Heterologous Resistance to Potato Virus Y in Transgenic Tobacco Plants Expressing the Coat Protein Gene of Lettuce Mosaic Potyvirus

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ABSTRACT

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Heterologous protection can provide resistance against virus infection in transgenic plants and was evaluated with two potyviruses, potato virus Y (PVY) and lettuce mosaic virus (LMV). PVY causes a serious disease of tobacco. Transgenic plants, *Nicotiana tabacum* cv. Xanthi, containing a modified coat protein (CP) gene of LMV have been generated and tested for resistance against PVY. Half of the 34 independent R_0 transgenic plants tested accumulated detectable levels of LMV coat protein (LMV CP+). These LMV CP+ plants were resistant to infection following sap inoculation with a necrotic strain of PVY (PVY-NV). Moreover, R_1 populations of five LMV CP+ lines tested were resistant to PVY-NV and to four other PVY strains. Different phenotypes were observed, in-cluding 1) complete resistance in two lines, 2) delay and attenuation of symptoms in two lines, and 3) delay in symptoms with no modification of symptoms in one line. In cases 2 and 3, the number of plants showing symptoms was reduced, and the PVY accumulation was greatly decreased in the infected plants. These lines were considered to be partially resistant to PVY infection. Although PVY and LMV are clearly distinct potyviruses (their CPs share 66% amino acid sequence homology), the expression of the coat protein gene of LMV can confer a high level of heterologous protection against PVY in tobacco plants. Results are discussed regarding the possibility of different modes of resistance.

Additional keywords: coat protein-mediated protection, virus resistance.

Potyviruses constitute the largest group of plant viruses. The viral RNA is encapsidated in flexuous particles composed of about 2,000 subunits of a coat protein (CP) with an M_r of 30,000–35,000. The genome consists of one single-stranded, positive-sense RNA molecule approximately 10 kb in length, which has a poly-adenylated tail at its 3′ end and encodes a polyprotein processed by several viral proteases (29). The CP is located at the carboxyl terminus (C terminus) of the polyprotein. Sequence data of the CP are now available for many members of the group (34).

The potyviruses are responsible for diseases in many economically important crops. We are interested in the control of diseases caused by two potyviruses, potato virus Y (PVY) and lettuce mosaic virus (LMV). PVY infects many cultivated plants throughout the world, including potato, tomato, pepper, and tobacco, and causes severe losses on these crops (31). PVY is the virus most frequently found on tobacco in France. Several distinct strains of PVY have been isolated from solanaceous plants. Some of these strains cause very mild symptoms, and some cause very severe symptoms, even death, of tobacco plants (13,31). The tobacco veinal necrosis disease, caused by the group of necrotic PVY (PVY-N) strains, is very destructive and may cause a complete loss of the tobacco crop (31). In France, strains of PVY-N are now predominant on the commercial tobacco cultivars Burley and Virginie. LMV is also found worldwide, and in Europe it is responsible for a major disease of commercial lettuce (7). LMV has a wide host range but does not infect tobacco. As with PVY, aggressive strains of LMV that overcome the known resistances in lettuce have recently been characterized (7).

As an alternative to traditional plant breeding for disease control, genetically engineered CP-mediated resistance against virus infection has been obtained for several plant viruses (1,16,26,27). This strategy has been successfully applied to potyviruses (9–11,19, 21,25,28). Several reports have also shown that the protection obtained with a potyvirus CP could be effective against different viruses within the potyvirus group (22,25,32). This phenomenon is called heterologous protection (32). In these reports, the appearance of symptoms in transgenic infected plants was delayed, and the percentage of plants showing symptoms was reduced. However, even in the lines showing an absence of symptoms, a low accumulation of virus was detected (32), and the level of heterologous resistance varied depending on the combination of the challenging virus and the potyvirus CP expressed in transgenic plants (22,25,32).

We recently cloned and sequenced the CP gene of LMV (8), and transgenic lettuces expressing the gene (LMV CP+ plants)

were obtained (J. Albouy and Y. Chupeau, *personal communication*) to control LMV infection. Because PVY is responsible for the most serious viral disease of tobacco in France, we also transferred the LMV CP gene into tobacco to determine if it would provide heterologous protection against PVY. In this paper, we report the production of LMV CP transgenic tobacco plants and the results of heterologous protection against PVY in these plants. The protection was evaluated by examining the severity of symptoms and the time of their appearance. Also, accumulation of CP was measured by enzyme-linked immunosorbent assay (ELISA) and used as an indication of viral titer. Different phenotypes of protection were observed, including complete or partial resistance. Five PVY strains were tested, four from the PVY-N group and one from PVY-O. The level of protection against these five strains was similar in the different lines tested. This shows that heterologous protection may be highly effective against severe strains of PVY as well as against mild strains. Furthermore, the different phenotypes observed suggest that the resistance may have resulted from at least two different levels of interference, virus multiplication and symptom expression levels.

MATERIALS AND METHODS

cDNA clones. Plasmid pLMV4 (referred to as S4 in ref. 8) contains a cDNA copy of part of the genome of the common strain of LMV, LMV O, corresponding to the 3' terminal region. This region has been sequenced (8) and contains the C terminus

region of the putative polymerase gene, the complete CP gene, and the 3' untranslated region (Fig. 1A). The 3' noncoding region is 211 nucleotides long and is followed by a polyadenylated tail. The nucleotide sequence data is available in the EMBL, Genbank, and DDBJ nucleotide sequence databases under the accession number X65652. All molecular biology techniques performed are essentially as described by Maniatis et al (24), unless otherwise indicated.

Construction of pBCP103, in vitro transcription, and translation. The CP coding region and the 3' untranslated region of LMV were subcloned into Bluescript KS+ vector (Stratagene Inc., La Jolla, CA), which had been modified to allow efficient initiation of the translation of the CP gene. The *Acc*I and *Sal*I sites of the polylinker were removed by digestion with *Xho*I and *Sal*I and filled in with the Klenow fragment of DNA polymerase I and religation. The resultant plasmid was then digested with *Hin*dIII and *Eco*RI, and the larger fragment was ligated with a *Hin*dIII-*Eco*RI fragment of pJMIOI (12) containing the tobacco mosaic virus (TMV) leader sequence (Ω'). This construct, called pBSΩ', was digested with *Sal*I (introduced by the insertion of the Ω' sequence) and *Pst*I. The larger fragment was then ligated with a synthetic oligonucleotide, 5'-TCGACTAAACCATG-GTAGACCTGCA-3' (6). This insertion created a restriction site, *Acc*I, directly downstream of an ATG codon (underlined in the oligonucleotide sequence). The resultant plasmid was called pBSΩ'C. Plasmid pBCP103 (Fig. 1A) was generated by insertion of the 1.2-kb *Acc*I-*Eco*RV CP fragment of pLMV4 into pBSΩ'C digested with *Xba*I (filled in) and then with *Acc*I. This construction inserted the coding region of the CP gene in frame and just downstream of the ATG codon of pBSΩ'C. In vitro transcription of pBCP103 was performed with T3 RNA polymerase (Stratagene) after linearization with *Sac*I. In vitro translation was performed either in rabbit reticulocyte lysate or in wheat germ extract (both from Promega Biotech, Madison, WI) in the presence of ^{35}S-methionine (Amersham, Buckinghamshire, England), using manufacturer's suggested procedures. The translation products were electrophoresed in sodium dodecyl sulfate (SDS)-poly-acrylamide gels and analyzed by fluorography.

Construction of pKFCP602. We used the plant expression vector pKFR9 (33) for direct transfer of the modified LMV CP sequence into plant cells (Fig. 1B). A 1.2-kb *Cla*I-*Sac*II fragment of pBCP103 was ligated into pKFR9 digested by *Sma*I and *Sac*II. The sequences inserted in the expression cassette of pKFR9 are illustrated in Figure 1A.

Plant transformation. Protoplasts were isolated (4) from *Nicotiana tabacum* L. cv. Xanthi XHFD8 (3) and transformed by electroporation (15) with either the control plasmid pKFR9 or with the construct pKFCP602. Calli were regenerated and cultured as described (3). Transformed cells and calli were selected with paromomycin (20 μg/ml). Shoots were rooted on plates containing kanamycin (70 μg/ml). Plants were transplanted into soil after the formation of roots and were acclimated into greenhouses.

Detection of LMV CP gene expression. Kanamycin-resistant transformants were analyzed for LMV CP gene expression by double antibody sandwich-ELISA (DAS-ELISA) (5). Leaf disks punched out with a no. 10 cork borer were ground and diluted 1:10 (w/v) in extraction buffer (0.02 M potassium phosphate, pH 7.5, 0.15 M NaCl, 0.05% Tween 20, and 2% polyvinyl-pyrrolidone) before being loaded onto precoated plates. Anti-LMV immunoglobulin G (IgG) (kindly provided by H. Lot) and anti-LMV IgG alkaline phosphatase conjugate were used at a concentration of 1 μg/ml. The reaction was monitored by measurement of the absorbance at 405 nm with a microplate reader (Molecular Devices, Menlo Park, CA).

Segregation of kanamycin resistance in the R$_1$ generation. The inheritance pattern of kanamycin resistance was determined by testing R$_1$ seedlings of self-fertilized R$_0$ transgenic plant lines. R$_1$ seeds were germinated under sterile conditions in the presence of kanamycin (70 μg/ml) at 25 C. Kanamycin resistance segregated in a 3:1 ratio, indicating that the gene was inserted at a single locus. The kanamycin-resistant seedlings were transplanted into
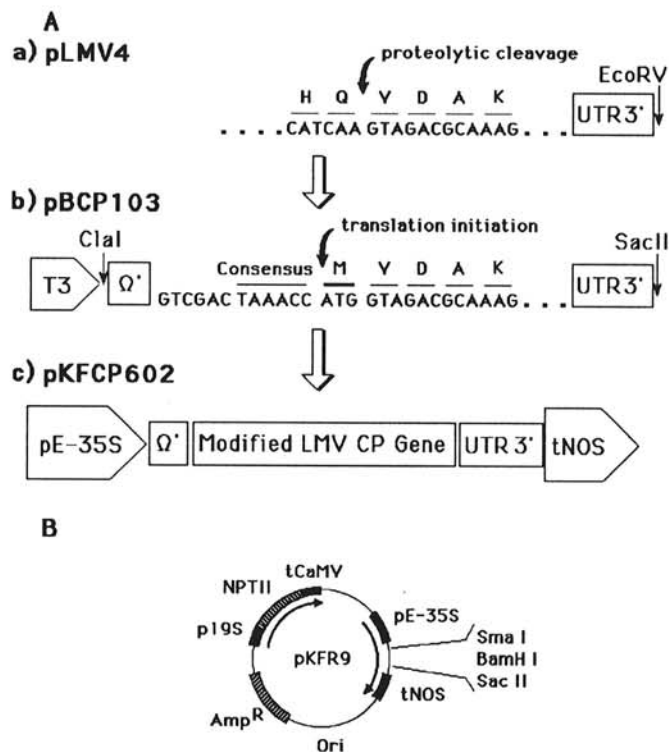
Fig. 1. Construction of the vector pKFCP602 used for the production of transgenic tobacco plants expressing the lettuce mosaic virus coat protein (LMV CP) gene. **A,** The LMV CP gene was modified from the wild-type sequence in pLMV4 (a) by the addition of an ATG codon at its 5' end, in a favorable context for translation initiation and insertion of the TMV leader sequence (Ω') downstream in pBCP103 (b). This sequence was introduced in pKFR9 under the control of an enhanced cauliflower mosaic virus (CaMV) 35S promoter (pE-35S) and the NOS terminator (t$_{NOS}$) to give pKFCP602 (c). UTR3' = 3' untranslated region. See Materials and Methods for details. **B,** The vector pKFR9, used for the construction of pKFCP602. The polylinker region is flanked by an enhanced CaMV 35S promoter and the NOS terminator. This vector also contains a plant-expressible neomycin phosphotransferase gene (NPTII) controlled by the CaMV 19S promoter (p19S) and the CaMV terminator (t$_{CaMV}$) and a bacterial ampicillin resistance gene (Amp^R).

soil 3 wk after germination and grown in greenhouses. Of the 17 R_0 CP+ lines that were tested, five were not fertile.

Western blot analysis. Fresh leaf tissue (0.5 g) was ground in 1.5 ml of sample extraction buffer (18). After heating at 100 C for 2 min, extracts were centrifuged at 1,000 rpm for 5 min, and 20 μl of supernatant was loaded onto 12% SDS-polyacrylamide gel. After electrophoresis, proteins were transferred to a nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany). Membranes were blocked in 2% powdered nonfat milk in TBS (0.02 M Tris HCl and 0.5 M NaCl, pH 7.5) at room temperature for 1 h and then incubated in a 1:10,000 dilution of anti-LMV serum overnight at 4 C. After they were washed in a solution containing TBS and 0.05% Tween 20, the membranes were incubated in alkaline phosphatase-conjugated goat anti-rabbit IgG (Biosys, Compiègne, France), and the enzyme activity was detected by fast red coloration.

PVY strains. Transgenic lines were tested by mechanical sap inoculation with five strains of PVY available in the INRA collection. Four PVY strains belonging to the group of necrotic strains were tested: the Versailles (France) strain PVY-NV, isolated from potato (30); an American strain PVY-NN, kindly provided by G. V. Gooding, Jr. (14); the Rennes (France) strain PVY-NR, more necrotic on tobacco than PVY-NV; and a strain PVY-NNT from Lebanon (20). Most of the experiments were performed with the PVY-NV strain, unless otherwise stated. We also used the Versailles (France) isolate of the common strain PVY-O. All the strains were originally isolated from potato plants in fields except PVY-NN, which was isolated from tobacco; all were propagated on Xanthi tobacco plants. These strains are all closely related serologically.

Infection of transgenic plants with PVY. The two youngest expanded leaves of young transgenic tobacco plants (at the five- to six-leaf stage) were dusted with Carborundum and gently rubbed with dilutions of the inoculum. The inoculum used was an extract made from tobacco leaves 3 wk after inoculation. The leaves were ground 1:1 (w/v) in the extraction buffer (0.05 M KH$_2$PO$_4$, 0.05 M Na$_2$HPO$_4$, pH 7.5, and 0.05% mercaptoethanol) and diluted in the same buffer to give an extract with a virus concentration of 50 μg of PVY per milliliter. These dilutions gave 100% infection of susceptible tobacco plants. For tests on R_1 plants, 30 plants from CP+ lines and 15 plants from control lines were inoculated, unless otherwise indicated. Symptom expression was recorded daily for 8 wk after inoculation. PVY CP accumulation was quantified by DAS-ELISA as described, using PVY-NV IgG produced in our laboratory. For each plant, three independent leaf samples were analyzed; the leaves sampled corresponded to the upper leaves at the time of the inoculation and presented typical symptoms on the control plants 14 and 28 days postinoculation (dpi). The amount of PVY CP antigen was assessed by comparison with dilutions of purified PVY-NV (0.05–2 μg/ml) included in each ELISA plate. The PVY-NV IgG does not cross-react with LMV antigen.

RESULTS

LMV CP expression in R_0 transgenic plant lines. The potyviral CP is translated as the C terminus protein of the viral polyprotein, and it was necessary to modify the 5' end of the LMV CP cistron sequence to allow its translation as an independent cistron. For that, we inserted a translational start site (Fig. 1) in a favorable context for efficient initiation (17,23). To further increase expression, the translational enhancer Ω' (12) was also placed upstream of the start site (Fig. 1A).

Cloning in a transcriptional vector allowed us to synthesize an in vitro transcript of this modified LMV CP gene. In vitro translation of this transcript in rabbit reticulocyte lysate or in the wheat germ extract system gave a 35-kDa product of the modified LMV CP gene similar in size to the purified LMV CP (data not shown). The modified LMV CP gene was subsequently introduced into the vector pKFR9, and this construct (pKFCP602; Fig. 1) was used to transform tobacco protoplasts. Thirty-four independent kanamycin-resistant tobacco plants were obtained

and assayed for LMV CP expression. As determined by DAS-ELISA, 50% of the R_0 plants accumulated detectable levels of LMV CP. Expression of the LMV CP was also confirmed by Western blot analysis (data not shown). pKFR9 was used to transform tobacco protoplasts, and the transformed plants were used as controls in subsequent experiments.

Resistance against PVY-NV in transgenic R_0 plants. To assess the protection against PVY, the R_0 LMV CP+ and LMV CP– transgenic tobacco plants were challenged with a necrotic strain, PVY-NV. Plants were scored daily for virus symptoms. Transgenic control and LMV CP– plants showed no delay in the appearance of symptoms when compared to nontransformed plants. Of the eight R_0 LMV CP+ plants tested, five plants did not show any symptoms throughout the 8 wk after inoculation, while the other three showed a 4- to 6-day delay in symptom expression. DAS-ELISA showed that asymptomatic plants did not contain detectable levels of PVY CP antigen at 4 and 6 wk after inoculation. Based on the response to PVY infection and on the accumulation of the LMV CP determined by ELISA, we selected one LMV CP– and five LMV CP+ lines for further analysis (Table 1). A control transgenic line was also included.

LMV CP expression in R_1 transgenic plant lines. R_1 plants derived from self-fertilized R_0 transgenic tobacco plants were tested for LMV CP expression. A protein with an estimated M_r of 35 kDa was detected in Western immunoblot analysis of plant extracts from plants of lines 69 and 106 (Fig. 2) and lines 7 and 25 (data not shown). This demonstrated the expression of LMV CP in R_1 progeny. No protein bands were observed in such blots (Fig. 2) in LMV CP– plants (line 2) or transgenic control plants (line 14). The accumulation of LMV CP in lines 7, 25, 69, and 106 was confirmed by DAS-ELISA (Table 2). LMV CP accumulation was also detected by DAS-ELISA in line 97, although at a low level (Table 2), but was not detected by Western blot analysis. R_1 seedlings, 30 plants per line, were tested in three independent samplings. The absorbance at 405 nm (Table 2) corresponds to an LMV CP accumulation ranging from 60 to 210 ng of CP per milligram of leaf wet extract (0.6–2% of total soluble proteins). An analysis of variance was done with these data ($P = 0.0001$, P significant at 0.05). A least significant difference analysis (LSD = 0.3780) (Table 2) showed the existence of two distinct groups, gathering lines 7, 25, 69, and 106 in one group (high LMV CP accumulation) and lines 2, 14, and 97 in

TABLE 1. Characterization of R_0 transgenic tobacco plants selected for further tests to determine resistance to potato virus Y (PVY)

R_0 line[a]	Phenotype[b]	Level of LMV CP accumulation[c]	Symptoms[d]
7	KR+ CP+	++[e]	None
25	KR+ CP+	++	None
69	KR+ CP+	+++	None
106	KR+ CP+	++	None
97	KR+ CP+	++	Mosaic (4-day delay)
2	KR+ CP–	–	Mosaic
14	KR+ ...	–	Mosaic

[a] Plants of lines 2, 7, 25, 69, 97, and 106 were transformed with pKFCP602, which is derived from pKFR9 and carries the lettuce mosaic virus coat protein gene within a plant expression cassette. Plants of line 14 were transformed with pKFR9 and used as a control. pKFR9 confers resistance to kanamycin.

[b] KR+ = Resistant to kanamycin; CP+ = expressing the coat protein gene; CP– = not expressing the coat protein gene.

[c] Lettuce mosaic virus coat protein.

[d] The plants were inoculated with the necrotic Versailles strain (NV) of PVY. The protection against PVY was assayed by visual observation of symptoms 8 wk after inoculation; the plants were inoculated with infected leaf extracts diluted to a viral concentration of 50 μg of PVY-NV per milliliter of extract.

[e] The amount of LMV CP accumulation was evaluated by double antibody sandwich–enzyme-linked immunosorbent assay. +++ = A$_{405nm}$, greater than fivefold difference above the control; ++ = A$_{405nm}$, two- to fivefold difference above the control; – = A$_{405nm}$, equivalent to the control.

a second group (low or no LMV CP accumulation). The average LMV CP accumulations in lines within these two groups are not significantly different (values less than the LSD value).

Protection against PVY infection in R_1 CP+ plants. Typically, tobacco plants infected with PVY-NV show symptoms of systemic veinclearing 5 dpi, which is followed by a mottling of the upper leaves 7–8 dpi and then by veinal necrosis. PVY-O causes similar symptoms but without vein necrosis, and symptoms usually appear 10–12 dpi. Plants from control line 14 and from LMV CP− line 2 inoculated with PVY-NV showed typical symptoms of systemic veinclearing and mottling of the upper leaves with no delay in symptom expression (Fig. 3A). Several other control lines and LMV CP− lines were also tested, and all developed typical infection symptoms without any delay in expression (data not shown).

In contrast, the LMV CP+ lines inoculated with PVY-NV showed either a complete absence of symptoms or a delay in the appearance of symptoms, and fewer plants in these lines were symptomatic (Fig. 3A). Two lines (69 and 106) were completely resistant to PVY infection (Fig. 3A). Indeed, all the plants were

Fig. 2. Western blot analysis of lettuce mosaic virus coat protein (LMV CP) expression in R_1 plants of tobacco cultivar Xanthi. Total sodium dodecyl sulfate-soluble proteins extracted from leaf tissue of nontransformed tobacco (lane 1) and transgenic tobacco plants in control line 14 (lane 2), CP− line 2 (lane 3), and CP+ lines 69 (lane 6) and 106 (lane 7). The extracts were blotted and incubated with anti-LMV serum. The migration of LMV CP from LMV-infected lettuce (lane 4) and prestained M_r standards (Amersham, Buckinghamshire, England) (lane 5) are also indicated.

TABLE 2. Accumulation of lettuce mosaic virus coat protein antigen in leaves of R_1 tobacco plants cultivar Xanthi

R_1 line[a]	Number of measurements[b]	Average A_{405nm}	Group[c]
25 (CP+)	90	1.072	A
7 (CP+)	90	0.969	A
106 (CP+)	90	0.941	A
69 (CP+)	90	0.810	A
97 (CP+)	90	0.440	B
2 (CP−)	90	0.363	B
14 (control)	45	0.286	B

[a] Lines were ordered as a function of the viral coat protein accumulation determined by double antibody sandwich–enzyme-linked immunosorbent assay. CP+ = expressing the coat protein gene; CP− = not expressing the coat protein gene.

[b] A_{405nm} values were averaged from 90 measurements (three samplings of 30 plants) for each of six transgenic R_1 tobacco lines, 2, 7, 25, 69, 97, and 106, and from 45 measurements (three samplings of 15 plants) for the control transgenic line 14.

[c] A least significant difference (LSD) analysis was performed on these data. The LSD value is 0.378, which defined two groups, A and B; each average within these groups differed by less than one LSD value from any other averages in the group.

asymptomatic throughout the 8 wk after inoculation, and ELISA tests were negative (see below). In the three other lines, 7, 25 (Table 3), and 97 (Fig. 3A), a significant number of plants were symptomless (i.e., about 50% of the inoculated plants in each of these lines were asymptomatic). These plants showed no symptoms in inoculated leaves or in upper leaves. The symptomatic plants in line 97 showed symptoms identical to those of the PVY-infected control plants, while the symptomatic plants in lines 7 and 25 showed atypical symptoms, with a mild mottle on the upper leaves and a few chlorotic spots. No veinclearing was observed on any plant, and the atypical symptoms described were barely detectable. Symptom expression in these three lines was delayed, occurring 2–10 days later than in the control plants (Fig. 3A).

Similar results of attenuation or absence of symptoms were observed when the plants were inoculated with PVY-O (Fig. 3B, Table 3) and with three other strains of PVY-N (Table 3). Plants from lines 69 and 106 were equally completely symptomless after inoculation with PVY-O (Fig. 3B, Table 3), PVY-NN, PVY-NR, or PVY-NNT (Table 3). The proportion of plants from lines 7 and 25 showing symptoms was reduced with PVY-NN and PVY-NNT, and the plants were asymptomatic with PVY-O (Table 3). The proportion of plants from line 97 that showed symptoms varied from 50 to 100% for the different strains (Table 3). Since 100% of controls were infected 25 dpi, this variability cannot be due to differences in inoculation efficiency. Only plants of this line presented typical symptoms.

The accumulation of PVY antigen was assessed with plants inoculated with PVY-NV. In the two asymptomatic lines, 69 and 106, no detectable PVY CP accumulated in any of the R_1 plants tested (30 plants per line) (Fig. 4). These two lines were completely resistant to PVY infection, based on the absence of detectable virus accumulation and the absence of symptoms. In lines 7, 25, and 97, the accumulation of the PVY was severely reduced compared to the control line, even in the plants showing symptoms (Fig. 5). About 80% of the plants accumulated less than 300 ng of PVY antigen per milligram of leaf extract, unlike control and LMV CP− transgenic lines, of which only 3% of the plants showed a PVY antigen accumulation less than this concentration (Fig.
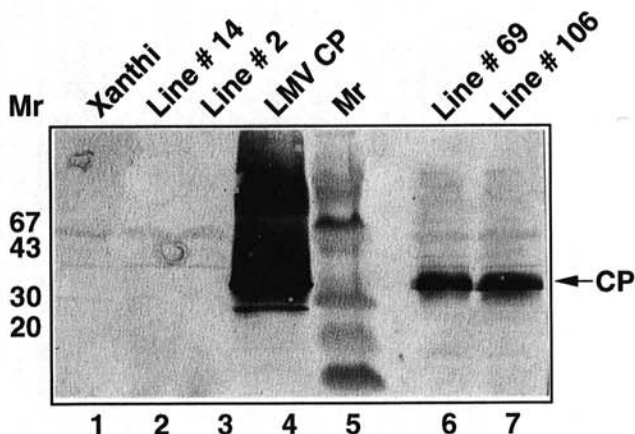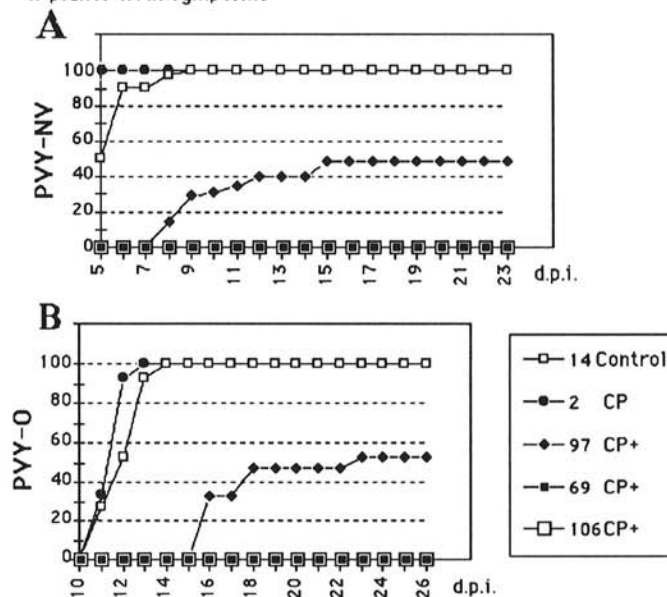

Fig. 3. Relative percentage of R_1 plants showing symptoms at successive days postinoculation (dpi) with 50 μg of virus per milliliter of leaf extract infected with potato virus Y strain NV (PVY-NV) (A) or strain O (PVY-O) (B). The average percentages were determined for 30 plants (A) or 15 plants (B) each of a transgenic line not expressing the coat protein gene of lettuce mosaic virus (LMV CP−) (line 2), three transgenic LMV CP+ lines (lines 97, 69, and 106), and a transgenic control (line 14).

5). This resulted from a significant number of symptomless plants in which PVY was undetectable by ELISA (47% for line 7, 40% for line 25, and 36% for line 97) in addition to symptomless plants with PVY accumulation of less than 100 ng/mg of leaf extract (approximately a fivefold reduction in accumulation compared to that of the control plants). Overall, about 50% of the plants showed undetectable or reduced PVY accumulation, and about 30% of the plants accumulated from 100 to 300 ng

of PVY CP per milligram of leaf extract (Fig. 5), which was still a low PVY accumulation.

DISCUSSION

We have demonstrated that heterologous protection against PVY is conferred by expressing the LMV CP gene in transgenic tobacco plants. The protection is correlated with the expression of the LMV CP. All LMV CP+ transgenic lines showed protection
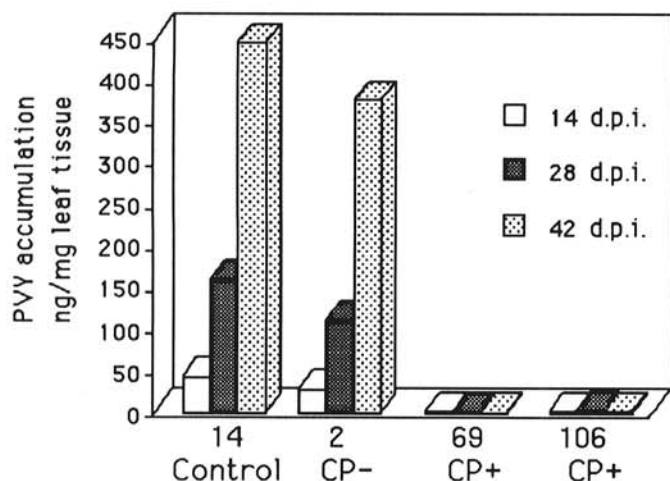
Fig. 4. Accumulation of potato virus Y (PVY) coat protein (CP) antigen in the leaves of transformed R_1 tobacco plants at various days postinoculation (dpi) with the necrotic Versailles strain (PVY-NV). Average PVY-NV CP concentrations were determined for 30 plants each of a transgenic line not expressing the CP gene of lettuce mosaic virus (LMV CP−) (line 2), two transgenic LMV CP+ lines (lines 69 and 106), and a transgenic control line (line 14). Plants were mechanically inoculated with PVY in infected leaf extract (50 μg of virus per milliliter of extract). PVY CP concentrations were determined by double antibody sandwich–enzyme-linked immunosorbent assay and expressed in nanograms of viral CP per milligram of fresh weight of tissue.
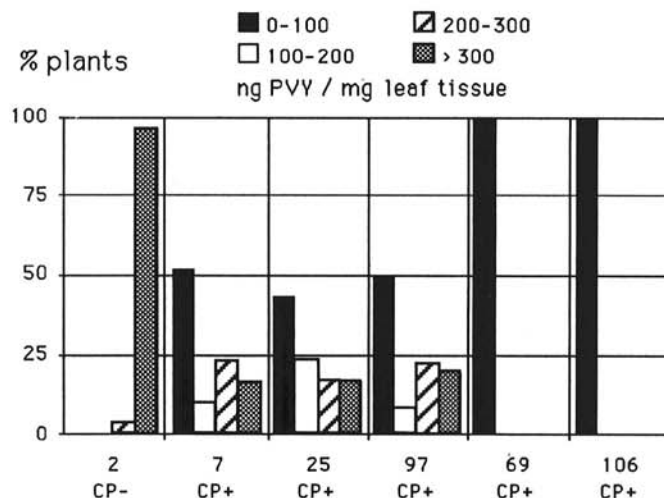
Fig. 5. Accumulation of coat protein (CP) antigen from the necrotic Versailles strain of potato virus Y (PVY) in R_1 transgenic plants. Thirty-six plants of line 97; 32 plants of line 7; 30 plants of lines 25, 69, and 106; and 15 plants of line 2 were assessed for virus accumulation 42 days after inoculation. Plants were mechanically inoculated with PVY in infected leaf extract (50 μg of virus per milliliter of extract). Viral CP concentrations were determined by double antibody sandwich–enzyme-linked immunosorbent assay and expressed in nanograms of viral CP per milligram of fresh weight of tissue.

TABLE 3. Resistance of transgenic R_1 tobacco plants containing transgene pKFCP602 lettuce mosaic virus coat protein (CP) to infection by five strains of potato virus Y (PVY)

R_1 line[a]	Days postinoculation	Plants with symptoms (%)[b,c]				
		PVY-NN	PVY-NR	PVY-NNT	PVY-O	PVY-NV
7 (CP+)	12	4	ND	0	0	6[d]
	18	13		8	0	16[d]
	25	50		45	0	50[d]
25 (CP+)	12	2	0	0	0	0[d]
	18	28	0	30	0	36[d]
	25	50	0	50	0	50[d]
69 (CP+)	12	0	0	ND	0	0
	18	0	0		0	0
	25	0	0		0	0
106 (CP+)	12	0	0	0	0	0
	18	0	0	0	0	0
	25	0	0	0	0	0
97 (CP+)	12	53	0	ND	0	40
	18	73	37.5		46	48
	25	100	100		53	50
2 (CP−)	12	100	92	93	93	100
	18	100	100	100	100	100
	25	100	100	100	100	100
14 (control)	12	66	40	80	53	100
	18	100	100	100	100	100
	25	100	100	100	100	100

[a] CP+ = expressing the coat protein gene; CP− = not expressing the coat protein gene.
[b] Four different necrotic strains belonging to the PVY-N group and one PVY-O strain were used for inoculation of young plants (see Materials and Methods). PVY-NN = American strain; PVY-NR = Rennes strain; PVY-NNT = Lebanese strain; and PVY-NV = Versailles strain.
[c] The numbers indicate the percentage of plants showing symptoms, as determined from 15 plants for each line inoculated with PVY-NN, PVY-NR, PVY-NNT, or PVY-O or 30 plants for each line inoculated with PVY-NV. ND = Not determined.
[d] Symptoms very attenuated and hardly detectable.

against five different strains of PVY. None of the transgenic LMV CP– lines showed resistance against PVY. Several phenotypes of protection were observed, including complete resistance, a delay in symptom expression, and a reduction in accumulation of viral PVY CP. Our objective now is to test the effectiveness of the protection under field conditions. We have not tested these transgenic plants for protection against LMV, because tobacco is not susceptible to this virus. Experiments are now in progress to evaluate the protection against LMV in crops susceptible to this virus.

This work reports complete resistance in two lines against a heterologous potyvirus. This is the first report of complete resistance in a case of heterologous protection in the potyviridae. Indeed, high resistance to PVY was reported only in cases of homologous protection (10,19), and PVY and LMV CPs are clearly distinct, with a 66% amino acid sequence homology (8,34). Such a heterologous complete resistance, however, was not observed against tobacco etch potyvirus (TEV) (data not shown), the CP of which shares 60% amino acid sequence similarity with the CP of LMV. The difference in the resistance observed against PVY and TEV infection involves a specific mechanism of resistance. This also confirms the observation that heterologous protection may vary drastically from virus to virus (32) and underlines the variation in the efficiency of genetically engineered protection.

The choice of the R_1 plant lines tested was based on the level of accumulation of LMV CP and the level of protection in the R_0 plants against PVY. In LMV CP+ R_1 plants, there were three distinct phenotypes: 1) lines with plants accumulating high levels of LMV CP and completely resistant to infection, 2) lines with plants accumulating high levels of LMV CP, showing no symptoms or developing highly attenuated symptoms that were delayed in appearance, and showing a significant decrease in viral accumulation, and 3) a line with plants accumulating a low level of LMV CP, showing no symptoms or developing normal symptoms that were delayed in appearance, and showing a significant decrease in viral accumulation. Thus, distinct resistance phenotypes were obtained, while the same transgene was expressed in these five lines.

Since the level of accumulation of the LMV CP may be involved in these different phenotypes of protection, closer examination of LMV CP accumulation was done in comparison with the level of protection. Statistical analysis of the LMV CP accumulation in R_1 plants showed that the level of accumulation constitutes two distinct groups, with lines 7, 25, 69, and 106 in one group and line 97 in a second one (Table 2). However, lines 7, 25, and 97 showed similar resistance to PVY accumulation after inoculation (Fig. 5). Consequently, the protective effect on viral accumulation (i.e., severe reduction of PVY in the infected leaves) does not seem to be directly affected by the level of LMV CP expressed in the R_1 transgenic plants. These results agree with the results of others (19,28,32).

However, and interestingly, the attenuation of symptoms seems to be positively correlated with the LMV CP accumulation. Indeed, lines 7, 25, 69, and 106 all showed good resistance to the expression of symptoms, with no symptoms or with atypical attenuated symptoms, regardless of the concentration of PVY accumulated in the plants. By contrast, in line 97, in which plants accumulated lower levels of LMV CP than in the other four lines, symptomatic plants presented typical severe symptoms. Furthermore, the symptom severity is not directly correlated with the virus accumulation in the plants. Indeed, although lines 7, 25, and 97 presented the same pattern of reduction of the accumulation of PVY (Fig. 5), plants in lines 7 and 25 developed atypical attenuated mosaic, whereas those in line 97 developed typical symptoms. Viral accumulation and symptom development may thus be controlled differently in the transgenic LMV CP+ plants, although we cannot rule out that the effect of the LMV CP on the initial stage of virus multiplication may directly or indirectly affect symptom development. Further analysis using a larger number of LMV CP+ lines is now in progress to confirm the hypothesis that resistance may result from two modes of interference, a first one acting directly on virus accumulation and a second one modifying the expression of symptoms.

Previous studies with heterologous protection reported a delay of symptom development, a reduction in the number of plants infected (22,25,32), and an attenuation of the severity of symptoms in the R_1 LMV CP+ plants that became infected (22,32). These protections are very similar to the protection observed in the resistant lines 7, 25, and 97. The delay in appearance of the symptoms for some plants and the apparent 35–50% of plants that escaped the infection may be caused by a reduction in the movement of the virus or a reduction in viral multiplication. This phenomenon, which is very similar to that described for CP-mediated protection with the TMV CP (1,16), may involve a similar mechanism of resistance. Little is known about the mechanism of CP-mediated protection for viruses other than TMV. Lindbo and Dougherty (21) suggested that the CP of TEV expressed in plants may prevent systemic spread of the virus in the plant. Heteroencapsidation has been observed between two potyviruses (2), and phenotypic mixing was recently described in PVY CP+ transgenic lines infected with a distinct strain (10). If packaging is required for protection, the propensity of potyviruses for heteroencapsidation may account for the heterologous protection reported in this and in other potyvirus studies. This hypothesis could also explain the modification of symptoms toward mild or asymptomatic expression when the amount of CP synthesized in the plant is sufficient for heteroencapsidation. However, we do not know whether the CP or its mRNA is responsible for the protection observed in our LMV CP+ lines.

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