DOI: http://dx.doi.org/10.1590/1678-992X-2016-0328

ISSN 1678-992X

SCIENTIA AGRICOLA

Molecular characterization of GSyV-1 and GLRaV-3 and prevalence of grapevine viruses in a grape-growing area

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Edited by: Emerson Medeiros Del Ponte

Received August 19, 2016
Accepted November 08, 2016

ABSTRACT: The aims of this study were to determine the prevalence of viruses in 119 samples from 32 grapevine cultivars, collected from nine vineyards in a specific grape-growing area in southeastern Brazil, perform a partial molecular characterization of 14 isolates of *Grapevine Syrah virus 1* (GSyV-1) and *Grapevine leafroll-associated virus 3* (GLRaV-3) and assess the coat protein genetic variability of these viruses. The detection of viruses was implemented by real-time RT-PCR (reverse transcription polymerase chain reaction) aiming to detect seven viruses and one viroid. With the exception of the Grapevine Cabernet Sauvignon reovirus (GCSV), the viruses and viroid that were evaluated were widespread in the sampled areas, often in high prevalence and multiple infections, ranging from 15 % up to 76 %. Eight isolates of GSyV-1 and six of GLRaV-3, partially characterized by complete coat protein gene nucleotide sequencing and a variability study showed nucleotide identities ranging from 91 % to 99 % (GSyV-1) and from 98 % to 100 % (GLRaV-3) among themselves, respectively. Comparisons between conventional and real-time RT-PCR detections were implemented for GSyV-1 and GLRaV-3 infections. Analysis of genetic variability indicated molecular differences between GSyV-1 and GLRaV-3 isolates and negative selection acting on the coat protein gene of both viruses. This is the first report of GSyV-1 in commercial vineyards in Brazil. The survey revealed widespread infections of seven important pathogens in one prominent Brazilian grape-producing region implying contaminated grapevine cuttings in the spread of disease.

Keywords: *Vitis*, diagnosis, variability, incidence, leafroll

Introduction

Grapevine (*Vitis* spp.) is one of the main fruit crops worldwide, in terms of its socioeconomic importance and area under cultivation. It is affected by several graft-transmissible agents that cause associated diseases. Its vegetative propagation has contributed to the worldwide spread of these pathogens and its perennial life cycle has accelerated the mixing of several pathogens in a single vine. These diseases cause crop losses and reduced plant vigor (Maliogka et al., 2015).

In Brazil, the occurrence of 18 viruses has already been reported in many viticultural areas (Basso et al., 2014). *Grapevine yellow speckle viroid 1* (GYSVd-1, *Apscaviroid*) infects grapevines in Brazil, among other viroid species infecting this host elsewhere (Fajardo et al., 2016). *Grapevine Syrah virus 1* (GSyV-1, *Marafivirus*) was first identified in the USA in connection with Syrah decline symptoms (Al Rwahnih et al., 2009). Since then, its presence has been reported in many countries (Glasa et al., 2015). This virus is phylogenetically related to *Grapevine fleck virus* (GFkV, *Maculavirus*) which has spread worldwide and is a non-mechanically transmissible virus associated with fleck symptoms (Sabanadzovic et al., 2001) that can be detected in latent infections in *V. vinifera* cultivars (Martelli, 2014). Grapevine leafroll disease (GLRD) is the most economically damaging and widespread viral disease of grapevine throughout the world (Almeida et al., 2013). It can cause up to 40 % yield losses (Naidu et al., 2015). Several viral species (GLRaV-1, -2, -3, -4 and its strains and -7) designated *Grapevine leafroll-associated virus* (*Closteroviridae*) are related to GLRD, which can occur alone or as a viral complex, amongst which GLRaV-3 stands out (Maree et al., 2013). Grapevine rugose wood disease (GRWD) is a complex disease occurring in several grapevine cultivating regions. Some of the agents associated with GRWD are *Grapevine rupestris stem pitting-associated virus* (GRSPaV) and *Grapevine virus A* and *B* (GVA and GVB, *Betaflexiviridae*) (Martelli, 2014). Grapevine Cabernet Sauvignon reovirus (GCSV, *Reoviridae*) was recently discovered infecting grapevines in the USA (Al Rwahnih et al., 2015).

There are few studies on the diversity and genetic variability of viruses and viroids in vineyards. Determining which species are prevalent in the main producing regions, as well as the characterization and genetic variability studies are important to understand how pathogens evolve, predict epidemics and recommend control measures. The aims of this study were to investigate the occurrence of viruses and viroids in a survey of samples from Brazilian vineyards, perform molecular characterization and molecular variability studies on the coat protein gene of local isolates of two virus species.

Materials and Methods

Plant material and insect vectors

For the purpose of taking a survey, symptomatic and asymptomatic leaves and mature canes of 119

grapevine plants including 32 cultivars were collected at random during the middle summer season in 2016 from nine vineyards (13.2 sample/vineyard and 3.7 sample/cv. in average) cultivated in the São Roque Municipality, in the eastern region of the State of São Paulo, Brazil (Latitude 23º 31' 45" S, Longitude 47º 08' 07" W and altitude 771 m). Table and wine cultivars of grapevines sampled were: *V. vinifera* (Alfrocheiro, Alicante, Alvarinho, Aragonês, Arinto D'ouro, Cabernet Franc, C. Sauvignon, Chardonnay, Fernão Dias, Malbec, Marselan, Moscato Setubal, Pinot Noir, Pinotage, Rebo, Sauvignon Blanc, Syrah, Tinta Cão, Tinta Roriz, Touriga Nacional, Verdelho), *V. labrusca* (Bordô, Bordô Grano D'oro, Concord, Isabel, Niagara Branca, N. Rosada) and hybrids (BRS cultivars: Carmem, Isis, Lorena, Margot, Violeta).

Twenty-eight compound samples of scale insects (11 samples) or mealybugs (17 samples) (ca. 20 adult individuals per sample) were collected inside the vineyards sampled in São Roque. Mealybugs and soft scale insects were identified as *Planococcus citri* (Risso) and *Pseudococcus longispinus* (Targioni Tozzetti) (Hemiptera: Pseudococcidae*)* and *Partenolecanium* spp. (Hemiptera: Coccidae). Both healthy and infected mealybugs and soft scale insects were included in the assays as negative and positive controls, respectively. These insects were maintained in non-hosts of grapevine viruses and in virus infected grapevines.

RNA extraction and quality

The total RNA extractions from 1 g of petioles, veins of leaves or scrapings of mature stems were obtained through the adsorption of nucleic acids on silica particles (Rott and Jelkmann, 2001), grinding plant tissues in liquid nitrogen, and following the prescribed protocol. Total RNA quality was monitored by evaluation using primers and probes for 18S rRNA (Osman et al., 2007). Total RNA was extracted from the mealybugs or scale insects using Trizol reagent (Invitrogen) according to the manufacturer's instructions.

Real time RT-PCR amplification

All plant samples were analyzed for the previously mentioned viruses and the viroid (GCSV, GSyV-1, GRSPaV, GVA, GVB, GLRaV-3, GFkV and GYSVd-1) by real-time RT-PCR (RT-qPCR). In the performed analyses, RNAse-free water, healthy grapevines and positive controls from mother stock plants and the viral collection at Embrapa Grape and Wine, respectively, were included. Real-time RT-PCR reactions (One Step RT-PCR) were carried out in 96-well plates using the TaqMan Fast Virus 1-Step Master Mix (Life Technologies) kit: 3 µL of the 4X TaqMan Fast Virus 1-Step Master Mix, 0.6 µL of the mixture of primers and probe (415 nM primer and 85 nM probe), 3 µL of total RNA (ca. 300 ng) to a final volume of 12 µL. Reactions were performed in a thermocycler StepOnePlus Real-time PCR System (Applied Biosystems) as follows: 45 °C for 35 min (for reverse transcription), 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s (denaturation) and 60 °C for 1 min (annealing and extension). The reaction data were analyzed in terms of presence/absence assays and graphically, using the StepOne Software program v.2.3 (Applied Biosystems), by determining the Cq (quantification cycle). The primers and probes used for viruses and viroid detections by real-time RT-PCR have been previously described (Osman et al., 2007; Osman et al., 2008; Osman and Rowhani, 2008; Bianchi et al., 2015) or designed in this work for GCSV, primers Ctg 468F (5′ACGTTGGATCAACTAGCCGAAG3′) (Al Rwahnih et al., 2015) and GCSV-CS103r (5′ACCCATGTAAATTACACGCCTTC3′) and GCSV-CS103 probe (5′TGCTCCTATGTTCGTTATGCCATG3′). All probes were labeled with 6-FAM or VIC and TAMRA in the 5′ and 3′ ends, respectively. All insect samples were tested for *Grapevine leafroll-associated virus 1* (GLRaV-1), GVA, GVB and GLRaV-3 infections by RT-qPCR.

Conventional RT-PCR amplification

For the molecular characterization of the coat protein (CP) genes, five (GSyV-1) and six (GLRaV-3) detected isolates were selected among infected plants. Additionally, other three isolates of GSyV-1 collected in an experimental vineyard in Bento Gonçalves, southern Brazil (Latitude 29º10'17" S, Longitude 51º31'09" W and altitude 691 m), were characterized. The primer pairs used to amplify GSyV-1 by one-step RT-PCR were GVQCP-R (5′GCATTGCTGCGCATTGGAGG3′), GVQCP-F (5′TCCCAGCTTCAGGGTGAATT3′) (Engel et al., 2010) and GLRaV-3 were LR3-9445c (5′CTACTTCTTTTGCAATAGTT3′) and LR3-8504v (5′ATGGCATTTGAACTGAAATT3′) (Fajardo et al., 2007), complementary and viral sense, respectively. The RT-PCR in a single step was carried out using the One Step RT-PCR kit (Qiagen) and reactions were carried out according to the manufacturer with 4 µL of total RNA (ca. 400 ng). The thermal amplification cycling was: 50 °C for 30 min, 95 °C for 15 min, 35 cycles: 94 °C for 50 s, 50 °C for 50 s, 72 °C for 1 min, and a final extension of 72 °C for 10 min. The RT-PCR products were analyzed on 1 % agarose gels prepared in a TBE buffer pH 8.0, in the presence of ethidium bromide and visualized under UV light.

Comparison of diagnostic tests

To compare real time RT-PCR (RT-qPCR) and conventional RT-PCR for GSyV-1 and GLRaV-3 indexings, seventeen samples, randomly selected among those previously determined as GSyV-1- or GLRaV-3-positive by RT-qPCR, were analyzed by conventional RT-PCR using aforementioned specific primers and reaction conditions.

Cloning and sequencing, nucleotide alignments and phylogenetic relationships

The expected DNA bands were cut from the gels and eluted using the Wizard SV Gel and PCR Clean-Up

System kit (Promega). The eluted DNA fragments were ligated into pGEM-T Easy vector (Promega). The recombinant plasmids were used to transform *Escherichia coli* DH5α competent cells by heat shock. The recombinant plasmids of transformed bacterial colonies were extracted using the Wizard Plus SV Minipreps DNA Purification System kit (Promega). The presence of the cloned viral fragment in the recombinant plasmids was confirmed by digestion with the *Eco*RI restriction enzyme (Sambrook and Russell, 2001). The automatic nucleotide sequencing (Sanger method) was carried out with two clones per isolate. Multiple sequence alignments of nucleotides (nt) and deduced amino acids (daa) and the matrix generation of nt and daa identities were carried out using Clustal X 1.8 (Thompson et al., 1997) and BioEdit 7.2.5 softwares. The sequences obtained for GSyV-1 were aligned with the reference sequence of GSyV-1 (GenBank NC_012484) and all complete coat protein gene sequences available in GenBank. Also GLRaV-3 sequences were aligned with the reference sequence of GLRaV-3 (NC_004667) as well as with all CP genes of 13 GLRaV-3 isolates with complete genome available in the GenBank, and with six other Brazilian GLRaV-3 isolates that had been previously characterized. Phylogenetic relationships were determined from the aligned sequences by using the maximum parsimony method (10,000 bootstrap replications) implemented in the MEGA 6.0 program (Tamura et al., 2013). The GenBank accession codes of the nucleotide sequences of the isolates used for phylogenetic analysis are presented in Table 1 and Figures 1 and 2. The molecular weight (MW) of the coat proteins of GSyV-1 and GLRaV-3 were calculated using the EXPASy software program (http://web.expasy.org/compute_pi/).

Description of the coat protein genetic variability and selection analysis

The molecular variability descriptors [total number of segregating sites (*S*), mean nucleotide differences among sequences (*K*), nucleotide diversity (π), haplotypes number (H), haplotype diversity (Hd) and Watterson's estimator for the population-scaled mutation rate] were estimated using the DnaSP software program v.5.10 (Rozas et al., 2003). The mean pairwise number of π per site was also calculated using a sliding window of 100 bases, with a step size of 25 bases across coat protein genes of GSyV-1 and GLRaV-3. Coat protein gene and site specific selection pressures were analyzed us-

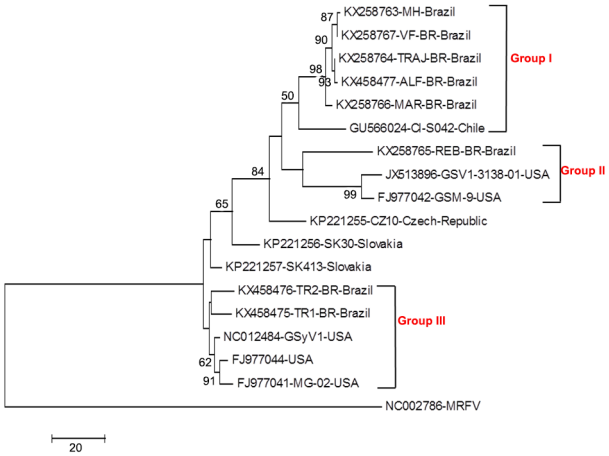

Figure 1 – Phylogenetic tree based on the alignment of nucleotide sequences of isolates REB-BR, MAR-BR, TR1-BR, TR2-BR and ALF-BR from São Roque and isolates MH, TRAJ-BR and VF-BR from Bento Gonçalves, southeastern and southern Brazil, respectively and other foreign isolates of *Grapevine Syrah virus 1* (GSyV-1, *Tymoviridae, Marafivirus*). The tree was constructed by the maximum parsimony method, using the MEGA 6.0 program, and bootstrap support from 10,000 replications. Names of GSyV-1 isolates and origins were included according to GenBank accession codes, and specific clusters are indicated. *Maize rayado fino virus* (MRFV), the type species of the genus *Marafivirus* (NC_002786) was used as outgroup. Bar = number of substitutions per site.

Table 1 – *Grapevine Syrah virus 1* (GSyV-1) and *Grapevine leafroll-associated virus 3* (GLRaV-3) molecularly characterized isolates.

Virus	DNA fragments	Cultivar (species and sample of grapevine)	Isolate	GenBank accession code
GSyV-1	723 bp (coat protein gene, 627 bp)	Rebo (*V. vinifera*, sample 19)	REB-BR	KX258765
GSyV-1	723 bp (coat protein gene, 627 bp)	BRS Margot (hybrid, sample 38)	MAR-BR	KX258766
GSyV-1	723 bp (coat protein gene, 627 bp)	Touriga Nacional (*V. vinifera*, sample 68)	TR1-BR	KX458475
GSyV-1	723 bp (coat protein gene, 627 bp)	Touriga Nacional (*V. vinifera*, sample 81)	TR2-BR	KX458476
GSyV-1	723 bp (coat protein gene, 627 bp)	Alfrocheiro (*V. vinifera*, sample 118)	ALF-BR	KX458477
GSyV-1*	723 bp (coat protein gene, 627 bp)	*Vitis flexuosa* (wild grapevine, sample 2M-BG)	VF-BR	KX258767
GSyV-1*	723 bp (coat protein gene, 627 bp)	Moscato de Hamburgo (*V. vinifera*, sample 6-BG)	MH	KX258763
GSyV-1*	723 bp (coat protein gene, 627 bp)	Trajadura (*V. vinifera*, sample 18-BG)	TRAJ-BR	KX258764
GLRaV-3	942 bp (coat protein gene)	Niagara Rosada (*V. labrusca*, sample 5)	NR-BR	KX499443
GLRaV-3	942 bp (coat protein gene)	BRS Margot (hybrid, sample 37)	MARG-BR	KX499444
GLRaV-3	942 bp (coat protein gene)	Chardonnay (*V. vinifera*, sample 48)	CH-BR	KX499445
GLRaV-3	942 bp (coat protein gene)	Touriga Nacional (*V. vinifera*, sample 82)	TN-BR	KX499446
GLRaV-3	942 bp (coat protein gene)	BRS Violeta (hybrid, sample 100)	VI-BR	KX499447
GLRaV-3	942 bp (coat protein gene)	Moscato Setubal (*V. vinifera*, sample 119)	MS-BR	KX499448

*Samples collected from Bento Gonçalves, southern Brazil with further samples from São Roque, southeastern Brazil.

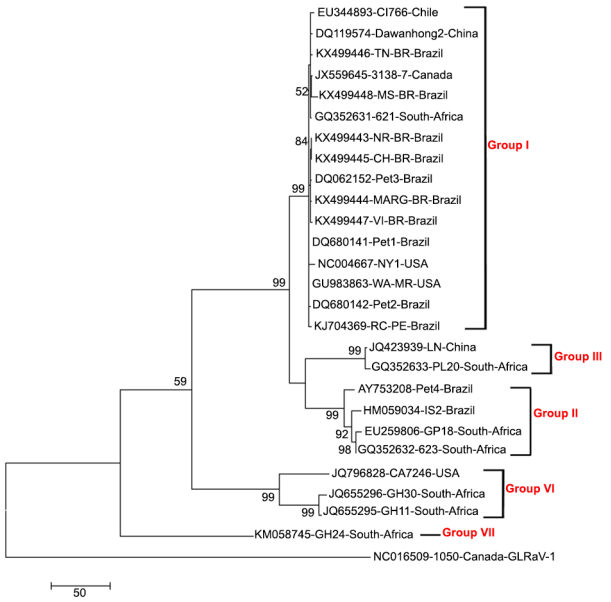

Figure 2 – Phylogenetic tree based on the alignment of nucleotide sequences of isolates NR-BR, MARG-BR, CH-BR, TN-BR, VI-BR and MS-BR from São Roque; isolates RC-PE, Pet-1, Pet-2, Pet-3 and Pet-4 from Petrolina and isolate IS2 from Bento Gonçalves, southeastern, northeastern and southern Brazil, respectively and other foreign isolates of *Grapevine leafroll-associated virus* 3 (GLRaV-3). The tree was constructed by the maximum parsimony method, using the MEGA 6.0 program, and bootstrap support from 10,000 replications. Names of GLRaV-3 isolates and origins were included according to GenBank accession codes, and specific clusters are indicated as defined by Maree et al. (2015). *Grapevine leafroll-associated virus 1* (GLRaV-1, *Closteroviridae, Ampelovirus*) (NC_016509) was used as outgroup. Bar = number of substitutions per site.

ing four algorithms, Single Likelihood Ancestor (SLAC), Fixed Effects Likelihood (FEL), Random Effects Likelihood (REL) and Partitioning for Robust Inference of Selection (PARRIS) within the HyPhy software (http://www.hyphy.org) implemented in the Datamonkey server (www.datamonkey.org) with default conditions. The nucleotide substitution model used was General reversible substitution (REV). To avoid the recombination events effect on the selection analysis, recombination analysis was carried out using the RDP program v.3.44 (Martin et al., 2010) and the GARD method (available at the Datamonkey server).

Results and Discussion

Incidence of viruses and viroid in grapevines

The viruses and viroid infecting grapevines in the São Roque municipality were unknown and the knowledge of the phytosanitary status of a defined region is important for proposing management and control procedures for grapevine diseases. Accordingly, 119 grapevine accessions assayed by real time RT-PCR revealed a high level of infection in the analyzed samples: 0 % (GCSV), 15 % (GSyV-1), 28 % (GVB), 61 % (GRSPaV), 62 % (GVA), 65 % (GLRaV-3), 66 % (GYSVd-1) and 76 % (GFkV). The results also showed 11 %, 8 % and 81 % of incidence of healthy samples (without eight pathogens tested for), infected with only one pathogen and infected with two or more viruses or viroid, respectively. To confirm the RT-qPCR results, two GYSVd-1 amplicons of 126 bp were sequenced resulting in 98 % nucleotide identities with other Brazilian isolates of this viroid (Fajardo et al., 2016).

In general, these pathogens were found widely distributed in the sampled vineyards. These results were supported by the fact that many grapevines (34 %) exhibited low vegetative vigor and general symptoms of virus infections [leafroll, unusual color (reddening or yellowing) or atypical appearance (coriaceous aspect) of the leaves, rugose wood (corky bark, grooving or pitting)] as a consequence of long-term maintenance (mean of 7 years) in vineyards. Grapevines infected by viruses may not exhibit noticeable symptoms because infections may be latent or asymptomatic in some host genotypes and commercial cultivars such as those usually observed in *Vitis labrusca* and hybrids; however, even in these cases, the virus infection can cause damage. Although crop losses were not assessed in the sampled vineyards, they probably occurred as has already been determined by other authors (Vega et al., 2011; Naidu et al., 2015). The correlation of virus and viroid infections and symptom expression might be related to other factors, such as temperature, certain virus isolates or strains, or multiple pathogen infections, making it impossible to diagnose precisely viruses and viroid infections based exclusively on symptomatology (Olmos et al., 2016). Despite these findings, there is a need for further evaluation of other grape-growing areas to determine the geographical distribution of these pathogens in Brazil. These results expand the knowledge about the incidence and virus distribution infecting grapevines in a representative Brazilian grape-growing region, and provide relevant information for the development of control strategies and management of these diseases, with emphasis on the importance of the use of propagation material of virus-free vine in the establishment of new vineyards.

Detection of viruses and a viroid (GRSPaV, GVA, GVB, GLRaV-3, GFkV and GYSVd-1) infecting grapevine in Brazil has already been reported (Basso et al., 2014) as well as another viral species (GSyV-1), not previously reported in commercial Brazilian vineyards. In many grape-growing countries and regions, virus surveys have been conducted on grapevines, for example, in Argentina (Volpe et al., 2010), Chile (Fiore et al., 2011), China (Liu et al., 2013), the United States (Jones et al., 2015), Slovakia, the Czech Republic (Glasa et al., 2015) and in two regions of northeastern Brazil (Catarino et al., 2015), in which the incidence of viruses was highly variable, depending on the viral species, grapevine cultivars and

sampled regions. However, invariably, significant infection rates were reported, similar to the observation made in the present survey.

The presence of, at least, six different viruses and one viroid infecting vineyards in São Roque may be attributable to the redistribuition of infected propagative materials from other Brazilian infected grape-growing regions or from other countries through the planting of imported, infected propagative materials and cuttings. In conclusion, it was shown by indexing that viruses and one viroid were able to infect 106 out of 119 samples analyzed comprising 32 different grapevine genotypes that included cultivars of American (*V. labrusca*) and European (*V. vinifera*) grapes, interspecific hybrids and rootstocks (Table 1).

Mealybugs and soft scale insects as vectors

RT-qPCR analyses of potential vectors collected in the same vineyards showed that 0 % (GLRaV-1), 4 % (GVB), 18 % (GVA) and 61 % (GLRaV-3) out of 28 samples of mealybugs and soft scales were infected. These rates were similar to those observed for the same viruses in the analyzed grapevine samples, suggesting that mealybugs and soft scale insects are spreading these virus species in the assayed vineyards. Another point to be considered is the availability of infected grapevines inside and outside the vineyard (source of inoculum) from which viruliferous vectors would perform transmission. Thus, the high frequency of virus-infected vines emphasizes the importance of clean plant materials as well as control of mealybugs (Jones et al., 2015).

Several mealybugs and soft scale species have already been reported as virus vectors in grapevine such as those mealybug species tested in this study and *Partenolecanium corni* (Almeida et al., 2013). GLRaV-1 and -3, GVA and GVB are transmitted by mealybugs and soft scale insects in a semipersistent manner (Tsai et al., 2010; Le Maguet et al., 2012), while GSyV-1 could be transmitted by leafhoppers (Al Rwahnih et al., 2009) and GCSV, GRSPaV, GFkV and GYSVd-1 have vectors that are still unknown or are not vector-transmissible. These viruses are transmitted by grafting with infected propagative materials (Martelli, 2014). In some cases, transmission modes may explain the observed incidence rates of viruses, in others there is no correlation. High incidence of GRSPaV, GFkV and GYSVd-1 has been found. However, since their vectors are not known, their spread is likely attributable to the use of infected cuttings or buds in the initial establishment of the sampled vineyards. Other cases, where high virus incidence rates were also observed (i.e. GLRaV-3 and GVA) could probably be attributed to mealybug transmissions in the sampled vineyards. Similar reasoning would be applied to the other assayed viruses (GCSV, GSyV-1 and GVB).

Comparison of diagnostic tests

In the assays carried out to compare RT-qPCR and conventional RT-PCR using specific primers to amplify two viruses, we detected only 35 % (GLRaV-3) and 29 % (GSyV-1) by conventional RT-PCR, out of seventeen positive samples (100 %) determined by RT-qPCR to both viruses. Divergences in nucleotide identities among isolates of these viral species have been related previously (Fajardo et al., 2007; Glasa et al., 2015), resulting from absence or mismatch of pairing of primers and probes with the virus sequence. This result highlights the relevance of knowledge of sequence variability when designing primers and probes for the reliable detection of a wide range of isolates of target viruses. Besides conventional RT-PCR, RT-qPCR was also used in this work to evaluate its capacity to detect a range of GSyV-1 and GLRaV-3 isolates, regardless of their variabilities. Based on the results obtained, it was demonstrated that primers and probes defined by Bianchi et al. (2015) and Osman et al. (2007) were suitable for a wide diagnosis of GSyV-1 and GLRaV-3, respectively. The GSyV-1 and GLRaV-3 sequence variabilities observed in the molecularly characterized samples could explain that the primers used for conventional RT-PCR were not suitable for accurately covering the sequences analyzed.

Sequencing, nucleotide alignments and phylogenetic relationships

DNA fragments of 723 bp comprising the complete gene of GSyV-1 capsid protein (627 nt and 208 daa) were amplified from five isolates collected in São Roque and three from Bento Gonçalves by RT-PCR using specific primers, and their nucleotide sequences were submitted to the GenBank (Table 1). The multiple alignment of the CP sequences of the Brazilian isolates of GSyV-1 revealed expressive nucleotide and amino acid divergences among certain isolates, suggesting high variability among them. The nucleotide sequences of the eight Brazilian isolates clustered in groups I (MH, VF-BR, TRAJ-BR, ALF-BR and MAR-BR isolates), II (REB-BR) and III (TR1-BR and TR2-BR) (Figure 1), showing nt and daa identities ranging from 91 % to 99 % and from 96 % to 100 % among themselves, respectively. The nt and daa identities between the type-isolate of GSyV-1 (NC_012484) and the Brazilian isolates ranged from 92 % to 98 % and from 97 % to 99 %, respectively, clustering this North American isolate in the group III together with other North American isolates. All Brazilian isolates clustered closer to North and South American isolates than to European isolates (Figure 1). Comparisons among Slovak, Czech and Hungarian strains of GSyV-1 also suggest high variability of European GSyV-1 strains (Czotter et al., 2015; Glasa et al., 2015). Variability among Chilean and foreign GSyV-1 isolates has already been reported by Engel et al. (2010). The calculated MW of the CPs of GSyV-1 Brazilian isolates was ca. 22 kDa, similar to that reported by Al Rwahnih et al. (2009).

DNA fragments of 942 bp comprising the complete gene of GLRaV-3 capsid protein (313 daa) from six isolates collected in São Roque were amplified by

RT-PCR using specific primers, and their nucleotide sequences were submitted to GenBank (Table 1). The multiple alignment of the CP sequences of Brazilian isolates of GLRaV-3 revealed restricted nucleotide and amino acid divergences among isolates, suggesting there is low variability among the analyzed isolates. The nucleotide sequences of the six Brazilian isolates (NR-BR, CH-BR, VI-BR, MARG-BR, TN-BR and MS-BR) clustered together (Figure 2), in group I as defined by Maree et al. (2015), and showed nt and daa identities ranging from 98 % to 100 % and from 98 % to 100 % among themselves, respectively. The nt and daa identities between the type-isolate of GLRaV-3 (NC_004667) and the Brazilian isolates ranged from 98 to 99 % and from 97 % to 99 %, respectively, placing this North American isolate in group I together with all GLRaV-3 isolates from São Roque, Brazil (Figure 2). The calculated MW of the CPs of Brazilian isolates of GLRaV-3 was ca. 35 kDa, similar to that reported by Fajardo et al. (2007). Variability among GLRaV-3 isolates had already been verified (Maree et al., 2015) including some isolates from southern and northeastern Brazil (Fajardo et al., 2007; Catarino et al., 2015). Other previously molecularly characterized Brazilian isolates of GLRaV-3 (Fajardo et al., 2007; Basso et al., 2010; Catarino et al., 2015) also clustered in group I (Pet-1, Pet-2, Pet-3 and RC-PE) and group II (Pet-4 and IS2) (Figure 2), suggesting that the restricted variability observed in São Roque, SP was wider with isolates from grape-growing areas in southern and northeastern Brazil, two Brazilian viticultural regions far apart from each other.

With regard to the genetic variability of the two viruses analyzed, a high level of deduced amino acid identities was found among isolates collected from different grape-growing regions in Brazil, emphasizing the validity of the information about possible common sources of infected propagative vines used in the country. Five GSyV-1 isolates from São Roque (southeastern Brazil) shared 96-100 % daa identities with three isolates from Bento Gonçalves (southern Brazil) (GenBank acession codes in Table 1). Six GLRaV-3 isolates from São Roque (Table 1) shared 94-100 % daa identities with five isolates from Pernambuco State (northeastern Brazil, GenBank KJ704369, DQ680141, DQ680142, DQ062152, AY753208) and one from Bento Gonçalves (GenBank HM059034) (data not shown).

Variability of the CP gene of GSyV-1 and GLRaV-3 isolates infecting a broad range of grapevine cultivars was demonstrated with expressive differences among themselves such as REB-BR, TR1-BR and TR2-BR isolates of GSyV-1. Thus, reliable detection methods for viruses and viroids, besides determining pathogen identity are important not only for taxonomical purposes, but also for constituting an essential requirement for the development of broader spectrum and precise detection methods (Olmos et al., 2016).

Coat protein genetic variability and selection analysis

The coat protein gene molecular variability of GSyV-1 and GLRaV-3 was evaluated using two datasets. The first consisted of both Brazilian and foreign isolates (the same isolates used in phylogenetic analysis for GSyV-1 and GLRaV-3, excluding the outgroup) and the second consisted of Brazilian isolates only. Descriptors for dataset consisting of both Brazilian and foreign isolates indicated higher genetic variability than the dataset consisting of Brazilian isolates only for both viruses. However, the differences verified between the two datasets were lower for GSyV-1 (Table 2). This was probably a consequence of the isolates that were analyzed together originating from different countries. These results corroborate the lowest nt and daa identities observed in the majority of comparisons between Brazilian and foreign isolates. The higher genetic variability is represented in turn by a higher number of segregating sites (S), nucleotide diversity (π), haplotype number (H) and haplotype diversity (Hd) (Table 2).

Nucleotide diversity (π) was lower than 0.089 for both viruses in the two datasets analyzed (Table 2). The GLRaV-3 virus (dataset with both Brazilian and foreign isolates) had the highest π value (0.08834 ± 0.02073), and the lowest π value was from the dataset with Brazilian isolates (0.02461 ± 0.00992). In general, when the variability descriptors for the dataset consisting of Brazilian isolates only are analyzed, the GSyV-1 virus had greater genetic variability. The π value detected in GSyV-1 and GLRaV-3 (for both datasets) is in concordance with the π values found by García-Arenal et al. (2001). These authors presented π values in the range of 0.002 to 0.224 for the coat protein gene of *Cotton leaf curl virus* (CLCuV), *Citrus tristeza virus* (CTV), *Cucurbit yellow stunting disorder virus* (CYSDV), *Groundnut rosette assistor virus* (GRAV),

Table 2 – Descriptors of molecular variability for *Grapevine Syrah virus 1* (GSyV-1) and *Grapevine leafroll-associated virus 3* (GLRaV-3) coat protein (CP) genes from Brazil and other foreign isolates.

Virus	Number of isolates*	CP length (nt)	S^a	K^b	π^c	H^d	Hd^e	Θ-W^f
GSyV-1	17	627	100	33.434	0.05572 ± 0.00423	17	1.0	0.04930
	8		68	26.750	0.04458 ± 0.01205	8	1.0	0.04371
GLRaV-3	26	942	357	83.215	0.08834 ± 0.02073	24	0.994	0.09931
	12		84	23.182	0.02461 ± 0.00992	11	0.985	0.02953

*Two datasets for GSyV-1 and GLRaV-3 were analyzed: the first dataset consisting of Brazilian and foreign isolates. The second dataset consisted of Brazilian isolates only. [a]Total number of segregating sites; [b]Mean number of nucleotide differences between sequences; [c]Nucleotide diversity; Standard deviation is indicated; [d]Haplotype number; [e]Haplotype diversity; [f]Watterson's estimate of the population mutation rate based on the total number of segreganting sites; nt = nucleotides.

Rice tungro spherical virus (RTSV), *Rice yellow mottle virus* (RYMV), *Sweet potato chlorotic stunt virus* (SPCSV) and *Yam mosaic virus* (YMV). Nucleotide diversity (π) was also analyzed throughout the length of the coat protein gene of GSyV-1 and GLRaV-3, using the two previously described datasets. The tendency for π values in the graphic was similar for the two datasets in each virus, differing only in absolute values of π (Figure 3). The GSyV-1 virus showed the greatest nucleotide diversity at positions 175 and 400, and the GLRaV-3 virus presented greater nucleotide diversity close to the N-terminal region (Figure 3). The Watterson's estimator for the population-scaled mutation rate (Θ-W) for the two viruses (both datasets) was in the order of 10^{-2} (Table 2).

Recombination events interfere with the selection analysis. Thus recombination was analyzed and no recombination events were detected in datasets used for site-specific selection analysis (data not shown). In this analysis we used only the datasets consisting of Brazilian isolates, which represent a population for each virus. The presence of positive and negative selection at each site of the coat protein gene was evaluated. The coat protein genes of GSyV-1 and GLRaV-3 showed dN/dS ratios (not synonymous/synonymous substitution ratios) lower than 1.0, indicating negative or purifying selection (Table 3). The coat protein gene of GLRaV-3 showed a lower dN/dS ratio [GLRaV-3 (0.050) and GSyV-1 (0.182)], suggesting it was more constrained. No sites under positive selection in all the methods used have been detected. The number of sites under negative selection varies according to the method employed (Table 3). Selection pressures can be associated with the maintenance of structural features of the virus (García-Arenal et al., 2001). For example, amino acids that are important in the assembly and stabilization of the coat protein are conserved in tobamoviruses (Altschuh et al., 1987). Viral proteins are multifunctional and may be involved in other processes such as replication, cell-to-cell, and long distance movement and transmission (García-Arenal et al., 2001). Therefore, in general, negative selection is predominant in the coding regions of viral proteins. The results presented here are consistent with the fact that the coat protein gene of arthropod-vectored viruses, such as GLRaV-3 and possibly GSyV-1, is the region under strongest negative selection and is the most constrained (Chare and Holmes, 2004; Zanardo et al., 2014).

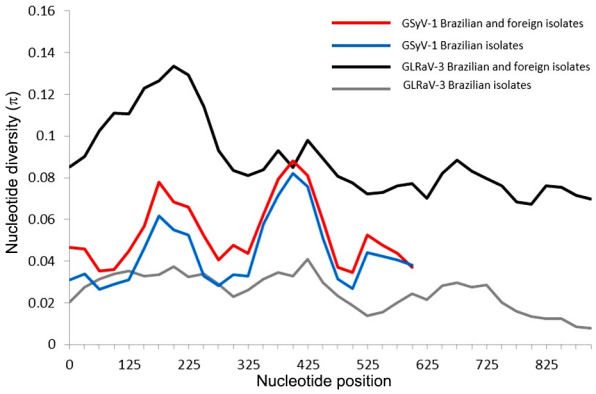

Figure 3 – Mean pairwise number of nucleotide differences per site (nucleotide diversity, π) calculated on a sliding window across coat protein gene of *Grapevine Syrah virus 1* (GSyV-1) and *Grapevine leafroll-associated virus 3* (GLRaV-3).

The results of this study revealed genetic variability in these two viruses in Brazilian grapevines that should be taken into consideration in symptomatological assessments, and biological and molecular indexing. Although the most remarkable changes were observed in the nucleotide sequences, the deduced amino acid sequences of coat proteins have a propensity for less variation among isolates from different origins (geographical regions, hosts). The capsid protein is structural. Thus evolutionary factors could restrict changes that might be deleterious for the virus as was observed for GSyV-1 and GLRaV-3. For example, changes in capsid proteins could result in loss of interactions with vectors or plant host factors. In addition, GSyV-1 and GLRaV-3 have grapevine as a single natural host, which would further restrict these changes (Catarino et al., 2015).

A survey and molecular characterization based on the methods used here allowed detection and further evaluation of the genetic variability and geographical distribution of viral and viroidal pathogens in a Brazilian grape-growing area. This provides valuable information for the implementation of regional management practices to control virus diseases, such as the monitoring of vector transmission in vineyards and establishment of new vineyards with pathogen-free propagative materials.

Acknowledgements

This research was financially supported by Embrapa (Project 02.13.14.002). The authors would like to acknowledge Marcos Fernando Vanni (Embrapa

Table 3 – Selection analysis for coat protein genes of Brazilian isolates of *Grapevine Syrah virus 1* (GSyV-1) and *Grapevine leafroll-associated virus 3* (GLRaV-3).

Virus	Number of isolates	dN/dS	SLAC[a]		FEL[b]		REL[c]		PARRIS[d]
			PS	*NS*	*PS*	*NS*	*PS*	*NS*	*PS*
GSyV-1	8	0.182	-	2	-	27	-	57	-
GLRaV-3	12	0.050	-	-	-	34	-	1	-

PS = number of positive selection sites; NS = number of negative selection sites; (-) no site under selection; [a]Single likelihood ancestor counting (SLAC); [b]Fixed effects likelihood (FEL); [c]Random effects likelihood (REL); [d]Partitioning for robust inference of selection (PARRIS); dN/dS = not synonymous/synonymous substitutions ratios.

Grape & Wine) for his technical support, Márcio Pereira (Federal Institute of Education, Science and Technology of São Paulo, São Roque) for his help in the sampling and Vitor Cezar Pacheco da Silva for taxonomic identification of the mealybugs. Marcelo Eiras is supported by a CNPq (Brazilian National Council for Scientific and Technological Development) research fellowship.

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