Transgenic Burley and Flue-Cured Tobacco with Resistance to Four Necrotic Isolates of Potato Virus Y

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ABSTRACT

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Transgenic burley (cultivars KY14, NC3433-33, and TN90) and flue-cured (cultivar K326) tobacco with resistance to four necrotic isolates of potato virus Y (PVY) were constructed. These tobacco cultivars were transformed with a chimeric gene designed to express the coat protein (CP) from the necrotic Chilean isolate of PVY. CP expression among R_0 plants was undetectable by indirect enzyme-linked immunosorbent assay and Western blot (immunoblot) analysis. Transgenic R_0 plants that harbored from one to five neomycin phosphotransferase II (NPT II) transgene loci were identified. Although the NPT II and the PVY CP chimeric genes were linked within a common T-DNA, no correlation was found between the number of NPT II and CP transgenes. Inoculation of 41 independent transgenic KY14 R_0 plants with PVY-Chilean identified

eight resistant plants, while inoculation of 30 transgenic K326 R_0 plants identified six resistant plants. Inoculation of 17 transgenic NC3433-33 R_0 plants with PVY-Chilean identified six resistant plants, while inoculation of 50 transgenic TN90 R_0 plants with PVY isolate VAM B resulted in nine resistant plants. Progeny derived from PVY-inoculated but symptomless R_0 plants were also resistant to the Chilean. Europe H, M^sN^r, N-Canada, and VAM B isolates of WY, demonstrating sexual transmission of the transgenes that condition resistance. We discuss the ability of the PVY-Chilean CP gene to protect transgenic tobacco K326, KY14, NC3433-33, and TN90 from infection by four necrotic isolates of PVY. The flue-cud tobacco cultivar K326, which carries a gene simultaneously conferring resistance against root-knot nematode and susceptibility to PVY strain M^sN^r, is protected against this strain when transformed with the PW-Chilean CP gene.

Additional keywords: capsid protein gene-mediated resistance, potyviruses, virus resistance.

Potato virus Y (PVY) is the type member of the *Potyvirus* genus within the Potyviridae, the largest family of RNA plant viruses (21). Infection of tobacco (*Nicotiana tabacum* L.) with PVY results in symptoms ranging from vein banding and mild mosaic to severe leaf necrosis and necrotic lesions on veins and stalks (8), depending upon the virus isolate and tobacco genotype. PVY infection also reduces tobacco leaf yields and quality (27,30,42). Infection of tobacco with PVY strains indigenous to the southeastern United States can result in yield reductions of 10 to 100% (14).

The occurrence of highly virulent necrotic PVY isolates bas been reported in countries throughout the world (1,13,14,17, 28,35,43,50). Most recently, a necrotic PVY isolate that exhibits mild symptoms in potato but causes severe necrosis in tobacco has been identified and is interfering with commerce in seed potatoes from Canada (*G.V.* Gooding, Jr., personal communication). Control of PVY is difficult, and the most viable approaches are the deployment of PVY-resistant cultivars and the use of cross-protection between PVY strains (11,25,29,38).

Unfortunately, acceptable sources of PVY resistance are rare in *Nicotiana* germ plasm (6). Tobacco breeding line NC 744 (7,12),

carrying the *va* homozygous recessive gene (16) derived from Virgin A Mutante (VAM) (26), confers either resistance or tolerance to strains of PVY indigenous to the southeastern United States. However, tobacco genotypes carrying the *va* gene in the homozygous condition are hypersensitive to blue mold disease (15,44). Furthermore, some strains of PVY, such as VAM B and ARG (11), overcome this gene. A specific strain of PVY, designated M^sN^r, produces necrotic symptoms in tobacco genotypes that carry a gene conferring resistance to root-knot nematodes (RKN) and mild symptoms in genotypes lacking this gene (5). This reaction is unaffected by the *va* gene. It has not been possible to break this negative correlation between RKN and PVY resistance, so the utility of the RKN resistance gene in areas where the M^sN^r strain is present is reduced. Therefore, identification of alternative sources of PVY resistance in commercial tobacco is desired.

Virus resistance from expression of the coat protein (CP) gene in transgenic plants, often referred to as pathogen-derived resistance, is potentially useful for developing PVY-resistant burley and flue-cured tobacco. The expression of the CP gene in transgenic plant strategy has been used successfully to protect *Nicotiana* sp. from viruses such as tobacco mosaic virus (TMV), tobacco etch virus (TEV), and potato virus X as well as PVY (20,31,32,37,46). Yet essentially no research has been performed to examine the use of this strategy against PVY in commercial tobacco cultivars. This report describes the virus-resistance effects afforded by integrating the necrotic PVY-Chilean CP gene into the commercial flue-cured tobacco cultivar K326 and burley

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tobacco cultivars KY14, NC3433-33, and TN90. Tobacco resistant to four necrotic isolates of PVY from throughout the world were identified among transgenic lines carrying the PVY-Chilean CP transgene. Finally, the use of CP gene transgenic resistance to expand protection against PVY infection afforded by the *va* resistance gene was evaluated.

MATERIALS AND METHODS

Materials. Restriction endonucleases and other DNA-modifying enzymes were obtained from Bethesda Research Laboratories, Bethesda, MD. [α-^{32}P]dATP was obtained from DuPont NEN, Wilmington, DE. A PVY monoclonal antibody (24) was kindly provided by R. L. Jordan, USDA-ARS, Beltsville, MD, and the polyclonal antibody against PVY-Chilean was a gift from G. V. Gooding, Jr., North Carolina State University.

PVY-Chilean CP clone and transformation vector. The *Agrobacterium tumefaciens* Ti plasmid-derived binary vector pBI121.1 (Clontech, Palo Alto, CA) (23) was modified by replacing the GUS open reading frame (ORF) with the PVY-Chilean CP cistron to form a chimeric gene construct composed of the CP sequence flanked by the cauliflower mosaic virus 35S promoter and the nopaline synthase-polyadenylation sequence. The PVY-Chilean CP ORF was created by polymerase chain reaction (PCR) amplification from a previously synthesized double-stranded cDNA template (47). The 5′ primer 5′-CGC-GGATCCATGGGAATGACACAATTGATGCA-3′ was composed of sequence from the beginning of the CP cistron from the PVY N strain (3,39,48) preceded by a *Bam*HI site (underlined) and a start codon (bold). The 3′ primer 5′-GGAATTCACATGTTC-TCAGTCCAAGTA-3′ was composed of sequence from the end of the CP gene of PVY N followed by an *Eco*RI site (underlined). The PCR product consisting of an engineered start codon (ATG), 801 bp of CP coding sequence, and 40 bp of 3′-untranslated sequence was cloned into pBS(+). After transformation and selection, an appropriate clone was digested with *Bam*HI and *Eco*RI, and the CP fragment was ligated into pBI121.1 in place of the GUS ORF to form the modified binary vector pBICh-491.

Plant transformation. The binary vector pBICh-491, maintained in *Escherichia coli* strain DH5αF′, was mobilized into *A. tumefaciens* strain LBA 4404 by triparental mating (9). LBA 4404 also harbors the plasmid pAL4404, which encodes the *vir* function necessary for T-DNA transfer. The resulting *A. tumefaciens* transconjugants carrying pBICh-491 were identified by Southern blot analysis (45).

Leaf disks of *N. tabacum* cultivars K326, KY14, and TN90 and haploid sporophytes from breeding line NC3433-33 were transformed with *A. tumefaciens*. Plantlets were regenerated by direct shoot regeneration from the leaf disks on Murashige and Skoog (MS) medium (Sigma, St. Louis, MO) as modified by Horsch et al. (22). Regenerated shoots were rooted on MS medium containing 2 µg of indole-acetic acid and 100 µg of kanamycin sulfate per milliliter. Approximately 50 independent primary transformants (R$_0$ plants) were regenerated from each tobacco cultivar, and each of these lines was duplicated clonally from shoot cuttings. All plants were transplanted into soil, placed in a growth chamber, and transferred to a glasshouse approximately 2 to 3 weeks after transplanting. A single clone of each R$_0$ plant was used to produce seed through self-pollination (R$_1$) and was backcrossed to the recipient cultivar to produce the backcross 1 (BC$_1$) seed generation. The remaining clones were used for virus inoculation experiments.

Haploid progeny were prepared from selected R$_0$ transgenic plants essentially as described by Burk et al. (4). In this procedure, tobacco (*N. tabacum* L.) plants are pollinated from *N. africana* Merxm. When the resulting seeds germinate, true hybrid progeny fail to develop true leaves and eventually die. A small proportion of the seedlings are haploid. These can be identified easily because they exhibit larger cotyledons, develop true leaves, and grow normally, although they are infertile at maturity.

Transgenic haploid sporophytes produced in this way represent a gametic array and would be expected to carry from none to all of the transgenic loci present in the R$_0$ from which they are derived. Chromosome doubling of these plants produces doubled haploid sporophytes in which all genes, including transgenes, are present in the homozygous condition.

Transgenic plant analysis. The presence of transgenes in R$_0$ plants and their progeny was confirmed by Southern blot analysis (45). Genomic DNAs were isolated from leaf tissues, and 10 to 20 µg was digested with *Hin*dIII, separated by electrophoresis, and transferred to nylon membrane. Blots were probed with [α-^{32}P]-labeled PVY-Chilean cDNA (36). PVY-Chilean CP expression among R$_0$ plants and their progenies was assayed by indirect enzyme-linked immunosorbent assay (indirect-ELISA) (34) and by enzymatic chemiluminescent (ECL) Western blotting (immunoblotting) (Amersham, Arlington Heights, IL). Leaf tissue samples were homogenized in 100 mM Tris (pH 7.5), 1 µl of 2-β-mercaptoethanol per milliliter, and 1 mM phenylmethylsulfonyl fluoride. Total protein in the homogenate was determined by Bradford assay, and 50 µg of the protein was precipitated with acetic acid and lyophilized. Protein pellets were resuspended in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (PAGE) buffer. Samples were subjected to PAGE followed by Western blot transfer to nitrocellulose membranes.

The number of neomycin phosphotransferase II (NPT II) transgene loci in independent R$_0$ plants was estimated by germinating 200 seeds of their respective progeny on Hoagland nutrient medium (19) solidified with 1% Bacto agar containing 200 µg of kanamycin sulfate per milliliter. Phenotypes of the seedlings were scored 4 weeks after germination. The segregation ratio for resistance (Kan^r) or susceptibility (Kan^s) to kanamycin was used to estimate the number of functional NPT II loci present in each R$_0$.

Viruses. All viruses used in these experiments were maintained by mechanical inoculation on Burley 21 tobacco. TEV, tobacco vein mottling virus (TVMV), and five PVY isolates were used to challenge the four tobacco cultivars containing the PVY-Chilean CP gene. Under glasshouse conditions, infection with PVY Chilean (NC isolate 178), PVY Europe H (NC isolate 189), and PVY N-Canada (NC isolate 204) resulted in vein clearing on younger leaves of K326, KY14, and NC3433-33 within 7 days postinoculation (dpi) followed by veinal necrosis, leaf necrosis, and severe mosaic within 10 dpi. PVY VAM B (NC isolate 182) produced vein clearing followed by severe leaf mosaic but no veinal necrosis on all cultivars used in these experiments, except K326. Inoculation of K326 and NC3433-33, which carries a gene conferring RKN resistance, with PVY M^{s}N^r (NC isolate 138) resulted in the formation of local lesions on inoculated leaves followed by plant death within 14 days. TEV (NC isolate 155) and TVMV (NC isolate 148) infections exhibited typical TEV and TVMV symptoms in tobacco (35).

Virus inoculation of transgenic lines. Transgenic R$_0$ transplants and their progeny were mechanically inoculated on each of the two expanded leaves at the two- to three-expanded-leaf stage with sap from virus-infected plants diluted (1:10) in 10 mM phosphate buffer. Four weeks after inoculation, phenotypic performance of the inoculated transgenic lines was recorded as wild-type (plants systemically infected as in the nontransformed control), modified (plants systematically infected but symptoms attenuated compared with those exhibited by susceptible controls), or resistant (plants with no symptoms typical of PVY infection). R$_0$ plants of K326, KY14, and NC3433-33 were initially screened by inoculation with PVY-Chilean. Transgenic R$_0$ TN90 plants carrying the homozygous-recessive *va* gene, which confers resistance to PVY, were screened by inoculation with PVY VAM B. As previously described, PVY VAM B can overcame the *va* resistance and infect the TN90 line (11).

To examine the efficacy of the PVY-Chilean CP gene to confer resistance against the four necrotic isolates of PVY and to demonstrate inheritance of the trait, approximately 20 randomly selected Kan^r progeny from K326 and KY14 R_0 plants were inoculated with the necrotic isolates PVY-Chilean, PVY Europe H, PVY N-Canada, and PVY VAM B and the nonnecrotic isolate PVY M^{s}N^r. Kan^r progeny of selected TN90 R_0 plants were challenged with PVY VAM B. To determine the efficacy of the genetic construct against other potyviruses, Kan^r progeny of K326-4C, KY14-5C, and TN90-38C were inoculated with TEV and TVMV. Inoculations were repeated in at least two sets of experiments.

RESULTS

Transgenic tobacco regeneration. All independently regenerated, putatively transformed shoots successfully formed roots on

rooting medium containing 100 µg of kanamycin sulfate per milliliter, suggesting that all plantlets carried at least one functional NPT II gene. The presence of the introduced sequence was also confirmed by hybridizing R_0 plant genomic DNA with an [α-^{32}P]-labeled PVY-Chilean CP ORF probe. Southern analysis revealed that the transgenic R_0 plants contained one to five PVY CP genes (Fig. 1). However, expression of CP among the transgenic K326, KY14, NC3433-33, and TN90 was undetectable
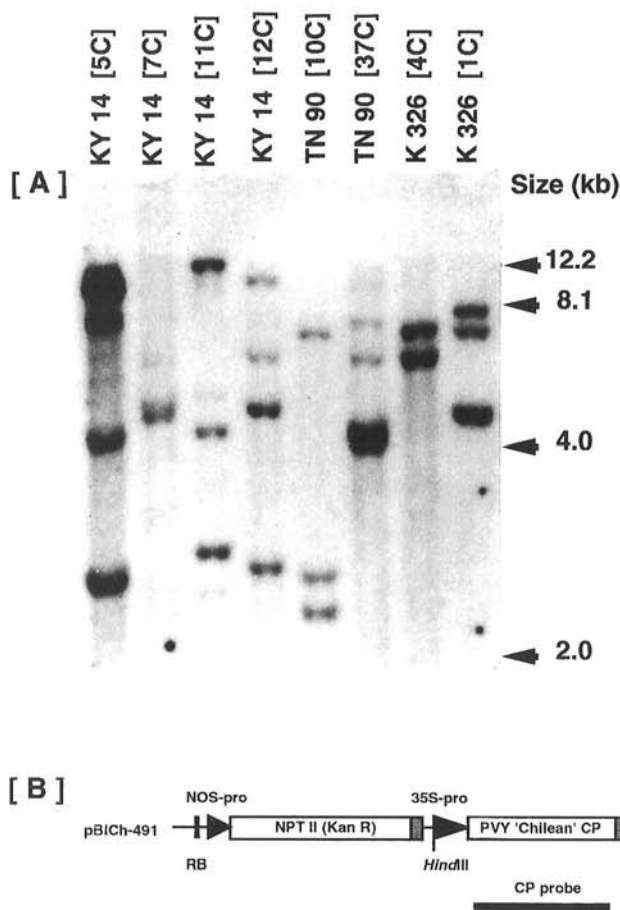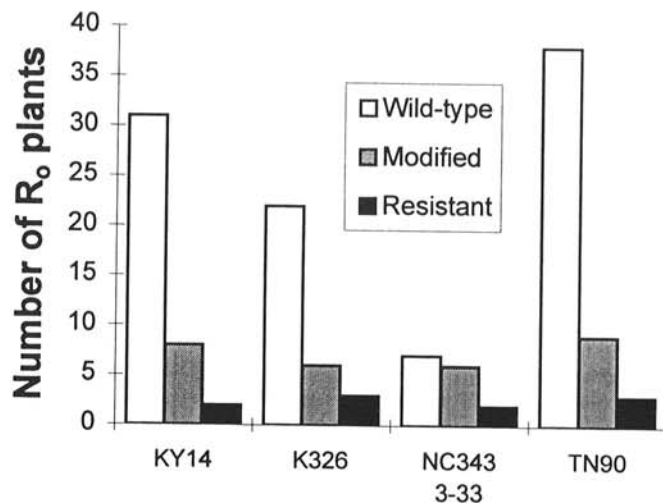

Fig. 1. A, Southern blot of representative transgenic K326, KY14, and TN90 R_0 plants. Genomic DNA (10 to 20 µg) digested with *Hind*III was loaded in each lane, and [α-^{32}P]-labeled potato virus Y (PVY) Chilean isolate coat protein (CP) open reading frame was used as the probe. **B,** Representation of pBICh-491 and PVY CP fragment used to probe DNA blots.


Fig. 2. Western blot of total protein from green leaf tissue obtained from independent transgenic R_0 plants carrying the potato virus Y (PVY) Chilean isolate coat protein (CP) gene. The blot was probed with PVY antiserum. Lanes 1 and 4, nontransformed KY14; lanes 2 and 3, KY14-5C-R$_1$; lane 5, NC3433-33-13C-2-S1; lane 6, NC 3433-33-1TH-S1; lane 7, nontransformed NC3433-33; and lane 8, 0.01 µg of PVY CP.

Tobacco genotypes

Fig. 3. Symptom response of independent transgenic R_0 plants carrying potato virus Y (PVY) Chilean isolate coat protein after PVY inoculation. One clone of each independent R_0 plant was mechanically inoculated at the two- to three-expanded-leaf stage with a 1:10 dilution of virus-infected plant sap and 10 mM phosphate buffer. Transgenic R_0 plants of K14, K326, and NC3433-33 were screened by inoculation with PVY-Chilean (NC isolate 178), and R_0 plants of TN90 were screened with PVY VAM B (NC isolate 182). Responses were recorded 28 days postinoculation and categorized as wild-type (plants systemically infected as control line); modified (plants systemically infected but showed attenuated symptom expression); or resistant (plants showed no symptoms typical of PVY infection).
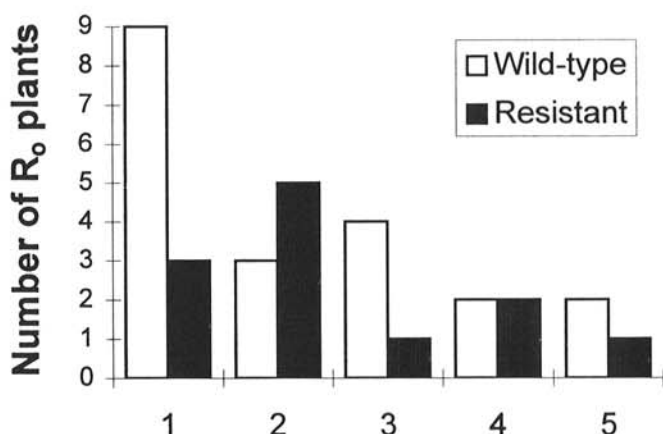
Number of functional NPTII loci

Fig. 4. Resistance to potato virus Y (PVY) in transgenic R_0 plants carrying a range of one to five transgene neomycin phosphotransferase II (NPT II) loci. A representative clone of each R_0 plant was inoculated with PVY isolate Chilean (K326 and KY14) or VAM B (TN90) at the two- to three-leaf stage, and the response of each plant was recorded 28 days postinoculation. Functional NPT II loci numbers were estimated by germinating seeds derived from respective R_0 plants on solidified Hoagland salt plates containing 200 mg of kanamycin sulfate per milliliter and determining the ratio of green and yellow seedlings.

by indirect-ELISA or by the highly sensitive ECL Western blot analysis (Fig. 2).

PVY resistance in transgenic R_0 plants. To assess resistance against PVY, transgenic K326, KY14, and NC3433-33 R_0 plants were challenged with PVY-Chilean and transgenic TN90 R_0 plants were challenged with PVY VAM B. Plants were visually scored for virus symptoms up to 28 dpi. The majority of transgenic R_0 plants were infected with PVY-Chilean or PVY VAM B by 7 dpi. However, a small number of plants were either symptomless or expressed intermediate PVY symptoms (modified) (Fig. 3). To confirm the resistance, symptomless R_0 plants were cut back to the crown at 28 dpi, axillary buds (suckers) were produced from rootstocks, and plants were reinoculated with PVY. In addition, a back-inoculation assay was conducted by taking a leaf sample from each symptomless R_0 plant and using it to inoculate the PVY-susceptible indicator Burley 21 tobacco. The presence of systemic symptoms on indicator plants at 28 dpi would indicate the presence of infectious virus. None of the indicator plants or suckers exhibited PVY symptoms. This confirmed the resistance of the symptomless R_0 plants tested and suggested the absence of virus within inoculated but symptomless R_0 plants (data not shown). Subsequently, all resistant and modified R_0 plants were allowed to grow to maturity in the glasshouse. None of the resistant R_0 plants tested exhibited PVY symptoms, and symptoms that occurred on some of the modified R_0 plants gradually disappeared as the plants aged.

In pBICh-491, the CP gene was linked within the same T-DNA to the NPT II gene (Fig. 1B). Consequently, the inheritance of the CP gene was assumed to follow that of NPT II. An array of transgenic R_0 lines containing one to five functional NPT II loci was identified whose PVY resistance ranged from immunity to wild-type levels of susceptibility (Fig. 4). However, no direct correlation was found between the presence of functional NPT II transgenes, or the number of NPT II inserts, and protection of the transgenic R_0 plants against infection by PVY.

Resistance to potyviruses in R_1 and BC_1 seedlings. Segregating R_1 and BC_1 progeny derived through self-pollination and backcrosses of R_0 plants to control cultivars, respectively, were inoculated with TEV, TVMV, and five isolates of PVY. Disease development among inoculated transgenic seedlings and nontransformed cultivars is presented in Figure 5. Nontransformed cultivars inoculated with the same potyviruses exhibited typical virus symptoms 6 to 10 dpi. Likewise, some segregating progeny derived from selected transgenic R_0 plants exhibited symptoms similar to those of nontransformed controls.

Plants harboring the PVY-Chilean CP gene with demonstrated resistance to PVY were not protected against TEV or TVMV. Transgenic KY14-5C and K326-4C, which are resistant to the PVY isolates tested, exhibited TEV and TVMV systemic symptom development at the same rate as the nontransgenic control after inoculation (Fig. 5). These observations suggest that the PVY-Chilean CP gene does not confer broad-spectrum protection against potyviruses, although it is effective against almost all PVY isolates tested (Table 1 and Fig. 5).

Segregating progeny derived from susceptible R_0 plants were susceptible to PVY infection. Resistant seedlings were not recovered from the segregating progeny (Table 1). However, R_0 plants that exhibited attenuated symptoms (KY14-11C) or ones that did not express symptoms (K326-4C; KY14-5C and 12C; and TN90-20C, -37C, and -38C) produced progeny that were resistant to PVY. Several resistant seedlings were recovered from segregating progeny derived from these R_0 plants inoculated with PVY (Table 1). Resistant plants were recovered from such progeny regardless of the PVY isolate used as inoculum. These data suggest that the resistant R_0 plants, modified R_0 plants, and their progeny carry PVY CP genes capable of conferring PVY resistance.

Interaction between CP transgenic resistance and natural resistance genes in tobacco. TN90 plants carrying the homozygous recessive *va* gene were transformed with pBICh-491.
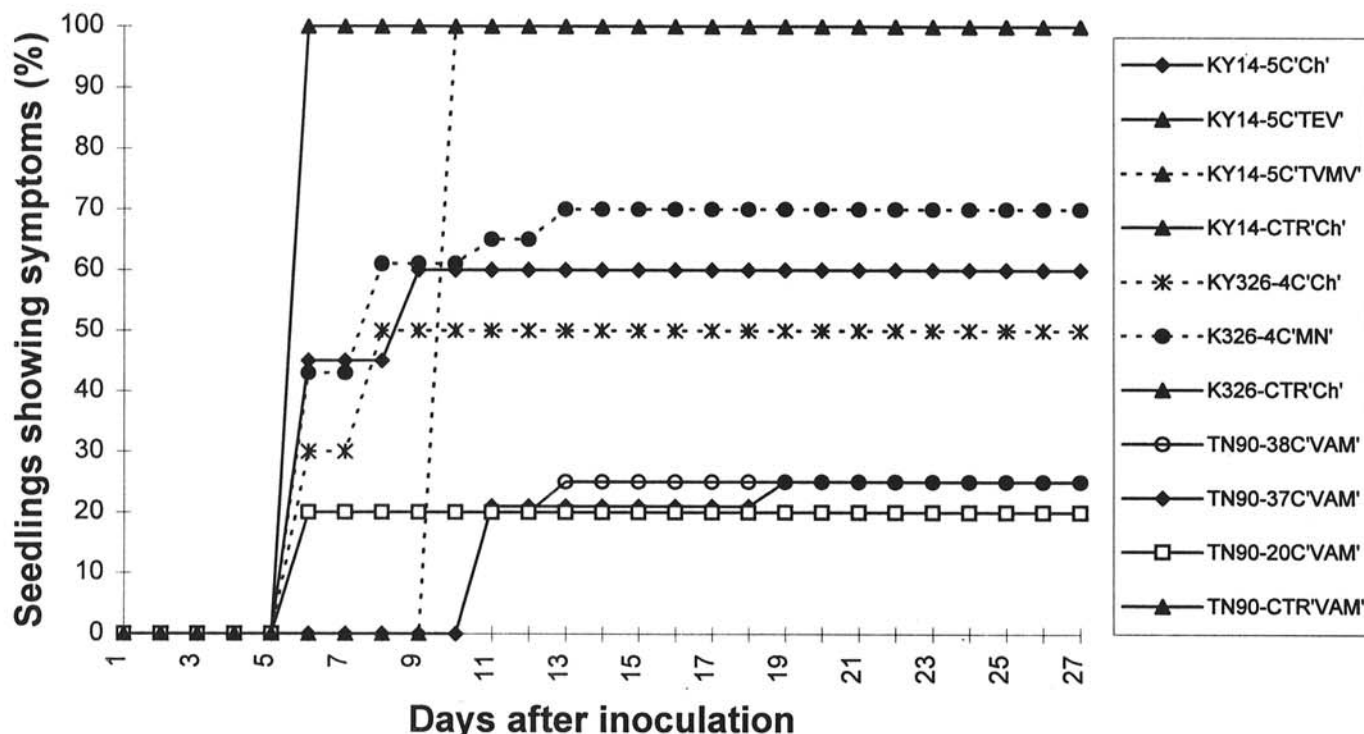

Fig. 5. The percentage of kanamycin-resistant (Kan^r) seedlings showing systemic symptoms typical of potyvirus infections on succeeding days postinoculation. At least 20 Kan^r R_1 (TN90- and K326-derived lines) or BC_1 (KY14-derived lines) seedlings were mechanically inoculated at the two- to three-expanded-leaf stage with a 1:10 dilution of virus-infected plant sap and 10 mM phosphate buffer. The plant genotypes followed by the PVY strain used to inoculate each are shown at right. KY14-5C'TEV', KY14-CTR'Ch', K326-CTR'Ch', and TN90-CTR'VAM' are assigned the same symbol (▲) because they have the same infectivity profile.

Transgenic TN90 R_0 plants and their progeny were inoculated with the VAM B isolate of PVY. Among 50 TN90 R_0 plants tested, three modified and nine symptomless R_0 plants were identified (Table 2). When progeny of VAM B-resistant R_0 plants (TN90-20C, -37C, and -38C) were inoculated with PVY VAM B, they segregated as wild-type and resistant. Progeny of susceptible R_0 plants were uniformly susceptible to PVY VAM B (Table 2). K326 and KY14, carrying the dominant Va allele, were initially susceptible to PVY. Transgenic K326-4C and KY14-5C, which are resistant to necrotic isolates of PVY, were also tested against PVY VAM B. Progeny derived from K326-4C and KY14-5C also showed resistance to PVY VAM B (Table 2). These observations suggest that the va gene is not required to obtain tobacco with resistance to PVY VAM B.

The presence of a dominant gene conferring RKN resistance has been reported in tobacco (51). The dominant allele of this gene has been associated with the severe necrotic response of some flue-cured tobacco to PVY M^sN^r infection (5,18,40,41). K326 and a haploid sporophyte of breeding line NC3433-33, carrying the RKN resistance gene, were transformed with PVY-Chilean CP construct. Transgenic K326 R_0 plants and their segregating progeny, carrying both the RKN resistance gene and the CP gene, were tested against an isolate of PVY M^sN^r. Previously, two independent transgenic K326 R_0 plants, K326-1C and K326-56C, exhibited a wild-type reaction when exposed to PVY-Chilean (Table 1). Segregating progeny derived from these plants were also uniformly susceptible to PVY M^sN^r infection. However, two other independent transgenic K326 R_0 plants,

K326-4C and K326-7C, were resistant to PVY-Chilean, and their progeny segregated as wild-type and resistant against PVY M^sN^r (Table 1).

K326 inoculated with PVY M^sN^r developed necrotic lesions on inoculated leaves (18). The infected plants developed severe veinal necrosis and died within 14 dpi. Development of these symptoms after infection with PVY M^sN^r is typical in tobacco carrying the dominant RKN allele. Transgenic K326 R_0 plants 4C and 7C and their progeny, which were resistant to PVY M^sN^r infection, did not develop necrotic lesions, and the plants survived to maturity. However, transgenic K326 R_0 plants 1C and 56C and their progeny, which were susceptible to PVY M^sN^r infection, showed typical necrotic lesions, and the plants died 14 dpi, as did control K326 plants.

DISCUSSION

A number of transgenic lines derived from four commercial tobacco cultivars containing the PVY-Chilean CP were resistant to infection by four necrotic PVY isolates. This resistance is displayed as attenuation or absence of symptoms. The symptoms on some of the lines showing attenuation gradually disappeared with maturity; in other lines, attenuated symptoms were observed throughout maturation. In contrast, all resistant lines remained free of virus symptoms throughout the life of the plants. Only resistant and modified R_0 plants produced progeny that were resistant to the six isolates of PVY tested. We have demonstrated that virus-specific protection against necrotic isolates of PVY is

TABLE 1. Percentage of resistant seedlings of transgenic K326 and KY14 tobacco lines after mechanical inoculation with four necrotic potato virus Y (PVY) isolates 28 days postinoculation (dpi)

Transgenic line[a]	R_0 line tested[b]	NPT II loci[c] (no.)	PVY isolate[d]			
			Chilean	Europe H	N-Canada	M^sN^r
K326-4C	R	1	50 (10)	50 (10)	20 (10)	29 (23)
K326-1C	WT	3	0 (21)	0 (10)	0 (10)	0 (20)
K326-56C	WT	5	0 (10)	0 (10)	0 (10)	0 (19)
KY14-5C	R	5	100 (20)	90 (20)	95 (20)	nt[e]
KY14-12C	R	4	93 (15)	80 (15)	40 (10)	nt
KY14-14C	WT	1	0 (20)	0 (20)	nt	nt
KY14-7C	WT	3	0 (20)	0 (20)	nt	nt
KY14-11C	Mod	4	10 (20)	15 (20)	nt	nt

[a] All selected transgenic lines were tested at the R_1 generation except K326-56C, which was tested at the BC_1 generation. The seedlings were subjected to kanamycin sulfate (200 mg ml^{-1}) selection for 28 days, and only kanamycin-resistant seedlings were tested against PVY.
[b] Responses were recorded 28 dpi and categorized wild-type (WT) (plants systemically infected as control line); modified (Mod) (plants systemically infected but showed attenuated symptoms expression); or resistant (R) (plants showed no symptoms typical of PVY infection for up to 28 dpi).
[c] Segregating loci were determined on the basis of the number of functional neomycin phosphotransferase II (NPT II) transgenes estimated in respective progeny.
[d] Percentage of resistant seedlings. Sample size is given in parentheses.
[e] Not tested.

TABLE 2. Percentage of resistant seedlings of transgenic TN90, K326, and KY14 tobacco lines after sap inoculation with the VAM B isolate of potato virus Y (PVY) 28 days postinoculation (pdi)

Transgenic line[a]	R_0 line tested[b]	NPT II loci[c] (no.)	Resistant R_1 seedlings (%)			Resistant BC_1 seedlings (%)		
			WT	Mod	R	WT	Mod	R
TN90-10C	WT	1	100	0	0	100	0	0
TN90-20C	R	1	20	0	80	55	0	45
TN90-37C	R	2	4	28	68	10	5	15
TN90-38C	R	2	25	0	75	50	0	50
K326-4C	R	1	70	10	20	nt[d]	nt	nt
KY14-5C	R	5	0	0	100	20	0	80
KY14-12C	R	4	70	20	10	100	0	0

[a] The seedlings were subjected to kanamycin sulfate (200 mg ml^{-1}) selection for 28 days, and only kanamycin-resistant seedlings (20 from each line) were tested against PVY.
[b] Responses were recorded 28 dpi and categorized as wild-type (WT) (plants systemically infected as control line); modified (Mod) (plants systemically infected but showed attenuated symptoms expression); or resistant (R) (plants showed no symptoms typical of PVY infection for up to 28 dpi).
[c] Segregating loci were determined on the basis of the number of functional neomycin phosphotransferase II (NPT II) transgenes estimated in respective progeny.
[d] Not tested.

conferred by integration of the PVY-Chilean CP gene into the genomes of one flue-cured and three burley tobacco cultivars. It appears that CP expression is not required for the protection observed. These observations are consistent with previously published results (31,49).

There was a positive correlation between virus resistance in R_0 plants and the recovery of resistant progeny. Progeny derived from wild-type R_0 plants were uniformly susceptible to PVY, while progeny of resistant R_0 plants segregated as wild-type and resistant when tested by PVY inoculation. These results indicate that PVY-resistant tobacco can be obtained by transforming tobacco with PVY-Chilean CP and screening the primary transgenics with PVY.

TN90-20C and -38C carried one and two NPT II loci, respectively. However, inoculation of PVY on seedlings that had been selected on kanamycin showed a further 1:1 segregation ratio between plants that were Kan^r and PVY susceptible and those that were Kan^r and PVY resistant (Table 2). These observations suggest that not all T-DNAs with functional NPT II inserts carry active CP genes. Despite the fact that some resistant R_0 plants exhibited complex insertion patterns by Southern blot analysis (Fig. 1), it is unclear whether the performance of the R_0 plants was caused by complex insertion per se, since some R_0 plants with complex integration patterns exhibited virus susceptibility identical to that of control plants. Moreover, some resistant R_0 plants had simple insertion patterns.

Obtaining stable tobacco genotypes with the complex insertion patterns observed through self-pollination may be difficult, since several generations of self-pollination and testing against PVY would be required to obtain pure lines that are resistant to PVY. To expedite this process, arrays of maternally derived haploid sporophytes (4) were generated from several of the resistant R_0 plants. A majority of haploid sporophytes derived from KY14-5C and -12C were resistant to PVY-Chilean in inoculation tests (data not shown). Spontaneous chromosome doubling of these haploid lines should produce genotypically stable dihaploid transgenic lines. Progeny of these dihaploids could be screened by inoculation with various isolates of PVY as a test for homozygosity of transgenic loci in the dihaploid lines. Subsequently, the homozygous dihaploid transgenic lines could be used for field evaluation of the resistance trait and released as improved commercial tobacco cultivars.

Transgenic plants derived from commercial tobacco cultivars carrying PVY-Chilean CP were resistant to PVY VAM B. This resistance was observed in transgenic tobacco, regardless of whether the native va gene was present in a homozygous condition.

M^sN^r is a strain of PVY indigenous to the southeastern United States (14), although its occurrence has been reported in other countries (17,29,50). The va resistance mechanism is not effective against PVY M^sN^r in flue-cured or burley tobacco carrying the RKN resistance gene (5). Therefore, PVY M^sN^r poses a significant threat to flue-cured tobacco production, since the majority of lines of this type of tobacco carry the RKN resistance gene. Transgenic K326 line 4C has been shown to be resistant against PVY M^sN^r, demonstrating the feasibility of combining RKN resistance and resistance against PVY M^sN^r into a single genotype.

After inoculation with PVY M^sN^r, the nontransformed K326 control developed local lesions on inoculated leaves followed by death of the inoculated seedlings by 14 dpi, while transgenic K326-4C were free of PVY M^sN^r symptom expression. Since the local lesions occur because of the interaction between RKN resistance and PVY M^sN^r, the observed phenomena may be explained by either inactivation of the RKN resistance gene by insertional mutagenesis or by the absence of replicating virus within transgenic plants.

Several hypotheses regarding the mechanisms of capsid protein gene-mediated resistance among potyviruses have been proposed (10,32,33,47). Unlike TMV capsid protein-mediated resistance, accumulation of CP in transgenic plants was unnecessary to obtain protection from potyvirus infection (31–33,47). It has been recently suggested (2) that the non-capsid protein-mediated protection so often observed with the potyviruses is the result of a cosuppression-like phenomenon. Similarly, all transgenic lines tested in this work expressed low or undetectable PVY-Chilean CP as measured by indirect-ELISA and the highly sensitive ECL Western blot (Fig. 2), and some of these transgenic plants were resistant to various isolates of PVY. It is possible that PVY resistance performance observed among transgenic lines in this work was the result of insertional mutagenesis. This is supported by the presence of some Kan^r progeny of the symptomless R_0 plants that were not PVY resistant and by the absence of correlation between insert number, CP expression, and the PVY resistance performance. However, the relatively frequent occurrence of the PVY-resistant phenotypes among transgenic lines argues against this scheme. On the other hand, PVY-resistant tobacco (the va resistance mechanism) has been obtained by physical mutagenesis (26).

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