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The *Agrobacterium rhizogenes* pRi TL-DNA segment as a gene vector system for transformation of plants

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Summary. A plant gene transfer system was developed from the *Agrobacterium rhizogenes* pRi15834 TL-DNA region. "Intermediate integration vectors" constructed from ColE1-derived plasmids served as cloning vectors in *Escherichia coli* and formed cointegrates into the TL-DNA after transfer to *A. rhizogenes*. An *A. rhizogenes* strain with pBR322 plasmid sequences replacing part of the TL-DNA was also constructed. Plasmids unable to replicate in *Agrobacterium* can integrate into this TL-DNA by homologous recombination through pBR322 sequences. No loss of pathogenicity was observed with the strains formed after integration of intermediate vectors or strains carrying pBR322 in the TL-DNA segment. Up to 15 kb of DNA have been transferred to plant cells with these systems. The T-DNA from a binary vector was cotransformed into hairy roots which developed after transfer of the wild-type pRi T-DNA. Tested on *Lotus corniculatus* the TL-derived vector system transformed 90% of the developed roots and the T-DNA from the binary vector was cotransformed into 60% of the roots. Minimum copy numbers of one to five were found. Both constitutive and organ-specific plant genes were faithfully expressed after transfer to the legume *L. corniculatus*.

Key words: *Agrobacterium rhizogenes* – Plant transformation – Intermediate integration vectors – Binary vectors

Introduction

The neoplastic diseases crown gall and hairy root are caused by the soil bacterium *Agrobacterium*. The pathogenic properties of the bacterium are encoded by two different large plasmids, tumour inducing (Ti) and root inducing (Ri), respectively. Both plasmids carry regions, T-DNA, which after transfer and expression from the plant genome cause the characteristic pathogenic traits (Hooykaas and Schilperoort 1984, and references therein). T-DNA regions from Ti plasmids, named by their opine type, have been characterized genetically (Leemans et al. 1982; Joos et al. 1983; Willmitzer et al. 1982, 1983), and physically (Gielen et al. 1984; Barker et al. 1983). Genes involved in opine synthesis and synthesis of morphogenic phytohormones auxin and cytokinins were identified (Joos et al. 1983; Barry et al. 1984; Inzé et al. 1984; Schröder et al. 1981, 1984; Akiyoshi et al. 1984). In the T-DNA only the 25 bp direct repeats bordering the T-DNA are required for the transfer of the DNA located in between (Zambryski et al. 1983; Wang et al. 1984), although transfer frequencies are increased by an adjacent short DNA sequence (Peralta et al. 1986). Ti-derived plant transformation vectors could therefore be constructed using the characterized T-DNA border sequences (Zambryski et al. 1983; Bevan 1984).

Like Ti plasmids Ri plasmids are characterised by their opine type (Petit et al. 1983). The agropine-type pRi has two T-DNA regions (De Paolis et al. 1985) called TL- and TR-DNA. The TL region is responsible for the hairy root phenotype and the TR for agropine synthesis as well as auxin biosynthesis. Four root morphogenic loci *rolABCD* are located in the TL segment and genes involved in auxin synthesis maps to the TR segment (White et al. 1985). Both the TL and TR segments are stably integrated into plant chromosomes and the 25 bp border repeats are found surrounding the TL segment (Constantino et al. 1984; Slightom et al. 1986). Presence of the TR is not required for the hairy root phenotype and root lines elicited by agropine-type *A. rhizogenes* strains do not always synthesize agropine (Petit et al. 1983; De Paolis et al. 1985).

The *A. rhizogenes* transformation regeneration system available for a number of plant species (Chilton et al. 1982; David et al. 1984; Tepfer 1984; Petit et al. 1987) makes it desirable to construct pRi derived plant transformation vectors. We have therefore constructed a set of integration vectors and an *A. rhizogenes* strain carrying pBR322 sequences within the TL-DNA of the agropine-type pRi plasmid 15834. Also a binary vector was tested. These systems allow plant transformation without changing the hairy root inciting properties of the *A. rhizogenes* donor strains.

Materials and methods

Microbiological techniques. Escherichia coli and *A. rhizogenes* strains were cultured in liquid LB medium or on LA plates, antibiotics were used at levels given by Van Haute et al. (1983). The plasmid helper system of Van Haute et al. (1983) was used for conjugational transfer of plasmids to *A. rhizogenes*.

Plasmid constructions and DNA analysis. Standard DNA techniques as compiled by Maniatis et al. (1982) were used for DNA manipulations. Bacterial DNA for Southern blot

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Table 1. Strains and plasmids

Strain		Reference
Agrobacterium rhizogenes		
C58C1 rif^r	pRi15834	Schell, Max-Planck-Institut, Cologne
AR1193	pRi1193 carrying pBR322 in the TL segment	This study
AR1134	pAR30 integrated into TL through *Eco*RI 40	This study
Plasmids		
pAR1	Ap^{r}Tc^r Sm^{r}Sp^r pGV710 derivative	This study
pAR2	Ap^{r}Tc^r Sm^{r}Sp^r pGV710 derivative	This study
pAR5	Km^{r}Ap^r pKC7 derivative	This study
pAR6	Km^{r}Ap^r pKC7 derivative	This study
pAR30	*Lbc$_3$5′3′-CAT* in pAR1	Stougaard et al. (1986)
pGV710	Ap^{r}Tc^r Cm^r Sm^{r}Sp^r	Deblaere et al. (1985)
pKC7	Km^r Ap^r	Rao and Rogers (1979)
pGV941	Ap^r Sm^{r}Sp^r pNOS-Km^r plant marker in T-DNA	Deblaere (1987)
pIV1	Sp^{r}Sm^r from R702 in pHC79	This study
pIV1b	*Eco*RI fragment 40 and 2.9 kb *Bam*HI/*Bgl*II TL fragments in pIV1	This study
pIV1c	pBR322 in pIV1b *Cla*I site	This study

analysis was extracted according to Dhaese et al. (1979). Plant DNA was extracted and digested according to Dellaporta et al. (1983).

Plant transformation. The legume *Lotus corniculatus* was transformed in wound site infections according to Petit et al. (1987).

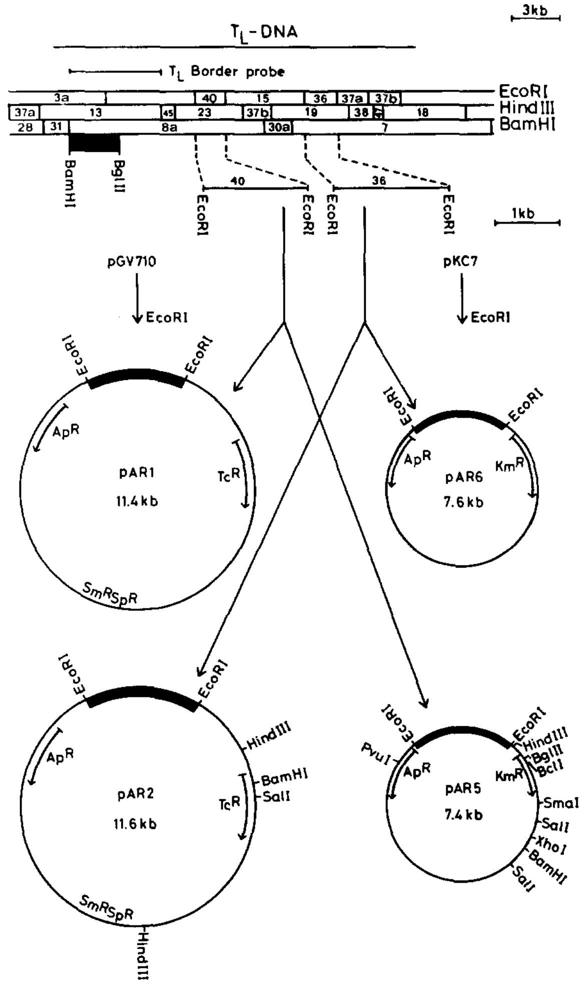

Fig. 1. Construction of the intermediate integration vectors for the *A. rhizogenes* TL segment. The *Eco*RI fragments 36 and 40 shown in the restriction map (Pomponi et al. 1983) for the TL region were subcloned into the ColE1 derived pGV710 and pKC7 plasmids. Restriction sites from pGV710 and pKC7 are indicated on plasmids pAR2 and pAR5, respectively. The Km^r and Sp^{r}Sm^r markers were used to select cointegrates after transfer to *A. rhizogenes*

Results

Intermediate integration vectors for the *A. tumefaciens* T-DNA have been constructed by Leemans et al. (1981). The T-DNA does however still carry the morphogenic phytohormone genes and the transformed plant cells develop into tumours. We have constructed integration vectors for the TL segment of the pRi15834 plasmid. The *Eco*RI fragments 36 and 40 (Pomponi et al. 1983) of the TL-DNA were chosen as integration sites and cloned into the ColE1 plasmids pGV710 and pKC7 (Fig. 1). The generated plasmids pAR5, pAR6 carry the Km^r marker from Tn*5* and pAR1, pAR2 the Sp^{r}Sm^r marker from R702. Cointegrates can therefore be selected in *A. rhizogenes* where ColE1 plasmids do not replicate. The plasmid helper system of Van Haute et al. (1983) was used for conjugational transfer of the plasmids from *E. coli* to *A. rhizogenes*.

Figure 2 shows the TL *Hin*dIII fragments generated after homologous recombination of the pAR1-derived pAR30 integration vector through *Eco*RI fragment 40. Frequencies of cointegrate formation were determined to be 5×10^{-7} for recombination through both *Eco*RI fragment 36 and 40. Integration generates a TL-DNA segment interrupted by the ColE1 cloning vector flanked by the *Eco*RI fragments 36 or 40 (Stougaard et al. 1986). *A. rhizogenes* strains with modified TL segments were not changed in functions essential for transformation or root growth.

A pBR322 acceptor strain

An *A. rhizogenes* strain capable of accepting pBR322 derived cloning vectors by homologous recombination through pBR322 sequences was constructed by replacement mutagenesis (Fig. 3). The plasmid pIV1b was constructed by subcloning of the 2.9 kb *Bam*HI/*Bgl*II TL fragment (shown as closed bar in Fig. 1) and the *Eco*RI fragment 40 into the pIV1 plasmid in the correct orientations. The pBR322 acceptor was subsequently cloned into the *Cla*I site of pIV1b to form pIV1c. Double recombination through the two homologous TL fragments on pIV1c, after conjugation to *A. rhizogenes*, placed the pBR322 acceptor within the TL segment. Double recombinants were identi-

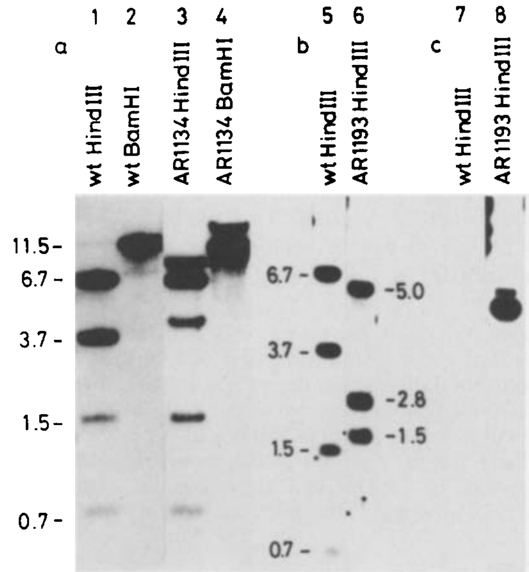

Fig. 2. Southern blot analysis of the AR1193 acceptor strain, and a strain AR1134, formed after cointegration of an intermediate vector through *Eco*RI fragment 40. Panel a shows the *Hind*III and *Bam*HI fragments hybridized to the *Bam*HI fragment 8a (Fig. 1) from the TL segment. Lanes 1 and 2, wild type, lanes 3 and 4, AR1134. Panel b shows the *Hind*III fragments hybridized to the 8a probe in wild type (lane 5) and the AR1193 strain (lane 6). Panel c is as panel b but probed with pBR322. The AR1193 *Hind*III fragments hybridizing to pBR322 and 8a are shown below the diagram of the AR1193 TL-DNA segment in Fig. 3

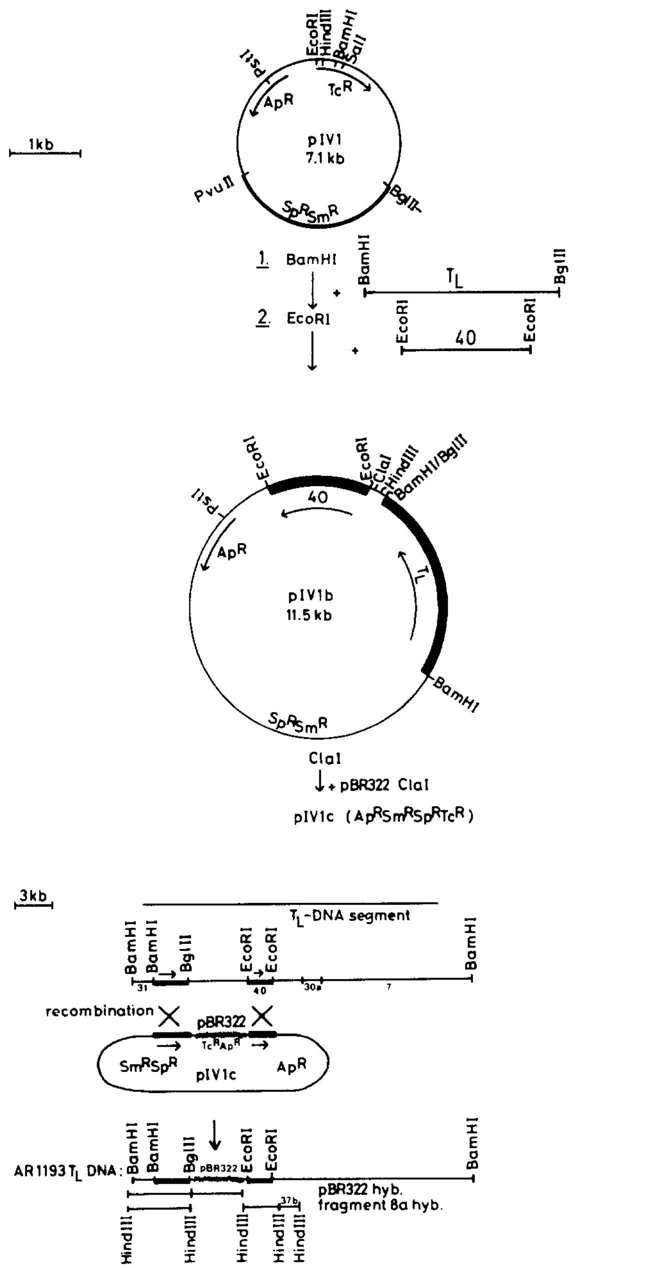

Fig. 3. Construction of the pBR322 *A. rhizogenes* acceptor strain AR1193 by replacement mutagenesis. The *Bam*HI/*Bgl*II and *Eco*RI fragments forming the flanking regions of the deleted TL segment, were cloned stepwise into pIV1 to create pIV1b. The pBR322 acceptor was subsequently cloned into the *Cla*I site to form pIV1c. AR1193 was identified as a Ap^rTc^r Sm^sSp^s double recombinant after transfer of pIV1c to *A. rhizogenes*. pIV1 was constructed by cloning of the R702 2.5 kb Sm^rSp^r, *Pvu*II *Bgl*II fragment into pHC79

fied as $Carb^r$ $Tc^rSp^sSm^s$ strains and checked by Southern blot analysis (Fig. 2). The pathogenicity of the resulting *A. rhizogenes* strain AR1193 was not changed, when tested on *L. corniculatus*. A construction similar to AR1193 has previously been described for the Ti vector system of *A. tumefaciens* (Zambryski et al. 1983).

It was necessary to determine whether the pBR322 sequences in AR1193 were transcribed, as transcripts from the TL segment have only been partially mapped (Taylor et al. 1985; Durand Tardif et al. 1985) and the DNA sequence (Slightom et al. 1986) revealed several putative promoter structures around the deletion carried by the AR1193 strain. Total RNA was therefore extracted from both an *A. rhizogenes* pRi15834 transformed and three AR1193 transformed root lines. Total RNA (20 μg) from each line was analysed by Northern blot analysis. No transcripts originating from pBR322 sequences were detected in the lines tested (data not shown).

Binary systems

The cotransfer of TL and TR segments from the agropine strains of *A. rhizogenes* into plant genomes (De Paolis et al. 1985) indicates that a T-DNA region from a binary vector would also be transferred if present. The broad host range pGV941 T-DNA vector (Deblaere 1987) was therefore conjugated into the *A. rhizogenes* pRi15834 strain selecting for Sp^rSm^r. Transconjugants were used to transform *L. corniculatus* in wound site infections. Hairy roots were taken into culture and screened for the $pNosKm^r$ marker located between the T-DNA borders of the binary vector. Kanamycin resistance to a level of 50 μg/ml was found in 60% of the root lines tested.

Copy numbers

The minimal copy number for a TL-DNA segment carrying the chimeric soybean leghemoglobin gene, *Lbc_3 5′3′-CAT*, was determined in the legume *L. corniculatus* with a TL border probe (Fig. 1 and Stougaard et al. 1986). Figure 4 shows the *Eco*RI and *Hind*III restriction fragments generated after insertion of the integration vector pAR30 into the plant genome. Copy numbers of one to five are typical for the TL segment. Similar copy numbers were found for

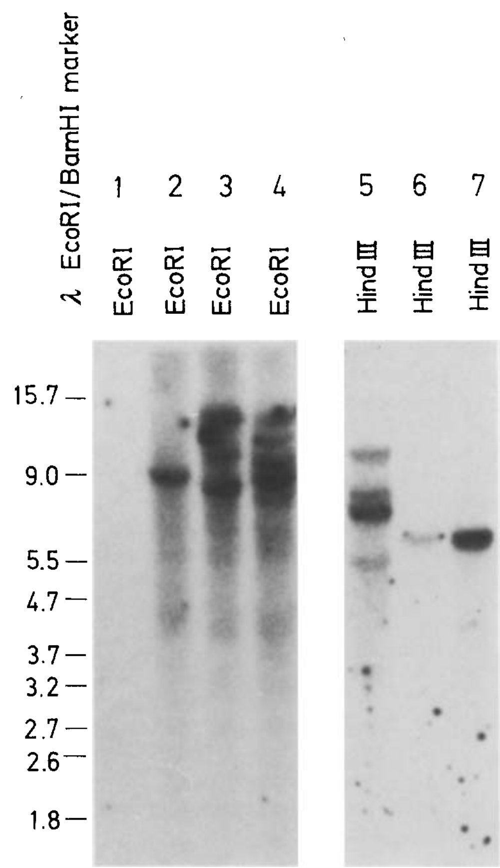

Fig. 4. Copy number estimation in transformed *L. corniculatus* plants. Total DNA from six independent plants was digested with *Eco*RI (lanes 2–4) or *Hind*III (lanes 5–7) and hybridized with the border probe shown in Fig. 1. Each band corresponds to at least one insertion site. Lane 1 contains DNA from untransformed plants

the T-DNA transferred from the binary vector pGV941 (data not shown).

Discussion

The convenient and effective transformation possible with *A. rhizogenes* makes it a very useful system for transfer of characterized genes into plants. To study soybean leghemoglobin gene expression in transgenic plants of the legume species *L. corniculatus* we therefore developed integration vectors for the *A. rhizogenes* TL-DNA region. These vectors pAR1, pAR2, pAR5, pAR6 allow characterization of genes in *E. coli* followed by integration into *A. rhizogenes* T-DNA. Cointegrate T-DNAs are transferred to plant genomes and integration through the *Eco*RI fragments 36 and 40 seems not to disturb virulence on *L. corniculatus* plants. The regeneration capacity of *L. corniculatus* root cultures was also unchanged. Durand-Tardiff et al. (1985) mapped two transcripts terminating within the *Eco*RI fragments 36 and 40, respectively. Neither of these transcripts would be disturbed by integration through these fragments. The ability of *L. corniculatus* to regenerate from roots incited with the AR1193 strain suggest that no genes required for any of these processes are located within the deletion created. Alternatively, functions from the TR region could complement the deleted functions.

Transformed *Nicotiana tabacum* plants carry shorter T-DNA regions encompassing only the central part of the TL segment (Durand-Tardiff et al. 1985). Both *Eco*RI fragments 36 and 40 are within the region transferred and the intermediate integration vectors can thus be used in this species. Genes integrated into the AR1193 strain might however not be transferred. Plant differences may therefore give preference to certain vectors. This problem could be circumvented by use of binary vectors as documented by the frequent cotransfer of T-DNA regions.

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