CHANGES IN THE GENETIC STRUCTURE OF *BEET NECROTIC YELLOW VEIN VIRUS* POPULATIONS ASSOCIATED WITH PLANT RESISTANCE BREAKDOWN

Charlie Rush, Rodolfo Acosta-Leal, and Jacob Price
Texas AgriLife Research, Amarillo, 79109

The genome of BNYVV consists of 4 to 5 single-stranded, RNA particles. RNA 1 and 2 encode the essential elements for virus replication, protein encapsidation, and cellular translocation, whereas RNA 3, 4, and 5 are involved in disease expression and vector transmission. Despite its multi-partite genome, and the potential of mixed infections with BSBMV, high genetic stability seems to be the norm. These observations suggest the existence of strong selective constraints on virus diversification, and effective isolation mechanisms operating among populations of BNYVV.

When a plant is infected by BNYVV, it is actually infected by a large collection of virus particles that are closely related, but not identical to each other. The specific molecular composition of these particles is referred to as virus population structure. Usually, when a virus is isolated from an infected plant, the virus is defined by the "average" genetic structure of all the infecting particles, and genetic variability of the virus population is not considered. However, when an infecting viral population whose specific genetic structure, rather than its average or dominant genotype, is discussed, it is referred to as a quasispecies. Few studies have investigated the quasispecies structure of plant viruses, even though it is likely that the quasispecies structure is the most important descriptive attribute of any specific virus isolate. In general, most infecting viral populations are composed of an arrangement of genotypes that are distinguished from each other by at least one mutation. Nonetheless, when the average genotypes of isolates from different infected plants are compared, the majority are almost identical. This suggests that in nature there is a strong selection pressure on infecting virus populations to maintain a state of equilibrium.

We believe that the genetic structure (quasispecies) of viral populations influences their biological properties, such as host range, pathogenicity, and transmissibility, but few efforts have been made in plant virology to test this idea. It has been found that the number of different genotypes in an infecting virus population can be altered by the host environment, including host genotype, but this variability has not been correlated to any other characteristic of the host or biological property of the infecting viral population. Our working premise is that widespread planting of *Rz1* resistant cultivars exerted selection pressure on BNYVV population structure which eventually led to emergence of resistance breaking isolates. The objective of this study was to identify and quantify the molecular changes that occur to an infecting BNYVV population when exposed to different host genotypes. Results of this study help explain how resistance breaking isolates evolve.

METHODS

BNYVV rarely infects foliar tissue, but root-infected plants often develop generalized yellowing that aids in the identification of plants with rhizomania. In this way, apparently healthy and diseased sugar beets were identified and then asymptomatic or symptomatic lateral roots were collected from 3-5 plants of the same condition. Isolates included in this study were from the Imperial Valley of California, Minnesota, and Texas.

RNA was extracted from root tissue using the RNAqueous®-Mini kit (Ambion Inc. Austin, TX) following manufacturer's instructions. Next, first strand cDNA was synthesized using the Omniscript® reverse transcriptase kit (Qiagen Inc., Valencia, CA). PCR was performed separately using Platinum® *Taq* high fidelity polymerase (Invitrogen, Inc., Carlsbad, CA) and 5.0 µl of the reverse transcription products. The amplified DNA fragment, composed of 974 or 1367 incorporated base pairs, was cleaned using the QIAquick kit (Qiagen Inc., Valencia, CA), quantified by spectrophotometry, and recombined with pCR®-Blunt (Invitrogen, Inc., Carlsbad, CA) vector. Plasmid DNA was extracted from individual clones using the QIAprep Spin Miniprep Kit (Qiagen, Inc.) and sequenced in both directions by a commercial company to analyze the genetic composition of the infecting populations. BNYVV titers in infected tissue were estimated by realtime RT-PCR quantification. The realtime reactions were performed by an ABI Prism 7000 system (Applied Biosystems, Inc., Foster City, CA).

The basic processing of sequences were performed with the DNAStar package v4.0 (Dnastar Inc., Madison, WI), and the chromatograms were inspected with Sequence Scanner v1.0 (Applied Biosystems, Inc.) to verify the presence of mutations. Out of 133 sequenced clones, 61 different genotypes were found. The specific type of variability in each of these genotypes was then determined.

RESULTS

The isolates included in this study were collected from 3-5 plants naturally infected in the field and clustered within a localized sampling area. These composite samples were grouped during the analyses according to plant response and host genotype. Thus, the compatible *Rz1*/RB group was composed of resistant breaking isolates of BNYVV which were taken from *Rz1* resistant cultivars showing severe rhizomania. The incompatible *Rz1*/AV group consisted of avirulent isolates that were collected from asymptomatic *Rz1*-resistant cultivars. Finally, the compatible *rz1*/WT group was comprised of wild type isolates of BNYVV obtained from diseased susceptible cultivars.

The use of realtime RT-PCR quantification revealed considerable variation in virus content among isolates. In compatible interactions, the amounts of amplifiable particles were 100 to 10,000 times higher than in the incompatible *Rz1*/AV interaction. In general, disease expression was associated with a virus content of at least 300,000 virus particles per nanogram of total RNA extracted from mature plants grown in the field. In some plant roots, the amount of virus was as high as 2.5 million particles per nanogram of total RNA.

Differences between isolates in this study focused on variability in RNA 3, which is responsible for disease severity. Sixty-one different genotypes were identified out of 133 samples. Phylogenetic analyses revealed that genotypes were clearly clustered based on their plant-virus interaction group. However, there were some cases where a genotype of one isolate was more closely related to genotypes of another isolate instead of its own. This genotype overlapping was common in the *rz1*/WT group despite the fact that these isolates were the most geographically separated (Minnesota and California isolates from 2005 and a Texas isolate from 1991). This finding supports the notion that they belong to a single North American BNYVV macro-population. The lineage that comprised isolates of the incompatible *Rz1*/AV group contained two genotypes from the *Rz1*/RB group. The genetic similarity between these overlapping genotypes supports the idea that RB variants evolved from existing avirulent populations from the same region rather than from an isolate that was externally introduced.

Analysis of molecular variance (AMOVA) indicated that each interaction group represented a separate genetic population of sequences. However, sequences derived from separate isolates did not always form populations that were significantly different from each other. For instance, within the *rz1*/WT group, none of the isolates were significantly different from each other. Similarly, within the *Rz1*/AV group, no significant difference existed between most of the isolates. In contrast, all *Rz1*/RB isolates represented distinct populations. Thus, resistance breaking isolates recovered from *Rz1* plants may not be derived from a single mutant strain or they have evolved separately to such an extent that any evidence of common ancestry was obscured. This finding helps explain our inability to identify a specific marker for RB isolates from Minnesota.

The greatest genetic diversity among isolates within a given group was found with the compatible *Rz1*/RB group. However, the degree of genetic diversity within individual isolates was 2-3 times higher in populations recovered from the incompatible *Rz1*/AV group than from either of the compatible interaction groups. The highest diversity was found in avirulent BNYVV and the lowest in wild type BNYVV. When the overall nucleotide diversities of the sequences included in each plant-virus interaction group were compared, only the less diverse *rz1*/WT group was significantly different from the others. However, when the overall diversity was broken down by diversity within and among isolates, the intra-isolate diversity was highest in the incompatible *Rz1*/AV interactions, whereas the differences among isolates were greatest in the *Rz1*/RB group.

DISCUSSION

BNYVV isolates derived from susceptible (*rz1*) sugar beets were characterized by the same dominant wild type (WT) genotype surrounded by a few mutant genotypes. However, in resistant *Rz1*-cultivars, the scenario was completely different: each isolate contained a different dominant genotype that was surrounded by a broad collection of mutant genotypes. Moreover, in the incompatible interaction between *Rz1*-plants and avirulent isolates (*Rz1*/AV), the infecting populations were 2-3 times more heterogeneous than in the compatible interactions *rz1*/WT and *Rz1*/resistant breaking (RB) isolates. *Collectively, these data suggest that sugar beet cultivars carrying the Rz1 allele altered the genetic structure of BNYVV in a way that promoted the generation and selection of RB variants. Furthermore, if high genetic diversity is the norm for avirulent isolates recovered from Rz1 cultivars, several different mechanisms for overcoming Rz1 resistance could emerge independently. In conclusion, we propose that resistance breaking isolates that evolve in different sugar beet production regions need be analyzed separately, instead of assuming that there is a unique cause of Rz1-mediated resistance breakdown.*