The Plant Cell, Vol. 4, 735–744, June 1992 © 1992 American Society of Plant Physiologists

Expression of Amino-Terminal Portions or Full-Length Viral Replicase Genes in Transgenic Plants Confers Resistance to Potato Virus X Infection

Carl J. Braun[1] and Cynthia L. Hemenway[2]

Monsanto Company, The Agricultural Group, Plant Protection and Improvement, Mail Zone AA3E, 700 Chesterfield Village Parkway, St. Louis, Missouri 63198

The first open reading frame (ORF 1) of potato virus X (PVX) encodes a putative replicase gene. Transgenic tobacco lines expressing ORF 1 are resistant to PVX infection when inoculated with either PVX or PVX RNA. Analyses of lines containing various portions of the ORF 1 gene demonstrated that resistance is conferred to plants by expressing approximately the first half of the ORF 1 gene. One line expressing the untranslated leader and first 674 codons of ORF 1 is highly resistant to PVX infection. Conversely, lines expressing either approximately the third or fourth quarter of the ORF 1 gene, which contain the conserved nucleotide triphosphate (NTP) binding motif and Gly-Asp-Asp (GDD) motif, respectively, are not protected from PVX infection. In the resistant full-length and amino-terminal lines, lower numbers of local lesions were observed, and the virus accumulation in the inoculated and upper leaves was reduced when compared with the nontransformed control. When the performance of the most resistant ORF 1 line was compared with the most resistant coat protein (CP) line in a resistance test, the best ORF 1 line was more resistant to PVX infection than the best transgenic line expressing the PVX CP gene. These findings define a promising new approach for controlling plant viral infection.

INTRODUCTION

The control of diseases caused by plant viruses is of considerable importance in modern agriculture. One approach utilized in the suppression of plant viruses is host-encoded control mechanisms, such as using cultivars with naturally occurring resistance genes. Another approach used to regulate plant viruses is to exploit virally encoded control mechanisms, such as classical cross-protection (Fulton, 1986). A successful alternative to these methods is genetically engineered resistance. The first example of genetically engineered virus resistance was reported by Abel et al. (1986), who demonstrated that plants expressing the coat protein (CP) gene from tobacco mosaic virus (TMV) were resistant to TMV infection. CP-mediated resistance has been successful with many viruses in both controlled environments and under field test conditions (reviewed in Beachy et al., 1990; Hanley-Bowdoin and Hemenway, 1992). Genetically engineered resistance has also been demonstrated in plants expressing viral satellite RNAs from cucumber mosaic virus and tobacco ringspot virus (Gerlach et al., 1987; Harrison et al., 1987) and in plants expressing the read-through portion of the putative replicase gene from TMV (Golemboski et al., 1990).

CP-mediated resistance to virus infection has been reported for potato virus X (PVX) in both transgenic tobacco and potato (Hemenway et al., 1988; Lawson et al., 1990). We wished to test whether portions of the PVX viral genome, other than the CP gene, could be used to genetically engineer PVX-resistant plants. PVX is a plus sense, single-stranded RNA virus that is a pathogen in commercial cultivars of potato. PVX is mechanically transmitted, and both the virion and viral RNA are infectious. PVX RNA resembles eukaryotic mRNA in that the 5′ terminus is capped and the 3′ end is polyadenylated (Sonenberg et al., 1978; Morozov et al., 1981).

The five open reading frames (ORFs) encoded by the PVX genome respectively contain the coding capacity for proteins of 165, 22, 12, 8, and 25 kD. ORF 1 may encode the viral replicase protein because it contains several conserved domains that are found in other known RNA-dependent RNA polymerase genes. For example, ORF 1 contains an NTP binding motif that could be the site of nucleotide triphosphate binding (Gorbalenya et al., 1988). In addition, ORF 1 contains the conserved Gly-Asp-Asp (GDD) motif that is thought to participate in the catalytic function of the replicase (Hodgman, 1988). ORFs 2, 3, and 4 are often referred to as the triple block genes because the reading frames overlap. For another potexvirus, white clover mosaic virus, the triple block proteins have been shown to be necessary for transport (Beck et al., 1991). The final ORF of PVX encodes the 25-kD capsid protein.

[1] To whom correspondence should be addressed.
[2] Current address: North Carolina State University, Department of Biochemistry, Raleigh, NC 27695-7622.

We have expressed the full-length putative PVX replicase gene and a series of defined fragments of the putative replicase gene in tobacco. We demonstrate that expression of full-length ORF 1 in transgenic tobacco confers resistance to PVX. In addition, we show that an amino-terminal fragment from the ORF 1 gene can confer PVX resistance. Finally, we compare the level of resistance in transgenic plants expressing PVX ORF 1 or PVX CP.

RESULTS

Construction of Vectors Containing ORF 1 and Subdomains of ORF 1

Figure 1 shows the genome organization of PVX. The putative replicase of PVX, ORF 1, is 4371 nucleotides in length and is preceded by an 84-nucleotide untranslated leader. We modified plasmid pMON8660, which is a PVX infectious clone (Hemenway et al., 1990), by site-directed mutagenesis. These modifications allowed us to clone the entire ORF 1 gene and portions thereof into pMON977, which is a single border plant transformation vector. Specifically, the addition of SacI restriction sites at the 5′ terminus of the cDNA clone and the 3′ end of ORF 1 facilitated cloning the untranslated leader and ORF 1 gene, resulting in plasmid pMON8439 (Figure 1B). To ensure that mutagenesis did not alter the sequence elsewhere in ORF 1, we completely resequenced ORF 1. Except for the addition of the SacI sites, the sequence of the ORF 1 gene was identical to the corresponding gene in pMON8660.

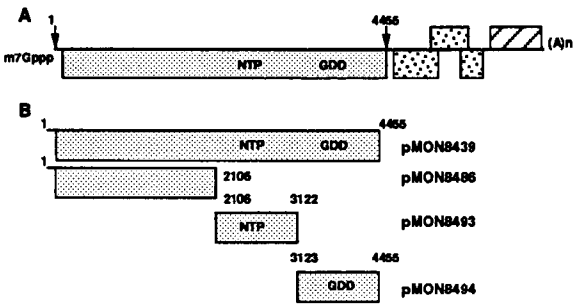

Figure 1. Genome Organization of PVX and Plant Transformation Vectors.

(A) The five ORFs of PVX are represented by boxes positioned along the 6436-nucleotide genome. The viral RNA cap structure and poly(A) tail are shown positioned at the 5′ and 3′ termini, respectively. ORF 1 is the lightly shaded box; approximate positions of the conserved NTP binding and GDD motifs are indicated. ORFs 2, 3, and 4 are referred to as the triple block genes because the reading frames overlap and are represented by a stippled pattern. ORF 5 is displayed as a striped box and encodes the 25-kD coat protein.

(B) Vectors pMON8439, pMON8486, pMON8493, and pMON8494 are all derivatives of pMON977 and contain either the complete ORF 1 (pMON8439) or portions thereof. All inserts are positioned between the promoter and 3′ signals in the sense direction. Positions of the small portions of ORF 1 are shown as genome base numbers above the fragment.

Additional mutagenesis of ORF 1, between codons 674 and 675 and between codons 1014 and 1015, allowed three portions of ORF 1 to be inserted into pMON977 to create plasmids pMON8486, pMON8493, and pMON8494 (Figure 1B). Plasmid pMON8486 contains approximately the first half of the ORF 1 gene, including the 84-nucleotide untranslated PVX leader and the first 674 codons of ORF 1. Because a stop codon is not present after codon 674, the ORF extends into the ribulose bisphosphate carboxylase small subunit (*rbsS*) region of the vector for an additional 33 codons. Plasmids pMON8493 and pMON8494 were created by inserting restriction sites upstream of internal, in-frame methionine codons. Plasmid pMON8493 contains a portion of ORF 1 beginning at the methionine at position 675 and ending at the histidine codon at position 1014; a UAA codon, also inserted during mutagenesis, terminates this partial ORF 1 construct. This plasmid includes the conserved NTP binding motif, which has the consensus sequence of Gly-X-X-X-X-Gly-Lys-X′, where X is any amino acid and X′ usually is either serine or threonine (Gorbalenya et al., 1988). The final 441 codons of ORF 1, which begin at the methionine residue at position 1015 and include the conserved GDD motif (Hodgman, 1988), are present in plasmid pMON8494 (Figure 1B). Plasmids pMON8439, pMON8486, pMON8493, and pMON8494 were used to transform tobacco (cv Samsun nn).

Expression Analysis of Transgenic Tobacco Lines

Primary transformants were analyzed for expression by RNA gel blot analysis (Thomas, 1980). Transgenic RNA was detected in approximately 80% of the lines tested. Figure 2A shows the expected 4.5-kb ORF 1 transcript from several plant lines transformed with pMON8439. A densitometry scanning of the autoradiogram shown in Figure 2A demonstrates that the steady state message levels vary approximately 10-fold (data not shown). Similarly, plants transformed with pMON8486, pMON8493, and pMON8494 also produced the expected 2.3-, 1.1-, and 1.4-kb transcripts, respectively (data not shown). From these analyses, it was clear that most of the transgenic lines produced detectable levels of transgenic RNA and that the RNA detected was of the expected length. The extent of ORF 1–encoded polypeptide expression levels in the transgenic lines remains to be determined. In preliminary experiments in which total plant proteins from crude leaf extracts were analyzed by probing protein blots with antisera raised against ORF 1 expressed in *Escherichia coli*, ORF 1–encoded protein was not detected in either transgenic lines or in PVX-infected control lines. Titration of the *E. coli*–derived antigen demonstrated that the sensitivity of detection on a protein gel blot was approximately 2 ng.

To determine whether the ORF 1 gene used to transform the transgenic plants was capable of being translated, this gene

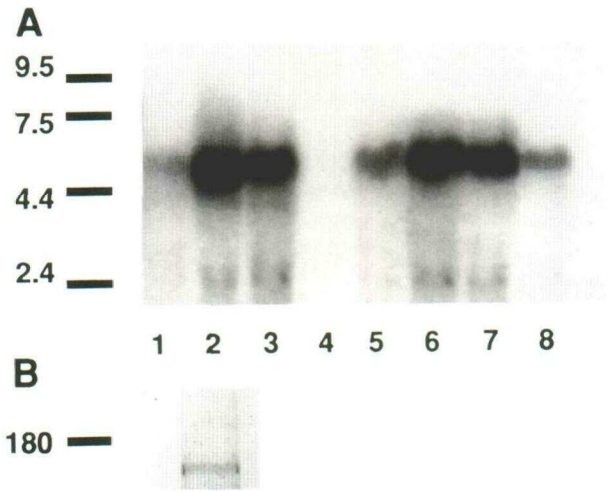

Figure 2. ORF 1 Expression Analysis.

(A) Forty micrograms of total RNA was extracted from young leaves of transgenic ORF 1 lines and electrophoresed on a 1.4% agarose gel. Lane 1, 30198; lane 2, 30199; lane 3, 30205; lane 5, 30214; lane 6, 30219; lane 7, 30220; lane 8, 30221. Lane 4 contains 40 μg of RNA from nontransformed tobacco. The gel was blotted and probed with a nick-translated ORF 1 cDNA fragment. The lengths of RNA markers (Bethesda Research Laboratories) are indicated at the left in kilonucleotides.

(B) Fluorogram of in vitro translated, ^{35}S-methionine–labeled ORF 1 protein that has been immunoprecipitated with antisera raised against *E. coli*–derived, ORF 1–expressed protein. One of the length markers (Sigma) is indicated at the left in kilodaltons.

was removed from pMON8439 and cloned into pGEM3Zf−; the resulting plasmid was transcribed and translated in vitro. A protein of the expected size, 165 kD, was detected by SDS-PAGE (Laemmli, 1970) analysis of these translation products. In addition, the 165-kD protein could be specifically immunoprecipitated (Figure 2B). A control translation reaction without RNA did not produce any protein in the 165-kD range. From these experiments, we conclude that the ORF 1 gene in transgenic plants is translationally competent and that the level of ORF 1–encoded protein, if present, is below our current detection level.

Expression of Full-Length ORF 1 Confers Resistance to PVX

Self-fertilized F_1 progeny from seven independently transformed pMON8439 lines were analyzed for resistance to PVX. Samples from F_1 seedlings were assayed by an ELISA for the product of the neomycin phosphotransferase II (NPTII) gene, which is the selectable marker gene for kanamycin resistance in pMON977. Because the gene of interest integrates adjacent to the marker gene, the NPTII gene and the viral transgene cosegregate in the progeny. For most lines, a 3:1 ratio of NPTII+ to NPTII− plants was observed; we have not determined whether these ratios were the result of a single insert or due to multiple integrations that were tightly linked.

For each protection test, 20 NPTII+ plants per line were inoculated with PVX. Ten plants per line were inoculated with a low viral inoculum concentration of 0.5 μg/mL PVX, and the remaining 10 plants were inoculated with 5.0 μg/mL PVX. Nontransformed tobacco (cv Samsun nn) plants were used as controls and were similarly inoculated. Plants were monitored daily for the development of lesions, which routinely appeared on the inoculated leaves of the nontransformed control between 4 and 7 days postinoculation (dpi). Figure 3A shows that by 8 dpi, lesions were clearly visible on the inoculated leaves of the nontransformed control plants. Systemic disease symptoms generally were observed 8 to 10 dpi on the control plants.

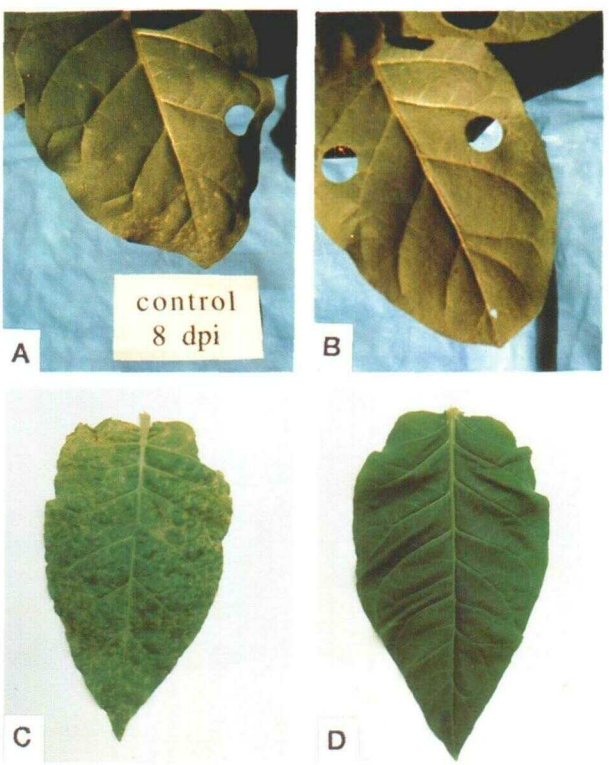

Figure 3. Viral Disease Symptom Comparison of Control and ORF 1 (29436) Lines.

(A) Inoculated leaf of control line, 8 dpi.
(B) Inoculated leaf of ORF 1 line, 8 dpi.
(C) Upper leaf of control line, 21 dpi.
(D) Upper leaf of ORF 1 line, 21 dpi.
Distinct lesions are visible on the inoculated leaf of the control plant, particularly near the tip of the leaf. In contrast, no lesions are present on the inoculated leaf of the ORF 1 line. In the upper leaves at 21 dpi, severe disease symptoms are manifested in the control, whereas the transgenic leaf shows no visible disease symptoms.

In one protection test, the performance of five pMON8439 lines that express ORF 1 RNA was compared to the nontransformed control. All five of the transgenic lines showed reduced numbers of lesions when compared to the control at 6 dpi. Because lesion count data did not follow a Gaussian distribution, a square-root transformation of the count data was used to equalize the variances so that a statistical analysis could be performed. Figure 4 shows that at P = 0.01 each of the pMON8439 transgenic lines showed a highly significant reduction in lesion numbers when compared with the control line at each viral inoculum concentration. Plants from line 29436 had the fewest total lesions (four lesions on 20 plants) compared with the control (909 lesions on 20 plants).

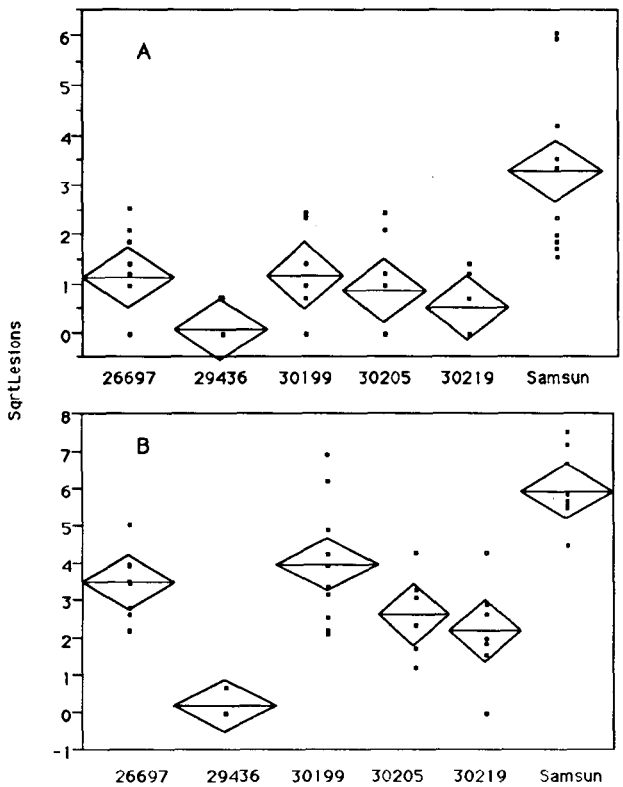

Figure 4. Statistical Comparison of Lesions in Control and ORF 1 Lines.

(A) Line performance when treated with 0.5 μg/mL PVX.
(B) Line performance when treated with 5.0 μg/mL PVX.
The square roots of the average number of lesions per inoculated leaf are plotted for five ORF 1 lines and the control. Data points are indicated by dots. A consequence of using a square-root transformation of count data is that sample variances tend to equalize, thus allowing for a statistical comparison. The top and bottom of the diamond-shaped box span the 95% confidence intervals. The bisecting line of the diamond represents the mean for the line. Least significant difference comparison tests show that all transgenic lines are significantly different from the control at both inoculum concentrations at P = 0.01.

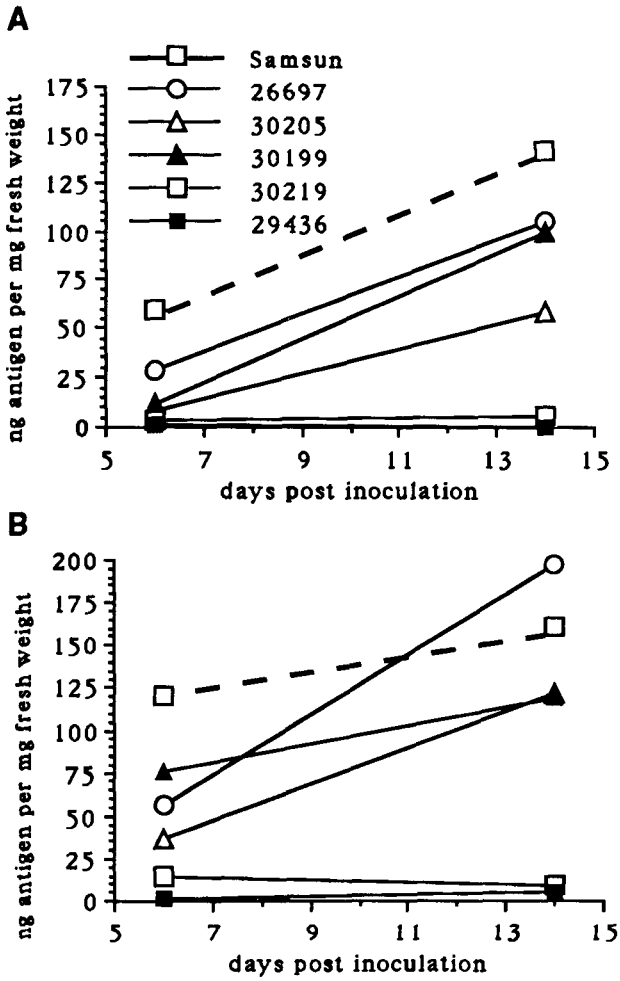

Figure 5. PVX Accumulation in the Inoculated Leaves of Control and ORF 1 Lines.

(A) Average antigen levels in five ORF 1 lines and a nontransformed control line inoculated with 0.5 μg/mL PVX.
(B) Average antigen levels in the same lines inoculated with 5.0 μg/mL PVX.
Virus antigen levels per milligram of fresh weight tissue are shown at 6 and 14 dpi. Antigen levels in the Samsun control line are indicated by a dashed line.

To estimate the extent of viral infection and spread in transgenic and control plants, inoculated and second upper leaves were sampled at various time points after inoculation and CP antigen levels were quantified by ELISA. Figures 5 and 6 show that the average levels of PVX CP antigen in the inoculated and upper leaves were substantially reduced in four out of five pMON8439 lines when compared with the control.

From these data, together with data from other experiments (data not shown), we categorized the performance of seven pMON8439 lines into three broad classes. The first class contains lines 26697 and 26693. These lines displayed a reduction

in the lesion numbers (Figure 4), but did not show a reduction in the estimated virus titer in either the inoculated or upper leaves. We classified these lines as displaying no virus resistance. Two pMON8439 lines, 30199 and 30205, fell into the second category. These lines displayed a type of resistance that can best be described as moderate resistance or a delay. In addition to having lower numbers of lesions than the control line, these transgenic lines showed a delayed accumulation of virus, particularly in the upper leaves (Figure 6). The final class contains three lines, 30219, 29436, and 30198, that displayed a high level of virus resistance (Figures 3 to 6; data not shown). These three lines consistently had reduced numbers of lesions and a great reduction in estimated virus titer levels in both the inoculated and systemic leaves when compared with control plants (Figures 4 to 6; data not shown). Line 29436 plants showed the highest level of resistance; most plants from this line failed to develop lesions on the inoculated leaves (Figures 3B and 4), and they did not display any visible signs of systemic disease symptoms (Figure 3D).

Table 1. Performance of Transgenic Tobacco Lines in Resistance Tests

Construct	ORF 1 Codons	Lines Analyzed	Resistant Lines Identified
pMON8439	1–1456	7	5
pMON8486	1–674[a]	6	1
pMON8493	675–1013	9	0
pMON8494	1014–1456	11	0

[a] This ORF extends an additional 33 codons past the 674th codon of ORF 1.

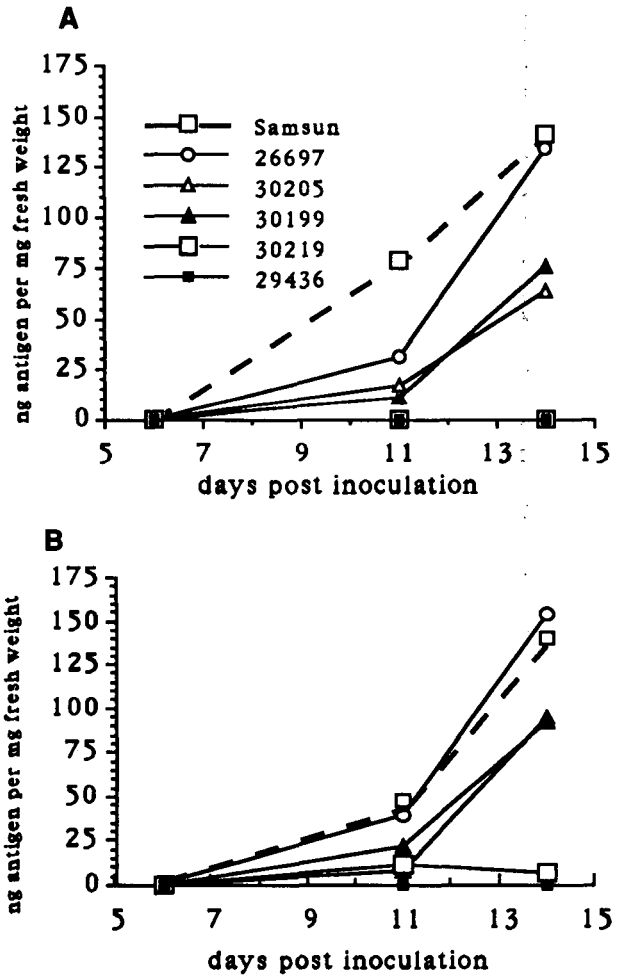

Figure 6. PVX Accumulation in the Second Upper Leaves of Control and ORF 1 Lines.

(A) Average antigen levels in five ORF 1 lines and a nontransformed control line inoculated with 0.5 μg/mL PVX.

(B) Average antigen levels in the same lines inoculated with 5.0 μg/mL PVX.

Virus antigen levels per milligram of fresh weight tissue are shown at 6, 11, and 14 dpi.

Several plants from line 29436 were grown for approximately 12 weeks postinoculation to obtain F_2 seed. During this time, no viral disease symptoms were observed. When the resistance test was repeated using a homozygous F_2 29436 line, the estimated virus present in the upper leaves at 14 dpi was approximately 25,000-fold lower than levels in the control line (data not shown).

Because the level of PVX resistance was so high in the ORF 1 lines, we inoculated the most resistant ORF 1 line, 29436, with a different virus to determine whether the resistance observed was limited to PVX or could be extended against other viruses. Line 29436 and nontransformed tobacco plants were inoculated with a low concentration, 0.3 μg/mL, of TMV (U1 strain). By 8 dpi, all of the plants showed signs of TMV infection on inoculated and upper leaves (data not shown).

Resistance Analysis of Transgenic Tobacco Containing Subdomains of ORF 1

At least six self-fertilized F_1 lines transformed with each of three partial ORF 1 constructs, pMON8486, pMON8493, and pMON8494, were tested in resistance tests, as described above. A summary of line performance is shown in Table 1. Plants from one resistant pMON8486 line, 26849, contained significantly fewer lesions than control plants. At 9 dpi, the average estimated virus level in the inoculated leaves of line 26849 plants inoculated with 0.5 or 5.0 μg/mL PVX was 3 and 6% of the control level, respectively (data not shown). Figure 7 shows that the levels of virus in the second upper leaf were also greatly reduced. Thus, the resistance observed in line 26849 was similar to the high level of resistance previously described for the pMON8439 lines. Of the three classes of

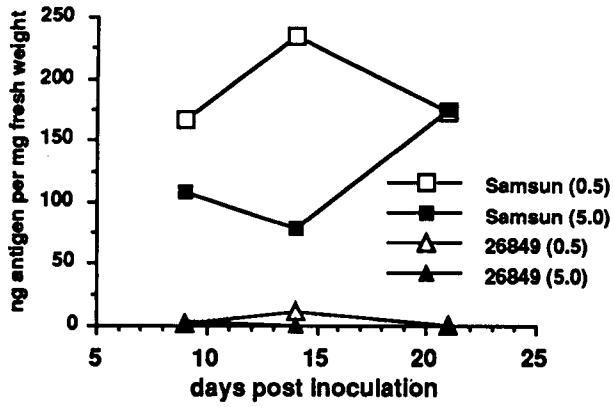

Figure 7. Comparison of Virus Levels in the Second Upper Leaves of Control and Truncated ORF 1 Lines.

Accumulation of average CP antigen levels are shown at 9, 14, and 21 dpi. Inoculum concentrations, which are either 0.5 or 5.0 μg/mL PVX, are shown parenthetically after the name of the line.

resistant plants observed with plants derived from pMON8439, line 26849 fell into the third class, which displayed a high level of resistance to PVX infection.

Nine pMON8493 lines and 11 pMON8494 lines were also analyzed in resistance tests. None of these 20 transgenic lines was resistant to PVX infection. Plants from these lines had approximately the same number of lesions as control plants and accumulated an equivalent amount of virus in the inoculated and upper leaves when compared with the nontransformed control plants (data not shown). Therefore, these results suggest that the amino-terminal half of ORF 1 is able to confer resistance.

Comparison of CP and ORF 1-Mediated Resistance

We have previously reported that expression of the PVX CP provides a high level of resistance to PVX (Hemenway et al., 1988). The most resistant CP line identified, 6665, was compared in a resistance test to line 29436, which was the most resistant ORF 1 line. In this test, the number of plants evaluated was increased to between 12 and 16 plants per treatment, and the plants were inoculated with either 5.0 μg/mL PVX or 5.0 μg/mL PVX RNA. Table 2 shows the average lesion counts per inoculated leaf for lines 6665, 29436, and the nontransformed control. Comparison of lesion counts, using a Mann-Whitney nonparametric test, demonstrated that the ORF 1 line consistently had a statistically significant reduction in the number of lesions when compared with the CP line. Indeed, only one lesion was observed on the 15 ORF 1 plants inoculated with 5.0 μg/mL PVX RNA; similarly, one lesion was observed on the 16 ORF 1 plants inoculated with 5.0 μg/mL PVX.

Protection in the CP and ORF 1 plants was also compared by estimating the relative virus accumulation in the inoculated and second upper leaves at 12 dpi by CP ELISA. To estimate more accurately the level of virus in the inoculated leaves, we harvested and ground whole inoculated leaves rather than sampling by leaf discs. Because the CP line contained an endogenous level of CP from the transgene, we also harvested uninfected leaves from line 6665 plants of the same age and used these samples to subtract the endogenous levels of CP from the level of CP present in line 6665 plants after inoculation. Antigen levels for the inoculated CP and ORF 1 lines are shown in Figure 8. Levels in infected control plants were very high and were not included in Figure 8 to emphasize the difference between the antigen levels in the transgenic lines. The control levels varied between 390 and 575 ng antigen/mg of fresh weight tissue in the inoculated leaves and between 375 and 400 in the second upper leaves. In contrast, antigen levels in both transgenic lines were significantly reduced when compared with the control. In addition, antigen levels in the inoculated leaves of the ORF 1 line were significantly lower than the CP line when plants were inoculated with either PVX virus or PVX RNA (Figure 8A). Similarly, antigen levels in the second upper leaves of the ORF 1 line were reduced compared to the CP line (Figure 8B).

DISCUSSION

Our results show that transgenic tobacco plants that express the putative replicase gene (ORF 1) from PVX are highly resistant to PVX infection. Resistant transgenic lines are phenotypically indistinguishable from the nontransformed plants and do not display any adverse effects due to expression of the putative viral replicase gene. The lines exhibit lower local lesion numbers and a reduction in virus levels in both the inoculated and upper leaves when inoculated with either

Table 2. Comparison of Lesion Numbers on CP, ORF 1, and Control Lines

Line	Inoculum	Mean Lesion Number[a]	Standard Deviation
Samsun	5.0 μg/mL PVX RNA	4.8	6.1
6665	5.0 μg/mL PVX RNA	0.25	0.44
29436	5.0 μg/mL PVX RNA	0.03	0.2
Samsun	5.0 μg/mL PVX	25.0	28.0
6665	5.0 μg/mL PVX	0.32	0.6
29436	5.0 μg/mL PVX	0.03	0.2

[a] Average number of lesions per line in which between 24 and 32 inoculated leaves were challenged. A Mann-Whitney nonparametric statistical test was used to compare lesion numbers. This test demonstrated a highly significant difference between the transgenic and control lines, and between the ORF 1 and CP lines.

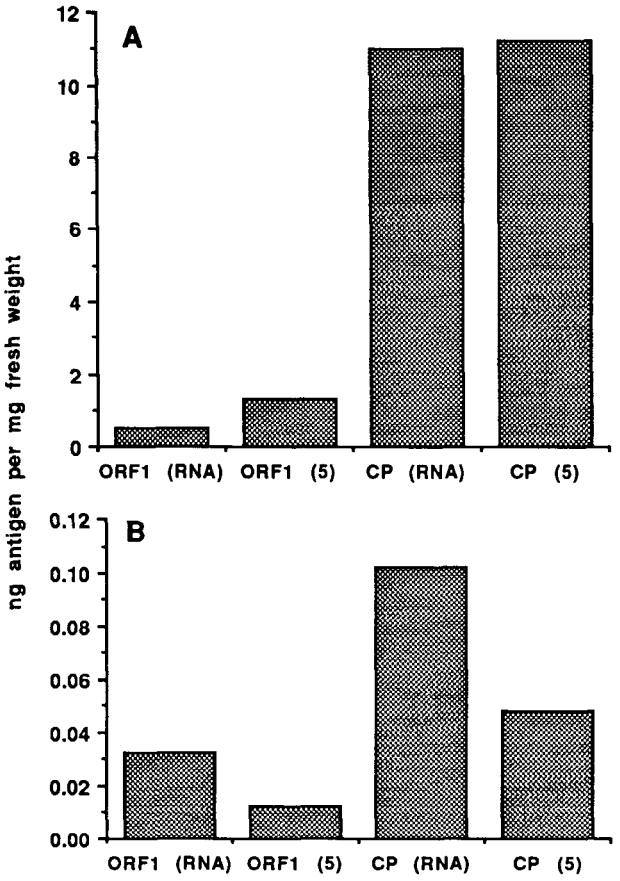

Figure 8. Antigen Levels in Transgenic ORF 1 and CP Lines.

(A) Average antigen levels in inoculated leaves.

(B) Average antigen levels in the second upper leaf.

Lines are labeled on the abscissa, and the inoculum used to inoculate plants is shown parenthetically after the line. RNA, 5.0 μg/mL PVX RNA; 5, 5.0 μg/mL PVX. Average antigen levels were determined at 12 dpi and are expressed in nanograms of antigen per milligram of fresh weight tissue. Antigen levels in the control lines, which are not displayed, ranged between 390 and 575 ng/mg fresh weight in the inoculated leaves and between 375 and 400 ng/mg fresh weight in the upper leaves.

a viral RNA or virus inoculum. Expression levels of ORF 1 RNA vary in the transgenic lines, and we have not observed a correlation between the extent of protection and transcript expression levels. The lack of correlation between transgene expression and viral resistance is not uncommon (Lawson et al., 1990; Hanley-Bowdoin and Hemenway, 1992) and is a poorly understood phenomenon.

We have identified one transgenic line expressing approximately the first half of the putative replicase gene that also is highly resistant to PVX infection. Conversely, transgenic tobacco lines expressing portions of the second half of ORF 1, which contain either the NTP binding or GDD motif, are susceptible to PVX infection. These results suggest that the portion of ORF 1 conferring resistance to PVX is localized within the first half of ORF 1.

Although expression of the transgene has been demonstrated at the RNA level, ORF 1–encoded protein has not yet been detected in transgenic plants. Given the steady state level of ORF 1 RNA in transgenic plants, we anticipate that ORF 1–encoded protein is present, but the level is below our current antibody detection limit of 0.002% of the total extractable protein. In vitro translated ORF 1 RNA produces a serologically detectable polypeptide of the correct size (Figure 2B), which indicates that the transgene is translationally competent. The possibility exists, however, that the ORF 1–encoded protein may not be stable in transgenic plants because an additional viral protein or binding to high-affinity sites on the viral RNA are required for stability. Alternatively, a host response may reduce the levels of ORF 1–encoded protein. Further experiments are necessary to determine whether ORF 1–encoded protein is present in the transgenic plants.

There have been many examples that describe the use of a viral CP gene to produce virus-resistant transgenic plants (for review, see Beachy et al., 1990; Hanley-Bowdoin and Hemenway, 1992). It is generally believed that the constitutive expression of the viral CP gene in the transgenic plant either prevents the inoculated virus from uncoating or affects another early step in the viral life cycle. We have shown previously that expression of the PVX CP gene provides a high level of resistance to PVX (Hemenway et al., 1988). In this report, we demonstrate that the most resistant ORF 1 line provides a higher level of resistance to PVX than our most resistant CP line. When inoculated with either viral RNA or virus, ORF 1 plants had significantly lower numbers of lesions and a reduced level of virus in the inoculated and upper leaves. From this comparison in transgenic tobacco, we conclude that constitutive expression of ORF 1 can provide a higher level of resistance against PVX than expression of PVX CP.

Golemboski et al. (1990) have reported that a portion of a nonstructural gene from TMV can be used to create transgenic plants that are resistant to TMV. TMV encodes two proposed replicase proteins of 126 and 183 kD; the larger protein is produced by reading through a UGA codon. Golemboski et al. (1990) have proposed that the read-through portion, which has the coding capacity for a 54-kD protein, is expressed as a separate TMV gene product. To assign function to the putative gene encoding the 54-kD protein, they transformed tobacco (cv Xanthi nn) with the 54-kD gene and unexpectedly found that the transgenic plants were highly resistant to TMV infection.

The report by Golemboski et al. (1990) is similar to our findings in that a nonstructural viral gene or portion thereof can provide virus resistance when constitutively expressed in transgenic plants. In both reports, however, expression analysis has only been confirmed at the RNA level; neither the full-length PVX ORF 1–encoded protein nor the 54-kD TMV protein has been detected in either transgenic plants or infected tissue. If these proteins are responsible for the resistance phenotype, then it is clear that they can be effective at low concentrations. Another similarity is that the level of virus resistance appears

to be higher in plants transformed with the nonstructural gene sequences that encode either PVX ORF 1 protein or the TMV 54-kD protein than that achieved by expressing the respective viral CP genes. Although no direct comparison has been made with plants containing the TMV CP and the gene encoding the 54-kD protein, separate reports have indicated the level of resistance achieved using these genes. In the original demonstration of genetically engineered resistance using TMV CP, Abel et al. (1986) found that four out of seven CP tobacco (cv Xanthi) plants eventually showed signs of viral infection when inoculated with 0.5 μg/mL of the U1 strain of TMV. Golemboski et al. (1990) found that the tobacco plants transformed with the sequence encoding the 54-kD protein, which were also cv Xanthi, did not develop any visible signs of infection when inoculated with 50 μg/mL of the U1 strain of TMV. Resistance in these plants was also observed when they were inoculated with 500 μg/mL U1 TMV.

Despite these similarities, it is clear that there are fundamental differences in the reports on TMV and PVX. The effective portion of the TMV replicase gene that confers virus resistance is the read-through portion of the gene, which is approximately the last quarter of the larger putative TMV replicase gene. Expression of the TMV gene encoding the 126-kD protein does not confer resistance to TMV (Golemboski et al., 1990). In contrast to TMV, the effective portion of PVX ORF 1 that confers virus resistance appears to be located in the first half of ORF 1. Both full-length PVX ORF 1 and the first half of the ORF 1 gene have been used to create PVX-resistant plants. Unlike TMV, however, expression of approximately the final quarter of the PVX ORF 1 gene does not confer virus resistance, despite the fact that the coding capacity for these portions of the PVX and TMV genes (51 kD versus 54 kD) are similar and that each region contains the conserved GDD motif which is present in many replicase genes.

Differences in CP-mediated protection also have been reported for TMV and PVX. Protection in transgenic plants expressing the TMV CP is largely overcome when the plants are inoculated with TMV RNA, suggesting that the endogenous CP blocks the uncoating step when the inoculum is virus (Nelson et al., 1987). In contrast, transgenic plants expressing the PVX CP are equally protected against both PVX and PVX RNA inocula (Hemenway et al., 1988). These data suggest that there may be multiple mechanisms of CP-mediated resistance.

To ensure the continuing existence of both host and pathogen, biological mechanisms are often used by plants and plant viruses that allow the host to regulate or control the extent of viral infection. In other, better defined systems, viral life cycles are strictly controlled by feedback mechanisms that utilize viral gene products to regulate viral gene expression. For example, bacteriophage R17 CP down-regulates replicase expression by binding to the viral template, thus prohibiting further replicase translation (Bernardi and Spahr, 1972). Kiho and Nishiguchi (1984) may have observed a similar type of viral control in their study of the $L_{11}A$ attenuated strain of TMV. Infection by $L_{11}A$ protects plants from further TMV infection. $L_{11}A$ virus replicates normally for approximately 4 days, but replication is diminished thereafter. This phenomenon has been termed autoregulation and is proposed to be due to the overproduction of the 183-kD putative replicase protein (Kiho and Nishiguchi, 1984). The alfalfa mosaic (AlMV) CP also can regulate viral replication. From the analysis of AlMV replication with CP mutants, Van der Kuyl et al. (1991) have recently shown that AlMV CP both enhances plus strand synthesis and is responsible for down-regulating minus strand synthesis.

The constitutive expression of the full-length PVX ORF 1 in transgenic plants may interfere with the viral infection process by one of the following mechanisms. First, an abnormally high ORF 1 protein concentration may override a feedback inhibitor. Perhaps, by analogy to AlMV, the ORF 1 concentration is sufficient so that CP or another protein cannot regulate replication. Second, ORF 1–encoded protein may bind to the viral RNA and inhibit either the translation of another viral gene product or the packaging of the viral RNA. Third, if the PVX ORF 1–encoded protein is the replicase protein, then overexpression of the protein may titrate host factors that normally participate in the viral replication complex. A similar mechanism has been suggested by Golemboski et al. (1990) to explain the TMV resistance conferred by the expression of the nonstructural sequences that encode the putative 54-kD protein. Recently, Carr and Zaitlin (1991) have further analyzed resistance in protoplasts derived from these transgenic plants. When these protoplasts were infected with TMV, both plus and minus strand synthesis of TMV was significantly reduced.

Assuming that ORF 1 is the replicase gene, the replicase protein that is expressed in the transgenic plants may not be the most active form of the enzyme. Our infectious clone is only 0.2% as infectious as PVX RNA (Hemenway et al., 1990). Perhaps the replicase derived from this clone is sufficiently different from the most active replicase in the viral population so that constitutive expression of this gene somehow interferes with infection. Alternatively, the molecule responsible for conferring PVX resistance could be the ORF 1 RNA. RNA-RNA duplexes formed between transgenic ORF 1 RNA and minus strand RNA could dramatically affect replication. Priano et al. (1987) have shown that a mutant Qβ bacteriophage can form replicase-inactive RNA-RNA duplexes. Moreover, once formation of these duplexes exceeds 50%, viral RNA synthesis continues to decrease and eventually approaches zero (Priano et al., 1987). Future experiments with PVX will address the mechanism of ORF 1–mediated resistance.

METHODS

Plant Transformations

Standard molecular techniques were used to mutagenize (Kunkel, 1985) and clone (Maniatis et al., 1982) various portions of the potato virus X (PVX) open reading frame (ORF) 1 gene into pMON977 in the sense orientation between a variant cauliflower mosaic virus 35S promoter (Kay et al., 1987) and the 3′ end of the pea *rbcS* gene (Cuozzo et al.,

1988). pMON977 is a single border plant transformation vector. Constructs were verified by sequencing (Sanger et al., 1977). Methods to mobilize plant transformation vectors into a binary *Agrobacterium tumefaciens* strain and to select transconjugants have been described previously (Klee et al., 1991). Transconjugants were used to transform tobacco (*Nicotiana tabacum* cv Samsun nn) by the leaf disc method (Horsch et al., 1985).

Expression Analysis

Total RNA was isolated from the primary transformed plants by the LiCl method (Goldberg et al., 1981) and used to detect transgene expression by RNA gel blot analysis (Thomas, 1980). The various-sized ORF 1 RNAs from plants transformed with pMON8439, pMON8486, pMON8493, and pMON8494 were detected using a nick-translated ORF 1 cDNA fragment as a probe (Maniatis et al., 1982). A pGEM3Zf– plasmid containing the ORF 1 gene from pMON8439 was used as a template for in vitro transcription and translation using the TNT™ coupled reticulocyte lysate system (Promega). ORF 1–encoded protein was immunoprecipitated from the translation reaction by a 1-hr incubation with serum raised against *Escherichia coli*–produced, ORF 1–encoded protein, followed by a 1-hr incubation with protein A coupled to Sepharose (Sigma). After incubation, the Sepharose beads were washed according to the manufacturer's instructions and electrophoresed on an 8% SDS-PAGE (Laemmli, 1970) gel. Prior to film exposure, the gel was infused with an organic scintillant.

Virus Resistance Experiments

Leaf samples from self-fertilized F_1 progeny of the primary transformed plants were analyzed immunologically for the presence of neomycin phosphotransferase II (NPTII) in an ELISA. Conditions for peroxidase conjugations and ELISA conditions have been described previously (Kaniewski and Jackowiak, 1989). Absorbance at 655 nm was measured using a plate reader (model No. 3550, Bio-Rad). The NPTII ELISA was also used to identify a homozygous ORF 1 29436 line in the F_2 generation. In the comparison of the full-length ORF 1, coat protein (CP), and control lines, between 24 and 32 homozygous plants per line were analyzed. In all other protection tests, 20 NPTII+ plants per line and 20 nontransformed control plants were inoculated with PVX. In all tests, plants were mechanically inoculated on two leaves when the plants were at the four- to six-leaf stage. Inoculation procedures and plant growth conditions have been described previously (Hemenway et al., 1988). Typically, local lesions were counted 6 days postinoculation (dpi). An analysis of variance was performed on the lesion count data to obtain a pooled estimate of variance, which was used in a least significant difference test to compare the average number of lesions between the control and transgenic lines. To perform statistical analyses, the square-root transformation of the lesion counts was performed to equalize the variances. Statistical analyses compared lines at the $P \approx 0.01$ level.

To estimate virus levels in the plants, two discs were sampled from all plants at various time points after inoculation using a no. 8 cork bore (1.15-cm diameter). These two discs, each weighing approximately 50 mg, were pooled and ground in 0.75 mL of PBSTO (0.15 M NaCl, 1.5 mM KH_2PO_4, 8 mM $Na_2HPO_4 \cdot 7H_2O$, 2.7 mM KCl, 0.5% [v/v] Tween 20, 0.2% [w/v] ovalbumin). Between 25 and 250 μL of the homogenate was loaded per well in a PVX ELISA (Hemenway et al., 1988) and used to estimate virus levels in the inoculated and upper leaves. In the inoculated leaves, one sample was taken in an area containing lesions and a second sample was taken in an area without lesions. In the second upper leaf, a sample was taken on both sides of the midrib. In the comparison of ORF 1, CP, and control line, the virus level in the inoculated leaves was estimated by harvesting both inoculated leaves at 12 dpi. Standard curves were drawn using at least 15 PVX standards per ELISA plate. An iterative process was used to solve a four parameter logistic equation that was used to determine the amount of PVX in the unknown samples (Ratkowsky and Reedy, 1986).

ACKNOWLEDGMENTS

We thank Drs. Robert Fraley, Ganesh Kishore, and Nilgun Tumer for supporting this project and for their helpful comments and suggestions. We also thank Jerry Anderson, Tim Coombe, Dr. Larry Holden, Nancy Hoffmann-Mathis, and Jim Weiss for their outstanding technical support.

Received February 26, 1992; accepted April 21, 1992.

REFERENCES

Abel, P.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.

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

Beck, D.L., Guilford, P.J., Voot, D.M., Andersen, M.T., and Forster, R.L.S. (1991). Triple gene block proteins of white clover mosaic potexvirus are required for transport. Virology **183,** 695–702.

Bernardi, A., and Spahr, P.F. (1972). Nucleotide sequence at the binding site for coat protein on RNA of bacteriophage R17. Proc. Natl. Acad. Sci. USA **69,** 3033–3037.

Carr, J.P., and Zaitlin, M. (1991). Resistance in transgenic tobacco plants expressing a nonstructural gene sequence of tobacco mosaic virus is a consequence of markedly reduced virus replication. Mol. Plant-Microbe Interact. **4,** 579–585.

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

Fulton, R.W. (1986). Practices and precautions in the use of cross protection for plant virus disease control. Annu. Rev. Phytopathol. **24,** 67–81.

Gerlach, W.L., Llewellyn, D., and Haseloff, J. (1987). Construction of a plant disease resistant gene from the satellite RNA of tobacco ringspot virus. Nature **328,** 799–802.

Goldberg, R.B., Hoschek, G., Tam, S.H., Ditta, G.S., and Breidenbach, T.W. (1981). Abundance, diversity, and regulation of mRNA sequence sets in soybean embryogenesis. Dev. Biol. **83,** 201–217.

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

Gorbalenya, A.I., Koonin, E.V., Donchenko, A.P., and Blinov, V. (1988). A conserved NTP-motif in putative helicases. Nature **333,** 22.

Hanley-Bowdoin, L., and Hemenway, C.L. (1992). Transgenic plants expressing viral genes. In Genetic Engineering with Plant Viruses, T.M.A. Wilson and J.W. Davies, eds (Boca Raton, FL: CRC Press), in press.

Harrison, B.D., Mayo, M.A., and Baulcombe, D.C. (1987). Virus resistance in transgenic plants that express cucumber mosaic virus satellite RNA. Nature **328,** 802–805.

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.

Hemenway, C., Weiss, J., O'Connell, K., and Tumer, N.E. (1990). Characterization of infectious transcripts from a potato virus X cDNA clone. Virology **175,** 365–371.

Hodgman, T.C. (1988). A new superfamily of replicative proteins. Nature **333,** 22–23.

Horsch, R.B., Fry, J.G., 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.

Kaniewski, W., and Jackowiak, N. (1989). Comparison of effectiveness of three methods of coupling peroxidase with immunoglobulines used in the ELISA test. Zesz. Probl. Post. Nauk. Rol. **381,** 55–61.

Kay, R., Chan, A., Daly, M., and McPherson, J. (1987). Duplication of CaMV 35S promoter sequences creates a strong enhancer for plant genes. Science **236,** 1299–1302.

Kiho, Y., and Nishiguchi, M. (1984). Unique nature of an attenuated strain of tobacco mosaic virus: Autoregulation. Microbiol. Immunol. **28,** 589–599.

Klee, H.J., Hayford, M.B., Kretzmer, K.A., Barry, G.F., and Kishore, G.M. (1991). Control of ethylene synthesis by expression of a bacterial enzyme in transgenic tomato plants. Plant Cell **3,** 1187–1193.

Kunkel, T. (1985). Rapid and efficient site-specific mutagenesis without phenotypic selection. Proc. Natl. Acad. Sci. USA. **82,** 488–492.

Laemmli, U.K., (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227,** 680–685.

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. Biotechnology **8,** 127–134.

Maniatis, T., Fritsch, E.F., and Sambrook, J. (1982). Molecular Cloning: A Laboratory Manual. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory), pp. 109–112.

Morozov, S.Y., Gorbulev, V.G., Novikov, V.K., Agranovskii, A.A., Kozlov, Y.V., Atabekov, I.G., and Baev, A.A. (1981). The primary structure of 5′- and 3′-terminal regions of potato virus X genomic RNA. Proc. Acak. Nauk. SSSR **259,** 723–725.

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.

Priano, C., Kramer, F.R., and Mills, D.R. (1987). Evolution of the RNA coliphages: The role of secondary structures during RNA replication. Cold Spring Harbor Symp. Quant. Biol. **52,** 321–330.

Ratkowsky, D.A., and Reedy, T.J. (1986). Choosing near-linear parameters in the four-parameter logistic model for radioligand and related assays. Biometrics **42,** 575–582.

Sanger, F., Nicklen, S., and Coulson, A.R. (1977). DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74,** 5463–5467.

Sonenberg, N., Shatkin, A.J., Riccardi, R.P., Rubin, M., and Goodman, R.M. (1978). Analysis of terminal structures of RNA from potato virus X. Nucl. Acids Res. **5,** 3325–3342.

Thomas, P.S. (1980). Hybridization of denatured RNA and small DNA fragments to nitrocellulose. Proc. Natl. Acad. Sci. USA **77,** 5201–5202.

Van der Kuyl, A.C., Neeleman, L., and Bol, J.F. (1991). Role of alfalfa mosaic virus coat protein in regulation of the balance between viral plus and minus strand RNA synthesis. Virology **185,** 496–499.