, functions required for conjugational transfer of plasmids; BAP, N^6-benzylaminopurine; NAA, α-naphthaleneacetic acid; CTAB, N-cetyl-N,N,N-trimethyl-ammonium bromide

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Escherichia coli cloning vectors allowing ready construction of binary vectors with different cloning properties.

In this cassette the T-DNA is coupled to a conditional mini-RK2 replicon containing only the ori_V and ori_T sequences of the broad host-range plasmid RK2. The replicon is conditional because the ori_V and ori_T elements alone, uncoupled from other regions of plasmid RK2, are not functional. When plasmid RK2 loci *trf* and *tra* (encoding functions required for replication at ori_V and conjugational transfer initiated at ori_T, respectively) are provided in *trans*, vectors carrying the cassette (or the cassette itself) can be maintained in *Agrobacterium* and in *E. coli* and can be efficiently mobilized between these hosts. The system is versatile. Each element of these cloning vectors can be modified easily and can be used coupled with the *vir*-region functions of many *Agrobacterium* strains.

The vector cassette contains two promoters derived from different T-DNA encoded genes, namely the T_L-DNA gene *5* and the nopaline synthase (*NOS*) gene. Since the function of gene *5* is not yet understood, we decided to exploit these vectors to compare the regulation of the promoter activity of gene *5* to that of the *NOS* promoter. Interestingly, it was thus found that the gene *5* promoter activity, but not the *NOS* promoter activity, is regulated in a tissue-specific fashion.

Materials and methods

Bacterial strains and plasmids. These are listed in Table 1.

Microbiological and cloning methods. Bacterial media, culture conditions, concentration of antibiotics, transformation and isolation of plasmid and bacterial chromosomal DNAs were as described previously (Koncz et al. 1983, 1984). Cloning procedures involving DNA fragment isolation, filling in protruding 3′- or 5′-ends of DNA fragments by Klenow fragment of *E. coli* DNA polymerase I or T4 DNA polymerase, phosphatase treatment of DNAs, ligations and addition of synthetic oligonucleotide linkers were as described by Maniatis et al. (1982).

Plant vector cassettes and cloning vectors. Figure 1 illustrates the general design of the proposed versatile plant vector cassettes. In order to construct the conditional replicon unit of plant vector cassettes, the RK2 ori_V sequence either by itself or coupled with a *cos* DNA sequence from phage lambda was subcloned and joined to the ori_T sequence of plasmid RK2. In the cassette this replicon unit is linked to the DNA to be transferred (T-DNA) which is defined by the 25 bp border repeats (Fig. 1).

For assembly of conditional mini-RK2 replicon units of plant vector cassettes, the *Eco*RI site of plasmid pLAFRI (Friedman et al. 1982) was converted to a *Hin*dIII site by addition of a synthetic linker. Subsequently the *Hin*dIII–*Bgl*II or *Hin*dIII–*Bst*EII fragments from this plasmid carrying the ori_V sequence of plasmid RK2 or the ori_V sequence coupled to the phage lambda *cos* sequence, were subcloned in plasmid pKC7 (Rao and Rogers 1979) cut by the same enzymes, thus yielding pKC710 and pKC720, respectively. The ori_T sequence of plasmid RK2 was subcloned from plasmid pSUP101 (Simon et al. 1983). The *Sph*I–*Eco*RV fragment of pSUP101 DNA was isolated, digested partially with *Sau*3A and treated with nuclease Bal31. After filling the recessed ends with the Klenow fragment of *E. coli* DNA polymerase I and ligation of a *Bam*HI linker, the mixture of fragments, of different lengths, was subcloned into the *Bam*HI site of plasmid pBR325 (Bolivar 1978) and transformed into *E. coli* strain SM10 (Simon et al. 1983) containing the mobilization helper functions of plasmid RK2 on its chromosome. Plasmid recombinants were tested for the presence of a functional ori_T by conjugation to *E. coli* strain HB101 Rif^r. A *Bam*HI fragment of 860 bp carrying ori_T was isolated from one of the transconjugants and ligated into the single *Bgl*II site of plasmids pKC710 and pKC720.

The right T-DNA border sequence derived from pTiC58

Table 1. Bacterial strains and plasmids

Bacterial strains	Relevant markers	References
Escherichia coli		
HB101	*pro leu his lac*Y *sup*F Sm^r r_K^- m_K^-	Boyer and Roulland-Dussoix (1969)
HB101 Rif^r		A. Kondorosi
AB1157	*arg his leu pro thr lac gal ara* Sm^r	D.J. Sherrat
C2210	*pol*A1 *his rha*	M. Kahn
SM10	*thi thr leu* su_{III}	Simon et al. (1983)
Agrobacterium tumefaciens		
GV3101	C58C1 Rif^r	Van Larebeke et al. (1974)
GV3103	C58C1 Sm^r Sp^r	Holsters et al. (1980)
GV3105	C58C1 Cm^r Ery^r	Holsters et al. (1980)
GV3101CT	GV3101::pCT153.1	This work
GV3103CT	GV3103::pCT153.1	This work
Plasmids		
PiAN7	*sup*F	H.V. Huang; Seed (1983)
pBR322	Ap^r Tc^r	Bolivar et al. (1977)
pBR325	Ap^r Tc^r Cm^r	Bolivar (1978)
pHC79	Ap^r Tc^r	Hohn and Collins (1980)
pKC7	Ap^r Km^r	Rao and Rogers (1979)
p45-2	Ap^r Gm^r	S. Kagan; Koncz et al. (1984)
pLAFRI	Tc^r	Friedman et al. (1982)
pRK2013	Km^r	Figurski and Helinski (1979)
pCT153.1	Km^r Tc^r	Thomas (1981)
pSUP401	Km^r Cm^r	Simon et al. (1983)
pGV23neo	Ap^r	Herrera-Estrella et al. (1983b)
pAGV40	Km^r	Herrera-Estrella et al. (1983a)
pGV0153	Ap^r	De Vos et al. (1981)
pGV354	Ap^r	Depicker et al. (1980)
pGV316	Ap^r	Depicker et al. (1980)
pGV2217	Km^r deletion of T_L-DNA of pTiB6S3	Leemans et al. (1982b)
pGV2275	Km^r, deletion of T_L+T_R-DNAs of pTiB6S3	R. Deblaere
pGV2201	Sm^r Sp^r	Leemans et al. (1982a)
pMP90	Gm^r, deletion of T-DNA of pTiC58	This work
pMP90RK	Gm^r Km^r	This work
R64drd11	Tc^r Sm^r	N. Datta
pGJ28	Km^r	Finnegan and Sherrat (1982)

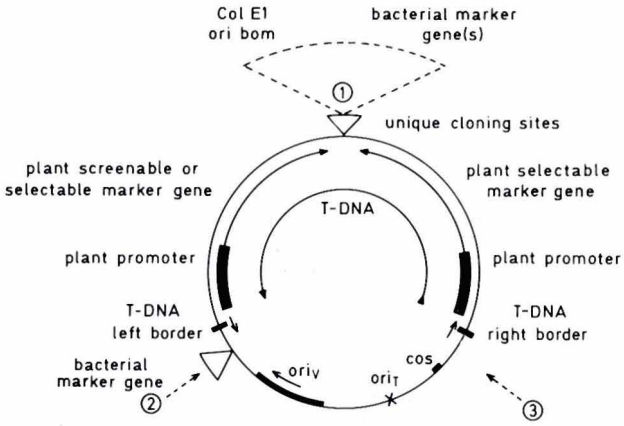

Fig. 1. Schematic design of plant gene vector cassettes. The position of a T-DNA unit, the polarity of T-DNA border sequences and the direction of replication initiated at ori_V sequences are labelled by *arrows* inside the circle. *Arrows* 1, 2 and 3 mark unique restriction endonuclease cleavage sites (1, *Bgl*II, *Sal*I, *Bam*HI; 2, *Kpn*I; 3, *Apa*I, *Xho*I) which can be used to introduce new elements into the cassettes. The vectors described in the text contain insertions of plasmid pBR322 derivatives in site 1

was integrated into the cassette along with a plant selectable marker gene. The vectors described here contain a chimaeric gene consisting of the promoter of nopaline synthase fused to the NPT-II coding sequence of transposon Tn*5* and to a polyadenylation signal sequence derived from the octopine synthase gene.

The right border sequence for the plant vector cassette T-DNA unit was initially isolated along with the *NOS* promoter-*NPT-II* chimaeric gene sequence as the *Bst*EII – *Sma*I fragment of plasmid pLGVneo23 (Herrera-Estrella et al. 1983b) and coupled to the *Pvu*II – *Hin*dIII fragment of plasmid pAGV40 DNA (Herrera-Estrella et al. 1983a) carrying 3′ polyadenylation sequences from the *OCS* gene and subcloned in plasmid pHC79, cut by restriction endonucleases *Hin*dIII and *Bst*EII.

This construction was further modified by replacing the *Eco*RI – *Hin*dIII fragment at the 3′-end of the chimaeric gene with a polylinker sequence derived from miniplasmid PiAN7. When the constructed plasmid was tested, it was noticed that transformed plants were not resistant to more than 50–60 μg/ml of kanamycin. The weak expression was due to a second, non-frame AUG codon preceding the *NPT-II* coding sequence (Beck et al. 1982; Bevan 1984). An extra *Bgl*II site was also found, in contrast to the expected sequence (Herrera-Estrella et al. 1983b), in the *NOS* promoter-*NPT-II* junction sequence. Therefore the construction was modified by replacing sequences downstream of the *NOS* promoter with a new *NPT-II* coding sequence derived from plasmid pKm109/2 (Reiss 1982; Herrera-Estrella, unpublished results) which was than coupled to a *Pvu*II – *Bst*EII fragment containing the 3′ polyadenylation sequences of the *OCS* gene (Gielen et al. 1984). In test experiments the new chimaeric gene allowed routine selection of transformed plant tissues at kanamycin concentrations of 100–200 μg/ml.

A *Hin*dIII – *Bgl*II fragment of pTiAch5 T_L-DNA carrying the left T-DNA border and gene *5* promoter sequences was subcloned from plasmid pGV0153 in plasmid pHC79. The *Bgl*II site interrupts the gene *5* promoter sequence 43 bp upstream from the translational initiation site (Barker et al. 1983; Gielen et al. 1984).

Vectors pPCV001 and pPCV002 (Fig. 2, Table 2) were assembled by inserting the *Eco*RI – *Bst*EII fragment containing the T-DNA right border sequence and the chimaeric *NPT-II* gene along with the *Hin*dIII – *Bst*EII fragments carrying the $ori_V - ori_T$ or the $ori_V - ori_T - cos$ replicon units into the *Eco*RI – *Hin*dIII sites of the plasmid containing the left T-DNA border and gene *5* promoter sequences. The *Hin*dIII site separating the left border from the replicon units was deleted by partial *Hin*dIII digestion followed by end-filling and religation of plasmid DNAs.

Primary plant vector cassettes carried by plasmids pPCV001 and pPCV002 were genetically marked by insertion of a Cm^r gene derived from plasmid pSUP401 (Simon et al. 1983). To obtain plasmids pPCV011 and pPCV021 (Fig. 2, Table 2) the ends of a 1.25 kb *Pst*I fragment carrying *CAT* coding sequences, derived from plasmid pSUP401, were made flush with T4 DNA polymerase and the fragment was cloned by blunt-end ligation into the *Kpn*I sites of plasmids pPCV001 and pPCV002 DNAs also filled in with T4 DNA polymerase. After *Bgl*II digestion, religation and transformation of plasmid DNAs into the *E. coli polA* mutant strain C2210 containing plasmid pCT153.1 (Thomas 1981) the mini-RK2 plasmids pRK011 and pRK021 were obtained (Fig. 2, Table 2).

Mini-RK2 replicons pRK011 and pRK021 can be maintained in *E. coli* strains carrying plasmids pCT153.1 or pRK2013 (Thomas 1981; Figurski and Helinski 1979) which provide the replication and maintenance functions of plasmid RK2 in *trans*. In these strains the copy number of plasmids pRK011 and pRK021 is low (5–10 copies/cell). These plant vector cassettes can be used as such as binary vectors. However, in this case purification of plasmid DNA from pRK011 and pRK021 would require an extra size selection step in order to separate them from helper plasmid DNAs.

Binary vectors independent of helper plasmids for their replication were constructed by combining the plant vector cassettes with multicopy *E. coli* plasmids. Unique sites (*Bgl*II, *Sal*I, *Bam*HI, *Apa*I, *Kpn*I and *Xho*I) in the cassette (labelled in Fig. 1 by arrows 1, 2, 3) were used to introduce the *E. coli* multicopy plasmids. Because a great number of *E. coli* plasmids are available, many different binary vectors can be constructed using this approach.

An additional property of pRK011 and its derivatives (Fig. 2, Table 2) is that because they contain the phage lambda *cos* sequence, these plasmids can also be used as cosmids (Hohn and Collins 1980).

As an example, a commonly used vector, pBR322, was inserted into the plant vector cassettes pRK011 and pRK021 (at insertion site 1, Fig. 1). After removal of plasmid pCT153.1 DNA by size separation, *Bam*HI-linearized pBR322 plasmid DNA was inserted into the *Bam*HI sites of plasmids pRK012 and pRK021 leading to plasmids pPCV012 and pPCV022 (Fig. 2, Table 2). Plasmid pPCV003 (Fig. 3, Table 2) was constructed by ligating *Bam*HI – *Bgl*II-linearized plasmid pRK011 DNA to the *Bgl*II fragment of a plasmid pHC79 derivative, carrying the Cm^r gene of plasmid pBR325.

Transcriptional fusions of the gene *5* promoter with sequences derived from the *OCS* gene were constructed with the plasmids pPCV012, pPCV022 and pPCV003. Plasmids, such as pAGV40 (Herrera-Esterella et al. 1983a), contain-

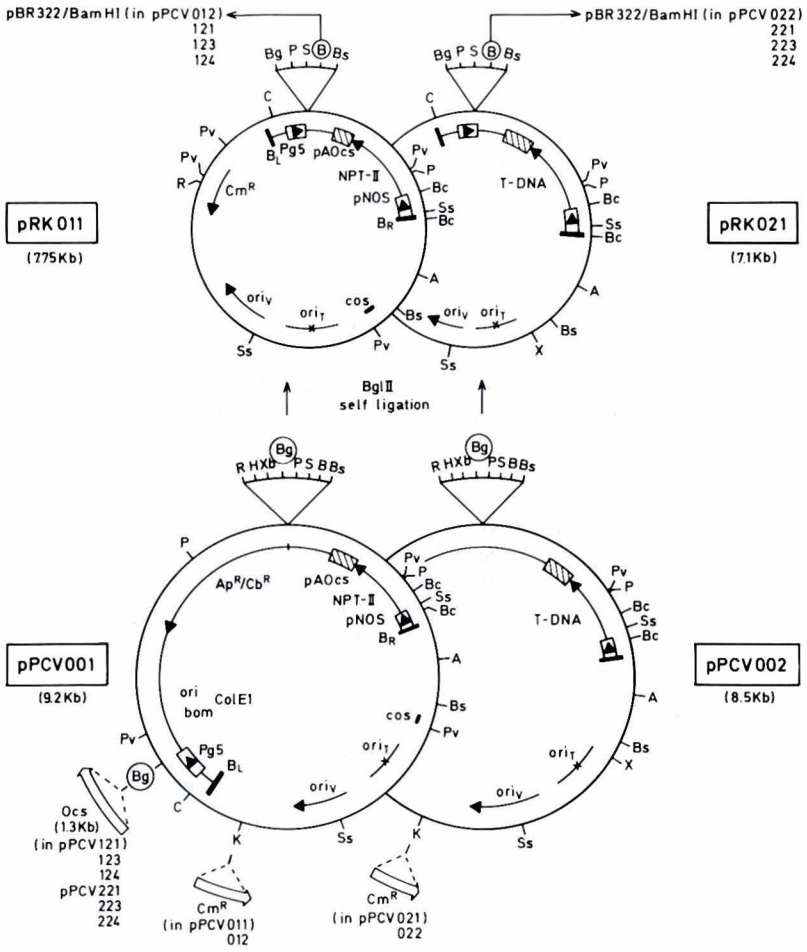

Fig. 2. Physical maps of plasmids pPCV001, pPCV002, pRK011, pRK021 and their derivatives. Positions of transferable segments (T-DNA units) are labelled inside the circular maps. Insertion of a Cm^r gene into the *Kpn*I site of plasmids pPCV001 and pPCV002 resulted in plasmids pPCV011 and pPCV021, respectively. Plasmids pRK011 and pRK021 were obtained by *Bgl*II digestion and self-ligation of pPCV011 and pPCV021 DNAs, respectively. Mini-RK2 replicons pRK011 and pRK021 were maintained in *Escherichia coli* strain C2210 (pCT153.1) or HB101 (pRK2013). Introduction of plasmid pBR322 into the *Bam*HI site of plasmids pRK011 and pRK021 led to plasmids pPCV012 and pPCV022, respectively. Plasmids pPCV121, 123, 124 and pPCV221, 223 and 224 were obtained by insertion of *Bam*HI fragments, carrying promoter deletion derivatives of the octopine synthase gene, into the unique *Bgl*II sites of plasmids pPCV012 and pPCV022, respectively (see Materials and methods). p*NOS*, promoter of nopaline synthase gene; p*AOCS*, polyadenylation sequence of octopine synthase gene; *NPT-II*, neomycin phosphotransferase gene of transposon Tn*5*; B_L and B_R, left- and right border sequences of vector T-DNAs; pg*5*, truncated promoter of T_L-DNA gene *5*. Restriction sites: A, *Apa*I; B, *Bam*HI; Bg, *Bgl*II; Bc, *Bcl*I; Bs, *Bst*EII; C, *Cla*I; R, *Eco*RI; P, *Pst*I; Pv, *Pvu*II; S, *Sal*I; Ss, *Sst*II; X, *Xho*I; K, *Kpn*I; Xb, *Xba*I

Table 2. Characteristics of constructed vectors

Plasmids	Cosmids	Bacterial markers	Cloning sites in the T-DNA	Plant markers	Size (kb)	Replicon
pPCV001	cos	Ap^r/Cb^r	*Eco*RI, *Hin*dIII, *Cla*I, *Xba*I, *Sal*I, *Bam*HI (*Kpn*I, *Apa*I)[a]	Km^r	9.20	pBR/RK2
pPCV002	–	Ap^r/Cb^r	As pPCV001 + (*Xho*I)	Km^r	8.56	pBR/RK2
pPCV011	cos	Ap^r/Cb^r Cm^r	*Hin*dIII, *Cla*I, *Xba*I, *Sal*I, *Bam*HI (*Apa*I)	Km^r	10.4	pBR/RK2
pPCV021	–	Ap^r/Cb^r Cm^r	As pPCV011 + (*Xho*I)	Km^r	9.76	pBR/RK2
pRK011	cos	Cm^r	*Cla*I, *Bgl*II, *Bam*HI, *Sal*I (*Apa*I, *Eco*RI)	Km^r	7.8	RK2
pRK021	–	Cm^r	As pRK021 + (*Xho*I)	Km^r	7.1	RK2
pPCV012	cos	Ap^r/Cb^r Cm^r	*Hin*dIII, *Bam*HI, *Sal*I (*Apa*I)	Km^r	12.2	pBR/RK2
pPCV022	–	Ap^r/Cb^r Cm^r	As pPCV012 + (*Xho*I)	Km^r	11.5	pBR/RK2
pPCV121	cos	Ap^r/Cb^r Cm^r	*Hin*dIII, *Sal*I (*Apa*I)	Km^r *OCS*	13.5	pBR/RK2
pPCV221	–	Ap^r/Cb^r Cm^r	As pPCV121 + (*Xho*I)	Km^r *OCS*	12.9	pBR/RK2
pPCV310	cos	Ap^r/Cb^r Cm^r Tc^r	*Hin*dIII, *Bam*HI, *Sal*I (*Kpn*I, *Apa*I)	Km^r *OCS*	14.2	pBR/RK2
pPCV311	cos	Ap^r/Cb^r Cm^r	*Sma*I, *Hpa*I, *Bam*HI (*Kpn*I)	Km^r *OCS ONC*	22.4	pBR/RK2
pPCV003	cos	Ap^r/Cb^r Cm^r Tc^r	*Eco*RI, *Bgl*II, *Hin*dIII, *Bam*HI, *Sal*I (*Kpn*I, *Apa*I)	Km^r	12.9	pBR/RK2

[a] Cloning sites outside of vector T-DNAs are given in brackets

ing promoter deletion derivatives of the *OCS* gene, were kindly provided by Dr. Ann Depicker. *Hin*dIII fragments of these plasmids (pAGV10, pAGV30 and pAGV40), carrying different deletion end-points determined by DNA sequencing (Depicker, unpublished) were modified by addition of *Bam*HI linkers and were inserted into single *Bgl*II sites of vectors pPCV012, pPCV022 and pPCV003 to obtain the derivatives: pPCV121, 123, 124; pPCV221, 223, 224; and pPCV310, 330 and 340. Plasmids of the pPCV310 series were further modified by inserting a *Bcl*I fragment of plasmid pGV354 DNA (Depicker et al. 1980) carrying the T-DNA genes *1, 2, 4* and *5* of pTiC58, into their *Bam*HI sites thus yielding the derivatives pPCV311, 331 and 341 (Fig. 3, Table 2).

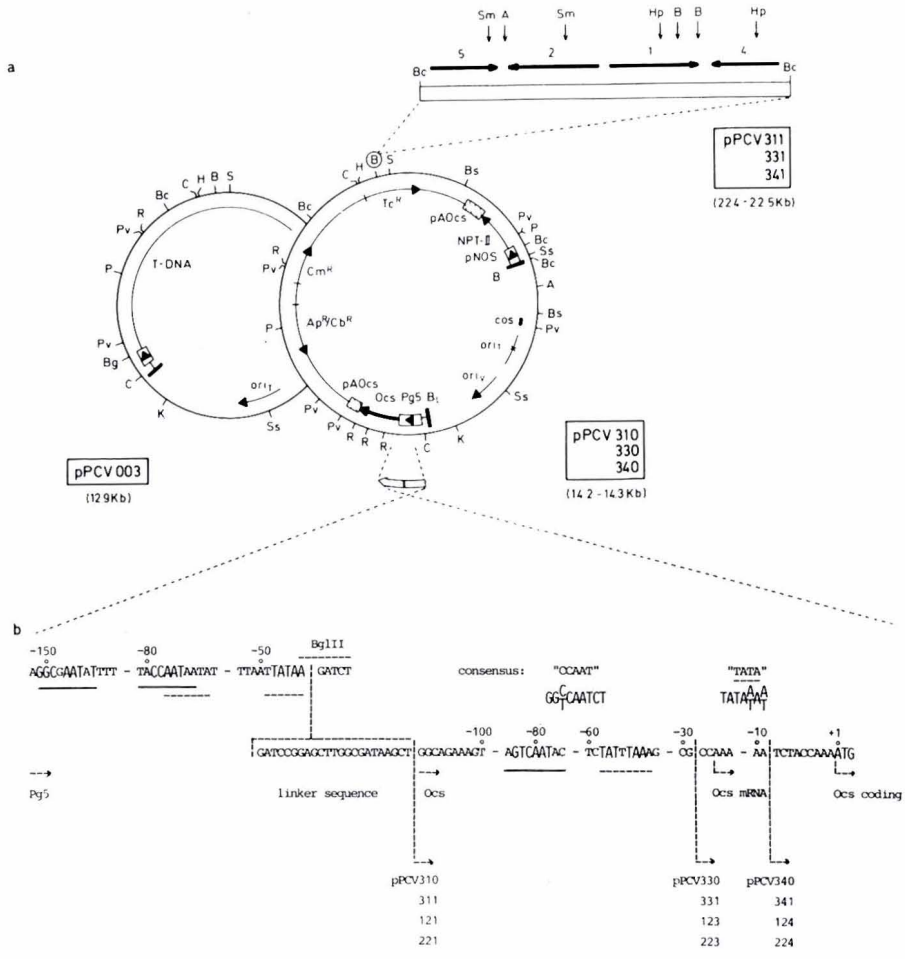

Fig. 3. a Physical maps of plasmids pPCV003, pPCV310, pPCV311 and their derivatives. (The construction of plasmids is described in Materials and methods). Abbreviations and labelling of T-DNA functions are as in Fig. 2; *OCS*, octopine synthase gene; Hp, *Hpa*I; Sm, *Sma*I.
b DNA sequence of gene *5* promoter – octopine synthase fusions in plasmids pPCV310, pPCV311, pPCV121, pPCV221 and their derivatives. The CCAAT and TATA boxes suggested by Gielen et al. (1984) and Barker et al. (1983) are *underlined* with *continuous* and *dashed lines*, respectively. Sequences identical to the CCAAT and TATA consensus sequences are written in *capital letters*. The *Bgl*II site was destroyed by the 23 bp linker sequence present in each fusion. *Dashed lines* show the last base pairs of the *OCS* sequences present in the different fusions. *OCS* mRNA, the 5′-end of the *OCS* wild-type transcript; *OCS* coding; the start of the *OCS* coding sequence

Agrobacterium hosts providing plasmid RK2 helper functions. pCT153.1 is a plasmid R6K derivative carrying a large fragment derived from RK2 and encoding replication and maintenance functions (Thomas 1981; Fig. 4). *Agrobacterium* hosts carrying plasmid pCT153.1 integrated into their chromosome were isolated by conjugation of *E. coli* strain C2210 (pCT153.1) with *Agrobacterium* strains GV3101, GV3103, GV3105 and LBA4301 cured of their Ti-plasmid (Van Larebeke et al. 1974; Holsters et al. 1980; Klapwijk et al. 1979). The conjugation and selection conditions have been described previously (Koncz et al. 1983, 1984). Since pCT153.1 cannot replicate in *Agrobacterium*, selection for its kanamycin resistance marker yields transconjugants carrying pCT153.1 insertions in their chromosome (Table 3). Chromosomal DNA samples isolated from selected non-auxotrophic transconjugants were cut with various restriction endonucleases, transferred onto nitrocellulose filters and analysed by Southern hybridization using nick-translated plasmid pCT153.1 DNA as a probe. Hybridization data (not shown) indicated that this integration of pCT153.3 in the *Agrobacterium* chromosome occurred at either the IS*8* or at the ori_T sequences of pCT153.1. (The frequency of integration mediated by IS*8* was found to be 5–10 times higher when compared to that occurring at ori_T sequences.) Transconjugants carrying plasmid pCT153.1 integrated into the *Agrobacterium* chromosome at ori_T were designated GV3101CT and GV3103CT.

The ideal *Agrobacterium* RK2 helper strain is one which provides the virulence functions essential for T-DNA transfer but without a wild-type T-DNA region which would induce tumour formation by transformed plant cells. This was achieved by introducing a number of Ti-plasmids from which the whole T-DNA region was deleted into strains GV3101CT and GV3103CT. The following T-DNA deleted Ti-plasmids were thus introduced into the RK2 helper strains pGV2217 (Leemans et al. 1982b), pGV2275 (Deblaere et al. 1985) or pMP90 (see below).

To have an alternative choice for easy reconstruction of the vector system in new *Agrobacterium* chromosomal backgrounds, the *trans*-acting replication and maintenance functions of plasmid RK2 were also inserted into a Ti-plasmid. This was achieved by stepwise modification of plasmid pTiC58 derivative pGV2201 (Leemans et al. 1982a) by using the marker exchange and gene replacement techniques described previously (Koncz et al. 1983, 1984). In order to delete the T-DNA region of plasmid pGV2201, which carries the Sm/Sp^r gene of plasmid R702 in its nopaline synthase gene (Leemans et al. 1982a) an intermediate vector was constructed providing a target for homologous recombination to the left and to the right of the T-DNA region. A sequence to the right of the T-DNA carried by plasmid pGV357 (Depicker et al. 1980), was marked by insertion of a gentamycin resistance gene, derived from plasmid p45-2 (Kagan, unpublished results; Koncz et al. 1984), at the junction of pTiC58 *Bam*HI fragments 2 and 3. Plasmid pGV357Gm1 was thus obtained. Its *Kpn*I frag-

Table 3. Conjugation/mobilization of plasmids between *Agrobacterium* hosts and *Escherichia coli* donors/recipients

	Matings			Conjugation/ mobilization frequencies
	Donors	Recipients		
(1)	*E. coli* C2210 (pCT153.1)	× *A. tumefaciens* GV3101		$1.2 - 1.65 \times 10^{-7}$
	E. coli C2210 (pCT153.1)	× *A. tumefaciens* GV3103		$1.3 - 1.86 \times 10^{-8}$
(2)	GV3101 (pMP90RK)	× GV3103	minA	$2.2 - 2.58 \times 10^{-3}$
			YEB	$0.8 - 2.0 \times 10^{-3}$
	GV3101 (pMP90RK)	× GV3105	minA	$1.3 - 2.6 \times 10^{-3}$
			YEB	$0.62 - 1.9 \times 10^{-3}$
(3)	*E. coli* AB1157 (R64drd11) (pGJ28) (pPCV310)	× GV3101 (pMP90RK)		$2.2 - 9.28 \times 10^{-5}$
	E. coli AB1157 (R64drd11) (pGJ28) (pPCV311)	× GV3101 (pMP90RK)		$1.4 - 3.4 \times 10^{-5}$ [a]
(4)	*E. coli* HB101 (pRK2013) (pPCV310)	× GV3101 (pMP90RK)		$1.6 - 4.75 \times 10^{-3}$
	E. coli HB101 (pRK2013) (pPCV311)	× GV3101 (pMP90RK)		$1.7 - 5.7 \times 10^{-3}$ [a]
(5)	*E. coli* SM10 (pPCV310)	× GV3101 (pMP90RK)		$0.64 - 2.35 \times 10^{-1}$
	E. coli SM10 (pPCV311)	× GV3101 (pMP90RK)		$0.54 - 1.11 \times 10^{-1}$
	E. coli SM10 (pPCV310)	× GV3101CT		$5.46 - 8.0 \times 10^{-2}$
	E. coli SM10 (pPCV311)	× GV3101CT		$3.27 - 5.64 \times 10^{-2}$
	E. coli SM10 (pPCV310)	× GV3103CT (pGV2275)		$0.96 - 1.46 \times 10^{-1}$
	E. coli SM10 (pPCV311)	× GV3103CT (pGV2275)		$1.26 - 2.04 \times 10^{-1}$
	E. coli SM10 (pPCV310)	× GV3103CT (pGV2217)		$0.96 - 1.36 \times 10^{-1}$
	E. coli SM10 (pPCV311)	× GV3103CT (pGV2217)		$0.93 - 1.36 \times 10^{-1}$
(6)	GV3103 (pMP90RK) (pPCV310)	× *E. coli* HB101 Rif^r		$0.74 - 1.68 \times 10^{-3}$
	GV3103 (pMP90RK) (pPCV311)	× *E. coli* HB101 Rif^r		$0.42 - 1.66 \times 10^{-3}$ [a]

minA, YEB, conjugation frequencies obtained on minimal and complete media, respectively

[a] Similar frequencies were obtained when *Agrobacterium* recipients/donors containing chromosomally integrated plasmid pCT153.1 were used

ment 12 was replaced by the *Kpn*I fragment 7 from plasmid pGV316 (Depicker et al. 1980) which overlaps the Ti-plasmid region to the left of the T-DNA. The constructed intermediate vector pTid37 was mobilized from *E. coli* to an *Agrobacterium* strain harbouring Ti-plasmid pGV2201 (Van Haute et al. 1983). Gm^r Sm/Sp^s transconjugants were obtained with a frequency of $2.1–4.0 \times 10^{-8}$. Each transconjugant was analysed in detail by Southern hybridization and found to contain a new Ti-plasmid, pMP90, from which the pTiC58 *Kpn*I fragments 3, 12 and 13 were absent and which carried an insertion of the Gm^r gene in *Hin*dIII fragment 2. The deletion-insertion event had also inactivated the nopaline catabolism genes, located to the right of the T-DNA on fragments *Kpn*I(12) and *Hin*dIII(2) (Holsters et al. 1980).

To introduce plasmid RK2 helper functions into Ti-plasmid pMP90 another intermediate vector was constructed by insertion of the 42.0 kb *Eco*RI fragment of plasmid pRK2013 (Figurski and Helinski 1979) into the unique *Xba*I site of plasmid pGV357 using *Xba*I linker addition. After conjugation of this intermediate vector, pRKGV20, to *Agrobacterium* strain GV3101(pMP90), and selection, Km^r transconjugants were isolated at a frequency of 1.05×10^{-2}. The Km^r (marker of pRK2013 DNA) transconjugants were then screened for the presence of the Ti-plasmid pMP90 Gm^r marker and for the loss of the Cb^r marker of the intermediate vector. Out of 48 Km^r transconjugants tested one contained plasmid pMP90RK which had arisen by a double crossover between plasmids pRKGV20 and pMP90 in the regions flanking the *Xba*I site located to the right of the Gm^r gene insertion in plasmid pMP90. The integrity of the pRK2013 DNA inserted in Ti-plasmid pMP90RK was proven by Southern hybridization. Further *Agrobacterium* hosts were obtained by conjugation of plasmid pMP90RK into different *Agrobacterium* recipients. These conjugations can result from either RK2 and Ti-plasmid Tra functions (on minimal media) or from RK2 Tra functions (on complete media) (Table 3).

Mobilization of vectors from E. coli to Agrobacterium. Three different *E. coli* donor strains were used to mobilize plasmids to *Agrobacterium* hosts. These were HB101 strains carrying either plasmids R64drd11 and pGJ28 (Van Haute et al. 1983) or plasmid pRK2013 (Ditta et al. 1980) and *E. coli* strain SM10 (Simon et al. 1983). Mobilization of vectors by *E. coli* donor strain HB101(R64drd11)(pGJ28) relies on the recognition of ColE1 *bom* sequences by the Mob function provided by pGJ28. Conjugation frequencies of $10^{-4}–10^{-5}$ were routinely obtained with this system (Table 3, section 3; Fig. 4). These frequencies could be much improved by providing the binary vectors with the ori_T sequence from plasmid RK2.

Two different ways of providing the necessary RK2 mobilization-helper functions were compared: in one the functions were provided in *trans* by plasmid pRK2013 (Ditta et al. 1980); in the other the RK2 mobilization helper loci were carried by the chromosome of the donor *E. coli* strain SM10. As can be seen from the results in Table 3, sections 4 and 5, both approaches yielded significantly higher mobilization frequencies. The use of *E. coli* SM10 was preferred because the high mobilization frequencies obtained with this donor allowed its direct plating onto *Agrobacterium* recipients spread on plates selective for *Agrobacterium* transconjugants.

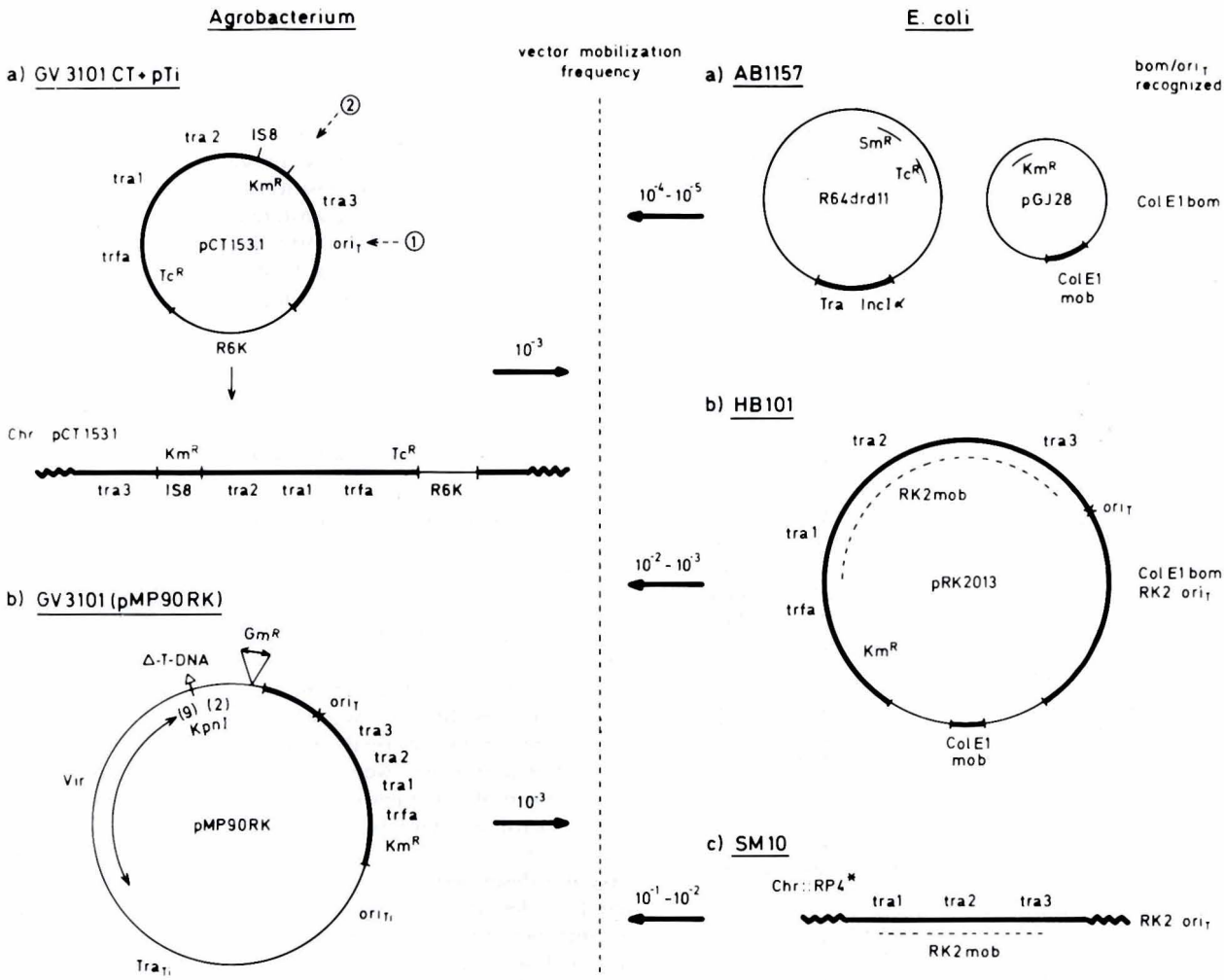

Fig. 4. *Agrobacterium* hosts and *Escherichia coli* donors for maintenance and mobilization of binary vectors. Characteristics of *Agrobacterium* hosts are summarized schematically in the *left-hand part* of the figure. A circular functional map and the structure of plasmid pCT153.1 integrated into the *Agrobacterium* chromosome are shown as an example in host GV3101CT (section a). Numbers 1 and 2 with *dashed line arrows* indicate the positions at which the integration of plasmid pCT153.1 into the *Agrobacterium* chromosome took place. In b the location of the Ti and RK2 plasmid functions are indicated on a schematic map of Ti-plasmid pMP90RK according to Holsters et al. (1980) and Thomas (1981). The position of the deletion which removed the T-DNA and joined *Kpn*I fragments 9 and 2 together, is marked by an *open arrow* crossing the circle. The position of the Gm^r gene insertion is shown outside the circle. The *right-hand part* of the figure summarizes the characteristics of *E. coli* donors used for vector mobilization. Section a shows schematic maps of plasmids R64drd11 and pGJ28. Section b shows a functional map of plasmid pRK2013 and section c, a schematic presentation of the helper functions of plasmid RK2 present in the chromosome of *E. coli* strain SM10. In each section the type of mobilization function provided by the different donors is indicated. Next to the figures vector sequences recognized by the donors are listed. Near the *dashed line* separating *Agrobacterium* hosts and *E. coli* donors *arrows* mark the direction of possible vector transfers and show the average of measured conjugation frequencies

Recovery of vectors from Agrobacterium by "ping-pong" conjugation. An interesting characteristic of this binary system is that the *Agrobacterium* RK2 helper hosts are not only able to maintain the cloning vectors but can also mobilize them back to *E. coli* with frequencies of about 10^{-3} (Table 3, section 6). To transfer plasmids back to *E. coli* *Agrobacterium* strains were plated onto *E. coli* recipients spread on plates selective for *E. coli* transconjugants. After 6–12 h of conjugation at 28° C plates were transferred to 37° C. At this temperature only *E. coli* transconjugants can grow. This "ping-pong" character of the conjugation system is schematically presented in Fig. 4.

Stability and copy number of vectors in Agrobacterium. The stability of vectors in *Agrobacterium* was tested according to Schmidhauser et al. (1983). The structural stability of each plasmid in *Agrobacterium* was tested by transferring the plasmid back to *E. coli* and analysing the plasmid DNA in 48 transconjugants by restriction endonuclease mapping and DNA hybridization.

In the absence of selection for vector-encoded antibiotic resistance they segregated with an average rate of 2.52% per generation. This segregation is probably due to the lack of elements in *cis*, which regulate the partitioning of mini-RK2 replicons. In contrast to the W-type Sa and Q-type RSF1010 plasmid derivatives (Leemans et al. 1982c; Simon et al. 1983), used as controls, the segregation rate of our vectors did not increase in *Agrobacterium* with increasing vector plasmid sizes from 7 to 50 kb. This segregation did not limit the practical application of the vectors. A popula-

tion of cells with the plasmid can be maintained by selection for a plasmid encoded antibiotic resistance. No alteration of the physical structure of the vector DNAs was observed under these circumstances.

The copy number of vectors was estimated from the yield of plasmid DNA in alkaline/SDS extracts or from DNA-DNA hybridization data. In *Agrobacterium* the copy number was found to be 5–8.

Transfer of binary vector T-DNAs from Agrobacterium to plant cells. Agrobacterium strains carrying binary vector constructions were used in co-cultivations with haploid *Nicotiana plumbaginifolia* and diploid *N. tabacum* (SR1) protoplasts, as well as for infections of *N. tabacum*, *N. plumbaginifolia* and *Kalanchoë daigremontiana* leaf discs, stem segments and plants.

Tumour induction on *K. daigremontiana, N. tabacum* and haploid *N. plumbaginifolia* plants and tissue culture conditions were as described previously (Márton 1984; De Block et al. 1984; Koncz et al. 1983, 1984). Leaf-disc infection, protoplast co-cultivation with *Agrobacterium* and regeneration of transformed plants were carried out according to established methods (Horsch et al. 1985; Márton et al. 1982; De Block et al. 1984; Márton 1984).

Transformants were obtained with all the various procedures. Regenerated plants or tumours could be maintained on media containing 100–200 µg/ml kanamycin sulphate which indicated that vector T-DNAs, carrying the dominant kanamycin resistance marker, were efficiently transferred. Callus cultures were initiated from *N. tabacum* leaf segments on media containing 0.5 mg/l NAA and 0.2 mg/l BAP or kinetin. Shoots were induced from calli in the presence of 0.5 mg/l BAP and 0.1 mg/l NAA. In co-cultivation experiments kanamycin-resistant calli or plantlets were obtained with an average frequency of 0.4%–14%, comparable to that reported for other vectors (De Block et al. 1984; Van den Elzen et al. 1985).

Plant DNA and RNA purification and hybridization conditions. Nuclear-enriched DNA was prepared from 0.1–0.5 g of leaf material according to Bedbrook (1981). After proteinase K digestion and phenol-chloroform extraction the DNA was cleaned by an additional precipitation with CTAB, according to Dellaporta et al. (1983). Alternatively, plant DNA and RNA was purified according to Taylor and Powell (1983). Larger amounts of poly(A)$^+$ RNA were purified as described by Willmitzer et al. (1982) and Eckes et al. (1985).

For northern analysis poly(A)$^+$ RNAs were separated on formaldehyde agarose gels and blotted onto nitrocellulose filters as described by Maniatis et al. (1982). For RNA dot-blots samples of 20 µg of poly(A)$^+$ RNA were denatured, diluted and blotted on nitrocellulose filters according to Eckes et al. (1985). Southern blots were prepared according to Maniatis et al. (1982). Both RNA and DNA blots were hybridized at 42° C in buffer containing 0.8 M NaCl, 50 mM Na phosphate (pH 7.0), 1 mM EDTA, 0.1% SDS, 0.2% Ficoll, 0.2% polyvinylpyrrolidone, 0.2% bovine serum albumin, 250 µg/ml denatured salmon sperm DNA and 50% formamide. Following hybridization, blots were washed at 67° C in 20 mM Na phosphate (pH 7.0), 50 mM NaCl, 1 mM EDTA and 0.1% SDS solution.

Octopine synthase and neomycin phosphotransferase assays. Octopine synthase enzyme activity was tested essentially as described by Otten (1982) and Otten and Schilperoort (1978). The test conditions were standardized by adjusting the samples to equal protein concentrations measured according to Bradford (1976), prior to testing. In tests using *Kalanchoë* stem segments or tobacco leaves, conditions were also standardized by taking stem segments or leaves of the same age and from similar positions on the plants.

The neomycin phosphotransferase activity was assayed according to Schreier et al. (1985) and extracts with equal protein concentrations were loaded on non-denaturing polyacrylamide gels to obtain comparable and reproducible results.

Results

Analysis of the promoter function of the T_L-DNA encoded gene 5

Gene *5* is one of the octopine Ti-plasmid T_L-DNA genes, the function of which is not yet understood. This gene is thought to be expressed at a low level because its transcript is barely detectable, if at all, in northern hybridizations driven by plant tumour RNAs (Willmitzer et al. 1982, 1983).

In order to test whether the low level of transcription of this gene is due to low unregulated promoter activity or to some transcriptional or post-transcriptional regulation, chimaeric genes were constructed linking the 5′ upstream promoter sequences of gene *5* to various segments of the octopine synthase gene of the pTiAch5 T_L-DNA used as an enzymatic promoter probe. In the plant vector cassettes described above the fragment containing the left-hand border of the T-DNA of pTiAch5 also contains the 5′ upstream promoter sequence of gene *5* (designated as pg*5* in Figs. 2 and 3a). From the known sequence of the octopine T_L-DNA (Gielen et al. 1984) it was deduced that the unique *Bgl*II site in pPCV003 (Fig. 3a) is located 43 bp upstream of the initiation AUG codon of gene *5*. This site was therefore used to insert a number of DNA fragments from the octopine synthase gene. Three different transcriptional fusions were constructed: in type I the gene *5* promoter sequence was fused, via a 23 bp linker sequence, to position -109, in type II to position -28 and in type III to position -8 of the promoter sequence of the octopine synthase gene (Gielen et al. 1984). (Position +1 marks the translational initiation site of the *OCS* coding sequence.)

It had been shown earlier that promoter elements extending to position -142 are not sufficient to promote octopine synthase gene expression to a level detectable by enzyme assays (Koncz et al. 1983). In the type I fusion, upstream sequences of both gene *5* and of the octopine synthase gene could provide CCAAT and TATA sequences. In fusion type II only the transcription initiation site from the 5′ upstream sequences of the *OCS* gene is retained while in fusion type III only 8 bp of the 5′ leader sequence of the *OCS* gene are present (Fig. 3b).

Plasmids pPCV121, pPCV221 and pPCV310 carry fusion type I, plasmids pPCV123, pPCV223, and pPCV330, fusion type II, while plasmids pPCV124, pPCV224 and pPCV340 contain fusion type III (Figs. 2, 3).

To analyse whether other T-DNA genes, responsible for synthesis of auxin and cytokinin as well as the product of gene *5* itself, regulate gene *5* promoter activity, the T-DNA region of pTiC58 encoding genes *1, 2, 4* and *5* (Joos

et al. 1983) was introduced into plasmids pPCV310, pPCV330 and pPCV340 resulting in pPCV311, pPCV331 and pPCV341, respectively (Fig. 3a). The properties of the vectors and details of their construction are listed and summarized in Table 2 and in Materials and methods.

Fifty-four kanamycin-resistant tobacco plants obtained from co-cultivations or leaf disc infections with *Agrobacterium* strains harboring plasmid pPCV310 were analysed for the presence of vector T-DNA by Southern hybridization. Plant DNA samples enriched for nuclear DNA and cut by restriction endonucleases *Eco*RI or *Bam*HI, were transferred to nitrocellulose filters and probed by nick-translated DNA fragments homologous to either the left border or to the *NPT-II* coding sequences at the right border of vector pPCV310 T-DNA (Figs. 3 and 5a). The estimation of T-DNA copy number met the requirement of obtaining equal numbers of fragments hybridizing to either probe in both digests. The hybridization data verified that all 54 plants selected on kanamycin were transformed and carried vector T-DNAs inserted into their nuclear DNA. The average copy number of T-DNA insertions was 1–2 and did not exceed 5 among the plants analysed. T-DNAs inserted in tandem array were not found. Because they contain two T-DNA border sequences our binary vectors (Fig. 1) can in principle give two different T-DNA intermediates (Koulikova-Nicola et al. 1985): one containing the expected T-DNA fragment and one containing the ori_V and ori_T sequences of plasmid RK2. However, the RK2-derived sequences of the vector were never detected in transformed plants as was shown in genomic blots probed by full length plasmid pPCV310 DNA.

These data also demonstrated that vector T-DNAs were transferred from *Agrobacterium* and integrated into the plant genome without any alteration or rearrangement in all 54 cases analysed.

Expression of gene 5 promoter – octopine synthase fusions

As an initial screening, tissues obtained by transformation with *Agrobacterium* strains harboring either vector pPCV310 or pPCV311 with the type I, type II and type III chimaeric genes were assayed for octopine synthase enzyme activity. Significant enzyme levels were detected in tobacco tumours transformed by the pPCV311, pPCV331 and pPCV341 vectors (Fig. 3; data not shown).

Interestingly, when tobacco tumour or *Kalanchoë* stem tissues, containing the three different types of gene *5* promoter – *OCS* gene transcriptional fusions were compared, no significant differences were found between their octopine synthase enzyme activities in standardized assays (Fig. 5b).

The transcription of the three types of fusions was quantitated and compared by using the nopaline synthase promoter-*NPT-II* gene fusion as an internal standard. The chimaeric *NPT-II* gene could be used as a standard because Southern hybridization data had indicated that the *NPT-II* and *OCS* genes were present in transformed cells at the same copy number. To standardize the analysis large samples of mixed tumour lines (at least 200 independently transformed calli), carrying the different fusions, were used for RNA isolation.

Poly(A)$^+$ RNA samples were probed in northern and RNA dot-blot hybridizations with DNA fragments homologous to the coding sequences of the *NPT-II* and *OCS* genes, similar in length and labelled to the same specific activity (Fig. 5c).

The *NPT-II* gene specific probe hybridized to mRNAs with a size of approximately 1.25 kb (Fig. 5c, 1–3). The hybridization signal had a similar intensity in all three extracts indicating that our approach of using at least 200 independently transformed calli for the assay of a given construction gave reproducible results for the internal p*NOS-NPT-II* standard.

On northern blots the *OCS* gene specific probe hybridized to mRNAs of about 1.4 kb in size (Fig. 5c, 4–6). Both northern gels and RNA dot-blot hybridizations showed signals that were essentially identical for all three promoter fusions (Fig. 5d, tracks b–d). These data indicated that all three fusion types were expressed to the same extent in tumours. Additional sequence elements derived from the *OCS* gene promoter neither enhanced nor inhibited transcription regulated by the gene *5* promoter.

Because the *NOS* promoter-*NPT-II* gene and gene *5* promoter – *OCS* gene constructions carried the same 3′ transcriptional signal sequence, the hybridization data, obtained by probing identical RNA dot-blots by *NPT-II* and *OCS* gene specific probes, allow a comparison between the nopaline synthase and gene *5* promoters (Fig. 5d). The amount of octopine synthase mRNA derived from the gene *5* promoter, estimated by quantitative comparison of dot-blot hybridizations, was found to be only 1.5–2 times less than that of *NPT-II* mRNA synthesized from the *NOS* promoter.

Tissue-specific activity of the gene 5 promoter

A systematic analysis of the level of expression of the gene *5* promoter – *OCS* fusion genes in different tissues of transformed tobacco plants revealed striking differences. The highest levels were detected in callus tissues or in crown gall tumours as well as in stem segments; petioles had reduced activity and no activity could be detected in fully developed leaves. Primordial leaves as well as roots showed a very low level of activity (Fig. 6a).

In order to determine the significance of these observations the same tissues were analysed for *NPT-II* activity, which was introduced by the vectors as a linked gene. The expression of this control gene (p*NOS-NPT-II*) did not show tissue-specific variation; in fact the activity increased in all tissues with age (Fig. 6b). It therefore appeared that the level of activity of the gene *5* promoter was correlated with the state of development of tissues harboring this gene.

The promoters of both the *NPT-II* and *OCS* genes are located close to the ends of T-DNAs and are joined to plant DNA sequences upon integration. It is therefore conceivable that plant DNA sequences flanking T-DNAs may have an effect on the expression of these genes. In order to prove that the lack of *OCS* enzyme activity in fully developed leaves was not due to a negative position effect suppressing gene *5* promoter activity, *OCS* activity was assayed in leaves of 450 individual transformed plants. Only a single plant was found expressing *OCS* in fully developed leaves. This finding indicated the significance of the results showing the tissue specifity of the gene *5* promoter (data not shown).

In order to test whether tissue-specific changes in gene *5* promoter activity were due to specific and reversible regulation, callus cultures were initiated from fully grown leaves. After 3 days on callus-inducing medium and therefore be-

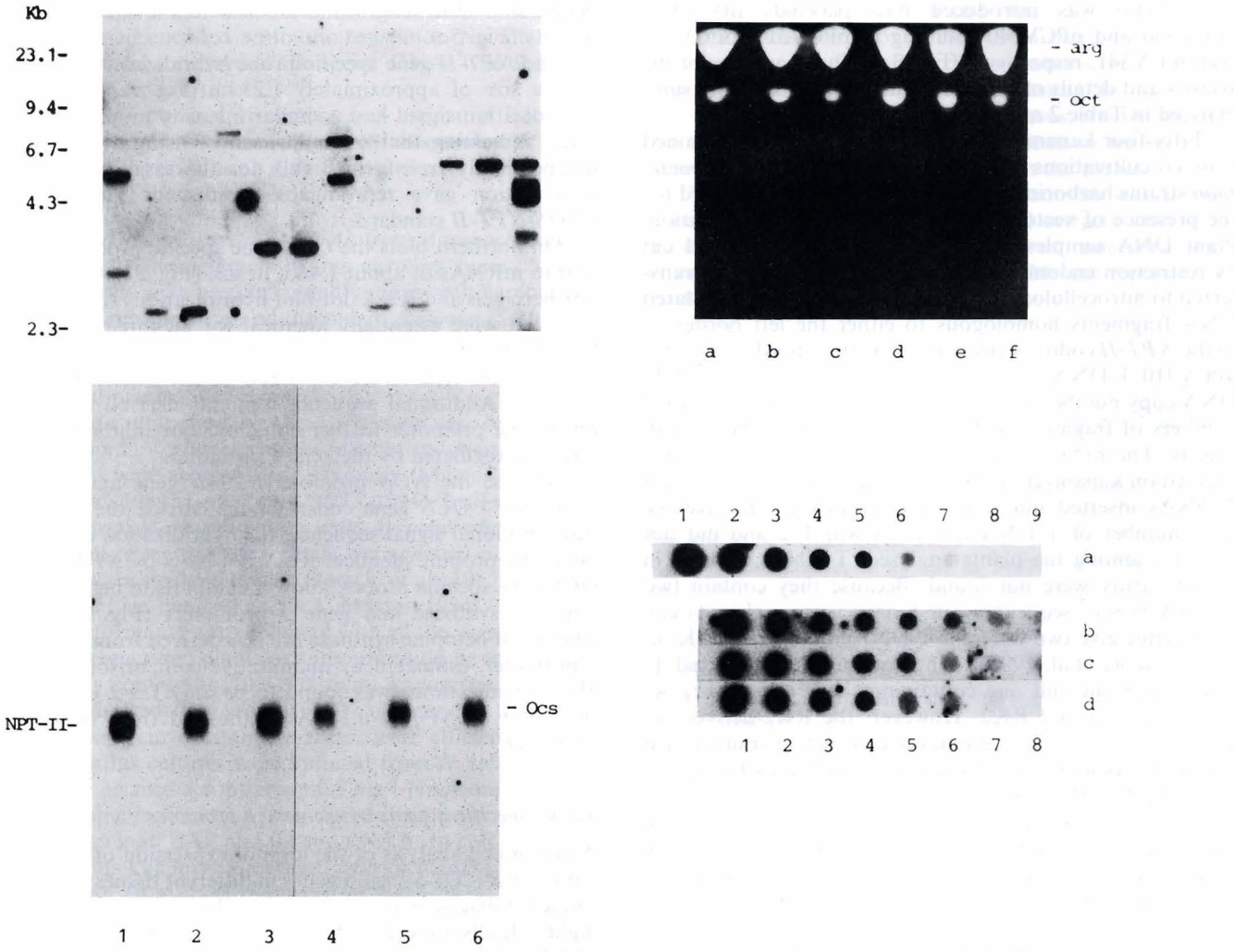

Fig. 5a–d. Analysis of gene *5* promoter – *OCS* gene fusions in plants transformed by vectors pPCV310 and pPCV311. **a** A representative sample of Southern gel DNA hybridizations. Nuclear-enriched DNA isolated from leaves of different tobacco plants transformed by vector pPCV310 was digested with *Eco*RI, blotted and probed with the nick-translated *Bgl*II – *Kpn*I fragment of plasmid pPCV003 DNA. An equal number of hybridizing fragments was observed when using a right-hand border specific probe (data not shown). **b** Octopine synthase activity in extracts prepared from *Kalanchoë* stem segments after 2 days of infection with *Agrobacterium* strain GV3101 (pMP90RK) containing plasmids pPCV310 (a), pPCV330 (b), pPCV340 (c), pPCV311 (d), pPCV331 (e), and pPCV341 (f) (Fig. 4). Positions of arginine and octopine are labelled as arg and oct. **c** Northern blots carrying 20 µg each of poly(A)$^+$ RNAs isolated from tumour tissues induced by *Agrobacterium* GV3101 (pMP90RK) strains harbouring plasmids pPCV311 (1,4); pPCV331 (2,5) and pPCV341 (3,6). Nick-translated probes: 1 kb *Bgl*II – *Sma*I fragment of plasmid pKC7 DNA containing *NPT-II* coding sequences (tracks 1 to 3) and 1.1 kb *Hin*dIII – *Pvu*II fragment of plasmid pAGV40 DNA representing coding sequences of the octopine synthase gene (tracks 4 to 6). **d** Dot-blot hybridization of poly(A)$^+$ RNA samples from tumour tissues containing T-DNAs of vectors pPCV311 (a, b), pPCV331 (c) and pPCV341 (d) to an *NPT-II*-specific probe as a control (track a) and to the *OCS* coding sequence (tracks b, c, d). (Dot-blot hybridizations of RNA samples isolated from tumours carrying plasmid pPCV331 and pPCV341 T-DNAs were identical to that of the vector pPCV311 shown in track a.) The dots carried 10,000 (1), 5,000 (2), 2,500 (3), 1,250 (4), 625 (5), 312 (6), 156 (7), 78 (8) and 39 (9) ngs of poly(A)$^+$ RNAs. Dots in track a are shifted by one position in order to indicate the difference between hybridizations to *NPT-II*- and *OCS*-specific probes

fore any visible change in tissue structure had occurred, a marked increase in *OCS* activity was detected in the leaf segments, whereas subsequent callus tissue contained full *OCS* activity. On the other hand when callus tissue was placed on shoot-inducing medium the appearance of shoot primordia was correlated with a decrease in *OCS* activity. Changes at the level of transcription, as measured by dot-blot hybridization, correlated well with changes of enzyme activity.

Preliminary observations have ruled out the possibility that the observed induction of gene *5* promoter activity in callus tissues is under direct auxin control since leaf fragments incubated with auxins alone did not show increased *OCS* activity (data not shown).

Discussion

We have described a novel *Agrobacterium* binary vector system and its application to the study of the promoter of gene *5* of T_L-DNA. The vector system developed differs in many respects from those described previously (Bevan 1984; Klee et al. 1985; An et al. 1985; Van den Elzen et al. 1985). An advantage of the system is that all plant vector functions are provided on a single, versatile plant vector

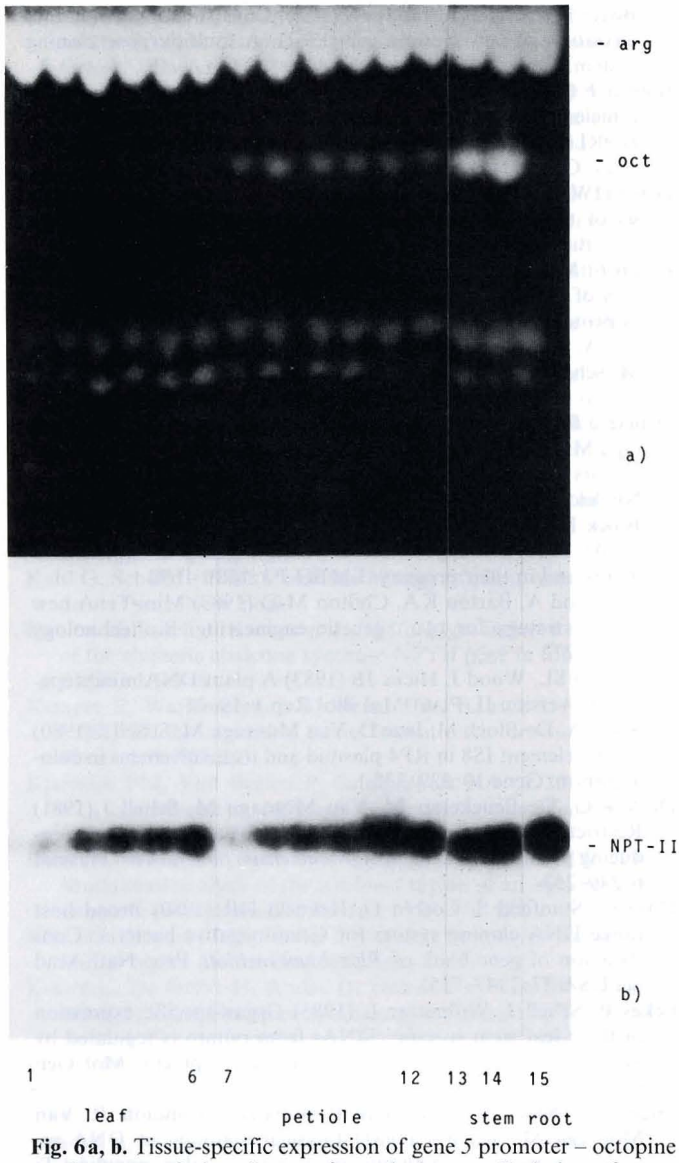

Fig. 6a, b. Tissue-specific expression of gene *5* promoter – octopine synthase gene fusions in transformed plants. **a** Variation of octopine synthase activity in leaves, petioles, stem sections and root tissues of a tobacco plant transformed by vector pPCV310. Equivalent extracts containing 1.5 μg of protein were incubated for 2 h and spotted onto adjacent lines. Leaves, petioles and stem segments were taken from the top to the bottom of a particular plant and the corresponding extracts were also used to assay *NPT-II* activity. **b** *NPT-II* activity (corresponding to 10 μg protein equivalent) assayed in similar tissues of the same transformed plant. Tissues of the same age show similar *NPT-II* activities. The age of tissue used for extraction increases in the order of sample numbers. Stem extracts (13, 14) correspond in age to leaf and petiole extracts 5, 6 and 11, 12, respectively. arg, arginine; oct, octopine; *NPT-II*, position of neomycin phosphotransferase

cassette. The basic element of this cassette is a conditional mini-RK2 replicon which is maintained and mobilized by *trans*-acting functions derived from plasmid RK2 and inserted into *Agrobacterium* hosts. Binary vectors can easily be constructed by combination of the plant vector cassette with *E. coli* cloning vectors. Thus plasmid pBR322 derivatives were coupled to plant vector cassettes to obtain binary vectors with different cloning properties. These vectors can be mobilized by at least three different *E. coli* donor systems and the mobilization frequencies achieved (1% to 25%) allow highly efficient transfer of vector recombinants to *Agrobacterium* hosts. The binary vectors can be maintained either in well-defined *Agrobacterium* hosts, with RK2 helper functions introduced into their chromosomes, or in *Agrobacterium* strains containing Ti-plasmids carrying the RK2 helper functions. Due to the presence of these RK2 helper functions the vectors can be mobilized back to *E. coli* which facilitates the analysis of the structural integrity of the vectors prior to plant transformation. This is important because certain plant DNA sequences cloned in wild-type *Agrobacterium* hosts may cause structural instability of plasmids (unpublished results).

The vectors are maintained in *Agrobacterium* at a low copy number and segregate in the absence of selection. This is not a unique property of our system, but is characteristic of all published vectors based on plasmid RK2 derivatives, such as plasmid pTJS75 derived vectors (An et al. 1985; Klee et al. 1985) and also pRK252 (Bevan 1984; Van den Elzen et al. 1985) which not only segregates but is also unstable in both *E. coli* and *Agrobacterium* (Schmidhauser et al. 1983; Thomas 1983; Schmidhauser and Helinski 1985). To overcome segregational loss of our vectors *Agrobacterium* strains can be grown under carbenicillin selection prior to plant transformation.

The transformed plant tissues were selected on the basis of their kanamycin resistance. The transformation frequencies obtained in co-cultivations with *N. tabacum* and *N. plumbaginifolia* protoplasts were similar to those reported for other Ti-plasmid-derived vectors (De Block et al. 1984; Van den Elzen et al. 1985). The majority of regenerated plants analysed carried low copy numbers of T-DNA insertions. In plants containing multiple T-DNA insertions, no T-DNA copies were found to be rearranged or to be present in tandem array.

Transformed tissues and plants carrying the promoter of gene *5* on their T-DNA were analysed in detail. The analysis of the promoter of gene *5* was of interest for the following reasons: the function of this gene is not yet known although genetical data suggest that it might act in concert with T-DNA-encoded genes *1* and *2* (Leemans et al. 1982b; Joos et al. 1983). These genes have recently been shown to code for enzymes establishing a new pathway for auxin synthesis (Schröder et al. 1984; Kemper et al. 1985).

In order to obtain data which could help in exploring the function of gene *5*, transcriptional fusions, containing the promoter of gene *5* coupled to promoter deletion derivatives of the octopine synthase gene, were constructed and studied in plant and tumour tissues. The most striking aspect of the observations made with these gene *5* promoter fusions is their tissue-specific mode of expression. Indeed the fusion genes appeared to be expressed at the highest levels in callus tissues and in stems of transformed plants and at barely detectable levels in fully developed leaves. Young leaves expressed the gene *5* promoter fusion gene at a low level. As an internal control the marker p*NOS-NPT-II* gene linked and co-transferred with gene *5* promoter fusions, was shown to be expressed in all tissues. The expression of gene *5* promoter fusion genes in fully developed leaves was fully restored by incubating leaf segments on high auxin, low cytokinin containing medium. On the other hand incubation of callus tissue on high cytokinin, low auxin medium resulted in a decrease in gene *5* promoter fusion gene expression. Dot-blot RNA analysis indicated

that the observed variations in the level of expression correlated with changes in RNA levels. This is the first documented instance of a gene derived from a procaryotic host (carried by Ti-plasmids of *A. tumefaciens*) the expression of which is tissue specific and apparently regulated by plant growth factors. This result is not entirely unexpected in view of the fact that other T-DNA genes code for enzymes involved in auxin and cytokinin synthesis. Our results suggest that the product of gene *5* will be produced only in tissues having a high level of internal auxins relative to the level of internal cytokinins.

By DNA hybridization and sequence analysis it was shown that the so-called core segment of the T-region, carrying genes *1, 2, 4* and *5* of the octopine pTiAch5 and nopaline pTiC58 plasmids are highly homologous (Engler et al. 1981; Gielen et al. 1984). Notwithstanding, analysis of T-DNA transcripts in crown gall tissues showed a considerable difference between steady-state levels of gene *5* transcripts in octopine- and nopaline-type tumours (Willmitzer et al. 1982, 1983).

However, steady-state levels of *OCS* mRNA derived from the octopine T_L-DNA gene *5* promoter fusions were found to be similar to that of *NPT-II* transcripts expressed from the promoter of the nopaline synthase gene in the same tumour tissues. These observations indicate that the low steady-state levels of gene *5* transcripts in octopine crowngall tumours are not due to weak promoter activity. In view of the fact that the overall homology between the DNA sequences of gene *5* in octopine and nopaline T-regions is very high in the coding regions as well as in the 5′ upstream regions, but breaks down 42 bp before the first polyadenylation site (Gielen et al. 1984; unpublished results), it is conceivable that the 3′-end of the octopine gene *5* may be responsible for its low level of expression.

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