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Differential inactivation and methylation of a transgene in plants by two suppressor loci containing homologous sequences

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Abstract

In a previous study on doubly transformed tobacco plants, we observed the unexpected inactivation *in trans* of T-DNA-I (encoding Kan^{r}NOS) following the introduction into the same genome of an unlinked copy of T-DNA-II (encoding Hyg^{r}OCS). This inactivation, which probably resulted from interactions between homologous regions on each T-DNA, was correlated with methylation in the nos_{pro}, which controlled the expression of both the *nptII* and *nos* genes. In this paper, we show that the inactivation and methylation of the nos_{pro}*nptII* gene in the presence of a suppressor T-DNA-II locus can be either complete (epistasis) or partial (cellular mosaicism). In plants showing partial suppression, the strength of the Kan^r phenotype, which apparently reflected the proportion of cells expressing the *nptII* gene, was inversely correlated with the degree of methylation of the nos_{pro}. The extent of nos_{pro} methylation decreased progressively in successive generations as suppressor T-DNA-II loci were crossed out. The strength of the Kan^r phenotype was improved and nos_{pro} methylation was less extensive in first generation Kan^r progeny obtained from outcrossing with untransformed tobacco than from self-fertilization.

Introduction

Accumulating evidence from a range of organisms indicates that the presence of multiple copies of homologous DNA sequences in a genome can, in certain cases, result in *trans* interactions which have negative effects on gene expression [8]. Recently, the possibility of such interactions in transgenic plants has been strikingly revealed following transformation with genes which had homology to endogenous sequences.

We found that the introduction of a T-DNA-II (encoding Hyg^{r}OCS) into the genome of a plant already transformed with an actively expressed T-DNA-I (encoding Kan^{r}NOS) could lead, in some double transformants, to suppression of the Kan^{r}NOS T-DNA. Expression of the Hyg^{r}OCS T-DNA was unaffected [11]. Homologous regions included two copies on each T-DNA of the nos_{pro}, which drove the expression of the *nos*, *nptI* and *ocs* genes, and was present as well at the right border of T-DNA-II. The nos_{pro} of the suppressed *nos* and *nptII* genes was methylated at an *Sst* II site in double transformants in which T-DNA-I was suppressed. In some progeny plants, methylation at this site was partially lost, and *nptII* and *nos* gene expression recovered to some extent, but only if these plants had not

inherited the suppressing T-DNA-II. In progeny plants inheriting both T-DNAs, the methylation and suppression of T-DNA-I genes in the presence of the nonallelic T-DNA-II was complete, a phenomenon termed epistasis. Particularly noteworthy was the fact that only some of the double transformants exhibited such interactions, leading to the suggestion that the proximity of the two unlinked T-DNAs was a factor in producing the effect [12].

In addition to epistasis, a second type of gene interaction, termed co-suppression by Napoli and coworkers, has also recently been reported [15, 19]. Co-suppression referred to the coordinate inactivation of both an introduced and endogenous chalcone synthase (*chs*) gene in transgenic petunia plants. The phenotypic effects of co-suppression could be visualized in petals of flowers, which are completely purple if the *chs* gene is expressed normally; however, co-suppression in transgenic plants led to the appearance of pure white or patterned flowers. The same coordinate inactivation was also observed between endogenous and introduced dihydroflavone-4-reductase (*dfr*) genes [19]. As in the case of T-DNA epistasis in tobacco, co-suppression was only observed in some of the transgenic petunia plants. However, in contrast to the epistatic T-DNA interactions, which were irreversible in somatic cells of a given plant, co-suppression showed somatic reversibility: a plant could switch from producing totally white to wild-type purple flowers. Although the somatic reversibility of co-suppression suggested the involvement of methylation, this has not yet been demonstrated.

Related findings were also recently reported by Linn and coworkers, who found that transgenic petunia plants containing multiple copies of a chimeric $35S_{pro}$-maize A1 gene (encoding DFR) had lower levels of expression and more extensive promoter methylation than was observed in plants containing single inserts of the same gene [9]. Similar suppressive effects in transgenic *Arabidopsis* plants have also been correlated with multiple copies and increased methylation of the transgene [17].

Although the interactions described above have been observed so far only in transgenic plants, there is evidence to suggest that similar interactions might also occur between endogenous homologous sequences. For example, the flower pigmentation pattern of a commercial variety of petunia is similar to that produced by co-suppression in transgenic plants, reflecting, perhaps, a natural case of co-suppression [19]. In addition, as Jorgensen has suggested [8], the cycling in activity of several different maize transposable elements [2, 3, 4, 6, 10, 18] might be due in part to homology-dependent interactions between multiple copies of the element.

Transgenic plants containing defined and easily altered DNA sequences can simplify the analysis of such phenomena. With this in mind, we have been studying the gene interactions which can occur in plants transformed with various combinations of two T-DNAs which encode easily distinguishable selection and screening markers, yet share some regions of homology. In this paper, we describe the different degrees of inactivation which can be caused by a suppressing T-DNA locus. We also present data to show that outcrossing is more effective than selfing in enhancing the loss of methylation and reactivation of a gene previously suppressed by an epistatic interaction.

Materials and methods

All procedures were carried out as described previously [11, 12]. To quantify the ratios of methylated and unmethylated fragments, autoradiograms were scanned using an Ultroscan XL Enhanced Laser Densitometer (Pharmacia).

The plants studied were the progeny of a doubly transformed tobacco plant which was homozygous (KK) at a single locus for T-DNA-I (encoding Kan^rNOS), and hemizygous (H–) at 2 unlinked loci for T-DNA-II (encoding Hyg^rOCS). One of these T-DNA-II loci was complete (H_1); the other truncated, containing only the *ocs* gene (H_2) (Fig. 1). A genetic analysis of the inheritance of selection and screening markers in first generation progeny suggested that

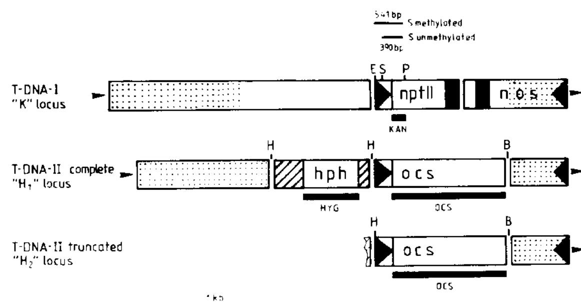

Fig. 1. Maps of T-DNAs present in the doubly transformed plant. Abbreviations: *nptII*, gene for neomycin phosphotransferase, which confers resistance to kanamycin (Kan^r); *nos*, gene for nopaline synthase (NOS); *hph*, gene for hygromycin phosphotransferase, which confers resistance to hygromycin (Hyg^r); *ocs*, gene for octopine synthase (OCS). The *nptII*, *nos* and *ocs* genes were under control of the *nos* promoter (nos_{pro}) (large black triangles). The black rectangles in T-DNA-I represent the *nos* terminator. Dotted areas designate additional regions of homology on each T-DNA (note that this includes an inactive, truncated version of the *nos* gene, plus promoter, on T-DNA-II). The *hph* gene was under the control of the cauliflower mosaic virus 35S promoter and terminator (hatched regions). Restriction enzymes used for DNA blot analysis: *Eco* RI (E), *Sst* II (S), *Pst* I (P), *Hin*d III (H) and *Bam* HI (B). For the analysis of methylation at the *Sst* II site in the nos_{pro} of the *nptII* gene, the expected fragment sizes are shown at the top.

negative effects were occurring, in which T-DNA-I was suppressed to a greater or lesser degree, respectively, by each of these T-DNA-II loci [12]. To analyse the stability of these effects in subsequent generations, and the involvement of methylation of the nos_{pro} (which drove the expression of both the *nptII* and *nos* genes in T-DNA-I) (Fig. 1), eleven Kan^r R1 (selfed) progeny, differing in the strength of their resistance to kanamycin, were chosen for analysis (Fig. 2A). To control for any differences which might be induced by kanamycin selection, 8 R1 progeny germinated in the absence of any antibiotics were also propagated (Fig. 2B). The latter were tested directly for opines; antibiotic resistances of these plants were subsequently determined by shooting leaf pieces on media containing either kanamycin, hygromycin, or both. The phenotypes of these plants are shown in Fig. 3A.

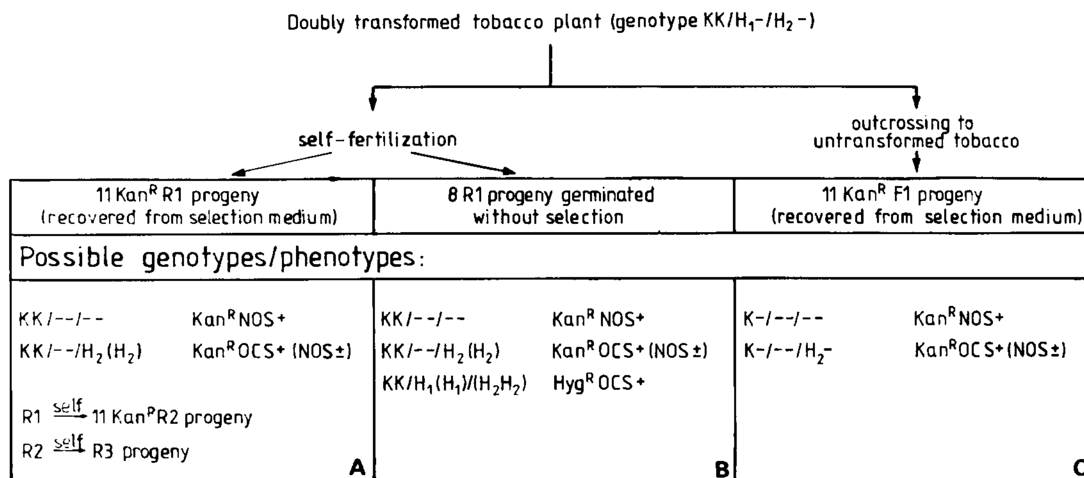

Fig. 2. Nomenclature of plants used in this study. In genotype designations, each T-DNA locus is separated by a slash. The K locus (T-DNA-I) could be homozygous (KK) or hemizygous (K–), as could each T-DNA-II locus (H_1 or H_2). If the exact genotype was not determined, this was placed in parentheses. The doubly transformed parent plant (top) was referred to as RO-3 in a previous publication [12]. The term 'NOS ±' indicates that NOS activity was usually only detectable in such a plant when kanamycin selection was applied [12].

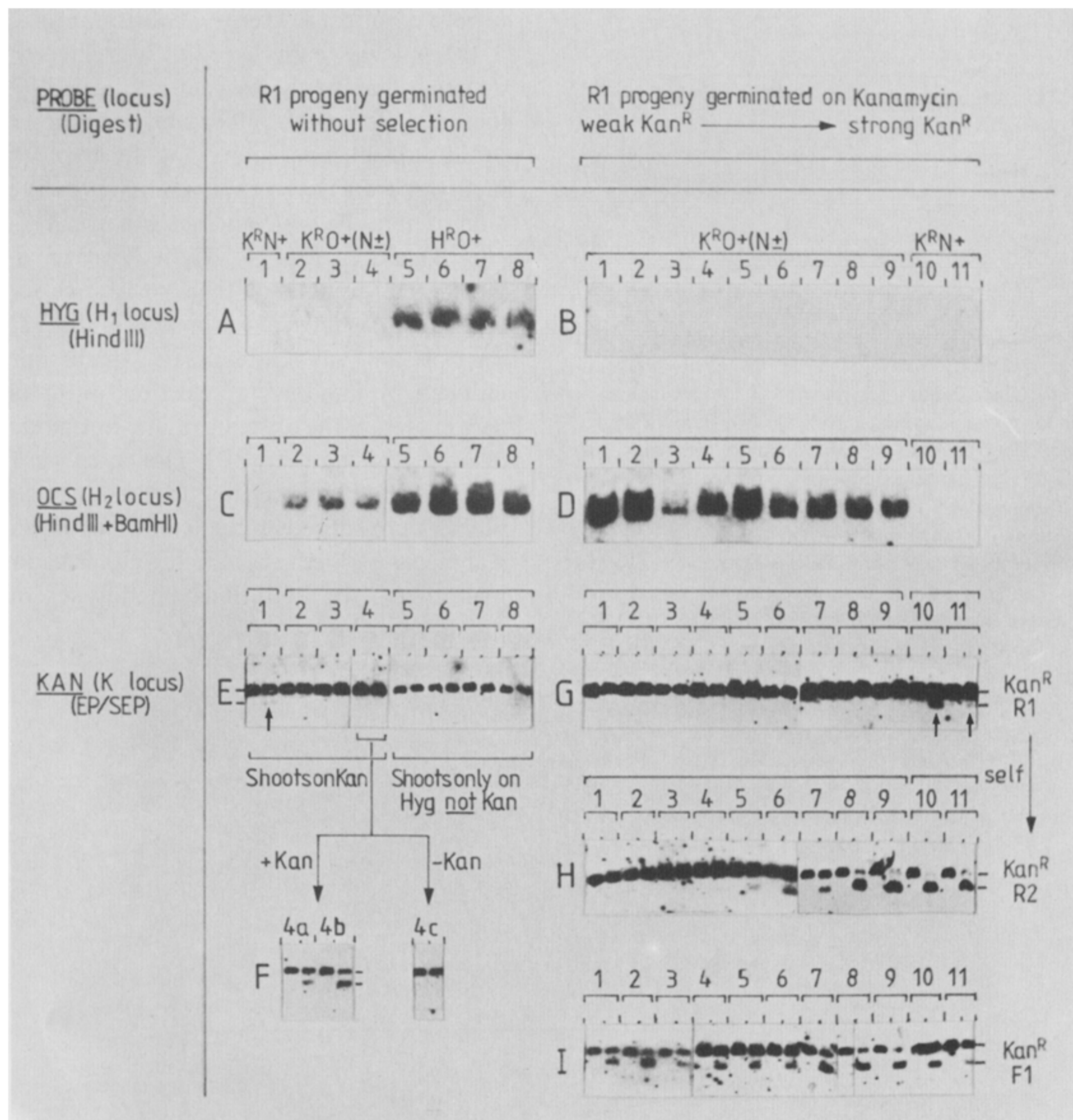

Fig. 3. DNA blot and methylation analyses. For an explanation of digests and probes, see Fig. 1 and the text. The plants numbered 1–8 are the same in parts A, C and E, as are the plants numbered 1–11 in parts B, D and G. In part H, each plant corresponds to a single Kan^r offspring of the identically numbered plant in part G. Analysis of methylation at the *Sst* II site in the nos_{pro} are shown in parts E, F, G, H and I. In each case, two adjacent lanes (referred to collectively in the text as a panel correspond to a *Eco* RI/*Pst* I double digest (EP) and *Sst* II/*Eco* RI/*Pst* I triple digest (SEP), respectively. The second lane of each panel therefore indicates the degree of methylation of the *Sst* II site of the nos_{pro} in a given leaf total DNA preparation. The positions of the 541 bp (methylated) and 390 bp (unmethylated) fragments are shown by the bars to the right or left of each blot. The percent methylation is listed in Tables 1 and 2. Since only the single *Sst* II was examined for methylation, any partial digestion with this enzyme indicates cellular mosaicism in methylation; i.e. the nos_{pro} in some cells had a methylated *Sst* II site, in other cells, this site was unmethylated. Although the *Pst* I site in the coding region of the *nptII* gene contains 2 potentially methylatable cytosines, these were apparently unmethylated since complete digestion with *Pst* I was always obtained. For parts E, F and G, methylation in the promoter of the intact *nos* gene was also examined, and in all cases, the results coincided with those shown: if the nos_{pro} of the *nptII* gene was unmethylated to any extent, then approximately the same amount of methylation was observed in the promoter of the intact *nos* gene (data not shown). Phenotypes of the plants are designated at the top as follows: K^r and H^r, kanamycin- and hygromycin-resistant, respectively; O$^+$, N$^+$, positive for octopine synthase and nopaline synthase activities, respectively. (N$^\pm$) indicates plants in which nopaline was usually only detectable when kanamycin selection was applied [12].

Results

A modifier T-DNA-II locus can either completely or partially suppress the Kan^r phenotype

To correlate the strength of the Kan^r phenotype with the T-DNA-II genotype of each R1 progeny plant (all of which were homozygous for T-DNA-I (K locus); Fig. 2A, B), we performed a DNA blot analysis using HYG and OCS probes (diagnostic for the H_1 and H_2 loci, respectively; Fig. 1). As shown in Fig. 3B (lanes 1–11), none of the Kan^r R1 progeny originally germinated on kanamycin-containing medium contained the H_1 locus, thus confirming results from a previous genetic analysis which indicated that T-DNA-I was only active in the absence of the H_1 locus [12]. Kan^r progeny could contain the H_2 locus (Fig. 3D, lanes 1–9), and were therefore OCS$^+$. However, the H_2 locus had some negative effect on *nptII* gene expression, resulting in weaker Kan^r phenotypes which were often mottled green/white and grew roots poorly on kanamycin ([12], Fig. 5A). The strongest Kan^r phenotypes, which were usually uniformly green but still failed to grow roots well on kanamycin (Fig. 5B), lacked even the H_2 locus (Fig. 3D, lanes 10, 11).

Consistent results were obtained for the 8 R1 progeny which were originally germinated without selection and then subsequently shown to be Kan^r or Hyg^r by shooting leaf pieces on antibiotic-containing media. Among these plants, only the single Kan^{r}NOS$^+$ plant contained neither the H_1 nor H_2 locus (Fig. 3A and C, lanes 1). However, OCS$^+$ plants which had only the H_2 locus (Fig. 3A and C, lanes 2–4) contained some Kan^r cells, as revealed by their ability to regenerate shoots on kanamycin-containing medium (Fig. 4–2). This was in contrast to OCS$^+$ plants containing the H_1 locus (Fig. 3, A, C, lanes 5–8), which regenerated shoots easily on hygromycin, but never on kanamycin (Fig. 4–1).

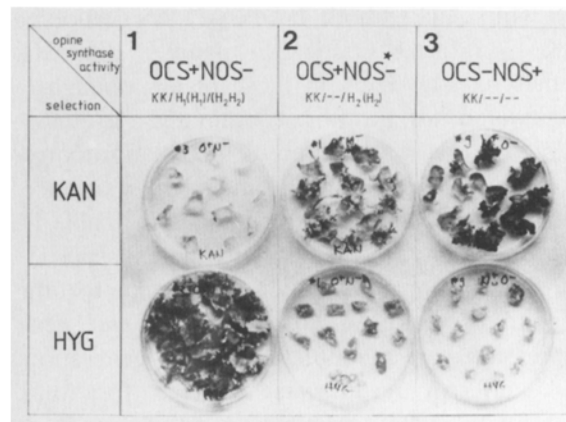

Fig. 4. Different effects of the H_1 and H_2 locus on shooting behavior of leaf pieces on antibiotics. Leaves of plants containing the H_1 locus were inhibited from shooting on kanamycin, although they shooted easily on hygromycin (column 1). In such plants the *nos*$_{pro}$*nptII* gene was completely and irreversibly inactivated and methylated (Fig. 3E, panels 5–8). In contrast, plants containing the H_2 locus could regenerate shoots on kanamycin (column 2); such plants were actually cellular mosaics for expression of the *nptII* gene, though, as was evident from the green/white mottled appearance of weak Kan^r progeny (Fig. 5A) and different methylation configurations in shoots regenerated either plus or minus kanamycin (Fig. 3E, parts 4a, b, c). Plants lacking both the H_1 and H_2 loci formed shoots easily on kanamycin (column 3). * Shoots regenerated from these plants on kanamycin often contained detectable levels of nopaline, even if the same plant grown without selection did not, indicating selection for proliferation of cells expressing T-DNA-I genes [12].

The presence of a modifier T-DNA-II locus can result in either complete or mosaic patterns of nos$_{pro}$ *methylation in R1 progeny*

The different degrees of suppression of the Kan^r phenotype by the H_1 and H_2 loci in R1 progeny could be correlated with differential methylation at an *Sst* II site in the *nos*$_{pro}$ of the *nptII* gene (Fig. 1). Three different categories of methylation which corresponded to the three general genotypes (Fig. 2B) were discernable. (Note that since only one site was examined for methylation, partial methylation patterns indicate mosaicism in methylation at this site.)

The only R1 plants in which a visible loss of methylation had occurred were Kan^{r}NOS$^+$ prog-

eny which lacked both T-DNA-II loci (genotype KK/--/--) (arrows, Fig. 3E, panel 1, and 3G, panels 10, 11). Even in these plants, though, a methylated nos_{pro} still predominated (85–98%; Table 1); this high degree of methylation correlated with the strength of kanamycin resistance, which was only moderate (Fig. 5B; Table 1). Therefore, even in plants which had lost all H_1 and H_2 suppressor loci, recovery to the unmethylated state and reactivation of *nptII* gene expression were incomplete in the first generation.

A different result was obtained for weak $Kan^r OCS^+$ R1 progeny of the genotype KK/--/H_2 (H_2) (i.e. either homozygous or hemizygous for the H_2 locus). In DNA isolated from these plants, the *Sst* II site appeared to be completely methylated (Fig. 3E panels 2–4, Fig. 3G panels 1–9, Table 1). However, despite the appearance of complete methylation, some cells in the $Kan^r OCS^+$ plants apparently contained an unmethylated nos_{pro}, as shown by the partial loss of methylation observed in plants regenerated from leaf pieces placed on kanamycin-containing medium (Fig. 3F, panels 4a and 4b). In contrast, the nos_{pro} in shoots regenerated from the same plant in the absence of kanamycin could still appear totally methylated (Fig. 3F, panel 4c).

In accord with results reported previously [11, 12], the *Sst* II site of the nos_{pro} in $Hyg^r OCS^+$ plants with the genotype KK/H_1 (H_1)/(H_2H_2) was found to be completely methylated (Fig. 3E, panels 5–8). This methylation was uniform and

Table 1. Correlation of H_2 genotype with percentage of methylation of the nos_{pro} in Kan^r progeny obtained from selfing.

Plant	R1 generation			R2 generation		
	Genotype	% methylation (Fig. 3G)	Kan^r phenotype (Fig. 5)	Genotype	% methylation (Fig. 3H)	Kan^r phenotype (Fig. 5)
1	KK/--/H_2H_2	100 a	A	KK/--/H_2H_2	100	A
2	KK/--/H_2H_2	100	A	KK/--/H_2H_2	100	A
3	KK/--/H_2H_2	100	A	KK/--/H_2H_2	100	A
4	KK/--/H_2H_2	100	A	KK/--/H_2H_2	98	B
5	KK/--/H_2H_2	100	A	KK/--/H_2H_2	95	B
6	KK/--/H_2-	100	A	KK/--/H_2-	88	B
7	KK/--/H_2-	100	A	KK/--/H_2-	78	B
8	KK/--/H_2-	100	A	KK/--/--	18	C
9	KK/--/H_2-	100	A	KK/--/--	12	C
10	KK/--/--	85	B	KK/--/--	10	C
11	KK/--/--	98	B	KK/--/--	21	C

All Kan^r progeny obtained from selfing were homozygous for the K locus, and lacked entirely the H_1 locus (Fig. 2). They could either be homozygous (H_2H_2) or hemizygous (H_2-) for the H_2 locus. This was determined by assaying seedlings for OCS, the H_2 screening marker (Fig. 1). H_2H_2 plants produced 100% OCS^+ seedlings; H_2- plants produced seedlings with a 3:1 ratio of OCS^+/OCS^-. The plants are numbered as in Fig. 3G and H. Percentage of methylation was determined by scanning the corresponding blots. Note the progressive recovery in strength of the Kan^r phenotype and the loss of methylation. Even after loss of all H_2 suppressor alleles, percentage of methylation is still quite high in first-generation (R1) progeny, and the strength of Kan^r only moderate (R1 plants 10 and 11). The R2 progeny of these plants have much less methylation and a correspondingly increased strength of Kan^r, even though the genotype remained the same as the R1 parents (R2 plants 10 and 11).

[a] The 100% methylation observed in many plants was probably deceptive: all of the plants shown formed shoots when leaf pieces were placed on kanamycin, indicating that the nos_{pro}*nptII* gene was not completely inactivated. Furthermore, if shoots regenerating on kanamycin were analyzed, some loss of methylation was observed (Fig. 3E and F, panels 4a, 4b). These results, as well as others showing the presence of nopaline in many shoots regenerated on kanamycin [12], were interpreted as indicating that two populations of cells existed in these plants; each population had a methylated or unmethylated nos_{pro}, respectively. In the absence of selection for *nptII*-expressing cells, the former population predominated, giving the appearance of complete methylation in a preparation of total leaf DNA.

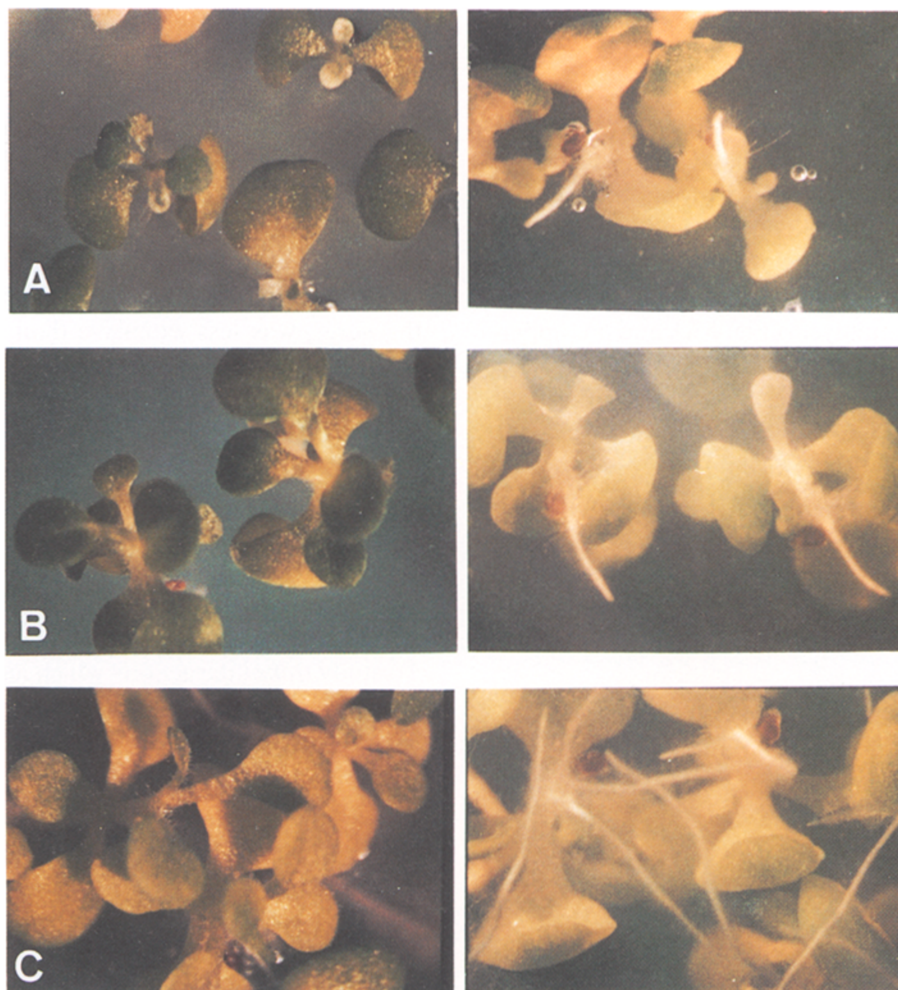

Fig. 5. Examples of different strengths of Kan^r in R2 progeny. These plants (as well as parent R1 plants) were all homozygous for the K locus, but differed in the presence of the H_2 locus. (The epistatic H_1 locus was always absent.) A. Weak Kan^r: resistant (green) and sensitive (white) regions in shoots (left), poor root growth (right); such plants were homozygous for the H_2 locus. B. Moderate Kan^r: better shoot growth and less mottling than in A, but still poor root growth; such plants were hemizygous for the H_2 locus. C. Strong Kan^r: good shoot and root growth; such plants lacked the H_2 locus entirely. Parent R1 plants usually showed phenotypes A and B (Table 1; [12]). For assignments of Kan^r phenotypes to R1 and R2 progeny, and the correlation with degree of nos_{pro} methylation, see Table 1.

irreversible in a given plant, as shown by the fact that such plants (as opposed to those only containing the H_2 locus) never regenerated shoots on kanamycin (Fig. 4–1).

The extent of nos$_{pro}$ *methylation decreases progressively in R2 progeny*

To study the inheritance of nos_{pro} methylation patterns, 11 Kan^r R2 progeny (one from each of the original 11 Kan^r R1 plants (Fig. 2A)) were examined as before for methylation at the *Sst* II site. For many R2 plants, the degree of methylation decreased and the strength of kanamycin resistance increased relative to the R1 parent plant. This was often due to crossing out of suppressor H_2 alleles, but could also occur even if the genotype of the R2 plant remained the same as that of the parent R1 (Table 1). These R1 generation to R2 generation differences again dem-

onstrated that loss of methylation and improvement in the strength of Kan^r took place progressively, and not completely in one step, even following the loss of all suppressor alleles.

As with the parent R1 plants, a clear correlation continued to be found between the strength of Kan^r, extent of methylation, and the presence of the H_2 locus. R2 progeny that were homozygous for the H_2 locus (Table 1), showed weak resistance to kanamycin (Fig. 5A), and an (apparently) completely or mostly methylated nos_{pro} (Fig. 3H, panels 1–5). R2 progeny that were hemizygous for the H_2 locus (Table 1) showed moderate Kan^r (Fig. 5B) and decreased nos_{pro} methylation (Fig. 3H, panels 6, 7). In plants lacking the H_2 locus entirely (Table 1), the strongest Kan^r phenotypes (Fig. 5C) and least extensive methylation were observed (Fig. 3H, panels 8–11).

Table 2. Percentage of methylation of the nos_{pro} in Kan^r progeny obtained from outcrossing.

F1 plant	Genotype	% methylation (Fig. 3I)
1	n.d.	64
2	n.d.	34
3	n.d.	69
4	K–/--/H_2-	81
5	K–/--/--	83
6	K–/--/--	85
7	K–/--/--	40
8	K–/--/H_2-	38
9	K–/--/--	26
10	K–/--/--	36
11	K–/--/--	78

All Kan^r F1 progeny were hemizygous for the K locus, and lacked the H_1 locus (Fig. 2). The H_2 locus, if present, was hemizygous (H_2-). Note the lack of correlation between the H_2 genotype and percentage of methylation. The H_2 genotype was determined by assaying for the H_2 screening marker (OCS) in progeny plants. n.d. = not determined. Plants are numbered as shown in Fig. 3I. Percentage of methylation was determined by scanning the blots shown in Fig. 3I.

Methylation of the nos$_{pro}$ *is less extensive in Kan^r progeny obtained from outcrossing than from selfing*:

To determine the effect of outcrossing on methylation of the nos_{pro} and the Kan^r phenotype, the original doubly transformed parent plant was crossed with untransformed tobacco, producing the F1 generation (Fig. 2C). In all 11 Kan^r F1 plants analysed (Fig. 3I, Table 2), methylation of the nos_{pro} was less extensive than in all but the strongest Kan^r R1 progeny tested (compare with Fig. 3G, panels 10, 11). In general, the appearance of F1 seedlings on kanamycin was similar to the moderately or strongly resistant R2 seedlings (Fig. 5B, C).

Discussion

To study *trans* interactions which lead to negative effects of gene expression, we analysed the progeny of a tobacco plant transformed in sequential steps with two different T-DNAs; these encoded unique selection and screening markers, yet shared some regions of homology. In these experiments, we found that the expression of the $nos_{pro}nptII$ on T-DNA-I could be suppressed to varying degrees by a modifier T-DNA-II locus: the presence of the H_1 locus caused complete suppression (epistasis) and irreversible nos_{pro} methylation; the presence of the H_2 locus resulted in partial suppression and methylation of the nos_{pro}. In this case, partial suppression was interpreted as reflecting expression in some cells and suppression in the rest (cellular mosaicism), and not a uniformly decreased rate of expression in all cells [1]. This mosaicism was suggested by the appearance of weak Kan^r progeny, which were often mottled green/white. Moreover, since only one methylation-sensitive restriction enzyme site was examined, the partial digestion patterns obtained were evidence for mosaicism, as was the fact that more than one methylation configuration was found in shoots regenerated from leaf pieces of the same plant. Such mosaic patterns of expression and methylation have also been observed for some transgenes in mice [13]. The reasons why

the partially suppressing H_2 locus resulted in mosaicism instead of alternative patterns of *nptII* gene expression remain to be established.

The effects we observed were not simply due to nonspecific methylation of foreign DNA introduced into a genome by transformation [5], since it was not the incoming T-DNA-II, but the *nptII* gene on the resident T-DNA-I which became methylated and inactivated. Furthermore, any loss of methylation and reactivation of the *nptII* gene always depended on segregating out the epistatic T-DNA-II (H_1) locus. Neither of the suppressor T-DNA-II loci encoded any *trans*-acting regulatory factor or DNA binding protein. Therefore, although the mechanism remains unknown, the presence of a T-DNA-II locus with homology to sequences on T-DNA-I was apparently sufficient to cause suppression and methylation of the nos_{pro}*nptII* gene.

It is not clear why the suppressing effects of H_1 and H_2, differed in intensity. Two possibilities come to mind. First, the truncated H_2 locus contained, in comparison to the H_1 locus, fewer homologous regions which could potentially interact with T-DNA-I, and/or compete with it for binding of transcription factors. However, if the interaction depended only on multiple copies of the nos_{pro} on each T-DNA (and not on the other region of homology at the left border), then the H_2 locus contained as many (two) as the H_1 locus (Fig. 1). A second possibility is that the different chromosomal locations of H_1 and H_2, and their different proximities to T-DNA-I, could have rendered them unable to interact equally with homologous sequences on T-DNA-I, the consequence being either complete or partial suppression, respectively. Future studies must clarify why a duplicated sequence should be a more effective suppressor in one location than in another one.

Another question concerns the relative proportions and distributions of expressing and non-expressing cells in cellular mosaics. In transgenic mice, different genetic backgrounds of a mosaically expressed transgene can apparently influence this ratio [13]. This also was true to a certain extent in our case. Weak Kan^r R1 progeny obtained from selfing (often cellular mosaics composed of varying proportions of resistant and sensitive regions) were homozygous for the K locus and always contained the H_2 locus. It became particularly clear in the R2 generation that both the strength of Kan^r (interpreted as indicating the proportion of cells expressing the nos_{pro}*nptII* gene) and the degree of methylation of the nos_{pro} depended on whether the H_2 locus was homozygous or hemizygous. Plants with an H_2H_2 genotype were less resistant to kanamycin and showed more extensive methylation than those with an H_2- genotype. In addition to this fixed genetic component, however, there was a random element involved as well because even progeny with the same H_2 genotype could have different degrees of methylation (Table 1). A random factor was also suggested by the different and erratic distributions of resistant and sensitive regions in genotypically identical sibling seedlings (Fig. 5A; [12]). Therefore, the H_2 background of the *nptII* gene (i.e. H_2- or H_2H_2) could determine the general boundaries within which a certain degree of methylation loss could take place in R1 progeny, but not determine exactly either this value or the cells in which it would occur.

Although slight decreases in methylation in selfed progeny could occur in a single plant generation even if the H_2 locus remained homozygous, substantial progress toward complete loss of methylation was observed only after it was segregated out entirely. In our experiments, this loss progressed over at least two generations. Such gradual loss of methylation after crossing out the suppressor alleles may suggest an explanation for the temporary persistence of the effects of paramutation even after the paramutable and paramutagenic alleles have segregated away from each other [8].

In comparison to most of the R1 progeny, the F1 offspring, which were hemizygous for the K locus, showed decreased nos_{pro} methylation and improved Kan^r. The H_2 locus (always hemizygous, if present at all, in F1 progeny) did not influence the percent methylation in the plants studied (Table 2). Holliday has suggested that outcrossing might help prevent the accumulation

of epigenetic defects, since they can be removed at meiosis when heterozygous (or hemizygous) but not when homozygous [7]. This is probably not the explanation for our results, however: even though the F1 progeny were hemizygous for the K locus, they were still the product of a KK (homozygous) transgenic parent. Therefore, the decreased methylation we observed in F1 versus R1 progeny was probably due either to more efficient demethylation or to less efficient maintenance methylation of a hemizygous locus in somatic cells.

Our findings document the complicated methylation and expression patterns which a single transgene can exhibit depending on the presence of modifier loci, which are apparently able to exert their negative effect simply because they are homologous with, and have a certain proximity to, the affected gene. Stable and uniform expression of foreign genes in transgenic plants will depend upon the absence of such potential modifier sequences. With respect to relevance for gene expression in general, it is possible that mosaic patterns of methylation and expression are quite widespread, contributing to phenomena such as incomplete penetrance and variable expressivity [16] and, perhaps, adaptive selection of epigenetic variants [12, 14].

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