# EFFECT OF HOST GENOTYPE ON GENETIC DIVERSIFICATION OF BNYVV

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Recent studies indicate that viral adaptation to specific hosts, a phenomenon known as 'host adaptation,' may be affected by the genetic structure of the original virus population (6), and selection pressure imposed by the potential host genotype (2, 9). The emerging picture is that some viruses can rapidly adapt to different host environments. For instance, resistance breaking (RB) variants can arise during the first encounter with a restrictive host (1, 2, 5). However, despite their potential for rapid host adaptation, most plant viruses exhibit high genetic stability, surviving in the same host species for many years. Purifying selection apparently plays a critical role in maintaining this genetic stasis. To understand the practical implications of viral host adaptation, we explored the relationship between strength of host resistance and genetic diversity of a benyvirus, *Beet necrotic yellow vein virus* (BNYVV).

p25 (RNA-3) accounts for most of the rhizomania syndrome, is one of the most variable BNYVV genes with strong positive selection acting on some of their amino acids, and encodes a determinant to overcome *Rz1* (3, 7, 8, 10, 11). For these reasons, RNA-3 was chosen to analyze the genetic diversity of BNYVV after its passage through resistant *Rz1*- and *Rz2*-cultivars and a susceptible control. Based on previous results from our lab (2), we hypothesize that certain populational changes taking place before the emergence of RB variants may follow predictable patterns. Our main objective was to identify populational parameters affected by host genotype that could be related to plant resistance durability.

## Materials and Methods

*The serial host planting experiment*. BNYVV-resistant sugarbeet cultivars carrying the dominant *Rz1* or *Rz2* alleles, and a susceptible (rz1rz2) control were grown in individual pots. Twelve plants of each cultivar were seeded into a commercial potting soil mix containing ca. 2 g of field soil infested with a wild type BNYVV (1, 2). As negative controls, the same number of plants of each cultivar was seeded into uninfested potting soil mix. The initial inoculum consisted of BNYVV-infested soil collected from the rhizosphere of susceptible plants cultivated in a commercial field near Climax, MN (Clx isolate, accession no. EU480492). Root tissue was harvested 12 to 14 weeks after planting and then, approximately 50% of the soil/root mixture from each pot was used as inoculum for the consecutive host planting. By following this experimental approach, it was expected that viruliferous sporosori of *P. betae* from the previously infected plants would be the sources to infect the following test plants, thereby creating virus lineages. However, the possibility that plants in the consecutive host planting also were infected by virions remaining from the original inoculum, rather than by virions from the previously infected plants, was not discarded. Therefore, truly serial host passages were not guaranteed, but this approach was preferred over mechanical inoculation of the virus from passage to passage because it more closely mimics what normally happens in the field from one cultivation cycle to the next.

*Total RNA extractions from root tissue.* Root tissue from the three host genotypes were collected on the same day for comparative purposes and stored in 2 ml microfuge tubes at -80ºC until processing. Total RNA extractions were performed from frozen 0.1 g of plant tissue according to the RNAqueous®-Mini kit protocol or the RNeasy Plant Mini Kit protocol. Both protocols gave similar reading of relative viral RNA content and did not affect the genetic composition of viral populations.

*Realtime RT-PCR viral RNA quantifications.* BNYVV titers in 20 ng of total RNA per sample were estimated by relative realtime RT-PCR quantifications using 18S ribosomal RNA as the endogenous control and a minimally infected sample as the calibrator. This procedure determines the number of times a target RNA is above or below the calibrator sample that is included as a second reference. Because we used a calibrator sample with the lowest detectable amount of viral RNA (ca. 100 molecules per ng of total RNA previously estimated by absolute quantification), all positive test samples were those with a virus titer above the calibrator sample. Primers 50F (5'-CCGTTTTCCACAGACACTAACTATGTA-3') and 51R (5'-TGCTAACCCTGAATCAGTTAAAGTACTT-3') plus TaqMan probe NYCP (6FAM-TGCACTTGTGTTATATGTTAATCTGTCTGACCCAG-TAMRA) were incorporated in one-step RT-PCR to target the core of the CP gene in RNA-2. Realtime reactions were performed by an ABI Prism 7000 system using the following parameters: 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 one min.

*Cloning, sequencing and sequencing analysis*. First strand cDNA was synthesized using the Omniscript® reverse transcriptase kit. 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.

The basic processing of cDNA sequences, such as assembling, correction, and alignment was performed with Lasergene package v8, and the chromatograms were inspected with Sequence Scanner v1.0 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 FST index of dissimilarity.

**Results**

*Diversification and survival of BNYVV from planting to planting.* In each of three serial host plantings, 12 inoculated and 12 non-inoculated plants of each sugar beet genotype were grown and harvested at 12 to 14 weeks after planting, to determine virus content in root tissue. Except for one non-inoculated *Rz2*-plant that may have been accidentally contaminated during the first planting, no BNYVV was detected by realtime RT-PCR in the negative controls. Only in resistant sugar beets, virus titer decreased from planting to planting to the point where the percentage of infected plants was, by the end of the experiment, 70 and 37 in *Rz1* and *Rz2* genotypes, respectively. Because virus titer in the susceptible controls was similar from planting to planting, this trend in resistant plants indicated that the reload of viable virus into the soil from the previously infected plants played a significant role in the amount of inoculum available for the following host planting, and that host resistance significantly affected this variable.

Consensus DNA sequencing of each single-plant isolate revealed that some carried a mutation undetected in the original wild type virus population. None of these mutations were passed to the progeny during the following host planting, which suggests that virus lineages were rarely, if ever fixed in the population, during the course of the experiment. Significantly, 1.8 to 4.9 times more mutations were detected in resistant than susceptible plants. This high frequency of mutations was more prevalent during the second host planting and in *Rz2*- than *Rz1*-plants. By the third host planting, all of the six sequenced isolates from *Rz1*-plants were wild type, which suggests that, at this stage, the low content of competent mutants in the inoculum was depleted or at very low frequency in the resting spores of *P. betae*.

*Host effect on the genetic diversity of BNYVV in the field.* To determine if a similar host genotype-virus variability relationship was taking place in the field, viral isolates extracted from *Rz1, Rz2*, and susceptible symptomatic plants collected from southern Minnesota were consensus sequenced on the same RNA-3 region. The lowest nucleotide diversity was among isolates from susceptible plants ($\pi = 0.00038 \pm 0.0002$) with an average nucleotide difference between isolates of $0.50 \pm 0.2$ (Table 4). From this baseline, the genetic diversity was around two and five times greater between isolates from *Rz1* and *Rz2* symptomatic plants, respectively. These values agree with the data obtained through the serial host planting experiment and indicate that the type and/or strength of sugar beet resistance against BNYVV accumulation also affect the diversification of BNYVV in the field.

**Discussion**

By comparing the genetic structure of BNYVV populations generated in susceptible and resistant plants from the same parental wild type population, we found that the same wild type haplotype predominated in most of the susceptible plants, which is consistent with the high genetic stability of BNYVV observed in the field. By contrast, resistant plants were more frequently infected by different predominant haplotypes that might have been randomly picked from the original soil inoculum. Once the test plants were infected by a founder haplotype, spontaneous mutations in the progeny gave place to a spectrum of mutants closely related to each other by descent, but vertical transmission was not detected for any of these mutants. In addition, the data presented in this work demonstrate that virus diversification was directly proportional to strength of plant resistance to virus accumulation in root tissue. This virus behavior also occurred in the field and may define the capability of BNYVV to eventually overcome host resistance through the incorporation of adaptive mutations. To our knowledge, this is the first empirical demonstration of a relationship between the strength of plant resistance to virus accumulation and the populational genetic diversity of a plant virus.

The differential responses between resistant and susceptible plants during the serial planting experiment indicate that reloading of the soil with viruliferous *P. betae* was important in maintaining the high incidence of BNYVV infections observed in susceptible plants. Therefore, the reduced virus occurrence in resistant plants by the end of the experiment could be explained by at least two possible non-exclusive hypotheses. First, infectious particles carried on by the vector from the source plant into the soil and then from the soil into the following test plant may have been drastically reduced. It has been demonstrated that partial sugar beet resistance to BNYVV accumulation in lateral roots decreases the proportion of viruliferous resting spores of *P. betae* without affecting the reproduction of the vector (12). Therefore, acquisition of BNYVV could have been reduced in the *Rz1* and *Rz2* source plants according to their virus titer. Second, most virus mutants may have had reduced capacity to survive out of the plant or be transmitted by *P.*

*betae*. At present, no evidence supports this possible scenario, but further studies comparing the genetic composition of virus populations extracted from source plants versus viruliferous zoospore suspensions might shed some light on this aspect.

The fact that none of the mutants was vertically transmitted through the same lineage was an unexpected finding but, at the same time, it provides a more realistic idea about the chances a BNYVV mutant might have to predominate in the crop from season to season. To be successful, a BNYVV mutant most likely needs to be positively selected and, moreover, be in numerical superiority against the parental wild type population residing in the soil. Thus, the type of relationship between size and complexity of BNYVV populations in restrictive host environments seems to be a suitable parameter to assess the risk of resistance breakdown.

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