Proc. Natl. Acad. Sci. USA
Vol. 85, pp. 4300–4304, June 1988
Botany

A stable bifunctional antisense transcript inhibiting gene expression in transgenic plants

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Communicated by Luis Sequeira, February 23, 1988

ABSTRACT **Tobacco plants expressing constitutive chloramphenicol acetyltransferase (CAT; EC 2.3.1.28) activity were obtained by transformation with a chimeric CAT gene driven by the cauliflower mosaic virus 19S promoter. Plants expressing different levels of CAT activity were retransformed with vectors containing CAT sequences transcriptionally fused in the antisense orientation between the coding region of the hygromycin-resistance gene and the 3′ end of the nopaline synthase gene. Several plants regenerated on high concentrations of hygromycin exhibited a loss of CAT activity, whereas plants retransformed with a vector conferring hygromycin resistance but lacking the antisense CAT sequence showed no reduction in CAT activity. RNA blot analysis revealed a strong correlation between the degree of CAT gene inactivation and the levels of stable antisense transcripts accumulated. The possibility that CAT gene inactivation was due to transferred DNA instability was discounted since a kanamycin-resistance gene contiguous with the CAT gene was expressed normally, and DNA blot analysis indicated no loss or rearrangements of the transferred DNA fragments. Thus, the imposed selection pressure enabled the selection of plants expressing high levels of stable bifunctional antisense transcripts that inhibited the activity of the targeted gene.**

Antisense RNA transcripts have been used to generate dominant mutations in eukaryotes by inhibiting the expression of specifically targeted RNA species (1–9), and the technique has been applied to address fundamental questions in animal molecular biology (10–16). The antisense approach in plants, if applicable, promises to make significant contributions to elucidating the functions of cloned plant genes and identifying genes with known phenotypes for which there are no biochemical markers. Moreover, it may prove useful for inhibiting the undesirable expression of genes, such as those involved in the production of harmful antimetabolites or disease-inducing genes encoded by plant viruses. In the only published report on the antisense approach in plants, transiently high levels of antisense RNA were generated by electroporating carrot protoplasts with a mixture of sense and antisense chloramphenicol acetyltransferase (CAT; EC 2.3.1.28) gene constructs and using an excess of the antisense plasmid (17). In that study, an input antisense:sense plasmid ratio of 50:1 reduced the expected level of CAT activity by 95%. However, such antisense:sense RNA ratios may be difficult to attain in stably transformed plants, and the necessity to synthesize high levels of antisense transcripts for the inactivation of genes in whole plants poses a formidable challenge.

This report describes the inhibition of CAT gene expression in transgenic plants by the stable production of abundant antisense CAT RNA and presents a strategy based on a procedure described by Kim and Wold (8) for preselecting "transgenotes" expressing high levels of the antisense mRNA transcript.

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MATERIALS AND METHODS

Construction of Transformation Plasmids. The plasmid pS6-4 (provided by N. Brisson) contains a 395-base-pair (bp) fragment of the cauliflower mosaic virus (CaMV) 19S promoter fragment (−380 to +15; see ref. 18) cloned into pUC8. The plasmid p19CATb contains a 780-bp *Taq* I CAT fragment from pBR325 cloned into the *Acc* I site of pUC19 in the sense orientation relative to the *lacZ* promoter. The 19S promoter (400-bp *Hin*dIII–*Sph* I fragment of pS6-4), the CAT sequence (780-bp *Pst* I–*Xba* I fragment of p19CATb), and the nos 3′ region (430-bp *Bam*HI–*Stu* I fragment of pMON200; ref. 19) were sequentially cloned into the multilinker of pUC18. The initiation codon of the 19S sequence was deleted by digestion with *Sph* I and T4 DNA polymerase; this was followed by blunt-end recircularization to give pUC-19S-CAT3′. The chimeric 19S-CAT-nos gene was transferred into the *Hin*dIII and *Kpn* I sites of pBin19 (20) to give p19S-C (Fig. 1*A*).

An 850-bp *Sac* I–*Eco*RV fragment from pUC-19S-CAT, comprising the CAT sequence and the 3′ proximal 65 bp of the 19S promoter sequence (including all 12 bp of the 19S 5′ untranslated region), was cloned in the antisense orientation next to the nos 3′ region in a pUC18 derivative to give pACN9. A 1.95-kilobase (kb) *Pvu* II fragment from pAGS120 (21) containing a chimeric HPT gene was inserted downstream from the antisense CAT-nos 3′ sequence in pACN9. The *rbcS* promoter (−1040 to +169) from *Nicotiana plumbaginifolia*, isolated as a 1.2-kb *Eco*RI–*Pvu* II fragment from pNPSS8B (a gift from N.-H. Chua), was cloned into the *Eco*RI and *Sma* I sites of pBin19 to give pBrbcS. A 3.3-kb *Xba* I–*Hin*dIII fragment containing the antisense CAT-nos 3′ fusion and the chimeric HPT gene was cloned into *Xba* I- and *Hin*dIII-linearized pBrbcS to give pRAC-H (Fig. 1*B*).

An *Eco*RI-blunted *Hin*dIII fragment from pUC13/35SCAT (22) containing the CaMV 35S promoter (−941 to +9) was cloned into the *Eco*RI and *Sma* I sites of Bluescript M13$^+$ (Stratagene, San Diego) to give pBS-35S. A 1300-bp *Xba* I–*Hin*cII fragment from pACN9 containing the antisense CAT-nos 3′ sequence was cloned into the *Xba* I and blunted *Sac* II sites of pBS-35S to give pBS-35S-AC. The HPT coding sequence, isolated as a 1070-bp *Xho* II fragment from pAGS120, was inserted between the 35S promoter and antisense CAT-nos 3′ sequence of pBS-35S-AC to give

Abbreviations: CAT, chloramphenicol acetyltransferase; HPT, hygromycin phosphotransferase; CaMV, cauliflower mosaic virus; kan, kanamycin; hyg, hygromycin; ^{r}, resistant; T-DNA, transferred DNA.

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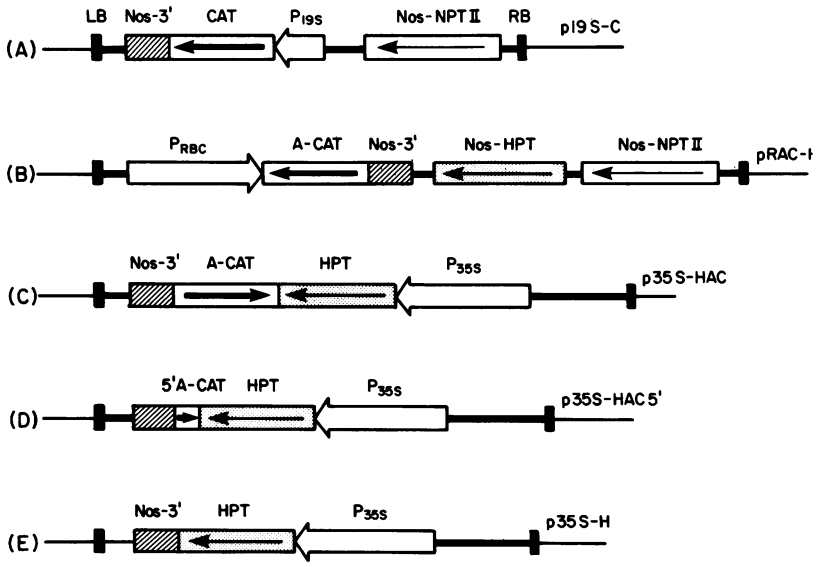

FIG. 1. Schematic structures of plant transformation vectors. Constructs *A* and *B* were made in the binary Ti-plasmid vector, pBin19 (20); *C–E* were constructed in a pBin19 derivative from which the nos-nptII gene had been deleted. Only the regions within the transferred DNA (T-DNA) border repeats are shown in detail. LB, left border; RB, right border; P_{19S}, CaMV 19S promoter; P_{35S}, CaMV 35S promoter; P_{RBC}, ribulose 1,5-bisphosphate carboxylase small subunit promoter; CAT, CAT sequence cloned in sense orientation; A-CAT, coding region of CAT cloned in antisense orientation; 5′ A-CAT, 5′ terminal (172 bp) CAT sequence cloned in antisense orientation; HPT, hygromycin phosphotransferase coding sequence (stippled); Nos-3′, 3′ flanking region of nopaline synthase gene (shaded); Nos-HPT, P_{Nos}-HPT-Nos 3′ gene; Nos-NPTII, P_{nos}-NPTII-Nos 3′ gene. Arrows indicate the orientation of promoter fragments and gene-coding sequences.

pBS-35S-HAC. Deletion of the chimeric nos-nptII gene from pBin19 by partial *Sph* I digestion gave pBMK19. The chimeric construct in pBS-35S-HAC was cloned into pBMK19 to give p35S-HAC (Fig. 1*C*). The plasmid p35S-HAC5′ (Fig. 1*D*) is similar to that of p35S-HAC except that it contains only 225 bp of the 5′ CAT sequence (including the 53 bp from the 3′ end of the 19S promoter). p35S-H (Fig. 1*E*) is also similar to p35S-HAC but lacks antisense CAT sequences.

Plant Transformations. Transformation vectors were mobilized from *Escherichia coli* DH5 to *Agrobacterium tumefaciens* LBA4404 (23), and leaf segments of *Nicotiana tabacum* cv. Wisconsin were transformed (24). Tissue transformed with p19S-C was regenerated on 100 μg of kanamycin (kan) per ml and that with pRAC-H was on media containing 10–15 μg of hygromycin (hyg) per ml. Kan-resistant (kan^r) tissue from p19S-C transformants was retransformed with p35S-HAC, p35S-HAC5′, and p35S-H and regenerated on 30 μg of hyg per ml and 100 μg of kan per ml.

CAT Assay. Leaf tissue (100 mg) was ground in liquid N_2 and suspended in 100 μl of 250 mM Tris·HCl, pH 7.6/1 mM EDTA/1 mM leupeptin/1 mM phenylmethylsulfonyl fluoride/0.1% ascorbic acid. The mixture was heated at 60°C for 10 min and then spun in a Microfuge for 5 min, and the protein concentration of the supernatant solution was determined (25): Aliquots containing 200 μg of protein were used for CAT assays using 0.1 μCi (1 Ci = 37 GBq) of [^{14}C]chloramphenicol (New England Nuclear) at 37°C for 30 min as described (26). The TLC plate was exposed to x-ray film for 20 hr, and CAT activity was quantitated by densitometry.

Nucleic Acid Hybridization. Plant genomic DNA was prepared essentially as described (27) and was further purified on a two-step CsCl/ethidium bromide gradient (28). For Southern analysis, 10-μg aliquots of *Kpn* I-digested DNA were subjected to electrophoresis through a 0.6% agarose gel and blotted onto a Zeta-Probe (Bio-Rad) nylon filter using 0.4 M NaOH (29).

The filter was hybridized in 0.225 M NaCl/0.015 M $NaH_2PO_4 \cdot H_2O$/1.5 mM EDTA, pH 7.4/1% $NaDodSO_4$/0.5% (wt/vol) "Crino" skimmed milk powder/100 mg of calf thymus DNA per ml with 0.6 mg of nick-translated [^{32}P]DNA with 600 ng of nick-translated probe (0.5–1.0 × 10^8 dpm/μg) for 20 hr at 65°C. Following hybridization, the filter was washed for 15-min periods at 65°C once in 0.15 M NaCl/0.015 M sodium citrate, pH 7.0/1% $NaDodSO_4$, three times in 75 mM NaCl/7.5 mM sodium citrate/1% $NaDodSO_4$, and once in 15 mM NaCl/1.5 mM sodium citrate. The filter was exposed to x-ray film at −70°C using an intensifying screen.

Leaf RNA was prepared as described (30) and poly(A)$^+$ RNA was isolated on oligo(dT)-cellulose. For RNA transfer blots, the RNA was subjected to electrophoresis through 1.2% agarose gels containing 2.2 M formaldehyde, blotted onto Zeta-Probe or GeneScreen filters as indicated in 25 mM phosphate buffer (pH 6.5), and hybridized as above.

RESULTS AND DISCUSSION

Transgenic Plants Expressing Constitutive CAT Activity. We used a model system to demonstrate the feasibility of inhibiting gene expression in plants by the stable production of antisense RNA. First, plants were transformed using kan selection with a chimeric P_{19S}-CAT-Nos 3′ gene (Fig. 1*A*), and kan^r regenerants were assayed for CAT activity. Three independently transformed plants, P12, P32, and P41, exhibited roughly similar levels of activity (Fig. 2*A*), and one of these (P41) was used as a test plant for inhibiting CAT activity by the antisense approach. CAT assays on a number of kan^r progeny from the self-fertilized P12 plant revealed that, whereas most of the progeny had levels of activity similar to the parent (e.g., plant S15 in Fig. 2*A*), some showed 4- to 5-fold higher CAT activity (e.g., plant S13 in Fig. 2*A*).

Plants expressing CAT activity constitutively (CAT$^+$ plants) were further characterized by RNA transfer and Southern blot analysis. The RNA transfer blot analysis showed that the varying levels of CAT activity paralleled the amounts of CAT RNA synthesized in the tested plants (Fig. 2*B*). Southern blot analysis (Fig. 2*C*) revealed that, whereas plants P32 and P41 had single T-DNA insertions (lanes 2 and 3), plant P12 contained multiple (≈6) copies of the CAT gene, apparently at a single locus (lane 1). Plant P12 and its progeny, S15, are T-DNA hemizygous, but another of the P12 progeny, plant S13, which exhibited elevated levels of CAT activity, clearly contained a doubled complement of the CAT

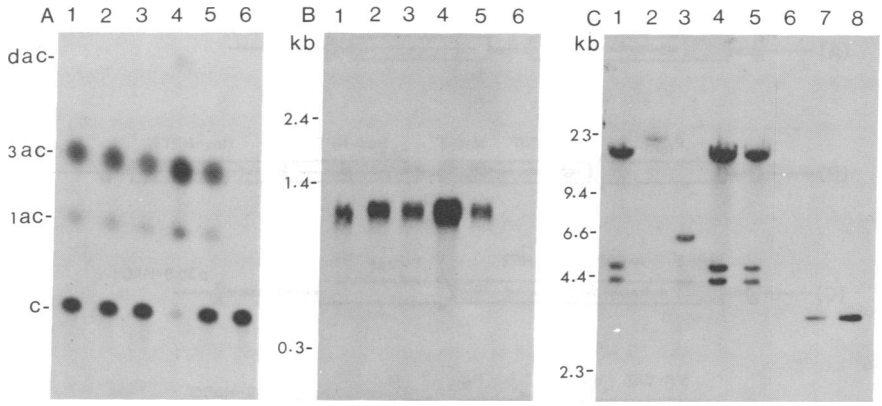

FIG. 2. Expression of CAT gene and gene organization in transgenic CAT$^+$ plants. (*A*) CAT activity in plants transformed with p19S-C (see Fig. 1). Lanes: 1, plant P12; 2, plant P32; 3, plant P41; 4, plant S13; 5, plant S15; 6, untransformed tobacco. P12, P32, and P41 are independent transformants. S13 and S15 are progeny of self-fertilized P12. Equal amounts of leaf protein extract were assayed in each case. c, Chloramphenicol; 1ac, 1-acetyl chloramphenicol; 3ac, 3-acetyl chloramphenicol; and dac, 1,3-diacetyl chloramphenicol. (*B*) RNA transfer blot of CAT$^+$ plants probed with the nick-translated CAT insert from p19CATb. Lanes 1–6 contain RNA from plants corresponding to those in *A*. Four-microgram aliquots of poly(A)$^+$ RNA were subjected to electrophoresis through a denaturing agarose gel and blotted onto a GeneScreen filter. The autoradiogram was exposed for 30 hr. RNA size markers are shown in kb. (*C*) Southern blot analysis of CAT$^+$ plants probed with the p19CATb insert. Lanes 1–6 contain *Kpn* I-digested genomic DNA from plants corresponding to those in *A* and *B*. Size markers (*Hin*dIII-digested DNA) are shown in kbp. Lanes 7 and 8 contain 7.2 and 14.4 pg of *Hin*dIII-linearized p19CATb, respectively, corresponding to one- and two-copy CAT gene equivalents per haploid tobacco genome. The autoradiogram was exposed for 48 hr.

genes (Fig. 2*C*, lane 4). This transgenote is presumably homozygous with respect to the T-DNA insertions. Plant S13, expressing a 5-fold higher level of CAT activity than P41, was also selected for further experiments aimed at inactivating the CAT gene(s).

Antisense RNA Produced at Low Levels Does Not Inhibit Gene Expression. In a preliminary attempt to block CAT gene expression, we used a construct pRAC-H (Fig. 1*B*), comprising the CAT sequence inserted in the antisense orientation between the promoter of a ribulose 1,5-bisphosphate carboxylase small subunit (*rbcS*) gene from *N. plumbaginifolia* and the nos 3′ region. The *rbcS* promoter was chosen since it is a very strong promoter and is light-regulated (31, 32). Plants P41 and S13 were retransformed with pRAC-H using hyg selection. After growth in continuous light for 1 week, 25 independent transformants were screened; however, none showed any antisense-mediated inactivation of the CAT gene. Transfer blot analysis of RNA from several pRAC-H-transformed plants indicated that the levels of antisense CAT RNA were lower by a factor of ≈10 than the amounts of sense CAT RNA transcribed in transgenic plants from the *rbcS* promoter under similar conditions (data not shown). These results suggested that the antisense CAT message may be relatively unstable and that the failure to inactivate the CAT gene was due to the inadequate levels of antisense RNA accumulated (see below).

A Strategy to Preselect Transformants Expressing High Levels of Antisense RNA. Another strategy based on a procedure described by Kim and Wold (8) was developed to achieve higher levels of antisense transcripts for the inactivation of the CAT gene(s). In this approach, a chimeric gene was designed to generate a single, bifunctional transcript encoding a selectable hyg-resistance (hyg^r) enzyme and the antisense CAT RNA. The rationale was that retransformed plants selected on high levels of hyg would necessarily contain high levels of the hyg RNA and thus high levels of the transcriptionally fused antisense CAT RNA. Since the promoter generating the antisense RNA would also be driving the synthesis of RNA coding for the selectable marker, it was necessary to use a constitutive promoter that would be active in transformed leaf cells as well as in the regenerating shoots and the roots. The CaMV 35S promoter was chosen since it shows no tissue specificity (22) and is the strongest constitutive plant promoter available (33), being at least 10 times stronger than the 19S promoter (34) used for the chimeric sense CAT target gene. To minimize the possibility of a spontaneous inactivation of the T-DNA genes (see refs. 35 and 36) in the CAT$^+$ plants used for retransformation, we imposed selection for kan resistance encoded by a chimeric nos-kan^r gene in tandem with the 19S-CAT gene. This necessitated the deletion of the kan^r gene from the vector used for the retransformation (see Fig. 1). Two different antisense CAT genes were constructed. In one, p35S-HAC (Fig. 1*C*), the entire CAT sequence (800 bp) was inserted in the antisense orientation, whereas the other, p35S-HAC 5′ (Fig. 1*D*), contained only the 5′ 172 bp of the transcribed CAT sequence. A control plasmid, p35S-H (Fig. 1*E*), containing a chimeric 35S-HPT-nos 3′ gene but lacking antisense CAT sequences, was used for comparison. Examples of results obtained are presented in Fig. 3.

High Levels of Antisense CAT RNA Repress CAT Gene Expression. CAT$^+$ plants were retransformed with the antisense CAT and control constructs and regenerated on medium containing kan and hyg. Leaf segments of approximately the same age were assayed. As shown in Table 1, CAT activity was reduced in a large proportion of the plants retransformed with the antisense CAT plasmids. A strong indication that this was not due to spontaneous gene inactivation was provided by the finding that plants retransformed with p35S-H, a plasmid lacking the antisense CAT sequence (Fig. 1*E*), showed no reduction in CAT activity in 39 independent transgenotes tested (35, 36).

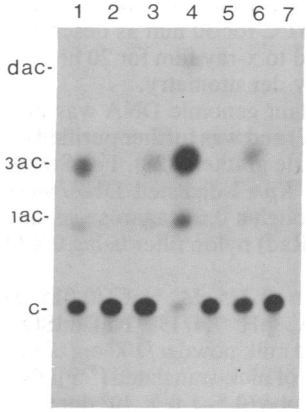

FIG. 3. CAT assays of CAT$^+$ and antisense-retransformed plants. Lanes: 1, plant P41; 2, plant AC13 (P41 retransformed with p35S-HAC showing complete loss of CAT activity); 3, plant AC22 (P41 retransformed with P35S-HAC showing partial reduction in CAT activity); 4, plant S13; 5, plant AC11 (S13 retransformed with p35S-HAC showing complete loss of CAT activity); 6, plant AC51 (S13 retransformed with p35S-HAC showing partial reduction in CAT activity); 7, untransformed tobacco.

Table 1. Results of retransformation with antisense plasmids on CAT activity

Target plant	Retransformation plasmid	Plants screened, no.	Partially inhibited activity*	Completely inhibited activity†
P41	p35S-HAC	32	9	6
	p35S-HAC5′	18	10	0
	p35S-H	32	0	0
S31	p35S-HAC	22	4	2
	p35S-HAC5′	15	5	0
	p35S-H	7	0	0

*Plants showing activity above the background (untransformed tobacco) level but <40% of the activity of the target plant.
†Plants showing only background levels of CAT activity.

About 47% of the P41 plants (expressing low CAT activity) retransformed with p35S-HAC (which contains the entire CAT gene-coding and 5′ untranslated sequences in the antisense orientation, Fig. 1*C*) showed significantly depressed levels of CAT activity (Table 1). In almost half of these plants, the amount of CAT activity was reduced to background levels. Following retransformation of S13 (a plant expressing high CAT activity) with p35S-HAC, 2 of 22 plants assayed showed complete loss of CAT activity, whereas CAT activity was partially inhibited in another 4 plants. Given that the concentration of CAT RNA in plant S13 was five times higher than in P41 (Fig. 2*B*), the less efficient gene inactivation observed in plant S13 compared to P41 is consistent with inactivation being mediated by the annealing of antisense transcripts with the target RNA.

Although the relationship between the length of the antisense transcript and the efficiency of gene inactivation was not systematically investigated in the present work, a gene construct, p35S-HAC5′, which generated transcripts containing only 172 nucleotides of 5′ terminal CAT sequence in the antisense orientation (Fig. 1*D*), was found to be less effective in the repression of CAT activity than the full-length antisense CAT construct (see Table 1; cf. ref. 4). It is possible that the transcriptional fusion of the antisense CAT sequences with the HPT gene sequence leads to the formation of secondary structures that reduce the accessibility of antisense CAT sequences for hybridization to the target RNA.

RNA transfer blot analysis (Fig. 4) showed that plants retransformed with p35S-HAC and that had exhibited a complete loss of CAT activity (lanes 2–5 and 7–9) contained appreciable amounts of a 2.3-kb RNA species corresponding to the composite HPT-antisense CAT-nos 3′ transcript. In the P41-derived plants, the level of antisense RNA was 2–20 times the P41 sense CAT RNA level. The amount of antisense RNA detected in the two CAT$^-$ S13-derived plants tested was approximately equal to the level of CAT RNA in plant S13. The absence of a distinct CAT transcript in the CAT$^-$ plants is consistent with the hypothesis that sense·antisense RNA hybrids may be rapidly degraded *in vivo* (8, 10). Also consistent with this is the observation that a P41-derived plant that exhibited only partially reduced CAT activity showed the most distinct sense CAT RNA band in addition to having the lowest level of antisense RNA (Fig. 4, lane 6).

The RNA transfer blot analysis (Fig. 4) further revealed that antisense retransformants of P41 and S13, which had shown no significant reduction in CAT activity, did not contain detectable amounts of the antisense transcript (lanes 10 and 12). Moreover, by contrast with the CAT$^-$ plants, these retransformants contained intact sense CAT RNA equivalent to the parent P41 and S13 plants. Since these plants were regenerated in the presence of hyg, the bifunctional transcript encoding hyg^r and the antisense CAT message was apparently synthesized at levels sufficient for phenotypic expression but below the detection limits of the

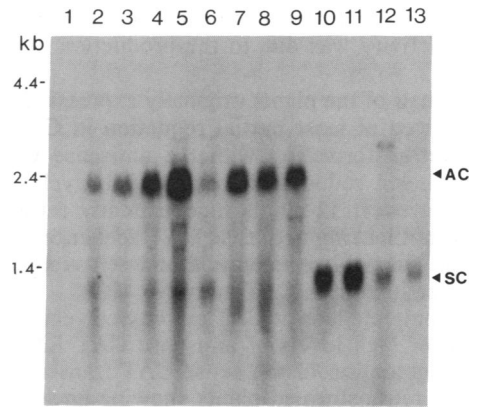

FIG. 4. RNA transfer blot of p35S-HAC-retransformed plants. Lane 1, RNA from untransformed tobacco. Lanes 2–13, RNA from the following plants: 2, AC9; 3, AC13; 4, AC16; 5, AC18; 6, AC22; 7, AC24; 8, AC11; 9, AC39; 10, AC12; 11, S13; 12, AC5; 13, P41. Plants AC9, AC13, AC16, AC18, and AC24 are P41 retransformants that exhibited a complete loss of CAT activity. AC22 and AC5 are P41 retransformants in which CAT activity was partially reduced and unchanged, respectively. AC11 and 39 are S13 retransformants in which CAT activity was completely lost. CAT activity was unchanged in plant AC12, another S13 retransformant. Twenty-five microgram aliquots of total RNA were subjected to electrophoresis, blotted onto a Zeta-Probe filter, and hybridized with the CAT insert from p19CATb. The autoradiogram was exposed for 48 hr. RNA size markers are shown in kb. The antisense CAT (AC) and sense CAT (SC) transcripts are indicated by arrowheads.

RNA transfer blot. Taken together, the above data reveal a strong correlation between the synthesis of high levels of the antisense transcript and the inhibition of CAT activity.

Inhibition of Gene Expression Is Not Due to Rearrangements or Loss of the CAT Genes. To test that the observed loss of CAT activity in antisense-retransformed plants was not due to gross structural alterations or loss of the T-DNA-borne CAT genes, DNA from CAT$^-$ plants was analyzed by Southern blotting. As shown in Fig. 5, all of the tested retransformed plants exhibited the same pattern of bands hybridizing to a CAT probe as the "parent" plants and, in addition, contained extra bands corresponding to the integrated antisense CAT genes. Thus, none of the sense CAT genes had been lost or visibly rearranged nor had the antisense T-DNA insert been integrated into the original T-DNA fragment by homologous recombination. Each transformation event appears random and independent with a variable number of copies of the gene integrated.

CONCLUSIONS

We have demonstrated that retransformation of transgenic plants expressing constitutive CAT activity with antisense CAT plasmids led to significant reduction(s) in CAT activity in a large proportion of the retransformants. The evidence

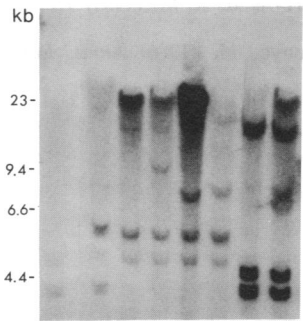

FIG. 5. Southern blot of p35S-HAC-retransformed plants. Lane 1, DNA from untransformed tobacco plant. Lanes 2–8, DNA from the following plants: 2, P41; 3, AC13; 4, AC18; 5, AC24; 6, AC5; 7, S13; 8, AC11. See legend to Fig. 4 for the derivation and CAT activity of the different plants. The blot was probed with the nick-translated p19CATb insert and exposed for 48 hr. Size markers (*Hin*dIII-digested DNA) are shown in kbp.

summarized below strongly indicates that the inhibition of CAT gene activity was due to the production of antisense CAT RNA.

(*i*) About half of the plants originally expressing low CAT activity showed at least partial reduction in CAT activity following retransformation with the antisense CAT gene, with some being reduced to background levels. In sharp contrast, however, 39 plants independently retransformed with a plasmid lacking antisense CAT sequences and taken through the identical regeneration and selection procedure showed no reduction in CAT activity.

(*ii*) The complete loss of CAT activity correlated with the production of high levels of antisense CAT RNA and the apparent degradation of the sense CAT transcript. On the other hand, retransformed plants showing unchanged CAT expression did not synthesize detectable amounts of antisense RNA but contained appropriate levels of intact CAT RNA. A plant in which CAT activity was only partially reduced contained some intact sense RNA and an intermediate level of the antisense CAT message.

(*iii*) The possibility that T-DNA instability might have led to inactivation of the CAT gene may be discounted since (*a*) DNA blot analysis revealed no detectable rearrangements or loss of the T-DNA insertions and (*b*) a chimeric kan^r gene contiguous with the CAT gene was expressed normally in all of the regenerated plants.

The exact mechanism(s) by which antisense RNA inhibits gene expression was not directly investigated. However, the results of RNA blot analysis are consistent with the hypothesis that the annealing of sense and antisense transcripts forms a hybrid molecule that is rapidly degraded *in vivo*, thus eventually preventing translation of the sense RNA.

In this work, a strategy was developed for effectively preselecting plants expressing high concentrations of antisense RNA by transcriptionally fusing the antisense sequence to the sequence of a selectable antibiotic-resistance gene. This strategy may have general applicability and, with the optimum level of the selective antibiotic, could enable even more efficient selection of plants with inactivated target genes. Coupled with strong tissue-specific and inducible promoters, this approach could be used for silencing undesirable genes in a temporal and spatial manner during plant development. The use of this approach to inactivate genes coding for nodule-specific proteins (nodulins) (37) may allow the deciphering of their functions in symbiosis. Since the completion of this work, a paper describing the antisense-mediated inhibition of nopaline synthase activity in stably transformed tobacco plants has been published (38).

We thank Normand Brisson for providing plasmid pS6-4, Nam-Hai Chua for pNPSS8B and pUC13/35S CAT, Michael Bevan for pBin19, pRK2013, and LBA4404, Steven Rogers for pMON200, Kathleen Lee for pAGS120, Truyen Nguyen for the construction of p19S-C, Christine Morrison for help with plant transformations, and Egidia De Michele and Patricia Monroe for typing the manuscript. This work was supported by operating and strategic grants from the Natural Sciences and Engineering Research Council of Canada.

1. Weintraub, H., Izant, J. G. & Harland, R. M. (1985) *Trends Genet.* **1**, 22–25.
2. Green, P. J., Pines, O. & Inouye, M. (1986) *Annu. Rev. Biochem.* **55**, 569–597.
3. Izant, J. G. & Weintraub, H. (1984) *Cell* **36**, 1007–1015.
4. Izant, J. G. & Weintraub, H. (1985) *Science* **229**, 345–352.
5. Melton, D. A. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 144–148.
6. Rosenberg, U. B., Preiss, A., Seifert, E., Jackle, H. & Knipple, D. C. (1985) *Nature (London)* **313**, 703–706.
7. Wormington, W. M. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 8639–8643.
8. Kim, S. K. & Wold, B. J. (1985) *Cell* **42**, 129–138.
9. McGarry, T. J. & Lindquist, S. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 399–403.
10. Crowley, T. E., Nellen, W., Gomer, R. H. & Firtel, R. A. (1985) *Cell* **43**, 633–641.
11. Holt, J. T., Gopal, T. V., Moulton, A. D. & Nienhuis, A. W. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 4794–4798.
12. To, R. Y.-L., Booth, S. C. & Neiman, P. E. (1986) *Mol. Cell. Biol.* **6**, 4758–4762.
13. Chang, L. J. & Stoltzfus, C. M. (1987) *J. Virol.* **61**, 921–924.
14. De Benedetti, A., Pytel, B. A. & Baglioni, C. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 658–662.
15. Cabrera, C. V., Alonso, M. C., Johnson, P., Phillips, R. G. & Lawrence, P. A. (1987) *Cell* **50**, 659–663.
16. Knecht, D. A. & Loomis, W. F. (1987) *Science* **236**, 1081–1086.
17. Ecker, J. R. & Davis, R. W. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 5372–5376.
18. Guilley, H., Dudley, R. K., Jonard, G., Baiezs, E. & Richards, K. E. (1982) *Cell* **30**, 763–773.
19. Fraley, R. T., Rogers, S. G., Horsch, R. B., Eichholtz, D. A., Flick, J. S., Fink, C. L., Hoffmann, N. L. & Sanders, P. R. (1985) *Biotechnology* **3**, 629–635.
20. Bevan, M. (1984) *Nucleic Acids Res.* **12**, 8711–8721.
21. van den Elzen, P. J. M., Townsend, J., Lee, K. Y. & Bedbrook, J. R. (1985) *Plant Mol. Biol.* **5**, 299–302.
22. Odell, J. T., Nagy, F. & Chua, N.-H. (1985) *Nature (London)* **313**, 810–812.
23. Hoekema, A., Hirsch, P. R., Hooykaas, P. J. J. & Schilperoort, R. A. (1983) *Nature (London)* **303**, 179–180.
24. Horsch, R. B., Fry, J. E., Hoffmann, N. L., Eichholtz, D., Rogers, S. G. & Fraley, R. T. (1985) *Science* **227**, 1229–1231.
25. Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254.
26. Gorman, C. M., Moffat, L. F. & Howard, B. H. (1982) *Mol. Cell. Biol.* **2**, 1044–1051.
27. Dellaporta, S. L., Wood, J. & Hicks, J. B. (1983) *Plant Mol. Biol. Rep.* **1**, 19–21.
28. Garger, S. J., Griffith, O. M. & Grill, L. K. (1983) *Biochem. Biophys. Res. Commun.* **117**, 835–841.
29. Reed, K. C. & Mann, D. A. (1985) *Nucleic Acids Res.* **13**, 7207–7221.
30. Jones, J. D. G., Dansmuir, P. & Bedbrook, J. (1985) *EMBO J.* **4**, 2411–2418.
31. Morelli, G., Nagy, F., Fraley, R. T., Rogers, S. G. & Chua, N.-H. (1985) *Nature (London)* **315**, 200–204.
32. Timko, M. P., Kausch, A. P., Castresana, C., Fassler, J., Herrera-Estrella, L., van den Broeck, G., van Montagu, M., Schell, J. & Cashmore, A. R. (1985) *Nature (London)* **318**, 579–582.
33. Sanders, P. R., Winter, J. A., Barnason, A. R., Rogers, S. G. & Fraley, R. T. (1987) *Nucleic Acids Res.* **15**, 1543–1558.
34. Rothstein, S. J., Lahners, K. N., Lotstein, R. J., Carozzi, N. B., Jayne, S. M. & Rice, D. A. (1987) *Gene* **53**, 153–161.
35. Fraley, R. T., Rogers, S. G. & Horsch, R. B. (1986) *CRC Crit. Rev. Plant Sci.* **4**, 1–46.
36. Peerbolte, R., Ruigrok, P., Wullems, G. & Schilperoort, R. (1987) *Plant Mol. Biol.* **9**, 51–57.
37. Verma, D. P. S., Fortin, M. G., Stanley, J., Mauro, V. P., Purohit, S. & Morrison, N. (1986) *Plant Mol. Biol.* **1**, 51–61.
38. Rothstein, S. J., DiMaio, J., Strand, M. & Rice, D. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 8439–8443.