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Variations in the VPg Protein Allow a Potyvirus to Overcome *va* Gene Resistance in Tobacco

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The *va* gene is used in commercial Burley tobacco cultivars including cv TN 86 to confer resistance to tobacco vein mottling virus (TVMV) and, to some extent, other potyviruses. A naturally occurring strain of TVMV (TVMV-S), which overcomes this resistance, was isolated from TN 86 plants. To investigate the mechanism by which TVMV-S overcomes *va* gene resistance, a cDNA clone encompassing the complete genome of TVMV-S was produced. Using chimeric transcripts combining regions of TVMV-S and regions of the wild-type strain (TVMV-WT) to which TN 86 is resistant, it was demonstrated that a domain within the VPg protein is responsible for overcoming *va* resistance in TN 86. DNA sequence analysis revealed six amino acid differences between the two strains of TVMV within this domain. Inclusion of sequences for four of the TVMV-S VPg amino acids was sufficient to confer the resistance-overcoming phenotype to all corresponding transcripts. Coinoculation experiments suggested that the resistance of TN 86 to TVMV-WT was not due to the induction of a general host defense response. The results are compatible with the hypothesis that VPg must assume an appropriate configuration in order to interact with appropriate host components and facilitate systemic virus movement.

INTRODUCTION

A single recessive gene designated *va* (Gupton and Burk, 1973) has been utilized to provide resistance or tolerance in commercial tobacco cultivars (*Nicotiana tabacum* L.) to the potyviruses tobacco vein mottling virus (TVMV), potato virus Y (PVY), and tobacco etch virus (TEV). Other than the N gene, which confers resistance to tobacco mosaic tobamovirus (TMV), the *va* gene is the most widely utilized gene providing resistance to viruses that infect tobacco. The irradiation-induced *va* gene was derived from the "Virgin A Mutante" (VAM) (Koelle, 1961), and seed of this resistance source was formalized as breeding line TI 1406. Genes allelic to this resistance source appear in a number of burley and cigar tobacco cultivars. Among them, *N. tabacum* cv TN 86, carrying a homozygous *va* allele, is highly resistant to TVMV and moderately resistant to TEV (Miller, 1987; Reddick *et al.,* 1991). However, one TVMV strain, designated TVMV-S (Gibb *et al.,* 1989), overcomes this resistance and produces symptoms and virus titers identical to those produced on a susceptible tobacco cultivar.

The molecular mechanism by which the *va* gene confers resistance to TVMV and to other potyviruses is unknown, but preliminary studies showed that the type strain, TVMV-WT, which does not "break" TN 86 resistance, replicates in TN 86 protoplasts but is restricted to a few isolated cells or groups of cells in TN 86 leaves after mechanical inoculation (Gibb *et al.,* 1989). Based on these data, it seems likely that the resistance of TN 86 is due primarily to restriction of virus cell-to-cell movement.

The current study was initiated to investigate two related questions: first, what molecular determinants of the TVMV-S genome are responsible for resistance-breaking in TN 86; and second, since TN 86 resistance appeared to be at the level of cell-to-cell movement, might elucidation of the determinants implicate them in normal aspects of potyvirus cell-to-cell movement? To date, evidence exists that at least two potyviral polypeptides — capsid protein (CP) and helper component protein (HC-Pro) — participate in potyvirus movement (Carrington *et al.,* 1996). In this report, we present evidence that the TVMV-S genome-linked viral protein (VPg), a protein covalently linked to the 5′-terminus of viral RNA, contains the determinant that confers the resistance-breaking phenotype to TN 86. Furthermore, this role implicates VPg as a factor in the more general phenomenon of potyvirus cell-to-cell movement.

MATERIALS AND METHODS

Construction and mutagenesis of chimeric TVMV cDNA clones

Plasmids containing a full-length cDNA copy of the TVMV-S or TVMV-WT genomes adjacent to a T7 RNA

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polymerase promoter were named pTVS-500.9 and pTV-WT, respectively. The construction and analysis of these plasmids have been described earlier (Nicolas *et al.,* 1996). Transcription plasmids derived from pTVS-500.9 and pTV-WT were given the prefixes pTVS and pTVWT, respectively. A number of chimeric full-length constructs were obtained by exchanging cDNA fragments between pTVS-500.9 and pTV-WT using common restriction sites (*Eco*RV, 5427; *Nde*I, 5813; *Hin*cII, 6322; *Csp* 45I, 7150; *Nco*I, directly following the poly(A) tail). Standard procedures were followed in all cloning steps (Sambrook *et al.,* 1989).

For mutagenesis of the TVMV-S transcription plasmid, a 2053-bp *Xba*I–*Hin*dIII fragment generated from pTVS-500.9 was cloned into M13 mp18 and single-stranded (ss) DNA was produced. For mutagenesis of the TVMV-WT transcription plasmid, a 473-bp *Xba*I–*Hin*cII fragment generated from pTV-WT was cloned into pBluescript II KS^+ (Stratagene), then subcloned as an *Xba*I–*Kpn*I fragment into M13 mp19, and ssDNA was produced. Occasionally, previously mutagenized ssDNA was used as template for a second round of mutagenesis. Mutations were generated by oligonucleotide-directed mutagenesis (Vandeyar *et al.,* 1988) using oligonucleotides listed in Table 1A. All mutations were confirmed by dideoxynucleotide sequence analysis using Sequenase (United States Biochemical Corp. Cleveland, OH). Double-stranded (ds) DNA was then produced from the mutagenized ssDNA and used to replace the analogous DNA fragment in the original clone. All mutations in the completed transcription plasmids were confirmed by sequencing. During the screening of chimeric recombinants, care was taken to select clones containing a poly(A) tail of approximately 110–130 residues in length as in pTVS-500.9 and pTV-WT (Nicolas *et al.,* 1996).

In vitro transcription of RNA and inoculation of plants

Prior to transcription, full-length cDNA clones were linearized by *Nco*I digestion followed by phenol/chloroform extraction and ethanol precipitation. Conditions for transcription reactions and plant inoculations were as described earlier (Nicolas *et al.,* 1996). For the chimeric virus studies, three independent experiments were conducted, each utilizing two KY 14 and eight TN 86 plants. For studies assessing the effect of specific mutations, various numbers of plants were inoculated in two to three independent experiments.

Detection of virus and analysis of progeny RNA

All KY 14 plants became infected when inoculated with any of the transcripts in this report. The first symptoms usually appeared between 6 and 7 days postinoculation (dpi). The number of TN 86 plants infected varied from clone to clone. Symptoms appeared within 7 dpi for pTVS-500.9 up to 48 dpi, depending on the source of the transcripts used for inoculation. All plants were tested for infection 11 dpi and/or 22 dpi or later when symptoms were delayed by more than 22 days. Two leaf disks (10 mg $\pm$ 1 mg) were taken from the youngest noninoculated leaf of each plant and TVMV CP was detected by indirect ELISA using an anti-capsid protein antiserum. Total RNA was extracted using the TRIzol protocol (Gibco BRL) from 10- to 15-mg samples of tissue from the youngest noninoculated leaf. DNA was generated by reverse transcription of viral RNA sequences in the total RNA preparations and amplified by PCR. First strand cDNA primed with oligo(dT) was synthesized using Superscript II reverse transcriptase and 1–5 μg of total RNA as recommended by the manufacturer (Gibco BRL). Specific primers (ON_{Xba} and ON_{78}; Table 1B) were used to amplify regions of the progeny RNA by PCR using one-tenth of the cDNA reaction mixture. PCR products (443 bp) were purified using the Wizard PCR Preps DNA purification system (Promega) and sequenced using Sequenase and either ON_{Xba} or ON_{78} as sequencing primer.

Coinoculation experiments

After *in vitro* transcription of cDNA clones pTV-WT and pTVS-500.9, an equal amount of each transcript was mixed with one volume of 3$\times$ inoculation buffer and used for inoculation of both KY 14 and TN 86 plants. At 8 and 22 dpi, plants were analyzed by RT-PCR for the presence of viral RNA using two sets of specific PCR primers (Table 1B and Fig. 2) for each of the two strains: ON_{78}/ON_{31} and ON_{80}/ON_{35} to detect specifically TVMV-WT, and ON_{78}/ON_{29} and ON_{80}/ON_{33} to detect specifically TVMV-S. Using ON_{78}/ON_{31} and ON_{80}/ON_{35} or ON_{78}/ON_{29} and ON_{80}/ON_{33} combinations of amplification primers, PCR products for TVMV-WT were expected to be 745 bp and 550 bp, and for TVMV-S, 685 bp, and 634 bp. The four different PCR analyses performed for each plant used one-tenth volume of the same cDNA reaction as template. PCR products were analyzed by agarose gel electrophoresis.

RESULTS

Localization of the resistance-breaking determinant of TVMV-S

In a previous investigation (Nicolas *et al.,* 1996), we reported the performance of transcripts from a cloned TVMV-S cDNA genome variant (designated pTVS-500.4) which contained a single amino acid difference from authentic TVMV-S. This variant, which encoded a Gly rather than Cys residue within the VPg domain (aa position 1928 of the TVMV-S polyprotein), displayed reduced infectivity on inoculated plants and required up to 35 days to initiate symptoms on TN 86 rather than the typical 7 days required for either TVMV-S RNA or transcripts which encoded the Cys residue. In the present study, TN 86 plants which ultimately developed symptoms following

TABLE 1

Transcription Vectors and the Respective Oligonucleotides Used for Site-Directed Mutagenesis of TVMV VPg Cistron (A) and Oligonucleotide Primers Used for the Detection and Identification of Progeny RNA Using PCR (B)

A

Transcription vector	Amino acid sequence[a]	Mutagenic oligonucleotide (5′ to 3′)[b]
pTVS-500.9	ICS--KS . . . S	—
pTVS-IIe/Met	**M**CS--KS . . . S	GTTGCGAGCAcATTGCGTCAGC
pTVS-Cys/Ser	**I**S**S**--KS . . . S	GCTCTTTTGTTGCGArkATATTGCGTCAGC
pTVS-Cys/Tyr	I**Y**S--KS . . . S	GCTCTTTTGTTGCGArkATATTGCGTCAGC
pTVS-Cys/Asp	I**D**S--KS . . . S	GCTCTTTTGTTGCGAGdbTATTGCGTCAGC
pTVS-Cys/Gly	I**G**S--KS . . . S	GCTCTTTTGTTGCGAGdbTATTGCGTCAGC
pTVS-Ser/Pro	I**C**P--KS . . . S	CCTTGCTCTTTGTTGCGbGCATATTGCGTC
pTVS-Lys/Arg	ICS--RS . . . S	GGATTCCCTTGCTCcTTTGTTGTGAGCA
pTVS-Ser/Asn	ICS--**K**N . . . S	GGATTCCCTTGtTCTTTTGTTGCG
pTVS-Ser/Ala	ICS--KS . . . A	TGACTCAAATgCGACCTCCTG
pTV-WT	MSP--RN	—
pTVWT-C	MCP--RN	CTTGTTCCTTTGTTGTGrgcAyATTGcATCAGCAAGTATAG
pTVWT-CKS	MCP--KS[c]	GCCTGGATTCCCTTGcTCtTTTGTTGTGrGCAyATTGC
pTVWT-CSKS	MCS--KS[d]	GYTCYTTTGTTGTGaGCAYATTGCATCAGC
pTVWT-CSS	MCS--RS[e]	GGATTCCCTTGCTCcTTTGTTGTGAGCA
pTVWT-CSK	MCS--KN[e]	CCTGGATTCCCTTGtTCTTTTGTTGTGAGC
pTVWT-SKS	MSS--KS[e]	GCTCTTTTGTTGTGAGgACATTGCATCAGC

B

Primer name	Strain specificity	Position at which primer anneals[b]	Oligonucleotides (5′ to 3′)[d]
ON_{Xba}	Both strains	5822–5844	TATCTAGACCCAGTCACAGGTGC
ON_{29}	TVMV-S	5579–5602	GAGAAAATGCATGAGCCGGTTCGG
ON_{31}	TVMV-WT	5518–5541	CTCATTGTTGTAAGTGGCGTCGGA
ON_{33}	TVMV-S	8264–8287	GAACCCTATAGAAGTCTCGCGGAT
ON_{35}	TVMV-WT	8348–8373	CCACTGACAATGAACTCACAGATTAC
ON_{78}	Both strains	6239–6264	CCAATGCCAAACAryCTCTCACTATG′
ON_{80}	Both strains	8875–8898	ATCTGACGTAATGAGGGATTCGCA

[a] Amino acids in bold differ from TVMV-S sequence and from TVMV-WT sequence in the pTVS and pTVWT series, respectively. Underlined amino acids are those mutagenized using the oligonucleotide primer described in the corresponding row.

[b] Lowercase letter indicates the mismatched nucleotide(s). b = T + C + G; d = A + T + G; k = T + G; r = A + G; y = C + T.

[c] DNA used for mutagenesis derived from pTVWT-C.

[d] DNA used for mutagenesis derived from pTVWT-CKS.

[e] DNA used for mutagenesis derived from pTVWT-CSKS.

inoculation with the variant transcripts were analyzed by direct sequencing of RT-PCR products which spanned the relevant region within the VPg cistron. The analysis revealed that among the systemically infected plants (total of 16 plants), Gly[1928] had reverted to Cys (11 plants) or Asp (5 plants). No such reversion was observed when infected KY 14 plants were analyzed using the same method and at the same postinoculation times (5 plants analyzed). The results suggested that VPg might be involved in the resistance-breaking phenomenon and that the observed genomic change was essential for progeny TVMV to systemically infect TN 86.

As a consequence of the above experiments, a series of chimeric viral cDNA genomes was constructed using shared restriction sites between transcription vectors that represented full-length clones of TVMV-S and TVMV-WT in order to locate the resistance-breaking determinant(s). Transcripts were then produced and used for inoculation of both KY 14 and TN 86. Progeny RNA samples isolated from both KY 14- and TN 86-infected plants were analyzed by RT-PCR followed by direct sequencing of the PCR products spanning "junction regions" to confirm that infection was due to the chimeric transcripts. Transcripts from two constructs (pTVS-Eco/Nco and pTVS-5′/Csp) (Fig. 1) induced symptoms on both KY 14 and TN 86 in less than 7 days, confirming their ability to break the resistance conferred by the *va* gene. All infected plants exhibited symptom type and time of appear-

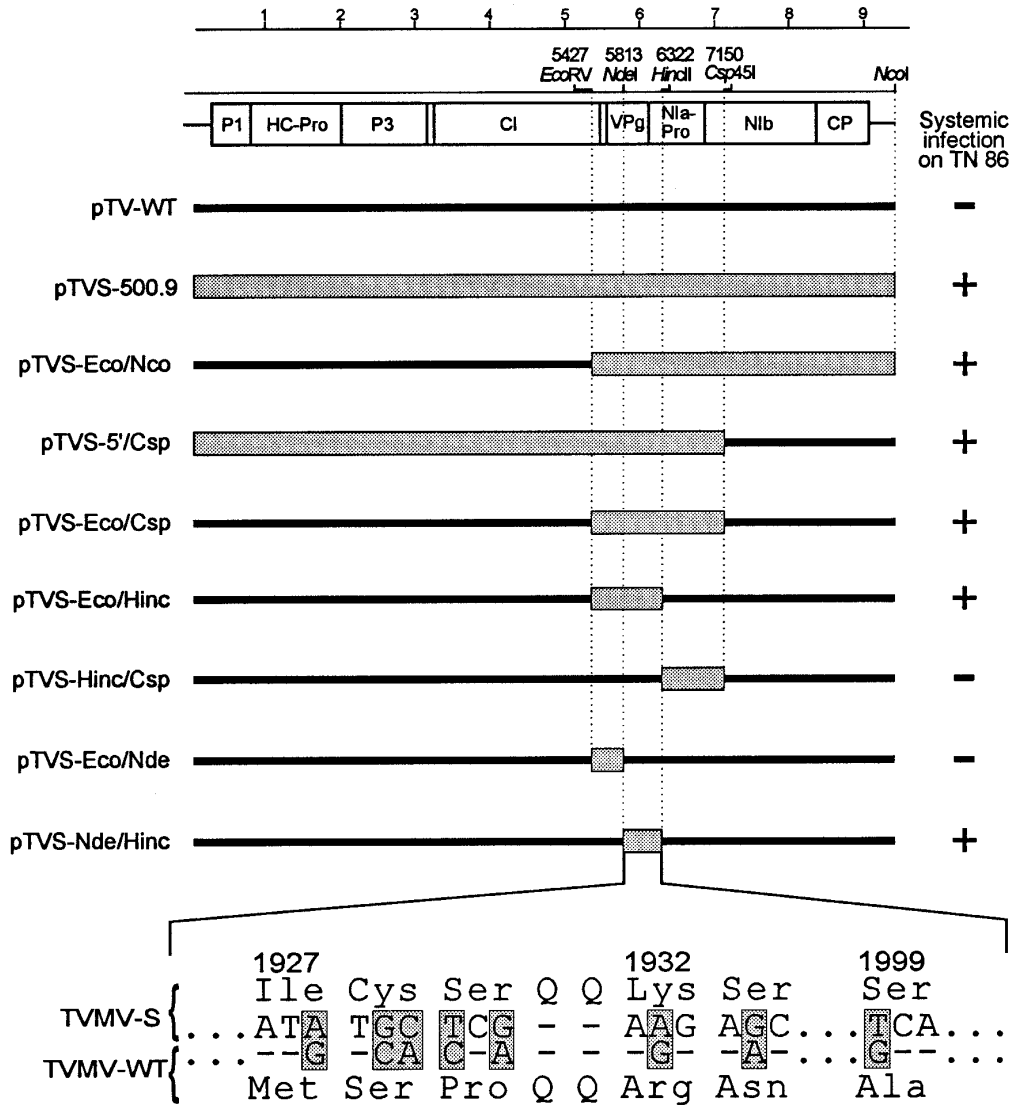


FIG. 1. Structure of *in vitro* transcripts and their performance following inoculation on tobacco cultivar TN 86. From top to bottom: Scale bar of nucleotide positions $\times 10^{-3}$; Location and nucleotide numbers of restriction sites utilized in the construction of chimeric vectors; Cistron map of TVMV cistrons designated by abbreviations for gene product names; Diagrammatic representation of chimeric transcripts; solid black bars represent TVMV-WT sequences; shaded boxes represent TVMV-S sequences. The ability of each type of transcript (named on left) to produce systemic symptoms on TN 86 at 7 dpi is indicated by a + or − indicated on the right. Below is a comparison of TVMV-WT and TVMV-S sequences located between *Nde*I (nt 5813) and *Hinc*II (nt 6322) sites. Only changes resulting in amino acid differences are shown. Differences in nucleotides are boxed and amino acid positions in the TVMV polyprotein are numbered.

ance on both cultivars which were indistinguishable from those caused by TVMV-S RNA or transcripts produced from pTVS-500.9 (Nicolas *et al.,* 1996). Infection was confirmed by ELISA analysis 11 dpi.

To further delineate the genomic location of the resistance-breaking determinant(s), additional constructs were produced which contained TVMV-S-derived sequences in the 1724-nt region common to both clones pTVS-Eco/Nco and pTVS-5′/Csp. Transcripts of clone pTVS-Eco/Csp (Fig. 1), in which the 6K2, VPg, and NIa-Pro cistrons as well as part of the NIb cistron were derived from TVMV-S, produced symptoms on TN 86 in less than 7 dpi, confirming the ability of this region alone to confer the resistance-breaking phenotype. Four other constructs were produced to narrow the location of the resistance-breaking determinant between *Eco*RV (nt 5427) and *Csp*45I (nt 7150) (Fig. 1). Transcripts derived from pTVS-Eco/Hinc as well as pTVS-Nde/Hinc, which contained a 510-nt segment between *Nde*I (nt 5813) and *Hinc*II (nt 6322) derived from TVMV-S, retained the resistance-breaking phenotype, whereas two other chimeras (pTVS-Eco/Nde and pTVS-Hinc/Csp), in which this region was derived from TVMV-WT, did not. All chimeric transcripts were equally infectious on KY 14, confirming that replication and systemic movement of the chimeras were not altered, at least in this susceptible cultivar.

TABLE 2

Effect of Amino Acid Substitutions in VPg on the Reaction of TN 86 and the Nature of Progeny RNA Recovered from TN 86

Transcription vector	Position and nature of amino acids	Infected/ inoculated[a]	Time of symptom appearance	Reversion observed (No. of plants analyzed)
A				
	1927 1932 1999			
pTVS-500.9	I C S -- K S . . . S	10/10	6–7 dpi	No (10)
pTVS-IIe/Met	[M] C S -- K S . . . S	8/8	6–7 dpi	No (8)
pTVS-Cys/Ser	I [S] S -- K S . . . S	11/16	30–48 dpi	Yes, S reverts to C (9), to Y (1), or F (1)
pTVS-Ser/Pro	I C [P] -- K S . . . S	13/16	9–18 dpi	Yes, P reverts to S (13)
pTVS-Lys/Arg	I C S -- [R] S . . . S	12/12	11–23 dpi	Yes, R reverts to K (12)
pTVS-Ser/Asn	I C S -- K [N] . . . S	12/12	11–22 dpi	Yes, N reverts to S (10), to C (1) or Y (1)
pTVS-Ser/Ala	I C S -- K S . . . [A]	8/8	6–7 dpi	No (8)
pTVS-Cys/Gly	I [G] S -- K S . . . S	2/8	20–30 dpi	Yes, G reverts to C (1), or to D (1)
pTVS-Cys/Asp	I [D] S -- K S . . . S	8/8	6–7 dpi	No (8)
pTVS-Cys/Tyr	I [Y] S -- K S . . . S	6/8	11–20 dpi	Yes, Y reverts to C (1) and no reversion for (5)
B				
pTV-WT	M S P -- R N . . . A	NA	NA	NA
pTVWT-CSKS	M [C S] -- [K S] . . . A	12/12	6–7 dpi	No (12)
pTVWT-SKS	M S [S] -- [K S] . . . A	0/16	NA	NA
pTVWT-CKS	M [C] P -- [K S] . . . A	2/20	25–35 dpi	Yes, P reverts to S (2)
pTVWT-CSS	M [C S] -- R [S] . . . A	1/20	10–12 dpi	Yes, R reverts to K (1)
pTVWT-CSK	M [C S] -- [K] N . . . A	1/12	30–35 dpi	Yes, N reverts to S (1)

Note. (A) Effect of TVMV-WT amino acid substitutions into TVMV-S genomic background. (B) Effect of TVMV-S amino acid substitutions into TVMV-WT genomic background. Only amino acids that differ between TVMV-WT and TVMV-S in the VPg cistron between positions 1927 and 1999 are shown. The numbers above the amino acids (in single-letter code) indicate their location in the TVMV polyprotein. Mutagenized amino acids are boxed. Capsid protein from the youngest systemically infected leaf was detected by ELISA at 11 and 22 dpi. Progeny RNA was analyzed by sequencing RT-PCR products which utilized total RNA extracted from the youngest leaf. No, no systemic infection of TN 86; inoculated plants were observed and tested for up to 60 days. NA, not applicable, since no symptoms appeared nor was virus detected using ELISA and RT-PCR.

[a] Transcripts produced from each of the clones in this table all produced systemic symptoms on KY 14 at 6–7 dpi. Progeny RNA from two to four KY 14 plants was analyzed at the same time as that from TN 86. No reversion was ever observed.

Precise domains representing VPg and NIa-Pro have not been experimentally determined for TVMV, but homology with TEV (Dougherty and Parks, 1991; Carrington *et al.,* 1993) and TuMV (Laliberté *et al.,* 1992) indicates that an NIa-Pro cleavage site separating these domains is located between amino acids Glu^{2001} and Ser^{2002} of the TVMV polyprotein. Comparison of TVMV-WT and TVMV-S sequences between positions nt 5813 and nt 6322, which contain approximately two-thirds of the VPg coding region as well as sequences at the 5′ end of NIa-Pro, showed 54-nt differences. Among these, 46 were silent while eight resulted in six amino acid changes within the VPg domain (Fig. 1). Interestingly, one of the six amino acids that differed between the two (Cys^{1928}) is the same residue whose change to a Gly (as in pTVS-500.4; Nicolas *et al.,* 1996) abolished the resistance-breaking phenotype of the corresponding transcripts. Based upon these findings we speculated that the resistance-breaking determinant(s) was in the VPg region, and that it could involve either a single or a combination of amino acid residues.

Analysis of TVMV-WT VPg amino acid sequence substitutions in TVMV-S genomic background

As noted above, six amino acid residues in the VPg region (between positions 1927 and 1999) differed between TVMV and TVMV-S. In this experiment, a set of constructs was made such that the effects of each of the six TVMV-WT residues could be tested, one by one, in the TVMV-S background (Table 2). Both TN 86 and KY 14 were inoculated with each of the resulting transcripts. The infectivity of the transcripts on each cultivar was observed, and the stability of the mutants was carefully assessed by analysis of all progeny RNA using direct sequencing of RT-PCR products that spanned the region between nt 5822 to nt 6264.

Two mutants (pTVS-IIe/Met and pTVS-Ser/Ala) per-

formed exactly as TVMV-S since all inoculated plants developed symptoms in less than 7 days (Table 2A). Analysis of progeny RNA from infected plants showed that these mutants were stable in both KY 14 and TN 86. Three mutants (pTVS-Ser/Pro, pTVS-Lys/Arg, and pTVS-Ser/Asn) produced delayed symptoms on TN 86. Symptoms appeared from 2 to 16 days after those produced by TVMV-S, and RNA analysis revealed that reversions had occurred in each case. Specifically, analysis of progeny RNA of pTVS-Ser/Pro showed that Pro^{1929} had reverted to a Ser as is present in TVMV-S (13 plants analyzed). Analysis of mutant pTVS-Lys/Arg showed that Arg^{1932} had reverted to Lys, as in TVMV-S (12 plants analyzed). Analysis of mutant pTVS-Ser/Asn showed that Asn^{1933} had reverted to Ser (10 plants out of 12) as in TVMV-S, but also to Cys (1 plant) and Tyr (1 plant). Interestingly, subsequent sap inoculation of TN 86, using material from the infected TN 86 plants in which a reversion was observed, resulted in the normal pattern of infection with no delay in symptom appearance. The most striking effect was with mutant pTVS-Cys/Ser in which Cys^{1928} was changed to Ser. This produced symptoms on TN 86 after a long delay (30–48 dpi) compared to plants inoculated with TVMV-S RNA. Progeny RNA analysis of infected plants (at 48 dpi) showed that Ser^{1928} had reverted to Cys as in authentic TVMV-S (9 of 11 plants), but also to Tyr (1 plant) and Phe (1 plant). Analyses of KY 14 plants at the same time as TN 86 plants (between 23 and 53 dpi) showed that no changes had occurred in the region under investigation (5 plants).

To further investigate the importance of the nature of the amino acid at position 1928, three additional clones were obtained in which Cys^{1928} was replaced with Gly (which was previously observed to alter resistance-breaking (Nicolas *et al.,* 1996)), and Asp or Tyr, two residues that were found after reversion when using transcripts produced from pTVS-Cys/Ser and pTVS-Cys/Gly. Using transcripts derived from pTVS-Cys/Gly, symptoms developed on only two of eight transcript-inoculated TN 86 plants and appearance was delayed (20 to 30 dpi). Analysis showed that Gly had reverted to Cys (1 plant) or to Asp (1 plant). The two KY 14 control plants developed symptoms within 7 days, and there was no change at this position. Transcripts derived from pTVS-Cys/Asp mimicked the performance of TVMV-S on TN 86, with symptoms appearing within 7 dpi, and no reversion was observed in progeny RNA extracted from TN 86-infected plants (8 infected plants/ 8 inoculated). Transcripts derived from pTVS-Cys/Tyr displayed an altered phenotype in which symptoms appeared from 11 to 20 dpi on 6 of the 8 plants inoculated. Reversion (Tyr to Cys) was found to have occurred in only one of these plants.

Analysis of TVMV-S VPg amino acid sequence substitutions in TVMV-WT genomic background

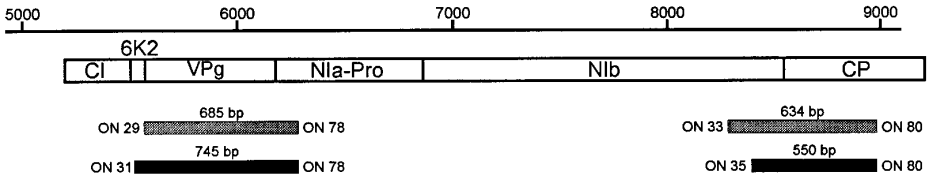
The previous results suggested that four amino acids in the TVMV-S polyprotein (positions 1928, 1929, 1932, and 1933-CSKS) were critical to the resistance-breaking phenomenon. The next step was to determine whether the inclusion of these four, or fewer, amino acids in the TVMV-WT background would be sufficient to provide the TVMV-S phenotype on TN 86. Therefore, a second series of constructs was produced in which three or four amino acids were replaced in pTV-WT with those found at the corresponding positions in TVMV-S (Table 2B). Transcripts produced from clone pTVWT-CSKS mimicked the performance of TVMV-S on TN 86 — 100% of inoculated plants developed symptoms in less than 7 days and no reversions were detected by progeny RNA analysis. This result confirmed that amino acids at position 1927 and 1999 were not crucial to the resistance-breaking phenotype.

One mutant (pTVWT-SKS), in which Ser^{1928} was not changed to the TVMV-S sequence (Cys), failed to infect TN 86 plants, while the three others infected TN 86, but at a very low rate, and following a long delay for symptom appearance. The analysis of the few symptomatic plants showed that in all cases the amino acid (of the four concerned in this study) that had not been changed to the TVMV-S sequence had reverted back to the TVMV-S residue at the same position: Pro reverted back to Ser with transcripts produced from pTVWT-CKS (2 plants); Arg reverted back to Lys with transcripts produced from pTVWT-CSS (1 plant); and Asn reverted back to Ser with transcripts produced from pTVWT-CSK (1 plant).

Coinoculation with TVMV-S and TVMV-WT infectious transcripts

A series of coinoculation experiments with transcripts of both TVMV-S and TVMV-WT was performed to gain insight as to how VPg from TVMV-S might provide resistance-breaking in TN 86 (and conversely, why TVMV-WT fails to move systemically in TN 86). We hypothesized that if the VPg of TVMV-WT induced an incompatible, movement-limiting response in TN 86, the movement of TVMV-S might also be impeded in doubly inoculated plants. Conversely, infection of TN 86 with TVMV-S might alter, for example, the size exclusion limits of plasmodesmata, allowing TVMV-WT to move out of cells which might also be infected with TVMV-S.

Using two sets of specific primers for each strain, one targeting the VPg coding region (ON_{78} and ON_{29} or ON_{31}) and one including the NIb/CP cleavage site (ON_{80} and ON_{33} or ON_{35}), we were able to detect and differentiate progeny RNAs of TVMV-S, TVMV-WT, or both in extracts from systemically infected leaves (Fig. 2). Two different sets of primers were used to reduce the possibility of not detecting TVMV-WT in the event that a recombination event had occurred between the two RNA species. Following coinoculation of KY14 (10 plants), RNA of both viruses was detected in systemically infected leaves. In contrast, only TVMV-S RNA was detected in TN 86 plants



	ON 29 / ON 78 (TVMV-S)	ON 31 / ON 78 (TVMV-WT)	ON 33 / ON 80 (TVMV-S)	ON 35 / ON 80 (TVMV-WT)
WT on TN 86 (10 plants)	-	-	-	-
WT + S on KY 14 (10 plants)	+	+	+	+
WT + S on TN 86 (30 plants)	+	-	+	-

FIG. 2. Analysis of TVMV-WT and TVMV-S systemic movement following inoculation of tobacco plants. From top to bottom: Scale bar of nucleotide positions; Cistron map of relevant region of TVMV genome targeted by PCR primers; location, size, and primers for predicted PCR products; shaded boxes are PCR products specific for TVMV-S; solid boxes are PCR products specific for TVMV-WT; tabular results of PCR analysis. −, Not detected using 35 cycles of PCR and a second round of reamplification of first PCR products; +, PCR products of predicted sizes were detected using agarose gel electrophoresis visualization.

coinoculated with equal amounts of each RNA species (30 plants analyzed).

DISCUSSION

Site-directed mutagenesis and inoculation of plants with chimeric RNAs demonstrated that a cassette of four amino acids located within a six amino acid domain of the TVMV-S VPg is responsible for the resistance-breaking phenotype of the recombinant viruses. Reciprocal experiments which included this cassette in TVMV-WT and TVMV-S genomic backgrounds indicated that these four amino acids (Cys^{1928}, Ser^{1929}, Lys^{1932}, Ser^{1933}) were necessary and sufficient for overcoming *va* resistance in tobacco. These data provide the first evidence for a virus resistance-breaking determinant residing within a viral VPg cistron.

These four amino acids, which have been shown to provide the difference between the two strains of TVMV in their response on TN 86, could affect cell-to-cell movement via two generalized types of mechanisms, both of which might result from conformational differences in the VPgs. The first involves differential reactions of TN 86 to the VPgs of the two strains. The coinoculation experiments argue against the elicitation of a general movement-limiting reaction in TN 86 by the TVMV-WT VPg, since movement of TVMV-S was not impeded. The evidence against the alternative, that the TVMV-S VPg affects, for example, plasmodesmatal size exclusion limits is less definitive, since coinfection of the same cells might be required for TVMV-WT to move; we have no direct evidence that such coinfection occurred. The second mechanism involves the direct involvement of the VPg in a "movement complex." TVMV-S VPg may assume a conformation which interacts, either alone or in combination with other viral proteins, with an unidentified TN 86 host factor which then facilitates movement from the initially inoculated cell. TVMV-WT VPg would not possess the required conformation and so be unable to engage in the same interaction, thus preventing its movement. Alternatively, the conformation of the VPg from TVMV-WT allows it to interact "improperly" with some TN 86 host component which then results in a restriction of movement. Such an interaction would most likely not restrict VPg's proposed role in replication, since replication of either virus strain remains unimpaired in TN 86 protoplasts (Gibb *et al.,* 1989).

It is reasonable to assume that during cell-to-cell movement of the virus (or viral RNA) VPg remains attached to the RNA. Therefore, it is likely that the VPg itself and not its precursor (6K-NIa or NIa) mediates the resistance-breaking property of RNA on which it is attached. Furthermore, considering the size of TVMV VPg (deduced to be approximately 22–24 kDa) it would seem likely that VPg is exposed, even in virions where it could not be accommodated by the regular helical structure of the repeating CP subunits. The participation of this exposed feature in functions other than replication thus make intuitive sense. It is similarly reasonable to propose that the role of the VPg of TVMV and other potyviruses is not limited specifically to resistance-breaking but that it is essential for the general phenomenon of cell-to-cell movement.

The nature of the presumptive conformational change(s) that result in the differential response of TN 86 remains to be clarified. It is unlikely, for example, that Cys^{1928} (as demonstrated using clone pTVS-Cys/Asp, Table 2B) is involved in disulfide bond formation, since the substitution of this residue with Asp did not alter the resistance-breaking phenotype. It is also noteworthy that although Ser, Cys, and Asn are all polar, uncharged, amino acids, they are not interchangeable at position 1928 or 1933 for providing systemic infectivity on TN 86. Similarly, Lys and Arg, two positively charged amino acids, are not interchangeable at position 1932.

Gibb *et al.* (1989) showed that TVMV-WT is able to replicate in TN 86 protoplasts and that individual epidermal cells or small foci of epidermal cells become infected upon mechanical inoculation. In our study, certain substitution mutants were able to systemically infect TN 86 after a considerable delay, and analysis of the viral RNA in the systemically infected leaves showed that a reversion to the TVMV-S genotype had occurred (Tables 2A and 2B). Evidently, sufficient rounds of replication of these mutants had occurred to allow the reversions to take place and to be selected. It is unclear why TVMV-S-derived transcripts, in which one amino acid had been mutagenized to the TVMV-WT residue, reverted more readily to provide the resistance-breaking phenotype than TVMV-WT-derived transcripts in which three amino acids had been changed to TVMV-S residues. In each case, only one mutation (one nucleotide change) is necessary for the progeny RNA to recover the resistance-breaking genotype.

Although our experiments have shown that the VPg from TVMV-S is involved in overcoming *va* gene resistance, further experiments are needed to elucidate the mechanism of this interaction. One important approach to investigating the interaction between VPg and putative host factors is the generation of transgenic plants which express VPg derived from TVMV-S. Inoculation of these plants with TVMV-WT may help to determine whether TVMV-S VPg can complement movement of a "defective" (TVMV-WT) strain. This approach would address the possibility that in coinoculation experiments, the two virus strains did not occupy the same cell. A lack of complementation of TVMV-WT in such plants would indicate that VPg needs to be attached to the RNA for viral movement to occur and that linkage of VPg to viral RNA is coupled with replication.

REFERENCES

Carrington, J. C., Haldeman R., Dolja, V. V., and Restrepo-Hartwig, N. A. (1993). Internal cleavage and trans-proteolytic activities of the VPg-proteinase NIa of tobacco etch potyvirus in vivo. *J. Virol.* 67, 6995–7000.

Carrington, J. C., Kasschau, K. D., Mahajan, S. K., and Schaad, M. C. (1996). Cell-to-cell and long-distance transport of viruses in plants. *Plant Cell* 8, 1669–1681.

Dougherty, W. G., and Parks, T. D. (1991). Post-translational processing of the tobacco etch virus 49-kDa small nuclear inclusion polyprotein: Identification of an internal cleavage site and delimitation of VPg and proteinase domains. *Virology* 183, 449–456.

Gibb, K. S., Hellmann, G. M., and Pirone, T. P. (1989). Nature of resistance of a tobacco cultivar to tobacco vein mottling virus. *Mol. Plant–Microbe Interact.* 2, 332–339.

Gupton, C. L., and Burk, L. G. (1973). Location of the factor for resistance to potato virus Y in tobacco. *J. Hered.* 64, 289–290.

Koelle, G. (1961). Genetishe analyse einer Y-virus-(Rippenbräune) resistenten mutante der tabaksorte Virgin A. *Züchter* 31, 71–72.

Laliberté, J.-F., Nicolas, O., Chatel, H., Lazure, C., and Morosoli, R. (1992). Release of a 22-kDa protein derived from the amino-terminal domain of the 49-kDa NIa of turnip mosaic potyvirus in Escherichia coli. *Virology* 190, 510–514.

Miller, R. D. (1987). Registration of TN 86 burley tobacco. *Crop Sci.* 27, 365–366.

Nicolas, O., Pirone, T. P., and Hellmann, G. M. (1996). Construction and analysis of infectious transcripts from a resistance-breaking strain of tobacco vein mottling potyvirus. *Arch. Virol.* 141, 1535–1552.

Reddick, B. B., Miller, R. D., and Gooding, G. V. (1991). Resistance in Nicotiana tabacum cultivar TN 86 to naturally occurring tobacco viruses. *Tobacco Sci.* Dec., 96–99.

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). *In* "Molecular Cloning: A Laboratory Manual," 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Vandeyar, M., Weiner, M., Hutton, C., and Batt, C. (1988). A simple and rapid method for the selection of oligodeoxynucleotide-directed mutants. *Gene* 65, 129–134.