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Reversible methylation and inactivation of marker genes in sequentially transformed tobacco plants

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Doubly transformed tobacco plants were obtained following sequential transformation steps using two T-DNAs encoding different selection and screening markers: T-DNA-I encoded kanamycin resistance and nopaline synthase; T-DNA-II encoded hygromycin resistance and octopine synthase. A genetic analysis of the inheritance of the selection and screening marker genes in progeny of the doubly tranformed plants revealed that the expression of T-DNA-I genes was often suppressed. This suppression could be correlated with methylation in the promoters of these genes. Surprisingly, both the methylation and inactivation of T-DNA-I genes occurred only in plants containing both T-DNAs: when self-fertilization or backcrossing produced progeny containing only T-DNA-I, expression of the genes on this T-DNA was restored and the corresponding promoters were partially or completely demethylated. These results indicated that the presence of one T-DNA could affect the state of methylation and expression of genes on a second, unlinked T-DNA in the same genome.

Key words: gene inactivation; promoter methylation; sequential transformation; T-DNA; transgenic plants

Introduction

Transformation vectors based on the T-DNA region of the *Agrobacterium tumefaciens* tumor-inducing (Ti) plasmid have been particularly useful for studying gene expression in higher plants (Klee *et al.*, 1987; Schell, 1987). With these vectors, genes can be transferred into the nuclei of susceptible plant cells, such as those of tobacco or petunia, and the expression of the transgenes can then be studied in regenerated plants (Kuhlemeier *et al.*, 1987; Willmitzer, 1988). For certain purposes, it could be advantageous to transfer several genes into a plant genome. However, the number of genes or size of a DNA fragment that can be inserted into T-DNA at one time is limited by the availability of unique restriction enzyme sites following each insertion. Two approaches for overcoming this limitation are co-transformation and re-transformation.

Co-transformation with more than one T-DNA has been accomplished by incubating plant cells either simultaneously with two different strains of *Agrobacterium*, each with a different Ti plasmid (Depicker *et al.*, 1985; Petit *et al.*, 1986; Simpson *et al.*, 1986; McKnight *et al.*, 1987; Prosen and Simpson, 1987), or with one strain of *Agrobacterium* containing two T-DNAs on separate plasmids (de Framond *et al.*, 1986; Simpson *et al.*, 1986; Hamill *et al.*, 1987). Direct gene transfer using a mixture of two plasmids has been used to co-transform plant cells with a selectable and a non-selectable gene (Schocher *et al.*, 1986).

Several genes can also be introduced into a plant cell during sequential transformation steps (re-transformation) if an alternative system to select transformants is available for the second round of transformation. Sequential transformation steps using kanamycin and hygromycin selection respectively, have produced transgenic tobacco plants containing both sense and antisense constructions of either the nopaline synthase gene (Sandler *et al.*, 1988) or the gene for chloramphenicol acetyltransferase (Delauney *et al.*, 1988).

For the present study, we have placed genes encoding kanamycin or hygromycin resistance, together with genes for unique screening markers, on two different Ti plasmid vectors, and used them to obtain doubly transformed plants in sequential transformation steps. In this paper, we describe a genetic and molecular analysis of marker gene expression in the progeny of several double transformants. Unexpectedly, this analysis revealed that the presence of one T-DNA could, in some cases, influence the degree of methylation and expression of a second, unlinked T-DNA in the same genome.

Results

(i) Production of doubly transformed plants by sequential transformation with two different T-DNAs

Following the first round of transformation, a Kan^{r}NOS$^+$ plant that contained one complete copy of T-DNA-I (which

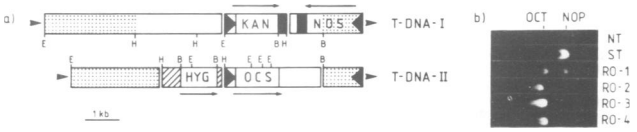

Fig. 1. (**a**) Maps of T-DNAs used in sequential transformation steps to obtain doubly transformed tobacco plants. T-DNA-I contained genes for neomycin phosphotransferase II (NPTII), which allowed selection of transformed plant cells on kanamycin (KAN); and nopaline synthase (NOS), so that nopaline (NOP) could be used as a screening marker. T-DNA-II encoded hygromycin (HYG) resistance and octopine synthase (OCS), so that octopine (OCT) could be used as a screening marker. Like the intact NOS gene, the NPTII gene and the OCS gene were under the control of the NOS promoter (large black triangles). The black rectangles in T-DNA-I represent the NOS transcription terminator. The hygromycin phosphotransferase gene was under the control of the 35S promoter and transcription terminator (hatched regions). The orientation of each gene is indicated by the arrows above and below the maps. The T-DNA borders are indicated by the small flanking arrowheads. The dotted areas indicate homologous regions (~2 kb on the left and 1 kb on the right) between the two T-DNAs. Only the restriction enzyme sites necessary for understanding the constructions are shown. E = *Eco*RI; H = *Hin*dIII; B = *Bam*HI. (**b**) Opine content of the original singly transformed plant (ST), which contained NOP, and four double transformants. One of these (R0-1) contained both NOP and OCT; the other three (R0-2, R0-3, R0-4) contained only OCT. NT = normal tobacco.

Table I. Inheritance of antibiotic resistance markers in R1 and F1 progeny of plants transformed sequentially with T-DNA-I (Kan^{r}NOS) and T-DNA-II (Hyg^{r}OCS)

Case 1. Homozygous T-DNA-I; heterozygous T-DNA-II (unlinked); KKHO genotype (plants R0-1, R0-2, R0-3).

Predicted genotypes	Predicted phenotypes	
	A. T-DNA−I active	B. T-DNA-I inactive in presence of T-DNA-II
KKHO × KKHO (selfed)		
25% KKHH	Kan^{r}Hyg^r	Hyg^r
50% KKHO	Kan^{r}Hyg^r	Hyg^r
25% KKOO	Kan^r	Kan^r
Totals	100% Kan^r; 75% Hyg^r; 75% Kan^{r}Hyg^r	25% Kan^r; 75% Hyg^r; 0% Kan^{r}Hyg^r
KKHO × OOOO (backcross)		
50% KOHO	Kan^{r}Hyg^r	Hyg^r
50% KOOO	Kan^r	Kan^r
Totals	100% Kan^r; 50% Hyg^r; 50% Kan^{r}Hyg^r	50% Kan^r; 50% Hyg^r; 0% Kan^{r}Hyg^r

Actual phenotypes	Selfed (R1 generation)	Backcross (F1 generation)	Phenotype
R0-1: Kan^r	300/300 = **100%**[a]	331/334 = **99%**	A
Hyg^r	230/300 = **77%**	240/458 = **52%**	
Kan^{r}Hyg^r	222/300 = **74%**	203/377 = **54%**	
R0-2: Kan^r	122/638 = **19%**[b]	273/604 = **45%**	B
Hyg^r	557/720 = **77%**	253/467 = **54%**	
Kan^{r}Hyg^r	0/639 = **0%**	0/328 = **0%**	
R0-3: Kan^r	199/715 = **28%**	443/897 = **49%**	B
Hyg^r	417/543 = **77%**	258/500 = **52%**	
Kan^{r}Hyg^r	0/509 = **0%**	0/570 = **0%**	

The number of resistant seedlings is shown over the total number tested. The percentage of resistant seedlings is shown in bold.
[a]For R1 seedlings of R0-1 germinated without selection, 100% contained NOS; 75% also contained OCS. Approximately 25% of the Kan^r R1 seedlings were NOS$^+$OCS$^-$; 75% contained both OCS and NOS. Hyg^r R1 seedlings were all NOS$^+$OCS$^+$, as were the Kan^{r}Hyg^r R1 progeny.
[b]For R1 seedlings of R0-2 germinated without selection, ~25% contained NOS, and the other 75% contained OCS. No seedlings contained both opine synthase activities. The Kan^r R1 progeny of R0-2 all contained only NOS; the Hyg^r R1 seedlings contained only OCS.

encoded these traits; Figure 1a) was chosen. Of the progeny obtained from selfing this plant, 75% were Kan^r. These Kan^r progeny were either homozygous (KK) or heterozygous (KO) for T-DNA-I. Leaf disks of plants from each T-DNA-I genotype were re-transformed using T-DNA-II, which encoded Hyg^r and OCS (Figure 1a). Of the 79 regenerated Hyg^r plants, ~50% contained both OCS and NOS; in 15% however, only OCS could be detected. To determine why NOS was absent from these plants, one OCS$^+$NOS$^+$ double transformant and three OCS$^+$NOS$^-$ double transformants were chosen for further study. Figure 1b shows the opine content of these four plants (designated the R0 generation) along with that of the original singly transformed plant. The remaining 35% of the regenerated plants, most of which were OCS$^-$NOS$^+$, were probably escapes from the hygromycin selection, and were not analyzed further.

(ii) Inheritance of antibiotic resistance markers in progeny of double transformants: genetic evidence that T-DNA-I was inactive in the presence of T-DNA-II

The absence of NOS in some of the double transformants was not due to the loss of T-DNA-I, but to inactivation of the NOS gene, since both it and a chimeric NPTII gene (conferring Kan^r) were present in all four double transformants [described in detail in Part (v)]. To determine whether a similar inactivation of the NPTII gene had occurred, progeny obtained from both self-fertilization of the four double transformants, and backcrossing them with untransformed tobacco (producing the R1 and F1 generations, respectively) were tested for resistance to either Kan or Hyg, or a combination of both (Table I).

Case 1: T-DNA genotype KKHO. The double transformants R0-1 (OCS$^+$NOS$^+$), and R0-2 and R0-3 (both OCS$^+$NOS$^-$) were derived from a singly transformed plant with a KK genotype. Since ~75% of the respective R1 seedlings were Hyg^r (indicating a single T-DNA-II locus which was inherited and expressed as a dominant Mendelian trait), their T-DNA genotype was KKHO. Table I shows the predicted genotypes of progeny produced by selfing and backcrossing of a KKHO plant, along with the actual phenotypes obtained for R0-1, R0-2 and R0-3.

Differences among these double transformants arose in the number of Kan^r and Kan^{r}Hyg^r progeny. Although R0-1 produced 100% Kan^r offspring as expected, only ~25% of the R1 progeny of R0-2 and R0-3 were Kan^r. In addition, no Kan^{r}Hyg^r progeny were obtained from selfing or backcrossing of R0-2 and R0-3, again in contrast to R0-1. Finally,

Table II. Inheritance of antibiotic resistance markers in R2 and F2 offspring and inferred genotypes of R1 plants

		Kan^r	Hyg^r	Kan^{r}Hyg^r
A. R1(R0-1) (Kan^{r}NOS$^+$OCS$^+$)	Selfed (R2)	172/172 = **100%**	138/183 = **75%**	127/174 = **73%**
Possible: KKHH or KKHO	Backcross (F2)	204/204 = **100%**	54/114 = **47%**	111/224 = **50%**
Actual: KKHO				
B. R1(R0-2) (Kan^{r}NOS$^+$OCS$^-$)	Selfed	131/131 = **100%**[a]	0/144 = **0%**	0/164 = **0%**
Probable: KKOO	Backcross	147/147 = **100%**	0/126 = **0%**	0/142 = **0%**
Actual: KKOO				
C. R1(R0-2) (Hyg^{r}OCS$^+$NOS$^-$)	Selfed	58/218 = **27%**[c]	161/201 = **80%**[d]	0/144 = **0%**
Possible: KKHH or KKHO	Backcross	169/338 = **50%**	192/376 = **51%**	0/349 = **0%**
Actual: KKHO[b]				
D. R1(R0-3) (Kan^{r}NOS$^+$OCS$^-$)	Selfed	212/212 = **100%**	0/150 = **0%**	0/138 = **0%**
Probable: KKOO	Backcross	371/371 = **100%**	0/288 = **0%**	0/290 = **0%**
Actual: KKOO				
E. R1(R0-3) (Hyg^{r}OCS$^+$NOS$^-$)	Selfed	0/203 = **0%**	217/217 = **100%**[e]	0/216 = **0%**
Probable: KKHH or KKHO	Backcross	0/77 = **0%**	184/184 = **100%**	0/120 = **0%**
Actual: KKHH				
F. R1(R0-4) (Kan^{r}NOS$^+$OCS$^-$)	Selfed	317/317 = **100%**	0/317 = **0%**	0/194 = **0%**
Possible: KKOO or KOOO				
Actual: KKOO				
G. R1(R0-4) (Hyg^{r}OCS$^+$NOS$^-$)	Selfed	0/316 = **0%**	314/314 = **100%**	0/203 = **0%**
Possible: KKHH, KKHO, KOHH, KOHO				
Actual: K?HH[f]				

The results were obtained after selfing, and in some cases backcrossing, of seven different R1 progeny: one from R0-1 (A) and two each from R0-2 (B and C), R0-3 (D and E) and R0-4 (F and G). The phenotypes are enclosed in parentheses. The number of resistant seedlings is shown over the total number tested. The percentage of resistant seedlings is shown in bold. The possible (or in the case of the Kan^{r}NOS$^+$OCS$^-$ plants in B and D, the probable) genotypes of the R1 plants (Table I) are also listed, along with the genotype most consistent with the data.

[a]100 of these seedlings tested were NOS$^+$OCS$^-$.

[b]This plant reproduced the results obtained for the original R0-2 and R0-3 double transformants (compare with Table I, phenotype B) and was presumably the same genotype: KKHO.

[c]100% of the seedlings tested were NOS$^+$OCS$^-$.

[d]100% of the seedlings tested were OCS$^+$NOS$^-$.

[e]Although no Kan^{r}NOS$^+$ progeny were obtained from this plant or R1(R0-4) (part **G**), both contained T-DNA-I, as demonstrated by hybridization to the KAN and NOS probes (Figure 3, bottom).

[f]From the data, it could only be concluded that this plant was homozygous (HH) for T-DNA-II. This plant obvious contained at least one T-DNA-I (K) allele, as illustrated by hybridization of KAN and NOS probes to DNA isolated from this plant (Figure 3, bottom), but no Kan^r progeny were obtained because of the inactivation of T-DNA-I in the presence of T-DNA-II.

more Kan^r progeny were obtained from the R0-2 and R0-3 plants after backcrossing with untransformed tobacco (~50%) than after selfing (~25%). The same result was obtained regardless of which parent was the transformed plant.

Given that both T-DNA-I and T-DNA-II were present in the R0-1, R0-2 and R0-3 plants, three states of T-DNA-I activity were possible (Table I): it could be fully active, as exemplified by plant R0-1 (phenotype A); it could be totally inactive (in which case no Kan^r seedlings should have been obtained); or it could be active only in the absence of T-DNA-II, resulting in some Kan^r seedlings, but no seedlings which were resistant to both antibiotics (phenotype B). As shown in Table I, the third alternative best explained the results obtained for plants R0-2 and R0-3. A DNA blot analysis of 10 Kan^r R1 and F1 progeny of R0-2 and R0-3 confirmed that T-DNA-II was indeed absent from these plants (data not shown).

Case 2: T-DNA genotype KOHO. The above effect was also observed with double transformants derived from a singly transformed plant which was heterozygous (KO) for T-DNA-I, as illustrated by the double transformant R0-4 (OCS$^+$NOS$^-$). Since 75% of the R1 progeny were Hyg^r, the T-DNA genotype of R0-4 was KOHO. Two observations were made, that backcrossing with untransformed tobacco produced a higher percentage of Kan^r progeny than did selfing and that there was a lack of any Kan^{r}Hyg^r offspring (data not shown), again suggesting that T-DNA-I was inactive in the presence of T-DNA-II.

(iii) Inheritance of OCS and NOS screening makers in progeny of double transformants: evidence for coordinate control of selection and screening marker genes

The presence or absence of the two screening markers in R1 and F1 seedlings germinated without selection closely

conformed to the expression of the corresponding antibiotic resistance markers, indicating coordinate control of the genes on each T-DNA [cf. the percentages of NOS^+, OCS^+ and NOS^+OCS^+ progeny (Table I, legend) with the percentages of Kan^r, Hyg^r and Kan^rHyg^r progeny (Table I), respectively]. For seedlings germinated on antibiotics, all Kan^r progeny of R0-2 contained only NOS and never OCS; conversely, all Hyg^r progeny of R0-2 contained only OCS and never NOS. In contrast, 100% of the Kan^r progeny of R0-1 contained NOS and in addition, 75% also contained OCS. All the Hyg^r and Kan^rHyg^r progeny of R0-1 contained both OCS and NOS (Table I, legend).

(iv) Inheritance of antibiotic resistance markers in R2 and F2 progeny: confirmation of genotypes of R1 plants

If, in the OCS^+NOS^- plants chosen for this study, T-DNA-I were only active in the absence of T-DNA-II, then the Kan^r R1 progeny of R0-2 and R0-3 could only have the genotype KKOO (Table I, phenotype B). Such plants should produce 100% Kan^r progeny and no Hyg^r or Kan^rHyg^r offspring following self-fertilization or backcrossing (which produce the R2 and F2 generations, respectively). Exactly these results were obtained for the progeny of the $Kan^rNOS^+OCS^-$ R1 plants tested (Table IIB and D), thus confirming that these plants had the required genotype. In contrast, the R2 progeny of R0-1 (in which both T-DNAs were expressed) included both Hyg^r and Kan^rHyg^r seedlings (Table IIA).

Hyg^r R1 progeny of R0-2 or R0-3 could have had genotypes of either KKHH or KKHO (Table I, phenotype B). If T-DNA-I continued to be inactive in the presence of T-DNA-II, then only the KKHO genotype would produce, following selfing, any Kan^r progeny (25% of the total); these would again contain only T-DNA-I (and have the genotype KKOO). The KKHH genotype would produce no Kan^r progeny, since 100% of the offspring would contain T-DNA-II (and be Hyg^r). Two examples, which illustrate both of these cases, are shown in Table IIC and E. Similar logic was used to determine the genotypes of R1 progeny of R0-4 (original genotype KOHO) (Table IIF and G).

These results demonstrated not only that the suppression of T-DNA-I in the presence of T-DNA-II was stably maintained in successive generations (Table IIC, E and G), but also confirmed that the T-DNA-I genes were not irreversibly inactivated, since double transformants which were originally $Hyg^rOCS^+NOS^-$ (and presumably Kan^s since no Kan^rHyg^r seedlings were observed) again produced some $Kan^rNOS^+OCS^-$ progeny (Table IIC). Also, since progeny with a KKOO genotype were obtained (Table IIB, D and F), it could be concluded that T-DNA-I and T-DNA-II were not genetically linked in the double transformants.

(v) Mechanism of inactivation of the NPTII and NOS genes: reversible methylation of promoters

A possible explanation for the reversible suppression of NPTII and NOS gene expression was that these genes became methylated in some plants following the introduction of T-DNA-II, and were demethylated when T-DNA-I was inherited independently of T-DNA-II in some progeny. An analysis using methylation-sensitive restriction enzymes indicated that this was indeed the case.

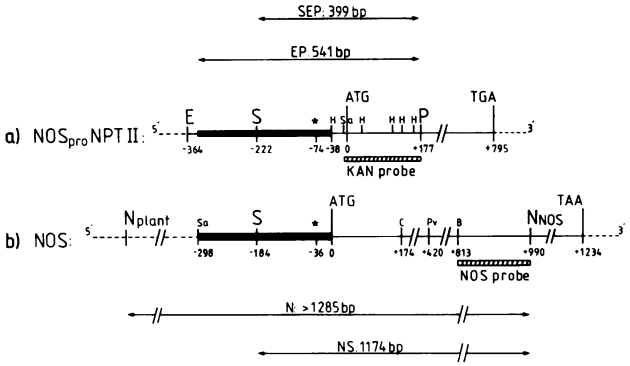

Fig. 2. Strategy for detecting methylation in T-DNA-I genes. Both the NPTII gene (**a**), conferring Kan^r and the NOS gene (**b**) were under the control of the NOS_{pro} (thick black line), which was ~300 bp in these constructions. The stars indicate the transcription start site of the NOS gene (Depicker *et al.*, 1985). The positions of restriction enzyme sites are numbered relative to the ATG start codons. The TGA and TAA stop codons are indicated. The NOS_{pro}–NPTII chimeric gene contained 38 bp upstream from the ATG not present in the intact NOS gene. Potentially methylatable sites in the genes are as follows: (**a**) NOS_{pro}–NPTII: in addition to an *Sst*II site (S; −222) in the NOS_{pro}, there were five *Hpa*II (H) sites. One, at −38, was contributed by a polylinker added during a construction step; the other four were in the NPTII protein-coding region between the ATG and a *Pst*I (P) site at +177. (**b**) NOS: methylatable sites included the *Sst*II site (S; −184) in the promoter and in the protein-coding region a *Cla*I site (C; +174) and a *Pvu*II site (P; +420). In preliminary experiments, none of the sites in the coding region of either gene appeared to be methylated. Therefore, only methylation at the *Sst*II site in the NOS_{pro} was studied in detail. This was done by digesting with either *Sst*II plus *Eco*RI (E; −364) and *Pst*I (for NOS_{pro}–NPTII); or (for NOS) *Sst*II plus *Nsi*I (N), for which there were sites at +990 (N_{NOS}) and upstream from the NOS_{pro} in plant DNA (N_{plant}). The fragment sizes expected from these digests are shown above or blow each map. The probes used are indicated by the hatched regions. Sa = *Sau*3a; B = *Bam*HI.

Maps of the NOS gene and the NPTII gene (under the control of the NOS promoter) indicating the positions of sites for methylation-sensitive restriction enzymes are shown in Figure 2. In preliminary experiments with each of these enzymes, only the *Sst*II site in the NOS_{pro} of each gene appeared to be methylated; therefore this site alone was examined in detail for all plants.

As Figure 3 shows, this *Sst*II site in both the NOS gene and the NOS_{pro}–NPTII chimeric gene (KAN probe) was totally or partially unmethylated in all plants containing an active T-DNA-I [the original singly transformed plant (top), the NOS^+OCS^+ double transformant R0-1 (middle) and the $Kan^rNOS^+OCS^-$ R1 progeny of all four double transformants (bottom)]. In contrast, this site appeared to be completely methylated in all plants in which T-DNA-I was inactive [the three $Hyg^rOCS^+NOS^-$ double transformants: R0-2, R0-3, R0-4 (middle) and their $Hyg^rOCS^+NOS^-$ R1 progeny (bottom)].

Discussion

We have obtained tobacco plants transformed sequentially with two T-DNAs encoding different screening and selection markers and found that in some of the double transformants, the genes for selection and screening markers on the first T-DNA became inactivated following the second transformation step. This inactivation was correlated with methylation at an *Sst*II site in the promoters of the affected

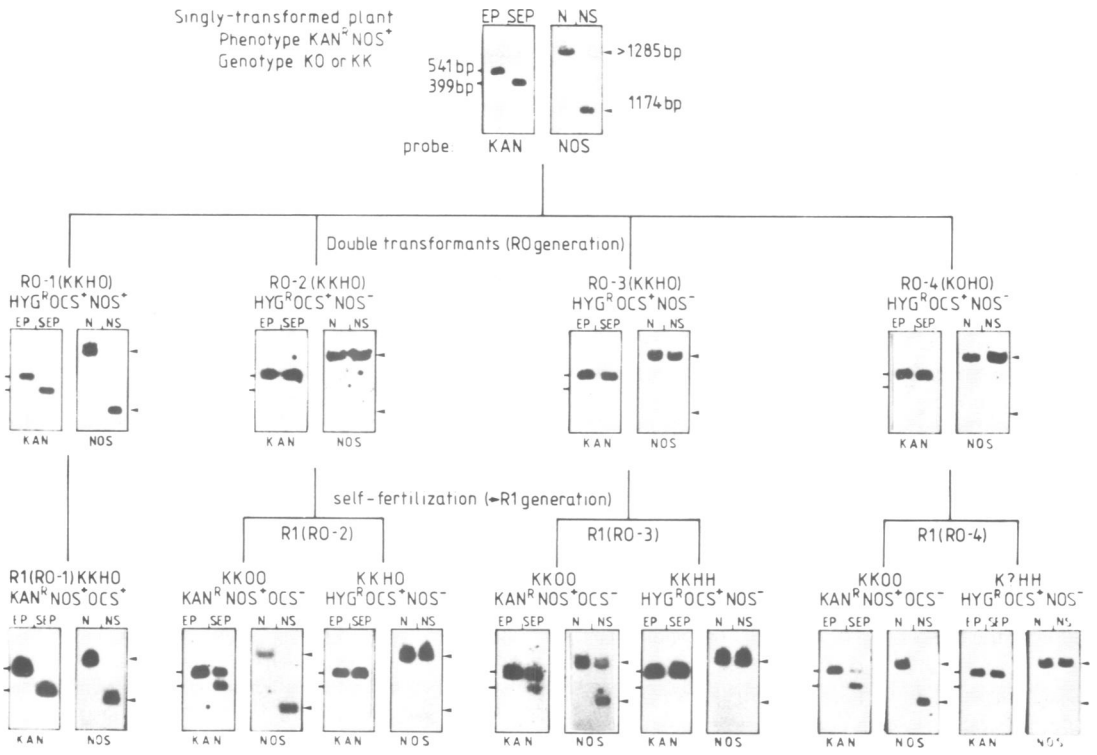

Fig. 3. Reversible methyation of promoters in T-DNA-I genes. DNA was isolated from the indicated plants and digested first with *Sst*II (S), followed by digestion with either *Nsi*I (N) or *Eco*RI/*Pst*I (EP) and then probed with either NOS or KAN, respectively. For an explanation of the fragment sizes expected from these digests, see Figure 2. The T-DNA-I in all plants shown was derived from the original singly transformed plant (**top**). In this plant, which was Kan^{r}NOS$^+$, the *Sst*II site in the NOS_{pro} of both T-DNA-I genes was unmethylated. In contrast, this site was methylated in the original Hyg^{r}OCS$^+$NOS$^-$ double transformants (R0-2, R0-3, R0-4) (**middle**), and Hyg^{r}OCS$^+$NOS$^-$ R1 progeny of these plants (**bottom**). For R0-2, R0-3 and R0-4 the methylation of T-DNA-I genes resulted from the introduction of T-DNA-II during the second transformation step. Although the Hyg^{r}OCS$^+$ plants were NOS$^-$ (and presumably Kan^s, since none of the progeny were resistant to both antibiotics) they clearly still contained T-DNA-I genes (see also Table IIC). For the double transformant R0-1 (middle), in which the expression of T-DNA-I genes was unaffected by the presence of T-DNA-II, the *Sst*II site in the promoters of T-DNA-I genes remained unmethylated. Kan^{r}NOS$^+$ R1 progeny of all double transformants, regardless of the parental phenotype, also contained T-DNA-I genes with at least partially unmethylated *Sst*II sites in the promoters (bottom). For Kan^{r}NOS$^+$ R1 progeny of R0-2, R0-3 and R0-4, this represented a reversal from the methylated states of these genes in the parent double transformants. This reversal was dependent on the absence of T-DNA-II from the genome of these R1 progeny (Table IIB, D and F). Such partial or complete demethylation of T-DNA-I promoters was also observed in a number of Kan^{r}NOS$^+$ F1 progeny of R0-2 and R0-3. These F1 progeny also lacked T-DNA-II and produced only Kan^r F2 offspring. The indicated genotypes were determined by analyzing the segregation of antibiotic resistance markers (Tables I and II). The EP/SEP and N/NS digests were run on 2.5 and 1.7% agarose gels, respectively. Slight differences in the migration of some bands were due to variations among individual blots, as were differences in band intensity.

genes. Furthermore, both the methylation and inactivation of T-DNA-I genes were dependent on the presence of T-DNA-II in the same genome: when selfing or backcrossing with untransformed tobacco produced progeny containing only T-DNA-I, the promoter methylation was reduced or abolished and the genes on T-DNA-I were again expressed. The methylation pattern of the NOS_{pro} and the expression of T-DNA-I genes could thus be traced in a pedigree from the Kan^{r}NOS$^+$ singly transformed plant (unmethylated and expressed), to the Hyg^{r}OCS$^+$NOS$^-$ double transformants of the R0 generation (methylated and not expressed), to Kan^{r}NOS$^+$OCS$^-$ plants of the R1 generation (these plants again contained only T-DNA-I which was unmethylated and expressed).

Further evidence for the decisive role played by T-DNA-II in the suppression of T-DNA-I genes was provided by the observation that plants which were regenerated directly from the singly transformed plant (i.e. without re-transformation with T-DNA-II) were all Kan^{r}NOS$^+$ (data not shown). In other words, the inactivation of T-DNA-I genes was only observed after the introduction of T-DNA-II into the genome of the singly transformed plant. This, along with the reversibility of the methylation and suppression of T-DNA-I genes, confirmed that a somatic mutation, which conceivably could have occurred in T-DNA-I genes during the regeneration process, was not responsible for the observed effect.

Even though the two T-DNAs did not have to be genetically linked to display such an interaction, it is likely that the site of insertion of T-DNA-II with respect to T-DNA-I played a role. Since all of the double transformants were derived from the same singly transformed plant, the chromosomal location of T-DNA-I and the genetic background into which T-DNA-II integrated should have been the same in all cases. However, since T-DNA integrates randomly into plant chromosomes (Ambros *et al.*, 1986; Chyi *et al.*, 1986; Wallroth *et al.*, 1986), the location of T-DNA-II presumably differed from plant to plant. There were obviously a number of sites into which T-DNA-II could integrate and not affect T-DNA-I expression, as illustrated by the double transformants in which both T-DNAs were expressed (50% of the total). However, the insertion of T-DNA-II into certain sites apparently created an arrangement which was incompatible with the expression of both

T-DNAs. In our study, T-DNA-II appeared to be dominant over T-DNA-I, but this was probably because hygromycin (the T-DNA-II selection marker) was used to select after the second transformation step.

We observed no differences in the expression of T-DNA-I genes in offspring obtained from the reciprocal backcrosses. Therefore, the effect we have described is not strictly analogous to genomic imprinting in animals, which is the differential modification and expression of the maternal and paternal genomes in progeny of sexual crosses (Monk, 1988). However, since there was a single specific factor (the presence or absence of T-DNA-II) which affected T-DNA-I methylation and expression in some plants, our results support the general concept that non-random patterns of differential methylation are established in plant genomes.

Although it remains to be seen whether other genetic elements are capable of influencing the degree of methylation of related elements or genes in the same genome, it has long been recognized that certain non-reciprocal interactions can occur between non-allelic genes. In such an interaction, termed epistasis (Lerner and Libby, 1976), one gene appears to suppress the expression of another. The molecular basis of epistasis is not yet understood. It is possible however, that a system such as ours, in which the expression of easily distinguishable, genetically unlinked transgenes can be studied in transformed organisms and their progeny, will be useful for investigating the molecular mechanisms involved in this phenomenon.

Although the two T-DNAs used in our study contained genes for different selection and screening markers, they nevertheless shared substantial regions of homology (Figure 1a). Two copies of the NOS_{pro} ($\sim$300 bp) were present on each T-DNA. In addition, homologous regions of $\sim$2 kb and 1 kb were present just inside the left and right borders of each T-DNA. It is possible that these homologous sequences were in some way responsible for the differential methylation of T-DNA-I in some doubly transformed plants. In our experiments, methylation of T-DNA-I genes apparently occurred in somatic cells during the second transformation step (i.e. in individual leaf disk cells of the singly transformed plant from which the double transformants were regenerated). Demethylation probably took place during gametogenesis, since it was first observed in Kan^r R1 and F1 progeny.

A correlation between cytosine methylation and inactivation of plant genes has also been observed for the NOS gene in flax tumors (Hepburn *et al.*, 1983), other T-DNA genes (Gelvin *et al.*, 1983; Amasino *et al.*, 1984; van Slogteren *et al.*, 1984; Peerbolte *et al.*, 1986), rRNA genes (Blundy *et al.*, 1987; Watson *et al.*, 1987) and maize zein genes (Bianchi and Viotti, 1988). Unlike others (Hepburn *et al.*, 1983; Amasino *et al.*, 1984; van Slogteren *et al.*, 1984; Peerbolte *et al.*, 1986), we have been unable to reverse the methylation of T-DNA-I genes by treating seedlings with 5-azacytidine (data not shown). The reversible inactivation we observed resulting from a methylation–demethylation cycle occurring in the time span of a single generation is similar to that reported for the maize transposable elements Activator (Ac) (Chomet *et al.*, 1987) and Robertson's mutator (Chandler and Walbot, 1986; Chandler *et al.*, 1988).

In practical terms, our results suggest that if sequential transformation steps using T-DNA are used to introduce genes into plants, resistance to all antibiotics should be selected for in later steps. Our findings might also partially explain why T-DNA copy number in transgenic plants is not a good indicator of the level of expression of transgenes, and why a high copy number of T-DNA is often associated with abnormal or non-Mendelian inheritance of selection marker genes (Deroles and Gardner, 1988a,b). An effect similar to the one we have observed could also possibly occur between multiple T-DNAs which have integrated during the same transformation step.

Materials and methods

Plasmids and vector constructions

(i) Plasmids: pGV99 is a pBR325 derivative containing the octopine synthase gene (OCS; DeGreve *et al.*, 1983). pDH51 is a pUC18 derivative which contains a polylinker flanked by the promoter and transcription terminator of the 35S transcript of cauliflower mosaic virus; $35S_{pro}$ and $35S_{ter}$ respectively (Pietrzak *et al.*, 1986). pGL2 is a pDH51 derivative containing the protein coding region of the bacterial gene for hygromycin B transferase (Hyg) (Gritz and Davies, 1983; van den Elzen *et al.*, 1985) placed between the $35S_{pro}$ and $35S_{ter}$ (J.Paszkowski, personal communication).

(ii) Assembly of the T-DNA in vector I (first round transformation vector): this binary vector was the same as BV1 described by Schernthaner *et al.* (1988). It contained as a selection marker the neomycin phosphotransferase II (NPTII) gene under the control of the nopaline synthase promoter (NOS_{pro}) and as a screening marker, the NOS gene. Into the *Eco*RI site of this vector, a vicilin gene, contained on a 5 kb *Eco*RI fragment from the pea genome (T.J.Higgins and E.Newbigin, personal communication), was inserted.

(iii) Construction of vector II (second round transformation vector): this binary vector was similar to the one described above, except that it contained as selection and screening markers Hyg^r and OCS, respectively. These components were added to the T-DNA region of the binary vector as follows:

(a) Construction of a NOS_{pro}–OCS chimeric gene. A *Sal*I linker was inserted into the *Sma*I site of pGV99. The resulting 2.5 kb *Bam*HI/*Sal*I fragment, which contained the OCS protein coding sequence and transcription terminator, was then inserted behind a 300 bp NOS_{pro} fragment (Matzke and Matzke, 1986) present in a pUC8 plasmid that had the *Sma*I site converted into a *Hin*dIII site. The *Bam*HI site was then destroyed and the *Sal*I site changed into a *Bam*HI site. The resulting plasmid contained the NOS_{pro}–OCS chimeric gene on a 2.8 kb *Hin*dIII/*Bam*HI fragment.

(b) Construction of a $35S_{pro}$–Hyg–$35S_{ter}$ chimeric gene: the Hyg protein-coding region was released from pGL2 with *Bam*HI and inserted in the correct orientation into a pDH51 plasmid in which the *Sal*I site in the polylinker had been destroyed and the *Nco*I site had been converted into a *Hin*dIII site. The resulting $35S_{pro}$–Hyg–$35S_{ter}$ chimeric gene was present on a 1.7 kb *Hin*dIII fragment.

(c) Assembly of T-DNA in vector II. The *Eco*RI/*Hin*dIII fragment '13L' which was present in the binary cassette (Matzke and Matzke, 1986) was replaced by the 2.5 kb *Eco*RI/*Hin*dIII promoter fragment of the 5 kb vicilin gene. The *Hin*dIII/*Bam*HI fragment '23L' in the binary cassette was replaced by the *Hin*dIII/*Bam*HI fragment containing the NOS_{pro}–OCS chimeric gene. In the single *Hin*dIII site of this construction, the 1.7 kb *Hin*dIII fragment containing the $35S_{pro}$–Hyg–$35S_{ter}$ was inserted in the orientation shown in Figure 1a.

All steps in the construction of the vectors and their introduction into *A.tumefaciens* were carried out as described by Matzke and Matzke (1986).

Transformation of plants

In the first round of transformation, leaf disks (Horsch *et al.*, 1985) of *Nicotiana tabacum* cv. petit havana SR1 were incubated with *A.tumefaciens* harboring a binary vector which contained T-DNA-I (Figure 1a). Transformed shoots growing from the leaf disks were selected on Murashige and Skoog (MS) medium containing 1 mg/l 6-benzoaminopurine, 500 mg/l claforan (a gift of Albert-Roussel Pharma, Vienna, Austria) and 50 mg/l kanamycin sulfate. In the second round of transformation, leaf disks from Kan^rNOS^+ progeny of one plant which contained a single complete copy of T-DNA-I, determined by Southern blotting (data not shown) and genetic analysis (selfing produced 901 Kan^r seedlings and 290 kan^s seedlings), were

incubated with agrobacteria harboring a binary vector which contained T-DNA-II (Figure 1a). Doubly transformed shoots were selected on the medium described above, except 40 mg/l hygromycin B was substituted for kanamycin. Southern blot analysis using T-DNA-II probes demonstrated that the four double transformants chosen for study were the products of different insertion events (data not shown).

Similarly to the nomenclature of Potrykus *et al.* (1985), the doubly transformed plants regenerated on hygromycin-containing medium were referred to as the R0 generation. Progeny obtained from self-fertilization of R0 plants were designated the R1 generation, selfing of R1 plants yielded the R2 generation. Backcrosses of R0 or R1 plants with untransformed tobacco produced the F1 or F2 generations respectively. The T-DNA genotypes are designated by K (Kan^r) and H (Hyg^r), for T-DNA-I or T-DNA-II, respectively. Although not specifically indicated in the genotype nomenclature, any plant with a K allele also contained the NOS gene and any plant with an H allele also contained the OCS gene.

Germination test

Seeds were surface sterilized by treating them for 30 s in a 10% solution of commercially available sodium hypochlorite solution (chloride content 12%) containing 0.2% SDS, followed by a 30 s wash in 70% ethanol and two rinses with sterile glass-distilled water. Treated seeds were placed on solid MS medium, containing either 50 mg/l kanamycin or 40 mg/l hygromycin or a combination of the two. After 6–8 weeks, seedlings were scored for resistance. Resistant seedlings, which were green and grew roots on the antibiotics, were easily distinguishable from sensitive seedlings, which never formed roots and turned white shortly after germinating. Approximately 200 seeds from at least two different seed pods (when available) of each plant were tested on each antibiotic. In all cases, similar results were obtained for groups of seeds from different pods.

Assays for screening markers

Extracts of leaves or seedlings were tested for octopine synthase (OCS) activity following the procedure of Otten and Schilperoort (1978). If nopaline (NOP) was still present in these extracts, it was easily visible on the paper electropherogram migrating just below octopine (OCT). If a putative double transformant was OCS$^+$NOP$^-$, leaves were tested for nopaline synthase (NOS) activity (Otten and Schilperoort, 1978). In all cases however, absence of NOP was shown to correlate with a lack of NOS activity.

Plant DNA isolation and Southern blot analysis

Plant DNA was isolated from leaves according to the procedure described by Taylor and Powell (1983) and digested with the appropriate restriction enzymes according to the manufacturers' instructions. All experiments with methylation-sensitive restriction enzymes were repeated at least twice. Agarose gel electrophoresis and Southern blotting were performed as described by Maniatis *et al.* (1982). Hybridization to ^{32}P-labeled RNA probes was carried out as described by Church and Gilbert (1984). The probes were synthesized from the desired DNA fragments (which were cloned next to a promoter for bacteriophage SP6 RNA polymerase in pGEM1 or pGEM2; Promega Biotec, Madison, USA) using an SP6 system and [^{32}P]UTP (both from Amersham, UK). The probes used were: a 188 bp *Sau*3A/*Pst*I fragment from the 5′ end of the NPTII protein-coding region; and a 177 bp *Bam*HI/*Nsi*I fragment from the 3′ half of the NOS protein-coding region (Figure 2).

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