Mol Gen Genet (1986) 205:34–41

MGG

T-DNA structure in transgenic tobacco plants with multiple independent integration sites

Albert Spielmann and Robert B. Simpson
Molecular Biology Group, ARCO Plant Cell Research Institute, 6560 Trinity Ct., Dublin, CA 94568, USA

Summary. Transgenic tobacco plants were produced by inoculation of leaf disks with *Agrobacterium tumefaciens* harboring a disarmed binary vector containing soybean leghemoglobin Lbc3 and glycinin G2 genes. Physical and genetic characterization of these plants indicated that one to six copies of DNA from the vector were transferred and maintained in the plant genome. Approximately 30% of the copies transferred were found to be incomplete or rearranged and in some cases joined as inverted repeats. The transferred DNA was found at multiple genetic loci in five of the six cases examined. In one plant, kanamycin-resistance traits were at four independent chromosomal positions, although two were genetically linked at about 3 centimorgans. Thus, *Agrobacterium*-mediated DNA transfer to plants has some characteristics in common with "natural" systems in animals, such as retroviral or P-element derived systems, some characteristics in common with "artificial" systems, such as microinjection, electroporation, or calcium phosphate coprecipitation techniques, and some novel characteristics.

Key words: *Agrobacterium tumefaciens* – Binary vector – *Nicotiana tabacum* – Kanamycin-resistance traits – Genetic loci

Introduction

Agrobacterium tumefaciens transfers DNA, the T-DNA, from the Ti plasmid to plant cells (as reviewed in Nester et al. 1984; Gheysen et al. 1985). Expression of the genes encoded by the T-DNA results in increased levels of hormones in the plant, causing the formation of a neoplasm called crown gall. Imperfect 25 bp direct repeat sequences flank the T-DNA in the Ti plasmid (Simpson et al. 1982; Yadav et al. 1982). The termini sequences are required for the transfer and probably direct the transfer process. However, some copies of T-DNA found in tumors are aberrant in that they contain fragments with less than the complete T-DNA (summarized in Simpson et al. 1986), and T-DNA sequences are sometimes rearranged (Zambryski et al. 1980; Simpson et al. 1982).

To date, detailed physical and genetic characterization of the T-DNA in plant cells has been hampered by three characteristics of the system. The presence of two different T-DNAs on many of the Ti plasmids, and the large sizes of the T-DNAs have complicated the detailed physical characterization of the transferred DNA (Nester et al. 1984). Also, the altered hormone balance interferes with plant regeneration (Yang and Simpson 1981), a step required for genetic analysis. Furthermore, generalizations about the structure of the transferred DNA are difficult because of differences in the experimental approach such as *Agrobacterium* strain, the member of the Ti plasmid family, the plant host and the tissue inoculated. Finally, there are two different approaches for using the Ti plasmid as a vector, the co-integration approach where an intermediate vector co-integrates into the intact Ti plasmid and the binary approach where the T-DNA is on a separate replicon from the remainder of the Ti plasmid.

Using *Agrobacteria* containing a disarmed, binary vector with a small T-DNA, we have transformed tobacco explants. The vector (Simpson et al. 1986) was derived from the Ti plasmid through the replacement of the hormone-synthetic genes with an antibiotic-resistance marker. The transgenic tobacco plants were characterized physically and genetically. In many cases, multiple independent DNA insertions have taken place giving rise to as many as four independent genetic loci in the resulting plants.

Offprint requests to: R.B. Simpson

Materials and methods

Plasmid constructions. The plasmid pKLBG2 is a derivative of the binary vector pARC8 (Simpson et al. 1986) and contains, in addition to a chimeric drug resistance marker (*Nos/Npt*) for plants, two soybean genes. One gene encodes the leghemoglobin Lbc3 (Brisson et al. 1982), a nodule specific protein involved in nitrogen fixation and the other gene encodes the glycinin G2, a seed storage protein (Fischer and Goldberg 1982). The plasmid pKLBG2 was constructed as illustrated in Fig. 1. The plasmid pLb11-3.7 was a gift from Dr. D.P. Verma and the phage λDA28-4 a gift from Drs. R.L. Fischer and R.B. Goldberg. The *E. coli* strain K514 (λ400) was a gift from Dr. M. Zabeau, EMBL, Heidelberg, Germany. The vector was transferred from *E. coli* to *A. tumefaciens* LBA 4404 (Hoekema et al. 1983) by conjugation, in the presence of *E. coli* strain HB-101 containing the plasmid pRK2013 (Ditta et al. 1980) to mobilize the vector.

Transformation of tobacco. Leaf explants from *Nicotiana tabacum* c.v. xanthi plants, grown *in vitro*, were inoculated

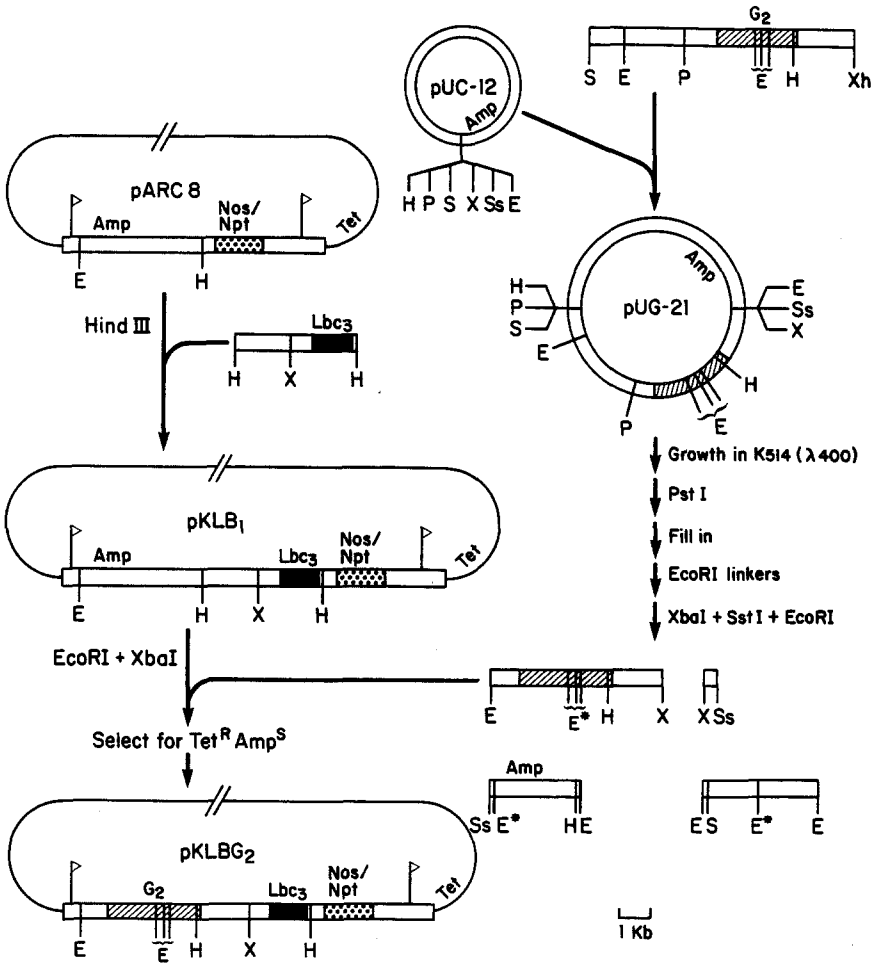

Fig. 1. Construction of the vector pKLBG2. The 3.7 kb insert of soybean DNA including the Lbc3 gene (*black bar*) from plasmid pLb11-3.7 (Brisson and Verma 1982) was digested with *Hin*dIII and ligated to pARC8 linearized with *Hin*dIII, creating pKLB1. A 9 kb *Xho*I/*Sal*I fragment from phage λDA28-4 (Fischer and Goldberg 1982), carrying the G2 gene (hatched) was subloned in pUC12 (*Sal*I site), creating pUG21. This plasmid was introduced into a E. coli strain K514[λ400], which constitutively expresses the *Eco*RI methylase. The DNA isolated from this strain was cut with *Pst*I, the ends were filled in, *Eco*RI linkers were added, and it was cut with *Eco*RI, *Xba*I and *Sst*I; the four products are shown. This mixture was ligated to pKLB1 linearized with *Eco*RI + *Xba*I to create pKLBG2. The flags stand for the termini sequences of the T-DNA borders. *E* = *Eco*RI, *H* = *Hin*dIII, *P* = *Pst*I, *S* = *Sal*I, *Ss* = *Sst*I, *X* = *Xba*I, *Xh* = *Xho*I, * = methylated restriction site

with *Agrobacterium* essentially as described (Horsch et al. 1985). Leaves were cut into small pieces (5–8 mm) and dipped in a culture of *Agrobacterium* grown overnight at 30° C in minimal A medium (Miller 1972) containing 5 mg/l of tetracycline. After 5 min of incubation, the leaf explants were blotted dry and incubated on nurse plates containing MS medium (Murashige and Skoog 1962) plus 1 mg/l benzyl amino purine and 0.1 mg/l indoleacetic acid. After 2–3 days, the leaf explants were transferred to plates containing the same medium, but without feeder cells or filter paper and containing cefotaxime (300 mg/l) and kanamycin (300 mg/l). After 2–4 weeks, shoots that developed were excised from calli and transferred to a root-inducing medium, TM5, (Shahin et al. 1985) containing cefotaxime (300 mg/l) and kanamycin (50 mg/l). Transformation of rooted plantlets was confirmed by assaying them for neomycin phosphotransferase II activity (Reiss et al. 1984) encoded by the *Nos/Npt* gene on the vector. The plantlets were finally transferred to soil.

Analysis of the progeny for resistance to kanamycin. Flowers of the regenerated plants were selfed or crossed with wild type tobacco. All flowers were bagged. Seeds were plated on LS medium (Linsmaier and Skoog 1965) containing 400 mg/l of kanamycin sulfate and incubated in unsealed dishes enclosed in transparent plastic boxes under continuous light at 25° C. Two weeks later kanamycin resistant seedlings were dark green and most of them had developed primary leaves, whereas the sensitive seedlings were white and never developed primary leaves.

DNA analysis. About 10 µg of plant DNA (Saghai-Maroof et al. 1984) was digested for 4–6 h in 300 µl of the appropriate restriction buffer using 75–100 units of each of the restriction enzymes. Before electrophoresis the mixture was extracted with phenol:chloroform (1:1) and the DNA was precipitated with ethanol. Gels were prepared for Southern transfer (Southern 1975) and hybridization essentially as described (Thomashow et al. 1980). Probes were nick translation products of either pNEO105, a derivative of pBR322 containing the chimeric *Nos/Npt* gene (Simpson et al. 1986), or a 2.0 kb EcoRI fragment, gel purified from pUG21 (Fig. 1), which is homologous to the 3′-end of the G2 gene (see Fig. 3B).

Genetic linkage of Nos/Npt genes in plant XSO-11. Our model (Fig. 4B) for the location in plant XSO-11 of the four copies of the *Nos/Npt* gene (labeled A, B, C and D) was based on experiments described in the Results. Copies B and C, which did confer kanamycin resistance, are on opposite members of a pair of homologous chromosomes but genetically close to one another. We assumed that unlinked copies A and D also conferred kanamycin resistance. Since [the frequency of kanamycin sensitive progeny in an outcross (6/1397)] equals [the product of finding neither A nor D (0.25)] times [the frequency of recombination between B and C] times [the probability of producing neither B nor C rather than both (0.5)], we estimate that the linkage between B and C is about 3 recombination units [(6/1397) × (4) × (2)]. This frequency would be reduced if the A or D loci did not confer kanamycin resistance.

Results

Transformation of tobacco

The plasmid pKLBG2 was constructed (Fig. 1) by inserting the Lbc3 leghemoglobin and G2 glycinin genes into the binary vector pARC8 (Simpson et al. 1986), a vector that lacks the Ti plasmid tumor inducing genes. In addition, pKLBG2 contains the selectable marker *Nos/Npt* which confers kanamycin-resistance on transformed plant cells through the production of the enzyme neomycinphosphotransferase II (NPT) and the termini sequences (flags in Fig. 1) required for the transfer of T-DNA to the plant genome. We transferred the plasmid to the *A. tumefaciens* strain LBA4404. This strain is disarmed in that it harbors a Ti plasmid which lacks the T-DNA region entirely but does contain the Vir genes required for the transfer of DNA from the bacterium to the plant cell (Hoekema et al. 1983). The resulting strain, LBA4404/pKLBG2, was used to inoculate segments of tobacco leaf. Of the 196 shoots that developed on kanamycin-containing medium, 78 subsequently rooted on a medium that also contained kanamycin. Each of the twenty independent plants selected for further study appeared normal and contained NPT enzyme activity (data not shown).

Molecular analysis of regenerated plants

The presence of certain T-DNA structures in the plant genome produces predictable restriction patterns from Southern blot hybridization. The presence of fully intact T-DNA sequences is indicated primarily by the detection of internal restriction fragments, of the same size and hybridization properties in the plant DNA as in the bacterial plasmid. The rest of the restriction fragments are border fragments, where T-DNA is joined to plant DNA or another copy of T-DNA. Our estimates of copy number are based on the numbers of border fragments. Incomplete or rearranged copies of T-DNA are indicated by the presence of border fragments of less than the minimum expected size or by the presence of fragments which do not correspond to any known internal fragment. When multiple T-DNA sequences are present, there is a possibility of having two or more T-DNA sequences integrated together as direct or inverted repeats. Each would yield predictable restriction patterns. The number of locations where T-DNA copies integrated into the plant genome was assumed to be the same as the copy number determined by Southern blot analysis unless there was evidence for the presence of T-DNA repeats.

Genomic DNA isolated from 9 kanamycin resistant plants was digested with the restriction enzyme *Bam*HI, *Bgl*II, *Eco*RI, or *Hin*dIII. The restriction fragments were fractioned by gel electrophoresis, transferred to nitrocellulose filters and subsequently hybridized to radiolabelled probes. Results for three representative plants are shown in Fig. 2, in which pNEO105 was used as a probe specific to the chimeric *Nos/Npt* gene, and in Fig. 3 where the probe used was a fragment specific to the 3′-end of the G2 gene. In other experiments (not shown), we used probes specific for the 5′-end of the G2 gene, the entire leghemoglobin gene, or the regions around the left and right termini sequences.

The structure of the transferred DNA in plant XSO-3 was relatively simple (lanes 2 of Figs. 2A and 3A). In each of the three restriction digests (*Bam*HI, *Hin*dIII or *Eco*RI),

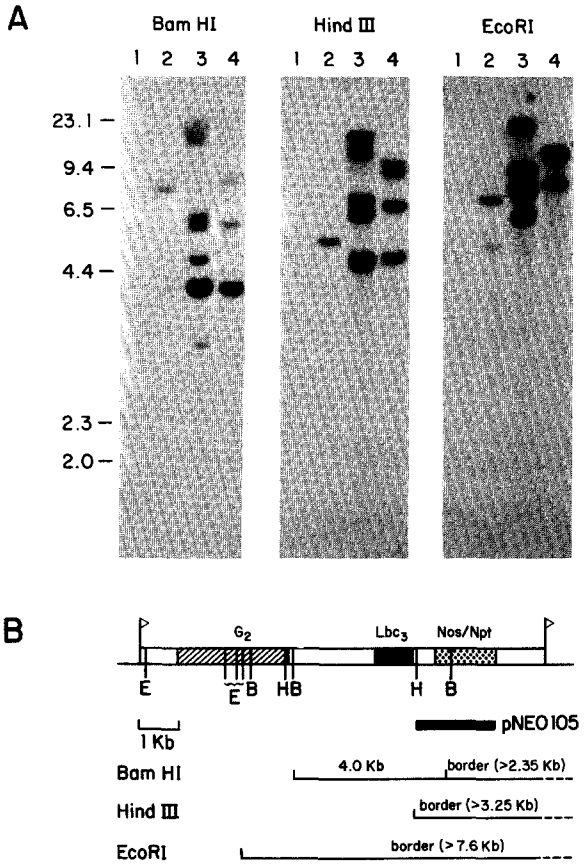

Fig. 2A, B. Analysis of the structure of the T-DNA in 3 independent transformants with the pNEO105 probe. **A** Southern blot analysis of DNA isolated from tobacco plants. Total DNA from an untransformed control plant (lanes 1) or transformants XSO-3 (lanes 2), XSO-5 (lanes 3) and XSO-11 (lanes 4) digested with *Bam*HI, *Hin*dIII or *Eco*RI, electrophoresed and blotted onto nitrocellulose. The probe was pNEO105, which is homologous to the *Nos/Npt* gene (see Fig. 2B). **B** The top line shows a schematic representation of the T-DNA of the plasmid pKLBG2 integrated in the plant genome. The symbols used are the same as in Fig. 1 with the addition of *Bam*HI (B). The size of the expected internal *Bam*HI fragment and the minimum expected sizes for the border fragments with each enzyme are shown below

only two bands hybridized when either pNEO105 or the 3′-end of the G2 gene was used as the probe. The bands represent border fragments with the exception of the 4.0 kb *Bam*HI internal fragment (Fig. 2) and the 2.0 kb *Eco*RI internal fragment (Fig. 3). The results of an *Eco*RI digest indicated the presence of an incomplete copy. First, the lower band revealed by the pNEO105 probe (Fig. 2A, lane 2) was shorter than the minimum expected size (7.6 kb, Fig. 2B) for a complete T-DNA copy. Second, when probed with part of the G2 gene (Fig. 3A), a 4.0 kb band was visible in addition to the single expected internal fragment product of 2.0 kb, indicating that the 4.0 kb fragment lacks one of the *Eco*RI sites. We conclude that XSO-3 contained 2 copies, one of which incomplete.

The situation was more complex in plant XSO-5, in which there was evidence for multiple copies. With the pNEO105 probe, a *Bam*HI digest (Fig. 2A and B) showed at least five hybridizing bands in addition to the expected internal fragment of 4.0 kb. This internal fragment hybridized more strongly than the others, confirming the presence

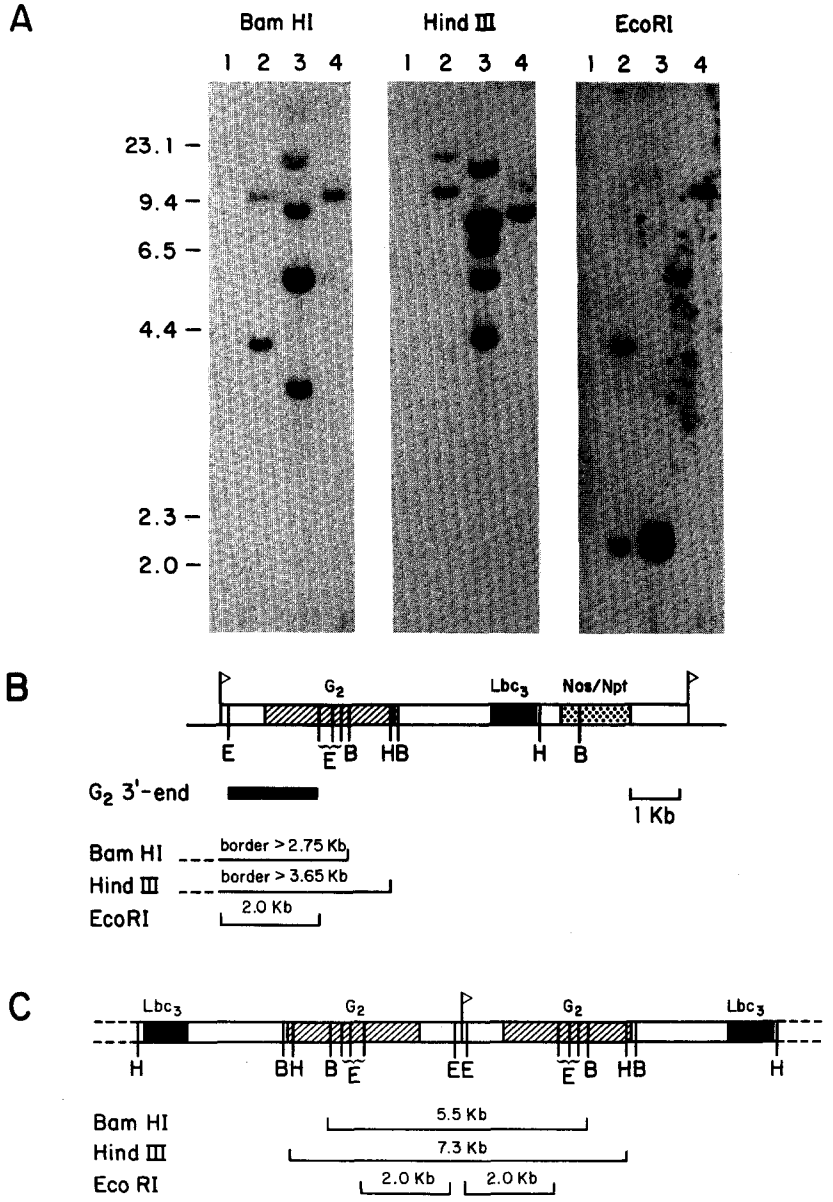

Fig. 3A–C. Analysis of the structure of the T-DNA in 3 independent transformants with the G2-specific probe. **A** Southern blot analysis of DNA isolated from tobacco plants. Total DNA prepared from an untransformed control plant (lanes 1), or transformants XSO-3 (lanes 2), XSO-5 (lanes 3) and XSO-11 (lanes 4) digested with *Bam*HI, *Hin*dIII or *Eco*RI, electrophoresed and blotted onto nitrocellulose. The probe was a 2.0 kb *Eco*RI fragment homologous to the 3′-end of the G2 gene (see Fig. 3B). **B** The top line shows a schematic representation of the T-DNA from the plasmid pKLBG2 integrated in the plant genome. The symbols used are the same as in Fig. 1. The size of the expected internal *Eco*RI fragment and the minimum expected sizes for the border fragments are shown below. **C** Schematic representation of an inverted repeat joined by left borders. The size of the expected junction fragments are shown below

of multiple inserts. The *Hin*dIII digest (Fig. 2A) showed six hybridizing fragments, while the *Eco*RI digest (Fig. 2A) showed only four. The fragments, which hybridize to the right border probe, are all larger than the minimum expected size (Fig. 2B). Thus, we estimated that this plant contained at least six copies of the T-DNA sequences which were integrated at different locations in the plant genome, each with an intact right T-DNA border. The hybridization data obtained with the G2 probe supported this model for the inserts in XSO-5. For example, the *Eco*RI digest (Fig. 3A) indicated that the six copies of the G2 gene were intact. No band, other than the expected internal 2.0 kb fragment homologous to the probe, was visible, and the hybridization signal was four to six times more intense than a one-copy signal. In either a *Bam*HI digest (Fig. 3A) or a *Hin*dIII digest (Fig. 3A), only 5 bands hybridized to the probe, but in each digest one of these bands was of double intensity. Plant XSO-5 apparently contained an inverted repeat of the T-DNA joined at the left border since the band of double intensity at about 5.5 kb in the *Bam*HI digest and the one at about 7.3 kb in the *Hin*dIII digest (Fig. 3A, lane 3) were of the sizes (Fig. 3C) predicted for such an event. Thus, we estimated that XSO-5 contained six intact copies, two of which were joined to form an inverted repeat.

Transformant XSO-11 is an illustration of a plant with multiple imperfect transfers. When DNA from this plant was probed with pNEO105 (Fig. 2A, lanes 4), at least four copies could be detected, as indicated by a *Hin*dIII digest (Fig. 2A, lane 4). However, when the 3′-end of the G2 gene was used as a probe (Fig. 3A, lanes 4), only one band hybridized in each of the three digests, demonstrating that at least three copies were incomplete, since only the fourth one had sequence homology with this probe. Moreover, the hybridization data from the *Eco*RI digest (Fig. 3A) indicated that even the fourth copy was imperfect, since the expected internal fragment of 2.0 kb (Fig. 3A and B) was replaced by a fragment of about 10.0 kb. Thus, we concluded that plant XSO-11 contained four incomplete or rearranged copies.

Table 1 summarizes the structure of the transferred DNA found in plants XSO-3, XSO-5 and XSO-11 plus

Table 1. Structure of transferred DNA in 9 independent transformants

	Copy number[a]	Aberrant copies[a]	Inverted repeats[a]	Location[a]	Loci[b]
XSO-2	2	1	–	2	nd
XSO-3	2	1	–	2	1
XSO-4	2	1	–	2	2
XSO-5	6	0	L	5	2
XSO-6	1	0	–	1	nd
XSO-7	1	0	–	1	nd
XSO-9	4	0	R	3	2
XSO-10	2	0	–	2	2
XSO-11	4	4	–	4	4[c]

R inverted repeats (right to right); L inverted repeats (left to left); nd not determined

[a] Based on Southern blot analysis (see text)
[b] Based on genetic analysis (Table 2)
[c] Based on DNA analysis of progeny (see text)

Table 2. Inheritance of kanamycin resistance in progeny of 5 independent transformants

Seed source	% germination	Number of seedlings		Suggested ratio	Chi square	Number of loci
		resistant	sensitive			
XSO-3 × self	93	427	155	3:1	0.83 ns	1[a]
XSO-3 × wt	95	254	242	1:1	0.29 ns	1[a]
XSO-4 × self	94	332	14	15:1	2.87 ns	2[b]
XSO-5 × self	nd	569	37	15:1	0.02 ns	2[b]
wt × XSO-5	95	373	114	3:1	0.66 ns	2[b]
XSO-9 × wt	97	187	64	3:1	0.03 ns	2[b]
XSO-10 × self	nd	335	16	15:1	1.71 ns	2[b]
XSO-11 × self	100	757	0	?	–	4[c]
XSO-11 × wt	97	629	3	?	–	4[c]
wt × XSO-11	97	762	3	?	–	4[c]

wt wild type parent, *Nicotiana tabacum* cv. xanthi. nd not determined; ns values not significantly different from expected ratio at 95% confidence

[a] Values for 2 loci are significantly different from expected ratio at 95% confidence
[b] Values for either 1 locus or 3 loci are significantly different from expected ratio at 95% confidence
[c] Based on DNA analysis of progeny (see text)

six other independent transgenic plants. One other feature, direct repeats, was not observed but has been reported by others (Lemmers et al. 1980; Zambryski et al. 1980).

Genetic analysis of transformants

To investigate the inheritance of the kanamycin resistance trait, six independent transformed plants were selfed, or backcrossed to the parent cultivar, and the number of seedlings resistant to kanamycin was determined (Table 2). In only one case, plant XSO-3, was the ratio of resistant to sensitive seedlings approximately 3:1 from a self and 1:1 from a backcross, the results expected if resistance were encoded by a single nuclear gene. Self pollinations of all

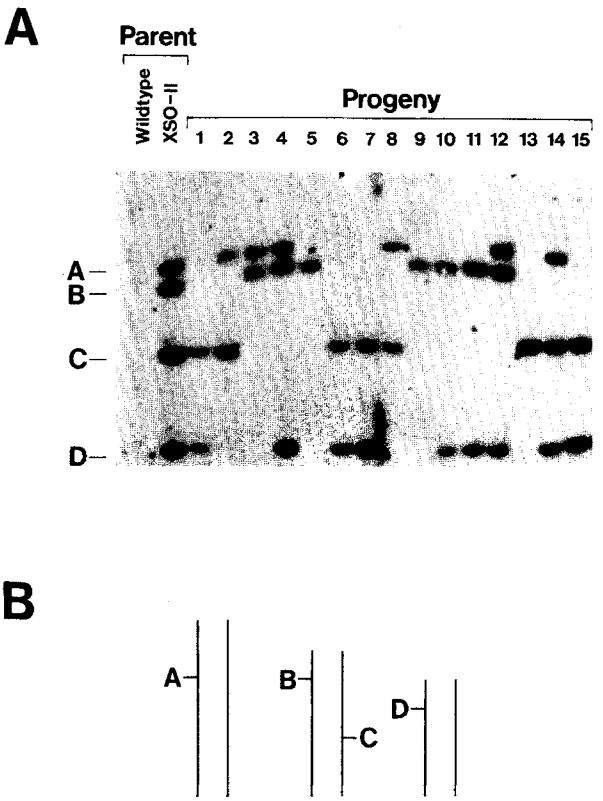

Fig. 4 A, B. Segregation of 4 loci in the F1 progeny of transformant XSO-11. **A** Southern blot analysis of the F1 progeny of transformant XSO-11 using the pNEO105 probe homologous to the *Nos/Npt* gene. Total DNA was prepared from an untransformed control plant (wildtype), transformant XSO-11 and 15 seedlings (germinated in the absence of kanamycin) representing the F1 progeny of XSO-11 crossed with wildtype (lanes 1–15). **B** Model for location of the 4 kanamycin-resistant loci in XSO-11. Each vertical line represents one of a pair of homologous chromosomes; only 3 of the 24 pairs of tobacco are shown. Note that the length of the lines serves only to distinguish the pairs and not to indicate chromosomal lengths

the other plants yielded a resistant to sensitive ratio of greater than 3:1, indicating that the transferred DNAs were inserted and functioning at multiple, unlinked genetic loci.

Seedling populations from self-pollinations of plants XSO-4 and XSO-10, respectively, segregated in a 24:1 and a 21:1 ratio of kanamycin-resistant to kanamycin sensitive seedlings. Since this is not significantly different from the 15:1 ratio expected for two unlinked loci (Table 2), these results agree with the DNA analysis which had shown two T-DNA copies at different locations (Table 1).

In contrast, the number of locations of T-DNA was greater than the number of kanamycin resistance loci in plants XSO-3, XSO-5, and XSO-9. In plant XSO-3, the kanamycin resistance trait segregated in the progeny from a self in a 3:1 ratio, the expected ratio for a single locus, even though the DNA analysis had showed two copies of the T-DNA. In plant XSO-5, the kanamycin trait segregated in a 15:1 ratio in the progeny from a self and in a 3:1 ratio in a reciprocal backcross to wildtype. These are ratios to be expected for independent segregation and assortment of two dominant unlinked genes. However, DNA analysis had showed that XSO-5 contained six copies of T-DNA. Similarly, plant XSO-9 contained four copies

of T-DNA in three locations yet transmitted the kanamycin resistance trait in a ratio expected for only two loci.

Transformant XSO-11 contained four T-DNA copies. All of the 757 seedlings from self-pollinations that were screened for kanamycin resistance were resistant, suggesting the segregation of at least four independent genes. However, of the 1397 seedlings from reciprocal backcrosses, only six were sensitive to kanamycin. This result is significantly different from the expected value of 92 sensitive seedlings if four independent genes were involved. One explanation is that there are actually more than 4 copies; 7 to 9 copies at separate loci should produce the observed result. The alternative is that there is a copy on each member of a pair of homologous chromosomes and that the copies are close together genetically. To distinguish between these alternatives, we analyzed the structure of the DNA in progeny of a backcross. Figure 4 shows the result of Southern blot analysis using pNEO105, a probe specific for the gene encoding kanamycin resistance. The parent plant XSO-11 resulted in four bands labeled A, B, C, D while the progeny (lanes 1–15) resulted in only two or three of the bands in various combinations. Thus, the four bands represent four loci that are segregating in the progeny. Bands B and C appear to be linked by repulsion, that is, B or C but not both were found in each progeny. This would happen if the T-DNAs corresponding to these loci were located in linked or allelic positions on homologous chromosomes. If they were located on homologous chromosomes but in non-allelic positions, some of the progeny would be sensitive to kanamycin because of recombination between B and C. The presence of 6 sensitive seedlings among 1400 outcross seedlings (Table 2) must be the result of such recombination. If we assume that both the A and D copies confer kanamycin resistance then we can estimate the recombination frequency between B and C from this figure to be about 3 recombination units, as described in Materials and methods.

Discussion

The results of the physical analysis of the structure of T-DNA transferred to tobacco from *Agrobacterium tumefaciens* strain LBA4404 containing the vector pKLBG2 are summarized in Table 1. The number of copies of the T-DNA sequence integrated in the plant genome varied from one to six. Seven of these copies were incomplete or rearranged out of an aggregate number of 24 in the nine plants examined. Two transformants (XSO-5 and XSO-9) contained two T-DNA copies joined as inverted repeats, structures seen previously in tumor cell lines (Simpson et al. 1982; Kwok et al. 1985). Seven of the nine plants contained T-DNA copies in more than one location.

The results of the genetic analysis of these plants confirmed that DNA transfer to multiple locations in the plant genome was common. Five of the six plants analyzed for the inheritance of the the kanamycin-resistance trait contained T-DNA at more than one genetic locus (Table 2). In two cases, XSO-4 and XSO-10 the number of loci, determined genetically, equaled the number of genomic locations, determined physically. In three other cases, XSO-3, XSO-5, and XSO-9, the number of functional loci was less than the number of genomic locations. In these latter plants, some copies of the *Nos/Npt* gene encoding kanamycin-resistance may be phenotypically silent due to mutation, methylation, or chromosomal position (Jaenisch et al. 1981; Jahner et al. 1982). Alternatively, all the *Nos/Npt* genes may be functional, but segregate as a single locus because of linkage on the same chromosome. In addition to the genetic analysis of the parent plant XSO-11, physical analyses of the progeny were necessary to establish that XSO-11 contained T-DNA at four genetic loci, two of which are on opposite members of a pair of homologous chromosomes but genetically close to one another.

The resulting structures of DNA transferred to plant genomes using the Agrobacterium system resemble the products of other "natural" transfer systems in some respects and the products of "artificial" transfer systems in others. As is true for natural retroviral and P-element mediated gene transfer to animal cells (Jaenisch et al. 1981; Rubin and Spradling 1982), *Agrobacterium* can transfer a single well-defined piece of DNA. Other times, *Agrobacterium* can transfer multiple copies in tandem array, or incomplete copies as do artificial systems for gene transfer in animals (Perucho et al. 1980; Scangos and Ruddle 1981). However, in addition to direct repeats, Agrobacterium mediated transfer can result in inverted repeats of the transferred DNA.

The integration of foreign DNA at multiple loci in a single transgenic organism is not common with either of the other systems. In experiments using direct DNA transformation or *Agrobacterium tumefaciens* to transfer DNA to tobacco, a single genetic locus has been found in 18/20 cases (Otten et al. 1981; Memelink et al. 1983; Wostemeyer et al. 1984; Paszkowski et al. 1984; De Block et al. 1984; Horsch et al. 1984, 1985; Sengupta-Gopalan et al. 1985; Hain et al. 1985; Potrykus et al. 1985; Deshayes et al. 1985; Peerbolte et al. 1985; de Framond et al. 1986; Czako and Marton 1986). The explanation for the occurrence of multiple loci cannot therefore be found in differences of experimental organism. The transformation protocol that we used was derived from one that had given rise to several plants with T-DNA at only a single locus, so this is an unlikely cause of a difference. We are currently testing the hypothesis that the copy number of the binary vector plasmid (four to seven based on the parent plasmid RK2) is an important difference from the single copy found in co-integration vectors. In support of this hypothesis, we note that of the transgenic plants which were derived from *Agrobacterium* transformation, the only one shown to contain more than one locus was the only one involving a binary vector (de Framond et al. 1986). In any case, the ability of Agrobacteria to transfer DNA to multiple locations in a plant genome emphasizes the higher efficiency of this natural system relative to the other systems, natural or artificial.

The presence of multiple loci of foreign DNA in a plant after one round of *Agrobacterium* transformation opens new approaches for experiments with transgenic plants. For example, a high number of loci per transgenic plant facilitates the use of the T-DNA as an insertion mutagen for gene isolation (Bingham et al. 1981) in dicotyledonous plants, much as the controlling element Activator has been used in a monocotyledonous plant (Fedoroff et al. 1984). Further, since more than one Agrobacterium can frequently transfer DNA to a single plant cell (Depicker et al. 1985; McKnight, Lillis, and Simpson, unpublished), it should be possible to isolate plants containing a selected T-DNA and an unselected T-DNA at different loci and then to identify progeny with only the unselected DNA. Thus, any undesir-

able effects of the selectable marker genes on a crop plant under field conditions could be eliminated. Further, the same selectable marker could be used repeatedly as desirable traits are added sequentially. The ability to transfer large numbers of DNAs from different vectors to the same plant cell could also facilitate gene isolation through genomic library transfer and complementation of function in plants. Segregation of the different inserts would greatly simplify the isolation of the responsible library member.

Acknowledgements. We thank Doug Hanahan (Cold Spring Harbor Lab), and our collegues at ARCO PCRI, Simon Bright in particular, for valuable discussions; Mike Freeling (University of California, Berkeley), Simon Bright, Jack Erion and Val Williamson for criticism of the manuscript; Dale Cetlinski for artwork; Karen Long for typing the manuscript; Bob Fischer, Bob Goldberg, Desh Pal Verma and M. Zabeau for strains; and Annick de Frammond and Mary-Dell Chilton for a preprint of their manuscript. For a portion of this work, A.S. was supported by the Swiss National Science Foundation.

References

Bingham PM, Levis R, Rubin GM (1981) Cloning of DNA sequences from the white locus of *D. melanogaster* by a novel and general method. Cell 25:693–704

Brisson N, Verma DP (1982) Soybean leghemoglobin gene family: Normal, pseudo, and truncated genes. Proc Natl Acad Sci USA 79:4055–4059

Czako M, Marton L (1986) Independent integration and seed-transmission of the TR-DNA of the octopine Ti plasmid pTi Ach5 in *Nicotiana plumbaginifolia*. Plant Mol Biol 6:101–109

De Block M, Herrera-Estrella L, Van Montagu M, Schell J, Zambryski P (1984) Expression of foreign genes in regenerated plants and in their progeny. EMBO J 3:1681–1689

de Framond AJ, Back EW, Chilton WS, Kayes L, Chilton MD (1986) Two unlinked T-DNAs can transform the same tobacco plant cell and segregate in the F1 generation. Mol Gen Genet 202:125–131

Depicker A, Herman L, Jacobs A, Schell J, Van Montagu M (1985) Frequencies of simultaneous transformation with different T-DNAs and their relevance to the *Agrobacterium*/plant cell interation. Mol Gen Genet 201:477–484

Deshayes A, Herrera-Estrella L, Caboche M (1985) Liposome-mediated transformation of tobacco mesophyll protoplasts by an *Escherichia coli* plasmid. EMBO J 4:2731–2737

Ditta G, Stanfield S, Corbin D, Helinski DR (1980) Broad host range DNA cloning system for gram-negative bacteria: Construction of a gene bank of *Rhizobium meliloti*. Proc Natl Acad Sci USA 77:7347–7351

Fedoroff NV, Furtek DB, Nelson Jr OE (1984) Cloning of the bronze locus in maize by a simple and generalizable procedure using transposable controlling element activator (Ac). Proc Natl Acad Sci USA 81:3825–3829

Fischer RL, Goldberg RB (1982) Structure and flanking regions of soybean seed protein genes. Cell 29:651–660

Gheysen G, Ghaese P, Van Montagu M, Schell J (1985) DNA flux across genetic barriers: the crown gall phenomenon. Hohn B, Dennis ES (eds) Genetic flux in plants. Springer, Wien New York, pp 11–47

Hain R, Stabel P, Czernilofsky AP, Steinbiss HH, Herrera-Estrella L, Schell J (1985) Uptake, integration, expression and genetic transmission of a selectable chimaeric gene by plant protoplasts. Mol Gen Genet 199:161–168

Hoekema A, Hirsch PR, Hooykaas PJJ, Schilperoort RA (1983) A binary plant vector strategy based on separation of vir- and T-region of the *Agrobacterium tumefaciens* Ti-plasmid. Nature 303:179–180

Horsch RB, Fraley RT, Rogers SG, Sanders PR, Lloyd A, Hoffmann N (1984) Inheritance of functional foreign genes in plants. Science 223:496–498

Horsch RB, Fry JE, Hoffmann NL, Eichholtz D, Rogers SG, Fraley RT (1985) A simple and general method for transferring genes into plants. Science 227:1229–1231

Jaenisch R, Jahner D, Nobis P, Simon I, Lohler J, Harbers K, Grotkopp D (1981) Chromosomal position and activation of retroviral genomes inserted into the germ line of mice. Cell 24:519–529

Jahner D, Stuhlmann H, Stewart CL, Harbers K, Lohler J, Simon I, Jaenisch R (1982) De novo methylation and expression of retroviral genomes during mouse embryogenesis. Nature 298:623–628

Kwok WW, Nester EW, Gordon MP (1985) Unusual plasmid DNA organization in an octopine crown gall tumor. Nucleic Acids Res 13:459–471

Lemmers M, De Beuckeleer M, Holsters M, Zambryski P, Depicker A, Hernalsteens JP, Van Montagu M, Schell J (1980) Internal organization, boundaries and integration of Ti-plasmid DNA in nopaline crown gall tumor. J Mol Biol 144:353–376

Linsmaier EM, Skoog F (1965) Organic growth factor requirements of tobacco tissue cultures. Physiol Plant 8:100–127

Memelink J, Wullens GJ, Schilperoort RA (1983) Nopaline T-DNA is maintained during regeneration and generative propagation of transformed tobacco plants. Mol Gen Genet 190:516–522

Miller J (1972) In: Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring, NY, pp 431–432

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473–497

Nester EW, Gordon MP, Amasino RM, Yanofsky MF (1984) Crown Gall: A molecular and physiological analysis. Ann Rev Plant Physiol 35:387–413

Otten L, De Greve H, Hernalsteens JP, Van Montagu M, Schieder O, Straub J, Schell J (1981) Mendelian transmission of genes introduced into plants by the Ti plasmids of *Agrobacterium tumefaciens*. Mol Gen Genet 183:209–213

Paszkowski J, Shillito RD, Saul M, Mandak V, Hohn T, Hohn B, Potrykus I (1984) Direct gene transfer to plants. EMBO J 3:2717–2722

Peerbolte R, Krens FA, Mans MW, Floor M, Hoge HC, Wullens GJ, Schilperoot RA (1985) Transformation of plant protoplasts with DNA: Cotransformation of non-selected calf thymus carrier DNA and meiotic segregation of transforming DNA sequences. Plant Mol Biol 5:235–246

Perucho M, Hanahan D, Wigler M (1980) Genetic and physical linkage of exogenous sequences in transformed cells. Cell 22:309–317

Potrykus I, Paszkowski J, Saul MW, Petruska J, Shillito RD (1985) Molecular and general genetics of a hybrid foreign gene introduced into tobacco by direct gene transfer. Mol Gen Genet 199:169–177

Reiss B, Sprengel R, Willi M, Shaller H (1984) A new sensitive method for qualitative and quantitative analysis of neomycin phosphotransferase in crude cell extracts. Gene 30:217–223

Rubin GM, Spradling AC (1982) Genetic transformation of *Drosophila* with transposable element vectors. Science 218:348–353

Saghai-Maroof MA, Soliman KM, Jorgensen RA, Allard RW (1984) Ribosomal DNA spacer-length polymorphisms in barley: Mendelian inheritance, chromosomal location, and population dynamics. Proc Natl Acad Sci USA 81:8014–8018

Scangos G, Ruddle FH (1981) Mechanisms and applications of DNA-mediated gene transfer in mammalian cells – a review. Gene 14:1–10

Sengupta-Gopalan C, Reichert NA, Barker RF, Hall TC, Kemp JD (1985) Developmentally regulated expression of the bean β-phaseolin gene in tobacco seed. Proc Natl Acad Sci USA 82:3320–3324

Shahin EA (1985) Totipotency of tomato protoplasts. Theor Appl Genet 69:235–240

Simpson RB, O'Hara PJ, Kwok W, Montoya AL, Lichtenstein C, Gordon MP, Nester EW (1982) DNA from the A6S/2 crown gall tumor contains scrambled Ti-plasmid sequences near its junctions with plant DNA. Cell 29:1005–1014

Simpson RB, Spielmann A, Margossian L, McKnight TD (1986) A disarmed binary vector from *Agrobacterium tumefaciens* functions in *Agrobacterium rhizogenes*: Frequent co-transformation of two distinct T-DNAs. Plant Mol Biol 6:403–415

Southern EM (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. J Mol Biol 98:503–517

Thomashow MF, Nutter R, Montoya AL, Gordon MP, Nester EW (1980) Integration and organization of Ti plasmid sequences in crown gall tumors. Cell 19:729–739

Wostemeyer A, Otten L, Schell JS (1984) Sexual transmission of T-DNA in abnormal tobacco regenerants transformed by octopine and nopaline strains of *Agrobacterium tumefaciens*. Mol Gen Genet 194:500–507

Yadav NS, Venderleyden J, Bennett DR, Barnes WM, Chilton M-D (1982) Short direct repeats flank the T-DNA on a nopaline Ti-plasmid. Proc Natl Acad Sci USA 79:6322–6326

Yang F-M, Simpson RB (1981) Revertant seedlings from crown gall tumors retain a portion of the bacterial Ti-plasmid sequences. Proc Natl Acad Sci USA 78:4151–4155

Zambryski P, Holsters M, Kruger K, Depicker A, Schell J, Van Montagu M, Goodman (1980) Tumor DNA structure in plant cells transformed by *A. tumefaciens*. Science 209:1385–1391

Communicated by R.B. Goldberg

Received April 17, 1986