DIFFERENCES IN RESISTANCE BREAKING ISOLATES OF BNYVV FROM MINNESOTA AND CALIFORNIA

Charles M. Rush, Regents Fellow and Professor, and Rodlofo Acosta-Leal, Assistant Research Scientist
Texas AgriLife Research, Amarillo 79109

Although most resistance genes (*R*-genes) deployed against virus infections have lasted more than 25 years, the effectiveness of *Rz1* that confers partial resistance against *Beet necrotic yellow vein virus* (BNYVV) was compromised in approximately 10 years in MN/ND by the emergence of resistance breaking (RB) strains of BNYVV (5). Using reverse genetics, Koenig *et al*. (3) demonstrated that for European A type isolates of BNYVV, valine at position 67 of the BNYVV p25 protein was required to overcome *Rz1*-mediated resistance and allow normal virus replication. This amino acid substitution was previously associated with breakdown of *Rz1* mediated resistance in field infected plants from the California Imperial Valley (CIV, 1, 2). However, Liu and Lewellen (4) did not find a correlation between p25 sequences of numerous North American isolates and virus titer in *Rz1*-plants in greenhouse assays. This suggested that BNYVV genetic background may affect the resistance breaking requirements of the p25 gene. Therefore, the objective of this work was to look for correlations between BNYVV p25 gene sequences and severity of rhizomania in *Rz1*-plants in the field.

Materials and Methods

Virus Isolates. BNYVV was baited from field soils, from sugar beet production regions around the USA. These 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. For virus quantification and genotyping using TaqMan specific probes, four to six *Rz1*-plants were collected from inside and outside of rhizomania spots, and individually analyzed for virus infection (2). Each sample consisted of around 0.1g of diseased hairy roots, or normal lateral roots from those plants without rhizomania. Samples from Minnesota (MN) were from four fields near Crookston and seven near Willmar.

Total RNA Extraction and Viral RNA Quantification. For RNA extraction, plant root tissue was powdered by immersing microfuge tubes in liquid nitrogen and then shaking them at 1600 rpm for 2 min in a high throughput homogenizer. Total RNA was extracted following the RNeasy Plant Mini Kit (Qiagen, Valencia, CA) protocol. 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) realtime RT-PCR. To estimate BNYVV RNA-2 titer, specific primers plus the TaqMan probe were incorporated in one-step RT-PCR to target the core of the CP gene. For detection and quantification of BNYVV RNA-3 p25, the allelic discrimination primers were used (2). Realtime reactions were performed by an ABI Prism 7000 system (Applied Biosystems, Inc., Foster City, CA) using the following sequential conditions: reverse transcription at 48ºC for 30 min, reverse transcriptase inactivation at 95ºC for 10 min, and amplification during 40 cycles of denaturing at 95ºC for 15 s and annealing at 60ºC for 1 min.

RT-PCR, cloning, sequencing and sequencing analysis. First strand cDNA was synthesized using the Omniscript® reverse transcriptase kit (Qiagen Inc., Valencia, CA). PCR was performed in a second tube and DNA amplification occurred during 30 cycles of denaturing at 94ºC for 30 s, annealing at 56ºC for 30 s, and extending at 68ºC for 1 min 30 s. Amplicons were cleaned, quantified by spectrophotometry, and submitted for consensus DNA sequencing and/or recombined with pCR-Blunt vector for sequencing individual cDNA clones. Amplicons and plasmid DNA were sequenced by Beckman Coulter Genomics Inc., Beverly MA.

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. Genetic relationships were determined by the neighbor-joining algorithm as implemented in MEGA 3.1. 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 F_{ST} index of dissimilarity.

Results

The relative titer of p25 with the $A_{67}C_{68}$ motif in relation to 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 MN and CA. Specific TaqMan probes, one targeting the RNA-3 p25 region encompassing the codons GCU and UGU for $A_{67}C_{68}$ and another the RNA-2 CP coding region, 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 typically contained significantly greater viral RNA-2 titer than green plants, even though BNYVV was infecting both groups. The greater RNA-2 titer in yellow plants was usually associated with lower titer or disappearance of the BNYVV RNA-3 $A_{67}C_{68}$ p25 motif in the infecting virus population. This suggested that most of these severely infected *Rz1*-plants were carrying a different, and consequently undetected, BNYVV RNA-3 p25-motif. Furthermore, by targeting the samples from MN with a

TaqMan probe for detection of the RB $V_{67}L_{68}$ p25-motif (2), it was revealed that these isolates were not carrying this specific RB allele. In BNYVV isolates from CA, the situation was different; RNA-3 p25 encoding $V_{67}L_{68}$ predominated in yellow plants and its titer was proportional to total virus content. By contrast, in the surrounding green plants, $V_{67}L_{68}$ p25-motif was generally undetected or in low concentration regardless of virus titer.

Breakdown of Rz1-mediated resistance in MN. Virus titer and consensus DNA sequences of WT and RB isolates from MN were analyzed to investigate the lack of RNA-3 p25 encoding $A_{67}C_{68}$ in yellow *Rz1*plants. Relative BNYVV quantification by realtime RT-PCR revealed that yellow plants contained the highest viral RNA-2 titers and green plants contained the lowest. Furthermore, sequences derived from yellow plants carried the $V_{67}C_{68}$ p25-motif whereas all isolates from green plants coded for WT $A_{67}C_{68}$. The genetic change behind this amino acid shift was a nucleotide transition from C to U at codon 67. Thus, the same mutation at RNA-3 p25 position 67 observed in RB variants from MN and the CIV accounted for the capability of BNYVV to cause rhizomania in the sampled yellow patches from MN-2007.

The phylogenetic differentiation between isolates from CIV and other parts of the USA, along with the high similarity between WT and RB isolates from MN, suggested that the determinant mutation to overcome *Rz1*(p25 V_{67}) occurred in parallel between CIV and MN isolates. To test this, genetic distances between WT and RB isolates from both regions were more precisely estimated. Consensus sequences of isolates from CIV and MN were grouped according to their pathogenicity in *Rz1*-plants to conform the groups WT_{MN}, WT_{CIV}, RB_{MN}, and RB_{CIV}. Then, the average number of nucleotide differences and percent of genetic differences was calculated between these four groups. This populational analysis revealed that WT and RB isolates from MN were more closely related each other than to either of the two groups from CIV. Similarly, RB and WT isolates from CIV were more closely related to each other than to any isolate from MN.

Discussion

The results presented in this paper indicate that BNYVV isolates encoding the amino acids $A_{67}C_{68}D_{135}$ in RNA-3 p25 predominated in most production regions of the USA infecting susceptible sugar beet genotypes lacking the dominant *Rz1* allele. Apparently, the consensus WT BNYVV genotype has been maintained to date with minimal variation since at least 1991, and perhaps before the massive commercialization of *Rz1*-cultivars. Also, WT BNYVV was consistently found in asymptomatic *Rz1*-plants from MN. However, in CIV, most WT isolates encoded $A_{67}L_{68}D_{135}$ instead. At present, it is unknown whether p25 evolved in CIV from $A_{67}C_{68}D_{135}$ to $A_{67}L_{68}D_{135}$ or both have been independently introduced in this region. On average, WT isolates from MN and CIV differentiated from each other by approximately 3.4 fixed nucleotide substitutions. Two of these mutations occurred at codon 68 where they caused an amino acid replacement that is under strong diversifying selection. The expected adaptive contribution that this amino acid change may have on virus fitness is still unknown. Surprisingly, the same nucleotide C to U substitution at codon 67, which confers to the ability of BNYVV to overcome *Rz1* was incorporated in RB variants from two highly variable production systems. 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 resistance durability of *Rz1* in the field.

Strong selection pressure seems to favor the occurrence of parallel nucleotide substitutions in distant virus populations. Therefore, it is possible that the $A_{67}V$ amino acid substitution in BNYVV p25 might have frequently occurred in parallel during distinct epidemic episodes. The agricultural relevance of this type of study is self explanatory: a high frequency of newly emerging RB variants of BNYVV will drastically limit the useful duration of *Rz1*-cultivars in the field.

Literature Cited

1. Acosta-Leal, R., Fawley, M. W., and Rush, C. M. 2008. Changes in the intraisolate genetic structure of *Beet necrotic yellow vein virus* populations associated with plant resistance breakdown. Virology 376: 60-68.
2. Acosta-Leal, R. and Rush, C. M. 2007. Mutations associated with resistance-breaking isolates of *Beet necrotic yellow vein virus* and their allelic discrimination using TaqMan technology. Phytopathology 97: 325-330.
3. Koenig, R., et. al. 2009. A single U/C nucleotide substitution changing alanine to valine in the *Beet necrotic yellow vein* virus P25 protein promotes increased virus accumulation in roots of mechanically inoculated, partially resistant sugar beet seedlings. J. Gen. Virol. 90: 759-763.
4. Liu, H. Y. and Lewellen, R. T. 2007. Distribution and molecular characterization of resistance-breaking isolates of *Beet necrotic yellow vein virus* in the United States. Plant Dis. 91: 847-851.
5. Rush, C. M., Liu, H. Y., Lewellen, R. T., and Acosta-Leal, R. 2006. The continuing saga of rhizomania of sugar beets in the United States. Plant Dis. 90: 4-15.

Acknowledgements

We thank Mark Bredehoeft (Southern Minnesota Beet Sugar Cooperative) and Al Cattanach (American Crystal Sugar Company) for their support and advice during field surveys. This work was funded in part by grants from the Beet Sugar Development Foundation, the Minnesota and North Dakota Research and Education Board, and USDA-NRI (Grant No. 2008-35319-04472).