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**IMPROVEMENT OF GRAPEVINE LEAFROLL-ASSOCIATED VIRUS 1
DETECTION THROUGH A NOVEL SPECIFIC QUANTITATIVE REAL-
TIME RT-PCR**

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RESUMEN

En la actualidad, la vid (*Vitis vinifera* L.) es uno de los cultivos más importantes del mundo por su impacto económico y social. Como en muchos otros cultivos, las plantas de vid son susceptibles a varios tipos de enfermedades causadas por microorganismos patógenos. El virus del enrollado 1 (GLRaV-1) está asociado a la enfermedad del enrollado de la vid y se considera a nivel nacional y europeo como un patógeno que debe estar ausente en el material vegetal propagativo. Por ello, es importante utilizar técnicas de detección específicas, sensibles y fiables que permitan determinar el estado sanitario de las plantas. El objetivo de esta investigación se ha centrado en el desarrollo de un nuevo método basado en una RT-PCR cuantitativa en tiempo real dirigida a la región genómica de la proteína de recubrimiento para mejorar la detección de GLRaV-1 en la vid, incluyendo un control interno del huésped en una reacción dúplex. Con este fin, se han recuperado tres nuevos genomas completos de GLRaV-1 mediante secuenciación masiva (High-throughput sequencing, HTS) y se han alineado con todas las secuencias disponibles en las bases de datos. Para la validación del método siguiendo los estándares de la EPPO, se han analizado 65 muestras naturalmente infectadas por GLRaV-1 de diferentes orígenes y variedades y se han comparado con otros dos métodos de detección descritos previamente basados en PCR. Los resultados muestran que el nuevo protocolo diseñado en este estudio es mucho más específico y sensible que los otros métodos. El método diseñado permite la cuantificación absoluta del título viral y es capaz de detectar tan solo 2 copias del virus. La nueva técnica se ha aplicado para el diagnóstico de 241 muestras de campo.

Palabras clave:

RT-PCR en tiempo real, detección, diagnóstico, GLRaV-1, legislación

ABSTRACT

Nowadays, grapevine (*Vitis vinifera* L.) is one of the most important crops in the world due to its economic and social impact. As in many other crops, grapevine plants are susceptible to various types of diseases caused by pathogenic microorganisms. Grapevine leaf roll associated virus-1 (GLRaV-1) is a virus associated with grapevine leafroll disease and it is considered at national and European level as a pathogen that must be absent in propagative plant material. For this reason, it is important to use specific, sensitive, and reliable detection techniques that allow determining the sanitary status of the plants. The objective of this research has focused on the development of a new method based on a TaqMan quantitative real time RT-PCR targeted to coat protein genomic region to improve the detection of GLRaV-1 in grapevine, including a host internal control in a duplex reaction. To this end, three new GRLaV-1 full genomes have been recovered by HTS and aligned with all sequences available in the databases. For the validation of the method following EPPO standards, 65 naturally infected samples from different origins and cultivars were analyzed and compared with two other PCR-based detection methods previously reported. The results show that the new protocol designed in this study is much more specific and sensitive than the other methods. The method designed allows the absolute quantitation of the viral titer, it is able to detect as little as 2 viral target copies and has been applied for the diagnosis of 241 field samples.

Keywords:

Real-time RT-PCR, detection, diagnosis, GLRaV-1, legislation

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ABBREVIATION LIST

DNA: Deoxyribonucleic acid

RNA: Ribonucleic acid

(+)ssRNA: Positive sense single-stranded RNA

cDNA: Complementary DNA

dNTPs: Deoxynucleotide Triphosphate

NA: Nucleic acid

ddNTPs: Dideoxynucleotides

ORFs: Open reading frames

CDS: Coding sequences

PPPs: plant protection products

RT: Reverse transcription

ELISA: Enzyme-linked immunosorbent assay

PCR: Polymerase chain reaction

qPCR: Quantitative PCR

LAMP: Loop-mediated isothermal amplification

C_T: Threshold cycles

HTS: High throughput sequencing

NGS: Next generation sequencing

CP: Coat protein

CPd2: Coat protein duplicate 2

HSP70: Heat-shock protein 70

SNPs: Single nucleotide polymorphisms

PEP: Phosphoenolpyruvate carboxylase

w:v: weight:volume

μL: Microliter

nM: Nanometer

°C: Celsius degree

bp: Base pair

kb: Kilobase

U: Unit

A: Absorbance

GLD: *Grapevine leafroll disease*

GLRaVs: *Grapevine leafroll-associated viruses*

GLRaV-1/2/3/4/5/6/10: *Grapevine Leafroll-associated Virus 1/2/3/4/5/6/10*

GFkV: *Grapevine fleck virus*

GFLV: *Grapevine fanleaf virus*

GAMaV: *Grapevine asteroid mosaic-associated virus*

GPGV: *Grapevine pinot gris virus*

GVA/B/E/F: *Grapevine Virus A/B/E/F*

GRsPaV-1: *Grapevine rupestris stem pitting-associated virus*

GRGV: *Grapevine redglobe virus*

GSyV-1: *Grapevine syrah virus 1*

GRSLaV: *Grapevine rootstock Stem Lesion-associated virus*

GRLDaV: *Grapevine roditis leaf discoloration-associated virus*

GYSV-1: *Grapevine yellow speckle Viroid 1*

GRVFV: *Grapevine rupestris vein feathering virus*

1. INTRODUCTION

Nowadays, with the projection of world population numbers reaching more than nine billion people in 2050, agriculture faces a huge challenge to produce enough food in a more sustainable way (FAO, 2017). In this scenario, crop losses due to pests and diseases are important threats to food production (Cerdeira *et al.*, 2017).

Pests and pathogens threaten crop production causing disease and therefore food losses (Delgado *et al.*, 2021; Farooq *et al.*, 2021). Factors involved in crop disease development include host susceptibility, pathogen virulence and environmental conditions (Islam *et al.*, 2017a).

Among plant pathogens, viruses, transmitted by many different ways including grafting, vectors, pollen, seeds, water and soil, cause one-third of plant diseases and are responsible for great impact on farmers' production around the world (Islam *et al.*, 2017b; Jones & Naidu, 2019) representing economic losses of more than 30 billion dollars per year.

1.1. Grapevine viruses

Grapevine (*Vitis vinifera* L.) is a crop that can be infected by many different viruses (Fuchs, 2020). This high viral diversity that englobes a total of 89 different viral species is due to the evolutionary history of this crop, its domestication and the coexistence with different pathogens and pests (Fuchs, 2020; Al Rwahnih *et al.*, 2021; Fan *et al.*, 2021a; ob).

Among them, 31 viral species have been associated with the four major disease complexes based on the type of symptoms caused by these pathogens: infectious degeneration, leafroll, rugose wood and fleck (Martelli, 2017).

The high complexity of the grapevine virome that commonly involves mixed infections by several viruses requires an accurate and reliable viral detection (Basso *et al.*, 2017; Mannini & Digiaro, 2017).

1.1.1. Grapevine Leafroll Disease

Grapevine leafroll disease (GLD) is caused by one of the most economically important widespread complexes among the grapevine viral agents (Maliogka *et al.*, 2015; Naidu *et al.*, 2015). GLD complex comprises five viral species belonging to the family

Closteroviridae and classified into two genera (*Ampelovirus*, and *Closterovirus*) (Table 1).

Table 1: Species of viruses associated to leafroll disease (GLD) in grapevine (*Vitis vinifera* L.).

Family	Genus	Specie	Genome	Particle shape	Vector	Disease
<i>Closteroviridae</i>	<i>Closterovirus</i>	GLRaV-2	(+)ssRNA	Filamentous	Unknown	Leafroll/Incompatibility
		GLRaV-1			Mealybugs	
	<i>Ampelovirus</i>	GLRaV-3	(+)ssRNA	Filamentous	and soft scales	Leafroll
		GLRaV-4				
		GLRaV-13	(+)ssRNA	Filamentous	Unknown	Leafroll

Source: Fuchs, 2020. GLRaV-2: *Grapevine leafroll-associated virus 2*; GLRaV-1: *Grapevine leafroll-associated virus 1*; GLRaV-3: *Grapevine leafroll-associated virus 3*; GLRaV-4: *Grapevine leafroll-associated virus 4*; GLRaV-13: *Grapevine leafroll-associated virus 13*.

Infection by these viruses promotes different symptoms depending on the target grapevine cultivar (Naidu *et al.*, 2015; Fuchs, 2020). Besides the usual symptom of downward rolling of leaf margins, viruses belonging to this complex also promote interveinal reddening in red grapevine cultivars and interveinal leaf chlorosis in white cultivars (**Fig. 1**) (Pojaari *et al.*, 2017; Elçi, 2019).

These symptoms are more present in late summer and autumn (Maliogka *et al.*, 2015). Nevertheless, the quality and yield of berries, significantly associated to plant vigor, are still compromised in 15-20 % in infected plants. Regarding fruits, important features related to wine production such as Brix level, maturation and pigmentation are severely affected (Elçi, 2019).

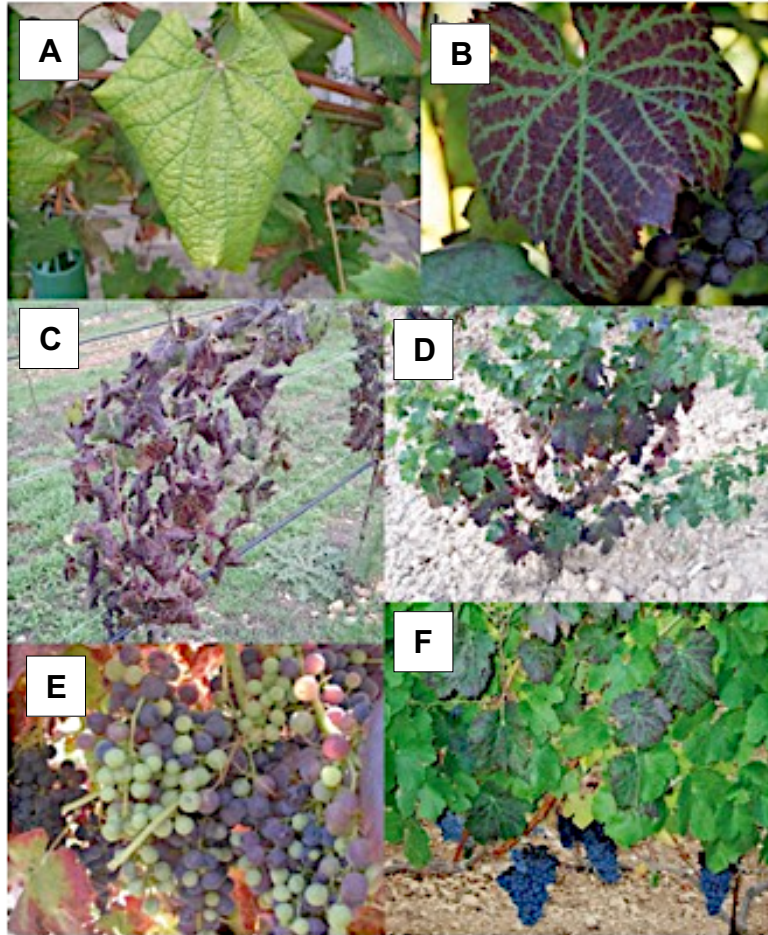


Figure 1: Symptoms associated to leafroll viruses in leaves and berries. (A) leaf rolling in a white variety; (B) interveinal reddening in a red variety; (C-D) leaf rolling and different types of alteration in coloration; (E) late and irregular ripening in berries, and (F) leaf symptoms without alteration in berries in a red variety. Source: Sociedad Española de Fitopatología, 2016.

GLD complex viruses transmitted by both propagation of infected plant material and insect vectors, such as mealybugs (Hemiptera: *Pseudococcidae*) and soft scales (Hemiptera: *Coccidae*) (Pojaari *et al.*, 2017).

1.1.2. Grapevine leafroll-associated virus 1 (GLRaV-1)

GLRaV-1 has a high worldwide distribution, as it has been reported infecting grapevines located in Africa, Asia, America, Oceania and Europe (Kumar *et al.*, 2012; Immanuel *et al.*, 2015; Zongoma *et al.*, 2018; Karthikeyan *et al.*, 2011; Habili *et al.*, 2007; Escobar *et al.*, 2008). In Europe and the Mediterranean basin, this virus has a wide distribution in important grapevine producing countries, such as Italy (Fortusini *et al.*, 1996), France (Hommay *et al.*, 2020), Spain (Bertolini *et al.*, 2010), Greece (Avgelis & Tzortzakakis, 1997), Germany (Messmer *et al.*, 2021), Switzerland

(Reynard *et al.*, 2015), Slovakia (Predajňa *et al.*, 2013), Tunisia (Mahfoudhi *et al.*, 2008) and Turkey (Akbaş *et al.*, 2007).

GLRaV-1 is transmitted by grafting and by several insect vectors, the mealybugs *Phenacoccus aceris* and *Heliococcus bohemicus* and the soft scales, *Parthenolecanium corni* and *Neopulvinaria innumerabilis* (Habibi *et al.*, 2007; Predajňa *et al.*, 2013).

GLRaV-1 belongs to the genus *Ampelovirus*. Its genome is encapsidated into flexuous particles and consists of a positive sense single-stranded RNA of approximately 18.7 to 18.9 kb in length (Donda *et al.*, 2017). Within this genome, nine open reading frames (ORFs) responsible for encoding methyl transferase/RNA helicase (ORF1a), RNA-dependent RNA polymerase (ORF1b), p7 (ORF2), p55 (ORF3), heat shock protein (ORF4), coat protein (ORF5), coat protein duplicate 1 (ORF6), coat protein duplicate 2 (ORF7), p21 (ORF8) and p24 (ORF9) (**Fig. 2**). These proteins are related to replication, cell-to-cell movement, RNA silencing suppression and long-distance movement (Esteves *et al.*, 2013; Naidu *et al.*, 2015; Donda *et al.*, 2017).

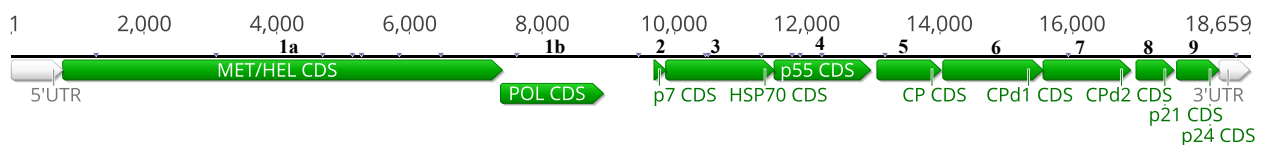


Figure 2: Genome organization of Grapevine Leafroll-associated Virus 1 (NCBI Reference Sequence NC_016509.1). **1a.** MET/HEL CDS: methyl transferase/helicase; **1b.** POL CDS: RNA dependent RNA polymerase; **2.** p7 CDS: protein 7; **3.** HSP70 CDS: heat shock protein 70; **4.** P55 CDS **5.** CP CDS: coat protein; **6.** CPd1 CDS: coat protein duplicate 1; **7.** CPd2 CDS: coat protein duplicate 2; **8.** p21 CDS: protein 21; **9.** p24 CDS: protein 24.

1.2. Detection of grapevine viruses

Due to the absence of effective plant protection products that can act directly on viruses, preventive control measures need to be adopted. Within these preventive measures are the use of resistant cultivars, vector control and use of healthy plant material for vegetative propagation (Messmer *et al.*, 2021). In this scenario, the use of

specific and reliable detection methods becomes a key factor in the management and control of the diseases (Morán *et al.*, 2018).

Traditional diagnosis of grapevine viruses has been based on biological indexing and enzyme-linked immunosorbent assay (ELISA). However, these methods have important limitations in viral detection, mainly due to the high cost and problems for large-scale analysis related to biological assays and the reduced sensitivity as well as unspecific cross-reactions that commonly affect ELISA tests (Bertolini *et al.*, 2010).

Polymerase chain reaction (PCR)-based methods overcome these disadvantages and have been shown to be powerful tools for viral epidemiological studies and viral detection, especially PCR techniques based on real time analysis (Morán *et al.*, 2018).

High throughput sequencing (HTS) has become a powerful tool in the plant virology area allowing the characterization of both known and unknown plant viruses (Massart *et al.*, 2014; Maliogka *et al.*, 2018; Villamor *et al.*, 2019). This technology has also become crucial in the designing of PCR detection methods as it can provide key information on the genetic variability of viral genomes.

1.2.1. Detection of GLRaV-1

Viruses from the leafroll complex can be detected by biological indexing (Rowhani *et al.*, 2017). These bioassays are performed to detect viruses in plant material, based on grafting buds of the plant to be tested in a susceptible host, woody indicator plants belonging to *Vitis vinifera* species. Red-berried cultivars such as Cabernet Franc, Cabernet Sauvignon, Pinot Noir, Barbera, Merlot, Mission and Gamay are known as “indicator plants” because of their susceptibility to leafroll-associated viruses and how easily seen are the symptoms promoted by them (Martelli, 1993; Rowhani *et al.*, 2017; Zherdev *et al.*, 2018).

Interveinal reddening on leaves, leaf margins rolled downwards, shortening of internodes and stunting appear between 4-6 weeks (greenhouse indexing) and up to 2 years (field indexing) (Martelli, 1993; Rowhani *et al.*, 2017). However, these symptoms are expressed in a different way depending on the cultivar of choice, as observed by Önder & Gümüş (2015) for Aegean vineyards. Unfortunately, these bioassays can detect leafroll as a disease complex, but not the specific virus responsible for the symptoms expressed (Martelli, 1993; Habili *et al.*, 2007; Al Rwahnih *et al.*, 2015).

Easy to conduct in large sample numbers, ELISA is a method that can be used either to detect one virus in different plants or different viruses in one plant, facilitating routine diagnostics (Youssef *et al.*, 2006). To do so, green organs, bark, or root tissues are ground in a buffer and get in contact with poly/monoclonal antibodies and enzyme-labelled specific antibodies, previously commercially produced (Martelli, 1993; Zherdev *et al.*, 2018).

Most of the studies (Habibi *et al.*, 1997; Akbaş *et al.*, 2007; Bertolini *et al.*, 2010; Guță *et al.*, 2010; Kumar *et al.*, 2013; Endeshaw *et al.*, 2014; Montero *et al.*, 2016) that used ELISA as method of detection of GLRaV-1 performed the test using available commercial kits.

For GLRaV-1, antigens can be peptide sequences located in the coat protein (CP) region, as proved by Esteves *et al.* (2013), while common antibodies are monoclonal antibodies conjugated to alkaline phosphatase enzyme (Rowhani *et al.*, 1997; Petersen & Charles, 1997). Double Antibody Sandwich ELISA (DAS-ELISA) is usually adopted as method of detection for grapevine viruses (Zherdev *et al.*, 2018).

Due to the lower sensitivity of ELISA tests using polyclonal antibodies and the frequent cross-reactions of this technology more reliable methods (such as polymerase chain reaction (PCR)-based methods) are recommended to increase the detection accuracy (Bertolini *et al.*, 2010).

Several studies have been performed addressing GLRaV-1 detection using conventional (Gambino & Gribaudo, 2006; Alabi *et al.*, 2011) or real-time amplification (Osman *et al.*, 2007; Bertolini *et al.*, 2010; Pacifico *et al.*, 2011; López-Fabuel *et al.*, 2013; Dubiela *et al.*, 2013; Bruissson *et al.*, 2017; Aloisio *et al.*, 2018). The most used regions targeted to this end are the heat shock protein 70 (HSP70) and the coat protein (CP).

Four different GLRaV-1 genomic regions (HSP70h, CP, CPd2 and p24) were tested by Alabi *et al.* (2011) through conventional RT-PCR. In their research, the detection method chosen by them showed the presence of genetically diverse GLRaV-1 populations in american grapevine cultivars.

Osman *et al.* (2007) detected GLRaV-1 and other leafroll-associated viruses in samples from different geographic regions using real-time RT-PCR. This set of primers/probe have been used by many different authors (Bertolini *et al.*, 2010;

Dubiela *et al.*, 2013; López-Fabuel *et al.*, 2013) and are reference for certification programs worldwide. However, with the high flow of information being constantly uploaded on GenBank database, it is important that detection protocols are updated and that they expand the level of detection to new isolates more recently registered.

2. OBJECTIVE

The objective of this study is the improvement of GLRaV-1 detection by the design and validation of a new real time quantitative RT-PCR protocol able to successfully detect all GLRaV-1 isolates currently known.

3. MATERIAL AND METHODS

3.1. Plant material

A total of 65 GLRaV-1 positive samples from different geographic origins: Spain (IVIA collection and samples provided by Dr. Diego Intrigliolo), Switzerland (samples provided by Dr. Jean Sebastien Reynard), Slovakia (samples provided by Dr. Miroslav Glasa), Tunisia (samples provided by Dr. Samia Daldoul), Thailand (samples provided by Dr. Thierry Wetzel), Greece (samples provided by Dr. Varvara Maliogka) and Germany (samples provided by Dr. Thierry Wetzel); and different varieties (Tempranillo, Bobal, Pinot Noir, Rèze, Räuschling, Veltliner, Muller-Thurgau, Gewurztraminer, Marsaoui, Roditis, Vertzami, Mavrothiriko, Geisenheim 26, Chardonnay, Pinot Blanc and Riestling) were used for validation of the new detection method. The complete description of these samples is detailed in **Table 2**.

In addition, 241 field samples from several random surveys from different Spanish grapevine growing regions (D.O. Priorato, D.O. Manchuela and D.O. Utiel-Requena) were analyzed.

Table 2: Description of the positive samples used for validation in this study.

Sample code	Host	Variety	Origin	Collection date
LR-1	Grapevine	Tempranillo	Spain	2021
100.4	Grapevine	Bobal	Spain	2021
100.25	Grapevine	Bobal	Spain	2021

100.32	Grapevine	Bobal	Spain	2021
100.42	Grapevine	Bobal	Spain	2021
100.44	Grapevine	Bobal	Spain	2021
100.47	Grapevine	Bobal	Spain	2021
100.48	Grapevine	Bobal	Spain	2021
100.50	Grapevine	Bobal	Spain	2021
88.1	Grapevine	Pinot Noir	Switzerland	2021
88.2	Grapevine	Rèze	Switzerland	2021
88.3	Grapevine	Räuschling	Switzerland	2021
91.1	Grapevine	Veltliner	Slovakia	2021
91.2	Grapevine	Veltliner	Slovakia	2021
91.3	Grapevine	Veltliner	Slovakia	2021
91.4	Grapevine	Veltliner	Slovakia	2021
91.5	Grapevine	Veltliner	Slovakia	2021
91.6	Grapevine	Veltliner	Slovakia	2021
91.7	Grapevine	Muller-Thurgau	Slovakia	2021
91.8	Grapevine	Muller-Thurgau	Slovakia	2021
91.10	Grapevine	Muller-Thurgau	Slovakia	2021
91.11	Grapevine	Muller-Thurgau	Slovakia	2021
91.12	Grapevine	Gewurztraminer	Slovakia	2021
52.1	Grapevine	Marsaoui	Tunisia	2019
35.2	Grapevine	Unknown	Thailand	2019
35.4	Grapevine	Unknown	Thailand	2019
35.6	Grapevine	Unknown	Thailand	2019
18.6	Grapevine	Unknown	Greece	2018
19.3	Grapevine	Mavrothiriko	Greece	2019
19.5	Grapevine	Vertzami	Greece	2019
19.6	Grapevine	Roditis	Greece	2019
29.4	Grapevine	Roditis	Greece	2019
29.6	Grapevine	Roditis	Greece	2019

29.8	Grapevine	Roditis	Greece	2019
29.11	Grapevine	Roditis	Greece	2019
98.2	Grapevine	Unknown	Greece	2021
98.3	Grapevine	Unknown	Greece	2021
98.8	Grapevine	Unknown	Greece	2021
98.9	Grapevine	Unknown	Greece	2021
98.10	Grapevine	Unknown	Greece	2021
98.11	Grapevine	Unknown	Greece	2021
98.12	Grapevine	Unknown	Greece	2021
98.13	Grapevine	Unknown	Greece	2021
98.14	Grapevine	Unknown	Greece	2021
98.16	Grapevine	Unknown	Greece	2021
98.17	Grapevine	Unknown	Greece	2021
102.1	Grapevine	Geisenheim 26	Germany	2021
102.2	Grapevine	Geisenheim 26	Germany	2021
102.3	Grapevine	Chardonnay	Germany	2021
102.4	Grapevine	Chardonnay	Germany	2021
102.5	Grapevine	Pinot Noir	Germany	2021
102.6	Grapevine	Pinot Noir	Germany	2021
102.7	Grapevine	Pinot Noir	Germany	2021
102.8	Grapevine	Pinot Noir	Germany	2021
102.9	Grapevine	Pinot Blanc	Germany	2021
102.10	Grapevine	Pinot Blanc	Germany	2021
102.12	Grapevine	Riesling	Germany	2021
102.13	Grapevine	Riesling	Germany	2021
102.14	Grapevine	Riesling	Germany	2021
102.15	Grapevine	Riesling	Germany	2021
102.16	Grapevine	Riesling	Germany	2021
102.17	Grapevine	Riesling	Germany	2021
102.18	Grapevine	Riesling	Germany	2021

102.19	Grapevine	Riesling	Germany	2021
102.20	Grapevine	Riesling	Germany	2021

3.2. Sample preparation and RNA purification

Branch and leaf tissues from each plant sample were placed in individual plastic bags (Bioreba, Switzerland) with extraction buffer (PBS containing 0.2 % of diethyldithiocarbamate and 2 % of polyvinylpyrrolidone-10) in a ratio of 1:5 (w:v). The samples were grinded on Homex 6 (Bioreba, Switzerland) (**Fig. 3**).



Figure 3: Example of grapevine branches processed (A) and grapevine tissue grinded (B) used in this study.

Total RNA was purified from 200 μ L of plant extract using a commercial Plant/Fungi Total RNA Purification Kit (Norgen Biotek Corporation, Thorold, ON, Canada) following the manufacturer's indications, with small modifications (**Fig. 4**). RNA was eluted in a total volume of 50 μ L.

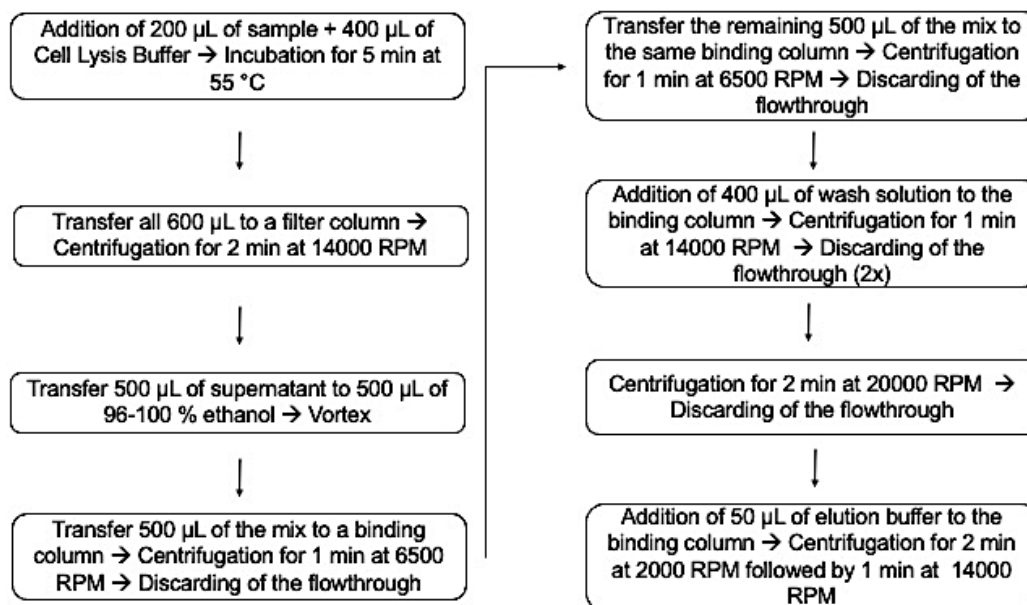


Figure 4: Flowchart of RNA purification performed from the crude extract.

Purified RNA was quantified using DeNovix DS-11 (DeNovix Inc., Wilmington, DE, USA) spectrophotometer to determine the concentrations and quality of the extraction, considering a nanometer ratio of A_{260}/A_{280} higher than 1.8 as satisfactory (Jalali *et al.*, 2017). All RNA purifications were stored at -80°C until subsequent analysis.

3.3. Sanger sequencing

All amplified samples using the protocol designed in this study were confirmed by Sanger sequencing. RT-PCR reaction products were purified using the mi-PCR Purification Kit (metabion international AG, Martinsried, Germany) and sequenced by Sanger in both directions (Eurofins Genomics Germany GmbH, Ebersberg, Germany). Data sequences obtained were trimmed and aligned using Geneious Software 10.0.7 (Biomatters Ltd., Auckland, New Zealand).

3.4. HTS analysis and genome recovery

HTS raw data of eight grapevine samples (**Table 4**) were analyzed using CLC Genomics Workbench 10.1.1 (Qiagen Bioinformatics, Hilden, Germany) and Geneious software 10.0.7 (Biomatters Ltd., Auckland, New Zealand). RNA quality control, library construction and HTS sequencing in a NextSeq 500 platform (paired 2x150nt) were performed at Macrogen Inc. (Seoul, Republic of Korea). Complementary DNA was synthesized from each RNA extraction for library

preparation using TruSeq Stranded Total RNA LT Sample Prep Kit (Plant). Library protocol preparation used for it was TruSeq Stranded Total RNA Sample Prep Guide, Part #15031048 Rev.

Raw reads were subjected to trimming of adapters and quality control using CLC Genomics Workbench 10.1.1 (Qiagen Bioinformatics, Hilden, Germany). Host genome subtraction was performed by mapping the reads against the reference sequences GCF_000003745.3_12X, FM179380 and DQ424856 corresponding to Grapevine's genome, mitochondria and chloroplast, respectively.

Grapevine unrelated reads were *de novo* assembled using CLC Genomics Workbench 10.1.1 (Qiagen Bioinformatics, Hilden, Germany). Generated contigs higher than 200 nt were analysed by BLASTN/BLASTX (e-value 10^{-4}). GLRaV-1 related contigs were exported to Geneious software 10.0.7 (Biomatters Ltd., Auckland, New Zealand) for further analysis.

Table 4: Number of reads after host genome subtraction, *de novo* contigs generated and originated from the HTS analyzed samples.

Sample	Number of reads	<i>De novo</i> contigs	Origin
19.5	827,368	1,047	Greece
Pin1	8,642,008	14,375	Spain
33.17	2,410,654	9,760	Spain
33.24	746,086	416	Spain
33.28	585,620	2,696	Spain
33.35	1,231,512	1,045	Spain
33.47	1,147,232	1,319	Spain
52.1	686,277	3,019	Tunisia

In order to recover full GLRaV-1 genomes contigs were extended by mapping the reads against the contigs using Geneious software 10.0.7 (Biomatters Ltd., Auckland, New Zealand).

3.5. Previously reported RT-PCR protocols for GRLaV-1 detection

The 65 positive samples were tested for the new real-time protocol designed in this study and two previously described protocols, a conventional RT-PCR (Alabi *et al.*,

2011) and one real-time RT-PCR (Osman *et al.*, 2007) with slight modifications. Primers and probe's sequences and descriptions are listed in **Table 5**.

Conventional RT-PCR (Alabi *et al.*, 2011) was performed in the Veriti 96 Well thermal cycler (Applied Biosystems, Foster City, CA, USA). The reaction mixture was performed in a total volume of 25 μ L using master mix AgPath-ID™ (Ambion Inc., Austin, TX, USA). containing 500 nM of each primer and 3 μ L of total purified RNA. Amplification conditions consisted of an initial reverse transcription step at 45 °C for 45 min followed by a denaturation step at 95 °C for 10 minutes and 40 cycles of amplification (30 s at 95 °C, 30 s at 50 °C and 25 s at 60 °C) with a final elongation step at 60 °C for 7 min.

Table 5: List of primers and probes used in the RT-PCR detection protocols.

Region	Primers/Probe	Sequence	T _m (°C)	Amplicon size (bp)	Reference
HSP70	HSP70-149 F	ACCTGGTTGAACGAGATCGCTT	58.7	168	Osman <i>et al.</i> (2007)
	HSP70-293 R	GTAAACGGGTGTTCTTCAATTCTCT	55.2		
	HSP70-225 P	ACGAGATATCTGTGGACGGA	54.9		
CPd2	GLRaV-1-CPd2/F	GTTACGGCCCTTTGTTTATTATGG	54.3	398	Alabi <i>et al.</i> (2011)
	GLRaV-1-CPd2/R	CGACCCCTTTATTGTTTGAGTATG	53.8		

T_m: melting temperature; HSP70: heat-shock protein 70; CPd2: coat protein duplicate 2.

Real-time RT-PCR protocol (Osman *et al.*, 2007) was carried out in StepOnePlus thermal cycler (Applied Biosystems, Foster City, CA, USA). The reaction was performed in a total volume of 12 μ L using master mix AgPath-ID™ One-Step RT-PCR Kit (Ambion Inc., Austin, TX, USA) containing 500 nM of each primer, 125 nM of TaqMan probe and 3 μ L of total purified RNA. Amplification conditions were an initial reverse transcription step at 45 °C for 10 min, a denaturation step at 95 °C for 10 min, followed by 45 cycles of amplification (15 s at 95 °C, 15 s at 49 °C and 45 s at 60 °C).

3.6. Newly designed real-time RT-PCR detection method

3.6.1. Primers and probe design

Primers and TaqMan probe design were performed using 659 GLRaV-1 sequences, including full and partial genomes registered in NCBI (accessed on Jul, 2021). In addition, two full genomes and one partial sequence recovered by HTS in this study (unpublished data) as well as one full genome from Slovakia provided by Dr. Miroslav Glasa (unpublished data) were included. Sequence alignment was performed using the algorithm Geneious included in Geneious software 10.0.7 (Biomatters Ltd., Auckland, New Zealand). OligoAnalyzer™ tool (Integrated DNA Technologies, USA) was used to determine the oligonucleotide parameters, specifically GC % content, melting temperature (T_m) and secondary structure.

3.6.2. RT-PCR conditions

The real-time RT-PCR was designed as a duplex reaction using the GLRaV-1 specific primers (GLRaV-1-F, 5'-GAATGGAAAGTTGAAGCCGAA-3', GLRaV-1-R1, 5'-TACTGAGCTTGTCACATTACT-3' and GLRaV-1-R2, 5'-AACCGAGCTTGTCACATTA-3') and probe (GLRaV-1S, 5'-6-FAM-TGCAGACCWTCTTAYTCTCARTTTAG-ZNA-4-BHQ-1-3') as well as a set of primers and probe amplifying the grapevine gene phosphoenolpyruvate carboxylase (PEP) used as a plant internal control, PEP-F1 (5'-GCCTCCTCCTCCAGATTGCT-3'), PEP-R1 (5' AGGCTTGCTTGATTCCATTATCTCTTTTCG-3') and PEP-probe (5'-Cy5-CGACCCATACTTGAAACAGAGACTCCGGC-ZNA-BHQ2-3') (Morán *et al.*, 2018). RT-PCR assays were carried out in a LightCycler 480 thermocycler (Roche, Basel, Switzerland), using PrimeScript™ One Step RT-PCR Kit (Takara Bio Inc., Kusatsu, Japan). Master mix contained 700 nM of each GLRaV-1 primer (GLRaV-1-F, GLRaV-1-R1 and GLRaV-1-R2), 100 nM of each internal control primers (PEP-F1 and PEP-R1), 125 nM of probe GLRaV-1S and 50 nM of PEP-probe. Reaction mixture was carried out in a total volume of 20 µL containing 3 µL of RNA template. Amplification conditions consisted of 45 °C for 10 min, 95 °C for 10 min and 45 cycles of amplification (15 s at 95 °C, 1 min at 60 °C). The default threshold set by the machine was slightly adjusted above the noise to the linear part of the growth curve at its narrowest point, according to the manufacturer.

3.6.3. Absolute quantitation

For the generation of real-time qPCR standard curves, the CP fragment targeted by the real-time RT-PCR was amplified by conventional RT-PCR from the positive sample LR-1 using GLRaV-1 primers designed in this study, Reaction mixture was composed by 1000 nM of each GLRaV-1 primers, 5U AMV Reverse Transcriptase (Promega Corporation, Madison, USA), 2.5U GoTaq® G2 Flexi DNA Polymerase (Promega Corporation, Madison, USA) and 5 µl of RNA template, in a final volume of 25 µL. Amplification conditions consisted of a reverse transcription step at 42 °C for 45 min and a denaturation step at 95 °C for 2 min, followed by 40 cycles of amplification (15 s at 95 °C, 30 s at 49 °C and 20 s at 72 °C) and a final step of 10 min at 72 °C.

The PCR product (185 bp) was purified using a commercial mi-PCR Purification Kit (metabion international AG, Martinsried, Germany) and inserted into a pGEM®-T Easy Vector (Promega Corporation, Madison, USA) following the manufacturer instructions and cloned in *Escherichia coli* XL1-Blue cells. Transformant colonies were selected by ampicillin resistance.

Plasmid extraction was performed using PureYield™ Plasmid Miniprep System (Promega Corporation, Madison, USA) following the manufacturer instructions and quantified with DeNovix DS-11 spectrophotometer (DeNovix Inc, Wilmington, DE, USA). Plasmid DNA was quantified (in DNA copy number) based on its length and mass with the mathematical equations 1 and 2. The total length of the plasmid DNA was estimated as 3200 bp considering the insert addition of 185 bp.

$$\text{DNA copy number} = \text{moles of dsDNA} \times 6.022\text{e}23 \left(\frac{\text{molecules}}{\text{mol}} \right) \quad (1)$$

$$\text{Moles of dsDNA} = \frac{\text{mass of dsDNA (g)}}{(\text{length of dsDNA (bp)} \times 617.96 \left(\frac{\text{g}}{\text{bp}} \right) + 36.04 \left(\frac{\text{g}}{\text{mol}} \right))} \quad (2)$$

Three replicates of serial dilutions from 2×10^9 to 2 plasmid copies were used to generate the standard curve. Amplification efficiency of the RT-PCR was evaluated based on the standard curve slope using equation 3.

$$\text{Amplification efficiency} = \left[10^{\left(-\frac{1}{\text{slope}} \right)} \right] - 1 \quad (3)$$

4. RESULTS

4.1. GLRaV-1 genome recovery by HTS

HTS datasets from 3 grapevine samples infected by GLRaV-1 from Greece, Spain and Tunisia were analyzed for further characterization of GLRaV-1 genetic diversity. Two GLRaV-1 full genomes of 18,725 nt and 18,872 nt as well as a partial genomic sequence of 14,439 nt were recovered (**Table 6**).

Table 6. GLRaV-1 genomic sequences recovered by HTS in this study.

Sample code	Origin	GLRaV-1	
		genomic sequence recovered (nt)	Other grapevine viruses and viroids present (¹)
19.5	Greece	18,725 nt (full genome)	GLRaV-3; GLRaV-4; GFkV; GRSPaV; GVA; GVE; GYSVd-1
Pin1	Spain	18,872 nt (full genome)	GLRaV-2; GFkV; GRSPaV; GRVfV; GVA; GYSVd-1; HSVd
52.1	Tunisia	14,439 nt (partial sequence)	GLRaV-2; GLRaV-3; GFLV; GRSPaV; GVA; GVB; GVE; GVF; GVL; GYSVd-1; HSVd

(¹) GLRaV-2: grapevine leafroll-associated virus 2; GLRaV-3: grapevine leafroll-associated virus 3; GLRaV-4: grapevine leafroll-associated virus 4; GFkV: grapevine fleck virus; GFLV: grapevine fanleaf virus; GVA: grapevine virus A; GVB: grapevine virus B; GVE: grapevine virus E; GVF: grapevine virus F; GVL: grapevine virus L; GRSPaV: grapevine rupestris stem pitting-associated virus; GRVfV: grapevine rupestris vein feathering virus; GYSVd-1: grapevine yellow speckle viroid 1; HSVd: hop stunt viroid.

4.2. Design of a new real time quantitative RT-PCR method for GLRaV-1 detection.

4.2.1. Sequence alignment and design of primers and probe

Based on the sequence alignment of 659 GLRaV-1 sequences, including complete and partial genomes, available in databases as well as two full and one partial genomic sequence recovered by HTS in this study and one full genomic sequence provided by Dr. Miroslav Glasa, the genomic region selected for the RT-PCR design was located in the CP.

Three primers and one probe were designed: primers GLRaV-1-F (5'-GAATGGAAAGTTGAAGCCGAA-3'), GLRaV-1-R1 (5'-TACTGAGCTTGTCACATTACT-3'), and GLRaV-1-R2 (5'-AACCGAGCTTGTCACATTA-3'), able to amplify a 185 bp sequence, and the TaqMan ZNA probe GLRaV-1S (5'-6-FAM-TGCAGACCWTCCTTAYTCTCARTTTAG-ZNA-4-BHQ-1-3').

4.2.2. *In silico* comparison of GLRaV-1 detection primers and probes

Primers and probe designed in this study were compared *in silico* to those used by two previously reported detection methods (Osman *et al.*, 2007; Alabi *et al.*, 2011). For this purpose, primers and probes were aligned with all available GLRaV-1 sequences. As a result of this analysis the number and frequency of mismatches and the presence of mismatches in critical 3'-end positions that might compromise primer binding severely affecting the sensitivity of detection were evaluated.

A score based on these parameters was created to better visualize the possible effect of mismatches in primers/probe performance. **Figure 6** represents the variant frequency respect to the sequence of primers and probe at each position. Four different colors have been used to represent the score mentioned above. According to their level of variability, nucleotides are marked in white (< 5%), yellow (5-20 %), orange (20-30%) or red (>30%). In addition, variable nucleotides located in the four primer positions closer to the 3'-end were also marked in red.

This *in silico* primers and probes comparison seem to indicate important limitations for the previously reported techniques (Osman *et al.*, 2007; Alabi *et al.*, 2011) in the universal detection of GLRaV-1, taking into account the current knowledge of the genetic variability of the virus. On the other hand, the primers and probe designed in this study are expected to be able to provide a very broad detection of all GLRaV-1 isolates.

Alabi et al. (2011)

GLRaV-1-CPd2/F	5'	G	T	A	C	G	C	C	T	T	T	G	T	T	A	T	T	A	T	G	G	-3'	
GLRaV-1-CPd2/R	5'	C	G	A	C	C	C	T	T	A	T	T	G	T	T	G	A	G	T	A	T	G	-3'

Osman et al. (2007)

HSP70-149 F	5'	A	C	C	T	G	G	T	T	G	A	A	C	G	A	G	A	T	C	G	C	T	-3'				
HSP70-293 R	5'	G	T	A	A	A	C	G	G	G	T	G	T	T	C	T	T	C	A	A	T	T	C	T	C	T	-3'
HSP70-225 P	5'	A	C	G	A	G	A	T	A	T	C	T	G	T	G	G	A	C	G	G	A	-3'					

This study

GLRaV-1-F	5'	G	A	A	T	G	G	A	A	G	T	T	G	A	A	G	C	C	G	A	-3'							
GLRaV-1-R1	5'	T	A	C	C	G	A	G	C	T	T	G	T	C	A	C	A	T	T	A	C	T	-3'					
GLRaV-1-R2	5'	A	A	C	C	G	A	G	C	T	T	G	T	C	A	C	A	T	T	A	-3'							
GLRaV-1S	5'	T	G	C	A	G	A	C	C	W	T	C	T	T	A	Y	T	C	T	C	A	R	T	T	T	A	G	-3'

Variant frequency: <5% 5-20% 20-30% < 30 and/or critical position*

Figure 6: Frequency of primers/probes mismatches present in GLRaV-1 detection methods.

4.3. Validation of the new real time quantitative RT-PCR technique

4.3.1. Technical sensitivity and absolute quantification

The absolute quantitation of GLRaV-1 was performed using known quantities of a plasmid carrying the CP fragment targeted by the real-time RT-PCR. A standard curve was obtained using three replicates of serial dilutions ranging from 2×10^9 to 2 target copies. The standard curve showed a slope of -3.33 which allowed to calculate an amplification efficiency of 99.79 % with a coefficient of correlation (R^2) of 0.98 (**Fig. 7**).

The GLRaV-1 detection method designed in this study was able to detect up to 2 copies or viral target, thus showing a very high sensitivity.

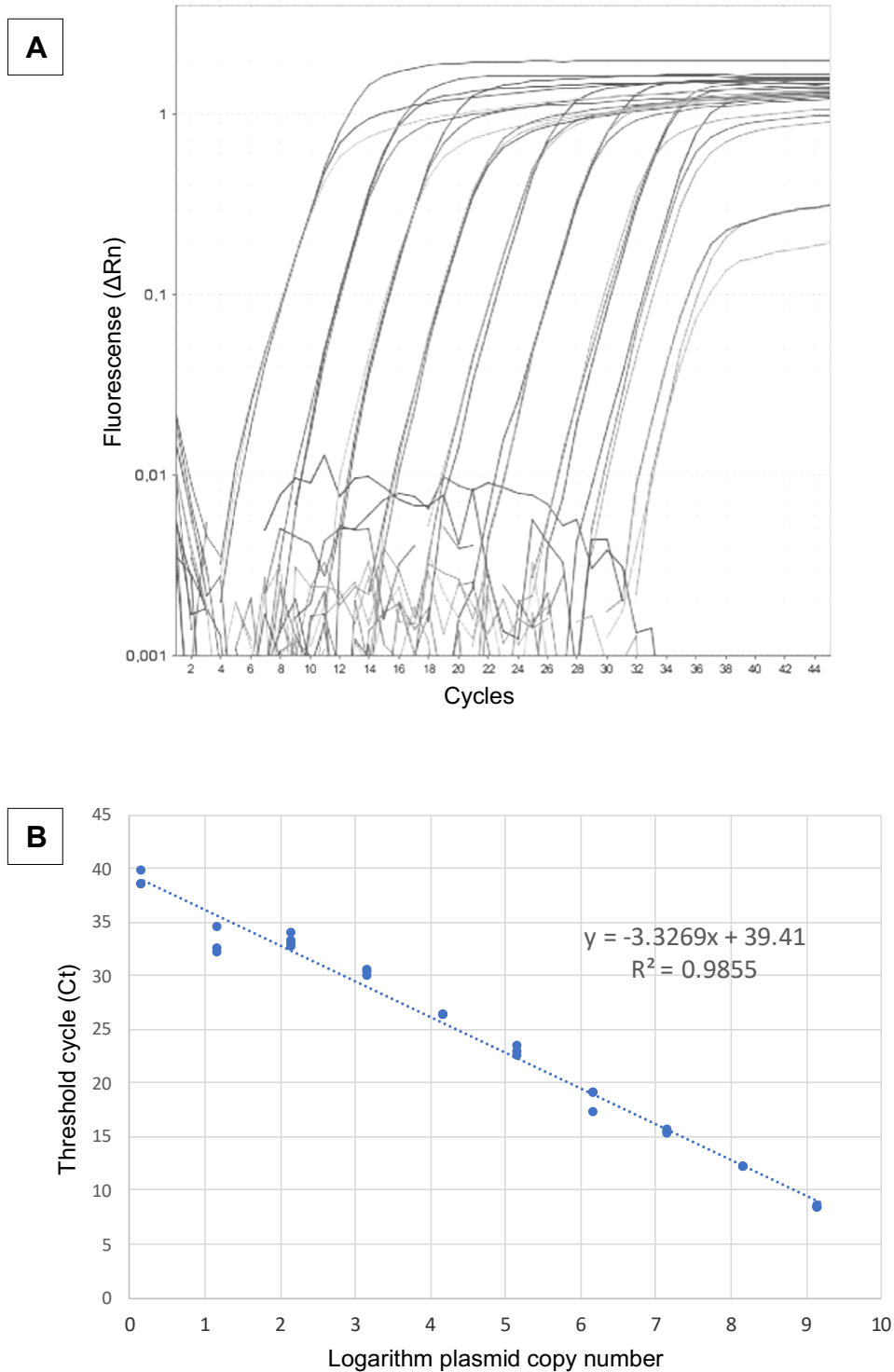


Figure 7: Absolute GLRaV-1 quantitation standard curve obtained from serial dilutions of plasmid DNA containing the RT-PCR target region. (A): Amplification plots for three replicates of serial dilutions of plasmid vector with the viral insert. (B): Threshold cycles (Ct) values obtained for three replicates of ten-fold serial dilutions. The mathematical equation of the standard curve and the coefficient of correlation (R^2) are indicated.

4.3.2. Analytical specificity and selectivity

Analytical specificity of the technique was evaluated considering both inclusivity and exclusivity. Inclusivity was evaluated by testing different GLRaV-1 isolates from different geographic origins. All positive samples tested, representing the GLRaV-1 genetic diversity, were successfully detected by the method herein developed. Exclusivity was evaluated by testing 5 grapevine GLRaV-1 negative plants infected by several common grapevine viruses, as determined by HTS (**Table 8**). None of these samples tested positive by the new real-time RT-PCR.

Table 8: Virome analysis by HTS of GLRaV-1 free grapevine samples.

Sample code	Origin	Virome (1)
33.17	Spain	GLRaV-3; GRSPaV; GRVfV; GAMaV; GFkV; GVA; GYSVd-1
33.24	Spain	GLRaV-4; GLRaV-3; GYSVd-1
33.28	Spain	GLRaV-3; GRSPaV; GRVfV; GAMaV; GVA; GYSVd-1
33.35	Spain	GLRaV-4; GLRaV-3; GYSVd-1; GRVfV
33.47	Spain	GLRaV-3; GRSPaV-1; GRVfV; GYSVd-1

(¹) GLRaV-3: grapevine leafroll-associated virus 3; GLRaV-4: grapevine leafroll-associated virus 4; GFkV: grapevine fleck virus; GAMaV: grapevine asteroid mosaic-associated virus; GVA: grapevine virus A; GRSPaV: grapevine rupestris stem pitting-associated virus; GYSVd-1: grapevine yellow speckle viroid 1; GRVfV: grapevine rupestris vein feathering virus.

On the other hand, selectivity was evaluated testing the presence of GLRaV-1 in 16 different cultivars. The new real-time RT-PCR protocol performance was not affected by these variations in the matrix.

4.4. Comparison of GLRaV-1 detection methods

The real-time quantitative RT-PCR designed in this study was compared to two previously reported detection methods (Osman *et al.*, 2007; Alabi *et al.*, 2011) in

experimental conditions. A total of 65 positive samples from different geographic origins and cultivars were tested by all three techniques (**Table 9**).

As expected, real-time PCR methods (Osman *et al.*, 2007 and this study) performed better than the conventional PCR protocol (Alabi *et al.*, 2011) which was only able to detect 23.1% of the total amount of positives. However, the real-time method described by Osman *et al.* (2007) gave a positive result for 35 out of 65 positives (53.8%) whereas all positive samples (100%) tested positive by our real-time RT-PCR, thus showing a significant improvement in GLRaV-1 detection.

In addition, the method reported in this study has been designed as a duplex reaction including a grapevine internal control, the housekeeping gene PEP, in order to detect putative false negative results and thus increasing the diagnostic sensitivity of GLRaV-1 detection.

Table 9: Comparison between the different GLRaV-1 detection protocols.

Sample code	Origin	Conventional RT-PCR (Alabi <i>et al.</i> , 2011)	Real-time RT-PCR (Osman <i>et al.</i> , 2007) (Ct)	New real-time RT-PCR protocol (This study) (Ct)	
				GLRaV-1	PEP
LR-1		+	28.8	25.0	20.1
100.4		-	-	29.2	21.7
100.25		-	-	28.7	20.3
100.32		-	-	33.3	24.8
100.42	Spain	-	-	28.2	25.1
100.44		-	-	30.0	23.2
100.47		-	-	28.5	22.6
100.48		-	-	27.9	23.2
100.50		-	-	28.2	23.4
88.1		+	17.7	17.5	20.7
88.2	Switzerland	+	17.9	18.5	21.8
88.3		+	18.6	18.6	23.9

91.1		-	-	28.9	22.3
91.2		-	-	29.0	23.7
91.3		-	-	17.2	21.6
91.4		+	-	18.9	20.3
91.5		-	-	18.3	21.7
91.6	Slovakia	-	-	34.0	22.3
91.7		-	27.4	33.0	23.5
91.8		-	15.0	14.9	24.6
91.10		-	33.0	31.8	20.4
91.11		-	-	32.8	25.3
91.12		+	-	23.3	24.1
52.1	Tunisia	-	-	36.8	21.5
35.2		+	22.8	24.9	21.9
35.4	Thailand	+	-	23.7	20.3
35.6		+	19.8	18.8	20.1
18.6		-	-	38.0	21.3
19.3		+	19.7	17.8	23.1
19.5		+	-	18.9	20.4
19.6		-	-	35.3	21.7
29.4		-	21.4	17.8	22.8
29.6		-	19.0	19.7	24.1
29.8		-	-	33.2	22.3
29.11	Greece	-	-	30.7	20.5
98.2		-	-	17.7	21.8
98.3		-	16.7	16.2	20.4
98.8		-	19.5	15.3	23.7
98.9		-	20.3	24.0	22.7
98.10		-	22.4	22.0	21.8
98.11		+	20.4	21.7	20.9

98.12		-	-	26.5	21.2
98.13		-	21.6	20.2	24.3
98.14		-	-	32.1	21.6
98.16		-	-	30.9	23.4
98.17		-	23.4	23.4	20.7
<hr/>					
102.1		-	-	35.0	23.8
102.2		-	21.9	22.2	21.9
102.3		-	18.6	19.7	24.6
102.4		-	22.6	22.9	21.0
102.5		-	20.8	19.6	20.1
102.6		-	17.6	19.7	20.4
102.7		-	14.1	18.2	21.5
102.8		-	16.7	21.9	20.8
102.9		-	-	34.8	21.1
102.10	Germany	-	-	33.9	22.3
102.12		+	20.4	16.6	21.3
102.13		+	21.2	21.3	22.1
102.14		-	20.4	20.3	21.6
102.15		-	22.5	33.7	23.5
102.16		-	18.5	18.6	25.1
102.17		-	19.1	18.8	23.5
102.18		-	26.6	23.7	24.3
102.19		-	21.6	18.3	21.8
102.20		+	23.4	19.4	23.1

(+): Presence of the virus; (-): Absence of the virus.

4.5. Performance of the new GLRaV-1 real time RT-PCR method in field samples

A total of 241 samples from several random surveys from different Spanish grapevine growing regions (D.O. Priorato, D.O. Manchuela and D.O. Utiel-Requena) that had previously tested negative by the RT-PCRs reported by Osman et al., 2007 and Alabi

et al., 2011 were analysed by the amplification protocol described in this study (**Table 10**). Interestingly, 24 samples tested positive for GLRaV-1 with Cts ranging from 22.4 to 36.9, thus representing false negative results by the previously described methods.

Table 10: Results of the survey performed with the new real-time RT-qPCR designed for GLRaV-1 detection.

Sample region	Year of collection	Total of sample collected	New real-time RT-PCR protocol (This Study)			
			Positives		Negatives	
Utiel-Requena	2015	48	5	(10.4%)	43	(89.6%)
Priorato	2016	29	0	(0.0%)	29	(100.0%)
Manchuela	2016	26	3	(11.5%)	23	(88.5%)
Utiel-Requena	2019	52	0	(0.0%)	52	(100.0%)
Utiel-Requena	2021	86	16	(18.6%)	70	(81.4%)
Total		241	24	(10%)	217	(90%)

5. DISCUSSION

Grapevine leafroll disease is one of the most important grapevine viral diseases, given its effect on grape and wine quality and production. As other grapevine viral diseases, GLD control requires efficient and reliable methods for the specific detection of the viruses associated to them, as GLRaV-1. These methods need to be improved and constantly revised as knowledge on the viral genetic diversity increases (Katsiani *et al.*, 2018; Diaz-Lara *et al.*, 2020). With the objective of improving the detection of GLRaV-1, in this study a new GLRaV-1 real-time quantitative RT-PCR detection method has been developed and validated according to EPPO standards (EPPO, 2008; EPPO, 2018).

The real-time RT-PCR method has been designed to target a genomic region in the the coat protein (CP) which is considered a conserved region for members of the *Closteroviridae* family, including GLRaV-1, (Ling *et al.*, 1997; Donda *et al.*, 2017; Agranovsky, 2021).

In fact, the *in silico* sequence analysis performed in this study, which has taken into account all the genomic variability currently known for this viral species, has shown

CP to be an appropriate region to detect all known isolates. Moreover, this hypothesis has been confirmed experimentally, as the technique has been able to detect all the positive controls from different origins analysed.

In addition, GLRaV-1 infected plants that had tested negative by previously reported detection methods have been successfully identified as infected by the virus. These results demonstrate an improvement in the inclusivity of the new technique, the performance of a test with a range of target organisms covering genetic diversity, different geographical origins and host, when compared to previous protocols.

Regarding analytical specificity, the designed method also shows a high exclusivity, the performance of the technique with regard to cross reaction with non-targets, as any false positive results have been obtained when analysing grapevine samples infected by common grapevine viruses and viroids.

The analytical sensitivity of the method, the smallest amount of targets that can be detected reliably, has been shown to be very high, allowing the detection of only 2 copies of viral targets.

Moreover, the detection technique has been designed including an internal RT-PCR control, a grapevine housekeeping gene (PEP), in a duplex reaction, in order to detect putative false negative results and thus increasing the diagnostic sensitivity, the proportion of true positives among the infected plants.

6. CONCLUSION

In conclusion, in this study a new protocol for GLRaV-1 detection has been designed and validated according to EPPO standards. The new method represents a clear improvement in the detection of this viral species compared to the current available methods. This new real time quantitative RT-PCR protocol can be used to successfully detect all GLRaV-1 isolates currently known, thus opening new possibilities in the management and control of this GLD-associated viral pathogen.

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