Inhibition of gene expression in plant cells by expression of antisense RNA

(chimeric genes/electroporation/plant transformation/transient chloramphenicol acetyltransferase assays/minus-strand RNA)

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ABSTRACT  Due to the paucity of mutations in biochemical pathways in plants, an alternative approach to classical genetics was tested by expressing antisense RNA in plant cells. A series of plasmids was constructed with the bacterial gene for chloramphenicol acetyltransferase (EC 2.3.1.28) linked in either the sense or antisense orientation to several different plant gene promoters. Various ratios of sense and antisense chloramphenicol acetyltransferase gene plasmids were introduced into plant protoplasts by electric field-mediated DNA transfer (“electroporation”) and the level of expression in each combination was monitored by chloramphenicol acetyltransferase assays. Transcription of antisense RNA was found to effectively block the expression of target genes. Thus, the observation that antisense RNA inhibits gene expression in bacteria and animal systems has been extended to the plant kingdom. Antisense RNA techniques have immediate practical applications in both basic research and in plant genetic engineering.

Recently, antisense RNA (minus-strand RNA) has been demonstrated to effectively inhibit gene expression in bacteria (1-7), *Dictyostelium* (8), *Xenopus* oocytes (9, 10), *Drosophila* (11), and mammalian cells (12-14). The use of antisense RNA techniques may offer a convenient alternative to classical genetic methods for the isolation of mutations or for reducing the activity expressed from "leaky" mutants. Because of the relative paucity of plant molecular biological techniques, similar studies of inhibition of gene expression in plant systems have not been reported. Recently, the successful adaptation of a technique called "electroporation" (15-17) for the uptake and expression of DNA in plant cells has been reported (18-20). We have used electroporation and chimeric plant genes to examine the effect of antisense RNA expression in transiently transformed plant protoplasts.

MATERIALS AND METHODS

Electroporation and Transient Assay Conditions. Wild carrot cell lines WOO1C (21) and RCWC-1 were grown in Murashige and Skoog (MS) medium containing 0.1 mg of 2,4-D (2,4-dichlorophenoxyacetic acid) per liter. Carrot protoplasts were prepared from rapidly growing cells that had been diluted 1:10 in fresh medium 4 days earlier. Briefly, carrot cells (10-ml packed volume) were resuspended in 50 ml of 1% cellulase (Cellulysin, Calbiochem-Behring) and 0.5% hemicellulose (Rhizomyze, Genencor), in 0.4 M mannitol/50 mM CaCl₂/10 mM sodium acetate, pH 5.8, for 2 hr at 25°C. Protoplasts were separated from cell clumps by passage through a 60-μm-mesh nylon screen (Tetko, Elmsford, NY), washed twice, and resuspended in electroporation buffer (10 mM Hepes, pH 7.1/150 mM NaCl/4 mM CaCl₂/0.2 M mannitol). Samples (1 ml) of protoplasts (~5 x 10⁴ ml) at 0°C were gently mixed with supercoiled plasmid DNAs and placed in ethylene oxide-sterilized disposable plastic cuvettes (Starstedt, Princeton, NJ). Fixed platinum electrodes (1 cm wide, 0.4 cm apart, and 0.125 mm thick were inserted into the DNA/protoplast mixture, and an electric pulse of 350 V/10 ms duration (1 time constant) was delivered to the cuvette by discharge of two 540-μF electrolytic capacitors (Sprague 36DX-540-450V, North Adams, MA) arranged in parallel. After incubation for 10 min on ice, the protoplasts were diluted with 4 ml of MS medium containing 3% sucrose, 0.4 M mannitol, and 0.1 mg of 2,4-D per liter and placed in sterile plastic flasks (T-25, Falcon). After 24-48 hr of incubation at 25°C, protoplasts were assayed for chloramphenicol acetyltransferase (CAT; EC 2.3.1.28) activity (22). Briefly, protoplasts were pelleted (150 x g for 5 min) and resuspended in 0.2 ml of 250 mM Tris (pH 7.8). Cell extracts were prepared by sonication for 2 min (cup sonicator; Branson, Danbury, CT), heated to 65°C for 10 min, and centrifuged for 2 min in a Microfuge to pellet cell debris. Acetyl coenzyme A and [¹⁴C]chloramphenicol (New England Nuclear) were added to the supernatant, and the mixture was incubated at 37°C for 1 hr. Radiolabeled reaction products were extracted with ethyl acetate, and the acetylated products were separated by TLC as described (22). An autoradiograph of the TLC plate was made, and the amount of acetylated products was determined by densitometry.

Construction of Sense and Antisense CAT Gene Plasmids. Plasmid constructions were made by standard techniques (23) into the polynucleotide of pUC8 (24). Briefly, a 250-base-pair (bp) fragment containing the nopaline synthase (NOS; EC 1.5.1.19) gene promoter (PNOS) (25) was obtained by digestion of pMON200 (26) with restriction enzymes *Pst* I and *Eco*RI. This fragment was subcloned into *Eco*RI/*Pst* I-digested pUC8 (Fig. 2, line a). A *Hind*III fragment containing a 780-bp CAT gene (27) and the 250-bp NOS gene poly(A) signal (designated pA in plasmid names) was inserted into the *Hind*III site of the PNOS-pUC plasmid in the sense (Fig. 2, line b) or antisense (Fig. 2, line c) orientation. The sense PNOS-CAT plasmid was cut with *Pst* I and religated. Digestion with *Pst* I results in two fragments, one containing the CAT gene coding region and one containing the NOS gene promoter and poly(A) signal. Religation of the two fragments results in the original plasmid (Fig. 2, line b) and a plasmid containing a flipped (antisense) CAT gene with a NOS gene poly(A) signal (Fig. 2, line d). Recombinant *Escherichia coli* containing these CAT gene plasmids were selected on LB plates containing chloramphenicol at 5 μg/ml. Recombinants containing the CAT gene in the sense orientation relative to the NOS gene promoter are resistant to chloramphenicol concentrations up to 30 μg/ml, whereas antisense CAT gene

Abbreviations: bp, base pair(s); CAT, chloramphenicol acetyltransferase; NOS, nopaline synthase; CaMV, cauliflower mosaic virus; PAL, phenylalanine ammonia-lyase; 2,4-D, 2,4-di-chlorophenoxyacetic acid.
plasmids are resistant to no more than 10 μg/ml. On plates containing chloramphenicol at 5 μg/ml, both recombinants will grow, but bacterial colonies containing CAT plasmids in the sense orientation are much larger; presumably such CAT gene transcripts are being driven by the lacZ promoter. A pUC8 plasmid containing a 450-bp fragment for cauliflower mosaic virus (CaMV) 35S RNA gene promoter (PcAmV) (19) was used to construct plasmids analogous to those described above (Fig. 2, lines e-g). Similarly, a pUC18 plasmid containing a 1100-bp fragment of the carrot phenylalanine ammonia-lyase (PAL; EC 4.3.1.5) gene promoter (PPAL) was used to construct plasmids similar to those described above (Fig. 2, lines h-j). Lastly, a control plasmid (Fig. 2, line k) was constructed containing an antisense CAT gene linked to the bacteriophage SP6 promoter (PSP6) (pGem-1, Promega Biotec, Madison, WI). This promoter is not active in plant cells.

RESULTS

Electroporation and transient CAT gene expression assays were used to examine whether transcription of antisense RNA could inhibit gene expression in plants. The protocol for these experiments is shown in Fig. 1. A series of plasmid constructions were made in which the CAT gene in either the sense or antisense orientation was placed downstream of several plant gene promoters (Fig. 2). Such plasmids were mixed at sense to antisense ratios of 1:1, 1:10, and 1:100 and were introduced into plant cell protoplasts with a brief electrical pulse. As shown in Fig. 3, lanes a-c, the level of CAT activity expressed in carrot protoplasts electroporated with a plasmid (Pnos-CAT-pA) containing the NOS promoter linked to the CAT gene and a NOS poly(A) signal was dependent on the input DNA concentration. This relationship was linear up to 400 μg of DNA per ml. When the amount of Pnos-CAT-pA DNA was kept at 5 μg/ml and additional carrier plasmid DNA lacking the CAT gene was added (Fig. 3, lanes g-i), then a dramatic increase in the level of CAT activity was observed. The amount of CAT activity observed with PNOS-CAT-pA plasmid at 40 μg/ml could be obtained by using the same plasmid at only 5 μg/ml in the presence of a non-CAT gene plasmid DNA at 50 μg/ml (compare lanes a and h in Fig. 3). Similarly, an amount of CAT activity equal to PNOS-CAT-pA plasmid at 125 μg/ml was obtained when non-CAT gene plasmid DNA at 500 μg/ml was added to PNOS-CAT-pA at 5 μg/ml. This enhancement of expression,

![Diagram](image)

FIG. 1. Schematic diagram of the protocol for antisense RNA experiments. Plant protoplasts were prepared and electroporated, and CAT assays were performed as described.

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FIG. 2. Structure of sense and antisense CAT gene plasmids. Plasmids were constructed in pUC8 or pUC18 (both designated here pUC) as described. Several different promoters (Pnos, lines a-d; PcaMV 35S RNA, lines e-g; PPAL, lines h-j; Psp6, line k) were fused to the CAT gene in the sense (CAT) or antisense (anti-CAT) orientation. Additionally, some plasmids contained a poly(A) (pA) signal derived from the NOS gene.

or “carrier effect,” was also observed when either salmon sperm DNA or yeast ribosomal RNA was mixed with the Pnos-CAT-pA plasmid DNA but not when several non-nucleic-acid polymers were used. However, when the carrier plasmid contained a CAT gene fused to the NOS gene promoter in an antisense orientation, such that the expected mRNA produced was complementary in base sequence to CAT mRNA, then a dramatic decrease in the expected level of CAT activity occurred. When the ratio of input sense CAT plasmid DNA to antisense CAT plasmid DNA was 1:100, the expected level of CAT activity was reduced by >95% (Fig. 3, compare lanes f and i). It is unlikely that the reduced activity resulted from the titration of a positive activator functioning in trans because increasing just the NOS gene promoter concentration does not cause inhibition (Fig. 3, lanes g-i).

The antisense CAT gene construction used in the above experiments did not contain a poly(A) signal. The effect of the addition of an efficient plant poly(A) signal, derived from the NOS gene, was examined in a similar experiment. In order to eliminate the carrier effect, the total plasmid DNA concentration in these experiments was kept constant. Fig. 4 shows the results of one such experiment. Analogous to the experiment described in Fig. 3, at a sense-to-antisense plasmid ratio of 1:100, a large inhibition of CAT activity was observed (compare lanes a and c in Fig. 4). When a NOS gene poly(A) signal was included on the antisense CAT construct, the amount of antisense plasmid DNA required to inhibit CAT activity to the same extent was reduced by at least a factor of 2 (Fig. 4, compare lanes c and d).
between sense and antisense DNAs, antisense CAT gene plasmids containing three different plant promoters of various efficiencies were constructed. In addition to the above described NOS gene promoter fusion, the gene promoters for CaMV 35S RNA and PAL were fused to the CAT gene (Fig. 2). When the NOS and CaMV gene promoters were joined in the sense orientation to the CAT gene, CAT enzyme was expressed in carrot protoplasts at approximately equal levels. However, a similar PAL gene promoter fusion expressed CAT at a level lower by a factor of 4 or 5. When the NOS- and PCaMV-antisense CAT plasmids were used in an experiment analogous to that described in Fig. 3, a similar level of inhibition of CAT activity occurred (Fig. 5, compare lanes b and c). When the PPAL-antisense CAT plasmid was used at the same ratio, less inhibition of CAT activity by a factor of 4 was observed (Fig. 5, compare lanes b and d). Thus, the degree of inhibition of CAT activity correlates with the strength of the antisense plasmid promoter. Similar experiments were carried out with a PPAL-CAT plasmid (Fig. 2, line h) as the target gene. The amount of NOS-antisense CAT plasmid or PCaMV-antisense CAT plasmid required to inhibit expression of the target PPAL-CAT gene plasmid was less than that required for the PPAL-antisense CAT plasmid (unpublished data).

**DISCUSSION**

The general applicability of antisense RNA technology is clear. This technique has been reported to effectively inhibit gene expression in bacteria, Dictyostelium, Drosophila, Xenopus oocytes, and cultured mammalian cells. We report antisense RNA inhibition of gene expression for plant cells. The molecular mechanism by which this occurs is unclear. Several different modes of inhibition have been described in eukaryotic systems. For mammalian cells, double-stranded RNA formation in the nucleus may block mRNA transport to the cytoplasm (14). Alternatively, as reported for the discoidin gene in Dictyostelium, RNA-RNA hybrids formed in the nucleus may be rapidly degraded (8). *In vitro* synthesized antisense RNA injected into Xenopus oocytes has been...
found to inhibit translation of mRNAs bound to polyribosomes in the cytoplasm (9, 10). Any or all of these mechanisms may be occurring in plant cells. Other possible modes of inhibition may also exist. However, inhibition of gene expression in plant protoplasts is unlikely because of heteroduplex formation between sense and antisense plasmids, since the degree of inhibition correlates with promoter efficiency and increases with the addition of a poly(A) signal.

The introduction of DNA into plant cells by electroporation provides a simple means to examine the effect of the simultaneous expression of sense and antisense RNAs on gene expression. The use of carrier DNA in electroporation experiments allows a high level of expression with low amounts of the particular promoter fusion DNA under investigation. Low amounts of plasmid DNA may be desirable for studies of transient gene expression. For example, Gorman et al. (28) found that large amounts of plasmid DNA containing an enhancer sequence, when introduced by transfection into mammalian cells, caused the titration of an enhancer-specific repressor protein. However, inhibition of CAT gene expression in plant cells was not due to DNA promoter titration of a positive trans-acting factor, since the expression of chimeric Pnos-CAT gene constructions was linear with input plasmid DNA and not affected by large amounts of additional NOS gene promoter DNA.

Inhibition of transient gene expression in plant protoplasts is a first step toward the eventual inhibition of gene expression in stably transformed plants. However, the effectiveness of antisense RNA technology may be gene dependent and may not be useful when complete loss of function is the goal. For example, the herpes simplex virus thymidine kinase gene contains sequences that interfere with the accumulation of antisense thymidine kinase RNA (14). Furthermore, transient gene expression assays may be optimal for RNA-RNA hybrid formation, since sense and antisense transcripts may be present in high local concentrations. One test of the utility of antisense RNA technology can be examined by the effect of inhibition of PAL gene expression on the disease resistance of plants. PAL, a key enzyme in the biosynthetic pathway of phytoalexins and lignin, is thought to play an important role in the ability of plants to resist infection (29–31). However, like many genes, the PAL gene may be essential for plant growth. Therefore, to avoid the potential lethal effects of high-level constitutive expression of antisense mRNAs of essential genes, it is necessary to use plant promoters that can be regulated. Inducible and tissue-specific plant promoters such as light-regulated promoters (32, 33), hormonally regulated promoters (36, 37), and stress-induced promoters (38) can allow the disrecretionary expression of antisense RNA.

Presently, the use of antisense RNA expression cannot completely shut off expression of a gene and, thus, may resemble a “leaky” mutation. Under these circumstances it will not be a reliable substitute for null mutations. Differences in phenotype are often observed between “leaky” and null mutations, causing misinterpretations if only “leaky” mutations are used in a study. Accordingly, antisense RNA may be used to investigate changes in phenotype by decreasing the activity of possible “leaky” mutations.

In addition to testing whether the expression of certain genes is essential for plant growth and survival, antisense RNA technology should be useful for the examination of cloned plant genes whose functions are unknown but are of particular interest because of their timing or pattern of expression during development. For example, antisense RNA experiments should be useful for examining the role in development of cloned genes of unknown function that are expressed during the early stages of somatic embryogenesis in carrot (39, 40). Experiments similar to those described for Drosophila embryos (11), in which “phenocopies” of Kruppel mutants were produced transiently by using microinjected antisense Kruppel RNA, should now be possible for plants because of recent advancements in the technology for microinjection of individual totipotent carrot cells (41). Finally, as many plant genes have been found to be members of multigene families, it was encouraging to note that the expression of a single antisense discoidin gene in Dictyostelium was found to inhibit the expression of all three members of the discoidin gene family (12). Thus, antisense RNA techniques may allow the simulation of mutations in single genes or small gene families in plants that previously could not be readily isolated by classical genetic methods.

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