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RNA-Mediated Resistance with Nonstructural Genes from the Tobacco Etch Virus Genome

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Sense RNA-mediated virus resistance has been described for transgenic plants expressing potyviral capsid protein sequences. This study was undertaken to determine if expression of other viral sequences could induce this type of virus resistance. Plants showing highly resistant or 'recovery' phenotypes were generated by expressing the tobacco etch virus (TEV) 6 kDa/21 kDa reading frames. Expression of translatable or untranslatable versions of this TEV sequence produced resistant lines. Highly resistant and recovery phenotype plants had biochemical hallmarks observed with resistant plants expressing TEV coat protein sequences. High transcription rates with low steady-state levels of the transgene transcript generally correlated with resistance. During recovery and induction of the resistance, RNA and protein steady-state levels decreased 5- to 20-fold, while transcription of the transgene continued at a similar level. A posttranscriptional, cellular system eliminating sequences contained in the transgene transcript and viral genome would be consistent with the results.

The expression of transgenes in various organisms has become commonplace over the past 5 years. This technical approach has often been used to increase the expression of a gene. However, the effective concentration of a gene product has also been decreased by expressing transgenes that contain a dominant negative mutation or via a sense-suppression strategy. Virologists have used similar approaches to engineer plants that either complement a virus mutation (Holt and Beachy 1991; Dolja et al. 1994; Li and Carrington 1995) or are resistant to plant virus infection (review, see Wilson 1993). The application of pathogen-derived resistance and dominant negative mutations has been pursued quite successfully with viruses that have an RNA genome. Representatives of most genes found in RNA virus genomes have now been expressed in plants and a continuum of resistance phenotypes, ranging from complete resistance to attenuation or temporal delay in symptoms appearance have been noted (for reviews, see Wilson 1993; Scholthof et al. 1993). The expectation in these cellular and viral studies is that the transgene-derived protein interferes with the normal function of the targeted gene product to produce the desired phenotype or resistance (Sanford and Johnston 1985; Herskowitz 1987; Szybalski 1991).

We have described a phenomenon referred to as sense RNA-mediated virus resistance and have speculated on a possible mechanism by which resistance is engendered (Lindbo et al. 1993; Dougherty et al. 1994, Smith et al. 1994). Sense RNA-mediated resistance appears to be a host-mediated response, may be analogous to selected cases of sense suppression of endogenous genes in transgenic organisms, and does not require a transgene-derived translation product. Our previous efforts have centered on expression of coat protein sequences from two viruses. Expression of translatable or untranslatable transgenes of tobacco etch virus (TEV) or potato virus Y (PVY) coat protein sequences could result in a highly resistant state, although the frequency of resistance was higher with the untranslatable version of the gene (Lindbo and Dougherty 1992; Smith et al. 1994). Here, we show that by expressing another part of a potyviral genome, namely the TEV 6- and 21-kDa open reading frames (ORFs), we are able to generate virus-resistant plants that have features consistent with sense RNA-mediated virus resistance. A strategy is discussed that will further enhance the efficacy and safety of RNA-mediated resistance.

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RESULTS

Construction and analysis of 6/21 transgenes.

TEV nonstructural gene sequences were examined in this study. Three different transgenes were constructed and are depicted schematically in Figure 1. The transgene that coded for a translatable (= T) mRNA that expresses the TEV 6- and 21-kDa proteins as a polyprotein was referred to as 6/21T. The transgene that coded for an untranslatable (= U) version of the same ORFs was referred to as 6/21U. The 6/21T and 6/21U transgenes differed at two nucleotide positions. Finally, a truncated version of the 6/21T transgene (6/21ΔN) was generated during our cloning into the binary plasmid vector. This construct contained nucleotide sequences corresponding to the C-terminal 113 amino acids of the 21-kDa ORF. The transcript derived from this transgene is likely untranslatable; the first two AUG codons were ~30 and 60 nucleotides into the ORF and out of frame, and the first in-frame AUG codon was ~70 nt from the beginning of the sequence and in a poor translation context.

Transgenic plant lines established.

Haploid *Nicotiana tabacum* cvs. Burley 49 and K326 were transformed, putative haploid transformants were identified, and chromosomes were doubled to generate homozygous doubled haploid (DH) transgenic lines. Multiple lines (2 to 5) were established for most of the transformation events and seeds were collected from a total of 361 DH transgenic lines. Progeny from 168 DH transgenic lines were analyzed in pathology assays (described below) and in Northern gel blot analyses. Of the 168 DH transgenic lines analyzed, 122 represented independent transformation events as follows: 45 expressed the 6/21T transgene, 29 expressed the 6/21U transgene, and 48 expressed the 6/21ΔN transgene. A preliminary resistance screen of haploid and DH plants was carried out by mechanically inoculating TEV onto the haploid plant and progeny of the DH transgenic plants. The resistance phenotypes and results are summarized in Tables 1 and 2. There were five notable observations. First, the resistance phenotype of the haploid state was not always an accurate predictor of resistance in the DH (= diploid) condition (Table 2). Lines that were symptomless in the haploid state often showed symptoms in the DH condition. (We recorded haploid inoculated plants as symptomless instead of highly resistant, as only a single plant could be inoculated and we could not exclude the possibility of an infection that did not take.) Conversely, some susceptible haploid lines displayed a TEV-resistant phenotype following chromosome doubling. Second, the recovery phenotype was the prominent resistance phenotype generated with DH plants containing transgenes that express the 6/21 ORF (Table 1). Third, there were a limited number of highly resistant lines generated (Table 1). Fourth, the truncated version of the 6/21 ORF was largely ineffectual in generating TEV resistant germplasm (Tables 1 and 2). Finally, there were a few lines that displayed a nonuniform response to TEV infection. Of the initial 122 DH lines examined, 52 were selected (with a bias in selecting resistance phenotypes) to be studied in greater detail. These lines are listed in Table 2.

Phenotypic analysis of the selected 6/21 DH transgenic lines.

The DH lines underwent an extensive challenge inoculation test with different isolates of TEV and PVY-nn, a PVY isolate that induces veinal necrosis on tobacco. Results of inoculation studies using the not-aphid transmitted isolate of TEV (TEV-NAT) are described in Table 1 and summarized in Table 2. A limited number of lines, such as TB-6/21T6.2, TK-6/21T22.1, and TK-6/21U23.2 displayed a high level of resistance to TEV replication. Virus-induced symptoms were never observed and virus could not be detected in ELISA or

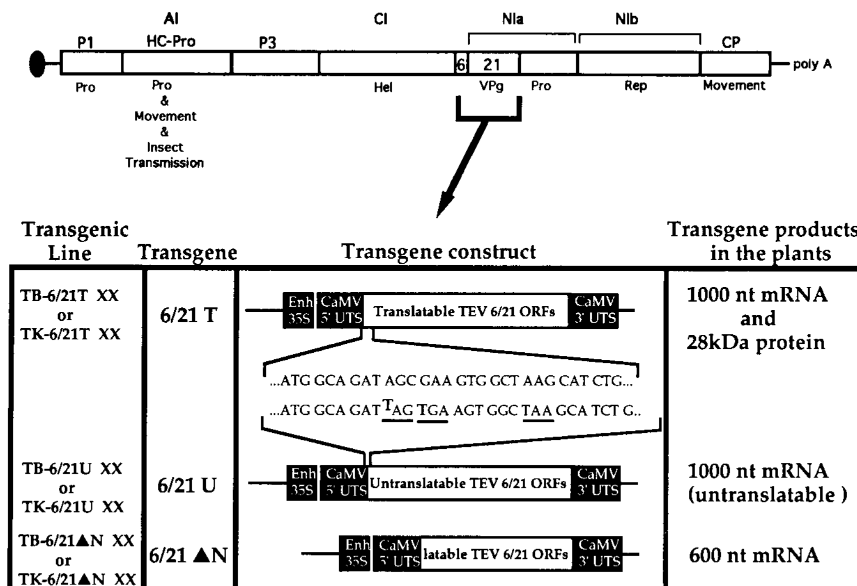


Fig. 1. A schematic representation of tobacco etch virus (TEV) genome and nonstructural transgenes. The TEV genome is presented at the top and various coding regions are labeled. The TEV genome is presented as a long rectangular box with thin lines at the 5′ and 3′ ends representing the untranslated regions. The genome-linked viral protein (VPg) ( ) and polyadenylate region (poly A) are labeled. At the top of the drawing, the gene products that constitute the various inclusion bodies produced during infection are labeled; AI, amorphous inclusion; CI, cytoplasmic cylindrical inclusion body, NIa and NIb, nuclear inclusion body proteins. Above the viral genome various gene products are labeled; P1, protein 1; HC-Pro, helper component-proteinase; P3, protein 3; CP, capsid protein. Below the viral genome, the different functions or activities associated with the various gene products are presented. Proteinase (Pro), helicase (Hel), putative replicase (Rep) activity, VPg, and proteins that participate in various movement activities are shown. An internal genome segment (above the bracket) coding for the 6- and 21-kDa proteins was copied into cDNA, ligated into a binary vector system, and introduced into plants using an *Agrobacterium* transformation procedure. The relevant portions of the inserted DNA containing TEV sequences are presented in the box at the bottom of the figure. Each virus-derived transgene has an enhanced version of the cauliflower mosaic virus (CaMV) 35S promoter (in bold) and 5′ and 3′ untranslated sequences (UTS)[ ] also derived from the CaMV genome. The TEV 6- and 21-kDa (6/21) ORFs were inserted just downstream of the 5′ CaMV UTS. Two complete versions were expressed: a translatable and an untranslatable version of the TEV sequences. Two nucleotide differences (in bold) and the resulting 3 stop codons (underlined) differentiate the translatable transgene (6/21T) from the untranslatable version (6/21U). Taking advantage of an internal *Bam*HI restriction endonuclease site, the N-terminal 103 codons were deleted to generate a transgene (6/21ΔN) that could encode the C-terminal 113 amino acids of the 21-kDa protein. Transgenic line nomenclature is as follows: TK or TB indicates the variety of tobacco transformed (TK = *Nicotiana tabacum* cv. K326; TB = *Nicotiana tabacum* cv. Burley 49); 6/21T, 6/21U or 6/21ΔN describes the transgene being expressed; the XX indicates the specific transgenic line number.

after back inoculation to *N. tabacum* cv. Burley 21. A high percentage of lines showed the recovery phenotype when inoculated with TEV. The asymptomatic recovered tissue could not be reinfected with TEV but could be infected with PVY (data not shown). We noticed a distinct difference in the time frame required for recovery to be manifested. Many lines showed complete recovery 7 to 10 days after a systemic infection was manifested; in others, recovery was noted only after 28 to 35 days and just prior to flowering. A number of lines were susceptible but showed altered symptoms when inoculated with TEV. Instead of a typical systemic mosaic, symptoms ranged from minor chlorotic vein banding to the appearance of many large (1 cm in diameter) chlorotic spots on a leaf (blotchy). Finally, in a limited number of lines, plants within a single line displayed responses ranging from susceptibility to slow recovery or recovery to the highly resistant state. These lines were DH and homozygous for the transgene(s). Line TB-6/21U1.3 displayed this phenotype. When a population of plants within this line was examined, plants with a highly resistant and a recovery phenotype were observed.

There was no difference in resistance when TEV-HAT or TEV-Oxnard strains were used as inoculum. All 52 of the selected 6/21 DH transgenic lines were susceptible to PVY infection.

Biochemical analysis of selected transgenic lines.

We next determined if the expected transgene-derived RNAs and/or protein were being expressed in the various transgenic lines. Northern blot hybridizations were conducted with total RNA samples extracted from leaf tissue from different transgenic lines. Representative hybridization gel blots are shown in Figure 2. The expected transcript was detected in most lines. Lines containing the 6/21T and 6/21U transgenes had an RNA with an estimated molecular size of 1,000 nt (Fig. 2), while plants expressing the 6/21ΔN transgene had a smaller transgene-derived transcript (600 nt, Fig. 2C). Varying levels of transcripts were detected in the different unchallenged lines, although the three highly resistant lines

Table 1. Response of tobacco germplasm expressing a tobacco etch virus (TEV) 6/21 transgene to infection with TEV

	Disease reaction[b]				
Genotype[a]	Susceptible	Altered	Recovery	Highly resistant	Nonuniform
TB-6/21T XX	5	1	7	1	2
TK-6/21T XX	14	0	13	1	1
TB-6/21U XX	4	2	2	0	1
TK6/21 U XX	7	0	11	1	1
TB-6/21 ΔN XX	11	2	0	0	0
TK-6/21 ΔN XX	33	1	1	0	0

[a] The nomenclature followed for the genotypes examined is explained in Figure 1.

[b] The reaction of the 6/21 transgenic plants to infection by TEV was grouped into one of five different responses. Susceptible: Plants became infected and displayed wild type, TEV-induced symptoms 4 to 6 days after inoculation. Altered: Plants became infected with TEV but showed symptoms not typically associated with TEV infection of burley or flue-cured tobacco. These included pronounced vein banding, mild mosaic, or large chlorotic spots (blotchy) up to a centimeter in diameter. Recovery: Plants initially became infected but as new leaves emerged virus-induced symptoms and virus were restricted to interveinal areas. Eventually a leaf emerged that was free of symptoms and virus. All subsequent tissue that developed was free of virus. Highly resistant: Transgenic plants could not be infected with TEV and displayed no symptoms. Nonuniform: A line that did not display a homogeneous symptom response after TEV inoculation.

Table 2. Summary of selected 6/21 transgenic lines

	Haploid transformant	Doubled haploid transformant	
Transgenic lines	Phenotype	Phenotype	RNA level
6/21T			
TB-6/21T5.1	Recovery	Recovery	High
TB-6/21T5.2	Recovery	Recovery	High
TB-6/21T5.3	Recovery	Slow recovery	High
TB-6/21T5.5	Susceptible	Recovery	High
TB-6/21T6.1	Symptomless	Recovery	High
TB-6/21T6.2	Symptomless	Highly resistant	Low
TK-6/21T5.7	Symptomless	Recovery	High
TK-6/21T6.1	Susceptible	Susceptible	Not detected
TK-6/21T8.1	Susceptible	Recovery	High
TK-6/21T8.2	Symptomless	Recovery	High
TK-6/21T8.3	Symptomless	Slow recovery	Low
TK-6/21T10.1	Symptomless	Recovery	High
TK-6/21T11.1	Symptomless	Susceptible	Not detected
TK-6/21T21.1	Recovery	Recovery	High
TK-6/21T21.2	Susceptible	Recovery	High
TK-6/21T22.1	Susceptible	Highly resistant	Low
TK-6/21T22.4	Susceptible	Susceptible	Low
TK-6/21T23.2	Susceptible	Susceptible	High
6/21U			
TB-6/21U1.1	Susceptible	Susceptible	Low
TB-6/21U1.2	Recovery	Recovery	High
TB-6/21U1.3	Susceptible	Nonuniform	Low
TB-6/21U1.4	Susceptible	Susceptible	Moderate
TB-6/21U2.1	Symptomless	Altered	Moderate
TB-6/21U3.1	Recovery	Slow recovery	High
TB-6/21U4.2	Symptomless	Recovery	High
TK-6/21U1.1	Symptomless	Recovery	High
TK-6/21U1.3	Susceptible	Recovery	High
TK-6/21U2.5	Susceptible	Susceptible	Not detected
TK-6/21U21.1	Symptomless	Recovery	Low
TK-6/21U21.2	Susceptible	Recovery	High
TK-6/21U22.1	Symptomless	Recovery	Moderate
TK-6/21U22.2	Symptomless	Recovery	High
TK-6/21U22.3	Symptomless	Recovery	High
TK-6/21U22.4	Altered	Susceptible	Low
TK-6/21U23.1	Symptomless	Susceptible	Low
TK-6/21U23.2	Symptomless	Highly resistant	Low
TK-6/21U25.2	Susceptible	Recovery	High
TK-6/21U25.5	Susceptible	Susceptible	Not detected
TK-6/21U26.1	Susceptible	Susceptible	Low
TK-6/21U26.2	Altered	Susceptible	Moderate
TK-6/21U26.3	Symptomless	Recovery	High
6/21ΔN			
TB-6/21ΔN1.1	Symptomless	Susceptible	Low
TB-6/21ΔN1.3	Susceptible	Slow recovery	Low
TB-6/21ΔN2.2	Susceptible	Slow recovery	Moderate
TB-6/21ΔN3.2	Susceptible	Susceptible	Moderate
TB-6/21ΔN8.1	Susceptible	Susceptible	Low
TK-6/21ΔN3.1	Susceptible	Susceptible	Moderate
TK-6/21ΔN4.2	Susceptible	Susceptible	Low
TK-6/21ΔN8.5	Susceptible	Susceptible	Very low
TK-6/21ΔN11.3	Susceptible	Slow recovery	Moderate
TK-6/21ΔN23.3	Symptomless	Altered	High
TK-6/21ΔN23.4	Susceptible	Susceptible	Low

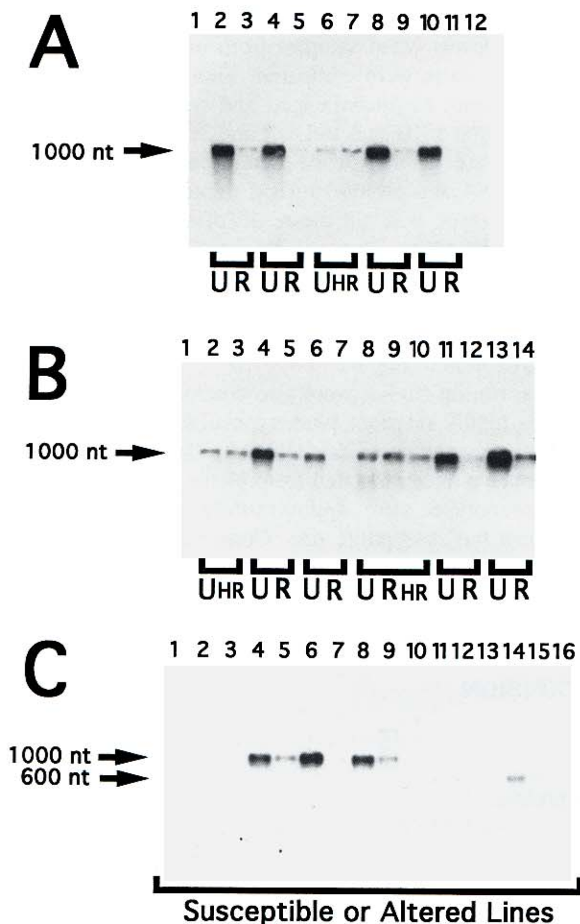


Fig. 2. Northern gel blot analyses of the steady-state levels of 6/21 transgene transcripts. Total RNA was isolated from leaf tissue of 6/21 doubled-haploid (DH) transgenic lines. The RNA (5 μg) was denatured andelectrophoretically separated in 1.2% agarose gels containing formaldehyde. The RNA was transferred to nitrocellulose and the blot was hybridized with ^{32}P-labeled RNA complementary to the TEV 6 and 21-kDa ORFs. Pictures of autoradiograms are presented. **A and B,** RNA from transgenic plants that have a resistant phenotype; **C,** RNA from susceptible plants. Molecular size markers were used to estimate transcript size (1,000 and 600 nt), shown on the left of each panel. The plant phenotype is presented beneath the figure as either unchallenged (U), recovery (R), or highly resistant (HR). **A,** Total RNA from leaf tissue of unchallenged plants and an inoculated sibling showing either a recovery or highly resistant phenotype. The following plants were used in this study: Lanes 1 and 12, untransformed *N. tabacum* cv. Burley 49; lanes 2 and 3, TB-6/21T5.1; lanes 4 and 5, TB-6/21T6.1; lanes 6 and 7, TB-6/21T6.2; lanes 8 and 9, TK-6/21T21.1; lanes 10 and 11, TK-6/21T21.2. **B,** Total RNA from unchallenged plants and their inoculated sibling showing either a recovery or highly resistant phenotype. The following plants were analyzed in this study: lane 1, untransformed *N. tabacum* cv. Burley 49; lanes 2 and 3, TK-6/21T22.1; lanes 4 and 5, TB-6/21U1.2; lanes 6 and 7, TK-6/21U4.2; lanes 8, 9, and 10, TB-6/21U1.3; lanes 11 and 12, TK-6/21U1.1; lanes 13 and 14, TK-6/21U25.2. **C,** Total RNA from various 6/21 transgenic plants that are susceptible to TEV infection. Upon infection with TEV, these transgenic lines display altered or wild-type TEV-induced symptoms. Plants used in this analysis were uninoculated and were: lane 1, TK-6/21T6.1; lane 2, TK-6/21T11.1; lane 3, TK-6/21T22.4; lane 4, TB-6/21U1.1; lane 5, TB-6/21U1.4; lane 6, TB-6/21U2.1; lane 7, TK-6/21U2.5; lane 8, TK-6/21U22.4; lane 9, TK-6/21U23.1; lane 10, TB-6/21ΔN1.1; lane 11, TB-6/21ΔN8.1; lane 12, TK-6/21ΔN4.2; lane 13, TK-6/21ΔN8.5; lane 14, TK-6/21ΔN11.3; lane 15, TK-6/21ΔN23.4; lane 16, untransformed *N. tabacum* cv. Burley 49.

always had low steady-state levels of the transcript. A few lines did not have detectable levels of the transgene transcript (Fig. 2C, lanes 1 to 3 and 13). An examination of genomic DNA of many of these lines revealed multiple copies of the 6/21 transgene were present (data not shown). These lines were always susceptible to TEV infection. The relative steady-state levels of the transgene-derived transcript are summarized in Table 2.

We examined a number of the 6/21T and 6/21ΔN transgenic lines for the presence of the expected translation product. Translation of the 6/21 ORFs should result in the accumulation of a ~28-kDa protein detectable with antiserum raised against the TEV nuclear inclusion a (NIa) polyprotein. A cocktail of monoclonal antibodies (Slade et al. 1989) that reacted with epitopes present in the N-terminal half of the TEV NIa polyprotein was used in this study. In nearly all transgenic plants expressing the 6/21T transgene, a protein with a molecular weight of ~28 kDa could be readily detected in Western blot studies (Fig. 3). However, no protein was detected in the two highly resistant lines that expressed the 6/21T transgene. The analysis of one of these lines (TK-6/21T22.1) in a Western gel blot is shown in Figure 3 (lanes 5 and 6). These lines also have low steady-state levels of transgene transcript (Figs. 2A and B).

An immunoreactive protein was never detected in transgenic plants expressing the 6/21U or 6/21ΔN transgenes (data not shown). These results were confirmed in ELISA analysis (Martin and Converse 1990) of selected lines.

Characteristics of plants with the recovery phenotype.

The most common virus resistance phenotype encountered was the recovery phenotype. Plants initially became infected and displayed typical TEV-induced symptoms. Protein and RNA steady-state levels were assessed in unchallenged and recovered leaf tissue of the same transgenic line. The trans-

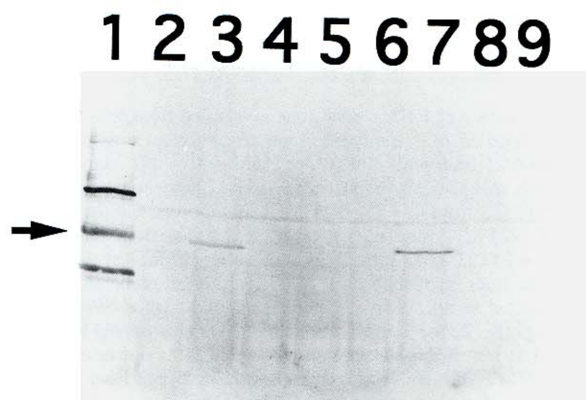
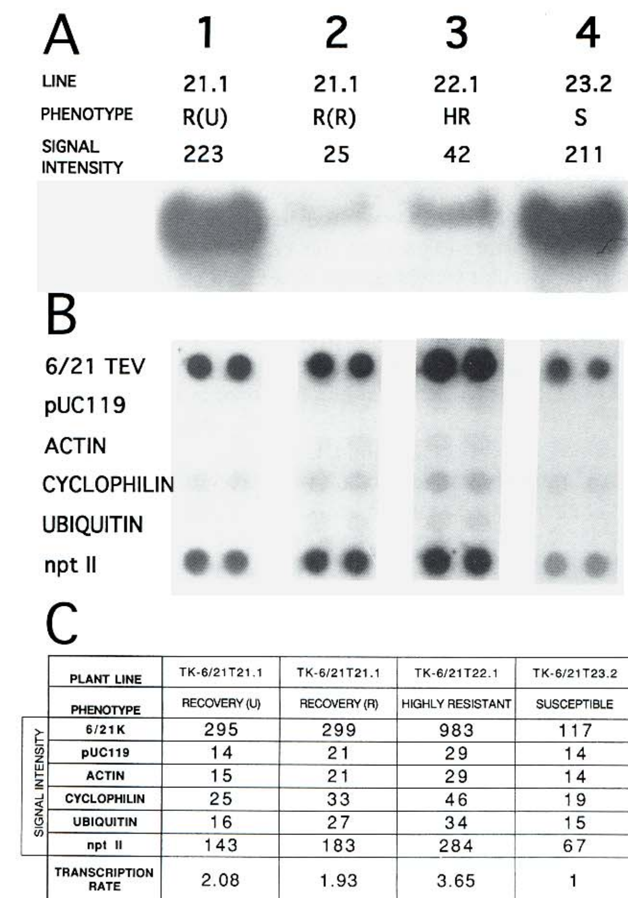


Fig. 3. Western gel blot analysis of 6/21T transgenic lines. Total protein samples were made from leaf tissue of various plants containing the 6/21T transgene. Proteins were separated by electrophoresis in a 12.5% polyacrylamide gel containing sodium dodecyl sulfate and electroblotted to nitrocellulose. The TEV6/21 polyprotein was detected using a cocktail of monoclonal antibodies specific for the 21-kDa TEV protein. The arrow on the left indicates the estimated position in the gel an ~28-kDa protein should migrate. Lane 1, 1 μg of a TEV nuclear inclusion preparation; lane 2, *N. tabacum* cv. Burley 49; lanes 3 and 4, unchallenged and recovered tissue from TK-6/21T21.1; lanes 5 and 6, unchallenged and highly resistant tissue from TK-6/21T22.1; lanes 7 and 8, unchallenged and recovered tissue from TK-6/21T21.2; lane 9, *N. tabacum* cv. Burley 49.

gene transcript always accumulated to readily detectable levels in unchallenged plants containing either the 6/21T or 6/21U transgene. However, transgene-derived transcript levels decreased 5- to 20-fold in tissue of the same plant line displaying the recovery phenotype (Fig. 2A and B). Lines expressing the 6/21T transgene and displaying the recovery phenotype also showed a significant decrease in 6/21-kDa polyprotein levels when samples from unchallenged and recovered leaf tissue were compared. This can be seen in the Western gel blot of unchallenged and recovered tissue presented in Figure 3 (lanes 3 and 4; 7 and 8).



	PLANT LINE	TK-6/21T21.1	TK-6/21T21.1	TK-6/21T22.1	TK-6/21T23.2
	PHENOTYPE	RECOVERY (U)	RECOVERY (R)	HIGHLY RESISTANT	SUSCEPTIBLE
SIGNAL INTENSITY	6/21K	295	299	983	117
	pUC119	14	21	29	14
	ACTIN	15	21	29	14
	CYCLOPHILIN	25	33	46	19
	UBIQUITIN	16	27	34	15
	npt II	143	183	284	67
	TRANSCRIPTION RATE	2.08	1.93	3.65	1

Fig. 4. Analysis of RNA steady-state levels and transgene transcription rates of selected 6/21 transgenic lines. Four tissue samples from three different 6/21 transgenic lines were examined in this study; leaf tissue from both an unchallenged (U) plant and a plant showing the recovery phenotype (R) from line TK-6/21T21.1; highly resistant leaf tissue from line TK-6/21T22.1 and unchallenged leaf tissue from the susceptible line TK-6/21T23.2. **A,** Total RNA was isolated from leaf tissue of three transgenic lines, separated on a 1.2% agarose gel containing formaldehyde, blotted to nitrocellulose, and hybridized with a ^{32}P-labeled TEV specific probe. Lanes 1 and 2, unchallenged and recovered tissue of TK-6/21T21.1; lane 3, highly resistant TK-6/21T22.1; and lane 4, susceptible TK-6/21T23.2. **B,** Nuclei were isolated from leaf tissue of the lines used in A and nuclear run-on studies were conducted to assess transgene transcription rates. The ^{32}P-labeled RNAs synthesized were hybridized to nitrocellulose membrane containing dot blots of DNA (5 μg) of the following genes contained in plasmids: TEV 6/21-kDa ORFs, pUC119, cyclophilin, actin, ubiquitin, and neomycin phosphotransferase (npt II). An autoradiogram of a typical experiment is presented. **C,** The nitrocellulose membranes with DNA and hybridized ^{32}P transcripts of nuclear run on reactions were analyzed in a phosphorimager. The level of 6/21 transgene transcription was compared between nuclei source by first normalizing transcription rates between lines using control gene levels. Based on these normalizations, relative TEV 6/21 transgene transcription levels were calculated and are presented as rate of transcription relative to line TK-6/21T23.2 which shows a TEV susceptible phenotype.

The decrease in the steady-state level of the transgene products (RNA and protein) in leaf tissue displaying the recovery phenotype was not the result of altered transcription rates of the transgene. This was determined in nuclear run-on studies. Nuclei from unchallenged and recovered leaf tissue of line TK-6/21T21.1 indicated a similar level of transgene transcription, while a ninefold difference in RNA steady-state level was exhibited (Fig. 4).

Nuclear run-on studies were also conducted with lines that showed a highly resistant phenotype (TK-6/21T22.1) and a susceptible phenotype (TK-6/21T23.2). Transgene transcription rates from nuclei isolated from plants with the highly resistant phenotype were approximately three- to fourfold higher than the susceptible line. Conversely, the susceptible line had RNA steady-state levels estimated to be ~5-fold higher than the highly resistant line. These results are presented in Figure 4.

DISCUSSION

This study was undertaken to assess whether transgene expression of a region of the potyvirus genome, other than the coat protein, could establish RNA-mediated virus resistance. The complete 6/21 ORFs were effective regardless of whether the transgene generated a translatable or untranslatable mRNA transcript. This was in contrast to our previous studies with different transgenes expressing potyviral coat protein sequences (Lindbo and Dougherty 1992; Smith et al. 1994). In these coat protein studies, the untranslatable version of the coat protein ORF elicited the highly resistant phenotype at a greater frequency. Transgenes containing TEV 6/21-kDa ORF sequences in the antisense orientation or lacking the TEV 6/21-kDa ORF sequences were not analyzed in this study; in our past studies with TEV and PVY, antisense sequences were largely ineffectual (Lindbo and Dougherty 1992; Smith et al. 1994). Two general types of resistance were observed; the highly resistant phenotype, a form of resistance that is 'on', and the recovery phenotype, an inducible form of the resistance. The highly resistant phenotype has been observed with plants expressing translatable and untranslatable coat protein sequences of TEV (Lindbo and Dougherty 1992; Dougherty et al. 1994) and PVY (Smith et al. 1994). The recovery phenotype has been reported for TEV (Lindbo and Dougherty 1992; Lindbo et al. 1993) and peanut stripe potyvirus (Cassidy and Nelson 1995). Both forms of resistance had biochemical hallmarks of RNA-mediated resistance. Highly resistant plants have transgenes that are transcribed at 'high' rates, but accumulate steady-state levels of the transgene products at a reduced or 'low' level. Induction of resistance, manifested as a recovery phenotype, correlated with a decrease in RNA (and protein) steady-state levels without a concomitant decrease in transgene transcription. A cytoplasmic host system that would recognize specific overexpressed RNA sequences and eliminate them has been proposed to account for the resistant state (Lindbo et al. 1993; Dougherty et al. 1994).

Other potyviral nonstructural protein gene sequences have been expressed from transgenes, resulting in resistant plant phenotypes. The NIa polyprotein of tobacco vein mottling virus (Maiti et al. 1993) and PVY (Vardi et al. 1993) has been expressed and a limited number of plants displayed some level of resistance. Audy and colleagues (1994) have also expressed the NIb protein of PVY. This protein has the sequence characteristics associated with replicases of RNA viruses. Lines expressing a complete NIb were shown to be resistant to PVY, while resistance was lost or decreased in lines with specific mutations or with truncated versions of the NIb ORF. The authors suggest from their data that a functional NIb protein is important in establishing the resistant state.

The 6/21 series of transgenic plants is part of a larger ongoing study to assess the effect of different viral sequences expressed alone or together. A series of plants expressing the NIa ORF (21 + 27-kDa ORFs; see Fig. 1) or the 3'-terminal half of the NIa (27-kDa ORF) have also been constructed and are being examined. Preliminary data suggest plants expressing the NIa ORF elicit the highly resistant state more frequently than the 6/21 ORF or the 27-kDa ORF alone (L. Silva-Rosales and W. G. Dougherty, unpublished). Additionally, the 6/21ΔN plants, which only contain ~60% of the 6/21 transgene sequence, were largely ineffectual at eliciting a resistance phenotype. The straightforward interpretation of this result is that the size of the transgene RNA is crucial in establishing the resistance response. However, we cannot rule out that there may also be nucleotide sequences or secondary structures which are 'more effective' at eliciting the response. It is likely that both aspects will contribute. Therefore, we conclude any viral ORF likely has the potential to elicit an RNA-mediated resistance response, but size and nucleotide sequence will affect the frequency at which it is established.

Transgene copy number also appears to be critical in eliciting RNA-mediated resistance and observations from this study form a corollary with previous studies. We have previously noticed that susceptible transgenic germplasm frequently has either one to two or many (>5) copies of the transgene (Smith et al. 1995; Dougherty et al. 1994). We suggest there is a level of transcript accumulation, with lower and upper limits, that must be attained to activate the post-transcriptional, cytoplasmic system responsible for sense RNA-mediated virus resistance. Lines containing one transgene copy, in general, appear unable to attain a sufficiently high level of transcripts in the cytoplasm, thus the lower threshold level is not reached and the cytoplasmic degradation system is not triggered. Lines expressing many copies likely exceed an upper limit and the cytoplasmic system cannot effectively regulate transcript level. Control of these transgenes would be relegated to a nuclear process and possibly a gene silencing strategy (reviews, Matzke and Matzke 1995a,b). Resistance phenotype changes following chromosome doubling may also be suggestive of critical transgene copy number. In haploid lines exhibiting resistance, the cytoplasmic system is activated. However, doubling the chromosomes (and the effective transgene copy number) results in the upper transcript threshold level being exceeded. At this point, a nuclear regulatory mechanism is engaged and these transgenes are transcriptionally inactivated. Hence, resistance is lost and a very low to undetectable steady-state level of the transgene transcript is observed. In lines where the haploid plant was susceptible but the DH derivative was highly resistant, we suggest the necessary level of transcription was achieved during chromosome doubling to activate the post-transcriptional process. Cytoplasmic and nuclear regulatory systems that control mRNA levels have been hypothesized for plants (review, Sullivan and Green 1993), but how these processes operate mechanistically and are coordinately regulated remains an interesting question with an elusive answer.

Our understanding of sense RNA-mediated resistance is approaching a level where it should be possible to design a transgene that will have increased efficacy at establishing the resistant state and will decrease the perceived risk to the environment (Rissler and Mellon 1993; Falk and Bruening 1994). What should be the characteristics of the 'ideal' viral resistance transgene?

A virus resistance transgene cannot adversely affect agronomic characteristics of the target crop. Expressing potyviral sequences affected the agronomic properties of transgenic tobacco (Whitty et al. 1994) and appeared to do so in a transgene dependent manner. These effects must be negated or minimized.

Additionally, transgenes that confer virus resistance should be designed to minimize possible interactions with other viruses. A number of possible virus/transgene interactions have been suggested that might pose environmental concerns (deZoeten 1991; Tepfer 1993). Some of these have been documented to occur (Farinelli et al. 1992; Green and Allison 1994). A transgene that codes for an untranslatable mRNA has significant advantages over transgenes that express a translatable mRNA and make a protein. With no viral protein present in the cell, the possible complementation of naturally occurring viral mutants and transencapsidation of other viruses are eliminated. Transgenes expressing an untranslatable RNA still produce an RNA species that could undergo recombination with a viral genome. However, by crafting a transgene with various safeguards it should be possible to negate this scenario also.

Viral sequences that are most effective at establishing resistance should be used. Preestablishing the highly resistant state and preventing virus replication is preferred to permitting virus replication followed by inducing the resistance. Such a scenario would decrease the chance of selecting for a mutant or recombination event that results in a virus that overcomes the resistance. A chimeric transgene, consisting of various segments of the viral genome that are most effective at establishing the resistant state would be preferred along with the insertion of stop codons throughout this sequence. The chimeric nature of the gene and the stop codons would help to ensure that any recombination event that did occur would not result in the formation of a viable virus. Additionally, the chimeric nature of the gene would provide multiple virus sequences to target.

Finally, concerns have also been expressed about the transfer of transgenes to weed species (Rissler and Mellon 1993; Regal 1994) and the increased competitive nature this might confer. Understanding the transgene requirement (i.e., copy number, promoter strength, chromosome location, etc.) needed to activate the process should identify homozygous transgene configurations and dosages that in the heterozygous (outbred) state no longer activate resistance. Strategies and technologies currently exist that should permit the safe and efficacious deployment of transgenic virus resistance.

MATERIALS AND METHODS

Construction of transgenes.

Transgenes were derived from cDNA of the TEV genome contained in the plasmid pTL-5473 (Carrington and Dougherty 1987) using specific primers and the polymerase chain reaction. The sequence for the translatable version of the 6- and 21-kDa TEV ORFs (6/21 kDa) was amplified. The 6-kDa protein has been speculated to have a membrane-binding domain and to serve a role in associating the viral replication complex to the plasma membrane. The 21-kDa protein is the genome-linked protein (VPg) covalently attached to the 5′ terminal nucleotide of the viral genome. The coding sequence for these two proteins was similar to the original TEV sequence with two exceptions. The N-terminal serine codon was replaced with methionine-alanine codons and the C-terminal glutamine codon was replaced with a stop codon. The untranslatable version of the 6/21-kDa ORFs was generated by site-directed mutagenesis, wherein two nucleotide changes downstream of the initiation codon were made; a thymidine was inserted and a cytidine was changed to a thymidine. These two changes resulted in the generation of three stop codons downstream of the initiation codon.

The cDNAs of the translatable and untranslatable versions of the 6/21 ORF were partially digested with the restriction enzyme *Bam*HI, DNA fragments were isolated from a gel, and ligated into the plasmid pPEV-6 (Lindbo and Dougherty 1992). The TEV sequence used to generate the 6/21ΔN transgene was obtained by a complete *Bam*HI digest of the 6/21T coding sequence. There is an internal *Bam*HI site in the 21-kDa ORF which is located at nucleotides 5917 to 5923 of the TEV genomic sequence (Allison et al. 1986). All three 6/21-related sequences in pPEV-6 were introduced into *Agrobacterium tumefaciens* by triparental mating (Ditta et al. 1980).

Construction of transgenic plants.

Haploid leaf tissue of tobacco cultivars Burley 49 (B49) and K326 was transformed by *Agrobacterium*-mediated leaf disk transformation (Smith et al. 1994). Regenerated 6/21 transgenic plants were predominantly (>95%) haploid and sterile. Doubled haploid plantlets were regenerated from haploid midvein cultures, as in Smith et al. (1994). More than 75% of these midvein-regenerated plants possessed the normal, diploid (2N = 48) chromosome complement, were fertile, and produced viable seed.

Whole plant inoculation experiments.

Plant leaves were lightly dusted with Carborundum and virus inoculum (50 to 100 μl) was applied with a cotton swab. Virus inoculum was a 1:10 dilution (w/v) of virus-infected plant tissue in deionized distilled water. Plants were typically observed for 30 to 45 days.

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 as described by Lindbo and Dougherty (1992). 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 6- and 21-kDa ORFs.

Nuclear run-on assays.

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

Western blot analysis.

Primary antibodies used to detect transgene-derived 6/21 proteins consisted of a cocktail of monoclonal antibodies 4901, 4902, 4904, 4906, 4907, and 4910 (Slade et al. 1989). All of these antibodies react with epitopes in the N-terminal half of the NIa protein (= 21-kDa protein). The cocktail was preadsorbed with partially purified B49 leaf proteins for 16 h at room temperature. The precipitate was removed by centrifugation, and the serum used at a 1:2,500 dilution to detect proteins as in the protein gel analysis procedure described for TEV coat protein (Lindbo and Dougherty 1992).

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