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Plants transformed with a tobacco mosaic virus nonstructural gene sequence are resistant to the virus

(plant virus/replicase)

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ABSTRACT ***Nicotiana tabacum*** **cv. Xanthi nn plants were transformed with nucleotides 3472–4916 of tobacco mosaic virus (TMV) strain U1. This sequence contains all but the three 3′ terminal nucleotides of the TMV 54-kDa gene, which encodes a putative component of the replicase complex. These plants were resistant to infection when challenged with either TMV U1 virions or TMV U1 RNA at concentrations of up to 500 μg/ml or 300 μg/ml, respectively, the highest concentrations tested. Resistance was also exhibited when plants were inoculated at 100 μg/ml with the closely related TMV mutant YSI/1 but was not shown in plants challenged at the same concentrations with the more distantly related TMV strains U2 or L or cucumber mosaic virus. Although the copy number of the 54-kDa gene sequence varied in individual transformants from 1 to ≈5, the level of resistance in plants was not dependent on the number of copies of the 54-kDa gene sequence integrated. The transformed plants accumulated a 54-kDa gene sequence-specific RNA transcript of the expected size, but no protein product was detected.**

The organization of the tobacco mosaic virus (TMV) genome is fairly well understood. However, one aspect of the genome strategy that has not been fully elucidated is the exact nature of the replicase enzyme responsible for the synthesis of the genomic and subgenomic RNAs. It is generally accepted that the virus codes for four proteins, two of which are translated from the genomic RNA and two from individual subgenomic RNAs (see review in ref. 1). The 5′ proximal region of the genomic RNA encodes two coinitiated proteins, the 126-kDa and 183-kDa proteins, considered to be components of the replicase (2). The 183-kDa protein is generated by a read-through of the UAG stop codon of the 126-kDa protein. The other two proteins, the 30-kDa cell-to-cell movement protein and the coat protein, are each synthesized from separate subgenomic mRNAs, on which each gene is 5′ proximal. What is not generally accepted, however, is our contention that there is a separate protein (the 54-kDa protein) for which there is an open reading frame in the read-through portion of the gene encoding the 183-kDa protein (1). Our principal evidence for the existence of such a protein comes from the finding that there is a third subgenomic RNA in TMV-infected plants, termed I_1 RNA (3), whose 5′ terminus has been mapped to nucleotide residue 3405 in the TMV genome. This subgenomic RNA contains the open reading frame for a 54-kDa protein, which starts with an AUG codon at position 3495 (4). Support for its function as a mRNA and as a subgenomic RNA is derived from the observation that it is found on polyribosomes (4) and that there is a double-stranded RNA of a size corresponding to the double-stranded version of the I_1 subgenomic RNA (5, 6).

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The 54-kDa protein has not been found in infected tissues, however. Saito *et al.* (7) prepared antibodies to a β-galactosidase fusion protein expressed in *Escherichia coli*, which contained 432 amino acids specific to the 183-kDa protein, and could not detect the 54-kDa protein in protoplast extracts by immunoprecipitation or Western blotting under conditions where the antibody would detect the 183-kDa protein. Likewise, we have not been able to detect 54-kDa protein in Western blots using antiserum made to the whole 183-kDa protein (8), although on occasion we have seen inconclusive faint bands in the region of the gel where such a protein would be expected. This is in spite of the fact that our antiserum is capable of precipitating the 54-kDa protein generated from *in vitro* translation products of either TMV RNA or T7 transcripts of the 54-kDa gene.

In an effort to attribute a function to the 54-kDa protein, we have transformed tobacco with the coding sequence for this nonstructural viral protein. Unexpectedly, these plants showed a complete resistance to replication of the U1 strain of TMV from which the 54-kDa gene sequence was derived. These findings and their implications for the production of virus-resistant plants are discussed.

MATERIALS AND METHODS

Plants and Virus Strains. TMV strain U1 was purified from infected *Nicotiana tabacum* cv. Turkish Samsun as described by Asselin and Zaitlin (9). Unless otherwise indicated, subsequent use of the term TMV implies strain U1. Before use, preparations of TMV strain U2 (10) and L (11), which had been stored as laboratory stocks at 4°C, were pelleted at 75,000 × *g* for 25 min and resuspended in water to enrich for full-length virions. A preparation of freshly prepared cucumber mosaic virus (CMV) strain Fny was a gift of M. J. Roossinck (Cornell University). Viral RNA was isolated by phenol extraction and ethanol precipitation. *N. tabacum* cv. Xanthi nn was used as a TMV-susceptible, systemic host and for transformation, and *N. tabacum* cv. Xanthi nc was used as a local lesion host. Virus was sometimes propagated in *N. tabacum* cv. Turkish Samsun. Protoplasts used in the studies to detect the 54-kDa protein were prepared from *N. tabacum* cv. Xanthi NN. The description and origin of these cultivars is discussed by Harrison (12). Plants were maintained in a greenhouse or in a growth chamber with a 14-hr light/10-hr dark cycle at 24°C.

Cloning the 54-kDa Gene. A clone of the TMV 54-kDa gene was obtained by using an oligonucleotide primer consisting of a *Bam*HI site linked to the 5′ end of a sequence complementary to nucleotides 4906–4923 of the TMV RNA sequence. First-strand cDNA was synthesized by Moloney murine leukemia

Abbreviations: AlMV, alfalfa mosaic virus; CaMV, cauliflower mosaic virus; CMV, cucumber mosaic virus; TMV, tobacco mosaic virus.

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virus reverse transcriptase and was rendered double stranded by sequential treatment with reverse transcriptase and the Klenow fragment of DNA polymerase I, relying on loop-back synthesis (13). The double-stranded cDNA was digested with *Bam*HI, which cleaves once within the TMV sequence at position 3321, and ligated into the *Bam*HI site of M13mp18. Sequencing showed that all the clones examined contained the sequence from position 3332 but lacked the *Bam*HI site provided by the primer. This resulted in the deletion of the 54-kDa termination codon and the extension of the 54-kDa protein at its C terminus by five amino acids derived from the vector M13mp18. The region of the insert encoding the 54-kDa protein was removed by digestion with *Hae* II, which cleaves at position 3467 in the TMV sequence, treated with Klenow fragment to blunt end the 3′ overhang, and digested with *Pst* I. The *Hae* II/*Pst* I fragment was ligated into *Pst* I/*Sma* I-digested pBS(−) (Stratagene), resulting in a plasmid termed pRTT-1. Sequencing showed that it contained the TMV sequence from nucleotides 3472 to 4916. The orientation of the insert was such that transcription from the T7 promoter gives plus-sense RNA transcripts (Fig. 1*B*).

The TMV 54-kDa gene sequence insert of pRTT-1 was excised by digestion with *Hin*dIII and *Sac* I, made blunt-ended by treatment with the Klenow fragment, and ligated into the *Sma* I or *Xho* I site of pMON316 (14). pMON316 contains a unique *Xho* I site in a polylinker region located between the cauliflower mosaic virus (CaMV) 35S promoter and the nopaline synthase 3′ untranslated region. A *Sma* I site is found in the polylinker region as well as within the Ti plasmid homologous region of pMON316. Plasmid pTS541A (Fig. 1*C*) was generated by insertion of the TMV sequence into the *Sma* I site, which resulted in the deletion of the nopaline synthase 3′ untranslated region and a portion of the Ti plasmid homology region. Insertion of the TMV sequence into the *Xho* I site resulted in the formation of pTS541. Clones containing the 54-kDa gene sequence in the sense or antisense orientation were characterized and isolated. Each construct was transferred to *Agrobacterium tumefaciens* strain GV3111 carrying pTiB6S3-SE by means of a triparental mating system (15), and transconjugates were selected by resistance to kanamycin and streptomycin.

Plant Transformation. Cut pieces of sterile *N. tabacum* cv. Xanthi nn leaves were transformed by the modified *A. tumefaciens* GV3111, containing the TMV 54-kDa gene coding sequence, as described by Horsch *et al.* (16). Transformed calli were selected on regeneration medium supplemented with kanamycin at a concentration of 300 μg/ml. Resistant calli were induced to regenerate shoots and roots, transferred to soil, and maintained in a greenhouse (16).

Nucleic Acid Analysis. DNA was isolated from leaves of plants by a modified procedure of Murray and Thompson (17). The DNA was digested with restriction enzymes, separated in 1.0% agarose gels, transferred to GeneScreen*Plus* (DuPont) nylon membranes, and hybridized to a ^{32}P-labeled probe (18) specific for the TMV 54-kDa gene sequence. RNA was isolated from leaf tissue by a modified procedure of Chirgwin *et al.* (19). Total RNA was separated in a 1.2% agarose/formaldehyde gel and transferred to nitrocellulose (13). The blot was hybridized to a ^{32}P-labeled DNA probe complementary to the 54-kDa gene coding sequence (18).

Immunological Analyses. An antiserum to the 54-kDa protein was made by injecting rabbits with tuberculin coupled to a synthetic polypeptide representing amino acid residues 243–257 of the 54-kDa protein (corresponding to residues 1407–1421 of the 183-kDa protein). An *in vitro* translation product of a T7 transcript from the 54-kDa gene was immunoprecipitable with this antiserum (data not shown). Antiserum to the read-through region of the 183-kDa TMV protein, and thus specific to the 54-kDa protein, was provided by T. Meshi (University of Tokyo). For Western blotting, total

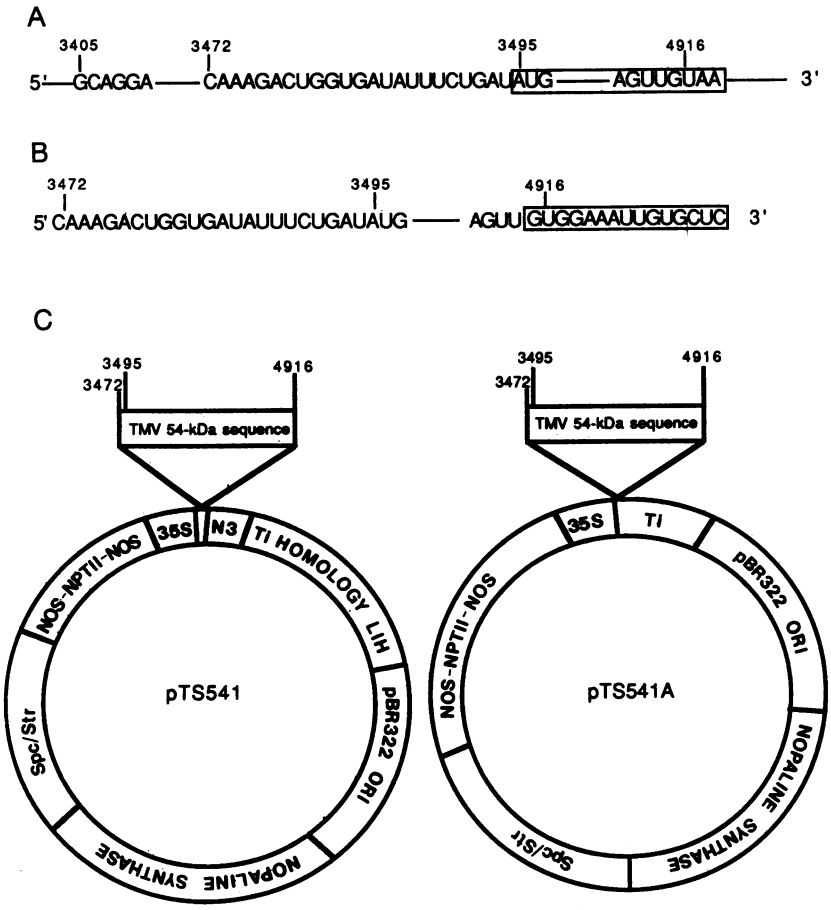

FIG. 1. (*A*) Important features of the sequence of a region of the TMV genome that contains the read-through portion of the 183-kDa protein gene. The I_1 subgenomic RNA begins at nucleotide 3405; the 54-kDa open reading frame extends from nucleotide 3495 to 4919. (*B*) TMV sequence used for plant transformation. The boxed region contains the sequence coding for the five additional amino acids added to the 54-kDa gene product. (*C*) Plant expression vectors containing the TMV 54-kDa gene sequence inserted between the CaMV 35S promoter and the nopaline synthase (NOS) polyadenylylation site. These plasmids were derived by insertion of the TMV cDNA into either the *Xho* I site (pTS541) or the *Sma* I site (pTS541A) in the polylinker region of pMON316. The numbers refer to nucleotides in the TMV genome. The neomycin phosphotransferase II (NPTII) gene confers a selectable kanamycin-resistance marker to transformed plants. LIH, Ti homologus DNA; ORI, origin of replication.

extracts of transformed and untransformed plants were prepared by homogenizing leaf samples in 50 mM Tris·HCl, pH 7.5/0.4 M sucrose/20% (vol/vol) glycerol/5 mM $MgCl_2$/10 mM KCl/10 mM 2-mercaptoethanol. The extracts were centrifuged at 30,000 × *g*. Both the supernatants and the resuspended pellet were subjected to electrophoresis in an SDS/12.5% polyacrylamide gel and transferred to nitrocellulose. The nitrocellulose filter was incubated first with specific antibodies followed by gold-conjugated anti-rabbit antibodies, and the detection was enhanced with silver (8).

In an additional approach to detect the 54-kDa protein, leaf strips (1–2 mm × 50 mm) of TMV-infected Turkish Samsun tobacco were vacuum infiltrated with [^{35}S]methionine at a concentration of 10 μCi/ml (1 Ci = 37 GBq) in 10 mM KH_2PO_4 (pH 7.0) containing chloramphenicol at 1 mg/ml. They were incubated in dim light for 20 hr at 25°C. Protoplasts were also labeled with [^{35}S]methionine; they were prepared from either transgenic *N. tabacum* cv. Xanthi nn or cv. Xanthi NN leaves as described by Hills *et al.* (8). The latter protoplasts were electroporated with TMV RNA at 10 μg/ml. Protoplasts (about 150,000 per ml containing 5–10 μCi of [^{35}S]methionine per ml) were incubated at 25°C in dim light for 40 hr. They were collected by low-speed centrifugation and lysed in 20 mM Tris·HCl, pH 7.5/2 mM EDTA/0.5% SDS/0.2% 2-mercapthethanol containing phenylmethylsulfonyl fluoride as a protease inhibitor. Leaf strips were extracted by grinding them in a mortar and pestle with a similar solution that did not contain the inhibitor. The extracts were centrifuged at 30,000 × *g*, and both the pellet and supernatant fractions were examined for the 54-kDa protein. The extracts of either the labeled leaves or protoplasts were incubated with the antisera described above and immunoprecipitated as described by Berry *et al.* (20). Further analysis was by polyacrylamide gels and autoradiography.

Inoculation of Transformed Plants. R1 seedlings from self-fertilized transgenic plants were routinely inoculated with either 100 μg of TMV (U1 strain) per ml of 50 mM phosphate buffer (pH 7.2), with Celite added as an abrasive, or TMV (U1 strain) RNA at a concentration of 300 μg/ml in 50 mM Tris phosphate buffer (pH 8.6) with Celite. Two leaves of each plant were inoculated. Excess inoculum was washed from the leaves. The volume of the inoculum was not standardized since inoculum concentration is the critical determinant as long as there is sufficient volume for adequate spread. In our more recent experiments, we used a closely related TMV mutant YSI/1 (mutant b6 in ref. 21), which is easier to score as a consequence of a bright yellow symptoms it elicits. Plants were scored daily by visual observation of symptom development. In some cases, presence of viral RNA in inoculated plants was determined by probing leaf extracts with ^{32}P-labeled cDNA to TMV (22).

RESULTS

TMV 54-kDa cDNA Gene Clone. A cDNA clone of part of the TMV genomic RNA was synthesized using a 22-base primer complementary to nucleotides 4906–4923 at the 3′ end of the 183-kDa gene sequence (23). The cloning procedure used resulted in the production of a cDNA clone, pRTT-1, which contains the entire 54-kDa open reading frame downstream from the T7 promoter in the vector pBS(−). The clone also contains an additional 12 nucleotides upstream of the AUG codon at position 3495, which is believed to be the initiator for the synthesis of the 54-kDa protein. The presence of an intact 54-kDa open reading frame in pRTT-1 was verified by synthesizing T7 transcripts *in vitro* and translating them in a rabbit reticulocyte lysate system (Promega). *In vitro* translation yielded a 54-kDa product, which confirmed the presence of an intact open reading frame and suggested that the AUG at position 3495 can function as an initiation codon. Immunoprecipitation with 54-kDa antiserum verified that the translation product is indeed the 54-kDa protein (data not shown).

Four transformed plants were generated with pTS541, and four plants were generated with pTS541A, which lacks the 3′ nopaline synthase untranslated region and a portion of the Ti homology region that is located immediately downstream from the 54-kDa open reading frame. This deletion did not interfere with integration of the chimeric TMV 54-kDa gene sequence into the plant genome. Progeny seed was collected from each self-fertilized plant. Additionally, plants were transformed with the chimeric TMV gene such that 54-kDa gene antisense RNAs were produced. Two independent antisense transformants were regenerated.

TMV 54-kDa Gene DNA Analysis. Six of the independently transformed plants (specified in Fig. 2) were analyzed for expression of the chimeric gene. Genomic DNA was isolated from transformed and untransformed *N. tabacum* cv. Xanthi nn. *Bam*HI digests of the genomic DNA were hybridized to a ^{32}P-labeled TMV 54-kDa gene sequence-specific probe. Hybridization to a 3.0-kilobase (kb) fragment verified the presence of a full-length 54-kDa gene coding sequence (data not shown). The 54-kDa gene sequence insert is 1.44 kb, and another 1.59 kb is contributed by flanking vector DNA. The copy number of the 54-kDa gene sequence in the transgenic plants, as determined by slot blot hybridization analysis, varied from one to five copies per diploid genome between different transgenic plants (Fig. 2). No copies of the 54-kDa gene sequence were detected in nontransformed plants or in plants transformed with pMON316 lacking the 54-kDa gene sequence insert.

TMV 54-kDa Gene RNA Analysis. RNA extracted from transformed plants was examined by Northern blot hybridization analysis (Fig. 3). RNA of the expected size for the chimeric mRNA of 1.6 kb was identified in total RNA from all eight transgenic plants containing the TMV 54-kDa gene sequence in the sense orientation. Plants containing the integrate plasmid that lacks the 3′ nopaline synthase untranslated region and the Ti homologous region (541A11, 541A12, 541A20, and 541A21) synthesized a 1.6-kb transcript in lower amounts (Fig. 3); in addition, a larger transcript was synthesized, which might result from the lack of the transcription termination sequence usually contributed by the nopaline synthase 3′ sequence. In all plants a number of smaller unidentified transcripts were also detected. Plants transformed with the vector alone did not produce any transcripts that hybridized with the probe for the 54-kDa gene sequence (lane 316 in Fig. 3).

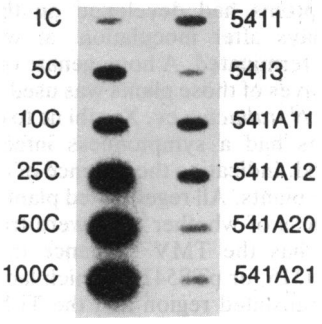

FIG. 2. Determination of the copy number of TMV 54-kDa gene sequences in transgenic tobacco plants. Ten micrograms of total DNA from each transgenic plant was spotted onto a nylon filter and hybridized to a ^{32}P-labeled probe specific for the 54-kDa gene sequence. Copy number was determined by comparison to reconstructions of 10 μg of tobacco DNA mixed with genome equivalents of the 54-kDa sequence insert for the different copy numbers. 1C, 5C, 10C, 25C, 50C, and 100C refer to 1, 5, 10, 25, 50, and 100 copies of the 54-kDa gene sequence per diploid genome, respectively.

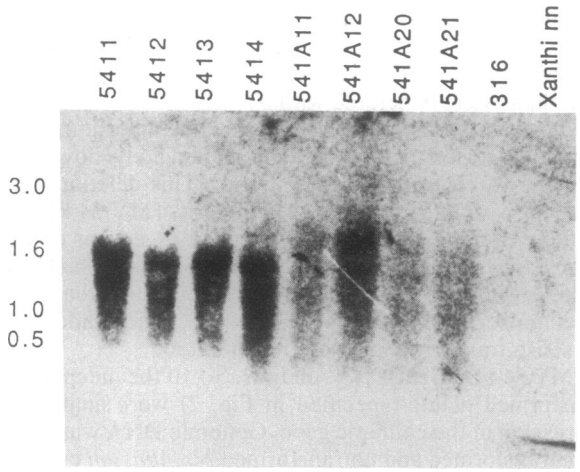

FIG. 3. Analysis of RNA in transformed tobacco plants. Total RNA was isolated from leaves of transgenic plants and separated on a 1.2% agarose/formaldehyde gel, transferred to GeneScreen*Plus*, and hybridized with a 54-kDa gene sequence-specific probe. RNA was isolated from pTS541-transformed tobacco (5411, 5412, 5413, and 5414), pTS541A-transformed tobacco (541A11, 541A12, 541A20, and 541A21), controls of tobacco transformed with pMON316 (316), and untransformed tobacco, Xanthi nn. The sizes of the RNAs (in kb) are given at left.

Analysis of Plants for the 54-kDa Protein. Leaf extracts of transformed plants and of infected untransformed controls were incubated with antiserum raised against a synthetic oligopeptide prepared to an internal region of the 54-kDa protein sequence and with antiserum specific for the 54-kDa read-through portion of the TMV 183-kDa protein. A 54-kDa protein could not be detected either by Western blotting or by immunoprecipitation of ^{35}S-labeled extracts from 54-kDa transgenic plants, protoplasts prepared from 54-kDa transgenic plants, or the infected untransformed control plants.

Resistance of Transformed Plants. In the first experiments to assay for resistance to infection by TMV, plants were inoculated with 50 μg of TMV (U1 strain) per ml. Four rooted cuttings from each of the eight independently transformed plants containing the 54-kDa coding sequence in the sense orientation, controls transformed with the vector alone, and several untransformed Xanthi nn were inoculated. At 5 days after inoculation, the transgenic controls and the untransformed controls had clearly developed characteristic mosaic symptoms, while the transformed plants containing the 54-kDa gene sequence showed no sign of symptom development. No symptoms had developed on these transgenic plants by 48 days after inoculation, at which point the experiment was terminated. A homogenate of the inoculated and the upper leaves of those plants was used to inoculate the local lesion host *N. tabacum* cv. Xanthi nc to determine if the transgenic plants had a symptomless infection. No local lesions developed, indicating the absence of detectable virus in the transgenic plants. All regenerated plants were resistant to TMV regardless of whether they were transformed with pTS541, which has the TMV sequence inserted into the complete pMON316, or pTS541A, which lacks the nopaline synthase 3′ untranslated region and the Ti homologous region. Typical plants showing resistance and susceptibility are shown in Fig. 4. Plants transformed with the chimeric gene in the orientation that resulted in synthesis of the 54-kDa gene antisense RNA were also challenged with TMV; these plants were not resistant, but in a single experiment, there was a delay in symptom development as compared to the vector-transformed control (data not shown). These plants were not examined further; we have concentrated our efforts on the plus-sense plants exhibiting complete resistance.

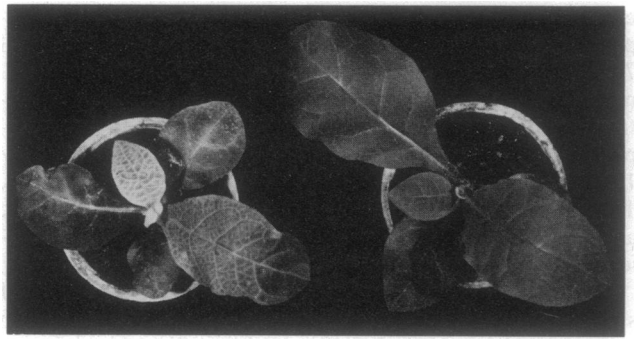

FIG. 4. Resistance of transformed tobacco plants to TMV infection. Shown are transgenic tobacco plants 15 days after inoculation with 100 μg of TMV, strain YSI/1, per ml. The plant on the left was transformed with the vector alone, and the plant on the right was transformed with the TMV 54-kDa gene sequence. Typical mosaic symptoms have developed only on the control plant.

Progeny seedlings from self-fertilized transgenic plants were also analyzed for inheritance of the resistance phenomenon. R1 seeds were germinated on tissue culture medium containing 300 μg of kanamycin per ml. Kanamycin-sensitive seedlings were considered to be those that were chlorotic and did not grow beyond the cotyledon stage. The segregation ratio of the seedlings expressing kanamycin resistance to those susceptible to kanamycin indicates that in each of the original transformants the neomycin phosphotransferase II (NPTII) gene (which confers resistance to kanamycin) was integrated at multiple loci. On the other hand, the resistance to TMV segregated at ≈3:1, even though some of the transformed plants contained multiple copies of the sequence (data not shown). Thus, the multiple inserts must all be on one chromosome. The large number of kanamycin resistant "escapes" makes this an unreliable means of screening progeny seedlings for expressors of the integrated chimeric TMV gene (24). All subsequent infection experiments described in this report were done with the segregating population of line 541A11-derived R1 seedlings.

Resistance was observed at inoculum concentrations up to 500 μg of TMV per ml or 300 μg of TMV RNA per ml (the highest concentrations tested). The resistant plants were maintained for 30 days after inoculation without any subsequent development of symptoms. To assay for virus replication and spread of the virus, extracts of the leaf samples were probed with cDNA prepared from purified TMV RNA. Viral RNA could not be detected in either the inoculated leaves or in the systemic leaves of the plants that demonstrated resistance; thus, the resistance was not merely a suppression of symptom development.

In another experiment, some plants were transferred immediately after inoculation to a growth chamber maintained at 31°C, to determine if the 54-kDa-induced resistance to TMV is temperature sensitive. Five of the seven inoculated plants in the segregating population, which carry the 54-kDa gene sequence, did not develop symptoms at 31°C, whereas all control plants developed symptoms typical of those kept at 24°C, indicating that the resistance was not temperature sensitive.

Resistance to Other TMV Strains and to CMV. Tobacco plants transformed with the 54-kDa gene sequence and the vector-transformed controls were inoculated with the U2 or L strains of TMV or with CMV. Each virus or strain was tested at concentrations of 2.5–100 μg/ml. No resistance was observed in any of the tests (data not shown).

DISCUSSION

Since the seminal paper of Powell-Abel *et al.* (25) showing that plants transformed with and expressing the coat protein gene of TMV are resistant to TMV, there have been other examples

of this concept, and it will undoubtedly have important implications for the protection of many crop species from many viruses. To date, coat protein-mediated resistance has been shown with alfalfa mosaic virus (AlMV) (26, 27), tobacco rattle virus (28, 29), potato virus X (30, 31), CMV (32), potyviruses (33, 34), and plants transformed with both potato virus X and Y coat protein (34). The findings reported here represent an entirely different approach to virus-induced resistance. In this study we have demonstrated that transgenic plants containing the TMV 54-kDa gene coding sequence are resistant to infection with TMV. Presence of the 54-kDa gene sequence prevents the development of local chlorosis and any systemic development of symptoms. There was no detectable virus in the inoculated or systemic leaves of resistant transgenic plants, whereas virus was easily detectable in all of the controls. The 54-kDa-induced resistance is absolute and is not simply a delay in symptom development. This resistance is also not as "fragile" as coat protein-induced resistance, which may break down when high concentrations of inoculum are used (25). In contrast, with the 54-kDa gene sequence, complete resistance is observed in plants challenged with high concentrations of virus or viral RNA. Protection mediated by the coat proteins of TMV and AlMV can be overcome by inoculating with viral RNA (26, 34, 35), whereas 54-kDa gene sequence-induced resistance remains uncompromised when challenged with RNA.

The level of resistance in plants transformed with the 54-kDa gene sequence does not appear to depend upon the number of copies of the inserted sequence. Plants with only one copy of the gene sequence did not show a decrease in resistance to intact virions or viral RNA. On the other hand, a single copy of the TMV coat protein gene was also sufficient to protect (25), whereas one copy of the AlMV coat protein did not provide protection (26). The absence of a polyadenylylation site immediately downstream from the 54-kDa gene sequence was also shown not to have any effect on expression of resistance.

The question remains as to whether the 54-kDa gene sequence-mediated resistance is due to the 54-kDa protein or its nucleic acid. Attempts to detect the 54-kDa protein in TMV-infected leaf tissue or in protoplasts have not been successful. Surprisingly, we have also not been able to detect a 54-kDa gene product in the 54-kDa transgenic plants. This is unexpected because the 54-kDa open reading frame is preceded by the CaMV 35S promoter, which is known to function quite well in the plant genome (14). It is possible that the 54-kDa protein is very labile and rapidly turned over in the cell or that it is made at such low levels that it is below our limit of detection. Based on the suggested role of the 183-kDa protein in replication of the TMV genome (2), one might hypothesize that the 54-kDa protein is also a component of a membrane-bound replicase. In addition, evidence suggests that the TMV 126-kDa protein is also a component of the TMV replicase complex (2). We have produced transgenic plants containing the complete 126-kDa gene sequence and have found these plants to be as susceptible to infection as untransformed plants (data not shown). The predicted 54-kDa protein contains a Gly-Asp-Asp motif that is conserved among many positive-strand viruses (36–38) and has been shown to be necessary for replicase activity in Q_β bacteriophage (39). Constitutive synthesis of either the 54-kDa RNA transcript or the 54-kDa protein, which includes the Gly-Asp-Asp sequence, probably interferes with TMV replication. If the 54-kDa protein itself is not a constituent of the replication complex, it could induce resistance by competing with the 183-kDa protein, which is most probably a constituent of that complex.

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1. Palukaitis, P. & Zaitlin, M. (1986) in *The Plant Viruses*, eds. Van Regenmortel, M. H. V. & Frankel-Conrat, H. (Plenum, New York), Vol. 2, pp. 105–131.
2. Young, N. D., Forney, J. A. & Zaitlin, M. (1987) *J. Cell Sci. Suppl.* **7**, 277–285.
3. Beachy, R. N. & Zaitlin, M. (1977) *Virology* **81**, 160–169.
4. Sulzinski, M. A., Gabard, K. A., Palukaitis, P. & Zaitlin, M. (1985) *Virology* **145**, 132–140.
5. Zelcer, A., Weaber, K. F., Balaczs, E. & Zaitlin, M. (1981) *Virology* **113**, 417–427.
6. Palukaitis, P., Garcia-Arenal, F., Sulzinski, M. A. & Zaitlin, M. (1983) *Virology* **131**, 533–545.
7. Saito, T., Watanabe, Y., Meishi, T. & Okada, Y. (1986) *Mol. Gen. Genet.* **205**, 82–89.
8. Hills, G. J., Plaskitt, K. A., Young, N. D., Watts, J. W., Wilson, T. M. A. & Zaitlin, M. (1987) *Virology* **161**, 488–496.
9. Asselin, A. & Zaitlin, M. (1978) *Virology* **91**, 173–181.
10. Siegel, A. & Wildman, S. G. (1954) *Phytopathology* **44**, 277–282.
11. Ohno, T., Aoyagi, M., Yamanashi, Y., Saito, H., Ikawa, S., Meishi, T. & Okada, Y. (1984) *J. Biochem. (Tokyo)* **96**, 1915–1923.
12. Harrison, B. D. (1987) *Ciba Found. Symp.* **133**, 130–131.
13. Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab., Cold Spring Harbor, NY).
14. Sanders, P. R., Winter, S. A., Barnason, A. R., Rogers, S. G. & Fraley, R. T. (1987) *Nucleic Acids Res.* **15**, 1543–1558.
15. Rogers, S. G., Horsch, R. B. & Fraley, R. T. (1986) *Methods Enzymol.* **118**, 627–640.
16. Horsch, R. B., Fry, J. E., Hoffman, N. L., Eichholtz, D., Rogers, S. D. & Fraley, R. T. (1985) *Science* **227**, 1229–1231.
17. Murray, M. G. & Thompson, W. F. (1980) *Nucleic Acids Res.* **8**, 4321–4325.
18. Feinberg, A. P. & Vogelstein, B. (1983) *Anal. Biochem.* **132**, 6–13.
19. Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J. & Rutter, W. J. (1979) *Biochemistry* **18**, 5294–5299.
20. Berry, J. O., Nikolau, B. J., Carr, J. P. & Klessig, D. F. (1985) *Mol. Cell. Biol.* **5**, 2238–2246.
21. Garcia-Arenal, F., Palukaitis, P. & Zaitlin, M. (1984) *Virology* **132**, 131–137.
22. Palukaitis, P. (1988) in *Methods for Plant Molecular Biology*, eds. Weissbach, A. & Weissbach, H. (Academic, New York), pp. 487–506.
23. Goelet, P., Lomonossoff, G. P., Butler, P. J. G., Akam, M. E., Gait, M. J. & Karn, J. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 5818–5822.
24. Lee, B., Murdoch, K., Kreis, M. & Jones, M. G. K. (1989) *Plant Mol. Biol. Rep.* **7**, 129–134.
25. Powell-Abel, P., Nelson, R. S., De, B., Hoffman, N., Rogers, S. G., Fraley, R. T. & Beachy, R. N. (1986) *Science* **223**, 738–743.
26. Loesch-Fries, L. S., Merlo, D., Zinnen, T., Burhop, L., Hill, K., Krahn, K., Jarvis, N., Nelson, S. & Halk, E. (1987) *EMBO J.* **6**, 1845–1851.
27. Tumer, N. E., O'Connell, K. M., Nelson, R. S., Sanders, P. R., Beachy, R. N., Fraley, R. T. & Shah, D. M. (1987) *EMBO J.* **6**, 1181–1188.
28. Van Dun, C. M. P. & Bol. J. F. (1988) *Virology* **167**, 649–652.
29. Angenent, G. C., van den Ouweld, J. M. W. & Bol, J. F. (1990) *Virology* **175**, 191–198.
30. Hemenway, C., Fang, R.-X., Kaniewski, W., Chua, N.-H. & Tumer, N. E. (1988) *EMBO J.* **7**, 1273–1280.
31. Hoekema, A., Huismann, M. J., Molendijk, L., van den Elzen, P. J. M. & Cornelissen, B. J. C. (1989) *Bio/Technology* **7**, 273–278.
32. Cuozzo, M., O'Connell, K. M., Kaniewski, W., Fang, R.-X., Chua, N.-H. & Tumer, N. (1988) *Bio/Technology* **6**, 549–557.
33. Stark, D. M. & Beachy, R. N. (1989) *Bio/Technology* **7**, 1257–1262.
34. Lawson, C., Kaniewski, W., Haley, L., Rozman, R., Newell, C., Sanders, P. & Tumer, N. E. (1990) *Bio/Technology* **8**, 127–134.
35. Van Dun, C. M. P., Bol, J. F. & Van Vloten-Doting, L. (1987) *Virology* **159**, 299–305.
36. Kramer, G. & Agros, P. (1984) *Nucleic Acids Res.* **12**, 7269–7282.
37. Goldbach, R. (1987) *Microbiol. Sci.* **4**, 197–202.
38. Habili, N. & Symons, R. H. (1989) *Nucleic Acids Res.* **17**, 9543–9555.
39. Inokuchi, Y. & Hirashima, A. (1987) *J. Virol.* **61**, 3946–3949.