

Monohybrid and dihybrid segregations in the progenies of tobacco transformed for kanamycin resistance with a Ti-vector system

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Abstract. A chimeric DNA construction having nopaline synthase promoter, coding sequences of neomycin phosphotransferase gene conferring resistance to antibiotic kanamycin and OCS (octopine synthase) polyadenylation sequences bracketed by T-DNA ends was transferred to tobacco. Leaf discs were infected with *A. tumefaciens* containing disarmed, cointegrate plasmid pGV3850::1103 and allowed to form a callus in the presence of kanamycin. Shoots regenerated from infected leaf discs either through the callus or arising directly were further selected for their ability to root in kanamycin-containing media. Among the nine transgenic plants that were progeny tested, the transferred bacterial gene segregated as monohybrid ratio (3 Kan^R: 1 Kan^S) in seven. Segregation data of two plant progenies indicated the presence of two independent loci of Kan^R DNA insertion (15 Kan^R: 1 Kan^S). Back-cross segregation data were consistent with the monohybrid or independent assortment of duplicate factors. Thus in the two cases, a minimum independent integration of two copies of T-DNA each with a Kan^R marker is inferred.

Keywords. *A. tumefaciens* (pTi Kan^R); tobacco leaf disc infection; genetic transformation; vertical transmission; monohybrid and dihybrid segregations.

1. Introduction

Methods for transferring specific single genes to plants using recombinant DNA techniques have been developed recently (see reviews by Herrera-Estrella *et al* 1985, Fraley *et al* 1986, and Bhatia *et al* 1986). Expression of the horizontally-transferred genes followed by their vertical transmission has been shown for *Agrobacterium*-mediated (De Block *et al* 1984; Horsch *et al* 1984) and direct transfers (Potrykus *et al* 1985; Hain *et al* 1985). Ti plasmid of *Agrobacterium tumefaciens* is eminently suitable for plants like tobacco that can be transformed *in vitro* by bacteria harbouring 'disarmed', engineered plasmid, and transformed cells can then be regenerated to form plants. Two types of vector systems have been developed; one utilizing an intermediate vector (Zambryski *et al* 1983; Herrera-Estrella *et al* 1983) and the other binary vectors (De Framond *et al* 1983). Recipient systems could either be isolated protoplasts (Marton *et al* 1979) or leaf discs (Horsch *et al* 1985). Spielmann and Simpson (1986) suggest that the number of genetic loci and copies integrated in the transgenic plants may depend on the use of intermediate or binary vectors. In the present study, tobacco leaf discs were infected with *A. tumefaciens* bacteria carrying an intermediate type of vector having a Kan^R selectable marker between the two ends of T-DNA. In the progeny of plants regenerated from transformed leaf discs, the Kan^R marker segregated both as single or as two independent genetic loci.

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2. Materials and methods

Bacterial strains: *E. coli* and *A. tumefaciens* strains, plasmids, their characteristics and their sources are given in table 1.

2.1 Plasmid transfer

pLGV *neo* 1103 was mobilized into *Agrobacterium* by triparental mating using *E. coli* strain GJ23 containing plasmids pGJ28 and R64*drd*11. The three strains C58C1, HB101 and GJ23 grown to midlog phase were mixed in equal amounts and 0.1 ml of the mixture was spread on YEB agar plates. After 16–18 hr growth, bacteria were resuspended in λ buffer (0.01 M MgSO₄, 0.01 M Tris-HCl, pH 7.2) and plated. Exconjugants were selected for rifampicin (Rif^R) and kanamycin resistance (Kan^R). Such clones of *A. tumefaciens* were inferred to carry the cointegrate pGV3850::1103.

2.2 Plant cell transformation

A simplified procedure essentially as prescribed by Horsch *et al* (1985) was used. Young leaves of tobacco (*Nicotiana tabacum* L) cultivars Petit Havana, and Jayashree, grown in pots were sterilized with 0.1% HgCl₂ containing a drop of Tween 20. Leaf discs were punched using a cork borer and incubated at 25°C in bacterial broth overnight (18–20 hr) with *A. tumefaciens* having the cointegrate pGV3850::1103. After incubation, leaf discs were washed in sterile water and placed in petri dishes or test tubes containing Murashige-Skoog (MS) medium (Murashige and Skoog 1962) having 0.1–1.0 mg/l naphthalene acetic acid (NAA), 0.1–2.0 mg/l benzylaminopurine (BAP), 100 mg/l kanamycin sulphate and 400–500 mg/l of cefotaxime (Claforan, Roussel).

2.3 Rooting of the shoots

Shoots formed directly from the leaf discs or from the callus on leaf discs were transferred to MS media containing 100 mg/l kanamycin.

Table 1. Bacterial strains and plasmids

Strains	Plasmids	Characteristics	Reference
<i>A. tumefaciens</i> C58C1	pGV3850	Cb ^R , Rif ^R , T-DNA border sequences, NOS gene	Zambryski <i>et al</i> (1983)
<i>E. coli</i> HB101	pLGVneo1103	Kan ^R , Cb ^R , Chimeric construction NOS promoter NPT II gene and OCS Poly A	Velten <i>et al</i> (1984)
<i>E. coli</i> GJ23	pGJ28 R64 <i>drd</i> 11	Kan ^R /Nm ^R , ColE1 <i>mob</i> , <i>bom</i> , Tc ^R , Sm ^R , <i>tra</i>	Van Haute <i>et al</i> (1983)

All the above strains were obtained from Professor M Van Montagu

2.4 Growing of transformed plants

Regenerated shoots that produced roots in kanamycin-containing media were transferred to paper cups filled with sterilized soil. Subsequently these were transferred to large pots and raised to maturity in the field.

2.5 Progeny testing for kanamycin resistance

Seeds harvested from transformed plants were planted in petri-dishes containing N6 medium (1/10th strength) with 100 mg/l kanamycin and 0.8% agar. Polyurethane sponge soaked in the above medium was found to be more convenient for raising seedlings for progeny testing. Kan^R plants were dark green and produced secondary leaves while Kan^S plants had light green cotyledonary leaves and did not form any secondary leaves.

2.6 Inheritance of kanamycin resistance

Regenerated, transformed plants in the first (R₁) generation were also pollinated with the pollen from the untransformed, parent cultivar to check the segregation for kanamycin resistance or sensitivity. Seeds obtained after crossing were grown like self-pollinated seeds.

2.7 Nopaline assay

Kan^R plants in the R₁ and R₂ generations were tested for the presence of nopaline following the methods of Otten and Schilperoort (1978).

3. Results and discussion

Leaf discs incubated with *Agrobacterium* and cultured on MS media containing kanamycin showed callus formation as well as shoot regeneration (figures 1,2). On subculturing the transformed callus showed further shoot regeneration. Direct as well as callus-mediated shoots were further selected for their ability to form roots in kanamycin-containing media. Plants that rooted in such media were raised to maturity and were nopaline positive.

Most of the transformed plants were phenotypically normal and fertile. A few plants showed marked elongation of the style (figure 3). Heterostyly has been reported previously in tobacco plants regenerated from tumour tissue transformed by oncogenic strains of *Agrobacterium* (Wullems *et al* 1981).

The selfed progenies of the transformants segregated into Kan^R and Kan^S plants (figure 4). Presence of nopaline was detected in both the transformed plants and their progenies (figure 5). Inheritance in the progeny of seven independent transformants, of which four were also back-crossed is given in table 2. Selfed seeds of seven transformants segregated into Kan^R and Kan^S seedlings in ratios that fit the expected 3:1 ratio considering the dominance of Kan^R and single or multiple tandem insertion(s) of the gene sequence as reported by De Block *et al* (1984). In two progenies, viz., 86-6 and 86-7, the number of Kan^R plants was considerably

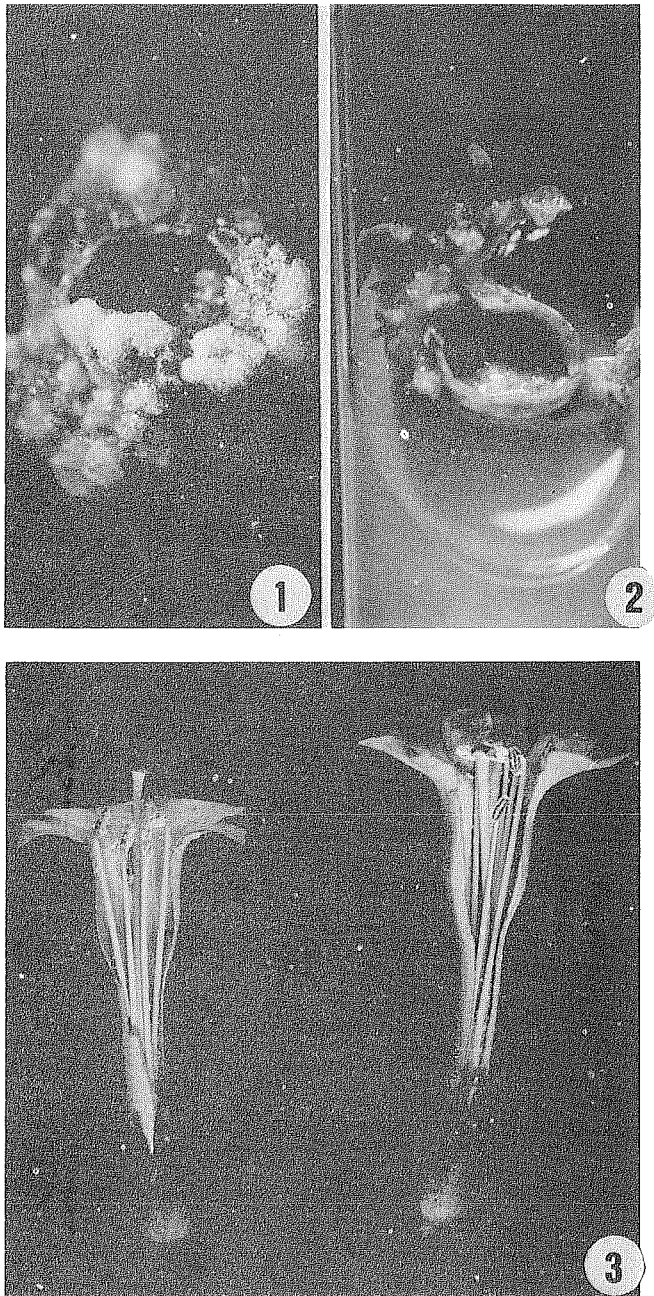


Figure 1. Callus formation from tobacco leaf discs incubated with *Agrobacterium tumefaciens* cultured on medium containing kanamycin.

Figure 2. Direct shoot regeneration from leaf discs on kanamycin-containing medium.

Figure 3. Flowers from a transformed plant showing heterostyly (left) and a normal style (right).

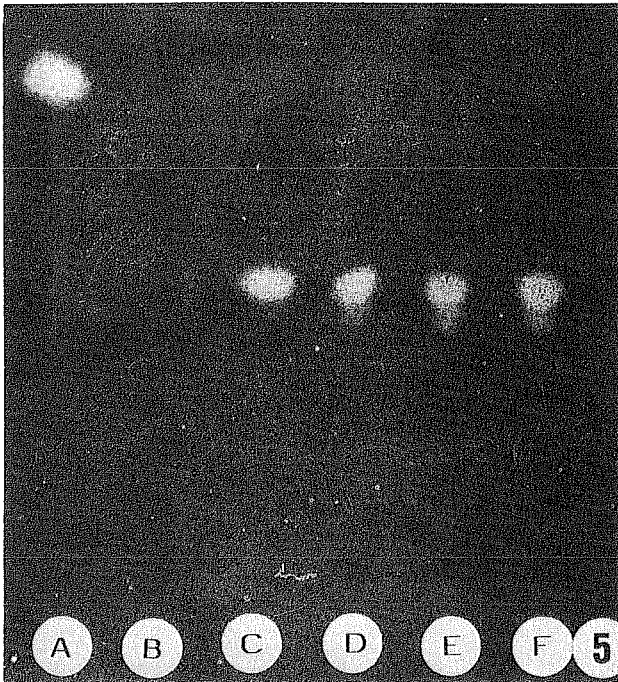
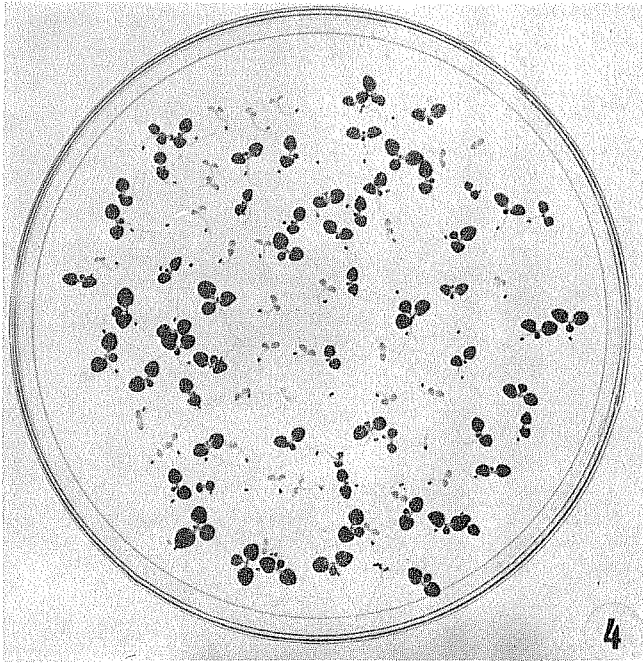


Figure 4. Segregation for kanamycin resistant and sensitive (bleached) plants in the progeny of a transformed plant.

Figure 5. Nopaline assay: A. Arginine standard; B. Untransformed tobacco leaf; C. Nopaline standard; D.E.F. Leaves from the progeny of transformed plants.

Table 2. Segregation of kanamycin resistance/sensitivity in the progeny of transformants

Pedigree number	Number of seedlings		χ^2		Probability
	Kan ^R	Kan ^S	for expected segregation		
86-1	865	248	3:1	4.2	0.05-0.02
86-2	328	83	3:1	4.8	0.05-0.02
86-3	180	50	3:1	1.13	0.90-0.70
86-4	50	23	3:1	1.49	0.30-0.20
86-5	290	77	3:1	2.93	0.10-0.05
86-6	552	46	15:1	1.88	0.20-0.10
86-7	253	10	15:1	2.63	0.30-0.20
86-8	918	296	3:1	0.21	0.70-0.50
86-9	777	263	3:1	0.04	0.90-0.80
86-5x	109	97	1:1	0.58	0.50-0.30
White Burley					
86-7x	156	62	3:1	1.37	0.30-0.20
Petit Havana					
86-9x	183	157	1:1	1.84	0.20-0.10
Petit Havana					
86-4x	114	116	1:1	0.04	0.70-0.90
Petit Havana					

higher than expected on the basis of monohybrid inheritance. The observed segregation in these two progenies fits the 15:1 ratio, expected on the basis of at least two insertions of the dominant Kan^R gene on different chromosomes or two unlinked sites on the same chromosome. The back-cross segregation of 1:1 and 3:1 supports monohybrid and dihybrid segregations in the selfed progenies.

In earlier reports, 1-16 copies of T-DNA insertions have been found (Ursic *et al* 1983; Hepburn *et al* 1983). Two questions arise: i) Does more than one original copy enter the nucleus or are the multiple insertions produced as a secondary event? and ii) Is there a propensity for the integrations to occur in tandem? Data of Spielmann and Simpson (1986) with leaf disc transformation of Kan^R marker and binary vector system suggest that in seven out of nine cases more than one T-DNA copy was integrated, and in five out of six plants analysed more than one genetic site was involved. Qualitatively our results are similar, although we do not have evidence for involvement of more than two loci. It is conceivable that the quantitative difference is due to the vector system used.

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