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Frequencies of simultaneous transformation with different T-DNAs and their relevance to the *Agrobacterium*/plant cell interaction

Ann Depicker, Lieve Herman, Anni Jacobs, Jozef Schell, and Marc Van Montagu
Laboratorium voor Genetica, Rijksuniversiteit Gent, B-9000 Gent, Belgium

Summary. We investigated whether the efficiency of transformation of plant cells by *Agrobacterium tumefaciens* during cocultivation is limited by the properties of the plant cells or by the infecting bacteria.

Therefore, tobacco protoplasts were infected by cocultivation with two different agrobacteria strains carrying Ti plasmids with distinguishable T-DNAs. These T-DNAs cotransform plant cells at a frequency equal to the product of their independent transformation frequencies, which indicates that all plant cells are equally competent. On the other hand, when these T-DNAs are located on the same Ti plasmid vector within one bacterial strain, the cotransformation frequency is significantly higher than the product of the single transformation frequencies. We interpret these results to indicate that transformation is limited more by the establishment of effective bacteria/plant cell interaction than by (i) the process of DNA integration and (ii) by the number of plant cells capable of being transformed by *Agrobacterium*. We found that most plant cells are transformed by only one or a few agrobacteria. Analysis of the number of T-DNA copies in these clonally transformed lines indicates amplification of the original, infecting T-region copy.

Introduction

Agrobacterium tumefaciens induces tumors by infecting wound sites on plants. During this process a segment of the Ti plasmid (T-DNA) harbored by these bacteria is transferred and stably integrated in the plant genome (for recent review, see Gheysen et al. 1985). Although the mechanism by which *Agrobacterium* integrates DNA into the plant cell is not yet elucidated, the process of infection can be subdivided in different steps: the attachment of bacteria to plant cells; induction of the virulence (*vir*) region, which encodes functions required for the transfer of the T-DNA to the plant cell; transfer of DNA out of the bacteria; transfer of DNA to the plant nucleus; integration of the T-DNA in the plant genome, and expression of T-DNA genes.

The attachment of bacteria to plant cells is mediated by an 11-kb portion of the *Agrobacterium* chromosome. The expression of this region is constitutive (Douglas et al. 1985). The specificity of *Agrobacterium*-binding results in 100 to 300 bacteria bound per plant cell at bacterial concentrations above 1×10^7 per ml (Neff and Binns 1985). During cocultivation, which is the infection of plant protoplasts with *Agrobacterium*, bacteria attach to single plant cells and transform them, so that clonal plant cell lines can be obtained (Márton et al. 1979). Routinely, frequencies of transformation are between 2% and 30% of the surviving calli, depending on the experimental conditions and the infecting strain used. The expression of the *vir* region of the Ti plasmid is induced by the presence of a plant product (Okker et al. 1984; Stachel et al. 1985). Only under these inducing conditions was it possible to isolate T-DNA circles (Koukolíková-Nicola et al. 1985), in which the T-DNA sequences were joined through the T-DNA end sequences. These T-DNA ends consist of a direct-repeat of a 25-bp sequence and must obligatory be present for the transformation of the T-DNA into plant cells to occur (Zambryski et al. 1982; Wang et al. 1984; Caplan et al. 1985).

In this paper we wished to determine whether a plant cell is transformed by one or by many agrobacteria during cocultivation, since there is a 100-fold excess of bacteria versus plant cells. Also, we wanted to know if the transformation frequency in cocultivation experiments is limited either by the number of plant cells capable of being transformed and/or by the frequency with which effective bacterial contacts are established with each plant cell. We approached these questions by measuring the cotransformation frequencies of two different selectable T-DNAs when present either in one and the same bacterium or in two different bacteria. The first T-DNA we used is the wild-type T-DNA of the plasmid C58; the hormone balance of the transformed plant cell is changed by expression of the T-DNA genes, allowing selection for hormone-independent growth. In addition, this T-DNA codes for nopaline synthase, an additional marker to detect plant cell transformation. The second T-DNA is a mini-T-DNA construction containing a selectable marker gene, consisting of fusions of plant promoters to the neomycin phosphotransferase coding sequence Tn*5* (Herrera-Estrella et al. 1983a, 1983b). The substitution of all the T-DNA sequences, except the ends, by genes expressing an antibiotic resistance allows the selection of transformed clonal plant lines containing such mini-T-DNAs (De Block et al. 1984; Deblaere et al. 1985).

The interpretation of the transformation frequencies is based on the following rationale. When two different T-

Offprint requests to: J. Schell – M. Van Montagu

DNAs are present in different bacteria which are mixed for infection by cocultivation, the fraction of doubly transformed plant cells will give us information about (i) whether or not a single plant cell can be transformed by two bacteria; (ii) whether there is a plant cell competence factor, in which case the fraction of doubly transformed cells would be expected to be high compared to singly T-DNA-transformed cells; and (iii) whether the bacteria/plant cell interaction is the limiting factor for transformation in which case we would expect the fraction of doubly transformed cells to be equal to the product of the number of singly transformed cells by each T-DNA. If, by contrast, the two independent, selectable T-DNAs are present on one and the same Ti plasmid, the cotransformation frequency of the two T-DNAs can give us information regarding the efficiency of stabilization of the T-DNA, after effective bacteria/plant cell contact has been made.

Materials and methods

Strains and plasmids. The *E. coli* and *Agrobacterium* strains, and the plasmids used in this work are summarized in Table 1.

E. coli strains were grown in LB (Miller 1972); *Agrobacterium* strains were grown either in MM medium (Holsters et al. 1980) or in rich YEB medium (Miller 1972).

Mobilization of plasmids from E. coli to Agrobacterium. pBR322-derived plasmids were mobilized to *Agrobacterium* by a triparental cross using GJ23 (Van Haute et al. 1983). Precultures of the *E. coli* donor, GJ23 and the *Agrobacterium* acceptor were mixed in equal proportions on a YEB plate and incubated at 28° C overnight. Appropriate dilutions of the conjugation mix were spread on YEB plates containing selective antibiotics for the *Agrobacterium* acceptor and the mobilized *E. coli* plasmid. pBR322-derived plasmids can be stabilized in *Agrobacterium* by a homologous recombination into a resident replicon (Van Haute et al. 1983).

Table 1. Bacterials strains and plasmids

	Relevant characteristics	Origin
MC1061		
GJ23		Van Haute et al (1983)
C58C1Rif^R		Holsters et al. (1980)
pGV3109	pTiC58::Tn*7*	Holsters et al. (1980)
pGV3188	pTiC58::Tn*1*	Holsters et al. (1980)
pGV2260	pTiB6S3ΔTL-TR =pBR322	Deblaere et al. (1985)
pGV720		De Greve et al. (1982)
pGV750		Deblaere et al. (1985)
pGVL32		This work
pGVL49		This work
pGVL081		Herrera-Estrella et al. (1983a)
pGVA1091		This work
pGVL136		Herman et al. (in preparation)

Restriction, cloning, and analysis of constructed strains. Restriction enzymes and T4 ligase were used according to the supplier's instructions (Boehringer, Mannheim, FRG). Analysis of *E. coli* plasmids was done by a modification of the alkaline denaturation method (Birnboim and Doly 1979). *Agrobacterium* transconjugants were controlled by total DNA preparations and hybridization with radioactive probes (Dhaese et al. 1979).

Plant DNA preparation and T-DNA analysis. Plant DNA was prepared on a large scale (more than 10 g wet plant tissue) by the method described by Lemmers et al. (1980), or on a small scale (less than 5 g wet plant tissue) by the method of Dellaporta et al. (1983).

Ten µg digested DNA per lane were transferred to nylon filters and hybridized with nick-translated radioactive probes.

Cocultivation. Protoplasts were prepared from 6-week-old sterile-grown plantlets (*Nicotiana tabacum* cv. Petit Havana mutant SR1), a streptomycin-resistant mutant tobacco line (Maliga et al. 1975) by an overnight incubation at 26° C in K3 0.4 M sucrose medium (Nagy and Maliga 1976), and the enzyme mix of 0.5% cellulase R10 (Yakult, Tokyo, Japan) and 0.2% macerozyme R10 (Yakult, Tokyo, Japan).

The protoplasts were washed 3 successive times in K3 medium, diluted to 2×10^5 cells/ml, and distributed in batches of 5 ml in petri dishes (diameter 90 mm). The protoplasts were allowed to regenerate their cell walls for 2 or 3 days at 26° C in the dark. When the first cell divisions were visible under the microscope, the plant cells were infected with *Agrobacterium* (50 µl of a fully grown *Agrobacterium* culture in MM medium). This results in 2×10^7 bacteria/ml with 2×10^5 plant cells/ml, or in approximately 100 bacteria per plant cell. We allowed the cocultivations to proceed for 60 h, after which cefotaximum (Claforan®, Hoechst, Frankfurt, FRG) was added to a final concentration of 500 µg/ml. After two days, the plant cells were diluted 2-fold with fresh K3 medium and incubated further for 5 days. The microcalli were embedded in 0.6% low-melting agarose by mixing 2.5 ml of the plant calli suspension and 2.5 ml of 1.2% low-melting agarose in K3 + 0.4 M sucrose medium in 45-mm petri dishes (Shillito et al. 1983). After settling for 3 h the agarose blocks were transferred to a 90-mm diameter petri dish and 10 ml of selective or non-selective medium was added. This medium was refreshed every week, and every two successive times the sucrose content was lowered by 0.1 M. The selection for hormone-independent growth was applied from the moment the plant cells were embedded in low-melting agarose by not adding plant hormones. The medium to select for kanamycin resistance contained 50 µg/ml. Km-resistant minicalli were discernible 4 to 5 weeks after embedding. The clonal calli can be picked and transferred to solid medium, after which growth, screening and regeneration to small plantlets can be performed. The hormone concentration during the whole course of the experiment amounted to 0.2 mg/l BAP and 0.1 mg/l NAA. The transformation frequency is the number of transformed calli per total number of calli regenerated without selection.

Opine tests. Octopine and nopaline tests were performed using a modification of the methods published by Aerts et al. (1979) and Otten and Schilperoort (1978). The tissue

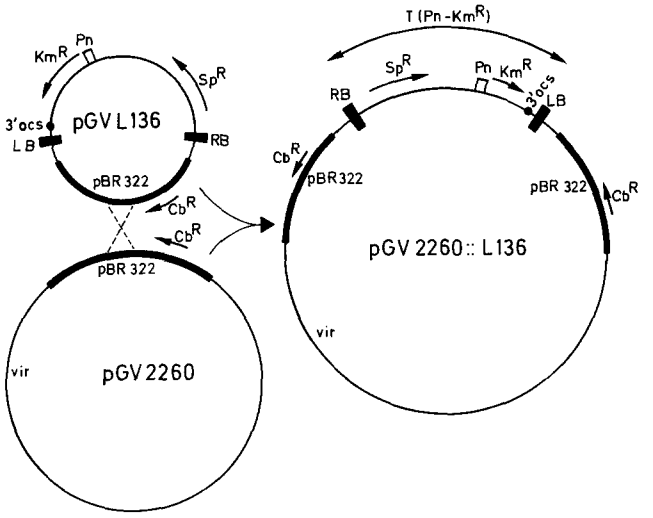

Fig. 1. Schematic illustration of the cointegrate pGV2260::L136. The plasmid pGV2260 is a derivative of pTiB6S3 with a substitution of the complete TL and TR regions by pBR322 sequences (Deblaere et al. 1985). The mini-T-region of pGVL136 contains a fused Pn-Km^R gene and a prokaryotic selectable marker, the SpSm^R gene of R702, in a pBR322-derived replicon. The construction and properties of pGVL136 will be described elsewhere (Herman et al. in preparation). By homology recombination through the pBR322 sequences, pGVL136 was stabilized in pGV2260; the crossed dashed lines symbolize the region of a single cross-over. Pn, *nos* promoter; 3′ *ocs*, polyadenylation sequence from the octopine synthase gene; LB and RB, left and right T-region border sequences

to be tested was incubated overnight at room temperature in K3 medium supplemented with 10 mM arginine and 10 mM pyruvate. The tissue was removed, rinsed in water, dried and transferred to a fresh microfuge tube. The tissues were frozen at −20° C for 10 min, crushed and spun down during 10 min. The supernatant was spotted on 3MM paper and electrophorized as described.

Results

Construction of an Agrobacterium strain containing a Ti plasmid with a single mini-T-region (Pn-Km^R)

We wished to determine the frequency with which two T-DNAs, each contained in separate *Agrobacterium* strains, can infect the same plant cell. Therefore, we needed two strains whose T-DNAs could be distinguished. For this purpose, we chose one strain containing a nopaline Ti plasmid with the wild-type T-DNA of C58 (see further). The second strain was constructed by introducing a mini-T-region into a Ti plasmid derivative that had no T-DNA of its own. The mini-T-region in-between the normal T-region border sequences (Herman et al., in preparation) contains a *nos* promoter-kanamycin resistance hybrid gene construction in the intermediate vector pGVL136 recombined in pGV2260 (Fig. 1). pGV2260 is an octopine Ti plasmid derivative of pTiB6S3 in which both the TL and TR regions are substituted by pBR322; the rest of the Ti plasmid including the *vir* region is intact (Deblaere et al. 1985). The plasmid pGVL136 was transferred to *Agrobacterium*; its stabilization by homology recombination through pBR322 sequences in pGV2260 can be selected for by the spectinomycin marker on pGVL136. The cointegrate pGV2260::L136 contains a mini-T-region (Pn-Km^R) flanked by direct-repeats of pBR322 sequences which are not transferred to the plant cell (see Fig. 1).

Coinfection of two Agrobacterium strains by cocultivation

Two strains with distinguishable T-regions were used for the coinfection of protoplasts. On the one hand, the strain C58C1Rif^R(pGV2260::L136) contains a Ti plasmid with an octopine *vir* region and a mini-T-region with octopine-derived border sequences; transformed plant cell lines can be screened or selected for by the kanamycin resistance marker. On the other hand, the strain C58C1Rif^R (pGV3188) was chosen because this C58 Ti plasmid derivative contains a wild-type nopaline *vir* region and T-region, and at the same time it is marked by an Tn*1* insertion in a functionally silent region, allowing specific titration of these bacteria. The nopaline T-DNA-transformed cell lines have a white compact appearance, grow hormone-independently (HI$^+$) and can be screened for by the nopaline synthase marker (Nos$^+$). Protoplasts cultured for 3 days were infected with a 1:1 mixture of both bacteria at a concentration of 10^7 bacterial cells/ml each. In order to control whether both strains grew identically during cocultivation (3 days), the titer of both bacteria was determined after cocultivation. The number of C58C1Rif^R (pGV2260::L136) bacteria was determined on SpSm-containing medium and the number of C58C1Rif^R(pGV3188) bacteria could be counted on plates containing carbenicillin and nopaline as sole N-source. Both bacteria grew to a titer of approximately 10^9 cells/ml in the cocultivation mixture. In order to follow the transformation and cotransformation events by different agrobacteria in one cocultivation experiment, the cocultivation mixture was divided in 4 fractions which were grown either without selection, or selection for hormone-independent growth (HI), or for kanamycin resistance, or for both.

In our hands the cocultivation method (See Materials and methods) leads on the average to the regeneration of 20% of the treated protoplasts to microcalli; of these about 25% are actually transformed by T-DNA. The kanamycin selection of plant microcalli is very efficient in the soft agar blocks (see Materials and methods); after about 5 weeks, the resistant calli outgrow nontransformed, sensitive microcalli. The hormone-independent selection does not work with the same efficiency, most likely because of the presence of hormones in the cocultivation medium and cross-feeding of growing mini-calli. Nevertheless, these calli can be picked out after 5 weeks and grown on hormone-free medium to screen for hormone independence.

Table 2 summarizes the results obtained in one experiment. The individual transformation frequencies of each T-DNA were similar: about 35% of the regenerating calli were transformed with one or the other of the T-DNA markers. Since direct selection for transformants within a dense population of regenerating protoplasts does not give an accurate estimate, transformation frequencies were determined by transferring calli, grown for 5 weeks on non-selective medium, to either kanamycin-containing or hormone-free medium. With both strains similar results were obtained: frequencies varying between 20%–40% in different cocultivation experiments were observed.

Table 2. Transformation frequencies of two different T-DNAs out of two different *Agrobacterium* strains

		Km^R calli[a]: 35%		
			HN^+ calli[b]: 36%	
	Km^SHN^-	Km^RHN^-	Km^RHN^+	Km^SHN^+
Predicted for independent events[c]	42%	22.3%	12.7%	23.3%
Observed[d]				
(a)	–	–	15%	–
(b)	–	19.2%	15.8%	–
(c)	–	–	16%	20%
(d)*	44%	20%	15%	21%

[a] The frequency of Km^R calli was determined by the transfer of 100 unselected calli to Km-selective medium

[b] The frequency of HN^+ (HI^+ and/or Nos^+ calli) was determined by (i) two successive transfers of 100 calli to B5 medium without hormones, and (ii) a test for the presence of nopaline in 100 calli

[c] Predicted percentage values for the different classes, when plant cell transformations from different bacteria would be independent events: $35\% \times 36\% = 12.7\%$ for Km^RHN^+ calli; $35\% - 12.7\% = 22.3\%$ Km^RHN^- calli; $36\% - 12.7\% = 23.3\%$ Km^SHN^+ calli, and $100\% - 22.3\% - 12.7\% - 23.3\% = 42\%$ for untransformed calli

[d] The observed frequencies were obtained (a) by the transfer of 200 unselected calli to Km-containing medium without hormones; (b) by cross-testing 150 Km^R calli for hormone independence and nopaline synthesis; (c) by cross-testing 50 HN^+ calli for the kanamycin-resistant marker; and (d) are the means of the experimental data

* The $\chi^2_{(2)}$ value is 0.943 which gives a probability of 0.65 that the differences between observed and expected frequencies are due to chance

One-hundred-fifty selected kanamycin-resistant calli were subsequently screened for hormone independence (H) and nopaline synthesis (N), which are the linked markers carried by the second T-DNA; 41 did grow in the absence of added hormones and contained nopaline; 16 grew on hormone-free medium, but did not contain nopaline; and 11 contained nopaline, but were hormone-dependent for their growth. Since the transformation frequency of the second T-DNA is the relevant figure for this experiment, all calli expressing either one or both of the T-DNA-linked phenotypes (*h*ormone-independent growth and *n*opaline synthesis) were grouped in one class, HN^+. Thus, 68 of the 150 Km^R calli were doubly transformed. Calculated on the total number of regenerating calli, this means that 15.8% of the calli were Km^RHN^+ and 19.2% were Km^RHN^- (Table 2b).

Similarly, 50 of the calli growing without hormones were cross-tested for kanamycin resistance; 22 grew for 3 successive transfers on Km-containing medium. On the total number of calli, this means that 16% were doubly transformed Km^RHN^+, and 20% were singly transformed HN^+ (Km^S) (Table 2c).

The means of these cross-testings gives 15% for doubly transformed calli vs 20% and 21% respectively for Km^R and HN singly transformed cells (Table 2d). The two populations of transformed lines overlap each other only by the product of their individual frequency. In other words, the transformation by different bacteria creates additive populations: the total percentage of plant cells transformed is 20% Km^R + 15% Km^RHN^+ + 21% HN^+ = 56% in this experiment. According to the null hypothesis, the difference between what is observed and what is expected is non-significant with a probability value of 0.65. Thus, transformations by two different bacteria of one plant cell are independent events.

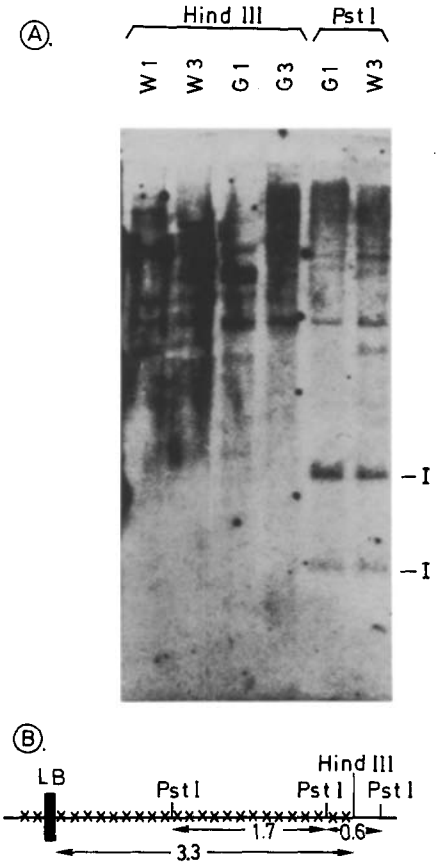

Fig. 2A, B. Genomic analysis of plant cell clones, transformed by two T-DNAs out of different bacteria. **A** W1, W2, G1 and G3 are the identification numbers of 4 different plant cell lines, transformed both by T(HI^+,nos) and T(Pn-Km). Total DNA was prepared, digested by *Hin*dIII or by *Pst*I, and blotted on nylon filters. A nick-translated probe was prepared from a purified *Hin*dIII fragment, extending over the left end of the T(Pn-Km^R) region of pGVL136 (indicated by the cross-hatched region in part **B** of the figure). This probe detects different left T-DNA/plant DNA composite fragments in a *Hin*dIII digest, from which the number of T-DNA integration sites can be derived. In a *Pst*I digest, the probe will detect two T-DNA internal fragments (indicated by I) of 0.6 and 1.7 kb, and left composite *Pst*I fragments. **B** Schematic drawing of the left end of the T-region in pGVL136. The *cross-hatched* area indicates the DNA sequence used as a radioactive probe. Only the restriction sites, relevant for the genomic blot shown in **A**, are indicated. *LB*, left border sequence. The numbers 1.7, 0.6 and 3.3 indicate the sizes of the DNA segments in kb

Analysis of doubly transformed plant cell lines

Four doubly transformed lines expected to result from a single infection by two different bacteria, were analyzed by genomic hybridizations for their T-DNA content. We were especially interested in knowing the number of genomic insertions of each of the two T-DNAs. Therefore, we

chose to make a probe of purified fragments, located over one of the ends of the T-DNAs. Hybridization to the digested genomic DNA thus reveals the number of T-DNA/plant composite fragments, allowing an estimate of the number of integration sites and copy number. A probe to the left end of the pGVL136 T-DNA (Pn-Km^R) hybridizes to at least 3 or 4 different *Hin*dIII composite fragments in the 4 different plant cell line clones (Fig. 2). Hybridization to *Pst*I digests confirms that the 2 internal *Pst*I fragments in the kanamycin resistance coding sequence hybridize more intensely than the border fragments (Fig. 2). A probe to the right end of the C58 nopaline T-DNA (fragment *Hin*dIII-23) detects two *Hin*dIII composite fragments in one tumor line and 3 composite fragments in the other 3 clonal plant lines (including a eventual composite fragment over tandem T-DNA insertions) (Lemmers et al. 1980).

Each of the 4 doubly transformed plant cell clones thus contains at least 3 or 4 copies of the pGVL136 T-DNA and 2 or 3 copies of the pTiC58 T-DNA. These T-DNA copies appear to be integrated independently. We found no evidence for joint integration events for the different T-DNAs.

Construction of an Agrobacterium strain with two different selectable T-DNAs on one Ti plasmid

In the previous experiment we determined the cotransformation frequency of two T-DNAs introduced via two different bacteria into the same plant cell, as being the product of the frequency of their individual transformation frequencies. This could be explained either if the number of bacteria actually capable of DNA transfer is low or if the probability of integration of T-DNA after transfer is low. Therefore, we measured the cotransformation frequency of two distinguishable T-DNAs introduced via the same bacterial host. In this way we hoped to determine the efficiency with which a second T-DNA is cotransformed when one selects for the stabilization of a first T-DNA out of the same bacterium. One of the T-DNAs was the native T-DNA of a pTiC58 derivative with a Tn*7* insertion in the *noc* region (pGV3109) (Holsters et al. 1980). The other T-DNA, as present on the intermediate vector pGVA1091, contains between the border sequences of the octopine TL-region a selectable kanamycin resistance marker and a screenable octopine synthase marker. The construction of pGVA1091 is outlined in the legend of Fig. 3. The mini-T-region contains a fused Pn-Km^R gene with which transformed plant calli can be selected. This particular hybrid gene was constructed by inserting the *nos* promoter (Herrera-Estrella et al. 1983a) into the *Bgl*II site 35 bp upstream from the initiation codon of the Tn*5*-derived Km resistance coding sequence. The insertion of the *nos* promoter fragment between the bacterial promoter and the remainder of the gene lowers the bacterial resistance level from 500 µg/ml to 10 µg/ml kanamycin. The mini-T-region in pGVA1091 contains, besides the Pn-Km^R gene, one other T-DNA-derived gene coding for octopine synthase, so that transformed calli can also be identified by screening for octopine production. Heteroduplex mapping (Villarroel et al. 1983) has shown that the spectinomycin-streptomycin (SpSm) resistance gene of Tn*7* is homologous to R702-derived SpSm resistance gene (Leemans et al. 1982) present in pGVA1091. The intermediate vector pGVA1091 which does not replicate in *Agrobacterium*, can

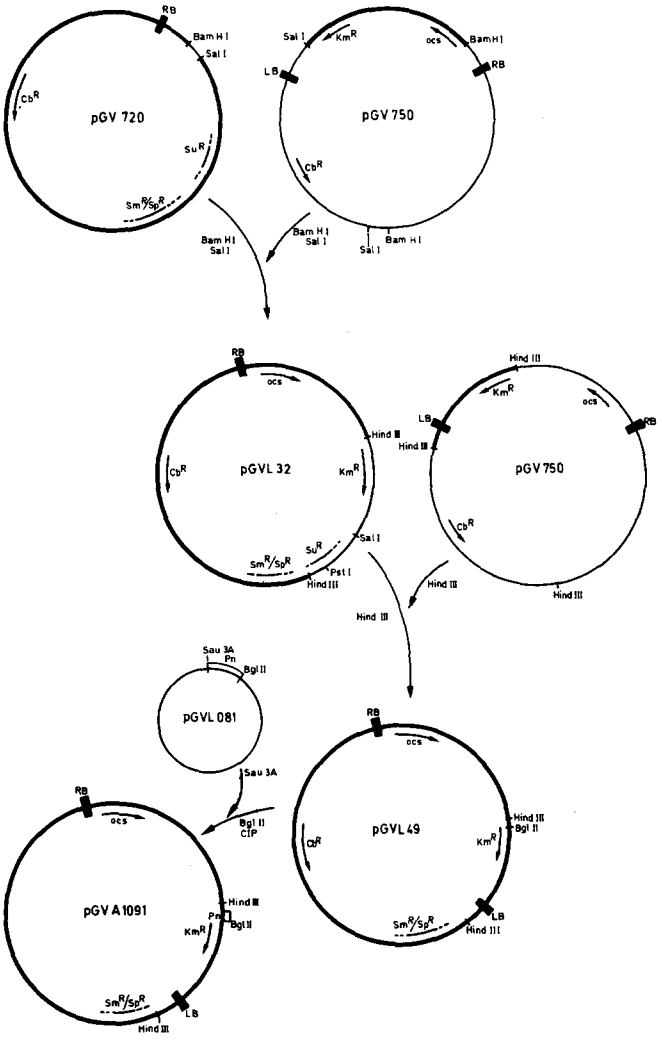

Fig. 3. Cloning strategy and structure of pGVA1091, an intermediate vector with a mini-T-region containing a screenable and selectable marker. pGV720 (De Greve et al. 1982) and pGV750 (Deblaere et al. 1985) were cut with *Bam*HI and *Sal*I, phenolized and ligated in equal proportions. The recombinant plasmid transformant (pGVL32) was isolated by SpKm selection and subsequent screening. DNA of pGVL32 and pGV750 were *Hin*dIII-digested. Transformants were selected on Sp and subsequently screened for the loss of the Su marker. The resulting plasmid, pGVL49, has a unique *Bgl*II site in the Tn*5*-derived DNA sequence 35 bp upstream of the start codon. The *nos* promoter was cloned by inserting the *Sau*3A fragment of 280 bp from pGVL081 (Herrera-Estrella et al. 1983b) into the unique *Bgl*II site of pGVL49. The resulting intermediate vector pGVA1091 confers a very reduced kanamycin resistance (10 µg/ml) to the bacteria as compared to pGVL49 (500 µg/ml)

thus be stabilized in C58C1Rif^R (pGV3109) by recombination through the homologous Sp^R/Sm^R genes of both plasmids (Fig. 4). This cointegrate plasmid, pGV3109::A1091 contains two T-regions: the resident nopaline C58 T-region conferring hormone independence (HI$^+$) and nopaline biosynthesis (Nos$^+$), and a mini-T-region conferring kanamycin resistance (Km^R) and octopine synthesis (Ocs$^+$). The orientation of this mini-T-region with respect to the orientation of the direct-repeat of the 25-bp border sequences was found to be counter-clockwise to that of the resident T-region of pTiC58. This assures the independent transfer

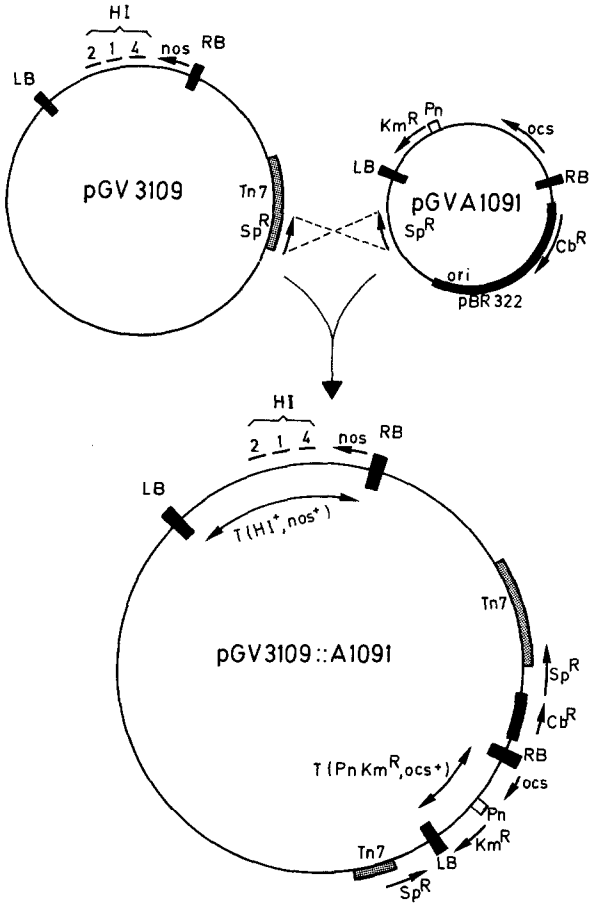

Fig. 4. Schematic representation of the formation of cointegrate pGV3109::A1091. The plasmid pGV3109 contains a Tn7 insertion in the *noc* region of pTiC58 (Holsters et al. 1980). The orientation of the Tn7 insertion and transcription of the $SpSm^R$ gene was determined by restriction analysis of the cointegrate (B. Claes, personal communication). The two selectable T-regions, $T(HI^+,nos^+)$ and $T(Pn\text{-}KM^R,ocs^+)$ are in opposite orientations with respect to the orientation of their T-region border sequences. The *dashed box* represents Tn7 sequences; the *arrows* indicate the orientation of transcription; *RB* and *LB*, right and left border sequences; the products of genes *1, 2* and *4* are responsible for hormone-independent growth (HI^+)

of these two T-regions from one Ti plasmid to the plant cell (Wang et al. 1984).

Cocultivation of tobacco SR1 protoplasts with Agrobacterium C58C1Rif^R (pGV3109::A1091) containing two T-DNAs

In order to know the frequency with which two different T-DNAs carried by the same bacterium are cotransferred to the same plant cell, regenerating protoplasts were infected by C58C1Rif^R(pGV3109::A1091). We first compared the efficiency with which each of the two T-DNAs can be selected for in regenerating protoplasts. The results of this cocultivation experiment are summarized in Table 3. Sixty calli out of 200 were able to grow in hormone-free medium and/or contained nopaline; 66 out of 200 survived the kanamycin selection (50 μg/ml). The respective transformation frequencies were thus 30% for HN^+ and 33% for Km^R.

The kanamycin-resistant calli were subsequently screened for the HI and Nos phenotypes (Table 3). Thirty-six calli out of 66 tested expressed the *nos* activity and grew without hormones. Four Nos^+HI^- calli may have been transformed by either incomplete or partially inactive T-DNAs. A similar explanation may account for two HI^+ Nos^- calli that were found. Both kinds of aberrations have been observed and studied (Van Lijsebettens et al. 1985). In total, we found that 42 calli (36+4+2) out of 66 kanamycin-resistant calli contained at least part of the C58 T-DNA.

Table 3. Tranformation frequencies of two different T-DNAs contained in one *Agrobacterium*

		$Km^R Ocs^+$ calli[a]: 33%		
			HN^+ calli[b]: 30%	
	$Km^S HN^-$	$Km^R HN^-$	$Km^R HN^+$	$Km^S HN^+$
Predicted for independent events[c]	47%	23%	10%	20%
Observed[d]				
(a)	–	11.6%	22.4%	–
(b)	–	–	22%	8%
(c)*	57%	11%	22%	8%

[a] The frequency of Km^R and/or Ocs^+ calli was determined by (i) transfer of 200 unselected calli to Km-containing medium, and (ii) a screening for octopine synthase activity of the Km^R calli

[b] The frequency of HN^+ calli (HI^+ and/or Nos^+ calli) was determined by (i) two successive transfers of 200 calli to B5 medium without hormones, and (ii) a test for the presence of nopaline in 200 calli

[c] Predicted value for the different classes: 30% × 33% = 10% for $Km^R HN^+$; 33% − 10% = 23% for $Km^R HN^-$; 30% − 10% = 20% for $Km^S HN^+$ calli; 100% − 10% − 23% − 20% = 47% for untransformed calli

[d] The observed values were obtained by (a) cross-testing the Km^R-transformed calli for hormone independence and nopaline synthesis; (b) cross-testing 30 HI^+ calli for growth on Km-containing medium; (c) are the means of the experimental data

* $\chi^2_{(2)} = 23$, whereby the probability that the differences between observed and expected values can occur by chance, is smaller than 0.0001

The hormone-independent calli were screened for the markers of the other T-region T(Pn-Km,ocs) (Table 3). Twenty-two out of 30 hormone-independent calli grew for 3 transfers on kanamycin-containing medium. These 22 calli were also screened for expression of the octopine synthase marker. Most were positive; however, the level of expression of the *ocs* marker varied over a wide range in these clonal calli for reasons not investigated.

The two types of T-DNA thus cotransform with a frequency of 60%–70% relative to the number of singly transformed cells. More strikingly, this high cotransformation frequency of 60%–70% was reproducibly found also in cocultivation experiments, in which the frequency of transformation for the single markers was not higher than 3%. The frequency of cotransformed plant calli in the experiment described in Table 3 (22%) is higher than the predicted value of 10% (33% × 30%) for two independent transformation events, and higher than the frequency of singly transformed calli. The probability that the differences between observed and expected values is due to chance is

smaller than 0.001. Therefore, we can assume that the transformations of two different T-DNAs from one bacterium to the same plant cell are not due to two independent events.

Discussion

At the onset of the experiments, we postulated that the cotransformation frequency of two T-DNAs coming from two different bacteria can indicate whether the transformation event is limited by the establishment of an effective bacteria/plant cell interaction or by the ability of the plant cell to acquire, integrate, and express a T-DNA. Therefore, we set up a cocultivation with two bacteria containing distinguishable T-DNAs.

If the frequency of doubly transformed plant cells would be equal to the product of the frequencies of each single transformation (see Table 3), then this would indicate that cotransformation is the result of independent transformation events, and that every plant cell is equally competent, and can equally well stabilize the introduced T-DNA copies presumably through integration. The competence of a plant cell can be defined as the ability to interact with *Agrobacterium* and to stabilize accepted T-DNAs during the course of cocultivation. If the number of cotransformed cells would be higher than the number of cells with only one type of T-DNA, this would imply that the plant cell competence to acquire, integrate, and express a T-DNA is more limiting than the *Agrobacterium* competence to transform a plant cell.

The results show that both the octopine *vir* region (in pGV2260::L136) and the nopaline *vir* region (in pTiC58::Tn*1*) in combination with their T-region ends are equally effective in transformation. The cotransformation frequency of two distinguishable T-DNAs from different bacteria by a mixed cocultivation infection is nearly identical to the frequency predicted for independent events. This observation seems to indicate that each plant cell has enough attachment sites for several bacteria. Under the presumption that there is no limitation for other bacteria to superinfect a transformed plant cell, suggested by the obtained cotransformation frequencies, one can also deduce from the experiment that during cocultivation most of the plant cells are transformed by only one or a few bacteria. The independence of the second infection suggests that there is no special competence or incompetence caused or detected by the first infection, such as induction of plant enzymes or an immunity against further transformations. The conformity of the distribution of singly versus doubly transformed cells to the expected one indicates that most regenerating plant protoplasts can efficiently stabilize the transferred T-DNAs. Moreover, since both groups of cells singly transformed by different bacteria, are additive, we believe that the bacteria are not saturating the cocultivation system. Therefore, the number of effective bacteria/plant cell interactions seems to be the limiting factor for the transformation frequency obtained by cocultivation.

The cotransformation frequency with two T-DNAs carried by the same bacterium was tested in order to determine the limitation on transformation once an effective bacteria/plant cell interaction has been made. We found that the cotransformation frequency of two different T-DNAs contained within one bacterium is much higher than expected for independent transformation events; in different experiments it ranges between 60% and 70% relative to singly transformed cells. Thus, a single bacterium can transfer and integrate two separate T-DNAs during one infection event. This indicates that plant cells are more likely to acquire several T-DNAs from one bacterium than one from each of several bacteria. The experiment demonstrates that, after a successful bacteria/plant cell interaction, the probability of transfer and/or stabilization of a second T-DNA is high ($>60\%$), but significantly less than 100%. Thus, the limitation factor on transfer and stabilization of a T-DNA is approximately 30%. Also, with the binary vector system, a cotransformation frequency of 60% has been reported of a Km^R T-DNA present on an independent replicon together with the wild-type T-DNA of the Ti plasmid (An et al. 1985).

From the observation that cocultivation with two different bacteria yields singly transformed cells (41%) at a higher frequency than cotransformed cells (15%), we deduce that half of the singly transformed plant cell clones were infected by one bacterium only. On the other hand, we observed that each of the four clonal lines contains two or more T-DNA copies, integrated at different sites of the plant genome. This observation led us to postulate that the different T-DNA copies in transformed plant cell clones are derived mainly from only one original bacterial T-region copy. This experiment does not allow to determine whether the multiplication of the original T-region copy occurs in the bacterium or in the plant cell. Further evidence for this replicative event has been obtained in a different way by Van Lijsebettens et al. (1985).

The apparent paradox between the physical number of agrobacteria – i.e. more than 100 bacteria per plant cell at the onset of cocultivation and 1000 bacteria at the end of the cocultivation – and the limitation of bacteria/plant cell contacts on the number of stably transformed plant cells, can only be explained by the assumption that surprisingly few bacteria/plant cell interations are effective, or that a cooperative effect is needed. In our cocultivation conditions, only 1/300 bacteria would be able to establish plant cell transformation. This argument is further substantiated by the observation that the number of transformed cells declines dramatically with the number of infecting agrobacteria. A cocultivation with 10^4 agrobacteria/ml containing a T(Pn-Km) region in a population of 10^7/ml agrobacteria with the same chromosomal background but without a T-region, resulted in 4 transformed plant cell lines per 10^5 infected plant cells (data not shown).

The limitation on transformation by the number of effective bacteria/plant cell contacts could be due to the fact that only a percentage of bacteria are induced to express the *vir* regions. On the other hand, the limitation could reside in specific requirements for attachment. If 20% of the bacteria attach (Douglas et al. 1982), it would mean that statistically only 1 in 70 bacteria attaches in the required way, while most bacteria (i.e. 69/70) have attached non-specifically. Whether these specific requirements for attachment consist of particular structures or of cell wall proteins is still an open question.

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