A Novel Genetic Variant of *Grapevine leafroll-associated virus-3* (GLRaV-3) from Idaho Grapevines

Brandon D. Thompson,¹ Jennifer Dahan,¹ Jungmin Lee,² Robert R. Martin,³ and Alexander V. Karasev^{1,†}

¹Department of Entomology, Plant Pathology, and Nematology, University of Idaho, Moscow, ID; ²Horticultural Crops Research Unit (HCRU; Corvallis, OR) Worksite, USDA-ARS, Parma, ID; and ³HCRU, USDA-ARS, Corvallis, OR

Abstract

Grapevine leafroll-associated virus-3 (GLRaV-3) is a major constraint on profitable grapevine cultivation. The virus is transmitted efficiently by mealybugs and soft scale insects, or through vegetative propagation by cuttings, and is present worldwide, wherever grapevines are grown. GLRaV-3 exists as a complex of genetic variants currently classified in several phylogenetic groups that can differ from each other by as much as 30% in nucleotide sequence of the whole genome. In the course of the GLRaV-3 testing of wine grapes in southern Idaho, plants of two grapevine cultivars were found to harbor a novel genetic variant of GLRaV-3, named ID45, which exhibited $\leq 80\%$ nucleotide sequence identity level to the known GLRaV-3 isolates in its most conserved HSP70h gene. The ID45 variant caused no foliar symptoms in ‘Cabernet Sauvignon’ in the fall,

and was demonstrated to have poor reactivity to commercial virus-specific antibodies. The entire 18,478-nt genome sequence of the GLRaV-3-ID45 was determined using a combination of high-throughput and conventional Sanger sequencing, and demonstrated to have typical organization for the genus *Ampelovirus* (family *Closteroviridae*), with only 70 to 77% identity level to the GLRaV-3 genomes from other established phylogroups. We concluded that ID45 represented a new phylogenetic group IX of GLRaV-3. Database search using ID45 nucleotide sequence as a query suggested that this novel ID45 variant is present in at least one other grape-growing state in the U.S., California, and in Brazil. An RT-PCR based test was developed to distinguish ID45 from the predominant GLRaV-3 phylogroup I found in Idaho in single and mixed infections.

Grapevine leafroll disease (GLD) is one of the main disorders of economic significance for the winegrape industry due to potential decrease in grape yield and quality that results in low quality wine (Maree et al. 2013; Naidu et al. 2014). GLD complex includes multiple viruses associated with symptoms of downward leaf rolling and various types of interveinal reddening visible in foliage of red-berried cultivars of grapevine (Martelli 2014; Naidu et al. 2014). *Grapevine leafroll-associated virus-3* (GLRaV-3) is the most common and most damaging virus of the GLD virus complex (Martelli et al. 2011), easily transmitted by mealybugs (Hemiptera: Pseudococcidae) and scale insects (Hemiptera: Coccidae) (Almeida et al. 2013; Golino et al. 2000; Martelli et al. 2011), and also transmitted through infected budwood (Martelli 2014; Naidu et al. 2014). Control of this virus is primarily based on prevention, through the use of certified GLRaV-free planting stock, systematic testing of the nursery budwood sources,

and vector management through insecticide application to limit in-field spread of the virus (Almeida et al. 2013; Martelli 2014).

GLRaV-3 is the type member of the genus *Ampelovirus* (family *Closteroviridae*) comprising mealybug-transmitted viruses with a single-stranded, positive-sense RNA genome (Karasev 2000; Ling et al. 1998, 2004; Martelli et al. 2002). The virus has long, flexuous filamentous, 1,800 × 12-nm particles built of a major capsid protein (CP), and is phloem-limited in grapevine (Martelli et al. 2011). GLRaV-3 has a large, approximately 18.5-kb genome coding for 11 to 13 open reading frames (ORFs); the genome expression includes translational frameshifting, polyprotein processing, and multiple subgenomic RNAs (Jarugula et al. 2010a; Ling et al. 1998, 2004). GLRaV-3 was found to represent a diverse group of genotypes or phylogroups, which differ by as much as 30% in their nucleotide identity, and which may differ in the number of ORFs encoded by the genome (Maree et al. 2015). The low level of the nucleotide sequence identity between these phylogroups suggested existence of multiple strains of the virus, and also possible difficulties in detection and diagnosis of some divergent isolates of GLRaV-3. Between six and seven phylogroups could be defined based on the CP gene sequences of GLRaV-3 isolates, but not all of the GLRaV-3 phylogroups had isolates with whole genomes sequenced and only six phylogroups held up when whole genomes available at the time were subjected to the phylogenetic study (Maree et al. 2015).

Due to movement of infected planting material, the ease of transmission, and wide availability of insect vectors, GLRaV-3 is present on all continents, except Antarctica, and in all grapevine production areas, and is the most prevalent virus in grapevines affected by GLD (Martelli 2014; Martelli et al. 2011; Naidu et al. 2014). GLRaV-3 is also the most prevalent virus in grapevines in the Pacific Northwest (PNW), in Oregon and Washington (Adiputra et al. 2018; Martin et al. 2005). The virus was reported to occur in Idaho about 10 years ago, found in 10 vineyards from two counties, and in six wine grape cultivars (Mekuria et al. 2009). However, only a partial sequence of one gene, HSP70h, was determined for several isolates of the virus, 22 individual sequences in total (Mekuria et al. 2009). The genetic diversity of GLRaV-3 in Idaho was not addressed specifically, beyond the conclusion that it was introduced into the state at least five times; GLRaV-3 genetic diversity in Idaho was deemed

†Corresponding author: Alexander V. Karasev;
E-mail: akarasev@uidaho.edu

B. D. Thompson and J. Dahan contributed equally.

Funding: This work was funded, in part, through grants from the Idaho State Department of Agriculture (ISDA) Specialty Crop Block Grant, Idaho Wine Commission, the Northwest Center for Small Fruits Research, USDA-ARS (2072-21000-047-00D, 2072-21000-052-00D, 2072-21220-003-00D, and 5358-21220-002-18G), the USDA National Institute of Food and Agriculture (Hatch project IDA01560), and the Idaho Agricultural Experiment Station. Data collection performed by the IBEST Genomics Resources Core at the University of Idaho were supported in part by NIH COBRE grant P30GM103324.

Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

*The e-Xtra logo stands for “electronic extra” and indicates that three supplementary tables are published online.

Accepted for publication 7 September 2018.

similar to the virus diversity in other states in the PNW (Mekuria et al. 2009).

Here, we report on an unusual genetic variant of GLRaV-3 found in Idaho, exhibiting $\leq 78\%$ genome nucleotide identity level to the closest match among GLRaV-3 isolates. This genetic variant, called ID45, was found in a symptomless 'Cabernet Sauvignon' vine where it displayed poor reactivity with the commercial ELISA kit specific to GLRaV-3. ID45 was found in two grapevine cultivars, in two vineyards in southern Idaho, planted in different years and having different owners. ID45 apparently represents a new genetic variant of GLRaV-3 that may have new biological and serological properties. The objective of the study was to characterize this novel genetic variant of GLRaV-3 and develop methods for its detection and differentiation. Recent releases of the GenBank sequences suggest that this same genetic variant of GLRaV-3 is present in California. A specific RT-PCR assay was developed to distinguish the ID45 genotype.

Materials and Methods

Origin of samples and sampling methodology. Foliar samples from grapevines were collected prior to harvest in the 2014–2017 seasons, in mid- to late September, near Caldwell, ID (Canyon County), in two commercially operating vineyards, designated 'vineyard A' and 'vineyard B.' The two vineyards were each about 1 ha in size, located approximately 5 km apart, and had separate owners. Two established vineyard blocks, 'Cabernet Sauvignon' and 'Merlot' (*Vitis vinifera* L.), 10 years of age, were sampled in vineyard A in each of the seasons, from 2014 to 2017, eight vines per cultivar, 16 individual vines total. In September 2014, these 16 vines were selected based on presence or absence of foliar symptoms for a study on how leafroll-affected and healthy grapevines influence berry quality; all were permanently tagged, numbered, and tested for the presence of grapevine viruses (see below). The same 16 vines were sampled again in each of the next three seasons. A young block of 'Tempranillo' (*V. vinifera*), 2–3 years of age, was sampled in vineyard B in 2015 and 2016, 35 vines in total. In September 2015, these 35 vines were also selected for a similar berry quality study, based on foliar symptoms; all vines were permanently tagged, numbered, and tested for the presence of grapevine viruses (see below). However, in this vineyard, the owner replaced all tagged vines with new vines of

the same cultivar (Tempranillo), presumably coming from the same nursery, between September 2015 and September 2016. These newly replaced vines were sampled and tested in September 2016. A single 'Cabernet franc' (*V. vinifera*) vine from a block adjacent to the 'Tempranillo' block in vineyard B was also selected based on foliar symptoms, tagged, and tested for viruses in 2015 and 2016; this vine was not replaced by the owner between the 2015 and 2016 seasons.

For virus testing, the sampling methodology followed the established convention (Kanuya et al. 2012; Martin et al. 2005; Mekuria et al. 2009). Four fully expanded leaves with complete petioles were collected per vine, from all sides of the canopy, and placed into a single plastic resealable bag labeled with the vine number and the name of the vineyard. These leaf samples were kept in a cooler with ice for 2 to 3 days until reaching the laboratory. The samples were kept in a cold room (4°C) until the final processing, which occurred 3 to 14 days after the leaf collection. Petioles were cut off from the leaves and used for subsequent extraction and analysis.

Virus testing by RT-PCR and ELISA. The extraction protocol and virus testing followed the general methodology described previously (Osman et al. 2007; Rowhani et al. 2000), with some modifications. About 0.2 to 0.3 g of petiole tissue, chopped using single edge razor blades, was homogenized in 5 ml of extraction buffer (1.59 g/liter Na_2CO_3 , 2.93 g/liter NaHCO_3 , 2% PVP-40, 0.2% bovine serum albumin, 0.05% Tween 20) in a meshed bag using the Homex 6 grinding machine (Bioreba, Reinach, Switzerland). These extracts were stored at -20 or -80°C for long-term storage. For a direct, one-step RT-PCR virus assay, an aliquot (8 μl) of the extract was added to 50 μl of GES buffer (0.1 M glycine at pH 9.0, 50 mM NaCl, 1 mM EDTA, 0.5% Triton X-100) and heated at 95°C . To minimize RNA degradation, 1% β -mercaptoethanol was added into GES before use and all operations that followed the denaturation step prior to RT-PCR were done on ice. For the RT-PCR, 2 μl of denatured extract was added to 23 μl of RT-PCR mix: forward primer, reverse primer, 100 mM DTT, 10 mM dNTPs, RNasin (Promega, Madison, WI), Superscript III reverse transcription (Invitrogen, Carlsbad, CA), GreenTaq DNA polymerase (Genescript, Piscataway, NJ). The PCR cycling conditions were as follows: initial single cycle at 52°C for 1 h (reverse transcription step for cDNA synthesis), then a single cycle of 94°C for 2 min followed by 35 consecutive cycles of the

Table 1. Primers used for RT-PCR and PCR based detection of several grapevine viruses

#	Target virus ^a	Primer name	Primer sequence, 5' to 3'	Amplicon size (bp)	Reference
1	GLRaV-1	GLRaV-1f	GAGCGACTTGCGACTTATCGA	321	Osman et al. (2007)
2		GLRaV-1r	GGTAAACGGGTGTTCTTCAAT		
3	GLRaV-4	GLRaV-4f	GGTATGAACAARTTCAATGC	371	Bahder et al. (2013)
4		GLRaV-4r	TAGACAACCATGTAYTCTATG		
5	GVA	GVA-f	GAGGTAGATATAGTAGGACCT	272	Goszczynski and Jooste (2003)
6		GVA-r	TCGAACATAACCTGTGGCTC		
7	GVB	GVB-f	GTGCTAAGAACGTCTTCACAG	460	Minafra et al. (1994)
8		GVB-r	ATCAGCAAACACGCTTGAACC		
9	GFkV	GFkV-f	TGACCAGCCTGCTGTCTCTA	179	Kanuya et al. (2012)
10		GFkV-r	TGGACAGGGAGGTGTAGGAG		
11	GRBV	GRLBV-f5	TGCAAGTGGACATACGTTTA	718–721	Unpublished
12		GRLBV-r9	GGGATCCCATCAATTGTTCT		
13	GLRaV-3	MP-HSP70F	GGGGDGGRACTTTCGAYGTSTC	600	Donda (2016)
14		MP-HSP70R	ATTGGACTRCCYTTYGGGAAAAAT		
15	GLRaV-3	MP-CPF	GATGGRAAGAAGATATA	280	Bester et al. (2014); unpublished
16		MP-CPR	CTAAACGCYTGTYGCTAG		
17	ArMV	ArMV-F	TGACAACATGGTATGAAGCACA	336	Gambino and Gribaudo (2006)
18		ArMV-R	TATAGGGCCTTTCATCACGAAT		
19	GLRaV-2	GLRaV2_L2F	ATAATTCGGCGTACATCCCCACTT	331	Osman et al. (2007)
20		GLRaV2_U2R	GCCCTCCGCGCACTAATGACAG		
21	GFLV	GFLV2231F	ACCGGATTGACGTGGGTGAT	322	Mekuria et al. (2009)
22		GFLV2533R	CCAAAGTTGGTTTCCCAAGA		
23	GFLV	GFLaV-F	ATGCTGGATATCGTGACCCTGT	118	Kanuya (2012)
24		GFLaV-R	GAAGGTATGCCTGCTTCAGTGG		

^a Abbreviations used: GLRaV, *Grapevine leafroll associated virus*; GVA, *Grapevine virus A*; GVB, *Grapevine virus B*; GFkV, *Grapevine fleck virus*; GRBV, *Grapevine red blotch virus*; ArMV, *Arabis mosaic virus*; GFLV, *Grapevine fanleaf virus*.

following profile: 94°C for 30 s, 54°C for 45 s, 72°C for 30 s; and followed by a single cycle at 72°C for 2 min, and then held at 4°C until further analysis. The following viruses were tested for: GLRaV-1, -2, -3, and -4; *Grapevine virus A* (GVA); *Grapevine virus B* (GVB); *Grapevine fleck virus* (GFKV); *Grapevine red blotch virus* (GRBV); *Grapevine fanleaf virus* (GFLV); *Arabid mosaic virus* (ArMV). Primers used and respective expected sizes for RT-PCR bands are shown in Table 1. Three additional primers were used for RT-PCR based differentiation of the two GLRaV-3 genotypes identified, GLR3_ID45_6119_F, GLR3_ID46_6331_F, and

GLR3_ID45_6719-R, listed in Supplementary Table S1. RT-PCR products were resolved on 1.2% agarose gels, stained with ethidium bromide, and visualized under UV light. Amplicons were cloned into a pGEM-T Easy plasmid (pGEM-T Easy Vector System, Promega), and three individual clones carrying inserts of the expected size per each PCR product were selected for the Sanger sequencing of the inserts using Genewiz Inc. (South Plainfield, NJ) or Elim Biopharmaceuticals (Hayward, CA). Cloned sequences of the GLRaV-3 HSP70h and CP fragments from the 2015 samples were deposited into GenBank.

Table 2. A summary of symptoms and virus status for the grapevine samples from Idaho tested in 2014–2017. *Grapevine leafroll-associated virus 3* (GLRaV-3) and *Grapevine virus A* (GVA) were tested for by RT-PCR.^a

Vine ID	Symptoms	Vineyard	Cultivar	GLRaV-3/HSP70				GLRaV-3/CP				GVA		
				2014	2015	2016	2017	2014	2015	2016	2017	2014	2015	2016
43	LR	A	Cabernet Sauvignon	-	+	-	NT	-	+	-	NT	-	-	-
44	LR		Cabernet Sauvignon	-	+	-	-	-	+	-	+	-	+	-
45	NS		Cabernet Sauvignon	-	+	+	-	+	+	+	+	-	-	-
46	LR		Cabernet Sauvignon	+	+	+	+	+	+	+	+	-	-	-
47	LR		Cabernet Sauvignon	+	+	+	+	+	+	+	+	-	-	-
48	NS		Cabernet Sauvignon	-	-	-	NT	-	-	-	NT	-	-	-
49	NS		Cabernet Sauvignon	-	-	-	NT	-	-	-	NT	-	-	-
50	NS		Cabernet Sauvignon	-	-	-	NT	-	-	-	NT	-	-	-
51	LR		Merlot	+	+	-	NT	+	+	-	NT	-	+	-
52	NS		Merlot	-	-	-	NT	-	-	-	NT	-	-	-
53	LR	B	Merlot	+	+	-	NT	+	+	-	NT	-	-	-
54	LR		Merlot	+	+	-	NT	+	+	-	NT	-	-	-
55	LR		Merlot	-	+	-	NT	+	+	-	NT	-	-	-
56	NS		Merlot	-	-	-	NT	-	-	-	NT	-	-	-
57	NS		Merlot	-	-	-	NT	-	-	-	NT	-	-	-
58	NS		Merlot	-	-	-	NT	-	-	-	NT	-	-	-
59	LR		Tempranillo	NT	+	+	NT	NT	+	+	NT	NT	-	-
60	LR		Tempranillo	NT	-	-	NT	NT	-	-	NT	NT	-	-
61	NS		Tempranillo	NT	-	-	NT	NT	-	-	NT	NT	-	-
62	NS		Tempranillo	NT	-	-	NT	NT	-	-	NT	NT	-	-
63	LR		Tempranillo	NT	+	-	NT	NT	+	-	NT	NT	-	-
64	LR		Tempranillo	NT	-	-	NT	NT	-	-	NT	NT	-	-
65	NS		Tempranillo	NT	-	-	NT	NT	-	-	NT	NT	-	-
66	LR		Tempranillo	NT	-	-	NT	NT	-	-	NT	NT	-	-
67	LR		Tempranillo	NT	-	-	NT	NT	-	-	NT	NT	-	-
68	LR		Tempranillo	NT	-	-	NT	NT	-	-	NT	NT	-	-
69	LR		Tempranillo	NT	-	-	NT	NT	-	-	NT	NT	-	-
70	LR		Tempranillo	NT	-	-	NT	NT	-	-	NT	NT	-	-
71	LR		Tempranillo	NT	-	-	NT	NT	-	-	NT	NT	-	-
72	LR		Tempranillo	NT	-	-	NT	NT	-	-	NT	NT	-	-
73	LR		Tempranillo	NT	-	-	NT	NT	-	-	NT	NT	-	-
74	LR		Tempranillo	NT	-	-	NT	NT	-	-	NT	NT	-	-
75	LR		Tempranillo	NT	-	-	NT	NT	-	-	NT	NT	-	-
76	LR		Tempranillo	NT	-	-	NT	NT	-	-	NT	NT	-	-
77	NS		Tempranillo	NT	-	-	NT	NT	-	-	NT	NT	-	-
78	NS		Tempranillo	NT	-	-	NT	NT	-	-	NT	NT	-	-
79	LR		Tempranillo	NT	-	-	NT	NT	-	-	NT	NT	-	-
80	LR		Tempranillo	NT	-	-	NT	NT	-	-	NT	NT	-	-
81	LR		Tempranillo	NT	-	-	NT	NT	-	-	NT	NT	-	-
82	LR		Tempranillo	NT	-	-	NT	NT	-	-	NT	NT	-	-
83	LR		Tempranillo	NT	-	-	NT	NT	-	-	NT	NT	-	-
84	LR		Tempranillo	NT	-	-	NT	NT	-	-	NT	NT	-	-
85	LR		Tempranillo	NT	-	-	NT	NT	-	-	NT	NT	-	-
86	LR		Tempranillo	NT	-	-	NT	NT	-	-	NT	NT	-	-
87	LR		Tempranillo	NT	-	-	NT	NT	-	-	NT	NT	-	-
88	LR		Tempranillo	NT	-	-	NT	NT	-	-	NT	NT	-	-
89	LR		Tempranillo	NT	-	-	NT	NT	-	-	NT	NT	-	-
90	NS		Tempranillo	NT	-	-	NT	NT	-	-	NT	NT	-	-
91	NS		Tempranillo	NT	-	-	NT	NT	-	-	NT	NT	-	-
92	NS		Tempranillo	NT	-	-	NT	NT	-	-	NT	NT	-	-
93	LR		Tempranillo	NT	-	-	NT	NT	-	-	NT	NT	-	-
94	LR		Cabernet franc	NT	+	-	NT	NT	+	-	NT	NT	-	-

^a Abbreviations used: LR, leaf reddening; NS, no symptoms; HSP70h, heat shock protein 70-homolog; CP, capsid protein; NT, not tested; - negative and + positive for the virus tested.

In 2016 and 2017, foliar samples collected in vineyards A and B were subjected to double-antibody sandwich (DAS)-ELISA testing using the commercial kit from Bioreba, with all buffers and antibodies provided and mixed according to the kit's specifications. The ELISA plate was coated at a 1:1,000 dilution with coating antibody and incubated overnight at 4°C. The following day, roughly 0.5 g of combined petiole and leaf tissue was chopped and homogenized at a 1:20 (w:v) ratio in extraction buffer inside a meshed Bioreba bag as with the PCR extraction mentioned above. Homogenized samples were left in the bag at 4°C for 3 h before being aliquoted to the coated and washed ELISA plate with four replicate wells per sample and incubated overnight at 4°C. The plate was again washed with the Bioreba washing buffer, loaded with alkaline phosphatase conjugated antibodies in buffer at a 1:1,000 dilution, and incubated at 4°C overnight. Finally, plates were washed and *p*-nitrophenyl phosphate (pNPP, Sigma-Aldrich, St. Louis, MO) dissolved in substrate buffer was added to each well and incubated in darkness at room temperature. Readings were taken using SpectraMax 190 (Molecular Devices, San Jose, CA) at 405 nm wavelength after 30 min, 2 h, and 4 h.

High-throughput and conventional sequencing, and sequence analysis. In 2017, three 'Cabernet Sauvignon' samples collected in vineyard A, #45, #46, and #47, were subjected to high-throughput sequencing (HTS) analysis. Between 500 and 800 mg of frozen tissue, consisting of a mixture of leaves and petioles, were powdered in liquid nitrogen using mortar and pestle,

and extracted following the procedure described by Gambino and Gribaudo (2006). Briefly, 1 ml of extraction buffer (4 M guanidine isothiocyanate, 0.2 M sodium acetate [pH 5.0], 25 mM EDTA, 2.5% PVP-40, 2% sarkosyl, 1% 2-mercaptoethanol) was added per 100 mg of powdered tissue. After addition of 0.5 volume of a chloroform/isoamyl alcohol (v:v, 24:1) solution, samples were thoroughly vortexed and centrifuged at 12,000 × *g* for 10 min at room temperature. The aqueous phase was recovered and subjected to RNA extraction following the Plant RNeasy Mini kit (Qiagen) instructions, including an on-column DNase treatment. Total RNA was depleted of ribosomal RNAs using the Ribo-zero rRNA removal (plant) kit (Illumina, San Diego, CA). Ribosomal RNA-depleted RNA extracts were finally cleaned-up on a column using the RNA Clean & Concentrator-5 kit (ZymoResearch Corp, Irvine, CA). Library preparation and sequencing were performed by the Genomics Resources Core (IBEST, University of Idaho). Sequencing was done on a MiSeq system, producing 300-bp paired-ends reads. Raw reads were trimmed to remove low quality and adapter sequences using Trimmomatic 0.36 (Bolger et al. 2014). Clean paired reads were mapped against the *Vitis vinifera* reference genome (https://plants.ensembl.org/Vitis_vinifera/Info/Index) using Bowtie2 2.3.4.1 in local mode to filter out host sequences (Langmead and Salzberg 2012). Unmapped read pairs were then de novo assembled using SPAdes 3.11 in rna mode, with default settings (Bankevich et al. 2012). Assembled contigs of over 1 kb were then subjected to a BLASTN search for virus sequences in GenBank. Alternatively,

Table 3. The list of partial sequences of *Grapevine leafroll-associated virus 3* (GLRaV-3) isolates found in Idaho determined in this work.^a Amplicons obtained by RT-PCR were cloned and sequenced as described in Materials and Methods. Bold indicates the samples of GLRaV-3 with the divergent sequence subsequently named ID45.

Vine ID ^b	Cultivar	GLRaV-3/HSP70h ^c	GLRaV-3/CP ^c	Phylogroup ^d	Accession numbers/HSP70h ^e	Accession numbers/CP ^e
44	Cabernet Sauvignon	+	+	III	MH667344 MH667345	MH667375 MH667376 MH667377
45	Cabernet Sauvignon	+	+	IX	MH667346 MH667347 MH667348	MH667378 MH667379 MH667380
46	Cabernet Sauvignon	+	+	I / IX	MH667349 MH667350 MH667351	MH667381 MH667382 MH667383
47	Cabernet Sauvignon	+	+	I / IX	MH667352 MH667353 MH667354	MH667384 MH667385 MH667386
51	Merlot	+	+	I	MH667355 MH667356 MH667357	MH667387 MH667388 MH667389
53	Merlot	+	+	I	MH667358 MH667359 MH667360	MH667390 MH667391 MH667392
54	Merlot	+	+	I	MH667361 MH667362	MH667393 MH667394 MH667395
55	Merlot	+	+	I	MH667363 MH667364 MH667365	MH667396 MH667397 MH667398
59	Tempranillo	+	+	IX	MH667366 MH667367 MH667368	MH667399 MH667400 MH667401
63	Tempranillo	+	+	I / III	MH667369 MH667370 MH667371	MH667402
94	Cabernet franc	+	+	I	MH667372 MH667373 MH667374	MH667403 MH667404 MH667405

^a Abbreviations used: HSP70h, the heat shock protein 70 homolog gene; CP, the capsid protein gene; +, positive amplification.

^b Vine numbers are from Table 2.

^c For all vines, results are given for 2015 season.

^d Phylogroup assignments following Maree et al. (2015); when HSP70h and CP assignments differed, both are given.

^e Accession numbers for partial sequences obtained for one to three clones of RT-PCR product from 2015 season sampling.

the unmapped read pairs were used in a Bowtie2 mapping against a custom database of all plant viruses and viroids sequences (<https://www.ncbi.nlm.nih.gov/genome/viruses>) retrieved from GenBank, and mapped read pairs were assembled using SPAdes. Resulting contigs were then used in a BLASTN search in GenBank to reveal additional viruses and viroid sequences.

To verify the nearly whole-genome sequence for the GLRaV-3-ID45, 16 primers were designed to amplify the ID45 genome in 8

overlapping RT-PCR fragments on total RNA extracted from the infected petiole tissue of the grapevine sample #45 collected in September 2017, essentially as described previously (Green et al. 2017). Amplified PCR fragments were treated with Exosap-It (Affymetrix, Cleveland, OH) and submitted for Sanger sequencing to Elim Biopharmaceuticals, Inc. (Hayward, CA) using 28 additional specific primers to sequence the entire amplified fragments. The 5' terminus was amplified using the 5' RACE Kit (Roche, Indianapolis, IN)

Fig. 1. Comparison of two vines, #45 and #46, of 'Cabernet Sauvignon' from the same vineyard in southern Idaho, observed (**A** and **B**) and sampled (**C**) for the ELISA assay on the same day in September 2017. (**A**) – Note asymptomatic appearance of the foliage on vine #45; (**B**) – note interveinal reddening of leaves on vine #46, characteristic of *Grapevine leafroll-associated virus 3* (GLRaV-3) infection. (**C**) – Double-antibody sandwich ELISA test on petioles collected from vines #45 and #46, compared with a sample from a healthy vine, and to a buffer (no leaf sample added). Four wells (replicates) were loaded with petiole extracts from each vine sample, and two wells were loaded with buffer. A commercial kit (Bioreba) was used for ELISA detection of GLRaV-3. X-axis represents samples tested in ELISA; Y-axis is an optical density (OD) reading at 405 nm.

according to the manufacturer's instructions, using primers SP1_LR3_600r and SP2_LR3_240r. To amplify the 3'-terminus of the genome, total RNA was polyadenylated using *Escherichia coli* Poly(A) Polymerase (New England Biolabs, Ipswich, MA) according to manufacturer's instructions. The first cDNA strand was synthesized using an anchored oligo(dT) primer, and then PCR was conducted using the SP3_LR3_18k-F forward primer in combination with the anchored oligo(dT) reverse primer. Resulting PCR products were submitted for direct Sanger sequencing to Elim Biopharmaceuticals. Individual sequence reads were assembled using the SeqMan program of the Lasergene 14 Suite (DNASTAR, Madison, WI). To assess the coding capacity and the overall genome organization of GLRaV-3_ID45, the sequence was analyzed using the Open Reading Frame (ORF) Finder program available at the National Center for Biotechnology Information, and identified ORFs were compared with the ORFs available in GLRaV-3 sequences from the database.

For phylogeny inference, whole genome sequences of GLRaV-3 were retrieved from GenBank and aligned with the two new genomes using MUSCLE with default settings implemented in MEGA version 7 (Kumar et al. 2016; Supplementary Table S2). Extra segments present at the 5' and 3' extremities outside the consensus in some sequences were trimmed. Phylogeny reconstruction was done on the alignment using the maximum likelihood method and the general time reversible model for nucleotide substitutions (GTR+G+I) as determined by the MEGA model selection test (Kumar et al. 2016; Nei and Kumar 2000).

Results

Virus status of the surveyed grapevines. Of the 52 vines surveyed between 2014 and 2017 in two vineyards, 11 were found positive for GLRaV-3, one positive for GVA, and two vines were found positive for both GLRaV-3 and GVA (Table 2). Again, due to the accompanying study on the impact of grapevine virus on berry quality, the same individual vines were observed and tested for virus presence for four (vineyard A) or two (vineyard B) growing seasons.

In vineyard A, three out of four asymptomatic 'Cabernet Sauvignon' and four out of four asymptomatic 'Merlot' vines were found free of all eight viruses tested for using RT-PCR, while eight vines that exhibited leaf reddening in September 2014 were found positive for GLRaV-3, of which two vines were also found positive for GVA in addition to GLRaV-3, in 2015. One single 'Cabernet Sauvignon' vine #45 that exhibited no symptoms in any of the four seasons of observations (Fig. 1A), consistently tested positive for GLRaV-3 in RT-PCR with HSP70h and CP-specific primers targeting the most conserved regions of the respective GLRaV-3 genes (Table 2). In September 2017, vine #45 was tested in ELISA using a commercial kit in parallel with vine #46, which was found to be GLRaV-3-positive using the same RT-PCR test and exhibited leaf reddening symptoms in each of the four seasons (Fig. 1B). To our surprise, vine #45 exhibited poor signal during this ELISA test, in contrast to vine #46, which was clearly determined as GLRaV-3-positive in ELISA (Fig. 1C).

In vineyard B, two of the 27 'Tempranillo' vines selected in September 2015 due to visible symptoms of leaf reddening, #59 and #63, were found positive for GLRaV-3 using RT-PCR (Table 2); the remaining 27 symptomatic and all eight asymptomatic 'Tempranillo' vines were found virus-negative. No other viruses were found in

vines #59 and #63 in 2015 using RT-PCR. Again, between September 2015 and September 2016, the owner of vineyard B replaced all 35 tagged 'Tempranillo' vines. All newly replanted 35 vines were tested again in 2016, and vine #59 was found GLRaV-3 positive using RT-PCR. A single 'Cabernet franc' vine also tested GLRaV-3 positive in RT-PCR in 2015 in vineyard B (Table 2). This 'Cabernet franc' vine exhibited clear interveinal leaf reddening and was adjacent to the 'Tempranillo' block.

Sequencing of GLRaV-3-positive samples and identification of a divergent GLRaV-3 isolate. All GLRaV-3-specific bands generated during the survey of the two vineyards in 2015 were cloned into a plasmid vector and sequenced to confirm the correct virus identification. Of the 12 samples found GLRaV-3-positive in vineyards A and B in 2014 and 2015 (Table 3), CP nucleotide sequences for four (#45, #46, #47, and #59) and HSP70h sequences for two (#45 and #59) were found unusual, displaying less than 80% nt identity with GLRaV-3 sequences in GenBank using BLASTn. On the other hand, the identity levels between sequences #45, #46, #47, and #59 were very high, ranging between 98 and 100%, for each gene fragment analyzed, HSP70h and CP. Six other Idaho GLRaV-3 samples produced HSP70h and CP sequences matching multiple GLRaV-3 entries from phylogroups I and III in GenBank, with nt identity levels at 98 to 99%, and 99%, respectively (Table 3). Interestingly, all 'Merlot' samples from vineyard A belonged to phylogroup I, indicating common origin of the GLRaV-3 isolates, while 'Cabernet Sauvignon' samples from the same vineyard A had GLRaV-3 isolates of three distinct genotypes, indicating multiple introductions of GLRaV-3 (Table 3). Three samples (#46, #47, and #63) produced HSP70h and CP sequences matching different phylogroups, suggesting mixed infections with at least two different GLRaV-3 variants for the corresponding plants (Table 3). We hypothesized that the four samples that produced amplicons with low levels of sequence identity to GLRaV-3 entries in GenBank represented a new, divergent strain of the virus, not described previously and potentially capable of escaping ELISA tests (Fig. 1C).

To determine the whole genome sequence for this hypothetical new genetic variant of GLRaV-3, foliar samples were collected from vines #45, #46, and #47 in September 2017, total RNA was extracted from all three samples, depleted of ribosomal RNA, and subjected to HTS on a MiSeq system to generate 300 bp paired-end reads. A summary of the analysis is given in Supplementary Table S3. After trimming, 1,255,387, 1,203,178, and 1,261,352 pairs of reads for samples #45, #46, and #47, respectively, were retained for analysis, of which 96.79 to 99.11% mapped to the *V. vinifera* genome. De novo assembly of the unmapped paired-end reads yielded numerous contigs, among which were one contig of 18,050 nt for #45, one 18,703 nt long contig for #47, and two contigs (18,477 and 18,487 nt) for #46. These lengths were close to what was expected to be near full-length GLRaV-3 genomes. An initial GenBank database search using the BLASTn program revealed that all four contigs represented nearly complete GLRaV-3 genomes. The 18,487- and 18,703-nt contigs from samples #46 and #47, respectively, exhibited 99% nucleotide sequence identity levels to the phylogroup I sequence JX559645 (isolate 3138-07), as described by Maree et al. (2015). On the other hand, 18,050- and 18,477-nt contigs from samples #45 and #46, respectively, exhibited only modest, $\leq 78\%$ nucleotide sequence identity levels to the closest GLRaV-3 match, GLRaV-3 isolate 621 from South Africa (GQ352631; Jooste et al. 2010), suggesting that indeed samples #45 and #46 harbored a novel genotype or genetic variant of GLRaV-3.

Inspection of the divergent GLRaV-3 contigs produced by HTS in the two samples suggested an incomplete genome, with missing 5' and 3'-terminal 9-419 nucleotides. To determine the correct genome sequence and acquire the exact 5' and 3' terminal regions, the entire 18,478-nt GLRaV-3 sequence was reamplified as overlapping DNA fragments from the total RNA extracted from sample #45 using a series of primers and sequenced directly as RT-PCR amplicons; the exact 5' and 3' terminal sequences were amplified using RACE methodology, cloned, and sequenced in recombinant plasmids. This whole genome sequence was named GLRaV-3_ID45 and deposited

Table 4. Percentage of coverage and nucleotide sequence identity of *Grapevine leafroll-associated virus 3* (GLRaV-3)-ID45 with each phylogroup. Representatives of each group were selected arbitrarily, and the analysis was done using the discontinuous Megablast.

Phylogroup	Accession number	Coverage (%)	Identity (%)
I	GU983863	79	76
II	EU259806	78	76
III	KY886362	80	76
VI	JQ796828	80	71
VII	KM058745	86	70

in the GenBank database under the accession number MH796136. ID45 sequence displayed a typical GLRaV-3 genome organization, encoding 11 ORFs of the 13 described for the consensus GLRaV-3 sequence (Ling et al. 2004; Maree et al. 2013). Two small ORFs encoding proteins with unknown functions and present in GLRaV-3 sequences from most phylogroups were missing in ID45: (i) p6 located downstream of the RdRp-encoding ORF 1b, and (ii) p4 usually found upstream of the 3'-proximal, small ORF, p7, at the 3' end of the genome. Pair-wise comparisons of the GLRaV-3_ID45 sequence with representative whole genomes from GLRaV-3 phylogroups I to VII indicated that the GLRaV-3_ID45 whole genome exhibited low identity levels all defined GLRaV-3 phylogroups that ranged between 70 to 76%, with coverage between 78 to 86% (Table 4).

Alignment of the phylogroup I-related GLRaV-3 contigs found in #46 (18,487 nt contig) and #47 with available full GLRaV-3 genomes indicated that the sequence in the #46 sample contig represented an almost complete virus genome, missing only 11 nt at the consensus 5' terminus, while the 3' terminus matched the exact 3'-end of the virus genome. The assembled contig in #47 was longer than expected for a GLRaV-3 genome, covering the entire GLRaV-3 genome including both extremities, but was extended beyond the consensus genome by 78 nt in the 5' and by 127 nt in the 3' directions; in both cases, extensions were identified as duplications of a part of the GLRaV-3 genome (not shown), probably due to sequence assembly errors. Aside from these differences at the ends, the two assembled genomes differed by only one nucleotide over

Fig. 2. Phylogenetic analysis of the *Grapevine leafroll-associated virus-3* (GLRaV-3) whole genome nucleotide sequences. The maximum likelihood method using the GTR+G+I model was used. Values at nodes are percent bootstrap support at 1,000 replications. The tree is unrooted. The phylogenetic groups indicated by black brackets are arbitrary, and some correspond to previously described phylogroups (Maree et al. 2015): groups I to III, groups VI and VII. Phylogroup IX is the new group proposed here. The dark blue brackets indicate the three out of four supergroups proposed by Maree et al. (2015). The two new variants described here are indicated in bold black for ID46, and bold red for ID45. Arrows point at additional sequences phylogenetically distant from other groups.

the whole 18,498-nt genome: an A in ID46 was substituted for T in ID47 at position 18,265, in the noncoding 3' untranslated region (UTR). The whole genome sequence obtained from the sample #46, similar to phylogroup I GLRaV-3 sequences, was named ID46; it included the eleven 5'-terminal, missing nucleotides which were recovered from the #47 contig. This GLRaV-3_ID46 sequence was deposited in the GenBank database under the accession number MH796135. The ID46 genome encoded all 13 ORFs described for the phylogroup I sequences (Ling et al. 1998, 2004), including the two ORFs missing in ID45.

Phylogenetic analysis of the GLRaV-3 whole genome. To determine phylogenetic relationships between the ID45 sequence and other GLRaV-3 phylogroups, whole genome sequences of GLRaV-3 available in the GenBank database were aligned with the ID45 sequence, and phylogenetic trees generated (Fig. 2). Several GLRaV-3 phylogroups defined previously based on capsid protein gene sequences (Maree et al. 2015) could be easily identified, such as phylogroups I, II, III, and VI (see Fig. 2). However, new phylogroups, distinct from the ones described earlier (Maree et al. 2015) became visible as well (Fig. 2). The two GLRaV-3 sequences determined here fell in two distinct lineages: ID46 was placed in phylogroup I according to Maree et al. (2015), while ID45 was placed in a new phylogroup IX (Fig. 2; not analyzed by Maree et al. [2015]). Recently, three new sequences were released by GenBank: KY707824, KY707825, and KY764333 (Al Rwahnih et al. 2018), exhibiting 99% identity to the ID45 sequence, and together with ID45 found here forming a distinct, novel GLRaV-3 clade (Fig. 2). Careful inspection of these three partial sequences from GenBank revealed some assembly errors close to 5' and 3' terminal regions in two of them (KY707824 and KY707825), resulting in segment duplications and inversions, and also missing exact termini (data not shown). Nevertheless, the high level of pair-wise sequence identities and phylogenetic placement into the same tight lineage with ID45 suggested these three isolates of GLRaV-3 from California belonged to the same novel genetic variant of the virus.

Development of a specific, RT-PCR based assay to detect GLRaV-3_ID45 variant. In order to quickly differentiate the two

GLRaV-3 genetic variants, ID45 and ID46, two primers were designed, GLR3_ID45_6119_F and GLR3_ID46_6331_F, targeting genetic variant-specific sequences in the HEL region in the ORF 1a, and used in RT-PCR in combination with the same nonspecific primer GLR3_ID45_6719-R. The ID45-specific primers GLR3_ID45_6119_F and GLR3_ID45_6719-R produced a band of 598 bp that was easily distinguishable from a 386-bp band produced by the ID46-specific primers GLR3_ID46_6331_F and GLR3_ID45_6719-R (Fig. 3). These primers could be used in a multiplex format and produced both ID45 and ID46-specific bands in samples where both sequences were present (sample #46; Fig. 3). As can be seen from Figure 3, for the 2017 season, the amounts of ID45 and ID46 genetic variants present in the three individual infected plants were at similar detectable levels. Since sample #47 from the 2015 season was initially found to harbor the ID45-like GLRaV-3 sequence, likely in a mixed infection with a ID46-type sequence, we retested all samples collected from the vine #47 in 2014 to 2017 seasons (stored at -80°C) using the ID45/ID46 differentiating primers. The presence of both ID45 and ID46 sequences in the sample #47 was confirmed only for the 2015 season, while samples from seasons 2014, 2016, and 2017 contained a single, ID46-type sequence matching GLRaV-3 isolates from phylogroup I (not shown). It is possible that the ID45 variant of GLRaV-3 was present at a low titer and/or was distributed unevenly in vine #47, and could not be detected in one season before and two seasons after 2015, either by RT-PCR with our new differentiating primers or by HTS (in 2017).

Other pathogens found in grapevine samples #45, #46, and #47. The raw reads for the three grapevine samples collected in 2017 were analyzed for the presence of sequences of other pathogens that may be present in grapevine samples. Host filtered reads from the HTS analysis for all three samples collected in September 2017, #45, #46, and #47, were mapped against a custom made database containing all vine viral and viroid sequences available in GenBank. Contigs were assembled based on mapped reads, and a BLASTN search retrieved full genomes of *Hop stunt viroid* (HSVd; 297 bases) and *Grapevine yellow speckle viroid 1* (GYSVd-1; 368 bases) in each of the three samples. The same strains of each viroid were found in all three samples that turned out to be 100% identical to the following sequences deposited in the GenBank database: HSVd isolate VV-SEM (accession number MF774873; unpublished), and GYSVd-1 sequence variant type 2 (accession number Z17225; Rigden and Rezaian [1993]).

Discussion

Genetic diversity is characteristic of RNA virus species, and was well documented previously in the PNW for several viruses of the grapevine leafroll complex, such as GLRaV-1 (Alabi et al. 2011), GLRaV-2 (Jarugula et al. 2010b), and GLRaV-3 (Maree et al. 2013, 2015). This genetic diversity ensures virus survival and evolution in different hosts and environments, but from a practical perspective, creates challenges for timely detection of the virus and development of control measures to limit its spread in a crop. Understanding the genetic diversity of GLRaV-3 is key to controlling the spread of the virus, through grapevine nursery screenings and distribution of GLRaV-free planting material. For effective control measures to be developed and implemented, both broad-spectrum detection tools and specific methodologies for differentiation of virus strains are needed to implement reliable virus detection and strain differentiation. Characterization of novel, divergent strains or variants of GLRaV-3, like ID45 described here, is the first necessary step in development of these detection tools.

The GLRaV-3_ID45 isolate described here may represent a novel genetic variant of the virus, exhibiting novel features. Indeed, vine #45 exhibited no foliar reddening symptoms (Fig. 1A; Table 2) characteristic of other GLRaV-3 positive vines infected with GLRaV-3 isolates belonging to phylogroups I and III (Fig. 1B; Table 2). This same vine exhibited poor reactivity in DAS-ELISA with at least one commercial kit (Fig. 1C). This poor reactivity in ELISA combined with the lack of foliar symptoms may result in an inadvertent spread of infected plants if no other methods of detection are used

Fig. 3. Agarose gel-electrophoresis analysis of RT-PCR products, after differential amplification of *Grapevine leafroll-associated virus-3* (GLRaV-3), ID45- and ID46-specific bands from three petiole samples of 'Cabernet Sauvignon' collected from the same vineyard in southern Idaho, using four GLRaV-3 specific primers. The sizes of the products amplified by primers GLR3_ID45_6119_F and GLR3_ID45_6719-R (598 bp), and GLR3_ID46_6331_F and GLR3_ID45_6719-R (386 bp) are indicated on the left. Numbers above the gel indicate the identity of the vines sampled; M = 100-bp markers.

for GLRaV-3 testing. Currently available degenerate primers targeting the most conserved genes of the virus (Table 1) may also produce inconclusive results, depending on the season and on the targeted gene, HSP70h versus CP (Table 2). ID45 represented a quite divergent genotype of GLRaV-3, distinct from the more prevalent genotypes in Idaho (under 78% nucleotide sequence identity), which were phylogenetically assigned to phylogroups I and III (Table 3). The 18,478-nt genome determined for the ID45 variant had the organization typical of GLRaV-3 variants missing two short ORFs, of the 13 characteristic of the type member of the GLRaV-3 species belonging to phylogroup I (Ling et al. 2004), and also present in the ID46 genome determined in this work. The amino acid sequence identity between the ID45 and ID46 CP sequences did not exceed 90% and could explain the poor reactivity of ID45 sample with the commercial ELISA kit.

In phylogenetic reconstructions, the GLRaV-3 ID45 sequence apparently clusters into a new lineage of GLRaV-3 isolates, which we propose to name 'phylogroup IX' (Fig. 2) continuing the nomenclature suggested by Maree et al. (2015). A recent conference report describing a California GLRaV-3 isolate 'Santa Barbara 138' (KY764333; Al Rwahnih et al. 2018) suggested an existence of a new GLRaV-3 lineage based on the analysis of the partial CP genes for two isolates, 'Santa Barbara 138' and '43-15' (Sharma et al. 2011), but did not propose any name for it. Given the low GLRaV-3 ID45 sequence identity level to all isolates from phylogroups I to VII, ranging between 70 and 76% (Table 4), and the clearly distinct position of the entire lineage in the phylogenetic tree of GLRaV-3 whole genomes (Fig. 2), we felt the naming of this lineage as phylogroup IX is fully justified.

The initial study of the GLRaV-3 in Idaho found that the 22 generated partial sequences of the HSP70h gene fell into five distinct clades suggesting multiple introductions of the virus into the state, perhaps brought in with the infected planting stock (Mekuria et al. 2009). Three of the clades comprising 15 of these sequences could now be matched with phylogroups I, II, and III presented in Figure 2, while the remaining seven of the Idaho sequences from two other clades (Mekuria et al. 2009) displayed less than 5% nucleotide sequence differences from group I isolates (not shown). Apparently, genetic diversity of GLRaV-3 revealed in Idaho previously was largely restricted to the GLRaV-3 supergroup A (Fig. 2; Maree et al. 2015). Samples collected during that initial survey were selected based largely on appearance of characteristic foliar symptoms and on ELISA reactivity using the Bioreba detection kit (Mekuria et al. 2009). Since the ID45 variant of GLRaV-3 may not express the characteristic interveinal reddening in 'Cabernet Sauvignon' (Fig. 1A and B), and may exhibit poor reactivity with the antibodies from the Bioreba ELISA kit (Fig. 1C), it might have been missed during the 2008 survey.

BLASTn search of the GenBank database, using the ID45 sequence as a query, revealed that the ID45 nucleotide sequence is 99% identical to three GLRaV-3 nearly whole genomes deposited recently (KY707824, KY707825, and KY764333; Al Rwahnih et al. 2018), and also to two small partial GLRaV-3 sequences MF947380 (Fajardo et al. 2005) and JF421951 (Sharma et al. 2011) spanning the HSP70h and CP genes of GLRaV-3, respectively. Four of these five GLRaV-3 sequences were deposited by research groups from California, and one (MF947380) originated from Brazil. Symptoms associated with these GLRaV-3 sequences closely related to ID45 were not described, and serology of these variants was not studied (Al Rwahnih et al. 2018; Fajardo et al. 2005; Sharma et al. 2011). The assumption can be made that the ID45 genetic variant or strain of GLRaV-3 is now distributed in various grapevine production areas, including at least two states in the U.S. (Idaho and California), and at least one additional country (Brazil). The relative prevalence of this ID45 variant will need to be determined, using differentiating primers developed here, as well as specific effects of this variant on grape berry yield and quality. New broad spectrum detection tools will need to be developed for the ID45 genotype to avoid possible false-negative results if ELISA or RT-PCR based specific for other phylogroups of GLRaV-3 are used.

Acknowledgments

We thank Gardenia Orellana and Lisa Tran (University of Idaho) for help in sample collection and ELISA testing. We thank Chris Rennaker (USDA-ARS) for aid in sample collection. The help of Idaho winegrape growers, Dale Jeffers of Skyline Vineyards, Ron Bitner of Bitner Vineyards, Greg Koenig of Koenig Vineyards and Fraser Vineyards, Mike Williamson of Williamson Orchards and Vineyards, Kat House of House of Wine, Keith Martin of Martin Vineyards, and Melissa Sanborn of Colter's creek vineyard and winery, who permitted our access to and sampling in their vineyards is greatly appreciated.

Literature Cited

- Adiputra, J., Kesoju, S. R., and Naidu, R. A. 2018. The relative occurrence of *Grapevine leafroll-associated virus 3* and *Grapevine red blotch virus* in Washington State vineyards. *Plant Dis.* 102:2129-2135.
- Al Rwahnih, M., Lara, A. D., Klaassen, V., Stevens, K., Arnold, K., Maree, H. J., and Golino, D. 2018. Molecular characterization of divergent *Grapevine leafroll-associated virus 3* isolates in California, USA. Pages 46-47 in: *Proceedings of the 19th Congress of ICVG*, Santiago, Chile.
- Alabi, O. J., Al Rwahnih, M., Karthikeyan, G., Poojari, S., Fuchs, M., Rowhani, A., and Naidu, R. A. 2011. *Grapevine leafroll-associated virus 1* occurs as genetically diverse populations. *Phytopathology* 101:1446-1456.
- Almeida, R. P. P., Daane, K., Bell, V., Blaisdell, K., Cooper, M., Herrbach, E., and Pietersen, G. 2013. Ecology and management of grapevine leafroll disease. *Front. Microbiol.* 4:94.
- Bahder, B. W., Alabi, O., Poojari, S., Walsh, D. B., and Naidu, R. A. 2013. A survey for grapevine viruses in Washington State 'Concord' (*Vitis × labruscana* L.) vineyards. Online. *Plant Health Prog.* doi.org/10.1094/PHP-2013-0805-01-RS
- Bankevich, A., Nurk, S., Antipov, D., Gurevich, A. A., Dvorkin, M., Kulikov, A. S., Lesin, V. M., Nikolenko, S. I., Pham, S., Pribelski, A. D., Pyshkin, A. V., Sirotkin, A. V., Vyahhi, N., Tesler, G., Alekseyev, M. A., and Pevzner, P. A. 2012. SPAdes: A new genome assembly algorithm and its applications to single-cell sequencing. *J. Comput. Biol.* 19:455-477.
- Bester, R., Maree, H. J., and Burger, J. T. 2012. Complete nucleotide sequence of a new strain of grapevine leafroll-associated virus 3 in South Africa. *Arch. Virol.* 157:1815-1819.
- Bester, R., Pepler, P. T., Burger, J. T., and Maree, H. J. 2014. Relative quantitation goes viral: an RT-qPCR assay for a grapevine virus. *J. Virol. Methods* 210: 67-75.
- Bolger, A. M., Lohse, M., and Usadel, B. 2014. Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* 30:2114-2120.
- Donda, B. P. 2016. Molecular biology and epidemiology of grapevine leafroll-associated viruses. Ph.D. dissertation. Washington State University, Pullman, WA.
- Engel, E. A., Girardi, C., Escobar, P. F., Arredondo, V., Domínguez, C., Pérez-Acle, T., and Valenzuela, P. D. T. 2008. Genome analysis and detection of a Chilean isolate of *Grapevine leafroll associated virus-3*. *Virus Genes* 37:110-118.
- Fajardo, T. V. M., Eiras, M., Schenato, P. G., Nickel, O., and Kuhn, G. B. 2005. Evaluation of Grapevine leafroll-associated virus 1 and 3 variability by nucleotide sequence analysis and single-strand conformation polymorphism. *Fitopatol. Bras.* 30:177-182.
- Fei, F., Lyu, M.-D., Li, J., Fan, Z.-F., and Cheng, Y.-Q. 2013. Complete nucleotide sequence of a Chinese isolate of *Grapevine leafroll-associated virus 3* reveals a 5' UTR of 802 nucleotides. *Virus Genes* 46:182-185.
- Gambino, G., and Gribaudo, I. 2006. Simultaneous detection of nine grapevine viruses by multiplex reverse transcription-polymerase chain reaction with coamplification of a plant RNA as internal control. *Phytopathology* 96: 1223-1229.
- Golino, D. A., Sim, S., and Rowhani, A. 2000. Experimental transmission of grapevine leafroll associated viruses by mealybugs. Pages 19-20 in: *Extended Abstracts 13th Meeting of ICVG*, Adelaide, Australia.
- Goszczynski, D. E., and Jooste, A. E. C. 2003. Identification of divergent variants of *Grapevine virus A*. *Eur. J. Plant Pathol.* 109:397-403.
- Green, K. J., Brown, C. J., Gray, S. M., and Karasev, A. V. 2017. Phylogenetic study of recombinant strains of *Potato virus Y*. *Virology* 507:40-52.
- Jarugula, S., Gowda, S., Dawson, W. O., and Naidu, R. A. 2010a. 3'-coterminal subgenomic RNAs and putative cis-acting elements of *Grapevine leafroll-associated virus 3* reveals "unique" features of gene expression strategy in the genus *Ampelovirus*. *Virol. J.* 7:180.
- Jarugula, S., Alabi, O. J., Martin, R. R., and Naidu, R. A. 2010b. Genetic variability of natural populations of *Grapevine leafroll-associated virus 2* in Pacific Northwest vineyards. *Phytopathology* 100:698-707.
- Jooste, A. E. C., Maree, H. J., Bellstedt, D. U., Goszczynski, D. E., Pietersen, G., and Burger, J. T. 2010. Three genetic grapevine leafroll-associated virus 3 variants identified from South African vineyards show high variability in their 5'UTR. *Arch. Virol.* 155:1997-2006.
- Kanuya, E. 2012. Survey of grapevine viruses in Idaho. M.S. Thesis, University of Idaho, Moscow, ID.
- Kanuya, E., Clayton, L. A., Naidu, R. A., and Karasev, A. V. 2012. First report of *Grapevine fleck virus* in Idaho grapevines. *Plant Dis.* 96:1705.
- Karasev, A. V. 2000. Genetic diversity and evolution of closteroviruses. *Annu. Rev. Phytopathol.* 38:293-324.

- Kumar, S., Stecher, G., and Tamura, K. 2016. MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. *Mol. Biol. Evol.* 33:1870-1874.
- Langmead, B., and Salzberg, S. L. 2012. Fast gapped-read alignment with Bowtie 2. *Nat. Methods* 9:357-359.
- Ling, K. S., Zhu, H. Y., Alvizo, H., Hu, J. S., Drong, R. F., Slightom, J. L., and Gonsalves, D. 1997. The coat protein gene of grapevine leafroll associated closterovirus-3: cloning, nucleotide sequencing and expression in transgenic plants. *Arch. Virol.* 142:1101-1116.
- Ling, K. S., Zhu, H. Y., Drong, R. F., Slightom, J. L., McFerson, J. R., and Gonsalves, D. 1998. Nucleotide sequence of the 3'-terminal two-thirds of the grapevine leafroll-associated virus 3 genome reveals a typical monopartite closterovirus. *J. Gen. Virol.* 79:1299-1307.
- Ling, K. S., Zhu, H. Y., and Gonsalves, D. 2004. Complete nucleotide sequence and genome organization of *Grapevine leafroll associated virus 3*, type member of the genus *Ampelovirus*. *J. Gen. Virol.* 85:2099-2102.
- 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.* 4:82.
- Maree, H. J., Freeborough, M.-J., and Burger, J. T. 2008. Complete nucleotide sequence of a South African isolate of *Grapevine leafroll-associated virus 3* reveals a 5'UTR of 737 nucleotides. *Arch. Virol.* 153:755-757.
- Maree, H. J., Pirie, M. D., Oosthuizen, K., Bester, R., Rees, D. J. G., and Burger, J. T. 2015. Phylogenomic analysis reveals deep divergence and recombination in an economically important grapevine virus. *PLoS One* 10:e0126819.
- Martelli, G. P. 2014. Directory of virus and virus-like diseases of the grapevine and their agents. *J. Plant Pathol.* 96(1 Suppl.):S1-S136.
- Martelli, G. P., Agranovsky, A. A., Bar-Joseph, M., Boscia, D., Candresse, T., Coutts, R. H. A., Dolja, V. V., Falk, B. W., Gonsalves, D., Jelkmann, W., Karasev, A. V., Minafra, A., Namba, S., Vetter, H. J., Wisler, G. C., and Yoshikawa, N. 2002. The family *Closteroviridae* revised. *Arch. Virol.* 147:2039-2044.
- Martelli, G. P., Saldarelli, P., and Minafra, A. 2011. Grapevine leafroll-associated virus 3. *AAB Description of Plant Viruses*, No. 422.
- 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.
- Mekuria, T. A., Karasev, A. V., Martin, R. R., and Naidu, R. A. 2009. First report of *Grapevine leafroll-associated virus-3* in six wine grape cultivars in Idaho. *Plant Dis.* 93:1218.
- Minafra, A., Saldarelli, P., Grieco, F., and Martelli, G. P. 1994. Nucleotide sequence of the 3' terminal region of the RNA of two filamentous grapevine viruses. *Arch. Virol.* 137:249-261.
- Naidu, R., Rowhani, A., Fuchs, M., Golino, D., and Martelli, G. P. 2014. Grapevine leafroll: A complex viral disease affecting a high-value fruit crop. *Plant Dis.* 98:1172-1185.
- Nei, M., and Kumar, S. 2000. *Molecular Evolution and Phylogenetics*. Oxford University Press, New York.
- 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.
- Prator, C. A., Kashiwagi, C. M., Vončina, D., and Almeida, R. P. P. 2017. Infection and colonization of *Nicotiana benthamiana* by *Grapevine leafroll-associated virus 3*. *Virology* 510:60-66.
- Rast, H. E., James, D., Habili, N., and Masri, S. A. 2012. Genome organization and characterization of a novel variant of *Grapevine leafroll associated virus 3*. Pages 61-63 in: *Proceedings of the 17th Congress of ICSVG*, 7-14 October. Foundation Plant Services, Davis, CA.
- Rigden, J. E., and Rezaian, M. A. 1993. Analysis of sequence variation in grapevine yellow speckle viroid 1 reveals two distinct alternative structures for the pathogenic domain. *Virology* 193:474-477.
- Rowhani, A., Biardi, L., Johnson, R., Saldarelli, P., Zhang, Y. P., Chin, J., and Green, M. 2000. Simplified sample preparation method and one-tube RT-PCR for grapevine viruses. Page 82 in: *Proceedings of XIII International Council for the Study of Viruses and Virus-Like Diseases of the Grapevine*, Adelaide, Australia.
- Seah, Y. M., Sharma, A. M., Zhang, S., Almeida, R. P., and Duffy, S. 2012. A divergent variant of *Grapevine leafroll-associated virus 3* is present in California. *Virol. J.* 9:235.
- Sharma, A. M., Wang, J., Duffy, S., Zhang, S., Wong, M. K., Rashed, A., Cooper, M. L., Daane, K. M., and Almeida, R. P. 2011. Occurrence of grapevine leafroll-associated virus complex in Napa Valley. *PLoS One* 6:e26227.