Investigation of genetic diversity of *Grapevine leafroll-associated virus-3* and *Grapevine red blotch virus* in Idaho grapevines

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Brandon Dee Thompson

Major Professor: Alexander V. Karasev, Ph.D.
Committee Members: Fangming Xiao, Ph.D.; Jungmin Lee, Ph.D.; Nora Olsen, Ph.D.
Department Administrator: Edwin Lewis, Ph.D.

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Authorization to Submit Thesis

This thesis of Brandon Dee Thompson, submitted for the degree of Master of Science with a Major in Plant Science and titled "Investigation of genetic diversity of *Grapevine leafroll-associated virus-3* and *Grapevine red blotch virus* in Idaho grapevines," has been reviewed in final form. Permission, as indicated by the signatures and dates below, is now granted to submit final copies to the College of Graduate Studies for approval.

Major Professor: ______________________________ Date: __________
Alexander V. Karasev, Ph.D.

Committee Members: ______________________________ Date: __________
Fangming Xiao, Ph.D.

______________________________ Date: __________
Jungmin Lee, Ph.D.

______________________________ Date: __________
Nora Olsen, Ph.D.

Department Administrator: ______________________________ Date: __________
Edwin Lewis, Ph.D.

Abstract

Wine grape production has steadily increased since planting of grape vines began in the 1870s. Although wine grape production is a relatively young industry within Idaho, the state now has over 50 wineries and an annual economic impact of nearly $170 million. Due to the significance of the industry to Idaho, it is important to understand the presence and diversity of viruses associated with diseases of grapevines that may put the industry at risk. The most common of these diseases is the grapevine leafroll disease caused by a complex of *Grapevine leafroll-associated viruses* (GLRaV), of which the most prominent is GLRaV-3. In Idaho, a novel genetic variant of GLRaV-3 was identified in separate vineyards infecting different wine grape cultivars. Recently, a new virus was also identified in Idaho vineyards for the first time, *Grapevine red blotch virus* (GRBV), already widely present in California, Washington, and New York. It is important to understand the characteristics of these viruses, the effects on vine health, and to develop methods for detection. Here a preliminary study of the GLRaV-3 and GRBV genetic diversity in Idaho is presented. The development of detection methods for screening of planted and nursery stock vines can have a substantial impact on the wine industry to ensure planting of virus-free stock and/or economical and agronomic reasons to remove infected commercial plantings.

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Dedication

To my family, without their unwavering love and support none of this would have been possible for me and is something that I will forever be blessed and grateful for.

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Chapter 1: Brief survey of grapevine and grapevine viruses in Idaho

1.1 Beginning and expansion of wine grape in Idaho

Grapevine is arguably one of the most valuable fruit crops in the world. It has a long documented history in civilization with both cultural and religious significance, along with being cultivated on every continent with the exception of Antarctica (Reynolds 2017). In Idaho, the grape industry began in the second half of the 19th century when the first vineyards were planted in Lewiston in 1864 (Idaho Wine Commission 2019). These were the first vineyards to be planted in the northwest, even before grape cultivation started in the states of Oregon and Washington. The industry continued to grow within the state until the passing of the 18th amendment to the United States Constitution and the beginning of prohibition. Wine production did not resume within the state until the 1970s, nearly 40 years after prohibition ended with the passing of the 19th amendment (Woodall et al. 2002).

The replanting of grapevines in Idaho primarily occurred in the Snake River Valley in southern Idaho, although vineyards have since been started in northern Idaho. As of 2016, the state of Idaho boasted up to 52 licensed wineries, which covered nearly 1,300 acres and produced an estimated 156,000 cases (Idaho Wine Commission 2019). This production has led to an economic impact of nearly $170 million in 2013 (Stonebridge Research 2014). This is more than double the economic impact the Idaho wine industry had only 5 years earlier in 2008 at $73 million with 38 wineries (Idaho Wine Commission 2008). With such a growing and profitable industry, it is important to study and understand grape diseases, which have the potential to significantly alter the revenue and value of the crop.

1.2 An overview of viruses tested in Idaho grapevine

With nearly 70 virus and virus-like disorders, which are recognized as affecting grapevine (Martelli 2017), it would be hard for any vineyard to be completely devoid of viral diseases. The primary focus of the research done was on *Grapevine red blotch virus* (GRBV) and *Grapevine leafroll-associated virus- 3* (GLRaV-3), although there were a total of seven grapevine viruses whose presence were tested for in samples from Idaho vineyards. Along with GLRaV-3 and GRBV, the additional viruses tested included: *Grapevine virus A* (GVA), *Grapevine virus B* (GVB), *Grapevine leafroll-associated viruses 1* and *4* (GLRaV-1 and GLRaV-4, respectively), and *Grapevine fleck virus* (GFkV).

Grapevine Leafroll Disease (GLD) complex is one of the most prominent of all diseases found in grapevine (Maree et al. 2013). It currently is known to be associated with a collection of five different viruses and multiple associated strains, collectively known as *Grapevine leafroll-associated viruses* (GLRaVs). GLD can account for an average loss of $25,000-$40,000 per hectacre but can increase to a reported $226,405, if uncontrolled, with decreased yields ranging 15-40%; the most prominent virus associated with GLD being GLRaV-3 (Martelli 2014; Naidu et al. 2014; Burger et al. 2017; Ricketts et al. 2015). All viruses associated with GLD are classified within the family *Closteroviridae*, with GLRaV-1, -3, and -4 placed in the genus *Ampelovirus,* while the species GLRaV-2 and GLRaV-7 are assigned to the genus' *Closterovirus* and *Velarivirus*, respectively (Martelli et al. 2012; Al Rwahnih et al. 2012; Naidu et al. 2014, 2015, 2017). Formerly, GLRaV-5, -6, -9, -Pr, -Car, and -De were classified as distinct species of GLD but have since been recognized as strains of GLRaV-4, referred to as "grapevine leafroll-associated virus 4-like viruses" or GLRaV-4LVs (Naidu 2017; Aboughanem-Sabanadzovic et al. 2017; Martelli et al. 2012). These viruses are restricted to the phloem tissue of the plant and are filamentous, positive sense, single stranded RNA viruses. GLRaV-3 has the largest genome with a length of approximately 18,500 nucleotides (Burger et al. 2017). All GLD associated viruses are graft transmissible and many can be transmitted by insect vectors, which includes mealybugs and soft scale insects (Martelli 2017).

Both GVA and GVB are classified in the family *Betaflexiviridae* and placed in the genus *Vitivirus* (Martelli 2017). *Vitiviruses* are filamentous, positive sense, single stranded RNA viruses with genome lengths of 7,351 nucleotides for GVA and 7,599 nucleotides for GVB, respectively (Du Preez et al. 2011). These viruses are associated with rugose wood disease complex, which also includes: rupestris stem pitting disorder, kober stem grooving disorder, and corky bark disorder (Du Preez et al. 2011). Both viruses are graft-transmitted and vectored by several mealybug and soft scale insects, which include: *Planococcus citri, Pl. ficus*, *Pseudococus longispinus*, *P. affinis*, and *Phenacoccus aceris* (Minafra et al. 2017; Du Preez et al. 2011).

GFkV is an isometric, positive sense, single stranded RNA virus (Martelli 2017). It has a genome of roughly 7,600 nucleotides and is classified in the family *Tymoviridae* in the genus *Maculavirus* (Martelli 2014). The virus is transmitted through grafting, primarily known to show symptoms in *V. rupestris*, and latent in European grapevine cultivars and most American rootstocks (Martelli 2014, 2017).

GRBV is a relatively new virus, recently discovered in 2008 and characterized in 2011 (Cieniewicz et al. 2017). Research of preserved tissue samples indicates the possibility that the virus has been present for more than 70 years prior to its discovery; as it was detected in archival material

from 1940 from California (Cieniewicz et al. 2017). Symptoms and characteristics are similar to those of GLD and GRBV is often misidentified as being GLD in plants showing visual symptoms of viral disease but negative laboratory results. After GRBV was determined to be a distinct and separate virus, it was characterized as a single stranded, DNA virus with a circular genome and a length of 3.2 kb. GRBV was placed in the genus *Grablovirus* of the family *Geminiviridae* (Zerbini et al. 2017). Though the virus had been characterized there is still much to learn about it. Currently, the only known and reliable method for detection of the virus is by the use of Polymerase Chain Reaction (PCR) and there current research is looking to create a detection assay by Enzyme-Linked Immunosorbent Assay (ELISA), though this has been unsuccessful so far. Distribution of the virus in the main grapevine-growing areas suggested it to be of North American origin; it was reported in South Korea and Switzerland from material brought from the United States (Cieniewicz et al. 2017). GRBV is graft-transmissible, which indicates infected propagation material may be the main source for spread of the virus (Cieniewicz et al. 2017; Al Rwahnih et al. 2013; Poojari et al. 2013). There is also evidence of local spread of the virus in the western United States. While a vector has not been confirmed with certainty, the three-cornered alfalfa treehopper, *Spissistilus festinus*, was shown to transmit the virus in laboratory and greenhouse conditions (Cieniewicz et al. 2017; Bahder et al. 2016).

1.3 Materials and Methods

1.3.1. Sample collection

Samples were collected during the two consecutive growing seasons of 2014 and 2015. During the initial season of 2014, vines that had been chosen for sample collection were tagged with non-corroding aluminum labels. These labels were imprinted with the vine and row number as well as the sample number and date of collection. In late September of 2014, 58 samples were collected and vines labeled from three different vineyards located in Canyon County in southern Idaho, which were designated as Vineyards ‘A’, ‘B’, and ‘C’. Samples were chosen based on visual symptoms of leaf reddening with roughly half of the samples being collected from vines that exhibited healthy characteristics and the rest from vines that showed symptoms of potential viral diseases.

In late August and early September of the following season of 2015, samples were collected from the same 58 vines which had been sampled during the previous season by finding and identifying the labeled tags which had been placed on the selected vines. Along with the original 58 samples, another 46 vines were identified, sampled, and tagged from an additional two vineyards, which were designated as vineyards ‘D’ and ‘E’. Vineyard D is located in Canyon County along with vineyards A, B, and C; however, vineyard E is located in Nez Perce County in northern Idaho.

Identification of vines for sampling was done exclusively within red grape cultivars, this was primarily due to infected white grape cultivars having less pronounced visual symptoms relative to their red grape counterparts. The samples were collected from multiple cultivars of red grapevines with an average of 6-10 samples per cultivar block per vineyard. Cultivars which were collected from include: ‘Merlot’, ‘Cabernet Sauvignon’, ‘Cabernet franc’, ‘Syrah’, and ‘Tempranillo’.

For virus testing, the sampling methodology followed the established convention (Martin et al. 2005; Mekuria et al. 2009; Kanuya et al. 2012). 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 2-3 days until reaching the laboratory. The samples were kept in a cold room (4°C) until the final processing, which occurred 3-14 days after the leaf collection. Petioles were cut off from the leaves and used for subsequent extraction and analysis.

1.3.2. Extraction of genetic material and detection by PCR and RT-PCR

The extraction protocol and virus testing followed the general methodology described previously (Osman et al. 2007; Rowhani et al. 2000), with some modifications. About 0.2-0.3 g of petiole tissue, chopped using single edge razor blades, was homogenized in 5 mL of extraction buffer (1.59 g/l Na_2CO_3, 2.93 g/l $NaHCO_3$, 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°C 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 transcriptase (Invitrogen, Carlsbad, CA), GreenTaq DNA polymerase (Genescript, Piscataway, NJ). The PCR cycling conditions were as follows: initial single cycle at 52°C for 1 hour (reverse transcription step for cDNA synthesis) then a single cycle of 94°C for 2 minutes followed by 35 consecutive cycles of the following profile: 94°C for 30 seconds, 54°C for 45 seconds, 72°C for 30 seconds; and followed by a single cycle at 72°C for 2 minutes, and then held at 4°C until further analysis. The following viruses were tested for: GLRaV-1, -3, and -4, GVA, GVB, GFkV*,* and GRBV.

For all viruses which required RT-PCR, the same protocol was used to detect presence of virus with the exception that each individual virus had its own set of forward and reverse primers

(Table 1.1.) to ensure that any bands which were detected were specific to the virus which was being surveyed.

Since GRBV is a DNA virus, reverse transcription was an unnecessary step. The same denatured extract that was used in RT-PCR was used in the detection of the virus with GRBV specific primers (Table 1.1.). Aliquots of 2 µL of denatured extract were added to 23 µL the PCR reagent mixture (forward primer, reverse primer, 10x Taq buffer, 10 mM dNTPs, Taq polymerase) with a PCR thermocycler protocol of an initial cycle at 94°C for 2 minutes, 30 cycles of steps: 94°C for 30 seconds, 54°C for 45 seconds, and 72°C for 45 seconds, followed by a single cycle at 72°C for 7 minutes, and lastly the resulting product was held at 4°C or stored long term at -20°C until analyzed by electrophoresis.

All RT-PCR and PCR products were analyzed by electrophoresis using 1% agarose gel stained with ethidium bromide and viewed under UV light. Samples which gave DNA product bands at the appropriate sizes pertaining to the virus and primers used in the mixture were deemed to be positive for infection by the virus.

1.4 Results

In the field, the primary symptoms found in the fall were leaf reddening and leaf-rolling, which were correlated with GLD. GLRaV-3 was the primary virus found to infect the collected samples with 26 positive vines during the 2014 season and 41 total positive samples during the following 2015 season with the additional vines (Table 1.2.). There were a total of three samples which tested positive for GLRaV-4, all of which came from vineyard E in northern Idaho during the 2015 season (Table 1.2.). However, there were no positive results for grapevines infected with GLRaV-1 for either of the two seasons.

For GVA, there were a total of two positive results during the 2014 season and 12 positive results for the 2015 season (Table 1.2.). All positive results came from samples collected in vineyards from southern Idaho. For GVB, there were no positive results for the 2014 season, though the following season three samples were found GVB-positive. All GVB positive vines were found in southern Idaho and all were found to also be positive for GVA. There were no positives for GFkV in either seasons.

GRBV-positives were found in three vines during the 2014 season and again during the 2015 season, GRBV-positives were found in only those same three vines (Table 1.2.). All three samples

which were positive for GRBV were from vineyard B in southern Idaho and all were from the same Syrah cultivar.

1.5 Discussion

The increase in vines positive for the presence of virus from the 2014 growing season to the 2015 growing season can partially be attributed to the increased number of vines which were sampled in the consecutive season. However, there was an increase in the number of vines with virus-positives from the original, tagged 58 vines, which had been identified and sampled in 2014 and then again in 2015, particularly for GLRaV-3. This may be attributed to the presence of a vector able to spread the virus, as seen in the increase of GLRaV-3 in 2015 in the same vineyards and cultivar blocks, which showed the highest occurrence of GLRaV-3 during the 2014 season (Table 1.2.). The removal of labeled vines by vineyard operators and replacement with new, presumably healthy vines could also account for discrepancies between seasons.

Another possible cause for differences in the presence of viruses within a vineyard could be the timing of sample collection. Sample collection in the 2014 season was done in late September while the samples for the 2015 season were collected in late August and early September with the exception of those from vineyard E which were done in early October. Typically, the optimal timing for observing GLD symptoms, and also for virus testing and detection in grapevines is in the fall for both *vitiviruses* and *closteroviruses*. This could account for fluctuations in results between seasons due to differences in the timing of sample collection and titer of virus within the vine. Disease symptoms on foliage can also vary depending on the cultivar, virus, and time of season. This variation could cause a misdiagnosis in the field whether it be as healthy or infected, which points out the importance of laboratory virus testing.

Table 1.1. A summary of primers used for screening of grapevine samples from Idaho by RT-PCR and PCR.

#	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 et al. (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	Thompson et al. (2019a)
12		GRLBV-r9	GGGATCCCATCAATTGTTCT		
11	GRBV	GRLBV-F OUT	AATGTTTTCTTCAGCCCACG	2,500	Thompson et al. (2019b)
12		GRLBV-R OUT	CACGCCATAATAAACAGC		
13	GLRaV-3	MP-HSP70F	GGGGDGGRACTTTCGAYGTSTC	600	Donda (2016)
14		MP-HSP70R	ATTGGACTRCCYTTYGGGAAAAT		
15	GLRaV-3	MP-CPF	GATGGRAAGAAGATATA	280	Bester et al. (2014); unpublished
16		MP-CPR	CTAAACGCYTGYTGYCTAG		

[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*.

Table 1.2. A summary of symptoms and virus status for all grapevine samples from Idaho tested in 2014-2015. [a)] [e)]

Vine ID	Symptoms	GLRaV-3 [b)]		GLRaV-4 [c)]	GVA		GVB [c)]	GRBV [d)]		Cultivar	Vineyard
		2014	2015	2015	2014	2015	2015	2014	2015		
1	LR	+	+	-	-	+	-	-	-	Merlot	A
2	NS	+	+	-	+	+	+	-	-	Merlot	A
3	NS	+	+	-	-	+	-	-	-	Merlot	A
4	NS	+	+	-	-	+	-	-	-	Merlot	A
5	LR	+	+	-	+	+	-	-	-	Merlot	A
6	LR	+	+	-	-	+	+	-	-	Merlot	A
7	LR	+	+	-	-	-	-	-	-	Cabernet Sauvignon	A
8	NS	+	+	-	-	-	-	-	-	Cabernet Sauvignon	A
9	NS	+	+	-	-	-	-	-	-	Cabernet Sauvignon	A
10	NS	-	-	-	-	-	-	-	-	Cabernet Sauvignon	A
11	LR	-	+	-	-	-	-	-	-	Cabernet Sauvignon	A
12	LR	+	+	-	-	-	-	-	-	Cabernet Sauvignon	A
13	LR	-	-	-	-	-	-	+	+	Syrah	B
14	LR	-	-	-	-	-	-	+	+	Syrah	B
15	LR	-	-	-	-	-	-	+	+	Syrah	B
16	NS	-	-	-	-	-	-	-	-	Syrah	B
17	NS	-	-	-	-	-	-	-	-	Syrah	B
18	NS	-	-	-	-	-	-	-	-	Syrah	B

19	LR	+	-	-	-	-	-	-	-	Cabernet franc	B
20	NS	+	+	-	-	-	-	-	-	Cabernet franc	B
21	LR	+	+	-	-	-	-	-	-	Cabernet franc	B
22	NS	+	+	-	-	-	-	-	-	Cabernet franc	B
23	LR	-	+	-	-	-	-	-	-	Cabernet franc	B
24	LR	+	+	-	-	-	-	-	-	Cabernet franc	B
25	NS	-	-	-	-	-	-	-	-	Cabernet franc	B
26	NS	-	-	-	-	-	-	-	-	Cabernet franc	B
27	LR	+	+	-	-	-	-	-	-	Cabernet Sauvignon	B
28	LR	+	+	-	-	+	-	-	-	Cabernet Sauvignon	B
29	NS	-	+	-	-	+	-	-	-	Cabernet Sauvignon	B
30	LR	-	+	-	-	-	-	-	-	Cabernet Sauvignon	B
31	NS	+	+	-	-	-	-	-	-	Cabernet Sauvignon	B
32	LR	+	+	-	-	-	-	-	-	Cabernet Sauvignon	B
33	NS	+	+	-	-	-	-	-	-	Cabernet Sauvignon	B
34	NS	+	+	-	-	-	-	-	-	Cabernet Sauvignon	B
35	LR	-	-	-	-	-	-	-	-	Merlot	B
36	LR	-	-	-	-	-	-	-	-	Merlot	B
37	LR	-	-	-	-	-	-	-	-	Merlot	B

38	NS	-	-	-	-	-	-	-	-	Merlot	B
39	NS	-	-	-	-	-	-	-	-	Merlot	B
40	NS	-	-	-	-	-	-	-	-	Merlot	B
41	NS	-	-	-	-	+	-	-	-	Merlot	B
42	LR	-	-	-	-	-	-	-	-	Merlot	B
43	LR	-	+	-	-	-	-	-	-	Cabernet Sauvignon	C
44	LR	-	+	-	-	+	-	-	-	Cabernet Sauvignon	C
45	NS	+	+	-	-	-	-	-	-	Cabernet Sauvignon	C
46	LR	+	+	-	-	-	-	-	-	Cabernet Sauvignon	C
47	LR	+	+	-	-	-	-	-	-	Cabernet Sauvignon	C
48	NS	-	-	-	-	-	-	-	-	Cabernet Sauvignon	C
49	NS	-	-	-	-	-	-	-	-	Cabernet Sauvignon	C
50	NS	-	-	-	-	-	-	-	-	Cabernet Sauvignon	C
51	LR	+	+	-	-	+	-	-	-	Merlot	C
52	NS	-	-	-	-	-	-	-	-	Merlot	C
53	LR	+	+	-	-	-	-	-	-	Merlot	C
54	LR	+	+	-	-	-	-	-	-	Merlot	C
55	LR	+	+	-	-	-	-	-	-	Merlot	C
56	NS	-	-	-	-	-	-	-	-	Merlot	C
57	NS	-	-	-	-	-	-	-	-	Merlot	C

58	NS	-	-	-	-	-	-	-	-	Merlot	C
59	LR	NT	+	-	NT	-	-	NT	-	Tempranillo	D
60	LR	NT	-	-	NT	-	-	NT	-	Tempranillo	D
61	NS	NT	-	-	NT	-	-	NT	-	Tempranillo	D
62	NS	NT	-	-	NT	-	-	NT	-	Tempranillo	D
63	LR	NT	+	-	NT	-	-	NT	-	Tempranillo	D
64	LR	NT	-	-	NT	-	-	NT	-	Tempranillo	D
65	NS	NT	-	-	NT	-	-	NT	-	Tempranillo	D
66	LR	NT	-	-	NT	-	-	NT	-	Tempranillo	D
67	LR	NT	-	-	NT	-	-	NT	-	Tempranillo	D
68	LR	NT	-	-	NT	-	-	NT	-	Tempranillo	D
69	LR	NT	-	-	NT	-	-	NT	-	Tempranillo	D
70	LR	NT	-	-	NT	-	-	NT	-	Tempranillo	D
71	LR	NT	-	-	NT	-	-	NT	-	Tempranillo	D
72	LR	NT	-	-	NT	-	-	NT	-	Tempranillo	D
73	LR	NT	-	-	NT	-	-	NT	-	Tempranillo	D
74	LR	NT	-	-	NT	-	-	NT	-	Tempranillo	D
75	LR	NT	-	-	NT	-	-	NT	-	Tempranillo	D
76	LR	NT	-	-	NT	-	-	NT	-	Tempranillo	D
77	NS	NT	-	-	NT	-	-	NT	-	Tempranillo	D
78	NS	NT	-	-	NT	-	-	NT	-	Tempranillo	D
79	LR	NT	-	-	NT	-	-	NT	-	Tempranillo	D

80	LR	NT	-	-	NT	-	-	NT	-	Tempranillo	D
81	LR	NT	-	-	NT	-	-	NT	-	Tempranillo	D
82	LR	NT	-	-	NT	-	-	NT	-	Tempranillo	D
83	LR	NT	-	-	NT	-	-	NT	-	Tempranillo	D
84	LR	NT	-	-	NT	-	-	NT	-	Tempranillo	D
85	LR	NT	-	-	NT	-	-	NT	-	Tempranillo	D
86	LR	NT	-	-	NT	-	-	NT	-	Tempranillo	D
87	LR	NT	-	-	NT	-	-	NT	-	Tempranillo	D
88	LR	NT	-	-	NT	-	-	NT	-	Tempranillo	D
89	LR	NT	-	-	NT	-	-	NT	-	Tempranillo	D
90	NS	NT	-	-	NT	-	-	NT	-	Tempranillo	D
91	NS	NT	-	-	NT	-	-	NT	-	Tempranillo	D
92	NS	NT	-	-	NT	-	-	NT	-	Tempranillo	D
93	LR	NT	-	-	NT	-	-	NT	-	Tempranillo	D
94	LR	NT	+	-	NT	-	-	NT	-	Cabernet franc	D
95	LR	NT	+	-	NT	+	+	NT	-	Merlot	A
96	LR	NT	+	+	NT	-	-	NT	-	Merlot	E
97	LR	NT	+	-	NT	-	-	NT	-	Merlot	E
98	NS	NT	-	-	NT	-	-	NT	-	Merlot	E
99	LR	NT	-	-	NT	-	-	NT	-	Merlot	E
100	LR	NT	-	+	NT	-	-	NT	-	Merlot	E

101	LR	NT	+	-	NT	-	-	NT	-	Merlot	E
102	LR	NT	-	+	NT	-	-	NT	-	Merlot	E
103	LR	NT	+	-	NT	-	-	NT	-	Cabernet franc	E
104	NS	NT	-	-	NT	-	-	NT	-	Cabernet franc	E

[a)] Abbreviations used: LR, leaf reddening; NS, no symptoms; NT, not tested; - negative and + positive for the virus tested; GLRaV, *Grapevine leafroll associated virus*; GVA, *Grapevine virus A*; GVB, *Grapevine virus B*; GFkV, *Grapevine fleck virus*; GRBV, *Grapevine red blotch virus*.

[b)] Represents combination of all results from RT-PCR using GLRaV-3 targeting primers

[c)] Results representing 2014 testing for GLRaV-4 and GVB were omitted due to absence of any samples being positive by RT-PCR

[d)] Represents combination of all results from PCR using GRBV targeting primers

[e)] Results representing testing for GLRaV-1 and GFkV were omitted due to absence of any samples being positive by RT-PCR

Chapter 2: A novel genetic variant of *Grapevine leafroll-associated virus-3* (GLRaV-3) from Idaho grapevines[1]

2.1. Introduction

Grapevine leafroll disease (GLD) is one of the main disorders of economic significance for the wine grape 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 infield spread of the virus, as well as the removal of infected vines (Almeida et al. 2013; Martelli 2014).

Grapevine leafroll-associated virus-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 11-13 open reading frames (ORFs); the genome expression includes translational frameshifting, polyprotein processing, and multiple sub-genomic 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 6 and 7

[1] This chapter represents a fragment of the published paper by Thompson et al. (2019a). A novel genetic variant of *Grapevine leafroll-associated virus-3* (GLRaV-3) from Idaho grapevines. Plant Disease 103: 509-518.

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 et al. 2011; Martelli 2014; Naidu et al. 2014). GLRaV-3 was also the most prevalent virus in grapevines in the Pacific Northwest (PNW), in Oregon and Washington (Martin et al. 2005; Adiputra et al. 2018). 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 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 ≤78% genome nucleotide identity level to the closest match among GLRaV-3 isolates. This genetic variant, subsequently named 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.

2.2. Materials and Methods

2.2.1. Origin of samples and sampling methodology

Foliar samples from grapevines were collected prior to harvest in 2014-2017 seasons, in mid- to late September, near Caldwell, ID (Canyon county), in two commercially operating vineyards, designated ‘vineyard C’ and ‘vineyard D’ (Chapter 1). The two vineyards were each about 1 hectare

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 C 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 D 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), 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 D 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 2015 and 2016 seasons. Sampling methodology followed as previously described in Chapter 1.

2.2.2. Virus testing by RT-PCR and ELISA

The extraction protocol and virus testing were done as previously described in Chapter 1 with the same viruses being tested for along with the following viruses: GLRaV-2; *Grapevine fanleaf virus* (GFLV); *Arabis mosaic virus* (ArMV). Additional primers used along with those given in Chapter 1 and their respective expected sizes for RT-PCR bands are shown in Table 2.1. 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 the Table 2.5. 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, under the accession numbers given in Table 2.3.

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 (Reinach, Switzerland), 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 hours before being aliquoted to the coated and washed ELISA plate with 4 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 minutes, 2 hours, and 4 hours.

2.2.3. High-throughput and conventional sequencing, and sequence analysis

In 2017, three 'Cabernet Sauvignon' samples collected in vineyard C, vines #45, #46, and #47, were subjected to the high-throughput sequencing (HTS) analysis done by Jennifer Dahan, Ph.D. 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 minutes at room temperature. The aqueous phase was recovered and subjected to RNA extraction following the Plant RNeasy Mini kit (Qiagen, Hilden, Germany) 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 (Zymo Research Corp, Irvine, CA). Library preparation and sequencing were performed by the Genomics Resources Core (IBEST, University of Idaho, Moscow, ID). 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, 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 (Table 2.5.) 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) according to the manufacturer's instructions, using primers SP1_LR3_600r and SP2_LR3_240r (Table 2.5.). 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 (Table 2.5.). Resulting PCR products were submitted for direct Sanger sequencing to Elim Bioparmaceuticals. 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 to 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; Table 2.6.). 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).

2.3. Results

2.3.1. Virus status of the surveyed grapevines

Of the 52 vines surveyed between 2014 and 2017 in two vineyards, eleven were found positive for GLRaV-3, one positive for GVA, and two vines were found positive for both GLRaV-3 and GVA (Table 2.2.). 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 C) or two (vineyard D) growing seasons.

In vineyard C, 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. 2.1., A), 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 2.2.). In September 2017, this vine #45 was tested in ELISA using a commercial kit, in parallel with vine #46 found GLRaV-3 positive using the same RT-PCR test and exhibiting leaf reddening symptoms in each of the four seasons (Fig. 2.1., B). 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. 2.1., C).

In vineyard D, 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 2.2.); 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 D 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 D (Table 2.2.). This 'Cabernet franc' vine exhibited clear interveinal leaf reddening and was adjacent to the 'Tempranillo' block.

2.3.2. 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 2.3.), 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% nucleotide 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 98-99%, and 99%, respectively (Table 2.3.). Interestingly, all 'Merlot' samples from vineyard C belonged to phylogroup I, indicating common origin of the GLRaV-3 isolates, while 'Cabernet Sauvignon' samples from the same vineyard C had GLRaV-3 isolates of three distinct genotypes, indicating multiple introductions of GLRaV-3 (Table 2.3.). 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 2.3.). 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. 2.1.C).

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, and subjected to HTS on a MiSeq system. 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, of 18,477 and 18,487 nt, for #46. These lengths were close to what was expected to be near full-length GLRaV-3 genomes. The 18,487-nt 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-nt and 18,477-nt contigs from samples #45 and #46, respectively, exhibited only modest, ≤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.

To determine the correct genome sequence and acquire the exact 5’ and 3’ terminal regions, the entire 18,478-nt GLRaV-3 sequence was re-amplified 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 in the GenBank database under the accession number MH796136. ID45 sequence displayed a typical GLRaV-3 genome organization, encoding eleven ORFs of the thirteen 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 70-76%, with coverage between 78 to 86% (Table 2.4.).

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 the whole 18,498-nt genome: an A in ID46 was substituted for T in ID47 at position 18,265, in the non-coding 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 thirteen ORFs described for the phylogroup I sequences (Ling et al. 1998, 2004), including the two ORFs missing in ID45.

2.3.3. 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. 2.2.). 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. 2.2.). However, new phylogroups, distinct from the ones described earlier (Maree et al. 2015) became visible as well (Fig. 2.2.). 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. 2.2.; not analyzed by Maree et al. (2015)]. Recently, three new sequences were released by the 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. 2.2.). Careful inspection of these three sequences from the 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.

2.3.4. 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 non-specific 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. 2.3.). 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. 2.3.). As can be seen from Fig. 2.3., 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 initially sample #47 from the 2015 season was found to harbor the ID45-like GLRaV-3 sequence, likely in a mixed infection with a ID46-type sequence, we re-tested all samples collected from the vine #47 in 2014 to 2017 seasons (stored at -80ºC), 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 of 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 the 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).

2.3.5. 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)].

2.4. 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.

GLRaV-3_ID45 isolate described here may represent a novel genetic variant of the virus, exhibiting novel features. Indeed, the vine #45 exhibited no foliar reddening symptoms (Fig. 2.1., A; Table 2.2.) characteristic of other GLRaV-3 positive vines infected with GLRaV-3 isolates belonging

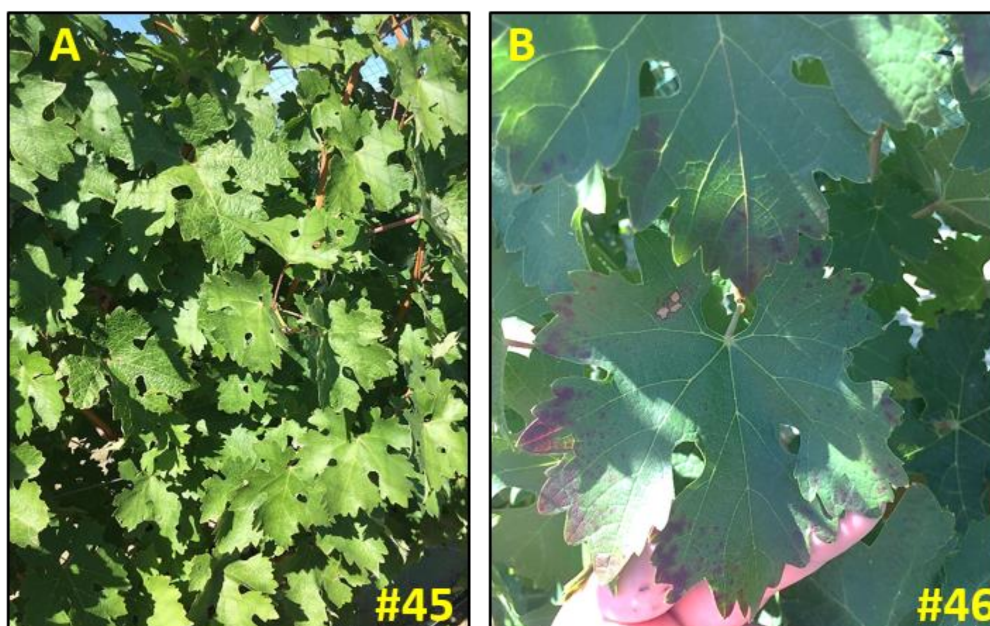
to phylogroups I and III (Fig. 2.1., B; Table 2.2.). This same vine exhibited poor reactivity in DAS-ELISA with at least one commercial kit (Fig. 2.1., C). This poor reactivity in ELISA combined with the lack of foliar symptoms may result in an inadvertent spread of infected plants if no other methods of detection are used for GLRaV-3 testing. Currently available degenerate primers targeting the most conserved genes of the virus (Table 2.1.) may also produce inconclusive results, depending on the season and on the targeted gene, HSP70h versus CP (Table 2.2.). 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 2.3.). 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 which are 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. 2.2.) 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 to 76% (Table 2.4.), and the clearly distinct position of the entire lineage in the phylogenetic tree of GLRaV-3 whole genomes (Fig. 2.2.), 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 on Fig. 2.2., while the remaining seven of the Idaho sequences from two other clades (Mekuria et al. 2009) displayed less than 5% nucleotide sequence differences from phylogroup I isolates (not shown). Apparently, genetic diversity of GLRaV-3 revealed in Idaho previously, was largely restricted to the GLRaV-3 supergroup A (Fig. 2.2.; 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. 2.1., A and B), and may exhibit poor reactivity with the antibodies from the Bioreba ELISA kit (Fig. 2.1., C), 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 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.



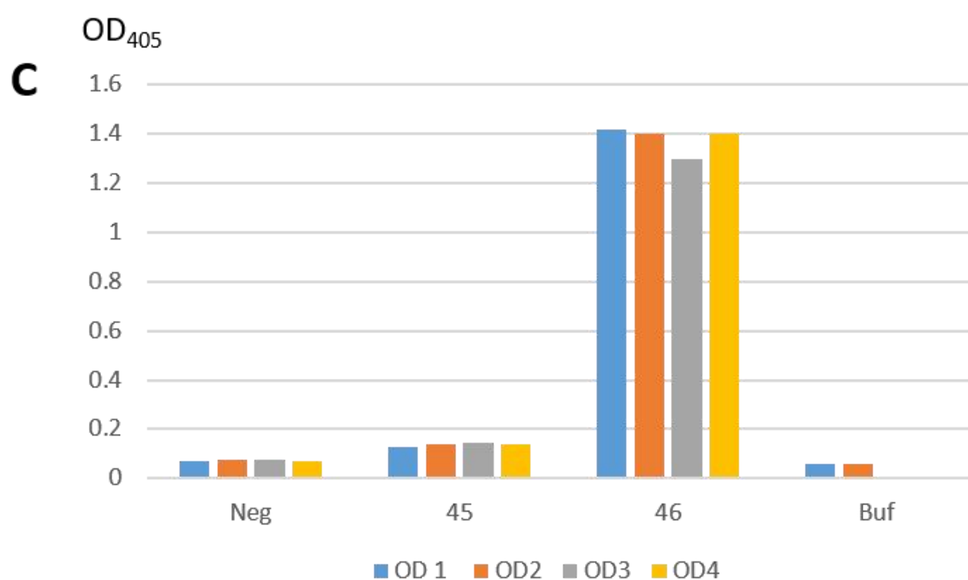


Figure 2.1. Comparison of two vines, #45 and #46, of 'Cabernet Sauvignon' from the same vineyard in southern Idaho, observed (A and B) and sampled (C) for the ELISA assay on the same day in September 2017.
(A) - note asymptomatic appearance of the foliage on vine #45;
(B) – note interveinal reddening of leaves on vine #46, characteristic of GLRaV-3 infection.
(C) – double-antibody 'sandwich' ELISA test on petioles collected from vines #45 and #46, compared to a sample from a healthy vine, and to a buffer (no leaf sample added). Four wells 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 *Grapevine leafroll-associated virus 3* (GLRaV-3). Y-axis is an OD reading at 405 nm.

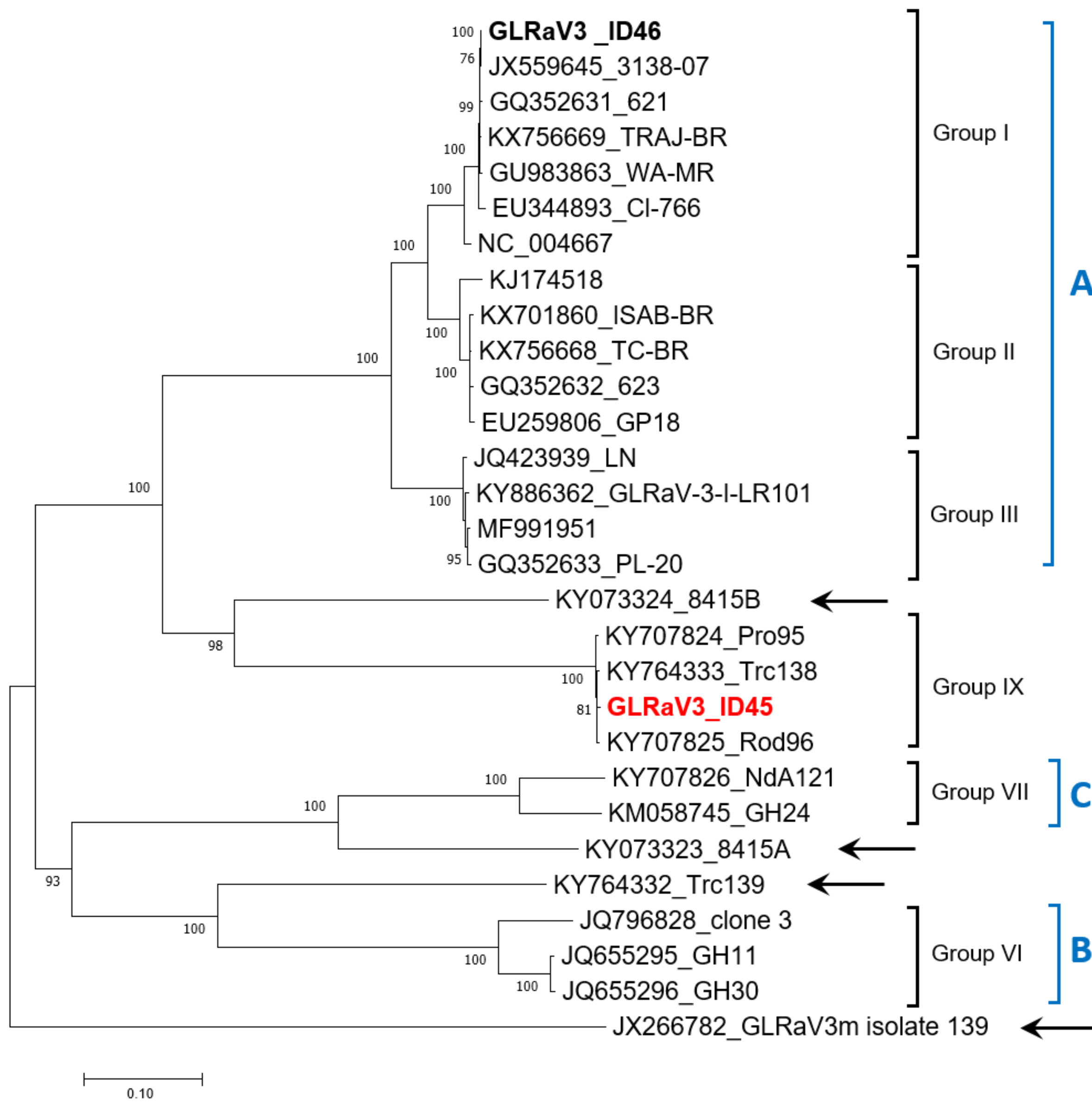


Figure 2.2. Phylogenetic analysis of the *Grapevine leafroll-associated virus-3* (GLRaV-3) whole genome nucleotide sequences. The maximum likelihood method using the GTR+G+I model was used. Values at nodes are bootstrap support at 1,000 replications. The tree is unrooted. The groups indicated by black brackets are arbitrary, and some correspond to previously described phylogroups (Maree et al. 2015): group 1 to group I; group 2 to group II; group 3 to group III; group 5 to group VII; group 6 to group VI. 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.

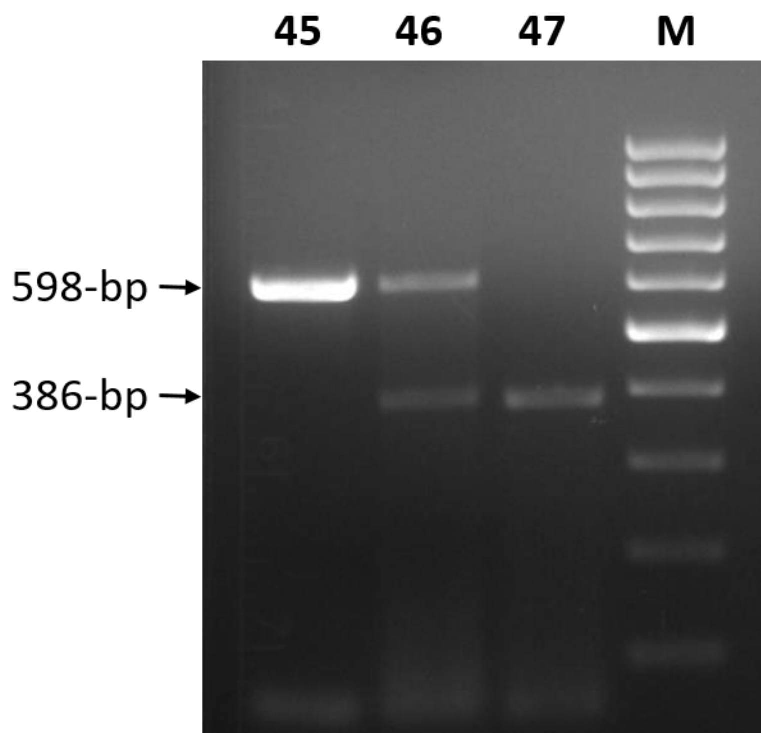


Figure 2.3. Agarose gel-electrophoresis analysis of RT-PCR products, after differential amplification of *Grapevine leafroll-associated virus-3*, ID45- and ID46-specific bands from three petiole samples of 'Cabernet Sauvignon' collected from the same vineyard in southern Idaho, using four GLRaV-3 specific 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 plants sampled; M – 100-bp markers.

Table 2.1. Additional primers used for RT-PCR based detection of multiple grapevine viruses.

#	Target virus [a)]	Primer name	Primer Sequence, 5' to 3'	Amplicon Size (bp)	Reference
1	ArMV	ArMV-F	TGACAACATGGTATGAAGCACA	336	Gambino and Gribaudo (2006)
2		ArMV-R	TATAGGGCCTTTCATCACGAAT		
3	GLRaV-2	GLRaV2_L2F	ATAATTCGGCGTACATCCCCACTT	331	Osman et al. (2007)
4		GLRaV2_U2R	GCCCTCCGCGCAACTAATGACAG		
5	GFLV	GFLV2231F	ACCGGATTGACGTGGGTGAT	322	Mekuria et al. (2009)
6		GFLV2533R	CCAAAGTTGGTTTCCCAAGA		
7	GFLV	GFLaV-F	ATGCTGGATATCGTGACCCTGT	118	Kanuya (2012)
8		GFLaV-R	GAAGGTATGCCTGCTTCAGTGG		

[a)] Abbreviations used: ArMV, *Arabis mosaic virus*; GLRaV, *Grapevine leafroll associated virus*; GFLV, *Grapevine fanleaf virus*.

Table 2.2. 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	C	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	C	Merlot	+	+	-	NT	+	+	-	NT	-	+	-
52	NS		Merlot	-	-	-	NT	-	-	-	NT	-	-	-
53	LR		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	D	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.

Table 2.3. 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. Grey shading highlights 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	MH667399

					MH667367 MH667368	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 2.2.
[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 1-3 clones of RT-PCR product from 2015 season sampling.

Table 2.4. Percentage of coverage and nucleotide sequence identity of 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

Table 2.5. Primers used in this work for RT-PCR amplifications and sequencing of *Grapevine leafroll-associated virus 3* (GLRaV-3) isolates ID45 and ID46. Non-GLRaV-3 specific primers are italicized.

#	Primers name	Primer Sequence, 5' to 3'	Notes on use
1	SP1_LR3_600r	GGAGAAGAAATGTTCAAATGACAAG	5' RACE
2	SP2_LR3_240r	CCTAAAAGACAAGACAAAGACGAGCTAG	5' RACE
3	SP3_LR3_18k-F	CTATTGCGTGGCGAGGAGCG	3' RACE
4	*Oligo d(T)-Anchor Primer*	GACCACGCGTATCGATGTCGACTTTTTTTTTTTTTTTTV	5' and 3' RACE (Roche)
5	*PCR Anchor Primer*	GACCACGCGTATCGATGTCGAC	5' and 3' RACE (Roche)
6	GLR3_ID45_1-F	CTAGTAGGTACCGAACACAGCATTTTTC	RT-PCR and sequencing
7	GLR3_ID45_1040-R	CTGGCCAGCAATTTGACCTTCTC	Sequencing
8	GLR3_ID45_815-F	CGACGTTAATAAGTTCATATTGGCCC	Sequencing
9	GLR3_ID45_1809-R	GCGCTACGTCGGTGCCTG	Sequencing
10	GLR3_ID45_1577-F	GTTAACACACTCCCAGGCAGTAATG	Sequencing
11	GLR3_ID45_2601-R	GGAACATAATCGCAATGTCGAATG	RT-PCR and sequencing
12	GLR3_ID45_2433-F	CTGAGGCGGCCTCCAAGTC	RT-PCR and sequencing
13	GLR3_ID45_3406-R	CCGGACGTGACAATATTCACAACTAC	Sequencing
14	GLR3_ID45_3209-F	GCCAATGACGGTAATTTGTTCG	Sequencing
15	GLR3_ID45_4220-R	CATTAGCGATATAAGCGCTAAAGGC	Sequencing
16	GLR3_ID45_4014-F	CGAGAAAGACGGCGGAGG	Sequencing
17	GLR3_ID45_5040-R	CGACCGCTTTCACTTCCTGC	RT-PCR and sequencing
18	GLR3_ID45_4898-F	GTACAGGAGCCGCTAAGAGAACG	RT-PCR and sequencing
19	GLR3_ID45_5886-R	CCCTGAGCCACTCGCACTTC	Sequencing
20	GLR3_ID45_5666-F	GGCATAGGGAAATCACTGACGC	Sequencing
21	GLR3_ID45_6719-R	CACCTTCTCCCTGTTTATGAATGG	RT-PCR, sequencing, and differentiating RT-PCR
22	GLR3_ID45_6522-F	GGGTCAATAGTGCAACAACCGTC	RT-PCR and sequencing
23	GLR3_ID45_7645-R	CCACCGAATCGTCACAGCC	Sequencing
24	GLR3_ID45_7442-F	CCAGTCTTTTCTCATCGAGGACG	Sequencing
25	GLR3_ID45_8547-R	GAGCTTCAGCGGGTCTGGAAC	RT-PCR and sequencing
26	GLR3_ID45_8303-F	CAGCCTGGTATTGTGCACTTTATTG	RT-PCR and sequencing
27	GLR3_ID45_9366-R	GTTGGCCCCCACCCCTTAC	Sequencing
28	GLR3_ID45_9157-F	CATCCGTGAGGGCGATACC	Sequencing

29	GLR3_ID45_10200-R	CGTCAAAACGACGAACAACGC	Sequencing
30	GLR3_ID45_10001-F	CCGAACATTCTTTTGTACAGAGGTC	Sequencing
31	GLR3_ID45_11029-R	CCGTTTAAAGGAGTTATAGTCCGCC	RT-PCR and sequencing
32	GLR3_ID45_10887-F	CGGACCCGACGTTTTATTGAG	RT-PCR and sequencing
33	GLR3_ID45_11893-R	CCTACGCTTGGATACACCTCGG	Sequencing
34	GLR3_ID45_11694-F	CACTTTGACCAATACACTGACGGAC	Sequencing
35	GLR3_ID45_12744-R	GCGCCGTTGCGACTCTC	Sequencing
36	GLR3_ID45_12538-F	CGCTTCGCGCTCAATAGACAG	Sequencing
37	GLR3_ID45_13560-R	CCTTTGAGGTTATACAATCTGCCG	RT-PCR and sequencing
38	GLR3_ID45_13359-F	CGCTACGGGGAGTGGAAGAC	RT-PCR and sequencing
39	GLR3_ID45_14400-R	GCGGCGGCTTGTTTGATAC	Sequencing
40	GLR3_ID45_14205-F	GGAGCTGTGCAAGAAGGTTATGG	Sequencing
41	GLR3_ID45_15319-R	GATAGAGGTTTTGGTCGACTGATACG	Sequencing
42	GLR3_ID45_15105-F	GGCGGGAGAGGGGTCG	Sequencing
43	GLR3_ID45_16158-R	CGAACCTTTACGCCTCAAATTG	RT-PCR and sequencing
44	GLR3_ID45_15983-F	GATTCAACCAGCGTACACAGCTC	RT-PCR and sequencing
45	GLR3_ID45_17044-R	CGGTGAGTATTAAGTCCCCAACG	Sequencing
46	GLR3_ID45_16867-F	CGTTCACTAATGAAACTGCTGTGC	Sequencing
47	GLR3_ID45_17875-R	GAGGTAACGTCTGTGGACGGAAG	Sequencing
48	GLR3_ID45_17606-F	GGAATTGTTCGAGGTGTGTAGTGG	Sequencing
49	GLR3_ID45_18466-R	CGATAAGTTAGCCTCATAAGAGGCC	RT-PCR and sequencing
50	GLR3_ID45_6119_F	GTCTAGCTCCGATCTCATCTATCACC	Differentiating RT-PCR
51	GLR3_ID46_6331_F	GCATGGTGGGCGAATCG	Differentiating RT-PCR

Table 2.6. Description of the *Grapevine leafroll-associated virus-3* (GLRaV-3) whole genomes used in this study. Geographic origin and grapevine cultivars are as described in GenBank files, or in associated publications, if available; n.s., not specified.

Isolate ID	Cultivar	Origin	Accession number	Reference
ID45	Cabernet Sauvignon	USA	MH796136	Thompson et al. (2019a)
ID46	Cabernet Sauvignon	USA	MH796135	Thompson et al. (2019a)
3138-07	n.s.	Canada	JX559645	unpublished
621	Sauvignon	South Africa	GQ352631	Jooste et al. (2010)
TRAJ-BR	Trajadura	Brazil	KX756669	unpublished
WA-MR	Merlot	USA	GU983863	Jarugula et al. (2010b)
Cl-766	Merlot	Chile	EU344893	Engel et al. (2008)
NY1	n.s.	USA	*AF037268*	Ling et al. (1997); Ling et al. (1998)
-	n.s.	Israel	*KJ174518*	unpublished
623	Ruby Cabernet	South Africa	GQ352632	Jooste et al. (2010)
GP18	Cabernet Sauvignon	South Africa	EU259806	Maree et al. (2008)
ISAB-BR	Vitis labrusca cv Isabel	Brazil	KX701860	unpublished
TC-BR	Vitis labrusca cv. Tardia de Caxias	Brazil	KX756668	unpublished
LN	Venus seedless	China	JQ423939	Fei et al. (2013)
I-LR-101	n.s.	USA	KY886362	Prator et al. (2017)
PL-20	Cabernet Sauvignon	South Africa	GQ352633	Jooste et al. (2010)
8415B	Riesling	Canada	KY073324	unpublished
Pro95	11184	USA	KY707824	unpublished
Trc138	Chardonnay	USA	KY764333	unpublished
Rod96	Roditis	USA	KY707825	unpublished
NdA121	Nero d'Avola	Italy	KY707826	unpublished
GH24	Cabernet Sauvignon	South Africa	KM058745	Bester et al. (2014)
8415A	Riesling	Canada	KY073323	unpublished

Trc139	Chardonnay	USA	KY764332	unpublished
clone 3	Merlot	USA	JQ796828	Seah et al. (2012)
GH11	Cabernet	South Africa	JQ655295	Bester et al. (2012)
GH30	Cabernet	South Africa	JQ655296	Bester et al. (2012)
139	Sauvignon blanc	Australia	JX266782	Rast et al. (2012)
-	Babica	Croatia	MF991951	Vončina et al. (2018)

Chapter 3: Sequencing and creation of infectious clones of *Grapevine red blotch virus* (GRBV)[2]

3.1. Introduction

Wine grape production in Idaho occurs on approximately 1,300 acres in predominately Canyon County in the Southwest and Nez Perce County in the Northwest (Idaho Wine Commission, 2019). Two viruses were previously reported to affect wine grapes in the state, *Grapevine leafroll-associated virus 3* (GLRaV-3; Mekuria et al. 2009; Thompson et al. 2019a) and *Grapevine fleck virus* (GFkV; Kanuya et al. 2012). *Grapevine red blotch virus* (GRBV) causes the devastating red blotch disease in wine grapes (Sudarshana et al. 2015), and belongs to the genus *Grablovirus*, family *Geminiviridae* comprising single-stranded DNA viruses with *ca.* 3.2-kb genome (Zerbini et al. 2017). GRBV was reported to occur in California and several other wine grape growing states (Krenz et al. 2014; Poojari et al. 2013; Sudarshana et al. 2015) and also in Canada, Mexico, and South Korea (Poojari et al. 2017; Gasperin-Bulbarela et al. 2019; Lim et al. 2016). In September of 2014-2015, a small survey of wine grapes was conducted in Canyon and Nez Perce counties of Idaho for the presence of GRBV, as described in Chapter 1. A total of 58 samples of red wine grape cultivars were collected in 2014 and again in 2015, along with an additional 46 collected in 2015 for a total of 104 vines sampled, based on visual symptoms of leaf reddening, and tested by PCR using GRBV-specific primers. Between 2014 and 2017, grapevines close to the original GRBV-positive plants were observed and tested for GRBV to determine if virus spread occurs in Idaho grapevines. Subsequently, several of the Idaho GRBV isolates were subjected to the whole genome sequencing and phylogenetic analysis.

After finding GRBV in Idaho grapevines for the first time, the next step was to clone and sequence Idaho isolates of GRBV, and attempt to build an infectious clone of the virus to study virus pathogenesis and evolution. In addition, we attempted to develop a laboratory host, *Nicotiana benthamiana*, for potential maintenance of the virus and for downstream use as a source of virus antigens for antibody production and formulating methods for future ELISA testing.

[2] This chapter represents a fragment of the published paper by Thompson et al. (2019b). First report of *Grapevine red blotch virus* in Idaho grapevines. Plant Disease. Published online May 16, 2019 (https://doi.org/10.1094/PDIS-04-19-0780-PDN)

3.2. Materials and Methods

3.2.1. Cloning and sequencing of entire GRBV genome

This work expands on a previous survey done in 2009-2011 (Kanuya et al. 2012), from which 434 previous samples were re-analyzed for GRBV, after identification of GRBV in 2014 (Chapter 1). Six additional GRBV-positive samples had been identified and partially sequenced. Of these six samples, two were fully sequenced along with the 2014 samples and included in this work. These were labeled as sample 'IDA8' which originated from 'Syrah' cultivar located at vineyard B (Chapter 1) and sample 'IDB8' from 'Merlot' cultivar located in a 5th vineyard in southern Idaho (Canyon county; not listed), both of which were from 2011.

The DNA products which were obtained by PCR for GRBV in three field samples from 2014 (ID13, ID14, and ID15), as described in Chapter 1, as well as two from 2011 (IDA8 and IDB8) were analyzed by electrophoresis then excised from the agarose gel and purified using a commercially available Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI). The purified PCR products were then ligated into the commercially available pGEM T-easy vector system (Promega), for TA cloning. Ligated plasmids were then transformed into the commercial XL2-Blue Ultracompetent Cells (Agilent Technologies, Santa Clara, CA). Transformed cells were spread plated on Luria-Bertani (LB) broth plates which included the reagents: ampicillin, Isopropyl β-D-1-thiogalactopyranoside (IPTG), and 5-bromo-4-chloro-3-indolyl β-D-Galactopyranoside (X-gal) to allow for blue/white colony selection.

The primer pair, GRLBV-f5 and GRLBV-R9, previously used to amplify GRBV (Chapter 1; Table 3.1.) only produced a DNA product which was roughly 720 bp in length. Therefore, it was necessary to amplify and clone the remaining 2.5 kb of the viral genome for each isolate. To do this, abutting primers were designed, named GRLBV-f Out and GRLBV-R Out (Table 3.1.), which would overlap the original primer pairs, running in opposing directions, and amplify the remaining genome. A Kapa HiFi PCR kit (Kapa Biosystems, Wilmington, MA) was used to create DNA products using high fidelity polymerase to minimize error rates. PCR was done by aliquoting 4 µL of denatured extract (described in Chapter 1) to 21 µL of the high fidelity kit reagents (10 mM Kapa dNTPs, forward primer, reverse primer, Kapa HiFi polymerase) and run in a PCR thermocycler with a protocol consisting of the following cycles: initial cycle at 95°C for 3 minutes, 35 cycles of steps: 98°C for 20 seconds, 61°C for 15 seconds, and 72°C for 2.5 minutes, followed by a single cycle at 72°C for 2.5 minutes, and lastly the resulting products were held at 4°C or stored long term at -20°C until analyzed by electrophoresis.

DNA products obtained by PCR were excised and purified from the agarose gel using the Wizard SV Gel and PCR Clean-Up System (Promega). Once the DNA products had been purified it was necessary to polyadenylate the amplified product, which will be necessary for efficient TA cloning. This step is required due to the proof-reading activity of the Kapa HiFi PCR kit (Kapa Biosystems) which cleaves this tail from the original PCR product. The polyadenylation consisted of adding 13 µL purified PCR product to a 7 µL mixture of reagents: 1 mM dATP, GreenTaq DNA polymerase (Genescript, Piscataway, NJ) and incubating at 70°C for 30 minutes. The polyadenylated PCR product was then purified again using the Wizard® SV Gel and PCR Clean-Up System (Promega) and could be used for cloning.

As with the previous 720 bp region which and been cloned, the purified and polyadenylated PCR products of the remaining 2.5 kb were then ligated into the pGEM T-easy vector system (Promega) and transformed into XL2-Blue Ultracompetent Cells (Agilent Technologies). These transformed cells were spread plated on LB broth plates with the reagents ampicillin, IPTG, and X-gal for blue/white colony selection.

Single colonies from the cloning of the 720 bp regions and the 2.5 kb regions for each of the 5 separate field samples from 2011 (IDA8 and IDB8) and 2014 (ID13, ID14, and ID15) were selected and used to inoculate 5 mL of liquid LB broth and grown overnight at 37°C. Plasmids were purified from 1-2 mL of overnight culture by plasmid mini-prep consisting of 3 solutions: solution I (25 mM Tris-HCl, 50 mM EDTA, 1% glucose), solution II (1% SDS, 0.2 N NaOH), solution III (1.67 M Potassium acetate, 3.33 M glacial acetic acid) followed by precipitation of nucleic acids using ethyl alcohol and re-suspension in sterilized H_2O.

One to three colonies per cloned region were selected for each isolate to be purified and sent for Sanger sequencing using Genewiz Inc. (South Plainfield, NJ) or Elim Biopharmaceuticals (Hayward, CA); additional primers were designed and used for sequencing of plasmids (Table 3.1.), along with universal primers provided by the commercial sequencing companies. Individual sequence reads were assembled using the SeqMan program of the Lasergene 12 Suite (DNASTAR, Madison, WI) to create the whole genome.

As with GLRaV-3 (Chapter 2) for phylogeny inference, whole genome sequences of GRBV available in the GenBank database were aligned with the ID13, ID14, ID15, IDA8, and IDB8 sequences, and a phylogenetic tree generated (Fig. 3.2.). Whole genome sequences of GRBV were retrieved from GenBank and aligned with the five new genomes using MUSCLE with default settings implemented in MEGA version 7 (Kumar et al. 2016; Table 3.2.). Phylogeny reconstruction was done

on the alignment using the maximum likelihood method and the Tamura-Nei model (NT93+G+I) as determined by the MEGA model selection test (Kumar et al. 2016; Nei and Kumar 2000).

3.2.2. Constructing infectious clones of GRBV

Once the whole genome of the GRBV samples had been fully sequenced, the next step was to identify unique restriction sites, which only occur within the genome a single time. Of these unique restriction sites, two would be chosen and used to digest the genome into two smaller fragments. These fragments would be ligated into a binary vector in tandem with a copy of the whole genome. Due to being a *grablovirus*, with circular DNA, the ligation of a partial fragment along with the whole genome is used in order to initiate rolling circle amplification (RCA) in plants which have been inoculated with the infectious clones (Poplawsky 2015).

The two unique sites chosen were *Kpn*I and *Pst*I. In order to proceed with cloning, PCR was used to amplify the entire genome and primers were designed, named GRBaV_KpnI-F and GRBaV_KpnI-R (table 3.1.), which overlap at the *Kpn*I restriction site and then continue on to amplify the entire genome of the virus. This overlap at the *Kpn*I site allows for this site to exist at both the 5' and 3' ends of the PCR product and can be used to digest and ligate the whole genome of GRBV. The *Pst*I exists roughly 500 bp from the *Kpn*I site and will be used in a double enzyme digestion to create the smaller, partial fragments of the genome.

Samples to be cloned were maximized by first amplifying all circular DNA present in the denatured extraction of the grapevine samples (Chapter 1) by using RCA, which was done using the illustra Templiphi amplification kit (GE Healthcare Life Sciences, UK). RCA product (1.0 µL) was then viewed by electrophoresis on agarose gel stained with ethidium bromide. The DNA product of the RCA was next used in PCR with the designed primers, GRBaV_KpnI-F and GRBaV_KpnI-R (Table 3.1.), which would then give a linear amplification of the entire genome of the GRBV, along with the *Kpn*I restriction site at both ends of the product. Kapa HiFi PCR kit (Kapa Biosystems) was used to minimize errors and maximize product. PCR was done by using 1.0 µL of RCA product added to a 24.0 µL mix Kapa HiFi reagents (10 mM Kapa dNTPs, forward primer, reverse primer, Kapa HiFi polymerase) and run on the thermocycler protocol with the following cycles: initial cycle at 95°C for 3 minutes, followed by 30 cycles of steps: 98°C for 20 seconds, 65°C for 15 seconds, and 72°C for 3.5 minutes, followed by a single cycle at 72°C for 3.5 minutes, and lastly the resulting products were held at 4°C.

The amplicons for all three samples were viewed using electrophoresis on agarose gel stained with ethidium bromide. The PCR of the RCA product gave two distinct amplicons with one at the

expected ~3,200 bp length and another at ~6,000 bp length. Two of the PCR products (ID14 and ID15) were chosen, based on their band intensity, to have the presumably correct band excised from the agarose and purified using the Wizard SV Gel and PCR Clean-Up System (Promega). These purified products were then cloned into the pGEM T-easy vector system (Promega) and three individual clones were selected, for both of the cloned samples, for digestion using the restriction enzymes *Kpn*I and *Pst*I (New England Biolabs, Inc., Ipswich, MA). Digestion gave the two partial fragments of the GRBV genome, one at length of ~500 bp and another at a length of ~2,700 bp. Due to the size of the vector being similar to that of the entire GRBV insert, this piece was double digested using *Kpn*I and *Fsp*I enzymes (New England Biolabs, Inc.) which gave several small products derived from the vector while leaving the whole GRBV genome insert intact. The partial and whole genome inserts digested from the vector were viewed and excised from agarose gel stained with ethidium bromide to be purified.

The two partial fragments of the GRBV genome were ligated separately into the pCambia1300 plant expression vector (Marker Gene Technologies, Inc., Eugene, OR) and cloned into XL2-Blue Ultracompetent Cells (Agilent Technologies). Several clones were chosen for mini-prep by blue/white colony screening and restriction enzyme digestion using KpnI and PstI (New England Biolabs, Inc.) was done to screen for correct inserts which were viewed by agarose gel electrophoresis. Plasmids which appeared to contain the correct inserts would then need to be opened for ligation of the whole genome with the partial GRBV genome. Digestion was done using KpnI, which was then deactivated according to manufacturer’s instruction once completed. Digested Plasmids were then treated using Antarctic phosphatase (New England Biolabs, Inc.) according to the manufacturer’s instructions. The dephosphorylated plasmids were then cleaned up using the Wizard SV Gel and PCR Clean-Up System (Promega). The whole GRBV genome was then ligated into the dephosphorylated plasmids containing the partial GRBV genome. Ligated plasmids were cloned into the XL2-Blue Ultracompetent Cells. Blue/white colony screening was not possible since the vector already contains the partial fragments from previous ligations, so several colonies were arbitrarily picked and plasmids were purified and checked for inserts using PstI enzyme digestion and viewed on agarose gel stained with ethidium bromide. Screened plasmids with both the partial and whole GRBV genomes ligated into them were then electroporated into *Agrobacterium tumefaciens C-58 GV2260* and plated on LB medium containing kanamycin and rifampicin antibiotics. Colonies were lastly screened by PCR and given a final label: ‘14-1(1.2)’ for ID14 whole genome with the small fragment, ‘14-3(1.8)’ for ID14 whole genome with the large fragment, ‘15-1(1.2)’ for ID15 whole genome with the small fragment, and ‘15-1(1.8)’ for ID15 whole genome with the large fragment. These colonies

were then used to inoculate liquid LB medium and prepared for infiltration of *Nicotiana benthamiana*, along with being mixed into a glycerol stock solution and stored at -80°C.

3.2.3. Inoculation of N. benthamiana

Once infective constructs were formed and the presence of inserts were confirmed, the next step was inoculation of *N. benthamiana*. The four screened constructs were grown on fresh LB medium plates along with the control *agrobacterium* containing only the pCambia 1300 vector and agrobacterium containing the HC-Pro gene (TuMV HCPro) to help facilitate infection by suppressing post-transcriptional gene silencing.

Colonies were used to inoculate 5 mL of liquid LB medium containing Kanamycin and Rifampicin antibiotics, which was grown for 16-24 hours at 28°C on an orbital shaker. The inoculated medium was then used to inoculate another 100 mL of liquid LB medium containing Kanamycin and Rifampicin antibiotics which was also grown at 28°C for 16-24 hours on an orbital shaker. Bacteria culture was recovered by centrifugation and the supernatant removed. The bacterial pellet was re-suspended in 30 mL of infiltration medium (10 mM MgCl2, 10 mM MES [pH 5.7], 150 µM acetosyringone) and incubated at room temperature for 16-20 hours on orbital shaker. Infiltration buffer was added until OD reached between 0.5 to 1.0 and continued incubation at room temperature for ~3 hours. The four constructs, along with the control agrobacterium, were mixed at a 1:10 ratio with TuMV HCPro.

Nicotiana benthamiana plants were grown for roughly two weeks, in greenhouse growth chamber, after planting and were selected for inoculation by *A. tumefaciens* containing the desired infectious clone constructs. Three fully expanded leaves towards the upper growing area of the plant were selected for infiltration. Leaves were inoculated by agro-infiltration and plants were left to grow on greenhouse bay benches until sampling was to be done. Plants were inoculated in two separate trials, which were done in succession of each other. The first consisted of six plants per infectious clone, as well as, two plants for the negative control consisting of empty pCambia vector and two plants which were left untouched as healthy controls. The second trial consisted of 10 plants per clone, along with, three plants each for the negative and healthy controls.

3.2.4. Sampling and extraction of genetic material

After inoculation, plants were left to grow and samples subsequently taken at 14, 25, and 32 day intervals for the first trial and 14, 22, and 28 day intervals for the second trial. Sampling was done using 1.5 mL micro-centrifuge tubes (VWR, Radnor, PA) to punch 3-9 cuttings from leaf tissues.

At 14 days post inoculation, samples were taken, separately, from the original inoculated leaves, as well as, the newer, upper, systemic leaves. After 21-24 days the same plants were sampled from three different positions with tissue again being taken from inoculated leaves, the upper leaves which were sampled at 14 days, and then newly grown, upper leaves. At roughly 28 days, random leaves from various positons on the plant were all combined and sampled together. All sampled tissues were kept on ice or stored at -80°C until they could be ground and the nucleic acids extracted.

Genetic material was extracted using a version similar to Dellaporta extraction protocol (Dellaporta et al. 1983). Tissue was frozen using liquid nitrogen and powdered using mortar and pestle with 500 mL of extraction buffer added (0.1 M Tris-HCl, 0.5 M NaCl, 50 mM EDTA, 0.7 µL/mL β-mercaptoethanol). Samples were vortexed after the addition of 140 µL 10% SDS and incubated at 65°C for 10 minutes; 250 µL 8 M potassium acetate was then added to the samples which were mixed well and incubated on ice for 5 minutes. Debris was removed by centrifuging the samples at 19,200 x g for 4 minutes and ≤1 mL of the supernatant was aliquoted to fresh 1.5 micro-centrifuge tubes. Nucleic acids were precipitated using 600 mL isopropyl alcohol and incubated overnight at -20°C before centrifuging for 5 minutes at 26,700 x g. The pellet was then washed using 1 mL 70% ethyl alcohol before left at room temperature for any remaining alcohol to evaporate. Dried pellet was then re-suspended in 60 mL sterilized H_2O and these Dellaporta extracts were prepared for use in PCR.

To verify infection of inoculated plants, PCR was done on Dellaporta extractions using the primer pair GRBaV_Out_3F & GRBaV_Out_3R (Table 3.1.). PCR was done by using 2.0 µL of Dellaporta extraction added to 23 µL of PCR mix: Forward primer, Reverse primer, 10 mM dNTPs, RNasin (Promega), GreenTaq DNA polymerase (Genescript). The PCR was run using the following cycling conditions: an initial cycle of 94°C for 5 minutes, followed by 30 consecutive cycles of 94°C for 30 seconds, 51°C for 30 seconds, 68°C for 1.5 minutes; and followed by a single cycle of 68°C for 7 minutes, and then held at 4°C until further analysis.

3.3. Results

3.3.1. Sequencing of GRBV-positive samples

The three initial, 720-bp PCR products were cloned into the plasmid vector, sequenced, and confirmed to represent a fragment of the GRBV CP gene between positions 1,313 and 2,031 in the genome of GRBV, displaying 99.8% identity to isolate GiGV-WA-MR (GenBank accession number KC427995). Similar to GLRaV-3 (Chapter 2), overlapping fragments of GRBV genome amplified by

PCR with abutting primers were cloned into a plasmid vector and sequenced. Complete genomes were assembled for GRBV found in the three samples collected in 2014 from vineyard 'B' (see Chapter 1), along with the two samples originally collected in 2011 and re-analyzed for the presence of GRBV in this work (IDA8 and IDB8). The assembled sequences from 2014 samples ID13, ID14, and ID15 had genomes with the lengths of 3,205 bp, 3,207 bp, and 3,208 bp, respectively; while 2011 samples IDA8 and IDB8 had genome lengths of 3,205 bp and 3,206 bp, respectively. When the genomes of these five samples were analyzed using BLASTn, they all displayed up to 99% nt identity with GRBV sequences available in GenBank.

3.3.2. Phylogentic analysis of GRBV

To determine phylogenetic relationships between the Idaho sequences and other GRBV isolates, whole genome sequences of GRBV available in the GenBank database were aligned with the Idaho isolates, and a phylogenetic tree generated (Fig. 3.1.). All whole genomes sequenced were assigned to clade 2 of GRBV (Krenz et al. 2014), most closely to a group of GRBV isolates from Washington state (Fig. 3.1.; Adiputra et al. 2018).

3.3.2. Observed symptoms of inoculated N. benthamiana

Post infiltration by *A. tumefaciens* infectious clones, *N. benthamiana* plants were observed multiple times a week for visual symptoms for up to 28-32 days. Throughout this time, the inoculated plants showed no visual changes when compared to healthy control plants or those which had been inoculated using negative control vectors. Leaves showed no mosaic, chlorosis, nor any other recognizable style of discoloration. Leaves also showed no type of curling or rolling, nor changes in leaf thickness nor brittleness. Inoculated plants also showed no discernable stunting or relative change in growth outside of what could be considered natural variations when compared to healthy plants. While it was unknown what symptoms would be induced through infiltration, it was hypothesized that discoloration or stunting would be the likely observed symptoms.

3.3.3. PCR status of inoculated plants

When Dellaporta extracts were tested by PCR, the results also indicated that the plants were likely negative for being infected by the clones. At 14 days post-inoculation in both trials, the majority of positive samples came from inoculated leaves for nearly all constructs give roughly 50% positive results from infiltrated leaves and 20% for upper systemic leaves. The exception for this is the 15-1(1.8) construct which gives roughly 80% positive results for systemic leaves and 20% positive in infiltrated leaves in both trials.

At 22-25 days post-inoculation, the positive PCR products drops to about 10-20% for infiltrated leaves and ≤10% for leaves the same leaves tested at 14 days; new upper leaves were all negative with the exception of a single plant in a single construct. At 28-35 days post-inoculation, all plants in both trials tested negative by PCR.

3.4. Discussion

The phylogeny of the Idaho GRBV isolates (Fig. 3.1.) revealed that all five Idaho GRBV isolates were placed in a tight lineage of the same Clade 2, and suggested the introduction of GRBV to Idaho from the same source, probably through infected planting material. Results showed that Idaho GRBV isolates were very closely related to several GRBV isolates found in the state of Washington (Fig. 3.1.), which may suggest that Idaho isolates originated in Washington state, where several large nurseries are used by Idaho producers to source planting material. Observations and PCR tests for the virus around GRBV-positive 'Syrah' plants in vineyard B conducted in 2011 and again from 2014-2016 suggested limited, if any, spread of the virus outside of the original three GRBV-positive vines.

Both the visual observations of inoculated plants and the PCR results of subsequent Dellaporta extracts taken over the course of multiple weeks indicated that the infectious clones failed to induce replication of the virus and systemic infection of *N. benthamiana*. There were positive results from PCR, however these were primarily in infiltrated leaves, which could be attributed to residual infectious clones left in the leaves. The lack of symptoms also can be seen as an indication that the clones failed to produce any viral products which moved systemically through the plant. Although, lack of symptoms is not necessarily an indication of a healthy plant, when combined with negative PCR results it can give credence to a lack of virus.

Previous checks of infectious clones during ligation and transformation by PCR and restriction enzyme digestion concluded that the infectious clones existed in the manner they were designed and intended. A potential cause for the lack of efficiency of the infectious clones could be an improper host. While *N. benthamiana* is considered to be a susceptible host for a wide range of pathogens, it may not be suited for use in all laboratory experiments. A more proper experiment using these infected clones would need to be done on a different host, which ideally would be grapevine or another plant known to be susceptible to this particular virus.

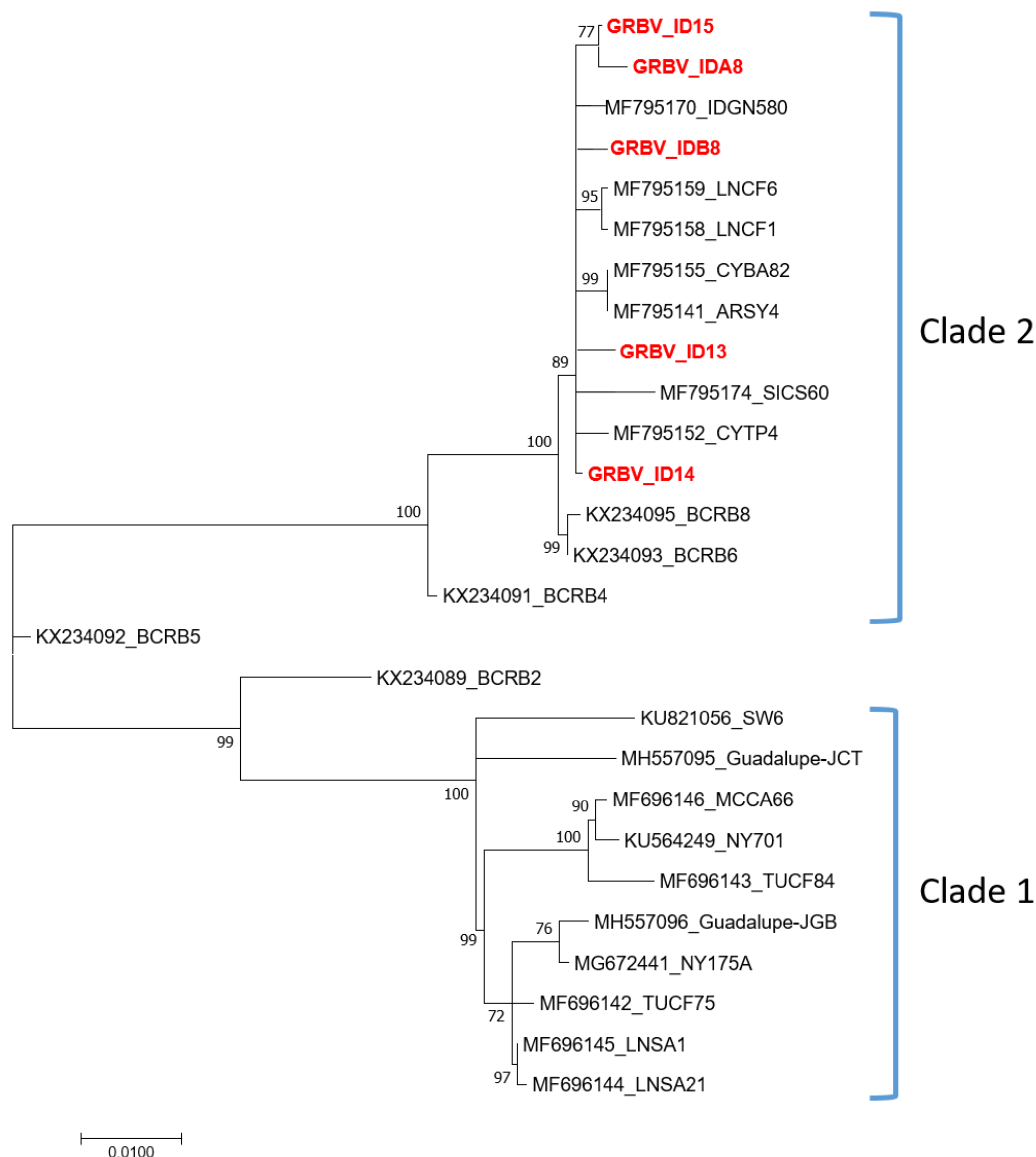


Figure 3.1. Phylogenetic analysis of nucleotide sequences of the whole genomes for the five isolates of *Grapevine red blotch virus* (GRBV) from Idaho and 22 additional, representative GRBV isolates, conducted in MEGA7 with the maximum likelihood method using the NT93+G+I model. Values at nodes are bootstrap support at 500 replications. Only nodes with bootstrap values of 70% or higher were retained. Brackets designate two clades of GRBV according to Krenz et al. (2014). GRBV isolates are highlighted in bold and red. Both an isolate name and a corresponding accession number in the GenBank database are given for each reference sequence.

Table 3.1. Primers used in this work for PCR amplifications and sequencing of *Grapevine red blotch virus* (GRBV) isolates IDA8, IDB8, ID13, ID14, and ID15.

#	Primers name	Primer Sequence, 5' to 3'	Notes on use
1	GRLBV-f5	TGCAAGTGGACATACGTTTA	PCR and sequencing
2	GRLBV-R9	GGGATCCCATCAATTGTTCT	PCR and sequencing
3	GRLBV-F OUT	AATGTTTTCTTCAGCCCACG	PCR and sequencing
4	GRLBV-R OUT	CACGCCATAATAAACAGC	PCR and sequencing
5	GRBaV_Out_3F	GAGTTGCAGAAACTCGTCT	PCR and sequencing
6	GRBaV_Out_3R	CCATGGAATGCAAACTGACA	PCR and sequencing
7	GRB_F-421	GGATCCGTTTATTCGTCGTCACA	Sequencing
8	GRB_F-2372	GCTGGACGCGTTTTGTTTCTTGT	Sequencing
9	GRBaV_KpnI-F	AGGGGTACCCCCAAAAGAAT	PCR and cloning
10	GRBaV_KpnI-R	GGGGGTACCCCTTCCCTTTT	PCR and cloning

Table 3.2. Description of the *Grapevine red blotch virus* (GRBV) whole genomes used in this study. Geographic origin and grapevine cultivars are as described in GenBank files, or in associated publications, if available; n.s., not specified.

Isolate ID	Cultivar	Origin	Accession number	Reference
ID13	Syrah	USA	MK928382	Thompson et al. (2019b)
ID14	Syrah	USA	MK928383	Thompson et al. (2019b)
ID15	Syrah	USA	MK928384	Thompson et al. (2019b)
IDA8	Syrah	USA	MK928386	Thompson et al. (2019b)
IDB8	Merlot	USA	MK928385	Thompson et al. (2019b)
IDGN580	Grenache	USA	MF795170	Adiputra et al. (2018)
Guadalupe-JGB	Pinot noir	Mexico	MH557096	Gasperin-Bulbarela et al. (2019)
Guadalupe-JCT	Nebbiolo	Mexico	MH557095	Gasperin-Bulbarela et al. (2019)
NY175A	n.s.	USA	MG672441	Yepes et al. (2018)
LNCF6	Cabernet franc	USA	MF795159	Adiputra et al. (2018)
LNCF1	Cabernet franc	USA	MF795158	Adiputra et al. (2018)
CYBA82	Barbera	USA	MF795155	Adiputra et al. (2018)
ARSY4	Cabernet franc	USA	MF795141	Adiputra et al. (2018)
SICS60	Cabernet Sauvignon	USA	MF795174	Adiputra et al. (2018)
CYTP4	Tempranillo	USA	MF795152	Adiputra et al. (2018)
BCRB8	Grenache	Canada	KX234095	Poojari et al. (2017)

BCRB6	Zinfandel	Canada	KX234093	Poojari et al. (2017)
BCRB4	Chardonnay	Canada	KX234091	Poojari et al. (2017)
BCRB5	Chardonnay	Canada	KX234092	Poojari et al. (2017)
BCRB2	Cabernet franc	Canada	KX234089	Poojari et al. (2017)
SW6	n.s.	South Korea	KU821056	Lim et al. (2016)
MCCA66	Carmenere	USA	MF696146	unpublished
NY701	Cabernet Sauvignon	USA	KU564249	Perry et al. (2016)
TUCF84	Cabernet franc	USA	MF696143	unpublished
TUCF75	Cabernet franc	USA	MF696142	unpublished
LNSA1	Sangiovese	USA	MF696145	unpublished
LNSA21	Sangiovese	USA	MF696144	unpublished

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