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CROP PROTECTION

Analysis of virome by high-throughput sequencing disclosed multiple infection in a single grapevine plant

Thor Vinícius Martins Fajardo[1*] **Antonio Nhani Júnior**[2] **Osmar Nickel**[1]

[1]Embrapa Uva e Vinho, 95701-008, Bento Gonçalves, RS, Brasil. E-mail: thor.fajardo@embrapa.br. *Corresponding author.
[2]Embrapa Agricultura Digital, Campinas, SP, Brasil.

ABSTRACT: Among phytosanitary problems of the grapevine, viruses stand out for their capacity of reducing the quality and yield of grapes. However, detecting and identifying viral infections in grapevines can be challenging. This study performed a high throughput sequencing (HTS) of the viral pathogens present in a vine showing virus-like symptoms to elucidate the etiology. HTS analysis reported in a hybrid grapevine with mild curling down of leaf edges, the presence of four viruses and viroids, which were probably implicated in the observed symptoms. The determined complete genomes showed high genetic identities with previously characterized isolates of homologous pathogens.
Key words: diagnosis, symptoms, HTS, diversity, virus.

Análise do viroma por sequenciamento de alto rendimento revelou infecção múltipla em uma planta de videira

RESUMO: Dentre os problemas fitossanitários da videira, os vírus se destacam pela capacidade de reduzir a qualidade e o rendimento da uva. No entanto, detectar e identificar infecções virais em videiras pode ser um desafio. O objetivo do estudo foi realizar um sequenciamento de alto rendimento (HTS) para determinar os patógenos virais presentes em uma videira com sintomas de virose e elucidar a etiologia. Com o HTS foi detectada, em uma videira híbrida com leve enrolamento dos bordos foliares, a presença de quatro vírus e viroides, os quais provavelmente estavam implicados com os sintomas observados. Os genomas completos determinados mostraram altas identidades genéticas com isolados previamente caracterizados de patógenos homólogos.
Palavras-chave: diagnose, sintomas, HTS, diversidade, vírus.

Grapevines are susceptible to over 86 different plant viruses (FUCHS, 2020). Multiple viruses may accumulate in a grapevine over its life span, and individual viruses or combinations of different viruses may have detrimental effects on the physiology of the vine, yield of harvested grape berries, and berry quality, thus negatively affecting vineyard performance. In Brazil, 21 different viruses and viroids have already been reported infecting grapevines (BASSO et al., 2017). Most viruses that infect grapevines have only the *Vitis* genus as their natural host, and often symptoms cannot be attributed to a single virus, as symptomatic vines usually have multiple infections. In grapevines, the expression of symptoms resulting from viral infection can be affected by the genotype (cultivar), the environment, multiple infections, virulence of the isolate or viral strains, host stage, and nutritional status of the plant (MANNINI & DIGIARO, 2017; SCHOELZ et al., 2021).

Depending on the virus-host interaction, a variety of viral symptoms can be observed such as abnormal coloring and general appearance of the leaves, presence of cork tissues in branches of the year, presence of grooving or pitting in the trunk wood or rootstock, in addition to reduction in photosynthesis and reductions in cluster number, cluster weight, berry weight, and vine size (MANNINI & DIGIARO, 2017; SONG et al., 2021). Leafroll and rugose wood diseases, which primary causal agents are grapevine leafroll-associated virus 3 (GLRaV-3) and, grapevine virus A (GVA) and grapevine virus B (GVB), respectively, are two of the most important virus diseases of grapevines,

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considering the detrimental effects they can cause (SCHOELZ et al., 2021).

An important aspect of viral infection is that infected and asymptomatic plants, kept within the vineyard, could serve as an unnoticed source of viral inoculum for healthy plants or vineyards in the neighborhood. In this case, viral transmission could occur through several types of vectors (e.g. mealybugs and soft scale insects, mites, nematodes). The management of viral diseases of the grapevine is based on the prevention and suppression of the viral inoculum (FUCHS, 2020). The prevention of virus introduction in new vineyards is achieved with the use of healthy propagation material. The reduction of the viral inoculum in infected vineyards is obtained by roguing of diseased plants (FUCHS, 2020). Thus, for control measures of grapevine viruses to be more effective, it is necessary to accurately identify plants with viruses and know about dynamics of symptom expression in different host genotypes.

Knowledge on the occurrence of grapevine viruses in Brazil and their characterization remains insufficient. First studies characterized grapevine viruses spread in Brazil based on standard genomic molecular tools usually targeting the viral coat protein gene (NICKEL et al., 2002), not designed for detecting the whole viral complexity present in grapevines. More recently, high-throughput sequencing (HTS) technology and bioinformatics have drastically improved identification and characterization of viral pathogens without prior knowledge of their primary structure (FAJARDO et al., 2017; GLASA et al., 2020). This research performed a massive sequencing and metagenomic analysis of a plant showing virus-like symptoms to associate symptoms with the presence of virus/viroids in this sample.

To achieve a more comprehensive picture of a particular virome, HTS of a sample of genotype CNPUV 776-25, a white-berry hybrid grapevine from a breeding program, exhibiting mild chlorosis and downrolling of leaf edges (Figure 1), collected in an experimental field in Canoinhas, SC, Brazil, was performed in 2019. Total RNA was extracted using the RNeasy Plant Mini kit (Qiagen) and ribosomal-depleted total RNA from the sample was used to prepare a sequencing library generated with TruSeq Stranded Total RNA with Ribo-Zero Plant kit (Illumina, USA), and then subjected to HTS on Illumina HiSeq X Ten platform. The total reads of HTS were quality trimmed with FastQ and Trim Galore softwares, and *de novo* assembled contigs were built by rnaSPAdes (SPAdes 3.14.1) software. Some generated contigs, after MetaQUAST (QUAST 5.0.2) analysis, corresponded to virus and viroid genomes, which

Figure 1 - Representative leaf symptoms of analyzed grapevine genotype CNPUV 776-25 with multiple infection.

were identified by comparison (Blastn/Blastx analyses) with viral/viroidal reference sequences available in the NCBI database. For HTS validation, total RNA was reextracted from the original source and was screened for identified pathogens by RT-PCR/RT-qPCR, according to previously reported protocols (OSMAN & ROWHANI, 2008; DUBIELA et al., 2013).

The sequences obtained were individually analyzed using NCBI's Blastn program (https://www.ncbi.nlm.nih.gov/) against the GenBank database. Multiple sequence alignments of nucleotides were performed using ClustalX 2.1 and a pairwise nucleotide sequence identity matrix was generated using SDT v.1.2 (MUHIRE et al., 2014). Phylogenetic relationships were determined from the aligned sequences by using the Maximum Likelihood method and Tamura-Nei model, and 1,000 bootstrap replications implemented in MEGA X software (KUMAR et al., 2018).

A single plant of genotype CNPUV 776-25 was selected for the HTS analysis in order to estimate the potential share of virome to the symptomatology observed in this hybrid grapevine genotype. HTS yielded 45,419,722 total reads, which after bioinformatics processing, generated 256,894 assembled contigs (>142 nucleotides, nt). Four genomes of pathogens, RNA viruses/viroids, common grapevine pathogens, were identified in a complex infection, and the complete genome sequences obtained were deposited at GenBank: grapevine rupestris stem pitting-associated virus (GRSPaV, *Foveavirus*, *Betaflexiviridae*), GRSPaV-BIB-BR isolate, 8,679 nt, GenBank accession MW357717; grapevine virus B (GVB, *Vitivirus*, *Betaflexiviridae*), GVB-BIB-BR isolate, 7,599 nt, MW357718; grapevine yellow speckle viroid 1 (GYSVd-1, *Apscaviroid*, *Pospiviroidae*), GYSVd1-BIB-BR isolate, 366 nt, MW357719, and hop stunt viroid (HSVd, *Hostuviroid*, *Pospiviroidae*), HSVd-BIB-BR isolate, 297 nt, MW357720. The highest pairwise nt sequence identities between these Brazilian and homologous isolates (with query cover 99-100%) showed 99.39% nt identity with Brazilian GRSPaV NUB1-BR isolate (MK804766); 97.81% nt identity with Brazilian GVB ISA-BR isolate (KX790785); 99.73% nt identity with Nigerian GYSVd1 UDSV1-4 isolate (MF576400), and 100% nt identity with Canadian and Chinese HSVd BacSB11_5310, BacMF3_243, BacMF3_783, and chi100Y-02-4-5 isolates (MT769774, MT769768, MT769769, AB219944), indicating low divergence among compared isolates of these homologous pathogens.

Grapevines harbor a core assembly of viruses and viroids which can be designated as background virome, including GRSPaV, HSVd, and GYSVd-1, probably involved as co-determinants in a synergistic system of disease etiology (SALDARELLI et al., 2017). According to these authors, disease itself is triggered by the really damaging viral species that disrupt the constitution of the natural virome in the plant. In fact, previous reports with samples of grapevines collected in Brazil usually detected GRSPaV, HSVd and/or GYSVd-1 and another virus that was actually implicated in the pathology of the viral disease, for example, GLRaV-3 as determined by FAJARDO et al. (2020). In the present research, GVB stands out in the virome determined by HTS. This supported carrying out of additional analyses on nucleotide sequence homology and phylogenetic relationships involving the studied GVB isolate.

Corky bark disease (CBD), a component of grapevine rugose wood complex, caused by GVB, induces decrease of production, incomplete ripening of grapes and progressive plant decline. Scion cultivars and rootstocks differ in their susceptibility to CBD. Depending on the infected genotype, symptoms may include dark red spotted leaves and mild curling down of leaf edges observed in cultivars of *V. vinifera*, bark swelling and longitudinal cracking of mature canes (*V. labrusca*), and asymptomatic reactions (mainly occurring in rootstocks) (NICKEL et al., 2002). As the genetic constitution of genotype CNPUV 776-25 is composed of 68.2% of *V. vinifera* and 31.8% of other, more rustic species of the genus *Vitis*, the observed mild symptoms are likely the result of the interaction of viral/viroidal pathogens with this particular genotype.

The pairwise nucleotide identity between the only two Brazilian GVB isolates with available complete genomes, GVB-BIB-BR (MW357718) and ISAB-BR (KX790785, 7,605 nt length), previously reported, was 97.81%, showing low variability, considering that these isolates were collected from different grapevine hosts (genotype CNPUV 776-25, a hybrid grape and cv. Isabel, a *V. labrusca*) and different Brazilian geographical regions (SC and RS states, respectively) (Figure 2). Phylogenetic analysis further confirmed the molecular homology analysis,

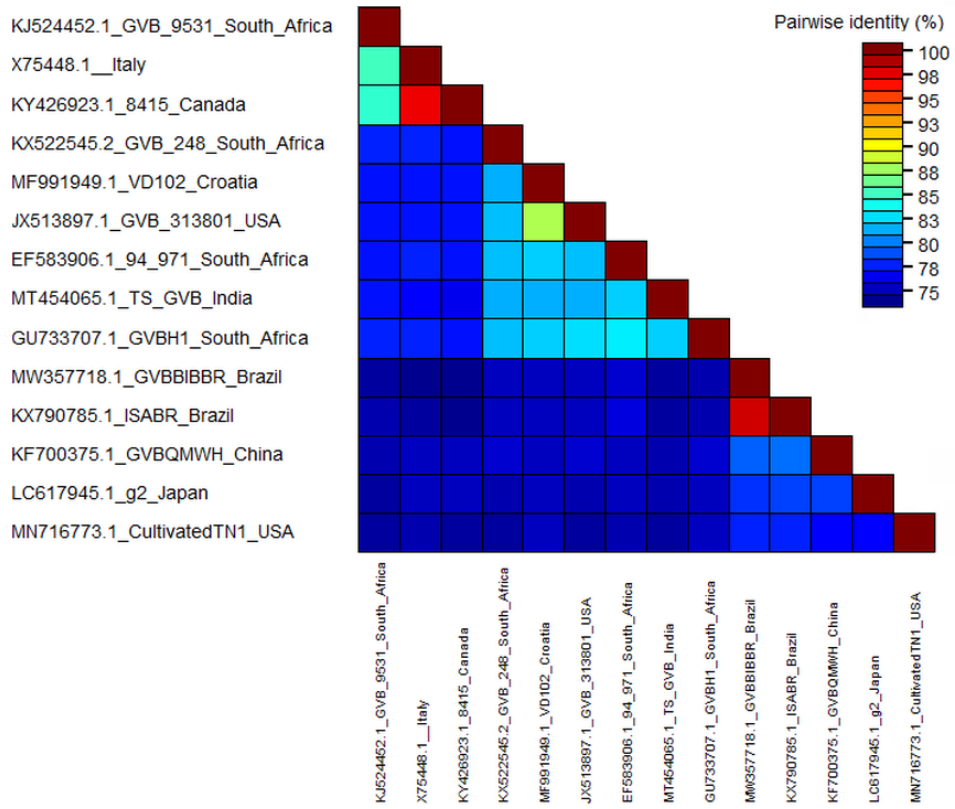

Figure 2 - Pairwise nucleotide sequence identity matrix of two complete genomes of GVB isolates from Brazil, including GVB-BIB-BR, and others from elsewhere retrieved from the GenBank, generated using SDT software. Isolates are identified by their GenBank accession number and country of origin.

clustering both Brazilian isolates in the same branch indicating the closest phylogenetic relationship. Some other GVB isolates from different countries (South Africa, India, USA, Croatia, Canada, Italy, Japan and China) and vine cultivars were distributed in the two main clusters (Figure 3). The close phylogenetic clustering of several isolates from different geographically distant countries (as observed e.g. for GVB) suggests a long term uncontrolled spread of these pathogens and their widespread dissemination, probably through the exchange and trade of infected propagation material (GLASA et al., 2020).

The aetiology of the disease observed in the plant of genotype CNPUV 776-25 cannot be properly elucidated, as the coexistence of several virus/viroid agents in a single grapevine plant (possibly acting in synergy or antagonism) hampers the establishment of a connection between the observed symptoms and a particular pathogen. HTS-based characterization of virus isolates highlights the need for a continual assessment of the grapevine virus molecular variability as a prerequisite to understand the virus variability (GLASA et al., 2020). Results demonstrated the complexity of grapevine viral diseases and showed that multiple virus/viroid infection of grapevine can be considered the rule, since this condition represents the vast majority of reported cases.

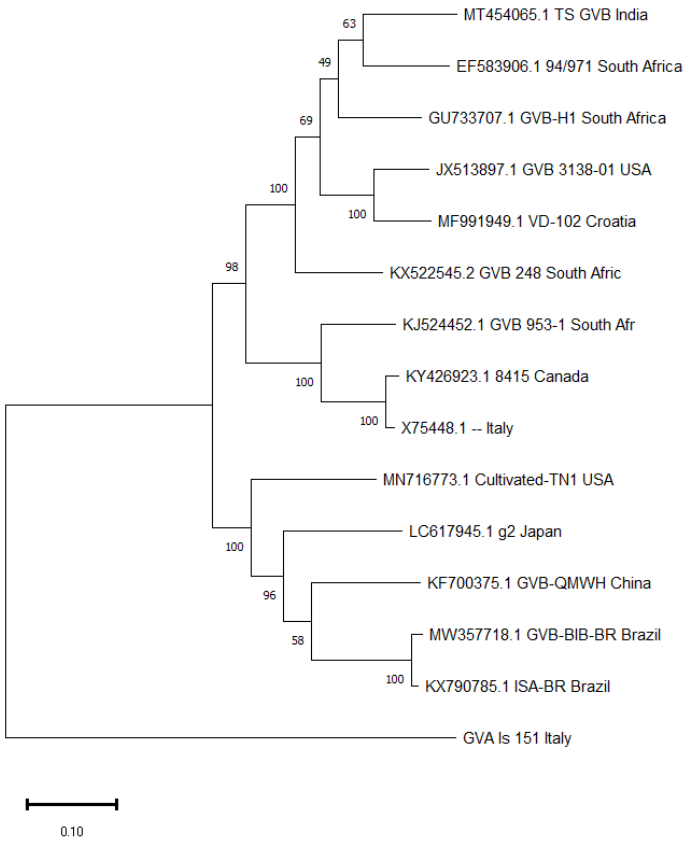

Figure 3 - Phylogenetic relationship based on the alignment of the complete genome sequences of GVB isolates from Brazil (GVB-BIB-BR and ISA-BR) and others retrieved from the GenBank. The tree was constructed by the Maximum Likelihood method and Tamura-Nei model, and 1,000 bootstrap replications implemented using MEGA X software package. Isolates are identified by their GenBank accession number and country of origin. Outgroup species is GVA. The scale bar indicates a genetic distance of 0.10.

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DECLARATION OF CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHORS' CONTRIBUTIONS

All authors contributed equally for the conception and writing of the manuscript. All authors critically revised the manuscript and approved of the final version.

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