Replicase-Mediated Resistance To Potato Virus Y in Transgenic Tobacco Plants

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Nicotiana tabacum **'Turkish Samsun NN' plants were transformed with nuclear inclusion b (NIb) gene sequences of potato virus Y, O strain (PVY^O). The full-length construct included an additional in-frame initiation codon contiguous to the putative N-terminal amino acid codon and a stop codon replacing the C-terminal amino acid codon. Of 13 independently transformed lines, four yielded 37 (out of 100) plants in the R_1 generation that were resistant to PVY^O infection. Progeny of 13 out of 15 of R_1 plants tested expressed resistance in the R_2 generation. Conversely, 30 independently transformed tobacco lines expressing essentially the same sequence but deleted for the Gly-Asp-Asp (GDD) motif were not resistant. Two other constructs encoding either the 5′-deleted or 3′-truncated NIb gene, but harboring the GDD motif, conferred resistance to PVY^O in some tobacco plants. Despite the high level of nucleotide and amino acid identity shared by strains PVY^O and PVY^N for the NIb gene, PVY^N replication was found in all PVY^O-resistant plants. However, plants of one R_2 line showed reduced PVY^N replication.**

Additional keywords: nonstructural gene, nuclear inclusion, polymerase, potyviruses, protection, virus replication.

The concept of pathogen-derived resistance (Sanford and Johnston 1985), which proposed that a host expressing particular parasite sequences may circumvent disease, was first applied in plant virology with the report that transgenic tobacco expressing tobacco mosaic virus (TMV) coat protein showed resistance to TMV infection (Powell-Abel *et al.* 1986). This type of resistance, now generally called "coat protein-mediated protection," has been described for more than 12 virus taxonomic groups including potyviruses (for review, see Hull and Davies 1992). Recently, a few reports have shown that virus resistance could also be introduced successfully into *Nicotiana* species using replicase sequences of tobamo- (Golemboski *et al.* 1990), tobra- (MacFarlane and Davies 1992), cucumo- (Anderson *et al.* 1992), and potexviruses (Braun and Hemenway 1992; Longstaff *et al.* 1993). This phenomenon has recently been reviewed by Carr and Zaitlin (1993).

Potato virus Y (PVY) is the type member of the potyvirus group, the largest and most widely distributed group of plant viruses (Francki *et al.* 1985). Its genome consists of a non-segmented positive-sense RNA strand of 9.7 kb encoding one large polyprotein, co- and posttranslationally processed into at least eight mature functional proteins (Riechmann *et al.* 1992). The core protein of the potyvirus replicase is thought to be the nuclear inclusion b (NIb) protein (Domier *et al.* 1987). PVY NIb protein is not expressed from a separate open reading frame but is processed from a full-length polyprotein. To determine if resistance to PVY can be engendered using replicase sequences, a full-length DNA copy of the PVY^O NIb gene generating translatable transcripts, and three modified constructs derived from that DNA were transformed into tobacco. Plants harboring the full-length NIb gene construct or two defective NIb gene constructs still encoding the GDD motif (characteristic of viral polymerases; Argos 1988) were resistant to PVY^O infection. However, no resistance was found in tobacco lines expressing a full-length NIb gene sequence in which the GDD motif had been deleted.

RESULTS

Cloning of the PVY^O NIb gene and sequence analysis.

Individual proteins of PVY (Fig. 1A) are normally generated from a large polyprotein following a cascade of proteolytic events, and thus most of the genes for the processed proteins lack initiation and termination codons. To express only the NIb gene, two primers were designed to amplify a full-length construct containing an in-frame initiation codon contiguous to the first NIb N-terminal amino acid, and a stop codon replacing the C-terminal amino acid (Fig. 1B). The sequence of the DNA fragment encoding the full-length NIb gene amplified by polymerase chain reaction (PCR) is shown in Figure 1C. Clone YNIb.FL DNA contains 1,560 nucleotides and encodes 519 amino acids and a stop codon. Alignment of this sequence with homologous sequences from the Japanese PVY^O-O (Hidaka *et al.* 1992), the Hungarian PVY-H (Thole *et al.* 1993), and the French PVY^N (Robaglia *et al.* 1989) reveals nucleotide sequence identities of 96.6, 83.5, and 92.7%, and amino acid sequence identities of 98, 93, and 93.4%, respectively. Typical conserved amino acid motifs of positive-strand RNA viruses (Koonin 1991) were identified (Fig. 1C, underlined): DxxxxD (positions 249–254), GxxxTxxxN (positions 312–320), GDD (positions 352–354) and K (position 390). Modifications in the GDD box region for the

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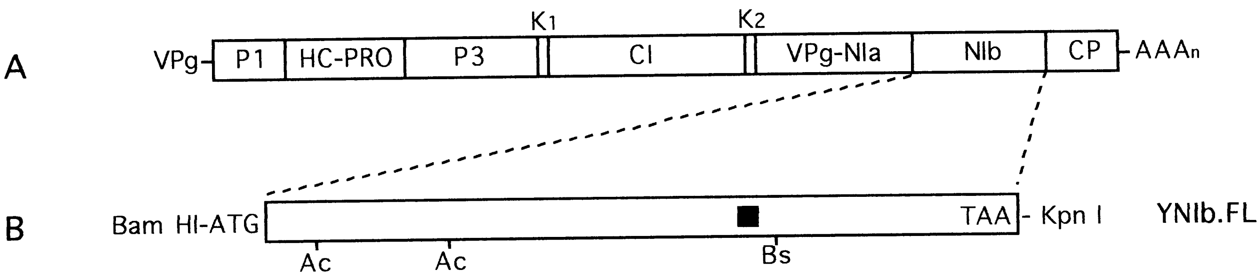
derived YNIb.Bsp and YNIb.GDD clones were confirmed by sequence analysis and are indicated in Figure 2A and C, respectively. An additional construct (YNIb.Acc) resulted in the deletion of 121 amino acids within the 5′ one-third of the NIb gene. Proteins encoded were ≈57 kDa (YNIb.FL), ≈39 kDa (YNIb.Bsp), ≈43 kDa (YNIb.Acc), and ≈57 kDa (YNIb.GDD) as predicted from the amino acid sequence (data not shown).

Ti-plasmid insertion and expression of NPTII protein in transgenic tobacco.

With the standard transformation protocol, more than 3 mo on rooting medium was necessary in some cases to allow development of a rooted plantlet that could tolerate transfer into soil. To enhance the efficiency of tobacco regeneration, the putatively transformed shoots were allowed to root on kanamycin-free medium. Selection of transformants was per-



C

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        M  A  K  H  S  A  W  M  F  E  A  L  T  G  N  L  Q  A  V  A  T  M  K  S  Q  L  V  T  K  H  V  V  K  G  E      35
1     ATGGCTAAGCATTCTGCGTGGATGTTTGAAGCCTTGACAGGAAATTTGCAAGCTGTCGCAACAATGAAGAGTCAATTAGTAACCAAGCATGTAGTTAAAGGAGAG

        C  R  H  F  K  E  F  L  T  V  D  A  E  A  E  A  F  F  R  P  L  M  D  A  Y  G  K  S  L  L  N  R  D  A  Y      70
106   TGTCGACACTTCAAGGAATTCCTGACTGTGGATGCAGAAGCAGAGGCATTCTTCAGGCCTTTGATGGATGCGTATGGGAAAAGCTTGCTGAACAGAGATGCGTAC

        I  K  D  I  M  K  Y  S  K  P  I  D  V  G  I  V  D  C  D  A  F  E  E  A  I  N  R  V  I  I  Y  L  Q  V  H      105
211   ATCAAGGACATAATGAAGTATTCAAAACCTTAGATGTTGGTATCGTGGACTGTGATGCATTTGAGGAAGCCATCAATAAGGGTTATCATCTATCTGCAAGTGCAC

        G  F  K  K  C  A  Y  V  T  D  E  Q  E  I  F  K  A  L  N  M  K  A  A  V  G  A  M  Y  G  G  K  K  K  D  Y      140
316   GGCTTCAAGAAGTGTGCATATGTCACTGACGAGCAAGAAATTTTCAAAGCGCTCAACATGAAAGCTGCGGTCGGAGCCATGTATGGTGGAAAAAAGAAAGACTAT

        F  E  H  F  T  D  A  D  K  E  E  I  V  M  Q  S  C  L  R  L  Y  K  G  L  L  G  I  W  N  G  S  L  K  A  E      175
421   TTTGAGCATTTCACTGATGCAGATAAGGAAGAAATAGTCATGCAAAGCTGTCTACGATTGTATAAAGGCTTACTCGGCATTTGGAATGGATCGTTGAAGGCAGAG

        L  R  C  K  E  K  I  L  A  N  K  T  R  T  F  T  A  A  P  L  D  T  L  L  G  G  K  V  C  V  D  D  F  N  N      210
526   CTCCGGTGTAAGGAAAAGATACTTGCAAATAAGACGAGGACATTCACTGCTGCACCTCTAGACACTTTGCTGGGTGGTAAAGTGTGTGTTGACGACTTCAATAAT

        Q  F  Y  S  K  N  I  E  C  C  W  T  V  G  M  T  K  F  Y  G  G  W  D  K  L  L  R  R  L  P  E  N  W  V  Y      245
631   CAATTTTATTCAAAGAATATTGAGTGCTGTTGGACGGTTGGGATGACTAAGTTTTATGGTGGTTGGGATAAACTGCTTCGGCGTTTACCTGAGAATTGGGTGTAC

        C  D  A  D  G  S  Q  F  D  S  S  L  T  P  Y  L  I  N  A  V  L  T  I  R  S  T  Y  M  E  D  W  D  V  G  L      280
736   TGCGATGCTGATGGCTCACAGTTTGATAGTTCACTAACTCCATACTTAATCAATGCTGTTCTCACCATCAGAAGCACATACATGGAAGACTGGGATGTGGGGTTG

        Q  M  L  R  N  L  Y  T  E  I  I  Y  T  P  I  S  T  P  D  G  T  I  V  K  K  F  R  G  N  N  S  G  Q  P  S      315
841   CAAATGCTGCGTAATTTATACACTGAGATTATTTACACACCCATTTCAACTCCAGATGGAACAATCGTTAAGAAGTTCAGAGGAAATAACAGTGGTCAGCCTTCT

        T  V  V  D  N  S  L  M  V  V  L  A  M  H  Y  A  F  I  R  E  C  I  E  F  E  E  I  D  S  T  R  V  F  F  V      350
946   ACTGTTGTGGACAACTCTCTTATGGTCGTCCTTGCTATGCACTATGCTTTCATCAGAGAATGCATTGAGTTTGAAGAGATTGACAGCACGCGCGTGTTCTTTGTC

        N  G  D  D  L  L  I  A  G  N  P  D  K  E  D  I  L  D  R  F  S  Q  H  F  S  D  L  G  L  N  Y  D  F  S  S      385
1051  AATGGTGATGATTTGCTGATTGCTGGGAATCCGGATAAAGAGGACATTCTTGACAGATTCTCACAACACTTCTCAGATCTTGGTTTAAACTATGATTTCTCGTCA

        R  T  R  N  K  E  E  L  W  F  M  S  H  W  G  L  L  I  E  G  M  Y  V  P  K  L  E  E  E  R  I  V  S  I  L      420
1156  AGAACAAGAAATAAGGAAGAGTTGTGGTTTATGTCTCATTGGGGCCTACTAATTGAGGGCATGTATGTGCCGAAACTTGAAGAAGAAAGGATTGTGTCCATTCTC

        Q  W  D  R  A  D  L  A  E  H  R  L  E  A  I  C  A  R  M  I  E  S  W  G  Y  S  E  L  T  H  Q  I  R  R  F      455
1261  CAATGGGACAGAGCAGACTTGGCTGAACACAGGCTTGAGGCGATTTGCGCACGTATGATAGAGTCCTGGGGTTATTCTGAACTAACACACCAAATCAGGAGATTC

        Y  S  W  L  L  Q  Q  Q  P  F  A  T  I  A  Q  E  G  K  A  P  Y  I  A  S  M  A  L  R  K  L  Y  M  D  R  A      490
1366  TACTCATGGTTATTGCAACAGCAACCTTTTGCAACAATAGCGCAGGAAGGGAAGGCTCCTTATATAGCAAGCATGGCATTAAGGAAACTGTATATGGATAGGGCT

        V  D  E  E  E  L  R  A  F  T  E  M  M  V  A  L  D  D  E  F  E  F  D  S  Y  E  V  H  H  *                     520
1471  GTGGATGAGGAAGAGTTAAGAGCCTTCACTGAAATGATGGTCGCATTAGACGATGAGTTTGAGTTTGACTCTTATGAAGTACACCATTAA
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Fig. 1. Schematic organization of the PVY RNA genome (**A**); PVY^O NIb full-length clone YNIb.FL (**B**). Restriction sites for *Acc*I (Ac) and *Bsp*EI (Bs) are indicated. The black box represents the relative position of the GDD amino acid motif. Nucleotide and amino acid sequence of clone YNIb.FL (**C**). Underlined nucleotides are part of the degenerate primers used in the PCR amplification of the NIb gene. Underlined amino acids are conserved motifs found in RNA virus RNA-dependent RNA polymerases (Argos 1988). Numbers in the left-hand column refer to nucleotides, those in the right-hand column refer to amino acids.

formed using PCR amplification of the genomic tobacco DNA for NPTII and PVY insert sequences (Fig. 3). This procedure was very reliable; 89 out of 92 of the original regenerated plantlets were transformants. Moreover, this approach allowed the rescue of 16 transformants harboring the C-terminally truncated construct YNIb.Bsp (called Bsp lines) which did not grow on kanamycin-rooting media, probably because of the poor expression of the NPTII protein in these lines (Fig. 4).

Detrimental effects due to the presence of NIb gene sequences were observed in some R_0 transformants harboring the full-length NIb gene (FL plants) and its C-terminally truncated derivative (Bsp plants). Plants belonging to these groups grew significantly more slowly than the non-transformed tobacco and a few were sterile. These characteristics were, however, not observed in the R_1– and R_2– progeny of fertile original transformants. Interestingly, only a few viable tobacco calli were regenerated on kanamycin-selection medium in six different transformation experiments involving the C-terminally-truncated construct (YNIb.Bsp DNA). The calli divided and grew very slowly and most of them died. Only a few shoots were rescued by transferring them onto an antibiotic-free medium after several unsuccessful attempts to make them root on kanamycin-bearing medium. These Bsp R_0 plants and selfed R_1 progeny did not have detectable levels of foliar NPTII protein expression and would have been scored as nontransformants using traditional screening procedures, but PCR analysis confirmed that they carried the NPTII and PVY gene sequences.

An NPTII ELISA assay was also used to assess expression of the NPTII protein in leaves of transformed and control plants. Figure 4 illustrates the typical range of NPTII protein expression levels monitored in leaf tissue of the original transformants. Three plants scored as nontransformants by PCR were also negative in the NPTII ELISA assay (data not shown). However, NPTII ELISA was less reliable than PCR as a screen for putative transformants. In the case of all Bsp lines, even though the NPTII gene sequences were integrated into the plant genome and could be amplified by PCR, NPTII protein expression was too low to be detected by ELISA (Fig. 4).

The full-length NIb gene confers resistance to PVY^O.

Self-fertilized R_1 progeny from 13 independently transformed FL R_0 lines were tested for their resistance to PVY^O using 25 plants per line (Table 1). R_1 transgenic pROK lines lacking PVY sequences were used as a control. No resistance was found for nine FL R_1 lines, but some individuals belonging to the FL5, 10, 12, and 13 lines exhibited resistance to PVY^O (Table 1). The FL12 line was particularly noteworthy with only 8% of plants infected. A few of these plants showing resistance were reinoculated and monitored again after 10 days; most of them did not show evidence of PVY^O replication. Five resistant individuals from the FL12 line listed in Table 1 were also assayed 40 days after inoculation with no virus detected by ELISA (data not shown).

Fifteen R_1 plants (selected out of the original 37 PVY^O-resistant R_1 plants) from lines FL5, 10, 12, and 13 as listed in Table 1, were allowed to flower and self-fertilize, and seeds of the R_2 generation were collected. R_2 progeny seedlings were challenged with PVY^O. Most of the R_2 plants, except those from the FL5 line, showed no PVY^O infection, whereas all control pROK plants became infected (Table 1). Sur-

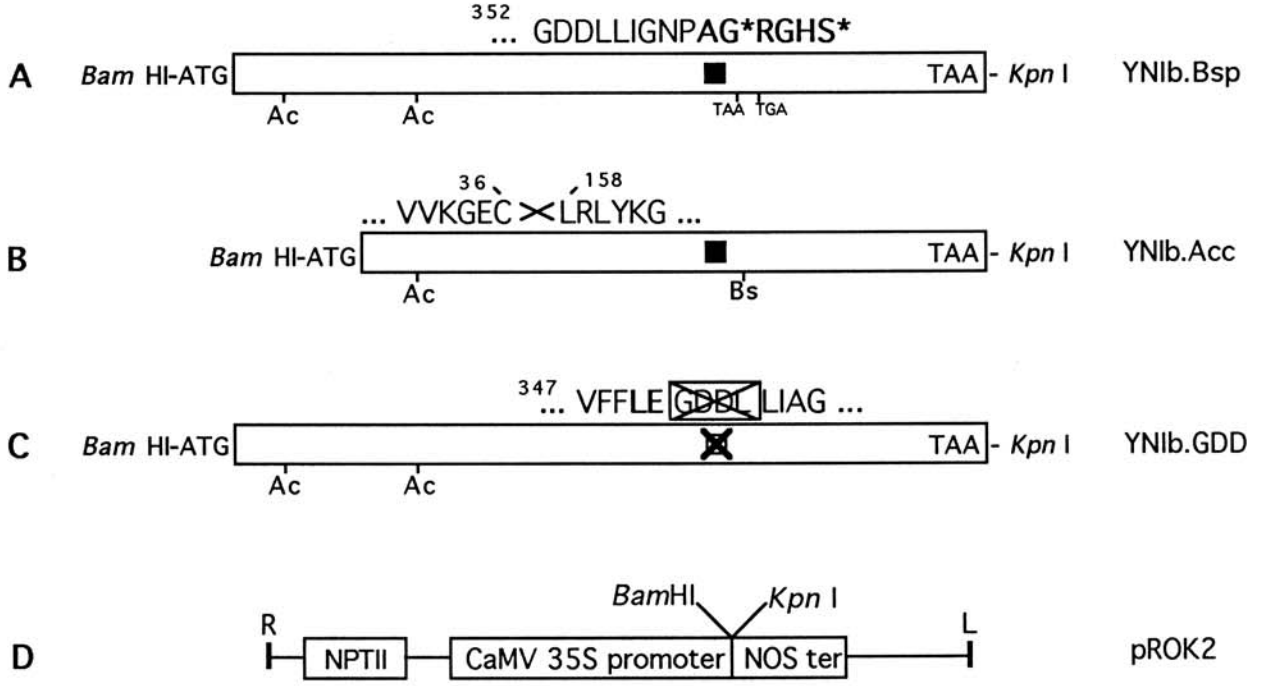

Fig. 2. Defective constructs YNIb.Bsp **(A)**, YNIb.Acc **(B)**, YNIb.GDD **(C)** of the full-length clone YNIb.FL (Fig. 1B). Restriction sites for *Acc*I (Ac) and *Bsp*EI (Bs) are indicated. The black square box represents the relative position of the GDD motif. Modifications of the NIb amino acid sequence are indicated for each construct: bold face characters indicate a substitution, cross bars a deletion, the sign * indicates a stop codon. Numbers refer to the amino acid position in the full-length NIb sequence (Fig. 1C). Left (L) and right (R) indicate border of Ti-plasmid DNA of the pROK2 plant expression vector (D). PVY NIb gene sequences were inserted in the unique *Bam*HI and *Kpn*I restriction sites between the CaMV 35S promoter and the NOS terminator of pROK2. NPTII (neomycin phosphotransferase II) is the selectable marker gene, under control of the nopaline synthase promoter.

prisingly, no resistance was found in R_2 FL5 plants (FL5-9 and FL5-16). The virus concentration as monitored by ELISA in these R_2 FL5 plants was essentially the same as that recorded in pROK plants (data not shown).

Resistance continued to be expressed in the other three lines in the R_2 generation. However, some progeny of specific plants were not resistant, suggesting continuing segregation for resistance within these populations. PVY^O replication in R_2 FL12 and FL13-infected plants was strongly reduced with an average ELISA reading (OD_{405}) of ≈0.080, compared to the value for healthy plants (≈0.030) and the value observed with control infected plants (≈1.50) (data not shown). In another experiment, a total of 12 inoculated R_2 plants from the FL10 and FL12 lines and four pROK plants were maintained in a growth chamber at 32° C in order to determine if resistance to PVY^O was temperature sensitive. After 10 days, virus was not detected by ELISA in NIb gene-engineered plants, whereas all pROK control plants were infected (data not shown).

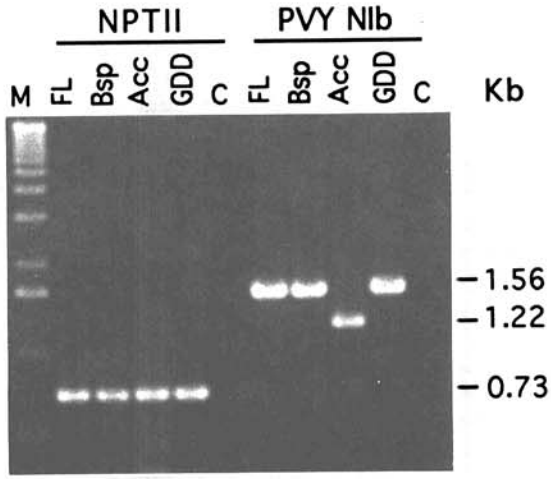

Fig. 3. Agarose gel electrophoresis of amplified NPTII and PVY NIb gene fragments. PCR products produced from genomic DNA from untransformed (C) and transformed tobacco plants harboring PVY NIb gene YNIb.FL (FL), YNIb.Bsp (Bsp), YNIb.Acc (Acc) and YNIb.GDD (GDD), were primed with oligonucleotides specific to the NPTII or PVY NIb sequences. Lane M is a 1-kbp ladder (Bethesda Research Laboratories, Gaithersburg, MD). Numbers on the right refer to the size (kbp) of the expected amplified fragments. Conditions used for PCR amplification are described in the text.

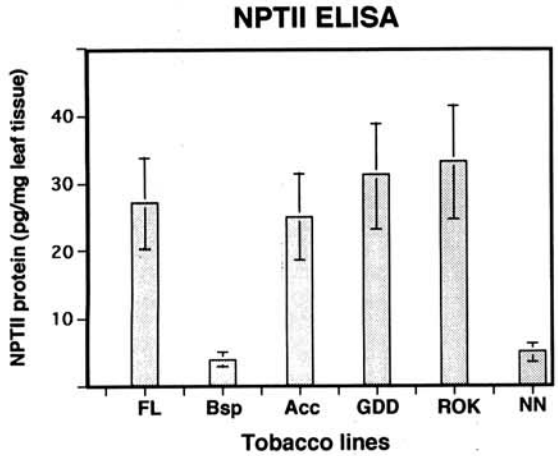

Fig. 4. NPTII protein concentration in leaf tissue of tobacco plants. Untransformed (NN) and transformed R_0 tobacco plants harboring pROK2 (ROK), YNIb.FL (FL), YNIb.Bsp (Bsp), YNIb.Acc (Acc), and YNIb.GDD (GDD) DNA sequences were assayed when the plants were approximately 3 inches tall. Each column represents the mean value and the standard error of a single determination of 5 untransformed plants (NN) and 13 FL, 16 Bsp, 27 Acc, 30 GDD or 5 pROK independently transformed plants.

Defective NIb gene constructs and resistance to PVY^O.

In contrast to the FL lines, initial assessment of resistance to PVY^O in plants transformed with the defective constructs was carried out with the original transformants (R_0). A total of 16 Bsp, 30 Acc, and 30 GDD transformants (described in Fig. 2) were regenerated, rooted, and tested for PVY^O resistance (Table 2). After a first set of inoculations, only five transformants (Bsp3, 9, and 10; Acc6 and 15) gave ELISA readings significantly lower than the pROK controls. These plants were reinoculated and monitored 10 days later, and all gave low ELISA readings.

Selfed R_1 progeny of the original Bsp and Acc resistant plants were challenged with PVY^O (Table 2). Resistance was still segregating in the R_1 progeny, in that there were both resistant and susceptible progeny from each R_0 plant. There was a striking difference in the virus concentration between susceptible and resistant R_1 plants within a given line.

Table 1. Assessment of replicase-mediated resistance to potato virus Y, O strain (PVY^O) or PVY^N inoculation in R_1 and R_2 generation transgenic full-length (FL) lines

	R_1 generation			R_2 generation		
		ELISA[a]				ELISA[a]
	PVY^O	1	2	PVY^O	PVY^N	1
pROK (control)	100/100[b]	1.303	1.231	50/50[b]	15/15[b]	1.017
Lines FL1,2,3, 4,6,7,8,9,11	225/225	1.041	ND			
Line FL5	23/25	0.970	ND			
Plant 5-9		0.048	0.013	10/10	3/3	0.936
Plant 5-16		0.005	0.005	10/10	3/3	1.088
Line FL10	22/25	1.052	ND			
Plant 10-7		0.020	0.013	0/12	3/3	1.030
Plant 10-11		0.081	0.041	0/12	3/3	1.071
Plant 10-16		0.006	0.033	0/12	3/3	1.023
Line FL12	2/25	0.554	ND			
Plant 12-4		0.003	0.003	0/10	3/3	0.477
Plant 12-8		0.004	0.009	3/10	3/3	0.628
Plant 12-17		0.006	0.013	0/10	3/3	0.694
Plant 12-19		0.028	0.004	0/10	3/3	0.472
Plant 12-24		0.003	0.004	0/10	3/3	0.167
Line FL13	16/25	0.723	ND			
Plant 13-2		0.004	0.014	5/10	3/3	0.922
Plant 13-3		0.008	0.009	0/10	3/3	0.854
Plant 13-10		0.018	0.008	2/10	3/3	ND
Plant 13-20		0.002	0.090	5/10	3/3	0.903
Plant 13-21		0.002	0.009	0/10	3/3	0.778

[a] For transgenic lines, ELISA reading represents the mean value (OD_{405}) of all infected plants in this specific group (ELISA background 0.00–0.03). For potentially resistant individual plants, ELISA readings represent the value of a single determination of a specific plant. 1, readings 10 days after the first inoculation; 2, Readings 10 days after the second inoculation. ND, not determined. ELISA for R_2 generation is for PVY^N inoculations only.

[b] Numbers of infected plants as determined by ELISA at 10 days after inoculation with PVY^O or PVY^N as a function of the total number of inoculated plants.

Infected plants supported a level of virus replication similar to the control pROK lines (ELISA OD_{405} ≈1.5), whereas resistant plants were essentially virus-free (≈0.020).

Resistance to other potyviruses and to CMV.

Heterologous viruses were used to challenge five plants each of R_2 FL and R_1 Acc and R_1 Bsp lines previously found to exhibit resistance to PVY^O. All plants inoculated with cucumber mosaic virus (CMV), tobacco etch virus (TEV), and pepper mottle virus (PepMoV) became infected and showed typical symptoms within 7 days. Neither differences nor delays in symptom expression were observed between control and transformed plants.

Resistance to PVY^N.

As the tobacco veinal necrosis strain of PVY (PVY^N) was symptomless in nontransformed tobacco 10 days after inoculation, ELISA was used to test for virus replication. Because of the quarantine safety requirement necessary for the use of this virus, only three plants from each line were inoculated and kept in a locked growth chamber. ELISA readings showed that PVY^N replication occurred in all inoculated plants, and generally, the concentration of virions was comparable in controls and NIb gene transformants (Tables 1 and 2). However, some R_2 FL12 lines yielded ELISA readings that were two to six times lower than the pROK control plants, suggesting a possible partial resistance (Table 1).

DISCUSSION

Since the first report describing extreme resistance to TMV disease in tobacco plants transformed with a putative viral-coded replicase component (Golemboski *et al.* 1990), an analogous approach has been successful in generating transgenic *Nicotiana* plants expressing resistance to pea early browning virus; (MacFarlane and Davies 1992), CMV (Anderson *et al.* 1992), and potato virus X (PVX; Braun and Hemenway 1992; Longstaff *et al.* 1993). In the first two cases, the DNA sequence transferred into plants was a replicase read-through open reading frame encoding a protein of 54 kDa. This replicase-mediated resistance was not restricted to viruses employing a read-through strategy, since recent reports with truncated CMV RNA2-encoded (Anderson *et al.* 1992) and full-length, truncated, or mutated PVX ORF1-encoded (Braun and Hemenway 1992; Longstaff *et al.* 1993) replicase sequences showed resistance induction. In our study, we were interested in determining if this striking form of replicase-mediated resistance could be extended to the economically important potyvirus group. Furthermore, we wished to extend the phenomenon to a virus group in which the replicase protein was normally processed from a polyprotein. The results presented in this paper show that tobacco plants transformed with the PVY^O NIb gene sequences express a high level of resistance to PVY^O.

A total of 325 R_1 plants (unselected segregating population), belonging to 13 independent lines transformed with the full-length NIb gene (FL lines), were challenged with PVY^O and some individuals from four FL lines exhibited resistance. None of these plants were infected following a second inoculation, confirming sustained resistance to PVY^O. For three of these lines, this resistance was retained in the R_2 progeny.

Interpretation of the induction of resistance by transformation of plants with replicase gene sequences is at times influenced by the procedure used to assay resistance. For example, our own experience with replicase-mediated resistance (Anderson *et al.* 1992) and that of others with coat protein-mediated protection (Anderson *et al.* 1989 as one example) indicated that plants which may appear to be resistant when tested with low concentrations of inoculum, no longer show resistance when challenged with higher concentrations. Thus, R_2 ratios of susceptible/resistant plants have little relevance. In this study we used high inoculum concentrations, thereby imposing a stringent test for resistance, accepting as resistant only those plants that showed no disease symptoms and which had background ELISA values. Furthermore, the number of copies of the inserted gene or gene fragment, and their relative positions in the host genome could influence both their capacity for resistance induction and the segregation ratios. It is also apparent that R_2 plants generated from resistant plants may still be segregating for

Table 2. Assessment of replicase-mediated resistance to potato virus Y, O strain (PVY^O) or PVY^N in original (R_0) and R_1 generation transgenic Bsp, Acc, and GDD lines

	R_0 generation			R_1 generation			
	Inoculated with PVY^O			Inoculated with PVY^O		Inoculated with PVY^N	
	No.	ELISA 1[a]	ELISA 2[a]	No.	ELISA 1[a]	No.	ELISA 1[a]
pROK (control)	30/30[b]	1.495	1.336	30/30[b]	1.371	15/15[b]	1.017
Bsp	13/16	1.123	ND				
Plant 3		0.184	0.005	6/10	1.462	3/3	1.245
Plant 9		0.005	0.005	3/10	1.182	3/3	0.788
Plant 10		0.191	0.011	5/10	0.958	3/3	ND
Acc	28/30	1.232	ND				
Plant 6		0.035	0.006	4/10	1.334	3/3	0.997
Plant 15		0.037	0.093	5/10	1.263	3/3	0.857
GDD	30/30	1.281	ND				

[a] For R_0 transgenic plants, ELISA readings represent the mean value (OD_{405}) of all infected plants in a specific group (Bsp, Acc, GDD). For potentially R_0 resistant individual plants, ELISA readings represent the value of a single determination of a given plant (ELISA background 0.00–0.03). For R_1 lines, ELISA values represent mean values for infected plants, with uninfected plants yielding background values of 0.00–0.03. 1, readings 10 days after the first inoculation; 2, Readings 10 days after the second inoculation. ND, not determined.

[b] Numbers of infected plants recorded by ELISA at 10 days after inoculation with PVY^O or PVY^N as a function of total number of inoculated plants.

resistance. This is evident in the plants of the FL12 and FL13 lines, and could explain why all of the R_2 plants in the FL5 line showed no resistance (Table 1).

This work represents the second example of replicase-mediated resistance resulting from the expression of a full-length replicase gene. Braun and Hemenway (1992) recently reported a dramatic reduction of PVX replication in tobacco plants expressing a full-length 165-kDa replicase gene of PVX. These findings were unexpected, taking into account the fact that tobacco plants expressing AlMV (Taschner *et al.* 1991) or BMV (Mori *et al.* 1992) full-length replicase genes did not exhibit resistance, but instead, complemented replicase-defective mutants. It remains to be determined, however, if the replicase sequences transformed in the case of PVX or PVY are fully functional and have not been inadvertently modified during the cloning and transformation processes.

PVY^O-resistant genotypes were also found among tobacco plants expressing two defective NIb constructs. One of those modified genes (YNIb.Bsp) encoding a truncated translation product 70% as large as the full-length protein, is similar to the truncated CMV replicase gene construct used by Anderson *et al.* (1992) to induce CMV resistance in tobacco, except that in PVY NIb, the GDD amino acid motif was retained. Three out of 16 Bsp R_0 tobacco plants exhibited resistance to PVY^O. The second defective construct (YNIb.Acc) carried a 20% deletion at the 5′ end of the NIb gene but still encoded all conserved motifs of viral replicases. Two out of 30 Acc R_0 transformants displayed resistance to PVY^O infection indicating that the 5′ portion of the NIb gene is not essential for the induction of resistance. None of the 30 R_0 lines of the GDD deletion construct showed resistance to PVY^O (Table 2).

Replicase-mediated resistance has been shown to have a narrower spectrum of specificity than coat protein-mediated protection, as shown with TMV (Golemboski *et al.* 1990). As expected, PVY^O resistant plants belonging to the FL, Bsp, and Acc lines did not exhibit resistance to CMV or to two distantly related potyviruses, TEV and PepMoV. Surprisingly, the closely related strain PVY^N was found to infect PVY^O-resistant plants. However, plants of the FL12 R_2 line did show a lower concentration of PVY^N virions compared to the pROK control plants 10 days after inoculation (Table 1). The potyviruses TEV and PepMoV share only 60–70% identity with PVY^O at both the nucleotide and amino acid levels for the NIb gene; however, in the case of PVY^N the percentage of identity is above 90%. The striking differences in response to PVY^O or PVY^N inoculation in transgenic PVY^O NIb plants suggest that NIb replicase-mediated resistance to PVY is indeed strain specific, but the finding of partial resistance in one line suggests that further selections may yield lines with an enhanced spectrum of resistance.

Farinelli *et al.* (1992) reported a similar phenomenon with transgenic potato plants expressing the PVY^N coat protein gene. Two PVY^N-transformed resistant potato lines were readily infected when challenged with PVY^O. These findings were surprising and unexpected since similarities between the PVY^O and PVY^N strains are even higher (≈98%) for the coat protein genes (Lawson *et al.* 1990). It is obvious from the above discussion that we have much to learn about the mechnism(s) underlying both coat protein- and replicase-mediated resistance induced by transformation of otherwise susceptible plant species.

MATERIALS AND METHODS

Plants and viruses.

Nicotiana tabacum 'Turkish Samsun NN' was used for virus propagation and transformation. The PVY^O strain used for cloning and inoculations was selected for its high titer in plant tissue; it was isolated from a *Solanum* genotype Mex 1035 (Griffiths *et al.* 1990). PVY^O was purified from systemically infected tobacco leaves 4 wk postinoculation (Yang *et al.* 1983).

Cucumber mosaic virus (Fny-CMV; Roossinck and Palukaitis 1990), the tobacco veinal necrosis strain of PVY (PVY^N; TVN-ONT; McDonald and Kristjansson 1993) and two heterologous potyviruses were used for challenge inoculation studies. Isolates of TEV and PepMoV kindly provided by John Murphy (Cornell University, Ithaca, NY), were used to generate infected plant tissue for inoculation.

Cloning and sequencing of the PVY^O NIb gene.

PVY^O RNA was extracted from purified virions (Dougherty and Hiebert 1980) and synthesis of the first cDNA strand was carried out with Moloney murine leukemia virus reverse transcriptase (Sambrook *et al.* 1989). The DNA-RNA duplex was isolated by phenol extraction and ethanol precipitation and subjected to PCR amplification with *Taq* polymerase (Ampli*Taq*; Perkin-Elmer Cetus). The upstream primer used for the amplification was 5′-GATCATGATGTAGTGATC-CC**ATG**GCTAAGCATTCTGC-3′, which corresponds in part to nucleotides 6986–7023 of the PVY^N RNA sequence (Robaglia *et al.* 1989). The original sequence was modified to create a *Bam*HI site (underlined) and an AUG start codon (bold face) adjacent to the first codon of the NIb gene. The downstream primer was 3′-CTTCATGTGGTA**ATT**CCAT-GGCTGTGTTAACTACG-5′ complementary to nucleotides 8558–8592 of the PVY^N RNA sequence. This oligonucleotide was designed to introduce a single base substitution (GTT to ATT) in the last 3′ codon of the NIb gene (bold face) to create an ochre stop codon (UAA) and to insert a *Kpn*I site (underlined). After 25 repeated cycles of amplification involving a denaturation step at 95° C for 1 min, an annealing step at 50° C for 1 min and a DNA synthesis step at 72° C for 3 min, the PCR products were visualized on a 1% agarose gel. Bands of the expected size (1.6 kb) were eluted from the gel, digested with *Bam*HI and *Kpn*I and ligated into a similarly digested pBluescript SK vector (Stratagene). Recombinant plasmids were transformed into *E. coli* strain XL-1 blue (Stratagene) using a $CaCl_2$ transformation procedure (Sambrook *et al.* 1989). This clone was called YNIb.FL (Fig. 1B). Subcloned YNIb.FL restriction fragments were sequenced by the dideoxynucleotide chain termination method (Sanger *et al.* 1977) using modified T7 DNA polymerase (Sequenase; U.S. Biochemicals).

Construction of defective PVY^O NIb genes.

Three defective NIb gene constructs were derived from the full-length YNIb.FL clone. An NIb replicase gene (YNIb.Bsp; Fig. 2A) was generated by digesting clone YNIb.FL with *Bsp*EI (position 1080–1085) followed by treatment with the Klenow fragment of DNA polymerase I to obtain a blunt-ended molecule. The plasmid, recircularized using T4 DNA ligase, contains a +4-frameshifting sequence that created two in-frame trans-

lational stop codons, 10 and 15 codons downstream from the GDD motif (Fig. 2A). A second defective construct (YNIb.Acc; Fig. 2B) was generated, resulting in an in-frame deletion of 363 nucleotides (position 110–472) between two *Acc*I recognition sites at the 5′ end of the YNIb.FL sequence. Finally, a third clone (YNIb.GDD; Fig. 2C) deleted for the sequence encoding the amino acids GDDL was generated using PCR mutagenesis (Kammann *et al.* 1989). T3 RNA polymerase derived transcripts of those pBluescript clones were assessed by *in vitro* translation using a rabbit reticulocyte lysate system (Promega) as described by Domier *et al.* 1989. Translation products were analyzed by SDS-polyacrylamide gel electrophoresis (Laemmli 1970).

Plant transformation.

The *Bam*HI-*Kpn*I fragments of the four YNIb clones were inserted into the *Bam*HI and *Kpn*I sites of the expression cassette of the binary vector pROK2 (Longstaff *et al.* 1993) to derive the corresponding plasmids pYNIb.FL, pYNIb.Bsp, pYNIb.Acc, and pYNIb.GDD (Fig. 2D). The pROK2 derivatives were transferred into the nononcogenic *Agrobacterium tumefaciens* strain LBA-4404 by triparental mating (Rogers *et al.* 1986) using pRK2013 as a helper plasmid. Transconjugants selected on kanamycin and streptomycin (50 and 125 μg/ml, respectively), were used to transform tobacco leaf disks as described by Horsch *et al.* (1985). Except where indicated in the Results section, transformants were regenerated on a selection medium containing kanamycin (300 μg/ml). Rooted plantlets were transferred to soil and maintained in a greenhouse with a 16 hr light/8 hr dark cycle at 25° C.

Selection of transformants.

The presence of the neomycin phosphotransferase II (NPTII) and PVY NIb gene sequences in the regenerated tobacco plants was determined by PCR analysis of genomic DNA isolated from each plant. Genomic DNA was isolated (Murray and Thompson 1980) and amplification of the NIb gene inserts was carried out using the degenerate primers used to clone the PVY^O NIb gene. For the NPTII gene, two oligonucleotide primers (5′-GGTTCTCCGGCCGCTTGGG-TGG-3′ and 3′-GCGGCGAGGGCTAAGCTCGCG-5′) were designed according to nucleotide positions 28–49 (+ sense) and 738–759 (– sense) of the NPTII sequence (Beck *et al.* 1982). PCR amplification was performed with *Taq* polymerase (Ampli*Taq*; Perkin-Elmer Cetus) and 0.5 μg of genomic plant DNA using a three-temperature program (95° C for 1 min, 60° C for 1 min, and 72° C for 2 min) for 30 cycles. Regenerated tobacco plants were also assayed for the NPTII protein using an ELISA-assay kit as recommended by the manufacturer (5 Prime–3 Prime, Inc.).

Virus resistance experiments.

Original transformants (R_0) and self-pollinated progeny plants (R_1 and R_2 generations) were inoculated with PVY^O at the three-leaf stage. In some cases, R_1 and R_2 progeny plants were also inoculated with PVY^N, TEV, PepMoV, or CMV approximately 6 wk after planting, when they had three or four leaves. All inocula were freshly prepared by grinding systemically infected tobacco leaves in a microtube without buffer. The sap collected after a brief centrifugation (10,000 *g*, 1 min) was used directly. The largest leaf was dusted with Carborundum, mechanically inoculated using a cotton applicator and washed with water. The concentration of virus in each inoculum was estimated at 50–100 μg/ml by ELISA using freshly purified virus preparations as standards. These inocula reproducibly gave 100% infection on control pROK plants. Control pROK plants were 'selfed' tobacco transformants (R_1) that harbored the NPTII gene and the expression cassette but not the PVY insert. The visual symptoms of PVY infection in the tobacco cultivar Samsun NN are very subtle, so ELISA was routinely used to assess virus replication. TEV-, PepMoV-, and CMV-inoculated plants were scored daily for visual symptoms. Double-antibody sandwich enzyme-linked immunosorbent assay (ELISA) was used to monitor PVY^O and PVY^N in leaf tissues 10 days, and in some cases, 20 and 40 days after inoculation. A PVY polyclonal antiserum (Agdia) was used to precoat the microtiter plates and ELISA was performed with the same polyclonal antibody conjugated with alkaline phosphatase.

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