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Stable and heritable inhibition of the expression of nopaline synthase in tobacco expressing antisense RNA

(chimeric gene/minus-strand RNA/plant transformation/stable expression)

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ABSTRACT **Antisense nopaline synthase (nos) (D-nopaline synthase; EC 1.5.1.19) RNA is stably expressed from the cauliflower mosaic virus 35S promoter in transformed tobacco plants. The expression of a previously introduced wild-type *nos* gene is inhibited by the antisense RNA, with less nos enzyme activity detected (by a factor of 8–50) depending on the tissue analyzed. The steady-state levels of nos mRNA are reduced in the presence of the antisense RNA, implying that mRNA degradation is probably the main mode of action for the decrease in expression in this system. The antisense RNA-expressing gene and its inhibition of *nos* expression are shown to be heritable, demonstrating that it is a potentially useful method for the modification of phenotype.**

A number of regulatory mechanisms have been recognized for the control of gene expression. Though many of these involve changes in the level of transcription initiation, several different posttranscriptional mechanisms have been delineated. These include mRNA stability, efficiency of mRNA translation, posttranslational modifications, and, more recently, inhibition of expression through the production of antisense RNA. Antisense RNA was originally found as a naturally occurring mechanism to control gene expression in bacteria (1, 2) and, unlike many of the other methods for controlling expression of a gene, can in theory be used to modify the expression of any gene without altering its structure. Antisense RNA has been shown to decrease the level of expression of genes in a variety of organisms in addition to bacteria, including *Dictyostelium* (3), *Drosophila* (4), and mammalian cells (5). It has also been demonstrated that it is possible to inhibit gene expression by using antisense RNA in transient expression experiments in *Xenopus* oocyte (6), mammalian cells (7), and carrot protoplasts (8).

The mechanism by which antisense RNA inhibits expression of a gene has been shown to vary depending on the system studied. In bacteria it has generally been found to inhibit translation, presumably by interfering with ribosome binding (1, 2). In transient expression in mammalian cells, an inhibition of translation has also been postulated (7). However, when the antisense RNA was transcribed from a gene integrated in a mammalian cell chromosome, the inhibition of expression was apparently due to the formation of double-stranded RNA in the nucleus and the subsequent failure of this RNA to be transported to the cytoplasm (5). In this case a large excess of antisense RNA was required to achieve a meaningful inhibition of synthesis of the target protein. A different result was found in *Dictyostelium*, where the mechanism of inhibition appeared to be the formation and subsequent degradation of double-stranded DNA (3). Here, the antisense RNA was expressed at a level comparable to that of the mRNA. Thus, the level of antisense RNA required to inhibit expression varies with the system and mechanism of inhibition. When a vast excess of antisense RNA is required to affect the level of expression, this technology becomes considerably less useful.

In most plants, it is difficult to isolate many types of mutants and it has not yet proved possible to use gene replacement by homologous recombination to construct defined mutations. It would be useful to make phenotypic alterations that would allow one to relate an isolated gene to a particular function. For example, one could inhibit expression of a single member of a gene family and determine the particular isozyme coded for by that gene and its function. In theory, it would be possible to use antisense RNA to alter the expression of any isolated gene, although the efficacy of the antisense RNA might have considerable variability.

In this work we demonstrate that it is possible to inhibit the expression of a nopaline synthase (*nos*) (D-nopaline synthase; EC 1.5.1.19) gene in tobacco plants by stably introducing an antisense gene into the tobacco genome. We also show coinheritance of the antisense gene with the altered phenotype.

MATERIALS AND METHODS

nos Assay. Conversion of [^{14}C]arginine to nopaline was assayed as described (9). Plant extracts were prepared by grinding leaf material in extraction buffer with a small pestle in a Microfuge tube. 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 (1 Ci = 37 GBq) of [^{14}C]arginine (Amersham, 340 mCi/mmol) and 2.5 μl of plant extract (usually 2–4 mg/ml). After incubation for 2 hr at 20°C, samples were subjected to electrophoresis on Whatman 3MM paper for 2 hr at 1500 V. The paper was then dried and subjected to autoradiography. Spots containing nopaline were cut out and assayed by liquid scintillation spectroscopy.

Detection of Nopaline in Tissues. Plant extracts were prepared as described above and spotted directly onto paper. Following electrophoresis, samples were visualized by staining with phenanthrenequinone as described (10) and photographed under an ultraviolet light source.

RNA Isolation. Total leaf RNA was isolated as follows. Between 3 and 5 g of leaf tissue (either fresh or frozen) was added to a mixture of 20 ml of buffer (50 mM Tris·HCl, pH 8.0/4% sodium *p*-aminosalycilate/1% sodium 1,5-naphthalenedisulfonate) and 20 ml of water-saturated phenol. The mixture was homogenized with a Polytron (Kinematica, Lucerne, Switzerland) for 2 min at maximum speed and then shaken at room temperature for 10 min at 300 rpm. After the addition of 20 ml of chloroform, the mixture was shaken for 10 additional min and centrifuged at 7000 rpm in a GSA rotor (Sorvall) for 20 min. The aqueous phase was reextracted with phenol/chloroform and then extracted with chloroform. The

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Abbreviations: nos, nopaline synthase; CaMV, cauliflower mosaic virus.

aqueous phase was adjusted to 2 M LiCl/2 M urea/1 mM EDTA and placed at 4°C overnight. The RNA was then pelleted by centrifuging in an SW 28 rotor (Beckman) for 2 hr at 25,000 rpm. The RNA pellet was resuspended in 1% $NaDodSO_4$/5 mM EDTA/20 mM NaOAc/40 mM Tris·HCl, pH 8.0, and precipitated with ethanol. The RNA pellet was reprecipitated with ethanol, and the RNA was resuspended in water and stored at −70°C.

RNase Protection. RNase protection experiments were performed as described (11). The nos DNA used as a probe was inserted into the *in vitro* RNA synthesis vector pGEM4 (Promega Biotec, Madison, WI). RNA was synthesized *in vitro* as described (12). *In vitro* labeled RNA (2×10^6 cpm) was added to 20 μg of total cellular RNA. The RNA mixture was precipitated with ethanol, resuspended in 30 μl of RNA hybridization buffer (80% formamide/40 mM Pipes, pH 6.4/400 mM NaCl/1 mM EDTA), heated to 85°C for 10 min, and hybridized overnight at 45°C. Three hundred microliters of RNase buffer (10 mM Tris·HCl, pH 7.8/5 mM EDTA/300 mM NaCl) containing 40 μg of RNase A per ml and 2 μg of RNase T1 per ml was added to each sample prior to incubation at 30°C for 30 min. The RNases were then inactivated by adding 10 μl of 20% $NaDodSO_4$ and 50 μg of proteinase K and incubating at 37°C for 15 min. The RNA was then extracted with phenol and the aqueous phase was reextracted with chloroform. Ten micrograms of carrier tRNA was added to each sample and the RNA was precipitated with ethanol. The sample was resuspended in loading buffer (80% formamide/40 mM Tris borate, pH 7.5), heated to 90°C for 10 min, and loaded on a 4% denaturing polyacrylamide gel.

***Agrobacterium*-Mediated Plant Transformation.** The plasmids pCIB743 and pCIB749 were transformed into the *Escherichia coli*-mobilizing strain S17-1 (13) and then mated into the *Agrobacterium* helper strain LBA4404. Four-week-old *in vitro* shoot tip cultures of *Nicotiana tabacum* cv. SR1 (T2-16, ref. 14) grown in Murashige–Skoog (MS) medium (15) without hormones (26°C, 16:8 photoperiod, 4000 lux) were used as source material for transformation. Experiments were performed essentially as described (16) with the following modifications. Nurse culture plates were omitted and the leaf discs were plated directly on nonselective MSBN medium (MS salts/B_5 vitamins/3% sucrose/1 μg of naphthylacetic acid per ml, pH 5.8/0.8% Phytagar; GIBCO) after a 10-min incubation with overnight cultures of *Agrobacterium*. Dishes were placed at 26°C and kept in the dark for 3 days.

Discs were then transferred to MSBN medium containing 500 μg of cefotaxime per ml (counterselective against *Agrobacterium*) and 20 μg of hygromycin per ml (selective for transformed plant cells) and incubated at 26°C in the light. Discs were subcultured weekly onto fresh antibiotic-containing medium. After shoots were ≈8 mm tall they were removed from the leaf discs and transferred to rooting medium [MS medium without hormones, containing 20 μg of hygromycin per ml in GA-7 containers (Magenta, Chicago)]. After ≈4 weeks, regenerated plantlets could be transferred to sterilized potting soil and into the greenhouse.

RESULTS

Construction of a *nos* Antisense Gene and Transformation of Tobacco. As a model system to test the usefulness of antisense RNA to stably inhibit gene expression in plants, we constructed a *nos* antisense gene and introduced this gene into a tobacco line that already contained the *nos* gene in its genome. The *nos* gene is present on the nopaline tumor-inducing (Ti) plasmid pTiT37 of *Agrobacterium tumefaciens* (17). To insert the desired portion of the *nos* gene into the antisense construct, we first introduced an *Xho* I restriction site ≈10 base pairs (bp) upstream of the start of transcription by way of *in vitro* mutagenesis (18). Following *in vitro* mutagenesis, an *Xho* I–*Hin*dIII fragment containing the *nos* gene was subcloned into pUC19, to make plasmid pCIB740, shown in Fig. 1. Approximately the first two-thirds of the *nos* gene can be isolated from this plasmid as an 860-bp *Bam*HI fragment (one of the *Bam*HI sites being derived originally from pUC19). This fragment was inserted into the cauliflower mosaic virus (CaMV) 35S promoter cassette pCIB710, which has a single *Bam*HI site between the promoter and the poly(A) addition site (19), in the correct orientation for the expression of nos antisense RNA (see Fig. 1). The resulting chimeric *nos* antisense gene was then inserted into the binary *Agrobacterium*-mediated plant transformation vector pCIB743 (19) to make pCIB749 (Fig. 1).

The wild-type *nos* gene had previously been transformed into the tobacco cultivar SR1 by deFramond *et al.* (14). The original transformant was self-pollinated and seedlings were tested for nos activity. One of these progeny, designated T2-16, was used in this study. The plasmids pCIB743, as a negative control, and pCIB749, containing the antisense gene, were used to transform T2-16 tobacco leaf disks. Hygromycin-resistant seedlings were grown in agar in GA-7

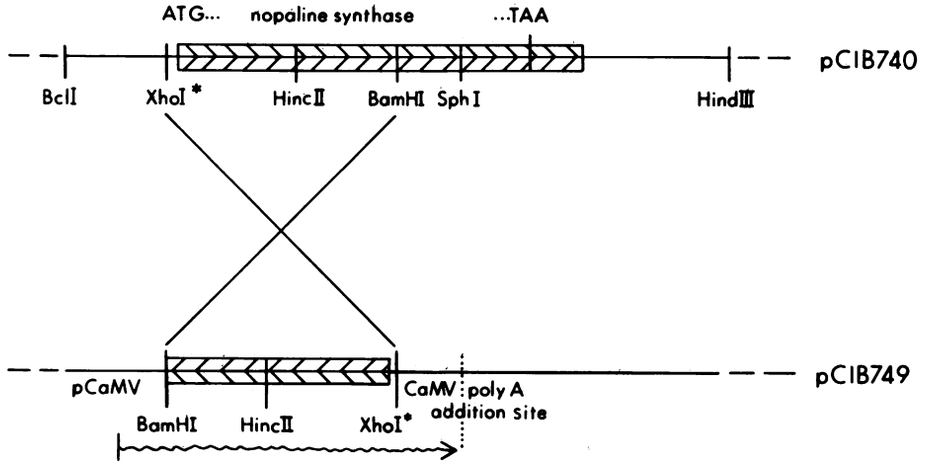

FIG. 1. Construction of the nos antisense RNA-expressing transformation vector. An *Xho* I site was inserted 10 bp upstream from the *nos* transcription initiation site. The DNA between this *Xho* I site and the *Bam*HI site in the *nos*-coding sequence was inserted into the CaMV 35S promoter cassette pCIB710 (18) so that nos antisense RNA would be expressed. This chimeric gene was then inserted into the plant transformation vector pCIB743 (19) to make pCIB749. ▭, *nos* transcript; ATG, translation start site; TAA, translation stop site; pCaMV, CaMV 35S promoter; ⇝, *nos* antisense transcript; *Xho* I*, inserted restriction site.

containers prior to being potted in soil and transferred to the greenhouse.

nos Activity of the Transformed Plants. The transformed plants were analyzed for nos enzyme activity. When analyzing the control plants (transformed with pCIB743), it became obvious that there was a considerable variability in the amount of nos activity detected for any one transformant. This variability was found to depend on the developmental stage of the leaf tissue from which the extract was made. In general, plants grown on tissue culture medium in GA-7 containers had higher levels of enzyme activity than larger greenhouse-grown plants. In addition, the larger, older leaves had more enzyme activity than younger leaves (unpublished results). This variability was not due to pool sizes of unlabeled arginine or some other small molecule, since desalting on Sephadex G-50 columns did not affect the levels of enzyme activity. We therefore found that it was essential to analyze leaves of as similar developmental stage as possible when comparing different transformed plants. As can be seen in Fig. 2 from the standard error, there still was some variability in the amount of enzyme activity detected in the control plants. Five plants transformed with plasmid pCIB749, containing the *nos* antisense gene, and six pCIB743-transformed control plants were analyzed [although only four of each (chosen at random) were studied in the results shown in Fig. 2*A*, experiment 1]. When plants grown *in vitro* in GA-7 containers were analyzed, the average decrease in enzyme activity for the nos antisense expressing plants was by a factor of 10 in one experiment (Fig. 2*A*, experiment 1) and by a factor of 50 in the other (Fig. 2*A*, experiment 2).

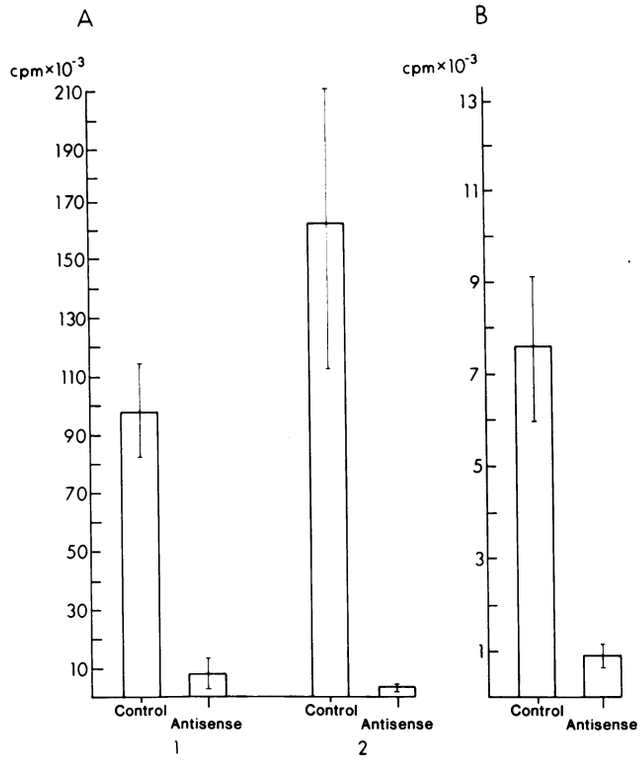

FIG. 2. Quantitation of the amount of nos activity. After paper electrophoresis, the [^{14}C]nopaline was autoradiographed and then the nopaline spot was cut out of the paper and assayed for radioactivity. The mean value for labeled nopaline is shown along with the standard error. (*A*) Quantitation of the enzyme activity of plants grown in GA-7 jars *in vitro*. Experiment 1: Four pCIB743-transformed control and four pCIB749-transformed nos antisense expressing plants were analyzed. Experiment 2: Six pCIB743-transformed plants and six pCIB749-transformed plants were analyzed. Four of each type of plant were from the same original transformants as tested in experiment 1. (*B*) Greenhouse-grown plants were analyzed. The same plants were analyzed as in *A*, experiment 2 (six control and five experimental).

However, these values probably understate the actual decrease in expression in most of the pCIB749-transformed plants since one of these had considerably higher levels of enzyme activity than the rest. All of the other plants had a decrease in enzyme activity by a factor of at least 50 when compared to the mean value for the control plants.

When larger greenhouse-grown plants were analyzed, the average decrease in activity was by a factor of ≈8 (see Fig. 2*B*). The amount of nos enzyme activity was considerably lower in the greenhouse-grown control plants. Therefore, even though the amount of activity found in the antisense RNA-expressing plants was extremely low (very close to background), the decrease in activity in comparison to the control plants was not as great as was found for the plants grown on tissue culture medium.

Steady-State nos mRNA Levels Are Decreased by Antisense Transcripts. To analyze the steady-state levels of sense and antisense transcripts in the transformed plants, RNase protection experiments were performed. Two fragments from the *nos* gene were cloned into the *in vitro* transcription vector pGEM4 for use as probes. One was a 510-bp *Hin*cII–*Sph* I fragment and the other was a 310-bp *Hin*cII–*Bam*HI fragment (see Fig. 1); the resulting plasmids are called pCIB772 and pCIB771, respectively. As can be seen in Fig. 1, all of the 310-bp fragment is present in the antisense construct, whereas part of the larger fragment is only present in the wild-type gene.

In the first experiment, the 510-bp fragment in pCIB772 was transcribed so that the resulting labeled RNA would hybridize to the sense mRNA. The labeled RNA was hybridized with total cellular RNA and treated with RNase. The results of the RNase protection are shown in Fig. 3. The amount of nopaline mRNA detected in the plants transformed with plasmid pCIB749, containing the *nos* antisense gene, was considerably lower than in the plant transformed with the pCIB743 control. By quantifying the bands on a scanning densitometer, the signal for the antisense RNA-expressing

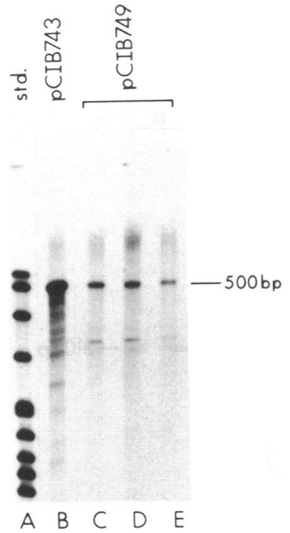

FIG. 3. RNase protection of nos mRNA. Twenty micrograms of total RNA was hybridized with *in vitro* labeled nos antisense RNA made from plasmid pCIB772 (1×10^6 cpm). RNase protection experiments were then performed. Lane A, molecular weight standard, pBR322 digested with *Hpa* II; lane B, wild-type nos mRNA levels from a plant transformed with pCIB743; lanes C–E, nos mRNA levels of plants transformed with pCIB749 and expressing nos antisense RNA.

plants was decreased by a factor of ≈8–10. Since the amount of labeled probe RNA is in considerable excess over the amount of the antisense and the sense RNA present in the total plant RNA, this decrease in mRNA signal is not due to competitive hybridization of unlabeled antisense RNA in the sample. This decrease in the nos mRNA in the pCIB749-transformed plants was also seen when greenhouse-grown plants were analyzed (data not shown).

A similar result was found when RNA transfer blot analysis (data not shown) was done on the plant RNA samples, although quantitation was not possible due to the low intensity of the signal for the pCIB749 plants. The nos mRNA for control plants could be detected, with the amount of mRNA detected being approximately the same for different control plants.

Steady-state levels of antisense RNA in the plant samples were determined in the same fashion. In this case, the 310-bp nos fragment in plasmid pCIB771 was used as a probe because the larger 510-bp probe was found to give unacceptably high background hybridization when transcribed to detect antisense RNA. As expected, antisense transcripts were detected in the pCIB749-transformed plants but not in the pCIB743-transformed control plants (Fig. 4). The steady-state level of the antisense RNA in these plants is approximately equal to the level of nos mRNA found in the control plants transformed with pCIB743. Since the CaMV 35S promoter is reported to yield as much as 30-fold more steady-state RNA than the *nos* promoter (20), the antisense RNA is presumably degraded at a higher rate than the mRNA. Some of this degradation most likely occurs when the mRNA and antisense RNA hybridize in the cell, although the antisense RNA may also be intrinsically more susceptible to degradation. The decrease in the level of steady-state nos mRNA undoubtedly accounts for much of the decrease in the nos enzyme activity in the pCIB749 plants. However, antisense RNA expression may also influence other factors that affect nos levels, such as inhibition of translation or transport of the mRNA from the nucleus.

Cosegregation of the Decrease in nos Expression with the Antisense Gene in Progeny Plants. A genetic analysis of the transformed plants was performed to demonstrate heritability of the antisense gene and phenotype. The pCIB749-transformed plants, which were nos$^+$/antisense$^+$ were crossed to wild-type (nos$^-$) tobacco. The T2-16 line originally transformed was heterozygous for the *nos* gene. If transformants are also heterozygous for the antisense gene, one would expect a progeny ratio of 1 nos$^+$/antisense$^+$:1 nos$^+$/antisense$^-$:1 nos$^-$/antisense$^+$:1 nos$^-$/antisense$^-$. Two of the pCIB749-transformed plants were crossed with Coker 176 line of tobacco. Sixteen progeny plants were tested for nopaline production and for hygromycin resistance (since the hygromycin-resistance gene was adjacent to the antisense gene on the DNA transferred to the plants, it should be very closely linked to that gene). For nopaline determinations, a soluble extract was made from leaf tissue from each of these plants and equalized with respect to protein concentration. After paper electrophoresis, the samples were stained for the presence of nopaline. The results for the nos$^+$/hygromycin-resistant and nos$^+$/hygromycin-sensitive plants, shown in Fig. 5, demonstrate a clear linkage between hygromycin resistance and a decreased level of nopaline in the progeny. Therefore, not only is the expression of antisense RNA effective in reducing the expression of the *nos* gene but also this effect is stably inherited.

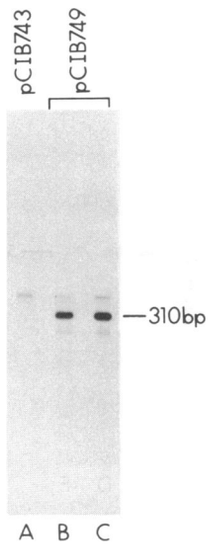

FIG. 4. RNase protection of nos antisense RNA. Twenty micrograms of total RNA was hybridized to *in vitro* synthesized nos$^+$ sense RNA made from the plasmid pCIB771 (1×10^6 cpm). RNase protection analysis was then performed. Lane A, levels of antisense RNA found in a control plant transformed with pCIB743; lanes B and C, antisense RNA found in plants transformed with pCIB749.

DISCUSSION

The inhibition of expression of the *nos* gene by antisense RNA demonstrates that it is possible to use this technology to alter phenotype at the whole plant level. This inhibition not only is maintained throughout the life of the plant but is stably inherited in progeny plants. It appears that the primary means by which the antisense RNA inhibits expression is through decrease of sense mRNA level. We propose the mechanism to be the formation of double-stranded RNA that is degraded more rapidly than free mRNA. It is possible that other mechanisms, such as decreased translation of the mRNA, are also important contributors to reduced gene expression.

The efficacy of a stably expressed antisense RNA for inhibiting gene expression has shown considerable variability for those few genes studied. In *Dictyostelium*, almost complete inhibition of expression of discoiden I protein was

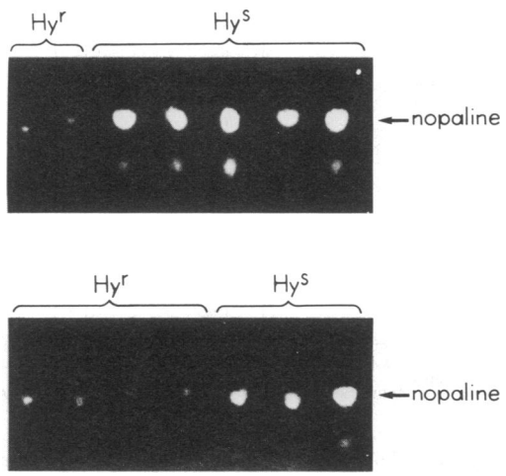

FIG. 5. Nopaline present in progeny plants. pCIB749-transformed T2-16 plants were backcrossed to wild-type tobacco. Sixteen progeny plants from each cross were tested for hygromycin resistance and for the presence of nopaline. Hygromycin-resistant (Hy^R) plants should contain the nos antisense RNA-expressing gene, whereas the hygromycin-sensitive (Hy^S) plants should not. Those plants that did not make nopaline were removed from the screen. The leaf material was homogenized and then each sample was equalized for its protein concentration. The samples were analyzed by paper electrophoresis and then stained for the presence of nopaline. Two sets of progeny plants are shown.

achieved without an excess level of the antisense RNA being produced (3). However, in experiments using mammalian cell culture, a vast excess of antisense RNA was required to achieve a meaningful inhibition of thymidine kinase activity (5). In our results, the inhibition of the *nos* gene falls somewhere between these two extremes. The steady-state level of the antisense RNA was approximately the same as the level of the nos mRNA in a control plant. However, this is considerably higher (8- to 10-fold) than the mRNA in the plants containing the antisense gene. Therefore, the steady-state level of the antisense RNA in this system apparently need not be in vast excess over the level of mRNA for a considerable decrease in expression to occur. In the inhibition of thymidine kinase expression, the antisense RNA was expressed as part of a larger mRNA that might have prevented efficient hybridization. Alternatively, there might be enough differences in the secondary structures of different mRNAs that the antisense RNA might not hybridize to some mRNAs efficiently. Finally, species might differ in how quickly and easily they degrade, transport out of the nucleus, and translate double-stranded RNA.

The stably inherited expression of antisense RNA to inhibit the expression of a gene is a potentially useful tool for making a wide variety of functional mutations. Although these will probably result in a decrease rather than a complete inhibition of expression of the target gene, in many cases this will still allow one to detect a change in phenotype. Furthermore, by placing the expression of the antisense RNA under developmental regulation, one could decrease the expression of a gene in specific tissues or developmental stages.

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