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Protection against tobacco mosaic virus in transgenic plants that express tobacco mosaic virus antisense RNA

(coat protein)

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ABSTRACT **Transgenic tobacco plants that express RNA sequences complementary to the tobacco mosaic virus (TMV) coat protein (CP) coding sequence with or without the tRNA-like structure at the 3′ end of the TMV RNA were produced. Progeny of self-pollinated plants were challenged with TMV to determine their resistance to infection. Plants that expressed RNA sequences complementary to the CP coding region and the 3′ untranslated region, including the tRNA-like sequences, were protected from infection by TMV at low levels of inoculum. However, plants that expressed RNA complementary to the CP coding sequence alone were not protected from infection. These results indicate that sequences complementary to the terminal 117 nucleotides of TMV, which include a putative replicase binding site, are responsible for the protection. However, the level of protection in these plants was considerably less than in transgenic plants that expressed the TMV CP gene and accumulated CP. Since the mechanisms of protection in the two systems are different, it may be possible to increase protection by introducing both sequences into transgenic plants.**

We have reported (1, 2) that expression of a tobacco mosaic virus (TMV) coat protein (CP) gene in transgenic tobacco plants confers protection against infection by TMV. The mechanism of protection depends on the presence of the CP molecule and not on the CP mRNA (3). It has also been suggested that protection against TMV might be conferred by expression of sequences complementary to the viral RNA (4). The purpose of the experiments reported here was to test this hypothesis.

A comprehensive review of the effect of antisense RNA on gene expression has been published (5). To date, naturally occurring antisense RNA known to function in gene regulation has been reported only for prokaryotes where transcription and translation are tightly coupled both spatially and temporally. Examples include regulation of the synthesis of outer membrane proteins in *Escherichia coli* (6) and the expression of the insertion element IS*10* (7). In these examples the antisense RNA presumably anneals with its corresponding mRNA and prevents translation (7, 8).

It has been shown that replication of the RNA coliphage SP can be inhibited by antisense RNA by expressing various antisense viral sequences in the host (9, 10). The antisense RNA for the maturase gene was the most effective molecule in these replication inhibition assays, and antisense RNA complementary to sequences at the 3′ end of the viral RNA had a lesser effect. Since the latter region contained only the 3′ one-third of the replicase gene and the 3′ noncoding region, the result indicates that antisense RNA may be effective at blocking replication in RNA viruses independent of its ability to block translation.

In human cell cultures oligonucleotides complementary to specific regions of the human immunodeficiency virus genome were effective in partially inhibiting replication of the virus (11). The most effective oligonucleotide was complementary to the splice acceptor site, although oligonucleotides complementary to a region close to the $tRNA^{Lys}$ primer binding site also reduced replication of the virus.

The capacity of antisense RNA to control gene expression in eukaryotes has been tested by introducing antisense RNAs into cells or by producing RNA molecules in transfected or transgenic cells. In the cases where antisense RNAs were injected into the cytoplasm, translational inhibition may be similar to that postulated for prokaryotes (12). However, in cases where antisense RNA was expressed by the host genome, there is evidence that most of the double-stranded RNA formed was in the nuclear fraction, suggesting that double-stranded RNA might be inefficiently transported into the cytoplasm (8).

To determine whether antisense RNA complementary to the TMV genome could effectively block TMV infection, we introduced chimeric genes encoding sequences complementary to the TMV CP gene and the 3′ untranslated region of TMV RNA into transgenic plants. Resistance to infection by TMV was tested in progeny of the transgenic plants and compared with protection in transgenic plants that accumulate TMV CP.

MATERIALS AND METHODS

Plasmid Constructions. The intermediate plasmids that contain TMV sequences were constructed in binary or integrative-type plasmids containing well characterized features. The intermediate plasmid containing a cloned cDNA encoding the TMV CP gene (bar A in Fig. 1*B*) has been described (1). Intermediate plasmids containing sequences represented in Fig. 1*B* by bars A or B were constructed so as to form chimeric genes that, when transcribed in transgenic plant cells, produced RNA in the sense or antisense orientations. To produce antisense RNA corresponding to sequence A (Fig. 1*B*), the sequence was excised from a pUC-type plasmid (13), end-filled with deoxynucleotides by using the Klenow fragment of DNA polymerase I, and then ligated to the intermediate plasmid pMON316 (14) that had been digested with *Xho* I and end-filled. Orientation of the insert with respect to the cauliflower mosaic virus (CaMV) 35S promoter was determined by digestion with diagnostic restriction enzymes. The intermediate plasmid containing sequences representing nucleotides 5707–6278 (i.e., lacking the tRNA-like sequence) was prepared as described (3) except that the

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Abbreviations: CP, coat protein; TMV, tobacco mosaic virus; CaMV, cauliflower mosaic virus.

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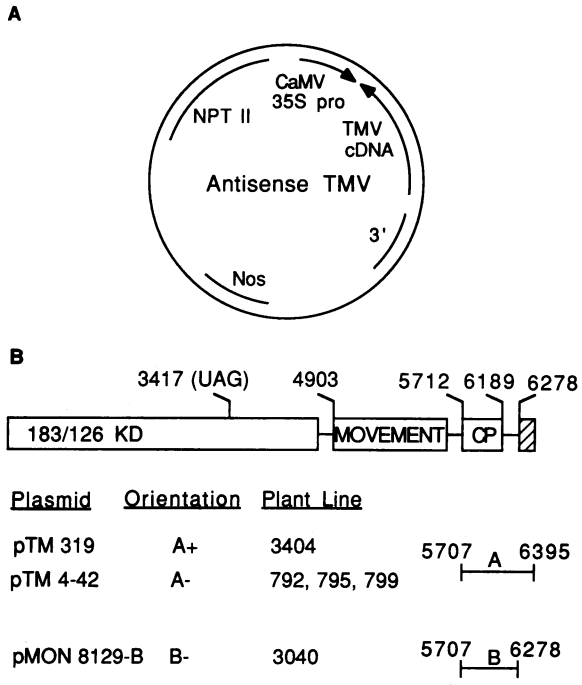

FIG. 1. (*A*) Position of the TMV cDNA sequence in each plasmid relative to the CaMV 35S promoter. Arrows indicate the normal direction of transcription. A cDNA for different regions of the TMV genome was ligated to the CaMV 35S promoter in the opposite orientation relative to normal transcription. The polyadenylylation regulation signal was provided either by a fragment from the α' gene of the 7S (β-conglycinin) seed storage protein or by a fragment from the nopaline synthase gene. NOS, nopaline synthase gene; NPT II, a chimeric eukaryotic gene encoding neomycin phosphotransferase. (*B*) Diagram of TMV RNA showing the regions represented in each of the plasmids. Region A is contained within pTM319 (1), and pTM4-42 and region B is in pMON8129-B. The cross-hatched box represents the tRNA-like structure at the 3' end of TMV RNA.

sequence was ligated into the binary intermediate plasmid pMON552 in an orientation to produce RNA complementary to TMV RNA. The plasmid pMON552, a derivative of pMON530 (15), contains the 35S promoter–polylinker–3' end from the α' gene of β-conglycinin. Fig. 1*A* shows the orientation of the CaMV 35S promoter in the intermediate plasmids pMON316 or pMON552 and the orientation of TMV cDNA sequences to produce antisense TMV RNA.

Transformation. Leaf disks of *Nicotiana tabacum* cv. Xanthi were inoculated with *Agrobacterium tumefaciens* cells containing either of the intermediate plasmids described carrying sequences A or B (Fig. 1*B*) to produce antisense RNA transcripts and transformed cells were selected for kanamycin resistance and regenerated into plants as described (16). Three independent transformants containing anti-A sequences and one containing anti-B sequences were used for analysis. Each transformant was self-pollinated and progeny seedlings were germinated and grown as described (2). Each independent transformant thus gives rise to a separate plant line. Plant line 3404 harboring a chimeric gene that expresses sequence A (Fig. 1*B*) to produce CP mRNA also accumulates CP and was described (1).

RNA Analysis. RNA was isolated from leaves of transgenic plants by the procedure of Haffner *et al.* (17) with minor modifications. Total RNA was separated on a 1.0% agarose gel containing formaldehyde and transferred to nitrocellulose (18). The blot was hybridized with a gel-isolated, ^{32}P-labeled nick-translated fragment containing the CP-coding sequence (18).

Analysis for Protection Against TMV. Two leaves of R1 seedlings bearing four or five leaves were mechanically inoculated with 0.01 or 0.05 μg of TMV per ml of inoculation buffer containing 20 mM potassium phosphate and 1 mM EDTA, brought to pH 7.4 with KOH. Plants were scored for the appearance of symptoms daily after inoculation. Vein clearing in systemically infected leaves, the first detectable symptom, was used as the criterion for positive disease diagnosis. Such symptoms are characteristic of infection by the U_1 strain of TMV.

Segregation of the transgenic trait in seedlings expressing antisense RNA (expressors) or not expressing antisense RNA (nonexpressors) was determined by nopaline analysis (19) for the lines 792, 795, 799, and 3404 or by kanamycin resistance (16) for line 3040. The nopaline synthase gene and the neomycin phosphotransferase gene are located on the intermediate plasmids and, therefore, cosegregate with expression of the antisense sequence in the transgenic plant lines. The control plants used (combined nonexpressors) included a transgenic line that contained pMON316 without TMV sequences and the nonexpressors from each of the lines used in the experiment.

RESULTS

Levels and Sizes of RNAs Produced. Various lengths of cloned cDNAs that encode RNAs complementary to the 3' region of the TMV genome were inserted into pMON316 or pMON552 in such a way that antisense RNAs (i.e., RNA complementary to TMV RNA) would be produced. These cDNAs were under the control of the CaMV 35S promoter, a strong constitutive promoter in transgenic plants.

The plasmids containing the chimeric genes were introduced into *A. tumefaciens* and subsequently introduced into tobacco cells by the leaf-disk cocultivation method (16), and transgenic plants were regenerated. Each transformed plant was self-pollinated to generate a line of transformed plants and RNA isolated from each plant line was analyzed by Northern blot hybridization reactions. Similar levels of RNA accumulated in lines 792, 795, and 799, each of which accumulated antisense RNA complementary to the CP gene and the 3' untranslated region of TMV RNA (Fig. 2). The level of RNA produced in these lines was 2–4 times lower than that produced by line 3404, a transgenic line that expresses CP RNA and accumulates CP (1). The size of the antisense RNA expressed by line 3040, although larger than in line 3404, reflected both the deletion of 117 nucleotides complementary to the sequences for the tRNA-like structure at the 3' end of the TMV RNA, and the difference in sequences contributed by the 3' polyadenylylation signals in the intermediate plasmids pMON316 and pMON552 (data not shown). The level of RNA in line 3040 was greater than for the other antisense lines (Fig. 2).

Assessing Protection Against Virus Infection. Seedlings from the initial transgenic plants described above were inoculated with TMV at two concentrations and observed daily for disease development. In this host/virus system there is a very tight correlation between symptom appearance and TMV replication. Therefore, in most experiments relative susceptibility was based upon observation of disease symptom development. Plant lines 792, 795, and 799 exhibited a low level of protection at inoculum concentrations of 0.01 and 0.05 μg of TMV per ml (Fig. 3 *A* and *B*, respectively). The protection was not as effective as in plant lines that accumulated CP (line 3404) but was significantly better than in control plants (combined nonexpressors). At these low levels of inoculum, protection was manifested as an escape of infection rather than a delay of symptom development. If the plants that did not become infected were not included in the analysis and only the percentage of plants that became infected were considered, the rate of symptom development for control and transgenic plants expressing antisense RNA

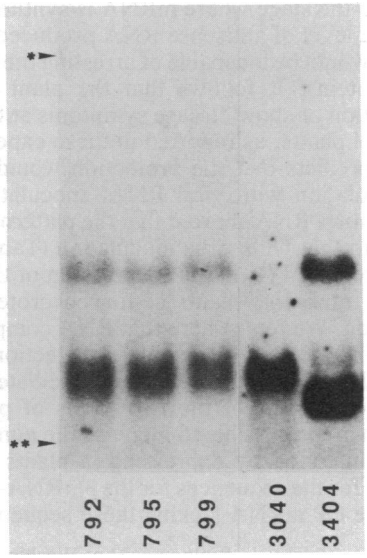

FIG. 2. Northern blot analysis of RNA isolated from transgenic tobacco lines. Plant line 3404 harbors the plasmid pTM319 (1), which carries TMV sequence A (Fig. 1*B*) in the sense orientation and accumulates TMV CP; lines 799, 795, and 792 harbor a chimeric gene containing sequence A (Fig. 1*B*) in the antisense orientation; line 3040 harbors a chimeric gene carrying sequence B (Fig. 1*B*) in the antisense orientation. Total RNA (25 μg) was loaded in each lane of a 1.0% agarose gel containing formaldehyde. Blotted RNAs were hybridized with a nick-translated DNA fragment containing the CP cDNA sequences. Migration of the RNA was from top to bottom. *, Position of the 6395-base full-length genomic TMV RNA; **, position of the 693-base TMV CP subgenomic mRNA.

were not significantly different from one another. A total of five plant lines expressing the CP antisense RNA were analyzed for protection against TMV in a total of seven independent experiments and the results were consistent with those presented here. In contrast, plants expressing antisense RNA lacking sequences complementary to the sequences for the 3′ tRNA-like structure of TMV RNA (line 3040) did not show protection against infection by TMV (Fig. 4).

Nonexpressing plants from each of the lines discussed above, as well as from a line containing the intermediate plasmid pMON316 without TMV sequences, were used as control plants. As seen in Fig. 3, not all of the control plants developed symptoms at the low level of inoculum used in the experiments. As expected, plant line 3404, which accumulates CP, showed a high level of protection at the concentration of TMV used in these experiments (Figs. 3 and 1*B*).

Assessing Protection Against TMV RNA. Plant lines 792 and 795, as well as control plants, were inoculated with TMV RNA to determine if antisense RNA protected the plants against unencapsidated viral RNA as it did against TMV. For these experiments it was important to use the same concentration of infectious units of TMV RNA as was used for virion inoculation. However, it was not possible to precisely quantitate the number of infectious units of RNA relative to that of virions as the concentration of virus used (0.01 and 0.05 μg of TMV per ml) was too low to produce a significant number of necrotic lesions on the local lesion host. Disease developed in control plants inoculated with 0.05 μg of TMV per ml at a slightly faster rate than in plants inoculated with 4.0 μg of TMV RNA per ml but more slowly than in plants inoculated with 8.0 μg of TMV RNA per ml. As shown in Table 1, there was protection in plant lines 792 and 795 against infection by TMV RNA applied at 4 μg/ml and less protection at 8 μg/ml. There was also a significant delay in disease development in the CP-expressing line 3404, compared to the vector control line 306 after inoculation with TMV RNA at 4.0 μg/ml (Table 1).

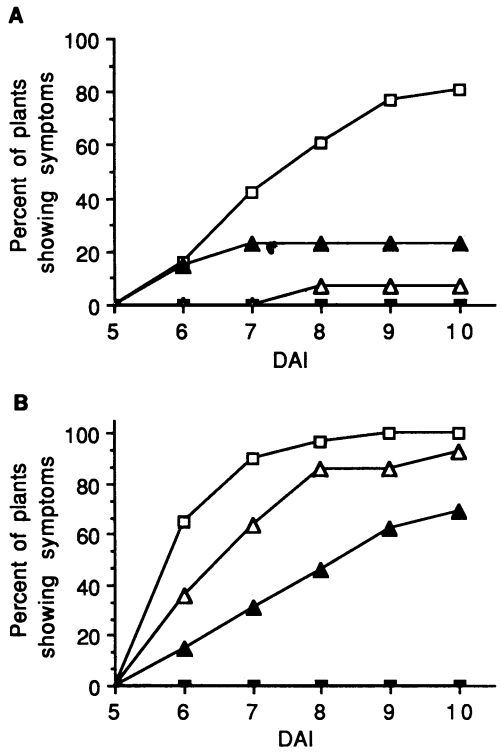

FIG. 3. Disease development (percent of plants showing symptoms) in transgenic plants after inoculation with TMV. Data from a total of 18 nonexpressing seedlings from three lines and 13 seedlings from a line harboring the intermediate plasmid lacking TMV sequences were combined to show disease development in nonexpressors (□). The remaining lines each contained between 10 and 14 TMV RNA-expressing plants that expressed TMV sequence. ▲, Line 792; △, line 795; ■, line 3404. (*A*) Plants were inoculated with 0.01 μg of TMV per ml. (*B*) Plants were inoculated with 0.05 μg of TMV per ml. DAI, days after inoculation.

DISCUSSION

We have shown that the accumulation of antisense RNA complementary to the TMV CP coding sequence and the 3′ noncoding region of the TMV genome in transgenic plants protects the plants from infection by low concentrations of TMV. The degree of protection is, however, considerably less than the protection in transgenic plants that accumulate

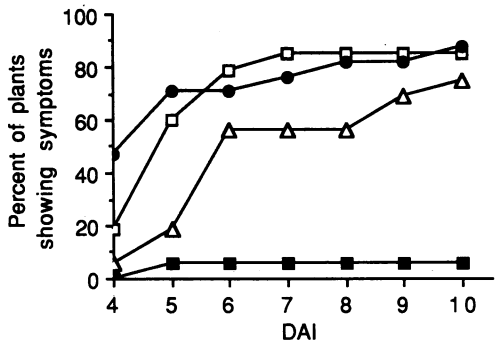

FIG. 4. Disease development in transgenic plants after inoculation with TMV. Seedlings were inoculated with 0.01 μg of TMV per ml and scored for symptom development daily. Disease development in 24 plants of line 3040 (●), containing antisense RNA lacking sequences from the 3′ end of TMV RNA (bar B, Fig. 1*B*), was compared with disease development in 16 plants of line 795 (△), which expresses antisense RNA that includes this sequence (bar A, Fig. 1*B*). Disease development in 18 plants of a line that accumulates CP (line 3404, ■) and in 47 combined nonexpressors (□) is also presented. DAI, days after inoculation.

Table 1. Disease development in transgenic plants inoculated with TMV or TMV RNA

Inoculum	Plant line	Disease development various days after inoculation					
		5	6	7	8	9	10
TMV							
(0.05 μg/ml)	3404	0	0	0	0	0	0
	306	0	2.31	3.69	4.54	5.00	5.00
	795	0	1.14	2.57	3.93	4.29	4.43
	792	0	0.46	1.00	1.92	2.77	3.15
TMV RNA							
(4.0 μg/ml)	3404	0	0.09	0.45	2.36	3.00	3.18
	306	0	0.69	2.15	3.62	4.08	4.23
	795	0.21	0.50	0.71	1.64	2.64	3.64
	792	0	0.69	1.85	2.46	3.31	3.54
TMV RNA							
(8.0 μg/ml)	3404	0	2.45	3.73	4.18	4.55	4.55
	306	0.23	3.77	4.69	5.00	5.00	5.00
	795	0	1.08	2.15	3.23	3.69	4.38
	792	0	1.77	3.00	4.23	4.23	4.23

Plants were scored for visual disease development 5–10 days after inoculation (as indicated) on a scale of 0–5. 0, No visible sign of infection; 1, no vein clearing but potentially infected; 2, vein clearing on one leaf; 3, vein clearing on more than one leaf; 4, dark green mottled leaves; 5, mosaic patterns in the leaf. The scores from 15 plants of each plant line were averaged for each day after inoculation. Line 3404 expresses a TMV CP gene and accumulates CP; line 306 harbors the intermediate plasmid without TMV sequences; lines 795 and 792 express antisense to TMV sequences shown as bar A in Fig. 1*B*.

high levels of TMV CP (e.g., plant line 3404). Antisense RNAs that lack sequences complementary to those of the tRNA-like structure of TMV RNA did not protect plants against TMV infection. The level of RNA in one such line, 3040, was greater than for the other antisense lines indicating that the lack of protection in this line was not due to differences in the amount of accumulated RNA. Therefore, RNA complementary only to the 5′ untranslated region and the coding sequence of the CP mRNA is not sufficient to confer protection. Other researchers have shown (20) that it may be more difficult to inhibit the translation of highly expressed genes (as would be the case for viral mRNA during infection) using antisense RNA.

In brome mosaic virus, deletion analysis has established that the tRNA-like structure is a replicase binding site (21); the tRNA-like structure of TMV RNA presumably serves a similar function for TMV. For the ColE1 plasmid it has been postulated that the tRNA-like cloverleaf structure of RNA I aids in the binding of RNA I to its target, RNA II (22). Perhaps the tRNA-like structure at the 3′ end of TMV, if it exists *in vivo*, is also a suitable target for binding the antisense RNA. It is proposed, therefore, that hybridization of the antisense RNA to the RNA genome of the challenge virus genomic RNA prevents viral replicase from attaching to the sense RNA, thereby inhibiting replication. Alternatively, the complementary sequence may itself bind replicase thereby reducing the chance for replication of viral RNA. However, we believe this is unlikely since there was not a difference in CP-dependent protection regardless of the presence or absence of the tRNA-like (i.e., pseudoknot) structure on the CP mRNA (3).

At low inoculum concentrations the protection against TMV caused by the antisense RNA is manifested as an escape of infection rather than a delay in disease development. This agrees with a model in which antisense RNA binds to the 3′ end of the incoming virus, possibly during uncoating of the virus, thereby preventing negative-strand synthesis. If replication is not inhibited at this stage, negative strands are synthesized and replication continues. Once replication progresses to the stage where mRNA is synthesized in large amounts, the level of antisense RNA produced in the transgenic plants would be incapable of arresting the translation of the viral proteins. It follows that the plant would either escape infection or show disease symptoms at the same time as the control plants, as reported in these experiments. The model also predicts that the protection would be effective against inoculation with viral RNA. Inoculation with low concentrations of RNA showed that the pattern of protection was similar to that with virus inoculation (Table 1).

We have reported (1, 2) that expression of the CP coding sequence in transgenic plants conferred protection against TMV infection. We report here that RNA complementary to the 3′ end of TMV RNA also confers protection, although at a substantially lower level than the CP-mediated protection. Since the mechanisms of the two forms of protection are different, it may be possible to increase the number of plants that escape infection by expressing in plants both the antisense RNA for the sequences for the 3′ tRNA-like structure as well as the CP mRNA lacking these sequences.

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