Field Resistance of Transgenic Tomatoes Expressing the Tobacco Mosaic Virus or Tomato Mosaic Virus Coat Protein Genes

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We would like to thank Dr. Jimmy Augustine and his team for their help in conducting the field test in Florida in 1988, Dr. Wayne Fowler of Asgrow Seed Company and his team for their help in conducting the field test in Florida in 1990, Dr. Mike Deom at Washington University for his help in raising ToMV C antibodies, and Cliff Lawson and Dr. Roger Beachy for helpful comments.
Accepted for publication 21 January 1992.

ABSTRACT

Sanders, P. R., Sammons, B., Kaniewski, W., Haley, L., Layton, J., LaVallee, B. J., Delannay, X., and Tumer, N. E. 1992. Field resistance of transgenic tomatoes expressing the tobacco mosaic virus or tomato mosaic virus coat protein genes. Phytopathology 82:683-690.

Under field conditions, transgenic tomato plants that express the coat protein (CP) gene of common (U1) strain of tobacco mosaic virus (TMV) showed a high degree of resistance to the U1 strain and to a more severe strain of TMV, PV230. Tomato fruit yields of inoculated control plants decreased 20% with U1 and 69% with PV230, whereas the CP+ line did not show any yield reduction after inoculation with U1 or PV230. In contrast, tomato plants expressing TMV CP showed either a low level of resistance or no resistance to several different tomato mosaic virus (ToMV) strains in the field. A ToMV CP gene was cloned from an isolate obtained from commercially grown tomatoes in Florida. Nucleotide sequence analysis showed that it was 88% homologous to TMV U1 strain at the amino acid level. Transgenic tomato plants expressing the ToMV CP showed a high level of resistance to ToMV in the field. These results demonstrate that although TMV and ToMV CP sequences are highly homologous, ToMV CP gene is more effective in control of ToMV in field-grown tomatoes than is the TMV CP gene.

Tobacco mosaic virus (TMV) and tomato mosaic virus (ToMV) are members of the tobamovirus group and can be distinguished by differences in their serological affinities and protein compositions. Although TMV can infect tomato, ToMV is the predominant virus worldwide in tomato crops (5). Infection by ToMV has been demonstrated to reduce yield in fresh market tomatoes by 10–50% (1,3,4,8,18). Yields are reduced because there are fewer fruits per plant as well as smaller fruits. The extent of the yield loss depends on the time of infection. Because ToMV is highly infectious, agronomic approaches for control, such as seed treatments, sanitation, and cross-protection with attenuated strains, have not been totally effective. The use of three ToMV resistance genes, Tm-1, Tm-2, and Tm-2^2, in commercial cultivars has been the most effective control measure against losses in fruit yield and quality caused by ToMV (5).

The introduction of ToMV-resistant cultivars resulted in the rapid emergence of strains that were able to overcome resistance (5). ToMV strains are classified according to their ability to overcome the different resistance genes. Strain 0 is unable to overcome any of the resistance genes, strain 1 can overcome the resistance gene Tm-1, strain 2 can overcome the resistance gene Tm-2, and strain 2^2 can overcome the resistance gene Tm-2^2. Many cultivars used in commercial production carry Tm-2 or Tm-2^2 resistance, although some do not contain these genes (13). The Tm-2^2 gene in homozygous cultivars is highly effective in controlling ToMV. However, in some cultivars it is difficult to separate this gene from undesirable linked traits.

In classical cross-protection experiments, the mild ToMV strain M11-16 protected against infection by the challenge virus, ToMV (1,6). TMV and ToMV do not cross-protect, and they can coexist in tomato (4).

Genetic engineering is an alternative tool that will allow breeders to make specific improvements in crops (such as viral disease resistance) while preserving the desirable characteristics of a tomato inbred parent. Expression of viral coat protein (CP) genes in transgenic plants provides resistance to the homologous virus or different strains of the same virus. Plants expressing CP genes show fewer lesions on inoculated leaves, a delay or absence of systemic disease symptoms, or reduced levels of virus in inoculated and systemic leaves (2).

Transgenic tomato plants expressing a gene encoding the TMV CP from common (U1) strain have been evaluated in the field. After inoculation with U1, no more than 5% of the CP-expressing (CP+) plants exhibited visual systemic disease symptoms compared with 99% of nontransformed control plants (22). In growth chamber studies, it has been shown that tobacco plants expressing the CP of U1 strain show fewer local lesions after inoculation by a severe strain of TMV, PV230, and ToMV L strain (20,21).

In this paper, we examine the level of resistance against different TMV and ToMV strains and field isolates in field-grown tomatoes expressing the TMV or ToMV CP gene. We demonstrate that ToMV CP expression is necessary for effective control of ToMV in the field. This is the first demonstration of CP-mediated resistance against ToMV in the field. We discuss these results in light of the possible mechanisms of CP-mediated resistance against ToMV.

MATERIALS AND METHODS

1988 Florida field test. The genotypes of tomato (*Lycopersicon esculentum* Mill. 'VF36') used in this trial were nontransgenic VF36 and the R5 homozygous progeny of transgenic line CRL306 expressing the TMV CP (22). There were 160 plants of each genotype in the test. The experiment was set up as a split block design, with two treatments as the main plots, two genotypes as the subplots, and four replications. Each subplot consisted of single rows, with 20 plants spaced 45 cm apart within the row. The main plots were blocks of three adjacent rows (subplots), spaced 1.5 m apart. The two treatments were no inoculation and mechanical inoculation with ToMV. The treatments were arranged in two adjacent strips across the replications to avoid contamination of uninoculated plots by the farm equipment. For transplant production, seeds were germinated in Todd Planter flats in the greenhouse on Feb. 10, 1988, and seedlings were hand-transplanted to the field on March 25, 1988.

ToMV was purified from leaves infected with *Nicotiana clevelandii* A. Gray by homogenization in 0.1 M phosphate buffer, pH 7.0, containing 0.05 M ascorbic acid and 0.02 M EDTA, using a tissue:buffer ratio of 1:3 (w/v). The homogenate was emulsified with 1/2 volume of chloroform:butanol (1:1). After low speed centrifugation, the aqueous phase was centrifuged in a 45Ti rotor (Beckman Instruments, Fullerton, CA) for 2 h at 63,000 *g*. The pellet was resuspended in 0.01 M phosphate, pH 7.0, with 0.001 M EDTA. Low and high speed centrifugation steps were repeated. Final purification was by sucrose density gradient centrifugation (10–40%) in a SW28 rotor (Beckman Instruments, Fullerton, CA) for 2 h at 70,000 *g*. The virus band was collected on ISCO model 640 gradient fractionator.

Two weeks following field transplanting, half of the field (80 plants of each genotype) was inoculated with a locally isolated ToMV strain, designated Naples C (ToMV C) and provided by Dr. J. Augustine (BHN Research, Naples, FL). Each plant was hand-inoculated on the youngest two terminal leaflets (at least 3 cm long) with 40 μl per leaflet of a 10 μg/ml preparation of purified Naples C in 10 mM potassium phosphate buffer, pH 7.0, 1 mM EDTA.

Plants were scored visually each week for virus symptom development. All plants were sampled at 4 wk postinoculation. The inoculated plots were sampled again at 8 wk postinoculation. The first sampling was done 10 days after the onset of visual symptoms. Three young terminal leaflets were sampled from separate branches of each plant. Three discs, 8 mm in diameter, were taken from all the plants, uninoculated and inoculated. The samples were homogenized in 750 μl of phosphate-buffered saline 0.05% Tween-20 and 0.2% ovalbumin (PBSTO). Enzyme-linked immunosorbent assay (ELISA) plates were coated with 1:1000 dilution of 1 mg/ml anti-ToMV IgG (Agdia, Inc., Elkhart, IN). Ten μl of the plant extract was added to 240 μl buffer and loaded on ELISA plates. The ToMV standards included on each plate were 1000 ng, 400 ng, 100 ng, 40 ng, 10 ng, 4 ng, and 1 ng per well. The plates were incubated at 4 C overnight. The plates were rinsed, horseradish peroxidase (Agdia, Inc.) conjugated anti-ToMV IgG (1:3000) was added, and then the plates were incubated for 4 h at 37 C, rinsed four times, and developed with the appropriate substrate. A plant was considered infected if 1 mg of leaf tissue contained at least 25 ng of virus as determined by ELISA.

Fruits harvested from each plot were weighed and recorded as kilograms per plot. Significant differences were measured at the 0.05 probability level using Ryan-Einot-Gabriel-Welsch multiple F test (10).

1988 Illinois field test. The genotypes of tomato used in this test were the R5 homozygous progeny of TMV CP line CRL306 and the nontransgenic VF36 control plants (22). The experiment was set up as a split plot design, with seven treatments as the main plots, the two genotypes as the subplots, and four replications. Each subplot consisted of single rows, with 20 plants spaced 30 cm apart within the row. The main plots were blocks of two adjacent rows (subplots), spaced 1.5 m apart. The seven treatments were no inoculation with virus, mechanical inoculation with two strains of TMV, and mechanical inoculation with four strains or field isolates of ToMV. The main plots were separated by a distance of 6 m to reduce the chances of cross-contamination between treatments. Seeds were germinated in Todd Planter flats in the greenhouse on April 21, 1988, and were transplanted in the field on May 31, 1988, using a mechanical transplanter. All TMV and ToMV strains were purified as described previously.

All transgenic plants were sampled 1 wk after planting in the field to verify the level of TMV CP. The TMV CP expression was determined by a 2-step ELISA. The sensitivity of the ELISA is 1 ng CP/25 mg fresh weight of plant tissue. The microtiter wells were coated with anti-TMV IgG (1:1000 dilution of 1 mg/ml preparation). A disc 15 mm in diameter from each plant was homogenized with a teflon tissue grinder in 250 μl of PBSTO. The tomato plant extract (150 μl) and alkaline phosphatase conjugated anti-TMV IgG (100 μl at 1:1200) were added and the plate was incubated overnight at 4 C. TMV virion standards were loaded at 100 ng, 10 ng, 5 ng, and 1 ng per well. The plate was rinsed four times and substrate was added (Sigma 104 phosphatase substrate). Color development was monitored at 405 nm using a Bio-Rad 3550 microplate reader.

Two weeks after transplanting to the field, the plants were hand-inoculated on the youngest two terminal leaflets with 40 μl per leaflet of a 10 μg/ml preparation of each virus. Infectivity of each virus was high and similar at this concentration as determined by titration on a local lesion host, *N. tabacum* L. 'Samsun NN' (data not shown). Eighty transgenic and 80 nontransgenic control plants were inoculated with each virus. The TMV strains U1 and PV230 and ToMV strain L were provided by R. Beachy (The Scripps Research Institute, La Jolla, CA). The other ToMV field isolates were Epcot, isolated from greenhouse pepper, provided by K. Pategas (Orlando, FL), and Naples C field isolate and the Aucuba strain, provided by D. Emmatty (H. J. Heinz Research, Bowling Green, OH).

The tomato plants were scored weekly for systemic disease symptom development. All 1120 plants were sampled for ELISA at 4 and 8 wk postinoculation. ELISA plates were coated with 1:1000 dilution of a 1-mg/ml preparation of anti-TMV or anti-ToMV IgG (Agdia, Inc.). Three discs, 8 mm in diameter, were sampled from each plant and homogenized in 750 μl of PBSTO. Ten or 50 μl of the plant extract was added to 240 or 200 μl of buffer per well, respectively. The TMV or ToMV standards included on each plate were 1000 ng, 400 ng, 100 ng, 40 ng, 10 ng, 4 ng, and 1 ng per well. The plates were incubated at 4 C overnight. The plates were rinsed, alkaline phosphatase conjugated anti-TMV IgG (1:3000) or horseradish peroxidase (Agdia, Inc.) conjugated anti-ToMV IgG (1:3000) was added, and the plates were incubated for 4 h at 37 C, rinsed four times, and developed with the appropriate substrate. A plant was considered susceptible if 1 mg of leaf tissue contained at least 300 ng of ToMV or 35 ng TMV as determined by ELISA at 8 wk postinoculation.

Fruits were harvested at three different times. Red fruits were harvested on 2 August and 1 September and a final harvest including all remaining fruits (red and green) took place on 25 September. Fruits harvested from each plot were weighed and recorded as kg/plot. Data from all three harvests were combined for each plot for yield analysis. Significant differences were measured at the 0.05 probability level using the Ryan-Einot-Gabriel-Welsch multiple F test (10). Because of incidence of natural virus infection in the uninoculated nontransgenic VF36 plots, yields for all treatments in transgenic and nontransgenic plots were compared to the uninoculated CRL 306 plots for analysis and determination of yield reduction. Nelson et al (22) previously showed that the yield of uninoculated CRL 306 did not differ significantly from that of uninfected VF36. The percent yield reduction was calculated using the ratio of the yield for each treatment/genotype combination to the yield of the uninoculated CRL 306.

Cloning of a ToMV CP gene and expression in transgenic tomato. A Florida field isolate of ToMV, Naples C, was selected for isolation of the CP gene. The isolate was purified as described for TMV and verified as ToMV using *N. sylvestris* Speg. as a host and antibodies (Agdia, Inc.) specific for the detection of ToMV (14). RNA was isolated from virions by incubation with 0.15 M Tris, pH 9.0, 0.5% sodium dodecyl sulfate, 100 μg/ml proteinase K for 30 min at 37 C, followed by extraction twice with phenol/chloroform and ethanol precipitation. A cDNA library was constructed in lambda ZAP (Stratagene, La Jolla, CA) using calf thymus random oligonucleotides (Stratagene) and screened with synthetic oligonucleotides made to the 5′ and 3′ ends of ToMV L sequence (26). The clone that hybridized to both probes was sequenced. A *Bgl* II cloning site was engineered upstream of the ATG using oligonucleotide site-directed mutagenesis (17).

The ToMV CP cDNA was cloned as a *Bgl* II-*Eco*RI fragment into plant expression vectors containing the 35S promoter from cauliflower mosaic virus (CaMV), a *Bgl* II site, followed by 5 bp of leader, 480 bp of CP coding region, and 170 bp of 3′ untranslated sequences. The construct pMON8413 contains the ToMV CP

DNA fragment in the pMON906 vector, a derivative of pMON200 lacking the nopaline synthase gene (11). In pMON 8413, the ToMV CP fragment is between the enhanced CaMV 35S promoter (16) and the ribulose-1,5-bisphosphate carboxylase (*rbcS*) E9 3′ end (7). Construct pMON8414 contains the ToMV CP cDNA between the CaMV 35S promoter and *rbcS* E9 3′ end. The TMV CP was also cloned between the enhanced CaMV 35S promoter and nopaline synthase 3′ end in pMON896, to generate the plant expression vector, pMON8169. The CP constructs were mated into *Agrobacterium tumefaciens* by the triparental mating procedure (9). UC82B tomatoes were transformed with *Agrobacterium* containing each TMV or ToMV CP gene construct. Approximately 30 kanamycin-resistant plants were produced for each construct.

The kanamycin-resistant transgenic tomato plants were assayed for TMV or ToMV CP expression by ELISA as described for the 1988 Illinois test.

The procedure for immunoblot analysis of TMV and ToMV CP in transgenic plants was as described by Tumer et al (27).

1989 Illinois field test. The four genotypes of tomato used in this trial were nontransformed UC82B, the homozygous R2 progeny from pMON8169 transformed line 2068, the R1 progeny of pMON8413 transformed line 3724, and the R1 progeny of pMON8414 transformed line 4174. The R1 progeny included only homozygous and heterozygous plants. The wild-type segregants (CP−) were eliminated by the ELISA for ToMV CP expression. There were 240 plants of each genotype, distributed in four replications.

The experiment was conducted as a split plot design, with three treatments as the main plots, four genotypes as the subplots, and four replications. Each subplot consisted of single rows, with 15 plants spaced 45 cm apart within the row. The main plots were blocks of eight adjacent rows (subplots) spaced 1.5 m apart. The three treatments were no inoculation with virus and mechanical inoculation with TMV U1 or ToMV C. The main plots were separated by a distance of 6 m to reduce the chances of cross-contamination between treatments. For transplant production, seed were germinated in Todd Planter flats in the greenhouse on April 10, 1989, and the seedlings were transplanted in the field on June 30, 1989, using a mechanical transplanter.

Five days after field planting, each plant was hand-inoculated with purified virus at 1 μg/ml as described previously. The inoculated UC82B plants (80 plants for each virus) were sampled 3 wk after inoculation to check for virus infection. ELISA analysis of the plant extracts indicated that a very low percentage of plants were infected: 12% for ToMV C and 16% for TMV U1. Because of this low level of infection in control plants, two of the four blocks were reinoculated (4 wk postplanting) with each virus at 50 μg/ml. Four weeks later, all plants were sampled and the samples assayed by ELISA as described for the 1988 Illinois field test. A plant was considered susceptible if 1 mg of fresh plant tissue contained more than 35 ng of virus as determined by ELISA.

1990 Florida field test. The tomato genotypes used in the 1990 Florida field test included nontransformed UC82B line, pMON8169 transformed line 2068 expressing the TMV CP, and pMON8413 transformed line 3724 and pMON8414 transformed line 4174, both expressing the ToMV CP. Homozygous seeds (R2 generation) were used throughout. The design was a modified split plot design with virus treatments as main plots, genotypes as subplots, and four replications. To avoid accidental virus contamination of the untreated plots, all replications of treated plots were grouped together, and a similar block contained all untreated plots. Each subplot consisted of single rows 4.6 m long, with 10 plants per row and 2.0 m between the rows. Each main plot consisted of two successive groups with six adjacent plots. A border row was planted at each side of the main plot. A 6.0-m alley separated the inoculated and uninoculated plots.

Seeds were germinated in the greenhouse and transplanted in the field on February 23, 1990. Seventeen days after transplanting, half of the field (40 plants of each genotype) was hand-inoculated as in the 1988 Illinois field test. The two treatments were no inoculation and mechanical inoculation with 10 μg/ml ToMV, Naples C isolate.

Samples from inoculated UC82B control plants were collected 14 days after inoculation to confirm infection of plants in the field. All plants were sampled 4 and 8 wk postinoculation and assayed for ToMV-C antigen using ToMV-C IgG raised in rabbits. Three discs 8 mm in diameter were sampled from young terminal leaflets on separate branches of each plant. The samples were homogenized in 750 μl PBSTO, and 50 μl of the plant extract was loaded on ToMV IgG-coated ELISA plates as described previously. To confirm ELISA-positive plant infections, sap from field samples was inoculated onto one of several local lesion hosts: *Chenopodium amaranticolor* Coste & Reynier, *N. sylvestris*, or *N. tabacum* 'Samsun NN'. A plant was considered infected if 1 mg of leaf tissue contained at least 12 ng of virus at the 4 wk timepoint as determined by ELISA; at the 8 wk timepoint, plants with at least 7.5 ng of virus per milligram of leaf tissue were considered infected. In addition to sampling for ELISA, plants were observed for symptom development. At the termination of the field test, fruits from the plots were harvested for yield determinations. Statistical analyses were done as for the 1988 Illinois field test.

RESULTS

1988 Florida field test. The purpose of this test was to evaluate the effectiveness of transgenic tomato (VF36), expressing the TMV U1 CP, in conferring resistance to ToMV infection in Florida field conditions. At 4 wk postinoculation, the uninoculated plots were nearly virus free; only one plant was infected with ToMV (Table 1). In the inoculated plots, the VF36 line was efficiently infected, with all plants showing systemic symptoms and ToMV antigen by the 8 wk timepoint. In contrast, 14.7% of the CRL306 line showed ToMV infection at 4 wk, which increased to 29.3% by 8 wk. This difference in the incidence of infection between the nontransformed VF36 line and TMV CP+ line CRL306 is statistically significant. The delay in infection in CRL 306 from 4 to 8 wk is also significant. In this trial, 70.7% of the CP+ plants escaped systemic infection; 14.6% showed a delay in systemic spread and 14.7% showed no inhibition of infection. These results demonstrated that TMV CP (U1 strain) conferred resistance to ToMV infection in Florida field conditions. However, resistance against ToMV was not as high as the resistance against TMV U1 strain in the 1987 field trial in Illinois (22).

The yield data showed no significant differences between the VF36 controls, which were 100% infected, and the TMV CP+

TABLE 1. Incidence of tomato mosaic virus (ToMV) infection and tomato fruit yield from transgenic (CRL306) and control (VF36) plants in 1988 in Florida

Plant line	Plants infected[y] (%)			Yield[z] (kg/plot)	
	Uninoculated 4 wk	ToMV C inoculated		Uninoculated	ToMV C inoculated
		4 wk	8 wk		
VF36	1.3 (80) Ab	95.4 (74) Aa	100 (73) Aa	45.1 a	46.0 a
CRL306	0 (79) Ac	14.7 (79) Bb	29.3 (79) Ba	46.6 a	49.4 a

[y] Incidence of infection as determined by double antibody sandwich enzyme-linked immunosorbent assay (ELISA) at 4 and 8 wk postinoculation is shown for all plants. The number of plants assayed for each group is shown in parentheses. VF36 is the nontransgenic (CP−) line, CRL306 is the TMV CP+ transgenic line. Two weeks following transplanting in the field, half of the field (80 plants of each genotype) was inoculated with 10 μg/ml ToMV Naples C. Three discs 8 mm in diameter were taken from three young terminal leaflets of each plant. ELISA plates were coated with 1:1000 dilution of 1 mg/ml anti-ToMV IgG. Ten μl of the plant extract was added to 240 μl buffer and loaded on ELISA plates. The plates were incubated at 4 C overnight, the bound virus was detected using horseradish peroxidase conjugated anti-ToMV IgG (1:3000). Data with the same upper case letter are not significantly different from other data in the same column ($P < 0.05$). Data with the same lower case letter are not significantly different from other data of the same row ($P < 0.05$).

[z] Fruits harvested from each plot were weighed and recorded as kg/plot.

line, which was only 29.3% infected. The VF36 line did not show a yield loss attributable to virus infection under Florida growing conditions. The 1987 Illinois field test of this line had demonstrated yield losses associated with TMV infection (22). The Florida field was subjected to drier weather than usual, strong winds, and heavy infestation of sweet potato whiteflies and leaf miner. The yield per plant on VF36 in this trial was 2.5 kg per plant versus 4.0 kg per plant in the Illinois trial. It is possible that the yield reduction attributable to the virus infection could not express itself under these unfavorable conditions.

1988 Illinois field test. The purpose of this test was to determine the level of resistance provided by transgenic tomato (VF36) expressing TMV CP against different TMV and ToMV strains in the field. The TMV CP levels determined by ELISA in 560 transgenic plants varied from 1 to 10 ng per sample. Three plants contained CP levels below 1 ng by ELISA assay.

Eight weeks after inoculation with TMV U1, 100% of the nontransformed VF36 plants were infected with TMV (Table 2). Only 1.2% of inoculated CP+ plants (one out of 80) accumulated TMV. This is one of the three plants that had undetectable levels of TMV CP by ELISA before inoculation. When a severe strain of TMV (PV230) was used, the incidence of infection in nontransformed VF36 plants was 100% and in CP+ plants was 2.5%. Only two out of 80 inoculated TMV CP+ plants contained detectable levels of TMV. Statistical analysis indicated that for both of these TMV strains, the protection observed in the TMV CP+ line is significant.

TABLE 2. Incidence of tobacco mosaic virus (TMV) or tomato mosaic virus (ToMV) infection and tomato fruit yield from transgenic (CRL306) and control (VF36) plants in the 1988 field test in Illinois

	Plants infected[y] (%)		Yield[z] (kg/plot)		Yield loss (%)	
Inoculum	VF36	CRL306	VF36	CRL306	VF36	CRL306
None	62.5 Ab	7.5 Bb	55.3 Aa	63.2 Aa	12%	0%
TMV						
U1	100 Aa	1.2 Bb	50.4 Aa	61.2 Bab	20%	0%
PV230	100 Aa	2.5 Bb	19.6 Ab	62.3 Ba	69%	0%
ToMV						
L	93.7 Aa	56.2 Aa	47.0 Aa	56.1 Aabc	26%	11%
Epcot	100 Aa	66.6 Ba	50.1 Aa	50.6 Aabc	23%	20%
C	100 Aa	78.7 Ba	50.9 Aa	48.9 Abc	19%	23%
Aucuba	100 Aa	88.7 Aa	53.6 Aa	47.4 Ac	15%	25%

[y] Incidence of infection as determined by double antibody sandwich enzyme-linked immunosorbent assay (ELISA) for TMV or ToMV at 8 wk postinoculation is shown for all plants. VF36 is the nontransgenic (CP−) control line, CRL306 is the TMV CP+ transgenic line. Eighty plants from line CRL306 and eighty VF36 plants were used for each treatment. Two weeks following transplanting in the field, plants were mechanically inoculated with 10 μg/ml of each virus. Three discs 8 mm in diameter were sampled from each plant at 4 and 8 wk postinoculation. ELISA was used to detect the challenge strain in each sample. ELISA plates were coated with 1:1000 dilution of 1 mg/ml anti-TMV or anti-ToMV IgG. Ten or 50 μl of the plant extract was added to 240 or 200 μl buffer per well, respectively. After incubation at 4 C overnight, the bound virus was detected using alkaline phosphatase conjugated anti-TMV IgG (1:3000) or horseradishh peroxidase conjugated anti-ToMV IgG (1:3000). The percentages shown for uninoculated plots represent ToMV analysis only. Analysis of these plots for TMV showed 2.5% TMV in VF36 and 2.5% TMV in CRL306. Data with the same upper case letter are not significantly different from other data of the same row (analysis between genotypes). Data with the same lower case letter are not significantly different from other data of the same column (analysis among treatments).

[z] Fruits harvested from each plot weighed and recorded as kg/plot. Fruits were harvested at three different times. Data from all three harvests were combined for each plot for yield analysis. Significant differences were measured at the 0.05 probability level using the Ryan-Einot-Gabriel-Welsh multiple F test (10). Because of incidence of natural virus infection in the uninoculated nontransgenic VF36 plots, yields for all treatments in transgenic and nontransgenic plots were compared to the uninoculated CRL306 plots for analysis and determination of yield reduction.

Uninoculated plots were checked for accidental TMV infection by ELISA. At 8 wk postinoculation, 2.5% of nontransformed VF36 plants and 2.5% of TMV CP+ plants contained detectable levels of TMV.

Disease symptoms on plants infected with U1 strain were mild. In contrast, PV230 caused severe symptoms, including bright yellow mosaic patterns on leaves and stunting on the control plants. The TMV CP+ plants were completely free of symptoms. The dramatic difference between the nontransformed VF36 plants and the TMV CP+ plants 8 wk after inoculation with PV230 is illustrated in Fig. 1.

The results of the ELISA assay for ToMV are also shown in Table 2. In contrast to the TMV data, 62.5% of the uninoculated VF36 plants accumulated ToMV 8 wk postinoculation. This could be the result of infection from natural sources of ToMV in the field, or accidental infection during plant handling following inoculations. The high level of ToMV infection in the uninoculated plots demonstrates the ease with which this virus can be disseminated to wild type plants. ToMV replicates and spreads within tomato plants much more quickly than does TMV (5). In contrast to the high incidence of ToMV infection observed in the uninoculated VF36 plants, only 7.5% of the uninoculated TMV CP+ plants contained ToMV at the final sampling. This accidental spread more closely mimics the natural level of ToMV exposure in the field environment than does the hand inoculation of each plant.

After inoculation with ToMV, 93.7–100% of the nontransformed VF36 plants became infected. A high percentage of TMV CP+ plants accumulated ToMV as well. After inoculation with ToMV Epcot or Naples C, the incidence of infection in CP+ plants was lower than the controls. However, after inoculation with ToMV L or ToMV Aucuba strains, the incidence of infection in TMV CP+ plants was not significantly different from the controls.

The plants inoculated with the ToMV strains and field isolates were checked in the TMV ELISA to determine if any plant was infected by TMV. Two nontransformed VF36 plants inoculated with ToMV L strain showed detectable levels of TMV. This could have occurred naturally or during handling. None of the other plots inoculated with ToMV showed any TMV. Similarly, the plants inoculated with the two TMV strains were checked by ToMV ELISA to look for ToMV infection. Fifteen percent of the nontransformed VF36 plants inoculated with either TMV strain contained detectable levels of ToMV, whereas only 8% of the TMV CP+ plants inoculated with either TMV strain contained detectable levels of ToMV. The inoculation of the VF36 plants with TMV may have reduced their susceptibility to superinfection by ToMV.

Because of the high incidence of ToMV infection in uninoculated nontransgenic plants, the yield from the uninoculated CP+

Fig. 1. Transgenic TMV CP+ line CRL-306 (left) and control VF36 (right) plants in the field after inoculation with 10 μg/ml TMV PV230. Plants were photographed 8 wk postinoculation.

plants was used as the standard for calculating yield losses (Table 2). Data from a similar test conducted in 1987 (22) showed that the yield of uninoculated CRL306 did not differ significantly from that of uninfected VF36. The yield of the TMV (U1 and PV230) inoculated VF36 plants was significantly reduced compared to the yield of the TMV CP+ line. The uninoculated VF36 line had a 12% yield loss possibly attributable to natural ToMV infection, since plants from this line did not contain TMV (data not shown). The nontransformed VF36 plants, which were inoculated with TMV U1, showed a 20% depression in yield, whereas the highly virulent strain, PV230, caused a 69% yield reduction. Thus, under the growth conditions in Illinois, a high incidence of TMV caused a significant reduction in tomato fruit yield. The TMV CP+ plants were protected from virus infection and therefore showed no yield loss after inoculation with TMV U1 or PV230.

The yield results for the plants inoculated with ToMV are also summarized in Table 2. The ToMV-inoculated plants, both nontransgenic and transgenic, showed yield losses between 11 and 26%. This yield loss data correlates with the observed levels of resistance to infection. In this test, there were no significant differences observed in yield between ToMV-inoculated VF36 and ToMV-inoculated CP+ plants. The maximum yield loss was 26%, even when 93.7% of the plants contained detectable levels of ToMV.

Cloning of the ToMV CP gene and expression in transgenic tomato. To enhance protection against ToMV, we have cloned the CP gene from a Florida field isolate of ToMV, Naples C. The ToMV cDNA was translated in vitro using the rabbit reticulocyte lysate system. A single protein comigrated with the CP from ToMV and reacted with ToMV antibodies (data not shown). The deduced amino acid sequence of the Naples C isolate is identical to the amino acid sequence of ToMV L strain (26). Comparison of the nucleotide sequences of the Naples C CP and the TMV U1 CP (12) showed 75% homology at the nucleotide level and 88% homology at the amino acid level.

The ToMV CP cDNA was cloned into plant expression vectors as described in Materials and Methods (Fig. 2) and transformed into tomato.

The percentages of kanamycin-resistant tomato plants that expressed the ToMV CP at levels detectable in the ELISA varied for each construct. When CP expression was driven by the enhanced CaMV 35S promoter in pMON8413, a greater percentage of CP expressors were obtained (84%) compared to when the CaMV35S promoter drove the expression of the CP in pMON8414 (41%). The average level of expression across the total population of transgenics analyzed was higher for the pMON8413 plants than for the pMON8414 plants. Therefore, it appears that the enhanced promoter increased the probability of ToMV CP expression above the minimal level of detection in the ELISA.

Primary tomato transformants, R_0 as well as R1 and R2 progeny, were analyzed by ELISA to quantitate ToMV CP expression. The level of ToMV CP expression as well as total leaf protein declined as the plants set fruit. The CP was readily detectable in young seedlings 1–1.5 mo old. The ToMV CP levels varied between 0.001 and 0.003% of total leaf protein by ELISA (data not shown).

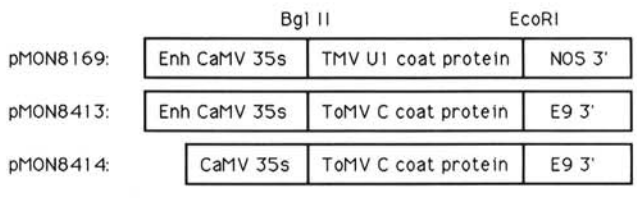

Fig. 2. Plant expression vectors containing TMV or ToMV coat protein (CP) genes. The promoters are derived from cauliflower mosaic virus (CaMV) 35S gene. Polyadenylation signals are derived from nopaline synthase or ribulose-1,5-bisphosphate carboxylase (rbcS) E9 gene. The vectors contain neomycin phosphotransferase II (NPTII) gene for kanamycin selection of transformed plants. The TMV CP was cloned between the enhanced (Enh) CaMV 35S promoter (16) and nopaline synthase 3′ end to generate pMON8169. In pMON8413, the ToMV CP fragment is between the enhanced CaMV 35S promoter and the rbcS E9 3′ end (7). In pMON8414, the ToMV CP fragment is between CaMV 35S promoter (11) and rbcS E9 3′ end.

The segregation of the ToMV CP in the R1 progeny of selfed CP+ plants was determined by ELISA. Three hundred and eighty R1 plants were analyzed from each line. The CP in progeny of line 3724, containing pMON8413 segregated with a ratio of 12:1 (CP+:CP−), indicating two independent integration loci (Chi-square analysis, 95% confidence of 15:1 ratio). The CP in progeny of line 4174 containing pMON8414 segregated at a ratio of 2:1, indicating insertion at a single locus (Chi-square analysis, 99% confidence of 3:1). The ELISA cut-off value for CP+ plants was skewed to select for definite positive plants rather than including very low expressors. The segregation may have more nearly equaled 15:1 and 3:1 if different minimal ELISA values had been used in the assay.

The results of immunoblot assays of homozygous plants (R2 generation) from TMV and ToMV CP+ lines are shown in Fig. 3. The pMON8414 line 4174 contains lower levels of ToMV CP than the pMON8413 line 3724. The CP in the transgenic plants comigrates with denatured virus protein standards. The level of expression estimated by immunoblot analysis is approximately 0.01–0.05% of total extractable protein.

1989 Illinois field test. The purpose of this test was to evaluate the level of resistance provided by transgenic tomato plants (UC82B) expressing CP from TMV U1 or ToMV Naples C against TMV U1 and ToMV Naples C.

All of the uninoculated plants remained virus free throughout the experiment (Table 3). One hundred percent of the UC82B nontransgenic plants were infected with TMV U1, whereas 93.3% were infected with ToMV Naples C at 4 wk postinoculation. A high incidence of infection was established even though the plants were reinoculated just before flower bud formation. The TMV CP+ tomato line 2068 was well protected against TMV; only 3.3% of the plants from this line contained TMV. When this line was inoculated with ToMV Naples C, incidence of ToMV infection was 30%. The incidence of TMV and ToMV infection in line 2068 is significantly different from the UC82B. Furthermore, the difference between incidence of TMV and ToMV infection in line 2068 is also significant. This confirms that the TMV CP+ line 2068 is more resistant to TMV than to ToMV C.

The ToMV CP+ tomato lines 3724 and 4174 showed some protection when challenged with TMV-U1 (Table 3). Incidence of TMV infection in these lines was 35.3 and 7.1%, respectively. None of the plants from either line became infected with ToMV. The differences in the incidence of infection between these lines and UC82B are significant. The difference between the incidence of TMV and ToMV infection in line 3724 is also significant. These results demonstrate that the ToMV CP+ line 3724 shows better resistance to ToMV, the homologous virus, than to TMV U1. Under Illinois field conditions, ToMV CP+ line 4174 showed a high level of resistance to both ToMV and TMV.

1990 Florida field test. At the 4 wk timepoint in uninoculated plots, ToMV was detected only in line 3724 and the UC82B control line (Table 4). Incidence of infection was 2.5% in line 3724 and 5.1% in UC82B. In the inoculated plots, 97.5% of the control UC82B plants were infected, whereas plants from line 4174 did not show any infection and only 5.1% of the plants from line 2068 and 17.5% of the plants from line 3724 were infected.

By the 8 wk timepoint in uninoculated plots, only lines 2068 and 4174 were free of ToMV infection. Incidence of ToMV infection in line UC82B and 3724 was 36.7 and 10.3%, respectively, in uninoculated plots. Incidence of infection in lines 4174 and 2068 in the uninoculated plots was significantly lower than the incidence in the control line UC82B. Eight weeks postinoculation, the inoculated UC82B plants were all infected. The TMV CP+ line 2068 showed 50.8% infection and the ToMV CP+ line 3724 showed 60% infection. Incidence of infection in both of these lines were significantly less than in nontransformed line UC82B.

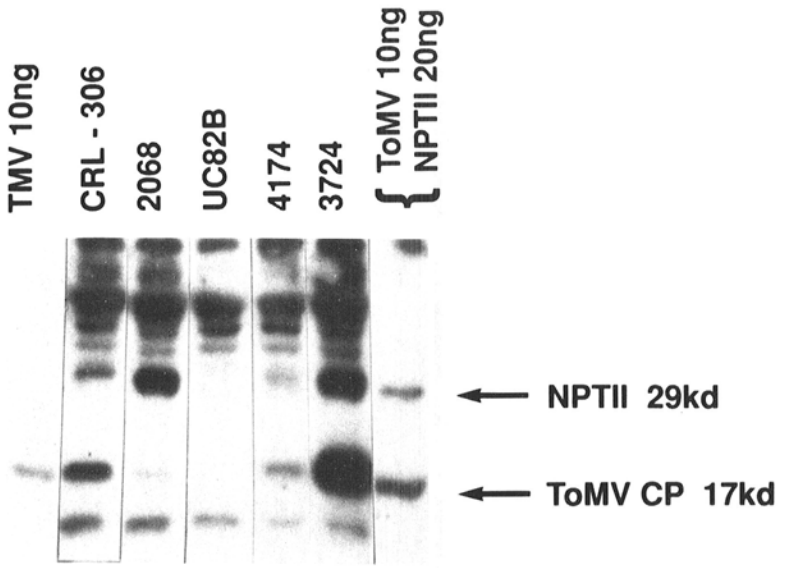

Fig. 3. Detection of TMV CP and ToMV CP by immunoblot analysis of transgenic tomato plants. One hundred micrograms of protein extracted from leaves of homozygous plants (R2 generation) from each line was separated on a 12.5% SDS-polyacrylamide gel, electroblotted, and probed with ToMV and neomycin phosphotransferase II (NPTII) IgG. The standards were 10 ng of purified ToMV and 20 ng of NPTII. Line CRL 306 is a TMV CP line containing pMON316 (22), line 2068 contains pMON8169 (TMV CP), UC82B is the nontransformed wild-type control, line 4174 contains pMON8414 (ToMV CP), line 3724 contains pMON8413 (ToMV CP).

TABLE 3. Incidence of tomato mosaic virus (ToMV) infection in the 1989 field test in Illinois

Plant line	CP	Plants infected[z] (%)		
		Uninoculated	Inoculated	
			TMV U1	ToMV C
UC82B	none	0	100 Aa	93.3 Aa
2068	TMV	0	3.3 Cb	30.0 Ba
3724	ToMV	0	35.3 Ba	0 Cb
4174	ToMV	0	7.1 Ca	0 Ca

[z]Incidence of infection as determined by double antibody sandwich enzyme-linked immunosorbent assay (ELISA) at 4 wk postinoculation is shown for all plants. UC82B is the nontransgenic (CP−) control line, 2068 is the TMV CP+ line, 3724 and 4174 are ToMV CP+ lines. The data represents 27–30 plants (two reps) for each genotype/treatment except for line 4174, of which there were 14 plants per treatment. Five days following field planting, each plant was hand inoculated with purified TMV U1 or ToMV Naples C at 1 μg/ml. The inoculated UC82B plants were sampled 3 wk following inoculation to check for virus infection. ELISA analysis indicated very low percentage of plants were infected. Because of this low level of infection in control plants, two of the four blocks were reinoculated with each virus at 50 μg/ml at 4 wk postplanting. Four weeks later all plants were sampled by taking three discs 8 mm in diameter from each plant. Ten or 50 μl of the plant extract was added to 240 or 200 μl buffer per well, respectively. After incubation at 4 C overnight, the bound virus was detected using alkaline phosphatase conjugated anti-TMV IgG (1:3000) and alkaline phosphatase conjugated anti-ToMV IgG (1:3000). Data is presented for the two reinoculated plots. Comparisons between all possible pairs of lines and treatments were conducted using the G-test of independence (25). Within each column, percentages with the same capital letter are not significantly different ($P < 0.05$). Within each row, percentages with the same lower case letter are not signficantly different ($P < 0.05$).

The ToMV CP line 4174 showed the lowest incidence of infection (20%), which is significantly different than the other three lines in this test. Furthermore, the titer of viral antigen in infected plants from line 4174 was at least 27-fold lower than the titer of viral antigen in the infected plants from the nontransformed UC82B line (data not shown).

Infected plants did not exhibit definite symptoms in the field. Although the CP+ lines showed significantly lower incidence of ToMV infection, analysis of tomato fruit yield failed to show significant differences between the inoculated and uninoculated lines (Table 4). The yield of uninoculated CP+ tomatoes was comparable to the yield of uninoculated nontransformed tomatoes, indicating that the transformation process did not affect tomato fruit yield.

DISCUSSION

Considerable effort has gone into obtaining resistance to plant viruses through genetic engineering. Although CP-mediated protection has been effective for a number of plant viruses, there is little information on the evaluation of CP-expressing plants under field conditions. Transgenic Russet Burbank potato plants expressing CP genes of potato virus X (PVX) and potato virus Y (PVY) have been evaluated in the field and have shown a high level of resistance to both viruses. Although the yield of control Russet Burbank plants was significantly reduced because of virus infection, the yield of CP+ plants was unaffected (15). We have tested homozygous progeny from a transgenic tomato line expressing TMV CP against a field isolate of ToMV in the field in Florida and against two different TMV strains and four different ToMV strains in the field in Illinois. Homozygous progeny from the same line were evaluated against TMV U1 in the field in 1987 and showed excellent resistance (22). In Illinois field trial, TMV CP+ plants showed excellent resistance to TMV U1 and TMV PV230. However, the same line showed little or no resistance against different strains and field isolates of ToMV. Because ToMV is the predominant virus problem in greenhouse and field-grown tomatoes, in an attempt to obtain high level of resistance to ToMV, we cloned the cDNA for the CP gene from a ToMV field isolate and expressed it in tomato plants. We tested progeny of nine different transgenic tomato lines expressing ToMV CP in growth chambers for resistance to TMV and ToMV. Progeny of two of these lines were tested in the field in Illinois and Florida. Both ToMV CP+ lines tested showed a high level of resistance to ToMV in the field in Illinois. In the same trial, the TMV CP+ line showed a higher level of resistance to TMV than to ToMV. In the 1990 Florida field trial, one of the two different ToMV CP+ lines tested showed a higher level of resistance to ToMV than did the TMV CP+ line. The results of the field tests described here demonstrate that under field conditions, the CP gene from the target virus confers better

TABLE 4. Incidence of tomato mosaic (ToMV) Naples C infection as determined by ELISA in the 1990 field test in Florida

Plant line	Plants infected[y] (%)				Yield[z] (kg/8 plants)	
	Uninoculated		Inoculated			
	4wk	8wk	4wk	8wk	Uninoculated	Inoculated
UC82B	5.1(39) A	36.7(38) A	97.5(39) A	100(40) A	30.5 Aa	24.2 ABa
2068	0(40) A	0(40) B	5.1(39) B	50.8(39) B	29.4 Aa	30.3 Aa
3724	2.5(39) A	10.3(39) AB	17.5(40) B	60(40) B	30.6 Aa	32.3 Aa
4174	0(40) A	0(40) B	0(40) B	20(40) C	32.3 Aa	28.7 ABa

[y] Incidence of infection as determined by double antibody sandwich enzyme-linked immunosorbent assay (ELISA) at 4 and 8 wk postinoculation. UC82B is the nontransgenic (CP−) control line, 2068 is TMV CP+ line, 3724 and 4174 are ToMV CP+ transgenic lines. Seventeen days after transplanting, half of the field was hand inoculated with 10 μg/ml ToMV Naples C. Three discs 8 mm in diameter were sampled from young terminal leaflets of each plant at 4 and 8 wk postinoculation. ELISA plates were coated with 1:1000 dilution of 1 mg/ml anti-ToMV IgG. Fifty μl of the plant extract was added to 200 μl buffer per well. After incubation at 4 C overnight, the bound virus was detected using alkaline phosphatase conjugated anti-ToMV IgG (1:3000). Within each column, the means with the same upper case letter are not significantly different ($P < 0.05$). Within each row, the means with the same lower case letter are not significantly different ($P < 0.05$).

[z] Fruits harvested from each plot were weighed and recorded as kg/8 plants.

resistance against the homologous virus than against a closely related virus. However, it is possible to identify CP+ lines that have good resistance to both viruses, as observed with the ToMV CP+ line 4174.

In field trials reported here, the transgenic tomato plants expressing the TMV CP have demonstrated complete resistance against two TMV strains in nearly 100% of the plants over the 8-wk duration of the trial. This indicates a lower incidence of initial virus infection in the CP+ plants compared to nontransformed control plants. It has been reported that the majority of tobacco plants expressing TMV CP exhibit a delay in virus symptom development (23). In contrast, the TMV CP+ tomatoes completely escaped infection after inoculation with TMV. Consequently, we did not see a delay in symptom development or virus accumulation. Furthermore, it has been reported that in growth chamber tests, transgenic tobacco plants expressing the CP of TMV-U1 show significant delays in systemic disease development after inoculation with ToMV compared to the nontransformed control plants (20). The TMV CP+ local lesion host plants (*N. tabacum* 'Samsun NN') showed greatly reduced numbers of necrotic lesions compared to the nontransformed control plants after inoculation with ToMV (20). In tomato, little or no protection was observed when TMV CP+ tomato plants were challenged with ToMV. The CPs of TMV and ToMV C are 75% homologous at the nucleotide level and 88% at the amino acid level. Although the two CPs are very similar, they are serologically distinguishable by ELISA. Interestingly, this small divergence at the protein level results in less than 100% protection in transgenic tomato when TMV CP+ plants are challenged with ToMV.

Assuming that a common resistance mechanism exists in CP+ tomato against TMV and ToMV, this mechanism can distinguish between these two very closely related viruses. Considering that the CP-mediated protection against TMV appears to be effective at or before uncoating of the virus (24), the surface structure of the virions could be recognized by the CP. It is possible that the amino acid substitutions in ToMV could be the sites of recognition or that they could alter the surface structure of virions such that it is no longer recognized effectively by the TMV CP. Further studies are needed to determine if these residues are involved in ToMV resistance.

In general, the growth chamber protection tests with tomato plants expressing ToMV CP and the field test results described here did not show good correlation between the level of expression of ToMV CP in tomato and the level of resistance to ToMV. The lack of correlation was observed with tomato plants containing either pMON8413 or pMON8414. In the Florida field test, the most resistant line 4174 contained lower levels of CP than line 3724, which showed less resistance. The lack of correlation between the amount of CP and the level of resistance has been observed previously in transgenic plants expressing potyvirus CP genes (2). One possible explanation for the lack of correlation is that the assays described measure the overall CP accumulation in the leaf; thus, they cannot detect differences in the level of expression of the CP at the sites of infection. Different transgenic plants could differ in their CP expression pattern at the site of infection because of the position of insertion of the foreign gene. The differences in the cell type or tissue-specific expression of the CP gene could be responsible for the lack of correlation between the amount of CP and the level of resistance.

The ToMV strain Tm-1, which overcomes the natural resistance gene, Tm-1, is a mutant of ToMV L, but the amino acid sequence of its CP is identical to the parent (19). Thus, the Tm-1 strain, which is a problem in commercial tomato production, has CP sequences identical to the ToMV Naples C protein engineered in tomatoes. Therefore, the excellent protection observed against Naples C strain should translate to the ToMV strain 1.

Although significant reduction in the incidence of infection was observed with transgenic lines expressing TMV or ToMV CPs in the Florida field test, no differences were seen in fruit yields between inoculated and uninoculated plots. Furthermore, the infected plants failed to show symptoms in the 1990 Florida field trial. ToMV is known to cause both symptom expression and yield loss in tomato. Symptom expression is highly dependent on environmental conditions, virus strain, and cultivar of tomato. Environmental factors present in this field trial may explain the lack of symptom expression and lack of differences in yield.

The results described here demonstrate the potential utility of CP-mediated protection to introduce ToMV resistance into commercial tomato genotypes, which is highly effective under field conditions. Future studies will determine if introduction of the ToMV CP gene into a variety of different commercial tomato genotypes will confer commercially acceptable levels of ToMV resistance while preserving other intrinsic properties of these lines.

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