Broad Resistance to Tobamoviruses Is Mediated by a Modified Tobacco Mosaic Virus Replicase Transgene

J. Donson[1,3] C. M. Kearney[2,3], T. H. Turpen[1,3], I. A. Khan[2,3], G. Kurath[3], A. M. Turpen[1], G. E. Jones[3], W. O. Dawson[2,3], and D. J. Lewandowski[2,3]

[1]Biosource Genetics Corporation, 3333 Vaca Valley Parkway, Vacaville, CA 95688 U.S.A.; and [2]Department of Plant Pathology, University of Florida, Citrus Research and Education Center, 700 Experiment Station Road, Lake Alfred 33850 U.S.A. [3]This work began at the Department of Plant Pathology, University of California, Riverside 92521 U.S.A.

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Tobacco plants made transgenic to express the wild type tobacco mosaic virus (TMV) 183-kDa replicase gene were not resistant to TMV. However, transgenic plants containing essentially the same sequences, but with an additional insertion that would terminate translation in the middle of the 183-kDa gene, were highly resistant to systemic infection by TMV and other tobamoviruses. The 1.4-kbp insertion in the replicase open reading frame (ORF) of the resistant plants was shown by DNA sequencing to be an IS10-like transposable element, which apparently inserted itself into the TMV sequence at nucleotide 2875 sometime during the propagation of this replicase ORF plasmid (pREP21). Because of four stop codons, in frame with the TMV replicase ORF on the immediate 5′ border of the IS insertion, REP21 effectively represents domain 1 (putative methylase domain) and a portion of domain 2 (putative helicase domain) of the TMV replicase ORF. REP21 Xanthi tobacco plants had a level of resistance to TMV similar to other reported transgenic replicase plants. No TMV was detected in upper leaves of these plants at 1-mo postinoculation. In addition, REP21 plants were resistant to an unusually broad range of tobamoviruses including tomato mosaic virus, tobacco mild green mosaic virus, TMV-U5, green tomato atypical mosaic virus, and ribgrass mosaic virus. These plants were not resistant to cucumber mosaic cucumovirus. The lack of systemic infection by TMV was due to reduced multiplication in inoculated leaves rather than complete prevention of replication. Inoculation of progeny of the cross of a resistant REP21 line with the TMV local lesion host, Xanthi nc tobacco, resulted in production of local lesions that were delayed, smaller, and less numerous.

Corresponding author: D. J. Lewandowski.
Present address of G. Kurath: National Fisheries Research Center, Building 204, Naval Station, Seattle, WA 98115.
Present address of I.A. Khan: Department of Horticulture, University of Agriculture, Faisalabad, Pakistan.

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A number of strategies involving transformation of plants with portions of viral genomes have been employed to produce virus-resistant plants. Virus resistance in plants has resulted from transformation with coat protein genes (Powell-Abel *et al.* 1986; for review see Beachy *et al.* 1990), satellite RNAs (Gerlach *et al.* 1987; Harrison *et al.* 1987), virus-specific antisense RNA (Cuozzo *et al.* 1988; Hemenway *et al.* 1988), and, most recently, nonstructural gene sequences (Golemboski *et al.* 1990; for review see Lomonossoff 1992). The latter strategy is distinguished by resistance to much higher levels of virus inoculum. However, the resistance is relatively narrow, being restricted to the homologous or closely related viruses (Golemboski *et al.* 1990; Braun and Hemenway 1992; MacFarlane and Davies 1992; Anderson *et al.* 1992; Longstaff *et al.* 1993).

In general, "replicase-resistant" plants, which have a genomic insertion of some part of a viral replicase gene, appear to be most effective when portions of viral replicase genes are expressed that would not result in a functional replicase subunit. This includes plants transformed with the readthrough region (54 kDa) of replicases from tobacco mosaic tobamovirus (TMV) (Golemboski *et al.* 1990) and pea early browning tobravirus (MacFarlane and Davies 1992), plants transformed with truncated replicase ORFs of cucumber mosaic virus (CMV) (Anderson *et al.* 1992) or potato virus X (PVX) (Braun and Hemenway 1992), or plants transformed with mutated PVX replicase genes (Longstaff *et al.* 1993). Transgenic plants expressing functional replicase genes of alfalfa mosaic virus (van Dun *et al.* 1988), brome mosaic bromovirus (Mori *et al.* 1992), and some isolates of PVX (Longstaff *et al.* 1993) are fully susceptible to viral infection. The exception appears to be plants transformed with an apparently wild-type replicase ORF of PVX that are highly resistant to PVX infection (Braun and Hemenway 1992). We report that tobacco plants containing the complete TMV 183-kDa replicase ORF had little or no resistance to infection

by TMV, but that tobacco plants transformed with TMV replicase sequences interrupted at nucleotide (nt) 2875 by the in-frame stop codons of a bacterial insertion element were resistant. Importantly, these plants are resistant to distantly related tobamoviruses.

RESULTS

Two constructs for plant transformation with the TMV replicase gene.

Two sets of plants derived from tissue independently transformed with different constructs containing the TMV 183-kDa replicase ORF (Fig. 1) were created and tested for resistance to systemic infection by TMV. Although designed somewhat differently, each transformation plasmid was constructed to cause the resulting plants to express the 126-kDa replicase protein and smaller amounts of the 183-kDa readthrough protein by suppression of the amber stop codon. Construct pBGC48 was made by placing TMV nts 1-5085 (the entire 183-kDa replicase ORF plus 183 nts of the 30-kDa transport protein gene) into the *Agrobacterium tumefaciens* Ti plasmid, pAP2034 (Velten and Schell 1985) adjacent to the cauliflower mosaic virus (CaMV) 35S promoter to initiate transcription at TMV nt 1 such that the native TMV leader would serve as the replicase mRNA leader. Construct pREP21 consisted of TMV nts 69-5463 (the 183-kDa replicase ORF plus 561 nts of the transport gene) inserted into the *A. tumefaciens* Ti transformation plasmid pMON530 adjacent to the cauliflower mosaic virus 35S promoter. Kanamycin-resistant shoots (R_0 generation) were selected, 16 from pBGC48-transformed tissue and 12 from pREP21-transformed tissue, rooted, transferred to soil, allowed to flower, self-fertilize, and seeds of the R_1 generation were collected. The resulting transgenic tobacco plants (R_1 generation) were tested for their ability to support systemic infection by TMV.

Sequence REP21, but not BGC48, induced TMV resistance in transgenic tobacco plants.

All BGC48 plants allowed TMV infection and produced symptoms essentially indistinguishable from those on untransformed tobacco. None of the resulting R_1 plants exhibited more than a minor delay in symptom development after being infected with TMV. By 10 days postinoculation, all plants had symptoms typical of TMV in the upper leaves.

In contrast, initial screening of REP21 plants revealed lines with a high level of resistance to TMV infection and symptom development (Fig. 2). Although nontransformed

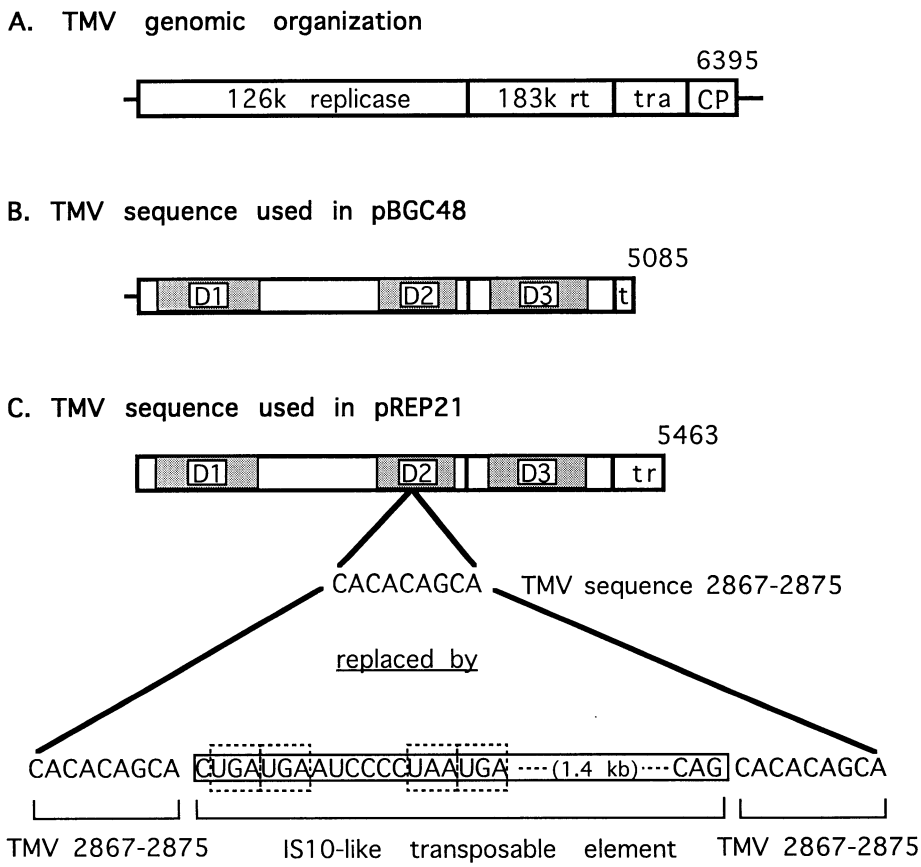

Fig. 1. Portions of the tobacco mosaic virus (TMV) genome included in the plant transformation plasmids pBGC48 and pREP21. **A,** Diagram of the TMV genome showing the 126-kDa replicase ORF and amber stop codon readthrough (rt) region forming a 183-kDa replicase ORF. Tra = 30-kDa transport protein gene; CP = coat protein gene. Replicase ORF portion included in **B,** pBGC48 (TMV nts 1-5085) and **C,** pREP21 (TMV nts 69-5463). **D1, D2,** and **D3** are conserved replicase domains. Insertion of the 1.4-kbp IS10-like transposon into the pREP21 sequence created four stop codons (dashed boxes) in frame with the TMV replicase ORF, as well as a direct repeat of nts 2867-2875.

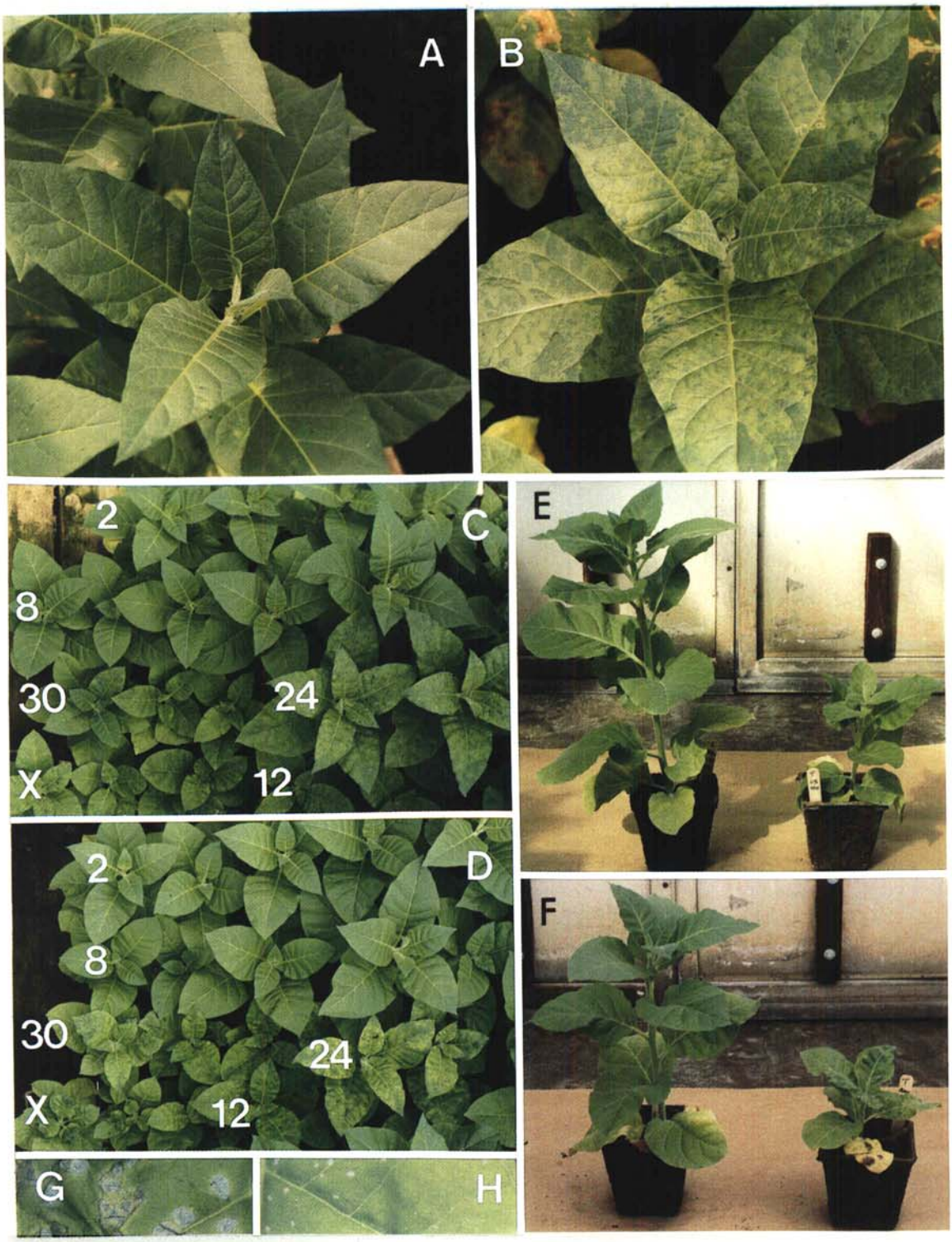

Fig. 2. Tobamovirus-induced symptoms in tobacco (*N. tabacum* cv. Xanthi) transgenic for the REP21 tobacco mosaic tobamovirus (TMV) replicase sequences and local lesion phenotype in REP21 cv. Xanthi nc leaves. Symptoms in upper non-inoculated leaves of **A,** a rare REP21 line 2 plant with chlorotic spots (the plant was cut back at 30 days postinoculation and a new shoot developed with symptoms) and **B,** nontransformed Xanthi tobacco inoculated with 100 μg/ml TMV. Symptoms of resistant (lines 2 and 8) and non-resistant (line 24) REP21 plants inoculated with **C,** 100 μg/ml TMV or **D,** 100 μg/ml green tomato atypical mosaic tobamovirus 28 days postinoculation. Lines are numbered to the left of each row: 2, 8, 24 = REP21 lines 2, 8, and 24, respectively; 12 and 30 = Xanthi tobacco transgenic for TMV 30-kDa gene; X = untransformed Xanthi tobacco. Symptoms of resistant REP21 line 2 (left) and untransformed Xanthi (right) inoculated with **E,** 100 μg/ml TMV-U5 or **F,** 100 μg/ml ribgrass mosaic tobamovirus 28 days postinoculation. TMV induced necrotic local lesions in **G,** Xanthi nc tobacco 3 days postinoculation or **H,** (REP21 line 2 ♂ × Xanthi nc ♀) 7 days postinoculation.

plants and plants transformed with the TMV 30-kDa transport gene developed vein-clearing and mosaic symptoms typical of TMV infections within 4–5 days, resistant REP21 lines remained symptomless for at least 30 days and infectivity assays of upper leaves detected no virus. Of the 12 R_1 lines developed from the 12 independent, kanamycin-resistant REP21 shoots selected, five had measurable resistance to TMV (lines 2, 4, 5, 7, and 8), although Southern blot analysis showed that 11 of the 12 lines harbored the REP21 ORF (Fig. 3). Western blot analyses using antiserum to TMV 126/183-kDa proteins (Lehto *et al.* 1990) did not detect replicase protein production in any REP21 line, but detected 126-kDa protein from most BGC48 plant lines (A. G. C. Lindbeck, personal communication).

One-month postinoculation with TMV, four plants each of lines 2 and 8 showed no symptoms of virus infection. At this time they were cut back to leave a few stem nodes above the level of the soil and allowed to develop new shoots for 2 mo. Most plants remained symptomless. One plant from line 8 developed mild mosaic symptoms and two plants (one each of lines 2 and 8) developed small chlorotic spots (Fig. 2A) and were still resistant to disease caused by TMV compared to control plants (Fig. 2B).

Chromosomal copies of REP21, but not BGC48, were disrupted by an insertion.

Chromosomal DNA from BGC48- and REP21-transgenic plants was digested with restriction endonucleases and analyzed in Southern blots probed with the entire TMV sequence. DNA from BGC48-transformed plants gave the predicted fragments, suggesting that these plants contained the entire wild-type TMV 183-kDa gene. Additionally, replicase sequences of pBGC48 were probably biologically active, because plasmids derived from pBGC48 which contained the entire genome of TMV (pBGC46) or the genome minus the coat protein gene (pBGC49) resulted in infected plants when transformed into tobacco plants (Turpen *et al.* 1993).

Southern blot analysis of pREP21 DNA after *Bam*HI digestion gave the expected bands of 2.3 and 3.3 kbp, but the REP21 sequences in the plant chromosomal DNA yielded a 2.3- and a 4.7-kbp band (Fig. 3), indicating insertion of a 1.4-kbp DNA fragment at some time before the plant transformation event, since all REP21 lines had the 2.3- and 4.7-kbp bands. Further restriction enzyme analysis (data not shown) confirmed the presence of a 1.4-kbp insert near TMV nucleotide 3000 in the transgenic REP21 sequences.

The insert region (TMV sequence 2624-3263) was PCR-amplified from plant chromosomal DNA from five REP21 lines of the R_1 generation (resistant lines 2, 4, 7, and 8, and nonresistant line 24). All lines produced a 2.0-kbp PCR band instead of the 0.6-kbp band expected, confirming the existence of a 1.4-kbp insertion. The PCR product above was present in minimal quantities, so it was reamplified by PCR, and then sequenced by a repetitive thermocycling scheme (Promega) using *Taq* I polymerase. The terminal 250 bases at each end of the insert had 98% identity to the 1.33-kbp prokaryotic transposable element IS10 (Halling *et al.* 1982). Further evidence for a transposition insertion event was provided by a nine-base direct repeat of TMV sequence 2867-2875 (Fig. 1C). If expressed, translation of the TMV replicase would be expected to terminate at the beginning of the insertion, since four stop codons in frame with the TMV replicase ORF are present in the first 2-18 nts of the inserted sequence (Fig. 1C).

N-gene tobacco plants transgenic for REP21 demonstrated reduced viral multiplication in inoculated leaves.

To determine the effect of the transgene of REP21 plants on initial infection and local replication by TMV, we inoculated mature leaves of a cross between Xanthi nc tobacco, which is homozygous for the dominant N resistance gene and gives local lesions to tobamoviruses, and REP21 line 2 (Xanthi nc [NN] × REP21 Xanthi). Lesion development in leaves of the kanamycin-resistant F_1 progeny inoculated with 30 µg/ml TMV was delayed and lesions were smaller and fewer (Fig. 2G and H). Lesions became visible later than 6 days postinoculation, compared with 2-3 days for control Xanthi nc tobacco plants lacking the REP21 sequences. Also, only one third as many lesions developed on REP21/N gene plants. By 7 days postinoculation with TMV, an average of 99.1 ± 44.6 lesions per leaf developed on REP21/N plants, while 289.3 ± 93.0 lesions per leaf developed on control NN plants. In contrast, Xanthi nc tobacco plants directly transformed with pBGC48 developed lesions after inoculation with TMV that were identical in size, timing, and number to that of nontransformed control plants (data not shown).

REP21 plants were highly resistant to a range of tobamoviruses.

Since R_1 Xanthi tobacco plants of REP21 lines 2, 4, 7, and 8 were found to be resistant to systemic infection by TMV, these lines (R_1 generation) were further screened

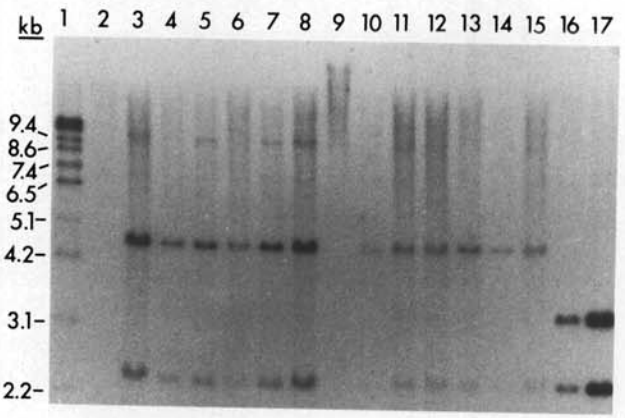

Fig. 3. Southern blot hybridization of total DNA from various REP21 transgenic tobacco lines following *Bam*HI digestion. Lane 1, molecular weight markers. Lane 2, untransformed Xanthi tobacco. Lanes 3-15, REP21 transgenic lines 2, 4, 5, 6, 7, 8, 17, 24, 29a, 29b, 30, 31, and 32. Lanes 16 and 17, untransformed Xanthi tobacco DNA plus the equivalent of one and five genomic copies of pREP21, respectively. The blot was probed with the 7.3-kbp *Pst*I fragment from pTMV204 labeled with [α-^{32}P] dATP.

with a variety of tobamoviruses that systemically infect tobacco (Fig. 2). Coat protein similarities, hybridization studies (reviewed by Gibbs 1986), and nucleotide sequence similarities (Ohno *et al.* 1984; Solis and Garcia-Arenal 1990) suggest an increasing evolutionary distance from TMV of tomato mosaic virus (ToMV), tobacco mild green mosaic virus (TMGMV), and ribgrass mosaic virus (RMV), respectively. One month after inoculation with 100 μg/ml of virions, systemic symptoms were absent from all REP21 plants inoculated with TMV, TMV-U5, TMGMV, or GTAMV (Table 1). With the tobamoviruses ToMV and RMV, most plants showed no symptoms. However, four of 20 plants exhibited delayed symptoms that consisted of isolated chlorotic spots in some leaves similar to those illustrated in Figure 2A. The amount of virus present in inoculated and upper leaves of these plants at 1 mo postinoculation was bioassayed in the local lesion host, *N. tabacum* 'Xanthi nc' (Table 2). No virus was detected in any of the upper leaves of REP21 plants inoculated with TMV or GTAMV, and only one plant allowed systemic replication of ToMV (Table 2, upper leaves). TMGMV and TMV-U5 were detected sporadically and in very low concentrations in upper leaves of REP21 plants. Even though three of 10 REP21

Table 1. Occurrence of chlorotic patches in upper leaves of REP21-transformed tobacco inoculated with a range of tobamoviruses

Transformant line	TMV[a]	TMV-U5	TMGMV	GTAMV	ToMV	RMV
2	0/8[b]	0/8	0/5	0/5	0/5	1[c]/5
4	0/4	0/4	...[d]	...	...	...
7	0/4	0/4	...	...	...	...
8	0/9	0/9	0/5	0/5	1[c]/5	2[c]/5
Untransformed tobacco	5/5	5/5	3/3	3/3	3/3	3/3

[a] TMV, tobacco mosaic virus; TMV-U5, the tobamovirus from *Nicotiana glauca*; TMGMV, tobacco mild green mosaic virus; GTAMV, green tomato atypical mosaic virus; ToMV, tomato mosaic virus; and RMV, ribgrass mosaic virus.
[b] Number of plants with systemic symptoms/number of plants inoculated. Plants were inoculated with virions at 100 μg/ml (100 μl/leaf = 10 μg/leaf = 20 μg/plant) and symptoms visually noted at 1 mo postinoculation.
[c] Delayed, chlorotic infected spots. Untransformed tobacco yielded strong mosaic (bottom line).
[d] Not determined.

Table 2. Local lesion assay for virus replication in inoculated and upper leaves of REP21-transformed tobacco challenged with several tobamoviruses

	TMV		TMV-U5		TMGMV		GTAMV		ToMV		RMV	
Transformant[a]	0.5[b]	100	0.5	100	0.5	100	0.5	100	0.5	100	0.5	100
Lesion bioassay of upper leaves												
2 A	0	0	91	28	2	0	0	0	0	0	0	0
B	0	0	0	0	0	0	0	0	0	0	0	0
C	0	0	0	0	0	0	0	0	0	0	0	0
D	0	0	0	0	0	1	0	0	0	0	0	>200
E	—[c]	—	—	—	0	0	0	0	0	0	0	0
8 A	0	0	0	0	1	0	0	0	0	0	0	0
B	0	0	13	0	0	0	0	0	0	0	1	0
C	0	0	0	15	0	0	0	0	0	0	>200	>200
D	0	0	23	0	0	0	0	0	0	>200	0	>200
E	0	0	0	0	0	0	0	0	0	0	0	0
Untransformed tobacco												
A	>200	>200	80	>200	>200	>200	>200	>200	0	>200	0	>200
B	>200	>200	>200	>200	25	>200	>200	15	>200	>200	0	>200
C	>200	>200	>200	>200	>200	>200	>200	>200	>200	>200	>200	>200
Lesion bioassay of inoculated leaves												
2 A	—[c]	32	>200	>200	—	>200	0	33	0	>200	0	>200
B	0	0	26	0	—	>200	0	60	0	—	0	>200
C	0	0	0	0	60	5	0	19	0	0	0	>200
D	0	17	0	0	0	70	0	>200	0	0	0	>200
E	—	—	—	—	0	1	0	4	0	0	0	0
8 A	0	120	0	0	—	>200	0	36	0	12	0	0
B	—	0	>200	0	0	53	0	5	0	>200	0	—
C	—	14	0	—	0	0	0	0	0	0	1	—
D	0	0	—	—	1	0	0	0	0	>200	0	—
E	0	0	0	0	0	60	0	100	0	8	0	3
Untransformed tobacco												
A	>200	>200	—	—	>200	100	—	>200	—	>200	0	>200
B	>200	>200	—	—	>200	>200	>200	>200	—	>200	0	>200
C	>200	>200	>200	>200	>200	>200	>200	>200	>200	>200	0	—

[a] These transformant plants (A–E of lines 2 and 8) are the same individuals from the same experiment yielding the data of Table 1.
[b] Transformant plants were inoculated with 0.5 or 100 μg/ml of virions and, at 30 days postinoculation, systemic symptoms were noted (Table 1) and virus replication was assayed (Table 2) by homogenizing at approximately 0.1 g/ml of either the initially inoculated leaves or new, upper leaves, and inoculating each homogenate onto the local lesion host *N. tabacum* 'Xanthi nc.'
[c] Not determined.

plants inoculated with 100 μg/ml RMV and one of 10 inoculated with 100 μg/ml ToMV contained higher levels of virus (>200 local lesions) in the upper leaves, these plants displayed only the sporadic chlorotic spots (Table 1) similar to that in Figure 2A, but otherwise grew like noninoculated control plants (Fig. 2F). Plants with lower levels of virus in upper leaves did not have visible symptoms (TMV-U5, TMGMV). For all of the viruses, the 100 μg/ml inoculations gave more critical tests since the 0.5 μg/ml challenge did not always infect untransformed control tobacco. All tobamoviruses replicated locally to some extent in the inoculated leaves of at least some REP21 plants, though generally there was inhibition of replication (Table 2, inoculated leaves).

REP21 lines 2 and 8 that are resistant to TMV were inoculated with cucumber mosaic virus to determine whether resistance extended to other virus groups. No reduction or delay in disease symptoms occurred in these plants (data not shown).

DISCUSSION

REP21 transgenic plants had resistance to a much broader range of viruses than previous reports of transgenic plants containing replicase genes. REP21 plants showed high levels of resistance to TMV and five other tobamoviruses. These viruses ranged in their relatedness to TMV (the origin of the REP21 replicase insertion) from relatively similar (ToMV) to distantly related (RMV). The transgenic TMV replicase plants of Golemboski *et al.* (1990) were highly resistant to the closely related TMV mutant YSI/1, but not to the closely-related tobamovirus, ToMV, or to other tobamoviruses. Similarly, plants transgenic for a pea early browning tobravirus (PEBV) replicase segment (MacFarlane and Davies 1992) were resistant to another isolate of PEBV and the closely related tobravirus, broad bean yellow band virus, which is serologically unrelated but appears to have substantial sequence identity to PEBV. However, these plants were susceptible to two more distantly related tobraviruses. The level of resistance of REP21 plants was comparable to that of the other reported transgenic replicase plants, allowing no systemic viral replication in all plants tested over 1 mo postinoculation with 100 μg/ml of TMV (Table 2), or 1:10 diluted sap from infected plants (data not shown).

Truncation of the replicase ORF of REP21 was achieved fortuitously and efficiently by the four in-frame stop codons of the IS10-like inserted element, beginning just one base from the insertion site. Thus, REP21 plants could potentially express only domain 1 (putative methyltransferase domain) and a portion of domain 2 (putative helicase domain) of the TMV replicase (Goldbach 1990). By contrast, the chromosomal insertions in BGC48 comprised all three complete domains. If expressed, a defective rather than an intact replicase insert might be responsible for the resistance found in this study.

In general, reports of plants transgenic for various portions of viral replicase sequences fall into three major groups. The first consists of plants transgenic for apparently functional replicase insertions, which remain susceptible. These include the 126-kDa ORF (Golemboski *et al.* 1990) or 183-kDa (BGC 48 of this study) of TMV, and RNA 1 and/or RNA 2 insertions of alfalfa mosaic virus (van Dun *et al.* 1988) and brome mosaic bromovirus (Mori *et al.* 1992). In contrast to the susceptibility of these plants is the high level of resistance obtained with a full-length replicase insertion of potato virus X (Braun and Hemenway 1992). This replicase ORF was derived from an infectious clone, though *in vitro* transcribed RNA from this clone was only 0.2% as infectious as wild-type viral RNA, possibly indicating a defective mutant sequence. A second group of transgenic replicase plants comprise inserts of various defective replicase ORFs and result in high levels of resistance. These include domain 1 and part of domain 2 of TMV (REP21, this study), RNA 2 of CMV (containing domain 3) from which an 84-base segment including the replicase motif GDD was deleted (Anderson *et al.* 1992), the full-length replicase ORF of PVX with a mutated GDD motif (Longstaff *et al.* 1993), and a 5′ portion of the PVX replicase ORF including domain 1 (Braun and Hemenway 1992). The third group consists of the domain 3 insertions derived from TMV (Golemboski *et al.* 1990) and pea early browning tobravirus (MacFarlane and Davies 1992) which yielded highly resistant transgenic plants. These insertions might be considered "defective" portions of the replicase. Mutations abolishing translatability of these constructs eliminated their ability to induce resistance, indicating that the domain 3 protein itself functions to provide the resistance (Carr *et al.* 1992; MacFarlane and Davies 1992).

Regardless of the diversity of replicase segments inducing resistance, it is possible that reduction in the rate of viral replication is the common mechanism behind the resistance, as demonstrated by Carr and Zaitlin (1991) with protoplasts derived from plants transgenic for domain 3 of TMV. Infection often occurs in inoculated leaves of the resistant transgenic replicase plants, though it rarely or never spreads systemically (REP21 plants of this study; Carr and Zaitlin 1991; Braun and Hemenway 1992; Anderson *et al.* 1992; Longstaff *et al.* 1993). The REP21/N plants of this study yielded much smaller and more delayed lesions than those of nontransformed plants, suggesting that resistance was due to a lower viral replication rate.

The observed systemic resistance seen in replicase-resistant plants may be due to a subthreshold level of virus produced in the inoculated leaves that is somehow insufficient in quantity or timing to allow for effective phloem loading and systemic spread. This is in parallel with systems in which alterations in viral genomes that reduce replication are often sufficient to prevent systemic infection. Deletion of portions of the replicase genes of brome mosaic bromovirus or barley stripe mosaic hordeivirus allow efficient replication in protoplasts, but prevent systemic infection of intact plants (Traynor *et al.* 1991; Jackson *et al.* 1991). These data suggest that a threshold of replication is required for systemic infection and modifications in either plant or virus that reduce replication can prevent disease.

MATERIALS AND METHODS

Plants and viruses.

Nicotiana tabacum (L.) 'Xanthi' and 'Xanthi nc' were the systemic and local lesion hosts, respectively, for the tobamoviruses used in this study. TMV (strain U1) was obtained by *in vitro* transcription of pTMV204 (Dawson *et al.* 1986). The tobamovirus from natural infections of *N. glauca* referred to as TMV-U5, tobacco mild green mosaic virus (TMGMV), green-tomato atypical mosaic virus (GTAMV), and tomato mosaic virus (fruit necrosis strain; ToMV-FN) were obtained from J. A. Dodds (Valverde *et al.* 1991). Ribgrass mosaic virus (Chinese cabbage strain; RMV; Oshima *et al.* 1974) was obtained from R. J. Shepherd. Each virus was purified from systemically infected Xanthi tobacco tissue essentially as described by Gooding and Hebert (1967).

Construction of transformation plasmids.

DNA manipulations were done essentially as described in Ausubel *et al.* (1988). TMV nucleotide numbering is according to Goelet *et al.* (1982).

pBGC48 (Fig. 1) was designed to contain TMV nts 1–5085 in *Agrobacterium tumefaciens* Ti plasmid pAP2034 (Velten and Schell 1985) such that transcription initiation from the cauliflower mosaic virus 35S promoter began at nt 1 of TMV (Turpen *et al.* 1993). pBGC45 (Turpen *et al.* 1993) contained TMV nts 1–3332 downstream of the cauliflower mosaic virus 35S promoter inserted into pAP2034. The 1.8-kbp TMV fragment (nts 3332–5080) was inserted into the *Bam*HI site of pBGC45 resulting in pBGC48. pBGC46 (Turpen *et al.* 1993), also derived from clone pBGC45 by insertion of TMV nts 3332–6395, contained the complete cDNA of TMV.

To construct plasmid pREP21, an *Nco*I restriction site at the sequence corresponding to the initiation codon of the ORF for the 126-kDa protein was created by mutagenesis of pTMV204 as described by Kunkel *et al.* (1987) with an oligonucleotide d(GTGTATGCCATGGTAATTGT) to give pTN2. The single-stranded ends of the 5.4-kbp *Nco*I fragment (TMV nts 69–5463) from pTN2 were filled with Klenow fragment and subcloned into *Sma*I digested pUC19 to give pREP20. pREP20 was digested with *Kpn*I and *Acc*I and the fragment containing the TMV sequences was inserted into *Cla*I/*Kpn*I digested pMON530 (Rogers *et al.* 1987) to produce pREP21.

Plant transformation.

pREP21 was mobilized into *A. tumefaciens* strains LBA4301 (Keane *et al.* 1970) and pBGC48 into C58C1 (Zambryski *et al.* 1983) by triparental mating (Rogers *et al.* 1986). Cut pieces of Xanthi tobacco leaves were transformed as described by Horsch *et al.* (1985). Transformed calli were selected on regeneration medium containing 100 μg/ml kanamycin sulfate. Shoots that developed were removed from the explant and induced to root. Rooted plantlets were transferred to soil and maintained in a glasshouse.

Pollen from REP21 transformed Xanthi tobacco line 2 (R_1 generation) was transferred to the stigma of emasculated flowers of *N. tabacum* 'Xanthi-nc.' Seed from these plants was germinated in the presence of 50 μg/ml kanamycin and resistant plantlets were transplanted to soil at the four leaf stage (after approximately a month).

Nucleic acid analysis.

Total nucleic acid was prepared from transgenic plants by the method of Dellaporta *et al.* (1983), digested with 50 μg/ml RNase A for 1 hr at 37° C, extracted with phenol-chloroform, and precipitated with ethanol. Aliquots of these DNA preparations were analyzed by *Bam*HI digestion followed by Southern transfer hybridization with the full-length TMV cDNA (Dawson *et al.* 1986) labeled by the method of Feinberg and Vogelstein (1984) with [α-^{32}P]dATP (New England Nuclear; Pharmacia) or with digoxigenin-labeled dUTP (Boehringer Mannheim).

The region of the transgene containing the IS10-like insertion was amplified by polymerase chain reaction (PCR) with oligonucleotide primers corresponding to TMV nts 2624–2646 and the complement of TMV nts 3243–3263. The product of the PCR was sequenced with the *fmol* DNA sequencing kit (Promega), using as primers TMV nts 2682–2702 for 5′ sequencing and the complement of TMV nts 2946–2967 for 3′ sequencing. The resulting sequence was used in a search of nucleotide sequence data obtained from the GenBank Genetics Sequence Data Bank (Release 73.1, October 1992), using the BLAST program (Altschul *et al.* 1990).

Plant virus resistance assays.

Two expanded leaves of each of the kanamycin-resistant seedlings (approx. 2–4 wk after transplanting to soil) were inoculated with 0.5–100 μg/ml TMV or 1:10 diluted sap from systemically infected leaves of TMV-infected plants in 0.5% sodium pyrophosphate buffer, pH 8.6, plus 0.5% bentonite and 0.5% Celite. Plants were scored for systemic symptoms up to 40 days postinoculation.

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