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Article

REAL-TIME RT-PCR HIGH-RESOLUTION MELTING CURVE ANALYSIS TO DETECT AND DIFFERENTIATE BRAZILIAN VARIANTS OF GRAPEVINE VIRUSES

ANÁLISE DAS CURVAS DE DISSOCIAÇÃO DE ALTA RESOLUÇÃO DE RT-PCR EM TEMPO REAL PARA DETETAR E DIFERENCIAR VARIANTES BRASILEIRAS DE VÍRUS DA VIDEIRA

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SUMMARY

Detecting and identifying viral infections in perennial plants, such as grapevines, can be challenging. Therefore, the aim of this study was to perform a real-time RT-PCR (RT-qPCR) high-resolution melting (HRM) curve analysis to detect and differentiate Brazilian variants of grapevine leafroll-associated virus 3 (GLRaV-3) and grapevine fanleaf virus (GFLV) in 74 and 10 infected plants, respectively, maintained in a collection block of grapevines. A single amplification curve was generated for each sample by RT-qPCR. Considering the amplified region of genomes of these two viruses, it was possible to identify and distinguish different variants of GLRaV-3 and of GFLV, which showed significantly different melting temperature (Tm) values between themselves, reflecting differences in the nucleotide sequences of the respective amplicons, and allowing discriminating variants and assess the viral diversity in grapevine accessions. The HRM analysis was validated by sequencing and nucleotide comparisons among Brazilian isolates of GLRaV-3 and GFLV.

RESUMO

Detetar e identificar infecções virais em plantas perenes, como videiras, pode ser um desafio. Portanto, o objetivo deste estudo foi realizar uma análise da curva de dissociação de alta resolução (HRM) por RT-PCR em tempo real (RT-qPCR) para detetar e diferenciar variantes do vírus do enrolamento foliar tipo 3 (GLRaV-3) e do vírus do urticado ou nó-curto (GFLV) em 74 e 10 plantas infetadas, respetivamente, mantidas em blocos de coleções de videiras. Uma única curva de amplificação foi gerada para cada amostra por RT-qPCR. Considerando a região amplificada dos genomas dos dois vírus, foi possível identificar diferentes variantes de GLRaV-3 e GFLV, que apresentaram valores de temperatura de dissociação (Tm) significativamente diferentes entre si, refletindo diferenças nas sequências de nucleotídeos dos respectivos DNA amplificados e, assim, constituindo uma forma simplificada de diferenciar variantes e avaliar a diversidade viral em acessos de videiras. A análise de HRM foi validada pelo sequenciamento e comparação de nucleotídeos de isolados brasileiros de GLRaV-3 e GFLV.

INTRODUCTION

Viticulture is an important socioeconomic activity in many regions and countries around the world. However, it can be affected by several phytosanitary disorders, such as infections with multiple systemic pathogens. Grapevines can host more than 90 viruses and viroids with some of them affecting its cultivation and fruit production worldwide (Fuchs, 2020).

Grapevine leafroll disease (GLD) is one of the most important grapevine viral diseases (Cabaleiro *et al.*, 2013). Typically, conspicuous symptoms of grapevine leafroll disease (GLD), leaf reddening or yellowing, downward curling in red- and white-berried cultivars, respectively, appear on mature leaves of *Vitis vinifera* infected by grapevine leafroll-associated virus 3

(GLRaV-3) (species classified in the family Closteroviridae; genus Ampelovirus), young sproutings being commonly asymptomatic. *V. labrusca*, hybrids and rootstocks may be asymptomatic or show mild symptoms only (Burger *et al.*, 2017). Grapevine fanleaf virus, GFLV (species classified in the family Secoviridae; genus Nepovirus) is regarded as the main causal pathogen among agents associated with Grapevine Degeneration (GD), severely affecting sensitive cultivars, inducing progressive decline, damaging yield and fruit quality (Andret-Link *et al.*, 2004). These two viruses have been reported to be widely distributed in many wine-growing regions of the world, including Brazil, infecting vineyards with varying occurrence frequency (Basso *et al.*, 2017). Both viruses are disseminated through the use of infected propagating materials and, transmitted by mealybugs or soft scale insects (GLRaV-3) and by the soil-borne nematode *Xiphinema index* (GFLV) (Digiaro *et al.*, 2017).

Real-time RT-PCR (RT-qPCR) has been successfully used to detect several plant viruses, being a rapid, reliable and quantitative detection method. The development of high-resolution melting (HRM) curve analysis, as an extension to RT-qPCR, provides a rapid, high-throughput, cost-effective and single tube approach to discriminate genotype strains of some phytopathogenic fungi, bacteria, and viruses (Chatzidimopoulos *et al.*, 2019) as well as being able to bring simple solutions for genotyping, mutation scanning and sequence matching in the field of medicine (Reed *et al.*, 2007) and also for detection of adulteration and mixture in agricultural products (Ay and Hürkan, 2023). Several studies have reported the use of HRM for detection of plant viruses in recent years, emphasizing its adequacy to distinguish plant virus variants (di Rienzo *et al.*, 2018; Rydzak *et al.*, 2020; Nie *et al.*, 2021).

The distinction of DNA variants is based on fluorescence melting curve analysis of PCR products, with the melting temperature (Tm) being a function of the GC/AT ratio, fragment length and nucleotide sequence assayed. It can also depend on the type of intercalant agent used and even on its brand. The differences between amplicons generate different melting patterns and distinguish between genotypes, which makes the method extremely sensitive, eliminating the need for electrophoretic analysis of amplicons, RFLP pattern determination or nucleotide sequencing (Varga and James, 2005; Bester *et al.* 2012).

Genetic variability among GLRaV-3 and GFLV isolates has been previously reported worldwide, including Brazil (Radaelli *et al.*, 2009; Oliver *et al.*, 2010; Catarino *et al.*, 2015; Digiaro *et al.*, 2017; Moura *et al.*, 2018; Fajardo *et al.*, 2020). This may have implications for biological properties, epidemiology, symptomatology, transmission, and detection of viruses, and highlights the importance to differentiate and identify viruses, preferably using a sensitive and accurate diagnostic assay that can detect different variants of a virus (Maree *et al.*, 2013). Despite the works already developed, the knowledge on the occurrence of grapevine viruses in Brazil, their molecular characterization and evaluation of Brazilian viral diversity remains insufficient.

The aim of this study was to detect and differentiate GLRaV-3 and GFLV sequence variants by RT-qPCR HRM curve analysis to assess viral genetic diversity in Brazilian grapevine accessions.

MATERIALS AND METHODS

The 74 and 10 grapevines with leafroll and degeneration viruses, respectively, collected in a collection block of viral isolates, maintained in a greenhouse (Embrapa Uva e Vinho, Bento Gonçalves, Brazil), were screened by RT-qPCR (Dubiela *et al.*, 2013). Leafroll-infected samples included the cultivars 'Cabernet Franc', 'Cabernet Sauvignon', 'Merlot', 'Italia', 'Isabel', 'Trajadura', 'Núbia', 'Tardia de Caxias', 'Red Meire', and 'Petite Syrah'. Grapevine Degeneration-infected samples included the cultivars 'Prosecco Tondo', 'IAC 514-6', 'Riparia do Traviú (106-8)', 'IAC 766', 'CNPUV 776-25', 'Chardonnay', 'Malvasia Aromatica', 'Merlot', 'Sauvignon Blanc' and, 'Sangiovese'. Total RNA extraction was performed by grinding one gram of mature cane scrapings in liquid nitrogen and thereafter using a silica adsorption protocol (Rott and Jelkmann, 2001).

The SYBR Green Quantitative RT-PCR kit (Sigma-Aldrich, USA) was used in the assays according to the manufacturer's recommendations. Reaction mixtures (20 μL final volume) contained 10 μL 2x SYBR Green Taq ReadyMix for Quantitative RT-PCR (containing buffer, Taq DNA polymerase and dNTP), 0.2 μL reference dye ROX (100x), 2.4 μL MgCl$_2$ (25 mM), 0.2 μL reverse primer (10 μM), 0.2 μL forward primer (10 μM), 0.1 μL M-MLV RT (200 unit/μL), 4 μL total RNA (ca. 100 ng/μL) and 2.9 μL water. Alternatively, the GoTaq 1-Step RT-qPCR System (Promega, USA) was also used according to the manufacturer's instructions, in this case reagents were BRYT Green dye, which has spectral properties similar to SYBR Green I and carboxy-X-rhodamine (CXR) reference dye, which is identical to ROX. The primers 56F and 285R (GLRaV-3) and CP2-671F1/CP2-671F2 and CP2-822R (GFLV) designed by Osman *et al.* (2007) and Osman *et al.* (2008), respectively, were used in the RT-qPCR reactions to amplify these viruses. The reactions were processed in the thermocycler StepOnePlus Real-Time PCR System (Applied Biosystems, ThermoFisher Scientific, USA).

The amplification cycling conditions of RT-qPCR reactions were 42 °C/35 min (for reverse transcription), 95 °C/10 min, 40 cycles [denaturation: 95 °C/15 sec, annealing/extension step: 58 °C/1 min], and 60 °C/10 sec. High-resolution melting curves of PCR amplicons were obtained with temperatures ranging from 60 °C to 95 °C with a 0.3 °C increase in temperature every second. The reactions were analysed using the StepOne Software v2.3 (Applied Biosystems, ThermoFisher Scientific, USA), which automatically provides the values of CT (cycle threshold) to the amplification curve and of melting temperature (Tm) to melt curve of analysed samples. Melting peaks were visualised by plotting the first derivative against the melting temperature. The Tm was defined as the peak of the curve, with the midpoint identified as the melting temperature. For electrophoretic analysis, PCR products (20 µL) were separated on a 1.5% low melting agarose gel in TBE buffer, at 90 V for 60 min, with ethidium bromide staining.

To further characterize some GLRaV-3 and GFLV variants, the amplified DNAs of isolates Pet-4 and RM-BR (GLRaV-3) and 776-25, Mer and IAC2 (GFLV) (Table I) were eluted from agarose gel, ligated into pGEM-T Easy vector (Promega), cloned in *Escherichia coli* DH5α and recombinant plasmids were sequenced by Sanger method (Dubiela *et al.*, 2013) or were directly sequenced. Pairwise comparisons between the obtained nucleotide sequences and homologous viral sequences retrieved from the GenBank (https://www.ncbi.nlm.nih.gov/) were performed using the BLASTn program at the NCBI site.

Six Brazilian isolates of GLRaV-3 and GFLV, whose nucleotide sequences covering the amplification target genomic regions and already available on GenBank, or which were obtained in this work (Table I), were aligned using the ClustalX 2.1 program, visualised with GeneDoc program and analysed in order to demonstrate the existing nucleotide divergence and relate it to the different variants.

Table I

Information on Brazilian GLRaV-3 and GFLV isolates whose nucleotide sequences were used for comparison to demonstrate the divergence in the region of the amplicons

Virus	GenBank accession code	Total nucleotide sequence length (nt)	Genome region analysed by HRM[1] (nt[2])	Grapevine cultivar	Isolate name
GLRaV-3	KC519443	230[*]	230 nt partial HSP70[3]	*V. vinifera* cv. 'Petite Syrah'	Pet-4
GLRaV-3	---	230[*]	230 nt partial HSP70	*V. vinifera* cv. 'Red Meire'	RM-BR
GLRaV-3	KX701860	18020	230 nt partial HSP70	*V. labrusca* cv. 'Isabel'	ISAB-BR
GLRaV-3	KX756668	18498	230 nt partial HSP70	*V. labrusca* cv. 'Tardia de Caxias'	TC-BR
GLRaV-3	MK804765	18498	230 nt partial HSP70	*Vitis* sp. cv. 'Núbia'	NUB-BR
GLRaV-3	KX756669	18313	230 nt partial HSP70	*V. vinifera* cv. 'Trajadura'	TRAJ-BR
GFLV	OP936993	152[*]	152 nt partial CP[4]	hybrid *Vitis* sp. 'CNPUV 776-25'	776-25
GFLV	OQ513992	152[*]	152 nt partial CP	*V. vinifera* cv. 'Merlot'	Mer
GFLV	---	152[*]	152 nt partial CP	*Vitis sp.* cv. 'IAC 766'	IAC2
GFLV	EU038294	1515	152 nt partial CP	*Vitis vinifera* cv. 'Prosecco Tondo'	RS
GFLV	EU258680	1515	152 nt partial CP	*Vitis* sp. cv. 'IAC 514-6'	RUP
GFLV	EU258681	1515	152 nt partial CP	*Vitis* sp. cv. '106-8'	IAC

(*) Isolates sequenced in this work to relate with the HRM analysis; (1) HRM (high-resolution melting) curve, (2) nt (nucleotide), (3) HSP70 (heat shock protein 70 gene), (4) CP (coat protein gene).

RESULTS AND DISCUSSION

Detecting and identifying viral infections in perennial fruit plants, such as grapevine, can be challenging, mainly due to the high genetic diversity of some viruses that infect this host (Vigne *et al.*, 2018). A single amplification curve was generated for each sample and virus by RT-qPCR, thus initially confirming the specific viral infections. Analysing all amplification plots for both viruses, the amplification curves showed CT values ranging from 15.95 up to 34.93 (average 25.27) for GLRaV-3 isolates and, from 15.13 up to 34.70 (average 24.09) for GFLV isolates, which are, as expected, compatible with samples infected by the evaluated viruses (Figures 1 and 2). By analysing the RT-qPCR HRM curve, it was possible to detect and differentiate the variants of each virus tested, GLRaV-3 and GFLV, considering 74 and 10 plants screened, respectively. Success was achieved in unraveling the variability of a wide range of isolates that so far was unknown.

The amplified genome target regions of these two viruses allowed to clearly identify different sequence variants of GLRaV-3 and GFLV, which showed distinct melting profiles and Tm values.

The identification of viral variants in a certain sample was possible by analysing the dissociation curves (melting curves) of the amplification products (Figures 1 and 2). Variations in dissociation curves and Tm values reflect differences in the nucleotide sequences of the respective amplicons and thus constitute a simplified, sensitive and reliable way of differentiating a range of variants of a virus. Several GLRaV-3 isolates showed different melting curves, with a melting peak produced for each variant, and the Tm values of the Brazilian GLRaV-3 isolates varied (lower and upper limits) from 82.01 to 84.39 °C (Figure 1). For GFLV isolates, the Tm values showed lower variation from 79.03 to 80.60 °C considering the range of GFLV isolates analysed (Figure 2).
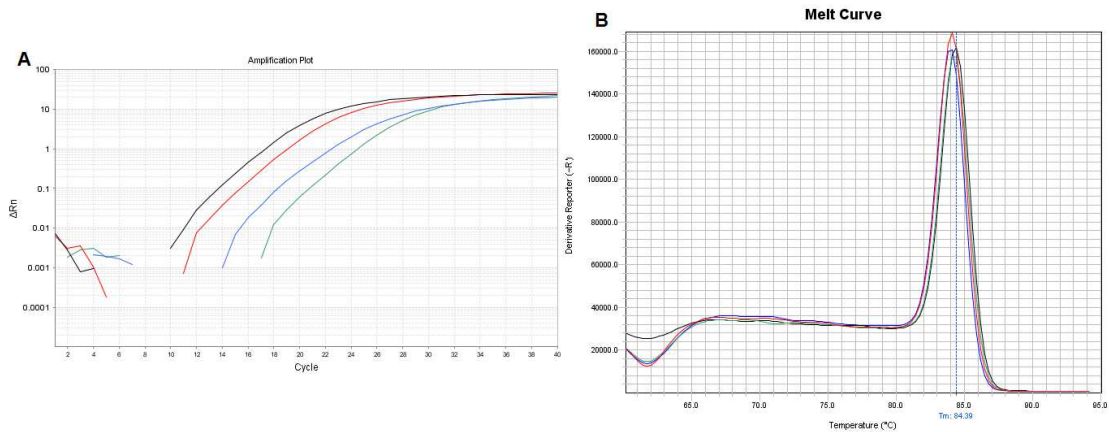

Figure 1. Amplification plots (curves) (**A**) and the corresponding melting curve analysis (**B**) of RT-qPCR products derived from GLRaV-3 amplifications, from sample 9 (blue line, melting temperature, Tm = 83.95 °C), sample 39 (black, Tm = 84.39 °C), sample 48 (green, Tm = 84.25 °C) and sample 74 (red, Tm = 84.10 °C).
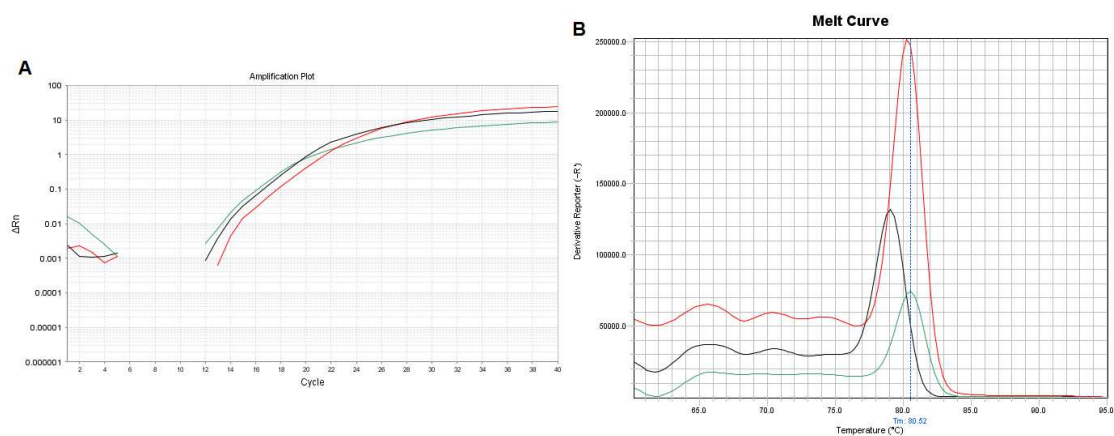

Figure 2. Amplification plots (curves) (**A**) and the corresponding melting curve analysis (**B**) of RT-qPCR products derived from GFLV amplifications, from sample 3 (black line, melting temperature, Tm = 79.03 °C), sample 4 (red, Tm = 80.23 °C) and sample 5 (green, Tm = 80.52 °C).

It is important to highlight that, for GLRaV-3, the sequence variant with Tm 84.10 °C was prevalent (23 isolates) among the analysed samples, followed by variants with Tm 83.95, 84.24, 84.09 and 82.99 with 17, 8, 6 and 5 isolates, respectively; other different Tm values were verified for groups composed of three or less isolates. For GFLV, the infection with sequence variant with Tm 80.52 °C was prevalent (two isolates). Variations in melting profile observed within GLRaV-3 and GFLV variants were 2.38 °C and 1.57 °C, respectively, according to temperature ranges between upper and lower Tm limits in the analysed isolates of these two viruses. A similar approach (melting point temperature interval) was adopted by Varga and James (2005) and Bester *et al.* (2012) to differentiate viral variants and strains, which were, in these cases, clustered into groups. The Tm variation observed between variants of the same virus can be explained by the high intraspecific genetic variability existing in these viruses that emerged from the high mutation rate of the viral genome (Bester *et al.*, 2012). To exemplify this feature, in the case of GLRaV-3, the comparison of sequences and classification in the six groups was proposed by Maree *et al.* (2013), and for GFLV phylogenetic analysis was performed by Panno *et al.* (2021) using sequences of Sicilian (Italy) isolates and established clades.

As the definition of variants is closely related to the variability of nucleotides present in the studied amplicon and applied to specific conditions (e.g. pairs of primers used), it is not possible to relate the variants found with those defined in similar studies based on other amplicons of viral genomes of GLRaV-3 and GFLV (Bester *et al.*, 2012).

In the amplification curve, the Y axis corresponds to ΔRn (normalised fluorescence) and the X axis to the number of cycles, while in the melting curve, the Y axis corresponds to derivative reporter (-R) and the X axis to temperature (°C). Thus, interpreting the graphs, the reached height of the curve or the melting peak means the amount of amplicon generated in the RT-qPCR reactions (Figures 1 and 2). For example, it is possible to observed that one variant of GFLV reaches higher viral titer than others (Figure 2B); the similar interpretation is applied to GLRaV-3 variants. Although several variables can influence the viral titer (e.g. host susceptibility, environmental conditions, among others), in general, more adapted variants could replicate more efficiently and then reach higher titers, possibly resulting in greater damage to the host.

A collection block, consisting of several grapevine accessions of different cultivars and sources, was sampled to evaluate genetic variabilities of GLRaV-3 and GFLV resulting in the determination of genetic variants of each evaluated virus. However, considering the different variables involved (cultivars, possible multiple viral infections, plant development conditions, among others), it was not possible to associate infection by a certain sequence variant with a specific symptom expression. In a next step, it could be interesting to establish such connection.

The finding of viral infections caused by GLRaV-3 and GFLV in Brazilian vineyards of different regions has already been widely reported (Catarino *et al.*, 2015; Moura *et al.*, 2018; Fajardo *et al.*, 2020), however, a more in-depth study on the occurrence of variants of these viruses, considering an expressive number of viral isolates, had not been described before.

Regardless of whether the analysed sample was infected with one or supposedly more viral variants, the agarose gels showed only one DNA band of the expected size resulting from the amplification of both viruses (Figure 3).
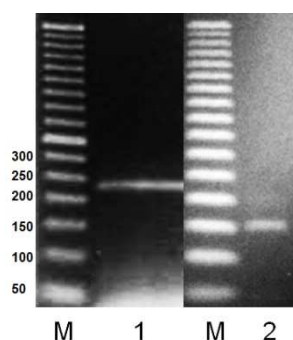

Figure 3. Agarose gel electrophoresis of amplification products by RT-qPCR from infected grapevine plants; (M) 50 bp (base pairs) DNA Ladder (Invitrogen, on the left and Promega, on the right); (1) 230 bp from a GLRaV-3 isolate; (2) 152 bp from a GFLV isolate.

High-resolution melting curve analysis is only reliable when the correct PCR product is amplified and used as a basis for analyses and due interpretations. Thus, to validate the HRM results, among the two determined Brazilian GLRaV-3 sequences, the GLRaV-3-amplicon sequence of 230 nucleotides, partially covering the heat shock protein 70 (HSP70) gene of Pet-4 isolate cv. 'Petite Syrah' (GenBank KC519443), was obtained, placed at nt 11060 to 11289 based on GLRaV-3 NY1 isolate (NC_004667). This sequence showed nt identities of 100% with two GLRaV-3 Brazilian isolates (GLRaV-3.NUB-BR, MK804765 and, TRAJ-BR, KX756669), and 94.8% nt identity with two other isolates from Brazil (ISAB-BR, KX701860 and, TC-BR, KX756668) (Table I, Figure 4). Among the three determined Brazilian GFLV sequences, the GFLV-amplicon sequence of 152 nucleotides, partially covering the capsid protein (CP) gene of isolate 776-

25 (GenBank OP936993) was obtained, placed at nt 671 to 822 based on GFLV CP of F13 isolate (NC_003623). This sequence showed the following nt identities with other three GFLV Brazilian isolates, whose CP sequences were retrieved from GenBank or were obtained in this work: 99.3% (Mer isolate, OQ513992) and, other two isolates, 98% (RUP, EU258680) and 98.6% (RS, EU038294) (Radaelli *et al.*, 2009) (Table I, Figure 4). The sequencing of GLRaV-3 and GFLV RT-qPCR amplicons and nucleotide comparisons additionally allowed to confirm the specificity of the curves observed in RT-qPCR amplification plots (Figures 1 and 2). Moreover, although the primer pairs used in this study have already been previously designed, it is considered that they were adequate to explore the existing variability in the genomic regions studied (HSP70 of GLRaV-3 and CP of GFLV) by RT-qPCR HRM assays.
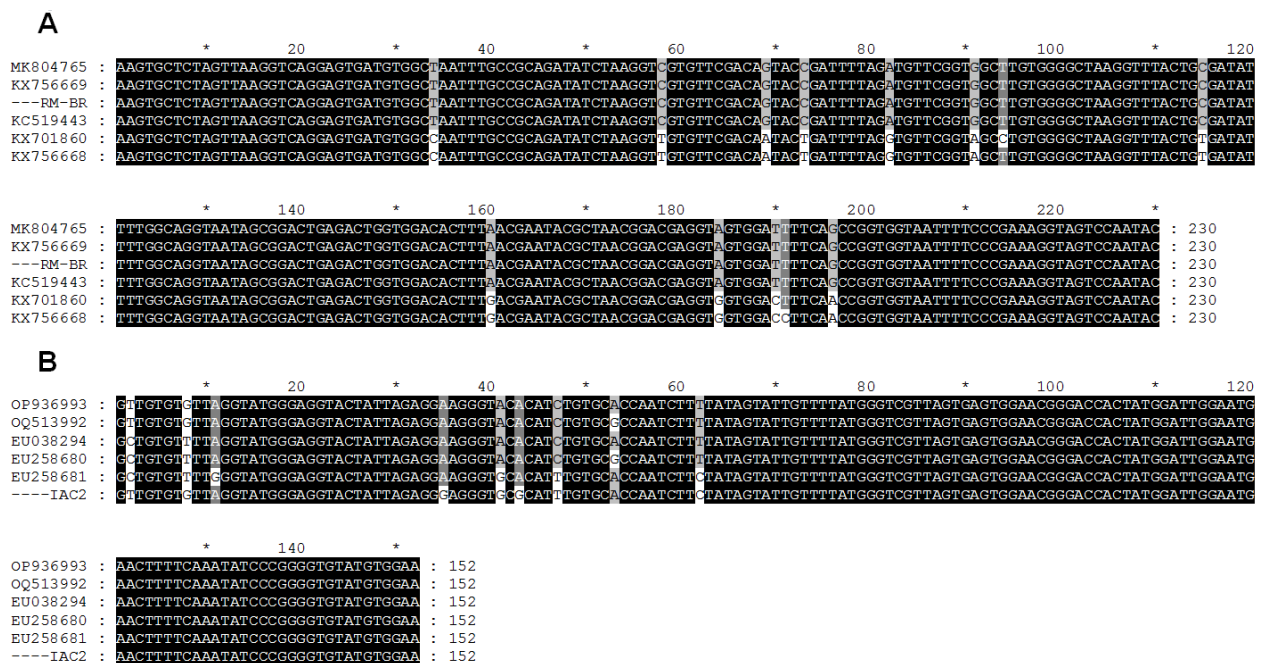

Figure 4. Multiple nucleotide sequence alignments of target regions of HRM analysis: the partial HSP70 gene (230 nt) of GLRaV-3 isolates (**A**) and the partial coat protein gene (152 nt) of GFLV isolates (**B**) demonstrating homologous and heterologous positions in the amplicons that determined different Tm of variants in HRM analysis. Nucleotide variation sites are shown as indicated by lighter highlighting. Numbers on the right of sequences indicate the positions, and codes on the left refer to the GenBank accessions.

The aligned nucleotide sequences allowed to observe the variability (changes of nucleotides by position) in some of the variants found. The alignment of the nucleotide sequences of the region corresponding to the amplicon analysed by HRM of the six Brazilian isolates of GLRaV-3 and GFLV revealed thirteen and nine positions of nucleotide divergence between homologous isolates, respectively (Figure 4). As

expected, high correlation was observed between high nucleotide identities between homologous viral variants showing similar Tm values and, likewise high nucleotide divergences among variants showing contrasting Tm values (Table I, Figure 4).

These nucleotide divergences would be involved in defining the range of observed sequence variants and which, in turn, would be associated with the different Tm values obtained.

In order to choose the most suitable diagnostic method for viral detection and characterization, some variables should be considered such as the number of indexed samples, cost, sensitivity, practicality and reliability of the test, qualified labor force, required equipment, genetic variability of the virus, and viral titer and distribution in the plant throughout the crop cycle (Fajardo *et al.*, 2021). The real-time RT-PCR HRM assay fulfils several of these aspects and, as demonstrated, is also suitable for the purpose of analysing and unveiling the existence of variants of two important grapevine viruses.

Although the genetic variability of GLRaV-3 and GFLV has been extensively studied in recent years and worldwide research showed the existence of several genetic variants of both viruses based on biological, serological and/or molecular methods (Maree *et al.*, 2013; Panno *et al.*, 2021; Kubina *et al.*, 2022), the RT-qPCR-HRM assay stands out for providing a sensitive and rapid tool, feasibility in routine analyses to detect and differentiate distinct variants in order to understand the molecular epidemiology of viral diseases and assist practices focused at managing and controlling the spread of some viruses (Bester *et al.*, 2012). In Brazilian vineyards, GLRaV-3 infections were found in asymptomatic plants, even in a sensitive genotype of *V. vinifera* cv. 'Cabernet Sauvignon' (Fajardo and Eiras, 2022). Asymptomatic and GLRaV-3-infected vines could be the result of infection with some specific variants of GLRaV-3 or other causes (recent infection, environmental conditions not favorable for the expression of symptoms, tissues not yet developed). Knowledge of the existing viral variability also allows the development and implementation of molecular diagnostic tests better adapted to detect a wider range of virus variants.

High-resolution melting curve analysis, which is a post-PCR process, was effectively applied to unveil and differentiate genetic variants, demonstrating the occurrence of mixed variant infections among a wide and diverse range of Brazilian isolates of two important grapevine-infecting viruses. This procedure is therefore a useful alternative for the rapid, cost-effective and reliable detection of viral variability. Demonstrating the existence and magnitude of viral genetic variability can raise concerns due to its potential negative consequences, such as affecting viral detection and the possibility of the emergence of more complex diseases or changes in the importance status of those that are already present in certain regions.

CONCLUSIONS

This work implemented an RT-qPCR-HRM assay capable of discriminating between the Brazilian variants of GLRaV-3 and GFLV. In addition, the genetic variability of a wide range of Brazilian isolates of GLRaV-3 and GFLV was determined, identifying the existence of several sequence genetic variants of both viruses. The methodology of RT-qPCR-HRM assay linked to an alignment of viral sequences (obtained in the work and GenBank available) was able to distinguish sequence variants. This could be an interesting approach to large-scale detection and classification of isolates due to the high intraspecific genetic variability existing in these viruses and the high-throughput approach of this assay.

CONFLICTS OF INTEREST: The authors declare no conflict of interest.

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