Coat Protein-Mediated Resistance in Transgenic Tobacco Expressing the Tobacco Mosaic Virus Coat Protein from Tissue-Specific Promoters

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Received 19 October 1992. Accepted 1 March 1993.

Coat protein-mediated resistance (CP-MR) was studied in transgenic *Nicotiana tabacum* 'Xanthi nn' and 'Xanthi NN' that express chimeric tobacco mosaic virus (TMV) coat protein (CP) gene constructs using two different tissue-specific promoters. The *Phaseolus vulgaris pal2* promoter leads to gene expression in the upper leaf epidermis and the xylem, while the *rolC* promoter from *Agrobacterium rhizogenes* leads to gene expression in phloem and leaf hair tip cells. Tissue-specific gene expression was verified using the gusA(*uidA*) reporter gene, while accumulation of TMV CP was verified by Western blot analysis. Transgenic Xanthi nn plants harboring the *pal2*-CP gene construct were partially resistant to TMV infection. On Xanthi NN plants that expressed the *pal2*-CP gene construct, fewer necrotic lesions were formed after TMV inoculation compared to nontransformed control plants. The level of resistance, however, was substantially less than in plant lines that expressed TMV CP from the cauliflower mosaic virus 35S promoter. By contrast, expression of the *rolC*-CP construct did not confer resistance in either Xanthi nn or Xanthi NN. The results provide further evidence that CP-MR to systemic TMV infection in tobacco is probably due to inhibition of infection rather than to effects on long-distance spread through the phloem.

Transgenic tobacco plants expressing tobacco mosaic virus (TMV) coat protein (CP) were shown to be resistant to TMV infection by Powell-Abel *et al.* (1986). The resistance was subsequently termed "coat protein-mediated resistance" (CP-MR) and has been demonstrated to be effective in a variety of host/virus combinations (Beachy *et al.* 1990). However, the mechanism(s) of virus resistance in transgenic plants is not yet completely understood. Furthermore, it is likely that there are different mechanisms of resistance in different host/virus combinations and that there may be more than one mechanism in a given system.

Most of the studies on the mechanism(s) of CP-MR have been carried out with the tobacco/TMV system. The systemic infection of tobacco plants with TMV proceeds in three phases, beginning with introduction of the virion into a wounded cell and disassembly of the virus particle, translation and replication of the viral genome, and the formation of progeny virions. The infection then spreads locally from the inoculated cell to adjacent cells through the plasmodesmata. This step involves a virus-encoded protein, the 30-kDa "movement protein" (Deom *et al.* 1987; Watanabe *et al.* 1987). Finally, systemic spread throughout the plant requires the assembly of virions (Saito *et al.* 1990) which probably move passively through the phloem (Esau and Cronshaw 1967). Tobacco plants bearing the N gene from *Nicotiana glutinosa* are resistant to systemic infection by TMV and react in a hypersensitive manner with the formation of necrotic lesions at the sites of initial infection (Weststeijn 1981).

CP-MR against TMV in tobacco plants results in a reduction of local necrotic lesions on leaves of N gene-bearing tobacco plants (Nelson *et al.* 1987) and a lower proportion of nn-genotype plants that become systemically infected (Powell-Abel *et al.* 1986). Neither of these effects occur when the plants are inoculated with TMV RNA rather than virions. There is, however, a delay in the development of systemic symptoms in transgenic plants inoculated with TMV RNA and in those plants that become infected upon inoculation with virions (Wisniewski *et al.* 1990). The delay could be explained by interference with phloem transport by the CP expressed in the transgenic plant. Alternatively, phloem loading may be delayed because there is slower spread through the inoculated leaf, and/or virus may unload slowly from phloem, resulting in reduced infection of upper leaves.

Accumulation of TMV and viral RNA is inhibited in inoculated protoplasts prepared from transgenic tobacco plants that express the TMV CP gene (CP[+]). When TMV RNA or partially disassembled virions were used as inoculum, CP-MR was overcome (Register and Beachy 1988). This suggests that one component of CP-MR is the inhibition of uncoating of the viral RNA. Nevertheless, the accumulation of TMV in transgenic protoplasts inoculated with up to 40 μg of viral RNA per milliliter is delayed (Register and Beachy 1988), suggesting that inhibition of the uncoating is not the only step in replication that is affected in CP-MR.

In most reported cases of CP-MR, the chimeric gene constructs included the promoter for the 35S transcript of cauliflower mosaic virus (CaMV). This promoter leads to high-level constitutive expression with little tissue spe-

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MPMI Vol. 6, No. 3, pp. 323-330

cificity. In previous work by Clark *et al.* (1990) the *Petunia* ribulose bisphosphate carboxylase small subunit (*rbcS*) promoter was used to drive CP gene expression. The *rbcS* promoter is active primarily in mesophyll cells.

Comparison of CP-MR between plant lines that accumulated similar amounts of CP from the 35S or the *rbcS* promoters showed that the 35S-CP gene construct conferred a much higher level of protection in whole plants than the *rbcS*-CP gene construct. Resistance was assessed as the percentage of plants showing systemic symptoms and the rate of symptom development. In contrast, accumulation of TMV was inhibited to an equivalent extent in mesophyll protoplasts prepared from either type of transgenic plant, suggesting that the different levels of resistance in whole plants are due to different patterns of expression by the two promoters.

In the current study we used two promoters with different patterns of tissue-specific expression to elucidate further the nature of CP-MR. The phenylalanine ammonia lyase (PAL) gene 2 promoter from *Phaseolus vulgaris* has been shown to drive expression of the reporter gene β-glucuronidase (*gusA*) in the upper epidermis and xylem of transgenic tobacco plants (Bevan *et al.* 1989; Liang *et al.* 1989). The *rolC* gene promoter of *Agrobacterium rhizogenes* is active primarily in phloem cells (Schmülling *et al.* 1989; Sugaya *et al.* 1989). Here, we investigated whether low level, but specific expression of the CP gene either in the epidermis and xylem or in the phloem of transgenic tobacco is sufficient to block or delay local and systemic infection by TMV.

RESULTS

Description of the transgenic plants.

Chimeric genes were constructed to express the CP or the reporter gene *gusA* under the control of the tissue-specific promoters of the *pal2* or *rolC* genes. To investigate the effects of CP accumulation on systemic and local infection of tobacco by TMV, the genes were introduced into plants of the systemically susceptible host *N. tabacum* 'Xanthi nn' and into the near-isogenic *N. tabacum* 'Xanthi NN'. Nontransgenic Xanthi nn plants develop mosaic symptoms on young leaves 4–5 days after inoculation, depending on environmental conditions. Xanthi NN plants begin to form local necrotic lesions on the inoculated leaf 40–48 hr after inoculation, confining the infection to the inoculated leaf. The plant lines that were used are described in Table 1.

Heterozygous R_1 progeny plants of the primary transformants that contained the *pal2*-CP or *rolC*-CP gene constructs were used in the inoculation experiments. Plants that accumulated NOS generally also accumulated CP. However, some plants of the lines PCN-NN, RoCN-NN, and RoCN-nn that were NOS (−) contained CP. Perfect cosegregation of NOS and CP accumulation in the R_1 progeny was only found in the PCN-nn lines. In these lines the segregation of NOS accumulation was 3:1, suggesting that the primary transformants contained a single active gene construct. Between 8 and 14 lines were generated for each gene construct. Their phenotypes are described in Table 2. The line 3646 is homozygous for NOS and CP accumulation, the line 306 is homozygous for NOS accumulation, and the line 748 contains more than one active locus of both the nos gene and CP gene construct.

Table 1. Description of the transgenic plant lines used

Designation of plant line	Tobacco cultivar	Gene construct	
		Promoter	Coding sequence
PCN-nn	Xanthi nn	*pal2*	TMV CP
PCN-NN	Xanthi NN	*pal2*	TMV CP
RoCN-nn	Xanthi nn	*rolC*	TMV CP
RoCN-NN	Xanthi NN	*rolC*	TMV CP
PGN-nn	Xanthi nn	*pal2*	*gusA*
PGN-NN	Xanthi NN	*pal2*	*gusA*
RoGN-nn	Xanthi nn	*rolC*	*gusA*
RoGN-NN	Xanthi NN	*rolC*	*gusA*
3646	Xanthi nn	CaMV 35S	TMV CP
748	Xanthi NN	CaMV 35S	TMV CP
G 6-1	Xanthi nn	CaMV 35S	*gusA*
306	Xanthi nn	CaMV 35S	none

Tissue-specific gene expression.

Expression of the reporter gene *gusA* was analyzed in several transgenic plant lines to verify the tissue-specific activity of the *pal2* and *rolC* promoters. Figure 1 shows leaf (A–D) and stem (E,F) cross sections of R_1 progeny after incubation with the GUS substrate X-Gluc. GUS activity resulting from expression of the *pal2* promoter (PGN plants) was restricted to the upper leaf epidermis and the xylem (Fig. 1D,F), whereas GUS activity resulting from expression of the *rolC* promoter (RoGN plants) could only be detected in the phloem and the hair tips (Fig. 1 C,E). In contrast, samples from plants expressing GUS from the CaMV 35S promoter (G 6-1 plants) were intensely stained with no apparent tissue specificity (Fig. 1B). The tissue-specific GUS expression from the *pal2* and *rolC* promoters could also be found in petiole cross sections and in 2- to 3- wk-old seedlings (data not shown). There were no differences in expression of the gene between Xanthi nn and Xanthi NN plant lines.

Induced GUS accumulation could be detected in plants harboring the *pal2-gusA* gene construct. Leaf samples taken 4 and 16 hr after mock-inoculation, TMV inoculation, or punching holes showed increased staining after incubation with X-Gluc (data not shown). The staining was confined to the inoculated leaves and the wound sites, respectively. Wound-induced activity of the *pal2* promoter has been reported earlier (Liang *et al.* 1989).

Transgenic plants that expressed the TMV-CP genes were tested for tissue-specific expression by Western blot analysis (Fig. 2) of total extractable proteins from the upper epidermis of leaves, from whole leaf samples, and from stems of the plant lines PCN-nn-3 and RoCN-NN-8, which

Table 2. Numbers of transgenic plant lines produced (see Table 1) and the phenotypes of the R_1 progeny

Promoter	Host	No. of lines	NOS(+)	TMV CP(+)
pal2	Xanthi nn	9	4	4
pal2	Xanthi-NN	10	3	2
rolC	Xanthi nn	14	7	7
rolC	Xanthi-NN	8	3	7

had the highest CP gene expression levels from the *pal2* and the *rolC* promoters. CP accumulation from the *pal2* and *rolC* promoters was much lower in all tissue samples compared to expression from the CaMV 35S promoter. In stem extracts of plants that expressed the TMV-CP sequence a second protein band of lower molecular weight than the CP reacted with the anti-TMV serum. We do not know whether this was a result of limited proteolysis of the CP. The band did not appear in significant amounts in leaf or epidermis extracts and was not detected in samples of control plants. In plants that expressed the *pal2*-CP construct (PCN lines), the highest levels of CP were detected in the upper epidermis. CP was also detected in samples of epidermal cells of plants that expressed the *rolC*-CP construct (RoCN lines). This could be due to the activity of the *rolC* promoter in hair tip cells that was apparent in the plants expressing the *rolC-gusA* gene construct (Fig. 1C). CP levels due to expression from both the *pal2* and the *rolC* promoters were higher in stem extracts than in total leaf extracts, where they were at or below the limit of detection. Since stems have a much higher proportion of vascular tissue than leaves, this was taken as an indication of tissue-specific activity of the promoters in the CP expressing transgenic plants. Expression patterns of the chimeric genes were not different in transgenic Xanthi nn and Xanthi NN plants.

Studies of CP-MR in Xanthi nn plants.

Four- to six-week-old transgenic Xanthi nn plants of several different parental lines were inoculated with several concentrations of TMV or TMV RNA. Young plants were chosen for these experiments because CP and GUS levels that accumulated due to expression of the *pal2* and *rolC* promoters were higher in younger plants than in older plants. All plants were tested for *nos* gene expression prior to inoculation. In the Xanthi nn lines NOS and CP accumulation cosegregated. In three plant lines that expressed the CP from the *pal2* promoter a lower percentage of the NOS(+)/CP(+) plants showed systemic mosaic symptoms, compared to the NOS(−)/CP(−) plants, at 14 days after inoculation. Table 3 summarizes the results of several experiments. Inoculum concentrations of 10–50 ng of TMV per milliliter were sufficient to infect 100% of the 306 [CP(−)] control plants and the NOS (−) PCN-nn plants and caused the formation of 20–60 local lesions on Xanthi NN leaves. The homozygous line 3646 which expressed the CaMV 35S-CP gene construct was essentially fully protected under these conditions. Of the PCN-nn lines, the line PCN-nn-3 showed the highest level of protection with at least 50% of the plants escaping systemic infection following inoculation with up to 50 ng of TMV per milliliter. Line PCN-nn-3 accumulated the highest levels of CP of the PCN-nn lines tested. Line PCN-nn-9 showed a lower level of CP accumulation and a lesser degree of protection. Inoculation with 100 ng of TMV per milliliter resulted in the breakdown of protection in the PCN-nn plants as well as a higher percentage (20%) of 3646 plants that became infected (Table 3). In one PCN-nn line in which the level of CP was at the limit of detection, and in another line which was NOS(−) and did not accumulate CP, there was no protection (data not shown).

The proportion of CP(+) plants of PCN-nn lines that escaped systemic infection varied between experiments. We attribute this to the heterozygosity of the plant populations and to variations in the inoculation procedure. The application of the inoculum on Carborundum-dusted leaves may occasionally have resulted in local destruction of the epidermal cell layer and infection of underlying palisade mesophyll cells which do not accumulate CP, in the case of plants that expressed the *pal2*-CP gene (Fig. 1D).

There was no TMV accumulation in either inoculated or upper leaves of plants that did not show symptoms, as determined by dot blot analysis of leaf samples collected at 14 days after inoculation (data not shown). In some experiments these plants were kept for up to 25 days and did not develop symptoms during that time.

Systemic mosaic symptoms appeared later on those CP(+) PCN-nn plants that became infected than on infected plants of line 306. Figure 3 shows development of disease symptoms in PCN-nn plants in one experiment. The proportion of plants that developed systemic symptoms did not change beyond 9 days after inoculation, whereas all the control plants showed symptoms by day 5. The time course of disease development varied between experiments depending upon greenhouse conditions. However, delay in symptom development was consistently observed.

When TMV RNA was used as inoculum instead of TMV there was no delay in symptom development, nor any reduction in the proportion of infected plants in the PCN-nn lines compared to the 306 plant line (data not shown). The plant line 3646 showed a 1- to 2-day delay in symptom development after inoculation with TMV RNA, as was reported earlier (Wisniewski *et al.* 1990).

Transgenic plants that expressed the CP from the *rolC* promoter (RoCN-nn lines) were equally susceptible to TMV as the 306 plant line. There was neither a reduction in the percentage of infected plants nor a delay in symptom development regardless of whether TMV or TMV RNA was used as inoculum in any of the seven lines tested (data not shown). Disease development in line RoCN-nn-10 after inoculation with TMV is shown in Figure 3.

Studies of CP-MR in Xanthi NN plants.

The formation of local necrotic lesions upon TMV infection of tobacco plants that carry the N gene has been well documented (Weststeijn 1981). In our studies, the number of local lesions formed was used as a measure of the number of successful infection events on CP(+) plants and nontransgenic Xanthi NN plants. Xanthi NN plants that accumulate high levels of CP from the CaMV 35S promoter (e.g., plant line 748) formed few or no lesions following TMV infection, depending on the concentration of the inoculum (Nelson *et al.* 1987). We studied the effect of expression of the CP gene from the *pal2* and *rolC* promoters on lesion numbers.

Four- to six-week-old transgenic CP(+) Xanthi NN plants were inoculated with TMV or TMV RNA on opposite leaf halves. Two plant lines harboring either the *pal2*-CP gene construct (PCN-NN lines) or the *rolC*-CP gene construct (RoCN-NN lines) were tested. Three days after inoculation the numbers of local lesions were de-

termined. Table 4 shows the results of three experiments. In only one of the experiments (experiment 3) was the infectivity of the RNA inoculum sufficiently high to cause the formation of lesion numbers comparable to the TMV inoculum. PCN-NN plants that were CP(+) showed reduced lesion numbers compared to nontransgenic Xanthi NN plants on the leaf halves that had been inoculated with TMV. The average lesion numbers were reduced by at least 47% on PCN-NN-2 plants and by at least 60% on PCN-NN-4 plants. On RoCN-NN plants (expressing the *rolC*-CP gene construct) the average numbers of lesions formed after TMV inoculation were reduced to a lesser extent (by 32–40%). Very few lesions were formed on the 748 plants which accumulate high levels of TMV-CP from the CaMV 35S promoter.

There was no significant reduction of lesion numbers on CP accumulating plants when TMV RNA was used as inoculum except in experiment 3 (line PCN-NN-2). In this case the PCN-NN-2 plants were slightly smaller than the other plants that were used in the experiment.

DISCUSSION

Previous work has shown that expression of the TMV CP gene in transgenic plants inhibits uncoating of the TMV

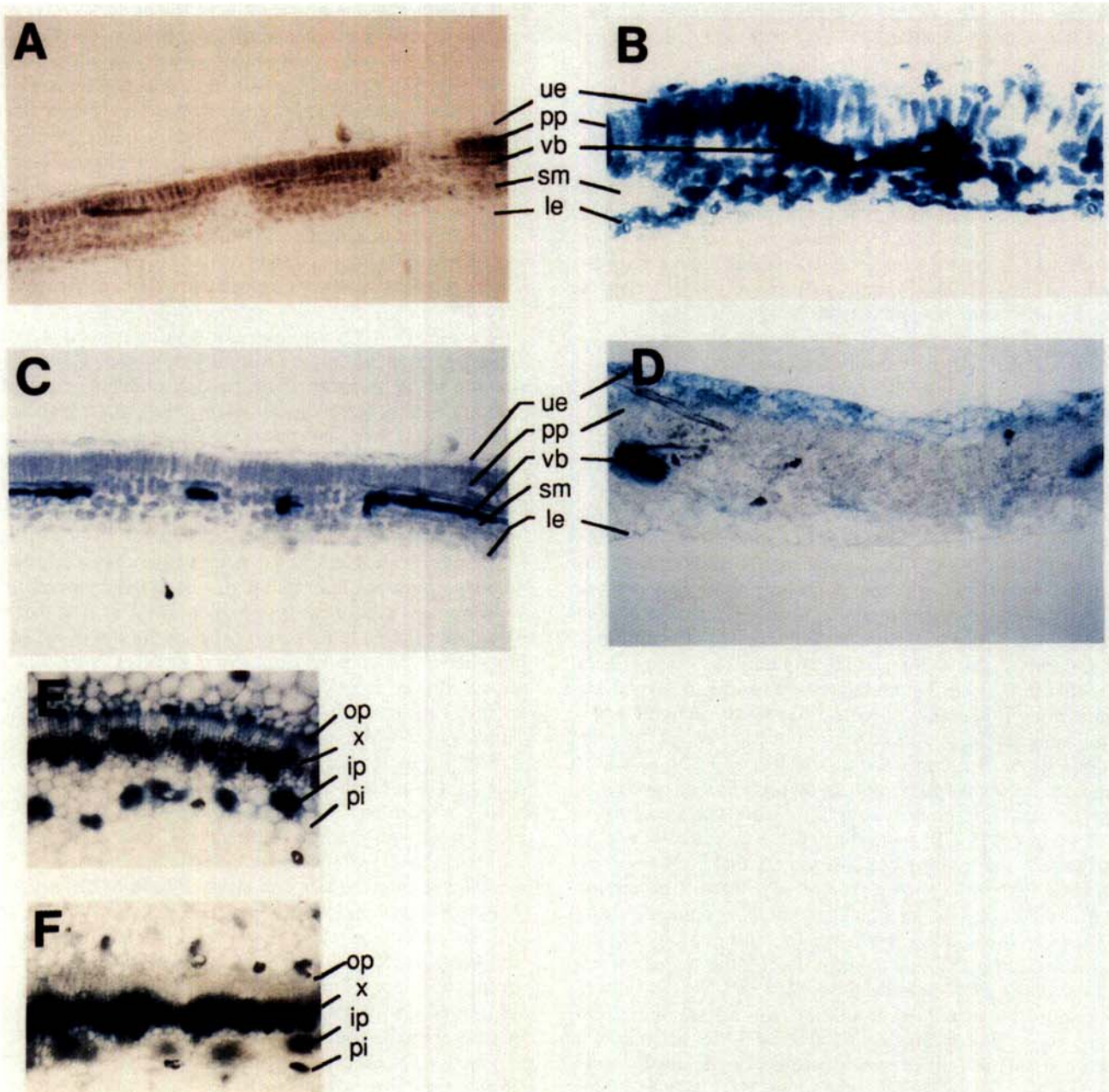

Fig. 1. Histochemical staining for GUS activity in leaf and stem sections of young tobacco plants expressing the *gusA* gene. Leaf **(A–D)** and stem **(E, F)** cross sections of transgenic plants expressing *gusA* from the cauliflower mosaic virus 35S promoter **(B)**, the *rolC* promoter **(C, E)**, or the *pal2* promoter **(D, F)** after incubation with the substrate X-Gluc. **(A)** Control plant (line 306). ip, inner phloem; x, xylem; op, outer phloem; pi, pith; pp, palisade parenchyma; sm, spongy mesophyll; ue, upper epidermis; le, lower epidermis; and vb, vascular bundle.

genome, resulting in inhibition of translation of the viral RNA (Wu *et al.* 1990). Furthermore, virus replication in CP(+) protoplasts infected with TMV RNA is delayed at low inoculum concentrations (Register and Beachy 1988). When transgenic CP(+) plants were inoculated with TMV RNA rather than virions they became systemically infected, although local and systemic spread of the infection was slightly delayed (Wisniewski *et al.* 1990). Here we studied the resistance of transgenic tobacco plants expressing TMV CP under the control of two different tissue-specific promoters to determine the role that the CP plays in initial compared to later events in CP-MR.

Expression of the TMV CP gene in Xanthi nn plants from the *P. vulgaris pal2* promoter resulted in a reduced number of plants that became systemically infected. In the plants that escaped infection there was no accumulation of TMV in either the inoculated leaves or upper leaves. Since the *pal2* promoter is expressed in the upper epidermis and xylem cells, these results could reflect resistance at either or both cell types. Those transgenic plants that became infected presumably contained insufficient amounts of CP to provide protection. It is also possible that the inoculum was introduced into nonexpressing subepidermal cells during the inoculation procedure. From these cells the infection would then spread throughout the plant.

In contrast, transgenic plants expressing the TMV CP gene from the *rolC* promoter did not escape systemic infection and did not show any delay in disease development. Apparently, expression of CP in the phloem alone is not sufficient to interfere with infection or disease development. It is possible, however, that expression levels in the phloem were insufficient to be effective. While the staining of the phloem in plants expressing the GUS gene from the *rolC* promoter appeared to be intense, we were unable to quantify CP or GUS expression levels in the phloem between plants bearing the different promoter constructs since we could not isolate the phloem for analysis.

A delay in disease development following inoculation with TMV RNA could only be seen in plant line 3646,

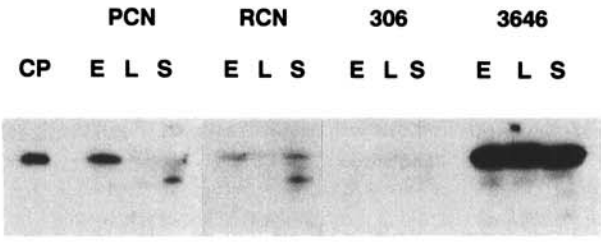

Fig. 2. Expression of tobacco mosaic virus coat protein (TMV CP) in transgenic plants. Total proteins extracted from the upper epidermis (E), whole leaves (L), and stems (S) of transgenic plants expressing TMV CP from the *pal2* promoter (plant line PCN-nn-3, PCN), the *rolC* promoter (plant line RoCN-NN-8, RCN), or the cauliflower mosaic virus 35S promoter (3646) have been separated by sodium dodecyl sulfate-polyacrylamide gel, transferred to nitrocellulose, and immunostained with anti-TMV serum. Samples taken from 10 plants of each line were combined and equal amounts of protein (90 g) or 10 ng of TMV CP (CP) were loaded on each lane. The control plants (306) do not carry a TMV CP gene construct.

Table 3. Evaluation of coat protein-mediated resistance in transgenic Xanthi nn plants

Plant line[a]	No. NOS(+) plants inoculated	Inoculum ng/ml TMV	% infected[b] NOS(+) plants
PCN-nn-3	10	10	30.0
PCN-nn-3	67	50	47.8
PCN-nn-3	28	100	82.1
PCN-nn-4	27	50	77.8
PCN-nn-4	14	100	100.0
PCN-nn-9	28	50	67.9
3646	10	10	0.0
3646	60	50	1.7
3646	15	100	20.0
306	10	10	100.0
306	51	50	100.0
306	15	100	100.0

[a] The plant lines are described in Table 1.
[b] Plants which showed systemic symptoms and virus accumulation at 14 days after inoculation.

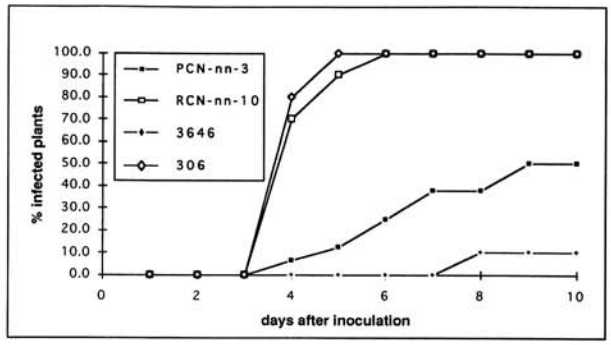

Fig. 3. Time course of appearance of systemic symptoms on CP(+) and CP(−) plant lines. CP(+)/NOS(+) progeny of the transformants PCN-nn-3 (-■-) expressing the tobacco mosaic virus (TMV CP) from the *pal2* promoter and RoCN-nn-10 (-□-) expressing the TMV CP from the *rolC* promoter were inoculated with 50 ng of TMV per milliliter. Other plant lines express no TMV CP (line 306, -◇-) or high levels of TMV CP (line 3646, -◆-). The plants were inspected daily for appearance of disease symptoms. Sixteen plants of the line PCN-nn-3, and 10 plants each of the other lines were inoculated.

Table 4. Evaluation of coat protein-mediated resistance in transgenic Xanthi NN plants

Exp.	Plant line[a]	No. of plants	No. of lesions/half leaf TMV inoc.[b]	No. of lesions/half leaf RNA inoc.[c]
1	PCN-NN-2	7	47.0 ± 26.4[d]	15.7 ± 9.8
	PCN-NN-4	9	33.0 ± 23.6[e]	8.2 ± 7.1
	Xanthi NN	6	82.0 ± 21.0	10.8 ± 2.9
2	PCN-NN-2	10	3.6 ± 3.8[e]	11.4 ± 4.8
	PCN-NN-4	9	13.9 ± 13.6[e]	19.0 ± 11.2
	RoCN-NN-2	5	30.4 ± 32.3	20.0 ± 14.6
	RoCN-NN-3	5	32.0 ± 8.4[e]	8.4 ± 5.8
	Xanthi NN	3	52.7 ± 19.5	4.3 ± 2.5
3	PCN-NN-2	10	5.9 ± 5.8[e]	56.5 ± 26.0[e]
	PCN-NN-4	10	25.2 ± 15.7[e]	95.5 ± 24.5
	RoCN-NN-2	10	79.4 ± 48.6[d]	69.2 ± 36.7
	RoCN-NN-3	10	79.2 ± 27.5[e]	97.4 ± 37.6
	Xanthi NN	10	116.3 ± 25.5	96.8 ± 26.3
	748	10	1.7 ± 2.4[e]	114.0 ± 23.9

[a] The plant lines are described in Table 1.
[b] The inoculum concentration was 0.2 μg TMV/ml.
[c] The inoculum was 20 μg TMV RNA/ml.
[d] Significant reduction of lesion numbers compared to Xanthi NN at $P < 0.05$ based on analysis of variance (student's *t*-test).
[e] Significant reduction at $P < 0.01$.

which accumulated high levels of CP from the CaMV 35S promoter. This could be due to interference by CP with long-distance movement in the phloem. However, reduced systemic spread in line 3646 might also be due to reduction in numbers of infection sites, reduced spread from the infected mesophyll cells into the phloem, or reduced spread from phloem cells into the uninfected cells in upper leaves.

Transgenic Xanthi NN plants that expressed TMV CP from the *pal2* promoter formed fewer local lesions following inoculation with TMV than the nontransgenic controls (Table 4). This could reflect reduced infection or replication in the infected cell or reduced spread to adjacent cells. The result again indicates that expression of CP in the epidermis is responsible for the decrease in TMV infection. There was considerable variability in the numbers of lesions produced on individual plants. This may have been a result of different expression levels of CP in the heterozygous plant populations and inconsistencies in the inoculation procedure. There were also some differences in the sizes of the inoculated leaves on the plants tested. While there appeared to be a reduction in lesion numbers on leaves of PCN-NN plants inoculated with TMV RNA, this reduction was not significant (using the Student's *t* test), except in one case (Table 4, Exp. 3, line PCN-NN-2). In this case the apparent differences may reflect differences in the sizes of the inoculated leaves rather than resistance to infection by TMV RNA.

On inoculated leaves of plants of the two RoCN-NN plant lines tested there was a slight reduction of local lesion numbers on the TMV inoculated leaf halves. The reduction was less significant than on the PCN-NN plants. These results suggest that expression of CP from the *rolC* promoter may result in fewer local infections by TMV, but not by TMV RNA. It is possible that accumulation of CP in the trichomes of the leaf epidermis prevented infections originating from introduction of TMV into these cells. Activity of the *rolC* promoter in the tips of the leaf hair could be observed in plants harboring the *rolC-gusA* gene construct (RoGN plants). Infection of tobacco with TMV through broken trichomes was reported by Zech (1952) and by Herridge and Schlegel (1962).

The TMV inoculum concentrations used in the protection experiments were relatively low. Susceptibility to systemic infection was generally very high in the young plants used in these experiments, and the levels of inoculum were sufficient to result in infection of all the CP(−) plants. Higher inoculation concentrations overcame protection in plants that expressed the CP from the *pal2* promoter. This is presumably due to the probability of infecting an epidermal cell having insufficient levels of CP expression to provide protection or to simultaneous infection of individual epidermal cells with large numbers of virions.

In addition to constitutive GUS activity in the upper leaf epidermis and the xylem (Liang *et al.* 1989), expression of the *gusA* gene from the *pal2* promoter was enhanced upon wounding, mock-inoculation, and TMV infection. It is therefore possible that induction of the *pal2* promoter might have contributed to the resistance in the CP-expressing plants. The lack of resistance to inoculation with TMV RNA, however, suggests that the CP expressed in the transgenic plants interferes with a step that precedes the full release of viral RNA (Register and Beachy 1988; Wu *et al.* 1990). This step is early in the viral infection cycle and probably precedes accumulation of CP as a result of wound-induced gene expression. Wound-induced expression of the *gusA* gene from the *pal2* promoter became visible 60 min after the treatment and continued to increase for several hours (data not shown).

The data presented here provide further evidence that CP-MR of tobacco to TMV is due to interference with an early step in the virus infection cycle in inoculated epidermal cells. We did not find an effect of CP expressed in the phloem on the rate of disease development. The results also indicate that partial protection of tobacco to TMV can be achieved by selective expression of CP in the upper epidermis and xylem. Tissue specific expression of coat proteins in transgenic plants might be a useful alternative to constitutive expression from the CaMV 35S promoter.

MATERIALS AND METHODS

Plant material and inoculation with TMV.

N. tabacum 'Xanthi nn' and 'Xanthi NN' were grown in the greenhouse. Four- to six-week-old plants were inoculated on one leaf, 5–8 cm in length. The inoculum was applied in 50 μl of 50 mM sodium phosphate buffer, pH 7.0, onto Carborundum-dusted leaves and spread by gentle rubbing. TMV (strain U1) was prepared according to Asselin and Zaitlin (1978). TMV RNA was prepared by heating virions in 0.5% (w/v) sodium dodecyl sulfate (SDS) to 80° C and subsequent phenol extraction.

The plant lines 3646 and 748, which express CP from the CaMV 35S promoter in Xanthi nn and Xanthi NN, respectively, have been described (Powell-Abel *et al.* 1986; Nelson *et al.* 1987). Line 306 is a Xanthi nn line that contains the vector plasmid pMON 316 (Rogers *et al.* 1987) only and was used as a control in several experiments. This line expresses the neomycin phosphotransferase II (*nptII*) and nopaline synthase (*nos*) genes. Seeds of a Xanthi NN plant line which harbors the *gusA* gene under the control of the CaMV 35S promoter (G 6-1) were kindly provided by Leigh G. Farrell, CSIRO, Canberra, Australia.

Plasmid construction.

The bean *pal2* promoter sequence was obtained from the genomic clone gPAL 2 (Cramer *et al.* 1989) as a 1,157-bp *Dra*I fragment. For generating the *pal2*-CP gene construct, the fragment was first ligated into the *Sma*I site of pMON 316 (Rogers *et al.* 1987). A *Bgl*II/*Hin*dIII fragment of the resulting plasmid containing the *pal2* promoter and the *nos* termination sequence was inserted into the *Bgl*II/*Hin*dIII sites of pMON 505, a binary plant transformation vector that contains the *nos* gene and the selectable marker gene *nptII* (Rogers *et al.* 1987) to give the plasmid pMON 505 PN.

The CP-coding sequence was isolated from pTM 102, which contains a 572-bp fragment comprising nucleotides 5707-6278 of TMV U1 in the *Sma*I site of pUC18. It was removed as an *Eco*RI/*Hin*dIII fragment and blunt-end ligated into the *Xho*I site of pMON 505 PN, resulting in the plasmid pMON 505 PCN.

The promoter sequence of the *A. rhizogenes rolC* gene was contained in an 870-bp *Hpa*I/*Hin*dIII fragment of pBR328 ORF12 (Oono *et al.* 1987; Sugaya *et al.* 1989). The fragment was blunt-end ligated into the *Sma*I site of pUC18, resulting in the plasmid pUC18 Ro. The CP-coding sequence and *nos* termination sequence were isolated from the plasmid pMON 319 (Powell-Abel *et al.* 1986) as a *Bgl*II/*Hin*dIII fragment and ligated into pUC18 Ro digested with *Bam*HI and *Hin*dIII to give pUC18 RoCN. The chimeric gene was then inserted as an *Eco*RI/*Hin*dIII fragment into the *Eco*RI/*Hin*dIII sites of pMON 505, resulting in the plasmid pMON 505 RoCN.

The *E. coli gusA* gene (*uidA*) was derived from the plasmid pRAJ 260 (Jefferson *et al.* 1986). The *rolC-gusA* and *pal2-gusA* gene fusions were constructed in the plasmid pGN 100. This plasmid contains the *gusA* sequence followed by the *nos* termination sequence and an upstream *Sma*I site. The *pal2* and *rolC* promoters were obtained as described above and ligated into the *Sma*I site. The chimeric genes were excised by *Eco*RI/*Hin*dIII digestion and inserted into the *Eco*RI/*Hin*dIII sites of pMON 505 resulting in the plasmids pMON 505 PGN and pMON 505 RoGN.

Tobacco transformation.

The plasmids described above were introduced into *A. tumefaciens* strain GV 3111 (Fraley *et al.* 1985) using triparental mating (Ditta *et al.* 1980). Leaf disks of *N. tabacum* 'Xanthi nn' and 'Xanthi NN' were transformed using standard methods (Horsch *et al.* 1985). The resulting plants were screened for either the accumulation of CP or β-glucuronidase (GUS) as well as for expression of the *nos* gene. Nopaline accumulation was determined by the method of Otten and Schilperoort (1978). In the plants harboring the *pal2* and *rolC* promoter constructs CP and GUS accumulation was highest in young plants (6 wk old or younger). F_1 progeny of the plants expressing the highest CP and GUS levels were used for further analysis and inoculation experiments.

GUS assay.

Whole seedlings, stem, and petiole sections, and young leaves were vacuum infiltrated with a solution containing 0.5 mg/ml X-Gluc in 50 mM sodium phosphate buffer, pH 7.0, 0.5 mM potassium ferrocyanide, 0.5 mM potassium ferricyanide, and 0.5 % (v/v) Triton X-100 (Jefferson 1987) and incubated overnight at 37° C. The samples were then transferred to 70% EtOH for destaining and storage.

Protein analysis.

Total proteins were extracted from whole leaf tissue (excluding the midribs) or peeled upper epidermis by homogenizing the samples in extraction buffer containing 62.5 mM Tris-HCl, pH 6.8, 1% (w/v) SDS and 20% (v/v) glycerol. Stem samples were powdered in liquid nitrogen prior to extraction. After boiling, proteins were precipitated with 4 volumes of cold acetone and dissolved in extraction buffer. Protein concentrations were determined using the BCA assay (PIERCE, Rockford, IL). Following SDS-PAGE (Laemmli 1970) and transfer onto nitrocellulose (Towbin *et al.* 1979), CP was detected using a polyclonal antiserum raised against TMV and ^{125}I-labeled anti-rabbit IgG antibodies from donkey (Amersham, Arlington Heights, IL). The limit of detection was below 1 ng of CP in a 50-μg protein sample. For analyzing TMV accumulation in leaves of inoculated plants two leaf disks, 5 mm in diameter, were ground in 100 μl of 50 mM sodium phospate buffer, pH 7.0. After centrifugation, 50 μl of the supernatant was dotted onto nitrocellulose using a BIO-DOT apparatus (Bio-Rad, Richmond, CA). CP was detected in the same way as in the Western blots.

ACKNOWLEDGMENTS

We thank C. Cramer (Virginia State University, Blacksburg) for providing the *pal2* promoter and H. Uchimiya (University of Tokyo) for providing the *rolC* promoter. We thank D. Droste for excellent tissue culture work, M. Dyer, and S. J. Leitner for plant production, and John Fitchen for critically reading the manuscript. Ulrich Reimann-Philipp was supported by fellowships from the German Academic Exchange Service (DAAD) and the German Research Society (DFG). Other support was provided by grant 2 RO1 AI27161-04A1 from the National Institute of Health.

LITERATURE CITED

Asselin, A., and Zaitlin, M. 1978. Characterization of a second protein associated with virions of tobacco mosaic virus. Virology 91:173-181.

Beachy, R. N., Loesch-Fries, S., and Tumer, N. E. 1990. Coat protein-mediated resistance against virus infection. Annu. Rev. Phytopathol. 28:451-474.

Bevan, M., Shufflebottom, D., Edwards, K., Jefferson, R., and Schuch, W. 1989. Tissue- and cell-specific activity of a phenylalanine ammonia-lyase promoter in transgenic plants. EMBO J. 8:1899-1906.

Clark, G. W., Register, J. C., III, Nejidat, A., Eichholtz, D. A., Sanders, P. R., Fraley, R. T., and Beachy, R. N. 1990. Tissue-specific expression of the TMV coat protein in transgenic tobacco plants affects the level of coat-protein mediated virus protection. Virology 179:640-647.

Cramer, C. L., Edwards, K., Dron, M., Liang, X., Dildine, S. L., Bolwell, G. P., Dixon, R. A., Lamb, C. J., and Schuch, W. 1989. Phenylalanine ammonia-lyase gene organization and structure. Plant Mol. Biol. 12:367-383.

Deom, C. M., Oliver, M. J., and Beachy, R. N. 1987. The 30 kilodalton gene product of tobacco mosaic virus potentiates virus movement. Science 237:389-394.

Ditta, G., Stanfield, S., Corbin, D., and Helinski, D. R. 1980. Broad host range DNA cloning system for gram-negative bacteria: Construction of a gene bank of *Rhizobium meliloti*. Proc. Natl. Acad. Sci. USA 77:7347-7351.

Esau, K., and Cronshaw, J. 1967. Relation of tobacco mosaic virus to the host cell. J. Cell Biol. 33:665-678.

Fraley, R. T., Rogers, S. G., Horsch, R. B., Eichholtz, D. A., Flick, J. S., Fink, C. L., Hoffmann, N. L., and Sanders, P. R. 1985. The SEV system: A new disarmed Ti plasmid vector system for plant transformation. Bio/Technology 3:629-653.

Herridge, E. A., and Schlegel, D. A. 1962. Autoradiographic studies of tobacco mosaic virus inoculations on host and non-host species. Virology 18:517-523.

Horsch, R. B., Fry, J. E., Hoffmann, N. L., Eichholtz, D., Rogers, S. G., and Fraley, R. T. 1985. A simple and general method for transferring genes into plants. Science 227:1229-1231.

Jefferson, R. A. 1987. Assaying chimeric genes in plants: The GUS gene fusion system. Plant Mol. Biol. Rep. 5:387-405.

Jefferson, R. A., Burgess, S. M., and Hirsh, D. 1986. β-Glucuronidase from *Escherichia coli* as a gene fusion marker. Proc. Natl. Acad. Sci. USA 83:8447-8451.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680-685.

Liang, X., Dron, M., Schmid, J., Dixon, R. A., and Lamb, C. J. 1989. Developmental and environmental regulation of a phenylalanine ammonia-lyase-gene fusion in transgenic tobacco plants. Proc. Natl. Acad. Sci. USA 89:9284-9288.

Nelson, R. S., Powell-Abel, P., and Beachy, R. N. 1987. Lesions and virus accumulation in inoculated transgenic tobacco plants expressing the coat protein gene of tobacco mosaic virus. Virology 158:126-132.

Oono, Y., Handa, T., Kanaya, K., and Uchimiya, H. 1987. The TL-DNA gene of Ri plasmids is responsible for dwarfness of tobacco plants. Jpn. J. Genet. 62:501-505.

Otten, L. A. B. M., and Schilperoort, R. A. 1978. A rapid micro scale method for the detection of lysopine and nopaline dehydrogenase activities. Biochim. Biophys. Acta 257:497-500.

Powell-Abel, P., Nelson, R. S., De, B., Hoffmann, N., Rogers, S. G., Fraley, R. T., and Beachy, R. N. 1986. Delay of disease development in transgenic plants that express the tobacco mosaic virus coat protein gene. Science 232:738-743.

Register, J. C., III, and Beachy, R. N. 1988. Resistance to TMV in transgenic plants results from interference with an early event in infection. Virology 166:524-532.

Rogers, S. G., Klee, H. J., Horsch, R. B., and Fraley, R. T. 1987. Improved vectors for plant transformation: Expression cassette vectors and new selectable markers. Methods Enzymol. 153:253-277.

Saito, T., Yamanaka, K., and Okada, Y. 1990. Long-distance movement and viral assembly of tobacco mosaic virus mutants. Virology 176:329-336.

Schmülling, T., Schell, J., and Spena, A. 1989. Promoters of the *rolA, B,* and *C* genes of *Agrobacterium rhizogenes* are differentially regulated in transgenic plants. Plant Cell 1:665-670.

Sugaya, S., Hayakawa, K., Handa, T., and Uchimiya, H. 1989. Cell-specific expression of the *rolC* gene of the TL-DNA of Ri plasmid in transgenic tobacco plants. Plant Cell Physiol. 30:649-653.

Towbin, M., Staehelin, T., and Gordon, J. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc. Natl. Acad. Sci. USA 76:4350-4354.

Watanabe, Y., Morita, N., Nishiguchi, M., and Okada, Y. 1987. Attenuated strains of tobacco mosaic virus. Reduced synthesis of a viral protein with a cell-to-cell movement function. J. Mol. Biol. 194:699-704.

Weststeijn, E. A. 1981. Lesion growth and virus localization in leaves of *Nicotiana tabacum* cv. Xanthi nc after inoculation with tobacco mosaic virus and incubation alternately at 22° C and 32° C. Physiol. Plant Pathol. 18:357-368.

Wisniewski, L. A., Powell, P. A., Nelson, R. S., and Beachy, R. N. 1990. Local and systemic spread of tobacco mosaic virus in transgenic tobacco plants. Plant Cell 2:559-567.

Wu, X., Beachy, R. N., Wilson, T. M. A., and Shaw, J. G. 1990. Inhibition of uncoating of tobacco mosaic virus particles in protoplasts from transgenic tobacco plants that express the viral coat protein gene. Virology 179:893-895.

Zech, H. 1952. Untersuchungen über den Infektionsvorgang und die Wanderung des Tabakmosaikvirus im Pflanzenkörper. Planta 40:461-514.