Sense and Antisense Coat Protein Gene Constructs Confer High Levels of Resistance to Tomato Ringspot Nepovirus in Transgenic *Nicotiana* Species

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The nucleotide sequence data of the coat protein gene and the 3′ untranslated region of RNA 2 of a peach isolate of tomato ringspot virus have been deposited in the GenBank under accession number U46022.

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ABSTRACT

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The coat protein (cp) gene and the 3′ end untranslated region of a peach isolate of tomato ringspot virus (TomRSV) were cloned from purified total viral RNA and sequenced. Reverse transcription and polymerase chain reaction (RT-PCR) were used to engineer the TomRSV cp gene so that it could be cloned into plasmid vectors designed for either in vitro transcription or plant expression. The cloned TomRSV cp gene was used to transform *Nicotiana benthamiana* and *N. tabacum* plants, a systemic and a local lesion host, respectively. After challenge inoculation with the TomRSV peach isolate, several R_0, R_1, and R_2 resistant transgenic lines containing sense and antisense cp constructs exhibited different levels of protection ranging from complete resistance to delay in symptom appearance or reduction in symptom severity. Interestingly, cp gene expression levels were undetectable by enzyme-linked immunosorbent assay (ELISA) in the resistant lines containing cp sense constructs, and levels of cp transcripts were low or undetectable by Northern blot on resistant sense and antisense lines. The high level of resistance obtained in *Nicotiana* spp. offers important prospects for the engineering of TomRSV resistance into several economically important fruit and berry crops susceptible to this nepovirus.

Additional keywords: engineered protection, pathogen-derived resistance.

Tomato ringspot virus (TomRSV) is a broad host range nepovirus (23,40) infecting numerous berry and fruit crops including apples, peaches, cherries, plums, raspberries, strawberries, and grapes. Serious diseases have been associated with this virus including peach stem pitting, peach yellow bud mosaic, apple union necrosis and decline, prune brown line, prune constriction and decline, raspberry crumbly berry, and grapevine decline (49). Symptoms of these diseases usually appear several years after the plants have been established in the field. TomRSV is transmitted by nematode species of the *Xiphinema americanum* Cobb group, which are endemic to the Great Lakes region of the United States and Canada, the Northeast and Pacific coast of the United States, and British Columbia, Canada (11).

The genome of TomRSV is bipartite, consisting of two single-stranded, positive-sense, RNA molecules separately encapsidated into isometric particles of about 28 nm in diameter (43). Both RNAs, RNA 1 of 8,214 nucleotides (nt) (37) and RNA 2 of 7,273 nt (38), are translated as two large polyprotein precursors that are cleaved to release functional proteins (22). Unlike most nepoviruses, TomRSV RNA 1 and RNA 2 have a large (1.5 kbp) 3′ end untranslated common region (39). The coat protein (cp) gene has been localized near the 3′ end of RNA 2 for the following nepoviruses: arabis mosaic (ArMV) (5), blueberry leaf mottle (BBLMV) (2), grapevine fanleaf (GFLV) (46), cherry leaf roll (CLRV) (44), grapevine chrome mosaic (GCMV) (10), raspberry ringspot (7), strawberry latent ringspot (SLRV) (15,29), tobacco ringspot (12), and tomato black ring (33). Rott et al. (38) suggested that the putative location of the cp gene of a raspberry isolate of TomRSV was at the 3′ end of RNA 2. Microsequencing of the N-terminal region of purified TomRSV cp allowed the identification of a Q–G cleavage site that was not previously reported for nepoviruses (22).

We were interested in engineering resistance against TomRSV using pathogen-derived genes (41). A number of laboratories have demonstrated in the past few years that introduction of specific viral genes into the plant genome confers protection in transgenic plants against the donor and closely related viruses (17,19,20). The feasibility of cp-mediated protection against nepoviruses has been demonstrated for ArMV (4), GCMV (9), and GFLV (3). To engineer resistance against TomRSV in *Nicotiana* spp., we cloned and sequenced the 3′ end terminal region of RNA 2 using a peach isolate of TomRSV, and subsequently cloned its cp gene. Transgenic *N. tabacum* L. and *N. benthamiana* Domin plants containing sense and antisense cp gene constructs were obtained via *Agrobacterium*-mediated transformation. Several transgenic sense and antisense lines obtained were highly resistant to mechanically inoculated TomRSV.

MATERIALS AND METHODS

Virus purification and RNA isolation. A peach isolate of TomRSV originally obtained from California was used in this study. This isolate induces peach yellow bud mosaic, and was propagated in cowpea and purified as previously described (6). Total viral RNA was extracted from purified virions (46) and fractionated by sucrose density gradient centrifugation. However, because of their similar molecular mass, RNA 1 and RNA 2 could not be separated. Therefore, total viral RNA was used as a template for oligo(dT)-primed cDNA synthesis.

Reverse transcription and polymerase chain reaction (RT-PCR), cloning, and sequencing of the 3′ end of RNA 2. cDNA clones containing the 3′ end sequences of RNA 1 and RNA 2 were constructed by cDNA synthesis using oligo(dT) primers and

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murine leukemia virus (MLV)-reverse transcriptase (Promega Corp., Madison, WI). The second cDNA strand was synthesized according to the method of Gubler and Hoffman (21), extremities were filled with T4 DNA polymerase (Promega Corp.), and *Eco*RI linkers were added using T4 DNA ligase (Promega Corp.). The cDNA molecules were digested with *Eco*RI and ligated into the *Eco*RI site of the plasmid pUC18 (Pharmacia LKB Biotechnology Inc., Piscataway, NJ). Recombinant plasmids were electroporated into *Escherichia coli* strain DH5α. The RNA species from which each oligo(dT)-primed clone was transcribed was determined by probing Northern blots of purified TomRSV RNA with labeled cloned cDNA. The cDNA clones corresponding to the 3′ end untranslated region of RNA 2 and the C-terminus of the cp gene were characterized by restriction digestion and sequenced using cesium chloride purified plasmid DNA and the dideoxynucleotide chain termination method (42). Sequence information was used to design primers for RT-PCR cloning.

Three cDNA clones were produced from total viral RNA by RT-PCR using MLV-reverse transcriptase (Promega Corp.), *Taq* DNA polymerase (Perkin-Elmer Corp., Norwalk, CT), and appropriate primers. A 1.5-kbp RT-PCR cDNA product was generated using primers 91-32 (5′-AGCACCATGGTCTGTCGAAAACAAAACTTGC-3′) and 91-34 (5′-AGCTGACCATGGCTTGGACAAAGTTCGACACTACG-3′), designed to prime the amplification of the untranslated 3′ end region of TomRSV. A 2.2-kbp RT-PCR product that was obtained using primers 91-33 (5′-AGCTGACCATGGAAGCTTCCATTAGAGCTTATC-3′) and 91-34 corresponded to the 3′ end noncoding region and the sequence coding for part of the C-terminal region of the cp. A third 1.7-kbp RT-PCR product was amplified corresponding to the full-length cp gene using primer 91-76 (5′-AGCTAG**TCTAGACCATGG**TTCAGGGCGGGTCCTGGCAAG-3′), designed at the start of the cp gene (based on the microsequencing data of the cp), and primer 91-77 (5′-GCAT-**GATCTAGACCATGG**TAAAAGCTAATTAAGAGGCCACC-3′), located 43 nt downstream of the end of the RNA 2 open reading frame as deduced from sequencing the RNA 2 cDNA clones.

The cloning primers contained an *Xba*I site (TCTAGA), followed by a *Nco*I site (CC**ATGG**) with the ATG initiation codon in the context of the plant translation consensus sequence (32). The *Xba*I site was used to facilitate cloning into the in vitro transcription vector and the in vivo plant expression vector. The RT-PCR products were ligated into the plasmid vector pGMM (a pBluescript derivative engineered in our laboratory, described below) used for sequencing and in vitro translation experiments. Manual ^{35}S-sequencing was done using the Sequenase T7 polymerase sequencing kit (United States Biochemical Corp., Cleveland, OH) and automated sequencing was done using the *Taq* DyeDeoxyterminator cycle sequencing kit (Applied Biosystems, Inc., Foster City, CA). All clones were sequenced completely in both directions using primers designed at 300- to 350-nt intervals. Sequence data analysis was done using the Genetics Computer Group, Inc., sequence analysis software package (Genetics Computer Group, Inc., University Research Park, Madison, WI) and DNASTAR biocomputing software (DNASTAR Inc., Madison, WI).

Protein purification and microsequencing of the N-terminus of purified cp. Purified virions (3 mg) were dialyzed overnight in 0.1 M KH_2PO_4 (pH 7.0) and dissociated by heating for 2.5 h at 55°C. Denatured protein was centrifuged at 5,000 × *g* for 10 min, washed twice with distilled water, and resuspended in 0.125 M Tris buffer (pH 7.0), 0.5% sodium dodecyl sulfate (SDS), and 10% glycerol. Undigested and *Staphylococcus aureus* V8 protease digested protein fractions were separated on a 12% denaturing SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to an Immobilon Millipore membrane (Millipore Corp., Bedford, MA) using a Bio-Rad miniblotter apparatus (Bio-Rad Laboratories, Richmond, CA). An Applied Biosystems model 470A protein sequencer with a model 120A PTH analyzer (Applied Biosystems, Inc.) was used to determine the amino terminal sequence of the cp by Edman degradation. The Pico-tag method was used for total amino acid analysis with a detection limit of 1 pmol. Amino terminal protein sequencing and total amino acid analysis of the cp were conducted at the Analytical/Synthesis Facility of the Biotechnology Center, Cornell University, Ithaca, NY.

In vitro transcription and translation. In vitro transcripts were synthesized using T7 RNA polymerase from *Not*I-linearized recombinant pGMM plasmids containing the TomRSV 1.7-kbp RT-PCR cDNA product. The pGMM plasmid is a pBluescript derivative engineered in our laboratory containing multiple cloning sites and the leader sequence of the cp gene of cucumber mosaic virus strain white leaf (CMV-WL). Transcripts were analyzed by electrophoresis on formaldehyde-containing agarose gels (35). Transcripts (1 μg/μl) were translated at 30°C in a nuclease-treated rabbit reticulocyte lysate system (Promega Corp.) containing ^{35}S methionine. Translation reactions were stopped after 1 h by the addition of denaturing buffer (10% SDS and 25% β-mercaptoethanol), and ^{35}S-labeled translation products were analyzed by SDS-PAGE.

Engineering of the cp gene constructs into plant transformation vectors. The 1.7-kbp RT-PCR cDNA product corresponding to the cp gene was digested with *Xba*I and ligated in both sense and antisense orientation into the *Xba*I site of the plant expression vector pNYS. This vector is a pUC18 derivative made in our laboratory by custom PCR engineering (47), and contains the cauliflower mosaic virus (CaMV) 35S promoter sequence, the CMV-WL leader sequence, a multiple cloning site, and the nopaline synthase (NOS) terminator sequences. Identification of sense and antisense constructs was done by *Bam*HI or *Kpn*I digestion. The TomRSV cp expression cassette was excised by partial *Hind*III digestion from pNYS and ligated into the *Hind*III site of the binary vectors pBI121 (Clontech Labs Inc., Palo Alto, CA) or pGA482GG, a derivative of pGA482 (1) modified by the insertion of the β-glucuronidase (GUS) gene in the *Bgl*I site, and a gentamycin resistance gene in the *Sal*I site (36) (Fig. 1). Both binary vectors contain the GUS gene and the neomycin phosphotransferase (NPT II) gene that confers resistance to kanamycin.

Transformation of *N. benthamiana* and *N. tabacum*. The binary vectors pGA482GG or pBI121 containing the TomRSV cp gene constructs in sense or antisense orientation were electroporated into the disarmed *A. tumefaciens* strains LBA4404 (Clontech Labs Inc.), C58Z707/C58sZ707 (25), and EHA101/EHA105 (26). Leaf disc-transformation by *Agrobacterium* was done following the procedure described by Horsch et al. (27). Leaves for transformation experiments were collected from seedlings germinated in vitro or in the greenhouse. Selection of transformants was done using kanamycin at 300 mg/liter. Some plants were transformed with binary vectors that did not harbor any cp gene constructs, and were used as controls for resistance evaluation.

Characterization of transgenic *N. tabacum* and *N. benthamiana* R_0 plants. Expression of GUS in transformed regenerants was assayed using the histological and fluorimetric assays (28). Expression of the NPT II gene was assayed by enzyme-linked immunosorbent assays (ELISA) using commercial γ-globulins (5′ Prime 3′ Prime, Inc., Boulder, CO). Coat protein expression in transgenic plants containing sense cp constructs was determined by direct double-antibody sandwich ELISA with γ-globulins against TomRSV produced in our laboratory (6). Extraction buffer, healthy *Nicotiana* tissue, TomRSV-infected tissue, and/or purified virus were included as controls in each ELISA plate (Immulon 2, Dynatech Labs Inc., Chantilly, VA). Replicated wells were loaded for each plant sample and optical density was read at 450 nm.

Detection of the TomRSV cp gene in selected transgenic plants was corroborated by PCR and Southern blot analysis after isola-

tion of plant genomic DNA (34). PCRs using oligonucleotide primers specific for the TomRSV cp, NPT II, and GUS genes were run using the reagents and instructions of the Perkin-Elmer PCR kit (Perkin-Elmer Corp.). Total plant RNA was extracted from actively growing leaves prior to inoculation (35), and used for Northern blot analysis. Southern and Northern blot analyses were conducted using a ^{32}P-labeled cloned probe for the cp gene that also contained the first 43 nt of the untranslated 3′ end common region of RNA 1 and RNA 2. Labeling was done using the oligolabeling method (16).

Challenge inoculation of transgenic *Nicotiana* plants. The TomRSV peach inoculum was prepared by harvesting symptomatic *N. benthamiana* leaves 5 days after inoculation and grinding them in phosphate buffer (0.01 M K_2HPO_4, pH 7.0). Several inoculum dilutions (wt/vol) were used to screen the transgenic plants, e.g., 1:25 (1 g of tissue/25 ml of buffer), 1:50, or 1:100 for *N. tabacum*, a local lesion host for TomRSV. Lower inoculum doses were used on the systemic host *N. benthamiana* because of the severity of the virus isolate, e.g., 1:50, 1:100, 1:250, 1:500, 1:1,000, 1:1,500, and 1:2,000 (wt/vol). Plants were inoculated when they were between 8 to 12 cm in height. The inoculum was applied by gently rubbing the upper surface of leaves predusted with Corundum (Universal Photonics, Inc., Hicksville, NY). For *N. benthamiana*, only the youngest, fully expanded apical leaves (three to four leaves/plant) were inoculated, while all the leaves were inoculated for *N. tabacum.* Seedlings of *Chenopodium quinoa*, a local lesion host for TomRSV, were inoculated at each inoculum dose to monitor inoculum strength from experiment to experiment. Plants were observed daily for symptom development. Number of lesions per leaf were counted for *N. tabacum*, and days required for necrosis to occur for *N. benthamiana*. Resistant R_0 and R_1 transgenic lines were carried on to R_1 and R_2 generations, respectively, for further evaluation and segregation studies. R_1 and R_2 seeds were germinated in vitro on kanamycin 300 mg/liter before establishing the plants in the greenhouse.

RESULTS

RNA sequence analysis. Three overlapping RT-PCR products (2.2, 1.7, and 1.5 kbp) were sequenced to determine the nucleotide sequence of the cp gene and the 3′ end untranslated region of a peach isolate of TomRSV (Fig. 2). The peach isolate cp gene was 1,689 nt in length with the following nucleotide composition: %A = 24.63, %G = 23.92, %U = 29.96, %C = 21.49 [%A + U = 54.59, and %C + G = 45.41]. Comparison of the nucleotide sequence for the cp of the peach isolate with the raspberry isolate of TomRSV sequenced by Rott et al. (38) indicated 96.9% identity at the nucleic acid level (51 nt differences) and 96.6% identity at the protein level (19 amino acid differences). From the deduced amino acid sequence, the molecular mass of the cp of the peach isolate was 62.0 kDa with 562 amino acids, 6.65 isoelectric point, and –2.18 charge at pH 7.0. The TomRSV raspberry isolate had the same number of amino acid residues and molecular mass, but a different isoelectric point (7.81) and charge (+3.80 at pH 7.0) because of 19 amino acid substitutions scattered throughout the cp (Fig. 2). Ten of the amino acid differences observed between the cp of the peach and raspberry isolates of TomRSV corresponded to changes in amino acid groups and charge (hydrophobic to acidic or polar/basic to polar or polar to hydrophobic) and accounted for the difference in isoelectric point and charge. The calculated size for the cp of the peach isolate was similar to that determined by SDS-PAGE (described below). Comparison of the nucleotide sequence for the 3′ end noncoding region of the peach (1,552 nt) and the raspberry (1,547 nt) isolates of TomRSV indicated only 90.3% identity (151 nt differences with 49 nt representing purine-pyrimidine changes, and 4 nt deletions plus 9 nt insertions accounting for the 5 nt difference in length). The nucleotide composition for the 3′ end untranslatable region of the peach isolate was as follows: %A = 24.61, %G = 24.23, %U = 30.48, and %C = 20.68 [%A + U = 55.09, %C + G = 44.91].

Coat protein analysis. SDS-PAGE of purified cp indicated that the TomRSV peach isolate had a cp with molecular mass of approximately 60 kDa. The amino acid composition obtained from acid hydrolysis of purified cp was in close agreement with the values expected from the RNA sequence (data not shown). The N-terminal region of the cp was microsequenced in order to identify the protease cleavage site and the amino acids at the cp N-terminus. The sequence determined for the first 22 amino acids at the N-terminal region was as follows:

GGSWQEGTEAAFLGKVT?AKDA

[The ? corresponds to the amino acid cysteine in the deduced sequence that could not be determined by the direct sequencing method used].

The cp cleavage site Q–G (Gln–Gly) was determined by comparing the cp N-terminal amino acid sequence with the residues deduced from the nucleotide sequence (Fig. 2). The predicted size for a protein released at the Q–G cleavage site was in agreement with the expected molecular mass of the cp. In addition, the amino acid sequencing data obtained for the N-terminal region of a V8 protease-generated polypeptide of molecular mass of 47 kDa matched perfectly the amino acid sequence for cp residues 133 to 155 (sequence underlined in Fig. 2). This confirmed that the open reading frame analyzed was indeed encoding the cp.

Characterization of the engineered cp constructs. In vitro translated ^{35}S protein products were observed for the sense TomRSV cp construct (data not shown), while antisense constructs gave no translated protein products as expected. The in vitro translation product comigrated with purified cp, supporting the proposed identity of the cp cleavage site and demonstrating the functionality of the cp sense constructs.

Characterization of transgenic *N. tabacum* and *N. benthamiana* R_0 plants. Transgenic *N. benthamiana* and *N. tabacum* plants containing sense and antisense cp constructs were obtained

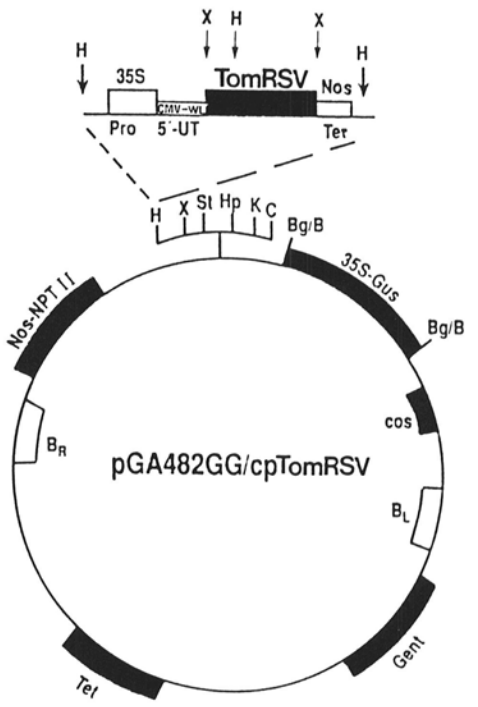

Fig. 1. Plant binary vector pGA482GG and expression cassette pNYS containing the TomRSV coat protein (cp) gene construct. Restriction sites on the pGA482GG vector are as described by An et al. (1), and restriction sites for the engineering of the cp gene are described in the text.

using *A. tumefaciens*. A total of 313 'putative' transgenic cp lines were recovered, from which 173 sense and 140 antisense transgenic lines were transferred to the greenhouse for resistance evaluation. Once transferred to the greenhouse, leaf samples were collected from each plant and tested by NPT II and GUS assays. Most tested plants (86%) were determined to be NPT II positive by ELISA prior to inoculation. GUS assays (X-Gluc and MUG) gave less consistent results for the same population of transgenic plants (73 and 82% positive, respectively). Coat protein gene expression was not detectable by ELISA in any of the transgenic

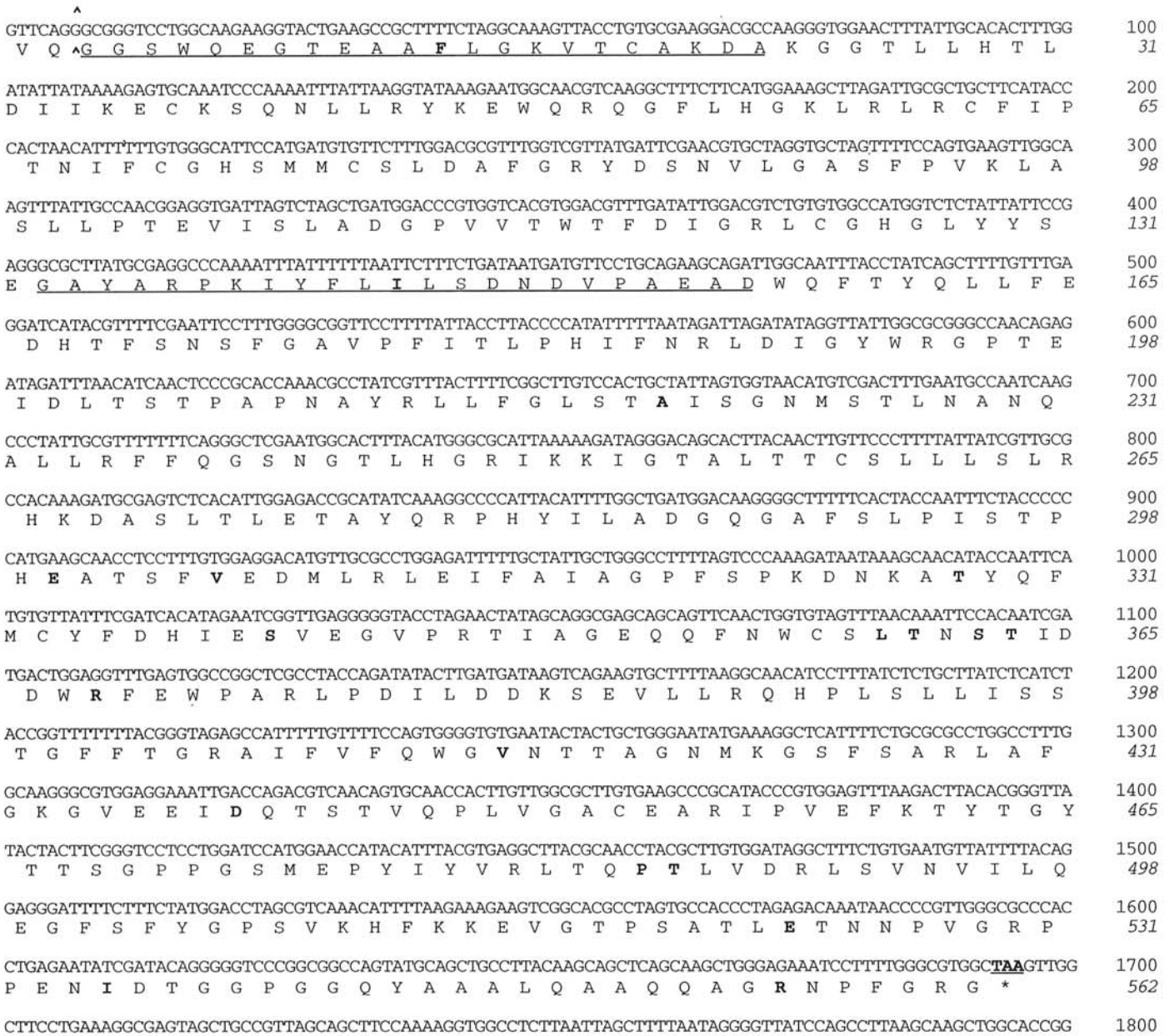

Fig. 2. Nucleotide sequence of the 3′ terminal region of RNA 2 of a peach isolate of TomRSV and deduced amino acid sequence for the polyprotein open reading frame. The sequence of the 3′ end untranslatable region (1,552 nucleotides) is given following the polyprotein stop codon (TAA). The '^' between the second and third amino acid residues of the polyprotein denotes the cleavage site (Q–G) between the putative movement protein and the coat protein (cp). The underlined amino acid sequences represent the first 22 amino acids at the N-terminus of the cp, and amino acids 133 to 155 obtained from microsequencing the purified cp and a protease-generated polypeptide, respectively. The 19 amino acid residues printed in bold (scattered throughout the cp sequence) represent differences between the peach and raspberry isolates of TomRSV.

N. benthamiana and *N. tabacum* sense lines tested. However, PCR analysis of several NPT II positive transgenic sense and antisense lines confirmed the presence of the transferred TomRSV cp gene (Fig. 3A). Similarly, Southern blot analysis demonstrated the presence of the TomRSV cp gene in several transgenic lines analyzed (data not shown). Interestingly, the cp transcripts were detectable in Northern blot analysis only at low levels in some, but not all, of the transgenic sense and antisense lines analyzed (Fig. 3B). Two hundred sixty-five independent transgenic cp plants that were NPT II ELISA positive were further analyzed for resistance in the greenhouse.

Evaluation of transgenic *N. benthamiana* for resistance. The systemic host *N. benthamiana* was highly susceptible to the peach isolate of TomRSV. Control (nontransformed and vector-transformed) plants developed local chlorotic lesions on the inoculated leaves 3 days after inoculation. Necrosis of the apical leaves was initiated 4 days postinoculation (Fig. 4A). Necrosis was observed on the apical tip after 4 to 5 days at 1:50 dilutions, and after 6 to 7 days at 1:250 to 1:2,000 dilutions. Severe necrosis of the apex led to plant death 7 to 8 days after inoculation regardless of the inoculum dose.

One hundred sixty-six R_0 transgenic *N. benthamiana* cp lines (representing 73 sense and 93 antisense lines) were inoculated with TomRSV at inoculum doses from 1:50 to 1:2,000. Most of the inoculated transgenic *N. benthamiana* R_0 plants (60 to 87%) showed no delay or a short delay in symptom development (2 to 5 days), but later on became necrotic and died. Approximately 10% of the 166 transgenic R_0 lines screened were resistant (16 lines = 7 sense and 9 antisense). These resistant lines never developed any visible symptoms or systemic necrosis, and the virus could not be detected by ELISA in the youngest, noninoculated leaves. All 16 R_0 resistant transgenic plants survived when reinoculated at 1:25 inoculum dose to ensure that they were not escapes, and were kept for seed production.

R_1 seedlings from 8 of the 16 R_0 resistant lines (four sense lines designated S1, S5, S9, and S12; and four antisense lines designated AS3, AS4, AS11, and AS13) were inoculated at 1:50 dilution (20 seedlings per line) to evaluate whether the resistance was inherited. Out of the eight R_1 lines tested, three lines (S5, S12, and AS4) were completely resistant with none of the tested plants developing symptoms (Fig. 5), three lines (S9, AS11, and AS13) were highly resistant with 80% of the plants remaining symptomless, and two lines (S1 and AS3) were intermediately resistant with 60% of asymptomatic plants (Fig. 5).

R_2 progeny of these R_1 resistant plants were inoculated to study the inheritance of their resistance. R_2 seedlings (20 per line) were inoculated at 1:25 or 1:50 dilution. Out of the eight R_2 lines screened, six lines (S1, S5, S12, AS4, AS11, and AS13) were completely resistant even at high inoculum doses with 100% of the plants remaining symptomless. One sense line (S9) was highly resistant with 75% asymptomatic plants, and one antisense line (AS3) was intermediately resistant with 30 and 60% asymptomatic plants following inoculation at 1:25 and 1:50, respectively. In addition, the other eight R_0 resistant transgenic lines were screened only at the R_2 seedling stage. Three of the lines (one sense and two antisense) were highly resistant with 75% of the plants remaining symptomless, and five lines (two sense and three antisense) were intermediately resistant with 40 to 60% asymptomatic plants.

Evaluation of transgenic *N. tabacum* for resistance. *N. tabacum* developed only local lesions and no systemic necrosis when inoculated with the TomRSV peach isolate. Chlorotic lesions appeared in control (nontransformed and vector-transformed) plants 3 days postinoculation, and became necrotic with typical rings around the central necrotic spot (Fig. 4B). The virions could not be detected by ELISA in the new, noninoculated leaves. Sixteen transgenic R_0 lines containing sense and antisense cp constructs were screened: 12 sense and four antisense R_0 lines. From the 16 R_0 lines screened at a 1:50 inoculum dose, only two lines (one sense [L7] and one antisense [L9]) displayed complete resistance with no local lesions developing. The remaining 14 R_0 lines screened developed local lesions ranging from numbers similar to the control to significantly reduced number of lesions (data not shown). No local lesions developed in the inoculated R_1 progeny from the two resistant R_0 lines L7 and L9.

In a separate experiment, 12 R_0 transgenic lines (seven sense and five antisense) were carried on to seeds without prior screening. The R_1 seedlings (average five plants per line) were inoculated at a 1:50 inoculum dose. Two sense transgenic lines (L3 and L38) and one antisense line (L42) showed no local lesions, while most other lines tested showed a significant reduction in the number of local lesions, as well as delay in symptom appearance (1 to 2 days), compared with the nontransformed controls. The number of local lesions for R_1 transgenic *N. tabacum* sense and antisense lines varied in range from numbers similar to the control (65 to 70 local lesions per leaf for the control versus 37 to 48 local lesions per leaf for the transgenic lines L33 and L11), to

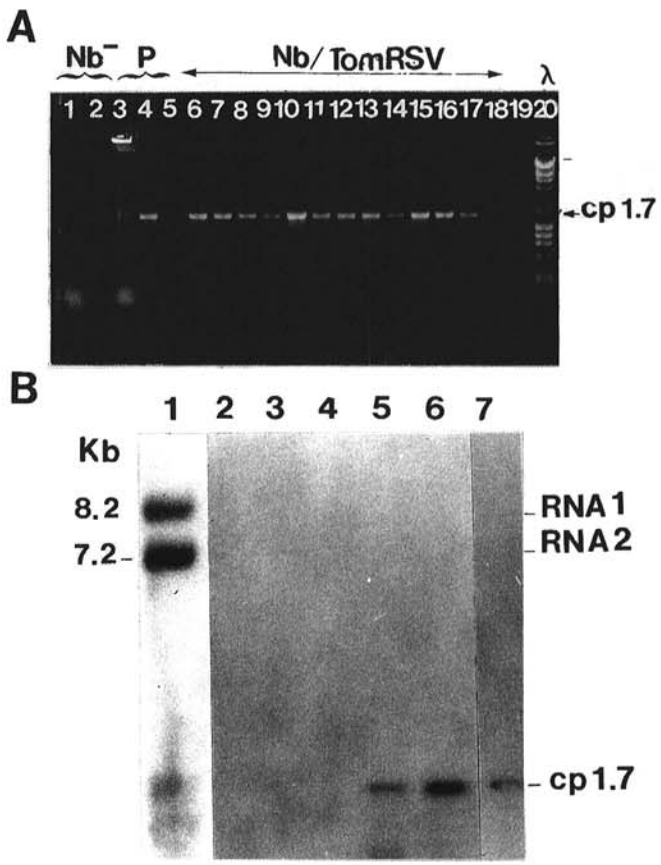

Fig. 3. Analysis of *Nicotiana benthamiana* transgenic lines. **A,** Polymerase chain reaction (PCR) analysis. Agarose gel representing the amplified products for the negative control (Nb–) (lanes 1 and 2), the plasmid control (P) (lanes 3 to 5; amplified from 1.0, 0.1, or 0.01 µg of DNA), and 14 transgenic R_0 lines (Nb/TomRSV) (lanes 6 to 19). From left to right, the lines tested were seven sense (S) lines (S1 to S7) and seven antisense (AS) lines (AS1 to AS7). The arrow indicates the location of the TomRSV coat protein (cp) PCR-amplified product (1.7 kbp). Lane 20 corresponds to the molecular size standards λ-*Hind*III and ϕ–*Hae*III. **B,** Northern blot analysis. All lanes were loaded with 5 µg of total plant RNA. Lane 1 corresponds to a nontransgenic control plant infected with the peach isolate of TomRSV. The probe used contained the cp gene and part of the untranslated 3′ end common region for RNA 1 and RNA 2, so it hybridized with both viral RNAs. Lane 2 corresponds to a healthy, nontransformed control plant, and lanes 3 to 7 correspond to five different noninoculated R_1 transgenic lines. Note that the transgenic lines expressed either low levels of the cp transcript (lanes 5 to 7 = lines S1, S16, and AS3, respectively) or undetectable cp transcripts (lanes 3 and 4 = lines AS4 and S5) even after long exposure times (5 to 7 days). The location of the cp transcript (1.7 kbp) is indicated in the figure.

intermediate numbers (25 local lesions per leaf for line L8), to lines with few local lesions (5 to 13 for lines L1 and L41), to no local lesions (L38) (Fig. 6). For *N. tabacum*, 12% of the R_0 lines screened (2/16) and 25% of the R_1 lines (3/12) were completely resistant to TomRSV, since they did not developed any local lesions.

DISCUSSION

We cloned and sequenced 3,247 nt at the 3′ end of RNA 2 of a peach isolate of TomRSV, determined the precise location of the polyprotein cleavage site, cloned the cp gene, and provided direct functional evidence of the engineered cp gene by in vitro transcription and translation. We found a high percent nucleotide identity (97%) between the nucleotide and amino acid sequences of the raspberry and the peach isolates for the cp gene. Interestingly, the percent identity for the 3′ end untranslated region (1.5 kbp) was significantly lower (90%). Comparison of the 3′ end untranslated and cp nucleotide and amino acid sequences among the different nepoviruses sequenced so far (2,4,7,8,10,12,13,15,29,33, 38,44,45,46) indicated higher similarity among members of the same nepovirus subgroups, with the exception of SLRV. Even though SLRV is transmitted by nematodes, its classification in the nepovirus group is debated because of the low similarity and the presence of two rather than one cp species (43 and 29 kDa) (15, 29).

Microsequencing data of the N-terminus of the cp of our peach isolate confirmed that the proteolytic cleavage site between the movement protein and the cp was Q–G at position 1,320 to 1,321 of the polyprotein, as recently reported (22). This cleavage site differed from the R–A, R–G, K–A, and C–A sites proposed for other nepoviruses in subgroups I and II (5,7,10,12,13,46), but was one of the proteolytic cleavage sites (Q–M, Q–S, Q–G, E–S, and E–G) described previously for como-, poty-, and picornaviruses (24). CLRV, which like TomRSV is in subgroup III, has also a picornavirus proteolytic site Q–S (44). Nepoviruses in general share similarities in genomic structure and translational strategies with the plant como- and potyviruses, as well as, the animal picornaviruses (18).

Previous attempts to engineer resistance to nepoviruses into *Nicotiana* spp. focused only on transgenic lines expressing high cp levels (3,4,9) using members of subgroups I and II. Bertioli et al. (4) inoculated *N. tabacum* cv. Xanthi plants cloned from two R_0 transgenic lines expressing the cp of ArMV, and observed that lesions developed only on the inoculated leaves, but no systemic virus infection was detectable on the newly formed leaves when challenged by virions or purified RNA and the resistance was durable with time. Interestingly, transgenic plants expressing the ArMV cp produced empty virus particles (5).

Evaluation of the resistance in transgenic *Nicotiana* lines expressing the cp genes of GFLV and GCMV was based on studying the inhibition of viral replication as assessed by dot blot (9) or Northern blot analysis (3), because GFLV and GCMV do not produce symptoms on *Nicotiana* spp. The use of dot and Northern blots greatly restricted the number of transgenic lines that were analyzed. Brault et al. (9) produced transgenic *N. tabacum*

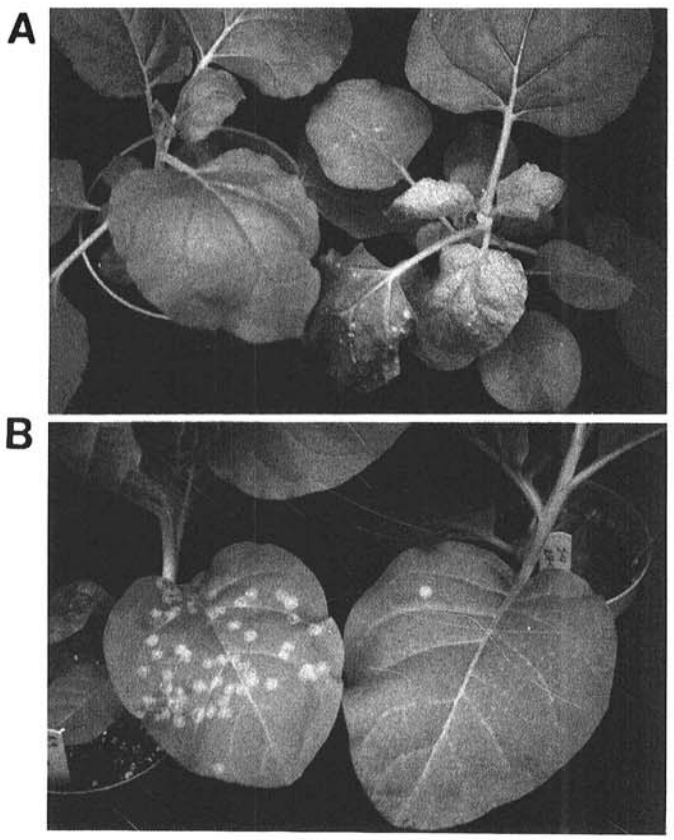

Fig. 4. Reaction of transgenic *Nicotiana* spp. to inoculation with TomRSV. **A,** *N. benthamiana* control nontransformed plant (right) showing local lesions on the inoculated leaves and systemic necrosis on the apical leaves that led to plant death after 7 to 8 days, while no symptoms were observed in the resistant transgenic R_1 plant (left). **B,** *N. tabacum* control nontransformed plant (left) showing numerous local lesions with typical necrotic ringspots, while the resistant transgenic R_1 plant (right) shows significant reduction in the number of local lesions.

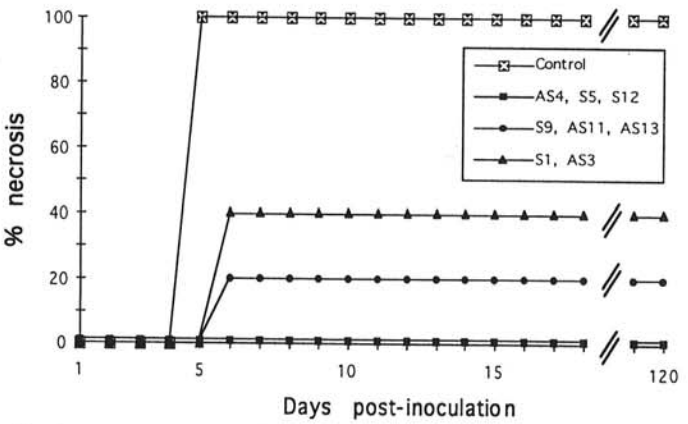

Fig. 5. Screening for TomRSV resistance of R_1 transgenic *N. benthamiana* lines. R_1 progeny were from self-pollinated R_0 plants that were resistant to TomRSV (AS = antisense line, S = sense line). Twenty plants were tested per line. Plants were mechanically inoculated with a 1:50 inoculum dilution.

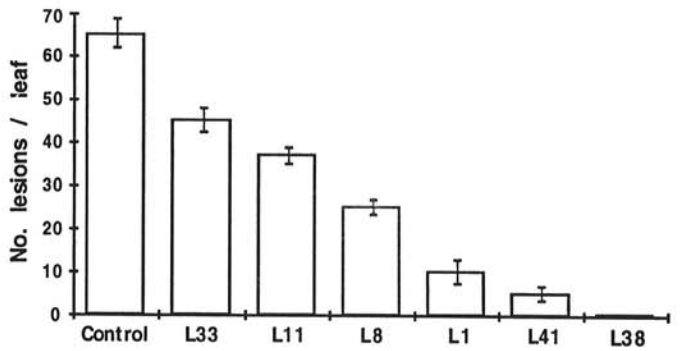

Fig. 6. Reactions of R_1 transgenic lines of *N. tabacum* to inoculation with TomRSV. *N. tabacum* is a local lesion host of the TomRSV peach isolate. The number of local lesions per leaf varied from susceptible transgenic lines (L33 and L11) that develop similar numbers of local lesions as the control (nontransformed and vector-transformed) plants to resistant transgenic lines with none (line L38) or significantly reduced number of local lesions (L8, L1, and L41). Five plants were tested per transgenic line. Vertical lines represent standard errors.

cv. Xanthi plants with a construct containing the cp gene and the complete 3′ end untranslated region of GCMV. Two out of nine transgenic lines characterized showed reduction in viral replication (monitored by counting the number of dot blot positive plants) at a low inoculum dose (1 μg/ml), but the difference vanished at a higher inoculum (10 μg/ml), although the overall accumulation of viral RNAs seemed to be reduced. Data were presented only for 10 days postinoculation, so evaluation of durability of the reduction in replication overtime could not be made. The authors suggested that reduced viral replication in those two transgenic lines was correlated with high cp expression levels. However, all the lines analyzed had high cp levels, and no lines with low or nondetectable cp levels were tested.

Bardonnet et al. (3) transformed *N. benthamiana* with the GFLV cp gene. Virus replication was assayed by Northern blot analysis or by immunodetection of the 5′ end genome-linked protein VPg. Only cp ELISA positive lines (11/40 transgenic lines obtained) were analyzed, from which one transgenic line that expressed the highest cp level by ELISA was selfed and its R_2 progeny characterized. Although significant delay in systemic infection occurred in this line, with nearly 65% of the plants protected 24 days after inoculation, the protection was finally overcome after 60 days when all the plants became infected.

Our study is the first report of cp-engineered protection against a nepovirus of subgroup III. We deliberately chose to analyze all our cp transgenic plants for resistance to TomRSV, regardless of their lack of detectable cp expression by ELISA. Nevertheless, the level of resistance obtained appeared to be much higher than that obtained so far for other nepoviruses. Direct evaluation of symptoms allowed us to analyze a large number of transgenic lines (over 250 independent sense and antisense transgenic lines). From the *N. tabacum* and *N. benthamiana* transgenic sense and antisense lines analyzed, we were able to obtain several lines that showed delay in symptom appearance, reduction of symptom severity, or appeared completely resistant. A high proportion of the resistant lines (40 to 56%) were antisense. Similar results have been reported for tobacco etch virus (TEV), for which antisense and truncated cp genes were in some way dysfunctional and more effective at disrupting the normal virus-host relationship than full cp genes (30).

Although the TomRSV cp gene was detected by PCR in DNA extracted from transgenic plants, Northern blot analysis showed very low levels of cp transcript and cp expression was not detectable by ELISA. The reasons for the lack of cp detection in our transgenic plants need further investigation. However, since we were able to detect the expression of the engineered cp gene in vitro, possible explanations are that the pNYS expression cassette gives very low transcription rates or the cp gene is cosuppressed as has been reported for TEV (30). Thus, the cp is produced at levels below the threshold level of detection by ELISA. Another possibility is that the cp is being degraded in the transgenic plants. Despite the lack of cp detection by ELISA, several of our transgenic lines were highly resistant to TomRSV.

There are other reports in the literature of transgenic plant lines that are highly resistant to virus inoculation, yet the expected protein product is not observed. In those instances, the resistant phenotype may be mediated through a defective RNA species and not the expected translation product (14,30,31,48). These studies hypothesized that RNA transcripts derived from antisense and cp genes produced in transgenic plants may interfere with viral replication by annealing to the viral RNA, thereby interfering with transcription or translation, or interfering with binding of the viral replicase or viral assembly. They also suggested that transgenic plants expressing defective RNAs or proteins will be among the most effective potyvirus control strategies.

Our results on resistance to TomRSV in *Nicotiana* spp. are encouraging and open important prospects for the possible use of engineered protection in economically important hosts. The cp constructs from this work are now being used in transformation of several important perennial fruit crops susceptible to TomRSV.

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