Transgenic Plants that Express the Coat Protein Genes of Tobacco Mosaic Virus or Alfalfa Mosaic Virus Interfere with Disease Development of Some Nonrelated Viruses

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ABSTRACT

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Transgenic tobacco (*Nicotiana tabacum* 'Xanthi') plants that express the coat protein (CP) gene from the U_1 strain of tobacco mosaic virus (TMV) are resistant to infection by TMV. To determine whether these plants also are protected against other viruses, they were inoculated with low concentrations of potato virus X (PVX), potato virus Y (PVY), cucumber mosaic virus (CMV), alfalfa mosaic virus (AlMV), and the cowpea strain of TMV (Cc-TMV). Although the accumulation of virus in inoculated leaves was equivalent in plants that express the CP gene (CP+) and plants that do not express the CP gene (CP−), there was a delay of 1 to 3 days in the development of systemic disease symptoms on CP(+) plants infected with PVX, PVY, CMV, and AlMV as compared with CP(−) plants. The magnitude of protection, however, was significantly lower than against TMV. Protection against Cc-TMV, assayed on a CP(+) local lesion host, also was much lower than against TMV-U_1. A delay in disease development also was observed when transgenic tobacco (*N. tabacum* 'Samsun') plants expressing the CP gene of AlMV were infected with PVX and CMV, but not when they were infected with TMV-U_1. The results of these experiments demonstrate that transgenic tobacco plants that express different CP genes have a low but significant degree of protection against other viruses.

Additional keywords: engineered protection.

Transgenic tobacco and tomato plants that express the coat protein (CP) gene from the U_1 strain of tobacco mosaic virus (TMV) were delayed in symptom development or escaped infection when inoculated with the U_1 and other strains of TMV (22,23,27). More recently, it was demonstrated that transgenic plants that express other coat protein genes are protected against infection by the virus from which the gene was obtained, that is, alfalfa mosaic virus (AlMV) (18,31,32), cucumber mosaic virus (CMV) (6), potato virus X (PVX) (14), tobacco rattle virus (32), and tobacco streak virus (33). In many of these examples, the numbers of chlorotic or necrotic lesions produced on the inoculated leaves were substantially lower than on control plants, and there was a reduction in the rate of systemic disease development. The resistance observed in these transgenic plants bears a striking resemblance to classical cross protection in which plants infected with a mild strain of a virus are protected against superinfection by a related strain of that virus (11,13,25). The protection engendered in transgenic plants has been referred to as engineered coat protein protection (4).

It is likely that multiple factors are involved in cross protection, and a variety of theories have been proposed to explain the phenomenon (7,25,28). It has been difficult to study the mechanism(s) of cross protection largely because of difficulties inherent in detecting small amounts of challenge virus in the presence of large amounts of protecting virus (36). Studies of engineered protection may be easier because they do not require the presence of a protecting virus. In an applied sense, protecting plants from viral infection by genetic transformation rather than by infection with intact virus can overcome objectionable problems of pre-infection. These include yield losses caused by the protecting virus, the potential for developing severe symptoms by synergism with a second virus, and the possibility of transfer of virus to other more susceptible plants in the vicinity (26). In cross protection, interference with nonrelated viruses is desirable but only rarely observed (13). Fulton (10) demonstrated that tobacco plants infected with tomato ringspot virus (TmRSV) were resistant to infection by cherry leafroll virus (CLRV). This observation was extended to eight CLRV isolates (17). Infection of tobacco plants by CMV delayed TMV accumulation in plants infected with CMV and vice versa; however, neither virus completely inhibited the replication of the other (12). In the majority of instances, infection by one virus does not interfere with replication of a nonrelated virus.

The objective of this study was to determine whether tobacco plants expressing the CP gene of TMV were protected against or showed any interference with the establishment of infection by viruses not related to TMV.

The viruses chosen for the study were type members of four different groups of single-stranded RNA viruses bearing significant structural differences from TMV, as well as a strain of TMV with little nucleotide homology or serological relatedness to the U_1 strain. TMV is a tobamovirus, a rigid, rod-shaped particle with a CP of mw = 17.5 kDa. PVX is a potexvirus, a flexuous, rod-shaped particle with a CP of mw = 31 kDa (8,19). Potato virus Y (PVY), a potyvirus, is also a flexuous rod with a CP of about 30 kDa (8). AlMV is a bacilliform virus composed of three RNA molecules encapsidated in separate particles with a CP of mw = 24 kDa (8,16). CMV, a cucomovirus, also is composed of three RNA molecules encapsidated in icosahedral particles with a CP of mw = 26 kDa (8). The cowpea strain of TMV (Cc-

TMV) differs from TMV-U_1 in its nucleotide sequence, including 96:156 differences in the amino acid sequence in the CP (35).

MATERIALS AND METHODS

Plants used. Progeny of transgenic tobacco (*Nicotiana tabacum* L. 'Xanthi') plant lines 3404, 3773, and 3648 that express the CP gene of the U_1 strain of TMV were used throughout this study (27). In local lesion studies, progeny of line 740, a transgenic line of *N. tabacum* 'Xanthi nc' that expresses the TMV CP gene, were used. Control plants for these experiments usually were progeny of transgenic line 306, which harbors the same vector used in the other lines but lacks the CP gene (27). Other control plants used were nontransformed Xanthi or Xanthi nc plants and segregating progeny of transgenic plants from line 3404 that did not express the CP gene. Control plants and transgenic plants usually were distinguished by assaying the accumulation of nopaline by the method of Otten and Schilperoort (24). In some cases plants also were sampled to immunologically determine the accumulation of CP by Western immunoblot analysis as described by Powell Abel et al (27).

Preparation of inocula and antibodies. PVX was obtained from R. Ford (University of Illinois, Urbana) and increased in Xanthi tobacco. The virus was purified by a method modified from those described by Francki and McLean (9) and McLean and Francki (20) and then maintained at 4 C. PVY also was obtained from R. Ford and was increased in Xanthi tobacco. Because virus purified by published methods had low infectivity, inoculum stocks were maintained as frozen desiccated leaf tissue, which was homogenized and diluted in buffer (20 mM $NaPO_4$, pH = 7.2, 1 mM ethylenediaminetetraacetic acid [EDTA], 0.1% Na_2SO_3) before each inoculation. AlMV was obtained from T. Godefey-Colburn (Strasbourg, France), maintained as frozen leaf tissue, and prepared as described for PVY before each inoculation (20 mM $NaPO_4$, pH = 7.2, 1 mM EDTA). Inocula concentrations of PVY and AlMV were determined by immunoblot analysis and comparison of the antibody reaction with a dilution series of purified virus. CMV (strain C) was obtained from H. Murakishi (Michigan State University, East Lansing) and increased in Xanthi tobacco. The virus was purified by a method modified from those described by Murant (21) and Scott (29) and maintained at 4 C. Cc-TMV was obtained from M. Zaitlin (Cornell University, Ithaca, NY), increased in cowpea (*Vigna sinensis* 'Blackeye') (5), and purified by the method used to purify TMV-U_1. TMV-U_1 also was obtained from M. Zaitlin, increased in Xanthi tobacco, and purified as described by Asselin and Zaitlin (2). The purified virus was maintained at 4 C.

Antibodies against purified PVX, PVY, and CMV were prepared in rabbits. Whole sera were used for immuno-dot-blot analyses at a 1:1,000 dilution and gave negligible cross-reaction with other plant proteins. Antibodies against AlMV and Cc-TMV were obtained from Agdia Inc. (Mishawaka, IN) and M. Zaitlin, respectively. Purified IgG was used for immuno-dot-blot analyses at a 1:1,000 dilution. Immunodetection was accomplished by reacting the primary antibody with ^{125}I-labelled F(ab')2 fragment of anti-rabbit IgG from donkey (Amersham Corp., Arlington Heights, IL).

Plant growth, virus inoculations, observations, and sampling of plants. Tobacco seedlings were transplanted 3 to 4 wk after seeding as described by Nelson et al (23). When plants reached the three-leaf stage (approximately 7–14 days after transplanting), leaf disks were taken from lower leaves for analyses of nopaline and CP accumulation as described previously. The gene segregation ratios obtained from the results of nopaline assays always complemented the results obtained for the CP assays for plant lines 3404, 3773, and 3648 (data not shown). When plants reached the five- to six-leaf stage (approximately 10–20 days after transplanting), they were inoculated and maintained in growth chambers as described by Nelson et al (23). Xanthi nc tobacco plants were inoculated at 48 days after planting after they had been topped and placed in low light for 20 hr.

Plants were observed daily for systemic disease symptoms beginning 4 days after inoculation and continuing for up to 14 days. Visual disease ratings were given to plants based on a scale of 0 to 5. Only ratings of 2 or more represented the development of systemic symptoms.

Plants were sampled periodically to verify that visual observations reflected virus accumulation, as well as to quantitate levels of virus. Sampling was done by harvesting either leaf disks or

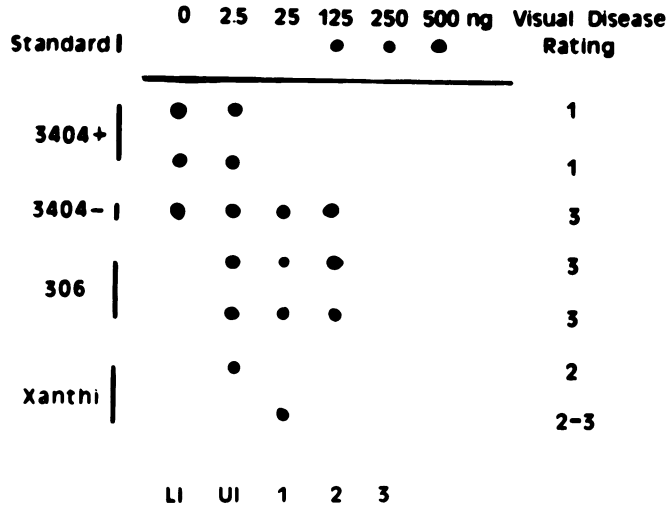

Fig. 1. Virus accumulation in the lower inoculated (LI), upper inoculated (UI), first systemic (1), second systemic (2), and third systemic (3) leaves of tobacco plants expressing the coat protein (CP) gene of the U_1 strain of tobacco mosaic virus (TMV) 8 days after inoculation with purified potato virus X. Half leaves from transgenic plants expressing TMV CP (3404+), nonexpressors (3404−), vector-only transformed control plants (306), and nontransformed *Nicotiana tabacum* 'Xanthi' plants were sampled and analyzed as described. Ten micrograms of protein was loaded per dot. Lower inoculated leaves from 306 and nontransgenic Xanthi were not sampled. The column on the right shows values for visual observations of disease symptom development assigned before harvesting leaves for other analyses. The disease rating is based on a 0–5 scale with a score of 1 representing symptoms on inoculated leaves and scores of 2 through 5 representing increasingly severe systemic symptoms.

TABLE 1. Percentage of plants that are systemically infected 8 days after inoculation

Virus	Inoculum concentration (μg/ml)	Plant type[a]	Plants with systemic infection 8 days after inoculation (%)
Potato virus X	1.0	CP− (13)	92[b]
		CP+ (10)	30
Alfalfa mosaic virus	1.4	CP− (17)	88[c]
		CP+ (14)	36
Potato virus Y	4.0	CP− (3)	100
		CP+ (6)	50
Cucumber mosaic virus	0.8	CP− (3)	100[c]
		CP+ (6)	33

[a] Plants either expressed the tobacco mosaic virus coat protein (CP) gene (CP+) or did not express the CP gene (CP−). The numbers of plants that were sampled in each case is shown in parentheses. Virus infection was determined by an immuno-dot-blot assay as described in the text. The virus used and the concentration of the inoculum are indicated.

[b] Significantly more CP− (nonexpressor) plants showed systemic movement of the virus than CP+ (expressor) plants 8 days after inoculation at the 0.005 confidence level as calculated by the Fisher Exact Probability Test.

[c] Significantly more CP− plants showed systemic movement of the virus than CP+ plants 8 days after inoculation at the 0.10 confidence level (Fisher Exact Probability Test).

half leaves; then the samples were frozen on dry ice. After protein extraction from leaf pieces, analysis of CP accumulation was done by a protein dot blot immunoassay as described by Nelson et al (23).

RESULTS

Virus replication and disease development in transgenic plants expressing the CP gene of TMV-U_1. When two leaves of transgenic tobacco (Xanthi) plants expressing the CP gene of TMV-U_1 (lines 3404, 3648, and 3773) were inoculated with any of the viruses other than TMV-U_1, there were no differences in virus accumulation in the inoculated leaves when compared with virus accumulation in leaves of control plants (representative data following inoculation with PVX are shown in Fig. 1).

To correlate virus accumulation in upper systemically infected leaves with visual disease symptom development, half leaves were harvested from up the canopy of inoculated plants and assayed for virus accumulation. The experiments demonstrated that, by 8 days after inoculation, 30 to 50% of the CP(+) plants showed virus accumulation in upper systemically infected leaves, whereas 65 to 100% of the CP(−) control plants contained virus in upper leaves (Table 1). The sample sizes in these experiments were small, and some variation in symptom development was observed.

TABLE 2. Systemic disease development after inoculation with potato virus X, cucumber mosaic virus, potato virus Y, or alfalfa mosaic virus

Virus	Inoculum concentration (μg/ml)	Plant type[a]	Plants (%) showing symptoms: 6 days pi[b]	7 days pi	8 days pi	10 days pi	12 days pi	14 days pi
Potato virus X	0.01	CP− (4)	0	0	0	25	100	100
		CP+ (8)	0	0	0	13	25	75[c]
	0.1	CP− (4)	25	50	100	100	100	100
		CP+ (8)	0	0	50	50	88	88[d]
	0.5	CP− (4)	0	75	100	100	100	100
		CP+ (8)	38	50	75	100	100	100
	1.0	CP− (4)	50	50	100	100	100	100
		CP+ (8)	38	38	75	88	100	100
	5.0	CP− (4)	50	50	100	100	100	100
		CP+ (8)	50	63	100	100	100	100
Cucumber mosaic virus	0.01	CP− (4)	0	0	0	75	100	100
		CP+ (8)	0	0	13	25	38	50[d]
	0.1	CP− (4)	0	0	0	50	50	100
		CP+ (8)	0	0	0	13	38	63
	0.5	CP− (4)	0	0	25	75	75	100
		CP+ (8)	0	0	0	38	38	75
	1.0	CP− (4)	25	50	100	100	100	100
		CP+ (8)	13	25	63	88	100	100
	5.0	CP− (4)	50	50	75	100	100	100
		CP+ (8)	50	75	88	100	100	100
Potato virus Y	0.5	CP− (4)	50	50	75	100	100	100
		CP+ (8)	13	13	13	25	38	63[c]
	0.8	CP− (4)	0	0	50	75	100	100
		CP+ (8)	13	13	25	50	75	100
	4.0	CP− (4)	75	75	100	100	100	100
		CP+ (8)	25	25	25	25	88	100[d]
	20.0	CP− (4)	100	100	100	100	100	100
		CP+ (8)	100	100	100	100	100	100
Alfalfa mosaic virus	0.7	CP− (4)	100	100	100	100	100	100
		CP+ (8)	50	50	100	100	100	100
	0.8	CP− (4)	75	75	75	100	100	100
		CP+ (8)	63	88	88	100	100	100
	1.3	CP− (4)	100	100	100	100	100	100
		CP+ (8)	88	100	100	100	100	100
	7.0	CP− (4)	100	100	100	100	100	100
		CP+ (8)	100	100	100	100	100	100

[a] Tobacco plants that express the tobacco mosaic virus coat protein (CP) gene (CP+) or do not express the CP gene (CP−) were inoculated with increasing concentrations of each virus and observed for the development of systemic disease symptoms 6–14 days after inoculation. The numbers in parentheses indicate the number of plants in each group in this experiment.

[b] pi = postinoculation.

[c] A delay in observed systemic symptoms was significant at the 0.05 confidence level (determined by Mann-Whitney U-Test for small samples).

[d] Delay was significant at the 0.1 confidence level (Mann-Whitney U-Test).

However, the results of the immunoassays always coincided with the disease rating given (representative data for PVX immuno-dot-blot assay results are shown in Fig. 1).

Based on these preliminary results, the data from visual observation studies were considered valid, and we undertook experiments using several virus inoculum concentrations and larger numbers of plants. Three visual observation experiments, and a minimum of two immuno-dot-blot assays, were done for each of the four viruses tested. In total, more than 2,000 plants were surveyed. The results shown in Table 2 are from single representative experiments. After inoculation with low-to-moderate concentrations of the nonrelated viruses, a delay of up to 3 days in disease development was observed in transgenic CP(+) plants compared with CP(−) control plants (Table 2). If inoculum concentrations were too low, some of the CP(−) plants did not become infected. Conversely, the delay in systemic symptom development was readily overcome in the CP(+) plants by increasing viral inoculum concentrations (Table 2).

In comparison, the delay in systemic spread of TMV-U_1 in plants grown under identical environmental conditions did not begin to break down until virus inoculum concentrations were greater than 10 μg/ml (Fig. 2). Experiments with Cc-TMV were carried out on the local lesion host, Xanthi nc, and resistance was determined by comparing the number of lesions produced on opposite half leaves of transgenic CP(+) plants and CP(−) plants by different levels of inocula (Table 3). Protection against Cc-TMV was equivalent to the protection against Cc-TMV RNA and TMV-U_1 RNA, but at least 10 times less than the protection against TMV-U_1.

To determine whether the interference in disease development in these transgenic plants was due to the presence of the CP gene of TMV, or if the expression of another foreign gene would give a similar plant response, we also inoculated purified TMV onto transgenic plants (Xanthi) that constitutively express the chloramphenicol acetyltransferase (CAT) gene (5). Disease symptoms developed in the CAT(+) plants at the same rate as in CP(−) control plants (Fig. 3), indicating that the resistance was associated with expression of the TMV CP gene.

To determine whether the interference of disease development was restricted to viral pathogens, plants were injected with several dilutions of a bacterial cell suspension. When plants were injected with *Pseudomonas syringae* pv. *pisi* (which caused an incompatible reaction on this plant), the hypersensitive response was observed in equivalent periods of time in both TMV CP(+) and CP(−) plants (data not shown).

TABLE 3. Local lesion numbers on half leaves of plants after inoculation with U_1 or the cowpea strain of TMV (Cc-TMV), or TMV-U_1 RNA or Cc-TMV RNA

Virus	Inoculum concentration (μg/ml)	Plant type[a]	Lesion number/ half leaf	Percent of coat protein control
Cc-TMV	0.5	CP− (18)	92 ± 15	
		CP+ (21)	39 ± 7	42
Cc-TMV	1.5	CP− (18)	158 ± 18	
		CP+ (22)	73 ± 13	46
Cc-TMV RNA	20.0	CP− (18)	89 ± 18	
		CP+ (21)	30 ± 5	34
TMV-U_1 RNA	10.0	CP− (18)	65 ± 11	
		CP+ (21)	31 ± 5	48
TMV-U_1	0.25	CP− (18)	102 ± 11	
		CP+ (21)	5 ± 1	5
TMV-U_1	0.75	CP− (18)	174 ± 18	
		CP+ (21)	7 ± 2	4

[a] Transgenic *Nicotiana tabacum* 'Xanthi nc' plants expressing the coat protein (CP+) of TMV-U_1 and nontransgenic (CP−) Xanthi nc plants were inoculated with Cc-TMV, Cc-TMV RNA, TMV-U_1 RNA, or TMV-U_1, as shown. Numbers in parentheses indicate the number of leaves inoculated within each treatment. Lesions were counted at 6 days after inoculation, and values represent the mean ± standard error for each treatment. The rightmost column represents a comparison (as a percentage) of lesion numbers on CP(+) vs. CP(−) leaves.

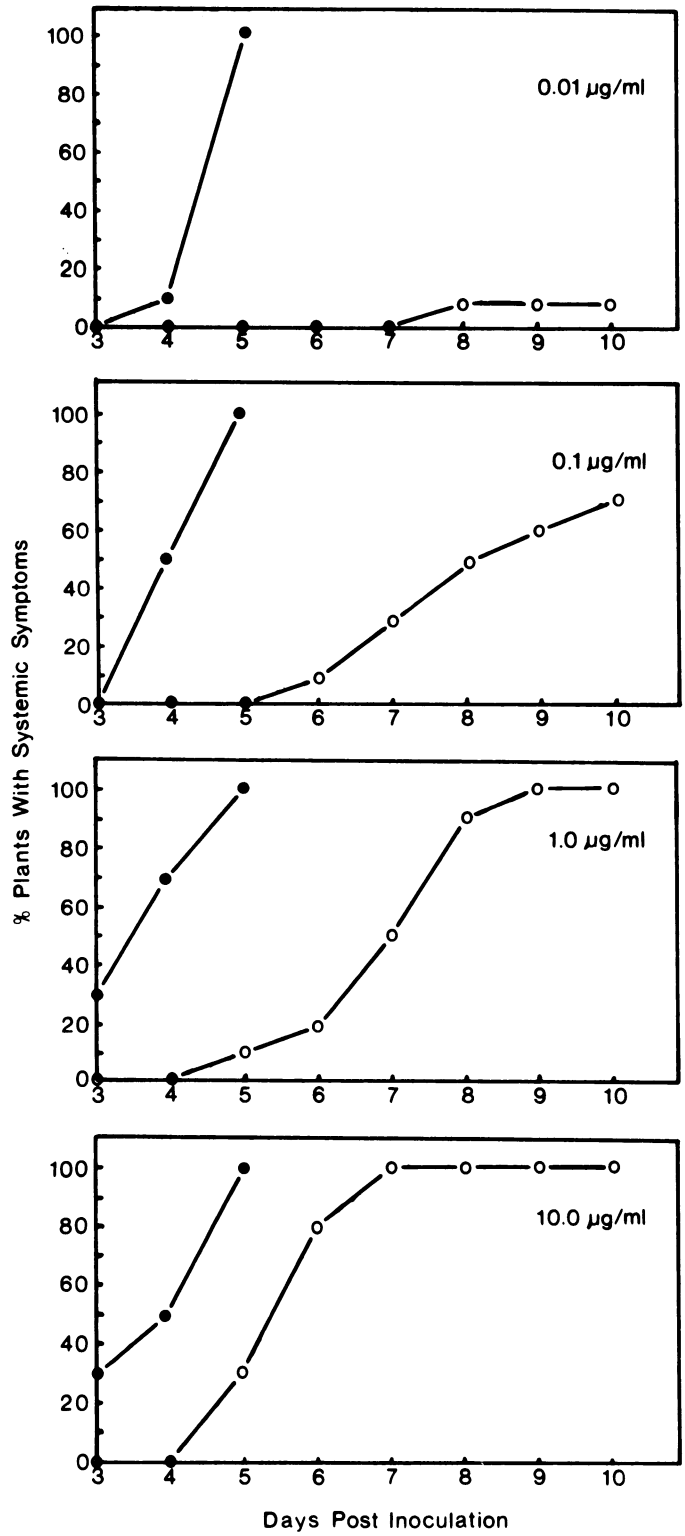

Fig. 2. Systemic symptom development caused by the U_1 strain of tobacco mosaic virus (TMV) on coat protein (CP)(+) transgenic plants (line 3404) that had been inoculated with four concentrations of the virus. Two leaves per plant were inoculated, and plants were maintained in a growth chamber. Ten plants of each line were used for each inoculum concentration, and observations were made beginning 3 days after inoculation and continuing daily for 10 days. The data represent the percentage of plants showing systemic symptoms at each day. ● = CP(−) control plants; ○ = CP(+) transgenic plants.

Infection of nonrelated viruses on transgenic plants expressing the CP gene of AlMV. To extend these observations to other transgenic plants, tobacco plants (*N. tabacum* 'Samsun') that express the CP gene of AlMV (31) were inoculated with PVX and CMV. As with the TMV CP(+) plants, there was a delay of 1.5 to 3 days in the development of systemic symptoms as compared with control plants (Table 4). However, when these plants were infected with TMV, there was no significant delay in disease development.

DISCUSSION

Previous workers (18,27,31,32) reported that transgenic tobacco plants expressing the CP genes from TMV or AlMV are resistant to infection by TMV or AlMV, respectively; that is, they are protected against the homologous virus. In this study we have demonstrated that transgenic tobacco plants expressing a TMV CP gene show a low level of resistance to, or interference with, systemic spread and disease development when infected with AlMV, CMV, PVX, and PVY, that is, heterologous viruses. However, there was little or no interference with the accumulation of these viruses in the inoculated leaves of transgenic plants. Likewise, transgenic tobacco plants expressing the AlMV CP gene caused a delay in systemic symptom development when infected with CMV and PVX, but not when infected with the U_1 strain of TMV. This result is in agreement with the results of Loesch-Fries et al (18) who also found that transgenic plants expressing the CP gene from AlMV were not delayed in symptom development when infected with TMV.

There are differences as well as similarities between homologous protection and heterologous protection. In homologous protection, there is a reduction in the number of sites of infection on the inoculated leaves (6,14,18,23,31) and a concomitant reduced rate of virus accumulation in those leaves (23). In contrast, inoculation with heterologous viruses led to the accumulation of equal levels of virus in transgenic CP(+), CP(−), and nontransgenic plants.

Systemic spread of virus and the development of disease symptoms on upper leaves was delayed in transgenic CP(+) plants infected with homologous and most heterologous viruses. However, transgenic plants expressing the TMV CP gene showed no statistically reproducible resistance to AlMV (Table 3), and transgenic AlMV CP(+) plants were not resistant to TMV (Table 4) (18). Like protection against homologous viruses, the rate of systemic spread of heterologous viruses was dependent upon virus inoculum concentration. However, protection against the homologous virus was expressed over a wide range of inoculum concentrations (Fig. 2) (3,6,14), whereas protection against heterologous viruses was limited to a narrow range of inoculum concentrations. Because of the differences in the degree of resistance, it is unlikely that the mechanism of protection against homologous and heterologous viruses is identical. The differences, however, could reflect differences in degrees of the same basic mechanism. Other studies are in progress to further characterize the protection against systemic spread of viruses in transgenic CP(+) plant lines.

Two types of experiments reflect the specificity of the protection. First, protection against both homologous and heterologous viruses was found in all plant lines tested (described in the Results section). However, only plant lines expressing the CP gene were protected; lines expressing the CAT gene or harboring the intermediate plasmid sequences without an additional gene were not protected. Second, protection apparently was limited to viruses; the bacterium *P. s. pisi*, which induces a hypersensitive response on tobacco, was equally inductive on both CP(+) and CP(−) plant types.

Other workers have reported that infections with viruses and other pathogens can induce a generalized resistance response that is associated with the accumulation of pathogenesis-related proteins (15,34) or an antiviral factor (1,30). Ross (28) suggested that an induced resistance would be effective against a challenge virus either related or unrelated to the virus that induced the protection. We have not determined whether the protection against infection or systemic spread of homologous or heterologous viruses is related to the induced resistance described by these workers.

TABLE 4. Systemic disease symptoms on plants that express the coat protein (CP) gene of alfalfa mosaic virus (AlMV) (CP+) or do not express the CP gene of AlMV (CP−)

Virus	Inoculum concentration (μg/ml)	Plant type[a]	Plants (%) showing symptoms: 6 days pi[b]	7 days pi	8 days pi	10 days pi	12 days pi
Potato virus X	0.01	CP− (5)	60	80	80	100	100
		CP+ (10)	0	0	0	70	100[c]
	0.5	CP− (5)	100	100	100	100	100
		CP+ (10)	40	50	70	100	100
Cucumber mosaic virus	0.01	CP− (5)	60	80	100	100	100
		CP+ (10)	20	20	40	90	100[d]
	0.5	CP− (5)	60	80	100	100	100
		CP+ (10)	20	40	40	100	100[d]
Tobacco mosaic virus U_1	0.001	CP− (8)	25	25	38	38	50
		CP+ (14)	14	29	29	36	50
	0.01	CP− (13)	92	92	92	92	92
		CP+ (24)	63	79	88	88	88
	0.1	CP− (5)	100	100	100	100	100
		CP+ (8)	88	100	100	100	100

[a] Plants were inoculated with increasing concentrations of potato virus X, cucumber mosaic virus, or the U_1 strain of tobacco mosaic virus observed for symptom development. CP− indicates progeny of plant line 6216 which were transformed with the vector lacking the CP gene of AlMV. CP+ represents progeny from three lines of transformed plants, 6100, 6103, 6342, that expressed the CP gene of AlMV.

[b] pi = postinoculation.

[c] A delay in symptom development is significant at the 0.025 confidence level as calculated by the Mann-Whitney U-test.

[d] A delay in symptom development is significant at the 0.05 confidence level (determined by Mann-Whitney U-test).

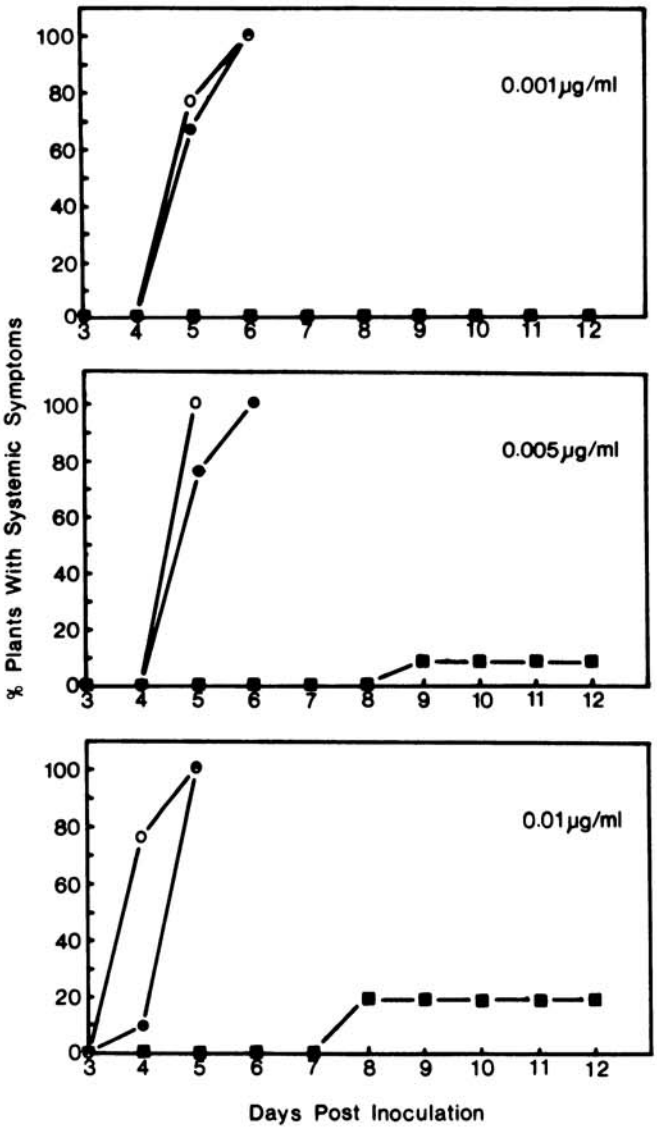

Fig. 3. Systemic disease symptom development on coat protein (CP) (−) (line 306), CP(+) (line 3404), and chloramphenicol acetyltransferase (CAT) (+) (line 37-B) transgenic plants that had been inoculated with three concentrations of the U_1 strain of tobacco mosaic virus. Nine plants of each line were used at each inoculum concentration, and observations were made beginning 3 days after inoculation and continuing until day 12. The points represent the percentage of plants showing systemic symptoms at each day. ○ = CAT(+) plants; ● = CP(−) plants; and ■ = CP(+) plants.

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