

Molecular variants of *Grapevine rupestris stem pitting-associated virus* infecting grapevines (*Vitis* spp.) in Brazil

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ABSTRACT: *Grapevine rupestris stem pitting-associated virus (GRSPaV)* is one of the most common viruses of grapevine. It is involved in the graft-transmissible disease *rupestris stem pitting* of the *rugose wood complex*. The objective of the research was to perform the molecular characterization of the coat protein (CP) gene of sixteen Brazilian GRSPaV isolates aiming to determine the occurrence of molecular variants (strains) of this virus. Nine grapevine samples were evaluated, from which dsRNA was extracted. Nucleotide sequences were generated by Next generation sequencing (NGS). Fifteen complete sequences of the GRSPaV CP gene were obtained and phylogenetically analyzed. Multiple alignments of the sequences showed identities of nucleotides ranging from 82% to 99%, suggesting high variability among the CPs of Brazilian isolates. The study revealed that genetic variability of GRSPaV comprising three molecular variants is also present in Brazilian grapevine genotypes.

Key words: GRSPaV, Foveavirus, strains, lineages, genetic variability.

Variantes moleculares de *Grapevine rupestris stem pitting-associated virus* infectando videiras (*Vitis* spp.) no Brasil

RESUMO: O GRSPaV é um dos vírus mais comuns da videira. Está associado à doença transmissível por enxertia denominada caneluras de *rupestris* que compõe o complexo do lenho rugoso. O objetivo do trabalho foi realizar a caracterização molecular do gene da proteína capsidial (CP) de 16 isolados brasileiros de GRSPaV visando determinar a ocorrência de variantes moleculares desse vírus. Nove amostras de videira foram avaliadas das quais foi extraído dsRNA. As sequências de nucleotídeos foram geradas pelo sequenciamento de nova geração (NGS). Quinze sequências completas do gene CP de GRSPaV foram obtidas e filogeneticamente analisadas. Os alinhamentos múltiplos entre as sequências mostraram identidades de nucleotídeos variando de 82% a 99%, sugerindo alta variabilidade entre as CPs de isolados brasileiros. O estudo revelou que a variabilidade genética de GRSPaV compreendendo três variantes moleculares também está presente nos genótipos de videira no Brasil.

Palavras-chave: GRSPaV, Foveavirus, estirpes, linhagens, variabilidade genética.

Among grapevine (*Vitis* spp.) pathogens, viruses stand out, considering that this crop is susceptible to numerous graft-transmissible agents that cause several diseases, reducing plant vigor, yield, productivity and also fruit quality. *Grapevine rupestris stem pitting-associated virus* (GRSPaV), genus *Foveavirus*, family *Betaflexiviridae*, is one of the most common viruses of grapevine and is widely distributed in many grape-growing areas of the world. GRSPaV is not mechanically transmissible and natural vectors are unknown. This virus is involved in the *rugose wood complex* as the agent of the disorder known as *Rupestris stem pitting*, and some of its strains have been shown to have a very close association with specific symptoms and disorders (BOUYAHIA et al., 2005). Since the

characterization of GRSPaV in 1998, a number of studies have revealed the remarkable genetic variability of this virus, which may reflect the unclear pattern of its biological effects (MENG & GONSALVES, 2007). At least, ten GRSPaV variants have been fully sequenced: GRSPaV (GenBank accession AF026278), RSPaV-1 (AF057136), -SG1 (AY881626), -BS (AY881627), -SY (AY368590), -PN (AY368172), -MG (FR691076), -PG (HE591388), -3138-07 (JX559646) and -GG (JQ922417) (HU et al., 2015). They are divided into four main groups depending on their complete genome sequences, and each group has the reference variant lineage (in bold) defined by NOLASCO et al. (2006) or MENG et al. (2006), respectively as follows: **GRSPaV-SY** and VS (1 or IV), **GRSPaV-SG1**, MG and 3138-

07 (2a or II), **RSPaV-1**, GRSPaV, GG and PG (2b or I) and **GRSPaV-BS** (3 or III) and an additional group GRSPaV-PN (V) (TERLIZZI et al, 2011). The positive-sense, ssRNA of GRSPaV is 8.7kb in length, and encodes five open reading frames (ORFs) in which the ORF in the 3' end encodes a 28kDa coat protein (CP) with 780bp (HU et al., 2015). Information about the variability of GRSPaV strains in Brazilian grapevines is very restricted (BASSO et al., 2010). Therefore, the aim of this research was to perform a molecular characterization of coat protein genes of GRSPaV to document the prevalence of molecular variants infecting Brazilian grapevines.

Nine genotypes were sampled (one sample/genotype) in three grapevine collections in Rio Grande do Sul, São Paulo and Pernambuco States, Brazil. The evaluated *V. vinifera* plants were symptomatic, exhibiting foliar symptoms related to viruses and other

genotypes were asymptomatic (Table 1). The GRSPaV CP isolates were characterized in enriched dsRNAs extracted from 30g of bark scrapings per sample using CF11 cellulose. Sequencing data was generated from a complementary DNA library that was constructed by Macrogen (Korea) or Eurofins (USA). The Illumina HiSeq2000 platform was used to generate the paired-end reads. CLC Genomics Workbench software was used for quality trimming and de novo contig assembly from the reads. All contigs were analyzed using NCBI's Blast program (<http://www.ncbi.nlm.nih.gov/blast>) against the viral RefSeq databases and also all contigs identified as GRSPaV were individually analyzed using Blastn against the GenBank database (FAJARDO et al., 2017).

Multiple sequence alignments of nucleotides (nt) and deduced amino acids (daa) were performed using ClustalX 2.1 and a pairwise

Table 1 - Information about sequences of GRSPaV included in study and available in GenBank.

Country*	Genotypes	Isolate	GenBank accessions	Nucleotide identity with sequence variant	Variant lineage
RS-Brazil	<i>V. vinifera</i> cv. Cabernet Sauvignon	CS-BR	KT008367	BS (96%)	BS
RS-Brazil	<i>V. vinifera</i> cv. Cabernet Sauvignon	VVCS2	KT008368	3138-07 (96%)	SG1
RS-Brazil	<i>V. flexuosa</i>	VF1	KT008369	BS (97%)	BS
RS-Brazil	<i>V. flexuosa</i>	VF2	KT008370	RSPaV-1 (99%)	RSPaV-1
RS-Brazil	<i>V. labrusca</i> cv. Isabel	VLIS1	KT008371	BS (95%)	BS
RS-Brazil	<i>V. labrusca</i> cv. Isabel	VLIS2	KT008372	BS (92%)	BS
SP-Brazil	<i>V. labrusca</i> cv. Isabel	VLIS3	KT008373	BS (99%)	BS
SP-Brazil	<i>V. labrusca</i> cv. Isabel	ISA-BR	KT008374	RSPaV-1 (99%)	RSPaV-1
SP-Brazil	<i>V. gigas</i>	VG	KT008375	RSPaV-1 (99%)	RSPaV-1
PE-Brazil	<i>V. vinifera</i> cv. Syrah	VVSY1	KT008376	BS (95%)	BS
PE-Brazil	<i>V. vinifera</i> cv. Syrah	VVSY2	KT008377	PG (92%)	RSPaV-1
PE-Brazil	<i>V. vinifera</i> cv. Tempranillo	TEMP-BR	KT008378	3138-07 (96%)	SG1
RS-Brazil	<i>V. vinifera</i> cv. Italia	VVIT	KT008379	BS (90%)	?
RS-Brazil	<i>V. vinifera</i> cv. CG90450	VVCG1	KT008380	BS (96%)	BS
RS-Brazil	<i>V. vinifera</i> cv. CG90450	VVCG2	KT008381	3138-07 (90%)	?
RS-Brazil	<i>V. berlandieri</i> x <i>V. rupestris</i> cv. 110R	VN-BR	KY321511**	PG (99%)	---
USA	<i>V. riparia</i>	RSPaV-1	NC_001948	--	RSPaV-1
Italy	<i>V. vinifera</i> cv. Moscato Giallo	GRSPaV-MG	FR691076	--	SG1
USA	<i>V. vinifera</i> cv. Syrah	GRSPaV-SY	AY368590	--	SY
Canada	<i>Vitis</i> sp. cv. Bertille Seyve 5563	GRSPaV-BS	AY881627	--	BS
USA	<i>Vitis vinifera</i> cv. Pinot Noir	GRSPaV-PN	AY368172	--	PN
USA	grapevine	GRSPaV	AF026278	--	RSPaV-1
USA	<i>V. rupestris</i> cv. St. George	GRSPaV-SG1	AY881626	--	SG1
Canada	<i>V. vinifera</i>	3138-07	JX559646	--	SG1
Italy	<i>V. vinifera</i> cv. Pinot Gris	PG	HE591388	--	RSPaV-1
USA	<i>V. riparia</i> cv. Grande Glabre	GRSPaV-GG	JQ922417	--	RSPaV-1
Portugal	<i>V. sylvestris</i>	Vs279-2	AY927684	--	SY
USA	<i>V. vinifera</i>	GRSPaV-WA	KC427107	--	SG1

*The Brazilian States of Rio Grande do Sul (RS), São Paulo (SP) and Pernambuco (PE), **partial CP gene and “?” not defined.

nucleotide sequence identity matrix was generated using Sequence Demarcation Tool version 1.2 (SDT v1.2) (MUHIRE et al., 2014). The matrix generation of nt and daa identities were also performed using BioEdit v.7.2.5 software. The 15 sequences obtained in this research were aligned with 12 representative isolates of GRSPaV from the GenBank, which accession numbers of the nt sequences of the GRSPaV CPs used for phylogenetic analysis are presented in Table 1. Phylogenetic relationships were determined from the aligned sequences by using the neighbor-joining (NJ) method using Kimura 2-parameter with gamma distribution (G) and 2,000 bootstrap replications implemented in Molecular Evolutionary Genetics Analysis (MEGA 7.0.21) software package.

Twenty rooted rootstocks of grapevine cv. 110R (*V. berlandieri* x *V. rupestris*), not inoculated, exhibiting severe vein necrosis on leaves were sampled for total RNA extractions using the RNeasy Plant Mini kit (Qiagen) from 100mg of petioles of symptomatic leaves, grinding plant tissue in liquid nitrogen. Primer pairs used to amplify the partial CP of GRSPaV for one-step RT-PCR were 48V/49C (LIMA et al., 2006). The RT-PCR in a single step was carried out using One Step RT-PCR kit (Qiagen) and reactions were performed according to the manufacturer with 4µL of total RNA (ca. 400ng). The RT-PCR reactions and amplification cycling, amplified DNA analysis, elution of DNA fragments, cloning, purification of recombinant plasmids from *Escherichia coli* and nucleotide comparisons were performed as described by BASSO et al. (2010). The automatic nucleotide sequencing was performed with two clones.

Fifteen complete sequences of the GRSPaV CP gene (780 nt and 259 daa length) were obtained by NGS from nine different grapevine genotypes including wild and commercial cultivars of wine and table grapevines (Table 1). Fifteen CP sequences for Brazilian isolates of GRSPaV were deposited in GenBank under accession numbers KT008367 through KT008381 (Table 1). Multiple alignments between these sequences showed identities ranging from 82% to 99% (Figure 1A) and, 93% to 100%, nt and daa; respectively, suggesting high variability among the CPs of Brazilian isolates.

The CP-based phylogenetic relationships of GRSPaV isolates from three Brazilian states were compared among themselves and with reference sequences from other grape growing regions. This analysis, using the NJ method, included a total of 15 CP sequences of local isolates of GRSPaV and 12 reference isolates (sequence variants) retrieved from the GenBank. Results showed segregation of 13 Brazilian GRSPaV CP sequences into three major lineages

represented by the GRSPaV-BS, -SG1 and RSPaV-1 isolates (Table 1, Figure 1B). Only two studied isolates (VVIT and VVCG2) did not cluster in the previously defined groups (lineages) (Figure 1B); although, these isolates showed high nucleotide identities of CP gene with reference isolates of GRSPaV, 90% with BS and 90% with 3138-07, respectively (Table 1). Each one of these lineages was assigned to a reference isolate to maintain a standardized nomenclature of GRSPaV sequence variant groups in analogy with a previous report by NOLASCO et al. (2006) and MENG et al. (2006). There have already been reports on the occurrence of sequence variants of GRSPaV in vineyards worldwide (LIMA et al., 2006).

GRSPaV is implicated in the rupestris stem pitting disease of the rugose wood complex responsible for graft incompatibility, delayed bud burst, severe decline, stem pitting, and even death of vines. This virus has been demonstrated to comprise a family of molecular variants, and a specific relationship between some of the viral variant groups and distinct *Vitis* species appears to exist. It seems that the RSPaV-1 and GRSPaV-SG1 lineages are more prevalent, whereas the GRSPaV-BS and GRSPaV-VS (SY) lineages are less common (MENG & GONSALVES, 2007). These authors also mentioned that viral variants that are distinct from these four variant groups (lineages) may exist in nature, and that additional distinct variants may be discovered later on.

From grapevines cv. 110R, exhibiting vein necrosis, it was possible to amplify a DNA fragment (330bp) (GenBank KY321511) with 100% nt identity between this and the CF210 (EF690384) Brazilian isolate and 99% with PG isolate (Table 1). Some information about biological properties associated with molecular variants of GRSPaV is available such as decline and incompatibility problems associated with GRSPaV-SY (AY368590) (LIMA et al., 2006).

Reliable and comprehensive detection and phylogenetic analysis of molecular variants of GRSPaV infecting grapevines were achieved after applying NGS and conventional RT-PCR. This result contributes to the improvement of the viral diagnosis, since it expands the knowledge on the genetic variability of GRSPaV in Brazil at the level of sequence variants. It is necessary to extend this study to sample productive Brazilian vineyards aiming to determine the incidence of representative variants in Brazilian grape growing areas. This study revealed that genetic variability of GRSPaV is also present in Brazilian grapevine genotypes and the information should be taken into consideration in symptomatological assessments and biological and molecular indexing.

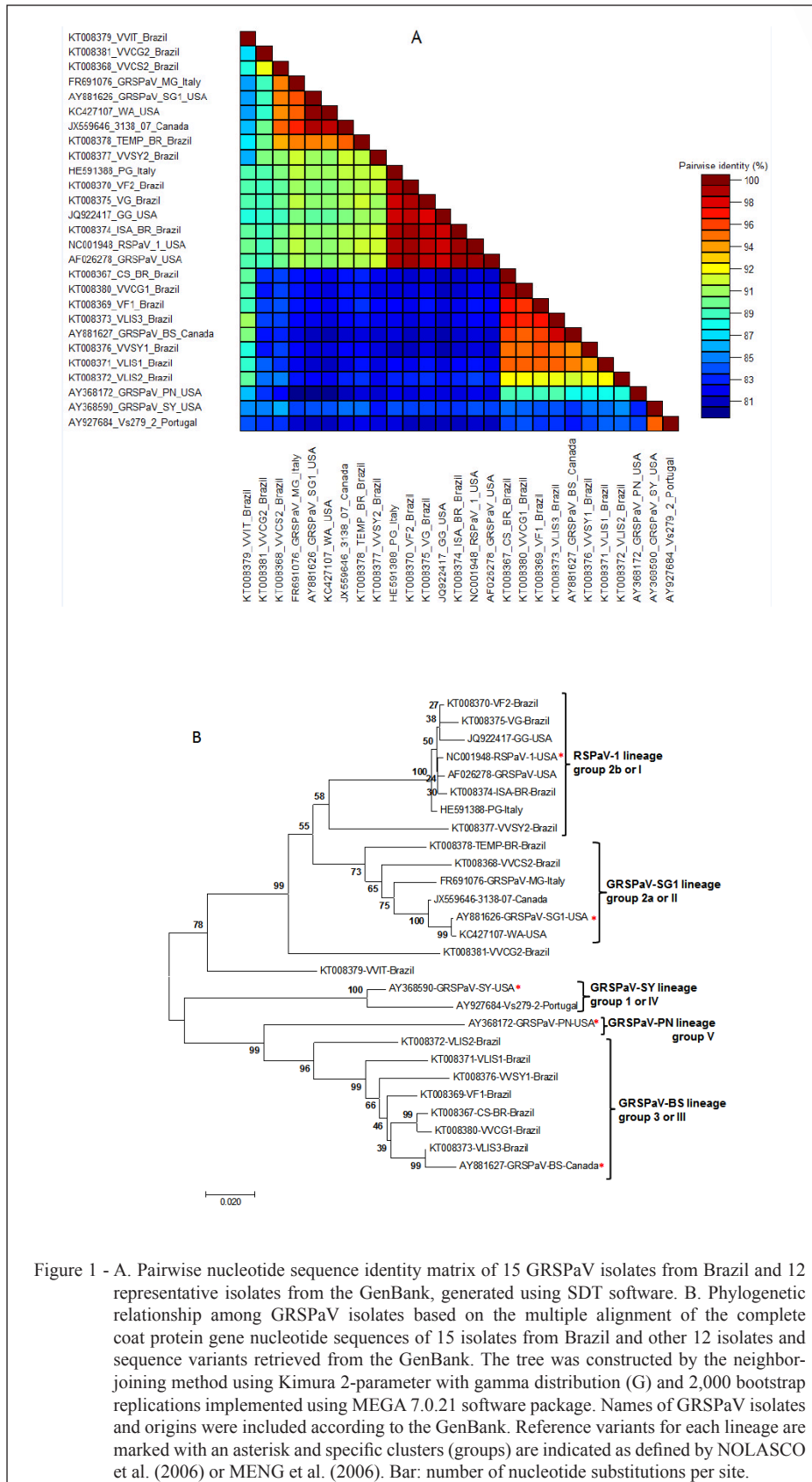


Figure 1 - A. Pairwise nucleotide sequence identity matrix of 15 GRSPaV isolates from Brazil and 12 representative isolates from the GenBank, generated using SDT software. B. Phylogenetic relationship among GRSPaV isolates based on the multiple alignment of the complete coat protein gene nucleotide sequences of 15 isolates from Brazil and other 12 isolates and sequence variants retrieved from the GenBank. The tree was constructed by the neighbor-joining method using Kimura 2-parameter with gamma distribution (G) and 2,000 bootstrap replications implemented using MEGA 7.0.21 software package. Names of GRSPaV isolates and origins were included according to the GenBank. Reference variants for each lineage are marked with an asterisk and specific clusters (groups) are indicated as defined by NOLASCO et al. (2006) or MENG et al. (2006). Bar: number of nucleotide substitutions per site.

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