Coat Protein Gene-Mediated Resistance to Potato Virus Y in Tobacco: Examination of the Resistance Mechanisms —Is the Transgenic Coat Protein Required for Protection?

Laurent Farinelli and Pia Malnoë

Federal Agricultural Research Station of Changins (RAC), CH-1260 Nyon, Switzerland; and University of Geneva, Department of Plant Biology, CH-1205 Geneva, Switzerland.

Received 17 August 1992. Accepted 15 January 1993.

We transformed SR1 tobacco with a cDNA fragment of the potato virus Y strain N (PVY^N) 605 genome which contained the 3′ end of the NIb polymerase gene, the coat protein (CP) coding sequence, and most of the viral 3′ untranslated region. Complete resistance to several PVY strains was obtained in plants of the R_2 generation. The resistance to PVY^N and PVY^O strains was maintained when the plants were inoculated dually with the potato potyviruses V or A (PVV or PVA). Only partial resistance to PVY^N 605 was observed in plants containing the CP coding sequence in an antisense orientation. Both the "sense" and "antisense" plants accumulated CP transcripts and possessed multiple copies of the transgene. Systemic spread of the virus was studied by grafting experiments. The transgenic PVY^N 605 CP was undetectable in healthy plants. This protein accumulated in plants of PVY-susceptible CP-transgenic line 4B5-31 when infected by PVY^O, PVA, or PVV, but not in plants of line 4B5-S4, that were resistant to PVY and tolerant to PVA or PVV. To assess the role of the transgenic PVY^N 605 CP in protection, we transformed plants with a construct bearing a frameshift mutation at the beginning of the CP coding sequence, and observed partial protection from PVY^N 605. Several hypotheses to explain these resistance mechanisms are discussed.

Additional keywords: coat protein-mediated protection, dual infection, mechanism, plant virus, potyvirus, transgenic tobacco.

Since the initial demonstration that expression of the coat protein (CP) gene of tobacco mosaic virus (TMV) in transgenic plants could provide effective protection against this virus (Powell Abel *et al.* 1986), CP-mediated protection has been used with success against several virus groups (for reviews, see van den Elzen *et al.* 1989; Beachy *et al.* 1990; Gadani *et al.* 1990; Nejidat *et al.* 1990). The role of CP in the infection process of TMV was studied extensively, and it was demonstrated that purified CP can confer transient protection against the virus in protoplasts from untransformed tobacco (Register and Beachy 1989).

MPMI Vol. 6, No. 3, 1993, pp. 284-292

In transgenic plants, the level of resistance was proportional to the amount of TMV CP accumulated in the cells (Powell Abel *et al.* 1986), and plants that accumulated CP gene transcripts but not the CP itself were not resistant to infection (Powell *et al.* 1990). Early events in the TMV infection process appear to be blocked by the prior accumulation of CP in the cell, e.g., as demonstrated *in vitro,* free CP might selectively block the cotranslational disassembly process (Wilson and Watkins 1986), and experiments performed with protoplasts from CP-transgenic plants suggest an inhibition of virus uncoating (Osbourn *et al.* 1989; Wu *et al.* 1990). Furthermore, CP-mediated protection is overcome by inoculation with TMV RNA or with partially uncoated virus particles (Nelson *et al.* 1987; Register and Beachy 1988). The CP might also interfere with the replication of viral RNA, because a small but significant protection is observed against TMV RNA in plants transgenic for the CP of the virus (Osbourn *et al.* 1989; Nelson *et al.* 1987; Register and Beachy 1988). Finally, CP-transgenic plants were shown to have a reduced rate of local spread of TMV within the inoculated leaves and a reduced rate of systemic spread of the virus from the inoculated leaves to the upper parts of the plant (Wisniewski *et al.* 1990).

Several of these observations proved valid for other viruses, but differences in CP-mediated protection were (also) observed sometimes. For example, with potato virus X (PVX) and potato virus S (PVS), the resistance was not overcome by inoculation with viral RNA (Hemenway *et al.* 1988; MacKenzie and Tremaine 1990). On the other hand, although a threshold level of CP accumulation is apparently required for protection, in the case of soybean mosaic virus (SMV), potato virus Y (PVY), cucumber mosaic virus (CMV), tobacco spotted wilt virus (TSWV), tobacco etch virus (TEV), or PVX, plants with a high level of CP (or nucleocapsid protein) expression were only poorly protected (Stark and Beachy 1989; Lawson *et al.* 1990; Quemada *et al.* 1991; MacKenzie and Ellis 1992; Gielen *et al.* 1991; Lindbo and Dougherty 1992; Jongedijk *et al.* 1992). It should be mentioned that for TEV, plants transgenic for a mutated frameshift construct that did not accumulate the CP were partially protected from the virus (Lindbo and Dougherty 1992). Similarly, the transgenic protein was undetectable in highly virus resistant transgenic tobacco expressing the 54-kDa nonstructural gene of TMV (Golemboski *et al.* 1990). However, in this case expression

of the 54-kDa protein is required, even if undetectable (Carr *et al.* 1992).

PVY is the type member of the potyviruses (see review by Riechmann *et al.* 1992). This virus is transmitted nonpersistently by aphids, and its host range is mainly limited to the Solanaceae. The PVY veinal necrotic strains (PVY^N) are relatively mild on potato, but can be very destructive on pepper and tobacco. In contrast, the common O strains (PVY^O) can cause severe necrosis on potato, depending on the cultivar (de Bokx and Huttinga 1981). The long and flexuous particles contain a single-stranded RNA of positive sense encapsidated by approximately 2,000 units of a single CP (Shukla and Ward 1989). The 9.7-kb genomic RNA has a VPg, is polyadenylated, and is translated as a single polyprotein that is subsequently cleaved via an autocatalytic pathway by virus-encoded proteases (Riechmann *et al.* 1992).

We show here that tobacco plants transgenic for the coat protein gene of PVY^N 605 are completely immune to both PVY^N and PVY^O strains and tolerant to potato viruses V and A (PVV and PVA), when the viruses are inoculated separately or dually. We observed that the transgenic PVY^N CP, which is undetectable in virus-free plants, appears in susceptible plants during infection by PVY^O, PVV, or PVA. We studied the spread of the virus by performing graft-mediated transmissions. Finally, to assess the role of the CP itself in protection, we transformed tobacco with a construct bearing a frameshift mutation at the beginning of the CP coding sequence. The implications of these observations for understanding the resistance mechanisms are discussed.

RESULTS

Characterization of the transgenic lines.

SR1 tobacco was transformed with a cDNA fragment of the PVY^N 605 CP region, which was inserted in sense or antisense orientation in the plant expression vector pPCV702. Southern hybridizations indicated that six "sense" and five "antisense" transformed lines carried CP sequences. Upon inoculation with PVY^N 605 (undiluted inoculum, see Materials and Methods), the "sense" plant 4B5 and the "antisense" plant 10B13 inhibited virus development. These plants were self-fertilized twice to obtain PVY^N 605-resistant R_2 homozygous lines, named 4B5-S4 and 10B13-S1. The 4B5 and 10B13 R_2 plants have a probable transgene copy number of five and four, respectively, and they both accumulate CP transcripts (Farinelli 1992). Interestingly, the transgenic CP remained undetectable in plants of the highly PVY-resistant line 4B5-S4 (see below).

The sense 4B5 and the antisense 10B13 R_2 plants were challenged with PVY^N 605, PVY^O 803, PVY^O 768, potato virus V 624 (PVV), or potato virus A 778 (PVA) to determine their resistance range. The strains are listed in order of serological relatedness to PVY^N 605. As shown in Table 1, all the plants of the line 4B5 were highly resistant to PVY^N 605, PVY^O 803, or 768. These viruses were neither detectable at the inoculation site nor in the systemic leaves by symptom observation or by ELISA. PVV and PVA were detected in the apical part of the 4B5 plants by ELISA, but PVV was symptomless (tolerance). In contrast, tertiary vein clearing appeared 6–8 days after inoculation on untransformed SR1 tobacco infected by PVY or PVV. After 2 or 3 days, severe necroses, associated with growth reduction and leaf deformations appeared on the PVY^N 605-infected untransformed plants, whereas the PVY^O strains only caused the systemic appearance of clear spots on the leaves. PVA was often symptomless on SR1 tobacco. The plants of antisense line 10B13 were only partially protected from PVY^N 605 and were susceptible to the other PVY strains tested. When appearing, the necrotic symptoms caused by PVY^N 605 were usually restricted to small portions of the stems or the central ribs of the leaves. The weak necrosis symptoms were correlated with a very slow accumulation of virus in the inoculated and in the systemic leaves of 10B13 plants. Aphid-mediated inoculations of the PVY strains were performed on very young plants (two leaves of 2–3 cm) with the same results.

Mixed infections.

Dual potato virus infections often occur naturally. It was of interest to determine whether the resistance observed against one virus was maintained when two potyvirus strains were inoculated at the same time in the combinations shown in Table 2. All the untransformed SR1 tobacco plants became infected by each virus, indicating that one strain is not hampering infection by the other strain. The 4B5-S4 plants were completely resistant to PVY^N 605, PVY^O 803, or PVY^O 768 inoculated in combination with PVV (Table 2). This experiment demonstrated that the resistance against PVY was maintained in the presence of an infection by PVV taking place in the same plants.

For the antisense 10B13-S1 plants, as with the 4B5-S4 plants, the protection against PVY^N 605 in double infections was not different from that against the virus inoculated alone.

Detection of the transgenic CP.

R_1 generation plant lines of 4B5 were challenged with PVY^O 768 (dilution 1:10), a strain serologically distantly

Table 1. Determination of resistance range

	Single potato virus inoculated[a]				
Plant lines	PVY^N 605	PVY^O 803	PVY^O 768	PVV 624	PVA 778
SR1 tobacco[b]	100[c]	100	100	100	100
Sense 4B5	0	0	0	100	100
Antisense 10B13	75	100	100	100	100

[a] The indicated virus strains were mechanically inoculated using a 1:10 dilution of fresh extract from infected tobacco (see text).

[b] SR1 tobacco, untransformed SR1 tobacco; Sense 4B5 and Antisense 10B13, homozygous R_2 plants of lines 4B5-S4 or 10B13-S1, transgenic for PVY^N 605 coat protein gene respectively in sense or in antisense orientation.

[c] Fifteen days after mechanical inoculation, the presence of virus was detected by ELISA in 15 plants for each line and each virus (see text). Values are shown as the percentage of plants containing a detectable level of virus accumulation ($A_{405} > 0.5$) to facilitate comparisons. Except for PVY^N 605-infected 10B13 plants, accumulation of virus was comparable in antisense transgenic, in PVV- or PVA-inoculated sense, and in untransformed plants ($A_{405} > 2.0$ after 30–60 min). The experiments with the PVY strains were repeated two or three times with the same results.

related to PVY^N 605. Ten plant lines out of the 17 inoculated were found to be susceptible to the virus. Surprisingly, by using a monoclonal antibody specific for detection of PVY^N but not PVY^O 768, it appeared that eight out of the 10 PVY^O-infected R_1 4B5 plant lines accumulated the transgenic PVY^N CP, with an A_{405} usually lower than in PVY^N-infected plants. PVY^N CP was not detectable in the healthy resistant or inoculated resistant 4B5 plant lines, or in untransformed SR1 control tobacco infected by PVY^O 768. To confirm that the plants accumulating PVY^N CP were not contaminated by PVY^N, or that a new PVY^N strain had not appeared by recombination between the PVY^O 768 genomic RNA and the transgenic messenger RNA of the CP gene, we inoculated extracts from these plants on untransformed SR1 tobacco. PVY^N CP was not detected in these plants (data not shown).

To determine if other potyviral strains also caused the appearance of the transgenic CP upon infection, three R_1 4B5 plant lines were self-fertilized: line 4B5-19 that was resistant to PVY^O 768, line 4B5-31 that was susceptible to this virus and accumulated the transgenic CP, and line 4B5-S4 that had not been inoculated. The R_2 progeny of the 4B5-S4 plant was used for characterization of the resistance range (Table 1). These plants were challenged with PVY^N 605, PVY^O 803, PVY^O 768, PVV, or PVA. Table 3 shows that the transgenic CP was detected in plants of line 4B5-31 infected by any of the viruses tested, but not in the 4B5-31 plants that remained uninfected. PVY^N CP was neither detectable in the 4B5-S4 or 4B5-19 plants, nor in the PVY^O 803-, PVY^O 768-, PVV-, or PVA-infected untransformed plants. The 4B5-S4 plants infected by PVV or PVA did not accumulate the transgenic CP, in contrast to 4B5-31 plants (Table 3).

These experiments show that both the phenotypes "PVY^O 768-susceptible" and "accumulation of the transgenic CP" are transmitted to the progeny of plant 4B5-31. Furthermore, the transgenic PVY^N coat protein was detected in virus-infected plants only.

Table 2. Dual inoculations of potyviruses

	Viruses or strains inoculated dually[a]				
Plant lines	**605:768**	**803:768**	**605:PVV**	**803:PVV**	**768:PVV**
SR1 tobacco[b]	100:100[c]	100:100	100:100	100:100	100:100
Sense 4B5	0:0	0:0	0:100	0:100	0:100
Antisense 10B13	75:94	100:100	82:100	100:100	100:100

[a] The two potyviruses indicated were mechanically inoculated as a 1:1 mixture onto 11–15 plants (final dilution 1:20). 605, 803, 768, and PVV are PVY^N 605, PVY° 803, PVY° 768, or PVV 624, respectively.
[b] The plant lines are as in Table 1.
[c] Values are percentage of plants containing virus specifically detected by ELISA 15 days after inoculation using monoclonal antibodies specific for each strain. The values correspond to the strains indicated at the top of each column.

Grafting experiments.

To find out whether the 4B5-S4 plants were protected once the virus had invaded the vascular system, we grafted the apical parts of 4B5 plants onto untransformed SR1 tobacco root stocks that were subsequently inoculated. Even though the lower part of these plants became severely infected, all the transgenic apical sections escaped infection (Table 4). In contrast, the untransformed apical parts of all control grafted plants were severely infected, showing that the graft did not block systemic infection by the virus.

The reverse experiment was also carried out with similar results, the untransformed apical part (that was inoculated) became infected normally and the transgenic root stock was free of virus (Table 4).

The grafts were also performed with stem sections instead of apical parts, to favor contaminations of very young axillary bud tissue. Even though an afflux of contaminated sap was feeding the developing axillary bud, all the transgenic shoots remained healthy. In contrast, young shoots from untransformed control stem scions were atrophied because of the severe infection.

We then carried out experiments with double grafts to determine whether the vascular transport of the virus was inhibited in the transgenic plants. We replaced a stem section of untransformed SR1 tobacco containing a leaf with a similar stem section originating from a 4B5 plant. After 10–12 days, PVY^N 605 was inoculated on the lower part of the grafted plant, and by monitoring the infection in the apical part, we could determine whether the virus was transported through the grafted section. In the first experiment, virus spread was blocked by the transgenic section (in 11 plants out of 14 tested), but during later experiments, in the spring, only a delay in infection was observed (Table 5). No virus was detected in the grafted transgenic leaf.

Table 3. Detection of the transgenic coat protein in R_2 plants[a]

	Potyvirus inoculated								
	PVY^N 605	PVY° 803		PVY° 768		PVV 624		PVA 778	
Plant line	**605[b]**	**803**	**CP-N**	**768**	**CP-N**	**PVV**	**CP-N**	**PVA**	**CP-N**
4B5-31	63[c]	57	57	73	73	100	100	100	100
4B5-19	0	0	0	0	0	NT[d]	NT	NT	NT
4B5-S4	0	0	0	0	0	100	0	100	0
NT-SR1	100	100	0	100	0	100	0	100	0

[a] The R_2 progeny of self-fertilized 4B5 plants was challenged with the potyviruses indicated. The mechanical inoculations were performed on 10–15 plants using a 1:10 dilution of fresh extract of infected leaves. The R_1 4B5-31 and 4B5-19 plants were, respectively, susceptible or resistant to PVY° 768. The line 4B5-S4 was previously shown to be completely resistant to the viruses tested here. NT-SR1 is untransformed SR1 tobacco.
[b] Viral strain specifically detected by ELISA carried out 15 days after inoculation. CP-N is a PVY^N-specific antigen corresonding to the transgenic CP.
[c] Values are presented as percentages of plants accumulating virus and/or the transgenic CP to facilitate comparisons.
[d] Not tested.

Control plants with a grafted section originating from untransformed tobacco became infected in the apical part as rapidly as nongrafted plants.

We have not been able to determine if the transgenic stem or the leaf was responsible for the delay in systemic infection. Indeed, neither grafts of a transgenic stem section without leaf, nor grafts of a leaf without stem (but with an axillary bud) showed any effect on systemic virus spread (Table 5). In both cases, PVY^N 605 was detected in the apical part as rapidly as in nongrafted untransformed SR1 tobacco.

Introduction of a frameshift mutation at the begining of the CP gene.

Previous experiments indicated that plants of the line 4B5-S4 were protected from PVY even though the transgenic CP was undetectable. It is still possible that the transgenic CP is synthesized but very rapidly degraded and thus neither detectable by ELISA nor by Western immunoblot analysis. To find out whether protection could indeed be provided by accumulation of the CP messenger RNA without the CP, or whether the CP itself had to be synthesized in the plant, we introduced a frameshift mutation in the begining of the CP gene of sense construct pCP4 (Fig. 1). SR1 tobacco was transformed with the mutated construct pCP4-Cla, the original sense construct pCP4, or with the pPCV702 expression vector. For each construct, 20 independent transformants (R_0) were regenerated and the plants lines were named stop, sense, or vector, respectively. The presence of the transgenic CP gene in the first two classes of these plants was shown by polymerase chain reaction analysis (data not shown). Cuttings of these R_0 transformants were challenged with PVY^N 605 (1:100 dilution), and, even if all transformants carried CP sequences, only one quarter of the sense transformants were resistant to the virus. All plants transformed with the pPCV702 expression vector accumulated the virus rapidly and developed typical PVY^N 605 necrotic symptoms, exactly as the untransformed SR1 tobacco plants.

During the first challenge experiment, two transformed lines, named 4Cla10 and 4Cla17, transgenic for the mutated construct pCP4-Cla, did not become infected by the virus. However when the inoculation was repeated on other cuttings of these plants, they accumulated the virus and attenuated necrotic symptoms were observed. This indicates that in the R_0 generation, the mutated construct pCP4-Cla is not sufficient to provide effective protection.

Four stop plants, one vector, and one sense plant were self-fertilized, to study the protection from PVY in the R_1 generation. When challenged with PVY^N 605, individuals of three stop lines were partially protected from the virus (Table 6). The most interesting line was 4Cla17: 1 mo after inoculation, four plants out of 10 were still virus-free, as monitored by ELISA, electron microscopy, and symptom observations (Fig. 2). Two other plants of this line exhibited a delay of 10–15 days in the systemic accumulation of the virus. The other four plants accumulated PVY^N 605 exactly as the untransformed or pPCV702-transformed plants (Fig. 2).

Northern blot analysis of total RNA extracted from nine of the 10 4Cla17 R_1 plants before inoculation revealed that transcripts of the expected size were detectable for each plant. The plants that were susceptible to the virus accumulated relatively high levels of transcripts (Fig. 3).

The R_2 progeny of six 4Cla17 plants that were protected from PVY^N 605 and the progeny of one susceptible plant (named 4Cla17-10) were challenged with PVY^N 605 to confirm the resistance phenotype. For each line except 4Cla17-10, several plants were found to be completely resistant to the virus. Indeed, PVY^N 605 was neither detectable visually, nor by ELISA or Northern blot hybridization (Fig. 4, and data not shown). All the plants of line 4Cla17-10 were susceptible to the virus, with symptoms undistinguishable from those of vector R_2 plants, or untransformed SR1 tobacco.

These experiments demonstrate that a partial protection from PVY^N 605 can be obtained in plants transgenic for a construct that contains a frameshift mutation in the CP gene.

DISCUSSION

We have obtained complete resistance to PVY in tobacco plants transgenic for the CP coding sequence of PVY^N

Table 4. PVY^N 605 transmission by simple grafting

Root-stock	Apical section	Number of plants[a]	PVY accumulation[b] Root-stock	Apical section
NT-SR1 (inoc.)[c]	4B5	22 (10)	22	0
NT-SR1 (inoc.)	NT-SR1	10 (5)	10	10
4B5	NT-SR1 (inoc.)	12	0	12
NT-SR1	NT-SR1 (inoc.)	15	15	15

[a] Total number of plants. In parentheses, grafts with truncated apical stem sections to favor the infection of young buds.
[b] Number of plants with infected root-stock or apical part, as detected by ELISA and confirmed by symptom observation 20 days after inoculation.
[c] NT-SR1:untransformed SR1 tobacco. 4B5:transgenic tobacco of line 4B5-S4, R_2, transgenic for the CP gene of PVY^N 605. Inoculated parts are indicated (inoc.).

Table 5. Assay of PVY^N 605 spread through a transgenic stem segment of tobacco plant of line 4B5 inserted in the middle of a untransformed SR1 tobacco plant

Grafted section[a]	Number of plants infected in the apical section: Stem and leaf[b]		Stem without leaf		Leaf without stem	
4B5	23[c]	(36)	7	(7)	10	(10)
Untransformed SR1	16	(16)	ND[d]		3	(3)

[a] Plants from which the grafted section originated (see Table 1).
[b] Three kind of grafts were carried out on untransformed SR1 tobacco: grafts of a stem section bearing a leaf, of a stem section without leaf, and of a leaf without any stem part.
[c] Number of infected apical sections as determined by ELISA and confirmed by symptom observation. In parentheses, number of plants tested. For the graft of 4B5 stem with leaf, the experiment was repeated three times with different results: 3/14, 13/15, and 7/7 plants became infected in the apical part (cumulative results shown). In these plants, the PVY accumulation was delayed by 2 days or more, and the symptoms were attenuated.
[d] Not determined.

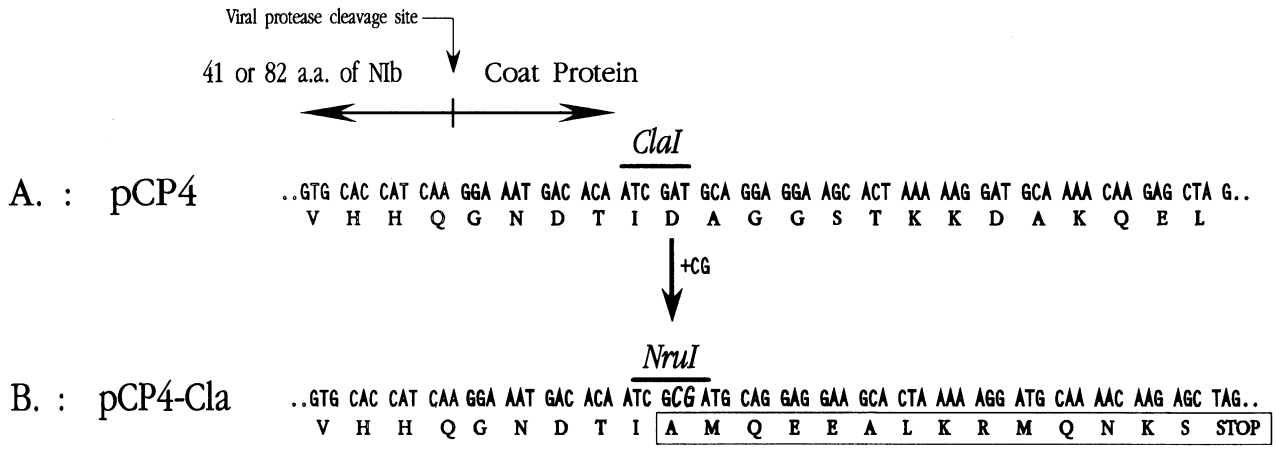

Fig. 1. Modification of the beginning of the coat protein (CP) coding sequence in construct pCP4-Cla. The introduction of nucleotides GC in the *Cla*I restriction site of pCP4 (Farinelli 1992) creates a frameshift mutation in the CP coding sequence. The *Cla*I site is then converted into a *Nru*I site. The mutated polypeptide encoded by this construct would comprise 82 or 41 amino acids of the end of the NIb polymerase gene, five residues of the CP gene, and 14 out-of-frame amino acids (boxed). The NIa protease site located between the NIb and the CP coding sequences is conserved.

Table 6. Resistance to PVY^N 605 in R_1 plants that contain a frameshift mutation in the coat protein (CP) gene

Plant line[a]	PVY-resistance (R_0)[b]	7 days[c]	24 days[c]
"Stop" 4Cla1	Susceptible	8	9
"Stop" 4Cla2	Susceptible	10	10
"Stop" 4Cla10	Resistant	6	9[d]
"Stop" 4Cla17	Resistant	4	5
"Sense" 4L5	Resistant	0	1[d]
"Vector" 702L2	Susceptible	10	10

[a] Progeny from self-fertilized R_0 plants, transgenic for construct pCP4-Cla (stop), pCP4 (sense), or pPCV702 (vector).
[b] Phenotype of the protection from PVY^N 605 at the R_0 generation. Plants 4Cla10 and 4Cla17 were only protected from the virus in one experiment out of three.
[c] Ten R_1 plants were challenged with PVY^N 605 inoculated as a 1:100 dilution of fresh extract from infected tobacco leaves. Values are number of plants in which virus was detectable by ELISA, 7 or 24 days after inoculation.
[d] These plants were tested 21 days after inoculation.

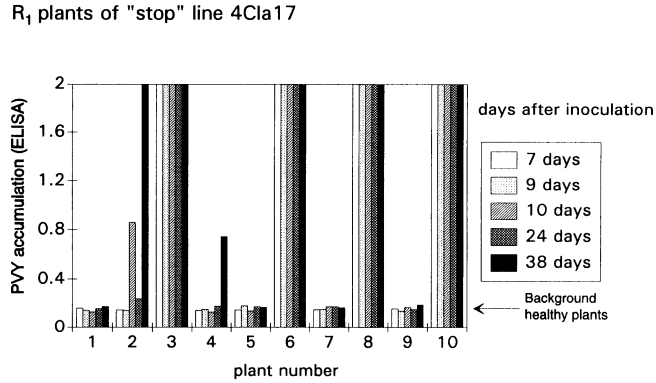

Fig. 2. Accumulation of PVY^N 605 in the apical part of "stop" R_1 plants of "stop" line 4Cla17, transgenic for frameshift construct pCP4-Cla. The virus was mechanically inoculated using a 1:100 dilution of fresh extract from infected leaves, and the infection was monitored by ELISA at the times indicated. Plants numbered 1, 2, 4, 5, 7, and 9 were symptomless, except for faint necrosis appearing with a delay of 10 days or more on the stem or on the leaves of plants numbered 1, 2, and 4. Vector transformed plants accumulated the virus as plant number 10.

605. As reported for other viruses, transgenic lines with the CP gene in the sense orientation showed better protection than lines with the CP gene in its antisense orientation (Hemenway *et al.* 1988; Cuozzo *et al.* 1988).

The R_2 plants of the sense line 4B5 were not only protected from the homologous PVY^N strain, but they were also completely resistant to serologically distant PVY^O strains. The resistance was not overcome by mechanical or aphid- or graft-mediated inoculations with high virus concentration. This phenotype contrasts with results reported for CP-mediated protection against TMV, where the resistance was reduced with high inoculum concentration or when distant strains were used (Powell Abel *et al.* 1986; Anderson *et al.* 1989). However, similarities were observed with the TMV 54-kDa gene-transformed plants (Golemboski *et al.* 1990), which could perhaps be explained by the NIb region present in our construct.

Plants of the transgenic line 4B5 were also tolerant to the potyvirus PVV, i.e., PVV spread in the plants but no symptoms appeared. When PVY^N or PVY^O were inoculated dually with PVV, PVV spread and the PVY strains were restricted as in separate inoculations. Infection by PVV certainly induces important changes in the cells to favor virus multiplication and transport, and it is surprising that PVY^N or PVY^O is unable to take advantage of this process.

The infection process of a virus can be blocked at several steps in a plant: First, in the inoculated cell, where the virus has to replicate, then during its movement from cell-to-cell through the plasmodesmata, and finally when the particles migrate into the rest of the plant through the vascular system.

We have indications that the early events of PVY infection are blocked in the transgenic 4B5 plants. Several grafting experiments demonstrated that the resistance is maintained when high concentrations of virus are transmitted from the vascular system to very young axillary

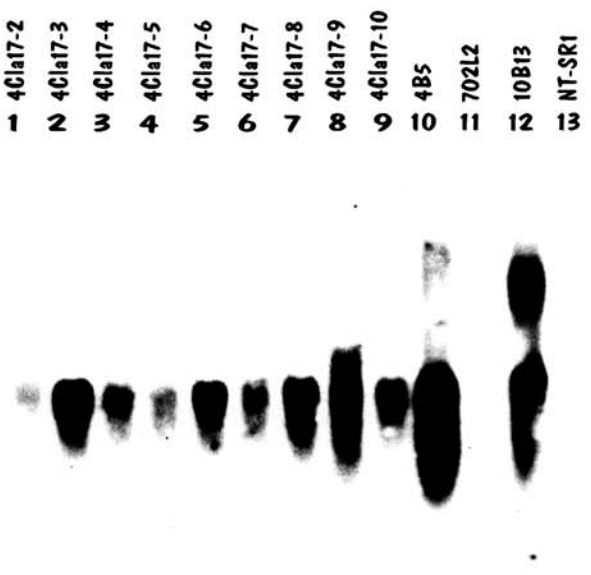

Fig. 3. Northern blot hybridization of total RNA from R_1 plants of "stop" line 4Cla17. The RNA was extracted just before inoculation with PVY^N 605 (see Fig. 2). Accumulation of coat protein transcripts of the expected size was observed. Staining of the samples with 1.5 μg of ethidium bromide before denaturation at 50° C verified the transfer efficiency, and that the same quantity of RNA was loaded on the different lanes (40 μg). Negative controls were the vector-transformed line 702L2 and the untransformed line NT-SR1. The positive controls were the transgenic plants (line 4B5-S4 and line 10B13-S1).

bud tissue. Furthermore, a delay in virus spread was observed when the virus was transported through a section of transgenic stem bearing a leaf. Without a leaf on the stem section, no delay was observed. Similar results were reported for TMV in tobacco, showing the importance of the leaf attached to the stem section for restriction of the systemic spread of the virus (Wisniewski *et al.* 1990). To assess the hypothesis that factors diffuse from the transgenic part to hamper the infection in the untransformed apical sections, we performed grafts of leaves without stem. Furthermore, we also grafted an untransformed apical section onto a transgenic root stock (Table 4). In neither case was the infection of the apical part reduced, indicating that in our case another mechanism must be involved.

Several reports indicate that in the cases of CP-mediated protection with potyviruses, the most resistant lines are not necessarily those that accumulate the highest levels of CP (Stark and Beachy 1989; Lawson *et al.* 1990; Ling *et al.* 1991). No AUG was engineered in our construct pCP4, and the transgenic PVY^N CP remained undetectable in healthy potato and tobacco transformants (Farinelli *et al.* 1992; Farinelli 1992). However, the transgenic CP mRNA is translatable because high accumulation of PVY^N CP was detected in PVY^O- PVV- or PVA-infected 4B5 plants of susceptible lines (e.g. 4B5-31, at the R_1 generation). The difference between the susceptible and resistant 4B5 lines is unexplained, but genetic rearrangements might have occurred during meiosis. However, the transgenic CP is nevertheless undetectable in these plants when they were

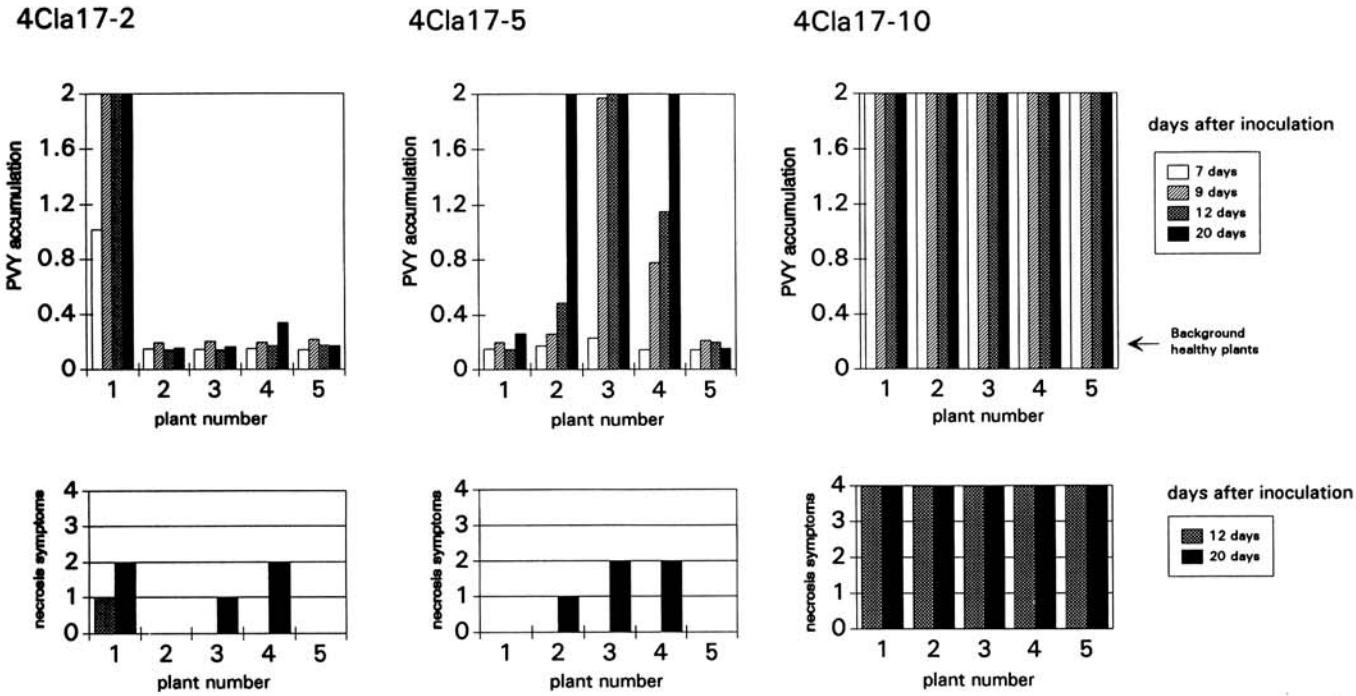

Fig. 4. Infection of PVY^N 605 in the "stop" R_2 progeny of plants 4Cla17-2, 4Cla17-5, and 4Cla17-10 (see Fig. 2). The accumulation of virus was monitored by ELISA on the apical leaves at the time indicated, and by symptom observations. The symptoms were very weak on the plants of lines 4Cla17-2 and 4Cla17-5, with faint necroses appearing on the stems and sometimes on the leaves. These plants were morphologically undistinguishable from healthy SR1 plants, i.e., no deformations or growth reduction were observed. The necrosis strength was rated from 0 to 4 with 0: healthy; 1: symptoms barely visible; 2: small brown spots mainly on the secondary veins; 3: the main vein of one leaf is entirely dark brown; 4: severe and systemic necroses with leaf deformations.

not infected by a virus. A hypothesis suggests that the transgenic CP could be stabilized, possibly by incorporation into virus particles. Indeed, we have observed heterologously encapsidated particles in infected transgenic potato plants that exhibited a similar phenotype (Farinelli *et al.* 1992). Another possibility is that the viruses in the infected plants might provide a translation factor, or induce a modification of the plant protein translation apparatus necessary for the synthesis of CP.

The 4B5-S4 plants (the completely resistant line) did not accumulate the transgenic CP when infected by PVV or PVA, which suggests that the putative viral factor might not be sufficient to allow synthesis of the viral or transgenic PVY CP. These data indicate that an additional factor (missing in the 4B5-S4 plants) might also be required. This hypothesis could explain the complete resistance of these plants, but other mechanisms are also certainly involved that might provide a partial protection, as observed for the antisense or the stop plants (see below).

The lack of transgenic CP accumulation in 4B5-S4 plants also suggests that the transgenic CP might not be synthesized at all. To determine whether a plant that did not synthesize the transgenic CP per se was nevertheless protected from PVY, we modified the pCP4 construct to introduce a frameshift mutation at the beginning of the CP coding sequence. In contrast to the results obtained for AlMV and TMV (van Dun *et al.* 1988; Powell *et al.* 1990), a partial protection from PVY^N 605 was observed (Fig. 4). Lindbo and Dougherty (1992) obtained a similar result for the potyvirus TEV with truncated CP genes, but no protection was observed with the complete CP gene. Three hypotheses could explain this result: 1) the CP mRNA is sufficient to provide resistance to the virus, perhaps by hybridization to the minus-strand, or because the 3′ untranslated region in the construct can be used to initiate abortive minus-strand synthesis; 2) a ribosomal frameshift or a reinitiation of translation takes place, and some CP is synthesized, in which case, only a very low level of CP would be synthesized; or 3) the 101 or 60 amino acid polypeptide that can still be synthesized would contain the NIb/CP cleavage site for the viral NIa protease. This specific site, in excess, might saturate the enzyme and reduce virus multiplication. However, the protease would probably only be inhibited in the presence of a modified substrate, e.g., because of an altered conformation of the polypeptide. This approach has been used in attempts to inhibit the infection of the retrovirus HIV with oligopeptides containing modified cleavage sites (Tözsér *et al.* 1991; Meek *et al.* 1990). Mutagenesis experiments of our constructs are under way to determine which sequence, from the NIb or the CP region, is required for protection.

MATERIALS AND METHODS

Virus strains.

PVY^N 605 (CH-1976 Bintje #605), PVY^O 803 (CH-1986 Bintje #803), and PVA 778 (CH-1984 Sirtema #778) were isolated in western Switzerland on potato (Gugerli and Fries 1983; P. Gugerli, unpublished results). PVV 624 (PVY^C Gladblaadje, J.A. de Bokx, Holland) and the atypical PVY^O 768 (PVY^c 768, S. Peressini, Italy) were from the collection of P. Gugerli. The viruses were propagated in a greenhouse on *Nicotiana tabacum* L. 'Xanthi'-nc or *N. tabacum* 'Petit Havana' line SR1 (SR1 tobacco).

Plasmid constructions and plant transformations.

cDNA was synthesized from PVY^N 605 genomic RNA and cloned into pUC19. A clone containing the CP gene was identified by serological means after expression in *Escherichia coli* and nucleotide sequence comparisons (P. Malnoë, L. Farinelli, G. F. Collet, P. Gugerli, and W. Reust, unpublished). A 1,298-bp *Sau3A* fragment of this clone was inserted into the *Bam*HI site of the plant expression vector pPCV702 (Koncz *et al.* 1989), either in sense orientation (construct pCP4) or in antisense orientation (construct pAS10), between the 35S cauliflower mosaic virus promoter and the nopaline synthase terminator sequences (Farinelli 1992). Initiation of translation is possible at two in-phase AUGs located 246 or 123 nt upstream of the CP gene, in the last 285 nt of the NIb polymerase gene, thus leading to the synthesis of a fusion protein containing 82 or 41 amino acids of NIb in addition to the 367 amino acids of the CP. The CP fragment also contained 212 nt of the viral 3′ untranslated region.

To introduce a frameshift mutation at the beginning of the CP coding sequence, the *Cla*I site of pCP4 was converted to a *Nru*I site by in-filling with the nucleotides GC using the Klenow fragment of DNA polymerase I (Fig. 1). The open reading frame of this construct, pCP4-Cla, is predicted to code for a short polypeptide comprising 82 or 41 amino acids of the NIb polymerase C-terminal end, the first five residues of the coat protein, and 14 out-of-frame amino acids.

Leaf disks from SR1 tobacco were cocultivated with *Agrobacterium tumefaciens* strain GV3101 (pMP90RK) (Koncz *et al.* 1989) carrying either pCP4, pCP4-Cla, pAS10, or pPCV702. Transformed plant cells were selected for kanamycin resistance and regenerated into plants as described (Horsch *et al.* 1985). Regenerated plants were then potted in soil and grown under greenhouse conditions, with a 16-hr day and 8-hr night cycle. Self-fertilizations were performed using paper bags to enclose the flower buds. Seeds were germinated on 1/2× Murashige and Skoog (MS) salts agar containing 100 μg of kanamycin (Sigma Chemicals, St. Louis) per milliliter. The number of resistant and sensitive seedlings was determined 4–5 wk after germination when the sensitive seedlings were chlorotic and unable to produce true leaves.

Virus transmission and infection.

For mechanical inoculations, two leaves of young plants at the four- to six-leaf stage were dusted with Carborundum and rubbed with the indicated dilutions (v/v) of inoculum prepared by grinding 1 g of young PVY-infected tobacco leaf tissue in 1.5 ml of 20 mM sodium phosphate, pH 7.6, 10 mM sodium diethyldithiocarbamate (DIECA). Inoculated plants were rinsed after 3–5 min. For aphid-mediated inoculations, *Myzus persicae* (Sulzer) were fed on PVY-infected tobacco. After 1 or 2 days, leaf fragments

with 20–50 apterous aphids were laid on each plant to infect. After another 2 or 3 days, the plants were treated with insecticide (Pirimor, ICI Chemicals).

Symptom appearance was recorded and the plants were tested by ELISA to detect PVY antigens, according to the protocol of Gugerli (1986). Typically, 0.25 g of leaf tissue from the youngest developed leaf was ground with 5 ml of PBS-Tween-PVP (phosphate-buffered saline, 0.05% [v/v] Tween 20, 2% [w/v] polyvinylpyrrolidone) in plastic bags internally coated with cheesecloth. Each sample was tested in duplicate. Polyclonal and monoclonal antibodies specific for each strain were obtained from P. Gugerli and from Bioreba AG, Basel (Gugerli and Fries 1983; P. Gugerli, unpublished results). All antibodies were used at a standard concentration of 1 μg/ml. The A_{405} was read 15, 30, 60, and 120 min after addition of *p*-nitrophenyl phosphate substrate solution. Background level for virus-free transgenic or untransformed plants was between 0.135 and 0.180. An A_{405} above 0.4 was considered clearly positive.

Grafts.

Grafts, with the shape of a "Λ", were performed on plants at the four- to six-leaf stage. The parts were joined using Parafilm, and the expanded leaves of the apical parts were cut off. The plants were then left for 7–12 days in a high humidity growth chamber before inoculation. During the experiment, the plants were kept in small chambers with 60–80% relative humidity.

Nucleic acids analysis.

DNA and RNA isolation from plants was carried out according to the protocol of Dellaporta and co-workers (1983) modified as follows: 0.5–1 g of young leaf tissue was ground in liquid nitrogen, transferred to Eppendorf tubes, and homogenized in 750 μl of hot (65° C) extraction buffer (100 mM Tris·HCl pH 8.0, 50 mM EDTA, 500 mM NaCl, and 20 mM 2-mercaptoethanol). After addition of 1% (w/v) sodium dodecyl sulfate (SDS), the extracts were heated for 10 min at 65° C. Proteins were precipitated at 4° C with 250 μl of 5 M potassium acetate, and nucleic acids were recovered by isopropanol precipitation. Samples were further purified by phenol extraction, and resuspended in 10 mM Tris·HCl, pH 8.0, 1 mM EDTA, containing 0.05% (v/v) diethyl pyrocarbonate for the RNA samples.

According to Maniatis *et al.* (1982), 5–20 μg of the purified DNA was digested with restriction enzymes in the presence of RNase, separated overnight on a 0.7% agarose gel (20 cm, 25 mA, in TBE buffer with recirculation), and transferred to nitrocellulose filters. Purified RNA was run on denaturing formaldehyde-agarose gels and transferred to nitrocellulose filters. Randomly primed ^{32}P-labeled probes were prepared according to Feinberg and Vogelstein (1983), and hybridizations were performed overnight at 42° C in 50% formamide, 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 5× Denhardts solution, and 100 μg/ml sheared calf thymus DNA. After washes in 2× SSC and 0.2× SSC in the presence of 0.1% (w/v) SDS, the filters were exposed for autoradiography at −70° C for 20–40 hr for Southern blots and for 3 days for Northern blots.

Western blots analysis of proteins.

Protein extractions and Western immunoblot analyses were carried out as described previously (Farinelli *et al.* 1992).

ACKNOWLEDGMENTS

We thank P. Gugerli for virus strains, antibodies, the use of electron microscope, and for stimulating discussion. Thank you also to the group of virology for help in the lab and in the greenhouse. We also grateful to J.-D. Rochaix and G. F. Collet for critical reading of the manuscript. This project was supported by the Swiss National Science Foundation, 31-26333.89 and European Program COST '88.

LITERATURE CITED

Anderson, E. J., Stark, D. M., Nelson, R. S., Powell, P. A., Tumer, N. E., and Beachy, R. N. 1989. Transgenic plants that express the coat protein genes of tobacco mosaic virus or alfalfa mosaic virus interfere with disease development of some nonrelated viruses. Phytopathology 79:1284-1290.

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

Carr, J. P., Marsh, L. E., Lomonossoff, G. P., Sekiya, M. E., and Zaitlin, M. 1992. Resistance to tobacco mosaic virus induced by the 54-kDa gene sequence requires expression of the 54-kDa protein. Mol. Plant-Microbe Interact. 5:397-404.

Cuozzo, M., O'Connell, K. M., Kaniewski, W., Fang, R.-X., Chua, N.-H., and Tumer, N. E. 1988. Viral protection in transgenic tobacco plants expressing the cucumber mosaic virus coat protein or its antisense RNA. Bio/Technology 6:549-557.

de Bokx, J. A., and Huttinga, H. 1981. Potato Virus Y. No. 242 in: Descriptions of Plant Viruses. CMI/AAB, Kew, England. 6 pp.

Dellaporta, S. L., Wood, J., and Hicks, J. B. 1983. A plant DNA minipreparation: Version II. Plant Mol. Biol. Rep. 1:19-21.

Farinelli, L. 1992. Coat protein gene-mediated resistance to the potato virus Y (PVY) in tobacco and potato. Ph.D. thesis. University of Geneva, Switzerland.

Farinelli, L., Malnoë, P., and Collet, G. F. 1992. Heterologous encapsidation of potato virus Y strain O (PVY^O) with the transgenic coat protein of PVY strain N (PVY^N) in *Solanum tuburosum* cv. Bintje. Bio/Technology 10:1020-1025.

Feinberg, A. P., and Vogelstein, B. 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. 132:6-13.

Gadani, F., Mansky, L. M., Medici, R., Miller, W. A., and Hill, J. H. 1990. Genetic engineering of plants for virus resistance. Arch. Virol. 115:1-21.

Gielen, J. J. L., de Haan, P., Kool, A. J., Peters, D., van Grinsven, M. Q. J. M., and Goldbach, R. W. 1991. Engineered resistance to tomato spotted wilt virus, a negative-strand RNA virus. Bio/Technology 9:1363-1367.

Golemboski, D. B., Lomonossoff, G., and Zaitlin, M. 1990. Plants transformed with a tobacco mosaic virus nonstructural gene sequence are resistant to the virus. Proc. Natl. Acad. Sci. USA 87:6311-6315.

Gugerli, P. 1986. Potato Viruses. Pages 430-446 in: Methods of Enzymatic Analysis. Volume XI: Antigen and Antibodies 2. H. U. Bergmeyer, ed. VCH Verlagsgesellschaft, Weinheim, Germany.

Gugerli, P., and Fries, P. 1983. Characterization of monoclonal antibodies to potato virus Y and their use for virus detection. J. Gen. Virol. 64:2471-2477.

Hemenway, C., Fang, R.-X., Kaniewski, W. K., Chua, N.-H., and Tumer, N. E. 1988. Analysis of the mechanism of protection in transgenic plants expressing the potato virus X coat protein or its antisense RNA. EMBO J. 7:1273-1280.

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

Jongedijk, E., De Schutter, A. A. J. M., Stolte, T., van den Elzen, P. J. M., and Cornelissen, B. J. C. 1992. Increased resistance to potato virus X and preservation of cultivar properties in transgenic potato under field conditions. Bio/Technology 10:422-429.

Koncz, C., Martini, N., Mayerhofer, R., Koncz-Kalman, Z., Körber, H., Redei, G. P., and Schell, J. 1989. High-frequency T-DNA-mediated gene tagging in plants. Proc. Natl. Acad. Sci. USA 86:8467-8471.

Lawson, C., Kaniewski, W., Haley, L., Rozman, R., Newell, C., Sanders, P., and Tumer, N. E. 1990. Engineering resistance to mixed virus infection in a commercial potato cultivar: Resistance to potato virus X and potato virus Y in transgenic Russet Burbank. Bio/Technology 8:127-134.

Lindbo, J. A., and Dougherty, W. G. 1992. Pathogen-derived resistance to a potyvirus: Immune and resistant phenotypes in transgenic tobacco expressing altered forms of a potyvirus coat protein nucleotide sequence. Mol. Plant-Microbe Interact. 5:144-153.

Ling, K., Namba, S., Gonsalves, C., Slightom, J. L., and Gonsalves, D. 1991. Protection against detrimental effects of potyvirus infection in transgenic tobacco plants expressing the papaya ringspot virus coat protein gene. Bio/Technology 9:752-758.

MacKenzie, D. J., and Ellis, P. J. 1992. Resistance to tomato spotted wilt virus infection in transgenic tobacco expressing the viral nucleocapsid gene. Mol. Plant-Microbe Interact. 5:34-40.

MacKenzie, D. J., and Tremaine, J. H. 1990. Transgenic *Nicotiana debneyii* expressing viral coat protein are resistant to potato virus S infection. J. Gen. Virol. 71:2167-2170.

Maniatis, T., Fritsch, E. F., and Sambrook, J. 1982. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Meek, T. D., Lambert, D. M., Dreyer, G. B., Carr, T. J., Tomaszek, T.A., Jr., Moore, M. L., Strickler, J. E., Debouck, C., Hyland, L. J., Matthews, T. J., Metcalf, B. W., and Petteway, S. R. 1990. Inhibition of HIV-1 protease in infected T-lymphocytes by synthetic peptide analogues. Nature 343:90-92.

Nejidat, A., Clark, W. G., and Beachy, R. N. 1990. Engineered resistance against plant virus diseases. Physiol. Plant. 80:662-668.

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

Osbourn, J. K., Watts, J. W., Beachy, R. N., and Wilson, T. M. A. 1989. Evidence that nucleocapsid disassembly and a later step in virus replication are inhibited in transgenic tobacco expressing TMV coat protein. Virology 172:370-373.

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.

Powell, P. A., Sanders, P. R., Tumer, N. E., Fraley, R. T., and Beachy, R. N. 1990. Protection against tobacco mosaic virus infection in transgenic plants requires accumulation of coat protein rather than coat protein RNA sequences. Virology 175:124-130.

Quemada, H. D., Gonsalves, D., and Slightom, J. L. 1991. Expression of the coat protein gene from cucumber mosaic virus strain C in tobacco: Protection against infections by CMV strains transmitted mechanically or by aphids. Phytopathology 81:794-802.

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

Register, J. C., and Beachy, R. N. 1989. Effect of protein aggregation state on coat protein-mediated protection against tobacco mosaic virus using a transient protoplast assay. Virology 173:656-663.

Riechmann, J. L., Lain, S., and Garcia, J.A. 1992. Highlights and prospects of potyvirus molecular biology. J. Gen. Virol. 73:1-16.

Shukla, D. D., and Ward, C. W. 1989. Structure of potyvirus coat proteins and its application in the taxonomy of the potyvirus group. Adv. Virus Res. 36:273-314.

Stark, D. M., and Beachy, R. N. 1989. Protection against potyvirus infection in transgenic plants: Evidence for broad spectrum resistance. Bio/Technology 7:1257-1262.

Tözsér, J., Bláha, I., Copeland, T. D., Wondrak, E. M., and Oroszlan, S. 1991. Comparison of the HIV-1 and HIV-2 proteinases using oligopeptide substrates representing cleavage sites in Gag and Gag-Pol polyproteins. FEBS Lett. 281:77-80.

van den Elzen, P. J. M., Huisman, M. J., Willink, D. P.-L., Jongedijk, E., Hoekema, A., and Cornelissen, B. J. C. 1989. Engineering virus resistance in agricultural crops. Plant Mol. Biol. 13:337-346.

van Dun, C. M. P., Overduin, B., van Vloten-Doting, L., and Bol, J. F. 1988. Transgenic tobacco expressing tobacco streak virus or mutated alfalfa mosaic virus coat protein does not cross-protect against alfalfa mosaic virus infection. Virology 164:383-389.

Wilson, T. M. A., and Watkins, P. A. C. 1986. Influence of exogenous viral coat protein on the cotranslational disassembly of tobacco mosaic virus (TMV) particles in vitro. Virology 149:132-135.

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. Plant Cell 2:559-567.

Wu, X.-J., 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.