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Transgenically Expressed Cucumber Mosaic Virus RNA 1 Simultaneously Complements Replication of Cucumber Mosaic Virus RNAs 2 and 3 and Confers Resistance to Systemic Infection

Tomas Canto* and Peter Palukaitis†[1]

*Virology Department, Scottish Crop Research Institute, Invergowrie, Dundee DD2 5DA, Scotland; and †Department of Plant Pathology, Cornell University, Ithaca, New York 14853

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Tobacco plants transformed with a cDNA copy of RNA 1 of the Fny strain of cucumber mosaic virus (CMV) promoted the asymptomatic accumulation of inoculated viral RNAs 2 and 3, which could be detected in noninoculated leaves, suggesting that the transgene also permitted viral long-distance movement. Typical symptoms of infection appeared later and correlated with the appearance of viral RNA 1 regenerated from the transgenic mRNA. Although all R_0-generation plants were susceptible to Fny-CMV, one line displaying variable susceptibility to the virus in R_1-and R_2-generations led to selected R_3-generation lines with systemic resistance to Fny-CMV. In the inoculated leaves of resistant plants, a dramatic decrease in the accumulation of viral RNA 1 was observed, relative to susceptible plants. No viral RNAs were detected in noninoculated leaves of the resistant plants, but such leaves were susceptible to infection. Furthermore, these leaves could sustain replication of inoculated CMV RNAs 2 and 3, indicating that a complete transgene-silencing had not been induced. Although a transgene-mediated, CMV RNA 1-suppression occurred in the inoculated leaf of resistant plants, the absence of a complete systemically acquired silencing suggests the existence of additional interferences with viral infection that prevented systemic infection by viral RNAs 2 and 3.

INTRODUCTION

Cucumber mosaic virus (CMV), a virus species within the family *Bromoviridae,* is a tripartite plant RNA virus with four major encapsidated RNAs (Palukaitis *et al.,* 1992). RNAs 1 and 2 encode viral components of the viral replicase: the 1a protein, a putative helicase, and the 2a protein, the viral polymerase (Hayes and Buck, 1990). In addition to these roles in replication, RNA 1 has a function in virus movement (Gal-On *et al.,* 1994), whereas RNA 2 also encodes a small protein of 13 kDa (2b), which may facilitate long-distance movement of the virus in certain hosts (Ding *et al.,* 1995). RNA 3 encodes the viral movement (3a) and coat (CP) proteins. The latter is expressed via subgenomic RNA 4. Both the 3a protein and the CP are required for virus cell-to-cell movement (Suzuki *et al.,* 1991; Boccard and Baulcombe, 1993; Canto *et al.,* 1997).

Transgenic expression of biologically active 1a or 2a proteins, capable of sustaining replication of the heterologous viral RNAs (1 or 2, plus 3) *in planta*, has been reported for three members of *Bromoviridae*: alfalfa mosaic virus (AlMV; van Dun *et al.,* 1988), brome mosaic virus (BMV; Mori *et al.,* 1992), and CMV (Suzuki *et al.,* 1996). Furthermore, when both proteins were simultaneously expressed in the same plant from transgenes encoding full-length viral RNAs, a viral replicon was generated (Mori *et al.,* 1992; Suzuki *et al.,* 1996).

No resistance has been reported so far in plants expressing either biologically active 1a or 2a proteins alone for any member within the *Bromoviridae*. However, resistance has been found in some transgenic plants that simultaneously express both proteins and seems to be related to the generation of a viral replicon. For example, transgenic tobacco plants expressing a BMV replicon showed a drastic suppression in the levels of accumulation of viral RNA in protoplasts (Kaido *et al.,* 1995). In contrast, little inhibition of replication was observed in protoplasts from plants expressing both BMV 1a and 2a proteins from transgenes lacking the 3′ end noncoding regions (NCRs) of the viral RNAs and thus unable to reconstitute a viral replicon (Kaido *et al.,* 1995). No resistance to viral infection was reported in plants expressing both biologically active 1a and 2a proteins of AlMV, which were also unable to reconstitute a viral replicon, due to deletions in the viral NCRs at the 5′ and 3′ ends (Taschner *et al.,* 1991). By contrast, some resistance was described in transgenic tobacco expressing a CMV replicon, although it was sensitive to the nature of the inoculum and effective only at very low doses of virus (Suzuki *et al.,* 1996). As in the BMV replicon-transgenic plants (Kaido *et* al., 1995), the resistance to CMV also involved suppression of the replication of the homologous virus in protoplasts.

[1] To whom reprint requests should be addressed. Fax: 44–1382 562426. E-mail: ppaluk@scri.sari.ac.uk.

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Resistance also has been described in plants expressing defective forms of the viral replicase. Strong resistance was found in tobacco plants expressing a defective form of the CMV 2a protein (Anderson *et al.,* 1992). In those plants, a marked reduction in the rate of virus replication in the inoculated cells was observed, but there also was evidence of a second interference with the systemic movement of the virus (Carr *et al.,* 1994; Hellwald and Palukaitis, 1995; Wintermantel *et al.,* 1997). The resistance was independent of the nature and dose of the inoculum but was effective only against the CMV strain from which the transgene originated and other closely related strains of CMV subgroup I but not strains of CMV subgroup II (Anderson *et al.,* 1992; Zaitlin *et al.,* 1994). Resistance was targeted to CMV RNA 2 (Hellwald and Palukaitis, 1995). Several transgenes expressing defective forms of the 2a protein of AlMV induced less extreme protection against the virus. In those plants, resistance correlated with high levels of transgene expression (Brederode *et al.,* 1995). It was suggested that the nonfunctional 2a protein encoded by the transgene could itself play a role in the resistance mechanism (Brederode *et al.,* 1995).

Transgenic tobacco expressing CMV RNA 1 did not show resistance to CMV but was able to complement infection of CMV RNAs 2 and 3 by the generation of biologically active CMV RNA 1 (Suzuki *et al.,* 1996). However, because the 1a protein of the Fny strain of CMV was not detectable in CMV-infected protoplasts, in contrast to the 2a, 3a, or CPs (A. Gal-On, C. H. Kim and P. Palukaitis, unpublished data), it appeared that very little 1a protein was required to support CMV replication. This made it likely that tobacco plants transgenic for Fny-CMV RNA 1, expressing low levels of biologically active 1a protein, would support replication of RNA 1-defective virus by complementation of viral RNAs 2 and 3. Moreover, based on the previous transgene-expression studies described above, CMV RNA 1-expressing transgenic tobacco that showed complementation of CMV replication would not be expected to show resistance to CMV, whereas transgenic plants that did show resistance to CMV, due to transgene silencing, would not be expected to show complementation. However, as shown here, contrary to expectations, transgenic tobacco expressing CMV RNA 1 could exhibit both resistance to CMV infection and complementation of CMV replication.

RESULTS

The 1a protein expressed from the transgene complements replication of CMV RNAs 2 and 3 in R_0-generation plants

Tobacco plants were transformed using a construct encoding a full-length RNA 1 of Fny-CMV, modified by the addition of sequences encoding six histidines at the 3′ end of the 1a gene. In addition, the construct contained nonviral sequences at both 3′ and 5′ termini (Fig. 1). Two calli were obtained: callus A, from which plant lines A1, A2, and A3 were regenerated, and callus B, from which plant lines B1, B2, B3, B4, and B5 were regenerated.

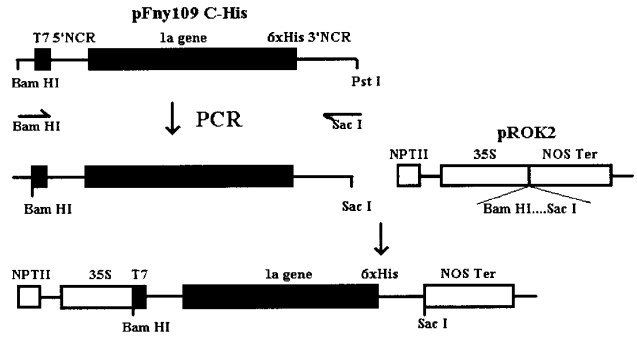


FIG. 1. Schematic representation of the expression construct used for the transformation of tobacco plants. A PCR fragment obtained from the RNA 1 full-length cDNA clone, pFny109 C-His, was inserted into the expression vector pROK2. NPTII, 35S, T7, and NOS Ter refer to neomycin phosphotransferase II, cauliflower mosaic virus 35S RNA promoter, T7 RNA promoter, and nopaline synthase gene transcriptional terminator, respectively.

The transgene could be detected by polymerase chain reaction (PCR) in all R_0-generation plants except A3 (not shown). Attempts to clearly detect the transgenic 1a protein in these plants by Western blotting were unsuccessful (not shown). Therefore, a complementation test was used to demonstrate transcription of the transgene into mRNA and translation into biologically active 1a protein by inoculation of the R_0-generation plants with Fny-CMV transcript RNAs 2 and 3. Replication of these RNAs was detected by reverse transcription PCR (RT-PCR) of viral sequences corresponding to RNAs 2 and 3 and/or Northern blot hybridization analysis of total RNA extracted from inoculated and noninoculated upper asymptomatic leaves. CMV RNAs were detected in all plants except plant A3 (Fig. 2, lanes 1 and 2 vs lanes 3 and 4; data not shown). Thus, the transgenic 1a protein complemented the RNA 1-deficient virus for replication. However, the levels of viral RNA detected by Northern blot hybridization analysis were considerably lower than the levels of viral RNAs detected in tissue inoculated with all three CMV RNA transcripts (Fig. 2, lanes 1 and 2 vs lane 6). Moreover, RNA 1 was not detected in the transgenic plants by the probe, which is specific to the 3′ terminal sequences common to each CMV RNA (Fig. 2, lanes 1 and 2).

Viral RNA 1 regenerates from the transgenic mRNA

Between 12 and 20 days postinoculation (d.p.i.), symptoms began to appear in the newly emerging leaves of R_0-generation plants showing complementation and replication of viral RNAs 2 and 3 (Fig. 3). Some foci of infection showed very mild symptoms visible only under transmitted light (Figs. 3B vs 3A, arrows). These symp-

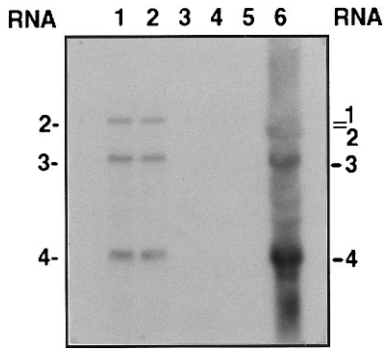


FIG. 2. Detection of viral RNAs in RNA 1 transgenic tobacco plants inoculated with Fny-CMV transcript RNAs 2 and 3. Northern blot hybridization analysis at 9 d.p.i. of total plant RNA. Samples are from inoculated leaf of plant line R_o-A1 (lane 1), upper, noninoculated leaf of plant line R_0-A1 (lane 2), inoculated leaf of plant line R_0-A3 (lane 3), upper noninoculated leaf of plant line R_o-A3 (lane 4), noninoculated tobacco (lane 5), and CMV-infected tobacco (lane 6). All the lanes contained the same amount of total RNA, although overloading of the viral RNA in lane 6 led to faster migration.

toms increased gradually in severity (Figs. 3A and 3B vs 3C), and eventually the symptoms in the new leaves became similar (arrow in Fig. 3C) to those induced by the wild-type (WT) virus. This represented a delay of ~1 week with regard to symptom appearance in tobacco inoculated with the 3 viral RNA transcripts (data not shown). The appearance of symptoms correlated both with increases in the levels of viral RNA and with the detection of RNA 1, not only in upper symptomatic leaves (Fig. 4, lanes 10 and 11) but also in the inoculated leaves (Fig. 4, lanes 6 and 8). Virus was not detected in some of the upper asymptomatic leaves (Fig. 4, lane 9), indicating that the phenomenon of cycling reported for Fny-CMV in tobacco (Gal-On *et al.,* 1995) also occurred in these transgenic plants. There also was some variation in the accumulation of viral RNA within the inoculated leaf on different days (Fig. 4, lanes 2 vs 5, 3 vs 6, and 4 vs 7), probably due to variation in the level of infection in different parts of the inoculated leaves (see Figs. 3A and 3B). When extracts from the upper leaves showing WT-like symptoms (analyzed in Fig. 4C, lanes 10 and 11) were inoculated to nontransformed tobacco, the plants became infected and showed the presence of all CMV RNAs (data not presented). To verify that this RNA 1 originated from the transgene, virus was isolated from these nontransformed tobacco plants, and the 3′ end of the 1a gene was sequenced by the dideoxynucleotide chain termination method. The sequence of the 1a 3′ end showed the presence of sequences encoding the six histidines, confirming that the RNA 1 regenerated from the transgene (data not shown).

Differential susceptibility to CMV in R_1- and R_2-generation plants of line B1: Occurrence of atypical infection symptoms

Because the initial purpose of this work was to study complementation and not resistance to CMV, seeds were only obtained from R_0-generation plants of lines A1 and B1. A genealogical representation of the different transgenic lines described in this article is shown in Figure 5. The R_1-generation plants (one for each line), which were used to obtain seeds for R_2-generation

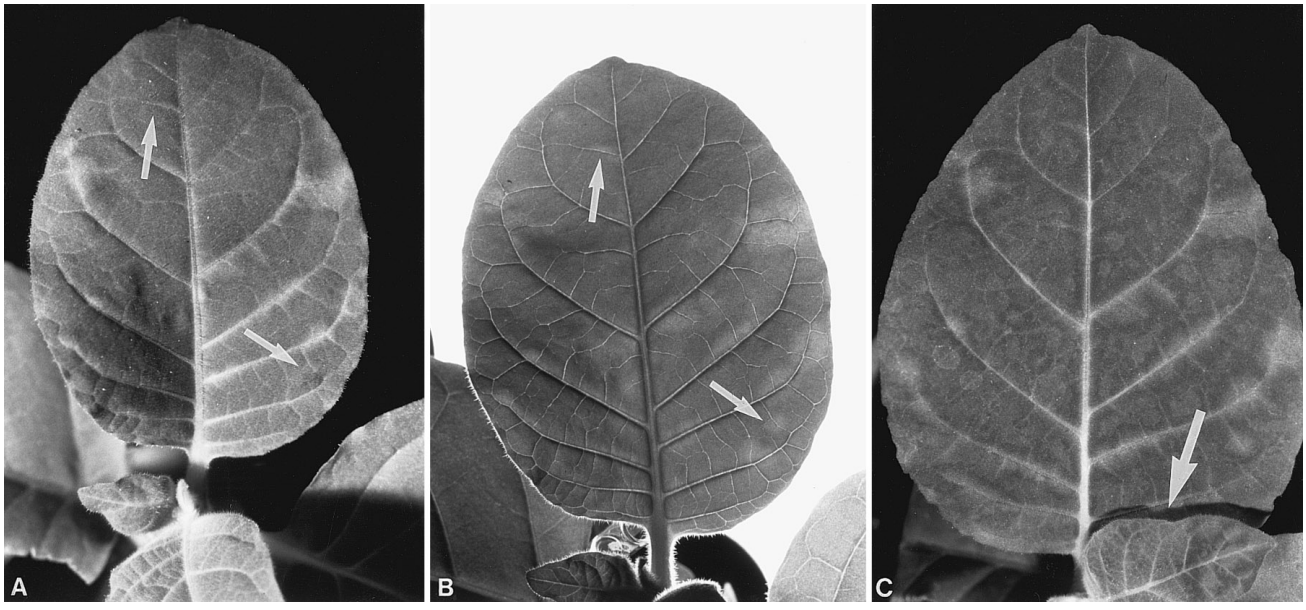


FIG. 3. Development of systemic infection symptoms in a plant of line R_0-A1 inoculated with CMV RNAs 2 and 3. (A and B) Initial chlorotic spots appeared in a noninoculated leaf at 12 d.p.i. (C) Symptoms increased in severity with time as the leaf expanded (16 d.p.i.). (C, arrow) Presence of intense symptoms in the small new leaf. Photographs were taken with either incidental light (A and C) or transmitted light (B), the latter of which shows lesions (B, arrows) not visible under incidental light (A, arrows).

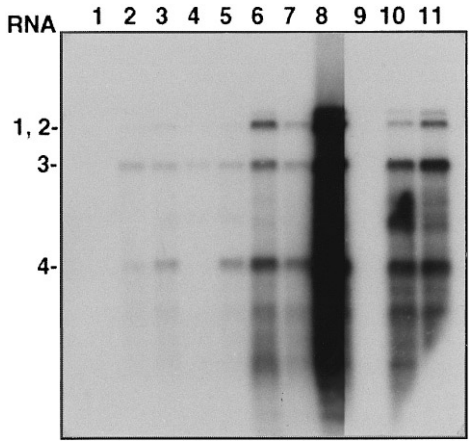


FIG. 4. Northern blot analysis of viral RNA accumulation at different d.p.i., after inoculation with viral transcripts of Fny-CMV RNAs 2 and 3 in plant line R_0-B4, in (two different) inoculated leaves and systemic leaves. Total RNAs were extracted from a healthy plant (lane 1); the first inoculated leaf at 4 (lane 2), 8 (lane 3), and 12 (lane 4) d.p.i.; or the second inoculated leaf at 4 (lane 5), 8 (lane 6), 12 (lane 7), and 18 (lane 8) d.p.i. or a systemic asymptomatic (lane 9) and two systemic symptomatic leaves (lanes 10 and 11), all at 18 d.p.i. Lanes 9–11 have one-tenth the RNA loaded in the other lanes.

plants, were not selected by any particular characteristic other than the presence of the transgene. Thereafter, three types of experiments were conducted using the R_1- and R_2-generation plants to test their susceptibility to CMV: (1) inoculation with transcript RNAs 2 and 3 and assessment for symptoms of systemic infection after the appearance of viral RNA 1, (2) inoculation with purified Fny-CMV and assessment of symptoms, and (3) inoculation with an extract from tobacco infected with a pseudorecombinant virus derived from Fny-CMV RNAs 1 + 2 and M-CMV RNA 3 (designated F1F2M3-CMV), and assessment of symptoms. Although Fny-CMV RNA 3 and M-CMV RNA 3 differ only by 1% in nucleotide sequence, the symptoms induced by the latter are more obvious, producing bright yellow/white chlorosis (Shintaku *et al.,* 1992). Thus, the pseudorecombinant virus F1F2M3-CMV allowed the detection of limited infection foci not visible after inoculation with Fny-CMV. The results of these experiments are summarized in Table 1.

Plants of line A1 (R_1- and R_2-generation; R_1-A1 and R_2-A1) were completely susceptible to infection by Fny-CMV (Fig. 5). The symptoms were WT-like in appearance with respect to infection in control plants (Table 1 and Fig. 6A, plant 3 vs plant 5). By contrast, plants of line B1 (R_1- and R_2-generation; R_1-B1 and R_2-B1) showed a variety of symptom responses among plants (Fig. 5) that could be classified in four categories: WT-like, WT-like plus necrotic rings, mild chlorosis and necrotic rings, and no systemic symptoms (Table 1). The necrotic rings (Fig. 6A, plant 1, and Figs. 6B and 6C) were not observed on nontransformed plants inoculated with Fny-CMV and thus represented a unique host response. When R_2-generation plants (Fig. 5) were inoculated with the pseudorecombinant virus F1F2M3-CMV, chlorotic yellow lesions appeared in the inoculated leaves of most control plants as well as R_2-A1 plants as early as 4 d.p.i. (as in plants photographed at 16 d.p.i. and shown in Fig. 7B, lower leaves). The diameter of the lesions was ~2 mm. In R_2-B1 plants, at 4 d.p.i., the lesions were less apparent and were ~1 mm in diameter (not shown). By 7 d.p.i., all except one of the R_2-A1 plants became infected systemically at the same time as the control plants (see Figs. 7A and 7B, center vs left plants or leaves). The one exception showed a delay of 2 days in the onset of symptoms, which were WT-like but with the addition of necrotic rings in some leaves (Table 1). By contrast, at 7 d.p.i. only two R_2-B1 plants were showing systemic symptoms, and by 24 d.p.i. and afterward, 7 of 20 R_2-B1 plants showed no systemic symptoms (Table 1), as for plants photographed at 16 d.p.i. (Figs. 7A and 7B, right vs left and central plants or leaves). There was considerable variability in the severity of symptoms among the remaining 13 line B1 plants (Fig. 7C, bottom row) vs nontransformed plants (Fig. 7C, top left).

Segregation of symptom responses in the R_2-generation of line B1: Identification of two R_3-generation lines with systemic resistance to CMV

The two line R_2-B1 plants, which remained asymptomatic after inoculation with Fny-CMV virus (50 μg/ml, Table 1), self-pollinated and generated seeds of R_3-generation lines R_3-B1A and R_3-B1B (Fig. 5). In addition, seeds were obtained from two of the line R_2-B1 plants that remained asymptomatic after inoculation with F1F3M3-CMV, in a experiment also described in Table 1, to produce R_3-

R_0-generation	Plant A1 (S)	Plant B1 (S)
	↓	↓
R_1-generation	Line A1 (S)	Line B1 (S/R)
	↓	↓
R_2-generation	Line A1 (S)	Line B1 (S/R)
		↓
R_3-generation		Lines B1A, B1B, B1C and B1D (R*)

FIG. 5. Genealogical representation of the different RNA 1-transgenic tobacco lines described in this report and their respective susceptibility (S) or resistance (R) to systemic infection by Fny-CMV. (S/R) Some plants of the line were susceptible, whereas others were systemically resistant to Fny-CMV. (R*), Systemic resistance to Fny-CMV, although with different degrees between lines (see text).

TABLE 1

Assessment of the Susceptibility of Lines A1 and B1, Plants of the R_1- and R_2-Generation, to Infection by CMV

Lines	Inoculum	Infectivity[a]	Pathology[b,c]
R_1-A1	Fny-CMV RNAs 2 + 3	3/5	3 WT
R_2-A1	Fny-CMV RNAs 2 + 3	5/5	5 WT
R_1-B1	Fny-CMV RNAs 2 + 3	4/5	2 WT; 1 WT + NR; 1 MC + NR
R_2-B1	Fny-CMV RNAs 2 + 3	5/5	1 WT; 1 WT + NR; 3 MC + NR
R_2-A1	Fny-CMV (10 μg/ml)	9/9	9 WT
R_2-B1	Fny-CMV (10 μg/ml)	4/9	3 WT; 1 MC + NR
NT tobacco[d]	Fny-CMV (10 μg/ml)	10/10	10 WT
R_2-A1	Fny-CMV (50 μg/ml)	9/9	9 WT
R_2-B1	Fny-CMV (50 μg/ml)	8/10	5 WT; 3 MC + NR
NT tobacco	Fny-CMV (50 μg/ml)	10/10	10 WT
R_2-A1	F1F2M3-CMV (sap)	18/18	17 WT; 1 WT + NR + Delay
R_2-B1	F1F2M3-CMV (sap)	13/20	4 [P1]; 1 [P2]; 4 [P3]; 4 [P4]
NT tobacco	F1F2M3-CMV (sap)	18/18	10 WT

[a] Number of plants showing symptoms divided by the number of plants inoculated.

[b] Systemic symptoms induced by Fny-CMV were classified into three categories: WT, wild-type-like; WT + NR, wild-type-like symptoms together with the presence of a varying number of necrotic rings in some of the leaves; MC + NR, plants with mild chlorotic symptoms and a varying number of necrotic rings in some of the leaves.

[c] Systemic symptoms induced by the pseudorecombinant F1F2M3-CMV were classified on a scale of severity of P1 to P4, shown in Figure 6C, P1–P4, respectively, to indicate the severity, and extent of systemic yellow chlorosis.

[d] NT tobacco; nontransformed tobacco.

generation lines R_3-B1C and R_3-B1D (Fig. 5). To test these plants for resistance, plants from these four lines were inoculated with extracts from tobacco infected with F1F2M3-CMV. The results are summarized in Table 2.

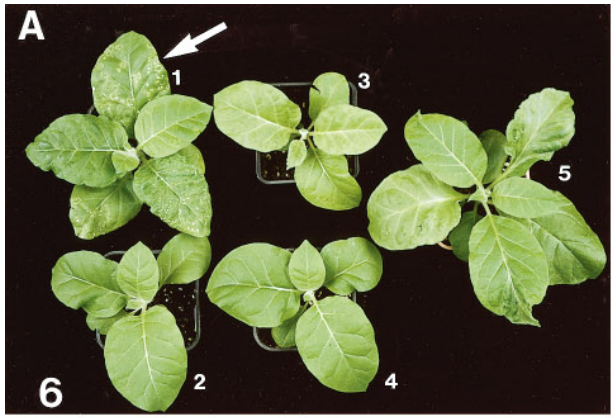
All plants from lines R_3-B1A and R_3-B1B were susceptible to infection by CMV. However, in contrast to what was seen in the line R_2-B1 plants, the variation in systemic symptoms among plants was much less. Systemic infection symptoms were mild, limited to chlorotic flecks in the systemic leaves, like those shown in Figure 7C leaves P2 and P3. By contrast, all plants of line R_3-B1C remained asymptomatic in the systemic leaves (Table 2). In some of the plants of line R_3-B1D, occasional, individual lesions were found in systemic leaves, but the plants otherwise remained asymptomatic (Table 2). In the plants of both lines, R_3-B1C and R_3-B1D, yellow, chlorotic lesions developed in the inoculated leaves (as in Fig. 8A).

The resistant lines, R_3-B1C and R_3-B1D (Fig. 5), showed complete susceptibility to the pseudorecombinant virus L1L2M3-CMV (Table 2), which contains RNAs 1 and 2 from the subgroup II strain LS-CMV and RNA 3 of the subgroup I strain M-CMV (Zaitlin *et al.,* 1994). The symptoms were indistinguishable from those shown by both the susceptible line R_2-A1 plants and nontransformed tobacco (Fig. 7A and Table 2). This suggests that the resistance was not effective against members of CMV subgroup II of CMV, such as LS-CMV, which show 25% nucleotide sequence divergence from subgroup I strains such as Fny-CMV (Palukaitis *et al.,* 1992).

To test the effect of inoculation with high doses of virus on the resistance, plants of the most resistant line (R_3-B1C) were inoculated with purified Fny-CMV at a concentration of 500 μg/ml. At 37 d.p.i., half of the plants remained asymptomatic, whereas the other half displayed various symptoms, ranging from WT to mild, localized chlorosis (Table 2). Thus, the resistance could be overcome in some, but not all the plants, by extremely high levels of inoculum.

Upper leaves of resistant plants are susceptible to infection and retain the ability to complement replication of inoculated viral RNAs 2 and 3

Inoculated plants from resistant line R_3-B1C were resistant to systemic infection by F1F2M3-CMV (Table 2). However, the asymptomatic, noninoculated, upper leaves of these plants were found to be susceptible to virus infection if mechanically inoculated with the same virus; that is, chlorotic lesions developed in these newly inoculated, upper leaves after inoculation with the pseudorecombinant F1F2M3-CMV, indicating virus cell-to-cell movement and accumulation (Fig. 8A). However, the plants retained resistance to systemic infection. Lesions were similar in size in plants that had (Fig. 8A, right) or had not (Fig. 8A, left) been inoculated previously with the same virus in the lower leaves. Moreover, localized fluorescence in single epidermal cells was detected after inoculation of upper leaves of R_3-B1C plants with Fny-CMV RNA 2 transcript plus a modified CMV RNA 3 transcript derived from the construct pL:3a/GFP, in which the gene encoding the green fluorescent protein (GFP) was substituted for the viral CP gene (Figs. 8B and 8C). This indicates that neither resistance nor transgene si-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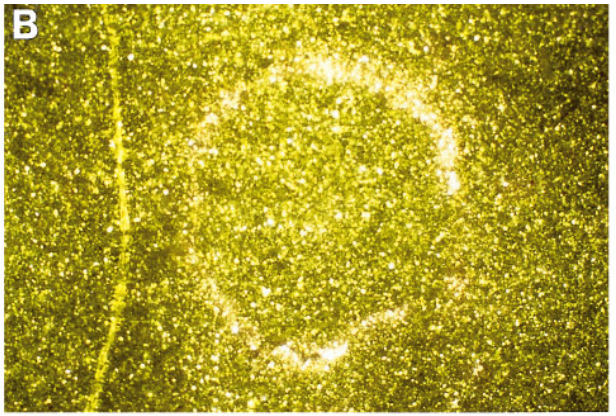
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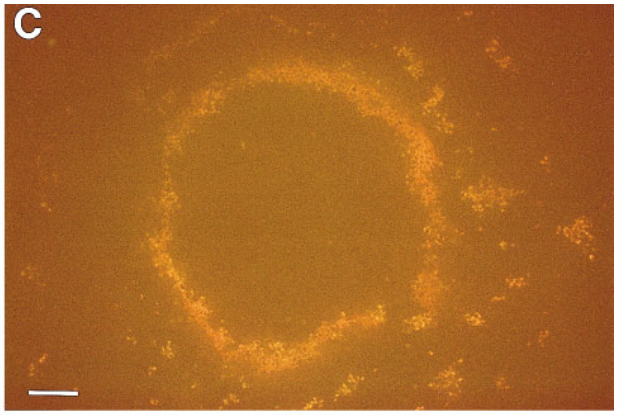
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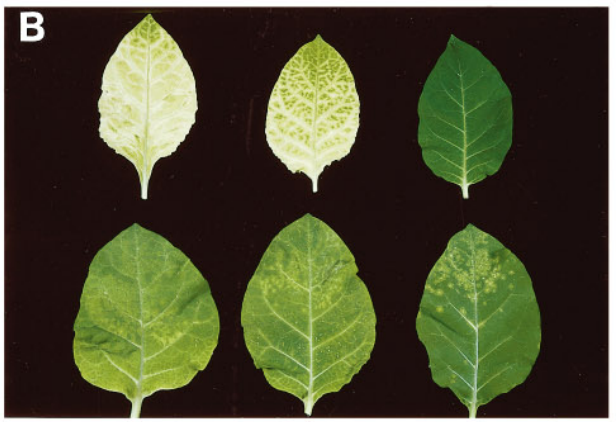
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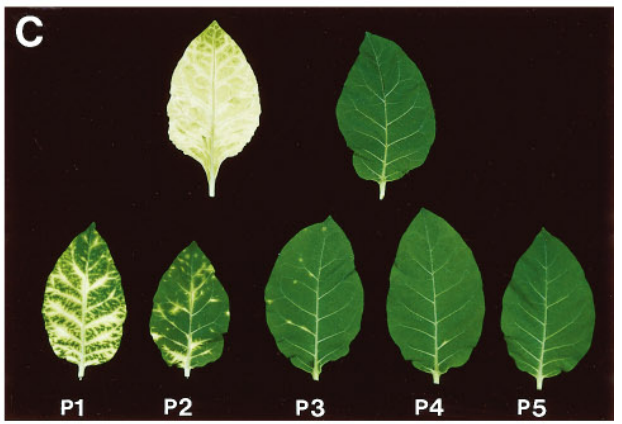
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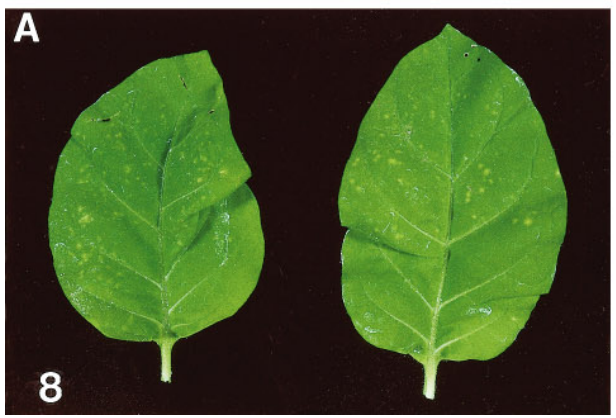
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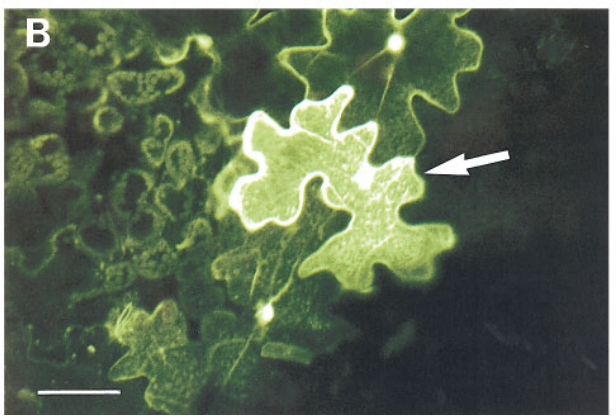
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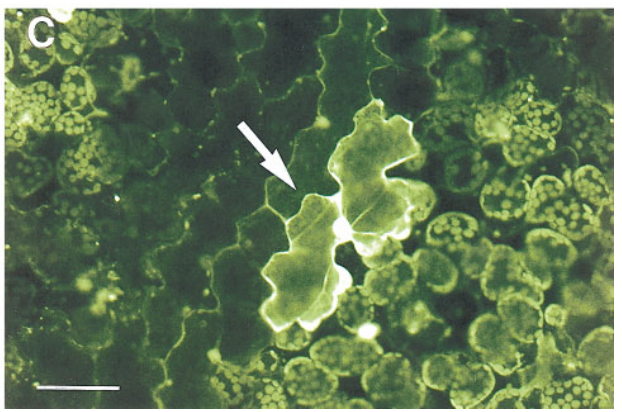
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TABLE 2

Assessment of the Susceptibility of Lines R_3-A–D to Infection by CMV

Lines	Inoculum	Infectivity[a]	Pathology[b,c]
NT tobacco[d]	F1F2M3-CMV (sap)	20/20	20 WT
R_3-B1A	F1F2M3-CMV (sap)	20/20	20 [P2; P3]
NT tobacco	F1F2M3-CMV (sap)	20/20	20 WT
R_3-B1B	F1F2M3-CMV (sap)	20/20	20 [P2; P3]
NT tobacco	F1F2M3-CMV (sap)	20/20	20 WT
R_3-B1C	F1F2M3-CMV (sap)	0/20	
NT tobacco	F1F2M3-CMV (sap)	16/16	20 WT
R_3-B1D	F1F2M3-CMV (sap)	10[e]/18	[P4; P5]
NT tobacco	L1L2M3-CMV (sap)	20/20	20 WT
R_2-A1	L1L2M3-CMV (sap)	20/20	20 WT
R_3-B1C	L1L2M3-CMV (sap)	20/20	20 WT
R_3-B1D	L1L2M3-CMV (sap)	20/20	20 WT
NT tobacco	Fny-CMV (500 μg/ml)	5/5	5 WT
R_3-B1C	Fny-CMV (500 μg/ml)	8/16	4 WT; 4 MC

[a] Number of plants showing symptoms divided by the number of plants inoculated.

[b] Systemic symptoms induced by Fny-CMV were classified as WT, wild-type-like, and MC, localized mild chlorosis in systemic leaves without leaf deformation.

[c] Systemic symptoms induced by the pseudorecombinant F1F2M3-CMV were classified on a scale of severity of from P1 to P5, shown in Figure 6C, P1–P5, respectively, to indicate the severity and extent of systemic yellow chlorosis.

[d] NT tobacco, nontransformed tobacco.

[e] In these 10 plants, only occasional isolated yellow flecks were detected in some systemic leaves, similar to P4 (Fig. 6C, P4).

lencing had been induced in the upper leaves by the prior inoculation of a lower leaf.

Resistant plants show a differential reduction in the accumulation of viral RNA 1 in the inoculated leaf

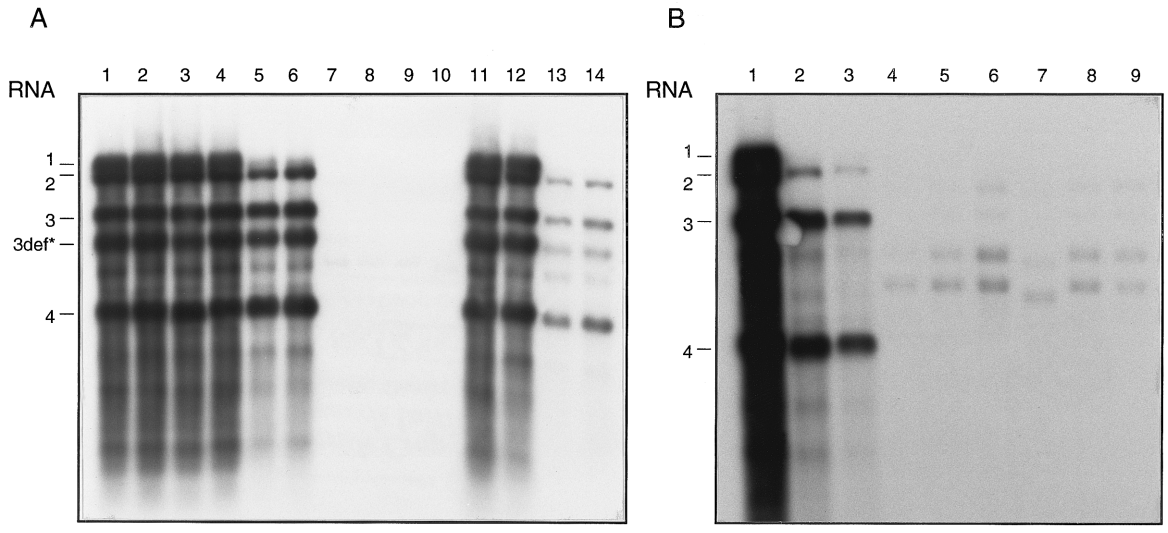
The analysis of viral RNA accumulation in lesions from the inoculated leaves of plants from both resistant lines R_3-B1C and R_3-B1D showed a dramatic decrease in the levels of viral RNA 1, ranging from 20-fold less (Figs. 9A and 9C, lanes 5 and 6) to almost undetectable (Figs. 9A and 9C, lanes 13 and 14), relative to the levels found in both susceptible transgenic (R_2-A1) and nontransformed tobacco plants (Figs. 9A and 9C, lanes 1–4 and lanes 11 and 12). This was found in several independent experiments (Fig. 9; data not shown). A proportional, but less pronounced, 2–13-fold decrease was also observed in the total levels of viral RNAs 2–4 (Figs. 9A and 9C, lanes 5 and 6 vs lanes 1 and 2 and lanes 13 and 14 vs lane 11, respectively). The analysis of viral RNA accumulation in the lesions (shown in Fig. 8A) from inoculated leaves of plants that had or had not been inoculated previously with virus in the lower leaves showed the same pattern of accumulation; that is, less total viral RNA accumulated in these resistant plants (Figs. 9B and 9D, lanes 2 and 3) than in nonresistant plants (Figs. 9B and 9D, lane 1). No trace of viral RNAs was found in leaves above the inoculated leaves (Fig. 9B, lanes 4–9).

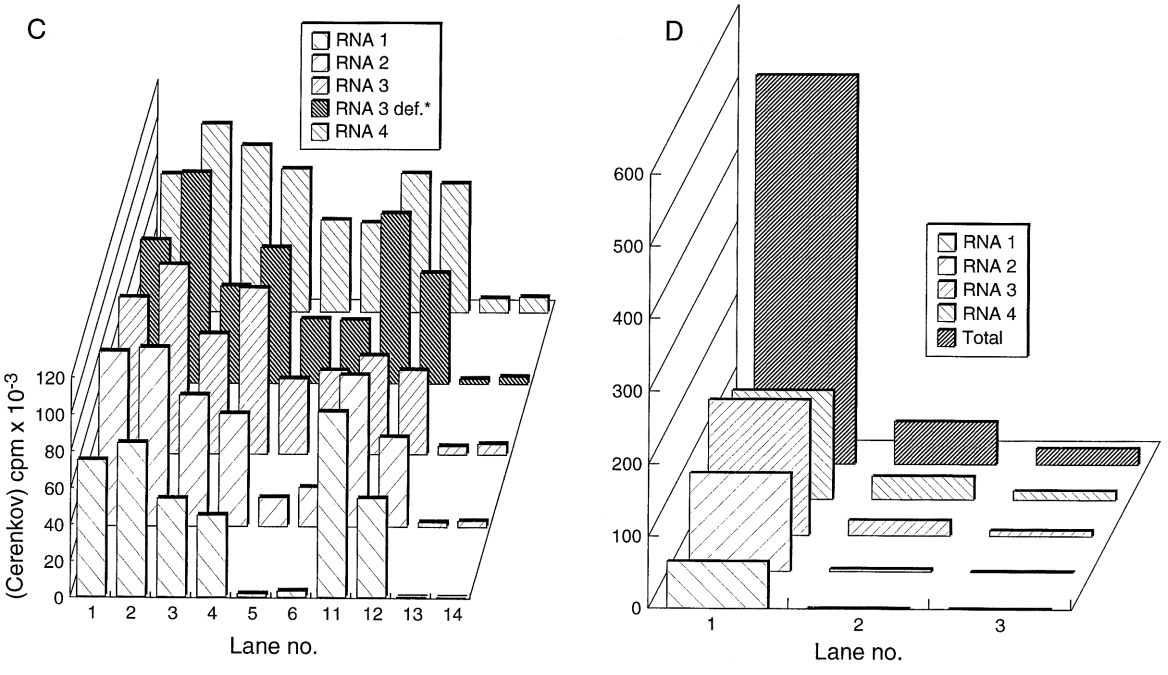
To assess the relative levels with which the 1a protein expressed from the transgenic mRNA sustained viral replication (and thus, an indirect measure of the levels of functional cytoplasmic, transgenic mRNA), total CMV RNA accumulation levels in the inoculated leaves of transgenic, susceptible (line R_2-A1) and resistant (line R_3-B1C) plants (Fig. 5) inoculated with viral RNAs 2 and 3 were compared with total CMV RNA accumulation after inoculation with the three viral transcript RNAs (Fig. 10A). Accumulation of the total Fny-CMV RNAs in plants of resistant line R_3-B1C (Figs. 10A and 10B, lane 1) was 10–12-fold lower than in plants of susceptible line R_2-A1 (Figs. 10A and 10B, lanes 3 and 4) when Fny-CMV RNAs 1–3 were used as inoculum, whereas when Fny-CMV RNAs 2 and 3 were inoculated to plants of line R_2-A1, the levels of total viral RNA accumulation varied from 0.5-fold (Figs. 10A and 10B, lane 5 vs lane 2) to 2-fold (Figs. 10A and 10B, lane 7 vs lane 2) to that observed in plants of line R_3-B1C. By contrast, the levels of accumulation of total viral RNAs were ~8-fold higher when Fny-CMV RNAs 1–3 were inoculated to plants of the resistant line

FIG. 6. Symptoms induced by Fny-CMV in R_1-generation plants. (A) Plants were either noninoculated (plants 2 and 4) or inoculated with Fny-CMV (plants 1, 3, and 5). Plants were from line B1 (plants 1 and 2), line A1 (plants 3 and 4), or nontransformed tobacco (plant 5). Note the presence of necrotic rings in the infected B1 plant (arrow), magnified in B and C. (B) Lesion under transmitted light. (C) Same lesion viewed using an FITC/TRITC epifluorescent filter, which highlights (in orange) the necrotized areas. (Bar) 1 mm.

FIG. 7. Symptoms induced in transgenic and nontransformed plants at 16 d.p.i. with sap from tobacco infected with the pseudorecombinant virus F1F2M3-CMV. (A and B) Nontransformed tobacco (left), a plant of line R_2-A1 (center), and a resistant plant of line R_2-B1 (right). Shown are the entire plants (A) and detached, systemic (upper row) and inoculated (lower row) leaves of the same plants (B). (C) Variability of symptoms induced in different plants of line R_2-B1. (Lower row, left to right) Responses designated by severity as less severe than WT (P1), large chlorotic flecks on and adjacent to the main veins (P2), multiple, small chlorotic flecks associated with main veins (P3), one or two small chlorotic flecks on a vein (P4), or no symptoms (P5). (Upper row) Infected (left) and healthy (right) nontransformed tobacco leaves.

FIG. 8. Susceptibility of line R_3-B1C to virus replication in the inoculated leaves. (A) Symptoms induced in the inoculated leaf of two plants of line R_3-B1C, at 20 d.p.i. with sap from tobacco infected with the pseudorecombinant virus F1F2M3-CMV. One of the plants (right leaf) had been inoculated 22 days previously to the above inoculation on a lower leaf with the same virus. The other plant (left leaf) had not previously been inoculated. (B and C) GFP fluorescence at 4 d.p.i. in epidermal cells (arrows) of two plants from line R_3-B1C inoculated with transcript Fny-CMV RNA 2 plus transcript RNA 3 derived from construct pL:3a/GFP with the CP gene replaced by the GFP gene. The plants had been either noninoculated previously (B) or inoculated 16 days before (C) on a lower leaf with Fny-CMV. F1F2L:3a/GFP yields infections limited to single cells (arrows) in the absence of the CP. (Bars) 100 μm.

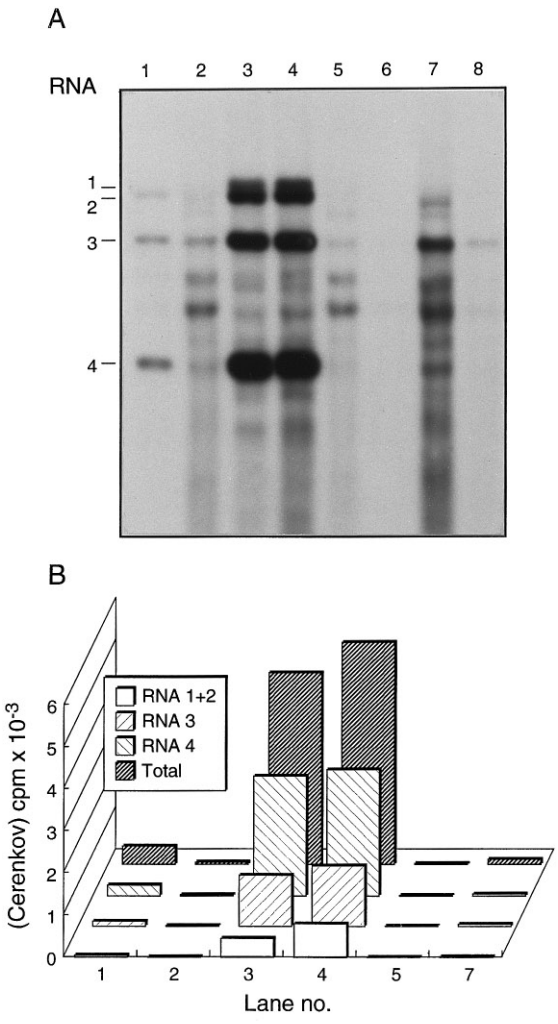




Lane	1/2	3/4	5/6	11	12	13	14
RNA 1	79.4	49.3	2.60	101.2	54.1	0.23	0.16
RNA 2	96.6	66.9	18.8	83.0	49.1	2.55	3.36
RNA 3	93.9	77.7	43.2	53.6	45.7	4.7	5.9
3def*	96.5	63.5	34.7	92.7	60.4	2.75	3.95
RNA4	89.0	84.7	49.6	75.9	70.4	7.8	8.56
Total	455.4	342.1	148.9	406.4	279.7	18.0	21.9

Lane	1	2	3
RNA 1	65.6	0.92	0.48
RNA 2	135.4	3.97	1.18
RNA 3	186.1	21.4	7.96
RNA 4	148.7	32.1	12.7
Total	535.8	56.3	22.3

FIG. 9. Northern blot hybridization analysis of the viral RNA accumulation in the lesions of leaves inoculated with sap from tobacco infected with the pseudorecombinant virus F1F2M3-CMV. (A) Experiment 1: total RNAs were extracted at 9 d.p.i. from two nontransformed tobacco (lanes I and 2), two plants of line R_2-A1 (lanes 3 and 4), and two plants of line R_3-B1C (lanes 5 and 6). Experiment 2: total RNAs were extracted at 10 d.p.i. from nontransformed tobacco (lane 11), a plant of line R_2-A1 (lane 12), a plant of line R_3-B1C (lane 13), and a plant of line R_3-B1D (lane 14). (Lanes 7–10) Total RNA from noninoculated plants: nontransformed tobacco (lane 7), plant line R_2-A1 (lane 8), plant line R_3-B1C (lane 9), and plant line R_3-B1D (lane 10). (B) Total RNAs were extracted at 20 d.p.i. from a plant line R_2-A1 (lane 1), and the two plants of line R_3-B1C (lanes 2 and 3) shown in Figure 8A. Before this inoculation, one of these plants (lane 3) had been inoculated on a lower leaf 22 days before with the same virus (corresponds to Fig. 8A, right); the other plant (lane 2) had not been inoculated previously (corresponds to Fig. 8A, left). Total RNA was analyzed from the three consecutive leaves above the inoculated leaf represented in lane 2 (lanes 4–6) or from the three consecutive leaves above the inoculated leaf represented in lane 3 (lanes 7–9). (C and D) The Cerenkov c.p.m. ($\times 10^{-3}$) for the viral RNAs in the corresponding lanes are shown in the graphs and charts. CMV RNAs are indicated on the left of A and B in descending order. (RNA 3 def.*) Spontaneously appearing defective form of viral RNA 3 with a deletion of 411 nucleotides in the 3a gene.



Cerenkov (cpm)

Lane	1	2	3	4	5	7
RNA 1+2	49	7.6	458	810	5.2	17.4
RNA 3	123	17.5	1232	1450	10.9	54.8
RNA 4	261	29.1	2867	3023	16.4	48.5
Total	433	54.2	4557	5283	22.5	120.7

FIG. 10. Northern blot hybridization analysis of the viral RNA accumulation in transgenic tobacco leaves inoculated with Fny-CMV transcript RNAs. (A) At 5 d.p.i., total RNA was extracted from two plants of line R_3-B1C (lanes 1 and 2) and four R_2-generation plants of line A1 (lanes 3–8). The plants were inoculated either with Fny-CMV transcript RNAs 1 + 2 and 3 (lanes 1, 3, and 4) or RNAs 2 and 3 alone (lanes 2, 5–8). Lanes 2, 5, and 7 contain 10 times the RNA loaded in the remaining lanes. (B) The Cerenkov c.p.m. for the viral RNAs from the samples analyzed in the corresponding lanes are shown in the graph and in the chart. The c.p.m. in lanes 2, 5, and 7 have been normalized to the c.p.m. expected for 10-fold less RNA.

R_3-B1C than when the inoculum was Fny-CMV RNAs 2 and 3 (Figs. 10A and 10B, lane 1 vs lane 2; lane 2 contains 10 times more RNA loaded than lane 1). Thus, RNA 1 derived from the inoculum was still supporting replication and was not completely suppressed. The accumulation of total Fny-CMV RNAs was much less in plants of susceptible line R_2-A1 inoculated with Fny-CMV RNAs 2 and 3 than in those inoculated with Fny-CMV RNAs 1–3, varying from 40-fold (Figs. 10A and 10B, lane 7 vs lanes 3 and 4) to 230-fold (Figs. 10A and 10B, lane 5 vs lanes 3 and 4). Therefore, it appears that the resistant line R_3-B1C was able to suppress Fny-CMV RNA accumulation 10–12-fold relative to the susceptible line R_2-A1 but did not show much difference in its ability to support viral RNA accumulation after inoculation with Fny-CMV RNAs 2 and 3 compared with the susceptible line R_2-A1. This indicates that the level of 1a protein probably was similar in the two transgenic lines. The apparent cross-hybridization to the ribosomal RNAs in Fig. 10 (and, to a lesser extent, in Figs. 2, 4, and 9) is a hybridization artifact caused by partial degradation of the genomic RNAs [described by Dougherty (1983) and Palukaitis *et al.* (1983)]. This extent of degradation does not appear to be related to the resistance.

DISCUSSION

We obtained transgenic tobacco plants that express biologically active 1a protein, able to complement the replication of viral RNAs 2 and 3 in the absence of viral RNA 1. Initially, the plants remained asymptomatic and the transgenic mRNA was undetectable by Northern blot analysis. However, RNAs 2–4 were readily detected in the same assay, not only in inoculated leaves but also in some systemic leaves. This suggests that the transgene not only complemented the replication of the heterologous viral RNAs but also allowed long-distance movement in the plant. On the other hand, it also indicates that the transgenic mRNA was not used efficiently as a template by the viral RNA-dependent RNA polymerase (RdRp), probably because the transgenic mRNA had additional nonviral sequences at both 5′ and 3′ ends. However, after a period of time, viral RNA 1 regenerated from the transgenic mRNA. This regeneration coincided with the appearance of RNA 1, an increase in the accumulation of the other viral RNAs, and the appearance of a WT viral phenotype in susceptible plants. Although we have not investigated the regeneration mechanism, it could have taken place by internal initiation of the viral RdRp on the transgenic mRNA, by use of partially degraded transgenic mRNA as a template by the viral RdRp, or by recombination during replication, between the common 3′ NCRs of RNAs 2, 3, or 4 and similar sequences in the transgenic mRNA.

Even though all the R_0-generation transgenic plants were susceptible to the virus, it was possible to obtain two R_3-generation lines (B1C and B1D) with systemic resistance to Fny-CMV, and another two lines (B1A and B1B) with attenuated systemic symptoms, through the selection of plants that showed differential susceptibility

to the virus in the R_1- and R_2-generations of line B1 (Fig. 5). The necrotic rings (see Fig. 6) observed in inoculated and systemic leaves of some plants of lines R_3-B1A, R_3-B1B, and R_3-B1D, but not in R_3-B1C (not shown), did not correlate with the establishment of resistance but appear to relate to a hypersensitive response that does not confine infection and may have some subtle effects on the infection process.

Plants exhibiting systemic resistance to CMV still showed virus replication and cell-to-cell movement in the inoculated leaves of these plants, as seen in Figures 8 and 9 for line R_3-B1C. However, the analysis of viral RNAs showed a drastic reduction in the levels of accumulation of viral RNA 1 (Fig. 9), compared with viral RNA accumulation in both susceptible transgenic and nontransformed plants. There was a proportional, but less pronounced, decrease in the levels of accumulation of the remaining viral RNAs. Hence, the level of 1a protein must have fallen below some threshold before the accumulation of the other viral RNAs was affected. Once this threshold was reached, the rate of replication and accumulation of the remaining viral RNAs, as well as the accumulation of their gene products, decreased. Relative to CMV infection in nonresistant plants, the accumulation levels would show further reductions in newly invaded cells.

There seems to be a specific mechanism that differentially and incompletely reduces viral RNA 1 accumulation in the inoculated leaf of resistant plants. One possibility is that the phenomenon of incomplete cosuppression (Lindbo *et al.,* 1993) was active in these plants, and after inoculation with viral RNA, the levels of viral RNA 1 would be affected by this cosuppression. However, after inoculation with viral RNAs 2 and 3, these plants sustained levels of accumulation of viral RNA comparable to those in transgenic, susceptible plants (Fig. 10). This indicates that the initial levels of transgenic 1a protein in the resistant line were not lower than those in the susceptible line.

Another possible mechanism for resistance engendered by CMV RNA 1 is that the inoculation with viral RNA triggered a mechanism of degradation that would cosuppress both transgenic mRNA and viral RNA 1 after a threshold level was reached (Lindbo *et al.,* 1993). The fact that this resistance did not operate against the pseudorecombinant L1L2M3-CMV suggests that the reduction in the levels of viral RNA 1 may be due to a homology-dependent effect. This is similar in some respects to resistance induced by a defective polymerase gene (Zaitlin *et al.,* 1994) but different from resistance induced by the expression of a CMV replicon in transgenic tobacco (Suzuki *et al.,* 1996). In the latter case, resistance was also effective against the distantly related tomato aspermy cucumovirus, which is not consistent with a homology-dependent gene-silencing mechanism. The limitation of the resistance engendered by the CMV replicon to low levels of inoculum (Suzuki *et al.,* 1996) also stands in contrast to the resistance observed against high levels of inoculum by transgenic plants expressing either the defective polymerase gene (Anderson *et al.,* 1992) or RNA 1 of CMV (present report). Although it is not clear whether similar mechanisms might be operating in the latter two situations, the incomplete inhibition of virus replication and the effect on systemic movement are other features common to both of these examples of resistance to CMV.

Remarkably, the resistant plants were able to complement the replication of viral RNAs 2 and 3 in upper leaves after reinoculation. Furthermore, these upper leaves were also susceptible to virus infection (Fig. 8). This shows that in the resistant plants, silencing of the transgene was not being induced systemically by the initial inoculation. Thus, despite the presence of virus replication and cell-to-cell movement in the inoculated leaf of resistant plants, this resistance cannot be considered a recovery phenotype in which an extreme systemic resistance is established, as has been described for other viral systems (Lindbo *et al.,* 1993; Guo and Garcia, 1997; Tanzer *et al.,* 1997). This latter response may involve the elicitation of some signal in the initially inoculated leaf that spreads through the plants, generating a systemic silencing (Palauqui *et al.,* 1997; Voinnet and Baulcombe, 1997; Jorgensen *et al.,* 1998). It is possible that the proposed suppression observed here is localized to the actual virus-infected tissue and that it does not affect noninfected tissue or that it is confined to the inoculated leaf. Alternatively, a signal from the inoculated tissue could be delivered that triggers an inefficient systemic silencing that does not silence the transgenic mRNA. This might be because the transgene mRNA concentration was too low (Jorgensen *et al.,* 1998), and thus the inhibitory effect in upper leaves after reinoculation would be barely discernible from suppression observed in a leaf of a plant inoculated *de novo*.

On the other hand, the presence of transgenic 1a protein in the systemic tissue of resistant plants after prior inoculation with virus requires the existence of a resistance mechanism other than the silencing of the viral RNA 1. This mechanism is needed to explain the lack of systemic infection in these plants inoculated with viral RNAs 2 and 3, against which there seems to be no targeted silencing. In the inoculated leaves of resistant and susceptible plants, RNAs 2 and 3 accumulated to comparable levels (Fig. 10A, lane 2 vs lanes 5 and 8) and systemic infection did not occur in resistant plants, whereas it did in susceptible plants (Fig. 4). Thus, there must be some mechanism inhibiting systemic infection. The nature of this mechanism and its relationship to the mechanism of inhibition of RNA 1 accumulation may or may not be related.

MATERIALS AND METHODS

Plants, viruses, and inoculation of plants.

Tobacco plants (*Nicotiana tabacum* cv. Turkish Samsun NN) were used for plant transformation and as susceptible plants in resistance tests. Plants were grown in the greenhouse or in a growth chamber at 25°C. CMV strain Fny (Roossinck and Palukaitis, 1990) was purified from infected tobacco as described (Palukaitis *et al.,* 1992). The pseudorecombinant virus made from Fny-CMV RNAs 1 and 2 and M-CMV RNA 3 and the pseudorecombinant virus made from LS-CMV RNAs 1 and 2 and M-CMV RNA 3 were obtained by mixing, respectively, Fny-CMV transcript RNAs 1 and 2 for the former combination and LS-CMV transcript RNAs 1 and 2 for the latter combination with M-CMV transcript RNA 3, derived from the corresponding full-length cDNA clones (Rizzo and Palukaitis, 1990; Zhang *et al.,* 1994; Shintaku and Palukaitis, 1990) to generate virus F1F2M3-CMV and virus L1L2M3-CMV. Plants were inoculated mechanically with purified virus, virus infected leaf extract (diluted 1:10 in 50 mM sodium phosphate, pH 7), or transcript RNAs by gently rubbing the inocula onto aluminum oxide-dusted leaves. Because particles of F1F2M3-CMV and L1L2M3-CMV do not store well after isolation, sap from infected plants, inoculated 10–12 days earlier, was used as the source of inoculum.

Generation of the plant expression construct and plant transformation

To generate plants expressing biologically active 1a protein, the cDNA sequence corresponding to the RNA 1 of Fny-CMV was inserted into the plant transformation vector pROK2 as shown in Figure 1. A DNA fragment corresponding to a full-length, biologically active, cDNA clone of Fny-CMV RNA 1 (pF109 C-His), modified by the addition of a hexahistidine-coding sequence at the 3′ end of the 1a gene and known not to adversely affect viral replication (unpublished observation), was amplified by the PCR. The primers contained restriction endonuclease sites at their 5′ ends, to facilitate cloning into the plant expression vector pROK2 digested with the same enzymes (*Bam*HI and *Sac*I). Besides the 1a gene, the insert contained the viral RNA 1 5′ and 3′ NCRs and a nucleotide sequence corresponding to the T7 promoter upstream the viral sequence (Fig. 1). After ligation into pROK2, the plasmid was electroporated into *Agrobacterium tumefaciens* strain LBA-4404, which was used to transform tobacco using the leaf disc method (Horsch *et al.,* 1985), with plants regenerating from calli in a kanamycin-selective medium.

Nucleic acid extraction and analysis

Leaves analyzed in Figures 2 and 4 were sampled by taking a single leaf disk per plant (~50 mg), whereas leaves analyzed in Figures 9 and 10 were sampled by taking 10 smaller leaf disks per plant (~50 mg). In inoculated leaves of Figure 9, the leaf disks corresponded to lesions. Total RNAs were extracted from plant tissues by grinding them in 300 μl of 50 mM Tris–HCl, pH 8.0, 10 mM EDTA, 2% SDS, and 0.5% 2-mercaptoethanol. The samples were extracted with phenol, and RNA was precipitated with ethanol as described previously (Palukaitis *et al.,* 1985). For Northern blot analysis, RNA samples were recovered by centrifugation, fractionated by electrophoresis in 1.5% agarose gels under denaturing conditions (6% formaldehyde), blotted to nitrocellulose membranes, and hybridized to [^{32}P] RNA probes complementary to the 3′ NCR of each Fny-CMV RNA (Gal-On *et al.,* 1994). The blots were washed and autoradiographed as described by Sambrook *et al*. (1989). After the blot analysis, to quantify the relative amounts of viral RNA, the areas of the nitrocellulose membranes at where each viral RNA had transferred were isolated, and the corresponding Cerenkov c.p.m. were obtained in a liquid scintillation counter.

Detection of biologically active transgene product

Direct detection of transgenic 1a protein in plant tissue was attempted by Western blotting. Proteins were separated by SDS–PAGE, blotted to nitrocellulose (Towbin *et al.,* 1979), and probed with a polyclonal antiserum against the 1a protein as described (Gal-On *et al.,* 1994). An indirect approach to confirm the expression of 1a protein was by detection of the products of the 1a proteins, biological activity; that is, plants were inoculated with CMV RNA transcripts 2 and 3 as described above, and replication of the inoculated RNAs was detected by three methods: (1) RT-PCR amplification of viral sequences using primers specific for RNA 2 (corresponding to nucleotides 1533–1549 and 2646–2660) or RNA 3 (corresponding to nucleotides 110–134 and 944–958), (2) detection of viral RNAs in plant tissue by Northern blot hybridization, as indicated above, or (3) with a modified RNA 3 transcript derived from construct pL:3a/GFP, in which the gene encoding the GFP was substituted for the viral CP gene, and with detection of GFP-derived fluorescence by confocal laser scanning microscopy as described previously (Canto *et al.,* 1997).

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