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A defective replicase gene induces resistance to cucumber mosaic virus in transgenic tobacco plants

(plant virus/replicase-mediated resistance)

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ABSTRACT **_Nicotiana tabacum_ cv. Turkish Samsun NN plants were transformed with a modified and truncated replicase gene encoded by RNA-2 of cucumber mosaic virus strain Fny. The replicase gene had been modified by deleting a 94-base-pair region spanning nucleotides 1857–1950; the deletion also caused a shift in the open reading frame, resulting in a truncated translation product ≈75% as large as the full-length protein. Upon transformation via _Agrobacterium tumefaciens_, transgenic plants were obtained that were resistant to virus disease when challenged with either cucumber mosaic virus virions or RNA at concentrations up to 500 μg/ml or 50 μg/ml, respectively, the highest concentrations tested. This resistance was absolute, as neither symptoms nor virus could be detected in uninoculated leaves, even after prolonged incubation (120 days after inoculation). These data suggest, therefore, that such a "replicase-mediated" resistance strategy may be applicable to other plant and animal viruses.**

Cucumber mosaic virus (CMV) is a small plus-sense RNA virus that is the causal agent for virus diseases affecting a variety of important crop species. There are many strains of CMV; it has been estimated that at least 775 plant species representing 85 families are natural hosts of this virus (1). The CMV genome is comprised of three RNAs that contain genes encoding four proteins (2). Two of the proteins, encoded by the two larger RNAs, are components of the RNA-dependent RNA polymerase, or replicase, responsible for CMV replication (3). Inhibition of the synthesis or function of replicase genes is a potential strategy to control virus disease. We have recently demonstrated that transformation of tobacco with an intact open reading frame of a replicase read-through component of tobacco mosaic virus (TMV) engendered resistance (4). MacFarlane and Davies (5) have extended this phenomenon to induce resistance in *Nicotiana benthamiana* to pea early browning virus, which, although in a different taxonomic group, also uses a read-through codon in its replicase gene. This form of resistance, which would be restricted to those viruses with such a replicase read-through open reading frame, requires the synthesis of a functional protein for resistance to be generated (5, 25). Another approach is to create "dominant negative" mutations in a gene that could compete with and disrupt the activity of the functional gene (6). To determine whether such a strategy might be effective for control of one of the many plant viruses that do not use the read-through strategy of TMV to produce two replicase proteins, a DNA copy of a defective replicase gene from RNA-2 of CMV strain Fny was used to transform tobacco plants. As reported here, plants transformed with this construct were resistant to CMV when the inoculum consisted of either virions or RNA.

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MATERIALS AND METHODS

Plants and Virus. *Nicotiana tabacum* cv. Turkish Samsun NN was used both for virus propagation and transformation. Plants were grown either in a greenhouse or in a growth chamber with a 14-hr light/10-hr dark cycle at 25°C when used for assessing resistance of transformants. CMV strain Fny was isolated and purified from infected plants as described (7). Viral RNA was isolated by phenol extraction and ethanol precipitation.

Cloning and Modification of the CMV RNA Replicase Gene. A cDNA clone of RNA-2 (8), which encodes a transcript of the replicase component of CMV subgroup I strain Fny was modified by deleting a 94-base-pair (bp) region spanning nucleotides (nt) 1857–1950 (Fig. 1, *A* and *B*). These 94 nt were deleted by digesting the Fny-CMV cDNA clone pFny206 (8), which encodes a full-length RNA-2, with *Nco* I and *Bst*EII and treatment with Klenow fragment of DNA polymerase I to obtain a blunt-ended molecule. This linear molecule was recircularized by T4 DNA ligase to generate a plasmid (pFny N/B-4) that contained a cDNA clone of Fny-CMV RNA-2 in which 94 nt from nt 1857–1950, inclusive, were deleted. This deletion also shifted the open reading frame, which caused an in-frame translational stop codon 15 codons downstream of the deletion site.

The modified gene was excised from this vector by the enzymes *Sph* I and *Bam*HI. Use of the *Sph* I restriction enzyme and retention of this site in the construct creates an AUG as a potential translational initiator 87 nt upstream of the AUG in the RNA-2 gene (Fig. 1, *C* and *D*). This result presents the likely possibility that translation of the truncated RNA-2 gene starts at this upstream AUG, adding an additional 29 amino acids at the amino terminus. The protein encoded by this modified gene is ≈75 kDa, as predicted from the sequence and determined by *in vitro* translation in a rabbit reticulocyte lysate system (data not shown), compared with a wild-type protein of 96.7 kDa.

Plant Transformation. A plasmid (pCMV N/B-23) was derived by inserting the modified Fny-CMV RNA-2 gene into the *Bam*HI site of pROK2 (10), a binary plant transformation vector (from M. W. Bevan, Norwich, U.K.). This insertion was accomplished by digesting pFny N/B-4 with *Sph* I, which cuts this plasmid at a site 5′ of the RNA-2 cDNA sequences. A *Bam*HI–*Sph* I adapter was ligated to this *Sph* I site and then digested with *Bam*HI, which cuts at a site 3′ of the RNA-2 sequences, thereby liberating the entire modified cDNA molecule. This 3-kilobase (kb) fragment was subcloned via standard techniques (11) into the *Bam*HI site of pROK2 (10). Before plant transformation, this construct was transferred to *Agrobacterium tumefaciens* strain LBA-

Abbreviations: CMV, cucumber mosaic virus; TMV, tobacco mosaic virus; nt, nucleotide(s); NOS, nopaline synthase.

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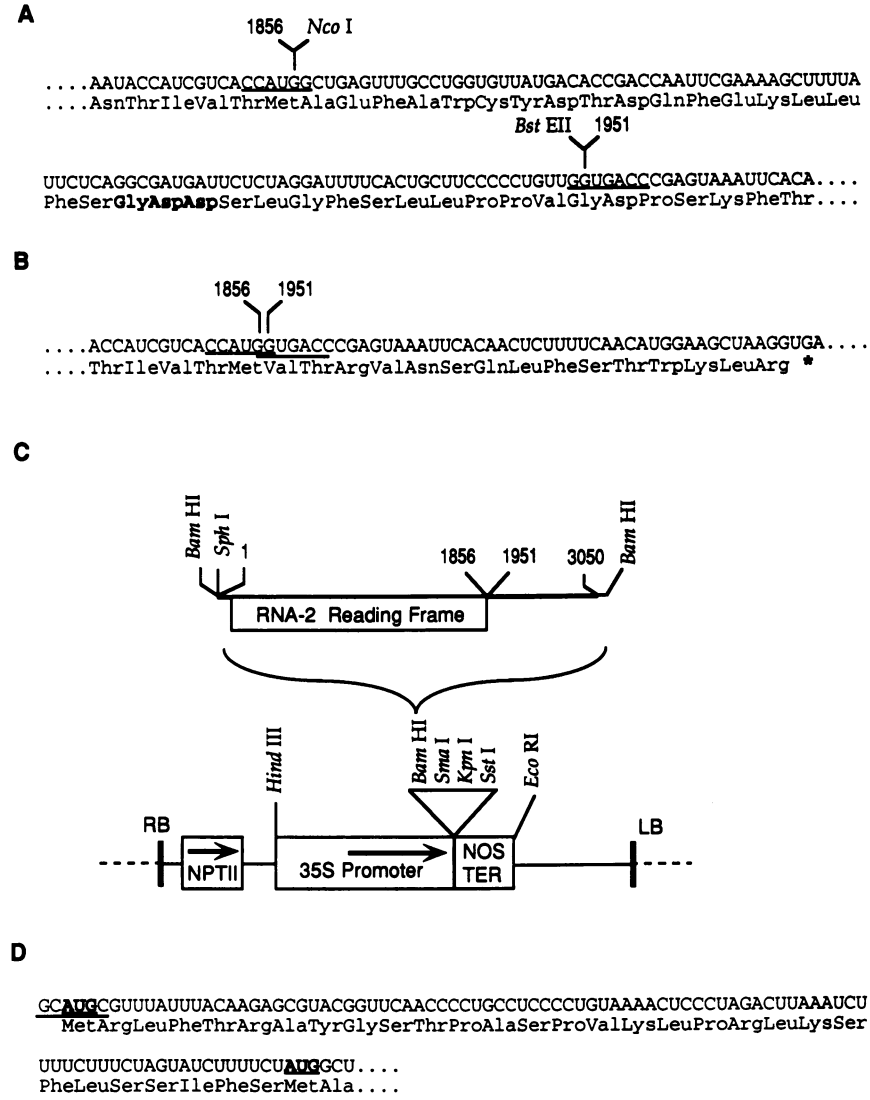

FIG. 1. (*A*) Partial nucleotide and amino acid sequence of the Fny-CMV RNA-2 cDNA clone (8), which was subsequently modified as described in text. The *Nco* I and *Bst*EII sites (underlined) were used to generate a 94-bp deletion. The Gly-Asp-Asp box common to replicases (9) is shown in boldface type. (*B*) Nucleotide and amino acid sequences of the modified Fny-CMV RNA-2 cDNA clone after 94 nt were deleted, showing additional amino acids translated as a result of the frame shift, terminating at the UGA indicated by *. (*C*) Plant expression vector containing the modified Fny-CMV RNA-2 gene sequence inserted between the cauliflower mosaic virus 35S promoter and the NOS polyadenylylation site (NOS TER). Numbers refer to nucleotides in the Fny-CMV RNA-2 sequence. LB and RB, left and right border of Ti plasmid DNA transferred to the plant genome during *A. tumefaciens*-mediated plant transformation; NPTII is the gene for the selectable marker neomycin phosphotransferase II; Ori, origin of replication. (*D*) Sequences of the 5′ end of the modified CMV RNA-2 clone depicting 29-amino acid residues potentially translated as a consequence of retention of an AUG codon by the *Sph* I restriction site (5 nt underlining).

4404 by tri-parental mating (12) mediated by *Escherichia coli* strain MM294-pRK2013. The transconjugants were selected by resistance to kanamycin and streptomycin at 50 and 125 μg/ml, respectively. The neomycin phosphotransferase II gene confers a selectable kanamycin resistance marker on transformed plants.

Nucleic Acid Analyses. For analysis of RNA transcripts, total RNA was isolated from plants (13), and 40 μg was electrophoresed in a 1.2% agarose gel containing formaldehyde (11), transferred to GeneScreenPlus membranes (DuPont) and hybridized using the nopaline synthase (NOS) terminator sequence (10) as a probe. The NOS terminator was used because it allows for the detection of the replicase gene transcript in both infected and uninfected plants. The presence of defective replicase genes was determined by Southern blot hybridization analysis (11) of genomic DNA isolated (14) from each plant. The 35S cauliflower mosaic virus promoter was used as a probe (15). These genomic DNAs were digested with either *Hin*dIII/*Eco*RI, which liberated a 3.8-kb fragment, *Pst* I, which liberated a 4.1-kb fragment as well as higher-molecular-mass fragments due to incomplete digestion of methylated DNA, or *Hin*dIII, which liberated fragments of various molecular weights, depending upon the insertion site of the modified replicase gene in the genome.

Plant Virus Resistance Tests. Fully expanded leaves were detached from the original transformants in a preliminary screen for virus resistance. They were inoculated with Fny-CMV at 50 μg/ml on the upper and lower surfaces and were kept with their petioles in water for 6 days to determine whether they supported virus replication. A fresh slice was removed from the cut end of the petiole each day to preclude callus formation, thereby ensuring water uptake. Virus replication was ascertained by dot-blot hybridization of leaf tissue using ^{32}P-labeled cDNA to CMV (16). Plants derived from the selfed transformants (R_1 generation) were assessed

for resistance by inoculation of small (<3 cm tall) seedlings on two leaves per plant, using an inoculum of virus in 50 mM $NaPO_4$ buffer, pH 7.2, with Celite (Fisher Scientific) as an abrasive. When CMV RNA was used as inoculum, it was suspended in 10 mM Tris·HCl buffer, pH 8.9. Control plants were either untransformed tobacco or plants of cell line 2-1, which, although transformed, did not exhibit resistance to CMV.

RESULTS AND DISCUSSION

Tobacco leaf tissue was transformed with the modified CMV RNA-2 replicase gene, and plants were derived from the shoots that developed on kanamycin. Eighteen independent transformants were screened initially for resistance to CMV infection in a detached-leaf assay. Seven of these 18 transgenic plants appeared resistant to CMV infection (data not shown). The plants were allowed to flower, self-fertilize, and seeds of the R_1 generation were collected.

R_1 generation seedlings from each of these seven putative resistant lines (1-1, 1-2, 1-8, 2-3, 2-5, 2-6, and 2-7), one transgenic line (2-1) that was not resistant by this detached-leaf assay, and nontransformed *N. tabacum* cv. Turkish Samsun NN were inoculated with Fny-CMV at 0.2, 1, 5, 10, and 100 μg/ml. All of the putative resistant lines either had a delay in symptoms (data not shown) or were resistant at inoculum doses between 0.2 and 1 μg/ml (Table 1). However, only two R_1 lines (1-2 and 1-8) contained plants that were resistant at inoculum concentrations of 5, 10, and 100 μg/ml. Because R_1 generation plant lines 1-2 and 1-8 had a very high degree of resistance to CMV, these lines were chosen for further analysis. It should be noted that not all R_1 generation plants from these lines were resistant, as there is segregation of the resistance trait within these R_1 populations. R_1 seedlings of lines 1-2, 1-8, 2-3, and the two control lines (NN and 2-1) were inoculated with CMV at 500 μg/ml. Fourteen of 32 inoculated line 1-8 plants and 21 of 26 inoculated line 1-2 plants developed symptoms (Table 1). Hence, it appears that very high inoculum doses may overwhelm some plants that might be resistant to lower inoculum doses. This presumption was confirmed in experiments with R_2 generation plants, where a few plants of line 1-2 (3 of 35) that were resistant at 100 μg/ml showed symptoms when superinfected at 500 μg/ml. Neither viral RNA nor virus could be detected in the uninoculated leaves of resistant plants with dot-blot hybridization and/or bioassay. This result showed that these plants exhibited true resistance and not merely symptom suppression. Chlorotic lesions were observed on the inoculated leaves of some resistant plants, which, nevertheless, did not develop systemic infections. Virus replication in these leaves was confirmed by dot-blot hybridization and bioassay. These observations suggest that resistance is due to an inhibition of viral movement and/or replication at the infection site. Judging from the spreading symptoms on the CMV-inoculated leaves, some viruses may move from cell to cell but do not invade or survive in the vascular system.

To determine whether resistance was temperature sensitive, R_1 seedlings of the resistant 1-2 and 1-8 lines and the two control lines were inoculated with CMV at 20 μg/ml and placed in a growth chamber maintained at 30°C. Within 4 days, all the control plants developed systemic symptoms, whereas many plants of lines 1-2 and 1-8 remained symptomless, indicating that the resistance was not temperature sensitive (Table 1).

Prior studies have shown that coat protein-mediated protection of TMV and alfalfa mosaic virus can sometimes be overcome by inoculation with viral RNA (17–19). Therefore, R_1 seedlings of line 1-8, the nonresistant 2-1 control line, and nontransformed *N. tabacum* cv. Turkish Samsun NN were inoculated with CMV RNA at 50 μg/ml. The controls developed systemic symptoms in 100% of the plants by 4 days. However, only 36% of the segregating line 1-8 plants developed symptoms even after 14 days (Table 1). It is apparent from these data that resistance to both virus and viral RNA at very high inoculum levels has been engendered.

Six of the plants of line 1-2 and six plants of line 1-8 were analyzed by Southern and RNA blot hybridization for the presence of the cauliflower mosaic virus promoter, detecting DNA (Fig. 2*A*), and for the NOS terminator on the poly(A)$^+$ RNA (Fig. 2*B*). In each line, three of the plants were infected (indicated by + on Fig. 2), and three were resistant to symptoms (indicated by −), and no virus was detected in these latter plants by dot-blot hybridization. The Southern blot hybridizations showed that all of the plants, whether susceptible or resistant, except for plant 3 of line 1-2, had DNA copies of the defective replicase gene. This result demonstrates that a copy of the gene is necessary for resistance but does not guarantee that a particular plant will be resistant. RNA blot hybridizations of these plants (Fig. 2*B*) as well as of the three control plants showed that the defective replicase gene transcript was also present in the transformed plants, except for plant 3 of line 1-2 and the three control plants.

Table 1. Assessment of replicase-mediated resistance to CMV infection in R_1 generation transgenic tobacco plants

	Inoculum concentration, μg/ml							
Line	0.2	1	5	10	100	500	20* (Temperature sensitivity test)	50† (RNA inoculum)
Resistant								
1-1	4/12 (33)	12/12 (100)	12/12 (100)	10/10 (100)	9/10 (90)	ND	ND	ND
1-2	1/12 (8.3)	13/34 (38)	2/12 (17)	17/33 (52)	16/36 (44)	21/26 (81)	8/10 (80)	ND
1-8	3/12 (25)	3/27 (11)	4/12 (33)	10/27 (37)	11/31 (35)	14/32 (44)	2/10 (20)	5/14 (36)
2-3	ND	12/23 (52)	ND	23/25 (92)	26/26 (100)	25/25 (100)	ND	ND
2-5	6/12 (50)	10/11 (91)	12/12 (100)	10/10 (100)	10/10 (100)	ND	ND	ND
2-6	4/12 (33)	7/12 (58)	11/11 (100)	10/10 (100)	10/10 (100)	ND	ND	ND
2-7	10/12 (83)	10/12 (83)	11/11 (100)	10/10 (100)	10/10 (100)	ND	ND	ND
Control								
2-1	12/12 (100)	31/31 (100)	11/11 (100)	28/28 (100)	26/26 (100)	27/27 (100)	10/10 (100)	10/10 (100)
N/N	3/3 (100)	4/4 (100)	4/4 (100)	ND	ND	3/3 (100)	4/4 (100)	4/4 (100)

A preliminary detached-leaf assay had indicated that all of these lines (except 2-1 and N/N) were resistant to CMV infection. Plants were inoculated with CMV in 50 mM $NaPO_4$ buffer, pH 7.2, with Celite as an abrasive. These data represent numbers of plants showing symptoms at 2 weeks after inoculation as a function of total number of inoculated plants; numbers in parentheses are the percentage of plants having systemic symptoms. ND, not determined.

*To determine whether temperature affected resistance, plants were inoculated with CMV at 20 μg/ml and incubated at 30°C for 2 weeks.

†Plants were inoculated with CMV RNA at 50 μg/ml in 10 mM Tris·HCl buffer, pH 8.9.

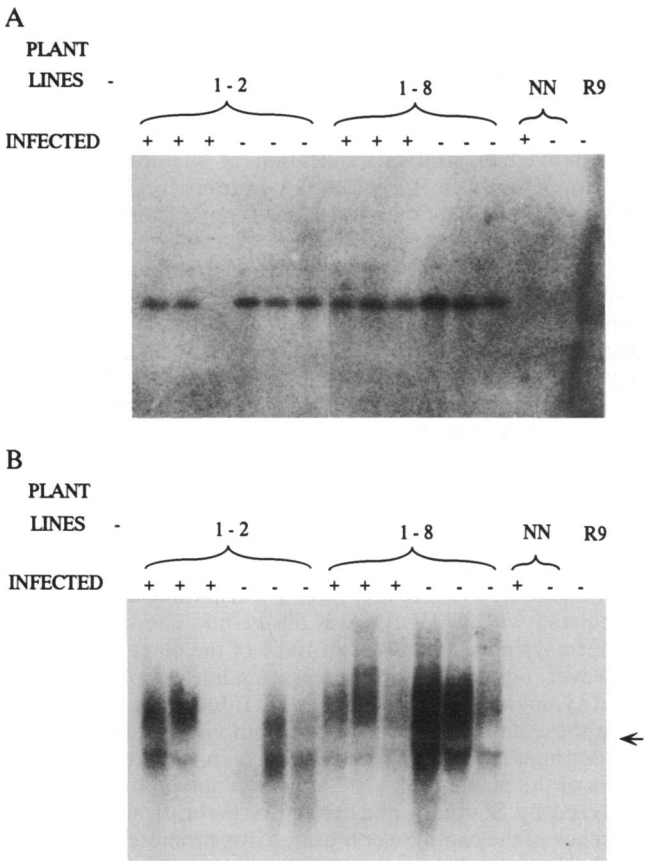

FIG. 2. Southern and RNA blot-hybridization analyses of susceptible and resistant R_1 plants of lines 1-2 and 1-8 inoculated with CMV at 100 and 500 μg/ml, respectively. The control plants were untransformed (NN) and transformed with the vector alone (R9). Of the control plants only the NN (+) plant was inoculated with CMV at 100 μg/ml. The + and − indicate the presence or absence, respectively, of virus in an uninoculated leaf, as determined by dot-blot hybridization. (*A*) Gel blot containing DNA digested with *Eco*RI/*Hin*dIII was probed with the 35S cauliflower mosaic virus promoter, as described in text. (*B*) Gel blot containing total RNA (40 μg/lane) was probed with the NOS terminator, as described. The signal produced by the probe is interrupted by the shadow of the 25S RNA band of 3.3 kb (arrow).

A reduction in virus replication possibly mediated by a defective replicase was also demonstrated by Marsh *et al.* (20). They constructed mutations in the RNA-2 replicase of brome mosaic virus and showed that virus replication was reduced in protoplasts electroporated with mutant RNA-2 transcripts together with wild-type brome mosaic virus genomic RNAs. The data of Marsh *et al.* (20) suggest that the RNA transcript is responsible for the reduction in replication in protoplasts but that there is an even greater reduction when the defective replicase transcript is capable of being translated. One possibility that should be investigated is whether CMV resistance is engendered by the overproduction of defective replicase proteins, in a manner suggested by Herskowitz (6), termed inactivation by "dominant negative mutations." This mechanism would, of course, require synthesis of a defective protein. At this juncture, we have no information about protein products; this investigation awaits the generation of a suitable antiserum to the RNA-2 gene product of Fny-CMV.

The sequences surrounding and including the deleted 94-bp region contain four domains that are highly conserved among putative replicase-encoding sequences in many plus-sense RNA plant and animal viruses (9). This deleted 94-bp region contained the third domain (Gly-Asp-Asp); this sequence has been shown essential for replication of the bacteriophage Qβ (21, 22). Thus, the resistance reported here is most likely a manifestation of the deletion of this region and the carboxyl-terminal third of the CMV RNA-2 protein. This resistance is probably not a result of the likely presence of 29 additional amino acids at the amino terminus of the replicase protein because it has been shown that a mutant of brome mosaic virus RNA-2 in which the amino terminus was extended had no apparent effect on virus replication (23).

We have shown that transgenic tobacco plants containing a modified CMV replicase gene are highly resistant to systemic infection by CMV. Furthermore, replicase-mediated resistance is maintained at an inoculum dose of 500 μg/ml, which is 10-fold higher than that previously shown for CMV coat-protein-mediated protection (24). Earlier, this laboratory reported the generation of TMV-resistant plants by transformation with a 54-kDa gene encoded in the readthrough of a replicase protein (4). An analogous case has recently been reported with pea early browning virus (5). In those situations and in the one reported here, replicase sequences were used for the plant transformation. However, with both the TMV (25) and pea early browning transformations (5) a functional protein seems to be required, whereas in the CMV resistance, a nonfunctional replicase gene is used. Thus the two systems, both of which involve transformation with viral replicase sequences, may operate by different mechanisms. We suggest, furthermore, that one or the other of these replicase-mediated resistances may be applicable to many plant and animal viruses.

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