Differences of Three Ampeloviruses' Multiplication in Plant May Explain Their Incidences in Vineyards

Leonardo Velasco, Instituto Andaluz de Investigación y Formación Agraria, Pesquera, Alimentaria y de la Producción Ecológica (IFAPA), 29140 Churriana, Málaga, Spain; **Josefina Bota** and **Rafael Montero**, Institut de Recerca i Formació Agrària i Pesquera de les Illes Balears, 07009 Palma de Mallorca, Spain; and **Enrico Cretazzo**, IFAPA, Spain

Abstract

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Grapevine leafroll ampeloviruses have been recently grouped into two major clades, one for *Grapevine leafroll associated virus* (GLRaV) 1 and 3 and another one grouping GLRaV-4 and its variants. In order to understand biological factors mediating differential ampelovirus incidences in vineyards, quantitative real-time polymerase chain reactions were performed to assess virus populations in three grapevine varieties in which different infection status were detected: GLRaV-3 + GLRaV-4, GLRaV-3 + GLRaV-4 strain 5, and GLRaV-4 alone. Specific primers based on the RNA-dependent RNA polymerase (*RdRp*) domains of GLRaV-3, GLRaV-4, and GLRaV-4 strain 5 were used. Absolute and relative quantifications of the three viruses were achieved by normalization of data to the concentration of the endogenous gene actin. In spring, the populations of GLRaV-4 and GLRaV-4 strain 5 were 1.7

$\times 10^4$ to 5.0×10^5 genomic RNA copies/mg of petiole tissue whereas, for GLRaV-3, values were significantly higher, ranging from 5.6×10^5 and 1.0×10^7 copies mg^{-1} . In autumn, GLRaV-4 and GLRaV-4 strain 5 populations increased significantly, displaying values for genome copies between 4.1×10^5 and 6.3×10^6 copies mg^{-1} , whereas GLRaV-3 populations displayed a less pronounced boost but were still significantly higher, ranging from 4.1×10^6 to 1.6×10^7 copies mg^{-1} . To investigate whether additional viruses may interfere in the quantifications the small RNA populations, vines were analyzed by Ion Torrent high-throughput sequencing. It allowed the identification of additional viruses and viroids, including *Grapevine virus A*, *Hop stunt viroid*, *Grapevine yellow speckle viroid 1*, and *Australian grapevine viroid*. The significance of these findings is discussed.

Grapevine leafroll disease (GLRD) is one of the most distributed viral diseases for the grapevine (*Vitis vinifera* L.; 2,17,28,31,46). Along with Grapevine fanleaf degeneration disease, it induces the major economic losses by virus infections in viticulture (28). To date, 11 *Grapevine leafroll associated viruses* (GLRaVs) have been described: GLRaV-1, -2, -3 -4, -5, -6, -7, -9, -Pr, -De, and GLRaCV, all belonging to the *Closteroviridae* family. Of these viruses, GLRaV-2 is the only member of the *Closterovirus* genus; a tentative genus called *Velavirus* has been created to classify GLRaV-7, while the rest of GLRaVs belong to the *Ampelovirus* genus (30). Among ampeloviruses, GLRaV-1 and GLRaV-3 are apparently the most common in vineyards and, together with GLRaV-2 (6), the main viruses responsible for GLRD damages. These are also much more extensively studied from any point of view. The others ampeloviruses (GLRaV-4, -5, -6, -9, -Pr, -De, and GLRaCV) belong to a common phylogenetic clade (subgroup II) in the *Ampelovirus* genera and may be classified as divergent variants of one virus, GLRaV-4, instead of distinct species, according to recent taxonomical classification (29). Thus far, there is not yet any specific study about the effects of GLRaV-4-like ampeloviruses on vine performance whereas only a few reports showed their transmission by some vectors in a nonspecific way (2,28).

The Commission Directive 2005/43/EC considers only GLRaV-1 and -3 among GLRaVs as harmful organisms in nursery stocks. However, the development of polymerase chain reaction (PCR) diagnostics tests has allowed more consistent detection of the GLRaV-4-like ampeloviruses all around the world (9,12,13,21,38). Particularly, GLRaV-4, -5, -6, and -9 have been recently identified

in Spain (34–36). Therefore, the genomic characterization of these viruses has acquired an increased interest (13,18,40,42). In addition, real-time quantitative PCR (qPCR) has been successfully used for detection and quantitation of GLRaVs (32,33,43). In this study, absolute and relative quantifications of GLRaV-3, GLRaV-4, and GLRaV-5 (henceforth, GLRaV-4 strain 5) have been achieved starting from leaf petiole extracts of vines displaying three different infection status (GLRaV-3+GLRaV-4, GLRaV-3+GLRaV-4 strain 5, and GLRaV-4 alone). Differences in concentration between GLRaVs were analyzed in order to understand the differences in their relative incidences in vineyards.

Materials and Methods

Plant material. Vines belonged to three traditional Spanish varieties: 'Rome' and 'Tintilla de Rota' from the Autonomous Community of Andalusia and 'Gorgollassa' from the Balearic Islands. For Rome and Tintilla de Rota, dormant cuttings from the grapevine germplasm collection at the Instituto Andaluz de Investigación y Formación Agraria, Pesquera, Alimentaria y de la Producción Ecológica (IFAPA) center of Rancho de la Merced (Jerez de la Frontera, Spain) were rooted and kept in pots and transferred to an insect-proof, temperature-controlled greenhouse at the IFAPA center in Churriana (Málaga) in 2010. For each variety, 12 plants were analyzed. All of them were double infected by GLRaV-3 and GLRaV-4 strain 5. For Gorgollassa, samples came from 10 clonal replicates of two vines, 5 each, infected by GLRaV-4 alone and by both GLRaV-3 and GLRaV-4, respectively. They were collected directly from the Balearic Islands grapevine germplasm collection at the Institut de Recerca i Formació Agrària i Pesquera de les Illes Balears (Palma de Mallorca). Preliminary serological or molecular assays to check for *Grapevine fanleaf virus*, viruses linked to rugose wood complex, and GLRaVs showed only infections by GLRaV-3, -4, and -4 strain 5. All plants exhibited only leafroll symptoms.

Ion Torrent sequencing for other viruses and viroids identification. Small RNAs (sRNAs) were isolated from petiole tissues collected in June 2013 and stored in RNAlater (Ambion). Samples

Corresponding author: L. Velasco,
E-mail: leonardo.velasco@juntadeandalucia.es

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corresponded to one individual plant from each of the varieties studied in this work. sRNA-enriched samples were obtained using the miRCURYTM RNA isolation kit (Exiqon) and quantified. The quality and profile of the sRNAs was examined with a Bioanalyzer 2100 using the RNA 6000 Nanokit (Agilent). The sRNAs from the four samples were pooled in the same amount (1 ng each) for subsequent massive sequencing. A library was constructed using the Ion AmpliSeq RNA Library Kit (Life Technologies) and sequenced as single reads with an Ion Torrent PGM 318 platform using the services provided by LifeSequencing. Sequencing adapters were trimmed off and reads shorter than 20 nucleotides (nt), as well as low-quality ones, were discarded from the sequence datasets using the CLC Genomics Workbench (CLC Bio). The remaining short reads were assembled into contigs larger than 50 nt using the de novo assembly function of the CLC Genomics software. The CLC contigs were subjected to BLASTx and Blastn analysis against the nonredundant database of the National Center for Biotechnology Information (NCBI) using the CLC Genomics software (<http://www.ncbi.nlm.nih.gov>). The E-value cut-off was set to 1.0×10^{-5} so that high-confidence matches could be reported. Organism taxonomy was retrieved by the CLC software from NCBI. Multiple hits were counted for each organism matched per query.

Isolation of total RNA and cDNA synthesis. Extraction of total RNA was performed in late April 2012 and October 2012 from 100 mg of medium leaf petioles using the Spectrum Plant RNA kit (Sigma-Aldrich). Previous disruption and homogenization of samples were carried out by a TissueLyser (Qiagen). A NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies) was used to determine the RNA concentrations in order to prepare the opportune dilutions for subsequent reverse transcriptions (RTs). The synthesis of cDNA by RT was performed in a 20- μ l reaction with 200 ng of RNA, random nonamers (Takara Bio), and Mu-MLV Reverse Transcriptase, following the manufacturer's instructions (Eurogentec).

Primer design for real-time RT-PCR. For all three viruses (GLRaV-3, -4, and -4 strain 5), a genome sequence within the RNA dependent RNA polymerase (*RdRp*) domain was amplified by conventional PCR. For GLRaV-3, primers P3U-2 and P3D (44) were used (Table 1). For GLRaV-4 and GLRaV-4 strain 5, primers (LR4-RP_F3, LR4-RP_R1, LR5-RP_F2, and LR5-RP_R3; Table 1) were designed using FastPCR software (22) based on GenBank accessions NC016416 and FR822696, respectively. All amplicons were cloned in vector pJET2.1 (Fermentas) and sequenced.

For the absolute quantitation, other sets of primers were designed for GLRaV-4 and -4 strain 5 within the *RdRp* domain (LR4-RP_F2, LR4-RP_R2, GLR5-RP_F1, and LR5-RP_R1; Table 1), based on the amplicons obtained by conventional PCR (984 and 916 bp, respectively; Table 1), using Primer3 on-line software tool (39). Primers LR3qrtF and LR3qrtR (43) were used for GLRaV-3 quantitation. In addition, the grapevine endogenous gene actin was amplified in parallel reactions using a specific primer pair (20) in order to provide a control for normalization.

Real-time RT-PCR. Quantitations were performed in white 96-well PCR plates using a Bio-Rad iQ5 Thermal cycler. Each reaction (20 μ l final volume) contained 1 μ l of cDNA, 10 μ l of KAPA SYBR Green qPCR mix (KAPA Biosystems), and 500 nM each primer. The cycling conditions consisted of an initial denaturation of 3 min at 95°C and 40 cycles of 30 s at 94°C and 15 s at 60°C. Each qPCR, including the actin gene internal control, was repeated three times. The specificity of the amplicons obtained was checked with Bio-Rad Optical System Software v.2.1 by means of melting-curve analyses (60 s at 95°C and 60 s at 55°C), followed by fluorescence measurements (from 55 to 95°C, with increments by 0.5°C) (8).

Standard curves generation. For each virus, a partial *RdRp* domain was amplified. Primers used were T7-LR4-RP_F3 and LR4-RP_R1 for GLRaV-4, T7-LR5-RP_F2 and LR5-RP_R3 for GLRaV-4 strain 5, and T7-P3U-2 and P3D for GLRaV-3 (Table 1), where T7 refers to the T7 promoter. Thus, for the synthesis of RNA, the amplicons from PCR were purified and used as templates in a reaction with T7 RNA polymerase (Life Technologies), according to the manufacturer's instructions. Synthetic RNA was quantified and serially diluted in virus-free 'Sugar Seedless' genomic RNA for RT and qPCR standard curve generation. Taking into account the molecular weight of synthetic RNAs, the number of genomic copies of each virus was calculated in each dilution. Thus, the absolute virus quantitation was inferred in each sample by interpolation. A standard curve for the actin gene was obtained for normalization. The efficiency (*E*) of amplifications was calculated from the standard curves by the equation: $E = 10^{(-1/\text{slope})}$ (8). In percentage, it is expressed by the formula $\% = (E - 1) \times 100$.

Statistical analyses. The relative and absolute amounts of viral genomic RNA detected by the RT-qPCR were compared as the means of the clonal replicates (12 Rome plants, 12 Tintilla de Rota plants, 5 Gorgollassa plants infected by GLRaV-4 alone, and 4 Gorgollassa plants infected by both GLRaV-4 and -3) of their respective mother vines. The differences among virus concentrations, depending on grapevine varieties and seasons, were investigated by analyses of variance and Student–Newman–Keuls (SNK) range tests. The software used was SPSS v. 13 (SPSS Inc.).

Results

Isolate *RdRp* domains characterization. Partial *RdRp* domains of GLRaV-3 isolates from each of the three varieties, GLRaV-4 strain 5 *RdRp* domains from Rome and Tintilla de Rota, and GLRaV-4 *RdRp* domain from Gorgollassa were sequenced and deposited in the GenBank under accession numbers KC561088, KC561089, KC561090, KC561092, KC561087, and KC561091, corresponding to GLRaV-3 (Gorgollassa), GLRaV-3 (Rome), GLRaV-3 (Tintilla), GLRaV-4 strain 5 (Rome), GLRaV-4 strain 5 (Tintilla), and GLRaV-4 (Gorgollassa), respectively. Amplicons of 984 and 916 bp were obtained for GLRaV-4 and -4 strain 5, respectively. For GLRaV-3, the amplicon length was 653 bp, as expected (43). Sequencing confirmed that primers LR3qrtF and LR3qrtR were appropriate for all three GLRaV-3 isolates of this

Table 1. Primers used in this work (see main text for description)

Primer	Sequence (5'–3')	Position in GenBank accession	Reference
ActinF	CTTGTCATCCCTCAGCACCTT	GU585869 (1)	20
ActinR	TCTGTGGACAATGGATGGA	GU585869 (62)	20
LR3qrtF	CTTCTACCACGGGATGGACACT	NC004667 (7,585)	43
LR3qrtR	GGTGTAGTATTGCCGGATGT	NC004667 (7,680)	43
P3U-2	GCTCATGGTGAAAGCAGAC	NC004667 (7,425)	Turturo et al. (2005)
P3D	TAGAACAAAAATATGGAGCAG	NC004667 (8,057)	Turturo et al. (2005)
LR4-RP_F2	GGCAGTGGAAATTGGAAGTGT	NC016416 (8,119)	This work
LR4-RP_F3	CTTTAGGGAGTGCTGGGTCA	NC016416 (7,287)	This work
LR4-RP_R1	GTATTGGCTGCACCTGTCTT	NC016416 (8,271)	This work
LR4-RP_R2	CTGCACCTGTCCTCTTTGT	NC016416 (8,264)	This work
LR5-RP_F1	ATCAAAATCTTGGCATCCAG	FR822696 (7,139)	This work
LR5-RP_F2	CTGGTTTGATTGACGGTGTG	FR822696 (6,823)	This work
LR5-RP_R1	TCTCAGCTTTAGCTGCGTCA	FR822696 (7,230)	This work
LR5-RP_R3	GCTGCCCAAGTGTCAGTAT	FR822696 (7,738)	This work

study and allowed us to design the specific primers needed for GLRaV-4 and -4 strain 5 qPCR quantitation. The nucleotide identities were also investigated. Regarding the *RdRp* domains for the three GLRaV-3 isolates, the nucleotide identities were 99.0% for the Tintilla de Rota isolate and 99.5 % for both Rome and Gorgollassa isolates with respect to GLRaV-3 isolate 623 (accession number GQ352632). With respect to the *RdRp* domain of the GLRaV-4 isolates identified in both Gorgollassa varieties, identities were 99.2% when compared with the GLRaV-4 isolate LR106 (accession number FJ467503). The nucleotide identities for the *RdRp* domains of the GLRaV-4 strain 5 isolates of Rome and Tintilla de Rota were both 93.3% when compared with the corresponding domain of the GLRaV-5 isolate 3138-03 (accession number JX559639).

Standard curves generation. Standard curves from in vitro synthesized RNA of GLRaV-3, -4, and -4 strain 5 *RdRp* partial gene were generated by means of serial dilutions of the amplicons. Thus, the amplification efficiency of each primer pair and target was calculated, resulting in 101.2, 104.12, and 104.3% for GLRaV-3, GLRaV-4, and GLRaV-4 strain 5, respectively (Fig. 1). Similarly, a standard curve was obtained for the actin gene and the efficiency of the amplification was 100.08 %.

Relative quantitation. For each variety and season, the mean $\Delta\Delta Cq = (Cq_{\text{virusA}} - Cq_{\text{actin}}) - (Cq_{\text{virusB}} - Cq_{\text{actin}})$ of GLRaV-3 versus GLRaV-4 or GLRaV-4 strain 5 was obtained, where Cq is the quantification cycle at a threshold level of fluorescence (7). The means $\Delta\Delta Cq$ were higher in April than in October in all cases. In Gorgollassa, $\Delta\Delta Cq$ mean between GLRaV-4 and GLRaV-3 was 2.11 in April but decreased to 0.51 in October. In Rome, $\Delta\Delta Cq$

between GLRaV-4 strain 5 and GLRaV-3 was 3.68 in April and 2.54 in October. In Tintilla de Rota, $\Delta\Delta Cq$ of GLRaV-4 strain 5 versus GLRaV-3 was 3.85 in April and 0.81 in October. In the conditions of this study, a $\Delta\Delta Cq$ of 3.3 corresponds to a difference by one magnitude order. Therefore, these $\Delta\Delta Cq$ s in April indicate differences higher than one magnitude order in Tintilla de Rota and Rome (GLRaV-3 versus GLRaV-4 strain 5), being less consistent in Gorgollassa (GLRaV-3 versus GLRaV-4). In October, the differences between ampelovirus titers were smaller in all three varieties.

Absolute quantitation. Standard curves (Fig. 1) allowed calculation of the absolute copy number of GLRaV-3, GLRaV-4, and GLRaV-4 strain 5 for each variety and season. In Gorgollassa vines, the mean copy number of GLRaV-3 in the petiole was 4.9×10^6 and $4.1 \times 10^6 \text{ mg}^{-1}$ in April and October, respectively, being not statistically different ($P = 0.564$). With respect to the quantitation of GLRaV-4, results did not display significant differences between vines with single (only GLRaV-4) and double (GLRaV-3 and -4) infections ($P = 0.885$): in April, the mean number of genome copies was $5.6 \times 10^5 \text{ mg}^{-1}$ in the former and $3.2 \times 10^5 \text{ mg}^{-1}$ in the latter. Similarly, in October, double-infected vines displayed values for GLRaV-4 similar to those for single-infected ones (9.0×10^5 versus $1.1 \times 10^6 \text{ mg}^{-1}$). In the analysis of the total virus population of GLRaV-4 in Gorgollassa vines (independently from the GLRaV-3 infection), small but significant differences were detected between seasons ($4.4 \times 10^5 \text{ mg}^{-1}$ in April and $9.9 \times 10^5 \text{ mg}^{-1}$ in October; Fig. 2; $P < 0.01$).

In Rome, vines were double infected by GLRaV-3 and -4 strain 5, and the mean number of genome copies was 5.6×10^5 for

Fig. 1. Evaluation of amplification efficiency and dynamic range of amplification of A, *Grapevine leafroll-associated virus 3*; B, *Grapevine leafroll-associated virus 4*; C, *Grapevine leafroll-associated virus 4 strain 5*, and D, actin as the internal reference gene cDNA templates. Mean quantification cycle values were obtained from serial $\log_{10}$ dilutions of reverse transcribed synthesized viral template RNAs. CT = cycle threshold.

GLRaV-3 and $1.69 \times 10^4 \text{ mg}^{-1}$ for GLRaV-4 strain 5 in April, increasing to $7.5 \times 10^6 \text{ mg}^{-1}$ and $4.1 \times 10^5 \text{ mg}^{-1}$, respectively, in October (Fig. 2). In Tintilla de Rota, vines were infected by both GLRaV-3 and -4 strain 5, resulting in mean copy number values of $1.0 \times 10^7 \text{ mg}^{-1}$ and $5.0 \times 10^5 \text{ mg}^{-1}$ in April for the former and the latter, respectively. Similarly to Rome, in Tintilla de Rota, the number of genome copies significantly increased in October for both viruses, being $1.6 \times 10^7 \text{ mg}^{-1}$ and $6.3 \times 10^6 \text{ mg}^{-1}$ for GLRaV-3 and GLRaV-4 strain 5, respectively (Fig. 2).

The SNK test of $\log_{10}(\text{genome copy number } \text{mg}^{-1})$ data showed significant differences depending on grapevine variety, virus, and their interaction (Table 2). Three groups were separated: the first included only the mean value of GLRaV-4 strain 5 from Rome; the second was composed of GLRaV-5 from Tintilla de Rota, GLRaV-4 from Gorgollassa, and GLRaV-3 from Rome; and the third collected the GLRaV-3s from Gorgollassa and Tintilla de Rota (Table 2). Taking into account October data exclusively, only two groups can be distinguished: one composed of GLRaV-4 strain 5 from Rome and GLRaV-4 from Gorgollassa and the second including all the rest of the mean genome copies (Table 2). This test also displays clear differences between seasons ($P < 0.001$) and viruses (GLRaV-3 versus GLRaV-4 or GLRaV-4 strain 5, $P < 0.001$).

Detection of viruses and viroids in grapevine samples using Ion Torrent sequencing. The Ion Torrent sequencer generated 4,527,060 single reads of 16 nt in length, on average, from the

pooled dataset composed from the four original samples. The sequence dataset was trimmed and filtered for quality and de novo assemblies were performed using CLC Genomics Workbench. In total, 478 contigs were assembled for the library. These contigs were subjected to BLASTx and BLASTn analysis in order to be characterized. In all, 48 contigs were identified corresponding to GLRaV-3, 9 to GLRaV-4, and 7 to GLRaV-4 strain 5 based on their homology with GenBank sequences. In addition, we identified from the pool of sRNA-enriched samples contigs corresponding to *Grapevine virus A* (GVA) and the viroids *Grapevine yellow speckle viroid-1* (GYSVd-1), *Hop stunt viroid* (HSVd-1), and *Australian grapevine viroid* (AGVd). Conventional PCR (*not shown*) allowed us to discriminate the presence of the organisms identified in the different varieties. GVA, GYSVd-1, and HSVd was detected in the plants of Gorgollassa. AGVd was present in the plants of the Rome variety. No other GLRaV species was identified in the pool of sequences, apart from the objects of this study.

Discussion

GLRaV-4-type ampeloviruses have been postulated as primitive members of the genera because of their genome organization (3,11,42). Although GLRaV-4s have been recently been the object of extensive genetic analysis (13,18,21,27,40), to date, there are no reports of GLRaV-4 dynamics in their hosts. Therefore, their differential incidence in the vineyards and their relative epidemiological impact still need research.

Real-time qPCR has replaced other methods such as enzyme-linked immunosorbent assay and molecular hybridization to estimate virus populations in their hosts, due to its higher sensitivity and ability to provide reliable, reproducible, and consistent results (5,47). In addition, an RT followed by qPCR allows the calculation of the number of specific RNA molecules per milligram of plant tissue that results in a fine estimation of the genome copy number in the case of RNA viruses (4,14,33,43). The *RdRp* domain is preferred for the calculation of virus genome copies to avoid the overestimation produced by genes that are shared between genomic and subgenomic RNAs, such as the heat shock protein 70 or coat protein ones (33,43). To reduce possible results of interference by RNA extractions and pipetting errors during PCR set up, the absolute number of viral genome copies was normalized in our analysis to the copy number of the endogenous gene actin.

The seasonal dynamics of GLRaV-3 already has been investigated in four grapevine varieties displaying virus populations within the same range of this study (43). In that report, it was shown that, in addition to 'Thompson Seedless', in which a slow boost was observed, the mean GLRaV-3 population in middle leaves did not increase in autumn with respect to spring, displaying a decrease even in 'Chardonnay'. Similar value ranges for GLRaV-3 are also described in another study (33). In the present study,

Fig. 2. Mean population of *Grapevine leafroll-associated virus* (GLRaV)-3, GLRaV-4, or GLRaV-4 strain 5. Virus concentration in leaf petioles indicates the $\log_{10}$ of the number of genome copies mg^{-1} host tissue collected from middle sections in grapevine shoots. Vertical bars indicate standard error of the means.

Table 2. Student-Newman-Keuls multiple-range test for grapevine and virus species grouping for $\log_{10}(\text{number of genome copies } \text{mg}^{-1} \text{ host tissue})^a$

Virus species	Variety	N	Subset for $\alpha = 0.05$		
			1	2	3
Total data					
GLRaV-4 str. 5	Rome	24	4.754	...	...
GLRaV-4	Gorgollassa	20	...	5.691	...
GLRaV-4 str. 5	Tintilla de Rota	24	...	5.918	...
GLRaV-3	Rome	24	...	5.921	...
GLRaV-3	Gorgollassa	10	...	...	6.602
GLRaV-3	Tintilla de Rota	24	...	...	6.736
	Significance		1	0.634	0.596
October data only					
GLRaV-4 str. 5	Rome	12	5.487	...	...
GLRaV-4	Gorgollassa	10	5.927	...	...
GLRaV-3	Rome	12	...	6.500	...
GLRaV-4 str. 5	Tintilla de Rota	12	...	6.525	...
GLRaV-3	Gorgollassa	5	...	6.596	...
GLRaV-3	Tintilla de Rota	12	...	6.962	...
	Significance	...	0.052	0.171	...

^a Group separation is obtained considering total data ($P < 0.05$) and October data only ($P < 0.05$). GLRaV = *Grapevine leafroll associated virus*.

differences in virus concentration between autumn and spring are evident in Rome and Tintilla de Rota, especially with respect to GLRaV-4 strain 5. In addition, the difference in genome copies among GLRaV-3 and -4 strain 5 is significantly higher in April (more than one magnitude order) than in October in both varieties. Although, in our conditions, interactions between virus species cannot be deduced due to the lack of vines infected by only GLRaV-3 or -4 strain 5, in a previous study, GLRaV-3 populations in vines infected by only this virus (43) displayed the same magnitude of order observed in Rome and Tintilla de Rota; thus, these viruses probably do not interact with one another, at least with respect to changes in virus populations. Furthermore, data from Gorgollassa vines can support such hypothesis because no significant differences in GLRaV-4 genome copies were found between double- and single-infected vines (*data not shown*). In addition, the GLRaV-3 population in October was in the same range as those quantified in Rome and Tintilla de Rota. In conclusion, our results support the idea that GLRaV-3 is able to establish more efficient multiplication rates in the host than GLRaV-4-type ampeloviruses.

In contrast to the Andalusian varieties, in Gorgollassa, both GLRaV-3 and GLRaV-4 populations did not show an appreciable variation between spring and autumn. These differential behaviors observed among varieties could be due to different experimental conditions because Rome and Tintilla de Rota vines were grown in pots in a controlled greenhouse whereas Gorgollassa vines were located in a germplasm collection in the field. However, varietal differences cannot be discarded, as reported previously (43).

Apparently, GLRaV-4-like ampeloviruses are less frequent in vineyards than GLRaV-1 and -3. In Spain, this has been repeatedly observed among candidate clone vines submitted from the different autonomous communities for the official certification (37). The sanitary analysis showed percentages of infections in virus infected samples of 21.6, 33.8, and 15.9% for the GLRaV-1, GLRaV-3, and GLRaV-4 clades, respectively. Most of the vines included in this study were simultaneously infected by more than one GLRaV. In another survey in the autonomous community of Valencia (Spain), GLRaV-3 was detected in 8.7% of the vines sampled, whereas GLRaV-5 and GLRaV-4 were found in only 0.8 and 3.2%, respectively (15).

One possible explanation for the differential incidence among ampeloviruses lies in the differences in transmission by insect vectors. A very extensive literature about the capability of many mealybugs (*Hemiptera: Pseudococcidae*) and soft scales (*Hemiptera: Coccidae*) to acquire and carry GLRaV-1 and -3 is available, including several examples of the natural virus spread in vineyards (2,28). In contrast, to date, only one report in field conditions and a few in experimental trials have shown the transmission of the other ampeloviruses by mealybugs and soft scales. In addition, no specificity between any vector and GLRaV-4-like ampeloviruses has been proven. All the vectors reporting GLRaV-4 and its variants are also vectors for GLRaV-3 (19,24,26,44) but more efficient in the transmission of the latter (26).

In addition to vector transmission, the spread of grapevine viruses by vegetative multiplication of infected stocks plays a very important role (7,23,25,30), especially in local varieties such as Rome, Tintilla de Rota, and Gorgollassa where, due to the absence of certified clones, the exchange of uncontrolled or standard category material between winegrowers is very common (10). Genetic analysis of GLRaV-4s supports that these viruses are well-adapted to vegetative propagation (13,27). Thus, the differences in incidence of GLRaV-3 with respect to GLRaV-4-type ampeloviruses can be explained in terms of its higher efficiency in the multiplication in the host and more efficient spread by vector.

The simultaneous infection of two or more viruses and viroids in the same plant is frequent in nature. Antagonistic or synergistic interactions could result between unrelated viruses and viroids (1,16,41,45). Antagonistic interactions have been previously observed for GYSVd and HSVd with GLRaV-3. However, the decrease in the GLRaV-3 sRNA concentration due to the presence of viroids reported in that study was small, fitting in the same order of

magnitude (1). Although there are differences in the concentrations of the GLRaVs for the different hosts studied in our work, these can be explained by host genetic background, environment, and the other virus and viroids found. Nevertheless, the fact that GLRaV-3 RNA concentration was around one magnitude order more abundant than GLRaV-4 and -4 strain 5 for the different virus–host combinations suggested that we postulate biological differences among the two ampelovirus members that contribute to explain their epidemiological distribution. Finally, the two Gorgollassa vines deferred only in the presence or absence of GLRaV-3, while sharing GLRaV-4, GVA, GYSVd, and HSVd. Given that the GLRaV-4 concentration in both varieties remained similar, regardless of the presence or absence of GLRaV-3, it may be that *Ampelovirus* spp. do not interact with each other. Finally, the real contribution to *Ampelovirus* populations made by the additional virus and viroids found in the vines will remain unknown until specific experiments are performed.

In this study, additional insight into the biological differences between two different members of the *Ampelovirus* genera belonging to subgroups I and II is provided, offering a complementary explanation for the differences in their relative incidences in field. To our knowledge, this is the first report of GLRaV-4 and -5 absolute quantifications. Also, this is the first detection of AGVd in Spain.

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Literature Cited

- Alabi, O. J., Zheng, Y., Jagadeeswaran, G., Sunkar, R., and Rayapati, A. N. 2012. High-throughput sequence analysis of small RNAs in grapevine (*Vitis vinifera* L.) affected by grapevine leafroll disease. *Mol. Plant Pathol.* 13:1060-1076.
- Almeida, R. P. P., Daane, K. M., Bell, V. a., Blaisdell, G. K., Cooper, M. L., Herrbach, E., and Pietersen, G. 2013. Ecology and management of grapevine leafroll disease. *Front. Microbiol.* 4:82.
- Al Rwahnih, M., Uyemoto, J. K., Falk, B. W., and Rowhani, A. 2007. Molecular characterization and detection of *Plum bark necrosis stem pitting-associated virus*. *Arch. Virol.* 152:2197-2206.
- Ambrós, S., El-Mohtar, C., Ruiz-Ruiz, S., Peña, L., Guerri, J., Dawson, W. O., and Moreno, P. 2011. Agroinoculation of *Citrus tristeza virus* causes systemic infection and symptoms in the presumed nonhost *Nicotiana benthamiana*. *Mol. Plant-Microbe Interact.* 24:1119-1131.
- Balaji, B., Bucholtz, D. B., and Anderson, J. M. 2003. *Barley yellow dwarf virus* and *Cereal yellow dwarf virus* quantification by real-time polymerase chain reaction in resistant and susceptible plants. *Phytopathology* 93:1386-1392.
- Bertazzon, N., Borgo, M., Vanin, S., and Angelini, E. 2010. Genetic variability and pathological properties of *Grapevine leafroll-associated virus 2* isolates. *Eur. J. Plant Pathol.* 127:185-197.
- Bertolini, E., García, J., Yuste, A., and Olmos, A. 2010. High prevalence of viruses in table grape from Spain detected by real-time RT-PCR. *Eur. J. Plant Pathol.* 128:283-287.
- Bustin, S. A., Benes, V., Garson, J. A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M. W., Shipley, G. L., Vandesompele, J., and Wittwer, C. T. 2009. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clin. Chem.* 55:611-622.
- Buzkan, N., Karadağ, S., Kaya, A., Baloglu, S., Minafra, A., and Ben-Dov, Y. 2009. First report of the occurrence of *Grapevine leafroll-associated virus-5* in Turkish vineyards. *J. Phytopathol.* 158:448-449.
- Cretazzo, E., Tomás, M., Padilla, C., Rosselló, J., and Medrano, H. 2010. Incidence of virus infection in old vineyards of local grapevine varieties from Majorca: implications for clonal selection. *Span. J. Agric. Res.* 8:409-418.
- Dolja, V. V., Kreuze, J. F., and Valkonen, J. P. 2006. Comparative and functional genomics of closteroviruses. *Virus Res.* 117:38-51.
- Escobar, P. F., Fiore, N., Valenzuela, P. D. T., and Engel, E. A. 2008. First detection of *Grapevine leafroll-associated virus 4* in Chilean grapevines. *Plant Dis.* 92:1474.
- Esteves, F., Teixeira Santos, M., Eiras-Dias, J. E., and Fonseca, F. 2012. Occurrence of *Grapevine leafroll-associated virus 5* in Portugal: Genetic variability and population structure in field-grown grapevines.

- Arch. Virol. 157:1747-1765.
14. Ferriol, I., Ruiz-Ruiz, S., and Rubio, L. 2011. Detection and absolute quantitation of *Broad bean wilt virus 1* (BBWV-1) and BBWV-2 by real time RT-PCR. J. Virol. Methods 177:202-205.
 15. Fiore, N., Zamorano, A., Sánchez-Diana, N., Pallás, V., and Sánchez-Navarro, J. A. Survey and partial molecular characterization of grapevine virus and viroids from Valencia, Spain. Pages 196-197 in: Proc. 17th Congr. Int. Counc. Study Virus Virus-like Dis. Grapevine (ICVG), Davis, CA.
 16. Flores, R., Hernández, C., Martínez de Alba, A. E., Daròs, J. A., and Di Serio, F. 2005. Viroids and viroid-host interactions. Annu. Rev. Phytopathol. 43:117-139.
 17. Fuchs, M., Martinson, T. E., Loeb, G. M., and Hoch, H. C. 2009. Survey for the three major leafroll disease-associated viruses in Finger Lakes vineyards in New York. Plant Dis. 93:395-401.
 18. Ghanem-Sabanadzovic, N. A., Sabanadzovic, S., Gugerli, P., and Rowhani, A. 2012. Genome organization, serology and phylogeny of *Grapevine leafroll-associated viruses 4* and *6*: taxonomic implications. Virus Res. 163:120-128.
 19. Golino, D. A., Sim, S. T., Gill, R., and Rowhani, A. 2002. California mealybugs can spread grapevine leafroll disease. Calif. Agric. 56:196-201.
 20. Gutha, L. R., Casassa, L. F., Harbertson, J. F., and Naidu, R. A. 2010. Modulation of flavonoid biosynthetic pathway genes and anthocyanins due to virus infection in grapevine (*Vitis vinifera* L.) leaves. BMC Plant Biol. 10:e187.
 21. Ito, T., Nakaune, R., Nakano, M., and Koichi S. 2013. Novel variants of *Grapevine leafroll-associated virus 4* and *7* detected from a grapevine showing leafroll symptoms. Arch. Virol. 158:273-275.
 22. Kalendar, R., Lee, D., and Schulman, A. H. 2011. Java web tools for PCR, in silico PCR, and oligonucleotide assembly and analysis. Genomics 98:137-144.
 23. Kominek, P., and Holleínova, V. 2003. Evaluation of sanitary status of grapevines in the Czech Republic. Plant Soil Environ. 49:63-66.
 24. Le Mague, J., Beuve, M., Herrbach, E., and Lemaire, O. 2012. Transmission of six ampeloviruses and two vitiviruses to grapevine by *Phenacoccus aceris*. Phytopathology 102:717-723.
 25. López-Fabuel, I., Wetzel, T., Bertolini, E., Bassler, A., Vidal, E., Torres, L. B., Yuste, A., and Olmos, A. J. 2013. Real-time multiplex RT-PCR for the simultaneous detection of the five main grapevine viruses. J. Virol. Methods 188:21-24.
 26. Mahfoudhi, N., Digiario, M., and Dhouibi, M. H. 2009. Transmission of Grapevine leafroll viruses by *Planococcus ficus* (Hemiptera: Pseudococcidae) and *Ceroptastes rusci* (Hemiptera: Coccidae). Plant Dis. 93:999-1002.
 27. Maliogka, V. I., Dovas, C. I., and Katis, N. I. 2008. Evolutionary relationships of virus species belonging to a distinct lineage within the *Ampelovirus* genus. Virus Res. 135:125-135.
 28. Maree, H. J., Almeida, R. P. P., Bester, R., Chooi, K. M., Cohen, D., Dolja, V. V., Fuchs M. F., Golino, D. A., Jooste, A. E. C., Martelli, G. P., Naidu, R. A., Rowhani, A., Saldarelli, P., and Burger, J. T. 2013. *Grapevine leafroll-associated virus 3*. Front. Microbiol. 4:94.
 29. Martelli, G. P., Abou Ghanem-Sabanadzovic, N., Agranovsky, A. A., Al Rwahnih, M., Dolja, V. V., Dovas, C. I., Fuchs, M., Gugerli, P., Hu, J. S., Jelkmann, W., Katis, N. I., Maliogka, V. I., Melzer, M. J., Menzel, W., Min-afra, A., Rott, M. E., Rowhani, A., Sabanadzovic, S., and Saldarelli, P. 2012. Taxonomic revision of the family *Closteroviridae* with special reference to the Grapevine leafroll-associated members of the genus *Ampelovirus* and the putative species unassigned to the family. J. Plant Pathol. 94:7-19.
 30. Martelli, G. P., and Boudon-Padieu, E. 2006. Directory of infectious diseases of grapevines. International Centre for Advanced Mediterranean Agronomic Studies. Opt. Méditerr. Ser. B Stud. Res. 55:59-75.
 31. Martin, R. R., Eastwell, K. C., Wagner, A., Lamprecht, S., and Tzanetakis, I. E. 2005. Survey for viruses of grapevine in Oregon and Washington. Plant Dis. 89:763-766.
 32. Osman, F., Leutenegger, C., Golino, D., and Rowhani, A. 2007. Real-time RT-PCR (TaqMan) assays for the detection of *Grapevine leafroll associated viruses 1-5* and *9*. J. Virol. Methods 141:22-29.
 33. Pacifico, D., Caciagli, P., Palmano, S., Mannini, F., and Marzachi, C. 2011. Quantitation of *Grapevine leafroll associated virus-1* and *-3*, *Grapevine virus A*, *Grapevine fanleaf virus* and *Grapevine fleck virus* in field-collected *Vitis vinifera* L. 'Nebbiolo' by real-time reverse transcription-PCR. J. Virol. Methods 172:1-7.
 34. Padilla, C. V., Cretazzo, E., López, N., García de Rosa, B., Padilla, V., and Velasco, L. 2010. First report of *Grapevine leafroll-associated virus 4* in Spain. New Dis. Rep. 21:21.
 35. Padilla, C. V., Cretazzo, E., López, N., Hita, I., Alcalá, M. J., Padilla, V., and Velasco, L. 2013. First report of *Grapevine leafroll-associated virus 9* in Spain. J. Plant Pathol. 95:662.
 36. Padilla, C. V., Cretazzo, E., López, N., Padilla, V., and Velasco, L. 2010. First report of *Grapevine leafroll-associated virus 5* in Spain. Plant Dis. 94:1507.
 37. Padilla, C. V., Garcia de Rosa, B., López, N., Velasco, L., Salmerón, E., Padilla, V., and Hita, I. 2012. Grapevine leafroll virus in candidate clones for plant certification in Spain. Pages 268-269 in: Proc. 17th Congr. Int. Counc. Study Virus Virus-like Dis. Grapevine (ICVG), Davis, CA.
 38. Pei, G.-Q., Dong, Y.-F., Zhang, Z.-P., Fan, X.-D. 2010. First Report of *Grapevine leafroll-associated virus 4* and *5* in grapevines in China. Plant Dis. 94:130.
 39. Rozen, S., and Skaletsky, H. J. 2000. Primer3 on the WWW for general users and for biologist programmers. Pages 365-386 in: Bioinformatics Methods and Protocols: Methods in Molecular Biology. S. Krawetz, and S. Misener, eds. Humana Press, Totowa, NJ.
 40. Saldarelli, P., Cornuet, P., Vigne, E., Talas, F., Bronnenkant, I., Dridi, A. M., Boscia, D., Gugerli, P., Fuchs, M., and Martelli, G. P. 2006. Partial characterization of two divergent variants of *Grapevine leafroll-associated virus 4*. J. Plant Pathol. 88:203-214.
 41. Syller, J. 2012. Facilitative and antagonistic interactions between plant viruses in. Mol. Plant Pathol. 13:204-216.
 42. Thompson, J. R., Fuchs, M., and Perry, K. L. 2012. Genomic analysis of *Grapevine leafroll associated virus-5* and related viruses. Virus Res. 163:19-27.
 43. Tsai, C.-W., Daugherty, M. P., and Almeida, R. P. P. 2012. Seasonal dynamics and virus translocation of *Grapevine leafroll-associated virus 3* in grapevine varieties. Plant Pathol. 61:977-985.
 44. Tsai, C.-W., Rowhani, A., Golino, D. A., Daane, K. M., and Almeida, R. P. P. 2010. Mealybug transmission of grapevine leafroll viruses: an analysis of virus-vector specificity. Phytopathology 100:830-834.
 45. Valkonen, J. P. T. 1992. Accumulation of *Potato virus Y* is enhanced in *Solanum brevidens* also infected with *Tobacco mosaic virus* or *potato spindle storage root viroid*. Ann. Appl. Biol. 121:321-327.
 46. Walter, B., and Martelli, G. P. 1996. Sélection clonale de la vigne: sélection sanitaire et sélection pomologique. Influence des viroses et qualité. 1ere partie: effets des viroses sur la culture de la vigne et ses produits. Bull. O. I. V. 69:945-971.
 47. Zhu, Y. J., Lim, S. T. S., Schenck, S., Arcinas, A., and Komor, E. 2010. RT-PCR and quantitative real-time RT-PCR detection of *Sugarcane yellow leaf virus* (SCYLV) in symptomatic and asymptomatic plants of Hawaiian sugarcane cultivars and the correlation of SCYLV titre to yield. Eur. J. Plant Pathol. 127:263-273.