

# VIRUS TOLERANCE, PLANT GROWTH, AND FIELD PERFORMANCE OF TRANSGENIC TOMATO PLANTS EXPRESSING COAT PROTEIN FROM TOBACCO MOSAIC VIRUS

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Two transgenic tomato plants that express the coat protein (CP) of the common ( $U_1$ ) strain of tobacco mosaic virus (TMV) were produced from cultivar VF36 using gene transfer techniques. CP-expressing plants were partially resistant to infection and symptom development caused by TMV and tomato mosaic virus (ToMV) strains L, 2, or 2<sup>2</sup>. Strains 2 and 2<sup>2</sup> normally overcome the natural resistance genes present in many commercial tomato cultivars. In the field, no more than 5% of the CP-expressing plants inoculated with TMV exhibited visual systemic disease symptoms by fruit harvest compared with 99% of the VF36 plants. Lack of visual symptoms was associated with lack of virus accumulation in the CP-expressing plants. In terms of agronomic traits, leaf and stem dry weight accumulation in greenhouse-grown uninoculated CP-expressing (line 306) and nonexpressing plants were essentially equal. In field analyses, tomato fruit yields of the VF36 plants decreased 26–35% due to virus infection, whereas yields of the CP-expressing plants were unaffected. Yields from one CP-expressing line were equal to that of the uninoculated VF36 plants suggesting that expression of the CP gene does not intrinsically cause yield depression. The results from these growth chamber, greenhouse and field experiments indicate the potential for use of genetically engineered protection in agriculture.

Virus infection can decrease yields by 20% or more in tomato plants grown under production conditions<sup>1</sup>. Although tobacco mosaic virus (TMV) can and does infect tomatoes, most of the yield loss is attributed to infection by strains of tomato mosaic virus (ToMV), a tobamovirus closely related to TMV<sup>2</sup>. Improved hygiene and the use of natural cross-protection (infection of plants with a mild strain of virus to protect against infection by more virulent strains) has helped to control severe outbreaks of virus infection. However, the use of virus-resistant tomato cultivars has provided the best control against ToMV, where a gene-for-gene relationship exists for resistance<sup>2–5</sup>. There are two loci for resistance to ToMV in tomato, Tm-1 and Tm-2; the Tm-2 locus has two alleles, Tm-2 and Tm-2<sup>2</sup>. Tomato lines carrying no genetic resistance develop symptoms when inoculated with strain O virus. The resistance genes Tm-1, Tm-2 and Tm-2<sup>2</sup> can be overcome by tomato mosaic virus strains 1, 2, and 2<sup>2</sup>, respectively. Most cultivars used in commercial tomato production carry Tm-2 or Tm-2<sup>2</sup> resistance. Although the Tm-2<sup>2</sup> resistance has proven durable to date, the appearance of more aggressive strains of 2<sup>2</sup> that reduce the usefulness of the Tm-2<sup>2</sup> locus is possible.

Recently it was shown that the expression of the coat protein (CP) of TMV<sup>6</sup> and of alfalfa mosaic virus (AIMV)<sup>7–9</sup> in transgenic plants results in protection of those plants against virus infection by TMV or AIMV, respectively. This “engineered” protection mimics cross-protection in that it is less effective at high levels of inoculum, largely overcome by inoculation with viral RNA, and exhibits some degree of strain specificity. An important manifestation of genetic cross-protection is that the inoculated leaves show fewer chlorotic or necrotic lesions compared with control plants<sup>7–10</sup>. There is also reduced rate of systemic spread of the virus if infection occurs<sup>10</sup>. Evidence to be published shortly indicates that CP derived resistance has been achieved for potato virus X and cucumber mosaic virus<sup>11–12</sup>.

In this paper we report that expression of the TMV coat protein gene in transgenic tomato plants results in the absence or decrease in disease symptoms in these plants after inoculation with TMV or ToMV, including those ToMV strains that overcome the natural resistance genes in tomato. We also report that protection is effective in

greenhouse and field experiments and that CP expression does not affect the agronomic characteristics of the CP-expressing tomato lines in the absence of virus inoculation.

## RESULTS

**Presence and expression of the coat protein gene in tomato.** Two plants (306 and 329) were regenerated from independent transformation events (see Experimental Protocol), self-fertilized, and the progeny analyzed for the presence of the TMV coat protein (CP) gene by Southern blot analysis. Line 306 plants (R1 generation) and a progeny line obtained by self-fertilization of line 306 (line 306-98, R3 generation), have one gene insert per haploid genome as determined by copy number reconstruction and border fragment analyses (data not shown). Line 329 plants (R1 generation) contained at least two inserts at different sites as determined by border fragment analysis (data not shown).

Plant lines 306 and 306-98 (R1 and R3 generation, respectively) were analyzed for CP mRNA accumulation by northern blot analysis (Fig. 1). The hybridization signal from the 0.9–1.0 kb RNA homologous to the CP gene in line 306-98 was approximately twice that observed for the RNA from line 306. Whereas transgenic tobacco lines that harbor the pTM319 plasmid accumulate a 2.1 kb RNA as well as the 0.9 kb RNA (Figure 1, lane 4), the larger RNA molecule was not observed in the tomato plants analyzed (Fig. 1).

Coat protein concentration in the youngest fully-expanded leaves from plant lines 306 and 329 (R1 generation) were approximately 0.05% of the total leaf protein extracted from plants grown under greenhouse conditions (data not shown). Although CP concentrations were similar, segregation ratios were dissimilar between lines 306 and 329, in which the CP segregated at 3:1 (expression:nonexpression) and 15:1 or greater ratios, respectively (line 306: 229 plants analyzed, 169 plants expressing; line 329: 29 plants analyzed, 28 plants expressing). These segregation ratios indicate one and two or more active loci in lines 306 and 329, respectively, and support the gene copy number analyses described above. Subsequent generations of 306-98 (R3 and R4 generations) were confirmed to be homozygous by CP analyses (data not shown).

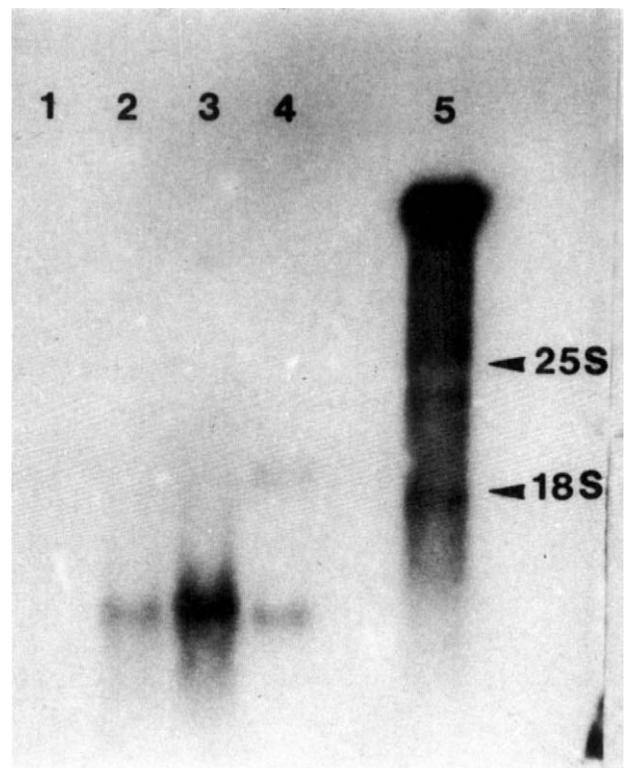
**Virus inoculation and symptom development in CP-expressing and control plants.** Seedling progeny of line 306 that express the CP-gene [CP(+)] and that did not express the CP-gene [CP(-)], of transgenic plants harboring genes other than the CP gene, and of the nontransformed parental line (VF36) were inoculated with increasing concentrations of U<sub>1</sub>-TMV (Table 1). Although increasing the virus concentration caused more rapid appearance of systemic disease symptoms in the control plants, the CP(+) plants showed no such increase. Protection was also observed against a highly virulent strain of TMV (PV230, ATCC designation) and to a lesser extent, against a strain of tomato mosaic virus (strain L, Table 1).

Experiments were also conducted to compare disease development in a CP(+) transgenic line (306), nontransgenic line (cv. VF 36) and near isogenic lines (cv. Craigella) that carried determinants for genetic resistance (Tm-1, Tm-2 or Tm-2<sup>2</sup>). Strain 2 not only caused rapid symptom development in VF36 plants that carry no resistance genes against ToMV, but also in plants carrying the Tm-2 resistance gene. CP(+) plants inoculated with ToMV 2 exhibited either a delay in symptom development or escaped disease. Similar results were obtained after ToMV 2<sup>2</sup> inoculation except that plants carrying the Tm-2<sup>2</sup> resistance were overcome and plants carrying Tm-

2 resistance were resistant. These data indicate that CP(+) plants protected fully or partially against all the TMV and ToMV strains tested. The other resistance genes (Tm-1, Tm-2, and Tm-2<sup>2</sup>) may confer more complete resistance against most strains, but are highly susceptible to specifically adapted strains.

In addition to visual observations, the accumulation of TMV (PV230) in inoculated and systemically infected leaves was determined by protein immunodot blot analysis 14 days post inoculation (data not shown). Both inoculated and systemic leaves of all VF36 plants contained high levels of TMV. Two of the five CP(+) plants escaped virus infection, while three of the five became infected and accumulated low levels of virus in the inoculated leaves. Only one CP(+) plants became systemically infected. None of the resistant lines (Tm-1, Tm-2, and Tm-2<sup>2</sup>) accumulated any virus and PV230 can be considered a type O strain. Thus, the correlation between visual observations (Table 2) and virus accumulation in the upper leaves was complete.

**Plant growth of CP-expressing and control plants under greenhouse conditions.** CP(+) plants from line 306 (R1 generation) and homozygous line 306-98 (R2 generation) were analyzed for leaf and stem dry weight accumulation through 88 days after planting in the greenhouse (Fig. 2). Leaf and stem dry weight accumulations in line 306 and 306-98 plants were, at minimum, equal to those in VF36 plants. It appears, therefore, that expression of



**FIGURE 1** Detection of TMV coat protein transcripts in transgenic tomato plants (line 306 and progeny). Total RNA was isolated from pooled leaf disks of 30 to 50 seedlings from VF36, 306 (R1 generation), and 306-98 (R3 generation). 40 µg RNA per plant type was fractionated on an agarose gel containing formaldehyde. After transfer to nitrocellulose the RNA was probed with a nick-translated TMV CP fragment. Lanes 1, 2, 3 and 4 contain RNA from VF36, 306, 306-98, and a tobacco plant (lines 3404, 20 µg total RNA) expressing the TMV CP, respectively. Lane 5 contains full length U<sub>1</sub>-TMV RNA (6.4 kb). The position to which the 25S and 18S (2 kb) plant ribosomal RNAs migrated is noted.

the CP gene in the transgenic plants (these plants did not express nopaline) does not alter the growth and development of these plants. By 88 days after planting (DAP) we observed that the VF36 plants had inadvertently become infected with TMV and thus terminated the formal experiment.

**CP expression and virus resistance under field conditions.** After receiving USDA-APHIS approval, the CP-expressing and nonexpressing plants (cv. VF36) were grown in the field to determine their agronomic characteristics. CP(+) plants from lines 306-98 (R4 generation) and 329-3 (R2 generation) were analyzed for CP expression under field conditions prior to inoculation with virus (Fig. 3). Although field expression levels were slightly lower than that observed under growth chamber or greenhouse conditions (0.02% vs. 0.05% of the total extracted leaf protein, respectively) we do not consider the difference significant.

Plants of each line were inoculated with different concentrations of the U<sub>1</sub> strain of TMV at 39 and 54 DAP. In

each case CP(+) plants expressed a delay or, in the majority, an absence of systemic disease symptoms compared with VF36 plants (Fig. 4a and b). By the beginning of fruit harvest for both inoculation dates only 3 of 96 of the 306-98 plants and 0 of 96 of the 329-3 plants bore disease symptoms compared with 95 of 96 VF36 plants. The visual observations were substantiated by quantitating virus accumulation in systemically infected leaves of these plants (Table 3). CP accumulated in all plants showing visual symptoms. CP(+) plants not showing symptoms did not accumulate TMV with the exception of two 306-98 plants that had weak visual symptoms and a small but measurable accumulation of virus. These experiments clearly demonstrate a high level of resistance to TMV strain U<sub>1</sub> under field conditions.

**Fruit yield under field conditions.** Mature fruit appeared on uninoculated VF36 and CP(+) plants at similar times in these field trials. Tomato yields from virus-inoculated CP-expressing plants were unchanged compared with the uninoculated CP-expressing plants, while

**TABLE 1** Protection against systemic symptom development in coat protein (CP) expressing transgenic plants in the presence of increasing virus inoculum concentrations. CP-expressing (line 306, R1 generation) and various control plants not expressing CP were inoculated with the U<sub>1</sub> or PV230 (ATCC designation) strains of TMV or with the L strain of ToMV and observed for symptom development in growth chambers (U<sub>1</sub> inoculations) or in the greenhouse (PV230 and L inoculations).

Plant Type <sup>a</sup>	Virus	Virus Inoculum Concentration (µg/ml)	Days Post Inoculation					
			5	6	7	8	9	30
<b>Percent Plants Showing Systemic Symptoms</b>								
+CP(7) <sup>b</sup>	U <sub>1</sub>	0.5	0	0	0	0	0	0
-CP(6)			17	17	17	17	50	83
-CP,ssu(9)			11	22	56	67	67	100
+CP(9)	U <sub>1</sub>	2.0	0	0	0	0	0	22
-CP(6)			0	17	67	83	100	100
-CP,ssu(7)			0	29	43	86	100	100
+CP(10)	U <sub>1</sub>	5.0	0	0	0	0	0	0
-CP(4)			0	50	75	75	100	100
-CP,ssu(8)			0	0	88	100	100	100
+CP(11)	U <sub>1</sub>	20.0	0	0	0	0	0	9
-CP(4)			25	50	75	75	75	100
-CP,ssu(8)			25	38	88	100	100	100
VF36(4)			50	75	75	100	100	100
<b>Days Post Inoculation</b>								
			7	8	9	10	13	29
+CP(10)	PV230	2.0	0	0	0	0	0	0
-CP(6)			17	67	83	100	100	100
-CP,ssu(5)			40	60	60	60	60	80
+CP(13)	PV230	20.0	0	0	0	8	23	54
-CP(3)			0	33	100	100	100	100
-CP,ssu(6)			17	50	100	100	100	100
<b>Days Post Inoculation</b>								
			8	9	10	12	20	29
+CP(12)	L	2.0	0	0	0	0	33	58
-CP(4)			0	0	0	50	100	100
-CP,ssu(6)			17	17	67	83	83	100
+CP(13)	L	20.0	0	0	0	0	46	62
-CP(3)			33	66	66	100	100	100
-CP,ssu(6)			33	50	100	100	100	100

<sup>a</sup>+CP, Segregating progeny expressing CP

-CP, Segregating progeny not expressing CP

-CP,ssu, Progeny of plants harboring the neomycin phosphotransferase gene driven by the promoter from the small subunit (ssu) of ribulose biphosphate carboxylase.

VF36, Nontransformed parental line.

<sup>b</sup>Numbers in parentheses equal the sample size for the particular treatment.

yields from virus-inoculated VF36 plants were substantially lower than yields of the uninoculated VF36 plants (Table 4). The major effect of virus infection on the VF36 plants was on number of fruits/plant rather than on weight/fruit. Tomato yields from 306-98 plants, regardless of virus inoculation, were equal to the yield of uninoculated VF36 plants. Thus the expression of the CP gene has not affected fruit production in this genetic background under these conditions. Yields of 329-3 were depressed presumably due to as yet undefined effects of plant transformation and/or regeneration.

## DISCUSSION

Two transgenic tomato plant lines (306 and 329) that express a chimeric gene encoding the coat protein (CP) of the common strain of TMV were produced. Line 306 contained and expressed a single gene, while 329 expressed two or more genes. Expression levels for CP were determined to be approximately 0.05% or less of the extractable leaf protein under our experimental conditions (data not shown; Fig. 3). Similar levels were determined for leaves of transgenic tobacco plants expressing the CP gene<sup>6,10</sup>. Expression of the TMV-CP gene was stable through all tomato generations produced to date (R2 generation for 329, R4 generation for 306). Müller et al.<sup>13</sup> have shown that homozygous transgenic lines of *Nicotiana tabacum*, produced using a binary vector, allowed for 0.06% or fewer revertants to occur after backcross to the parental line. They concluded that meiotic instability in their plants was not greater than the spontaneous mutation rate of other plant genes and therefore acceptable for commercialization. Other workers<sup>14</sup> have noted some genetic instability after backcrossing transgenic *L. esculentum* × *L. pennellii* plants with *L. esculentum*. The instability, however, may be due to the interspecies cross or more likely to the complicated arrangement of the T-DNA itself in the particular transformant.

In addition to the high degree of protection against TMV (U<sub>1</sub> and PV230), protection was observed against three strains of ToMV (L, 2, and 2<sup>2</sup>) in the CP(+) tomato plants (Tables 1 and 2). Although the protection provided by the CP of the U<sub>1</sub> strain of TMV against ToMV strains

was somewhat less than that provided against the TMV strains, it is novel in that previous reports using classical cross-protection suggested that TMV could not protect against ToMV<sup>15,16</sup>. Our data demonstrate that it may be useful to introduce CP-expression into other resistant lines to add breadth to existing resistance genes. In addition, it will be interesting to determine if expression of a ToMV-CP will increase the protection against these strains in transgenic plants.

Analysis of virus accumulation within CP(+) plants after inoculation with TMV (PV230) showed that these plants accumulate less virus in the inoculated and systemic leaves compared with VF36 plants, and that many plants escaped infection (data not shown). Thus, there is blockage in infection and/or replication of virus in the inoculated leaves. Previous reports with both TMV and AIMV CP-expressing tobacco plants<sup>7-10</sup> and AIMV expressing tomato plants<sup>7</sup> indicate a similar blockage in the inoculated leaves. Thus, there may be a commonality in the protection mechanism across several plant species expressing unrelated virus CP genes.

Expressing the TMV CP gene did not affect vegetative traits since the presence and expression of this gene did not alter seed germination percentages (data not shown), the rate of stem elongation (data not shown), leaf or stem dry matter accumulation, or dry matter partitioning between leaf and stem (Fig. 4), in line 306 and its progeny. In addition, reproductive traits such as the number of flowers/plant (data not shown), fruit maturation date (data not shown), and fruit yield (Table 4) were also unaffected. Future experiments will address processing and fruit quality questions as well as the expression level of the CP gene in transgenic fruit. Expression of the CP gene in the tomato fruit of the transgenic plant is not novel since it has been known for many years that tomato plants infected with TMV contains CP-enshrined virus in their fruit<sup>15,17</sup>.

In terms of pathogen protection, both of the CP-expressing lines, 306 and 329, displayed nearly complete protection against TMV infection in the field (Fig. 4, Table 3), and the yields of tomato fruits from these plants were equal to those of uninoculated CP-expressing plants.

**TABLE 2** Comparison of symptom development in CP-expressing, non-expressing, and genetic resistant lines after virus inoculation under greenhouse conditions. VF36, nontransformed parental cultivar; 306, CP-expressing line (R1 generation); Tm-1, Tm-2, and Tm-2<sup>2</sup>, genetic resistant lines of the cv. *Craigella*. Seedlings were inoculated with ToMV strains (2 or 2<sup>2</sup>) or a TMV strain (ATCC designation PV230). Numbers in parentheses indicate the sample size. PV230 inoculum concentration was 2 µg/ml while those for ToMV-2 and 2<sup>2</sup> were approximately 3 times the infectivity of PV230 as determined by comparison of lesion numbers on local lesion host plants (*Nicotiana tabacum* cv. Xanthi 'nc').

Plant Line	Virus Strain	Days Post Inoculation						
		4	5	6	7	8	11	14
		Percent Plants Showing Systemic Symptoms						
VF36 (5)	ToMV-2	80	100	100	100	100	100	100
306 (5)		0	0	0	20	20	60	60
Tm-1 (5)		0	0	0	0	0	0	0
Tm-2 (5)		40	60	100	100	100	100	100
Tm-2 <sup>2</sup> (5)		0	0	0	0	0	0	0
VF36 (5)	ToMV-2 <sup>2</sup>	0	0	20	100	100	100	100
306 (5)		0	0	0	0	0	20	20
Tm-1 (5)		0	0	0	0	0	0	0
Tm-2 (5)		0	0	0	0	0	0	0
Tm-2 <sup>2</sup> (5)		0	40	40	40	40	60	60
VF36 (5)	TMV-PV230	60	80	100	100	100	100	100
306 (5)		0	0	0	0	0	0	20
Tm-1 (5)		0	0	0	0	0	0	0
Tm-2 (5)		0	0	0	0	0	0	0
Tm-2 <sup>2</sup> (5)		0	0	0	0	0	0	0

In contrast, fruit yields of VF36 plants infected with TMV were 26–35% lower than the uninoculated VF36 plants (Table 4). It is apparent that, as has been found in natural cross-protection experiments<sup>16,18</sup>, the genetically engineered cross-protection has the potential for maintaining yields even under the severe infection pressures in these experiments. The protection effect would be considerably greater if the challenge virus would have been a more severe strain such as PV230 which causes marked chlorosis and stunting in tomatoes.

The results of these growth chamber, greenhouse and field experiments indicate that expression of the CP gene of U<sub>1</sub>-TMV provides a high level of protection against several different strains of TMV and ToMV. As depicted in Figure 4 and Table 3 not only do most plants escape disease symptom development, but less virus accumulates in the transgenic CP(+) plants than in the non-expressing

CP(-) controls. Recently evidence has been presented<sup>19,20</sup> that expression of viral satellite RNAs in transgenic plants suppresses replication and/or symptom expression caused by infection of the helper virus. While this approach to plant protection may be a second method for protecting plants against some virus diseases, it suffers from two problems: (1) satellite RNAs have been identified in a very limited number of virus situations; and (2) in some situations disease symptoms resulting from infection by helper viruses alone can be dramatically aggravated by the presence of satellite RNA as in the case of tomato lethal necrosis<sup>21</sup>. The breadth of application of the CP derived protection and its relative safety, as well as efficacy, therefore appear to have advantages over that provided by satellite RNAs.

**EXPERIMENTAL PROTOCOL**

**Sources of plants and virus strains.** *Lycopersicon esculentum* cv. VF36 seed was obtained from the Tomato Genetics Stock Center, UC, Davis. Near isogenic lines (cv. *Craigella*) carrying the genetic resistances Tm-1, Tm-2, or Tm-2<sup>2</sup> were obtained from the Glasshouse Crops Research Institute, Sussex, England. Tobacco mosaic virus strains U<sub>1</sub> and PV230 were obtained from Milton

**TABLE 3** Accumulation of U<sub>1</sub>-TMV after inoculation of CP-expressing or nontransformed plants under field conditions. Inoculations were carried out as described under Materials and Methods on CP-expressing plants from lines 306-98 (R4 generation) and 329-3 (R2-generation) and nontransformed plants from the parental cv. VF36. Leaves from plants inoculated at 44 days after planting (DAP, early inoculation, 10 µg/ml U<sub>1</sub> virus) were harvested at 49 days post inoculation (DPI) while leaves from plants inoculated at 59 DAP (late inoculation, 40 µg/ml U<sub>1</sub> virus) were harvested at 39 DPI. One subterminal leaflet from each of 3 branches per plant were harvested and each sample was analyzed for U<sub>1</sub>-TMV CP by ELISA. Sampled leaves were greater than 5 cm in length with the next younger leaf (if present) less than 5 cm in length. For VF36 plants each value represents the mean ± S.D. for 4 replicates with each replicate containing values from 4 plants. For 306-98 and 329-3 plants, values are given in ranges with the number of plants within the range in parentheses following the value. Values are in ng CP per 500 ng protein. The limit of detection was 0.25 ng per 500 ng of protein in the assay.

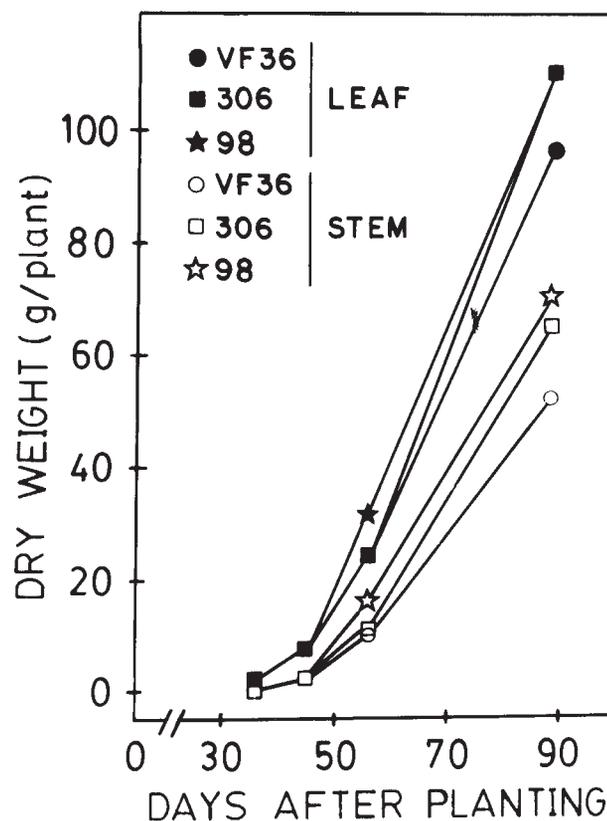
Plant Line	Time of Virus Inoculation	
	Early	Late
VF36	46±9	47±16
306-98	6(1)	15(1)
	0.25-1(3)	0.25-1(3)
	<0.25(12)	<0.25(12)
329-3	0.25-1(7)	0.25-1(3)
	<0.25(9)	<0.25(13)

**TABLE 4** Fruit weight (Mg/ha) of CP-expressing or non-expressing field grown plants under various virus inoculum regimes. Inoculations were carried out on plants as described in Table 3 and Figure 4. VF36, nontransformed parental cultivar; 306-98, R4 generation of a CP-expressing line; 329-3, R2 generation of a CP-expressing line. Harvest was begun at 98 DAP and continued through 128 DAP. Fruit was harvested at the physiological stage of color break and fruit number and fresh weight determined. Twelve plants per replicate were harvested. There were four replicates per plant line-virus treatment, except for the uninoculated VF36 fruit yield which was composed of three replicates. Each value represents the mean ± S.E. for the replicates. The experimental design was a split plot and statistical analysis was by analysis of variance.

Plant Line	Time of Virus Inoculation		
	Uninoculated	Early	Late
VF36	84.0±6.4	62.0±3.4*	54.6±2.3*
306-98	88.3±2.0	85.8±6.2	77.6±5.5
329-3	29.9±2.2 <sup>+</sup>	33.3±2.1	34.1±2.9

\*Significantly different from uninoculated treatment (p<0.05).

<sup>+</sup>Significantly different from VF36 uninoculated treatment (p<0.05).



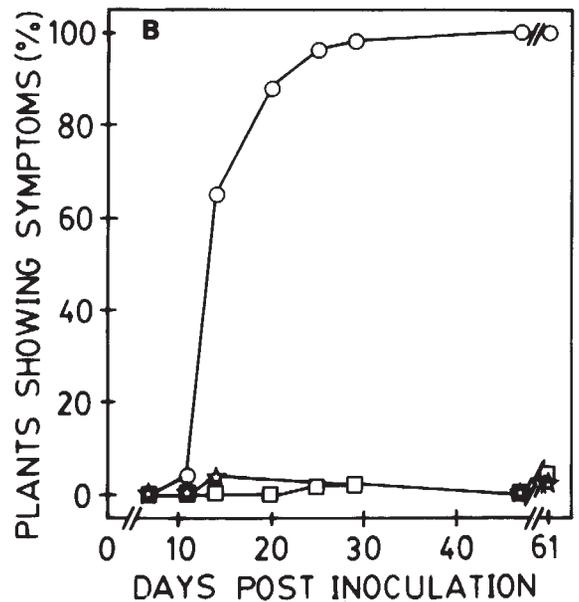
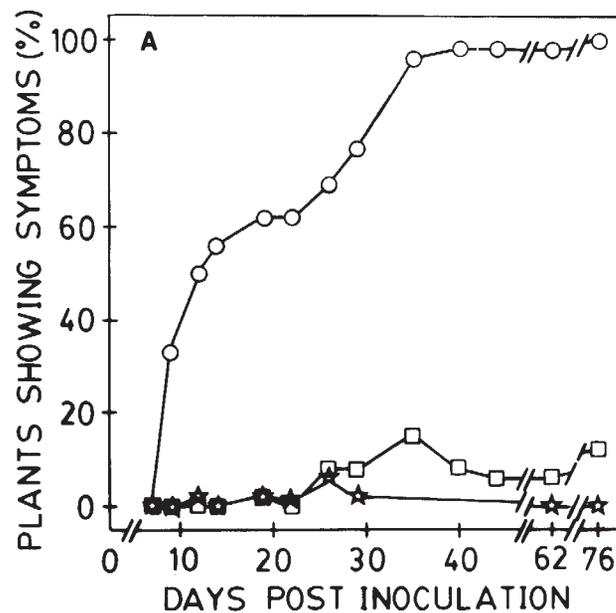
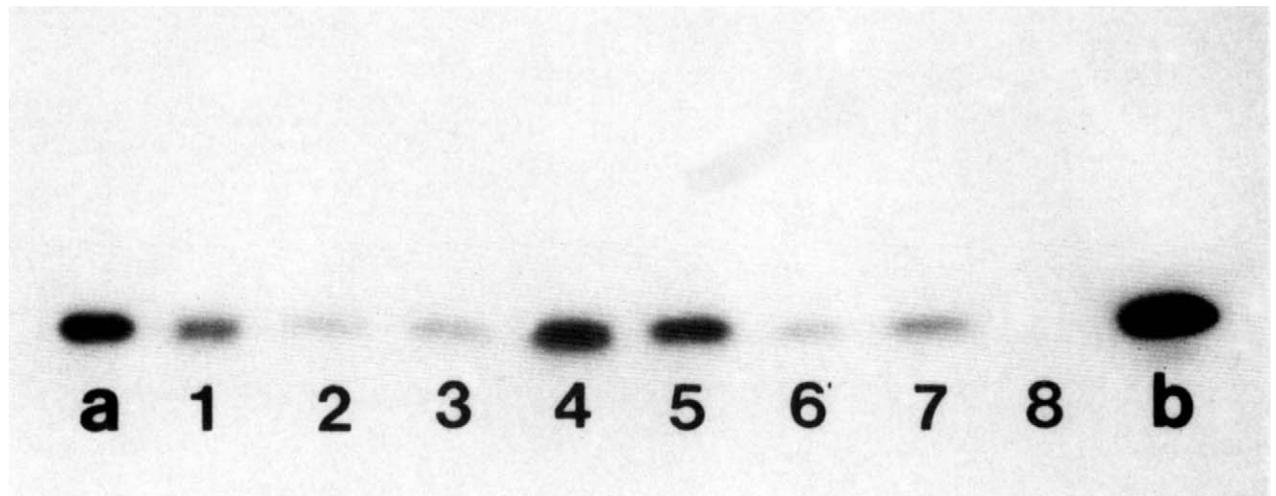
**FIGURE 2** Analysis of leaf and stem dry weight accumulation in transgenic and nontransformed plants under greenhouse conditions. The experimental design was a randomized complete block with three replicates for nontransformed cv. VF36 and CP-expressing plant line 306 (R1 generation) and two replicates for the CP-expressing homozygous line 306-98 (98, R2 generation). Each replicate contained 3–4 plants. No statistically significant decrease in dry matter accumulation was observed for plants expressing the CP gene compared with VF36. Statistical analysis was by F test followed by an lsd determination at the 0.05 level with a correction made for the missing replicate for line 306-98. The only statistically significant observation was a decrease in stem weight of VF36 compared with the CP-expressing plants at 88 days after planting.

Zaitlin (Cornell University, Ithaca, NY) and the American Type Culture Collection, respectively. Tomato mosaic virus strains 2 and 2<sup>2</sup> were obtained from A. Th. B. Rast (Institute of Phytopathological Research, Wageningen, the Netherlands). Tomato mosaic virus strain L was obtained from M. Zaitlin.

**Plant transformation.** VF36 tomato leaf pieces or cotyledons were transformed with *A. tumefaciens* harboring pTM319<sup>6</sup> as described by McCormick et al.<sup>22</sup> and transgenic plants were regenerated. Progeny (R1 generation) of the self-fertilized primary transformant, plant number 306, were scored for kanamycin resistance by the leaf callus assay<sup>22</sup>, and for the presence of TMV coat protein (CP) by immunoblot analysis<sup>6</sup>. Although the nopaline synthase gene was carried on pTM319, the parent and

the progeny of plant 306 were silent for nopaline expression. There was a 100% correlation in the progeny between kanamycin resistance and CP expression (40 plants analyzed). Homozygotes in line 306 were identified by self-fertilizing R1 generation plants and analyzing progeny for kanamycin resistance. Progeny of a second self-fertilized primary transformant, plant number 329, were scored for nopaline synthase activity<sup>23</sup> and TMV coat protein. Nopaline and CP expression segregated together in the 19 R1 progeny of plant 329.

**Isolation of plant RNA and northern blot analysis.** Total RNA was isolated from pooled leaf disks from 30 to 50 seedlings from VF36, 306, or 306-98 as described by Powell Abel et al.<sup>6</sup> The RNA was fractionated by electrophoresis to nitrocellulose<sup>6</sup>. The



**FIGURE 3** Accumulation of coat protein (CP) expression in transgenic plants under field conditions. Leaf samples from single plants were taken eight days after transplanting from the greenhouse to the field. 40 µg of protein extracted from one expanded leaflet of either VF36, 306-98 (R4 generation), or 329-3 (R2 generation) plants were analyzed by SDS-PAGE and immunoblot. Lanes 1, 4, and 5 contain samples from 306-98 plants; lanes 2, 3, 6, and 7 contain samples from 329-3 plants; lane 8 contains a sample from a greenhouse grown VF36 plant; and lane a and b contain, respectively, 7.5 ng and 30 ng of U<sub>1</sub> virus standard.

**FIGURE 4** Systemic symptom development in CP-expressing and nontransformed plants under field conditions after virus

inoculation. Panel A shows disease development in nontransformed plants of cv. VF36 (○), and CP-expressing plants of line 306-98 (R4 generation, □) and 329-3 (R2 generation, ☆) inoculated with 10 µg/ml of strain U<sub>1</sub> TMV on terminal leaflets of three successive leaves. The youngest inoculated leaf was greater than 3 cm in length. Plants were inoculated at 44 days after planting (eight days after transplanting to the field) and observations were made on 48 plants per plant line. Panel B shows results from plants inoculated with 40 µg/ml of strain U<sub>1</sub> TMV on terminal leaflets of two successive leaves. The youngest inoculated leaf was greater than 5 cm in length. Plants were inoculated at 59 days after planting (23 days after transplanting) and observations were made on 48 plants per plant line.

blot was probed with a <sup>32</sup>P-labeled DNA fragment containing only the CP sequence.

**Isolation of plant protein and immunological analyses.** Protein was extracted from leaves of plants from lines 306, 329, and 306-98 and analyzed by immunoblot reactions for the presence or absence of CP (+CP and -CP, respectively) in the transgenic plants as described by Powell Abel et al.<sup>6</sup> In experiments in which virus accumulation after inoculation was determined, analyses were by dot blot immunoassay<sup>10</sup> or enzyme linked immunosorbent assay (double sandwich ELISA)<sup>24</sup>.

**Growth, inoculation, observation and sampling of plants.** Seeds were germinated in a greenhouse under natural light conditions, supplemented in winter months with light (~75 μE.m<sup>-2</sup>.sec<sup>-1</sup>) to produce 14 h days. Seedlings were transplanted into 4 in. pots approximately 7 days after planting. Approximately 7 days following transplanting, leaf tissue was taken from the 1st and/or 2nd leaf above the cotyledons, frozen in liquid N<sub>2</sub>, and later analyzed for CP expression. In addition, fresh leaf tissue was analyzed for kanamycin resistance (lines 306 and 306-98) or the presence of nopaline (line 329). In growth chamber and greenhouse experiments involving virus inoculations, approximately 21 days after planting, two unsampled terminal leaflets were dusted with carborundum (330 grit, Fisher Scientific) and inoculated with purified virus diluted to designated concentrations with inoculation buffer (20 mM sodium phosphate, pH 7.2, 1 mM EDTA). After inoculation, the leaflets were rinsed with water and the plants placed either in growth chambers under previously reported conditions<sup>10</sup> or left in the greenhouse. Individual plants were scored daily for systemic disease symptom development (chlorotic areas on the leaves above the inoculated leaves leading to mosaic symptoms in later developing leaves). To determine virus accumulation, leaf tissue was sampled from both inoculated and systemic leaves, frozen in liquid N<sub>2</sub>, and then ground in extraction buffer (see previous section) in ratios of 1:2 or 2:1 (ml buffer:g fresh wt. of tissue), respectively. In the greenhouse experiment to determine leaf and stem dry weight accumulation, plants were transplanted at 8 days after planting (DAP) into 4 in. pots, and then at 36 DAP into 3 gallon pots and allowed to grow under greenhouse conditions. Leaf tissue was collected at various dates, and consisted of leaf lamina, midrib, and petioles, while stem tissue consisted of main plus side stems from the cotyledon node upward. After sampling, tissue was dried in paper bags at 80°C for 48 h in forced air ovens and then weighed. The experimental design was a randomized complete block design with three replicates per plant genotype and one missing replicate for line 306-98. Field experiments were initiated by germinating seed (VF36; 306-98, R4 generation; 329-3, R2 generation) in transplanting trays. The seedlings were grown in a greenhouse followed by transplanting to the field at 36 DAP. The field test was located in Jersey County, Illinois, on a Muscatine silt loam soil. Permission to conduct this first field test of transgenic tomatoes was granted by the Biological Assessment Support Staff, Plant Protection and Quarantine within the Animal and Plant Health Inspection Service, United States Department of Agriculture on June 1, 1987 (application #000038)<sup>25</sup>. The experimental design was a randomized split plot design with virus treatments assigned to the whole plots and genotypes assigned to the subplots. There were four replications. Each subplot consisted of a single row ~3.6 m in length containing 12 plants 31 cm apart. Distance between rows was 1.5 m within the main plots. The main plots were separated by a 6 m border. Plants were not staked and irrigation was provided as needed. The virus treatments included an early inoculation of plants at 44 DAP with 10 μg/ml of the U<sub>1</sub> strain of TMV on three leaves per plant, a late inoculation of plants at 59 DAP with 40 μg/ml of U<sub>1</sub>-TMV on two leaves per plant, and uninoculated plants. Virus inoculations were executed by abrading leaves with a sponge soaked in a suspension of virus containing 1% carborundum. In addition to visual observations, virus accumulation in plants was quantitated immunologically. Fruit harvest began at 98 DAP and continued through 128 DAP. Fruit was harvested on a continuous basis after reaching the color break stage. Fruit fresh weight, number of fruits, and date of maturation were determined from this data.

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