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Transformation of a partial nopaline synthase gene into tobacco suppresses the expression of a resident wild-type gene

(gene silencing/gene expression/DNA methylation/transgenic plants)

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ABSTRACT **A portion of the nopaline synthase gene under the control of the cauliflower mosaic virus 35S promoter was used to transform a tobacco plant that had previously been transformed with a wild-type nopaline synthase (*nos*) gene. Unexpectedly, in all nine primary transformants tested the wild-type *nos* expression was virtually completely suppressed. In contrast, plants transformed with the control vector DNA, which differed only in the absence of the partial *nos* gene, did not show any inhibition of *nos* expression. Progeny plants were analyzed for the stability of the gene-silencing phenotype. All of the progeny that carried both the wild-type and partial *nos* genes had no detectable nopaline synthase activity. In addition, wild-type *nos* mRNA could not be detected in these plants. In most plants in which the wild-type gene was segregated away from the partial *nos* gene, wild-type levels of activity were detected. Although DNA methylation has been shown to be correlated with a decrease in promoter activity in plants, none of the progeny appeared to carry a methylated *nos* promoter. The underlying mechanism causing this gene suppression phenomenon is unclear at this time.**

The introduction of genes into plants and their subsequent expression is an important tool for the analysis of a wide range of fundamental processes. Such experiments have involved either the expression of genes not normally found in plants or modifications in the expression of endogenous genes. In theory, the production of a particular gene product should be straightforward, since one can simply place the gene of interest under the transcriptional control of a plant regulatory sequence that produces high levels of mRNA. However, for several reasons, these expectations have not always been borne out by experimentation.

In experiments involving the transformation of foreign reporter genes, individual transformed plants were found to exhibit different levels of expression even though the same piece of DNA was inserted into the plant's genome (for example, see ref. 1 and references therein). This phenomenon has been attributed to differences in the state of the surrounding DNA into which the foreign DNA has been inserted. The exact mechanism(s) underlying this "position" effect is unknown at this time. More recently, attempts to overexpress genes already present in the plant genome have led to variable results. While in some experiments overexpression due to the transformed gene has occurred as expected (2, 3), in other studies gene transformation has led to the production of plants that not only do not express the transformed gene but also inhibit expression of the corresponding endogenous genes in the plant genome (4, 5).

In this work, part of the nopaline synthase (*nos*) gene under the control of the cauliflower mosaic virus (CaMV) 35S promoter was used to transform a tobacco plant that had previously been transformed with a wild-type *nos* gene. Nopaline production was analyzed in nine primary transformants and was found to be virtually completely inhibited after the introduction of the partial *nos* gene. We found that this lack of expression is due to the suppression of *nos* mRNA levels. In contrast, plants transformed with vector DNA carrying the same T-DNA borders but lacking the *nos* gene showed no decrease in nopaline synthase activity (EC 1.5.1.19). We found that the inhibition of *nos* expression is stably inherited and that segregation of the partial *nos* gene away from the wild-type gene in general leads to the production of wild-type levels of *nos* mRNA and nopaline.

MATERIALS AND METHODS

Detection of Nopaline in Tissues. Plant extracts were prepared by grinding leaf material in extraction buffer with a small pestle in microcentrifuge tubes and were rendered equal in protein concentration. Two methods were used to analyze for nopaline. In the first, the extract was spotted onto Whatman 3MM paper and electrophoresed for 2 hr at 1500 V. Samples were visualized by staining with phenanthrenequinone as described (6) and photographed under an ultraviolet light source. Alternatively, the samples were analyzed by thin-layer chromatography.

Nopaline Synthase Assay. Conversion of [^{14}C]arginine to nopaline was assayed as described (7). Plant extracts were prepared as described above. Each reaction mixture (5 μl) was 4.5 mM in NADH and 17 mM in α-ketoglutarate (pH 6.8) and contained 0.5 μCi of [^{14}C]arginine (Amersham, 340 mCi/mmol; 1 Ci = 37 GBq) and 2.5 μl of plant extract (2–4 mg of protein). After incubation for 2 hr at 20°C, samples were subjected to electrophoresis. The paper was dried and subjected to autoradiography.

RNA Isolation. Total leaf RNA was isolated as follows. Approximately 500 mg of leaf tissue was added to a mixture of 1 ml of buffer (50 mM Tris·HCl, pH 8.0/4% sodium *p*-aminosalicylate) and 1 ml of buffer-saturated phenol. This mixture was homogenized for 1 min in an Ultra-Turax grinder (Ika-Werk Instruments, Cincinnati). One milliliter of chloroform/isoamyl alcohol (24:1, vol/vol) was then added and the sample was mixed for 1 min on a Vortex stirrer at high speed. After centrifugation, the aqueous phase was transferred and reextracted with 2 ml of chloroform/isoamyl alcohol. The aqueous phase was transferred to an Eppendorf tube and made 2 M in LiCl, and the RNA was allowed to precipitate overnight at 4°C. The RNA was then pelleted for 30 min in a cold Microfuge and the pellet was resuspended in 40 mM Tris·HCl, pH 7.5/20 mM NaOAc/5 mM EDTA/1% SDS. Debris was removed by centrifugation. The aqueous phase was then precipitated with ethanol and the RNA was resuspended in distilled water and stored at −70°C. RNA blot hybridization was done as described (8).

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Abbreviation: CaMV, cauliflower mosaic virus.

DNA Isolation and Blot Hybridization. Leaf tissue (0.5–1.0 g) was frozen in liquid nitrogen and ground to a fine powder with a mortar and pestle. The powder was transferred to 700 μl of proteinase K buffer (50 mM Tris·HCl, pH 8.0/100 mM EDTA/100 mM NaCl/1% SDS) containing proteinase K at 500 μg/ml and incubated overnight at 60°C. RNase A was added to a final concentration of 280 μg/ml and the mixture was incubated at 37°C for 2 hr. After a series of organic extractions (once with phenol; once with phenol/chloroform/isoamyl alcohol, 25:24:1, vol/vol; and once with chloroform/isoamyl alcohol), the genomic DNA was precipitated by adding an equal volume of isopropyl alcohol. The pellet was washed with 70% (vol/vol) ethanol and resuspended in TE buffer (10 mM Tris·HCl/1 mM EDTA, pH 8.0).

For the DNA blot analysis, 5 μg of DNA was digested with restriction enzymes, fractionated on 0.7% gels, and transferred to Zetabind membranes (AMF Cuno). Hybridization of membranes was carried out in 50% (vol/vol) formamide/5× SSC/5× Denhardt's solution/20 mM sodium phosphate, pH 6.5/0.1% SDS/10% dextran sulfate, containing salmon sperm DNA at 100 μg/ml, at 42°C, overnight (1× SSC is 0.15 M NaCl/0.015 M sodium citrate, pH 7; 1× Denhardt's solution is 0.02% bovine serum albumin/0.02% Ficoll/0.02% polyvinylpyrrolidone). DNA probes were labeled by random priming (9). The blots were washed in 1× SSC/0.1% SDS at room temperature and then in 0.1× SSC/0.1% SDS at 65°C. To determine which plants contained the wild-type and partial *nos* genes, the blots were probed with the *nos* promoter, which detects only the wild-type *nos* gene; the hygromycin-resistance gene, which detects only the plasmid carrying the partial *nos* gene; or the *nos* coding region, which hybridizes to both constructs. The *nos* promoter fragment was also used to detect the wild-type *nos* gene in the methylation studies. Since the partial *nos* gene contains only the *nos* coding region under the direction of the CaMV 35S promoter, the *nos* promoter fragment does not detect this construct.

***Agrobacterium*-Mediated Plant Transformation.** The plasmids pCIB743 and pCIB750 were used to transform the *Escherichia coli* strain S17-1 (10) and then mated into the *Agrobacterium* helper strain LBA4404. The resulting transconjugant bacteria were inoculated into a *Nicotiana tabacum* cv. SR1 plant that had previously been transformed with a wild-type *nos* gene (11). The transformation procedure used was as described previously (12).

RESULTS

Construction of the Partial *nos* Gene and Transformation of Tobacco. A *Bam*HI site was inserted 20 base pairs upstream of the *nos* transcription initiation site as described (12). There is a single *Bam*HI site in the *nos* coding sequence, making it possible to isolate an 860-base-pair *Bam*HI fragment containing approximately the first two-thirds of the *nos* gene. This fragment was inserted into the CaMV 35S promoter cassette pCIB710 (13), and the correct orientation for the production of a partial sense mRNA was determined through the analysis of internal restriction enzyme sites (Fig. 1). This chimeric gene was then inserted into the *Agrobacterium*-mediated plant transformation vector pCIB743 (13) to make pCIB750.

The wild-type *nos* gene had previously been used to transform the tobacco cultivar SR1 by deFramond *et al.* (11). One of the self-progeny plants from this original transformant was designated T2-16 and was the plant material used for transformation. The plasmids pCIB743 (a negative control) and pCIB750 were used to transform T2-16 leaf disks. The hygromycin-resistance gene present on pCIB743 was used to select for resistant seedlings, which were grown in agar in GA-7 containers (Magenta, Chicago) prior to being potted in soil and placed in the greenhouse. The original transformants were back-crossed to a wild-type (*nos*$^-$) tobacco plant and the progeny seeds were collected for further analysis.

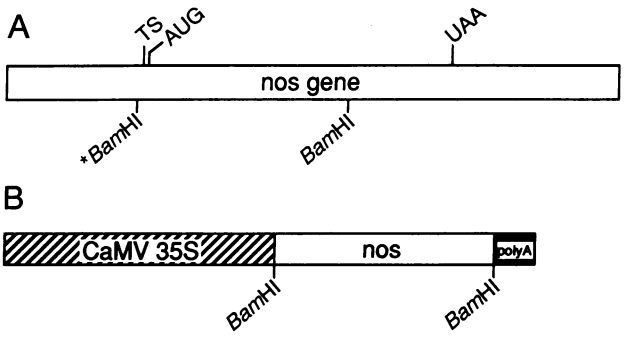

FIG. 1. Partial *nos* gene construct. A *Bam*HI site was inserted just upstream of the transcription initiation site in the plasmid pCIB740 (13). A *Bam*HI fragment that contains approximately the first two-thirds of the *nos* coding region was cloned in the CaMV 35S promoter cassette pCIB710 (13). The chimeric CaMV 35S promoter–*nos* chimeric gene was then inserted into the *Agrobacterium*-mediated plant transformation vector pCIB743 (13) to make pCIB750. (*A*) *nos* gene in pCIB740. (*B*) Chimeric CaMV 35S promoter–*nos* gene present in pCIB750. TS, transcription start site; AUG, translation initiation site; UAA, translation termination signal; polyA, poly(A) addition signal from CaMV.

Presence of Nopaline and Nopaline Synthase Activity in the Primary Transformants. Nine independent transformed plants (from different leaf disks) were analyzed for the presence of pCIB750 by DNA blot hybridization and assayed for the presence of nopaline. Surprisingly, none of the plants transformed with the partial *nos* chimeric gene had any nopaline detectable under the assay conditions utilized. This was not simply a tissue culture effect or a general effect of transformation, since each of 12 T2-16 transformant plants transformed with the control constructs pCIB743 or pCIB715 exhibited wild-type levels of nopaline. Vector pCIB715 has the CaMV 35S promoter transcribing the hygromycin-resistance gene instead of the partial *nos* gene (13).

Four of the pCIB750 primary transformants were tested in detail for the presence of nopaline synthase activity. No enzyme activity was detectable in any of the leaf material analyzed in plants transformed with the partial *nos* gene (Fig. 2). On the other hand, the plants transformed with pCIB743 had high levels of nopaline synthase activity (Fig. 2). In

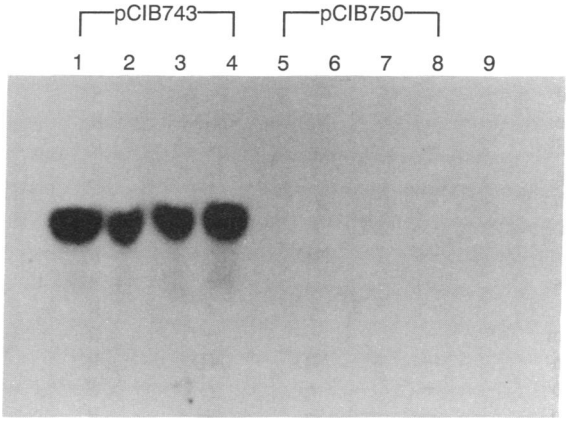

FIG. 2. Nopaline synthase enzyme activity in the original transformed plants. After paper electrophoresis, the [^{14}C]nopaline was autoradiographed. Four of the plants transformed with pCIB750, which carries the partial *nos* gene, were compared with four plants transformed with the vector pCIB743. Lane 9 contained a nontransformed plant.

comparison to these results, plants transformed with the same portion of the *nos* gene inserted in the antisense orientation relative to the CaMV 35S promoter, while having greatly reduced nopaline synthase activity, still had detectable nopaline (12). Therefore, transforming the partial *nos* gene in the sense orientation was even more effective in inhibiting expression from the resident wild-type *nos* gene than was introducing an antisense gene.

Inhibition of Nopaline Production in Progeny Carrying the Partial *nos* Gene. To determine the heritability of suppressed *nos* gene expression, five different primary transformants carrying pCIB750 were selfed and 16 progeny from each plant were analyzed for the presence of nopaline and for hygromycin resistance. All of the plants that were resistant to hygromycin, and therefore carrying the partial *nos* gene, had no detectable nopaline (Table 1 and unpublished results). In contrast, a number of the hygromycin-sensitive progeny exhibited wild-type levels of nopaline.

Two of the sets of progeny plants were analyzed by DNA blot hybridization for the presence of wild-type *nos* gene, partial *nos* gene, and hygromycin-resistance gene. As expected, none of the hygromycin-sensitive progeny carried either the gene for hygromycin resistance or the linked partial *nos* gene (Table 1). Thus, in this limited sample, there were no examples of plants having the hygromycin-resistance gene and not expressing it properly due to an epigenetic alteration. As shown in Table 1, 14 of 32 progeny plants tested carried both the wild-type *nos* gene and the partial *nos* gene from pCIB750. None of these plants produced any detectable nopaline. Therefore, the inhibition of the expression of *nos* expression cosegregated with the partial *nos* gene in the progeny plants.

Table 1. Nopaline synthase activity and genes present in progeny of transformants

Progeny	Nopaline	*nos* gene	Partial *nos* gene	Hygromycin	hyg gene
		Transformant 1			
1a	−	+	+	R	+
1b	+	+	−	S	−
1c	−	−	−	S	−
1d	−	+	+	R	+
1e	−	+	−	S	−
1f	−	+	−	S	−
1g	−	+	+	R	+
1h	+	+	−	S	−
1i	−	+	+	R	+
1j	+	+	−	S	−
1k	+	+	−	S	−
1l	−	−	+	R	+
1m	−	ND	ND	S	ND
1n	−	+	+	R	+
1o	−	−	+	R	+
1p	−	+	+	R	+
		Transformant 2			
2a	−	+	+	R	+
2b	−	+	+	R	+
2c	−	+	+	R	+
2d	−	−	−	S	−
2e	−	ND	ND	R	ND
2f	−	+	+	R	+
2g	−	+	+	R	+
2h	−	−	+	R	+
2i	−	+	+	R	+
2j	−	ND	ND	R	ND
2k	−	−	+	R	+
2l	−	+	+	R	+
2m	−	+	+	R	+
2n	−	ND	ND	R	ND
2o	+	+	−	S	−
2p	−	−	+	R	+

Progeny plants from two of the original transformed plants were analyzed for the synthesis of nopaline, for the *nos* gene and the partial *nos* gene from pCIB750, for resistance to hygromycin, and for the hygromycin-resistance gene. R, hygromycin-resistant; S, hygromycin-sensitive; hyg gene, hygromycin-resistance gene; ND, not determined.

Six hygromycin-sensitive progeny plants were found by DNA blot hybridization analysis to carry the wild-type *nos* gene. Of these, four had wild-type levels of nopaline while two had no detectable levels of nopaline (Table 1). Therefore, in some cases segregation of the *nos* gene from the inhibitory partial *nos* gene led to a restoration of expression, while in other cases the wild-type gene expression was still inhibited. As will be shown below, this inhibition at the transcript level was partial when compared with that seen when the partial *nos* gene was present.

Steady-State Wild-Type mRNA Levels Are Decreased in Progeny Carrying the Partial *nos* Gene. To determine whether the decrease in nopaline synthase activity was accompanied by altered steady-state mRNA levels in the presence of the transformed partial *nos* chimeric gene, a set of the progeny plants was analyzed by RNA blot hybridization. The progeny from transformant 1 (Table 1) had the entire range of *nos* phenotypes, and these were chosen to measure the *nos* transcript levels. Total RNA was isolated and probed for the production of *nos* transcripts. As shown in Fig. 3, plant 1k (lane 1), which does not have the partial *nos* gene and produces high levels of nopaline, does have significant levels of *nos* mRNA. The progeny plant 1f, which does not have a partial *nos* gene, actually has an intermediate level of mRNA (about 1/5th of wild-type levels, based on densitometric scanning of the autoradiogram). This can correspond to an even greater decrease in the nopaline synthase enzyme activity (12), which presumably accounts for the failure to detect any nopaline in this plant. In contrast, none of the five plants that contained both the wild-type and partial *nos* genes produced any detectable *nos* mRNA (Fig. 3, lanes 4–8). The original transformants whose progeny were analyzed were also found to have the same decrease in *nos* mRNA (data not shown). These results are similar to those of other investigators (4, 5), who found that the introduction of a new chalcone synthase gene led to the inhibition of the resident

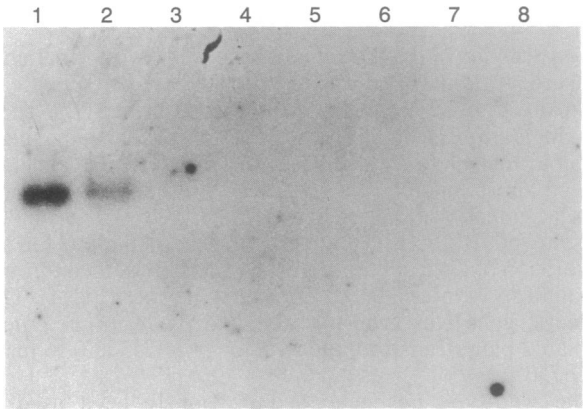

FIG. 3. Transcript levels of the *nos* gene. Total RNA was isolated from progeny plants derived from transformant 1 and analyzed by RNA blot hybridization. The following plants were analyzed: lane 1, wild-type *nos* gene producing nopaline and having no partial *nos* gene (progeny 1k); lane 2, wild-type *nos* gene producing little if any nopaline and having no partial *nos* gene (1f); lane 3, no *nos* gene present (1o); lanes 4–8, progeny carrying both the wild-type and partial *nos* genes (1a, 1d, 1g, 1n, 1p).

gene through a reduction in steady-state mRNA levels. In our experiments, there is no evidence for production of a partial *nos* transcript in the RNA blot shown in Fig. 3. This might be due to several factors, including the rapid degradation of the partial transcript or the low level of transcription initiation from the CaMV 35S promoter in these plants.

Analysis of Methylation in the *nos* Promoter. Increased DNA methylation has been shown to be highly correlated with decreased transcriptional activity in several plant transposable elements (14–16). Furthermore, it has previously been shown that methylation of an *Sst* II site in the *nos* promoter can be correlated with the lack of transcriptional activity of this gene (17). Therefore, it seemed reasonable to suspect that methylation at this site might occur in those plants also carrying the partial *nos* gene.

To test this hypothesis, DNA samples from a number of plants carrying both the *nos* and partial *nos* gene (as well as plants having only the wild-type gene) were digested with *Sst* II and *Hin*dIII and probed with a *nos* promoter fragment which hybridizes only with the wild-type *nos* gene. If methylation has not taken place at the *Sst* II site in the *nos* promoter, the probe should hybridize to a *Hin*dIII–*Sst* II 2.1-kilobase (kb) fragment. If the site is methylated, the probe should hybridize to a 7.8-kb *Hin*dIII fragment (Fig. 4). From the two lines, 18 plants carrying the wild-type *nos* gene were analyzed. Of these, 11 also contained the partial *nos* gene. Surprisingly, none of the plants tested was found to be methylated at the *Sst* II site (Fig. 4). Since there were no other known methylation-sensitive restriction sites in the promoter region, it was not possible to test for other methylated sites in this fashion.

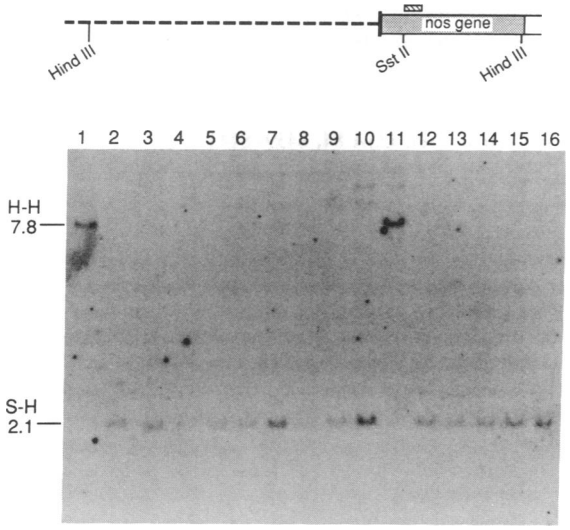

FIG. 4. Methylation analysis of the *nos* promoter. A representation of the integrated wild-type *nos* gene is shown on top. There is a *Hin*dIII site in the chromosome that is 7.8 kb upstream from a *Hin*dIII site at the 3′ end of the *nos* gene. The methylation-sensitive *Sst* II site in the *nos* promoter is 2.1 kb away from the 3′ *Hin*dIII site and 150 base pairs upstream from the transcription initiation site in the *nos* promoter. If the *Sst* II site is methylated, one should see the 7.8-kb *Hin*dIII fragment (H-H) when the genomic DNA is digested with *Hin*dIII + *Sst* II and probed with the *nos* promoter fragment (hatched box). If there is no methylation, only the 2.1-kb *Sst* II–*Hin*dIII fragment (S-H) will be seen, as the *nos* promoter probe lies 3′ to the *Sst* II site. The partial *nos* gene, which contains only *nos* coding sequences, is not detected by the *nos* promoter probe. Genomic DNA was digested with *Hin*dIII (lanes 1 and 11) or *Hin*dIII + *Sst* II (lanes 2–10 and 12–16). Lanes 1–10 and 11–16 represent progeny plants from transformants 1 and 2, respectively. The DNA in lanes 2–5 and 12 (progeny 1h, 1b, 1e, 1f, and 2o) contains only the wild-type *nos* gene. Lanes 6–10, 13–16 (1a, 1d, 1g, 1n, 1p, 2a, 2c, 2f, and 2g) represent progeny carrying both full and partial *nos* genes.

DISCUSSION

The introduction of a transgene carrying part of the *nos* coding region under the control of the CaMV 35S promoter leads to a drastic suppression in the expression of a resident *nos* gene. This inhibition does not appear to be caused by gross alterations of the wild-type gene as shown by DNA blot hybridization and the fact that some progeny plants could regain wild-type levels of expression once the partial *nos* gene was no longer present. The gene-silencing effect does not occur when the plant is transformed with a vector lacking the partial *nos* gene. Thus the presence of additional T-DNA sequences and the CaMV 35S promoter does not appear to be involved in the suppression of the resident *nos* gene.

The much lower steady-state levels of *nos* gene mRNA in plants transformed with a partial *nos* gene suggest that down-regulation occurred at the level of transcription initiation, mRNA processing, or mRNA stability. We envision three possible mechanisms by which this could occur. The first would involve the autogenous regulation of the wild-type *nos* gene through the synthesis of a truncated *nos* polypeptide. In this model, the partial protein would act as a negative regulator of gene transcription. Alternatively, the wild-type protein (which is present as a tetramer) would act as a positive regulator of transcription whose structure is disrupted by the partial protein. A second mechanism would invoke some type of epigenetic modification of the wild-type gene upon the introduction of the transgene. A third possibility is that the production of the partial length mRNA might in some way increase the degradation rate of the wild-type mRNA. However, it should be noted that no partial *nos* transcript was detectable in any of the progeny plants.

Although it is formally possible that the nopaline synthase protein regulates its own expression, this seems fairly unlikely. This model would imply that nopaline synthase has DNA-binding activity or that it can modify another transcription factor. Two recent publications have shown that transformation of a gene has led to the silencing of the resident gene [either chalcone synthase (4, 5) or a dihydroflavonol-4-reductase gene (5)]. However, in these cases full-length cDNA clones were used to transform petunia plants, rather than the partial gene used in this study. Assuming that a similar mechanism of action operates in all of these cases, it seems implausible that this effect is due to all three of these genes being autoregulated. This model is further rendered unlikely by the finding that two of the progeny plants that carried the wild-type *nos* gene (1e and 1f) but no longer contained the partial *nos* gene did not have wild-type levels of nopaline synthase activity. A simple model of autoregulation would predict that segregation of the wild-type gene away from the partial *nos* gene should always lead to wild-type level of *nos* expression.

The epigenetic factor that has been most closely correlated with transcriptional activity is DNA methylation. The activity of transposable elements appears to be determined by their methylation state (14–16). Furthermore, it has been shown that an inactive *ipt* gene in cell culture is hypermethylated and can be activated by treatment with 5-azacytidine, which leads to demethylation of the *ipt* gene (18). Finally, the *Sst* II site in the *nos* promoter has been shown to be methylated occasionally when two T-DNAs were present in the same plant. Such methylation was found to be correlated with decreased expression (17). We were therefore surprised that no methylation was detectable at this restriction site in plants carrying both the *nos* and partial *nos* genes. There are two possible explanations for this result. Either there are other important sites in the *nos* promoter that are methylated or some factor other than methylation is inhibiting the expression of this gene. At this point it is difficult to determine which of these hypotheses is correct.

Whatever model is used to explain these results must explain two phenomena. The first is the inhibition of expression upon transformation. The second is the reversion to full activity in a portion of the progeny in which the wild-type gene segregated away from the partial *nos* gene. In this context, it is interesting to compare the phenotype of plants transformed with the partial *nos* gene and that of those transformed with a chimeric gene that expresses *nos* antisense RNA (12). While the plants expressing antisense RNA have a marked decrease in the expression of nopaline synthase, there were detectable levels of nopaline in all of the different transformed lines. The silencing by antisense expression of nopaline synthase was leaky, with 2.5–10% of activity remaining in different individual transformants. In the case of the plants transformed with the partial *nos* gene, no detectable nopaline was present in any of the transformed plants tested. The level of mRNA present also was reduced in comparison with the antisense plants. However, even though there were clear differences in the level of inhibition, the question remains whether the gene-silencing mechanism is actually different in the two cases. If it is different, it means that the transformation with the piece of DNA homologous to that already present in the genome has a different effect depending on its orientation with respect to the CaMV 35S promoter. (This was the only difference between the two vectors.) This would then imply that the transcription of the transgene might be required for the sense suppression phenomenon to occur, while antisense RNA might work through the formation of double-stranded RNA (12). It is interesting in this context that in the case of chalcone synthase the phenotypes of plants expressing antisense RNA are different from those transformed with the sense construct (4, 5). Paradoxically, as mentioned earlier, no partial *nos* transcript was detectable in these plants. It is certainly possible that these transcripts need to be present only at very low levels or only at certain crucial times for the suppression phenomenon to occur. These times would include the transformation process and seed germination.

The variety of results that have been achieved in experiments involving plant gene transformation is certainly a curious phenomenon. In some experiments, the transgene expression is as expected with the endogenous gene(s) remaining unaffected (2, 3). In others, the expression of the endogenous plant gene is suppressed (4, 5, this work). Occurrence of these unexpected effects presumably depends upon the gene in question, and possibly also its chromosomal location. The involvement of transcription in the suppression phenomenon may be better understood by transforming plants with a gene coding region in the absence of a promoter. Furthermore, the role of a protein encoded by the transgene could be analyzed by introducing a nonsense mutation in the coding region which would prevent its synthesis. The underlying fundamental question is whether the mechanism controlling suppression by transgenes is related to gene suppression during normal plant development.

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