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RNA-Mediated Virus Resistance: Role of Repeated Transgenes and Delineation of Targeted Regions

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Resistance to cowpea mosaic virus (CPMV) in transgenic *Nicotiana benthamiana* plants is RNA mediated. In resistant CPMV movement protein (MP) gene-transformed lines, transgene steady state mRNA levels were low, whereas nuclear transcription rates were high, implying that a post-transcriptional gene-silencing mechanism is at the base of the resistance. The silencing mechanism can also affect potato virus X (PVX) RNAs when they contain CPMV MP gene sequences. In particular, sequences situated in the 3′ part of the transcribed region of the MP transgene direct elimination of recombinant PVX genomes. Remarkably, successive portions of this 3′ part, which can be as small as 60 nucleotides, all tag PVX genomes for degradation. These observations suggest that the entire 3′ part of the MP transgene mRNA is the initial target of the silencing mechanism. The arrangement of transgenes in the plant genome plays an important role in establishing resistance because the frequency of resistant lines increased from 20 to 60% when transformed with a transgene containing a direct repeat of MP sequences rather than a single MP transgene. Interestingly, we detected strong methylation in all of the plants containing directly repeated MP sequences. In sensitive lines, only the promoter region was found to be heavily methylated, whereas in resistant lines, only the transcribed region was strongly methylated.

INTRODUCTION

Transgenic plants expressing virus-derived sequences can be resistant to plant virus infections. Pathogen-derived resistance is mediated either by the protein encoded by the transgene or by the transcribed mRNA (Wilson, 1993; Lomonossoff, 1995). Protein-mediated resistance generally offers moderate protection against a broad range of viruses, whereas RNA-mediated resistance results in immunity in the cell for closely related viruses.

RNA-mediated virus resistance has been interpreted as an example of homology-dependent gene silencing (Flavell, 1994; Smith et al., 1994; Dougherty and Parks, 1995; Meyer, 1995; Mueller et al., 1995; English et al., 1996; Prins et al., 1996). Gene silencing can operate transcriptionally or post-transcriptionally (Finnegan and McElroy, 1994; Jorgensen, 1995; Matzke and Matzke, 1995; Meyer and Saedler, 1996) and is detected as either the absence of nuclear transcription or the decreased accumulation of specific mRNAs, respectively. For RNA-mediated virus resistance, post-transcriptional silencing seems plausible, because no homologous endogenous gene is present and viral RNA replication is merely restricted to the cytoplasm. This was indeed demonstrated in several cases of viral resistance (Lindbo et al., 1993a; Smith et al., 1994; Mueller et al., 1995; Swaney et al., 1995; Prins et al., 1996) in which low transgene mRNA steady state levels relative to transcriptional activity was observed for resistant lines.

RNA-mediated virus resistance is a highly specific process. The mechanism is induced in only some of the transgenic lines, and only very specific sequences are a target for the resistance mechanism. To explain sequence-specific cytoplasmic RNA degradation, researchers have suggested a role either for antisense RNAs formed in the nucleus or for complementary RNAs formed in the cytoplasm by a plant RNA-dependent RNA polymerase (RdRp) (Flavell, 1994; Dougherty and Parks, 1995; Meyer, 1995). The duplex RNA structures, which are consequently formed, would serve as a target for nucleolytic cleavage by a cellular factor, after which exonucleases could continue the degradation process (Dougherty and Parks, 1995). Various factors have been suggested to play a role in triggering the silencing mechanism, for example, exceeding an RNA threshold level (Lindbo et al., 1993a; Smith et al., 1994), the formation of aberrant mRNAs (Dougherty and Parks, 1995; Meyer, 1995; English et al., 1996), or the presence of an unknown genomic feature at or near the silencing transgene locus (Mueller et al., 1995). This genomic feature could relate to a complex organization of T-DNA insertions at a locus (Van Blokland et al., 1994) or the methylation of the transgene (Smith et al., 1994; English et al., 1996). These aspects are not mutually exclusive and might cause aberrations in processes, such

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as mRNA transcription, processing, or export, that could trigger gene silencing (Flavell, 1994).

Previously, we obtained engineered resistance to cowpea mosaic virus (CPMV) in *Nicotiana benthamiana* (Sijen et al., 1995). CPMV consists of two genomic RNA molecules: RNA1, which encodes the viral replicase and is able to replicate independently, and RNA2, which encodes the movement protein (MP) and the coat proteins (CPs), and is dependent on the expression of RNA1 for its replication. When the viral replicase and MP genes were expressed, several transgenic plants carried resistance that specifically blocked the cellular replication of the viral segment from which the transgene was derived (Sijen et al., 1995). These characteristics suggested, for the MP gene–containing plants in particular, that an RNA-based rather than a protein-based mechanism underlies resistance.

In this study, the RNA-based nature of resistance is confirmed for both MP- and replicase-mediated protection. In transgenic plants expressing defective versions of the MP or replicase genes, a similar type of resistance was detected. In addition, we show that a CPMV CP gene can confer cellular, segment-specific resistance, supporting the idea that transgenic expression of a CPMV RNA sequence rather than expression of a specific CPMV protein is involved in generating this type of resistance to CPMV. In resistant MP-transformed lines, the steady state transgene mRNA levels were low relative to the high nuclear transcription rates, thus supporting the concept of RNA-mediated viral resistance as a sequence-specific post-transcriptional RNA degradation process. To obtain more information about the mechanism of post-transcriptional silencing, we examined some aspects of the recognition of the sequences that are targeted for degradation as well as some features of the resistance-conferring locus in the experiments described in this article.

In MP gene–resistant plants, not only RNA2 but also heterologous RNA molecules can be subject to the resistance mechanism if they contain the sequence corresponding to the MP gene sequences of the transgene. The genomic RNA of potato virus X (PVX) (Chapman et al., 1992) was used as the recipient heterologous RNA molecule. The PVX expression system was further exploited to determine whether the resistance mechanism is targeted to a specific region, sequence, or structure of the transgene mRNA. By studying the fate of recombinant PVX genomes containing sequences corresponding to different parts of the transcribed region of the MP transgene, we show that in three independent resistant MP-transformed lines, sequences representing the 3′ part of the transcribed region are specific targets of resistance, as was shown previously for a post-transcriptionally silenced β-glucuronidase (*GUS*) transgene (English et al., 1996). We generated PVX derivatives that contained sequences corresponding to various smaller or larger portions of the 3′ part of the transcribed region. When inoculated onto resistant MP-transformed plants, these PVX derivatives revealed that the entire 3′ part is a target for the resistance mechanism rather than a specific sequence or structure within this 3′ part.

To examine more directly whether integration of transgenes as repeated sequences can trigger post-transcriptional silencing, we constructed transgenic plants expressing transgenes with either inverted or direct repeats of the MP gene sequences. These transgenic plants revealed not only that transgenes with directly repeated sequences at the transcribed region can confer resistance at increased frequency but also that methylation at the transcribed region of the transgene can play a role in establishing resistance. A refined model (Dougherty and Parks, 1995; English et al., 1996) of the mechanism of RNA-mediated virus resistance, taking into account these novel observations, is discussed.

RESULTS

Transgenic Plants and the Resistance Phenotype

Resistance to CPMV in transgenic plants expressing full-length CPMV MP or replicase genes is present in the cell and is specifically directed against the accumulation of the viral segment from which the transgene was derived (Sijen et al., 1995). This observation suggests an RNA-based rather than a protein-based mechanism. To test this hypothesis, we generated transgenic *N. benthamiana* plants expressing defective versions of the MP and replicase genes that produce the full-length mRNAs but only truncated proteins (Table 1, MP48ΔN and

Table 1. Transgenic Plants Examined for Resistance to CPMV

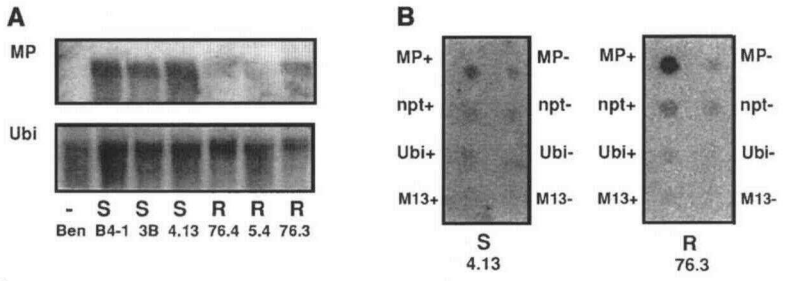
	Plant[b]		Protoplast[c]	
Transgene[a]	R_0	R_1	RNA1	RNA2
MP48ΔN	4/19	4/19	+	–
Rep200ΔX	3/26	3/26	–	–
MP48PL	0/24	0/6	ND	ND
VP23	ND	1/16	+	–
VP23-cotransformed VP37	6/20	3/6[d]	+	–

[a] The viral sequences used to transform *N. benthamiana* were a defective version of the 48-kD MP gene (MP48ΔN), a defective version of the 200-kD replicase gene (Rep200ΔX), a promoterless 48-kD MP gene (MP48PL), and full-length versions of 37- and 23-kD viral CP genes (VP37 and VP23).

[b] The values represent the number of resistant R_0 plants or R_1 lines of the total number tested for resistance to CPMV.

[c] From plants that appeared to carry a resistant phenotype, protoplasts were made and transfected with CPMV RNA. In subsequent immunofluorescence assays, two different antisera were used to monitor the replication of the two RNA segments, RNA1 and RNA2, individually. (+), replication of the examined CPMV RNA segment; (–), inhibition of replication; ND, not determined.

[d] The six R_0 generation plants that were selected for examination of resistance in the R_1 generation comprised three resistant and three sensitive plants, which were scored as three resistant and three sensitive lines in the R_1 generation.



C

Plant line	B4-1	3B	4.13	76.4	5.4	76.3
Phenotype	S	S	S	R	R	R
MP+/Ubi+	3.0 ± 1.0	5.0 ± 1.2	2.8 ± 2.0	15.3 ± 8.0	18.4 ± 9.7	11.3 ± 5.0

Figure 1. RNA Gel Blot Analysis and Nuclear Run-On Analysis to Determine Steady State Transgene mRNA Levels and Transcription Rates in Both Resistant and Sensitive Full-Length MP Gene-Transformed Plants.

(A) RNA gel blot analysis was performed with total RNA extracted from nontransformed (Ben) plants and full-length MP-transformed resistant (R) lines 76.4, 5.4, and 76.3 and sensitive (S) lines B4-1, 4.13, and 3B (Sijen et al., 1995). For each line, ~18 μg of RNA was used, and the blot was probed with a double-stranded probe specific for either the MP transgene (MP) or the constitutively expressed plant ubiquitin (Ubi) gene. **(B)** and **(C)** Run-on transcription analysis was performed with isolated nuclei of the same lines as those given in **(A)**. The synthesized ^{32}P-labeled RNAs were hybridized to a membrane with spots of 1 μg of various single-stranded phagemid DNAs containing sequences specific for the sense (+) or antisense (−) sequence of the MP transgene (MP), the kanamycin resistance selection marker (npt), the constitutively expressed plant ubiquitin gene (Ubi), or the M13mp18/19 vector (M13). The hybridized ^{32}P-labeled transcripts were quantified using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). For each line, the sense (+) MP transgene transcription rates were normalized to the sense (+) ubiquitin transcription rate. In **(B)**, typical results of run-on experiments with either sensitive (S) line 4.13 or resistant (R) line 76.3 are shown. In **(C)**, the average results of three or four independent run-on analyses are represented by the value MP+/Ubi+ and the concomitant standard deviation.

Rep200ΔX). The defective versions were made by introducing frameshifts downstream of the AUG codon, leaving reading frames for 71 of the 342 amino acids of the MP and 233 of the 1866 amino acids of the replicase, respectively. The genes were under the control of an enhanced cauliflower mosaic virus (CaMV) 35S promoter and a nopaline synthase 3′ termination signal.

Plantlets that contained the transgene sequence, as determined by polymerase chain reaction (PCR) analysis, were transferred to the greenhouse and inoculated with CPMV. When a plant remained free of symptoms 10 days postinoculation (DPI), protoplasts were prepared and infected with CPMV RNA to examine resistance in the cell. When protoplasts are infected with CPMV RNA, it is possible to monitor individually the replication and subsequent expression of the two viral segments (RNA1 and RNA2) by using two distinct and specific antisera in immunofluorescence assays. Several transgenic plants expressing the defective MP or replicase genes revealed a resistant phenotype similar to the phenotype observed in resistant transgenic plants expressing full-length sequences (Table 1; Sijen et al., 1995). Resistance was complete in the plant, maintained in the cell, and specifically directed against the replication of the viral segment from which the transgene was derived. These results confirm an RNA-based nature of resistance.

RNA gel blot analysis of uninfected plants carrying a full-length MP transgene (Sijen et al., 1995) revealed relatively low steady state transgene expression levels for the resistant lines (Figure 1A). However, nuclear run-on experiments (Figures 1B and 1C) showed that the nuclear transcription rates of the MP transgene normalized to the transcription rates of a constitutively expressed ubiquitin gene (Figure 1C) were not lower in the resistant lines than in sensitive lines. In resistant lines, the standardized transgene transcription rates were found to be two- to sixfold higher (Figure 1C). These results support the concept that post-transcriptional RNA degradation underlies RNA-mediated virus resistance. The RNA degradation process is primarily directed toward the transgene mRNAs, but homologous, incoming viral RNA molecules can also be eliminated, resulting in a resistant phenotype. For both resistant and sensitive lines, low levels of antisense transgene transcripts were detected (Figure 1B) that are barely above the background level, indicating that in resistant and sensitive lines, genomic enhancer sequences around the integration site do not result in significant levels of antisense transcripts.

When transformed with a construct containing a promoterless MP gene, none of the 24 transgenic plants showed any resistance (Table 1, MP48PL), indicating that transcription is likely to be essential for establishing RNA-mediated resistance to CPMV. This would agree with the assumption of a post-transcriptional basis for resistance.

To investigate whether the other viral genes, in addition to

the replicase and MP genes, could confer resistance of a similar type to transgenic plants, the two viral CP genes, encoding a 23- (VP23) or a 37-kD (VP37) protein, respectively, were separately cloned into binary vectors. Transgenic plants were generated that expressed the two CP genes either separately or together. Double transformants could be easily obtained by cotransformation because the CP genes were present on two different binary vectors containing different plant selection markers (kanamycin or methotrexate). As shown in Table 1 (VP23 and VP23-cotransformed VP37), resistance to CPMV was observed in transgenic plants carrying the VP23 gene alone and in the transgenic plants containing both the VP23 and VP37 genes. Resistance was maintained cellularly and was specifically directed against the replication of RNA2, also suggesting an RNA-based nature for resistance with this gene.

Sequences Corresponding to the 3′ Part of the Transcribed Region of the MP Transgene Are the Target of the Resistance Mechanism

The full-length MP-transformed lines 76.4 and 5.4 (Sijen et al., 1995), for which homozygous resistant R_2 lines were obtained, were chosen to investigate the RNA-mediated resistance to CPMV in more detail. In addition, plants of the nonhomozygous resistant R_2 line 76.3 were used in some of the experiments. Resistant plants of this line were first identified by examining protoplasts from one leaf of a plant for resistance to infection with CPMV RNA.

In the MP-transformed plants, only RNA molecules highly homologous to the transgene mRNAs are eliminated, particularly the CPMV RNA2 molecules. Accordingly, the plants are not resistant to the distinct potexvirus PVX. However, when the sequences corresponding to the full-length MP CPM transgene were inserted into the genome of PVX (Chapman et al., 1992) and infectious transcripts of this PVX derivative were inoculated onto resistant MP-transformed plants, the plants remained free of PVX-specific symptoms (Figure 2, insert 1). Nontransformed *N. benthamiana* plants, inoculated with recombinant PVX full-length MP RNA, showed clear mosaic symptoms 8 DPI. This represents a 2-day delay in symptom appearance compared with wild-type PVX. This delay is probably due to the increased length of the genome (from 6500 to 8300 nucleotides), as described previously by Chapman et al. (1992).

The apparent resistance of the plants transformed with the full-length MP gene for the PVX full-length MP derivative was confirmed by reverse transcriptase–PCR (RT-PCR) analyses at 12 DPI. RT-PCR provided no amplified fragment specific for the recombinant PVX molecules (results not shown) when a primer pair (pvxF and pvxR) specific to sequences flanking the integration site was used. For the nontransformed plants,

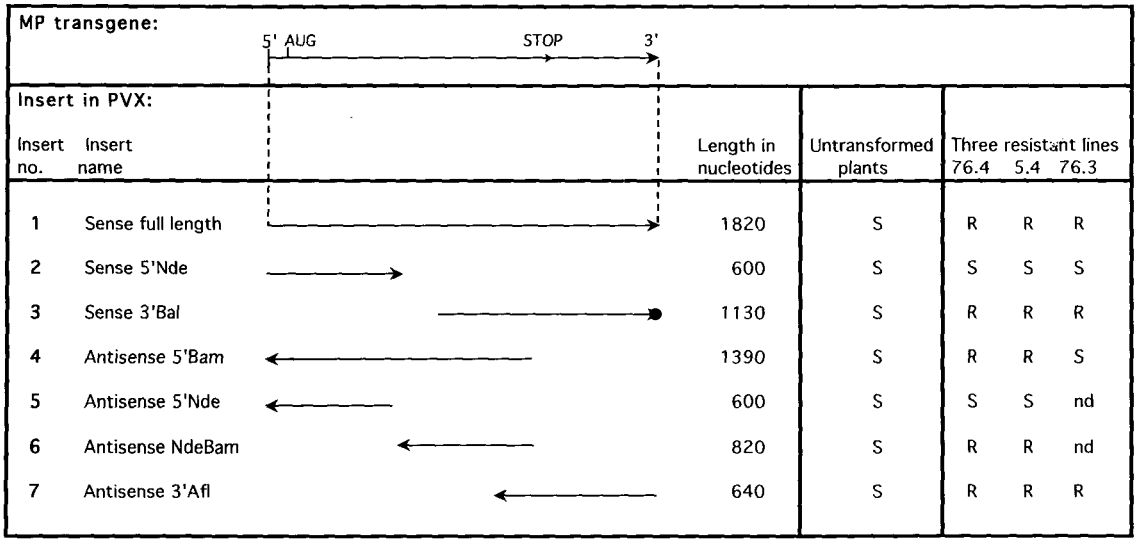


Figure 2. Recombinant PVX Genomes Tested as Targets for Resistance on Resistant Full-Length MP Gene–Transformed Plants.

In addition to the sequence homologous to the full-length MP transgene, sequences corresponding to smaller parts of the MP gene were inserted into the genome of PVX. The sequences were inserted in either the sense or the antisense orientation. The full-length MP transgene contains an open reading frame of 1380 nucleotides (from AUG to STOP codon) encoding the CPMV 48-kD MP, a 5′ leader sequence of 45 nucleotides, and a 3′ nontranslated region of 400 nucleotides. Untransformed *N. benthamiana* plants and three individual resistant MP-transformed lines (76.4, 5.4, and 76.3) were inoculated with infectious transcripts of the recombinant PVX genomes. For each line, at least six plants were tested. Sensitive plants (S) developed severe mosaic symptoms 6 to 8 DPI. Resistant plants (R) either remained free of symptoms or developed symptoms later than 12 DPI, which was shown in RT-PCR analyses to result from an infection with wild-type PVX, which occurred after recombination of the PVX derivatives. nd, not determined.

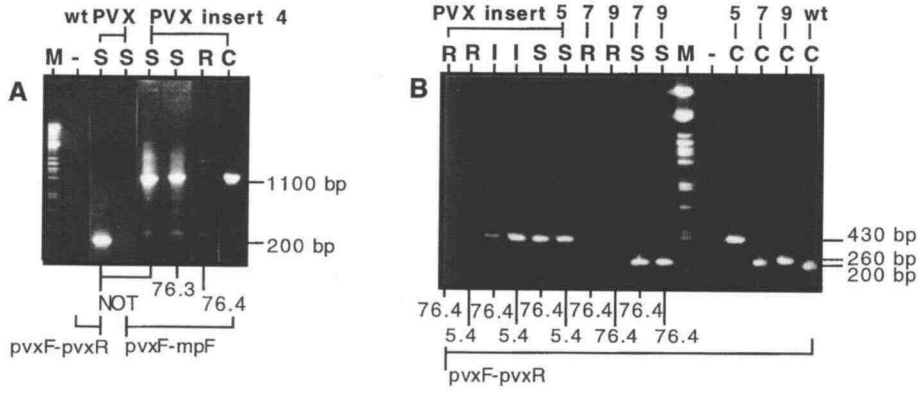


Figure 3. RT-PCR Analyses of Transgenic Plants Inoculated with in Vitro Transcripts of Recombinant PVX Genomes Carrying Sequences Homologous to Regions of the MP Transgene.

(A) RT-PCR analyses of total RNA isolated from plants inoculated with wild-type PVX or PVX antisense 5′Bam (PVX insert 4 as given in Figure 2, insert 4) at 9 DPI. Either untransformed *N. benthamiana* plants (NOT) or plants of MP-transformed lines 76.4 or 76.3 were used. Plants were denoted as either resistant (R) or sensitive (S). Water (−) was used as a negative control. Plasmid DNA (C) was used as a positive control. A combination of primers was used (pvxF–mpF) of which one is specific to sequences in the PVX genome upstream of the insertion site (pvxF) and the other is specific to sequences of the MP gene (mpF). This primer pair results in a fragment of 1100 bp for PVX insert 4 genomes; no fragment is amplified for wild-type PVX. Primer pair pvxF–pvxR is specific to sequences flanking the insertion site and results in a fragment of 200 bp for wild-type PVX genomes. The approximate lengths of the amplified fragments are indicated. A marker (M) is provided that indicates fragments of 11, 5, 2.8, 2.4, 2.1, 2.0, 1.7, 1.1, 1.0, 0.8, 0.5, 0.45, 0.3, 0.2, 0.15, and 0.1 kb.

(B) RT-PCR analyses of total RNA isolated from plants inoculated with wild-type PVX, PVX sense 3′Nae (PVX insert 5 as given in Figure 4, insert 5), PVX sense XhoBam (PVX insert 7 as given in Figure 4, insert 7), or PVX antisense XhoBam (PVX insert 9 as given in Figure 4, insert 9) at 13 DPI. Plants of MP-transformed lines 76.4 or 5.4 were used. Plants are denoted as resistant (R), intermediate resistant (I), or sensitive (S). The water control (−), plasmid DNA control (C), and a marker (M) are as given in **(A)**. Primer pair pvxF–pvxR was used and resulted in amplified fragments of 200 bp for wild-type (wt) PVX, 430 bp for PVX insert 5, and 260 bp for PVX insert 7 or 9. The approximate lengths of the amplified fragments are indicated.

RT-PCR analyses demonstrated that at 12 DPI, not only recombinant PVX molecules but also wild-type PVX molecules were present (results not shown). These molecules presumably resulted from the deletion of the inserted sequences as a result of homologous recombination among the duplicated CP promoters in the recombinant PVX RNA (Chapman et al., 1992). These experiments revealed that a heterologous RNA molecule can become victim of the silencing mechanism if it harbors sequences homologous to the transcribed region of a transgene that is post-transcriptionally silenced (Figure 2, insert 1).

In addition, we examined whether minus strands of PVX would be accessible to the resistance mechanism. Viral minus strands play an essential role in viral replication and are thought to occur only in viral replication complexes. To obtain PVX minus strands containing sequences representing the transcribed region of a sense CPMV MP transgene, we cloned sequences corresponding to the MP gene in the antisense orientation into the PVX vector. The designed RNA molecules are produced when this PVX derivative replicates. As shown in Figure 2, insert 4, the MP-transformed plants were also resistant to these recombinant viruses, and the resistance appeared similar to that of the PVX derivative carrying the sequence of the MP gene in the sense orientation. This suggests that for PVX, the genomic plus strands as well as the minus strands can serve as targets for a sense suppression mechanism existing in resistant lines, provided that they contain sequences representing the transcribed region of the transgene.

Surprisingly, in contrast to plants of resistant lines 76.4 and 5.4, plants of resistant line 76.3 were not resistant when inoculated with the PVX recombinant carrying sequences of the CPMV MP gene in an antisense orientation (Figure 2, insert 4). RT-PCR analyses (Figure 3A), using a combination of primers for which one was specific to PVX and the other to MP gene sequences, confirmed this different response for plants of lines 76.3 and 76.4. In this PVX recombinant, the inserted sequences represent a partial MP gene that is lacking the extreme 3′ 400 nucleotides (Figure 2, insert 4). We were prompted to examine whether in line 76.3 resistance would occur to a PVX derivative containing sequences corresponding to the 3′ terminal 640 nucleotides of the MP gene sequences present in the transgene. All three lines displayed resistance to this PVX derivative (Figure 2, insert 7). These results suggest that only RNA sequences corresponding to a limited region of the transgene can specifically direct the elimination of recombinant PVX genomes (Figure 2, inserts 4 and 7, plant line 76.3).

This hypothesis was analyzed in more detail with additional PVX recombinants containing sequences representing either only the 5′ or the 3′ part of the MP gene in the sense orientation. Plants of all three tested lines (5.4, 76.4, and 76.3) were sensitive to a PVX recombinant containing sequences homologous to the 5′ part of the MP gene but resistant to the PVX recombinant carrying sequences corresponding to the 3′ part (Figure 2, inserts 2 and 3). Thus, elimination seems restricted for RNA sequences corresponding to the 3′ part of the transcribed region of the transgene, indicating that recognition of the degradation process primarily occurs at the 3′ part of the transgene mRNA molecules. This 3′ part can be different for individual lines carrying the same transgene (Figure 2, insert 4, plant lines 76.3 and 5.4). The results with PVX derivatives containing sequences representing the 5′ or middle part of the transcribed region of the transgene in the antisense orientation further support this observation (Figure 2, inserts 5 and 6).

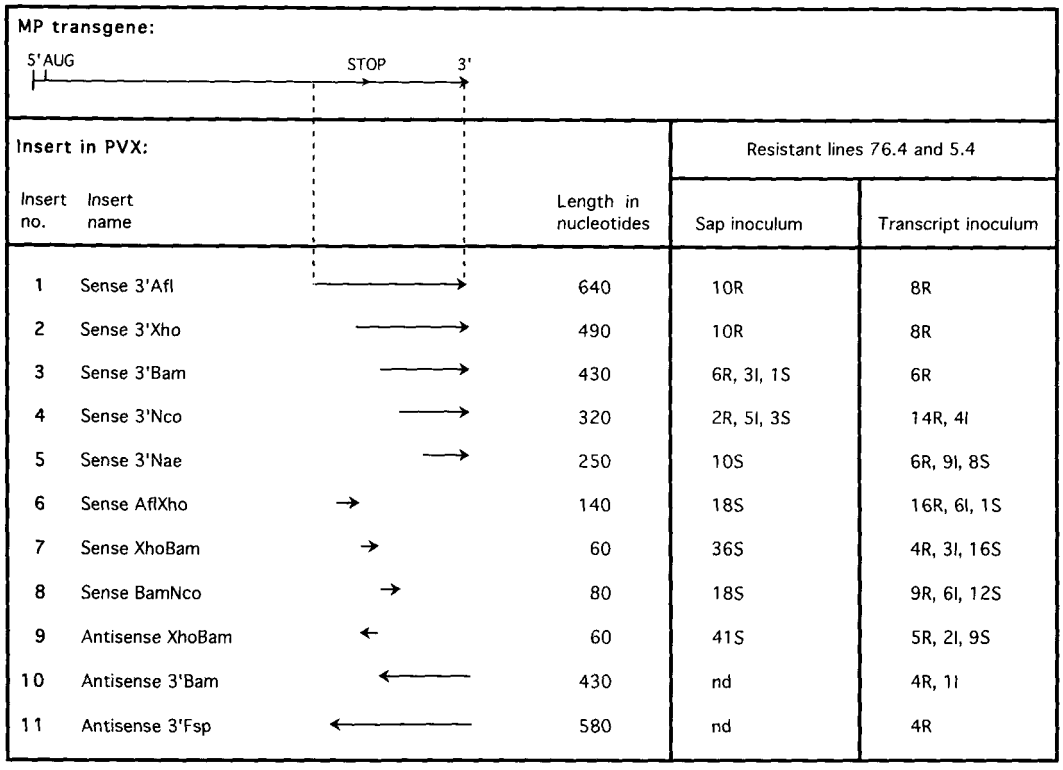
Fate of Heterologous RNAs Containing Small Insertions Homologous to the Transcribed Region of the Transgene

In line 76.3, the sequences of the transgene mRNA that are specific targets of the degradation process appeared to differ from the sequences specifically targeted in lines 5.4 and 76.4. For the transgene mRNA molecules in line 76.3, either of the sequences corresponding to a less extended 3′ part or specific sequences located more to the extreme 3′ end seemed to be the specific targets. To examine in more detail to which portion of the 3′ part of the transgene mRNA the degradation mechanism was specifically aimed, we inserted sequences corresponding to different portions of the 3′ part into recombinant PVX genomes and examined degradation of these PVX derivatives. The inserted region representing the 3′ part of the full-length MP transgene was divided into portions in two ways. The first approach involved a stepwise reduction in the size of the insert corresponding to the extreme 3′ part of the MP gene from 1130 to 230 nucleotides (Figure 4, inserts 1 to 5, 10, and 11). In the second approach, small fragments, ranging in size from 60 to 140 nucleotides, corresponding to small internal sequences within the 3′ part of the MP gene, were inserted in PVX and examined (Figure 4, inserts 6 to 9). All of these newly generated PVX derivatives were as infectious as wild-type PVX when inoculated onto untransformed *N. benthamiana* plants. In addition, the stability of these recombinant PVX RNAs strongly increased because of the reduction in length of the insert, and deletion of inserted sequences by homologous recombination was not detected in infected plants 2 weeks after inoculation. Consequently, for the PVX derivatives described in Figure 4, reliable sap inoculum could be obtained from transcript-inoculated nontransformed plants at 7 DPI. These plants contained only virions of the recombinant PVX and no PVX molecules that had lost the insert, as was confirmed by RT-PCR analyses.

The sap inoculum was used to test the plants for resistance to higher inoculum concentrations of the PVX recombinants. At these high inoculum doses, systemic mosaic symptoms emerged very rapidly (4 to 5 DPI), and thus, the observations using sap inoculum were done within a period in which PVX derivatives carrying these small insertions are stable. Because lines 76.4 and 5.4 responded similarly to the inoculated PVX derivatives, the responses of plants of these lines are recorded together in Figure 4. For line 76.3, no homozygous line was obtained; therefore, individual plants had to be examined for resistance at the protoplast level before inoculation with PVX derivatives. Consequently, not enough plants of line 76.3 were examined to obtain data sufficiently reliable to be included in Figure 4.

Remarkably, when inoculated with PVX derivatives containing inserts as listed in Figure 4, some of the plants of the homozygous lines 76.4 and 5.4 became rapidly infected, whereas other plants appeared resistant. A third type of response was also observed. Some plants showed a delay of several days in the appearance of symptoms and became systemically infected only 8 to 11 DPI, whereas all of the nontransgenic control plants showed systemic mosaic symptoms at 5 to 7 DPI. Such a delay in symptom appearance can be regarded as an intermediate resistance response (Figure 4). Plants carrying phenotypes denoted resistant or sensitive or of intermediate resistance were examined by RT-PCR analyses using a primer pair specific to sequences in the PVX genome flanking the integration site (pvxF and pvxR), as shown in Figure 3B for plants inoculated with transcripts of the PVX derivatives with inserts 5, 7, or 9 (Figure 4). As shown in Figure 3B, in the first, second, seventh, and eighth lanes, plants that were designated resistant were truly resistant to PVX recombinants because no amplified fragment was obtained. In plants denoted sensitive (Figure 3B, fifth, sixth, ninth, and tenth lanes) or of intermediate resistance (Figure 3B, third and fourth lanes), recombinant PVX genomes were replicating, and because for all plants only a fragment specific for the PVX recombinants was amplified, it is evident that the symptoms observed for the sensitive plants or the plants of intermediate resistance were the result of an infection with only recombinant PVX genomes. The frequency of sensitive plants and plants of intermediate resistance appeared to depend on both the length of the inserted sequence (Figure 4, transcript inoculations) and the inoculum concentration (Figure 4, transcript to sap inoculations).

The variability in responses of plants of one homozygous line suggests that a variability in recognition of the inoculated recombinant PVX RNA molecules occurs in infected plants or cells. The intermediate phenotype should probably be regarded as the result of a difference in the response of individual cells of one plant to the infection with a PVX derivative. The efficiency with which recombinant PVX RNAs are targeted by the resistance mechanism appeared to decrease when the size of the inserted region was reduced or when the inoculum dose was increased. However, the presence of only 60 nucleotides homologous to sequences within the 3′ part of the transcribed region of the full-length MP transgene seemed to be sufficient to tag a heterologous RNA, presumably PVX plus strands and



Insert in PVX: Insert no.	Insert name	Length in nucleotides	Resistant lines 76.4 and 5.4: Sap inoculum	Transcript inoculum
1	Sense 3'AfI	640	10R	8R
2	Sense 3'Xho	490	10R	8R
3	Sense 3'Bam	430	6R, 3I, 1S	6R
4	Sense 3'Nco	320	2R, 5I, 3S	14R, 4I
5	Sense 3'Nae	250	10S	6R, 9I, 8S
6	Sense AflXho	140	18S	16R, 6I, 1S
7	Sense XhoBam	60	36S	4R, 3I, 16S
8	Sense BamNco	80	18S	9R, 6I, 12S
9	Antisense XhoBam	60	41S	5R, 2I, 9S
10	Antisense 3'Bam	430	nd	4R, 1I
11	Antisense 3'Fsp	580	nd	4R

Figure 4. Recombinant PVX Genomes Containing Small Inserts Homologous to the 3′ Part of the MP Transgene Tested as Targets for Resistance on Resistant Full-Length MP Gene–Transformed Plants.

Two resistant full-length MP gene–transformed lines (76.4 and 5.4) were inoculated with various recombinant PVX genomes containing small portions of the 3′ part of the transcribed region of the MP transgene (see the legend to Figure 2) either in the sense or the antisense orientation. Untransformed *N. benthamiana* plants were inoculated as controls and indicated that all of the PVX derivatives were as infectious as wild-type PVX (results not shown). Because the two resistant lines responded similarly, the results are recorded together. Plants were inoculated either directly with transcripts or with sap obtained from a systemically infected nontransformed plant at 7 DPI with transcripts. When systemic mosaic symptoms appeared 5 to 7 DPI with transcript inoculum or 4 to 6 DPI with sap inoculum, plants were denoted sensitive (S). When no symptoms were observed, plants were called resistant (R). Plants showing a delay in symptom appearance, with symptoms occurring 8 to 11 DPI with transcript inoculum or 7 to 10 DPI with sap inoculum, were denoted as being of intermediate resistance (I). The values indicate the number of plants showing a specific response. nd, not determined.

PVX minus strands as well, for elimination by the silencing mechanism present in the resistant plants transformed with the full-length MP gene (Figure 4, inserts 7 and 9, transcript inoculations).

A Transgene Containing a Direct Repeat of CPMV MP Gene Sequences Induces Resistant Lines with High Frequency

Van Blokland et al. (1994) reported a correlation between post-transcriptional silencing and integrations of multiple T-DNA copies, preferably arranged in a complex locus containing an inverted repeat. To examine the T-DNA arrangement in our transgenic plants, we subjected two resistant and two sensitive transgenic lines carrying the full-length MP gene (Sijen et al., 1995) to DNA gel blot analyses (results not shown). Sensitive line 4.13 contained two transgenic loci, each with a single-copy T-DNA insertion. Sensitive line 3B contained one locus harboring two T-DNAs arranged in an inverted repeat. Resistant line 76.3 contained two transgenic loci, one having a single T-DNA insertion and one having two T-DNAs inserted as a direct repeat. Resistant line 76.4 contained four T-DNA copies at two loci. Two of the T-DNAs were inserted as a direct repeat, and another two were integrated as an inverted repeat. Although we tested only a limited number of lines, our results are consistent with the general finding that transgenic plants that carry gene-silencing processes often contain multiple copies of the transgene that are often arranged in complex loci.

To examine the possible significance of repeated transgenic

sequences more directly, we constructed transformation vectors in which CPMV MP gene sequences were cloned as repeated sequences within the expression cassette formed by an enhanced CaMV 35S promoter and a nopaline synthase 3′ termination signal. We used a CPMV MP gene with a 3′ deletion of 436 nucleotides (400 nucleotides of the 3′ nontranslated region and the 36 nucleotides encoding the 12 C-terminal amino acids) (MP48ΔB). This truncated MP48ΔB transgene conferred resistance to CPMV in ~20% of the transgenic lines (three of 14) (Table 2, MP48ΔB). This frequency is comparable to the 20% of resistant lines (four of 19) obtained when transforming plants with a defective version of the MP gene (Table 1, MP48ΔN). A construct carrying two inverted repeats of the MP gene (denoted IR transgene), situated within the expression cassette with the orientation sense followed by antisense, was made. Also, a construct containing two direct tandem repeats of an MP gene (denoted TR transgene) located within the expression cassette, both oriented in the sense direction, was constructed. DNA gel blot analyses revealed that the TR transgenes were transferred properly into the plant genome by Agrobacterium but that the IR transgenes had recombined in different ways during or after integration into the plant genome. However, at least a partially invertedly repeated MP gene was still left. In some of the plants, a portion of the antisense-oriented MP gene was deleted (IRΔ transgene). In other plants, the transgene occurred in an integration of three successive MP genes oriented sense, antisense, sense within the expression cassette. This can be regarded as an inverted plus inverted repeat (IIR transgene) (data not shown).

From the 20 transgenic plants that contained an IR transgene, either as an IRΔ or an IIR transgene, only one plant showed resistance to CPMV infection (Table 2, MP48IR). On the other hand, when plants containing a TR transgene were tested for resistance, resistance occurred in ~60% (19 of 31) of the lines, which is a strong increase compared with the frequency of 20% (3 of 14) at which resistant plants occur when transformed with a single CPMV MP transgene (Table 2, MP48ΔB and MP48TR). For all of the lines, cellular resistance was confirmed in protoplast experiments and found to be specifically directed against the accumulation of CPMV RNA2 (Table 2), which is indicative of an underlying post-transcriptional silencing mechanism. Strikingly, DNA gel blot analysis of IR-transformed plants revealed that the one resistant plant carried a transgenic locus consisting of two directly repeated T-DNA insertions, whereas none of the seven sensitive lines examined contained such a complex locus. Interestingly, two TR-transformed lines (TR6 and TR12) both carried two independently integrated, single T-DNA inserts, whereas another transgenic line (TR11) carried one single T-DNA insertion. Thus, a TR-transformed plant can show a resistant phenotype when a single copy of the TR transgene is integrated, as the plants were tested in the hemizygous state.

We assume that a transgene with a transcribed region of two identical directly repeated sequences can be regarded as carrying two copies in the genomic DNA, which is transcribed into one RNA molecule. This suggests that the mechanism underlying RNA-mediated resistance to CPMV does not follow a model based merely on exceeding a threshold level of transgene mRNA, because this does not explain the increased frequency of CPMV-resistant lines when plants are transformed with a TR transgene instead of a single MP transgene. Instead, this observation suggests that the arrangement of the integrated transgene sequences is important in generating RNA-mediated resistance to CPMV.

Table 2. Transgenic Plants with Repeated Sequences Tested for Resistance to CPMV

	Plant[b]		Protoplast[c]	
Transgene[a]	R_0	R_1	RNA1	RNA2
MP48ΔB	3/14	3/14	+	−
MP48IR	1/20	1/8	+	−
MP48TR	19/31	5/8	+	−

[a] Transgenes containing repeats of CPMV MP gene sequences were used for transformation of *N. benthamiana*. The single MP gene sequences encoded a truncated MP (MP48ΔB). Both an inverted repeat (MP48IR) and a direct tandem repeat (MP48TR) of these sequences were used.

[b,c] As given for footnotes b and c in Table 1.

Methylation at the Transcribed Region of the Transgene Strongly Correlates with Resistance

DNA gel blot analyses with genomic DNA of both resistant and sensitive full-length MP gene–transformed plants, that was digested with Sau3AI, BamHI, or XhoI, indicated that bands due to partial digestion of the DNA were specifically present in the resistant lines (results not shown). This partial digestion was only observed for methylation-sensitive restriction sites within the MP gene sequence, and it was mainly for sites situated in the middle and 3′ part of the transcribed region. This partial digestion could have been due to limited methylation at cytosine residues in the DNA sequences.

This correlation between resistance and methylation of transgene sequences was examined further by DNA gel blot analyses of the transgenic plants containing transgenes with repeated MP genes (IIR, IRΔ, and TR transgenes). In the one IIR and the four TR-transformed resistant lines that were analyzed, restriction analysis of the four HindIII sites in the sequence of the repeat-carrying transcribed region revealed extensive methylation (Figure 5A, for TR-transformed lines). The HindIII site flanking the expression cassette was barely methylated (digestion of the flanking EcoRI site is not sensitive to methylation at the C residue). In the five IR-, two IRΔ-, and three TR-transformed sensitive lines that were analyzed, no methylation or only very limited methylation of either the transcribed sequences or the sequences flanking the expres-

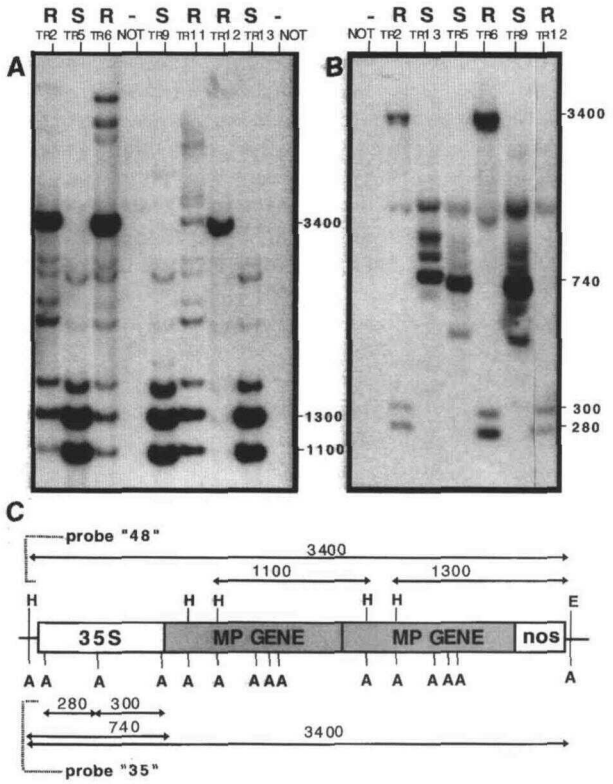


Figure 5. DNA Gel Blot Analysis of Genomic DNA of Plants Containing a Transgene of a Directly Repeated CPMV MP Gene.

(A) DNA gel blot of genomic DNA of the transgenic lines TR2, TR5, TR6, TR9, TR11, TR12, and TR13 carrying a direct tandem repeat of the CPMV MP gene within the transgene expression cassette and of nontransformed plants (NOT) digested with EcoRI and HindIII and probed with a fragment specific for the MP gene. Resistant (R) are lines TR2, TR6, TR11, and TR12. Sensitive (S) are lines TR5, TR9, and TR13. Untransformed plants do not contain a transgene (–).

(B) DNA gel blot of genomic DNA of a nontransformed plant (NOT) and the transgenic lines TR2, TR13, TR5, TR6, TR9, and TR12 digested with AluI and probed with a fragment specific for the the CaMV 35S promoter.

(C) Diagram showing the positions of the AluI (A), EcoRI (E), and HindIII (H) sites. Major fragments (approximate size indicated in bp) that resulted from either full or partial digestion hybridized with either a probe specific for the CaMV 35S promoter (probe "35"; **[B]**) or specific for the CPMV MP gene (probe "48"; **[A]**) and are indicated here and at right in **(A)** and **(B)**. nos, nopaline synthase.

sion cassette was observed (Figure 5A, for TR-transformed lines). Accordingly, in the DNA gel blot analyses of TR-transformed lines (Figure 5A) for the three sensitive lines that were analyzed, basically two bands of 1100 and 1300 bp (Figure 5C), representing full digestion of each MP gene, were observed. For all of the four resistant lines that were analyzed, basically one band of 3400 bp, which is characteristic of the entire expression cassette (Figure 5C), was detected.

Resistant and sensitive IR- and TR-transformed plants were also analyzed for methylation of sequences within the promoter region. Surprisingly, in this AluI restriction analysis, extensive methylation at the three AluI sites in the promoter region was found for only the three sensitive plants containing a TR transgene (Figure 5B). In the resistant TR-transformed plants, two bands of 280 and 300 bp, which are characteristic of fully digested promoter sequences (Figure 5C), were clearly detected, although some methylation of the promoter region was observed as a hybridizing fragment of ~3400 bp representing the entire expression cassette. This latter observation strengthens the finding that for resistant lines, specific and strong methylation at transcribed transgene sequences occurs, because full methylation was found for 10 AluI sites within the transcribed region. For the IR transgene–transformed lines, neither the resistant nor the sensitive lines displayed methylation at the promoter region (results not shown).

Methylation in the promoter region is known to be involved in establishing transcriptional silencing (Meyer, 1995). RNA gel blot analysis of poly(A)$^+$ RNA of sensitive TR-transformed plants in which specifically methylation in the promoter region was found (Figure 6, TR5 and TR9; for TR13, result not shown) confirmed that no transgene mRNA could be detected, implying that transcriptional silencing occurred. In the resistant TR-

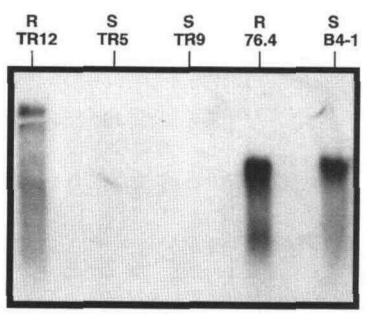


Figure 6. RNA Gel Blot Analysis of Both Transcriptionally and Post-Transcriptionally Silenced Lines Containing a TR Transgene.

Poly(A)$^+$ RNA was isolated from lines TR12 (TR transgene, resistant [R], extensively methylated specifically at transcribed transgene sequences), TR5 and TR9 (both TR transgenes, sensitive [S], extensively methylated specifically at promoter sequences), 76.4 (full-length MP transgene, resistant), and B4-1 (full-length MP transgene, sensitive). The blot was hybridized using an MP gene–specific probe. The size of a full-length MP transgene transcript is ~1750 nucleotides, and the size of a TR transgene transcript is ~2700 nucleotides. No MP gene–specific transcript was detected in sensitive lines TR5 and TR9. The amounts of MP-transformed mRNAs detected are not directly indicative of the relative steady state levels of MP-transformed mRNAs of each line, because variable quantities of poly(A)$^+$ RNA were isolated and analyzed for the lines. Approximately four times less poly(A)$^+$ RNA from line TR12 and ~10 times less poly (A)$^+$ RNA from lines TR5 and B4-1 than from lines TR9 and 76.4 were analyzed. This was determined when the blot was hybridized using a ubiquitin probe (results not shown).

transformed plants (Figure 6, TR12; for TR2 and TR6, results not shown), transgene mRNAs could be detected, albeit at low levels. The amounts of MP-transformed mRNAs that were detected, as shown in Figure 6, are not directly indicative of the relative steady state levels of the transgenic mRNAs of each line because variable quantities of poly(A)$^+$ RNA could be isolated and analyzed for the lines. Approximately four times less poly(A)$^+$ RNA from line TR12 and ~10 times less poly(A)$^+$ RNA from lines TR5 and B4-1 than from lines TR9 and 76.4 were analyzed, as determined by hybridization of the blot with a ubiquitin probe (results not shown), which detects the poly(A)$^+$ levels of the constitutively expressed plant ubiquitin gene. Taking into account the variable amounts of poly(A)$^+$ RNA that were analyzed, the blot (Figure 6) confirmed the relatively low steady state level of transgene mRNAs for the resistant full-length MP-transformed line 76.4 compared with the sensitive full-length MP-transformed line B4-1 (Figure 1A).

In summary, the striking observation is that in all of the plants analyzed containing a transgene carrying two directly repeated MP genes, methylation was detected. In all three sensitive plants, methylation was found specifically at all three of the sites examined in the promoter region. In all four resistant plants, methylation was detected specifically at all 10 of the sites examined in the transcribed region. Furthermore, in all of these plants, silencing of transgene expression had occurred. In sensitive plants, it occurred transcriptionally; in resistant plants, it occurred post-transcriptionally.

DISCUSSION

Resistance to CPMV and Post-Transcriptional Silencing

Resistance to CPMV has been observed in transgenic *N. benthamiana* plants expressing either a full-length CPMV replicase, MP, or CP gene or a defective version of the replicase or MP gene. Resistance is complete in the plant and is maintained cellularly. In the cell, resistance is found to be specifically directed toward the viral segment from which the transgene was derived. Specifically, in resistant MP-transformed plants, relatively low steady state transgene mRNA levels are found, whereas the nuclear transcription rates of the transgene are high. These characteristics imply that resistance to CPMV can be regarded as RNA-mediated virus resistance that is based on a post-transcriptional silencing mechanism (English et al., 1996), which is primarily directed at the transgene mRNA. In addition, the silencing mechanism can eliminate incoming, homologous viral RNA molecules. Hence, transcription of the transgene is essential for inducing virus resistance, and indeed, no resistance was observed in 24 lines transformed with a promoterless MP transgene.

This observation at first sight contrasts with the results of Van Blokland et al. (1994), who found that in transgenic petunia plants carrying chalcone synthase (*chs*) coding sequences without promoter sequences, cosuppression of the endogenous *chs* gene occurred. However, an essential difference between the two systems should be noted. In the case of cosuppression using the *chs* coding sequences, an endogenous *chs* gene is present in the nucleus, whereas in the case of RNA-mediated virus resistance, no endogenous homologous gene is present. A *chs* promoterless transgene might impose an effect on the endogenous gene that is under the control of an endogenous promoter. Thus, in the case of a promoterless transgene, post-transcriptional silencing could be induced upon transcription of the endogenous gene that has been affected, in a yet unidentified manner, by the promoterless transgene.

The resistant, full-length MP-transformed plants showed higher nuclear transcription rates of the transgene than did the sensitive lines, suggesting that the rate of transgene transcription could play a role in inducing post-transcriptional silencing. The observed difference between the transcription in sensitive and resistant plants can be partly attributed to the presence of more copies in the genome of the resistant lines (lines 76.3 and 76.4 contain three or four copies, respectively) than in the sensitive lines (lines 3B and 4.13 both contain two copies). Previously, a role for the level of RNAs in inducing resistance has been assumed because a recovery phenotype was found among transgenic plants displaying RNA-mediated resistance to potyviruses (Lindbo et al., 1993a; Dougherty et al., 1994; Smith et al., 1994; Swaney et al., 1995). This recovery phenotype suggested that viral RNA molecules contributed to raising the level of RNAs above a threshold required for establishing resistance. On the other hand, Van Blokland et al. (1994), Mueller et al. (1995), and English et al. (1996) did not find a significant correlation between the nuclear transcription rates of transgenes and the establishment of post-transcriptional gene silencing.

The Silencing Mechanism Is Directed toward a Defined Region of the Transgene mRNA and Can Act with Variable Efficiency

By using the PVX vector system (Chapman et al., 1992), we have shown that heterologous RNA molecules are the targets of the resistance mechanism in full-length MP-transformed plants if they contain sequences homologous to the MP gene sequences of a post-transcriptionally silenced CPMV MP transgene. Furthermore, in all three of the lines examined, the silencing mechanism was found to be specifically targeted to the 3′ region of the MP transgene mRNA. These results agree with the findings of English et al. (1996), who have shown that in three different types of transgenic plants in which transgenes (carrying either *GUS*, polygalacturonase, or neomycin phosphotransferase coding sequences) are post-transcriptionally silenced, the accumulation of PVX molecules that harbor insertions homologous to these transgene coding regions is inhibited. The silencing of the *GUS* transgene was also found to be targeted specifically to the 3′ region of the transgene mRNA. In our system, the extent of the 3′ region that is the target of the resistance mechanism was found to differ for in-

dividual transgenic lines, because one of the three examined lines showed no resistance to recombinant PVX genomes containing sequences representing the transcribed region of the transgene lacking the 3′ terminal 400 nucleotides. Furthermore, we showed that a small insert of only 60 nucleotides, corresponding to a sequence within the 3′ region of the transcribed region of the transgene, was sufficient to tag a recombinant PVX molecule for the elimination process.

PVX derivatives containing small inserts of 140, 60, 80, or 320 nucleotides corresponding to successive, nonoverlapping sequences within the 3′ terminal 600 nucleotides of the MP gene are all prone to elimination by the resistance mechanism present in full-length MP-transformed plants. This result suggests that recognition of the elimination process can happen at various sites within the 3′ region of the transgene mRNA and that a specific sequence or structure within the 3′ region of the transgene mRNA is not of predominant importance in the initiation of degradation. This interpretation goes against the suggestion of Goodwin et al. (1996), who proposed that selected sequences or structures of the transgene mRNA are sites of initial cleavages. By using RNA gel blot and primer extension analyses, they detected smaller internal fragments of transgene mRNA specifically in resistant lines. These smaller fragments might, however, reflect specific products of further degradation rather than specific sites of initial cleavage.

Recombinant PVX RNAs containing MP gene sequences in the antisense orientation were also found to be eliminated in resistant full-length MP plants. Strikingly, no essential differences were observed in the fate of recombinant PVX genomes carrying sequences homologous to various parts of the transcribed region of the transgene with either a sense or antisense orientation. This indicates that the same mechanism underlies resistance to PVX derivatives containing MP gene sequences in the sense and antisense orientations. This suggests that viral minus strands, even though they are presumably located in viral replication complexes, are accessible to the suppression mechanism. Otherwise, this observation could suggest that the elimination mechanism recognizes sequences corresponding to either polarity of the transgene mRNA in the PVX plus strand. In either case, the result invites the suggestion that the RNA-mediated resistance conferred by antisense expression of viral genes, such as the tobacco etch virus CP gene (Lindbo and Dougherty, 1992; Lindbo et al., 1993b), could be regarded as sense suppression of viral minus strands rather than as antisense inhibition of the viral genomes. Antisense inhibition has been proposed to result from the direct interaction of the transgene antisense transcripts with the viral RNA molecules.

Remarkably, we observed that individual plants of the same homozygous line responded differently to inoculation of recombinant PVX genomes containing small inserts corresponding to the 3′ region of the MP gene. Some of the plants were completely resistant, some plants were fully sensitive, and other plants displayed a phenotype of intermediate resistance. In these cases, when inoculated with higher inoculum concentrations, more plants were found to be sensitive, suggesting dose-dependent resistance. These results imply that the resistance mechanism is variable in its ability to eliminate RNA molecules efficiently. Whether all RNA molecules are eliminated, resulting in full resistance, depends on both the length of the target region present in the RNA molecule and the concentration of RNA molecules in the inoculum. Thus, the resistance mechanism holds quantitative aspects. A positive correlation between the level of resistance and the length of the insert, corresponding to sequences of the transcribed region of the transgene that are present in a heterologous RNA molecule, was also reported by Hellwald and Palukaitis (1995). Resistance to cucumber mosaic virus was studied using chimeric constructs of two cucumber mosaic virus strains, which can be regarded as a homologous and a heterologous virus (similar to CPMV and PVX in our studies) because resistance only holds against the strain from which the transgene was derived.

Direct Repeats of the CPMV MP Gene Induce Resistance at High Frequency and Show Strong Methylation

DNA gel blot analyses of several resistant and sensitive full-length MP-transformed plants indicated that the resistant lines contained more transgene copies than did the sensitive lines. The multiple transgene copies are partly present in complex loci. When *N. benthamiana* plants were transformed with a direct repeat of the CPMV MP gene that was placed within the expression cassette under the control of the same promoter as the single full-length MP transgene, we found a frequency of 60% of resistant lines. This frequency is considerably higher than the frequency of 20% of resistant lines that was found when plants were transformed with a single MP transgene. For one of the resistant TR lines, we showed that one single T-DNA insertion was integrated. The primary transformant of this line was inoculated with CPMV and found to be completely resistant. The hemizygous plant carried two copies of the CPMV 48-kD sequences in the genomic DNA, but these two copies were transcribed into one RNA molecule. These observations suggest that a direct tandem arrangement of CPMV MP gene sequences is important for inducing the resistance mechanism. Thus, it appears that establishing RNA-mediated resistance can be influenced by qualitative traits of the transgene mRNA rather than only by quantitative aspects.

Remarkably, plants containing an inverted MP gene repeat (IRΔ transgene or IIR transgene) showed resistance at a lower frequency than did plants carrying a single MP gene. This is in contrast to the TR-transformed plants that showed resistance at a higher frequency than did plants containing a single MP gene. This strengthens our suggestion that specific qualitative aspects have a role in inducing the resistance mechanism, because both TR and IR transgenes resulted in two copies of the MP gene in the genomic DNA that, when transcribed, are present on one mRNA molecule. In addition, this finding suggests that mRNAs containing inverted repeat sequences

are less able to generate RNA-mediated resistance. This could be because they are (partially) double-stranded RNAs that could be prone to degradation by cellular double-stranded RNases and deliver high instability to the IR transgene transcripts.

Resistant plants containing a CPMV MP gene displayed specific methylation at several sites in the transcribed region of the transgene. This methylation was not detected in sensitive full-length MP-transformed plants. It is known that multiple copies, integrated either as concatamers or at ectopic positions, are preferred targets for methylation (Meyer, 1995). In resistant full-length MP-transformed plants, more copies of the transgene were present than in sensitive lines. Methylation of the restriction sites that were examined in these plants was found to be limited to some integrated copies or to some cells, because the inhibition of cleavage by the restriction enzymes was not complete. In resistant TR MP gene–transformed plants that all carry at least twice the MP gene in the genome, however, the restriction sites that were examined in the transcribed transgene sequences were found to be methylated in all integrated copies of all cells in the plants. It would be interesting to confirm the specific methylation for resistant TR-transformed lines by studying methylation at several more sites within the transgene sequence for the lines studied in this article and by adding more TR-transformed lines in these analyses. Methylation of the C residue in the HindIII and AluI sites is not at a CG or CXG sequence, representing the symmetrical target sequences for maintenance DNA methylation in plants, and can thus be regarded as de novo methylation.

Recently, Hohn et al. (1996) have clearly shown that methylation at the coding region alone can lead to a reduction of reporter gene expression in transiently transformed plant protoplasts. For various cases of post-transcriptional silencing of transgene mRNAs, a correlation with methylation of transcribed transgene sequences has been reported previously (Hobbs et al., 1990; Ingelbrecht et al., 1994; Smith et al., 1994). English and co-workers (1996) have even connected the region of the transcribed transgene sequences that is methylated to the region of the transgene mRNA that is the specific target of the RNA elimination process. Several groups have not detected a correlation between post-transcriptional gene inactivation and transgene methylation (Hart et al., 1992; Van Blokland et al., 1994; Judelson and Whittaker, 1995; Goodwin et al., 1996). However, in these cases, either methylation could have occurred only in a limited number of the integrated copies of the transgene (as suggested above for the full-length MP transgenes) or it was restricted to a limited region of the transgene (English et al., 1996). We have tried to determine whether methylation at transcribed sequences of the transgene has a direct role in establishing RNA-mediated resistance. We germinated and cultivated seedlings of resistant TR lines on 5-azacytidine (Weber et al., 1990; Bochardt et al., 1992; Palmgren et al., 1993). However, at concentrations of 5-azacytidine (20 to 60 μM), at which plants just survived, only very limited demethylation had occurred (DNA gel blot analyses not shown) and the plants remained resistant.

In conclusion, the striking observation from our study is that in all TR MP–transformed plants examined, methylation at introduced sequences correlates with silencing of transgene expression. In all of the sensitive lines tested, only the promoter sequences were methylated, resulting in transcriptional silencing. In the resistant lines examined, methylation specifically at the transcribed transgene sequences occurred together with post-transcriptional silencing. This striking correlation between methylation and silencing could imply that de novo methylation occurs to repress expression of repeated transgene sequences. If promoter regions are affected by triggered de novo methylation, transcriptional silencing is obtained (Matzke et al., 1989) and the plants remain sensitive. Plants carrying transcriptional silencing are analogous to the transgenic plants containing a promoterless MP transgene, of which we found none to be resistant. If sequences in the transcribed region of the transgene are affected by methylation, post-transcriptional silencing occurs (English et al., 1996), and the plants show resistance when inoculated with a homologous virus. Remarkably, we have not come across lines in which both the promoter and the transgene sequences were extensively methylated, suggesting that methylation of either the promoter or the transcribed transgene sequences is sufficient to silence expression of a directly repeated CPMV MP gene.

A Refined Model for RNA-Mediated Virus Resistance

A model of RNA-mediated virus resistance (Figure 7) should explain all features of the process. The most striking characteristic of the resistance mechanism is the high specificity of recognition of sequences that are targets of the degradation process. The viral RNA molecules are degraded in the cytoplasm; however, for initiating the resistance process, active transcription of the introduced transgene is required. The resistance mechanism is specifically directed toward sequences corresponding to a defined region of the transcribed sequences of the transgene. Integration of transgenes carrying a transcribed sequence of direct repeats of CPMV MP gene sequences can stimulate the induction of resistance, and the appearance of resistance is accompanied by extensive methylation of sequences of the transcribed region of the transgene.

The most widely held assumption is that complementary RNA (cRNA) molecules are involved in accomplishing the specificity of the RNA degradation (Dougherty and Parks, 1995; Meyer, 1995; English et al., 1996). The cRNA molecules could arise as antisense RNA molecules formed in the nucleus upon transcription from promoter sequences near the genomic integration site. Alternatively, the cRNA molecules might be produced in the cytoplasm by the action of a plant-encoded RdRp (Lindbo et al., 1993a), which has been shown to exist in plants (reviewed in Fraenkel-Conrad, 1986) and to be capable of unprimed transcription at the 3′ terminal nucleotides of single-stranded RNA templates (Schiebel et al., 1993). We favor the hypothesis involving a role of a cellular plant RdRp over the nuclear production of antisense RNAs because in the

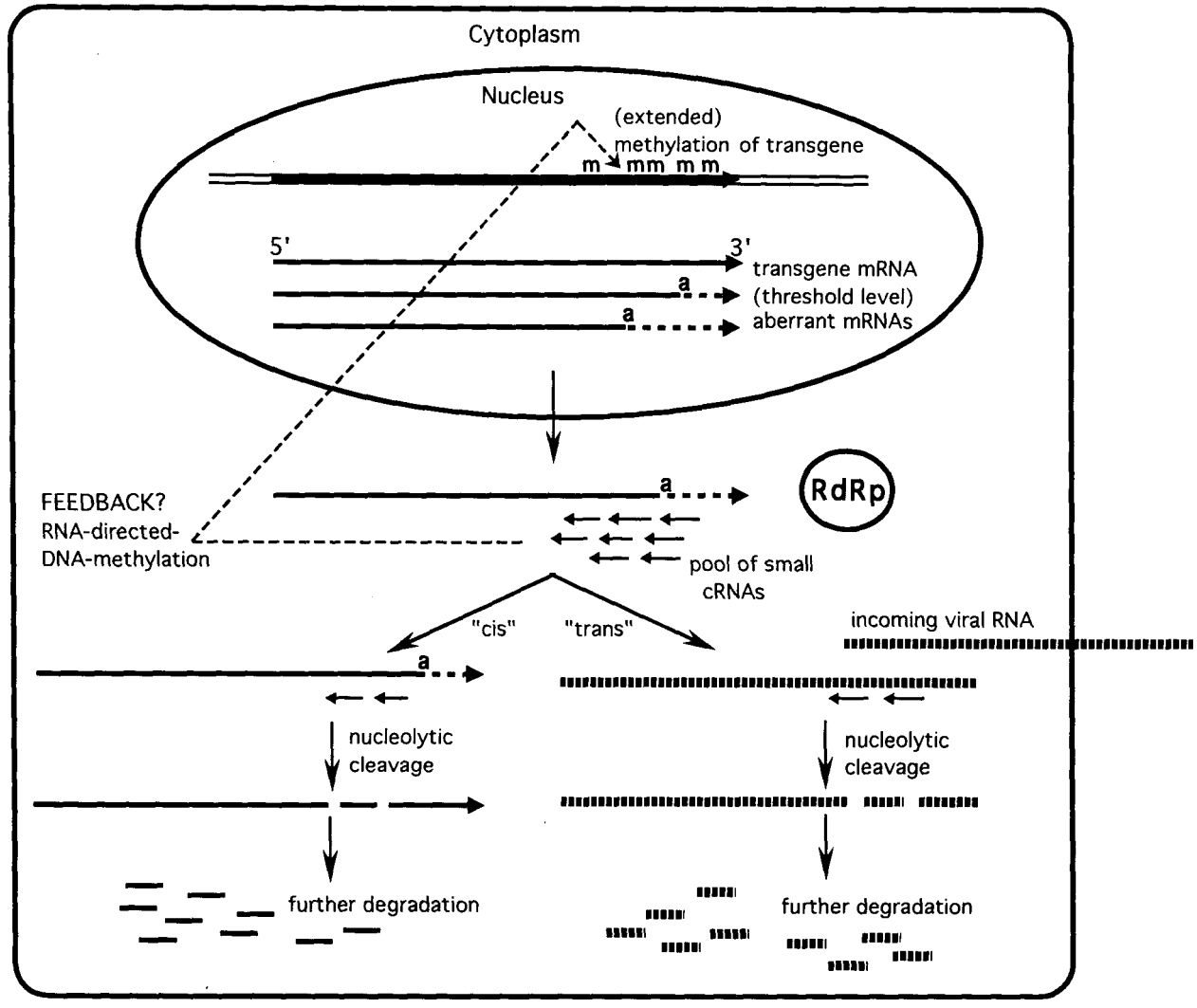


Figure 7. Model of RNA-Mediated Virus Resistance.

This refined model (adapted from Dougherty and Parks, 1995) of RNA-mediated virus resistance could also account for cases of post-transcriptional silencing of transgenes or endogenous genes. We propose that in the nucleus, aberrant (a), abnormal, or tagged RNA molecules are formed, possibly because of (limited) methylation (m) of the transgene sequence. Methylation could be due to integration of transgenes or T-DNAs in a special arrangement, for instance, as a direct repeat as described in this study. The form of an aberrant RNA remains to be determined. The presence of an amount of aberrant RNAs above a certain threshold level could have a role in the efficiency of the process that follows. The aberrant RNAs can serve in the cytoplasm as a template for a plant-encoded RdRp, which produces a pool of cRNA molecules. Per cell, this pool can vary in composition, availability, or efficiency of interaction with the target RNA molecule. The cRNA molecules can form duplex structures either in *cis* on the template mRNAs or in *trans* on homologous mRNAs or incoming viral RNAs. Such a duplex structure could induce a nucleolytic cleavage that would render RNA molecules accessible for further degradation by exonucleases. Possibly the cRNA molecules have an additional feedback effect in the nucleus, like a previously described process of RNA-directed DNA methylation (Wassenegger et al., 1994), that could lead to an extended methylation of the transgene sequence.

nuclear run-on experiments presented in this study, the transcription of antisense transgene RNAs is hardly above background and thus these RNAs do not outnumber the sense transgene RNAs. The cRNAs can either act in *cis* on their template RNAs or in *trans* on nontemplate transgene mRNAs or incoming viral RNAs. Subsequently, duplex structures might be formed by the binding of the cRNAs to target RNAs, which could effect nucleolytic cleavage by a double-stranded RNase with additional degradation by cytoplasmic exonucleases (Dougherty and Parks, 1995).

In this study, we showed that plants of one homozygous line can respond in a different way to inoculation with recombinant

PVX genomes containing inserts corresponding to various parts of the transcribed sequences of a MP transgene. Therefore, we propose that the RdRp produces a pool of small cRNA molecules (Figure 7). For individual cells, this pool can vary in composition, availability, or efficieny of interaction with the target RNA molecules. If the target region in a heterologous RNA molecule is made larger or the inoculum dose is increased, the chance increases that all of the RNA molecules interact with the cRNA molecules and are eliminated, resulting in full resistance. Thus, the resistance mechanism definitely acts with quantitative features. Because the cRNA molecules have to act in *trans* on viral RNA molecules, we concur with Dougherty and Parks (1995) in their assumption that the cRNA molecules are rather small in size, although large enough to be sequence specific. In *Caenorhabditis elegans*, a role for small cRNAs (21 or 61 nucleotides) in downregulating translation has been described (Lee et al., 1993), thus illustrating that small cRNA molecules can have specific regulatory effects.

In inducing the specific RNA turnover (Figure 7), both quantitative (Lindbo et al., 1993a; Smith et al., 1994; Jorgensen, 1995) and qualitative (Meyer, 1995; English et al., 1996) aspects of transgene mRNAs have been suggested to play a role, because both high levels of transcription of the transgene (Lindbo et al., 1993a; Smith et al., 1994) and methylation at transcribed transgene sequences (Smith et al., 1994; English et al., 1996) were found to correlate with resistance. These aspects are not mutually exclusive, and in our study, resistant full-length MP-transformed plants showed both specific methylation at sequences in the transcribed region of the transgene and high transgene transcription rates. Also, our experiments using the various PVX derivates indicated quantitative aspects of the resistance mechanism. However, the increased frequency of resistant lines that was observed when a transgene carrying directly repeated MP gene sequences at the transcribed region is integrated into a plant genome strongly suggests that qualitative traits of the transgene mRNAs can also play a role. Therefore, this observation seems to argue against a simple threshold model in our system.

Nevertheless, it would be interesting to study whether use of a double enhanced CaMV 35S promoter (Elmayan and Vaucheret, 1996) to control expression of a CPMV MP TR transgene further stimulates the frequency of generating resistant lines. Likewise, it would be of interest to determine whether duplication of the number of direct repeats of the CPMV MP gene, expressed from a single enhanced CaMV 35S promoter, increases the frequency of resistant lines above the 60% reported in this study. In addition, it would be interesting to develop experiments in which methylation is either stimulated or prevented, thereby permitting us to study whether methylation can indeed have a causative role in post-transcriptional silencing. In conclusion, both quantitative and qualitative aspects of the transgene mRNAs might define these molecules as preferred templates for the RdRp and effect the synthesis of cRNA molecules (Dougherty and Parks, 1995), resulting in a post-transcriptional correction of the expression of the foreign transgenes. The proposed pool of small cRNAs can be produced either as a result from a divergent pool of template RNAs or because of an intrinsic character of the RdRp.

The increased frequency of resistant lines when the CPMV MP gene was integrated as a direct repeat within an expression cassette suggests that establishing resistance is influenced by the arrangement of the transgenes in the genome. Furthermore, all sensitive and resistant TR-transformed lines showed both extensive methylation of transgene sequences and silencing of transgene expression. This observation might suggest that methylation of the transgene sequences is a cause rather than a consequence of resistance (Figure 7), as was suggested previously by English et al. (1996), who showed that for a silenced *GUS* transgene, the region in the transgene corresponding to the target sequence for the silencing mechanism is the same region of the transgene at which methylation occurred.

In mouse cells, methylation of genomic DNA has been shown to alter an active chromatin structure (Keshet et al., 1986; Kass et al., 1993) into a type of structure that resulted in transcriptional inactivation (Keshet et al., 1985). Dorer and Henikoff (1994) showed for Drosophila that the integration of transgene repeats could induce heterochromatin formation, resulting in gene silencing. In the plant genome, methylation of transgene sequences might also affect chromatin structure, as previously suggested by Ingelbrecht et al. (1994). Ten Lohuis et al. (1995) showed that in petunia, repetitive DNA that carries a hot spot for methylation induces variegated expression of adjacent sequences, which was suggested to be due to heterochromatin-mediated gene inactivation. Genes in inactive or heterochromatin domains are not considered to be accessible to the regulation, either activation or repression, that is afforded by DNA elements in promoters (Lewin, 1994; Rivier and Pillus, 1994).

However, in the process of RNA-mediated virus resistance, transcription is not inhibited, as was demonstrated in our experiments and earlier by Lindbo et al. (1993a), Mueller et al. (1995), and English et al. (1996), and the resistance process acts post-transcriptionally. Therefore, the question is how a methylated DNA sequence in the transcribed region of a gene can render an RNA molecule tagged in such a way that it is recognized as a molecule to be eliminated or, in our model, as a template for the RdRp. If methylation can induce an alteration of chromatin structure, a similar alteration may be achieved independent of whether the DNA has a function as a coding or a promoter region. Furthermore, several proteins are known to bind specifically to methylated DNA (Zhang et al., 1989; Lewis et al., 1992). It is conceivable that both an altered chromatin structure and the binding of methylated DNA binding proteins could affect typical transcription by RNA polymerase II. Barry et al. (1993) have shown that methylation in *Ascobolus immersus* results in a low level of smaller transcripts as a result of premature transcription termination and low stability of the transcripts. Lang et al. (1994) have proposed that in yeast, a relatively nonspecific signal can cause RNA poly-

merases to pause. In this system, the pausing is not sufficient for the termination of transcription, although pausing is a prerequisite in the process of transcript release. Possibly, alteration of the chromatin structure or specific binding of methylated DNA binding proteins impedes RNA polymerase II, which could tag the produced mRNA molecule, either by inducing a specific sequence or structure on the mRNA molecule or by triggering another process that modifies the mRNA molecule. Possibly, features other than methylation of transcribed transgene sequences could result in the formation of truncated, modified, or aberrantly structured transgene mRNA molecules, as was shown in several cases of post-transcriptional gene silencing in which no correlation with methylation has been reported (Hart et al., 1992; Van Blokland et al., 1994; Judelson and Whittaker, 1995; Goodwin et al., 1996).

In the model presented in Figure 7, a role of a possible feedback mechanism from cytoplasm to nucleus is included (Dougherty and Parks, 1995; English et al., 1996). Wassenegger et al. (1994) have shown that upon RNA–RNA replication of viroid genomes, occurring when introduced transgene sequences are expressed, the corresponding DNA sequences become methylated (RNA-directed DNA methylation). In our model, the cRNA molecules might have such a feedback effect on de novo methylation of transgene DNA in addition to their role in RNA degradation. RNA-directed DNA methylation could amplify and/or extend an initially limited methylation, thereby reinforcing the resistance mechanism.

Our model also applies to cases of transgene-induced post-transcriptional silencing of endogenous genes (cosuppression). In these cases, mRNAs transcribed from a homologous endogenous copy of the transgene are eliminated in *trans* but not incoming viral RNA molecules. Although much about the mechanism of post-transcriptional gene silencing needs to be elucidated, deliberate integration of repeated transgenes might provide a general strategy for efficiently downregulating the expression of endogenous genes or for engineering virus resistance.

METHODS

Construction of Transgenes and Transgenic Plants

Plasmid pBINM48ΔNde, containing a defective version of the cowpea mosaic virus movement protein (CPMV MP) gene, was constructed by NdeI digestion of pBINM48 (Sijen et al., 1995), followed by blunt-end formation when treated with the Klenow fragment of DNA polymerase I and religation. The binary plasmid pBINB200ΔXba, containing a defective version of the CPMV replicase gene, was constructed similarly by XbaI digestion of pBINB200 (Sijen et al., 1995). Plasmid pBINM48ΔB, encoding a truncated CPMV MP, was obtained when a BamHI fragment of 450 bp was deleted. This 450-bp fragment contains the 36 nucleotides encoding the last C-terminal 12 amino acids of the MP gene and 400 nucleotides of the 3′ nontranslated region from plasmid pBINM48 (Sijen et al., 1995). The plasmids pBINM48IR and pBINM48TR, containing within the expression cassette inverted or direct repeats of the truncated CPMV MP gene, respectively, were made when a 1.1-kb BglII-BamHI fragment was ligated with BamHI-digested pBINM48ΔB in either the antisense or the sense orientation.

Plasmid pBINM48PL, containing a promoterless MP gene, was obtained, and a BglII-SstI fragment of 1.5 kb containing the full-length MP gene was ligated into BamHI-SstI–digested binary vector pBIN19. pBINM23 encodes the small viral coat protein (CP), and it was made by replacing the HindIII-BamHI fragment containing the enhanced cauliflower mosaic virus (CaMV) 35S promoter and CPMV replicase gene of plasmid pBINB200 with a 1.2-kb HindIII-BamHI fragment from pMMVP23 (Wellink et al., 1996) containing an enhanced 35S promoter and the small CP gene. pMEXM37, containing the large viral CP gene, was made by ligating a 1.1-kb BglII-XbaI fragment from pMMVP37 (Wellink et al., 1996), containing the large CP gene, with BamHI-XbaI–digested pMEX001, a binary vector carrying on the T-DNA region a methotrexate resistance gene and a multiple cloning site that is flanked by a nonenhanced 35S promoter and a 35S 3′ termination signal.

The pBIN19-derived binary plasmids were transferred to *Agrobacterium tumefaciens* LBA4404 with the aid of *Escherichia coli* RK2013. The pMEX001-derived plasmids were electroporated into competent cells of Agrobacterium GV3101.

Transformation of explants of in vitro–cultivated *Nicotiana benthamiana* plantlets was performed as described by Sijen et al. (1995). pBIN19-transformed shoots were selected on 150 mg L^{-1} kanamycin, and pMEX001-transformed shoots were identified by selection on 0.05 mg L^{-1} methotrexate. Double transformants were selected on medium containing both 150 mg L^{-1} kanamycin and 0.05 mg L^{-1} methotrexate.

Regenerated shoots were cultivated on medium without cefotaxime for ~2 weeks to ensure that no agrobacteria were present. DNA from one leaf was isolated as described by Sijen et al. (1995), and polymerase chain reaction (PCR) analysis was performed to identify transgenic shoots. Positively identified plantlets were transferred to the greenhouse and placed into soil.

Inoculation of Transgenic Plants and Testing of Resistance to CPMV

Within 1 week after the transfer of primary transformants to the greenhouse, the plants were inoculated with the sap of CPMV-infected tissue. If no symptoms occurred 10 days postinoculation (DPI), protoplasts were prepared from the transgenic plants, as described by Sijen et al. (1995). The protoplasts were infected with 2 μg of CPMV RNA, and ~18 hr after infection, immunofluorescence assays were performed (Sijen et al., 1995). Two different antisera were used to allow separate monitoring of the replication and subsequent expression of RNA1- and RNA2-specific proteins.

The primary transformants were selfed to obtain R_1 generation plants. In cases in which R_1 lines were tested for resistance, 20 individual plants were analyzed for the occurrence of symptoms after inoculation with sap of CPMV-infected tissue.

Construction of Recombinant Potato Virus X Genomes and Testing on Plants

Potato virus X (PVX) mutants were cloned into the PVX expression vector pPC2S, a derivative of pGC3 (Chapman et al., 1992) in which the

β-glucuronidase (*GUS*) coding sequence was replaced by unique restriction sites to allow convenient cloning. The vector was generously provided by D. Baulcombe (Sainsbury Laboratory, Norwich, UK). The CPMV MP gene was cloned in the sense orientation into the ClaI-EcoRV–digested PVX vector as a 1.8-kb ClaI-SstI fragment (the SstI overhang was blunted with the Klenow fragment) derived from pMM58/48 (Wellink et al., 1993), resulting in pPVXS58. The 5′ region of 600 bp of the CPMV MP gene was cloned in the sense orientation as a ClaI-NdeI fragment (the NdeI overhang was blunted with the Klenow fragment) into the ClaI-EcoRV–digested PVX vector, resulting in plasmid pPVXS58Nde5′. A truncated CPMV MP gene was cloned in the antisense orientation into the ClaI-EcoRV–digested PVX vector as an 1.1-kb ClaI-BglII fragment (the ClaI site was obtained when the BamHI site was filled in and the BglII overhang was blunted with the Klenow fragment), resulting in plasmid pPVXAS48Bam5′.

To clone the extreme 3′ region of 640 bp in the antisense orientation into the PVX vector, we ligated an AccI-AflII fragment (the AflII overhang was blunted with the Klenow fragment) from pPVXS58 into the ClaI-EcoRV–digested PVX vector (the AccI overhang of the insert is compatible with the ClaI overhang of the vector), resulting in plasmid pPVXAS48Afl3′. Cloning of plasmids pPVXAS48Fsp3′ and pPVXAS48Bam3′ was similar to the cloning of pPVXAS48Afl3′. For pVXAS48FSp3′ and pPVXAS48Bam3′, a FspI or blunted BamHI overhang instead of a blunted AflII overhang was used. The constructs containing smaller CPMV inserts were made from plasmid pPVXS58 or pPVXAS48Bam5′ (or the subsequent derivatives of these plasmids) by the exchange of fragments containing CPMV and PVX sequences with fragments containing fewer CPMV and the same PVX sequences. In the PVX vector–derived sequences, the unique ClaI, ApaI, or SstI site was used. In the CPMV derived- sequences, the NdeI, BalI, AflII, XhoI, BamHI, NcoI, or NaeI site was used. The plasmids that were generated in this way were pPVXS58Bal3′, pPVXS58Afl3′, pPVXS-58Xho3′, pPVXS58Bam3′, pPVXS58Nco3′, pPVXS58Nae3′, pPVXS-58AflXho, pPVXS58XhoBam, pPVXS58BamNco, pPVXAS48Nde5′, pPVXAS48NdeBam, and pPVXAS48XhoBam.

Infectious transcripts were produced from these clones, as described by Chapman et al. (1992), with minor modifications. The DNA was isolated by Qiagen Quick Spin Columns (Westburg, Leusden, The Netherlands), and linearization with SpeI was performed during the transcription reaction. No purification steps were performed, and before plant inoculations, bentonite and sodium phosphate, pH 7.2, were added to concentrations of 5 μg μL^{-1} and 40 mM, respectively, to the transcription reaction mixture. Plants were inoculated with ~5 μg of infectious transcripts distributed over three leaves.

When plants were infected with PVX transcripts, systemic mosaic symptoms occurred ~5 to 7 DPI. After inoculation with recombinant PVX genomes containing large inserts at several days postinoculation, RNA was extracted from the diseased plants and reverse transcriptase–PCR (RT-PCR) analyses (Sijen et al., 1995) were performed. The primers that were used corresponded to sequences around the insertion site in PVX or to the MP gene. In this way, we could establish that the symptoms were due to the replication of the recombinant PVX genomes and not to wild-type PVX.

Nucleic Acid Analyses of Transgenic Plants and Nuclear Run-On Analysis

Total RNA was extracted from ground leaf material with a 1:1 mixture of phenol and extraction buffer (100 mM LiCl, 100 mM Tris, pH 8.0, 10 mM EDTA, and 1% SDS) preheated to 80°C. After the addition of half of a volume of chloroform–isoamyl alcohol (24:1), the samples were centrifugated for 15 min at 4°C. RNA was precipitated from the aqueous phase with one volume of 4 M LiCl (upon incubation for 1 hr at −70°C), collected by centrifugation for 30 min at 4°C, and washed with 70% ethanol. Poly(A)$^+$ RNA was isolated from total RNA by using Qiagen Oligotex mRNA columns (Westburg). RNA was separated by electrophoresis on 1% (w/v) agarose–formaldehyde gels, blotted onto GeneScreen Plus membranes (New England Nuclear, Boston, MA), according to standard procedures (Sambrook et al., 1989), and hybridized using ^{32}P-labeled PCR-amplified fragments as probes (Sambrook et al., 1989).

Genomic DNA was purified with Qiagen genomic tips by using the isolation protocol provided by the manufacturer (Westburg). Digestion of genomic DNA was performed by incubation twice for 2 hr in the presence of 1 μM spermidine. Gel electrophoresis, DNA gel blotting, and hybridization procedures were performed according to standard procedures (Sambrook et al., 1989). Nuclear run-on analysis was performed essentially as described by Van Blokland et al. (1994).

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REFERENCES

Barry, C., Faugeron, G., and Rossignol, J.-L. (1993). Methylation induced premeiotically in *Ascobolus*: Coextension with DNA repeat lengths and effect on transcript elongation. Proc. Natl. Acad. Sci. USA **90,** 4557–4561.

Bochardt, A., Hodal, L., Palmgren, G., Mattsson, O., and Okkels, F.T. (1992). DNA methylation is involved in maintenance of an unusual expression pattern of an introduced gene. Plant Physiol. **99,** 409–414.

Chapman, S., Kavanagh, T., and Baulcombe, D. (1992). Potato virus X as a vector for gene expression in plants. Plant J. **2,** 549–557.

Dorer, D.R., and Henikoff, S. (1994). Expansions of transgene repeats cause heterochromatin formation and gene silencing in Drosophila. Cell **77,** 993–1002.

Dougherty, W.G., and Parks, T.D. (1995). Transgenes and gene suppression: Telling us something new? Curr. Opin. Cell Biol. **7,** 399–405.

Dougherty, W.G., Lindbo, J.A., Smith, H.A., Parks, T.D., Swaney, S., and Proebsting, W. (1994). RNA-mediated virus resistance in transgenic plants: Exploitation of a cellular pathway possibly involved in RNA degradation. Mol. Plant-Microbe Interact. **7,** 544–552.

Elmayan, T., and Vaucheret, H. (1996). Expression of single copies of a strongly expressed *35S* transgene can be silenced post-transcriptionally. Plant J. **9,** 787–797.

English, J.J., Mueller, E., and Baulcombe, D.C. (1996). Suppression of virus accumulation in transgenic plants exhibiting silencing of nuclear genes. Plant Cell **8,** 179–188.

Finnegan, J., and McElroy, D. (1994). Transgene inactivation: Plants fight back! Bio/Technology **12,** 883–888.

Flavell, R.B. (1994). Inactivation of gene expression in plants as a consequence of specific sequence duplication. Proc. Natl. Acad. Sci. USA **91,** 3490–3496.

Fraenkel-Conrad, H. (1986). RNA directed RNA polymerases of plants. Crit. Rev. Plant Sci. USA **4,** 213–226.

Goodwin, J., Chapman, K., Swaney, S., Parks, T.D., Wernsman, E.A., and Dougherty, W.G. (1996). Genetic and biochemical dissection of transgenic RNA-mediated virus resistance. Plant Cell **8,** 95–105.

Hart, C.M., Fischer, B., Neuhaus, J., and Meins, F. (1992). Regulated inactivation of homologous gene expression in transgenic *Nicotiana sylvestris* plants containing a defense-related tobacco chitinase gene. Mol. Gen. Genet. **235,** 179–188.

Hellwald, K., and Palukaitis, P. (1995). Viral RNA as a potential target for two independent mechanisms of replicase-mediated resistance against cucumber mosaic virus. Cell **83,** 937–946.

Hobbs, S.L.A., Kpodar, P., and DeLong, C.M.O. (1990). The effect of T-DNA copy number, position and methylation on reporter gene expression in tobacco transformants. Plant Mol. Biol. **15,** 851–864.

Hohn, T., Corsten, S., Rieke, S., Mueller, M., and Rothnie, H. (1996). Methylation of coding region alone inhibits gene expression in plant protoplasts. Proc. Natl. Acad. Sci. USA **93,** 8334–8339.

Ingelbrecht, I., Van Houdt, H., Van Montagu, M., and Depicker, A. (1994). Posttranscriptional silencing of reporter transgenes in tobacco correlates with DNA methylation. Proc. Natl. Acad. Sci. USA **91,** 10502–10506.

Jorgensen, R.A. (1995). Cosuppression, flower color patterns, and metastable gene expression states. Science **268,** 686–691.

Judelson, H.S., and Whittaker, S.L. (1995). Inactivation of transgenes in *Phytophthora infestans* is not associated with their deletion, methylation, or mutation. Curr. Genet. **28,** 571–579.

Kass, S.U., Goddard, J.P., and Adams, R.L.P. (1993). Inactive chromatin spreads from a focus of methylation. Mol. Cell. Biol. **13,** 7372–7379.

Keshet, I., Yisraeli, J., and Cedar, H. (1985). Effect of regional DNA methylation on gene expression. Proc. Natl. Acad. Sci. USA **82,** 2560–2564.

Keshet, I., Lieman-Hurwitz, J., and Cedar, H. (1986). DNA methylation affects the formation of active chromatin. Cell **44,** 535–543.

Lang, W.H., Morrow, B.E., Ju, Q., Warner, J.R., and Reeder, R. (1994). A model for transcription termination by RNA polymerase I. Cell **79,** 527–534.

Lee, R.C., Feinbaum, R.L., and Ambros, V. (1993). The *C. elegans* heterochronic gene *lin-4* encodes small RNAs with antisense complementarity to *lin-14.* Cell **75,** 843–854.

Lewin, B. (1994). Chromatin and gene expression: Constant questions, but changing answers. Cell **79,** 397–406.

Lewis, J.D., Meehan, R.R., Henzel, W.J., Maurer-Fogy, I., Jeppesen, P., Klein, F., and Bird, A. (1992). Purification, sequence, and cellular localization of a novel chromosomal protein that binds to methylated DNA. Cell **69,** 905–914.

Lindbo, J.A., and Dougherty, W.G. (1992). Pathogen-derived resistance to a potyvirus: Immune and resistance phenotypes in transgenic tobacco plants expressing altered forms of a potyvirus coat protein nucleotides sequence. Mol. Plant-Microbe Interact. **5,** 144–153.

Lindbo, J.A., Silva-Rosales, L., Proebsting, W.M., and Dougherty, W.G. (1993a). Induction of a highly specific antiviral state in transgenic plants: Implications for regulation of gene expression and virus resistance. Plant Cell **5,** 1749–1759.

Lindbo, J.A., Silva-Rosales, L., and Dougherty, W.G. (1993b). Pathogen derived resistance to potyviruses: Working, but why? Semin. Virol. **4,** 369–379.

Lomonossoff, G.P. (1995). Pathogen-derived resistance to plant viruses. Annu. Rev. Phytopathol. **33,** 323–343.

Matzke, M.A., and Matzke, A.J.M. (1995). Homology-dependent gene silencing in transgenic plants: What does it really tell us? Trends Genet. **11,** 1–3.

Matzke, M.A., Priming, M., Tronovsky, J., and Matzke, A.J.M. (1989). Reversible methylation and inactivation of marker genes in sequentially transformed tobacco plants. EMBO J. **8,** 643–649.

Meyer, P. (1995). Understanding and controlling transgene expression. Trends Biotechnol. **13,** 332–337.

Meyer, P., and Saedler, H. (1996). Homology-dependent gene silencing in plants. Annu. Rev. Plant Physiol. Plant Mol. Biol. **47,** 23–48.

Mueller, E., Gilbert, J., Davenport, G., Brigneti, G., and Baulcombe, D.C. (1995). Homology-dependent resistance: Transgenic virus resistance in plants related to homology-dependent gene silencing. Plant J. **7,** 1001–1013.

Palmgren, G., Mattson, O., and Okkels, F.T. (1993). Treatment of Agrobacterium or leaf disks with 5-azacytidine increases transgene expression in tobacco. Plant Mol. Biol. **21,** 429–435.

Prins, M., de Oliveira Resende, R., Anker, C., van Schepen, A., de Haan, P., and Goldbach, R. (1996). Engineered RNA-mediated resistance to tomato spotted wilt virus is sequence specific. Mol. Plant-Microbe Interact. **9,** 416–418.

Rivier, D.H., and Pillus, L. (1994). Silencing speaks up. Cell **76,** 963–966.

Sambrook, J., Fritsch, E.F., and Maniatis, T.A. (1989). Molecular Cloning: A Laboratory Manual, 2nd ed. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory).

Schiebel, W., Haas, B., Marinkovic, S., Klanner, A., and Sänger, H.L. (1993). RNA-directed RNA polymerase from tomato leaves. J. Biol. Chem. **268,** 11858–11867.

Sijen, T., Wellink, J., Hendriks, J., Verver, J., and van Kammen, A. (1995). Replication of cowpea mosaic virus RNA1 or RNA2 is specifically blocked in transgenic *Nicotiana benthamiana* plants expressing the full-length replicase or movement protein genes. Mol. Plant-Microbe Interact. **8,** 340–347.

Smith, H.A., Swaney, S.L., Parks, T.D., Wernsman, E.A., and Dougherty, W.G. (1994). Transgenic plant virus resistance mediated by untranslatable sense RNAs: Expression, regulation, and fate of nonessential RNAs. Plant Cell **6,** 1441–1453.

Swaney, S., Powers, H., Goodwin, J., Silva-Rosales, L., and Dougherty, W.G. (1995). RNA-mediated resistance with nonstructural genes from the tobacco etch virus genome. Mol. Plant-Microbe Interact. **8,** 1004–1011.

Ten Lohuis, M., Mueller, A., Heidmann, I., Niedenhof, I., and Meyer, P. (1995). A repetitive DNA fragment carrying a hot spot for de novo methylation enhances expression variegation in tobacco and petunia. Plant J. **8,** 919–932.

Van Blokland, R., Van der Geest, N., Mol, J.N.M., and Kooter, J.M. (1994). Transgene-mediated suppression of chalcone synthase expression in *Petunia hybrida* results from an increase in RNA turnover. Plant J. **6,** 861–877.

Wassenegger, M., Heimes, S., Riedel, L., and Saenger, H.L. (1994). RNA-directed de novo methylation of genomic sequences in plants. Cell **76,** 567–576.

Weber, H., Ziechmann, C., and Graesmann, A. (1990). *In vitro* DNA methylation inhibits gene expression in transgenic tobacco. EMBO J. **9,** 4409–4415.

Wellink, J., van Lent, J.W.M., Verver, J., Sijen, T., Goldbach, R.W., and van Kammen, A. (1993). The cowpea mosaic virus M RNA-encoded 48-kilodalton protein is responsible for induction of tubular structures in protoplasts. J. Virol. **67,** 3660–3664.

Wellink, J., Verver, J., van Lent, J., and van Kammen, A. (1996). Capsid proteins of cowpea mosaic virus transiently expressed in protoplasts form virus-like particles. Virology **224,** 352–355.

Wilson, T.M.A. (1993). Strategies to protect crop plants against viruses: Pathogen-derived resistance blossoms. Proc. Natl. Acad. Sci. USA **90,** 3134–3141.

Zhang, D., Ehrlich, K.C., Supakar, P.C., and Ehrlich, M. (1989). A plant DNA-binding protein that recognizes 5-methylcytosine residues. Mol. Cell. Biol. **9,** 1351–1356.