Breakdown of Host Resistance by Independent Evolutionary Lineages of *Beet necrotic yellow vein virus* Involves a Parallel C/U Mutation in Its p25 Gene

Rodolfo Acosta-Leal, Becky K. Bryan, Jessica T. Smith, and Charles M. Rush

ABSTRACT


Breakdown of sugar beet *Rz1*-mediated resistance against *Beet necrotic yellow vein virus* (BNYVV) infection was previously found, by reverse genetics, to be caused by a single mutation in its p25 gene. The possibility of alternative breaking mutations, however, has not been discarded. To explore the natural diversity of BNYVV in the field and its effects on overcoming *Rz1*, wild-type (WT) and resistance-breaking (RB) p25 genes from diverse production regions of North America were characterized. The relative titer of WT p25 was inversely correlated with disease expression in *Rz1* plants from Minnesota and California. In Minnesota, the predominant WT p25 encoded the A<sub>67</sub>C<sub>68</sub> amino acid signature whereas, in California, it encoded A<sub>67</sub>L<sub>68</sub>. In both locations, these WT signatures were associated with asymptomatic BNYVV infections of *Rz1* cultivars. Further analyses of symptomatic resistant plants revealed that, in Minnesota, WT A<sub>67</sub>C<sub>68</sub> was replaced by V<sub>67</sub>C<sub>68</sub> whereas, in California, WT A<sub>67</sub>L<sub>68</sub> was replaced by V<sub>67</sub>L<sub>68</sub>. Therefore, V<sub>67</sub> was apparently critical in overcoming *Rz1* in both pathosystems. The greater genetic distances between isolates from different geographic regions rather than between WT and RB from the same location indicate that the underlying C to U transition originated independently in both BNYVV lineages.

Additional keywords: *Beet soilborne mosaic virus*, BNYVV, convergent evolution, rhizomania.

To overcome plant resistance genes, viruses generally need to replicate under restrictive host conditions to incorporate adaptive genetic changes. The type, number, order, and speed of these viral changes impact plant resistance durability (3). Although most resistance (*R*) genes deployed against virus infections have lasted more than 25 years in the field (7), the effectiveness of *Rz1*, which confers partial resistance against *Beet necrotic yellow vein virus* (BNYVV), the causal agent of rhizomania in sugar beet, has been compromised by the reemergence of the disease after 15 years of commercial field deployment of *Rz1* in North America (18,19,27).

The *Bnyvirus* BNYVV is a multipartite, single-stranded, positive-sense RNA virus. RNA-1 and -2 encode the essential elements for replication, encapsidation, and cellular translocation, whereas RNA-3, -4, and isolate-specific, RNA-5, encode proteins involved in pathogenesis, vector transmission, and suppression of gene silencing (17,25,31). Despite its divided genome and the potential of mixed infections with different strains (13), high genetic stability seems to be the norm between spatiotemporally separated populations (11). In an otherwise genetically stable BNYVV genome, *p25* (encoded by RNA-3) and *p26* (RNA-5) genes are the most variable genomic regions, with strong positive selection acting on some of their codons (28). These genes operate synergistically to exacerbate symptoms in certain sugar beet cultivars (10,17), although *p25* accounts for most rhizomania expression (32).

Rhizomania is characterized by profuse lateral root proliferation, taproot constriction, root necrosis, and leaf chlorosis without foliar virus infection. The resistance mechanisms governed by *Rz1* are phenotypically expressed by restricting virus accumulation in taproots and suppressing rhizomania development (32). At the biochemical level, resistance is associated with differential expression of genes involved in pathogenesis and hormone-mediated plant development (4,15,29). Despite these plant defense responses, BNYVV still accumulates at low levels in asymptptomatically infected roots of *Rz1* plants.

By reverse genetics, Koenig et al. (12) demonstrated, that for European *A* type isolates E12 and S8, valine at position 67 of the BNYVV p25 protein is required to overcome *Rz1*-mediated resistance and allow normal virus replication. This amino acid substitution was previously associated with breakdown of *Rz1* in field-infected plants from the California Imperial Valley (CIV) (2). However, Liu and Lewellen (18) did not find a correlation between *p25* sequences of numerous North American isolates and their titer in soil-inoculated *Rz1* plants in greenhouse assays. This observation suggested that BNYVV might mutate in different ways to overcome *Rz1* in North America. Therefore, the objective of this work was to explore the genetic diversity of the BNYVV *p25* gene that might be associated with expression of rhizomania in *Rz1* plants in the field.

MATERIALS AND METHODS

**Field sampling.** For initial investigation of BNYVV sequences, the virus was baited from field soil samples as described by Acosta-Leal and Rush (2). These infested soil samples, some of which had been collected as early as 1991, were from the rhizosphere of symptomatic and asymptomatic *Rz1* plants, and symptomatic susceptible (*rz1*) plants from different sugar beet production regions around the United States. The name of these isolates provides information about their origin (i.e., field name, when it
was known, state, and collection year). BNYVV rarely infects the aerial part of sugar beet plants but root-infected plants normally develop upright leaves with generalized chlorosis during matura-
tion. This foliar symptom facilitates identification of plants with
rhizomania, which frequently have a cluster distribution in the
field (27). For virus quantification and genotyping using TaqMan-
specific probes, four to six Rz1 plants were collected from both
inside and outside a yellow patch of plants with rhizomania and
individually analyzed for virus infection. Each sample consisted of
0.1 g of diseased hairy roots, or normal lateral roots from
those plants without any root expression of the disease. Samples
from Minnesota (MN) were from Crookston (four fields, 2005),
Willmar (seven fields, 2005), and Roseland (one field, 2007),
whereas samples from CIV were from four fields surveyed in
2006.

Total RNA extractions. Root samples were collected in 2-ml
microfuge tubes that contained a sterile 4-mm stainless steel
grinding ball and then stored at −80°C until processing. During
RNA extraction, the plant tissue was first powdered by immersing
the unopened 2-ml tubes containing the sample in liquid nitrogen
and then immediately shaking them at 1,600 rpm for 2 min in a
Talboys high-throughput homogenizer (Thorofare, NJ). Then,
total RNA was extracted following the RNasey Plant Mini Kit
(Qiagen, Inc., Valencia, CA) protocol. All filtrations and filter
drying were performed by centrifugation at 16,000 × g for 1 min
at room temperature. This protocol usually yielded total RNA per
sample at 200 to 500 ng µl−1.

Real-time reverse-transcription polymerase chain reaction
viral RNA quantifications. The concentration of nucleic acids in
total RNA-preparations was estimated by spectrophotometry and
adjusted to 20 ng µl−1 for viral RNA quantification. The amount of
viral RNA encoding sequences recognized by specific TaqMan
probes was estimated by relative quantification (RQ) real-time
reverse-transcription polymerase chain reaction (RT-PCR) or
directly by the cycle threshold (Ct) values generated without the
incorporation of standards in the real-time RT-PCR reaction.
RQ was calculated by the ΔΔCt method (20) using 185 ribosomal
RNA as an endogenous reference (Applied Biosystems, Inc.,
Foster City, CA) and a plant RNA sample with the lowest
detectable virus titer as the calibrator. This procedure determined the
times a TaqMan-targeted viral molecule was above the cali-
brator sample. To estimate the BNYVV RNA-2 titer, primers 5′-CCGTTTTTCCACAGACACTAACTATGTA-3′ and 51R (5′-
TGCTAACCCTGGAATCATGTTAATCTGGCTAACCCTGAA TCAGTTAAAGTACTT-3′) plus the TaqMan probe
NYCP (6FAM-TGCACTTGTGTTATATGTTAATCTGGCTAACCCTGAA TCAGTTAAAGTACTT-3′-CCGTTTTTCCACAGACACTAACTATGTA-
TAMRA) were incorporated in one-step RT-PCR to target the core of the coat protein (CP) gene (2). For de-
tection and quantification of viral RNA-3 encoding specific
sequences in the hypervariable coding region of p25, the allelic
discrimination primers and 3′ minor groove binder TaqMan
probes described by Acosta-Leal and Rush (2) were utilized.
Real-time reactions were performed by an ABI Prism 7000 sys-
tem (Applied Biosystems, Inc.) using the following sequential
conditions: reverse transcription at 48°C for 30 min, reverse
transcriptase inactivation at 95°C for 10 min, and amplification
during 40 cycles of denaturing at 95°C for 15 s and annealing at
60°C for 1 min.

RT-PCR, cloning, and sequencing. First-strand cDNA was
synthesized using the Omniscript reverse-transcriptase kit (Qiagen,
Inc.) according to the manufacturer’s recommendations. Total
RNA (0.5 µg) was denatured at 65°C for 5 min before annealing
with 0.5 µg of oligo-dT12-18 primer and incubating for cDNA
polymerization at 37°C for 1 h. PCR was performed in a second
tube by incorporating 1 unit per 50-µl reaction of Platinum
Taq high-fidelity polymerase (Invitrogen, Inc., Carlsbad, CA), 5.0 µl
of single-stranded cDNA product, primers NYP25-F1 (5′-TTCCTGACCCACCCAAATCACA-3′) and NYP25-R1 (5′-GTAAACGGACGCGGAAACACCA-3′) at 0.5 µM each, and the rest of the
reagents at concentrations recommended by the manufacturer.
DNA amplification occurred during 30 cycles of denaturing at
94°C for 30 s, annealing at 56°C for 30 s, and extending at 68°C
for 1 min 30 s. Amplicons were cleaned using the QIAquick kit
(Qiagen, Inc.), quantified by spectrophotometry, and submitted
for consensus DNA sequencing or recombined with pCR-Blunt
(Invitrogen, Inc.) vector for sequencing individual cDNA clones.
Plasmid DNA was extracted from individual clones using the
QIAprep Spin Miniprep Kit (Qiagen, Inc.). To minimize experi-
mental error, cloned DNA was never exposed to mutagens (i.e.,
UV or ethidium bromide) and bacterial colonies were not
replicated. Amplicons and plasmid DNA were sequenced in both
directions by a private company (Beckman Coulter Genomics
Inc., Beverly, MA) using high-throughput capillary electro-
phoresis DNA-sequencing instruments.

Viral RNA sequence analyses. The basic processing of cDNA
sequences, such as assembling, correction, and alignment, was
performed with Lasergene package v8 (Dnastar Inc., Madison,
WI), and the chromatograms were inspected with Sequence
Scanner v1.0 (Applied Biosystems, Inc.) to verify the presence of
mutations. Sequence alignments were saved in Fasta format to be
exported into the different applications used in this study.
Phylogenetic trees were reconstructed by the neighbor-joining
algorithm as implemented in MEGA 3.1 (14). This software was
also used to calculate genetic distances between individual
sequences and groups of sequences. Genetic differentiation between
pairs of populations was statistically estimated by the Wright’s
Fst index of dissimilarity as implemented in Arlequin v3.11 (5)
fornhaploidy data. In brief, Fst measures the degree of differ-
entiation between two putative subpopulations by comparing the
agreement between two haplotypes drawn at random from each
subpopulation with the agreement obtained when the haplotypes
are taken from the same subpopulation.

RESULTS

The BNYVV wild-type AγCα p25 motif predominates in
susceptible sugar beet genotypes from different regions of
North America. Phylogenetic analysis of partial RNA-3
consensus sequences of 24 isolates collected from several regions
of the United States, and one sequence from France as outgroup,
revealed a cluster of nine almost identical BNYVV isolates (i.e.,
Willmar [MN 2005], wt15 [MN 2000], Salinas [CA 2005],
Kammerzal [CO 1999], Idaho [ID 2003], Glendon [MN 2004],
Climax [MN 2005], Christian [TX 1991], and TexasKF [TX
2001]) (Fig. 1). These isolates shared the biological feature of
having been isolated from severely infected susceptible sugar beet
cultivars. Otherwise, they were from different production regions
of the country, and collected as much as 14 years apart. Based on
this information, these nine isolates were considered contemp-
orary representatives of at least one of the originally introduced
wild-type (WT) BNYVV genotypes. At the genomic level, WT
isolates encoded alanine and cysteine (i.e., GCU UGU) at the
polyorphic amino acid positions 67 and 68 of p25. This AγCα
amino acid signature was undetected by consensus sequencing of
BNYVV isolates collected from resistant Rz1 plants showing
severe symptoms of rhizomania.

Except for Tecker (CA 2005), isolates collected from Rz1-
resistant plants in CIV encoded leucine instead of cysteine or
histidine at position 68 of p25 (Fig. 1). The phylogenetic clus-
tering of these CIV isolates correlated with pathogenicity in Rz1
plants. Thus, RB isolates (i.e., DWest149 [CA 2005], Tamarack
[CA 2004], Mag10 [CA 2005], Spruce [CA 2005], and Onion [CA
2003]) formed a clade that differentiated from WT CIV isolates
(Rockwood [CA 2006], Tuberose [CA 2006], Wtciv [CA 2005],
B5 [CA 2006], F5 [CA 2006], and R156 [CA 2003]). As expected from previous studies (2,12), all RB isolates, including Tecker
(CA 2005) and those from MN (i.e., Blinker83 [MN 2004] and
Pathogenicity of BNYVV in Rz1 plants is associated with a reduction or disappearance of RNA-3 molecules encoding A67C68. The relative titer of p25 encoding the A67C68 motif in relation with total BNYVV titer was estimated in asymptomatic (i.e., green plants generally without rhizomania) and symptomatic (i.e., yellow plants with rhizomania) Rz1 plants collected from three locations in MN and four fields from CIV during 2005 to 2007. These production regions represent two contrasting pathosystems. In MN, sugar beet crops are grown from April to October, mainly without irrigation and, in addition to BNYVV, the crop frequently is infected by another Benyvirus, Beet soilborne mosaic virus (BSBMV) (9,26,36). In CIV, sugar beet plants are cultivated from September to July under irrigation, and BNYVV is apparently the only Benyvirus present.

Specific TaqMan probes, one targeting the RNA-3 region encompassing the codons GCU and UGU for A67C68 and another the CP-coding region on RNA-2 (2), were used to estimate WT p25 and total virus titers, respectively. In total, 37 green and 50 yellow plants infected by BNYVV were analyzed. As expected, roots from yellow plants usually contained greater viral RNA-2 titer than green plants (Fig. 2). Statistical comparisons were done between titers of the same targeted RNA sequence from the same sampled location. Except for those bars in Figure 2 with the same letter, the differences in content of the targeted RNA between green and yellow plants were statistically significant (P < 0.001). Thus, the difference in RNA-2 titters between green and yellow plants was statistically significant except for the samples from Willmar, MN (Fig. 2B). Coincidently, only these samples were infected in high incidence by BSBMV, which was co-infecting with BNYVV in 100% of the green and 60% of the yellow Rz1 plants. Also, BSBMV titer was greater in green than yellow Rz1 plants (data not shown). These observations support previous

![Fig. 1. Phylogram of 24 North American isolates of Beet necrotic yellow vein virus (BNYVV) rooted by an isolate from France (accession no. AY34501) included as outgroup, and their predicted amino acid motif at positions 67 and 68 of p25.](image_url)

![Fig. 2. Relationship between titers of Beet necrotic yellow vein virus (BNYVV) RNA-2 (coat protein [CP] coding region) and RNA-3 encoding A67C68 in Rz1 plants collected from three locations of Minnesota (MN) and four fields from the California Imperial Valley (CIV). Viral RNA (vRNA) titers were semiquantitatively estimated by the cycle threshold values generated by real-time reverse-transcription polymerase chain reaction minus 40, which was the maximum number of amplification cycles. In this way, the y-axis values are directly proportional to RNA titer. N is the number of infected green (g) and yellow (y) plants included in the analyses. Bars of the same targeted vRNA from the same location with the same letter are not significantly different (P > 0.05).](image_url)
reports of an antagonistic interaction between BNYVV and BSBMV (21,35). In this case, BNYVV apparently decreased the incidence of BSBMV in yellow plants. Nevertheless, it also suggests that BNYVV titer increased in green plants (Fig. 2B) as a result of its interaction with BSBMV. Some of these green plants exhibited root symptoms different from the typical rhizomania symptoms.

A greater RNA-2 titer in yellow plants was usually associated with lower titer or disappearance of V67L68 p25 in the infecting virus population (Fig. 2). This observation suggested that most of these severely infected Rz1 plants were carrying a different and, consequently, undetected BNYVV p25 motif. Furthermore, by targeting the samples from MN with a TaqMan probe for detection of the RB V67L68 p25 motif (2), it was revealed that these isolates were not carrying this specific RB allele (data not shown). In CIV, the situation was different; RNA-3 encoding V67L68 predominated in yellow plants and its titer was proportional to virus content (Fig. 3A). By contrast, in the surrounding green plants, V67L68 p25 motif was generally undetected or in low concentration regardless of virus titer (Fig. 3B).

The same C to U nucleotide substitution in the coding region of p25 is associated with breakdown of Rz1-mediated resistance in MN and CIV. Virus titer and consensus DNA sequences of sympatric WT and RB isolates were analyzed to investigate the lack of TaqMan detection of RNA-3 encoding AcC68 using the GCU UGU codons in yellow plants from MN-2007 (Fig. 2C). Green and yellow plants were sampled approximately every meter along an 8- to 10-m transect passing through a yellow spot of diseased plants. The three yellow spots sampled in this way were ≈200 to 400 m apart in the same field, and two to three isolates from green and yellow plants per transect were randomly selected for amplicon sequencing.

Relative BNYVV quantification by real-time RT-PCR revealed that yellow plants in the center of the yellow spots contained the highest viral RNA-2 titers (RQ Log10 = 6.48 ± 0.64) and green plants located at the extremes of the transects contained the lowest (RQ Log10 = 4.89 ± 1.33) (Fig. 4A). Furthermore, phylo-
genetic analysis indicated that sequences derived from yellow plants configured a separate clade (Fig. 4B). Plants that were close together occasionally were infected by the same consensus RNA-3 sequence that differentiated from the other sequences (e.g., 2–4y and 2–5y). Interestingly, all isolates infecting yellow plants carried the V67C68 p25 motif whereas all isolates from green plants codified for WT A67C68. The genetic change behind this amino acid shift was a nucleotide transition from C to U at codon 67 (Fig. 4B). Thus, the same mutation observed in RB variants from MN and CIV (Fig. 1) accounted for the capability of BNYVV to cause rhizomania in the sampled yellow patches from MN-2007.

Asymptomatic plants in CIV are mostly infected by a distinct WT BNYVV genotype. Despite the ample dispersion of WT A67C68 p25 motif in North America, these genotypes were scarcely detected in green Rz1 plants from CIV (Fig. 2D). Previously, we observed that most asymptomatic infections were caused by WT BNYVV isolates encoding the A67L68 p25 motif (2). Also, in this particular pathosystem, a polymorphism at amino acid position 135 of p25 was associated with pathogenesis. To determine the genetic composition of WT BNYVV populations in CIV, viral RNA-3 cDNA clones derived from nine asymptomatic Rz1 plants were sequenced. Viral RNA-3 encoding WT A67C68 D135 was found in just one plant, and it was coinfecting with molecules encoding V67L68E135 (Table 1). As expected, the WT A67L68 D135 p25 was detected alone in 78% of the plant samples. Also, RB haplotypes with the V67L68E135 signature were detected only in the isolate Tub(7g). This may have resulted from a recent RB infection that later may have developed typical disease symptoms.

RB variants from MN and CIV apparently originated from different BNYVV lineages during independent events. Except for BNYVV-Tecker, the phylogenetic differentiation between isolates from CIV and other parts of the United States (Fig. 1), along with the high similarity between WT and RB isolates from MN (Fig. 4B), suggested that the determinant mutation to overcome Rz1 occurred in parallel between CIV and MN groups of isolates (i.e., the occurrence of same nucleotide substitutions at the same site in two or more independent lineages) (8). To test this hypothesis, genetic distances between WT and RB isolates from both regions were more precisely estimated. Consensus sequences of isolates from CIV and MN used for Figure 1 and all sequences used for Figure 4B were grouped according to their pathogenicity in Rz1 plants to form the groups WT MN, WT CIV, RB MN, and RB CIV. Then, the average number of nucleotide differences and percentage of genetic differences was calculated between these four groups. This populational analysis revealed that WT and RB isolates from MN were more closely related to each other (i.e., 1.8 ± 1.0 nucleotide differences) than to either of the two groups from CIV (i.e., 3.4 to 5.6 nucleotide differences) (Table 2). Similarly, RB isolates from CIV were more closely related to WT isolates from the same region than to any isolate from MN.

### DISCUSSION

This study revealed that BNYVV isolates infecting susceptible sugar beet genotypes, which lack the dominant Rz1 allele, encode the amino acids A67C68 D135 in p25 (RNA-3) in most sugar beet production regions of the United States. Apparently, this consensus WT genotype has been maintained with minimal variation since 1991, before the massive commercialization of Rz1 cultivars. Also, WT BNYVV was consistently found in asymptomatic Rz1 plants from MN and some production areas in California. However, in CIV, most WT isolates encoded A67L68 D135 instead of A67C68 D135. It is unknown whether p25 evolved in CIV from A67C68 D135 to A67L67E135 or if both were independently introduced into this region.

Isolates from the groups WT MN (i.e., encoding A67C68 D135) and WT CIV (i.e., encoding A67L68 D135) differed from each other by ≈3.4 fixed nucleotide substitutions in the p25 gene (Table 2). Two of these mutations occurred at codon 68 where they caused an amino acid replacement that is under strong diversifying selection (1,28) but the effect of this amino acid change on virus fitness is unknown. Surprisingly, the same nucleotide C to U substitution at codon 67, which confers to BNYVV the capability to overcome Rz1 in greenhouse assays (12), was identified in RB strains from two distantly separated and contrasting pathosystems in the United States. The fact that this nucleotide substitution was a C to U transition in a hypervariable region suggests that RB variants could easily originate multiple times by convergent evolution, which may explain the reduced durability of Rz1-mediated resistance in the field.

At least two possible evolutionary scenarios could explain the origin of RB isolates encoding V67L68D135 (RB MN group) and V67L68E135 (RB CIV group). In the first scenario, the determinant A67V amino acid substitution may have originated in a common p25 ancestor and then diversified in Rz1 plants into V67C68 D135 in MN and V67L68 E135 in CIV. In the second scenario, RB progenitors of the RB MN and RB CIV groups derived directly from WT A67C68 D135 and A67L68 E135 isolates, respectively, by a single parallel nucleotide substitution. The first hypothesis is less likely because it requires a greater number of mutational events (6) (Table 2), whereas the second hypothesis only involves a single parallel C to U nucleotide substitution in the hypervariable coding region of p25. Although WT isolates encoding A67L67E135 have

### Table 1. Number of Beet necrotic yellow vein virus (BNYVV) haplotypes with specific amino acid motif in p25 (positions 67, 68, and 135) detected in isolates from asymptotically infected Rz1 sugar beet plants from California Imperial Valley

<table>
<thead>
<tr>
<th>Isolate</th>
<th>ACD</th>
<th>ALD</th>
<th>VLE</th>
<th>Total per plant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Roc(1g)</td>
<td>0</td>
<td>7</td>
<td>0</td>
<td>7</td>
</tr>
<tr>
<td>Roc(2g)</td>
<td>0</td>
<td>4</td>
<td>9</td>
<td>13</td>
</tr>
<tr>
<td>Roc(1g)</td>
<td>4</td>
<td>0</td>
<td>5</td>
<td>9</td>
</tr>
<tr>
<td>Roc(2g)</td>
<td>0</td>
<td>3</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Roc(1g)</td>
<td>0</td>
<td>6</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>Tri(2g)</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Tub(2g)</td>
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<td>8</td>
<td>0</td>
<td>8</td>
</tr>
<tr>
<td>Tub(7g)</td>
<td>0</td>
<td>8</td>
<td>0</td>
<td>8</td>
</tr>
<tr>
<td>Tub(8g)</td>
<td>0</td>
<td>8</td>
<td>0</td>
<td>8</td>
</tr>
<tr>
<td>Total per motif</td>
<td>4</td>
<td>38</td>
<td>22</td>
<td>64</td>
</tr>
</tbody>
</table>

*Total number of sequenced cDNA clones derived from viral RNA isolated from asymptomatic lateral roots of individual Rz1 sugar beet plants.

### Table 2. Genetic distances between wild-type (WT) and resistance-breaking (RB) groups of Beet necrotic yellow vein virus (BNYVV) isolates collected from California Imperial Valley (CIV) and Minnesota (MN)

<table>
<thead>
<tr>
<th>Pairwise comparison</th>
<th>Nucleotide differences (±SE)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number</td>
</tr>
<tr>
<td>WT CIV versus WT MN</td>
<td>3.40 ± 1.40</td>
</tr>
<tr>
<td>WT CIV versus RB MN</td>
<td>4.40 ± 1.72</td>
</tr>
<tr>
<td>WT CIV versus RB CIV</td>
<td>4.08 ± 1.42</td>
</tr>
<tr>
<td>WT MN versus RB MN</td>
<td>1.80 ± 1.00</td>
</tr>
<tr>
<td>WT MN versus RB CIV</td>
<td>5.65 ± 1.92</td>
</tr>
<tr>
<td>RB MN versus RB CIV</td>
<td>4.65 ± 1.70</td>
</tr>
</tbody>
</table>

* Each isolate was represented by the consensus sequence of a 975-nucleotide fragment encompassing the complete p25 gene and adjacent 3′ untranslated regions in RNA-3.

In total, 10, 10, 6, and 4 isolates were grouped in WT MN, RB MN, WT CIV, and RB CIV, respectively. Average genetic distances between groups, encoded by number of nucleotide differences and percentage of nucleotide differences, were calculated by MEGA 3.1 (14); SE = standard error. All four groups were genetically different (P < 0.005) to each other based on the FST index of disequilibrium calculated by Arlequin version 3.11 (5).
not been found in CIV. RB isolates encoding V$_{67}$L68E$_{135}$ were recently detected in CIV (unpublished data). This suggests that BNYVV may have evolved in this region from WT A$_{67}$V to RB V$_{67}$L68D$_{135}$ and then to V$_{67}$L68E$_{135}$. This possibility is supported by the observation that positive selection was detected on the replacement of aspartic acid by glutamic acid at position 135 of p25 (1).

Strong selection pressure, such as the widespread introduction of resistant cultivars, seems to favor the occurrence of parallel mutations in individual virus populations. Parallel mutations have been detected in high frequency during host adaptation of phage cultures (34), and parallel mutations better explained the ability of two groups of _Potato virus X_ strains to overcome Nb-mediated potato resistance (22). Therefore, it is possible that the A$_{67}$V amino acid substitution in BNYVV isolates from MN of resistant cultivars, seems to favor the occurrence of parallel mutations in the CP of RB isolates of _Pepper mild mottle virus_. Genetic analyses of a larger number of sympatric WT and RB isolates from around the world may clarify this issue, and also may reveal a greater number of alternative mechanisms by which BNYVV overcomes Rz1.

At present, it appears that the mutational options for BNYVV to overcome Rz1 resistance are limited. However, in other virus–plant interactions, the mutational choices to overcome host resistance are greater. For instance, a number of amino acid substi tutions in the central region of the VPg gene in _Rice yellow mottle virus_ can result in RB isolates (24). Likewise, different mutations in the CP of RB isolates of _Pepper mild mottle virus_ allow it to overcome the dominant L$_{3}$-mediated resistance (33). The agricultural importance of this study is that a high frequency of newly emerging RB variants of BNYVV will drastically limit the useful duration of Rz1 cultivars in the field.

Initially, we hypothesized that A$_{67}$ in p25 was the elicitor of a generalized BNYVV-induced Rz1-mediated resistance, as has been previously reported in other incompatible gene-for-gene interactions, including viruses (30). However, the following observations in this particular BNYVV–Rz1 interaction suggest that A$_{67}$ is not eliciting a generalized defense response. First, viral haplotypes encoding alanine or valine at position 67 of p25 can coinfet the same Rz1 plant and rhizomania is still expressed. Second, no amino acid other than valine at position 67 has been associated with the ability of BNYVV to overcome Rz1. Therefore, a specific p25–Rz1 interaction may be required for disease development rather than defense activation. This putative p25–Rz1 interaction could be necessary, for instance, to deactivate or delay Rz1-mediated defense responses. Viral suppression of gene silencing is the best example of molecular virus–plant recognition leading to plant susceptibility (16). However, similar recognition events may suppress some other defense mechanisms (23). Given the dominant inheritance of Rz1-mediated resistance, it is possible that the recessive r1 allele either lacks or encodes a defective defense mechanism. This could explain why susceptible r1 plants can be severely infected by haplotypes of BNYVV that encode alanine and several other amino acids, including valine, at position 67 of p25. Thus, in this compatible plant–virus interaction, the specific p25–r1 molecular coupling might be unnecessary for the virus to cause severe infection.

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**LITERATURE CITED**


