Genetic and Biochemical Dissection of Transgenic RNA-Mediated Virus Resistance

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RNA-mediated virus resistance has been observed in transgenic plants at varying frequencies, suggesting that a nuclear requirement or other pre-condition must be met. This study was undertaken to characterize genetically transgenes that confer a highly resistant state to infection by tobacco etch virus (TEV). Transgenic tobacco line 2RC-6.13, expressing an untranslatable mRNA containing the TEV coat protein open reading frame, had three distinct transgene integration events that segregated as two linkage groups. A genetic series of plants that contained zero, one, two, or all three transgene inserts in both homozygous and heterozygous conditions was produced and examined. Genetic and biochemical data suggested that RNA-mediated virus resistance is a multigenic trait in line 2RC-6.13; three or more transgenes were necessary to establish the highly resistant state. One or two transgene copies resulted in an inducible form of resistance (i.e., recovery). Transcription rates and steady state RNA levels of the transgene-derived transcript present in different members of the genetic series supported a post-transcriptional RNA degradation process as the underlying mechanism for transgene transcript reduction and virus resistance. This degradation process appeared to initiate via cleavage of specific sites within the target RNA sequence, as determined by RNA gel blot and primer extension analyses of transgene-derived mRNA from various transgenic plant lines.

INTRODUCTION

The expression of a transgene in a plant usually results in plants with the predicted phenotype. However, unexpected phenotypes have been generated at varying frequencies. One example, suppression of endogenous gene expression by a transgenic exogenous counterpart, has been described in a number of reports. A variety of gene regulatory pathways can be operational yet result in essentially the same suppression phenotype (Finnegan and McElroy, 1994; Flavell, 1994; Matzke and Matzke, 1995a, 1995b). Two mechanisms appear to account for most examples of host gene suppression by exogenous transgenic counterparts. One of the mechanisms is gene silencing (Matzke et al., 1989, 1994), an apparently nuclear-based process during which transcription is down-regulated. The other is sense suppression (de Carvalho et al., 1992; Ingelbrecht et al., 1994). Detailed analyses of these transgene-related phenomena should extend our understanding of coordinate gene expression.

Sense RNA-mediated virus resistance is another unexpected transgenic phenotype that may provide a system to examine the regulation of gene expression. Plants expressing a transgene derived from the genomic sequence of an RNA virus can be completely resistant to that virus (de Haan et al., 1992; Lindbo and Dougherty, 1992a, 1992b; van der Vlugt et al., 1992; Pang et al., 1993). The transgene-derived RNA may be either translatable or untranslatable, indicating that the translation product of the transcript is not involved in eliciting resistance. Plants displaying this virus-resistant phenotype transcribe the transgene(s) at a high rate yet accumulate the transgene transcript at low levels (Dougherty et al., 1994; Smith et al., 1994; Mueller et al., 1995; Swaney et al., 1995). Plants with these characteristics do not permit the cytoplasmically localized virus to replicate to a detectable level. It has been suggested that a post-transcriptional RNA targeting system could be activated, resulting in the elimination of both viral RNA and transgene transcript from the cytoplasm (Lindbo et al., 1993; Dougherty and Parks, 1995).

We have suggested that a threshold level of transgene-derived transcripts must be exceeded to activate the cytoplasmic post-transcriptional RNA degradation process and elicit virus resistance (Smith et al., 1994). Therefore, the number of transgenes and their relative level of expression should be important in determining the activation state of this cytoplasmic system. This study was undertaken to assess a transgene configuration that established sense RNA-mediated virus resistance. A genetic array of tobacco plants was generated from...
a progenitor transgenic line that expressed an untranslatable tobacco etch virus (TEV) coat protein (CP) open reading frame (ORF). This parental line was highly resistant to infection by TEV and had the hallmarks of RNA-mediated virus resistance. Genetic and molecular studies determined that three transgenes were sufficient to preactivate the system to a highly resistant state. The presence of one or two transgenes permitted the recovery phenotype, an inducible form of the highly resistant state occurring after TEV infection. In both highly resistant and recovered plants, transgene transcript steady state levels were low despite the significant rate of transgene transcription, suggesting a post-transcriptional RNA degradation process. Molecular analyses showed that transgene transcript degradation was most likely initiated by specific cleavages of the transgene mRNA in those plant lines resistant to virus infection.

RESULTS

Molecular Genetic Analysis of Parental Lines

Two tobacco lines served as the starting genetic material for this study. *Nicotiana tabacum* cv Burley 49 (B49) is a commercial Burley tobacco cultivar susceptible to TEV infection. Transgenic line 2RC-6.13, highly resistant to infection by TEV, is a transformed version of B49 expressing an untranslatable mRNA composed of the TEV 5' untranslated region (UTR), a mutated version of the TEV CP ORF containing three stop codons immediately downstream of the initiation codon, and a 3' UTR derived from the tumor morphology large gene (*tml*) of Agrobacterium (Lindbo and Dougherty, 1992a; Dougherty et al., 1994). Cells (protoplasts) isolated from 2RC-6.13 leaf tissue do not support a detectable level of TEV replication (Lindbo and Dougherty, 1992b). 2RC-6.13 and B49 were used as parental lines and were designated line 1 and line 2, respectively (Figure 1A and Table 1).

Genomic DNA from lines 1 and 2 was isolated and digested with the restriction enzyme EcoRI. There is a single EcoRI restriction site in the transferred DNA, mapping downstream of the *tml* 3' UTR. DNA fragments were analyzed in a DNA gel blot hybridization study, using 32P-labeled TEV CP gene sequences as a probe. Three hybridizing bands were identified in line 1, whereas none were observed in line 2 (Figure 1B, lanes 3 and 2). The upper two bands have estimated molecular lengths of 13.2 and 9.1 kb; the lower band was estimated to be 5.4 kb.

Establishment and Characterization of Genetic Material

A genetic array of plant lines was established as described below and schematically diagrammed in Figure 1A. Line 1 (2RC-6.13) was hybridized with line 2 (B49) to generate an F1 hybrid (line 3) heterozygous for the transgenes. The F1 hybrid

Figure 1. Generation and DNA Gel Blot Analysis of a DH Array of Transgenic Plants.

(A) A schematic diagram of two of the 24 tobacco chromosomes during the production of a genetic array of 2RC-6.13-derived plants is shown. 2RC6.13 (line 1) and untransformed B49 (line 2) were crossed to generate an F1 hybrid (line 3). A haploid plant array was produced by crossing the F1 progeny with *N. africana*. Selected haploid plants were chromosome doubled. Progeny from these homozygous DH plants were either selfed (S), backcrossed to B49 (B), or intercrossed (X) to generate various transgene combinations. Two of the TEV transgenes (open boxes) always segregated together, whereas a third segregated independently. Plant line numbers are indicated below the genotypic indicators.

(B) Genomic DNA was isolated from parental and DH plant lines, digested with the restriction enzyme EcoRI, and subjected to DNA gel blot hybridization with a TEV-specific probe. The resulting autoradiogram is presented. Lane 1, λ DNA markers; lane 2, line 2; lane 3, line 1; lanes 4 through 9, DH plants that displayed a symptomless phenotype after inoculation with TEV; lanes 10 through 15, DH plants that showed symptoms after inoculation with TEV. The plants analyzed in lanes 10 through 12 underwent recovery 1 to 2 weeks after symptom
**Table 1.** Plant Lines, Genotypes, Molecular Characteristics, and Phenotypes of Materials Used in This Study

<table>
<thead>
<tr>
<th>Line or Hybrid</th>
<th>Transgene Copy No.</th>
<th>RNA Accumulation</th>
<th>Relative RNA Levels</th>
<th>Transcription Rate</th>
<th>Normalized Transcription Rate</th>
<th>Resistance Phenotype</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>Line 2RC6.13</td>
<td>6</td>
<td>Low</td>
<td>10</td>
<td>None</td>
<td>Highly resistant</td>
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<tr>
<td>2</td>
<td>Cultivar B49</td>
<td>0</td>
<td>None</td>
<td>0</td>
<td>None</td>
<td>Susceptible</td>
</tr>
<tr>
<td>3</td>
<td>F₁-1 X 2</td>
<td>3</td>
<td>Low</td>
<td>11</td>
<td>None</td>
<td>Mixed</td>
</tr>
<tr>
<td>4</td>
<td>DH (0)</td>
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<td>None</td>
<td>0</td>
<td>None</td>
<td>Susceptible</td>
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<td>5</td>
<td>DH (3)</td>
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<td>Low</td>
<td>13</td>
<td>Very high</td>
<td>Highly resistant</td>
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<tr>
<td>6</td>
<td>F₁-9 X 2</td>
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<td>7</td>
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<td>7</td>
<td>DH (3)</td>
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</tr>
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<td>DH (3)</td>
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<tr>
<td>10</td>
<td>F₁-9 X 2</td>
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<td>Low</td>
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<td></td>
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<td>11</td>
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<td>9</td>
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<tr>
<td>12</td>
<td>F₁-11 X 2</td>
<td>2</td>
<td>High</td>
<td>85</td>
<td></td>
<td>Recovery</td>
</tr>
<tr>
<td>13</td>
<td>DH (2)</td>
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<td>Low</td>
<td></td>
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</tr>
<tr>
<td>14</td>
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<td></td>
<td>Moderate</td>
<td>Recovery</td>
</tr>
<tr>
<td>15</td>
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<td>Recovery</td>
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<td>100</td>
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<td>Recovery</td>
</tr>
<tr>
<td>18</td>
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<td>1</td>
<td>Moderate</td>
<td>43</td>
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<td>Slow recovery</td>
</tr>
<tr>
<td>19</td>
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<td>High</td>
<td>70</td>
<td></td>
<td>Recovery</td>
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<td>39</td>
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<td>Slow recovery</td>
</tr>
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<td>21</td>
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<td>High</td>
<td>70</td>
<td></td>
<td>Recovery</td>
</tr>
<tr>
<td>22</td>
<td>F₁-21 X 2</td>
<td>1</td>
<td>Moderate</td>
<td></td>
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</tr>
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</tr>
<tr>
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<tr>
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<td>22</td>
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</tr>
<tr>
<td>27</td>
<td>F₁-11 X 21</td>
<td>3</td>
<td>Low</td>
<td>30</td>
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<tr>
<td>29</td>
<td>F₁-11 X 17</td>
<td>3</td>
<td>Low</td>
<td>12</td>
<td>High</td>
<td>Highly resistant</td>
</tr>
</tbody>
</table>

*Plant line nomenclature is as described in the text and Figure 1.

Numbers in parentheses indicate the transgene copy number in the original haploid plant.

RNA steady state levels were quantified using a Phosphorlmager; signal strength was translated into a number between 0 and 100. The average of two experiments is presented. Genotypes with RNA levels of 1 to 30 were considered low; 30 to 60, moderate; and above 60, high. Rows without values were analyzed once.

Transcription rates were normalized as described by Smith et al. (1994). The number given represents a ratio of the normalized rate of transcription to the rate of transcription determined for line 18, a plant line containing a single transgene copy.

This plant line was generally highly resistant, although one test plant became infected, as described in the text.

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was pollinated with *N. africana*, and a large number of haploid plants (*n = 425*) was produced (Burk et al., 1979). Hybridization of tobacco with pollen of *N. africana* results in abundant seed that germinate readily. The interspecific hybrid seedlings are highly lethal and die in the cotyledonary stage of germination. A low frequency (0.001) of seedlings survive, and these consist of approximately equal numbers of phenotypically distinguishable tobacco haploids and aneuploid interspecific hybrids. After inoculation of the haploid plants with TEV, 50% (*n = 213*) exhibited TEV-induced symptoms; the remaining 50% were symptomless. Among the haploid group expressing symptoms, 50% of the plants recovered, whereas the remaining plants exhibited wild-type TEV systemic symptoms throughout their life cycle.

Haploid plants representative of the various phenotypes were chromosome doubled and self-pollinated (Kasperbauer and Collins, 1972) to produce doubled haploid (DH) lines. A DNA gel blot hybridization analysis of these lines is presented in Figure 1B. Four banding patterns were noted. Plants that were symptomless after TEV inoculation had three or two bands,
whereas DH lines showing symptoms had one or no bands. In all cases, the two larger bands (~13.2 and ~9.1 kb) were present together, indicating a close genetic linkage of the two transgenes.

S2 progenies of three independent DH lines representing each of the molecular genotypes (one, two, or three bands) were selected, self-pollinated, and crossed to line 2 (B49). In addition, lines homozygous for the 13.2- and 9.1-kb bands (lines 11, 13, and 15) were crossed with lines 17, 19, and 21, homozygous for the 5.4-kb band. Collectively, these crosses resulted in a series of transgenic plants heterozygous or homozygous for various transgene combinations (Figure 1A). These plants and their genotypes are listed in Table 1 and were used in a comprehensive assessment of genotype to phenotype relationships.

Pathology of Genetic Series after Inoculation with TEV

A total of 29 lines and hybrids were analyzed in this study. Four distinct phenotypes were noted among S2 generation plants in five challenge inoculation tests with TEV. Many of the lines displayed the highly resistant phenotype. These plants showed no symptoms of TEV infection, and TEV could not be detected in these plants via ELISA or biological assays. All S2 generation DH lines and F1 hybrids were homogeneous in their response to TEV with the exception of line 11. (In this line, one plant out of 35 showed a rapid recovery phenotype instead of the highly resistant phenotype.) A number of lines and hybrids showed a typical recovery phenotype (i.e., lines 12, 14, and 21). These plants became infected with TEV and exhibited typical wild-type TEV systemic symptoms. However, ~10 days after infection, the plants began to recover from infection. Three weeks later (days 19 to 24), new leaf tissue emerged that was free of symptoms and detectable virus. A third phenotype, a slow recovery, was observed with lines 18, 20, and 22. These plants showed signs of recovery ~18 to 21 days after inoculation, and recovered tissue emerged ~21 days later, just before terminating the test (at approximately day 45). The fourth phenotype, characterized by systemic wild-type TEV symptoms and no apparent recovery, was observed in lines 2 and 4, which both lack a TEV-derived transgene. These results are summarized in Table 1.

Molecular Analysis of the Genetic Series

DNA Analysis

Genomic DNA was extracted from leaf tissue of individual lines within the genetic series of plants. DNA was digested with the restriction enzyme EcoRI and analyzed in DNA gel blot hybridization studies. The results of one such study are presented in Figure 2. Parental-derived banding patterns were found in all lines. Lines with three, two, and one hybridizing band were confirmed. In addition, there was a clear and consistent difference in signal intensity between homozygous and heterozygous lines (i.e., Figure 2, lane 6 versus 7 or lane 12 versus 13). The signal intensity of a homozygous line was approximately twice that found in the corresponding heterozygous condition, as determined by PhosphorImager analysis.

We attempted to characterize molecularly the linkage among these three bands. A λ genomic library was generated by using DNA isolated from line 1. Eighteen clones that hybridized to TEV CP-specific sequences were identified. Based on restriction enzyme digestion patterns, three different groups of clones could be identified. Representatives of each group were then subcloned into a plasmid vector for more detailed mapping analyses. Results from these studies indicated that each group corresponded to one of the three TEV-hybridizing EcoRI bands shown in a typical DNA gel blot (data not shown). No genomic sequences inserted into λ DNA and containing the two genetically linked transgenes together were identified. However, none of the clones contained an entire T-DNA insert; all clones terminated within the right border portion of the transferred cassette in the area of tml or neomycin phosphotransferase II (nptII) sequences. Several attempts were made to generate a cosmid library to determine the exact linkage distance; however, a usable library was not obtained because the recombination required would have been too extensive.

Extensive gel blot analysis of the genomic DNA of lines 1 and 15 (a line homozygous for the top two bands), probed with both TEV and nptII sequences, revealed that the two trans-
genes were adjacent to each other in a tail-to-tail (right border-to-right border) orientation, probably with no more than 300 bp separating the two insertions (data not shown). This agrees with the transgenes contained in the 15.2- and 9.1-kb DNA fragments segregating as a single unit in genetic studies.

RNA Analysis

Leaf tissue from this same genetic array of plants was used in an RNA gel blot analysis of transgene transcript steady state levels. The transgene-derived transcript was detected with a 32P-labeled TEV-specific RNA probe, and the signal was quantified using a PhosphorImager. An autoradiogram is presented in Figure 3A. There is a correlation between transgene copy number, resistance phenotype, and RNA steady state levels. Germplasm containing three or more transgenes displayed the highly resistant phenotype and accumulated the transgene transcript at low levels. In Figure 3A, this can be observed in lanes containing RNAs from lines 5, 6, 15, 23, 26, and 29. Transgene numbers of less than three did not confer the highly resistant state, and transcripts accumulated at a higher level. This could be observed with plants from lines 16 to 18. Lines possessing one or two transgenes accumulated the transgene transcript in direct proportion to the number of transgenes present. Although these plants were initially susceptible to TEV infection, they did display a recovery phenotype; the highly resistant state was induced, and new leaf tissue emerged free of virus and symptoms.

Comparisons of the transgene-derived transcript in unchallenged and recovered plants within the same lines were also made. Lines (12, 19, and 21) that contained two transgenes in different configurations were examined by RNA gel blot analyses. The steady state level of the transgene transcript was estimated to decrease two- to fourfold with the induction of the highly resistant state (Figure 3B). A similar but often larger (approximately six- to 15-fold) decrease in transgene transcript steady state level has been noted with other virus transgenes that rapidly induce the recovery phenotype (Lindbo et al., 1993; Dougherty et al., 1994; Swaney et al., 1995). The difference observed for FL-44.4, a TEV CP-producing line, is ~12-fold (Lindbo et al., 1993) and is shown in lanes 3 and 4 of Figure 3B. Therefore, the highly resistant state in this genetically related series of plants, whether preestablished or induced after TEV infection, correlates with low steady state levels of transgene transcript. These results are summarized in Table 1.

The association of low steady state levels of the transgene transcript with the virus-resistant state could be due to transcriptional inactivation of the TEV-related transgenes or to a post-transcriptional process. Likely involvement of a post-transcriptional process was shown by the collective analysis of data generated using three different experimental approaches.

Methylation Studies

Methylation of genomic DNA sequences has been shown to correlate with gene inactivation (Matzke and Matzke, 1991; Matzke et al., 1994). Genomic DNA from various lines was digested with restriction enzymes to identify methylation differentially at specific adenosine or cytosine residues within the transgene. Most of the enzymes used are sensitive to methylation residues; however, DpnI cleaves only when its recognition site is methylated. Therefore, increased methylation within the transgene sequences might decrease restriction by Aval, Avall, HincII, Mbol, or Sau3AI, depending on the specific site of addition. Conversely, Mbol cleavage might increase if its recognition sequence is altered. These restriction sites
within the transgene are presented in Figure 4A, with the results of one experiment presented in Figure 4B. Transgene sequences were not methylated differentially in most members of the related genetic series, although some methylation was detected in the TEV CP transgene any time the upper two bands were in a homozygous condition. This was readily noted in line 5, which is homozygous for all three bands (six transgenes), and, to a lesser degree, in line 15, which is homozygous for only the upper two bands (four transgenes).

**Nuclear Run-on Studies**

We next examined the transcription rate of the various transgene configurations. Nuclei were isolated from leaf tissue of selected lines and used in nuclear run-on studies to assess the transcription rate of TEV-derived transgenes. Multiple experiments were conducted with nuclei isolated from three different sets of plants. Transgene transcription rates among plant lines were compared by normalization of constitutively expressed actin, cyclophilin, and ubiquitin, as described by Smith et al. (1994). The results are presented in Table 1. There was a good correlation between the number of transgenes present and the rate of transcription. The greater the number of transgenes present, the higher the transcription rate. This was in direct contrast to steady state levels of the transgene transcript determined from RNA gel blot hybridization studies (Figure 3 and Table 1). Highly resistant lines, accumulating low levels of transcript, transcribed the transgenes at rates higher than those found in lines that accumulated high levels of the transcript. Viewed with the results of the methylation studies (Figure 4), these findings strongly suggest a post-transcriptional process requiring a minimal level of transgene transcription for activation.

**Elimination of the Transgene-Derived Transcript**

RNA-mediated resistance is a remarkably specific process; resistance is displayed only against the virus from which the transgene is derived. We have speculated that the entire process is post-transcriptional and occurs in the cytoplasm and that specificity is driven by small complementary RNAs or proteins (Lindbo et al., 1993; Smith et al., 1994). The transgene-derived transcript serves to “prime” the cellular surveillance system to target the transgene transcript and any other RNA species that might contain the targeted sequence. We have suggested (Lindbo et al., 1993) that the entire transcript sequence would be available to elicit this cellular process. Therefore, we examined various transgenic germplasm to determine whether there were differences in transgene-derived RNA populations in resistant and susceptible germplasm.

Total RNA was isolated from line 1 (2RC-6.13; highly resistant), FL-44.4 (unchallenged and recovered tissue) (Lindbo et al., 1993), and RC-7 (highly resistant) and RC-9 (susceptible) (Lindbo and Dougherty, 1992b). RNA was analyzed in a gel blot hybridization study, and the results are presented in Figure 5. The expected full-length transgene-derived transcript was detected in each plant line by using either total or poly(A) RNA. The RNA gel blot was then quantitated, using a Phosphorlmager. Equivalent areas of each lane containing total RNA samples were scanned, and data are presented in Table 2. All lines with a susceptible phenotype had ~80% of...
the signal associated with the expected full-length transcript. The remaining 20% of bound radioactivity was associated with less-than-full-length RNA. However, in highly resistant (inducible or preinduced) lines, only 40 to 50% of the hybridized counts were associated with the full-length transgene transcript; the remaining signal was associated with less-than-full-length RNA species. This would be consistent with a cytoplasmic degradation process present in lines exhibiting resistance to virus infection.

We had hypothesized that the entire RNA transcript would be available for recognition by the RNA surveillance system and that degradation would be via random cleavage of the transcript (Lindbo et al., 1993). In this scenario, we predicted that breakdown products of the transgene-derived transcript would be heterogeneous in size. In RNA gel blot analysis of total and poly(A)⁺ RNA, a smear of random breakdown products was observed, but to our surprise, clearly identifiable bands were also present, indicating that preferred sites might be targeted initially in different RNAs (Figure 5). Lines FL-44.4 (recovered, lanes 5 and 6), RC-7 (highly resistant, lanes 7 and 8), and 2RC-6.13 (highly resistant, lanes 1 and 2) had multiple faster migrating bands in RNA gel blot hybridization analyses, using a radiolabeled probe derived from the entire TEV CP ORF. Some of these bands were more readily apparent in the poly(A)⁺ RNA preparations. The hybridization experiment was repeated with total and poly(A)⁺ cellular RNA from these various lines, using probes derived from the 5' one-third, the middle one-third, or the 3' one-third of the TEV CP ORF. In addition to the full-length transcript, different lower molecular weight bands could be detected consistently with these different riboprobes (data not shown).

**Figure 5.** Gel Blot Analysis of RNA from Various Transgenic Plant Lines. 
Total (T) and poly(A)⁺ RNA samples were isolated from the various TEV transgenic plant lines indicated at bottom. Line 2RC-6.13 (2RC-) is a highly resistant line. Line FL-44.4 (FL-44) is a TEV CP-producing line, and RNA was isolated from unchallenged (u) or from recovered (rec) leaf tissue. Line RC-7 is a highly resistant line, and RC-9 is a susceptible transgenic line; both produce an untranslatable mRNA. RNAs were electrophoresed in formaldehyde-containing agarose gels, blotted to nitrocellulose, and hybridized with a radiolabeled TEV CP probe. An empty lane separates the RNA samples from each plant type.

**Table 2.** Percentage of Transgene-Derived Transcript Present as Full Lengtha

<table>
<thead>
<tr>
<th>Plant Line</th>
<th>Resistance Phenotype</th>
<th>% Full Lengthb</th>
</tr>
</thead>
<tbody>
<tr>
<td>RC-7</td>
<td>Highly resistant</td>
<td>45</td>
</tr>
<tr>
<td>RC-9</td>
<td>Susceptible</td>
<td>84</td>
</tr>
<tr>
<td>FL-44 (unchallenged)</td>
<td>Susceptible</td>
<td>80</td>
</tr>
<tr>
<td>FL-44 (recovered)</td>
<td>Highly resistant</td>
<td>46</td>
</tr>
<tr>
<td>2RC-6.13</td>
<td>Highly resistant</td>
<td>35</td>
</tr>
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a Percentages are based on the amount of signal present in a gel blot containing RNA isolated from the plant line indicated. Quantitation was accomplished with PhosphorImager analysis.
b Numbers represent an average derived from quantitation of three RNA gel hybridization blots.

Putative cleavage regions within the TEV CP ORF were identified based on fragment length and source of the radiolabeled probe. Oligonucleotide primers were synthesized and used in primer extension analysis of total RNA isolated from the various plant lines. Results of one such analysis are shown in Figures 6A and 6B. In general, primer extension results were difficult to obtain because we were attempting to map the 5' terminus of an RNA cleavage product in a population of degrading transgene-derived RNA species. Background was significant in these experiments, but certain primer extension products, unique to resistant lines, were detected consistently. Figure 6A shows the primer extension results of the predominant cleavage site in the 3' half of TEV CP-derived transgenes. This extension product was detected consistently in three studies using five different resistant lines and absent in all tested susceptible lines. One other site was mapped that was consistent with the RNA gel blot hybridization studies. These two sites are presented in Figure 6B.

**DISCUSSION**

A genetic array of plants was generated to examine systematically the role of transgene copy number and transcription in establishing and maintaining the highly resistant state against virus infection. This study suggested that the highly resistant and recovery phenotypes are mediated by the same cytoplasmic system. The induction of this system was controlled by transgene copy number in a dosage-dependent fashion. The transcription of one or two transgenes did not provide a level of transcript sufficiently high to exceed the proposed lower threshold and activate the post-transcriptional system in this transgenic series. However, the expression of these transgenes did permit manifestation of the recovery phenotype, again in a dosage-dependent fashion. One transgene conferred a "slow" recovery phenotype on the tobacco germplasm, whereas two transgenes permitted a "rapid" recovery. Three or more
The multigenic nature of highly resistant lines has been noticed in our past studies. Most of our susceptible transgenic plants have a single or many (more than eight) bands when examined in DNA gel blot hybridization studies (Smith et al., 1995; S. Swaney and W. G. Dougherty, unpublished data). We suggest that transcription of a single transgene, using the enhanced cauliflower mosaic virus 35S promoter, in most cases does not produce a level of RNA transcript sufficient to activate the RNA surveillance system. Susceptible transgenic lines with a very high transgene copy number usually had undetectable to very low levels of transgene transcript. This may be indicative of transgenes having been “turned off” at the nuclear level by a gene-silencing mechanism. Highly resistant lines usually contain two to five bands when examined (although two lines, out of a sample population of ~300 plants, have been identified that apparently possess a single hybridizing band). These transgenes appear to produce a level of transcript sufficient to activate the system. Therefore, for RNA-mediated virus resistance to be engaged, there appears to be a window of transgene expression, perhaps with both upper and lower limits.

The genetic and biochemical data suggest that the different transgenes in 2RC-6.13 are transcribed at approximately the same level. It may be that this genotype is typical of most transgenic lines, and elements or sequences that increase transcription are not operational. Hence, multiple transgenes are needed to activate the cytoplasmic surveillance system. In those limited number of transgenic lines in which only a single transgene is found, the multiple transgene requirement may be supplanted by optimal positioning of the single transgene near an enhancer sequence or some other sequence or structure (i.e., scaffold attachment site; Allen et al., 1993) that might increase transcription. The enhanced 35S promoter is used in all of our gene constructions, and promoter strength (stronger or weaker) may also affect transgene dosage requirements. Therefore, it is reasonable to expect that many variations in transgene number and expression could provide a transcript level sufficient to preactivate the post-transcriptional surveillance system.

The transcription rates and RNA steady state levels of the transgenes in this study provided additional support for the hypothesis that a post-transcriptional RNA targeting system had been activated in these plants and was responsible for the low steady state level of transgene transcript and the virus-resistant phenotype. A post-transcriptional mode of RNA degradation is also supported by the presence of discrete lower molecular weight bands in resistant lines yet absent in susceptible material. The detection of these less-than-full-length RNA species was not anticipated. We had suggested that the entire RNA sequence would be available for the surveillance system and that only a smear of randomly degraded fragments would be detected in RNA gel blot hybridization studies (Lindbo et al., 1993). This was not the case; instead, selected sequences or structures within the TEV CP sequence appear to be sites of initial cleavage. Inspection of the cleavage site sequence, however, did not reveal a consensus sequence or

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**Figure 6.** Primer Extension Analysis of Various Transgenic Plant Lines.  
(A) Autoradiograms of a dideoxy chain termination sequencing reaction (left) and primer extension reactions (right) are shown. In all lanes, an oligonucleotide complementary to TEV genomic nucleotides 9159 to 9180 was used as a primer. For the sequencing reaction, dideoxy nucleotide mixtures used are shown at the top and the plasmid template is at the bottom. The sequence shown at the far left is complementary to the TEV coding strand (nucleotides 9078 to 9058) and was derived from a double-stranded cDNA contained in pTC-FL (Lindbo and Dougherty, 1992a). Primer extension reactions were conducted using RNA derived from the following plant lines: lane 1, B49 (line 2); lane 2, line 5; lane 3, line 3; lane 4, line 4; lane 5, 2RC-7; lane 6, 2RC-8; lane 7, FL-44.4 (unchallenged tissue); lane 8, FL-44.4 (recovered tissue). The phenotype of the plant line (S, susceptible; I, highly resistant) is shown at bottom. An arrow indicates the position of a primer extension product unique to the highly resistant phenotype.  
(B) A schematic drawing of the transgene transcript in 2RC-6.13 (line 1) is presented. The 5′ (cross-hatched box) and the 3′ (diagonally hatched box) UTRs are shown, with the coding sequence for the TEV CP designated by the open white box and the polyadenylated tail indicated (AAA). Numbers below the diagram refer to TEV genomic nucleotides: 8515 and 9310 represent the nucleotides for the 5′ and 3′ termini of the TEV CP ORF; 8953 and 9071 are nucleotides that map as 5′ termini of major RNA products found only in highly resistant plants. Sequences surrounding these positions are shown at bottom, with the primer extension product termination points indicated by arrowheads. UTL, untranslated leader.
structure that could be a hallmark of this system (Figure 6B). The same sequences or similar structures must also be found in the viral RNA genome, and cleavage at these sites would result in a genomic RNA molecule unable to replicate. This sequence or structure requirement adds another level of complexity to the elicitation of RNA-mediated resistance.

Transgenic virus resistance in plants can be achieved by incorporation of viral sequences other than CP genes, yet similarities in expression modes of different transgenes are noteworthy (Mueller et al., 1995; Swaney et al., 1995). Mueller et al. obtained tobaccos with high resistance to potato virus X (PVX) by transforming plants with the RNA polymerase gene from this virus. PVX-resistant plants accumulated the RNA transcript at a much lower level than susceptible transgenics, incomplete resistance was gene-dosage dependent, and a cytoplasmic degradation mechanism also appeared to be operating.

Not all experimental studies dealing with the post-transcriptional suppression of endogenous gene expression are in agreement with our threshold model (Lindbo et al., 1993; Smith et al., 1994) of excess transcript levels activating a post-transcriptional surveillance system that specifically targets an RNA sequence. Van Blokland et al. (1994), examining chalcone synthase in petunia, did not find a correlation between transcription and sense suppression. Indeed, they were able to elicit the suppressed state using transgenes that were lacking a promoter. De Carvalho Niebel et al. (1995), examining the suppression of β-1,3-glucanase genes, also were unable to establish a simple relationship between transgene copy number and sense suppression in a number of genetic crosses they made. As suggested by these authors, it is likely that some of the transgene loci are not actively transcribed.

Developmental and environmental conditions have also been suggested to be important in establishing the suppressed state (Hart et al., 1992; De Carvalho Niebel et al., 1995). We have indirectly examined this with respect to virus resistance in greenhouse (Lindbo and Dougherty, 1992; Smith et al., 1994) and field (Whitty et al., 1994) settings and have not observed the resistant state to be influenced by either parameter.

In summary, the highly resistant state of germplasm derived from 2RC-6.13 is a multitransgene trait in which the RNA targeted for elimination appears to be attacked initially at selected sites. Most but not all examples of RNA-mediated virus resistance appear to be multigenic. This study also genetically links the recovery phenotype and the highly resistant state as manifestations of the same cellular mechanism. Understanding the gene dosage requirement and genetic makeup is important in assessing the stability of the virus resistance and should permit the selection of transgenic plants in which the multigenic virus resistance behaves as a single dominant allele in inbred lines. Selection of plants with a transgene copy number that will lose the trait in outbred lines, as the critical transgene copy number is not met, also should mitigate the introgression of virus resistance into weedy relatives of domesticated crop species.

**METHODS**

**Generation of Plants**

Generation of haploid and doubled haploid (DH) tobacco plants was accomplished as described by Smith et al. (1994). Tobacco females were pollinated with Nicotiana africana. Haploid plants were selected among the low percentage of surviving progenies in this interspecific cross (Burk et al., 1979). Chromosome doubling of haploid plants was achieved by the in vitro leaf midvein techniques of Kasperbauer and Collins (1972).

**DNA Analyses**

**Gel Blot Hybridizations**

DNA extractions, digestions, electrophoresis, and gel blot hybridizations were performed as described by Smith et al. (1994). Blots were hybridized with radiolabeled sequences corresponding to the tobacco etch virus (TEV) coat protein (CP) open reading frame (ORF). These probes were generated with a random prime labeling kit (Du Pont). Quantification of signal was accomplished using a Phosphorlmager (model PSI-486; Molecular Dynamics, Sunnyvale, CA).

**Genomic Cloning**

Total DNA from line 1 was partially digested with Sau3AI, and the Klenow fragment of DNA polymerase I was filled in using dATP and dGTP (Sambrook et al., 1989). Fragments ranging in length from 9 to 23 kb were isolated and ligated into a λ, FI×11 vector (Stratagene). This vector had been digested with Xhol and partially filled in using dCTP and dTTP. After packaging and amplification, the resulting library was screened, and plaques hybridizing with a TEV CP probe were purified. DNA was purified from plate lysates using the Wizard λ DNA purification system (Promega), according to the manufacturer's directions. After initial restriction analysis, representative clones were digested with NotI and subcloned into a pBluescript KS+ plasmid (Stratagene) for detailed mapping.

**RNA Analyses**

**Gel Blot Analysis**

Total RNA was isolated from transgenic plants for RNA blot analysis 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 32P-labeled RNA probes generated from SP6/T7-based cell-free transcription reactions of a plasmid containing a cDNA copy of the TEV CP gene (Lindbo and Dougherty, 1992a; Lindbo et al., 1993).

**Nuclear Run-on Assays**

An estimation of relative transcription rates was obtained through nuclear run-on assays. Isolation of nuclei from transgenic plant tissue, in vitro labeling of run-on transcripts, and blot hybridizations were as described by Smith et al. (1994). Hybridization signals were quantified using a Phosphorlmager.
**Primer Extension Analysis**

Total plant RNA was isolated by LiCl precipitation as described above. For each sample, 50 µg of total RNA was analyzed with Promega's avian myeloblastosis virus reverse transcriptase primer extension system, according to the manufacturer's recommendations. The oligonucleotide primers used were complementary to TEV CP coding sequence from nucleotides between 8618 and 8648, 8911 and 8940, 9050 and 9080, and 9151 and 9180 (Allison et al., 1986). An additional primer, complementary to sequences found in the tml 3' untranslated leader, was also used to prime extension of transgene sequences.

After the reverse transcription reaction, samples were treated with 1 µL of a 10 mg/mL RNase A, 2000 units per mL RNase T1 cocktail at 37°C for 30 min. SDS (final concentration of 1%) and 5 µL of 10 mg/mL proteinase K were added, and incubation at 37°C proceeded for 30 min. Samples were then ethanol precipitated in the presence of carrier tRNA.

Sequencing reactions were performed using pTC.FL as a template (Lindbo and Dougherty, 1992a), using Sequenase version 2.0 DNA sequencing kit (U.S. Biochemical) according to manufacturer's specifications.

Primer extension and sequencing samples were denatured at 90°C for 10 and 2 min, respectively, and separated by electrophoresis in 8% polyacrylamide/8 M urea gels. Gels were exposed to Kodak X-Omat AR film for 2 to 7 days.

**Whole-Plant Inoculations**

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

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