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Transgenic Plant Virus Resistance Mediated by Untranslatable Sense RNAs: Expression, Regulation, and Fate of Nonessential RNAs

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Haploid leaf tissue of tobacco cultivars K326 and K149 was transformed with several transgenes containing cDNA of the potato virus Y (PVY) coat protein (CP) open reading frame (ORF). The various transgenes containing the PVY CP ORF sequence produced (1) the expected mRNA and CP product, (2) an mRNA rendered untranslatable by introduction of a stop codon immediately after the initiation codon, or (3) an antisense RNA that was untranslatable as a result of the incorrect orientation of the PVY CP ORF behind the transcriptional promoter. Homozygous doubled haploid (DH) (diploid) plants were generated, and selfed progeny from these plants were examined. Resistance was virus specific, functioning only against PVY. An inverse correlation between transgene-derived PVY transcript steady state levels and resistance was generally noted with lines expressing the untranslatable sense version of the PVY CP ORF. A collection of DH lines, derived from a single transformation event of a common haploid plant and isogenic for the PVY transgenes expressing untranslatable sense RNA, displayed different levels of PVY resistance. Lines with actively transcribed, methylated transgene sequences had low steady state levels of transgene transcript and a virus-resistant phenotype. These results are discussed within the context of sense suppression in plants.

INTRODUCTION

Germplasm that does not display disease symptoms when infected by a plant virus can be readily generated using molecular genetic approaches (for reviews, see Beachy, 1993; Wilson, 1993). Expression of viral gene sequences from a transgene frequently results in a plant resistant to both the virus and its disease symptoms. In a number of examples, a translation product does not appear to be necessary for the resistant state (de Haan et al., 1992; Lindbo and Dougherty, 1992a, 1992b; van der Vlugt et al., 1992; Pang et al., 1993). Mechanistic models proposed to explain transgenic virus resistance range from the transgene-derived virus protein product interfering with viral processes (Reimann-Philipp and Beachy, 1993) to transgene product activation of a cellular process (Lindbo et al., 1993a, 1993b).

Expression of an untranslatable mRNA effectively generates transgenic plants resistant to tobacco etch virus (TEV) (Lindbo and Dougherty, 1992a, 1992b; Dougherty et al., 1994). The resistance can be either preestablished or induced and is specific and complete. The resistance does not function against potato virus Y (PVY), a virus that shares ~60% nucleotide homology, or a collection of other RNA viruses tested. TEV and PVY are members of the potyvirus family, which is part of the picorna-like superfamily of RNA viruses (Riechmann et al., 1992; Koonin and Dolja, 1993). The virus resistance generated in these plants appears to function in the cytoplasm (Lindbo et al., 1993b) and may share features with a phenomenon observed in transgenic plants referred to as sense suppression (Napoli et al., 1990; van der Krol et al., 1990; for reviews, see Matzke and Matzke, 1993; Flavell, 1994). Sense suppression often results from attempts to increase expression of a particular endogenous plant gene by transforming the plant with an exogenous copy of the gene, usually under the control of a constitutive plant promoter. However, instead of the expected increase in gene expression, expression of both the endogenous and exogenous genes is often reduced. A number of different mechanisms have been proposed to explain this phenomenon, including methylation of DNA sequences (Matzke et al., 1989; Hobbs et al., 1990; Matzke and Matzke, 1991; Ottaviani et al., 1993), ectopic pairing of homologous DNA sequences and subsequent heterochromatin formation (Assaad et al., 1993), fortuitous transcriptional expression of a complementary antisense RNA (Grierson et al., 1991; Mol et al., 1991), and an undefined post-transcriptional regulation of RNA levels (Smith et al., 1990; de Carvalho et al., 1992).

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Transgenic plants expressing viral sequences and displaying resistance to an RNA virus that replicates in the cytoplasm may provide a unique system to examine the fate of specific ribonucleotide sequences nonessential for the host plant. We have generated a collection of doubled haploid (DH) transgenic plants expressing different versions of the PVY coat protein (CP) open reading frame (ORF) in an attempt to unravel the underlying mechanism of transgenic plant virus resistance. A number of plants expressing the untranslatable mRNA displayed an extreme resistance and could not be infected. Surprisingly, a number of DH lines that were derived from the same haploid plant and genotypically identical for the transgene displayed different steady state RNA levels and levels of resistance. Resistance correlated with high transcription rates and low steady state levels of RNA. These lines provide an isogenic series of plants to examine the cellular pathway responsible for both resistance and the decrease or suppression of transgene transcript levels.

RESULTS

PVY Transgene and Generation of DH Transgenic Plants

A mutated cDNA copy of the PVY CP ORF contained either an initiation codon or an initiation codon followed by a stop codon. These sequences were ligated adjacent to regulatory sequences derived from cauliflower mosaic virus (CaMV) such that the PVY sequences would be efficiently transcribed and translated (Figure 1). The plasmid containing the translatable version of the PVY CP ORF (designated Y-CP) differed from the untranslatable sense version (designated Y-RC1) at five nucleotide positions. Inserting the PVY CP ORF in the reverse orientation generated an antisense version of the ORF (designated Y-AS). The various transgene constructs were introduced into haploid tobacco leaf tissue using Agrobacterium-mediated

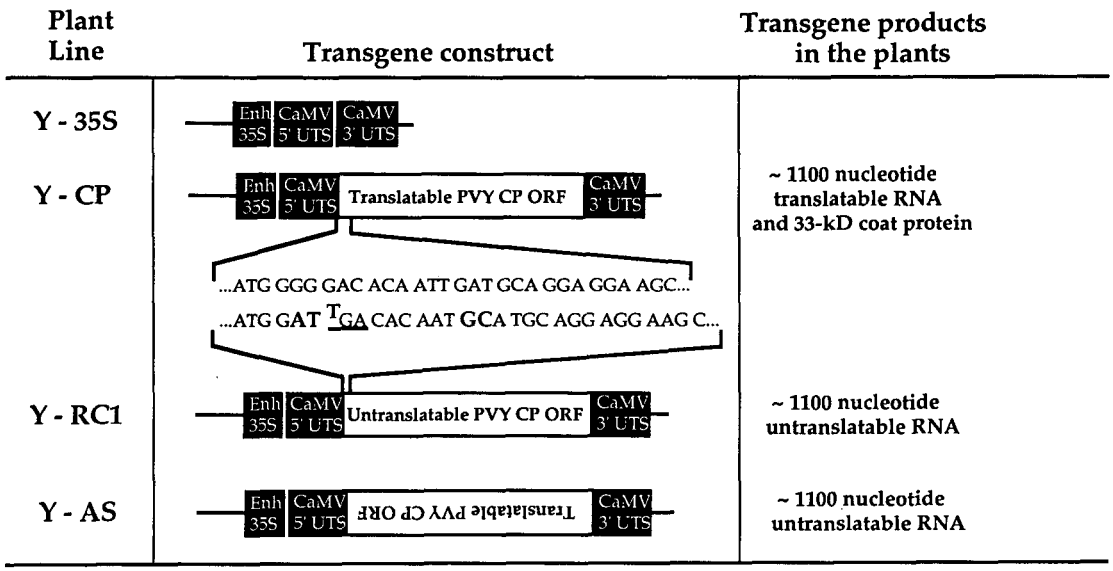


Figure 1. Schematic Drawing of the Transgenes Expressed and Their Nomenclature.

The CP ORF of PVY was copied into cDNA, altered by mutation, and inserted into a plasmid vector that would allow Agrobacterium-mediated transformation of tobacco tissue. All transgenes contain sequences derived from CaMV (black boxes) and include the enhanced 35S mRNA promoter (Enh 35S), as well as the 5′ (5′ UTS) and 3′ (3′ UTS) untranslated regions of the 35S CaMV mRNA. A BamHI restriction site is located between the 5′ and 3′ CaMV UTS in Y-35S and is the insertion site of double-stranded cDNAs that encode mutated versions of the PVY CP ORF. Transgenic plants expressing only CaMV gene regulatory sequences are referred to as Y-35S lines. Lines containing a transgene that codes for a PVY CP mRNA that is translated into a 33-kD PVY CP are referred to as Y-CP lines. Transgenic lines that contain a transgene that expresses an untranslatable PVY CP mRNA are referred to as Y-RC1 lines. The nucleotide sequence of the Y-CP and Y-RC1 genes immediately downstream of the initiation codon is presented. Y-CP and Y-RC1 lines differ from each other at five nucleotide positions (shown in boldface), and this results in the formation of a nonsense stop codon (underlined), which is two codons downstream of the initiation codon of the PVY CP ORF. Insertion of the translatable version of the PVY CP ORF in the reverse orientation results in the formation of transgenic plant lines that produce an untranslatable antisense version of the PVY CP mRNA and are called Y-AS lines. The RNA and protein products detected in transgenic plant tissue are presented on the right.

Table 1. Transgenic Germplasm Examined

Germplasm Designation[a]	Gene Construct[b]	Total Number	Response to Challenge with PVY[c]			
			HR	HR%	R	S
Tobacco cultivar K149						
Y-35S-149-XX	35S	3				3
Y-AS-149-XX	AS	6				6
Y-CP-149-XX	CP	15	3		4	8
Y-RC1-149-XX	RC1	17	11	2		4
Tobacco cultivar K326						
Y-35S-326-XX	35S	6				6
Y-AS-326-XX	AS	15				15
Y-CP-326-XX	CP	19	3	1	5	10
Y-RC1-326-XX	RC1	47[d]	13	10		24

[a] Two flue-cured tobacco cultivars, K326 and K149, were used in this study. Our transgenic plant designation is presented below each cultivar name. Our line nomenclature (i.e., Y-RC1-149-XX) indicates the virus from which the transgene is derived (Y, PVY), the transgene (RC1, a transgene containing an untranslatable PVY CP mRNA) (see footnote b), the tobacco cultivar transformed (149, K149; 326, K326), and the individual line number (XX = 1,2,3, and so forth).

[b] Four different gene constructs were introduced into the tobacco cultivars. A schematic drawing of the four gene constructs and their designation is presented in Figure 1. 35S, transgene that contains a cauliflower mosaic virus transcriptional promoter and 5′ and 3′ untranslated regions of the 35S RNA; AS, antisense version of the PVY CP ORF; CP, a translatable version of the PVY CP ORF; RC-1, an untranslatable sense version of the PVY CP ORF.

[c] Number of transgenic lines showing a particular response to PVY challenge inoculation is presented. Highly resistant (HR), no detectable PVY or PVY-induced symptoms in any plant; high percentage, highly resistant (HR%), most but not all plants showed the HR response; resistant (R), plants became infected but symptoms were attenuated and delayed; susceptible (S), typical PVY-induced symptoms.

[d] The total number of Y-RC1-326-XX lines examined is an overestimation of the number of independently selected lines. Multiple doubled haploid selections were made from two of the haploid RC1 lines. Therefore, of the 47 lines listed, only 32 represent independently selected transformation events of haploid tissue.

leaf disc transformation. Transgenic haploid plants were identified in a preliminary screen using RNA gel blot hybridization analysis. Midvein sections were removed from leaves of positive transgenic haploid plants, surface sterilized, and placed in tissue culture. Following callus formation, plantlets were regenerated. A high percentage (>75%) of the plantlets derived from midvein tissue culture developed into fertile DH plants (Kasperbauer and Collins, 1972), homozygous for the transgene(s). Typically, a single DH plant was propagated for each haploid line; however, in two instances multiple DH lines were generated from a single transformed haploid plant. We refer to these DH lines as comprising an isogenic "family." This approach was used for two different varieties of tobacco and permitted the production of homozygous transgenic plants within 16 months (Burk et al., 1979).

All DH plants were initially assessed for accumulation of the appropriate PVY-related RNA transcript. A total of 113 transgenic DH lines were identified from different haploid transformation events (data not shown). In addition, multiple DH Y-RC1 lines were generated from two Y-RC1-326 haploid plants. This resulted in a total of 128 lines examined in this study.

Seed from these DH transgenic plants were germinated, the R1 plants (10 to 20 cm in height) inoculated with PVY, and their response to PVY evaluated (Table 1). The response of Y-CP and Y-RC1 transgenic plant lines to PVY infection was line dependent. Resistance response ranged from complete susceptibility to complete resistance. No PVY-resistant lines were observed with plants of Y-35S lines or of Y-AS lines. A total of 33 representative lines were selected from the 128 transgenic lines for detailed analysis (Tables 2 and 3).

Analyses of Representative Transgenic Plants Correlate Resistance with Low Steady State RNA Levels

The representative transgenic plants were analyzed in biological and biochemical studies to determine if there were features shared by independent transformants showing resistance. Transgenic lines were challenge inoculated with three isolates of PVY (PVY-nn, PVY-H, and PVY-A26) and related potyviruses, tobacco vein mottling virus (TVMV) and TEV. Four plant responses to challenge inoculation with PVY were noted and are as follows: (1) highly resistant (HR, no detectable PVY or PVY-induced symptoms in any plant tissue); (2) a high percentage, highly resistant response (HR%, most plants had HR phenotype, but 2 to 30% of the plants exhibited normal PVY-induced symptoms); (3) resistant response (R, PVY symptoms were attenuated and temporally delayed in their appearance); and (4) a susceptible (S) plant response during which typical PVY-induced symptoms were noted on >90% of the plants challenged. Figure 2 presents a photograph comparing a leaf from a Y-RC1 plant showing an HR phenotype with leaves from the

Table 2. Characteristics of Selected Transgenic Plants

Transgenic Line Designation[a]	Response to Challenge Inoculation[b]					Transgenic Copy Number[c]	RNA Accumulation[d] (pg/μg)	CP Accumulation[e] (ng/g)
	PVY-A26	PVY-nn	PVY-H	TEV	TVMV			
Y-35S-326-04	S	S	S	S	S	ND	ND	
Y-35S-326-07	S	S	S	S	S	ND	ND	
Y-AS-326-01	S	S	S	S	S	2	5.4	
Y-AS-326-05	S	S	S	S	S	ND	3.0	
Y-CP-149-04	S	S	S	S	S	>4	28.4	0.055
Y-CP-149-10	R	S	S	S	S	ND	13.0	1.150
Y-CP-149-18	R	R	R	S	S	3	3.4	1.500
Y-CP-326-02	R	R	R	S	S	2	58.0	1.720
Y-CP-326-10	HR%	HR%	R	S	S	>4	12.3	0.027
Y-CP-326-11	S	S	S	S	S	1	21.0	0.910
Y-CP-326-14	S	S	S	S	S	1	28.0	1.150
Y-RC1-149-01	S	S	S	S	S	1	31.0	
Y-RC1-149-04	HR	HR	HR	S	S	3	7.6	
Y-RC1-149-09	HR	HR	HR	S	S	4	7.0	
Y-RC1-149-18	HR	HR	HR	S	S	2	9.0	
Y-RC1-149-19	HR	HR	HR	S	S	2	13.4	
Y-RC1-326-02	S	S	S	S	S	1	31.4	
Y-RC1-326-03	S	S	S	S	S	1	10.0	
Y-RC1-326-08	HR%	HR%	HR%	S	S	3	9.4	
Y-RC1-326-11	S	S	S	S	S	1	18.4	
Y-RC1-326-12	S	S	S	S	S	2	13.0	
Y-RC1-326-26	HR	HR	HR	S	S	3	4.0	
Y-RC1-326-34	S	S	S	S	S	2	33.0	
Y-RC1-326-35	HR%	HR%	HR%	S	S	3	2.0	
Y-RC1-326-58	HR%	HR%	HR%	S	S	4	3.4	
Y-RC1-326-46	HR	HR	HR	S	S	4	12.2	
Y-RC1-326-32	HR	HR	HR	S	S	3	13.2	

[a] The plant line nomenclature is as described in Table 1.

[b] The phenotypic responses have been grouped into one of four catagories described in the text and Table 1. Potyviruses used in this study were PVY isolate A26 (PVY-A26), PVY-nn, PVY-H, TEV, and TVMV.

[c] Transgene copy number was estimated from DNA gel blot hybridization analyses. The number presented may actually be an underestimation if individual bands detected by hybridization are comprised of multiple inserts. ND, not determined.

[d] RNA accumulation is a reflection of steady state levels of the transgene transcript. Total RNA (5 μg) was examined in slot blot hybridization studies. Total RNA levels were standardized between plants using actin mRNA levels. The values reported are picograms of transgene transcript per microgram of total RNA.

[e] CP accumulation levels were determined in double-antibody sandwich ELISA using purified PVY CP as a standard. Plants of the same developmental age (30 cm in height) were used in these studies. Values reported are nanograms of PVY coat protein per gram of leaf tissue.

Table 3. Characteristics of a Family of DH Transgenic Plants[a]

Transgenic Line Designation	Response to Challenge Inoculation with					Transgene Copy Number	RNA Accumulation (pg/μg)
	PVY-A26	PVY-nn	PVY-H	TEV	TVMV		
Y-RC1-326-58	HR%	HR%	HR%	S	S	4	3.4
Y-RC1-326-15	HR%	HR%	HR%	S	S	4	2.6
Y-RC1-326-17	S	S	S	S	S	4	13.4
Y-RC1-326-18	HR%	HR%	HR%	S	S	4	5.0
Y-RC1-326-51	S	S	S	S	S	4	28.4
Y-RC1-326-54	S	S	S	S	S	4	26.0
Y-RC1-326-56	HR%	HR%	HR%	S	S	4	5.4

[a] See footnotes in Table 2.

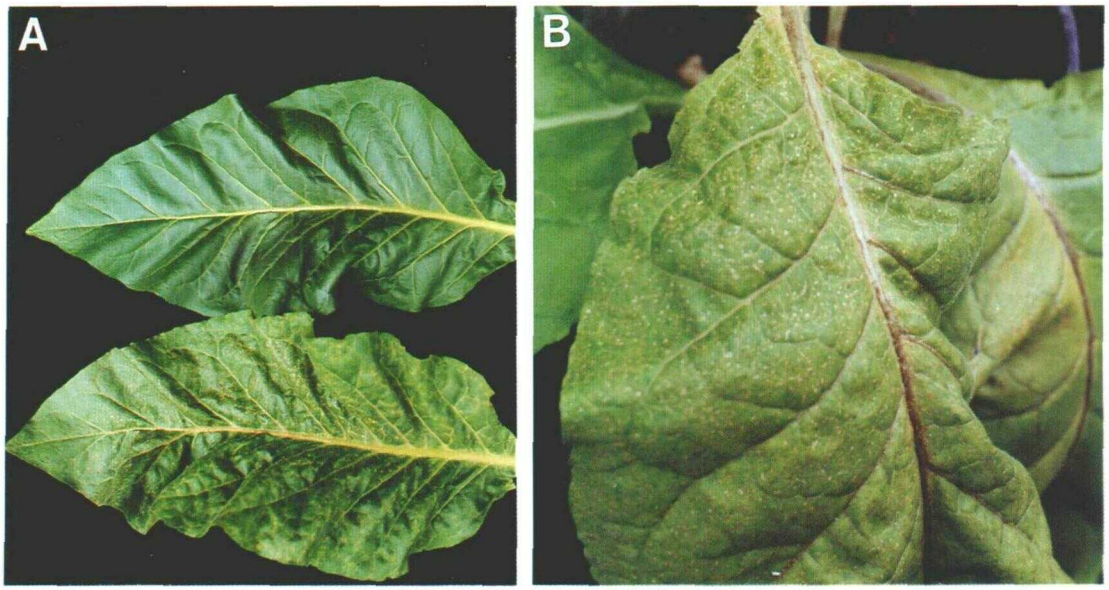


Figure 2. PVY-Induced Symptoms in Transformed and Untransformed Tobacco Plants.

(A) Leaves from a similar position on a Y-RC1-326-32 plant (top) showing an HR phenotype and from an untransformed tobacco cultivar K326 plant (bottom) are shown. Both plants were inoculated with a PVY-nn isolate, and 1 month later, upper leaves were removed and photographed. **(B)** A close-up of a leaf from a tobacco cultivar K326 plant infected with PVY-nn. Veinal necrosis and etching of the leaf surface are very pronounced and characteristic of this PVY isolate on the tobacco cultivar K326.

untransformed parental K326 line. All leaves are from plants inoculated with the PVY-nn isolate. The resistance in all cases was PVY specific, and no resistance was noted against TVMV or TEV (Tables 2 and 3).

Different responses were observed in transgenic plants that produced either translatable or untranslatable mRNAs of the PVY CP transgene (Table 2). The HR phenotype was only noted with Y-RC1 lines, and virus resistance was displayed against all three isolates of PVY. No symptoms were observed in the HR plants. ELISA analysis and back inoculation studies of tissue extracts from these lines to susceptible tobacco cultivar Burley 21 indicator plants did not detect virus in inoculated or upper leaves.

Lines that displayed the HR% phenotype usually displayed extreme resistance; however, a minor percentage of the plants would display an S or R phenotype. Typically, 2 to 30% of the plants would become infected and show virus disease symptoms. A number of Y-RC1 lines and one Y-CP line (Y-CP-326-10) displayed this phenotype (Table 2).

Lines that displayed the R phenotype had virus symptoms that were attenuated and often temporally delayed. This form of resistance was evident in Y-CP lines infected with the necrotizing isolates PVY-H or PVY-nn. Normal veinal necrosis was not observed in some of these transgenic lines; instead, only a mild mosaic symptom was noted. Virus could be readily recovered; however, ELISA indirectly suggested that there was less virus in some of these lines (data not shown).

Finally, the majority of the transgenic plants (Table 1) expressing the different transgenes displayed typical symptomatology when inoculated with the different PVY isolates and were considered to have an S phenotype.

The DH plants and their homozygous transgene(s) provided an excellent genetic background to assess resistance, transgene copy number, and steady state levels of transgene RNA and CP (Figures 3A and 3B and summarized in Table 2). Transgene copy number was estimated from DNA gel blot hybridization analysis of genomic DNA digested with restriction enzymes that cut the DNA adjacent to the transgene. There was a tendency for HR and HR% lines to have multiple hybridizing bands (two to four) in gel blot analyses. Steady state RNA levels were determined by slot blot hybridization and RNA gel blot hybridization analyses. A correlation between the R phenotype and steady state levels of the untranslatable Y-RC1 transgene transcript was observed in the gel blot hybridization analysis of total RNA extracted from various transgenic lines. A comparison of the Y-RC1 transcript levels (Figure 3A and Table 2) with the PVY-resistant phenotype revealed that HR lines always accumulated small amounts of the transgene transcript. Susceptible Y-RC1 lines generally accumulated more transgene transcript RNA, although a low

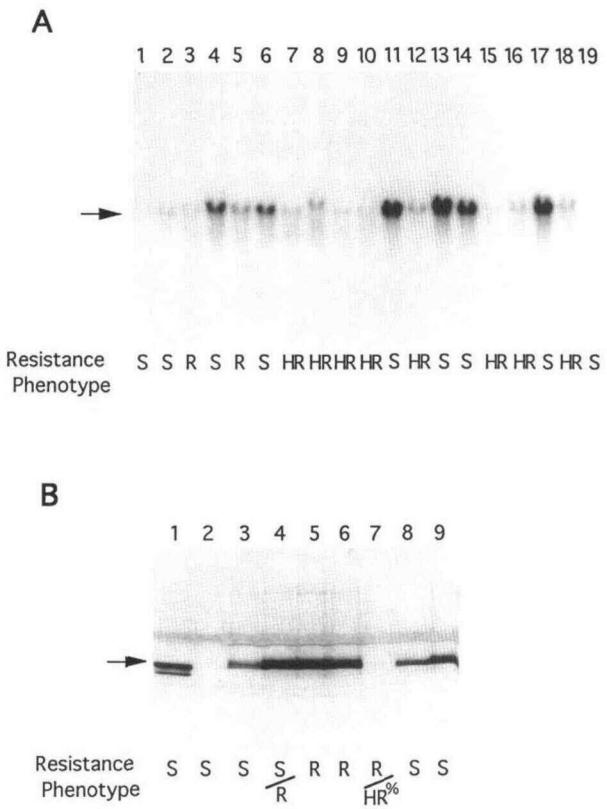


Figure 3. Analysis of Transgene Products in Y-CP and Y-RC1 Tobacco Leaf Tissue.

(A) An autoradiogram of an RNA gel blot showing steady state RNA levels. Total RNA (5 μg) from various transgenic plant lines was electrophoretically separated in agarose gels containing formaldehyde and blotted onto nitrocellulose membrane. The membrane was hybridized with a ^{32}P-labeled riboprobe corresponding to the antisense form of the PVY CP coding sequence, washed, and exposed to x-ray film. The position of the transgene transcript is indicated by the arrow at left. The resistance phenotype of each plant was determined and is presented at the bottom of the autoradiogram. Plants had a sensitive (S), resistant (R), or highly resistant (HR) phenotype. Some plant lines had a variable phenotype (i.e., S/R, sensitive/resistant **[B]**). The different resistance responses are described in detail in Table 1. RNA samples were purified from leaf tissue of the following plant lines: lane 1, untransformed tobacco cultivar K326; lane 2, cultivar K326 to which 100 pg of a T7 polymerase–synthesized PVY CP ORF transcript was added; lane 3, Y-CP-149-18; lane 4, Y-CP-149-04; lane 5, Y-CP-326-02; lane 6, Y-CP-326-11; lane 7, Y-RC1-149-04; lane 8, Y-RC1-149-09; lane 9, Y-RC1-149-19; lane 10, Y-RC1-149-18; lane 11, Y-RC1-149-01; lane 12, Y-RC1-326-58; lane 13, Y-RC1-326-12; lane 14, Y-RC1-326-03; lane 15, Y-RC1-326-35; lane 16, Y-RC1-326-26; lane 17, Y-RC1-326-11; lane 18, Y-RC1-326-08; and lane 19, cultivar K326. The phenotypic response to PVY challenge of each plant line is indicated below each lane.
(B) Protein gel blot analysis of selected Y-CP lines. A total protein sample from a leaf extract was separated by electrophoresis in 10% polyacrylamide gels containing SDS and electroblotted to nitrocellulose. PVY CP derived from the Y-CP transgene was detected using polyclonal antiserum raised against the PVY CP. The arrow at left indicates the position at which purified PVY CP migrates. Protein extracts were examined from the following plant lines: lane 1, untransformed tobacco cultivar K326 with 10 ng of purified PVY CP added; lane 2, untransformed cultivar K326; lane 3, Y-CP-149-04; lane 4, Y-CP-149-10; lane 5, Y-CP-149-18; lane 6, Y-CP-326-02; lane 7, Y-CP-326-10; lane 8, Y-CP-326-10; lane 9, Y-CP-326-14. The phenotypic response to PVY challenge of a plant line is indicated below each lane and abbreviations are as given in **(A)**.

steady state level of transgene transcripts was noted for a few susceptible Y-RC1 lines. A correlation of the R phenotype with accumulated CP was not noted when PVY CP levels in Y-CP lines were estimated in ELISA or protein gel blot hybridization studies (Figure 3B and Table 2).

Analyses of an Isogenic DH Family of Plants Reveal Surprising Differences in Resistance and Gene Expression Levels

Typically, we selected a single DH plant to be representative of a transformation event. In two cases, multiple DH lines were saved. One of these families of plants is listed in Table 3. DNA gel blot hybridization studies of the genomic DNA from these seven lines confirmed that they were genotypically identical for the transgene (Figure 4). The genomic DNA digested with the restriction enzyme XbaI revealed three bands in each line. No differences between lines were noted when the genomic DNA from these lines was digested with other restriction enzymes (HindIII, EcoRI, or HpaI) followed by DNA gel blot hybridization (data not shown). Subcloning of the genomic DNA of Y-RC1-326-54 into a λ DNA library suggested there are four copies of the transgene in this family of plants.

This family of DH plants did not respond to PVY infection in the expected uniform fashion. Four of the lines displayed an HR$^{\%}$ phenotype and three had an S phenotype. The plants showing extreme resistance in lines Y-RC1-326-15, Y-RC1-326-18, Y-RC1-326-56, and Y-RC1-326-58 were similar to plants from lines possessing an HR phenotype in Table 2, in that a high level resistance to all PVY isolates tested correlated with a low steady state level of the transgene transcript (Table 3 and Figure 5). Transgenic lines Y-RC1-326-17, Y-RC1-326-51, and Y-RC1-326-54 were susceptible to PVY infection. Y-RC1-326-51 and Y-RC1-326-54 had significantly higher steady state levels of the transgene transcripts than the level found in the lines with an HR phenotype (Table 3 and Figure 5). Line Y-RC1-326-17 also accumulated higher steady state levels of transgene transcript than did lines displaying the HR phenotype (Table 3), however, at only half the level found in lines with an S phenotype. The susceptible plants in the HR$^{\%}$ line, Y-RC1-326-15, showed a normal PVY-induced disease progression and had high steady state levels of transgene transcript (Figure 5, lane 3).

Our DNA gel blot hybridization analyses of the genomic DNA of members of this DH family of plants revealed that the transgene copy number and organization were similar. Could a

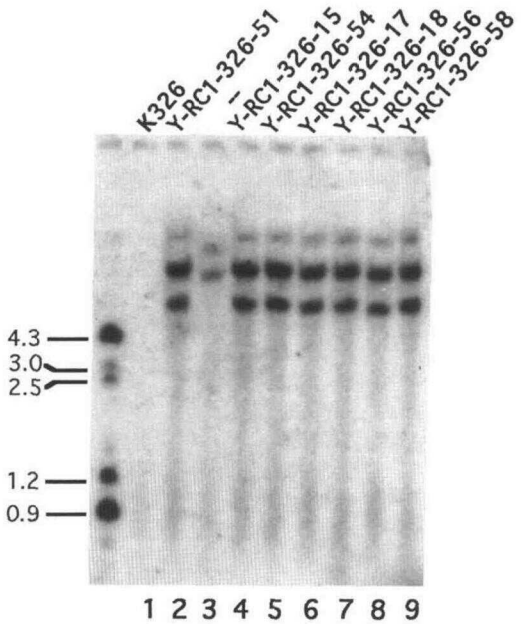


Figure 4. Gel Blot Analysis of the Genomic DNA from an Isogenic DH Family of Plants.

Genomic DNA (40 μg) was digested with the restriction endonuclease XbaI, separated by electrophoresis in an 0.8% agarose gel, and blotted onto nitrocellulose. Membranes were hybridized with a ^{32}P-labeled DNA probe specific for PVY CP ORF sequence, washed, and exposed to x-ray film. The plant lines examined are given at top. Genomic DNA from the untransformed tobacco cultivar K326 is in lane 1. Lane 2 and lanes 4 through 9 contain genomic DNA from seven DH lines derived from the same haploid transformant. Lane 3 contains genomic DNA from the line Y-RC1-326-27 (S phenotype) digested with XbaI. Molecular length markers are given at left in kilobases.

difference in the level of transgene DNA methylation in these lines account for the observed differences in steady state RNA levels? Genomic DNA was digested with either the restriction enzyme BamHI or EcoRI, followed by digestion with a restriction enzyme sensitive to DNA methylation at cytosine and adenosine residues, such as AvaI or HpaII. MspI, a restriction endonuclease insensitive to methylation at cytosine residues, was used as a positive control. The results were consistent with all three enzymes; only the AvaI digestion patterns are presented (Figure 6). The three lines with an S phenotype had limited methylation of their transgene DNA sequences, as indicated by the ability of the restriction enzyme AvaI to digest the genomic DNA (lanes 2, 4, 6, 8, 10, and 12). However, genomic DNA from lines Y-RC1-326-56 and Y-RC1-326-58 (HR$^{\%}$ phenotype) was poorly digested by AvaI (lanes 14, 16, 18, and 20), suggesting more methylation of the transgene DNA than detected in the lines with an S phenotype.

Methylation of the transgene sequences correlated with low steady state RNA levels, suggesting that transgene transcript levels simply were a reflection of reduced transcription rates. To assess this, two lines with an HR$^{\%}$ phenotype and low steady state RNA levels, Y-RC1-326-56 and Y-RC1-326-58, and two lines with an S phenotype, Y-RC1-326-51 and Y-RC1-326-54, which had high steady state levels of the transgene transcript, were selected to examine transcription rates in nuclear run-on studies (Figure 7). (The specific plants selected from the HR$^{\%}$ lines displayed extreme resistance based on inoculation studies after nuclei were harvested.) The two HR$^{\%}$ lines (Y-RC1-326-56 and-Y-RC1-326-58) with methylated transgene sequences had similar transcription rates. Surprisingly, these rates were approximately twofold higher than the PVY transgene transcription rate found in susceptible Y-RC1-326-51 nuclei and three- to fourfold higher than that in susceptible Y-RC1-326-54 nuclei. Therefore, plants containing methylated Y-RC1 transgenes were transcribed at "high" levels, had "low" steady state transgene transcript levels, and displayed extreme resistance to PVY. Conversely, plants containing Y-RC1 transgenes with little to no methylation were transcribed at "lower" levels, had "high" steady state transgene transcript levels, and were susceptible to PVY.

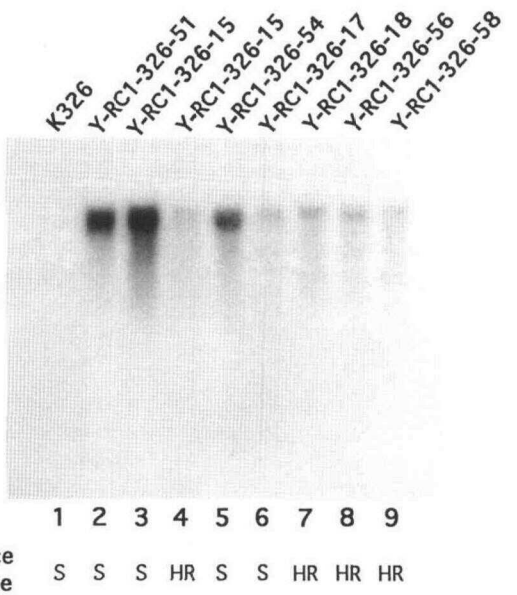


Figure 5. RNA Gel Blot Analysis of a DH Family.

Total RNA from leaf tissue of the plant lines indicated above the lanes was prepared, separated by electrophoresis in an agarose gel under denaturing conditions, and transferred to nitrocellulose. A ^{32}P-labeled riboprobe was used to detect the untranslatable PVY CP RNA. Total RNA (5 μg) was analyzed in each lane. The virus resistance phenotype (S, sensitive; HR, highly resistant) of each plant examined is presented below. RNA from two Y-RC1-326-15 plants (lanes 3 and 4) showing different virus resistance phenotypes is presented.

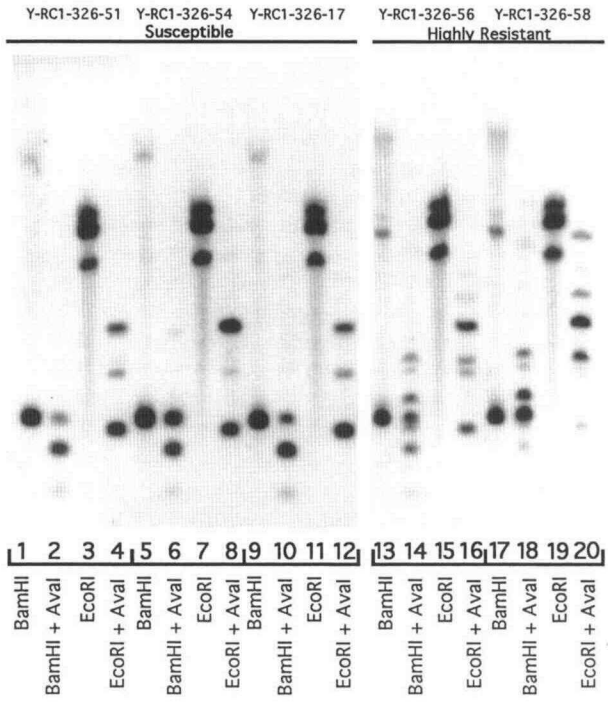


Figure 6. DNA Gel Blot Analysis of Differential Methylation Patterns in Genomic DNA from a DH Family.

Genomic DNAs from three PVY susceptible and two highly resistant plant lines were digested with the restriction endonucleases BamHI (lanes 1, 5, 9, 13, and 17) or EcoRI (lanes 3, 7, 11, 15, and 19). Double digests of genomic DNA from these same lines were performed with BamHI and AvaI, a restriction endonuclease that does not cut if certain methylated cytosine and adenosine residues are present (lanes 2, 6, 10, 14, and 18), or EcoRI and AvaI (lanes 4, 8, 12, 16, and 20). The AvaI site is located in the PVY transgene 259 bp downstream from the initiation codon. Plant line designations and phenotypes are shown above the gels.

DISCUSSION

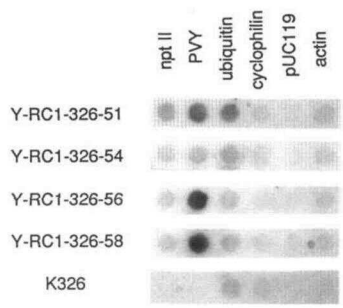
We have extended our ability to generate virus-resistant plants to another plant virus by altering plants to produce an untranslatable mRNA. We have previously suggested that TEV transgenic resistance may be mediated by a cellular pathway normally involved in the elimination or down-regulation of aberrant or overexpressed mRNAs (Lindbo et al., 1993b; Dougherty et al., 1994). Consistent with these observations, transgenic plants resistant to PVY usually have multiple PVY transgenes that appear to be highly transcribed, yet accumulate low levels of the transgene transcript. A cytoplasmic-based, post-transcriptional cellular surveillance system that targets specific RNAs for elimination could account for these results.

Production of DH plants, which were homozygous for the transgene(s), simplified both biochemical and molecular genetic analyses of the transgenic plants containing multiple transgenes. We were able to generate transgenic plants that displayed an R phenotype by producing an mRNA and a functional CP, but there was not a clear correlation between CP accumulation and the degree of virus resistance. We have observed similar results with TEV (Lindbo and Dougherty, 1992a). In addition, Dolja et al. (1994) have shown that full-length TEV or PVY CP, expressed from a transgene, was able to complement TEV mutants deficient in movement. These observations, coupled with the ability to generate HR germplasm by only expressing an untranslatable RNA, suggests that CP is not an integral factor in the establishment of the HR state that precludes all potyvirus replication.

We suggest that transgenic plants expressing an untranslatable sense RNA will provide insights into the coordinate regulation of RNA in plant cells. We consistently observed a correlation between the HR phenotype and low steady state level of the Y-RC1 transgene transcript. Y-RC1 lines that had an S phenotype often contained high steady state levels of the transgene transcript; however, we also noted that a few Y-RC1 lines had low steady state levels. We suggest that plant cells have an as yet undefined "threshold" below which they can accommodate a specific RNA species. This threshold may be defined by both qualitative and quantitative aspects of the RNA transcript (Dougherty et al., 1994). The untranslatable mRNA (Y-RC1) species may attain this threshold at a lower effective concentration than the translated mRNAs (Y-CP) because of its aberrant nature. Once this threshold is exceeded, a cytoplasmic-based system is activated and lowers the effective concentration by specifically targeting and eliminating the RNA species. Activation of this system also results in a virus-resistant phenotype. In contrast, the threshold level in susceptible germplasm has not been exceeded, and the cytoplasmic-based system is not activated. Therefore, steady state RNA levels of untranslatable RNA transcripts may be lower, the same, or higher than levels observed in the highly resistant germplasm and would be directly proportional to the transcription rate of the transgenes. We have also described a recovery phenotype in which virus resistance is activated (Lindbo et al., 1993b; Dougherty et al., 1994). In this case, we suggest that the additive level of the TEV transgene transcript and the TEV genomic RNA exceeds the threshold level and activates the system. These concepts are presented in a schematic model in Figure 8.

Disease reaction variability of the DH family (genotypically identical with transgenes in identical chromosome sites) after challenge inoculation with PVY and the clear differences in steady state transgene-derived RNA levels were unexpected (Table 3 and Figure 5). This series of plants suggests another level of gene regulation in addition to the previously proposed relationship between transgene expression and location in the chromosome (Allen et al., 1993). Some undefined host component in these transgenic plants is partially responsible for

A



B

Source of nuclei	Resistance Phenotype	cpm bound /5ug plasmid npt II	PVY	ubiquitin	cyclophilin	pUC119	actin
Exp 1							
Y-RC1-326-54	S	230	256	169	151	104	152
Y-RC1-326-58	R	218	962	165	196	134	171
Exp 2							
Y-RC1-326-54	S	94	97	75	83	61	66
Y-RC1-326-58	R	113	350	90	98	75	77
Exp 3							
K326	S	80	89	202	149	104	135
Y-RC1-326-51	S	354	472	458	191	146	203
Y-RC1-326-54	S	225	278	314	183	123	172
Y-RC1-326-56	R	233	824	244	177	148	184
Y-RC1-326-58	R	239	824	300	204	154	201
Exp 4							
K326	S	49	55	55	60	55	55
Y-RC1-326-51	S	122	160	68	65	62	60
Y-RC1-326-54	S	118	118	68	64	57	67
Y-RC1-326-56	R	141	483	72	84	65	74
Y-RC1-326-58	R	147	473	90	83	74	74
Exp 5							
K326	S	ND	73	80	100	65	81
Y-RC1-326-51	S	ND	450	120	120	83	110
Y-RC1-326-54	S	ND	285	168	124	101	122
Y-RC1-326-56	R	ND	1544	199	148	109	136
Y-RC1-326-58	R	ND	1411	162	151	106	134

C

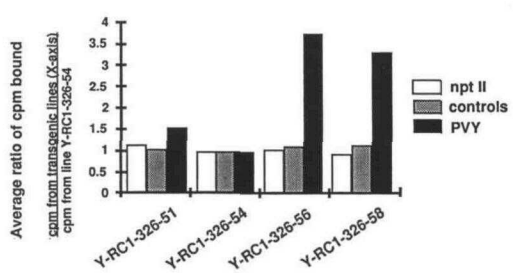


Figure 7. Analysis of Transcription Rates for Selected Members of a DH Family.

Isolated nuclei were used in nuclear run-on assays. The ^{32}P-labeled RNAs synthesized were hybridized to nitrocellulose membranes containing dot blots with 5 μg of various plasmid DNAs affixed in particular locations. The plasmid DNAs contained a cDNA version of the entire PVY CP ORF (PVY), cDNAs of constitutively expressed plant genes (actin, cyclophilin, and ubiquitin) used as internal controls, pUC119 plasmid DNA, or the gene coding for neomycin phosphotransferase (*nptII*).

(A) An autoradiogram of a typical experiment is shown. Plant lines used as a source of nuclei are shown on the left of each blot. Nuclei from four transgenic lines and tobacco cultivar K326 were used. Genes contained in plasmid DNAs are listed above. Following autoradiography, the individual hybridization dots were excised, placed in 5 mL of liquid scintillation cocktail, and counted in a liquid scintillation counter.

transcription levels. Such families of plants may provide an experimental system to examine the ubiquitous but poorly defined products of these "modifier genes" (Hartl, 1991).

A question not resolved by this study is whether this putative host effect is mediated via the methylation of transgene sequences or some other process. In our analysis of this family of plants, high transcription rates in nuclear run-on studies occurred in nuclei in which the PVY transgene sequences displayed elevated levels of methylation compared to transgene sequences with low levels of methylation that were transcribed at a reduced comparative rate. This suggests a direct causal relationship, with methylation leading to increased transcription rates. However, clear differences in DNA methylation were observed between the two highly transcribed lines with an HR phenotype (Figure 6, lanes 13 to 20). An alternative explanation for the DNA methylation observed is that it actually represents an attempt to reduce transgene transcription. In this scenario of coordinate gene regulation, cytoplasmic and nuclear pools of RNA would dictate gene expression levels. RNA-directed methylation of transgene sequences has recently been described in an elegant study by Wassenegger et al. (1994) and could be mediated by a sense or antisense RNA product of the gene. If the cytoplasmic concentration of an mRNA was high, this might result in an increased nuclear level of this mRNA, signaling methylation of DNA sequences from which it was derived. Alternatively, methylation could be directed by small RNAs that are complementary to the mRNA sequence. Lindbo et al. (1993b) have suggested that mRNAs may be targeted for elimination by small complementary RNAs generated by the enigmatic, host RNA-dependent RNA polymerase (Schiebel et al., 1993a, 1993b). If these hypothetical small complementary RNAs accumulate to a sufficient level in the nucleus, they could potentiate methylation of the target transgene DNA. Therefore, the concentration of any particular RNA in a cell could be self-regulated at both a cytoplasmic and nuclear level.

Does the transgenic virus resistance we have described here and in previous studies provide an opportunity to study the molecular mechanisms underlying sense suppression in plants and how multiple copies of the same gene are coordinately

(B) Total counts per minute (cpm) contained in each excised dot are given. The data from five different assays, using nuclei isolated from two different sets of plants, are presented. The autoradiogram in **(A)** corresponds to experiment 3. ND, not determined.

(C) The combined results from the experiments shown in **(B)** are represented graphically. In each experiment, the hybridization signals (counts per minute) of control genes (actin, cyclophilin, ubiquitin, and pUC119) were used to normalize transcription rates between lines. Based on these normalizations, PVY and *nptII* transcription levels were determined and compared between lines. The data are a summation of all five experiments and expressed as the rate of transcription of a particular transgenic line relative to transcription in line Y-RC-326-54 (line with the lowest level of PVY transgene transcription and a susceptible phenotype). For simplicity, the averages of all internal control genes are depicted.

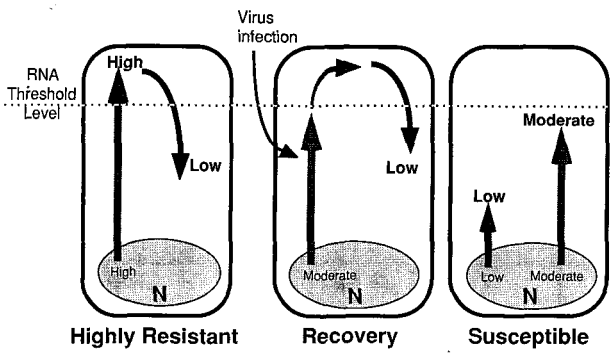


Figure 8. A Model for a Potential Resistance and Sense Suppression Mechanism.

Transcription of the transgene within the nucleus (N) produces varying amounts of RNA among the different transgenic lines. Plants with high transcription rates generate RNA levels that exceed a certain threshold level, which activates a cytoplasmic-based, cellular process that specifically targets this RNA for elimination and results in low steady state levels of the transgene mRNA. If the transgene shares nucleotide homology with a plant virus, the plants are phenotypically resistant to the challenging virus (left). Alternatively, in lines where transcription rates are lower and the threshold level is not exceeded, the putative cytoplasmic degradation mechanism is not induced. Steady state levels of the mRNA will be proportional to the transcription rate of the gene. The cytoplasmic regulatory system is off and a susceptible phenotype will be manifested (right). We have also described an "inducible" resistance (Lindbo et al., 1993b) referred to as a recovery phenotype. We suggest the additive level of transgene mRNA and the viral RNA containing the target sequence exceed the threshold level, resulting in an activation of the cytoplasmic system, which is manifested as a lowering of the transgene mRNA steady state levels and the concomitant establishment of the resistance phenotype (center).

expressed in a plant cell? We believe it does and suggest many (not all) examples of sense suppression (Smith et al., 1990; de Carvalho et al., 1992; Hobbs et al., 1993; Seymour et al., 1993) are mediated by a post-transcriptional cytoplasmic-based system. Many sense suppression studies have concluded that methylation or some other alteration of the physical state of the transgene sequence is responsible for the decrease in transgene transcript accumulation, although transcription has not been examined in most of these studies. The cytoplasmic replicating virus in our studies provided an additional target RNA molecule to examine along with the nuclear-transcribed transgene transcript. This system enabled us to clearly divorce nuclear events from cytoplasmic events. Coupled with the results from the DH family of plants, we suggest methylation of the Y-RC1 transgene sequences in this family of plants cannot be solely responsible for a decrease in steady state RNA levels. If decreased transgene transcript accumulation and virus resistance are coupled, then an understanding of transgenic virus resistance will yield important insights and aid in the general understanding of sense suppression and the control of gene expression in plants.

METHODS

Virus Isolates and Tobacco Germplasm

The tobacco etch virus TEV-H (highly aphid transmissible) isolate and the tobacco vein mottling virus (TVMV) isolate were originally obtained from T. Pirone (University of Kentucky, Lexington). Two of the potato virus Y (PVY) isolates, PVY-nn and PVY-H, were obtained from G. Gooding (North Carolina State University, Raleigh). Both isolates cause veinal necrosis on many tobacco cultivars. The third PVY isolate, PVY-A26, was obtained from infected potatoes at the Oregon State University Research and Education Complex, Hermiston, OR. PVY-A26 induces mild symptoms on potato and tobacco and does not induce necrosis. Viruses were maintained in the tobacco cultivar Burley 21 and purified as described by Dougherty and Hiebert (1980).

Tobacco cultivars K326 and K149 are commercial varieties of fluecured tobacco. Tobacco cultivars Burley 21 and Burley 49 are commercial burley tobacco varieties.

Construction of Transgenes

The PVY coat protein (CP) open reading frame (ORF) used in this study was derived from PVY-A26. A cDNA copy of the PVY CP ORF was made from partially purified PVY-A26 RNA by reverse transcription coupled with the polymerase chain reaction and inserted into the cloning vector pTC (Lindbo and Dougherty, 1992a) to generate the plasmid pPVYCP. The untranslatable form of the PVY CP ORF was generated by mutating the plasmid pPVYCP using the method of Taylor et al. (1985a, 1985b). PVY CP gene constructs were inserted between the 5′ and 3′ genomic untranslated sequence of cauliflower mosaic virus (CaMV) in the plasmid pPEV-6 (Lindbo and Dougherty, 1992a). Pertinent features of the transgene construct, the distinctions between CP, RC1, and AS transgenes, and transgenic plant nomenclature are presented in Figure 1.

Construction of Transgenic Plants

Haploid plants were generated by crossing the tobacco cultivars K326 and K149 with pollen of *Nicotiana africana*. The interspecific hybridizations result in abundant seed, but the species possess a complementary genetic lethality system (Burk et al., 1979). Euploid interspecific hybrid seedlings (99.9%) are lethal at germination. Surviving progenies (0.1%) consist of mixtures of aneuploid interspecific hybrids (plants that have lost a chromosome possessing a lethality allele) and tobacco parthenogenetic haploids. The two seedling classes are readily phenotypically distinguishable. The haploid plants are maintained in tissue culture. Leaf tissue of a haploid plant was transformed by the Agrobacterium-mediated leaf disc transformation procedure of Horsch et al. (1985), as modified by Lindbo and Dougherty (1992a). Transformed plants containing the PVY CP related gene sequences were predominately (>95%) haploid and sterile. A section of leaf midvein, 15 to 20 mm in length, from a transformed haploid plant was surface sterilized and cultured in vitro (Kasperbauer and Collins, 1972). Plantlets regenerated from midvein cultures were rooted, then transplanted to soil. More than 75% of the midvein-regenerated plants possessed the normal, diploid (2n = 48) chromosome complement, were fertile, and produced abundant, viable seed.

Analysis of Genomic DNA in Transgenic Plants

Plant genomic DNA was extracted as described by Rogers and Bendich (1988). Genomic DNA was digested with various restriction enzymes using the manufacturer's recommended procedures. DNA gel blotting procedures were performed as described by Sambrook et al. (1989). Gel blots were probed with α-^{32}P-dCTP–labeled PVY CP DNA fragments. CP DNA probes were synthesized by the random prime method of Feinberg and Vogelstein (1984) using a random prime extension labeling kit (Du Pont).

Estimation of Transcription Rates: Nuclear Run-Off Assays

Isolation of nuclei from transgenic plant tissue, in vitro labeling of run-on transcripts, and blot hybridization were as originally described by Cox and Goldberg (1988) and modified by Dougherty et al. (1994).

Analysis of RNA in Transgenic Plants

Total RNA was isolated from transgenic plants by LiCl precipitation (Verwoerd et al., 1989). Denaturing RNA gels and RNA gel blotting were described by Lindbo and Dougherty (1992a, 1992b). Gel blots were hybridized with strand-specific ^{32}P-labeled RNA probes generated from SP6/T7-based cell-free transcription reactions of a plasmid containing a cDNA copy of the PVY CP gene (Lindbo and Dougherty, 1992a; Lindbo et al., 1993b).

Slot blot hybridization was used to estimate the amount of transgene transcript in R1 and R2 plants. Plus or minus sense PVY CP transcripts were generated from SP6/T7-based cell-free transcription vector containing the PVY CP ORF and used to generate a standard curve. Duplicate slot blots were prepared and probed with a PVY strand-specific probe or an actin transcript-specific probe as an internal standard. The slot blots were hybridized with the appropriate ^{32}P-labeled riboprobe, washed, and exposed to Kodak X-Omat x-ray film. The amount of radioactivity hybridizing was estimated by densitometric analysis of exposed x-ray films with a Zeineh soft laser scanning densitometer (model SL-DNA; Biomed Instruments Inc., Fullerton, CA).

Analysis of Transgene-Encoded PVY CP Proteins

The fusion protein used to generate antibody to PVY CP was derived from the bacterial expression vector pGEX-CS (Parks et al., 1994) in *Escherichia coli*. The glutathione *S*-transferase–PVY CP fusion protein was purified using affinity chromatography and glutathione conjugated to agarose beads. The purified glutathione *S*-transferase–PVY CP fusion was injected into rabbits, and antiserum was collected and stored at −20°C (Dougherty and Hiebert, 1980).

Protein Gel Blot Analysis

The PVY CP antiserum was preadsorbed with partially purified tobacco (K326) leaf proteins for 16 hr at room temperature. The precipitate was removed by centrifugation, and the serum was used at a 1:1000 dilution to detect transgene-derived PVY CP in a protein gel analysis procedure described for TEV CP (Lindbo and Dougherty, 1992a).

ELISA

A double-antibody sandwich ELISA (Converse and Martin, 1990) was used to detect PVY CP in plant extracts. A 1:1000 dilution of polyclonal PVY CP antiserum produced in rabbits was used to coat plates, and a 1:500 dilution of PVY CP antiserum conjugated to alkaline phosphatase was used to detect PVY CP antigen.

Whole Plant Inoculation Experiments

Plant leaves were lightly dusted with carborundum, and virus inoculum (50 μL) was applied with a cotton swab. Virus inoculum was at a 1:10 dilution (w/v) of virus-infected plant tissue in deionized distilled water. Plants were typically observed for 30 to 45 days.

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REFERENCES

Allen, G.C., Hall, G.E., Jr., Childs, L.C., Weissinger, A.K., Spiker, S., and Thompson, W.F. (1993). Scaffold attachment regions increase reporter gene expression in stably transformed plant cells. Plant Cell **5,** 603–613.

Assaad, F.F., Tucker, K.L., and Signer, E.R. (1993). Epigenetic repeat-induced gene silencing (RIGS) in Arabidopsis. Plant Mol. Biol. **22,** 1067–1085.

Beachy, R.N. (1993). Transgenic resistance to plant viruses. Semin. Virol. **4,** 327–416.

Burk, L.G., Gerstel, D.U., and Wernsman, E.A. (1979). Maternal haploids of *Nicotiana tabacum* L. from seed. Science **206,** 585.

Converse, R.H., and Martin, R.R. (1990). ELISA methods for plant viruses. In Serological Methods for Detection and Identification of Viral and Bacterial Plant Pathogens, R. Hampton, E. Ball, and S. de Boer, eds (St. Paul, MN: American Phytopathological Society), pp. 179–196.

Cox, K.H., and Goldberg, R.B. (1988). Analysis of plant gene expression. In Plant Molecular Biology: A Practical Approach, C.H. Shaw, ed. (Washington, DC: IRL Press), pp. 1–35.

de Carvalho, F., Gheysen, G., Kushnir, S., Van Montagu, M., Inze, D., and Castresana, C. (1992). Suppression of β-1,3-glucanase transgene expression in homozygous plants. EMBO J. **11,** 2595–2602.

de Haan, P., Gielen, J.J.L., Prins, M., Wijkamp, M.G., van Schepen, A., Peter, D., van Grinsven, M.Q.J.M., and Goldbach, R.W. (1992).

Characterization of RNA-mediated resistance to tomato spotted wilt virus in transgenic tobacco plants. Bio/Technology **10,** 1133–1137.

Dolja, V.V., Haldeman, R., Robertson, N.L., Dougherty, W.G., and Carrington, J.C. (1994). Distinct functions of capsid protein in assembly and movement of tobacco etch potyvirus in plants. EMBO J. **13,** 1482–1491.

Dougherty, W.G., and Hiebert, E. (1980). Translation of potyvirus RNA in rabbit reticulocyte lysate: Identification of nuclear inclusion proteins as products of tobacco etch virus RNA translation and cylindrical inclusion protein as a product of the potyvirus genome. Virology **104,** 174–182.

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

Feinberg, A.P., and Vogelstein, B. (1984). A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. **137,** 266–267.

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.

Grierson, D., Fray, R.G., Hamilton, A.J., Smith, C.J.S., and Watson, C.F. (1991). Does co-suppression of sense genes in transgenic plants involve antisense RNA? Trends Biotechnol. **9,** 122–123.

Hartl, D.L. (1991). Basic Genetics, 2nd ed. (Boston: The Jones and Bartlett Publishing Co.).

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.

Hobbs, S.L.A., Warkentin, T.D., and DeLong, C.M.O. (1993). Transgene copy number can be positively or negatively associated with transgene expression. Plant Mol. Biol. **21,** 17–26.

Horsch, R.B., Fry, J.E., Hoffman, N.L., Eichholtz, D., Rogers, S., and Fraley, R.T. (1985). A simple and general method for transferring genes into plants. Science **227,** 1229–1231.

Kasperbauer, M.J., and Collins, G.H. (1972). Reconstitution of diploids from leaf tissue of anther-derived haploids in tobacco. Crop Sci. **12,** 98–101.

Koonin, E.V., and Dolja, V.V. (1993). Evolution and taxomony of positive-strand RNA viruses: Implications of comparative analysis of amino acid sequences. Crit. Rev. Biochem. Mol. Biol. **28,** 375–430.

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

Lindbo, J.A., and Dougherty, W.G. (1992b). Untranslatable transcripts of the tobacco etch virus coat protein gene sequence can interfere with tobacco etch virus replication in transgenic plants and protoplasts. Virology **189,** 725–733.

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

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

Matzke, M.A., and Matzke, A.J.M. (1991). Differential inactivation and methylation of a transgene in plants by two suppressor loci containing homologous sequences. Plant Mol. Biol. **16,** 821–830.

Matzke, M.A., and Matzke, A.J.M. (1993). Genomic imprinting in plants: Parental effects and *trans*-inactivation phenomena. Annu. Rev. Plant Physiol. Plant Mol. Biol. **44,** 53–76.

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

Mol, J., van Blokland, R., and Kooter, J. (1991). More about cosuppression. Trends Biotechnol. **9,** 182–183.

Napoli, C., Lemieux, C., and Jorgensen, R. (1990). Introduction of a chimeric chalcone synthase gene into petunia results in reversible co-suppression of homolgous genes *in trans.* Plant Cell **2,** 279–289.

Ottaviani, M-P., Smits, T., and Hanisch ten Cate, C.H. (1993). Differential methylation and expression of the β-glucuronidase and neomycin phosphotransferase genes in transgenic plants of potato cv. Bintje. Plant Sci. **88,** 73–81.

Pang, S.-Z., Slightom, J.L., and Gonsalves, D. (1993). Different mechanisms protect transgenic tobacco against tomato spotted wilt and impatiens necrotic spot *Tospoviruses.* Bio/Technology **11,** 819–824.

Parks, T.D., Leuther, K.K., Howard, E.D., Johnston, S.A., and Dougherty, W.G. (1994). Release of proteins and peptides from fusion proteins using a recombinant plant virus proteinase. Anal. Biochem. **216,** 413–417.

Reimann-Philipp, U., and Beachy, R.N. (1993). The mechanism(s) of coat protein-mediated resistance against tobacco mosaic virus. Semin. Virol. **4,** 349–356.

Riechmann, J.L., Lain, S., and García, J.A. (1992). Highlights and prospects of potyvirus molecular biology. J. Gen. Virol. **73,** 1–16.

Rogers, S.O., and Bendich, A.J. (1988). Extraction of DNA from plant tissues. In Plant Molecular Biology Manual, S.B. Gelvin, R.A. Schilperoort, and D.P.S. Verma, eds (Dordrecht, The Netherlands: Kluwer Academic Publishers), pp. A6-1–A6-10.

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

Schiebel, W., Haas, B., Marinković, S., Klanner, A., and Sänger, H.L. (1993a). RNA-directed RNA-polymerase from tomato leaves. I. Purification and physical properties. J. Biol. Chem. **263,** 11851–11857.

Schiebel, W., Haas, B., Marinković, S., Klanner, A., and Sänger, H.L. (1993b). RNA-directed RNA polymerase from tomato leaves. II. Catalytic *in vitro* properties. J. Biol. Chem. **263,** 11858–11867.

Seymour, G.B., Fray, R.G., Hill, P., and Tucker, G.A. (1993). Down-regulation of two non-homologous endogenous tomato genes with a single chimaeric sense gene construct. Plant Mol. Biol. **23,** 1–9.

Smith, C.J.S., Watson, C.F., Bird, C.R., Ray, J., Schuch, W., and Grierson, D. (1990). Expression of a truncated tomato polygalacturonase gene inhibits expression of the endogenous gene in transgenic plants. Mol. Gen. Genet. **224,** 477–481.

Taylor, J.W., Schmidt, W., Cosstick, R., Odruszed, A., and Eckstein, F. (1985a). The use of phosphorothioate-modified DNA in restriction enzyme reactions to prepare nicked DNA. Nucl. Acids Res. **13,** 8749–8764.

Taylor, J.W., Ott, J., and Eckstein, F. (1985b). The rapid generation of oligonucleotide-directed mutations at high frequency using phosphorothioate-modified DNA. Nucl. Acids Res. **13,** 8765–8785.

van der Krol, A.R., Mur, L.A., Beld, M., Mol, J.N.M., and Stuitje, A.R. (1990). Flavonoid genes in petunia: Addition of a limited number of gene copies may lead to a suppression of gene expression. Plant Cell **2,** 291–299.

van der Vlugt, R.A.A., Ruiter, R.K., and Goldbach, R. (1992). Evidence for sense RNA-mediated protection to PVY^N in tobacco plants transformed with the viral coat protein cistron. Plant Mol. Biol. **20,** 631–639.

Verwoerd, T.C., Deker, B.M.M., and Hoekema, A. (1989). A small scale procedure for the rapid isolation of plant RNAs. Nucl. Acids Res. **17,** 2362.

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

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