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Transgenic Tobacco Plants Expressing a Coat Protein Gene of Tobacco Mosaic Virus Are Resistant to Some Other Tobamoviruses

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Transgenic tobacco plants expressing the coat protein (CP) gene of tobacco mosaic virus were tested for resistance against infection by five other tobamoviruses sharing 45–82% homology in CP amino acid sequence with the CP of tobacco mosaic virus. The transgenic plants (CP^+) showed significant delays in systemic disease development after inoculation with tomato mosaic virus or tobacco mild green mosaic virus compared to the control (CP^-) plants, but showed no resistance against infection by ribgrass mosaic virus. On a transgenic local lesion host, the CP^+ plants showed greatly reduced numbers of necrotic lesions compared to the CP^- plants after inoculation with tomato mosaic virus, pepper mild mottle virus, tobacco mild green mosaic virus, and Odontoglossum ringspot virus but not ribgrass mosaic virus. The implications of these results are discussed in relation to the possible mechanism(s) of CP-mediated protection.

Additional keywords: amino acid sequence homology, systemic symptoms.

Powell Abel *et al.* (1986) reported that transgenic tobacco plants which express the coat protein (CP) gene of tobacco mosaic virus (TMV) are resistant to infection by TMV. CP-mediated protection, as this type of resistance is termed, was later confirmed with a number of other viruses (Loesch-Fries *et al.* 1987; Van Dun *et al.* 1987, 1988; Van Dun and Bol 1988; Hemenway *et al.* 1988; Cuozzo *et al.* 1988; Hoekema *et al.* 1989; Stark and Beachy 1989). CP-mediated protection confers resistance against infection by the homologous virus (the virus from which the gene was isolated) but provides little (Anderson *et al.* 1989) or no protection against unrelated viruses (Loesch-Fries *et al.* 1987; Van Dun *et al.* 1988).

CP-mediated protection is also effective against closely related strains or viruses. For example, the CP of TMV (U1 strain) protects against infection by the severe strain of TMV, PV230 (Nelson *et al.* 1987), and against tomato mosaic virus (ToMV) (Nelson *et al.* 1988). Van Dun and Bol (1988) showed that the CP of tobacco rattle virus, strain TCM, protects against infection by TCM but not against infection by the PLB strain with which it shares only 39% homology in CP amino acid sequence. On the other hand, the CP of TCM protects against infection by an isolate of pea early-browning virus that shares a high degree of homology with the CP of TCM.

Stark and Beachy (1989) showed that the CP gene of soybean mosaic virus (SMV) expressed in transgenic tobacco plants confers high protection against infection by two other potyviruses, tobacco etch virus and potato virus Y. Tobacco etch virus shares 61% and potato virus Y shares 58% homology in CP amino acid sequence with SMV. These plants were not protected against TMV.

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Based upon these reports, one can suggest that to obtain high levels of CP-mediated protection a minimum degree of CP amino acid sequence homology is required between the protecting CP and the CP of the challenge virus. To address this hypothesis, we examined the degree of protection obtained with the CP of TMV expressed in transgenic tobacco plants against five other tobamoviruses sharing from 45 to 82% sequence homology with the CP of TMV.

MATERIALS AND METHODS

Plant material. Tobacco (*Nicotiana tabacum* L.) cultivars Xanthi and Xanthi-nc were used as systemic and local lesion hosts, respectively. Transgenic plants of Xanthi tobacco expressing the TMV CP gene (CP^+) are progeny of lines 3646 and 3404 (Powell Abel *et al.* 1986) and Xanthi-nc line 748 (Nelson *et al.* 1987). CP^- (nontransformed) Xanthi and Xanthi-nc were used as controls. Plants were grown in a greenhouse under natural lighting conditions, supplemented in the winter with artificial light to provide 14-hr days. Plants at 25–27 days after seed germination (systemic host) or 35–40 days after germination (local lesion host) were used in all experiments.

Sources and purification of viruses and RNA. Strain L of ToMV was obtained from M. Zaitlin (Cornell University, Ithaca, NY). Tobacco mild green mosaic virus (TMGMV) or the U2 strain of TMV (Wetter 1986); Odontoglossum ringspot virus (ORSV), also known as the orchid strain of TMV (Edwardson and Zettler 1986); and ribgrass mosaic virus (RMV) were obtained from the American Type Culture Collection (ATCC PV228, PV318, and PV229, respectively.) Pepper mild mottle virus (PMMV) was kindly supplied by B. L. Subba Rao (Chemtex, Port Arthur, TX). All viruses were passed through a single local lesion host and subsequently propagated in an appropriate systemic host as indicated in Table 1. All viruses were purified as described by Asselin and Zaitlin (1978). RNA was isolated as described by Bruening *et al.* (1976).

Plant inoculation. Two leaves of each plant were dusted with Carborundum (330 grit, Fisher Scientific, Fair Lawn, NJ) and inoculated with purified virus diluted in 20 mM potassium phosphate, pH 7.2, 1 mM EDTA. After inoculation, the plants were rinsed with water. Plants of Xanthi tobacco were scored as diseased when vein-clearing symptoms were observed on leaves above the inoculated leaves. Necrotic lesions were scored 3–4 days after inoculation. Necrotic rings (following inoculation with ORSV) were scored 10 days after inoculation.

RESULTS

TMV CP protection against ToMV. Nelson *et al.* (1988) reported that tomato plants which express the TMV (U1 strain) CP gene are resistant to infection by the L strain of ToMV. The CP sequence of ToMV has 82% amino acid sequence homology with the CP sequence of TMV (Gibbs 1986). To quantitate the degree of resistance of tobacco plants that express the CP gene of TMV to infection by ToMV, seedlings of CP^+ lines 3646 and 3404 were inoculated with increasing concentrations of ToMV-L. The results summarized in Figure 1 show that the CP^+ plants are resistant to disease development after inoculation with ToMV. Most of the CP^- plants developed systemic disease symptoms within 7 days after inoculation with 0.01 μg of ToMV per milliliter. On the other hand, the CP^+ plants developed disease symptoms by 14 days after inoculation when inoculated with virus concentrations of 100 μg/ml but not 10 μg/ml (Fig. 1).

Following inoculation with ToMV, local lesion host CP^+ plants (line 748) showed less than 1% of the necrotic lesions shown on the control plants (Table 2). No additional lesions were detected by the starch lesion assay (Thomas and Zielinska 1983). The CP^+ plants were less resistant to infection by ToMV RNA and showed 77% of the necrotic lesions shown on the CP^- plants (Table 2). Similar results were obtained when CP^+ plants were inoculated with TMV RNA (Nelson *et al.* 1987).

TMV CP protection against PMMV. Although the CP amino acid sequence of PMMV has not yet been determined, it has been reported that based upon amino acid composition PMMV is less closely related to TMV than is ToMV (Gibbs 1986; Wetter *et al.* 1984). PMMV does not produce systemic disease on Xanthi tobacco (Wetter *et al.* 1984) but produces local lesions on Xanthi-nc. As shown in Table 2, PMMV produced less than 1% of the necrotic lesions on CP^+ as were produced on the CP^- plants. When PMMV RNA was used as the inoculum, CP^+ plants were less resistant and showed 71% of the necrotic lesions shown on the CP^- plants (Table 2). The results of these experiments indicate a high level of resistance against infection by PMMV in plant line 748.

TMV CP protection against TMGMV. Based on the CP amino acid sequence, TMGMV shares about 67% homology with TMV (Gibbs 1986) or 72% as corrected by Altschuh *et al.* (1981) and as derived from nucleic acid sequence analysis (Nejidat and Beachy, unpublished data). TMGMV produces systemic disease symptoms in Xanthi and necrotic local lesions in Xanthi-nc. At least 10^3-fold more virus was needed to produce systemic disease symptoms on CP^+ plants (lines 3646 and 3404) than on CP^- plants at 7 or 14 days after inoculation (Fig. 2). In

Table 1. Plant hosts used to propagate viruses

Virus[a]	Local lesion host	Propagation host
ToMV	*Nicotiana tabacum* cv. Xanthi-nc	*N. tabacum* cv. Xanthi
PMMV	*N. tabacum* cv. Xanthi-nc	*Capsicum frutescens* cv. Long Red Cayenne
TMGMV	*N. sylvestris*	*N. tabacum* cv. Xanthi
ORSV	*N. tabacum* cv. Xanthi-nc	*N. benthamiana*
RMV	*N. tabacum* cv. Xanthi-nc	*N. tabacum* cv. Xanthi

[a]ToMV, tomato mosaic virus; PMMV, pepper mild mottle virus; TMGMV, tobacco mild green mosaic virus; ORSV, Odontoglossum ringspot virus; and RMV, ribgrass mosaic virus.

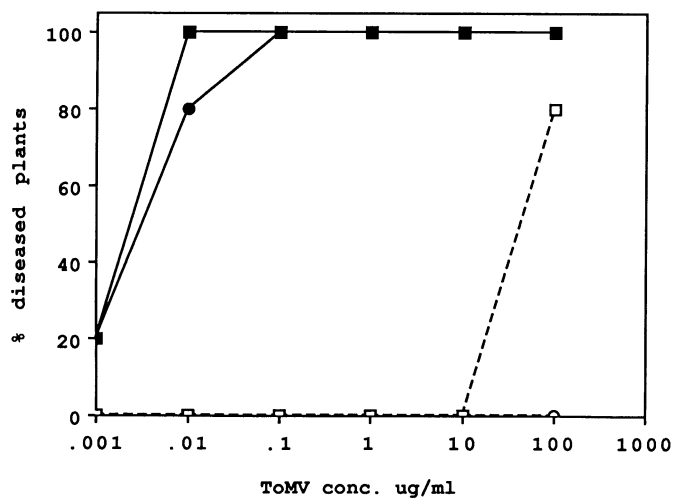

Fig. 1. Disease development in CP^+ (lines 3646 and 3404) and CP^- tobacco plants after inoculation with tomato mosaic virus (ToMV). Disease symptoms were scored daily; the results show the percentage of plants that developed systemic disease symptoms at 7 and 14 days after inoculation. At least 20 plants were inoculated with each virus concentration. (—●—) CP^-, 7 days after inoculation; (—■—) CP^-, 14 days after inoculation; (- - -○- - -) CP^+, 7 days after inoculation; and (- - -□- - -) CP^+, 14 days after inoculation.

Table 2. The number of necrotic lesions on control (CP^-) and transgenic (CP^+) tobacco plants (line 748) after inoculation with different tobamoviruses or their RNAs

Inoculum[a]	Concentration (μg/ml)	Necrotic lesions per plant[b] CP^-	Necrotic lesions per plant[b] CP^+	%[c]
ToMV	1.0	771 ± 66	1	0.13
ToMV RNA	10.0	191 ± 31	148 ± 17	77.0
PMMV	1.0	308 ± 12	1	0.3
PMMV RNA	10.0	380 ± 49	272 ± 25	71.0
TMGMV	1.0	906 ± 103	1	0.1
TMGMV RNA	10.0	264 ± 42	107 ± 19	40.0
RMV	0.01	13 ± 2	6 ± 1	46.0
	0.1	143 ± 19	62 ± 7	43.0

[a]Abbreviations are as given in Table 1.
[b]For each treatment, the number of necrotic lesions were scored on two leaves of each of five plants at 4 days after inoculation. The numbers are mean ± SE. The results are representative of four repeats at different concentrations with similar results.
[c]The numbers are the percentage of the necrotic lesions produced on the CP^+ plants compared to those produced on the CP^- control plants.

addition, very low numbers of local necrotic lesions were produced on CP^+ plants even when the virus inoculum produced more than 1,000 lesions on control plants (Table 2). No additional microscopic or starch lesions were detected on the CP^+ plants. The protection against infection in line 748 is partially but not fully overcome by inoculation with TMGMV RNA (Table 2). Line 3646 showed a 2-day delay in disease symptom development when inoculated with 10 μg/ml of TMGMV RNA (Fig. 3), a level of inoculum that caused high numbers of necrotic lesions on the local lesion host (Table 2).

TMV CP protection against ORSV. The CP amino acid sequence of ORSV shares approximately 60% sequence homology with TMV (Gibbs 1986). ORSV is incapable of systemic infection of Xanthi tobacco and produces local necrotic rings and local necrotic lesions on the inoculated leaves of Xanthi-nc. The necrotic local lesions occur primarily on older inoculated leaves. Transgenic tobacco plant line 748 showed 0–20% of the necrotic rings shown on the CP^- plants (Table 3). The data in Table 3 are the results of two independent experiments, but similar results were obtained from other experiments.

Under greenhouse conditions the necrotic lesions appeared at 6 days after inoculation, and necrotic rings formed 10 days after inoculation on both CP^- and CP^+ plants. In some experiments additional chlorotic or necrotic rings were produced on the CP^+ plants at 20 days after inoculation. Virus extracts from chlorotic lesions induced necrotic rings on CP^- plants typical of infection by ORSV (data not shown).

TMV CP protection against RMV. The CP sequence of RMV has 45% amino acid sequence homology with the CP of TMV (Gibbs 1986). RMV produces systemic disease symptoms in Xanthi and necrotic local lesions in Xanthi-nc. After inoculation with RMV, the CP^+ plants showed only a 1-day delay in systemic disease development compared to the CP^- plants after inoculation with low but not high concentrations of inoculum (Table 4). On the local lesion host, the CP^+ plants showed about 45% of the necrotic lesions shown on the CP^- control plants (Table 2).

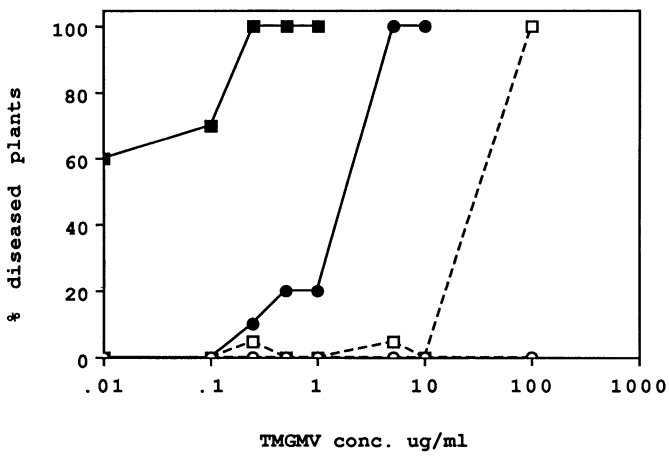

Fig. 2. Systemic disease development on CP^+ and CP^- tobacco plants after inoculation with tobacco mild green mosaic virus (TMGMV). The plants were inoculated and scored as described in Figure 1. (—●—) CP^-, 7 days after inoculation; (—■—) CP^-, 14 days after inoculation; (- - -○- - -) CP^+, 7 days after inoculation; and (- - -□- - -) CP^+, 14 days after inoculation.

DISCUSSION

Following the report of Powell Abel *et al.* (1986) of CP-mediated protection against infection by TMV in transgenic tobacco plants, a number of other research groups showed that expression of a viral CP gene in transgenic plants confers resistance to the homologous virus (Loesch-Fries *et al.* 1987; Van Dun *et al.* 1987, 1988; Hemenway *et al.* 1988; Cuozzo *et al.* 1988; Hoekema *et al.* 1989).

The results of our study show that the CP of TMV confers a high degree of protection against tobamoviruses having different levels of amino acid sequence homology with the CP of TMV. In the CP^+ plant lines (3646 and 3404) of the systemic host, 10^3 to 10^4 greater concentrations of ToMV and TMGMV were needed to cause disease symptoms as compared to CP^- plants (Figs. 1 and 2). These viruses share, respectively, 82 and 72% homology in the CP amino acid sequence with TMV. This high degree of protection is essentially the same as reported for the protection against TMV (Beachy 1988). Protection is somewhat less complete against ORSV (60% homology) where the CP^+ plants showed up to 20% of the necrotic rings shown

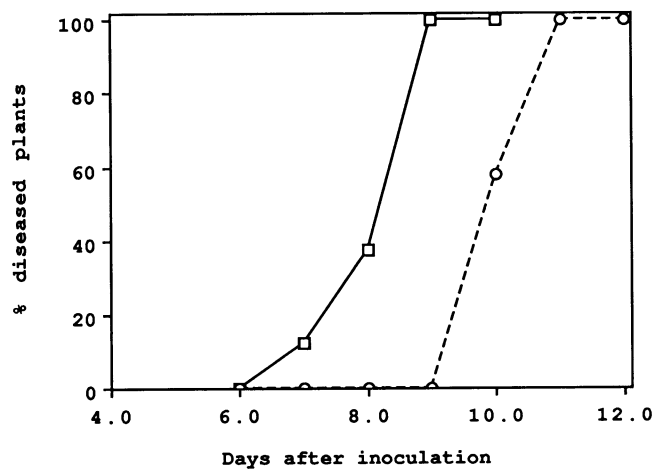

Fig. 3. Systemic disease development in CP^+ (line 3646) and CP^- tobacco plants after inoculation with 10 μg/ml of tobacco mild green mosaic virus RNA. (—□—) CP^- and (- - -○- - -) CP^+.

Table 3. The number of necrotic rings on control (CP^-) and transgenic (CP^+) tobacco plants (line 748) after inoculation with Odontoglossum ringspot virus

Virus concentration (μg/ml)	Experiment 1		Experiment 2	
	Necrotic rings per plant[a]		Necrotic rings per plant[a]	
	CP^-	CP^+	CP^-	CP^+
0.25	120 ± 15	25 ± 3	180 ± 31	2
0.5	217 ± 20	25 ± 10	286 ± 18	0
2.0	...	...	514 ± 94	69 ± 34

[a]For each virus concentration, the number of necrotic rings were scored on two leaves of each of five plants at 10 days after inoculation. The numbers are mean ± SE.

on the CP^- plants (Table 3). No protection was observed against RMV in the CP^+ systemic host plants (Table 4), while the local lesion CP^+ plants showed about 45% of the local lesions shown on the CP^- plants (Table 2).

The TMV CP-mediated protection against ToMV, PMMV, and TMGMV is largely (but not fully) overcome when viral RNA was used as the inoculum (Table 2) as was reported for TMV RNA (Nelson *et al.* 1987). However, it is worth noting that when the CP^+ plants became systemically infected due to high levels of inoculum (Figs. 1 and 2) or after inoculation with viral RNA (Fig. 3), the symptoms produced were much less severe than those on CP^- plants (data not shown).

Although inoculation with viral RNA significantly overcomes the protection in the inoculated leaves (as indicated by the number of necrotic lesions), the systemic host shows significant delay in systemic disease development, even when TMGMV RNA is used (Fig. 3). These results support the suggestion of multiple levels of protection in CP^+ plants against TMV (Wisniewski *et al.*, in press) and other tobamoviruses. However, although the CP^+ plants showed only 45% of the necrotic lesions shown on the CP^- plants (Table 2) following inoculation with RMV, there were only slight delays in systemic disease development (Table 4) and the severity of the disease was the same on both plant types (data not shown). This may indicate that low numbers of infection sites following inoculation with RMV are sufficient to overcome the initial protection and that there is little resistance against the systemic spread of RMV as compared to the other viruses tested.

Stark and Beachy (1989) reported that when the CP gene of SMV, a potyvirus which is nonpathogenic to tobacco, was expressed in transgenic tobacco plants, the plants had a high degree of protection against tobacco etch virus and potato virus Y, two potyviruses which share approximately 60% homology in CP amino acid sequences with the CP of SMV. From the data of Stark and Beachy (1989) and the data presented here, it is difficult to conclude whether the absolute amino acid sequence homology of the "protecting" CP with the CP of the challenger virus or sequence homology within particular regions of the CP is most important for protection. Indeed, it is possible that structural similarities, as determined by conserved primary amino acid sequences, are important in conferring protection against infection. Thus the CP of TMV provides good protection against ORSV (60% homology) but poor protection against RMV (45% homology) and sunn-hemp mosaic virus (Cc-TMV), which shares about 38% homology with the CP of TMV (Anderson *et al.* 1989). It is tempting to suggest that the approximately 60% homology in the CP amino acid sequences of tobamoviruses and potyviruses is sufficient to confer significant structural similarity to provide protection against related viruses.

Register and Beachy (1988) reported that the CP of TMV interferes with an early event in infection by TMV which normally leads to the uncoating of viral RNA. It is, therefore, suggested that 60% homology between the protecting CP expressed in the transgenic plants and the CP of the challenger virus provides sufficient conformational similarity for the protecting CP to block the uncoating process. Whether the protecting molecule is the subunit form of the CP or aggregated states of the CP (that is, small aggregates or helical rodlets of CP) remains to be determined. It is noteworthy that Wilson (1989) recovered rodlets of TMV CP from CP^+ line 3404.

Although the mechanism of CP-mediated protection requires additional study, our results confirm those from other studies (Van Dun and Bol 1988; Stark and Beachy 1989) and show that a single type of CP can confer resistance against several members of a virus group.

Table 4. Systemic disease development in control (CP^-) and transgenic (CP^+) tobacco plants (line 3636) after inoculation with ribgrass mosaic virus

Plant type	Inoculum (μg/ml)	Days after inoculation 4	5 (% diseased plants)[a]	6
Xanthi CP^-	0.01	0	30	100
	0.1	0	100	100
	1.0	0	100	100
	10.0	0	100	100
Xanthi CP^+	0.01	0	0	100
	0.1	0	0	100
	1.0	0	80	100
	10.0	0	100	100

[a] For each virus concentration, 10 plants were inoculated and scored daily for systemic disease symptoms.

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